Analysis of aptamer discovery and technology

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Abstract | Aptamers are nucleic acid molecules that mimic antibodies by folding into complex 3D shapes that bind to specific targets. Although some aptamers exist naturally as the ligand-binding elements of riboswitches, most are generated *in vitro* and can be tailored for a specific target. Relative to antibodies, aptamers benefit from their ease of generation, low production cost, low batch-to-batch variability, reversible folding properties and low immunogenicity. However, the true value of aptamers lies in the simplicity by which these molecules can be engineered into sensors, actuators and other devices that are often central to emerging technologies. This Review examines changing trends in aptamer technology by analysing the first quarter century of aptamer data that is available in the scientific literature (1990–2015). We highlight specific examples that showcase the use of aptamers in key applications, discuss challenges that have impeded the success of aptamers in practical applications, provide suggestions for choosing chemical modifications that can lead to enhanced activity or stability, and propose standards for the characterization of aptamers in the scientific literature.

Phenotypes
Observable characteristics, such as binding or catalytic activity, that are encoded in the sequence of a nucleic acid or protein.

Genotypes
Genetic sequences that encode phenotypes.

Antibodies
Protein affinity reagents produced by the immune system that can be made to recognize a wide range of targets called antigens.

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Nucleic acids (DNA and RNA) are commonly thought of as the genetic blueprint of life because they carry the instructions on how an organism can grow, develop and replicate[1,2]. However, these molecules can also fold into complex 3D structures (known as ribozymes) that catalyse reactions[3], control gene expression[4], communicate cellular responses[5] and mediate protein synthesis[6]. Although the biological importance of folded RNA structures has long been recognized[7], the *in vitro* evolution of nucleic acid molecules with non-biological functions was achieved only when it became possible to generate large populations of degenerate oligonucleotides by solid-phase synthesis and amplify individual members using the polymerase chain reaction (PCR)[8,9]. These technologies enable the isolation of functional nucleic acid molecules that can bind to a specific target or catalyse a chemical reaction[10]. Affinity reagents based on either DNA or RNA are referred to as aptamers, the Latin root of which means 'to fit' (REF. 11) (BOX 1). Aptamers can be synthesized to bind to a wide range of chemical and biological targets from small molecules to whole cells.

The process used to isolate aptamers from large random-sequence libraries is called *in vitro* selection, which is also termed SELEX (systematic evolution of ligands by exponential enrichment)[11–13]. Similar to natural selection, SELEX is an iterative process of selection and amplification in which large pools of nucleic acid molecules (typically >1 trillion distinct sequences) are challenged to bind to a desired target under a defined set of conditions (for example, temperature and salt concentration). Molecules bound to the target are separated from the unbound pool and amplified to generate a new population of molecules that is enriched in members that share a common functional property[14]. Nucleic acids are ideally suited for this purpose because they can fold into shapes with a defined function (phenotypes) and their sequences (genotypes) can be replicated *in vitro* to produce progeny molecules with similar characteristics to the parent sequence[10]. The ability to amplify individual molecules with desired phenotypes and optimize their functions by directed evolution is a distinguishing feature that separates nucleic acids from other organic molecules, most of which cannot replicate because they lack a genotype–phenotype connection[15,16].

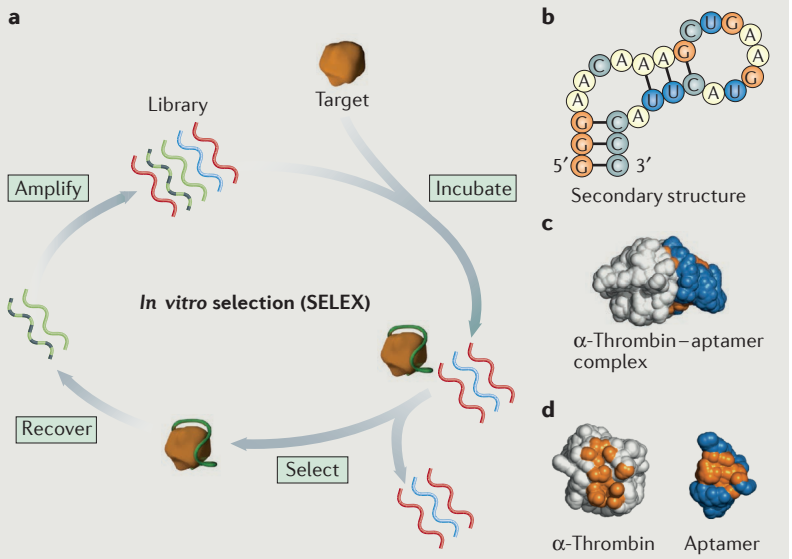
Aptamers are often compared with antibodies, as both molecules function as affinity reagents[17]. However, unlike antibodies and other protein-based affinity reagents (including single-chain variable fragment antibodies, affibodies and designed ankyrin repeat proteins)[18–22], aptamers have unique advantages that make them powerful tools in the arsenal of affinity reagents. At the time of writing, aptamers can be produced on larger scales than antibodies, and the retained genetically encoded sequence of aptamers can be expressed *in vivo* in cultured cells[23]. As aptamer production is a chemical process rather than a biological process, it avoids the problem of viral or bacterial contamination that can occur during antibody manufacturing and reduces the potential

for batch-to-batch variability that plagues the antibody market and frustrates researchers seeking to reproduce data[24]. As therapeutics, aptamers generally exhibit less of an immune response than do proteins, and their small size (<30 kDa versus ~150 kDa for a full-size antibody) increases their chances of availability to biological areas that are inaccessible to antibodies[25,26]. In addition, their ease of chemical modification allows extended control over their renal clearance and half-life[27]. Therapeutic aptamers can also be rapidly deactivated with antisense oligonucleotides (referred to as antidotes) that have been designed to base pair with the binding domain of the folded structure[27,28]. Aptamers may unfold during prolonged storage at ambient temperature; however, this does not affect their function, as aptamers can refold into a functional state using a simple annealing procedure of heating and cooling in an appropriate buffer. Overcoming the cold-chain problem will lower the cost of shipping aptamers relative to antibodies. The ability to engineer aptamers into ligand-responsive devices that activate only in the presence of a desired analyte has led to a surge of research activity aimed at designing sensors and other genetically controlled elements for diagnostic applications[29]. Finally, because aptamers are oligonucleotides, they can be used as reagents that can be seamlessly integrated with other technologies involving nucleic acid-based systems, such as amplification systems, DNA nanotechnology or DNA computing[30–32]. Additional comparisons between aptamers and antibodies may be found in previous reviews[17,25].

In this Review, we examine the changing trends in aptamer technology by analysing the first quarter century of aptamer data that is available in the scientific literature (1990–2015). In particular, we highlight recent examples that showcase the use of aptamers in key applications, discuss challenges that have impeded the success of aptamers in practical applications, provide suggestions for choosing chemical modifications that can lead to enhanced function or stability, and propose recommendations for aptamer characterization. Certain specialized topics are beyond the scope of this Review; accordingly, we direct readers interested in learning more about aptamers to several excellent reviews that cover a wide range of topics from chemical biology to therapeutics[27,33–36]. It is our hope that this Review will stimulate chemists to think about new ways in which aptamers can be used to push the boundaries of science beyond what is currently known or to solve major problems that affect human health and the environment.

Box 1 | **Introduction to aptamers**

Aptamers are single-stranded nucleic acid molecules that can fold into discrete 3D structures with ligand-binding sites that are complementary in shape and charge to a desired target. Aptamers are generated by a process known as *in vitro* selection or SELEX (systematic evolution of ligands by exponential enrichment), which is a Darwinian evolution process in which iterative rounds of selection and amplification are used to enrich for sequences that can perform a desired function, such as target binding or catalysis. A schematic representation of a typical SELEX cycle used to generate aptamers with affinity for an arbitrary target is shown in part **a** of the figure. To apply the principles of Darwinian evolution, a large population (library) of nucleic acid sequences (typically up to 10^{15} unique molecules) is incubated with a target. Molecules that bind to the target are separated from the pool of non-functional sequences through the use of physical methods, such as gel electrophoresis or affinity chromatography. Functional sequences are recovered and amplified to create a new population of molecules that is now enriched in sequences that can perform the chosen function. The process of selection and amplification is repeated until the pool is dominated by sequences that exhibit high affinity to the desired target. Aptamers isolated by SELEX are analysed for possible secondary structural motifs using computational programs that identify regions of the sequence that are capable of Watson–Crick base-pairing. Aptamers can form several types of secondary structure; one example is shown in part **b** — the predicted secondary structure of a DNA aptamer binding to human α-thrombin. The structure of an α-thrombin–aptamer complex (shown in part **c**), which includes the protein and the complementary aptamer, was solved by solution NMR (Protein Data Bank identifier: 3DD2)[129]. The NMR structure shows that nucleic acid sequences can fold into compact tertiary structures with discrete ligand-binding sites. Residues at the protein–aptamer interface (highlighted in orange, part **d**) form strong intermolecular interactions that enable the aptamer to recognize a particular site on the protein surface. These interactions are similar to the types of interactions found at the interface of a protein–protein quaternary structure. Images in part **c** and **d** courtesy of W. Van Horn, Arizona State University, USA.



The primary literature (1990–2015)

The number of citations per year is a common metric that is used to provide evidence for growth in a field of science. However, this measure of scientific productivity uses databases that can inflate the scientific literature by including duplicate citations, non-peer-reviewed articles and commentaries. Instead, a more accurate reflection of scientific productivity would be the number of non-redundant peer-reviewed publications generated per year, which can be compared with other scientific disciplines as well as the normal growth pattern of the scientific literature. For example, in the case of aptamers, interrogating Web of Science, Google Scholar and PubMed for the term 'nucleic acid aptamer' with the year range set to 1990–2015 returned >45,000 entries (FIG. 1a), most of which are duplicates or non-primary literature (defined here as original peer-reviewed publications, excluding commentaries and books). The total number of publications is reduced by one order of magnitude if only the non-redundant publications are considered.

