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Aptamers in Biomedicine: Selection Strategies and Recent Advances

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Abstract:	Aptamers have come in the spotlight as bio-mimetic molecular recognition elements in the field of biomedicine due to various applications in diagnostics, drug delivery, therapeutics, and pharmaceutical analysis. Aptamers are composed of nucleic acid strands (DNA or RNA) that have the ability to specifically interact in a three-dimensional tailored design with the target molecule. The basic method to generate aptamers is Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Recent technology designs used in the selection of aptamers enable faster and cheaper production of high-affinity new generation aptamers compared to traditional SELEX which can last up to several months. Still, some aptamers are more prolific than others. Rigorous characterization performed by multiple research groups endorsed several well-defined aptamer sequences. Binding affinity, nature of the biomolecular interactions and structural characterization are of paramount importance for aptamer screening and selection. However, remarkable challenges of aptamers from the biomedical field still need to be dealt with. Poor specificity and sensitivity, questionable clinical use, low drug loading, and toxicity are only a few examples. Even though high-throughput sequencing is far more excellent than cloning and Sanger sequencing, low use is reported because of a lack of cost-effectiveness. Hence, post-selection modification enables improved substitutes. This review accounts for the 30 th celebration of the SELEX technology underlining the most important advances of aptamers' achievements in the biomedical field within mostly the past five years. Aptamers' advantages over antibodies are discussed. Because of possible clinical translational utility, insights of remarkable developments in aptamer-based methods for diagnosis and monitoring of disease biomarkers and pharmaceuticals are discussed focusing on the recent studies (2015-2020). The current challenges and promising opportunities for aptamers for therapeutic and theragnostic purposes are also presented.

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Aptamers in Biomedicine: Selection Strategies and Recent Advances

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Keywords: aptamer selection, dissociation constant, SELEX, biomedical applications, aptamer therapy, electrochemical aptasensors

Abstract

Aptamers have come in the spotlight as bio-mimetic molecular recognition elements in the field of biomedicine due to various applications in diagnostics, drug delivery, therapeutics, and pharmaceutical analysis. Aptamers are composed of nucleic acid strands (DNA or RNA) that have the ability to specifically interact in a three-dimensional tailored design with the target molecule.

The basic method to generate aptamers is Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Recent technology designs used in the selection of aptamers enable faster and cheaper production of high-affinity new generation aptamers compared to traditional SELEX which can last up to several months. Still, some aptamers are more prolific than others. Rigorous

characterization performed by multiple research groups endorsed several well-defined aptamer sequences. Binding affinity, nature of the biomolecular interactions and structural characterization are of paramount importance for aptamer screening and selection.

However, remarkable challenges of aptamers from the biomedical field still need to be dealt with. Poor specificity and sensitivity, questionable clinical use, low drug loading, and toxicity are only a few examples. Even though high-throughput sequencing is far more excellent than cloning and Sanger sequencing, low use is reported because of a lack of cost-effectiveness. Hence, post-selection modification enables improved substitutes.

This review accounts for the 30th celebration of the SELEX technology underlining the most important advances of aptamers' achievements in the biomedical field within mostly the past five years. Aptamers' advantages over antibodies are discussed. Because of possible clinical translational utility, insights of remarkable developments in aptamer-based methods for diagnosis and monitoring of disease biomarkers and pharmaceuticals are discussed focusing on the recent studies (2015-2020). The current challenges and promising opportunities for aptamers for therapeutic and theragnostic purposes are also presented.

1 Introduction

Nucleic acids (deoxyribonucleic acid, DNA or ribonucleic acid, RNA) consist of a linked series of nucleotides. A pentose sugar (2-deoxyribose or ribose), a phosphate group and one of the nitrogen-containing bases (pyrimidines and purines) compose each nucleotide. Nucleic acids play a vital role in encoding, transmitting, and expressing genetic information, but they can also act as functional molecules, exhibiting ligand-binding properties, or even enzyme-activity.

Aptamers are single or double-stranded nucleic acids that make a strong interaction with a specific target molecule, mirroring natural interaction antigen-antibody. Aptamers were first discovered in early 1990 by two separate groups, Ellington & Szostak [1] and Turck & Gold [2],

which isolated RNA sequences to bind specifically to different target molecules. The given name aptamer comes from the Latin *aptus* (to fit) and the Greek *meros* (part). The aptamer is selected from a large pool of sequences, formed approximatively of 10^{15} different sequences, through an *in vitro* selection process named Systematic Evolution of Ligands by Exponential enrichment (SELEX) technology. Aptamers can make a strong interaction with a broad range of target molecules such as small organic and inorganic molecules [3], proteins [4], nucleotides [5], antibiotics [6], toxins [7]. Besides, aptamer has been developed for complex target molecules as cancer cells [8,9], viruses [10], bacteria [11], they can even cross physiological barriers, for example penetrating the brain [12], presenting high affinity and high specificity [13]. Another important role of nucleic acid aptamers is the ability to act as the ligand-binding component of riboswitches [14]. As the aptamers are folding around the target molecule, a 3D structure is generated. A property that distinguishes aptamers from other synthetic receptors is that they are characterized by a phenotype-genotype connection [15]. Not only their specific sequences (genotype) but also the property of folding into different shapes with specific functions (phenotypes), determines their recognition properties. Besides the structural complementarity, short-range non-covalent bonds such as hydrogens bonds, van der Waals and electrostatic interactions, as well as the arrangement of aromatic rings [16] stabilize the complex that forms between the aptamers and the target molecule (Figure captions

Figure 1).Figure captions

Figure 1**Insert Figure captions**

Figure 1

The strength of the interaction between two molecules is named also as affinity and is measured and reported by the dissociation constant (K_d): as the dissociation constant value decrease, the binding affinity is higher for its target molecule. Aptamers' K_d value typically ranges between low micromolar to high picomolar levels.

Since the aptamers were first discovered, the SELEX procedure has been used for the selection of over 2000 sequences [17] gaining valuable attention from an economical perspective. As stated by Market Research in 2018 in a published book, the global aptamer market is estimated to reach more than 400 billion dollars by 2023 [18]. Promising aptamers for therapeutic, diagnosis and imaging

biomedical procedures are herein described. However, several challenges like low screening and selection efficiency of aptamers need to be addressed to gain more reliability among researchers and end-users [19]. Therefore, improved and effective tools in the selection and screening of aptamers are highly required to ensure promising biomedical aptamer-based applications. Several reviews reported in the literature offer a comprehensive report of aptamer screening and the selection and multiple applications [17,19–27]. This review covers (1) introductory information related to aptamers and their properties and advantages over antibodies, (2) the latest selection strategies of aptamers highlighting the SELEX principle and advantages and challenges of Protein-based SELEX, Cell-based SELEX, Capture-SELEX, and capillary electrophoresis SELEX. Because of clinical translational utility, (3) insights of remarkable developments in aptamer-based methods for biomedicine applications will be discussed focusing on the recent studies (2015-2020). We aim to give an insight of the aptamers selection strategies and related medical and pharmaceutical applications based on the most recent discoveries.

2 Aptamers - promising tools in biomedicine

2.1 The tendency in aptamer use: evolution of aptamers reported publications (1990-2019)

The progress that occurs in the aptamer-research field of science is measured by the number of citations stated in one year. Moreover, we examined reviews and articles within the chemistry field, generated per year, for more accurate scientific data concerning productivity. For the advanced search of the term “nucleic acid aptamer” with the timeline set between 1990 and 2019 on PubMed, Scopus, and Web of Science databases returned ~8255 peer-reviewed and non-redundant publications. It should be mentioned that the aptamers generated at private companies were not included in this analysis. As observed the number of publications increased exponentially, which is unusual for a growth pattern within the scientific community (

Figure 2A). Therefore, the interest in aptamers research is highly appreciated, although, translation to medical applications is still yet to be developed to move these discoveries from the researcher’s bench to the patient’s bedside and community. The main topics presenting a keen

interest using aptamers are therapy and biosensors research, according to Scopus database in the set of specified year range of 2015-2019 (

Figure 2B).

Insert

Figure 2

2.2 Aptamers versus antibodies

As both, aptamers and antibodies, have the function to act as reagents for molecular recognition with high affinity, it is not unexpected that they are often compared. Before nucleic acid aptamers were discovered, antibodies used to be the top leader among target recognition molecules. Although similar to antibodies, aptamers have several advantages and disadvantages. Ease of generation and chemical modifications, no variability between batches, and a broad spectrum of target molecules are a few benefits that aptamers have when compared to antibodies. Even though the molecular size of the aptamers is considerably smaller than that of antibodies, the aptamers are large enough to recognize and bind its target molecule. However, the information regarding the affinity and selectivity properties of the aptamer-target complex is not always available because of the incomplete characterization of some of the developed aptamers [28].

Heat stability as well as stability on a wide range of pH and salt concentrations are other high marks of aptamers. Cell cultures or animal hosts aren't needed because aptamers are chemically synthesized throughout a standard process [19]. A weak point is the limited number of aptamers discovered by now, in contrast with the number of antibodies generated by plasma cells. The selection of aptamers occurs *in vitro* within a variety of conditions, while antibodies are limited to physiologic conditions by animal immunization. The aptamers don't generate an immunogenic response as antibodies do, with an increased response with repeated dosage.

The production of aptamers can be provided at a larger scale compared with antibodies. Because aptamers and antibodies have different production ways, nucleic acid aptamers have great cost advantages compared with antibodies. Furthermore, unlike antibodies, aptamers can be reused in applications, as they are capable of being reversibly denatured, which will also reduce the manufacturing costs. Though the aptamers have numerous advantages against antibodies, there is a lack of aptamers interlaboratory studies that don't give enough confidence to the users or commercial companies to apply the product. A list of major advantages and disadvantages of aptamers and antibodies is presented in Table 1 [29].

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2.3 Challenges of aptamers for in vivo applications

For therapeutic applications, aptamers have more access to organs and higher bioavailability due to their smaller sizes compared with monoclonal antibodies [30]. Although aptamers have these favorable benefits, the biggest obstacles over the use of aptamers as therapeutic agents arise from their *in vivo* stability. Because of their small size (about 8-30 kDa, ~1-2 nm diameter vs. >150 kDa, ~ 10-15 nm diameter for antibodies), the osmosis rate from body liquids by kidney or different organs is high, by this means the therapeutic potential and pharmacokinetic lifetime are severely reduced [31].

Aptamers' chemical stability in the presence of (exo- or endo-) nucleases is dramatically affected as unmodified aptamers can last *in vivo* for only up to 10 minutes. Therefore, functionalization is highly recommended to increase their stability and bioavailability. One approach is the "In-SELEX" methodology which uses nucleotides modified with different chemical groups (e.g. modifications at 2′ sugar position using 2′-O-methyl pyrimidine nucleosides or 2′-amino pyrimidine nucleosides or 2′-fluoropyrimidine nucleosides, and locked nucleic acids) within the

aptamer selection process to produce the desired modification. A simpler modification can be represented by the conversion of D-form to L-form of aptamers within the "Post-SELEX" methodology to obtain the so-called spiegelmers [32]. For recognizing natural biomolecules, a synthetic L-enantiomer must be used as a target, in such a way that after selection with natural nucleic acids (D-form), the mirror-image aptamer can recognize the natural target.

The functionalities should also provide appropriate linkages to enable tissue penetration, cell recognition, and internal delivery. Acid-cleavable hydrazine, *N*-succinimidyl-4-(2-pyridyldithio) pentanoate (SPP) and *N*-succinimidyl-4-(2-pyridyldithio) butyrate (SPDB) are some examples of cytosolic cleavable linkers [29]. Several modifications that hinder the renal filtration are represented by attachments of PEG [33], cholesterol [34], or dialkyl lipids [35].

2.4 Binding equilibrium

The molecular recognition process is described, from a quantitative point of view, as the specific interaction between the aptamer (A) and target (T) to obtain the complex (AT), assuming a 1:1 stoichiometry according to the equilibrium: A+T ⇋AT. The intensity of the bond realised between oligonuleotides and target molecule is known as affinity, being evaluated as an association (K_a) and dissociation constants (K_d) [26]. Association constant K_a is the ratio of $k_{forward}$ (on-rate, in units of M^{-1} s^{-1}) and $k_{reverse}$ (off-rate, in units of s^{-1}) constants (Eq.1). A quantitative description of the energetics involved in these interactions requires data obtained from binding assays under equilibrium conditions.

$$\mathbf{K}_a = \frac{[\mathbf{AT}]}{[\mathbf{A}][\mathbf{T}]} = \frac{1}{K_d} = \frac{k_{forward}}{k_{reverse}} \quad \mathbf{(1)}$$

A common method to measure *K* is to maintain a constant concentration of one ligand while titrating it with increasing concentration of the other one. The concentration of the complex [AT] can

be determined after the equilibrium is obtained. If the concentration of initial aptamer remains constant, then f_a (fraction of bound aptamer) is described by Langmuir isotherm (Eq.2.) The asymptote of f_a equaling 1 takes in the first quadrant the general form of a rectangular hyperbola. Using graphic representations, K_d can be estimated with a nonlinear regression analysis. Variable forms of f_a have been introduced to describe different graphic plots, used to estimate K_d [26].

$$fa = \frac{[T]}{K_d + [T]} \quad \textbf{(2)}$$

The thermodynamic feature of the AT interaction requires studies of the temperature dependence of affinity constant. The change in free binding energy (ΔG) characterizes the enthalpy (ΔH) and the entropy (ΔS) of the interaction between the aptamer and target molecule (Eq.3.). A more comprehensive overview and detailed study about the thermodynamic perspective and characterization of aptamer–ligand complexes are comprised in a chapter of Miranda-Castro *et al.* [36].

$$\Delta G = \Delta H - T\Delta S \quad \textbf{(3)}$$

It is conceivable to use automated analytical instrumentation to measure binding constants, such as High-Performance Liquid Chromatography (HPLC), a strategy dependent on the guideline of the partition of bound and unbound aptamer between the stationary and mobile phase, capillary electrophoresis (CE) based on differential migration performed inside capillaries when a potential is applied. Also, surface plasmon resonance (SPR) is commonly used, which makes it possible to measure the binding kinetics, as well as the affinity between an aptamer and its conjugate target. The most important analytical techniques designed to measure affinity binding constants are presented in **Error! Reference source not found.** [26,37,38].

