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The interactions between DNA nanostructures and cells: A critical overview from a cell biology perspective

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Abstract

DNA nanotechnology has yielded remarkable advances in composite materials with diverse applications in biomedicine. The specificity and predictability of building 3D structures at the nanometer scale make DNA nanotechnology a promising tool for uses in biosensing, drug delivery, cell modulation, and bioimag-ing. However, for successful translation of DNA nanostructures to real-world applications, it is crucial to understand how they interact with living cells, and the consequences of such interactions. In this review, we summarize the current state of knowledge on the interactions of DNA nanostructures with cells. We identify key challenges, from a cell biology perspective, that influence progress towards the clinical translation of DNA nanostructures. We close by providing an outlook on what questions must be addressed to accelerate the clinical translation of DNA nanostructures.

Statement of significance—Self-assembled DNA nanostructures (DNs) offers unique opportunities to overcome persistent challenges in the nanobiotechnology field. However, the interactions between engineered DNs and living cells are still not well defined. Critical systematization of current cellular models and biological responses triggered by DNs is a crucial foundation for the successful clinical translation of DNA nanostructures. Moreover, such an analysis will identify the pitfalls and challenges that are present in the field, and provide a basis for overcoming those challenges.

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Keywords

Nanotechnology; DNA nanotechnology; Cytotoxicity; Cellular uptake; Protein corona; Bionano interactions

1. Introduction

Advances in nanotechnology have enabled interesting applications and techniques in various fields, ranging from engineering to pharmacology and medicine [1-5]. The unique physicochemical properties of nanocarriers, in combination with their multifunctional capacity, allows these nanomaterials to be implemented in multiple biomedical applications [2]. In fact, distinct nanoparticles (NPs) were found to be highly useful in drug delivery, diagnosis, and imaging [3,6-8], thanks to improvement of the biodistribution and pharmacokinetics of the active pharmaceutical ingredients [9,10]. Numerous chemically distinct NPs (e.g. gold, metal oxides, silica, polystyrene, etc.) have been synthesized and are now being utilized as drug delivery vehicles, imaging enhancers, biosensing platform components, and other therapeutic and diagnostic uses [3,6-8,11,12]. Successful implementation of nanotechnology in medicine has resulted in clinical approval of 27 nanoparticle-based medicines by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) [13]. Although FDA approval indicates some clinical success of nanomedicines, thus far patients offered these nanomedicines have showed only minor improvement in survival rates [14-16]. Additionally, nanomedicine formulations possess the risk of activating the immune system, which may lead to premature clearance from the body, as well as toxic side effects [17]. Emerging evidence highlights the following major challenges that hamper the clinical success of nanoparticles: difficulties in overcoming various biological barriers, low targeting efficiency, and safety issues [2,5,6,15,16]. As a result, medical applications of NPs are often criticized for their extremely low rate of clinically successful outcomes, despite their long research history and large investments [16,18-21]. Additionally, the lack of detailed understanding of the basic biological foundations of NP-cell interactions have also resulted in poor clinical translation of nanomedicines [6,16,19,21-23].

To circumvent translational challenges, it is crucial to reproducibly form nanomaterial complexes, retaining high precision in the nanometer range [2,24]. Indeed, production of complex functionalized NPs on this scale usually lacks a tight control over size, shape, and surface chemistry [24-26]. Self-assembly motifs, which are based on predictable and specific molecular interactions, represent an important direction in nanotechnology, with a promising foundation to overcome the challenges with structural precision [27-29]. Specifically, DNA nanotechnology bears tremendous potential in constructing complex 3D structures with nanometer precision [27,30-32]. DNA nanostructures (DNs), are being extensively investigated and applied in various research fields, such as chemical sensing, nanoelectronics, and biomedicine [27,30-34]. Their architectural diversity is exemplified by the variety of methods that have been developed to assemble these structures, including wireframe DNA structures (Fig. 1A and B) [35-37], DNA origami (Fig. 1C and D) [38-40], DNA brick [41] and tile [42,43] motifs (Fig. 1E and F, respectively). Dynamic, actuatable

DNs [44,45] have also been developed (Fig. 1G and H) in addition to larger, hierarchically assembled structures (Fig. 1I and J) [46,47].

Biomedical DN research is progressing impressively quickly, specifically in the direction of diagnostics and therapeutics [2,24,27,32,34,48]. DNs possess several key advantages in biomedical applications over conventional NPs [2,24,27,32,34]. For example, conventional NPs have been shown to induce various adverse reactions [21,49,50]. By contrast, DNs typically exhibit great biocompatibility and thus far lack toxicity in preliminary studies [2,24,27,32,34]. Furthermore, the capacity of DNs for self-assembly allows their construction into well-defined 3D architectures of arbitrary shape and size at the nanoscale. This in turn enables the biological activity of DNs to be finely tuned and modified [2,24,27,32,34,44,51,52]. The surfaces of DNs can be functionalized accurately and precisely using the properties of DNA self-assembly [2,24,27,32,34,44,51,52]. These unique properties of DNs have opened doors to numerous biomedical applications. Thus far, DNs represent great nanomedical potential and are being actively studied as platforms for controlled release of various therapeutic compounds, as imaging modules, and as vehicles for targeted delivery [2,24,27,32,34,44,51,52].

Despite these promising initial results, the translation of DNs to the clinic is still in its infancy. There are only a handful of studies that use DNs under *in vivo* conditions [34], and the field still lacks a thorough knowledge about the precise molecular determinants that modulate DN-cell interactions [34]. Verifying the key principles of DN-cell interactions is important to understanding the molecular mechanisms underlying therapeutic approaches. Clear knowledge of how certain treatments work is crucial in forthcoming clinical trials, and represents a roadmap for successful implementation of the treatment [53]. Understanding the mode of action at the cellular and molecular level will aid in determining the therapeutic window of a treatment, enabling better dosing, stratifying clinical trials, and eventually helping patients [21,53-57]. Thus, in this review we present an analysis of current knowledge on DN-cell interactions. We discuss challenges currently limiting DNs translation towards real-world applications. Finally, we highlight strategies that may help to overcome these challenges and maximize the biomedical potential of DNs.

