Chapter 8
From Natural to Artificial Biorecognition Elements: From Antibodies to Molecularly Imprinted Polymers

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8.1 Introduction

The observation of nature that surrounds us and the effort to simplify everyday tasks led hand in hand to the discovery of many inventions and novel technologies, many of which are based on natural principles. But why should they be limited only to objects of a macroscopic world that can be seen by the naked eye? With the progress in science and discoveries of processes taking place in living cells, people started to be interested how life works. Consequently, they realized that life is based on specific interactions among cells and molecules, which exhibit some kind of molecular complementarity. They found out that molecular recognition is crucial in a number of processes, such as: (1) cell recognition where the protein-based surface

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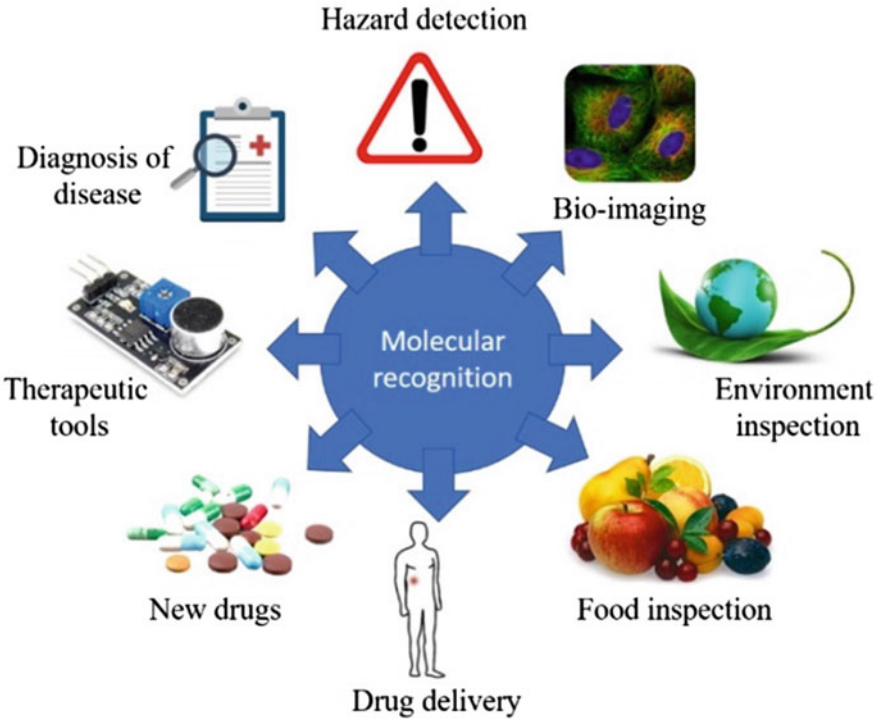

Fig. 8.1 Schematic overview of areas of applications of molecular recognition

receptors are able to recognize and respond to external stimuli; (2) catalytic activity of enzymes, which recognize substrate and enable to implementation of a wide range of reactions; (3) defensive ability of antibodies that are able to recognize antigens and trigger various protective processes; (4) cell division where specific proteins are able to recognize certain part of DNA and initiated replication; (5) cell differentiation which is based on recognition of stimuli leading to changes in gene expression and finally to specialized cells. So, why should not we get inspired by biological processes occurring in living cells and try to apply them in technologies that make our life easier?

The role of molecular recognition between biomolecules was first described as a "lock-and-key" mechanism by E. Fisher in 1894. Nowadays, molecular recognition draws a lot of attention in many scientific disciplines (e.g., efficient purification, analytical methods, imaging and clinical applications, etc.). Moreover, understanding and use of molecular recognition lead not only to the creation of novel assays and sensors but also to the development of new materials for drug delivery and treatment. The overview of scientific fields in which molecular recognition is important is schematically shown in Fig. 8.1.

Initially, biological macromolecules (such as antibodies or aptamers) have been widely used in molecular recognition-based technology. However, their limitations,

such as high production costs and low stability, lead to the development of other completely novel recognition components that are able to overcome the limitations of biological macromolecules.

In this chapter, the molecular elements (specifically antibodies, aptamers, and molecularly imprinted polymers) that have an enormous potential to be used as antibody alternatives in chemistry and medicine are discussed in detail.

8.2 Development and Production of Recognition Elements

8.2.1 Antibodies

Antibodies (Abs) are Y-shaped glycoprotein molecules formed by two heavy and two light polypeptide chains. They are produced by B-cells to recognize and neutralize antigens such as a variety of pathogens [1]. Abs were discovered in 1890 by Emil von Behring along with K. Shibasaburo, who for the first time described the presence of "neutralizing substances" in the blood, which could counter infections [2]. At the beginning of the twentieth century, antibodies were labeled by Paul Ehrlich as "magic bullets" that selectively target an area of disease in the human organism, and he predicted their application in medical therapy [3].

However, the first experiments focused on medical therapies that were performed with **polyclonal antibodies** (PAbs) were not as effective as predicted [4]. The majority of antigens have a highly sophisticated structure and contain several epitopes that can be recognized by different lymphocytes. As each lymphocyte produces an antibody against a different epitope, the resulting antibody response was polyclonal. This means that obtained PAbs are a mixture of heterogeneous antibodies, which are able to recognize and interact with a variety of epitopes within a single antigen. Because of low specificity and significant cross-reactivity, PAbs are not suitable for antibody-based therapy in medicine. Nevertheless, PAbs are applicable in the detection of unknown antigens, so, for example, they are utilized as primary antibodies in many immunoassays (incl. ELISA, Western blotting, etc.) or are useful in immunohistochemistry [5].