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In general, aptamers show strong interaction and low dissociation constant with the target molecule. Therefore, aptamers can interact with low amounts of target samples, making them reagents to develop analytical assays with high sensitivity. Aptamers are also useful in the discovery of diagnostic biomarkers, and the development of high-affinity aptamers makes possible the identification of new biomarkers that are less abundant in body fluid [39].

3 The generation of DNA and RNA aptamers

Aptamers are generally selected through an interactive method mimicking natural evolution, which is named SELEX (Systematic evolution of ligands by exponential enrichment). Incredible upgrades to the original method were accomplished since the SELEX innovation was created thirty years ago [1,2]. Generally, the conventional *in vitro* selection process takes weeks up to months until an aptamer with high affinity is obtained. For better time production and higher achievement rates, modified SELEX methods were proposed. Furthermore, to increase stability and enlarge the spectrum of aptamer applications, it is possible to introduce post-SELEX chemical modifications to the aptamer sequences. Below, we describe some variants of the selection method, highlighting advantages as well as disadvantages of each method [40].

3.1 Principle of the classic SELEX method

The process starts with a library of DNA or RNA single-stranded sequences containing around 20 to 80 randomized nucleotides with 6–30 kDa molecular weights, flanked by specific sequences at both 5′ and 3′ ends, which define the primers to be used during the amplification step using the polymerase chain reaction (PCR). The library contains ~10^{15} unique sequences (~1-2 nmol), giving information about the primary structure of the sequences. SELEX process includes several steps to successfully generate an aptamer against a specific target molecule. These steps include incubation of the aptamer with the desired target molecule and binding affinity reaction, partitioning of unbounded sequences. This step is proceeded by the amplification of bound sequences by PCR and the obtention of ssDNA to start a new cycle [41,42]. 4-10 rounds of selection are needed

to enrich an aptamer with high affinity to the target molecule, thereafter cloned and sequenced to select the primary structure of the oligonucleotides (Figure captions

Figure 1B). Alternatively, high-throughput sequencing of the last pool may improve aptamer identification, increasing the aptamer selection rate [43]. Differences among the selection processes of RNA and DNA aptamers which arise from the chemical nature of the used library should be mentioned. In the case of RNA aptamers selection, an additional step is necessary, known as reverse transcription PCR (RT-PCR) method. The RNA is first reverse transcribed into its complementary DNA (cDNA) using reverse transcriptase, and futher amplified using the standard PCR procedure. Even though DNA and RNA present similar functionalities, both possess different key advantages: DNA aptamers are more cost-effective and more chemically stable, whereas RNA aptamers generally present higher specificity and binding affinity with their target molecule[44,45].

SELEX targeting proteins is one of the most common forms of SELEX. One condition for protein-based SELEX is the preparation of a sufficient quantity of high-purified protein. Aptamers generated by protein-based SELEX have been mostly developed for cell-membrane associated enzymes, cell-adhesion molecules, tyrosine kinase receptors, T-cell receptors, surface transmembrane glycoproteins, etc. MUC-1 [46], L-selectin [47], tyrosine kinase receptors EGFR [48], HER-2 [49], are examples of biomarkers subjected to protein-based SELEX, with variation in the design of the library, the conditions during the selection steps and the number of rounds of selection. In general, high-affinity aptamers were evolved against these targets, with affinity constants in the low nanomolar range. The main disadvantage is that the targets employed for selection: peptides, solubilized membrane proteins, or recombinant proteins, require improvements in the selection process due to their length. Some aptamers failed to recognize their targets in the whole cells; also, co-receptors are required for some biomarkers for proper folding. Consequently, the medical applications of these aptamers are limited [50]. Further improvements were addressed in the

separation step of the process, such as cell-based SELEX using whole living cells as the target to diminuish these bottlenecks.

A critical step for obtaining high-quality aptamers is the separation between the bounded and unbounded fractions of the nucleic acid library. To facilitate the separation, either the target molecule or the nucleic acid library can be immobilized onto a solid support. It is also possible to perform the interaction step in solution, taking advantage of the differences in their physicochemical properties to separate the free-oligonucleotide sequences from their complex with the target. Another important question, especially for the selection of aptamers against proteins, is to consider if the target must be isolated and purified, or instead used in its native form. Taking into account these issues, we categorize the SELEX approaches into four classes [51]:

i) The interaction between the aptamer and the library doesn't involve the immobilization of any of the two components: Capillary electrophoresis -SELEX

ii) Immobilization of the library: Capture-SELEX

iii) Immobilization of the purified target: Magnetic-bead-SELEX

iv) The target is not purified: Cell-SELEX

3.2 Capillary electrophoresis SELEX (CE-SELEX)

The improvement that the CE-SELEX method confers is the applied procedure for the separation between bound and unbound sequences, which is made by differences in the electrophoretic mobility. The high-resolution separation that is achieved reduces the number of selection rounds. However, the resolution depends on the size of the target. For large target molecules (e.g. proteins), the specific shift in the electrophoretic mobility after complex formation is easily achieved; however, the shift is not seen for small molecules or molecules with similar charges. Different CE separation modes have been proposed to extend CE-SELEX applications, which have

been recently reviewed [19]. Because the injected amount in CE is very small, the size of the library is typically reduced to ~10^{12} sequences, and/or requires higher target and aptamer concentration which can end to non-specific interactions [52]. CE-SELEX shows noteworthy enhancements accomplished while utilizing a micro free-flow electrophoresis device (μFFE). With the help of μFFE, the time associated with fraction collection (target immobilization, elongated incubation, or negative selections) in CE-SELEX is eliminated. Time production increases, as in four days, four-round cycles are performed. Low nanomolar affinity sequences were identified after a single round of selection, implying the possibility that aptamers can be obtained even faster [53].

3.3 Capture-SELEX

Another possibility to avoid target immobilization is the so-called capture-SELEX. It was first developed for the selection of structure-switching signaling aptamers [54] and then named by B. Strehlitz *et al.* [55]. The capture-SELEX process starts with a randomized ssDNA oligonucleotides library, designed with an additional central part named docking sequence, fixed and complementary to a sequence used for capturing the library on a solid support. An aliquot of magnetic beads modified with capture oligos is incubated with the library, in such a way that its immobilization takes place by hybridization between the docking sequence of the initial library and the capture oligos on the magnetic beads. Further, the ssDNA library immobilized on the beads is incubated with the target molecule. The oligonucleotides with an affinity for the target molecule are released from the DNA-beads-complexes and fold into the three-dimensional structure to the target in solution. The supernatant is collected by magnetic separation and amplified by PCR, to be subjected to the next selection round [56]. The capture-SELEX process was successfully applied for selection of DNA aptamers for small organic molecules such as the aminoglycoside antibiotics kanamycin A [55], paramomycin [57] and tobramycin [58], quinolone antibiotics [56], vanillin [59], penicillin [60], and spermine [61].

3.4 Magnetic-bead-SELEX

During the SELEX process, the bound and unbound oligonucleotides target separation is a crucial step for successful aptamer selection. Using magnetic beads for target immobilization improves the efficiency of the separation. This procedure requires just small amounts of the target molecule, which also is easy handling and time improving. In the magnetic-beads SELEX method, the starting DNA pool is incubated with the magnetic beads modified with the target molecule to form a chemical interaction. Unbound oligonucleotides are magnetically separated and the sequences that are bound with the target are then extracted from the target-magnetic beads complex by heat treatment or denaturing. The selected oligonucleotides are amplified by PCR using specific primers. The relevant sequences are further purified and used in the next SELEX round [62]. Recently, different biomedical applications of aptamers selected by the magnetic-bead SELEX methodology have been introduced [63–67]. For lung tumor biomarkers, a recent study presents the selection process of six new aptamers using magnetic beads-based SELEX, using the serum of lung cancer patients, presenting also the binding affinity characterization as well as their secondary structure modelling [68]. FluMag SELEX is another improvement of magnetic beads-based SELEX procedure where fluorescein-labeled ssDNA is used to evaluate the enrichment of target-aptamer during the selection steps. An example of a successful selection of streptavidin-specific aptamers using FluMag-SELEX is presented by Stoltenburg *et al.*in a peer-reviewed paper [69].

3.5 Cell-Based SELEX

This variant of SELEX uses live cells as aptamer selection targets. In this way, aptamers are selected to distinguish proteins, or a common protein that has been modified, present on the surface of the specific cell. The cell-SELEX method involves positive selection for target cells and negative selection for non-target cells. This method led to aptamers that are useful in the diagnosis and therapeutic applications, but also to biomarkers discovery [70]. Even though, negative effects still

occur such as the selection of aptamers toward unwanted targets due to dead cells in the starting target population leading to artifacts [71]. It is restricted to molecules present on the cell surface. Therefore, it is crucial to have healthy cells for a successful selection. Some techniques have been developed to minimize the non-specific binding or to eliminate the dead cells, by means of magnetic beads separation [72] and fluorescence-activated cell sorting (FACS) [73]. The cell-SELEX procedure does not need a special preparation or purification of target molecules. Therefore, many DNA aptamers designed to diagnose and image cancer tissue have been selected with cancer cells. It includes pancreatic [74], gastric [75], prostate [76], colon [77], breast [76,78], and glioblastoma [79] cancer cells. More examples can be found in a recently published review [80].

In 2016 it was introduced a method named Ligand Guided Selection (LIGS), as a new strategy for identifying highly specific aptamers against complex targets, as known cell surface receptors on a particular cell [81]. To direct the selection toward a specific target on the cell surface a competitive elution with a secondary ligand, such as a monoclonal antibody, is introduced. The first aptamer developed within the LIGS strategy is an aptamer against membrane-bound IgM (mIgM) [82]. The advantage that LIGS brings within is the high adaptability which will increase the number of applications in biomedicine towards low-cost, highly stable therapeutic agents and diagnostic devices.

3.6 Post-SELEX modifications

As already presented in section 2.3, one crucial step in the aptamer synthesis and use in biomedical applications is related to the *in vivo* stability, to ensure the therapeutic effect. Once the aptamers sequences are *in vitro* identified and characterized by their affinity and selectivity, several strategies have been developed to enhance the properties of the aptamers for *in vivo* experiments. To protect the degradation of both DNA or RNA aptamers from exo- or endo- nucleases [83,84], increasing they're *in vivo* half-life [85] or even enhancing their binding affinity [86], a set of post-

SELEX chemical modifications were proposed for the selected aptamers. However, the chemical modifications of aptamers may lead to a decrease in the binding affinity constant, and so the optimization in every particular case is very challenging, making the process highly laborious.

The complex three-dimensional structures, the presence of an additional hydroxyl group at 2' position of ribose in RNA and their single-stranded structure predominantly occurring in nature fortunate the RNA aptamers against DNAs. However, their cleavage by ubiquitous RNAses significantly limits RNA-aptamer applications. Most commonly, RNA aptamers are chemically modified at 2' position of the sugar moiety with: –F, $–NH_2$ and $–OCH_3$, which helps to overcome their resistance to nucleases [42]. From the first studies published, a significant increase was reported in the nuclease resistance of RNA aptamers. In the same way, as ribozymes modified with 2'-fluoro- or 2'-amino nucleotides at all cytidine and uridine positions are stabilized against degradation by a factor of 10^3 compared to unmodified ribozyme in rabbit serum [87,88]. After selection, the aptamers can be modified by incorporating different 2'-modified residues. As the size of the aptamer is smaller, the susceptibility to renal filtration increases as compared to higher molecular weight antibodies. It was reported that the clearance time of 2'-fluoro aptamers was 5-15 h in plasma. This is a suitable, but laborious strategy, which can be overcome by employing a starting library composed of 2′-fully modified RNA aptamers, provided by a mutated RNA polymerase, used in such a way that non-canonical ribonucleotides are incorporated during the *in vitro* transcription process. It was demonstrated a facile transcription of a fGmH RNA library by a T7 RNA polymerase mutant, which is composed of 2′-F-dG and 2′-*O*Me-dA/dC/dU residues with high hydrophobicity. The aptamer was selected against *Staphylococcus aureus* Protein A (SpA) target. It has been showned how the fGmH RNA aptamers had the capacity to functionalize, balance out, and further deliver aggregation-prone silver nanoparticles (AgNPs) to the bacteria *S. aureus* with SpA-dependent antimicrobial implacts [89].

In addition to sugar ring modifications mentioned above, a set of modifications can be applied to the phosphodiester linkage such as its replacement with methylphosphonate, phosphorothioate [90], and triazole [91] or other chemical compounds [92]. Post-SELEX modifications were made by substituting almost all purine nucleotidic residues with 2'-O-methyl units after selection by solid-phase synthesis, inverting 3'terminal nucleotide, and poly(ethylene glycol) moiety was then added to 5' termini. Even though Macugen was replaced by more recently discovered *recombinant protein therapies* [93], and monoclonal antibodies [94], it remains a milestone in the aptamer and SELEX technology. Spiegelmers consisting of non-natural *L*-ribose nucleotides can present high resistance to nuclease degradation maintaining their binding affinities. Based on the sequences of the respective *D*-aptamer, the *L*-enantiomeric oligonucleotide aptamers are then chemically synthesized [95,96]. Nevertheless, this approach increases the cost of the aptamer selection process.

4 Aptamers for biomedical applications

In this section, we highlight some of the most recent and significant achievements of aptamers in disease diagnosis, therapy, and theragnostic (

Figure 3).

Insert

Figure 3

4.1 Diagnostic and treatment monitoring

Correct diagnosis is extremely important to enjoy a healthy life as patients generally experience pain. To successfully achieve this, various cutting-edge technologies have been designed and manufactured to diagnose a specific disease. Among these recently developed technologies, aptamers or aptamer conjugated with different hybrid nanomaterials have received considerable

attention as they have numerous advantages, such as low immunogenicity, good biocompatibility as well as controllable selectivity.

For diagnosis purposes, aptamers are for the most part utilized as biorecognition elements of target molecules as part of an aptasensor design. Due to their binding properties that make them extremely selective ligands, aptamers have been used to detect at nanomolar levels different types of pharmaceuticals and biological molecules paving the way to alternative analytical methods.

