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Review

DNA Nanostructures as Drug Carriers for Cellular Delivery

WU Na and ZHAO Yongxi*

Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an 710049, P. R. China

Abstract Drug delivery systems have been widely developed for enhancing target activity and improving drug functions. Liposomes, high-molecular polymer, gold nanoparticles and carbon nanomaterials, etc., are all the candidates of drug carriers. However, immunotoxicity, heterogeneity and low solubility generally exist and hamper their applications. As a kind of biological materials, DNA has its unique advantages in biomedical applications, including excellent biological compatibility and programmability. DNA nanostructures have been proved to possess high cellular uptake efficiency, which sheds new light on DNA-based drug delivery system. In this review, we summarize the influence factors of DNA nanostructure internalization efficiency, including cell lines, and the size and the shape of DNA nanoparticles. Uniformity of DNA nanostructures in appearance and properties ensures the stability in research, which makes DNA carriers stand out from other nanomaterials. Next, we focus on the functionalization of DNA carriers, which endows DNA nanostructures with the potential to construct integrated drug delivery platforms. We also discuss the internalization pathways of DNA nanostructures and their fate in cells. The deeply understanding about the endocytic pathways provides new sight for the further design strategy on changing the transportation routes of DNA carriers in cells. Finally, the challenges in further applications are discussed, and suggestions are proposed.

Keywords DNA nanostructure; Drug delivery; Cellular uptake; Internalization pathway; DNA-based drug carrier

1 Introduction

In recent years, applications of nanomaterials in biomedical fields have been widely studied, involving clinical diagnosis[1], drug delivery[2], gene transfection[3] and cell tracking[4], etc. Kinds of nanomaterials, such as liposomes[5], polymers[6], gold nanoparticles[7], magnetic nanoparticles[8], carbon materials[9], etc., were exploited and great progress has been made. However, immune toxicity[10] and heterogeneous distribution in size constituted major problems[11]. DNA, as the genetic material of living systems, possesses good biocompatibility and unique programmability, which empowers DNA-based nanomaterial full potentials. Various kinds of DNA manipulation tools and methods guarantee the realization of relevant applications.

With the development of DNA nanotechnology, its advantages in constructing delicate, complex and dynamic nanostructures are increasingly prominent, which greatly promotes the studies and applications in biomedical fields[12,13]. Plasma membrane is a barrier for cells and plays crucial roles at the communications between cells and external environments. The interaction between DNA and biological membrane is the first step for DNA materials participating in cellular functions[14]. Electronegativity and hydrophilia of DNA inhibit its insertions in biomembrane. However, DNA nanostructures show new features, which have been proved to have efficient cellular uptake even without the assist of transfection reagent[15]. This provides precedents for systematic studies of DNA nanostructures as nanodrug carriers. Compared with other nanomaterials, DNA nanostructures have unique superiorities, including good biocompatibility, low toxicity, homogeneity in size, programmability and controllable environmental responsiveness[16,17], which will be meaningful in drug delivery systems[3,18,19]. The preparation principle of DNA nanostructures determines the homogeneity of particles in size and physical/chemical properties, which is crucial for systematic studies and future applications, and is hard to be realized on other nanomaterials. The addressability endows DNA nanostructures with precise positioning and multicomponent modifications to enhance their targeting and controllable release[20].

The cellular uptake efficiencies of DNA nanostructures rely on different cell lines, and also can be influenced by the morphology and surface properties of nanoparticles[11,21]. The endocytosis pathway of DNA nanostructures and their fate in cells also cause researchers' attention, which helps the future design and optimization of DNA nanostructure-based drug delivery systems. In this review, we conclude and discuss the recently correlative work. Of course, this field is still in the

*Corresponding author. Email: yxzhao@mail.xjtu.edu.cn

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early stage, and in need of constant exploration and development. In the last part of this review, the existing problems are analyzed and suggestions are given for the future development.

