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Review Article

DNA tetrahedron as nanoparticulated delivery system in combating diseases

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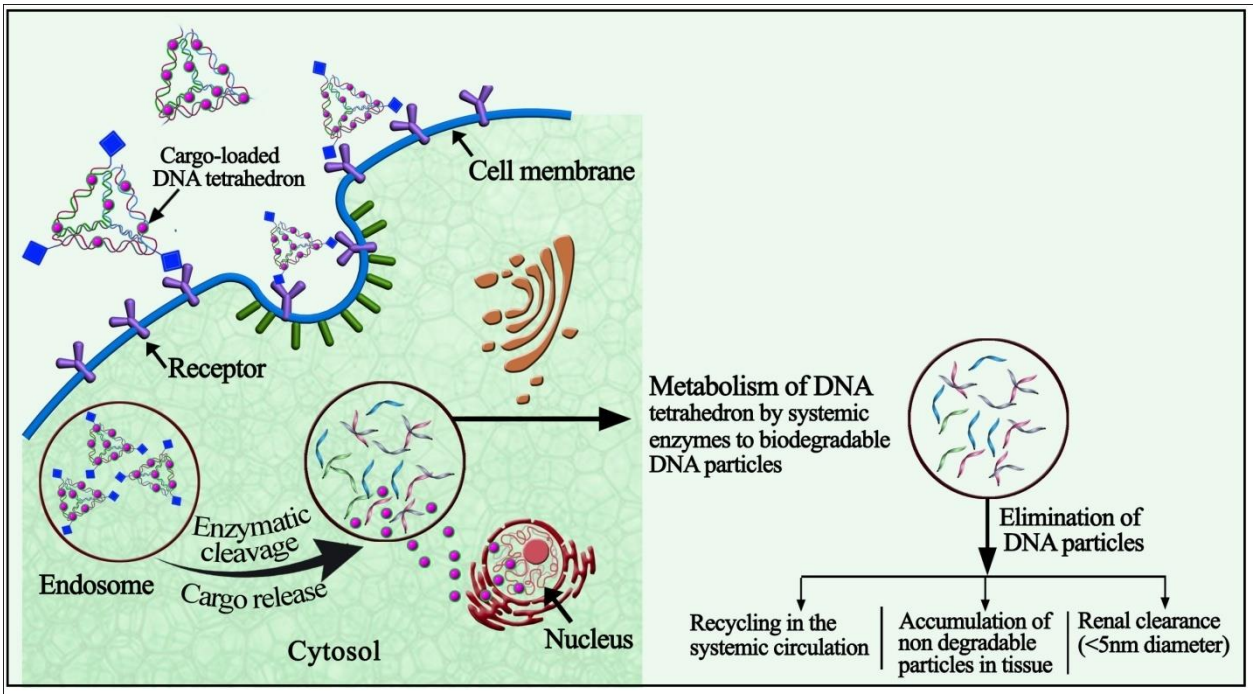
Abstract

Many diseases suffer from drug resistance and nucleic acid cargo delivery. To optimize pharmaceutics and to enhance their efficiency of cellular uptake, DNA nanomaterial tetrahedrons, owing to their precise control in size, shape, excellent biocompatibility and cellular permeability, reduced cytotoxicity, good stability, ease synthesis and multiple sites for targeting design, have attracted attention for targeting cargos delivery. Their nanostructural binding efficiency with many cargos depends on their electrostatic attractions among free electrons of phosphate oxygen, sugar and base nitrogen. Self-assembled DNA tetrahedrons (DTs) alone also can regulate cellular processes to some extent, especially, on migration, differentiation, proliferation and autophagy, and their modifications with the attachment of aptamers, peptides, nucleic acids, antibodies, different low-molecular-weight drugs and other components, make them a novel targeted delivery system as effective nanomedicine. This review demonstrates the current progress of DTs towards their synthesis, characterization, biomedical applications, biodistribution, elimination and toxicity as possible nanoparticulated delivery system.

Keywords: Diseases; Drug resistance; DNA tetrahedron; nanoparticulated delivery system; Nanomedicine

Graphical Abstract



Introduction

Presently, the demand for developing preventive, predictive and non-invasive patient-oriented medicines as therapeutics is being increased for the treatment of a specific disease with power to leverage qualitative medical care in the life-threatening diseases [1-3]. Both biomolecular and chemical drugs as conventional therapy face their obstacles in poor solubility, systemic toxicity, enzymatic degradation, cell membrane-impermeability, drug resistance and non-specific targeting. To overcome these barriers, it is needed to develop active targeted system for delivering drug molecules to specific site of interest. In recent decades, several artificial molecular devices such as applications of viruses, liposomes, polymers, metallic nanomaterials, peptides, proteins, antibody, DNA, siRNA and synthetic inorganic molecules at the nanoscale have been developed to overcome multidrug resistance, therapeutic degradation, cytotoxicity, insolubility of the hydrophobic drugs, cell barricades, and to target cells with higher biological efficiencies and controlled drug release [4-10]. Many of these nanotechnology-devices are recently under clinical trials and several are approved by Food and Drug Administration (FDA) as clinical therapeutics for human applications [11]. In spite of the advances in the development of the nanotechnology-based delivery system, some of them have still few limitations, such as, short DNAs viral delivery into the cells showing random insertion sites, mutagenesis and cytopathic effects, inherent cytotoxicity and immune toxicity by cationic dendrimers, and cytotoxicity of many non-degraded inorganic nano-elements or residuals in the biological system [12-16]. In this context, three-dimensional (3D) DNA-nanotechnology has been emerged as attractive drug delivery system to get maximum efficacy with the minimum toxicity [17]. Based on the A-T, G-C Watson-Crick base pairing, natural DNA nanostructure, stabilized by strong hydrogen bond, shows excellent characteristics, such as, precise control in shapes and sizes, non-toxicity and biocompatibility, less susceptibility to nuclease and cell lysate, easy targeting design in multiple sites, and smart cargo delivery [18,19]. The most efficient DT, consisting of four or more single-chain DNA self-assembled by base pairing in a specific solution, becomes rigid-structure, highly stable and productive [20,21]. As a cargo-carrier, DT exists three main criteria to conjugate cargos, such as, pre-linking of the components mostly nucleic acids at the 5' or 3' end of single strands before self-assembly, decorating of an overhang for not interference with the DT formation following bondage of the materials via the complementary sequence with the overhang, and setting of the components in the DNA double helices by physical conjugates.

