Interfacing DNA nanodevices with biology: challenges, solutions and perspectives

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Abstract
The cellular machinery performs millions of complex reactions with extreme precision at nanoscale. From studying these reactions, scientists have become inspired to build artificial nanosized molecular devices with programmed functions. One of the fundamental tools in designing and creating these nanodevices is molecular self-assembly. In nature, deoxyribonucleic acid (DNA) is inarguably one of the most remarkable self-assembling molecules. Governed by the Watson–Crick base-pairing rules, DNA assembles with a structural reliability and predictability based on sequence composition unlike any other complex biological polymer. This consistency has enabled rational design of hundreds of two- and three-dimensional shapes with a molecular precision and homogeneity not preceded by any other known technology at the nanometer scale.

During the last two decades, DNA nanotechnology has undergone a rapid evolution pioneered by the work of Nadrian Seeman (Kallenbach et al 1983 Nature 205 829–31). Especially the introduction of the versatile DNA Origami technique by Rothemund (2006 Nature 440 297–302) led to an efflorescence of new DNA-based self-assembled nanostructures (Andersen et al 2009 Nature 459 73–6, Douglas et al 2009 Nature 459 414–8, Dietz et al 2009 Science 325 725–30, Han et al 2011 Science 332 342–6, Iinuma et al 2014 Science 344 65–9), and variations of this technique have contributed to an increasing repertoire of DNA nanostructures (Wei et al 2012 Nature 485 623–6, Ke et al 2012 Science 338 1177–83, Benson et al 2015 Nature 523 441–4, Zhang et al 2015 Nat. Nanotechnol. 10 779–84, Scheible et al 2015 Small 11 5200–5). These advances have naturally triggered the question: What can these DNA nanostructures be used for?

One of the leading proposals of use for DNA nanotechnology has been in biology and biomedicine acting as a molecular ‘nanorobot’ or smart drug interacting with the cellular machinery. In this review, we will explore and examine the perspective of DNA nanotechnology for such use. We summarize which requirements DNA nanostructures must fulfi! function in cellular environments and inside living organisms. In addition, we highlight recent advances in interfacing DNA nanostructures with biology.

For a molecular device to act in vivo it should preferably (i) constitute a well-defined structural entity, (ii) be biocompatible, (iii) target the relevant bodily compartment or cells, and (iv) execute a desired function (figure 1). The motivation for each requirement will be addressed in further detail in the following sections.

1. A structural scaffold

A well-formed homogenous structure is essential for any device to ensure reproducibility and rational control. Several classes of in vivo nanoparticle systems exist today [1, 2], e.g., lipid-based, polymer-based, protein-based and inorganic nanoparticles. However, these nanoparticle systems are often characterized by being poorly structural defi ned, heterogeneous in size and with low spatial addressability.
In contrast, DNA nanostructures hold tremendous structural powers. Rothemund [3] demonstrated these capabilities by forming a diverse set of DNA nanostructures, each homogenous in population and with fully addressable surfaces. Especially the latter property is a key attribute of DNA structures' success as it enables decoration of assemblies with any desired functionality in complex patterns. In this way, DNA nanostructures can serve as a molecular peg-board, where the engineering scientist freely can add new properties to his device.

Today a wide array of pre-designed structures ranging from complete solid rigid structures [4], twisted [5], hollow boxed [6, 7] to curved spherical structures [8] (figure 2) have been reported. Especially structures with inner cavities could potentially serve as carriers of therapeutic payload, keeping the cargo protected from the external environment throughout its journey.

2. Biocompatibility

For any artificial molecular device aimed for in vivo applications, a primary requirement is stability and biocompatibility. The nucleic acid structures must remain structurally intact during the functional relevant time period when exposed to bodily fluids. At the same time the devices should be sufficiently biocompatible such that they can be excreted from, or naturally degraded in, the human body to avoid toxicity. Furthermore, the devices must be able to evade immune-recognition to avoid rapid clearance and reduce the risk of acute anaphylaxis shock.

