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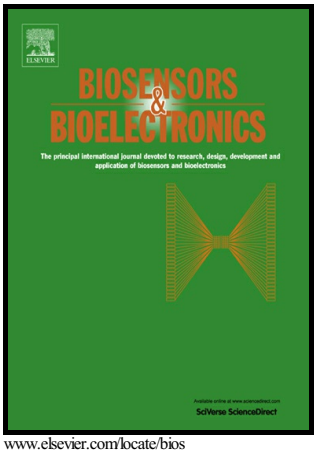

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Aptamers, Antibody scFv, and Antibody Fab' Fragments: An overview and comparison of three of the most versatile biosensor biorecognition elements

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Abstract

The choice of biosensing elements is crucial for the development of the optimal biosensor. Three of the most versatile biosensing elements are antibody single-chain Fv fragments (scFv), antibody fragment-antigen binding (Fab') units, and aptamers. This article provides an overview of these three biorecognition elements with respects to their synthesis/engineering, various immobilization techniques, and examples of their use in biosensors. Furthermore, the final section of the review compares and contrasts their characteristics (time/cost of development, ease and variability of immobilization, affinity, stability) illustrating their advantages and disadvantages. Overall, scFv fragments are found to display the highest customizability (i.e. addition of functional groups, immobilizing peptides, etc.) due to recombinant synthesis techniques. If time and cost are an issue in the development of the biosensor, Fab' fragments should be chosen as they are relatively cheap and can be developed quickly from whole antibodies (several days). However, if there are sufficient funds and time is not a factor, aptamers should be utilized as they display the greatest affinity towards their target analytes and are extremely stable (excellent biosensor regenerability).

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Keywords: Biosensors; scFv fragments; Fab' fragments; Aptamers; Biorecognition elements

1. Introduction

Through the development of biosensor technology, biosensors have received significant attention as tools in analytical and diagnostic applications. Biosensors are analytical devices composed of three parts: a biosensing (or biorecognition) element, a transducer, and a signal processing unit (Skoog et al., 2007). In general, a biosensing element is chosen to specifically interact and sequester the target analyte from solution. These elements are bound to the transducer surfaces, which allow for the conversion from a chemical to electrical signal. Since biosensors are developed to provide rapid and reliable analyses of target analytes, one crucial step in the optimization of a biosensor is the choice of the biosensing element. Four of the most prominent biorecognition elements are whole monoclonal antibodies (mAb), fragment antigen-binding (Fab') units, scFv fragments, and aptamers (**Fig. 1**).

Immunoglobulins (Ig), or antibodies, are large proteins produced by the immune system that have extremely high affinities and specificities for their target analytes (Crivianu-Gaita and Thompson, 2015b).

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Of the many classes of immunoglobulins (i.e. IgE, IgM, IgG, etc.), the immunoglobulin G (IgG, ~150 kDa) is the most prominently used class in the field of biosensing. The structure of an IgG antibody consists of two heavy protein chains and two light protein chains. The two antibody halves (each half containing one heavy and one light chain) are held together via disulfide bonds in the hinge region (**Fig. 1**) (Adlersberg, 1976). The number of disulfide bonds in the hinge region varies depending on antibody species and antibody class (Crivianu-Gaita et al., 2015a). The paratope of the antibody – the region that recognizes and binds to the target analyte (or antigen) – involves the top of the V_L and V_H domains.

The antibody contains two Fab fragments, each one consisting of the V_L, V_H, $C_{L,}$ and C_{H1} domains. These two fragments are held together by the key hinge disulfide bridges (Adlersberg, 1976). Fab fragments may be obtained in one of two possible ways: via recombinant synthesis (Choe et al., 1994) or proteolytic cleavage of the parent antibody (Ryan et al., 2008). Fragments including disulfide bridge thiols (**Fig. 1**) are called Fab' fragments whereas those lacking the thiol functional group are termed Fab fragments. The thiol functional group of the the Fab' fragments allows for easy immobilization onto biosensor surfaces (Crivianu-Gaita and Thompson, 2015b).

Even smaller than the Fab fragment is the antibody Fv fragment (**Fig. 1**), consisting of only the V_H and V_L domains. These fragments can only be obtained reliably via recombinant synthesis (Ward, 1992) and are held together by relatively weak non-covalent interactions (Owens and Young, 1994). As a result, several modified types of Fv fragments have been developed including, but not limited to, single-chain Fv (scFv) (Tsumoto et al., 1998), disulfide-stabilized Fv (dsFv) (Reiter et al., 1994), diabodies (divalent dimers) (Lawrence et al., 1998), and permutated Fv (pFv) fragments (Brinkmann et al., 1997). This review will focus on the scFv fragments as they are the most prominent of the Fv-derived antibody fragments used as biosensing elements.

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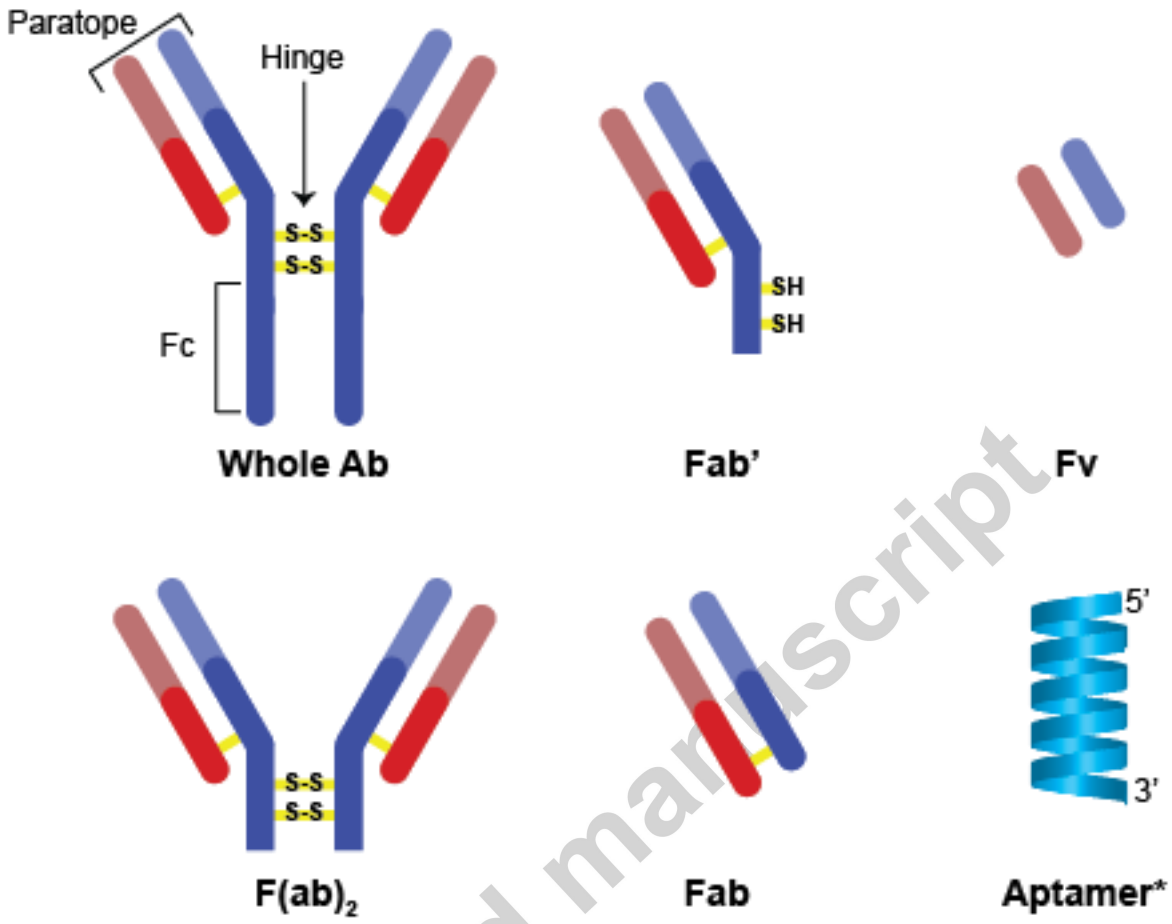

Fig. 1. An illustration of whole antibodies (Ab), $F(ab)_2$, Fab', Fab, Fv, and aptamers. *Compared to antibodies and their fragments, aptamer shape and size varies from one aptamer to another. The blue helix has been chosen to designate a general aptamer in this review, however, it should be noted that not all aptamers form helices.

Compared to Fab' and scFv fragments, aptamers are not derived from antibodies. Aptamers are single stranded ribonucleic acid (RNA) or 2'-deoxyribonucleic acid (DNA) chains that have affinities and specificities for their target analytes on orders of magnitude comparable to or better than antibodies (Jayasena, 1999). The size of aptamers (~1-2 nm), however, is much smaller than that of whole antibodies (~10-15 nm) allowing them to be immobilized in higher densities on surfaces, resulting in higher sensitivities and lower limits of detection (LOD) in biosensors (So et al., 2005). The same phenomenon is observed with Fab' fragments (Crivianu-Gaita and Thompson, 2015b) and scFv fragments (Kumada, 2014a). For this reason, whole antibodies will not be discussed in this review as Fab' fragments and scFv fragments are considered to be superior biosensing elements.

This review consists of the analysis of scFv fragments, Fab' fragments, and aptamers as biosensing elements. Each of these three major sections are subdivided into three smaller subsections discussing the synthesis of the particular biosensing element, a sample of immobilization techniques, and various biosensor examples. The final section of this review compares and contrasts the three biosensing elements, illustrating advantages and disadvantages for each.

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2. Single-chain Fv fragments (scFv)

2.1. Synthesis and engineering of scFv fragments

As stated previously, Fv fragments are inherently unstable since they are held together by only non-covalent interactions (Owens and Young, 1994). Expression and elution of Fv fragments can result in the dimerization of the V_L domains, leading to Bence Jones proteins and V_L dimers (Essen and Skerra, 1993; Stevens et al., 1991). The variable stability of Fv fragments is due to the difference in the sequences of the third hypervariable loops (CDR3) between antibodies, affecting the stability of the V_L-V_H interaction (Ward, 1992).

