Application of multivalent aptamers in tumor diagnosis, analysis and therapy (Review)

HONGYUE ZENG, WENJING ZENG and YAOYAO LIANG

Department of Oncology, The Second Affiliated Hospital of China Medical University, Shenyang, Liaoning 110004, P.R. China

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Abstract. Cancer remains one of the leading causes of mortality worldwide, making early diagnosis and precise treatment crucial for enhancing patient survival rates. Previously, nucleic acid aptamers have emerged as promising tools in tumor diagnosis, analysis and therapy, owing to their high specificity, cost-effectiveness and ease of modification. Unlike monovalent aptamers, multivalent aptamers markedly improve target affinity and specificity through multipoint binding, demonstrating superior efficacy in the detection, capture and treatment of tumor cells. The present study reviews the construction methods of multivalent aptamers, their applications in tumor diagnosis and therapy and their challenges and prospects. Constructed through nucleic acid nanostructure self-assembly, bio-coupling, nanomaterial loading and chemical cross-linking, multivalent aptamers can effectively detect circulating tumor cells, enabling the non-invasive release of tumor cells. They serve as alternatives to bispecific antibodies in tumor immunotherapy. Despite challenges in specificity, *in vivo* delivery efficiency and large-scale production in the tumor microenvironment, multivalent aptamers hold promise for future research in dynamic response technology, artificial intelligence-driven development and clinical application. The present systematic review summarizes advancements in multivalent aptamers in oncology over the past decade and their potential application in precision medicine.

Correspondence to: Dr Hongyue Zeng, Department of Oncology, The Second Affiliated Hospital of China Medical University, 36 Sanhao Street, Shenyang, Liaoning 110004, P.R. China
E-mail: bfzwzhy@163.com

Abbreviations: CTCs, circulating tumor cells; VEGF, vascular endothelial growth factor; XNA, xeno-nucleic acid; LNA, locked nucleic acid; PNA, peptide nucleic acids; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; HNA, anhydrohexitol nucleic acid; SELEX, Systematic Evolution of Ligands by Exponential Enrichment; CE-SELEX, capillary electrophoresis-SELEX; MUC1, mucin 1; PCR, polymerase chain reaction; PEG, polyethylene glycol; EpCAM, epithelial cell adhesion molecule; EGFR, epidermal growth factor receptor; ssDNA, single-stranded DNA; pAPNC, nanocarrier based on polyvalent aptamer-protein; BSA, bovine serum albumin; ApDC, aptamer-drug conjugate; RCA, rolling circle amplification; PSMA, prostate-specific membrane antigen; AuNP, gold nanoparticle; PAMAM, polyamidoamine dendrimer; TMAO, amphiphilic trimester amine N-oxide; TEXs, tumor-derived exosomes; HCR, hybridization chain reaction; HGF, hepatocyte growth factor; FGFR1, fibroblast growth factor receptor 1; CRS, cytokine release syndrome

Key words: multivalent aptamers, tumor cells, capture, detection, therapy

Contents

1. Introduction

Cancer has been a major threat to human health for >5 decades and remains the primary cause of mortality in China (1). Early detection, diagnosis and treatment are vital for slowing tumor progression and improving survival rates. Therefore, rapid detection of tumor cells serves a notable role in the occurrence, treatment and prognosis of malignant tumors (2). However, the complexity of clinical samples presents challenges for several methods in detecting low-abundance tumor cells, notably hindering the specificity and sensitivity of multiple detection techniques.

Currently, techniques such as histochemistry, immunohistochemistry and flow cytometry are utilized to identify tumor cells in clinical laboratories (3). However, histochemical staining often lacks sensitivity for low-abundance tumor cells, while immunohistochemistry is complex and time-consuming. Flow cytometry offers sensitive quantification of several markers but requires costly reagents and advanced equipment. Consequently, there is a pressing need for a simple, cost-effective method with high sensitivity and specificity for detecting low quantities of tumor cells in specimens.

Previous years have seen a surge in interest in nucleic acid aptamers, which are small molecules of RNA or single-stranded DNA (ssDNA) molecules with unique three-dimensional structures, typically comprising 20-80 bases and possessing a relative molecular mass ranging from 6,000 to 30,000 Da. Similar to antibodies, nucleic acid aptamers recognize targets specifically through shape complementarity and are used in several applications, including novel therapeutics, drug delivery, tumor cell detection and biological imaging (4). Aptamer-based tumor detection methods are flexible and cost-effective, leading to the creation of numerous highly sensitive detection techniques (5). However, monovalent aptamers face challenges in selectivity and targeting efficiency when identifying their targets, which can result in suboptimal detection and therapeutic outcomes, thereby hindering advancements in tumor detection and treatment.

In natural biological systems, multivalent synergistic interactions are frequently employed, wherein multiple ligands on a single biological entity simultaneously interact with receptors on a different biological entity to achieve high affinity and hyper selectivity. Currently, molecular biologists are increasingly leveraging the mechanism of multivalent interactions to develop novel molecular assemblies that can either introduce new functionalities to aptamers or enhance the efficacy of existing ones (6,7). Compared with monovalent aptamers, multivalent aptamers offer several advantages. Firstly, they can markedly enhance binding affinity. By engaging with the target molecule through multiple binding sites, multivalent aptamers establish multipoint interactions that strengthen overall binding, a phenomenon known as the multivalent effect (8). When several aptamer molecules simultaneously bind to the target, the resulting synergistic effect further amplifies binding strength, akin to a zipper mechanism (9). Secondly, multivalent aptamers can enhance binding specificity. Their multiple binding sites accurately match various epitopes of target molecules, thereby minimizing interactions with non-target entities and improving specificity (10). In addition, the diversity of binding sites enables multivalent aptamers to recognize different regions of the target molecule, further reducing the cross-reaction with non-target molecules (11). Thirdly, multivalent aptamers exhibit kinetic advantages. The presence of multiple binding sites increases the likelihood of contact between the aptamer and the target molecule, thereby enhancing the binding rate. The probability of simultaneous dissociation from multiple binding sites is low, resulting in a reduced overall dissociation rate and prolonged binding duration. Finally, multivalent aptamers possess functional advantages. In the context of biosensing, multivalent aptamers can simultaneously bind multiple signaling molecules, thereby amplifying the detection signal and enhancing sensitivity (12). In targeted therapy, multivalent aptamers can simultaneously bind multiple target molecules, thereby enhancing the efficiency and accuracy of drug delivery (13). Therefore, the development of multivalent aptamers is of great importance.

Multivalent aptamers offer notable advantages over conventional and bispecific antibodies in cancer diagnosis and treatment, particularly in addressing the challenges associated with antibody technology (14). Firstly, multivalent aptamers can transcend structural limitations and effectively target molecules often elusive to antibodies (15). Due to their larger molecular size, antibodies struggle to infiltrate dense tumor tissues or cross the blood-brain barrier, while aptamers, being notably smaller, can efficiently access the tumor microenvironment to identify low-expression or concealed epitopes (16). Numerous tumor-associated targets exhibit low immunogenicity or possess intricate structures, complicating the generation of effective antibodies. By contrast, aptamers can recognize small molecules, ions and complexly folded transmembrane proteins through their ability to adapt conformationally. They demonstrate a heightened sensitivity to conformational alterations in the target. They can dynamically bind to changing biomarkers on tumor cell surfaces in real time, a capability that antibodies may lack due to fixed epitopes (17,18).

