Constrained global sensitivity analysis for bioprocess design space identification

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Abstract
The manufacture of protein-based therapeutics presents unique challenges due to limited control over the biotic phase. This typically gives rise to a wide range of protein structures of varying safety and \textit{in vivo} efficacy. Herein we propose a computational methodology, enabled by the application of constrained Global Sensitivity Analysis, for efficiently exploring the operating range of process inputs \textit{in silico} and identifying a design space that meets output constraints. The methodology was applied to an antibody-producing Chinese hamster ovary (CHO) cell culture system: we explored >8000 feeding strategies to identify a subset of manufacturing conditions that meet constraints on antibody titre and glycan distribution as an attribute of product quality. Our computational findings were then verified experimentally, confirming the applicability of this approach to a challenging production system. We envisage that this methodology can significantly expedite bioprocess development and increase operational flexibility.

\textbf{Keywords:} monoclonal antibodies; glycosylation; Chinese hamster ovary cells; design space identification; global sensitivity analysis; kinetic modelling

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\textbf{Abbreviations} CHO: Chinese hamster ovary; mAb: monoclonal antibody; QbD: quality by design; NSD: nucleotide sugar donor; GSA: global sensitivity analysis; LSA: local sensitivity analysis; cGSA: constrained global sensitivity analysis; MPC: model-predictive control; DS: design space; HDMR: High-Dimensional Model Representation, RBF: Radial Basis Functions; GI: galactosylation index; FS: feeding strategy
1. Introduction

The development of potent treatments for cancer and autoimmune disorders is shifting away from small molecules towards protein therapeutics, also known as biopharmaceuticals [1, 2], which are produced in living cells. Nearly 70% of these therapeutics are glycoproteins, i.e. they have sugar residues (oligosaccharides) attached to them, which affect their safety and efficacy [3]. Monoclonal antibodies (mAbs), which have been driving double-digit growth in this sector [2], belong to this glycoprotein product category. In a manufacturing context, a key challenge is how to control which sugars are added (glycoform) and ensure that all or most proteins produced have the same sugar structures on them (homogeneous glycoform profile). Current production methods yield a heterogeneous mix of glycoforms [4]. In addition, different glycoforms interact with the immune system in different ways therefore affecting the protein's efficacy as a pharmaceutical product [5-9]. It would therefore be of benefit to be able to produce certain glycoforms over others depending on the intended in vivo function of the protein therapeutic.

Controlling glycoform distribution requires an understanding of the intracellular process and the manufacturing process factors that affect it. The sugar residues are attached to the protein backbone in a series of enzyme-mediated trimming and addition reactions, collectively known as the glycosylation process, which starts in the endoplasmic reticulum and is completed in the Golgi apparatus. The rates of these enzymatic reactions depend on the concentration, activity and localization of individual enzymes, which are dependent on the culture temperature and pH [10-12].

The reaction rate also depends on the availability of the monosaccharides to be added to the growing oligosaccharide chain, which take part in the form of nucleotide sugar donors (NSDs). These are produced metabolically in the cytosol using key nutrients such as glucose and glutamine and are then transported into the Golgi apparatus using an antiport protein-mediated mechanism. The involvement of these metabolic products as co-substrates for the glycosylation process creates another avenue for the manufacturing conditions to influence the glycosylation process, through the depletion of nutrients extracellularly and subsequent reduced availability of NSDs intracellularly [13, 14]. A common strategy to overcome this problem is to supplement the culture medium or feed with the metabolic precursors, i.e. sugar monomers and nucleosides, of the NSDs of interest [12, 15]. Although this is easy to implement, it comes with certain trade-offs as cell growth on sugars other than glucose can be slower, while high extracellular concentrations of nucleosides can also reduce growth, leading to an overall reduction in product yield.
Two final determining factors are the specific rate of recombinant protein synthesis and specific growth rate of the cell population. The first determines the protein residence time in the Golgi apparatus while the latter determines the rate at which new cellular proteins, among them several endogenous glycoproteins, are being produced [16]. Metabolic demand for NSDs is therefore not limited to the recombinant product, but a significant proportion is channeled to cellular glycosylation, particularly at high cell growth rates.

The manufacturing challenge is that product quality is usually not factored into the early stages of process development. We therefore do not always have a quantitative understanding of how cell line and process-level decisions affect protein glycosylation early enough in the decision-making process. This is important because when optimising a production platform, we need to consider resource allocation: the trade-off of fast cell growth versus productivity also implies a competition for resources for host cell protein versus product synthesis and glycosylation. Mathematical modelling is one of the underpinning approaches for the implementation of the Quality-by-Design (QbD) framework adopted by the US Food and Drug Administration and European Medicines Agency [17]. QbD applications in cell culture processes for biopharmaceuticals production often involve statistical approaches [18, 19], which do not necessarily build fundamental product or process understanding. In contrast, mechanistic modelling can include details of the underpinning phenomena and, for this reason, has been shown to offer a strong basis for process design and optimisation [20].

In this study we present a cost-effective methodology to explore the bioprocess design space computationally and determine the range of inputs under which the desired glycoform profile can be produced without negatively affecting cell growth or mAb productivity. To do this we have used mathematical modelling to describe the interactions between the upstream production conditions and the mAb product glycoform. This forms the basis of a platform that is then used to explore in silico a large number of possible dynamic feeding strategies of NSD precursors and compute which of these strategies will yield products within acceptable limits of quality and quantity using a novel methodology for constrained Global Sensitivity Analysis (cGSA). It also identifies conditions that do not meet product quality requirements. A number of these strategies were tested experimentally and the validity of model predictions was confirmed. We envisage that this approach will speed up the development of new production processes by substituting some of the experimental effort with this rapid and low cost computational approach.

