Therapeutic Protein-Based Vaccines

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Abstract

Infectious diseases are reported worldwide, and the emergence of highly mutated antibiotic-resistant strains is a major concern globally. Developing efficient vaccines is the only way to prevent and treat diseases effectively. Though developing conventional vaccines is an intricate and time-consuming process due to several rate-limiting steps, these vaccines help treat an array of existing diseases. There is a dire need for new forms of vaccines as many incidents of resistance are reported, and the efficacy of newly developed vaccines must be enhanced to treat the infections well in time. The human immune system fights against several infections utilizing antibody and the non–antibody-based immune mechanism, providing significant protection against identified pathogens. Nowadays, much effort is being made to develop vaccines focussing on the role of cellular responses to clear several complicated infections. This chapter concentrates on strategies for designing therapeutic protein-based vaccines and their diverse clinical and nonclinical applications.

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13.1 Introduction

Over 17 million individuals die annually from preventable infectious or communicable diseases globally. The high mutation rates in pathogens and the development of antibiotic-resistant strains are the key barriers to the prevention and treatment of such diseases. Vaccines offer a safer and faster method to overcome infectious diseases over a large population scale. However, the high cost of conventional vaccines in developing nations is a major concern, which is attributed to a lack of proper storage and supply facilities [1–3]. Hence, there is a vital need to develop new methods of vaccine development with low cost and high specificity. Vaccines are usually an inactivated pathogen or its component (DNA, RNA, protein) that stimulates a benign effect in an immune reaction to generate defense against an infection/disease on subsequent exposure to the pathogen when introduced to the host [2, 4–6]. Vaccines usually provide neutralizing activity in the body by generating protective antibodies against infections, and these antibodies develop in a few weeks to several months. Therefore, vaccines deliver the antigens to induce specific protecting antibodies to control, eliminate, and protect humans from pathogens and associated diseases [6–8]. Vaccinology is the conjunction of epidemiology, microbiology, immunology, and pharmacy principles. The ever-changing high mutation rates in pathogens are the vital challenges associated with safe and effective vaccination. Moreover, the efficacy and immune response of vaccination depends on numerous factors such as disease complexity, host immunity (cell- and antibody-mediated responses), gender, age, genetic variations, medical conditions of the host, etc. [2].

13.2 Types of Vaccines

The desired properties of any vaccine comprise safety, efficacy, specificity, long-lasting neutralizing activity against pathogens, lack of autoimmunity, storage, and ease of administration to the host. The use of different kinds of vaccines (DNA-based, RNA-based, protein-based) resulted in successful and long-lasting immunity; however, in many cases, the immunity is not long-lasting enough to cure the disease [9]. The advantages and disadvantages of various vaccines, based on their development, are summarized in Table 13.1.

Table 13.1 Summary of mechanisms, advantages, and limitations of different types of vaccines

Types of vaccine	Mechanism of action	Advantages	Limitation	References
DNA-based vaccine	Also called the third-generation vaccines. Induces an immunologic response in the individual against bacteria, parasites, viruses, and potential cancer by using engineered DNA.	It can induce both cellular and humoral responses at the same time. Non–live-cell approach. DNA molecules are more stable over time. Removes the need for protein purification and increases safety and efficacy.	The immune effect is very low as only a small amount can enter intracellular space.	[6, 10, 11]
mRNA-based vaccine	To achieve the expression of target antigens, they need to enter the cytoplasm.	Theoretically safe.	Some immune responses like headache, muscle pain, and fatigue can be there.	[5, 12]
Protein-based vaccine	Includes an inactivated bacterial toxoid protein, and induces an immune response, e.g., human papillomavirus (HPV) vaccines.	Cost-effective, easy to administer, and stable.	Cannot be produced through MHC-I.	[4, 13–15]
Pure polysaccharide vaccine	Includes polysaccharide molecules (sugar/carbohydrate) found on the outside of some bacteria, e.g., some vaccines to protect against *Pneumococci.*	Less expensive.	Not able to offer long-lasting herd immunity due to hyporesponsiveness.	[16]
Live attenuated vaccine	Functional/alive and weakened virus or bacteria is used. Also, it can replicate in the body to produce	Produces a strong and lasting cell-mediated immune response.	In some cases, the disease can develop due to the multiplication of weakened viruses or bacteria. Production and	[2, 5, 12]

(continued)

Table 13.1 (continued)

Types of vaccine	Mechanism of action	Advantages	Limitation	References
	an immune response without causing the disease, e.g., chickenpox.		maintenance are complex.	
Dead/ inactivated vaccines	Viruses or bacteria in these vaccines are inactivated/ dead, e.g., polio.	Cause a humoral immune response. Easy to prepare.	Always require repeated doses for immunity.	[5, 14, 17]

13.2.1 Protein-Based Vaccines

DNA- and RNA-based vaccines are better choices in terms of effectiveness and long-term immunity; however, the limitation with using these vaccines is the presence of gene coding materials, which can induce health issues in the host. It is well known that antigens are solely responsible factors for generating the adaptive immune response. Most antigens are either proteins, polysaccharides, or peptides. The variations in the structure of different proteins lead to distinct immune responses in individuals. Recent studies against various infections provided detailed knowledge about viral envelop, protein conformation, and epitope information. This can be incredibly advantageous for designing specific vaccines against these harmful viruses. Due to these facts, it is essential to focus on protein-based vaccines (PBVs) [4, 7, 8, 14]. The advancement in genetic sequencing, microbiology, X-ray crystallography, nuclear magnetic resonance (NMR), spectroscopy, and genetic engineering provides a better and more detailed understanding of the structure of proteins with explicit knowledge of why some proteins are more immunogenic than others proteins [8, 18, 19]. PBVs are designed using weakened or inactivated proteins that can trigger immune responses inside the host. These protein antigens are obtained from the pathogen by isolation and purification. Further, the advantage of this method is that it confiscates the after side effects of the dose. At the same time, this method requires multiple doses to enhance a more potent and durable effect. The antigen-presenting cells (APCs) are responsible for producing adaptive immune responses in the host [10, 12, 20]. The first PBV vaccine was a bacterial toxin vaccine that was made from antitoxin isolated from an animal immunized with the unmodified toxin in a small amount. Later it was realized that the success rate of this active immunization could be increased if, before administration, the toxin was chemically or thermally treated or coadministered with proper antitoxin. The human trials of PBV are in progress against SARS-COV-2 [21]. The safety and efficacy were the primary reason for PBV use over live attenuated and inactivated vaccines, as the immune response produced by PBV is usually based on the antigen used. However, the safety can be influenced due to genetic modification or mutations

in antigen structure, as in the case of SARS-CoV-2, both positively and negatively (autoimmunity). To avoid the problem related to autoimmunity or efficacy, the conjugated vaccine is a better option. The conjugated vaccine is designed using the unnatural amino acids (p-nitrophenylalanine) incorporated with PBV structure, for example, vaccines against RANKL and TNFα.

