BIOMEDICINE AND CHEMICAL SCIENCES
AN INTERNATIONAL PEER-REVIEWED JOURNAL

BIOMEDICINE AND CHEMICAL SCIENCES
Website: https://journals.irapa.org/index.php/BCS
e-ISSN: 2790-296X | p-ISSN:2957-5826

Original Article

A FRAMEWORK OF MONOCLONAL ANTIBODIES AND RELATED PRODUCTION ENGINEERING

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Article History:
Received on: Jan 15, 2025
Revised on: Feb 17, 2025
Accepted on: Feb 28, 2025
Published on: Mar 15, 2025

Citation:
Navneet Kumar Verma *et all*. A Framework of Monoclonal Antibodies and Related Production Engineering. Biomedicine and Chemical Sciences, 5 (1): 36-44, 2025

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ABSTRACT
Monoclonal antibodies (mAbs) have revolutionised modern medicine as potent therapeutic agents. This comprehensive review traces the history of monoclonal antibodies (mAbs) from their beginnings to their present-day prominence, illuminating key moments along the way and investigating their various medical applications. We begin by outlining the molecular composition and mechanisms of action of mAbs, and then we get into the processes of hybridoma and recombinant DNA production and manufacture. The initial approach, called hybridoma technology, fused B lymphocytes with myeloma cells to create single monoclonal antibodies (mAbs) against a specific antigen. Several novel and distinct methods have been established to improve mAb synthesis, alongside hybridoma technology. These encompass electrofusion as well as the introduction of brand new technologies such as phage, yeast, bacterial, ribosome, and mammalian display systems; DNA/RNA encoded antibodies; transgenic animals; and single B cell technology.

Keywords: Monoclonal antibodies (mAbs), Hybridoma, Recombinant DNA.

Introduction
To mimic the immune system's ability to fight off viruses and damaged cells, scientists create molecules in the lab called monoclonal antibodies (mAbs) [1]. Being engineered to specifically target specific antigens, these antibodies offer a potent and targeted treatment approach for several medical conditions [2]. The advent of monoclonal antibodies and the tremendous efficacy they bring to the treatment of cancer, autoimmune diseases, infectious diseases, and countless more conditions has revolutionised healthcare. Because of their specificity and versatility, they provide medical personnel with powerful options to confront complicated medical problems, making them valuable diagnostic and therapeutic tools [3]. Abs, produced by differentiated B cells, serve multiple functions in the immune system. The ability to produce protective Abs in response to Ag-driven lymphocyte development and subsequent germinal center processes is an example of the host immune response's inherent intelligence that can be used to develop monoclonal antibodies (mAbs) for use in prevention and treatment [4]. Antigens can originate from a variety of sources, including bacteria, viruses, fungi,

parasites, pollen, or even nonliving substances such as toxins, chemicals, medications, or foreign particles that the body perceives as foreign, such as epitopes expressed on cancer cells. Antibodies are one of the body's natural defence mechanisms against these threats. As a destiny for the Ag-Ab immune complex, attaching to their targets either renders them inactive and prevents them from causing harm or marks them for elimination. In the fields of medicine, pharmaceuticals, and even veterinary science, antibodies have emerged as a game-changer for the diagnosis, treatment, and prevention of numerous serious diseases [5–12]. The advent of monoclonal antibodies and their tremendous therapeutic potential in the treatment of cancer, autoimmune diseases, infectious diseases, and countless others has revolutionised the medical field. With their specificity and versatility, they provide medical personnel with significant options to approach challenging medical problems, making them valuable diagnostic and therapeutic tools [13]. On the other hand, monoclonal antibodies (mAbs) have the ability to attach selectively to specific antigen-functionally essential epitopes, which were their initial point of research. Optimising their binding, functional activity, or half-life can also help them make a bigger splash in the global therapeutic market. Significant strides have been achieved in the treatment of non-communicable diseases since the introduction of mAbs [14].