DNA is a naturally occurring molecule in any known organism, and thus, in it's nature, highly biocompatible. However, inside the eukaryotic cell DNA is spatially confined to the nucleus and smaller organelles. Occurrence of DNA outside these compartments is often recognized as a danger signal by the vertebrate innate immune system triggering its activation through nucleic acid immune receptors such as TLR9. Another challenge for the stability of DNA-based devices in vivo, resides in the ion concentrations of both intra- and extracellular body fluids being well below the relative high cationic concentration required for keeping many DNA assemblies intact.

Hahn et al [10] addressed this latter issue in 2014. The researchers found that DNA nanostructures often denatured owing to low cation concentration in cell media. In addition, the presence of nucleases in serum led to degradation and digestion of structures due to nuclease activity. Recently, Benson et al [11] published a set a new scaffolded DNA nanostructures built on polygonal meshes (figure 2(c)). These structures, in contrast to the ones tested by Hahn et al [10], consisted of a more open conformation with only one helix per edge and proved stable at lower ionic conditions relevant for biological assays. However, the more open structure could also pose disadvantages, especially in regard to nuclease vulnerability and exposed interior leaving potential cargo unprotected.

The cellular stability of DNA origami has also been investigated by Mei et al [12]. Rather surprisingly, and in contrast to the above mentioned studies, the researchers found that both two- and three-dimensional DNA
Origami were relatively stable in cellular lysates when incubated at temperatures up to 25 °C. However, the study was conducted in the presence of various nuclease inactivating components including SDS, deoxycholic acid, and trypsin, thus lowering the relevance to physiological conditions. Furthermore, the stability of the structures was only assayed at temperatures well below the biological relevant 37 °C.

Castro et al. [13] tested the vulnerability of multilayered DNA origami structures to various nucleases and, interestingly, found a relatively high structural resistance towards degradation by several of the tested nucleases. Moreover, for nucleases that succeeded in degrading structures, kinetic studies showed a ten-times slower degradation of origami structures than a double-stranded DNA (dsDNA) plasmid of similar size.

In 2014, Steven Perrault and William Shih [14] presented an interesting approach for improvement of the stability and pharmacokinetic behaviour of DNA nanostructures. Inspired by viral particles, the researchers decorated their DNA nanostructure with lipophilic tails to direct the assembly of an adjacent PEGylated lipid bilayer around their structure (figure 3(a)). The encapsulation was shown to confer protection against DNase I digestion, reduce stimulatory cytokine production and evade uptake by splenocytes. In addition, the researchers examined the in vivo pharmacokinetic properties of the formulation in muridae animals comparing three DNA species: a non-encapsulated origami structure, the encapsulated origami, and a naked oligo. Interestingly, the circulation half-life of encapsulated structure was found to increase nine times compared to the non-encapsulated origami and with the naked oligo being rapidly cleared by renal secretion.

Recently, Cassinelli et al. [15] attempted to ‘chain-armour’ their DNA structures in order to resist cation depletion, nuclease attacks and adverse pH conditions. Utilizing click chemistry, the researchers catenated a six-helix bundled DNA tube through covalently linking single-stranded DNA tiles. The procedure conferred increased resistance to DNase digestion and complete structural stability of the assemblies in pure water.

While addressing many of the individual challenges, the field of DNA nanotechnology, however, still lacks an easy, scalable, and efficient method that ensures structural stability and desirable pharmacokinetic properties of DNA-based nanostructures.

3. Targeting

To alleviate side effects and increase the concentration of nucleic acid nanostructures at the relevant tissue it is desirable to implement a targeting system. Naked DNA nanostructures transfect cells rather poorly [17], thus numerous protocols based on lipid and polymer formulations have been described to facilitate this process. However, many of these reagents may interfere with the function of DNA nanodevices by charge competition and condensation of structures. During the last decade, several reports investigating the addition of targeting ligands to DNA nanostructures to facilitate less disruptive translocation of DNA nanostructures over the cell membrane have surfaced [18–20]. Examples of such cell targeting moieties comprise antibodies, aptamers, or receptor ligands including proteins, peptides, and small molecules. In cancer therapy, for example, common targets include overexpressed receptors related to angiogenesis (e.g. VEGF), uncontrolled cell-proliferation (e.g.
folate), or metabolic demands (e.g. transferrin)\(^1\). Binding to such cellular receptors may cause receptor-mediated endocytosis enabling the release of the drug inside the cell.