2. The protein corona and its impact on DNA nanostructures

Generally, it has been found that contact with physiological fluids results in the formation of a protein corona around many types of nanoparticles [58-60]. Proteins and other biomolecules interact with the surface of the particles, forming a multilayered shell [58-60]. The presence of the corona may shield surface modifications (e.g. chemical moieties, targeting ligands, antibodies, etc.) and affect their function or efficiency [58-60]. Not surprisingly, a protein corona has been shown to form on DNs as well [27,34,61]. Overall, adsorption of proteins onto a particle surface occurs rapidly, approximately one hour after exposure to physiological fluids [58-60]. Accumulating evidence suggests that multiple factors play a role in the composition of the protein corona and biomolecule binding efficacy [58-60,62,63]. NP physicochemical properties (i.e. chemical composition, size, shape, surface functionalization), physiological fluid composition, and exposure time determine the makeup and nature of the protein corona [58-60,62,63]. In turn, protein

binding to the NP surface changes the physicochemical properties of the particle itself (e.g. hydrodynamic diameter, zeta potential, solubility), and the protein properties are also altered (e.g. misfolding, aggregation, conformational changes, alteration in enzymatic activity) [58-60,62-65]. These structural and functional changes of proteins upon binding to NP surface may lead to cellular injury [58-60,62,63]; furthermore, the protein corona may greatly hinder the targeting capabilities of NPs by shielding surface functionalization [66]. On the other hand, tuning the surface modification of NPs may affect protein corona composition in a way to improve circulation half-time, mitigate toxic effects, and/or ameliorate targeting issues [60,67].

The considerations outlined above demonstrate why it is crucial to study the protein corona formation around DNs in detail. Although DNA nanostructures have now been studied for decades, research on applications in biomedicine (e.g. DNs as tools for imaging and vehicles for gene delivery) and therapeutics (e.g. targeted drug delivery) for DNs are quite recent (Fig. 2). Consequently, little attention has been given thus far to the analysis of DN-protein corona composition and the corona's functional consequences for DNs [27,34,61]. Recently, more research has been devoted to how protein corona affects DN stability [27,34,61]. Indeed, the limited stability of DNs in physiological fluids represents a challenge for their successful biomedical application [27,34,61]. Nucleases are predominantly responsible for *in vivo* degradation of DNs [61], and to mitigate this problem, peptides and proteins have been used to create nuclease-protective coatings that give DNs a longer half-life in biological environments [61]. Synthetic protein coronae may also be utilized to create nuclease-resistant DNs [61,68]. For example, bovine serum albumin (BSA)-dendron conjugates attached to DNs protected nanostructures from exposure to 10 U of DNase I (Fig. 3A) [68]. The BSA corona also significantly reduced the immune response against DNs and improved their transfection efficacy [68]. Protein polymers and diblock polypeptides have also been shown to be effective for shielding DNs from enzymatic degradation [69,70]. Another strategy to enhance DN stability is to create stable and enzymatically resistant DNs that simultaneously reduce particle-protein interactions, such as coating the nanostructures with poly(ethylene glycol) (PEG) [2,27,34,61], while ensuring that DN surface functionality is not compromised. One way to achieve PEG passivation is via an electrostatically-adhered oligolysine–PEG coating, which was found not to interfere with the functionality of surface-displayed ligands on DNs [71]. While PEG conjugation is a widely used surface modification for various other types of NPs, some PEG-based nanomaterials have been shown to be immunogenic, resulting in release of anti-drug antibodies [72,73]. PEG itself can trigger anti-PEG IgG and IgM antibody responses [72,73], and high titers of these antibodies may lead to severe allergic reactions such as anaphylaxis [72-74]. Thus, PEG-based coatings of DNs must be designed in a controlled and cautious manner. An additional consideration is that DN coatings (e.g. PEG, the protein corona) may undermine compatibility and functionality of switchable and dynamic DNs [34].

Importantly, studies that analyze how a protein corona would affect biological and therapeutic properties of DNs are rather rare [75]. However, it is critical to assess not only the stability of DNs in physiological fluids, but rather how those fluids may modify the surface, and impact or hamper the desired function of DNs. We recently showed that

the protein corona greatly affects the intracellular function of specifically designed DNs [76]. In absence of serum proteins, DNs coated with aurein 1.2 (a peptide that facilitates endosome escape [77]) showed marked endolysosomal escape in different cell lines (Fig. 3B) [76]. However, upon exposure to serum-containing medium, a protein shell formed around the DNs, significantly hampering the efficiency of endolysosomal escape and leading to accumulation of DNs in lysosomal compartments (which is the usual fate for unmodified nanostructures) [76]. Therefore, protein corona formation over DN particles should be taken into account for successful and clinically relevant design and optimization of DNs.

3. Physical background on the interaction of DNA nanostructure ligands with cell surface proteins

In a light of above discussed, it is crucial to analyze how functionalized DNs physically interact with the surface receptors of cells. Generally, ligand interactions with cell surface proteins predispose subsequent cell entry of exogenous materials and regulate to a large extent the intracellular fate of various materials [78-81]. Interestingly, DNA molecules alone do not cross the plasma membrane of the cell. However, 3D DNA nanostructures are able to efficiently enter the cellular cytosol [82]. Therefore, a study of the physical parameters that modulate cellular interaction and processing of DN represents an important milestone for efficient targeting of cell surface receptors.

Indeed, current progress in understanding nanoparticle-cell interactions revealed several possibilities for the modulation of targeting efficacy and cellular uptake [83]. Those possibilities comprise the orientation, mobility, and surface density of ligands on the nanoparticle [84-88]. Furthermore, accumulating evidence revealed that particle geometry parameters, e.g. size, shape, and aspect ratio, affects largely their uptake and to a larger extend therapeutic efficacy [89-91]. For example, particles having a rod-like geometry showed higher cellular binding efficacy in comparison with spherically-shaped particles [92]. By contrast, spherically-shaped particles showed a higher uptake efficiency compared with rod-shaped ones [93].

