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Glycoengineering Can Reframe Therapeutic Protein Function Without Changing Its Primary Sequence

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ABSTRACT

Therapeutic proteins are commonly interpreted through primary sequence, higher-order structure, and dose, yet glycosylation can change function while leaving the encoded amino-acid sequence intact. This perspective argues that glycan state should be treated as a configurable functional layer whose meaning is conditional on biosynthetic context, molecular topology, receptor mechanism, product heterogeneity, and manufacturing history. Evidence from therapeutic antibodies, Fc-containing proteins, and other glycoprotein platforms shows that glycan composition, positional isomerism, pairing, and terminal processing can alter Fc-receptor engagement, complement activity, circulatory persistence, and immune-cell handling. The same literature also exposes an important limit: analytically detectable glycan differences do not automatically propagate into clinically meaningful differences, and engineering success cannot be inferred from a desired glycan shift alone. Cellular engineering, process manipulation, model-based control, and predictive proxies can modify or estimate glycan quality within defined domains, but transfer across products, scales, sites, and clinical contexts requires separate validation. We therefore develop a mechanistic perspective in which glycoengineering is interpreted as controlled movement through linked biosynthetic, product-quality, molecular-interaction, biological-response, and translational boundaries. The central claim is not that one glycoform is universally superior, but that primary-sequence-identical proteins can occupy functionally different states whose relevance depends on where and how the glycan difference propagates.

Keywords: Glycoengineering, Therapeutic proteins, Fc glycosylation, CHO cells, Critical quality attributes, Bioprocess control

INTRODUCTION

For recombinant proteins, primary sequence defines the encoded scaffold but not the complete functional state delivered to a patient. Glycosylation can modify stability, recognition, trafficking, efficacy-related behavior, and safety-relevant interactions across protein, gene, and cell-therapy platforms without changing the underlying amino-acid sequence [1]. This creates a pharmaceutical-science problem: two molecules that are sequence-identical can nevertheless differ in receptor engagement, persistence, immune handling, or manufacturability because their glycans occupy different structural and compositional states.

Modern glycoengineering makes that problem actionable. Mammalian host engineering, chemoenzymatic remodeling, and alternative expression strategies can deliberately reshape antibody glycans to alter selected functions [2]. Yet technical ability to generate a glycoform is not equivalent to evidence that the glycoform is

optimal, robustly manufacturable, clinically superior, or transferable across antibody scaffolds. Upstream synthesis control and downstream separation can shift therapeutic-antibody glycan distributions, but control remains multifactorial and process dependent [3]. Heterogeneity therefore cannot be treated simply as manufacturing noise that disappears once a preferred glycan is specified.

This perspective develops a narrower argument than a general review of therapeutic glycosylation. It asks when a glycan change becomes functionally meaningful, where that meaning is generated, and where inference must stop. The proposed synthesis separates five linked but non-equivalent layers: biosynthetic generation, measured product glycan state, molecular interaction, biological consequence, and translational interpretation. Evidence-supported links are distinguished from associations, predictions, and context-limited preclinical observations. The objective is to reframe glycoengineering from “changing a glycan” to controlling a state whose pharmaceutical relevance depends on mechanism, product context, time, heterogeneity, and validation.

Glycans as functional information

The strongest reason to treat glycans as functional information is that identical protein backbones can support nonequivalent glycoforms. Structural and in vivo work with afucosylated IgG1 demonstrated that core-fucose state can alter glycoform recognition and FcγRIIIa-related interaction without changing the antibody sequence [4]. The relevant information is therefore not genetic information in the strict sense; it is a post-translational state that changes the interaction possibilities available to the encoded protein.

That state is also multidimensional. Quantitative analysis of IgG1 glycovariants showed that effector functions can reflect combinations of glycan features rather than a single isolated attribute [5]. A one-motif rule can therefore conceal covariance and interaction among fucosylation, galactosylation, sialylation, and other features. Even bulk composition can be incomplete. Analysis of glycan pairing in antibodies, bispecific formats, and Fc-fusions showed that two products with similar aggregate glycan abundance can differ in how glycans are paired across Fc sites, with pairing influenced by molecular format, backbone, cell line, and process [6].