The most common solution for this problem is to attach a linker peptide between the V_H and V_L domains, creating the scFv fragment (Berry and Pierce, 1993; Deyev and Lebedenko, 2008). Protein linker chains between 15-30 amino acids display the greatest propensity towards monomeric behavior, whereas dimeric or higher molecular weight forms are associated with linkers between 0-10 amino acids (**Fig. 2**) (Desplancq et al., 1994; Deyev and Lebedenko, 2008). The most common peptide linker is $(Gly_4\text{-}Ser)_3$ due to its flexibility, although it is possible to incorporate charged amino acids (i.e. lysine, glutamic acid) in order to increase solubility (Reiter and Pastan, 1996). Comparison of binding orientation using the same linker has indicated that the V_L-linker-V_H orientation displays greater binding capacities (direct ELISA) compared to the V_H-linker-V_L orientation (Desplancq et al., 1994). Potentially, the N-terminus of the V_L domain and the C-terminus of the V_H are involved in antigen binding.

It is also possible to connect the two Fv fragment domains with other types of linkers. For example, Arndt et al. (2001) connected the V_H and V_L domains using a heterodimeric coiled coil (WinZip A2B1), creating a fragment that is nearly as stable as the scFv fragment under the same conditions. This alternative to the peptide linker may be useful in cases where multimerization of the scFv fragment is a problem.

Dimerization of scFv fragments containing short peptide linkers (0-10 amino acids) involves the head-to-tail (**Fig. 2**) association of the two fragments (McCartney et al., 1994). The non-covalently linked dimers (diabodies) are difficult to create in controlled environments as trimers (triabodies) and higher molecular weight forms are also in equilibrium (Petrov et al., 2004). However, it is sometimes desirable to achieve dimerization in a controlled manner. McCartney et al. (1994) engineered $(scFv')_2$ dimers using scFv fragments containing C-terminal (Gly_4Cys) chains, which were then allowed to form disulfide bridges. The resulting dimers displayed affinity constants that were comparable to whole antibodies due to the increase in functional avidity. Natarajan et al. (2007) linked two scFv fragments together using a polyethylene glycol (PEG)-based alkyne chain and a PEG-based azide chain in a copper-catalyzed 1,3-dipolar cycloaddition. Other groups have created dimers involving two different scFv fragments, resulting in bispecific antibody fragments (De Jonge et al., 1995).

In terms of synthesis, the two most prominent expression systems for scFv fragments are bacterial systems (i.e. *E. coli*, *Pichia pastoris*) and phage display systems (Jung et al., 2008; Kumada, 2014a). In the former system, a DNA sequence of interest is incorporated into vectors for transfection into the bacterial cells. Phage display systems utilize bacteriophages, where the gene encoding the protein of interest is incorporated into the phage coat protein gene. The protein of interest is then produced and displayed on the phage coat. For biosensor applications, where the protein of interest is an scFv fragment, bacterial systems are preferred as they allow the isolation of the produced fragments. Both *E. Coli* and *Pichia pastoris* bacterial systems have been used, however, there have been reports of undesirable glycosylation from the latter system (Kumada, 2014a).

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One benefit of bacterial expression systems is that modifications to the proteins can be performed by the bacteria themselves, avoiding potentially destructive chemical modifications. For example, Piervincenzi et al. (1998) fused a streptavidin-binding peptide to the C-terminus of scFv fragments for immobilization onto biotin surfaces. Kumada et al. (2014b) fused C-terminal polystyrene-binding peptides to scFvs, allowing for more efficient refolding of the fragments on polystyrene. Bacterial incorporation of fused proteins ensures a strict 1:1 ratio of scFv fragment to fused protein. Bacterial expression systems are also relatively cheap, efficient, and can produce large amounts of antibody products.

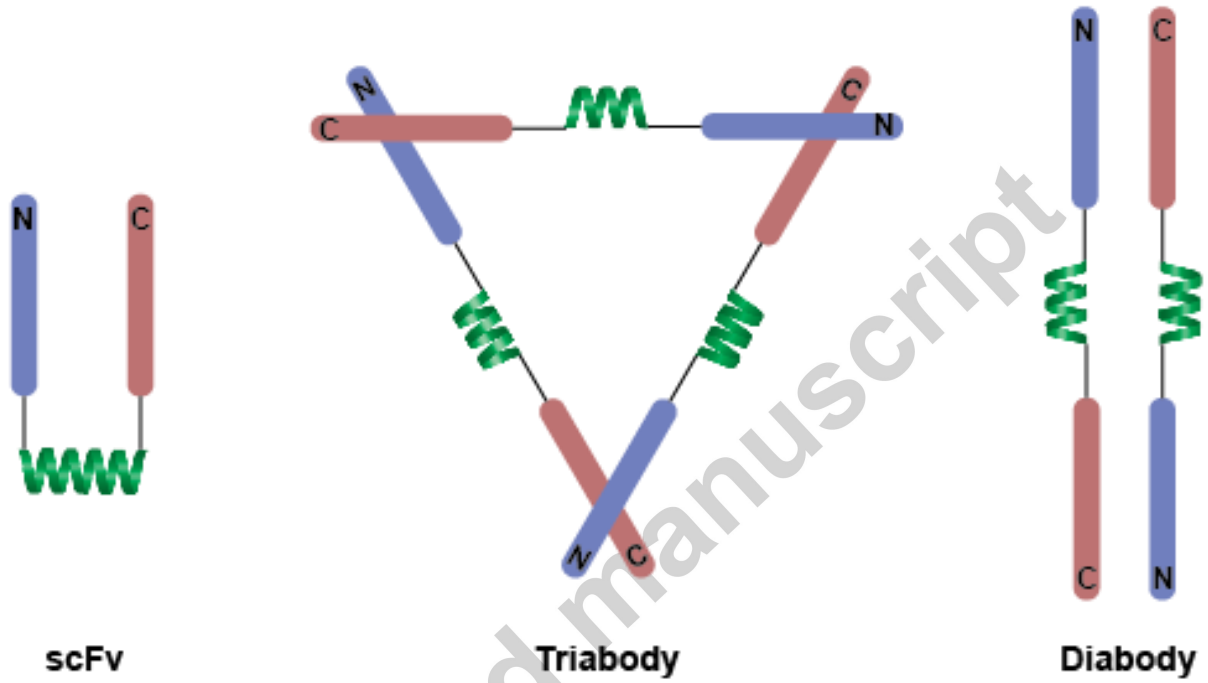

Fig. 2. A comparison between single-chain Fv fragments (scFvs), non-covalent Fv dimers (diabodies), and non-covalent Fv trimers (triabodies). The latter two occur prominently when the interdomain protein linker chains (green) are less than 10 amino acids in length.

2.2. Immobilization techniques of scFv fragments

The customizability of scFv fragments is tremendous, allowing for scFv fragments to be immobilized onto surfaces in a variety of different ways. One of the earliest methods of immobilization involved the use of hexa-histidine (hexa-his) tags on both the V_H and V_L domains of the Fv fragment, binding the two domains to surfaces using a Zn^{2+}/iminodiacetic acid (IDA) method (Essen and Skerra, 1993). Similar to this method, Maly et al., (2002) immobilized scFv fragments onto nickel-nitrilotriacetic acid (Ni-NTA) coated gold surfaces. Methods such as these involve a non-covalent immobilization of the scFv fragment to the surface – an undesirable trait for the development of regenerable biosensors.

One potential exception to this is the use of streptavidin-biotin interactions. Piervincenzi et al. (1997) fused a streptavidin-binding biotin-mimetic peptide (SerAlaTrpArgHisProGluPheGlyGly) to the C-terminus of scFv fragments in order to bind them to streptavidin-rich surfaces. The streptavidin-biotin interaction is widely used due to the exceptionally high affinity (K_D ~10^{-14} M) of the two reagents for each other (Deyev et al., 2003). More recently, other systems of comparable affinity have been discovered for the immobilization of scFv fragments. For example, Deyev et al. (2003) discovered that the interaction between the ribonuclease barnase (~12 kDa) and its inhibitor barstar (~10 kDa) has comparable affinity

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(K_D ~ 10^{-14} M). Similarly, Hosse et al. (2009) immobilized scFv fragments using the nuclease domain of the bacterial toxin colicin E7 (DNase E7) and its partner the immunity protein 7 (Im7) (K_D ~10^{-14} M).

In order to create more robust surfaces, scFv fragments are immobilized in a covalent fashion. One of the simplest strategies for this involves the genetic engineering of C-terminal thiols on scFv fragments (Ros et al., 1998) (**Fig. 3**). These fragments were immobilized directly onto gold surfaces using the C-terminal thiols and were mixed with mercaptoethanesulfonate to prevent denaturation. The high surface energy of gold surfaces denatures many proteins, including antibodies (Kumada et al., 2011). Therefore, it is necessary to add a chemical agent that will reduce the interaction between the gold surface and the immobilized antibody. Similar immobilization techniques using C-terminal thiols have been developed for maleimide-rich surfaces (Torrance et al., 2006). Another simple method involves the engineering of scFv fragments with an N-terminal serine (**Fig. 3**). Surfaces rich with cyclooctyne moieties were used to react with the N-terminal serines, which participated as nitrones, in strain-promoted azide-nitrone cycloadditions (SPANC) (Colombo et al., 2012).

Coupling proteins via free amines to carboxylic acid-rich surfaces can be accomplished using *N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Howell et al. (1998) randomly immobilized scFv fragments and whole antibodies to carboxymethyl dextran using this carboxylate coupling strategy. Surface plasmon resonance (SPR) and scanning electron microscopy (SEM) analyses indicated a higher binding density of scFv fragments compared to whole antibodies. The problem with this random immobilization strategy is that some of the antigen-binding domains will be oriented away from solution, decreasing immunoreactivity (Crivianu-Gaita and Thompson 2015b; Howell et al., 1998).

The most effective methods involve the fusion of a C-terminal peptide, which are then used to covalently immobilize the scFv fragments to surfaces. For example, recombinant scFv fragments were engineered with a cutinase fusion protein that covalently binds to phosphonate groups on gold SPR surfaces (Kwon et al., 2004). SPR analyses showed that 73% of bound fragments were able to detect analyte. Sugimura et al. (2007) fused a T26 transglutaminase substrate-like peptide (T26TS) onto scFv fragments (**Fig. 3**). Transglutaminase covalently links glutamine amino acids in proteins with primary amines or peptide-bound lysines. Incubation of the fragments with primary amine-rich surfaces and transglutaminase covalently binds the scFv fragments in an oriented fashion (Sugimura et al., 2007). Hu et al. (2012) fused DQNAT glycosylation sequences at the C-terminals of scFv fragments. Upon treatment with *C. Jejuni* PglB oligosaccharyl transferase, the DQNAT sequence allowed for glycosylation of the asparagine side chain. Further treatment with sodium metaperiodate yielded aldehyde groups on the saccharides, which then reacted with primary amine-rich surfaces (Hu et al., 2012).