2. Experimental Materials and Methods

2.1 Cell line and maintenance
An IgG-producing Chinese hamster ovary CHO-T cell line (kindly provided by MedImmune, UK) was revived and cultured in CD-CHO medium (Invitrogen, UK) where 50 μM methionine sulfoximine (MSX, Sigma-Aldrich, Dorset, UK) was supplemented during the first and second passages only, and was shaken at 150 rpm in humidified 36.5°C incubator with 5% CO₂ supply. Cells were subcultured in fresh medium every three days at a seeding density of 3 x 10⁵ viable cells/mL. After 3 passages, experiments were set up in 500mL Erlenmeyer shake flasks at a seeding density of 2*10⁵ cells/mL. All cultures were supplemented with 1μM manganese(II) chloride solution (Sigma-Aldrich, Dorset, UK) and were fed with 10mL of Feed C (Invitrogen, UK) every other day starting from day 2. Feed C was supplemented with varying concentrations of galactose and uridine according to Table 1. Experiments were carried out in biological triplicates.

### 2.2 Analytical assays

Cell concentration and viability were determined with the *Viability and Cell Count* assay on NucleoCounter NC-250™ (ChemoMetec A/S, Allerod, Denmark) using DAPI and acridine orange (AO) staining. Antibody concentration of the supernatants was quantified using the BLItz system (Pall ForteBio Europe, Portsmouth, UK) and the Dip and Read™ Protein A (ProA) Biosensors (Pall ForteBio, Portsmouth, UK). The antibody was prepared for glycan analysis by trypsin digest. mAb samples were diluted to a concentration of 3 mg/mL in 100 mM Tris pH 7.6 and 8M Urea. DTT (Sigma-Aldrich, Dorset, UK) was added to a concentration of 10 mM. The protein was reduced and denatured for 10 minutes at 50°C. Mass spectrometry grade trypsin (Sigma-Aldrich, Dorset, UK) was reconstituted in 100 mM Tris pH 7.6. Trypsin was added to the denatured protein at a 1:20 trypsin to antibody ratio, the sample was then incubated at 37°C for 4 hours. Trypsin digested samples were analysed on a triple quadrupole mass spectrometer (Waters, Milford, Massachusetts, USA)

### 3. Computational Framework

#### 3.1 Description of mathematical model

We have adapted the recently published mathematical model of antibody-producing hybridoma cell culture [21] to an antibody producing system of CHO cells. The proposed model was trained to a set of five feeding experiments of galactose and uridine, including the control experiment. The maximum likelihood formulation in gPROMS was used for parameter estimation, with the objective function being the maximization of the probability that the model will successfully predict the experimental data.

The set of experiments used for model construction and parameter estimation is described in [22] and
is unrelated to the experiments used in the current work for DS identification. The results of the model presented herein therefore represent predictions. The original training dataset included feeding of $50 \text{m} \leq \text{Urd} \leq 200 \text{mM}$ and $100 \text{mM} \leq \text{Gal} \leq 200 \text{mM}$. Model calibration resulted in a concordance correlation coefficient (CCC) $\geq 0.95$ for the majority of the experimental data for viable cell density and antibody concentration, indicating a good overall fit.

The model links extracellular culture conditions, NSD metabolism in the cytosol and antibody production and glycosylation. Briefly, the cell growth model uses Monod kinetics to describe growth dependence on key metabolites. Intracellular NSD metabolism is described based on a reduced set of reactions and as a function of the extracellular environment as in [23]. The Golgi glycosylation model employed herein was developed specifically to describe the reactions relevant to N-linked glycosylation of the Fc region of monoclonal antibodies [24]. The model has been trained using data from a series of dynamic feeding experiments with uridine and galactose in the presence of manganese chloride based on the experimental design of Grainger and James [15]. The model equations are summarized in the supplementary file.

For the sake of simplifying notations in the following sections on model analysis methodology, we will refer to the model as a functional $f(\mathbf{x})$ with one or more outputs $f_s(\mathbf{x}), s = 1, \ldots, n_f$ dependent on the vector $\mathbf{x} = (x_1, \ldots, x_n) \in H^n = [0,1]^n$ of $n$ process design and/or operational parameters. Without loss of generality, we can assume that model inputs $x_i, i = 1, \ldots, n$ vary between 0 and 1 (which can be achieved through an appropriate shifting and scaling of real operational parameters varying between their respective lower and upper bounds). In the model of antibody production, the model outputs of interest are the mAb titre and the galactosylation index, defined in (1), while the inputs are feed concentrations of Gal and Urd at the end of days 4, 6, 8 and 10 of cell culture, so that the vector of model inputs is denoted as $\mathbf{x} = (\text{Gal}_4, \text{Gal}_6, \text{Gal}_8, \text{Gal}_10, \text{Urd}_4, \text{Urd}_6, \text{Urd}_8, \text{Urd}_{10})$.

The GI (mg·L$^{-1}$) was defined with respect to mAb concentration, to ensure product quality and quantity:

\begin{equation}
\text{GI} = (1 \times \% \text{ monogalactosylated glycoforms} \\
+ 2 \times \% \text{ digalactosylated glycoforms} ) \text{ [mAb]}
\end{equation}

The definition of GI in (1) is based on previous work by Siemiatkoski et al. [25] and Grainger and James [15] and later modified to account for mAb concentration by Jedrzejewski [26]. Although, NSD precursor feeding is expected to increase the percentage of the produced galactosylated glycoforms, it can limit cell growth and mAb production. Therefore, integrating mAb
concentration in the index calculation ensures that both the minimum quantity and the desired product quality are considered.

