Aptamers as promising agents in diagnostic and therapeutic applications

D. Kubiczek[1], **N. Bodenberger**[1] and **F. Rosenau**[1]

[1] Center for Peptide Pharmaceuticals, University Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany

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1. Introduction

With increasing struggle to treat infectious diseases, for example due to multiple resistances against antibiotics, it becomes more and more important to develop new approaches in terms of therapy and diagnosis to allow an effective treatment. According to the world health organization especially carbapenem resistant *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and *Enterobacteriaceae* like *Klebsiella pneumonia* or *Escherichia coli* are classified as critical [1]. A promising agent for the treatment and diagnosis of such pathogens are so called aptamers. The possible aptamer selection approaches and their application will be discussed throughout this chapter.

The term aptamer is derived from the latin word “aptus”, meaning “to fit” and the greek word “meros”, which means “part”. Aptamers are short single stranded nucleic acids like ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) and consist of up to 100 nucleotides. Through the formation of loops, stems and hairpins aptamers develop a three dimensional structure, which allows them to bind specifically to a certain target similar to an antibody [2]. However, aptamers have several advantages in comparison to antibodies making them a promising alternative for diagnostics and treatment. An outstanding advance of aptamers is their chemical and physical stability. While proteins are known to denature irreversibly at higher temperatures, aptamer reanneal to their original conformation and regain their ability to specifically bind to their target. For this reason aptamers are also stable during long term storage making them suitable for many applications. Furthermore, the identification and production of aptamers is more cost efficient. The identification and production of antibodies involves the immunization of animals and later big scale biotechnological fermentation for their recombinant production [3]. In addition to that, activity differences of the antibody may occur between individual batches making additional testing of each batch necessary. On the other hand, aptamers can be selected completely *in vitro*, even under non *in vivo* conditions, allowing the proper adjustment of the selected aptamer to the desired conditions depending on the desired purpose. The production of the aptamer is then achieved by synthesis by well-defined chemical reactions, making it highly reproducible and thus less differences in their activity appear. This also allows the modification of aptamers easily during the chemical synthesis. This modification can simply regard its stability but also fluorophores or other labels can be introduced to use aptamers as highly specific biosensors. Another important point is that antibodies usually exhibit a high immunogenicity. In contrast aptamers are much less immunogenic or toxic. For example a vascular epithelial growth factor (VEGF) specific aptamer has been reported, which can be given to monkeys at a 1000 fold higher dose than the necessary dose for treatment, detecting only a little immunogenic response [4,5]. Last but not least the generation of specific antibodies is limited due to the fact that they are normally gained through the immunization of animals. Consequently, the isolation of antibodies against toxic targets or molecules that trigger a high immune response is not practicable at all. Furthermore, it is not possible to find antibodies against very small targets which cannot be detected by the immune system. However, aptamers are selected *in vitro*, thus making the toxicity or immunogenicity of the target irrelevant and they are also able to bind very small targets like ions specifically.

2. Isolation of aptamers

The typical method for the isolation of aptamers was first described in 1990 [6–8] and is known as the “systematic evolution of ligands by exponential enrichment” (SELEX). This procedure starts with a randomized library of oligonucleotides, which have known flanking regions. This randomized library is incubated with the target of choice under desired conditions. Aptamers exhibiting an adequate three-dimensional structure under the given conditions will then bind to the target. All remaining unbound aptamers are subsequently removed by appropriate washing steps. The bound aptamers are then eluted from the target and amplified by a polymerase chain reaction (PCR) using primers complementary to the flanking constant regions. In case of RNA aptamers, a reverse transcription is necessary to generate DNA prior to amplification and a successive transcription of the amplified DNA to RNA. In case of DNA aptamers the undesired complementary strand has to be removed to regain a single stranded aptamer. A common method to achieve this is the use of streptavidin-coated magnetic beads and a biotinylated reverse primer. In this way, a biotin residue is introduced to the 5′-end of the unwanted strand. The product is then immobilized to magnetic beads and the aptamer can be released by alkaline strand separation. Another technique is the generation of single stranded aptamers by asymmetric PCR [9]. Hereby the forward primer is added in excess to favor the synthesis of the single

stranded aptamer coding strand while the lower concentrated primer is incorporated into double stranded DNA. The product consisting of single stranded aptamer and double stranded DNA can then be separated by a non-denaturing polyacrylamide gel electrophoresis (PAGE) and the aptamers are eluted from the gel. A further possibility is to generate strands with different length during the PCR. This is achieved by using a primer consisting of the complementary region necessary for the PCR and a spacer like hexaethylen glycol, which functions as a terminator, as well as a polyA extension at its 5´-end [10,11]. The resulting DNA strands can then be again separated by alkaline gel electrophoresis and the desired strand can be purified. Last but not least single stranded aptamers can be generated by digesting the undesired strand with lambda exonuclease. For this purpose, the primer used for the synthesis of the undesired strand is phosphorylated while the other remains unphosphorylated. As the enzyme exhibits a specific 5´→3´-exodeoxyribonuclease activity the phosphorylated strand is removed while the desired ssDNA remains [12]. All this methods are suited for the generation of ssDNA aptamers. However, the usage of streptavidin coated magnetic particles and biotinylation of one strand is the most widely spread, as it does not include time consuming gel purification steps.

