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Review

Research and development of next generation of antibody-based therapeutics

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Monoclonal antibodies (mAb) are emerging as one of the major class of therapeutic agents in the treatment of many human diseases, in particular in cancer and immunological disorders. To date, 28 mAb have been approved by the United States Food and Drug Administration for clinical applications. In addition, several hundreds of mAb are being developed clinically by many biotech and pharmaceutical companies for various disease indications. Many challenges still remain, however, and the full potential of therapeutic antibodies has yet to be realized. With the advancement of antibody engineering technologies and our further understanding of disease biology as well as antibody mechanism of action, many classes of novel antibody formats or antibody derived molecules are emerging as promising new generation therapeutics. These new antibody formats or molecules are carefully designed and engineered to acquire special features, such as improved pharmacokinetics, increased selectivity, and enhanced efficacy. These new agents may have the potential to revolutionize both our thinking and practice in the efforts to research and develop next generation antibody-based therapeutics.

Keywords: antibody therapeutics; antibody design; antibody engineering; novel antibody format; next generation of antibody; antibody efficacy enhancement

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Introduction

One of the major milestones in the history of antibody research and development was the invention of hybridoma technology to create monoclonal antibodies (mAb) in 1975 by Georges Kohler and Cesar Milstein[1], who were awarded a Nobel Prize in 1984. In 1986, OKT3, the first antibody derived from mouse hybridoma, was approved by the United States Food and Drug Administration (FDA) for use in patients to prevent transplant rejections. The mouse hybridoma derived antibody, however, can be recognized by the human immune system as foreign and induce human anti-mouse antibody (HAMA) response, resulting in short half-life, reduced efficacy, and in some cases increased toxicity in patients. To this end, various antibody discovery and engineering technologies have been developed in order to reduce the immunogenicity of mouse antibody: for example, antibody chimerization[2] and humanization[3, 4], using recombinant DNA technology were created in 1980s, by replacing portions of murine antibody with the human counterparts. Further, technologies to generate fully human antibodies, such as phage display libraries[5] and transgenic mice[6–8], were established in early 1990's. The first chimeric and the first humanized antibodies were approved by the FDA for human use in 1993 and 1997, respectively. Since 2002, seven fully human antibodies generated from phage display and transgenic mice have been approved for therapy applications. These genetically engineered antibodies have proven to be much less immunogenic in patients across various disease indications[9]. Today, a total of 28 therapeutic antibodies have been approved by the FDA for marketing in the United States (see Table 1). In addition, four other mAb are available for human use in non-US markets. Worldwide sales of therapeutic mAb have risen dramatically in recent years from about $4.0 billion in 2001 to over $30 billion in 2008. The market of therapeutic mAb represents the fastest growing sector in the pharmaceutical industry.

The major focus of antibody engineering technology development in the past three decades has been to reduce immunogenicity of murine antibody and to improve manufacturability. As more therapeutic mAb make their way through research and clinical development, the field has clearly shifted from murine towards humanized and fully human products (see Table 1). Technology advancement in protein production, both in microbial and mammalian cell systems, has enabled us to produce therapeutic antibodies at a level that is

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Table 1. Monoclonal antibodies approved for therapeutic use.