We have downloaded and catalogued the 4,795 non-redundant articles into a manually curated database

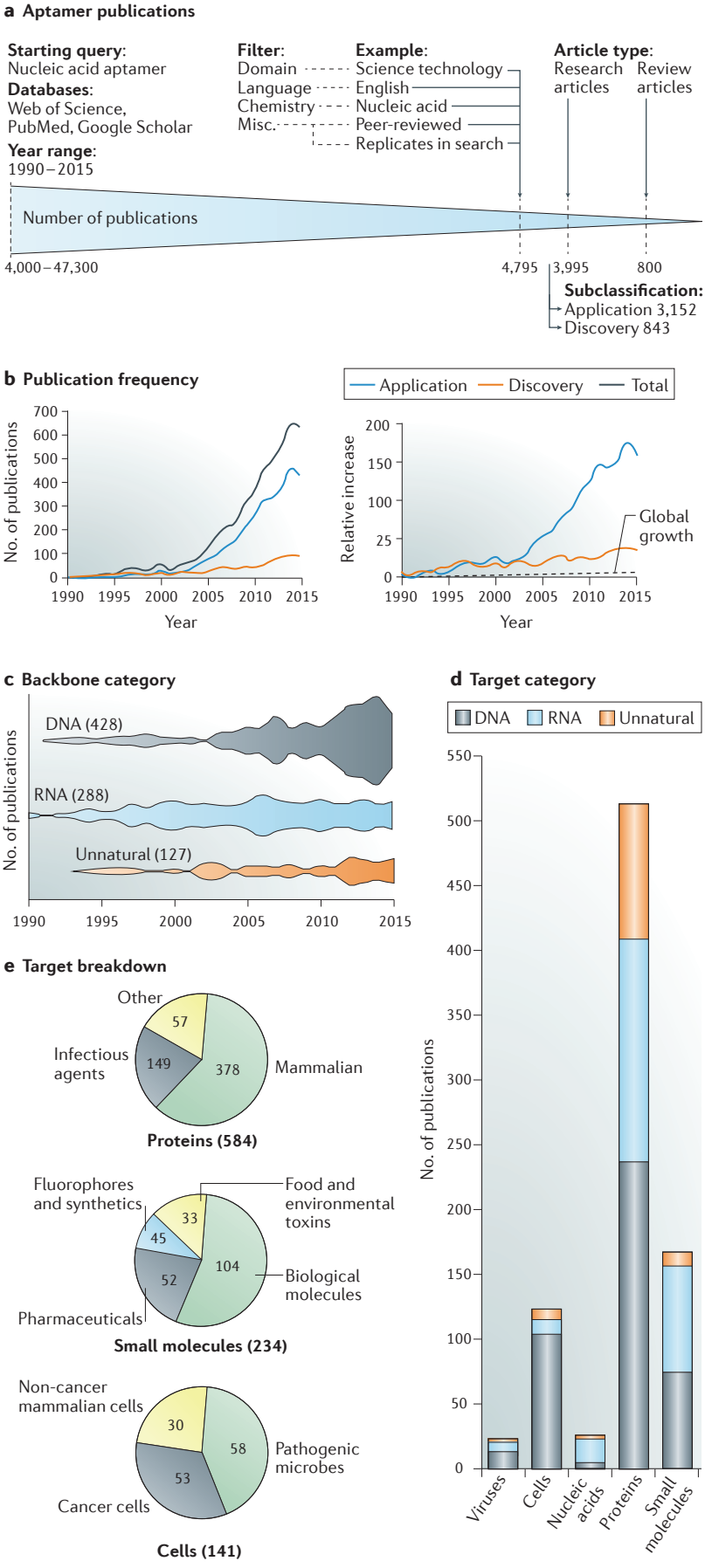


Figure 1 | **Trends in aptamer publications.** **a** | Non-redundant articles published in the aptamer field between 1990 and 2015. **b** | The annual frequency of aptamer articles is shown on the left. The growth of the aptamer literature relative to the scientific literature is shown on the right. The number of the aptamer articles has been normalized taking into account the total number of annual scientific publications. These data were obtained from the PubMed database, which had the least number of duplicate and non-primary literature hits for searches performed using the term 'nucleic acid aptamer' (7,114 hits compared with 4,795 observed publications; Supplementary information S1 (table)). **c** | Distribution of aptamer publications per year organized by backbone chain. **d** | Bar chart showing the distribution of aptamer targets and backbone structures reported in the set of 843 aptamer articles discussing systematic evolution of ligands by exponential enrichment (SELEX). **e** | Breakdown of the top three target categories based on a total of 1,003 independent aptamer selections. The difference between aptamer publications and selections (843 versus 1,003) is due to a small number of cases in which multiple SELEX experiments are described in the same article. Misc., miscellaneous.

(FIG. 1a), and a summary of the information obtained from the database along with a complete list of references is provided in the Supplementary information S1–S21 (tables). Of the non-redundant articles, 843 are research articles reporting the discovery of new aptamers (discovery articles), 3,152 are research articles describing the application of aptamers (application articles) and 800 are review articles (FIG. 1a). A plot of the number of articles published per year (FIG. 1b) reveals an exponential growth pattern that began in 2005 and continued through to 2015. When normalized against the number of publications recorded annually in the PubMed database (chosen because of its low redundancy), it becomes clear that the number of aptamer publications is increasing at a faster rate than the normal growth pattern observed for the scientific literature.

Trends in aptamer discovery

The set of aptamer discovery articles was queried for specific information about backbone chemistry, target selection, characterization and downstream application. To simplify our analysis, the selection process for one target was counted as a single entry. Articles describing selections for multiple targets were given one entry per selection. Missing information was listed as unreported.

Backbone chains. From the 843 discovery articles, we identified 1,003 *in vitro* selection experiments performed against 705 unique targets that ranged in complexity from small molecules to whole cells. It should be noted that these values do not include aptamers that were generated at private companies. Although the dominant backbone chain used for aptamer discovery is DNA-based (>50%) — chosen presumably for its increased chemical and biological stability relative to RNA, commercial availability and ease of handling — the past few years have witnessed a rise in unnatural nucleic acids as polymers for the development of new aptamers (FIG. 1c,d).

The category of unnatural polymers consists primarily of nucleic acids with chemically modified nucleobase and sugar moieties, which have been synthesized with the aim of enhancing the function or nuclease stability of aptamers for clinical applications. This important area of aptamer development is addressed in later sections.

Target distribution. Our analysis of 1,003 *in vitro* experiments reveals new insights into the targets chosen for aptamer selections. Proteins constitute by far the largest target category with 584 entries, followed by small molecules and cells with 234 and 141 entries, respectively (FIG. 1d,e). In addition, a few aptamer species that have been generated against viruses and nucleic acid molecules were also identified (22 entries each). The top five targets chosen for aptamer development are human α-thrombin, streptavidin, vascular endothelial growth factor (VEGF), influenza haemagglutinin and adenosine-5′-triphosphate (ATP).

Although the inherent properties of an aptamer can be influenced by the selection process and backbone chemistry, it is not always possible to determine whether an aptamer can be generated to bind to a given target[10]. Consequently, studies that focus on methodology developments tend to favour targets that have been used successfully and that are considered to be highly aptagenic. Common examples of these targets include human α-thrombin, VEGF and influenza haemagglutinin, which have clear implications for aptamer therapeutics[25], whereas ATP and streptavidin benefit from selection protocols that are straightforward to implement and known to produce aptamers after just a few cycles of selective amplification[37,38].

Characterization. One of the most important properties of an aptamer is how well it binds to its designated target and distinguishes it from others that may be present in a biological mixture. The key parameters to consider are the binding affinity and specificity. The binding affinity is typically reported as a solution equilibrium dissociation constant (K_d), with a low value of K_d corresponding to a high binding affinity. The specificity is quantitatively measured as the ratio of K_d for the cognate target versus K_d for a non-cognate target. Specificity is often reported for just a few off-target proteins that either have broad affinities for nucleic acids in general or represent homologues of the target protein. It would be impractical to measure the affinity constants for many off-target molecules. In general, K_d values for most protein aptamers are in the low- to sub-nanomolar regime, whereas K_d values for small-molecule aptamers are in the low- to sub-micromolar regime.

Our analysis has led to several surprising revelations regarding the level of aptamer characterization in published scientific articles. We find that ~20% of the analysed articles do not include a binding affinity value of any kind and <25% report a specificity test. Among the articles that do report a specificity test, only ~10% use a complex biological mixture, such as human serum or total *Escherichia coli* lysate, to evaluate off-target binding. We also find that ~10% of the articles reporting aptamer discovery and >75% of those reporting aptamer application do not disclose the sequence of the aptamer discovered or used in the study. Furthermore, of the aptamer discovery articles that do report a sequence, ~20% report only a single aptamer sequence, which is startling as selection processes generally produce many aptamer sequences. As sequence information is essential for reproducing previously published data, this information along with the binding conditions should be considered minimum requirements for publication (see below for more on suggested standards). Despite an unexpected number of poorly characterized aptamers in the scientific literature, we have identified several excellent publications in which *in vitro*-selected aptamers were characterized in great detail. One straightforward example comes from Szostak and co-workers on the isolation of an ATP-binding RNA aptamer in which the natural uridine residues found in RNA were replaced with the unnatural residue 5-(3-aminopropyl)uridine (UNH_2)[39]. UNH_2 expands the chemical diversity of RNA by offering a primary amine that exists in the ammonium form (pK_a ~9.5) at physiological pH, enabling favourable electrostatic interactions with the negatively charged phosphates on the ATP ligand. The UNH_2 residue satisfies all of the conditions for *in vitro* selection, namely: the modified nucleotide triphosphate serves as a substrate for RNA polymerase, and reverse transcriptase is able to copy UNH_2-modified RNA back into complementary DNA (cDNA) for amplification by PCR.