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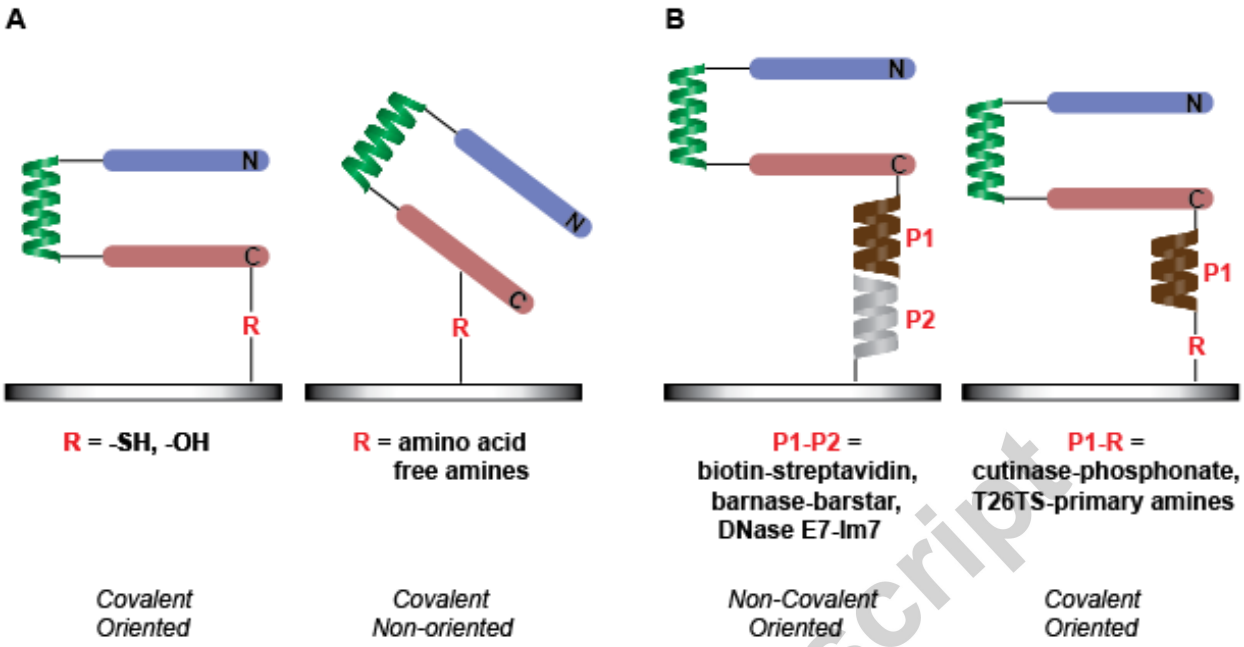

Fig. 3. R = functional group, P1-P2 = protein-protein immobilization pair, P1-R = protein-functional group immobilization pair. (A) scFv fragments can be immobilized in an oriented fashion onto surfaces using C-terminal small functional groups (thiols, hydroxyls) or in a non-oriented fashion using free amines from amino acid chains. (B) C-terminal fusion proteins are commonly used to immobilize scFv fragments in an oriented fashion. The proteins can either non-covalently associate with other surface bound proteins or can covalently react with surface functional groups.

Recently, an immobilization method involving UV-irradiated scFv fragments was developed (Petersson et al., 2014). A *p*-benzoyl-L-phenylalanine (*p*Bpa) group was incorporated into a constant framework domain of the scFv fragments. Irradiation of the scFv fragments at 350-360 nm allowed for the excitation of the *p*Bpa group, resulting in the formation of C-C covalent bonds with surface-bound β-cyclodextrin. There was little difference between the analyte binding affinities of the bound wild (K_D = 2.0 nM) and mutant-types (K_D = 3.1 nM).

2.3. scFv fragments and their use in biosensors

It is common for biosensors to use scFv fragments as biosensing elements due to the many benefits they offer: high customizability, wide variety of immobilization techniques available, and immobilization can be achieved in high surface densities compared to whole antibodies. There are several major categories of biosensors that incorporate scFv fragments as biorecognition elements: piezoelectric biosensors, optical biosensors, immunoassays, and to a lesser extent electrochemical biosensors.

One of the earliest recorded piezoelectric biosensors involving scFv fragments was the quartz crystal microbalance (QCM) sensor developed by Horacek et al. (1998). This reverse-biosensor was developed such that the analyte, parathion, was immobilized onto gold-coated piezoelectric disks. Anti-parathion scFv fragments were then allowed to flow over the surfaces, binding to the parathion and creating detectable frequency shifts (LOD = 9.5 µg/mL). However, the most common types of QCM biosensors involve the direct immobilization of scFv fragments, followed by the detection of the analyte. For example, Larsson et al. (2005) immobilized anti-cholera toxin scFv fragments onto 1-palmitoyl-2-oleyl-

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sn-glycero-3-phosphocholine (POPC) surfaces containing 5% Ni-NTA groups using C-terminal hexahis-tags. In buffer, the limit of detection was calculated to be 750 pM for cholera toxin. Detection in fetal bovine serum (FBS) proved difficult as the scFv fragments were replaced by serum proteins since they were not covalently bound. The addition of a secondary hexahis-tag stabilized the scFv fragments, resulting in a 5-fold signal increase. Torrance et al. (2006), engineered anti-cowpea mosaic virus (CPMV) scFv fragments that also contained the antibody C_L domain as well as a C-terminal cysteine residue. The fragments were immobilized onto N-maleoyl propionic acid succinimidyl ester-covered gold surfaces. The biosensor displayed a LOD of 2.5 µg/mL and only a 3% signal loss after 10 regeneration cycles.

From the optical biosensor category, surface plasmon resonance (SPR)-based biosensors are commonly encountered. Unlike QCM biosensors, most scFv-based SPR biosensors generally operate in a reverse fashion where the analyte is bound to the surface. One of the earliest reported SPR biosensors utilizing scFv fragments was the study by Pearce et al. (1997). This reverse biosensor once again worked based on the random immobilization of the analyte, avian influenza virus neuraminidase (NC10), onto amine-covered gold surfaces. Flow of different concentrations of anti-NC10 over the surfaces created the calibration curve for detection. Dillon et al. (2003) developed an inhibition-based SPR biosensor for the detection of morphine-3-glucuronide (M3G) using recombinantly engineered scFv fragments. After immobilization of M3G onto carboxymethyl dextran using EDC/NHS coupling, standard solutions (urine) of M3G and varying concentrations of anti-M3G scFv fragments were allowed to flow over the surfaces (LOD = 3 ng/mL). Similarly, Welbeck et al. (2011) immobilized N-acetyl-β-D-glucosaminidase (NAGase) onto carboxymethyl dextran surfaces using EDC/NHS coupling. Spiked milk samples of NAGase and varying concentrations of anti-NAGase scFv fragments allowed for a LOD of 1 µg/mL for this inhibition-based biosensor.

Immunoassays offer the constant benefit of yielding exceptionally low limits of detection compared to other biosensors. Wang et al. (2006) developed a fluorescence-based immunoassay using alkaline phosphatase (AP) or cyanine (Cy3)-labeled bis-scFv fragments. Detection of *Bacillus anthracis* protective antigen (PA) was accomplished down to 10 pg for AP-labeled fragments and 1 pg for Cy3-labeled fragments. Compared to monomeric scFv fragments, the bis-scFv dimer displayed a 5-fold lower dissociation constant ($K_{D,monomer}$ = 770 pM, $K_{D,dimer}$ = 154 pM). Park et al. (2006) engineered anti-hepatitis B virus (HBV) preS2 scFv fragments with C-terminal polyhydroxyalkanoate (PHA) depolymerase substrate-binding domains. Once the fragments were immobilized onto PHA surfaces, fluorescently-labeled (red fluorescent protein) presS2 antigen was detected with an LOD of 2.7 ng/mL.

Finally, there has been a recent rise in the use of scFv fragments in electrochemical biosensors. For example, Grewal et al. (2014) engineered anti-*E. histolytica* antigen (EHA) scFv fragments with a fused C-terminal hemagglutinin (HA) peptide. Gold surfaces were initially treated biotin-labeled bovine serum albumin (BSA), followed by strepavidin and biotin-labeled anti-HA antibodies. The scFv fragments were then immobilized via their capture to the anti-HA antibodies. This Faradaic electrochemical impedance spectroscopy (F-EIS) biosensor displayed an LOD of 10 pg/mL in buffer and 500 pg/mL in stool samples.

3. Fragment antigen-binding (Fab') units

3.1. Synthesis and engineering of Fab' fragments

As stated earlier, Fab' fragments contain V_L, V_H, C_L, and C_{H1} domains as well as C-terminal thiols – remnants from the antibody hinge disulfide bridges. The number of C-terminal thiols varies between different antibody species (Crivianu-Gaita et al., 2015a). These thiols are extremely useful for the oriented immobilization of Fab' fragments onto biosensor surfaces (Crivianu-Gaita and Thompson, 2015b). The

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primary method for the production of Fab' fragments is via enzymatic/chemical modification of the whole antibody. An alternative, although less common method is through the recombinant synthesis of $F(ab)_2$ antibody fragments, followed by chemical reduction of these fragments to yield Fab' units (Pezzini et al., 2009).

In order to produce Fab' fragments from whole antibodies, a two step protocol is generally employed – enzymatic cleavage of the whole antibodies to form $F(ab)_2$ fragments followed by reduction of those fragments to yield Fab' fragments (Crivianu-Gaita et al., 2015a). Enzymes such as papain, pepsin, matrix metalloproteinases (MMPs), elastases, cathepsins, bromelain, ficin, and lysyl endopeptidase are commonly used to cleave whole antibodies (Brezki and Jordan, 2010; Mariani et al., 1991). However, not all of these enzymes are able to cleave and form $F(ab)_2$ fragments. For example, antibody cleavage with papain yields two Fab fragments (no C-terminal thiols) plus the remnants of the antibody Fc tail (Benny et al., 1987). The most common enzymes for the formation of $F(ab)_2$ fragments are pepsin, bromelain, ficin, and elastase (Mariani et al., 1991). These enzymes cleave below the hinge disulfide bridges, allowing for the formation of $F(ab)_2$ fragments. The difficulties with enzymatic cleavage of whole antibodies are that the cleavage protocols must be optimized for every antibody and that not all enzymes work for every class/species of antibody. For example, Mariani et al. (1991) showed that mouse IgG1a antibodies are resistant to pepsin cleavage, whereas mouse IgG2a and IgG2b can be cleaved in high pepsin to antibody ratios.

