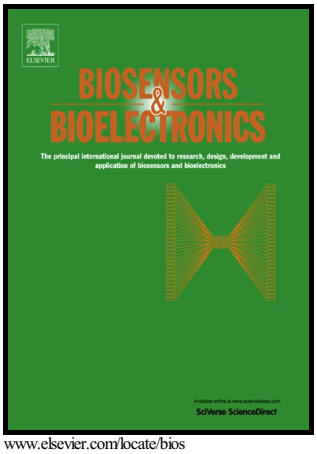

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New biorecognition molecules in biosensors for the detection of toxins.

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Abstract:

Biological and synthetic recognition elements are at the heart of the majority of modern bioreceptor assays. Traditionally, enzymes and antibodies have been integrated in the biosensor designs as a popular choice for the detection of toxin molecules. But since 1970's, alternative biological and synthetic binders have been emerged as a promising alternative to conventional biorecognition elements in detection systems for laboratory and field-based applications. Recent research has witnessed immense interest in the use of recombinant enzymatic methodologies and nanozymes to circumvent the drawbacks associated with natural enzymes. In the area of antibody production, technologies based on the modification of *in vivo* synthesized materials and *in vitro* approaches with development of "display "systems have been introduced in the recent years. Subsequently, molecularly-imprinted polymers and Peptide nucleic acid (PNAs) were developed as an attractive receptor with applications in the area of sample preparation and detection systems. In this article, we discuss all alternatives to conventional biomolecules employed in the detection of various toxin molecules We review recent developments in modified enzymes, nanozymes, nanobodies, aptamers, peptides, protein scaffolds and DNazymes. With the advent of nanostructures and new interface materials, these recognition elements will be major players in future biosensor development.

Keywords: Bioreceptors, novel designs, biosensors, toxin molecules, future development

1. Introduction

A biosensor can be defined as a compact analytical device or unit incorporating a biological or biologically derived sensitive 'recognition' element integrated or associated with a physio-chemical transducer" (Turner, 2000). The bioreceptor recognizes the target analyte, while

transducer converts the recognition event into a measurable signal. The uniqueness of a biosensor is that the two components are integrated into one single sensor (Fig.1). This combination enables the measurement of target analyte without using extensive volume of reagents. For example, the glucose concentration in a blood sample can be measured directly by simply filing a mini drop of blood on the glucose biosensor. The main advantage of a biosensor is the simplicity and the quickness of measurements without requiring specialized laboratory skills.

In principle, any biomolecules and molecular assemblies that have the capability of recognizing a target analyte can be used as a bioreceptor. The first bio recognition element used in biosensor design was from living system. Depending on the nature of bioreceptor, catalytic or affinity biosensors were developed in the literature. Enzymes were the first recognition element integrated in biosensor designs with wide spread sensing applications. However, other bioreceptors molecules such as antibodies and protein affinity systems were introduced very shortly in the construct of biosensors.

Due to the emergence of bioengineering techniques, and the difficulties to obtain recognition element against small size molecules such as toxins or pollutants, many novel biosensor recognition elements have been developed and synthesized in laboratory.

2. Modified Enzyme based recognition

Enzymes are the most widely used recognition element in the fabrication of various biosensors due to their specific binding affinities and catalytic activities. Recently, the catalytic enzymatic sensors have attained significant attention in biosensing applications due to their various measurable reaction products (light, electrons, protons and heat) arising from catalytic processes. Considering the needs of the reliable and robust sensing application, much effort has been done to design innovate and novel enzymatic sensor designs. To address the upcoming medical needs, researchers are interested to commercialize these sensors for the point of care testing. Among the developed enzyme based sensor, until now only the few methods have attained commercialized success such as glucose biosensors (Clark *et al*., 1962) and microfabricated electrophoresis chips (Chambers *et al*., 2008). However, the several disadvantages of enzyme sensor such as poor stability, critical operational condition, pH and temperature variation restrict their wider utility for real time applications. For example, the protein phosphates (PP1A and PP2A) are the enzymes which play a key role in various chemical reactions of cell physiology (Fig. 2). They are inhibited by a number of biotoxins (okadaic acid and its analogues, dinophysistoxins; DTX1, DTX2 and DTX3) produced by marine algae and freshwater. The PP2A purified from animal tissues has limited applications due to lower

enzyme activity and stability fluctuations. In thrust of improve stability, reliability, selective and sensitive measurements, scientific community explored the field of genetic engineering to develop the recombinant enzyme or to modify the catalytic/allosteric enzymatic sites. Various constructs of the PP2A inhibition-based biosensor have been reported for electrochemical monitoring of MC-LR in cyanobacterial cell samples (Campàs *et al.*, 2005, 2007 (b)) or for okadaic acid in marine samples (Volpe et al. 2009; Campàs and Marty, 2007 (c)). In the same context, Rubiolo *et al.*, (2013), genetically modified the PP2A catalytic site to enhance the enzyme stability and activity.

Acetylcholinesterase (AChEs), obtained from electric eel is used in 98% of the bioassay and biosensor designs for the detection of carbamates and organophosphates insecticides. The enzyme is considered very stable but lack of sensitivity against many insecticides restricts its applicability. To address this issue, Fournier (2005), produced hundreds of mutants of AChEs to improve the selectivity of enzyme towards the majority of the insecticides, and ability to discriminate in between insecticides such as organophosphate (OPs), carbamates and natural neurotoxic (anatoxin-a) (Villatte *et al*., 2002). Similarly, the functional expression of recombinant AChEs have been studied in rat, COC cells, baculovirus-insect cell system (Schulze *et al.*, 2003). AChE-immobilized transducer surfaces have been used for detecting AChE inhibitors including organophosphorus and carbamate pesticides. The conducting transducer platforms based on carbon electrode, gold and platinum electrode (Guler *et al.*, 2016, Luan *et al.*, 2016, Nesakumar *et al.*, 2016), glassy carbon electrode (Shamagsumova *et al.*, 2015), chitosan (Warner and Andreescu, 2016, Timur and Telefoncu, 2004) or Prussian blue-modified electrode (Sun and Wang, 2010) and nanocomposite (El-Moghazy *et al.*, 2016, Ye *et al.*, 2016, Kestwal *et al*., 2015) have been employed in the construction of AChE biosensors.

Site directed mutagenesis is another approach to increase assay sensitivity towards toxins and insecticides analysis. The genetic modification of the enzyme has been shown to yield more active and stable bioreceptor molecules (Schulze *et al.*, 2003). For example, the sensitivity of AChEs from *Drosophila melanogaster* was observed 12-fold higher after mutation at position 408 (Villate *et al.*, 1998). These enzyme variants were used in biosensor application for sensitive detection of Anatoxin-a in water samples with a detection limit of 1.0 µg/L (Villatte *et al.*, 2002; Devic *et al.*, 2002). The recombinant enzyme was used for detection of aflatoxin B1 (AFB1) (Puiu *et al.*, 2012; Moscone *et al.*, 2011; Hansmann *et al.*, 2009; Arduini *et al.*, 2007) with the aim to enhance the stability and sensitivity of assay. Mycotoxin induced inhibition of genetically modified AChE was integrated in surface plasmon resonance (SPR) assays which provided improved analytical figures with the detection limits (LOD) of 0.008 µM (2.5 ng/ml) for AFB1 analysis(Puiu *et al.*, 2012).

Several strategies based on genetically engineered enzyme with alternative merits have been reported in the literature. The perusal of recombinant enzymes of different origin in various cell systems is highly important. Depending upon the host system, several type of PP2A have been produced such as trimeric, dimeric (e.g. PP2A(Aα/Cα)) or monomeric catalytic (PP2ACα) form. In general, the first choice for the expression of recombinant proteins is *E. coli*, however, the method was not fruitful to obtain expression of active human PP2A. On the other side, the enzyme in its active form was successfully expressed in yeast, mammalian cells (Swiateck *et al.*, 2000), insect cells (Myles *et al.*, 2001) and insect larvae (Rubilio *et al.*, 2012). Very low quantities of active enzyme were produced in yeast and mammalian cells, while a high expression level was achieved in insect cells and insect larvae (Rubiolo *et al.,* 2013). Both systems, insect cell driven expression and insect larvae expression were characterized with high quantities of the catalytic subunit of PP2A with enhance activity and inhibition by okadaic acid (OA), similar to that observed for the enzyme purified from animal tissues (Rubilio *et al.*, 2012). Various other studies have also demonstrated the potential advantages of recombinant enzymes as compared to the natural one in term of sensitivity and stability for detection of biotoxins (Garibo *et al.*, 2012; Sassolas *et al.*, 2011). The recombinant PP2A (rPP2Ac) expressed in baculovirus system has been shown to detect various microcystin analogs, microcystin-LR, -RR, -YR, -LF, and -LW, and nodularin. rPP2Ac activity was strongly inhibited with IC_{50} values of 0.048, 0.072, 0.147, 0.096, 0.114, and 0.54 nM, respectively for above mentioned toxins (Ikehara *et al.,* 2008). The recombinant human AChE is generally unstable, however, work by Mor *et al.*, (2001) described the expression of human AChE in transgenic tomato with increased activity and stability (Mor *et al.*, 2001). A similar phenomenon was observed in other plants (Geyer *et al.*, 2005; 2010). In conclusion, modified based enzyme recognition elements are characterized with high selectivity, specificity, stability and sensitivity, but complicated modification process increases the cost per assays and may also impact their recognition characteristics' to some extent.

