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Review

Tetrahedral DNA nanocages as delivery agent for biological and biomedical applications

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Abstract: Tetrahedral DNA nanocages have emerged as highly versatile tools for delivering a wide range of biological agents by leveraging their unique structural properties and functional adaptability. This review critically examines the field of tetrahedral DNA nanocages as delivery agents, communicating key findings and insights from existing literature. An extensive examination of the advantages of tetrahedral DNA nanocages as drug-delivery vehicles is outlined, with specific emphasis on their exceptional cargo encapsulation efficiency and controlled release capabilities. An in-depth exploration of in vivo studies and preclinical models is provided, encompassing comprehensive assessments of therapeutic efficacy, pharmacokinetics, toxicity, safety, and targeting capabilities. Moreover, the potential of tetrahedral DNA nanocages in regenerative medicine applications is highlighted. To address future challenges and directions in the field, the review emphasizes the importance of optimization of large-scale synthesis and translational studies. The significant role of tetrahedral DNA nanocages as delivery agents is underscored, showcasing their potential to revolutionize the landscape of targeted and programmable therapeutic interventions.

Keywords: tetrahedral DNA nanocages; delivery agents; biological applications; drug delivery; imaging; targeted therapies

1. Introduction

In the ever-evolving landscape of nanotechnology, DNA nanotechnology has proven itself as a fascinating molecular platform with immense potential for revolutionizing a wide range of biological applications. DNA nanotechnology has captivated the dynamic realm of nanotechnology, proving to have profound implications for diverse biological applications. Exceptional properties such as its biocompatibility, cellular invasion potential, and inherent programmability offer a promising pathway for groundbreaking advancements. By harnessing the remarkable self-assembly capabilities and structural precision of DNA, scientists can engineer intricate nanostructures with unparalleled control and accuracy. The allure of DNA nanotechnology lies in its ability to manipulate the fundamental building blocks of life itself, unlocking a realm of tailored solutions to address complex challenges.

DNA nanotechnology is a novel and interdisciplinary field that harnesses the inherent programmability and self-assembly properties of DNA molecules to create intricate nanoscale structures [1]. Through the precise design and arrangement of DNA strands, researchers have successfully constructed a diverse range of

nanoarchitectures, including DNA nanotubes [2], DNA origami [3], and most notably DNA nanocages [4]. These structures can be tailored to exhibit specific geometries and functionalities, enabling their application in areas such as drug delivery, sensing, imaging, and nanomedicine. DNA nanocages are small, self-assembling structures made of DNA that can be used to carry cargo, typically constructed from several strands of DNA that are designed to fold into a specific shape, forming a cage-like structure as shown in **Figure 1** [5]. These structures show immense promise within a wide variety of applications in medicine, nanotechnology, and biotechnology, as they serve as an inherently biocompatible delivery agent due to their composition.

Figure 1. Visualization of the fundamental components of DNA nanocages, including individual DNA strands, a representative four-way junction, and a range of diverse shapes that can be achieved through DNA self-assembly. The figure provides a visual overview of DNA structure and demonstrates the remarkable versatility of DNA in forming various configurations and shapes. Made with BioRender.

Drug delivery is a highly promising application of DNA nanocages, offering potential advancements in targeted therapeutic interventions. Conventional chemotherapy approaches often exhibit undesirable side effects, leading to detrimental impacts on normal cellular function [6]. However, the development of nanocages with the ability to selectively recognize and bind to specific target molecules, such as cancer cells [7], holds great promise in enabling precise drug delivery exclusively to the intended sites of action. This targeted approach could significantly enhance treatment efficacy while concurrently minimizing off-target effects on healthy cells. Consequently, the utilization of DNA nanocages as carriers

for delivering therapeutic agents represents a pivotal advancement in the field of drug delivery, with profound implications for improving patient outcomes.

Biosensing and Bioimaging technologies have also witnessed remarkable advancements with the emergence of DNA nanocages as the functionalization capabilities of the cages allow for the attachment of imaging markers for accurate targeted fluorescence emission testing and thus [8]. This capability enables the development of sophisticated sensing platforms that offer exceptional selectivity and sensitivity. The integration of DNA nanocage-based sensors holds significant promise in diverse applications, ranging from environmental monitoring to disease diagnosis, where accurate and rapid detection of target molecules is paramount.

Various geometries of DNA nanocages, including tetrahedron, cube, buckyball, and icosahedron, have been explored in the field of DNA nanotechnology. Among these shapes, the tetrahedral DNA nanocage (TDN) has emerged as a particularly well-studied and promising geometry, exhibiting inherent stability and ease of synthesis, making them highly attractive for diverse applications. Furthermore, TDNs have demonstrated superior cellular uptake capabilities, allowing for efficient internalization by cells and potential applications in targeted drug delivery and gene therapy [9].

The remarkable potential of DNA nanocages, including tetrahedral DNA nanocages (TDNs), extends to the field of regenerative medicine and tissue engineering. TDNs have demonstrated the ability to deliver cargo to enhance the differentiation of stem cells, showing promise in addressing tissue regeneration defects [10]. Additionally, aptamer-modified TDNs have shown remarkable capabilities in promoting angiogenesis and vascularization, offering new possibilities for tissue engineering applications [11]. Furthermore, TDNs have exhibited immunomodulatory effects and the ability to facilitate tissue healing, making them attractive candidates for therapeutic interventions in inflammation resolution and tissue regeneration [12]. These findings highlight the versatility and potential of TDNs in advancing regenerative medicine and tissue engineering approaches.

In the subsequent sections of the paper, we delve into the intricacies of tetrahedral DNA nanocages (TDNs). This includes their design, synthesis, characterization techniques, functionalization, and modification strategies. We explore their remarkable potential in drug delivery and biological applications, such as their advantages as drug-delivery vehicles, controlled cargo release capabilities, and in vivo studies utilizing TDNs. Additionally, we discuss the exciting prospects of TDNs in regenerative medicine, specifically their role in enhancing stem cell differentiation and promoting angiogenesis. Finally, we address the challenges and future directions in optimizing TDNs and highlight the potential translational studies to further advance their applications.