But one has to keep in mind that the alkaline treatment of the streptavidin coated beads could lead to the dissociation of the biotin leading to a contamination of the sample with the complementary strands meant to be removed [13]. Nevertheless, purified single stranded aptamers are finally prepared to a new library, which is subsequently used in a further round of selection. This leads to the exponential enrichment of specific aptamer ligands over usually 6 to 20 rounds. The general SELEX procedure is illustrated in figure 1.

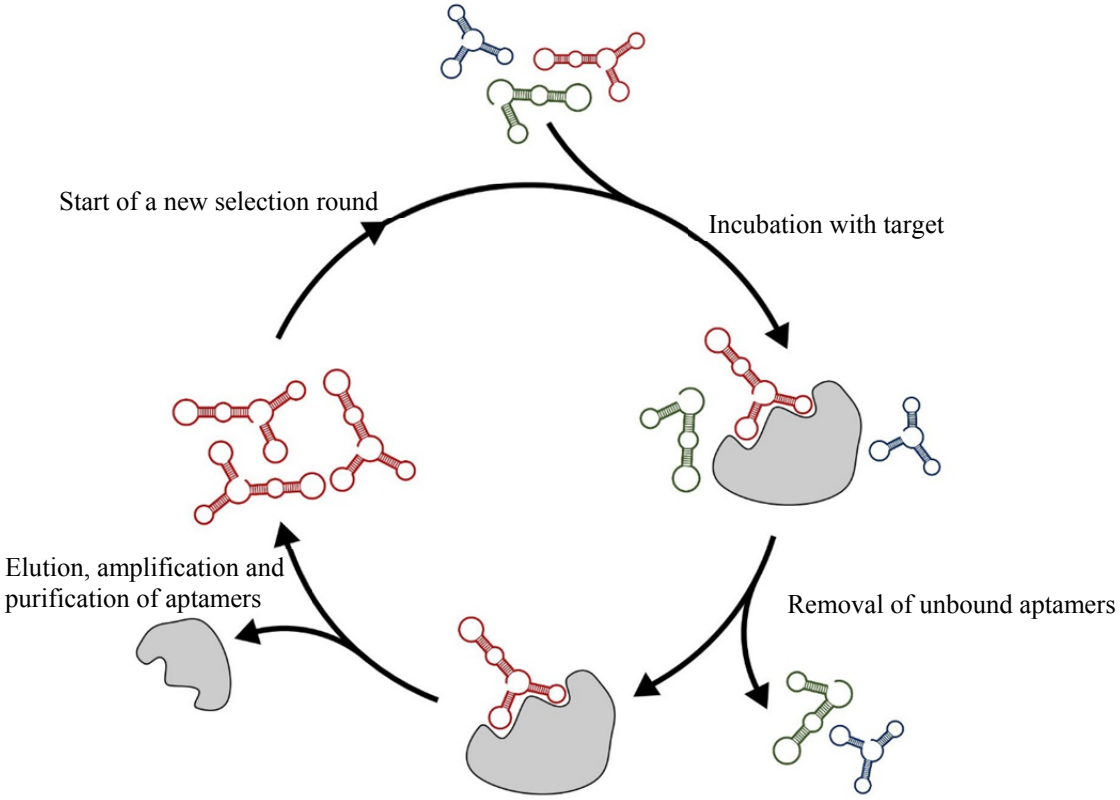

Figure 1. Schematic illustration of the SELEX procedure

After a sufficient number of SELEX rounds the enriched aptamers are sequenced. Therefore, amplified sequences can be cloned into appropriate vectors using restriction enzymes, if the respective restriction sites are present in the constant flanking regions. Therefore, the PCR product and the vector are restricted with the same enzymes, ligated and transformed into bacteria for subsequent sequencing [14]. Another very popular cloning method is based on the addition of a 3´-adenine overhang by the *Taq* polymerase, which is often used during the SELEX procedure. While the PCR products bear the 3´-adenine overhang, the linear vector is designed to have a 3´-thymine overhang. In addition to that topoisomerase I from vaccina virus is used, which specifically recognizes a pentameric sequence and covalently couples to a phosphate group attached to the 3´-thymine of the vector [15]. Upon hybridization of the complementary overhands of the PCR product and the vector, the ligation is catalyzed by the attached enzyme and then released from the vector.