Once the $F(ab)_2$ fragments are obtained, a reduction step must be performed to break open the disulfide bonds linking the Fab' fragments together. Commonly encountered disulfide bond reducing agents (**Fig. 4**) are mercaptoethylamine (MEA), tris(2-carboxyethyl)phosphine (TCEP), β-mercaptoethanol (β-ME), dithiothreitol (DTT), and dithiobutylamine (DTBA) (Burns et al., 1991; Cleland, 1963; Crivianu-Gaita et al., 2015a; Whitesides et al., 1977). Monothiol reducing agents such as MEA and β-ME are termed "sticky" reducing agents as they sometimes adhere to the molecules they are trying to reduce (Lukesh et al., 2012). DTT offers the benefit of being a dithiol reagent capable of performing an intramolecular attack of the disulfide once it has attached to the molecule being reduced, eliminating the problem of "sticky" reducing agents (Cleland, 1963). TCEP reduces disulfides rapidly and completely in water at pH 4.5, preferentially reducing strained disulfides (Burns et al., 1991). Although TCEP is more reactive towards small molecules, it is significantly less reactive (up to 2000-fold) than DTT towards proteins around neutral pH (Cline et al., 2004). DTBA is a relatively new dithiol reducing agent that has been found to be more efficient at cleaving $F(ab)_2$ fragments than DTT and MEA by 213 times and 71 times, respectively (Crivianu-Gaita et al., 2015a).

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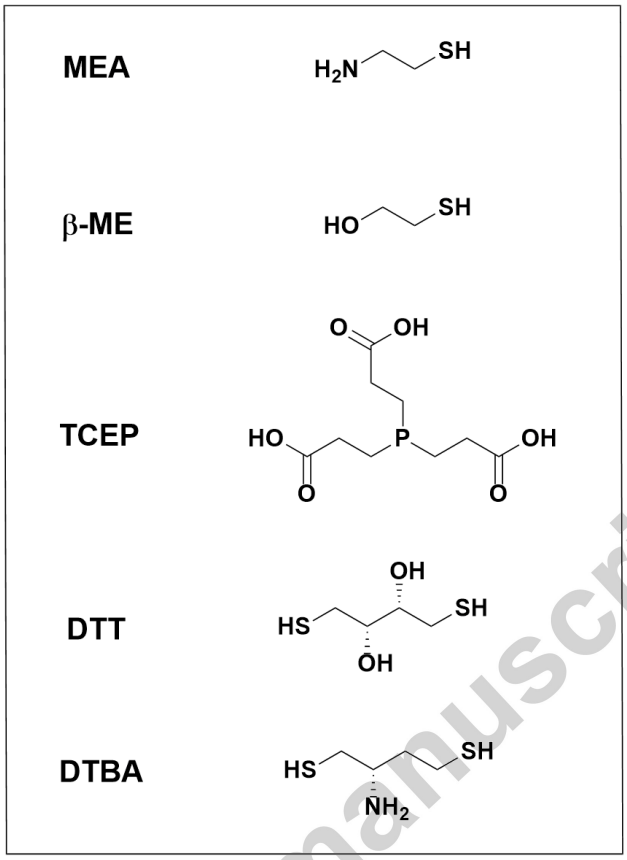

Fig. 4. Chemical structures of various thiol reducing agents that are used in protein biochemistry.

The largest problem with enzymatic/chemical cleavage of antibodies is the destruction of some of the antigen-binding domains and/or destruction of the fragments themselves. For example, Mariani et al. (1991) showed that even after optimization of the enzymatic cleavage of whole antibodies, the resulting $F(ab)_2$ fragments displayed immunoreactivities between 35% and 85%. Similarly, Crivianu-Gaita et al. (2015a) found that upon cleaving $F(ab)_2$ fragments with DTBA and DTT, some of the resulting Fab' fragments were degraded into their heavy/light chain components if left for too long in the reducing environments.

The majority of Fab/Fab' fragment-based biosensors utilize Fab' fragments as they can be made cheaply, quickly, and can be used within one week of receiving the whole antibodies. Also, the Fab' fragments can be immobilized in an oriented fashion easily through their C-terminal thiols (Crivianu-Gaita and Thompson, 2015b). A disadvantage of recombinant Fab synthesis is that the light chains tend to aggregate and form Bence Jones proteins (Hust et al., 2007). That being said, recombinant synthesis of Fab fragments is extremely common and is very similar to recombinant scFv fragment synthesis. For example, it is possible to add functional fusion proteins (i.e. immunotoxins, his-tags, biotin-acceptor proteins, etc.) to the C-terminals of Fab fragments (Choe et al., 1994; Czerwinski et al., 2009; Ylikotila et al., 2006). Biosensors from these fragments do exist, however, this review will focus on Fab' fragments as they offer significant advantages from their enzymatic/chemical cleavage protocols.

3.2. Immobilization techniques of Fab' fragments

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Although Fab' fragments are less customizable than scFv fragments, many types of immobilization techniques have been developed over the last 30 years. Fab' fragments have been immobilized onto gold, inorganic, plastic-based, polysaccharide-based, silicon-based, and magnetic surfaces (Crivianu-Gaita and Thompson, 2015b). This has allowed for the development of a wide range of biosensors having very different surface chemistries.

One of the oldest methods of Fab' fragment immobilization involves the direct adsorption of the fragments onto surfaces (Nisnevitch and Firer, 2001). The weak non-covalent interactions binding the Fab' fragments to surfaces are not strong enough when liquid flow occurs, resulting in the leaching of the biosensing elements (Nisnevitch and Firer, 2001). The development of covalent immobilization techniques centered around the functional C-terminal thiols prevent fragment leaching and allow for oriented immobilization of the fragments onto surfaces (Crivianu-Gaita and Thompson, 2015b).

As the C-terminal thiols are nucleophilic, one popular method for immobilization involves the reaction with maleimide-rich surfaces. For example, Prisyazhnoy et al. (1988) activated AH-sepharose 4B with N-maleonyl-β-alanine to form maleimide-terminated surfaces, which were then used to covalently immobilize Fab' fragments (**Fig. 5, A**). Viitala et al. (2000) immobilized the antibody fragments onto gold surfaces containing a polymerized lipid matrix containing *N*-(ϵ-maleimidocaproyl)dilinoleoylphosphatidylethanolamine (**Fig. 5, B1/B2**). One benefit of polymer matrices is that they avoid the phase separation and disruption of the monolayer during deposition. Similarly, Vikholm et al. (1999) used *N*-(ϵ-maleimidocaproyl)dipalmitoylphosphatidylethanolamine (DPPE) to immobilize Fab' fragments onto quartz surfaces through a mixed polymer matrix containing cholesterol. Surfaces without cholesterol exhibited Fab' binding constants that were 10-fold less than those containing cholesterol. Medina-Casanellas et al. (2013) used sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC) (**Fig. 5, C**) to immobilized the antibody fragments onto amine-terminated silica particles (125 Å).

Another common immobilization technique involves the reaction of the Fab' C-terminal thiols with surface disulfide or surface thiosulfonate groups. For example, Iwata et al. (2008) copolymerized glycidyl methacrylate (GMA) and 2-metacrylolyloxyethyl phosphorylcholine (PMPC) on silicon wafers, which were then reacted with DTT and 2,2-dithiopyridine (2TP) (**Fig. 5, D**). The resulting pyridyl disulfide moieties were used to react, via thiol-disulfide interchange, with the Fab' C-terminal thiols for oriented fragment immobilization. Crivianu-Gaita et al. (2016) used S-(11-trichlorosilyl-undecanyl)-benzothiosulfonate (TUBTS) (**Fig. 5, E**) to immobilize Fab' fragments in an oriented fashion on piezoelectric quartz disks. TUBTS contains a thiosulfonate group, containing one sulfide and one sulfone sulfur atom. The nucleophilic C-terminal thiols on the Fab' fragments react with the thiosulfonate group, creating a benzenesulfonyl leaving group.

Although maleimide-rich surfaces are most common, other techniques exist that involve the reaction between the C-terminal thiols and surface electrophilic functional groups. For example, Bright et al. (1990) immobilized Fab' fragments onto tresyl-terminated quartz surfaces using (3-glycidoxypropyl)trimethoxysilane and 2,2,2,-trifluoroethanesulfonyl chloride (tresyl chloride) (**Fig. 5, F**). Immobilization at pH 5.0 avoided the non-specific interaction between free amines on the antibody fragments and the surface tresyl groups.

Alternatively, it is possible to block the C-terminal thiols, creating an electrophilic group that is able to react with nucleophilic surface functional groups. Delair et al. (1994) reacted latex particles with cationic surfactants followed by basic buffers for the deprotection of surface thiols. Fab' fragments activated with 5,5'-dithiobis-(2-nitrobenzoic acid), or Ellman's reagent, were then allowed to undergo thiol-disulfide interchange with the surface thiols for immobilization. Catimel et al. (1997) activated carboxylic

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acid-terminated dextran using ethanolamine (EA) and EDC/NHS coupling chemistry (**Fig. 5, G**). Under basic conditions, Fab' fragments activated with Ellman's reagent were immobilized onto the nucleophilic surfaces.

Therefore, it can be concluded that many different strategies have been developed to immobilize Fab' fragments in an oriented fashion. These fragments are popular in the field of biosensors as they have been immobilized in higher binding densities compared to whole antibodies, resulting in higher sensitivities and lower limits of detection (Crivianu-Gaita and Thompson, 2015b; Kumada, 2014).

