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Abstract

The usage of oligonucleotide aptamers as biosensors presents a novel and promising approach for the detection of pathogen-associated antigens. Based on the non-covalent binding of DNA or RNA sequences, selective and sensitive assays for the detection of various target molecules have been proposed. Autonomous Detection Nucleic Acids (AUDENAs) exploit the recognition properties of aptamers through combination with to date widely unconsidered DNAzymes. Herein, an aptamer sequence for a *Staphylococcus aureus*-derived target protein, namely staphylococcal enterotoxin B (SEB), was used as target-binding unit and joined to various G-quadruplex (G4) DNA sequences possessing biocatalytic properties. Several differently designed AUDENAs were used to indicate and quantify the presence of SEB. Upon addition of hemin, the G4 structures integrated into the AUDENAs are capable of forming a G4-hemin DNAzyme. These enzymatically active units mimic the commonly known horseradish peroxidase (HRP) and catalyze the oxidation of ABTS by H_2O_2 to the corresponding radical cation $\text{ABTS}^{+\cdot}$ which possesses a distinctive green color.

Within the scope of this study, the experimental parameters of the detection assay were examined and optimized. In particular, the influence of the concentration of reactants as well as incubation times was analyzed. In addition, different AUDENA designs were evaluated. The variation of reactant-specific incubation times turned out to largely influence catalytic activity, which was reflected by changes in absorbance obtained through UV-Vis spectroscopic measurements. The incubation time of the proposed biosensors with hemin for the formation of a fully functional DNAzyme as well as the presence of ionic species (NaCl and/or KCl) in the aqueous reaction mix was found to play an important role in the overall efficiency of the AUDENAs. Out of all designs tested within the present study, the highest catalytic activity - which was obtained by means of measuring initial reaction rates the relative activity of the AUDENA in the presence and the absence of SEB - was observed using a linear design. The results indicate applicability of AUDENAs for the detection of SEB. However, further optimizations of the experimental set-up are necessary to enhance the efficiency of target detection through the colorimetric detection system presented in this study. The G4 HRP-mimicking DNAzymes enabled the production of colorimetric signals upon detection of SEB in the sample solution.

Overall, the oligonucleotide-based biosensors developed within this study demonstrated the potential for SEB screening in clinical as well as in food or environmental samples using different designs of the DNA probes presented. Therefore, this assay could be applied for the fast and reliable detection of bacterial pathogens which represents an ever-increasing challenge in modern diagnostics.

Kurzfassung

Aptamere als Sonden zu nutzen stellt eine neuartige und vielversprechende Herangehensweise zur Detektion von Pathogen-assoziierten Antigenen dar. Basierend auf nicht-kovalenten Wechselwirkungen von DNA- oder RNA-Sequenzen wird der selektive und sensitive Nachweis von verschiedensten Molekülen realisiert. Autonome Detektions-Nukleinsäuren (AUDENAs) kombinieren sowohl die von Aptameren bereits bekannte Eigenschaft, Zielmoleküle erkennen und spezifisch binden zu können, also auch die bis dato oft unterschätzten DNAzyme. Im Rahmen dieser Arbeit wurde eine Aptamersequenz für ein von *Staphylococcus aureus* stammendes Protein, Staphylokokken Enterotoxin B (SEB), mit unterschiedlichen G-Quadruplex (G4) DNA Sequenzen, die in Anwesenheit eines Cofaktors biokatalytische Eigenschaften besitzen, kombiniert. Mehrere Designs an AUDENAs wurden entwickelt um das Vorliegen von SEB sowohl zu verifizieren als auch zu quantifizieren. Durch die Anwesenheit von Hemin, welches als Cofaktor mit der G4-Einheit der AUDENAs interagiert, werden vollwertige und funktionsfähige G4-Hemin DNAzyme ausgebildet. Diese enzymatisch aktiven Einheiten entsprechen in ihrer Funktion einer herkömmlichen Meerrettichperoxidase (HRP) und katalysieren die Oxidation von ABTS unter Verwendung von H_2O_2 . Das dabei entstehende ABTS^{•+} Radikalkation besitzt eine charakteristisch grüne Farbe und ist UV-Vis spektrophotometrisch nachweisbar.

Im Rahmen dieser Arbeit, wurden die experimentellen Parameter des Ansatzes untersucht und optimiert. Vor allem der Einfluss von Reaktanden-Konzentration sowie den jeweiligen Inkubationszeiten wurde analysiert und ein Vergleich der unterschiedlichen AUDENA-Designs wurde aufgestellt. Die Variation der jeweiligen Inkubationszeiten schlug sich erheblich in der beobachteten katalytischen Aktivität der jeweils verwendeten DNA-Sonde nieder, was sich wiederum in einer veränderten gemessenen Absorbanz offenbarte. Die Inkubationszeit der hier beschriebenen Biosensoren mit Hemin sowie die Zugabe von NaCl und/oder KCl zum Reaktionsansatz zeigten sich dabei als entscheidende Faktoren für die katalytische Effizienz der AUDENAs. Die höchste katalytische Aktivität wurde dabei mit einem linearen AUDENA-Design erzielt.

Die Ergebnisse der vorliegenden Studie suggerieren die Möglichkeit die hier beschriebenen AUDENAs für den Nachweis von SEB einzusetzen. Nichtsdestotrotz obliegen die experimentellen Parameter einer weiteren Optimierung im Hinblick auf ihre Spezifität und Sensitivität. Eine Effizienzsteigerung des Detektionssystems könnte durch weitere Variation der Bedingungen, unter denen die Messungen stattfinden erzielt werden.

Die Aptamer-basierten Biosensoren, die im Rahmen der vorliegenden Arbeit entwickelt wurden, zeigen Potential zur Nutzung der Detektion von SEB in Proben aus der Klinik, Lebensmitteln oder der Umwelt. Der beschriebene experimentelle Ansatz könnte demnach zum schnellen und verlässlichen Nachweis von bakteriellen Pathogenen, der eine immer größer werdende Herausforderung modernen Diagnostik darstellt, eingesetzt werden.

1. Introduction

1.1. Bacterial Pathogens

Bacteria capable of causing infectious diseases in mammals are designated to be *pathogenic*. In contrast to bacteria that are harmless or even beneficial, such as commensal bacteria residing in the digestive tract, pathogenic bacteria are the cause of adverse effects on their hosts. Along with infectious diseases caused viruses or by non-bacterial microorganisms, such as parasites and fungi, pathogenic bacteria are the cause of millions of infections and thousands of deaths each year. According to the World Health Organization (WHO), these numbers are primarily a result of emerging antimicrobial resistance throughout the world (Organization, 2018). Due to the extensive use of antimicrobial agents for the treatment of diseases caused by bacteria, antibiotic resistance goes hand in hand with the occurrence of bacterial infections and represent an ever-increasing threat to clinical medicine. Hence, fast and reliable detection systems for bacteria and bacterial-derived pathogens need to be established in order to contain the spread of untreatable infections.

1.1.1. Staphylococcal Enterotoxin B (SEB)

Staphylococcus aureus (*S. aureus*) is one of the most abundant hospital germs with a high prevalence in the upper respiratory tract and on the skin (Coates, Moran, & Horsburgh, 2014; Ortegon, 2015). As a member of the *Firmicutes* phylum, it is a Gram-positive, facultative anaerobic bacterium with a round shape. In healthy individuals, *S. aureus* is usually part of the commensal human microbiome. Nevertheless, it is also known for being an opportunistic pathogen able to cause adverse medical conditions. Pathogenic staphylococcal strains are involved in the clinical occurrence of various infectious diseases including particularly skin abscesses, infections of the respiratory system and food poisoning. In recent years, more and more antibiotic-resistant strains of *S. aureus* evolved, creating a major concern of modern medicine. Most notably, the clinical treatment of methicillin-resistant *S. aureus* (MRSA) emerged to be a worldwide issue.

Along with other virulence factors, including adherence factors (adhesins) and gene regulatory segments, *S. aureus* produces and secretes so-called exotoxins (Bien, Sokolova, & Bozko, 2011). Exotoxins are proteins involved in the activation and

modulation of the host's immune system. Among these exotoxins, there is a special group of secreted proteins called staphylococcal enterotoxins (SE). SEs are classified as pyrogenic toxin superantigens (PTSAgs) which cause an extensive antigen-independent activation of T-lymphocytes by direct binding to the major histocompatibility complex (MHC) class II proteins on antigen-presenting cells as well as to the β -chain of the T cell receptor (TcR) (H. Li et al., 1998; Sundberg, Deng, & Mariuzza, 2007). The fixation of the interaction between the MHC protein and the TcR results in a massive secretion of pro-inflammatory cytokines as well as of other immunomodulatory factors, which in turn cause an excessive response of the host's immune system. Thereby, SEs play an important role in the development of toxic shock syndrome (TSS). Frequently found in food, staphylococcal enterotoxins are a primary cause for food poisonings (Argudín, Mendoza, & Rodicio, 2010; Tallent, DeGrasse, Wang, Mattis, & Kranz, 2013).

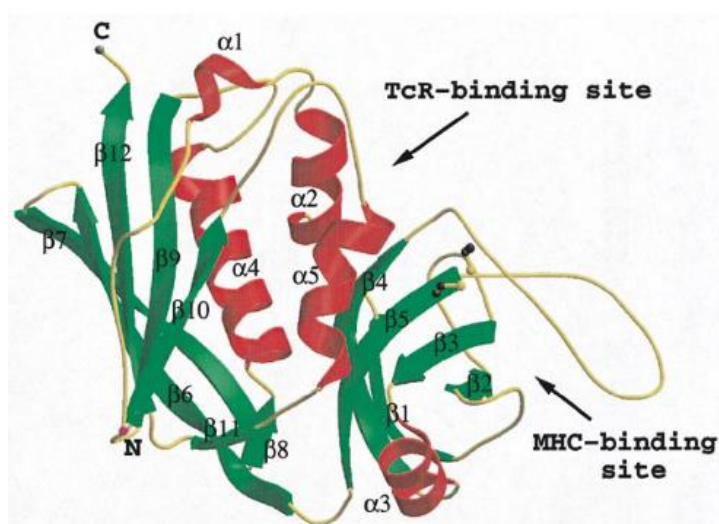


Figure 1 Schematic representation of the crystal structure of SEB and its TcR- and MHC-binding sites indicated by the black arrows. Similar to other known proteins of the staphylococcal enterotoxin family also SEB is composed of two domains containing α -helices (red) as well as β -sheets (green). Amino (N)-terminal and carboxy (C)-terminal segments are marked respectively (Papageorgiou, Tranter, & Acharya, 1998).

Staphylococcal enterotoxin B (SEB) is one of the most immunogenic staphylococcal virulence factors. The protein has a molecular weight of 28.366 kDa and consists of 239 amino acids, which constitute a single polypeptide chain. The structure of SEB is known to an atomic level as depicted in Figure 1 (Papageorgiou et al., 1998). It is

assembled by two unequally-sized domains, an N-terminal oligosaccharide/oligonucleotide binding domain and a C-terminal β -grasp domain, which are formed by a composition of α -helices and β -sheets each (Dinges, Orwin, Schlievert, Biology, & The, 2000; H. Li et al., 1998). SEB shares some distinct chemical properties with the other members of the enterotoxin family (SEA, SEB, SECn, SED, SEE, SEG, SEH, and SEI). Due to their complex and compact tertiary structures staphylococcal enterotoxins tend to be highly resistant to harsh environmental conditions such as heat and low pH and proteases, including trypsin, chymotrypsin, and papain. These proteolytic enzymes are usually found in the digestive tract where they protect the gastro-intestinal epithelium from protein toxins by proteolysis. The high toxicity of SEB becomes even more evident as it has been considered a potent biological weapon in recent years (Janik, Ceremuga, Bijak, & Bijak, 2019).

1.2. Aptamers

Aptamers are molecules capable of binding to a target with high affinity and specificity owing to their particular three-dimensional structures that they adopt to upon the presence of their targets (Ruscito & DeRosa, 2016). DNA and RNA aptamers are usually short (from 20-60 nucleotides), single-stranded oligonucleotides. Today, aptamers based on nucleic acids have spread in many different fields of application, ranging from detection probes to therapeutics. Given the fact that aptamers display several favorable features, such as relatively low cost-production, high stability and their ability to target small molecules to name but a few, they took hold in biotechnological and medical applications (Jayasena, 1999)

1.2.1. Systemic Evolution of Ligands by Exponential Enrichment (SELEX)

Aptamers that specifically bind to a target molecule are generated and selected *in vitro* through a process called SELEX (Systematic Evolution of Ligands by EXponential enrichment) (AD & JW, 1990; Darmostuk, Rimpelova, Gbelcova, & Ruml, 2014; Gold & Tuerk, 1990; W. Li et al., 2016). SELEX enables the production of single-stranded DNA or RNA-aptamers that specifically bind to a target molecule. Starting with an initial library consisting of a large pool of different, chemically synthesized and randomly generated oligonucleotides, the process is based on binding of the nucleic acid sequences to the selected target followed by the removal of unbound sequences

(Figure 2). Oligonucleotides binding tightly enough are eluted from the target and amplified by polymerase chain reaction (PCR). These amplicons serve as new binding probes for the target molecules. Repeating these steps of selection leads to the identification of the most efficient binding oligonucleotides.

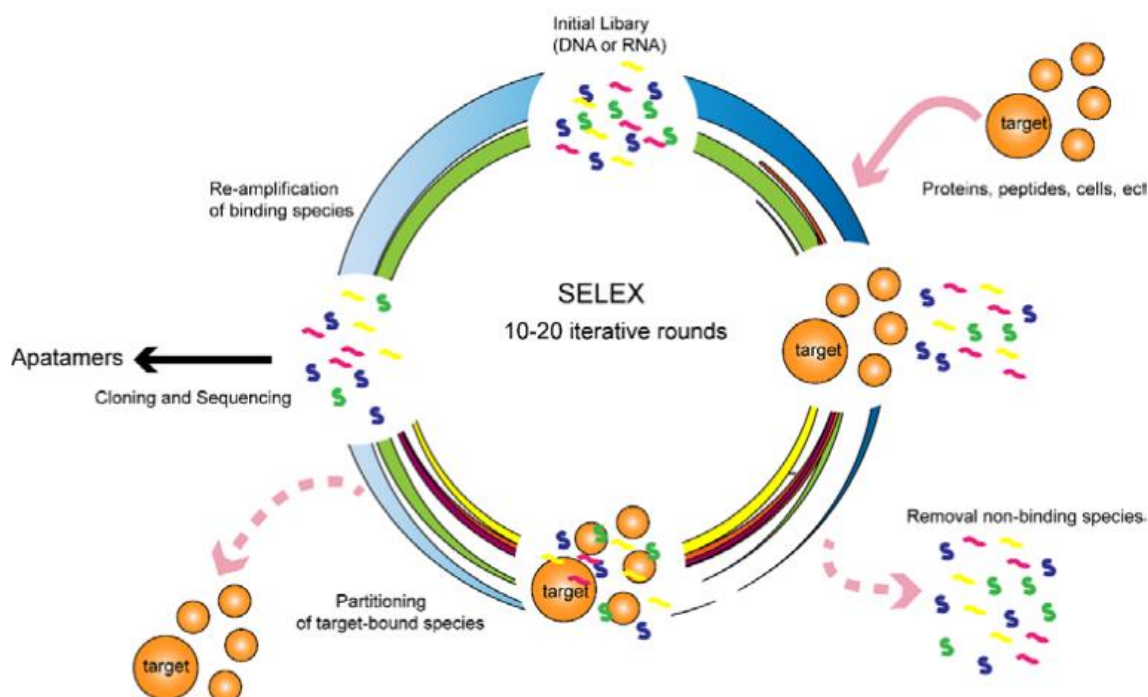


Figure 2 Schematic illustration of the conventional SELEX process (Zhuo et al., 2017). An initial DNA or RNA library containing up to 10^{15} different oligonucleotide sequences serves as the starting point for the selection process (Stoltenburg, Reinemann, & Strehlitz, 2007). The desired target molecule is exposed to the oligonucleotide pool. Non-binding sequences are removed from the selection process followed by segregation of the oligonucleotides from the target and hence a reamplification of the released sequences. After up to 20 rounds of target-binding, removal of non-binding species and amplification of the binding sequences the remaining specifically target-binding oligonucleotides are being segregated for cloning and sequencing. As of now, these sequences qualify for the designation of aptamers.

Targets against which aptamers can be generated are very diverse and range from ions to small organic compounds and even whole proteins.

1.2.2. Aptamer-Target Interactions

Usually, the binding of an aptamer to its target is mediated by more than just one binding force (Cai et al., 2018). Among them, the formation of hydrogen bonds was found to be essential for aptamer-target binding (Mairal et al., 2008). Considering proteins as underlying targets, interactions mostly involve amino acid side chains functioning as H-bond donors. The nucleic acid aptamer contributes to the hydrogen bonding via negatively charged backbone phosphates which act as H-bond acceptors. Several studies demonstrated the dominance of hydrogen bonding in the interaction of the aptamer with the target (Cowan, 2000; Nagatoishi, Isono, Tsumoto, & Sugimoto, 2011).

Nevertheless, also electrostatic forces contribute significantly to the binding between the aptamer and the target. Studies carried out using RNA aptamers binding specifically to proteins revealed that the main interaction between the oligonucleotide and the target was based on electrostatic interactions (Long, Long, White, & Sullenger, 2008; Wurster, Bida, Her, & Maher, 2009). Here, electrostatic forces refer to the interaction of the negatively charged sugar-phosphate backbone of the nucleic acid and positively charged residues of target proteins.

Finally, also hydrophobic effects influence the binding of aptamers to their targets. It was even proposed by *Kimoto et al.* to introduce a more hydrophobic unnatural base in addition to the conventional four nucleotide bases used when generating aptamers through the SELEX process in order to improve binding affinities (Kimoto, Yamashige, Matsunaga, Yokoyama, & Hirao, 2013). Just like single-stranded nucleic acids in solution, also aptamers strongly tend to fold into secondary structures based on the before-mentioned hydrogen-bonds and also on base-stacking of adjacent bases. Shape complementarity with the surface of a protein target strongly relies on the motility of the phosphate backbone of the aptamer. The actual spatial surface of an aptamer interacting with a target is comparable to that of an antibody (Gelinas, Davies, & Janjic, 2016).

Binding mechanisms and interaction surfaces of aptamers have been studied with a variety of target molecules (Cowan, 2000; Huang et al., 2017; Keefe, Pai, & Ellington, 2010; Miller, Tuske, Das, DeStefano, & Arnold, 2016; Presnell & Alper, 2018;

Zimmermann, Wick, Shields, Jenison, & Pardi, 2000). It was shown by *Lin et al.* and later also by *Russo Krauss et al.*, that the presence of ions influence the ability to bind to the target protein of thrombin-binding aptamers (TBAs) (Lin et al., 2011; Russo Krauss et al., 2012). Understanding the structure-based interaction of aptamers with their targets poses a major challenge in the development of effectively binding and selective biosensors.