As a nano-sized delivery vehicle, DT may penetrate independently the negatively charged cell-membrane through receptor-mediated endocytotic internalization [22,23] with its inherent capability of resisting nuclease attack to retain its structural integrity for a long time owing to steric hindrance and non-toxic biocompatibility. In this concern, folate or peptide-anchored ligand specific DTs loaded with different cargos by covalent attachments show their efficiencies against tumors [24,25].

As monoclonal antibodies have limited capability to liberate drugs for covalent bonding, penetrating cells, immune responsive property and high cost, small peptides mimicking antibodies of smaller sizes and biological specificities like affibody molecules exhibit their efficiencies in drug targeting [26,27]. Affibody molecules consisting of three α-helix bundle domains with fifty eight amino acids obtained from the immunoglobulin G protein Z-domain scaffolds lacking cysteins and disulfide bridges are used to form affibody-DT nanoparticles for the treatment of HER2 over-expressing cancers, while DNA-affibody nanoparticles contain one DT and two affibody molecules mimicking one Fc and two Fab regions of the structured antibody for their binding activities [28-33]. In addition to acting as a scaffold for anchoring two affibody molecules, DT also is utilized as a carrier to bind multiple small molecular cargos non-covalently for specific targeting.

Aptamers, short, single stranded DNA and RNA oligonucleotides -ligands, are useful for forming complicated three-dimensional structures with DT, and higher binding capability with a target MUC1 molecule over-expressed in tumor cells [34-37]. Furthermore, the binding of tumor-targeting aptamer with a DT through DNA complementary base pairing loaded with drug within its DNA strands may be an effective approach for their specific target drug delivery [21,38-40]. When cytosine-phosphate-guanine (CpG) motifs, the short oligonucleotides where 2'-deoxycytidine is connected to 2'-deoxyguanosine by a phosphodiester bond, are appended to the DNA nanostructures, they show agonist property of Toll-like receptor 9 (TLR9) present in plasmacytoid dendritic cells and B cells through their bindings for boosting the immune response to treat cancer and allergic diseases [41-43]. In addition to DNA nanoparticles binding to specific ligands, siRNAs and other cargos also can be loaded for their delivery to specific target site/s [44-47]. This review demonstrates mainly the therapeutic efficacies of DT for the treatment of cancer and other diseases to judge as very effective delivery vehicle.

Synthesis and purification of DNA tetrahedron

DT consists of four isometric single stranded DNAs [21]. According to Watson-Crick's hybridization-principle, each single stranded DNA possesses three blocks utilized for hybridizing with the other three strands respectively to shape rigid DNA helices triangles into one of the DT –sides, with two terminals of oligonucleotides joined covalently at the vertex [48]. Each DT –side is splited up by several non-hybridized nucleotides for providing enough flexibility to bend. For the synthesis of DT (Fig.1), each equimolar single stranded DNA sequences is dissolved in 0.5 x TE buffer (10 mmol/L Tris-HCl [pH 8.0] and 50 mmol / L $MgCl_2$) to form one triangle of DTs while every edge is formed through the specific Watson-Crick base pairing by two different single stranded DNAs [49-51], where the corresponding DNA optical density (OD) value is determined at 260 nm by UV Spectrophotometer. In this way, four chains are made with the addition of TE buffer at the same concentration. The mixing ratios of four single strand-DNAs (1:1:1:1) at 1μM / 100 μL in TM buffer is performed for the reaction in a polymerase chain reaction (PCR) machine with the cycling conditions: denatured at 95 ◦ C for 10 min and annealed by natural cooling to 4 ◦ C [21]. In this context, all of the single stranded DNAs are purified by HPLC with 260 nm distinctive absorption peak, while the peak time of DNA tetrahedron in the HPLC spectrum becomes faster than that of single strand, and the yield is collected at the accompanying time point.

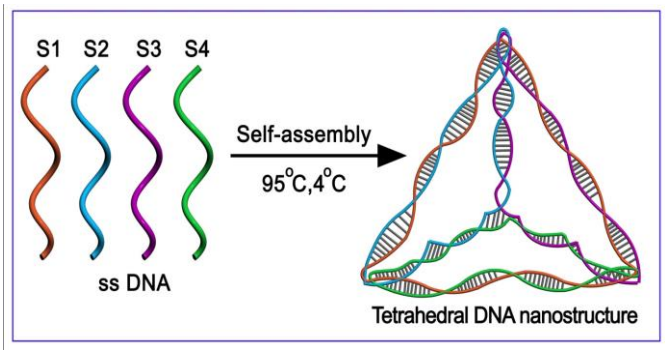


Figure 1: Schematic diagram of the synthesis of DNA tetrahedrons.

Functionalization of DNA tetrahedron with folate / aptamer / affibody and drug

Free hydrogen groups of drug molecule and folate are modified with azide groups and coupled with 3'-OH of single stranded DNAs through click chemistry reactions, while addition of different amounts of functional group tagged single stranded DNAs may stoichiometrically control the ratios of functional groups through specific side chains -hybridization [52]. For the synthesis of folate-DT, DT-drug and folate-DT-drug, the molar ratios are set respectively as 1:1, 4:1 and 1:1:3, while all the synthesis are accomplished at micromolar levels at 37 ◦ C, and kept at 4 ◦ C [53] (Fig.2).

