MAXIMIZING NEOTISSUE GROWTH KINETICS IN A PERFUSION BIOREACTOR: AN IN SILICO STRATEGY USING MODEL REDUCTION AND BAYESIAN OPTIMIZATION

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Abstract

In regenerative medicine, computer models describing bioreactor processes can assist in designing optimal process conditions leading to robust and economically viable products. In this study, we started from a (3D) mechanistic model describing the growth of neotissue, comprised of cells and extracellular matrix, in a perfusion bioreactor set-up influenced by the scaffold geometry, flow-induced shear stress and a number of metabolic factors. Subsequently we applied model reduction by reformulating the problem from a set of partial differential equations into a set of ordinary differential equations. The quality of the reduction step was assessed by comparing the reduced model results to the mechanistic model results and to dedicated experimental results. The obtained homogenized model is 10^5 fold faster than the 3D version, allowing the application of rigorous optimization techniques. Bayesian optimization was applied to find the medium refreshment regime in terms of frequency and percentage of medium replaced, that would maximize neotissue growth kinetics during 21 days of culture. The simulation results indicated that maximum neotissue growth will occur for a high frequency and medium replacement percentage, a finding that is corroborated by reports in the literature. This study demonstrates an *in silico* strategy for bioprocess optimization paying particular attention to the reduction of the associated computational cost.

Introduction

Bone tissue engineering (TE) is a field combining expertise from medical and engineering sciences to create living implants to treat large or non-healing bone defects. One of the strategies often used to grow these living implants is to combine skeletal progenitor cells with 3D scaffolds. Specific *in vitro* culture strategies are used to induce cell proliferation or neotissue production inside the scaffold (neotissue being the combination of cells and the proteinaceous extracellular matrix they produce). In order to expose cells to the proper dynamic *in vitro* culture environment ensuring the supply of nutrients to and removal of metabolic waste from the system, (perfusion) bioreactors are necessary (Grayson et al. 2011; Papantoniou et al. 2013). In addition, bioreactors provide mechanical stimulation to the cells to help maintain their viability and stimulate biological activity (Papantoniou et al. 2014; Sonnaert et al. 2015b). Regular 3D porous scaffolds are often used in this context because of their ability to induce reproducible culture conditions that can control specific cell behavior such as proliferation and differentiation. Furthermore, scaffolds can provide fundamental patterns for subsequent cell growth and neotissue formation (Melchels et al. 2011). See figure 1A for a schematic representation of such a bioreactor set-up with 3D scaffold for cell culture.

Figure 1: (A) Schematic representation of the perfusion bioreactor and the 3D scaffold used in this study. The green volume inside the Gyroid scaffold (center) represents the neotissue. (B) Schematic representation of the workflow of this study.

Despite extensive research, translating TE products from bench to bedside remains a challenge (Srijaya et al. 2014; Steeves 2015). For example, the majority of the reported bioreactor studies rely on manual media changes at historically determined time points without evaluating the impact of this on cell and neotissue growth kinetics. This usually results in suboptimal bioprocesses for the formation of TE implants. Computational models could play a significant role in deciphering the complexity entailed in 3D dynamic biology such as the models describing neotissue growth within scaffolds (Guyot et al. 2015; Hossain et al. 2015b). Additionally, these models could enable bioreactor process optimization by defining optimum operating conditions (e.g. medium flow rate, nutrients concentration and medium refreshment rate). Guyot (2015) developed a mechanistic model for 3D neotissue growth in perfusion bioreactors, taking into account influences of geometry, flow-induced shear stress, oxygen, glucose, lactate and pH. One of the main limitations of this model is its computational cost, which makes the optimization problem intractable.

An optimization problem maximizes or minimizes an objective function subject to a defined domain of the input space. To mention just a few of them, Kabra et al. (2013) used a mixed integer linear programming optimization technique to address the production and scheduling of biopharmaceutical manufacturing process, and Liu et al. (2016) used the “chance constrained programming” approach, a technique that specifically deals with uncertainties in engineering applications, to optimize the bioreactor sizing, column sizing and to minimize the total cost of goods in the process of producing monoclonal antibodies. However, the field of tissue engineering and stem cell bioreactor culture is not yet in full development and only now starts to explore the possibilities of rigorous *in silico* optimization. There are different classes of optimization algorithms. One of these classes is evolutionary algorithms, e.g. genetic algorithms (GA), particle swarm optimization (PSO), simulated annealing (Civicioglu and Besdok 2013). Evolutionary algorithms browse the entire input space starting from a random initial population of model solutions that are evaluated using a cost function that encompasses the elements that need to be optimized (e.g. minimization of culture time or cost of culture). Subsequently specific evolutionary algorithms, often inspired by biological processes, select the best solutions and create a new population to be tested with the objective function. These algorithms require a large amount of function evaluations, making them intractable for most detailed mechanistic models (including the model of Guyot (2015)). In contrast, Bayesian optimization is a powerful technique designed to deal with expensive-to-evaluate objective functions as it moves toward the optimum solution with minimum number of function evaluations (Brochu et al. 2010; Jones et al.

