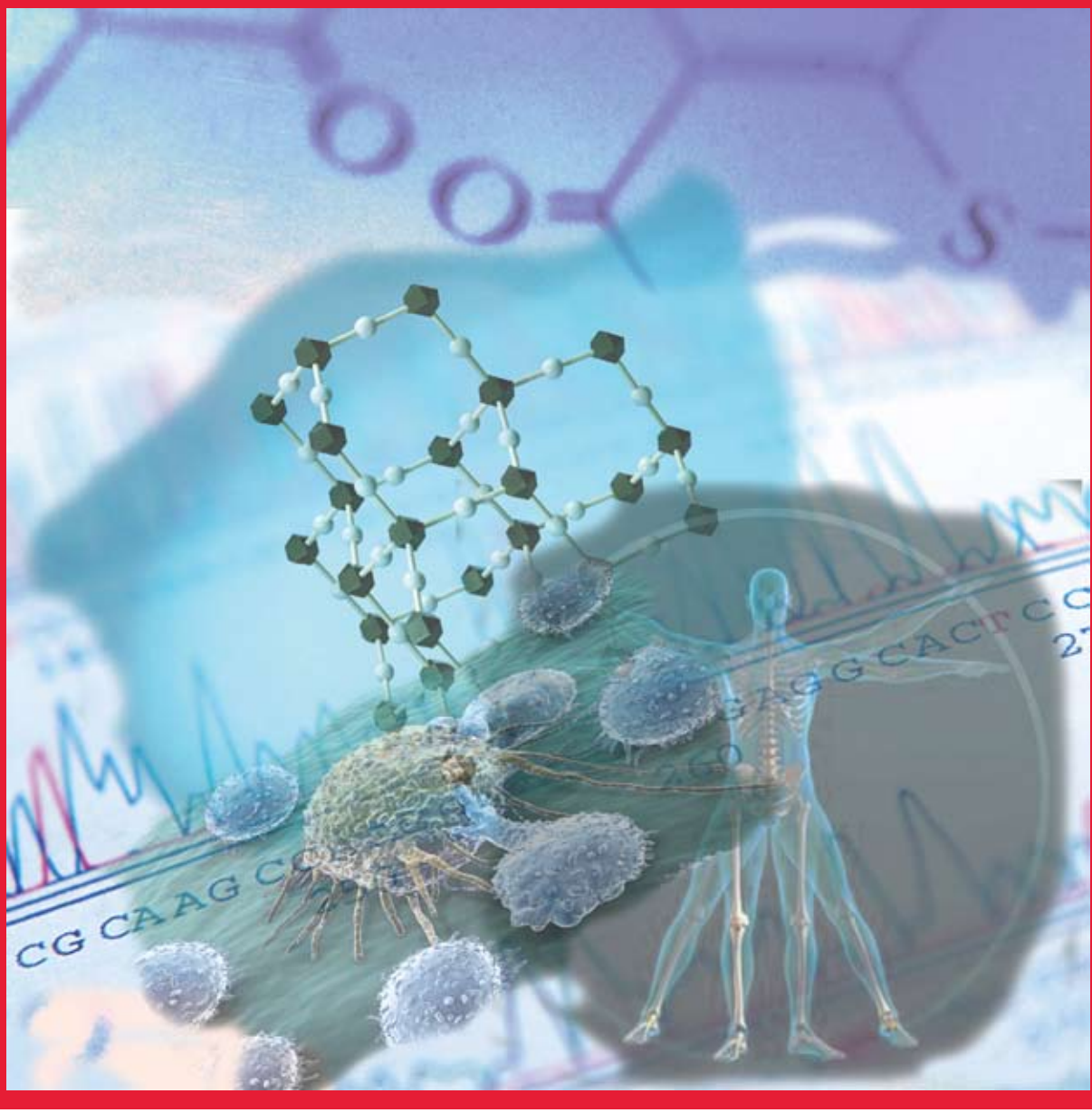
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Man-made antibodies and immunoconjugates with desired properties: function optimization using structural engineering

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The review outlines progress and problems in the design of non-natural antibodies for clinical applications over the past 10–15 years. The modular structure of natural antibodies and approaches to its targeted modifications and combination with other structural elements and effector molecules are considered. The review covers modern methods for immunoglobulin engineering and promising strategies for the creation and applications of monoclonal antibodies, their derivatives and analogues, including abzymes and scaffolds, oriented to the use in the diagnosis and targeted therapy of cancer and other socially significant diseases.
The bibliography includes 225 references.

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I. Introduction

In the past decade, tremendous progress has been made in the construction of monoclonal antibodies (mAbs) and their derivatives for the diagnosis and therapy of cancer and other serious autoimmune and infectious diseases. About 40 monoclonal antibody-based drugs have been approved for the clinical use,[1,2] their number being doubled during the last five years. Besides, the percentage of drugs intended for the treatment of a spectrum of non-tumour diseases significantly increased, and two antitumour immunotoxins were licensed for the first time for use in clinical practice.[3] Dozens of drugs based on mAbs and their derivatives, which are intended for the treatment of such serious diseases as stomach cancer, hepatocellular carcinoma, multiple sclerosis, Alzheimer's disease, osteoporosis, hemophi-

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lia A and B, homozygous familial hypercholesterolemia, asthma, chronic obstructive pulmonary disease and so on, are in the last phase of clinical trials.[4]

Historically, tumours were the main targets for such drugs, and antibodies, owing to their high specificity, were regarded as a 'magic bullet' that would selectively hit disease focus in the the human body (see Paul Ehrlich's Nobel Lecture,[5] 1908). However, it took a century for this genius idea to be realized after tremendous research efforts, primarily due to the work by Köhler and Milstein published in 1975,[6] which laid the foundation for hybridoma technique for mAb production. The hybridoma technology can be used to produce, as a result of the immune response to a particular antigen, an antibody of a single specificity (mAb), which is directed against one specific antigen, instead of a set of various immunoglobulin molecules (natural antibodies). This technology remains the keystone in antibody engineering.[7]

First attempts to use mouse mAbs for clinical purposes failed and revealed the following seemingly insurmountable disadvantages of mAbs: in some cases, low affinity as compared to polyclonal antiserum; high immunogenicity and, as a consequence, rapid elimination from the body; inability to activate the complement system and cellular immune response mechanisms in a foreign system. The unique potential of immunoglobulins, which have a modular structure and functions related to certain structural modules, was realized and modifications of antibodies for various clinical applications became possible due to advances in protein and carbohydrate chemistry, as well as owing to genetic and protein engineering methods and techniques for the creation of transgenic animals.[8–12] Depending on the practical problem, researchers can vary the size of antibodies, their specificity, affinity and valency, reduce the immunogenicity and optimize the pharmacokinetic properties and effector functions.[10, 13–18]

Due to high affinity and specificity for antigens, mAbs can be used not only as individual therapeutic agents but also as targeting modules providing the targeted delivery of diagnostic and therapeutic agents or own immune system cells to target cells. For this purpose, mAbs and their fragments are chemically conjugated to toxins or radioisotopes and are produced as recombinant-fused proteins, which include antibodies of another specificity, cytokines, protein toxins, enzymes and fluorescent proteins.[19–24] A great diversity of methods and approaches for mAb engineering fully reflects the universality of natural antibodies as platforms for the design of therapeutic agents for diagnosis and targeted therapy.[11]

The development of protein engineering of antibodies takes place in parallel with the growing interest in the design and application of recombinant binding molecules based on alternative scaffold proteins (scaffolds). The use of these proteins is particularly important when the application of immunoglobulins is limited by their large size and insufficiently high stability. In addition, the expression in a bacterial system significantly reduces the cost of the production of such reagents as compared to full-size antibodies. This class of proteins is often referred to as antibody mimics because, despite the different structural basis of recombinant binding proteins and designed antibodies, modern strategies for their production and fields of applications are, to a large extent, the same. Scaffolds successfully and, in some cases, more efficiently perform some functions of antibodies, in particular, therapeutic. Therefore, it seems reasonable to include a brief consideration of this class of compounds in the present review.

In the last decade, the engineering of monoclonal antibodies for clinical applications has been widely discussed and was covered in numerous reviews published in international and Russian journals (see for example, Refs 1, 2, 11, 12, 14 and 23). A number of reviews concerned with certain problems related to the improvement of physicochemical and therapeutic properties of mAbs, the reduction of their side effects and the search for new targets.[9, 10, 24–27] It should be noted that certain aspects, for example, new methods for the site-specific conjugation of toxins to antibodies were described in detail, whereas little attention has been given to fully genetically encoded immunotoxins and the use of mAbs as targeting modules for supramolecular multifunctional structures. The present review covers main approaches and methods of engineering of monoclonal antibodies, their derivatives and analogues, including abzymes and scaffolds, for the diagnosis and targeted therapy of cancer and other serious diseases, which were published in modern literature and developed by the authors of this review.

II. Modular structure of natural immunoglobulins. Shortened antibody fragments

Natural antibodies, or immunoglobulins (Ig), are highly specific protein molecules, which have a complex modular structure and can recognize and eliminate foreign antigens. This modular organization of antibodies, which possess a number of functions related to individual structural modules, allows the body to respond rapidly to foreign invasion. It should be noted that immunoglobulins are very stable molecules, due to which they can circulate in the bloodstream and perform their functions over a long period of time.

Different classes of antibodies in mammals are structurally similar and are tetrameric glycoproteins with two antigen-binding sites [see, for example, human immunoglobulin G (IgG), Fig. 1]. Immunoglobulins are complex Y-shaped molecules consisting of two identical heavy chains ($\sim$50 kDa) and two identical light chains ($\sim$25 kDa). The molecule is stabilized by four interdomain disulfide bonds. Individual globular domains have a characteristic β-fold, are stabilized by intradomain disulfide bonds and are divided into variable (V) and constant (C) domains. The N-terminal light and heavy chain domains, so-called variable domains VL and VH, substantially vary in different antibodies, whereas the other parts of both polypeptide chains referred to as constant domains CL and CH, are different in different classes of antibodies and are species-specific for antibodies of one class. The structural domains of immunoglobulins are not only spatially separated but also perform different functions in the course of the immune response.

The unique specificity and high affinity of an individual antibody for the self antigen (dissociation constants (K_D) are 10^{-8}–10^{-11} M) are provided by the complementarity of the antigen-binding site of the antibody and a particular region of the antigen molecule (epitope). The antigen-binding site is composed of two variable domains, VH and VL, and each domain consists of three hypervariable complementarity determining regions (CDR) responsible for the

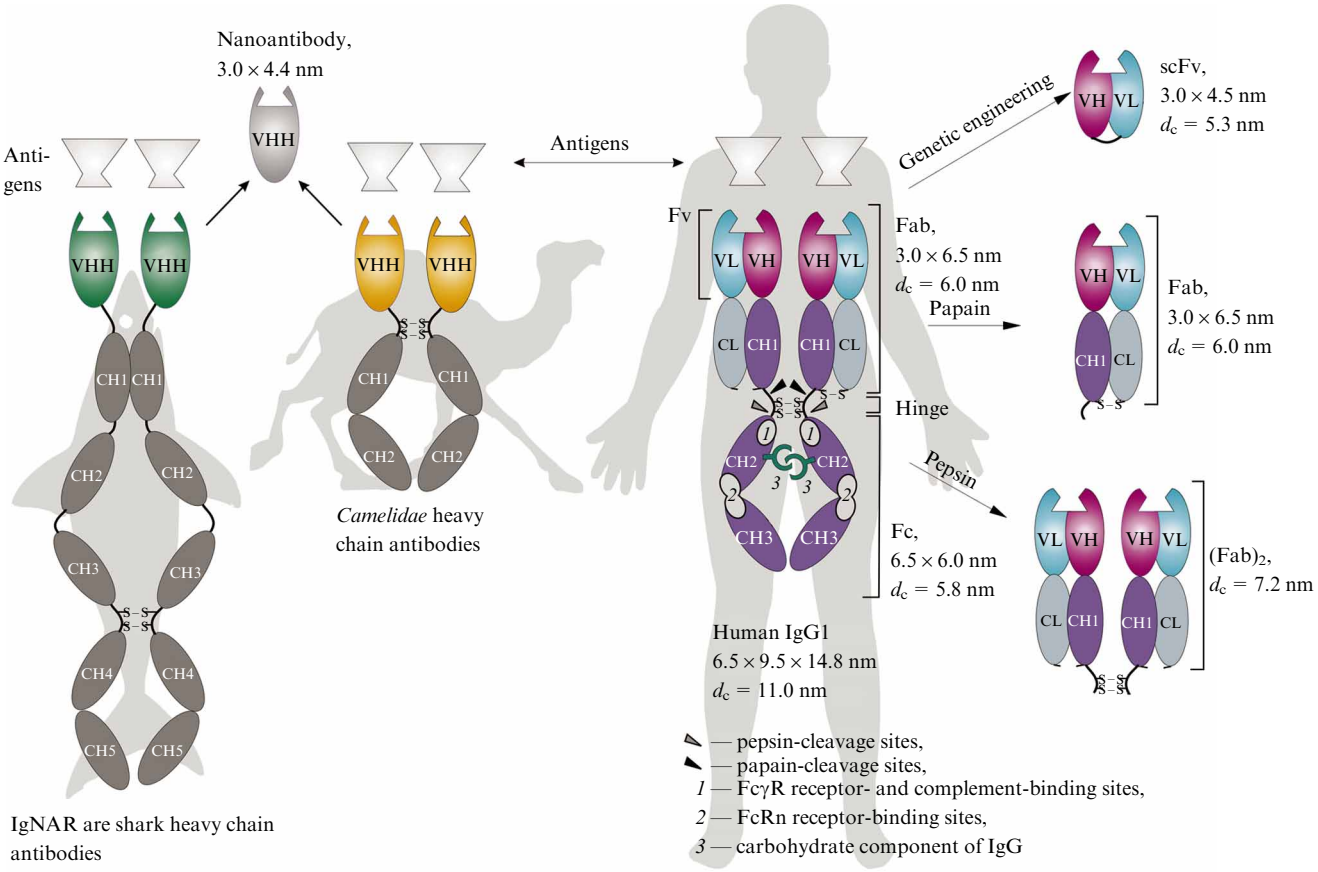


Figure 1. Natural antibodies and their fragments.[15]
Fab and $(Fab)_2$ are the antigen-binding IgG fragments produced by papain and pepsin digestion, respectively; Fc is the C-terminal part of IgG composed of the heavy chain constant domains CH2 and CH3 responsible for effector functions; Fv is the variable domain composed of variable light chain (VL) and heavy chain (VH) fragments; scFv is the single-chain variable domain composed of VL and VH conjugated by the genetic engineering technique; VHH is the nanoantibody, the variable domain of heavy chain antibodies emerged in cartilaginous fishes and animals of the *Camelidae* family; S—S is a disulfide bond. The linear dimensions of antibodies and their fragments were measured by atomic force microscopy; the hydrodynamic diameter d_c was calculated by the Stokes–Einstein equation.

molecular recognition and the complementarity of the interaction. Each CDR is surrounded by framework regions (FR), which are not directly involved in the antigen binding but perform the structure-forming role, providing the correct three-dimensional functional topography of CDR and stabilizing the structure of the antibody variable fragment Fv *via* contacts at the VH/VL interface.