However, this so called TOPO-TA cloning approach also has a major disadvantage. It has been shown that up to 90% of the resulting clones generated double sequence results upon automated DNA sequencing runs making the gained information useless. This is probably due to unwanted cleavage by topoisomerase I leading to an excision of a small fragment from the vector and subsequent recircularization [16]. Furthermore, the system involves a blue white screening of clones. Upon insertion of the PCR product the expression of the *lac* Z gene, which is coding for β-

galactosidase, is disrupted. As a consequence, clones, which contain an insert are not able to cleave 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and the colony remains white, while colonies bearing an empty plasmid turn blue due to the formation of a blue product. But in case of cloning aptamers blue colonies can also bear successfully cloned PCR products being thus false negative. This is due to their small size (often below 100 bp), which may not be sufficient to disrupt the expression of the *lac* Z gene [17,18]. To circumvent this problem and to gain more insight into selected sequences, next generation sequencing (NGS) has emerged as a powerful tool for the analysis of the diversity of enriched aptamers. As the conventional cloning approach results in a few hundred sequences, many sequences are lost or simply not captured [19]. On the other hand, NGS gives millions of sequence reads and thus allows the analysis of the enrichment of single sequences compared to sequences already found in early stages of the SELEX procedure.

Due to this reason NGS has become more and more important in the field of aptamer identification especially with the prices for these techniques drastically decreasing from year to year [20–27].

Aside from this general description of the SELEX procedure, many different variations have been developed to adjust it to the intended approach. Below, some of these variations are highlighted to illustrate the variability and adjustability of the SELEX procedure.

2.1 capillary electrophoresis SELEX

In order to select aptamers against a specific protein, an immobilization of the target is necessary to allow a separation of unbound sequences from the bound ones. There are many different matrices like functionalized agarose or sepharose materials, which have been used for this purpose. Another approach for sequence separation was the filtration of the proteins through an appropriate filter. These methods usually need large amounts of target protein and also possible aptamer binding sites for aptamers might be blocked through the immobilization. Furthermore, washing steps are involved to eliminate unbound or unspecific bound sequences. In capillary electrophoresis SELEX (CE-SELEX) the binding is directly performed in solution. Upon binding of specific aptamers, the target undergoes a electrophoretic mobility shift [28]. During a subsequently performed capillary electrophoresis unbound aptamers leave the capillary first while bound sequences are retained by the target molecule and can then be amplified from the respective fraction. Without any washing steps this procedure allowed the isolation of specific aptamers within only four selection rounds [29].

2.2 FluMag SELEX

The enrichment of aptamers throughout the SELEX process has to be monitored in order to verify its success. This was traditionally done by the introduction of radioactive labels. By measuring the amount of radioactive bound and unbound fractions, conclusions can be drawn about the specificity of the used aptamer pool. However, the radioactive labelling of DNA makes the SELEX procedure quite expensive and in addition to that the process has to be performed under special laboratory conditions for the handling of isotopes. A SELEX modification termed “FluMag SELEX” circumvented this issue by introducing fluorescent labels to the aptamers. This allows to readout the success of the specific aptamer enrichment by the increasing fluorescence signal throughout the procedure. A further advantage of the FluMag SELEX is the use of magnetic particles for the immobilization of a target. This also circumvents the previously mentioned problem of large target protein amounts necessary [10].

2.3 cell-SELEX

While SELEX mainly targets purified proteins, the cell-SELEX approach targets whole eukaryotic or prokaryotic cells in order to isolate aptamers directed towards a specific type of cells. More specifically, the complete cell surface and the including proteins serve as a complex target for the aptamer selection [14]. In case of cells in suspension, several centrifugation steps are necessary for removing unbound aptamers during the selection process. In addition to that often counter selection steps are performed towards other (control) cells. This assures that the selected aptamers specifically target the desired cell type instead of non-specific cell surface structures, which are common for different types of cells including the control. In other words, the counter selection assures the discrimination of the selected aptamers between two closely related targets [30]. A further variation of the cell-SELEX approach was termed “target expressed on cell-surface” SELEX (TECS-SELEX). This approach also targets whole cells, but the selection is driven towards one specific protein displayed on the cell surface. This is achieved by using cells, which express the desired target protein to their surface. The respective counter selection is performed against the same cells, but these cells do not display the target protein. In this way aptamers against the target can be enriched, without a previous purification, which can be challenging especially for membrane proteins [31]. While this procedure was mainly performed with mammalian cell lines, it was also demonstrated for yeast cells. Using a “yeast surface display” SELEX (YSD-SELEX) it was possible to identify target motifs of a certain class of homing endonucleases, which recognize long sequences. In this special case, the SELEX process is performed with a randomized library consisting of double stranded DNA. Subsequently the library is incubated with yeast cells which displayed the homing endonucleases on their surface so that fitting sequences can bind, while others are washed away. Sequences recognized by the homing endonucleases are then isolated and

