Modern affinity reagents: Recombinant antibodies and aptamers

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ABSTRACT

Affinity reagents are essential tools in both basic and applied research; however, there is a growing concern about the reproducibility of animal-derived monoclonal antibodies. The need for higher quality affinity reagents has prompted the development of methods that provide scientific, economic, and time-saving advantages and do not require the use of animals. This review describes two types of affinity reagents, recombinant antibodies and aptamers, which are non-animal technologies that can replace the use of animal-derived monoclonal antibodies. Recombinant antibodies are protein-based reagents, while aptamers are nucleic-acid-based. In light of the scientific advantages of these technologies, this review also discusses ways to gain momentum in the use of modern affinity reagents, including an update to the 1999 National Academy of Sciences monoclonal antibody production report and federal incentives for recombinant antibody and aptamer efforts. In the long-term, these efforts have the potential to improve the overall quality and decrease the cost of scientific research.

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Contents

1. Introduction

There is a strong desire within the scientific community to see an improvement in the reproducibility of biomedical research. Last year, Francis Collins M.D., Ph.D., Director of the United States National Institutes of Health (NIH), and Lawrence Tabak, D.D.S., Ph.D., Principal

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Deputy Director of the NIH, wrote that the NIH is concerned about the lack of reproducibility in biomedical research and shared actions the NIH was exploring to address this problem (Collins and Tabak, 2014). In response, a workshop organized by the NIH, Science, and Nature Publishing Group was convened to identify principles to increase reproducible, robust, and transparent research (McNutt, 2014). Among these principles is the recommendation to establish best practice guidelines for reporting on antibodies used in research, including the source, dilution used, and how the antibody was validated (NIH, 2015).

Similarly, there is a growing awareness within the scientific community of the need to improve the quality of commercial antibodies, which often show poor specificity or fail to recognize their targets. Recent publications cite documented evidence of the lack of quality and reproducibility of animal-derived antibodies and describe how their use has wasted tremendous amounts of money, time, and experimental samples (Baker, 2015; Bradbury and Plückthun, 2015). One study found that only 49% (2726 out of 5436) of commercial, animal-derived antibodies could be validated to recognize only their targets (Berglund et al., 2008). It has been estimated that half of the $1.6 billion spent worldwide on protein-binding reagents is used on unreliable antibodies and that these antibodies may be the laboratory tool most commonly contributing to irreproducible research (Baker, 2015; Bradbury and Plückthun, 2015).

Alternative affinity reagents offer increased quality, speed of production, and return on investments in research. The existence of aptamers and recombinant antibodies (rAbs), two much-discussed modern non-animal affinity reagents, makes the replacement of conventional animal-based monoclonal antibody (mAb) production methods an attractive and achievable goal. One of the impediments to the replacement of animal-derived antibodies has been that the research community is largely unaware of the benefits associated with rAb and aptamer technologies. This review aims to familiarize antibody users with the state-of-the-science of these non-animal-based methods, how rAbs and aptamers can be incorporated into protocols that require affinity reagents, and how to gain momentum in the transition to these reagents. Greater awareness of the technical advantages of these non-animal alternatives among academia, industry, regulators, and funding bodies will help to facilitate wider funding, development, and use.

1.1. Background on antibodies

In their native role as components of the adaptive immune system, antibodies—also called immunoglobulins (Ig)—are large, complex glycoproteins capable of binding substances, termed antigens, that may elicit a larger immune system response. Antibodies recognize small structural elements, or epitopes, on an antigen, thereby marking them for phagocytosis or other biological processes. Epitopes recognized by antibodies are typically short amino acid sequences within foreign proteins.

There are five mammalian antibody classes: IgA, IgD, IgE, IgG, and IgM. Antibodies belonging to the IgG class are the predominant immunoglobulin in human serum and the most important from a research perspective. They are generally represented as Y-shaped molecules consisting of two heavy and two light chains (Fig. 1). The shorter light chains interact with the N-terminus of the heavy chains to form the two "arms," or antigen-binding (Fab) domains, which are composed of both constant and variable regions. Six variable amino acid loops at the termini of the Fab domains, also called the complementarity determining regions (CDRs), are responsible for binding to the antigen (Kierny et al., 2012). The tail of the Y-shape, the Fc domain, mediates the antibody's interaction with macrophages and other cells expressing Fc receptors.

The ability of antibodies to precisely bind their target antigen is the principal characteristic making antibodies an irreplaceable component of the immune system and particularly useful in research
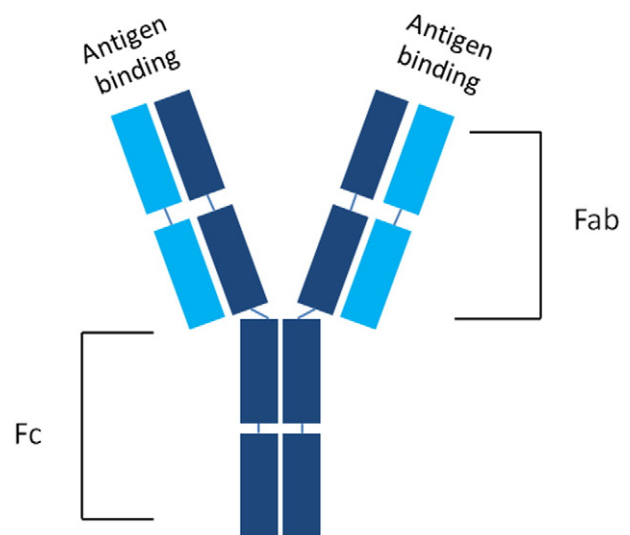

Fig. 1. General structure of an IgG antibody showing the heavy and light chains, the Fab, and Fc domains, and antigen binding sites.

applications. Both polyclonal (derived from multiple lines of antibody-producing cells) and monoclonal (derived from a single line of antibody-producing cells) antibodies are used in research. Monoclonal antibodies are defined by their capacity to selectively bind a single antigen.

1.2. Historical methods of monoclonal antibody discovery and production

Monoclonal antibodies are generated using either animal or recombinant DNA methods. Many technical advances have been made in mAb production technology in the four decades since Köhler and Milstein published their manuscript on hybridoma technology in 1975 (Köhler and Milstein, 1975). Their report describes the hybridization of antibody-producing B cells from the spleens of immunized mice with an immortal mouse myeloma tumor cell line, enabling the production of mouse mAbs for use as an investigational tool. The two general ways to discover and produce mAbs in animals, the ascites method and the "*in vitro*" method, share initial discovery steps. First, an animal (usually a mouse) is immunized with an antigen of interest. The mouse is often immunized multiple times over several weeks and, ultimately, killed to extract the spleen. Antibody-producing spleen cells from the mouse (immunocompetent B cells, which have a limited life span) are fused with immortalized myeloma tumor cells *in vitro* to produce a hybridoma. Hybridomas can be expanded in two ways: (1) by injection into the peritoneal cavity of a second mouse (called the *in vivo* ascites method) or (2) by culturing the hybridoma cells *in vitro* (called the "*in vitro*" method). While both methods use animals in the initial immunization step, the ascites method uses additional animals in procedures recognized to cause considerable pain and distress (Fig. 2) (Animal Welfare Division of OPRR, 1997; Marx et al., 1997; NRC, 1999). Historically, the ascites method produced more concentrated antibodies than the "*in vitro*" method without the need for expertise in cell culture methods; however, technological advancements have led to the "*in vitro*" production of more concentrated antibodies and non-animal affinity reagents (Hendriksen, 2006; Marx and Merz, 1995).