Generic name	Trade name	Antibody format	Antigen	Approved indication	FDA approval	EMEA approval	Sponsor
Muromomab	Orthoclone	murine IgG2a	CD3	Allograft rejection in allogeneic renal transplantation	6/19/86	NA	Ortho Biotech Inc
Abciximab	Reopro	chimeric Fab	GPIIb/IIIa receptor	Prevention of cardiac ischemic complications	12/16/93	NA	Centocor
Rituximab	Rituxan	chimeric IgG1κ	CD20	Non-Hodgkin's Lymphoma, chronic lymphocytic leukemia and rheumatoid arthritis	11/26/97	6/2/98	Genentech and Biogen Idec
Daclizumab	Zenapax	humanized IgG1κ	IL-2Rα	Prophylaxis of acute organ rejection in renal transplants	12/10/97	2/26/99	Hoffman-La Roche
Basiliximab	Simulect	chimeric IgG1κ	IL-2Rα	Prophylaxis of acute organ rejection in renal transplantation	5/12/98	10/9/98	Novartis
Palivizumab	Synagis	humanized IgG1κ	RSV F protein	Respiratory syncytial virus infection	6/19/98	8/13/99	Medimmune
Infliximab	Remicade	chimeric IgG1κ	TNFα	Crohn's disease and rheumatoid arthritis *etc*	8/24/98	8/13/99	Centocor
Trastuzumab	Herceptin	humanized IgG1κ	Her2	Breast cancer	9/25/98	8/28/00	Genentech
Gemtuzumab Ozogamicin	Mylotarg[1]	calicheamicin-humanized IgG4κ	CD33	Acute myeloid leukemia	5/17/00	NA	Wyeth/Pfizer
Alemtuzumab	Campath	humanized IgG1κ	CD52	B-cell chronic lymphocytic leukemia	5/7/01	7/6/01	Ilex/Genzyme
Ibritumomab Tiuxetan	Zevalin	Y^{90}-murine IgG1κ	CD20	B-cell non-Hodgkin's lymphoma	2/19/02	1/16/04	Biogen Idec/Spectrum
Adalimumab	Humira	human IgG1κ	TNFα	Rheumatoid arthritis and Crohn's disease *etc*	12/31/02	9/1/03	Abbott
Omalizumab	Xolair	humanized IgG1κ	IgE	Moderate to severe persistent asthma	6/20/03	10/25/05	Genentech
Tositumomab	Bexxar	I^{131}-murine IgG2aλ	CD20	Non-Hodgkin's lymphoma	6/27/03	NA	Corixa/GSK
Efalizumab	Raptiva[2]	Humanized IgG1κ	CD11a	Moderate to severe plaque psoriasis	10/27/03	9/20/04	Genentech
Cetuximab	Erbitux	chimeric IgG1κ	EGFR	Head and Neck cancer, colorectal cancer	2/12/04	6/29/04	ImClone/BMS/ Merck kGa
Bevacizumab	Avastin	humanized IgG1κ	VEGF-A	Various solid tumors	2/26/04	1/12/05	Genentech
Natalizumab	Tysabri	humanized IgG4κ	α4-integrin	Multiple sclerosis and Crohn's disease	11/23/04	6/27/06	Biogen Idec/Elan
Ranibizumab	Lucentis	humanized Fab	VEGF-A	Age-related macular degeneration	6/30/06	1/22/07	Genentech
Panitumumab	Vectibix	human IgG2κ	EGFR	Metastatic colorectal carcinoma	9/27/06	12/19/07	Amgen
Eculizumab	Soliris	humanized IgG2/4κ	C5	Paroxysmal nocturnal hemoglobinuria	3/16/07	6/20/07	Alexion
Certolizumab Pegol	Cimzia	Peglated humanized Fab	TNFα	Crohn's disease and rheumatoid arthritis	4/22/08	10/1/09	UCB, Inc
Golimumab	Simponi	human IgG1κ	TNFα	Rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis	4/24/09	10/1/09	Centocor Ortho Biotech
Canakinumab	Ilaris	human IgG1κ	IL-1β	Cryopyrin-associated periodic syndromes	6/17/09	10/23/09	Novartis
Ustekinumab	Stelara	human IgG1κ	IL-12/ IL-23	Plaque psoriasis	9/25/09	1/16/09	Centocor Ortho Biotech
Ofatumumab	Arzerra	human IgG1κ	CD20	Chronic lymphocytic leukemia	10/26/09	NA	Glaxo Grp Ltd
Tocilizumab	Actemra	humanized IgG1κ	IL-6R	Rheumatoid arthritis	1/8/10	NA	Roche/Chugai
Denosumab	Prolia	human IgG2κ	RANK ligand	Postmenopausal women with risk of osteoporosis	06/02/10	05/28/10	Amgen
Catumaxomab	Removab	murine/rat hybrid IgG	EpCAM and CD3	Intraperitoneal treatment of malignant ascites in patients with EpCAM-positive carcinomas		04/20/09	TRION Pharma
Edrecolomab	Panorex[3]	murine IgG2a	EpCAM	Colon cancer			Wellcome/ Centocor
I^{131}-TNT	Cotara[4]	I^{131}-chimeric IgG1	DNA	Lung cancer			MediPharm Biotech
Nimotuzumab	Theracim[5]	humanized IgG1	EGFR	Nasopharyngeal carcinomas and head and neck tumors			CIM/CIMAB/ YM Bioscience

[1] On June 21, 2010, Pfizer announced the voluntary withdrawal from the US market of Mylotarg® (gemtuzumab ozogamicin) for patients with acute myeloid leukemia, at the request of the US FDA after results from a recent clinical trial raised new concerns about the product's safety and the drug failed to demonstrate clinical benefit to patients enrolled in trials.
[2] Efalizumab (Raptiva®) has been withdrawn from US market due to side effect. As of 06/09/09, it is no longer available in the United States. It also has been withdrawn from EU market since 08/05/09.
[3] Edrecolomab (Panorex®) was approved in Germany in 1995, but was subsequently withdrawn from market.
[4] I^{131}-TNT (Cotara®) was approved in China in 2003.
[5] Nimotuzumab (Theracim®) was approved in Cuba, Argentina, Colombia, India and China in 2005 and 2006.