2 Development of DNA Nanotechnology

With the development of DNA nanotechnology, the realization of various elaborated and complicated nanostructures has promoted relevant fundamental researches and applications in biological and medicine fields[22—25]. In 1982, Ned Seeman[26] proposed an idea for protein-crystallisation with the assistance of 3D periodic lattice assembled by DNA Holliday structures, which opened the door of DNA nanotechnology. Since then, construction methods based on DNA-tile assembly have been deeply exploited. Like LEGO toys, kinds of 3D nanostructures were fabricated, including nanotube, tetrahedron, dodecahedron, icosahedron, buckyball, nanocage and so on[27—30][Fig.1(A)]. On this basis, Yin's group[31] developed a single-stranded DNA tile(SST) method, which pushed the complexity of DNA nanostructures to a new level[Fig.1(B)]. Inspired by the work of William Shih, in 2006, Rothemund[32] used a single-strand DNA composed of 7429 bases, as the scaffold strand, to form various 2D structures with the help of more than 200 short strands, which was called "DNA origami" technology[Fig.1(C)]. This invention is a milestone in the development of DNA nanotechnology.

One of the advantages of DNA origami reflects in its unique addressability and convenient design procedures. Target molecules could be modified on DNA origami at nanoscale precision and multicomponent modifications are available, which enhances the targeting of DNA carriers and makes the DNA nanostructure possible to be a multifunctional drug delivery platform[33]. Good homogeneity in size and properties distinguishes DNA nanostructures from other traditional nanoparticles, and ensures the constancy and stability of DNA-based drug delivery systems. Open-source softwares, such as caDNAno, Tiamat, etc.[34], are developed to help the convenient design and fabrication of complicated 3D DNA nanostructures[Fig.1(D)][35]. It is easy to acquire complex nanostructures even for researchers not in this field, which benefits the promotion and application of DNA nanotechnology in biological and medical fields.

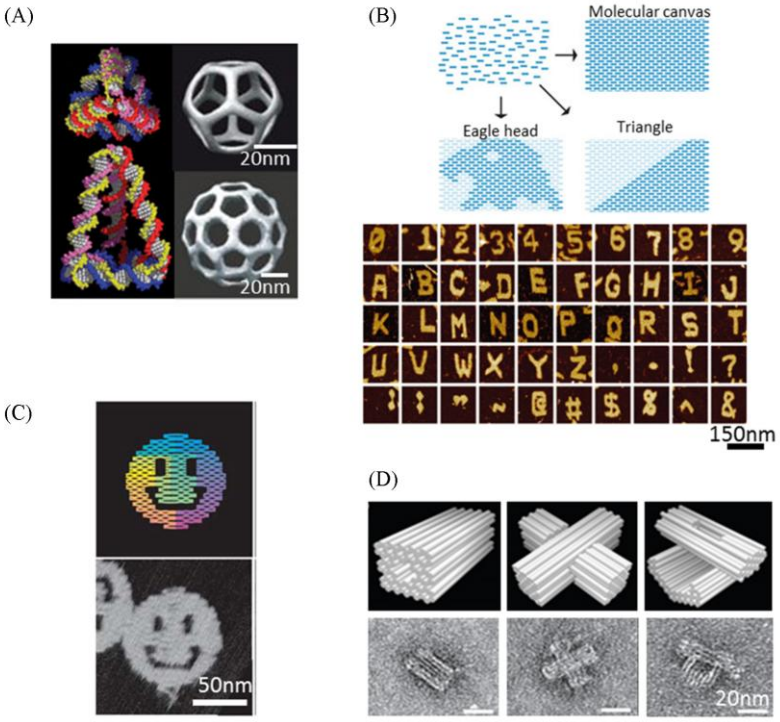

Fig.1 Sophisticated DNA nanostructures based on self-assembly technology

(A) DNA tetrahedron, dodecahedron and buckyball structures, tetrahedron was consisted of DNA strands with 3×20/3×30-bp. Reprinted from refs.[29,30]. Copyright 2005 American Association for the Advancement of Science. Copyright 2008 Springer Nature; (B) complex nanostructures assembled *via* single-stranded DNA tile(SST) strategy. Reprinted from ref.[31]. Copyright 2012 springer Nature; (C) 2D structure manufactured based on DNA origami self-assembled strategy, which was invented by Rothemund in 2006. Reprinted from ref.[32]. Copyright 2006 Springer Nature; (D) hollow or solid 3D DNA origami structures constructed by packed helices. Reprinted from ref.[35]. Copyright 2009 Springer Nature.