Even though there are a plethora of aptasensors capable of targeting different types of drugs or molecules involved in different pathologies, the detection step remains an important area of application, due to its practical assessment [97]. Depending on the type of the transducer and sensor technology, several readout techniques are usually reported among aptasensors: optical [98–100], piezoelectrical [101], electrochemical [102–104], etc.

Electrochemical detection methods have been successfully used due to their most important characteristics as increasing sensitivity and selectivity. Another advantage of electrochemical methods is related to the easy-to-use and cost-effective lab instrumentation.

Their development has been growing as shown by the increasing number of scientific publications since 2004 when the first electrochemical aptasensor was developed [105]. Electrochemical aptasensors presents considerable advantages over optical, piezoelectric, or thermal detection, being cost-effective and simple-to-operate [106]. Some of the most representative electrochemical aptasensors for biomedical applications (between 2015 and 2020) containing the aptamer sequences and most important figures of merit are summarized in **Error! Reference source not found.**.

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4.1.1 Aptasensors for cancer biomarkers

To improve the rate of survival in cancer patients, early monitoring of cancer biomarkers by detection strategies is mandatory. In recent years, early cancer detection using aptamers have been developed in a large number, against cancer-specific biomarkers. These molecules are generally over-expressed by tumour cells in the bloodstream or at the cell surface.

Recently, aptamers against specific cancer biomarkers were selected for the development of selective and sensitive sensors for cancer biomarkers as human epidermal growth factor receptor 2 (HER-2) [127], circulating tumor cells (CTCs) [128,129], alpha-fetoprotein (AFP) [117,130], vascular endothelial growth factor ($VEGF_{165}$) [131,132], epidermal growth factor receptor (EGFR) [133], mucin 1 (MUC1) [134].

MUC1 is a large glycoprotein that can be over-expressed and -glycosylated in a plethora of malignancies like breast, ovarian, lung, prostate, hepatic, and colorectal cancers [135]. However, aberrant levels of MUC1 are commonly associated with breast cancer. A recently published paper presents the successful implementation of a competitive electrochemical aptasensor developed for the detection of MUC1 cancer biomarkers. The ferrocene-labeled complementary DNA (cDNA-Fc) is first bound on the surface of MXene (Ti_3C_2) to form a cDNA-Fc/MXene probe and the MUC1 aptamer is fixed to the electrode by Au-S bonds. The competitive strategy between the cDNA-Fc probe and MUC1 on the cDNA-Fc/MXene/Apt/Au/GCE aptasensor also improved the selectivity. The new proposed competitive electrochemical aptasensor was applied in human serum samples with good recovery rates and low LOD [136].

Due to their characteristics and functional traits, exosomes extracted from various tumor cells become a focus in the research area, mainly being used in early identification of prostate cancer, lung cancer as well as other studies. With the aim of fast identification of non-small cell lung cancer (NSCLC)-derived exosomes, a low-cost lateral flow aptamer (LFAA) test strip based on an aptamer against CD63 protein was successfully developed [137].

CTCs are a type of cancer cell circulating in the bloodstream from primary or metastatic tumors and can lead to the development of primary tumors and metastatic lesions. Nowadays, aptamer-based biosensors for quick and precise detection of CTCs, have gained huge attention. A diagnosis method using an aptamer-based microfluidic system was developed for CTCs detection, proving good discrimination amongst positive and false-positive results [138]. Non-coding RNAs (ncRNAs) are integrated into a broad spectrum of regulatory RNA molecules such as ribozymes, small interfering RNAs, or aptamers that are found naturally in different types of cells either synthesized to target specific genes and therapy control. Most long non-coding RNAs (lncRNA) exhibit significant cell type-specific expression which is associated with the pathological stage. It was discovered that lncRNA has potential as a biomarker for cancer prognosis [139]. Also, lncRNA can be recognized by aptamers based on interactions that are not, at least exclusively, complementary interactions between bases. lncRNA prostate cancer antigen 3 (PCA3) was investigated for the determination of structural ncRNAs employing an aptamer-modified MNPs, thus indicating that the elongation process to lncRNA occurs only after the transcription step [140].

HER-2 is an important prognostic factor for invasive breast cancers which influences the type of treatment prescribed for breast cancer patients. An example of a sandwich format electrochemical aptasensor for the detection of HER-2 is presented. In the development of this aptasensor, a DNA primer on HER-2 aptamer was used to initiate auxiliary DNA self-assembly at the electrode surface to form a long one-dimensional DNA. The resulting long-dimensional DNA is then reacted with molybdate to generate electrochemical current. This aptasensor was successfully tested in standard solutions as well as human serum [141].

Regardless their promising future, cancer diagnostics based on aptamers usage has still many years of early-stage research ahead as most of the synthesized aptamers didn’t get the approval after *in vivo* trial experiments. As cancer cells vary in composition across both tumor type and subtype with complex membrane proteins, accurate identification of complex tumor cells is still difficult to

achieve. New SELEX methodologies are highly requested for the synthesis of aptamers with increased features in terms of production, stability, and affinity. A new strategy to be more explored is the introduction of informatics technology coupled with next-generation sequencing (NGS) within SELEX techniques which could make it possible to pushing aptamers into clinical settings [142].

4.1.2 Aptasensors for bacterial pathogens

Bacterial pathogens are responsible for water and food poisoning and nosocomial infections, being one of the most notorious microorganisms. Their detection is much needed and critical for the health status of the patient. Several research groups addressed this challenge, and have developed new aptasensors to detect various bacterial pathogens, including, *Escherichia coli* (ATCC 25922 antigen), *Pseudomonas aeruginosa* (ATCC 27853 antigen), *Staphylococcus aureus* (ATCC 29213 antigen) [143]; *Mycobacterium tuberculosis* (MPT64 protein) [144].

A recent study reports the detection of lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 determined through voltammetric analysis. To that, glassy carbon electrodes (GCE) were first modified with reduced graphene oxide and gold nanoparticles (AuNPs) to act as support for antiLPS aptamer immobilization. It was demonstrated that the sensor had good selectivity for LPS, also in a complex matrix with good RSD% [145]. Another published paper on the detection of bacterial pathogens, in this case, protein MPT64 which is secreted by *Mycobacterium tuberculosis,* presents the use of an impedimetric aptasensor based on interdigitated electrodes to selectively capture the immunogenic protein. The DNA aptamer was synthesized with a length of 40 nucleotides, modified with a thiolated long linker (HS-$(CH_2)_6$-$OP(O)_2O$-$(CH_2CH_2O)_6$-TTTTT-aptamer). It was concluded that the optimal ssDNA sequence for MPT64 detection was the long linker aptamer/MCH. The aptasensor was then tested in spiked human serum showing a LOD of 81 pM, which is also relevant for clinical setup [144].

Staphylococcus aureus (*S. aureus*) is a pathogenic bacterium responsible for multiple types of infections in humans. A sensitive assay for the identification of *S. aureus* was developed by means tailored release of fluorophore molecules through nuclease resistant nucleic acid gates. To decrease the matrix effect in blood samples, a pre-treatment step was introduced to capture the target by affinity interaction with the aptamer-functionalized magnetic nanoparticles. The aptasensor provides a new and effective strategy to monitor *S. aureus* infections directly in the blood samples and with ease incorporation into automated systems in portable devices [146]. An electrochemical approach for fast analysis of *S. aureus* was recently developed using synthesized nanocomposite based on cellulose nanofibers nanocomposite (CNFs) and gold nanoparticles/carbon nanoparticles (AuNPs/CNPs) immobilized at GCE. The EIS measurements were performed both in PBS buffer and human serum samples allowing the quantification of *S. aureus* as low as 1 CFU mL^{-1} in the linear domain between 1.2×10^1 and 1.2×10^8 CFU mL^{-1}.

4.1.3 Aptasensors for small molecules

In the last decades, aptamers have been synthesized and selected towards a variety of small molecules among biomolecules (e.g. ATP [147]), pharmaceuticals (e.g. steroid hormones [148,149], anti-inflammatory drug [150], antibiotics [98,102,103,151,152]), heavy metals, and other contaminants.

A blu-ray competitive aptasensor was recently developed for the detection of Adenosine triphosphate (ATP) using two types of MNPs and a blu-ray optical pickup unit. Two mutually exclusive binding reactions were designed: (i) the affinity recognition of the target by a specific aptamer and (ii) the hybridization reaction between this bioreceptor and its complementary DNA sequence. Both DNA sequences were first immobilized at different MNP's surface. In the presence of ATP, it's binding to the aptamer inhibits the clustering of the particles because of the

complementarities between the immobilized sequences, proving thus the practical application of the optomagnetic aptasensor for small molecule detection [147].

A label-free impedimetric aptasensor was designed based on a newly synthesized aptamer and gold electrodes for progesterone (P4) sensing. The correlation between the P4 concentration and the increase in the charge transfer resistance enabled the detection of the hormone in the range between 10 to 60 ng/mL with a low LOD of 0.90 ng/mL. Hence, the sensor was applied in spiked tap water samples showing no cross-reactivity against 17β-estradiol (E2) and norethisterone [148]. Zhu *et al.* developed a similar design for the ultra-low (fM) electrochemical quantification of 17β-estradiol (E2) hormone in human urine samples [149].

Anti-inflammatory pain relievers, such as ibuprofen (IBP), are known as non-steroidal anti-inflammatory drugs (NSAIDs). The study presents the hybrid modification of GCE with a carbon-based nanocomposite, namely carbon nanotubes mixed with chitosan and an ionic liquid, to serve as an immobilization platform for the aptamer specific to IBP. Then, a redox-active molecule (methylene blue - MB) was put in contact with the Apt-modified electrode to enable the intercalation onto the Apt and to further realize the electrochemical assessment of IBP. MB is released upon ibuprofen-aptamer binding with a concomitant decrease in the electrochemical signal. The DPV measurements enabled the IBP detection as low as 20 pM in the linearity domain from 70 pM up to 6 μM. The label-free electrochemical aptasensor was successfully applied in complex biological matrices such as pharmaceutical formulations, spiked human blood serum, and wastewater samples [150].

Antibiotics are antimicrobial medicines, widely used to treat bacterial infections and to promote animal growth. The misuse and the overuse of the antibiotics cause deleterious health outcomes for consumers from allergic reactions to the progress of antibiotic-resistant bacteria [153]. As a result, it is of high importance to develop sensitive and selective strategies for antibiotics detection in biological, food, and environmental samples to minimize the side effects of antibiotics.

Another label-free electrochemical approach was realized for chloramphenicol (CAP) detection using an aptamer-SH-modified screen-printed electrode (AuNPs/functionalized mesoporous silica) and hemin (HEM) as a redox probe. Upon HEM oxidation, the quantification of CAP was achieved using voltammetric techniques in two linear ranges of 0.03 – 0.15 μM and 0.15 – 7.0 μM with a LOD of 4.0 nM [151].

Two optical strategies for ampicillin detection based on aptamer-conjugated (A) silver [98] and gold nanoparticles [152], employing colorimetric and fluorescent sensing techniques, respectively (

Figure 4A and B). The sensors allowed for sensitive detection of ampicillin in the picomolar range and were successfully applied in biological samples.

Figure 4C presents another aptasensor for ampicillin but related to the electrochemical label-free detection by means of electrochemical impedance spectroscopy measurements at an aptamer-modified Co- and terephthalonitrile-based organic frameworks platform. The electrochemical aptasensor showed lower LOD than the optical approaches, being of 0.217 fg mL^{-1} [102]. It is also possible to use a displacement assay, where the aptamer is immobilized by hybridization with a complementary sequence on the electrode surface. This smart design was used for detecting tetracycline (TET) based on the capture of aptamer complementary structures, which leads to an insulating M-shape structure formation (

Figure 4D). Upon TET addition, the M-shape structure is displaced making the electrode surface available for the redox probe diffusion and enabling the exonuclease to act for the removal of the immobilized probe, leading to a noticeable increase in the electrochemical signal. This design successfully achieved the ultra-low sensing of TET in serum samples in the pM range (LOD = 710 pM) [103].

Insert

Figure 4

A non-conventional 'PEP-onDEP' electrochemical sensor was developed based on a peptide-aptamer strategy to detect molecule less accessible, such as renin. Renin acts as an emerging biomarker protein in the regulation of arterial blood pressure to predict cardiovascular predisposition. A selected peptide-aptamer was linked to AuNPs and embedded in a one-step competitive assay using a disposable electrochemical printed chip (DEP) and a USB powered portable potentiostat system. The detection range of this peptide aptamer-based competitive assay was from µg-to-ng mL^{-1} [154].

Although scientists have put a lot of effort in the synthesis of new aptamers for small molecules, it is still challenging to obtain the "perfect" aptamer configuration to selectively bind such molecules.

4.2 Therapeutic uses of aptamers

Generally, DNA aptamers can be used in two ways for therapeutic purposes either by their (1) intrinsic curative properties or by (2) targeted delivery of drugs which could be straightforwardly connected to the drug molecules or in a mix with nanoparticles to shape the conveyance platform [3].

The conventional dosage forms become a real struggle for researchers through the years. To defeat this issue, targeted and modified drug delivery systems were studied and implemented as valuable tools to treat the disease and increase the patient's health condition. A significant increase in the efficacy of drug delivery has been made recently, introducing innovative procedures by nanotechnology [25,155].

4.2.1 Aptamers for in vitro and in vivo therapy and intracellular imaging

Recent studies have shown the great potential of aptamers in the treatment of diseases as cancer [156–159], immune diseases [160–163], acute myeloid leukemia [164], metabolic skeletal disorders [165] or thrombosis [166] due to their specific binding ability to different biological molecules.

For example, AS1411 is an antinucleolin aptamer that proved antiproliferative properties in breast cancer. It is a G-rich phosphodiester oligonucleotide with increased nuclease resistance [167] and a particular affinity towards nucleolin (K_d is in pM to low nM range) [168].

Several studies from the past five years have shown the use of aptamers to regulate the receptors/ligands involved in immune reaction in cancer patients. Of these can be mentioned: T cell immunoglobulin-3 (TIM-3) [160], cytotoxic T-lymphocyte associated protein 4 (CTLA-4) [161], programmed death receptor 1 (PD-1) or its ligand programmed death-ligand 1 (PD-L1) [162], and 4-1BB [163]. One interesting aptamer-guided therapy approach is based on the successful development of a novel high-affinity DNA aptamer (K_d=11.84 nM) against cytotoxic T lymphocyte antigen-4 (CTLA-4). The aptamer was synthesized by cell-based SELEX and selected by high-throughput sequencing. The aptCTLA-4 aptamer showed to be stable in serum and exhibited inhibition in tumour development, both *in vitro* and *in vivo* setups [161].