Because they are highly target specific and organised into distinct structural and functional domains, antibodies are superior therapeutic prospects. When developing an antibody for medicinal use, it is important to maximise its immunogenicity while simultaneously ensuring its stability, effector functions, half-life, penetration, and distribution patterns throughout the body. To develop antibodies with desired therapeutic characteristics, antibody engineers use a variety of methods within the realm of antibody properties. The number of antibodies now being studied in clinical trials exceeds 150, with 19 of these antibodies having been granted clinical approval by the FDA. Recombinant MAbs and their derivatives are being studied for a wide range of medical conditions, including but not limited to: autoimmune and allergic diseases; angioplasty complications; sepsis; a number of inflammatory conditions; a plethora of viral and bacterial infections; organ transplant rejection; and solid and haematological tumours [15].

Discovery

There are several pivotal moments in the history of immunology and medicine that occur with the development of antibodies. Antibodies, Y-shaped proteins made by B cells, are essential for the immunological response that the body mounts against infectious microbes and viruses. When Emil von Behring and Shiba Saburo Kitasato demonstrated that serum therapy effectively treated diphtheria in 1890, it marked a significant turning point in antibody research [16-17]. In 1897, the German researcher Paul Ehrlich used the term "antibody" to describe branched molecules that attach to toxins. Understanding the function of antibodies in immunity was paved the way for by this definition [18]. The molecular structure of antibodies was discovered by Rodney Porter and Gerald Edelman, who were awarded the Nobel Prize in Physiology or Medicine in 1959 for their outstanding work. The enzyme-linked immunosorbent assay (ELISA) was revolutionised in 1971 by Swedish scientists Peter Perlman and Eva Engvall, who also made it easier to detect hormones and viruses. The mystery of how a tiny set of genes could haphazardly rearrange themselves to produce a vast array of antibodies was addressed by the Japanese scientist Susumu Tonegawa, who was awarded the Nobel Prize in Physiology or Medicine in 1987 [19]. From their original therapeutic usage in serum therapy to our current understanding of antibodies' molecular structure and genetic basis, these turning events demonstrate how our understanding of antibodies has advanced. Thanks to this tendency, monoclonal antibodies have been developed and have many applications in modern medicine [18–19].

Molecular Structure

Monoclonal antibodies have a "Y-shaped" structure that consists of four polypeptide chains: two heavy chains and two light chains that are an exact match. The molecular weight of a monoclonal antibody is around 150 kDa. Both "Y" arms, which are called the Fab (antigen-binding fragment) regions, contain the variable domains that bind antigens. Mediating effector functions and establishing the antibody's class or isotype, the "Y" stem is called the Fc (fragment crystallisable) region. The antibody is able to bind to a specific antigen epitope with great affinity because of complementarity-determining domains (CDRs) located in the Fab region. According to research [20–23], the light chain is responsible for antigen binding and creates the upper arms of the "Y" shape, whereas the heavy chain is the constant area and forms the lower part of the "Y" shape.

Mechanisms of Action

Signaling-mediated cell death: Surface antigen cross-linking can trigger cell death. To kill cells, monoclonal antibodies (mAbs) trigger apoptotic signalling pathways. Intercepting growth-promoting signals, monoclonal antibodies (mAbs) successfully halt tumour cell proliferation by blocking activation signals. The process of antibody-dependent cellular cytotoxicity (ADCC) involves monoclonal antibodies (mAbs) binding to Fc receptors on immune cells such Natural Killer (NK) cells, leading to the targeted killing of certain cells. By binding to their Fc receptors, mAbs can cause complement-mediated cytotoxicity (CMC), which in turn causes the formation of the membrane attack complex and the lysis of target cells. To improve immune responses against tumours, monoclonal antibodies (mAbs) may alter the cytokine milieu. To improve anti-tumor immunity, some monoclonal antibodies (mAbs) can act as agonists, boosting T

cell receptors like ICOS. To stop tumours from growing and boost cell survival, mAbs may work by blocking tumour cells' interactions with growth factors and receptors [24-25].