3 Biomembrane Properties and Interactions with DNA

Biomembrane is an important component of cells, which plays an important role in cell behaviors and functions, such as endocytosis, exocytosis, cell signal transduction and cell migration. Biomembrane consists of phospholipids, cholesterol, sugars and various membrane proteins, and possesses liquidity, complexity and high degree of cooperation. The aggregation of phospholipid molecules generates rich phase states and phase behaviors, which are mainly divided into liquid ordered phase, liquid disordered phase and solid gel phase[36]. The phase behaviors of phospholipid not only maintain the fluidity of cell membrane, but also correlate with the physiological functions of biomembrane[37]. Phase separation forms locally

microregions due to the reframing of compositions on cell membrane. A kind of microregion, which is rich of cholesterol, sphingomyelin, a large number of receptors and signal molecules, and exists in liquid ordered phase, is called lipid raft. The unique physicochemical properties of lipid raft make it crucial areas for physical behaviors occurring.

DNA is less prone to getting close to biomembrane due to electrostatic repulsion. Generally, there are mainly three methods to promote the interactions between DNA and biomembrane. One of them is electrostatic adsorption. DNA with negative electricity can generate mutual attractions with positively charged biomembrane, such as DOTAP and DOTMA. The second one is that hydrophobic molecules are modified on DNA, such as cholesterol[38,39], porphyrin[40,41] and tocopherol molecules[42], etc. The third one is receptor-ligand interaction, which is a kind of specific recognition method[43]. Among them, hydrophobic molecule-modification helps to extend the stay time of DNA on membranes, which is usually applied on the studies of DNA nanostructures' behaviors on membranes. Due to the 3D configurations, DNA nanostructures are proved to posses high cell-penetrating ability by endocytosis, even though without the help of transfection reagents.

4 DNA Nanostructures as Drug Carriers

Nanoscale drug delivery systems have shown great potentials with improved targeting and enhanced efficacy. DNA nanostructures as drug carriers display unique superiorities, and have the potential to be multifunctional delivery platforms to achieve combined/collaborative therapy[44,45]. At present, a lot of works on DNA nanostructure-based drug delivery have been reported. Small molecule chemotherapeutics are usually hydrophobic and hard for direct administration. Aiming at this problem, Zhang and Zhu's groups[46,47] applied chemical reactions to graft small molecule-drug to DNA strands and further to form DNA tetrahedral structures for enhanced cellular uptake and aggregation of drugs in cells. Using DNA nanostructures, Liedl *et al*.[48] and Fan *et al*.[49] constructed an efficient and non-toxic CPG oligonucleotide-based delivery system and triggered the strong immune response[Fig.2(A) and (B)]. Kim[50], Jiang[33], Zeng[51], Zhao[20], and Chan[52] *el al*. loaded anthracycline doxorubicin(Dox) on DNA nanostructures (tetrahedral frame, plane triangle and other structures with various curvatures) to induce the apoptosis of human breast cancer cells[Fig.2(C)]. Shin group[53] built rectangular and tubular DNA nanostructures with different sizes using DNA brick assemble method to help the delivery of siRNA in tumour cells[Fig.2(D)]. Factors that affect the efficiency, rate and route of cellular uptake of DNA nanostructures are generally concerned problem for researchers in this field[46,11,54,55].