1.2.3. Aptamers as biosensors and therapeutics

Due to their specific binding properties aptamers are frequently used as sensors, often also referred to as aptasensors (Baldrich, Restrepo, & O'Sullivan, 2005). A fully functional biosensor can be engineered by combining the aptamer as a recognition unit to a sensing unit, which can be electrochemical, optical or mass-sensitive to name but a few (Song, Lee, & Ban, 2012). Among the most current applications of these biosensors are clinical diagnostics of biomarkers in cancer and other diseases and detection of infectious microorganisms in highly diverse sample matrices (H. Hong, Goel, Zhang, & Cai, 2011; P. Hong, Li, & Li, 2012; Ku et al., 2015; Lakhin, Tarantul, & Gening, 2013).

Apart from their usage as biosensors, aptamers were shown to be potential therapeutics in various diseases due to the specific binding to their target. In 2004, the first aptamer was approved by the FDA to be used for the treatment of age-related macular degeneration (AMD) (Gragoudas, 2004). Here, the aptamer functions as an inhibitor directed against vascular endothelial growth factor (VEGF)-165 (Ng et al., 2006).

1.3. G-Quadruplex

G-quadruplex (G4) structures arise from RNA or DNA sequences rich in repetitive guanines. The structural basis of a G4 is four guanine molecules forming a planar tetrad that is intermolecularly stabilized by Hoogsteen bonding and monovalent metal ion coordinated in the center (Figure 3).

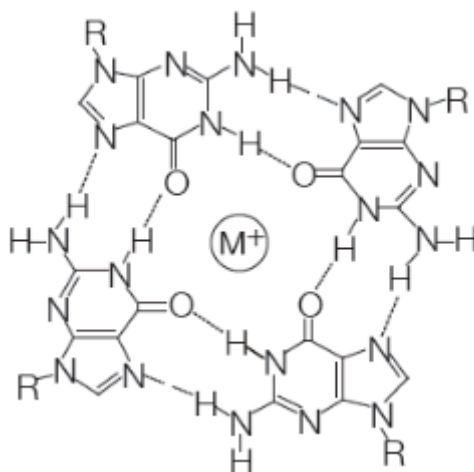


Figure 3 Illustration of a G tetrad consisting of four adjacent G bases coordinated to a central cation (Lerner & Sale, 2019).

Typically, strands with potential G4-forming sequences build up four-stranded helical structures through Hoogsteen base pairing of adjacent G tetrads. In contrast to the far more common Watson-Crick base pairing, which is characterized by the distance between the two C1 atoms being greater than 10 Å, the same C1 atoms in Hoogsteen base pairs display a distance of no greater than 8.3-8.5 Å (Figure 4). Hydrogen bonds in Hoogsteen base pairs are formed between the N3 position of the pyrimidine and the N7 position of the purine, whereas in Watson-Crick base pairs, these bonds are formed through the purine's N1-atom. As a result of the increased distance of the interacting bases, Hoogsteen base pairing enlarges the angles of the glycosidic bonds of complementary bases. Taken together, these spatial differences of Hoogsteen base pairing compared to standard Watson-Crick base pairs ultimately result in changes in the nucleic acids secondary and tertiary structure.

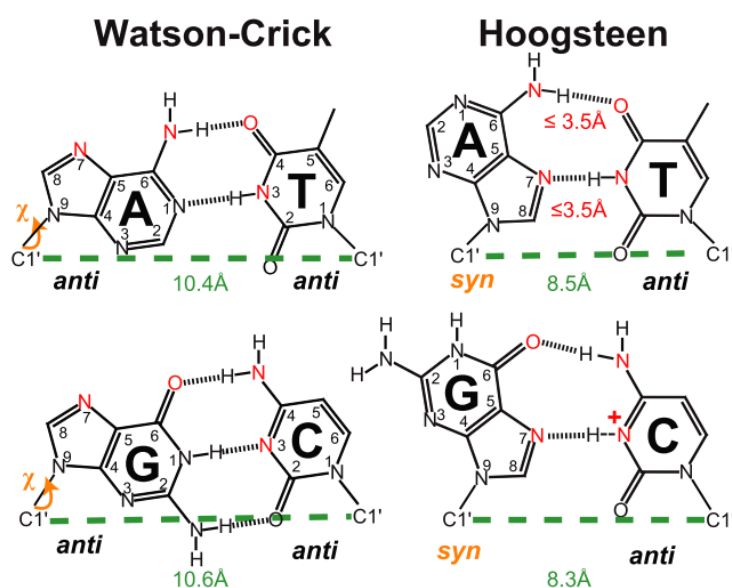


Figure 4 Geometric differences between Watson-Crick and Hoogsteen base pairing (Zhou et al., 2015).

Furthermore, guanines can adopt two different conformations within a tetrad, namely *anti* or *syn* (Cang, Šponer, & Cheatham, 2011). This is referring to the steric position of the base linked to a furanose ring via an N-glycosidic bond. The base can rotate about this bond, which results in *anti* or *syn* conformations, respectively (Figure 4).

Other molecular interactions stabilizing quadruplex structures are stacking base stacking phenomena between the involved guanine bases as well as electrostatic forces (Moon, Han, Kim, Jung, & Kim, 2015). Similar to conventional DNA double helices base stacking was found to contribute even more to overall stability than hydrogen bonding (Yakovchuk, Protozanova, & Frank-Kamenetskii, 2006). In a fully formed G4, the planar tetrads of guanosines are stacked one above the other. Base-stacking occurs on an inter- as well as an intramolecular basis due to hydrophobic and electrostatic forces between adjacent bases (Yakovchuk et al., 2006). This interaction is not only decisive for the formation of G4 structures but also contributes to overall DNA stability (Mak, 2016).

The three-dimensional secondary structure of a G4 is built up by either one, two or four single strands of DNA, referring to as unimolecular, bimolecular or tetramolecular G4s, respectively (Lerner & Sale, 2019) (Figure 5). On a molecular basis, these DNA strands

are formed by at least four adjacent repeats of guanine bases containing a minimum of three up to seven bases each, which are separated by regions not directly involved in the formation of the quadruplex structure itself. Todd et al. defined a universal formula for the in vitro formation of G4 structures: $G_{3-5} NL_1 G_{3-5} NL_2 G_{3-5} NL_3 G_{3-5}$ with NL_{1-3} indicating regions one up to seven nucleotides in length (Todd, Johnston, & Neidle, 2005).

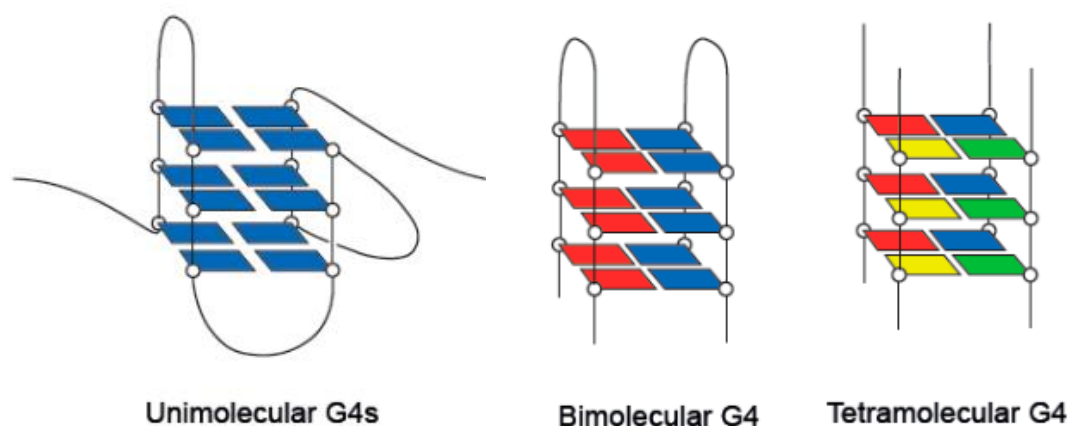


Figure 5 Classification of different types of G4s depending on the number of (different) strands involved in G4 formation (Lerner & Sale, 2019).

1.3.1. Topology of G4s

1.3.1.1. Strand orientation

The three-dimensional molecular structure of G4s is determined by orientations of the strands contributing to a G4. Based on that different terms for classification of G4s exist (Bhattacharyya, Mirihana Arachchilage, & Basu, 2016). If all the strands involved in G4 formation are orientated in the same direction a G4 is designated to be parallel. Contrary to parallel G4s also antiparallel G4s are observed, which possess strands of opposite polarity relatively to one another. Furthermore, also mixed parallel-antiparallel topologies of G4s were found (Esposito et al., 2007) (Figure 6).

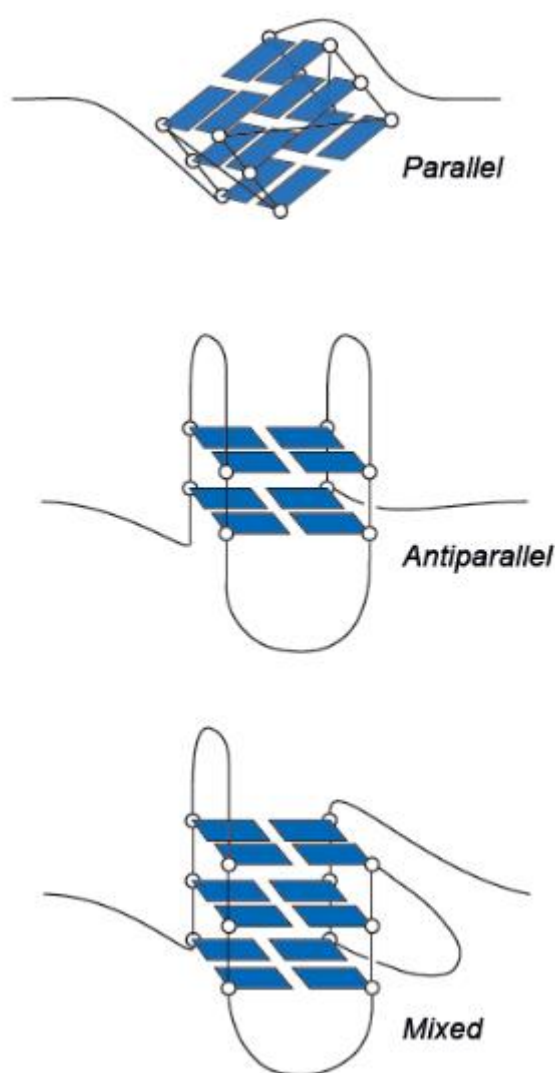


Figure 6 Possible topologies of G4-forming sequences. Depending on the orientation of the strands involved in the formation of the G4, the ultimate structures are either termed parallel, antiparallel or mixed parallel-antiparallel (Lerner & Sale, 2019).

Enzymatically active G4 structures can either arise by the inter- or intramolecular assembly of one up to four DNA strands in either parallel or antiparallel direction (Kong, Wu, Wang, Yang, & Shen, 2009). Transitions between the different states are also possible and depend strongly on various environmental factors.

1.3.1.2. Loops

Adjacent G-repeats within a potential G4 forming sequence are usually separated by linker sequences. These sequences are involved in the formation of so-called loops that are located in-between the actual G4 forming regions (Cheng et al., 2018; Guédin, Gros, Alberti, & Mergny, 2010; Russo Krauss, Spiridonova, Pica, Napolitano, & Sica, 2016). Length and nucleotide composition of loops were found to be crucial in terms of overall conformation and stability of G4s (Agrawal, Lin, Mathad, Carver, & Yang, 2014; Guédin, Alberti, & Mergny, 2009; Hazel, Huppert, Balasubramanian, & Neidle, 2004; Kumar & Maiti, 2008; Manuscript, 2008; Rachwal, Brown, & Fox, 2007; Risitano & Fox, 2004; Sattin et al., 2013).

1.3.1.3. Effect of Metal Ions on G4 Topology

G4-hemin DNAzymes generally rely on the presence of metal ions to be fully active. As a consequence of the polyanionic nature of DNA in solution, positively charged ions reduce the repulsion of negatively charged phosphate backbones. This enables the approximation of intra- or intermolecular guanines of available DNA strands leading to the formation of integral G4s. A G4 tetrad comprising of four square planar guanines is stabilized by centrally located monovalent ions, such as physiologically prevalent potassium and sodium cations (Schultze, Hud, Smith, & Feigon, 1999; Williamson, Raghuraman, & Cech, 1989; S. Zhang, Wu, & Zhang, 2014). These ions, located at the center of the tetrad, are coordinated with the O6 atoms of the four guanine molecules (Figure 7).

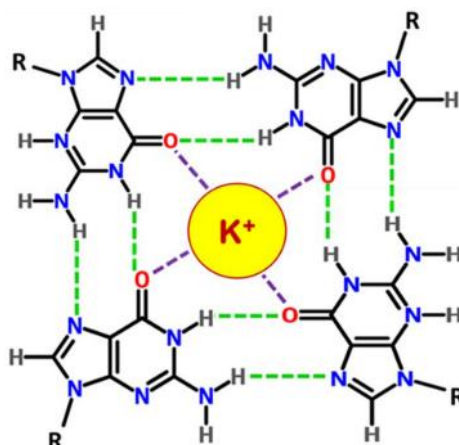


Figure 7 Monovalent potassium cation located in the center of a G tetrad (Bhattacharyya et al., 2016).

The coordinated ions within the center of a G tetrad play an important role in the stability of G4s (Bergues-Pupo, Arias-Gonzalez, Morón, Fiasconaro, & Falo, 2015; Lane, 2012). *Ab initio* quantum chemistry studies showed that the thermodynamic stability of G tetrads depends on the presence of ions even more than on the above-mentioned hydrogen bonds or base stacking interactions (Gu, Leszczynski, & Bansal, 1999).

Apart from stability, the distinct topology that a G4 will adopt to also strongly depends on the type of ion it is interacting with. The very same G4 sequence can form different structures in the presence of one ion or another owing to their coordinated position relative to the G tetrads (Cevec & Plavec, 2005; Črnugelj, Hud, & Plavec, 2002; Gray, Li, & Chaires, 2009; Parkinson, Lee, & Neidle, 2002). NMR and X-Ray crystallography studies of a G4 forming telomeric sequence from *Oxytricha Nova* revealed coordination with five equidistant K^+ cations whereas only four Na^+ were observed to coordinate within the same sequence (Haider, Parkinson, & Neidle, 2002; Horvath & Schultz, 2001). Thus, the distinct three-dimensional structure that a potential G4 DNA-sequence will form upon the presence of cations can also be used for their detection of in biological samples (Yang et al., 2016).

1.3.2. Structure Determination

As structural properties of G4s were found to strongly influence final enzymatic activity, many studies have been carried out to find out more about the effect of topology on

the catalytic behavior of these distinct DNA or RNA secondary structures. The determination of G4 structures can be accomplished through several different methods. Especially spectroscopic methods, such as Raman and CD spectroscopy, provide useful information about structural features and topology of G4s (Vorlíčková et al., 2012).

1.3.3. G4s *in vivo*

Studies on G4s have frequently been carried out *in vitro*, whereas the investigation of their existence and roles *in vivo* is still ongoing (Eddy & Maizels, 2006; Maizels & Gray, 2013; Rhodes & Lipps, 2015; Tarsounas & Tijsterman, 2013). G4 motifs were found to be highly conserved across related species, indicating their biological importance (Capra, Paeschke, Singh, & Zakian, 2010).

According to Huppert and Balasubramanian, over 370 000 potential G4 sequences can be predicted within the human genome (Huppert & Balasubramanian, 2005, 2007). More recent research based on a genome-wide screening revealed that there might exist even more than 700 000 putative G4-forming sequences (Chambers et al., 2015). There, G4s tend to occur predominantly in telomeric regions and regulatory segments. Especially at gene promoters were found to be a frequent source for the occurrence of G4 sequences (Huppert & Balasubramanian, 2005, 2007).

In terms of functionality, G4 sequences were shown to counteract the activity of telomerase at telomeric sites of DNA (Fletcher, Sun, Salazar, & Hurley, 1998; Zahler, Williamson, Cech, & Prescott, 1991). Recent studies revealed that G4 motifs frequently occur in proto-oncogenes making them a relevant target for potential anti-cancer therapeutics (Eddy & Maizels, 2006; Han & Hurley, 2000). Due to their biological role in cell cycle and aging, there is growing interest to aim at G-quadruplexes as potential therapeutic targets (Balasubramanian & Neidle, 2009; Cree & Kennedy, 2014).

1.4. DNAzymes

In recent years the concept of nucleic acids as molecular storage and carrier molecule of genetic information has broadened by the discovery of their enzymatic properties which have increasingly gained importance for practical applications in research (Nayak, Roth, & McGavern, 2014). Catalytically active DNA, also referred to as

deoxyribozyme or DNAzyme, is capable of triggering chemical reactions similar to enzymes comprising of protein domains (Nayak et al., 2014). Besides acting as catalysts for oligonucleotide substrates, there are many other reactions that can be performed by DNAzymes. Due to their many advantages, DNAzymes have gained a lot of attention lately. Cost efficiency, relatively high chemical stability and uncomplicated synthesis are just a few of the outstanding features making them a favorable alternative to conventional protein enzymes.

1.4.1. Horseradish Peroxidase-mimicking G4-hemin DNAzymes

DNA sequences capable of catalyzing the H_2O_2 -mediated peroxidation of organic substrates in the presence of respective cofactors are generally referred to as horseradish peroxidase (HRP)-mimicking DNAzymes (Albada, Golub, & Willner, 2016; Golub, Albada, Liao, Biniuri, & Willner, 2016).

A combination of potential G4 forming DNA sequences with a metalloporphyrin cofactor gives rise to a fully enzymatically active HRP-mimicking DNAzyme (Kosman & Juskowiak, 2011a). Peroxidases and other oxidative enzymes commonly use hemin ($\text{C}_{34}\text{H}_{32}\text{ClFeN}_4\text{O}_4$) as their active cofactor. Hemin interacts with G4-DNA noncovalently via end-stacking (Ghahremani Nasab, Hassani, Mohammadi Nejad, & Norouzi, 2017). Hemin itself displays noteworthy peroxidative properties even in absence of the inherent apoenzymatic unit of any DNAzyme.

However, the noncovalent interaction of G4-DNA with hemin increases peroxidase activity of the latter leading to oxidation of the target molecule via hydrogen peroxide (Kosman & Juskowiak, 2011b). Ultimately, enzymatic properties of HRP-mimicking G4-hemin DNAzymes are used to indicate the presence of various target molecules, which is accomplished through a colorimetric reaction. Oxidation of chromophores, such as ABTS (2,2'-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid)) by peroxidase-mimicking DNAzymes, finds its use in colorimetric assays monitoring changes in absorbance through ABTS radical cation ($\text{ABTS}^{+\bullet}$) formation (Figure 8) (Rodríguez-López et al., 2001; Stefan, Denat, & Monchaud, 2012; L. Zhang, Li, Qin, Chu, & Zhao, 2014). G4-hemin DNAzymes have been used as sensing platforms for various targets including nucleic acids, proteins but also small molecules and metal ions (Paridah et al., 2016).