Variability in Monoclonal Antibody Specificity and Affinity

For several reasons, the specificity and affinity of monoclonal antibodies (mAbs) may vary substantially. Studies have shown that monoclonal antibodies (mAbs) can react with a variety of normal tissues, and that their binding specificities can range from very specific to exceedingly cross-reactive. One study that targeted carcinoembryonic antigen (CEA) with 52 well-characterized mAbs discovered varied binding patterns. While most monoclonal antibodies (mAbs) demonstrated cross-reactivity with several normal tissues, a small percentage exhibited very selective reactions, positively interacting with only one of nine discriminating tissues. Specificity testing revealed that just 10% of the mAbs could distinguish between normal gastric foveola, colon cancer, and colon mucosa. The study found no clear correlation between antibody specificity and affinity for CEA, as indicated by the binding constant for the mAb-CEA interaction. Thus, it is possible for mAbs to exhibit varying degrees of affinity, and it is also possible for mAbs to exhibit varying degrees of specificity [26]. When optimising mAbs for therapeutic purposes, it is vital to balance affinity and non-specific binding, according to research. According to studies, most antibodies that have a high affinity also have a high level of non-specific binding. On the other hand, antibodies that have a low level of non-specific binding often have bad affinity [27].

Classification of Antibodies

B lymphocytes produce a wide range of polyclonal antibodies that bind to different antigen surface epitopes in response to foreign antigen exposure. Low antigen affinity is a common feature of antibodies produced during the early stages of an immune response. Antibodies produced by B cells undergo affinity maturation as part of germinal center responses, a process that progressively increases bonding affinity. However, avidity differs from affinity and characterises the overall binding strength of interactions between multivalent antibodies and antigens. Polyclonal antibodies are present in serum from humans and other animals because several B cell clones create them [28]. Polyclonal antibodies were the first of their kind and were utilised to treat a variety of diseases and disorders, eliminate harmful substances from the body (such as digoxin), and prevent organ rejection after transplantation. Produced by a single B cell clone, monoclonal antibodies first appeared towards the end of the twentieth century [29]. There are five categories into which antibodies fall. Different immunoglobulins have different heavy chain structures, which allow them to be categorised into five types: IgG, IgM, IgA, IgD, and IgE. Despite extensive research on IgM antibodies, IgG antibodies remain the most common and clinically significant class. Medications in this class are commonly prescribed for the treatment of cancer, autoimmune diseases, metabolic disorders, infectious diseases, and genetic disorders, among others. Recent years have seen progress in the development of antibodies apart from monomeric IgG. It is more difficult to design, produce, and purify pentameric or hexametric IgM molecules due to their size compared to IgG. Nevertheless, methods have been devised to manufacture antibodies that surpass 95% purity. Roughly twenty IgM antibodies have been used in human trials. There has been no evidence of major negative effects, and these drugs showed minimal immunogenicity. However, a positive assessment is not feasible because the IgM antibodies tested so far had very little efficacy. As a result, further research is needed to improve their therapeutic efficacy [30]. Using antibodies from mice was the first line of treatment, but those were highly immunogenic to people. Presently, recombinant antibodies are employed to mitigate this impact. There is a minor protein fraction translated by mouse gene sequences and the second major component, which is transcribed by the human immunoglobulin genes, both of which are present in these.

There are four types based on the proportion of these sequences in the antibody molecule:

- Antibodies made entirely of mouse sequences.
- Chimeric antibodies with mouse-variable sections that make up around 25% of the total sequence; human sequences make up the remaining portions.
- The majority of human sequences are retained in humanized antibodies, along with CDRs originating from mice and occasionally certain framework residues.
- Human antibodies are free of alien sequences [31-33].

Techniques for producing antibodies

Hybridoma Technology

One common way to make mAbs is using hybridoma technology, which has been around since 1975 [34]. Isolated Ab-producing B cells are combined with immortal myeloma cell lines after mice are vaccinated with a specific Ag. Cultivation of the resultant hybridoma cells is necessary for the production of mAbs [35]. One advantage of hybridoma technique is the capacity to generate mAbs against nearly any Ag of interest. Having said that, fusion efficiency is often low when using this method [36]. Another big drawback is that Ag could be subjected to proteolytic degradation, which would mean that the mAbs will not be able to recognise the native form of Ag. Furthermore, mAbs generated from different species increase the danger of disease transmission, and the resultant mAbs may not be pathogen-free even after purification. In addition, there have been several reports of immunogenic reactions to murine-derived mAbs. When immunogenicity issues arise, humanisation treatments such as CDR graft, Ab cholerization, and human Ig transgenic rats