In DNA nanotechnology, an interesting folate-siRNA-conjugated DNA tetrahedron\(^16\) was reported by Lee and co-workers in 2012 (figure 3(b)). The group demonstrated targeted delivery accompanied with gene silencing in HeLa cells and an \textit{in vivo} mouse model. Notably, the researchers found that only structures displaying at least three folate molecules exhibited efficient delivery and gene silencing underlining the importance of multivalency and pattern-recognition in cellular uptake. A similar DNA structure coated with DNA aptamers showed specific uptake into cancerous cells and selectively inhibited cell proliferation\(^21\).

Recently, Schaffert \textit{et al}\(^22\) demonstrated efficient uptake of a planar DNA structure decorated with the iron transfer protein transferrin. The researchers were able to increase intracellular uptake by up to 22-fold compared to bare structures and depending on number of transferrin molecules attached.

Another interesting approach for cellular targeting was published by Mikkila \textit{et al}\(^23\). The researchers electrostatically coated their DNA origami structures with a positively charged virus capsid coating protein. Using this approach, the group found a 13-fold improved delivery efficiency into human embryonic kidney cells compared to bare origami. The addition of the cationic viral protein likely neutralized the negative charge of the DNA to some extend, making it more prone to uptake. Similarly, Brglez \textit{et al}\(^24\) showed how increasing the surface hydrophobicity, using acridine-based DNA intercalators, can enhance cellular uptake. Importantly, the addition of these tailor-made intercalators could be added without disturbance of the structural integrity of the DNA nanostructures. The above findings reinforce the prospective of DNA-based nanostructures for therapeutic applications.

### 4. Functional DNA nanostructures

DNA is commonly introduced into cells with the functional purpose of encoding RNA or proteins. However, DNA nanostructures have taken on more diverse roles like for instance dynamic structural switches, facilitating enzymatic activity, inducing therapeutic responses and incorporating computational and sensing capabilities.

#### 4.1. Dynamic DNA devices

Dynamic behaviour often requires an energy input to generate mechanical forces. Many of nature’s molecular motors thus utilize the energy-rich triphosphate bonds in NTPs to drive movement and/or enzymatic reactions. Another example is the duplex formation of DNA driven by a cooperative interplay of hydrogen-bonding, \(\pi\)-stacking, hydrophobic, and electrostatic interactions. These latter energy sources are currently among the most frequently used in artificial DNA devices.

The very first mechanical controllable DNA-based device was introduced by Mao \textit{et al}\(^25\) in 1999. The researchers succeeded in switching structural conformation of their DNA device between B- and Z-DNA conformation depending on ionic-conditions, leading to rotary motion. Recently, changes in ionic-conditions were also used to drive dynamic behavior in larger DNA assemblies\(^26\). Utilizing shape-complementarity and base-stacking between structures, the researchers created highly complex macromolecular devices with fully reversible movements.
Another approach for directed dynamic behavior of DNA-based structures, was made by Yurke et al. [27], who exploited single stranded DNA overhangs, so-called 'toeholds', to initiate and drive structural oscillations by strand displacement using external DNA oligos. Thereby, the researchers could perform reversible tweezer-like movement of a DNA nanodevice fueled by a set of input DNA strands. This utilization of DNA as fuel molecules has since been fundamental in driving multiple dynamic DNA-based machines, both in strand displacement cascades and in enzymatically driven motors [28].

A special subset of dynamic DNA machines, that often utilizes strand displacement to control dynamic behavior, is DNA walkers. A DNA walker is a molecular complex with 'legs' made up from DNA that can move along a pre-designed DNA-based track. The first reported DNA walkers [29, 30] utilized DNA as fuel molecule for their movement using a strand displacement cascade. However, the use of DNA as both track and fuel limits the walker to unidirectional behavior due to destruction of the track. To overcome this problem, various approaches have been used to fabricate fully autonomous walkers built on DNAzymes [31], photolysis [32, 33], ionic [34] or enzymatic approaches [35, 36]. With the introduction of DNA origami, larger devices [37, 38], including the tripped walker from Gu et al [39], began to appear. In addition to its three 'legs', Gu’s structure contained three separate 'hands', which could be programmed to collect cargo at specific locations along its track like a molecular assembly line. Directed movements have also been achieved using the cellular motor proteins, Kinesin and Dynein, which in nature 'walks' along the cellular microtubule filaments [40].