13.3 Design and Development of Protein-Based Vaccines

Initially, when protein-based vaccines were designed, they relied on natural sources for the antigens [22, 23]. In recent decades, the technical approaches for developing and producing new vaccines have grown exponentially, especially during the COVID-19 pandemic. The vaccine design methodology combines the various interrelated fields like genetics/reverse vaccinology, molecular biology, polysaccharide chemistry, protein biology, virology, immunology, bacteriology, fermentation technology, macromolecular purification, formulation of the complexes, etc. [24]. A significant fraction of the previously developed vaccines is from the preventive category (prophylaxis) of infectious disease rather than therapy of infections [23]. Modern technological developments have facilitated the development of vaccines for noninfectious diseases, such as autoimmune disease, cancer allergy, drug addiction, and therapeutic vaccines for a specific group of infectious and noninfectious diseases. The significant development in this area in the last decade redefined the vaccine development process. Due to the recent achievements in vaccine design, several vaccines could be created in just a few months against SARS-CoV-2. Vaccines can be classified into active and inactive vaccines. Active vaccines stimulate the immune system and produce either specific antibodies or cellular immune responses [25]. In some cases, both the responses are activated concurrently and help to treat the disease condition. While in the passive vaccination, preformed antibodies can bind to a human cellular antigen and, thus, completely neutralize a pathogen. An inactive vaccine is administered before or around the time of exposure to a pathogen or a subject showing initial symptoms of infection. The vaccine design strategies can be divided into several categories that are discussed below.

13.3.1 Glycoconjugate Vaccines

In the early and late twentieth century, polysaccharide vaccines were prepared that protect against *Haemophilus influenzae*, pneumococcal, and meningococcal infections. These vaccines are derived using capsular polysaccharides (CPS) from the surface of these bacteria. The high abundance and surface exposure of CPS provoke immune responses and, thus, result in bactericidal activities. A significant improvement is required in the formulation of these polysaccharide vaccines, because they are only effective in adults compared to infants and young children. Only a single serotype caused most *H. influenzae* type b (Hib) diseases, while several

immunologically distinct serogroups circulate during infections; thus, more complex epidemiology exists for other pathogens. A broadly protective glycoconjugate vaccine that can improve the immune response to polysaccharide antigens can be designed for pathogens by including multiple CPS serogroups in a multivalent formulation [22, 23, 25]. The glycoconjugate vaccine (7-valent) against *Streptococcus pneumoniae* showed a significant reduction in pneumococcal disease across all age groups. Though such multivalent vaccines broadly offer protection, due to the discovery of more than 90 distinct disease-causing pneumococcal serotypes, there is a dire need to develop alternative pneumococcal vaccines based on one or a few highly conserved protein antigens.

13.3.2 Protein Subunit Vaccines and Structure-Based Antigen Design

The initial success of glycoconjugate vaccines paved the way for scientists to develop alternate methods of vaccine design using modern techniques. In the early twentieth century, toxoid protein-based vaccines were developed against diphtheria, tetanus, influenza, etc. Vaccines were also created using hemagglutinin as the primary antigen. Hemagglutinin is the glycoprotein that plays a crucial role in the early stage of the infection in the influenza virus [22, 25]. Similarly, various protein-based vaccines for different disease targets started developing. Many vaccines exist for different serogroups against *Neisseria meningitides I*, but the existing arsenal of vaccines could not provide a universal solution for serogroup B (MenB) patients. A multicomponent vaccine, 4CMenB, was developed against MenB by applying principles of reverse vaccinology. This initiative greatly appreciated and accelerated the vaccine formulation using computational identification and reversed vaccinology techniques. These initial developments resulted in the first genome-derived recombinant protein-based vaccine, Bexsero®, against MenB. Initially, the Bexsero® vaccine was approved by the European Medicines Agency in 2013, and later on, it received approval in more than 35 countries worldwide.

In an attempt to provide proper antigen characterization, advances in structural biology methods such as X-ray crystallography, cryogenic electron microscopy (cryo-EM), NMR spectroscopy, and computational studies are making an immense contribution to designing and optimizing new vaccine antigens. Recently, various approaches to vaccine antigen design in combination with structural biology techniques have been reported. This multidisciplinary approach is also termed "structural vaccinology." There are three ways in which structural vaccinology is helping in vaccine research. Firstly, poor biochemical behaviour is resolved using structural biology approaches where potential weakness can be highlighted in an antigen. Secondly, these structural studies can identify conformational heterogeneity in an antigen, leading to the formulation of different mutated antigen forms. Thirdly, when this approach is combined with the epitope mapping, the antigens' multiple regions can be identified, which is necessary for elevating protection or neutralizing antibody responses [26]. Structural vaccinology is being used in many vaccine

research against the human immunodeficiency virus (HIV), where they focus on designing immunogens capable of producing protective antibody responses against gp120 or gp41 segments of HIV envelope glycoprotein trimer [27]. Structural vaccinology has also collaborated with nanobiotechnology to create self-assembling protein nanoparticles that present many copies of an antigen in an ordered array. A large antigen nanoparticle is more immunogenic than the recombinant proteins [8, 25]. This multidisciplinary combination of structural vaccinology and nanobiotechnology has shown multiple benefits. The technological advances in human B-cell cloning and antibody production have made it feasible to develop an effective structure-based antigen design.

13.4 Delivery and Mechanism of Action of Protein-Based Vaccines

13.4.1 B-Cell Repertoires, Antibody Discovery, and the Human Immune Response

Antibody-mediated immune responses play a decisive role in preventing infection, and T cell-mediated responses are crucial in killing the cells infected with a virus. Adaptive immunity mainly relies on the production of specific antibodies. The maintenance of protective levels of antibodies is critical for eliciting an adequate immune response after vaccination. The B-cell receptors (BCRs) present on the surface of B-cells process the antigen, and interactions between the antigen-specific T-cells and B-cells are mainly responsible for initiating specific B-cell responses. This cognate T-cell and B-cell interaction leads to the expansion of antigen-specific B-cells and their differentiation into short-lived plasma cells. This results in the production of unmutated antibodies, mostly of IgM isotype, and provides the first-line defense against the infection. These responses are followed by the formation of the germinal centre (GC) in the lymphoid organs. Plasma cells with a higher affinity for the antigen come from the GC of the bone marrow. This pool of plasma cells has a long life that will continuously release antibodies, and they are thus responsible for nourishing antibody levels even if the antigen is absent. Memory B-cells generated through a GC reaction start recirculating in the secondary lymphoid organs and peripheral blood. Thus, their affinity for BCRs makes them highly competent in capturing antigens. Usually, the production of new plasma cells attains its highest level in the blood on the 7th day of antigenic boost. This results in a continuous rise in antibody titer in serum; however, not all antibody titers are equally competent. T-cell-independent antibody responses to free polysaccharides have a short life, whereas T-cell-dependent antigens can evoke immunity for decades or a lifetime. Almost all licensed vaccines provide protection against disease by producing antibodies by B-cell. However, the underlying nature of promising antibody response has been challenging to generate. Antibodies of heavy (m, a, g, d, ε) and light (k, l) chains are linked by disulfide bonds and contain variable and constant domains. One of the most remarkable developments in understanding antibody

