A therapeutic battle: Antibodies vs. Aptamers

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Abstract
The goal of creating drugs that specifically target their pathogen has been around since the concept was first introduced in 1900. The last three decades antibodies have been studied as targeting agents for therapeutics. They have been very successful in targeting pathogens and have been used in clinics to treat multiple diseases with success. However, several problems relating their high costs, cumbersome discovery of new antibodies using animals, and problems regarding homogeneous production are still left unsolved. Recently a new class of molecules has entered the market. Due to new observations regarding the function of oligonucleotides a new perspective for RNA and DNA strands has formed regarding targeted therapy. As the structural diversity of RNA and DNA is large, forming complementary structures to an antigen should be possible causing specific binding similar to antibodies. Screening methods for these oligonucleotides, called aptamers, have been developed and new research is progressively conducted. As studies are expanding the abilities of aptamers, they are starting to compete with the tradition antibodies. Although research relating aptamers just emerged compared to antibodies, it is gaining momentum. Aptamers surely possess advantages over current monoclonal antibodies. But currently aptamer research is still underdeveloped. Therefore overshadowing antibodies in clinics is in the far future. Until then, aptamers could proof very useful in specific fields or in conjunction with antibodies.

Keywords
Monoclonal antibodies — Aptamers — Therapeutics

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Introduction

In 1900, Dr. Paul Ehrlich came up the concept of the ”magic bullet” regarding therapy. The idea is that a drug (the bullet) enters the body and specifically distributes to the pathogen and only treat the diseased tissue leaving the healthy tissue unharmed. Similar to a missile following its target until impact. Ehrlich though that this would be the ideal solution for numerous diseases. This concept has gained momentum and became a very popular goal in pharmacy.

Most therapeutics used in clinics, such as radiotherapy, chemotherapy, immunotherapy, etc. do not target the specific pathogen that should be treated. Therefore the drug is not effectively delivered at the target but also distributed to healthy tissue. This may a ffect the healthy tissue causing unwanted side effects.[1] Therefore the search for drugs targeting only diseased cells is still a hot topic in pharmacy. Such a drug would have multiple advantages such as lower concentrations needed for successfully treating patients and less side effect as healthy tissue would be damaged less. The toxicity would be increased specifically at the diseased cells.

Antibodies were rapidly envisioned as possible candidates for this ”missile”. Antibodies are proteins that bind very specifically to certain receptors on the surface of cells. They are part of our immune system and help with protecting our body against bacteria and viruses. These antibodies are able to ”sense” whether intruders are harmful or not. Every antibody

binds only one antigen. As the body needs to be protected against many different antigens, a large amount of different antibodies are already present.

Since the first production of monoclonal antibodies (mAbs) in 1975 by Georges Kohler and Cesar Milstein, research on antibodies for targeted therapy has boomed. Just one year after the discovery of Kohler and Milstein, Ron Levy already found a monoclonal antibody specifically recognizing cancer cells. Just four years later, again a monoclonal antibody was found by Lee Nadler recognizing a different diseased cell, namely a non-Hodgkin lymphoma cell.[2]

The future for antibody therapeutics looked bright and expectations were high. Unfortunately the therapeutics did not seem to work as effectively as anticipated. Numerous problems were encountered limiting approval of antibody therapeutics. It was not until 1992 until the first monoclonal antibody was approved for therapeutic use. A lot of research has been done one mAbs in order to optimize their properties and efficacy. In the year 2015 and 2016 ten antibodies were approved by the FDA. The total of approved monoclonal antibodies is currently 68. Not all problems regarding mAbs are solved and antibodies as the "magic bullet" is still not feasible for every disease.

The problems encountered with antibodies, together with the realization that oligonucleotides possessed more functionalities than previously expected caused RNA and DNA strands to become another possible candidate as therapeutic "missile". Research on these oligonucleotides, called aptamers, emerged and showed promising results. A selection procedure is designed in order to select specifically binding oligonucleotide strands against certain antigens. This process is called SELEX and has produced multiple specifically binding aptamers with high affinities.

Currently, one aptamer has been approved for clinical use and several others are in approval. The expectations for aptamers are very high as rivals for antibodies. It is unknown if these small "chemical antibodies" are able to fulfill their high expectations. Although results are promising, aptamers applicable in clinics are not largely produced yet.

In this article an extensive description of antibodies and aptamers is given regarding their properties for therapeutic use. A review is given concerning the recent progress in the techniques used. The most important advantages and drawbacks concerning both are highlighted and the future prospect and use of both types of molecules is given.

1. Antibodies

1.1 Structure of antibodies

Antibodies (Ab) are Y-shaped proteins that belong to the immunoglobulins, a family of globular proteins. They are naturally used in the immune system of the human body to protect the body against infectious agents (e.g. viruses, bacteria, fungi etc.) also called pathogens. The host is able to produce a large collection of diverse antibodies all possessing a similar Y shape. The difference antibodies have different sequences of amino acids in their variable domain. The large amount of amino acids (20) used by the body makes the amount of possible sequences enormous.

The function of antibodies is closely related to their structure. The antibody is used in the body to recognize particular proteins on the membrane of the pathogen, called antigens. As there is a vast amount of different antigens the Abs need to be versatile in order to protect the body from all of them. Secondly they need to bind very specifically. The immune system only needs to be stimulated when the body is infected by pathogens. Also the biological activity is dependent on the antibody structure. After antibody-antigen binding a certain region of the antibody decides what response is stimulated against the antigen (e.g. phagocytosis).

Studies on antibodies performed in the 1940s and 1950s showed that the size of different antibodies varies. Their molecular weight ranges from 150 to 1000 kDa (kilo Dalton).[3] In the 1950s and 60s Rodney Porter and Gerald Edelman studied the structure of antibodies fo r which they eventually received the Nobel Prize in medicine in 1972. They found that the antibody consists of two subunits. A large subunit, now called the heavy (H) chain and a smaller subunit, called the light (L) chain. The original molecule used by Edelman had a molecular weight of 150 kD. As the light chain and heavy chain had a molecular weight of 23 kDa and 50 kDa respectively, he concluded that the antibody consists of two heavy chains and two light chains.

The immunoglobulins are divided in to five different so called isotypes, namely IgA, IgD, IgE, IgM and IgG. These isotypes have different heavy chains labeled as $\alpha, \delta, \varepsilon, \mu$ and γ respectively. To be more specific, the constant regions (C-regions) of their heavy chains differ. The C-region is indicated in figure 1 with the letter C. Most of the IgGs, IgDs and IgEs are monomers, but IgM and IgA are pentamers and dimers respectively. The five different classes are schematically illustrated in figure 1.

IgG is by far the most abundant antibody in the human serum as approximately 70-85% of the immunoglobulin pool is IgG.[5] It also is the most widely used for therapeutic purposes.

Figure 2 shows a schematic of a typical igG antibody monomer. The antibody consists of four polypeptide chains, two heavy chains (50 kDa) and two light chains (25 kDa).[6,7] These chains are held together by disulphide bonds.[8] Most IgGs have two bonds connecting the heavy chains in the middle. This region is called the hinge region. The two heavy and light chains are also connected by disulphide bonds.[7] As can be seen from figure 2 the heavy and light chain also have two intrachain disulphide bonds. These extra bonds stabilize the domains.[7] Different types of antibodies share part with the approximate same sequence in both the heavy and light chain. The other parts are highly variable. Both the light and the heavy chain can therefore be subdivided in two regions called the variable region (V) and the constant region (C). The regions are labeled as V_L and C_L for the light chain and

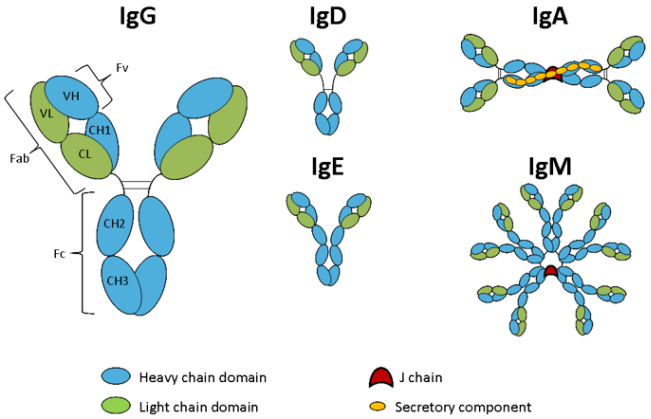

Figure 1. A schematic of the five different classes of antibodies. The green ellipsoids correspond to the light chain while the blue ones represent the heavy chain. The di-sulphide bonds are indicated with the black lines. The regions indicated with Fab, Fv and Fc represent the fragment anti-gen binding region, fragment variable region and fragment crystallizable region respectively. Note that some classes of antibodies possess different valencies.[4]

The regions in between these amino acids are called the framework residues FR1, FR2, FR3 and FR4. These regions form the β-sheets and determine the position of the CDRs. Their sequences do not vary greatly between different antibodies. About 85% of the V-region comprises of these framework residues. The CDRs of the heavy and light chain together form the antigen binding site.