3. Recombinant antibody fragments and nanobodies

Antibodies of the immunoglobulin G (IgG) class are preferably employed in the development of immunosensors for environmental toxins (Fig 3). Their use has been demonstrated for the detection of mycotoxins such as ochratoxin A, aflatoxins, DON (Meulenberg, 2012; Bazin *et al.*, 2010; Pietro-Simón *et al*., 2012) and some cyanotoxins (McNamee *et al.*, 2013; Campas *et al*., 2007; Feng *et al.*, 2014). Although, antibodies are the most successful binder, they exhibit limitations associated to their biophysical properties such as poor solubility, low thermal stability, thermal induced aggregation and retention of binding affinities at high temperature.

As a consequence, derived antibody molecules with smaller size such as recombinant antibody fragments Fab, Fv (variable fragment), scFv (single chain variable fragment) and the VH containing all specific recognition sites of the antibodies have been explored for the fabrication of immunosensors.

Recombinant antibodies (rAb) isolated by enzymatic action or from in vitro combinatorial phage display libraries are an exciting alternative due to (1) circumventing the need for experimental animals, (2) speed of production in commonly used *in vitro* expression systems and (3) subsequent molecular enhancement of binder performance. Enzymes such as papain and pepsin are used to digest the intact antibody to generate small antigen binding fragments (Fig.3). A new approach based on reduction via tris(2-carboxyethyl) phosphine (TCEP) was described for detection of toxin produced by group of *Escherichia coli*. Fv fragments are the smallest entities that can also bind with antigen to some extent. They comprise of both VH and VL variable regions assembled in functional form to interact with their monovalent antigen. However, the interaction between the two modules is very low and Fv is unstable. While two chains are held as heterodimers through interaction of VH and VL domains, especially between the CH1 and CL domains in case of Fab fragment. BIRD in 1988 proposed methodology to link the two DNA domains by a flexible linking sequence of 10 to 25 aa (Linker), to avoid the dissociation process upon dilution (Hudston *et al.*, 1988; Worn and Pluckthun, 2001). ScFv (25 kDa) are heterodimeric antibodies fragment, consisting of variable light and heavy chain joined by synthetic linker rich in serine and glycine (Fig.3.)

While representing less than 20% of the size of an intact antibody, fragments consisting of heavy and light-chain variable domains (scFv) have been shown to retain specificity and sensitivity for the target antigen. In this context, scFv have been the most commonly employed rAb reagents for hapten biotoxin detection over the last two decades. Phage display technology has facilitated the selection and enrichment of these recombinant antibody fragments from large antibody libraries against a range of toxins. These library can be screened for bacterial environmental toxins like Shiga (Neri *et al.*, 2011; Zhang *et al.*, 2012; Wang *et al.*, 2012), fumonisin B1 (Hu *et al.*, 2013),deoxynivalenol (Wang *et al.*, 2007; Choi *et al.*, 2004)and zearalenone (Chang *et al.*, 2008). Antibody fragments selected against particular antigens can be expressed in the bacterial host such as *Escherichia coli* (Glockshuber *et al.*, 1990). As an example, ScFv have been developed for the detection of tetrodotoxin, a small molecular weight neurotoxin (Wang *et al.*, 2014 (a)). Moreover, development of a single-chain variable fragment has been obtained with monoclonal antibodies for the development of an immunosorbent assay for determination of fumonisin B_1 (Min *et al.*, 2010 and 2015; Hu *et al.*, 2013 and 2015; Zou *et al.*, 2014) and deoxynivalenol (Ramanazzo *et al.*, 2010). Test

performance clearly depends on the selected scFv sequence. For example, the scFv obtained with monoclonal antibody against fumonisin B1 has a binding activity 12-fold lower than the monoclonal antibody (LOD > 110 µg/kg) (Min *et al.*, 2010). Zou *et al.*, used another type of scFv in ELISA based assay for quantitation of fumonisin B1 in naturally contaminated corn samples (Zou *et al.*, 2014). scFv antibody can also competitively bind to free FB1, FB2, and FB3 with zero cross-reactivity against deoxynivalenol, nivalenol and aflatoxin (Hu *etal.*, 2015., 2010). Recently scFv for citrinin has been selected by *in vitro* eukaryotic ribosome display method. The selected scFv was used to perform an indirect ELISA based assay for citrinin analysis. The eukaryotic ribosome display method operates through the formation of libraries of antibody-ribosome-mRNA complexes that are selected on immobilized antigen, followed by recovery of the genetic information from the mRNA by RT-PCR (Fig. 4)(Cheng *et al.*, 2015).

So far, different techniques are in progress to create multimerization of ScFv. A short peptide is preferred to (11 amino acid residues or less) induces multimerization of the fragment because steric constraints prevent the association of the heavy and light variable domains of one subunit. Several subunits can then associate the variable domain of a heavy subunit pairing with light variable domain of another subunit. The nature and number of amino acid residues composing the arm and the arrangement of two variable domains influence the stable form and proportions of various oligomers (Kortt *et al.*, 2001). ScFv can directly self-assembly in diabodies or Bis-ScFv (dimers ScFv, 55kDa) with the linker length from three to eleven residues (Holliger *et al.*, 1993). Reducing the link within three residues promotes formation of triabodies (3ScFv, 80kDa) (Pei *et al.*, 1997) or tetrabodies ScFv (Power *et al.*, 2003). The multimerization process increases the number of antigen binding site and subsequently enhance the sensitivity of the immunoassays by increasing the avidity and stability of the ScFv antigen interaction (Dolezal *et al.*, 2003). To date, these novel antibody fragments are mainly used for gene therapy, but this new architect could be easily extended to the detection of toxins in a similar fashion that has been reported for simultaneous analysis of fluoroquinolones and sulfonamides in milk sample (Chen *et al.*, 2014 (a)) or detection of virus (Chen *et al.*, 2007). The direct pairing of two independent antibody specificities to create a new bispecific antibody may also offer the enhanced detection of toxins in the future (Spiess *et al.*, 2013). Considering the potential impact of these novel bioreceptors, a ScFv based optical-planar waveguide immunoassay has been designed for the detection of cyanobacterial toxin. The assay provided a limit of less than 0.2 ng/mL$^{(-1)}$for microcystin-LR analysis(Murphy*et al.*, 2015)

Recently, heavy chain antibodies (hcAbs), found in camelids or immunoglobulin new antigen receptor (Ig NAR) from shark, have gained considerable attention due to their unique structure, being composed of only heavy chains (Fig 5). These single-chain antibodies or hcAbs

(heavy Chain Antibody) lack light chains, and are composed of a VHH variable domain different from the VHs domain (Hamers-Casterman *et al.*, 1993). They are also known as nanobodies (nbAb) or single domain antibodies (sdAb) due to their small size and variable domain VHH of camels. Nanobodies are robust, stable and can be stored at 4°C for several months and for a longer period at -20°C and even several months at 37°C without affecting their binding capacity (Ghahroudi *et al.*, 1997). They can also resist to high stringent condition with denaturation occurring around 3 M guanidinium and at 70°C (Dumoulin *et al.*, 2002). Nanobody fragments of hcAb, have been investigated for the detection of toxin molecules such as Bacillus thuringiensis (Bt) Cry1Ac and Cry1Fa in environment (Li *et al.*, 2014; Wang *et al.*, 2014 (b)).The current tendency is to use these new bioreceptors for detection of environmental toxins and mycotoxins such as aflatoxin (Wang *et al.*, 2013(a) and 2016(a)) and ochratoxin A (Liu *et al.*, 2014 and 2015(a)). Interestingly, nanobodies have also been developed to mimic the target analyte and are named as anti-idiotipic nanobody. Recently, an alpaca nanobody was isolated against fumonisin B1 to design a Nb-ELISA for the detection of FB1 (Shu *et al.*, 2015) or citrinin (Xu *et al.*, 2015 (a), (b)). On the other hand, the potential use of anti-idiotypic VHH as surrogate for small molecules is demonstrated to improve assay performance for food and environmental safety monitoring. Other cartilaginous fish species such as nurse shark with similar Ig-NAR have variable domain similar to VHH, but VHH have a higher degree of sequence identical to human VHs (Nuttall, 2012).

Nbs can also be chemically modified for site-specific immobilization onto a transducer surface (Saerens *et al.*, 2005); branched to nanoparticle (Van de Broek *et al.*, 2011) and express in fusion to magnetic nanoparticle (Pollithy *et al.*, 2011). Nbs have great potential and are worth to explore as a biorecognition element in the construction of immunosensors for toxins detection.

Although, rAb-based biosensors have been reported to detect sub-regulatory levels of fungal (mycotoxins), marine (phycotoxins) and aquatic biotoxins in a wide range of food and environmental matrices, however, this technology is yet to surpass the performances of the equivalent mAb- and pAb-based formats. The full potential of such rAb technology in toxin detection is yet to be achieved, but we can foresee the inherent advantages of engineered rAb to provide the next generation of ultra-high performing binder reagents for the rapid and specific detection of hapten and toxins.

4. Aptamers

Aptamers are oligonucleic acids (DNA or RNA) which can be synthesized against a wide range of target analytes with high binding affinities. While the development of antibodies depends on the immunization of animals and their production via cell culture techniques, aptamers are much easier and cheaper to produce through in vitro process, without requirement of cells or animals. Consequently, they can be selected against all type of target including those with high toxicity or low immunogenicity. Aptamers are considered as the amino acid analogue of antibodies and have a strong affinity to the targets with a dissociation constant close to the nano-picomolar (Wang *et al.*, 2015; Wilson and Szostak, 1999).