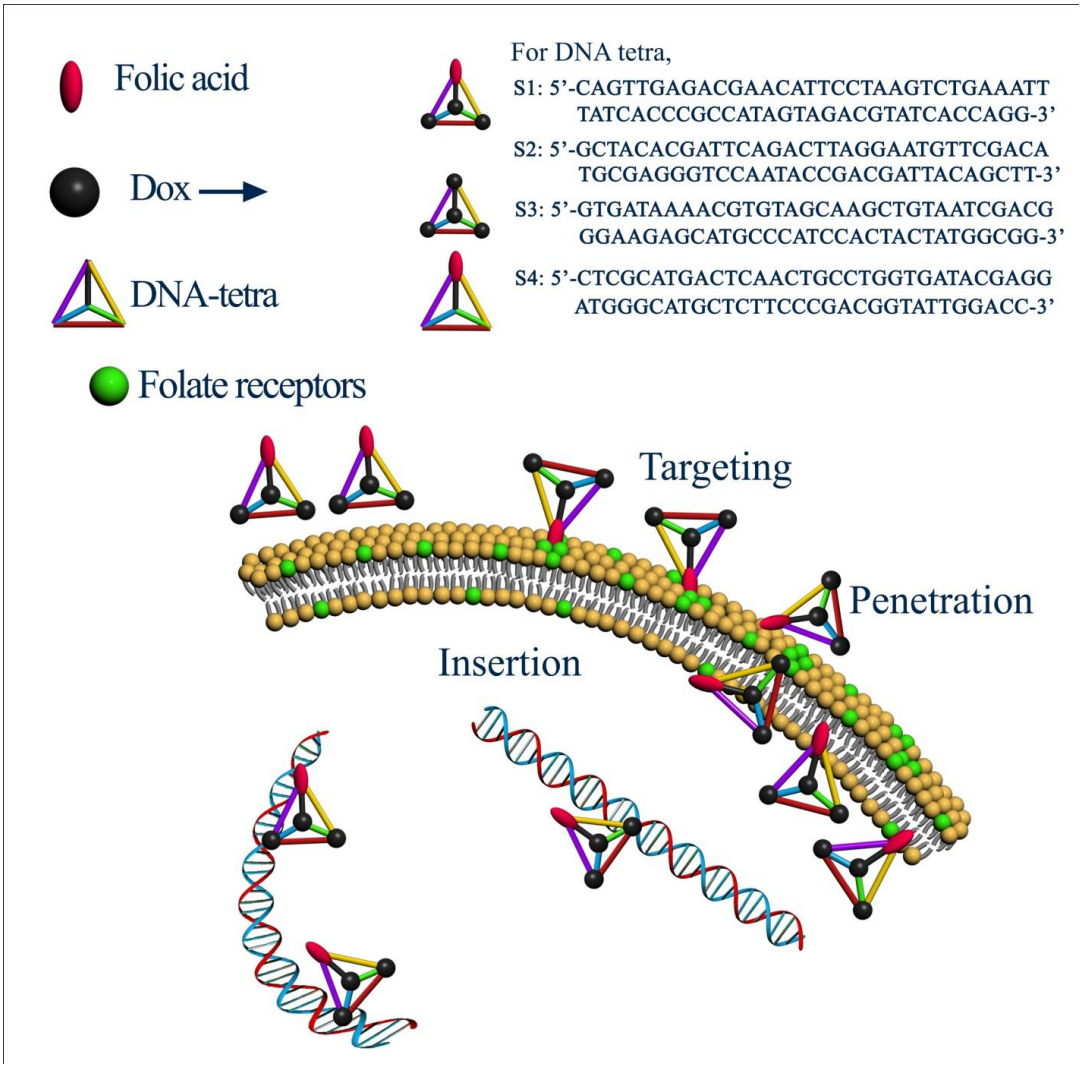


Figure 2: Schematic diagram of DNA tetra-Dox, folic acid-DNA tetra and folic acid-DNA tetra-Dox. S1, S2, S3 and S4 indicate the single stranded DNA sequences of DNA tetrahedron. The figure denotes the process of targeting of inserted DNAs to tumor cells through the cell membrane penetration.

Aptamer Sgc8c, a DNA sequence with 42 nucleotides, or other aptamer-modified DNA tetrahedron, known to bind to cell membrane protein tyrosine kinase 7 (PTK-7) / MUC1 protein over-expressed respectively on human T-cell ALL and tumors / MCF-7 cells may also be fabricated under the same conditions as DTs using aptamer sequences [54-57] (Table 1) (Fig.3).

Table 1. The specific sequences of each single-stranded DNA.

Single-stranded DNAs	Directions	Detail sequences
S1	5'→3'	ATTTATCACCCGCCATAGTAGACGTATCACCA GGCAGTTGAGACGAACATTCCTAAGTCTGAA
S2	5'→3'	ACATGCGAGGGTCCAATACCGACGATTACAGC TTGCTACACGATTCAGACTTAGGAATGTTCG
S3	5'→3'	ACTACTATGGCGGGTGATAAAACGTGTAGCAA GCTGTAATCGACGGGAAGAGCATGCCCATCC
S4	5'→3'	ACGGTATTGGACCCTCGCATGACTCAACTGC CTGGTGATACGAGGATGGGCATGCTCTTCCCG
S5	5'→3'	ATCTAACTGCTGCGCCGCCGGGAAAATACTGTA CGGTTAGATTTTTACATGCGAGGGTCCAATACCG ACGATTACAGCTTGCTACACGATTCAGACTTAGG AATGTTCG