are employed. While humanisation has the potential to reduce immunogenicity, it is common for affinity to decrease as a byproduct [37]. Regardless of these limitations, hybridoma technique enables the consistent and large-scale production of mAbs with a desired specificity. This method is still preferred over others because of its inherent ability to preserve knowledge about natural cognate Ab pairings, maintain innate immune cell capabilities, and undergo in vivo affinity maturation. High-throughput mAb synthesis methods have also been developed. The restrictions at the species level have been surpassed by ab-engineering technology, which can isolate hybridomas across phylogenetically different species. One potential solution to the problem of poor fusion efficiency is the use of electroporation/electrofusion technology, which has several advantages such as a high fusion yield, low cytotoxicity, simplicity of operation, standardisation, lack of chemicals or viruses, and excellent controllability [38].

Bacterial Display

Bacterial display often shows Ab fragments on the surface of bacterial cells, typically with lipoproteins on the outer or inner membrane of the bacteria. Bacterial display libraries mine the surface of microbes for target peptides and proteins, which can number in the billions. The Gram-negative bacterium Escherichia coli is the basis of one of the most heavily researched bacterial systems for displaying proteins, peptides, and a range of natural surface proteins [39]. We use a display strategy based on Gram-positive Staphylococcus in addition to Gram-negative systems. Amplification and the generation of huge Ab libraries are made possible by effective transformation and operational expression methods in E. coli. Using flow cytometry, it was shown that single-chain variable fragments (scFvs) are functionally exhibited on the surface of E. coli [40]. The target protein in surface-based display systems in bacteria must fuse to either the N- or C-terminal of the anchoring segment. Since most of the Abs selected by library scanning cannot be manufactured independently, fusion proteins must be utilised in conjunction with them [41]. The use of bacterial display techniques to fully isolate target-specific Abs from naive libraries is still in its infancy. Among these Ab forms are Fabs, scFvs, and full-length IgGs [42]. In contrast to yeast expression systems, which allow for post-translational modification, bacterial expression systems do not. Less efficient transitions are another drawback. But since they multiply so quickly and have such a large repertoire, bacterial cells are a good option for in vitro display. Bacterial display isn't as common as other methods, but it has advantages like low cost and easy expression. Bacterial surface display has many applications, including affinity-based screening, antibody epitope mapping, peptide substrate identification, cell-binding peptides, and vaccine development [43].

Phage Display

Phage display relies on multiple rounds of Ag-guided selection and phage propagation, as well as genetic editing of bacteriophages. It was created in 1985 by fusing foreign peptides with the M13 bacteriophage's coat protein (p III) by DNA recombination, which caused the peptides to be displayed on the bacteriophage's surface [44]. In order to select Ag-specific Abs from a pool of more than 108 phages, phage vectors were subsequently created [45]. To date, three different kinds of phage libraries have been created:

- immune
- naïve
- synthetic [46].

Making an Ab library is the initial stage in mAb identification using a phage display library. An initial step is the extraction of RNA from PBMCs or immunised animals. After cDNA synthesis, mAb clones are examined by amplifying the variable parts of the H and L chains using PCR and ligating them onto a phage vector. You can get Fab fragments or scFvs, and then you can convert them into complete IgG. One potential issue is that while creating libraries, the coupling of VH and VL chains may not accurately reflect in vivo Ab pairing in patients. Second, because of intrinsic toxicity or disruption of phage assembly, not all phage clones from a given library will display a protein. As a result, phage libraries may not represent all Abs properly. Thirdly, insufficient RNA recovery or DNA loss could cause the clones of interest to be missed. Lastly, because of the limited mAb sampling that follows panning, some relevant clones may be lost. An advantage of phage-displayed libraries is shown by the way the phage particles relate Ab genotype and phenotype. The small size and high solubility of phage particles allow for the effective production and demonstration of up to 1011 distinct clones in a single library. [47].

