Aptamer-dendrimer bioconjugate: a nanotool for therapeutics, diagnosis, and imaging

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Introduction: Aptamers hold great promise as molecular tool in biomedical applications due to the therapeutic utility exhibited by their target specificity and sensitivity. Although current development of aptamer is hindered by its probable *in vivo* degradation, inefficient immobilization on probe surface, and generation of low detection signal, bioconjugation with nanomaterials can feasibly solve these problems. Nanostructures such as dendrimers, with multivalency and nonimmunogenicity, bioconjugated with aptamers have opened newer vistas for better pharmaceutical applications of aptamers.
Areas covered: This review covers brief overview of aptamers and dendrimers, with specific focus on recent progresses of aptamer-dendrimer (Apt-D) bioconjugate in areas of targeted drug delivery, diagnosis, and molecular imaging along with the discussion on the currently available conjugates, using their *in vitro* and *in vivo* results.
Expert opinion: The novel Apt-D bioconjugates have led to advances in targeting cancer cell, have amplified biosensing, and offered *in vivo* cell imaging. Because of the unique properties and applications, Apt-D bioconjugate propose an exciting future. However, further research in synthesis of new target-specific aptamers and their conjugation with dendrimers is required to establish full potential of Apt-D bioconjugate.

Keywords: aptamer, aptamer-dendrimer bioconjugate, dendrimer, diagnosis, imaging, targeted drug delivery

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1. Introduction

Recent developments in nanotechnology have made an enormous impact in the fields of drug discovery, therapeutics, diagnosis, and imaging. The drug delivery systems are designed with the aim of spatial placement and temporal delivery of drug in a desired quantity [1]. However in diagnostic and imaging applications, there is a need in providing target recognition ability to nanomaterials by using biomolecules to get specific detection and precise quantification [2]. Recently developed aptamers bioconjugated with nanomaterials can satisfactorily achieve the above goals.

Aptamers are single stranded DNA/RNA oligonucleotides, which bind to their target molecules like proteins, drugs, organic, and inorganic molecules, or even cells with high affinity and specificity through structure compatibility due to hydrogen bonding, electrostatic forces, or van der Waal's interaction [3-6]. They are synthesized by cost-effective biopanning method called Systematic Evolution of Ligands by Exponential Enrichment (SELEX), which involves screening of combinatorial oligonucleotide libraries followed by iterative *in vitro* selection and amplification [7,8]. Unlike antibodies, aptamers are smaller in size (~ 1 – 2 nm), stable, have better tissue penetration and uniform activity regardless of batch synthesis [9-11]. These factors make them popular candidate in areas of drug delivery and

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Article highlights.

- The aptamer-dendrimer (Apt-D) bioconjugate, in which aptamer exhibit target specificity, whereas dendrimer acts as an excellent nanocarrier, is emerging as novel category of nanohybrid with exceptional promise in the field of biomedical sciences.
- In targeted drug delivery for cancer treatment, Apt-D bioconjugate offers high drug-loading capacity, reduced toxicity, and higher therapeutic effect.
- In diagnostic field, Apt-D bioconjugate demonstrates better immobilization of aptamers and amplified detection signal.
- Apt-D bioconjugate has proven itself better candidate for imaging purpose due to its specificity for target cells and capacity to conjugate excellent florescent agents with less systemic toxicity.

This box summarizes key points contained in the article.

therapeutics. In addition to these they possess ease of synthesis, modification, and manipulation [12]. Also, they are widely used in diagnosis due to their ability to get immobilized on various probe surfaces [13]. Although aptamers are emerging as strong and versatile candidates in biomedical applications, they have certain limitations such as probable *in vivo* degradation, inefficient immobilization on probe surface, and need for development of method to convert aptamer-target recognition into detectable signals [14]. One solution to overcome this conundrum is chemical modification of aptamer; however, bioconjugation of aptamer with nanostructure like dendrimer is recently demonstrated to be of superior resolution owing to unique properties of dendrimer.

Dendrimers are a unique class of synthetic macromolecules, which comprises of an inner core with radial series of branches around it [15,16]. High level of control possible over the architectural design, high ligand density, surface functionality, and the ability to cross cell membranes and to reduce the risk of premature clearance clearly distinguish dendrimers as excellent nanocarriers [17-19]. Unlike classical polymers, they possess multivalency, well-defined molecular weight, monodispersity, high aqueous and nonpolar solubility, high drug-loading capacity, reproductive pharmacokinetic properties, ease of preparation, and functionalization [20,21]. Dendrimer finds its application in cancer therapy owing to its lack of immunogenicity, good biocompatibility, and low toxicity [22]. Dendrimers can form conjugates with genes and nucleic acids such as aptamers through various forces of interactions, which allows more efficient and safer gene transfer as compared to liposomes and other polymers [23,24]. They have greater transfection efficiency not only due to specific geometry but also by low pK values [25]. They also allow controlled release of bioactive agents by using different biodegradable linkers [26,27].

Aptamer-dendrimer bioconjugates (Apt-D bioconjugates) combine the advantageous features of both entities and have opened stimulating avenue for exploration. This article summarizes recent progresses of Apt-D bioconjugate in areas of targeted drug delivery, diagnosis, and molecular imaging along with the discussion on the currently available conjugates, using their *in vitro* and *in vivo* results.

2. Aptamer-Dendrimer (Apt-D) bioconjugate and applications

2.1 Application in drug delivery and therapeutics

2.1.1 Targeted chemoimmunotherapy using drug-loaded Apt-D bioconjugate

Cancer treatment possesses complicated challenges. Cancer is very complex disease; the malignancy of tumors is detected only at advanced stages when administration of chemotherapeutic drugs is toxic to healthy cells. Moreover, conventional therapy such as surgical resection is highly invasive and nonspecific. It is proposed that cancer arises through clonal development from cells that build up a series of mutations. If the pathway, on which cancer cell relies heavily, can be blocked then one can selectively inhibit growth of cancer cell [28-30]. The promise of Apt-D bioconjugate lies in the potential to perform multitasking such as detection, diagnosis, imaging, and drug delivery in a single molecule selectively to the cancer cells.

