life science research and diagnostic assays rely heavily on the use of antibodies as affinity reagents, yet significant drawbacks are still associated with their use in these in vitro contexts. Because of these drawbacks, a range of alternative affinity reagents are now entering the arena, offering scientists the opportunity to explore avenues hitherto closed to them. Where these new reagents have been specifically engineered for detection, they hold significant promise of improved research assays and, ultimately, an expanded range of diagnostic tests.

The problems with antibodies

Costs

The high costs of antibodies are linked to their complexity. Each molecule is composed of two heavy chain and two light chain proteins, intricately folded, covalently linked and post-translationally glycosylated. Such a complex molecule cannot be generated easily within microbial cells, requiring folding chaperones and specific glycosyltransferases, which are only naturally present in mammalian cells. These cell factories are expensive to culture and cannot be scaled up as easily or as cheaply as their microbial counterparts resulting in high costs for these everyday research tools. Moreover, as the identification and production of a target-specific antibody which binds with high affinity is a lengthy process, custom monoclonal antibodies typically take six months to a year to generate. This may result in significant delays in research timescales and discourage many scientists from branching into new areas where research antibodies are not readily available.

Availability

It was estimated that between 1950 and 2009, three-quarters of the published protein research focused on a mere 10% of proteins. Research antibodies to certain targets are not available, not because they haven’t yet been generated, but because they cannot be generated. The introduction of a target protein into an animal typically results in the animal recognising the protein as foreign and raising antibodies against it. These antibodies may then be captured and utilised by scientists to target the protein of interest in their experiments. However, if the target required is too toxic to be injected into the animal host as an
immunogen, the animal may die before antibodies can be raised. Similarly, if the required target protein is highly conserved across species, the animal's immune system may not recognise it as foreign, and the failure to elicit an immune response means no antibodies will be generated. If a target is a conformational or post-translational epitope it may be lost during antigen processing, again resulting in the failure to generate a specific antibody. In the case of rare antigens, it may be difficult to source enough target to provide an immunogenic dose to an animal host.

The case of the ubiquitin-mediated protein degradation system highlights the difficulties which may be encountered in antibody generation. Despite the obvious biological importance of the cellular pathway for cell signalling and protein degradation and its being a key target in drug development, only two antibodies have been generated to the highly related members of the family of ubiquityl chains in the last decade. These are difficult targets, but the dearth of available research antibodies is holding back understanding and innovation in this field.

Chemical stability
Problems don’t always end with the successful production of an antibody to the desired target. Cellular and molecular assays to examine the function and activity of different proteins often require conditions such as extremes of pH, which can cause the delicate structure of the antibody to be affected, resulting in a loss of function. The presence of structural elements such as the disulphide bridges that hold an antibody together precludes exciting applications such as live cell imaging in which the reducing nature of the cell interior would destroy antibodies.

References
Specificity
Despite the widely-held belief that antibodies are specific to only their target protein, many antibodies do cross-react with proteins other than their target, which can cause additional headaches for bench scientists.

Size
The large size of the antibody molecule means that the density with which they can be packed on a sensor surface is limited. Their large size also means that it is more difficult to get them across certain barriers which can be a problem in some detection assays and may also restrict the delivery of an antibody therapeutic. For cytosolic and nuclear proteins the cell membrane represents an initial barrier. In normal immunocytochemistry this is permeabilised through cellular fixation and the use of selected detergents, but in live cell imaging this is not a possibility as the procedure would kill the cells under investigation. Beyond this, even for surface proteins being studied in this manner, the addition of large bulky antibodies to a protein may alter their function and localisation. Antibody fragments, such as scFV and Fab fragments, have been utilised by many to overcome the problems of the large size of antibodies, and they can also be made into recombinant libraries, overcoming the limitations of relying on an animal host. However, as many of these molecules still rely on intra-domain disulphide bonds for stability, they are unstable in the cell interior, and expensive mammalian cells are again required for their production.

Alternative affinity reagents: what are they and do they work?
In view of the inherent limitations of antibodies, alternative affinity reagents are now emerging for use by life scientists, some in the form of nucleic acids (known as aptamers) and others that are protein based (including Affibodies, Affimers, Affilins and many more, which have sometimes been generically termed peptide aptamers or protein scaffolds). The key to success for an affinity reagent appears to be the ability to adopt a defined conformation that...
matches a binding surface on the target, allowing high affinity, highly specific interactions. Here we will describe some of the key technologies and provide evidence of their successes.

