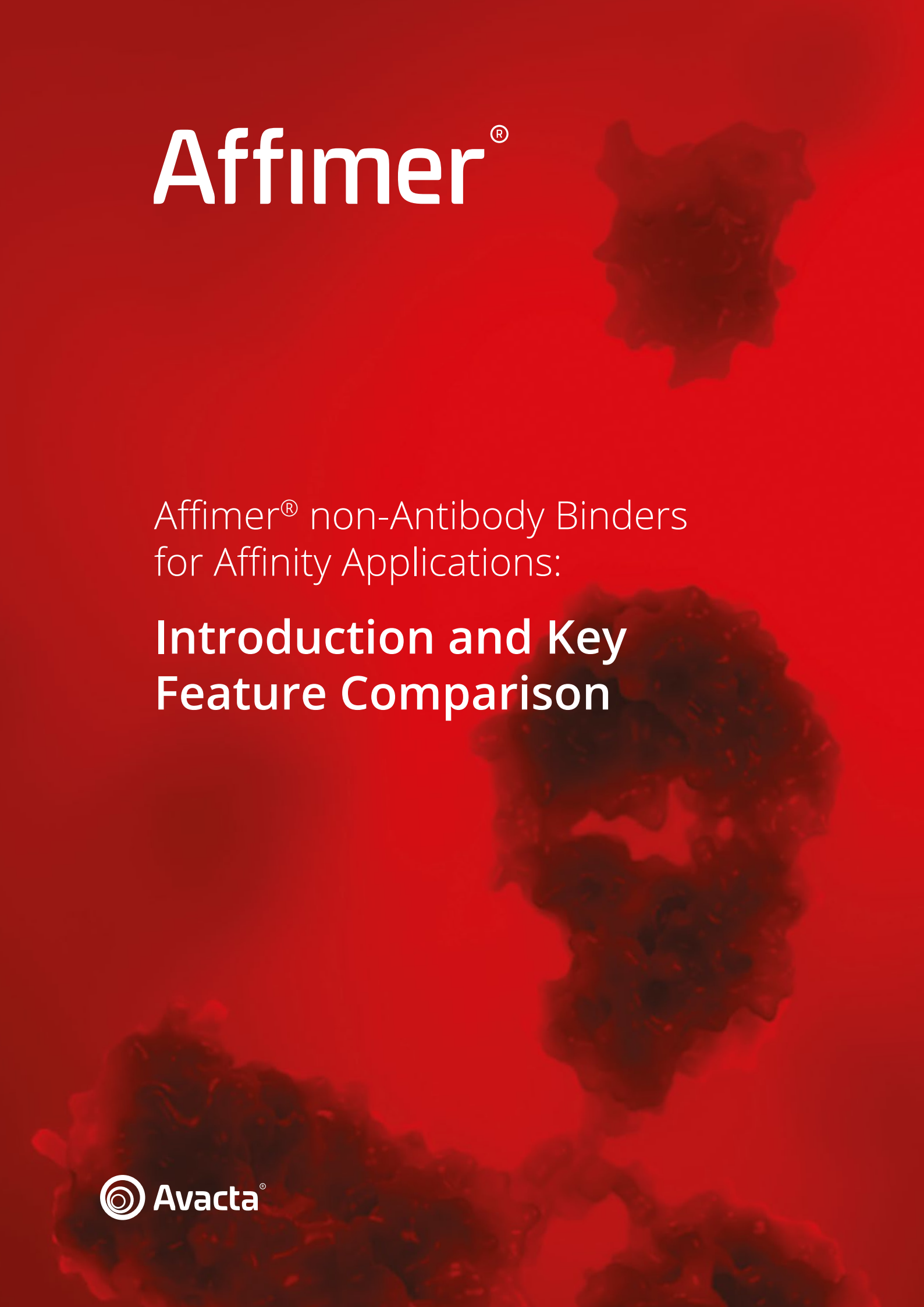
Affimer®

Affimer® non-Antibody Binders for Affinity Applications:

Introduction and Key Feature Comparison



Avacta®

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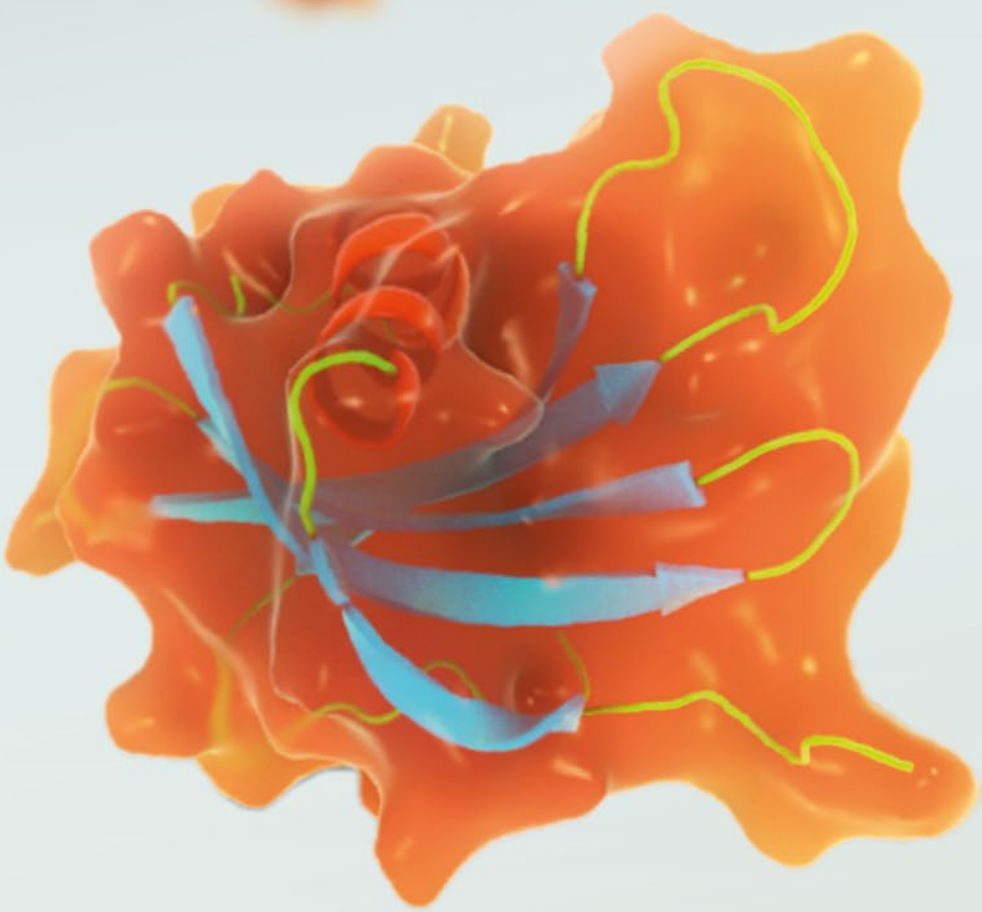


Abstract

Affimer® reagents and therapeutics are non-antibody protein scaffolds that display equivalent molecular recognition characteristics to antibodies, but exhibit superior stability, versatility and ease of production. They offer potential benefits across all the traditional applications of antibodies, with particular advantages in the more sophisticated and challenging, such as rapid diagnostics, point of care and biosensing, and as biotherapeutics. In these applications, their flexibility in binding site generation and selection, stability and size properties, ability to form multimers and their ease of production combine to maximum effect.

Introduction

Affinity binders such as antibodies recognise and bind specific proteins of interest, and as such have found many applications in the analysis and manipulation of different protein targets. These applications range from protein detection and quantitation to purification, mode-of-action analysis, subcellular localisation and use as therapeutics.

Traditionally, the antibody has been the workhorse of biological experiments and for some applications, antibodies remain the gold standard. As the sophistication of applications has increased, so have the demands on affinity binders, and this has made the limitations of antibodies much more apparent. In response, in recent years, a number of alternatives have surfaced, including antibody fragments, nucleic acid aptamers and engineered non-antibody protein scaffolds such as the Affimer platform.

This paper reviews the main applications of affinity binders and the requirements placed on them according to application. Looking in particular at the Affimer non-antibody protein scaffold, it examines the features that make this scaffold uniquely versatile and an ideal solution to address production and technical challenges across a broad range of *in vitro*, diagnostic and therapeutic applications.

Affinity-based applications in the detection, analysis and manipulation of protein targets

The ability of an affinity binder to bind with specificity to a target protein, even within a complex assay, cellular or *in vivo* environment, opens the door to many applications.

In vitro applications

A non-exhaustive list of *in vitro* applications is given in Figure 1. For all applications, the underlying principle is the specific attachment of the affinity binder to a target protein or protein complex, followed by detection (direct or indirect) and / or isolation and purification of the protein.

This target-specific affinity binder can be:

- free in solution
- immobilised on a chromatography matrix (for purification applications)
- immobilised on surfaces for lateral flow, diagnostic and biosensor or high throughput assays
- labelled for direct detection (e.g. Immunohistochemistry (IHC), Immunocytochemistry (ICC) or Immune-mass spectrometric (Immune-MS) measurements)
- tagged with a capture tag for separation or pull-down experiments (e.g. chromatin immunoprecipitation)
- paired with a labelled secondary reagent that binds to the affinity binder itself for indirect detection (such as Enzyme Linked ImmunoSorbent Assay (ELISA), or Enzyme ImmunoAssay (EIA))

In vitro applications are diverse and place wide-ranging demands on the affinity binder to be used.