After ten cycles of selection and amplification, RNA molecules that remained in the pool were cloned and sequenced[39]. Members of representative families were screened for ATP binding to identify clones that were dependent on the presence of the amino modification for target binding. One clone, 10N23, showed 40-fold higher binding to ATP when transcribed with UNH_2 compared with uridine triphosphate (UTP), and was 5′ and 3′ truncated by end-mapping deletion analysis to identify the boundaries of the core binding domain. Secondary-structure analysis indicated a certain stem–loop with a bulge to be a region possibly responsible for this high affinity. To identify the nucleotides that were crucial for binding, a doped library was constructed such that a portion of the sequence was partially randomized and carried through additional rounds of *in vitro* selection. UNH_2 residues essential for binding were identified by individually replacing each UNH_2 residue with cytidine and screening for binding to ATP. Finally, affinity and specificity values were obtained by measuring the K_d of the core binding domain for ATP and a panel of ATP analogues. This study demonstrates many of the key features of a well-characterized aptamer, which includes sequence information, mutational analysis, secondary-structure prediction, as well as qualitative and quantitative binding assays under well-defined binding conditions.

Post-SELEX modifications. Several strategies have been developed to improve the function of *in vitro*-selected aptamers. This approach, which is generally referred to as post-SELEX optimization, involves modifying *in vitro*-selected aptamers with functional groups that

Aptagenic
A target that is known to produce an aptamer by *in vitro* selection.

Dissociation constant
The equilibrium constant for the dissociation of the target–aptamer complex (K_d = [target][aptamer]/[target–aptamer]).

Lysate
The contents of a cell produced by cell lysis.

cDNA
The complementary DNA sequence that results when RNA (or xeno-nucleic acid (XNA)) is reverse transcribed into DNA.

Doped library
A nucleic acid library that contains variants of a single sequence, which feature a small fraction of non-wild type residues at each position. For example, if the wild-type residue is A, then the library would contain mostly A with a mixture of C with T and G.

Recombinant protein therapies
Therapies that are produced through recombinant DNA technology, which involves expressing and purifying a protein from bacterial or mammalian cells. Many biologics, such as monoclonal antibodies, are recombinant protein therapies.

Phosphodiester linkage
The chemical linkage, connecting the monomeric units in a nucleic acid polymer. In general, the linkage takes the form RO(O)P(O$^-$)OR′.

Induced-fit
A biochemistry model in which the initial interactions of an enzyme–substrate complex (or antibody–target complex) are strengthened by conformational changes that increase the strength of the intermolecular interactions involved in substrate or target recognition.

were not present in the original selection[40]. RNA aptamers developed for diagnostic and therapeutic applications, for example, are routinely modified with nuclease-resistant analogues that protect the 2′-hydroxyl groups from hydrolytic attack[41]. Pegaptanib sodium (Macugen; Pfizer/Eyetech) (FIG. 2a), an RNA aptamer selected against $VEGF_{165}$, is a prime example of a SELEX product modified for enhanced biological stability[42]. Macugen was selected from a library of 2′-fluoropyrimidines and modified post-SELEX by substituting nearly all of the purine residues with 2′-*O*-methyl analogues, inverting the 3′ terminal nucleotide and adding a 40 kDa poly(ethylene glycol) moiety to the 5′ end[43]. Together, these substitutions enabled Macugen to become an effective treatment for neovascular age-related wet macular degeneration (AMD)[42]. Although Macugen has since been replaced by more effective monoclonal antibodies[44] and recombinant protein therapies[45], its distinction as the first therapeutic aptamer to be approved by the US Food and Drug Administration (FDA) remains an important milestone in aptamer technology.

Most post-SELEX modifications are designed to enhance the biological stability of *in vitro*-selected aptamers[40], as chemical modifications that lead to enhanced functional activity are harder to predict. Yang and co-workers have challenged this trend by demonstrating that a single phosphorodithioate linkage (PS_2, FIG. 2b) inserted into the phosphodiester backbone can lead to a ~1,000-fold increase in the binding affinity of known RNA aptamers[46]. Similar enhancements in binding affinity have been achieved by bivalent affinity reagents, which combine two ligands that can independently recognize different sites on a protein surface[47].

In contrast to the gains in binding affinity, small structural changes to an aptamer sequence rarely result in substantial gains in functional activity. However, in two separate examples (α-thrombin and VEGF), Yang and co-workers showed that a simple PS_2-walking experiment, in which each phosphodiester linkage along the oligonucleotide backbone was individually replaced by a PS_2 linkage, could reveal the location of the PS_2 substitution responsible for transforming a modest affinity aptamer (K_d ~1–2 nM) into an ultrahigh affinity binder (K_d ~1–2 pM)[46]. In the case of the α-thrombin aptamer, analysis of the binding interactions by X-ray crystallography (FIG. 2b) revealed that the increase in binding affinity was caused by an induced-fit rearrangement of the aptamer at the protein–RNA interface[46]. Given the simplicity of PS_2-walking experiments, this technique represents a powerful approach for improving the binding affinity of *in vitro*-selected aptamers.

Trends in aptamer applications

Aptamers have had a notable effect on the development of new chemical tools with practical applications across a wide range of scientific disciplines[48]. To help quantify these efforts, we have categorized each of the 3,152 articles that discuss aptamer applications into the five technological groups that broadly define the application areas (FIG. 3a). More than 95% of the analysed articles belong to

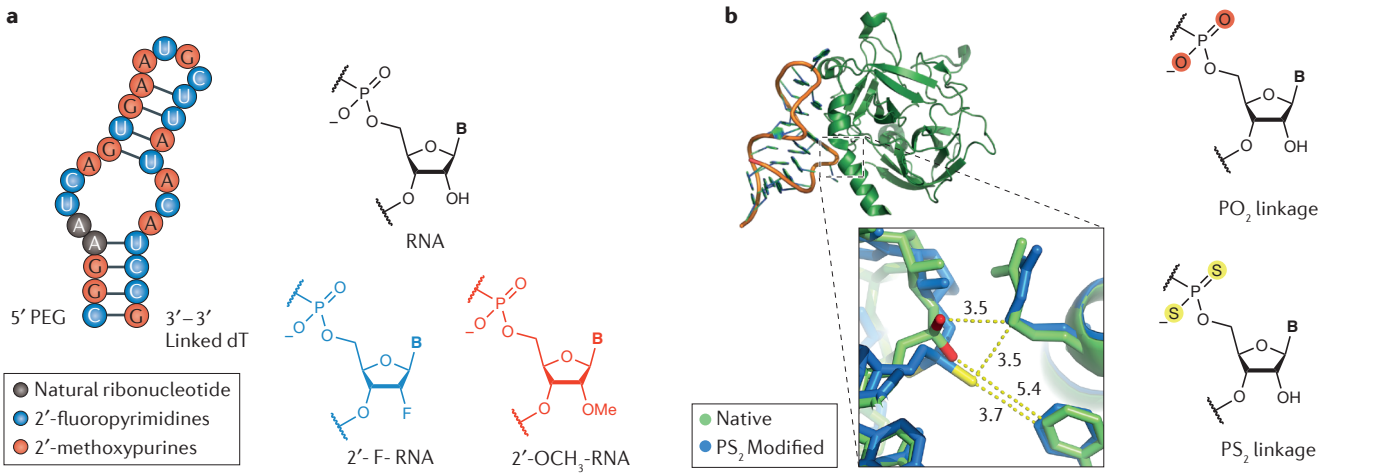


Figure 2 | **Post-SELEX modifications. a** | Macugen (pegaptanib sodium) is a therapeutic RNA aptamer, the sequence and secondary structure of which are presented on the left. The molecule has a stem–loop structure with a bulge, with the 5′ terminus bearing a PEG group and the 3′ terminus having an inverted nucleotide. These modifications, as well as the derivatization of most of the 2′-hydroxyl sites, are performed post-SELEX and confer stability towards nucleases, such that is was the first FDA-approved aptamer[42]. Macugen comprises natural ribonucleotides (shown in black on the right) as well as unnatural nucleotides, with the latter having their 2′-hydroxyls replaced with fluoro (blue) or methoxy groups (red). **b** | Comparing the X-ray structures of human α-thrombin (green ribbon) complexed to either an RNA aptamer or its PS_2-modified analogue (orange ribbon) enables the structural features that underlies the tighter affinity binding of the latter to be identified[46]. The zoomed region shows a superimposition of the native (green sticks) and PS_2-modified (blue sticks) aptamer–thrombin complexes, highlighting that the PS_2 linkage at position 18 (sulfur in yellow; Protein Data Bank identifier: 5DO4) can approach Phe232 on the thrombin surface more closely than can the native PO_2 linkage (oxygen in red; PDB ID: 3DD2); the bond lengths of the intermolecular interactions are in angstroms. The chemical structures of the PO_2 and PS_2 linkages are given on the right. dT, 2′-deoxythymidine; FDA, US Food and Drug Administration; PEG, poly(ethylene glycol); SELEX, systematic evolution of ligands by exponential enrichment.

Fluorescent reporter
Molecules that elicit a fluorescent signal.

Fluorescence resonance energy transfer
A photophysical effect in which energy is transferred between fluorescent or light-sensitive moieties.

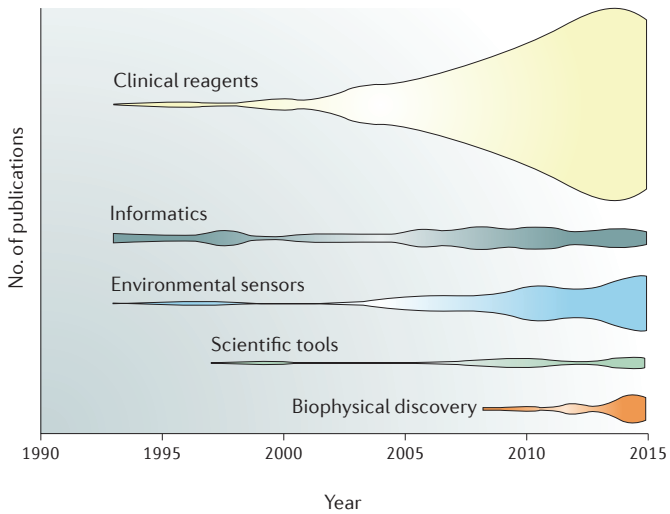
three of the five technological categories: scientific tools (comprising gene regulatory elements, nanotechnology, affinity chromatography and non-clinical sensors), clinical reagents (comprising therapeutics, diagnostics, drug delivery systems and clinical biosensors) and environmental sensors (comprising reagents for food and water analysis). The remaining publications fall into the technological groups of informatics and biophysical discovery.