Nucleic acid aptamers

It has long been recognised that short, single-stranded RNA molecules can form 3D structures within the ribosome and in some mRNA molecules to selectively recognise and bind relevant metabolites9-11. Aptamers are made up of two constant sequences, used for PCR amplification, that flank an ‘insert’ sequence of varying length. The insert sequence is randomly varied giving rise to different possible conformations. Aptamer libraries are then screened using a technique called SELEX to identify molecules that can bind selectively to the desired target protein with affinities similar or in rare cases surpassing those achieved by good antibodies12,13.

Advantages of aptamers are the ease with which they can be chemically synthesised and modified with dyes, labels and surface attachment groups14. The original aptamers were developed as RNA-based molecules. Further chemical modification of these molecules has increased their resistance to degradation by nucleases and improved their storage stability. A common modification is the substitution of the 2’ hydroxyl group with a fluorine modification of the 2’OH to increase nuclease resistance, and cleavage of the molecular backbone15. An RNA aptamer therapeutic, Pegaptanib (Macugen), which utilises the fluorine modification of the 2’OH to increase nuclease resistance, was discovered by NeXstar Pharmaceuticals, later acquired by Gilead Sciences, and licensed for late stage development and marketing to OSI Pharmaceuticals within the US and Pfizer outside the US. It achieved FDA approval in 2004 for the treatment of neovascular age-related macular degeneration. However, this treatment was superseded by Ranibizumab, a monoclonal antibody-based therapy from Novartis in 2011. NOXXON Pharma is currently invested in the development of enantiomeric RNA aptamers, termed Spiegelmers, as therapeutics. NOXXON Pharma currently has a number of Spiegelmers in the pipeline including treatments for diabetic nephropathy, chronic lymphocytic leukaemia and multiple myeloma all in Phase II trials16.

Despite the advantages associated with nucleic acid aptamers, the limited conformational diversity of nucleic acid libraries remained a constraining factor in the success of nucleic acid aptamers. Scientists have worked to increase the diversity available by modifying the side chains of the nucleotides to include non-native, frequently hydrophobic functional groups resembling amino acid side chains, typically at positions oriented away from the hydrogen bonding face of the bases, such as the C-5 position of pyrimidines and the C-8 position of purines17. This strategy was used successfully in the generation of SOMAmeres and the SOMAscan platform, by SOMALogic. Since its

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drug discovery in 2000, SOMAlogics has raised $200 million to advance its technology for use primarily in diagnostics. It has recently described biomarkers for diseases including non-small cell lung cancer, mesothelioma and Alzheimer’s disease17.

**Protein scaffolds**

With 20 naturally occurring amino acids rather than four nucleotides, the diversity of interaction and conformation offered by protein structures is significantly larger than that of nucleic acids. This increases the probability of isolating an engineered protein affinity reagent that can bind with high affinity and specificity to the desired target. This idea is borne out by the data: libraries of 10^13 nucleic acid aptamers need to be screened (through as many as 20 rounds of sampling) to identify binders with nM KD, whereas this can be achieved in as few as three panning rounds of a phage display library of 10^10 of a protein scaffold. Considering these advantages over nucleic acid scaffolds, scientists are now turning to engineered protein scaffolds as a source of suitable affinity reagents in answer to the limitations of antibodies.

**Why use a scaffold protein?**

While short peptide sequences may possess an affinity for a target, their performance tends to be poor as they are able to adopt a range of conformations in solution. Constraining the peptide, or multiple peptides, using a large protein structure – the ‘scaffold’ protein – produces better affinities and specificities and begins to mimic the way that the variable peptide insert lengths to create specific binding sites to the target molecule. Much of this technology has been targeted at the development of therapeutics with many scaffold proteins now in the final phases of clinical trials or having been licensed for use in diagnosis or treatment.