The ability of antibodies to bind simultaneously two antigens results in a substantial increase in their functional affinity (avidity), as well as in the retention time on cell surface receptors and other polyvalent antigens. A total of five immunoglobulin classes (IgM, IgG, IgE, IgD and IgA) were found in mammals. Two classes can form dimers (IgA) and pentamers (IgM) capable of efficiently neutralizing pathogenic microbes and preventing their penetration into tissues. Some organisms (camels, llamas, sharks) have non-canonical antibodies completely devoid of light chains with two antigen-binding sites, each site being formed by a single variable heavy chain domain. The camel variable domains are referred to as nanobodies (see Fig. 1).

The light chains of immunoglobulins have one constant domain (CL), and their heavy chains consist of three or four (depending on the antibody class) constant domains (CH). The hydrophobic regions located between the CH1 and CH2 domains retain relative flexibility and form a hinge region, which provides the displacement of the Fab fragments and their rotation about the hinge. The constant heavy chain domains are usually glycosylated. The type of glycosylation substantially varies in different organisms and influences the effector functions of immunoglobulins. Moreover, even antibodies of the same isotype in one type of organisms may have heterogeneous glycosylation patterns. The CH2 and CH3 domains form a crystallizable fragment (Fc), which mediates the effector (secondary) functions of immunoglobulin G. The Fc fragment contains binding sites for the C1q protein of the complement system and the cellular receptors for the Fc fragments (FcγR). The interactions of IgG with C1q and FcγR trigger the mechanisms of complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) (Fig. 2). The ability of the constant domains of immunoglobulins G and M to bind the neonatal Fc receptor (FcRn) plays an important role in the control of the level of antibodies and the duration of their circulation in the serum.

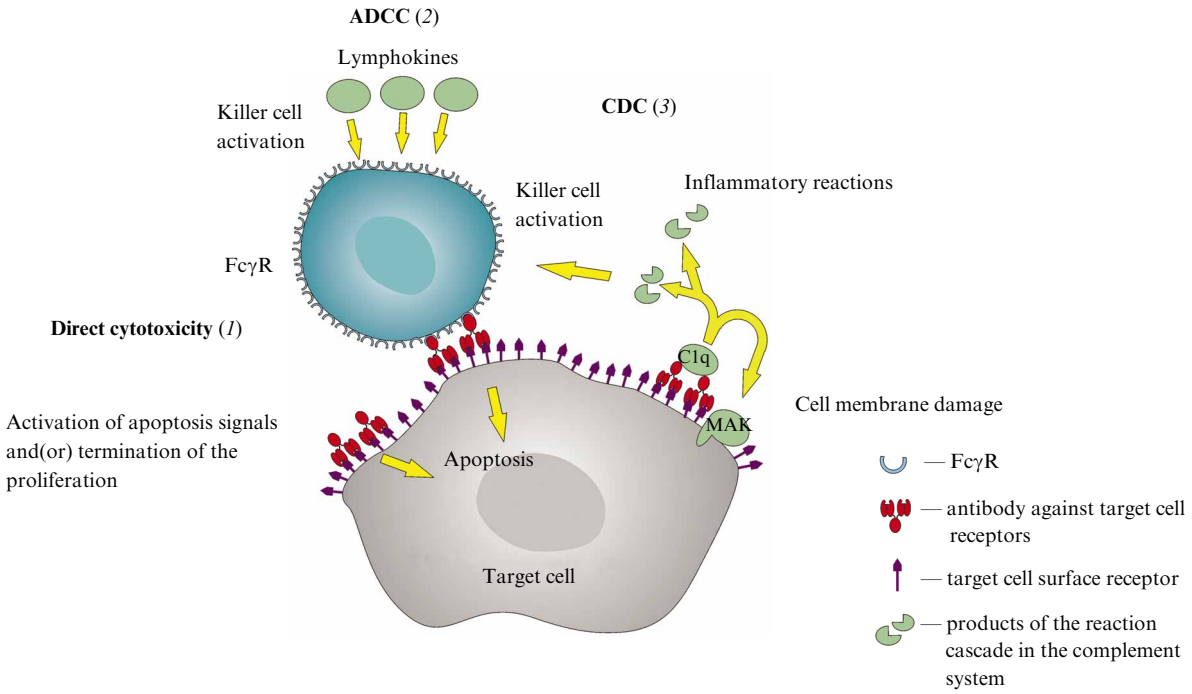


Figure 2. Interaction pattern of unloaded antibodies with a target cell.[1]
(*1*) Direct cytotoxicity. Antibodies can induce apoptosis or terminate the proliferation of target cells by binding to membrane antigens on their surface; (*2*) antibody-dependent cellular cytotoxicity (ADCC). Killer cells bearing the receptors FcγRI (CD16), FcγRII (CD32) and FcγRIII (CD64) for IgG constant domains (natural killers, lymphokine-activated killers, macrophages and phagocytes) and the receptors FceRI and FceRII (CD23) for IgE constant domains (acidocytes) attack the target cell, the surface antigens of which bind antibodies; (*3*) complement-dependent cytotoxicity (CDC). Antibody pairs bind to the protein complex C1q and trigger a cascade of reactions in the complement system, resulting in the membrane damage. Certain products of this reaction cascade involve immune cells or cause anaphylactic shock.

The immune response in the body gives rise to a complex set of various immunoglobulin molecules, which can trigger several cellular mechanisms of elimination of a foreign antigen and the relevant cascade of biochemical reactions intended for this purpose. The diversity of antibodies is generated through recombination of V(J)D segments of the encoding gene,[28] as well as through numerous somatic mutations in hot spots of the antibody gene during affinity maturation *in vivo*.[29] These processes were described in detail in the review.[18]

The hybridoma technology proposed in 1975 by Köhler and Milstein [6] made it possible to produce, as a result of the immune response to a particular antigen, one type of immunoglobulin molecules specific for this antigen (monoclonal antibodies), rather than a complex set of antibodies of different composition characteristic of polyclonal serum. This work laid the basis for the molecular engineering of antibodies and provided a further progress in the use of antibodies for both research and practical purposes (diagnosis *in vitro*). However, at early stages, the expectations of researchers to design specific and highly effective drugs based on mAbs and antibodies conjugated to active agents and to substitute serum-derived vaccines with mAbs against pathogenic microorganisms and their toxins were not met. The use of mAbs for the delivery of radioactive isotopes to target cells was not highly effective.[30]

First-generation monoclonal antibodies produced from a pool of mouse hybridomas have shortcomings, which make the use of mAbs in clinical practice ineffective and hazardous. Interactions between individual mAbs and antigens are often much less efficient as compared to the polyclonal antiserum titre. This is due to specificity for a single epitope of the antigen and probably due to partial inactivation of antibodies during their isolation. Poor pharmacokinetic properties of full-size mAbs interfere with their use as targeting molecules. The xenogenic nature of mAbs results in their rapid catabolism in the human body and impedes the involvement of the complement system and activation of cellular mechanisms of elimination of the foreign antigen (CDC, ADCC, ADCP, see Fig. 2) due to the poor recognition of mouse mAbs by Fc receptors of the the human immune system cells. Because of high immunogenicity of mouse mAbs in human patients, the clinical application of mAbs is extremely hazardous since the repeated administration may cause hyperimmune reactions up to anaphylactic shock. In addition, serious side effects related to the nature of the bound toxin were observed for first-generation immunotoxins. Therefore, it became evident that modifications of mAbs, often mutually opposite, are required for particular clinical applications (Table 1).

Shortened antibody fragments, monomeric Fab and dimeric $(Fab)_2$, intended for the application in radioummunotherapy as targeting agents were produced by the treatment of intact IgG by the proteolytic enzymes papain and pepsin, respectively (see Fig. 1). Since these fragments are devoid of the Fc domain, they do not exhibit effector properties of IgG (see Section III.3), which are undesirable in this case, and are incapable of recirculating from lysosomes, resulting in a decrease in the dose-limiting myelotoxicity and the nonspecific radioactive background as

Table 1. Necessity for optimizing the properties and functions of monoclonal antibodies by molecular engineering methods.

Type of modification	Therapeutic implication of mAbs	Diagnostic implication of mAbs: *in vitro* diagnosis/ *in vivo* visualization
Immunogenicity	yes	no/yes
Binding to an antigen	yes	yes/yes
Binding to FcγR receptors	yes	no/no
Effector functions: ADCC, ADCP, CDC	yes	no/no
Physicochemical characteristics: solubility, stability, chemical resitance, charge (isoelectric point)	yes	yes/yes
Pharmacokinetics: half-life in the body (blood serum)	yes	no/yes
Biochemical properties: glycosylation	yes	no/no
Conjugation with other molecules (effector, visualizing)	no and(or) yes	yes/yes

compared to full-size IgG. It should be noted that shortened antibody fragments retain the parent three-dimensional structure and antigen-binding properties. The main disadvantages of the enzymatic method for the production of antibody fragments are the complicated purification of the desired product and the impossibility of the further optimization of physicochemical properties of the antibody without employing chemical modifications. Besides, first-generation Fab fragments are of mouse origin and retain high immogenicity, despite the removal of a larger part of constant domains.[31, 32]

Another type of truncated antibody derivatives, so-called single-chain variable fragments (scFv) or mini-antibodies, were produced by genetic engineering techniques. Mini-antibodies are encoded by a single gene and contain one antigen-binding site composed of variable light chain (VL) and heavy chain (VH) domains connected by a flexible peptide linker[33] (see Fig. 1). Mini-antibodies are monovalent and often have lower affinity than the parent antibody. Due to the rapid clearance and the homogeneous localization at the target, mini-antibodies are more suitable for the delivery of radioisotopes than the parent full-size IgG. Another very important advantage of single-chain antibodies is their compatibility with bacterial expression systems and lower immunogenicity.

The cloning of single-chain antibodies with the retention of antigen-binding functions had extremely important consequences. From that time, great progress has been made in the development of technologies for the production of recombinant antibodies of virtually any specificity. Various collections (libraries) of recombinant antibody fragments were constructed, modern methods were proposed to improve their diversity and technologies allowing for an increase in the valency[15] and affinity of antibodies and for the efficient selection of required variants were developed.[34, 35] Selection platforms for immunoglobulin libraries were elaborated using phage, yeast and ribosome display.[18, 36, 37] Non-canonical antibodies of cartilaginous fishes and animals of the *Camelidae* family (camels and llamas) (see Fig. 1), in which a single antigen-binding site has good solubility, high stability and ability to bind cryptic epitopes, serve as a promising source of antigen-binding fragments with unusual properties.[38, 39]

The constant fragment Fc was also subject to antibody engineering, which made it possible to utilize the powerful potential of mAbs more fully, *i.e.*, employ, if necessary, their effector functions.[9, 17]

III. Targeted modifications of monoclonal antibodies for optimization of their functions

III.1. Reduction of the immunogenicity of mAbs for clinical applications

Almost all therapeutic agents of the protein nature, including monoclonal antibodies, possess, to a certain extent, immunogenicity. Many factors can influence the immunogenicity of protein therapeutics, including external factors, such as the route, dose and frequency of drug administration, the state of the immune system of the patient and interactions with other drugs, as well as the drug-inherent factors related to the characteristic properties of a particular protein medication.[40] One of the first and serious problems encountered in the construction of antibodies for clinical applications is high immunogenicity of mAbs xenogenic to a human recipient[31] due to the foreign type of glycosylation and other species-specific post-translational modifications, as well as due to the presence of variable and constant domains of antibody molecules of T-cell epitopes, the presentation of which on CD4-positive T cells (T helpers) activates the immune response process.[41] The human anti-mouse antibody response (HAMA response) causes severe systemic reactions up to anaphylactic shock.[31, 42] Therefore, one of the main goals of engineering of antibodies for clinical applications is to reduce their immunogenicity by the partial substitution or removal of certain regions of the molecule of a foreign nature (antibody chimerization and humanization) or by the identification and removal of T-cell epitopes (deimmunization).