Yeast Display

Yeast display, which was developed in 1997, allows for the surface expression of recombinant proteins by fusing them to the C-terminus of the mating agglutinin protein AGA-2 [48]. This method has been used to identify specific binding-active Ab fragments, including scFvs, VHH, Fabs, nanobodies (Nbs), and full-length IgGs. As the most common type of antibody, scFvs can be found in as many as 100,000 yeast cells [49]. The conventional approach to displaying Fab fragments involves creating separate plasmids encoding the H chain variable (VH)-CH1 and L chain variable (VL)-CL regions through homologous recombination in haploid yeast strains. After that, when the yeast cells mate, they can undergo a transformation from haploid to diploid, with functional Fabs attached to their surface. One common application of yeast surface display is screening Fab immune or naïve libraries for binders using soluble Ag. By changing the Ag concentration, it is possible to select Abs according to their affinity [50]. Yeast displays can be modified post-

translationally to synthesise and fold complex eukaryotic proteins [51]. Because of its glycosylation characteristics, yeast also shows that entire Abs are more soluble in yeast. Furthermore, the endoplasmic reticulum of yeast is responsible for producing chaperone proteins, which aid in the proper folding of proteins. The yeast display method has a few flaws. The first drawback is that it doesn't have a very large repertoire compared to other methods of display, such as those based on bacteria and phages [52]. It only has around 107-109 distinct clones.

Mammalian Cell Display

In order to display DNA in mammalian cells, transfection of these cells with Ab-encoded DNA is required, either temporarily or permanently [53]. The feasibility of employing mammalian cells to link an in vitro selectable phenotype to genotype was demonstrated in early research using transient expression on the surface of COS, CHO, and HEK-293 cells [54]. Improved expression and stability are just two of the many benefits it provides, along with functionally glycosylated mAbs on the surface of the cell. It is possible to use small libraries biased towards a particular Ag, but screening larger libraries has proved a challenge in mammalian cell display, making it hard to directly isolate high-affinity binders from naive libraries. Through the use of mammalian cell display and in vitro activation-induced cytidine deaminase (AID)-induced somatic hypermutation (SHM), it is possible to isolate human mAbs that resemble critical components of adaptive immunity, thus circumventing this limitation [55]. Basically, AID delaminates cytidine to uridine, which causes nucleotide transitions, transversions, double mutations, and nonsynchronous mutations on both the transcribed and nontranscribed strands of the V-region DNA. This in vivo method can be reproduced in vitro because AID expression is enough to simulate important SHM features in B and other mammalian cells [56]. Most human B cell lines aren't ideal for mammalian cell display because of how slowly they mature and how difficult they are to transfer. Two mammalian cell display-ready cell lines, CHO and HEK-293, can undergo SHM in vitro with rates of AID-induced variable gene mutation equivalent to B cells, but [57].

Transgenic Animals

The area of recombinant immunoglobulins has made use of transgenic animal technology in no less than three separate cases. The first application is in the bioreactor industry, specifically in the production of antibodies from milk by transgenic animals. Transgenic animals' milk has long been recognised for its abundance of therapeutically relevant human proteins. Various domestic species, including goats, sheep, pigs, and cattle, produce milk with average yields of grams per litre. Antibodies are produced by means of promoters that are particular to mammary tissues and which ensure proper assembly and function of the foreign substances found in milk [58]. Animals engineered to produce recombinant antibodies or antibody fragments that can neutralise common diseases of the species were the second use of transgenic technology. A second approach used germline-arrangement of human constant and variable gene regions in mice. After conventional vaccination procedures, human antibodies with modifications are produced by the B cells of transgenic mice [59].