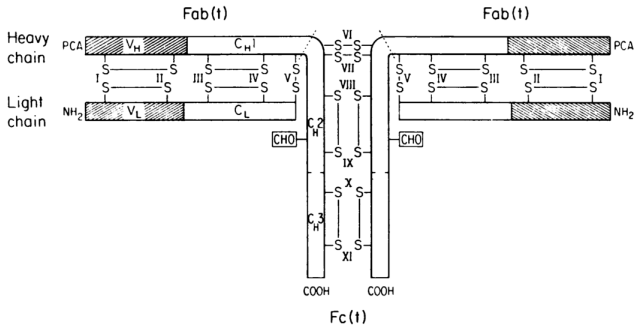

Figure 2. A schematic representation of the structure of an IgG antibody. Both the inter- and intrachain disulphide bonds are indicated.[8]

V_H and C_H for the heavy chain as indicated in figure 1. The V-region of the heavy and light chains have approximately the same length.[9] Also the V_L-region has the same length as the C_L-region. The C_H-region however is approximately three times longer.[8] The C_H-region can be subdivided in two three domains of equal length: C_H1, C_H2, C_H3. Each domain is approximately 110 amino acids long[10] and folds in to a tertiary structure. This tertiary structure is composed of two β-sheet structures with an hydrophobic interior. The interaction between the domains cause the whole molecule to fold in to three spherical shapes creating a Y or a T shape.[11]

The variable parts of both the H and L chain can be divided in three hypervariable sequences (HV1, HV2, HV3). In the light chain the HVs are located at amino acid 28 to 35, 49 to 59 and 92 to 103 respectively.[7] These three HV sequences are also called complementary determining regions (CDR1, CDR2 and CDR3) as these are the parts that bind to antigens. The part of the antigen that is recognized by the antibody is called an epitope. When the V domain fold in to their three dimensional structure the CDRs are displayed on the surface of the domain at the end of the antibody. The approximate surface of the CDRs available for binding is 2800 Å. The structure of an IgG antibody against HIV-1 was determined by Saphire *et al.* using X-ray crystallography. The distance between the two binding sites on the arms of the antibody was determined to be approximately 171 Å.[12] The angle between the arms was 143°. Figure 14 shows the physical dimensions of an IgG antibody. The sequence of the amino acids determines the three dimensional structure of the whole antibody. The sequences of the CDRs can vary greatly which causes a large diversity of structures and ionic properties which define the high specificity of antibodies.[3]

1.2 Antibody-antigen interactions

Antibodies thus bind to a specific part of the antigen called epitopes. The compliment structure of the CDRs cause for high specificity. Antibodies themselves help with passively protecting the body against pathogens by binding to them. However, they do not defuse the pathogens. On binding they block receptors on the surface of the virus, inhibiting certain functions.

Furthermore antibodies are able to involve other parts of the immune system. Single domains in the C-region of the heavy chains define the biological activity after antibody-antigen binding. By binding to pathogens, the antibodies effectively mark these pathogens, which enables the immune system to react accordingly. Binding of the antibody and subsequent recognition of the C-region causes the immune system to break down bacteria (bacterial lysis) or accelerate the uptake by macrophages (phagocytosis).[3] The function of the C-region is affected by the V region. Changing the variable region affects the binding properties of the Fc region.[13]

The region where the disulphide bonds link the two heavy chains (the hinge region) is important in the function of the antibody as well. This region creates flexibility during antigen binding. It was shown that antibodies without a hinge region were not able to bind and stimulate an immune response.[14]

Multiple interactions are involved in the binding of antibodies with their antigen. High affinity between antibody and antigen is obtained when both possess opposite charges and when there is a good fit between the surfaces of both the molecules in their ground state.[15] From equilibrium constant measurements on antibodies with their antigens it can be concluded that bonding is not covalent.[16] There are multiple non-covalent interactions that can form a bond between an

antibody and an antigen. These non-covalent interactions are mainly Coulombic or van der Waals bonds while hydrogen bonds are found more scarcely but have been shown to occur as well.[17] At 37°C the average kinetic energy of the water molecules are higher than the weakest bonds. As the average kinetic energy is a distribution of the kinetic energy of an ensemble of molecules, some molecules possess an energy higher than the strongest weak interaction. In order to obtain stable binding between the antibody and antigen several weak interactions have to work in concert at physiological temperatures.[18] The several weak interactions between antibodies and antigens are discussed in more detail below.

Coulomb interactions - Due to electrostatic attraction between opposite charges a bond can be formed. Several of the 20 amino acids comprising the proteins are charged. The charged amino acid residues include lysine (+), arginine (+), aspartate (-) and glutamate (-). Interactions between the oppositely charged amino acids in the antibodies and antigens form the most common bonds.[16] Coulombic interactions are relatively strong compared to other non-covalent interactions (see table 1).

Hydrogen bonds - Similar to Coulombic interactions, hydrogen bonds find their origin in attraction between opposite charges. Hydrogen bonds are formed between polar molecules. An important difference between the two is that hydrogen bonds only act on small distances. Typical hydrogen bonds are formed in the order of 1.5 to 5 Å[16] while Coulombic interactions are active in the order of 100 Å. In aqueous solutions hydrogen bonds between antibodies and antigens are weakened due to hydrogen bond formation with the solvent. Hydrogen bonding is therefore not a primary bond formed between antigen and antibody. Only when the two are brought in close proximity due to other forces, hydrogen bonds will be formed. They play an important role in strengthening antigen-antibody interaction. Hydrogen bonds are thus regarded as secondary bonds.

Van der Waals bonds - Van der Waals bonds originate from attraction between a fluctuating dipole in one molecule, inducing an opposite dipole in a different molecule. They are active on very short ranges. Compared to Coulombic interactions their interaction energy is normally small (see table 1). These interactions are, however, active in all molecules. A good complementary fit increases the amount of Van der Waals interactions between the two resulting in a stronger bond. The surface area active in binding is generally just a few amino acids long and comprises of 0.4 to 8 nm^2.[18] As Van der Waals interactions decay exponentially with distance a good fit also ensures that the the antigen and antibody can come in close proximity of each other.

Ca^{2+}-bridges - Ca^{2+}-ions are able to function as a coordination centre, binding surrounding negatively charged molecules. These bonds are called Ca^{2+}-bridges and are common in several biological systems. Therefore it is reasonable to expect these bridges in the formation of antigen-antibody complexes. However, it was shown that these bonds are not commonly formed between antibodies and their antigen.[16]

Hydrophobic interactions - Hydrophobic interactions are described as the tendency of non-polar molecules to bind together in aqueous solutions. The interaction is a result of the fact that water molecules rather form hydrogen-bonds with itself than interact with the non-polar molecule that cannot participate in hydrogen bonding.

Table 1. The interactions between antigen and antibody and their approximate energies.[18]

Bond	Energy (kJ/mol)
Van der Waals	4 (1-20)
Hydrogen	20 (5-40)
Hydrophobic	<40
Coulombic	20 (10-50)

Binding between antibodies and their antigen can shortly be described as follows. When antibody and antigen are separated by approximately 100 Å, long-range forces start to bring them closer together. These bonds are labeled as primary bonds. These long-range forces are Coulombic forces and hydrophobic forces as these work on larger separation. When the two are brought in closer proximity other forces start to increase and act on the antigen and antibody increasing the interaction. These bonds are labeled as secondary bonds. The bonds are active on a short range like hydrogen bonds and Van der Waals forces. The specificity of the antibody-antigen bond it mainly due to interactions that are early present in the binding process. Primary bonds thus play a very important role. Hydrogen bonding and Van der Waals interactions are therefore not very important regarding the specificity of the bond. Although these primary bonds play an important role in specificity the total binding energy (primary + secondary) of the antigen and antibody is significantly higher than the interaction energy of the primary bonds alone.[16] The main contributor to secondary bonds are the forces between the interfaces of the antigen and antibody.

1.3 Factors affecting epitode binding

Temperature - The optimum temperature for epitope binding is very dependent on the chemical structure of the antibody and antigen and thus on the non-covalent interactions between the two. Hydrogen bonds are more stable at low temperatures.[18] An in increase in temperature will decrease the strength and therefore importance of this interaction during binding. Hydrophobic interactions show the opposite behaviour and increase with increasing temperature. At low temperatures the hydrophobic interactions are reduced. These interactions are important during protein folding. At low temperatures the antibodies are unable to fold causing large hydrophobic part to be in contact with the hydrophilic solvent. In order to minimize this undesirable interaction the antibodies will aggregate.[7] Measurements on the equilibrium constants between different red cell antibodies and antigens were performed in a range of 2 C° to 40 C°.[18] Some antigen-antibody bonds showed a decrease in equilibrium constant while other showed

no significant difference or even an increase. This indicates that the temperature dependence is very much dependent on the specific antibody and antigen. Above a temperature of 70 C° the antibodies will denaturate.

pH - Antibody-antigen interactions are weakened upon an decrease of pH. For red blood cell antibodies a maximum of the equilibrium constant was found between a pH of 6.5 and 8.4.[18] At both sides from the maximum the equilibrium constant was decreased.

The fact that antigen-antibody interactions are weakened can be used to ones advantage. It was observed that antibodies are able to extend the endurance of their target due to a buffering effect.[19] Endosomes have a slightly acidic environment. Antibodies bound to their antigen are able to penetrate into the endosome where their interaction is weakened. Therefore antibodies will let go from and leave their antigen in the endosome. This increases lysosomal degradation of the antigen and decreases the buffering effect of antibodies on their antigen. Subsequently the antibody can be recycled and reused in order to transfer more antigens to the endosome. This decreases the concentration of antibodies needed to successfully treat a patient.

Very low or high pH values cause conformational change and might even cause denaturation of the antibody completely removing the interaction with its antigen.

Ionic strenght - Changing the ionic strength of the solution can have an effect on the interaction. Mainly the interactions caused by Coulombic forces are affected. Attraction between negatively and positively charged amino acids is weakened with an increase in ionic strength.[16] The dissociation between antigen and antibody is therefore increased for these systems. However, when hydrophobic interactions or hydrogen bonds dominate the antigen-antibody bond the opposite is true and dissociation is decreased.

1.4 Antibody Therapeutics

The first hybridoma, obtained by fusing a B cell with an immortal B cell cancer cell, was successfully produced in 1975 by George Köhler and César Milstein.[20] The production of this cell line was the start of monoclonal antibody (mAb) therapeutics. For their discovery they received the Nobel Prize in Medicine and Physiology in 1984. It was not until 1997 before the first anti-tumor mAb was approved. Since then mAbs are a fast growing class of therapeutics. Currently it is the second largest class of drug after vaccines.[21] Although antibodies are already used in clinics, active research is still conducted to improve their applicability and efficacy. Multiple new antibody drugs are being studied in order to optimize them.

Monoclonal antibodies (mAbs) - Monoclonal antibodies are antibodies which are all made by the same immune cell. Different immune cells are able to produce different antibodies. In order to obtain identical antibodies using multiple immune cells, a parent cell is cloned to obtain multiple identical immune cells all producing the exact same antibody. Therefore the mAbs are in fact all clones and thus have the exact same chemical structure. Due to their same structure they bind to the same epitope. The first Ab therapeutics used in clinics were monoclonal antibodies. They possess the natural IgG structure and work in a similar fashion. Just as with natural antibodies these mAbs protect the body directly by binding to the antigen. This may result in inhibition of critical functions of the pathogen. Also indirect protection is obtained by activating an immune response by inducing phagocytosis due to recognition of its Fc region.