Approaches involving genetic engineering techniques, which were proposed back in the 1980s, made it possible to partially replace immunogenic sites of mouse mAbs (Fig. 3, *1*) by the corresponding fragments of human antibodies, thus substantially reducing their immunogenicity. In chimeric mAbs (Fig. 3, *2*), all constant domains of both chains of the mouse antibody were replaced by the constant domains of human immunoglobulin (the percentage of human amino acid sequences in the amino acid sequence of the recombinant antibody, *i.e.*, the degree of humanization, is $\sim$70%).[43, 44] In humanized mAbs (Fig. 3, *3*), only hypervariable regions responsible for complementary interactions with the antigen (complementarity determining regions, CDR) are of mouse origin (the degree of humanization is $\sim$90%).[45] Single gene-encoded constructs of humanized antibodies can also be produced using genetic engineering techniques.[46] Currently, fully mouse antibodies are used in low doses only for radioimmunotherapy due to the necessity of the rapid clearance from the bloodstream.[1] Although these technologies for reducing the immunogenicity are commonly used in antibody engineering, the clinical applications of chimeric and humanized mAbs are still associated with a complex of severe immune responses to their administration in patients.[32] Therefore, an urgent problem is to develop methods for the construction of

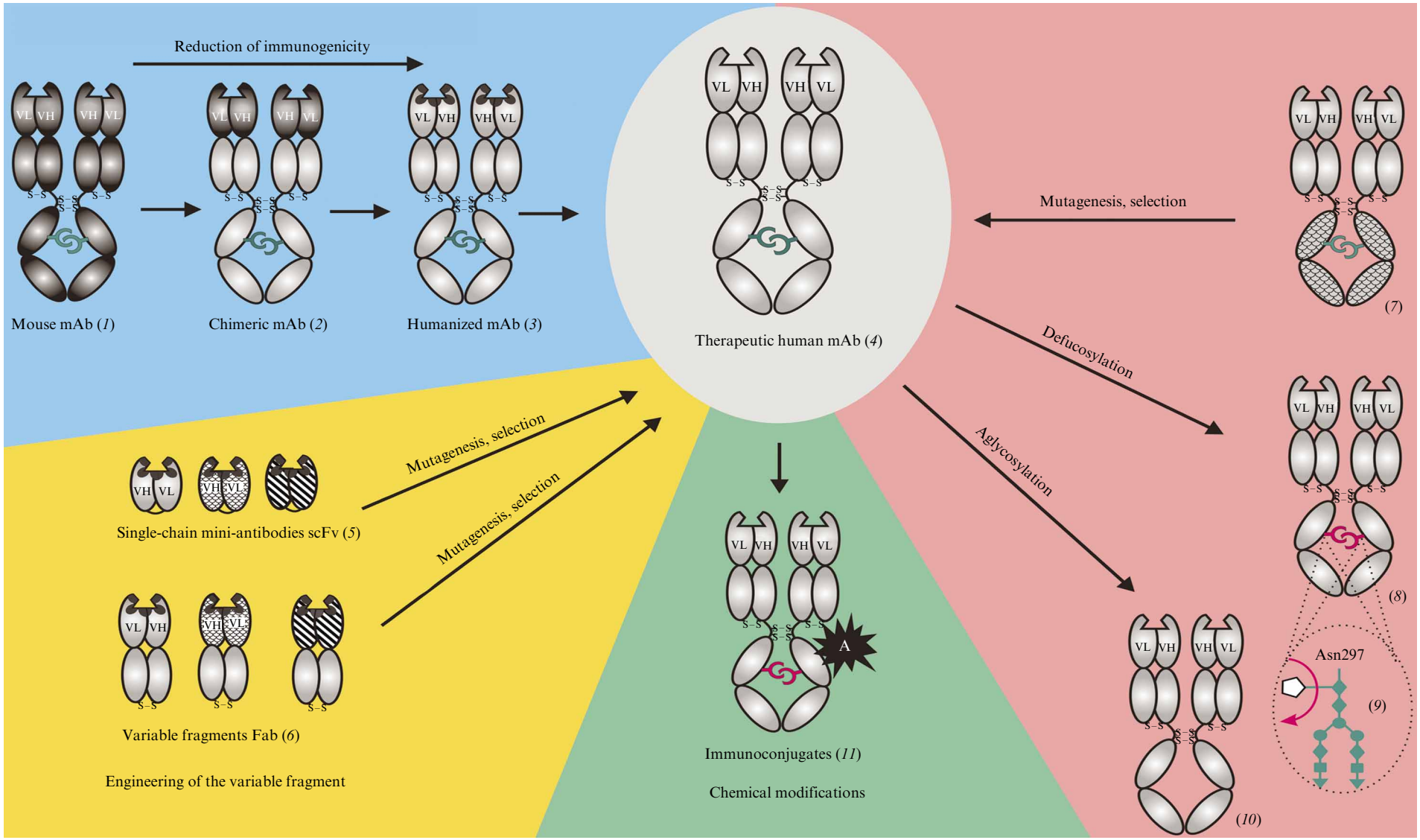


Figure 3. Main modifications of monoclonal antibodies for clinical applications.
Human mAb containing modified fragments (*7*), defucosylated human mAb (*8*) containing a modified carbohydrate component (*9*), aglycosylated human mAb (*10*), mAb chemically conjugated to a biologically active agent (*11*). The notations of antibodies and their fragments are the same as in Fig. 1; the hypervariable regions of variable domains (CDR) are in black, the mouse antibodies and their fragments are in dark gray, the human antibodies are in light gray, the variable domains of different specificity (*5*, *6*) and modified constant domains (*7*) are dashed. A is a biologically active agent (toxin, enzyme, fluorescent protein, *etc.*).

fully human mAbs (Fig. 3 *4*) having the antigen-binding affinity as high as that of mouse mAbs produced by hybridomas.[37, 47, 48]

There are diverse technologies for the production of fully human mAbs.[10] Methods for screening scFv or Fab fragments of antibodies of desired specificity (see Fig. 3 *5*, *6*) from combinatorial libraries (phage, ribosome and yeast display) followed by the reconstruction of human full-size immunoglobulins do not require the preliminary immunization. For instance, the drug Adalimumab (Humira®) commonly used in the therapy of autoimmune diseases was initially produced as the scFv fragment by the Cambridge Antibody Technology (MedImmune) and was then reconstructed to the full-size antibody.[12] Fully human mAbs can be produced also in transgenic mice expressing genes of human immunoglobulins.[48] The technology involves the replacement of mouse IgG gene loci by the corresponding human genome regions. An example is the therapeutic anti-EGFR (epidermal growth factor receptor) agent Panitumumab (Vectibix®), which was constructed using the HuMouse technology (Abgenix).

This field of antibody engineering is still being actively developed, and the whole diversity of approaches applied to humanize antibodies can be arbitrarily divided into rational and empirical.[49] The rational approaches involve the so-called design cycle, including the generation of a few variants based on the data on the structures of the antibody and its gene followed by their analysis and the selection of the hits. The rational methods involve the following procedures: the grafting of hypervariable regions of mouse mAbs responsible for the complementarity of the interaction (CDR grafting) or only of their small part (20% – 30%) responsible for the specificity (SDR grafting) onto the human immunoglobulin framework;[50] the resurfacing of mouse mAbs to make them complementary to the surface of human immunoglobulin;[51] the superhumanization based on the detection and elimination of potential epitopes from the mouse immunoglobulin molecule for the major histocompatibility complex and T cells (human string content optimization);[52] the production of human antibodies by mutagenesis of amino acid residues in hypervariable regions of the mouse antibody and in the vicinity of these regions based on the semi-automatic computer-assisted analysis of the relevant sequences from the human embryonic cell line.[47] For example, three human mAbs recognizing dif-

Table 2. USAN nomenclature of therapeutic medicines with antibodies and their fragments.[a]

Prefix	Disease or target class	Source/origin of antibody	Suffix	Examples
Distinct compatible syllable(s)	**vir** — viral **bac** — bacterial **lim** — immune system **les** — infectious lesion **cir** — cardiovascular **fung** — fungal **ner** — nervous system **kin** — interleukins **mul** — musculoskeletal system **os** — bones **toxa** — toxins **anibi** — angiogenesis *Tumours* **col** — colon **mel** — melanoma **mar** — mammary **got** — testis **gov** — ovary **pro** — prostate **tum** — misc	**u** — human **o** — mouse **a** — rat **e** — hamster **i** — primate **axo** — rat/mouse chimera **xi** — chimeric **zu** — humanized **xizu** — humanized	**mab**	
ca	**pr**	**o**	**mab**	**Capromab pendetide** (Prostascint®) — ^{111}In-labeled monoclonal mouse antibody conjugate for diagnosis of prostate cancer
ab	**ci**	**xi**	**mab**	**Abciximab** (ReoPro®) — chimeric mAb for prevention of coronary thrombosis
tras	**tu**	**zu**	**mab**	**Trastuzumab** (Herceptin®) — humanized mAb against tumours
cana	**kin**	**u**	**mab**	**Canakinumab** (Ilaris®) — fully human anti-interleukin IL1beta mAb

Note. To create a unique name, a distinct, compatible syllable or syllables should be selected as the starting prefix; then key elements of a monoclonal antibody name should be added in the following order: an infix representing the disease (tumour type) or target class, an infix indicating the source and the stem used as the suffix mab; to simplify the pronunciation, the last consonant in the infix representing the target can be omitted. [a] USAN stands for US Adopted Names; www.ama-assn.org.

ferent therapeutic targets (the proteins CD25, VEGF and TNFα) were produced by the replacement of 59, 46 and 45 residues, respectively, in the variable domains of the parent mouse mAbs. It should be noted that some of these substitutions were made in the CDR regions, which usually remain unaltered. The *in vitro* functional activity of the constructed fully human mAbs is comparable with that of the relevant therapeutic humanized or chimeric mAbs (Daclizumab, Bevacizumab and Infliximab, respectively).[47]

In recent years, camel single-domain antibodies (nanobodies) (see Fig. 1) have drawn the attention of researchers because, due to the unique physicochemical and immunochemical properties, nanobodies can serve as an efficient tool for biomedical research, diagnosis and therapy.[38, 53] In order to use nanobodies in clinical practice, the general strategy was developed for their humanization.[54] Researchers revealed amino acid residues responsible for the differences between nanobodies and the relevant variable heavy chain domain of human IgG. Then, in order to make the structure of nanobodies as similar to that of the corresponding domain of human immunoglobulin as possible, nanobodies were extracted from the extensive library and humanized by the fine tuning using the sequential replacement of some regions in the molecule. The resulting nanobodies remained stable and retained the initial affinity.

As opposed to rational methods, empirical methods of antibody humanization are based on the creation of large combinatorial libraries[34, 35, 37] and the selection of the required variants using such processing technologies as the phage, ribosome or yeast display or employing high-throughput screening.[36] Most of these methods are applicable to single-chain antibodies (scFv), which can, if necessary, be transformed into single gene-encoded mAb[46] through the fusion with the Fc constant domain and the dimerization and be reconstructed to the full-size antibody of a canonical structure.

In practice, the rational and empirical approaches usually complement each other. Currently, a number of humanized and human mAbs obtained using new selection methods are in the last phase of clinical trials.[4]

The nomenclature of therapeutic agents approved in the USA (US Adopted Names; www.ama-assn.org) is employed in the construction of antibodies for clinical applications. This nomenclature indicates the origin or the degree of humanization of a particular antibody, as well as the target disease (Table 2).

III.2. Engineering of mAb variable domains

In terms of the design of a magic bullet, the most attractive property of antibodies is their specific binding to an antigen, which should provide the targeted effect of the antibody or the delivery of the active agent (for example, a radioactive isotope or toxin) to the target cell. The affinity and specificity of the antibody for the antigen, as well as its physicochemical properties, such as the valency, surface charge, size and stability that influence the pharmacokinetics, play a key role in the efficacy of the antibody as a therapeutic agent or a targeting agent.[15, 55] Consequently, the goals of molecular engineering of antibody variable domains are to perform targeted modifications and optimization of the binding parameters (affinity and specificity), enhance the stability of the variable fragment and improve the pharmacokinetics of the antibody.

III.2.a. Improvement of antibody affinity

During antibody affinity maturation, rather high antigen-binding affinity values characterized by the dissociation constant in the range of 10^{-7}–10^{-10} M can be achieved in the mammalian immune system *in vivo* due to a secondary immune response.[29] Primary antibodies produced by the recombination of the V(J)D segments in the encoding gene (see above) possess rather low affinity and moderate specificity for the self antigen. The multistep process involving the generation of the diversity of antibody variants through spontaneous somatic hypermutations of the variable fragment followed by the selection of high-affinity clones gives rise to matured antibodies. The interaction of the antigen with the membrane form of immunoglobulin on the surface of immune system B cells producing antibodies leads to the internalization of this complex followed by the activation of this B-cell clone (antibody affinity maturation was described in detail in the review[18]). The activation of B cells producing antibodies depends on the ratio of the dissociation rate of the antigen to the internalization rate of the antigen–antibody complex. If the affinity of immunoglobulin is insufficiently high, the antigen is dissociated during the period of time required for internalization, and the activation of B cells does not occur. At affinities $> 10^{-10}$ M, the half-life of the antigen–antibody complex is 2–3 times longer than the time of its internalization, and the further improvement of affinity does not lead to an increase in the efficiency of the selection. Therefore, the *in vivo* production of antibodies with affinity in the low picomolar range (low picomolar affinity) is generally impossible in the B-cell immune response because of the known affinity limit.[56, 57]

These limitations can be reduced by elmploying the technological approach of *in vitro* affinity maturation to produce antibodies with femtomolar affinity. It should be noted that the affinity of antibodies used in the therapy of solid tumours is in the nanomolar concentration range (from 10^{-8} to 10^{-10} M), which is optimal for most of purposes. It was also shown that an increase in the antibody affinity to 10^{-11} M impairs the penetration of the antibody into the target tumour and leads to a decrease in the targeting selectivity.[58] On the other hand, high-affinity antibodies may find use in the *in vitro* diagnosis for the development of highly sensitive methods of immunodetection of rare antigens and those present at low concentrations, as well as for certain therapeutic applications, for example, for neutralization of toxins.[59]

The following three main approaches are used for antibody affinity maturation *in vitro*: random mutagenesis, targeted mutagenesis and shuffling of variable domain regions or CDR regions in the parent antibody.[18, 60] A knowledge of the fine structure of antibodies and their interactions with antigens, in particular, the availability of abundant X-ray diffraction data for antibodies and their complexes with antigens, allows researchers to apply *in silico* approaches for the improvement of antibody affinity, which resulted in a substantial acceleration and optimization of this process.[61, 62] For instance, the affinity of the therapeutic antibody specific for the subunit of the VLA1 integrin was increased tenfold (up to 1.9 nM) using the computer-assisted design and the introduction of three calculated mutations.[63] A therapeutic antibody with an affinity of 58 nM, which is 10 times higher than that of the parent therapeutic antibody Cetuximab specific for the

tumour marker EGFR, was constructed using several cycles of the computer-assisted analysis and the introduction of single mutations taking into account electrostatic interactions.[64]

The further expression and selection of appropriate antibody variants are performed by means of phage, yeast and ribosome display.[36, 60] These combinatorial approaches employed for the affinity maturation *in vitro* require the creation of extensive molecular libraries, which is not always possible because of the limited cell transformation efficiency. A ribosome display is a cell-free system containing up to 10^{14} clones in the library and it can be used to construct antibodies with picomolar affinity.[65] An advantage of an yeast display is that the post-translational modifications and processing are similar to the corresponding processes in mammals, due to which useless variants can be rejected by FACS (fluorescence-activated cell sorting). An antibody with the absolutely best affinity (48 fmol litre^{-1}), which is 10 000 times higher than the affinity of the parent antibody, was obtained with a yeast display.[66]

