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Thomas Tiller *Editor*

Synthetic Antibodies

Methods and Protocols

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Synthetic Antibodies

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Dedication

Dedicated to Isi, Josh & Family.
...and Rea.
Thanks for inspirational, wise, and humorous support.

Preface

Antibodies are important tools that are used extensively in basic biomedical research, in diagnostics, and in the treatment of diseases.

Traditionally, the production of antibodies relies on the immunization of an animal. For example, for the generation of monoclonal antibodies by the hybridoma technology, usually mice and rats are preferred. For polyclonal antibody production, larger mammals (e.g., rabbits, sheep, and goats) are used as the relatively huge amount of serum that can be collected from these animals serves as a rich source for antibody purification. These antibodies are all based on an immunoglobulin scaffold and are derived from a genuine in vivo immune response. Despite their widespread applications as detection, diagnostic, and therapeutic agents, in vivo-generated polyclonal and monoclonal antibodies bear some limitations. For example, polyclonal antibodies as detection reagents are not only prone to batch-to-batch variability but also contain significant amounts of nonspecific antibodies. Furthermore, due to their inadequate characterization, it is not surprising that many experimental results that are obtained with polyclonal antibodies are often not reproducible. In contrast, hybridoma-derived monoclonal antibodies are considered to be perfectly defined reagents with unique specificities. Very often, however, they secrete additional light and/or heavy chains, which makes it cumbersome to evaluate if the binding behavior of the hybridoma-derived mAb is intrinsic to the mAb from the target B cell or due to artificial chain combinations caused by the presence of the additional chains derived from the fusion cell line. Furthermore, hybridoma cells can lose expression, are prone to mutations, and thus require frequent retesting.

The restrictions of these traditional in vivo-generated antibodies have been overcome by modern synthetic recombinant in vitro antibody technologies.

One of the most significant difference between naturally occurring and *synthetic immunoglobulins* per se is the way these two groups are generated. Naturally occurring immunoglobulins are generated in vivo by processes of V(D)J recombination and somatic hypermutation of the B cell antigen receptor during B cell development and differentiation and its secretion as soluble immunoglobulin by plasma cells. *Synthetic antibodies* on the other hand can be defined in general as affinity reagents engineered entirely in vitro, thus completely eliminating animals from the production process. (Although this definition might get blurred, e.g., by processes such as antibody humanization, which basically is the replacement of frameworks of a murine antibody generated in vivo with their human counterparts by recombinant genetic engineering in vitro. Therefore, a humanized antibody could be considered as "semisynthetic").

Synthetic affinity reagents include recombinantly produced *immunoglobulin antibodies derived from combinatorial antibody libraries* (i.e., antibody libraries built on in silico-designed and chemically defined diversity on the basis of synthetic oligonucleotides) and so-called *antibody mimetics* that are based on alternative protein/polypeptide scaffolds.

In addition, the term "*synthetic antibody*" is also often used to describe affinity reagents that are different from protein/polypeptides but share typical antibody characteristics such as diversity and specific binding affinities. For example, *aptamers* as a class of small nucleic

acid ligands are composed of RNA or single-stranded DNA oligonucleotides. Like antibodies, *aptamers* interact with their corresponding targets with high specificity and affinity.

An example of synthetic "plastic antibodies" are *molecularly imprinted polymers* (MIPs), which are polymeric matrices obtained by a technique called *molecular imprinting technology* to design artificial receptors with a predetermined selectivity and specificity for a given analyte. MIPs are able to mimic natural recognition entities, such as antibodies and biological receptors.

This volume on Synthetic Antibodies aims to present a set of protocols useful for research in the field of recombinant immunoglobulin and alternative scaffold engineering, aptamer development, and generation of MIPs. Part I includes methods that deal with amino acid-based synthetic antibodies. Brief protocols about the generation of antibody libraries are detailed, as well as techniques for antibody selection, characterization, and validation. This section is completed by a brief description of a bioinformatics platform that supports antibody engineering during Research and Development. Part II contains basic procedures about the selection and characterization of aptamer molecules, and Part III describes fundamental processes of MIP generation and application.