2. Tetrahedral DNA nanocages

As researchers continue to explore the vast capabilities of these nanoscale architectures, tetrahedral DNA nanocages (TDNs, otherwise known as the tetrahedral framework nucleic acid (tFNA) or tetrahedral DNA Nanostructures (TDNs)) have garnered significant attention for their unique properties and

promising prospects as a delivery agent in the field of biological applications. These nanocages, characterized by their tetrahedral shape composed of DNA strands, offer a myriad of exciting possibilities for various biological applications. Their well-defined structure, stability, and programmability make them highly versatile platforms for precise cargo delivery, sensing, and imaging [9]. With these distinct properties in mind, their immense potential became apparent, prompting in-depth investigations into their unique functionalities and therapeutic capabilities. The tetrahedral shape of TDNs provides a robust framework for encapsulating and delivering therapeutic cargoes, such as drugs or nucleic acids, to specific target sites [13]. The well-defined structure of TDNs allows for precise control over cargo loading and release kinetics, enabling efficient and controlled delivery.

2.1. Design and synthesis of TDNs

The design of TDNs is a critical stage that lays the foundation for their successful synthesis and subsequent utilization in diverse applications. This design stage encompasses aspects such as sequence selection, secondary structure prediction, and consideration of desired functionalities. Through careful consideration of these aspects concerning the specific synthesis route, researchers have been able to achieve the desired tetrahedral architecture and functional properties [4]. Building upon this DNA design framework, the subsequent stage focuses on the synthesis and assembly techniques employed to transform these designed DNA sequences into functional TDNs.

Among the various synthesis methods available, the one-pot synthesis approach stands out as an exemplary method that best showcases the self-assembly of these nanocages (**Figure 2a**). This method allows for the simultaneous mixing of individual DNA strands, prompting their spontaneous folding and assembly into the desired tetrahedral structure. By carefully controlling reaction conditions and optimizing the concentration ratios of the constituent DNA strands, researchers can achieve an efficient and reproducible synthesis of TDNs without the need for latches or staples [14].

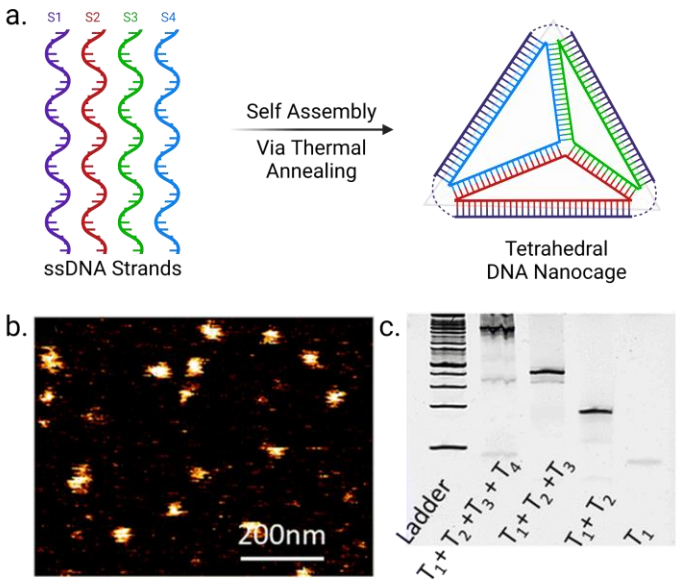

Figure 2. Synthesis of tetrahedral DNA nanocages **a)** visualization of the synthesis and structure of tetrahedral DNA nanocages. It displays the four individual DNA strands that comprise the nanocage, each represented by a distinct color and label. The figure highlights the symmetrical arrangement of the three-way junctions at each vertex. Made with BioRender; **b)** 200nm 2D AFM image of TDN reproduced from Singh et al. [15] with permission from RSC; **c)** 10% native PAGE of TDN synthesis reproduced from Singh et al. [15] with permission from RSC.

To effectively synthesize the TDNs, many factors that influence the folding kinetics and yield must be considered, the most influential being the concentration of DNA strands, the use of auxiliary agents or additives, and the environment of synthesis. Higher DNA strand concentrations can promote faster self-assembly but may also increase the likelihood of unwanted aggregation, misfolding, and unwanted precipitation of structures. Lower DNA strand concentrations, however, can adversely affect the yield of fully formed structures. Typical synthesis concentration has been found to occur around concentrations ranging from a low end of 200 nM to a more typical 10 μM per strand (equimolar) resulting in solution concentrations of 50 nM to 2.5 μM tetrahedron respectively [16].

Various experimental conditions, including temperature, ion presence, and pH highly influence the self-assembly processes of TDNs. Higher temperatures generally accelerate DNA strand hybridization and promote faster self-assembly; however, extreme temperatures can denature the DNA strands and disrupt the formation of desired nanostructures [17]. Different ions present in the solution can influence the screening of electrostatic repulsion between DNA strands, thereby modulating the hybridization kinetics and stability of the assembled structures. Changes in pH can alter the ionization states of DNA strands, affecting their hybridization behavior [18]. Furthermore, different buffer systems can influence the nanocages' overall stability and assembly kinetics [19]. As a preventative measure for complications due to the above factors, nuclease-free water is typically used for synthesis [5,9,20].

TDNs exhibit unique structural features and geometrical properties that contribute to their functional versatility. The tetrahedral DNA nanocage has a highly symmetrical structure, with each of the four vertices containing a three-way DNA junction. The stability of the nanocage is due to the complementary design of the DNA strands which allow for the formation of strong Watson-Crick base-pair interactions [14]. Additionally, the presence of specific structural motifs such as aptamers can enhance the stability and resistance to degradation in vivo as demonstrated by Han et al. in their study of aptamer-modified TDNs for anti-tumor therapy [21].

2.2. TDN characterization techniques

Characterization techniques play a crucial role in verifying the successful generation of TDNs. These techniques encompass various approaches for visualizing, measuring, and analyzing different aspects of TDNs. Transmission Electron Microscopy (TEM) is employed to visualize the nanoscale morphology and structure of TDNs [22,23]. It provides detailed insights into the arrangement and overall shape of the nanocages. Atomic Force Microscopy (AFM) enables high-resolution imaging and precise measurements of TDNs [4,9] (**Figure 2b**). It offers a topographical view of the nanocages, allowing researchers to examine their surface features and dimensions. Dynamic Light Scattering is utilized to analyze the size distribution and stability of TDNs in solution [4,9]. This technique provides information about the hydrodynamic size and aggregation state of the nanocages. Native polyacrylamide gel electrophoresis (PAGE) [4] serves as a method for assessing the purity and homogeneity of TDNs. It allows researchers to separate and analyze the nanocages based on their migration patterns in a gel matrix (**Figure 2c**). Cryo-electron microscopy (Cryo-EM) [3] is a powerful technique used to obtain three-dimensional structural information of TDNs. By rapidly freezing the samples, Cryo-EM captures the nanocages in their native state, revealing their intricate architecture and organization.