Earlier, we have shown that the VH domain of the human antiferritin antibody F11, despite the partially disordered structure, retains the ability to bind the self antigen.[67] Advances in the engineering of antibody fragments made it possible to use this property of the variable heavy chain domain of human antibodies for the construction of high-affinity single-domain antibodies (eAds;V-based eAds, variable domain-based engineered antibody domains) specific for different targets.[68] Due to a rather low molecular mass (12–15 kDa), the VH variable domain is a promising scaffold protein for the design of therapeutic agents against targets, which are inaccessible to full-size antibodies with a molecular mass of 150 kDa. Preliminary data were obtained on the possible use of single-domain antibodies against HIV-1 possessing resistance mechanisms to full-size antibodies. Due to rather small sizes, single-chain antibodies can efficiently reach sterically shielded epitopes and more easily penetrate into densely packed tissues, where the virus replication occurs.[69]

III.2.b. Alteration of antibody specificity

The specificity of an antibody constructed for therapy is determined by the selection of an appropriate target. In the past decade, tremendous progress has been made in understanding the mechanisms of action of antibodies and in searching for new targets for the therapy of different diseases. Monoclonal antibodies approved for the clinical use by the beginning of 2014 are specific for 23 types of targets, 2/3 of which are cell surface antigens, including the commonly used tumour markers HER1, HER2/neu and PSMA (prostate specific membrane antigen). In the last five years, the percentage of drugs for the treatment of a variety of non-tumour diseases, particularly of autoimmune and infectious diseases, was substantially enlarged (Table 3).[3, 4] New approaches were proposed for the treatment of tumour diseases using antibodies, which are not directly related to the effect on the tumour cell surface markers. The therapeutic antibody Ipilimumab (Yervoy®) (see Table 3) targeted to one of the main immune checkpoint modulators, *viz*., the cytotoxic T-lymphocyte antien-4 (CTLA-4), was the first such antibody approved for the clinical use.[70] The search for new targets, including intracellular targets, for the disease-specific action, as well as the optimization of the properties of antibodies, are to a large extent responsible for the progress in the therapeutic and diagnostic applications of drugs based on monoclonal antibodies, their fragments and analogues.[70–72]

The specificity of the binding of a therapeutic antibody, as well as its affinity, can be subject to the optimization. Methods involving targeted and random mutagenesis and molecular display technologies are commonly used for this purpose. The main goal of the engineering of antibody specificity is to decrease the cross-reactivity with other antigens. For instance, the cross-interaction of the anti-progesterone antibody with 5α- and 5β-dihydroprogesterones was substantially reduced, while high affinity for the parent antigen was retained, using the phage display technology by means of mutagenesis of amino acid residues in the 2H and 3L complementarity-determining regions.[73]

In some cases, researchers may need to extend the spectrum of antibody specificity or increase the cross-reactivity of the human antibody with the corresponding antigens from other organisms. In order to increase the therapeutic potential of the neutralizing antibody against the botulinum neurotoxin A1, a yeast surface display was employed to obtain a variant of this antibody that interacts also with the botulinum neurotoxin A2, the amino acid sequence of which has 90% homology to neurotoxin A1.[74] The engineering of species cross-specificity of a therapeutic human or humanized antibody is employed as an alternative approach to preclinic trials of this antibody in model animals instead of the construction of a surrogate antibody. For example, a humanized anti-human CD11a antibody was reconstructed into the antibody having cross-specificity for rhesus macaque CD11a using the computer-assisted design followed by the introduction of four mutations in the CDR region of the parent antibody.[75]

According to the commonly accepted views, a monoclonal antibody can simultaneously interact with a single antigen, the interface between the antigen and the antibody can be described by a lock-and-key model. Recently, an antibody, a variant of the therapeutic antibody Trastuzumab commonly used in cancer therapy, was constructed. This antibody has high affinity and simultaneously interacts with two different antigens, *viz*., the human epidermal growth factor receptor 2 (HER2) and the vascular endothelial growth factor (VEGF).[76] It was shown that this antibody with improved affinity inhibits HER2- and VEGF-mediated tumour cell proliferation *in vitro* and retards tumour progression in mouse models. The two-in-one antibody casts doubt on the validity of the current paradigm of the single specificity of mAbs and provides new potential for variable domain engineering.

III.2.c. Improvement of antibody pharmacokinetics

Class G immunoglobulins have a much longer half-life in the body compared to other known protein-based therapeutics due to FcRn receptor-mediated recyclization.[77] The rate of nonspecific IgG clearance can be increased by engineering the Fc constant domain (see Section III.3). Recently, two other mechanisms of IgG clearance were found, and it was shown that their rate can be changed by means of variable domain engineering.[78]

One of these mechanisms involves nonspecific pinocytosis of mAbs followed by the rapid degradation of mAb, which does not bind to the FcRn receptor and depends on the electrostatic interaction of mAbs with the negatively charged cell membrane. Therapeutic antibodies, the iso-

Table 3. Monoclonal antibody drugs approved by the FDA for the clinical use in the past five years (data by 01.03.14) and potential side effects.[a]

Field of application	Brand name	USAN[b] name	Type of antibody	Target	Application/mechanism of action [enhancement (↑) or weakening (↓) of the effect is indicated]	Company and approval date[c]	Potential side effects [http://www.fda.gov/Drugs/default.htm]
Therapy of tumour diseases	Adcetris®	Brentuximab vedotin	chimeric IgG1 immunotoxin	CD30	Hodgkin's lymphoma/binding to the receptor, intracellular release of toxin; inhibitor of the cell division	Seattle Genetics, 2011; EC, 2012	neuropathy, neutropenia, hypersensitivity reactions
	Arzerra®	Ofatumumab	human IgG1 kappa	CD20 (B-cells)	B-cell chronic lymphocytic leukemia/complement activation	Glaxo Group Limited, 2009; EC, 2010	neutropenia, hypersensitivity reactions, risk of hepatitis B reactivation
	Gazyva®	Obinutuzumab	humanized IgG1 kappa	CD20	B-cell chronic lymphocytic leukemia/complement activation	Genentech, 2013	reaction to intravenous injection, neutropenia
	Kadcyla®	Trastuzumab emtansine	IgG1 immunotoxin conjugated to DM1	HER2, subdomain IV	HER2-positive metastatic breast cancer/inhibitor of meiosis	Genentech, 2012; Roche, 2012	sickness, musculoskeletal pain, headache
	Perjeta®	Pertuzumab	humanized IgG1, mAb 2C4	HER2, subdomain II	HER2-positive metastatic breast cancer/inhibitor of dimerization	Genentech, 2012; Roche, 2012	cardiomyopathy
	Removab®	Catumaxomab	mouse gG2a/rat IgG2b bispecific antibody	EpCAM/CD3	malignant ascites/activation of antitumour immunity	EU, 2009; not approved by the FDA	hypersensitivity reactions, dyspnea, abdominal pain
Therapy of autoimmune diseases, transplantology, immune system	Actemra®	Tocilizumab	humanized IgG1	human IL-6 receptor	rheumatoid arthritis, systemic juvenile idiopathic arthritis	Genentech, 2010	infectious diseases, tuberculosis, viral infection reactivation
	Benlysta®	Belimumab	human IgG1 lambda	BLyS (BAFF) belonging to TNFα family	systemic lupus erythematosus/blockade of TNFα activity, inflammation↓	Human Genome Sciences, 2011	respiratory tract infections, urogenital infections, neuropathy
	Cimzia®	Certolizumab pegol	humanized Fab′ conjugated to PEG (mol. mass ~91 kDa)	TNFα	rheumatoid arthritis, psoriasis/blockade of TNFα activity, inflammation↓	UCB, 2008	risk of invasive fungal infections and tuberculosis
	Simponi®/ Simponi Aria®	Golimumab	human IgG1 kappa	TNFα	rheumatoid arthritis, psoriasis, ankylosing spondylitis/blockade of TNFα activity, inflammation↓	Centocor Ortho Biotech, 2009; Janssen Biotech, 2009	risk of invasive fungal infections and tuberculosis
	Yervoy®	Ipilimumab	human IgG1 kappa	CTLA4	inoperable stage III and IV melanoma/antitumour activity of T-cells↑	Bristol Myers Squibb, 2010	immune reactions, enterocolitis, endocrinopathy

Table 3 (continued).

Field of application	Brand name	USAN [b] name	Type of antibody	Target	Application/mechanism of action [enhancement (↑) or weakening (↓) of the effect is indicated]	Company and approval date [c]	Potential side effects [http://www.fda.gov/Drugs/default.htm]
Cytokines	Ilaris®	Canakinumab	human IgG1 kappa	human IL-1β	gouty arthritis, cryopyrin-associated periodic syndromes/immunodepressant; production of inflammatory mediators IL-6 and COG2↓	Novartis AG, 2009	risk of bacterial, viral and fungal infections, including tuberculosis, hypersensitivity reactions, dyspnea, giddiness
	Stelara®	Ustekinumab	human IgG1 kappa	p40 subunit of interleukins IL12 and IL23	chronic plaque psoriasis/blocks the interaction with the IL-12R beta-1 receptor	Centocor Ortho Biotech, 2009	risk of bacterial, viral and fungal infections, including tuberculosis
Bacterial infections	Raxibacumab	Raxibacumab	human IgG1 lambda	protective antigen of the causative agent of anthrax *Bacillus anthracis*	pulmonary anthrax form (combined with antibiotics)/inhibits the interaction of *B. anthracis* toxins with the protective antigen on the bacterial cell surface	Human Genome Sciences, 2012	hypersensitivity, rash
Bone diseases	Xgeva ®/ Prolia ®	Denosumab	human IgG2	RANKL	malignant bone tumours/activation of the RANKL receptor on the osteoclast cell surface↓; bone resorption↓	Amgen, 2010	hypocalcemia, hypophosphatemia, hyperhidrosis, dyspnea, diarrhea, dedentition, osteonecrosis of the jaw

[a] Data on therapeutic monoclonal antibodies which have been approved for the clinical use earlier are summarized in the review.[1]
[b] The nomenclature of monoclonal antibodies and their fragments approved in the USA (see the note to Table 2) is currently used around the world.
[c] The EU and the date are included, if the drug is approved at least in one of the European Union countries; FDA stands for Food and Drug Administration (USA).

electric points (pI) of which are in the range from 5 to 9, have the optimal surface charge. An increase in both the positive and negative charge during the modification of mAbs may have a substantial effect on their pharmacokinetic properties and the distribution in the body.[79] Thus, an increase in the pI value of mAbs by more than one pH unit leads to their enhanced clearance from the blood plasma, an increase in the distribution in tissues and an increase in the nonspecific capture by target tissues. On the contrary, a decrease in the pI value of mAbs leads to reduction of their clearance and results in lower nonspecific accumulation in tissues.[79] A decrease in the pI value of immunoglobulin IgG1 by engineering its variable region made it possible to decrease the nonspecific clearance of the antibody by a factor of 3.1,[60] which is comparable with the results of optimization of the specific binding of the antibody constant region to FcRn (see Section III.3).

Another mechanism involving the antigen-mediated clearance contributes to the total clearance of the antibody, if the latter is able to be internalized *via* interaction with the cell membrane bound antigen. After the binding of the antibody to the self antigen, the antigen–antibody complex is internalized and then undergoes lysosome-mediated degradation. A new approach based on the pH-dependent antigen–antibody binding has been recently proposed for the optimization of antigen-mediated clearance.[78] The therapeutic anti-interleukin-6 receptor antibody Tocilizumab (Actemra®) (see Table 3) was modified in such a way that the new antibody was able to bind with high affinity to the receptor in the blood plasma (pH 7.4), but could rapidly dissociate from the complex with the receptor in an acidic medium of the endosome (pH 6.0) for subsequent recyclization. For this purpose, histidine residues were inserted into the antigen-binding site of the therapeutic antibody Tocilizumab, resulting in a substantial reduction of its antigen-mediated clearance.[78] This pH-dependent clearance holds promise in overcoming limitations associated with high affinity of the antibody (see above) and makes it possible to reduce the drug dose or frequency of administration when using high-affinity antibodies.

The mAb size plays an important role in the antibody engineering, since it determines the possibility of the excretion of antibodies through the kidneys. The half-excretion time of proteins through the kidneys correlates with the size of the molecule. The glomerular filtration limit in the kidneys is estimated at 60–65 kDa.[80] Full-size IgG molecules (~150 kDa) (see Fig. 1) are too large to be excreted through the kidneys, whereas the half-excretion time of single-domain variable fragments (12–15 kDa)[68] and scFv fragments (~30 kDa) is, on the contrary, as short as 0.5–2 h, and these fragments are rapidly eliminated from the body.[81] Monomeric Fab fragments (48 kDa) hold an intermediate position, having the half-excretion time of 14–15.5 h.[81] Due to this diversity of antibody fragments having different characteristics, the required size can be selected for a particular clinical application. For example, the Fab-format antibody, the angiogenesis-blocking agent Ranibizumab (Lucentis®), is used in the treatment of age-related macular degeneration. Due to the reduced format, this agent, as opposed to full-size antibodies, can easily penetrate the retina and, consequently, can be employed in clinical practice.[82] On the other hand, for many clinical purposes, it is necessary to modify monovalent antibody fragments produced with the use of molecular libraries by increasing their valency.

The valency of the recombinant antibody toward the target antigen plays an important role in retaining mAbs in target cells.[15] Most of natural mammalian antibodies are bivalent. The ability to bind simultaneously two antigens substantially increases the functional affinity (avidity) of immunoglobulins and is responsible for the long-term retention on cell surface receptors and other polyvalent antigens. This property is employed in wildlife. Thus, tetra- and decavalent complexes of A and M class antibodies have enhanced affinity of multiple binding of polyvalent antigens and can serve to efficiently protect against such natural polyvalent antigens as pathogenic microorganisms.