The production of mAbs starts with injecting a mammal, usually a mouse, with an antigen for which one wants to produce an antibody against. The antigen causes an immune response in the mouse. The white blood cells, B cells, of the mouse that produce antibodies against the antigen are subsequently collected. By fusing this B cell with an immortal B cell cancer cell, myeloma, a new class of cell is obtained called a hybridoma. This hybridoma possess both the antibody producing capabilities of the B cell of the mouse and the long lifetime of the myeloma. The B cell that produces the desired antibody can be extracted after screening the hybridomas. The selection procedure is usually done via a technique called enzyme-linked immunosorbent assay (ELISA). The B cells are subsequently cloned in order to get a many identical B cells which are all producing identical antibodies against the specified antigen. The final step is harvesting the antibodies.

In 2015, 39 mAbs are approved for therapeutic use.[22] They have been extensively studied the last few decades and have been shown to possess drawbacks as well. Solid tumors are relatively resistant to mAbs.[23] The mAbs do not accumulate well in these tumors. Therefore large concentrations are needed in order to have a significant therapeutic effect. In order to solve these issues new strategies in therapeutics regarding antibodies are explored.

Single-chain Fv (scFv) - In the 1990s new research was conducted on fragments of antibodies. One example is the use of single-chain Fv(scFv). This therapeutic consists only of one variable region of the light and heavy chain instead the whole antibody molecule linked by a peptide linker. It was shown that these molecules possessed better pharmacokinetics than whole antibodies.[24] Also tumor penetration was enhanced. This was attributed to the smaller size of the scFv in comparison with whole mAbs. One structure that is intensively studied is the Tandem scFv (TaFv).[25] This antibody consists of two scFv fragments which are linked by a peptide linker. This extra peptide linker is flexible. With natural antibodies, the Fc region of a mAb is able to interact with an Fc receptor (FcRn) which protects the antibody from degradation in intracellular liquids. As the scFv does not possess the Fc region this protection is lost. This causes the scFv to be cleared from the blood at higher rates than normal antibodies. Additionally their small weight of 25-30 kDa and the fact that they are monovalent causes the off-rates to be large. Also renal filtration is significant due to their small size. The threshold limit for renal filtration is 70kDa.[23] The off-rates

and renal filtration are decreased with the use of TaFvs and Dbs as both their molecular weight and valency is increased. In order to increase the pharmacokinects of these fragments of antibodies they have been linked with other molecules. Poly(ethylene glycol) (PEG) has been used in order to increase the molecular weight.[26] Also albumin was used as linker as albumin involves the FcRN receptor again for protection.[24] Both strategies were able to increase the half-life of the antibody fragment. An advantage of the use of Ab fragments also includes the fact that a new technique called phage display could be used. This technique allows one to bypass the use of hybridoma's and produce Ab fragments using V genes which are obtained in vitro.[27] Therefore animals are no longer needed for the production of these antibodies.[28]

Multivalent antibodies - Another way of increasing the tumor penetration, decrease the off-rates and increase the distribution of the antibody therapeutics is via the use of multivalent antibodies. Natural antibodies are bivalent as at the ends of both of the arms of the Y shaped molecules CDR regions are present, able to bind to an epitope. In natural occurring antibodies these CDR regions on both of the arms are identical. Therefore they bind the same epitope which makes the antibody monospecific. By bonding fragments of antibodies together via a linker multivalent antibodies are obtained. Due to the multivalency the interaction energy increases between the molecule and its target. This decreases dissociation[28] as multiple binding interactions hold the molecules in place. At low concentrations the multivalent antibody will accumulate more at its binding site than a monovalent antibody. Therefore the amount of therapeutics needed to treat a patient is lowered which entails cheaper treatments and less side effects.

Bispecific antibodies (bsAbs) - Alongside increasing the valency of the antibody also increasing the specificity of the antibody is being explored. A relative new method in increasing the efficacy of antibodies as therapeutics is via the use of bispecific antibodies (bsAbs).

bsAbs consists of different binding sites and can be formed by non-covalent bonding of V_HA-V_LB and V_HB-V_LA.[29] The bsAbs can be divided in to two different categories based on their structure: IgG-like (orthodox) and non-IgG-like (heterodox).[30]

The orthodox antibodies are engineered antibodies which imitate the natural structure of IgG.[23] An example is the fusion of scFv and Fc to obtain scFv-Fc antibodies.[31] This antibody, with a molecular weight of 100-105 kDa, showed high viral-neutralizing capabilities.[29] Another example is the IgG-like antibody obtained by fusing scFv with the single C_H-domain. This antibody is called a miniantibody and has a molecular weight of 80 kDa just above the threshold for renal filtration.

Engineered antibodies that do not imitate natural IgGs are called heterodox antibodies. A heterodox antibody can be obtained by decreasing the length of the peptide linker in a TaFv.[32] The short linker forces two different Fvs to pair.[25] The V_H and V_L cannot bind intramolecular anymore so intermolecular pairing between variable regions into dimers is stimulated. These antibodies are able to bind two different antigens when two different Fvs are linked and are subsequently called a diabodies (Dbs). In combination with cytokines, Dbs have been shown to have great potency. The Dbs-cytokine fused proteins increase the therapeutic index. This means that smaller amounts of the therapeutic are needed in order to get the desired therapeutic effect which will minimize side effects.[33]

The field of antibodies for therapeutic and diagnostic applications is benefiting immensely from the design of novel recombinant antibody constructs, which have overcome many of the limitations of native IgG. Certain implemented modifications have been able to improve antibody pharmacokinetics, blood clearance, tumor penetration, and tumor retention, by customizing antibody size or valence, or through removal of Fc regions. Furthermore, the advent of various technologies for the generation of multispecific recombinant antibodies has yielded a variety of formats and designs that are currently being explored. By blocking two or more pathways simultaneously, multispecific antibodies could provide synergistic effects comparable to those obtained by com- bination of single agents.

Several limitations regarding natural IgGs have been overcome with designing new antibody constructs. By cleverly chancing the valency and specificity of the therapeutics using fragments of antibodies improvements are found in pharmacokinetics, renal filtration, blood clearance, penetration of tumors and off-rates. As these proteins are highly customizable they seem ideal for therapeutics as one can tune them for specific pathogens.

Although the first bispecific antibodies were already created mid 1980,[34] still few bsAbs are currently used in clinics. Large production and use in clinics is limited by the fact that it is difficult to produce vast amounts of homogeneous bsAbs using current techniques.[25] Therefore new techniques are explored in order to produce these antibodies at larger scale.

Antibody-drug conjugates (ADCs) - In order to increase the anti-cancer activity of monoclonal antibodies it was suggested to conjugate antibodies to toxic molecules (cytotoxic agent). In this way the antibody improves the selectivity while the toxin increases the effectiveness of the drug. The antibody-drug conjugates have a rather long circulation time. When the antibody bind the antigen on the surface of a specific cell. The antibody-drug conjugate is subsequently internalized by the cell. Inside the cell the drug is released from the antibody. Here the drug is able to treat the target. In order for this drug to work optimally, both the antibody as the drug and linker linking the two need to be optimized. Several combinations have been studied. The most successful antibody-drug conjugates were formed by linking the cytotoxic agent to a humanized IgG antibody via disulphide bonds or peptide linkers. Currently one conjugated complex is approved in the US. Multiple candidates are in still in clinical trials.[35]

2. Aptamers

Another relatively new strategy in therapeutics consists of using aptamers, also known as chemical antibodies,[36] as targeting ligands. Aptamers are oligonucleotides that are possible of targeting specific molecules. The name aptamer is derived from the Latin word "aptus" which means to fit. A very interesting property of aptamers for therapeutic use is the ability of aptamers to bind with high selectivity. They are small double or single-stranded DNA or RNA molecules. The function of nucleic acids was only regarded to store genetic information in DNA for a long time. The role of RNA was mainly related only to translation of genetic information to actual expression. Recently new functions of these nucleic acids have been found such as enzymatic catalysis.[37] Due to these observations in the early 1980s[38] a new view on the function of these molecules and their role in the evolution of life arose which led to a theory called "the RNA world theory". According to this theory nucleic acids are able to perform multiple functions. It is believed that they were catalyzing reactions for a period before proteins took over. This theory was supported by later studies that showed that oligonucleotides can bind various molecules and perform multiple functions.

Aptamers usually consist of 15 to 50 nucleotides and have an molecular weight ranging from 5 to 15 kDa.[37] Multiple aptamers have been generated so far successfully binding to a wide variety of different objects such as small molecules,[39] proteins[40] and cells.[41] Similar to the antibody-antigen interaction, the recognition between aptamers and their target is very specific. The selectivity of the aptamer finds its origin in the three dimensional structure of the aptamer[42] which allows it to bind their target via non-covalent interactions.

As there are multiple challenges with using antibodies as targeting ligands alternative ways of targeting pathogens are investigated. Aptamers are now investigated to check whether these new ligands might have advantages over antibody ligands. Aptamers are seen as potential replacements for these antibodies.

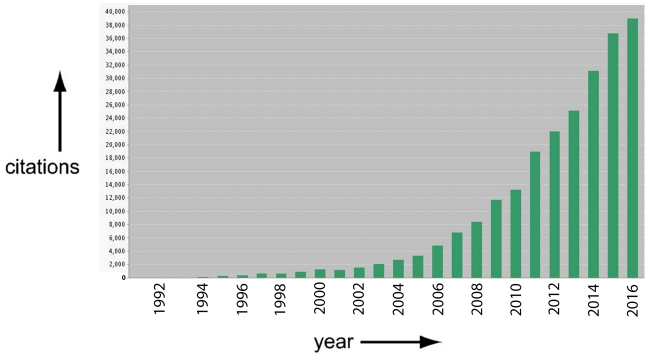

Figure 3. Citations found on the Web of Science when searching the keyword "aptamer" (status:21-03-2017).

2.1 Selection of aptamers

A new method was introduced in 1990[43] to screen large libraries of oligonucleotides for certain properties such as binding affinity for specific targets. Tuerk and Gold created an RNA pool by randomizing the sequence at a certain position and screened for a nucleic acid that was able to bind to a T4 DNA polymerase. They called their selection method SELEX (Systematic Evolution of Ligands by Exponential enrichment). Independently, Ellington and Szostak, used a similar method to find a RNA molecule which was able to bind a small ligand due to its specific three dimensional structure. They called their isolated RNA molecule "aptamer".[44]

This method of combinatorial chemistry is very important for pharmaceutical research. Using combinatorial chemistry it is possible to synthesize large amounts of chemicals which are related but structurally different. This method is now commonly used in the search for applicable aptamers. So called libraries of 10^{15} different oligonucleotides can be produced. These different nucleic acids are able to fold in to different secondary and tertiary structures. By screening the library for specific binding properties and functionalities, ...