I would like to express my sincere thanks to all contributing authors for sharing their research expertise. Without their support, this volume would not have been possible. Many thanks to John M. Walker for the invitation to edit this volume on "Synthetic Antibodies" and to Monica Suchy and Patrick Marton from Springer for helpful advice and for publishing this book.

Planegg, Germany ***Thomas Tiller***

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Part I

Amino Acid-Based Synthetic Antibodies

Chapter 1

Antibody Mimetics, Peptides, and Peptidomimetics

Xiaoying Zhang and Thirumalai Diraviyam

Abstract

In spite of their widespread applications as therapeutic, diagnostic, and detection agents, the limitations of polyclonal and monoclonal antibodies have enthused scientists to plan for next-generation biomedical agents, the so-called antibody mimetics, which offer many advantages compared to traditional antibodies. Antibody mimetics could be designed through protein-directed evolution or fusion of complementarity-determining regions with intervening framework regions. In the recent decade, extensive progress has been made in exploiting human, butterfly (*Pieris brassicae*), and bacterial systems to design and select mimetics using display technologies. Notably, some of the mimetics have made their way to market. Numerous limitations lie ahead in developing mimetics for different biomedical usage, particularly for which conventional antibodies are ineffective. This chapter presents a brief overview of the current characteristics, construction, and applications of antibody mimetics.

Key words Antibody mimetics, Protein engineering, Monoclonal antibodies (mAbs), Therapeutics, Diagnostics

1 Introduction

A revolution has been made in the biological science through the development of the hybridoma technique to generate monoclonal antibodies (mAbs) [1]. In the meantime, advancements in genetic engineering revolutionized the methods to select, humanize and produce recombinant antibodies. The accomplishment of fabricating antibody fragments in different host systems (e.g., bacteria and yeast) and selection technologies, such as phage and ribosome display, permitted the production of antibody-based reagents for varied applications. On the other hand, animal-sourced antibodies faced some challenges such as ethical concerns to use animals for experiments, the penetration difficulty for large sized antibodies in solid tumors, immunogenicity [2], presence of six hypervariable loops that are difficult to manipulate at once, if generation of a large synthetic library is required [3], complex multi-chain architecture and glycosylation of the heavy chains [4]. Besides, some studies reported that, some antibodies have lost their activity when

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used in microarrays [5], are required in high doses to achieve clinical efficacy [6], exhibit poor pharmacokinetic behavior and costly manufacturing processes [7].

The tremendous advancements of biotechnology and cutting-edge protein engineering have made it possible to synthesize antibody-like molecules, the so-called antibody mimetics. The process of producing antibody mimetics upholds the precepts of 3Rs (replacement, reduction, and refinement) for using laboratory animals [8]. They mimic natural antibodies and functionally exhibit many advantages than conventional antibodies. To date, several antibody mimetics such as, affibodies, anticalins, avimers, bicycles, DARPins, fynomers, iBodies, and nanofitins, have been developed and many more are under development. These novel approaches are gaining acceptance by offering versatile advantages to combat with clinically important diseases such as cancer, autoimmune diseases, and acquired immunodeficiency syndrome.

2 Steps Involved in Constructing Antibody Mimetics

Antibody mimetics are mainly constructed by two methods, protein-directed evolution and fusion of complementary determining regions (CDRs) through cognate framework regions (FRs) in different sequences.

Presently, the protein-directed evolution is employed to harness the power of natural selection to evolve proteins with preferred properties. In principle, it involves four key steps as illustrated in Fig. 1a: (1) Identification: the sequence of interest is chosen on the basis of its perceived proximity to the desired function and its evolvability [9]; (2) Diversification: the parent sequence is