However, we have to state that despite this progress, it is still not fully understood how DNs influence the interaction between ligands functionalizing the DN surface and cell surface receptors. Indeed, it was shown that DN functionalization with a protein ligand does not reduce the protein’s ability to bind its receptor [94,95]. Another recent study identified that the affinity of anti-programmed cell death protein 1 antibody (aPD1) incorporated onto DN remains unchanged compared with the free antibody [96]. Interestingly, this study further revealed that the absolute number of bound DNs was significantly lower in comparison with the free antibody, which in turn resulted in lower binding efficiency [96]. In fact, the cell surface composition plays the role of a natural barrier, resulting in limited receptor accessibility for functionalized DNs [96]. As a result, DN orientation and size represent crucial parameters for effective binding to the receptors [96]. In other words, the efficacy of cellular targeting by functionalized DNs is predisposed by an interplay of receptor affinity and accessibility of receptors [96]. Such knowledge is critical in designing

programmable DNs for improved applications of nanomedicines towards targeted cell signaling modulation.

DNs offer programmable precision for decorating their surfaces with biomolecule nanopatterns, enabling precise spatial separation between ligands on the nanoscale [36,39,51,96-98]. Furthermore, DNs have been decorated with varying nanopatterns of biomolecules, e.g. ephrin-A5 [94,99], immunogen eOD-GT8 [100], caspase-9 variant [101], antigens of human IgGs and IgMs [102], and Fas ligands [103]. Of note, regulation of the spatial organization of surface receptors at the nanoscale provides a route for controlling cellular responses [104]. A recent study revealed the use of DNs for regulated death receptor 5 (DR5) clustering and subsequent triggering of apoptosis [105]. Furthermore, the study revealed that the required inter-ligand distance for initiation of apoptotic events was less than 10 nm [105]. Interestingly, this approach of DN-mediated clustering of DR5 was effective even against resistant breast cancer cells [105].

Overall, nanometer precision in patterning of various DNs with specific surface ligands offers a significant boost to the potential of DN-based nanomedicines. We see in this technology an opportunity to study also fundamental cell biological questions of receptor function.

4. Analysis of DNA nanostructure cytotoxicity

To bolster the biomedical applicability of DNs, researchers commonly stress that DNA is a natural biological molecule [24,34,61,106], and is therefore readily biodegradable and biocompatible, with minimal toxicity [24,34,61,106]. Therefore, DNs made of DNA molecules are generally assumed to be biocompatible as well as nontoxic [24,34,61,106]. However, this is the so-called “naturalistic fallacy” [107,108]: The “natural” origin does not directly correspond to “safe” or “biocompatible” [107-109], as plenty of “natural” molecules are toxic or immunogenic [107-109]. Specifically, cell-free DNA is known to be present in blood plasma of healthy individuals [110], yet high levels of circulating cell-free DNA are also associated with multiple pathologies, including systemic lupus erythematosus, metastatic cancers, atherosclerosis, primary Sjögren’s syndrome, and rheumatoid arthritis [110-113]. Elevated levels of donor-derived cell-free DNA during transplantation may lead to adverse post transplantation events such as allograft rejection [113-115]. Furthermore, it has been proposed that cell-free DNA may possess cytotoxic properties [110,113,116]. DNA can be released during cellular injury as damage-associated molecular patterns (DAMPs) [117,118]. Injury-issued DAMPs, including extracellular DNA, can result in the activation of innate immunity [117]. For example, circulating cell-free mitochondrial DNA was shown to induce inflammasome-dependent caspase-1 activation and IL-1β and IL-18 release [119]. Therefore, careful assessment of the toxicological and immunogenic potential of DNs is imperative for successful clinical translation of DN-based technologies.

It is worth noting that preliminary studies indicate some DN biocompatibility and potentially favorable clearance kinetics [120]. In light of the aforementioned DNA-related adverse cellular effects, it is important to systematically analyze the available literature regarding DN toxicity, and to our knowledge there is no systematic analysis of their toxic

potential [24,27,34,61,75,106]. Thus, we briefly summarize available accounts of *in vitro* toxicological responses to the DN architectures most studied thus far (Table 1).

Overall, from Table 1 it is clearly seen that in the majority of studies, DNs that have not been loaded with drugs show low to no cytotoxicity. However, the maximum exposure time used in majority of studies is only 48 h (Table 1). Indeed, designed nuclease-resistant DNs may withstand harsh biological environment for more than 48 h [61]. Thus, longer-term cytotoxic effects have yet to be fully elucidated. Of note, the U.S. Environmental Protection Agency (EPA) explicitly mentions that biodegradability does not guarantee low toxicity of a compound [121]. A number of compounds that showed rapid biodegradability were found to be carcinogenic, mutagenic, or toxic [121-123]. During degradation, decomposition products and/or adducts of initial compounds might be highly reactive and possess significant toxicity [121-123]. A classic example is drug-induced liver injury (DILI) triggered by products of acetaminophen metabolization [124,125]. Indeed, drug-protein adducts, occurring drug metabolism in hepatocytes, may act as neoantigens, triggering an immune response and resulting in cell injury [124,125]. Idiosyncratic (unpredictable) DILI pathology does not require high doses of the drug, and can be profound with relatively low but chronic doses of >50–100 mg per day [124,126]. Specifically, both short oligonucleotides and long DNA pieces show undesired toxic and immunogenic responses [127,128], and it is well known that dsDNA induces a number of autoimmune pathologies [129-131]. An increase in serum DNA concentration is a straightforward marker of systemic inflammatory reaction and sepsis [132,133]. Therefore, it is important to consider not only toxicity of entire DNs but as well, their degradation products and/or adducts forming upon metabolization by cells, and to probe potential side effects of these materials for extended exposure and circulation times.