In 1975, Kohler and Milstein demonstrated a process of production of **monoclonal antibodies** (MAbs) [6]. MAbs are generated by a single B-lymphocyte clone, and thus they have a monovalent affinity and are able to recognize only one specific and always identical epitope (small antigen part) of an antigen [5]. The production of MAbs is based on an administration of the chosen antigen into an appropriate organism (e.g., mouse). After the development of the immune response within the animal, B-lymphocytes are extracted from the spleen. Subsequently, the isolated B-cells are fused with a myeloma cell line, resulting in the creation of immortalized B-cell/myeloma hybridomas. The created hybridomas have properties of both the fused cells; they are capable of fast continuous growth in a culture like a myeloma cell line and also of production of Abs as B-cells. Finally, hybridomas producing only one clone of antibodies (MAbs) are separated from the culture and used for the

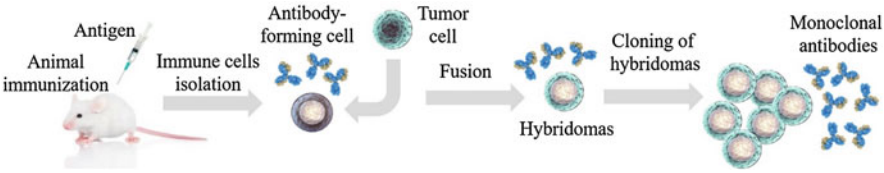

Fig. 8.2 Scheme of the procedure leading to the preparation of monoclonal antibodies

production of chosen MAbs [7]. For easier understanding of the process, preparation of MAbs is schematically shown in Fig. 8.2. Unfortunately, the main drawback of the hybridoma technology is the risk that the hybridoma cells lose the ability to produce the desired antibodies over time, or the antibodies may undergo unwanted changes, which may affect their functionality [8].

It was widely believed that these MAbs would be ideal reagents for imaging, drug delivery, and/or therapy. However, due to their animal origin, these MAbs were recognized as alien and eliminated by the patient's immune system when they were used as therapeutics. Notwithstanding, MAbs enable very selective or specific separation and identification of a wide range of different targets, and they are indispensable tools in many analytical approaches such as ELISA, Western blot, affinity chromatography, antibody-based sensors, and so on [9].

Recombinant phage antibodies (RAbs) are small proteins that consist of domains with variable heavy and light chains, and they may be connected via a flexible peptide chain. These antibody fragments retain the ability to recognize and bind the target epitope of an antigen. A major breakthrough in the field of RAbs was reported in 1990 by McCafferty et al., who presented the display of protein fragments on the surface of a filamentous bacteriophage. The filamentous phage is a virus-like particle that can infect bacteria (commonly *Escherichia coli*). Upon infection, the bacterial cells start to produce the phage particles with antibody fragments on their surface and secrete them into the culture media. RAbs produced by this way have several benefits compared to traditional MAbs (e.g., small penetrable size, high standard of specificity, low immunogenicity, and rapid production) [10]. Currently, RAbs are utilized in a number of clinical trials for diagnostic as well as therapeutic purposes [11]. Characterized RAbs can be used as chemicals to replace standard MAbs used for flow cytometry or immunohistochemistry. Thanks to their low immunogenicity, they are also applicable in cell targeting and imaging, as well as in vaccine development [12]. However, it is necessary to realize that in the case of RAbs there is only one antigen binding site (in comparison with two sites in the case of native antibodies), and therefore the affinity to antigen must be very high to avoid losses during the purification process [10]. Another limitation due to the small size of antibody fragments rests in their shorter in vivo half-lives, and therefore a more frequent drug dosing is necessary, which can increase the risks of undesirable effects.

The high immunogenicity of MAbs was one of the problems for their utilization in clinical use. However, progress in gene engineering enabled the partial

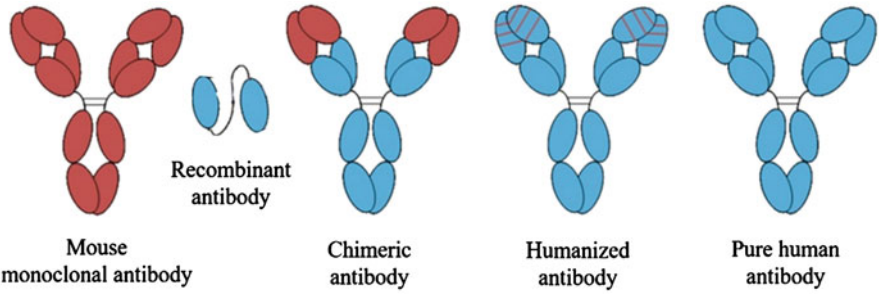

Fig. 8.3 Differences between a variety of types of antibodies. Scheme available at [16]

replacement of immunogenic sites in mouse MAb with the appropriate fragments of human antibodies. In 1984, chimeric Abs were developed by Morisson et al. to overcome the immunogenicity of MAbs [13]. Chimerization permits the connection of the whole antigen-specific domain of a mouse antibody with constant domains of a human antibody. These **chimeric Abs** are recognized as of own body and therefore are not eliminated by the immune system, and thus can be used in medical therapy [14]. Later, **humanized MAbs** were produced by gene engineering: here, only sites enabling selective interaction with antigen are of mouse origin. And finally, **pure human MAbs** began to be manufactured using transgenic mice producing human immunoglobulins. The approach takes advantage of the ability to replace mouse antibody gene locuses with their corresponding human genome sites [15]. Differences between the above-mentioned Abs are shown in Fig. 8.3.