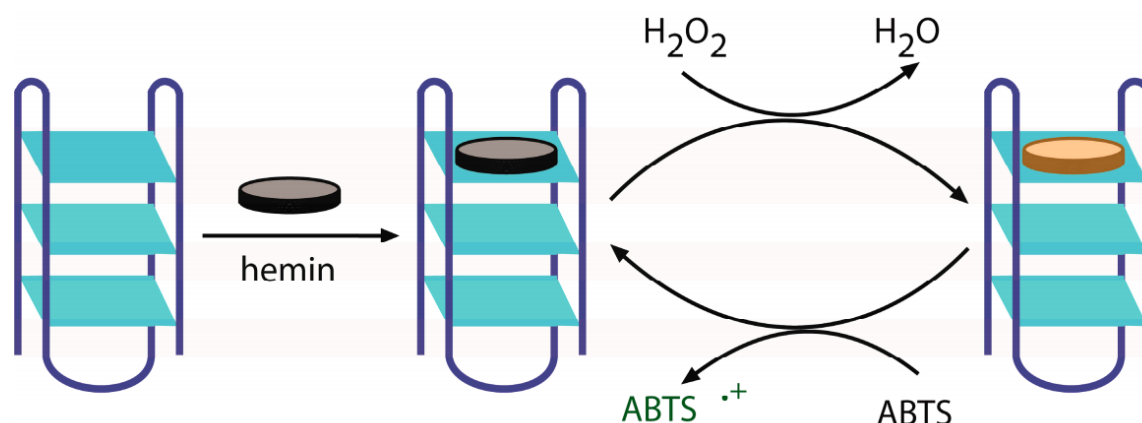


Figure 8 Colorimetric assay based on H_2O_2 -mediated peroxidation of ABTS catalyzed by the G4-hemin DNAzyme (Dnazymes et al., 2019). The hemin molecule that is stacked within the G4 reacts with H_2O_2 to form the very reactive $\text{ABTS}^{\bullet+}$ radical cation. The colorimetric detection is visible to the naked eye and can also be detected spectrophotometrically.

The H_2O_2 -mediated peroxidation reaction, resulting in the intense turquoise-colored product, is visible to the naked eye and represents a widely-used and reliable indicator for monitoring the enzymatic activity of peroxidase-mimicking G4-hemin DNAzymes (Figure 9).

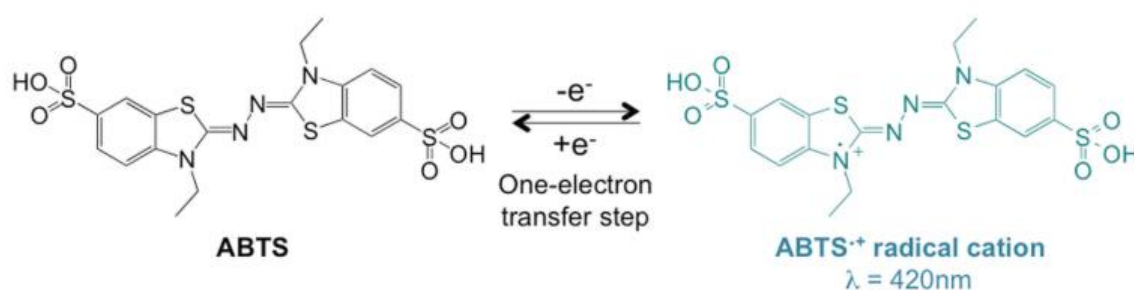


Figure 9 H_2O_2 -mediated peroxidation of ABTS to $\text{ABTS}^{\bullet+}$. The resulting radical cation exhibits an absorbance maximum at $\lambda = 420 \text{ nm}$ (Stefan et al., 2012).

2. Materials and Methods

2.1. Design of AUDENAs

Different designs comprising varying approaches of aptamer binding and G-quadruplex formation were created. The three indispensable building blocks of an AUDENA covered: (i) the aptamer sequence interacting with the SEB target, (ii) a linker sequence, (iii) varying G-quadruplex sequences, which – through the addition of hemin – catalyzed the peroxidation reaction leading to a colorimetric signal.

The functional sequences of the aptamer and varying G4s were obtained from the literature. Thus, an already established aptamer sequence for SEB was used for the design of different AUDENAs: **5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTT GTTTCGT-3'** (DeGrasse, 2012).

G4 sequences were obtained likewise (Chen & Yang, 2012; Kong et al., 2009, p. 464; R. Li, Xiong, Xiao, & Ling, 2012; T. Li, Dong, & Wang, 2009; T. Li, Wang, & Dong, 2010) (Table 1).

Table 1 G4 sequences used for designs of different AUDENAs.

G4	Sequence (5'-3')
AGRO100	5'-GGTGGTGGTGGTTGTGGTGGTGGTGG-3'
Hum12	5'-TTAGGGTTAGGGTTAGGG-3'
Hum21	5'-GGGTAGGGTTAGGGTTAGGG-3'
Myc2345	5'-GGGTGGGGAGGGTGGG-3'
Oxy12	5'-GGGGTTTTGGGG-3'
PS2.M	5'-GTGGGTAGGGCGGGTTGG-3'
PS5.M	5'-GTGGGTCATTGTGGGTGGGTGTGG-3'
PW17	5'-GGGTAGGGCGGGTTGGG-3'
T30695	5'-GGGTGGGTGGGTGGGT-3'

Final AUDENA designs comprising of the SEB-aptamer sequence linked to varying G4s via linker sequences, as described in the following section, were ordered and purchased from Integrated DNA Technologies (IDT), BVBA (Leuven, Belgium).

Secondary structures of the final AUDENA designs were predicted using the Mfold web server (<http://www.bioinfo.rpi.edu/applications/mfold/>) (Zuker, 2003). Mfold provides

thermodynamic calculations for the prediction of folding and hybridization of nucleic acids.

2.1.1. Linear AUDENAs

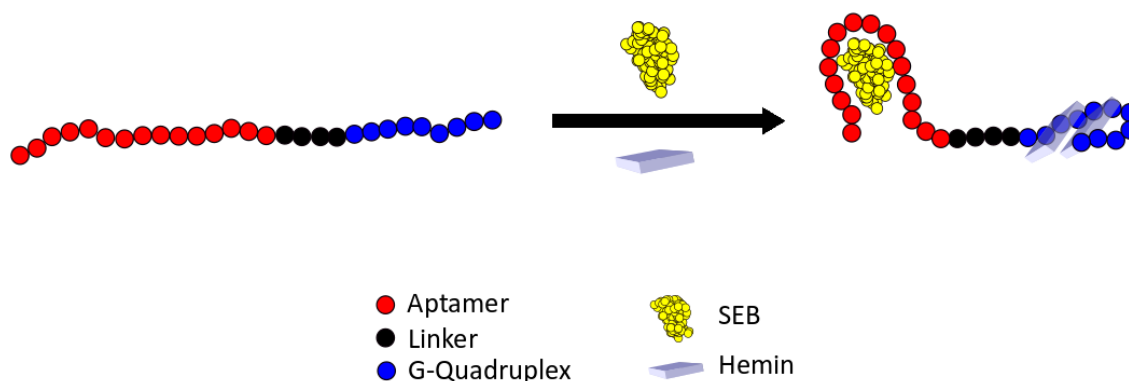


Figure 10 Schematic illustration of the interaction of a linear AUDENA with SEB.

The linear AUDENA design comprised of the aptamer connected to the G4 by a linker sequence in 5'-3' direction. Linker sequences with different lengths and nucleotide compositions were implemented (Table 2). Incubation with SEB followed by the addition of Hemin was thought to lead to the formation of a fully functional G4-hemin DNAzyme and the initiation of the H₂O₂-mediated oxidation of ABTS (Figure 10).

Table 2 Sequences of linear AUDENA designs (red: aptamer, black: linker, blue: G4).

AUDENA	Sequence (5'-3')	Length (b)
Linear_A_L_PS2.M#1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTTGTGGGTAGGGCGGGTTGG-3'	63
Linear_A_L_PS2.M#2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTTTTTTTGTGGGTAGGGCGGGTTGG-3'	68
linear_Hum21 (telomer)_#1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTTGGGTTAGGGTTAGGGTTAGGG-3'	66
linear_Hum12_#2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTTTATAGGGTTAGGG-3'	57
linear_Oxy12_#3	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTTGGGGTTTTGGGG-3'	57
linear_T30695_#4	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTTGGGTGGGTGGGTGGGT-3'	61
linear_Myc2345_#5	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTTGGGTGGGGAGGGTGGG-3'	61
linear_PW17_#6	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTTGGGTAGGGCGGGTTGGG-3'	62

linear_PS2.M_#7	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTGTGGGTAGGGCGGGTTGG-3'	63
linear_PS5.M_#8	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTGTGGGTCATTGTGGGTGGGTGTGG- 3'	69
linear_AGRO100_#9	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTGGTGGTGGTGGTGTGGTGGTGGT GG-3'	71
linear_Hum21_#10	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTTTGGGTTAGGGTTAGGGTTAGGG-3'	65
linear_Hum21_#11	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTATAGGGTTAGGGTTAGGGTTAGGG-3'	65
linear_Hum21_#12	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTCTCGGGTTAGGGTTAGGGTTAGGG-3'	65
linear_Hum21_#13	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTATTAGGGTTAGGGTTAGGGTTAGGG-3'	67
linear_Hum21_#14	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTCTTCTGGGTTAGGGTTAGGGTTAGGG-3'	67
linear_PS2.M_#15	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTGTGGGTAGGGCGGGTTGG-3'	60
linear_PS2.M_#16	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTATAGTGGGTAGGGCGGGTTGG-3'	62
linear_PS2.M_#17	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTCTATAATCGTGGGTAGGGCGGGTTGG-3'	67
linear_PS2.M_#18	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTTATTAGTGGGTAGGGCGGGTTGG-3'	64
linear_PS2.M_#19	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG TTTCGTTCTTCTGTGGGTAGGGCGGGTTGG-3'	64

2.1.2. Linear Complementary AUDENAs

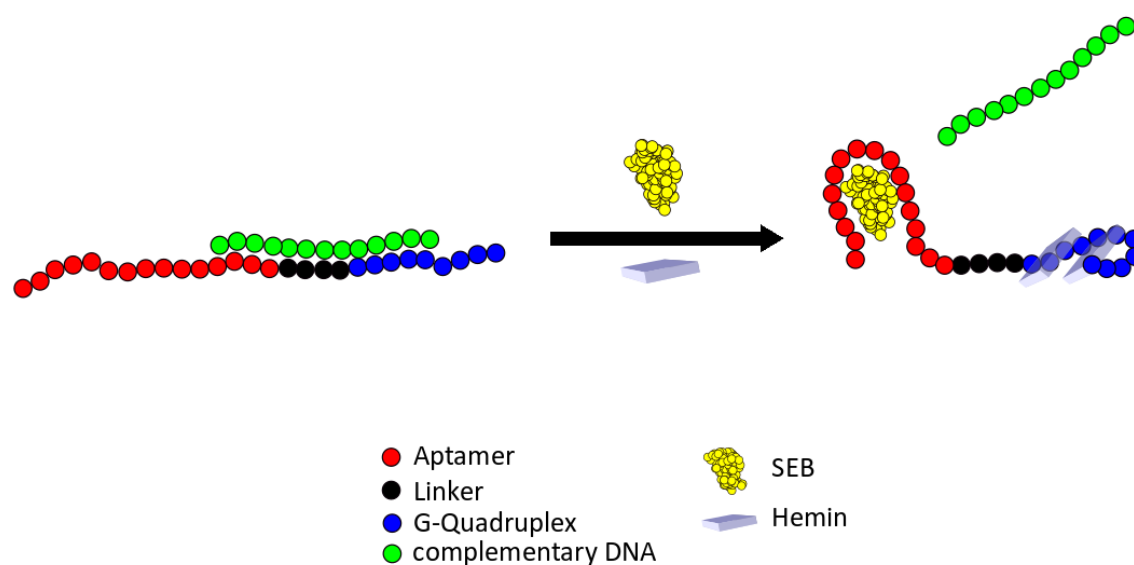


Figure 11 Schematic illustration of the interaction of a linear complementary AUDENA with SEB.

AUDENAs consisting of a linear complementary design were based on the hybridization of two complementary DNA strands. One of the linear AUDENA strands served as the actual detection strand (linear_complementary_PS2.M#1). Its overall constitution was in principle equal to the linear AUDENA design described above, comprising of a single DNA strand with the three building blocks connected to each other in a linear manner. The second DNA strand, which partially hybridized with the main strand, served as the inhibitory component. Upon interaction of the detection strand with SEB, the inhibitory strand disengaged and hence allowed the peroxidation reaction to take place (Figure 11). The inhibition of the aptamer binding domain as well as of the G4 was assumed to result in higher specificity compared to using a single linear strand alone.

Complementary sequences were designed varying in the size of the AUDENA-portion they were intended to cover with regard to the detection strand. Different complementary strands covered either longer or shorter parts of the aptamer and G4 sequence of the main strand. Furthermore, the complementary strands also differed in the length and composition of the linker sequences used to connect the parts complementary to the aptamer and the G4 (Table 3).

Table 3 Sequences of linear complementary AUDENA designs (red: aptamer, black: linker, blue: G4). The detection strand (linear_complementary_PS2.M#1; shaded in grey) had a length of 64 nucleotides, whereas its complementary strands were significantly shorter ranging from 18-32 nucleotides in length.

AUDENA	Sequence (5'-3')	Length (b)
Detection Strand: linear_complementary_PS2.M#1	5'-CACTGGTCGTTGTTGTCTG TTGTCTGTTATGTTGTTTCGTTTTTTT GTGGGTAGGGCGGGTTGG-3'	64
linear_complementary_PS2.M_#1.1	5'-CGCCCTACCCACCACTATACGAAACAACAT-3'	30
linear_complementary_PS2.M_#1.2	5'-GCCCTACCCACCACTATACGAAACAACA-3'	28
linear_complementary_PS2.M_#1.3	5'-CCCTACCCACCACTATACGAAACAAC-3'	26
linear_complementary_PS2.M_#1.4	5'-CCTACCCACCACTATACGAAACAA-3'	24
linear_complementary_PS2.M_#1.5	5'-CTACCCACCACTATACGAAACA-3'	22
linear_complementary_PS2.M_#1.6	5'-TACCCACCACTATACGAAAC-3'	20

linear_complementary _PS2.M_#1.7	5'- ACCCGCCCTACTATC CACTATACGAAACAACAT- 3'	33
linear_complementary _PS2.M_#1.8	5'-CGCCCTACCCACTTTTTTACGAAACAACAT-3'	30
linear_complementary _PS2.M_#1.9	5'-GCCCTACCCACTTTTTTTACGAAACAACA-3'	28
linear_complementary _PS2.M_#1.10	5'-CCCTACCCACTTTTTTTACGAAACAAC-3'	26
linear_complementary _PS2.M_#1.11	5'-CCTACCCACTTTTTTTACGAAACAA-3'	24
linear_complementary _PS2.M_#1.12	5'-CTACCCACTTTTTTTACGAAACA-3'	22
linear_complementary _PS2.M_#1.13	5'-CCCGCCCTACTATCTTTTTTTACGAAACAACAT- 3'	32
linear_complementary _PS2.M_#1.14	5'-GCCCTACCCACAAAAAAAACGAAACAACA-3'	28
linear_complementary _PS2.M_#1.15	5'-CCCTACCCACAAAAAAAACGAAACAAC-3'	26
linear_complementary _PS2.M_#1.16	5'-CCTACCCACAAAAAAAACGAAACAA-3'	24
linear_complementary _PS2.M_#1.17	5'-CTACCCACAAAAAAAACGAAACA-3'	22
linear_complementary _PS2.M_#1.18	5'-TACCCACAAAAAAAACGAAAC-3'	20
linear_complementary _PS2.M_#1.19	5'-ACCCACAAAAAAAACGAAA-3'	18

2.1.3. Hairpin AUDENAs

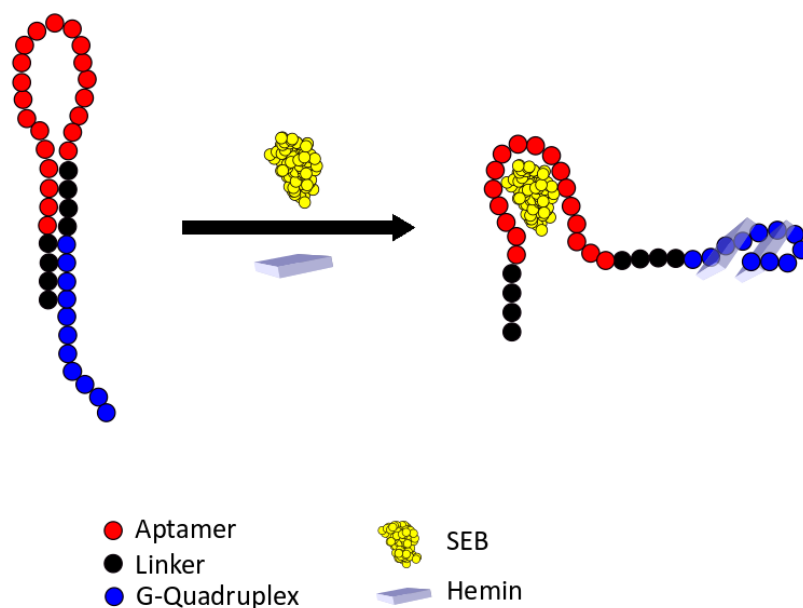


Figure 12 Schematic illustration of the interaction of a hairpin AUDENA with SEB.

A DNA strand being capable of forming of a hairpin-like structure possesses a sequence with partial intramolecular complementarity and another one with unpaired bases forming a loop-like structure at the end of the double helix (Table 4). Like the above mentioned linear complementary AUDENA design, the intramolecular hybridization leads to an inhibition of the aptamer as well as the G-quadruplex sequence. Upon interaction of the aptamer domain with SEB, the hairpin structure is supposed to unfold, making the interaction sites of the G4 with hemin accessible and therefore enabling the peroxidation reaction to take place (Figure 12). Likewise, this was supposed to increase specificity for the target.

Table 4 Sequences of hairpin AUDENA designs (red: aptamer, black: linker, blue: G4).