Cardiovascular diseases pose a serious threat to human health [169]. A recent study presents the comparison between the REG1 system with anticoagulant properties with a new strategy that specifically bind the blocking Factor IXa and bivalirudin. The system contains an RNA aptamer (pegnivacogin) and its complementary oligonucleotide sequence (anivamersen). Both designs were tested against the side-effects exhibited after percutaneous coronary intervention. [166]. A new strategy to prevent in-stent restenosis which often occurs after endovascular stent placement is further presented based on a gene-eluting stent. The system involves gene therapy (complexes of nucleic acid vector and plasmid vascular endothelial growth factor) coupled with stent implantation. The *in vivo* efficacy was evaluated by implanted stents into rabbit aortas proving thus promising results in preventing ischemic fatal episodes [170].

As for intracellular imaging, one of the latest successful targeted delivery aptamer sensors to mitochondria for the monitoring of ATP levels was recently realized by Hong *et al.*. The principle is based on a photo-controlled ATP sensing strategy coupled with cationic DQAsomes to monitor the *in*

vivo ATP activity in mitochondria (3.7 μM LOD), being successfully delivered to subcellular organelles [171]. However, different strategies of aptamer post-SELEX modifications are likely to be exploited to ensure (1) enzymatic degradation resistance, (2) optimal pharmacokinetics, or (3) chemical integrity and bioavailability under physiological conditions [172]. A new procedure for non-invasive tumour cell-targeted delivery relies on photodynamic therapy (PDT) by the employment of photo-generated singlet oxygen (SO) and reactive oxygen species (ROS). *In vivo* imaging and PDT was realized based on a new approach comprised of tumor microenvironment (TME)-activatable circular pyrochlorophyll A (PA)-aptamer-PEG (PA-Apt–CHO–PEG) nanostructures. Deep penetration into the solid tumor and specific recognition of cancer cells was demonstrated (Figure 5) [173].

Insert Figure 5

Key features as relatively easily reproducible synthesis, tailored design, and cost-effective chemical modifications make these nanocarriers the prospect of targeted therapy. Nevertheless, the combination of these aptamers with immunosuppressive drugs can lead to targeted immunotherapy which will be further presented.

4.2.2 Aptamer-based drug delivery systems

Aptamer-based drug delivery schemes received increased attention in oncology due to their targeted drug delivery efficiency. However, to improve tumour-targeted therapy and decrease the side effects, also toxicity, different NPs have been used as drug nanocarriers. Other weak points of conventional dosage are repetitive administration with a shorter half-life, patient compliance diminution, high peak, and typical peak-valley plasma concentration and therefore specificity of the targeting is not or well achieved. Likewise, aptamers, antibodies, or peptides are generally used as

targeting agents [97,174]. Recent advances in aptamer technology reveal that aptamers can molecularly recognize intra- or extra- cellular expressed tumor markers [175] being able to distinguish among very similar target molecules. In targeted drug delivery and nanocarriers, aptamers could be used as guiding molecules towards tumor cells. The size of aptamers is also extremely important: they are in the range of 5 to 20 kDa, which is relatively small compared to antibodies (IgG 150 kDa) being more capable of penetrating tissues and tumors than antibodies.

DNA/RNA aptamers can be conjugated directly to drug molecules either the drug molecule is packed into an aptamer-folded structure. To realize the targeted delivery of the aptamer and drug, different loading systems are realized. On this ground, one representative type of targeted drug delivery systems is NPs, which provide site-specific drug delivery. As a subclass of colloidal particles, NPs range from 1 to hundreds of nm, divided into different groups. NPs have an increased specificity for the targeted location considerably reducing the side effects upon uncontrolled drug release. The bio-distribution rate in the human body of chemotherapeutic drugs is improved by the size and surface characteristics of NPs. Nowadays the most researched type of NPs is polymeric NPs. Polymer-based NPs come in many shapes, as dendrimers, polymeric micelles, ligand-based NPs, PEGylated NPs, etc. Polymeric nanostructures are being widely used in four broad areas covering the most important aspects of biomedical and pharmaceutical fields. Improvements are made at the particle size, the clearance time becomes longer, therapeutic efficacy is improved and toxicity is reduced [97]. Some examples in which polymer NPs are used as the target to varied diseases, are cancer diagnosis, gynecological disorders, etc, presenting various benefits over other conventional chemotherapeutic drug therapy. Thus, it derives from this that polymeric nanocarriers have a highly promising future in the field of medicine and biomedical application. If polymeric NPs are successfully functionalized, tremendous advances will be made in stem cell technology. Presently, these polymers already have extended applications in gene therapy and drug delivery systems that deliver nucleic acid as a treatment regime. The preparation of the polymeric drug delivery system can

be challenging at times since there can be some biocompatibility and biodegradability issues. However, due to their extensive application, polymers produce significant developments in several research areas [174]. Although many studies have passed the academic requirements as proof of concept of efficient nanocarriers for cancer therapy, just a few have been approved by the FDA academy [176]. Nonetheless, NPs as poly (d, l-lactide-co-glycolide, PLGA) have gained considerable attention from their role in delivery systems. As PLGA has biocompatible and biodegradable properties, breaking into more products that are rapidly metabolized in the human body, the US FDA approved PLGA for clinical use [30]. The biggest challenge of the polymeric NPs is that sometimes they suffer from toxic degradation when the residual material associated with them is toxic. In some instances, the extensive accumulation of the polymers can also cause huge toxicity.

Doxorubicin [177–181], fluorouracil [182–185], epirubicin [186–188], gemcitabine [189] and vemurafenib [190] are several chemotherapeutic drugs conjugated to DNA aptamers that have been mostly employed for *in vitro* and *in vivo* therapeutic purposes.

Doxorubicin (DOX) is largely used in chemotherapy. The functionalization of aptamers with DOX and their loading on different types of NPs was used as a drug delivery system against human lung cancer. In this approach, PLGA combined with poly (N-vinylpyrrolidone) NPs loaded with aptamer and DOX were spherical with an average size of approximately 87 nm. These NPs triggered the cancer cell and their efficiency was proved by cytotoxicity assay. The authors reported that this aptamer drug delivery system induced apoptosis through the activation of the apoptosis-related proteins and improved the therapeutic efficiency through the nucleolin receptor endocytosis targeted drug release [177]. Another NPs-based approach using PGLA was realized for DOX and an aptamer that specifically binds CD30 protein which is overexpressed in anaplastic large cell lymphoma (ALCL) cells. These NPs were larger than the previously described ones being of about 168 nm [178]. However, the clinical use of this procedure could be constrained by helpless take-up into malignancy cells. Biodegradability and carrier stability are of high importance. To this, Baneshi *et al.*

imagined a delivery system by the tailored use of albumin-based carriers coupled with iron oxide and AuNPs for AS1411 aptamer and DOX. The nanocarrier was synthesized by a desolvation cross-linking method and resulted in spherical-shaped NPs of 120 nm in size [179]. Although the nano-carrier system seemed to be promising for *in vivo* applications also due to its possible slow and controllable release of the dual complex (Apt-DOX), only *in vitro* studies on MCF7 breast cancer cells were presented. A new strategy to design a drug-targeted platform was realized by Chaithongyot *et al*. based on molecular-engineering of only double-stranded oligonucleotides (ODNs) and DOX to act as an antagonist towards targeted transcription factors from the pancreatic tumor [180].

A recent study reports a DNA origami nanosphere was modified with a specific aptamer for selective DOX delivery. The specificity of the targeted nanocarrier was investigated against three cell lines with different levels of Mucin 1 expression. The experimental data proved that the DOX-loaded, MUC1 aptamer-functionalized nanosphere preferentially delivered drugs and exhibited cytotoxic effects at low DOX concentration in Mucin 1-high MCF-7 cells (

Figure 6) [181].

Insert

Figure 6

5-fluorouracil (5-FU) is commonly utilized for the treatment of breast, colon, rectum, stomach, and pancreas cancers [191]. In a very interesting approach, the functionalization of nanocapsules based on carboxymethyl chitosan with AS1411 aptamer and loading with 5-FU was conducted. The results of cytotoxicity and hemolysis tests showed that these original 5-FU-loaded nanocapsules were able to induce a more pronounced cytotoxic effect on neoplastic MCF-7 cells, the occurrence of dead cells being more rapidly than in the case of free 5-FU [183]. A 5-FU loading carrier based on aptamer conjugated with hyaluronan/chitosan NPs was prepared to target MUC 1 biomarker from colorectal cancer. The prepared NPs were about 181 nm in size and showed

acceptable stability. By in vitro cytotoxicity assays and confocal scanning microscopy in (MUC1+) human adenocarcinoma was observed that the toxicity of the NPs was significantly higher compared with a free drug in both cell lines [184].

Epirubicin (EPI) belongs to the anthracyclines group of anticancer drugs [192]. Several studies report its use in co-delivery systems with DNA/RNA aptamers in the treatment of breast, lung, and colon tumors. For instance, Jalalian *et al.* show the focus on co-delivery of EPI and NAS-24 aptamer to malignant growth cells utilizing selenium nanoparticles (SeNPs). These edifices empowered the aggregation of both medication and aptamer into disease cells for upgrading tumor reaction *in vitro* and *in vivo* (mice) exhibited by the fluorescence imaging and flow cytometric investigation procedures [187].

Hence, a recent study proved the antioxidant properties and antitumoral activity of SeNPs against lung cancer [193] allowing for synergistic anticancer effect when combined in delivery frameworks [194,195]. Another methodology joined the antitumoral effect of EPI and antimir-21 co-delivery system in treating breast and colon cancer cells. The targeted system uses two polymers: (1) poly(β amino ester) as a core for the delivery of antimir-21 and (2) poly (lactic-co-glycolic acid) (PLGA) modified with MUC1 aptamer to enable the encapsulation of EPI [192]. Hybrid delivery carrier based on PLGA NPs modified with chitosan for EPI drug encapsulation and 5TR1 DNA aptamer was demonstrated against the MUC1 receptor. *In vivo* anticancer activity of the targeted NPs was realized on MCF7 cells (breast cancer cell) and in BALB/c mice bearing C26 cells (murine colon carcinoma cell) [196]. Dimeric or dendrimer DNA aptamers linked to drug molecules enhance the efficiency of the delivery, as research studies show [188,197]. Taghdisi *et al.* effectively realized the *in vitro* and *in vivo* focus on targeted delivery in MCF-7 cells (breast cancer cell) and C26 cells (murine colon carcinoma) of EPI in association with three aptamers (MUC1, AS1411, and ATP aptamers) by DNA dendrimers loading system. Multiple building blocks of aptamers on the surface of dendrimer enabled the differentiation amongst target and non-target cells [188].

Gemcitabine is another antineoplastic chemotherapeutic drug being also classified as an antimetabolite drug. It has been considered a first-line chemotherapy agent for the treatment of pancreatic cancer, however it has been additionally utilized to treat other malignaces in lungs, bladder, sarcoma, breast or ovars. In any case, the initial response rate of gemcitabine is low and chemoresistance frequently occurs [189]. In that particular case, aptamers can be effectively targeting the cancer cells via binding to target molecules found on the membranes with high affinity and specificity. In an examination reported by Park *et al.*, an aptamer-based gemcitabine delivery framework was created and its therapeutic effects on pancreatic malignant growth cells *in vitro* and *in vivo* were tried. The outcomes acquired on various lines recommend that the gemcitabine-joined aptamer might be a promising targeted therapeutic strategy for pancreatic malignancy [189]. The principle challenge of using aptamer-drug conjugates for therapeutic purposes is their little capacity to enter cells as they are commonly negatively charged, impedimenting thus the penetration over the cell membrane. Additionally, they tend to degrade rapidly. One answer to this issue can be found in the utilization of natural and manufactured NPs as delivery vehicles for nucleic acids. Viral vectors that incorporate retroviruses, adeno-related infections, and lentiviruses, are proficient vehicles. However, they can create mutagenesis and carcinogenesis, and repeated administration can trigger safe reactions that hinder payload delivery. Subsequently, non-viral vectors (for example lipoplexes) are an alluring option [187].

4.3 Theragnostic

Theragnostic combines two terms “Therapeutics” and “Diagnostics,” as a single system, reflecting on the field of molecular therapeutics, molecular imaging, image-guided therapy, multifunctional NPs platforms, and translational nanomedicine. Hence, this strategy delivers new tools to enhance diagnostic specificity and therapeutic effectiveness. It requires an interdisciplinary

system gathering medicine, chemistry, pharmaceutical science, material science, nanotechnology, molecular imaging, molecular cell biology, and so forth [198].

Aptamers can be used as diagnostics systems by acting as key ligands for targeting the cancer cell or attaching them on various drug delivery systems directed against specific membrane proteins. Nanomaterials are the key in theragnostic approaches, therefore different NPs are used, especially for image-guided therapy. AuNPs [199] and quantum dots (QDs) [200] have intrinsic diagnostic/therapeutic properties. Also, magnetic NPs have shown potential as nanocarriers in the mechanism of targeted drug delivery to specific tumor areas [201]. Ultrasensitive detection using MNPs derives from the fact that most of the biological samples don't have a magnetic background, MRI traceability [202]. A disadvantage of magnetic nanoparticle usage would be potential cytotoxicity as a consequence. Moreover, carbon nanotubes are successfully used for theragnostic applications since they can decrease the effect of chemotherapeutic agents and are transcribed to various clinical applications [203]. Slow biodegradation is the issue that needs to be overcome while using carbon nanotubes in theragnostic.