Whether these areas will experience continued growth is unclear, as >50% of the articles describe the same set of well-characterized aptamer–target pairs. The most common targets for application development are α-thrombin, adenosine nucleosides and nucleotides (ATP, ADP, AMP and adenosine), VEGF, platelet-derived growth factor BB (PDGF-BB, which features two B subunits), cocaine and theophylline (FIG. 3b). In addition to historical reasons, these targets were chosen because the aptamers that were generated to bind to these targets fold into a privileged stem–loop motif that can be easily engineered into ligand-responsive elements that produce signal changes that are compatible with a wide range of detection modalities (for example, optical or electrochemical)[34]. Based on this analysis, it appears that the application field would benefit from the study of other examples of aptamer–target pairs. Furthermore, the development of robust multiplexed platforms that use combinations of aptamers to simultaneously detect many analytes would help to expand the application of aptamers in sensor-based technology.

Sensors. One area of aptamer-based research that has received substantial attention is the field of optical sensors[34]. Numerous studies have shown that fluorescent dyes attached to conformationally flexible regions of an aptamer can transduce ligand-binding events into an optical signal[34] (FIG. 4). Strategies that rely on more than one fluorescent reporter have become particularly valuable, as they enable signal transduction based on fluorescence resonance energy transfer (FRET). Stojanovic and co-workers have developed a mix-and-measure assay that responds with high sensitivity and selectivity to small-molecule targets that are chelated by *in situ* derivatizing agents[49]. Chelated targets were found to have high-affinity binding interactions with aptamers, whereas the unchelated molecules proved to be resistant to selection owing to their conformational heterogeneity (linear and cyclic forms of the sugar). The selection used a structure-switching approach that involved the release of active library members from an immobilized complementary strand upon ligand recognition[49]. Owing to the structure-switching design, aptamers isolated from the selection could be readily converted into optical sensors by adding a quencher to the 3′ end of the complementary strand and a fluorophore to the 5′ end of the aptamer (FIG. 4c,d). In this configuration, aptamers undergo conformational change in the presence of the small-molecule analyte, which produces a dose-dependent fluorescence signal as the quencher strand is released from the aptamer. The generality of this approach opens the door to highly sensitive detection systems for clinical and environmental applications. For example, the real-time monitoring of analytes in whole blood[50].

Environmental screening. The development of aptamer-based sensors for environmental screening is one of the newest and fastest growing areas of aptamer-based

a Application breakdown



b Top ten most applied targets

Target	Count
α-Thrombin	665
Adenosine nucleoside and/or nucleotide	374
Vascular endothelial growth factor (VEGF)	144
Platelet derived growth factor BB (PDGF-BB)	86
Cocaine	77
Theophylline	71
Lysozyme	65
Nucleolin	65
Immunoglobulin E (IgE)	60
Ochratoxin A (OTA)	60

Figure 3 | **Distribution of aptamer applications and targets. a** | The annual number of articles discussing possible aptamer applications is shown as a violin plot illustrating the growth across five broadly defined subcategories: biophysical discovery, which includes structural and thermodynamic analyses; clinical reagents, including therapeutics, drug conjugates, diagnostic agents and clinically tailored biosensors; informatics, which accounts for *in silico* modelling and selections, machine learning and software development; scientific tools, including chromatography, non-clinical sensors, gene regulation and nanotechnology; and environmental sensors, accounting for food and water sample analysis. **b** | Table listing the top ten targets for application development. The complete target list is provided in Supplementary information S16 (table). BB denotes that two B domains are present.

research. When normalized for sample size, this category contains the most diverse set of aptamer–target pairs with a strong emphasis on reagents that can detect pathogens, toxins, antibiotics and pesticides in food, water and soil samples[51,52]. Investment by companies such as NeoVentures Biotechnologies has resulted in commercial kits for detecting ochratoxin A and aflatoxins in food samples. Given the need to safeguard domestic food stocks and the environment, this area of aptamer-based application could grow rapidly in the coming years.

Therapeutics. To date, the FDA has approved one aptamer for the treatment of AMD, and ten aptamers have undergone clinical trials for the treatment of diseases such as AMD, coagulation disorders, cancer and inflammation[27]. All of the aptamers that have entered clinical trials thus far fall into the general category of antagonists (BOX 2) because they act by disrupting the function of a pathological target protein. For example, Macugen, the only FDA approved aptamer therapeutic, prevents VEGF from stimulating blood vessel growth and eventual vision loss by inhibiting the binding of $VEGF_{165}$ (the major pathological VEGF isoform) to its cognate receptor[42]. Another interesting example is the aptamer-based anticoagulation system, REG1 (RB006 plus RB007; Regado Biosciences), which is a drug–antidote pair that acts on coagulation factor IXa (REFS 53,54). RB006 is an aptamer that prevents blood clots by inhibiting factor IXa-mediated coagulation, and RB007 is a complementary antidote sequence that reverses the effects of RB006 in a dose-dependent manner. Together, RB006 and RB007 prevent arterial thrombosis by providing a rapid and tightly regulated system for controlling the coagulation cascade mechanism[55,56]. A third example is NOX-A12 (NOXXON Pharma), an L-RNA aptamer that prevents tumour proliferation by inhibiting CXC chemokine ligand 12 (CXCL12)[57,58]. L-RNA and L-DNA aptamers represent a general class of aptamers known as spiegelmers, which are mirror-image aptamers that are not recognized by nucleases present in human blood and serum[59], as nucleases are specific for D-sugar moieties. Spiegelmers are created using the principle of mirror-image symmetry, in which natural D-RNA aptamers are generated against the mirror image of a desired target and then, after the selection is complete, the resulting RNA sequences are chemically synthesized with L-RNA phosphoramidites to produce the desired L-RNA sequence (FIG. 5), which recognizes the natural target[60]. In clinical trials, NOX-A12 showed favourable activity against a range of solid tumours and multiple myeloma. Joyce and colleagues have shown that selected L-RNA aptamers can also be used to inhibit the processing of specific microRNAs[61]. As L-RNA cannot base pair with D-RNA, these aptamers function through highly specific tertiary structures rather than Watson–Crick base-pairing.

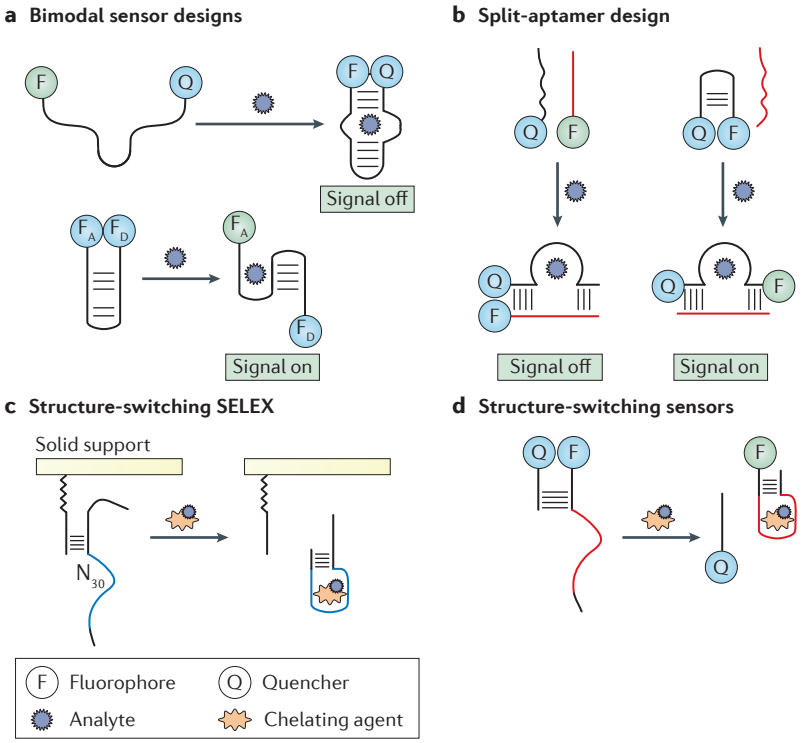
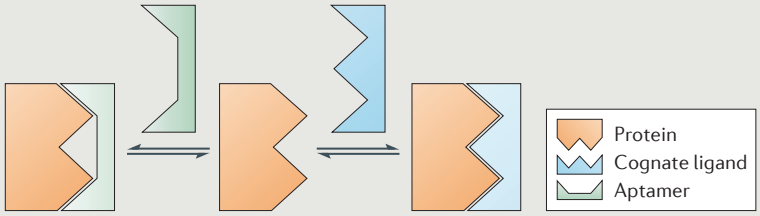


Figure 4 | **Aptamer-based optical sensors. a** | In the bimodal design, unimolecular aptamer sensors either emit or quench a fluorescent signal based on a conformational change induced by an analyte. Binding to a target may result in a fluorophore and quencher coming into proximity, or may involve a fluorescent donor–acceptor pair (F_D and F_A) separating to produce a fluorescent signal[34]. **b** | In the split-aptamer design, aptamer sequences are separated into two strands that come together in the presence of an analyte to either emit or quench a fluorescent signal. **c** | Structure-switching SELEX promotes the discovery of aptamers that can be used as optical sensors with minimal sequence engineering. In this example, functional aptamers are released from a solid support when they bind to a desired analyte, which, in turn, is bound to a chelating agent. The N_{30} random region is shown in blue. **d** | Aptamers discovered through structure-switching SELEX can be converted into sensors by replacing the solid support at the end of the DNA capture probe with a quencher and adding a fluorescent dye to the end of the aptamer strand[49]. SELEX, systematic evolution of ligands by exponential enrichment. Parts **c** and **d** are adapted with permission from REF. 49, Macmillan Publishers Limited.