AUDENA	Sequence (5'-3')	Length (b)
hairpin_PS2.M_#1	5'-CGCCCTACCCACCACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTTCGTCAACGACCAGTGGTGGGTAGGGC GGGTTGG-3'	82
hairpin_PS2.M_#2	5'-GCCCTACCCACCACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTTCGTAAACGACCAGTGGTGGGTAGGGCGG GTTGG-3'	80
hairpin_PS2.M_#3	5'-CCCTACCCACCACTGGTCGTTGTTGTCTGTTGTC	78

	TGTTATGTTGTTTCGTACGACCAGTGTGGGTAGGGCG GGTTGG-3'	
hairpin_PS2.M_#4	5'-CCTACCCACCACTGGTCGTTGTTGTCTGTTGTCT GTTATGTTGTTTCGTGACCAGTGTGGGTAGGGCGG GTTGG-3'	76
hairpin_PS2.M_#5	5'-CTACCCACCACTGGTCGTTGTTGTCTGTTGTCTGTTA TGTTGTTTCGTGACCAGTGTGGGTAGGGCGGGTTGG- 3'	74
hairpin_PS2.M_#6	5'-TACCCACCACTGGTCGTTGTTGTCTGTTGTCTGTTA TGTTGTTTCGTACCAAGTGTGGGTAGGGCGGGTTGG-3'	72
hairpin_PS2.M_#7	5'-ACCCACCACTGGTCGTTGTTGTCTGTTGTCTGTTATG TTGTTTCGTCCAGTGTGGGTAGGGCGGGTTGG-3'	70
hairpin_PS2.M_#8	5'-CCCACCACTGGTCGTTGTTGTCTGTTGTCTGTTATG TTGTTTCGTGAGTGTGGGTAGGGCGGGTTGG-3'	68
hairpin_PS2.M_#9	5'-CCACCACTGGTCGTTGTTGTCTGTTGTCTGTTATGTT GTTTCGTAGTGTGGGTAGGGCGGGTTGG-3'	66
hairpin_PS2.M_#10	5'-CGCCCTACCCACCACTGGTCGTTGTTGTCTGTTGTCT GTTATGTTGTTTCGTCCAGTGTGGGTAGGGCGGGTTG G-3'	76

2.1.4. Proximity Ligation Assay-like (PLA-like) AUDENAs

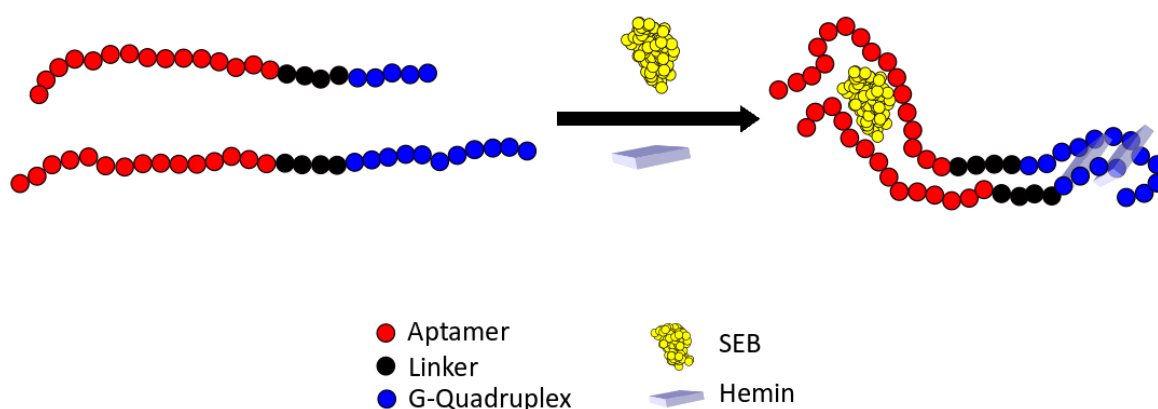


Figure 13 Schematic illustration of the interaction of a PLA-like AUDENA with SEB.

AUDENAs were designed inspired by the concept of proximity ligation assay that is frequently used for the detection of proteins (Battey, James, Greenland, & Brownlee, 2007; Gullberg et al., 2004; Gustafsdottir et al., 2005). Each of the two PLA-like oligonucleotide probes consisted of the aptamer sequence for binding to the SEB-target linked to a G4 sequence each. Notably, each of the two probe strands did only contain parts of the total G4 sequence (Table 5). Upon binding of the aptamers to SEB, the two probe strands and consequently also the two parts of the G4 got in proximity

to each other, thus being able to form a fully functional G4 (Figure 13). Through the usage of PLA-like AUDENAs sensitivity and specificity of the detection might be increased.

Table 5 Sequences of PLA-like AUDENA designs (red: aptamer, black: linker, blue: G4).

AUDENA	Sequence (5'-3')	Length (b)
PLA_a_PS2.M_#1.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTGGGT-3'	47
PLA_a_PS2.M_#1.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTGGGTAGGGCGGG-3'	55
PLA_a_PS2.M_#2.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTGGGT-3'	48
PLA_a_PS2.M_#2.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTGGGTAGGGCGGG-3'	56
PLA_a_PS2.M_#3.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTGGGT-3'	49
PLA_a_PS2.M_#3.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTGGGTAGGGCGGG-3'	57
PLA_a_PS2.M_#4.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTGGGT-3'	50
PLA_a_PS2.M_#4.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTGGGTAGGGCGGG-3'	58
PLA_a_PS2.M_#5.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTTGGGT-3'	51
PLA_a_PS2.M_#5.2	CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTTGGGTAGGGCGGG-3'	59
PLA_a_PS2.M_#6.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTTGTGGGTAGGGCGGG-3'	60
PLA_a_PS2.M_#6.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTTBTGG-3'	50
PLA_s_PS2.M_#1.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTGGGTAGGG-3'	50
PLA_s_PS2.M_#1.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTGGGTGGG-3'	50
PLA_s_PS2.M_#2.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTGGGTAGGG-3'	51
PLA_s_PS2.M_#2.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTGGGTGGG-3'	51
PLA_s_PS2.M_#3.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTGGGTAGGG-3'	52
PLA_s_PS2.M_#3.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTGGGTGGG-3'	52
PLA_s_PS2.M_#4.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTGGGTAGGG-3'	53
PLA_s_PS2.M_#4.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTGGGTGGG-3'	53
PLA_s_PS2.M_#5.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTTGGGTAGGG-3'	56

PLA_s_PS2.M_#5.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTTGGGTTGGG-3'	54
PLA_s_PS2.M_#6.1	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTTGTGGGTAGGG-3'	82
PLA_s_PS2.M_#6.2	5'-CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTTT CGTTTTTTTCGGGTTGG-3'	80

2.1.5. Split G4 AUDENAs

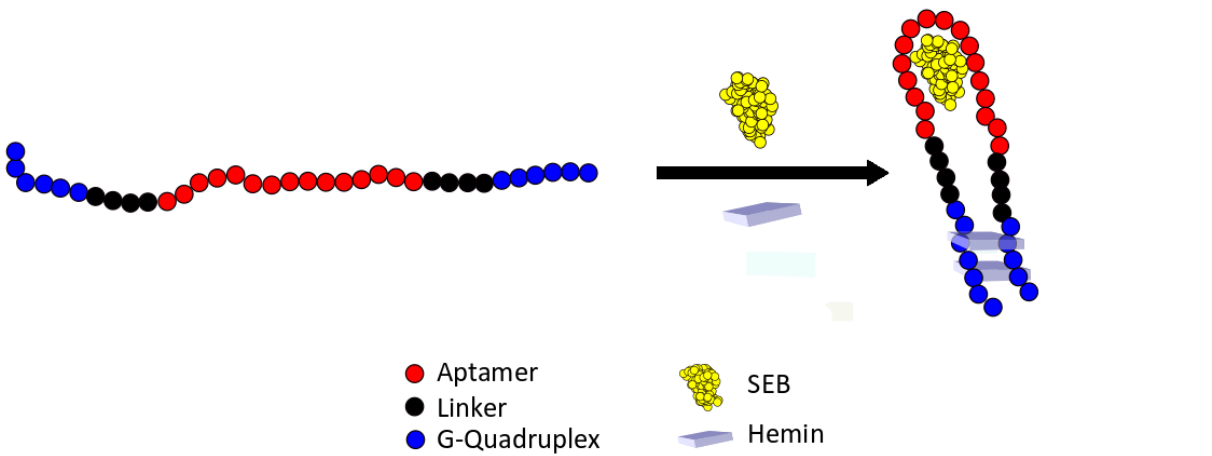


Figure 14 Schematic illustration of the interaction of a split G-quadruplex AUDENA with SEB.

Herein, the building blocks of the AUDENA were again lined up one after another on a single molecule, similar to the linear AUDENA design described above. However, here the G4 was split at different positions within its sequence, placing one part at the start and the other part at the end of the AUDENA (M. Deng, Zhang, Zhou, & Zhou, 2008; Zhu, Zhang, Dong, & Wang, 2015). Through the binding of the aptamer to SEB, the two parts of the G4 might be able to form a fully functional DNAzyme (Figure 14). Depending on the sites a G4 sequence was split and its resulting portions, theses sequences were either termed symmetrically (s) or asymmetrically (a) split G4 (Table 6).

Table 6 Sequences of split G-quadruplex AUDENA designs (red: aptamer, black: linker, blue: G4).

AUDENA	Sequence (5'-3')	Length (b)
split_a_Hum21	5'-GGGTTAGGGTTAGGGTTTTTCACTGGTCGTTGTTGTCTGTT GTCTGTTATGTTGTTTCGTTTTTTATGGG-3'	71

split_a_	5'-TGGGT	TTTTT	CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTT	68
PS2.M_#1	GTTTCGT	TTTTT	TGGGTAGGGCGGG-3'	
split_a_	5'-TGGGT	TTT	CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTT	62
PS2.M_#2	TCGT	TTT	TGGGTAGGGCGGG-3'	
split_a_	5'-			
PS2.M_#3	TGGGT	TTTT	CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGTT	64
	TCGT	TTT	TGGGTAGGGCGGG-3'	
split_a_	5'-			
PS2.M_#4	TGGGT	TTTT	CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTGT	66
	TTTCGT	TTTT	TGGGTAGGGCGGG-3'	
split_a_	5'-TGGGT	TATA	CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTTG	66
PS2.M_#5	TTTCGT	TATAT	TGGGTAGGGCGGG-3'	
split_a_	5'-TGGGT	TATAATATT	CACTGGTCGTTGTTGTCTGTTGTCTGTTA	76
PS2.M_#6	TGTTGTTTCGT	TTATAATATT	TGGGTAGGGCGGG-3'	
split_a_	5'-			
PS2.M_#7	TGGGT	TATACT	CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTT	70
	GTTTCGT	TCATAT	TGGGTAGGGCGGG-3'	
split_a_	5'-TGGGT	TTTATAT	CACTGGTCGTTGTTGTCTGTTGTCTGTTATG	70
PS2.M_#8	TTGTTTCGT	TATATT	TGGGTAGGGCGGG-3'	
split_a_	5'-GTGGGT	AGGGCGGGTATATT	CACTGGTCGTTGTTGTCTGTT	70
PS2.M_#9	GTCTGTTATGTTGTTTCGT	TTATATT	TGG-3'	
split_s_	5'-GGGT	AGGGTTTTTTT	CACTGGTCGTTGTTGTCTGTTGTCTG	71
Hum21	TTATGTTGTTTCGT	TTTTT	AGGGTAGGG-3'	
split_s_	5'-GGGT	TGGGTTTTT	CACTGGTCGTTGTTGTCTGTTGTCTGTTAT	66
PS2.M_#1	GTTGTTTCGT	TTTTT	TGGGTAGGG-3'	
split_s_	5'-GGGT	TGGGTT	CACTGGTCGTTGTTGTCTGTTGTCTGTTATGTT	60
PS2.M_#2	GTTTCGT	TTT	TGGGTAGGG-3'	
split_s_	5'-GGGT	TGGGTTTT	CACTGGTCGTTGTTGTCTGTTGTCTGTTATGT	62
PS2.M_#3	TGTTTCGT	TTT	TGGGTAGGG-3'	
split_s_	5'-GGGT	TGGGTTTT	CACTGGTCGTTGTTGTCTGTTGTCTGTTATG	64
PS2.M_#4	TTGTTTCGT	TTTT	TGGGTAGGG-3'	
split_s_	5'-GGGT	TGGGTATA	CACTGGTCGTTGTTGTCTGTTGTCTGTTA	64
PS2.M_#5	TGTTGTTTCGT	TATAGGGTAGGG-3'		
split_s_	5'-GGGT	TGGGAATAATATT	CACTGGTCGTTGTTGTCTGTTGTC	74
PS2.M_#6	TGTTATGTTGTTTCGT	TTATAATAAGGGTAGGG-3'		
split_s_	5'-GGGT	TGGGATACT	CACTGGTCGTTGTTGTCTGTTGTCTGTT	66
PS2.M_#7	ATGTTGTTTCGT	TCATAGGGTAGGG-3'		
split_s_	5'-GGGT	TGGGTTATAT	CACTGGTCGTTGTTGTCTGTTGTCTG	68
PS2.M_#8	TTATGTTGTTTCGT	TATATT	TGGGTAGGG-3'	
split_s_PS	5'-			
2.M_#9	GTGGGT	AGGGTTATAT	CACTGGTCGTTGTTGTCTGTTGTCTGTT	70
	ATGTTGTTTCGT	TATATT	CGGGTGG-3'	

2.2. Activity measurements of AUDENAs

Changes of absorbance intensity upon oxidation of ABTS in the presence of H₂O₂ through the enzymatically active G4-hemin DNAzyme was monitored at 421 nm on an EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, USA). Prior to absorbance measurements, AUDENAs were incubated in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) and heated for 15 min. at 95 °C in order to avoid oligonucleotide

aggregates. Subsequently, the oligonucleotide solution was transferred to a 96-well plate. The solution was then incubated with SEB for 1 h at RT in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) to ensure binding of the AUDENA to the target protein as well as the proper formation of a fully enzymatically active G4-hemin DNAzyme. Different experimental set-ups concerning the final concentration of reactants, time point of reactant-addition and total incubation times were carried out. Hemin (0.5 mM) was either added at the beginning (0 min) or at the end (60 min) of incubation time of the AUDENA with SEB. The same experimental set-ups were examined in terms of the addition of KCl (20 mM) and/or NaCl (200 mM). Finally, ABTS (18 mM) was added to every well with a multichannel pipette and mixed by pipetting. In order to start the peroxidation reaction H_2O_2 (0.6 mM) was added to every well in the same manner as ABTS. Concentrations of ABTS as well as of H_2O_2 were kept constant during all experiments as they could be presumed not to be limiting factors of the reaction (Dnazymes et al., 2019). Changes of absorbance intensities were measured immediately after as a function of time every 10 s for up to 1800 s in total. All the above-mentioned concentrations of reactants indicate their final concentrations used in the assay.

2.2.1. Control Experiments

Control experiments were carried out to monitor the effects of experimental variables other than the target on the G4-hemin DNAzyme detection system. The following procedures were tested: (1) To check for the ability of the AUDENA to reliably detect the SEB-target measurements using AUDENAs in the presence of hemin, ABTS and H_2O_2 in the absence of SEB were carried out. (2) Additionally, the background catalytic activity of hemin itself was determined by measuring changes in absorbance in the presence of SEB, hemin, ABTS, and H_2O_2 . When added to the experiment, each reactant was present at the same concentration as in the experimental set-up described above. The consequential missing volumes were filled up with HEPES buffer to maintain constant total volumes in all experiments.

2.2.2. Selectivity

The selectivity of the assay was assessed by measuring absorbance changes in the presence of BSA instead of SEB. The experimental procedure was the same as

described above, except for the replacement of SEB by BSA. Hence, the peroxidative activity of AUDENAs was measured after incubation with equimolar concentrations of BSA with regard to the SEB concentration used.

2.2.3. Quantification and Method Validation

In order to address the performance and reliability of the proposed detection system for SEB, generally established parameters indicating the quality of the analytical method were assessed. Different concentrations of SEB were used to verify the linearity of the enzymatic activity of the AUDENA in a certain working range. Following final SEB concentrations were tested: 5, 10, 15, 20, 25, 30 ng/μL (176, 353, 529, 705, 881, 1058 nM respectively).

2.2.3.1. Limit of Detection (LOD)

The limit of detection (LOD), referring to the smallest concentration of an analyte in a sample that can still be detected by the given method, was determined using a linear AUDENA design. The ability of the proposed detection system to distinguish signals obtained in the presence of the analyte from background noise was calculated by means of the standard deviation (STD) of the lowest concentration used in the assay and the slope of the linear calibration curve obtained (Equation 1). As a generally accepted threshold, the value obtained in the experiment needed to be a minimum of three times bigger than the background signal of the assay.

Equation 1 Calculation of LOD.

$$LOD_{SEB} = \frac{3 * STD_{(5 \text{ ng}/\mu\text{L})}}{Slope}$$

2.2.3.2. Limit of Quantification (LOQ)

Likewise, the limit of quantification, expressing the lowest concentration of analyte that can reliably be quantified by the applied analytical approach, was calculated (Equation 2).

Equation 2 Calculation of LOQ.

$$LOQ_{SEB} = \frac{10 * STD_{(5\text{ ng}/\mu\text{L})}}{Slope}$$

2.2.4. Determination of Initial Reaction Rate (V_0)

The initial reaction rate (V_0) is expressed as the change in absorbance per time ($\Delta A_{421\text{nm}}/s$), which corresponds to the slope of the initial linear portion in graphs displaying absorbance versus time.

Equation 3 Calculation of initial reaction rates (V_0) using arbitrary units of absorbance (A) and time points of absorbance measurement (t).

$$V_0 = \frac{(A_1 - A_0)}{(t_1 - t_0)}$$

t_0 ...Start of reaction (s)

t_1 ...Timepoint of reaction (s)

A_0 ... Absorbance at time point t_0

A_1 ...Absorbance at time point t_1

In this study, the initial reaction rates were calculated from the slope of the initial linear portion (the first 60 s) of the increase in the absorbance values.

The concentration of ABTS oxidized to the ABTS radical cation ($ABTS^{*+}$) by the G4-hemin DNAzyme was determined using the calculated V_0 value and the *Lambert-Beer* equation. An ϵ value of $ABTS^{*+}$ at $\lambda = 421\text{ nm}$ used was $\epsilon = 36\,000\text{ M}^{-1}\text{cm}^{-1}$ (Travascio, Li, & Sen, 1998).

Equation 4 Conversion of initial reaction rate (V_0) to the total amount of ABTS-substrate turned over by the G4-hemin DNAzyme to the measured $ABTS^{*+}$ - product after the predefined reaction time.

$$c = \frac{V_0}{\epsilon * l}$$

2.2.5. Determination of Relative Activity (%)

Hemin is known for its ability to catalyze the ABTS-H₂O₂ oxidation reaction without peroxidases being present. To take the weak but present catalytic effects of hemin on overall oxidation of the ABTS-substrate into account, relative reaction rates were determined. Hence, relative activity (%) of the G4-hemin DNAzyme in the presence of SEB to activity in the absence of SEB was calculated.

Equation 5 Relative activity (%) of the G4-hemin DNAzyme to hemin only.

$$\text{Relative activity (\%)} = \frac{V_{0(\text{hemin}+\text{SEB})}}{V_{0(\text{hemin})}} * 100$$

Equation 6 Relative activity (%) of the G4-hemin DNAzyme in the presence of hemin and SEB to the G4-hemin DNAzyme in the presence of hemin and BSA.

$$\text{Relative activity (\%)} = \frac{V_{0(\text{hemin}+\text{SEB})}}{V_{0(\text{hemin}+\text{BSA})}} * 100$$

3. Results

3.1. Quantitative detection of SEB

A linear AUDENA design (linear_A_L_G4_PS2.M#1) was used to obtain a calibration curve by incubation of the AUDENA with different concentrations of SEB (5-20 ng/ μ L). Absorbance values received from the linear section of the increase in absorbance were plotted as a function of SEB concentration shown in Figure 15.