Castro et al recently expanded the landscape of dynamic DNA-based devices [41, 42]. Analogous to macroscopic machines, the group introduced discrete single-stranded DNA joints in their structures allowing for specified rotational and/or translational movement of more rigid dsDNA components. Doing so, the researchers succeeded in constructing several interesting molecular motors resembling common macroscopic tools such as a crank-slider, hinge, and scissor-lift [43].

4.2. Biosensing DNA devices

An important characteristic of any molecular device is the ability to sense and respond to surrounding signals. Several types of nucleic acid based biosensors have been constructed throughout the years with a wide range of sensor capabilities usually leading to structural rearrangements. Many DNA-based devices with various abilities to detect, for instance, ions [44–47], small molecules [48–52], proteins and peptides [53, 54], nucleic acid sequences [55–57], and pH [58–61] have been designed. One of the first and most successful DNA sensors developed is the 'molecular beacon' published by Tyagi and Kramer [62]. Upon hybridization with its target, the device shifts conformation from a fluorescein-quenched hairpin-like structure to a fluorescence linear state. The original design could detect specific nucleic acid sequences e.g. miRNAs, single-nucleotide polymorphism (SNP), products of real-time PCR reactions. Subsequently, the sensing capabilities have been further extended to include detection of temperature [63] and macromolecules including antibodies [64]. In addition, molecular beacons have also been incorporated into larger DNA structures [65].

One of the first examples of a larger DNA device responding to an external signal was the three-dimensional DNA origami box reported by Andersen et al [6]. The top face of the box could be opened in a controlled manner as a response to specific DNA or RNA oligos as 'keys'. In the original design the opening of the top face was non-reversible, but, has since been extended with a reversible locking system [7] and Boolean logic-based gating [66]. Today, several reports have successfully demonstrated the ability to encapsulate molecules inside DNA nanostructures [18, 67] enabling controlled delivery of enzymatic or therapeutic payload.

4.3. Enzymatic DNA devices

The addressability of DNA nanostructures makes them extremely suitable for arranging and displaying molecular patterns and arrays on their surface [68]. One approach has been to arrange enzymes in spatially proximity to speed up multistep enzymatic processes [69–71].

One interesting example is the modular nanoreactor recently published by Linko et al. [72]. The researchers dimerized two who dimerized two tube-like DNA origami structures, enzymatically-functionalized with either three glucose oxidase (GOx) or three horseradish peroxidase (HRP) enzymes in a barrel-like three-dimensional geometry. The barrel-like structure confined any entering substrate in the barrel, thereby increasing the likelihood of interacting with the enzymes in a serial pattern and thus the turnover rate.

Another interesting dynamic DNA structure with the ability to control enzymatic activity was reported by Liu et al [73] in 2013. The researchers fabricated a large tweezer-actuated nanostructure and functionalized both of its arms separately: one with the enzyme glucose 6-phosphate dehydrogenase, and the other with the enzymes, essential cofactor, NAD+. Using DNA-fuel strands, the structure could open and close, bringing the enzyme and cofactor into sufficient proximity to allow for the enzymatic reaction to occur. The team was able to show
reversible inhibition/activation cycling of the enzyme. Similar tweezer-like DNA structures have been utilized for protein capturing using aptamers as fangs, thereby regulating protein activity [74].

