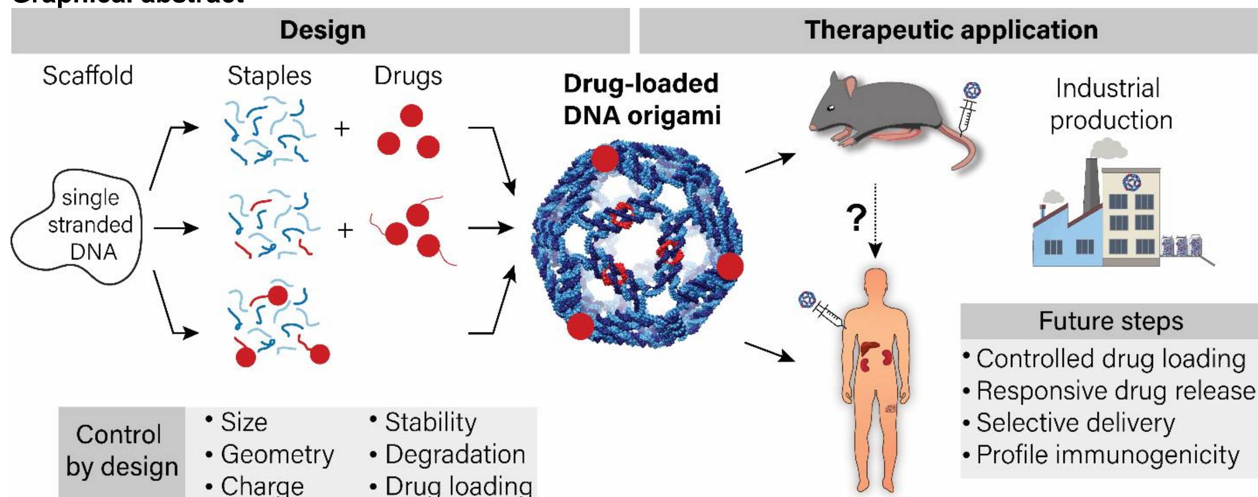
DNA Origami Nanostructures for Controlled Therapeutic Drug Delivery

Jorieke Weiden[1], Maartje M.C. Bastings[1,*]

1. Programmable Biomaterials Laboratory (PBL), Institute of Materials (IMX), Interfaculty Bioengineering Institute (IBI) School of Engineering (STI), École Polytechnique Fédérale Lausanne (EPFL), EPFL-STI-IMX-PBL MXC 340, Station 12, Lausanne, 1015, Switzerland.
Email: jorieke.weiden@epfl.ch; maartje.bastings@epfl.ch
* Correspondence to: maartje.bastings@epfl.ch

Abstract
DNA nanostructures are emerging as a versatile platform for controlled drug delivery as a result of recent progress in production yield and strategies to obtain prolonged stability in biological environments. The construction of nanostructures from this unique biomaterial provides unparalleled control over structural and functional parameters. Recent applications of DNA origami-based nanocarriers for therapeutic drug delivery in preclinical phases highlight them as promising alternatives to conventional nanomaterials, as they benefit from the inherent favorable properties of DNA including biocompatibility and precise spatial addressability. By incorporating targeting aptamers and responsive properties into the nanocarrier design, more selective DNA origami-based nanocarriers are successfully prepared. On the other hand, current systems remain poorly understood in terms of biodistribution, final fate and controlled drug release. As such, advances are needed to translate this material platform in its full potential for therapeutic applications.

Graphical abstract



Keywords
Drug delivery, DNA origami, DNA nanomaterials, nanotechnology, selective targeting, controlled release, translational therapy, nanomedicine

1. Introduction

One of the major challenges in medicine is the controlled delivery of drugs to diseased tissues or cells. While a poor drug will not improve its function through the addition of a biomaterial carrier, precision and performance of good drug can be augmented using a well-designed drug-delivery system. An ideal drug delivery system maximizes the therapeutic effect of the drug whilst minimizing off-target toxic effects. Important benefits of using nanocarriers include facilitating the delivery of insoluble drugs, enhanced drug stability, more selective delivery of drugs, improved drug safety, enhanced transport across biological barriers and improved bioavailability and pharmacokinetics[1,2]. Liposomes, polymeric or inorganic nanoparticles, dendrimers, nanocrystals, micelles, nano-emulsions and polymer-drug conjugates have all successfully been applied as carrier systems to deliver therapeutic drugs with improved selectivity and efficacy[1]. A wide variety of nanomaterial-based drug delivery vehicles are clinically approved or have reached the clinical trial stage, in particular for cancer treatment but also as vaccines or for gene

therapy[1,3,4]. Despite these promising advances, currently available nanocarriers often show a lack of homogeneity in geometry, suboptimal biocompatibility and insufficient selectivity for diseased cells, resulting in the need for higher drug doses and potential toxicity. Additionally, many nanocarriers lack control over precise drug dosing, drug release rate and degradation behavior. As such, there is a motivation to design improved nanomaterial carriers with favorable and above all controllable properties to improve selectivity, efficacy and thus safety.
Over the past two decades, the potential of DNA as a programmable material rather than a carrier of genetic information, has gained considerable interest and initiated the field of DNA nanotechnology. The molecular recognition resulting from programmed hybridization of complementary sequences allows for the construction of DNA nanostructures with high precision and efficiency. Such DNA nanostructures can be created using a wide variety of methods building on various levels of complexity. With short single strands of DNA or RNA, tiles or bricks can be self-assembled, which subsequently can undergo hierarchical organization into larger architectures. Alternatively, DNA origami nanostructures (DONs) are obtained via the nanoscale folding of a long single stranded (ss)DNA scaffold strand (typically derived from length and sequence variations of the M13 bacteriophage genome) using complementary synthetic staple strands[5]. With the scaffold present as a guide through the entire object, these stable DONs hold potential for biomedical and therapeutic applications.The DNA origami technique has simplified the creation of programmable nanostructures and typically allows for achieving structures of a larger size, increased stability and superior robustness compared to bottom-up assembly methods of DNA nanostructures using DNA tiles or bricks[6]. A wide range of 2D and 3D DNA origami nanostructures can be produced that are exceptionally uniform in size and shape, finding use for applications in plasmonics[7], bioimaging[8], bioengineering[8], and biosensing[9].At present, the design, stability, production process and scalability of DONs have reliably been established[10]. DONs are now being applied as vehicles for controlled drug delivery, thereby capitalizing their inherent favorable characteristics such as their structural versatility, programmability, spatial addressability with nanometer precision, biocompatibility, and biodegradability. This opinion paper will explore and comment on the potential of DONs for therapeutic drug delivery applications. We will address the most important hurdles towards clinical translation, notwithstanding the progress made over the last 5 years.