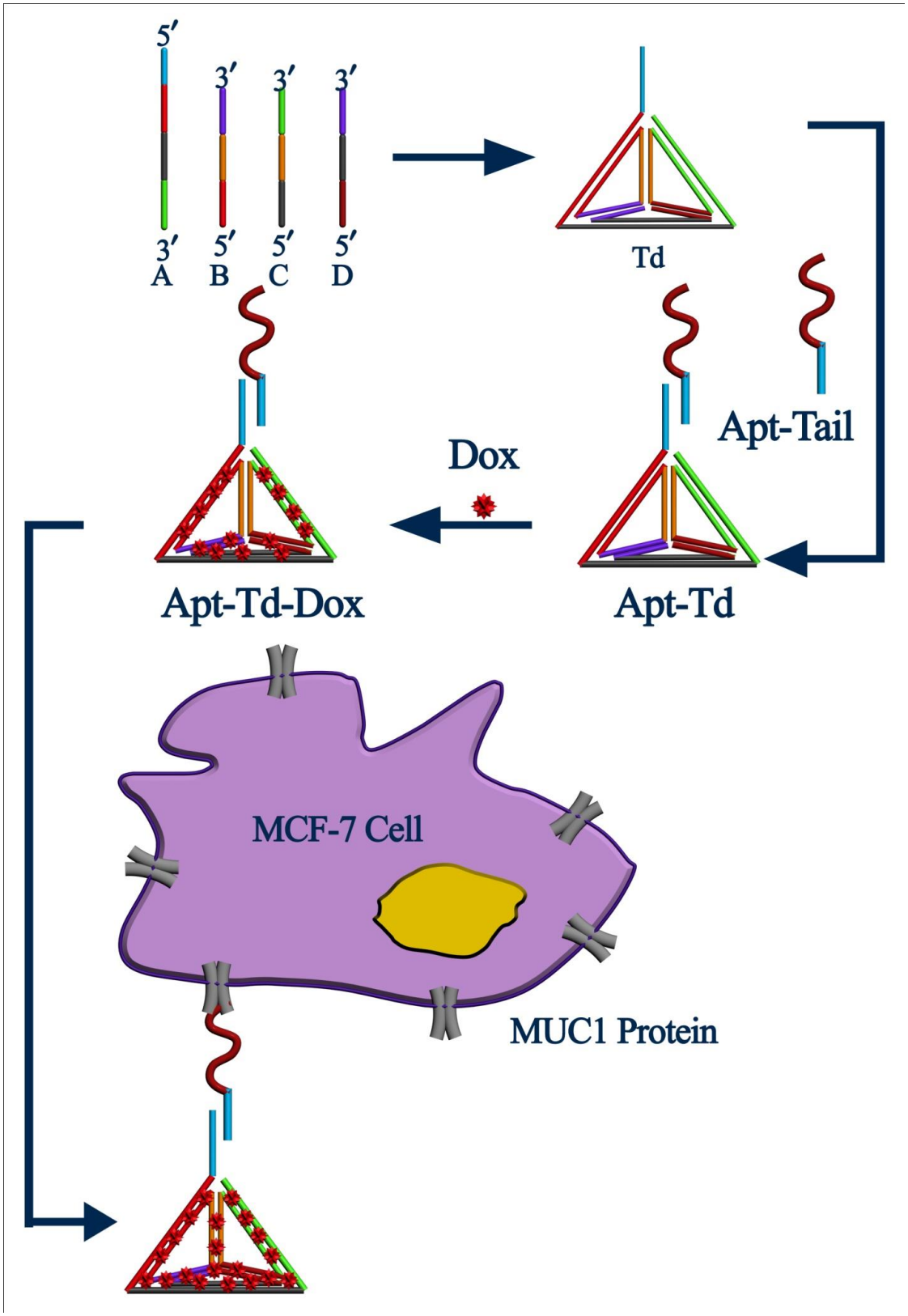


Figure 3: Schematic design of the aptamer-decorated DNA tetrahedron for selective targeting of doxorubicin to MUC1-overexpressed breast cancer cells. Four DNA single strands of DNA tetrahedron with a modified MUC1 aptamer (Apt-tail) indicate strand A, 5'-ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA-3', strand B, 5'-TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC-3', strand C, 5'-TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTC-3', strand D, 5'-TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT-3' and Apt-tail, 5'-AGGAAGAGAGAAGGAAGGGAATTTTTACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA-3'. The four DNA single strands have been assembled into a DNA tetrahedron through DNA complementary base pairing. One of the four strands has been extended with a sticky end exposed outside the tetrahedron. The MUC1 aptamer extended with an Apt-tail can pair with the sticky tetrahedron end. The formed aptamer-tetrahedron complex becomes mixed with doxorubicin for forming apt-tetra-dox to bind MCF-7 cancer cells for targeted drug delivery.

Two 5'-NH_2 labeled DNAs ($DNA_{1,2}$) are dealt with N^{ϵ}-maleimidocaproyloxy succinimide ester (EMCS) for generating two N^{ϵ}-maleimidocaproyloxy-DNAs ($I_{1,2}$) [58] (Fig.4). The obtained DNAs are dealt with an affibody containing a cysteine residue at the C-terminus for affording DNA-affibody chimeras ($II_{1,2}$). The affibody possessing a hexa histidine tag at its N-terminus is explicited in *E Coli* BL21 cells and purified utilizing a Ni-NTA column [59-61]. The coupling reaction yields between $I_{1,2}$ and the affibody do not differ for the incubation time ranging from 1-5 h. The produced DNA-affibody chimeras are then purified utilizing DEAE-Sepharose CL-6B column for removing the surplus affibody in the reaction mixture following a procedure for oligonucleotides-purification [62]. After this chromatography, the un-reacted DNAs in the eluate are removed by Ni-NTA chromatography for specific binding of the hexahistidine peptide to the attached affibody. After purification, the DNA-affibody chimeras are treated with Coomassie Brilliant Blue R-250 and ethidium bromide to stain and detect protein and DNA, respectively. Afterthat, the two pure DNA-affibody chimeras ($II_{1,2}$) are merged with two single stranded DNAs (DNA_3 and DNA_4) for forming an affibody-tetrahedron structure (III) containing one DT particle with two affibody molecules. These affibody-tetrahedron structure III particles are incubated with excess drug for non-covalent binding associations at room temperature for 10 min to get DT-affibody-drug nanoparticles (IV) [63], which are purified further utilizing a Sephadex G-25 column to assess the number of drug molecules in the nanoparticles determined by UV-vis spectrophotometry.