more economic than ever. To date, in addition to more than two dozen mAb products either in Phase III clinical trials or awaiting FDA approval, there are several hundred antibodies being tested in early stage clinical trials in a variety of disease indications. Albeit the clinical and commercial success and the current hype among major biotech and pharmaceutical companies continue, many challenges in developing antibody therapeutics remain and the full potential of therapeutic antibodies has yet to be realized. To date, most FDA approved antibodies are full-length unmodified antibodies, which are large proteins with a molecular weight of around 150 kDa. With new genetic engineering technology development and our further understanding of disease biology and mechanisms of action of antibodies, an array of classes of novel antibody formats or antibody derived molecules is emerging as promising new generation therapeutics. These new antibody formats or molecules are carefully designed and engineered to acquire special features, such as improved pharmacokinetics, increased selectivity, and enhanced efficacy.

Bispecific antibody (BsAb)

The incurrence of a disease is rarely due to a single point of deregulation, rather most likely multiple mechanisms are developed in the process of disease to reinforce its pathogenesis. Quite often a monotherapy targeting a specific node of biology network in the cell cannot eradicate the disease or prevent the disease from recurrence. An obvious strategy to overcome disease resistance to a single therapy is to simultaneously attack multiple components of the cellular pathways by a combination of drugs that act on different targets and/or mechanisms. This has been greatly validated in cancer treatment as most of the chemotherapy regimens are comprised of a combination of several cytotoxic agents. The combinational strategy has also been shown to be efficacious when combining low molecular weight drugs with therapeutic antibodies, as well as simultaneously administrating two or more therapeutic antibodies. For examples, the combination of cetuximab (Erbitux®, anti-epidermal growth factor receptor, EGFR) and bevacizumab (Avastin®, anti-vascular endothelial growth factor, VEGF) in the treatment of metastatic colorectal cancer[10], and rituximab (Rituxan®, anti-CD20) and epratuzumab (anti-CD22) in Non-Hodgkin's lymphoma patients[11], have both demonstrated the benefits of antibody combination, when compared to a single antibody therapy, in preliminary clinical studies. However, several critical issues, including high development cost, significant development timelines, and complex regulatory approval path, present high hurdles to developing two individual therapeutic mAb for combination therapy in the future. A promising alternative to antibody combination is to create a bispecific antibody (BsAb), a single antibody molecule capable of strong and specific binding simultaneously with two different target antigens[12]. Since the conception of the BsAb in late 1970's, early research in the field has been heavily focused on the construction of BsAb for effector cell targeting, *ie*, a BsAb that can bind to both a tumor associated antigen on cancer cells and an activating molecule such as CD3 on T cells and CD16 on NK cells. In this context, the BsAb can not only cross-link the tumor and the effector cells but also simultaneously activate the effector cells, leading to tumor cell killing/lysis[13, 14]. Recently, a new concept has emerged, which is quickly gaining significant enthusiasm among major biotech and pharmas, that is to create dual-targeting BsAb that is able to simultaneously bind and also modify two disease-relevant targets[12].

Based on the mechanisms of action, dual-targeting BsAb can be constructed to bind to different targets of the signaling pathways within the same diseased cells. Simultaneous modifying (*eg*, blocking or neutralizing) two disease associated targets should provide the benefits of both enhancing the therapeutic efficacy of, and also blocking the compensatory mechanisms associated with, the individual antibody therapy, thus circumventing resistance to monotherapy[15–17]. By simultaneously binding to different cell surface targets, BsAb may result in enhanced binding avidity, leading to preferential (strong) binding to only cells that express both targets but not cells that only express a single target, thus fine-tuning the antibody selectivity[18]. BsAb can also be designed to bind to different targets expressing on different cell populations within the diseased tissues to achieve synergistic therapeutic effects and/or to enhance specific tissue distribution[19]. Since each binding arm of a BsAb is functionally active independent of the other, the BsAb should be, in theory, able to exert its biological activity towards diseased cells that express either both the target antigens or just one of the two, thus potentially expanding the therapeutic disease indications of the BsAb[16, 17]. Further, BsAb can be created from two antibodies that bind to different epitopes on the same target (*ie*, bi-paratopic binding) to enhance binding avidity[20] and to increase antibody load on tumor cells for enhanced effector functions, such as antibody dependent cellular cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC)[17]. It has been demonstrated that, bi-paratopic BsAb, by simultaneously binding to two different epitopes on the same target molecule, could even potentially acquire new functionality that could not be achieved with the parent antibodies when used alone or in combination[20].