Extrapolating experience from other nanomaterial studies, some particles may be retained in the human body for weeks before excretion [21,134]. In fact, emerging evidence suggests that many different nanomaterials may possess time-delayed toxicity [135-140], so it is important to carefully and systematically analyze such long-term toxicity.

Another challenge apparent from the analysis in Table 1 is that the toxicological assessments for the majority of studies have been primarily been carried out in only a handful number of standard cell lines. For example, MCF-7 cells are frequently used as a model breast cancer cell line, and HeLa cells are abundantly utilized as a general “cancer” cell model. However, a thorough investigation of different MCF-7 cell line strains revealed substantial genetic heterogeneity among them [141]. When those strains were challenged with 321 anti-cancer compounds, they showed dramatic variability in response. Strikingly, 75% of compounds that induced marked toxicity in some strains were completely ineffective in others tested [141]. Another thorough study demonstrated that different strains of HeLa cells possess great genetic and phenotypic variability, e.g. variations were found in genome-wide copy numbers, mRNAs, proteins, and protein turnover rates [142]. Those studies highlighted an important question regarding the reproducibility of research conducted using MCF-7 and HeLa cells. It is worth noting that cell line authentication is crucial for conducting reproducible and reliable research [143]. Avoiding this authentication can easily lead to unreliable outcomes, resulting in the loss of time, money, and trustworthy publication data [143]. It has been reported that over 20% of cell lines are misidentified or mislabeled, often

due to cross-contamination [144]; indeed, HeLa cells are the major contributors to such instances of cross-contaminations [144,145].

Another key challenge in the assessment of DNs toxicology is the scarcity of studies implementing primary cell cultures (Table 1). Although cell lines are very powerful for initial screening, they do not fully recapitulate tissue-specific functions and have limited predictive value towards *in vivo* applications [146,147]. In this regard, primary cell cultures could mitigate these problems and provide results more closely related to *in vivo* conditions [146-148]. Even cell lines phenotypically related to primary cells can possess substantial gene expression differences and be functionally distinct by comparison [149-151]. Additionally, highly proliferating tumor-derived cell lines such as HeLa cells tend to redistribute the nanomaterial among daughter cells, resulting in a lower particle load per cell and thus overlooked toxic effects [21,152,153]. By contrast, primary cell cultures with limited proliferative activity may provide more reliable results on nanomaterial toxicity [21,152,153]. Therefore, there is an unmet need to boost research on DN toxic effects utilizing primary cell culture models.

5. Analysis of DNA nanostructures interactions with cells

Aside from toxicological assessments of DNs, a deep analysis of DN-cell interactions and identification of target proteins and/or pathways mediating the cellular effects of DNs is necessary for successful translation of DN technology into any biomedical application. To help meet this end, we summarize the currently most studied DN-induced cellular effects and interactions (Table 2).

It is evident from Table 2 that a deep analysis of signaling pathways involved in cell-DN interactions is underrepresented in the current literature. Current research has been primarily focused on revealing DN uptake and subcellular localization with minimal attention towards functional changes that DNs may elicit in cells (Table 2). This is likely because research efforts towards biomedical applications for DNs are still relatively new [27,34,61]. Despite this, there has already been substantial progress in understanding how the size and shape of DNs affect cellular uptake and subcellular distribution (Table 2 and Fig. 4). However, it has yet to be seen whether signaling is biased in cells upon DN treatment. We may take lessons from NP studies in which it has been suggested that NPs trigger substantial cellular responses that bias lysosomal function without triggering a cytotoxic response [21,154]. Additionally, current studies on DN-cell interactions suffer from same problems described in the previous section, i.e. lack of primary culture use in research, usage of spurious cell lines, and a lack of studies on their long-term effects.

We would like to stress that the current developments in the field of DNA nanotechnology are considerable, intriguing, and provide great perspective. Specifically, in biomedically driven studies, DNs have shown promising results in biosensing, drug delivery, cell modulation, and bioimaging [24,27,34,61]. For instance, DN-based biosensors proved advantageous in precise design, specificity, and low-cost synthesis [155,156]. DNs can be designed and functionalized to bear various drug cargos, which opens a route for improved drug delivery applications [24,27,34,61]. Cell behavior and activity can

also be altered in a controlled manner using smartly designed DNs [157]. DNs are indispensable for super-resolution DNA-PAINT (DNA-based point accumulation for imaging in nanoscale topography) imaging applications [158]. However, for advanced and successful implementation of these various functional DNs, an understanding the detailed mechanisms of DN-cell interactions and their consequences is vital. Knowledge of the long-term effects, signaling mechanisms, immunogenicity, and excretion of DNs (Table 2) has yet to be fully elucidated.

It is worth noting here, that in addition to nanoparticles, DNA nanotechnology has been applied to tunable hydrogel systems [159,160]. Such systems represent 3-D hydrophilic networks featuring DNA as a part of the system [159-161]. DNA hydrogels are scalable from bulk hydrogels to nanogels [159,160,162]. As DNA hydrogels contain programmable and complementary DNA strands as part of the network, this feature allows resultant hydrogels to be easily manipulated to create different DNA building block with precise geometries, leading to a predictable and controlled resultant DNA networks [159,160,162,163]. Due to the structural programmability of DNA hydrogels, these systems allow to exert various interaction with cells in controlled manner [159,160,162,164]. For example, immunostimulatory CpG DNA hydrogels may be potent in enhancing the antigen-specific antitumor immunity [165]. Additionally, DNA-based hydrogels allow for control of interactions between cells and the extracellular matrix interactions with nanoscale precision [166]. This possibility makes DNA hydrogels a promising platform for programmed tissue engineering [159,160,162]. However, there are still considerable challenges needed to be addressed in the development of DNA-based hydrogels, e.g. cost-effective upscaling, potential for degradation by secreted nucleases, and possible toxic or immunogenic effects [159,160,162].