The natural aptamers were discovered in 2002 in a gene regulatory element based nucleic acid riboswitch. Riboswitch is a part of an mRNA molecule that is transcribed from a gene, which binds directly to a small target molecule. Aptamers were produced independently for the first time in 1990 in two different laboratories at the University of Colorado at Boulder and Massachusetts General Hospital in Boston in the USA. Larry Gold (Tuerk and Gold, 1990) used the term SELEX (systematic evolution of ligands by exponential enrichment) for the process selection of RNA binding to the T4 DNA polymerase, and Jack Szostak (Ellington and Szostak, 1990) selected the RNA binding to different organic dyes and coined the term "selection in vitro "and" aptamer". Aptamer selection process is based on the repetition of successive steps of target binding and removal of unbound oligonucleotides, followed by the steps of elution, amplification, and purification of the selected oligonucleotides. SELEX process enables the selection of a specific ligand from a random library of 10^{15} different sequences against wide size range target analytes (Cheng *et al.*, 2009) including ions (Wrzesinski and Jóźwiakowski, 2008), amino acid (Yang *et al.,* 2015(b)), peptides (Nieuwlandt *et al.*, 1995), proteins (Huang *et al.*,2014), viruses (Liang *et al.*, 2014) , whole cell (Wang *et al.*, 2014 (c)) and bacteria (Duana *et al.*, 2013*).* The use of aptamers for analytical applications for various toxins has been extensively explored in the recent literature. A multitude of aptamers have been developed since first aptamer sequence recognition and were integrated in aptasensors development for the detection of mycotoxins (Cruz-Aguado and Penner 2008, Yang *et al.*, 2011, Rhouati *et al.*, 2013(a), Liu *et al.*, 2015(b)), bacterial toxins (Tok *et al.*, 2008; Luo *et al.*, 2014), and cyanotoxins (Eissa *et al.*, 2013; 2014).We have summarized the aptasensing methodologies proposed against various toxins in Table 1.

Aptasensors have been characterized with similar or even better performance compared to antibody based assays in laboratory analysis. Aptamer-based methods have been widely applied to replace the immunochemical methods in toxin biosensing applications. They offers various advantages including but not limited to reduced cost, ease of chemical

modification,long term storage stability, transportation at ambient temperature, regeneration of biosurface with retain affinity and site selective modification.

As aptamers mimic the properties of antibodies, aptamer-based bioassays are analogous to immunoassays and can adopt different assay configurations to transduce bio-recognition events (Song *et al.*, 2008). Aptamer-based assays have been designed from simple optical bioassays (Chen *et al.*, 2012; Elshafey *et al.*, 2014) to sophisticated electrochemical aptasensors (Eissaa *et al.*, 2015; Rhouati *et al.*, 2013) in toxins monitoring

The recent progress in aptasensing has permitted the multiplexed detection of various toxin analytes simultaneously. (Yue *et al.*, 2014; Saberian-Borujeni *et al.*, 2014) The novel aptamer based multiplexed FRET system was established for the first time in detection of mycotoxins (Wu *et al.*, 2012) and enterotoxin B (Wu *et al.*, 2013). In a step towards more sophistication, Berezovski *et al.*, introduced a technique to sequence high binding affinity clones in a selective manner. Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), consist of repetitive partitioning step of clones without amplification step, was employed to shorten the procedure to one week in comparison to several weeks of SELEX approach.

5. Ribozymes/DNAzymes

The discovery of natural RNA catalysts has prompted chemical biologists to pursue artificial catalytic nucleic acid. Such artificial nucleic acid enzymes may comprise either RNA (ribozymes) or DNA (deoxyribozymes). The term “ribozyme was first introduced in 1982 with the discovery of natural catalytic activity of RNA (Kruger *et al.*, 1982) and subsequently used universally for both artificial or natural catalytic RNAs. The term “deoxyribozyme” was first used in 1994, with the first report of artificial DNA catalyst by Breaker and Joyce (Breaker and Joyce, 1992).

Combinatorial techniques “in vitro selection” have enabled the identification of functional RNA and DNA sequences with a wide range of catalytic activities. Both ribozymes and deoxyribozymes can be used for practical purposes such as sensing the presence of analyte in various mediums. In this context, numerous efforts have been focused on nucleic acid enzymes as the basis for biosensors applications. One of the most productive approaches combines *in vitro* selection with rational design to create allosteric nucleic acid enzymes. In such a case, catalytic activity is regulated by binding of a small-molecule ligand remote to the catalytic site in a modular fashion (Fig. 6). In particular, catalytic RNA structures, such as the hammerhead and hairpin ribozymes have been used in biosensor engineering for the detection of metabolites, antibiotics and toxins like ricin A (Muller *et al.*, 2006; Roday *et al*., 2008). Liu *et al.*, have developed deoxyribozyme sensors for metal ions and small organic

molecules, based on the fluorescence or colorimetric detection methodologies (Liu *et al.*, 2003; Liu and Lu, 2004; 2005).

DNA enzymes or deoxyribozyme have already demonstrated their capacity for the development of biosensors for various toxins. DNAzyme have been used to create fluorescent biosensors by employing fluorophore and quenching element in the detection medium where the signal was generated upon cleavage of DNA (Wu *et al.*, 2013; Yu *et al.,* 2013). The coupling of gold nanoparticle with DNAzyme was also explored to design colorimetric biosensors. The specific construction allowed DNAzyme to selectively catalyse the luminol/H_2O_2in the presence of hemin as peroxidase mimic to generate a chemiluminescence response(Xiao *et al.,* 2010) for the detection of ochratoxin A (Teller *et al.*, 2009; Travascio *et al.,* 2001, Yang *et al.*, 2012*)*and oxidize 2,2-azino-bis-(3-ethylben-zthiazoline-6-sulfonic acid) (Travascio *et al.,* 1998*)*.Yang *et al.* have implemented DNAzyme-aptamer methodology for the analysis of ochratoxin A in wine samples (Yang *et al.*, 2013) The OTA-specific aptamer sequence was coupled with the horseradish peroxidase (HRP)-mimicking DNAzyme sequence to design the colorimetric aptasensor. Other studies have demonstrated that DNAzyme can also be integrated in electrochemical (Zhang *et al.*, 2013) and SERS biosensors (Wang *et al.*, 2011).Chen et al have recently investigated this approach for the detection of ochratoxin A (Chen *et al*., 2014(b)). Similarly, resonance energy transfer (CRET) using DNAzyme-linked aptamers was applied for very sensitive detection of ochratoxin A with a LOD of 0.27 ng/ml (Mun *et al.*, 2014). DNAzymes offers many advantages such as higher stability in serum samples as compared to that of SiRNA, relatively easy and inexpensive synthesis, non-toxicity in preclinical studies, increased stability both in vitro and in vivo, improved activity and elevated sequence specificity. DNAzymes are prone to nucleolytic degradation in body fluids, however, this problem can be resolved by the integration of modified nucleotides.

6. Peptides and Artificial protein scaffold (affibody)

Peptide receptors have many advantages over antibodies, and can be obtained for any target from toxic species to low immunogenicity analytes. Peptides can be chemically synthesized, offering a wide variety of targeted modifications, such as fluorescent or affinity tags. Due to their small molecular weight, they are more stable in a wide range of buffer solutions and less prone to activity loss. Phage display technique is the most recently reported combinatorial approach to select specific peptides. This technique allows to study protein–protein or protein–peptide interactions using bacteriophages (viruses that infect bacteria) to connect proteins with the genetic information for encoding. Affinity selection of peptide by panning (Fig.7) and subsequent reinfection into *E. coli* greatly enhances the number of specific and high

affinity-binding peptides. To perform the panning process, the phage library is incubated with specific antigen in an immunoplate. The unbound phage particles are washed away and the remaining bound phages are then removed under strict elution conditions. Successive rounds of panning are carried out to enrich peptide binders, and to ensure the isolation of peptide fragments with the strongest binding affinities.

Peptides are used to mimic the mycotoxins as immunochemical reagents (Yuan *et al.*, 1999; Thirumala-Devi *et al.*, 2001; Yu *et al.*, 2005; Liu *et al.*, 2013; Wang *et al.*, 2013(b); He *et al.*, 2013). Recently, this combinatorial approach has been employed to select short peptide libraries that have been specifically synthesized to target specific molecules, including endotoxins and mycotoxins like ochratoxin A and aflatoxin B1 (Giraudi*et al.*, 2007; Tozzi *etal.*, 2003). In the latter case, peptide was used to mimic the antigenic region of the toxin for the development of a specific biosensor (Mujika *et al.*, 2014). In a complementary approach, the structure-based receptor design was built on the known target structure (Bazin *et al.*, 2013) that permitted detection of ochratoxin A at 1ng/ml level upon integration in biosensor design (Soleri *et al.*, 2015). In the same sequence, some computational modeling programs, such as SYBYL (technique of representing molecular structures numerically and simulating their behavior with quantum equations and classical physics) have been successfully employed for selection of ochratoxin A specific peptide ligand (Heurich *et al.*, 2013). However, the major issue in the use of small peptide is difficulty in control of the scaffold structure. To overcome this problem, the development of protein scaffolds has been reported in the literature, particularly in selection of peptides against small proteins. They have already been shown as a possible bioreceptor in the detection of proteins and some small molecules.