amplified for the next selection round as usual [32]. Although the YSD-SELEX did not screen for aptamers that specifically bind a target, it perfectly illustrates the broad variability and applicability of the SELEX process.

2.4 AEGIS-SELEX

Compared to antibodies which gain their structural variety from a sequence composed of twenty different amino acids, DNA or RNA aptamers gain their structural differences from four different nucleotides. To increase the variety of possible aptamer folding additional natural not occurring nucleotides are introduced in the aptamer library [33]. The "artificially expanded genomic information system" (AEGIS) adds eight additional nucleobases to DNA or RNA besides the natural occurring adenine, thymine, uracil, guanine and cytosine [34]. As these additional nucleotides are able to pair with each other and furthermore can be amplified by a usual polymerase chain reaction it met all the requirements to be used in a SELEX process. It was also observed that in such a reactions natural occurring bases mutate into artificial ones and vice versa, allowing evolution accordingly to the Darwinian idea further supporting the idea that during SELEX only the best fitting ligands are enriched [35]. In the first AEGIS-SELEX two additional nucleosides P and Z were introduced to the aptamer library which are illustrated in figure 2.

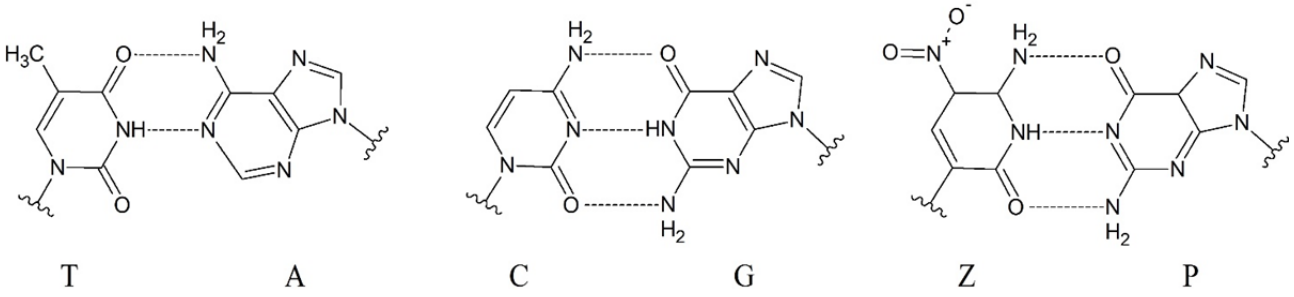

Figure 2. Nucleobases used in the AEGIS-SELEX. Besides the natural occurring bases T, A, C and G, the artificial bases Z and P were added, which are able to pair by forming three hydrogen bridges.

In this AEGIS-SELEX approach a whole cell-SELEX was performed towards a breast cancer cell line. Although, the selected aptamer did not show a higher affinity compared to conventional aptamers, the authors stated that high affinity aptamers were enriched in fewer number of rounds. This conforms with higher diversity resulting from the artificially expanded genetic code, so that the used aptamer library contains more possible aptamers with nanomolar affinities which are consequently enriched faster. It was also observed that the replacement of the artificial nucleobases by natural occurring ones the specificity was almost completely lost [36]. Taken together the AEGIS-SELEX provides an improved method to select genetically expanded aptamers whenever an increased diversity for the SELEX procedure is desired or necessary.

2.5 Genomic SELEX

The starting library of a SELEX process consists of synthetic randomized sequences with constant flanking primer binding sites. For the genomic SELEX approach the library is derived from the genome of an organism. Therefore, genomic DNA is isolated from the desired organism and sheared to obtain fragments. Subsequently constant regions are introduced by random priming and used for the synthesis of the library. After a denaturing gel electrophoresis the library with the desired size is eluted from a gel and a T7 promoter is introduced by an overhang extension PCR to allow transcription and thus the generation of a RNA aptamer library [37]. The genomic SELEX thus helps to identify protein binding RNAs, which would have been missed by cDNA based approaches due to a low transcription level also resulting from different physiological conditions [38].

