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A Synthetic Biology Strategy: Genetic and Metabolic Orthogonality for Overcoming the Immunogenicity of Therapeutic Proteins

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Abstract:

With the widespread application of therapeutic protein drugs, numerous issues associated with these medications have been increasingly serious. The most prevalent one is immunogenicity, that is antigens stimulate an immune response in the body. It would limit efficacy and safety. To overcome this challenge, this paper built a dual orthogonal system via synthetic biology to reduce protein drug immunogenicity without interfering with host cellular functions. It includes both genetic and metabolic orthogonality. Codon reprogramming was employed to mask drug immunogenic epitopes for concealing immune recognition sites, enabling the drug to achieve "molecular invisibility" in the body. Furthermore, by reconstructing glycosylation to modify non-human glycans and establishing a human glycan synthesis pathway, the potential for immunogenicity caused by xenogeneic glycans was reduced. Using insulin as a model, this strategy produced modified molecules capable of evading MHC-II binding and antibody-mediated neutralization. The dual orthogonal framework therefore offers a promising route to generate therapeutic proteins that are functionally active yet immunologically "invisible". Besides insulin, this approach also can be extended to other biotherapeutics. Taken these, this finding establish safer, de-immunized therapeutic proteins through synthetic biology contributing to the future of biologic drug design and personalized therapy.

Keywords: Bioorthogonal; Immunogenicity; Therapeutic proteins; Codon reprogramming; Glycosylation.

1. Introduction

Therapeutic proteins often specifically target pathogens [1]. This characteristic of therapeutic protein drugs can greatly enhance efficacy and cure rates especially in the treatment of certain complex diseases. Common therapeutic proteins, such as monoclonal antibodies and growth factors, have become crucial weapons in treating cancers and autoimmune diseases [2]. In the fields like oncology and immunology, monoclonal antibody drugs have achieved remarkable clinical success, with a global market exceeding $100 billion [3].

Compared to small-molecule drugs, therapeutic proteins are highly efficient recognition, substantially improving treatment outcomes. Despite their clinical advantages, therapeutic proteins also cause a major limitation-immunogenicity, characterized by the induction of anti-drug antibodies following administration, because therapeutic proteins often possess larger sizes and more intricate structures. Protein drugs exhibit primary, secondary, and tertiary structures, and some may even form quaternary structures through folding and binding. Following folding, proteins undergo further modifications such as acetylation and glycosylation, making the structural characterization of protein drugs exceptionally complex. Such complex structures make it more likely to identify protein drugs as foreign bodies and launch immune attacks for the immune system, which is damaging to neutralizing the drug, reducing its efficacy, and potentially adverse reactions that could threaten the patient's life in severe cases. This unintended immune response may pose serious clinical safety concerns. Therefore, in spite of the promising prospects of therapeutic proteins, navigating immunogenicity challenges remains an inevitable hurdle in their clinical application.

A commonly employed approach to reducing protein drug immunogenicity is to use immunogenic epitope databases to predict potential immunogenic risks in the drug, and selectively remove these epitopes. However, this method requires a thorough assessment of the drug to avoid introducing new epitopes or diminishing its therapeutic effectiveness [4]. Other researchers have utilized chemical modification techniques, such as polyethylene glycol (PEG) modification. PEG is covalently linked to protein or peptide molecules through chemical methods for modification. PEG polymers, with their hydrophilic, chemical inert properties and steric hindrance effects, can shield immunogenic epitopes, making the therapeutic drug molecule "invisible", leading to enhanced efficacy, reduced dosing frequency and fewer side effects. However, due to the presence of anti-PEG antibodies in certain populations, this method cannot fully eliminate immunogenicity [5].

To tackle the challenges posed by immunogenicity, this study introduces a synthetic biology strategy: a dual orthogonal system. Codon reprogramming is used to cover immunogenic epitopes and reconstructing glycosylation enables the establishment of a human-specific glycan biosynthesis pathway. The combined actions of both approaches can significantly reduce the potential for unpredictable immune responses triggered by the protein drug. More importantly, the system operates without disturbing the host's native metabolic processes, achieving complete orthogonality and noninterference between the system and the host.