The SELEX method consists of multiple steps. Figure 4 shows the different steps of the selection procedure. The first step in a SELEX process is to chemically synthesize a pool of randomized DNA oligonucleotides. This pool normally consists of 10^{13} to 10^{15} different sequences.[45] The specific molecule that one wants to target using aptamers is added to the pool. In obtaining RNA aptamers an additional step is needed. For RNA aptamers the DNA library needs to be converted to a RNA library.

After a certain incubation time the unbound oligonucleotides are separated from the weakly bound oligonucleotides. The weakly bound oligonucleotides are then amplified using a technique called PCR for DNA aptamers and reverse transcription (RT)-PCR for RNA aptamers.

The obtained sequences are double stranded (ds) DNA molecules. In order to increase the affinity and specificity of the aptamers a new pool, obtained from this first SELEX round, is again incubated with the target molecule. This second pool is created by separating the dsDNA in to single strand (ss) DNA. By doing multiple cycles of SELEX the finally obtained pool only consists of a few DNA or RNA sequence motifs that specifically bind the target with high affinity. The number of rounds performed depends on multiple criteria. It depends for example on the amount of binding features the target has and the ratio of target molecules to oligonucleotides.[45] Also the composition of the starting DNA pool is important. Normally the tested DNA pool is screened more strictly. This can be done by reducing the concentration of the target in following rounds or by changing the binding conditions.[45]

The SELEX method described above is the most basic process. Multiple studies have added steps to this procedure in order to increase the quality of the final aptamer. Negative selection steps may be added and are recommended in order

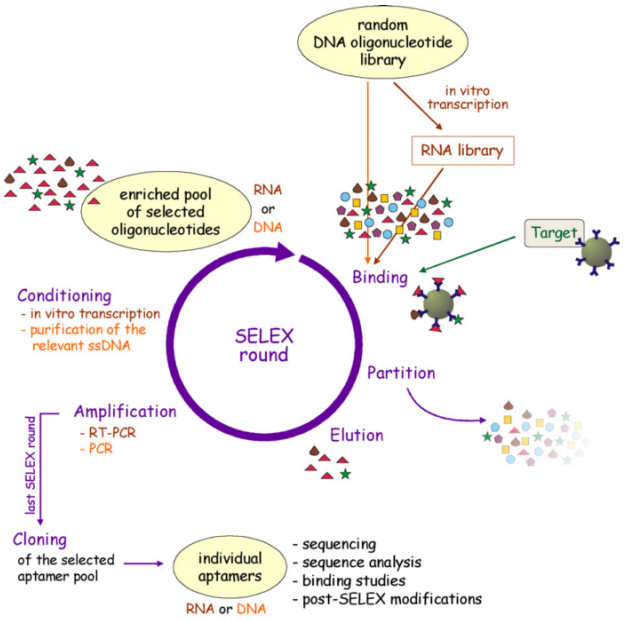

Figure 4. An overview of the different steps performed in SELEX rounds. The process starts with synthesizing a random DNA oligonucleotide library. This library consists of a diverse pool of ssDNA fragments ($\sim 10^{15}$). When selecting RNA aptamers the library needs to be converted in to an RNA library. Subsequently the target is introduced in the pool, non binding fragments are removed by several washing steps and the remaining fragments are amplified by PCR or RT-PCR. A new pool of oligonucleotides is created using the selected fragments and another round is performed. Usually eight to fifteen rounds are performed in order to obtain a high affinity aptamer.[45]

to minimize cross reactivity.

After multiple SELEX rounds, the final step is stopped after PCR amplification. The obtained aptamers are then cloned. These clones are sequences and analyzed. Structure determination can be performed on these aptamers in order to investigate the binding properties of the specific aptamer.

Targets - Different types of molecules can be targeted with SELEX. Aptamer selection can be performed for inorganic or small organic molecules, proteins, peptides, carbohydrates, antibiotics but also large complexes like whole cells and organisms.[45] One of the smallest molecules targeted using aptamer selection is ethanolamine.[46]

The amount of publications concerning aptamers as therapeutics form a big share of the total publications regarding aptamers. The main target for therapeutic aptamers are proteins. Proteins are large molecules consisting of a sequence of amino acids which are able to fold in complex spatial structures. Therefore proteins normally possess surfaces with multiple binding sites. This makes them an excellent target for aptamers. The first protein successfully targeted with aptamers is the protein thrombin.[40] The aptamer targeting this protein folds in to a so called G-quadruplex. By binding to thrombin, the execution of its functions was hindered.

In order to successfully find an aptamer that specifically targets a certain molecule with high affinity some requirements have to be met. The target should be present in sufficient purity and concentration.[45] This prevents the enrichment of non-specific aptamers in later SELEX rounds. Positively charged groups or atoms are easily targeted with aptamers. Primary amino groups for example are able to bind via Coulombic interactions with aptamers. Another feature is the presence of hydrogen bond donors and acceptors. Also aromatic molecules are able to bind via electrostatic interaction (dipole-quadrupole or quadrupole-quadrupole interaction).

Hydrophobic molecules are harder to target as specific binding cannot easily be obtained using aptamers. Aptamers have a more hydrophilic character as their building blocks (oligonucleotides) are hydrophilic molecules. Also negatively charged molecules are harder to target than positively charged molecules.

2.2 Structure of aptamers

An aptamer is a single or double stranded RNA or DNA molecule. DNA and RNA is normally illustrated as a double stranded helical molecule where nucleotides with bases adenine and thymine or guanine and cytosine are positioned at opposite sites bonded via hydrogen bonds. This structure is not the only stable conformation of DNA or RNA. Another stable structure is formed in a guanine rich environment.[47] This structure is a four stranded motifs called a G-quadruplex and is illustrated in figure 5. The guanines are able to associate with themselves via non-covalent interactions (also illustrated in figure 5). This allows the strand of DNA/RNA to fold in stable two or three dimensional structures (a.k.a. secondary structure) maximizing the amount of favorable interactions between the nucleotides. A large amount of aptamers occupy such a G-quadruplex structure.

There are multiple techniques for determining the structure of protein-aptamer complexes. Both NMR spectroscopy and X-ray crystallography can be used for structure determination of high resolution.

One example of such an aptamer is the anti-thrombin aptamer shown in figure 6a. The structure is retrieved using NMR. The schematic structure of the G-quadruplex is usually represented as illustrated in figure 6b. In figure 14 another representation is given for the same structure of the thrombin binding aptamer.

The three dimensional structure of the aptamer is dependent on the sequence of the nucleotides. As there are an enormous amount of different sequences possible, aptamers can occupy a vast amount of different three dimensional structures. The interactions between aptamers and their targets are discussed in more detail in section 2.3.

2.3 Aptamer-antigen interaction

Before looking at the factors affection the binding of an aptamer with an epitope, first the non-covalent interactions an aptamer can participate are discussed.

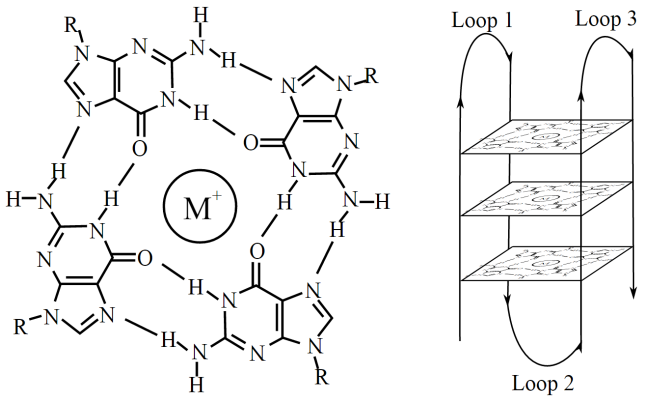

Figure 5. (left) Planar guanine tetrad formed via Hoogsteen base-pairing. The circle in the middle represents a positive metal ion. (right) A G-quadruplex is formed by multiple stacks of these planar structures on top of each other.[47]

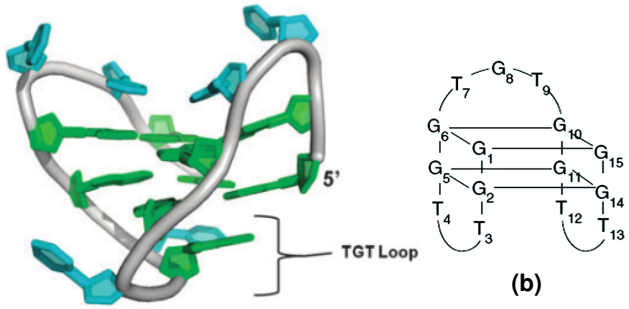

Figure 6. (a) Thrombin binding aptamer (PDB Id 148D) with sequence d(GGTTGGTGTGGTTGG) forming a G-quadruplex structure. The structure was obtained using NMR in 1993.[47] (b) Common representation of the G-quadruple structure for the trombin binding aptamer.[48]

By looking at the binding of aptamers with small molecules the different interactions can be studied more easily. For example an aptamer has been selected to bind the small molecule theophylline illustrated in figure 7a. This molecule is very similar to caffeine. The only difference is that caffeine possesses a methyl group instead of the circled hydrogen atom. Although this is a minor change, the aptamer selected for theophylline has an affinity for theophylline 10,000 times higher than for caffeine.[49] The theophylline ligand surrounded by the aptamer is shown in figure 8. Binding is obtained by stacking interactions (aromatic) between the aromatic molecule and the planar surface of the aptamer (indicated with cyan). Also a hydrogen bond is formed between the circled hydrogen bond and the oxygen of a neighboring cytosine base. For the caffeine molecule, the methyl group would hinder H-bonding bonding with the cytosine which explains the smaller affinity.[39]

In proteins, often the amino acid arginine plays an important role in aptamer selection. The structure of arginine is shown in figure 7b. The guanidinium group, the groups consisting of the three blue nitrogen atoms, interacts with the

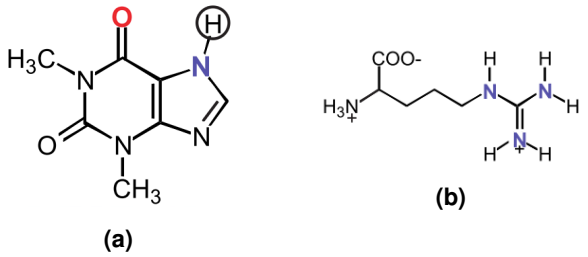

Figure 7. (a) Molecular structure of theophylline. The only difference with caffeine is the circled hydrogen atom which is a methyl group for caffeine. Although a minor change, aptamer affinity for theophylline is 10,000 times higher than for caffeine. The polar oxygen (red) and nitrogen (blue) are able to participate in hydrogen bonding.[39] (b) The structure of the amino acid arginine.[39]

bases in both RNA and DNA.[39] The positive nitrogen in the guanidinium group is able to interact electrostatically with the negative phosphate group of the nucleotides.[50] Figure 9 is obtained from a structure analysis of an arginine-aptamer complex amino acid.[39] The amino acid is aligned coplanar to a cytosine base with which it forms two hydrogen bonds. This interaction is stacked in between two adenine-thymine bonded nucleotides. The tight binding of the aptamer around the specific ligand explains the high specificity of the aptamer for this ligand.