In fact, DNA assembly into complex customized 3D structures with desired functions has seen great advancements in recent years [24,27,34,61]. It is now possible to produce more stable DNs at a faster rate, while precisely varying the size and shape with higher production yields [24,27,34,61]. However, for successful clinical adaptation of DNA nanotechnology we need to overcome several challenges arising from a biological point of view. We describe and discuss those challenges in the following section.

6. Challenges and future perspectives

DNs as biomolecule-based nanoparticles possess advantages over standard nanomaterials in terms of controllable size, shape, and surface functionality [2,24,27,34,61]. Undoubtedly, those advantages will enable even more therapeutic and diagnostic use of these nanostructures, but as DNs become increasingly complex, more efforts must be taken to overcome hurdles to clinical translation. In this regard, thorough studies of DN-cell interactions are of paramount importance. Below, we summarize current studies of DNs in biological contexts, then identify challenges and pitfalls in their biomedical implementation in order to provide a roadmap for overcoming them.

The first major challenge is the stability of DNs under physiological conditions. Substantial progress in this direction has been already achieved [2,24,27,34,61], but often these

approaches are reported as stand-alone studies. It will be critical to integrate these stabilizing approaches with specific applications of nanoparticles for biomedical applications, in conjunction with primary cells or *in vivo* models. For example, most of the coatings reported were not tested for how they may change bare DN immunogenicity, biodistribution, or pharmacokinetics; probing these factors will aid in more sophisticated, real-world applications of functionalized DNs.

Drug delivery, specifically of cancer therapeutics, is certainly the most investigated and notable DN application [2,24,27,34,61]. Thus, DN-cancer cell interactions must be more thoroughly interrogated to reach the greatest potential of anticancer DN use. For example, both toxicological assessments and analyses of DN interactions with cells are still fragmented and unstandardized (Tables 1 and 2). There are very few studies employing primary cell cultures to investigate these two parameters, and a substantial number of studies still use potentially problematic cell lines, like HeLa and MCF-7 (Tables 1 and 2), that could affect the reproducibility of their results. We also draw attention on the importance of cell line authentication [143]. Apart from these concerns, we propose that a thorough justification of the choice of biological model should be provided, especially when DN research is directed toward therapeutic use. Guidelines for selecting and justifying cancer models already exist [167-169], since human tumor cell lines that are routinely used may possess considerable differences in comparison with primary tumors [168,170]. Misidentification, contamination with mycoplasma, genetic drift, and phenotypic instability in frequently used cell lines are often neglected by many researchers [146]. In order to reliably compare results of DN function within cells, guidelines of cell line selection, authentication, and maintenance must be applied in future studies [146].

Recently, it has been noted that there is substantial variability in the field regarding DN characterization techniques and experimental design [2,24,27,34,61]. Thus, we propose that the biomedical research of DNs adopt a “minimal reporting standard” derived from an already existing one from the field of bionanotechnology [171]. This minimal reporting standard combines guidelines for nanomaterial characterization, biological model justification, and standardized experimental protocols [171]. Implementation of such standards will improve reproducibility and significantly boost quantitative comparisons of results on DN-cell interactions.

Another opportunity to boost biomedical research of DNs lies in the implementation of more sophisticated *in vivo* models than conventional rodent models. Current research shows that animal model systems currently abundantly used in the biomedical field poorly recapitulate human counterparts, often leading to unreliable results [172-174]. Some researchers suggest that “more complex human conditions” should be used in biomedical research in place of rodent models [172]. For this reason, the U.S. Environmental Protection Agency has plans to dramatically reduce or even eliminate the use of animal models for testing research by 2035 [175]. Organoids—complex multicellular systems that recapitulate *in vivo* structure and functions of the selected tissue—may help to overcome those challenges and provide systems that are more relevant for use in humans [176-178]. Organoids have already been implemented in biomedical nanoparticle research, showing reliable and progressive results [179,180]. The use of organoids in DN research is still quite fragmented [2,24,27,34,61],

so using them for screening toxicity and performing studies on the interactions of DNs with cells would provide important foundation for future clinical translation of DNA nanotechnologies.

A final challenge that must be addressed prior to DN use in the clinic is that of liver sequestration. Cumulative evidence suggests that the liver sequesters up to 99% of intravenously injected nanoparticles [21,23,181,182]. Long-term accumulation in the liver results in adverse effects and greatly limits the clinical efficacy of nanoparticles [21,23,181,182]. Generally, drug-induced liver injury (DILI) is described as a harmful and unexpected impact of drugs on the liver [124]. In fact, DILI represents a serious problem, being one of major causes of acute liver failure in Western countries [124,125]. Moreover, many nanoparticles have been shown to possess hepatotoxicity and display DILI properties that were initially overlooked [21,183-186]. Indeed, studies carefully addressing the hepatotoxic potential of DNs remain unaddressed in current literature. Examining the hepatotoxic properties of DNs will help optimize the design and synthesis of clinically suitable DNs to avoid off-target effects of DNs.

In summary, we would like to emphasize that DNs possess great biomedical potential. We expect to see more diverse applications of DNs capable of translating towards clinical use, but understanding the hurdles and limitations of therapeutic DNA nanotechnology is crucial for its clinical success. Consequently, the critical analysis provided herein will help researchers to establish a roadmap for overcoming these challenges.