Secondly, bispecific aptamers can address the shortcomings of bispecific antibodies (19). The development of bispecific antibodies necessitates complex protein engineering, a process that is both time-consuming and expensive, whereas aptamers can be synthesized rapidly and cost-effectively through chemical methods, achieving cost reductions of >90% (20). Furthermore, although bispecific antibodies can induce the development of drug-resistant antibodies, aptamers, which are based on nucleic acids, demonstrate low levels of immunogenicity (21). Moreover, aptamers can be chemically modified to enhance resistance to nuclease degradation, extending their *in vivo* half-life. Multivalent aptamers can integrate multiple targeting units and non-protein targets, unlike bispecific antibodies, which are limited by the compatibility of the two target proteins (22). Thirdly, multivalent aptamers can modularly integrate diagnostic and therapeutic functionalities. For instance, they can be linked with fluorescent moieties or nanoparticles for tumor imaging or liquid biopsy (23). They can also serve as drug carriers or be combined with immune checkpoint molecules for targeted delivery and immunomodulation (24).

Previous advancements in aptamers are exemplified by Macugen®, the first approved aptamer targeting vascular endothelial growth factor (VEGF) for age-associated macular degeneration (25). This has driven research into antitumor aptamers. While studies show that tumor-targeted aptamers can markedly inhibit tumor growth in animal models, clinical trials have lagged, with most remaining in preliminary phases and requiring further phase III trials to validate safety and efficacy. Ferreira *et al* (26) screened Apt2, a nucleic acid aptamer capable of specifically binding to triple-negative breast cancer (TNBC) MDA-MB-231 cells, by cellular Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technology combined with high-throughput sequencing. The target molecule of Apt2 has not yet been identified; however, it exhibited high affinity, specificity and low toxicity, and showed targeting ability in breast cancer tissue sections, indicating it has potential to serve as a therapeutically and diagnostically targeted ligand for TNBC. Hwang *et al* (27) performed a phase I/II clinical trial evaluating the safety and potential efficacy of combined vitreous cavity injections of the VEGF inhibitor ranibizumab and the platelet-derived growth factor inhibitor E10030 for the treatment of severe ocular-type von Hippel-Lindau disease-associated retinal hemangiomas. The results demonstrated that the combination had a reasonable

safety profile but had limited results in terms of improvement in vision, shrinkage of the tumor or reduced exudation.

To ensure a comprehensive collection of relevant literature, the PubMed (https://pubmed.ncbi.nlm.nih.gov), Web of Science (https://webofscience.clarivate.cn/wos/alldb/basic-search) and Scopus (http://www.scopus.com) databases were searched for the present review. The timeframe of the search was set from 2010 to 2025, which covers the various stages of this research topic from initial exploration to gradual in-depth development, providing a comprehensive and time-sensitive literature base for the review. Based on the research topic, the following search keyword combinations were used: 'Multivalent aptamers', 'tumor cells', 'capture' and 'detection'. A comparison of key information in the literature was initially performed to remove duplicates from the search results. Subsequently, non-research literature was excluded, such as conference papers and conference abstracts, and the present review focused on review articles, clinical trials and other types of literature that could provide substantive research data and in-depth analysis. The titles and abstracts of the preliminarily screened literature were read one by one to exclude those that were clearly inconsistent with the topic, and those that were too broad or narrow in terms of research content. Finally, the studies that passed the screening process were read in full to further assess their quality and relevance and to finalize the selection of literature to be included. The present review examined the basic principles of aptamers, the obstacles and potential solutions encountered in their development, the methodologies for constructing multivalent aptamers, their applications in oncology and the challenges and future prospects associated with multivalent aptamers.

2. Basic principles of aptamers

Aptamers are oligonucleotide or peptide sequences obtained by *in vitro* screening techniques. They bind target molecules with a high affinity and specificity, functioning similarly to antibodies in their ability to recognize and bind to these targets. They are often called chemical antibodies (28,29) and can be categorized into groups such as DNA, RNA, peptide, xeno-nucleic acid (XNA), locked nucleic acid (LNA) and peptide nucleic acid (PNA) aptamers, as well as complexes with nanomaterials and fluorescence labeling.

DNA aptamers, made from ssDNA, are stable, easy to synthesize and modifiable, making them valuable in biosensing and diagnostics. Liu *et al* (30) designed a non-G-Quadruplex DNA aptamer targeting nucleolin for bladder cancer diagnosis and treatment. RNA aptamers, composed of single-stranded RNAs, have complex secondary and tertiary structures, making them suitable for intracellular application and gene regulation. Yang *et al* (31) used RNA aptamers targeting the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) structural protein for antiviral research. Peptide aptamers, consisting of short peptide sequences typically derived from phage display technology, can specifically bind to proteins or other biomolecules. Shen *et al* (32) developed a peptide aptamer-paclitaxel conjugate for tumor-targeted therapies to enhance drug delivery precision while minimizing damage to normal cells. XNA aptamers are composed of unnatural nucleic acids, such as 1',5'-anhydrohexitol nucleic acid and 2'-fluoroarabinonucleic acid, which offer greater stability and chemical diversity. LNA aptamers, composed of locked nucleic acids, whose ribose rings are locked into a rigid structure, exhibit high stability, affinity and specificity. PNA aptamers, consisting of peptide nucleic acid with a peptide chain replacing its phosphate backbone, also demonstrate remarkable stability and binding capabilities. The diversity of aptamers allows for a broad spectrum of applications in disease diagnosis, therapy, biosensing and basic research. With the advancement of chemical modification and screening technologies, the design and application of aptamers are expected to become increasingly precise and diversified.

Aptamers are mainly identified using an *in vitro* evolutionary approach called phylogenetic exponential enrichment of ligands. Current screening methods include the conventional SELEX technique and its enhanced variants [such as capillary electrophoresis-SELEX (CE-SELEX), Cell-SELEX, Capture-SELEX], non-SELEX techniques and computer-assisted design, each with unique advantages and disadvantages.

The conventional SELEX technique serves as the standard approach for aptamer screening, encompassing a four-step process: Construction of random oligonucleotide libraries, isolation of target-bound and unbound sequences, enrichment of high-affinity sequences by polymerase chain reaction (PCR) amplification and the execution of several cycles of screening until highly specific aptamers are identified. However, the traditional SELEX technique has some limitations in screening small molecule aptamers (33).

