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Nucleic Acid Aptamers in Cancer Research, Diagnosis and Therapy

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Abstract

Aptamers are single-stranded DNA or RNA oligomers, identified from a random sequence pool, with the ability to form unique and versatile tertiary structures that bind to cognate molecules with superior specificity. Their small size, excellent chemical stability and low immunogenicity enable them to rival antibodies in cancer imaging and therapy applications. Facile chemical synthesis, versatility in structural design and engineering, and the ability for site-specific modifications with functional moieties make aptamers excellent recognition motifs for cancer biomarker discovery and detection. Moreover, aptamers can be selected or engineered to regulate cancer protein functions, as well as to guide anti-cancer drug design or screening. This review summarizes their applications in cancer, including cancer biomarker discovery and detection, cancer imaging, cancer therapy, and anti-cancer drug discovery. Although relevant applications are relatively new, the significant progress achieved has demonstrated that aptamers can be promising players in cancer research.

Cancer is a complex disease that varies widely in its roots and progression. This dynamic disease involves many different deregulated or mutated genes and related proteins within the malignant cells and their surrounding tissues. These deranged, defective and rather dominating molecules are major players for cancer diagnosis and are potential targets for personalized medicine.[1] Antibodies have made tremendous contributions in understanding cancer molecular mechanisms, diagnosis and therapy. However, off-target cross-reactivity, batch-to-batch variation, immunogenicity, denaturation, and limited chemical modifications are common shortcomings.[2] Additionally, antibodies with large size and high affinity do not penetrate deep into tumors and thus may result in poor therapeutic efficacy and imaging efficiency.[3-6] Although antibodies and their derivatives (e.g., nanobodies) prepared by recombinant and engineering methodologies address some of these problems,[7] they still face major challenges to provide the specifications and functionalities needed for state-of-the-art cancer diagnostics and therapies.

Nucleic acid aptamers have emerged as alternative biorecognition elements with distinctive properties. Aptamers are single-stranded oligonucleotides (DNA or RNA) which form unique tertiary structures that bind to target molecules.[8-14] They are normally identified from a random library comprised of approximately 10^{12}-10^{14} unique oligonucleotides of 20-80 bases by a procedure known as Systematic Evolution of Ligands by EXponential enrichment (SELEX).[15-17] This process involves enriching the initial pool of oligonucleotides by selective amplification of sequences that possess the desired characteristics and especially tunable specificity and affinity that can be pre-defined in the selection process. Since its introduction in 1990, approximately 20 variants of the original SELEX process have been developed.[18, 19] A number of aptamers have been identified with the capability of binding to targets such as metal ions, small molecules, proteins, viruses, cells, etc.[20] Some of these aptamers are archived in a database (aptamer.icmb.utexas.edu) and the International Society

on Aptamers (aptamersociety.org). Compared to antibodies, aptamers are chemically stable at a wide range of pH, temperature and ionic conditions with lower immunogenicity.[21, 22] They can be reversibly denatured and refolded without loss of activity.[23] Moreover, aptamers can be chemically synthesized in large quantity with high reproducibility.[24, 25] Aptamers can also be site-specifically modified.[26] For instance, through facile solid state phosphoramidite chemistry, one can conjugate a vast range of functional moieties (e.g., thiols, dyes, and Polyethylene glycol (PEG) etc.) to aptamer at any selected position.[27, 28] Recently, nucleotides modified with hydrophobic bases were incorporated at predetermined positions in a random sequence library, and the yielded DNA aptamers at binding affinity is over 100-fold higher than unmodified ones.[29] In addition, the intrinsic ability of natural nucleic acids to hybridize with a complementary strand can be exploited to produce a versatile range of molecular architectures and functionalities.[30]

Due to these excellent properties, aptamers have been applied to both preclinical and clinical cancer research and applications. While reviews on specific topics including SELEX, aptamer sensors, aptamer therapeutics, and aptamer-based drug delivery are available elsewhere[9, 14, 18, 31] including especially from Tan and colleagues,[32, 33] we present a comprehensive, updated aptamer review with a specific focus in the cancer field. We summarize the recent progress of aptamer applications in cancer studies, especially in cancer biomarker discovery and detection, cancer diagnosis, cancer therapy, and anti-cancer drug discovery. Finally, we discuss the challenges and future perspectives in the aptamer field including their inherent deficiencies, the difficulties of the discovery of new aptamers, undefined biochemical characteristics, and their potential roles in cancer research in the near future.

1. Aptamers in Cancer Proteomic Biomarker Discovery

Identification of cancer biomarkers can facilitate cancer risk assessment, diagnosis, prognosis, and treatment monitoring and guidance.[34] These biomarkers may include genetic, epigenetic, proteomic, and cellular factors.[34] In particular, specific proteins and variations in their expression are the most attractive cancer biomarkers because they are dynamic and reflective of cellular physiology, which may not be revealed at the gene level.[35] However, the identification and validation of protein biomarkers have been a challenge due to cancer heterogeneity, intraindividual variation in proteome, nonspecific overlaps between cancer and normal tissues, complexity of biofluids, and low abundance.[36, 37] Therefore, a high throughput, sensitive, specific and robust approach for identification of protein biomarkers is urgently needed.

Several techniques for protein biomarker discovery have been reviewed.[36, 38] Mass spectrometry (MS) and its associated systems are the leading cancer proteomics technologies. However, they suffer from poor reproducibility, limited capacity to process proteins within serum, and inherent qualitative nature of MS.[39] Bioaffinity-based proteomic assays using aptamers can provide sensitive means of discovering cancer indicators.[40] Enzyme-linked aptamer assay (ELAA) is a sandwich-type assay involving one or several aptamer binders where proteins are captured and then analyzed (Fig. 1).[41] Its sensitivity and specificity are comparable with enzyme-linked immunosorbent assay.[42, 43] Moreover, multiplex and high-throughput analysis of ELAA can be further achieved by robotic systems.[44] Following this introduction of aptamer-based protein binding and analysis, we further review several exciting aptamer-based techniques that have been used for cancer protein biomarker discovery below.

SELEX is a powerful technique that can facilitate biomarker discovery. The essential step towards biomarker discovery is using a library of oligomers to identify the potential proteins from a complex mixture, while harvesting the binding aptamers. The target entity

could be protein, cell, and tissue. In Protein-SELEX (Fig. 2A), the oligomers library is firstly incubated with proteins from healthy cells for negative selection. The unbound oligomers are then incubated with purified proteins, collected from cancer cell secretomes[45] or lysates.[46-48] Through this strategy, cyclophilin B, a candidate pancreatic cancer biomarker, and bound aptamer were simultaneously identified.[45] The drawback of this method is that the conformation of the isolated proteins might be changed during purification, which may mask the physiologically relevant binding domains and render them inaccessible to the aptamers.