**Do scaffold protein technologies work?**

Kalbitor is a 6kDa scaffold protein-based therapy, produced by the American biopharmaceutical company Dyax. Targeted to treat sudden angioedema attacks, it is based on the Kunitz domain of trypsin inhibitors and was licensed by the FDA for treatment in 2009. Approximately 10,000 people in the United States have hereditary angioedema. When brought to market Kalbitor was one of two approved therapies to treat attacks of this disorder and remains the only subcutaneous treatment for sudden attacks that targets and blocks plasma kallikrein.

Other promising protein scaffolds abound. Affibody has utilised the 6kDa Z domain of protein A as its scaffold for the presentation of peptides21,22. Its anti-HER2 Affibody is currently in use as an imaging agent in Phase II clinical trials and it has additional targets, such as IL-1 for the treatment of inflammation and autoimmune disorders, in its pipeline. Developed from a University of Zurich spin-out company, Molecular Partners has established its scaffold based on the ankyrin repeat motif. These designed ankyrin repeat peptides (DARPins) vary in molecular weight from 10 to 19kDa and have resulted in a pipeline including DARPins for VEGF which is due to start Phase III trials in the second quarter of 2015, HER2 and PDGF, which are currently in early stage clinical trials23. With collaborations to date including Johnson and Johnson, Roche and Allergan, Molecular Partners recently underwent an IPO raising $100 million to further expand and develop its protein therapeutics platform. Pieris AG is commercialising a further scaffold, based on lipocalin proteins and called Anticalins. These have the unusual property of being able both to bind to target proteins and to encapsulate small molecules, potentially leading to combined approaches to treatment. Pieris now has partnerships with Daiichi-Sankyo and Sanofi Group, all of which are at stages varying from discovery to pre-clinical.

**Next generation protein scaffolds?**

Spun out from the University of Leeds in 2012, the Affimer scaffold platform is based on cystatin proteins24-27 (Figure 1). Featuring all the desired characteristics of a scaffold including easy intracellular folding, a small size and a biochemically inert and biophysically stable (Tm=80°C) backbone, Affimers are produced by Avacta Life Sciences for use in research and diagnostics, with a longer-term goal of developing Affimers with therapeutic properties. Avacta Life Sciences’s near term focus on

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Proteomics

The reduced reliance on antibodies may offer new tools to researchers. Figure 3: Direct ELISA demonstrating the unique anti-diubiquitin K33 Affimer. Antibody vendors have failed to generate many antibodies to the difficult ubiquityl chain targets, but the reduced reliance on antibodies may offer new tools to researchers. Continued from page 21.

Affimers in drug discovery

Affimers contain no disulphides or post-translational modifications making them simple and cheap to manufacture with high yields. Importantly, this also makes them suitable for applications inside the reducing interior of a living human cell, whether as inhibitors of protein interactions or as probes for live cell imaging. The availability of a reagent that can both be used to study the protein in fixed cells or in biochemical assays and to probe its biology in living cells may yield a step change in the validation of candidate drug targets before chemical library screening even starts. Intra-cellular applications are particularly suitable, because Affimers also appear to be particularly adept at recognising conformational epitopes, which opens up the possibility of investigating conformations and interactions stabilised by post-translational modifications within cells. Finally, the stability of Affimers on surfaces and their small size (they are just 3nm in globular diameter) means that they pack tightly on surfaces improving sensitivity of assays (Figure 2). This has made possible the development of Affimer microarrays that can be used for the discovery of differentially expressed proteins in cell or tissue lysates. Any of these differentially expressed proteins may represent a new drug target or biomarker and the availability of Affimers to these proteins should allow researchers to determine whether changes in protein expression are a cause or a consequence of the disease they are studying.

A neat demonstration of the benefits of moving away from antibodies and the use of animals’ immune systems towards the use of in vivo screening and a protein scaffold such as an Affimer is that Avacta Life Sciences has already generated Affimers to several of the array of di-ubiquitin chains for which antibody vendors have failed to generate antibodies (Figure 3).

It is clear that a new generation of affinity reagents will drive an acceleration of discovery in the life sciences, with the ability to use the same tool for in vitro biochemical studies of a protein and to inhibit its function inside cells potentially leading to a step change in the efficiency of drug target discovery and validation. Together with their potential as new biological therapeutics, we can safely predict that there is a bright future ahead for non-antibody affinity reagents.

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