Recently, combination of chemotherapy with immunotherapy (chemoimmunotherapy) has proven as a novel and efficient therapeutic strategy in the cancer treatment. The synergistic effect of this combination therapy requires carriers that incorporate and simultaneously deliver both immune-stimulating and cytotoxic chemotherapeutic agents [31]. Doxorubicin is an anticancer drug originally isolated from bacteria found in soil samples taken from *Castel del Monte*, an Italian castel. It acts by intercalating and inhibiting macromolecular biosynthesis. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed, and thereby stopping the process of replication. Bagalkot *et al.* developed combined chemoimmunotherapeutic complex using a plasmid bearing unmethylated Cytosine-peptide-Guanine (CpG) nucleotides and Doxorubicin (Dox). CpG oligonucleotides acted as an immune-stimulating agent and as a carrier for the chemotherapeutic drug, Dox. This plasmid–Dox complex resulted in greater antitumor efficacy with lower systemic toxicity as compared to the same amount of free Dox [32]. However, plasmid–Dox complex lacked specificity for cancer targeting; nevertheless, such targeting can be achieved by using aptamer [33]. Studies carried out by Kaminskas *et al.* showed that when dendrimers or liposomes were used to carry Dox, they had long residence time in plasma as compared to Dox in saline. But in both cases, concentration of free drug remained low. Pharmacokinetic studies showed that all three formulations resulted into same degree of reduction in tumor growth but dendrimer- and liposome-conjugated drug had better residence in tumor environment. Moreover, dendrimer-Dox caused less severe systemic toxicity in comparison with other

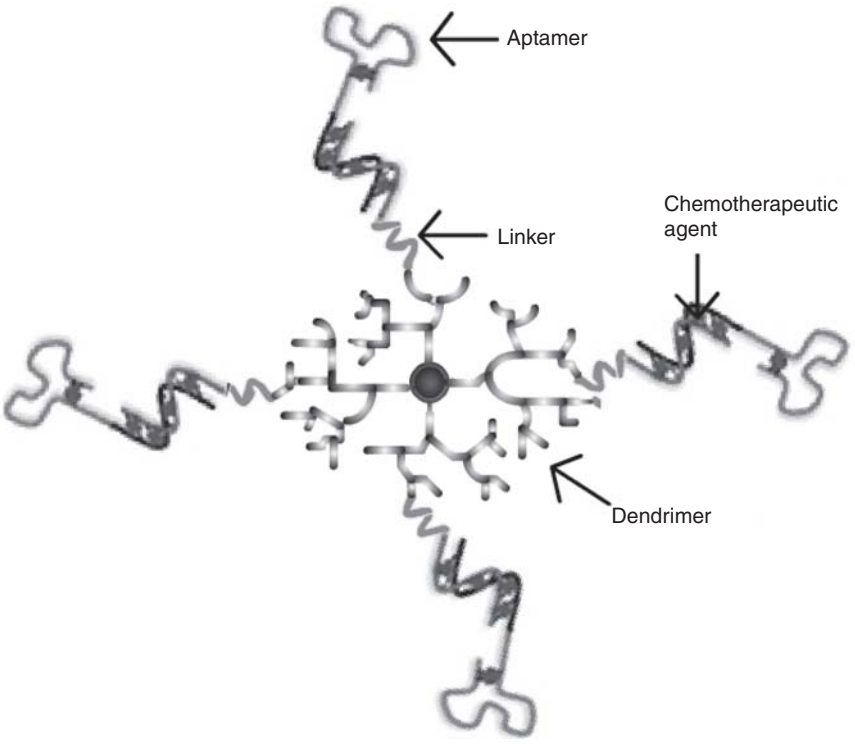

Figure 1. Dox@Apt-dONT-DEN (Doxorubicin intercalated between aptamer-deoxynucleotide-dendrimer) bioconjugate for targeted chemoimmunotherapy of prostate cancer.

two. These preliminary evidences proved that Dox-conjugated dendrimers showed similar *in vivo* chemotherapeutic activity but reduced toxicity as compared to liposomal Dox owing to smaller size of dendrimer (16 – 20 nm) than liposome (20 – 250 nm) [34]. Hence, targeted chemoimmunotherapy approach based on aptamer–dendrimer bioconjugate can be more advantageous with greater antitumor efficacy, cancer-specific targeting ability, and much lower toxicity.

Lee *et al.* reported an Apt-D bioconjugate using prostate-specific membrane antigen (PSMA) specific A9-RNA aptamer (A9-RNA apt) as targeting moiety, G4 Polyamidoamine (PAMAM) dendrimer as carrier, Dox as chemotherapeutic agent and duplex oligonucleotides (dONT) as immunostimulant for prostate cancer chemotherapy [35]. In Apt-D bioconjugate developed by Lee and his coworkers (**Figure 1**), PAMAM dendrimer was attached to CpG oligonucleotides through biodegradable adenine linker. Target specificity was imparted by binding PSMA-specific aptamer to the formed moiety. Linking was done through chemical bonding between complementary nucleotide sequences of elongated 3′ end of aptamer and that of oligonucleotides. Anticancer activity of Apt-D bioconjugate was due to Dox intercalated between resultant double stranded CG base pairs. Dox was selected as a chemotherapeutic agent because it is known to form stable complex with duplex oligonucleotides (dONTs) by intercalating favorably into consecutive CG base pairs [35,36].

PSMA is a type 2 integral membrane glycoprotein. It is expressed on the surface of prostate carcinomas abundantly at all stages of disease but importantly absent on membranes of healthy tissues [37-40]. Thus an RNA aptamer that specifically recognizes PSMA can be used as a prostate cancer–targeting ligand [41-45]. Cancer-specific targeting ability of this aptamer restricts drug distribution to the noncancerous cells, and thereby decreases potential systemic toxicity associated with the conventional chemotherapy [46]. Being composed of DNA/RNA oligonucleotides, aptamer can form linkages with other oligonucleotides (ONTs) having base sequences complementary to each other. It is the PSMA aptamer that allows the formation of duplex nucleotide, which permits delivery of immune-stimulating agent—CpG oligonucleotide. Moreover, double strand formed due to bonding of aptamer and CpG oligonucleotides increases the number of cytosine-guanine base pairs, where Dox preferentially intercalates and increases drug-loading capacity [36,47].

Dendrimers possess advantageous features such as confined nanometer size (~ 16 – 20 nm) and high *in vivo* stability. They also show controlled degradation and high ligand density [16]. Because of their small size, dendrimers readily interact with biomolecules both on surface and within the cells resulting in increased circulation time in the bloodstream [48,49]. Also, they possess enhanced permeability and retention (EPR) effect making them attractive candidate as nanocarrier. Drug-loaded