By contrast, in Cell-SELEX (Fig. 2B), aptamers are selected for cell surface biomarkers which are in their native structures and complexes. These targets provide more accurate information on disease states because they are a better representation of *in vivo* conditions. Moreover, prior knowledge of the target proteins such as structure, conformation, or expression levels is not required.[49] After typically 8-20 rounds of selection using cancer cells and healthy ones as positive and negative targets, respectively, aptamers and their bound molecular biomarkers that distinguish cancer from healthy control can be identified.[50-65] Several cancer biomarkers including protein tyrosine kinase 7 (PTK7, aptamer sgc8) for T-cell acute lymphoblastic leukemia,[50] immunoglobulin heavy mu chain molecule (aptamer TD05) for Burkitt's lymphoma,[63] tenascin-C (aptamer GBI-10) for U251 glioblastoma cell line[62] have been identified. We further refer review articles that describe more on the cancer biomarkers and corresponding aptamers that have been discovered.[66, 67] It should be noted that protein biomarker identification through Cell-SELEX is not always successful. A high degree of non-specific background binding of oligomers to cells causes slow convergence.[68] Additionally, the process heavily relies on well-studied cancer cell lines, whereas its feasibility in real biological and clinical samples[69] that are complex and heterogeneous has yet to be well validated.[70]

Aptamer-facilitated Biomarker Discovery (AptaBiD) has been developed to further improve biomarker discovery efficiency.[65] In contrast to Cell-SELEX, AptaBiD employs biotinylated aptamers to efficiently isolate target cells with streptavidin coated beads after several round of positive and negative selection. Aptamer-biomarker complexes are then separated from lysed cells followed by MS identification. Accordingly, six biomarkers were found for immature dendritic cells (DC), of which CXorf17, transmembrane glycoprotein NMB, lipoprotein lipase, sulfated glycoprotein 1, and serine β-lactamase-like protein were previously unknown.[65] Three biomarkers were identified for mature DC, of which Copine-2 was previously unidentified.[65]

Tissue-SELEX, which can be performed on fixed tissue sections (Fig. 2C) or in living systems (Fig. 2D), has been used for cancer biomarker discovery as well.[71, 72] By using paraffin-embedded cancerous tissue sections and adjacent normal tissue sections from the same breast cancer patient as target and control, respectively, both aptamer BC15 and overexpressed biomarker, hnRNP A1, were identified in tissue sections and in cultured MCF7 and MDA-MB-231 cells.[72] Another study identified an overexpressed nuclear protein, RNA helicase p68, in intrahepatic colorectal tumor-bearing mice which were intravenously injected with a library of nuclease-resistant RNA oligomers followed by many cycles of tumor mass collections and oligomer amplification.[71] The *in vivo* identified biomarker is a more physiologically-relevant indicator of the cancer state than biomarkers identified by artificially cultured cancer cells.

In SELEX, slow convergence limits processing of a large number of samples.[73] By comparison, Slow Off-rate Modified Aptamer (SOMAmer) based proteomic assay (SOMAscan assay) is able to simultaneously detect more than 1,000 proteins, and hundreds of different samples can be screened per day. It provides a rapid way to determine the signatures of protein biomarkers.[74] The assay uses well-established aptamers to discover

biomarkers through detecting fluctuations of protein expression. The core concept relies on the use of DNA microarrays to quantify SOMAmer concentrations, which is taken to be a proxy for target protein concentrations (Fig. 3). SOMAmers incorporate hydrophobic moieties (i.e. benzyl, naphthyl, tryptamino, and isobutyl), which mimic amino acid side chains, at the 5'-position of uridine. These modifications expand the chemical diversity of canonical aptamers resulting in the generation of SOMAmers with higher affinities (dissociation constants (K_d) of 1 nM or lower, and show slow dissociation off-rates, ranging from 10^{-4} to 10^{-5} s^{-1}).[75] The binding interactions between SOMAmer and target are similar to antibody-antigen interactions.[76] The overall success rate for SOMAmer selections to human proteins is approximately 84%.[77] SOMAscan assay has been reported to measure 813 proteins with low limits of detection (LOD), overall dynamic range of 7 logs, and 5% median coefficient of variation. Using this assay, 60 proteins that varied significantly between early and later cancer stages were successfully identified.[75] In another study, 44 proteins of interest in non-small cell lung cancer were identified with approximately 90% accuracy.[78] Plate-based SOMAPanel assay addressed the problem of reagent cross-reactivity and non-specific surface adsorption by satisfying streamlined multiple proteins analysis, which further improved the detection specificity and sensitivity.[79] In the future, the expansion of the variety of SOMAmer for cancer-specific proteins is imperative.[80, 81]

2. Aptamers in Cancer Diagnosis

Theoretically, aptamers are able to specifically recognize almost any oncoproteins, cancer metabolites, and cancer cells. This makes aptamers an appealing tool for cancer diagnostics,[82] especially for the biomarkers for which antibodies are not available or lack the required selectivity.

2.1 Aptamers in Cancer Cellular and Proteomic Biomarkers Detection

Overall, the potential of aptamer-based biosensing is immense, and related research is seeing exponential growth.[30] Aptamers as target recognition moieties have been coupled to various transduction platforms to demonstrate their applicability for biosensing.[30] Generally, optical assays, electrochemical assays, and mass difference assays are the most popular platforms.[14] We summarize them in Table 1 and recommend the corresponding review articles.[14, 18, 26, 30, 33, 83, 84] Some aptamer-based modalities have shown ultrasensitivity, with great promise for cancer cell(s) detection in biological samples.[30, 84-86] Magnetic relaxation switching sensing, which relies on the size of magnetic nanoparticles (MNPs) cluster, has the potential to be developed with extremely low noise, owing to the fact that most biological samples do not have a magnetic background.[87] The presence of cancer cells can be detected with as few as 10 cancer cells bound with aptamer conjugated MNPs in 250 μl of real biological sample.[88] Adaptation of aptamers to nucleic acid amplification platforms can also improve the sensitivity of detection.[89] Specifically, aptamers are amplified after aptamers bind to HL-60 cells, and the PCR products are quantitatively analyzed. The LOD of cancer cells can be pushed down to a single tumor cell in 8 ml of cell suspension by the immuno-aptamer PCR method.[90] Electrochemical detection methods also display extremely high sensitivity in cancer cell detection. A recent study reported that TLS11a aptamers grafted gold disk electrode can detect 2 HepG2 cancer cells in 1 ml of cell suspension using electrochemical impedance spectroscopy.[91] In another study, glassy carbon electrodes that are immobilized with dual aptamers, TLS1c and TLS11a, can successfully detect as few as a single MEAR cancer cell in 10^9 whole blood cells. The detection sensitivity is higher than those modified with a single type of aptamer alone, showing a very promising potential for rare cells-related clinical applications.[92] Regarding cancer protein biomarker detection, the LOD of the present aptamer-based assays can easily go down to target protein concentration in the picomolar range.[93, 94] A study has shown electrochemical detection of unlabeled target

protein at 5 femtomolar using aptamer-conjugated gold nanoparticles (GNPs).[95] Single molecule target protein detection has also been achieved on chemically derived graphene field effect transistors using amplified signal transduction.[96]

Cancer cell capture and isolation at the single-cell level is important as it permits the investigation of heterogeneity among cancer cells and as cancer diagnostics. Rare cancer cells in blood, i.e. circulating tumor cells (CTC) can be enriched by aptamers. In microfluidic devices, a dense aptamer layer can be achieved to match the overexpressed target proteins on cell membranes. To increase capture efficiency, microchannels with small height, low flow rate, and chaotic flow, etc., that can increase the odds of binding are preferred.[97-99] Furthermore, it was reported that a 3D aptamer network, produced by rolling circle amplification (RCA) comprised of repeated anti-PTK7 aptamers in herringbone microfluidic device significantly enhanced the capture efficiency of CCRF-CEM cells over monovalent aptamers and antibodies.[98] Recently, nanostructured substrates, with surface roughness ranging from a few nm to hundreds of nm, have emerged as a very promising technique for CTC detection and isolation.[100] These topologies provide more surface area for aptamer immobilization and cell focal adhesion sites, which reduced cell rolling velocity in microchannels.[101-104] The latest review summarizes, an average of 90% isolation efficiency can be obtained on nanostructured substrates although the specificity is decreased.[105] Aptamers can also be conjugated to isolation agents such as MNPs that use external pulling forces to separate target cancer cells from the cell mixture. In one study, 40% of CCRF-CEM cells were recovered from whole blood using aptamer-tethered MNPs, which was consistent with that of antibody-mediated separation.[106, 107]