Comparing the structures of aptamer-protein complexes it was shown that in some cases the peptide could rearrange its shape on aptamer binding while in other cases the shape of the peptide was unaffected on binding. It was shown that the ligand of a 17-residue peptide from a HIV protein could interact with an aptamer due to binding in to a deep groove of the aptamer. The deep groove was wide enough to insert the peptide due a purine-purine base pair in the aptamer. A purine-purine pair is the ”widest” allowed base pair geometry.[51] By insertion of the peptide in the deep groove multiple hydrogen bonds are able to form between the guanidinium group in the arginine amino acid and the phosphate groups of the nucleic acid aptamer.

With many proteins forming protein-RNA complexes, so called non-Watson-Crick base pairs play an important role in aptamer binding. Watson-Crick base pairs are the normal usual base pairs adenine-thymine and cytosine-guanine. Due to these non-Watson-Crick base pairs wide grooves are present in the aptamer allowing insertion of peptide ligands where they can form unique sets of hydrogen bonds.[52] The tight wrapping of the nucleic acid around a large part of the ligand causes the specific binding. For small molecules this was pointed out with the large difference in affinity between theophylline and caffeine, where the extra methyl group prevents strong binding.

Furthermore aptamers often have unpaired bases forming a loop region. These disordered loops are able to fold in a specific conformation wrapping around a ligand promoting specific binding as well.

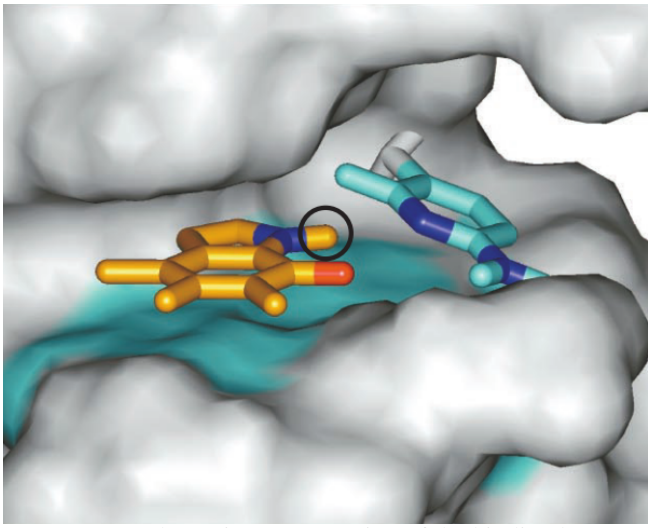

Figure 8. A schematic representation of the binding between the theophylline and an aptamer. The circled group is the hydrogen atom which is replaced for a methyl group in caffeine. The planar surface of the aptamer (cyan) and the aromatic theophylline gives rise to favorable stacking interaction. The cyan ligand is a base with which the theophylline forms hydrogen bonds. The polar nitrogen (blue) and the oxygen (red) form hydrogen bonds.[39]

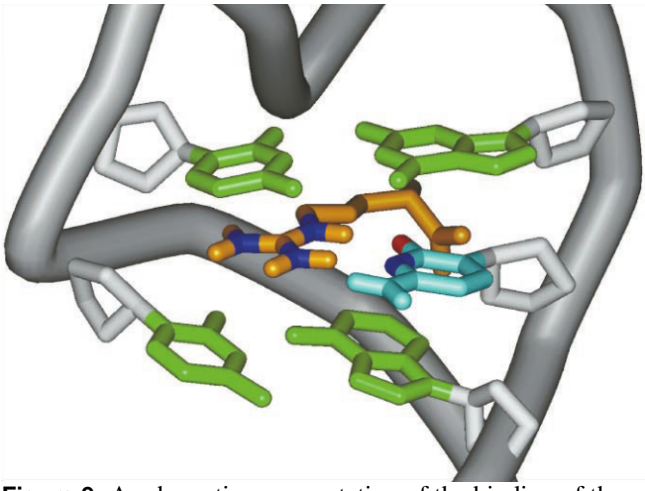

Figure 9. A schematic representation of the binding of the amino acid arginine by a DNA aptamers. The amino acid side chain (orange) forms hydrogen bonds with the bases (cyan) of the DNA aptamer. Also the polar nitrogen (blue) and oxygen (red) atoms forming hydrogen bonds are marked.[39]

So aptamers bind to specified receptors on the surface of cells. By specifying the aptamer to a receptor that is over expressed on a dangerous cell (e.g. cancerous cell) one can target the cancer cell specifically.

The receptors on the surface of a cell consists of a chains of amino acids (proteins). Proteins are normally large molecules and therefore have a large surface. Secondly they possess ridges, grooves, projections and depressions all possessing multiple H-bond donors and acceptors.[53] Due to these H-bonds aptamers are expected to bond non-covalently quite easily with the receptors.

Convery *et al.* were the first to determine the structure of coat protein-aptamer complexes using X-ray crystallography.[54] They showed that not only the sequence of the bases but also the spatial separation of the bases are important for selective binding. It was shown that adenine at position 4 and 10 as well as their spatial arrangement was important in binding to a MS2 protein

2.4 Increasing affinity

Although aptamers seem to be able to target a wide variety of molecules, it has been shown that aptamers do not have high affinities for all types of molecules. Their oligonucleotide structure can also limits certain interactions between the aptamer and the target molecule. From multiple studies it is concluded that some high affinity aptamers are not selected via SELEX. After changing the sequence or removing parts of the sequence of the selected aptamer, it was shown the new aptamer sometimes possessed higher binding affinities.[55]

The oligonucleotides are hydrophilic molecules. This limits possible hydrophobic interactions.[56] Adding hydrophobic parts to the aptamer may increase the number of interactions that are formed between the aptamer and the target.

These chemically modified aptamers can be obtained via the normal SELEX method. Gold *et al.* studied an artificial nucleotide instead of using the natural occur nucleotide 2'-deoxyuridine-5'-triphosphate (dUTP).[57] Three different modifications were on dUTP were studied and are shown in figure 10. The added side chains are hydrophobic chains allowing for hydrophobic interactions. Normal SELEX could be performed using these modifications. Thirteen different proteins were tested. These specific proteins were selected as high affinity aptamers consisting of normal nucleotides could not be found to bind to these proteins with high affinity. Using these artificial nucleotides multiple high affinity aptamers were selected. This shows that the range of targets can be extended using artificial nucleotides. Adding hydrophobic interacting groups to nucleotides may also increase the variety of structures of aptamers[56] which increases the amount of proteins that can be targeted.

So addition of hydrophobic group increases binding affinity both by increasing the amount of interactions and increasing the structural complementary to epitopes on proteins. A problem however using modified nucleotides is the fact that the certain steps in the SELEX method limit the efficiency significantly. Especially the PCR amplification and cloning are problematic with regard to these hydrophobic nucleotides. Therefore further studies have to be conducted in order to optimize the SELEX method for these new nucleotides in order to obtain higher throughput before large production for clinical purposes is possible.

Secondly the interactions between aptamer and target molecule is stronger when the available surface of the aptamer is large. Increasing the contact area between the aptamer and

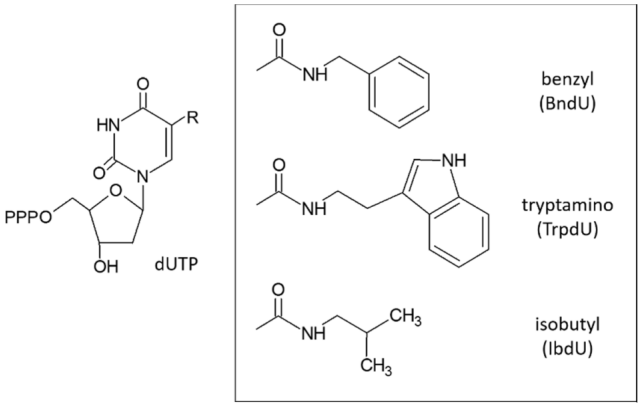

Figure 10. Three artificial nucleotides created by adding additional groups at the 5th position of the base (indicated by R) to the natural occuring dUTP.[57] The added side chains are hydrophobic increasing the number of interactions between the aptamer and target which increases the binding affinity.

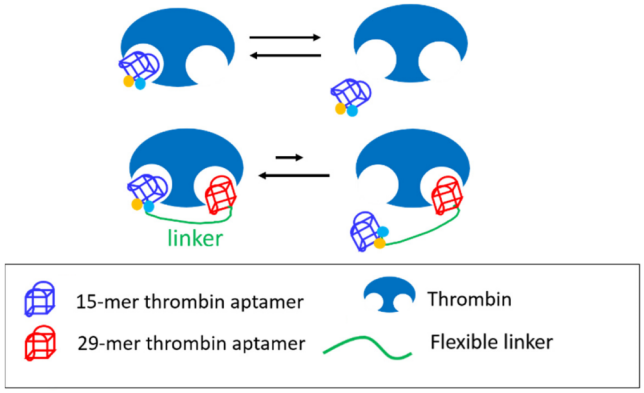

Figure 11. Drawing of the bivalent aptamer created by linking a 15- and 29-monomer pair by a flexible linker. Note the small arrow for the bivalent binding reaction representing a small k_{off} value.[58]

its target is likely to increase the binding affinity. The aptamers selected after multiple SELEX rounds, however, are usually small. The common length of an aptamer is around 30 monomers despite the fact that the SELEX library consists of aptamers ranging from 30 to 60 monomers.[56]

One way of increasing the contact area between the aptamer and the target could be joining multiple aptamers together. This would result in a multivalent aptamer which could produces higher binding affinity than the single aptamer. So called bivalent constructs have been produced and studied.[58] In this study a 15 monomer aptamer and a 29 monomer aptamer which both target the protein thrombin were dimerized. This bivalent aptamer possessed a dissociation constant (K_D) of $1/10$ of the K_D of the 15 monomer aptamer. A schematic drawing is shown in figure 11. Note that the smaller k_{off} value for the bivalent aptamer is indicated by a smaller arrow.