responses is the development of technologies to produce human monoclonal antibodies (mAbs) by using Epstein-Barr (EB) virus transformation by performing phage displays in genetically modified mice. Since 2008, next-generation sequencing (NGS) technologies have provided a way of amplification and cloning vectors of heavy and light chain immunoglobin genes from B-cells. This advancement is mainly used to identify high-affinity influenza-specific antibodies and segregate broadly neutralizing antibodies (bnAbs) against HIV [8]. The advances in NGS technologies have led to sequencing antibody genes from millions of cells, thus providing a detailed characterization of the antibody sequence repertoire and reactions that occur after vaccinations. The antibody repertoires are examined after immunization with influenza and tetanus using NGS technologies. Various analyses of the antibody repertoire allowed tracing the evolutionary paths, resulting in bnAbs. The presence of new methods to separate human mAbs and study the atomic details of their protein structures has helped in describing the antigen-antibody interactions comprehensively. Various developments in this field focus on the vaccine against HIV; however, it should also be done for other pathogens, including influenza and respiratory syncytial viruses (RSVs), which are responsible for high morbidity and mortality in children. In recent studies, the immunoglobin gene repertoire information is combined with the antigen-specific repertoire information that consists of human serum polyclonal response [28]. Recent studies also suggest that many peripheral B-cell-encoded antibodies are not present in the blood or secretions, so they cannot contribute to humoral immunity. Overall, these studies are taking us toward an era in which antigen-specific immunological research on various antigens can increase the pace of vaccine development and design more effective vaccine antigens.

13.4.2 Nucleic Acid Vector Vaccine Delivery Systems

The human immune system is a redundant, non–antibody-based immune mechanism that can provide significant protection against pathogens alone or with antibodies. Considering this, many efforts are made to design vaccines focussing on cellular responses to clear challenging pathogens such as HIV, hepatitis C, Ebola, etc. The CD8+ responses can be increased using DNA vectors that harbor the genes responsible for encoding intracellular antigen expressions. Many attempts are being made to achieve this, such as using naked DNA fragments or systems based on alphavirus, poxvirus, vaccinia virus, or lentivirus. The human adenovirus 5 (Ad5) has been used for gene delivery in many vaccine development studies. The use of Ad5 showed favourable results in preclinical and clinical trials. Since most humans are already exposed to Ad5, this affects the immunological potency of these vector delivery systems. Recent studies in the viral-based delivery of genetic vaccines have a prime-boost strategy that combines the Chimpanzee adenoviral (ChAd) vector with the modified vaccinia virus Ankara (MVA) vector. Favourable results come from the high levels of both CD8+ and CD4+ T-cells, especially for the antigens delivered genetically for HCV. The clinical efficacy of the ChAd vectors is yet to be fully

established. After the outbreak of the West African Ebola virus, which resulted in more than 8500 deaths, a vaccine development program started a clinical trial to study the working of the monovalent ChAd3 vaccine encoding the peripheral glycoprotein of the Zaire Ebola virus. RNA-based vaccines have several advantages compared to DNA vaccines. Using DNA increases the possibility of integrating plasmid DNA into the genome of the immunized host, which can then be directly translated into the cytoplasm [23, 29]. The RNA-based vaccines have better antigen expression during acute infection and can generate more robust antigen-specific immune responses. The effectiveness of the RNA vaccine is also dependent on the fact that RNA is a rich stimulator of innate immunity, and the results in animal models have been highly favourable. Earlier, RNA vaccines were not preferred due to the presence of unstable naked RNA in the tissue fluids. Several studies have been performed to increase the efficiency and stability of RNA-based vaccines. Clinical works in metastatic melanoma and renal cell carcinoma have shown a rise in antigen-specific immune responses (both antibodies and T cells). Currently, RNA vaccines against prostate cancer, melanoma, rabies, influenza, HIV, tuberculosis, etc., are in clinical trials [29], and RNA vaccines against infectious diseases are under assessment. However, the future of RNA vaccines relies on new and synthetic delivery systems.

13.4.3 Synthetic Viral Seeds for Rapid Generation of Influenza Vaccines

With the global emergence and rapid spread of new SARS-CoV-2 variants in the human population, health organizations and pharma companies rapidly developed responses to provide well-matched vaccines against the variants. In a pandemic, there is little hope that any pre-existing vaccines will boost the immune responses of human populations worldwide. Nowadays, scientists are trying to improve the vaccine responses against the emergence of new influenza variants. Multiple influenza strains are used to design universal influenza vaccines and develop new methods to speed up vaccine production. The advances in synthetic biology enable rapidly identifying genes responsible for encoding new influenza variants. Recently, scientists constructed a synthetic seed virus with hemagglutinin (HA) and neuraminidase (NA) genes taken from influenza (H7N9) virus sequence, using Madin-Darby canine kidney (MDCK) cell lines. The combined approaches significantly improved vaccine production rates compared to existing methods. The cell-culture-derived H7N9 vaccine was found to be safe and immunogenic in the phase I trial. After two doses, the vaccine shows potentially significant immune responses in most subjects with no pre-existing immunity against the H7N9 virus. These observations have provided a strong rationale for further clinical development of synthetic vaccine reagents.

13.5 Advantages and Limitations of Protein-Based Vaccines

The protein-based vaccines display several advantages over the other vaccine platforms. Nonetheless, there are associated limitations too. Both the advantages and limitations are detailed below:

1. One of the most important advantages of protein-based vaccines is that they can be easily accessible to low- and middle-income countries. Also, protein-based vaccines against some diseases such as hepatitis B are made locally in Brazil, Indonesia, and India.
2. Researchers from the University of Liverpool and the MRC Laboratory of Molecular Biology in Cambridge stated that protein-based subunit vaccines are good alternatives to mRNA-based vaccines [30]. Currently, few protein-based vaccines are approved for COVID-19 disease and are reported better in terms of ease and cost production, transportation, administration, and effectiveness of protection.
3. Unlike inactivated whole-cell vaccines, protein-based vaccines do not contain live parts of the pathogens. They only have antigenic characteristics of the pathogen, so they are considered safe comparatively.
4. The major limitation of protein-based vaccines is that they require adjuvants and booster shots to generate an effective immune response. Adjuvants are ingredients being used for decades in vaccines to enhance their immunogenicity. Some adjuvants can lead to more local reactions (like redness, swelling, itching, and pain at the injection site) and systematic reactions (like fever and body ache) in the patients.
5. In addition, the design of protein-based vaccines may also take time to determine the perfect antigen combination.