Following isolation, it is important to be able to recover the captured cells for subsequent biological studies and drug development. The challenge in post-isolation analysis is detachment of captured cells from substrates, while keeping them unperturbed.[108]

Typically, captured cells are lysed or fixed on the device surface for subsequent molecular analysis, which makes it difficult to explore comprehensive and dynamic information of the cell.[109] Various detachment methods have been developed.[110] Among them, aptamer-mediated detachment by destruction of its tertiary structure using nuclease digestion,[111, 112] thermoregulation,[113] and paired oligomers,[114] have been applied (Fig. 4). Nucleases have been shown to release 97% of captured cells but with a cell viability of 90%, indicating that nucleases could damage the cells. Moreover, nuclease degradation is not effective on chemically stabilized aptamers.[115] Although increasing temperature over 48 °C has shown the release efficiency is approximately 80%, fragile cancer cells would be damaged at elevated temperature.[116] Paired oligomers release 92% of captured cells by opening up the hairpin structure of the aptamer without perturbing the cells. Moreover, an alternate method used competitive binding and formation of DNA duplexes at non-functional regions of aptamers to dissociate them from an immobilized capture probe. Over 95% of aptamer-bound cells were released with over 99% viability.[117]

Although promising, very few aptamer-based assays have unequivocally shown clinical validity or utility, and most of these methods remain in the laboratory settings. The high-abundance non-target blood proteins which comprise 95% of the bulk mass of proteins but represent less than 0.1% of total number of proteins, produce significant noise signals in most proteomic approaches,[118] and thus interfere with the detection and isolation of target cancer cells or proteins that exist in relatively low amount.[119] Moreover, cancer cell heterogeneity and/or associated protein diversity such as splice variants and post-translational modifications post extra challenges. The critical distinctions between samples prepared in buffer and real biological samples from tumor patients also require the existing aptamer-based detection and isolation approaches to be fully validated before applying to clinical settings.[9] Noted that the affinity of some identified aptamers only have medium affinity,

which may impair cancer cells or proteins detection and isolation.[120] Therefore, careful selection of aptamers with desirable properties to the target proteins is necessary for accurate diagnosis. In addition to the aptamer affinity, the sensing platform itself has a great impact on the sensing performance. Taking thrombin, one of the most widely studied proteins with regards to aptamers (although not a cancer marker per se), as an example, a survey of literature demonstrates that the detection sensitivity appears to depend on the type of assay (Table 1). In these papers, the same 15-nt thrombin aptamer was used, which allows us to directly compare the influence of detection platform on sensing performance (i.e., sensitivity). For example, individual aptamer grafted single-walled carbon nanotubes (SWCN) can detect single thrombin through the analysis of the change in conductivity of the SWCN in a field effect transistor; in contrast, by measuring the change in capacitance using potentiometry the limit of detection (LOD) of anti-thrombin aptamers conjugated SWCN drops to 80 nM. Moreover, we acknowledge that other reaction conditions including sample matrix (buffer vs. serum) and temperature may also determine the performance of aptamers since these parameters affect on aptamer binding and conformations. Therefore, fully understanding the biochemistry of aptamers including K_d, conformational state, buffer conditions, temperature, and detailed technical requirements, etc. are fundamental for aptamer-based assays.[14]

2.2 Aptamers in Cancer Imaging

Imaging probe-labeled aptamers can interact and bind to the target proteins on and in cell membranes,[121] the interior of cells,[122] or the extracellular matrix.[123] The molecular imaging techniques enable us to visualize, trace, and measure the expression and activities of target molecules that influence tumor behavior and/or response to therapeutics.[124, 125] Anti-epidermal growth factor receptor (EGFR) aptamer conjugated with dye or GNPs have been used to determine the expression of wild-type EGFR and EGFRvIII mutation, and to directly

monitor aptamer-mediated GNPs internalization into cells.[124, 126] AS1411, TTA1, and MUC-1 aptamers labelled by quantum dots (QDs) that have distinct emission wavelengths have been used for parallel analysis of co-localization and co-expression of nucleolin, tenascin-C and mucin, respectively, within a single tumor cell.[125] Molecular beacon aptamers have been used to monitor protein-protein interactions *in vitro* in real-time. For instance, the thrombin-antithrombin III (AT3) interactions can trigger release of the beacon aptamer from thrombin, leading to detectable fluorescent signals. The detailed kinetics of thrombin-AT3 binding, the interaction mechanism, and the binding site of the interaction thus can be elucidated.[127] Furthermore, using aptamers for monitoring of cellular interactions with the microenvironment was reported.[128] Mesenchymal stem cells attached with aptamers containing a pair of dyes were used to quantitatively detect platelet derived growth factor (PDGF) secretion from MDA-MB-231 cells. Binding of the aptamer to the protein induced a conformational change that brought the dyes within close proximity of each other, producing an optical signal. Such cell sensors can be used to study intercellular signaling and cellular microenvironments in real time, at single-cell resolution, and potentially in living animals. Fluorescence-based methods that offer single-molecule sensitivity towards detection of tumor lesions *in vivo* have been accomplished using aptamers as homing agents and single photon detectors.[129-133] Far-red fluorescent probes that have minimum photo-toxicity, deep tissue penetration, and minimal background interference from scattering, absorption, or auto-fluorescence of samples may hold potential for *in vivo* cancer imaging applications in the future.[134, 135]

In vivo cancer imaging still heavily relies on biomedical imaging tools including magnetic resonance imaging (MRI), computed tomography, and others. These techniques combined with immunochemistry have proven fruitful both in clinical cancer diagnosis and in cancer research settings.[136] Aptamers offer an additional tool for *in vivo* molecular imaging

owing to their excellent tumor penetration, superior signal-to-noise ratio, and sub-centimeter resolution in solid tumors.[137, 138] Intravenously injected TTA1 aptamers containing radioactive isotopes showed rapid penetration of U251 and MDA-MB-435 tumor xenografts within 10 min and high tumor-to-blood signal ratio within 60 min.[5] However, the aptamers displayed rapid blood clearance due to their small size. It was recently reported that a cyclen-based aptamer with a sulfur-containing arm offered increased stability of the aptamer-metal complex, and perceptibly stayed in the tumor for 6 hours.[139] To date, aptamer-conjugated MNPs[140, 141] and GNPs[142] as contrast agents have been tested *in vitro*. However, due to limited studies, information on their safety still remains inconclusive and thus limits *in vivo* applications. For example, AS1411 aptamer-conjugated nanoparticles showed nonspecific accumulation in liver by the mononuclear phagocytic system.[143]

3. Aptamers in Cancer Therapy

3.1 Aptamers as therapeutic agents

In loss-of-function phenotypic knockdown approaches, aptamers can regulate gene expression and modulate protein function, which can be valuable in cancer molecular studies and therapy.[144] siRNA or miRNA downregulates gene expression and protein levels via target mRNA degradation or by blocking translational initiation. However, such antisense approaches become ineffective because of the secondary or tertiary mRNA structures and/or the proteins bound to the mRNA.[145] In contrast, aptamers directly bind to and further block or modulate the biological activities on the protein targets which are involved in pathogenesis.[32] Aptamer for cancer therapeutics can generally be divided into four categories. (1) Inhibition of cancer cell adhesion and invasion through blocking involved molecules (e.g., E-selectin and P-selectin). (2) Modulation of the immune system which could be deactivated by tumor cells. For instance, inhibition of CTLA-4 can prevent immune system inactivation and promote tumor elimination. (3) Blockage of signaling pathways by inhibiting kinases,

phosphatases, or carboxypeptidases etc. to stop downstream activation and signaling for tumor growth.[146-148] Anti-EGFR aptamers can block EGFR autophosphorylation, inhibit recruitment and phosphorylation of intracellular substrates in signal pathways, and thus inhibit cancer cell proliferation, migration and apoptosis resistance.[149, 150] (4) Binding to target proteins that have a close connection with tumor development. For example, intracellularly applied aptamers (intramer) targeting β-catenin can inhibit multiple oncogenic functions of target proteins in HCT116 colon cancer cells.[151] These examples provide simple principles for the use of aptamers for cancer therapy.[122, 152] We have summarized the mechanisms along with the aptamer targets and their binding affinities in Table 2.