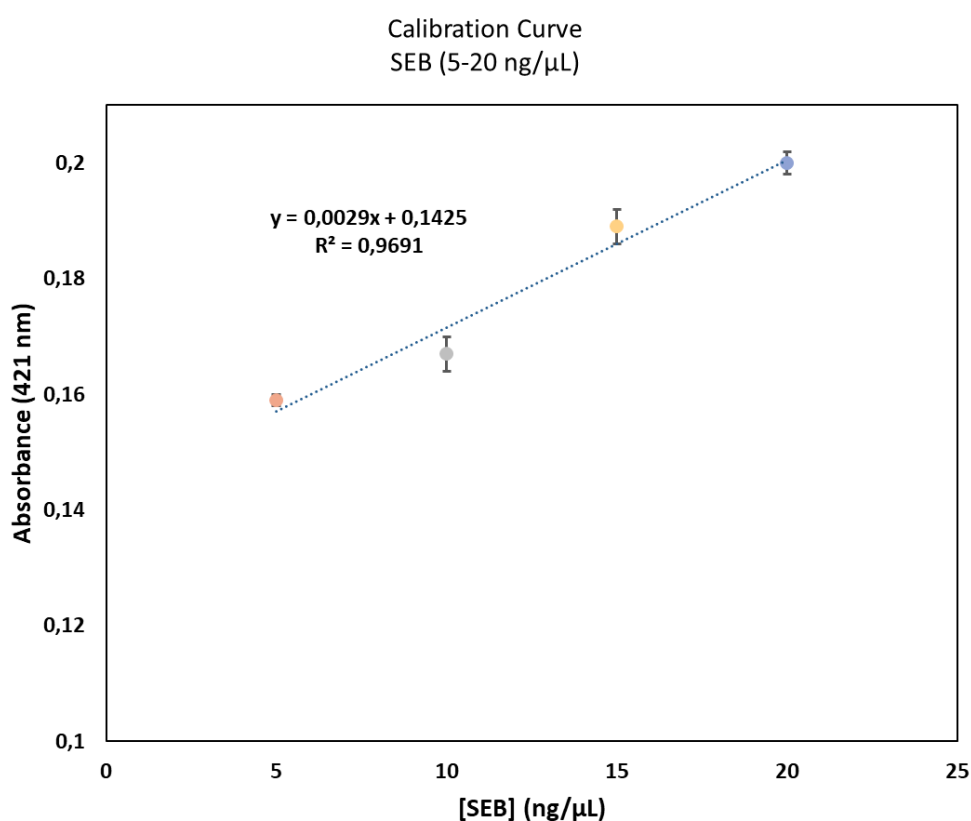


Figure 15 SEB concentration-dependent change in the absorbance signal at a fixed time point of 3 min of reaction time using a linear AUDENA design: linear_A_L_G4_PS2.M#1 (A, 5'- CAC TGG TCG TTG TTG TCT GTT GTC TGT TAT GTT GTT TCG TTT TTT GTG GGT AGG GCG GGT TGG - 3').

500 nM AUDENA was mixed with different concentrations of SEB (5, 10, 15, 20 ng/ μ L) in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, 20 mM KCL, 200 mM NaCl, pH 7.4). The reaction mixture was incubated for 60 min. 500 nM Hemin was added at the end (60 min) of incubation time. Experiments were performed in the presence of 18 mM ABTS and 0.6 mM H₂O₂, which were added to every well successively at the end of incubation time and

mixed by pipetting. Absorbance was measured at 421 nm after 3 minutes of reaction time. Measurements were carried out in duplicates.

The observed increase of absorbance was consistent with rising concentrations of SEB and showed linearity in a range of final concentrations of SEB ranging from 5 ng/ μ L- 20 ng/ μ L. The resulting linear relation that was obtained was $A_{421\text{nm}} = 0.1425 + 0.0029c_{\text{SEB}}(\text{ng}/\mu\text{L})$. The correlation coefficient of absorbance to SEB concentration is 0.9691, showing that concentration measurements of solutions containing SEB according to the calibration curve obtained within this assay are feasible for quantitative detection of SEB. The proposed detection system yielded a LOQ of 3.45 ng/ μ L and a LOD of 1.03 ng/ μ L SEB referring to 121.56 nM and 26.47 nM SEB respectively.

3.2. Optimization of incubation time with hemin

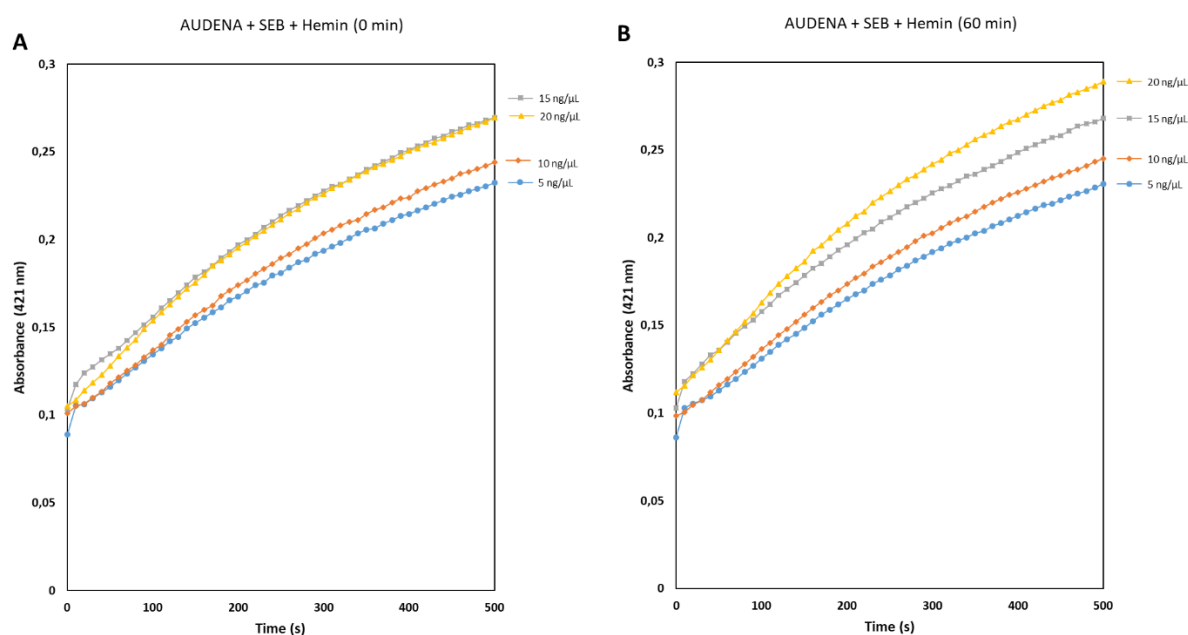


Figure 16 Time-dependent absorbance changes as a result of H_2O_2 -mediated oxidation of ABTS by the peroxidase-mimicking G4-hemin DNAzyme in the presence of different concentrations of SEB given two different incubation times of the detection system with hemin. A linear AUDENA design was used: linear_A_L_G4_PS2.M#1 (A, 5'- CAC TGG TCG TTG TTG TCT GTT GTC TGT TAT GTT GTT TCG TTT TTT GTG GGT AGG GCG GGT TGG - 3')

500 nM AUDENA was mixed with different concentrations of SEB (5, 10, 15, 20, 25, 30 ng/ μ L) in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, 20 mM KCL, 200 mM NaCl,

pH 7.4). The reaction mixture was incubated for 60 min. 500 nM Hemin was either added at the beginning (**A**, 0 min) or at the end (**B**, 60 min) of incubation time. Experiments were performed in the presence of 18 mM ABTS and 0.6 mM H₂O₂, which were added to every well successively at the end of incubation time (60 min) and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time for 1800 s in total (first 500 s are shown). Measurements were carried out in duplicates and agreed within an RSD of $\leq 6.70\%$.

The changes of absorbance induced by the catalytic activity of an AUDENA are influenced by the total amount of time that hemin is present in the assay as seen in Figure 16. Pre-incubation of the AUDENA with hemin, by which the association of a fully enzymatically active G4 is induced, leads to overall slightly lower intensities in absorbance (Figure 16, A). Interestingly, the differences of absorbance intensities for the given concentrations of SEB appear to be greater and more distinct when hemin is added just before starting the enzymatic reaction by addition of ABTS and H₂O₂ (Figure 16, B).

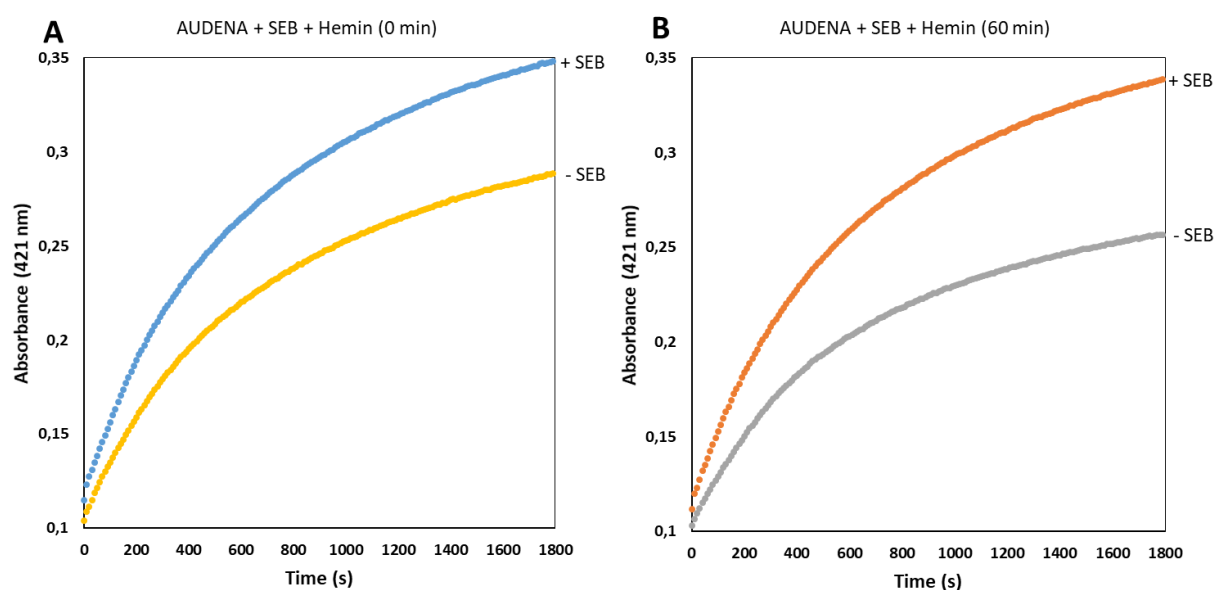


Figure 17 Effect of time-point of hemin addition on time-dependent absorbance changes as a result of H₂O₂-mediated oxidation of ABTS by the G4-hemin DNAzyme in the presence (+ SEB) and absence (-SEB) of the SEB-target given two different incubation times of the detection system with hemin. A linear AUDENA design was used: linear_A_L_G4_PS2.M#1 (5'- CAC TGG TCG TTG TTG TCT GTT GTC TGT TAT GTT GTT TCG TTT TTT GTG GGT AGG GCG GGT TGG - 3').

500 nM AUDENA was mixed with 529 nM (15 ng/ μ L) SEB in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, 20 mM KCL, 200 mM NaCl, pH 7.4) and incubated for 60 min. 500 nM Hemin was added either at the beginning (A, 0 min) or the end (B, 60 min) of incubation time. Experiments were performed in the presence of 18 mM ABTS and 0.6 mM H₂O₂, which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time for 1800 s in total. Measurements were carried out a total of 8 times and agreed within an RSD of $\leq 25.11\%$.

Figure 17 compares the absorbance intensities of assays employing two different incubation times of the AUDENA with hemin in the presence or absence of SEB. Apparently, the total amount of time that hemin is present in the assay exerts a bigger influence on changes of absorbance in experiments lacking SEB (Figure 17, A and B). In the presence of SEB, both experiments display similar changes of absorbance, whereas the difference between the two control experiments lacking SEB is more evident. In other words, the relative activity of experiments carried out in the presence of SEB compared to those carried out with no SEB present in the assay was greater, when hemin was added just before the start of the actual peroxidation reaction (Table 7).

Table 7 Initial reaction rates (V_0) of linear_A_L_G4_PS2.M#1 in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (- SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Different experimental conditions were tested with regard to incubation time of hemin (0 min or 60 min) with the G4-hemin DNAzyme prior to starting the actual peroxidation reaction. Error-values represent the standard deviation of eight replicate measurements.

Experimental Condition	$V_{0(+SEB)}$ (μ M min ⁻¹)	Relative activity (%) $V_{0(+SEB)}/V_{0(-SEB)}$
Hemin (0 min)	0.53 ± 0.05	133
Hemin (60 min)	0.53 ± 0.08	161

3.3. Effect of linker sequences within linear AUDENAs on catalytic activity

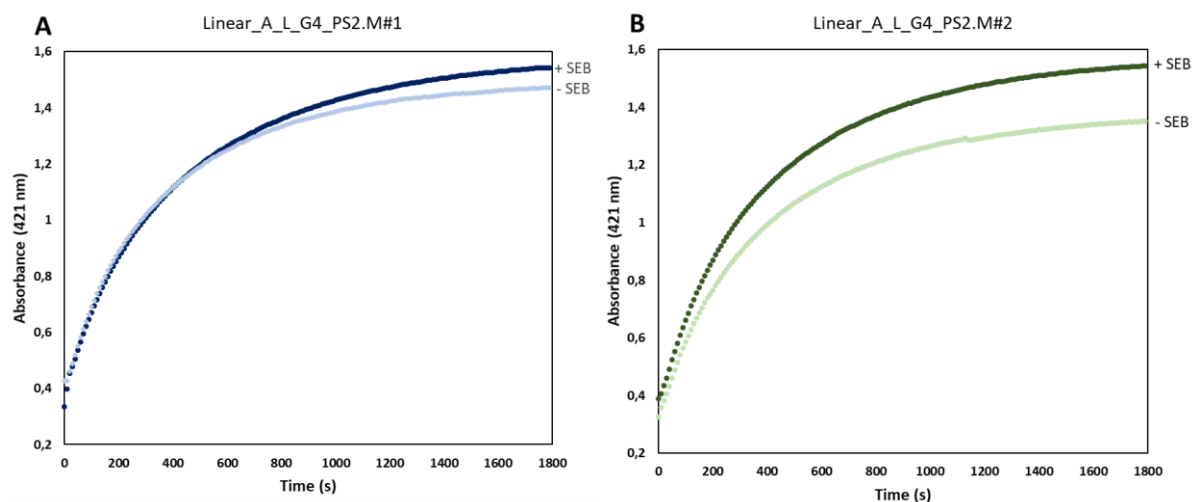


Figure 18 Comparison of time-dependent absorbance changes as a result of H_2O_2 -mediated oxidation of ABTS by the peroxidase-mimicking G4 DNAzyme in the presence (+ SEB) or absence (-SEB) of SEB using two different linear AUDENA designs: linear_A_L_G4_PS2.M#1 (A, 5'- CAC TGG TCG TTG TTG TCT GTT GTC TGT TAT GTT GTT TCG TTT TTT GTG GGT AGG GCG GGT TGG - 3') and linear_A_L_G4_PS2.M #2 (B, 5'- CAC TGG TCG TTG TTG TCT GTT GTC TGT TAT GTT GTT TCG TTT TTT TTT TTG TGG GTA GGG CGG GTT GG - 3').

500 nM AUDENA were mixed with 881 nM (25 ng/ μL) SEB in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, 20 mM KCL, 200 mM NaCl, pH 7.4) and incubated for 60 min. 500 nM Hemin was added at the beginning (0 min.) of incubation time. Experiments were performed in the presence of 18 mM ABTS and 0.6 mM H_2O_2 , which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time for 1800 s in total. Measurements were carried out in duplicates and agreed within an RSD of $\leq 9\%$ for linear_A_L_G4_PS2.M#1 and $\leq 4\%$ for linear_A_L_G4_PS2.M#2 respectively.

Figure 18 shows rates of absorbance changes plotted versus time in the presence or absence of SEB. Both linear AUDENA designs have greater catalytic activity in the presence of SEB compared to experiments carried in the absence of the target protein. Nevertheless, the difference between these two experimental set-ups is more evident when using *Linear_A_L_G4_PS2.M#2*, which differs from *Linear_A_L_G4_PS2.M#1* only regarding the length of the poly-T-linker sequence used to connect the aptamer to the G4 sequence. It was proven by several authors, that stability and also catalytic

activity of G4s depends on the final topology, especially in terms of loop formation (Hazel et al., 2004; Risitano & Fox, 2004; Tippiana, Xiao, & Myong, 2014). Here, the presence of additional thymine nucleotides separating the aptamer from the G4 within the AUDENA sequence might, therefore, influence overall G4 properties including catalytic activity.

3.4. Effect of KCl and NaCl on the activity of linear AUDENAs

The way of how the AUDENAs were affected by the presence or absence of K^+ and Na^+ cations was investigated by either adding each of them separately or both of them in combination at two different time points of incubation. Here, incubation time refers to the incubation of KCl and/or NaCl with the AUDENA, SEB, and hemin. In all experiments described in the following section, KCl and/or NaCl were unexceptionally added before the start of the actual peroxidation reaction in order to ensure the proper and best possible formation of respective ion-coordinated G4 structures without premature oxidative reactions.

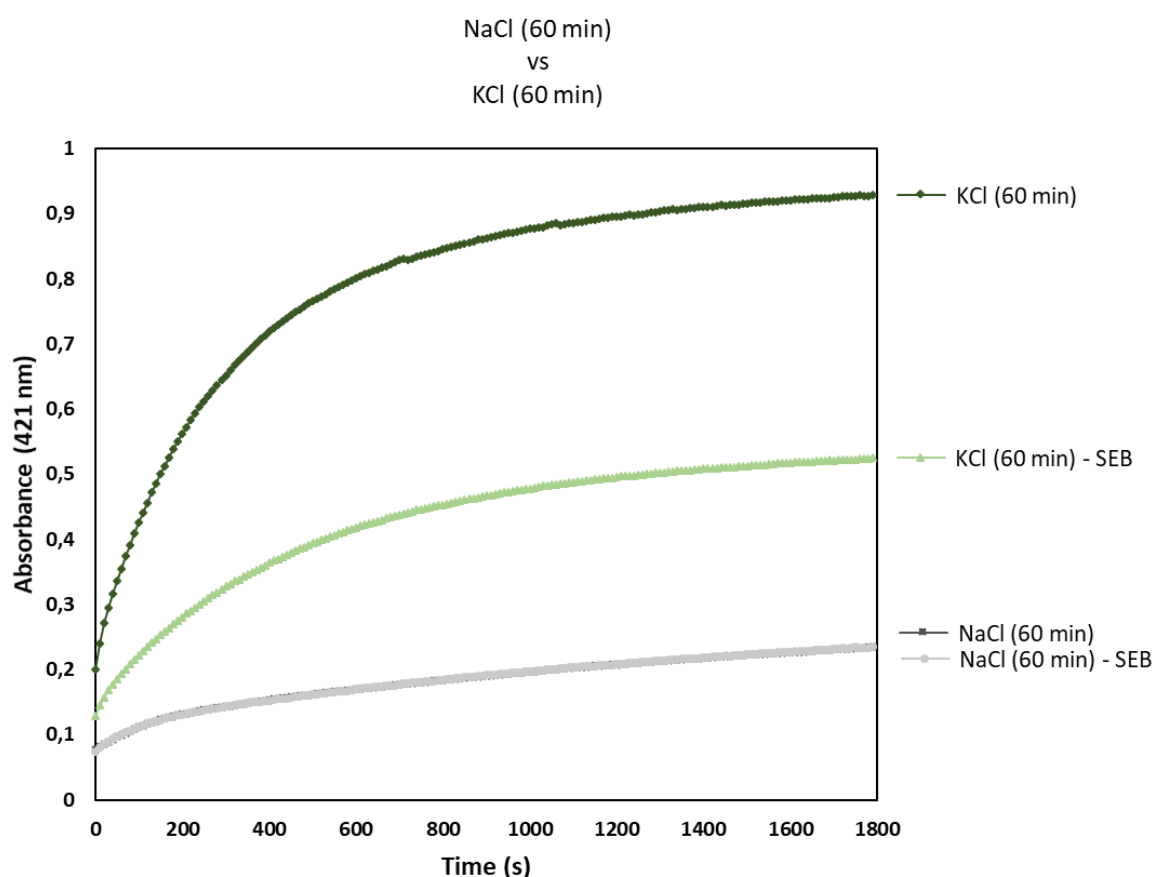


Figure 19 Time-tracing curves of ion-dependent absorbance changes in the presence of either KCl or NaCl by the hemin-G4 DNAzyme and the presence (+ SEB) or absence (-SEB) of SEB using a linear AUDENA design: linear_A_L_G4_PS2.M#2 (5' - CAC TGG TCG TTG TTG TCT GTT GTC TGT TAT GTT GTT TCG TTT TTT TTT TTG TGG GTA GGG CGG GTT GG - 3').