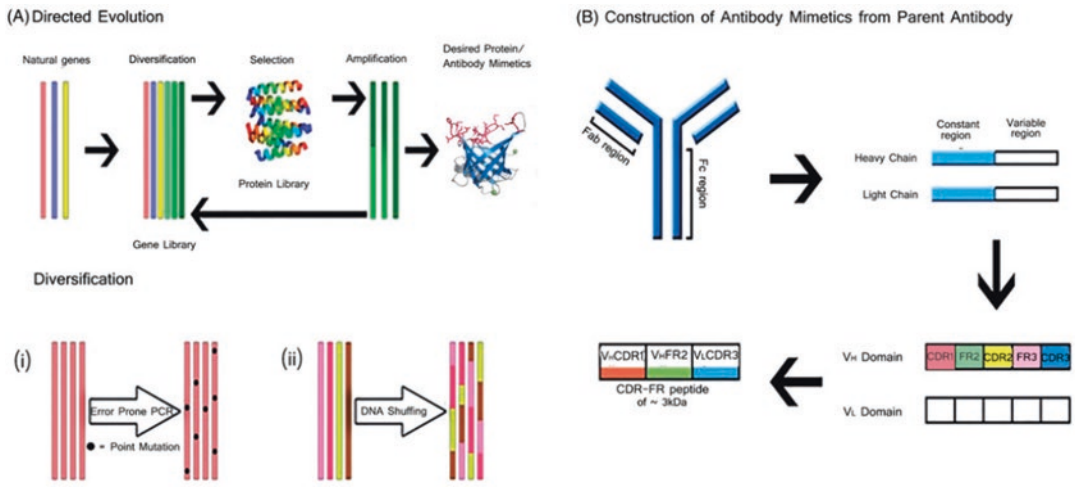

Fig. 1 Construction strategies of antibody mimetics

subjected to diversification by error-prone PCR and DNA shuffling; (3) Selection: the screening or selection is used to test the presence of mutants in the generated library; and (4) Amplification: the variants are screened, selected and replicated many-fold to harvest a variant with the desired properties. Massive combinatorial libraries have been constructed by randomizing amino acid positions in structurally variable loops of proteins [10] or by exon shuffling and phage display [11]. The mutant libraries specific against desired antigens are screened by phage display or ribosome display selection (Fig. 1).

By fusing two CDRs through a cognate framework region (FR) the CDR-FR peptides are constructed. Protein antigens are generally recognized by all six CDRs from both the VL and VH domains of the intact antibody combining site. The CDRH3 loop is considered the most indispensable part of the mimetic, as it is often the most accessible of the CDR loops, and is almost always involved in antigen binding to the greatest extent due to its greater sequence diversity. The C-terminus of the selected CDR1 or CDR2 loop and the N-terminus of the selected CDRH3 loop are joined with a FR chosen from VH or VL [12] (Fig. 1b). On the basis of these principles numerous antibody mimetics have been developed.

3 Antibody Mimetics as Therapeutic Agents

Human epidermal growth factor receptors (HER1, HER2, HER3, and HER4) dysregulation and overexpression may cause different types of cancers, and therefore the HER proteins are considered as reliable biomarkers for cancer progression and treatment [13]. The FDA approved anti-HER2 mAb Trastuzumab (Herceptin) is successfully used for the treatment of breast cancer; however, Trastuzumab application may also result in side effects such as cardiac dysfunction in some patients [14]. Small, non-immunogenic, stable, and specific affibody molecules named ZHER2:342 with good tissue penetration are successfully used as imaging and treatment agents as alternatives to mAbs [15]. ZHER2:342 has been fused with a truncated form of Pseudomonas exotoxin A, and the fusion protein was found to bind successfully to HER2-expressing cells [16]. It was also evaluated whether albumin binding domain (ABD) conjugation with the anti-HER2 affibody could improve its pharmacokinetics and enable radionuclide therapy for small tumors expressing HER2. This conjugation strategy [177Lu-CHX-A00-DTPA-ABD-(ZHER2:342)2] exhibited significantly enhanced half-life and reduced the kidney uptake [17]. The affibody molecule ZEGFR was encapsulated in liposomes to prevent degradation from metabolizing enzymes and was successfully delivered to EGFR-expressing cells [18]. The effects of two other affibodies (Z05416 and Z05417) were investigated against HER3 on

different cell lines and these molecules completely inhibited heregulin (HRG)-induced cancer cell growth in an in vitro assay. The antiproliferative effect of these affibodies on cells was caused by blocking the physiological interaction between HER3 and HRG [19].