More specifically, ascites antibody production often involves injecting animals' abdominal linings with a priming solution (such as Pristane or Freund's Incomplete Adjuvant) to induce inflammation and interfere with drainage of peritoneal fluid. Priming is followed by injection of the hybridoma cell suspension. Hybridoma cells multiply and produce antibody-containing fluid, which accumulates in the abdominal cavities of the mice. As tumors grow, animals' abdomens distend as they fill with antibody-containing fluid;
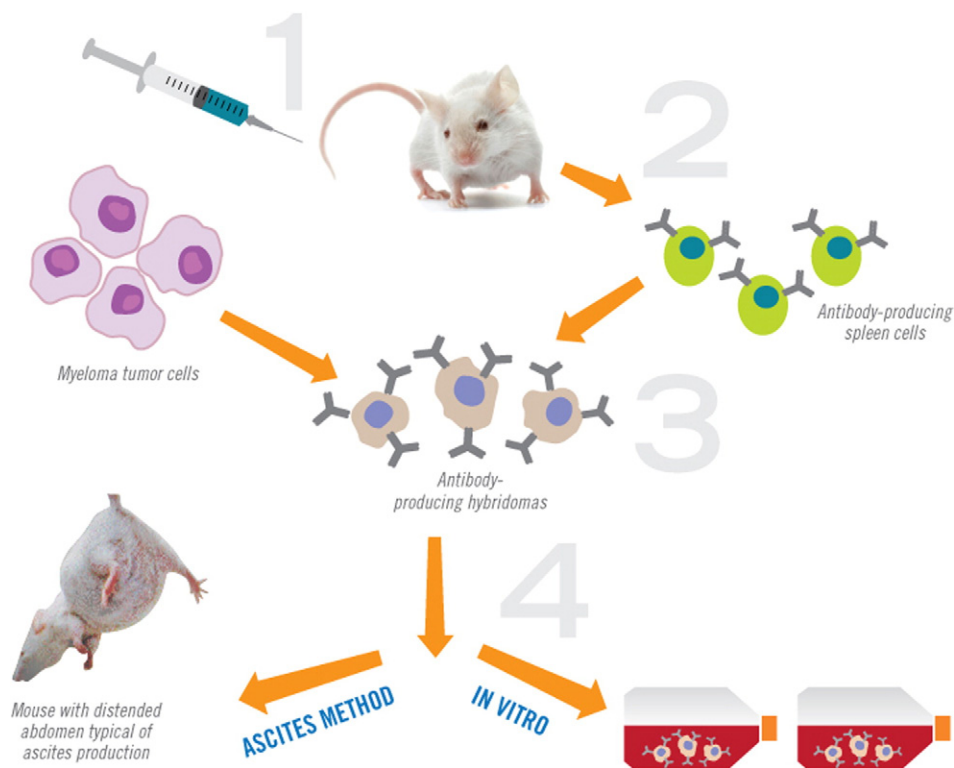
Fig. 2. Historical methods of monoclonal antibody production. Monoclonal antibodies have historically been produced using the ascites or "*in vitro*" methods. The methods' steps include 1) immunization of a mouse with the antigen; 2) isolation of antibody-producing cells from the mouse's spleen; 3) fusion of immortal myeloma cells with the antibody-producing spleen cells to make hybridomas; and 4) screening and selection of the desired antibody-producing hybridoma, followed by expansion of the hybridoma cells either in a mouse (ascites method) or *in vitro*.

animals have been reported to suffer from hunched posture, rough hair coat, reduced food consumption, emaciation, inactivity, difficulty in walking, and respiratory problems during this process—signs associated with pain and distress (Animal Welfare Division of OPRR, 1997; de Geus and Hendriksen, 1998; Hendriksen and de Leeuw, 1998; Howard and Kaser, 2013; Jackson et al., 1999; Marx et al., 1997; McGuill and Rowan, 1989; NIH Office of Animal Care and Use, 1996; NRC, 1999; OECD, 2002; Peterson, 2000). The fluid is typically extracted one to three times before the animal is killed, and the extraction procedure can lead to circulatory shock, as indicated by pale eyes, ears, and muzzle as well as breathing difficulties (Howard and Kaser, 2013; Jackson et al., 1999; NIH Office of Animal Care and Use, 1996; Peterson, 2000). Without intervention, an animal will die within two to four weeks after suffering from weight loss, muscle atrophy, dehydration, and complications associated with the tumor (NRC, 1999).

Overall, the "*in vitro*" hybridoma method is a refinement that uses fewer animals than the ascites method because animal use is not involved in the production phase. However, it still requires animal use in the discovery phase and, consequently, presents animal welfare concerns. In addition to animal welfare issues, these animal-based methods pose scientific issues, including the inability of an animal to mount an immune response to antigens that are highly conserved orthologs; the risk of contamination with viruses, rodent plasma proteins, bioreactive cytokines, murine immunoglobulins, and other contaminants; the destruction of antigens that are particularly labile in animal systems; and the inability to develop antibodies against toxic antigens that kill the animals prior to antibody production (Frenzel et al., 2014; Geyer et al., 2012; Hairul Bahara et al., 2013; Marx et al., 1997; McArdle, 1997; Scholler, 2012; Thiviyanathan and Gorenstein, 2012). Additionally, there are issues with the reproducibility of animal-derived antibodies (Baker, 2015; Bradbury and Plückthun, 2015; Marx et al., 1997). In 2015, 111 academic and industry scientists called for an international shift to the use of recombinant antibodies for reasons that include

increased reliability and reduced lot-to-lot variability in affinity reagents (Bradbury and Plückthun, 2015).

Hybridoma-derived antibodies are time-consuming to create and often cause immune reactions in humans, limiting their usefulness as clinical therapies (Geyer et al., 2012). There is also an issue of practicality; in this era of proteomics, it is not feasible to use such animal-intensive and lengthy processes to create antibodies for the growing number of available variations of human proteins and their animal orthologs.

2. Recombinant antibodies

Recombinant antibodies are produced *in vitro* and can be used for the same purposes as mAbs produced *in vivo*. Recombinant antibodies are selected from libraries of genes encoding slightly different antibody proteins for their affinity to bind to target antigens. In 1985, George Smith demonstrated that foreign proteins could be expressed on the surface of a bacteriophage, a virus that infects *Escherichia coli* (*E. coli*), by fusing the gene of the peptide to be displayed with the gene of the minor phage coat protein, pIII (Smith, 1985). In 1990, McCafferty and colleagues showed that variable domains of antibodies could be displayed in the same way (McCafferty et al., 1990). In the 25 years since this discovery, researchers have made great strides in optimizing the specificity and affinity achieved with this technology.

Various rAb display platforms have been developed that present the antibody on the surface of phages, mammalian cells, yeast, bacteria, insect cells, or ribosomes (Harel Inbar and Benhar, 2012). Phage display in bacteria is one of the oldest and most commonly used techniques (Even-Desrumeaux and Chames, 2012; Frenzel et al., 2014). Phage display involves the generation of an antibody library, selection of specific antibodies, and affinity maturation, as discussed below (and shown in Fig. 3) (Even-Desrumeaux and Chames, 2012; Frenzel et al., 2014).

2.1. Construction of recombinant antibody libraries and phage display

There are four main types of libraries that differ by the source of the sequences used to build the library: immune, naïve, synthetic, and semi-synthetic (Geyer et al., 2012; Harel Inbar and Benhar, 2012). Immune antibody libraries are generated using active B cells from an immunized human donor or animal, usually a mouse, and consist of more than 10^{10} different antibody clones (Lloyd et al., 2009; Rader, 2012). A new immune library must be generated for each antigen of interest (Geyer et al., 2012). Naïve antibody libraries are generated using resting B cells from healthy, non-immunized humans and have been reported to consist of up to 10^{11} clones (Lloyd et al., 2009; Thie et al., 2009).