Engineering approaches to multivalent antibodies were described in detail in the review.[15] The proposed strategies involve the chemical conjugation of antibody fragments, the use of self-associated peptides and the domain shuffling when two antibodies of different specificities are mixed. Bivalent tandem and bispecific antibodies are generated by linear genetic fusion. The use of protein or partially protein heterodimerization modules, including the streptavidin–biotin system, the barnase–barstar module,[83–85] the dimer of immunoglobulin CH3 domain with asymmetric single-residue substitutions (knob-into-hole technology),[86] a module of cAMP-dependent protein kinase (cAMP is cyclic adenosine monophosphate) and peptide anchoring domains of the A-kinase anchor protein (dock-and-lock method, DNL method),[87] *etc.*, is more universal. The valency of multivalent antibodies constructed in this fashion, *i.e.*, the number of antigen-binding domains in the antibody, varies from 2 to 10. Each strategy has advantages and drawbacks, but none of them is universal. The systematization and comparative analysis of the experimental data on the pharmacokinetics of multivalent antibody derivatives of different formats targeted to the cancer markers HER2/neu and CEA and the extra domain B (ED-B) of fibronectin serving as a marker of angiogenesis showed that, in most cases, the pharmacokinetic characteristics and biodistribution are substantially improved when using the bivalent (multivalent) antibody format instead of monovalent one.[15,83]

An alternative way of improving the pharmacokinetics and biodistribution of mini-antibody derivatives is based on the conjugation of polyethylene glycol[88] or albumin,[89] resulting in an increase in the molecular mass of the compound and, correspondingly, the retardation of excretion through the kidneys. In the case of albumin, the additional mechanism of neonatal receptor-mediated recirculation probably takes place.[90] The therapeutic antibody Certolizumab pegol (Cimzia®) (see Table 3), which is the anti-tumour necrosis factor (TNFα) Fab′ fragment conjugated to polyethylene glycol with a molecular mass of ~40 kDa (the resulting molecular mass is ~91 kDa), may be referred to as an example of this approach.

Physicochemical properties of antibodies are also important for their clinical applications. Despite the fact that the variable region accounts for only one-third of the mAb molecule, the structure of the antibody and its tendency to aggregate exert a substantial effect on such pharmaceutical properties of immunoglobulin as the temperature resistance, solubility, chemical stability and heter-

ogeneity, which also can be optimized by the variable domain engineering.[60, 91, 92]

III.3. Engineering of mAb constant domains

The mAb constant domains CH1 and CL are parts of the Fab and Fab′ variable regions and are not involved in the Fc constant region, which is composed of the CH2 and CH3 constant domains in immunoglobulin G (see Fig. 1). These domains are rarely subject to engineering, despite the fact that they contribute to stabilization of variable regions.[93] For example, it was shown that the introduction of point mutations into the CH1 and CL domains leads to a more dense packing of the amino acid residues in their interaction region, thus increasing the thermodynamic stability of the variable region Fab.[94] The switch from the kappa to lambda light chain of the reactibody (catalytic antibody) A17 that cleaves the organophosphorus pesticide paraxon was shown to increase the thermal stability and improve antigen-binding properties, but not the activity of the reactibody.[95]

The mAb CH1 and CL domains can undergo glycosylation along with the Fc region through somatic mutations during the construction of a therapeutic antibody.[96] In most cases, this results in the undesired heterogeneity of the mAb sample, which should be avoided.[97]

The biological role of an antibody in the vertebrate immune system is determined by its effector functions mediated by the Fc constant fragment (see Fig. 2).[98] The human IgG Fc fragment is responsible for the binding to immune cells *via* interactions with different activating (hFcγRI, RIIA, RIIC and RIIIA) or inhibitory (hFcγRIIB) receptors and also interacts with the FcRn receptor and the protein of the complement system. The engineering of the IgG Fc fragment in order to enhance the efficacy of its action is aimed at improving pharmacokinetics of the agent and optimizing the following effector functions: complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis.

III.3.a. Modification of the Fc fragment for optimization of the mAb biodistribution and pharmacokinetics

An important procedure used to vary the pharmacokinetic characteristics of mAbs involves fine site-specific modifications of the Fc constant domain depending on the particular application of the immunoglobulin to be constructed.[99] The neonatal Fc receptor (FcRn) is responsible for the translocation of IgG across epithelial and endothelial barriers, in particular, for the mother-to-child transmission of immunoglobulins, and protects IgG and albumin from catabolism in lysosomes of the vessel endothelium.[100] The FcRn receptor has high affinity for IgG at pH 6.0 and does no bind the latter at pH 7.2. Therefore, the mechanism of IgG protection from catabolism relies on the FcRn–antibody binding in lysosomes at pH 6.0 and their recycling back to the bloodstream.[101] Antibody variants can be produced through targeted mutagenesis of the Fc fragment by randomly varying their half-life in the bloodstream.[102] The substitution of the key residues in the constant domain regions of mouse anticancer antibodies involved in the interaction with FcRn was employed to produce a series of mutants with half-lives from 8 to 80 h in the mouse body, whereas this parameter for wild-type antibodies is ~12 days.[103] These antibodies are promising for the use in radioimmunotherapy due to rapid clearance of the radioactive agent not bound to the target and in scintigraphy due to higher resolution. On the contrary, mutant antibodies with enhanced binding affinity for FcRn at pH 6.0 have a longer half-life in the body.[104] An increase in the circulation time is required in the treatment of infectious diseases with therapeutic antibodies or in the treatment of acute poisoning with neutralizing antibodies. For example, the introduction of the triple mutation M252Y/S254T/T256E into the constant domain of the anti-respiratory syncytial virus monoclonal antibody resulted in a tenfold increase in its binding to the human and rhesus macaque FcRn receptors at pH 6.0. Besides, mutant mAbs were easily separated from FcRn at pH 7.4. An investigation of the pharmacokinetic properties of mutant mAbs in model cynomolgus (crab-eating) macaques demonstrated that their half-life in the bloodstream and the accumulation in the lungs increased fourfold.[77] Recently, it has been shown that an increase in the half-life of targeted antibodies increases their therapeutic efficacy.[105] It should be noted that in most cases, the mutagenesis of residues of the constant domain responsible for the binding to FcRn does not cause changes in other effector properties of the antibody. Therefore, the targeted modification of the FcRn-binding regions is a promising method for controlling the pharmacokinetic properties of mAbs constructed for different purposes.

The X-ray diffraction study of the molecular complex of the FcRn receptor with peptides revealed the 16-mer peptide QRFVTGHFGGLYPANG necessary and sufficient for the interaction with this receptor. For the fluorescent protein mKate, it was shown that the insertion of this peptide into the protein molecule by genetic engineering imparts the properties of immunoglobulin G to this protein, which are determined by the interaction with the FcRn receptor. This is a promising strategy for the improvement of pharmacokinetics, biodistribution and epithelial transgenic expression applicable for the optimization of the properties of a series of proteins, hormones, growth factors, as well as small fragments of antibodies and their derivatives, intended for the clinical use.[106]

III.3.b. Optimization of antibody effector properties

There are several ways for optimizing effector functions of antibodies. In some cases, it is necessary to completely switch off these functions for clinical applications. Different variants of truncated antibodies which are devoid of the Fc constant region responsible for the binding to the C1q component of the complement system and killer cells are used for this purpose (see Section II). Another method is based on the switch from the IgG1 antibody subclass to IgG2. An example is the anticancer agent Pertuzumab (Perjeta®) (see Table 3), which is used as an inhibitor of the dimerization of the tyrosine receptor HER2 with other receptors of this family, HER1 and HER3. Since the IgG2 constant domain virtually does not bind the FcγR receptors of killer cells, antibodies of this subclass cannot cause ADCC.

A more versatile approach involves targeted mutagenesis of the binding sites of the constant domains for immune cell C1q and FcγR receptors. The targeted mutagenesis became possible due to the fine mapping of the FcγR-binding sites on IgG1 constant domains.[107] The whole spectrum of mutant mAbs was obtained: from antibodies, which virtually do not bind FcγR and C1q and, consequently, cannot cause ADCC and CDC, to high-affinity mAbs, which cause the whole set of reactions following

these two mechanisms.[108, 109] In addition, mAb variants, which cannot bind C1q and, consequently, do not cause a complement cascade, but retain high binding affinity for FcγR receptors and the ability to cause ADCC, were constructed.[108] An important result of this study was the construction of mutant mAbs (S239D/I332E/A330L), which can be easily bind not only the rare homozygous alleles FcγRIII-158V/V (20% of patients) but also the more common alleles FcγRIII-158V/F (45% of patients) and FcγRIII-158F/F (35% of patients). The construction of therapeutic antibodies having effector functions regardless of the patient polymorphism status allows for the successful treatment of the whole patient population with such drugs as Herceptin® (Trastuzumab) and Rituxan® (Rituximab).

Recent studies showed that interactions of antibodies with immune cell activating (hFcγRI, RIIA, RIIC and RIIIA) or inhibitory (hFcγRIIB) receptors play a key role in the *in vivo* functioning of antibodies.[110] It was shown that different *in vivo* efficacy of antibodies belonging to different IgG subclasses correlates with the ratio of the activating to inhibitory FcγR receptor affinity (so-called A/I ratio).[111] An increase in the affinity of the therapeutic antibody for activating FcγR receptors considerably enhances its cytotoxic effect (Fig. 4 *a*).[112] As opposed to the activating FcγR receptors, which play an important role in the efficacy of cytotoxic antibodies, the inhibitory FcγRIIB receptor plays a key role in the *in vivo* functioning of immunomodulatory agonist anti-CD40 antibodies (Fig. 4 *b*).[113] Similar results were obtained for the CD95 cellular receptor from the tumour necrosis factor superfamily. Thus, the presence of the inhibitory FcγRIIB receptor is required for the agonist CD95 antibody to induce liver inflammation and initiate apoptosis of mouse lymphoma cells.[114] This unexpected fact opens up new prospects for the optimization of the activity of immunomodulatory mAbs (see Fig. 4 *b*).

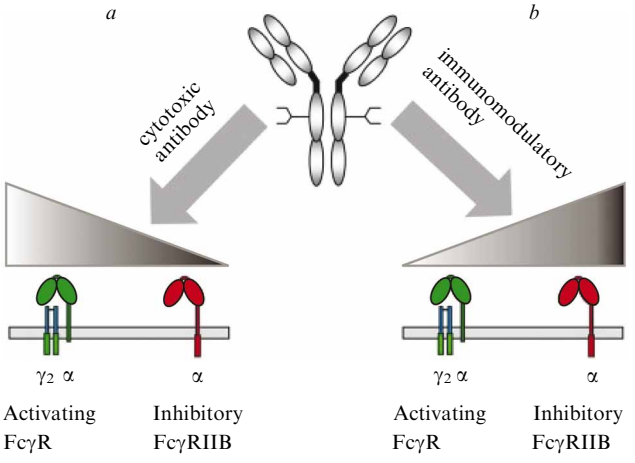


Figure 4. Engineering of the Fc constant fragment for enhancing cytotoxic or immunomodulatory properties of therapeutic antibodies *in vivo*.[110]
The schematic representation of the increase in the *in vivo* activity of cytotoxic antitumour antibodies with an increase in their affinity for the activating cell receptors FcγR (*a*). On the contrary, the *in vivo* activity of immunomodulatory agonistic antibodies increases with an increase in their affinity for the inhibitory cell receptor FcγRIIB (*b*).

The carbohydrate component of mAbs accounts for only 3% of the total molecular mass (see Fig. 1); however, it plays an important role in antibody effector functions. Immunoglobulin G contains two N-linked branched oligosaccharide chains at the residue Asn297 in the CH2 domain of the constant fragment (see Fig. 3, *8*). The composition of the carbohydrate component of immunoglobulins displays high species specificity and varies within one species. The structure of the carbohydrate component was shown to influence the cytotoxic or immunomodulatory activity of the antibody.[115] For example, if the latter contains a large number of terminal sialic acid residues, the IgG molecule has low affinity for classical immune cell receptors and exhibits considerable antiinflammatory activity.[116] For the therapeutic antitumour antibody, this type of glycosylation has an adverse effect because it reduces the cytotoxicity of the antibody. The removal of the core fucose residue from the carbohydrate component of the antibody enhances ADCC by a factor of more than 50.[107] It was shown that the lower efficacy of antibodies in the body as compared to *in vitro* experiments, resulting in the necessity of the administration of the drug at higher doses, is due to the inhibitory effect of fucosylated immunoglobulins of the blood plasma.[117] Non-fucosylated therapeutic antibodies have higher affinity for FcγR receptors as compared to fucosylated antibodies, due to which the former can withstand the inhibitory effect of immunoglobulins G that circulate in the plasma and exhibit activity in lower concentrations.[118]

All therapeutic antibodies approved for the clinical use are currently produced in CHO cells, in the mouse myeloma cell lines NS0 and SP2/0 and in mouse hybridomas. Almost all these antibodies contain a fucosylated carbohydrate component and, as a consequence, ADCC of the products is far from optimal.[119] The carbohydrate component of IgG is fucosylated in mammalian cells by the enzyme α-1,6-fucosyltransferase (FUT8). Some mouse cell lines express a lower level of this enzyme than CHO cells. However, the most efficient way of removing fucose involves the irreversible inactivation of the *FUT8* gene. This approach was used to construct *FUT8* knockout CHO cell lines allowing for the production of non-fucosylated therapeutic antibodies having 50–100 times higher ADCC (see Fig. 3, *8*).[25] An understanding of the role of the carbohydrate component of IgG gave a powerful impetus to a new line of research in antibody engineering. In 2012, the first therapeutic non-fucosylated humanized antibody Mogamulizumab was approved for the clinical use in Japan for the treatment of patients with recurrent or relapsed adult CCR4-positive leukemia/lymphoma.[120] A new chemical enzymatic method of glycoengineering of intact antibodies was developed in order to improve their therapeutic properties.[121] However, the recent study on the production of aglycosylated mAb derivatives (see Fig. 3, *10*) casts doubt on the necessity of this sophisticated technology for the production of antibodies with different types of glycosylation. It was shown that the introduction of only two amino acid substitutions into the Fc fragment completely devoid of the carbohydrate component allows the antibody to bind FcγR receptors with high affinity and to exhibit effector properties.[122] These new data hold great promise for the production of functionally active human antibodies in biotechnological expression systems, for example, in bacterial systems, as well as in cell-free systems.[123]