13.6 Recombinant Production of Protein-Based Vaccines

13.6.1 Bacterial Systems

E. coli bacteria was the first recombinant expression system. It helps understand molecular biochemistry, offers a large yield of defined proteins, has a fast growth rate, and requires a short production time with low cost, simple process scale-up, upstream processing, and high productivity [17, 31]. However, the *E. coli* system lacks machinery for posttranslational modifications (PTMs) such as glycosylation and multimer assembly. It is essential to focus on the PTMs, as it is the primary reason for protein misfolding, low solubility, and nonfunctionality. To resolve the issue of PTMs, engineered bacteria is a better choice [32]. Furthermore, the development of molecular biology, biopharmaceutical applications, and bioinformatics tools helps predict potential expression issues. Leucogen® (Virbac, Carros, France), a purified recombinant p45 FeLV-envelope antigen, was the first recombinant veterinary vaccine successfully produced in *E. coli*. For recombinant protein

production, the cytoplasm and periplasm are the possible targets in the *E. coli* cells. The success rate of recombinant protein production usually depends on the total metabolic load imposed on cells and the ability of the host cell to produce proteins [21]. Three pathways are exploited for recombinant protein production: (1) the twin-arginine translocation (TAT) pathway, (2) SecB-dependent pathway, and (3) SRP-mediated pathway. Among these, SecB-dependent pathway is the most popular method [33]. However, the limitation of using the standard Sec pathway is its incapability to transport folded proteins. In such cases, the TAT pathway is a better choice as it can export fully folded proteins and cofactor substrates with a limit of size up to 150 kDa [34]. However, the limitation of the TAT pathway is its low product yield due to the low abundance of TAT apparatus. Therefore, this system has not been used for industrial production [35], and further technological advancement is required for its successful use.

13.6.2 Yeast System

For routine expression of proteins with PTMs, yeast has emerged as a preferred choice for clinical or veterinary use. The use of yeast offers an opportunity for an extensive range of substrates, advanced genome analysis, and specific responses against genetic manipulations [36]. With all these factors, yeast displays a straightforward method for developing a nontoxic vaccine. There are an array of applications of yeast systems (Fig. 13.1), and various techniques can be used to

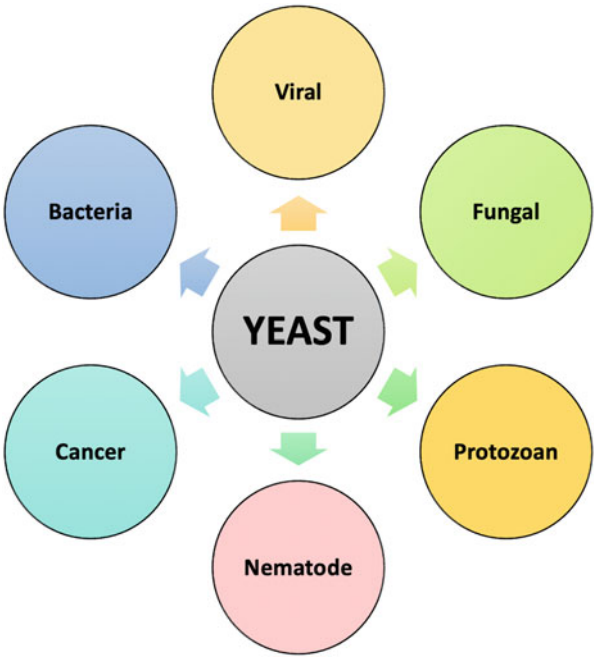

Fig. 13.1 Schematic diagram showing different applications of yeast system

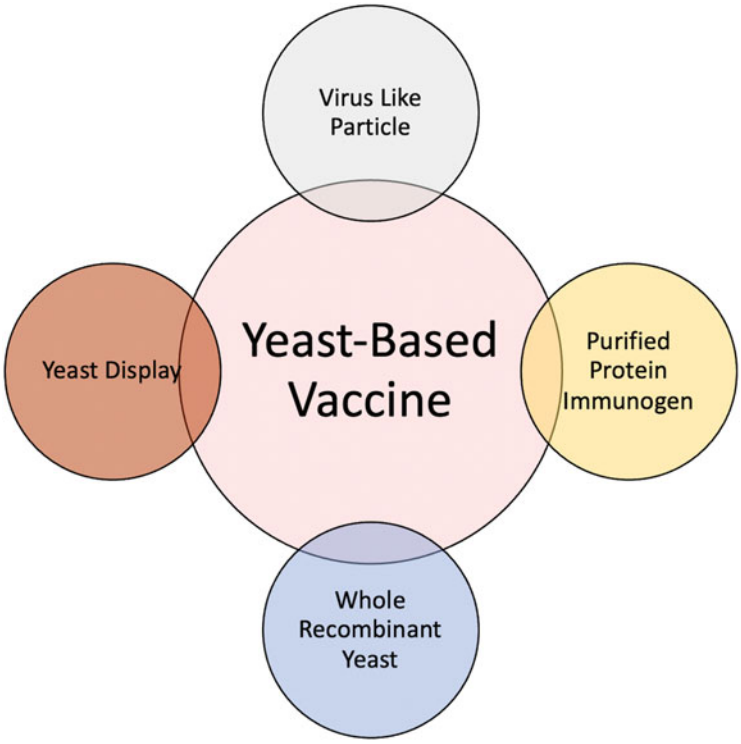

Fig. 13.2 Various methods to design yeast-based vaccines

design yeast-based vaccines (Fig. 13.2). The nonpathogenic nature of yeast is already known, but in recent studies, yeast has also shown an immunologic response in animals and is taken up via dendritic cells (DCs) and macrophages [37]. Earlier it was assumed that due to polysaccharides, such as beta-1,3-D-glucan (BG) and mannan, yeast cells possess an immunogenic nature through antigen-presenting cells (APCs), including DCs, accompanied by employing the technology of threat signals through microbial infection. Due to the robust adjuvant nature of these carbohydrates moieties, the infection can be detected utilizing sample popularity receptors like toll-like receptors (TLRs) and mannan receptors on APCs, which help activate T-cells through interaction and recognition of antigen peptides through MHC molecules [36–38]. For cell-mediated immune response, T-cells activation is essential. The major advantage of yeast display is that the soluble antibodies in the blood can directly recognize the antigen present in yeast cells and produce an immune response [39].