The discovery of chimerization and humanization of antibodies led to a new era of antibody-based therapeutics. It has been expected that by 2020 approximately 70 MAbs products will be commercialized for therapeutic purposes, mainly for therapy of cancer and immune disorders [17] based on the fact that a total of 61 MAbs were approved by the end of 2017, which demonstrates the enormous impact of this technology.

The first MAbs-based therapeutic (Orthoclone OKT3) was approved by the Food and Drug Administration (FDA) in 1986 [18]. Since then, a wide range of other antibodies and antibody derivatives have been developed and approved for use, and new ones are still being developed. An overview of Abs-based drugs approved by FDA is presented in Table 8.1.

Moreover, George P. Smith and Gregory P. Winter were awarded Nobel Prize in Chemistry in 2018 for the development of their contributions to antibody therapy by utilization of Phage display technology [19, 20]. For a very detailed overview, readers should refer to [21].

Nevertheless, MAb-based treatments still face several obstacles that limit their widespread application as therapeutics. The main limitation is given by the high production costs associated with the need of mammalian cell cultures for the fabrication of MAbs, followed by a cleaning process under the Good Manufacturing Practice regime. This process is very time-consuming and expensive, which limits

Table 8.1 The overview of Abs-based drugs approved by FDA or EMA (The European Medicines Agency)

International name	Brand name	Target	Format	Disease	EMA approval	FDA approval
Sacituzumab govitecan	TRODELVY	TROP-2	Humanized IgG1	Breast cancer	NA	2020
Isatuximab	Sarclisa	CD38	Chimeric IgG1	Multiple myeloma	NA	2020
Eptinezumab	VYEPTI	CGRP	Humanized IgG1	Migraine prevention	NA	2020
Teprotumumab	Tepezza	IGF-1R	Human IgG1	Thyroid eye disease	NA	2020
[fam-]trastuzumab deruxtecan	Enhertu	HER2	Humanized IgG1	Metastatic breast cancer	NA	2019
Enfortumab vedotin	Padcev	Nectin-4	Human IgG1	Urothelial cancer	NA	2019
Crizanlizumab	Adakveo	CD62	Humanized IgG2	Sickle cell disease	In review	2019
Brolucizumab	Beovu	VEGF-A	Humanized scFv	Neovascular age-related macular degeneration	2020	2019
Romosozumab	Evenity	Sclerostin	Humanized IgG2	Osteoporosis	2019	2019
Polatuzumab vedotin	Polivy	CD79b	Humanized IgG1	Diffuse large B-cell lymphoma	2020	2019
Risankizumab	Skyrizi	IL-23 p19	Humanized IgG1	Plaque psoriasis	2019	2019
Caplacizumab	Cablivi	von Willebrand factor	Humanized nanobody	Acquired thrombotic thrombocytopenic purpura	2018	2019
Moxetumomab pasudotox	Lumoxiti	CD22	Murine IgG1 immunotoxin	Hairy cell leukemia	In review	2018
Emapalumab	Gamifant	IFN	Human IgG1	Primary hemophagocytic lymphohistiocytosis	In review	2018
Ibalizumab	Trogarzo	CD4	Humanized IgG4	HIV infection	2019	2018
Ravulizumab	Ultomiris	C5	Humanized IgG2	Paroxysmal nocturnal hemoglobinuria	2019	2018
Fremanezumab	Ajovy	CGRP	Humanized IgG2	Migraine prevention	2019	2018
Tildrakizumab	Ilumya	IL-23	Humanized IgG1	Plaque psoriasis	2018	2018
Mogamulizumab	Poteligeo	CCR4	Humanized IgG1	Mycosis fungoides or Sézary syndrome	2018	2018

Lanadelumab	Takhzyro	Plasma kallikrein	Human IgG1	Hereditary angioedema attacks	2018	2018
Burosumab	Crysvita	FGF23	Human IgG1	X-linked hypophosphatemia	2018	2018
Galcanezumab	Emgality	CGRP	Humanized IgG4	Migraine prevention	2018	2018
Erenumab	Aimovig	CGRP receptor	Human IgG2	Migraine prevention	2018	2018
Gemtuzumab	Mylotarg	CD33	Humanized IgG4	Acute myeloid leukemia	2018	2017
Durvalumab	IMFINZI	PD-L1	Human IgG1	Bladder cancer	2018	2017
Benralizumab	Fasenra	IL-5R α	Humanized IgG1	Asthma	2018	2017
Ocrelizumab	OCREVUS	CD20	Humanized IgG1	Multiple sclerosis	2018	2017
Emicizumab	Hemlibra	Factor Ixa	Humanized IgG4	Hemophilia A	2018	2017
Avelumab	Bavencio	PD-L1	Human IgG1	Merkel cell carcinoma	2017	2017
Sarilumab	Kevzara	IL-6R	Human IgG1	Rheumatoid arthritis	2017	2017
Guselkumab	TREMFYA	IL-23 p19	Human IgG1	Plaque psoriasis	2017	2017
Inotuzumab	BESPONSA	CD22	Humanized IgG4	Acute lymphoblastic leukemia	2017	2017
Dupilumab	Dupixent	IL-4R α	Human IgG4	Atopic dermatitis	2017	2017
Brodalumab	Siliq	IL-17R	Human IgG2	Plaque psoriasis	2017	2017
Obiltoxaximab	Anthim	*B. anthracis*	Chimeric IgG1	Prevention of inhalational anthrax	In review	2016
Atezolizumab	Tecentriq	PD-L1	Humanized IgG1	Bladder cancer	2017	2016
Bezlotoxumab	Zinplava	*Clostr. difficile* enterotoxin B	Human IgG1	Prevention of *Clostr. difficile* infection recurrence	2017	2016
Olaratumab	Lartruvo	PDGFRα	Human IgG1	Soft tissue sarcoma	2016	2016
Reslizumab	Cinqaero	IL-5	Humanized IgG4	Asthma	2016	2016
Ixekizumab	Taltz	IL-17a	Humanized IgG4	Psoriasis	2016	2016
Elotuzumab	Empliciti	SLAMF7	Humanized IgG1	Multiple myeloma	2016	2015
Daratumumab	Darzalex	CD38	Human IgG1	Multiple myeloma	2016	2015
Alirocumab	Praluent	PCSK9	Human IgG1	High cholesterol	2015	2015
Mepolizumab	Nucala	IL-5	Humanized IgG1	Severe eosinophilic asthma	2015	2015
Secukinumab	Cosentyx	IL-17a	Human IgG1	Psoriasis	2015	2015
Dinutuximab	Unituxin	GD2	Chimeric IgG1	Neuroblastoma	2015	2015