2.6 Conjugate SELEX

Usually aptamers are selected as single molecules, which are subsequently modified for example by attaching desired cargos. A modified aptamer was e.g. used to coat liposomal nanoparticles for drug delivery purposes. Aptamers are thus used for the recognition of specific cells, while the cargo delivers a drug to the cell [39]. The conjugate SELEX approach demonstrated that it is possible to apply the SELEX process also when the aptamers are coupled to liposomal nanoparticles already in the beginning of the selection process. The aim thereby was to screen only for aptamers which were internalized into cells. Therefore, a nanoparticle coupled aptamer library is incubated with the desired target cells and internalized aptamers are isolated from the cytosol. After amplification, the aptamers are again coupled to liposomal nanoparticles and subjected to a new round of selection. In summary the conjugate SELEX procedure drives the selection towards target specific aptamers, which fulfill predefined properties, in this case for drug delivery purposes [40].

3. Modification of aptamers

When talking about application of aptamers in terms of diagnosis or therapeutics, usually modifications of the aptamers are necessary in order to introduce e.g. labels which are used for readouts or to increase its stability. As oligonucleotides are easy to synthesize, there is a range of different modification which can be introduced, without changing its specificity and affinity towards its respective target. First of all, it is possible to reduce the size of an aptamer to a shorter length, for example by removing the primer binding sites to guarantee a cost-efficient chemical synthesis, without reducing its affinity [41,42]. It was observed that a shortened aptamer can even exhibit a strongly increased affinity [43,44]. However, the truncation of aptamers has to be performed carefully. Although structures formed by the constant regions of the aptamer are less likely to be the ones which are responsible for the specific interaction, as they appear in all aptamers and thus no sequence would be specifically enriched. On the other hand interactions between the constant region and the randomized region can surely be important for the specific folding of the variable region and thus for the affinity towards the target. So, as imaginable there are also examples of aptamers, which exhibited lower or complete loss of their affinity upon truncation [41]. Due to this, the truncation has to be well thought-out by analyzing the enriched conserved motifs and the secondary structures previously. Therefore also bioinformatical tools like ValFold are available, which help to predict secondary structures for the shortened aptamers [45]. Further modifications are made to increase the stability of the aptamer in order to make it suitable for e.g. therapeutic applications. Especially RNA is prone towards degradation by nucleases. The nuclease resistance can be increased by modifications of the sugars in the backbone of the aptamer by introduction of fluoride, primary amino or methoxy groups at their 2´-position [46–50]. Besides that, the use of so called locked nucleic acids (LNA) can increase the nuclease resistance and thermal stability of aptamers. In LNA the 2´-oxygen of the sugar is coupled to the 4´-carbon [51,52]. In addition to its increased stability it was also observed that the usage of an LNA can increase an inhibitory effect of an aptamer in comparison to a conventional counterpart [53]. These locked nucleotide variants can already be implemented during the SELEX process, as an amplification by PCR is possible [54,55]. Another approach to increase the resistance especially towards exonucleases can be the ligation of the 3´-hydroxy and the 5´-phosphate end of the aptamer or of different aptamers to generate a ring structure with several specificities [56]. An extremely high resistance can be also achieved by generating so called “Spiegelmers”. In this special form of aptamers the D-form sugars of the backbone are replaced by their respective L-configurated form. This modification is only possible if the previous SELEX process was performed with the unnatural D-form of the protein using natural D-form aptamer libraries. The selected aptamer can then be synthesized in its L-configuration and binds to the natural L-protein target. Spiegelmers are usually highly stable towards degradation, because the L-aptamer is not a target of nucleases [57–59].

In order to make aptamers applicable as therapeutics they often have to be PEGylated to increase their bioavailability. Due to their small size aptamers can be quickly cleared out from the blood stream through renal filtration. The conjugation of PEG residues to the aptamer leads to a decreased renal excretion so that the aptamer remains in the blood stream for up to several days. A further positive effect of the PEGylation is that the efficiency of the delivery into tissues and organs is increased [60,61]. The nucleobases of aptamers are usually modified at the C5 position of pyrimidines for example to further improve its affinity [62]. Therefore, a range of for example hydrophobic or hydrophilic groups are capable to be introduced to tune the binding ability of the aptamer. It has been shown that already a single modification of a base can lead to a 37 fold increased binding affinity [63]. Other modifications like 5-iodouracil or bromouracil nucleotides can be used to generate aptamers which can be crosslinked to their target by a photochemical reaction, which helps to identify the interaction site between aptamer and target [64–66]. Finally, the introduction of fluorescent dyes to the 5´ or 3´-end or over a functional group introduced to a base of the aptamer make them a powerful tool especially in diagnostic applications, as will be discussed later on. Figure 3 gives a short overview of possible modifications that are used to improve aptamer stability, affinity and functionality.