Numerous enhanced SELEX technologies have evolved from the original methodology. Notably, the CE-SELEX technique employs CE to differentiate between aptamers and unbound sequences of target molecules, leveraging variations in electrophoretic mobility for effective screening. This method necessitates a smaller sample size, markedly reducing the number of screening iterations required (1-4 rounds) (34). Researchers have developed advanced CE-SELEX techniques to address the limitations of traditional SELEX methods. For instance, low pH-CE-SELEX technology mitigates electroosmotic flow and the adsorption of basic proteins, thereby enhancing screening efficiency (35). Additionally, single-step CE-SELEX technology facilitates a one-step, online reaction, making it particularly suitable for valuable, rare and difficult-to-prepare proteins. Furthermore, synchronized competition CE-SELEX technology allows for simultaneous competition to screen two target proteins, thereby improving both screening efficiency and aptamer affinity (36). Cell-SELEX technology permits the direct screening of aptamers on the surface of living cells, targeting cell membrane proteins or surface markers, such as cancer cell-specific receptors. This approach maintains the target's natural conformation and post-translational modifications, eliminating the need for protein purification (37). Capture-SELEX technology immobilizes the target on a solid-phase carrier (such as magnetic beads or microarrays) and performs aptamer screening in a capture-elution format. This method effectively addresses the limitations of the traditional SELEX method for small molecule target screening (33,38) and is noted for its straightforward operation.

In addition to SELEX technology, alternative methods such as non-SELEX technology can be employed for aptamer

screening. For instance, microfluidic SELEX leverages a microfluidic chip to consolidate the processes of binding, isolation and amplification, thereby facilitating high-throughput and automated screening. Currently, the main microfluidic chip SELEX technologies that have been developed include magnetic bead and sol-gel methods. The combination of microfluidic chip and SELEX technology enhances the screening efficiency of aptamers while simultaneously lowering associated costs (39). Furthermore, *in vitro* display technologies, such as ribosome or mRNA display, directly link aptamer sequences to functional proteins (such as green fluorescent protein) using *in vitro* translation systems. This approach negates the need for PCR and mitigates the impact of amplification bias on library diversity (40).

It is also possible to predict aptamer-target interactions and optimize sequences using computer-aided design, which integrates bioinformatics, molecular docking and machine learning techniques (such as deep learning). This approach can reduce the number of experimental screening phases and expedite the development timeline. Jeddi and Saiz (41) developed a computational model to explore the effect of different system designs on the efficacy of aptamer-based biosensors, utilizing an anti-mucin 1 (MUC1) aptamer alongside a silica biosensor substrate. The authors investigated different aptamer attachment termini, surface densities, orientations and solvent solutions. The results showed that the 5'end fixation was superior to the 3'end; high surface density ($8x10^{12}/cm^2$) could reduce the fluctuation, but spatial site-blocking needed to be avoided; and 0.8 M NaCl solvent could optimize the electrostatic environment and stabilize the aptamer conformation.

3. Challenges and solutions in aptamer development

Challenges in optimizing aptamer properties. Aptamers offer several advantages over antibodies, including straightforward synthesis, enhanced stability and ease of modification, making them suitable for diverse applications in biosensors, drug development and diagnostics (42). However, the three-dimensional structure of aptamers is susceptible to environmental influences, resulting in issues such as inadequate affinity, low specificity, limited functional activity and reduced detection sensitivity. Consequently, individual optimization of each aptamer is essential.

To address the issue of inadequate aptamer affinity, particularly at low target concentrations, the binding strength of the aptamer to the target often falls short of requirements (43). Firstly, the development of multivalent aptamers is proposed, as their apparent high affinity allows for effective signal capture even at low target concentrations while resisting interference from the sample matrix (such as salt ions and pH fluctuations) (44). The detection limits of multivalent aptamers can achieve levels as low as fg/ml, which can be comparable with or even exceed those of antibodies. For instance, a DNA hydrogel-based bivalent aptamer detects apolipoprotein A1, a bladder cancer marker, at 0.01 nm without interference from urea and creatinine (45). Secondly, sequence truncation and reconstruction mitigate spatial site resistance by removing non-critical nucleotides and retaining the core binding sequence. Thirdly, chemical modifications or the introduction of organic compounds are utilized to incorporate phosphorothioate bonds or 2'-fluorine modifications, thereby enhancing the rigidity of the binding site. Research indicates that the binding capacity of aptamers can be adjusted by adding specific organic compounds (46). For instance, tumor microenvironment-activated drug conjugation markedly reduces the binding of all aptamers, whereas polyethylene glycol (PEG) 8000 markedly improves the binding signal of certain aptamers (such as salicylic acid-aptamer) (46). Subsequently, structural optimization is performed, wherein secondary structures such as hairpins and G-quadruplexes are designed to enhance target binding efficiency through pre-folding (47). Furthermore, buffer conditions are optimized, revealing that an increase in NaCl concentration leads to a reduction in the binding of all aptamers, indicating that aptamer binding is primarily mediated by electrostatic interactions, with enhanced stability observed within a neutral pH range (48). The binding signals of certain aptamers (such as pyruvate dehydrogenase/lactate dehydrogenase-aptamer) show reduced binding signals without divalent cations, whereas the addition of Ca^{2+} and Mg^{2+} doubles these signals (48). This highlights the importance of divalent cations in aptamer binding affinity. Lastly, higher affinity variants can be identified using mismatch PCR or targeted mutation libraries (49).

Aptamer specificity presents notable challenges. Firstly, aptamers may exhibit cross-reactivity with non-target molecules (such as structural analogs and serum proteins), leading to false-positive results. Secondly, prolonged incubation can lead to non-specific binding of the aptamer to proteins or lipids on cell surfaces due to charge interactions (50). Numerous existing aptamers are affected by these specificity issues, which may hinder the advancement of aptamer-based diagnostics onto the market. The development of multivalent aptamers is anticipated to address the limitations of low specificity through synergistic effects and structural enhancements. This approach can notably improve the specificity of aptamers, particularly in complex biological samples (such as blood and urine) by reducing non-specific binding (43). The use of multivalent aptamers in complex samples can effectively mitigate non-specific adsorption, thereby fulfilling the requirements for clinical testing. For instance, in cancer exosome detection, the construction of heterologous bivalent aptamers targeting cluster of differentiation 63 and epithelial cell adhesion molecule (EpCAM) can eliminate the interference from normal cellular exosomes, achieving a specificity of >95% (51). In serum epidermal growth factor receptor (EGFR) assays, a trivalent aptamer sensor demonstrated the ability to distinguish between EGFR and human EGFR 2 in 10% serum, whereas the monovalent aptamer exhibited a false-positive rate of >30% due to non-specific adsorption (52). In addition, Kelly *et al* (53) found that the addition of the adding non-specific competitors, such as ssDNA, inhibited the non-specific binding of aptamers.

Specificity can also be improved through counter-screening, where non-target molecules are added to eliminate cross-binding sequences during the screening phase. Computer-assisted methods can also predict binding sites via molecular docking, allowing for the optimization of complementary regions (54). Chemical modifications, such as PEGylation, can be employed to introduce charge or spatial resistance outside the binding region, further reducing

non-specific adsorption. Modifying buffer conditions has shown that proteins such as interferon-γ and thrombin tend to non-specifically bind to aptamers at low pH levels. At a pH level <5, all aptamers exhibit this non-specific binding, which is further enhanced by divalent cations (55).

