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Bacteriophage Endolysins - Challenges and their Application Approaches

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Abstract: ***Antimicrobial resistance (AMR) is a global public health concern that jeopardizes the effective prevention and control of emerging infectious diseases. Bacteriophage-derived proteins, such as endolysins, could provide an efficient remedy. Endolysins are bacteriophage-encoded peptidoglycan hydrolases that lyse bacterial cells by targeting their wall, especially Gram-positive bacteria with a peptidoglycan layer. In recent years, the scientific community has particularly focused on lytic enzymes due to their specific selectivity, mode of action, engineering potential, and lack of resistance mechanisms. Recombinant endolysins have proven effective against well-known infections including MRSA, Staphylococcus strains that create biofilms, and Pseudomonas aeruginosa. Endolysins have been explored in combination with other antimicrobials to have a synergistic activity. The Engineered endolysins with outer membrane penetration can help manage multidrug-resistant Gram-negative bacteria and play a vital role in ameliorating widespread bacterial diseases. The Present review examines endolysins' current state and highlights their active potential in controlling bacterial infections.***

Keywords: Bacteriophages, Endolysins, Anti-microbial resistance, Antimicrobials, Endolysin therapy, Bacteriophage enzymes.

I. INTRODUCTION

Skin and mucous membranes comprise the body's first line of defense, which are easily exposed to pervading microbes and get damaged by virulent microorganisms that enter the body and cause diseases. Wound Infections can prolong the healing process and increase the risk of dehiscence. Among hospitalized patients, trauma is the most common cause of wounds. Traumatic injuries are categorized as purposeful or unintentional, purposefully induced wounds include those acquired in hospitals, such as surgical incisions or wounds from intravenous medical devices[1]. On the other hand, non-intentionally induced wounds include injuries like decubitus ulcers. Surgical procedures are the primary source of acquired wound infections in hospitals[2,3].

Alexander Fleming was the first person to notice that microorganisms might quickly become resistant to penicillin after extended exposure to the antibiotic. Since the 1940s, when they were first employed in therapeutic settings, antibiotics have been overprescribed and misused for both human and animal populations. That led to the emergence of antibiotic-resistant bacteria and its determinants to uncontrollably spread among bacterial infections; some of them are now highly resistant to all available antibiotics such as MRSA, VRE, and MDR-TB.

Resistance mechanisms emerge from antibiotic-inactivating enzymes, efflux pumps, and permeability/target changes. Novel pathways underlying the development of AMR are being discovered, making this field of medical microbiology relevant. In addition to genetic components of AMR, additional behaviors such as inaccurate diagnosis, broad-spectrum antibiotic exposure, and delayed diagnosis also contribute to resistance development[4] . The necessity for new, potential as antibacterials (such as phages and phage lysins) to address the global rise of antibiotic resistance in human and animal diseases is well acknowledged, as the growing worry over this issue[5]. Phages can be detected either directly connected with their bacterial hosts or in enormous quantities as free virions in the environment. Because of their widespread distribution, phages may be the most abundant biological entities on Earth[6].

A. *Bacteriophages*

Bacteriophages, or phages, are viruses that are naturally occurring and specifically target bacteria. Therefore, Phages are thought to be efficient in fighting against bacteria. They have an impact on physiological and immunological processes, which could be crucial to their therapeutic advantages. Phages can weaken the immune system by activating immunological responses including cytokine release. Phage's reproduction process might be either lytic, lysogenic (temperate), or chronic.

Bacteriophages may be used instead of antibiotics to treat multidrug-resistant bacteria infections. Research on how phages and antibiotics work together is becoming increasingly important, particularly in light of antibiotic resistance[7]. Unlike antibiotics, which have a considerably broader spectrum, phages are very specific to their hosts and are unlikely to induce secondary infections. Furthermore, the time and financial expenses associated with isolating and selecting novel phages are lower than those associated with developing antibiotics[8].

Lytic phage has a brief life cycle, which destroys the host by lysis, viral particles are shed continuously by membrane protrusion, a non-lethal effect of chronic replication. The Phage display their life cycle steps by adsorption, penetration, uncoating (DNA injection and replication), biosynthesis , maturation, and release as shown in Figure 1. Host cells are lysed by tail-like phages using an endolysin & holins. There are two different life cycles possible for temperate phages: lysis or recombination of the genome at a chromosomal attachment site, which is carried out by an integrase encoded by the phage genome. Transcriptional repressors control the phage transition to an infectious or lytic state of the phages inside the host chromosome. The switching of the lytic cycle triggers the excision which is facilitated by the favourable environmental conditions of the host[9, 10]. Since temperate phages often carry virulence proteins that pathogenic bacteria use, as and when infecting humans and animals, they are essential for understanding the evolution of bacterial pathogenesis.