1998; Snoek et al. 2012; Luna and Martínez 2014; Martínez et al. 2013). For that reason it is known to be highly applicable to biological systems (Ulmasov et al. 2016).

Due to the complexity of the biological systems (Bellouquid and Delitala 2006), mathematical modeling of such systems will usually result in complex models which are expensive to evaluate, as is indeed the case for (Guyot 2015), making them impractical to be used in real life applications such as the aforementioned optimization. Therefore, deriving a reduced order model with a substantial decrease in computational cost while maintaining the functionality of the full model behavior is a step forward toward the application of such model approaches in each domain. The reduced order model developed in this study enables us to perform detailed process optimization for neotissue growth, which is novel in the field of stem cell bioprocessing. Different techniques for model reduction exist, including variable lumping, timescale separation, sensitivity analysis and model reformulation (Eriksson and Tegnér 2016). Variable lumping is the process of reducing the dimensions of a system by merging variables with similar properties (e.g. protein concentrations with similar dynamic behavior). Timescale separation can be applied in cases where there is a clear distinction in the speed of the different processes encompassed in a single model with one process considered constant whilst the other is being studied. Model reformulation pertains to replacement of detailed description of specific processes in the model with a high-level qualitative description of the major features. (Eriksson et al. 2004; Halász et al. 2007). The strategic choice depends on the problem at hand.

In this study, for the first time we give an example of an *in silico* model used for rigorous optimization of a perfusion bioreactor culture (see schematic overview Figure 1B). We start by developing a reduced version of the previously published 3D model describing neotissue growth inside a perfusion bioreactor (Guyot 2015). All the details of the 3D model equations and their implementation are provided in the Supplementary Material. Given the nature of the mechanistic model, we applied model reduction using the model reformulation technique. Reduced model reparametrization is carried out using a genetic algorithm fitting procedure, followed by a global sensitivity analysis of the reparametrization parameters. Subsequently, Bayesian optimization was applied on the 3D and reduced models to find the optimal medium refreshment strategy. Corroboration of the model findings was performed by comparison with the 3D model results obtained as well as dedicated experiments (Fig. 1B). Finally, the ability of the model to describe neotissue growth for all triply periodic structures using a single set of parameters for all scaffold shapes is shown. To our knowledge, this study is the first to describe all the

aforementioned steps (reduction, reparametrisation, sensitivity analysis, optimization) on one and the same model of a TE bioreactor set-up, guided by dedicated experimental data. Hence, this study provides a blueprint for the incorporation of other mechanistic models into the TE research and development pipeline.

Methods

Model set-up

In the model developed by Guyot (2015), neotissue growth is described using the level-set method which is a technique to track a moving interface between two subdomains, applied here to the interface between neotissue and void space. The speed of the neotissue growth, represented by the advection of the interface, depends on local geometrical factors, flow-induced shear stress, obtained through a computational fluid dynamic analysis, and local concentrations of oxygen, glucose and pH. All of these variables are calculated through dedicated diffusion-advection-reaction equations. This coupled set of equations makes the model computationally very expensive (on average requiring 3 days on an Intel® Xeon® CPU @2.60GHz, 24 cores machine with 132 GB RAM) and makes rigorous neotissue production optimization an almost intractable problem. Details of the 3D model can be found in the supplementary material. In the current study, we have adapted this model to reduce the cost but maintain a substantial level of mechanistic detail by essentially removing all references to spatial dimensions and heterogeneity. The following sections describe the different steps of model reduction, reparametrization, sensitivity analysis and optimization. We will refer to the original model of Guyot by its reference ‘(Guyot 2015)’ or as ‘3D model’ and we will refer to the model developed in this study as the ‘reduced’ model.

Model reduction

Given the characteristics of the 3D model and the envisaged goals for the reduced model (optimization), model reformulation was deemed the best model reduction strategy. As the experimental medium read-outs provide only a single value for the model variables (oxygen, glucose, lactate, neotissue volume), spatial heterogeneity was not a crucial element in the optimization of the culture strategy. Therefore, during the model reduction step all references to spatial distribution of the neotissue and metabolites were removed and the set of partial

differential equations was reformulated into a set of ordinary differential equations. The resulting equations are described in detail below, together with the model reparametrization information and details on the sensitivity analyses.