These observations require an analytical boundary. Biosimilar characterization has become sufficiently sensitive to detect glycan differences that may or may not carry a clinical consequence [7]. Conversely, mechanistic evidence shows that N-glycosylation can affect protein stability, clearance, pharmacokinetics, and immune interactions [8]. The correct inference is neither that every difference matters nor that analytical differences are irrelevant. Instead, a measured glycan feature becomes functionally informative only when its connection to a molecular or biological mechanism is supported for the relevant product and context. In this article, that distinction becomes the first element of the proposed glycan-to-function logic: measurement describes state; mechanism assigns possible consequence; validation determines decision relevance.

Biosynthetic origins of glycan heterogeneity

Glycan heterogeneity is generated by a network, not by an isolated terminal-decoration step. CHOglycoNET reconstructed a comprehensive CHO glycosylation reaction network from 200 glycan datasets and showed that observed glycoprofiles are constrained by shared, sequential biosynthetic reactions while retaining cell- and product-dependent variation [9]. This network view matters because perturbing one precursor, enzyme, or regulatory node can redistribute several connected glycan species rather than move a single independent variable. The ordered character of glycosylation is also experimentally tractable. A gene-engineered human glycome atlas demonstrated that sequential glycosylation pathways can be dissected through controlled manipulation of glycosylation genes, revealing pathway requirements for specific glycan structures [10]. That system is not a direct substitute for therapeutic CHO production, but it establishes the logic of hierarchical biosynthetic dependence.

CHO engineering studies then show how this logic can be exploited without implying complete orthogonality. Engineering nucleotide-sugar synthesis pathways enabled selective and combined modulation of N-glycan galactosylation and fucosylation under defined production conditions [11]. Regulatory engineering with natural and artificial microRNAs provided another route to alter CHO glycosylation, including fucosylation, without editing the therapeutic protein sequence [12]. Together, these studies support controllability but not invariance. The relevant manufacturing unit is therefore a distribution generated by a host-specific biosynthetic network. A glycoengineering intervention should be interpreted as shifting that network into a new product-quality state, with the possibility of coupled changes, clone dependence, and process sensitivity.

Receptor binding and effector function

Functional consequences emerge when a glycan state changes a molecular interaction that is relevant to the therapeutic mechanism of action. Antibody engineering experiments show that protein context and glycan state can interact: the same broad glycan modification need not generate the same effector output on every scaffold [13]. This is a key boundary for a sequence-centered interpretation of function. Glycans can reframe the behavior of the sequence, but the sequence and higher-order Fc context still constrain how the glycan is expressed functionally.

Resolution below gross composition can also matter. In a defined afucosylated antiviral antibody, galactosylation on the $\alpha 6$ antenna increased Fc γ RIIIa binding whereas galactosylation on the $\alpha 3$ antenna did not produce the same effect [14]. Thus, “amount of galactose” can be an insufficient descriptor when positional isomerism changes receptor interaction. The claim should remain product specific; the study does not establish a universal $\alpha 6$ -galactose rule.

A different pathway illustrates why effector functions should remain analytically separate. In a complement-dependent IgG1 context, Fc galactosylation was linked to altered Fc organization, enhanced IgG hexamerization, and stronger complement activation, with the process relation supported by more than 800 historical CHO samples and orthogonal mechanistic assays [15]. That mechanism is directly relevant to complement-dependent cytotoxicity but should not be transferred automatically to antibodies whose desired pharmacology depends on other effector routes.

Even the language of an “optimal” glycan is therefore conditional. A defined structure–activity study found strong effector-function performance for an $\alpha 2,6$ -sialylated biantennary Fc glycan in a specific anti-SSEA4 antibody model [16]. This is evidence that a glycan state can be favorable relative to a defined antibody and desired endpoint, not evidence for a universal Fc optimum. Glycoengineering should consequently target mechanism-matched function rather than a context-free ideal glycoprofile.