The idea to use protein scaffolds as molecular binders was to copy what nature had already designed, but with improvements. The structure of IgG molecules allows antigen recognition due to the combination of a structurally-conserved framework supporting a spatially-defined binding site. The binding sites are composed of peptide segments that are hypervariable in both sequence and conformation. By selection of a very stable protein structure that can tolerate substitution, insertion or deletion of variable domains, specific binding regions can be selected through recombinant-protein techniques (Beste *et al.*, 1999). The selected protein scaffolds are usually smaller than IgG in size. Over 50 types of scaffolds have been identified in the literature, and some of them such as affibodies, ankyrin repeat protein (DARPin) or protein lipocalin have been used in the biosensing applications (Binz *et al.*, 2004;Pluckthun, 2015) . The binding domain contains some randomized amino acids which is responsible for the production of disulphide bonds in loops. For example, Ramoni *et al.* investigated the binding capacities of four forms of lipocalin odorant-binding protein (OBP) for the detection of

explosives components (Ramoni *et al.*, 2007). Affibody molecules have intrinsic small size, fast folding ability and simple but robust non-cysteine containing structure. Based on their 13 amino acid positions variation between binding members specific for different receptors and proteins, most of the modulation and labeling technologies of one affibody molecule can be extended to another. This new approaches can pave a way as an alternative for the development of small peptides in detection of toxins.

7. Synthetic alternatives to antibody-based molecular recognition: Molecularly Imprinted Polymers (MIPs)

In parallel to biological-based receptor for toxins and small molecules, researchers are also interested in the potential of synthetic materials to achieve selective recognition of toxins.

Molecularly Imprinted Polymers (MIPs) offer an accessible method of creating substrate-specific materials. Molecular imprinted polymers offer great promise for development of very stable "solid-state like" artificial sensing elements. Three different methodologies to prepare MIPs have been reported: covalent, non-covalent and semi-covalent approaches. The non-covalent approach is the most widely used technique for the preparation of MIPs, thanks to its versatility (Fig 8). In recent years, the technology of molecular imprinting has proliferated as an inexpensive, accessible and effective strategy for the development of sorbent materials, exhibiting high specificity for selected substrate materials. MIPs are developed for a wide range of small compounds and toxins. The use of MIPs as artificial antibodies for sample pretreatment was described for detection of mycotoxins (Cigić and Prosen, 2009). There are also reports on the integration of MIPs in immuno-based sample preparation methods for trace analysis (Xu *et al.,* 2011; Ge and Turner, 2009).

Recent years have witnessed increasing attraction in MIPs as substitutes for immuno-analysis (e.g., binding assays, biosensors, and solid-phase immune-extraction). As early as 2004, a two-dimensional extraction procedure combining solid phase extraction (SPE) and MIP was used for the extraction of OTA (Maier *et al.*, 2004). The same approach was extended to extraction and pre-concentration steps prior to HPLC analysis of ochratoxin A (Giovannoli *et al.*, 2014; Lee *et al.*, 2012; Yu *et al.*, 2010; Ali *et al.,* 2010), zearalenone (Lucci *et al.*, 2010; fumonisin B (De Smet *et al.*,2009) or aflatoxins (Szumski *et al.*, 2014). Some of the recent and novel applications include the development of MIP-based sensing protocols such as piezoelectric biosensing techniques. SPR has been proven to be one of the most adaptable techniques in the area of MIP-based sensing, and has been equally employed for quantitative analysis of the mycotoxinsandT-2 Toxin (Choi *et al.*, 2011, 2009; Gupta *et al.*, 2011). Additionally, MIP based

sensor have also been reported for cyanotoxins detection like mycrocystin LR (Chianella *et al.*, 2003; He *et al.*, 2015). The MIP offers some advantages included but not limited to their stability under varying temperature conditions and physiological pH, low cost and ease of preparation. But their specificity and affinity are generally considered lower in comparison to the binding affinities of antibodies.

8. Peptide nucleic acid based recognition

Peptide nucleic acids (PNA) are synthetic DNA analogues or mimics with a polyamide backbone instead of a sugar phosphate bone. Both double and triple stranded complexes are capable of being formed by PNA in association with nucleotides (Nakamura and Karube, 2003; Sawata *et al.*, 1999).The negatively charged ribose-phosphate backbone of nucleic acids is replaced by an uncharged N-(2-aminoethyl)-glycine scaffold to which the nucleobases are attached via a methylene carbonyl linker (Fig. 9). Because the intramolecular distances and configuration of the nucleobases are similar to those of natural DNA molecules, specific hybridization occurs between PNAs and cDNA or RNA sequences.

PNA can be integrated into microarrays and other biosensor formats. Specific hybridization of PNA to complementary DNA or RNA is generally used to quantify the toxin producing. For example, the development of a highly sensitive and specific PNA enabled detection of E. coli O157 (non-O157 Shiga toxin-producing Escherichia coli (STEC)) with a detection limit of 1 CFU/25 g or ml in food samples (Almieda *et al*., 2013).Some researchers have used specific peptide nucleic acid probes to detect Alexandrium rRNA for detection of the marine dinoflagellate Alexandrium (Duy *et al.*, 2014). With the progress in the field of PNA, it is also possible to measure toxic chemicals with indirect approach. For example, a sensor system was designed for indirect detection of atrazine, based on the quantification of Saccharomyces cerevisiae (Oyama *et al.*, 2001). Similarly, this technique also enaled the indirect detection of some specific gene producing environmental toxin like verotoxin-producing Escherichia coli O157:H7 through measure of Shiga toxin-2 (stx-2) gene (Kai et al. 2000). Past PNA research has mainly focused on the detection of toxin producing organisms, however, future research can be directed towards detection of some mycotoxins and cyanotoxins. PNA offers advantages in the design of DNA biosensors because they are cleaved by nucleases or protease, making them stable biorecognition element. However, PNA are limited to only for the analysis DNA sequences, and are not explored for other type of target analytes yet.

9. Nanozymes; next-generation artificial enzymes

With the advent of nanotechnology, nanomaterials based enzyme mimics have been emerged as alternative catalysts to natural enzymes in the domain of chemical analysis. In this context, inorganic nanomaterials as a single component or multi component (composites and doped materials) have been explored as artificial enzyme with high surface to volume ratio, enhanced catalytic activity and abundance of surface reactive species(Wei and Wang 2013). Most of these materials act as oxidase, per-oxidase and catalase mimetics to replace the biological catalysts. Some of these materials have been employed as active materials in bioassays, biotechnology and biomedical field (Gao *et al.*, 2007; Tang *et al.*, 2011). They offer the advantages of low cost, high stability, easy of production and tenability of catalytic activity. To date, these materials are mainly used to replace horseradish peroxidase enzyme in the monitoring of hydrogen peroxide (Xiang *et al.*, 2015), glucose(Wang *et al.*, 2016(b)), antioxidants (Sharpe *et al.*, 2013), cholesterol (Hayat *et al.*, 2015 (b)) and d-alanine (Haider *et al.*, 2015), but there are only few reports on the detection of toxin analytes. Figure 10 shows a principle of nanoenzyme; the replacement of natural enzyme by nanozyme in the detection of hydrogen peroxide (Fig 10). For example, oxidase like properties of nanoceria particles was explored in the development of colorimetric assays for the detection of catechol and dopamine to replace the commonly used tyrosinase enzyme in a recent study(Hayat *et al.*, 2015 (a)). Similarly, metal contaminants have been observed to specifically quench the fluorescence of nanomaterials in the development of fluorescence assays (Lu *et al.*, 2012). Analytes specific nanomaterials have been incorporated on the transducer surface to perform the direct electrochemical detection of various target molecules(Liu *et al.*, 2013). Although this field of research is relatively new, but future work may consider such methodologies to replace the biological receptors in designing analytical assays for toxin analytes. However, nanomaterials have not real enzyme like properties, as it is very difficult to regenerate the nanomaterials surface for subsequent measurement, limiting their applications in the amperometric biosensors. Moreover, it is very difficult task to control the reactivity of nanomaterials against certain interfering molecules. To replace enzyme for biosensing applications, it is highly desirable to design selective and specific nanomaterials to overcome the matrix interferences.

10. Conclusion

There has been a rapid growth in the development and application of naturally occurring molecular recognition elements in the field of biosensors. However, in parallel, different approaches are under investigation to produce alternatives to conventional bioreceptors that can be used to detect small-size analyte like toxins for environment and food analysis. Table 2

provides an overview of different receptors used in detection of such toxins. Clearly, enzyme, antibodies and DNA aptamer are dominating the field and with hindsight, the literature has shown that some receptors offers advantage but also have disadvantages. This is presented in Table 3. Sophisticated chemical modification and biotechnical techniques have been applied in the refinement of their binding, chemical and thermal stability and in their immobilization properties. In the same context, there has been a recent rise in the development of synthetic molecular recognition elements. The latter promises to offer more robust recognition but to date lack the specificity offered by their natural counterparts. As discussed in previous section, biosensors employ a broad diversity of different bioreceptors (natural or artificial). And now to overcome the lack of specificity, the future trend will be a combination of various bioreceptors. Recent paper showed the used of dual-aptamer for detection. Additionally, it can be concluded that combination of the DNAzymes and the aptamers may offer wide spread applications as a new class of functional nucleic acids known as allosteric DNAzymes or aptazymes. By using these receptors molecules, a variety of sensing methodologies can be designed.