Neotissue volume

The neotissue volume is a key parameter in this study. The neotissue volume produced inside the scaffold is a function of several geometrical, chemical and physical factors.

$$\frac{dV_n}{dt} = A f_s(\tau) f_c(\kappa) h_1(C_o) h_2(C_g) h_3(pH) \frac{V_n}{K_{V_n} + \lambda V_n} \quad (1)$$

Equation (1) expresses the neotissue volume (V_n) as a function of oxygen (C_o), glucose (C_g), pH level (pH), mean curvature (κ) of the neotissue-void interface inside the 3D scaffold and the shear stress (τ) caused by the medium flow that is perfused through the scaffold as described in Guyot (2015). The last factor on the right hand side of the Eq. (1) was added in the reduced model in order to limit the growth when the neotissue filling reaches the saturation level with parameters λ and K_{V_n} describing the saturation function. Parameter A is a general amplification factor. Additional details on these functions and parameters will be provided in the following sections.

As neither flow-induced shear stress nor mean curvature can be calculated in a spatially homogenized model, relationships between these variables and the neotissue volume filling ($\eta f\%$) were derived from the mechanistic heterogeneous model developed by Guyot et al. (2016). By plotting the shear stress behavior as a function of neotissue filling percentage (Fig. 2A) for the five triply periodic structures discussed above, a similar trend could be observed for all of them. The shear stress experienced by the cells decreases to a certain turning point (dependent on the scaffold filling percentage) after which it increases as the neotissue fills the remaining scaffold. Similar behavior has been found for the relationship between the curvature and the filling percentage (Fig. 2B) where the experienced curvature by the cells increases to a certain turning point reflecting the increase in curvature due to the gradual filling of the majority of the pores inside the scaffold. After around 70% of scaffold filling, most of these pores are filled completely with neotissue, and the average curvature value is determined by the less favorable parts of the geometry that do not fill up easily and keep a rather small value of local curvature throughout the entire simulation.

Figure 2: (A) Shear stress and (B) local curvature distribution for five different scaffold internal structures as a function of the neotissue filling percentage generated using the 3D model (Guyot 2015).

These relationships for the shear stress and curvature have been implemented in the reduced model using the following equations:

$$\tau = \begin{cases} s_1 V_n, & nf\% < T_1 \\ s_2 V_n, & nf\% \geq T_1 \end{cases} \quad (2)$$

$$\kappa = \begin{cases} c_1 V_n, & nf\% < T_2 \\ c_2 V_n, & nf\% \geq T_2 \end{cases} \quad (3)$$

Parameters T_i , s_i and c_i are determined through a fitting procedure using genetic algorithms, similar to the procedure used to determine the model parameter values as explained in the model implementation section below. T_1 and T_2 are the two turning points for the shear stress and curvature, respectively.

With these homogenized relationships between neotissue volume and average shear stress or curvature, the functions describing their influence on neotissue growth as described in Guyot (2015) can be maintained in the reduced model. For the shear stress influence, Chapman et al. (2014) introduce a growth model for cell aggregates in hollow fiber bioreactors where cell population growth varies depending on wall shear stresses experienced by cells. These findings have been incorporated in Guyot (2015) through Eq. (4) and transferred to the reduced model. This function enables taking into account an optimal shear stress (τ) range enhancing growth (between a_1 and a_2) as well as a detrimental value of τ ($\geq a_3$) that inhibits growth. The values of the parameters a_1 , a_2 and a_3 used in this study are shown in Table 2.

$$f_s(\tau) = \begin{cases} 0.5 + \frac{0.5\tau}{a_1}, & 0 \leq \tau < a_1 \\ 1, & a_1 \leq \tau < a_2 \\ \frac{\tau - a_2}{a - a_3}, & a_2 \leq \tau < a_3 \\ 0, & a_3 \leq \tau \end{cases} \quad (4)$$

The function describing the effect of curvature on growth is expressed using a linear function:

$$f_c(\kappa) = \begin{cases} \kappa, & \kappa > 0 \\ 0, & \kappa \leq 0 \end{cases} \quad (5)$$

The influence of oxygen and glucose concentrations on the produced neotissue in Eq. (1) is taken into account through the functions h_1 and h_2 where neotissue volume reduces when the species level decreases. The values of K_o and K_g used in this study are shown in Table 2.