Additionally, spectroscopic techniques such as UV-Vis spectroscopy, fluorescence spectroscopy, and circular dichroism (CD) spectroscopy can provide information about the optical properties and secondary structure of TDNs, allowing researchers to study the absorption, emission, and chiroptical properties of the nanocages [1]. These characterization techniques enable a comprehensive understanding of the structural features and properties of TDNs and provide insights into their structural stability and conformation. Further utilization and development of these techniques could lead to advancements in the utilization and insights into the nature of TDNs as a Delivery Agent for Biological Applications.

2.3. TDN characterization techniques

The unique shape and surface characteristics of TDNs facilitate enhanced cellular uptake, allowing for efficient internalization by cells [9]. This superior cellular uptake capability of TDNs is particularly significant as it opens possibilities for targeted drug delivery and gene therapy. The efficient internalization by cells enables the TDNs to deliver therapeutic payloads directly to the desired cellular

locations, enhancing treatment efficacy while minimizing off-target effects. The well-established synthesis protocols for TDNs further contribute to their widespread exploration and potential applications in biomedical research.

TDNs offer significant potential for targeted drug delivery and customization through functionalization with ligands, enabling precise targeting of specific cell types and receptors [24]. This section explores various methods for introducing nanocages with functional groups, modifications, or ligands, facilitating their tailored applications further highlighted by a visual overview in **Figure 3**. Several methods are available for introducing functional groups, modifications, or cargo molecules into TDNs, enabling their customization for specific applications.

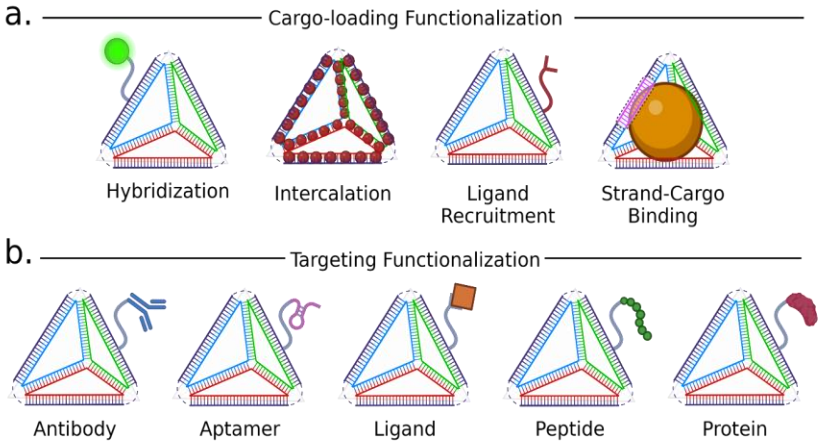

Figure 3. Visualization of the functionalization of the tetrahedral DNA nanocage. It visually represents the modified nanocage with **a)** various cargo functionalization methods; **b)** targeting functional groups. Made with BioRender.

One approach involves the modification of preformed nanocages by introducing functional groups or cargo molecules post-assembly. The versatile nature of DNA plays a crucial role in this process, as the functional groups present on DNA strands provide opportunities for binding various types of groups and cargo. Functional molecules can be covalently attached to the nanocages through specific chemical reactions, establishing a stable and long-lasting linkage [25]. This capability allows for the incorporation of a wide range of functional molecules, including targeting ligands, imaging agents, and therapeutic payloads, expanding the potential applications of DNA nanocages in areas such as targeted drug delivery, bioimaging, and biosensing. Common covalent attachment techniques include bioconjugation methods such as amide bond formation, thiol-ene chemistry, click chemistry and carbodiimide coupling. Click chemistry such as azide-alkyne cycloaddition (CuAAC) or strain-promoted alkyne-azide cycloaddition (SPAAC) provide highly efficient and selective attachment of functional groups through biorthogonal reactions performed under mild conditions [7]. These techniques provide robust and irreversible attachment, ensuring the functional molecules remain associated with the nanocages throughout the desired application.

Other approaches relying on non-covalent interactions to achieve functionalization present an alternative means of modifying TDNs. These

interactions include intercalation and intermolecular interactions (such as electrostatic interactions and hydrogen bonding), which enable the attachment of functional groups with complementary properties. Intercalators, such as Doxorubicin (Dox) for example, can intercalate (insert themselves) between the DNA base pairs. This results in the integration of the intercalator into the nanostructure itself as a method for treating targeted cancer cells in vitro studied by Xia et al. [24]. Additionally, non-covalent interactions, such as electrostatic interactions [7,23,26], π-π stacking [1,27], and hydrogen bonding [1,18,27] have been explored for functionalizing TDNs. These interactions allow for a reversible non-covalent attachment of functional groups onto the nanocage surface, offering versatility in modification. Electrostatic interactions enable the binding of oppositely charged functional groups [26], π-π stacking facilitates the attachment of aromatic molecules [27], furthermore, hydrogen bonding exploits the inherent hydrogen bonding property to attach functional groups that contain appropriate hydrogen bond acceptors or donors to the nanocage surface [27].

The ability for functional modification of the properties of TDN opens many avenues for their clinical application. Drug delivery is one such avenue in which these nanocages can be tailored to target specific cells, tissues, or organs within the body. This targeted approach enhances the delivery efficiency of drugs while minimizing off-target effects, providing a highly effective strategy for therapeutic interventions [7]. Encapsulation strategies provide an additional avenue for modification by incorporating cargo molecules inside the cavities of TDNs, enabling the controlled release of the cargo and protection against degradation [28]. Various techniques, including intercalation, electrostatic interactions, or specific binding [29] can be employed to encapsulate cargo molecules, offering flexibility and customization options [26]. These strategies expand the functionality of TDNs and broaden their potential applications.

Immunotherapy benefits significantly from the use of these modified nanocages, as they can be harnessed to deliver immunomodulatory agents, such as peptides or antibodies to specific cells at target sites. By precisely modulating the immune response, nanocages enhance the efficacy of immunotherapeutic treatments, offering new avenues for precision medicine as analyzed by Cremers et al. (2021) [30]. The parameters that influence nanostructure performance were explored and evaluated, highlighting the controllability of receptor activation with TDNs on a nanoscale. Furthermore, functionalized nanocages have emerged as promising carriers for antigens in vaccine development. By encapsulating antigens within the nanocage structure, they can improve immune response and vaccine efficacy. The controlled release and targeted delivery of antigens enhance the immune system's recognition and generate a robust immune response [31]. In gene therapy, functionalized nanocages can be utilized as carriers for delivering therapeutic genes to target cells. By incorporating specific ligands or functional groups, nanocages can facilitate the targeted delivery of gene therapies, offering potential treatments for genetic disorders or diseases [26].