The first therapeutic aptamer targeting VEGF was FDA approved in 2004 for the treatment of age-related macular degeneration. Currently, there are a few aptamers for cancer treatment that are being tested in clinical trials. AS1411/AGRO001 (Antisoma) for acute myeloid leukemia and NOX-A12 (NOXXON Pharma) for multiple myeloma and non-Hodgkin's lymphoma are being evaluated in Phase-II and Phase-I clinical trials, respectively.[9, 153, 154] Even so, the translation for clinical applications still faces challenges. Improvements have been made in the stability of aptamers and circulating time in the body. To increase resistance, the required modifications either in the base, sugar ring or the backbone of aptamers are summarized in Table 3.[155] Replacing the 2'-OH with fluoro, amino or hydroxymethyl groups can result in at least 1000-fold longer half-lives of aptamer in plasma.[155] Incorporation of inverted end caps at the 3'terminus,[156] phosphorothioate and methylphosphonate modifications also reduce aptamer degradation.[157, 158] To reduce fast renal filtration of aptamers due to their small mass (<15 KDa),[8] biocompatible and biodegradable polymers such as PEG have been used to increase the mass to reach over 50 KDa which is the cutoff for renal glomerulus resulting in 2-10 fold increase in circulating half-life in animal models.[159] Tagging with lipids, such as cholesterol,[160] dialkyl glycerol and liposome,[161] may

achieve similar effects. However, these tags may increase inflammatory responses.[160, 162] On the other hand, the potential toxicity of aptamers, especially intramers, needs to be verified. The immune complement system can be triggered by high doses of aptamers and nonspecific, off-target protein interactions.[163] The toxic effects may cause serious damage to the cardiovascular system.[164] Low-affinity interactions of aptamers with a tenase complex of coagulation can result in prolonged coagulation time.[165] A decrease in red blood cell mass along with a decrease in reticulocytes have been found after repetitive administration of high doses of therapeutic aptamers.[8] The accumulation of aptamer at very high concentrations in the cytoplasm of renal proximal tubular epithelial cells and other cells or organs may cause degeneration and measurable organ dysfunction.[166] Aptamers remain to be an unpredictable class of therapeutics due to the limited prevalence of published information.

3.2 Aptamers as Delivery Agents

Aptamers against cancer cell surface biomarkers or extracellular matrix proteins has led to the application of aptamers for delivery of therapeutic agents both *in vitro* and *in vivo.*[9] We summarize various carriers and scaffolds that can be used together with aptamers for drug delivery (Fig. 5).[167] Some chemotherapeutics including doxorubicin (Dox), Docetaxel (Dtxl) and antisense oligonucleotides can be non-covalently or covalently conjugated to aptamers for targeted delivery.[46, 168-174] Protein therapeutic agents e.g. gelonin have been fused with aptamers for cancer therapy.[175, 176] Aptamer covalently-grafted photosensitizer including Chlorin e6 and TMPyP4[177, 178] or photothermal nanoparticles i.e. GNPs, gold-silver nanorods, carbon nanotubes (CNTs) in gold cage, etc.,[179-182] have also been tested for cancer treatment. It is noted that therapeutic drugs can easily detach from non-covalent conjugated aptamers. Dox that physically binds to dsDNA through intercalation can detach from aptamers before arriving at cancer lesions. Covalent conjugation of aptamers to drug residues, although avoids early detachment of drug, frequently impairs drug activity. Moreover, during multiple steps

of conjugation, the amount of loaded drug is often compromised. Easy denaturation of gelonin and technical difficulties in fusion with the aptamer limit the application of gelonin-aptamer chimeras.[175] A similar dilemma also exists in siRNA-aptamer chimeras.[171]

Compared to direct conjugation of aptamer to therapeutic agents, the large surface-to-volume ratio and interior space of nanocarriers enable larger number of agents to be delivered by aptamers (Fig. 5).[167] Moreover, evidence from literature suggests that covalent attachment of aptamers on surfaces and encapsulation of therapeutic agents ensure safe delivery of drugs while maintaining delivery specificity.[183-187] Although non-specific delivery happens in surface attachment of aptamers due to nuclease digestion,[188, 189] chemical modifications mentioned preveiously can alleviate this chanllenge. Aptamer micelles are aptamers attached with lipid tails that act not only as cell targeting factors but also building blocks, providing interior space for drug loading. The decreased K_d and dissociation rate suggest that the micellization can also be used as a general strategy to promote binding of low-affinity aptamers through multivalent effect.[190] Several aptamer-conjugated nanocarriers including gold-silver nanorods,[179] CNTs,[191] GNPs,[192] MNPs,[193] have been tested for targeted cancer thermochemotherapy. Additionally, theranostic applications of aptamer-grafted nanocarriers enable full therapeutic efficacy while reducing the degree and frequency of invasive interventions.[194] For example, porous hollow MNPs as contrast agents loaded with Dox while being conjugated with aptamers have been used for cancer therapy and MRI.[195] Upon specific uptake of these MNPs to the target cancer cells, the acid-labile pores in the lysosomes are subjected to acid etching and thus rapidly release Dox. A few nanocarriers including drug loaded aptamer-QDs,[196] aptamer-GNPs,[197] and others[198] can also simultaneously perform imaging, phototherapy and chemotherapy.

Currently, to address heterogeneity among cancer subtypes for targeted drug delivery and to enhance functional binding affinity, multivalent aptamers have been used.[168, 174, 199

Another achievement is the selection of cell-specific internalizing aptamers.[200] Aptamer-mediated targeting of drugs to cell membrane epitopes is widely used to gain site-specific delivery. Both internalizing and noninternalizing epitopes can be targets. However, if aptamers bind to noninternalizing ones, drugs may accumulate around the cells and enter into cells either by passive diffusion or normal transport mechanisms. In contrast, when aptamers bind to internalizing epitopes, the binding triggers receptor-mediated uptake and hence drug uptake is more effective.[201] A recent study reported the use of prostate cancer cell-specific internalizing aptamers in a physiological environment for drug delivery, significantly improved drug concentration inside the cells.[202]

4. Aptamers in Anti-cancer Drug Discovery

Molecularly targeted therapeutics is currently the focus of anti-cancer drug development. The drugs should specifically inhibit or modulate the functions of target molecules, however their identification poses significant challenges to the conventional discovery process.[203] Aptamers that directly modulate the function of protein targets therefore present appealing new opportunities for discovery of molecularly targeted drugs. Several recent pioneering studies through elucidating the tertiary structure of aptamer or utilizing the competitive replacement of aptamers from target molecule have validated this possibility of aptamer-based drug design and screening.