Synthetic or semi-synthetic libraries contain either exclusively manmade CDRs or both natural and artificial CDRs, respectively. Because the region on the antibody that binds to the antigen (the CDR) is manmade, synthetic libraries are not limited to natural CDRs existing in animals. Examples of synthetic libraries are MorphoSys' Human Combinatorial Antibody Library (HuCAL) (available through Bio-Rad AbD Serotec) and AxioMx's library, each of which consists of more than 10^{10} antibody clones (AxioMx, 2015; Prassler et al., 2011; Ylera, 2010).

In phage display, libraries consist of antibody gene fragments presented on phage particles. Because the full length IgG cannot be displayed on the phage surface, Fab fragments or single chain Fv (scFv) are often used (Frenzel et al., 2014). The complete antigen-binding site is retained; therefore, these fragments still have high affinity for their targets and can be modified to increase their affinity (Donzeau and Knappik, 2007). For some downstream applications, the Fab or scFv fragment is preferable over the full length IgG (e.g., to decrease nonspecific binding or interference from other parts of the molecule and in applications where their smaller size may be advantageous) (Donzeau and Knappik, 2007; Holliger and Hudson, 2005; Kierny et al., 2012).

2.2. Panning

There are multiple variations to the panning process. In general, the antigen of interest is immobilized on solid supports, such as magnetic beads, immunotubes, or microtiter plates (Kotlan and Glassy, 2009). The antibody library is then incubated with the immobilized antigen (Hairul Bahara et al., 2013). During this process, the selection conditions can be precisely controlled by presenting specific conformations of the target antigen or by including competitors to direct selection toward epitopes of interest. This is useful, for example, in the generation of phospho-specific antibodies, those specific to the ubiquitin-bound or guanosine triphosphate (GTP)-bound form of proteins, and those that specifically recognize post-translational modifications (Eisenhardt et al., 2007; Gao et al., 2009; Hoffhines et al., 2006; Kehoe et al., 2006; Nizak et al., 2003; Nizak et al., 2005). Furthermore, because integral membrane proteins need a membrane or detergent environment to maintain their native conformation, conformation-specific rAbs can be selected for in the presence of a detergent, which is not possible *in vivo* due to the denaturing effect of the serum (Rothlisberger et al., 2004). Following incubation, nonspecific antibodies are washed away, and specific antibodies are eluted and amplified by infection in a bacterial host. This selection processes, called panning, generally occurs several times (Kierny et al., 2012). With each iteration, the pool of selected antibodies becomes enriched (Coomber, 2001).

2.3. Cloning, selection, and screening

After panning, the resulting antibody DNA is cloned into expression vectors, transformed into *E. coli*, and then plated on agar plates (Kotlan and Glassy, 2009). Colonies—each of which represents one mAb—are picked and grown. Antibody molecules are screened for specificity and sequenced to identify unique antibodies, which are then expressed and purified.
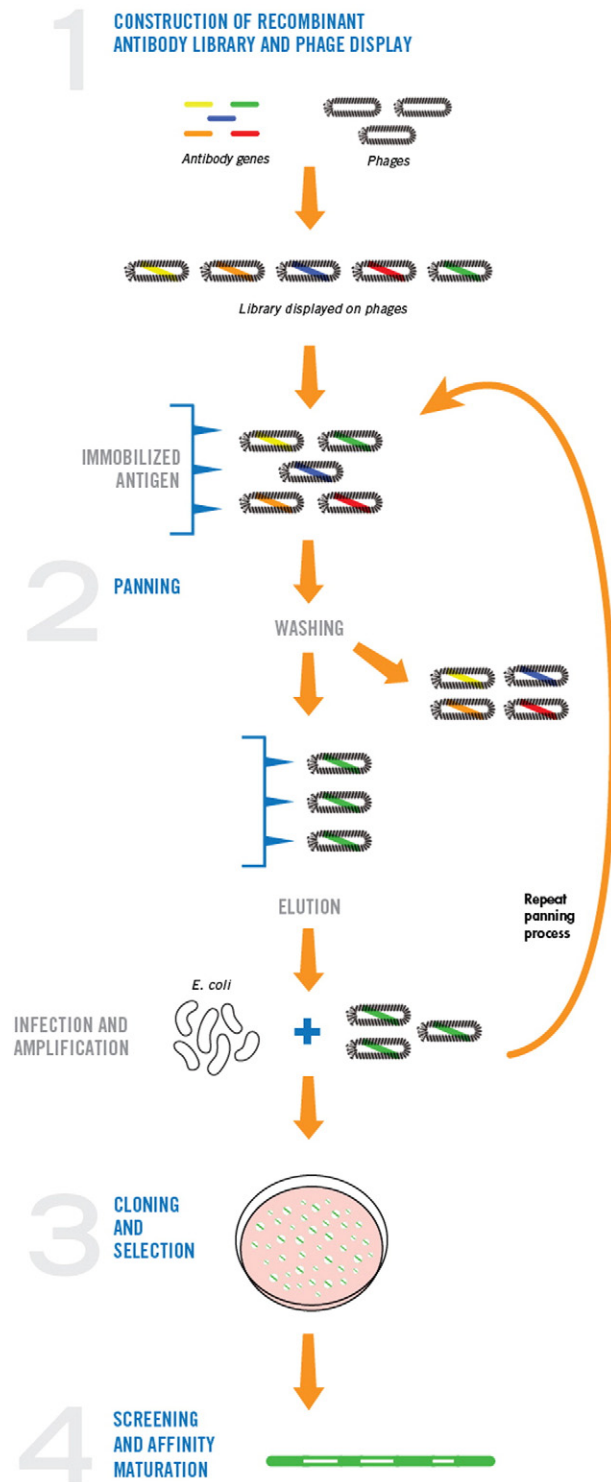
Fig. 3. Recombinant antibody generation in phage. Recombinant antibody generation consists of: 1) antibody genes are fused with a gene of the phage coat protein, causing the phage to display the antibody protein on its surface. 2) Panning involves a) selecting an antibody of interest by combining the antibody library with an antigen immobilized on a solid support; b) washing antibody-antigen complexes to remove nonspecific or low affinity antigens/phage; and c) eluting the bound phage and infecting *E. coli* cells to amplify selected clones. The panning process is generally repeated several times. 3) the antibody-DNA-containing expression vector transformed into *E. coli* is plated on agar plates. Colonies representing individual mAbs are picked and grown. 4) antibody molecules are screened for specificity and sequenced to identify unique antibodies, which are then expressed and purified. Sequence diversity is introduced and antibody clones are evaluated for improved affinity.

2.4. Affinity maturation

Antibody affinity describes the strength of the interaction between an epitope on an antigen and one antigen-binding site on an antibody. In animals, affinity maturation is the process in which B cells produce antibodies with increasing ability to bind an antigen. The nature of the B cell response in animals, including the incubation time of the antigen with the B cell surface antibody, somatic hypermutation, and the presentation of the antigen to T cells, limits the affinity of animal-derived antibodies (Foote and Eisen, 2000). Because these biological constraints do not exist for rAbs, they can reach affinities higher than that of natural antibodies (Foote and Eisen, 1995, 2000; Geyer et al., 2012).

In general, larger antibody libraries can be used to reach higher affinities. Recombinant DNA-derived antibodies reach high antigen affinity through the introduction of sequence diversity into an antibody by either targeted or non-targeted methods. Non-targeted techniques, such as error-prone PCR and DNA shuffling, introduce diversity randomly into the whole antibody, which can lead to the introduction of deleterious mutations that compromise antibody stability. Targeted techniques, such as site-directed mutagenesis or CDR exchange and combination, allow the mutations to be directed within the CDR, the region most likely to increase antibody affinity without introducing deleterious mutations (Geyer et al., 2012; Ponsel et al., 2011; Steidl et al., 2008). Following the in vitro affinity maturation process, antibody clones are evaluated for improved affinity, and antibody affinity in the picomolar to femtomolar range can be obtained (Boder et al., 2000; Hanes et al., 2000; Kierny et al., 2012; Lee et al., 2004; Razai et al., 2005; Schier et al., 1996). Advantages of recombinant antibody technology and relevant considerations for its use are noted in Tables 1 and 2, respectively.