Moreover, functionalized TDNs have shown promise in stem cell applications. They can serve as carriers for delivering growth factors, signaling molecules, or other therapeutic agents to regulate stem cell behavior and enhance their

differentiation potential. These characteristics are exemplified by studies such as one done by Zhou et al. (2021) [32] that showed that TDNs decreased the release of pro-inflammatory cytokines and levels of cellular reactive oxygen species in periodontal ligament stem cells, which promoted osteogenic differentiation. Similarly, a study conducted by Li et al. 2022 [33] demonstrated that functionalized TDNs facilitated the targeted delivery of specific microRNA molecules, such as MiR335-5p, to mesenchymal stem cells (MSCs). This delivery strategy resulted in enhanced osteogenic differentiation of MSCs and showed potential for addressing bone regeneration challenges. The controlled release of therapeutic molecules by functionalized TDNs provides a tailored approach to manipulate stem cell behavior and promote tissue regeneration in applications such as bone tissue engineering and regenerative medicine. Nanocages provide a controlled and precise environment for stem cell manipulation, offering new possibilities for regenerative medicine and tissue engineering.

The continuous advancements in functionalized TDNs underscore their remarkable potential for future applications. By developing efficient synthesis methods, expanding functionalization techniques, refining controlled release strategies, and advancing targeted delivery systems, these nanocages are poised to revolutionize medical interventions and enable highly efficient and personalized approaches to healthcare.

3. Drug delivery and biological applications

3.1. The advantages of using TDNs as drug-delivery vehicles

TDNs offer significant advantages as drug delivery vehicles. One of their key strengths lies in their high stability, which ensures the integrity and effectiveness of encapsulated therapeutic agents. The rigid and stable framework of TDNs provides robust protection to the payload, shielding it from enzymatic degradation and harsh physiological conditions [34]. This enhanced stability extends the shelf life of the encapsulated drugs and improves their efficacy upon delivery.

Another advantage is the biocompatibility of TDNs. As these nanocages are composed of DNA, a naturally occurring biomolecule that is well-tolerated by the human body, the nanocages are similarly biocompatible. This biocompatibility minimizes the risk of adverse immune reactions and offers a favorable safety profile for drug delivery applications. The use of DNA as the building material for nanocages enhances their biocompatibility and supports their potential for clinical translation as studied by Bhaskar et al. and supported by various in vivo studies to be discussed [35].

Furthermore, functionalized TDNs enable targeted drug delivery, which is a significant advantage in therapeutic interventions. By attaching targeting ligands, such as antibodies, aptamers, or peptides, to the nanocage surface, specific recognition and binding to target cells can be achieved as previously discussed in section 2.2. This targeted approach enhances the delivery efficiency of drugs while minimizing off-target effects. By precisely directing the therapeutic agents to the desired cells or tissues, TDNs maximize the therapeutic effect and reduce potential side effects. The versatility of functionalized TDNs becomes further evident in

biosensing applications. These nanocages serve as dynamic platforms for the detection of biomolecules and analytes, enabling sensitive and specific sensing capabilities. By incorporating appropriate ligands or probes, nanocages can selectively interact with target molecules, facilitating accurate and reliable detection methods [8,30,36]. The integration of these sensing or imaging agents onto drug-delivery functionalized cages gives rise to exciting possibilities in nanomedicine; the dual-functionalized nanocages can act as multifunctional carriers, delivering therapeutic payloads while simultaneously allowing for real-time monitoring of therapeutic responses. One notable study that utilizes this concept was done by Han et al. in 2019 [21]; DOX was encapsulated in n-apt TDN and administered in vivo. The tissue distribution was analyzed in real-time through fluorescent imaging techniques and resulted in the visualization of tumor uptake of DOX and reduction of tumor size throughout a 13-day observation period. This theranostic approach enables precise treatment monitoring and paves the way for a precise personalization of medicine as represented in **Figure 4**.

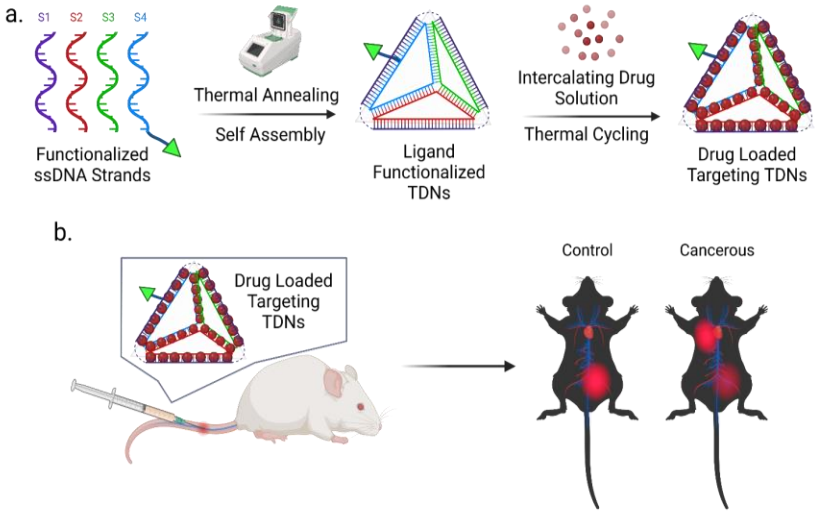

Figure 4. Visualization of theranostic capabilities of functionalized tetrahedral DNA nanocages. This visual representation emphasizes the multi-functionality of the nanocages, enabling the delivery of therapeutic payloads while facilitating real-time monitoring or imaging of the therapeutic responses, showcasing the potential of functionalized tetrahedral DNA nanocages in theranostic applications. **a)** functionalization steps for a possible combination therapy utilizing TDNs. **b)** administration of combination therapy and in vivo effects demonstrated by representation of fluorescence imaging in control and positive (cancerous) mouse models. Made with BioRender.

Moreover, TDNs exhibit efficient cellular uptake and internalization; in comparison to other geometries TDNs showed maximum internalization as studied by Gada et al. (2023) [20] and Rajwar et al. [9]. They are readily taken up by cells and can be internalized into the cytoplasm or specific organelles, allowing for intracellular delivery of encapsulated cargo, and ensuring effective drug action at the desired site.

3.2. Controlled cargo release capabilities of TDNs

Tetrahedral DNA nanocages exhibit remarkable controlled release capabilities, making them versatile and efficient drug delivery vehicles. Incorporating responsive elements such as pH-sensitive linkers into the nanocage design enables tight control over cargo release in response to the desired stimulus.