However, the latest studies are focusing more on a complex multi-component system, where are assembled in a single smartly engineered nano-system for targeting, imaging, diagnostic, and therapeutic functions. A novel activatable theragnostic nanoprobe (ATNP) strategy based on Au@Ag/AuNPs complex with activatable aptamer probes (AAPs) was developed and applied as a "nano-doctor" for image-guided cancer therapy *in vitro* as well as *in vivo*. Au@Ag/AuNPs serve as fluorescence quencher and optical heater, while the S6 aptamer is used against the A549 cancer cell line. The AAP sequence showed excellent target recognition ability and successfully attained selective fluorescence activation. The multifunctional nanoplatforms constructed as ATNP accomplished its goal regarding theragnostic, including active targeting, activatable imaging, and guided site-specific near-infrared photothermal therapy (NIR PTT) (Figure 7A) [204]. By changing the bimetallic Au@Ag/AuNPs core with a magnetic one (Fe_3O_4@carbon), a synergistic antitumoral

effect was observed when coupled with an aptamer strand and DOX for the *in vitro* chemo–photothermal therapy in lung adenocarcinoma cells (A549). Besides, the Apt-Fe_3O_4@C@DOX NPs shown a decrease in contrast enhancement of recorded magnetic resonance (MR) signals. Consequently, this system can work as a potential contrast agent to realize the visualization of T2-weighted MR in tumor tissues (Figure 7B) [205].

Insert Figure 7

Another study reported the elaboration of a theragnostic plasmonic magnetic nanomaterial-based model. The targeted molecules in this study are tumor cells from blood sample. The experimental results showed that synthetic molecule of Cy3 attached by S6 aptamer conjugated to the nanomaterial can be used for fluorescence imaging and magnetic separation in blood sample spiked with SK BR-3 cancer cell. Furthermore, diagnosis and photothermal destruction were successfully achieved. A photothermal experiment was done using near-IR light (1064 nm) at 3 W outlining the selective irreparable cellular damage to SK-BR-3 cancer cells. This theragnostic nanotechnology has vast potential for applications, mostly used as a contrast agent and therapeutic activator for cancer disease [206]. A study for human breast cancer cells (MCF-7) was performed, where an anionic linear globular dendrimer is used as a suitable carrier for delivery and AS1411 aptamer is the targeting agent to carry Iohexol specifically to the cancer cells. This approached showed promising effects on the accumulation of Iohexol in the cancer tumors, reducing the number of cancer cells, and decreasing the toxicity of it on normal cells [207]. A correlation between monoclonal EpCAM antibodies and EpCAM aptamers was made by Xiang *et al.* featuring the prevalence of the aptamers. The tests were realised *in vivo*, utilizing xenograft mouse models. Firstly, the EpCAM-targeting aptamer was modified with a DY647 fluorophore molecule and injected intravenously into the tumor-bearing mice. This study shows the superior ability of aptamers to antibody marking invaluable characteristics of aptamers for molecular imaging and targeted therapy

[208]. Recently, Ghahremani *et al.* demonstrated a new strategy for effective *in vivo* tumor targeting and megavoltage radiosensitizing by utilization of AS1411-aptamer conjugates with gold nanoclusters (GNCs) that are synthesized through BSA as the capping agent. This method exhibited 39% of radiotherapy efficacy by taking advantage of flow cytometry and fluorescence microscopy. Importantly, the survival of the mice increased in 9 days [157].

Selective and sensitive recognition of a particular molecule has been achieved by aptamers, great targeting ligands. Aptamer-conjugated nanomaterials used as platforms for both diagnostic and therapeutic approaches, will increase the number of applications in the biomedical field and improve theragnostic outcomes, at the same time reducing the costs of production [209].

5 Aptamers from laboratory to industry

Over the most recent few years, antibodies showed high variability between different batches including cross-reactivity, perhaps it is the correct time for aptamers to enter into the stage as higher affinity tools and become widely used in medical applications. Some examples of "successful stories and applications of routinely used aptamers" of which the only one yet FDA approved can be consulted in the review of T.K. Sharma *et al.* [20].

As the aptamer market is continuously growing, the diagnostic industry expects to receive a great contribution. Instead of being just a research tool for academia, one could envisage aptamers to surpass their real potential as high alternative affinity tools. AptaBiosciences (Singapore), Aptahem (Sweden), Berlin Cures GmbH (Germany), Apta Biotherapeutics (South Korea), and Aptitude Medical (USA) are several relatively new companies involved in aptamer synthesis and commercialization for medical applications. Aptamer-Linked Immobilized Sorbent Assay (ALISA), nanoparticle-based assays, fluorescence-based assays, electrochemiluminescence (ECL) assays, electrochemical sensors, and glucometer-based assays are several point-of-care diagnostic platforms

used over the past years, serving for the detection of small or complex molecules and even more to whole cells [210].

When used as therapeutic agents, aptamers are targeting growth factors or angiogenic factors such as angiopoietin-1 and angiopoietin-2, fibroblast growth factor, integrins, hepatocyte growth factor, transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), etc. As well-known, Macugen® (Pegaptanib sodium) is the first RNA aptamer to make it to clinical use. The aptamer was selected against human vascular endothelial growth factor isoform 165 (VEGF165) for neovascular age-related macular degeneration treatment [211]. This protein stimulates vasculogenesis and angiogenesis and prevents choroidal neovascularization causing blindness in elderly people in developed countries, diabetic macular edema, and diabetic retinopathy [212]. The synthesis of Pegaptanib sodium RNA aptamer started to form a library of 2'-fluoropyrimidines, and to increase its biological stability, the post-SELEX modification was performed. After the FDA approval in 2004 and the European commission in 2006 [213], Macugen® ownership moved between several companies. In the early 1990s, it was initially developed by NeXstar Pharmaceuticals, a company founded by Lary Gold, which later merged with Gilead Sciences. Macugen® was then acquired by Eyetech Pharmaceuticals, which in collaboration with Pfizer performed the randomized clinical trials and managed to license Macugen in 2004. Eyetech Pharmaceuticals in turn was acquired by OSI Pharmaceuticals in 2005. Other changes occurred between companies, but currently, the drug is marketed by Bausch and Lomb, which was acquired in 2013 [172].

Two aptamers with antitumoral effects have yet experienced clinical trials. AS141 is a 26-nucleotide G-quadruplex DNA oligonucleotide [214] being the first aptamer for the treatment of human malignant growth. Its choice doesn't follow a traditional SELEX technique as being created from a screen of antisense oligonucleotides with antiproliferative capacity. Further, to expand its security for, *in vivo* applications, PEG-ylation of the strand was figured out. Attributable to its G-

quadruplex structures, AS1411 is impervious to nuclease degradation. This aptamer is commercially available from Antisoma.

The second aptamer is an RNA-based L-structure 45-nucleotide, specifically NOX-A12, which was created against chemokine C-X-C theme ligand 12 (CXCL12) which is mainly involved in tumor development and metastasis [95,215]. NOX-A12 binds to two key sites fighting against the CXCL12 activity, thereby hindering the tumor development in malignancies located at respiratory, digestive or central nervous systems. NOXXON Pharma AG, founded in 1997 (Berlin, Germany), is one of the leading companies in aptamer discovery, which uses aptamer-based technology for diagnostics [216]. NOX-A12 is produced by NOXXON Pharma AG. Several other aptamers developed by the same company are either in the clinical pipeline (NOX-36 (CCL2); NOX-H94 (hepcidin)) or in the preclinical pipeline (NOX-S93 (S1P); NOX-D21 (C5a); NOX-G16 (glucagon); NOX-L41 (CGRP)).

An anticoagulation-aptamer based system (REG1-RB006 in addition to RB007) was realesed by Regado Biosciences which showed improvements in treating different cardiovascular disorders [217]. It is comprised by two RNA aptamers: the drug (RB006), a 35-mer chemically modified at 2'-end with purine or fluoropyrimidine, and the antidote (RB007), a 17-mer modified at 2´-end with O-methyl and a 40 kDa PEG.

SomaLogic, founded in 1999 (Boulder, Colorado, USA) by Larry Gold, focuses more on diagnosis and proteomic analysis. SOMAscan® is a Slow Off-rate Modified Aptamer (SOMAmer)-based biomarker, an exceptionally adaptable kit with the capacity to recognize and evaluate 1,305 protein analytes found in the human fluids with high accuracy and sensitivity [218,219]. The relatively new entry, Base Pair Biotechnologies (2012, Pearland, Texas, USA), focuses on both custom aptamer identification and characterization service for therapeutics and development of diagnostics and environmental testing.

6 Conclusions and future perspectives

This review covers the most recent advances (2015-2020) in the selection of aptamers and challenges among various methods. Aptamers' potential as powerful tools in biomedicine applications are of high interest. Nonetheless, a retrospective presentation focusing on the synthesis and selection procedures is briefly introduced providing more dedicated review articles to address each section.

The most well-known comparison to being in the spotlight is aptamers versus antibodies. Indeed, some key features of aptamers as (1) lower-cost production (no-host animals), (2) reproducibility among batches (chemical synthesis), and oftentimes (3) higher affinity and specificity will likely expend the aptamer's marketplace in the next years to come. It can be seen from the report we conducted based on the literature that aptamers' interest has increased with more than 220% in the last decade (

Figure 2A). Hence, several aptamers were successfully introduced in clinical trials or the more so pipelines expecting promising results from the next generation of therapeutic and diagnosis aptamers.

Recent technology designs used in the selection of aptamers enable faster and cheaper production of high-affinity new generation aptamers compared to traditional SELEX which can last up to several months. Still, some aptamers are more prolific than others. Rigorous characterization performed by multiple research groups endorsed several well-defined aptamers sequences. Binding affinity, nature of the biomolecular interactions, and structural characterization are of paramount importance for aptamer screening and selection. However, the remarkable challenges of aptamers from the biomedical field still need to be dealt with. Poor specificity and sensitivity, questionable clinical use, low drug loading, and toxicity are only a few examples. Although high-throughput sequencing has considerably more features than cloning and Sanger sequencing, its use is still very little due to very high costs. Hence, post-selection modification enables improved substitutes.

As for the low loading capacity and target selectivity, targeted drug delivery may be the answer to these limitations. It is worthwhile to mention that also the cost of the SELEX strategy of *in vivo* aptamer synthesis should not be neglected as aptamer-bioconjugates are mostly employed mainly due to the present *in vivo* nucleases. Unfortunately, besides higher costs, possible toxic side-effects can be initiated. To tackle down this issue, more clinical trials on animal models should be addressed to evaluate the safety and efficiency of these aptamer-bioconjugates. The investment in improving the targeted delivery systems is of paramount importance as these conjugates have already demonstrated multifunctional capability for cancer therapies and diagnostics the possibility. Some of the most significant aptamer-based delivery systems were reported in this review, which are commonly founded on conjugates as aptamer-chemotherapy operators, aptamer-nanoparticles, aptamer-protein, aptamer-siRNA/shRNA/miRNA, aptamer-immune response.

Nevertheless, while the aptamer-based sensing strategies are in exponential development, a large gap between proofs-of-concept and commercialized biomedical applications of such affinity sensors is observed. Hopefully, electrochemical aptasensors will break the ice into the diagnostic marketplace due to their small, reproducible, and disposable features which allow for point-of-care/use analysis.

In summary, based on the progress of SELEX technology and of nanomaterials, new tailored aptamers are yet to be envisaged. Whilst improvements in the efficiency and cost are greatly recommended, more advanced aptamer-based strategies for diagnosis, therapeutic and sensing applications are likely to be developed in the near future.

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Figure captions

Figure 1. Schematic representation of (A) aptamer-target interaction and (B) SELEX method: (1) an initial pool of different nucleic acid sequences is incubated with target-molecule; (2) after incubation, a separation step is applied to set apart the nucleic acid sequences bound to target molecules from unbound sequences; (3) bound sequences are removed and amplified by PCR, for the next selection cycle; (4) after several selection rounds the aptamers are cloned, sequenced and subjected to evaluation of their affinity toward the target.

Figure 2. (A) Evolution of aptamer-based publications. Peer reviewed "nucleic acid aptamer"-related papers published between 1990 and 2019 (Web of Science, Scopus and PubMed databases). The unusual growth frequency of "nucleic acid aptamers" articles is encountered; **(B)** Literature report of aptamers used for medical applications between 2015 and 2019 (Scopus database).

Figure 3. Schematic illustration of DNA-aptamers' use in biomedicine.

Figure 4. Aptamer-based sensors for antibiotic detection. Schematic representation of two **optical strategies** for ampicillin detection based on aptamer-conjugated **(A)** silver and **(B)** gold nanoparticles, by means of colorimetric and fluorescent sensing techniques, respectively. Adapted with permission from Ref. [98] and Ref. [152]. Schematic representation of electrochemical aptasensors for **(C)** ampicillin label-free detection by means of electrochemical impedance spectroscopy measurements at an aptamer-modified Co- and terephthalonitrile-based organic frameworks platform and **(D)** tetracycline indirect detection by means of cyclic voltammetry measurements in $[Fe(CN)_6]^{4-/3-}$ enabled to evaluate the degradation rate of an M-shape structure (3

complementary DNA strands + aptamer) in the presence of the antibiotic. Adapted with permission from Ref. [102] and Ref. [103].

Figure 5. Aptamer-guided *in vivo* therapy. *In vivo* fluorescence imaging of mice bearing MCF-7 tumors after intravenous injection of two aptamers (PA-Apt–NHS–PEG or PA-Apt–CHO–PEG). Reproduced with permission from Ref. [173].

Figure 6. Aptamer-based drug delivery system. (A) Schematic illustration of MUC 1 aptamer-sphere loading system for Doxorubicin and **(B)** loading capacity determination by means of confocal microscopy of QD-labeled MUC1 aptamer, empty sphere, or Apt-sphere **(a)** and quantitated **(b)**. Reproduced with permission from Ref. [181].