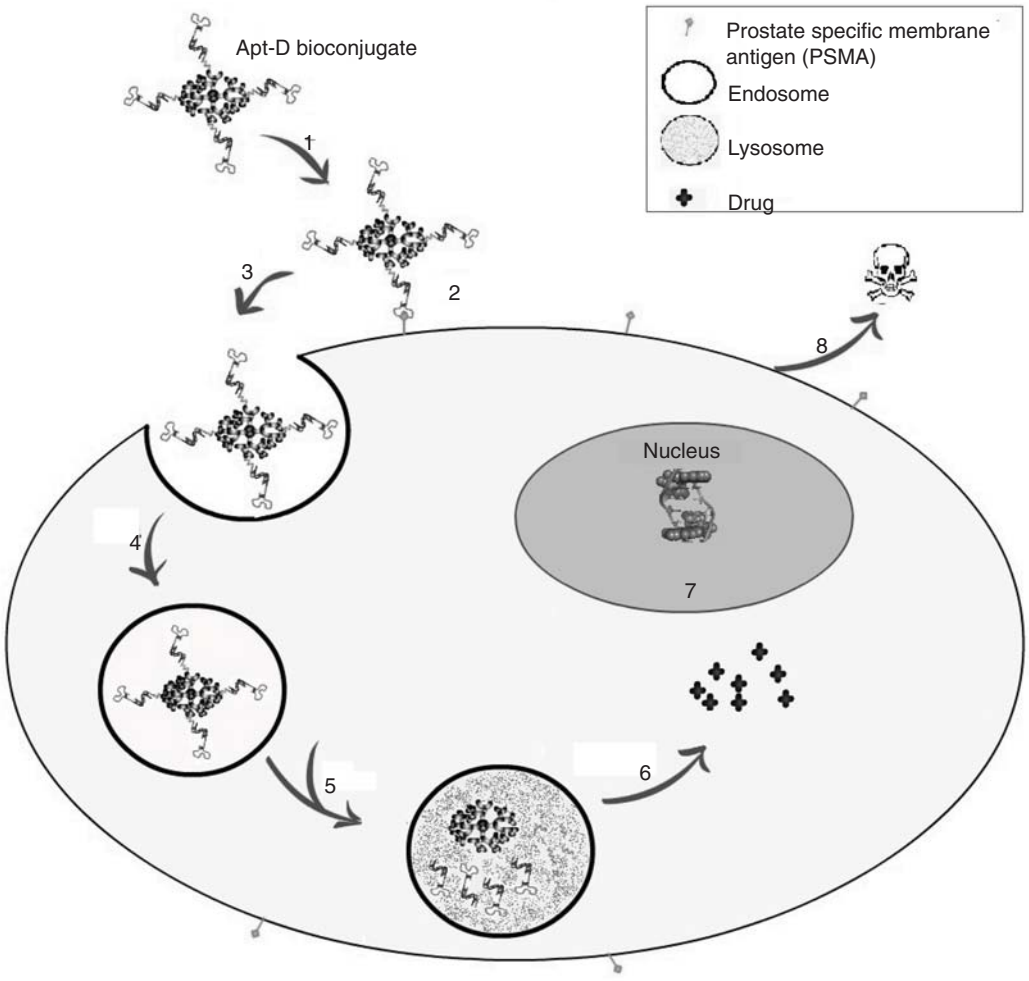

Figure 2. Illustration showing targeting and death of cancer cell by Apt-D bioconjugate. 1. Apt-D bioconjugate circulating in blood reaches site of action, carcinoma cell, through recognition by aptamer; 2. Apt-D bioconjugate binds to Prostate-Specific Membrane Antigen (PSMA) through active targeting and detection by PSMA-specific aptamer; 3. Internalization of Apt-D bioconjugate; 4. Invaginating plasma membrane envelopes the complex of the receptor and Apt-D bioconjugate to form an endosome; 5. Lysosome formation as the pH value in the interior of the endosome becomes acidic and lysozymes are activated; 6. The drug is released from bioconjugate and enters cytoplasm; 7. Doxorubicin enters nucleus and intercalates DNA; 8. Eventually, cell death [35,50].

dendrimers accumulate in cancer cells without being recognized by P-glycoprotein efflux pump responsible for multidrug resistance. This leads to high intracellular concentration of drug [50]. Furthermore, dendrimer allows the use of different biodegradable linkers to control drug release in a desired controlled manner. In the above Apt-D bioconjugate, an oligonucleotide of A10 adenine was used as a linker. This acid-labile linker releases complex specifically within the acidic environment of a solid tumor [34,46].

The concept underlying targeting and death of cancer cell is illustrated in **Figure 2**. Aptamer recognizes a PSMA present specifically on prostate cancer cell surface. Internalization of Apt-D bioconjugate follows through receptor-mediated endocytosis to form endosome. From endosome Apt-D bioconjugate transit through several stages of transport and finally ends up in a lysosome. Acidic environment of lysosome triggers release of drug, here Doxorubicin. Doxorubicin then intercalates with DNA in the nucleus of cancer cell and thereby inhibits its macromolecular biosynthesis. This eventually causes death of cancer cell [35,50].

Serum stability studies performed by Lee *et al.* clearly indicated increased stability of RNA aptamer in serum due to dendrimer. This is attributed to the steric hindrance generated around Apt•dONTs attached to dendrimer core, which prevented access of nucleases to the cleavage site in the conjugate [35,36]. Importantly, results of growth inhibition assay

Table 1. Summary of properties of different conjugates.

	Therapeutic efficacy	Target specificity	Systemic toxicity	Ref.
Free Dox	✓	×	✓✓✓	[34]
Plasmid-Dox	✓✓	×	✓✓✓	[32]
Liposome-Dox	✓✓✓	×	✓✓	[34]
Apt-DEN-Dox	✓✓✓	✓	✓	[35]

Note: Number of ✓ represents severity of characteristic. This table represents qualitative comparison between different factors based on the *in vivo* and *in vitro* results found in the literature.

showed greater cytotoxicity of Dox@Apt•dONT-DEN toward PSMA-positive cell lines LNCaP and 22RV1 [36]. Free Doxorubicin's most serious adverse effect is life-threatening cardiotoxicity. But, histopathological analysis of heart tissues carried out by researchers showed that histological appearance was less disordered when Doxorubicin was delivered through dendrimer. This proved that dendrimer decreases cardiotoxicity of Doxorubicin when it was used as a carrier in the system [36].

Table 1 summarizes the comparison between properties of different conjugates. When free Dox is delivered alone, not only it lacks target specificity but at the same time it causes more systemic toxicity. Plasmid-Dox and liposome-Dox conjugates have better therapeutic efficacy and comparatively lower toxicity, but they also lack specificity toward target. However, Apt-DEN-Dox bioconjugate show all three desired results namely better therapeutic efficacy, target specificity, and significantly low systemic toxicity.

Aptamer contributes cancer targeting specificity, whereas dendrimer contributes greater transfection efficiency, high drug-loading capacity, enhanced delivery of drug complex to carcinoma cells, and importantly reduced toxicity. Also, dendrimer of Apt-D bioconjugate helps to increase *in vivo* stability of oligonucleotides and other RNA- or DNA-based drugs against exonucleases. This proof-of-concept demonstrates the potential of Apt-D bioconjugate as a new approach to improve chemotherapy.

2.2 Application of aptamer-dendrimer bioconjugate in diagnostics

The detection and quantification of proteins has become essential in fields of bioanalysis and medicine. Biosensor is simple, inexpensive, integrated receptor-transducer device having the ability of providing escalating quantities of protein information and thus can be used for early diagnostic purposes. The biosensor consists of a biological recognition element acting on a biochemical mechanism and of a transducer relying on electrochemical, mass, optical, or thermal principles (**Figure 3**). The direct spatial contact between the biomolecule and the transducer makes biosensor a critical tool for diagnosis [51,52].

Existing techniques of diagnosis such as Enzyme Linked Immunosorbent Assay (ELISA), radioimmunoassay (RIA), immunohistochemistry are used to detect number of biological molecules. In such methods proteins, enzymes, antibodies, microorganisms, or nucleic acids serve as typical bioreceptors [51,53]. Despite extensive development and number of advantages, these techniques suffer from certain limitations as they are labor-intensive, time-consuming, require sophisticated instrumentation, and possess lack of specificity as well as affinity for target molecules. Therefore, there exists considerable scope and potential for newer generation of biosensors with novel bioreceptor, which can overcome limitations of existing biosensors [54].