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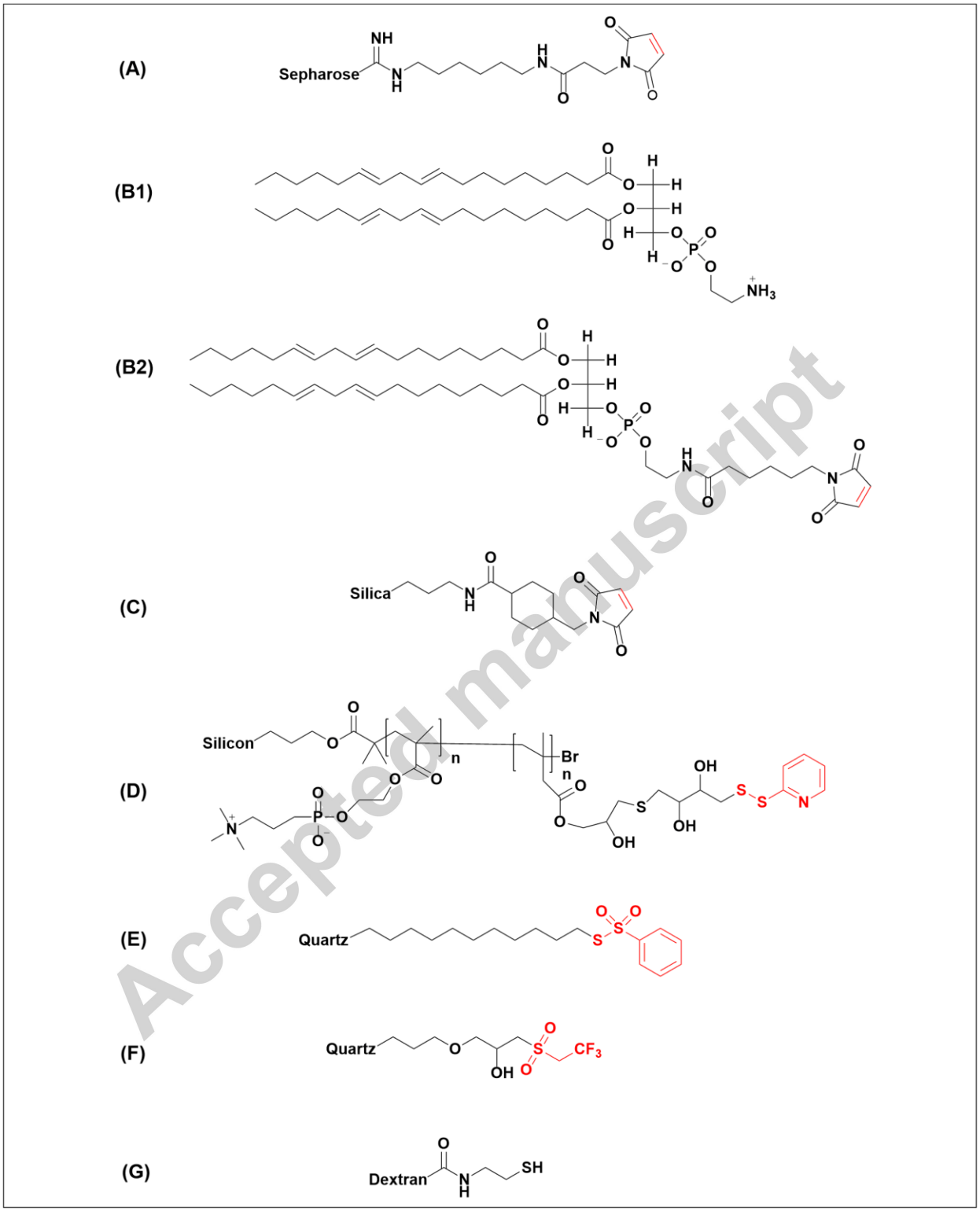

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Fig. 5. Chemical structures of the Fab' fragment immobilization linkers discussed in this review. The highlighted (red) portion of the linker indicates where the Fab' C-terminal thiols react.

3.3. Fab' fragments and their use in biosensors

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Fab' fragments have been used in optical, electrochemical, and piezoelectric biosensors, with a minimal prevalence in immunoassays. The latter are primarily dominated by Fab fragments containing C-terminal fusion proteins that help immobilize them to secondary proteins (such as serum albumin) on the plastic wells (Konig and Skerra, 1998). Direct immobilization of antibody fragments onto plastic can easily denature the fragments and render their biosensing capabilities useless (Kumada et al., 2014b).

The large majority of optical biosensors utilizing Fab' fragments are SPR-based. For example, Lee et al. (2005) directly immobilized anti-insulin Fab' fragments onto gold surfaces. This biosensor displayed an LOD of 0.1 µg/mL with a linear range up to 10 µg/mL. This study shows that it is possible to detect analyte from simple solutions even if there is nothing else on the SPR surfaces except for the antibody fragments. One difficulty of using SPR-based biosensors in whole serum or complex solutions is that gold surfaces tend to allow proteins to non-specifically adsorb (Crivianu-Gaita and Thompson, 2015b). Thus, it is necessary to add an anti-fouling agent to these surfaces to minimize non-specific adsorption. Vikholm-Lundin et al. (2011) directly immobilized Fab' fragments against 3,4-methylenedioxymethamphetamine (MDMA) onto gold surfaces. A hydrophilic non-ionic polymer, *N*-[tris-(hydroxymethyl)methyl]acrylamide, was used to help reduce non-specific binding. This SPR-based biosensor displayed an LOD of 0.02 ng/mL in buffer and 0.4 ng/mL in dilute saliva.

A few fluorescence/luminescence-based optical biosensors have also been developed using Fab' fragments. For example, Erikaku et al. (1991) used Fab' fragments to detect tumor necrosis factor-α (TNF-α) down to the attomole level in a bioluminescent assay. First, polystyrene cuvettes were coated with whole murine anti-human TNF-α antibodies and then TNF-α was allowed to bind. Secondary anti-TNF-α Fab' fragments were bound to luminescent apoaequorin using an SSMCC linker. When these conjugated Fab' fragments flowed over the TNF-α rich surfaces, immobilization occurred and luminescence was detected. Another example is the study by Schiel et al. (2011) where phenytoin was immobilized onto a Nucleosil Si-100 support using a glutaraldehyde linker. Anti-phenytoin Fab' fragments labeled with IRDye 800 CW were allowed to bind to the surface-immobilized phenytoin. When a solution containing phenytoin flowed over the surfaces, the analyte would displace the Fab' fragments and the fluorescence of the resulting solution was detected. The detection limit of the biosensor was in the picomolar range. Benefits of using fluorescence/luminescence-based biosensors include exceptionally low limits of detection as well as the minimization of signal-interfering non-specific adsorption effects observed in other types of biosensors.

Unlike scFv fragment-based electrochemical biosensors, those featuring Fab' fragments are more common. Nassef et al. (2009) developed a sandwich electrochemical amperometric immunosensor for the detection of gliadin. Anti-gliadin Fab' fragments were immobilized directly onto gold and the surfaces were back-filled with 1-(mercaptoundecyl-11-yl)-tetra(ethyleneglycol) as an anti-fouling agent. After incubation of the electrodes with gliadin, secondary whole anti-gliadin antibodies conjugated with horseradish peroxidase (HRP) were introduced into the solution followed by hydrogen peroxide. The LOD of this electrochemical biosensor was determined to be 3.29 ng/mL with a linear range up to 30 ng/mL.

Finally, one of the earliest applications of piezoelectric biosensors using Fab' fragments was the study by Vikholm et al. (1998). Anti-human IgG Fab' fragments were immobilized onto gold surfaces using the linker DPPE, and bovine serum albumin (BSA) was used as an anti-fouling agent. This biosensor utilized the QCM sensor platform for the detection of analyte. The QCM dominated as the leading piezoelectric sensor platform for a long period time. However, recently an electromagnetic piezoelectric acoustic sensor (EMPAS) was developed that is superior to the QCM as an analytical device (Ballantyne and Thompson, 2004). Unlike the QCM, the EMPAS uses an external magnetic field to stimulate the quartz disk remotely, operating as an electrode-free sensor (Thompson et al., 2003). The EMPAS is also able to operate at ultra-high frequencies (> 1 GHz), resulting in a significantly greater sensitivity (signal

increase outweighs the increase in noise associated with larger frequencies) compared to the QCM (Ballantyne and Thompson, 2004). Crivianu-Gaita et al. (2016) used the EMPAS to develop the first biosensor capable of detecting breast and prostate cancer metastasis. Fab' fragments against the parathyroid hormone-related peptide (PTHrP) were immobilized onto piezoelectric quartz disks using the linker TUBTS. Using a secondary whole anti-PTHrP antibody to mass-amplify the signal, the LOD of this biosensor was determined to be 441 ng/mL with a linear range up to 100 ug/mL.

4. Nucleic acid aptamers

4.1. Synthesis and engineering of nucleic acid aptamers

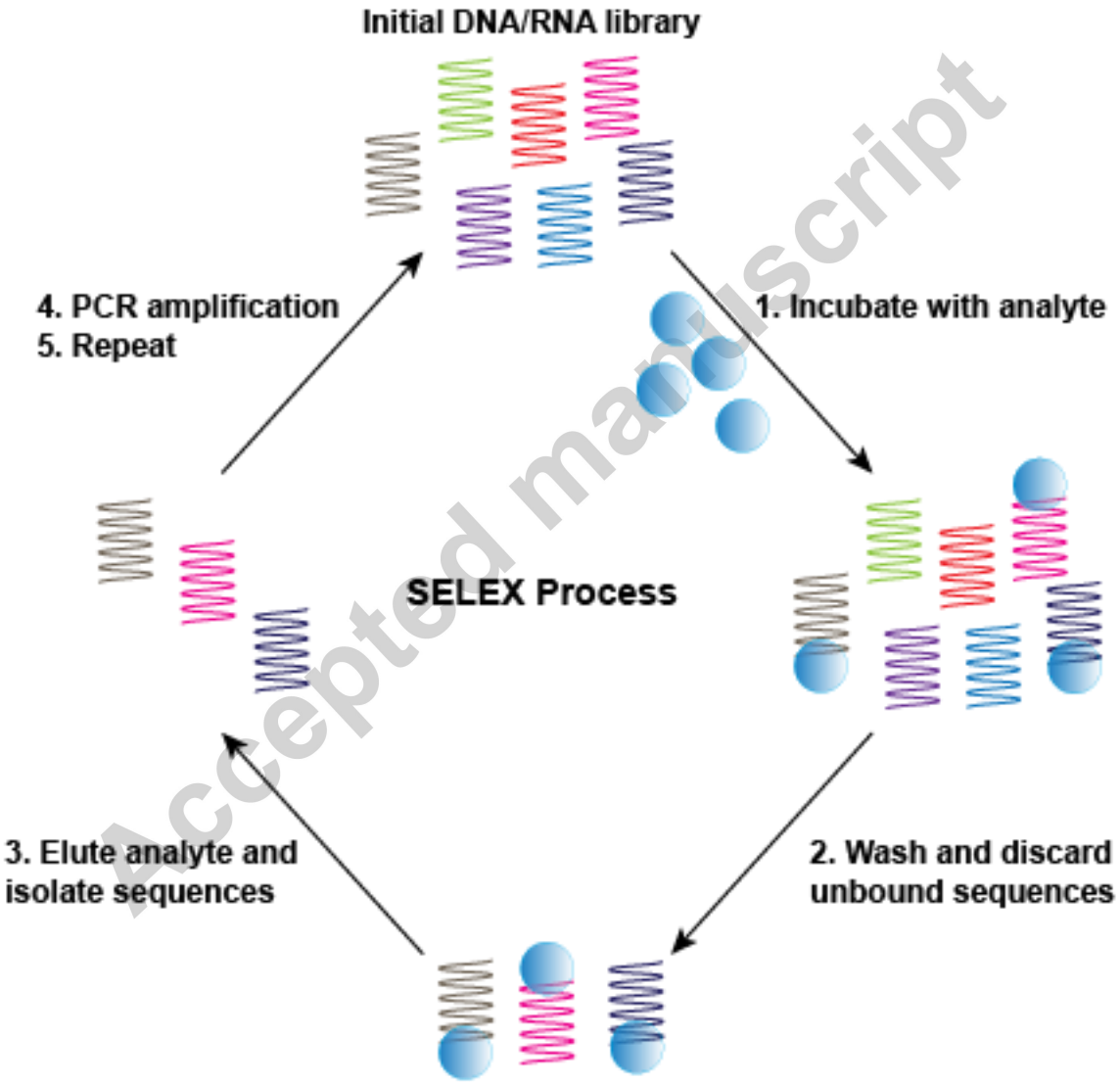

Fig. 6. Systematic evolution of ligands by exponential enrichment – SELEX. The process begins with a large DNA/RNA library and allows binding of the analyte. Aptamers that bound the analyte are then amplified and taken to the next round for further selection. In the end, the ideal aptamer will only bind its target with high selectivity and specificity.