3.2 Constrained Global Sensitivity Analysis

As process models grow in complexity and dimensionality, the analysis of dependences of their outputs on inputs and parameters becomes increasingly complicated especially when taking into account associated aleatory uncertainty. GSA is a comprehensive framework that allows quantifying how variations in model inputs (including potential uncertainties in these quantities) affect the variation (uncertainty) of model outputs [27]. The difference between the global (GSA) and local sensitivity analyses (LSA) is that LSA quantifies the effect of small changes in model inputs in the vicinity of a given (“nominal”) point $x^*$ in the input space while GSA explores the whole input space by taking into account simultaneous changes in all model inputs and their (joint) distributions. Variance-based methods of GSA based on Sobol’ indices have been widely adopted owing to their ease of interpretation and efficiency in discriminating between influential and non-influential model inputs [28-33]. Using output values computed at $N$ (quasi)random $n$-dimensional sampling points $X = \{x^j, j = 1, ..., N\}$ (Sobol’s low discrepancy sequence was used in this work [34]), they generate sensitivity data in the form of main effect, $S_i$, and total effect indices, $S_{iT}$ for every input $x_i$, $i = 1, ..., n$. The former reflect the contributions of individual parameter variability into that of the output(s) while the latter also include joint effects by a given input and any other inputs. Total effect indices can be used to identify key parameters whose variation or uncertainty affects outputs to the largest extent as well as to fix nonessential inputs and thus reduce model dimensionality [27, 35].

We have recently proposed an extension of the GSA methodology to cover a wide range of problems involving inequality constraints imposed on model inputs [36, 37]. In presence of inequality constraints the sensitivity of model outputs $f_s(x), s = 1, ..., n_f$ to variability in the inputs $x$ has to be quantified within an implicitly defined non-rectangular multidimensional domain defined by $n_c$ constraints of the general form:

$$\Omega^n: \{x \in H^n: g_k(x) \geq 0, k = 1, ..., n_c\} \quad (2)$$

The latter introduce additional structural dependences between model inputs besides potential correlations between them. Thus, the variation of a model input near the boundary of the feasible domain may not be independent of changes in other inputs which is reflected in the resulting sensitivity indices.

In the present case of antibody production, inequality constraints correspond to the titre and quality requirements. The resulting set of acceptable input values constitutes the ‘design space’
(represented by the subset $X^+ = \{x^i \in X: g_k(x^i) \geq 0, k = 1, ..., n_c\} \subset X$ consisting of $N_{feas}$ ‘feasible’ sampling points) while any input combinations from outside the design space would fail to yield satisfactory output (we denote the subset of $N_{infeas}$ ‘infeasible’ sampling points in this region as $X^- = \{x^i \in X: g_k(x^i) < 0, k = 1, ..., n_c\} \subset X$, so that $X = X^- \cup X^+$). Consequently, any further optimisation of the model output in favour of certain process or productivity requirements must be performed within the design space $X^+$ in agreement with the QbD paradigm.

Multidimensional models like the one considered here (with eight inputs considered in the QbD approach) usually have few influential parameters (i.e., those with total sensitivity indices exceeding a problem-dependent threshold $S_{m,n}^T$ typically ranging between 0.05-0.1. Note that this threshold is arbitrary and is determined by the modeller based on the nature of the model and perceived importance of inputs. Changing this ‘importance’ threshold simply amounts to a different classification of the inputs and a different fraction of the total output variance that can be attributed to the variability of identified influential inputs) with the remaining ones being either non-independent (statistically or structurally) or non-discriminant (i.e., their variation does not result in tangible changes in model outputs) [27]. When this is the case, the model is amenable to substantial dimensionality reduction while the design space can also be represented using lower-dimensional projections as described in the following paragraphs 3.4 and 3.5. This is important both from regulatory and operational perspectives.

3.3 Setting the constraints for cGSA

mAb concentration was constrained based on the control experiment, where no galactose and uridine were fed. The feeding of galactose limits cell growth while uridine is inhibitory for both growth and mAb productivity. Therefore, a reasonable mAb concentration constraint was deemed to be up to 5% lower than the day 12 value of the control experiment, i.e. 430 mg·L$^{-1}$. Moreover, the GI threshold was set at 180 mg·L$^{-1}$ as the highest observed galactosylated fraction of the control experiment was 42%. This fraction and the constraint on mAb concentration were therefore used to calculate the respective GI constraint:

$$G_{I\text{constraint}} = \text{MAX}(\text{galactosylation}_{\text{control}}) \cdot m_{\text{Ab\text{constraint}}}$$

(3)

3.4 Design Space description

In line with the QbD framework, product quality characteristics should be determined prior to the design of the manufacturing process and its operational strategy. The corresponding set of inequality constraints explicitly defines the desired ranges of product characteristics. These
ranges should then be mapped onto the set of design and/or operational process parameters in order to define the process ‘design space’ \cite{38, 39}.