IV. Catalytic antibodies (abzymes)

Abzymes (catalytic antibodies) represent a new class of biocatalysts and belong, from the structural point of view, to the immunoglobulin superfamily. Their antigen-binding hypervariable regions contain amino acid residues involved in the catalytic event. Therefore, abzymes can be considered as antibodies-enzymes, which explains the etymology of the term 'abzymes' (AntiBody-enZYME). From the point of view of the primary function of antibodies as participants of the humoral response, abzymes can specifically bind antigens but they simultaneously can trigger targeted antigen degradation. The discovery of abzymes overturned one of the main paradigms of immunology, according to which antibodies are considered only as highly specific antigen acceptors of the protein nature. It was shown that antibodies can be used as biocatalysts alternative to enzymes. This area of research is at the intersection between immunology and chemistry (immunochemistry).[124] The views of the functioning of abzymes are based on ideas of world science classics, including the principle of molecular complementarity, the lock-and-key concept, the transition state theory and the idiotypic network theory. In relation to these ideas, it is necessary to mention E Fischer, L Pauling, W Jencks and N Jerne.[125] Abzymes can be produced as monoclonal antibodies against stable transition state analogues of reactions.[126] Like enzymes, abzymes have the highest affinity not for the product and substrate but for the transition state of the reaction. They can reduce the activation energy of the biochemical reaction, thus performing catalytic transformations. Abzymes can also be produced as anti-idiotypic antibodies against enzymes.[127] Antibodies extracted from the blood serum of patients with autoimmune disorders and the corresponding model animals developing the autoimmune process can exhibit activity of natural abzymes.[128, 129] This approach can also be used for the targeted induction of the catalytic response towards the antigen of interest.[130] The research group headed by Lerner in the Scripps Research Institute (USA) proposed a method for the production of abzymes by reactive immunization. In this case, the antigen has an activated reactive group, which can modify the active site of the antibody. This approach was used to induce abzymes that catalyze complex chemical transformations.[131]

Abzymes catalyze a broad spectrum of esterase and oxidase reactions (Fig. 5).[132] Abzymes having protease, DNase or RNase activity were constructed.[133 – 135]

Abzymes can also catalyze non-enzymatic reactions. Therefore, the employment of abzymes becomes a promising new frontier in biotechnology and medicine in particular, they can be regarded as potential effective catalytic vaccines capable of catalyzing the degradation of pathogens, pesticides, narcotic drugs and toxins.[136, 137] The construction of abzymes capable of catalyzing the degradation of biopolymers, such as proteins, nucleic acids and carbohydrates, holds great promise. The abzyme-mediated decrease in the concentration of a pathogenic protein should become the effective therapy of antibody-based specific binding. In this case, an excellent example would be the construction of a catalytic antibody against the tumour necrosis factor. A relevant therapeutic agent would be more effective than binding antibodies commonly used in the therapy of rheumatoid arthritis. The natural factor interfering with the development of this line of

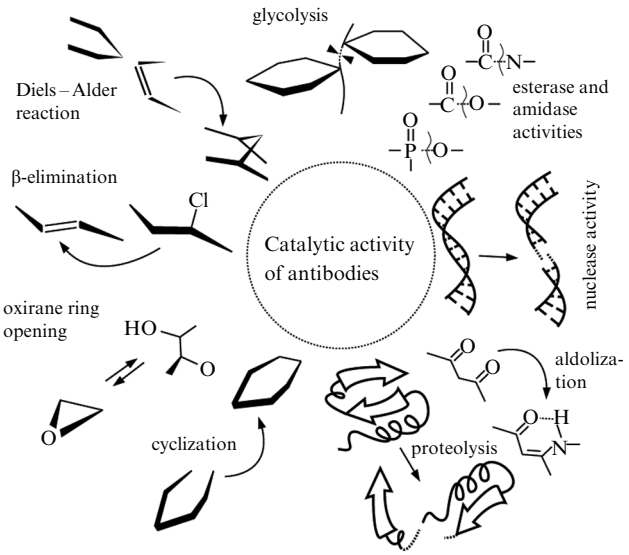


Figure 5. Chemical reactions catalyzed by abzymes.[132]

research is the ambiguity in the selection, modelling and synthesis of the corresponding transition state analogue, the construction of an antibody panel and its screening for catalytic activity. Similar difficulties are encountered in an alternative approach using the induction of anti-idiotypic abzymes.[138, 139] Besides, a considerable drawback of this approach is that it cannot be used to produce catalytic antibodies, the catalytic efficacy of which is comparable to that of natural enzymes. This is primarily due to the fact that the antibody cannot simulate all stages of the enzyme dynamics performed by the evolutionary perfect enzyme. Despite all the aforesaid, the use of abzymes may have substantial advantages over enzymes as potential therapeutic agents. The research group headed by Professor Nevinsky[140] characterized the protein kinase activity of abzymes. This is in fact the only example of abzyme-catalyzed bisubstrate reaction described in the literature. It should be noted that since the mechanism of the phosphoryl transfer requires the evolutionary complex active site of the biocatalyst, detailed studies of the discovered activity are required.[141, 142]

The employment of conventional drugs, including antibiotics designed before the era of biotechnology, for the treatment of certain bacterial and infectious diseases has not met with success. Numerous attempts to use therapeutic agents for the HIV treatment were not effective. The surface antigen, the viral coat protein gp120, can be one of the targets in the development of a new approach to the treatment of infections. The specific cleavage of this protein can lead to considerable changes in the immune reaction in the body and its ability to bind the lymphocyte receptor CD4. This problem cannot be solved by enzymatic therapy, but there is an alternative approach employing abzymes.[143] The surface antigen gp120 of the HIV virion plays an important, probably key, role in the blockade of the ability of the host immune system to prevent the penetration of the pathogen. Vaccination is the most efficient strategy in the struggle against viral infections, and its efficacy was proved for many viral diseases, including smallpox and poliomyelite. In Russia, the prophylactic vaccine was developed by the

research team of the Institute for Immunology. It should be noted that the therapy of HIV remains an unsolved problem.[144, 145]

It is very difficult to directly destroy the virus using the anti-gp120 antibody, because the neutralizing antibodies cannot bind to this antigen having a 3D spike structure. One of approaches that would prevent the virus invasion involves the disruption of the interaction between the viral particle and CD4. In this case, the specific cleavage of the protein gp120 plays an important role. Previously, it has been shown that the protein gp120 is cleaved by trypsin at the residue 432 and by the protease V8 at the residue 269, due to which it cannot interact with CD4. However, after the cleavage of the protein gp120, the buried regions of this cell surface antigen can become accessible to the immune system or new antigenic determinants can appear, resulting in the effective neutralization of this virus and destruction of infected cells.[146]

Data on the conformational plasticity of the main surface HIV antigens and their relationship with the immune reaction and receptor binding [147] suggest that the cleavage of the accessible constant region of the protein gp120 can lead to the following effects:

— conformational changes in the structure of the surface antigen and the accessibility of the conserved immunogenic epitopes, resulting in that the buried antigenic determinants become also accessible to recognition by the immune system;[144]

— the ability of neutralizing antibodies to recognize buried epitopes and interfere with the infection by binding the virus and(or) cells producing viral particles;[148]

— the cleavage of particular regions and violation of the functions of the internalization apparatus, resulting in the direct inhibition of the virus infection;[145]

— the appearance of radically new immunogenic epitopes during the proteolysis followed by the enhancement of the productive immune response to HIV;[130]

— the degradation of viral coat proteins and, as a consequence, their shedding from the virus surface and the accessibility of the previously buried epitopes of the HIV protein gp41.

The cleavage of the protein gp120 holds promise as an approach that would allow for a change in the immunological properties of the infection-competent virion surface and induction of the stable neutralizing antiviral immune response.[130]

We proposed an approach based on the possibility of the induction of the catalytic response against the background of the development of autoimmune pathology. As opposed to simple enzymes, a catalytic antibody can have antigen-specific properties. In addition, the antibody does not act as a stimulator for the host immune system. Due to this property, abzymes can be utilized in the human body. Moreover, the relatively low catalytic efficiency and high ability of abzymes to bind antigens can provide stronger binding of the biocatalyst to the target antigen and reduce the rate of non-specific proteolysis hazardous to the organism.[125]

An interesting line of research in modern abzymology is the search for catalytic antibody clones by screening combinatorial libraries of immunoglobulin genes. This approach has recently been used to find antibodies capable of forming specific covalent interactions with organophosphorus poisons and catalyzing their metabolism.[149, 150] Considerable attention is being given to reengineering of catalytic antibodies and the construction of optimal scaffolds, which are able to serve catalytic functions most efficiently.[95, 151] The specificity and catalytic functions of abzymes can be varied by the replacement of antibody chains, for example, of κ by λ. The substitutions of amino acid resides not only in the active site region but also in the outer sphere hold considerable promise. Apparently, in the nearest future it would be possible to predict routes to the construction of more perfect biocatalysts based on antibody templates relying on X-ray diffraction data for abzymes, as well as on the results of molecular dynamics methods.

V. Immunotoxins and immunoconjugates

Sub-optimal efficiency of the first-generation unloaded antibodies as therapeutic agents stimulated researchers to conjugate antibodies to other effector molecules (see Fig. 3), such as radioisotopes, toxins, cytokines, drug-activating enzymes, *etc*. In immunoconjugates (antibody drug conjugates, ADC), antibodies serve as the targeting component that delivers the active (cytotoxic) or diagnostic agent to the target. As opposed to the treatment of tumour diseases with chemotherapeutic agents (chemoagents), which have low therapeutic indices [ratio of the maximum tolerated dose (MTD) to the minimum effective dose (MED)], the use of immunotoxins makes it possible to increase the therapeutic index and expand the therapeutic window (Fig. 6).

Chemical conjugation methods are commonly used for the conjugation of low-molecular-mass agents, for example, radioisotopes or low-molecular-mass fluorescent dyes.[152] The target cell specificity of such conjugates, as well as their efficacy and safety to the body, depend both on the targeting agent and the toxic component, as well as on the conjugation method used.[24] The active agents are usually conjugated to an antibody at reactive amino groups of lysine residues (a total of ~40 lysine residues per IgG molecule) or at SH groups of cysteine residues obtained by reducing four intramolecular disulfide bridges. There are,

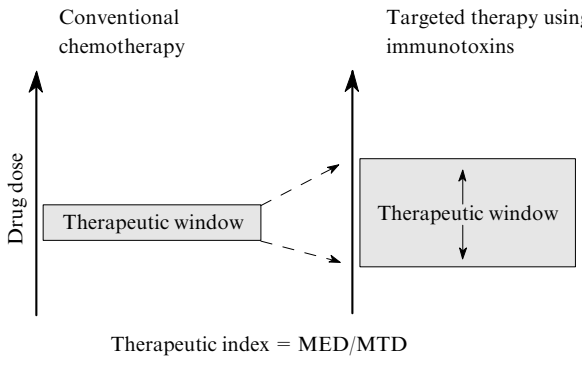


Figure 6. Expansion of the therapeutic window using immunoconjugates.
The selective delivery of the active agent to tumour cells results in an increase in the percentage of the drug that reaches the target cells, thus decreasing the minimum effective dose. The maximum tolerated dose increases due to a decrease in the percentage of the drug damaging normal non-tumour cells.

on the average, from 0 to 8 conjugated molecules per antibody molecule, and the number of variants with different properties may be >100. The only two immunotoxins approved for the clinical use, *viz*., Adcetris® and Kadcyla® (see Table 3), have a heterogeneous composition because of different toxin to antibody ratios. Therefore, the main disadvantages of the chemical conjugation of antibodies to active agents is the heterogeneity, resulting in the non-optimal efficacy of the agent, and the impossibility of standardizing the processing technology. The following approaches have been recently proposed for the solution of this problem: several site-specific conjugation techniques involving the preliminary introduction of cysteine in the position Ala114 of the antibody heavy chain or the introduction of synthetic amino acids (selenocysteine, acetylphenylalanine), as well as employing the enzymatic conjugation using glycotransferases and transglutaminases.[24]

In order to improve the penetration of radioimmunoconjugates into the tumour, functionally active peptides, which can penetrate across the membrane and translocate other proteins into the cell, are conjugated to antibodies by genetic engineering techniques. For instance, it was shown that radioimmunoconjugates, which were constructed based on mini-antibodies specific for the tumour antigen TAG72 and supplemented with penetratin or the TAT oligopeptide, are accumulated 2.5–3 times better in xenograft mouse tumours.[153]

In oncology, the following two agents were approved for radioimmunotherapy of non-Hodgkin's lymphoma: Bexxar®, which is mouse IgG2a conjugated to the medium-energy β-emitter ^{131}I (the penetration radius is 1 mm), and Zevalin®, which is mouse IgG1 conjugated to the high-energy β-emitter ^{90}Y (the penetration radius is 11 mm). Currently, investigations are being aimed at replacing mouse antibodies as targeting agents for radioimmunotherapy with less immunogenic fragments; antibodies are also employed to develop pre-targeting technologies for delively of radioactive therapeutic agents in order to decrease the total radiation load of the body.

A heterodimerization module is required for pre-targeting, as well as for the construction of bispecific antibodies (see below). For instance, the streptavidin–biotin system,[154] the promising dock-and-lock method[87] and the barnase–barstar module, which was shown to be efficient in a number of analytical applications,[85, 155, 156] are used for this purpose. The commonly used streptavidin–biotin system requires covalent modifications, resulting in heterogeneity of the agent. This drawback can be alleviated using the site-specific biotinylation of proteins with biotin ligase; however, these techniques require cotransfection of a special plasmid into mammalian cells.[157, 158] In addition, the employment of the streptavidin–biotin system in the human body is limited by the presence of a large amount of endogenous biotin, which may compete with biotinylated components. We proposed a strategy for the two-step delivery based on the interaction between two small bacterial proteins, *viz*., barnase and its natural inhibitor barstar; the stability of their complex ($K_D \approx 10^{-14}$ mol litre^{-1}) is comparable with that of the streptavidin–biotin system.[83] The two-step delivery of the active agent to cancer cells was demonstrated by the following examples: the delivery of recombinant mini-antibodies specific for the cancer surface antigen HER2/neu and delivery of different visualization components, such as the recombinant fluorescent protein EGFP (enhanced green fluorescent protein), quantum dots, gold nanoparticles and nanosized anti-Stokes phosphors.[84, 159–164] Important advantages of the barnase–barstar module are the exact 1 : 1 ratio of the components in the complex and the complete absence of their self-aggregation, as well as high affinity of the interaction, which is the best of all known dimerization systems, except for the streptavidin–biotin system.[165, 166] As opposed to the streptavidin–biotin system, the use of the heterodimerization barnase–barstar module combined with targeting mini-antibodies relies on genetic engineering techniques and does not require covalent modifications.