The development of molecular engineering technologies in the past years has enabled us to design and construct various formats of recombinant BsAb. Early BsAb research and engineering have been concentrated on the creation of bispecific fragments, and significant progress has been made in the past two decades in this field[21, 22]. BsAb fragments do not require glycosylation, thus they can be produced in high yield in microbia such as bacteria. BsAb fragments are smaller than full length IgGs, so they may have better solid tumor penetration rates. However, their small size and lack of an intact Fc also result in their being cleared rapidly from circulation, leading to a short *in vivo* half-life. Recently there has been an increased interest on the design and construction of IgG-like BsAb[23, 24]. These molecules contain an intact Fc, which endows them with the effector functions such as ADCC and CDC, and a half-life of normal IgG, but permute variable

domain organizations to endow them with bi-specificity, and in many cases tetravalent binding[16, 17, 24]. The engineering and application of various BsAb formats have been reviewed extensively[21–26].

A major technological obstacle in the successful development of BsAb has been the difficulty of producing the materials in sufficient quality and quantity for both preclinical and clinical studies. The major challenge in the development of IgG-like BsAb is to construct a recombinant molecule with good pharmaceutical properties comparable to those of the conventional mAb, such as good molecule characteristics (*eg*, homogeneity, stability, low aggregation propensity, *etc*), and ease of manufacturing and downstream processing including high level productivity, simple purification process and no special need in formulation. On the biology front, it has long been known that not every single combination of cytotoxics or other disease modifying agents would necessarily lead to additive or synergistic therapeutic effect. Developing highly effective BsAb will require clear elucidation and understanding of the molecular details in the aberrant signaling pathways that lead to various diseases to guide the selection of the target pairs for co-targeting.

Antibody drug conjugates (ADC)

Another new approach in therapeutic antibody development is to use the target specificity of an antibody to deliver therapeutic payloads, typically radioactive isotopes, chemotherapeutic drugs or toxins, to target cells. This is particularly useful for therapeutic antibodies in oncology, the area which sees the most growth of mAb treatments. The non-specific toxic side-effect of chemotherapy on normal tissues is a major issue that severely limits the applications of chemotherapeutic drugs in the clinic. Antibody conjugates can deliver the toxin or chemotherapeutic drugs to specific tumor tissues, thus reducing systemic toxicity and increasing efficacy. Three antibody drug conjugates (ADCs) have been approved by the FDA: gemtuzumab ozogamicin (Mylotarg®, see Table 1, footnote 1, for recent development of the conjugate), a humanized anti-CD33 antibody conjugated to calicheamicin for acute myeloid leukemia[27], ibritumomab tiuxetan (Zevalin®), an ^{90}Y-labeled murine anti-CD20 antibody, and tositumomab (Bexxar®), an ^{131}I-labeled murine anti-CD20 antibodies, for non-Hodgkin's lymphoma[28]. There is clearly a significantly revived interest in the field at this time, as many major biotech and pharmas are intensifying their efforts in promoting ADCs in both discovery research and clinical trials[29]. For example, Genentech is performing multiple clinical studies with trastuzumab-DM1 (T-DM1), an anti-HER2 mAb-maytansinoid drug conjugate, which had showed greater antitumor activity compared with nonconjugated trastuzumab (Herceptin®, anti-HER2) while maintaining selectivity for HER2-overexpressing tumor cells[30]. Genentech initiated a Phase III study evaluating T-DM1 for second-line advanced HER2-positive breast cancer in 2009. On July 6, 2010, Genentech submitted a Biologics License Application to the US FDA for T-DM1 in people with advanced HER2-positive breast cancer who have previously received multiple HER2-targeted medicines and chemotherapies. Another ADC in pivotal clinical trial is Brentuximab Vedotin (SGN-35), an anti-CD30 mAb linked to monomethyl auristatin E (MMAE) for Hodgkin lymphoma, from Seattle Genetics. Currently, there are over a dozen of other ADCs, for example, SGN-33 (anti-CD33-MMAE), inotuzumab (anti-CD22)-ozogamicin, IMGN242 [HuC242 (anti-CanAg)-DM4], CDX-011 (anti-GPNMB-MMAE), MDX1203 (anti-CD70-duocarmycin) and MEDI-547 (anti-EphA2-MMAE), AVE9633 (anti-CD33-DM4), in various stages of clinical trials[31, 32].