It is well known that, targeting cytotoxic T lymphocyte associated antigen-4 (CTLA-4) has opened new avenues in immunotherapy of cancer, HIV as well as other infectious diseases. A novel engineered antibody mimetic anticalin (lipocalin), derived from neutrophil gelatinase- associated lipocalin (NGAL), is a potential candidate for immunotherapy of cancer and infectious diseases by blocking the activity of CTLA-4. A combinatorial library of ~2×10^{10} variants was constructed by randomizing the positions of 20-aa in a structurally variable loop of NGAL. The mutant library was then subjected to phage display selection. Lipocalin (Lcn) selected by phage display competitively inhibited physiological interaction between CTLA-4 and B7.1/B7.2, and interestingly, selected lipocalins showed no cross-reactivity with CD28, a structurally related T-cell coreceptor [10]. The anticalin complex with its target CTLA-4 is shown in Fig. 2.

PRS-190, a bi-specific anticalin (Duocalin), was developed with the dual specificity to target IL-17 and IL-23 (members of Th17 cytokine family involved in autoimmunity and inflammation). DigA16 (H86N) anticalin functions as a digoxin antidote when administrated intravenously in rats, dramatically decreasing the free digoxin concentration in plasma and rapidly reducing its toxic effects [20]. Anticalins are also demonstrated to be suitable candidates for treatment of digitalis intoxications [21]. The other anticalin programs from Pieris Pharmaceuticals such as PRS-050, PRS-110, PRS-080 are developed to target VEGF-A, c-Met oncogene, and chemotherapy-induced anemia (CIA) and chronic kidney disease (CKD), respectively. The PRS-060 is an advanced anticalin program developed to target IL-4 for treating asthma (http://www.pieris.com/).

The E7 protein is well known for inactivation of pRb (tumor suppressor) and is a strong element involved in rampant growth of cervical cancer [22]. It is identified that inhibition and functional knockout of E7 protein leads to arrest of cell proliferation and/or cell growth and apoptosis. The intracellular protein E7 was the target for inhibition by anti-E7 affilin molecules, which were able to arrest cellular growth and were confirmed to be highly specific for E7+ mammalian cells [23].

It was demonstrated that different cytokines including IL-5, IL-6, IL-13, and TNFa, produced by cultured human mast cells, were cleaved by chymase [24]. Fynomers bind chymase with a KD of 0.9 nM and k_{off} of $1.1 \times 10^{-3}s^{-1}$ selectively inhibiting chymase activity with an IC_{50} value of 2 nM [25]. The D3 fynomer was discovered from a Fyn Src Homology3 (SH3) phage library that