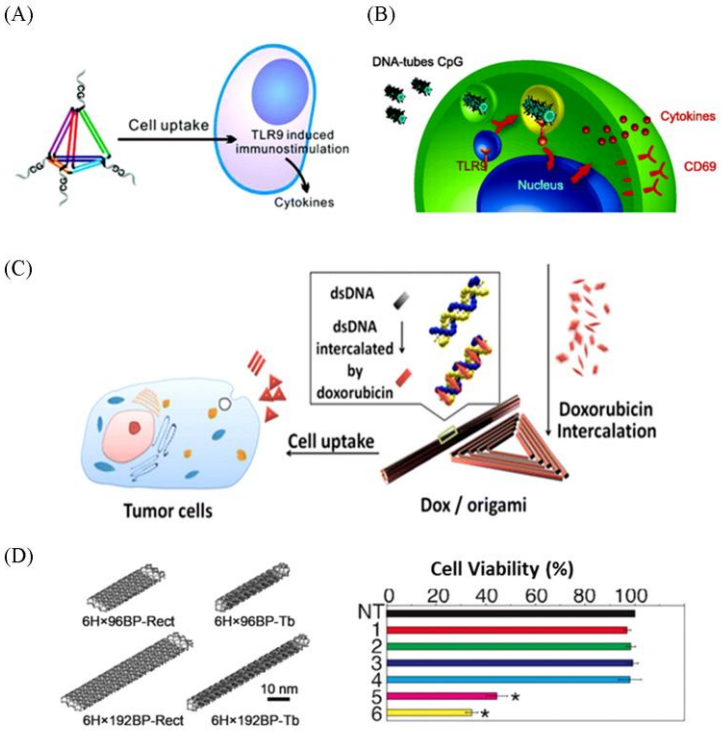

Fig.2 Drug targeting delivery by DNA nanostructure carriers

(A) DNA tetrahedra drug carriers with multivalent cytosine-phosphate-guanine(CpG) were proved to have high cellular uptake efficiency, and this process was noninvasive. Reprinted from ref.[49]. Copyright 2011 American Chemical Society; (B) DNA tubes functionalized with 62 CpG sequences transferred into spleen cells and triggered a strong immune response, which was realized by TLR9 recognition-dependent. Reprinted from ref.[48]. Copyright 2011 American Chemical Society; (C) doxorubicin was efficiently intercalated into DNA carriers, and this system exhibited prominent cytotoxicity to doxorubicin-resistant cancer cells, further inducing an obvious reversal of phenotype resistance. Reprinted from ref.[33]. Copyright 2012 American Chemical Society; (D) various shapes of DNA nanostructures were constructed to transport the siRNA into tumors by targeting anti-apoptotic protein Bcl2, demonstrating that the tumor growth was suppressed. Reprinted from ref.[53]. Copyright 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

5 Efficiency of Cellular Uptake and Functionalization

It is known that the way and efficiency of a pathogen entering a host cell is affected by its shape. Previous studies about nanoparticle-based drug delivery systems have also demonstrated that the composition, size and surface charge of the nanomaterial influence its entry rate and efficiency. Inspired by this, is the cellular uptake process of DNA nanostructures adjusted by its morphology and surface properties? DNA self-assembly technology can realize various 2D and 3D configurations, which offers material basis for the researches of correlation between morphology of DNA-based drug carriers and cellular uptake. Mou *et al.*[46] constructed DNA tetrahedrons, dodecahedrons and buckyballs with different sizes and symmetry as drug carriers. The results showed that the efficiency of cellular uptake increased with the growing in size, and among those structures, buckyballs showed optimal uptake efficiency. Wang *et al.*[11] designed DNA origami carriers with four morphologies for human lung cancer cell lines H1299 and DMS53, and confirmed that the size and shape of DNA nanostructure would affect the uptake. Larger structures offered more chances to contact with receptors on cells, which contributed their high internalization efficiency. They also proved that rod-shape structures had higher cellular uptake efficiency, compared with tetrahedral structures[Fig.3(A)][11]. Bastings *et al.*[54] also found the similar result, which was that more compact structure(low aspect ratio) with higher molecular weight was inclined to be taken by cells. Wang and Bastings *et al.* verified that cellular uptake efficiency was mainly decided by cell types. Fan and Ma's groups[55] applied single-particle tracking experiments and computer simulations to study the behaviors of DNA tetrahedrons into cells[Fig.3(B)]. By the polygon-shape, they found that the corner attack of tetrahedrons and the charge redistribution of the membrane played crucial roles in this process. The results demonstrated that DNA tetrahedrons often get close to cell membranes by the "corner attack" mode, and the ability of "attack" would change with the shape. For example, the form of tetrahedron dimers influenced the internalization efficiency and route. This work provided new ideas for the design of DNA-based nanomaterials in drug delivery systems and gave research basis on the internal mechanism studies for Viruses taken by cells in nature.