(continued)

Table 8.1 (continued)

International name	Brand name	Target	Format	Disease	EMA approval	FDA approval
Necitumumab	Portrazza	EGFR	Human IgG1	Cell lung cancer	2015	2015
Idarucizumab	Praxbind	Dabigatran	Humanized Fab	Reversal of dabigatran-induced anticoagulation	2015	2015
Evolocumab	Repatha	PCSK9	Human IgG2	High cholesterol	2015	2015
Alemtuzumab	Lemtrada	CD52	Humanized IgG1	Multiple sclerosis	2013	2014
Blinatumomab	Blincyto	CD19, CD3	Murine bispecific tandem scFv	Acute lymphoblastic leukemia	2015	2014
Pembrolizumab	Keytruda	PD1	Humanized IgG4	Melanoma	2015	2014
Nivolumab	Opdivo	PD1	Human IgG4	Melanoma, non-small cell lung cancer	2015	2014
Vedolizumab	Entyvio	α4β7 integrin	Humanized IgG1	Ulcerative colitis, Crohn disease	2014	2014
Ramucirumab	Cyramza	VEGFR2	Human IgG1	Gastric cancer	2014	2014
Siltuximab	Sylvant	IL-6	Chimeric IgG1	Castleman disease	2014	2014
Obinutuzumab	Gazyva	CD20	Humanized IgG1	Chronic lymphocytic leukemia	2014	2013
Raxibacumab	Pending	*B. anthracis*	Human IgG1	Anthrax infection	NA	2012
Ado-trastuzumab	Kadcyla	HER2	Humanized IgG1	Breast cancer	2013	2012
Pertuzumab	Perjeta	HER2	Humanized IgG1	Breast cancer	2013	2012
Brentuximab vedotin	Adcetris	CD30	Chimeric IgG1	Hodgkin lymphoma, systemic anaplastic large cell lymphoma	2012	2011
Ipilimumab	Yervoy	CTLA-4	Human IgG1	Metastatic melanoma	2011	2011
Belimumab	Benlysta	BLyS	Human IgG1	Systemic lupus erythematosus	2011	2011
Denosumab	Prolia	RANK-L	Human IgG2	Bone loss	2010	2010
Tocilizumab	RoActemra	IL-6R	Humanized IgG1	Rheumatoid arthritis	2009	2010
Ofatumumab	Arzerra	CD20	Human IgG1	Chronic lymphocytic leukemia	2010	2009
Golimumab	Simponi	TNF	Human IgG1	Rheumatoid and psoriatic arthritis, ankylosing spondylitis	2009	2009

Canakinumab	Ilaris	IL-1β	Human IgG1	Muckle-Wells syndrome	2009	2009
Ustekinumab	Stelara	IL-12/23	Human IgG1	Psoriasis	2009	2009
Certolizumab pegol	Cimzia	TNF	Humanized Fab	Crohn disease	2009	2008
Eculizumab	Soliris	C5	Humanized IgG2	Paroxysmal nocturnal hemoglobinuria	2007	2007
Ranibizumab	Lucentis	VEGF	Humanized IgG1 Fab	Macular degeneration	2007	2006
Panitumumab	Vectibix	EGFR	Human IgG2	Colorectal cancer	2007	2006
Natalizumab	Tysabri	a4 integrin	Humanized IgG4	Multiple sclerosis	2006	2004
Bevacizumab	Avastin	VEGF	Humanized IgG1	Colorectal cancer	2005	2004
Cetuximab	Erbitux	EGFR	Chimeric IgG1	Colorectal cancer	2004	2004
Omalizumab	Xolair	IgE	Humanized IgG1	Asthma	2005	2003
Ibritumomab	Zevalin	CD20	Murine IgG1	Non-Hodgkin lymphoma	2004	2002
Adalimumab	Humira	TNF	Human IgG1	Rheumatoid arthritis	2003	2002
Trastuzumab	Herceptin	HER2	Humanized IgG1	Breast cancer	2000	1998
Infliximab	Remicade	TNF	Chimeric IgG1	Crohn disease	1999	1998
Palivizumab	Synagis	RSV	Humanized IgG1	Prevention of respiratory syncytial virus infection	1999	1998
Basiliximab	Simulect	IL-2R	Chimeric IgG1	Prevention of kidney transplant rejection	1998	1998
Rituximab	MabThera	CD20	Chimeric IgG1	Non-Hodgkin lymphoma	1998	1997
Abciximab	Reopro	GPIIb/IIIa	Chimeric IgG1 Fab	Prevention of blood clots in angioplasty	1995	1994

Data were obtained from https://www.antibodysociety.org/

the widespread use of this type of medicaments [9]. Moreover, Abs very often suffer from batch-to-batch variations [22].