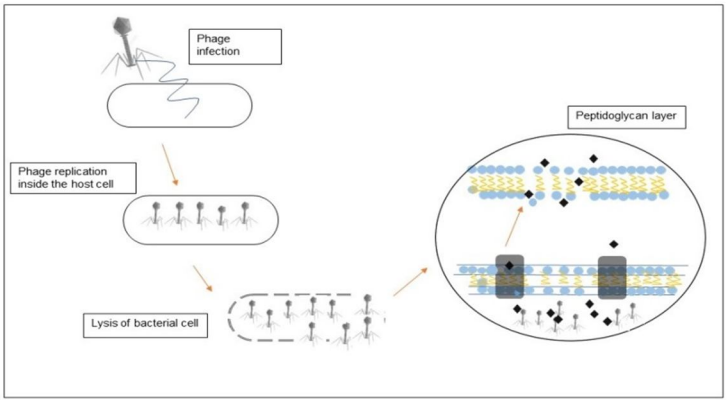

Figure 1: Life cycle of bacteriophage

Many studies have been conducted on the same lines, where the lytic enzymes - bacteriophages worked as possible therapeutics for treating bacterial infections in a wide range . Provided the exogenous recombinant proteins as shown in table 1 worked as phage lytic products, can target the Pathogenic microorganisms. However, there is a growing realization that the intrinsic bactericidal activity of these drugs can be considerably improved by altering existing functional domains or by adding new domains[11].

Protein	Functions	References
DNA polymerase	Replication, DNA cloning, and other molecular applications	[12]
Ligases	genetic engineering, virus metabolism, genome replication, recombination and repair	[13]
Endolysins	degrade the peptidoglycan of the bacterial host	[14]
Coat proteins	Binding and incorporation of specific RNA	[15]

Table 1: Bacteriophage proteins and their functions

This is rather understandable that the coevolution of bacteriophages and specific host bacteria reduces the likelihood of resistance development as a natural process. However, there are some limitations for using phages as a short shelf life, restricted host range, the possible development of resistance, lack of broad-spectrum activity, and complex interactions with the host. Furthermore, rather than entering the target cell, endolysins act on the bacterial cell wall, and potential resistance mechanisms such as efflux pumps and decreased membrane permeability do not affect them, making them an excellent targeting option in place of phage.

Owing to these reasons, there has been growing interest in virus-encoded proteins (endolysins) that can effectively destroy bacteria. Instead of comprehensive phage therapy, the focus has shifted towards extracting and manipulating viral proteins. Endolysins are capable of combating the microbes, which has gained a significant attention as an alternative to complete phage therapies[16].

II. ENDOLYSINS

Endolysins are phage-encoded proteins that target the peptidoglycan layer of the bacterial cell wall and are therefore, dubbed as peptidoglycan hydrolases enzymes, along with a related holin protein, build up within the host cell in the absence of the phage virus. Because endolysins lack their signal sequences, holins allow access to the bacterial peptidoglycan by generating pores in the cytoplasmic membrane. This is a carefully regulated sequence of events that occurs only when the holin concentration reaches a certain threshold. Endolysins can now access and degrade peptidoglycan, disrupting the cell's osmotic balance, leading to lysis and, finally, death. To reach the cell wall and carry out their lytic action, endolysins produced in the cytoplasm of infected cells, must pass through the cell membrane. Based on the route, these enzymes facilitate their entry into the cell membrane, they are categorized as either exported endolysins (e-endolysins) or canonical endolysins (c-endolysins). To leave the cytoplasm, endolysins need the help of the holin, another phage-encoded function is required. When holes are genetically programmable, they oligomerize in the cell membrane and create holes, which causes the proton-motive force to collapse. Moreover, c-endolysin from the cytoplasm can escape via these pores and reach the cell wall, which is necessary for lysis to continue. This mechanism defines the conventional lysis paradigm, which is prototyped by the Escherichia coli phage λ. As endolysins, C-endolysins have been investigated. The holin's job is to make holes in the cytoplasmic membrane so that the endolysin can access the bacterial peptidoglycan, as endolysins are devoid of their signal sequences. Only when the holin concentration hits a specific threshold does this tightly controlled series of events. Then the endolysins get access to peptidoglycan, initiate the breakdown, and also upset the osmotic equilibrium of the cell, ultimately leading to lysis/cell death. Holins and endolysins cooperate to guarantee efficient phage infection. Endolysins have a broad range of species-specific lytic action that reduces the pressure of selection on commensals.[17].