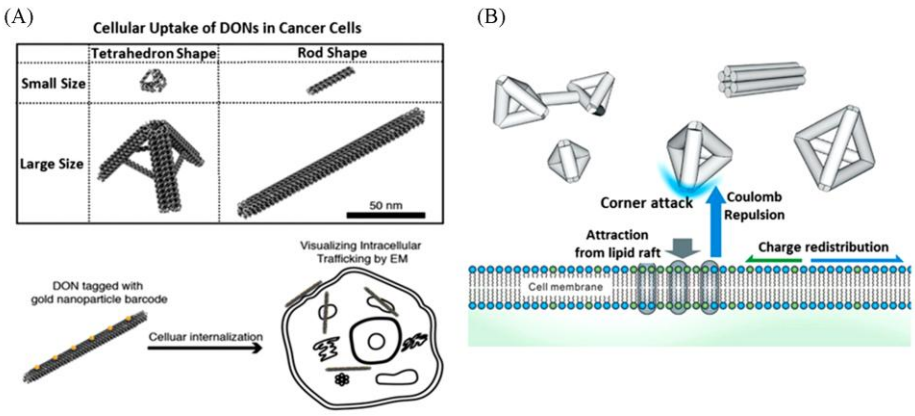

Fig.3 Size/shape-dependent cellular uptake efficiency of DNA nanostructures

(A) The cellular uptake of four DNA origami structures-rod and tetrahedron with different sizes in multiple human cancer cells was investigated. They proved that the internalization of DNA nanostructures was influenced by cell line(the main factor), sizes and shapes. Reprinted from ref.[11]. Copyright 2018 American Chemical Society; (B) single-particle tracking technique and molecule simulations revealed that DNA tetrahedral internalization here mainly depended on caveolin-mediated lipid-raft pathway. DNA tetrahedral got close to cell membrane at "corner attacking" manner to minimize electrostatic repulsion and induced the redistribution of charge on membrane, which was easy to entry into cell. Reprinted from ref.[55]. Copyright 2018 American Chemical Society.

Controllable modification is a convenient manner to increase the stability, biocompatibility and targeting of drug carriers. Homogeneous decoration is relatively easy to be acquired, but the decoration at fixed points and quantity is a challenge. DNA nanostructures show unique superiorities in precise modification due to its programmability, which endows them great potential in high efficiency delivery and controllable drug release[52,56—58]. Kjems's group[59] modified transferrin on flat DNA origami to realize the increase of cellular uptake efficiency by 22 times. Shih's group[60] constructed DNA nanooctahedron structure wrapped with PEGylated lipid bilayer, simulating viral particles for enhanced stability, to protect DNA particles from nuclease digestion[Fig.4(A)]. Modifications of folate[56,57], aptamers[61] and tumor-overexpressed protein receptors[62] endow DNA-based drug carriers specific ability to target tumors. The groups of Biocca, Lin, Ding and Fan have made meaningful explorations in the above field. Zhang's group[46,47] used a chemical method to graft chemotherapeutic small molecules onto DNA nanostructures, and achieved controllable release by introducing disulfide bond. Zhao *et al.*[20] designed DNA nanocarriers with varying degress of global twist to acquire the stacked structures of double helixes with different degrees of looseness, which determined the efficiency of drug encapsulation and release. Qian's group[63] applied the spermidine-mediated DNA self-assembled nanotechnology to realize the magnesium-free assembly of DNA nanostructures. Compared with traditional self-assembly techniques, spermidine DNA composite nanostructure displayed higher thermal stability and better anti-enzyme activity, and had higher cellular uptake efficiency in several researched cancer cell lines. L-DNA is a mirror form of natural D-DNA, which not only possesses the similar programmability as natural D-DNA, but

also displays unique characteristics different from natural DNA. For example, L-DNA can resist catabolic enzymes of nucleic acid and demonstrates enhanced stability in serum. Beyond that, the disturbance with sequences of DNA-based carriers can be effectively avoided[64,65]. The results demonstrated that compared to conventional delivery materials, the mirror DNA nanostructures had the promising potential in drug delivery systems due to enhanced cellular and tissue permeability and greater anticancer effects.

Multi-valence and quantity-controlled modifications could be realized using DNA nanotechnology, which endows DNA nanostructures with the potential to be a multi-functional drug delivery platform for co-delivery and combination therapy[44,66,67]. Ding's group[43] made a series of work in this field, which promoted the application of DNA nanostructures as the integrated platform for drug delivery. They presented a strategy to deliver the platinum drug-56MESS using DNA tetrahedron carrier by intercalating to DNA duplexes[Fig.4(B)]. For target delivery, an anti-EGFR nanobody was chosen to be decorated on DNA tetrahedron to increase the drug accumulation in the tumor region by specifically recognizing. Meanwhile, the signal transduction of EGFR in tumor would be blocked. Compared with other types of antibodies, the camelid-derived single-domain antibody(nanobody) possesses the smallest size, which endows it with higher affinity and specificity for epitope recognition, as well as higher stability. This work provided a promising strategy for the development of a DNA-based versatile platform for targeted and combined tumor therapy. Another DNA-based multifunctional platform example is to integrate a chemotherapeutic drug(doxorubicin), gold nanorods and a tumour-specific aptamer MUC-1 to develop synergistically the chemotherapy combined with photothermal therapy. Song *et al.*[45] applied this system in mucin protein overexpressed MCF-7/ADR cells with the integration of multiple functional elements, including targeting, chemotherapeutics, imaging and photothermal therapy. Li *et al.*[68] developed nanoscale robots with molecular triggers using DNA origami technology [Fig.4(C)]. The robot was a tubular structure loading drugs in its interior, and would be opened to form flat rectangular sheet to release drugs after initiating the molecular triggers. In this system, thrombin molecules were delivered as drugs to tumor-associated blood vessels for blocking tumor blood