Aptamers can guide structure-based rational design (Fig. 6A). Small molecule drugs have a predilection of binding to functional sites on target proteins. Similarly, aptamers also recognize and bind to specific sites of the targets. Through elucidating the mode of aptamer-protein complex formation, rational aptamer modifications and chemical drug design could be achieved.[204, 205] The aptamer-protein complex yield crucial information, such as overall 3D morphology, the precise bases engaged in aptamer-protein interactions, the localization of these bases, aptamer-protein interface, and engaged subunits of proteins, etc.[206] The

information can be derived from crystallographic analysis. Additionally, crystallography further reveals potential ionic interactions, hydrogen bonds, base stacking, and structural factors (such as distorted hairpin structure of aptamer,[204] hydrophobic interactions in SOMAmers,[207] G-quadruplex,[208] or cation-π interaction with von Willebrand factor[209]), which may contribute to aptamer affinity. So far, very few studies have resolved the 3D structure of aptamer-protein complexes. These include the crystal structures of aptamer-thrombin,[210] aptamer-NFκB,[211] and aptamer-HIV TAR[212] and others.[213-215] Most recently, the structure of SOMAmers bound to PDGF-B and lactate dehydrogenase were solved.[204, 207] Nevertheless, the number of high-quality crystallographic 3D structures of nucleic acid sequences and proteins has increased significantly, and the resolution of the subunit crystal-structure and computer modeling also take major steps towards a better understanding of aptamer tertiary structure, motifs, and interactions with respective protein epitopes.[206] However, the binding sites and folding structures of both aptamers and targets are still poorly predicted by theory or by experience. Firstly, the conformational space available to a biomolecule can be large and grows exponentially with the increased number of residues.[216] The existing structures from experimental databases of known nucleic acid structures may have poor performance on prediction of novel motifs. Additionally, algorithms to accurately predict conformation at an atomic level are not available.[217]

Aptamer can also be used as a drug screening tool through the displacement of aptamers by molecular drugs from target protein surfaces in the form of competitive binding (Fig. 6B). Aptamer-tagged PDGF-B have been used for drug screening, in which aptamers were displaced by an anti-cancer agent, suramin, from the PDGF binding site.[218] This approach does not require the labeling of targets or compounds or the prior knowledge of ligand/receptor structures. Moreover, the application of aptamers as displacement agents to

study the interactions between different protein molecules can be an alternative method for monoclonal antibodies screening, which may not be dependent on epitope dominance.[127, 219]

Aptamer-based drug discovery may increase the effectiveness of anti-cancer drug discovery, shorten the development process, and decrease the failure rate of drug candidates through increased specificity, predictability, and high-throughput screening capability. However, there are multiple hurdles needed to address before it achieves fruitful clinical applications. Selection of cancer specific targets for cancer therapy is difficult given the complexity of molecular signaling pathways.[220] Even if the key molecular mechanism has been well explored, the separation of target proteins, identification of the aptamers through SELEX, their binding mode, respective 3D structures are still demanding challenges. In addition, the high-throughput screening of drugs using aptamers may also be associated with false-negative or false-positive results.[218] For example, false-positive results would be obtained if test compounds prevented the protein-aptamer interaction by binding to the aptamer. Finally, the therapeutic activity and toxicity of these preselected compounds, along with the patients' heterogeneity in drug response and toxicity, are inherent challenges before their clinical use.

5. The Future of Aptamers in Cancer Research

Aptamers have been selected against a number of cancer targets over the last two decades, and the variety, affinity, chemical diversity and half-lives are expected to be further improved. Compared to antibodies that have 20 amino acids carrying a full range of chemical substituents, aptamers have only four bases as building blocks,[221] resulting in their relatively inadequate variety. The discovery and validation of new aptamers mainly relies on SELEX which is tedious and inefficient. Automated systems which can achieve successful selections within one week offer a promising solution to these obstacles,[222, 223] and the quality of yielded aptamer is comparable with that of manual *in vitro* selection. After discovery, large scale

synthesis and cost remain to be primary manufacturing challenges. Existing nucleic acid technology is capable of synthesizing long oligos consisting of 200 nt.[224] However, the high cost of large-scale, modified and high-quality aptamers may be prohibitively expensive for some applications. Although PCR amplification followed by denaturation and transcription offer liberal quantities, the primer design can potentially disrupt aptamer structures and result in undesirable primer-dimer formation.[225] In practical applications, a new generation of aptamers with binding affinity in the picomolar range and rich chemical diversity are desired. A full understanding and improvements of the pharmacokinetics, pharmacodynamics, cytotoxicity, and systemic reaction of aptamers are also required. The biochemistry of aptamers needs to be characterized as well. Some fundamental questions, such as their structures, folding patterns, binding affinity, and regulation of protein and/or cell function need to be explored.[226, 227] Moreover, it has been demonstrated that aptamers can regulate gene expression at four different levels (transcription, intron splicing, mRNA stability, and RNA interference) in the natural eukaryotic systems.[228] Such aptamers have neither been discovered in human nor studied in cancer research. It is likely that the properties of aptamers are far more complicated than our present knowledge.

Together, most of the aptamer-based techniques have remained at the proof-of-concept level. The commercialization of these unique molecules has been slow mainly due to the complexity of real biomedical samples and above mentioned limitations which have not yet been overcome, and thus aptamers have not realized the full scope of their potential. Nevertheless, the inherent advantages of aptamers provide incentive towards drive their use in a variety of cancer-related applications in the near future. By addressing the current concerns, aptamers can rise to be key players in the diagnosis and treatment of cancer.

Figure Captions

Figure 1. Three kinds of sandwich-type ELAA. Target molecule is captured by immobilized aptamer followed by analysis using labelled antibodies (A) or labelled aptamer that recognizes a different epitope on the target molecule (B). Target molecule can also be captured by immobilized antibody and analyzed by labelled aptamer.

Figure 2. SELEX in cancer protein biomarker discovery. In Protein-SELEX, proteins collected from cancer cells and healthy ones, respectively, are incubated with the oligomer library. After multiround selection, cancer protein biomarker and bound aptamer can be simultaneously identified (A). Similarly, in Cell-SELEX (B) and Tissue-SELEX (C and D), cells or tissues as target entity can be used for cancer protein biomarker discovery.

Figure 3. Principle of multiplex SOMAmer affinity assay. SOMAmers and samples are mixed (A) to allow formation of cognate and non-cognate SOMAmer-target protein complexes (B). Since SOMAmers are modified with biotin, the complexes can be separated by beads coated with streptavidin (C). Further, proteins are tagged with NHS-biotin for the second round separation of complexes (D). The following photocleavage can release complexes from the bead surface, meanwhile non-cognate complexes preferentially dissociate during the kinetic challenge (E). The released complexes are captured by streptavidin-coated beads again through the biotin tag on the protein (F). Next, SOMAmers are released from complexes (G) followed by quantitative analysis using DNA microarray technique (H). Reprinted with permission from (Gold et al. Plos One, 2010 5(2): e15004).

Figure 4. Various aptamer-mediated cell detachment. Captured cells can be released through nuclease degradation of aptamer (A), high temperature (B) or paired oligomer-induced conformational change of aptamer (C). Captured cell also can be released through competitive hybridization at non-functional regions of aptamers (D).