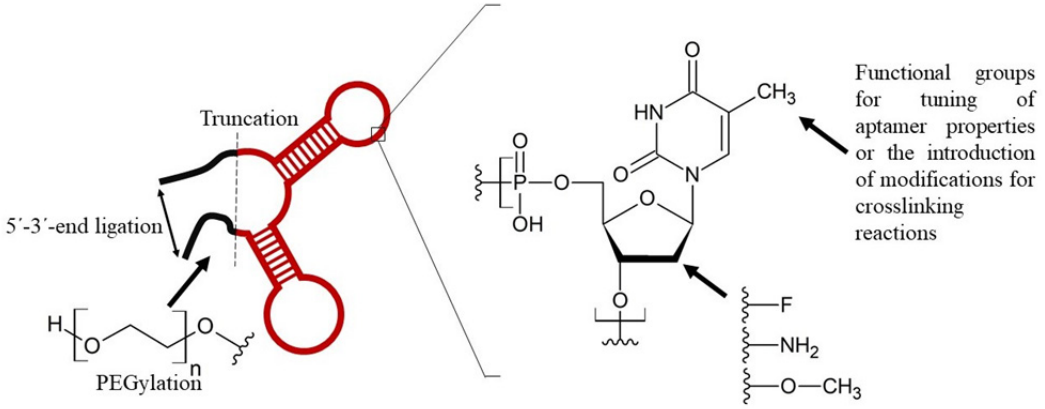

Figure 3. Modification of a selected aptamer. Possible modification approaches and specific modification sites are indicated by arrows.

4. Application of aptamers in diagnostics

In public health and in food industry the detection of pathogens is extremely important. Due to their high specificity, chemical stability and their easy and cost efficient synthesis, aptamers are a perfect reagent in diagnosis. Therefore, numerous biosensors based on aptamers have been developed of which some are subsequently highlighted to illustrate the broad applicability of aptamers in diagnostic assays. Aptamers can be coupled to fluorescent nanoparticles, which then selectively and specifically bind to target cells. This was already shown for aptamers directed against *Escherichia coli*. The nanoparticle labelled cells can be detected using a microfluidic system on a single cell level [67]. Other approaches rely on the directly on the fluorescent labelling of the aptamer. For example, a molecular beacon was constructed from an aptamer, which specifically binds and inhibits the tat protein of HIV-1. More precisely two oligonucleotides were developed based on the aptamer. Thereby the first oligonucleotide formed a hairpin structure and was labelled with a fluorescent dye and a respective quencher. In the presence of the tat protein the hairpin structure was disrupted and the oligonucleotide formed a duplex structure with the second one. This structural change leads to the separation of the fluorescent dye and the quencher so that the presence of the target can be detected by the appearing fluorescent signal [68]. A quite invers approach is to introduce a complementary strand to form a double stranded oligonucleotide with the aptamer. One strand thereby is labelled fluorescently while the other bears the quencher. If the target is present the dissociation of the strands is forced and fluorescence can be measured [69,70]. Another detection technique in which aptamers can be applied is the surface plasmon resonance (SPR) spectroscopy. This method detects the change of the refractive index of a metal surface, which is highly dependent on the medium close to the surface.

Consequently changes in this area for example due to the interaction of two components can be measured [71]. Based on this principle it was possible to detect the pathogenic *Staphylococcus aureus* on a single cell level by surface enhanced Raman scattering (SERS). A specific aptamer was coupled to gold nanoparticles which then bound to the bacteria. A further specific aptamer was coupled to magnetic nanoparticles, which allowed the purification of the pathogen and thus resulted in an amplification of the signal [72]. Besides fluorescent dyes the signal for an optical readout can also be generated by enzymatic reaction. This has been implemented for the detection of *Salmonella parathyphi* A. The specific ssDNA aptamer was coupled to a catalytic DNA sequence (DNAzyme) and wrapped around single walled carbo nanotubes (SWNT) by a self-assembly reaction. Upon binding of the bacterial cells to the specific aptamer, the structural change allows the release from the SWNT and the binding of hemin to the DNAzyme sequence and thus formation of a horse radish peroxidase mimicking complex. This complex then generates a chemoluminescent signal through the oxidation of luminol [73]. Furthermore, aptamers targeting the outer membrane protein C from *Salmonella typhimurium* were implemented in an aptamer immobilized enzyme linked immunosorbent assay (ELISA) and an aptamer linked precipitation assay. For the aptamer immobilized ELISA the aptamer was biotinylated and immobilized in a streptavidin coated plate. Bacterial cells were then bound to the immobilized aptamer and incubated with a specific antibody. The subsequently added secondary antibody was conjugated to horseradish peroxidase to generate a luminescent signal as previously described for the DNAzyme based application. Although antibodies were used in this assay it is imaginable to perform this assay exclusively using aptamers in a so called enzyme linked oligonucleotide/oligosorbent assay (ELONA/ELOSA). The readout signal would thereby be generated by enzyme linked or fluorescent labelled aptamers [74,75]. The aptamer linked precipitation assay was also performed using the luminescent signal generation, by hybridizing an extended version of the aptamer with a biotin labeled oligo-dT.