Glycosylation and circulatory persistence

Circulatory persistence shows particularly clearly why molecular difference and clinical relevance must be separated. A retrospective analysis of licensed monoclonal-antibody biosimilar programs found that highly sensitive analytics could detect high-mannose differences, including differences that did not translate into an observable pharmacokinetic difference in the reviewed programs [17]. This does not make high mannose irrelevant. It shows that the existence of a difference and the sufficiency of that difference to alter human exposure are separate questions.

High-mannose glycans remain mechanistically and operationally important because they can influence monoclonal-antibody clearance and are sensitive to manufacturing conditions [18]. The magnitude and consequence of a shift depend on product context, baseline distribution, and the clearance mechanisms that dominate for that molecule. A universal high-mannose penalty or transferable acceptance threshold is therefore not supported.

The same principle extends beyond canonical monoclonal antibodies. In soluble ACE2-Fc decoy receptors, increasing sialylation of native glycans altered preclinical exposure [19]. However, the fully optimized constructs in that work also incorporated sequence engineering and additional glycosylation changes. The title-level claim of function without primary-sequence change is therefore justified only for comparisons that isolate native-glycan sialylation, not for the entire optimized construct. This distinction is central: glycosylation can reshape persistence, but attribution must track exactly which molecular variables changed.

Immunological consequences

Glycan state can also change how a therapeutic protein is recognized and processed by the immune system. Defined rituximab glycovariants altered dendritic-cell uptake, receptor-dependent handling, antigen processing, T-cell-related responses, and antibody-response readouts across human-cell assays and a mouse model [20]. These findings support a mechanistic route from glycan state to immune handling, but they do not demonstrate that a particular glycoengineering strategy prevents clinically meaningful antidrug-antibody formation in patients.

That boundary is important because immunogenicity can otherwise become an undifferentiated endpoint. N-glycans can influence immune interactions through several mechanisms [8], while broader therapeutic-platform evidence shows that the direction and consequence of glycosylation depend on the product, site, and biological context [1]. Sequence epitopes, aggregates, impurities, disease state, receptor expression, exposure history, and concomitant therapy may coexist with glycan-mediated effects. Glycoengineering therefore changes one layer of immune recognition rather than replacing the rest of the immunogenicity problem.

A second boundary lies between analytical comparability and immune consequence. Biosimilar glycan analysis can reveal fine structural differences, but analytical resolution does not itself establish clinical immune nonequivalence [7]. The proposed interpretation is temporal: a release-state glycan distribution becomes immunologically relevant only if it survives through successive states of receptor encounter, uptake or processing, adaptive priming, and—where relevant—repeat exposure.

The temporal distinction is useful because an engineered glycan can be stable as a product CQA yet conditional as an immune signal. An early uptake difference may not persist into an adaptive response, and a preclinical immune readout may not transfer quantitatively to human treatment. Comparing these transitions in prose alone obscures where evidence changes meaning; **Table 1** therefore separates the successive qualification states and their different validation requirements.

Table 1. Time-Dependent Immune Interpretation of Engineered Glycan States in Therapeutic Proteins