2. DNA origami for therapeutic delivery: control by design

Design parameters that impact the performance of nanomaterials used for therapeutic delivery include their size, geometry, charge, stability, degradation and drug loading[11]. The highly specific interaction between complementary DNA strands forms the foundation of the programmable and addressable nature of DNA origami[11]. As such, the use of DNA allows for an exceptional *"control by design"* over the parameters that impact performance of a drug delivery vehicle compared to traditional nanomaterials.

2.1 Size and geometry

Particle size and geometry influence the biodistribution and clearance of DONs *in vivo*[12,13], their ability to cross biological barriers[14], their ability to be taken up by cells[15], but also the strategy with which drugs can be incorporated (Figure 1A). The optimal particle size greatly depends on the drug that it delivers (including its target and mode of action), the cell type or tissue that is targeted, the route of administration and the desired application[16]. For instance, the delivery efficiency of particles to solid tumors after systemic administration seems to be higher when the particles are smaller than 100 nm in size (but >6 nm to prevent early clearance through renal filtration)[17], although particles with larger sizes are retained in tumor tissue more effectively than those with smaller sizes[18]. The accumulation of particles in lymph nodes for vaccination purposed is favored by nanoparticles that are smaller than 100 nm as well[19]. In heterogenous nanocarrier preparations, the observed therapeutic effect could be caused by only a minor sub-population of the nanocarriers. In contrast to conventional nanocarriers such as liposomes or polymeric nanoparticles, DONs have an exceptionally high size and shape uniformity as they are folded from identical template scaffold strands (Figure 1A). DONs can be assembled within the optimal size range for therapeutic delivery[20,21], ranging from as small as 20nm up to submicron dimensions[22], both in 2D as well as 3D configuration. 2D nanostructures are constructed of linear arrangements of DNA helices (Figure 1B), whereas 3D DONs can be made by stacking multiple DNA helices on various lattices [6]. DNA nanostructures can be created in solid, hollow or wireframe format (Figure 1C), a feature which has been

shown to affect cellular entry efficiency, where more compact and low aspect-ratio geometries are internalized favorably[15].

2.2 Charge

The charge of nanocarriers directly impacts their *in vivo* biodistribution, which can be exploited to direct nanocarriers towards specific organs or cell types after systemic delivery. For example, positively charged liposomes are predominantly directed to the lungs of mice after intravenous (i.v.) administration, in contrast to near-neutral or negatively charged lipid particles that accumulate in the spleen[23]. Furthermore, surface charge also impacts the interaction of nanocarriers with the negatively charged cell membrane, cellular uptake and intracellular localization[24]. DONs are intrinsically negatively charged as a result of the phosphate backbone; however, the surface charge can be easily tuned by surface coating modifications to manipulate cellular interactions.