Other issues are the elimination of mAb therapeutics that can occur in the body and the fact that antibody-drug antibodies (ADAs) can be formed, binding competitively to the active site of the therapeutic MAbs, thus compromising the drug efficacy. Also, the drug pharmacokinetic properties, biological effects, and the toxicity can be unpredictably changed by ADAs [23]. Another issue is a misleading prediction of human response using animals due to the different levels of immunogenicity between animal models and humans [24].

Last but not least, enzymatic degradation may arise. It is generally known that MAbs may undergo enzymatic degradation, which may occur either at the site of the administration or in the bloodstream. Owing to the presence of high concentrations of enzymes in the gastrointestinal tract, oral delivery systems are challenging to develop [24]. Therefore, the means of administration of MAbs drugs is usually the parenteral injection. However, MAbs can still suffer from enzymatic degradation (e.g., by proteases or hydrolases) because they are abundant throughout the body [17].

Despite the high potential of Abs in medical therapy, these limitations lead to efforts to develop some novel recognition elements which will be more stable and less expensive.

8.2.2 APTAMERs

In 1990, two ground-breaking studies were performed simultaneously and independently confirmed that ligands of nucleic acids can selectively interact with virtually any protein. A.D. Ellington and J. W. Szostak isolated those RNA subpopulations from a population of RNA molecules with random sequences that specifically interacted with a variety of organic dyes and named them aptamers [25]. At the same time, C. Tuerk and L. Gold created an RNA chain that was able to selectively bind to T4DNA polymerase and used the term SELEX (Systematic Evolution of Ligands by Exponential Enrichment) for the process of its selection [26].

Aptamers are usually short single-stranded chains of DNA or RNA (50–100 base-long) that are able to bind to protein targets by folding into a three-dimensional conformation [27]. The above-mentioned SELEX is a process that enables the identification of an aptamer selective for a chosen target. The process consists of three main repetitive steps: selection, partitioning, and amplification (see Fig. 8.4). The entire procedure begins with the creation of a large "library" containing a variety of nucleotide sequences, which can theoretically adopt a specific three-dimensional structure.

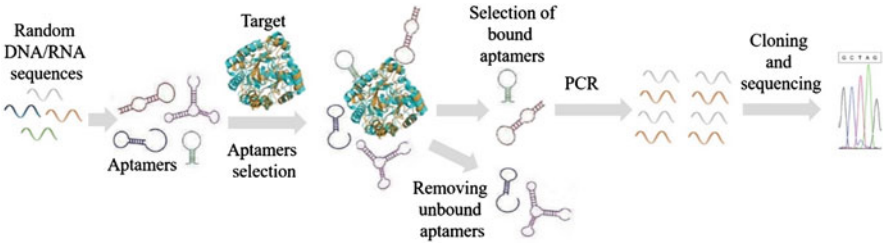

Fig. 8.4 Scheme of a process called SELEX, which ensures the choice of specific aptamers against the desired target

- In the **selection phase**, the nucleotide sequences from the "library" are incubated in the presence of the target for a required period of time. The sequences with weak or no affinity for the target remain unbound in solution, in difference to those with a higher ability to interact with the target that will be attracted to it.
- The **partitioning phase** ensures the physical separation of the aptamer–target complexes from the unreacted components of the mixture.
- In the **amplification phase**, the bound aptamers are released from the target. The captured and purified sequences are amplified by polymerase chain reaction (PCR) to generate a new "library" of aptamers that contains increased amounts of those effectively binding the target.

To reduce the very large number of unique sequences (in the order of trillions) to a small number of unique sequences that are able to selectively interact with the target, the whole process is repeated with a new "library" enriched in each step, repeated ca 5–15 times. The individual aptamers are subsequently extracted and sequenced. Their binding affinity and specificity are also determined [28] (Fig. 8.4).

In 2004, the first aptamer-based medicament was approved by the FDA (Macugen), focusing on the vascular endothelial growth factor and the therapy of age-related macular degeneration [29]. Unfortunately, no other aptamer-based drug has been developed since then, which is likely caused by insufficient knowledge of their structure, target interactions, and pharmacokinetics.

During the last few years, a wide range of experiments were carried out that led to improvements in the stability and efficiency of aptamers. This opens multiple new directions for therapeutic applications of aptamers. In this part of the chapter, we will discuss the benefits and limitations of aptamers, their significance for therapeutic applications, as well as the advances and directions of future research.

Aptamers are characterized by high affinity and excellent specificity toward desired targets [30], and therefore they offer new very attractive prospects in diagnostics [31], sensors [32], (bio)analytical assays [33], and also in medicine, where they have opened novel avenues for the development of therapeutics [34], and targeted drug delivery [35].

In contrast to Abs, they have a number of benefits, such as the possibility of automation and monitoring of the SELEX process that allow to produce specific aptamers with high selectivity and prevent batch-to-batch variability

[36]. Nevertheless, achieving required aptamer properties is challenging due to the short in vivo half-life, risk of immunogenicity, and entrapment in cellular organelles [37].