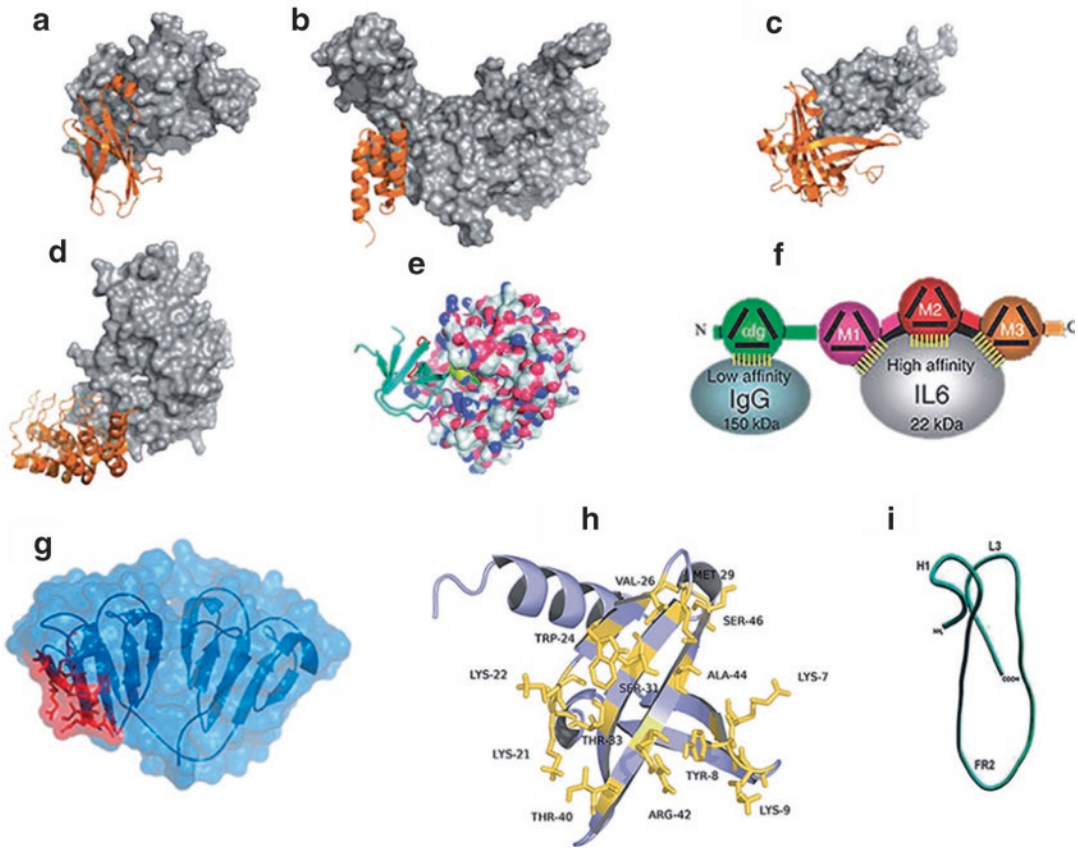

Fig. 2 Structures of antibody mimetics and their parent proteins. (**a**–**f**) Antibody mimetics in complex with their targets. (**g** and **h**) Parent proteins of the antibody mimetics. (**a**–**d**) the *orange color* represents the antibody mimetics while the targets are shown as *gray surfaces*. (**a**) Engineered Adnectin/Monobody (10Fn3) in complex with human estrogen receptor alpha binding domain. PDB: 2OCF. (**b**) Affibody molecule in complex with HER2 extra domain cellular region. PDB: 3MZW. (**c**) Anticalin in complex with the extracellular domain of Human CTLA-4. PDB: 3BX7. (**d**) DARPin in complex with aminoglycoside phosphotransferase. PDB: 2BKK. (**e**) Fynomer 4C-A4 (ribbon diagram) in complex with human chymase (space filling model). For Fynomer: The *magenta* represents RT-loop and *red* represents n-src-loop. The accession numbers for six Fynomer-chymase complexes in PDB are: 4afq, 4afs, 4afu, 4afz, 4ag1, and 4ag2. (**f**) Schematic representation of anti-IL6 Avimer (C326), in a tetramer construct. The first domain binds monovalently with human IgG in serum to prolong half-life while the remaining three domains bind to various epitopes on the surface of IL-6. (**g**) Representation of bovine g-B-crystallin, which is used to model the human g-B-crystallin scaffold (Affilins). The *red color* shows the eight selected amino acid residues (Positions 2, 4, 6, 15, 17, 19, 38, and 38) used to construct the library (PDB: 1AMM). The bovine molecule consists of 174 amino acid residues with a molecular weight of 20 kDa. (**h**) Schematic representation of wild-type Sac7d, the parent protein of nanofitins (PDB: 1AZP). (**i**) Ribbon diagram (model) of CDR-FR mimetics. The VHFR2 that links VHCDR1 and VHCDR2 in native Fab here plays the role of connecting VHCDR1 and VLCDR3, keeping them in a "quasi-physiological" binding site orientation (refer: 11, 12, 26, 27, 33, 46, and 47)

binds extra-domain B (EDB) but no other structurally related proteins [26]. The COVA301, a dual TNF/IL-17A inhibitor has also been developed, in which the fynomer against IL-17A was fused with an approved anti-TNF antibody [27].

Deregulation of IL-6 gene expression is implicated in the pathogenesis of several autoimmune diseases, e.g., rheumatoid arthritis and plasma cell neoplasias [28]. The functionality of avimer C326 in vivo was determined, and the results suggested that it completely abrogated acute phase proteins induced by human IL-6. The same mimetic showed no effect on acute phase proteins induced by human IL-1, demonstrating that the inhibitory effect of C326 is highly specific [11]. It was also demonstrated that mimetics against IL-6 with an IC_{50} of 0.8 pM were biologically active in two animal models.