Bacteria were then centrifuged and resuspended in a horseradish peroxidase coupled to streptavidin conjugate containing solution. The incubation with the substrate then generated the luminescence signal for the readout. In another approach the aptamer was labelled with radioactive phosphate and the bacteria-aptamer complexes were detected by measuring the radioactivity with a liquid scintillation counter [76]. Other approaches utilized aptamers in combination with an electrochemical readout. In an approach to detect *Staphylococcus aureus* electrochemically, specific aptamers were immobilized on magnetic beads to allow the capture of bacterial cells. A second specific aptamer was immobilized on silvernanoparticles. With these constructs bound to the bacterial cells, *S. aureus* was separated from a sample and the silver nanoparticles were then dissolved in a diluted nitric acid solution to produce silver ions. The amount of silver ions, which then could be determined by voltammetric measurements, was directly proportional to the bacterial cell concentration [77]. Another biosensor relying on electrochemical measurements was developed for the detection of *Salmonella*. For this purpose a carbon electrode was modified with graphene oxide and gold nanoparticles to improve its electrochemical properties. A thiolated specific aptamer was then coupled to the surface of the electrode. By incubation with *Salmonella* containing solutions the bacteria were consequently bound to the electrode and after washing to remove unbound cells the electrode was transferred into an electrolyte solution. Depending on the number of bacteria bound, the electrochemical properties between the electrode and the electrolyte changed. With increasing number of bound cells a drop of the current and an increased resistance was determined. Thus, the presence and amount of *Salmonella* could be determined by the changes of these parameters with a detection limit of 3 colony forming units per milliliter [78]. A quite similar approach was performed using diazonium modified carbon electrodes which were again coated with a specific aptamer. In these experiments, again a very high sensitivity was demonstrated with an detection limit of 6 colony forming units per milliliter [79]. Finally, it has to be mentioned that besides the direct detection of microorganisms also important microbial toxins should not be neglected when talking about public

security. The anthrax toxin from *Bacillus anthracis* for example, which is a threatening, terroristic bioweapon due to its high stability and lethality, is involved in an early state of infection, at which it is very hard to detect. But as the first symptoms of such an infection are unspecific it is necessary to have a sensitive diagnosis system to allow a quick treatment before it reaches the lethal stage [80]. A highly sensitive aptamer based sensor was developed for such purposes. The aptamer was immobilized to already mentioned single walled carbo nanotubes which connected two gold electrodes. Upon binding of anthrax toxin to the aptamer the high increase of the resistance could be determined electrically delivering a device which allows the detection of the toxin with a limit of 1 nM [81]. Another toxin which detection is important in food safety and also because of its potential as bioweapon is the botulinum neurotoxin from *Clostridium botulinum* [82]. Also for this bacterial toxin a highly sensitive electrochemical sensor was developed. An aptamer was isolated and immobilized on an electrode. Upon binding of the botulinum neurotoxin a structural change of the aptamer leads to an increased electrochemical current signal [83]. Although this summary of aptamers applied for the detection of bacteria is not complete it strongly demonstrates the power of aptamers in biological sensor systems.

5. Aptamers as therapeutics

The first aptamer approved for treatment by the food and drug administration is called pegaptanib and is distributed by Pfizer under the name Macugen. Pegaptanib is an PEGylated RNA aptamer with fluoro-modified sugar, which inhibits the vascular endothelial growth factor and is therefore used to treat wet age related macula degeneration [84]. Further aptamers like the emapticap pegol (NOX-E36) from NOXXON Pharma, which could be used for the treatment of type 2 diabetes, are already in clinical trials [85]. Most of these aptamers target important molecular structures involved in cancer, heart diseases etc. [86–88]. Nevertheless, the potential of aptamers in antimicrobial treatment has not remained unexplored. Besides the previously described use of aptamers for the detection of botulinum neurotoxin also three RNA aptamers were reported, which are able to inhibit the action of this bacterial toxin, which is known as the most potent poison known to mankind while no antidotes are available [89]. The reported inhibitory aptamers exhibit a high potential as such antidotes for the treatment of deadly botulism [90]. Further therapeutic promising aptamers were isolated by a cell SELEX approach. Specific aptamers were selected against *Salmonella typhimurium* and *Salmonella enterica* and respective aptamers were analyzed by the surface viable counting method [91]. Therefore, bacteria were incubated with the aptamers and then plated. In this way, an antibacterial effect was observed. Although the molecular mechanism remained unknown, the aptamers appear to lead to the depolarization of the cell wall membrane, which was irreversible in some cases, and thus the suppression of colony formation [92]. This demonstrates the direct applicability of aptamers as antibiotics. Another approach for aptamer assisted antimicrobial treatment targets the evolved antimicrobial resistances. β-Lactamases for example are able to hydrolyse and thereby inactivating β-lactam antibiotics.