The dock-and-lock method[87] employs the specific protein–protein interaction between the dimeric regulatory subunit RII of the cAMP-dependent protein kinase and the anchoring domain (the amphipathic helix consisting of 14–18 residues) of the anchoring protein kinase A. These polypeptides and Fab fragments of two different antibodies were used to construct a trivalent bispecific antibody recognizing the tumour antigen CEA (carcinoembryonic antigen) and the histamine-succinyl-glycine hapten. In the first step of the targeted delivery, the docking component provides the bivalent interaction with the tumour antigen. In the second step, the lock linked to the dock by the anchoring domain binds the radioactive ^{177}Lu-labeled hapten. First positive results of clinical trials of this technology were obtained in the treatment of metastatic colon cancer.[167]

The construction of conjugates of antibodies with toxins of different nature for the targeted action on cells is a very important line of research in antibody engineering.[24] More than a half of antibody-based anticancer agents in clinical trials are immunoconjugates or recombinant proteins. Only the following two immunotoxins were approved for the clinical use: Brentuximab vedotin (Adcetris®), which is a chimeric anti-CD30 antibody conjugated to the cell division inhibitor, monomethyl auristatin E, and Trastuzumab emtansine (Kadcyla®), which is a humanized anti-HER2 antibody conjugated to the toxin emtansine having a similar mechanism of action. These drugs are used for the treatment of lymphoma and breast cancer, respectively (see Table 3). The previously approved drug Mylotarg®, which is the humanized anti-CD33 antibody Gentuzumab chemically conjugated to the cytotoxic antibiotic calicheamicin, was removed from the clinical use in 2000 because of high toxicity. This disproportion reflects all objective problems of the development of such compounds. Since the drug efficacy is directly related to the level of toxicity of the compound, toxins with high IC_{50} values varying in the range of nano- and picomolar concentrations should be used for the design of therapeutic drugs. Currently, researchers have at their disposal auristatins, maytansinoids, calicheamicins and protein toxins (ricin, pseudomonas and diphtheria toxins).[168]

The mechanism of action of pseudomonas exotoxin A and diphtheria toxin is based on the catalytic ADP ribosylation of the elongation factor 2 that induces translation inhibition. The first and second generations of these multidomain proteins have extreme systemic toxicity, which was substantially reduced after the removal of the domains responsible for the binding to cells. The advantage of these proteins is high toxicity in picomolar concentrations. Thus, a few molecules are required to kill the cell. The drawbacks are the immunogenicity and the capillary leak syndrome as

the side effect. To eliminate immunogenicity, the research group headed by Pastan,[169] a pioneer in studies of immunotoxins, carried out a comprehensive work on the deimmunization of the truncated pseudomonas exotoxin PE38 with the full retention of toxicity.

Bacterial ribonuclease barnase is yet another promising agent for the construction of anticancer immunotoxins.[170] This small, stable and well soluble protein is toxic to cells but is insensitive to the natural human RNase inhibitor. The supplementation of the toxic agent barnase with a targeting mini-antibody substantially increases its efficacy and specificity for cancer cells.[170–173] In addition, the targeting anti-HER2 mini-antibody is internalizing and provides the delivery of barnases into the target cells.[163] It was shown that immunodibarnase taken in a concentration of 1.8 nmol litre^{-1} induces apoptosis in 50% of HER2/neu-positive cells, whereas a 250–300 times higher concentration is required for a similar effect on HER2/neu-negative cells. The cytotoxic effect of barnase devoid of the targeting mini-antibody is non-specific and is $\sim$1000 times lower than the cytotoxic effect of imminodibarnase.[170]

Ribonuclease barnase circulating in the bloodstream is non-toxic to the body and induces cytotoxicity only when penetrated into the cancer cell, because it is, in a certain sense, a precursor of toxin (pro-toxin).[174] The idea of the construction of inactive prodrugs, which can be delivered to the target and then converted into the active form, is being developed in different versions of the ADEPT (antibody-directed enzyme prodrug therapy) technology. The latter involves the pre-targeted delivery of the antibody–enzyme conjugate to target cells. The specificity of the antibody for the cell surface antigen provides targeted delivery. In the next step, an inactive prodrug is introduced, and the latter is converted into the active form by the previously delivered enzyme only in the microenvironment of the target, resulting in the killing of adjacent cells. β-Lactamase, carboxypeptidase G2, *etc*., are employed as enzymes in this approach.[175]

Cytokines, which are natural protein regulators of intercellular and intersystem interactions, also hold promise as active components of immunoconjugates. Some cytokines, for example, IL-2, IL-12, TNFα, IFNγ and GM-CSF, have immunomodulatory and antitumour effects. These proteins cannot be directly used as drugs because of high systemic toxicity in active concentrations, rapid metabolism and excretion from the body, as well as because of the non-targeted and non-specific effect on tumour cells. These limitations can be reduced by employing immunocytokines created by the conjugation of cytokines to targeting antibodies. For example, promising results were obtained in clinical trials of recombinant immunocytokines, which were constructed based on interleukin 2 and humanized antibodies against the cancer markers GD2 and EpCAM, as well as against the angiogenesis marker EDB, for the treatment of melanoma, neuroblastoma and non-Hodgkin's lymphoma [176, 177] and also in clinical trials of immunocytokines based on interleukin 2 conjugated to antibodies against the cell marker CD20, for the treatment of malignant B-cell leukemia.[178] Besides, the employment of immunocytokines leads to enhancement of therapeutic effects in chemotherapy.[179]

The design of immunophotosensitizers — hybrid totally protein molecules consisting of a targeting antitumour mini-antibody and a protein phototoxin — is a new promising line of research in the construction of immunoconjugates for photodynamic therapy of cancer. These agents have obvious advantages over conventional chemical conjugates. Thus, the genetic engineering of immunophotosensitizers can be used to produce molecular conjugates, which have a strictly constant composition, properties and characteristics (high affinity for the tumour antigen, high cytotoxicity of the effector domain) and, consequently, their functional properties can be exactly reproduced. We were the first in the world to construct two fully genetically encoded immunophotosensitizers [180, 181] containing the mini-antibody 4D5scFv specific for the tumour marker HER2-neu as a targeting component. The phototoxic fluorescent protein KillerRed [182] and the highly toxic flavoprotein miniSOG, which can generate singlet oxygen with quantum yield $\Phi^{\mathrm{miniSOG}} = 0.47$ upon irradiation in the presence of the cofactor FMN, were used as the photosensitizing component.[183]

It was shown that both constructed totally protein immunophotosensitizers specifically bind to the breast adenocarcinoma cells SKBR3 overexpressing the cancer marker HER2-neu; the binding affinity (7–10 nmol litre^{-1}) is similar to that of the parent mini-antibody. As opposed to the KillerRed-based protein, which has insufficiently high phototoxicity, the immunophotosensitizer 4D5scFv-miniSOG induces cytotoxicity in SKBR3 cells with $IC_{50} = 160$ nmol litre^{-1} upon blue light irradiation with an intensity of 55 mW cm^{-2}. It was shown that the effect of the immunophotosensitizer 4D5scFv-miniSOG is considerably enhanced when it is used in combination with the antitumour drug (Taxol) and the recently discovered protein JO-1, which improves the penetration of antibodies into the target tumour.[184] The addition of the drug Taxol in a concentration of 1 μmol litre^{-1} led to the 100% death of SKBR3 cells after the treatment with the immunotoxin 4D5scFv-miniSOG, and the addition of the protein JO-1 in a concentration of 5 μg ml^{-1} led to a decrease in the IC_{50} value for the immunotoxin 4D5scFv-miniSOG from 160 to 5 nmol litre^{-1} (Ref. 167). Therefore, it was shown that it is, in principle, possible to construct totally protein (genetically encoded) immunophototoxins of constant composition having cytotoxic properties better than those of photosensitizers chemically conjugated to antibodies.

VI. Bispecific antibodies

Natural antibodies are monospecific, *i.e*., they bind antigens of a single type. The diversity of mechanisms of pathogenic processes, including neoplastic transformation, limits the efficacy of the use of highly selective antibodies as monodrugs. The construction of bispecific antibodies capable of binding two targets simultaneously is one of approaches to the solution of this problem.[185] Bispecific antibodies are used for the retargeting of cytotoxic immune cells to pathological targets,[186] the increase in avidity and selectivity of interactions with different antigens of tumour cells [187] and the delivery of cytotoxic agents to the target tumour.[87]

A veriety of antibody engineering strategies were proposed for the construction of bispecific antibodies for different clinical applications, by combining specificity for two different antigens in a single molecule.[188] First bispecific antibodies, which were produced by fusion of two hybridoma cell lines, were random mixtures of the target bispecific antibody and parent antibodies and, conse-

quently, the complicated procedure was required to isolate the desired variant. The following approaches were proposed in order to increase the yield of bispecific molecules: the production of interspecies mouse–rat quadromas with the predominant coupling of light and heavy chains from one species [189] and the elegant knob-into-hole technology.[86] Chemical conjugation is still commonly used for the production of bispecific antibodies [190] despite such drawbacks as the necessity of performing chemical modifications, which are sometimes hazardous to the coupled antibodies, and the difficulty of the separation of the resulting variants.

Recently, a new approach to the construction of chemically programmed antibodies was proposed based on the site-specific conjugation of an antibody serving as a scaffold to the chemically synthesized targeting peptide modules.[191] The synthesized bifunctional heterodimeric peptide module (named by the authors pharmacophore), which can simultaneously bind with high affinity and specificity the vascular endothelial growth factor (VEGF) and angiopoietin 2 (Ang2), was used to construct a bispecific antibody that inhibits the functions of both factors.[21] In the course of the conjugation process, the azetidinone fragment of the heterodimeric peptide module interacted with the residue Lys93 of the heavy chain of both Fab fragments, resulting in the ring opening of the azetidinone linker and the formation of a stable amide bond between the peptide heterodimer and the scaffold antibody (Fig. 7). The specificity of the binding of azetidinone to the antibody ensured the stoichiometric conjugation of two peptide heterodimers per antibody molecule.[21] Like the above-described two-in-one bispecific antibody generated by variable domain engineering,[76] the latter antibody is immunoglobulin, in which each variable domain binds two antigens. The simple chemical method for the creation of this site-specific conjugate enables one to produce sets of antibodies with a broad specificity spectrum by varying the structure of the pharmacophore.[8]

Genetic engineering techniques can be used to produce bispecific mAbs and their fragments bypassing the problem of the separation of a complex mixture of products.[15] The streptavidin–biotin system, characterized by the highest stability of the complex with the dissociation constant of the components $K_D = 10^{-15}$ mol litre^{-1}, is commonly used for this purpose. The barnase–barstar protein pair proposed by the authors as a heterodimerization module for the construction of bispecific antibodies is almost as stable as the streptavidin–biotin system ($K_D = 10^{-14}$ mol litre^{-1}), but it allows one to produce bispecific antibodies with the exact ratio of the components equal to 1 : 1 or 1 : 2 (trimer),

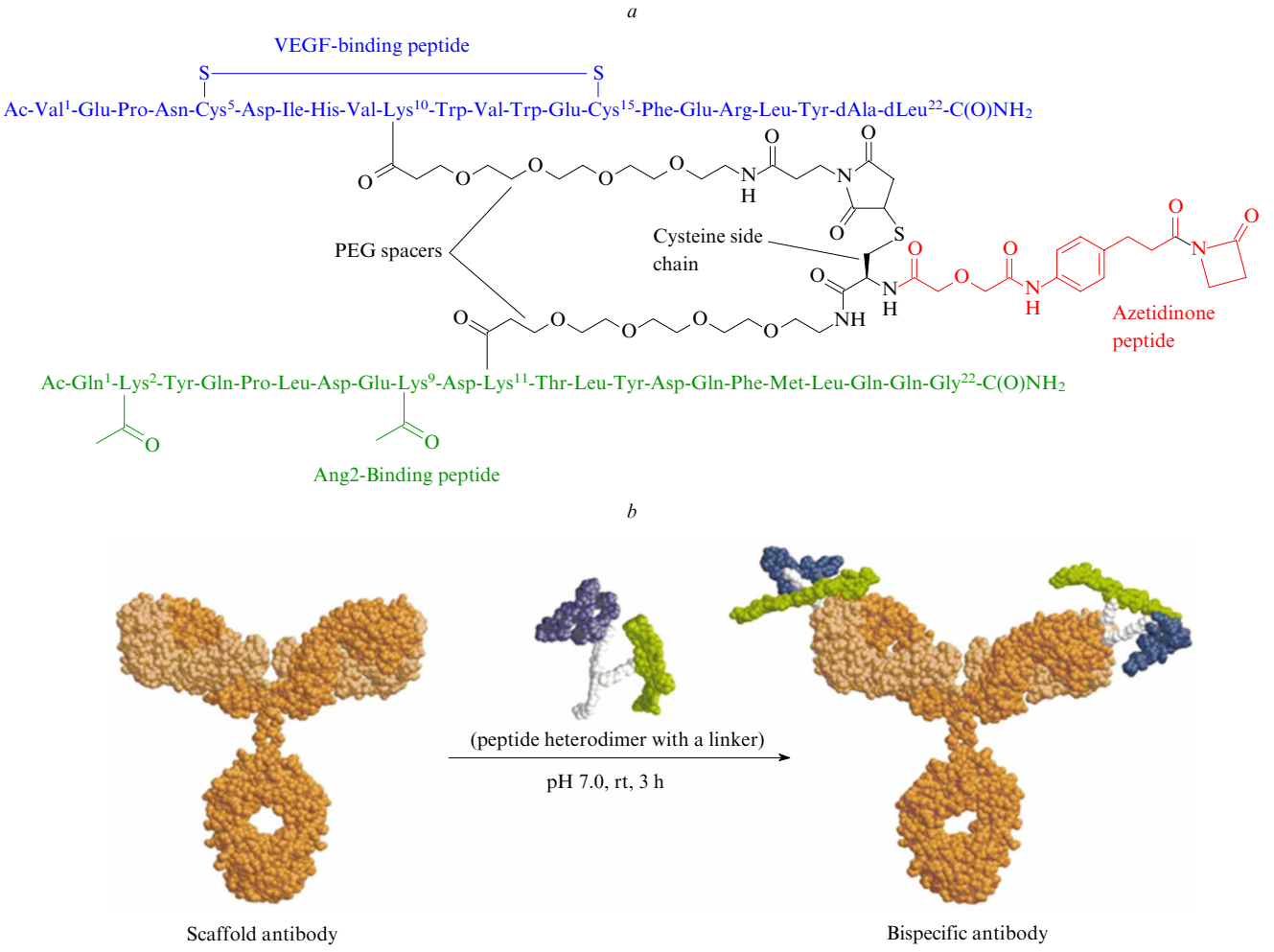


Figure 7. Construction of chemically programmed antibodies. The structure of the VEGF- and Ang2-binding peptide heterodimer with the azetidinone linker (*a*) and the scheme of its conjugation to a scaffold antibody (*b*).[21]

no chemical modifications being required.[192] The elegant dock-and-lock method[87] can be employed to construct bispecific antibodies characterized by good pharmacokinetic properties and the bivalent binding to the surface antigen. The antibodies produced by this method are promising agents for radioimmunotherapy.[167] The drawback of this method is relatively low affinity of the complex ($K_D \approx 10^{-9}$ mol litre^{-1}), due to which it should be additionally stabilized by disulfide bonds.

