Aptamers: An emerging class of affinity reagents

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Aptamers are short nucleic acid oligomers selected from a random, chemically synthesised oligonucleotide library that are able to bind with very high affinity and specificity a wide range of molecular targets through a process called "Systematic evolution of ligands by exponential enrichment"(SELEX). Their binding capabilities are comparable to those of antibodies, making them a valuable alternative to the use of protein-based affinity reagents. Aptamers exhibit a variety of desirable characteristics as affinity reagents, but their main advantages over antibodies are their chemical synthesis (relatively easy, cheaper and exhibiting little batch to batch variation), their stability (which translates in longer shelf-life and less stringent storing conditions) and the potentially limitless amount of targets aptamers can be selected for. Despite this, some issues with both the isolation platform and the pharmacokinetic properties of aptamers exist, and only one aptamer-based drug has currently been approved for clinical use. The dawn of a variation in the aptamer isolation procedure called cell-SELEX is opening exciting new possibilities for therapeutic and diagnostic uses of aptamers.

Introduction

What are aptamers?

When one thinks of nucleic acids and their functions in a living cell, the first thing that comes to mind is their role (central to life) as carriers of heritable information that codes for proteins. However, this is not the whole story. Over two decades ago,

another interesting (and, as it turns out, extremely useful) property of nucleic acids was discovered: Nucleic acid molecules can fold into complex three-dimensional shapes that are capable of binding with very high affinity and specificity to a vast variety of molecular targets. This was first observed thanks to the development of an in vitro selection procedure called "Systematic Evolution of Ligands by Exponential enrichment", or SELEX (Tuerk and Gold, 1990, Ellington and Szostak, 1990). This essentially consists in the incubation of a chemically synthesised library of short, single stranded oligonucleotides (either ssDNA or ssRNA) with a target molecule and collecting those nucleic acid molecules that bind to the target. The binding oligonucleotides are then amplified (typically through PCR or RT-PCR) and the whole procedure is repeated on the new, enriched library obtained. Through the iteration of these steps eventually only ligands that bind very tightly and with a high degree of specificity remain (Steps are further explained in Figure 1) (Darmostuk *et al*., 2015). Oligonucleotide molecules obtained through this method are termed "aptamers" from the Latin "*aptus*" which means "to fit" and "*merus*", which means "particle". With binding constants (K_D) usually in the low nanomolar or high picomolar range (Keefe *et al*., 2010), aptamers exhibit binding properties comparable to those of antibodies and their potential as diagnostic, therapeutic and analytical tools was immediately recognized upon their discovery.

Aptamers and other affinity reagents

Antibodies are currently one of the most important and most widely used tools in the biomedical sciences, utilized for a wide range of applications for which specific molecular recognition is required. However, the isolation and production of novel antibodies is a difficult and expensive process, with some inherent limits due to their animal origins and chemical properties: Antibodies have a short shelf life, are hard to mass-produce and often give rise to immune reactions *in vivo*. Furthermore, as the antibody isolation procedure starts from an animal, antibodies capable of binding to molecules toxic or harmful to animals are difficult to isolate. This is also true for targets that exhibit intrinsically low immunogenicity (Jayasena, 1999). Another emerging issue with commercially available antibodies is that a significant amount of these (up to 51% according to a recent study (Berglund *et al*., 2008)) exhibit either non-specific binding or lack the ability to recognize the target altogether (Groff *et al*., 2015). On the other hand, aptamers are quicker and relatively cheaper to isolate, very stable chemically and can be readily modified through medicinal chemistry techniques. Their chemical stability, coupled with their *ex vivo* isolation procedure means that aptamers can be engineered to bind potentially any molecule over a much wider range of conditions than those accessible to antibodies. Because of these desirable characteristics, aptamers capable of binding many different molecular targets have already been isolated and tested. These range from proteins (Savory *et al*., 2010, Wang *et al*., 1993), low molecular weight metabolites (Miyachi *et al*., 2009), sugars (Boese and Breaker, 2007) and even a variety of different cell types (Li *et al*., 2014, Shangguan *et al*., 2006, Homann and Goringer, 1999).