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Figure caption

Figure 1: Biosensor configuration showing biorecognition, interface, and transduction elements.

Figure 2: Structure of PP2A. The quaternary protein structure is shown, composed of the catalytic subunit Cα, the 65 kDa regulatory subunit Aα, and the 55 kDa regulatory subunit Bα (Rubilio et al., 2013).

Figure 3: Recombinant antibodies used for immunoassay, immunoglobulin G (IgG) and related fragments. In orange is represented the linker. CH : constant heavy ; VH : variable heavy ; CL : constant light ; VL : variable light.

Figure 4: Ribosome display. a. Ribosome-mRNA complexes. b. Ribosome display technique: a native library of DNA sequences coding for polypeptides is transcripted and translated. The

ribosome stopped at the end of the mRNA because of the absence of a stop codon and does not release the protein. The ribosome-mRNA complexes are used for affinity selection on an immobilized target. mRNA of bound complexes is recovered after washing and the disruption of ribosome complex. The mRNA obtain are the reverse transcribed and amplified by PCR, they can be used directly for analysis of single clones after cloning into expression vectors or for the next cycle of ribosome display.

Figure 5: human conventional monoclonal antibodies (mAbs) and single chain Fv (scFv); single heavy chain antibodies from camelid (HCaB) and Sharks (Ignar) and corresponding single domain fragments (dAb). CH : constant heavy ; VH : variable heavy ; CL : constant light ; VL : variable light.

Figure 6: Schematic view of nucleic acid enzyme catalysis, showing separate binding and catalytic regions of the ribozyme or deoxyribozyme (Silverman et al. 2008)

Figure 7: Phage display technique: phage display screening to identify polypeptides that bind with high affinity to desired target toxin.

Figure 8: Formation of molecularly imprinted polymers

Figure 9: PNA structure and Watson-Crick base pairing with DNA

Figure 10: Replacement natural enzyme by nanozyme in the detection of hydrogen peroxide

Table 1 : Aptamers for mycotoxins and toxins

Aptamer type	Target toxin	Technique involved in bosensor	reference
DNA	OTA	Impedimetric Electrochimiluminescence	Cruz-Aguado et al. 2008, Rivas et al. 2015 ; Yang et al. 2015(a)
	Aflatoxin M1	Electrochemical	Malhotra et al. 2014 ; Nguyen et al. 2013
	Fumonisin B1	Colorimetric and Chemiluminescence	McKeague et al. 2010 ; Hosseini et al. 2015
	Zearalenone	indirect competition enzyme-linked oligonucleotide assay (ELONA)	Chen et al. 2013 ; Wang et al. 2015
	Microcystine LR	Colorimetric, voltammetric, electrochemical	Lin et al. 2013 ; Li et al. 2016 ; Eissa et al. 2014 ;
	Okadaic Acid	Voltammetric, direct competitive enzyme-linked aptamer assay (ELAA).	Eissa et al. 2013 ; GU et al. 2016
	Saxitoxin	ELISA	Zhen et al. 2015 ; Handy et al. 2013
	Enterotoxin	Electrochemical, fluorescence resonance energy transfer (FRET) Electrochemical	Bruno JG and Kiel JL 2002 ; Deng et al. 2014; Wu et al.

	Toxin A	Electrochemical, impedance	2013
	Endotoxin	Electrochemical	Luo et al. 2014
	Brevetoxins (BTXs)	Electrochemical, quantum dots (QD) fluorescence detection	Ding et al. 2009 ; Bai et al. 2014, Su et al. 2013 and 2012
	Botulinum neurotoxin		Eissa et al. 2015
			Tok et al. 2008, Wei and Ho 2009, Wei et al. 2011; Fetter et al. 2015; Bogomolova and Aldissi 2015
RNA	Shiga Toxins	–	Challa et al. 2014
	Botulinum neurotoxin.	surface plasmon resonance	Chang et al. 2010 ; Janardhanan et al. 2013 ;
	microcystin-LR	–	Hu et al. 2012

Table 2: Receptors used for toxin detection

Binder	**target**	**Performances example**	**Biosensor type**	**Ref**
Modified enzyme	AFB1	LOD 0.008µM (2.5 (ng/ml)	SPR	Puiu et al. 2012
	AFB1	LOD 3µM	Amperometric biosensor	Hansmann et al. 2009
	Anatoxin-a	LOD 1µg/l	electrochemical detection	Villatte et al. 2002
	microcystin-LR	0.0039 µg L(-1) and IC(50) value of 0.21 µg L(-1).	Colorimetric detection	Sassolas et al. 2011
Antibody	OTA	LOD 5.0 ng/mL	sandwich dot-ELISA	Venkataramana et al. 2015.
	OTA	limit of detection was 1.39pgmL(-1),	chemiluminescence (CL) immunoassay	Kim et al. 2015
	Aflatoxin	detection limits of 3 ng/ml of AFB1 and 43 ng/ml for FB1	Antibody-based microarrays	Lamberti et al. 2009
	Okadaic acid	LOD 0.36 ng/ml	multiplex surface plasmon resonance (SPR)	McNamee et al. 2013
	Domoic acid	LOD 1.66 ng/ml		
	paralytic shellfish poisoning (PSP)	LOD 0.82 ng/ml,		
scFv	Tetrodotoxin (TTX)	affinity constant 1.1 × 10(6) L/mol	ELISA	Wang et al. 2014 (a)
	Fumonisin B1	LOD 0.11 µM	Direct competitive	Hu et al. 2015

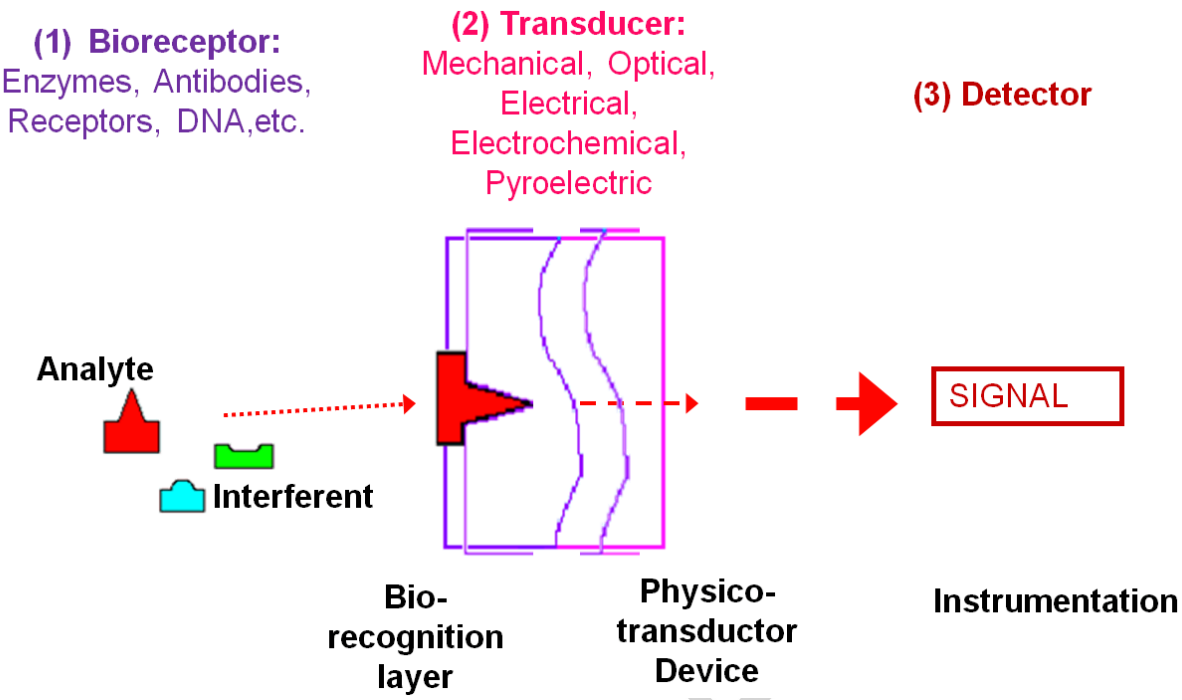
	Fumonisin B1	Limit of detection was 8.32 µg kg(-1).	ELISA indirect competitive ELISA	Zou et al. 2014
	Citrinin	LOD 0.003 µg/ml	indirect ELISA	Cheng et al. 2015
	Zearalenone	IC 14 ng/ml	indirect competitive ELISA	Yuan et al. 1997
nanobody	Cry1Fa toxin	linear range from 1 to 100 ng/mL detection limit of 0.88 ng/mL	SPR	Wang et al. 2013 (a)
	Cry1Fa toxin	Detection limit of 0.005 µg·mL-1 and a working range 0.010-1.0 µg·mL-1.	biotin-streptavidin based double antibodies (nanobodies) sandwich-ELISA (DAS-ELISA) assay	Li et al. 2014
	OTA	LOD 0.04 ng/mL linear range of 0.06-0.43 ng/mL	competitive fluorescence enzyme immunoassay (dc-FEIA)	Liu et al. 2015
	OTA	LOD 3.7 pg/L linear range of 0.01-1000 pg/mL	VHH phage-based real-time immuno-PCR (RT-IPCR).	Liu et al. 2014(a)
Aptamer (DNA/ RNA)	OTA	LOD 0.05 ng/ml Linear range 0.05–2.56	electrochemical aptamer-based assays	Rhouati et al. 2013
	OTA	LOD 0.00012 Linear range 0.00012–0.0055	electrochemical aptamer-based assays	Hayat et al. 2013
	Okadaic acid	a linear range 100 pg/mL and 60 ng/mL detection limit of 70 pg/mL	label-free electrochemical biosensor	Eissa et al. 2013
	microcystin-LR	LOD 1.9 pM	Label-free voltammetric aptasensor	Eissa et al. 2014
DNAzyme/ Ribozyme	Ricin Toxin A-Chain (RTA)	LOD 14 ng/mL		Roday et al. 2008
	OTA	limit of detection of 4 nM	label-free colorimetric aptasensor based on DNAzyme-aptamer	Yang et al. 2013
	OTA	LOD of 0.27 ng/ml	resonance energy transfer (CRET	Mun et al. 2014
	Microcystin-LR	Linear range of 0.1-10 ng/ml; LOD 0.05 ng/ml	gold nanoparticles Immunosensor	Zhu et al. 2011
Peptide/ Affibody (protein scaffold)	OTA	(K(eq)=3.4 x 10(4) M(-1))	SPE	Giraudi et al. 2007
	OTA	K(D) ~15.7 µM	SPR	Heurich et al. 2013 Soleri et al. 2014
	Aflatoxine B1	Kd:10(4) M(-1))	SPE	Tozzi et al. 2003
MIPs	Zearalenone	LOD = 0.3 ng/g	SPR	Choi et al.2009
	Deoxynivalenol	range of 0.1-100	SPR	Choi et al.2011