Figure 7. Aptamer-guided theranostic nanomedicine. (A) Schematic illustration of the novel activatable theranostic nanoprobe (ATNP) strategy for aptamer-mediated *in vivo* imaging and guided photothermal therapy **(a)**. *In vivo* aptamer-related activatable fluorescence imaging **(b)** and imaging-guided site-specific NIR PTT **(c)** of SPCAi (cyan circles) and A549 (pink circles) tumor following ATNP administration. Reproduced with permission from Ref. [204]. **(B)** Schematic ilustration of the preparation of Apt-Fe_3O_4@C@DOX NPs and internalization of Apt-Fe_3O_4@C@DOX NPs into cancer cells for chemo–photothermal combination therapy **(a)**; *In vivo* aptamer@DOX-mediated citotoxicity in A549 cells under different laser power densities and T2-weighted MR images of the tumor sites after intravenous injection with Apt-Fe_3O_4@C@DOX NPs **(b)**. Reproduced with permission from Ref **.** [205]**.**

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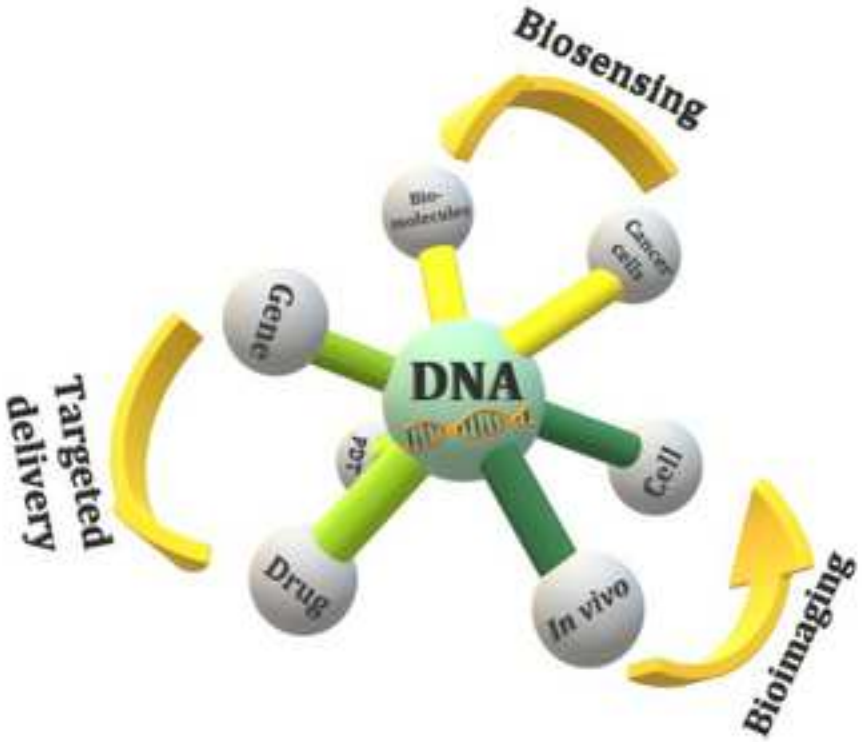
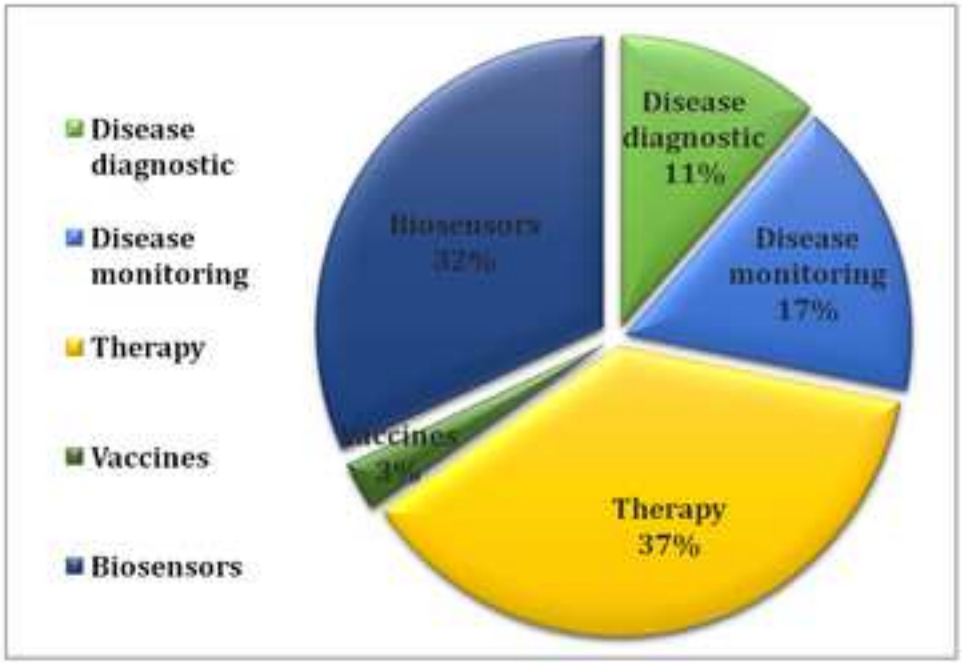
Graphical Abstract (for review)





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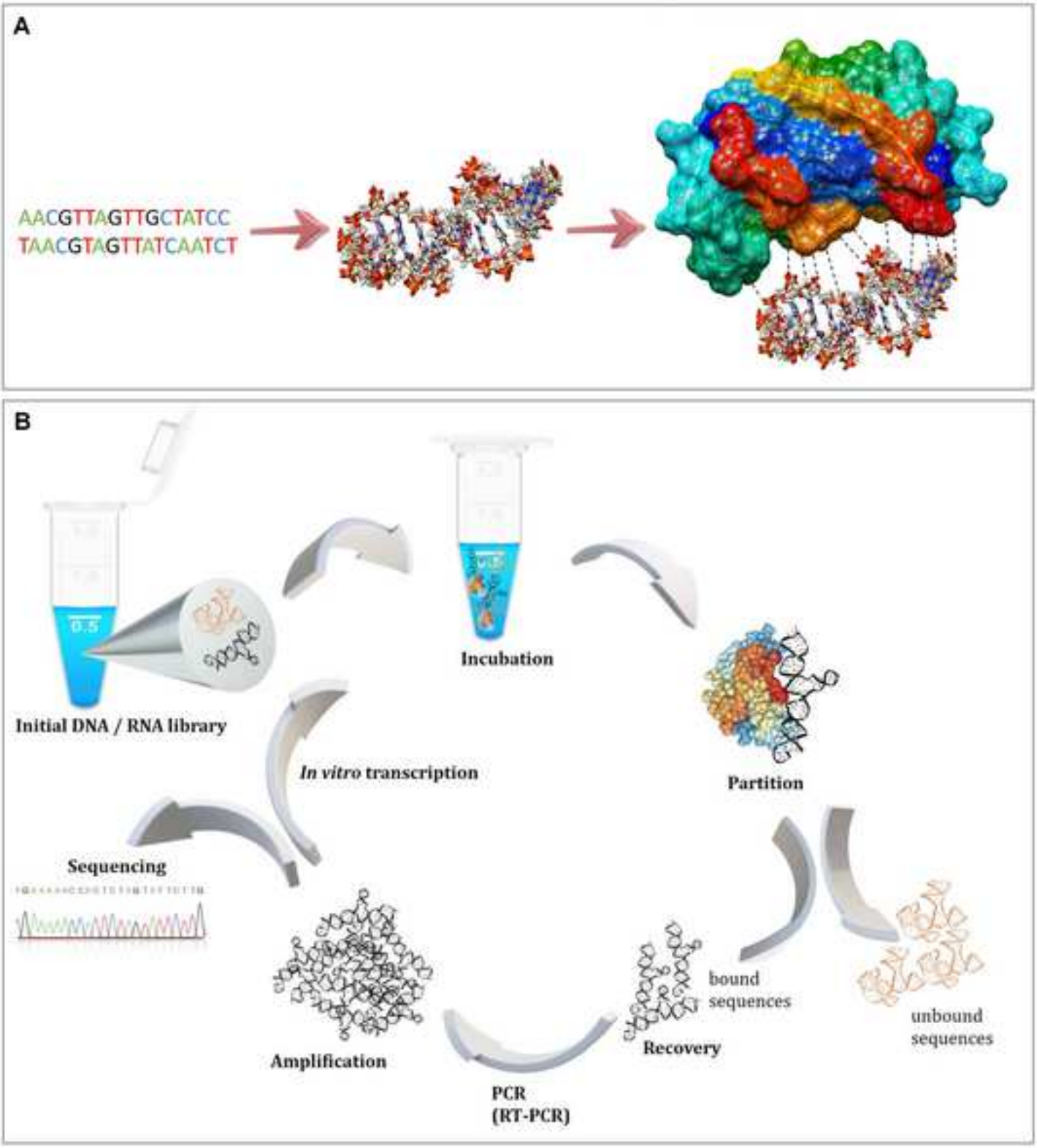

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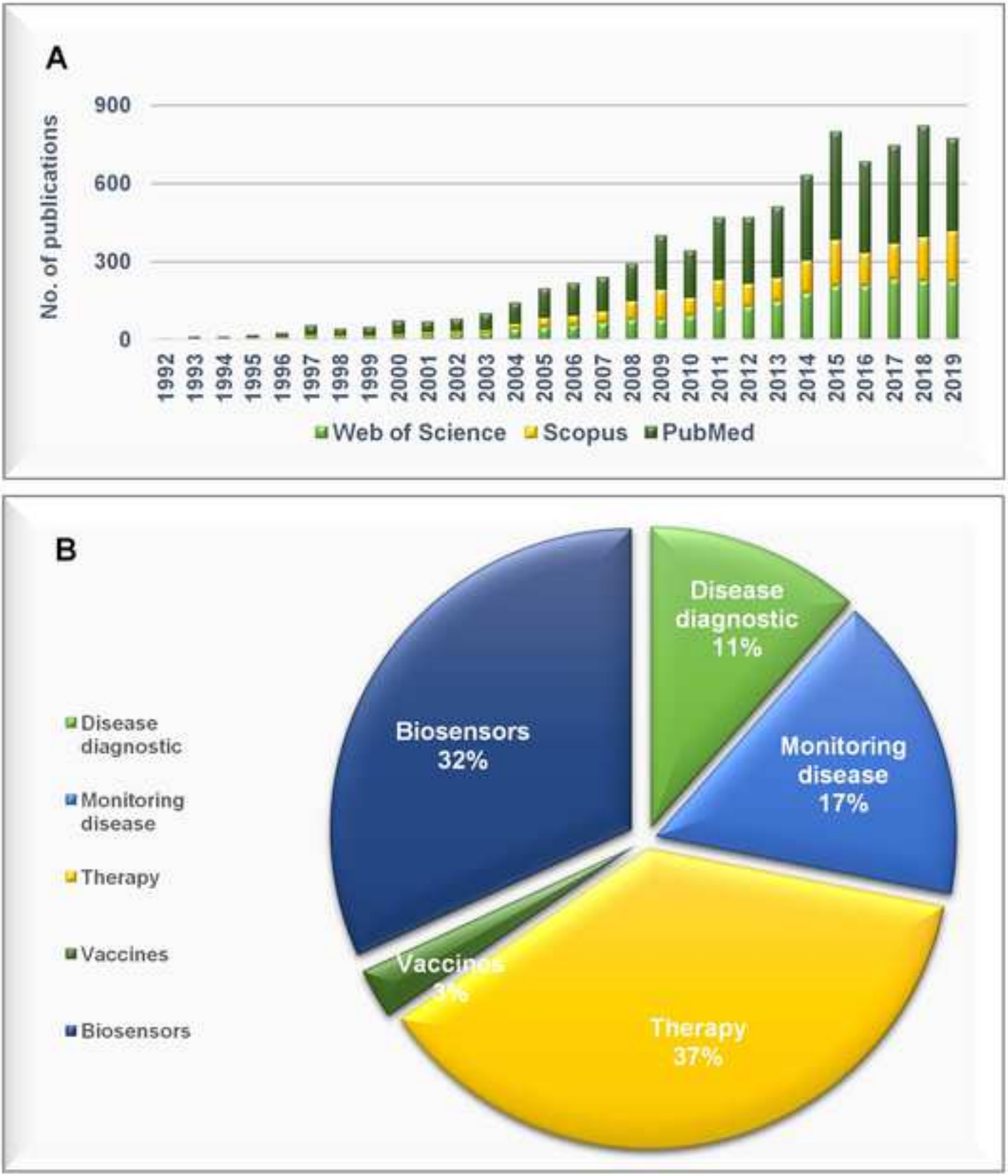

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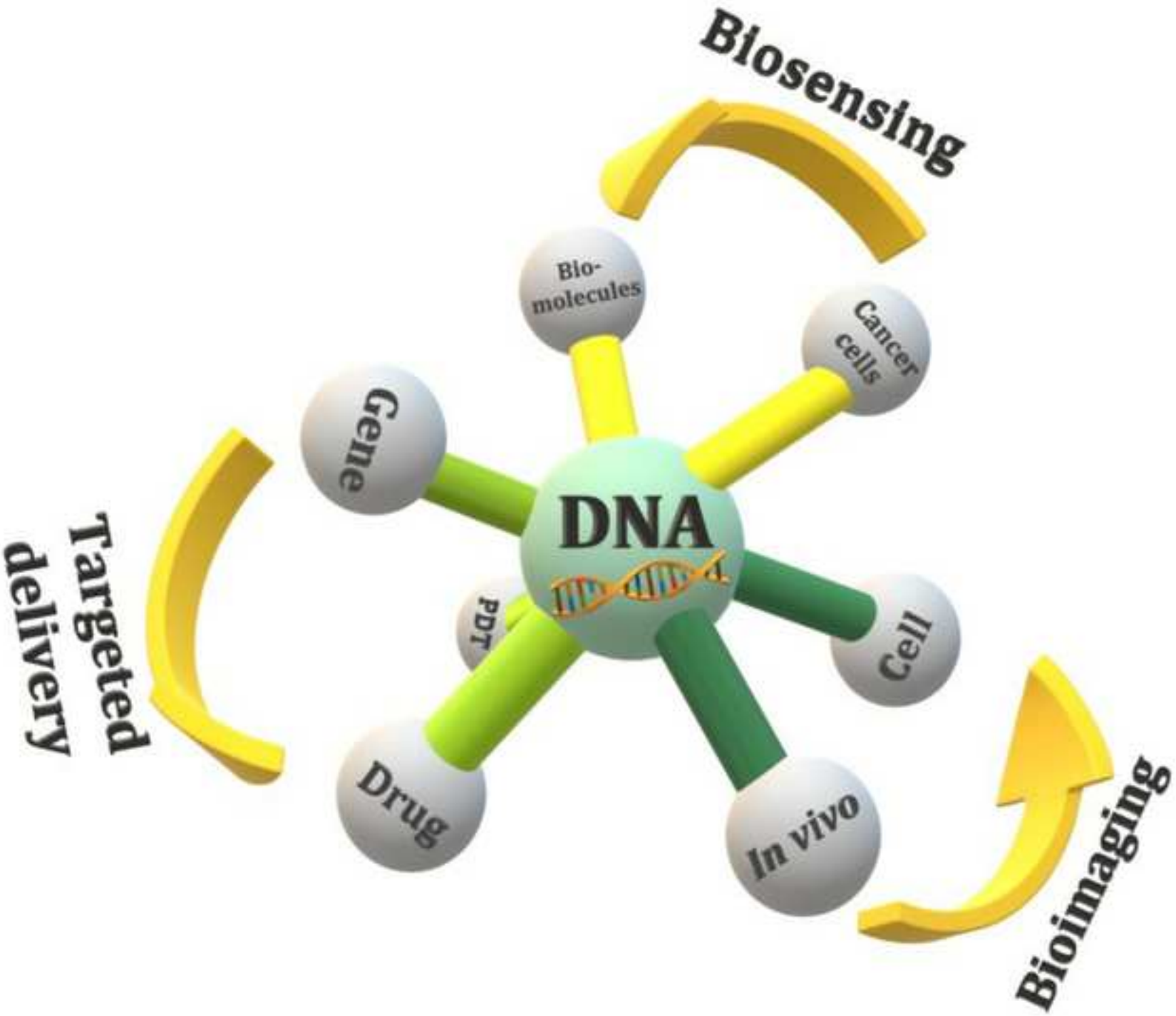