$$h_1(C_o) = \frac{C_o}{K_o + C_o} \quad (6)$$

$$h_2(C_g) = \frac{C_g}{K_g + C_g} \quad (7)$$

Lactate production in the medium is in direct relation with the medium pH level and has a negative influence on the neotissue growth. In Wuertz et al. (2009) a detrimental effect of pH on cell fate was described using Eq. (8) where the neotissue growth rate decreases linearly when the medium pH level decreases.

$$h_3(pH) = \begin{cases} 1, & pH > 7.1 \\ \frac{4}{3}pH - 8.5, & 7.1 \leq pH < 6.375 \\ 0, & pH \leq 6.375 \end{cases} \quad (8)$$

Nutrients and waste transport

One of the advantages of a perfusion bioreactor set-up is the ability to continuously supply nutrients such as oxygen and glucose and remove metabolic waste such as lactate. The influence of these variables on neotissue growth is described in Eq. (9)-(11). In these equations, the right-hand side terms show the production or consumption of the species by the cells, modeled using Michaelis-Menten kinetics with ϕ_{cells} being the cell density within the neotissue, V_i the consumption rate and K_i the Michaelis-Menten constants for oxygen and glucose. Compared to the equations of the 3D model, the diffusion and advection terms were omitted and in the reaction term for each specie, a dependence on the neotissue volume (V_n) was introduced to compensate the effect of spatial distribution. In the reparametrization step following the reduction, parameters α , β , γ and λ were added to the equations to calibrate the model with the results of the experiments and the 3D model. These parameters

$(\alpha, \beta, \gamma, \lambda)$ have been determined using a genetic algorithm to minimize the differences between the 3D and reduced model results.

$$\frac{dC_o}{dt} = -\alpha V_n \phi_{cells} V_o \frac{C_o}{K_o + \gamma C_o} \quad (9)$$

$$\frac{dC_g}{dt} = -\beta V_n \phi_{cells} V_g \frac{C_g}{K_g + C_g} \quad (10)$$

$$\frac{dC_{la}}{dt} = 2 V_n \phi_{cells} V_g \frac{C_g}{K_g + C_g} \quad (11)$$

$$pH = 7.4 - 0.0406C_{la} \quad (12)$$

The simulation of medium refreshment, taking place in a sequential batch manner in the experiments, was simulated by reinitializing the model variables (C_g , C_{la}) to specific amounts at the specific time points. For complete medium refreshment, this means the aforementioned model variables were set to the values of the refreshed medium. For partial medium refreshment, a percentage weighed average was calculated with the current values of the variables and the medium values.

Model implementation

The reduced model was implemented in MATLAB (MathWorks®). For implementation details of the 3D model, we refer to Guyot (2015) and the supplementary material. Table 1 provides the overview of all parameters used in this study and their origin. The capacity of predicting the evolution of the neotissue growth using the reduced vs. the 3D model was tested on five scaffolds with triply periodic internal structures. Three different types of scaffolds were used, namely Gyroid, Dschwarz and Pschwarz, with two different values of internal periodicity for Gyroid and Dschwarz (G10/D10 having an average pore size of roughly 1000 μm and G7/D7 of 700 μm).

Table 1: Overview of all parameter values used in this study

Parameter	Value	Reference
A	$1.8e^{-17}$	This study (GA, G7, Table 2)
α	238	This study (GA, G7, Table 2)
β	$3.41 e^5$	This study (GA, G7, Table 2)

γ	0.4514	This study (GA, G7, Table 2)
λ	0.6892	This study (GA, G7, Table 2)
s_1	$-3e^{-5}$	This study
s_2	$1.5e^{-4}$	This study
c_1	20	This study
c_2	-250	This study
T_1	60	This study
T_2	70	This study
a_1	0.01	(Chapman et al. 2014)
a_2	0.03	(Chapman et al. 2014)
a_3	0.05	(Chapman et al. 2014)
V_o	$1.09e^{-17}$ mol/cell/s	(Lambrechts et al. 2014)
V_g	$9.5e^{-17}$ mol/cell/s	(Zhou et al. 2013)
K_o	$1.82e^{-3}$ mM	(Carlier et al. 2014)
K_g	0.3 mM	(Hossain et al. 2015a)
K_{Vn}	$1e^{-8}$	This study
ϕ_{cells}	$2.5e^{13}$ cells/m ³	(Guyot 2015)

Parameter fitting using a genetic algorithm

The numerical parameter values A , α , β , γ , λ used in Eq. (1) & (9) – (11) were obtained by performing a genetic algorithm (GA) fit implemented in MATLAB to find the best fit between the simulation results from the 3D and the reduced version of the model. The ability of the GA method to perform parameter estimation was demonstrated by Chatterjee et al. (1996). Based on natural selection and genetics (Malhotra et al. 2011), GA searches for the global maximum or minimum of a function without having any knowledge of the problem. Starting from multiple guess points, called individuals, forming the initial population, GA fits (evaluates) each individual directly to the objective function. Then it selects parents, by an appropriate method, and reproduces

through natural operators (crossover, mutation) evolving to a new and better fitting population. This procedure continues until the population has converged or a predefined maximum number of generations has reached.