3. Aptamers

Aptamers are similar to antibodies in that they can bind to proteins and modulate their function, and they are referred to as chemical antibodies due to their synthetic production (Ahmadvand et al., 2011; Bouchard et al., 2010). Aptamers are short, single-stranded DNA or RNA oligonucleotides that can bind to their targets with high specificity and affinity through van der Waals forces, hydrogen bonding, salt bridges, and hydrophobic and other electrostatic interactions. Aptamers have the ability to fold into complex and stable three-dimensional shapes, which allows them to fold within or around their targets. DNA aptamers have greater chemical stability, while RNA aptamers produce more structural shapes due to their greater flexibility (Radom et al., 2013; Toh et al., 2015). Aptamers have been identified against targets that include small organic and inorganic molecules, such as dyes, nucleotides, amino acids, and drugs; biopolymers, such as peptides, proteins, and polysaccharides; ions; phospholipids; nucleic acids; viruses; bacteria; cell fragments; and whole cells (Kong and Byun, 2013; Li et al., 2011; Nezlin, 2014; Ni et al., 2011; Pei et al., 2014; Thiviyanathan and Gorenstein, 2012; Zhu et al., 2012, 2014).

Aptamers can be used to detect and characterize their targets and modify the activity of their targets. First reported in 1990, aptamers were conceptualized independently by three groups of researchers, Ellington and Szostak (1990), Robertson and Joyce (1990), and Tuerk and Gold (1990). Tuerk and Gold named the production process the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Fig. 4).

3.1. SELEX

The process of screening large nucleic acid pools to develop aptamers, SELEX, has been optimized since its development to meet a variety of needs (Radom et al., 2013; Tan et al., 2011). Mimicking natural selection, SELEX involves screening pools of random-sequence nucleic acid libraries for oligonucleotides that bind a particular target (Citartan et al., 2012; Radom et al., 2013). Automated DNA synthesizers prepare the libraries of up to approximately 10^{17} random sequence oligonucleotides using equimolar mixtures of nucleotide bases during generation of the random regions (Zaher and Unrau, 2005).

Table 1
Advantages of recombinant antibody technology.

High affinity: The in vitro affinity maturation process allows for the production of antibodies with affinities in the picomolar to femtomolar range. Antibody properties, including affinity, can be controlled using methods such as increasing the number of panning iterations, adjusting washing stringency, presenting different selection conditions, and adjusting the amount and presentation of antigens (Donzeau and Knappik, 2007).

High specificity: Altering antigens during panning iterations can select for cross-reactive rAbs and negative selection techniques (competition with related molecules) can be used to generate highly specific antibodies that can differentiate between similar targets (Ayriss et al., 2007; Mersmann et al., 2010; Parsons et al., 1996; Pershad et al., 2010; Donzeau and Knappik, 2007). For example, antibodies have been generated that recognize only fetal and not adult hemoglobin (Geyer et al., 2012).

Variety of targets: Recombinant methods allow for the generation of antibodies against tissue samples, whole cells, small molecules, specific protein conformations, integral membrane proteins, RNA, post-translational modifications, and complexes, such as biotinylated major histocompatibility complex/peptide complexes (Geyer et al., 2012; Yau et al., 2003; ten Haaf et al., 2015; Lev et al., 2002; Pavoni et al., 2014; Moutel et al., 2009).

Independent of a biological immune response: Unlike animal methods, recombinant techniques allow for the generation of antibodies to unstable, toxic, volatile, immunosuppressant, or non-immunogenic antigens (Chen and Sidhu, 2014; Frenzel et al., 2014; Hairul Bahara et al., 2013; Thie et al., 2009). For example, rAbs have been developed to target West Nile virus and Botulinum neurotoxins (Scholler, 2012). In addition, phage display technology requires less antigen to isolate an rAb than is needed to immunize an animal (Barkhordarian et al., 2006; Harper and Ziegler, 1999).

Reduced immunogenicity: Recombinant antibodies developed from human-derived libraries elicit a reduced immune response compared to animal-derived antibodies when used in therapeutic applications. This is especially relevant for the treatment of chronic diseases that require repeated doses over long periods of time (Rader, 2012).

Precisely controlled selection conditions: Antibody selection conditions, such as the presence of salts, buffers, detergents, or a specific pH, can be controlled (Kierny et al., 2012; Thie et al., 2009). Control over selection parameters in the panning process has resulted in the ability to create antibodies with high affinity against proteins that have very similar sequences (Hairul Bahara et al., 2013).

Known sequence: Because the amino acid sequence of the antibody is known, its generation is reproducible, recombinant analogs of existing antibodies can be created, and modifications can be inserted into an antibody's sequence, such as to increase its affinity or modify biochemical properties (Altshuler et al., 2010; Bradbury and Plückthun, 2015). For example, rAbs have been conjugated with peptide tags and enzymes (Donzeau and Knappik, 2007). In addition, because the DNA sequence encoding the antibody is known, modifications such as biotinylation, multimerization, and the addition of epitope tags are easy to incorporate using simple cloning procedures (Casey et al., 2000).

Versatility: Recombinant antibody technology allows for the generation of smaller antibody fragments, such as monovalent or bivalent Fab or the complete immunoglobulin format, depending on downstream applications. Because the sequence of a rAb is known, fragments can be converted into and switched between any of the antibody classes using simple cloning techniques (Dozier, 2010).

Faster production: The phage display selection process is largely automated. Once an antibody library is established, it takes approximately two to eight weeks to produce a rAb, in contrast to the four or more months in animals (Dozier, 2010; Geyer et al., 2012; Ylera, 2010). Recombinant antibodies also are compatible with high-throughput methods (Chen and Sidhu, 2014; Hairul Bahara et al., 2013; Kotlan and Glassy, 2009).

Dependability: Unlike hybridoma cell lines, recombinant antibody supplies are not at risk of dying off. Because rAb's sequences are known, they can be reproduced by DNA synthesis if necessary. Additionally, standardized rAb production results in low lot-to-lot variability (Bradbury and Plückthun, 2015).

Existing infrastructure: Recombinant antibodies can leverage the structural and intellectual infrastructure already in place for antibodies.

Animal welfare: There are no animal welfare issues with the production of rAbs (e.g., no animals are used, unless immune antibody libraries are derived from animals).

Table 2
Considerations of recombinant antibody technology.

Fragment conversion: For some applications, such as many therapeutic uses, researchers will need to convert the antibody fragment selected during phage display into the full length immunoglobulin form by gene engineering (Hu et al., 2010).

Construction of rAb libraries: Immune libraries may be derived from humans or animals; however, the animal-derived libraries present scientific disadvantages, such as the production of antibodies that may need to be humanized to avoid side effects if administered to humans (Clementi et al., 2012).

Intellectual property: Intellectual property (IP) rights and the specific technological skills needed to create rAbs have slowed their adoption and are still impediments to rAb development (Echko and Dozier, 2010; Storz, 2011). IP issues may include patents providing exclusive rights to sell an antibody, which may be defined by its target, its functional properties, an epitope of a target, its sequence, or other attributes; patents for new indications for an already existing antibody; patents for antibodies used in conjunction with another agent; and patents for antibody formats, such as fragments or rearranged components of antibodies (Storz, 2011). However, development processes are now more routine, and development kits can be purchased. In addition, as more universities have started working with rAbs, more libraries have become available in the public domain (Shoemaker, 2005).