The 'ideal' affinity binder would show:

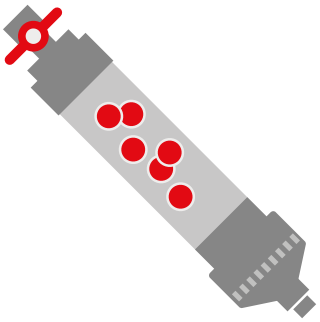
- high specificity and tailored affinity for the target
- a large and versatile binding surface — not just loops, surfaces or grooves but combinations of these
- tolerance to randomisation or mutagenesis, to enable highly diverse library generation and universal application; it should be possible to generate high affinity binders to all targets, even challenging functional epitopes
- high discriminatory powers, for example to distinguish between isoforms that differ only by a single amino acid at the binding epitope
- easy conjugation with a reporter tag without affecting binding to the target (for direct detection) or have a validated secondary detection partner that is labelled
- structural stability (including absence of disulphide bonds), functionality and correct orientation when immobilised onto solid supports
- absence of cysteine residues, to allow high yield expression in *E. coli* and allow controlled insertion of a single cysteine to permit site-specific chemical ligation if required
- chemical stability in the assay matrix, across a wide range of temperatures, salt concentrations and pH values
- options for cost effective, scalable and easy production

Figure 1:

While antibodies still have many uses in many *in vitro* applications, they have intrinsic limitations including size, stability in assay solution and / or when immobilized to a surface and host toxicity. Affimer binders offer significant advantages over antibodies as applications become more sophisticated and stringent in their demands.

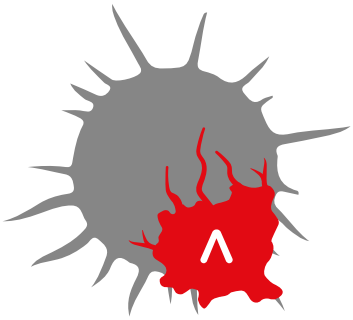
Affimers: from discovery to drug delivery

This unique scaffold has application in every step of the drug discovery process, from *in vitro* discovery assays to *in vivo* imaging and administration as a therapeutic



In vitro:

- ELISA, EIA
- Western blot
- Chromatin immunoprecipitation
- Pull-down (e.g. magnetic bead)
- Affinity chromatography
- Immuno-mass spectrometry
- Multiplexed protein array
- Multiplexed bead assay



In vivo:

- Immuno-imaging
- Targeted therapeutic (direct)
- Targeted modulator
- Targeted drug delivery

Cellular & ex-vivo:

- Flow cytometry
- Immunocytochemistry
- Immunohistochemistry
- Monitor post-translational modifications
- Protein structural determination

Diagnostic applications

In vitro assays for clinical diagnostics are increasing in sophistication. For many, speed to result is paramount. This has driven demand for point of care and biosensor assays. We define a biosensor as a small device which uses a biological agent to capture the target onto the sensor surface. A transducer then converts this capture event into a signal (e.g. electrical or fluorescence) that is picked up by a detector. Success of affinity biosensors, where the capture is mediated by an affinity binder, relies on the immobilisation of that affinity binder such that its structure and binding activity are maintained. The binding domain must be accessible and active to minimise non-specific adsorption to the surface. Similarly, non-specific adsorption of matrix molecules to the affinity binder must be minimised, so unfolding of the scaffold should be minimal. These all affect the detection limit, specificity and reproducibility of the assay.[1] The scale of benefit is significant: increases of between twenty and two hundred-fold in performance parameters have been shown with correct antibody orientation,[2] although the limitations of antibodies in biosensors are discussed below.

Chemical stability is a pre-requisite for biosensors. Rapid, point of care diagnostic assays cannot demand complex sample pre-purification steps prior to detection, so the binder must work across all biological sample matrices and should withstand harsh conditions to permit biosensor regeneration. Ideally, affinity binders will also have long shelf life and tolerate ambient temperature storage.

For biosensor applications, the affinity binder should be:[1]

- stable when immobilised on a solid surface
- easy to control in orientation for binding site availability
- small enough to allow uniform and dense coverage of the surface to minimise non-specific binding
- stable under harsh sample matrix, assay and regeneration conditions
- stable to permit easy transport and storage in the field

Therapeutic applications

Antibodies were the first affinity agents to be licensed as therapeutics.[3] They can work in a number of ways: the first is direct modulation of protein or cell activity through the specific binding of the antibody to the target protein. The function of the target protein is consequently modulated by the bound antibody.

The second is indirect, where the antibody binds to the cell, and then the Fc (conserved tail) region recruits effector cells to bind. These effector cells stimulate phagocytosis and removal of the cell or antibody-mediated cellular cytotoxicity and cell death. Indirect effects can also occur through activation of the complement cascade, or through the conjugation of the antibody to toxins, cytokines or other drugs, which can then be delivered to specific cells through antibody binding.[3]

Therapeutic use therefore requires that the affinity binder should be:

- able to reach the site-of-action in sufficient quantity to exert its intended therapeutic effect
- able to bind specifically and effectively to the target to interfere with that target's natural action to exert therapeutic effect
- 'tuneable' in its half-life where a controlled rate of clearance from the body is required for the application
- have a minimal level of toxicity or immunogenicity
- simple to produce to Good Manufacturing Protocol (GMP) standards; there needs to be process control throughout production and validation, to meet with regulatory requirements, and this requires batch-to-batch consistency and long term stability / shelf life
- purifiable to sufficient levels to minimise contaminants, e.g. cellular contaminants from the recombinant production process, in clinical formulations
- amenable to direct conjugation with other proteins or small molecules and / or the formation of multimers with single or multiple specificities

Antibodies and their limitations as affinity binders

Antibodies are still the main tool used for many protein analysis applications. However, they do have limitations in some applications.