ADC has three components, the mAb, the cytotoxic payload, and the linker connecting the payload to the antibody. The antibody carrier should be highly specific and efficiently internalized once binding to its target antigen on the cell surface. The desired properties for a toxic payload include high potency towards tumor cells, a suitable functional group for linkage to an antibody, reasonable solubility in aqueous solutions to enable the reaction with antibodies, and prolonged stability in aqueous formulations commonly used for antibodies. The linker between the antibody and the payload should be designed in a manner that ensures ADC stability during storage and in circulation *in vivo* but allows for a rapid release of the cytotoxic payload in its fully active form once inside the target cells. In the past several years, significant progress has been made in optimizing each of the three components of an ADC. Less immunogenic and more selective high affinity antibody carriers have been designed and selected. Toxic payloads have evolved from radio isotope and conventional chemotherapeutics to more potent cytotoxic agents, such as calicheamicin, maytansinoids and auristatins. Several types of cleavable (labile) or non-cleavable (stable) linkers, for example, disulfide linkers and acid- and peptidase-labile linkers, have been developed[31]. The conjugation technologies have advanced to a point where both the site and stoichiometry of drug attachment to the carrier antibody can be controlled. In the near future, the research focus of the area will be to identify even more potent payloads and to develop better conjugation strategies including further improvement in linker design and conjugation chemistry and efficiency. Other areas that critically need to be addressed include establishment of analytic platforms for manufacturing and process development (chemistry, manufacturing and control, CMC) and clinical pharmacokinetic/pharmacodynamic assays, and development of preclinical toxicology and pharmacology assessment protocols to satisfy the regulatory and safety requirement.

Antibody with modified Fc functions (Fc engineering)

In addition to the direct effect of binding to an antigen, antibodies can mediate a variety of "effector" functions such as ADCC and CDC, via their Fc regions. By fixing complement or interacting with the Fc receptors (FcRs) thus activating immune cells such as NK cells, macrophages, and T cells, the antibody can mediate additional cell killing against target cells. These effector mechanisms are particularly relevant when the antibodies are used to treat cancer and certain inflammatory diseases. ADCC as part of the mechanisms of

action for therapeutic antibodies has been strongly implicated in several clinical trials. For example, a better clinical response to rituximab is observed in non-Hodgkin's lymphoma patients carrying an IgG FcγRIIIa of V158 allotype, an allotype with higher affinity binding to the Fc region of an IgG, compared to that in patients who carry the F158 allotype[33]. Similarly, patients carrying the 158 VV genotype of FcγRIIIa were also associated with a better clinical response to trastuzumab[34] and cetuximab[35]. Based on these clinical observations, it is plausible to further enhance the therapeutic efficacy of a mAb by optimizing (increasing) its Fc interaction with the FcRs on effector cells, via molecular engineering and/or manufacturing process modification[36–38].

By combining various molecular engineering methods, including alanine scanning, site-directed mutagenesis, computational structure-based design/algorithm and experimental selections[36, 37, 39, 40], a large set of Fc variants has been generated that provides a spectrum of FcγR binding profiles. Several variants have been identified that provide up to 100-fold greater affinity for FcγRIIIa, resulting in an enhanced ADCC, for up to two to three logs higher, than what can be achieved by the wild-type antibody[38, 41]. For example, a Fc-engineered anti-CD19 antibody, with 100–1000 fold increased ADCC, has shown more potent antitumor activity than its IgG1 analogue in prophylactic and established mouse xenograft models[42]. In addition to ADCC, an IgG1/IgG3 chimeric Fc, constructed via alternative domain shuffling, has demonstrated markedly increased CDC activity both *in vitro* and *in vivo* in a cynomolgus monkey model[43]. Albeit of all the encouraging observations, specific design and selection of Fc variants with preferentially enhanced binding to individual FcγR, either the activating FcγRIIIa, FcγRIIa, and FcγRIa, or the inhibitory FcγRIIb, still remain a challenge. It also remains to be seen whether it is possible to fine-tune the Fc domain of a defined mAb agent to selectively activate (or inhibit) a sub-population of immune cells and/or to optimize the CDC activity for intended therapeutic application.

The glycosylation of the antibody Fc domain also has a major influence on its binding affinity for FcγRs. It is well known that the carbohydrates at the position Asn297 is critical for FcγR binding. The presence of fucose and its content at this position can negatively influence ADCC activity of a mAb. To this end, new cell lines capable of producing defucosylated mAb have been established, such as CHO cell lines that are genetically engineered (knock-out) to delete the *FUT8* gene coding for the enzyme α-1,6-fucosyltransferase[44]. Alternatively, CHO cell lines that over-express a recombinant β-1,4-N-acetylglucosaminyltransferase III (GnTIII) have also been established. Production in these cell lines resulted in antibodies enriched in bisected and non-fucosylated oligosaccharides[45]. Antibodies produced in these modified cell lines have been shown to be more potent than their counterparts produced in the wild-type CHO cell lines in mediating ADCC towards target cells[44, 45]. Similar approaches have also been attempted in non-mammalian expression systems, including yeast, plants, and moss, in parallel to engineering away non-human (thus potentially immunogenic) glycoforms[46–48]. Other glycoform modifications, such as sialylation of the Fc carbohydrate, has also been suggested as a biological mechanism for regulation of FcγR affinity and cytotoxicity[49].