Aptamers have drawn considerable attention as significant recognition elements for the construction of various kinds of biosensors due to their exclusive properties. Few important ones are small molecular weight, simple structure, excellent target versatility, biomolecular recognition ability. In addition to this, aptamers have capacity to distinguish between chiral molecules and to recognize a distinct epitope of a target molecule with high efficiency. Their ability to get preserved for longer time makes them very useful in practical applications [55-58]. Aptamers possess several advantages in diagnostic area over antibodies such as ease of synthesis, easy labeling, and ease of manipulation of binding affinities or specificities, and good stability [51,54]. Moreover, aptamers are capable of being reversibly denatured, which facilitates capture and release of target compounds in reusable applications [59].

Biosensors using aptamers as bioreceptors for recognition are termed as aptasensors. In 1998, Potyrailo designed first aptasensor and his report showed that biosensors using DNA/RNA aptamers can detect various protein molecules present even in quantity upto nanomolecular concentration with increased thermostability and raised tolerance to pH and salt composition [60]. Many aptasensors exhibiting most of the common biosensing strategies like optical, electrochemical, and mass sensitive approaches are then developed and studied [56,57]. For example, Ying Lu *et al.* developed DNA-aptamer-based electrochemical biosensor for interferon gamma detection [61]. Kyung Mi Song *et al.* have invented optical aptasensor for ampicillin using gold nanoparticle (GNP)-based dual fluorescence-colorimetric methods [62,63]. While developing nanostructure with GNP, it is vital to heed few aspects of toxic biological effects. Biocompatibility, physical, chemical, and optical properties of GNP is greatly influenced by their size. GNPs of 10 to 100 nm tend to accumulate in organs of the reticuloendothelial system. The biodistribution homogeneity is in direct proportion to the size of GNPs. Pan *et al.* demonstrated that

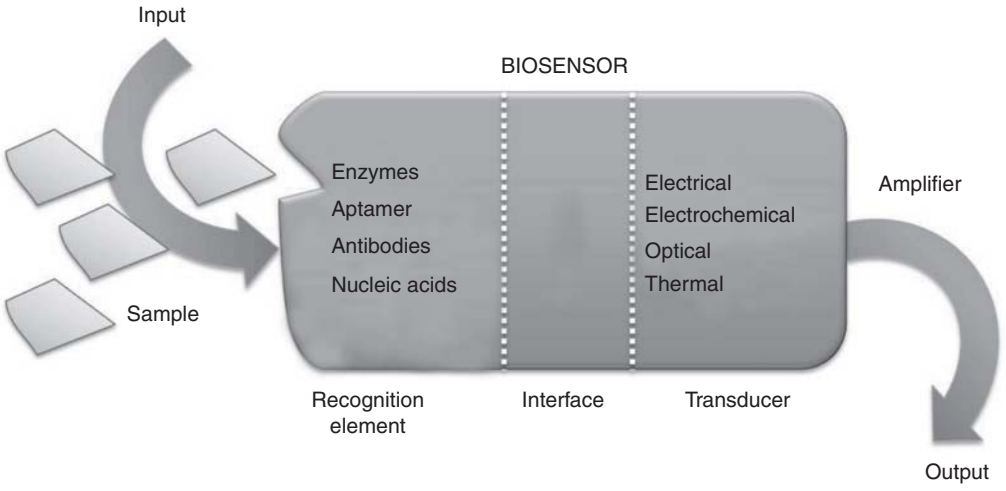

Figure 3. Schematic representation of biosensors showing biorecognition system—typically aptamer, antibodies, nucleic acid—immobilized on surface of transducer. Specific interactions between target molecule and biorecognition system produces a physicochemical change which is detected by transducer. The transducer can be of variety of types depending on the parameter being measured-electrical, electrochemical, optical, and thermal are the popular universal parameter.

GNPs of diameter 1 – 2 nm can induce cytotoxicity, but larger GNPs of diameter 15 nm cannot. This can be attributed to possibility of irreversible binding of GNPs to the biopolymers in cell [64,65]. Nevertheless, Niidome *et al.* have shown that toxicity is triggered by the surface modification of GNPs [66]. On the other hand, Connor *et al.* showed that neither the surface characteristics nor the size of GNPs seemed to play a role in inducing cytotoxicity [67]. Data on *in vivo* experiments is rather incoherent and more studies are needed to understand biodistribution and toxicity of GNPs.

Although aptasensors are valuable, they possess problems with stability and signal registration. The practical application of aptamer-based recognition is limited because immobilization of aptamers to the supported film coupled with their microenvironment affect aptamer structure and aptamer–ligand interactions [68]. Levels of signal and noise in case of biosensors based on electrochemical properties depend on interface between the sensor's surface on which biomarkers are immobilized and sample solution. When aptamer is directly immobilized on the metal electrode, the surface-aptamer interaction causes its denaturation and thereby results in reduced activity and low signal production. But, if interface is covered with suitable conducting polymer, it will prevent conformational change in aptamers and reduce the problem of low detection signal [69]. Furthermore, there is a need of elimination of interferences with other proteins and cells. Immobilization of aptamers onto nanomaterials, for example, dendrimers for biosensing have found to give excellent results to circumvent the above described problems [53,59].

Many strategies utilizing dendrimers alone as biosensor materials have been studied [70,71]. For example, biosensor was developed by Li *et al.* based on self-assembled gold electrode with G4 PAMAM dendrimer to immobilize DNA [72]. Fei *et al.* introduced nano sized dendrite structure composed of short sequence of DNA oligonucleotides into interface of biosensors with the aim of improving performance of detector [69]. Number and nature of functional groups can be adjusted on the surface of dendrimer for the purpose of chemical fixation. This, in addition to their good biocompatibility, allows dendrimers to be used for modifying electrode surface of aptasensors [69,73]. Importantly, dendrimer alleviate the problem of low activity and low signal, which are caused by denaturation of aptamers [74]. Thus, nanoparticles such as dendrimers serve not only as solid phases but also as catalyst used to amplify detection signal of aptasensors [59]. Consequently, a new approach based on aptasensor conjugated to dendrimer is emerging as promising tool for detection and quantification of biomaterials. Some of those applications are discussed here in brief.