In pH-responsive release, tetrahedral DNA nanocages exploit pH variations to initiate cargo release as studied by Liu et al. [18]; by integrating pH-sensitive linkages or moieties, the nanocages undergo conformational changes or disassembly in response to pH fluctuations. This pH responsiveness offers the advantage of targeted release within specific acidic environments, such as tumor tissues or intracellular compartments, thereby enhancing therapeutic efficacy while minimizing off-target effects.

The development of new release methods and the exploration of additional external cues provide avenues for further improving targeting specificity and advancing drug delivery strategies. These controlled release capabilities, coupled with the advantages discussed earlier, set the stage for in-depth exploration of tetrahedral DNA nanocages in vivo and in preclinical models as promising drug delivery vehicles. In the following section, we delve into the exciting realm of in vivo studies and preclinical models to evaluate the therapeutic efficacy, biodistribution, and safety profiles of tetrahedral DNA nanocages as drug delivery vehicles.

3.3. Exploration of In vivo studies and preclinical models of tetrahedral DNA nanocages as drug delivery vehicles

In vivo studies and preclinical models have demonstrated the potential of tetrahedral DNA nanocages as drug delivery vehicles, evaluating their therapeutic efficacy, biodistribution, and safety profiles. The results have shown promising outcomes, indicating the potential of these nanocages for clinical translation. Some of the main categories of research are explored below; the selection of studies is not extensive but rather dictated solely on notability and novelty. Further research on the fields of interest is suggested to have a complete understanding of the field in its current state.

3.3.1. Toxicity and safety studies

Evaluating the safety profile of TDNs is crucial for their clinical translation. Toxicity studies assess the potential adverse effects of the Td nanocages on various organs and systems. These studies investigate parameters such as acute toxicity, immunogenicity, and long-term safety to ensure the biocompatibility and tolerability of the nanocages.

In a recent comprehensive study conducted by Wamhoff et al. [37], the safety profile of wireframe DNA origami nanostructures was thoroughly investigated. The study employed mouse models to evaluate the acute toxicity and biocompatibility of these nanostructures. Various doses of DNA nanostructures were administered to assess their potential impact on vital organ functions, general health, and behavior. Notably, the results of this study revealed no significant acute toxicity or adverse effects associated with the administered DNA nanostructures. While the focus of the

evaluation was not specifically on TDNs, these findings provide valuable insights into the overall safety and biocompatibility of wireframe DNA nanostructures. Consequently, it is reasonable to infer that TDNs, being a type of wireframe DNA nanostructure, are likely to demonstrate similar characteristics. However, it is crucial to emphasize the necessity for further research specifically targeting TDNs to establish a more direct assessment of their safety profile.

In another recent preclinical study by Wamhoff et al. [37], the immunogenicity of TDNs was investigated to assess their potential for safe clinical application. The study involved the administration of TDNs to animals, followed by the evaluation of immune system activation. This evaluation encompassed the analysis of pro-inflammatory cytokine production and the presence of immune cell infiltration to determine whether TDNs elicited an immune response that could potentially restrict their clinical utility. Encouragingly, the study revealed minimal immunogenicity associated with TDNs, indicating their promising prospects for safe use in clinical settings.

3.3.2. Therapeutic efficacy studies

In these studies, the effectiveness of TDNs as drug carriers is evaluated by assessing their ability to deliver therapeutic agents and achieve the desired therapeutic outcomes. This includes investigating the nanocages' ability to enhance the efficacy of the encapsulated drugs in treating specific diseases or conditions.

TDNs have been utilized for the delivery of chemotherapeutic drugs such as doxorubicin. In studies by Liu et al. [39], Xia et al. (2019) [24], and Xu et al. (2022) [38], doxorubicin was intercalated with TDNs, resulting in enhanced cellular uptake and cytotoxicity against cancer cells. The nanocages efficiently delivered doxorubicin to the nucleus of cancer cells, leading to increased therapeutic efficacy. Additional chemo-drugs such as daunorubicin [40], curcumin [41], and antisense [42] have been utilized, resulting in similar outcomes.

Therapeutic efficacy studies were conducted to evaluate the potential of TDNs as carriers for chemotherapeutic drugs by Liu et al. at the University of Chinese Academy of Sciences, Beijing in 2018 [39]. In a mouse model of breast cancer, the nanocages were loaded with the drugs and assessed for their ability to deliver the therapeutic agents effectively. Remarkably, the nanocages demonstrated efficient drug delivery to the tumor cells, leading to substantial inhibition of tumor growth and notable improvements in survival rates, surpassing the efficacy of free drugs. These findings underscore the promising therapeutic potential of TDNs as advanced drug carriers in the treatment of specific diseases or conditions.

TDNs have also demonstrated potential for delivering small interfering RNA (siRNA) to silence specific genes in cancer cells. In studies by Han et al. [22] and Lee et al. [43], siRNA targeting ECE-1 was loaded onto TDNs. The functionalized nanocages effectively delivered siRNA into cancer cells, resulting in gene silencing and inhibition of tumor growth in a xenograft mouse model.

Combination therapy is one conceptual application for TDNs, as its functionalization allows for multiple therapeutic agents to be delivered simultaneously to enhance treatment efficacy. In a study by Liu et al. [39], TDNs loaded with both doxorubicin and siRNA were used for combination therapy against

cancer cells. The synergistic effect of dual drug delivery led to significantly improved cytotoxicity and apoptosis induction through the downregulation of Pgp and surviving expression compared to individual treatments.

3.3.3. Pharmacokinetics studies

Pharmacokinetics studies aim to understand how TDNs are distributed throughout the body after administration. These studies investigate the clearance, accumulation, and localization of nanocages in various organs and tissues over time. They provide important information about the nanocages' behavior and potential targeting capabilities. The biodistribution of fluorescently labeled TDNs was investigated in a mouse model in vivo by Zhang et al. (2022) [23]. The nanocages were intravenously injected through the tail vein, and their distribution in various organs and tissues was assessed using fluorescence imaging-assisted histology. The results showed a preferential accumulation of nanocages in the kidney followed by urinary excretion, indicating their clearance pathways to follow natural excretion pathways unless targeted to a specific tissue, in which they exhibited an accumulation at that tissue. Targets specifically tested in this study include skin cancer showing tumor-specific accumulation when functionalized with siRNA.