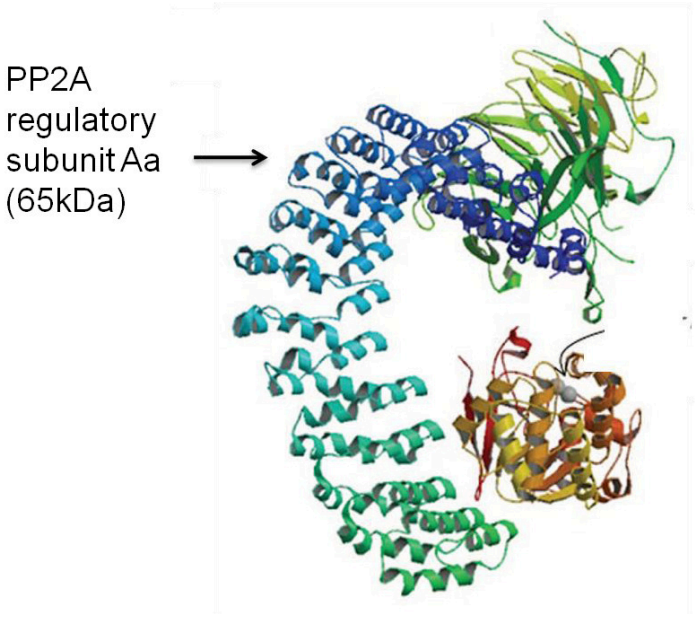
	Domoic acid Microcystin-LR	ng/mL; LOD>1 ng/ml LOD for = 5 µg/L LOD 0.04 nM	SPR quartz crystal microbalance (QCM) sensor	Lotierzo et al. 2004 He et al. 2015
PNAs	verotoxin-producing Escherichia coli O157:H7	10(2) cfu per 0.1 g	SPR	Kai et al. 2000

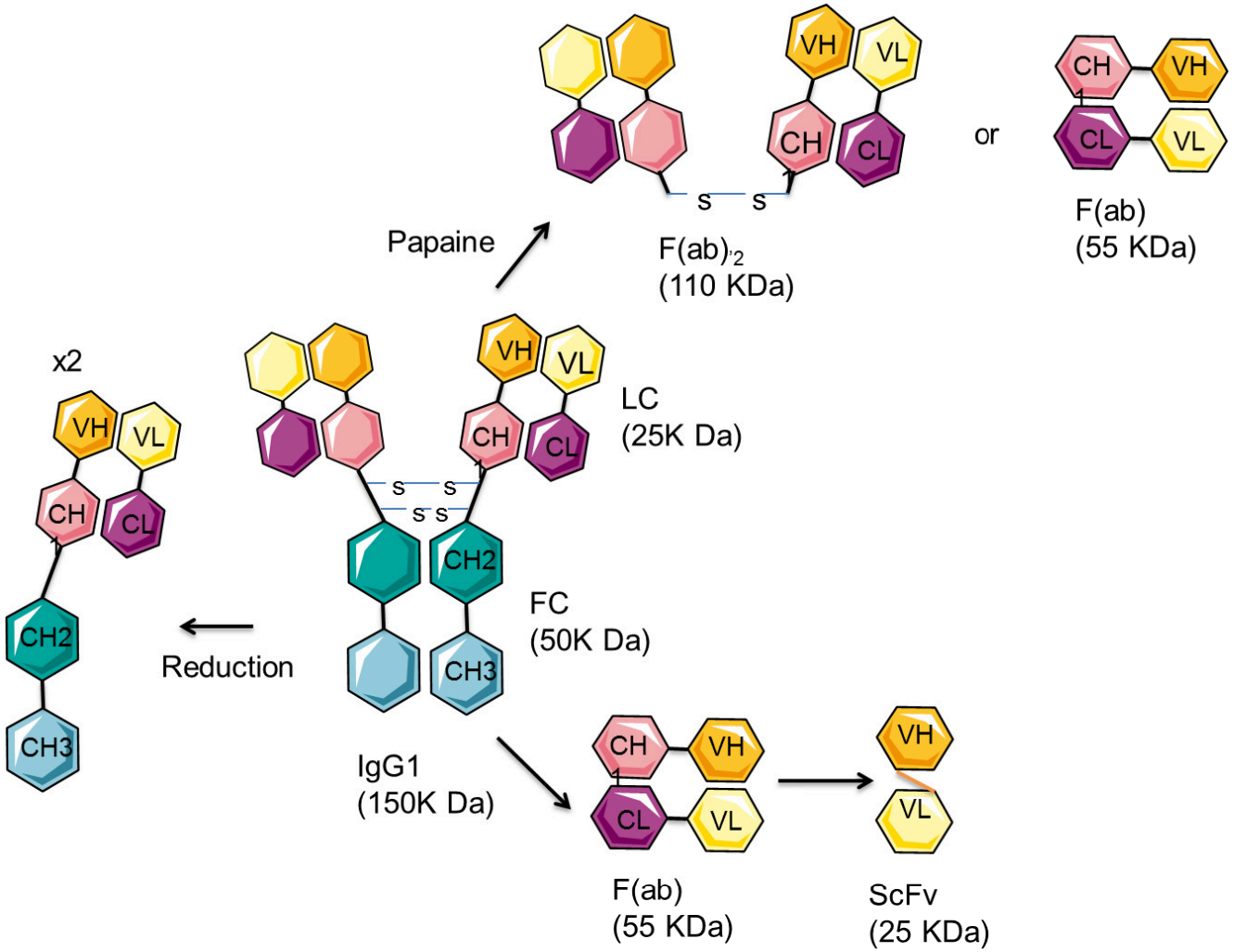
Table 3. Comparative study of the properties of the three bioreceptors