Temporal qualification state	Entry glycan condition	State-specific readout	Hidden biological state	Disturbance or persistence source	Propagation path	CQA meaning at that state	Delayed endpoint and validation need
Release-state exposure	Defined product glycan distribution and heterogeneity	Glycan composition, pairing, terminal features	Structural accessibility of the glycan on the therapeutic protein	Clone, process history, storage, or batch variation	Product state enters biological exposure	Confirms what was manufactured, not what the immune system will do with it	Validate stability of the relevant glycan state over the intended product and dosing interval
Early receptor encounter	Circulating glycoform reaches Fc- or lectin-bearing cells	Receptor binding, cellular association, or uptake	Receptor abundance, competition, and cell activation state	Host phenotype, disease context, and concurrent inflammatory signals	Glycan-dependent recognition modifies probability of cellular entry	CQA becomes functionally interpretable only if linked to a relevant recognition mechanism	Establish that the interaction occurs at clinically relevant exposure and cell context
Processing window	Internalized therapeutic protein enters antigen-processing pathways	Uptake route, intracellular processing, and presentation-related readouts	Endosomal routing and antigen-presenting-cell state	Cell type, maturation state, and co-stimulation	Recognition can alter processing without guaranteeing adaptive amplification	Product glycan state may influence immune handling while remaining one determinant among several	Demonstrate persistence of the signal beyond uptake and across relevant human immune models
Adaptive-response window	Processed antigen reaches T-cell and B-cell response pathways	T-cell-related or antibody-response readouts	Pre-existing repertoire, tolerance, and immunological history	Sequence epitopes, aggregates, impurities, and host susceptibility	Earlier glycan-dependent handling may or may not propagate into adaptive response	Glycan CQA has possible immunogenic relevance, not established clinical consequence	Require human-relevant evidence before inferring an antidrug-antibody effect
Repeat-exposure window — proposed synthesis	The same or shifted glycan distribution is encountered across dosing cycles	Longitudinal immune and exposure readouts	Immune memory, tolerance, disease evolution, and treatment co-medication	Batch drift, process change, or altered patient state	Product and host histories interact over time	The same release specification may have different biological meaning across treatment states	Reassess comparability and clinical relevance when product state or treatment context materially changes

The article's central problem is therefore not simply whether a glycan difference exists, but whether it crosses successive molecular and biological boundaries without being neutralized, redirected, or rendered clinically

irrelevant. **Figure 1** compresses this propagation logic so that evidence-supported molecular routes remain visually distinct from proposed transfer relationships and from the boundary between experimental signal and clinical interpretation.