500 nM AUDENA was mixed with 529 nM (15 ng/ μ L) SEB in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) and incubated for 60 min. 500 nM Hemin was added at the beginning (0 min) of incubation time. 20 mM KCl or 200 mM NaCl were added at the end of incubation time (60 min). Experiments were performed in the presence of 18 mM ABTS and 0.6 mM H₂O₂, which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time for 1800 s in total. Measurements were carried out in triplicates and agreed within an RSD of ≤ 15.93 %.

The linear AUDENAs used in the present experiment exhibited strongly variable enzymatic activities in the presence of different ions (Figure 19). In the presence of SEB and KCl only, absorbance intensities rose up to 0.93 AU whereas in the presence of SEB and NaCl only absorbance data obtained reaches a maximum value of 0.23 only. To be more accurate, absorbance signals observed in the presence of KCl were more than 4 times higher compared to those in the presence of NaCl despite its 10-fold higher final concentration in the assay, respectively. Interestingly, in the reaction solution containing KCl the difference of absorbance between experiments carried out on behalf of detection of SEB was distinguishably higher compared to the control experiment lacking SEB. In the presence of NaCl, this effect was basically non-existent. Here, absorbance values for both the assay containing SEB as well as the control experiment carried out in the absence of SEB exhibited almost similar absorbance-behavior, with the control experiment yielding surprisingly higher relative catalytic activity (Table 8).

Table 8 Initial reaction rates (V_0) of linear_A_L_G4_PS2.M#2 in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (-SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Different experimental conditions were tested with regard to ions (KCl or NaCl) and incubation time applied in the assay. Error-values represent the standard deviation of three replicate measurements.

Experimental Condition	$V_{0(+SEB)}$ ($\mu\text{M min}^{-1}$)	Relative activity (%)
		$V_{0(+SEB)}/V_{0(-SEB)}$
KCl (60 min)	3.03 ± 0.07	235
NaCl (60 min)	0.43 ± 0.03	84

Enhancement of the G4-hemin-DNAzyme-catalyzed reaction in the presence of K^+ has already been reported by several authors. Travascio et al. found, that the ameliorating effects of K^+ on the overall enzymatic activity were most eminent when using final concentrations ranging from 10-25 mM KCl (Travascio et al., 1998). Here, a final concentration of 20 mM KCl, which is moving along with these findings, was used.

Possible explanations for the preference of K^+ over Na^+ as well as the mechanistic basis have been proposed by a number of studies so far. It was shown that in solution K^+ is bound by G-tetrads more likely than Na^+ (H. Deng & Braunlin, 1996; Hud, Smith, Anet, & Feigon, 1996; Xu, Deng, & Braunlin, 1993). The preference for K^+ over Na^+ binding was linked to a better fit of the potassium ion within a G4 by coordination to the carbonyls of the guanines (Williamson et al., 1989). Moreover, kinetic studies examining the formation of telomeric G4s reported that lifetimes of G4s increased in K^+ containing solutions compared to Na^+ solutions (Bardin & Leroy, 2008).

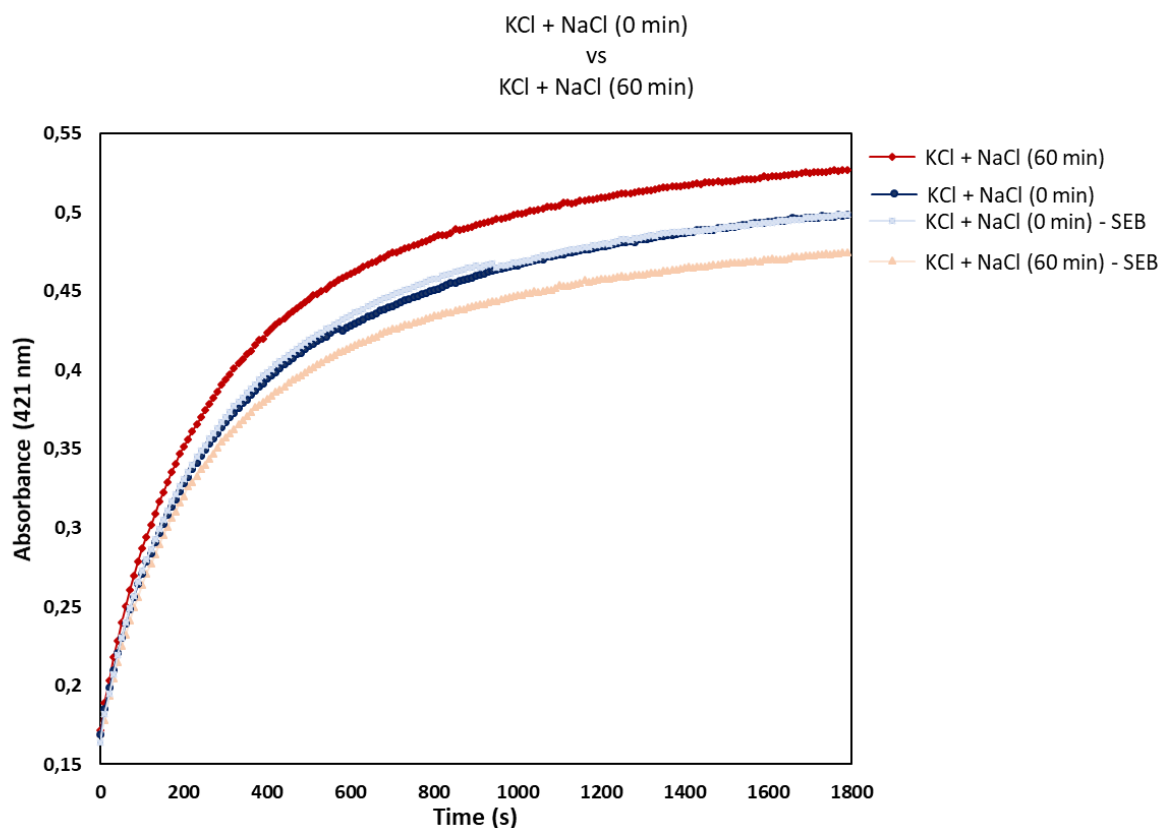


Figure 20 Effect of incubation time of the hemin-G4 DNAzyme with ions on absorbance changes in the presence of both KCl and NaCl in the presence (+ SEB) or absence (-SEB) of SEB using a linear AUDENA design: linear_A_L_G4_PS2.M#2 (5' - CAC TGG TCG TTG TTG TCT GTT GTC TGT TAT GTT GTT TCG TTT TTT TTT TTG TGG GTA GGG CGG GTT GG - 3').

500 nM AUDENA was mixed with 529 nM (15 ng/ μ L) SEB in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) and incubated for 60 min. 500 nM of Hemin was added at the beginning (0 min) of incubation time. 20 mM KCl and 200 mM NaCl were added simultaneously either at the beginning (0 min) or the end (60 min) of incubation time. Experiments were performed in the presence of 18 mM ABTS and 0.6 mM H_2O_2 , which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time for a total of 1800 s. Measurements were carried out in triplicates and agreed within an RSD of $\leq 13.57\%$.

When added simultaneously to the reaction either at the beginning or the end of incubation time, induction of enzymatic activity was greater when both KCl and NaCl were added after 60 min of incubation of the AUDENA with hemin and SEB (Figure

20). This effect might mostly be owed to the presence of KCl as seen in prior experiments (Figure 19).

Table 9 Initial reaction rates (V_0) of linear_A_L_G4_PS2.M#2 in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (-SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Different experimental conditions were tested with regard to ions (KCl **and** NaCl) and incubation time applied in the assay. Error-values represent the standard deviation of three replicate measurements.

Experimental Condition	V_0 (+SEB) ($\mu\text{M min}^{-1}$)	Relative activity (%)
		$V_0(+\text{SEB})/V_0(-\text{SEB})$
KCl + NaCl (0 min)	1.38 ± 0.11	93
KCl + NaCl (60 min)	1.53 ± 0.19	124

Competition between K^+ and Na^+ ions for coordination sites within a G4 might be a reasonable explanation, as the presence of different ions leads to aberrant conformations of G4 sequences (Schultze et al., 1999). Thus, the peroxidative strength of the G4-hemin DNAzyme might be affected, resulting in altered absorbance values.

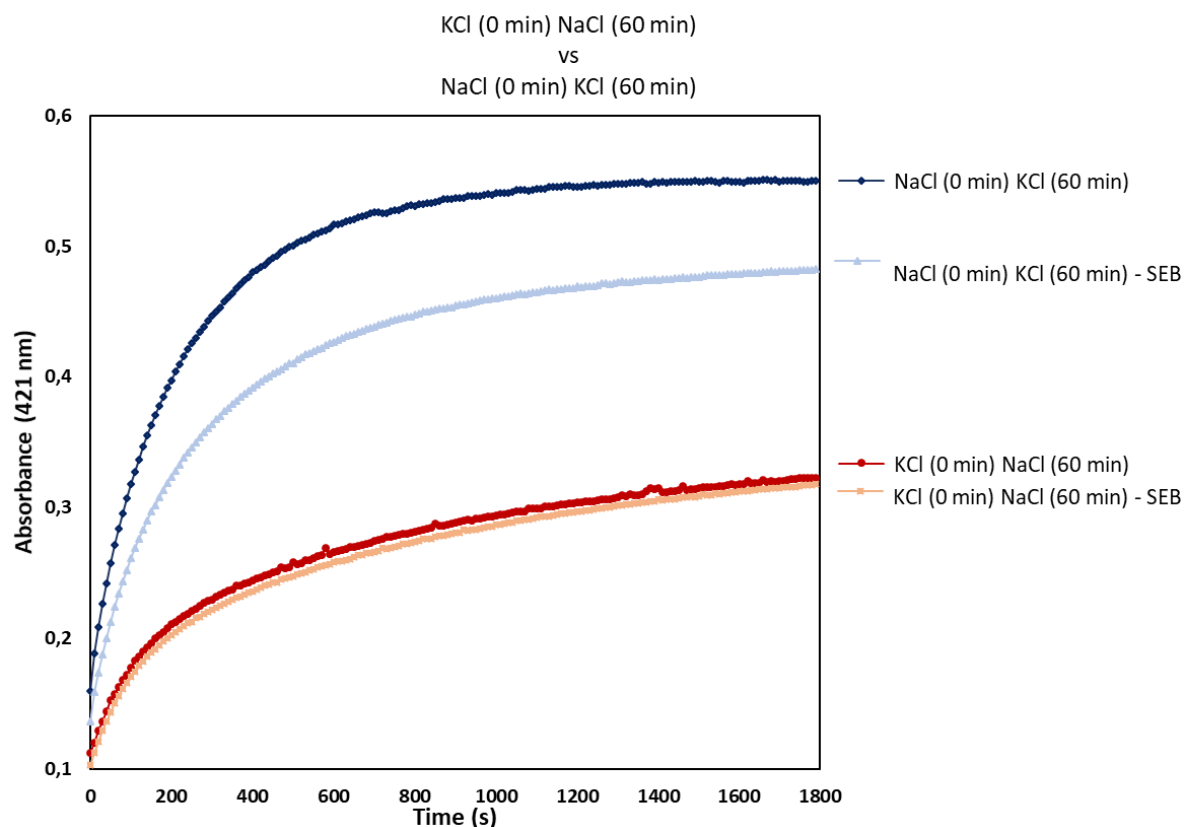


Figure 21 Effect of incubation time of the hemin-G4 DNAzyme on absorbance changes in the presence of both KCl and NaCl in the presence (+ SEB) or absence (-SEB) of SEB using a linear AUDENA design: linear_A_L_G4_PS2.M#2 (5' - CAC TGG TCG TTG TTG TCT GTT GTC TGT TAT GTT GTT TCG TTT TTT TTT TTG TGG GTA GGG CGG GTT GG - 3').

500 nM AUDENA was mixed with 529 nM (15 ng/ μ L) SEB in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) and incubated for 60 min. 500 nM Hemin was added at the beginning (0 min) of incubation time. 20 mM KCl and 200 mM NaCl were added at the beginning (0 min) or at the end (60 min) of incubation time. Experiments were performed in the presence of 18 mM ABTS and 0.6 mM H_2O_2 , which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time for a total of 1800 s. Measurements were carried out in triplicates and agreed within an RSD of $\leq 16.82\%$.

Table 10 Initial reaction rates (V_0) of linear_A_L_G4_PS2.M#2 in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (-SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Different experimental conditions were tested with regard to ions (KCl **and** NaCl) and

incubation time applied in the assay. Error-values represent the standard deviation of three replicate measurements.

Experimental Condition	$V_0 (+\text{SEB}) (\mu\text{M min}^{-1})$	Relative activity (%)
		$V_0(+\text{SEB})/V_0(-\text{SEB})$
KCl (0 min) + NaCl (60 min)	1.47 ± 0.26	81
NaCl (0 min) + KCl (60 min)	1.82 ± 0.01	102

The presence of KCl and the absence of NaCl at the very beginning of incubation time of the AUDENA with SEB and hemin (0 min) results in lower absorbance compared to when added just before the start of the peroxidation reaction (60 min) (Figure 21). Very similar to ion-dependency measurements shown in Figure 19, the difference between absorbance changes in the presence of SEB and the control experiment is again almost neglectable.

The addition of NaCl at the beginning (0 min) and KCl at the end (60 min) of incubation time lead to a nearly linear rise of absorbance in the first 60 s of reaction. The absorbance curve of samples containing SEB is more distinct to the control lacking the target.

3.5. Comparison of different AUDENA designs

All of the AUDENA designs exhibited oxidative properties among ABTS. The particular sequences presented within the following section contain the most significant outcomes of absorbance measurements carried out during the study.

3.5.1. Linear Complementary AUDENAs

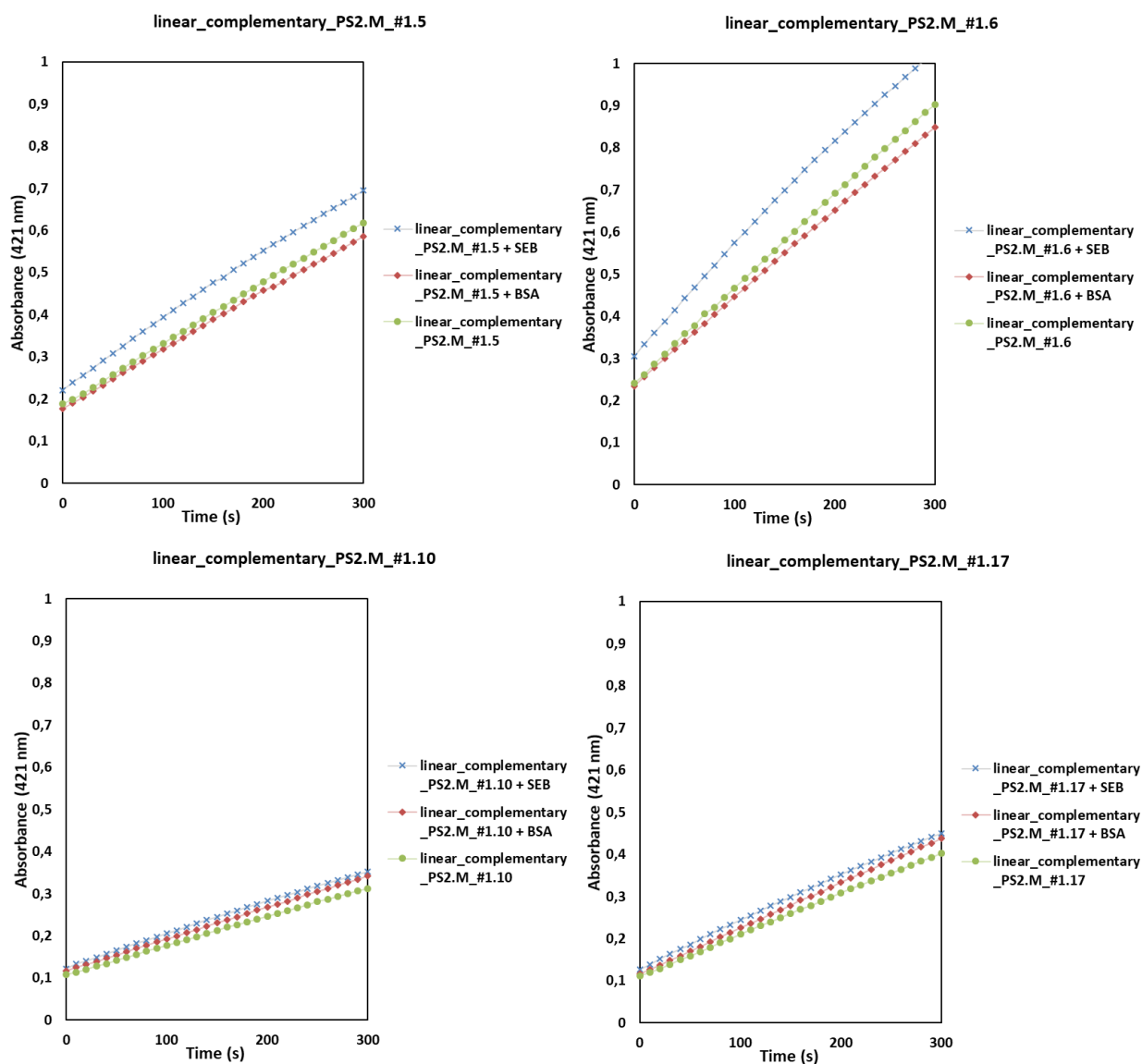


Figure 22 Time-dependent absorbance changes as a result of H_2O_2 -mediated oxidation of ABTS by the peroxidase-mimicking G4-hemin DNAzyme using linear complementary AUDENA designs. The specificity of the reaction was determined by carrying out experiments in the presence of either SEB or BSA or measuring the peroxidative activity of solely the AUDENA in the absence of any protein target.

1 μM AUDENA was mixed with 500 nM of either SEB or BSA in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) and incubated for 60 min. 20 mM KCl and 200 mM NaCl were added followed by 1.8 μM hemin. Experiments were carried out in the presence of 1.4 mM ABTS and 0.6 mM H_2O_2 , which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time for 1800 s in total (first 300 s are shown). Measurements were carried out in triplicates and agreed within an RSD of $\leq 14.26\%$.

Depending on the portion of the functional part of a linear complementary AUDENA that was shielded by its complementary sequence respectively different changes of absorbance were observed (Figure 22). The highest enzymatic activity was observed using linear_complementary_PS.2M_#1.6. The most evident distinction of absorbance changes in the presence of SEB compared to control experiments was observed with linear_complementary_PS.2M_#1.10 (Table 11).