13.6.3 Mammalian Cells

Protein-based therapeutics are rapidly growing due to their advance and specific applications. For recombinant protein-based vaccine production, mammalian cell lines are dominantly used to generate safe and human-like glycoproteins. Mammalian cells host mAbs, enzymes, hormones, and cytokines [40, 41] and are a better choice over the other systems for recombinant protein production due to their ability to generate complex PTMs, stability over deviations in oxygen, temperature, and pH in the production stage, high productivity, and heterologous secretion of protein molecules in the site of extraction via cell lysis. However, with all these advantages, there is a challenge with mammalian cells associated with low production speed, very high cost, the requirement of supplementation of growth factors, amino acids, vitamins, and the risk of contamination during the production process. The risk of virus contamination can be reduced by following regulatory guidelines, selecting low-risk raw materials, and in-process manufacturing control to prevent contamination in the final product. An appropriate method and cell lines are needed to transport the gene of interest in the host cells; mAbs are used in more than 60% of cases. Continuous cell lines (CCLs) are used for virus propagation to develop virus-based vaccines. For this, Vero (African monkey kidney epithelial) cell line is practiced worldwide and used to produce polio and rabies virus vaccines [42]. The cell lines derived from mammalian cells can synthesize large and complex protein molecules. Mouse myeloma, human embryonic kidney 293 (HEK293) cells, and Chinese hamster ovary (CHO) cell systems are standard cell lines used for recombinant vaccine production. The human cell lines offer a greater advantage, as they could also have PTMs characteristics of human proteins [14, 40–42]. These cell lines are developed in adherent cultures or suspension cultures. Suspension culture has greater application as it is easier to scale up and is adaptable to automated processes. To express the foreign genes over these cell lines, stable or transient expression processes (a large amount of protein) can be used. CHO cells are primarily used for stable cell line expression, whereas HEK-293 cells are used for transient expression due to their high transfection efficiency. The transient method provides rapid protein production in a short period making it suitable for recombinant vaccine production. The optimization of vaccine development using mammalian cells continues; a human vaccine produced with CHO cells has been approved for use [7, 8, 14, 43].

13.6.4 Insect Cells

Insect cells are another alternative host platform for recombinant protein-based vaccine production due to the high cost of mammalian cell lines. The baculovirus expression vector system (BEVS) has emerged as a better choice. It provides a high yield of recombinant protein as it has a strong late viral polyhedrin (polh) promoter, less production time, bypassing the requirement of developing stable cell lines, no contamination by prions, and oncogenic DNA. Figure 13.3 summarizes the characteristics of the BEVS system that makes it a better option for recombinant

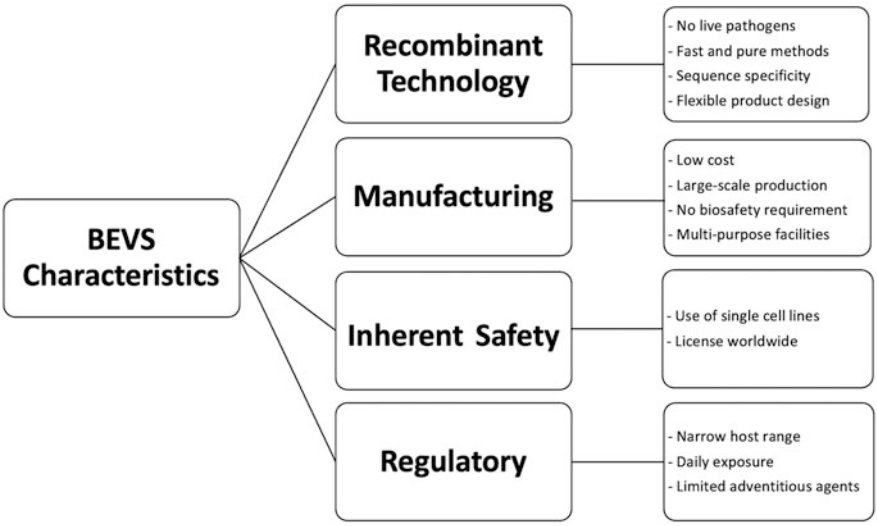

Fig. 13.3 The characteristics of the baculovirus expression vector system (BEVS)

protein-based vaccine development [41, 44]. The major limitations of the insect baculovirus expression are the lack of homogenous human-like glycosylation and cell lysis [15, 38, 45]. The insect cell growth contains two phases: (1) the insect cells are multiplied to desired cell density, and (2) infected with suitably modified baculovirus containing the gene of interest [18]. Another issue related to the insect cells is their inability to carry out N-glycosylation. However, to solve this problem, two steps are followed: (1) the mammalian glycosyltransferases can be introduced into insect cells, or (2) the coexpression of these enzymes with the gene of interest in baculoviruses.

Sf9 is the most popular cell line for the baculovirus expression system [44]. Other cell lines commonly used are S2, Sf21, Tn-368, and High-Five™ cells. The first commercially available veterinary vaccine produced in insect cells was the classical swine fever virus (CSFV) vaccine based on the E2 antigen [45]. Overall, the BEVS possesses flexibility, efficacy, safety, specificity, and single-cell line use in manufacturing multiple products.

13.6.5 Plant-Based System

The advancements in promoter selection, plasmid transformation, codon optimization, transgenic and transformation approaches, and recombinant protein-based vaccine designing using plant sources have become easy and more cost-effective than eukaryotic systems [14, 46, 47]. The expression studies of vaccine antigens in plants include whole plants, roots, moss, suspension cells, microalgae, and duckweeds. The plant-based vaccine offers high protein stability, low cost, safety,

stability over pH or temperature, the capability of producing N-glycosylated proteins, and easier and more economical storage of engineered drugs. Plant-based systems have minor differences in glycosylation patterns compared to mammalian cells. The terminal galactose and sialic acid residues are common in animals, whereas plant-based systems are deficient, and instead, the plant proteins contain α-(1,3) fucose and β-(1,2) xylose, which animal proteins lack. Therefore, glycoproteins produced in plants can affect the pharmacokinetic properties and generate immune reactions. Also, controlling transgene expression levels in plants is difficult, and the purification stage is more complex, posing a greater challenge in eliminating the secondary metabolites and pesticide residues from plant sources. To avoid this issue, glycoengineering approaches are used. Currently, two major strategies are used for obtaining the desired therapeutic protein where the protein is first extracted from a plant source and then purified and examined to check its immunogenic activity [47]. These are (1) *Agrobacterium*-mediated transformation, where a stable transgene expression is acquired, and (2) via plant viral vectors, where a transient expression of the foreign gene is obtained. The stable transgene expression is advantageous but time consuming and results in low expression yields. At the same time, transient expression is easy to manipulate and quick but less stable. After evaluating and examining the production of the functional protein level at the laboratory stage, large-scale industrial production in a plant-based system is preferred. An example of such an industrial scale is the production of therapeutic protein in carrot cell cultures (ProCellEx™) to treat Gaucher disease using the human recombinant β-glucocerebrosidase (taliglucerase alfa) [14, 41, 47]. The eligible dose and combination requirements to target plant and transgenic protocol, and proper and safe procedure for cultivation, manufacturing, and processing are essential points that must be taken care in order to design a effective plant-based vaccine.