The length of the linker and its flexibility are important parameters effecting the binding properties of the multivalent aptamer. Positive chelate cooperative binding is observed only when the length of the linker is long enough. Linkers that are too small resulted in low binding affinities.[56] Flexible linkers are used as the distance between binding sites on the protein are not always well known. Using flexible linkers one is able to compensate for uncertainties in the structure. A drawback of this method is the fact that not all targets have multivalent binding sites. Furthermore, aptamer pairs that are able to bind to different epitopes on the same target are rarely found. Therefore further studies need to be performed to find more aptamer pairs.

2.5 Aptamer drug delivery systems

Instead of using aptamers as therapeutics they can also be used solely for their targeting properties as ligands conjugated to a drug. There are two main delivery systems using aptamer as targeting ligands. The first is a aptamer-drug conjugate. Here the aptamer is directly linked to the drug via a linker. The second system is called an aptamer-nanomaterials system. Here nanocarriers are used to deliver the drug to the target while the aptamers on the surface of the nanocarrier increase the specificity of the complex. In these systems the aptamer causes for recognition. In the ideal case, after binding of the aptamer to the antigen, the whole complex will be entering the cell where the drug is released.

There are numerous aptamer-drug conjugates being studied in the first system. Several drugs have been conjugated to the aptamer. An example is oligonucleotide conjugation. Figure 12a shows an illustration of such a chimera. Here the aptamer is conjugated to a small RNA strand interfering with the expression of genes (siRNA). In treatment of mice the siRNA conjugated to an aptamer interference of gene expression was observed while for naked siRNA it was not. This indicates that the conjugation to the aptamer increases specificity of the chimera. Further studies have created chimeras using different RNA and DNA therapeutics such as short hairpin RNA (shRNA) or splice-switching oligonucleotides (SSO) chimeras. Using DNA aptamers resulted in more stable chimeras compared to RNA aptamers.

Other therapeutics conjugated to aptamers are produced as well. Doxorubicin has been used extensively in research. Multiple techniques are used to conjugate the aptamer with the drug. Again a linker has been used to covalently bind the aptamer. Also intercalation has been studied. Here the doxorubicin is placed in between the nucleotides of the aptamer as illustrated in figure 12b

An advantage of the use of nanomaterials is that they are relatively big. Therefore multiple drugs can be carried per nanoparticle. Due to the large surface of the nanoparticles, several ligands can be conjugated to the surface increasing affinity. Multiple nanomaterials have been studied such as dendrimers, polymers, micelles, quantum dots, gold nanoparticles, carbon nanotubes all in combination with aptamers in order to target and deliver the drug to specific pathogens.

Gold (Au) nanoparticles (NP) are frequently used as the

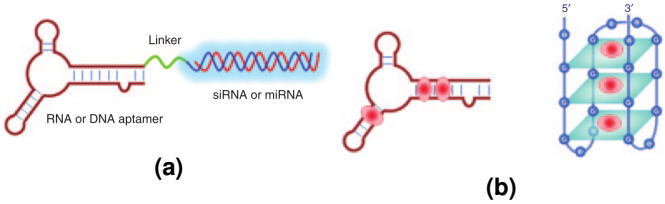

Figure 12. (a) Illustration of an aptamer conjugated to a siRNA via a linker. (b) Illustration of intercalation of a drug in between the RNA or DNA nucleotides. The right image shows intercalation between a G-quadruplex.[59]

are inert, very stable, have no toxicity and are easily conjugated using gold-thiol chemistry.[59] These properties make them ideal as nanomaterial for delivering drugs. An illustration of a Au nanoparticle conjugated to hairpin DNA (hpDNA) and a DNA aptamer is shown in figure 13. The drug was again loaded as described before by intercalating between the nucleotides of the DNA strand. The Au nanoparticle conjugated to the aptamer showed an increase in release of DOX compared to the nanoparticle without the DNA aptamer.

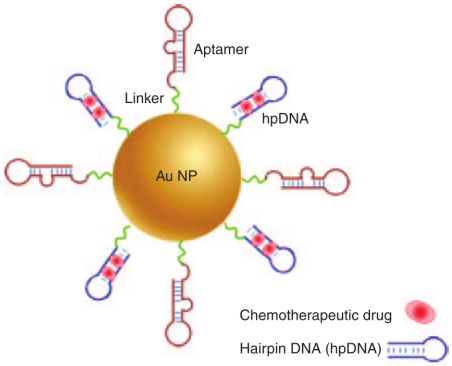

Figure 13. Illustration of a gold particle conjugated to two different ligands, namely a DNA aptamer and a hpDNA. Doxorubicin is intercalated inbetween the nucleotides of the hpDNA. Using this nanoparticle multiple molecules of doxorubicin are transported and delivered at the target.

3. Aptamers and antibodies as therapeutics

As the demand of targeted therapy is rising, the research for an ideal candidate as recognition ligand is booming. The fast amount of research on possible candidates is conducted on antibodies. A lot is known about this class of molecules as they have been around for quite a while now. Antibodies are a result of evolution so one might expect that these substances are work quite effective. The new class of recognition ligands, aptamers, are relatively unfamiliar. As seen from figure 3 research on aptamers is also rapidly increasing. Aptamers are regarded as possible replacements for antibodies as promising results have been shown. Expectations for aptamers are high, however, still research is conducted on new advances in antibodies as not everyone is convinced.

3.1 Drawbacks of antibodies

Antibodies are a big part of current therapeutics. They have been deployed in various fields and with success. Due to the discovery of monoclonal antibodies, large scale production of unique therapeutic antibodies has been made possible, boosting their popularity. A lot of research has been conducted ever since in order to optimize their pharmacokinects and affinities. Although the effectiveness of antibodies has been proven in some cases, a few drawbacks are preventing large scale application and progress of antibody therapeutics today.

The main reason of limited application of antibodies is the fact that a lot of antibodies induce an immune response of the body. This response, called the human anti-murine antibody (HAMA) response, is triggered especially when artificially produced antibodies are being used. Therefore antibodies used today need to be human or humanized to suppress this response. Recently new advances in mAbs have reduced this effect by binding variable domains to fragments constant regions of human antibodies. This results in a more human-like antibody. Studies however have shown that still an immune response is provoked by the variable domain. Subsequent studies have tried to introduce the specific CDR regions in to human framework regions. These modifications were able to suppress an immune response but the affinity of the antibody to its target was significantly reduced.[10]

Some severe drawbacks are found in the production process of antibodies. Animals are used to produce the antibodies against a specific target for example, which is cumbersome. Moreover, antibodies can only be produced against substances that provoke an immune response. The produced antibodies in the animal are extracted, cloned and amplified. A problem occurs however if one wants to produce an antibody against a molecule that does not provoke an immune response in the animal. The substances that are suitable targets are therefore limited notably. Using antibody fragments instead of natural whole antibodies may be a solution for circumventing animals.[28] Research still needs to be conducted on these engineered antibodies to prove their efficacy.

Furthermore the costs of producing antibodies are reasonably high. The full treatment with the antibody trastuzumab, which is a monoclonal antibody used for treating breast cancer, now costs $70,000.[60]

It is hard to produce large, homogeneous batches for antibodies and the quality of the produced antibodies differ significantly from batch-to-batch.[25] For the use of therapeutic a constant throughput of high quality antibodies is necessary in order to insure equal patient treatment. This means that every produced batch of antibodies needs to be tested in order to make sure that high quality antibodies are used in treatment. This makes working with antibodies more inconvenient.

The pharmacokinects of antibodies are not tunable as their production is limited to physiological conditions. The kinetic parameters can therefore not be changed on demand.[61]

Antibodies are sensitive molecules. By changing the conditions (e.g. pH, temperature, salt concentration) denaturation may occur. For antibodies this in an irreversible process which means that after denaturation the antibodies are lost and cannot be reproduced. The storage time for antibodies is therefore limited.

3.2 Benefits of aptamers

Aptamers are foreseen as possible candidates for possible additions to the shortcomings of antibodies or replacing antibodies completely. In comparison to conventional therapeutics such as antibodies and small molecules, aptamers offer some benefits. Some of the major benefits of aptamers are described below.

Aptamers are synthesized in vitro. This means that no animals are needed to obtain new aptamers as is the case with monoclonal antibodies. The selection process is independent of cells or other in vivo conditions.[61] Performing modifications to the aptamer is therefore easier. Also non-physiological conditions (high salt concentrations, high/low pH, temperatures) can be used during selection of aptamers. This allows one to select a broader range of aptamers. Aptamers selected under extreme conditions may be useful in targeting a specific pathogen under physiological conditions.

The non-physiological conditions cannot be used for antibodies as they would denaturate irreversibly. Just as antibodies denaturation of aptamers occurs. An important difference between the two however is that the denaturation process for aptamers is reversible while for antibodies it is not. Aptamers could be reproduced in just a few minutes. The ”shelf-life” of aptamers is therefore very high which makes aptamers superior to antibodies on ground of long term storage. Also transport will be possible at normal conditions which is more cumbersome with antibodies.

As no animals are used for production of the aptamers, toxic substances and molecules that do not induce an immune response can be used to produce aptamer therapeutics as well. The low immunogenicity of aptamers are an advantage as no unwanted immune response is induced. Antibodies may induce an unwanted immune response due to their Fc region. Aptamers lack this region. Using aptamers could therefore lead to a reduce in side effects.[62] However, the safety issues with aptamers are not very well known yet due to the short time they have been around. Long-term studies still have to be conducted in order to say with confidence that aptamers do not stimulate an immune response.

Aptamers are synthesized in a chemical manner. Therefore their reproducibility is expected to be very accurate. By using very pure targets in a unnatural environment during SELEX the selected aptamers show little batch-to-batch variation.[36] Therefore large scale production with aptamers is expected to be easier.