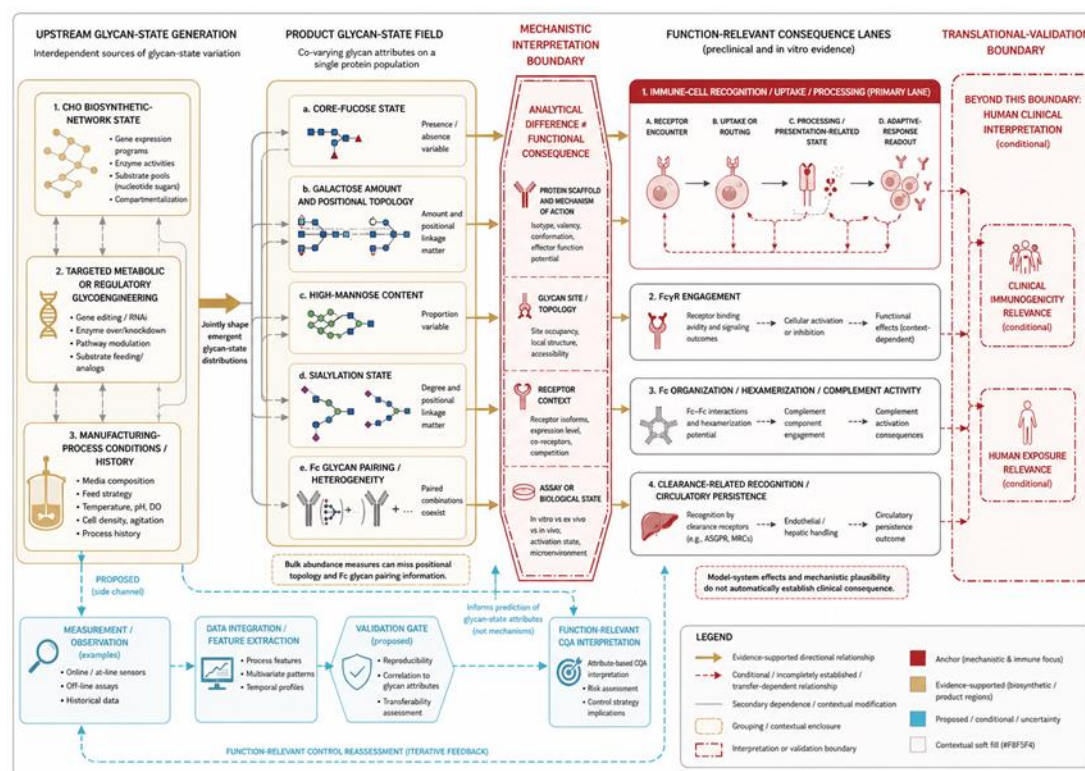


Figure 1. Glycan-State Propagation Across Product, Receptor, and Immune Interpretation Boundaries

Cellular and process-level glycoengineering

Once glycan function is treated as conditional rather than intrinsic, the manufacturing question changes. The objective is not merely to create a preferred motif but to maintain a function-relevant glycan distribution inside a validated cell-line and process domain. Model-based bioprocess literature shows that culture conditions, cellular metabolism, and glycosylation can be linked computationally to support process understanding and optimization [21]. Such models are useful within their domain, but prediction should not be described as mechanistic proof or assumed to transfer automatically across products, clones, scales, or facilities.

Artificial intelligence extends the predictive layer rather than erasing this boundary. Reviews of AI for glycosylation analysis emphasize both its capacity for pattern discovery and its vulnerability to sparse, interdependent data and limited interpretability [22]. A concrete CHO example used spent-media markers and machine learning to predict antibody glycan quality across defined anti-HER2 culture conditions, reporting strong within-study regression and classification performance [23]. Those results support bounded soft sensing; they do not establish a universal PAT surrogate or causal digital twin.

Glycoengineering also operates inside the wider manufacturing system. Mammalian recombinant-protein production couples cell-line properties, media, culture strategy, process development, and product characterization, so a glycan target cannot be optimized independently of the rest of the product-quality and productivity landscape [24]. The importance of process history becomes even clearer in continuous manufacturing. Residence-time-distribution work showed that a glycosylation perturbation can be spread and delayed as material moves through an integrated continuous monoclonal-antibody process [25]. A process may therefore satisfy a mean CQA target while still carrying time-dependent excursions that matter for diversion, control, or lot interpretation.

The engineering evidence supports deliberate movement of CHO glycan state through network, metabolic, and regulatory interventions [9, 11, 12]. What it does not support is a universal control hierarchy. Comparing these situations as topics would obscure the decision boundary between what is known and what can safely be done. **Table 2** therefore treats cellular and process control as a sequence of decision states, each permitting only the action justified by the information currently available and specifying what observation should force reassessment.

Table 2. Decision States for Converting Cellular and Process Glycan Control into Function-Relevant Product Quality

Decision state	Information available	Propagation path being controlled	Function-relevant CQA	Unresolved uncertainty	Permissible action	Reassessment trigger	Article-specific endpoint
Biosynthetic-network state — proposed classification	Host glycosylation network and product glycoprofile are characterized	Enzyme and precursor network to product glycan distribution	Product-specific glycan features linked to a plausible mechanism	Which network nodes dominate for this clone and protein	Select candidate engineering or process levers for experimental qualification	Unexpected coupled glycan shifts or non-glycan product effects	Move the product toward a mechanism-relevant state without assuming orthogonality
Engineered-cell state — proposed classification	Targeted metabolic or regulatory intervention shifts the glycan distribution	Cell intervention to secreted-product state	Intended motif distribution plus broader heterogeneity	Clone stability, cellular burden, and interaction with productivity or other CQAs	Advance only changes that preserve acceptable product and process behavior	Drift across passages, altered productivity, or newly emergent heterogeneity	Retain functional gain without converting it into an unstable cell-state phenotype
Calibrated process-model state — proposed classification	Mechanistic or data-driven model reproduces observations in the development domain	Culture variables and metabolism to predicted glycan state	Glycan CQA represented by validated model outputs	Extrapolation beyond training or calibration space	Use the model for bounded optimization, scenario testing, or control support	New scale, clone, media regime, or prediction residual outside qualification logic	Improve process decisions while keeping prediction separate from causal certainty
Soft-sensing state — proposed classification	Media or process markers predict glycan quality within a defined product and process	Sensor or proxy signal to estimated glycan CQA	Predicted glycan distribution or class	Proxy stability and external validity	Use as supplementary monitoring or development support after fit-for-purpose qualification	Marker–CQA relationship shifts with site, scale, product, or process change	Shorten information delay without treating a proxy as the glycan mechanism
Dynamic continuous-process state — proposed classification	Residence-time behavior and disturbance propagation are characterized	Upstream perturbation through integrated unit operations	Time-resolved glycan-quality excursion	Duration, dilution, and downstream mixing of the disturbance	Define monitoring, diversion, or investigation logic specific to the architecture	Change in residence-time behavior, flow regime, or unit-operation configuration	Prevent temporally distributed glycan excursions from being hidden by average-state reporting