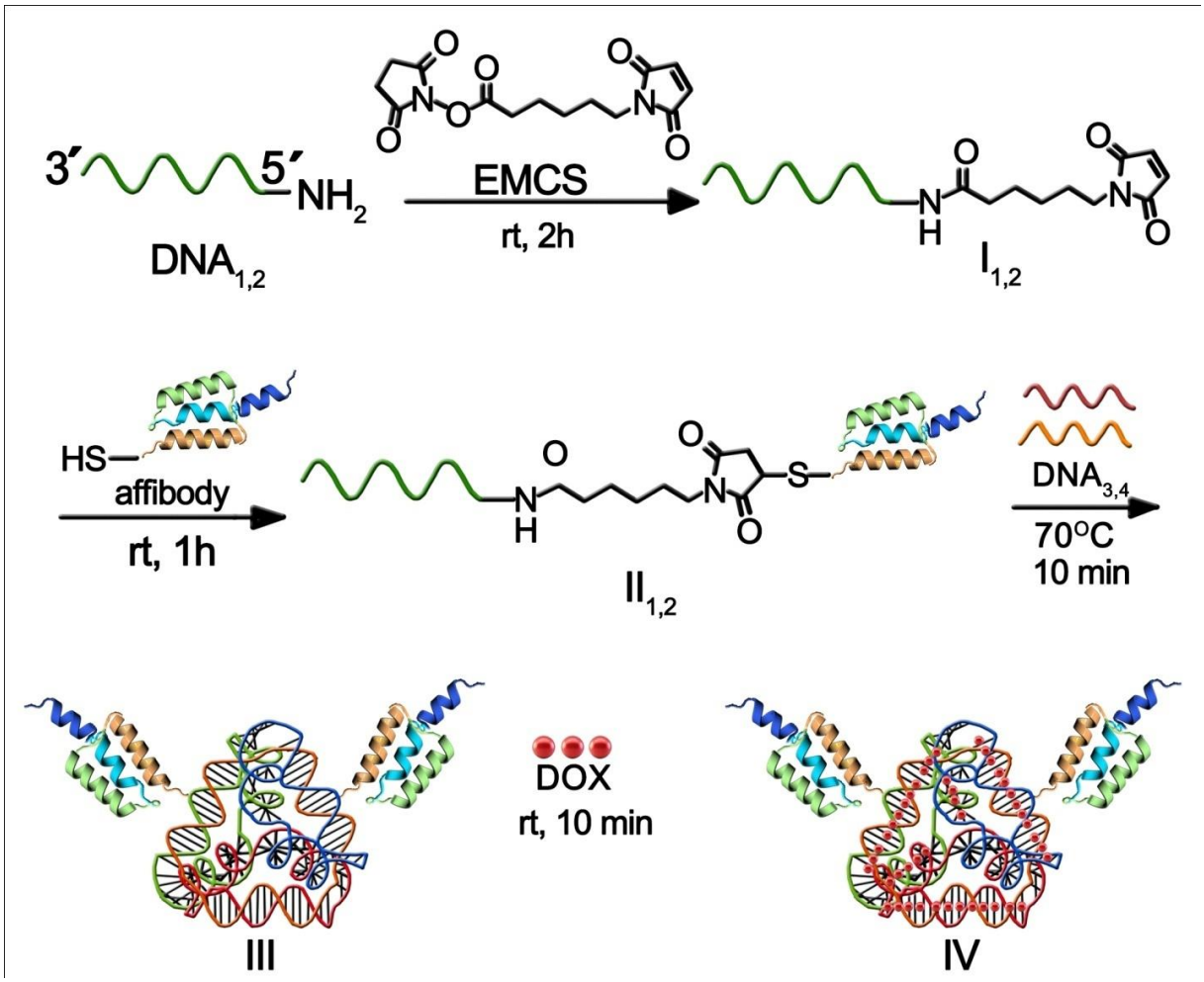


Figure 4: Schematic strategy to prepare DNA tetrahedron-affibody nanoparticles (III) and DNA tetrahedron-affibody-drug nanoparticles (IV).

Characterization

To evaluate whether DNA strands and protein are assembled in DT –folate / aptamer / affibody -drug moiety, gel electrophoresis is conducted, followed by ethidium bromide and / or Coomassie Brilliant Blue staining. To determine the structure, size and zeta potential of the DT nanoparticles, atomic force microscopy and a dynamic light scattering study are performed. Transmission electron microscopy may also be preformed for observing the morphology of the DT nanoparticulated moiety.

DNA tetrahedron as delivery vector

DT can specifically locate and permeate into plasma membrane and deliver cargos mainly through actin-driven clathrin and cavolae -mediated endocytosis as well as macropinocytosis, phagocytosis and clathrin and caveolin -independent endocytosis [64]. Its high flexibility in various sizes enables its high capability of cargos-loading with enhanced killing efficacy. The programmability of DT may be modified as vertex, capsule, mosaic and cantilever functional moieties with small molecules, oligonucleotides, antibody, affibody, protein, peptides, ligands and photosensitizers to fulfill suitable targeted therapies such as chemotherapy, immunotherapy, gene silencing and photodynamic therapy [65-67] (Figs.5&6) (Table 2).