2. Orthogonal System

An orthogonal system refers to an artificially synthesized system that coexists with the host cell's own synthetic system without interference, enabling specific synthetic activities desired in the host cell [6]. Such synthetic processes may be beyond the natural capacity of the host cell or may be incompletely expressed due to intracellular inhibitory mechanisms. The various elements of the orthogonal system can act either synergistically or independently within the host cell. Meanwhile, it does not disrupt the host's native biochemical reactions or metabolic processes. Regarding the immunogenicity of therapeutic proteins, establishing orthogonal systems modifies drugs for rendering them unrecognizable by the immune system, which can significantly reduce immunogenicity. The dual orthogonal system ensures humanized protein production while reducing the risk of immunogenicity to the lowest possible level. This system operates at two levels-genetic orthogonality and metabolic orthogonality. Genetic orthogonality involves manipulation at the level of the genetic code (DNA) by altering the meaning of specific codons, endowing them with new functions for human application. Metabolic orthogonality is reflected in the manipulation of metabolic processes such as glycosylation. Upon completion of protein translation, glycosylation serves as a crucial post-translational modification, with the resulting glycan chains directly influencing protein structure, properties, and function. At this level, immunogenicity reduction is achieved by modifying the glycosylation pathway.

2.1 Genetic Orthogonality

The first level of orthogonality, genetic orthogonality, is primarily achieved through codon reprogramming. During the elongation of protein translation, tRNA molecules are aminoacylated by aminoacyl-tRNA synthetases (aaRS) and subsequently delivered to the ribosome. As the ribosome translocates along the mRNA, a polypeptide chain

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is synthesized through the sequential addition of amino acids. Typically, organisms employ 64 triplet codons to encode 20 standard amino acids, with most amino acids being represented by multiple synonymous codons. Codon reprogramming refers to remove one or several codons assigned to a specific amino acid, followed with their corresponding tRNAs eliminating that amino acid from the system and introducing a noncanonical amino acid (ncAAs) and its cognate tRNA [7]. This is effectively to reassign the codon with a new function. Noncanonical amino acids exhibit diverse structures and functions. Some occur naturally as metabolites, signaling molecules, or biosynthetic intermediates, while others are entirely synthetic. In this strategy, a bulky ncAA is selected to cover native immunogenic epitopes and leverage steric hindrance to block interactions between the drug molecule and immune-recognition components.

The theoretical and practical foundation of this approach originates from the genetic code expansion technology pioneered by Schultz and colleagues [8]. Implementation of this technology requires two artificially engineered orthogonal components. The first is the orthogonal tRNA, which typically derives from archaeal species. Due to the evolutionary distance between archaea and humans, their tRNAs and aaRSs differ substantially, allowing for the construction of an orthogonal tRNA/aaRS pair that operates independently of the host. The orthogonal tRNA can be recognized by the host ribosome but not by endogenous aaRSs, ensuring that it exclusively delivers ncAAs to the growing peptide chain without misincorporating natural amino acids-thus avoiding interference with native protein synthesis.

The second component is the orthogonal aminoacyl-tRNA synthetase (O-aaRS), which was obtained through directed evolution [9]. It exhibits strict substrate specificity, selectively recognizing and activating a particular ncAA and attaching it to its cognate orthogonal tRNA, while remaining inert toward the host's natural amino acids.

In practical implementation, a target codon within the gene sequence is selected and reassigned to encode an ncAA at first. The genes encoding the orthogonal tRNA, O-aaRS, and the target protein are then co-introduced into the host cell. The O-aaRS specifically captures the externally supplied ncAA and loads it onto the orthogonal tRNA, forming an O-tRNA/O-aaRS complex. During translation of the target protein, the ribosome pauses at the reprogrammed codon site, where the complex inserts the ncAA into the nascent peptide chain. Incorporation of the ncAA simultaneously prevents the release factor from terminating translation, resulting in the replacement of the original amino acid residue with the ncAA.

This technique allows for the site-specific substitution of key native amino acids within immunodominant epitopes by ncAAs, thereby masking epitopes recognized by the immune system and introducing steric hindrance to block interactions between antigen-presenting cells and drug peptides.

In applied contexts, an ncAA can be inserted into an immunogenic epitope region of the therapeutic protein to shield the site from recognition by B-cell receptors (BCRs), which are responsible for triggering immune responses through antigen-specific binding. This modification enables the drug to evade immune recognition. In previous research, insertion of ncAAs has been shown to generate superimmunodominant epitopes, enhancing antigen strength of BCR binding and improving therapeutic efficacy [10]. In contrast, by introducing ncAAs that lack cross-linking properties within the epitope region, the binding between the drug and BCRs can be effectively prevented, thus reducing immunogenicity.

2.2 Reconstruction of Glycosylation

The second dimension of orthogonality is metabolic orthogonality. Most therapeutic proteins are expressed in non-human mammalian cell lines, whose glycosylation profiles differ intrinsically from those of humans. As a result, traditional biopharmaceuticals often carry non-human glycan moieties. It can be recognized as foreign by the human immune system and elicit undesired immune responses, compromising both productivity and therapeutic efficacy.