In addition to its effector activities, another major function of the antibody Fc domain is to bind to the neonatal Fc receptor (FcRn), the major mechanism responsible for the long circulation half-life of an antibody, compared to other proteins of similar size. A long serum half-life is generally desirable as it would reduce the frequency of dosing, thus potentially reducing both the inconvenience and the total cost of the treatments. It has been demonstrated in several reports that various mutations within the Fc domain of an IgG1 led to significantly improved binding to FcRn at pH 6.0 (but no changes in binding at pH 7.4). The enhanced FcRn binding resulted in increased antibody circulation half-life in cynomolgus monkeys and improved *in vivo* dosing regimen[50–55]. It has been suggested that the kinetics of Fc/FcRn interaction may also have an effect on the extent to which improved binding translates into extended serum half-life[56]. Besides IgG, a half-life enhanced Fc fragment can also be used as the fusion partner to extend the half-life of antibody fragments, alternative scaffolds, and other protein therapeutics.

Antibody fragments and single domain antibodies

Full length IgG has good *in vivo* half-life and effector functions, but its large size limits antibody tissue penetration, especially in solid tumor, and complicates manufacturing process. Antibody genetic engineering has enabled us to produce various antibody fragments, with defined size, valency, and desired pharmacokinetics profiles, that retain the binding activity of the full-length molecule, to suit various *in vivo* applications[57]. For example, it is relatively straight-forward to create and produce antibody fragments with different size and valence, such as Fab, single chain Fv (scFv)[58], diabody[59], and minibody[60] *etc*. It has been demonstrated that, in some species, even a single variable antibody domain could bind to antigen with high affinities. Camelids and sharks are two species that naturally express heavy chain only antibodies, called heavy chain antibodies (HcAbs)[61] and new antigen receptor antibodies (IgNAR)[62], respectively. The single variable domains of their antibodies, called VHH in camelids and V-NAR in sharks, have been engineered to retain high affinities towards a large spectrum of antigens. Recently, single domain antibody (dAb) based on a single human antibody variable domain, VH or VL, has also been generated with high affinity and specificity[63]. To date, three antibody Fab fragments have been approved for clinical applications, including abciximab (Reopro®, anti-GPIIb/IIIa receptor), ranibizumab (Lucentis®, anti-VEGF) and certolizumab pegol (Cimzia®, anti-tumor necrosis factor α, TNFα). Several single domain antibodies are currently in various stages of clinical development, including two dAbs (anti-TNFα and anti-IL-1R) and three nanobodies (anti-vWF, anti-RANKL, and anti-TNFα), the humanized camelid (llama)-derived VHH domains.

Because of their reduced size, antibody fragments usu-

ally penetrate solid tissue much more rapidly and efficiently than the full length IgG, but this advantage is countered by a very short serum half-life that eventually could decrease the overall tissue uptake of these fragments. Several approaches are being explored to increase the serum half-life of antibody fragments. One approach is PEGylation [chemical addition of polyethylene glycol (PEG) to increase the size of the fragments], exemplified by certolizumab pegol, a recently approved anti-TNFα PEGylated Fab fragment[64]. Other alternative approaches include HSA fusion (fusion of recombinant antibody fragments to human serum albumin)[65] and anti-HSA fusion (fusion of antibody fragments to HSA-binding peptides or proteins)[66–68]. Multimerization of antibody fragments has also been explored. For example, diabodies, triabodies and tetrabodies have been produced by multimerization of scFvs harboring a short or no linker between the VL and the VH, leading to high molecular weight and multivalent fragments with increased serum half-lives[57].

Alternative protein scaffold

Antibodies gain their universal antigen recognition function by the combination of a structurally conserved framework with a spatially defined combining site composed of peptide segments that are hypervariable both in sequence and conformation[69], referred to as the complementarity determining regions, or CDRs. Alternative protein scaffolds provide a potential avenue for developing a new class of biotherapeutics. Dozens of small independently folding proteins and domains have been evaluated as antibody mimics in attempts to obtain high affinity binders with therapeutic potential.