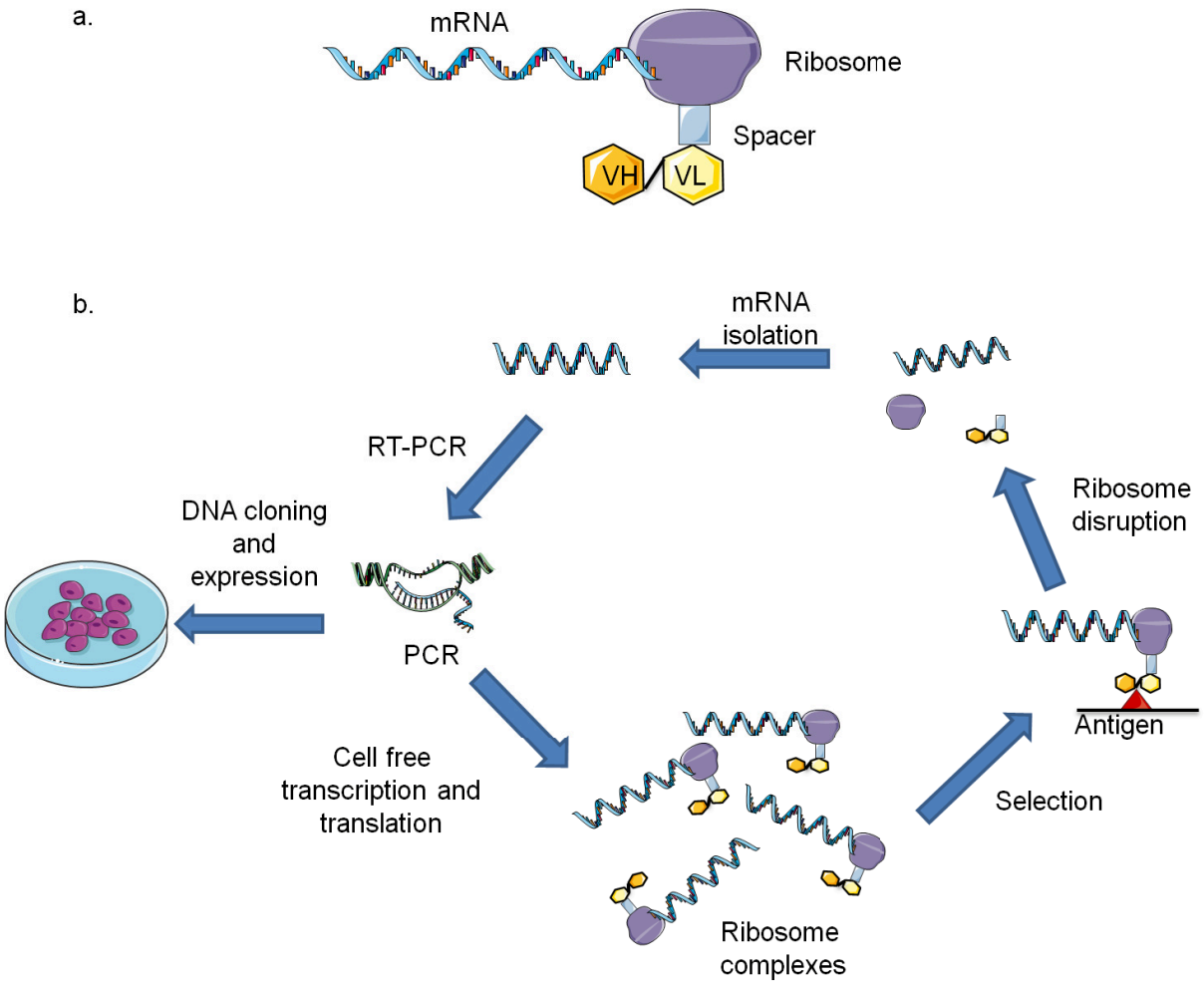
Property	Enzyme	antibody	Aptamer
Acquisition	• In vitro process no animals are involved • All the toxins don't inhibit enzymatic activity or not substrate of enzyme • Manipulation is not possible • Physiological conditions obligatory	• In vivo process -animals are involved • Difficult to obtain antibodies against targets that are non-immunogenic or toxic • Manipulation of selection hardly or not possible • Physiological conditions obligatory • Difficult to expose different epitopes of the same target for selection • Identification laborious • the immunogen must be the major fraction in the immunization reagent	• In vitro process no animals are involved • Possible to obtain aptamers against targets that are non-immunogenic or toxic • Manipulate selection to obtain binding and kinetic properties desirable for specific assays • Non-physiological conditions acceptable • Expose different epitopes of the same target for selection • Identification easy and rapid process performed on automated platform • the target used for selection can be a small portion in the target preparation
Stability	• Increased by modifications • Loss activity over time • Narrow stability in terms of pH, ionic strength and temperature • Unstable at room temperature	• Cannot be increased by modification • Relatively stable over time • Narrow stability in terms of pH, ionic strength and temperature • Unstable at room temperature	• Increased by modifications • Relatively stable over time • Stability over a wide range of pH, ionic strength and temperature • Long room temperature shelf lives
Specificity and selectivity	• Less selective and specific	• Binding constants for target species comparable with aptamers	• Binding constants for target species comparable with antibodies
Modifications	• Can be engineered	• Biological and hardly engineered	• Chemically and easy to engineer • Side specific attachment • Efficient and exact - of reporter molecule - of spacers - of functional groups • Homogeneous product
Denaturation	• Enzyme accelerates the reaction and does not consume	• Mild conditions needed to prevent irreversible denaturation • Sometimes difficult to separate from antibody-target-complex	• Regeneration after denaturation possible • Easy to separate from aptamer-target-complex
Sensors	• Immobilization at defined densities and locations difficult • Immobilization can denature enzyme	• Immobilization at defined densities and locations sometimes difficult • Molecular recognition functionalities hardly possible	• Immobilization at defined densities at precise locations on solid surfaces (microarrays) • Conformational changes on binding providing molecular-recognition functionalities

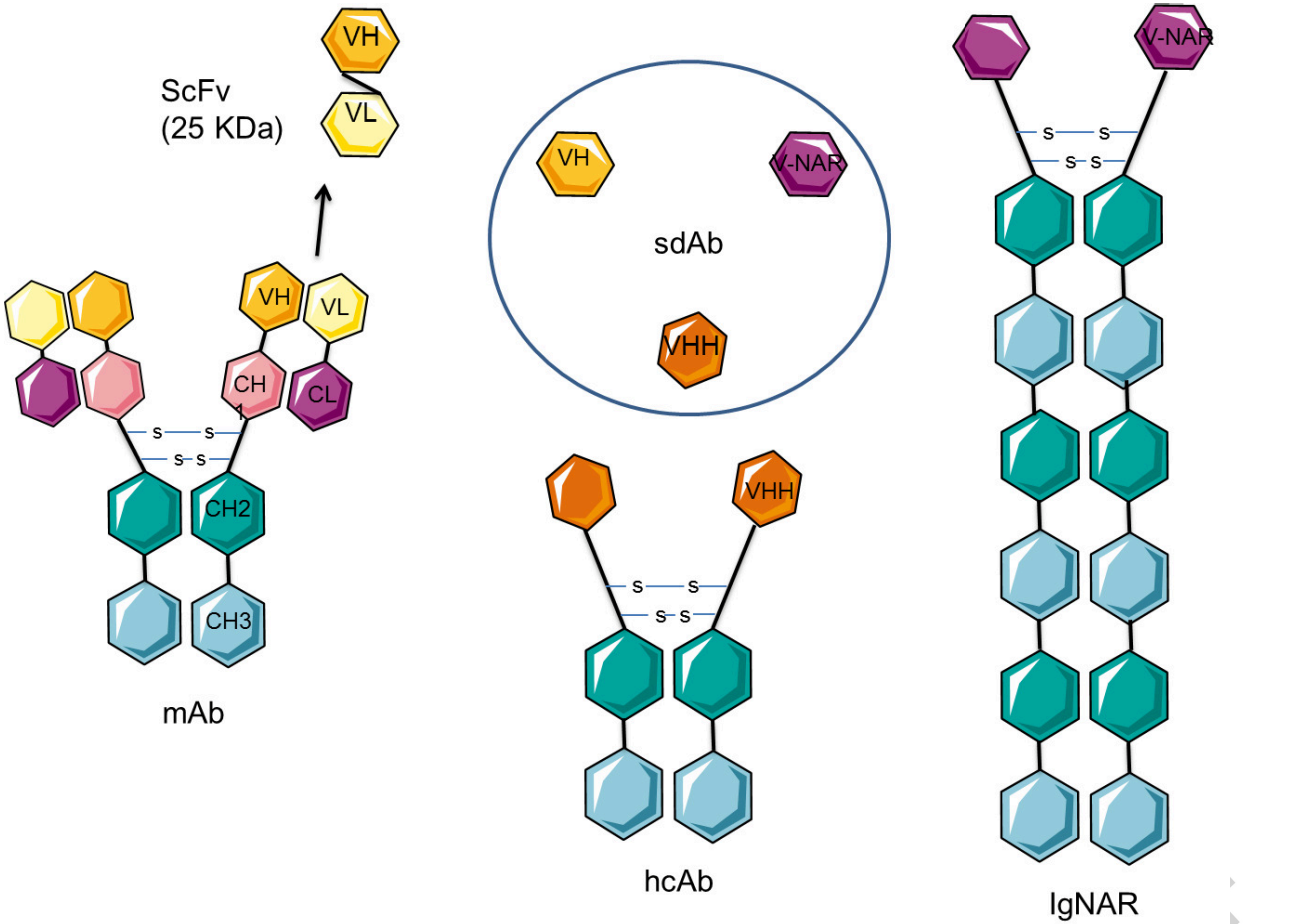
		• Irreversible cross-linking usually not possible	• Irreversible cross-linking with target protein possible-second ligand for detection not needed