Thrombin has been the most studied target and its aptamer sequences have been used as biological recognition agents in the design and development of biosensors [75]. Thrombin is a specific serine protease and acts as a major stimulus of both procoagulant and anticoagulant reactions [55,58]. Generally, normal blood concentration of thrombin in human body during coagulation process varies from nanomolar to molar. The controlled thrombin level in blood is essential because of its major role in hemostasis. Therefore, a sensitive, specific, and highly efficient bioanalytical detection method is required to be developed. Zhang *et al.* have developed a sensitive impedimetric thrombin aptasensor conjugated with polyamidoamine (PAMAM) dendrimer based on electrochemical impedimetric principle [76]. Biosensor acting on electrochemical impedance spectroscopy (EIS) is a device that transduces interfacial changes between

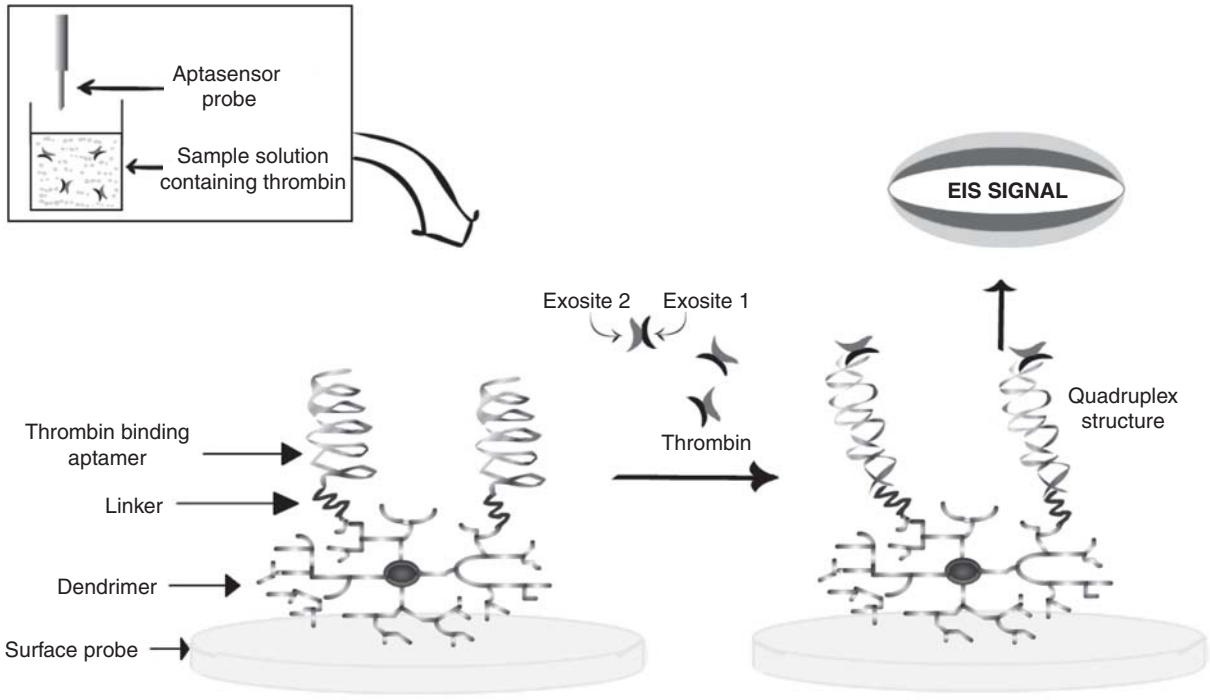

Figure 4. Schematic illustration of aptamer-dendrimer biosensor for thrombin detection. 15-mer-single-stranded DNA is used as thrombin-binding aptamer (TBA) which is bioconjugated to G4 PAMAM dendrimer. Aptamer undergoes target-induced conformation change for detection of thrombin. TBA maintains its unfolded structure and allows electron transfer to occur causing generation of electrochemical signal. TBA, in presence of thrombin, binds with amino acids on exosite I of thrombin and forms quadruplex structure, which restricts electron transfer, resulting in an increase of the interfacial electron transfer resistance detected by Electrochemical Impedance Spectroscopy (EIS). The principle behind quantification of thrombin is the change of electron-transfer resistance, as magnitude of electrochemical signal depends on degree of electron transfer, concentration of thrombin can be quantified.

the electrode and the electrolyte because of bioreceptor hybridization, conformational changes, or biomolecule damages to an electrical signal [69]. In this approach, amino-modified thrombin binding-aptamer (TBA) probe was immobilized onto G4 PAMAM dendrimer layer, which was covalently attached to gold electrode surface. Zhang *et al.* used 15-mer single-stranded DNA as a thrombin-binding aptamer and G4 PAMAM dendrimer (**Figure 4**) [77].

It is known that thrombin has two positively charged sites termed Exosite I (the fibrinogen recognition exosite) and Exosite II (the heparin-binding exosite) on the opposite sides of the protein [77]. The 15-mer TBA increases the specificity for fibrinogen recognition exosite. In the absence of target molecule, aptamer could maintain its unfolded structure and electron transfer occurs from electrode surface, generating electrochemical signal. When TBA binds with thrombin, it forms an intermolecular quadruplex structure and restricts the electron transfer (**Figure 4**). Based on the extent of electron transfer, degree of electrochemical signal is generated, which ultimately depends on the amount of thrombin present in the sample [78]. PAMAM dendrimer increases immobilization of aptamer probes due to the surface effect of nanomaterials [76].

Surface plasmon resonance measurements have proved that 15-mer short-chain DNA molecule, that is, TBA improves the assembly capacity of probe molecule greatly. EIS characterization confirmed that attachment of TBA onto electrode surface led to increase in charge transfer resistance (R_{ct}) due to lot of negative charges on the TBA sugar-phosphate backbone [76,79]. Further, formation of the TBA–thrombin complex on the electrode surface contributed to a significant increase in the R_{ct} value [76]. This is because thrombin and its aptamer complex prevent the electron transfer between the electrode surface and the electrolyte solution. Positive linear relationship of R_{ct} value with the concentrations of thrombin in the range of 1–50 nM, and the detection limit as low as 0.01 nM indicated that TBA increases the sensitivity of detection of targeted biomaterial thrombin. Also, stable covalent immobilization strategy and TBA molecules

insusceptibility to fast degradation make aptasensor favorable for regeneration, keeping same sensing activity. In addition to these, use of specific aptamer enhances selectivity, reproducibility, and life stability of modified electrode used for diagnosis [76]. Higher ΔR_{ct} values of the immobilization capacity of TBA molecules and the response signals of thrombin of the dendrimer-modified electrode when compared to those of the non-dendrimer modified electrode indicate that dendrimer layer not only remarkably improves the immobilization capacity of probe molecules but also magnifies the response signals greatly [76]. This is because an individual PAMAM dendrimer molecule can bind several TBA molecules owing to 64 amino groups on dendrimer's surface. Also, electrochemical impedance spectroscopy illuminate the potential application of aptasensor conjugated with dendrimer in real samples [60].

Arotiba *et al.* carried out electrochemical studies of neopterin on poly(propyleneimine) (PPI) dendrimer–gold nanocomposite screen printed carbon electrode using voltammetry and electrochemical impedance spectroscopy. Scientists developed DNA aptamer for neopterin (biomarker of tuberculosis) using SELEX and used it as probe surface of biosensor. It was found that neopterin aptamers could be suitably immobilized onto the Au-PPI dendrimer nanocomposite by electrostatic attraction or streptavidin–biotin interaction. It was seen that neopterin was easily oxidized on the newly formed conjugate and could be detected in the sub-nanomolar concentration level (chemical sensor). The impedance profiles of the aptasensor on binding with neopterin gave a remarkable detection limit lower than 1 nm and the aptasensor was also found to be stable [60].

Altogether the dendrimer immobilization on the electrode surface improved the probe-binding capacity and amplified the response signals of the aptasensor. Also, Apt-D bioconjugate used for diagnosis exhibited high sensitivity, favorable specificity, stability, and detectability in biological fluid. Thus, the aptasensor in conjugation with dendrimer could lead to the development of advanced biosensor platforms for the expansion of fast and efficient detection systems.