Here are some plausible reasons why resistance to endolysins is unlikely to develop. Bacteria cannot alter the phage endolysin's ability to bind and break highly conserved linkages and connections in cell wall components, which lessens the possibility that endolysin resistance would evolve. Antibiotic resistance is prevented by the two catalytic domains present in most endolysins, which cleave the connections inside the peptidoglycan layer. Endolysins avoid the mechanisms of antibacterial resistance, such as reduced membrane permeability and active efflux from the cell, because most of the phage lysins work externally and target the cell wall without entering bacterial cells. Additionally, it has been proposed that endolysins and antibiotics can be utilized to treat infections to increase the therapeutic potential of antibiotics and promote the emergence of antibiotic resistance[18].

A. Structure of Endolysins

Because of the differences in their cell wall compositions, positive (Gram-positive) and negative (Gram-negative) lysins have distinct structures. The two unique domains that make up a positive lysin's modular structure are the cell wall-binding domain (CBD) at the end and the enzymatically active domain (EAD) at the beginning. The linker is usually short and flexible, targets are identified by the CBD by binding to certain molecules on the cell wall. The disruption of the cell wall occurs when specific PG bonds are broken down by the EAD. Several CBDs or EADs were discovered and were present in various sequential orders inside the lysins. Lysin's specificity is often determined by the CBD and the selectivity may be aided by the interaction between CBD and EAD, which is discovered at the cell wall receptors, as demonstrated. Lysins have an advantage over conventional antibiotics due to their high specificity because they do not disrupt the microbiota[19]. Comprehension of endolysin structure may result in variants exhibiting enhanced activity, stability, or host range, hence facilitating superior therapeutic applications.

B. Properties of Endolysins as Antimicrobials

The endolysin enzymes are linked with Amidase and Peptidase domains for exerting cell breakage activity. Functionally the endolysins may classified into three types based on mode of action: (a) glycosidases, which cleave the glycan portion of peptidoglycan (MurNAc-GlcNAc); (b) amidases- which cleave the amide bond between the glycan moiety (MurNAc) and the

peptide moiety (L-alanine); and (c) endopeptidases- cleaves the peptide bond between two amino acids of the stem peptide. Endolysins exhibit broad-spectrum antimicrobial activity, which may disrupt the microbiota; however, their antimicrobial efficacy can be adjusted by altering the endolysin concentration. They can target both dormant and proliferating cells, and no bacterial resistance to endolysins has been documented. Additionally, they demonstrate efficacy against bacterial biofilms, exhibit a reduced degree of antibody neutralization, and possess well-defined pharmacokinetics[20]. However, CHAP domains are an exception, as they are classified according to their catalytic mechanism rather than the peptidoglycan link which they slice. In their active region for substrate cleavage, CHAP domains consist of conserved cysteine and histidine residues. The table 2 shows the types of compounds and their features.

S.No	Type of compound	Features	References
1	EDTA	Outer membrane permeabilizer	[21]
2	Artilysins(Genetically engineered endolysin)	Increases lytic activity by passing through the outer membrane barrier	[22]
3	Aminoacids addition to the C-terminal of endolysin	Improves antimicrobial activity	[23]
4	ε-poly-L-lysine	Outer membrane permeabilizer	[24]
5	Organic acids	Improves antimicrobial activity	[25]
6	Liposomes	Encapsulation of endolysin	[26]

Table:2 The table represents the Methods for overcoming the barrier of the outer membrane of the Gram-negative bacteria.

III. IMMUNOLOGICAL RESPONSE

Protein therapies have laid a considerable success in medical treatments. However, they encounter a significant challenge, profusely related to immune responses elicited by non-human proteins. These immune responses include the IgG response, which is meant for identifying and neutralizing foreign proteins, as well as the IgE response, which can initiate allergic reactions and mediate type 1 hypersensitivity. These immune responses may restrict the effectiveness of non-human protein therapy. Presumably, it is ensured when a person is exposed to a foreign protein towards the medication, consequently, their immune system may produce neutralizing IgG antibodies. If the same therapy is used repeatedly, the presence of these antibodies may lead to inactivation, which results in treatment failure. At this juncture, a few of the antibodies can recognize specific epitopes on foreign antigens simultaneously. This phenomenon highlighted the complexity of creating effective protein therapies and emphasizes their need for further research and development in the present specified area[27].