13.7 Current Status of Protein-Based Vaccines

The design, expansion, and delivery of protein-based vaccines are still a challenge in the fields of vaccine development. However, an array of protein antigens capable of inducing adequate immune responses against specific pathogens have been discovered. The development of protein-based vaccines is still in its naive phase because of existing delivery problems. There is an absence of a complete understanding of the basic requirements for formulating and delivering protein-based therapeutics. Yet proteins have recently proven to be very effective as vaccines as they can mount immunogenic responses owing to stimulation of the body's natural mechanism [48]. Several protein-based vaccines have been developed against diseases like influenza, cancer, COVID-19, etc. [49].

13.7.1 Influenza Vaccine

The development of influenza vaccines aims to elicit a broader immunity, because the seasonal influenza vaccines lack efficacy against pandemic influenza strains [50]. Though seasonal influenza vaccines have always been promising and saved countless lives, the continuous genetic mutation and immune escape mechanism in this virus need regular upgradation of vaccines. Recombinant protein vaccines are among the universal vaccine approaches that utilize innovative technologies [51]. Immunological and virological advances, along with knowledge of structural biology and bioinformatics, are boosting the development of novel vaccine approaches [52–54]. The influenza virus membrane contains two critical proteins: haemagglutinin (HA) and neuraminidase (NA). They are crucial for the entry and release of the virus from infected cells [55]. Apart from these two proteins, other structural components, such as the RNA-binding matrix protein M1, the nucleoprotein (NP) that coats the viral RNA, and the ion channel M2 protein, can be recognized by our immune systems. However, HA and NA are more accessible antibody targets than other components owing to their increased prevalence and accessibility on the viral envelope.

Protein-based vaccines contain viral haemagglutinin and neuraminidase proteins. The viruses used for vaccine production are typically grown in chicken eggs, which makes the reliability of vaccine production on a steady supply of embryonated eggs [56]. To avoid this need, a newer technology that employs cell culture for virus growth has been used. A recent report showed the increased efficacy in healthy adults and improved protection in elderly subjects upon administering recombinant HA-subunit vaccine produced from insect cells [57]. Recently vaccines based on neuraminidase, matrix protein 2 ectodomain (M2e), and nucleoprotein have proven effective [58]. The next-generation subunit protein vaccines open new avenues for meeting the escalating demand for safe, affordable, and effective influenza vaccines.

13.7.2 Cancer

Though protein-based vaccines for cancer treatment have not been that successful so far, delivering these proteins within caged protein nanoparticles has shown promise in improving the vaccine efficacy [59]. The protein nanoparticles are required to increase the immunogenicity of the tumor microenvironment. Since immune escape is the hallmark of cancer, it becomes essential to elicit better immune responses while administering any vaccine for cancer. For a cancer vaccine to be effective, it must also impart long-term immune memory to prevent tumor recurrence [60]. The vaccine must also recognize the tumor-associated antigens present specifically on the surface of cancer cells. Hence, the vaccines used for cancer treatment should recognize these antigens and destroy the cancer cells. Protein vaccines are made from tumor-associated antigens in cancer cells that can elicit immune responses quickly. For example, cervical cancer cells express the human papillomavirus HPV E7 oncoprotein (E7), which plays a crucial role in cellular transformation and

maintaining the transformed phenotype. The E7 protein is a potent target for developing therapeutic subunit vaccines against cervical cancer. However, it has a limitation of having low antigenicity, so there is a need to add suitable adjuvants to increase its efficacy. A novel chimeric form of the 4-1BBL costimulatory molecule engineered with core streptavidin (SA-4-1BBL) has been developed [61, 62]. The utility of SA-4-1BBL as the immunomodulatory component of HPV-16 E7 recombinant protein-based therapeutic vaccine in the E7-expressing TC-1 tumor as a model of cervical cancer in mice showed that the results are encouraging and offer 70% efficacy in eradicating established tumors in the mice model.

13.7.3 COVID-19

Despite the administration of safe and effective COVID-19 vaccines worldwide, researchers are working to develop different vaccine strategies that could provide longer immunity. The administration of COVID-19 vaccines aims to generate neutralizing antibodies against SARS-CoV-2, particularly the antibodies against the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein [63]. The spike protein is responsible for facilitating viral entry through its interaction with the epithelial angiotensin-converting enzyme 2 (ACE2) receptors. The titer of antibodies reactive to the RBD/spike protein and neutralization of viral infectivity is the primary measures of response to COVID-19 vaccines. Although the initially approved vaccines were based on mRNA, they targeted only the SARS-CoV-2 spike protein. Moreover, protein-based vaccines offer advantages over mRNA vaccines in terms of the ease and cost of production, the robustness of the material, and potency. A recent report showed that an archaeon-based ferritin-like protein coupled with different antigens from SARS-CoV-2 was highly effective in generating a stable immune response [30]. These highly stable vaccine nanoparticles completely protected the mice from SARS-CoV-2-associated pneumonia in just a single immunization. Richmond et al. tested a stabilized trimeric spike subunit protein vaccine (SCB-2019) [64]. This vaccine is unique from those approved as it uses a stabilized protein trimer as the antigen. Another group used Trimer-Tag, a protein derived from the C-terminus of human type I procollagen, which preserves the trimeric conformation of the SARS-CoV-2 spike protein [65]. The Trimer-Tag technology provides an alternative trimer stabilization strategy to the molecular clamp derived from HIV proteins [66]. This technology can be used for scalable production and rapid development of safe and effective protein-based vaccines.

13.7.4 Other Diseases

Various protein-based drugs produced by recombinant technologies are now readily available therapeutics at reasonable prices for treating chronic diseases. Therapeutic proteins are increasingly prominent because they have been effective in treating many potentially fatal diseases like diabetes, heart disorders, and cancer [48, 67,

68]. Moreover, proteins have been proven effective even as vaccines to help stimulate the body's natural defense mechanism for an immunogenic response. Therapeutic proteins are booming in the pharmaceutical industry through the cloning and expression of cDNA that encodes heterologous proteins [69]. Protein-based vaccines have been developed for Hepatitis B surface antigen (HBsAg) for hepatitis B infection and antirhesus (Rh) IgG vaccine for routine postpartum prevention of Rh (D) immunization in Rh(D)-negative women [70].