2.3 Dendrimer-modified quantum dots-aptamer bioconjugate for biomedical imaging

Quantum Dots (QDs) are semiconductor nanoparticles made up of elements from the group II–VI or III–V of periodic table. QDs are usually in size range of 2 to 10 nm in diameter As a result, QDs possess similarities with biological macromolecules like nucleic acids and proteins in case of dimensions, and thus they are attracting great interest of researchers. Optical and electronic properties of QDs, which can be accurately tweaked by fine-tuning their size and composition, are result of the quantum confinement effect due to mobility of charge carrier-electrons and holes [80,81].

Inorganic QDs are typically bright with quantum efficiency of around 20 – 80% and stable under comparatively hard environments as compared to organic fluorescent dyes [82,83]. Conventional dyes suffer from narrow excitation spectra, requiring excitation by light of a specific wavelength, whereas broad absorption spectra of QDs allow simultaneous excitation of multiple different colored QDs using a single wavelength [83]. QDs can be designed for the emission of light on incidence of various precise wavelengths from ultraviolet (UV) to infrared (IR). Organic fluorophores bleach readily within minutes on exposure to light, whereas QDs are extremely stable. Due to these unique properties, QDs are being extensively studied as a new class of nanoparticle probes for cellular and *in vivo* imaging [83,84].

First generation of QDs was originally stabilized in water using small ligand surfactants, but these thin layers deteriorate swiftly. Therefore, QDs were modified using thick organic bilayer coatings for stabilization in water. However, these are limited by signal intermittence (blinking), thick organic coating resulting in bulky size [85,86]. To reduce the size of QDs, first-generation QDs were tailored using a compact monolayer of multidentate ligands, such as di-thiols conjugated to ethylene glycol oligomer or low molecular weight multidentate polymer [87]. These novel types of coating gave rise to second generation of QDs, and tremendously reduced overall size of QDs [86]. Also in second-generation QDs, bandgap between the core and shell layers is being modified and optimized for higher quantum yield efficiency. Nevertheless, synthesis of ideal QD with tunable emission having continuous (nonblinking) light emission, defined valency, and compact size is still a challenge [85].

Use of QDs, however, is limited due to few challenges. First, large surface area-volume ratio makes QDs core very reactive and because of which QDs undergo very strong unspecific interactions with macromolecules causing variation in fluorescence and particle aggregation. Second, due to surface oxidation and heavy metal composition QDs have inherent cytotoxicity, which strongly depends upon physicochemical properties such as size, surface charge, capping material, surface functional groups, oxidative, photolytic, and mechanical stability, although progress is made in overcoming this problem by developing cadmium-free QDs [88]. Also, conjugation of QDs with biomolecules may change their diffusivity, disturb original conformation of protein or affect fundamental pharmacokinetic and pharmacodynamic properties of conjugated nanocarrier. Third, body clearance of QDs can be major concern as they accumulate in mouse kidney, liver, spleen after administration for several months. Also, negatively charged QDs reduce clearance of larger particles from kidney [81], [89-93]. Nonetheless, surface modification of QDs using dendrimers has found to surmount some of these challenges [83,94,95]. Algarra *et al.* used diaminobutane dendrimers with thiolated surface functionalities to obtain CdSe quantum dots nanocomposite. Thiolated group not only increased affinity of dendrimer toward QDs but also increased stabilization of QDs in water [96]. QDs were modified with PAMAM dendrimer, having large number of primary amine groups, to augment cellular uptake, resulting in strong fluorescence intensity [97]. Huang *et al.* synthesized PAMAM dendrimer possessing carboxylic acid surface group,

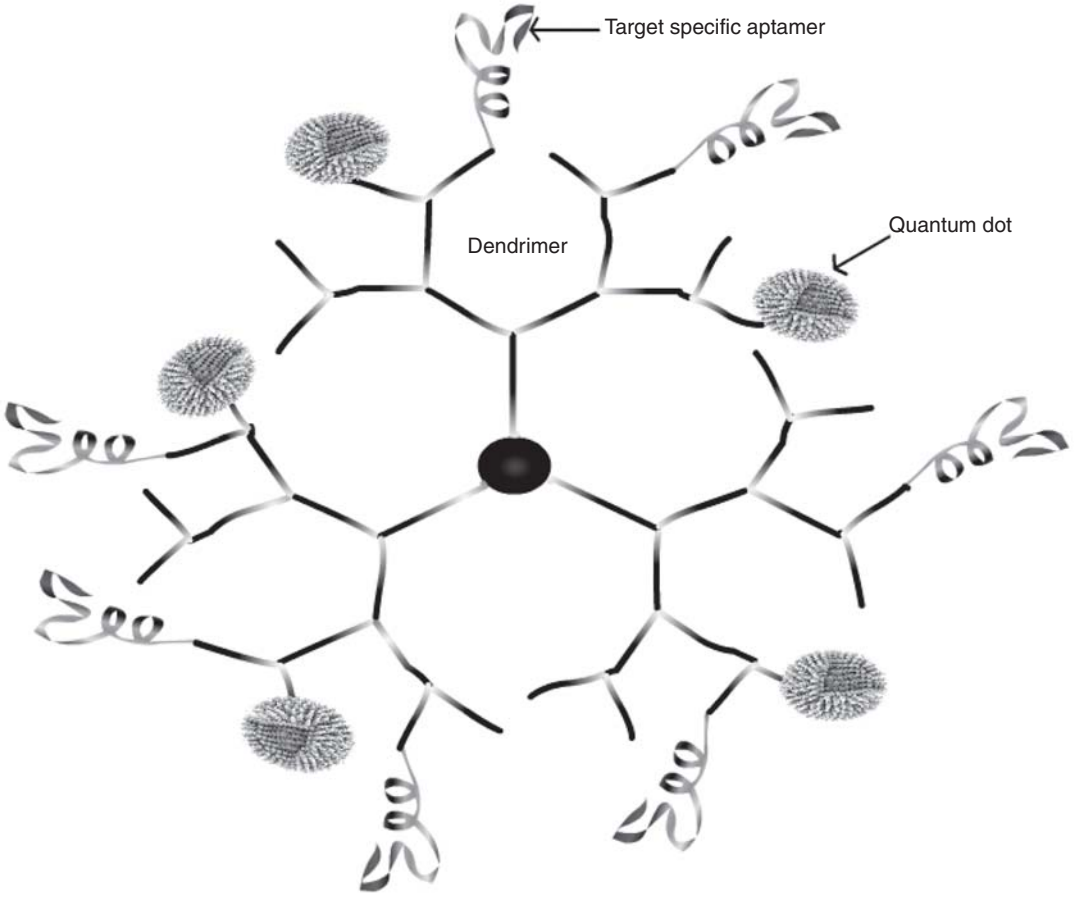

Figure 5. Schematic illustration of Apt-D-QD bioconjugate.

which was reduced with dithiothreitol to yield single site, thiol core, functionalized PAMAM. These dendrons were used to surface modify CdSe/CdS QDs to increase fluorescence intensity [98].