In a study by Jiang et al. 2016 [44], the pharmacokinetics of TDNs functionalized with Dylight 755 fluorescent dye, folic acid, and radioactive isotope technetium-99m were evaluated in mice through single-photon emission computed tomography (SPECT) and near-infrared (NIR) fluorescence imaging. Pharmacokinetic parameters such as clearance, half-life, and volume of distribution were calculated to understand the systemic behavior of the nanocages. It was found that TDNs had twofold longer circulation time in the blood system than double-stranded (ds)DNA. In a review of the in vivo utilization of DNA nanostructures by Okholm et al. [45], the pharmacodynamics of TDNs were examined. TDNs were investigated in a multitude of animal experiments, particularly in mice. Studies showed that siRNA-decorated TDNs could accumulate in tumor xenografts and effectively knock down gene expression. However, higher dosages were required for efficient delivery. The biodistribution analysis revealed accumulation primarily in the liver and kidneys, with the liver being the main organ for accumulation and the kidneys responsible for excretion through the urine.

3.3.4. Targeting and specific delivery studies

In these studies, researchers explore the ability of functionalized TDNs to achieve targeted drug delivery. They investigate the nanocages' capability to selectively accumulate and deliver therapeutic agents to specific cells, tissues, or disease sites, thereby improving treatment outcomes and minimizing off-target effects.

One notable study by Jorge et al. [46] focuses on utilizing DNA nano scaffolds as delivery vehicles for 5-fluoro-2'-deoxyuridine (FdUn) oligomers in colorectal cancer treatment. Considering the limitations of fluoropyrimidines like 5-fluorouracil (5-FU) in terms of poor specificity and tumor cell resistance, the researchers employed DNA tetrahedron (Td) and rectangle DNA origami as nano scaffolds. They incorporated FdUn oligomers into these DNA nanostructures and enhanced cellular uptake by attaching cholesterol moieties to the Td and DNA origami staples.

The study demonstrated that the functionalized DNA nanostructures exhibited heightened cytotoxicity and increased induction of apoptosis in colorectal cancer cells compared to conventional 5-FU and FdU treatments, particularly with cholesterol as an internalization helper. The cytotoxicity of the FdUn nanostructures correlated significantly with the cholesterol content. Furthermore, the DNA nano scaffolds loaded with FdUn effectively addressed the low sensitivity of colorectal cancer cells to 5-FU. Both DNA nanostructures displayed comparable cytotoxic effects, with Td demonstrating superior antiproliferative activity, primarily dependent on the concentration of DNA nanostructures.

These findings highlight the potential of self-assembled DNA nanoparticles as efficient carriers for delivering fluoropyrimidines, opening new avenues for the development of promising therapeutics in cancer treatment, specifically targeting colorectal cancer. Moreover, the success of this study emphasizes the significance of functionalized nanocages in achieving targeted drug delivery, as they exhibited enhanced accumulation in tumor tissues compared to non-targeted controls, showcasing their potential for specific delivery of therapeutic agents to cancer cells.

Tian et al. [47] provide an extensive investigation of the application of TDNs for targeted drug delivery to the CNS. The researchers aimed to address the challenge of delivering therapeutic agents to the CNS by utilizing Angiopep-2 (ANG) modified TDNs as a platform for efficient and targeted drug delivery to the brain and spinal cord. As ANG acts as a ligand for the lipoprotein receptor-related protein-1 (LRP-1), a common receptor expressed on the surface of various cells that form the blood-brain barrier (BBB) and neural cells in the central nervous system, the researchers hypothesized the ANG functionalization of the cages would increase their ability to pass through the BBB. The study demonstrated the successful accumulation of the modified TDNs within the brain and spinal cord, indicating their potential to overcome the blood-brain barrier and specifically target neural cells. This targeted accumulation highlights the promising role of TDNs as a platform for the precise and effective delivery of therapeutic agents in the treatment of neurological disorders.

3.3.5. Regenerative medicine applications

The structural stability, biocompatibility, and cargo-loading capacity of TDNs make them extremely suitable for delivering growth factors, cytokines, and other signaling molecules to enhance tissue restoration; an array of qualities that have prompted extensive studies in both in vitro and in vivo experimentation. Numerous studies have demonstrated the efficacy of TDNs in various regenerative medicine scenarios, showcasing their applications in regenerative medicine and tissue engineering.

As briefly mentioned in section 2.3, TDNs have the potential to transport and deliver MiR335-5p, a specific microRNA molecule, to enhance the osteogenic differentiation of mesenchymal stem cells (MSCs), challenging bone defects in steroid-associated osteonecrosis (SAON). MiR335-5p functions as a regulatory molecule that modulates gene expression at the post-transcriptional level by targeting and downregulating the expression of the Dickkopf-1 (DKK1) gene [48], which is implicated in inhibiting the Wnt signaling pathway. The Wnt pathway plays a pivotal

role in bone metabolism and regeneration, of which modulation through the targeted delivery of MiR335-5p holds significant promise in promoting the osteogenic differentiation of MSCs. This approach has demonstrated encouraging outcomes in preclinical studies such as those by Li et al. [33] and Zhang et al. [49], showing its potential to enhance the generation of bone tissue. By effectively manipulating gene expression within MSCs, this innovative method opens new possibilities for developing therapies to address challenging bone defects, such as those associated with steroid-associated osteonecrosis (SAON).

Recent advancements on the subject were made by Gada et al. [20], finding that TDNs and other Self-Assembled DNA Nanocages had the potential to promote cell migration and differentiation of Human Umbilical Vein Endothelial Cells in vitro. This study paves the way for further research in vivo applications of regenerative medicine through TDNs. One study that laid the groundwork for the concept of the role of TDNs in the promotion of vascularization and angiogenesis was Zhao et al. [50]. The study aimed to investigate the potential of TDNs in delivering pro-angiogenic factors and emulating the native extracellular matrix to enhance blood vessel formation. The findings demonstrated that aptamer-modified TDNs, specifically "tFNA–Apt02" and "tFNA–AptVEGF", exhibited remarkable capabilities in accelerating the proliferation and migration of endothelial cells, facilitating tubule formation, spheroid sprouting, and promoting angiogenesis both in vitro and in vivo. The study employed a range of in vitro and in vivo assays to evaluate the proliferation and migration of endothelial cells, the formation of tubular structures, spheroid sprouting, and the development of micro-vessels. The results revealed that tFNA–Apt02 and tFNA–AptVEGF demonstrated superior angiogenic potential compared to aptamers or TDNs alone. These modified TDNs effectively facilitated endothelial cell proliferation and migration, stimulated the formation of capillary-like structures, and enhanced microvessel formation in vivo. Moreover, the combination of aptamers and TDNs synergistically enhanced angiogenesis, presenting a promising avenue for vascularization in tissue engineering. The study highlighted the advantages of employing TDNs over traditional angiogenic growth factors in promoting angiogenesis. Aptamer-modified TDNs, characterized by their small molecular weight, high stability, and ease of synthesis, hold significant potential for tissue engineering vascularization. They possess the ability to efficiently deliver pro-angiogenic factors and emulate the native extracellular matrix, thereby promoting blood vessel formation. The findings suggest that TDNs, particularly tFNA–Apt02 and tFNA–AptVEGF, hold considerable promise in supporting the development of functional vasculature within engineered tissues.