Functional gain versus product heterogeneity

Glycoengineering becomes most difficult when a desired functional shift is interpreted as though it were an isolated molecular event. Glycan abundances are biosynthetically interdependent: a computational framework that accounts for shared biosynthetic steps showed why conventional glycomics can misread sparse, correlated glycan measurements as independent variables [26]. A change in one reported species can therefore reflect redistribution through a connected pathway rather than a single autonomous adjustment. Functional gain should consequently be evaluated against the altered glycoprofile that accompanies it.

Cell engineering makes that trade-off concrete. Multiplexed targeted integration of glycosyltransferase genes in CHO cells generated antibodies with diverse complex-type N-glycans, while some interventions also affected

production-related phenotypes [27]. Achieving a desired galactosylation, sialylation, or other glycan state is therefore not sufficient evidence that the engineered cell state is preferable. The relevant question is whether the resulting distribution preserves product quality, productivity, clone behavior, and process robustness while producing the intended mechanism-relevant function.

This perspective proposes a distinction between functional gain and glycan-state burden. Functional gain is the product-specific biological advantage supported for a defined mechanism; glycan-state burden is the additional heterogeneity, biosynthetic coupling, measurement complexity, or manufacturing sensitivity created while obtaining that advantage. The distinction is conceptual rather than validated as a quantitative decision rule. Multivariate IgG1 evidence indicates that several glycan features can jointly shape effector behavior [5], while Fc glycan-pairing work shows that bulk abundance can conceal structurally different states [6]. Thus, a larger engineered shift is not necessarily a cleaner or more interpretable one. A successful intervention is one whose function-relevant consequence can be separated from collateral glycan redistribution and maintained within an adequately characterized product and process domain [27].

The distinction also prevents a common analytical shortcut: equating reduced heterogeneity with improved function. A narrower distribution may simplify characterization yet remove glycoforms that contribute to the intended mechanism, whereas a broader distribution may be acceptable when the functionally sensitive region is understood and controlled. Conversely, an engineered mean can look favorable while hiding pairwise, positional, or low-abundance states that remain mechanistically relevant. The appropriate control target is therefore not minimal heterogeneity as such, but a product-specific distribution whose functionally important dimensions are identified and whose residual variation is bounded by evidence. That requirement becomes increasingly demanding when the same glycan feature participates in several receptor, clearance, or immune pathways, because improvement along one route can coexist with neutrality or disadvantage along another.

A glycan-to-function mechanistic map

The article's proposed glycan-to-function map is an evidence-boundary architecture rather than a universal pathway. Its first layer is biosynthetic generation: host-cell networks constrain which glycan states are reachable and which changes are coupled [9]. The second is measured product state, where abundance, topology, pairing, and heterogeneity must be represented at the resolution needed for the mechanism under study. The third is molecular interaction. Positional galactosylation can change FcγRIIIa binding in a defined antibody [14], whereas Fc galactosylation can alter hexamerization and complement activity in a complement-dependent context [15]. These are different mechanistic routes and should not be collapsed into a generic "effector function" arrow.