Cost: Cost has been a barrier to the adoption of rAbs. Production costs for rAbs are similar to those for animal-derived antibodies and should decrease over time (Bradbury and Plückthun, 2015). Animal-derived antibodies have been produced for decades; therefore, the technology and knowledge are widespread, and many antibodies do not have to be custom-developed. Currently, fewer rAbs have been developed and are available for production, making the initial investment in a new, custom-made rAb, as well as fit-for-purpose validation, costly. In the short-term, developing new technologies may be more expensive, but the price should decline because more universities and companies are becoming involved in rAb development. Additionally, the potential cost-savings associated with the more reproducible research that will result from using higher quality antibodies should be considered.

Larger libraries allow for greater sequence diversity and increased ability to develop high-specificity and high-affinity aptamers. Library oligonucleotides have a central randomized sequence of approximately 30 to 80 bases with defined terminal binding sites on each end for capturing and enzymatic manipulation. An immobilized target is incubated with the library, and the oligonucleotides that bind to the target are isolated, eluted, and amplified. This is accomplished by filter assay, chromatography, electrophoresis, or another separation method followed by polymerase chain reaction (PCR) for DNA or reverse transcription PCR (RT-PCR) for RNA (Ahmadvand et al., 2011; Bouchard et al., 2010; Bunka et al., 2010; Kong and Byun, 2013; Ni et al., 2011; Radom et al., 2013; Sundaram et al., 2013).

The cycle of isolation, elution, and amplification is repeated with increased stringency in order to select for oligonucleotides with greater sensitivity and/or specificity; this usually requires seven to fifteen iterations with increasing binding affinities (Bouchard et al., 2010; Bunka et al., 2010; Toh et al., 2015). In addition, selection parameters may be modified to screen for aptamers that meet particular conditions (e.g., pH, temperature, and buffer composition) (Radom et al., 2013). The pool of oligonucleotides that bind the target is then cloned, sequenced, and tested for target binding ability.

Finally, aptamer candidates are characterized and modified for their anticipated use. For example, aptamer modification can increase retention time in the body by imparting resistance to nuclease degradation or can incorporate a variety of functional groups for the purpose of conjugation with drug molecules, antibodies, fluorescent tags, or nanoparticles for diagnostic and therapeutic applications (Ahmadvand et al., 2011; Bouchard et al., 2010; Dassie and Giangrande, 2013; Kong and Byun, 2013; Nezlin, 2014; Šmuc et al., 2013; Sundaram et al., 2013).

There are a variety of modifications of the SELEX method, including negative-SELEX and counter-SELEX (Darmostuk et al., 2015). In negative-SELEX, oligonucleotides are incubated with the matrix used to immobilize the target before incubation with the actual target in order to reduce nonspecific binding (Aquino-Jarquin and Toscano-Garibay, 2011; Lakhin et al., 2013). In counter-SELEX, oligonucleotides are incubated with a target analog or with a range of structurally-similar but undesirable molecules before the actual target, thereby allowing for the removal of these potential interfering targets (Aquino-Jarquin and Toscano-Garibay, 2011). Other modifications, which reduce the time required for aptamer selection, include bead-based and microfluidic approaches. In the bead-based method, the oligonucleotide library is synthesized on noncleavable beads, and aptamers are identified in one step. In the microfluidic approach, researchers are able to select and characterize aptamers to multiple targets in days or weeks by carrying out the selection process on microfluidic chips (Dassie and Giangrande, 2013; Thiviyanathan and Gorenstein, 2012; Xu et al., 2010).
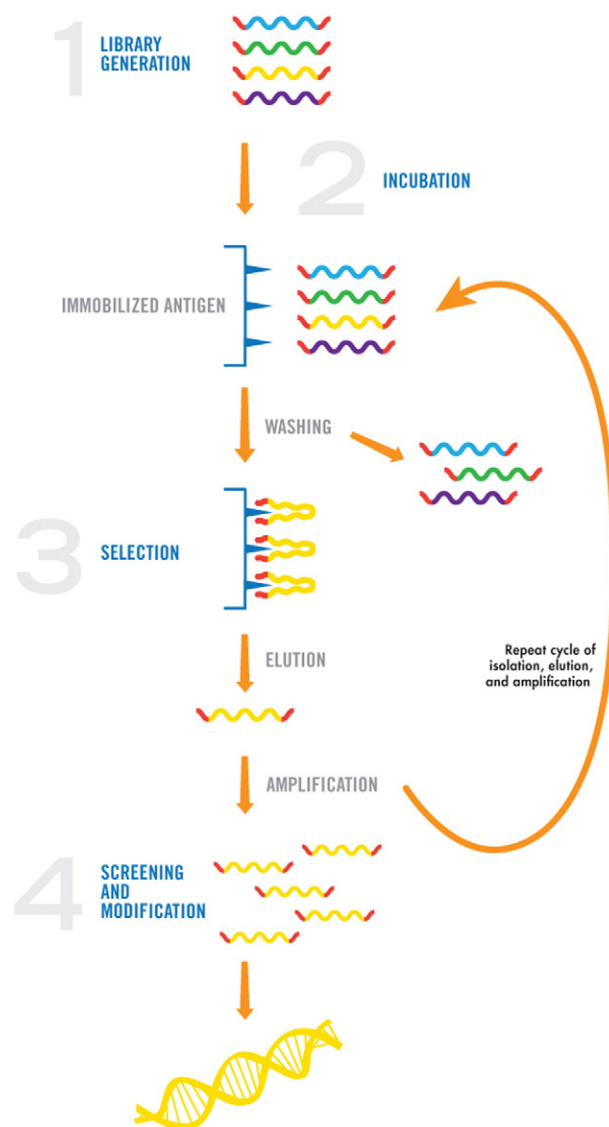
Fig. 4. Basic concept of SELEX, of which there are various modifications. SELEX involves: 1) random sequence oligonucleotides are synthesized with a central randomized region and terminal binding sites. 2) an immobilized target is incubated with the oligonucleotide library. 3) oligonucleotides that bind to the target are isolated, eluted, and amplified. This cycle is repeated until oligonucleotides with high binding affinity are selected for. 4) oligonucleotides that bind the target are cloned, sequenced, verified for binding ability, and modified for their intended use.

Another optimized process, Cell-SELEX, uses living cells to select for aptamers without prior knowledge of the molecular properties of the target. In this method, oligonucleotides are incubated with target cells, such as cancer cells. Oligonucleotides that bind with the target cells are selected, cloned, and sequenced in order to identify aptamers. The Cell-SELEX method has the ability to discover biomarkers of disease and novel cell-surface targets and to profile the molecular characteristics of target cells (Pei et al., 2014; Ray et al., 2013; Zhu et al., 2012, 2014). Due to their ability to distinguish between diseased and healthy cells, aptamers are a promising avenue for cancer treatment and personalized medicine (Sundaram et al., 2013). Aptamers have been selected for by Cell-SELEX for several types of cancer cells, including lymphocytic leukemia, myeloid leukemia, liver cancer, small and nonsmall-cell lung cancer cells, and hepatocellular carcinoma (Pei et al., 2014; Tan et al., 2011; Zhu et al., 2014). See Table 3 for advantages of aptamer technology and Table 4 for considerations of this technology.

4. Applications of recombinant antibodies and aptamers

Monoclonal antibodies are used extensively in basic biomedical research, in diagnosis of disease, and in treatment of illnesses. Recombinant antibodies and aptamers can be used in the same applications as mAbs produced in animals, including in basic research, regulatory testing, and clinical applications.

4.1. Basic research

In basic research, rAbs and aptamers can be conjugated with peptide tags, proteins, and nanoparticles to give them fluorescent properties to identify and detect the concentration of molecules, biological compounds, viruses, residues in food, and diseased cells (Esposito et al., 2014; Geyer et al., 2012; Hairul Bahara et al., 2013; Kierny et al., 2012; Pei et al., 2014; Penner, 2012; Zhu et al., 2012). They can be used in commonly-performed assays, such as immunofluorescence microscopy, microarrays, immunocytochemistry, immunohistochemistry, flow cytometry, ELISA, and blotting assays (Cho et al., 2011; Deng et al., 2014; Hairul Bahara et al., 2013; Martin et al., 2013; Ramos et al., 2007, 2010; Schirrmann et al., 2011; Shin et al., 2010; Simmons et al., 2012; Toh et al., 2015; Webber et al., 2014; Zeng et al., 2010).