To be used for biomedical applications high-quality QDs must be made water-soluble. QDs synthesized directly in water suffers from few problems namely, narrow size range and wide size distribution of QDs leading to wide full width at half maximum (FWHM). Whereas, QDs synthesized from organic solvent are monodisperse leading to narrow FWHM but they are insoluble in water. Water solubilization can be achieved by (a) Ligand exchange: QDs produced using organic solvents have hydrophobic surface groups such as tetradecylphosphonic acid (TDPA) and trioctlyphosphonic acid (TOP). These hydrophobic groups can be replaced by water-soluble bifunctional molecules, one end of which is connected to QDs and other end has hydrophilic moiety. Example of such bifunctional molecules are 2-aminoethanethiol, dithiothreitol and dihydrolipoic acid. (b) Formation of micelles using phospholipids having both hydrophobic and hydrophilic ends or by using long chain length amphiphilic polymer like poly(acrylic acid) partially grafted with octyamine to form micellar structure, and (c) Silica encapsulation of QDs [83,91].

For fully exploiting water-soluble QDs for molecular imaging and other biomedical applications, they were conjugated to biomolecules such as aptamer to render specificity for biological targets [81]. Several approaches have been used to link biological molecules, as discussed below [99].QD surface was conjugated with biomolecules containing thiol groups using a mercapto linkage [100,101]. However, this type of conjugation can readily dissociate from the nanoparticle surface as the bond between Zinc and thiol is not very strong causing QDs to precipitate from the solution [99]. Researchers have found that absorption of small molecules like oligonucleotides [102] and various serum albumins [103] on the surface of water-soluble QDs is nonspecific and depends on many factors like pH, ionic strength, and surface charge of molecules. Mattoussi *et al.* reported conjugates of protein on QD surfaces through electrostatic interaction, but it was found to be inappropriate for *in vivo* or *ex vivo* cell labeling. Possible explanation for unstable interaction is interference from positively charged proteins [99].

Table 2. Summary of applications of aptamer-dendrimer bioconjugate.

	Name of Conjugate	Aptamer Used	Dendrimer Used	Application	Ref.
Therapeutics	Dox@Apt•don't-DEN	PSMA-specific A9 RNA aptamer	G4 PAMAM dendrimer	Targeted chemoimmunotherapy for prostate cancer	[35]
Diagnosis	Thrombin aptasensor	15-mer TBA	G4 PAMAM dendrimer	Diagnosis of thrombin	[76]
	Neopterin aptasensor	Neopterin-specific aptamer	PPI dendrimer	TB detection	[60]
Imaging	Apt-D-QD	GBI-10 Aptamer	PAMAM dendrimer	Imaging of glioblastoma cells	[106]

Intricacies involved while conjugating QDs using non-covalent interactions demand researcher to explore covalent interaction for conjugating QD. Jiang *et al*. developed PAMAM dendrimer-modified QDs through covalent binding using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling agent and investigated their biocompatibility and cytotoxicity in PK15 cells. Characterization of CdTe-PAMAM nanocomposition using transmission electron microscopy showed good stability and dispersion. Biocompatibility and cytotoxicity of nanocomposition was evaluated by incubating PK15 cells with CdTe-PAMAM dendrimer. Results of incubated cells showed cells with red color nanocomposition scattered in cytoplasm that could not be washed away, which indicated that the QD-Dendrimer nanostructure could penetrate into the cell membrane. Cell viability was assessed by using MTT assay after incubation. Results of MTT assay indicated that viability of cells incubated with QD-Dendrimer nanostructure was significantly high as compared to cells that where incubated with QD alone, which proved that nanocomposite had no significant cytotoxicity [104]. In the study by Das *et al.* comparison of several approaches to achieve conjugation with QD was done, which concluded that conjugates linked through amide-linked chains and poly(ethylene glycol) (PEG) had low solubility and lacked receptor affinity and the most efficient approach was to use PAMAM D5 dendrimer [105].

Li *et al*. utilized the benefits of QDs, dendrimer, and aptamer by preparing aptamer-conjugated dendrimer-modified QD (Apt-D-QD) (**Figure 5**). Single stranded DNA (ssDNA) sequence was selected for synthesis of GBI-10 Aptamer. Functional PAMAM dendrimers in methanol was mixed with CdSe QDs and dendrimer-modified QD was prepared. Excess of glutaric anhydride was used to convert surface amine groups of dendrimer to carboxylic acid. Active ester of dendrimer-modified QD was bioconjugated with aptamer. Agarose 2% gel electrophoresis was used to characterize the completion of conjugation of aptamer with dendrimer-modified QD. Apt-D-QD nanostructure was characterized using high-resolution transmission electron microscopy (HR-TEM) and photoluminescence spectra. Results of HR-TEM indicated good stability and dispersibility of newly formed nanocomposite. Reports of photoluminescence spectra suggested minor decline in the photoluminescent intensity of QD, nevertheless the emission pattern exhibited typical Gaussian distribution. This group also utilized the same method used by Jiang *et al*. to evaluate targeting capability of Apt-D-QD. U251 glioblastoma cells incubated with Apt-D-QD showed a strong red color, which could not be washed away, suggesting that Apt-D-QD can bind with U251 glioblastoma cells with high binding affinity. On the other hand, Scb-10-dQD, which was incubated with U251 glioblastoma cells as control, could not bind with U251 glioblastoma cells, indicating that tenascin-C interacts with GBI-10 but not with the control Scb-10, which further validate that APT-D-QD can target desired cells specifically [106].

Above research undoubtedly shows that Aptmer-dendrimer-QD bioconjugate combines target specificity of aptamer, unique features of dendrimer as carrier and fluorescent properties of QDs.

Apt-D bioconjugates have also been explored in the other areas. Wei *et al.* have developed dendrimer-interfaced aptasensor for the detection of Botulinum neurotoxin [107]. This technology can be developed for detecting live/dead *Staphylococcus aureus* cells [108] and codelivery of hydrophobic and hydrophilic drugs [109].

Table 2 summarizes various applications of Aptamer-dendrimer bioconjugate discussed in this article.

3. Challenges

Some researchers have described nanotechnology as "double-edged sword." The same unique properties making nanomaterials attractive can make them potentially harmful. Factors determining their potential toxicity include size, dose, structure, shape, surface chemistry, opsonization, solubility, aggregation, and chemical composition. Nanotoxicity can result from interaction of nanoparticles with host cells or proteins, through cell entry pathways, by leaching toxic ions to cells, by blocking receptors on cell membranes, or by activating defense cells as done by bacteria and viruses. However, reactive oxygen species (ROS) and free radical production after active (through phagocytes) or passive cellular uptake of nanomaterials is found to be major mechanism of nanotoxicity. Quantum size effects and large surface area to volume ratio make nanoparticles chemically extremely reactive, which leads to increased production of ROS and free radicals. Unremitting production of ROS can cause inflammatory responses, tissue degeneration, damage

to proteins or mitochondria, and DNA mutation. ROS can also potentially create defects in lipid membranes such as physical disruptions, formation of holes, or thinned regions. All these lead to oxidative stress and eventually cell death. Antioxidants generated during homeostatic activity of cells can neutralize free radicals and can partially alleviate problem [110-114]. Fine-tuning properties of nanoparticles like shape, size, and so on can help to reduce nanotoxicity, however, more *in vivo* studies are required to understand factors affecting nanotoxicity. Following are strategies towards the production of greener nanotechnology: 1) Modification of the nanoparticles by coating with biocompatible material to preclude their interaction with water and other biological materials. 2) Mixing nanoparticles with benign biocompatible particles [115]. Nonetheless, there is dire need of detailed assessment of biodistribution of Apt-D bioconjugate, its interaction with cellular and immune system, its metabolism, and fate of its degraded products for its safe use.