Table 11 Initial reaction rates (V_0) of linear complementary AUDENAs in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (-SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Error-values represent the standard deviation of three replicate measurements.

AUDENA	$V_0 (+\text{SEB})$ ($\mu\text{M min}^{-1}$)	Relative activity
		(%) $V_0 (+\text{SEB})/V_0 (-\text{SEB})$
linear_complementary_PS2.M_#1.5	2.04 ± 0.11	124
linear_complementary_PS2.M_#1.6	3.20 ± 0.21	120
linear_complementary_PS2.M_#1.10	1.01 ± 0.15	126
linear_complementary_PS2.M_#1.17	1.41 ± 0.16	124

In Table 11 the two designs listed below, linear_complementary_PS.2M_#1.10 and linear_complementary_PS.2M_#1.17, which both possess a linker composed of repetitive bases of either T or A in the inhibiting DNA strand, seem to be impaired in either aptamer binding or enzymatic activity of the functional part of the AUDENA when comparing V_0 values to the other AUDENA designs tested. Nevertheless, their relative activities resemble the two other AUDENAs, linear_complementary_PS2.M_#1.5 and linear_complementary_PS2.M_#1.6, described in Table 11

3.5.2. Hairpin AUDENAs

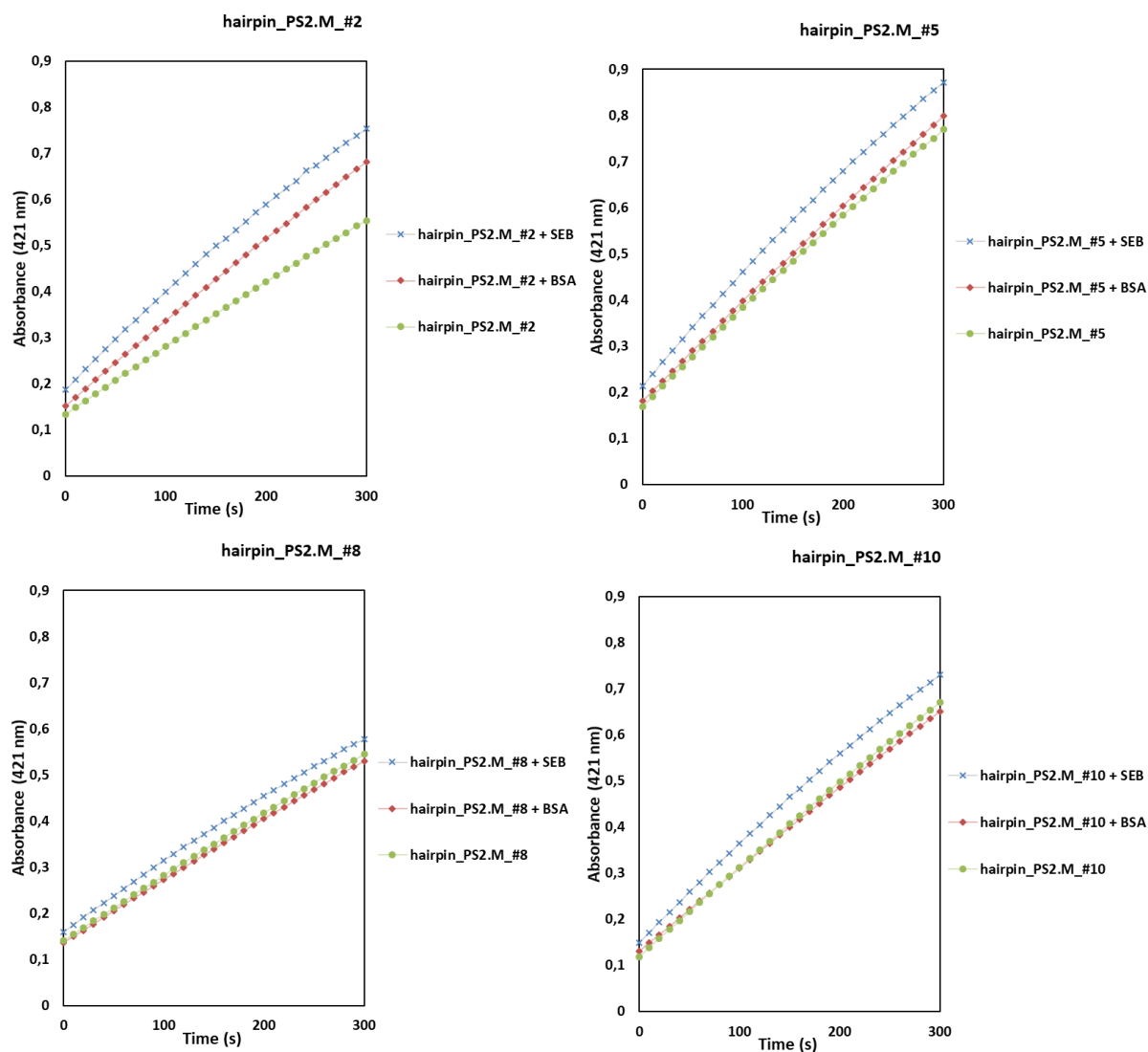


Figure 23 Time-dependent absorbance changes as a result of H_2O_2 -mediated oxidation of ABTS by the peroxidase-mimicking G4-hemin DNAzyme using hairpin AUDENA designs. The specificity of the reaction was determined by carrying out experiments in the presence of either SEB or BSA or measuring the peroxidative activity of solely the AUDENA in the absence of any protein target.

1 μM AUDENA was mixed with 500 nM of either SEB or BSA in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) and incubated for 60 min. 20 mM KCl and 200 mM NaCl were added followed by 1.8 μM hemin. Experiments were carried out in the presence of 1.4 mM ABTS and 0.6 mM H_2O_2 , which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time for

1800 s (first 300 s are shown). Measurements were carried out in triplicates and agreed within an RSD of $\leq 24.39\%$.

Among hairpin AUDENA designs, the peroxidative activity of the G4-hemin DNAzyme varied notably depending on the respective design used in the assay (Table 12). Apparently, the portions of the aptamer and the G4 that are hydrogen-bonded to a corresponding linker within the AUDENA sequence are crucial for the eventual interaction with the target as well as for peroxidative activity (Table 4). The number of bases of the linkers contributing to the hairpin structure by their complementarity to either the aptamer or the G4 influences the activity of the AUDENA. Depending on whether bigger parts (hairpin_PS2.M_#2) or smaller ones (hairpin_PS2.M_#10) are blocked by the linkers within the sequence, the hairpin designs display varying rates of activity. V_0 values of experiments carried out using hairpin_PS2.M#9 are more than twice as high when compared to V_0 values of hairpin_PS2.M#2. The whole sequences of the latter contain more than twice as much bases than hairpin_PS2.M#10. The coverage of the functional sequences might, therefore, be too strong and result in a decrease in binding and/or peroxidative activity of the AUDENA. Nevertheless, it has to be mentioned that the difference of the SEB-binding AUDENA to the control experiment containing BSA is not very strong with hairpin_PS2.M#10 indicating relatively low selectivity of the sensing probe. Due to relatively high RSD values of as high as 24.39 %, the usage of hairpin AUDENAs for the detection of SEB causes the risk of not being reliable enough, compared to other designs used.

Table 12 Initial reaction rates (V_0) of hairpin AUDENAs in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (-SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Error-values represent the standard deviation of three replicate measurements.

AUDENA	V_0 (+SEB) ($\mu\text{M min}^{-1}$)	Relative activity (%) $V_{0(+SEB)}/V_{0(-SEB)}$
hairpin_PS2.M_#2	2.55 ± 0.07	147
hairpin_PS2.M_#5	2.95 ± 0.39	117
hairpin_PS2.M_#8	1.85 ± 0.26	110
hairpin_PS2.M_#10	2.55 ± 0.30	111

3.5.3. Proximity Ligation Assay-like AUDENAs

3.5.3.1. Symmetric Proximity Ligation Assay-like AUDENAs

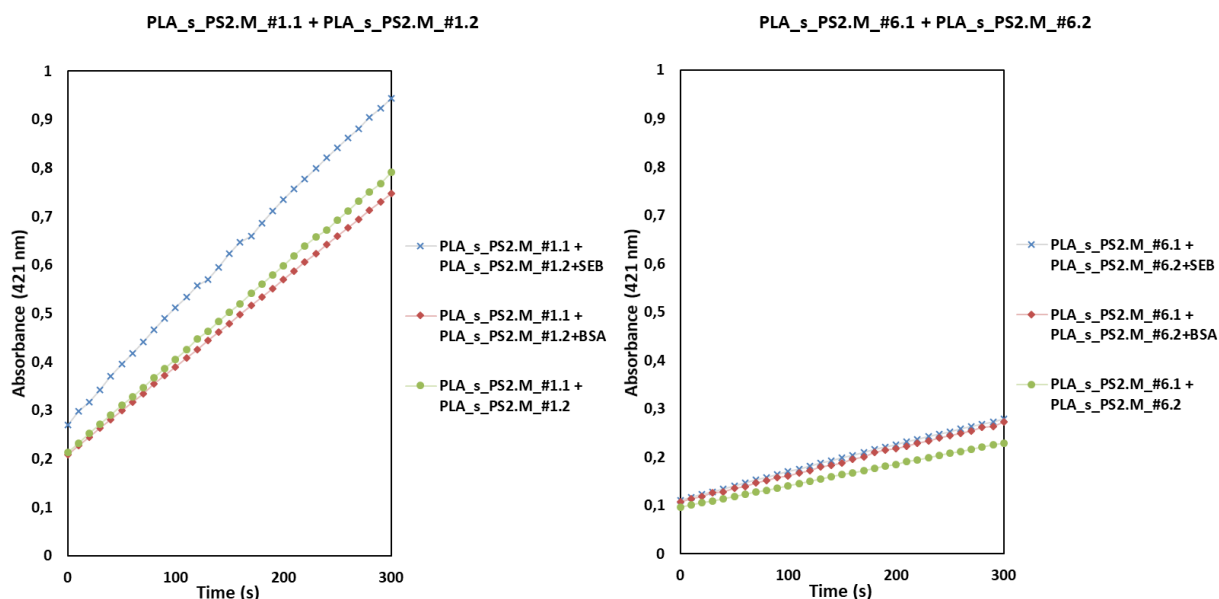


Figure 24 Time-dependent absorbance changes as a result of H_2O_2 -mediated oxidation of ABTS by the peroxidase-mimicking G4-hemin DNAzyme using symmetric PLA-like AUDENA designs. The specificity of the reaction was determined by carrying out experiments in the presence of either SEB or BSA or measuring the peroxidative activity of solely the AUDENA in the absence of any protein target.

1 μ M AUDENA was mixed with 500 nM of either SEB or BSA in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) and incubated for 60 min. 20 mM KCl and 200 mM NaCl were added followed by 1.8 μ M hemin. Experiments were carried out in the presence of 1.4 mM ABTS and 0.6 mM H_2O_2 , which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time for 1800 s in total (first 300 s are shown). Measurements were carried out in triplicates and agreed within an RSD of ≤ 22.66 %.

Symmetric PLA-like AUDENAs exhibited significantly different catalytic activities in terms of the AUDENA design used (Figure 24). The two detection approaches, PLA_s_PS2.M_#1.1 + PLA_s_PS2.M_#1.2 and PLA_s_PS2.M_#6.1 + PLA_s_PS2.M_#6.2 differed from each other only in terms of linker sequences used. Each of the strands of the two designs contains a poly-T-linker sequence, comprising of two or six adjacent Ts respectively. Remarkably, maximum absorbance values reached as well as relative activity differed distinctly for both AUDENA designs (Table 13).

Table 13 Initial reaction rates (V_0) of symmetric PLA-like AUDENAs in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (-SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Error-values represent the standard deviation of three replicate measurements.

AUDENA	V_0 (+SEB) ($\mu\text{M min}^{-1}$)	Relative activity (%)
		$V_{0(+SEB)}/V_{0(-SEB)}$
PLA_s_PS2.M_#1.1 + PLA_s_PS2.M_#1.2	2.92 ± 0.15	125
PLA_s_PS2.M_#6.1 + PLA_s_PS2.M_#6.2	0.69 ± 0.13	138

3.5.3.2. Asymmetric Proximity Ligation Assay-like AUDENAs

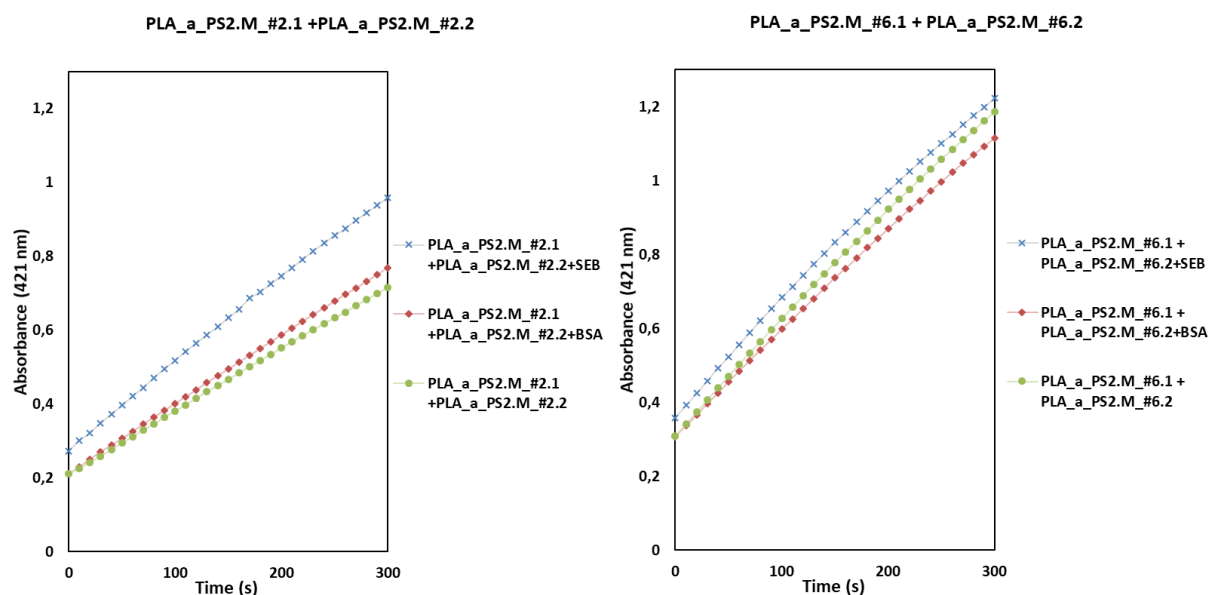


Figure 25 Time-dependent absorbance changes as a result of H_2O_2 -mediated oxidation of ABTS by the peroxidase-mimicking G4-hemin DNAzyme using asymmetric PLA-like AUDENA designs. The specificity of the reaction was determined by carrying out experiments in the

presence of either SEB or BSA or measuring the peroxidative activity of solely the AUDENA in the absence of any protein target.

Similar to the above described AUDENAS from Table 13, asymmetric PLA-like AUDENAS showed different initial reaction rates as well as relative activities besides the two designs compared only differed in the length of their poly-T-linker. Contradicting to the symmetric PLA-like AUDENAS, the usage of a shorter linker sequence in PLA_a_PS2.M_#2.1 + PLA_a_PS2.M_#2.2 yielded higher relative activity when compared to PLA_a_PS2.M_#6.1 + PLA_a_PS2.M_#6.2 (Table 14).

1 μM AUDENA was mixed with 500 nM of either SEB or BSA in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) and incubated for 60 min. 20 mM KCl and 200 mM NaCl were added followed by 1.8 μM hemin. Experiments were carried out in the presence of 1.4 mM ABTS and 0.6 mM H_2O_2 , which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time. Measurements were carried out in triplicates and agreed within an RSD of $\leq 24.49\%$.

Table 14 Initial reaction rates (V_0) of asymmetric PLA-like AUDENAS in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (-SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Error-values represent the standard deviation of three replicate measurements.

AUDENA	V_0 (+SEB) ($\mu\text{M min}^{-1}$)	Relative activity
		(%) $V_{0(+SEB)}/V_{0(-SEB)}$
PLA_a_PS2.M_#2.1 + PLA_a_PS2.M_#2.2	3.00 ± 0.17	165
PLA_a_PS2.M_#6.1 + PLA_a_PS2.M_#6.2	3.89 ± 0.10	105

3.5.4. Split AUDENAs

3.5.4.1. Symmetrically Split AUDENAs

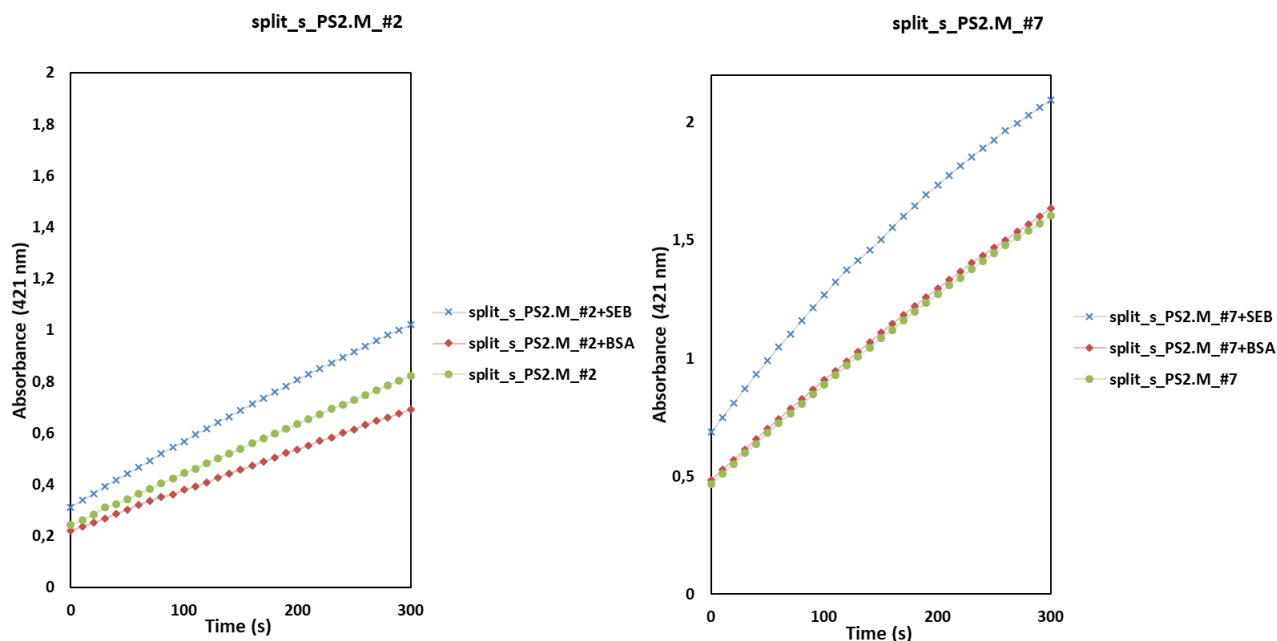


Figure 26 Time-dependent absorbance changes as a result of H_2O_2 -mediated oxidation of ABTS by the peroxidase-mimicking G4-hemin DNAzyme using symmetrically split AUDENA designs. The specificity of the reaction was determined by carrying out experiments in the presence of either SEB or BSA or measuring the peroxidative activity of solely the AUDENA in the absence of any protein target.