Drug delivery. In addition to serving as drugs, aptamers have been developed to selectively deliver therapeutic agents to the surface or cytoplasm of human cells[62]. In this case, aptamers function as targeted drug delivery vehicles that can be used to increase the efficacy of a drug and reduce the side effects of traditional non-targeted approaches such as chemotherapy and radiotherapy, which are commonly used to treat patients with cancer. Human prostate-specific membrane antigen (PSMA), a transmembrane protein associated with prostate cancer that is overexpressed on the surface of solid tumours and constitutively internalized into the cell, was the first model system developed for aptamer-based drug delivery[63]. In 2002, Coffey and co-workers showed that aptamers selected to bind to the extracellular domain of PSMA could be internalized by a clathrin-dependent pathway[64]. In numerous subsequent studies, PSMA-specific aptamers have been used to deliver small-molecule therapeutics and small interfering RNAs (siRNAs) that are covalently or non-covalently bound to the aptamer[27]. The efficacy of this approach has improved, and, for example, we now have access to a PSMA-specific aptamer drug conjugate that facilitates tumour regression following systemic administration[65]. This general concept of aptamer-based drug delivery has been extended to

Box 2 | **Therapeutic aptamers function as antagonists**

Aptamers developed for therapeutic purposes function as antagonists of the binding interaction of a protein with its cognate ligand. Although non-competitive interactions are possible, most aptamers function by directly competing with the natural ligand for the ligand-binding site on the protein surface.



nanoparticles; in this approach, nanoparticles encapsulating therapeutic agents are coated with aptamer recognition elements for aptamer-mediated internalization and drug release[66].

Another important application of aptamer-based drug delivery involves developing strategies to fight HIV. In patients with HIV, viral entry into immune cells occurs when envelope glycoprotein gp120 — a glycoprotein located on the exposed surface of the viral particle — interacts with the CD4 receptor and CC-chemokine receptor 5 (CCR5), both of which are located outside the host cell[67]. As this interaction is crucial for viral invasion, individuals that carry certain CCR5 mutations can be immune to some HIV strains[68]. However, these mutations are rare; therefore, drugs that interfere with the binding process could provide a possible route for therapeutic intervention. Pioneering work by Rossi and co-workers has led to the development of gp120 aptamers that function with dual activity: first, to inhibit the interaction between gp120 and the CD4 receptor and, second, as a cell-type specific delivery agent for siRNA[69,70]. The gp120 aptamer was reported to deliver anti-HIV siRNA into HIV infected cells and inhibit HIV activity *in vitro*. Subsequent work performed in a humanized mouse model showed that systemic administration of gp120 aptamer–drug conjugates suppressed HIV replication by several orders of magnitude[71], indicating that aptamer–drug conjugates offer a promising treatment for HIV infection.

Allosteric modulation. The affinity and selectivity with which SELEX-derived aptamers bind to a given target are governed by the stringency of the selection process[10]. Lefkowitz and colleagues have developed an iterative approach combining *in vitro* selection with next-generation sequencing to discover RNA aptamers that stabilize several functionally distinct conformations of a model G-protein-coupled receptor[72] (GPCR, FIG. 6a). A highly diverse library of 2′-fluoropyrimidine-modified oligonucleotides afforded a series of RNA aptamers that stabilize the active, inactive and ligand-specific receptor conformations of β_2-adrenoceptor (β_2AR), a protein that is a well-characterized member of the GPCR family[73]. Of particular utility in this study were comparative bioinformatic analyses, which helped in the identification of target-specific aptamers according to the fold enrichment of individual clones. This analysis provides a useful method for distinguishing target-specific aptamers from highly abundant members that function with non-specific activity[72]. Selectivity of the aptamers for specific β_2AR conformations was demonstrated using biochemical, pharmacological and biophysical approaches. The aptamers A1, A2 and A13 exhibited strong conformational selectivity for the high-affinity agonist (BI167107)-bound active β_2AR conformation, whereas aptamer A16 showed conformational selectivity for the inactive β_2AR conformation of the inverse-agonist ICI-118,551. The discovery that aptamers can serve as allosteric GPCR modulators adds to the diversity of ligands available to study the structural and functional regulation of GPCRs and represents a basis for the design of GPCR ligands with improved safety and enhanced therapeutic efficacy.

Natural product synthesis. Natural product synthesis is an important aspect of drug discovery because many FDA approved drugs are analogues of small-molecule natural products[74]. In pursuit of new drugs, chemists have taken 'top-down' approaches by modifying natural products isolated from nature as well as 'bottom-up' approaches that require the total synthesis of a desired compound from simple commercially available precursors. Regardless of the approach taken, functional group selectivity invariably becomes a problem, as many natural products are structurally complex. Herrmann and co-workers have used aptamers as protecting groups to facilitate the highly chemo- and regioselective derivatization (>99%) of natural antibiotics in a single, high yielding (83%) synthetic step[75]. This technique relies on the aptamers to fold into well-defined shapes with discrete ligand-binding sites that recognize and shield certain regions of a molecule from chemical derivatization. The possibility of using aptamers to distinguish equivalent functional groups provides a simple and cost-effective strategy for selectively modifying complex natural products.

Barriers to commercial success

The promise of rapid and low-cost production of reagents that are compatible with any target imaginable has led researchers to embrace aptamers as a chemical alternative to antibodies. Once selected, these molecules provide a renewable source of affinity reagents based on genetically encoded sequences that exhibit high ligand-binding affinity and low batch-to-batch variability. However, like most new technologies, aptamers took years to develop and followed a Gartner hype cycle, which is defined by a 'peak of inflated expectations', followed by a 'trough of disillusionment', a subsequent 'slope of enlightenment' and, finally, a 'plateau of productivity'. After nearly three decades of intense research, aptamers now seem ready to enter the long-awaited period of strong productivity. The past few years alone have witnessed tremendous growth, with new technologies overcoming many of the problems that have limited the use of aptamers in practical applications.

Mirror-image symmetry
Symmetry with respect to a reflection or plane of symmetry. Mirror-image molecules are non-superimposable. For example, your right hand cannot be superimposed on top of your left hand.

von Willebrand factor
A glycoprotein found in the blood that is involved in haemostasis (blood clotting).

Click chemistry
A general class of high-yielding cycloaddition reactions that occur between azide and alkyne functional groups. Click chemistry is often used to add new functional groups to biomolecules.

Chemical diversity. The limited chemical functionality of natural genetic polymers (DNA and RNA) generally explains the dominance of proteins over nucleic acids as scaffolds for biological receptors and catalysts[10]. For example, when compared with antibodies, aptamers have fewer monomer units that can be used to mediate target recognition events (4 bases versus 20 amino acids). This weakness is compensated for by aptamer libraries being very large ($\geq 10^{15}$ unique sequences), with each molecule being a water-soluble species with the potential to fold into a tertiary structure with a well-defined ligand-binding site. Despite this, many targets have proved to be resistant to aptamer selections. The synthesis of nucleic acid libraries that carry a wider range of chemical groups seems to be a solution to this problem.

One of the first approaches to increasing the functional diversity of nucleic acid libraries involves expanding the genetic alphabet to include additional bases that achieve Watson–Crick base-pairing through alternative arrangements of hydrogen-bond donor and acceptor groups. Benner and colleagues, for example, developed an artificially expanded genetic information system, termed AEGIS, that was used to isolate aptamers with low nanomolar affinity for a human adenocarcinoma breast cell line (MDA-MB-231)[76]. In addition to the standard nucleobases of A, C, G and T, entries in the AEGIS library also contain the nonstandard nucleotides Z (6-amino-5-nitro-2(1*H*)-pyridone) and P (2-amino-imidazo[1,2-*a*]-1,3,5-triazin-4(8*H*)one), which were found to be essential for aptamer binding (FIG. 6b). Although the information capacity of the AEGIS library is higher than a standard nucleic acid library, the functional similarity of AEGIS bases to natural bases suggests that the AEGIS system explores a relatively small region of chemical space.

In an effort to search larger regions of chemical space, several groups have developed new strategies for introducing functional groups at specific positions in the sequence. In this regard, Hirao and co-workers have constructed a library in which an unnatural hydrophobic base was positioned at several predefined locations in the random region of the DNA library[77] (FIG. 7a). The genetically expanded library was then used to isolate aptamers with affinity for the human proteins $VEGF_{165}$ and interferon-γ. Despite the requirement for the modified base to occupy predefined positions, the resulting aptamers exhibit substantially stronger (>100-fold increase) binding relative to known aptamers isolated from natural libraries[77]. The improved binding affinity was attributed to the increased hydrophobic character of the unnatural base. This approach was extended using genetically expanded bases that were not constrained to specific library positions to create high-affinity aptamers against von Willebrand factor[78]. As expected, the chemically modified aptamers showed higher affinity than previous von Willebrand factor aptamers isolated from unmodified DNA libraries.

Mayer and co-workers established a conceptually simpler approach, termed click-SELEX, in which functional groups are added to specific nucleotides in a DNA library after the library has been enzymatically synthesized[79].