Several strategies can be employed to address the issues of inadequate functional activity, particularly for the aptamer to efficiently modulate its function (such as inhibiting enzyme activity or blocking receptor signaling) after binding to the target. Firstly, by utilizing binding site-directed design, the functional domain of the target can be identified using structural biology to create aptamers that specifically target this region. Secondly, developing multimeric aptamers or aptamer-nanocomplexes can enhance target conformation through synergistic binding. Finally, functionalized coupling can link aptamers with effector molecules (such as anti-PD-1 antibody) to enhance downstream effects (56).

To address the issue of inadequate detection sensitivity, characterized by the weak signal response of aptamers in sensing or diagnosis, two approaches can be employed. Firstly, implementing signal amplification strategies, combined with nanomaterials or enzyme-catalyzed reactions, can enhance sensitivity. Secondly, the design of 'molecular beacon' aptamers, which induce fluorescence or electrical signal changes upon binding to the target, presents another viable solution (57).

Challenges at the application level. Aptamers have a wide range of applications due to their specific binding capabilities to target molecules, particularly in drug development and diagnostic fields. However, a notable challenge for the *in vivo* application of aptamers is their susceptibility to degradation by nucleases (58). Conventional methods for aptamer modification, such as the incorporation of a phosphorothioate backbone or modifications to the sugar structure, can enhance their resistance to nuclease activity. However, these modifications may affect the binding affinity and specificity of the aptamer. To address this issue, Tabuchi *et al* (59) introduced the sulfur-fluoride exchange reaction, which facilitates the covalent attachment of the aptamer to the target protein. This covalent binding not only increased the binding strength of the aptamer to the target protein but also conferred relative nuclease resistance of the aptamer. Zhang *et al* (60) developed a nanocarrier based on polyvalent aptamer-protein (pAPNC) utilizing a bovine serum albumin (BSA) core with a multivalent XQ-2d aptamer shell. By binding multiple XQ-2d aptamers to the surface of BSA, pAPNC not only bolstered the stability of the aptamers but also enhanced the drug loading capacity through a synergistic charge effect. This increased resistance to nucleases allows pAPNC to maintain a prolonged circulation time *in vivo*, thereby augmenting its potential for targeted therapeutic and imaging applications.

Aptamers face limitations in pharmacokinetics, including a short half-life or poor tissue penetration *in vivo* (61). PEGylation can increase their molecular weight, effectively prolonging kidney clearance (62). Designing bispecific aptamers that simultaneously target cell surface receptors and targets can prolong the local duration of aptamer action (63). Furthermore, the accumulation of aptamers in tissues can be improved through aptamer-mediated targeting of delivery vehicles, such as exosomes and liposomes (64). Xiao *et al* (65) developed a novel method to covalently attach aptamer-drug conjugates (ApDC) to the surface of attenuated *Salmonella* using click chemistry. This approach markedly enhanced the stability of the ApDC *in vivo*, prolonged its half-life in serum and reduced premature degradation and clearance of the drug.

The application of aptamers also faces the risk of immunogenicity. *In vivo*, aptamers may be identified by the immune system, leading to the activation of an antibody response (66). To mitigate this issue, aptamers can be humanized to eliminate the use of sequences that are readily detected by the immune system, such as CpG sequences (67). Chemical modifications can also be used to conceal immunogenic properties, such as modifying PEG or sugar chains on the surface of the aptamer (68).

Challenges at the screening phase. Screening phase issues arise from the limited diversity of the initial aptamer library, resulting in a narrow evaluation range. This can be addressed by using longer random sequence regions or chemically modified nucleotides to increase structural diversity. Additionally, next-generation sequencing can monitor library diversity, allowing for adaptive refinement of the screening process (69).

4. Method for constructing multivalent aptamers

Nucleic acid nanostructure self-assembly. The self-assembly of nucleic acid nanostructures relies on the principle of base complementary pairing among DNA molecules, facilitating the construction of multivalent aptamers by designing specific DNA sequences and structures. This process results in the formation of precise DNA templates and auxiliary strands that contribute to the development of nanostructures. Due to the highly specific interactions of Watson-Crick base pairing, DNA molecules can self-assemble into well-defined DNA nanostructures, such as dimer, trimer, tetrahedron, hexamer and origami in a highly precise and predictable manner. Peng *et al* (70) designed a dual-recognition controlled electrochemical biosensor containing two aptamer hairpin probes that form DNA dimers that can bind to MUC1 and EpCAM proteins located on the cell membrane. When both probes simultaneously bind to the target cell, a roll-over amplification reaction is initiated, resulting in the production of numerous DNA products. These products include G-quadruplex forming sequences and complementary sequences of tetrahedral DNA structures. The tetrahedral DNA structure is affixed to the surface of the electrode and can capture the rolling circle amplification (RCA) products, thereby notably amplifying the electrochemical signal. Li *et al* (71) designed a trimeric aptamer with triple rotational symmetry that aligns perfectly with the trimeric structure of the SARS-CoV-2 spiny protein for coronavirus disease 2019 detection. This symmetrical design enables the aptamer to bind simultaneously to all three subunits of the spiny protein, leading to a substantial increase in binding affinity. This symmetrical trimeric aptamer demonstrates a two-order-of-magnitude enhancement in binding affinity compared with traditional monomeric or linear multimeric aptamers. Ge *et al* (72) constructed a novel drug delivery system targeting prostate-specific membrane antigen (PSMA)-positive prostate cancer cells utilizing multivalent

aptamers through the DNA origami technique. This system exhibits a high loading capacity, precise targeting capabilities and a slow drug release profile compared with conventional antibody-drug conjugates.

Bio-coupling. Bio-coupling is a chemical and biological method that employs non-covalent bonding between biological macromolecules (such as proteins and nucleic acids) or covalent bonding to link multiple aptamers, thereby creating multivalent aptamers. This process enhances the affinity and specificity of multivalent aptamer-target binding. Wang *et al* (73) developed multivalent aptamer nanodrug couplers with a nucleosome-like structure, based on copper alkalosis in the form of cell death, to improve tumor cuproptosis treatment by leveraging mitochondrial copper overload and glutathione depletion. The resulting multivalent aptamer, featuring molecular aptamers for tumor targeting and repetitive polyT sequences for copper chelation, facilitates efficient loading and targeted delivery of copper peroxide-elesclomol nanodots and improves the affinity and specificity of the multivalent aptamers to the target. However, its diversity in loading remains constrained due to the limited range of drugs suitable for coupling, and an excess of multivalent aptamers may potentially diminish the targeting efficacy.