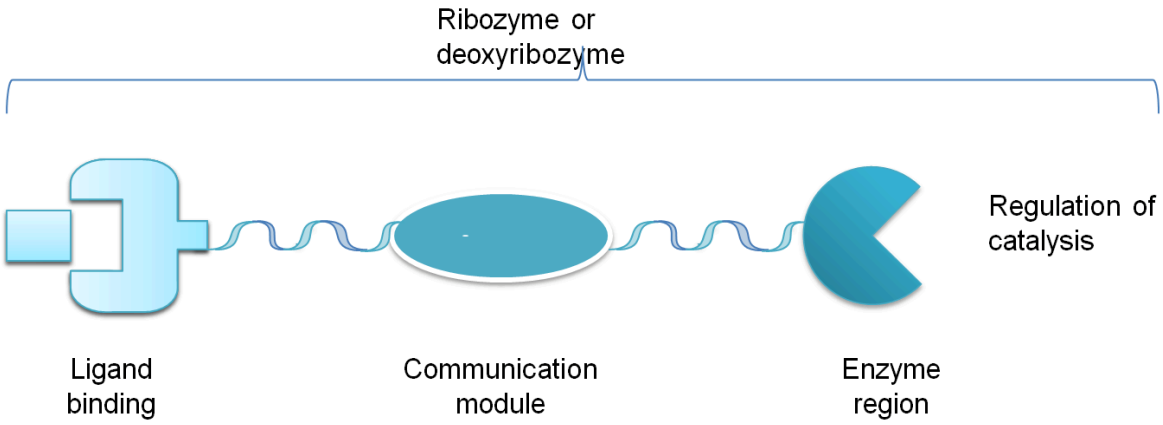


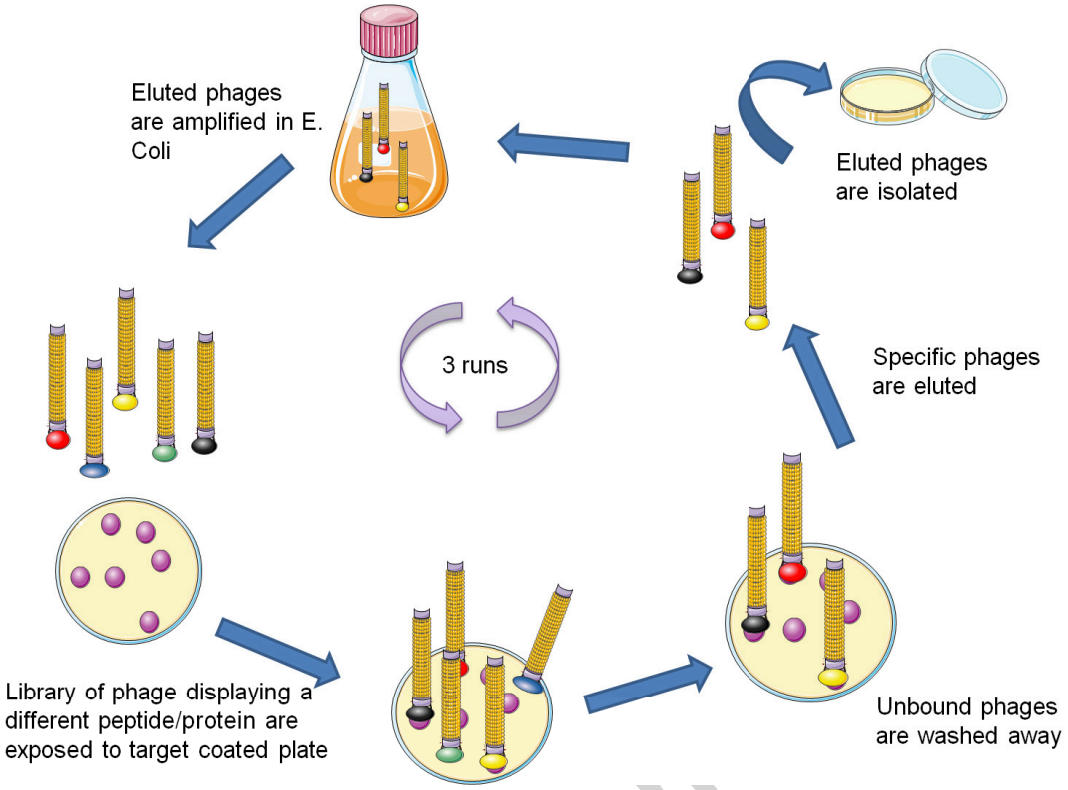


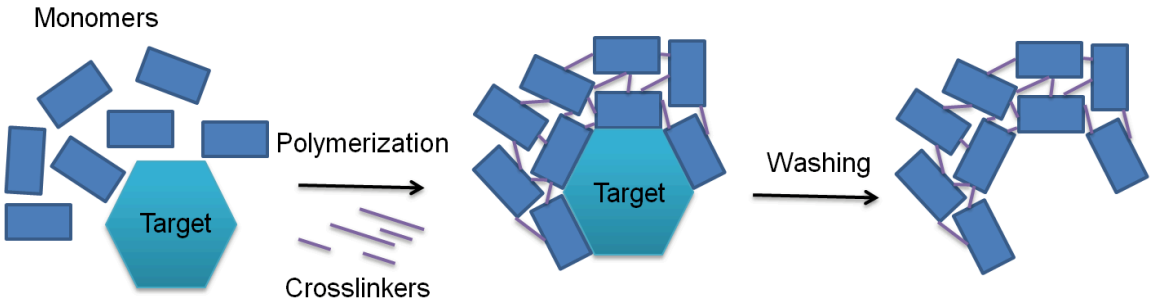




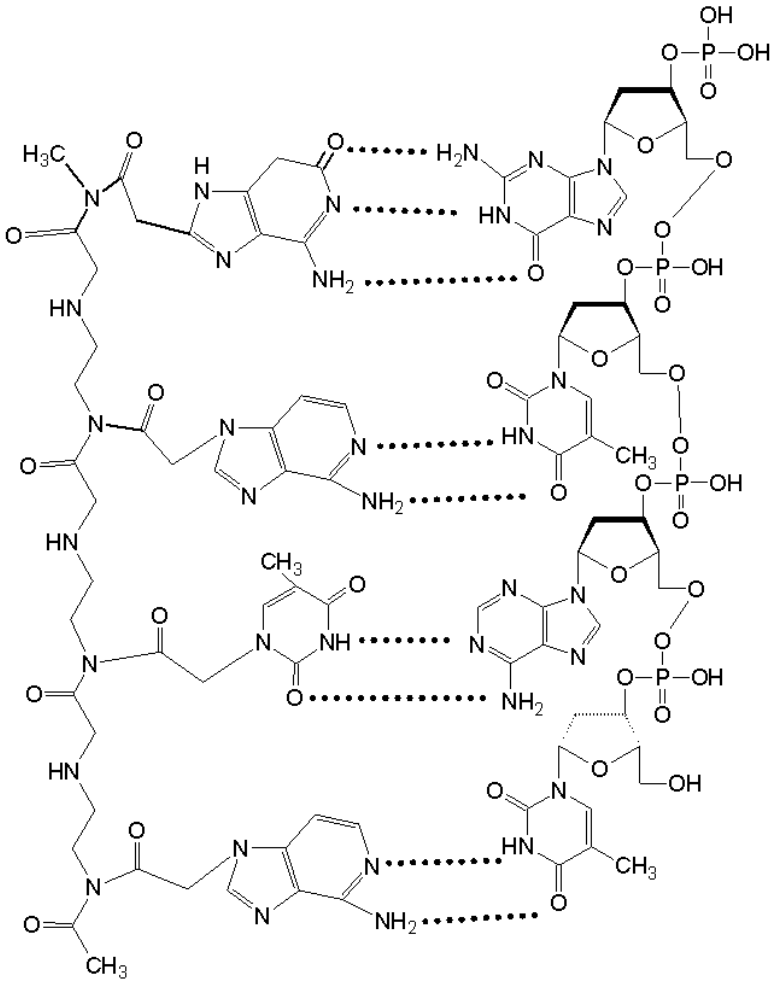








PNA



DNA

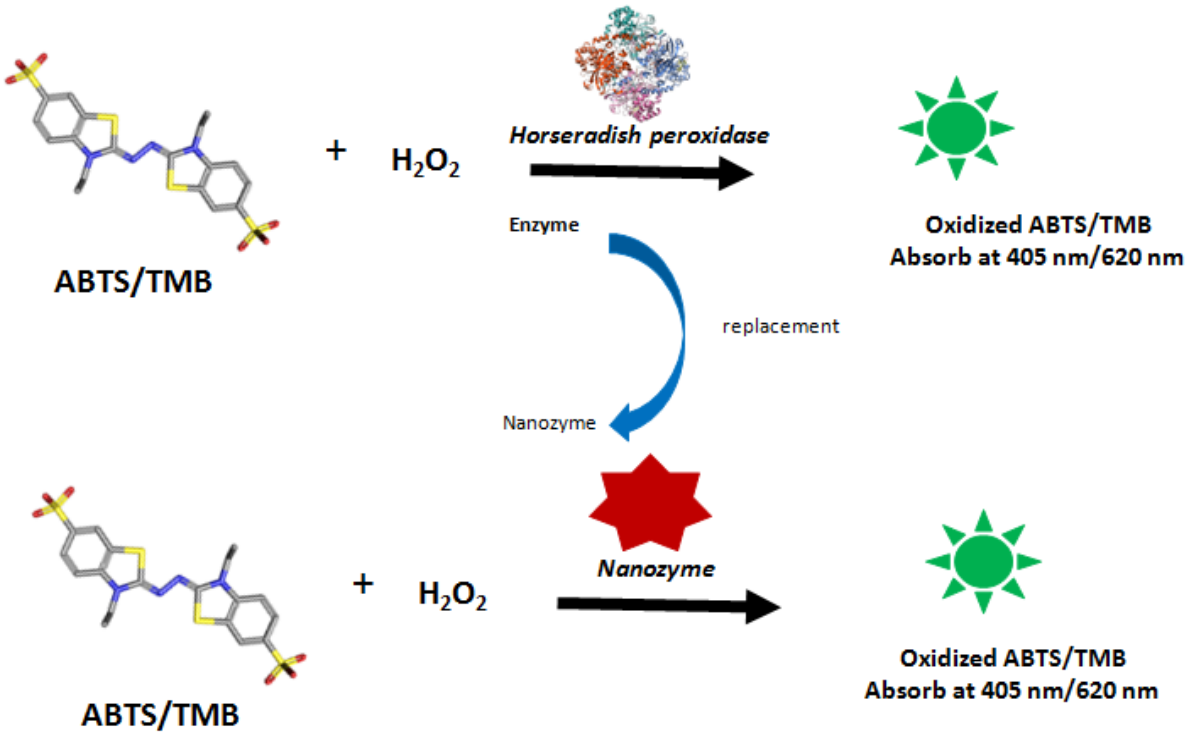


Figure 10