Due to the small size compared to antibodies, aptamers can access protein epitopes that cannot be accessed by antibodies.[45] A comparison of the size of an antibody relative to an aptamer is shown in figure 14. Subsequently their relative small size makes them easier to synthesize and chemically modify.[63]

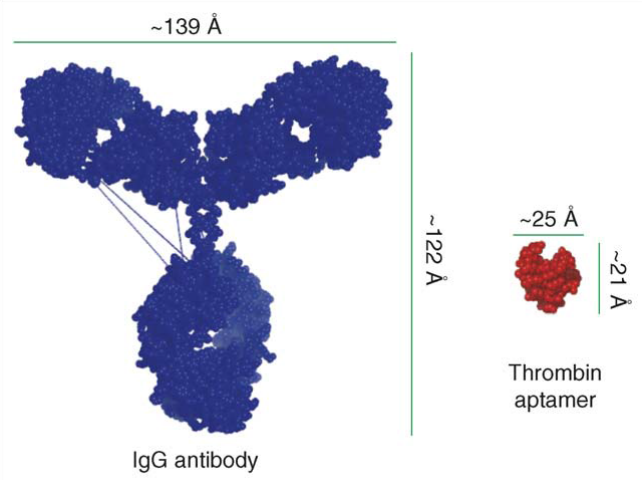

Figure 14. Comparison of size of an antibody and aptamer. The antibody shown is a normal IgG antibody. The aptamer shown is an anti-thrombin DNA aptamer with a length of 17 residues. It should be noted that this is a relatively small aptamer as aptamers of 70 residues are used as well. The usual length of an aptamer however is in between 15 to 50 residues. The amount of residues does not always correlate with the size of the aptamer. The conformation to which is folds also affects the size significantly. As example an anti-streptomycin aptamer with 40 residues approximately possessed the same size as this anti-thrombin aptamer.[63]

For proteins the difference in binding affinity is due to structural difference between the different proteins. As the building blocks of proteins are 20 different amino acids, a large library of different combinations of these molecules can be constructed. This enables proteins to structure very complementary to their binding ligand. Compared to oligonucleotides, which have only four different building blocks, the amount of different combinations is significantly less. This causes the complementary of the aptamer to be structurally less compared to proteins. Antibodies have a much more chemical diversity.[64] Therefore it is expected that antibodies should be able to find a more complementary structure to the antigen. However binding affinities are similar (dissociation constants on nano molar range) for structures that antibodies can target.[65] The deep grooves present in some aptamers compensates for the lack in the complementary structure, causing similar binding strength and specificity compared to proteins.

Although the chemical diversity of antibodies is larger, for aptamers the diversity can be increased by chemical modifications. Because aptamers are being produced in vitro, chemical modifications are more easily performed on aptamers as compared to antibodies. This allows for more freedom while working with aptamers as on- and off-rates of can be modified on demand for example[61] and there pharmacokinects can be

altered as well. Also other functional groups can be added more easily. An example is biotin or fluorescence groups which allow one to determine the position of aptamers with high accuracy. Multiple biotin labels have already been added to an aptamer successfully.[66] Adding functional groups also allows one to study activation of aptamers (see 3.3).

The production of aptamers is expected to be significantly cheaper compared to antibodies.[37] However treatment with the first therapeutic aptamer approved (Macugen) still costs $12,500.[62] In order to improve the cost effectiveness of aptamers still a lot of research needs to be conducted. Automated SELEX already would decrease the costs as the production of aptamers would take less time. It is believed that in the foreseeable future aptamer production costs will decrease.

The large surface of aptamers compared to small molecule therapeutics cause for multiple interactions between the aptamer and its antigen. This not only increases the aptamers affinity and specificity compared to small molecules but it has also been shown that it is harder for viruses and other pathogens to avoid the aptamers via mutations.[63] In addition, due to their relative small size compared to antibodies, aptamers can bind certain epitopes that antibodies cannot. Due to their size, aptamers thus possess advantages over both smaller and larger therapeutics.

3.3 Challenges of aptamers as therapeutics

Nuclease degradation - Antibodies usually have long circulating half-lives compared to aptamers. In normal blood plasma aptamers possess a circulating half-life of only 2 minutes.[67] The main cause is nuclease degradation. For the use of therapeutic the half-life should be increased significantly. Among other things, antibodies possess a longer circulating half-live due to their Fc region. The Fc region causes incorporation of the FcRn receptor inducing FcRn-mediated recycling which increases their half-lives. Secondly their molecular weight is large compared to aptamers which reduces filtration by the kidney.

The targets of aptamers are mostly present in blood plasma or they are present on the surface of cells that are accessible from blood plasma. Therefore aptamers are exposed to nuclease degradation, renal filtration, liver or spleen uptake.[41]

Different methods have been studied to enhance the half-lives of aptamers. The half-lives can be increased by chemical modifications of the nucleotides. In figure 15 the chemical modifications are illustrated. When fluoro,[68] amino[69] or O-methyl[70] is placed at the 2' position an increased nuclease resistance is observed. Macugen, the first aptamer approved for medical applications, used these modified nucleotides to enhance the nuclease resistance.[37] The chemical modifications can be performed before but also after SELEX screening. Adding these functional groups to the aptamer after SELEX could affect the aptamers affinity for the target but this is not always the case.

Another method of increasing nuclease resistance in blood is inverting the nucleotide at the 3'-terminus.[41] In this way the

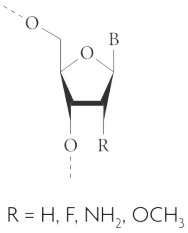

Figure 15. Chemical modification of the nucleotide to increase nuclease resistance.[41]

aptamer has two 5'-termini. As nuclease of the 5'-terminus is lower than the 3'-terminus the resistance is increased. Also the three-dimensional structures of the aptamer could enhance resistance by protecting the termini. When renal filtration also has been limited by covalently bonding to 40 kDa PEG, see paragraph below, aptamer half-lives of 10 days were obtained in humans[41] due to these chemical modifications of the

A relative new method of avoiding degradation of aptamers in vivo is via the use of so called Spiegelmers. These aptamers differ from normal aptamers as there backbone consists of L-ribose (RNA) or L-deoxyribose (DNA) oligonucleotides while natural RNA and DNA consists of D-ribose and D-deoxyribose respectively (the chemical structures of the D- and L-ribose are illustrated in figure 16). Nucleases effectively cleave only the natural occurring oligonucleotides and does not degrade the unnatural Spiegelmers. Therefore transforming a D-aptamer in to a L-aptamer increases nuclease resistance.

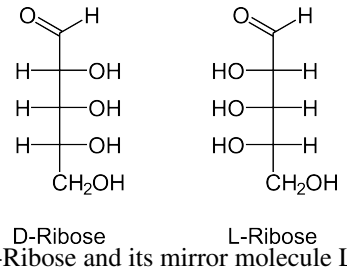

Figure 16. D-Ribose and its mirror molecule L-Ribose. L-ribose nucleotides are used to construct an L-aptamer.

Translating the D-aptamer in to an L-aptamer by sequencing the D-aptamer and rebuilding it using L-oligonucleotides will result in the L-aptamer. However, the binding properties of these L-aptamers differ from the natural D-aptamers. If the D-aptamer binds the natural occurring L-protein, the obtained L-aptamer will bind the unnatural D-protein. In order to target the right L-protein using L-aptamers an addition step needs to be taken. The process of Spiegelmer production is illustrated in figure 17. The L-protein, which is the target, is first mirrored via chemical synthesis in order to obtain the D-protein version of the target. Subsequently SELEX is performed using a library of normal D-oligonucleotides. The selected aptamer is thus a D-aptamer which targets the D-version of the protein. The sequence of this aptamer is identified. The L-aptamer is than produced by rebuilding the identified sequence using

L-oligonucleotides. The L-aptamer obtained binds the natural occurring L-protein. This Spiegelmer has shown to be very resistance against nuclease degradation.[37]

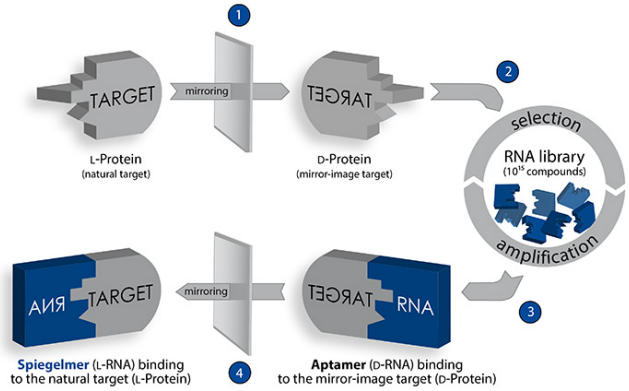

Figure 17. Spiegelmers[71]

Renal filtration - Another problem which should be overcome before aptamers can be used successfully as therapeutic drugs is renal filtration. Compared to IgGs (150 kDa) aptamers (30-60 kDa) are quite small. The kidney removes substances with a molecular weight below 50 kDa easily.[37] Therefore renal filtration of aptamers is a problem.

A relatively simple solution to this problem has been found. By conjugating a large molecule to the aptamer, the molecular mass is increased so that renal filtration is decreased. polyethylene glycol or PEG (40 kDa) has been used most frequently in this regard. Also for antibodies PEG has been used to increase circulation times in the bloodstream. The filtration of PEG-aptamers by the kidney was decreased resulting of circulation times in the order of days. A plot of the concentration of aptamer versus time is shown in figure 18. Here one can see that the aptamers conjugated with PEG show significantly longer circulation half-lifes. A study on the use of cholesterol molecules instead of PEG have shown to increase circulation duration.[72] More recently this has a new study however showed the opposite, as the half-life was decreased using cholesterol.[73] Research on renal filtration is still being conducted and no definite solution has been found yet.

Pharmacokinectis - The duration time in which a drug has therapeutic effects is very important in drug design. Different factors play a role in the pharmacokinetics of a drug such as degradation and filtration. These factors for aptamers have already been discussed.