The first challenge to overcome was the short half-life of aptamers *in vivo*. It was expected that the small size of aptamers will allow to achieve higher aptamer penetration into tumors, in comparison with the structurally larger Abs. However, the majority of aptamers have sizes in the range of 5–30 kDa (the average diameter of aptamers is less than 5 nm), and thus they are susceptible to renal clearance [38]. One possible way to overcome the rapid renal excretion may be an increase of their total size through linkage with some suitable high molecular weight moieties (e.g., polyethylene glycol [39], cholesterol [40, 41], some proteins [42], or nanomaterials [43]).

Another limitation is due to nucleases (enzymes cleaving phosphodiester bonds of oligonucleotides) abundantly present in biological fluids that cause aptamer decay in several minutes [38]. An improvement in the stability of aptamers in serum can be resolved by their chemical modification. For example, locked nucleic acids that contain a 2-O, 4-C methylene bridge can be used. This modification has a high resistance to nucleases [44]. Another possibility is based on the replacement of the -OH group of ribose for amino [45] or -fluoro [46] moiety that can increase the resistance of aptamers.

On the one hand, the above-mentioned modifications protect the aptamers against degradation and extend their in vivo half-life, but, on the other hand, modified aptamer sequences may resemble pathogen-associated molecular patterns and activate the innate immune system, and thus they could cause immunogenicity or toxicity [47]. In some cases (e.g., in cancer treatment), it might be even beneficial because aptamers have a dual effect—they serve as the delivery agents of a chemotherapeutic drug and simultaneously, they re-activate the anti-tumor immunity. However, the risk of undesirable and harmful side effects is increased. Moreover, aptamers can accumulate in some organs (such as in the liver, kidney, or spleen) and might cause toxicity [37]. Therefore, deeper investigations of the correlation between the aptamer structure, the administration route, and adverse effects in vivo are urgently needed.

The most serious impediment in effective aptamer-based treatments is probably due to cellular organelles. The aptamers specific for cell-surface receptors are often endocytosed and trapped in the endo-lysosomal vesicle. For the endosomal escape, a number of approaches can be applied, such as a combination of aptamers with other delivery vehicles (liposomes [35], viruses [48], etc.), or aptamer attachment to a protein part that disrupts the endosome membrane [49]. Unfortunately, these strategies are not applicable for therapeutic aptamers because they might be toxic or immunogenic [37]. Therefore, it is necessary to develop non-toxic molecules enhancing the ability of endosomal escape compatible with the aptamer drug formulation.

Recently, a number of potential aptamers-based drugs that can be used in the treatment of infectious diseases started to appear. Among very promising works are those of application of aptamers as inhibitors of viral nucleic acid replication. So far, a cholesterol-conjugated aptamer able to enter into the cell

Table 8.2 Examples of aptamer-based drugs, which are currently in the phase of clinical trials

Aptamer	Molecular target	Disease	Phase
Blood and heart disease			
ARC1779	Specific inhibitor of Von Willebrand factor	Von Willebrand disease	II
ARC19499	Inhibitor of tissue factor pathway inhibitor	Hemophilia	I
NOX-H94	Inhibitor of hepcidin	Anemia of chronic disease	I
NU172	Inhibitor of thrombin	Heart disease	II
REG1	Inhibition of factor IX in the coagulation cascade	Coronary artery disease	II
Ophthalmology			
ARC1905	Inhibitor of factor C5 of the complement cascade	Age-related macular degeneration	I
E10030	Inhibitor of platelet-derived growth factor	Age-related macular degeneration	II
Cancer treatment			
NOX-A12	Inhibitor of cell-derived factor-1	Multiple myeloma; chronic lymphocytic leukemia	II
AS1411	Inhibitor of nucleolin	Leukemia, myeloid	II

Data were obtained from https://clinicaltrials.gov/

infected by Hepatitis C virus has been developed, and currently, this aptamer is investigated as a promising anti-viral drug [40, 41]. The aptamer inhibited virus RNA replication while not changing the gene expression profiles including genes related to innate immune response. Another promising study in the area of treatment of virus diseases was focused on the inhibition of HIV replication by using aptamers which were able to block viral protein Rev essential to the regulation of HIV protein expression [50].

In recent years, enormous progress has been made in the development of aptamers for anticoagulant, antithrombotic, and prohemostatic indications, and several aptamer-based medicaments are currently in the phase of clinical trials [51]. Another immensely important area of potential aptamer application is that in cancer treatment. So far, a wide range of aptamers capable of targeting different cancer cell biomarkers has been developed and reached the phase of clinical trials [30]. An overview of some selected promising aptamers in clinical trials is summarized in Table 8.2. The large number of aptamers-based medicaments is predicted soon to compete with antibody-based drugs for therapeutic applications.

8.2.3 Molecularly Imprinted Polymers (MIPs)

In 1931, M. V. Polyakov performed an experiment that led to the discovery of novel artificial materials with recognition ability. He found out that polymers prepared in the presence of another molecule were able to recognize and selectively interact with

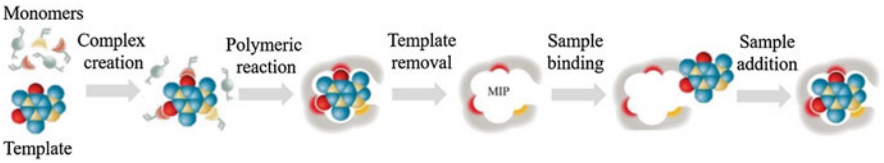

Fig. 8.5 Scheme of preparation and application of MIPs. Information available at [63]

this molecule [52]. It was the beginning of MIPs era. Due to their unique features, MIPs are sometimes dubbed artificial antibodies, enzyme mimic or synthetic receptors. Owing to their excellent physical and chemical stability, tuneable properties, and low production cost, they came to be competitors to commonly used biological macromolecules—antibodies [53].