A representative example is the yeast expression system, widely employed among eukaryotic hosts. Yeast typically produces high-mannose N-glycans, which are immunogenic and lead to reduced protein stability, activity, and half-life [11]. To improve its suitability for therapeutic protein production, early studies employed *Pichia pastoris* as a chassis with inherently lower mannosylation and subsequently humanized its glycosylation pathway.

This modification aimed to remodel its N-glycan structure toward a human-like core, specifically the $Man_5GlcNAc_2$motif [12]. As a result, the engineered *Pichia pastoris* system can express human-compatible glycoproteins, effectively avoiding the immunogenic high-mannose glycans that previously triggered immune reactions [11].

Although humanized Pichia-based glycosylation systems have achieved remarkable progress and reached commercial application, they remain patent-restricted and largely follow conventional design paradigms. Consequently, developing novel platforms for human-type glycoprotein synthesis remains of great significance for next-generation biologics [11].

The emergence of synthetic biology offers new opportuni-

ties for reconstructing glycosylation pathways. This process entails deleting endogenous non-human glycosylation genes and introducing human glycosylation modules to establish an orthogonal biosynthetic circuit insulated from host interference. Such orthogonalized metabolic systems provide a versatile chassis for advanced biotherapeutic production, particularly in autoimmune disease and cancer immunotherapy.

The reconstruction process consists of several steps, including the identification of key targets responsible for non-human glycan synthesis in the host, the knockout of essential enzymes through genome-editing techniques to remove non-self-antigenic determinants, the integration and optimization of human glycosyltransferases to enable human-like glycan biosynthesis, and the enhancement of nucleotide sugar precursor pools to ensure sufficient substrate supply and maximize conversion efficiency. Through this synthetic remodeling, non-human glycans can be effectively eliminated, leading to a substantial reduction in immunogenic potential.

By combining genetic and metabolic orthogonality, the dual-orthogonal framework enables the construction of therapeutic proteins equipped with a molecular "invisibility cloak". Such proteins can bypass immune surveillance during biosynthesis and function precisely within the human body, thus moving closer to the realization of molecular invisibility in therapeutic design.

3. Case Study: Insulin

Insulin, a classic therapeutic protein requiring long-term administration and exhibiting significant immunogenicity, serves as an ideal theoretical model to validate this strategy. In the treatment of type 1 diabetes, conventional therapy inevitably induces antibody-mediated neutralization following exogenous insulin administration. Thus, it necessitates strategies to prevent immune attacks against foreign insulin. Previous studies have primarily focused on reducing insulin immunogenicity via screening for low-immunogenicity mutants or through chemical modification. However, these approaches remain limited by the stochastic nature of epitope mutations and allergic responses in certain individuals.

This study envisions integrating bioorthogonal systems by combining codon recoding and glycosylation reconstruction techniques for addressing immunogenicity from multiple dimensions. It establishes a dual-orthogonal platform for insulin modification with precise therapeutic control.

In the immune process triggered by insulin within patients, antigen-presenting cells (APCs), such as dendritic cells, internalize proinsulin, process it into peptides, and present them in complex with major histocompatibility complex class II (MHC-II) molecules, which mediate antigen processing and presentation [13]. Among all insulin-derived peptides capable of presentation, immunodominant epitopes exhibit the strongest and most stable binding to MHC-II molecules. APCs presenting these epitopes most efficiently recognize and activate naïve T cells carrying corresponding T-cell receptors, thereby initiating immune responses.

At the level of genetic orthogonality, this strategy aims to alleviate sequence-derived immunogenicity. Using codon recoding technology, key codons within known immunodominant T-cell epitopes of insulin are reassigned to encode noncanonical amino acids. This design differs from simple amino acid substitution by introducing a physical barrier into the core region of epitope. Such modification sterically disrupts the binding between antigenic peptides and MHC-II molecules, preventing the initiation of T-cell immune responses at the source. Consequently, upon administration, the immunogenic epitopes of modified insulin can no longer bind MHC-II molecules, blocking antigen presentation and theoretically preventing anti-drug antibody (ADA) induction. Moreover, because the modification is confined to the insulin molecule, normal immune responses within the host remain unaffected.

At the metabolic orthogonality level, the objective is to reduce post-translational modification of derived immunogenicity. According to J. Ma et al., a multiplex Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9 (CRISPR-Cas9) gene-editing strategy was applied in *Pichia pastoris* to knock out key genes responsible for non-human glycan synthesis [11], while simultaneously introducing enzymes to reprogram the yeast glycosylation pathway. This approach reconstructs a humanized glycosylation orthogonal pathway dedicated to insulin modification. The engineered pathway is metabolically insulated from the host's endogenous glycosylation network, which ensures glycoform uniformity and immune compatibility and theoretically eliminates B-cell recognition caused by glycan heterogeneity. Significant advances have been achieved in N-glycosylation engineering of *Pichia pastoris*, enabling partial mammalian-like modifications though the efficiency and uniformity of complex glycan synthesis still require optimization.