With the exception of a small number of models for which the inverse is identifiable analytically, this mapping can only be done through repetitive solution of the model for a large number of combinations of input values, although there are different approaches for doing this, which entail different computational demands. Regardless of the approach taken, model-based definition of the DS is a computationally intensive task. However, it generally allows exploring the space of model parameters at a lower overall cost compared with the Quality-by-Testing approach (involving extensive and expensive experimentation) and with high resolution and accuracy. It is also amenable to speedup through parallel computation. As a result, the feasible design/operational space can be described as a set of (non)linear inequalities involving only the model inputs (explicit constraints). Such a description, as opposed to simple range constraints (double-sided inequalities) on each of the model inputs, allows for a significantly larger feasible operational space, especially in higher dimensions. Indeed, the volume of the largest cuboid inscribed into the feasible space (corresponding to simple range constraints onto each of the inputs) decreases exponentially with increasing dimensionality compared with the volume of the actual DS $\Omega_n$ even if they are extremely close in shape.

The difficulty of describing the DS in high dimensions through sampling is exacerbated by the fact that, even with the use of space-filling sample points (such as the pseudo-random Sobol’ sequence \cite{34}), the distances between sampling points increase as $\sqrt{n}$ with increasing dimensionality $n$ for the same sample size $N$. Hence the effective sampling resolution decreases which makes it much more difficult to capture the features of the function (output) of interest. Sampled input values (which can be the same as those used for cGSA) and corresponding function output(s) can be used either (i) directly to construct a description of the DS, or (ii) indirectly, to approximate the model output(s) with a simpler, easier to compute function (or metamodel), which can then be substituted into model constraints to find a description of the DS. The accuracy of both approaches may depend strongly on sampling resolution.

Although the latter approach, utilising e.g. High-Dimensional Model Representation (HDMR), Radial Basis Functions (RBF), Kriging, etc. \cite{40-43}, may yield a reasonably good approximation of function output(s) there are a number of drawbacks associated with this approach. First, the resulting description of the DS is again implicit which makes locating the DS boundary, computing its dimensions and volume a complicated task to be achieved by some sort of sampling. Second, nonlinear function approximations (e.g. polynomials) are known to produce oscillatory features
as a result of overfitting, which is often the case in function sampling. As a result, important properties of the function of interest, particularly of constraints $g_k(x), k = 1, \ldots, n_c$ describing the DS, such as e.g. convexity, may not be preserved. In the latter case, misrepresenting a convex DS by a non-convex approximation may have negative implications on the existence of solutions of optimal model-predictive control (MPC) problems within the DS [44].

Direct use of sample points in the DS description implies the construction of a polyhedral (piecewise linear) approximation to its boundary in a certain way. If convexity of the set of feasible sampled points can be established, the DS itself can be assumed to be convex and a convex hull approximation [45, 46] can be used to describe the DS. This has a number of advantages including (i) the well-developed methodology for the construction of convex hulls, (ii) the fact that the DS is represented by a polytope whose dimensions and volume can be easily computed, and (iii) a simple representation of the DS using a simultaneous system of linear inequalities $Ax \leq b, A \in \mathbb{R}^{N_f \times n}, b \in \mathbb{R}^{N_f}$ describing the $N_f$ facets of the convex hull. On the other hand, if the set of feasible points is nonconvex, its boundary can still be described using a polyhedral approximation such as an alpha shape [47, 48] or, equivalently, a set of conjunctive-disjunctive linear inequality constraints [49]. The parameter $\alpha$ determines how ‘tight’ the alpha shape is as it changes from $N$ disjoint feasible points ($\alpha \to 0$) to the convex hull of those points ($\alpha \to \infty$). This is illustrated in Fig. 1 to emphasize the importance of the choice of $\alpha$. The shape of the resulting polyhedron can be relatively easily assessed visually in up to three dimensions, but may prove difficult to interpret in higher dimensions.

It should be noted that a DS description based on a set of linear or nonlinear inequalities (2) may lead to a DS with multiple disjoint components, a non-simply connected domain (i.e. one with one or more ‘holes’), an empty set or other degenerate situations (e.g. a lower-dimensional set embedded in the $n$-dimensional input space), if no conditions are imposed on functions $g_k(x), k = 1, \ldots, n_c$. Each of these situations presents significant challenges that would require the development of specific approaches.

In particular, since the constraint functions $g_k(x), k = 1, \ldots, n_c$ are not known explicitly, it is important to ascertain whether the feasible domain is simply connected. The use of low discrepancy sequences (e.g. Sobol’ [34]) for sampling the input space offers the advantage of uniformity of the sample which enables relatively straightforward identification of disjoint components within feasible sampling points $X^+$ using, e.g., basic complete-linkage clustering [50] with Euclidean distance and a threshold based on the ‘dispersion’ of the sequence $d_n(n) =$
\[
\sup_{x \in H^n} \min_{1 \leq j \leq N} \|x - x_j\|_2 \quad [51]
\]
(that can be estimated or computed for a particular sample \(X\)).

Polyhedral approximations can then be applied to each of the identified disjoint components. The discrimination between disjoint components depends on the resolution of the sample \(X\) measured by sequence discrepancy \(d_N(n)\): individual components can be distinguished only if distance between them significantly exceeds \(d_N(n)\). On the other hand, non-simple connectedness of the DS (i.e., presence of ‘holes’) is more difficult to establish since disconnectedness of the infeasible set \(H^n \setminus \Omega^n\) does not guarantee that \(\Omega^n\) is non-simply connected.

Thus, detection of non-simple connectedness in higher dimensions requires further investigation. However, approaches described here are valid for connected domains as is the case for the bioprocess considered here and can be readily extended to disjoint domains. Because of this and negative implications of non-convexity for MPC alluded to above, in this work, we will be considering only convex approximations to the DS.