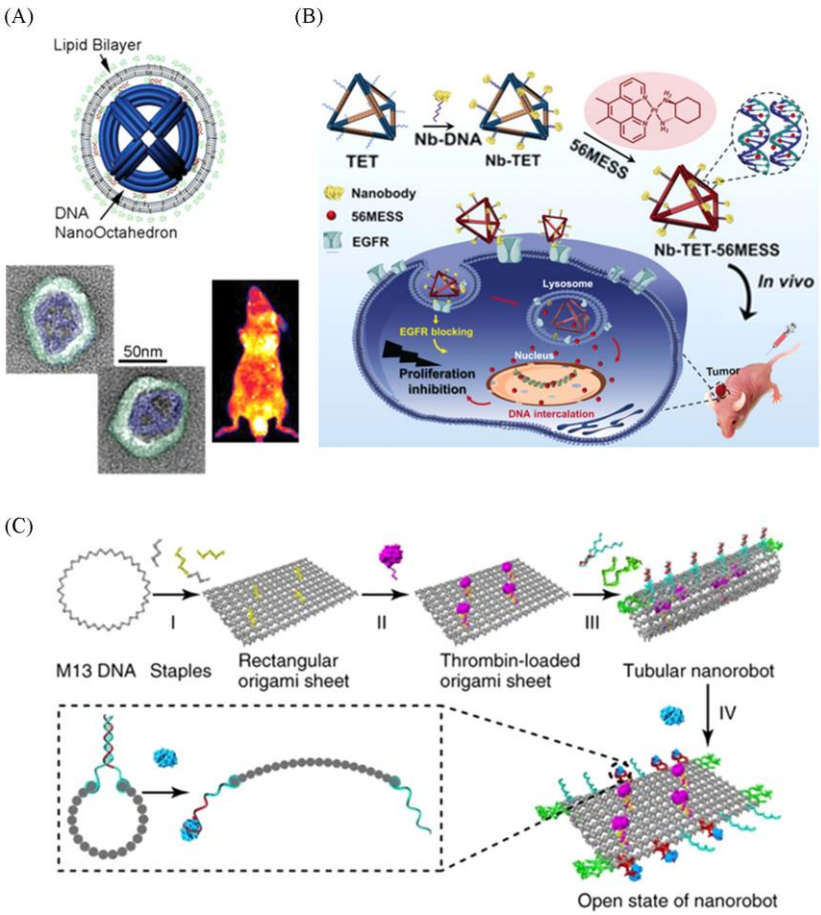

Fig.4 Functionalization improving the properties of DNA nanostructure carriers in drug delivery system

(A) PEGylated lipid bilayer was enveloped on DNA nanooctahedron to resist nuclease digestion and immune activation, meanwhile modification also increased the pharmacokinetic bioavailability. Reprinted from ref.[60]. Copyright 2014 American Chemical Society; (B) multifunctional DNA nanoplatform was designed to delivery the platinum drug-56MESS, and an anti-EGFR nanobody was integrated on this nanocarrier that both targeted and blocked EGFR. Reprinted from ref.[43]. Copyright 2019 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim; (C) DNA nanorobot sensitively responded to the surrounding environments, which was applied to controllable drug release. Nucleolin-targeting aptamer modified on this platform was as targeting domain and as a molecular trigger. After the nanorobot opened at targeting sites, thrombin molecules were exposed to activate coagulation and induce intravascular thrombosis. Reprinted from ref.[68]. Copyright 2018 Springer Nature.

supply and inhibiting tumor growth. This platform displays the application prospect of multifunctional DNA-based drug delivery, by integrating tumor-targeted delivery, recognition of tumor microenvironmental signals and controllable drug release. In a melanoma mouse model, this robot inhibited proliferation of tumor cells.