13.8 Assistance of Artificial intelligence in Vaccine Development

Artificial intelligence (AI) has revolutionized and transformed the field of medicine. The power of AI's automatic feature learning, combined with the massive volume of data, contributed to its role in its wide applications. In the medical field, the two most crucial areas, drug discovery and vaccine discovery, are immensely benefited by AI technology [41, 71–73]. In recent decades, machine learning (ML), the subfield of AI technology, also helped to improve vaccine design. The AI/ML employs an algorithm structure to interpret and learn the features from the data given in the input form. It makes independent decisions for completing specific objectives. The prominent role of AI technology is basically to analyze the existing data and use it for prediction purposes [74]. Apart from the prediction, it also helps understand and suggest the paradigm for developing future vaccines based on the case studies against a disease. The essential feature of AI is speed and accuracy, which impacts diagnosis and vaccine development processes [24].

VaxiJen is the first server that implemented ML in reverse vaccinology approaches and showed promising results for antigen prediction using its physicochemical properties [75, 76]. The recent web-based reverse vaccinology program, Vaxign-ML, is used to predict bacterial protective antigens. These pipelines, which consist of feature extraction, feature selection, data augmentation, and cross-validation, are implemented to predict the vaccine candidates against various infectious diseases [77]. Other pipelines, such as the immune epitope database (IEDB) and BlastP, use the recurrent neural network (RNN) approaches to study different pathogenic viruses [78, 79]. Recently, some pipelines have been developed that work based on the graph theory method and represent antibodies with expert-designed features. A subset of AI, namely, deep learning (DL), is also widely used on graph-based features to speed up accurate vaccine development [80]. Thus, DL-based approaches revolutionized the field of vaccinology through improved prediction methods [81–83]. Autoencoders of the DL approach have shown promising enhancement in mining the features from data, which could be utilized in vaccine discovery [84]. The critical aspects of the development of vaccine therapy are safety and reliability. The vaccine adverse event reporting system (VAERS) and vaccine safety databank (VSD) are the most popular immunization strategy for tracking, recording, and predicting the safety of vaccines. Earlier, computational simulation and mathematical modelling techniques were significantly used to

Table 13.2 Some prediction tools used for vaccine design

Vaccine types	Prediction tools
B-cell epitope	ABCpred, ElliPro, Pep-3D-Search, MimoPro, BEPro (PEPITO), BEST, SVMTriP, Pep-3DSearch
T-helper epitope	IL4pred, IFNepitope
MHC Class I binders	ProPred1, RANKPEP, nHLAPred, MMBPred
MHC Class II binders	Propred, MARIA, EpiDOCK, HLA DR4Pred, MHC2Pred, Consensus, HLA DR4Pred
Endogenous antigen processing	NetChop, CTLPred, FRED, TAPreg, TAPhunter, NetTepi, Pcleavage
Allergenicity of peptides	AllerHunter, Allertop, Hemolytik, Toxinpred, AHTPDB
Antigenicity	SVMTriP, ANTIGENpro, VaxiJen

improve the trade-off between the assessment of safety and efficacy [84, 85]. Natural language Processing (NLP) technology is now widely used to identify adverse events related to vaccine development [86]. Many prediction tools are available for vaccine design that are listed in Table 13.2.

In summary, AI has been applied in the drug discovery and vaccine development subfields. The advances in DL algorithms are significant for the rapid discovery of vaccines and drugs. The DL-based models can extract important features from the dataset with high accuracy without any manual intervention. The generative ability of DL-based models is exploited for better epitope prediction, which may lead to lower chances of failure of the designed vaccine in the trial phases. Thus, AI is a novel approach to vaccine development that uses transfer learning and leverages the learned knowledge from existing data.

13.9 Challenges and New Approaches for Protein-Based Therapeutics

Protein-based therapeutics are exceptionally effective in the clinic. Computational methods to analyze small molecule drug development use mathematical calculations to scan the underlying information and integrate it into the target molecules [87, 88] with the assumption that they will regulate its action [89]. This is an essential first step toward high-throughput screening and a suitable therapeutic approach. In general, small molecules that are not naturally occurring can be significantly more dangerous than human proteins. Despite the limitations associated with their pharmacokinetic features, therapeutic proteins are increasingly being used for a wide range of treatments [90]. The success of protein-based therapeutics is mainly due to the application of ideas and techniques developed, which resulted in significant improvements in three critical aspects of competitor therapeutics that are required for FDA approval: safety, efficacy, and quality [91]. These three are vital to the success of any treatment and are discussed in detail below.

13.9.1 Safety

Therapeutic protein-induced side effects could be associated with either interaction with expected targets or interaction with accidental targets. The organization of suppressor therapeutic proteins could have a variety of unintended consequences. Overstimulating the immune system can lead to more severe diseases [92]. Restricting to a specific goal can result in unintended consequences, such as immunomodulatory antibodies, which can either inhibit or stimulate the immune system [93]. One significant distinction between counteracting agent-based restoratives containing Fc and other helpful proteins is antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [94]. The cardiotoxicity associated with trastuzumab is amplified when the antibody is taken simultaneously or sequentially with an anthracycline [95]. A common example is the adverse acute infusion reactions after protein administration, where cytokine discharge plays a critical role, but other unidentified players could also be involved; such responses were observed for some protein therapeutics such as infliximab, rituximab, trastuzumab, and panitumumab, insulin, and interferon [96]. The leakage of cell debris from lysed harmful B cells can result in cumulative effects of rituximab [92]. Protein structure can also cause sensitivity responses such as anaphylactic shock and serum ailment. Previous IgEs that cross-respond with proteins can increase the intensity of such reactions [95].

Immunogenicity of therapeutic proteins can be a big issue [92, 97, 98]. For example, the discovery of less immunogenic proteins was crucial to the success of mAb-based treatments. In the 1980s, murine mAbs were tested as prospective treatments, but their high immunogenicity resulted in large titers of human antimouse antibodies (HAMAs), toxicities, and limited effectiveness. The development of the less immunogenic new mAbs, which contain human Fc sections, and humanized mAbs, which have mouse complementarity-determining regions (CDRs) joined into the human antibody system, showed clinical success. Fully humanized antibodies, on average, have low immunogenicity and are the most widely used form of antibody in development, despite the fact that the majority of the therapeutic antibodies approved for clinical usage are still artificial and humanized mAbs.