Nanomaterial loading. Nanomaterial loading methods leverage the surface properties and internal structure of nanomaterials to bind to aptamer molecules, resulting in the formation of multivalent aptamers. Commonly used nanomaterials include metallic [such as gold nanoparticles (AuNPs)], carbon-based, semiconductor and polymer nanomaterials. Due to their distinctive attributes, including diminutive size, unique optical characteristics and ease of biofunctionalization, nanomaterials have found applications in biosensing, imaging, cancer diagnostics and therapeutic interventions (74). Nanomaterials measuring <100 nm can circulate in the bloodstream for long periods and accumulate non-specifically in tumors with low selectivity through enhanced permeability and retention effects (75). The high surface area to volume ratio of these nanomaterials allows for the loading of numerous aptamers, facilitating the construction of multivalent aptamers. These multivalent aptamers enhance the specific recognition capabilities of nanomaterials, thereby improving the efficiency of cancer cell identification and targeted delivery. In addition, the incorporation of nanomaterials increases the density and molecular weight of the aptamers, which improves the binding affinity, nuclease resistance and aptamer circulation time within the bloodstream. Zhang *et al* (76) developed a biofunctionalized AuNP probe featuring ~3000 6-FAM-Sgc8 aptamers per AuNP, specifically designed to bind to the protein tyrosine kinase 7 on target cells. The binding constants of this probe to MCF-7 cells were 170-fold higher compared with those of the monovalent Sgc8 aptamers, ensuring stable binding to the target cells. Multivalent aptamers developed using nanomaterial-loading methods can interact with targets through multiple binding sites, markedly improving their affinity and specificity. However, these approaches can be expensive and certain nanomaterials (such as nano sliver and nano titanium dioxide) may exhibit bio-toxic properties.

Chemical cross-linking. Chemical cross-linking is the process of chemically linking multiple aptamer molecules together to form a complex with multiple binding sites, which notably improves its binding ability and specificity toward the target. A critical consideration in designing effective multivalent aptamers using chemical cross-linking methods is determining the optimal distance for linking monovalent aptamers to maximize the affinity of the multivalent aptamer. The effectiveness of this aptamer attachment method is primarily influenced by the specificity of the aptamer-target interaction and the desired functionality of the multivalent aptamer. Generally, the inclusion of flexible junctions of varying lengths and materials is the effective operation for multivalent constructs. These junctions help minimize the formation of unintended secondary structures within individual aptamers and can enhance the affinity of multivalent aptamers by mitigating spatial site barriers posed by adjacent aptamers. Commonly used junctions include oligonucleotide-based junctions, such as those for ssDNA and double-stranded DNA, non-nucleotide linkers, PEG-derived linkers and polyacrylamide (10).

Aptamer-protein fusion. The aptamer-protein fusion represents a method that leverages the inherent stability and functionality of proteins to integrate nucleic acid aptamers with proteins to form multivalent aptamers (9). Amini *et al* (77) established an aptamer-protein fusion strategy to identify a bivalent aptamer with ultra-high affinity for the SARS-CoV-2 spike protein. This was achieved using structured libraries of double random structural domains, highlighting the potential of aptamer-protein fusion in enhancing binding affinity. While this technique enhances the stability and targeting capabilities of the aptamer, the resulting fusion protein may exhibit immunogenic properties, potentially triggering an immune response and raising safety concerns.

5. Application of multivalent aptamers in tumor diagnostic analysis

Application in the efficient detection of tumor cells. The monitoring of circulating tumor cells (CTCs) in human bloodstreams offers notable insights for diagnosing metastasis, assessing prognosis and managing cancer therapies. However, their low abundance in blood complicates detection. Multivalent aptamers have demonstrated promise in addressing this challenge.

Application in the aptamer biosensors. Yang *et al* (78) developed an electrode interface modified with an *in situ*-produced multivalent aptamer network for effective capture and sensitive detection of CTCs in whole blood using electrochemical methods. The authors synthesized long ssDNA strands with repetitive aptamer fragments, immobilized on the electrode using RCA, to efficiently isolate rare CTCs. Functional AuNPs, modified with antibodies and horseradish peroxidase, specifically bind to CTCs, amplifying the electrocatalytic signal and increasing current output. This approach markedly enhances the sensitivity of CTC detection, achieving a detection limit as low as five cells. The interface with a multivalent aptamer network effectively distinguished target cells from control cells, enabling precise detection of CTCs (Fig. 1A).

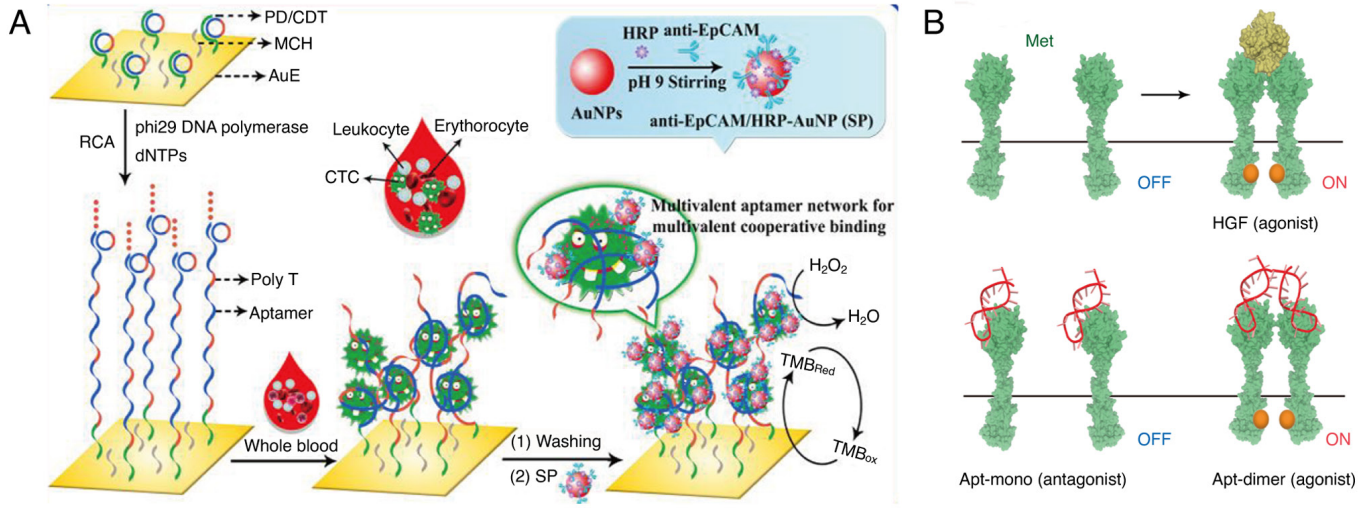

Figure 1. Examples of multivalent aptamers in tumor diagnostic analysis. (A) An electrode interface modified with an *in situ* produced multivalent aptamer network to efficiently capture and sensitively detect CTCs in whole blood. Reprinted with permission from (77). Copyright © 2020, American Chemical Society. (B) Schematic representation of HGF-induced Met activation and the Met activation potential of the Apt-mono and the Apt-dimer. Reprinted with permission from (83). Copyright © 2020 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. CTC, circulating tumor cell; HGF, hepatocyte growth factor; AuNP, gold nanoparticle; RCA, rolling circle amplification; EpCAM, epithelial cell adhesion molecule; Met, MET proto-oncogene, receptor tyrosine kinase; Apt, aptamer; HRP, horseradish peroxidase; TMB, 3,3'5,5'-tetramethylbenzidine; SP, anti-EpCAM/HRP-AuNP, anti-EpCAM antibodies and horseradish peroxidase co-conjugated gold nanoparticle; dNTPs, deoxyribonucleotides mixture; PD, primer DNA; CDT; circular DNA template; MCH, 6-mercapto-1-hexanol; AuE, gold electrode.