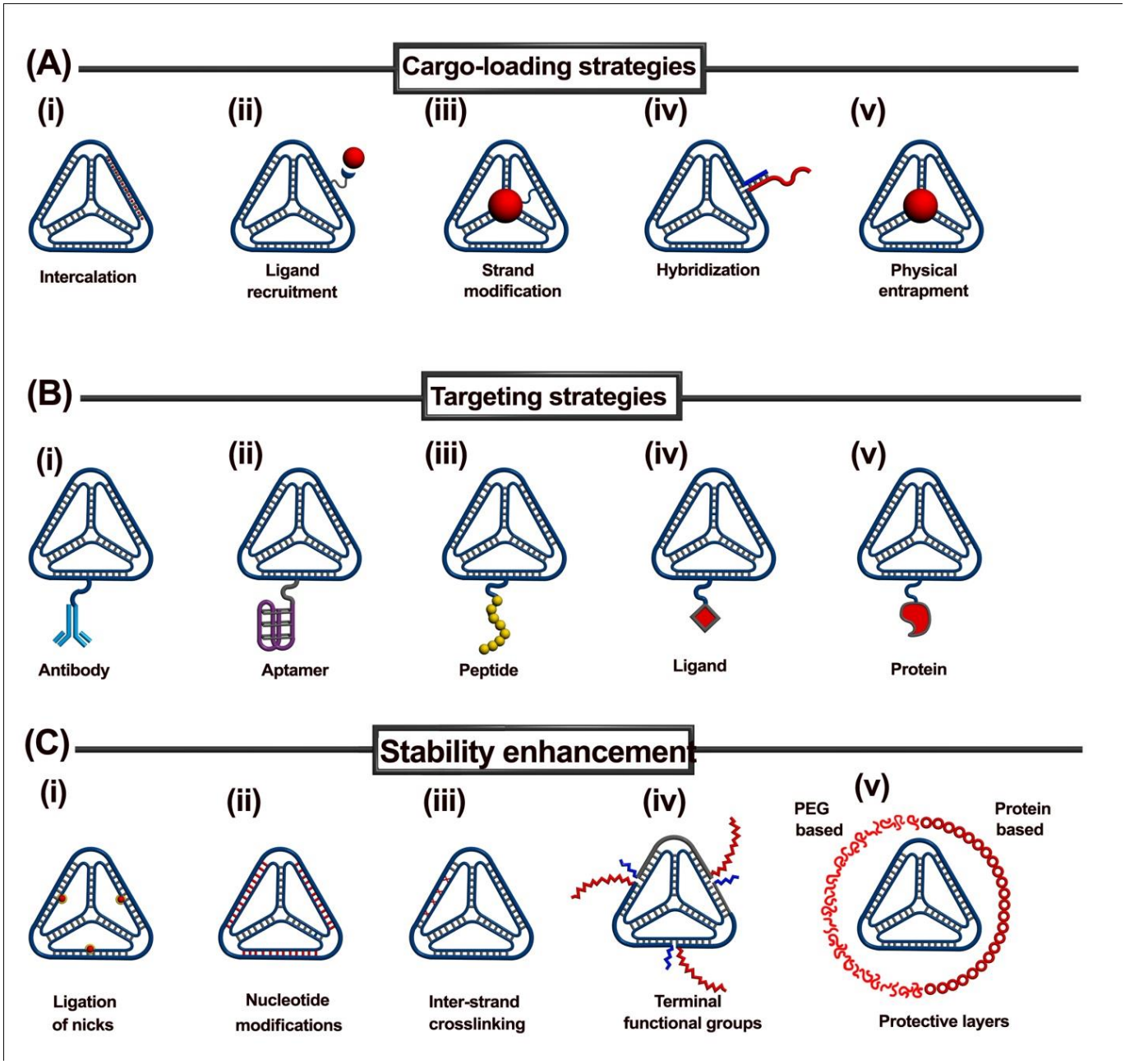


Figure 5: Characteristics of DNA nanostructures for cargo delivery. A. Drug-loading strategies: Cargos may be encapsulated in the nanostructures by ligand recruitment, intercalation, hybridization, entrapment and strand modification. B. Targeting strategies: Drug-loaded nanostructures may be designed to reach specific locations by utilizing cell-specific peptides, aptamers, ligands, antibodies or receptor-specific proteins. C. Strategies for improving biostability: Modifications for improving the stability of DNA nanostructures include nucleotide modifications, ligation of nicks, inter-strand cross-linking, hexane diol and hexaethylene glycol functional groups, and protein or polyethylene glycol -based protective layers.

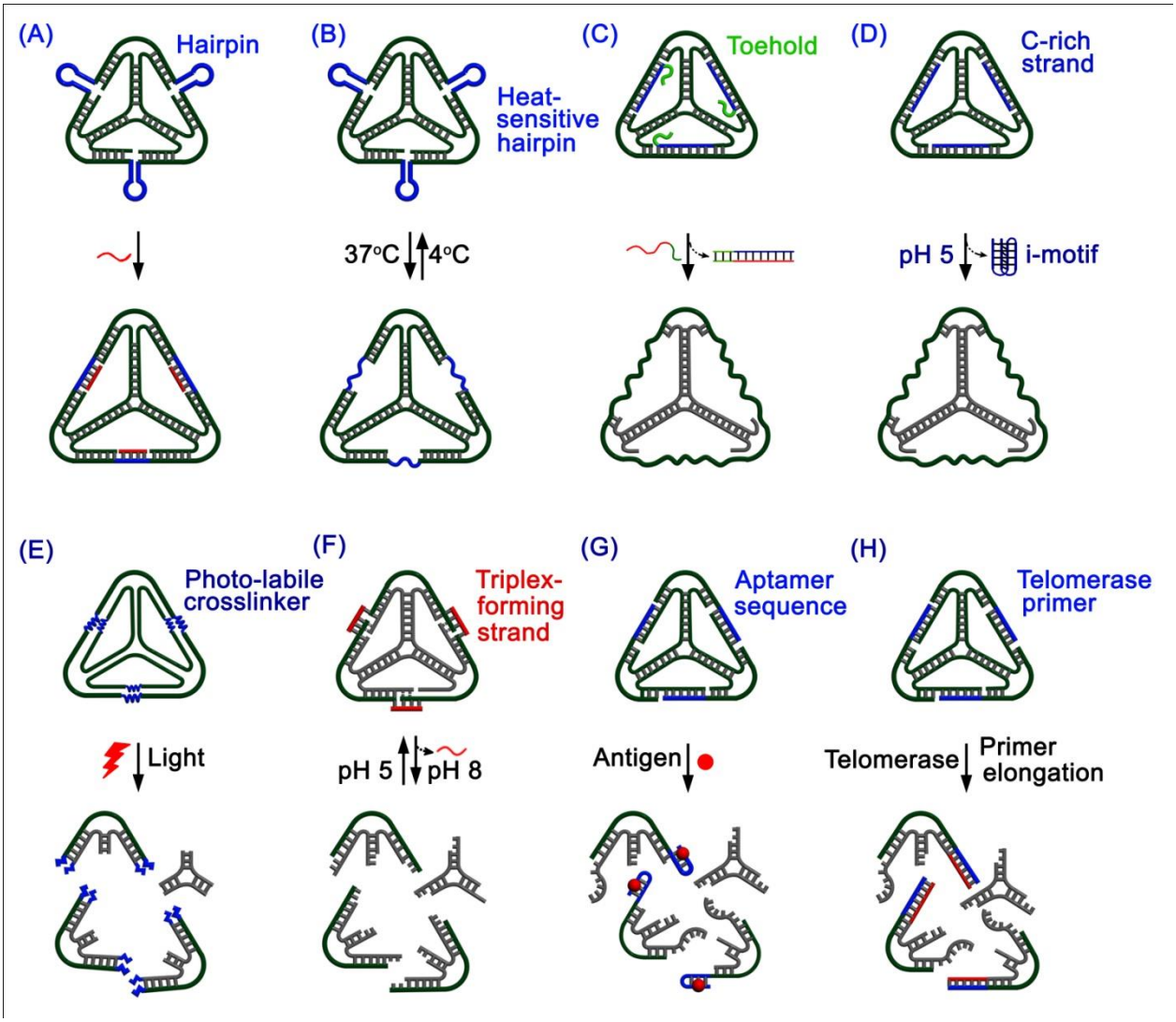


Figure 6: Reconfigurable DNA carriers. The nanostructures may be triggered for releasing the cargos after reaching the target site by (A) an oligonucleotide, complementary to a hairpin nanocarrier region to expand the structure, (B) temperature-triggered nanostructure-expansion, (C) toehold-mediated strand exchang to yield single stranded regions to destabilize the nanocarrier, (D) cytosine-rich strands forming an i-motif at low pH to destabilize the carrier, (E) nanostructures stabilized by photo-labile crosslinkers dissociate on light-exposure to release cargo, (F) nanostructures stabilized by triplex to form oligonicleotide dissociate on pH change, (G) dissociation of nanocarriers owing to aptamer sequences remodeling in sticky ends on recognizing antigens, and (H) primer strands elongation at sticky ends owing to telomerase activity to yield carrier dissociation. Here, modifications have been shown only on the front-faced edges of the tetrahedra.

Table 2. Modifications and biomedical applications of DNA tetrahedrons in the field of cargos-delivery.