4. Future prospects

Despite the fact that commercial exploitation of the Apt-D bioconjugate is lagging behind the research discoveries, it is rapidly maturing from a simple research tool into a major technology with commercial potential. Promising therapeutic targets for this technology can be divided into two classes, intracellular targets, such as transcription factors, and extracellular targets, such as invading viruses. Also, more studies with Apt-D bioconjugate should be carried out for the treatment and diagnosis of other major diseases. For example, Apt-D bioconjugates can be developed against Ab peptide associated with Alzheimer's disease, abnormal protein found in prion diesases such as scrapie and Creutzfeldt–Jakob disease. Other active areas of research could be chronic viral infections such as hepatitis C and HIV. Many applications of antibodies can, now, be brought to fruition using Apt-D bioconjugate as this nanohybrid shows higher target affinity, more stability in biological environment, and lack of immunogenicity.

Major efforts are required in the field of Apt-D bioconjugate for successful clinical translation like: i) development of more efficient selection methods to generate new aptamers for different diseases with very high affinity, ii) development of easier and more stable bioconjugation strategies for dendrimer joining to aptamer, iii) understanding biodistribution of Apt-D bioconjugate and assessment of cytotoxicity, and iv) research to learn consequence of parameters, such as particle size and surface charge of Apt-D, on clearance of particles from the tumor after intratumoral delivery.

Recently developed other nanocarriers like mesoporous silica [116] and graphene [117] can be, possibly, explored for the development of nanohybrid systems with Apt-D bioconjugate to overcome its limitations. Graphene, two-dimensional crystal lattice of sp^2 bonded carbon, is especially attractive because few nanohybrids of aptamer, dendrimer, and graphene have been developed [118,119] However, some serious hindrance in realization of graphene as nanocarrier is lack of method of mass production and extremely high cost of synthesis.

5. Conclusion

Aptamers have become valuable and increasingly developed research tool in areas of therapeutics, diagnosis, bioanalysis, and other biomedical applications. The brief observations described in this article demonstrate the applications of novel approach of integrating target-specific aptamer with an excellent nanocarrier-dendrimer. Results showed that the unique aptamer-dendrimer bioconjugate can be used as a highly specific and sensitive agent in drug delivery, targeted cancer therapy, diagnostic, and imaging fields. Combination of exceptional characteristics of both aptamer and dendrimer makes this bioconjugate excellent alternative to antibody-nanomaterial conjugate-based biomedical applications.

There are six major advantages of Apt-D bioconjugate: 1) the specificity of aptamers guarantees a targeted detection or binding, which is the most important and preliminary step in case of efficient drug delivery and detection of biomolecules; 2) the ease of synthesis and modification make aptamers appealing in various kinds of practical applications; 3) definite and flexible architecture, inherent desired pharmacokinetic properties of dendrimers allows their use almost in every area of research; 4) toxicity problems associated with conventional cancer therapy can be alleviated with the use of Apt-D bioconjugate; 5) dendrimers increase detection signal level of aptasensors which when used alone have advantages of aptamers solitary; 6) *in vivo* imaging is more suitable using proposed bioconjugate due to reduction in undesired effects. Aptamer-dendrimer bioconjugate not only circumvents the limitations of individual components of nanohybrid (i.e., aptamer and dendrimer) but it also potentiates their properties and increases the spectrum of application. However successful realization of this bioconjugate also lies in proper attention to nonspecific adsorption which is important in terms of detection level of biosensor and toxicity issues in drug delivery. Also, surface of dendrimers must be optimized to bind required number of aptamers maintaining its biological activity.

With continued research in future, we can expect more application of Apt-D bioconjugate in biomedical field making Apt-D bioconjugate as emerging luminary entity.

6. Expert opinion

Aptamers have materialized as promising candidates in targeted drug delivery, diagnosis, and imaging primarily due to key aspects of target specificity and sensitivity. Their major limitations of *in vivo* degradation, generation of low detection signals, and difficulty of immobilization on probe surface found to be overcome by nanocarrier-dendrimers efficiently. It's been 20 years since aptamers were invented and till date

only Macugen has reached market of US and EU. Eight other aptamers are in different phases of development cycle. Market survey and prediction by BCC research indicated that global market for aptamers is on the edge for significant new market candidate in the near future. Aptamer market was worth $10 million in 2009 and is expected to be worth $1.9 billion [120,121]. And when emerging aptamer is explored with equally potential nanostructure dendrimer, the combination of both is expected to reach pinnacle of success in the field of therapeutics, diagnosis and imaging.

Despite rapid advances, aptamer-dendrimer bioconjugates are still in its infancy. More research work is required to realize and establish potential of this bioconjugate. Also, further studies needs to be done to synthesize diverse number of aptamers specific for different targets and conjugate them with dendrimers for practical utility. More numbers of multifunctional optimized Apt-D bioconjugates are required to design with extensive research to treat diseases like cancer where targeting drug delivery is desired. Apt-D bioconjugate consisting of dendrimers carrying aptamer specific for many types of carcinoma cells is still to be exploited. These types of Apt-D bioconjugates will have multifunctional activities of targeted drug delivery and diagnosis that will help oncologist gain upper hand against cancer. Apt-D bioconjugate incorporating different imaging agents and anticancer agents needs to be developed. Fundamental interactions of developed bioconjugates with targets especially malignant cancer cells need to be explained in detail. These results will speed up the optimization of several types of Apt-D bioconjugates. Furthermore, toxicity and pharmacokinetic aspects of Apt-D bioconjugate are rarely studied in detail. These areas of investigation are very important to use Apt-D bioconjugate clinically.

Although aptamers and dendrimers are relatively easy to synthesize but synthesis of Apt-D bioconjugate is still expensive and economic considerations can be hurdle in development of this novel approach. Therefore, newer methods for the synthesis of Apt-D bioconjugate that are equally proficient but relatively less expensive, easy, and time efficient must be explored by the researcher in coming years. This will not only enhance the speed of development of newer bioconjugates for diverse number of diseases but also increase the clinical use of this useful approach. Looking at the speed with which newer and newer investigations are being made in the areas of aptamer, we are very optimist about growth of Apt-D bioconjugate and believe that it will emerge as unique nanohybrid, and we will have wide variety of Apt-D bioconjugates for treatment and diagnosis of disorders like Parkinson's disease, endometriosis, osteoporosis to name few.

In short, while there is a great deal possibility for improvement, we expect that aptamer-dendrimer bioconjugate will eventually become a real-world nanotool, which can overcome limitations of current available approaches and truly become the magic bullet of 21st Century.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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