One of the most common basic research uses of antibodies is in the detection of proteins by Western blotting. The routine complications associated with Western blotting—including nonspecific binding that results in either cross reactivity with similar proteins or a lack of detectable signal compared to noise—have been extensively reported (Baker, 2015; Bradbury and Plückthun, 2015). The use of rAbs and aptamers overcomes many of the hurdles associated with the use of animal-derived antibodies. When using rAbs, it is important to remember that, if a Fab format is used instead of an immunoglobulin format, the secondary antibody cannot be directed against the Fc domain that is lacking in Fab antibodies. Instead, an anti-human Fab secondary antibody or antibodies against appropriate epitope tags can be used. Alternately, some companies have developed methods that allow for the direct detection of rAbs in the absence of a secondary antibody, for example by conjugation to a fluorescent dye (Ylera, 2010). Conjugation of an rAb or aptamer directly to a reporter unit such as a dye allows proteins to be identified in much of the same way as a traditional antibody, while allowing for greater specificity and sensitivity (Shin et al., 2010; Strehlitz et al., 2008). Multiple laboratories have reported protein blotting results using rAbs or aptamers to be comparable or superior to blots probed with traditional antibodies (Cho et al., 2011; Lazzarotto et al., 1997; Martin et al., 2013; Ramos et al., 2007, 2010; Shin et al., 2010; Webber et al., 2014). Others have published on the advantages, such as faster turn-around time. For example, Shin et al. (2010) detected their protein of interest via Western blot following a shorter incubation time (2 h versus overnight incubation) with an aptamer than with the animal-derived antibody. This same lab has developed an aptamer-based biochip technology that can be used for specific and sensitive detection and quantification of proteins (Lee and Hah, 2012).

4.2. Regulatory testing

The properties of rAbs and aptamers are ideal to aid safety and efficacy testing for regulatory purposes. For example, rAb technology has been used to develop an assay to determine vaccine potency, and a study conducted by the U.S. Food and Drug Administration (FDA) demonstrated that aptamers can be used during the quality control testing of therapeutic proteins by detecting small differences

Table 3
Advantages of aptamer technology.

High affinity and specificity: Aptamers can have high affinity and binding specificity for their targets and recognize both intracellular and extracellular targets (Amaya-González et al., 2013; Citartan et al., 2012; Esposito et al., 2014; Ni et al., 2011; Pei et al., 2014). They are able to discriminate between highly similar molecules, and specificity can be adjusted by sequence modifications (Banerjee and Nilsen-Hamilton, 2013; Toh et al., 2015). In addition, because small changes in aptamer sequence can alter target specificity or the range of targets recognized, closely related aptamers can distinguish between similar targets (Gopinath et al., 2006).

Small size: At about one-tenth the molecular weight of antibodies, aptamers have the ability to access targets, such as viruses and other pathogens that escape recognition by the immune system (Bunka et al., 2010; Thiviyanathan and Gorenstein, 2012). They are also able to rapidly penetrate tissues (Tan et al., 2011; Zhu et al., 2012).

Flexible structures: Their flexible structures permit aptamers to bind to hidden epitopes, which antibodies are unable to reach (Pei et al., 2014; Zhu et al., 2014).

Modifications: Aptamers have the ability to be easily modified to chemically conjugate with other molecules, such as imaging labels and siRNAs, and obtain new properties from the addition of functional groups (Amaya-González et al., 2013; Gold et al., 2010; Kong and Byun, 2013; Ni et al., 2011; Zhang et al., 2011; Zhu et al., 2014). Modifications include optimizing the aptamer's recognition sequence or secondary structure, and changing the binding reaction conditions. Modifications can be introduced at any position in the nucleotide chain (Thiviyanathan and Gorenstein, 2012).

Stable: Aptamers are stable in harsh chemical and physical conditions, such as at high temperatures (Šmuc et al., 2013; Thiviyanathan and Gorenstein, 2012; Zhang et al., 2011).

Reproducible: Aptamer technologies allow for easy access to underlying gene sequence information for characterization and optimization of binding and functional characteristics (Bannantine et al., 2007; Ramos et al., 2007; Shin et al., 2010).

Independent of a biological immune response: Aptamers can be developed to bind to highly toxic or non-immunogenic antigens, unlike animal immunization technologies (Ahmadvand et al., 2011; Pei et al., 2014; Zhu et al., 2012, 2014).

Reversible: In human therapeutic applications, the addition of reverse complementary oligonucleotides can reverse an aptamer's action or allow for controlled drug release and, thus, minimize side effects (Sundaram et al., 2013). In addition, aptamers have the potential to reduce side effects through targeted treatment or targeted delivery of therapies, such as siRNAs, chemotherapeutics, toxins, and nanoparticles (Dassie and Giangrande, 2013; Esposito et al., 2014).

Repeat use: Denaturation of aptamers is reversible and, thus, aptamers can be repeatedly used under certain conditions (Šmuc et al., 2013; Toh et al., 2015). For example, heat, concentrated salt solutions, acidic or basic solutions, surfactants, and other methods can be used to release an antigen from an aptamer; the aptamer can then be refolded into a functional configuration (Toh et al., 2015; Yue et al., 2013).

Reliable: Because aptamers do not rely on biological processes, batch to batch variation is limited (Kong and Byun, 2013; Zhang et al., 2011).

Storage: Aptamers have the ability to be dehydrated and stored for years (Pendergrast et al., 2005).

Faster production: Aptamers are synthesized and modified *in vitro* and are relatively quick and inexpensive to produce compared to animal-based antibodies, allowing for large-scale production (Amaya-González et al., 2013; Nezlin, 2014; Noma et al., 2006; Zhu et al., 2014). Aptamers can be synthesized within days (Baird, 2010).

Animal welfare: There are no animal welfare issues with the production of aptamers.

Table 4
Considerations of aptamer technology.

Library synthesis: Large oligonucleotide libraries must be synthesized or purchased prior to aptamer development (Darmostuk et al., 2015).

Small size: Because of their small size, aptamers are subject to faster renal filtration and nuclease degradation than antibodies (Lakhin et al., 2013; Taylor et al., 2014). Therefore, in therapeutic applications, aptamers are modified either during or after production to increase their half-lives, for example, by chemical modification of the phosphate backbone, sugars, or the bases or end-capping at the 3′ or 5′ termini (Bunka et al., 2010; De Souza et al., 2009; Nezlin, 2014; Thiviyanathan and Gorenstein, 2012; Zhu et al., 2014). Other modifications can be made to stabilize aptamer structures and to prolong aptamer circulation times. Modifications must be tested to ensure that they do not affect the aptamer's activity (Kong and Byun, 2013; Ni et al., 2011).

Limited functional groups: Consisting of a sugar phosphate backbone and four bases, DNA and RNA have a limited number of functional groups; therefore, it can be difficult to develop aptamers for some targets (Taylor et al., 2014). For example, because it can be difficult to select an aptamer targeted to very acidic proteins, functional groups must be added to the oligonucleotide bases in these circumstances (Thiviyanathan and Gorenstein, 2012).

Aptamer selection: The identification process for aptamers that bind to a target is not as well developed as it is for antibodies. Often, the ligand-binding domain of the selected aptamers does not involve all the nucleotides in the random region. Amplification of small concentrations of short molecules can result in artifacts, and the immobilization of the target onto resin can lead to the identification of aptamers for components of the resin if appropriate measures are not taken to counter this (Penner, 2012). This is an area requiring more research.