A fourth layer contains computational and process inference. Mechanistic and data-driven process models can support glycosylation optimization inside validated domains [21]. AI can identify predictive glycosylation patterns, but data sparsity, interdependence, and interpretability constrain what those predictions mean [22]. Likewise, media-marker machine learning predicted glycan quality within a defined anti-HER2 CHO dataset [23]. The proposed map therefore places prediction behind a validation boundary: predictive accuracy within a development domain does not by itself establish the biological mechanism represented downstream.

The final layer is translational interpretation. Human biosimilar experience shows that detectable high-mannose differences need not always propagate into measurable PK differences [17]. Preclinical ACE2-decoy data show that native-glycan sialylation can alter exposure while also illustrating how sequence changes confound broader construct-level attribution [19]. Immune studies demonstrate glycan-sensitive uptake and response-related readouts but do not establish clinical immunogenicity outcomes [20]. The map therefore ends not in a universal clinical endpoint but in a set of conditional exits. A glycan difference acquires decision relevance only when the evidence bridge appropriate to the product, mechanism, assay, exposure, and clinical question has been crossed.

Manufacturing and translational constraints

Manufacturing translation begins with the recognition that glycan control and glycan interpretation are separate tasks. Upstream and downstream strategies can shift antibody glycosylation, and high-resolution analytics can reveal architectures that bulk measurements miss [3]. This does not imply that every detectable feature requires active control. The analytical representation should be sufficiently detailed to capture the glycan attributes plausibly connected to the product's mechanism, without turning analytical resolution itself into a surrogate for clinical importance [6].

Robustness is equally conditional on process architecture. Mammalian production integrates cell-line properties, media, culture conditions, process development, and product characterization, so a glycan specification cannot be

interpreted independently of the manufacturing system that generates it [24]. Continuous processing adds explicit temporal structure: residence-time distributions can spread a glycosylation perturbation through integrated unit operations [25]. Scale-up, scale-out, site transfer, or a change in operating mode can therefore alter the relationship between an upstream disturbance, the measured glycan state, and the material eventually assigned to a batch or continuous product stream.

The same caution applies to model-enabled control. AI and soft sensors may shorten the interval between process change and glycan-quality inference, but prediction remains bounded by the data and process state in which it was developed [22]. The anti-HER2 media-marker model is therefore evidence for product- and process-specific predictive feasibility, not for universal deployment across biologics manufacturing [23]. More broadly, manufacturing control, glycan analytics, and human PK evidence together show why a detected or controllable difference must still pass a clinical-relevance test [3, 7, 17]. Regulatory interpretation cannot be inferred from controllability alone.

The proposed translational principle is therefore “mechanism-matched control”: control the glycan attributes that have a defensible path to the intended product function, characterize the surrounding heterogeneity needed to interpret that path, and validate transfer whenever the host, process, scale, product, assay, or clinical context changes. This principle does not define a regulatory standard or universal acceptance threshold. It is a way to prevent glycoengineering from outrunning the evidence that connects manufactured state to therapeutic consequence.

CONCLUSION

Glycoengineering expands the functional design space of therapeutic proteins because primary-sequence identity does not guarantee functional identity. Glycan state can alter receptor engagement, complement activity, persistence, immune handling, and product behavior, but those effects are conditional on molecular topology, scaffold, biosynthetic network, manufacturing history, and biological context. The central contribution of this perspective is a proposed evidence-boundary view in which glycan state moves through biosynthetic, analytical, mechanistic, biological, and translational layers rather than directly from manufacturing change to clinical consequence. This framing also exposes the limits of optimization: a desirable glycan shift can create coupled heterogeneity, analytical complexity, or process sensitivity, and a measurable difference may fail to produce a meaningful pharmacological effect. Glycoengineering is therefore most defensible when the engineered state is linked to a specific mechanism, its surrounding heterogeneity is characterized, and its interpretation is revalidated across relevant process and translational boundaries. The protein sequence remains unchanged, but the pharmaceutical state—and potentially the function it supports—does not.

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