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Aptamers are single stranded RNA or DNA chains created to mimic the selectivity and specificity of antibodies (Famulok et al., 2007; Jayasena, 1999). The affinities (K_D) of aptamers are on the order of nanomolar and picomolar – comparable, if not better than monoclonal antibodies (Jayasena, 1999). These nucleic acid chains can be developed for an extremely wide range of molecules and can achieve binding affinities greater than those exhibited by whole monoclonal antibodies (Jayasena, 1999). The development process begins with a massive random library of RNA or DNA that is then subjected to bind the target analyte (Tuerk and Gold, 1990). The nucleic acid chains binding the analyte successfully are then isolated, amplified, and sent towards the next round of enrichment (**Fig. 6**). Multiple rounds of this process (~8-15 cycles) result in the exponential increase of the nucleic acids displaying the greatest binding affinities towards the analyte. This iterative process is termed the "systematic evolution of ligands by exponential enrichment", or SELEX, and it can take several months to accomplish (Jayasena, 1999). Generally, aptamers developed by the SELEX process are around 70-80 nucleotides long, although it is possible to create functional aptamers smaller than 40 nucleotides long (Lin et al., 1995; Tasset et al., 1997). It is possible to shorten the length of aptamers through the removal of fixed sequence primer regions as most of the time they do not participate in aptamer function (Jayasena, 1999). Once the sequence of the aptamer is determined, chemical synthesis of the aptamer can be accomplished relatively quickly in 2-3 days.

Traditional SELEX development required the use of a pure and soluble analyte, devoid of impurities. However, due to recent developments it is now possible to isolate functional aptamers in complex mixtures such as plasma and cell-surface proteins (Shamah et al., 2008). It is now also possible to increase the affinity of the aptamer further by using specific subdomains of the target analyte during the SELEX process (Keefe et al., 2010). In addition, many SELEX processes also offer counter-SELEX – the discarding of potential nucleic acid aptamers that bind efficiently to the analyte and to closely related structural analogs (Jayasena, 1999). This ensures that the developed aptamer has extremely high selectivity and specificity for only the target analyte.

Aptamers have been developed for a wide range of molecules: metal ions (Ciesiolka and Yarus, 1996), amino acids (Famulok, 1994), organic dyes (Ellington and Szostak, 1992), enzymes (Chen et al., 1996), antibiotics (Wallace and Schroeder, 1998), cofactors (Burgstaller and Famulok, 1994), antibodies (Wiegand et al., 1996), viral particles (Pan et al., 1995), and many more. These nucleic acids have the ability to differentiate between closely related analogs of the target analyte. For example, aptamers can differentiate between two molecules differing by the presence or absence of methyl groups (Jenison et al., 1994), hydroxyl groups (Mannironi et al., 1997), and between enantiomers (D- versus L-) (Geiger et al., 1996).

Unmodified RNA and DNA aptamers tend to be susceptible to nuclease degradation (Keefe et al., 2010. Several strategies have been developed to combat aptamer nuclease degradation, increasing the stability of aptamers when in contact with whole blood or serum. For example, it is possible to add a 5'-PEG moiety or to introduce modified internucleotide linkages (i.e. phosphorothioate linkages) (**Fig. 7A**) (Kang et al., 2007; Vaught et al., 2004). The addition of fluorine or amine groups at the sugar 2' positions has shown to increase the stability of RNA aptamers in biological fluids (**Fig. 7A**) (Pieken et al., 1991). Synthesis of RNA aptamers with L-ribonucleotide triphosphates, termed spiegelmers, can also impart nuclease resistance (Faulhammer et al., 2004; Helmling et al., 2004). Nucleases are highly stereoselective enzymes that recognize and target D-ribonucleotide chains, thus allowing spiegelmers to have increased stability by several orders of magnitude in comparison. In order to produce spiegelmers, SELEX must be performed using D-ribonucleotide triphosphates and the enantiomer of the target analyte since wild-type RNA polymerases only recognize nucleotides with D-riboses. After SELEX, chemical

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synthesis of the L-ribose aptamers will allow binding to the target analyte. This method is limited to relatively small targets whose enantiomers can be produced synthetically for the SELEX process.

Modifications can also be made to aptamers that do not affect their stability but impart additional characteristics. An example is the introduction of unnatural base pairs, such as IsoG/IsoC or ^{Me}isoC/6-AH-isoG, for the expansion of the genetic code diversity in the aptamer (**Fig. 7B**) (Piccirilli et al., 1990; Tor and Dervan, 1993). Aptamers with 5'-bromouracil or 5'-iodouracil can form covalent linkages with other chemical species in close proximity after light activation of those groups (Jensen et al., 1995).

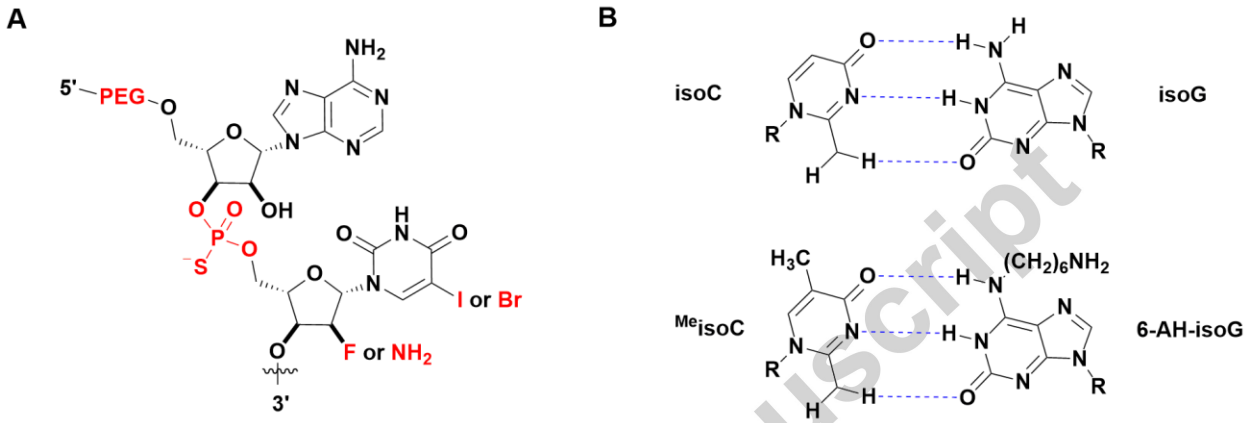

Fig. 7. (A) Modifications of aptamers can be made to increase their stability in serum (2'-fluorine/amine, phosphorothioate linkages, 5'-PEG moieties) or to create immobilizing functional groups (5'-bromo/iodouracil). (B) Unnatural base pairs, such as isoC/isoG and ^{Me}isoC/6-AH-isoG, are used to expand the genetic code diversity of the aptamer.

4.2. Immobilization techniques of nucleic acid aptamers

Aptamers, like Fab' fragments, have been immobilized on a wide variety of different surfaces. For example, the thrombin aptamer has been immobilized onto quantum dots (Hansen et al., 2006), gold nanoparticles (Pavlov et al., 2004), silica (Lee and Walt, 2000), glass (Potyrailo et al., 1998), and carbon nanotubes (So et al., 2005). However, compared to scFv fragments, aptamers are not as customizable since their modifications must be performed chemically. Also, any modifications that are made must not interact with the analyte-binding ability of the aptamers. Generally, aptamer modifications consist of the attachment of 3'- or 5'- functional linkers that are used for immobilization onto surfaces (Balamurugan et al., 2008). The effects of linker location (3'- versus 5'-) have been studied and it was determined that the optimal position varies from one aptamer to another (Cho et al., 2006). Many aptamer modifications are performed by the company selling the aptamers, reducing the number of in-lab modifications that are required by the researcher.

One such modification involves the attachment of a linker containing a thiol or disulfide functional group (Balamurugan et al., 2008). The latter functional group is commonly used to immobilized aptamers directly onto gold nanoparticles (Balamurugan et al., 2008). Most thiol-functionalized aptamers are obtained as asymmetric disulfides with one sulfur attached to the aptamer and the other to a hexanol moiety. Reduction of the disulfide with DTT and the purification with a size-exclusion column yields the thiol terminated aptamer that is ready for immobilization (Neves et al., 2015). For example, Lai et al. (2007) immobilized 3'-methylene blue, 5'-thiol modified aptamers directly onto gold surfaces along with 6-mercapto-1-hexanol (anti-fouling agent). Unlike the denaturing interactions between gold surfaces and

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proteins, aptamers are not affected to the same extent and display significantly better stability on the surfaces.

The attachment of a 3'- or 5'- linker containing an amine group is another common modification of aptamers. Wang et al. (2011) immobilized 5'-amine modified (C6 linker) onto polyacrylic acid-derived upconverting phosphor particles using EDC/NHS coupling. Stadtherr et al., (2005) modified glass slides using 3-aminopropyltrimethoxysilane (APS) followed by glutaraldehyde and sodium cyanoborohydride. Aptamers reacted with sodium cyanoborohydride were immobilized in an oriented fashion on the glass surfaces. It is also possible to immobilize amine-modified aptamers onto amine-derived surfaces. Walter et al. (2008) modified aldehyde-coated glass slides to form surface amines with polyethylenimine. Aptamers with a 3'-amine group were activated using cyanuric chloride in sodium borate buffer. Cyanuric chloride reacts with amines and was used in this case to link the amine-terminated aptamers with the amine-rich surfaces.