6 Internalization Processes

The deeply understanding of the internalization pathways of DNA nanostructures in cells and their fate after ingestion is crucial important to the design of DNA-based drug carriers, which has received extensive attention from researchers[69]. Xia *et al.*[70] studied the internalization of tetrahedral DNA nanostructures in different cell lines, selected cells including normal bronchial epithelial cells(BEAS-2B), carcinoma cells(HeLa), and macrophage(RAW264.7). Their work confirmed that DNA nanostructures possessed good biocompatibility in various cells and furthermore endocytic tetrahedron would not disturb the cell physiological functions and cycle progression. They also observed that in HeLa/ BEAS-2B cells, DNA nanostructures firstly moved slowly across the cell membrane(60/80 s), and then suddenly entered into the cells. The internalizing behavior in RAW264.7 cell was different from the above two kinds of cells, and its retention time on the membrane was only 8 s [Fig.5(A)]. Wang *et al.*[11] investigated the internalizing process of DNA rod structure in H1299 cells by decorating 5 nm gold nanoparticles on the surface of rods for convenient observation[Fig.5(B)]. The researchers found that the internalization was divided into four distinct stages: stage I, the nanorods were aligned longitudinally onto the cellular membrane, which helped maximize the contact area with the membrane scavenger receptors; stage II, the nanorods were investigated into the membrane by rotating 90° to minimize energy expense; stage III, nanostructures were transported into early endosome and kept relatively intact in this stage; stage IV, the nanorods were transported into late endosome or lysosome, and severe degradation occurred in such a highly acidic and enzymatic environment. No AuNPs were observed to escape from endosomes or lysosomes, which posed challenges to the applications of DNA nanostructure- based drug delivery systems.

Liang *et al.*[71] studied the translocation of DNA tetrahedrons across cell membranes and the process of intracellular