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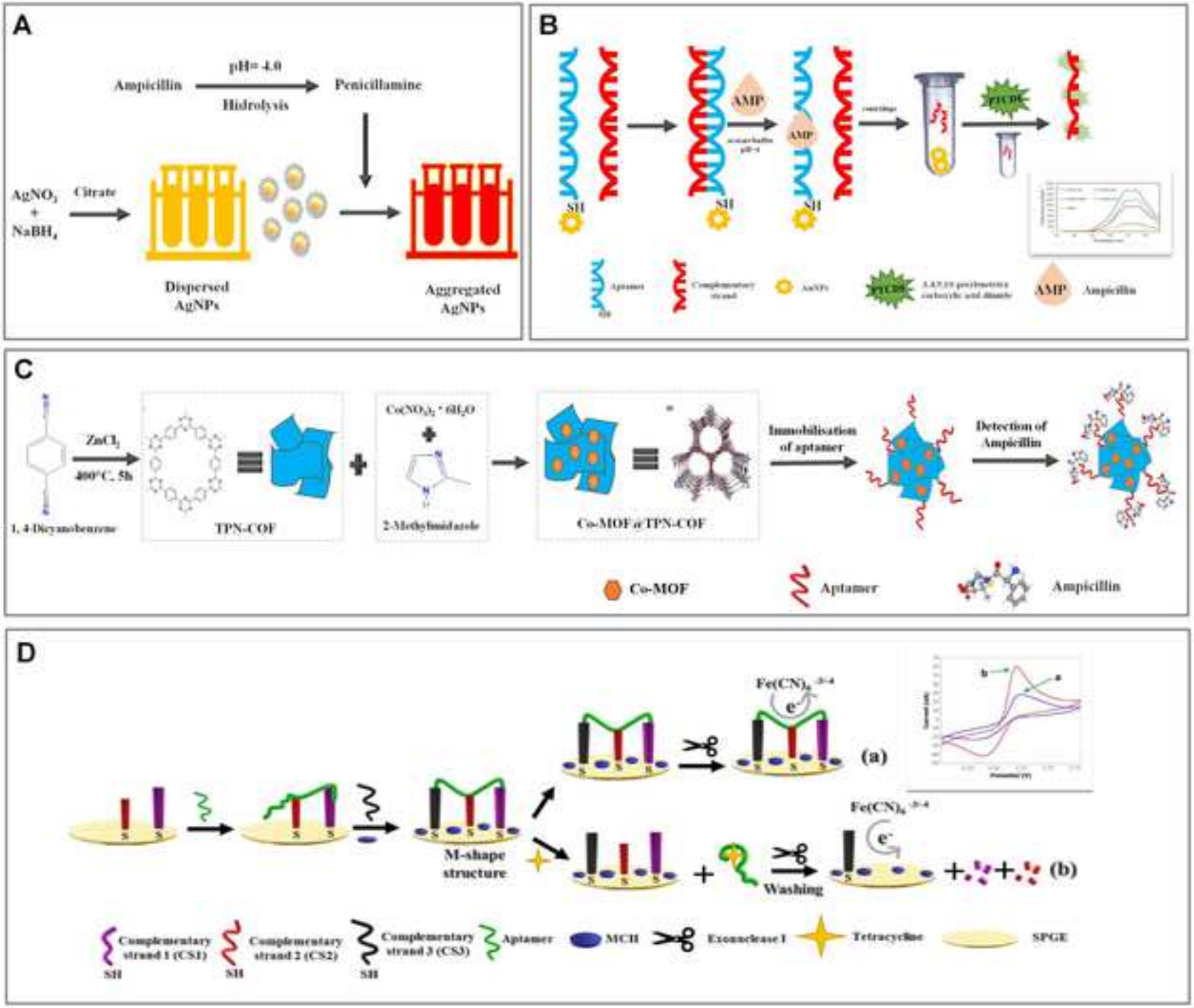

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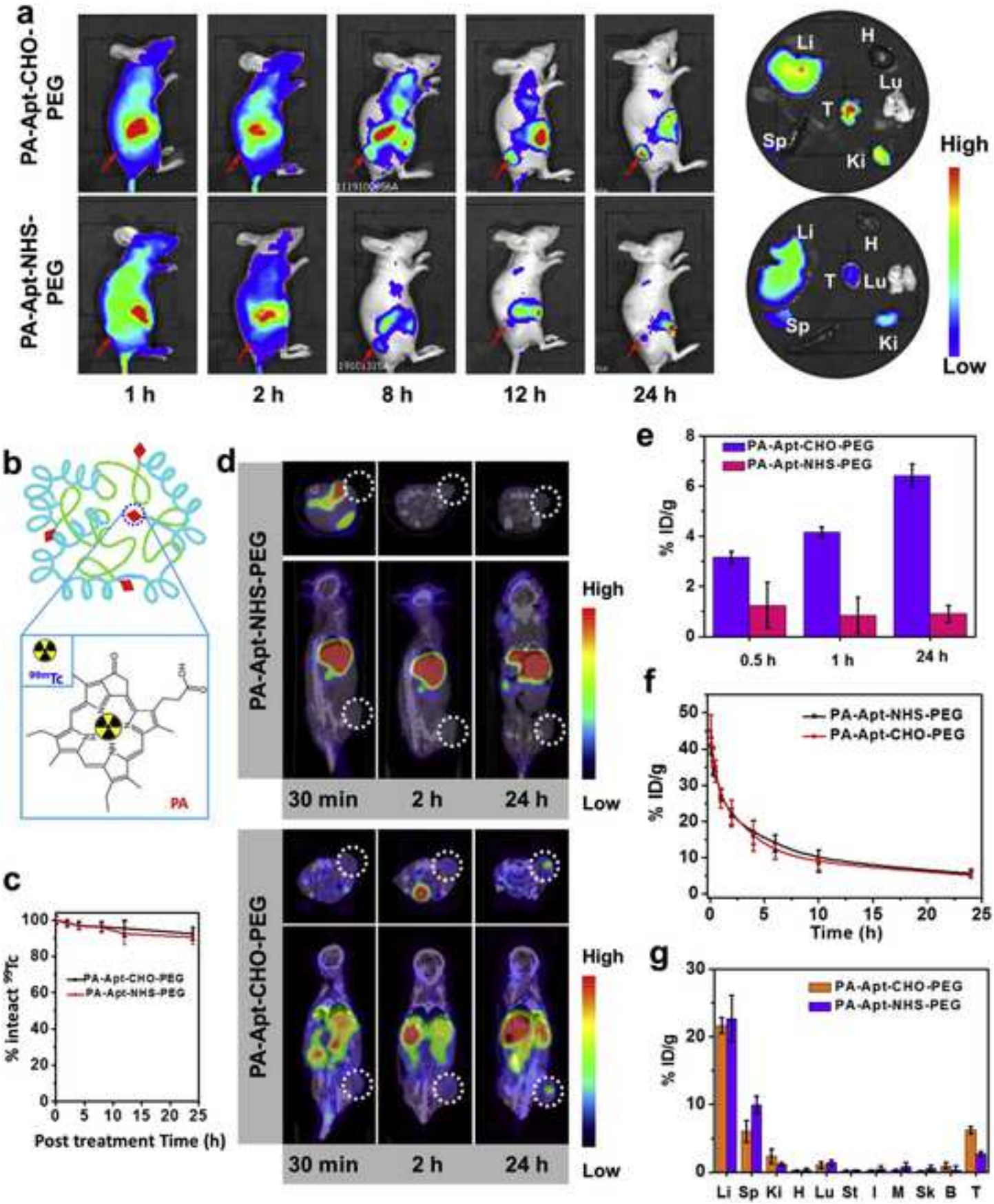

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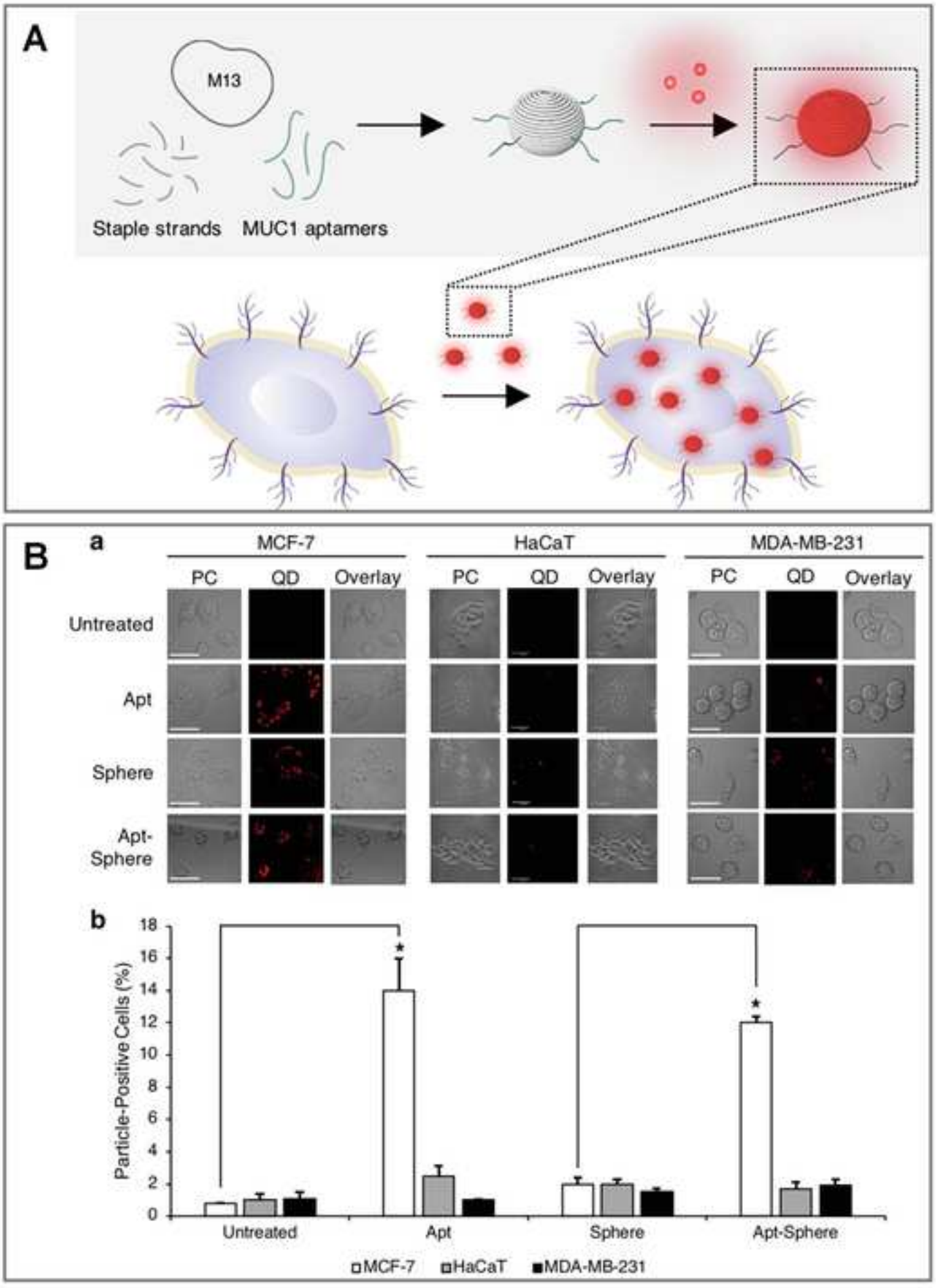

Figure(s)