Figure 5. Examples of aptamers as homing agents for anti-cancer drug delivery. Drugs can be covalently or non-covalently conjugated to aptamers in a direct way. Meanwhile, a wide range of nanocarriers with a targeting aptamer conjugated to the surface can be loaded with therapeutic drugs. These drugs can be bound to the nanocarriers or be entrapped inside the nanocarriers.

Figure 6. Aptamers in drug discovery. Through elucidating the mode of aptamer-protein complex formation and mimicking 3D structure of aptamer, rational chemical drug design could be achieved (A). Drugs can also be selected through the displacement of aptamers from target protein surfaces in the way of competitive binding (B).

Table 1. Various aptamer-based assays for thrombin detection and respective sensitivities. Note that aptamer sensor performance is highly dependent on sensing platform. LOD: limit of detection.

Table 2. Aptamers to targets of therapeutic interest.

Table 3. Strategies of modifications to aptamers.

Figure 1

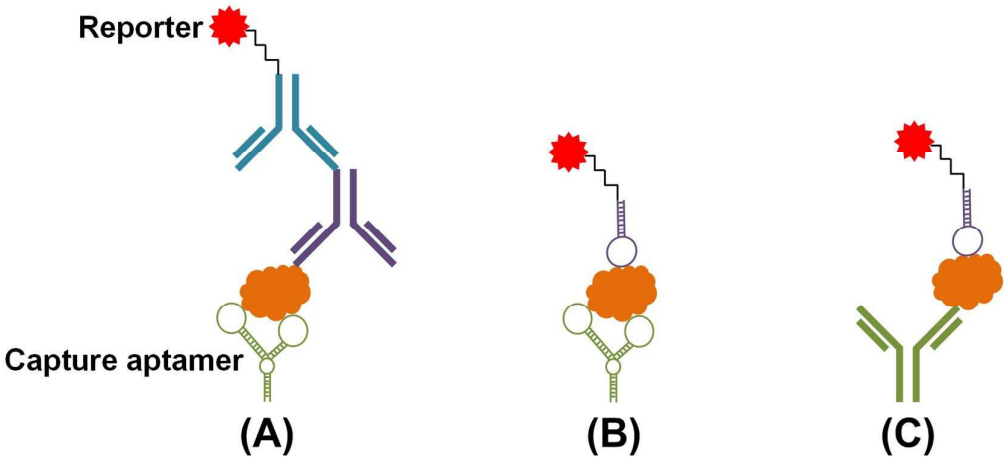

Figure 2

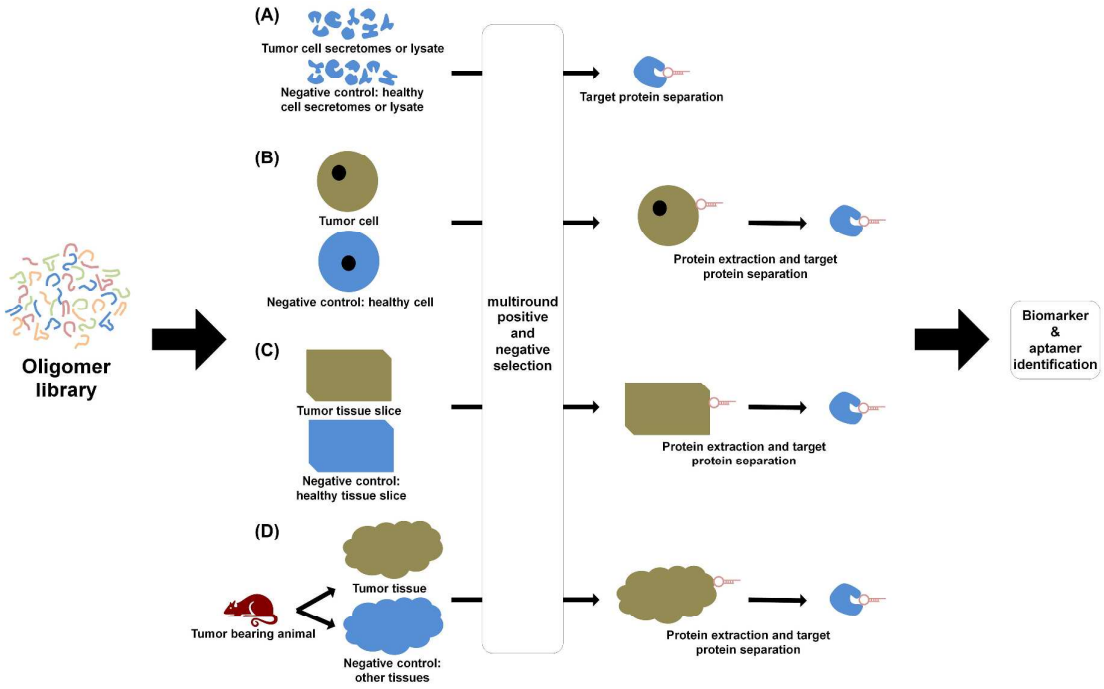
(A)
Tumor cell secretomes or lysate
Negative control: healthy cell secretomes or lysate
Target protein separation
(B)
Tumor cell
Negative control: healthy cell
Protein extraction and target protein separation
Oligomer library
multiround positive and negative selection
(C)
Tumor tissue slice
Negative control: healthy tissue slice
Protein extraction and target protein separation
Biomarker & aptamer identification
(D)
Tumor tissue
Tumor bearing animal
Negative control: other tissues
Protein extraction and target protein separation