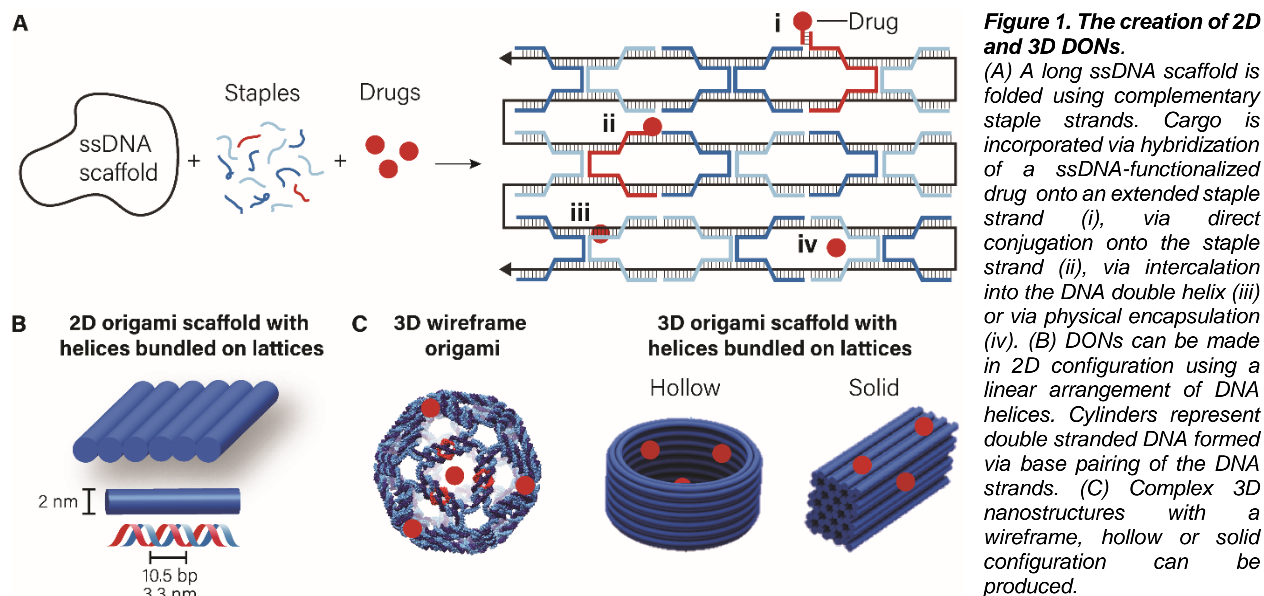


***Figure 1. The creation of 2D and 3D DONs**. (A) A long ssDNA scaffold is folded using complementary staple strands. Cargo is incorporated via hybridization of a ssDNA-functionalized drug onto an extended staple strand (i), via direct conjugation onto the staple strand (ii), via intercalation into the DNA double helix (iii) or via physical encapsulation (iv). (B) DONs can be made in 2D configuration using a linear arrangement of DNA helices. Cylinders represent double stranded DNA formed via base pairing of the DNA strands. (C) Complex 3D nanostructures with a wireframe, hollow or solid configuration can be produced.*

It has been shown that coatings can not only enhance DON stability, but also screen their overall negative charge. Coatings that have been used for DONs include virus capsid proteins[25] or cationic polymers such as polyethylene glycol (PEG) poly-lysine[26], polyethyleneimine[27] and chitosan[27]. The benefits and drawbacks of these different coatings for DONs have been reviewed before[28].

2.3 Stability and degradation

Therapeutic carriers and the drugs that they transport need to be stable after *in vivo* administration to effectively deliver the drug to the target site, followed by clearance or degradation. DNA nanostructures are inherently vulnerable to nucleases present in the blood, the extracellular milieu and the cytoplasm, which offers the ability to control nanocarrier stability and degradation by tuning their design[28]. Compact and stiff DONs limit access of nucleases and show enhanced resistance against degradation[29], as in particular ssDNA regions, internal nicks and flexible regions are sensitive to nuclease activity. DONs can furthermore be stabilized at physiological cation levels[30] and confer resistance against nucleases by changing the staple strand length and arrangements[31], through enzymatic ligation of staple strands[32], by covalent crosslinking[33], through micellization[34], and by coating the structures with PEG-conjugated cationic polymers[26], lipids[35], (virus capsid) proteins[25,36] or peptoids[37]. As stability is inversely correlated to degradation, particle lifetime can be controlled by modifications in DON design and the protection strategy. For instance, structurally distinct DONs release DNA intercalating drugs in different rates when exposed to nucleases[38]. Stabilization of DONs with peptoid coatings has furthermore been reported to reduce the release rate of intercalated drugs from DONs[37]. However, a detailed study that investigates DON stabilization and degradation by design with respect to of drug loading and drug release from DONs has

not yet been performed. We believe this will provide an additional level of spatiotemporal control over drug release which may further enhance selectivity.
Following cellular uptake of DONs, they are ultimately degraded in the endo-lysosomal pathways even when stabilizing coatings are applied. This can be an effective way to release cargo (e.g. physically incorporated or intercalating drugs) that may subsequently escape the endosomes[39]. However, the endosomal escape of intact DONs can be beneficial for certain applications. Although a few studies investigated strategies to promote endosomal escape, including (virus capsid) protein-coating of DONs [25,40] or attachment of cell penetrating peptides[41], it remains unclear whether substantial amounts of DONs are able to escape the endosomes intact[42].

2.4 Strategies to incorporate drugs
Drug carriers should incorporate sufficiently high amounts of drug which need to be loaded and released in a controlled manner. The cargo incorporation strategy depends on the properties of the drug, the type of DNA origami (e.g. hollow 3D versus 2D) and the need to retain drug activity after release (Figure 1A). Small molecules such as anthracyclines and metal complexes can be intercalated within the DNA duplex[43,44], although care should be taken to not disrupt or destabilize the folded DNA[45]. Nucleic acid cargoes such as CpG motifs or interference RNA (RNAi) can be easily introduced into the staple sequence design, or directly hybridized onto a complementary strand[46]. Larger biomolecules[47,48] and single stranded DNA-coated gold nanorods[49] can be tethered onto staple strand extensions using complementary DNA oligonucleotides. An alternative strategy to incorporate larger molecules is chemical modification of staple strands to covalently attach ligands (e.g. biotin) to recruit (e.g. streptavidin-)modified drugs[50]. Finally, 3D DONs can physically entrap cargo and thereby simultaneously protect cargo from digestion to augment bioactivity. In this way, large biomolecules such as enzymes can be attached to DON-based 'half-cages', which can then be combined into a full cage using complementary linker strands [51]. The stabilizing coatings for DONs may also be exploited to load cargo, as drugs (such as monoclonal antibodies) can for instance be conjugated to reactive groups incorporated into peptoid coatings[37]. As such, the method of incorporation needs to be carefully selected based on the envisaged application.

3. DNA origami for therapeutic drug delivery: current successes and remaining hurdles
With ongoing improvements in mass production and various strategies to stabilize DONs in physiological fluids, the potential of DONs for biomedical applications is rightfully being explored. In the last three to five years, a wide variety of DONs have been applied as vehicles for therapeutic drug delivery both *in vitro* and *in vivo* (Table 1 and Figure 2). Careful analysis of these studies is important to identify the unique strengths that DONs offer as therapeutic carriers but at the same time identify limitations, unexplored parameters and hurdles that need to be addressed to improve efficacy and facilitate clinical translation in the years to come. Here, we will dissect the parameter-space for DNA origami-based drug delivery to identify successful approaches and challenges that remain.