Chemical modifications to the aptamer may increase the pharmacokinetics but for aptamers there is another way of controlling the duration of action. Several studies have been performed on synthesizing antidotes for aptamers.[74] These antidotes have the complementary oligonucleotide sequences which causes strong binding between the aptamer and its antidote. The three dimensional conformation of the aptamer changes on binding. Therefore the affinity and specificity of the aptamers towards its original target is completely lost. For antibodies such antidotes are not available. Also low-

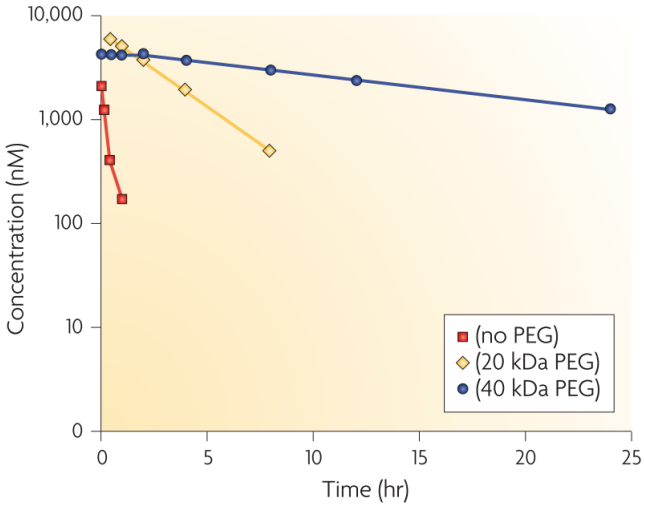

Figure 18. Plot of the concentration of aptamer in the blood versus time. Three data sets for aptamer only, aptamer-PEG(20kDa) conjugates and aptamer-PEG(40kDa) conjugates are plotted. The aptamer used in this experiment was a 39-mer 2'-deoxy purine, 2'-O-methyl pyrimidine composition aptamer. The aptamers were injected in to CD-1 mice at 10 mg per kg.[41]

molecular weight molecules do not have such an option. So aptamers offer a great advantage compared to other therapeutics towards controlling the duration of action in the body. Other methods have been found for controlling the duration time. A new method uses photosensitive modifications that activates the aptamer.[75] Aptamers bound to a photosensitive ligand is inactive. But on exposure of the photosensitive ligand to a certain wavelength, the aptamer loses this ligand which changes the conformation of the aptamer and makes it active.

Membrane penetration - Aptamers are small compared to antibodies but relatively big compared to traditional non-protein drugs. Due to their increased size they do not penetrate membrane as easily. This forms a problem especially for reaching intracellular targets. However, compared to antibodies, aptamers penetrate membranes more easily.

SELEX - In the SELEX process the target molecule is added in order to find aptamers that specifically bind this target. The target should be very pure before adding it to the library of oligonucleotides. This will ensure that aptamers do not bind any contamination and are also selected during SELEX. The purification of the target is very time consuming and cumbersome and sometimes even impossible for some protein targets. For selecting an high affinity aptamer usually eight to fifteen rounds of SELEX are required. One round approximately needs two days.[76] A fast production process for aptamers is desirable for mass producible therapeutics.

A new methods trying to optimize the SELEX method are being studied. An example of such an alternative SELEX procedure is automated SELEX. In 2001 Cox and Ellington increased the production rate of aptamers by a factor of 10-100

compared to manual selection. They implemented a robotic work station to select the nucleotides.[77] They were able to produce aptamers for 120 targets within 1 month. Although this allows for more rapid selection, the chance for selection due to contamination is increased as there were no ”quality control steps” conducted during automated SELEX.

Cross reactivity - Cross reactivity is another obstacle which is sometimes encountered with aptamers. Again the SELEX method produces high specific aptamers. Ideally these aptamers only bind the target. In practice the selected aptamers may still bind to other molecules with comparable structures to the target. This could be advantageous as this could make it possible to target a family of structures rather than one specific molecule. But cross reactivity, however, may also lead to unwanted side effects and should be controlled properly in order to minimize these side effects and enrichment of unspecific bound aptamers.

In order to minimize this process SELEX conditions need to be optimized. By introducing a negative selection step in to the SELEX method for example, cross reactivity is minimized. Similar structures to the target are introduced in the pool of nucleotides. The ones that bind to the structures are subsequently removed from the pool. Only the non-binding aptamers are left behind. These strict selection steps have shown to increase the affinity and specificity of the selected aptamers.[37]

Limited target range - Not all molecules can be targeted with aptamers. Molecules containing positively charged groups (e.g. primary amino groups), hydrogen-bond donors and acceptors and aromatic compounds due to their planarity can be targeted. It is more difficult to target molecules that are hydrophobic or negatively charged (containing phosphate groups).[65]

Addition of hydrophobic parts have been raised as a possible solution in order to solve this problem as was mentioned in section 2.4. Results are promising but this research is still at its start.

4. Discussion

Antibodies have been very successful in the clinic. However, they are not yet the ideal molecules for targeting as not all molecules can be targeted, production is cumbersome, expensive and not easily tunable, batch-to-batch variation causes significant quality differences and immune responses against these therapeutics have prevented large production and application of these therapeutics. These problems have led to the search for new, more ideal substances for therapeutics. In this regard aptamers have been envisioned as superior candidates. But will they be able to realize these high expectations?

As discussed before aptamers have multiple advantages. They have shown to have similar binding affinities, but also outperform antibodies at some instances. Due to their small size they penetrate membranes more easily and have been able to target antigens that could not be targeted with antibodies. In addition, molecules can be targeted that do not trigger an immune response using aptamers. As they are produced in vitro they are easier to work with and chemically modify on demand. This is a big advantage over antibodies, especially in the field of personalized therapy where every patient is being given a personalized treatment depending on their specific needs. The fact that production of large amounts of homogeneous batches is not a problem in combination with long storage times and reversible denaturation makes working with aptamers significantly easier and cheaper compared to antibodies. This makes aptamers both attractive for clinical applications as for industrial synthesis.[42]

More research needs to be conducted on possibly one of the biggest advantages of aptamers. The use of antidotes creates a whole new way of controlling the pharmacokinetics compared to antibodies. This is a big advantage especially because the pharmacokinetics of antibodies are hard to control. This feature may shift the odds of therapeutic use in the favor of aptamers. Still, research is ongoing and real application of antidotes, if it will ever happen, is most likely far down the road.

Aptamers have been around or quite a while now. Since the discovery of their selective binding they have been envisioned as possible replacements for antibodies. Although expectations have been high, thus far aptamers have not been able to live up to these high expectations. When one simply looks at the amount of aptamers used in clinics today a disappointing number of 1 is found, which is small compared to the use of antibodies. Some new aptamers are still in clinical trials waiting to be approved but antibodies are currently more widely available than high-affinity aptamer.[45] Therefore the question arises if aptamers will ever find the light of day in clinics.

New therapeutics are always extensively being studied before clinical use is approved. Of course this is understandable as people’s health might be at risk so extreme caution has to be taken. The introduction of monoclonal antibodies show a nice example of new therapeutics entering the market. They were discovered in 1975 (see section 1.4) but it was not until 1992 when the first monoclonal antibody drug was approved for use in clinics. The subsequent antibody therapeutic was introduced on the marked in 1994.[37] After almost 20 years only two antibodies had entered the market.

Aptamers are considered as possible therapeutics since the 1980s. Now, more than 35 years later, still only one aptamer is available on the market. The reason for their problems entering this market mainly lies with the fact that these new ligands have not been studied as extensively as antibodies. Research towards antibodies goes further back than that of aptamers. Of course DNA and RNA were studied long before the term ”aptamer” was introduced but they were not examined for the use of therapeutics. Antibodies however have their roots in the immune system of mammals, already protecting the body. Therefore a lot more was already known about their binding, structure and function regarding therapeutics before they were even considered as therapeutics. This certainly delays

entrance of aptamers on to the market.

Some problems have to be solved regarding aptamers before clinical use became even feasible. As aptamers are quite small, renal filtration and fast degradation by nucleases were limiting the power of aptamers. Recent advances on these grounds have given reasonable results. PEGylation of the aptamer showed significant longer circulation half-lifes and chemical modifications increased both degradation resistance and binding affinity. However, recent studies have shown that anti-PEG antibodies were induced when using PEG-aptamer conjugates in vivo.[42] Therefore alternative techniques are needed. The new method using Spiegelmers shows promising results regarding nucleases resistance and may make aptamers more suitable for therapeutic use.

Other problems also do not have a clear solution yet. Also aptamers have shown to be limited regarding their range of targetable molecules. Still hydrophobic and negatively charged parts of molecules are problematic targets for aptamers. Chemical modification have tried to minimize these problems. Nevertheless, aptamers are able to target molecules that antibodies are not which enlarges the range of targets.

SELEX is still a very time consuming technique and advances in this regard have not shown very clear solutions. Automated SELEX might be a solution in increasing throughput and decreasing production costs but in compromise to its high-affinity.

Furthermore cross reactivity forms a problem for some aptamers. Aptamers in physiological conditions need to be studied in more detail in order to know if cross reactivity may interfere with the effectiveness and distribution of the therapeutic. Negative selection as additional step during the SELEX procedure has already increased the control over cross reactivity. But still new strategies regarding SELEX need to be found and optimized before aptamers will flourish at their real potential. Most of the aptamer therapeutics are currently tested in vitro. Before real application is feasible in vivo studies need to be performed. Therefore one can expect that the vast amount of aptamer application for therapeutics is not around the corner.

5. Conclusion

The problems with antibodies lie deeply rooted in their natural role in protecting the body. Altering antibodies in order to circumvent the system that also tries to protect our body seems less effective than working with substances that are allowed in our body. With aptamers, solving their problems is more efficient, cheaper and less cumbersome. The use of aptamers therefore surely makes more sense. Looking at the advantages of aptamers it seems inevitable for aptamers to enter the market at some point. As this may take some time, research on antibodies could definitely be useful. Research regarding antibodies may result in temporary solutions for therapeutics that could work very well. Still a lot of potential of therapeutic antibodies is unused. Also, disadvantages of aptamers may be solved by using antibodies instead. Secondly, choosing both instead of one may produce the best results. Antibodies could be used in combination with aptamers in order to get synergistic effects. Amplifying each other's strengths. In the near future both aptamers and antibodies show promising results. Especially combinations of the two could result in very promising therapeutics entering the market at relatively short time scales. However, on the long haul, if one has to choose which of the two the superior one is, future prospect indicate that aptamers are more likely to be the dominant future of therapeutics. Working alongside the system that tries to protect our body may be more natural than working against it.

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