Lysibodies, a designed lysin, are opsonins the S. aureus cells by activating to activate the classically the immune complement system. This can lead to phagocytosis and the removal of the pathogenic organisms. Endolysin can be employed similarly to direct an immune response against harmful microorganisms. Therefore, a similar technique without the use of antibody fragments for a protein, produced from the CBD of the endolysin PlyV12 fragment as shown in table 3. When some of the Gram-negative endolysins are programmed pre-clinically, that generates the issues with immunogenicity, toxicity from lipo-polysaccharide leakage during the bactericidal process. Subsequently, several pharmacokinetic obstacles may arise due to the complexity of the Gram-negative cell wall, which must be addressed[28,29].To minimize the complexity of immune responses that are triggered by endolysins, it is very much essential for the researchers to explore authentically and a specific method provided to involve and incorporate human immune cells in the development of engineered endolysins. This novel approach aimed to reduce the likelihood of immunological interaction to the foreign proteins and slow down their cell clearance from the body. By utilizing human immune cells in the construction of these endolysins, Recombinant proteins are recognized as part of the body, which potentially leads to a paradoxically decrease in immunological responses. Since endolysins are proteins, they may provoke an immune reaction when administered through mucosal or systemic routes, potentially impacting their activity. To investigate such a target in the future, there is a necessity to go for trials, and it has been conducted both *in vitro* and *in vivo*

Endolysin	Catalytic domain		Cell wall binding domain	References
LysK	cysteine-histidine dependent amido-hydrolase/peptidase domain (CHAP(K))	It has a Central amidase domain	SH3b cell wall-binding domain	**Error! Bookmark not defined.**
Ply500	Endopeptidase	-	Cell wall binding domain	**Error! Bookmark not defined.**
Cpl-7	Muramidase	-	Cpl-7(3x)	[30]
KZ144	Lytic transglycosylase	-	Cell wall binding domiain	[31]
B30	CHAP endopeptidase	Muramidase	SH3b cell wall-binding domain	[32]

Table 3: Modular structure of different types of endolysins

In the current scenario, several researchers found that inhibiting the enzyme lithotripsin Cpl-1 in the lab did not stop its activity when rabbit hyperimmune serum was used. Similar results were seen with lysins from B. anthracis and S. pyogenes. In other studies, work performed on mice, most of them represented that the Cpl-1 showed scanty impact on some immune responses, but it had only a small effect on the enzyme's activity. Another enzyme, lysostaphin, was shown to have reduced immune response and improved drug delivery provided the attachment of polyethylene glycol (PEG), but the results noted slightly decreased its activity[33].

IV. ENDOLYSIN THERAPY

The table 4 represents the types of lysins and their domains and functions.

lysin	N terminal	C terminal	Function	Examples	References
Endolysin	Natural catalytic domain	Natural cell wall binding domain	Cleaves the Peptidoglycan layer of the bacterial cell wall	PlyC, PL-3	[34]
Chimeric lysin	Catalytic domain	Cell wall binding domain	Natural domains were shuffled	LysK, PlyV-12	[35]
Artilysin	LPS disruptor binds to catalytic domain	Cell wall binding domain	LPS disruptor added to disrupt the gram-negative outer membrane	SMAP29-GSA-LysPA26, SMAP29-$(GGGGS)_3$-LysPA26	[36]
Virion associated lysin	Natural catalytic domain	Cell wall binding domain	Addition of cell wall binding domain to target specific bacteria	KMV36-C	[37]
Truncated lysin	Catalytic domain	-	Removal of the C-terminal to increase the activity	MV-L, LysH-5	[38]

Table 4: Different types of lysins, their structural differences, and examples

A. Limitations of Endolysin therapy

The clinical implications of endolysin delivery methods present a number of difficulties, despite their great therapeutic promise. Each endolysin requires them to be carefully planned and optimized, which might raise the cost and effort required to scale up the development of such formulations[39]. Endolysin's execution in a short half-life *in vivo*, which is by neutralizing antibodies and cytokines' inflammatory reaction, is one of its drawbacks. When endolysin is used frequently, the immune system reacts, which may lead to loss of its enzyme-mediated lytic activity in vivo. Exclusively, an innovative method for identifying immune-derived

engineered endolysins was proposed by integrating or the fusion of several endolysin-containing CBDs to the human IgG antibody at the Fc receptor[40,24].