Limitations exist

This being said, only one aptamer-based drug (pegaptanib, a vascular endothelial growth factor targeting drug developed for the treatment of age-related macular degeneration, the oligonucleotide was conjugated to polyethylene glycol to avoid renal filtration) has been approved by the FDA since aptamer discovery (Gragoudas *et al*., 2004, Ruckman *et al*., 1998). This because there are still some difficulties associated with the use of aptamers that need to be addressed: As aptamers are essentially low-molecular weight oligonucleotides, they are subject to renal filtration and degradation by serum nucleases, which means they have a short circulating half-life.

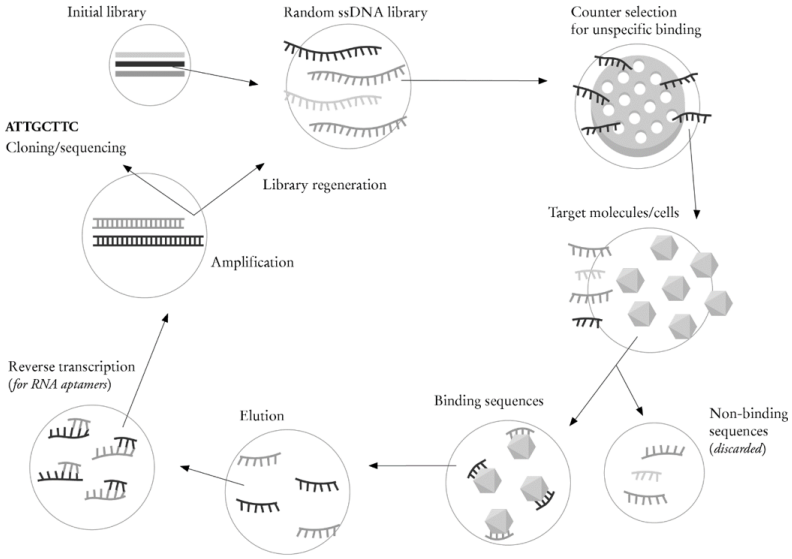

Figure 1. ***Schematic representation of the classic SELEX procedure***. *Often a counter-selection step is added (this step is especially necessary in some variants of the SELEX procedure, such as cell-SELEX), which is needed to ensure that the aptamers bind only to the target molecule. It consists in the opposite of the positive selection step, aptamers demonstrating non-specific binding towards molecules other than the target (what molecules are used for the negative selection step depends on the prospective use of the aptamer) are discarded, while unbound aptamers are collected. The rest of the steps are as illustrated: An oligonucleotide library is incubated with a target molecule, unbound aptamers are discarded, while oligonucleotides exhibiting selective binding properties towards the target molecule are isolated and amplified, in order to produce a new, enriched library for the next round of selection. Iteration of these step produces aptamers binding with the desired affinity and specificity, which are then cloned and sequenced. Adapted from: Darmostuk et al., 2015*

There are also some issues with methods for aptamer isolation, such as the use of PCR during the selection process, which is inefficient for the task of amplifying an extremely diverse library of different DNA templates, as it is biased towards certain sequences rather than others, which in turn can unnecessarily lengthen the SELEX procedure (Rozenblum *et al*., 2015). The focus of this review will be describe the main features of aptamers and outline the current prospects and challenges in their use as affinity reagents, comparing them with antibodies and highlighting pros and cons of both.