In animal models of tissue injury, TDNs have shown promising results in promoting tissue healing by modulating immune responses, resolving inflammation, and improving tissue regeneration. Zhang et al. [51] demonstrated the ability of TDNs to regulate immune cells and cytokine production, resulting in a decrease in the release of inflammatory cytokines such as IL-1β, IL-6, and TNF-α upon administration to mice via tail injection. Moreover, TDNs exhibited therapeutic potential beyond immunomodulation by mitigating oxidative stress and facilitating tissue healing. Through the activation of the Akt/Nrf2 pathway in separate mouse models with neurodegenerative diseases, TDN administration reduced ROS-induced

apoptosis, highlighting their neuroprotective role in oxidative-related diseases. The study overall demonstrated that TDNs promote enhanced tissue regeneration and functional outcomes by modulating immune responses and creating a favorable microenvironment, highlighting the potential of TDNs for therapeutic interventions in Immunomodulation and Inflammation Resolution.

In addition to their immunomodulatory and tissue-healing properties, TDNs have shown promise in targeted bioimaging applications. A study by Kansara et al. [52] explored the cellular targeting and intracellular fate of DNA nanocages in a zebrafish model. They found that tetrahedral DNA nanocages exhibited significant internalization in zebrafish embryos and larvae without disrupting the expression of genes involved in development. This understanding of time-, tissue-, and geometry-dependent uptake provides valuable insights into the biocompatible potential of TDNs for biomedical applications. These findings further highlight the versatility and potential of TDNs in tissue regeneration and targeted bioimaging, expanding their scope for therapeutic interventions in immunomodulation and inflammation resolution.

There has been an influx of research on the application of TDNs in regenerative medicine, to learn more a comprehensive review by Dou et al. [53] provides an in-depth exploration of the applications of TDNs in regenerative medicine, offering valuable insights and a broader understanding of this field.

4. Challenges and future directions

4.1. Potential translational studies for optimization and focus on TDNs

As many applications concerning TDNs as a delivery agent for biological agents remain to be explored, there are many avenues for future directions as well. To move forward most efficiently, the basic synthesis of the nanocages must be optimized, as the production of large quantities of nanocages with consistent quality and characteristics is still a challenge. Improving the yields and cost-effectiveness of nanocage synthesis would increase the availability of the research subject and decrease the experimentation pressure imposed by the cost of materials.

The application of TDNs has shown promising results in terms of biocompatibility in mouse models, exhibiting stability in blood and body, minimal immune rejection, efficient clearance through the kidney, and the ability to cross the blood-brain barrier*; h*owever, further testing is required before considering human trials. It is crucial to conduct comprehensive research to address key aspects such as pharmacokinetics and biodistribution to understand how TDNs are absorbed, distributed, metabolized, and excreted in the human body. Thorough toxicological evaluations are also necessary to determine systemic and local toxicity, assessing the impact on vital organs and tissues *and* their potential to induce immune responses *or* any associated adverse effects. Moreover, the potential for off-target effects and the optimal dosage of TDNs need to be investigated to ensure safe and effective use. Lastly, compatibility studies should be conducted if TDNs are intended to be used in combination with conventional therapies. By addressing these areas through further testing, researchers can gather comprehensive data to inform the design and

implementation of human trials, ensuring the responsible translation of TDNs as therapeutic agents.

Table 1. Potential translational studies in drug delivery with TDNs.

Chemical	Function	Previous framework	References
Tirapazamine	Hypoxia-activated Prodrug. Image tissue hypoxia/normoxia maps during the chemotherapeutic process and indicate alleviated tumor hypoxia.	Triangular DNA Nanocage	[54]
Methotrexate	Antimetabolite; inhibits folate metabolism and DNA synthesis.	ss-Oligonucleotide aptamer	[55]
Gemcitabine	Nucleoside analog; inhibits DNA synthesis and cell proliferation. Historically used in pancreatic cancer treatment.	DNA Nanogel	[56]
		ss-Oligonucleotide aptamer	[57]
		AuNP co-delivery with miR-21	[58]
Etoposide	Topoisomerase II inhibitor; induces DNA damage and cell apoptosis. Requires Encapsulation. Historically used in the treatment of testicular cancer, small cell lung cancer, and certain types of lymphomas and leukemias.	Albumin NP Encapsulation	[59]
		32-arm star polymers (stPCL-PEG32 and stPLA-PEG32)	[60]
Docetaxel	Anticancer agent; promotes microtubule stabilization and cell death. Historically used in the treatment of various solid tumors, including breast, lung, prostate, gastric, and head and neck cancers.	Albumin NP Conjugation with AS1411 Aptamer	[61]
		AuNP Encapsulation	[62]
Vinblastine	Anticancer agent; inhibits microtubule formation and cell division. It will likely require encapsulation. Historically used for Hodgkin's lymphoma, non-Hodgkin's lymphoma, testicular cancer, breast cancer, and certain types of lung cancer.	PNA-modified Liposome Encapsulation	[63]
		Triticum Liposome Encapsulation	[64]
Irinotecan	Topoisomerase I inhibitor; induces DNA damage and cell apoptosis. It has been historically used to treat colorectal cancer, lung cancer, and certain types of brain cancer such as glioblastoma.	Thermosensitive Magnetic Liposome Encapsulation	[65]
		Graphene Oxide NP Adsorption	[66]
Mitoxantrone	Topoisomerase II inhibitor. It will likely require encapsulation. Historically used to treat metastatic breast cancer, advanced prostate cancer, and acute nonlymphocytic leukemia (ANLL).	PEGylated Hollow AuNP Encapsulation	[67]
		pillar [6] arene (**WP6**) Encapsulation	[68]
Genistein	Inhibits the activation of NF-κB and Akt signaling pathways, leading to reduced cell survival and preprogrammed cell apoptosis. Historically used to treat breast, prostate, ovarian, and colorectal cancer	Genistein-loaded folic acid-conjugated chitosan NPs	[69]
		TPGS-b-PCL copolymer NPs	[70]

To increase the application potential for TDNs, its cargo loading efficiency must be improved. Areas of research towards this include translational studies of drugs being delivered by inorganic nanocages and non-Tetrahedral DNA nanocages, functionalization and loading technique development, and stimuli-responsive systems for cargo loading and release. In addition to cargo functionalization, further research on ligand functionalization for specific tissues needs to occur to enhance the targeting capabilities of TDNs. **Table 1** provides examples of specific studies that could be translated to the tetrahedral framework from various other drug delivery systems for ideal delivery.