3.1 Drug loading and release
3.1.1. Controlled drug loading
A variety of drugs have been incorporated into DON carriers in recent years, including anthracyclines, small molecules, enzymes, antibodies, proteins, nanoparticles, RNAi and anti-sense oligonucleotides (ASOs) (Table 1). The vast majority of studies focus on introducing doxorubicin (DOX) into DONs (17 out of 28 studies where a drug was incorporated). This anthracycline is used for cancer chemotherapy as it triggers cellular DNA damage and cell death[55]. DON-DOX complexes are readily created through intercalation of DOX between G-C base pairs, and via minor groove binding in A-T rich areas of double stranded DNA[56]. Other anthracyclines such as daunorubin can be incorporated in a similar manner[57]. Although DNA intercalation of drugs is straightforward, it does not provide quantitative or qualitative control over drug loading. The currently available in vivo literature does also not address the accessibility and delivery of DOX in the context of stabilizing coatings. Future experiments will tell whether or not these coatings interfere with drug loading or release kinetics.
After cellular uptake, DONs are degraded in the endo-lysosomal pathways, effectively releasing DOX and facilitating its translocation to the nucleus[39]. While the release of DOX from DONs preceding cellular uptake has been studied in vitro, current data is not leading to a confident conclusion regarding the correlation between drug release kinetics and carrier degradation rates in vivo. We expect to see more in-

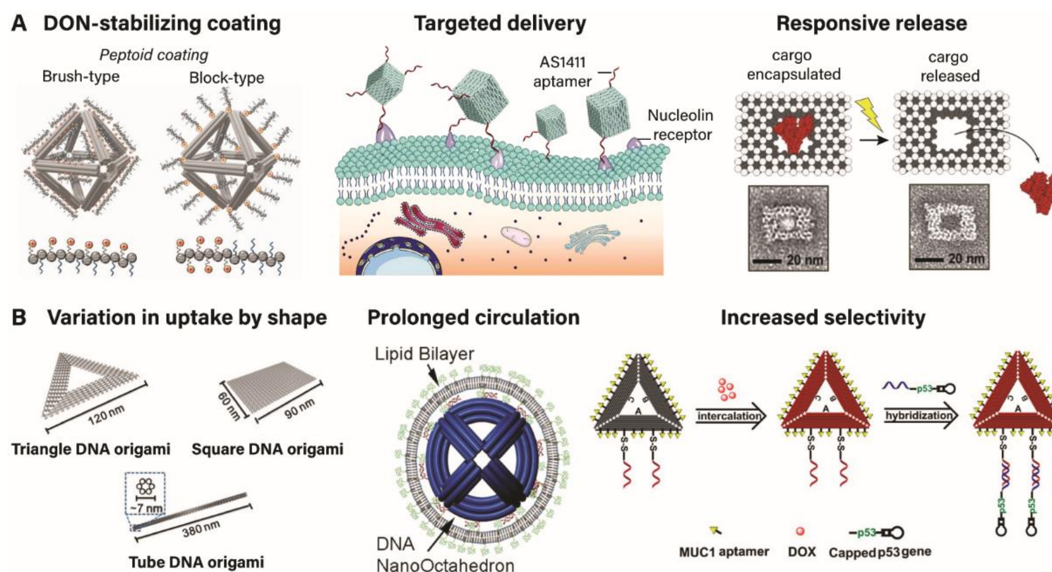


Figure 2. A selection of different DONs designs used for drug delivery in vitro (A) or in vivo (B). *(A) Left: Peptoid coatings are applied to stabilize DONs and provide handles for drug-loading. Reprinted (adapted) with permission from [37]. Middle: Nucleolin receptor-specific aptamers can be used to target the delivery of DONs to nucleolin receptor-expressing tumor cells. Reprinted (adapted) with permission from [52]. Right: Responsive release of drugs from DONs by making use of light-cleavable linkers. Reprinted (adapted) with permission from [53]. Copyright (2016) American Chemical Society.*
(B) Left: Design of DONs with varying shapes can induce differential tumor accumulation. Reprinted (adapted) with permission from [43]. Copyright (2014) American Chemical Society. This is an unofficial adaptation of an article that appeared in an ACS publication. ACS has not endorsed the content of this adaptation or the context of its use. Middle: Coating of DONs with lipid bilayers can enhance their circulation in the bloodstream. Reprinted (adapted) with permission from [35]. Right: Aptamer-functionalized DONs can be preferentially delivered to MUC-1-overexpressing cells for targeted gene delivery. Reprinted (adapted) with permission from [54]. Copyright (2018) American Chemical Society.

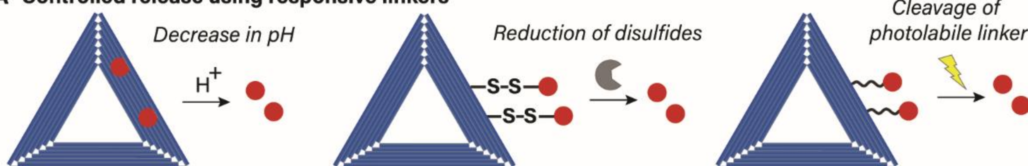
depth quantification of controlled drug-loading and drug-release in light of carrier stability in the years to come.
To achieve control over the exact number of drugs per carrier, ssDNA-functionalized drugs can be tethered onto extended handles on DON staple strands [58,59]. For instance, Li et al. could functionalize >70% of rectangular DONs with exactly four thrombin molecules at specific locations[58]. This strategy provides a tool to study and control the effect of drug loading on treatment efficacy, but this has unfortunately not yet lead to an extensive characterization on the effect of controlling DON drug loading on efficacy or safety. Recently, a number of studies exploiting DONs have underlined the importance of spatial organization in modulating antigen binding of antibodies[60], cancer cell signaling[47], and tuning immune activation[61]. Nevertheless, we have noticed a lack of studies exploiting the unique potential of DONs to control the spatial organization of drugs, which could offer an important opportunity to optimize drug efficacy and enhance the safety profile.