The amine functional group may also be used to link aptamers with functional peptides. For example, Chu et al. (2006) reacted unmodified aptamers with N-succinimidyl-3-(2-dithiopyridylthio) propionate (SPDP), allowing random amine groups to reach with the NHS moiety. The toxin gelonin (protein), reduced with DTT, was allowed to react with the dithiopyridine group on the aptamers. This type of immobilization is acceptable in a non-biosensor application where orientation of the aptamers is not critical. Similarly, Carter and LaBean (2011) used EDC/NHS chemistry to couple 5'-amino modified aptamers and gold-binding peptides (C-terminus). In this case, the gold-binding peptides allowed for oriented immobilization of the aptamers onto gold surfaces. It can be concluded that the amine functional is extremely versatile and can be used to immobilize aptamers on a wide variety of surfaces – any electrophilic surface capable of reacting with amines.

Non-covalent immobilization methods exist for aptamers and are very similar to those used for scFv and Fab' fragments. Tombelli et al. (2005) used 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) and NHS to immobilize streptavidin to carboxylated dextran. Biotinylated aptamers were then allowed to interact and bind to the surfaces. One benefit of this method is that it is possible to purchase biotinylated aptamers, thus eliminating the potentially damaging chemical coupling of biotin to the aptamers. However, a disadvantage to using the biotin/streptavidin immobilization technique is the potential denaturation of the streptavidin protein. Sultan et al. (2009) immobilized unmodified aptamers onto a multilayer polyelectrolyte film consisting of poly(diallyldimethylammonium chloride) (PDDA) as the polycation and with poly(sodium 4-styrene-sulfonate) (PSS) and the aptamers as polyanions. Although this is a non-covalent/non-oriented approach towards aptamer immobilization, QCM and confocal microscopy studies indicated no loss of specificity and only a modest reduction in binding affinity.

Therefore, one can conclude that although aptamers are less customizable than scFv fragments, a wide variety of immobilization techniques have been developed, allowing them to be immobilized onto many different types of surfaces.

4.3. Nucleic acid aptamers and their use in biosensors

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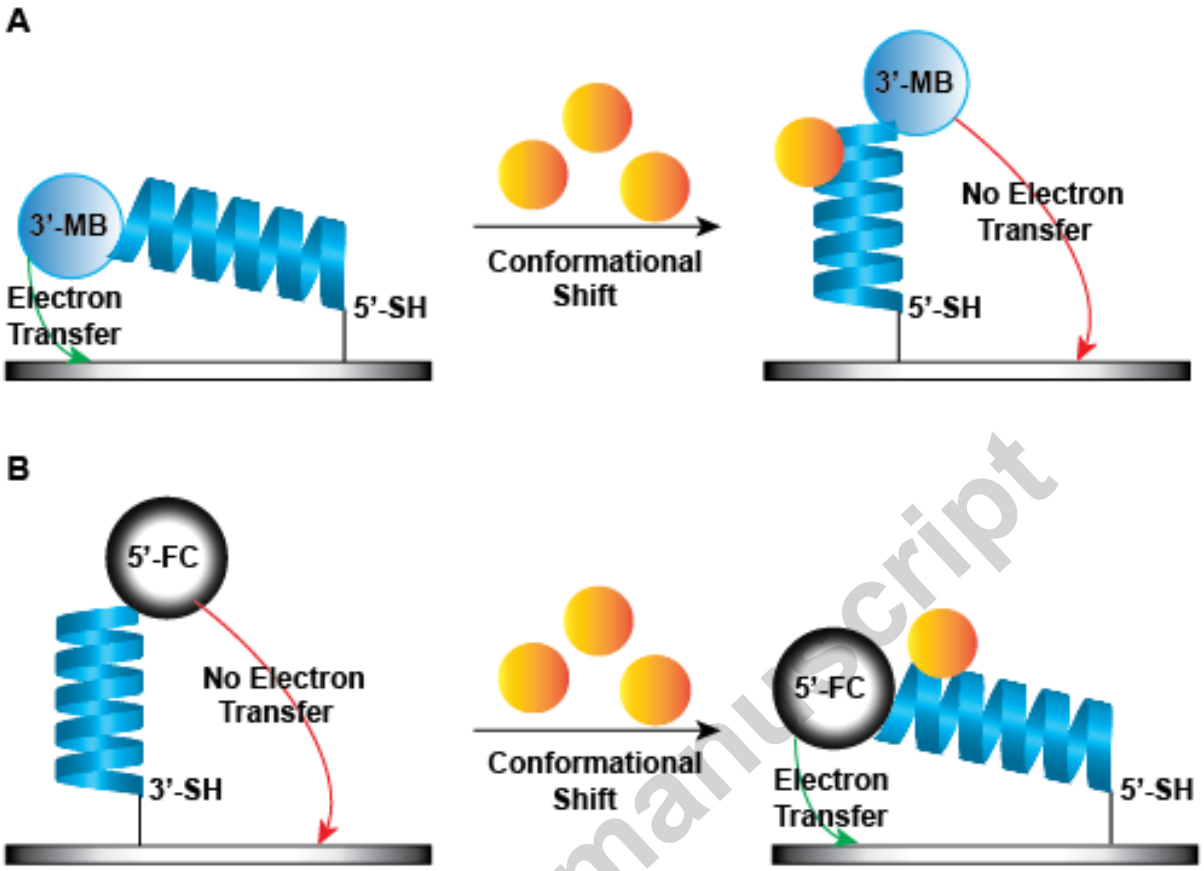

Fig. 8. MB = methylene blue, FC = ferrocene. (A) A negative signal electrochemical biosensor using 3'-methylene blue-labeled aptamers. Analyte binding creates a conformational shift in the aptamers, resulting in the distancing of the MB units from the surface, preventing electron transfer. (B) A positive signal electrochemical biosensor using 5'-ferrocene-labeled aptamers. Analyte binding creates a conformational shift in the aptamers, moving the FC units closer to the surface and enhancing electron transfer.

Since the discovery of the aptamer in 1990, many biosensors have been developed using these antibody-mimetic biosensing elements. However, electrochemical and optical biosensors dominate the literature, with an increasing prevalence of mass-sensitive biosensors (Schoukroun-Barnes et al., 2015; Song et al., 2008).

Electrochemical biosensors utilizing aptamers are based on the changes of the conformation of the aptamer, which modify the electron transfer efficiency between the electrode surface and a reporter redox molecule (Schoukroun-Barnes et al., 2015). For example, Maehashi et al. (2007) engineered a carbon nanotube field-effect transistor aptasensor for the detection of IgE. Anti-IgE aptamers with 5'-amine modifications were immobilized onto surfaces that were previously altered using 1-pyrene butanoic acid succinimidyl ester. The linear dynamic range of this electrochemical aptasensor was determined to be from 250 pM to 20 nM of IgE. Xiao et al. (2005) developed an electrochemical biosensor for the detection of thrombin using 3'-methylene blue, 5'-thiol modified aptamers that were immobilized directly onto the gold electrodes (**Fig. 8A**). Binding of thrombin to the aptamer resulted in an aptamer conformational shift, moving the 3'-methylene blue unit away from the gold surface, decreasing the detected signal. The linear range of the biosensor was between 10 nM and 768 nM. Similarly, Radi and

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O'Sullivan (2006) created an electrochemical biosensor for the detection of potassium ions using 3'-thiol, 5'-ferrocene (FC) modified aptamers that were immobilized directly onto the gold electrodes (**Fig. 8B**). Unlike the previous example, upon binding of the analyte in this biosensor, a conformational shift of the aptamer resulted in the ferrocene unit coming closer to the surface, enhancing electron transfer, and resulting in an increased signal.

Similar to immunoassays, optical aptamer biosensors are termed bioassays and can be engineered as either single-site binding or dual-site binding modes (Song et al., 2008). Bioassays detecting small molecules can only be engineered as single-site binding assays since the analytes tend to be engulfed in the aptamer binding pocket (Song et al., 2008). Common optical bioassays involve the use of fluorescent detection due to the ease of labeling aptamers, the ability for real-time detection, and the low limits of detection that can be achieved. One common format of these fluorescence bioassays involves the use of aptamer-based molecular beacons (aptabeacons) (Yamamoto et al., 2000). Aptamers in these assays are engineered into hairpin structures with the ends labeled with a fluorophore and quencher. Binding of the analyte disrupts the aptamer conformation, separating the fluorophore and quencher, leading to fluorescence detection. One such example is the use of the fluororophore-labeled, quencher-labeled, cocaine aptamer by Stojanovic et al. (2001). This aptasensor operated in serum and was able to achieve a linear dynamic range from 10 µM to 4000 µM, binding only cocaine and none of its metabolites. One limitation of these aptabeacon biosensors is the high background fluorescence signal, leading to higher than desirable limits of detection.

Some optical bioassays involve the use of DNA molecular light-switching complexes (Jiang et al., 2004; Zhou et al., 2006). These complexes are not luminescent in aqueous solution, however, upon binding to double-stranded DNA they radiate intense luminescence. Although aptamers are single-stranded RNA or DNA chains, double-stranded sections form due to the conformational shift associated with analyte binding. Zhou et al. (2006) used the unsymmetrical cyanine DNA intercalating dye TOTO as a molecular light-switching complex to detect oncoprotein platelet derived growth factor BB (PDGF-BB). The combination of the high affinity of the aptamer towards PDGF-BB and the intense fluorescence produced upon binding of TOTO, allowed the biosensor to reach an LOD of 0.1 nM.

One specific category of optical aptamer biosensors are classified as nanosensors due to the use of surface-bound or free metal nanoparticles. For example, Wang et al. (2015) developed a fluorescence-based aptamer nanosensor for the detection of thrombin through the use of the cyanine dye PicoGreen (PG) as a signal molecule and surface-bound metal-enhanced fluorescence substrates (silver island films) as enhancers. This label-free aptasensor presented with very high sensitivity and a low limit of detection (73 pM). Similar fluorescence-based aptamer nanosensors were developed by Chen et al. (2015), Mei and Zhang (2012), and Yi et al. (2014). These optical nanosensors offer high sensitivities, rapid responses, large signal-to-background ratios, and the option of real-time detection of the target analyte.