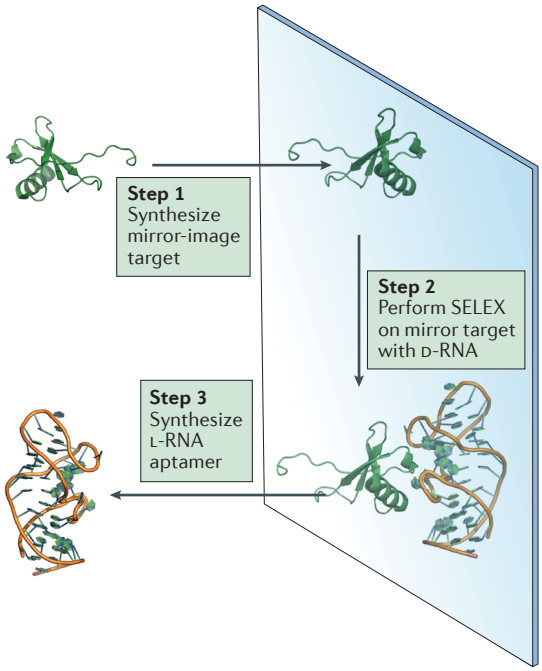


Figure 5 | **L-RNA aptamers or spiegelmers.** A cartoon showing the general strategy used to isolate L-RNA aptamers (spiegelmers) by *in vitro* selection[59]. In step 1, the mirror image of the desired target is chemically synthesized. In the case of proteins, targets are generated by solid-phase synthesis using unnatural D-amino acids. For small molecules, the target is generated by chemical synthesis. In step 2, standard *in vitro* selection is performed using a natural RNA (or DNA) library to isolate aptamers that recognize the mirror-image version of the desired target. In step 3, L-RNA (or DNA) aptamers are generated by solid-phase synthesis using L-phosphoramidites to construct the mirror-image version of a sequence isolated from the selection. Based on the rules of mirror-image symmetry, the unnatural L-RNA aptamer will bind the natural L-protein with an affinity constant that is identical to the binding interaction observed between the natural D-aptamer and unnatural D-protein. SELEX, systematic evolution of ligands by exponential enrichment. Adapted with permission from from REF. 59, Elsevier.

For each round of selection, the alkyne-modified DNA pool, prepared by substituting thymidine triphosphate (TTP) with C5-ethynyl-2′-deoxyuridine (EdU), is further modified with an azide-containing compound using a copper-mediated alkyne–azide cycloaddition reaction (FIG. 7c), commonly referred to as click chemistry. Following several rounds of selection and amplification, the click-SELEX strategy generated an indole-modified aptamer with low-nanomolar affinity for the green fluorescent protein (GFP) — a target known to have low aptagenic activity[80]. GFP binding activity was shown to be dependent on the presence of the indole modification, demonstrating that in addition to increased binding affinity, chemical modifications can also improve the overall likelihood of isolating aptamers for difficult targets[79]. The compatibility of this approach with other functional groups provides a powerful method for exploring new regions of chemical space.

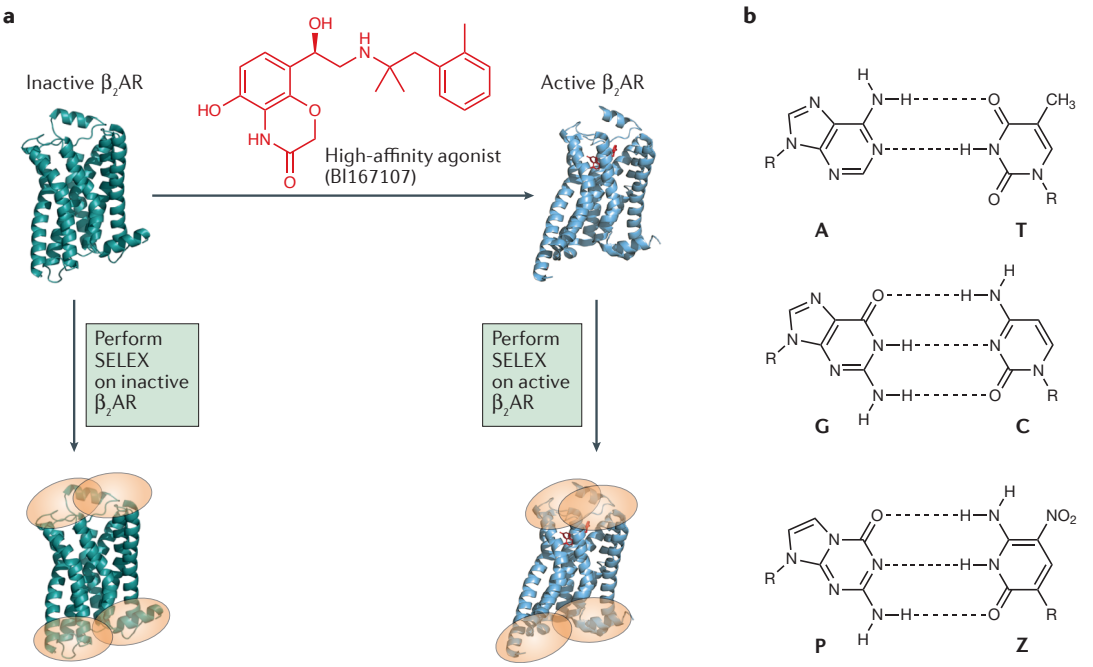


Figure 6 | **Examples of aptamer applications and chemical modifications. a** | The G-protein-coupled receptor β_2-adrenoceptor (β_2AR) exists in inactive (shown on the left; Protein Data Bank identifier: 2RH1) and active conformations (shown on the right; PDB ID: 3SN6)[72]. The inactive form is converted to the active form on treatment with the agonist BI167107 (shown in red). The two forms can be separately subjected to SELEX, and in this case the inactive conformation is stabilized by three different aptamers, denoted A1, A2 and A13, whereas the active conformation was stabilized by another aptamer, A16. The possible sites at which the aptamers bind to β_2AR are indicated by orange ovals. This stabilization enabled structural analysis of this model membrane receptor by cryo-electron microscopy. **b** | Artificially expanded genetic information systems (AEGIS). Based on the premise of complementary hydrogen bonding, new Watson–Crick base pairs, such as P•Z, can be generated by creating new combinations of hydrogen-bond donor and acceptor groups that recognize each other through new arrangements of hydrogen-bond complementarity[76]. R = 1′-(5′-phosphatylribose); SELEX, systematic evolution of ligands by exponential enrichment. Adapted with permission from REF. 72, Macmillan Publishers Limited.

The use of slow off-rate modified aptamers (SOMAmers), developed by SomaLogic, provides access to aptamers with still greater chemical diversity[81]. In the original strategy, diversity-enhancing functional groups were added to the 5 position of deoxyuridine residues, which were subsequently converted to nucleotide triphosphates[82] (FIG. 7b). Polymerase-mediated synthesis produces a combinatorial library of DNA molecules in which each thymidine residue is uniformly replaced by a specific type of functional group. Among the types of chemical moieties tested, functional groups that resemble the side chains of hydrophobic amino acid residues have proved to be successful against a wide range of protein targets[82,83]. Results from several protein–SOMAmer cocrystal structures indicate that these functional groups have a role in target binding and aptamer folding[84]. This strategy has substantially improved the percentage of effective *in vitro*-selected aptamers, as exemplified by the generation of aptamer–target pairs that were previously difficult to observe. The technology platform of SomaLogic was expanded by the inclusion of DNA libraries that carry two types of modified bases[85]. Shorter random-sequence libraries of doubly modified aptamers have higher binding affinity for a target than traditional libraries (30-mer versus 40-mer random regions)[85]. According to SomaLogic, SOMAmers have been generated against >3,000 proteins and these reagents are currently used as a point-of-care technology for monitoring protein levels in human serum[86].

Methods for evolving DNA aptamers with modified bases are currently limited to one or two types of chemical modifications per library. Liu and co-workers overcame this problem using a ligase-mediated translation system that makes it possible to synthesize nucleic acid polymers that carry a rich assortment of different functional groups in the same library[87]. In this approach, T4 DNA ligase is used to mediate the DNA-templated polymerization of short 5′-phosphorylated oligonucleotides containing a wide variety of functional groups (FIG. 7d). The resulting library of single-stranded nucleic acid polymers is a suitable template for primer extension using an archaean thermophilic B-family polymerase, which enables regeneration of the starting template by copying functionally rich sequences back into their encoding cDNA[87]. Hili and co-workers applied the same approach to synthesize DNA libraries that were modified with peptides and a wide range of small molecules with diverse functionality[88,89]. Despite the fact that this methodology has not been used to generate an affinity reagent by *in vitro* selection, the high level of

Primer extension
A DNA (or xeno-nucleic acid (XNA)) replication step in which a nucleic acid primer is extended by annealing the primer to a template and copying the template with a polymerase.

technical optimization performed thus far suggests that sequence-defined polymers generated by template-directed ligation offer a rich source of chemical diversity for generating high-quality aptamer-like reagents.

Specificity. The long-held adage of 'you get what you select for' has been one of the great truisms of the aptamer world[90]. Consequently, after nearly three decades of *in vitro* selection, it should come as no surprise that aptamers selected to function in one environment may not function well in other environments. This observation is not unique to aptamers; for example, antibodies generated against whole proteins tend to function better in pull-down assays, whereas those produced against short peptide epitopes are better suited for western blots[91,92]. The obvious solution to this problem is to develop selection strategies that more closely resemble the desired downstream applications. However, isolating aptamers that exhibit both high affinity and high specificity can be difficult to achieve using conventional selection methods, as these approaches either enrich for affinity (positive selection) or enrich for specificity (negative selection), but rarely do both simultaneously.