1 μM AUDENA was mixed with 500 nM of either SEB or BSA in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) and incubated for 60 min. 20 mM KCl and 200 mM NaCl were added followed by 1.8 μM hemin. Experiments were carried out in the presence of 1.4 mM ABTS and 0.6 mM H_2O_2 , which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time. Measurements were carried out in triplicates agreed within an RSD of $\leq 15.57\%$.

AUDENA molecules possessing a symmetrically split G4 part diverge significantly in their catalytic activity depending on the design used in the experiment (Table 15). Results of absorbance measurements carried out using two symmetrically split AUDENA designs are shown in Figure 26. V_0 values reveal that the two designs display different enzymatic activities even though their sequences differ from each other only regarding the length and the composition of the linker sequence used (Table 6). Using

split_s_PS2.M_#7 for peroxidation of ABTS results in more than twice as high V_0 values when compared to split_s_PS2.M_#2. Furthermore, the application of split_s_PS2.M_#7 in the assay seems to be more specific as the difference between SEB-recognition and the binding to BSA is higher than with split_s_PS2.M_#2.

Table 15 Initial reaction rates (V_0) of symmetrically split AUDENAs in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (-SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Error-values represent the standard deviation of three replicate measurements.

AUDENA	V_0 (+SEB) ($\mu\text{M min}^{-1}$)	Relative activity (%) $V_0(+\text{SEB})/V_0(-\text{SEB})$
split_s_PS2.M_#2	2.99 ± 0.23	132
split s PS2.M_#7	7.2 ± 0.51	143

3.5.4.2. Asymmetrically Split AUDENAs

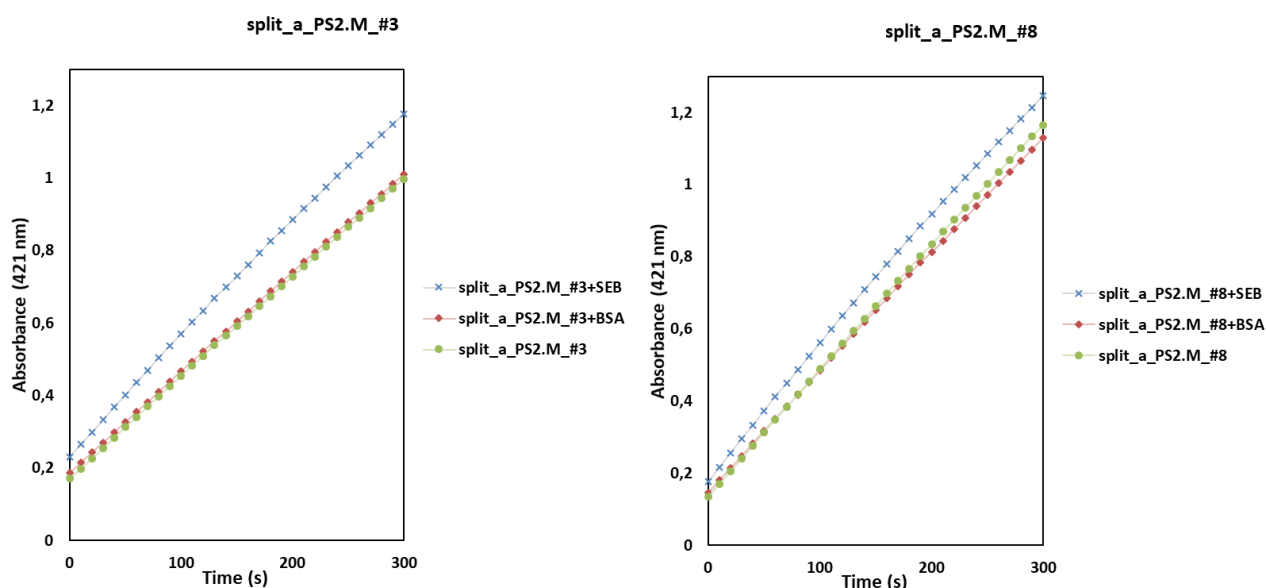


Figure 27 Time-dependent absorbance changes as a result of H_2O_2 -mediated oxidation of ABTS by the peroxidase-mimicking G4-hemin DNAzyme using asymmetrically split AUDENA designs. The specificity of the reaction was determined by carrying out experiments in the presence of either SEB or BSA or measuring the peroxidative activity of solely the AUDENA in the absence of any protein target.

1 μM AUDENA was mixed with 500 nM of either SEB or BSA in HEPES buffer (25 mM HEPES, 0.05% Triton-X-100, 1% DMSO, pH 7.4) and incubated for 60 min. 20 mM KCl and 200 mM

NaCl were added followed by 1.8 μM hemin. Experiments were carried out in the presence of 1.4 mM ABTS and 0.6 mM H_2O_2 , which were added to every well successively and mixed by pipetting. Absorbance was measured at 421 nm immediately after as a function of time. Measurements were carried out in triplicates agreed within an RSD of $\leq 9.89\%$.

Among the asymmetrically split AUDENAS, relative catalytic activity obtained with split_a_PS2.M_#3 was slightly higher compared to split_a_PS2.M_#8 (Table 16). Again, sequences differed solely in the linker sequences, with split_a_PS2.M_#3 containing linkers consisting of three Ts whereas split_a_PS2.M_#8 possessed longer ones containing six T or A nucleotides.

Table 16 Initial reaction rates (V_0) of asymmetrically split AUDENAS in the presence of SEB (+SEB) and relative activity (%) compared to control experiments carried out in the absence of SEB (-SEB). V_0 was calculated from absorbance values obtained after one minute of reaction time. Error-values represent the standard deviation of three replicate measurements.

AUDENA	$V_0 (+\text{SEB})$ ($\mu\text{M min}^{-1}$)	Relative activity
		(%) $V_0(+\text{SEB})/V_0(-\text{SEB})$
split_a_PS2.M_#3	4.02 ± 0.23	120
split_a_PS2.M_#8	4.59 ± 0.55	110

3. Discussion

As a matter of fact, bacterial pathogens have been evolving into a serious concern of modern society. The prevention of the spread of pathogenic bacterial species and development of hypervirulent strains, which is due to excessive use of antibiotics, might be accomplished through fast and feasible detection systems. Due to their many favorable features, aptamers have been gaining increased popularity over the past few years. Especially when compared to antibodies, for which they are envisaged to be feasible substitutes, the number of advantages of aptamers is evident. Both aptamers and antibodies possess a high affinity for their targets. To date, antibodies are still widely accepted as being the gold standard for antigen detection. However, the generation of antibodies is less cost- and resource-efficient as it is usually carried out *in vivo*. Moreover, antibody generation against a specific target is a time-consumable process. As a consequence of their production in animals, the production of antibodies inevitably relies on the immune response of a host, often impeding the generation of antibodies especially against non-immunogenic targets. Contrary, the whole *SELEX* process takes place *in vitro* circumventing the necessity of an animal model and also enabling the generation of aptamers for often non-immunogenic small molecule targets (Ruscito & DeRosa, 2016). Here, an aptamer sequence binding specifically to SEB as a target is used. Due to the fact that SEB is actively secreted by *S. aureus* and therefore is naturally present in the bacterial environment, the preceding lysis of staphylococcal cells is obsolete. Therefore, SEB presents an easily accessible target for potentially binding aptamer sequences of AUDENAs. As a next step and for a more thorough analysis of staphylococcal samples, surface proteins of *S. aureus* such as cell-wall anchored (CWA) proteins, could be addressed as potential targets. The SEB-binding part of the AUDENAs used in this study is thereby to be replaced by the corresponding aptamer sequences (Foster, Geoghegan, Ganesh, & Höök, 2014; Geoghegan & Foster, 2015). Aptamers specifically binding surface proteins as well as whole bacterial cells of *S. aureus* have already been generated through *SELEX* (Ramlal, Mondal, Lavu, Bhavanashri, & Kingston, 2018; Stoltenburg, Krafčíková, Víglaský, & Strehlitz, 2016). Therefore, the spectrum of application fields of detection could be broadened meeting the needs of a more distinct analysis of *S. aureus* isolated from clinical or food samples.

A critical feature of the colorimetric assay presented within this study is the fact that the cofactor of the G4-hemin DNAzyme, namely hemin, possesses itself catalytic activity towards the oxidation of ABTS via H_2O_2 . Several approaches have been made so far to reduce the background noise of hemin resulting in an increased sensibility of the detection system (Y. Zhang & Li, 2011). What is more, it was also shown, that ABTS is oxidized very slowly by H_2O_2 alone, even in the absence of any catalyst (Travascio et al., 1998). Alternatively, the here proposed detection system based on a G4-hemin DNAzyme could be replaced by any other detection system that is realizable within reasonable time expenses and financial efforts.

As shown by Chang et al., that the capability of DNAzymes to catalyze the oxidation of H_2O_2 increased with a higher binding affinity for hemin, which in turn depends on the nucleotide composition of the G4-forming sequences used (Chang et al., 2016). Especially those regions within the sequence flanking the G4 are thought to be decisive in terms of hemin interaction and overall activity of the DNAzyme (W. Li et al., 2016). Based on these findings, it is, of course, questionable to which extent AUDENA designs implementing a complementary sequence binding to parts of the G4, such as linear complementary and hairpin AUDENAs, are affected.

The universality of the colorimetry-based sensing system using ABTS as an indicator substance is to be proven by using e.g. TMB (3,3',5,5'-Tetramethyl[1,1'-biphenyl]-4,4'-diamine) as alternative chromogenic substrate. Analogous to ABTS, TMB acts as a hydrogen donor in the presence of peroxidative enzymes and causes a colorimetric change of H_2O_2 -containing reaction solutions due to an oxidatively formed yellow diimine measurable at 450 nm (B. Li, Du, Li, & Dong, 2009). As shown by *Li et. al*, oxidation of TMB is comparable to ABTS when used in a colorimetric assay based on a G4-hemin DNAzyme. What is more, the high performance of the AUDENA-based diagnostic test is evident due to the possibility of parallelizing measurements of up to 96 samples as proposed within this study using a 96-well plate. In high-throughput diagnostics, the simultaneous measurement of several hundred or thousands of samples is a time-critical as well as a financial factor that has to be considered.

SEB was captured by the proposed AUDENA in a linear range from 176-705 nM, with a resulting LOD of 26.47 nM. Studies also anticipating the usage of aptamer

sequences for the detection of various protein target molecules, such as thrombin and IgE, showed that the detection limit obtained relied strongly on the type of reporter unit rather than on the aptamer used (Strehlitz, Nikolaus, & Stoltenburg, 2008). Therefore, an increase in the detection efficiency of the AUDENAs might be achieved by optimizing or even exchanging the G4 sequences.

Furthermore, the induction of G4 formation was proven to be highly dependent on experimental factors. In particular, the presence of ionic species in the assay was shown to evidently impact the G4-hemin DNAzyme detection system. One of the decisive criteria when testing potent G4-stabilizing ions is their ionic radius (Bhattacharyya et al., 2016). Contrary to the findings in the present study, K^+ with an ionic radius of 1.33 Å was previously declared to be too big for proper coordination within a G4, whereas Na^+ was found to fit better due to its ionic radius of only 0.95 Å. Several studies on how the presence of different ions influences the ultimate structure a G4 adopts to were carried out in the past (Cáceres, Wright, Gouyette, Parkinson, & Subirana, 2004; Haider et al., 2002; Horvath & Schultz, 2001; You et al., 2017). Activity, as well as the stability of G4s, is clearly influenced by the presence of ions. It should be pointed out that increased stability is not necessarily accompanied by increased activity of G4s and vice versa. *Li et al.* proved that the addition of Pb^{2+} to the K^+ -coordinated PS2.M G4-sequence lead to stabilization of the G4 accompanied by a simultaneous decrease in catalytic activity (T. Li et al., 2010). In terms of final concentrations of ions used in the experiment, it has to be considered that increasing salt concentrations lead to a stabilization of the otherwise repulsive charge-mediated forces between adjacent DNA phosphate backbones (Yakovchuk et al., 2006). It is widely accepted, that mainly cations contribute to the generation of G4 secondary and tertiary structures. Nevertheless, studies suggest that e.g. ethanol leads to even better stabilization of quadruplex structures than potassium cations, which showed comparatively best results in the present study (Kuzuhara, Fujiwara, & Hori, 2007). These findings indicate that stabilization does not only rely on cations but also strongly on other components constituting the reaction solution which has to be taken into account when performing G4-based detection assays.

It was shown, that an excess of reagents present in the assay can lead to a functional impairment of the detection system based on a G4-hemin DNAzyme. Hence, the

optimization of experimental parameters with special emphasis on final concentrations of reagents and incubation times needs to be pursued. As described by Wang et al., the interference of metal ions, such as K^+ and Na^+ , which were originally found to be vital for the peroxidase-mimicking detection system, was circumvented by fabrication of metal-ion free G4-hemin DNAzymes (Wang, Zhao, Bao, & Dai, 2016). Through covalent bonding of the hemin to the terminal of the G4-forming sequence the exogenous addition of the cofactor was avoided. Consequently, the catalytic background activity of hemin was reduced.

Apart from the unambiguous effects that ions have on the G4-forming part of the AUDENAs regarding stability as well as activity, also the aptamer might be influenced likewise. Lin et al. carried out a study that showed how the binding capacity of an aptamer was impaired by the increase of Na^+ concentration used in the experiment (Lin et al., 2011). Shielding effects of cations on aptamer binding sites might interfere with the aptamer's ability to properly recognize and bind its target. In order to be able to consider the proposed ion-dependent AUDENAs for fast and feasible detection of SEB in clinical or food samples, a special focus has to be put on the stability and reliability of the DNA probes.

The weakness of PLA-like AUDENA designs might be the fact, that for the formation of an entire and fully active G4-hemin DNAzyme approximation of two DNA probe strands containing an aptamer each is required with the aptamer-binding site of SEB as the restricted element. Therefore, two aptamer sequences competing over one SEB-binding site might lead to a decrease in enzymatic activity measured.

As opposed to this, increased aptamer binding affinity could be enforced by the introduction of hydrophobic moieties through modified or artificial nucleotides during the course of the SELEX process (Hasegawa, Savory, Abe, & Ikebukuro, 2016). The exact mechanism of how aptamers bind to their targets is still to be elucidated. Hence, the aptamer sequence persevered in its original sequence when designing different AUDENAs unlike the G4 sequences used, which were partially used in split variants for split G4 AUDENA designs.

Regarding overall enzymatic activity and distinguishability of the different AUDENA designs tested, linear AUDENAs are to be preferred over the others for application of SEB detection in the here proposed 96 well colorimetric assays. Hairpin AUDENAs exhibited the least favorable properties, including high RSD values as well as comparably low relative activities.

SEB-contaminated samples, whether they derive from the food industry or the clinic, are expected to be composed of highly diverse and complex matrices. By adapting the experimental parameters of the detection assay, this complexity should be taken into account regarding future experiments. The creation of an authentic environment in which measurements take place should ensure that the AUDENA-based detection system is widely applicable for real samples containing SEB.

4. Conclusion

In this study, it was shown that sensing probes based on the binding-capacity of a SEB-specific aptamer and the enzymatic activity of G4-DNA, implemented by different AUDENA designs, exhibited the potential for the colorimetric detection of SEB. Several designs varying in G4 and linker sequences, secondary structures and therefore also in the way of G4-hemin DNAzyme formation, were obtained by connecting the aptamer unit to the enzymatically active moiety of the AUDENA. A comparison of the different AUDENA designs was drawn by their overall catalytic activity and their performance under various experimental conditions.

One of the linear AUDENA-based detection systems was validated by the determination of quality variables, namely LOQ and LOD. The obtained values of 121.56 nM and 26.47 nM respectively, were compared to resembling studies found in the literature. Relative activity of the AUDENAs in the presence of the SEB-target compared to experiments carried out in the absence of SEB was calculated and was found to be highest in one of the linear AUDENA designs, (linear_A_L_G4_PS2.M#2: 5' - CAC TGG TCG TTG TTG TCT GTT GTC TGT TAT GTT GTT TCG TTT TTT TTT TTG TGG GTA GGG CGG GTT GG - 3') making it a feasible candidate for future detection assays. To benefit future applications of the proposed sensing system, the following experimental parameters were examined and optimized: (1) Incubation time of the AUDENA and SEB with hemin was found to play an important role in the subsequent peroxidation of ABTS. Compared to pre-incubation of the AUDENA and SEB with hemin 60 min prior to the start of the peroxidation reaction, the addition of hemin just before the very start of the reaction resulted in higher relative enzymatic activity. (2) Experiments carried out using linear AUDENAs proved, that the catalytic activity of the G4-hemin DNAzyme strongly depends on the ions present in the reaction solution. Here, the addition of KCl leads to a significant enhancement of peroxidation by the G4-hemin DNAzyme, whereas NaCl resulted in comparatively lower absorbance rates.

Moreover, the results obtained in this work suggest that especially the composition and length of linker sequences used within different AUDENAs designs might impact overall aptamer binding und G4-hemin DNAzyme activity due to possible structural

changes of the G4 entity as proposed by preceding studies. Nevertheless, the initial reaction rates and relative activities of different AUDENAs are somewhat contradicting and need to be verified in further studies.

One of the striking advantages of the proposed system is the collection of biosensor data in a multiwell format, therefore enabling simultaneous high-throughput analysis of a great number of different samples. To prove the universality of the AUDENAs proposed in this study, detection of other targets than SEB through the usage of respective aptamer sequences should be considered and elaborated within further experiments.

Taken together, the findings received within the present study provide revelatory information for ongoing research on biosensor designs based on aptamers and G4-DNA sequences. In the light of emerging antibiotic resistance, the need for fast and reliable detection of microorganisms and their pathogens is evident. Hence, research on oligonucleotide-based detection systems is to be carried on vigorously.

Abbreviations

A	Adenine
ABTS	2,2'-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid)
AMD	Age-related macular degeneration
AUDENA	Autonomous Detection Nucleic Acid
BSA	Bovine Serum Albumin
C	Cytosine
C-terminal	Carboxy-terminal
CWA	Cell Wall-Anchored
EDTA	Ethylenediaminetetraacetic acid
G	Guanine
G4	G-quadruplex
HRP	Horse Radish Peroxidase
LOD	Limit of Detection
LOQ	Limit of Quantification
MRSA	Methicillin-Resistant <i>S. aureus</i>
MHC	Major Histocompatibility Complex
min	Minutes
N-terminal	Amino-terminal
PCR	Polymerase Chain Reaction
PLA	Proximity Ligation Assay
PTSAgs	Pyrogenic Toxin Superantigens
RSD	Relative Standard Deviation
RT	Room temperature
s	Seconds
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SE	Staphylococcal Enterotoxin
SEB	Staphylococcal Enterotoxin B
SELEX	Systematic Evolution of Ligands by EXponential Enrichment
STD	Standard Deviation

T	Thymine
TcR	T cell receptor
TMB	3,3',5,5'-Tetramethyl[1,1'-biphenyl]-4,4'-diamine
Tris	Tris(hydroxymethyl)aminomethane
TSS	Toxic Shock Syndrome
UV-Vis	Ultraviolet-Visible
VEGF	Vascular Endothelial Growth Factor
V₀	Initial Reaction Rate
WHO	World Health Organization

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