Cargos	**Connective approaches**	**Modifications**	**Cell lines** ***In vitro***	***In vivo***	**Ref.**
Doxorubicin	Inserting	L-DNA	Sec7/HeLa	Yes	68,69
		Aptamer	MCF7	No	70
		Aptamer and Folic acid	HT29	No	71
		Tumor-penetrating peptide	U87MG	No	25
		D/L-Sugar	Cancer	Yes	72
Actinomycin D	Inserting	-	*Escherichia coli* / *Staphylococcus aureus*	No	73
Methylene blue	Inserting	Photodynamic	SCC7	Yes	68
			B16F10		
			MDA-MB231		

Pyro	Inserting	Photodynamic	SMMC7721	Yes	67
Floxuridine oligomers	Inserting	Floxuridine oligomers, Cholesterol conjugated ODNs	Colorectal cancer	Yes	74
CpG	Pre-linking	-	RAW 264.7	No	75
CpG ODNs and Streptavidin	Overhang	Biotin-CpG ODNs, CpG ODNs and Phosphorothioate ODNs	Vaccines	Yes	76
siRNA	Overhang	Folic acid Tumor targeting ligands and 2′-O-methyl-ODNs	HeLa Cancer	Yes	24 24
ASOs	Loop Inserting	Lipofectamine 2000 PNA	HeLa MCF7 C2C12 *Escherichia coli*	No No	77 78
Aptamers	Overhang Pre-linking Overhang Overhang	- L-DNA - Folic acid	HeLa NIH3T3 NIH3T3 HeLa A549 MCF7 HT29 HT29	No No No No	79 80 70 71

Chemotherapy

Traditional chemotherapy is utilized to destroy infected or cancerous cells by delivering small molecular drugs such as doxorubicin, actinomycin D, paclitaxel, cisplatin and adinamycin into infected or tumor tissues specifically through inserting a DNA duplex and hindering the biomolecular biosynthesis associated strong anticancer efficacy with poor selectivity, drug resistance, low uptake and strong adverse effect [81-83]. As a promising nanovehicle, cage-like spacious DT, capable in inserting doxorubicin in GC-regions of DNA, showed its higher efficiency compared to free drug to overcome drug resistance avoiding P-glycoprotein and multi drug resistance (MDR) efflux pumps [84]. Paclitaxel, capable to promote tumor cell apoptosis through activating the polymerization of microtubules and inhibiting their depolymerization and ending normal mitosis, was conjugated with DT to treat drug resistant tumor cells for getting higher therapeutic efficacy as antitumor agent in comparison to free drug treatment [51,85,86]. Actinomycin D loaded DT showed its higher uptake and killing efficiency of bacterial cells after entering cells with its degradation by DNase and liberation of drug by RNA synthesis inhibition [73]. An aptamer, a short stretch of single stranded DNA, RNA or polypeptide, having the capability of binding to the corresponding ligand with high specificity and affinity, has been utilized for site specific active cargos targeting. AS1411, a 26-mer DNA aptamer, modified with DNA tetrahedron loaded drug, have been used to treat and kill most efficiently MCF-7 breast cancer cells through the specific binding to nucleolin over-expressed on the surface of tumor cells [25,87,79,88]. MUC1 aptamer-guided DNA tetrahedron, hybridized with an extended sequence at one vertex, was utilized for a targeted doxorubicin delivery into Mucin1-positive breast cancer cells [70]. SL2B, a 26-mer DNA strand, capable to target specific heparin binding domain (HBD) of vascular endothelial growth factor ($VEGF_{165}$), after functionalization with doxorubicin loaded DT and folate caused efficient growth inhibition of HT-29 cancer cells through their surface recognition of VEGF and folate receptors [71]. Tumor-penetrating peptide (TPP) aptamer, capable to bind neuropilin-1 receptor over-expressed on the surface of U87MG human glioma cells, was anchored to one of the vertices of a DT for forming a conjugate with drug for inhibition of tumor cells proliferation with enhanced cellular uptake and killing efficiency [25,89,90]. Nuclear localization signals (NLSs), the amino acid sequences existed in some macromolecular proteins, are needed for some proteins for active transporting to the nucleus through recognition by karyopherins and interacting with nucleoporins. The NLS peptide-modified DNA tetrahedron was utilized to transport to the nucleus of HeLa cells through NLS peptide-specific binding as nuclear targeting from lysosomes to the nucleus [91,23]. Nowadays, drug loaded DT modified by two affibody molecules has shown greater selective efficacies in cellular uptake and killing ability towards HER2 over-expressed breast cancer cells compared to free drug [92].

Immunotherapy

Immunotherapy is an effective treatment technique to cure diseased cells chiefly by the stimulation and activation of host immune system [93-95]. CpG oligodeoxynucleotides (ODNs), derived from viral or bacterial genomes, are capable to link covalently to the lysine or cysteine residues of an antibody to provide strong immune-stimulatory activities recognized by TLR9 [96-99]. Phosphorothiolate modified CpG-DT having stability in serum from enzymatic degradation, showed its higher target efficiency and strong immune response in macrophage-like RAW264.7 cells [75]. The small biotin (vitamin H) molecule, exhibiting a strong binding affinity with avidin or streptavidin protein, may be utilized for site specific loading of cargos in DNA assemblies and their site-selective cellular uptake and controlled release [100-102]. In this context, biotinylated DNA tetrahedron was also used as vehicle to

deliver antigen streptavidin into mice to stimulate strong and continuous antibody responses against the antigen compared to free antigen relating DNA-based delivery system for synthetic vaccines [76]. Furthermore, DNA tetrahedron was utilized as a platform to prepare another type of synthetic vaccines where DT, modified with streptavidin antigen and CpG ODNs-adjuvant, delivered both assembled antigen and adjuvant to diseased cells, followed with the higher level of anti-streptavidin IgGs and the induction of effective immune responses triggering the secretions of IL-6, IL-12 and TNF-α to induct cancer cell apoptosis and necrosis [76,103].