High malleability: The high malleability of aptamers' structures in response to the local environment can decrease their affinity for targets in solution and *in vivo*. This is a key issue since the environment in which the aptamer undergoes the SELEX process may influence its utility. However, aptamer structure has been shown to be a function of the ionic environment, and knowledge of the environment in which the aptamer will be employed allows aptamer specificity and affinity to be better controlled (Ilgu et al., 2013).

Hybridization and nonspecific binding: Hybridization of the random region in aptamers with the primer regions, which are adjacent to the central random domain and function to amplify the target-bound sequences via PCR, can alter the aptamer structure, and, therefore, its affinity for a target. Primer regions and/or regions distal to the ligand-binding domain of the aptamer may also cause nonspecific binding or compromise affinity (Alsager et al., 2015). Modifications of standard SELEX protocols have been implemented to address these issues (Pan and Clawson, 2009; Radom et al., 2013).

Modifications: Traditional SELEX does not optimally select for aptamers that recognize cell surface receptors in their native state, undergo cellular uptake, and release in the cytoplasm of target cells. For example, a hurdle to using aptamers for siRNA therapeutics is cytoplasmic delivery into cells. These hurdles can be overcome by variations of the SELEX method and modifications of the aptamers (Dassie and Giangrande, 2013).

Pharmacokinetic profile: In therapeutic applications, the pharmacokinetic profile, toxicity, and other properties of aptamers upon systemic delivery are poorly understood (Zhang et al., 2011).

Intellectual property: Aptamers may be subject to intellectual property patents (Keefe et al., 2010). However, core patents that apply broadly to aptamers and aptamer discovery and development expired recently or will be expiring in the next couple of years (Archemix Corp., 2008).

Protocol and reagent availability: While antibody assay protocols and reagents are widely available in laboratories, a large part of the scientific community is not yet familiar with aptamer protocols and secondary reagents.

among protein products that went undetected by animal-based mAbs. Additionally, the U.K.'s Department of Environment, Food, and Rural Affairs (DEFRA) conducted a project to develop aptamers for rabies batch potency testing with the aim of replacing mice used to develop antibodies for the detection of contaminants in vaccine preparations (Department for Environment Food and Rural Affairs; Weidanz, 2014; Zichel et al., 2012).

Similarly, aptamers and rAbs can replace the use of animal-derived antibodies to detect environmental contaminants. For example, researchers at the University of Illinois and the University of Waterloo developed an aptamer-based colorimetric sensor test kit to detect an analyte in an aqueous test sample (Lu and Liu, 2013).

4.3. Clinical applications

4.3.1. Imaging

Aptamers' small size, reduced immunogenicity relative to animal-derived antibodies, and rapid diffusion and clearance make them ideal for imaging (Ni et al., 2011; Toh et al., 2015). Clinical applications for rAbs and aptamer-based imaging agents are widespread, including for treatment monitoring and cancer detection (Hong et al., 2011). For example, aptamers targeting cancer biomarkers can be conjugated with nanomaterials for tumor imaging (Lao et al., 2015). Due to the specificity and binding affinity of aptamers to their targets, cancer cells can be detected at low levels (Pei et al., 2014).

4.3.2. Therapeutics

Recombinant antibodies and aptamers have been used in therapeutic applications to alter target activity, for example, by binding to cell surface receptors, or by delivery of therapeutic agents to target cells via conjugation to antibiotics, RNA interference, toxins, enzymes, or drugs (Ahmadvand et al., 2011; Bunka et al., 2010; Dassie and Giangrande, 2013; Esposito et al., 2014; Nezlin, 2014; Pei et al., 2014; Sundaram et al., 2013; Zhu et al., 2012). Aptamer and rAb development methods in which cells (instead of purified proteins) are used as targets are useful for developing aptamers and antibodies for drug delivery to targeted

cells (Geyer et al., 2012). Aptamers also have the ability to deliver agents that are otherwise impermeable to cells because they can target internal cell surface receptors in some circumstances (Xiao et al., 2008). Recombinant antibodies and aptamers are potential tools to treat a range of conditions, including the following:

- *Autoimmune conditions*: For example, an RNA aptamer has been developed that suppresses activity of sphingolipid S1P, a signaling molecule implicated in autoimmune conditions (Purschke et al., 2014).
- *Toxins*: Recombinant antibodies have been developed to neutralize toxins, such as botulinum neurotoxin A, staphylococcal enterotoxin B, and snake venom (Hu et al., 2010; Unkauf et al., 2015).
- *Chronic diseases*: Aptamers have been shown to have the ability to modify T-cell reactions (Nezlin, 2014).
- *Diseases caused by pathogens*: Includes human immunodeficiency virus (HIV), hepatitis B and C viruses, influenza virus, and many others (Feng et al., 2011; Gao et al., 2014; Wandtke et al., 2015; Zhu et al., 2012). Using immune libraries created from B cells isolated from donors, phage display has been used to create rAbs to block the entry of influenza into cells (Geyer et al., 2012). Aptamers are small and have a flexible structure and, therefore, are more likely to penetrate into a viral particle than antibodies. In one case, an aptamer was reported to prevent viral entry and replication in cultured human cells by up to 10,000 fold by binding a core region of the HIV viral protein (Banerjee and Nilsen-Hamilton, 2013).
- *Cancer*: Aptamers have been developed to target the extracellular domain of transmembrane receptors over-expressed in tumors and can potentially deliver therapeutic agents to targeted tissues. Both aptamers and rAbs can be conjugated to imaging probes, siRNAs, and therapeutics to be delivered specifically to the cancer cells, thereby increasing treatment efficacy and decreasing side effects (Esposito et al., 2014).

Currently marketed rAbs can be used to treat a variety of diseases. For example, ecallantide is used for the treatment of acute attacks of hereditary angioedema, and belimumab is used to treat the

autoimmune disease systemic lupus erythematosus (Center for Drug Evaluation and Research, 2011; Seymour, 2009). Recombinant antibodies have been produced for the treatment of other diseases, such as cancer or autoimmune conditions (e.g., necitumumab and ramucirumab), and the neutralization of toxins (e.g., raxibacumab for the treatment of inhalation anthrax) (Center for Drug Evaluation and Research, 2012; Nixon et al., 2014).

The aptamer drug pegaptanib was approved by the FDA in 2004 for the treatment of macular degeneration (NDA 021756). Other aptamers are currently being assessed in clinical trials, and aptamers for hundreds of targets have been discovered (Darmostuk et al., 2015; Dassie and Giangrande, 2013; Ni et al., 2011). As seen in the wide range of ongoing clinical trials, aptamers can be used to treat a variety of conditions from vision loss to cancer (NIH Office of Animal Care and Use, 1996).

5. Discussion

Recombinant antibodies and aptamers are two types of affinity reagents that can replace animal-derived mAbs and offer a range of technological advantages. From a scientific perspective, it is clear that there is a strong need for more reliable, specific, and versatile affinity reagents (Baker, 2015; Bradbury and Plückthun, 2015; NIH, 2015). Recombinant antibodies and aptamers meet that need; they can be sequenced and reproduced, selected for in controlled conditions, modified to fill a specific purpose, bind a variety of targets with high affinity, and offer financial and time-saving benefits over animal-derived mAbs.

Animal welfare principles provide further impetus to the use of rAbs and aptamers because they stress replacement, reduction, and refinement of the use of animals by seeking, considering, and implementing modern alternatives to the use of animals. Hundreds of thousands of animals are used in the production of affinity reagents every year. The number of animals used in antibody production in the U.S. is unavailable because the numbers of mice and rats used in testing are not publically available, and, for species whose numbers are available, the manner in which they are used is not reported. However, in 2013 alone, 9522 animals were documented to be used in the production of monoclonal or polyclonal antibodies in Great Britain (Home Office, 2014). Thus, in addition to meeting a scientific need, the development and further use of rAbs and aptamers satisfies a recognized ethical need.