Structural features

Binding mechanisms

Aptamers are short nucleic acid molecules selected from an initial nucleic acid library typically consisting of oligonucleotides containing a random sequence of about 20-100 bases in length, with constant regions at the 3′ and 5′ ends needed to manipulate them enzymatically. A library will generally contain between 10^{13} and 10^{15} different individual sequences. For other nucleic acid-based therapeutic agents (such as antisense oligonucleotides and short interfering RNAs) the effect of the compound depends directly on the oligonucleotide sequence, as it must interact with other nucleic acids through classical Watson-Crick base pairing (Wagner, 1994, de Fougerolles *et al*., 2007). The mechanism of action of aptamers, instead, depends directly on their 3D structure and thus the aptamer's ability to bind with the target molecule through a combination of Van der Waals interactions, hydrogen bonding, classical base pairing mechanisms and electrostatic interactions (Rozenblum *et al*., 2015). This means that while other nucleic acid based therapies must necessarily act intracellularly, aptamers can act intracellularly, extracellularly or upon cell-surface receptors. Various common structural motifs involved in the binding of aptamers to specific ligands have been identified, including a variety of loops, stems, hairpins and some more specific structures like the pseudoknot and the G-quadruplex (Radom *et al*., 2013) (Figure 2). It is interesting to note that, although aptamers are synthetic molecules that were first isolated through combinatorial chemistry methods, oligonucleotides that function in a similar manner also exist in nature. Untranslated mRNA regions commonly found in eubacteria and termed riboswitches have been shown to regulate gene expression through their binding to metabolites. As the process does not require protein factors, riboswitches work essentially as natural aptamers (Tucker and Breaker, 2005).

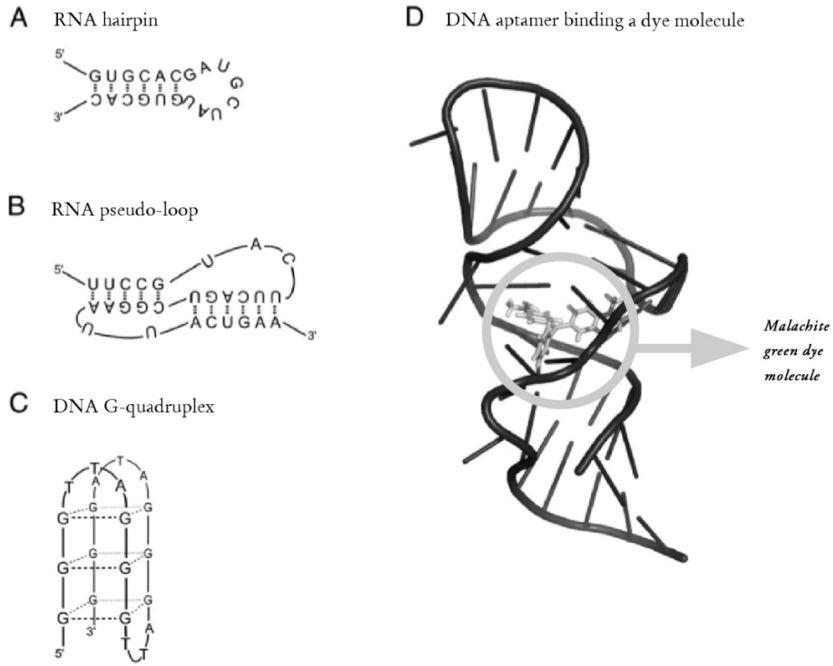

Figure 2. ***Some common structural motifs involved in the aptamer-ligand binding process.*** *It can easily be seen that single stranded nucleic acid structures fold following the same base-pairing rules which determine the structure of double stranded oligonucleotides, with the addition of some unconventional base-pairing. Typical RNA hairpin* ***(A)****, an RNA pseudo-knot* ***(B)****, and a more exotic DNA G-quadruplex* ***(C)*** *are shown.* ***(D)*** *Is a computer generated three-dimensional model of an aptamer binding its ligand (in this case malachite green), showing how the aptamer folds and snugly "fits" around the ligand (this if a small molecule such as a dye is the target, if the ligand is a large macromolecule like a protein, the aptamer may fit inside it's active site). Adapted from: Radom et al, 2013*

A wide range of structures is available.