3.1.2 Responsive drug release

An important tool to promote selective delivery to the tissue or cell type of interest is the responsive release of cargo in a predictable manner from DONs upon external triggers or changes in the environment such as pH, redox activity or light[62] (Figure 3A). Various studies focus on drug release in response to a change in pH, as this could enable selective delivery into the acidic microenvironment of tumors. Research into pH-responsive drug release from DONs so far exclusively focuses on rapid release of DOX at lower pH (e.g. pH 4.5-5.5)[39,63–65]. However, the extrapolation of these observations remains to be established as the performance and selectivity of these pH-responsive DON systems have only been studied *in vitro*. Also, analytical techniques need to be evaluated critically and the effect of pH on the spectroscopic properties of DOX itself need to be addressed carefully to prevent wrongful interpretation[38].

Similarly, redox-responsive DONs could favor release of cargo in tumors displaying increased intracellular reduction activity by attaching cargo such as ASOs[63], RNAi[66] or functional genes[54] via disulfide linkage to DONs. For instance, this approach was succesfully applied to co-deliver DOX and the p53 gene to cancer cells[54]. Light-responsive DONs enable a high level of spatiotemporal control over drug release. Strategies have been designed that exploit drug release from DONs via a photo-labile crosslinker[53], enable photodynamic therapy for cancer therapy by attachment of a photosensitizer to DONs[67], or support photothermal therapy through attachment of gold nanorods[49,65]. Although these studies highlight the feasibility of creating light-responsive DONs *in vitro*, the ability to enhance drug selectively and efficacy *in vivo* remains to be established.

Besides attaching cargo to DONs with responsive linkers, an important advancement with the DON drug delivery field is shielding cargo within 3D DONs that are closed using aptamer locks[48,58,68,69] or pH-responsive latches [70](Figure 3B). Seminal work by Li *et al.* exploited nucleolin-targeted aptamer strands to configure a rectangular origami sheet into a tubular configuration, thereby shielding thrombin within the DON from circulating platelets and plasma fibrinogen in circulation until interaction with nucleolin proteins selectively expressed on tumor vascular endothelial cells[58]. This approach induced thrombosis in tumor vessels and effectively inhibited tumor growth in different tumor models. Combinations of aptamer locks can be used to make logic gates, thereby enhancing the selectivity of the drug delivery system[48,71]. Now that a number of studies have exploited this promising approach, it will be essential to perform detailed investigation of DON integrity and responsiveness in immunocompetent animals in the future.



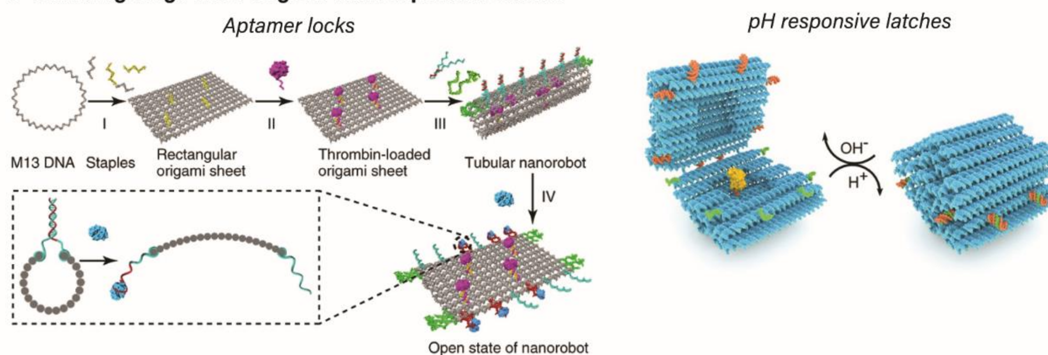


Figure 3. Responsive drug release from DONs. *(A) Drugs can be release from DON in a pH responsive manner (e.g. for intercalating drugs) (left), in response to reduction activity (middle) or by light-mediated cleavage of photolabile linkers (right). (B) Responsive latches on 3D origami can be exploited to deliver cargo selectively in response to an aptamer target (left – Reprinted by permission from Springer Nature from* [58]*, copyright (2018)), or a change in pH (right – Reprinted with permission from* [70]*).*

3.2 *In vivo* behavior and safety

3.2.1 Biocompatibility and immunogenicity

Recent studies have increasingly focused on dissecting the biocompatibility and immunogenicity of DONs, both *in vitro* [39,57–59,63,72–76] and *in vivo*[35,43,54,58,64,66,77–79], which is pivotal to establish DONs as a feasible and safe platform for drug delivery. These studies affirm the excellent biocompatibility of DONs by revealing a lack of cytotoxicity in representative cell lines [15,39,42,63,72–74], although this will need to be confirmed in primary cells as well. The modest number of *in vivo* studies with DONs investigate biocompatibility by performing body weight measurements[43,66], analyzing hematological indices[43,77],

Table 1. An overview of recent literature on DONs applied for drug delivery *in vitro* (top) and *in vivo* (bottom). Abbreviations: Yes (+), No (-), not tested/not applicable (N.A.), nanoparticles (NP), antibody (Ab), antisense oligonucleotide (ASO), fragment antigen binding (Fab), doxorubicin (dox), streptavidin (strep), bovine serum albumin (BSA), RNA interference (RNAi).