Initially, the interest in molecular imprinting remained quite low. However, a discovery of non-covalent imprinting by Mosbach et al. in 1984 allowed a significant simplification in the preparation of MIPs, which led to an enormous upswing of this scientific field and exponential growth of publications in this area [54]. During the last decade, the progress in molecular imprinting enabled the development of novel types of diagnostic tools, sensors [55, 56], and assays [57, 58]. In addition, recently, the vast potential of molecular imprinting was discovered in therapeutic use (cell recognition [59], drug delivery [60], and regulation of cell behavior [61], etc.).

Molecular imprinting is a process that includes co-polymerization of functional monomers and cross-linkers in the presence of a template (the imprinted molecule). The functional monomers bind the template predominantly by non-covalent bonds and form a template–monomers complex. After initiation, the polymerization step leads to the creation of a highly cross-linked polymeric net. In the next step, template removal reveals the binding sites complementary to the imprinted molecules in terms of spatial structure as well as chemical availability of functional moieties. The obtained polymer matrix has molecular memory and enables rebinding of the imprinted molecule (analyte) with a very high specificity [62]. The principle of MIP preparation and subsequent application is schematically shown in Fig. 8.5**.**

In spite of the tremendous progress in the technology of imprinted polymers, imprinting of larger structures such as proteins, bacteria, viruses, etc., is still quite a big challenge. The main reason is that in the case of these structures creation of polyclonal MIPs with a broad range of binding sites with different affinities and specificities may occur. This may be caused by difficulties in maintaining the conformation and space orientation of native protein during the polymerization process. Besides, the large size of the imprinted structure causes difficulties in the template removal from the polymerized net, and the large binding sites may have reduced selectivity features because a range of smaller polypeptides can interact with them [64, 65].

In 2000, a completely new strategy termed epitope imprinting was discovered [66]. The epitope imprinting is based on imprinting of a small analyte/template fragment which is characteristic for the chosen large template instead of the

imprinting of the whole template structure [67]. In principle, this approach is very similar to the recognition of antigen by antibody, where the antibody is able to recognize only an epitope (small antigen part) and not the whole antigen structure. Imprinting of epitope in comparison with the whole protein imprinting permits relatively easy removal of the template ensures a uniform production of binding sites (because the small fragments have a less complicated structure and so maintaining their conformation during the polymerization is easier), and the synthesis cost is reduced, especially in the case of expensive protein templates [68]. The main drawback is associated with the identification of appropriate epitopes for the large molecule that requires detailed knowledge of template conformations [69].

However, recently, a novel protocol for protein epitopes identification suitable for molecular imprinting has been reported. The protocol is based on the synthesis of MIP nanoparticles in the presence of a whole target protein. The low concentration of polymeric mixture leads to the formation of MIP particles only around small parts of the protein. Partial proteolysis of the protein causes proteolytic cleavage of parts of the protein that are not protected by the created MIPs. The peptides surrounded by the polymer net are subsequently released and sequenced [70]. In brief, this approach enables the identification of surface protein regions appropriate for recognition by imprinted polymers.

Conventional polymerization approaches (e.g., bulk, emulsion, or suspension polymerization) exploit templates that are dissolved in the polymerization solution. These templates can rotate and freely move in the solution, which leads to the creation of random binding sites that suffer from heterogeneous polyclonal distribution and problematic batch-to-batch reproducibility. However, these drawbacks may be overcome by introducing an approach called solid-phase synthesis [62].

In this approach, the template is linked to a solid support (the most common are glass beads). This ensures its consistent orientation and leads to a decrease in polyclonality of the imprinted sites. Subsequently, the solid supports with covalently bound template molecules are incubated with a mixture of functional monomers, cross-linkers, and initiator, and the polymeric reaction is initiated. The unreacted components of the polymerization mixture and MIPs particles with low affinity are eluted at room temperature; meantime, the high affinity MIP nanoparticles stay bound to the template, and they are eluted in the next step by increasing the temperature. The obtained MIP particles have monoclonal binding sites, and therefore can be applicable to the preparation of commercially available MIPs [71, 72].

In spite of all the benefits that MIPs offer, the number of MIP-based commercial products is largely confined to a handful of products predominantly intended for selective solid-phase extraction and a few sensors. For the commercial application of MIPs, it is necessary to control the fabrication process and optimize it in such a manner that the obtained MIPs have a good batch-to-batch reproducibility, which was initially quite problematic. Therefore, it was necessary to focus on novel tools enabling to overcome bottlenecks in the production and characterization of MIPs-based materials. In 2013, an automated chemical reactor for solid-phase synthesis of MIP NPs in an aqueous environment was developed by the research group of

S. Piletsky [72]. The developed device was able to produce "ready to use" MIP nanoparticles with sub-nanomolar affinity in only 4 hours. Significant advantages of this automated chemical reactor rest in its full automation that enable the reactor to operate for 24 h, which eliminates human error and ensures high batch-to-batch reproducibility [72, 73]. These new advances may be the first step in the spread of commercial MIP-based products.