Fibronectin is perhaps the most advanced scaffold in development. Adnectin is one of the examples. It is based on the 10th fibronectin type III domain, a highly stable (T_m=90 °C) 94-residue member of the Ig superfamily which does not contain any cysteines. Three loops of the protein can be randomized in similar fashion to the CDRs of a single domain antibody, and the resulting library has been used to select therapeutically useful binders to various targets[70]. CT-322, a pegylated anti-VEGFR2 adnectin, is currently in Phase II clinical development as an angiogenesis inhibitor in the United States in recurrent glioblastoma multiforme (rGBM) and in a Phase Ib/II study for first line GBM.

Avimers is another advanced case in this category. They are based on 40-amino-acid-long human A-domains, which occur as strings of multiple domains in several cell-surface receptors. Although each of the domains separately has low target-binding activity, in tandem arrays very high avidities can be achieved. C326, a subnanomolar IL-6-blocking avimer has been successfully generated. It showed 0.8 pmol/L IC_{50} in cell-based assays and was biologically active in two animal models[71]. C326 has been in phase I clinical trial in adults with Crohn's disease since 2006.

Another alternative scaffold being developed is ankyrin repeat proteins (DARPins), which are designed based on a combination of sequence and structure consensus analyses of one of the largest highly conserved protein families in nature[72]. A 33 amino acid residue ankyrin repeat (AR) module with seven randomized positions can provide a theoretical diversity of 7.2×10^7. Different numbers of this module, *eg* four to six repeats, can be assembled together between the N and C-terminal capping repeats to further increase the theoretical diversity. DARPins with picomolar affinities have been isolated and affinity matured against human EGFR[73], as well as eukaryotic kinases JNK2 and p38[74], underlining their therapeutic potential as intracellular inhibitors. The lead product MP0112, a DARPin which inhibits all relevant forms of VEGF with high potency, is currently in Phase I/IIa testing in wet age-related macular degeneration (wet AMD) and diabetic macular edema (DME).

In addition, alternative scaffolds based on staphylococcal protein A[75], lipocalins[76], thioredoxin[77] and many others[78] have also been explored. Compared to the conventional IgG antibodies, small protein scaffolds may provide several advantages that render them good candidates for the development of therapeutic agents. For example, due to their small size and generally high stability, the scaffold proteins may be more amendable for alternative delivery routes, *eg*, by inhalation or subcutaneous injection. Further, owing to their single domain structure, the scaffold proteins may represent excellent building blocks for the construction of bi- and multi-specific/valent therapeutics. Taken together, the alternative protein scaffolds have generated high interests and expectations in recent years, and many of the promising scaffolds are under serious research and development. There are several major issues remaining to be addressed, in particular, the potential immunogenicity, the lack of effector functions, and the short circulation half-life in the context of therapeutic use of the scaffold proteins. These issues will no doubt be the focus of the field in the near future.

Intrabody

Antibody is naturally secreted into extracellular space and binds antigen extracellularly, so it is natural that all the targets for current therapeutic antibodies and antibody derivatives are extracellular proteins. Recent advances in antibody engineering have allowed the specific, high affinity interaction of antibodies with antigens to be directed intracellularly through the creation of intracellular antibodies, or so-called "intrabodies". Intrabody expands the pool of targets for therapeutic antibody beyond the traditional extracellular proteins. The most commonly used intrabodies are in the form of scFv as a single polypeptide. Other antibody formats have also been used, including Fab fragments[79], single domain antibodies[80], conventional IgG antibodies, and multivalent bispecific fragments (for example, "intradiabody" [81]). Intrabodies, through the use of N- or C-terminal tags that encode natural intracellular trafficking signals, can direct the antibody-antigen interaction to a specific cellular compartment[82]. As a result, intrabodies can exert their biological activities via interfering with the intracellular transport or misdirecting the localization of proteins, as demonstrated in the cases of VEGFR2 intrabody[83] and EGFR intrabody[84]. Intrabodies can also achieve their therapeutic

effect by directly interfering with enzyme function intracellularly, as shown in the cases of intrabodies against Akt[85] and epithelial tyrosine kinase (Etk)[86]. Further, intrabodies may function by blocking protein-DNA interactions in the nucleus. A nuclear-targeted intrabody has been developed that binds to cyclin-E, interferes with its function, and inhibits the growth of a breast cancer cell line[87].