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Data availability

All data needed to support the conclusions are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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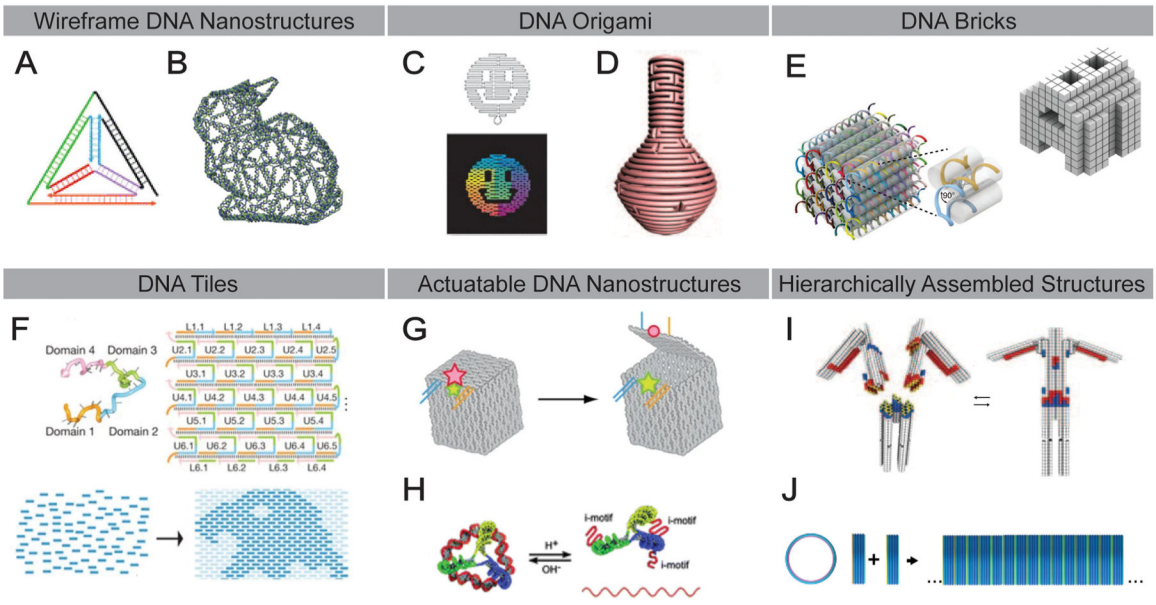

Fig. 1.

Key examples of various DNA nanostructure designs. (A) DNA tetrahedron [35]. (B) Three-dimensional wireframe rabbit-shaped DNA structure designed from a polygonal mesh architecture [36]. (C) Two-dimensional DNA origami in the shape of a smiley face [38]. (D) Three-dimensional DNA origami vase structure featuring complex curvature [39]. (E) Modular DNA structures composed of 32-nucleotide “brick” motifs [41]. (F) Single-stranded DNA “tiles” acts as pixels in a two-dimensional array [187]. (G) A DNA box designed to be opened via toehold strand displacement to release a cargo of interest [44]. (H) pH-sensitive DNA i-motifs allow the assembly and disassembly of a DNA tetrahedral structure [45]. (I) Heteromultimeric assembly of complex DNA architectures via shape complementarity [46]. J) Homomultimeric assembly of DNA barrel structures into a hollow DNA tube via sticky end adhesion [47].

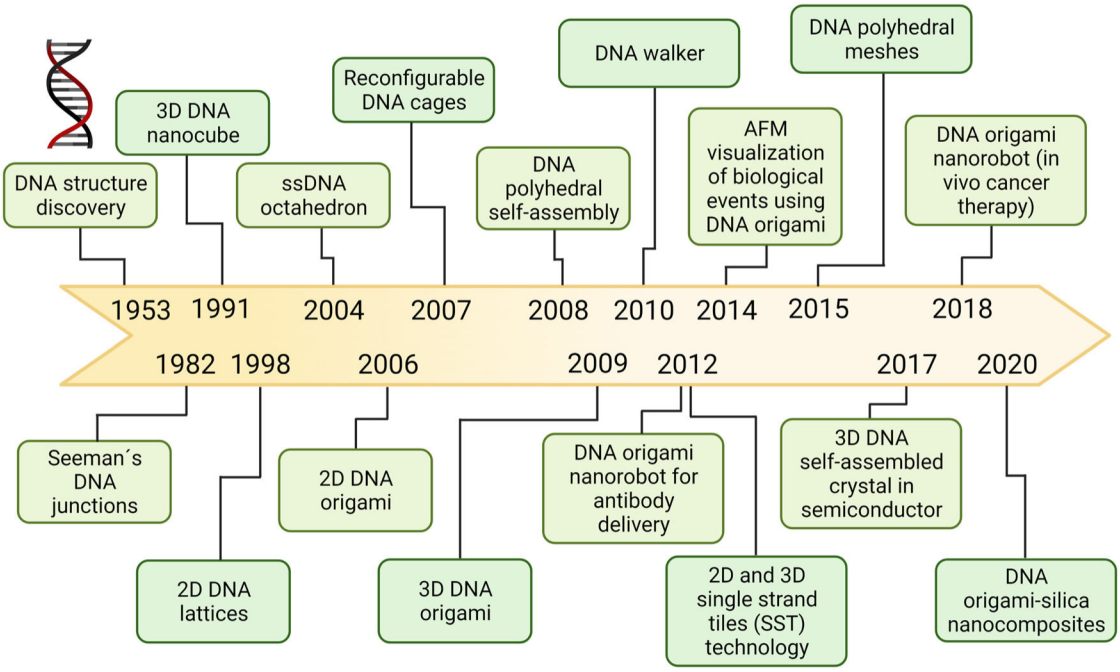

Fig. 2.

Historical timeline of the advancements in DNA nanotechnology research [2,24,27,34,61].