Polyclonal antibodies are relatively inexpensive to generate. By their nature, a mixture of polyclonal antibodies will have multiple recognition epitopes, which can increase sensitivity, but is also likely to reduce specificity, as the chances of sequence and structural homology with other proteins increases with the number of different antibody paratopes present. Monoclonal antibodies overcome this, to some extent, through the selection of the most efficient, specific recognition epitope. Monoclonals can therefore offer higher specificity, lower batch-to-batch variation and potentially higher stability. There are, however, intrinsic features of all antibodies that limit their utility for more demanding applications, such as the need for stable di-sulphide binding to maintain their conformation, including that of the large constant region that mediates many interactions with proteins that occur naturally in biological samples (especially blood, plasma and serum).

Production considerations: ease, speed, cost and process control

Classically, polyclonal and monoclonal antibodies are produced in animals. Monoclonals are generated by immunisation with the antigen, isolation of the host spleen cells (immune B-cells) and fusion of these cells with a myeloma cell to produce an 'immortal' hybridoma. There are significant drawbacks to this.

Large quantities of antigen are often needed to elicit an immune response, and for native proteins these amounts can be difficult to achieve. While protein fragments or peptides can be used to generate the antibody, these may not be recognised in the fully-folded native protein assay.

Some antigens are non-immunogenic, especially when they are closely related to the host, and those that are toxic cannot be used.

The production process for both monoclonal and polyclonal antibodies takes several months to complete and is challenging - single cell clonal selection for monoclonal antibody production in particular is difficult. Polyclonal antibodies are non-renewable owing to the fact that they are the product of different polyclonal responses in different animals of the same species. The likelihood of generating identical batches via the immune response of different animals is thus incredibly small.

Hybridoma cells can be unstable, and will often fail to grow, or stop secreting the monoclonal antibody because of gene loss. When this happens, that monoclonal source is lost. This is problematic for applications where batch-to-batch reproducibility of the antibody is essential.

Many researchers have employed recombinant techniques to produce monoclonal binders and these have been partially successful in overcoming the issues posed by animal production. *In vitro* selection from phage display libraries does not benefit from the significant affinity maturation processes that happen relatively rapidly *in vivo*, although this can be partially overcome by making the library from the B-cells of an immunised animal.

Success in recombinant expression of the resulting antibody can be compromised by issues of aggregation of the recombinant protein, plasmid loss and the failure of prokaryotic and lower eukaryotic organisms to carry out complex glycosylation.[4]

Size and structural complexity

Antibodies are large, multimeric, multi-domain proteins with complex tertiary and quaternary structures *(Figure 2A)*. This can result in a propensity to aggregate when concentrated during purification and delivery.[5]

Large size can also cause problems of steric interference with smaller target molecules and epitopes. In general, antibodies will only recognise planar surfaces, not grooves and other 3D structures. Studies show that 75% of examined polyclonal sera contain antibodies that overwhelmingly favour linear epitopes, a stretch of continuous amino acids in the protein target sequence, compared to conformational epitopes typically found in grooves, that are composed of key amino acids brought together by folding.[6,7] Large size is a hindrance for *in vivo* applications where tissue penetrance is required. Finally, large size restricts the number of antibodies that can be immobilised in a given area, which can limit the capacity of antibody-coated beads or the sensitivity of detection in biosensing applications, or in ELISA.

Stability under assay conditions

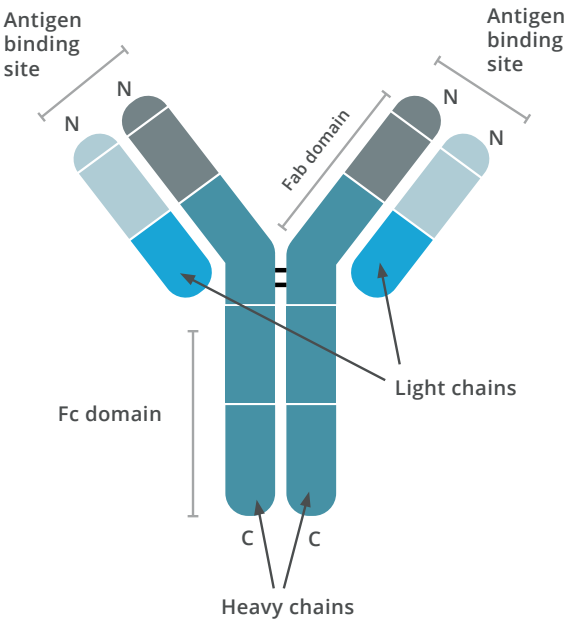
The conformation of different antibody subclasses has an impact on their stability under variable pH and ionic strength.[8, 9] Antibodies have hinge regions that are particularly susceptible to pH, salt and temperature.[10, 11] They are therefore sensitive to assay conditions and sample matrix effects.