Type of drug	Drug	Stability	Degradation	Controlled drug loading	Characterized drug release	Controlled drug release	Cellular uptake	Targeting	Ref	Year
In vitro										
NP, Ab	Gold NP, Fab	N.A.	N.A.	+	N.A.	+	-	+	[48]	2012
NP, anthracyclines	Quantum dots, dox	N.A.	N.A.	-	-	-	+	N.A.	[41]	2015
Proteins	Strep, BSA, glutamic acid	N.A.	N.A.	+	+	+	N.A.	N.A.	[53]	2016
Photosensitizers	BMEPC	+	+	-	-	+	+	N.A.	[67]	2016
Anthracycline	Daunorubicin	+	+	-	+	-	+	N.A.	[57]	2016
Enzyme	Luciferase	N.A.	N.A.	+	N.A.	-	+	N.A.	[82]	2016
Anthracycline, small molecules	Dox, afatinib	N.A.	+	-	+	+	N.A.	N.A.	[65]	2017
-	-	N.A.	N.A.	N.A.	N.A.	+	N.A.	+	[68]	2018
Anthracycline	Dox	N.A.	N.A.	-	+	+	+	N.A.	[39]	2018
NP	Gold NP	+	N.A.	+	N.A.	+	N.A.	+	[69]	2018
Anthracycline	Dox	+	N.A.	-	+	-	+	+	[83]	2018
NP, enzymes	Metal NP, horseradish peroxidase	+	+	+	N.A.	+	N.A.	N.A.	[70]	2019
Enzymes	RNAse A	N.A.	N.A.	+	N.A.	-	+	+	[59]	2019
Proteins	BSA	+	+	+	N.A.	-	+	N.A.	[75]	2019
Proteins	Lysozyme	N.A.	N.A.	+	N.A.	-	-	+	[76]	2020
Anthracyclines	Dox	N.A.	N.A.	-	-	-	+	+	[52]	2020
Anthracyclines, proteins, Ab	Dox, BSA, trastuzumab	+	+	-/ +	+	+	N.A.	N.A.	[37]	2020
Anthracyclines	Dox	+	N.A.	-	+	+	+	+	[84]	2020
Anthracyclines, ASOs	Dox, ASOs	+	N.A.	-/ +	+	+	+	+	[63]	2020
Anthracyclines	Dox	+	+	-	+	-	+	+	[72]	2020
Anthracyclines	Dox	N.A.	N.A.	-	+	-	+	+	[73]	2020
Anthracyclines	Dox	+	N.A.	-	+	-	+	+	[74]	2020

Type of drug	Drug	Route of administration	Model system	Stability	Degradation	Controlled drug loading	Characterized drug release	Controlled drug release	Pharmaco-kinetics	Ref	Year
In vivo											
Anthracycline	Dox	i.v.	Mice	+	N.A.	-	+	-	+	[43]	2014
-	None	Into blood	Cockroach	N.A.	N.A.	N.A.	N.A.	+	N.A.	[71]	2014
-	None	i.v.	Mice	+	N.A.	+	N.A.	-	+	[35]	2014
-	None	i.v.	Mice	N.A.	N.A.	+	N.A.	-	N.A.	[49]	2015
Anthracycline, RNAi	Dox + RNAi (2x)	i.v.	Mice	+	N.A.	- / +	+	+	+	[66]	2018
Protein	Thrombin	i.v.	Mice / Pigs	+	N.A.	+	+	+	+	[58]	2018
-	None	i.v.	Mice	+	+	N.A.	N.A.	-	+	[77]	2018
Anthracycline, gene	Dox, p53	i.v.	Mice	+	N.A.	-	+	+	+	[54]	2018
Anthracycline	Dox	Topical	Mice / Pigs	+	+	-	-	-	+	[78]	2019
Anthracycline	Dox	i.v.	Mice	+	N.A.	-	+	+	N.A.	[64]	2019
Anthracycline	Dox	i.v./i.p.	Mice	N.A.	N.A.	-	-	+	N.A.	[79]	2019

measuring kidney function and liver enzymes in blood[77], and extensive histological examinations[43,54,58,66,77] including spleen, kidney, lungs, liver and skin[78] after administration of DONs. These analyses consistently indicate that DONs display excellent biocompatibility after i.v. or topical administration.
Preventing unwanted immune reactivity is a challenge for any nanocarrier, but this poses an even more prominent threat for DNA origami[80]. Both exogenous and intracellular DNA can trigger innate immune sensors[81], and thus strategies are required to minimize immune activation. Co-culture of DNA nanotubes[46] or DNA origami octahedrons[35] *in vitro* with splenocytes can lead to stimulation of immune cells. *In vivo* administration of DONs results in production of inflammatory cytokines indicative of an innate immune response (IFNα, TNFα and/or IL-6) in immunocompetent mice in some studies[35,64], but not in others[43,54,58,66,77]. Clearly, a more comprehensive assessment of the immunogenic potential of various DON designs needs to be performed and should include the consequences of repeated administration of DNA origami. This could lead to identification of design parameters that regulate DON immunogenicity. As an example, coating of DONs with lipids has been shown to reduce immunogenicity[35,64], yet this remains a highly structure-dependent and complex strategy.

3.2.2 Biodistribution
The efficacy of drug nanocarriers is determined by their ability to selectively target cargo to the tissue of interest. The physicochemical properties of DONs greatly impact their tissue selectivity. Almost all (10/11) studies that apply DONs for drug delivery focus on i.v. administration, of which some investigate DON biodistribution in mice (Table 1). In general, DONs are cleared rapidly from the body within a time span of ~8–24 hours via the kidneys and liver[35,43,54,58,66,77]. Jiang et al. observed increased liver retention of non-folded ssDNA or partially folded DONs, suggesting recognition and clearance by phagocytes of these structures but not of completely folded DONs[77]. This contradicts other studies that show localization to the kidneys for non-stable DONs and liver localization for intact DONs[26], suggesting that the exact role of DON design parameters and stability on *in vivo* biodistribution remains unclear. Importantly, DONs can accumulate in tumors of tumor-bearing mice, which peaks between 6-8 hours and remains high up to 24 hours[43,54,58]. It seems that tumor accumulation can be enhanced by changing the geometry of DONs as triangular DONs showed superior tumor localization compared to square and tube DON[43], or by attaching tumor-targeting aptamers[54,66], although this will need to be explored in more detail. Notably, one study investigated transdermal administration via topical application of DONs[78]. They observed that compact DONs with a low aspect ratio displayed the highest tissue penetration while maintaining structural integrity, indicating that DON geometry is an important design parameter[78]. Delivery of DOX-loaded DONs localized the drug to the skin and prevented off-target serum and organ accumulation.
Altogether, these studies provide preliminary insights into the fate of DONs after i.v. and topical administration and suggest that DONs can safely be administered for drug delivery purposes and reach tumor sites. However, a systematic characterization of the complex relationship between DON design parameters (DON sequence, size, geometry, charge and coating strategy) and biodistribution is warranted, as well as a quantitative readout on the proportion of DONs that actually reach the tumor site.