An aptamer selective towards a metallo-β-lactamase from *Bacillus cereus* was selected and showed an inhibitory effect on the enzyme. The isolated DNA aptamer was shortened to 10 nucleotides without changing its binding characteristics. Due to changes in the active site of the enzyme upon aptamer binding, the hydrolysis of β-lactam antibiotics was inhibited. Thus, incubation of resistant *Bacillus cereus* with a combination of the aptamer and β-lactam antibiotics leads to an inhibited bacterial cell growth. Summarized, aptamers can be used to circumvent antibiotic resistances to allow the treatment of drug resistant bacteria [93]. Other approaches rely on the conjugation of specific aptamers to other components as effectors. For example, aptamers were isolated, which bind to lipopolysaccharide from *Escherichia coli*. This aptamer was conjugated to the C1qr protein, which is the first component of the complement system. In this way it was possible to bind the aptamers to bacteria and the conjugated C1qr triggered the activation of the complement system way above the natural response mediated by non-specific activation induced by lipopolysaccharides [94]. A comparable approach was chosen using an aptamer directed towards poly-D-glutamic acid, which can be found in the capsule of *Bacillus anthracis*. The aptamer was then conjugated to the Fc-portion present in antibodies to use the conjugate for opsonization. This opsonization lead to the internalization of magnetic particles coated with poly-D-glutamic acid and thus demonstrated possible use of aptamer hybrids for the treatment of encapsuled bacteria [95]. A different utilization of aptamers against bacterial pathogens was targeted towards pathogenic pathways. *Mycobacterium tuberculosis*, the cause of the lethal disease tuberculosis, is also developing more and more antibiotic resistances. The pathogenic pathway of *M. tuberculosis* includes the invasion of bacteria into alveolar macrophages to escape the immune defense mechanisms. Consequently, an inhibition of the invasion of the bacteria would be a powerful approach for treatment of tuberculosis. Therefore, aptamers were isolated against *M. tuberculosis* in a cell SELEX approach [96]. These aptamers were able to prevent the invasion of bacteria into macrophages and T-cell. As an interesting side effect it was observed that the specific aptamers also increased the production of interferon-γ, which is responsible for a further decrease of *M. tuberculosis* invasion efficiency. The potential of these aptamers as possible anti-tuberculosis agents was finally strengthened by animal studies [97,98].

Other bacteria are able to form biofilm, which can mediate an up to 1000-fold increased resistance towards antibiotics compared to bacteria in solution. Aptamers specifically selected against the biofilm forming *Salmonella choleraesuis* were isolated in a whole SELEX approach. Bacteria incubated with the selected aptamers were not capable anymore to attach to surfaces and thus the biofilm formation was inhibited [99]. Consequently, these studies not only demonstrated a further possible application of aptamers in biofilm associated disease treatment, but as biofilm

formation in clinical tubing is also a threatening source of infection, also the coating of such instruments with antibiofilm aptamers might be a potential tool to limit the infection in hospitals.

Taken together, aptamers are a powerful and promising agent for future treatment of bacterial infections. Besides its inherent potential as antibiotics, aptamers can also be used to inhibit crucial intermediates which trigger pathogenicity.

6. Conclusion

Although aptamers will probably not replace already approved antibodies, they are a promising agent for future approaches. Due to their low immunogenicity, their high stability and their cost-efficient production aptamers exhibit several advantages over antibodies. Furthermore many different isolation methods of aptamer isolation have been developed, delivering a toolbox to precisely apply the SELEX process to the desired needs. As modifications can be easily introduced during the synthesis of aptamers, they can be perfectly fine tuned according to the desired application.

With their high specificity aptamers are powerful agents in diagnostic approaches, which can help to improve biological safety and diagnosis in clinics and food industry. Also the potential as antimicrobial agents has already been clearly demonstrated. With the first therapeutic aptamer already approved for clinical use and further aptamers in clinical trials, we currently experience the beginning of aptamer based therapeutic treatment.

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