Nucleic acid libraries can provide an immense amount of diversity, a typical 25 residue nucleic acid sequence made up by A, C, G and T (U if RNA is being used) will in fact give rise to 4^{25} possible combinations which means about $\approx 1.12 \times 10^{15}$ unique sequences, producing a vast array of different three dimensional structures. This means there is an astounding number of different structures available for selection during a typical SELEX experiment, more, in fact, than those of any other known combinatorial library (Gloekler *et al*., 2010).

Aptamers and antibodies

Monoclonal antibodies and aptamers

Monoclonal antibodies have, since their discovery (Kohler and Milstein, 2005), revolutionized research methods used in the biological sciences. Nucleic acid aptamers exhibit similar binding affinities and specificities to antibody based affinity reagents. Aptamers also have a variety of desirable qualities that make them an interesting alternative to antibodies for analytical, diagnostic and therapeutic applications. Possibly the most important advantage of aptamers over antibodies is that they are synthetized chemically, eliminating the use of animals from the isolation and production steps (Tombelli *et al*., 2005). This greatly extends the variety of targetable molecules, as animal-based antibodies cannot be isolated against toxic compounds or molecules with a very low native immunogenicity. Antibodies also work only at physiological conditions, while conditions in which aptamers are capable of binding can be modified and tweaked by changing SELEX parameters (This may be useful if an aptamer for an *ex vivo* assay is required). It must be noted though that the use of recombinant antibodies can also partly overcome these limitations, as animals are not directly involved in their production (Groff *et al*., 2015). Another advantage is that aptamer isolation through SELEX is cheaper and faster than monoclonal antibody isolation, and bulk production of aptamers can be achieved with relative ease through solid phase phosphoramidite chemistry (Caruthers, 1985) . Further limitations of antibodies are their short shelf-life (and careful storage is required, as they are susceptible to irreversible denaturation if measures are not taken to avoid this). High batch-to-batch variability in efficiency is another major limitation, as recent data suggests that about half of antibodies commercially available may not be capable of actually recognizing their targets with the required degree of affinity and specificity (Groff *et al*., 2015) . Aptamers can overcome these, as they are very stable molecules and can thus be stored with little precautions for long periods of time. Furthermore, as they are produced through chemical synthesis, there is virtually no batch-to-batch variation associated with aptamer production. The nucleic acid nature of aptamers also makes them relatively straightforward to modify through medicinal chemistry techniques, making conjugation to drugs and reporter molecules, as well as pharmacokinetic tailoring, possible. Interestingly, developing an antidote for a specific aptamer also appears to be very straightforward, as oligonucleotides with a complementary sequence to the aptamer have been shown to inhibit it's effects (Rusconi *et al*., 2002). Antibody modifications (such as humanisation to reduce immunogenicity, site specific modifications to enhance pharmacokinetics or antibody-drug conjugation) are instead non-trivial, require the investment of substantial time and effort and have some inherent limitations to the modifications achievable (Keefe *et al*., 2010). More specifically to *in vivo* therapeutic and diagnostic applications, antibodies are inherently immunogenic molecules, and the risk of adverse effects is always present, while aptamers have in general shown low or no immunogenicity (Rusconi *et al*., 2000, Martin *et al*., 2002). It must be noted

though that adverse effects observed during clinical trials of REG1, an aptamer based drug targeting coagulation Factor IXa as a reversible anticoagulation system, suggest that low immunogenicity may not be an intrinsic property of all aptamers (Rusconi *et al*., 2002, Rozenblum *et al*., 2015). Finally, the use of cell-SELEX offers the opportunity of creating aptamers for molecular targets of unknown structure by selecting the aptamers against whole cells rather than highly purified molecules, which may also aid in the identification of novel biomarkers.