5.1. Antibody regulations in the European Union and the United States

In general, two European laws regulate the use of animals in research: (1) Council Directive 2010/63/EU (formerly 86/609/EEC) and (2) the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, ETS 123 (Council of Europe, 2006; European Parliament, 2010). These laws require that

Table 5
Names and websites for companies offering commercially available (pre-made and custom) rAbs and aptamers. Note that this list is not comprehensive, and a number of the companies listed sell animal-derived antibodies in addition to rAbs.

Company Name	Website
Catalog recombinant antibodies	
Bio-Rad AbD Serotec (host HuCAL)	http://www.abdserotec.com/monoclonal-antibodies.html
Absolute Antibody	http://absoluteantibody.com/catalog/
Creative Diagnostics (including Creative BioMart)	Diagnostics: http://www.creative-diagnostics.com/CommonTypeList_185.htm; BioMart: http://www.creativebiomart.net/Recombinant-Antibodies_140.htm
RayBiotech, Inc.	http://www.raybiotech.com/products/reagents/antibodies/
Toronto Recombinant Antibody Centre	http://trac.utoronto.ca/index.html
University of Geneva	https://www.unige.ch/medecine/phym/en/antibodies/see-our-antibodies/
Custom recombinant antibodies	
Bio-Rad AbD Serotec	http://www.abdserotec.com/hucal-monoclonals.html
Absolute Antibody	http://absoluteantibody.com/custom-services/
Abgent	http://www.abgent.com/ELITE-Services-for-Drug-Discovery
Avantgen	http://avantgen.com/partner.html
AxioMx	http://axiomxinc.com/services/custom-antibodies/
Toronto Recombinant Antibody Centre	http://trac.utoronto.ca/index.html
University of Geneva	https://www.unige.ch/medecine/phym/en/antibodies/order-a-new-recombinant-antibody/
Catalog aptamers	
Amsbio	http://www.amsbio.com/aptamers.aspx
Aptagen	http://www.aptagen.com/aptamer-index/aptamer-list.aspx
AptSci	http://www.aptsci.com/product/product.html
Base Pair Biotechnologies	http://www.basepairbio.com/project-phases-2/existing-aptamers/
CD Genomics	http://www.cd-genomics.com/Aptamers-list-40.html
Creative Biogene	http://www.creative-biogene.com/Product/Aptamers-list-40.html
GeneLink	http://www.genelink.com/newsite/products/modoligosINFO.asp
OTC Biotech	http://www.otcbiotech.com/aptamers.php
SomaLogic	http://estore.somalogic.com/Category/2_1/Reagents.aspx
Custom aptamers	
AM Biotech	http://am-biotech.com/services/
Aptagen	http://www.aptagen.com/research-and-development/project-inquiry.aspx
AptaMatrix	http://www.aptamatrix.com/company/
AptSci	http://www.aptsci.com/pro/pro_1.html
Aptasol	http://www.aptamergroup.co.uk/Solutions/Services/Ordering
Base Pair Biotechnologies	http://www.basepairbio.com/services/
CD Genomics	http://www.cd-genomics.com/Aptamers-Service.html?gclid=Cj0KEQiA8rilBRDZu_G8hszXraoBEiQABlB9Y3BfTCCzFZ8S1eyKp6gD-OO4xgkAuEKfydJB4uJ0YOQaAmZ68P8HAQ
Creative Biogene	http://www.creative-biogene.com/Services/Aptamers
GeneLink	http://www.genelink.com/newsite/products/aptamers.asp
LC Sciences	http://www.lcsciences.com/applications/genomics/dna-rna-aptamer-arrays/ordering-information/
NeoVentures Biotechnology	http://neoventures.ca/custom-aptamer-identification/
SomaLogic	http://www.somalogic.com/Products-Services/SOMAmer-Discovery-Service.aspx
TriLink	http://www.trilinkbiotech.com/aptamers/aptamersynthesis.asp

alternatives to animals be used when "reasonably and practically available."

Furthermore, the use of the ascites method specifically has been discouraged by regulatory and oversight bodies for decades due to the pain and distress it causes the animals used in the process. In fact, a report from a 1996 European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) organized workshop stated that the ascites method should not be used and suggested a two-year transition period to phase it out (Marx et al., 1997). A number of countries, such as Australia, The Netherlands, the United Kingdom, Germany, Switzerland, and Canada, have restricted or banned the production of antibodies via the ascites method due to animal welfare concerns (Canadian Council on Animal Care, 2002; Hendriksen, 2006; National Health and Medical Research Council, 2008).

As far back as 1997, the NIH Office of Laboratory Animal Welfare encouraged the use of "*in vitro*" methods for producing mAbs as opposed to the use of the ascites method (Animal Welfare Division of OPRR, 1997). In April 1997 and March 1998, the American Anti-Vivisection Society (AAVS) petitioned the NIH to prohibit the use of animals in the production of mAbs (NRC, 1999). In response, the NIH commissioned a study from the National Research Council (NRC) to determine whether there is a scientific necessity for producing mAbs using the mouse method. Completed in 1999, the NRC report called for the replacement of ascites-derived mAbs with "*in vitro*" methods wherever possible (NRC, 1999). At that time, the NRC determined that the use of ascites was only scientifically justified in three to five percent of proposed uses. Information about rAbs and aptamers was not included in the original NRC study. In the more than 15 years since this report was published, significant advances have been made in rAb and aptamer technology, highlighting the need for an updated version of this NIH guidance.

5.2. Recommendations

Multiple paths forward have the potential to assist researchers in the transition from animal-derived antibodies to non-animal sources, such as the following:

- In light of tremendous scientific advancements since the publication of the NRC report *Monoclonal Antibody Production* in 1999, NIH should commission an updated review that includes the merits of non-animal-derived antibodies and other affinity reagents (NRC, 1999).
- Researchers should familiarize themselves with and use non-animal research methods such as those offered by aptamer and rAb technologies whenever possible and, when using antibodies, should search for existing rAbs from companies, such as those listed in Table 5.
- Federal funding incentives should be made available for researchers interested in the development, use, and fit-for-purpose validation of rAbs or aptamers.
- Universities should provide monetary assistance to researchers who are interested in using rAbs but do not have the funds to purchase custom-made antibodies.
- A consortium of industry, academia, government, and nongovernmental organizations should fund the development of commonly used affinity reagents to be made available to all researchers.
- For industry, the use of rAb and aptamer technologies for regulatory testing purposes must be considered. In 1999, the NRC reported that the FDA estimated that it would cost between two and ten million dollars to prove equivalence of an "*in vitro*" antibody to a mAb previously produced via the ascites method (NRC, 1999). While fit-for-purpose validation is likely less expensive today, seemingly small procedural changes can lead to significant costs for companies conducting regulatory testing, and incentives should be implemented so that companies are not penalized for switching to a more sophisticated and humane technology.
- To help rAb and aptamer developers identify key areas of interest, a list of ascites-produced antibodies purchased by researchers and the justification for using the ascites method of production should be made publicly available. This will allow developers of alternative technologies to focus efforts on the development of these antibodies via recombinant methods.
- To take advantage of resources already spent on developing and validating existing hybridoma monoclonal antibodies, existing hybridomas should be sequenced and produced using recombinant technology moving forward (Bradbury and Plückthun, 2015).

As outlined, once technological knowledge and the supporting infrastructure are established, rAbs and aptamers require less time to produce, require less purified antigen, and can be created against a larger number of targets than mAbs (Petrenko and Vodyanoy, 2003). These technologies are the way forward to increased scientific validity and reproducibility and to accelerated research in the life sciences.

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