Figure 3

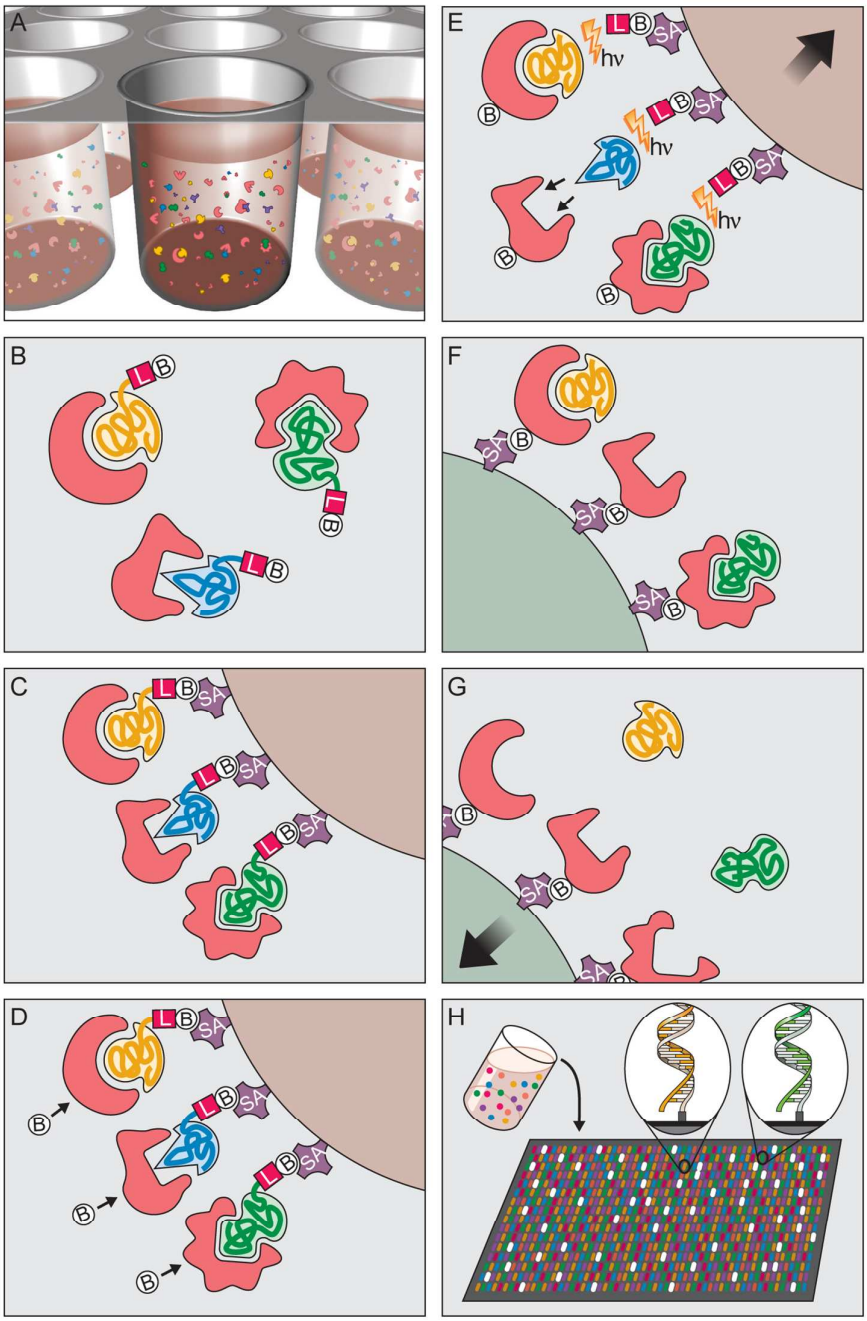

Figure 4

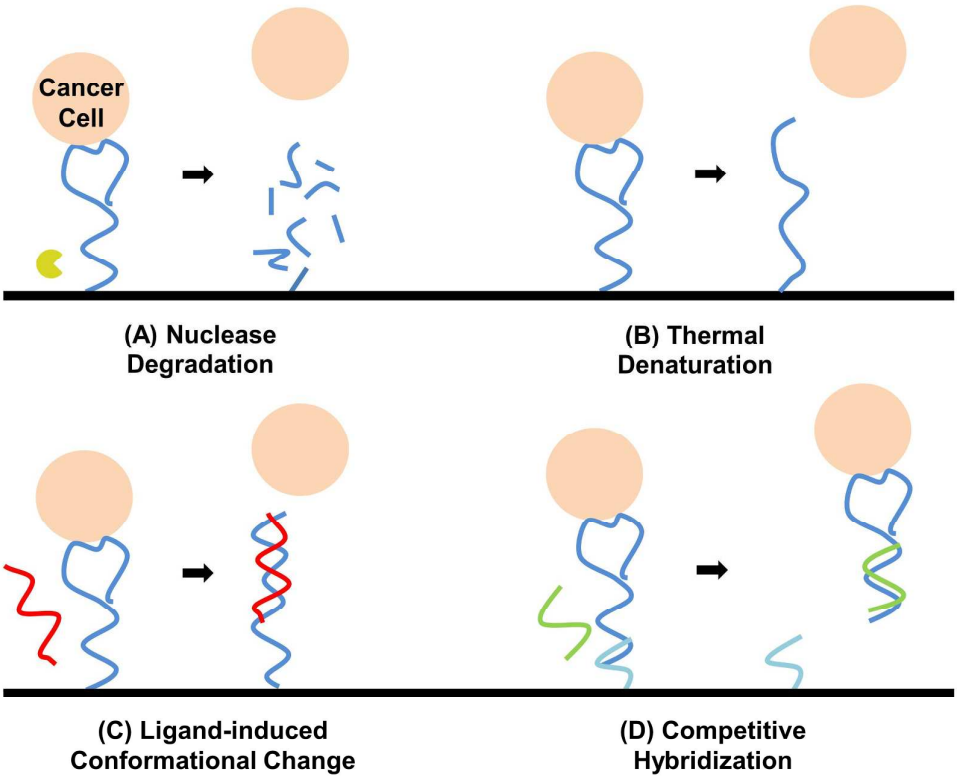
Cancer Cell
(A) Nuclease Degradation
(B) Thermal Denaturation
(C) Ligand-induced Conformational Change
(D) Competitive Hybridization

Figure 5

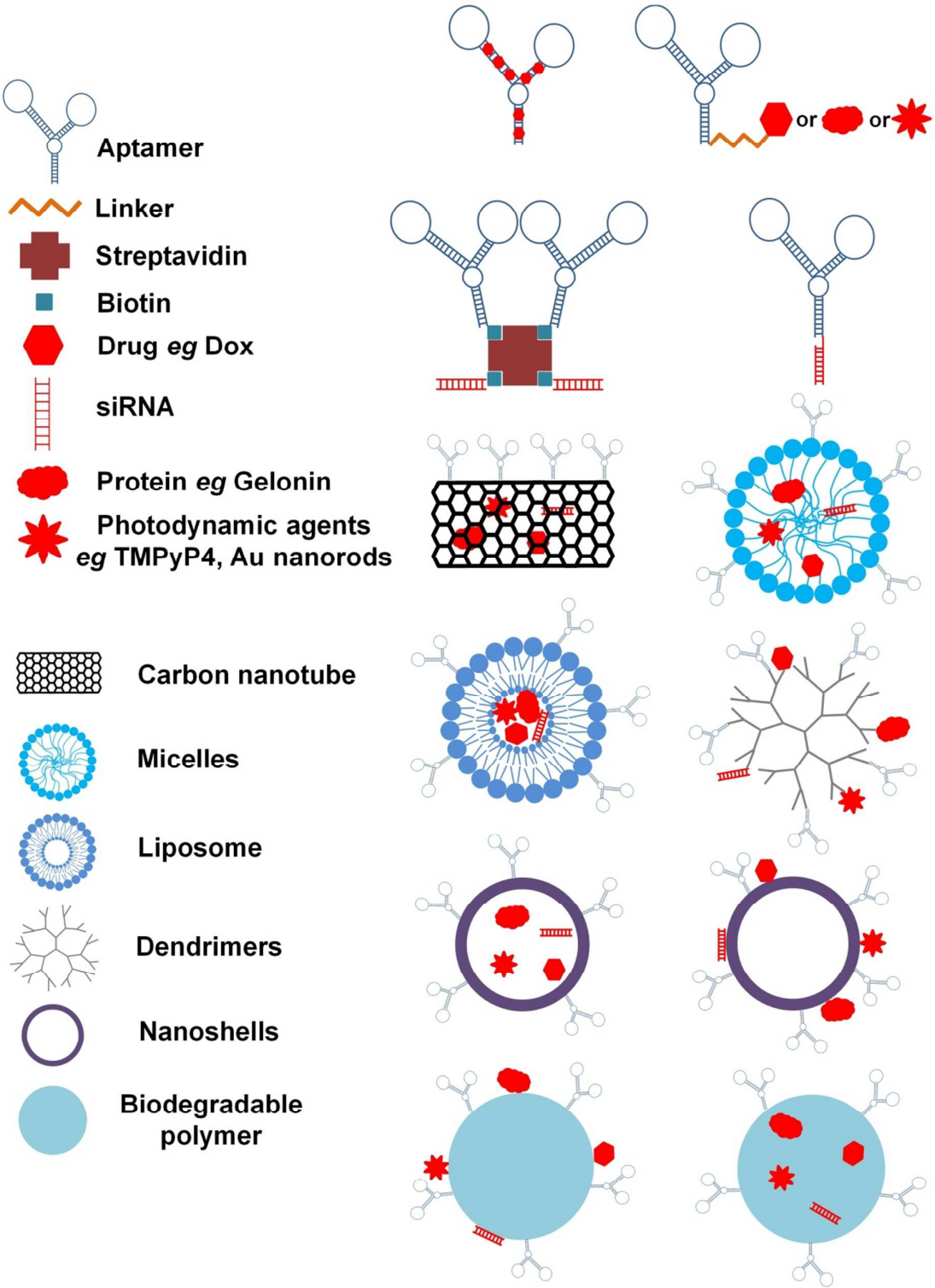
Aptamer
Linker
Streptavidin
Biotin
Drug eg Dox
siRNA
Protein eg Gelonin
Photodynamic agents
eg TMPyP4, Au nanorods
Carbon nanotube
Micelles
Liposome
Dendrimers
Nanoshells
Biodegradable
polymer
or
or