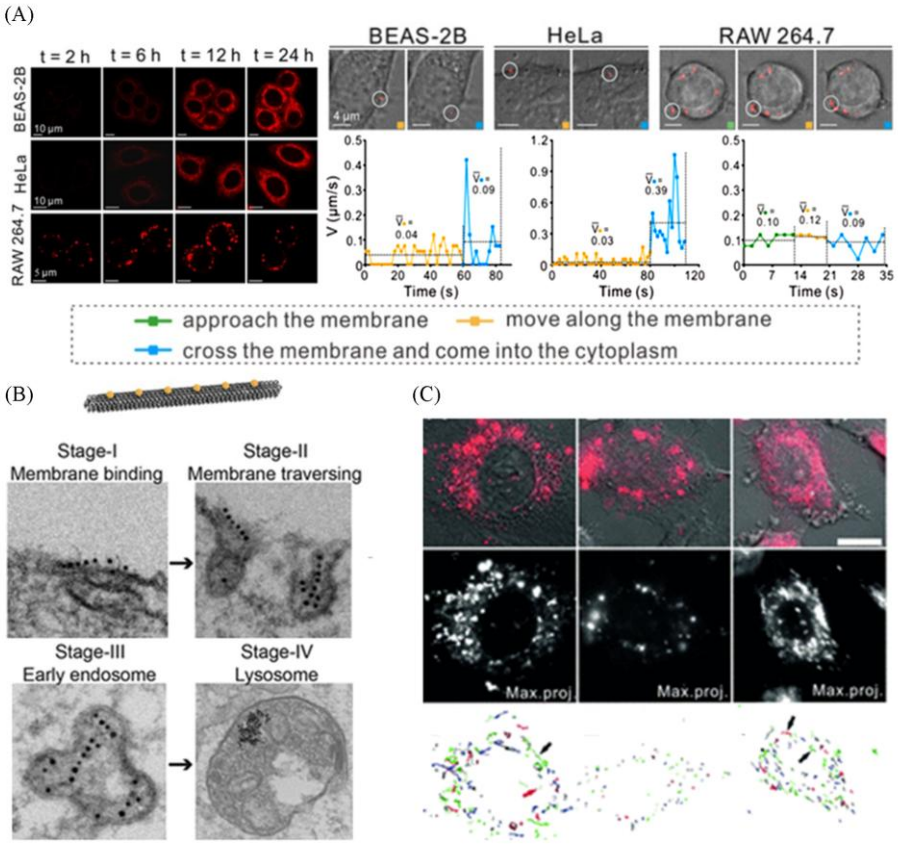

Fig.5 Endocytic pathways of DNA nanostructures in various cell lines

(A) Different cells were incubated with tetrahedral DNA nanostructures for 24 h, including BEAS-2B, HeLa and RAW264.7 cells. Real-time live-cell imaging with a DV Elite microscope revealed the representative trajectories. Green: approaching the membrane; yellow: moving along the membrane; blue: crossing the membrane and coming into the cytoplasm. Reprinted from ref.[70]. Copyright 2018 American Chemical Society; (B) the internalization process of DNA nanorods in H1299 cells was investigated by TEM and 5 nm AuNPs were tagged on the rods for convenient observation. The results revealed that internalization process went through four stages, including: membrane binding, membrane traversing, transported into early endosome and the last stage-aggregation in late endosome and lysosome. Reprinted from ref.[11]. Copyright 2018 American Chemical Society; (C) single-particle tracking technique was used to study the endocytotic internalization and transport of tetrahedral DNA nanostructures in cells. The representative trajectories of 100 randomly selected DNA particles were recorded. Nocodazole and cytochalasin were applied to deduce the internalization pathway. The results demonstrated that the intracellular motility of DNA tetrahedra was dependent on microtubules. Reprinted from ref.[71]. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

transportation by using total internal reflection microscopy, single particle tracing and other techniques[Fig.5(C)][71]. The results demonstrated that the endocytosis was accomplished through a caveolin-dependent pathway, and the entire process was completed within one minute. After internalization, DNA tetrahedrons were transported to the lysosomes by microtubule-dependent and actin filament independent manner. Of course, keeping in the lysosomes is not the perfect state in delivery systems. In order to modulate the cellular fate of the nanostructure, nuclear localization signaling peptides were functionalized to induce DNA tetrahedrons to escape from the lysosomes and transfer to the cellular nuclei. This work improved our understanding of the endocytic pathways of DNA nanostructures and provided new insight for modulating nanoparticle fates in cells. Zeng *et al*.[51] studied the cellular internalization of Dox/DNA origami complexes by using the integrated time-lapse live-cell imaging technology, and tracked the process of drugs release for up to 3 d at the first time. Compared with 2D structures, 3D DNA origami triangle frame exhibited an increasing accumulation of DOX in nuclei. Turberfield's group[72] confirmed that the DNA tetrahedron basically remained intact after cellular uptake for 48 h. These studies have shed new light on the researches and applications of DNA nanostructures in drug delivery systems, deepened our understanding of the cell entry and transport pathways, and presented new sight on the design of DNA drug carriers.

7 Discussion

The development of DNA nanotechnology brings new opportunities for its applications in biomedicine field. DNA nanostructure-based drug delivery system displays unique superiorities, compared with other nanomaterials. Firstly, each DNA nanoparticle formed is completely the same in molecular weight, size and properties, which is unmatched by any other nanomaterials and ensures the stability in treatment. Secondly, DNA nanostructures possess the programmability, especially for DNA origami. Its unique addressability endows DNA carriers with functionalization at precise locations, which provides the potential for constructing integrated DNA drug delivery platforms. Many meaningful research works have been reported, and some drugs have been successfully transferred into cells, including small molecule chemotherapy drugs, CPG sequences, siRNA and so on.

It has been proved that DNA nanostructures could be efficiently internalized by cells without the assist of transfection reagents, and the efficiency is related with the size and shape of nanostructures. Researchers have investigated the entry manner, international pathway and the subsequent fates in cells. The endocytic pathways in various cell lines are different, and also modulated by the morphologies and sizes of nanoparticles, some of which are caveolin-dependent. After entering into cells, most of these DNA carriers with drugs will transfer into lysosomes. In order to escape from being digested, offorts have been made. For example, functionalization targeting organelles are applied to change the transportation pathway in cells.

Though some promising results have been achieved, more efforts needs to be done to promote the efficient delivery of drugs into the target sites in cells. High tumor targeting, low cellular toxicity and immune responses are the advantages of DNA carriers, most of which require the help of functionalized modifications. This may be a double-edged sword, which means that functionalization improves the properties of DNA carriers, while large-scale modifications may hamper the effective load and release of drugs. This issue needs to be considered and balanced. Another considerable problem is the huge cost. DNA is in great demand as drug carriers, but still expensive now. Reducing costs by developing standardized manufacturing processes is a prerequisite to promote the applications of DNA-based drug delivery systems.

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