Intrabodies have been isolated to a diverse array of targets that are involved in the pathogenesis of various human diseases and have demonstrated efficacies in a variety of *in vitro* and *in vivo* studies. These include intrabodies that inhibit cancer growth[88], HIV infection[79, 89, 90], HPV infection[91], protein misfolding in neurologic disorders including Huntington's disease, Parkinson's disease, Alzheimer's disease and Prion disease[92], and knockout of MHC class I expression in an attempt to prevent graft rejection[93]. The ER-targeted anti-erbB-2 intrabody has been in phase I clinical trial for cancer treatment[94]. Most approaches using intrabodies to treat a specific disease are in essence a gene therapy approach, involving the introduction of genes into target cells to correct the aberrant molecular processes responsible for the diseased state. Compared to other gene therapy approaches, such as antisense oligonucleotides (AnO)[95] and RNA interference methods (RNAi)[96], the intrabody approach may offer several advantages, such as higher specificity, longer half-life, and an ability to target proteins with particular post-translational modifications.

There are two major technical issues associated with the development of intrabodie as therapeutics. One involves the efficiency and specificity of delivery of the intrabody or the genetic material encoding the intrabody to disease sites and the expression of intrabodies inside the target cells at a stable and high enough level needed to obtain a therapeutic effect. Several groups have successfully used adenovirus to deliver intrabodies against cancer-related targets in animal models[81, 94]. However, the risk of potential genetic modification[97], immnogenicity and selectivity of delivery have been the concern for viral-based DNA delivery, though several other approaches are being explored[98, 99]. Another system for intrabody delivery has been developed that involves transporting the intrabody (at the protein level) across the plasma membrane of a cell using membrane translocating sequence (MTS) or protein transduction domains (PTD)[85, 100, 101]. However, it still remains to be determined if such protein translocation approaches can obtain a high enough level, activity, and half-life of an intrabody in a target cell to achieve a therapeutic effect, especially *in vivo*. Another issue associated with the clinical development of intrabodies is the correct folding or stability of intrabodies and their tendency to aggregate when expressed in the reducing environment of the subcellular compartments. Many groups are actively addressing this issue, with most of the studies focusing on isolating functional intrabodies from large libraries using selection conditions mimicking the intracellular environment, creating stable antibody frameworks for intrabody construction, and exploring alternative intrabody formats, *eg* using Intracellular Antibody Capture (IAC) system[102], direct phage to intrabody screening (DPIS) system[103], modified yeast two-hybrid system[104], or using scFv, single domain antibody[80, 86]. The lessons learned from all of these studies and progress on other forms of gene therapy should facilitate the development of intrabodies as therapeutics.

Perspectives

Compared to low molecular weight chemical drugs, therapeutic antibodies have high target specificity, lower systemic toxicity, longer half-life, and potentially higher barrier for generic (or biosimilar) competition. The future growth of mAb based therapeutics is still strong. Among all biologics that are being studied in clinical trials, 85% are mAb or antibody based molecules. Antibodies and antibody-based therapeutics consist of more than one third of all new agents currently under both preclinical and clinical development at biotech/pharma companies around the world. Consensus sales forecasts predicts that, by 2014, six out of the world's top 10 best-selling drugs will be therapeutic antibodies or antibody fusion protein, and the total sales of mAb based therapeutics will approach $58 billion dollars[105].

There are many challenges remaining in the continuous successful development of mAb therapeutics. Despite the completion of the human genome project more than ten years ago, new therapeutic target discovery/identification and validation, remain elusive and challenging tasks. Competition on a limited number of validated targets is fierce. For example, for the top 8 mostly pursued therapeutic targets, 32 biotech/pharma companies had active mAb development programs in 2009. Novel antibody formats engineered for enhanced therapeutic efficacy, such as BsAb and ADC, may open new target space for the development of mAb therapeutics. To this end, certain targets previously deemed not viable for therapeutic development, for example, targets overexpressed on diseased tissue but not associated with known major pathophysiologic functions for intervention (*eg*, CD33), may prove to be good candidates for ADC development. Further, there are individual targets (pathways) that may not provide sufficient therapeutic benefit when being modified alone, but if combined with other targets (pathways), could produce additive and/or synergistic activity. These targets thus represent excellent candidates for the development of dual-targeting BsAb therapeutics. Besides the targets, development of new technology platforms in antibody discovery/engineering and production to increase the efficacy of therapeutic mAb, and at the same time, to reduce the cost of the therapy, represent the other critical components in winning the competition. We expect to witness more novel mAb and mAb-like molecules with carefully engineered features, as those discussed in this article, to enter clinical development in the near future. Many issues remain to be carefully researched and addressed, for example, manufacturing and downstream process development (purification and formulation), analytical and quality control, preclinical toxicology and pharmacology studies, regulatory and clinical development pathways, *etc*, before we see these new antibody

formats become bona fide mainstream therapeutics.

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