Importantly, genetic and metabolic orthogonality acts not as a simple additive combination but as a synergistic enhancement. The former rewrites the genetic instructions, while the latter purifies the modification of environment. Together, they theoretically convert insulin from an exogenous "antigen" into an immunologically "invisible" self-molecule, potentially reducing both cellular and humoral immune responses. The proposed dual-orthogonal system establishes a technological foundation for

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ultra-low-immunogenic, long-acting insulin development and serves as a versatile, programmable platform for designing de-immunized therapeutic proteins of high clinical value, carrying significant theoretical and translational potential.

4. Other Application Cases

Numerous additional studies employing synthetic biology-based bioorthogonal design have validated its potential for clinical translation. In research on the antitumor immune function of pyroptosis, investigators have constructed a bioorthogonal system based on phenylalanine trifluoroborate (Phe-BF_3). The Phe-BF_3-catalyzed bioorthogonal desilylation reaction precisely cleaves silent Gasdermin protein precursors loaded on nanoparticles. These precursors subsequently induce tumor cell rupture and death, locally activating the pyroptosis program and initiating antitumor immune responses. The Phe-BF_3-based bioorthogonal system effectively resolves key issues associated with antibody, drug conjugates (ADCs), uncontrolled and inefficient drug release [14].

Importantly, the desilylation reaction between Phe-BF_3 probes and silyl ether linkers is not naturally present in biological systems. Since no endogenous enzymes exist to catalyze this reaction, it proceeds orthogonally within cells, exerting no unintended physiological effects. Furthermore, the Gasdermin precursor in this system can be replaced with other therapeutic proteins, highlighting the principle of "modular design" that lies at the core of synthetic biology.

Beyond therapeutic delivery, bioorthogonal chemistry also enables in vivo biomolecular labeling and tracing. When studying viral invasion mechanisms, the bioorthogonal system combined with metabolic engineering permits the labeling of viral proteins or nucleic acids without disrupting viral structure or interfering with viral metabolic or physiological processes. This allows precise tracing of viral infection dynamics, offering valuable insights into the mechanisms by which viral infections lead to complex diseases [15].

Collectively, these examples demonstrate that bioorthogonal systems developed via synthetic biology offer promising avenues for addressing specific clinical challenges, ranging from precise drug activation to real-time molecular tracking.

5. Conclusion

In summary, this study proposes a synthetic biology, a dual bioorthogonal system, integrating genetic and metabolic orthogonality to address the immunogenicity of therapeutic proteins. Through codon reassignment based on genetic code expansion, the immunodominant epitopes of therapeutic proteins can be masked to evade unpredictable immune responses. Concurrently, reconstruction of glycosylation pathways eliminates non-human glycotypes and establishes humanized glycoforms, thereby reducing immunogenicity at its molecular source.

The two components of this dual bioorthogonal strategy complement and reinforce each other, cooperatively creating a functionally independent cellular module that operates without perturbing host physiology. This theoretically enables the production of "invisible" therapeutic proteins. For existing therapeutic proteins with immunogenicity concerns, this strategy offers an innovative, integrative solution with significant implications for both novel protein drug development and immunogenicity mitigation of marketed biologics.

In the future, gene-editing technologies will continue to advance and integrate deeply with artificial intelligence. The design of orthogonal systems in synthetic biology is expected to reach new milestones. On one hand, AI-driven predictive modeling will enable multidimensional optimization of immunogenic epitopes, protein folding energy landscapes, and metabolic pathway interactions, markedly enhancing the efficiency and performance of orthogonal component design. On the other hand, advances in CRISPR and related genome editing tools will facilitate efficient, precise integration of multigenic modifications and complex genetic circuits, enabling the construction of more sophisticated and robust orthogonal systems in diverse host platforms.

With continued technological advances, multidisciplinary integration will drive this strategy from a customized approach for individual proteins toward a versatile, broadly applicable platform technology adaptable to various therapeutic proteins. Ultimately, this paradigm will transcend immunogenicity mitigation alone, enabling on-demand customization of pharmacokinetic behavior, tissue targeting, and stability profiles. It will guide biopharmaceutical research from traditional "screen-and-discover" paradigms into a new era of "rational design", offering more precise and effective therapies for cancer, autoimmune disorders, and genetic diseases.

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