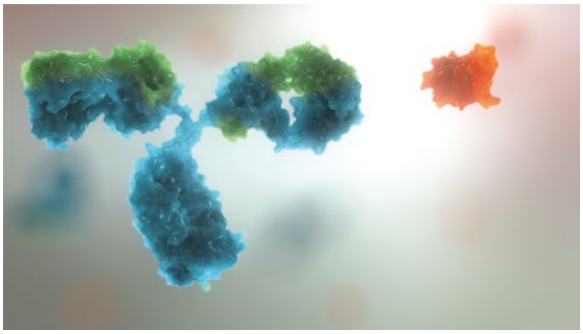
Immobilisation onto solid support

Immobilisation onto a solid support is a pre-requisite for many advanced, multiplexed assays such as microarray or bead-based detection, and for biosensor and diagnostic assays. Antibody microarrays have seen limited success for a number of reasons. One contributing factor is their lack of structural stability: when immobilised on a hydrophobic solid support surface they tend to collapse.[12] The same is true for biosensor assays, with the added disadvantage of non-specific binding to gold surfaces.

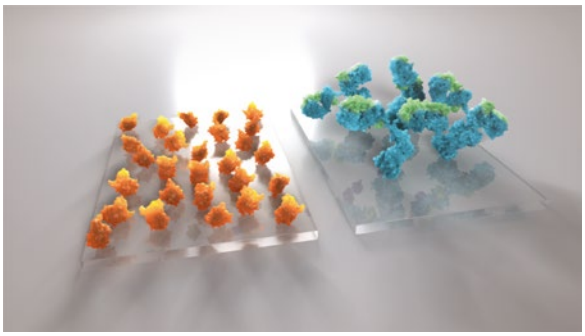
Figure 2:

A:





B: An Affimer molecule (orange) is shown to scale to show the difference in size.



C: Due to the small size of the Affimer protein, when immobilized on a surface as in a biosensor assay, on an ELISA plate or on beads, many more Affimer protein molecules can bind to the surface than antibodies, which brings sensitivity advantages. This also minimises non-specific binding.[13]

Success rate

One of the major technical limitations of antibodies is the need to generate high affinity and high specificity binders to any antigen. In practice, it often proves impossible to generate and select antibodies that exhibit sufficient sensitivity for a given target. The same applies to recombinant antibodies that are limited by the low potential for diversity in the selection library. This can result in failure to generate a candidate with sufficient affinity, specificity or reactivity against certain targets.

Limitations of antibodies as biosensors – stability, size and orientation

Antibody biosensors are limited by the poor stability of the antibody under assay conditions, the size of the immobilised antibody, and by their orientation on the surface. As discussed previously, they are sensitive to high salt, high temperatures or extremes of pH. Their sheer bulk limits the number of capture molecules that can be immobilised in a given area *(pg.7, Figure 2C)*,[13] which impacts assay sensitivity and sample volumes required and can also cause increased background through non-specific binding.

More crucial is the control of orientation of the immobilised antibody. Immobilisation strategies include covalent and affinity-mediated immobilisation, but all carry disadvantages – covalent immobilisation can be random and inefficient, and can result in antibody loss and modifications to the antibody that reduce its binding affinity for the target. Affinity-immobilisation through the Fc domain on the antibody can be mediated by Protein A or Protein A Gold (PAG) or avidin-streptavidin interactions. Although the latter may result in a degree of controlled orientation, these steps all add complexity and time to the assay protocol, and are limited in success. None offers the ideal solution.[1]

Limitations of antibodies as therapeutics – immunogenicity, toxicity and 3D structure recognition

The limitations of antibodies as therapeutics are well documented.[3] Antibodies raised in animals and then injected into humans will be recognised as 'foreign' by the human immune system and cleared from circulation. This issue has been partially resolved by the production of 'humanised' chimeric antibodies that use the human antibody conserved regions, into which just the hyper-variable domain of the specific animal antibody is cloned and subsequently humanised as far as possible. However, this adds significantly to the complexity and speed of production.

For therapeutic antibodies used as direct modulators of protein function, it is often desirable to target the functional epitopes of a protein directly rather than just binding anywhere on the protein. Due to the size of many targets and problems of epitope dominance, antibodies often do not show the required level of resolution.[14]

At 150 kDa in size antibodies are large proteins. Except in the case of haematological cancers, the large size of therapeutic antibodies is a significant drawback, as they are often unable to penetrate the target tissue of solid tumours and dense extracellular matrix. This severely limits the therapeutic efficacy of such treatments, and is reflected in the proportionally small number of therapeutic antibodies developed that target solid tumours.[15, 16]

Antibody engineering: future directions

Much effort is being applied to recombinant antibodies in an effort to optimise stability, enhance pharmacokinetic properties and manufacturability and introduce multi-specificity, particularly for therapeutic applications. Variable regions containing the paratope can be engineered to improve epitope recognition specificity. Grafting of complementarity-determining regions (CDRs) onto human variable region sequences prevents immune recognition of the antibody by the human host. Recent strategies include efforts to isolate monoclonal antibodies from human memory B cells and plasma cells[17, 18] and the development of humanised mice, by injecting immunodeficient mice with human stem cells to generate human immune systems in these animals, for example, to generate tumour-specific antibodies.[19] Many other engineering approaches are under development to try to overcome key technical limitations of native antibodies, and to introduce application-specific performance attributes.[20]

Recombinant antibody engineering will undoubtedly produce many more success stories for antibodies as therapeutics. Even as this work progresses, so does the development of antibody alternatives that are viable contenders. These alternatives have been designed specifically to overcome many of the intrinsic weaknesses of antibodies, and they offer much simpler and more versatile architecture for manipulation and application.