Figure 6

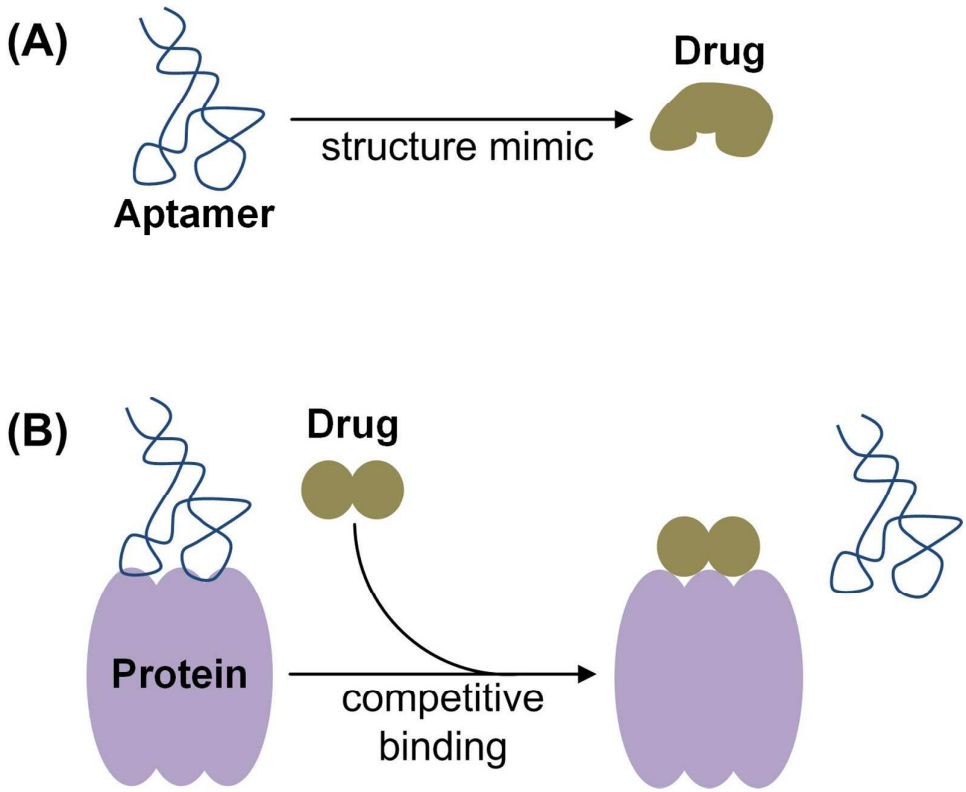
(A)
Drug
structure mimic
Aptamer
(B)
Drug
Protein
competitive
binding

Table 1

Techniques	Sub-classification	LOD of Thrombin
Optical Assays	Fluorescence	10 pM[229]
	Colorimetry	3.2 fM[230]
	Chemiluminescence	6.2 pM[231]
	Surface Plasmon Resonance	500 fM[232]
	Surface Enhanced Raman Scattering	100 pM[233]
	Phosphorescence Energy Transfer	13 pM[234]
	Fluorescence Resonance Energy Transfer	31.3 pM[235]
	Resonance scattering	13 pM[236]
Electrochemical Assays	Voltammetry	7.82aM[237]
	Electrochemical Impedance Spectroscopy	30fM[238]
	Field Effect Transistor	Single molecule[96]
	Impedimetry	10 fM[239]
	Capacitance	80 nM[240]
	Amperometry	5.5fM[241]
	Potentiometry	140 pM[242]
	Electrochemiluminiscence	26aM[243]
Mass difference Assays	Surface Acoustic Wave	9 pM[244]
	Quartz Crystal Microbalance	780 zM[245]
	Microcantilever	200 pM[246]

Table 2

Function	Target Name	K_d (nM)
Inhibit cancer cell adhesion	E-selectin[186, 247]	47
	P-selectin[248]	0.04
	L-selectin[249]	3
	Sialy Lewis X[250]	0.085
	Cytohesin 2[251]	115
	Epidermal cell adhesion molecule[252]	12
Modulate immune system	Cytotoxic T-lymphocyte-associate protein 4[253]	10
	Interleukin-4 receptor α chain[147]	14
	Chemokine (NOX-A12)[154]	N/A
Modulate enzyme activity	Receptor tyrosine kinase RETC634Y[254]	35
	Protein tyrosine phosphatase[255]	18
	Prostate-specific membrane antigen[256]	2.1
	Axl[257]	12
	ErbB1[146]	10
	HER2[258]	3.49
	HER3[259]	45
	Epidermal growth factor receptor variant III[260]	33
	Epidermal growth factor receptor[149]	23
	Protein tyrosine kinase 7[261]	0.8
	Raf-1[262]	18
	Transforming growth factor-β type III receptor[263]	1.52
Inhibit cancer cell activity	Alpha-fetoprotein[264]	33
	Fibroblast growth factor 2, basic[265]	0.35
	Vascular endothelial growth factor[266]	0.14
	Platelet-derived growth factor[267]	0.1
	Keratinocyte growth fator[268]	0.0002
	Neutrophil elastase[269]	N/A
	Nuclear factor-κB[270]	1
	Lymphocyte function-associated antigen 1[271]	500
	αvβ3 integrin[272]	2
	Tenascin C[68]	4
	Gonadotropin-releasing hormone 1[273]	50
	E2F transcription factor[274]	15
	Neurotensin 1[275]	1.5
	Angiopoietin 1[276]	2.8
	Angiopoietin 2[277]	0.06
	Plasminogen activator inhibitor 1[278]	N/A
	Amylin[279]	3
	Mucin 1[280]	0.135
	Substance P[223]	40
	Ghrelin[281]	35
	Nucleolin (AS1411)[153]	N/A
	Tumor necrosis factor super-family member 4-1BB[282]	40
	Tumor necrosis factor super-family member OX40[283]	8
	Wilms tumor protein[284]	700
	B-catenin[285]	5

	Glutathione[286]	41.8
	Osteopontin[287]	57.2
	p68[71]	30.8

Table 3

Modification	Details
2'-OH on sugar residues	2'-fluoro,[288] 2'-NH_2,[289] 2'-*O*-methyl,[290] 2'-*O*-methoxyethyl,[291] or 2'-*O*-dimethylallyl[292]
Phosphate	phosphorothioate,[293] or methylphosphonate[294]
Nucleotide base	propenyl, 5-(*N*'-aminoalkyl) carbamoyluracil methyl, trifluoromethyl, phenyl, or 2-thiopyrimidine[295]
Terminus	3'-3'-linked dinucleotide caps,[156] PEG chains,[296] or cholesterol group[160]

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