MIPs as artificial receptors in the field of medical therapy have a number of benefits in comparison with natural biorecognition materials. The major advantages are the possibility of automatized fabrication allowing rapid and low-cost production, batch-to-batch uniformity [72], and fast and homogenous functionalization by different probes providing unique features (e.g., fluorescence, magnetic properties, electric conductivity, etc.) [74]. Another enormous advantage is the possibility to design MIPs tailor made for almost any target from ions and small molecules to larger structures such as proteins by using computational modeling [75]. Their extremely high stability makes MIPs compatible with thermal sterilization and ensures their resistance against enzymatic or pH-dependent degradation, which is also beneficial [76].

Clearly, the potential of MIPs in cell biomedical fields is huge, spanning across many areas such as recognition or regulation of cell behavior. However, so far, the main interest has been in the creation of adsorption or separation materials and sensing tools and the application of MIPs in the therapeutic area was rarely reported. Nevertheless, in the last years, the interest in MIPs has been consistently growing, and the numbers of research publications in the area of MIPs have been increasing. MIPs, as well as various other nanomaterials, have been successfully used in medicine as part of diagnostic devices [77, 78] and sensors [79], drug delivery elements [77, 78], and cell imaging probes [80]. However, it has been found that only MIPs could also be used as medicaments. This means that the huge potential of MIPs in the therapeutic area is still in their infancy. The following part provides an overview of several ground-breaking works focused on the utilization of MIPs as pharmaceuticals, which can change the main areas of their traditional application.

The work published in 2010 by Hoshino and his co-workers completely changed the view of MIPs' utilization and directed many researchers to investigate the potential of MIPs in finding and creating novel medicaments. The authors utilized for the first time MIPs as an anti-venom, when MIPs they developed allowed to capture and clear a target bee peptide toxin from the bloodstream of living mice [81]. Since then, other studies on MIPs-based anti-venom have begun to appear [82].

The first study confirming the possibility of MIPs' application as anti-virotics was performed in 2019 by Xu et al. The study focused on the preparation of MIPs targeting a specific peptide motif situated on the surface of the human immunodeficiency virus (HIV). This structure is responsible for the decline of $CD4^+$ T-cells and the resulting deterioration of the immune system during HIV infection. Blocking the function of this peptide by the developed MIP nanoparticles is a promising therapeutical approach for counteracting HIV [61].

Currently, studies focusing on MIPs that may have the potential to be applied as immunotherapeutic or sensitizing agents to improving chemotherapeutic anti-tumor effects appear highly attractive. These MIPs may, namely, participate in the establishment of a novel therapeutic platform in cancer treatment. For example, the approach published by Rangel and co-workers in 2020 seems to be very promising. This work is based on MIPs that could block the function of cadherins and thus completely disrupt three-dimensional tumor spheroids as well as inhibit invasion of healthy cells [83].

In spite of the impressive results, the applications of MIPs in medical therapy are still in their infancy, and there is a long way to achieve their commercial utilization in medicine. There are many unanswered questions to which solutions are needed to be found and many issues that need to be resolved before successful practical applications of MIPs. These issues include safety and biodegradability, which have to be investigated in detail, and the optimal properties for their biodistribution and clearance have to be found. However, it is very likely that MIPs will attract progressively more attention and perhaps in a few years maybe will be available as first MIP-based medicaments approved by FDA.

8.3 Conclusions

Currently, the most utilized recognition elements in medicine are Abs, and a number of Abs-based medicaments are commercially available. However, the treatment by Abs-based drugs is very expensive. Besides, Abs still suffer from several issues that limit the widespread use of this type of medicaments. Therefore, there is a huge effort in finding some Abs alternatives that enable to overcome these limitations. Aptamers and MIPs seem to be very promising candidates. In the case of aptamers, one aptamer-based medicament has been already approved by FDA. However, insufficient understanding of the structure, target interactions, and pharmacokinetics led to the production of novel aptamer-based drugs having been halted for some time. Nowadays, there is a number of promising pharmaceuticals based on aptamers in the clinical phase, which predicts that most likely they will soon compete with Abs-based drugs for therapeutic applications. As for MIPs, owing to their excellent physical and chemical stability, tuneable properties, and low production costs, they seem to be very attractive as Abs alternatives. In the last years, interest in these therapeutics has been growing, and the number of research publications focused on this topic has been increasing as well. Despite many excellent laboratory results, there is a wide range of unanswered questions and unsolved issues that have to be resolved before MIPs practical therapeutic application. A summary of the properties of the individual recognition elements is shown in Table 8.3.

Table 8.3 Comparison of different recognition elements

Production			
Characteristics	Antibodies	MIPs	Aptamers
Formation	Immune response in animal host	Computational modeling	SELEX
Manufacture	Mammalian cell systems	Chemical synthesis	Chemical synthesis
Batch-to-batch variability	Very high	Low (in the case of automated reactor)	Low
Average lead time (incl. development, production, validation)	>6–8 months	2–4 weeks (usually one day for production)	2–4 months (few days for production)
Cost for development of a new entity	15,000–25,000 $	4000–13,000 $ (depending on template cost)	6000–10,000 $
Production cost	Very high	Low	Medium
Range of targets	Medium	Wide	Wide
Ease of functionalization	Low	Very high	Very high
Availability of monomers	Limited to the number of amino acids	>4000 of variety functional monomers	Limited to the number of nucleic bases
Properties			
Characteristics	**Antibodies**	**MIPs**	**Aptamers**
Size (nm)	10–20	10–300 (hydrodynamic)	2–8
Affinity	Pico- to nanomolar	Pico- to nanomolar	Pico- to nanomolar
Immunogenicity	High	Unknown	Low
Thermal stability	Low	Very high	High
Organic solvent stability	Low	Very high	Low
pH stability	Low	Very high	Low
Enzyme stability	Low	Very high	Low

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