Application in the molecular capture probes. Su *et al* (79) developed a novel immunomagnetic substrate, Fe_3O_4@polyamidoamine dendrimer (PAMAM)@amphiphilic trimester amine N-oxide (TMAO)@Aptamer, for efficient exosome detection and capture. This was achieved through the sequential modification of the fifth-generation PAMAM, TMAO and EpCAM aptamer onto a magnetic core. The substrate leverages the strong magnetic properties of magnetite (Fe_3O_4), the abundant affinity sites of PAMAM, the strong hydrophilicity of TMAO and the enhanced affinity of EpCAM aptamer. It demonstrates an impressive capture efficiency of up to 90.5% for tumor-derived exosomes (TEXs) within 30 min, while the non-specific adsorption of non-TEXs remains low at only 8.2%. Song *et al* (80) introduced a novel approach for the detection and *in situ* imaging of MUC1, utilizing aptamer conformational changes and hybridization chain reaction (HCR). By creating a specialized aptamer-trigger probe, the aptamer selectively binds to MUC1, initiating the HCR reaction and resulting in signal amplification. This approach not only detects MUC1 in solution but also allows for *in situ* imaging at the cellular level, making it suitable for the localization and quantitative analysis of tumor cells.

Application in efficient capture and non-invasive release of tumor cells. Detection and capture of tumor cells in blood or other bodily fluids has been proposed to be an important approach for improving early diagnosis of tumors and helping to determine tumor metastasis. Currently, target cells are isolated based on their size, surface adhesion, cell surface or distinctive marker proteins using affinity ligands that selectively recognize these surface markers. Such ligands include antibodies, aptamer molecules or peptide compounds. Conventional immunomagnetic-based cell capture techniques demonstrate poor sensitivity and specificity, with suboptimal overall efficiency and economic feasibility (81). By contrast, multivalent aptamers have demonstrated high potential to efficiently capture and release tumor cells. Liu *et al* (82) designed novel dual aptamer-modified nitrogen-doped carbon quantum dot probes to improve the capture of CTCs. The probes contained EpCAM and vimentin dual aptamers, exhibiting potential to capture CTCs with different programmed death-ligand 1 (PD-L1) expression levels (such as H1299 and A549 cells). It was observed that the dual aptamer modification markedly improved the capture efficiency and purity of CTCs surpassing the traditional single EpCAM capture method, particularly for CTCs undergoing epithelial-mesenchymal transition.

Application in tumor cells' behavior characterization. Besides their use in detecting and enriching tumor cells, multivalent aptamers on cell surface can be used to promote the receptor dimerization, activation and inhibition mechanisms.

Ueki *et al* (83) designed a DNA aptamer that mimics the function of hepatocyte growth factor (HGF) through dimerization (Apt-dimer). Previous studies have demonstrated that HGF stimulates the downstream signaling pathways by binding to and inducing dimerization of the receptor MET proto-oncogene, receptor tyrosine kinase (Met). It was observed that the Apt-dimer, designed by dimerization, successfully activated the Met receptor and triggered cell signaling similar to that of HGF (Fig. 1B). Nuclease stability is considered a major limitation to the *in vivo* application of DNA aptamers. Through serum stability experiments, i.e., Apt-dimer and monomeric aptamer were dissolved in serum-containing solution respectively, while serum-free buffer was set up as a negative control; samples were taken at different time points and changes in the integrity of the nucleic acid bands were observed by electrophoresis, it was observed that the Apt-dimer showed high

nuclease stability in serum, whereas the monomeric form of the DNA aptamer was rapidly degraded (84). Therefore, it was postulated that the 3' end of the Apt-dimer forms a stem-loop structure that protects the DNA aptamer from 3' exonuclease degradation. This finding provides new ideas for designing highly stable DNA aptamers.

Using the SELEX technology, Menon *et al* (85) reported RNA aptamers that can specifically bind CD3 to improve the efficacy of overt T-cell therapy. Unlike conventional CD3 agonistic antibodies, the designed aptamers expanded T cells in an antigen-specific manner and avoided non-specific T-cell expansion. These aptamers markedly enhanced T-cell activation and the proliferation of low-affinity T-cell receptors, improving the treatment of solid tumors, which have low affinity. In addition, the aptamers promoted T-cell activation and proliferation, but increased T-cell persistence *in vivo*, achieving antitumor effects.

Zlinska *et al* (86) designed a DNA aptamer named VZ23, which could specifically inhibit fibroblast growth factor receptor 1 (FGFR1) signaling. The aptamer demonstrated good efficacy in treating human diseases, including growth disorders, degenerative diseases and cancer. Currently, the treatment of FGFR is mainly based on small-molecule tyrosine kinase inhibitors, but these inhibitors are not sufficiently specific and inhibit multiple FGFR isoforms and other receptor tyrosine kinases simultaneously, inducing side effects and toxicity (87). Therefore, the VZ23 aptamer, which specifically inhibits FGFR1 signaling without interfering with other FGFR subtypes or non-FGFR receptors, may be an effective treatment for cancer.

6. Application of multivalent aptamers in tumor therapy

Currently, the lack of efficient and accurate delivery systems for targeting tumor cells is a major limitation to the effective treatment of tumors. Researchers have proposed the tumor-immune bispecific antibody to improve cancer treatment strategy by enhancing the immune system's ability to attack tumor cells (88). This approach leverages bispecific antibodies to target two different molecules simultaneously (88). A major advantage of tumor-immune bispecific antibodies is their high specificity and few side effects. However, bispecific antibodies are associated with certain limitations (89). For instance, their production requires complex genetic engineering and purification processes, which are costly. It has been shown that they may induce immunogenic effects such as cytokine release syndrome (CRS). In addition, the macromolecular structure of bispecific antibodies restricts their penetration into the microenvironment of solid tumors, resulting in inadequate penetration into solid tumors. Multivalent aptamers, on the other hand, exhibit high chemical stability, low immunogenicity and their synthesis is highly flexible. Thus, they are expected to be an alternative to bispecific antibodies for cancer treatment. Multivalent aptamers can mimic the 'bridging' function of bispecific antibodies by binding to multiple targets (such as tumor antigens and immune cell receptors) owing to their modular design, which allows greater freedom of design. Aptamers have a low molecular weight and can efficiently penetrate solid tumors. Based on the solid-phase synthesis approach, large-scale production of aptamers can be achieved at a notably lower cost compared with antibody-based drugs. Specifically, being nucleic acid molecules, multivalent aptamers are less likely to trigger antibody-dependent immune responses and may avoid side effects such as CRS. Their stability and half-life can be chemically modified (such as phosphorothioate backbone or PEGylation). The potential application scenarios of multivalent aptamers in tumor therapy are discussed in the present review.