An important drawback of polyhedral approximations described above (both convex and non-convex) is that they suffer from the ‘curse of dimensionality’ in the sense that the number of facets of a convex hull or an alpha shape may grow exponentially with the dimensionality of the model input space [45]. This may prove computationally prohibitive even for models with relatively few inputs (<10).

### 3.5 Use of cGSA for dimensionality reduction in DS description

In order to alleviate the potentially enormous computational burden and avoid unnecessarily voluminous descriptions of the multidimensional DS we propose to take advantage of cGSA and reduce the dimensionality of the DS description by considering the subspace of influential inputs whose total sensitivity indices exceed a given threshold (see Section 3.2 above):

\[
\hat{x} = \{x_{i_t} : S_{i_t}^T \geq S_{min}^T, t = 1, ..., n_{imp}\}
\]

where \(n_{imp} < n\) is the number of influential inputs. Inputs with total sensitivity indices below the chosen threshold \(S_{min}^T\) can be deemed as having a negligible effect on model outputs regardless of their values. Hence, their ranges of variation need not to be restricted as this would not bring about a better attribution of output variance to sources of uncertainty in the model. Fig. 2 shows a simplified illustration of the use of lower-dimensional projections to describe the DS. The feasible (blue) and infeasible (orange) spaces are separated with a plane in \(n = 3\) dimensions. Considering 2D projections, the \(yz\) projection does not allow any discrimination between the feasible and infeasible sets while the \(xy\) projection includes a considerable overlap between the
projected feasible and infeasible sets (light orange regions). On the other hand, the $xz$ projection affords perfect discrimination between the projections of the feasible and infeasible sets and its consideration is sufficient to construct a description of the DS in 3D. It is also evident, that input $y$ does not contribute to the description of the DS and the contribution to its total effect index $S_T^T$ due to structural dependences on other inputs through constraints is zero (although $S_T^T$ itself may in principle be significant depending on the effect of $y$ on model outputs through the functional $f(x)$ itself). Therefore, the subset of influential variables in this case is likely to consist of variables $x$ and $z$ in accordance with graphical observations.

A description of the DS can then be sought in the form of a convex hull $CH^{n_{imp}} = \text{conv}(\Pi_{n_{imp}}(X^+))$ of the subset of feasible points $X^+$ projected onto the set of influential dimensions $\hat{x}$ (where $\Pi_{n_{imp}}(\cdot)$ is a projection operator from $\mathbb{R}^n$ onto $\mathbb{R}^{n_{imp}}$). If no infeasible points fall within $CH^{n_{imp}}$ a convex approximation of the DS has been found. Alternatively, if the convex hull does contain infeasible points (i.e., if $CH^{n_{imp}} \cap X^- \neq \emptyset$), which can be a consequence of the real $n$-dimensional DS being non-convex or due to less significant model inputs being ignored, an inscribed convex polytope can be constructed by successively adding linear constraints and maximising the volume of the polytope $V_M$ while ensuring that all the infeasible points lie outside of it. Formally, a convex polytope $P(A_M, b_M)$ in the subspace of influential inputs (i.e., within a unit hypercube $H^{n_{imp}} = [0,1]^{n_{imp}}$) is defined by a system of $M$ linear inequalities $a_k \hat{x} \leq b_k$, $a_k \in \mathbb{R}^{n_{imp}}$, $k = 1, \ldots, M$, and those defining the ‘sides’ of the hypercube: $0 \leq \hat{x} \leq 1$. $A_M$ denotes the matrix consisting of coefficient vectors $a_k$, $k = 1, \ldots, M$ and $b_M$ is a vector of respective constant terms.

The following optimisation problem can then be formulated to maximise the volume $V_M$ of $P(A_M, b_M)$ by varying the coefficients of the $M$ constraints:

$$V_M := \max_{A_M, b_M} \text{Vol}(P(A_M, b_M))$$

s.t. $A_M \hat{x}_{r_{infeas}}^\text{feas} > b_M, r = 1, \ldots, N_{infeas}$

$a_k \hat{x} \leq b_k, \quad k = 1, \ldots, M$

$\|a_k\| = 1, \quad k = 1, \ldots, M$

$|b_k| \leq \sqrt{n_{imp}}, \quad k = 1, \ldots, M$

$0 \leq \hat{x} \leq 1$  \hspace{1cm} (5)

where $\hat{x}_{r_{infeas}}^\text{feas}$ are the projections of infeasible points onto the subspace $\hat{x}$.

By solving optimisation problem (5) for an increasing number of constraints $M$, a compromise
can be attained between the achieved volume $V_M$ of the polytope approximation and the number of linear constraints required for its construction. As convergence of $V_M$ towards the volume of $\Omega^n$ is monotonic in $M$ a stopping criterion for this iterative process can be formulated as

$$\frac{V_M - V_{M-1}}{V_{M-1}} \leq \varepsilon$$

where $\varepsilon$ is the minimum relative improvement of the DS volume approximation. Alternatively, if the volume $V$ of the convex hull of feasible points (if $CH^{\text{imp}}$ is determined to be convex, see above) or that of the $\alpha$-shape over $\Pi_{\text{imp}}(X^+)$ is known, $V_M$ can be considered to be a reasonable approximation of $V$ if $V_M/V \geq 0.8$. Iterations stop when either of these two criteria are satisfied.

The set of constraints in (5), guarantees that an inscribed convex polytope can be constructed without prior assumptions about the geometry of $\Omega^n$ as long as it is connected. However, if $\Omega^n$ is non-convex its convex polytope approximation may have a substantially lower volume so that $V_M/V \ll 0.8$. Also, as mentioned above, if $\Omega^n$ is disconnected the same approach can be applied to each of the disjoint components. However, this would result in a significantly higher number of linear constraints required to accurately describe the DS.