Efforts to overcome this problem have led to innovative strategies for generating aptamers that retain high target binding affinity in the presence of complex biological environments. One approach involves the *in vivo* selection of nuclease-resistant aptamers in a live animal model developed to target a specific disease or condition. Sullenger and co-workers demonstrated that tumour-specific aptamers could be generated by administering a 2′-fluoropyrimidine RNA library intravenously to the animal, harvesting the organ of pathological interest and extracting the library members that bound the organ[93]. This technique of *in vivo* selection was used to isolate nuclease-resistant aptamers for an animal model of colorectal cancer metastasis. More recently, the *in vivo* selection approach has been used to generate aptamers that can penetrate the parenchyma tissue of the brain in a wild-type mouse model[94]. A second approach for generating aptamers with high specificity involves exploiting the capacity of fluorescence-activated cell sorting (FACS) to simultaneously screen for affinity and specificity. Using a strategy called multiparameter particle display (MPPD), Soh and co-workers developed several examples of DNA aptamers that function with low- to subnanomolar affinity in human serum[95]. This level of binding activity was achieved using droplet PCR to create 'monoclonal' aptamer particles, a process whereby streptavidin-coated beads are decorated with many copies of the same aptamer sequence[96]. The aptamer particles are then incubated with a complex mixture comprising the desired target labelled with AlexaFluor 488 (a green dye) and competing serum proteins labelled with Alexa Fluor 647 (a red dye). Following incubation, FACS is used to isolate aptamers that exhibit both high target binding affinity and specificity, based on a dual-colour gating parameter. The resulting DNA aptamers were shown to outperform monoclonal antibodies in a standard enzyme-linked immunosorbent assay (ELISA)[95]. Although the MPPD strategy is limited to nucleic acid modifications that can be amplified using PCR, the ability to isolate aptamers with high affinity and specificity outside an animal model represents an important advancement in the development of high-quality aptamers.

Stability. *In vitro* selection has produced several aptamers that can bind to a wide range of targets, from small molecules to whole cells[10]. However, aptamers composed

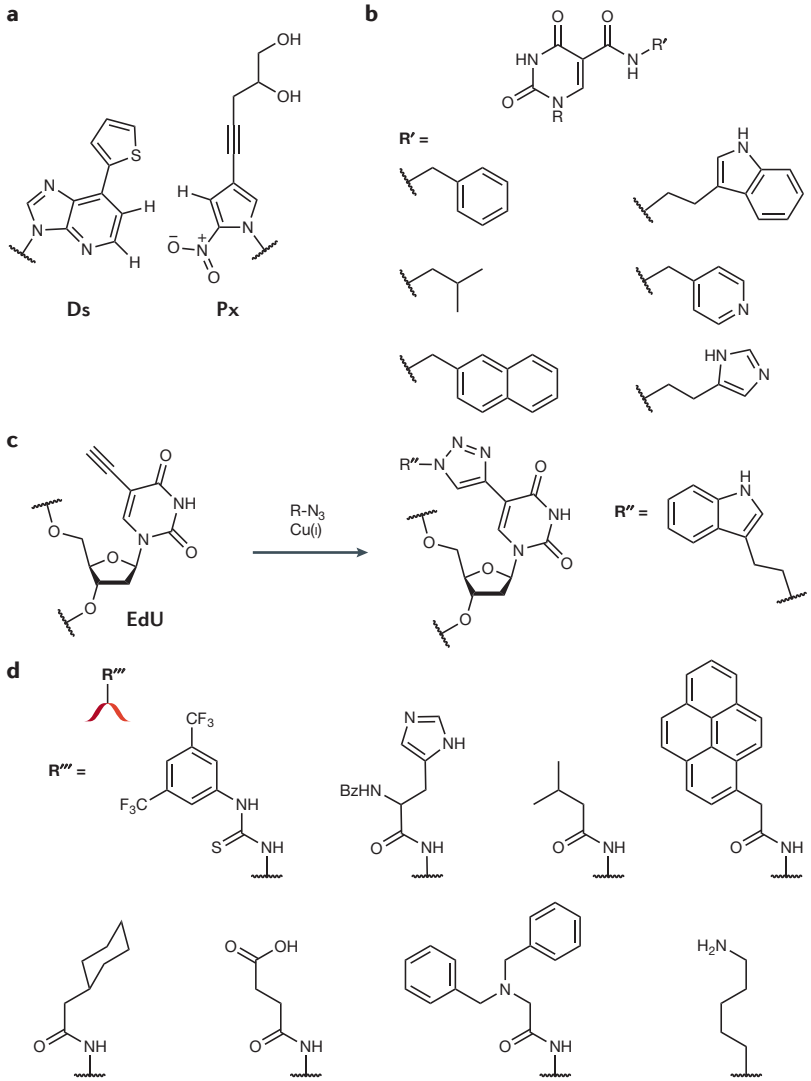


Figure 7 | **Chemical modifications used in aptamer selections.** Numerous types of chemical modifications have been developed to increase the functional diversity of DNA aptamers. **a** | One example includes expanding the genetic alphabet with hydrophobic base analogues that recognize one another through shape complementarity, such as the 7-(2-thienyl)imidazo[4,5-*b*]pyridine (Ds) and diol-modified 2-nitro-4-propynylpyrrole (Px) base pair[77]. **b** | SOMAmers, or slow off-rate modified aptamers, contain hydrophobic side chains at the C5 position of pyrimidine nucleotides that can enhance the functional properties of DNA aptamers[82]. **c** | Click-SELEX uses the base analogue C5-ethynyl-2′-deoxyuridine (EdU) as a substrate for DNA polymerases and modifies the library post-synthesis using click chemistry to endow the library with enhanced chemical functionality[79]. **d** | In an effort to create functionally rich DNA molecules bearing many different side chains, template-directed ligation was developed to synthesize DNA molecules from shorter segments of sequence-defined members[87]. The red ribbon represents a short DNA oligonucleotide that functions as a synthetic codon for the assembly of highly functionalized unnatural nucleic acids. R = 1′-(2′-deoxy -5′-phosphatylribose); SELEX, systematic evolution of ligands by exponential enrichment.

Fluorescence-activated cell sorting
A development of flow cytometry that enables sorting of a mixture of cells into two or more fractions based on the light scattering and fluorescence signal of each cell.

Enzyme-linked immunosorbent assay
A diagnostic test that uses affinity reagents, typically antibodies, to detect a substance.

of natural DNA and RNA are poor candidates for diagnostic and therapeutic applications owing to their limited stability in biological environments. For example, an unmodified DNA aptamer developed as an α-thrombin inhibitor exhibited an *in vivo* half-life of <2 minutes when assayed in a primate animal model[97]. Introducing chemical modifications that stabilize the nucleic acid structure against nucleic acid degrading enzymes can enhance the utility of aptamers in practical applications. In particular, substitution of the 2′-hydroxyl group of ribonucleotides — fundamental for phosphodiester bond cleavage by nucleases — with amino, fluoro or methoxy groups can confer resistance[98]. Many 2′-modified aptamers have been produced by *in vitro* selection[43,69,99–102]; however, these modified sites are still prone to attack by nucleases, demonstrating that the biological stability of an aptamer can range from being slightly more stable than natural DNA and RNA to biologically inert[103,104].

Efforts to create aptamers that are immune to nuclease digestion have focused on the development of replication systems that can facilitate the synthesis of artificial genetic polymers (commonly referred to as xeno-nucleic acids or XNA) with backbone structures that are distinct from natural DNA and RNA[105]. L-RNA and L-DNA aptamers, also known as spiegelmers (discussed above), were one of the first types of alternative genetic polymers developed for aptamer production[60]. Although spiegelmers represent a powerful approach for generating biologically stable aptamers, this strategy is limited to the subset of targets that can be generated by chemical synthesis. For example, this number is <1% of all human proteins, as most human proteins are not amenable to solid-phase synthesis[106]. However, this situation appears to be changing with the development of mirror-image polymerases that can synthesize L-DNA and L-RNA[107,108]. Such enzymes have the potential to expand the number of L-RNA aptamers that can be used in clinical trials by overcoming the target generation problem.

The advent of new polymerase engineering techniques[109–112] have made possible the development of XNA polymerases that can copy genetic information back and forth between DNA and XNA. Using these enzymes, XNA aptamers have been produced through an *in vitro* selection strategy that involves copying degenerate DNA libraries into XNA and applying *in vitro* selection methods to isolate only those XNA molecules that bind to a desired target. The selected XNA molecules are then copied back into DNA for amplification by PCR[113]. As with regular *in vitro* selection, the process is iterated until the pool becomes dominated by sequences with affinity for the target. To date, three different XNA polymers (1,5-anhydrohexitol nucleic acid (HNA)[114], 2′-deoxy-2′-fluoroarabino nucleic acid (FANA)[115] and α-L-threose nucleic acid (TNA)[116,117]) have been replicated in a Darwinian evolution system to produce aptamers against a target of interest (FIG. 8). Of these, TNA has a backbone repeat unit that is one atom shorter than the repeat unit of DNA or RNA[118,119], making it one of the more difficult XNAs to replicate *in vitro* but also one of the most biologically stable genetic polymers developed to date that is capable of Darwinian evolution[120]. Although XNA aptamers are still in their infancy, this technology platform is poised to witness substantial growth in the coming years[121], as therapeutic aptamers require extreme biological stability. Indeed, XNA polymers are now being developed with expanded chemical functionality, which should increase their binding affinity and targetability[122].