Alternative T-cell splicing dual antibodies. Bispecific aptamers can bind two different target molecules simultaneously, usually an antigen on the surface of cancer cells and a receptor on the surface of immune cells (90). Based on this mechanism, bispecific aptamers can form an artificial immune synapse between cancer cells and immune cells, stimulating T-cell activation and cancer cell lysis, while overcoming the penetration limitations of large molecule antibodies, making them ideal bispecific antibodies. For instance, Sun *et al* (91) designed a bispecific aptamer, Ap3-7c, that can target both programmed cell death protein 1 (PD-1) and PD-L1. Mechanistically, it not only inhibits the PD-1/PD-L1 interaction, but also promotes the physical contact between the T cells and the tumor cells, thereby potentiating the antitumor activity of the T cells. The bispecific aptamer binds to dibenzocyclooctyne through a novel 'recognize-then-conjugate' mechanism, in which it covalently binds to the target cells (Fig. 2A). This therapeutic maneuver extends the retention time of the aptamer at the tumor site and exerts an optimal therapeutic effect.

Dual signaling pathway inhibition. Cancer immunotherapy is increasingly transforming the treatment of tumors. Furthermore, the emergence of immune checkpoint inhibitors [such as anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and anti-PD-1/PD-L1 antibodies] has been a major breakthrough (92). However, existing immune checkpoint inhibitors exhibit limited response rates and immune-related side effects (92,93). Therefore, it is crucial to formulate novel immunotherapeutic strategies with high specificity and lower toxicity. Ayass *et al* (94) designed a bifunctional aptamer that could target CTLA-4 and natural killer group protein 2 (NKG2A) to mimic dual immune checkpoint inhibitors. Designed using computational biology approaches, this aptamer selectively targets CTLA-4 and NKG2A, relieving T and natural killer cell inhibition and thereby enhancing their antitumor activity (Fig. 2B).

Multifunctional carriers. ApDCs have been extensively investigated with respect to their potential to treat cancer, with previous studies showing that aptamers can specifically deliver drugs to cancer cells without affecting normal cells. In the study by Chen *et al* (95), a multivalent hydrophobic polymer was conjugated with an sgc8 aptamer to construct an ultra-stable nano-micellar system that can maintain the structural integrity in physiological environments. The system exhibited good targeting and drug delivery efficiency. The nano-micelle system was loaded with photosensitizer Ce6 for photodynamic therapy and other hydrophobic drugs (such as Adriamycin and paclitaxel), demonstrating its potential as a multifunctional platform for drug delivery. In addition, Choi *et al* (96) constructed a novel pancreatic ductal adenocarcinoma-targeted therapeutic

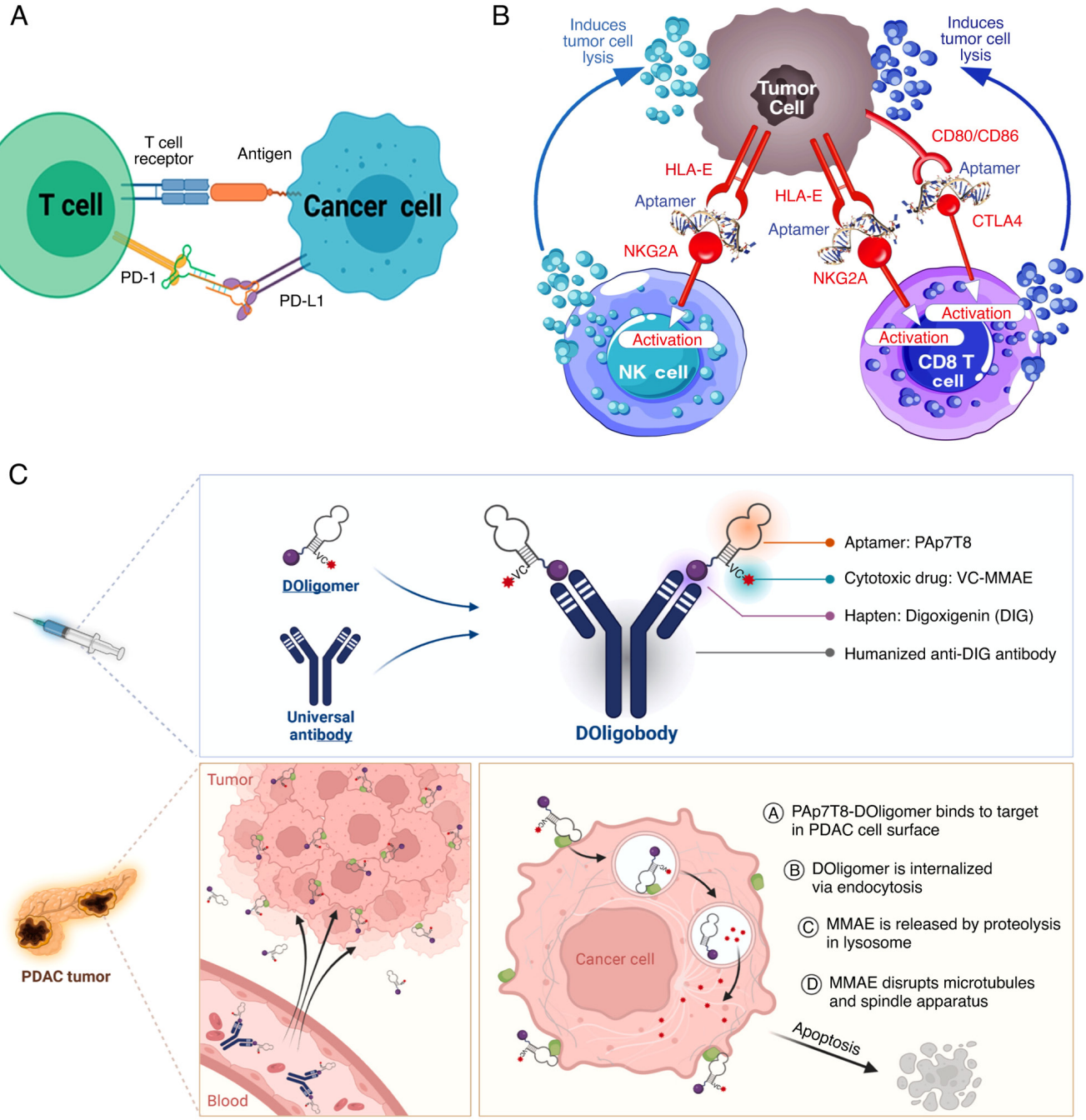

Figure 2. Examples of multivalent aptamers in tumor therapy. (A) Schematic representation of the mechanism by which Ap3-7c blocks the PD1/PD-L1 axis. Reprinted with permission from (91). Copyright © 2022, American Chemical Society. (B) A comprehensive mechanistic representation of the dual CTLA4/NKG2A aptamer (AYA22T) targeting immunotherapy. Reprinted with permission from (94). Copyright © 2024 by the authors. (C) Proposed mechanism of anticancer effect by PAp7T8-DOligobody in PDAC. Reprinted with permission from (96). Copyright © 2023 The Authors. Published by Elsevier B.V. PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; CTLA4, cytotoxic T-lymphocyte associated protein 4; NKG2A, natural killer group protein 2; HLA, human leukocyte antigen; PDAC, pancreatic ductal adenocarcinoma; MMAE, monomethyl auristatin E; CD80/CD86, cooperative B7-1/2; Doligomer, drug-conjugated oligomer; DOligobody, drug-conjugated oligobody.