Alternatives to antibodies

Engineered antibody fragments

The functional domains of antibodies can be cloned and produced on their own, for example, the Fab fragment *(pg.7, Figure 2)* which is the 'arm' of the antibody that contains the binding site, or that binding site itself, the single chain variable region fragment (scFv).

Advantages:

In comparison to whole antibodies, antibody fragments are:

- smaller in size
- generated using phage display libraries for more rapid selection and subsequent expression in plant, prokaryotic, yeast, mammalian or insect cells
- not susceptible to interference from non-specific Fc region binding

Limitations:

Antibody fragments are:

- hampered by low affinity for the target, especially if the clones are generated from naïve libraries —libraries not constructed in B cells donated from hosts immunised with the antigen[21]
- still susceptible to misfolding and aggregation if expressed in the periplasmic space in E.coli, which can result in poor expression efficiency, particularly with genetically fused multimers. Options to overcome this include cloning with a signal peptide to facilitate export from bacterial cells, or the re-folding of the protein *in vitro* to ensure the correct structure
- cleared very rapidly from the body via the kidneys because they lack the Fc region;[21] this can be restrictive for some applications where prolonged activity is required
- limited in epitope recognition — due to a planar binding interface, as in the case of whole antibody molecules, it is often not possible to raise antibody fragments against grooves, catalytic domains and other small 3D structures

Aptamers

Aptamers are short (usually 20 to 60 nucleotide) single-stranded DNA or RNA oligonucleotides that can in theory bind a range of targets with high affinity and specificity. Aptamers have been shown to bind nucleic acids, small compounds, proteins, protein complexes, and even entire cells. On paper, they offer strong benefits that should make them viable alternatives to antibodies. In practice, the generation, manipulation and application of aptamers has been more challenging than anticipated and the success stories fewer.[22]

Advantages:

In comparison to antibodies, aptamers are:

- small — typically 1/10th to 1/100th the size of an antibody
- non-immunogenic and non-toxic
- tolerant of wider pH and temperature ranges than proteins for storage — see *Limitations* right for a note on the effect of these variables on binding behaviour
- easier and more economical to identify using an *in vitro* screen of oligonucleotide pools — systemic evolution of ligands by exponential enrichment (SELEX) is an iterative, competitive selection process where aptamers are selected on the basis of their competitive affinity for a target molecule; multiple rounds increase specificity
- more economical for use in scaled manufacturing as they can be generated via basic chemical reactions rather than requiring expensive complex biological processes
- able to offer higher potential for randomisation than antibodies — depending on the size of the aptamer, the variable domains can be 30-50 nucleotides in length and each position can be occupied by any one of the four nucleotides
- easy to manipulate — variable regions are flanked by constant domains, which can be used to manipulate the variable region, by insertion of expression or fusion elements; this allows some tuning, for example of pharmacokinetic properties, by conjugation

Aptamer engineering – future enhancements

Current aptamer development work focuses on overcoming these limitations. A modified, high-fidelity or HiFi SELEX protocol with digital PCR amplification blocks retention of non-specific aptamers, reduces amplification artefacts and increases the diversity of the selection library.[23] Modifications have been introduced to the chemistry of the aptamers themselves for improved stability and enhanced function, for instance, in the creation of a nuclease-stabilised backbone and the incorporation of modifications into the bases that mimic amino acid side chains.[24]

These efforts are testament to the fact that aptamers show some potential as viable antibody alternatives, but not in their current format. Unfortunately, the improvements also come at a cost – modified backbones and nucleic acid chemistries add complexity, time and cost to production.

Limitations:

Aptamers are:

- prone to amplification artefacts which can reduce specificity
- hydrophobic and negatively charged — these unfavourable biophysical characteristics mean that oligonucleotides do not bind to acidic protein domains with ease or high affinity
- unstable *in vivo* — both DNA and RNA are susceptible to nuclease attack
- sensitive in their binding behaviour to altered salt, temperature, pH and other sample matrix components in the assay mix
- limited in their conformational repertoire — recent advances of the introduction of non-natural side chains that mimic amino acids have increased the number of possible conformations available with aptamers, by making them appear more protein-like

Non-antibody protein scaffolds

Protein scaffolds are small, stable and highly soluble protein structures that usually lack disulphide bonds and glycosylation sites. More than 45 different types have been introduced in recent years and 10 have already been tested in clinical trials.[25] The best are readily expressed in bacterial systems, because they have no issues with misfolding or aggregation. Sequence diversity is introduced by integrating short random peptide sequences into surface accessible loops to create a stable, diverse selection library for phage display selection. There are several available commercially, including Affibodies, DARPins, Nanofitins and Affimer scaffold proteins. Accurate differentiation/comparison between these is hampered by the fact that most data is proprietary and has been generated using differing experimental conditions,[16] but some differences can be observed *(Table 1)*.