Throughput. Recent advances in aptamer selection techniques have increased the quality and throughput of *in vitro*-selected aptamers. In conventional methods, nonspecific binding and PCR amplification problems can negatively affect the efficiency of aptamer selections by limiting the enrichment of functional molecules. As expected, an assay with a 100-fold partitioning efficiency (meaning 100-fold enrichment of functional molecules over non-functional library members) will require many more rounds of selection than will an assay that can achieve a partitioning efficiency of 100,000-fold per round of selection. Unfortunately, most bead-based aptamer selections have partitioning efficiencies in the ~500-fold range, which is why traditional processes often require ten or more rounds of selection. New solution-based selection approaches have been developed to avoid the problem of nonspecific aptamer binding to a solid support matrix[123]. For selections performed on a solid surface, such as an ELISA plate, surface passivation techniques that include surfactants have shown great promise for reducing nonspecific binding of the library to the plastic surface[124]. These techniques are often

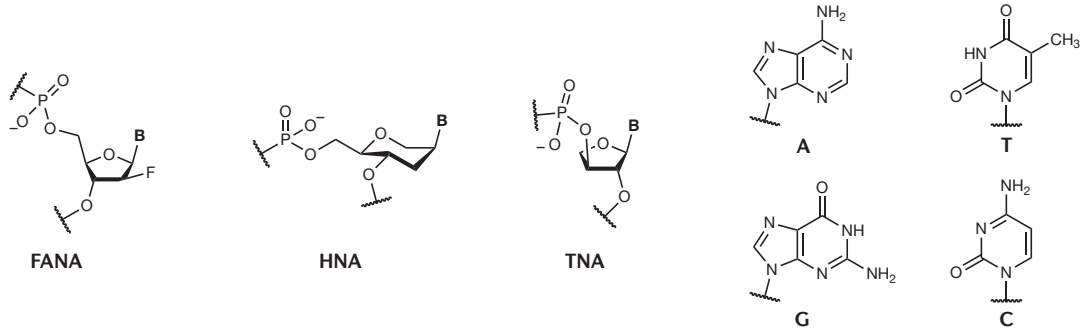


Figure 8 | **Xenonucleic acids.** Recent advances in polymerase engineering have enabled the coding and decoding of genetic information in artificial genetic polymers commonly referred to as xenonucleic acids (XNAs). Like DNA, these polymers are formed from A, T, G and C. Examples of XNA aptamers generated by *in vitro* selection include 2′-fluoroarabino nucleic acid (FANA), 1,5-anhydrohexitol nucleic acid (HNA) and α-L-threose nucleic acid (TNA)[114–117].

Emulsion PCR
A variant of the polymerase chain reaction (PCR) that is performed in water-in-oil droplets.

coupled with quantitative real-time PCR and emulsion PCR techniques that minimize the accumulation of PCR artefacts[125]. Finally, because of the recursive nature of *in vitro* selection, protocols have been developed that accelerate aptamer production using liquid handling robots that can perform incubation, washing and chromatography steps[126]. The use of automated protocols minimizes human error and maximizes throughput, accuracy and reproducibility.

Choosing the right chemical modification

The preceding section described new approaches that have been taken to overcome some of the barriers that have limited the success of aptamers in commercial and clinical applications. As discussed, the most challenging barriers to aptamer development include limited chemical diversity, poor selectivity, low biological stability and low throughput. Fortunately, recent advances have produced new aptamer technologies that are vastly superior to traditional approaches. Many of the newer technologies are based on chemical modifications and polymerases that are commercially available. However, other technologies require extensive knowledge of organic synthesis and polymerase engineering, as these building blocks and catalysts are not commercially available. In turn, many engineered polymerases require expression and purification from *E. coli*.

Choosing the right chemical modification depends on several factors, including the desired downstream application, commercial availability of the chemical modification and polymerase, activity of the polymerase for the modified unit when present in the template or as a nucleoside triphosphate (xNTP), and the chemistry and molecular biology experience of the user. For users interested in developing chemically modified aptamers, we have outlined the strengths and weaknesses of the chemical modifications described in this Review (TABLE 1). Although we anticipate that most researchers would prefer to use chemical modifications and polymerases that are commercially available, others may wish to pursue more exotic types of chemical modifications that require chemical synthesis and use polymerases that have been developed in specialized laboratories. In general, applications that require extreme biological stability would benefit most from XNA systems, including L-RNA and L-DNA, that are resistant to nuclease digestion. Applications that require specific target binding would benefit instead from modifications that increase the chemical diversity of the library repertoire. If high biological stability and high target binding affinity and selectivity are required, then it may be necessary to explore techniques that expand the chemical functionality of XNA libraries.

Suggested standards

The growing demand for aptamers as high-quality affinity reagents warrants a discussion on steps that can be taken to increase the quality, reproducibility and transparency of studies describing *in vitro*-selected aptamers. Analogous efforts in the antibody community are aimed at preparing high-quality protein capture reagents with known sequences and dependable functions[127]. As discussed earlier in this Review, many aptamers described in the literature are poorly characterized in terms of their sequence, binding properties, structure and stability. On the basis of these observations, we suggest that authors, editors and reviewers consider the following factors when preparing and reviewing aptamer manuscripts.

1. ***Sequence information.*** Does the manuscript contain the sequence of each nucleic acid aptamer described in the study? Are the sequences clearly annotated with information about the primer binding sites, random region boundaries and chemical modifications? If chemical modifications are used, is it clear which modifications were used (vendor and catalogue number) and where the modifications are located in the sequence? If the aptamers were truncated or otherwise modified, are the resulting sequence variants clearly defined?
2. ***Characterization.*** Does the manuscript include characterization of the binding interaction of the *in vitro*-selected aptamer(s) to the cognate target? Were affinity measurements determined with relevant controls or validated using independent techniques? Are the data believable or could it be the result of avidity rather than affinity? Are the binding conditions (for example, salt concentration, pH and temperature) reported? If necessary, could the binding assay be repeated by an independent laboratory? Are comparisons made between the affinity of the final aptamer and the starting library, which are required to show gain-of-function activity?
3. ***Structure.*** Do the authors predict the secondary structure of the *in vitro*-selected aptamer? Was the structure validated with a doped library selection or were key residues tested by mutagenesis? Was structure probing performed to elucidate structure–activity relationships?
4. ***Specificity***. Did the authors measure aptamer specificity against non-cognate targets? Affinity measurements made to homologues, analogues, chemical mimics or common biological molecules present in complex mixtures can provide a strong indication of the potential for off-target binding. These assays are particularly important for aptamers that are developed for practical applications that require the aptamer to function in a complex biological medium. A strong indicator of aptamer specificity can be a pull-down assay performed in a relevant biological environment, such as total *E. coli* lysate or human serum.
5. ***Stability.*** Did the authors measure the biological, chemical or thermal stability of the aptamer? Biological stability assays are particularly important for aptamers developed for diagnostic or therapeutic applications. In such cases, the authors should consider measuring the stability of the aptamer in relevant environments, such as concentrated human liver microsomes[128] (which contain a high abundance of both endo- and exo-nucleases) or 50% human serum in media. If degradation occurs, it may be worthwhile for the authors to determine the identity of the metabolites produced by enzymatic digestion so that more stable chemical derivatives can be constructed.

Table 1 | **Chemical modifications and their contributions to aptamer technologies**

Type	Targets	Characteristics	Availability
2′-F RNA	Numerous proteins	$t_{1/2}$ ~45 min in HLM[113]	NTP analogues available
		$t_{1/2}$ ~45 min in 50% HS[113]	Natural or engineered polymerases
2′-OCH_3 RNA	Numerous proteins	$t_{1/2}$ ~24 h in SVPE[97]	NTP analogues available
			Natural or engineered polymerases
Click-SELEX	Green fluorescent protein	Side chains increase the functional diversity	Reagents available
			Natural polymerases
SOMAmers	About 3,000 protein targets	Hydrophobic groups improve aptamer binding and targetability	NTPs require synthesis
			Natural polymerases
AEGIS	Breast cancer cell line (MDA-MB-231)	AEGIS increases the sequence diversity	ZTP and PTP available
			Natural polymerases
Spiegelmers	Numerous proteins and some small molecules	Refractory to nucleases[55]	Reagents available
		Require targets that can be synthesized	Natural polymerases
FANA	HIV reverse transcriptase	$t_{1/2}$ ~45 min in HLM[113]	fNTPs are available
		$t_{1/2}$ ~4 h in 50% HS[113]	Requires engineered polymerases
		Fluorine increases the hydrophobicity of DNA	
HNA	HIV TAR RNA motif	Refractory to nucleases[107]	hNTPs are not available
	Hen egg lysozyme	Requires specialized knowledge	Requires engineered polymerases
TNA	Human α-thrombin	Refractory to nucleases[113]	tNTPs are not available
		Requires specialized knowledge	Requires engineered polymerases

2′- F, 2′- deoxy-2′- fluoro; 2′-OCH_3, 2′- deoxy-2′- methoxy; AEGIS, artificially expanded genetic information system; FANA, 2′-deoxy-2′- fluoroarabino nucleic acid; fNTPs, FANA nucleotide triphosphates; HLM, human liver microsomes; HNA, 1,5-anhydrohexitol nucleic acid; hNTPs, HNA nucleotide triphosphates; HS, human serum; NTPs, nucleotide triphosphates; PTP, (2-aminoimidazo[1,2-*a*]-1,3,5-triazin-4(8*H*)one) triphosphate; SOMAmers, slow off-rate modified aptamers; SVPE, snake venom phosphodiesterase; TAR, trans-activation response element; TNA, α-L threose nucleic acid; tNTPS, TNA nucleotide triphosphates; ZTP, (6-amino-5-nitro-2(1*H*)-pyridone) triphosphate.

Outlook

The past quarter century has witnessed tremendous growth in the discovery and application of *in vitro*-selected aptamers. The specific quantifiable metrics discussed in this Review provide an opportunity to reflect on the accomplishments of aptamers as a powerful class of synthetic affinity reagents. However, relative to antibodies, aptamers remain an early-stage technology that will require further development to ensure sustained growth over the next 25 years. Key areas of development that will help close the gap between aptamers and antibodies include new selection techniques that enable aptamers to function in their desired downstream application, biologically stable scaffolds that are refractory to nuclease digestion and new sensors that can be tailored to any given target. In addition, when considering new aptamer projects, greater emphasis should be placed on functions that are not currently accessible to antibodies or that antibodies have difficulty performing, such as the recognition of small-molecule targets or targets that are toxic to cells, as well as the use of next-generation sequencing techniques that can accelerate the search for high-quality aptamers. Given the importance of chemical modifications to next-generation aptamers, the field as a whole would benefit from innovations in nucleic acid chemistry that make chemically modified nucleotides more accessible. In particular, new advances that increase the scale and purity of modified nucleotide triphosphates (xNTPs) would have an important and lasting effect on the field. By focusing on these areas and learning from past results, we are confident that the promise of creating an inexpensive renewable source of high-quality affinity reagents is finally within our grasp and could be realized in the next few years.

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