platform, which could efficiently combine cytotoxic drug monomethyl auristatin E with an aptamer and was delivered through a generic semi-antigenic antibody (anti-digoxin antibody) to extend the half-life of the aptamer *in vivo*. The aptamer specifically recognizes pancreatic cancer cells, achieving specific tumor delivery of drugs, which reduces toxicity to normal cells (Fig. 2C). The use of a universal antibody allows easier replacement of different aptamers and improves simultaneous targeting of multiple targets.

7. Challenges and prospects

Challenges. Studies have demonstrated that multivalent aptamers have unique advantages, but they also have several limitations (9,56,97,98). Firstly, the use of multivalent aptamers requires balancing between target specificity and affinity. Notably, aptamers bind to targets through three-dimensional structures, but they are likely to introduce risks due to non-specific binding. For instance, for highly heterogeneous

targets in the tumor microenvironment, multivalent aptamers may trigger off-target effects as a function of excessive cross-linking, decreasing their therapeutic effects. Secondly, although they have a low molecular weight, aptamers have a multivalent structure, which increases the size and limits its penetration ability into solid tumors. In addition, nucleic acid molecules may be degraded by serum nuclease, have a short half-life and rely on nanocarriers or liposome encapsulation for effective delivery. For instance, in glioma treatment, aptamers must effectively penetrate the blood-brain barrier; however, most research remains at the *in vitro* or animal model stage, with limited success in clinical translation (99). Thirdly, the efficacy of aptamers is limited by the interference of the complex tumor microenvironment. Factors such as acidic pH, hypoxia and high interstitial pressure in the tumor microenvironment may affect the binding efficiency of aptamers and targets. In addition, the dynamic expression of antigens on the surface of tumor cells (such as drug-resistant mutations after treatment) may decrease the efficacy of the aptamer. Therefore, dynamically responsive aptamers need to be developed to cope with the hostile microenvironment. Fourthly, large-scale production and quality control may be important challenges. Although chemical synthesis of aptamers is relatively low-cost, the precise assembly of multivalent structures (such as dimer or trimer design) may be a complex production process. The length and flexibility of the linker arm can influence aptamer binding efficiency, necessitating optimization through high-throughput screening. Finally, clinical studies have primarily tested the efficacy of single-target applications (26,100,101), but few systematic investigations have explored combination therapy strategies for the use of multivalent aptamers (such as with immune checkpoint inhibitors). In addition, little attention has been paid to the identification of biomarkers that can be used to accurately predict patient response.

Prospects. In the future, research into multivalent aptamers should aim to explore the following aspects. Firstly, there is a need to adopt multivalent design and dynamic response technologies to establish efficient environment-responsive aptamers (such as pH or enzyme-sensitive) to achieve selective activation of the tumor microenvironment. Multimodal co-design of aptamers with other therapeutic modules (such as photo-thermolysis, chemotherapeutic agents or gene editing tools) are needed to build an 'all-in-one' platform. For instance, aptamers should be integrated with proteolysis-targeting chimeras technology to degrade difficult-to-adhere targets (such as transcription factors) via the ubiquitin-proteasome system. Secondly, researchers should optimize nanocarriers by leveraging liposomes, exosomes or metal-organic frameworks to encapsulate aptamers, which will potentially enhance their *in vivo* stability and tumor accumulation. Penetration enhancement strategies are carried out to enhance solid tumor penetration by aptamer-modified penetrating peptides or using physical means such as ultrasound/magnetic fields. Thirdly, artificial intelligence-driven aptamer development should be investigated in future to integrate machine learning to predict aptamer-target binding conformations, thereby accelerating the SELEX screening process. For instance, leveraging AlphaFold-based protein structure prediction could facilitate the reverse design of aptamer sequences. Furthermore, it is imperative to integrate multi-omics using spatial transcriptome technology to resolve tumor heterogeneity and enhance the precise selection of aptamer targets. Fourthly, investigators should aim to expand the target range of aptamers and develop combination therapies. For membrane proteins, which cannot be targeted efficiently using traditional small molecules (such as delta-like ligand 3 or PSMA), multivalent aptamer-ADC or bispecific chimeras may be used to address the drug resistance limitation. Coupling aptamers with immune agonists (such as IL-15) to remodel the tumor immunosuppressive microenvironment and enhance T cell infiltration. Future research should prioritize clinical translation and personalized medicine by integrating aptamer therapy with liquid biopsy techniques, such as circulating tumor DNA testing, to identify and stratify potential beneficiary populations for more precise and effective treatment. Based on the tumor-specific antigen profiles of patients, multivalent aptamer combinations could be customized to achieve precision therapy. Future research should focus on advancing multivalent aptamers for oncology, exploring their potential from diagnosis to therapy, targeted drug delivery and dynamic regulation. This will require interdisciplinary collaboration and further technological innovations to overcome existing challenges and enhance their clinical applicability. Furthermore, efforts to integrate aptamers and tumor biology are warranted to develop strategies to improve the clinical application of aptamers.

The present review has the following limitations in terms of content and scope of research: The study primarily focuses on literature from the past decade and fails to cover research achievements from earlier periods. This may lead to the neglect of the historical background and foundational research of multivalent nucleic acid aptamers in the early development of tumor diagnosis and treatment. Although a comprehensive search in the PubMed, Web of Science and Scopus databases was performed, some relevant literature may still have been overlooked. For instance, conference papers or preprints not indexed in these databases may contain valuable cutting-edge research findings. Although the review mentions the potential applications of multivalent aptamers in tumor diagnosis and treatment, most studies are still at the *in vitro* or animal model stage, with limited clinical trial evidence, making it difficult to accurately assess their efficacy and safety in real-world medical practice. The present review includes only a limited discussion on the differences in the application of multivalent aptamers in various types of tumors (such as solid tumors and hematological malignancies). The biological characteristics and microenvironmental differences among different tumors are substantial, which may necessitate different aptamer design strategies. The present review also has limited discussion on the range of biomolecules that multivalent aptamers can target, particularly in terms of certain membrane proteins or intracellular targets that are difficult to target. This may result in an inaccurate assessment of the potential and challenges of multivalent aptamers in expanding their application scope.

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Authors' contributions

HYZ conceived the study, wrote the original manuscript and made subsequent revisions to the article. WJZ and YYL searched the literature and participated in writing the manuscript. All authors have read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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