Limitations of aptamers and possible solutions

Renal filtration

For all their desirable properties as affinity reagents, aptamers also have some limitations, especially for *in vivo* applications. Due to their small size, aptamers are subject to renal filtration, which greatly reduces their circulating half-life. Rapid degradation by serum nucleases is also a problem, further reducing their availability in the bloodstream. Renal filtration can be efficiently avoided through conjugation with molecules of high molecular mass such as polyethylene glycol (the effects of PEG-conjugations are further illustrated in Figure 2) with aptamer circulating half-lives going from 5-10 minutes for unconjugated oligonucleotides up to a day for PEG-conjugated molecules (Healy *et al*., 2004, Boomer *et al*., 2005). Cholesterol can also be used for the same purpose, although it appears to be less effective then PEG (Keefe *et al*., 2010).

Nuclease attack

It is possible to reduce aptamer attack from serum nucleases by pre- or post-SELEX base modification. This is generally done through the addition of a variety of functional groups to the nucleotide bases (mostly on the pyrimidine bases, preferred target of serum nucleases), capping of oligonucleotide termini and the use of purine rich aptamers (Lapa *et al*., 2015). Further chemical base modifications can also enhance the aptamer's binding parameters (some functional group additions to oligonucleotides are shown in Figure 3). Other, more exotic methods are also being investigated, such as the use of spiegelmers, aptamers in which the sugars are the opposite enantiomer of the wild-type sugar (so RNA spiegelmers, for example, would be made using L-ribose rather than D-ribose), but there are some additional synthetic difficulties associated with these (Bilik *et al*., 2007).

Unknown properties, patenting and funding allocation.

Further problems are that the pharmacokinetic properties of a specific aptamer are hard to predict in advance, although they can be somewhat tailored during the SELEX process or through medicinal chemistry techniques. The extensive

patenting of SELEX technology is also a discouraging element, and may very well be the main reason why many companies choose to allocate financial resources towards the development of other types of affinity reagents, as the intellectual property of most monoclonal antibodies has instead expired by now (Keefe *et al*., 2010). This, coupled to the relative inefficiency of the SELEX procedure deriving from PCR bias may also be slowing down the development of aptamer technology (Rozenblum *et al*., 2015).

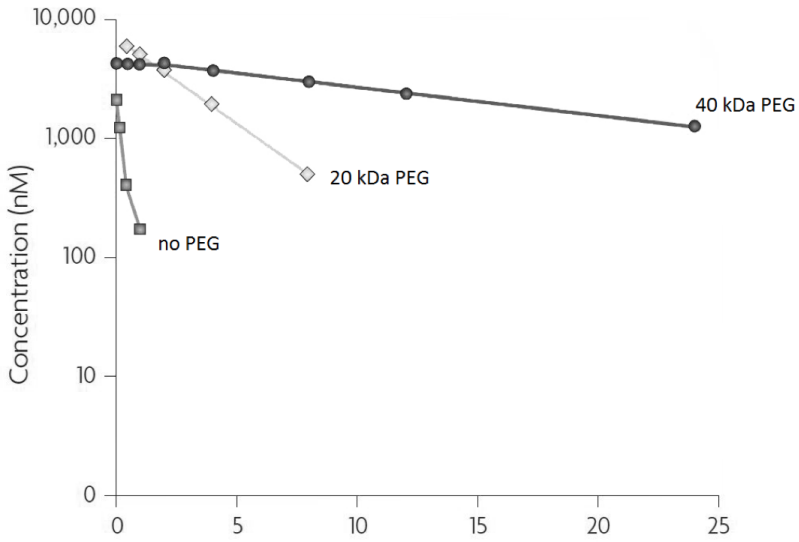

Figure 3. ***Plot illustrating the dramatic increase of the circulating half-life of Polyethylene glycol-conjugated aptamers when compared to non-conjugated oligonucleotides.*** *The aptamers used in this experiment are composed by 39 residues of 2'deoxypurines and 2'-O- methyl pyrimidines, conjugated (20 kDa PEG or 40 kDa PEG) and were injected in CD-1 mice (triplicate samples) in a concentration of 10 mg kg^{-1}. Adapted from: Keefe et al, 2010*