Non-antibody scaffold binders are diverse in their origin and attributes. The lists to the right are generalised – not all features are common to all scaffold binders. Table 1 gives more detail on individual attributes.

Advantages:

In comparison to antibodies, non-antibody scaffold binders are:

- expressed in bacterial hosts, which reduces the initial identification and production time from months to weeks and cuts cost
- smaller in size (typically < 15 kDa) than antibodies or antibody fragments *(pg. 7, Figure 2B and 2C)*
- more stable than antibodies or antibody fragments across a wider range of assay conditions
- particularly suited to the generation of 'tuneable' multimers by genetic fusion or chemical ligation: multi-specific affinity agents, which can allow increased binding specificity, enhanced receptor blocking capacity, or specificity for two different biomolecules, in which case the second might enable effector cell recruitment or prolong serum half-life for therapeutic use
- structurally stable even when immobilised on a solid support
- amenable to precise orientation such that binding epitopes are available

Limitations:

Non-antibody scaffold binders:

- lack Fc mediated properties of antibodies such as long serum half-life and effector cell function which are useful in therapeutic applications; however counter strategies have been developed, including fusion to serum albumin for stability or half-life extension, or Fc fusions for effector functions as well as half-life extension[26]
- are, in most cases, proprietary to biopharmaceutical companies[16]
- display melting temperature / thermal stability that can be affected by the sequence of the recognition insert[27]
- typically require some form of *in vitro* affinity maturation to achieve very high affinity binding
- are limited in the degree of randomisation possible where insert sizes are small

Table 1:

Key features comparison for non-antibody protein scaffolds. Affimer binders are unique among scaffolds with a combination of proven stability, monomeric protein design without disulphide bridges or post translational modification and ease of production in *E. coli*.

	Template	Production	Tm	Size of variable region (amino acids)	Size of scaffold
Affibody®	Z domain of protein A	*E. coli* or peptide synthesis	42-71°C	13	5-6 kDa
DARPin®	Natural Ankyrin repeat proteins	*E. coli*, up to 200 mg/mL	66-89°C	18 (split across 3 conjugated DARPin units)	18-20 kDa
Nanobody®	Single-domain HC-only antibody fragments	*E. coli*, up to 150 mg/mL (post-translational modifications & disulphide bridges)	60-80°C	9-12	15 kDa
Affimer®	Human protein Stefin A and plant Cystatin	*E. coli*, up to 200 mg/mL	42 -100°C	18	12-14 kDa

Affimer binders: what's different?

There are currently two Affimer scaffolds; both use the cystatin protein fold, one is derived from the human protease inhibitor Stefin A[28] the second from consensus of plant Cystatin A proteins.[29]

The folded structure of both Affimer scaffolds are virtually identical *(Figure 4)* and the interaction with the target is mediated via two loops into which variable binding domains can be inserted, with target-specific binders being selected from a highly diverse library using phage display.

Affimer binders have specific advantages over antibodies, aptamers and over other non-antibody scaffold proteins:

- they are structurally stable, even when immobilised onto a solid support, and form dense monolayers on the surface[30, 31]
- their orientation can be controlled with precision, to ensure presentation of the correct paratope
- they lack post-translational modifications and cysteine residues, and are expressed efficiently in *E.coli*
- the size of the inserts in the loop regions is large in comparison to some other scaffold proteins, which may confer an advantage in diversity of the selection library
- they show superior thermal and chemical stability including over a wide pH range (pH 2-13)
- for biosensor assays they allow direct assay from unpurified samples such as urine, stomach acid and environmental samples[32]
- this is very beneficial for point of care, rapid biosensor assays
- Affimer binders are also stable for prolonged periods at room temperature, and at higher temperatures, which will facilitate shipment and storage to remote, point of care locations

Figure 4:

Structure of the Affimer scaffold protein, and key features that offer benefit over antibodies.

Affimers: advanced protein scaffold engineering integrates six key features essential for next generation affinity applications:

Small size, no modifications

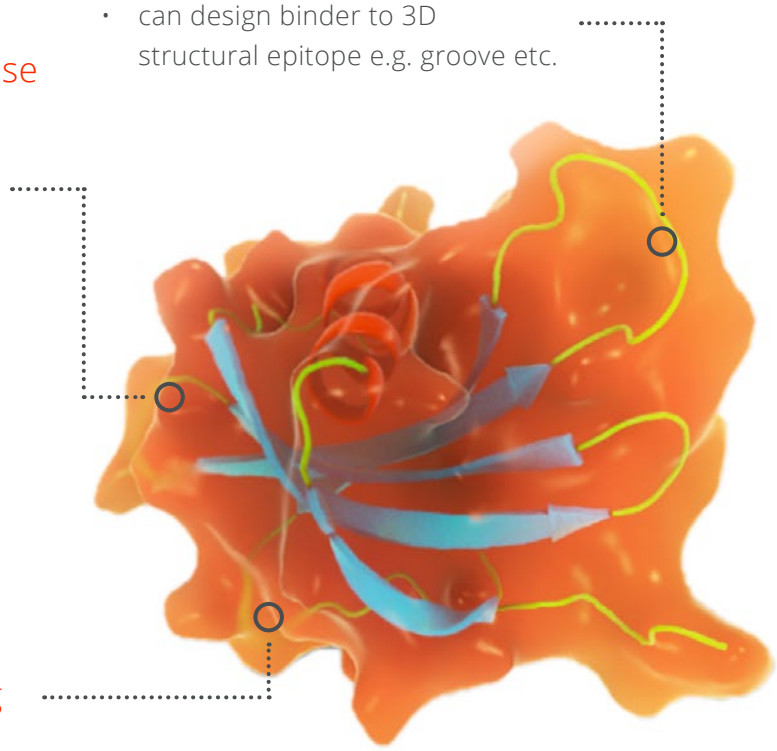
- can readily be selected by phage display
- does not aggregate in *E. coli*
- enhanced tissue penetration in therapeutic use
- can be multimerised for bispecificity

Two loops for versatile design

- insert sequences can be highly randomised, makes very diverse selection library
- suitable for ALL targets
- applicable to proteins, peptides, small molecules
- can design binder to 3D structural epitope e.g. groove etc.

Does not induce immune response or toxicity in humans

- stable under a wide range of salt concentrations, pH and temperatures
- can be immobilised on solid supports



Can easily form multimers

- bestows huge advantage in enhanced function of primary target interaction, or addition of a second specificity, for example to enhance *in vivo* half life

Structurally very stable, including under harsh assay conditions

- stable under a wide range of salt concentrations, pH and temperatures
- can be immobilised on solid supports
- no disulphide bridges

N terminus free

- allows for direct conjugation to reporter tag OR drug for targeted delivery

Affimer binders can be generated to almost any protein target, and show high discriminatory powers

Large (>6x1010), high diversity, *in vitro* Affimer phage display libraries were generated by incorporating random peptides into the scaffold's loop regions. These libraries can be screened in a three-or-four-round phage display process, to select individual Affimer binders that display the desired affinity and specificity for the target molecule.

The length of each of the two binding loop peptides is normally nine amino acids, but this can be varied to change the size or shape of the recognition surface without destabilising the scaffold, which is valuable when targeting protein pockets or other structural motifs. Affimer proteins can be generated to almost all protein targets, even in cases where antibodies fail. The specificity of target recognition is high. Affimer binders can distinguish between proteins that differ by only one amino acid [Avacta Application Note – Difficult Targets], between active and inactive conformations of the same protein and between a protein-ligand complex versus the protein or ligand individually.

In practice: Development of novel Affimer diagnostic reagents for Zika virus outbreak management

Affimer binders were generated which were capable of binding to a recombinant form of the Zika virus NS1 protein, which is a candidate diagnostic marker of early, acute infection.[33] Three Affimer binders were developed. They showed high specificity for the NS1 protein, and were able to distinguish it from NS1 proteins from closely related viruses: Dengue (all 4 major serotypes), Yellow Fever, West Nile and Japanese and Tick-borne Encephalitis. There are no antibodies available that can offer this level of specificity. The total time for production and characterisation was just 13 weeks from receipt of viral protein. Infectious disease outbreak and epidemic response requires speed. Affimer binders meet this need, and are ideal for front line outbreak detection and monitoring.

In practice: Affimer binders can be targeted to extracellular effector domains and allosteric regions of receptor proteins

Affimer proteins have been generated that bind to the extracellular domains of receptor proteins with sufficient affinity to completely block ligand binding.

Affimer binders can be developed to be very specific, for example, such that they don't bind homologous receptor proteins. They therefore show significant potential as direct therapeutics, and since they can identify allosteric regions of proteins, they could also help inform drug design strategy.[34]

In practice: The ability of Affimer proteins to form multimers and fusion proteins allows fine tuning of therapeutic activity and stability *in vivo*

Affimer binders are highly amenable to the formation of multimers. Dimer, trimer and tetramer Affimer constructs have been generated and retain high expression yields close to the monomer. Multimeric Affimer structures were generated against the human Programmed Cell-Death Ligand 1 (PD-L1)[35] which displayed an avidity effect showing that each sub-unit in the multimer retained the ability to engage target when presented as an Fc fusion. This, combined with the possibility of conjugating to drugs, peptides, or even antibodies, for fine tuning of therapeutic activity and half-life, offers broad potential in therapeutic applications.

Conclusion

As affinity assays become more sophisticated and the use of affinity binders for therapeutic applications increases, performance and production requirements become more stringent and the limitations of antibodies and antibody fragments become more pronounced.

The Affimer non-antibody protein scaffold offers significant advantages in terms of speed and cost of production, the diversity of targets that can be bound, the affinity and specificity of binding and inherent stability and 'tuneability' of the scaffold itself. Affimer binders offer superior performance that can help shape the future of medicine from *in vitro* discovery assays to therapeutic use and companion diagnostics *(pg.3, Figure 1)*.

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