Gene therapy

Therapeutic ODNs such as small interfering RNAs (siRNAs), micro RNAs (miRNAs), antisense oligonecleotides (ASOs) and CRISPR-Cas9, are capable to target their genes following various mechanisms with high selectivity for the treatments of disease-related genes [104,105]. siRNAs act by targeting and inducting the cleavage of certain complementary mRNAs leading to the shutdown of the expressions of mRNA-encoded proteins within the eukaryotic RNA interference (RNAi) pathway [106]. DT, hybridized with siRNA and decorated with the folate molecules, showed their higher selective delivery-efficacy of siRNAs and gene silencing *in vivo* in tumors [24]. Similarly, miRNAs, loaded on DNA nanostructures through hybridization, exhibited their therapeutic efficacies by suppressing tumor growth and blocking cell invasion and metastasis [107,108]. DT, modified with anti-bla CTX-M-group1 antisense PNA (PNA4), showed reduced inhibitory concentration (to CTX) of *E. coli* carrying bla CTX-M-3 [78]. CRISPR-Cas9, a prokaryotic immune system, utilized to resist foreign plasmid and phage DNAs, acts through the recognition of complementary DNA sequences flanked by a 5'-NGGPAM motif by a single guide RNA (sgRNA) for directing Cas9 to cleave the recognized DNA [109-112]. In this concern, DNA nanostructures are being designed with Cas9/sgRNA for their efficient therapeutic deliveries as future human therapeutics [113,114].

Photodynamic therapy

Photodynamic therapy (PDT), a cytotoxic treatment utilized to kill cancer cells by the liberation of singlet oxygen upon irradiation of photosensitized drugs [115,116]. Doxorubicin loaded and pyropheophorbide (pyro) attached DT showed its synergistic efficacies not only to destroy target tumor cells by disturbing gene biosynthesis but also to brighten targeted cells and produce cytotoxic singlet oxygen upon light irradiation [67]. Differently, fluorescent methylene blue loaded DT exhibited its higher therapeutic uptake and cell cytotoxic efficiencies in tumor, propotional to the amount of delivered methylene blue [68]. Furthermore, fabrication of DNA nanostructure with metallic gold nanoparticles exhibited higher cellular accumulation with enhanced antitumor efficacy in tumor cells through photothermal ablation [117-119].

Biodistribution, pharmacokinetics and elimination

All the factors such as size, shape, susceptibility to digestion by enzymes, attachment of ligands, encapsulation, animal model and routes of administration of DNA nanostructures influence their blood residence, tissue distribution and mechanisms of elimination. The labeled tetrahedral nanostructures decorated with folate ligands and loaded with siRNA were exploited to treat tumor through attaching folate receptors over-expressed in Luc-KB cells [24,120]. The *in vivo* fluorescence molecular computed tomography in a Luc-KB xenograft model in athymic Balb/c mice after intravenous injection from 5 min to 24 h and 12 h post injection *ex vivo* organ fluorescence analysis showed that the targeted nanostructures were accumulated primarily in the tumor and kidney and a little accumulation in the liver, spleen, lung or heart. The blood half-life of the nanostructures was ~25 min which was longer than the administered siRNA alone (~6 min). The half-life of the tetrahedrons was longer possibly due to the enhancement in their hydrodynamic radius size caused by the appended siRNA ligands from normal ~7 nm per edge to ~20 nm. Another folate-anchored tetrahedral nanostructures labeled with a near-infrared (NIR) emitter and a radioactive isotope for single-photon emission computed tomography (SPECT) imaging and *ex vivo* analysis showed a greater accumulation in the tumors especially for the folate receptors and less in the stomach, spleen, lungs and heart, whereas free tetrahedrons bearing only the NIR emitter after intravenous injection exhibited their accumulation in the bladder within few minutes with a blood half-life of ~5-3 min in normal healthy ICR mice [121]. The high resolution of SPECT imaging exhibited the accumulation of the nanostructures in the gallbladder and intestines after 2 h intravenous injection, whereas combined NIR and SPECT analysis showed their major accumulation in the bladder within 2 h of intravenous injection [122]. The intravenous injection of biotinylated DT loaded with ruthenium polypyridyl complexes (RuPOP) into nude Balb/c mice bearing HEPG2 tumors exhibited the accumulation of nanostructures primarily in the tumor cells after 6 h injection, assessed by the fluorescence imaging from 6-24 h. After 24 h, the accumulation was also observed in the mice liver [123].

The *in vivo* administered DNA nanostructures are internalized into cells by endocytosis and phagocytosis and degraded in phagolysosomal compartment by lysozymes, DNases, metabolized in liver, degraded in the blood, extracellular milieu and other cells by nucleases specifically at pH 8.0 [18,124,125]. They undergo biliary excretion and kidney elimination through glomerular filtration (< 5 nm diameter), while larger particles may be sequestered in tissue for longer time or re-entered into the systemic circulation in reduced sizes [16,122].

Toxicity

DT nanostructures decorated with folate ligands and siRNAs showed a minimal immune response of marker IFN-α secretion in the blood after 6 h post intravenous injection in C57BL/6 mice [24]. RuPOP loaded biotinylated DT exhibited normal levels of blood biochemical parameters compared to tumor free mice based on the estimations of glucose, aspartate aminotransferase, alanine aminotransferase, total protein, globulin, albumin, albumin-globulin, urea, creatinine, high and low -density lipoproteins, cholesterol, triglyceride, creatine kinase and lactate dehydrogenase in a HEPG2 xenograft model of Balb/c nude mice injected every 2 days for a total of 28 days [123]. The *ex vivo* tissue histopathology exhibited minimum cellular damage, while administration of the RuPOP alone caused pulmonary hemorrhage, indicating DNA nanostructures had insignificant cellular toxicity as a drug delivery carrier.

Conclusions and future perspectives

In general, linear DNA nanostructures are vulnerable to nucleases and lysozymes in cytoplasm and serum, associated with low ionic concentration and pH8.0. However, non-immunogenic three dimensional programmable structures of DT have made them more resistant to easier disassembly, while L-DNA shows more stability than natural D-DNA [68]. For passive targeting, L/D –DT loaded with cargos and / or coated with poly ethylene glycol (PEG) or other vesicles may be more effective due to their favorable site-oriented targeting, membrane penetration capability, suitable biostability and

biocompatibility as delivery vehicle to destroy diseased cells [68,72,126]. For active targeting, DTs may also be decorated with small molecules, ligands and cargos to conjugate, intercalate, encapsulate or bind covalently or non-covalently for enhancing their biostability, elonging their circulation time and changing their appropriate surface and mechanical features to reach to specific target cells. In this context, a thorough systematic investigation specifically on prolonged repeated doses regarding bio-distribution, pharmacokinetics, eliminations, toxicities and effective biological efficiencies especially for oral and intravenous administrations for all differently functionalized DTs in *in vivo* animal models is needed for their proper pharmaceutical and biomedical applications as future therapeutic nanomedicine in clinics to benefit the human beings.

Conflict of interests

The author declares no conflicts of interest.

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