The incremental approach to the construction of a polytope approximation of the DS is also beneficial when the convex hull approximation to the DS involves a large number of linear constraints many of which may be redundant. For engineering purposes (e.g. for the implementation of MPC, regulatory compliance, bioprocess design), a more concise, convex description of the DS would be desirable.

4. Results and Discussion

4.1 Unconstrained and constrained GSA of model outputs

The model outputs, mAb and GI, were subjected to global sensitivity analysis first without any quality constraints and then with the following constraints, representing KPIs in the QbD framework, based on the previously introduced lower thresholds on mAb titre and galactosylation index:

$$mAb \geq 430 \text{ mg/L}$$

(6)

$$GI \geq 180 \text{ mg/L}$$

(7)

Possible feed concentration ranges for both $Urd$ and $Gal$ were chosen to be between zero and these upper limits $Urd_{\max} = 100$ mM and $Gal_{\max} = 500$ mM:

$$0 \leq Urd_i \leq Urd_{\max}$$

(8)

$$0 \leq Gal_i \leq Gal_{\max}$$

(9)

where index $i = 4, 6, 8, 10$ denotes the days cell culture is fed. The upper galactose concentration limit is based on the compound’s solubility in aqueous media, while the upper uridine limit is based on previous reports of growth inhibitory levels [15]. Uniform distributions within the
above limits were assumed for these inputs corresponding to their possible ranges of variation rather than distributions of aleatory uncertainty around some nominal values. Both GSA and cGSA analyses were performed using a sample of $8192 = 2^{13}$ points in the 8D input space with the help of SobolGSA software [52] and our recently developed approach to cGSA [36, 37], respectively.

The results for the unconstrained case (Fig. 3) demonstrate the effect on the variance of the model outputs (mAb titre and GI) of variability in the amounts of Gal and Urd fed to the cell culture on different days. It can be seen that, without imposing any thresholds on those outputs the most influential inputs are the Urd feed concentrations on days 4 and 6. This clearly confirms that the amount of Urd fed early on during the culture has the strongest effect on the final mAb titre and GI. Moreover, the main and total effect indices are practically identical which indicates the absence of ‘interactions’ between the inputs in determining the outputs in this case.

When quality constraints (6)-(7) are imposed, the results change significantly (Fig. 4). While $U_{rd4}$ is still an influential parameter, the importance of $U_{rd6}$ and $U_{rd8}$ drastically increases. The importance of $U_{rd10}$ remains low even under quality constraints since feeding towards the end of the culture is too late to affect its outcome.

4.2 Design Space Description

cGSA results help identify model inputs most affected by the introduction of output quality constraints. They identify inputs whose sensitivity indices change the most as a result of structural dependencies imposed by those constraints. Fig. 4 shows clearly that only three parameters (namely, the concentrations of Urd in the feed on days 4, 6 and 8) are tangibly affected by the output constraints (4)-(5) This allows for a drastic simplification of the analysis and the description of the DS because the dimensionality of this problem can be reduced significantly (from 8D to 3D) as shown below.

The sampling of the input space for (c)GSA revealed that out of 8192 sampled points 556 satisfied both constraints. Therefore, we can estimate the volume of the DS to be approximately $V_{DS} \approx 556/8192 = 0.068$. This is the value of the normalised volume, which corresponds to all inputs being normalised with respect to their ranges. This value can be used as a benchmark for comparing different approximations to the DS below.

In the constrained case, the analysis of the outputs of interest, mAb concentration and galactosylation index, revealed that only the mAb constraint is active, i.e. the GI constraint is less
stringent and is satisfied whenever mAb concentration is above its threshold value. Based on the cGSA results in Fig. 4, only three of the inputs are influential according to the values of their total effect indices. Therefore, according to the methodology described in paragraphs 3.4 and 3.5, we will consider a projection of the sampled data onto the three-dimensional subspace defined by $\mathbf{x} = (\bar{U}_{rd_4}, \bar{U}_{rd_6}, \bar{U}_{rd_8})/\bar{U}_{rd_{\max}}$. Note that the components of $\mathbf{x}$ are normalised to their maximum possible value to yield variables in the range $[0,1]$. The projection of both feasible and infeasible subsets of sample points onto this subspace is shown in Fig. 5(a), where it is immediately observable that the two subsets appear to be separable. Next to this plot, a projection of the same feasible and infeasible points onto a subspace of one influential and two uninfluential inputs is shown for comparison (Fig. 5(b)). It is clear that discrimination between the two subsets in this case is impossible owing to the fact that the variations in two uninfluential inputs ($Gal_4$ and $Gal_6$) do not result in distinguishable values of the outputs.

To describe the 3D projection of the DS encompassing the blue points in Fig. 5(a) we first attempt to build a simplicial convex hull around them. The resulting shape shown in Fig. 6(a) has 114 facets and therefore can be described using a set of 114 simultaneous linear inequalities. One thing to note, however, is that its volume is 0.075, which significantly exceeds the expected value of 0.068. This convex hull in fact contains 218 infeasible points of the original sample and is therefore not a useful approximation of the DS. It is worth noting that if a convex hull were to be constructed in the original 8-dimensional space, because of the 'curse of dimensionality' it would have $\sim 7 \times 10^5$ facets rendering this description computationally intractable for most practical situations.