Diverse bispecific antibodies, in which one recognition domain binds to a particular tumour cell surface marker and another domain binds to the activating receptor of cytotoxic cells, were constructed for retargeting cytotoxic immune cells on pathological targets.[185, 193] The cell surface marker CD64 (FcγRI receptor of the immunoglobulin G Fc fragment), which is characteristic of monocytes, macrophages and dendritic cells, and the marker CD89 (FcαRI receptor of the immunoglobulin A Fc fragment), which is expressed mainly in neutrophils, macrophages and eosinophils, as well as the CD3 marker for cytotoxic T-lymphocytes, are used as receptors targeting cytotoxic cells. It should be noted that T-lymphocytes, which are among the most powerful killer cells, cannot be targeted by antibodies, since they are devoid of Fcγ receptors. Unfortunately, clinical trials demonstrated inefficiency of this approach. Immune cells required additional activation; the effect was manifested in the presence of a (40 – 100)-fold excess of the effector over the number of target cells and a high drug dose; high dose-limiting toxicity of the drugs was observed.[193] The use of bispecific antibodies for the targeting of cytotoxic T cells to cancer targets *via* the binding to CD3 was effecient only in the case of the local use[194] or in the case of adoptive transfer of T cells treated with bispecific antibodies *ex vivo*.[195]

The construction of new-generation antibodies of the BiTE (bispecific T-cell engager) format, which are recombinant bispecific single-chain antibodies consisting of two scFv fragments specific for the target cell surface antigen and the T-cell antigen CD3, made it possible to overcome almost all these limitations. Antibodies of the BiTE format do not need additional activation of the targeted immune cells and induce cytotoxicity in substantially lower concentrations.[186] Evidently, bispecific immune cell-retargeting mAbs hold promise, especially for the treatment of blood cancer diseases. Thus, clinical trials showed that the antibody Blinatumomab (anti-CD19/anti-CD3) in a concentration of 0.005 mg m^{-2} day^{-1} leads to the complete removal of cancer B-lymphocytes from the bloodstream in patients with non-Hodgkin's lymphoma.[196]

A similar approach employing bispecific antibodies, which simultaneously recognize the stem cell receptor CD45 and the antigens from the damaged myocardium, was used for the retargeting of stem cells for the vascularization and restoration of heart function in myocardial infarction.[190] This approach makes it possible to deliver much more cells to the injury site as compared with the injection of a suspension of stem cells directly to the myocardium.

Yet another area of research which requires the construction of bispecific antibodies is the simultaneous binding of two different antigenic determinants on the same target cell. An excessive presence of other tyrosine kinase receptors (IGF-1R, c-MET, Ron, *etc.*), which trigger the same signalling pathways bypassing the blocked receptors, is one of the mechanisms of tumour resistance to inhibitors of the overexpressed HER1 and HER2 receptors.[197] The use of bispecific antibodies for the simultaneous blockade of EGFR and IGF-1R considerably enhances the response of cancer cells as compared to the effect of antibodies of one type.[198] This combined effect on several cell surface antigens can block redundant signalling pathways in the cancer cell and give more favourable clinical results.

Due to abundant new information on the structures of antibody recognition regions, the latter can be introduced into a scaffold antibody as additional modular recognition domains (MRD) by genetic engineering to produce so-called Zybodies, full-size antibodies with a specificity set from 2 to 5.[199] It was shown that these antibodies against five tumour receptors (ErbB2, EGFR, IGF-1R, Ang2 and integrin αvβ3) disrupt multiple intracellular signalling pathways, inhibit cell proliferation and are comparable with the parent antibodies in the efficacy in tumour xenograft models.[199]

VII. Alternative scaffold proteins

Extensive studies of molecular constructs alternative to binding antibody domains were carried out with the aim of finding more effective agents stable under different conditions and capable of targeting active agents to molecular targets. These constructs are referred to as scaffolds.[200] The term scaffold was proposed by Plückthun and co-workers[200] to describe a protein framework that could carry altered amino acids or short sequence insertions that confer on protein variants different functions, usually the possibility of binding to specific targets. The advantages of such proteins are small sizes, the stable structure, good penetration into tissues, the possibility of additional functionalization and expression in a cost-effective bacterial system.[202]

Numerous natural and synthetic scaffold proteins are described in the literature. For example, let as refer to affibody, peptabody, ankyrin repeats, lipocalins, *etc.* (Fig. 8).[201, 203–205] These proteins can be divided into two large groups according to structural elements that are subject to randomization. In one case, this element comprises one or several variable loops (fibronectin domain,

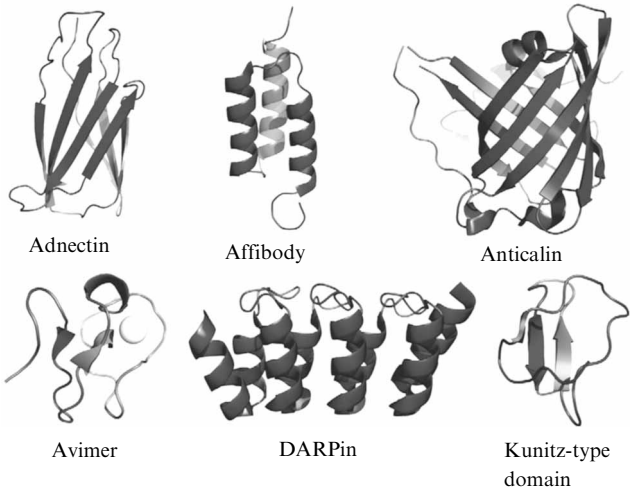


Figure 8. Bispecific antibody
Examples of alternative scaffold proteins.[203]

lipocalin). For example, the tenth human fibronectin type III domain (^{10}Fn3) has a small size (10 kDa), is extremely thermally stable and highly soluble. Synthetic binding proteins based on this domain (called adnectins or monobodies) are usually constructed by varying the sequence of three or two loops of ^{10}Fn3, which are structural analogues of immunoglobulin CDR.[206, 207] Proteins of the lipocalin family consist of 160–180 residues, which form 8 strands of the antiparallel β-barrel linked by variable loops.[208]

In another group of scaffold molecules, single amino acid residues involved in relatively rigid surface-exposed secondary structure elements are varied.[202] This group includes affibody molecules and ankyrin repeats. Randomization of 13 surface-exposed residues of two α-helices of the protein A from *Staphylococcus aureus* gave so-called affibody molecules that bind a number of protein ligands.[202] Natural ankyrins consist of 33-mer repeat domains. Their stable structure is composed of a β-turn and two α-helices. The ankyrin repeat serves as a basis for the construction of DARPin molecules. The latter usually contain three synthetic consensus repeat sequences, six residues being varied in each repeat.[205] So-called ~40-mer WW domains that link linear peptide sequences should be mentioned as new scaffold proteins.[209] Nine residues of these domains can be randomized in order to construct the synthetic binding surface.

Synthetic binding proteins based on alternative scaffolds can be generated using the same main approaches as for the construction of recombinant antibodies.[210] Yeast and phage display, as well as mRNA display, are most commonly employed for this purpose. The development of synthetic binding molecules based on alternative scaffold proteins holds promise for the design of new formats of compounds which specifically bind molecular and cellular targets, are intended for biomedical applications and are particularly suitable for robotic technologies and construction of supramolecular nanostructures. Thus, a number of synthetic binding proteins, which have high affinity for the epidermal growth factor receptor, the carcinoembryonic antigen (CEA) and other ligands, were constructed based on the fibronectin domain.[211, 212] Variants of ^{10}Fn3 that bind Erk-2 mitogen-activated protein kinase were selected by the yeast display method. High-affinity proteins recognizing the target epitopes and suppressing the activity of Erk-2 *in vitro* and in mammalian cells were produced.[213] A recombinant protein, which can bind to this cytokine and decrease its *in vitro* cytotoxicity against the TNFα-sensitive cell line L929 by a factor of 50, was constructed by grafting hypervariable regions of the anti-TNFα F10 antibody onto ^{10}Fn3.[214] Recombinant immunotoxin against EpCAM (epithelial cell adhesion molecule) was constructed based on the DARPin Ec4, and its *in vitro* and *in vivo* efficacy against certain types of tumour cells was confirmed.[215]

For the combined effect, scaffold proteins that bind different molecular targets can be assembled into hybrids. In particular, bispecific and bivalent DARPins that bind different epitopes involved in the α-chain of the human immunoglobulin E receptor were proposed. Variants, which can serve as a basis for the preparation of inhibitors of IgE-mediated allergic reactions, were identified by screening.[216] Recently, bispecific molecules targeting both EGFR and IGF-1R were constructed based on ^{10}Fn3; these antinodies can be used in the cancer therapy. Such heterodimeric ^{10}Fn3 variants facilitate a 80% decrease in the level of EGFR on the surface of different cell lines.[217] Triple-specificity hybrid affibody molecules than bind HER2, HER3 and albumin block the ligand-induced phosphorylation of HER receptors and, consequently, have an antiproliferative effect.[218]

Scaffold proteins can be functionalized also by the formation of hybrids with enzymes. For example, biomolecules can be detected using digoxigenin-specific anticalin fused with alkaline phosphatase.[219] Anti-human IgA affibody molecules produced as tetrameric β-galactosidase hybrids in the *Escherichia coli* cytoplasma were constructed. These proteins can be used in the enzyme-linked immunosorbent assay (ELISA), as well as in dot-blot and immunohistochemistry assays.[220]

Alternative scaffold-based binding proteins are most commonly employed for the detection and visualization of target ligands. Due to a small size, such molecules can easily penetrate into tissues and are rapidly removed from the body. Thus, radioactive isotope-labelled HER2- and EGFR-binding affibody molecules, as well as labelled anti-EGFR variants, were successfully used for the detection of mouse tumour xenografts expressing the corresponding proteins.[217, 221] The amino acid sequence of the *Ecballium elaterium* trypsin inhibitor was modified by the addition of residues providing the binding of integrin αvβ3 followed by mutagenesis in order to enhance affinity. Reagents for the visualization by positron emission tomography were prepared by labelling with radioactive ^{64}Cu and ^{18}F isotopes.[222] A new class of hybrid proteins containing the ^{10}Fn3 variant that binds integrin αvβ3 and the red fluorescent protein mCherry was developed.[223] It was shown that this protein can be used for the visualization of target molecules — markers for neoangiogenesis — on the cell surface.

VIII. Conclusion

The last decade has witnessed great progress in the engineering of antibodies with specified properties.[1, 2] According to the definition given in the review, monoclonal antibodies are typical natural scaffold proteins, or scaffolds. The modular structure of natural immunoglobulins is not only responsible for a great diversity of these molecules produced in the body against foreign antigens and the multiplicity of mechanisms by which the body responds to their invasion, but is also an inexhaustible source for the construction of synthetic molecules with required properties.

Both full-size antibodies[8] and individual domains[68] are used as scaffold proteins for the construction of new compounds. Numerous rational and empirical approaches to the engineering of mAb variable fragments can be employed to perform targeted modifications and optimization of antibody binding parameters.[60] Different technologies for targeted modifications of the constant domain are suitable for controlling effector functions of antibodies.[110]

Let us mention the following new promising lines of research in the antibody engineering lying at the interface between biology and chemistry: the development of methods of site-specific chemical conjugation, which can be applied to supplement mAbs with additional effector molecules and even recognition peptide modules; reengineering of catalytic antibodies and construction of optimal scaffolds capable of performing catalytic functions most efficiently;[95, 151] engineering of individual domains of human mAbs and alternative scaffold proteins for the creation of

new formats of compounds, which specifically bind molecular and cellular targets, intended for biomedical applications and particularly suitable for robotic technologies and construction of supramolecular nanostructures.[68, 162, 201, 224]

The successful development of numerous mAb engineering technologies becomes possible due to an enormous volume of information on their structures and the available computer and combinatorial methods allowing for the use of this information. However, the main driving force for the development of this area of research is still the idea of a magic bullet, *i.e.*, the design of highly selective and effective drugs for the treatment of diseases that are hard to cure by conventional therapies. Monoclonal antibodies against markers of tumour cells and immune system cells provide an impressive example of the realization of this idea. These antibodies serve as a basis for compounds constructed for diagnosis, targeted therapy and monitoring of the disease course[11, 12, 27, 196] and are important components of the therapeutic regimen in the complex treatment of many types of tumour diseases.[70, 72, 76]

It should be noted that, despite the introduction of mAb-based drugs into the clinical use and considerable commercial success,[3, 4] the tremendous potential of the structure and functions of immunoglobulins is not fully used in diagnosis and therapy. Only a small part of compounds constructed based on mAbs and their derivatives comes to clinical practice. Evidently, the expansion of their use as therapeutic and diagnostic agents requires further fundamental investigations of the molecular mechanisms of different diseases, the search for new diagnostic markers and therapeutic targets, the development of systematic approaches for personalized medicine[225] and further development of technologies for mAb engineering to create new selective drugs with improved pharmacological properties.[10, 62, 70, 123]

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