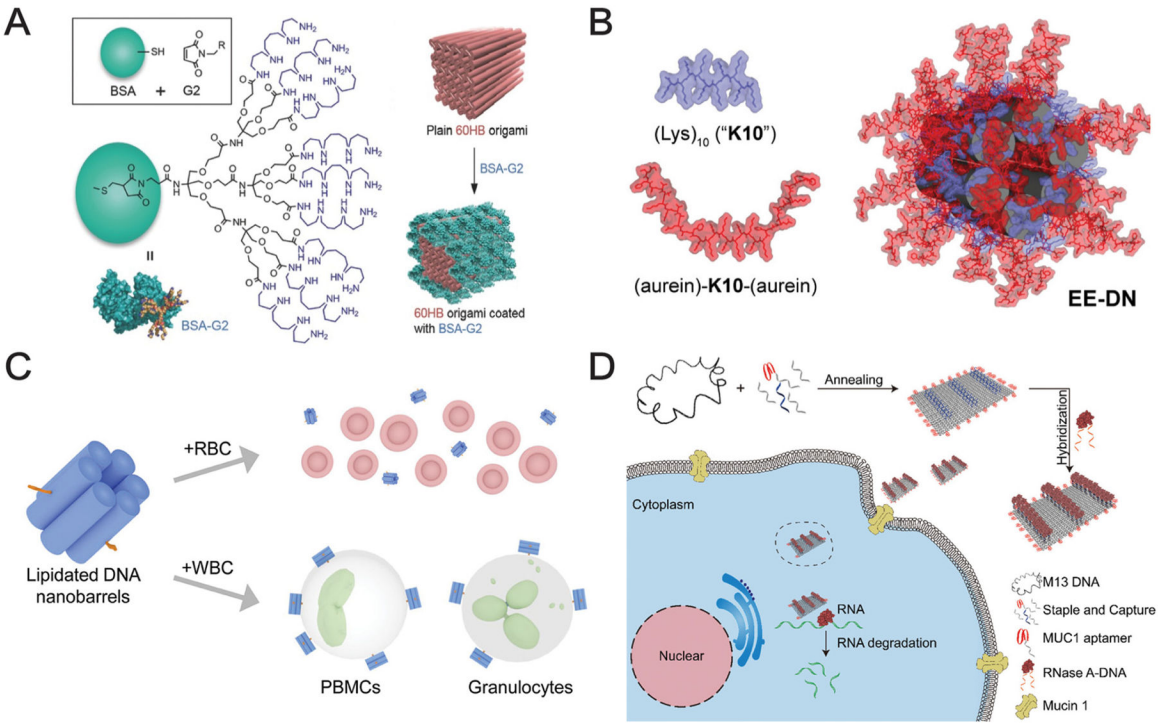

Fig. 3.

DNA nanostructures for biological applications. (A) BSA modified with positively charged dendrimers to adhere to a 60-helix bundle (60HB) nanostructure enables enhanced nanostructure stability, uptake, and immunoquiescence [68]. (B) Oligolysine-based peptide coating featuring two functional aurein 1.2 sequences that exhibits endosomal escape of the coated DNA nanostructure (EE-DN) in the absence of serum proteins [76]. (C) Cholesterol-bearing 6-helix bundle DNA nanostructures facilitate targeted uptake in white blood cells compared to red blood cells [188]. (D) A DNA origami sheet bearing MUC1-targeted aptamers capable of targeted intracellular delivery of active RNase A [189].

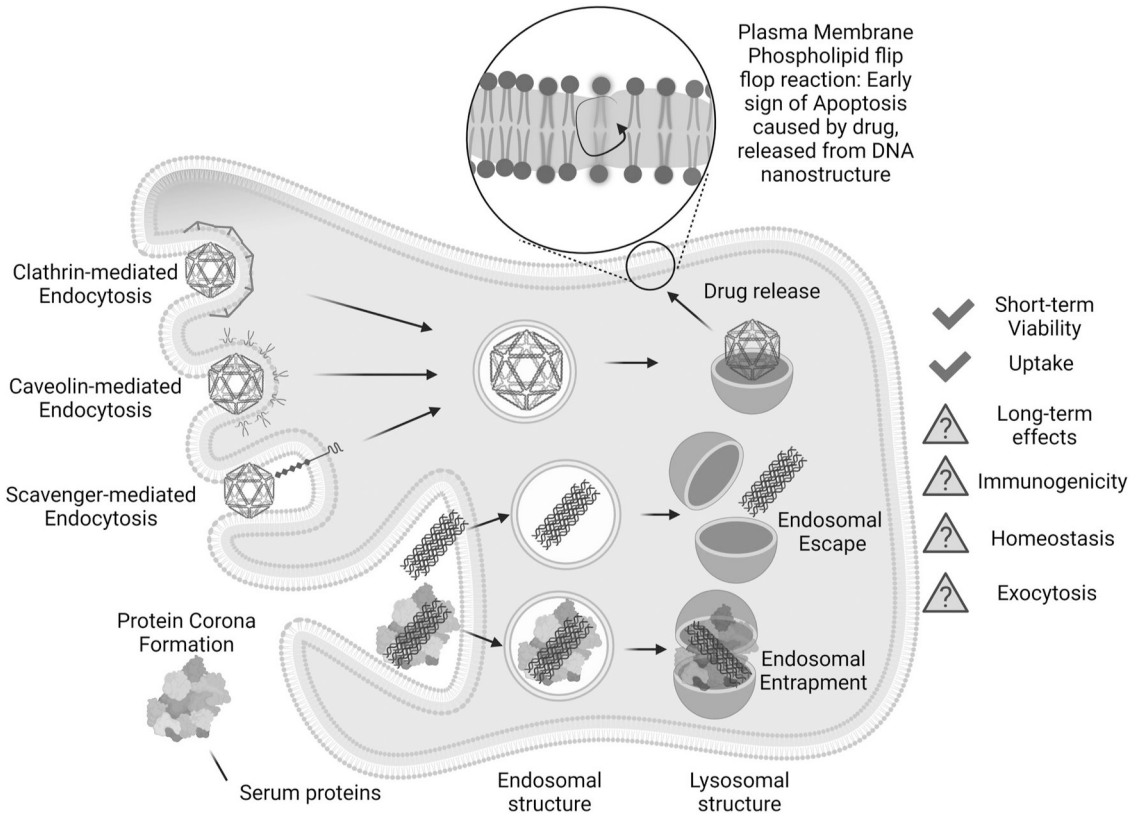

Fig. 4.

Schematic brief summary of DNA nanostructures interaction with living cells.

Table 1

A brief summary of *in vitro* toxicity assessments of different DNs.