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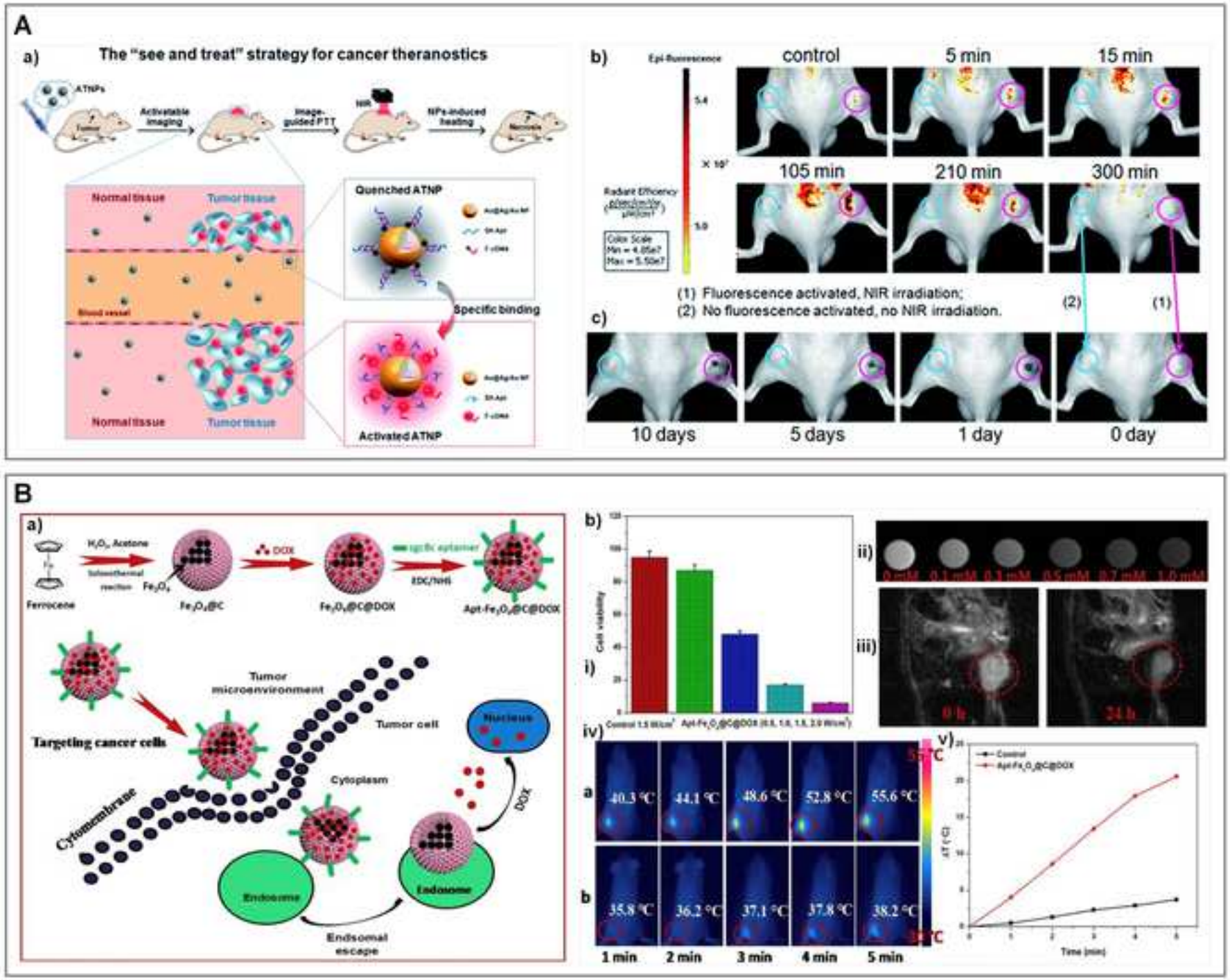

Table 1. Advantages and disadvantages of aptamers over antibodies [29]

Criteria	Aptamers	Antibodies
Chemical nature	• ssDNA and RNA	• proteins
Molecular weight and size	• 6–30kDa (20–100nt) • ~2nm	• 150–180 kDa • ~15 nm
Secondary structure	• hairpin, loops, G-quadruplex, psudoknots	• four polypeptide chains joined to form a Y shaped molecule
Targets	• ions, organic and inorganic small molecules, proteins, toxins, whole cells, organs and live animals	• limited to immunogenic molecules
Batch to batch variation	• not or low	• significant
Affinity	• high; multivalent aptamers increase affinity	• high; it depends on the number of identical epitopes on the antigen
Specificity	• high	• high
Synthesis	• chemical after *in vitro* SELEX (2–16 selection cycles) • ~2–8 weeks	• biological after *in vivo* process • ~6 months or longer
Physical and thermal stability	• heat stability and long lifetime • resistant to high temperatures; allow denaturation and renaturation	• short lifetime • not resistant to high temperature; irreversible denaturation
Chemical modification and conjugation	• accessible and controllable • aptamers can be modified without loss of binding affinity	• restricted and uncontrollable • limited types and chemical reactions

Table(s)

Table 1. Comparison of different techniques used to measure aptamer–target binding [26,37,38]

Technique	Sample consumption	Experiment time	K_d limit	Equipments
UV-Vis spectroscopy	~15-25 µL for each sample	~10 min per sample	10^{-6} M	Spectrophotometer, quartz cuvette
Gel electrophoresis (GE)	From 10 µL to 50 µL for each sample	~3 h separation.	10^{-13} M	GE Electrophoresis power supply and autoradiography
Capillary electrophoresis (CE)	~10 µL for each sample	~10 min per sample injection	10^{-9} M	CE system; CE with LIF detection for capillary DNA sequencing
HPLC	100 µL to 18 mL, dependent on column type	~10-30 min per sample injection	10^{-6} M	HPLC with UV detection
Surface plasmon resonance (SPR)	10-20 µL for each sample	~20 min per each sample	10^{-12} M	Biacore SPR instrument and chip sensor
Fluorescence intensity	~150 µL	~10 min per sample; break needed after each titration	10^{-10} M	Spectrofluorometer and cuvette
Isothermal titration calorimetry (ITC)	~200–500 µL for each injection	~1.5-2 h	10^{-9} - 10^{-8} M	ITC calorimeter

Table(s)

Table 1. Several examples of electrochemical aptasensors for biomedical applications

Target	Sensing platform	Sensing Enhancement	Utilized method	Detection linear range	Limit of detection	Incubation time	Stability	Sample	Ref.
MUC-1	AuE	-	SWV	10 pM - 1µM	4 pM	60 min	93.75% after 4 weeks	Synthetic	[107]
	AuE	AuNPs	EIS	0.5−10 nM	0.1 nM	-	-	Human blood serum	[108]
HER-2	silicon/silicon oxide wafers	Interdigitated electrodes	CV/EIS	1 pM−100 nM	1 pM	30 min	-	Human serum	[109]
	Au IDE	Interdigitated microelectrodes	nFIS	0.2– 2 ng/mL	0.2 ng/mL	-	-	Human serum	[110]
PSA	GCE	AuNPs/multi-walled carbon nanotubes	DPV	0.005- 20 ng/mL	1.0 pg/mL	40 min	95%–92% after 30 days	Human serum	[111]
	Au interdigitated electrode	Self-assembled monolayer	EIS	0.5- 5000 ng/mL	0.51 ng/mL	60 min	90% after 6 days	Human serum	[112]
Thrombin	GCE	$Ru(bpy)_3^{2+}$/ β-cyclodextrin/Au NPs/nanographene nanocomposite	ECL	0.4–1000 pM	0.23 pM	15 min	-	Human serum	[113]
	GCE	CD/AuNF	ECL	0.5–40 nM	0.08 nM	-	-	Synthetic	[114]
Cytochrome c	GCE	Electro-polymerized neutral red and decacarboxylated pillar	CV/EIS	80 pM−80 nM	0.02- 1.0 nM	20 min	85% after 14 days	Human serum	[115]
Interleukin 6	SPGE	Polypyrrole/AuNPs	CV/EIS	1 pg/mL−15 µg/mL	0.33 pg/mL	30 min	93% after 10 days	Human serum	[116]
Alpha-fetoprotein	SPCE	TH/RGO/Au NPs	DPV	0.1 µg/mL - 100.0 µg/mL	0.050 µg/mL	-	95.76% after 7 days - 90.86% after 14 days	Human urine	[117]
	SPGE	-	CV/DPV	7–500 pg/mL	2 pg/mL	90 min	-	Human serum	[118]
IFN- γ	Microfluidic chip	MB	CV/SWV	10–500 pg/mL	6 pg/mL	-	92 ± 2% after 30 days at room temperature	Culture medium; blood serum	[119]
Valrubicin	AuE	AuNPs/en/MWCNTs	CV	5 – 60 µM	18 nM	-	-	Human	[120]

								urine; blood serum	
Oxytetracycline	AuE	Ce-MOF@COF	EIS	20 x 10 pM – 1.0 x 10 nM	0.035 pM	-	-	Urine samples	[121]
Streptomycin	GCE	AgNPs/GQDs-N-S/AuNPs	CV/EIS	0.01– 812.21 pg/mL	0.0033 pg/mL	-	87% after 14 days	Serum samples	[122]
Ampicillin	AuE	Co-MOF-on-TPN-COF	EIS	1.0 fg/mL - 2.0 ng/mL	0.217 fg/mL	-	15% after 15 days	Human serum	[102]
Tetracycline	SPGE	-	CV/DPV	-	0.71 nM	75 min	-	Human serum	[103]
Hepatitis C virus	GCE	GQD	CV/DPV/EIS	10–70 pg/mL – 70–400 pg/mL	3.3 pg/mL	40 min	96% after 10 days	Human serum	[123]
Mycobacterium tuberculosis antigen MPT64	AuE	AuNPs-C_{60}-PAn	CV/EIS	0.02 - 1000 pg/mL	20 fg/mL	-	-	Human serum	[124]
Adenosine	AuE	MB/ $Ru(NH_3)_6^{3+}$	CV/EIS	-	0.032 nM	-	-	Human serum	[125]
Hemoglobin	GCE	AMSN	DPV	10 aM – 1.0 × 10 µM	650 aM	60 min	-	Human blood	[126]
Hemin	GCE	AMSN	DPV	10 aM – 1.0 × 10 µM	750 aM	60 min	90% after three days	Human blood	

AMSN- Amino–functionalized mesoporous silica nanoparticles; AuE- gold electrode; AuNPs- gold nanoparticles; AuNPs-C_{60}-Pan- gold nanoparticles- coil-like fullerene-doped polyaniline; CD/AuNF- carbon dot/ gold nanoflower; COP- porous organic framework; Ce-MOF- Ce-based metal organic framework; Co-MOF- Co-based metal-organic frameworks; CV- Cyclic voltammograms; DPV- Differential pulse voltammetry; EIS- Electrochemical impedance spectroscopy; ECL- electrochemiluminescence; GCE- glassy carbon electrode; GQD- graphene quantum dots; GQDs-N-S- graphene quantum dots functionalized with amine and thiol; IDE- Interdigitated microelectrodes; IFN- γ- interferon-gamma; MB- magnetic beads; Mn-ZnS QDs- Mn-doped ZnS quantum dots; MWCN- Multi-wall carbon nanotubes; nFIS- nonfaradaic impedance spectroscopy; SNP- silica nanoparticles; SPCE- Screen printed carbon electrode; SPGE- Screen printed gold electrode; TH/RGO - Thionin/reduced graphene oxide; TPN-COF- terephthalonitrile-based covalent organic framework;

Target	**Aptamer sequences**
MUC-1	5′- MB-GCAGTTGATCCTTTGGATACCCTGG-3’
	5’ –HS-$(CH_2)_6$ -GCAGTTGATCCTTTGGATACCCTGG-3’

HER-2	5′-SH-$(CH_2)_6$ - AACCGCCCAAATCCCTAAGAGTCTGCACTTGTCATTTTGTATATGTATTTGGTTTTTGGCTCTCACAGACACACTACACACGCACA-3′
	5’-NH2-$(CH_2)_6$ -GGGCCGTCGAACACGAGCATGGTGCGTGGACCTAGGATGACCTGAGTACTGTCC-3’
PSA	5′-HS-$(CH_2)_6$ –TTT TTAATTAAAGCTCGCCATCAAATAGCTTT-3′
	5′-COOH-C_6-TTTTTAATTAAAGCTCGCCATCAAATAGCTTT-’3
Thrombin	Apt 1: 5′-Fc-GGTTGGTGTGGGTTGG-3′ Apt 2: 5′-Fc- AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3′
	Apt 1: 5′–SH–$(CH_2)_6$-GGTTGGTGTGGTTGG-3′ Apt 2: 5′–SH–$(CH_2)_6$-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3′
Cytochrome c	5’-NH_2-CCGTGTCTGGGGCCGACCGGCGCATTGGGTACGTTGTTGC-3’
Interleukin 6	5’-GGTGGCAGGAGGACTATTTATTTGCTTTTCT-3’
Alpha-fetoprotein	5'GTGACGCTCCTAACGCTGACTCAGGTGCAGTTCTCGACTCGGTCTTGATGTGGGTCCTGTCCGTCCGAACCAATC -3'
	SH-GTTGGGCACGTGTTGTCTCTCTGTGTCTCGTGCCCTTCGCTAGGCCCACA
IFN- γ	5′λ-Fc-GGGGTTGGTTGTGTTGGGTGTTGTGTCCAACCCC-biotin-3′
Valrubicin	5′ SH-$(CH_2)_6$ -CGCGGGCCGGGCCGG-3′; 5′ SH-$(CH_2)_6$ -(CH2)6-ATATTATAAATTATA-3′; 5′ SH-$(CH_2)_6$ -CGAGTATGCATGACC-3′; 5′ SH-$(CH_2)_6$ -CGCGCGGG-3′
Oxytetracycline	5’-CGTACGGAATTCGCTAGCCGAGGCACAGTCGCTGGTGCCTACCTGGTTGCCGTTGTGTGGATCCGAGCTCCACGTG-3’

Streptomycin	5′-TAGGGAATTCGTCGACGGATCCGGGGTCTGGTGTTCTGCTTTGTTCTGTCGGGTCGTCTGCAGGTCGACGCATGCGCCG-SH-3′
Ampicillin	5′-TTAGTTGGGGTTCAGTTGG-3′
Tetracycline	5’-CGTACGGAATTCGCTAGCCCCCCGGCAGGCCACGGCTTGGGTTGGTCCCACTGCGCGTGGATCCGAGCTCCACGTG-3’
Hepatitis C virus	5’-ACTATACACAAAAATAACACGACCGACGAAAAAACACAACC-3’
Mycobacterium tuberculosis antigen MPT64	MBA I: 5’-SH-$(CH_2)_6$-TGGGAGCTGATGTCGCATGGGTTTTGATCACATGA-3’ MBA II: 5’-SH-$(CH_2)_6$-TTCGGGAATGATTATCAAATTTATGCCCTCTGAT-3’
Adenosine	5’- NH_2-TTTTTTACCTGGGGGAGTATTGCGGAGGAAGGT-3’
Hemoglobin **Hemin**	5′–NH_2–GTGGGTAGGGCGGGTTGG–3′

Credit Author Statement

Geanina Stefan: Investigation, Methodology, Visualization, Writing - Original Draft; **Oana Hosu:** Conceptualization, Investigation, Visualization, Writing - Original Draft & Review & Editing; **Karolien De Wael:** Writing - Review & Editing; **María Jesús Lobo-Castañón:** Conceptualization, Writing - Review & Editing; **Cecilia Cristea:** Writing - Review & Editing, Funding acquisition