Next, let us consider constructing an alpha shape on the feasible points. Choosing the highest value of parameter $\alpha$ ensuring that no infeasible points are contained within the alpha shape (thus affording it the highest volume of 0.055) yields a DS description shown in Fig. 6(b), which contains 486 facets. The shape of this domain suggests that the projection of the design space is not convex, and therefore the original 8-dimensional DS may also be non-convex. Note that, owing to the nature of this alpha shape, 486 linear inequalities are required to describe it. However, these inequalities form a conjunctive-disjunctive system of constraints, which may be difficult to construct and use in applications.

The last description of the DS was constructed using the incremental approach represented by the optimisation problem (5), in which constraints are sequentially added to improve the description of the DS. The resulting description consists of just one linear constraint (in addition to the obvious requirement of input non-negativity and upper bounds (6)-(7) on the inputs):
shown in Fig. 6(c). Interestingly, the volume of the resulting DS approximation is 0.58, which is higher than that of the alpha shape although the single constraint rejects 83 out of 556 feasible points located near the boundary. Nevertheless, with just a single linear constraint it is possible to describe 85% of the expected DS volume.

To put this finding into context, a typical approach to the DS definition would be to find the largest ‘box’ (i.e., a set of ranges for all of the inputs) ensuring the fulfilment of the quality constraints (4)-(5). Such a ‘box’ inscribed into the non-rectangular DS has the following dimensions:

\[
\begin{align*}
Urd_4 / Urd_{\text{max}} &\leq 0.107 \\
Urd_6 / Urd_{\text{max}} &\leq 0.238 \\
Urd_8 / Urd_{\text{max}} &\leq 0.536
\end{align*}
\]

and the volume of 0.014, which is less than one quarter of the volume afforded by a convex polytope description in the subspace of influential model inputs, although it does allow independent control of the input variables.

### 4.3 Comparison of model predictions with experimental data

For experimental validation, five feeding strategies from the 556 indicated to be inside the Design Space and two feeding strategies that fall outside the Design Space were chosen to be implemented in the laboratory (precursor concentrations presented in Table 1). Fig. 7 presents the predictions of mAb concentration that were obtained using the mathematical model and the respective experimental measurements on day 12 of cell culture. The data are plotted in ascending order with respect to the experimental mAb concentration values. The experimental measurements confirm the ability of the mathematical model to quantify mAb concentration (within the operating ranges explored) and propose a realistic Design Space. Although the average mAb concentration of the FS5 experiment is below the imposed constraint ($\geq 430$ mg·L$^{-1}$), the feeding strategy is not rejected from the DS when the standard deviation is taken into consideration.

Fig. 8 depicts the model predictions and experimental points for the GI. The data are plotted in ascending order with respect to the experimental GI values. With the exception of FS2 all the experimental measurements fall within the DS. All feeding strategies resulted in a higher value of GI compared to the control experiment, indicating that galactosylation was improved with feeding both in terms of percentage (Table 2) and in terms of cell-specific galactosylation.
capacity. Although FS2 resulted in a GI below the imposed constraint, the standard deviation of the experimental value indicates that the value is not significantly different from the accepted range. Hence, all the points suggested by the mathematical model to meet the GI constraint are successfully confirmed experimentally.

The selected strategies are illustrated in the 3D projection of the input parameter space along with the Design Space in Fig. 9. A summary of key performance indicators obtained experimentally is presented in Table 2. Surprisingly, the desired GI was met by all the experimental conditions, indicating that the GI was perhaps not sufficiently constrained. Experimental results indicate that FS6 and FS1 (control experiment) show the highest IVCD \((10^6 \text{cell·day·mL}^{-1})\) while the highest achieved mAb concentration \((\text{mg·L}^{-1})\) is observed in FS7. The discrepancies between model predictions and the experimental results for FS7 could be attributed to the inability of the glycosylation model to account for upregulation of the glycosylation enzymes – in this particular case potentially \(\beta-1, 4\)-galactosyltransferase – under different operational conditions. Moreover, the data set used for model training indicated that the addition of galactose and uridine has a monotonous negative impact on antibody concentration, an observation that contradicts with the currently presented experimental data. Therefore, the experimental data presented herein could be used for further model training and refinement. FS1 reasonably presents higher IVCD and mAb concentration compared to the majority of the other feeding strategies as the addition of galactose and uridine is expected to reduce cell growth and protein production.

The FS1 experiment shows limited degree of galactosylation, as evidenced by the low percentage of the G1F, G2 and G2F glycoforms (Table 2). In comparison, strategies FS4–FS7 show a considerable increase in the percentage of galactosylated structures as a result of the addition of galactose and uridine. Importantly, feeding also resulted in an increase in the specific production rate of galactosylated mAb, \(q_\text{mAb,gal} \ (\text{pg·cell}^{-1}·\text{day}^{-1})\), for the majority of the feeding strategies (Supplementary Material Figure S1).

5. Conclusions
Although there is an increasing number of publications using model-based methods to analyse and rationalise the behaviour of biological systems, their application in industrial environments is still limited. Despite the increasing emphasis on QbD implementation, most cases of industrial practice in biologics process development use experiment-heavy statistical techniques. There is an opportunity to combine models with limited experimentation for model development and
validation rather than for direct discovery of the design space and, thus, support system exploration, design space dimension reduction and design space identification in silico.