4.4. Therapeutic DNA devices
Several notable approaches have been made using DNA nanostructures for therapeutic purposes as well. Among the more widespread approaches is the delivery of chemotherapeutic agents [75–79] such as doxorubicin (dox) to cancer cells utilizing the intercalating properties of the agents to trigger apoptosis. Jiang et al [77] and Zhao et al [76] both demonstrated increased apoptotic effect in vitro on a breast cancer cell line upon delivery of dox-loaded origami carriers. Intriguingly, the study from Yan’s lab [77] showed that origami carriers could help circumvent drug resistance gained by cancer cells. Recently, a similar study, using another DNA intercalator similar to dox, also reported the ability to circumvent drug resistance [79]. In the study by Zhao et al [76], the designed structures accounted for the helical twist induced by intercalation of dox by having 12.0 bp/turn instead of the standard 10.5 bp/turn (figure 4(a)). Using TEM images, the researchers demonstrated how the assembly of these heavily underwounded structures greatly improved in the presence of dox. In addition, loading efficiency and drug release kinetics were shown to be improved in twisted origami structures compared to standard origami and dsDNA plasmid.

As tools for immunotherapy, DNA structures were used to enhance immune expression. Acting as adjuvants, the structures carried several strands with CpG rich motifs on their surface for stimulation of TLR9 both in vitro [56, 57] and in vivo [80]—a topic which have been extensively reviewed elsewhere [81]. Another exciting approach in DNA nanotechnology is the cytotoxic pore-forming lipid coated DNA nanostructures first demonstrated in cell culture by the group of Friedrich Simmel [82] and later Burns et al [83]. One could easily imagine coupling these pore-forming structures with a logic based gate, dynamically controlling the exposing of the cytotoxic-machinery only when applicant.

One of the most impressive therapeutic DNA nanodevices is the logic-gated DNA nanorobot (figure 4(b)) constructed by Douglas et al [84]. Instead of relying on delivery of a therapeutic cargo to the interior of targeted cells, the researchers induced cell-signaling pathways through binding of exterior receptors yielding a desired therapeutic response. Douglas’ origami was of a hexagonal barrel-shaped design. On the interior, protruding DNA handles functioned as attachment sites for various fluorescently labeled antibody fragments (Fab’). The structure was kept closed through an aptamer-based lock system forming duplexes in one end and hinged through the scaffold at the other end. The lock system constituted a logic-based AND-gate, made up of three different aptamers, which only upon binding of two cancer specific extracellular membrane proteins would be disrupted. The dehybridization resulted in a drastic conformational change of the device acting similar to an entropic spring, exposing the interior of the barrel. The therapeutic potential was demonstrated by showing its ability to discriminate target cells from a complex background of whole-blood leukocytes. The device proved capable of arresting cell growth in an aggressive natural killer cell line or induce activation of T cells in vitro, depending on the choice of Fab’ molecules. The system successfully demonstrated the capability of DNA nanostructures to sense and deliver molecular signals to biological systems in a programmed manner. Recently, the device have been reported working in vivo in a DNA-based biocomputing setup [85]. Similar approaches
have been adapted by other groups to increase sensitivity to growth hormones [86] and inhibit tumour metastasis [87].

5. Future perspective and conclusion

The development of a stable in vivo molecular DNA device carrying the above-described characteristics could open up a new frontier in medical treatment. By combining these ideas, researchers could build injectable macromolecular devices capable of sensing diseased tissue followed by therapeutic intervention at sub-cellular level.

As an interesting prospect, future molecular machines could incorporate functional domains to perform active locomotion. Instead of relaying on passive diffusion, the device could thus traffic its way around the body. Coupled with a sensing functionality, the devices could move in response to a chemical stimulus, seek out their specific target and initiate a relevant action when in reach (similarly to chemotaxis) yielding a highly sophisticated nanorobot.

The realization of such devices would enable us to treat diseases or molecular dysfunctions at an entire new level, which would lead to great improvement to human health.

As summarized above, DNA nanotechnology holds many of the properties required for the realization of such devices. Throughout the last three decades, researchers have succeeded in addressing key challenges and developed both dynamic and therapeutic DNA devices capable of responding to external stimuli. The modularity of DNA nanostructures will enable researchers to build even more complex multi-functional devices as already demonstrated [88–90].

Although challenges still lie ahead, research continues to push the technological limits beyond what anyone would have imagined possible just a few years ago. DNA nanodevices have become established as one of the most promising multifunctional gadgets and will undoubtedly become even more widespread in the future.

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