Some well-known examples of proteases implicated in disease progression are the proteasome, HIV proteases and neutrophil elastase, for cancer growth and progression, HIV infection and cystic fibrosis, respectively [29]. Kunitz-types protease inhibitors are designed to address certain types of diseases: DX-88 and DX-890 have been developed to treat hereditary angioedema and cystic fibrosis with excellent inhibition of plasma kallikrein and neutrophil elastase, respectively [7], and recently DX-88 (Ecallantide) has been approved by the FDA for the treatment of hereditary angioedema [30].

The secretin Pu1D is a major component of Type II secretion systems (T2SSs) of gram-negative bacteria and it has gained much attention as a therapeutic target. The Pu1D binding nanofitins have been derived from Sac7d proteins and demonstrated to bind with the bacterial outer membrane secretin Pu1D, thus blocking the type II secretion pathway [31].

HUMIRA (Adalimumab), a human monoclonal antibody directed against TNFa was approved by the FDA to treat rheumatoid arthritis in 2002 and later for some other diseases. However, as HUMIRA suppresses the immune response; consequently, patients receiving HUMIRA treatment are also more prone to diseases like hepatitis B infections, allergic reactions, nervous system problems, heart failure and psoriasis [32]. Adnectins have been developed against the same pharmacological target but without aforementioned side-effects. Adnectins are mainly selected by phage, mRNA and yeast display technologies, and yeast two-hybrid techniques [33]. As another example, Adnectin Ct-322 binding to vascular endothelial growth factor receptor 2 (VEGFR-2) displays antitumor activities and results also suggest that adnectins can be developed for the treatment of various others diseases [34]. Adnectins are generated and selected to target Src SH3, Abelson (Abl) kinase SH2 domain lysozyme, TNF-α, and estrogen receptor a ligand [33]. Polyethylene glycol (PEG) has been used as a flexible scaffold molecule to link two Fabs together to generate Fab-PEG-Fab (FpF) molecule that is capable to act as IgG mimetic. Anti-VEGF and anti-Her2 FpFs molecules have successfully been prepared and evaluated. The prepared FpFs displayed similar affinities to their parent IgG molecule. In vitro antiangiogenic

properties of anti-VEGF FpFs were evaluated and it was found that these properties were comparable to or even better than bevacizumab (monoclonal antibody used to treat various cancers) [35].

The CDR-FR peptides retain the antigen recognition function of their intact parent molecule IgG but have superior capacity to penetrate solid tumors. The mimetics that are fused with the C-terminus of bacterial toxin Colicin Ia are called pheromonicins. Therapeutic efficacy of such fusion proteins was tested for their killing effects against Epstein-Barr virus (EBV)-induced BL, AIDS-related body-cavity lymphoma and nasopharyngeal cancer cells, and results showed the killing effects of PMC-EBV within solid tumors bearing specific surface antigens. The bacterial toxin used as a payload has many significant advantages such as solubility, heat stability and absence of cystine residues; through indirect ELISA and assessment in normal mice, it was also shown that the cancer killing toxin was non-immunogenic [12]. The mimetic-Fc small antibodies were generated by using CDR and FR sequences from trastuzumab, a humanized anti-HER2 monoclonal antibody, fused with the Fc domain of IgG. The designed fully functional mimetic-Fc small antibody called HMTI-Fc successfully inhibited the binding of trastuzumab with HER2-overexpressing SK-BR3 cells, thus showing its potential to treat cancer [36]. The Fc part of the antibody participates in recruiting the immune cells in antibody-dependent cell-mediated cytotoxicity (ADCC) [37], and intriguingly the HMTI-Fc effectively mediated ADCC against HER2-positive breast-cancer cells [36]. Other anti-HER2 [38] and anti-CD4 [39] antibody mimetics known as DARPins have also been developed, and anti-CD4 DARPins with pM affinity blocked the entry of HIV into cells by competing with binding of gp120 to CD4. The CD4+ cells are a type of white blood cell (lymphocyte) and are critical to the immune system. The MP0112 DARPin is perhaps the most advanced program, and has been developed as a VEGF-A inhibitor (IC_{50} less than 10 pM) to treat ocular neovascularization. MP0112 has been demonstrated to be safe and well tolerated in wet age-related macular degeneration (wet AMD) and diabetic macular edema (DME). The therapeutic effect of MP0112 lasted for 16 weeks and several studies have revealed that MP0112 is long-acting and highly efficacious [40].