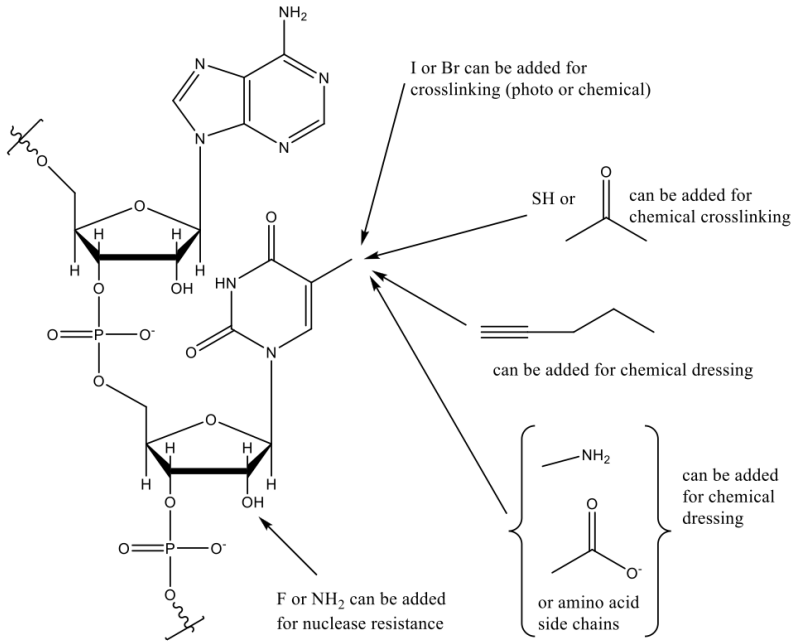

Figure 4. ***Summary of some common chemical modifications commonly carried out on nucleic acid aptamers, used to produce modified oligonucleotide libraries for the SELEX procedure.*** *Functional groups added to the 2′ sugar carbon are important for nuclease resistance, while additions made on the 5′ pyrimidine carbon can enhance binding of the oligonucleotide to its target or help crosslinking to it. Adapted from: Jayasena, 1999*

Conclusions

Great therapeutic and diagnostic potential

Since the possibility of cell-SELEX (a SELEX procedure in which whole live cells are used as targets for selection) was theorized (Vant-Hull *et al*., 1998) and subsequently proven possible through the isolation of aptamers targeting red blood cell ghosts (Morris *et al*., 1998), a plethora of SELEX experiments against targets of therapeutic interest has arisen. Just to cite a few notable examples, aptamers for pancreatic cancer cells (Champanhac *et al*., 2015, Li *et al*., 2014), prostate cancer cells (Kim *et al*., 2010), metastatic colorectal cancer cells (Li *et al*., 2014) and even live eukaryotic parasites (Goeringer, 2012) have been successfully isolated, demonstrating their therapeutic potential either by exhibiting intrinsic cytotoxic capabilities or through their use as vectors for cell-specific drug delivery. Cell-

SELEX, coupled with aptamer chemical modification techniques, has also caused a bloom of research into aptamer-based diagnostics, for example in the detection of circulating tumour cell and cancer biomarkers (Sun *et al*., 2015). As exemplified by these studies, aptamers show great potential as affinity reagents, possibly even surpassing antibodies: They are cheap, easy to store and readily produced on an industrial scale. More importantly, aptamers can go where antibodies cannot, with a much wider range of molecular targets available for selection.

Issues must be solved

But for aptamers to unleash their full potential as affinity reagents, some steps have yet to be taken: Synthetic methods for generating and producing aptamers need to be improved, as, although cheaper than antibodies to produce, they are still more expensive than classic small molecule pharmaceuticals due to their large size and complexity. To this end a variety of "one-round" SELEX procedures are currently under scrutiny (Darmostuk *et al*., 2015). Their pharmacokinetic properties also need to be extensively studied, as the current lack of knowledge in that respect greatly lengthens development times and costs. If these conditions are met, and unless unforeseen undesirable properties of aptamers are discovered, their use as therapeutic and diagnostic agents should flourish in the following years, hopefully fulfilling and exceeding expectations.

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