The protein structure, composition, PTMs, contaminations, and heterogeneity can all affect immunogenicity, along with the patient's susceptibility and disease status, following medication, course, period, and recurrence of the disease, mainly when controlled as varied dosages for a long time [98]. Human proteins can trigger antihuman antibodies in humans. Treatment with the human mAb adalimumab resulted in antibodies against the therapy ranging from 1% to 87% for different cohorts of patients, procedures, diseases, and measurement methods in one of the most researched cases of anti-TNFa mAbs [99]. The antibody sequences that contribute to antigen binding and specificity but may appear foreign are a potential explanation for human mAb immunogenicity. Human therapeutic proteins can also disrupt immunological tolerance, and antibody elicitation can be influenced by aggregation [98]. Aggregation can lead to structures that do not necessarily require T cell assistance. Protein immunogenicity may potentially influence efficacy via the

pharmacokinetic or neutralizing effects of antibody responses, which are controlled by several parameters, including the affinity, specificity, and concentration of the produced antibodies [97]. Because immunogenicity affects both safety and efficacy, researchers are working hard to predict and reduce immunogenicity in therapeutic proteins [100–102].

Individual safe reactions to therapeutic proteins fluctuate generally. Despite rigorous efforts to identify critical factors associated with immunogenicity, it is difficult to predict the immunogenicity of therapeutic proteins in human subjects. However, less is known about the individual antibodies that compensate for the polyclonal reaction to therapeutic proteins. Because the germline antibody repertoire, at any given time, could be a major determinant of individual differences, knowledge of a large pool of antibodies produced by the human immune system, preferably the entire set, that is, the antibodyome, is essential [103], and could ultimately assist in predicting individual insusceptible reactions to therapeutic proteins. Therapeutic proteins have a significant benefit over small molecule therapies, which are often less selective and can attach to many molecules nonspecifically. However, there are major adverse effects in some circumstances, and safety concerns can result in therapeutic proteins being withdrawn from the market [104].

13.9.2 Efficacy

Besides safety, the FDA considers efficacy the essential factor in granting approval. Many therapeutic proteins, including insulin for diabetes, epoetin for anaemia, and rituximab for non-Hodgkin's lymphoma [96], are very effective in vivo and have changed the landscape of disease therapy. Additional examples are alemtuzumab, a drug used to treat hematological cancers [105], and trastuzumab, the human epidermal growth factor receptor type 2 (HER2) positive breast cancer adjuvant systemic medication [106]. The addition of trastuzumab to non–trastuzumab-based adjuvant treatment lowers recurrence by roughly 50% and improves overall survival by 30%, according to results from six studies involving over 14,000 women with HER2-positive early breast cancer [107]. Therapeutic mAbs and other therapeutic proteins have low overall effectiveness, and there is a lot of individual heterogeneity. Trastuzumab (Herceptin) has completely transformed the management of HER2-positive patients; most patients still have nonreacting cancers, and infection movement occurs in most cases within a year [108]. Antiangiogenic treatments, such as bevacizumab, that target the vascular endothelial growth factor (VEGF) and the VEGF receptor (VEGFR), are useful adjuncts in treating solid tumours and are usually regulated in blend with cytotoxic chemotherapy. Regardless, many patients fail to respond to angiogenic treatment of gliomas, and the response term is brief and variable [109].

New techniques, such as improved effector functions, are being explored to enhance the efficacy of mAb and other therapeutic proteins by working on the half-life, expanded cancer and tissue availability, and, more importantly, stability. The improvement in efficacy involves both protein engineering and glycoengineering fields [109–111]. The mAbs that do not interact with innate

immunity are being created [112]. Antibodies with many targets are being produced and evaluated in clinical studies. Modulation of immune responses by mAbs targeting T cell immune response regulators is also a viable approach. The inhibitory regulator of such responses is the cytotoxic T lymphocyte antigen 4 (CTLA-4) expressed on activated T cells. Human antibodies and Fc fusion proteins that block CTLA-4 function have been evaluated in the clinic and proven to have antimelanoma activity [113, 114].

Second- and third-generation mAbs against already established targets, including HER2, CD20, and tumor necrosis factor-α (TNF-α), are now in clinical trials or have already been authorized. Several methodologies have been employed to find new, relevant targets, but progress has been slow. An enhanced selection of cross-reactive antibodies by sequential antigen panning [115] and competitive antigen panning for focused selection of antibodies targeting a specific protein domain or subunit have been described as modifications to normal panning processes [116, 117]. To enable greater tissue penetration and concealed epitope access, a variety of modestly designed antibody domains (approximately tenfold smaller than IgG) are being developed [118, 119]. Antibodyome information might be utilized to create semi-synthetic libraries for selecting high-affinity binders with small sizes and low immunogenicity [103]. The development of antibody-based therapeutics means that existing antibodies are gradually improving in characteristics and being designed. A continuous upgradation is required in the properties of existing therapeutic proteins and in identifying novel therapeutic protein targets. The future challenge is how to increase the efficacy of therapeutic antibodies and how to go for their mass production without compromising standard protocols. Developing successful personalized antibody-based treatments and predicting toxicity or potentially poor efficacy in vivo are other key obstacles [91].

13.9.3 Quality

The FDA considers quality to be a critical factor in approving any pharmaceutical. The heterogeneity of mAbs and other biologics is a key feature that separates them from small-molecule medications. Modifications, such as incomplete disulfide bond formation, glycosylation, N-terminal pyroglutamine cyclization, C-terminal lysine processing, deamidation, isomerization, oxidation, amidation of the C-terminal amino acid, modification of the N-terminal amino acids by maleuric acid, as well as noncovalent associations with other molecules, conformational diversity, and aggregation, cause heterogeneity [120–122]. A vast number of variations with a similar arrangement might exist together. Improving excellent protein therapeutics with negligible heterogeneity and defilement is fundamental for their security and endorsement by the FDA [123]. The possibility of using molecular cloning and genetically engineered approaches for manufacturing low-cost therapeutic proteins in plants and delivering therapeutic proteins by in vivo methods are other methods to improve quality and reduce the treatment cost [91].

13.10 Conclusion and Future Perspectives

DNA and RNA vaccines are generally preferred in terms of effectiveness and long-term immunity. To avoid problems related to autoimmunity or efficacy, the conjugated vaccine is a better option. The conjugated vaccine is designed using unnatural amino acids (p-nitrophenylalanine) incorporated into the protein-based vaccine structure, for example, vaccines against activator of NF-κB ligand (RANKL) and TNF-α. However, the design and development of protein-based vaccines remain challenging. The advancement in technical approaches leads to the identification of new protein antigens that can induce immunity to infectious pathogens. The availability of new methods would allow investigators to focus on best-suited resources for different applications in the field of vaccine research. A lot of quick progress made in recent decades toward developing effective therapeutic proteins gives hope for the future. Antibody treatments will benefit immensely from studies evaluating the synergistic effects of antibodies with chemotherapeutic drugs, radiation, or other biologic agents in the future. Furthermore, the discovery of new biomarkers can potentially increase the efficacy and specificity of antibody-based therapies for human diseases.

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