DN type	Specifications	Cell model	Exposure time	Outcome	Ref.
Deoxyribonucleic acid-nanothread (DNA-NT)	Diameter: 50–150 nm; Length: 300–600 nm; CPT-DNA-NT Attachment of cisplatin	HeLa	48 h	CPT-DNA-NT reduced cell viability; Signs of apoptosis; DNA-NT No effect on cell viability	[190]
DNA nanobarrels (NB)	Six DNA duplexes forming a six-helical bundle (9 × 5 × 5 nm) NB-3C, NB-1C, and NB-0C containing 3, 1, or 0 cholesterol anchors	Red blood cells (RBC); white blood cells (WBC); granulocytes; peripheral blood mononuclear cells (PBMC)	6 h	No effect on viability	[188] Fig. 3C
DNA nanoscaffolds, DNA tetrahedron (Td) rectangle DNA origami	Incorporation of 5-fluoro-2′ - deoxyuridine; (FdUn) oligomers; Attachment of cholesterol	HTB-38; HCC2998	24 or 48 h	Reduced proliferation of the HTB-38 cells; Signs of apoptosis higher in HTB-38 cells	[191]
DNA duplexes	Attachment of cholesterol; Attachment of alkyl-phosphorothioate (PPT) belt	HeLa; MyrPalm-EGFP HeLa	5 min	No data on viability	[192]
Nanotoroids	3 types of nanotoroids of different size $\rho = 6$, $\rho = 2$, and $\rho = 1.5$ [1]	SMMC-7721; HeLa	6 h	Slight decrease of cellular viability with higher concentration of nanotoroids	[193]
DNA nanopores	NP- EP pore- 6-helix bundle with hydrophobic belt containing ethyl phosphorothioate (EP) groups; 3 negative controls of nanobarels without EP-belt formation	HeLa	1 and 24 h	Decreased viability of cells after incubation with NP-EP	[194]
DNA nanopore	Six DNA duplexes modified with phosphorothioate (PPT) group	MCF-7	48 h	No significant effect on cellular viability; Decrease viability after incubation DNA nanopores with doxorubicin	[195]
Rectangular DNA origami nanosheets	Binding of RNase A to DNs; With/without decoration with protein MUC1	MCF-7	48 h	Colocalization with lysosomes after 1 h; No effect of bare DNs on cell viability; Increased cell death post incubation with MUC1-modified RNase A loaded DNA origami	[189] Fig. 3D
DNA origami nanobox (DON)	Cuboid structure; 36 × 36 × 42 nm; Attachment of AS1411 aptamers and incorporation of doxorubicin (DOX)	HeLa; MCF-7	2 h	Decrease viability after incubation DON with doxorubicin; Signs of apoptosis	[196]

[1] The aspect ratio of the nanotoroid is derived as $\rho = R/r$, where R - the radius of the nanotoroid and r - the radius of the nanotoroid cavity. A larger ρ value defines a smaller cavity [193].

Table 2

A brief summary of DNA nanostructures-induced cellular effects and interactions.

DN type	Specifications	Cell model	Incubation conditions	Major results	Ref.
Tetrahedral DNA nanostructure (TDN)	Four 55-base ssDNA strands; each vertex of TDN labeled with cyanine-3 (Cy3)	HeLa; COS-7	Up to 12 h for uptake; Incubation at 4 °C and 37 °C for 6 h	Time-dependent uptake; Caveolin-dependent endocytosis; Microtubule-dependent transport; Lysosomal internalization after 12 h of incubation	[197]
6-helix bundle (6HB) nanostructure	7 × 6 nm; attachment of (Lys)10 peptide (K10) and aurein 1.2	HepG2; Alexander; Huh7	Up to 24 h at 37 °C in complete medium for uptake and cytotoxicity assays; incubation with/ without serum for 6 h 37 °C	Colocalization of DNs with lysosomes; Protein corona formation post incubation in the presence of serum; Reduced endosomal escape of DNs	[76]
Framework nucleic acids (FNAs)	3 different shapes: tetrahedron, triangular prism, and cube labeled with cyanine-5 (Cy5)	HeLa	In FBS-free culture medium at 37 °C for 3 h	Partially clathrin-mediated endocytosis; Scavenger receptor (SR)-mediated endocytosis; Cellular uptake dependent on DN geometry	[198]
DNA origami nanostructures (DONs)	2 different shapes: tetrahedron and rod Cy5 labeled with distinct size: small tetrahedron (ST) 4 × 2 × 11 per edge; small rod (SR) 4 × 4 × 32; large tripod tetrahedron (LT) 7.2 × 12 × 47 per arm; large rod (LR) 8 × 8 × 127	H1299; DMS53	Up to 8 h at 37 °C for uptake analysis	Cellular uptake dependent on DN shape and size; larger and rod-shaped structures have higher efficiency of uptake; scavenger receptor-mediated uptake; endolysosomal accumulation of LR after 24 h	[199]
DNA origami nanoparticles (DONs)	11 distinct DNA-origami shapes Cy5-labeled; range of the size: 50–400 nm	HUVEC; HEK293; BMDCs	At 37 °C for 12 h for uptake analysis	Higher uptake of larger DNs with better compactness	[200]
DNA-based nanostructure ChloropHore	61-base pair DNA duplex Cl^- reporter domain-Clensor and a pH reporter domain (I-switch)	Primary human dermal fibroblasts	Up to 23 h for stability analysis	Scavenger receptor-mediated endocytic pathway; use for evaluation of pH and Cl^- in lysosomes	[201]
DNA nanobundles (NB)	6-duplex nanobundle Hight: 9 nm Width: 6 nm; NB-3C, NB-1C, and NB-0C containing 3,1 or 0 cholesterol anchors	HeLa	For 2 h/24 h in OptiMEM or DMEM+ 10% FCS	Higher cellular uptake of NB-3C, nanobundles containing 3 cholesterol anchors; colocalization of DN with endolysosomal compartments after 24 h	[202]
Tube DNA nanostructures	YOYO-1 labelled tube DNs	NIH 3T3	For 4 h/24 h	Colocalization of DNs with lysosomes	[203]