Finally, mass-sensitive aptamer biosensors are becoming more common due to the use of label-free detection techniques and the ease of immobilization of aptamers. One such example uses a label-free microfabricated cantilever-based sensor for the detection of Taq DNA polymerase (Savran et al., 2004). Two adjacent cantilevers, one covered in aptamers and the other blocked with single-stranded DNA, detected the polymerase analytes via the differential cantilever bending upon analyte binding. Another category of mass-sensitive aptamer biosensors involves the piezoelectric biosensor platforms, such as the QCM and the EMPAS (Liss et al., 2002; Neves et al., 2015; Yao et al., 2010). As the EMPAS is a superior detection platform the QCM, only the aptasensors developed with it will be discussed. Neves et al. (2015) immobilized anti-cocaine 5'-thiol modified aptamers onto TUBTS covered piezoelectric quartz disks. Currently, this is the best cocaine biosensor that has been developed having a limit of detection of

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0.9 µM in complex buffer (contains select added serum proteins). Unlike electrochemical and some optical aptamer biosensors that rely on the conformational shift of the aptamer for detection, the EMPAS aptasensor does not – detection occurs upon analyte binding to the aptamer via a change in the resonance of the piezoelectric disk.

5. A comparison of scFv fragments, Fab' fragments, and aptamers and their roles in the biosensor world

The previous sections have provided a well-rounded overview of the three biosensing elements. Using this information the biorecognition elements can be compared in order to determine their roles in the biosensor world. **Table 1** illustrates advantages and disadvantages to using all three of the biosensing elements. The optimization of the biosensing elements and their immobilization onto transducer surfaces are arguably the most important steps in the development of a biosensor. Biosensing elements need to be judged for their cost and ease of development, variability and ease of immobilization, affinity towards the analyte (selectivity and specificity), and stability.

Of the three biosensing elements compared in this review, Fab' fragments are the cheapest and quickest to obtain. Once the whole antibodies are received, it is only a matter of days before they are cleaved and Fab' fragments are obtained. The only downside is that there is usually some loss of immunological activity of the antibody fragments via chemical cleavage. Due to the use of recombinant antibody technology, scFv fragments are significantly more expensive and require several weeks of development before their use. The development process is also very laborious and requires skilled researchers that are well versed in cell culture practices. Overall, aptamers are the most costly and require the longest optimization time. This is largely due to the SELEX process, which lasts several months and costs tens of thousands of dollars. The actual synthesis of the aptamers is very fast (a few days) and can be quite cheap (on the scale of dollars per gram) when synthesized in large quantities (Jayasena, 1999).

In terms of biosensing element immobilization, scFv fragments have the potential to be immobilized onto the largest variety of surfaces due to how highly customizable they are (ex. addition of a C-terminal thiol or a functional immobilizing C-terminal peptide, etc.). However, it is arguable that Fab' fragments and aptamers can be immobilized onto surfaces with greater ease. The most common modification of scFv fragments is the addition of a C-terminal fusion peptide that is used for immobilization. Thus, the researcher has to worry about avoiding scFv denaturation and fusion peptide denaturation. Also, many of these immobilizing peptides require specific substrates that need to be on the surfaces. In comparison, Fab' fragments and most aptamers only have one small functional group (ex. thiol, amine, etc.) that can react with surface functional groups for immobilization in a simple manner. In terms of surface density, aptamers can be immobilized with the greatest number of binding sites per unit area due to their small size (~1-2 nm) (So et al., 2005) compared to Fab' fragments (~7 nm) (Droz et al., 1996) and scFv fragments (~4 nm) (Saerens et al., 2008).

The optimal biosensing element has an affinity that is highly selective and specific for the target analyte. Aptamers dominate this category as their affinities can be improved through more rounds of SELEX and through the use of counter-SELEX. This ensures that the best aptamers have affinities in the nanomolar/picomolar range and do not bind to close derivates of the analyte. Fab' fragments are in second place for affinity as they have been shown to have smaller dissociation constants compared to scFv fragments (Kikuchi et al., 2005). Compared to whole antibodies, aptamers have similar or better affinities for their analyte whereas Fab' fragments and scFv fragments have lower affinities (Jayasena, 1999). However, the affinity of scFv fragments can be increased through dimerization (increased avidity)

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or through affinity maturation in the recombinant process. For example, Torrance et al. (2006) showed that after three affinity maturation cycles, the affinity of the scFv fragments was increased by 100-fold. In terms of selectivity and specificity, scFv fragments come in second place behind aptamers. Recombinant synthesis of the scFv fragments ensures that they are highly specific for their target analyte, binding to only one epitope. On the other hand, Fab' fragments can be obtained from monoclonal or polyclonal antibodies. The latter involve antibodies that bind to one target analyte, but through multiple potential epitopes. These antibodies are prone to non-specific binding to close derivatives of the analyte (Jayasena, 1999).

Table 1. A comparison of the advantages and disadvantages of scFv fragments, Fab' fragments, and aptamers as biosensing elements.

	scFv	**Fab'**	**Aptamer**
Advantages	• Highly customizable due to recombinant technology • Functional avidity can be increased via dimerization • Affinity can also be increased via affinity maturation • Low batch to batch variability • Immobilization can be achieved on a wide variety of surfaces • Smaller size compared to whole antibodies results in higher sensitivities and lower limits of detection in biosensors	• Cheap • Synthesis (from the whole antibody) is fast on the scale of several days • Synthesis is simple and not very intensive • Immobilization can be achieved on a wide variety of surfaces • Smaller size compared to whole antibodies results in higher sensitivities and lower limits of detection in biosensors	• Can be developed for almost any analyte • Chemical synthesis of the aptamers is fast (several days) and cheap • No batch to batch variability • Minimally immunogenic • Analyte affinity can be increased through more rounds of SELEX • Moderate customizability allows for immobilization onto many surfaces • Smaller size compared to whole antibodies results in higher sensitivities and lower

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			limits of detection in biosensors • Are able to renature and regain analyte binding ability after denaturation (biosensor regenerability possible) • Can be developed for use in non-traditional solvents, temperatures, buffers
Disadvantages	• Synthesis is slow on the scale of several weeks • Synthesis requires significant expertise and labour • Monomers have lower affinities towards analyte compared to whole antibodies • Susceptible to permanent denaturation • Cannot be produced for any analyte (i.e. small molecules)	• Minimal customizability • Monomers have lower affinities towards analyte compared to whole antibodies • Susceptible to permanent denaturation • Cannot be produced for any analyte (i.e. small molecules, toxins or anything that will kill the animal producing the antibodies) • Antigen-binding sites can	• Aptamer sequence determination via SELEX is very slow on the scale of several months • SELEX is extremely expensive • Unmodified aptamers are susceptible to nuclease degradation in serum

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	• Contain immunogenic regions • Expensive • Biosensor detection requires solution to be compatible and non-denaturing • Minimal biosensor regenerability	be damaged upon enzymatic or chemical cleavage of the whole antibody • Contain immunogenic regions • Biosensor detection requires solution to be compatible and non-denaturing • Minimal biosensor regenerability

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The final category of comparison involves the stability of the three biosensing elements. Once again, aptamers dominate this category as they are capable of regaining their analyte binding abilities even after being denatured. This allows aptamers to be stored for long periods of time and allows them to be used in regenerable biosensors. In comparison, Fab' fragments and scFv fragments are not able to regain their analyte binding abilities once they have been denatured. These antibody fragments cannot be stored for long periods of time and their biosensor regeneration capabilities are minimal.

6. Conclusions and Future Perspectives

This review illustrates a thorough comparison between antibody scFv fragments, antibody Fab' fragments, and aptamers. Antibody scFv fragments, composed of the antibody V_L and V_H domains as well as a linking peptide, are the smallest of the three biosensing elements. These recombinantly-derived fragments can be modified to include various immobilization groups (i.e. linking peptides, reactive functional groups). For this reason, scFv fragments are the most customizable compared to Fab' fragments and aptamers. Antibody scFv fragments have been used in piezoelectric biosensors, optical biosensors, immunoassays, and to a lesser extent electrochemical biosensors.

Antibody Fab' fragments, composed of the antibody V_L, V_H, $C_{L,}$ and C_{H1} domains, are generally derived via chemical modification of the whole antibody. The cleavage of the whole antibody to yield the Fab' fragment is very quick and cheap, however, does suffer from a loss of immunological activity. The C-terminal thiols of the Fab' fragments allow for simple oriented covalent immobilization onto electrophilic surfaces (i.e. maleimide-rich, thiosulfonate-rich). These antibody fragments have also been utilized in a

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variety of biosensors: optical, electrochemical, and piezoelectric biosensors, with a minimal prevalence in immunoassays.

Aptamers – single stranded ribonucleic acid (RNA) or 2'-deoxyribonucleic acid (DNA) chains – are derived to have extremely high affinities for specific analytes. Through the use of SELEX and counter-SELEX, it is possible to obtain affinities in the picomolar range – similar or better than those observed in whole antibodies. The customizability of aptamers is limited as it must be done chemically after the aptamer has been synthesized. Common modifications include the addition of functional amine or thiol groups for immobilization onto surfaces. Aptamers are extremely prevalent in the electrochemical and optical biosensor fields, consistently reaching very low detection limits and very high sensitivities.

Two reasons for why a researcher wouldn't begin the engineering of a biosensor with aptamers are the time and cost associated with the aptamers' development. As the popularity and use of aptamers increases with time, an associated decrease in price should be expected. The largest time and cost allocations for aptamer development are in the optimization stages while aptamer synthesis itself is very quick and cheap.

The biosensor world will continue to see more and more biosensors developed with aptamers due to the high affinities and excellent stabilities achieved with these nucleic acid biosensing elements. However, this does not mean that we will see the complete disappearance of other types of biosensing elements (i.e. scFv and Fab' fragments). These other biosensing elements offer too many benefits to be completely overlooked by the biosensor community. For example, if one wishes to develop a biosensor, one should initially use Fab' fragments as they are cheap, quick, and easy to use. After this proof of concept biosensor is developed, one can then choose to either use scFv fragments or aptamers depending on the time and money available. Only if detection of the analyte via Fab' fragments or scFv fragments is not possible should one begin developing a biosensor using aptamers. It is important for a researcher to be well-versed in all three of these biosensing elements in order to determine the best-suited option for their particular analyte detection.

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Highlights:

- scFv fragments, Fab' fragments, and aptamers are common biorecognition elements
- scFv fragments are the most customizable due to recombinant synthesis methods
- Fab' fragments are the cheapest and quickest to develop for biosensor use
- Aptamers have the highest affinity for their target analytes and are most stable

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