B. Applications of Endolysin therapy

Endolysin therapy shows potential as a viable alternative to antibiotics, particularly in combating antibiotic-resistant infections and biofilms. It has promising applications in medicine, food safety, and other fields as shown in figure 2.

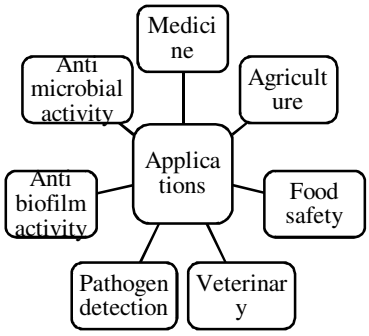

Figure 2: Applications of Endolysin therapy

1) Veterinary

Lysins can be produced and used for various purposes other than treating humans with infectious diseases. There is a wide scope in the veterinary industry as well. Sometimes it would also stop a pathogen from getting into food or from spreading zoonotic diseases. The most frequent cause of mortality for adult dairy cows is bovine mastitis, which results in large economic losses for dairy farmers across the globe annually. In addition, food contamination may result from its presence, especially when raw milk cheese is involved. The lysin LysH5 possesses the remarkable ability to actively break down and destroy *staphylococci bacteria*, which are known to cause mastitis, particularly in pasteurized milk. Conversely, the Ply700 enzyme has demonstrated its capacity to effectively target and break down streptococci bacteria, the culprits behind mastitis in cows' milk[41].

2) Food Applications

Ply3626 provides evidence that lysins may be used in the food industry. This enzyme has proven to be lytic against a range of C. perfringens, which are frequently responsible for food poisoning and a significant source of financial losses for the poultry industry[42]. Endolysin has been the subject of research in vegetable products to mitigate and prevent the presence of pathogenic microorganisms that contribute to antibiotic resistance in vegetables. Notably, the endolysin LysP53 has demonstrated its efficacy against a broad spectrum of bacteria, exhibits thermal stability, and is considered as safe. This specific endolysin is an excellent biocontrol agent applied for effectively reducing bacterial contamination in fresh vegetable products. It has definitively demonstrated its ability to lyse *Salmonella enteritidis* on fresh romaine lettuce[43,44].

The shortened form of Ply511 lysin shows a higher lytic activity than the original enzyme. Furthermore, recombinant starter cultures have been developed to prevent L.monocytogenes contamination during soft cheese ripening. A lactococcal starter strain was created by cloning a phage holin-lysin system for the controlled release of host intracellular enzymes. These enzymes generate flavor during cheese ripening and plants for their defense against phytopathogens.

3) Therapeutic Protein Delivery

The existing techniques for identifying B. anthracis infection in humans are cumbersome and unsuitable for application in settings other than labs. Its critical role is to have quicker detection methods because the spore-exposed individuals have only 48 hours to receive treatment. Using such a novel method that can show benefits in as little as 15 minutes, PlyG lysin makes treatment far more successful. In a third investigation, magnetic beads equipped with the cell wall-binding domains of the Listeria phage lysins Ply118 and Ply500 were employed effectively to eliminate the bacteria from contaminated food samples. By using this technique, almost 90% of the L. monocytogenes cells were recovered. Another lysin, Ply118, has been created for use in molecular biology. It may effectively recover native intracellular proteins, DNA, or RNA from small-scale Listeria bacteria, according to studies by Loessner et al. Furthermore, it has been shown that for proteome-based investigations of cell wall-anchored proteins in GAS, the multimeric phage lysin PlyC dissolves the S. pyogenes cell wall more effectively than mutanolysin[45].

V. CONCLUSION

Endolysin research has gained popularity in the last decade, with phage treatment resurfacing as a promising subject. Endolysins are potent antimicrobials and have synergistic effects, but faced obstacles in becoming widely used. They are safe for medicine but need more research to confirm clinical reliability and commercialization.

Metagenome studies uncover numerous phages and their endolysin sequences, providing a vast resource for discovering new endolysins. These sequences help assemble modular chimeric endolysin domains. Bio-informatic and proteomic investigations may lead to domain swapping, chimera creation, and designing lysins for various applications. Endolysins represent a fresh hope in the era of antimicrobial resistance.