4 Applications of Antibody Mimetics Diagnosis and Imaging

Antibody mimetics could be labeled and used to image metabolite pathways, intracellular targets such as kinases and polymerases, and other proteins associated with cancers. Studies have revealed that affibodies are promising among the tracers for HER2-specific molecular imaging [41]. ZHER2:342 affibody molecules with the chelator sequence of maEE were synthesized and labeled

with technetium-99 m. The synthesized molecule 99mTc-(mercaptoacetyl-Glu-Glu-Glu) maEEE-ZHER2:342 appeared to be a better tracer for clinical imaging of HER2 overexpression in tumors and metastases [42]. Affibodies have been used in protein capture microarrays and due to their high specificity they can be used for affinity capture in analyses of complex samples, e.g., human serum or plasma [5]. Anticalins, due to their small size when conjugated with radioactive isotopes, can be used for in vivo diagnostics [3] and images of high contrast have been obtained soon after administration [43]. Anticalin C26 was developed with high binding affinity for rare-earth metal–chelate complexes, and further improvement in this anticalin by in vitro selection yielded CL31 with fourfold slower dissociation (more than 2 h). Oncofetal isoform of extracellular matrix protein fibronectin carries the EDB and is exclusively expressed in neovasculature, and has gained significant interest for tumor diagnosis. The human Lcn2 has been employed as a small non-immunoglobulin scaffold to selecting EDB-specific anticalins, and anticalins showing low nanomolar affinities for EDB were isolated and biochemically characterized. When these isolated anticalins were used in immunofluorescence microscopy, they showed specific staining of EDB positive tumor cells, and the analysis of BIAcore affinity data showed that they recognized distinct epitopes of EDB, suggesting that these EDB specific anticalins could provide potential biomolecules both in research and biomedical drug development [44].

The CDR-FR peptides have been used for in vivo fluorescence imaging and these antibody mimetics also conferred enhanced intracellular delivery, thus rendering the mimetics potent candidates for cancer diagnostic applications [12]. The nanofitins have been designed to selectively bind to a wide range of targets and have been reliable tools for targeting (immunolocalization, in vivo neutralization), capture (affinity chromatography, protein removal) and detection (immunoassays, western blot). The DARPins H6-2-B3 and H6-2-A7 have been used for in vivo tumor imaging and tumor targeting and were shown to localize at the tumor [38].

5 Future Prospects for Antibody Mimetics

Due to their high target retention, rapid tissue penetration and blood clearance, antibody mimetics are gaining importance both in therapeutics and diagnostics, especially in tumor targeting and treatment. Antibody mimetics can be generated against a range of biomarkers associated with specific diseases for the development of electronic and other formats of multiplex biosensors, reagents for detection in routine immunological analysis such as ELISA and Western blot. Other small molecules called aptamers (about 10 kDa) [45] have attracted the attention of scientific community

due to their merits such as thermal stability, cost-effectiveness, and unlimited applications. Therapeutic efficacy and continuing advances in the production of human-derived molecules suggest a promising future for antibody mimetics; however, some questions remain relating to both therapeutic and diagnostic uses, principally their short half-life. The mimetics exhibit shorter half-lives because they lack the Fc region and have much lower molecular weights. However, when antibody mimetics are engineered with some functional antibody part such as Fc [36], they better mimic the real antibody and combine the advantages of both natural antibodies and antibody mimetics. With the recent advancements of bioengineering, the biological activity of mimetics can be increased by many-fold. Despite their reduced size and increased affinity, the effects of mimetics in treating diseases other than solid tumors and autoimmune diseases still need to be further assessed.

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