3.3 Selective targeting
Drug selectivity can be enhanced by targeting drug-loaded nanocarriers to favor uptake in specific cell types. Although this remains a relatively unexplored field, DONs can be passively targeted to tumor cells by optimizing their size and geometry[43,49,77]. Active targeting has been applied to DONs mainly by incorporating aptamers[48,52,54,58,59,63,66,68,69,72,73,76,83,84] or by attaching cell surface receptor ligands[74]. Alternatively, peptide coatings could be employed to modify DONs with functional biomolecules for targeting purposes[37]. Aptamer sequences can be easily incorporated into the staple strand design of DONs, or they can be conjugated on the DON surface via hybridization. The cell surface protein ligand that is most frequently engaged is MUC-1 [54,59,63,66,69,73], a mucin that can be overexpressed on tumor cells. Liu et al.[54] attached MUC-1 targeting aptamers onto triangular DONs and observed enhanced accumulation of DONs in tumors and a higher DOX uptake in tumor cells after i.v. administration.
Recent work has advanced our understanding regarding the design parameters that impact the efficacy of aptamer-mediated targeting of DONs, which exploit the exceptional control that DONs offer over ligand density and spatial organization. Sun et al. site-specifically attached C2NP aptamers onto rectangular

DONs, which bind CD30 receptors on T cell lymphoma cells but also trigger apoptosis[83]. Increasing the density of aptamers on the surface from 4 to 16 aptamers per DON enhanced uptake and cell killing *in vitro*. Chen et al. included C2NP-targeting aptamers into the staple strand design and varied the aptamer spatial distribution and stoichiometry[72]. DONs with 30 aptamers at 6 nm inter-aptamer spacing induced stronger cell inhibition compared to DONs with 15 aptamers and/or 3 nm inter-aptamer spacing. Finally, Liu et al. designed cuboid DONs loaded with DOX onto which 2 or 4 aptamers were attached in an adjacent or diagonal orientation[52]. Enhanced uptake, internalization and tumor cell inhibition was observed when aptamers were presented *in cis*, possibly because of the smaller inter-aptamer spacing (36 nm vs 42.9 nm). Similarly, the efficacy of DOX-loaded DONs to inhibit prostate cancer cells overexpressing prostate-specific membrane antigen (PSMA) depends on the DUPA ligand density and/or spatial distribution, as the cell index decreased linearly with increasing number of DUPA ligands that were spaced more closely together[74]. These findings illustrate the importance of characterizing and controlling aptamer loading on DONs for selective targeting.

3.4 Uptake by cells and intracellular fate

For many therapeutic drugs, uptake by target cells is required to deliver cargo to the appropriate intracellular compartment and decrease off-target toxicity[85,86]. Although the anionic and hydrophilic nature of DNA restricts entry of linear ssDNA and dsDNA into cells, DNA nanostructures appear to be readily internalized by cells via the endocytic pathway. Cellular uptake of DNA origami differs per cell type and is dictated by the compactness and shape of DNA origami, highlighting the importance of uniform material geometry and controlled design[20,42]. Despite the importance DONs size and geometry, recent literature on therapeutic drug delivery exploits DONs consisting of a wide variety of different sizes and geometries. The importance of careful side-by-side analysis is underpinned by the few studies that compared DONs of varying sizes and geometries [39,43,49,72,77,78], and in some cases compared their performance to unfolded ssDNA M13 scaffolds[43,77]. The impact of geometry was shown by Zeng et al., who compared the performance 2D cross-shaped, 2D rectangular and 3D triangular DONs *in vitro* with comparable drug loading efficiency of DOX[39]. The 3D triangle displayed increased internalization, enhanced drug release and a lower IC50 than both 2D DONs, probably as a result of the rigid edges and sharp vertices of the nanocarrier. Similarly, Zhang et al. also observed that 2D triangular DONs demonstrated the most pronounced selective tumor accumulation as a result of passive tumor targeting following i.v. injection, thereby outcompeting 2D square DONs, 2D tubular DONs and ssDNA M13[43]. On the other hand, a study into the biodistribution of various DONs after i.v. administration did not display substantial differences in their *in vivo* clearance or kidney uptake of rectangular, triangular or tubular DONs[77], indicating that the impact of DON geometry might be target and DON-specific. This warrants a comprehensive comparison between DONs of varying size and geometry, where a distinction will need to be made between their *in vivo* biodistribution, cellular uptake and, ultimately, intracellular fate.

3.5 Performance compared to other nanocarriers

An instrumental step in advancing DONs for drug delivery towards clinical application is the recognition of their benefits over alternative nanocarriers. So far, only two studies compared the performance of DONs to 'standard' nanocarriers. Perrault et al. compared the *in vivo* biodistribution of lipid-coated DONs with liposomes of the same size, formulation and concentration after i.v. administration[35]. The distribution of both nanocarriers across organs was similar, but DONs were cleared slower than the liposomes. On the other hand, Wiraja et al. compared DOX-loaded DONs with liposomes and polymeric poly(lactic-co-glycolic acid) (PLGA) nanoparticles, which are commonly used topical drug carriers[78]. Interestingly, the larger liposomes and PLGA NP allowed for two-time higher drug loading compared to DONs, but at the same time displayed significant drug leakage. Following topical application on pig skin, DOX-loaded DONs penetrated into the skin the furthest, reaching up to 400 μm in the dermis instead of 300 μm (liposomes) or 100 μm (PLGA NP). These studies might suggest that DONs could provide unique advantages over other nano-sized delivery systems, and in the years to come it will be critical to identify the specific medical challenges in which these benefits are particularly useful. In future studies, inclusion of a comparable "gold standard" is crucial in order to convincingly demonstrate the advantages of using DONs.