Ribosome Display

To halt the loss of ribosome, protein, and m-RNA, a cell-free in vitro translation approach known as ribosome display technology forms an Ab-ribosome-mRNA combination [60]. In a ribosome display construct, a T7 promoter is followed by a ribosome binding site, which attracts the ribosome to a start codon. Since the used vector does not contain a stop codon, the polypeptide cannot be released from the ribosome or mRNA. The first step in obtaining the necessary polypeptides is to ligate the C-terminal spacer/tether region to a DNA library [61]. Proteins are formed by the translation of messenger RNA (mRNA) generated by transcription of this DNA. Gathering and purifying ribosomal complexes after target addition removes weak or nonspecific binders. During the elution process, strong target-binding sequences are retrieved. In the steps that follow bio panning, mRNA is extracted, the ribosome is broken up, and RT-PCR is carried out. The cDNAs of the Ab fragments are amplified using polymerase chain reaction (PCR) [62] to collect DNA for subsequent selection cycles. Ribosome display has multiple advantages. For starters, eukaryotic cell-free systems can carry out post-translational modifications, screening huge libraries becomes more easier, and the technology improves upon previous approaches when it comes to choosing high-affinity combining sites. The fact that no cell culture is necessary makes it both efficient and fast. The ribosome display method for scFv optimisation is one of the most effective display ranges, usually 1012 molecules [63]. However, a major restriction is the amount of functional and accessible ribosomes in the reaction, which is dependent on the size of the library. Nonetheless, ribosome display is still among the top in vitro selection strategies for mAbs [64] because of its outstanding ability to directly create complete proteins, especially scFvs.

DNA-encoded Antibodies

By introducing genetic constructs that encode the essential mAbs into the host cells, the DNA-encoded mAb approach does away with the need for conventional production and purification processes. Viral vectors, especially adeno-associated virus (AAV), have been successful in delivering monoclonal antibodies (mAb) [65]. It is possible for AAV to infect both dormant and fast-growing cells without incorporating into the host cell's DNA. Its advantages include a genetic cassette that remains persistent for a long time after a single injection and a relatively low immunogenicity.

However, anti-vector immunity is an additional layer of complexity that might arise since AAV can provoke immunological reactions. Antibodies that neutralise the AAV capsid have the potential to halt transduction and the delivery of the gene therapy vector in question [66]. Another method for gene delivery that allows for the synthesis of mAbs in vivo is the use of DNA plasmids to transmit critical genes. The capacity to insert genes more efficiently, avoid immunological responses to the vector, and inject the patient many times are all advantages. Furthermore, DNA exhibits reduced immunogenicity and is more stable when kept at room temperature [68]. Last but not least, this method is cost-effective due to the fact that DNA can be mass-produced with little to no need for refrigeration or special shipping. One drawback is the time required to obtain maximal expression, which usually takes 7 to 14 days. This can be too long for immediate therapeutic uses. Two or three months later, the expression will be noticeable. The possible risks of DNA-based treatments, including insertional mutagenesis and the start of immune reactions to DNA following repeated delivery, need to be studied [69], even if they have not been demonstrated in clinical settings as yet.

RNA-encoded Antibodies

The production of monoclonal antibodies (mAbs) requires the in vitro transcription of mRNA encoding abs from a DNA template. Transcription of a promoter-containing linear DNA template with 5′ and 3′ untranslated regions and an open reading frame is the first step in mRNA production. This template is mediated by RNA polymerase. A polyadenylation tail and a triphosphate cap are necessary for the mRNA to have biological activity. After mRNA is transported to the cytoplasm, post-translational modifications begin the production of the encoded mAbs. Within hours of completion of this process, mRNA-encoded mAbs reach their maximum expression level, which is a few days later. The genetic information transmitted by messenger RNA is transient until it is degraded [70]. One benefit is the ability to express proteins quickly (within hours), which allows for dosing schedules to be repeated. This method confirms the feasibility of producing large quantities of clinical-grade mAb. Not only does this technology have a solid track record of clinical safety, but its cell-free design streamlines production and cuts costs [71]. Due to their instability within cells, mRNA-encoded mAbs require carrier platforms for effective transport and expression. Lipid nanoparticles are now the most flexible and effective delivery system for mAbs encoded by messenger RNA [72].

Conclusion

In modern medicine, the discovery of monoclonal antibodies (mAbs) was a game-changer. Monoclonal antibodies (mAbs) have emerged as powerful therapeutic agents with numerous medical uses since their development, thanks to innovations in technology such as hybridomas and recombinant DNA. Infectious diseases, autoimmune disorders, cancer, and many more have found effective treatments thanks to their precise targeting abilities and action modes, which have revolutionised the therapeutic landscape. There is great hope for the future of mAbs as research focuses on next-generation treatments, personalised medicine techniques, and integration with cutting-edge modalities like as gene therapy and immunotherapy.

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