We have developed a novel method of constrained sensitivity analysis that can find application in design space identification ensuring product quality (or other) constraints are met with modest computational effort. This has been verified against an antibody production process with constraints on antibody concentration and glycosylation, the latter as a measure of quality. We believe that this method can present significant time and cost savings in process development and support operational flexibility in bioprocessing. A further development of the method of reduced-order representation of multidimensional design spaces would include quantification of uncertainty of respecting the constraints (especially their linear approximations) depending on probability distributions of operational and other model parameters.

Acknowledgments

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Figure 1. Polyhedral approximations of a set of points: (a) convex hull (or an alpha shape with $\alpha \to \infty$); (b) alpha shape ($\alpha=0.3$); (c) another, disjoint alpha shape ($\alpha=0.2$).

Figure 2. A graphical representation of DS description in lower-dimensional projections. See text for details.
Figure 3. Results of unconstrained GSA given the ranges of Urd and Gal feed concentrations in (6)-(7).
Figure 4. Results of Constrained GSA (cGSA) with the range constraints on feed concentrations imposed along with product quality constraints (4)-(5).

Figure 5. Projections of feasible (blue) and infeasible (red) sampled points onto lower-dimensional subspaces: (a) the subspace of influential inputs Urd_4, Urd_6 and Urd_8, and for comparison (b) a subspace of two uninfluential and one influential inputs (Gal_4, Gal_6 and Urd_6).
Figure 6. Four descriptions of the 3D projection of the Design Space: (a) convex hull, (b) alpha shape (α=0.15), and (c) a single linear constraint, and (d) a cuboid inscribed into the real DS (equivalent to imposing 'feasible ranges' onto each input). Blue dots in (c) represent feasible points that lie outside the approximate domain boundary.
Figure 7. Experimental measurements and model predictions of mAb concentration. The red line indicates the lowest accepted limit of mAb concentration (430 mg·L⁻¹).
Figure 8. Experimental measurements and model predictions of GI. The red line indicates the lowest accepted limit of the GI (180 mg·L⁻¹). Student’s t-test (Two Sample Unpaired t-test assuming Equal Variances) was used to evaluate the significant differences between the control experiment (FS1) and the feeding strategies and was represented by: p ≤ 0.05 (*) and p ≤ 0.01 (**).
Figure 9. Location of experimental feeding strategies relative to the Design Space: blue points are feasible while red points are infeasible. FS1 is the control experiment.
### Table 1. Concentrations of galactose and uridine used for culture feeding strategies. FS1 is the control experiment; FS2 and FS3 fall outside the DS; FS4-FS7 fall inside the DS.

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Galactose concentration in Feed C (mM)</th>
<th>Uridine concentration in Feed C (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 6</td>
</tr>
<tr>
<td>FS1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FS2</td>
<td>189.3</td>
<td>219.9</td>
</tr>
<tr>
<td>FS3</td>
<td>267.5</td>
<td>47.0</td>
</tr>
<tr>
<td>FS4</td>
<td>10.1</td>
<td>194.0</td>
</tr>
<tr>
<td>FS5</td>
<td>446.9</td>
<td>306.0</td>
</tr>
<tr>
<td>FS6</td>
<td>22.8</td>
<td>27.0</td>
</tr>
<tr>
<td>FS7</td>
<td>203.9</td>
<td>266.4</td>
</tr>
</tbody>
</table>

### Table 2. Experimental results for key performance indicators for each feeding strategy. IVCD: integral of viable cell density. FS1 is the control experiment; FS2 and FS3 fall outside the DS; FS4-FS7 fall inside the DS.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>FS1</th>
<th>FS2</th>
<th>FS3</th>
<th>FS4</th>
<th>FS5</th>
<th>FS6</th>
<th>FS7</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVCD ((10^6 \text{cell} \cdot \text{day} \cdot \text{mL}^{-1}))</td>
<td>42.7</td>
<td>31.0</td>
<td>36.5</td>
<td>41.0</td>
<td>36.5</td>
<td>43.3</td>
<td>40.1</td>
</tr>
<tr>
<td>[mAb] ((\text{mg} \cdot \text{L}^{-1}))</td>
<td>479.4</td>
<td>375.1</td>
<td>412.9</td>
<td>471.6</td>
<td>401.6</td>
<td>487.2</td>
<td>619.3</td>
</tr>
<tr>
<td>q_mAb ((\text{pg} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}))</td>
<td>11.2</td>
<td>12.1</td>
<td>11.3</td>
<td>11.5</td>
<td>11.0</td>
<td>11.3</td>
<td>15.4</td>
</tr>
<tr>
<td>G0 (%)</td>
<td>6.1</td>
<td>4.6</td>
<td>4.9</td>
<td>4.4</td>
<td>3.8</td>
<td>4.2</td>
<td>4.1</td>
</tr>
<tr>
<td>G0F (%)</td>
<td>53.7</td>
<td>53.8</td>
<td>52.3</td>
<td>44.0</td>
<td>40.9</td>
<td>43.5</td>
<td>41.9</td>
</tr>
<tr>
<td>G1F (%)</td>
<td>34.2</td>
<td>33.5</td>
<td>34.4</td>
<td>40.4</td>
<td>43.3</td>
<td>41.2</td>
<td>42.0</td>
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<tr>
<td>G2 (%)</td>
<td>0.4</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6</td>
<td>0.8</td>
<td>0.6</td>
<td>0.6</td>
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<tr>
<td>G2F (%)</td>
<td>4.8</td>
<td>4.5</td>
<td>4.6</td>
<td>6.9</td>
<td>7.8</td>
<td>7.0</td>
<td>7.7</td>
</tr>
<tr>
<td>Man5 (%)</td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
<td>0.9</td>
</tr>
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