In the realm of DNA nanotechnology, temperature-sensitive DNA strands have emerged as a valuable tool for achieving precise control over the encapsulation and release processes. A notable study conducted by Juul et al. in 2013 [71] demonstrated the utilization of temperature-sensitive DNA strands within a non-

tetrahedral DNA nanocage framework to enable temperature-controlled encapsulation and release of the enzyme horseradish peroxidase. This groundbreaking research showcased the potential of temperature-responsive DNA components in orchestrating dynamic cargo encapsulation and controlled release mechanisms.

The findings from this study provide a significant foundation for further translational work in the field of TDNs. By building upon the insights gained from temperature-sensitive DNA strand encapsulation, researchers can explore novel strategies and methodologies to enhance the encapsulation efficiency and cargo release kinetics of TDNs. The knowledge acquired from this translational research can pave the way for the development of improved encapsulation methods, facilitating the design of more efficient and tailored TDNs for various biomedical applications.

4.2. Long term delivery

Achieving greater stability is paramount for enabling the slow and sustained release of therapeutic cargo, a crucial requirement for many therapeutic interventions. To address this challenge, researchers are encouraged to embark on comprehensive investigations aimed at optimizing the design, synthesis, and structural properties of DNA nanocages. By exploring innovative strategies such as incorporating stabilizing modifications, exploring alternative structural motifs, or utilizing protective coatings, we can pave the way for the development of more stable DNA nanocages that can effectively deliver therapeutics over extended periods. Moreover, conducting systematic studies to evaluate the stability profiles of DNA nanocages under various physiological conditions and in different storage conditions is imperative. By doing so, we can gain a deeper understanding of the factors that influence their stability and devise practical solutions to enhance their long-term performance.

One intriguing avenue for exploration in enhancing long-term delivery is the concept of remote-control ability, which has not yet been extensively explored in the context of TDNs. A notable study by Amon et al. in 2016 [72] demonstrated the potential of remote-control mechanisms, such as magnetism and temperature, to facilitate cargo release from DNA nanocages. This concept opens exciting possibilities for achieving precise and on-demand cargo release in TDNs. By harnessing the principles of magnetism and temperature, researchers can develop strategies to remotely trigger the release of therapeutic cargo from TDNs. Magnetic properties could be incorporated into the nanocages, allowing for targeted manipulation and control using external magnetic fields. Additionally, temperature-responsive components could be integrated into the structure of TDNs, enabling precise modulation of cargo release in response to changes in temperature.

Another novel concept for the application of TDNs is their use in biomaterial scaffold functionalization. In the study by Ko et al. (2020) [73], TDNs were utilized as a tool for biomaterial scaffold functionalization. To achieve this, the researchers first designed and synthesized TDNs functionalized by attaching specific functional molecules or ligands to their surfaces. Next, the functionalized TDNs were incorporated into a DNA hydrogel matrix via rolling circle amplification (RCA),

where a circular DNA template was amplified using a DNA polymerase enzyme. During the amplification process, the TDNs were distributed within the growing DNA hydrogel network resulting in the functionalized TDNs becoming integral parts of the DNA hydrogel structure. The DNA hydrogel served as a scaffold or matrix to support and stabilize the functionalized TDNs, allowing the TDNs to retain their functional properties.

DNA hydrogels themselves possess unique characteristics such as biocompatibility, biodegradability, and the ability to encapsulate or entrap other biomolecules or therapeutic agents. The incorporation of functionalized TDNs into the DNA hydrogel could enable targeted drug delivery by selectively binding to specific receptors on the target cells or tissues. Additionally, the use of the scaffolds in regenerative medicine could be amplified via the utilization of TDNs as a carrier for growth factors and nutrients within the scaffold over time.

4.3. Long-term safety of TDNs

To address the crucial knowledge gap regarding the long-term safety of TDNs, it is imperative to initiate comprehensive studies focused on their chronic effects. Considering the current lack of data in this area, one proposed course of action could involve the following steps:

a. Design and plan a long-term study: Develop a study protocol that includes repeated administration of TDNs over an extended duration in an appropriate animal model.
b. Assess toxicity parameters: Implement rigorous toxicity assessments, including comprehensive organ function evaluations, hematological analyses, and histopathological examinations. These assessments will help identify any potential toxicological concerns associated with prolonged exposure to TDNs.
c. Monitor for long-term adverse effects: Conduct thorough monitoring of the animals for an extended period, with particular attention to the development of delayed or cumulative adverse effects. This may involve regular observation of general health parameters, behavioral assessments, and long-term organ function monitoring.
d. Analyze and interpret the data: Collect and analyze the data obtained from the long-term study, employing appropriate statistical analyses. Interpret the findings to determine the presence or absence of significant long-term toxicity and adverse effects associated with TDDN administration.

By undertaking these proposed steps, researchers can contribute to a better understanding of the long-term safety profile of TDNs. The resulting data will be invaluable for assessing the potential risks and benefits associated with their prolonged use and will provide crucial insights for the responsible development and clinical translation of TDNs.

5. Challenges and future directions

To summarize, the findings and insights presented in this paper have illuminated the remarkable potential of TDNs across a range of scientific and technological domains. With their unique structural properties and functional

versatility, these nanocages have emerged as promising candidates for various applications. The significance and potential impact of TDNs cannot be overstated; their robustness, stability, and programmability offer unprecedented control over their structure and function, opening new avenues for targeted drug delivery, molecular sensing, and nanoscale assembly.

Looking ahead, the prospects for continued research in this field are highly promising. By further exploring the properties and applications of TDNs, researchers can unlock innovative solutions in biomedicine, nanotechnology, and materials science. Collaborations and interdisciplinary approaches will play a crucial role in harnessing the full potential of these nanocages. Considering the insights provided, it is evident that TDNs hold immense importance for advancing scientific knowledge and technological advancements. By leveraging their unique attributes and building upon the foundations of DNA nanotechnology, researchers can drive transformative breakthroughs in nanoscience and nanotechnology.

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