A. List of Abbreviations

MRSA - Methicillin-resistant Staphylococcus aureus
VRE - Vancomycin-Resistant Enterococci
MDR-TB - multidrug-resistant tuberculosis
AMR – Antimicrobial Resistance
DNA – Deoxy ribonucleic acid
CBD – Cell Wall binding domain
EAD – Enzymatically active domain
CHAP - cysteine, histidine-dependent amidohydrolases/peptidases
EDTA - Ethylenediaminetetraacetic Acid
IgG - Immunoglobulin G
IgE - Immunoglobulin E
LPS – Lipopolysaccharides

B. Declarations

- Ethics approval and consent to participate: Not Applicable
- Consent for publication: Not Applicable
- Availability of data and material: We collected the data through a literature review from PubMed, Research Gate, Google Scholar, and other Google sources. The pictures were created using PowerPoint.
- Competing interests: The authors declare that they have no competing interests.
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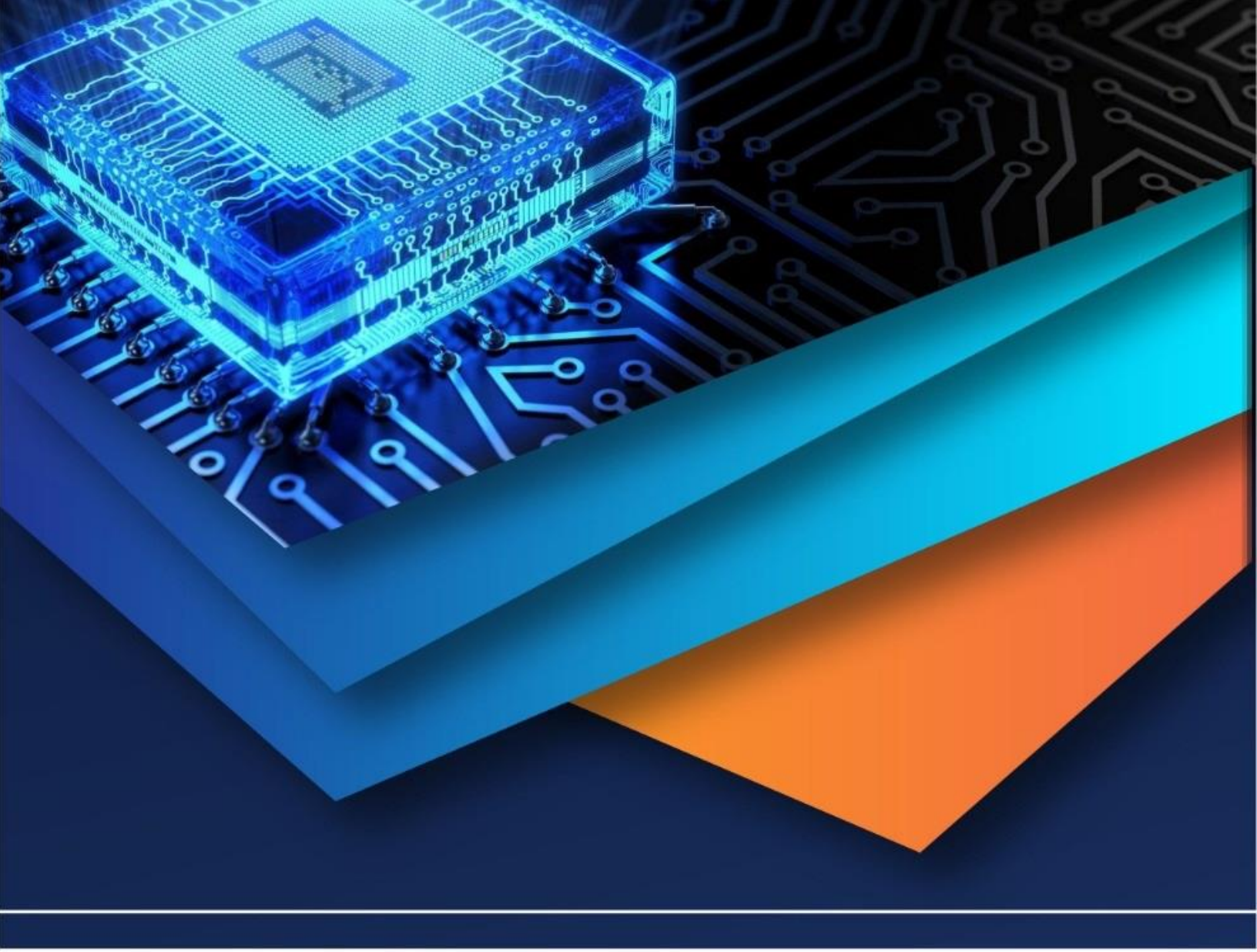

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