4. Conclusions

Precision and performance of a good drug can be augmented using well-designed drug delivery systems. Following the dawn of DNA nanotechnology, the field is rapidly exploring applications in medicine. Drug delivery vehicles require a plethora of parameters to be precisely controlled for optimal performance, a role exquisitely fitting for DONs. Over the past years, we have seen demonstrations of DON application for therapeutic delivery *in vitro* and *in vivo*, made possible through the extensive work on stabilizing the DNA helix in biological environments. While successes can be seen in terms of drug loading and targeting, many parameters remain to be explored in detail (Figure 4).

The potential of applying DONs for drug delivery depends on their behavior in vivo. Studies on the biodistribution of DONs suggest rather rapid clearance within 8 – 24 hours following i.v. administration, which is the same range as other nanostructures of a similar size[13,16]. As in vivo models suggest effective tumor accumulation of DONs, these circulation times are sufficient to support extravasation in the tumor vasculature. For therapeutic applications that require prolonged dosing and therapeutic action, one can envision to encapsulate the DONs into complementary protective and slowly degrading environments such as nanogels or lipid- or polymer-based capsules.

We observe a collective effort in recent literature to use the DNA-intercalating cancer drug DOX, thereby aiming for improvement of cancer therapy by diminishing toxicity and off-target action. A common strategy is observed using DOX and aptamer-mediated targeting of DONs to tumor sites. However, the comparison with current gold standard DOX delivery systems, and a systematic evaluation of all design parameters inherent to DONs remain to be presented. In the near future, we expect to see efforts along these routes in order to narrow the wide parameter space outlined above, and to arrive to a consensus structure that identifies itself as potential commercialize-able delivery platform for DOX-mediated therapies.

But is it commercially feasible? Can this technology be competitive with current formulations? In the case of DOX as an anti-cancer treatment, the current protocol is a three-week cycle at an indicated dose of 60-75 mg/m^2 [87]. Assuming an estimated body surface area of 1.62 m^2 for humans, this requires 122mg DOX per treatment cycle[88], which costs ~€200 in soluble form[89]. Benefitting from the strong DNA-intercalating property of DOX, a loading of 2500 DOX molecules per DON can be assumed[38], demanding ~450 mg of DONs per cycle. Large scale production of DONs can now be performed at an estimated cost of €0.18 per mg of folded DON[10], yielding an estimated material cost per treatment of €80. With this number possibly diminishing over time as a result of optimized large-scale production advances, we here demonstrate that material costs will not be a limiting factor to get DONs in the clinic. As comparison, the commercial cost of current liposomal-DOX nanocarrier formulations is ~€4500 per treatment cycle [90]. Regarding the use of DONs as drug delivery vehicles for non-intercalating drugs, we believe that the incorporation of nucleic acid cargo by inclusion into the staple sequence design could also become feasible with respect to cost efficiency and scalability. The caging of cargo in 3D DONs or the tethering of ssDNA-modified drugs onto ssDNA staple extensions will however be a lot more time and cost demanding. Modification of drugs with ssDNA could alter their activity and pharmacokinetic parameters, thus the use of dynamic covalent chemistry is preferred which will naturally dissociate and leave a pristine drug after release from DONs. For these drugs, it will be even more important to identify applications in which the unpreceded control over the exact spatial orientation of drugs or their lock-mediated responsive delivery is instrumental to enhance efficacy or safety of drug delivery.

Concluding, we expect the field of DON-mediated drug delivery to move toward more *in vivo* studies focusing on controlled drug loading and release, as well as verifying DON biodistribution, degradation and particle fate. Together with improved understanding on the structure-function relationship of DON-cell interactions and DON behavior *in vivo*, we expect that the field settles down on a new "standard" in design geometry. This architecture can then be optimized for mass production and enter translation toward clinical test phases in the next decades.

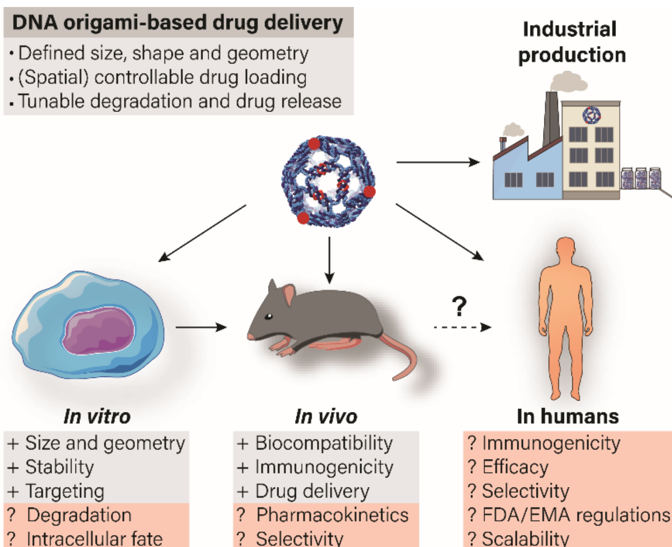


Figure 4. The current status (grey) and insufficiently explored areas (red) for application of DONs as vehicles for therapeutic drug delivery.

Author contributions

JW and MB both conceptualized and visualized the manuscript. JW wrote the original draft, which was reviewed and edited by MB.

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