In order to determine the best parameter set (A , α , β , γ , λ), we have defined the objective function for the GA as the minimum of the trapped area between the reduced model's neotissue volume evolution over time and that from the 3D model for every scaffold geometry. Table 2 shows the best parameter combination found using GA for each of the scaffold geometries.

Table 2: The best parameter values found using GA for every scaffold geometry.

	A	α	β	γ	λ
G7	1.8 e^{-17}	238	3.41e^5	0.4514	0.6892
G10	1.43 e^{-17}	262	3.65 e^5	0.5602	0.7503
D7	1.81 e^{-17}	283	3.55 e^5	0.4601	0.7270
D10	1.57 e^{-17}	242	2.86 e^5	0.5529	0.5286
P10	1.24 e^{-17}	289	2.92 e^5	0.4194	0.5346
Averaged	1.57 e^{-17}	262	3.28 e^5	0.4888	0.6459

Model sensitivity analysis

A sensitivity analysis (SA) of the parameters was performed using the Global Sensitivity Analysis Toolbox (GSAT) in MATLAB®. The method used in this toolbox for analyzing the sensitivity of the model to its parameters is a variance-based method (Sobol 2001), which quantifies the contribution of each input parameter, or combinations thereof, to the total variance of the output. Variance-based measures of sensitivity are very useful tools because they enable measuring sensitivity across the whole input space ,global sensitivity, and can deal with nonlinear responses (Aloisi et al. 2012). In the context of our study, the input parameters are A , α , β , γ and λ , and the output is the neotissue filling percentage of the scaffold at 21 days of culture. The input parameter space is defined by 20,000 samples where each parameter is varied between 50% to 150% of the value obtained in the GA procedure.

Model optimization

In the bioreactor experiments performed for studying the neotissue growth in the scaffold (Sonnaert et al. 2015b), the entire medium supply flowing through the perfusion bioreactor in a closed loop is refreshed every 3 days based on a long-standing protocol, which might be suboptimal. As both the refreshment frequency and the fraction of medium being refreshed strongly impact the neotissue produced, we performed an optimization procedure for finding the best refreshment strategy, yielding the maximum amount of neotissue in 21 days of culture. The computational cost of the reduced model developed in this study is very low (around 2 seconds to calculate), which is a 10^5 fold reduction in calculation time compared to the 3D model. Hence, the reduced model allows a rigorous optimization procedure to be executed without running into computational challenges. In this study however, a Bayesian optimization approach was followed allowing for a direct comparison of the results of the optimization process obtained with the reduced and the 3D model.

Bayesian optimization

Bayesian optimization is a powerful technique for finding the extrema of an objective function that is expensive to evaluate in terms of computational costs as this technique is one of the most efficient techniques in terms of the required number of function evaluations (Brochu et al. 2010; Jones et al. 1998; Kushner 1964; Snoek et al. 2012). This property is necessary if we want to compare the results of the optimization routines executed on the reduced model and on the 3D model. This strategy is applied mainly to problems where a closed-form expression of the objective function is not available, but observations of the function at sampled values can be obtained. This efficiency in Bayesian optimization comes from using prior knowledge about the problem (previously sampled data) to choose the optimal next point for sampling. In this technique, the famous Bayes' theorem is used to direct the sampling of the objective function. A direct result of Bayes' theorem is stated mathematically in Eq. (13): the posterior probability of a model M given evidence (observations) E is proportional to the likelihood of E given M , multiplied by the prior probability of M :

$$P(M|E) \propto P(E|M)P(M) \quad (13)$$

Bayesian optimization constructs a Gaussian process model of the system based on the previous function evaluations. A Gaussian process is defined as a collection of random variables, any finite subset of which are

jointly Gaussian distributed. The collection of random variables are the values of the function at different points.

We assume that the function values are jointly Gaussian distributed.

$$\begin{bmatrix} y_A \\ y_B \end{bmatrix} \approx N \left(\begin{bmatrix} \mu_A \\ \mu_B \end{bmatrix}, \begin{bmatrix} \Sigma_A & \Sigma_{AB} \\ \Sigma_{BA} & \Sigma_B \end{bmatrix} \right) \quad (14)$$

This means that, given an observation $y_A=a$, we can compute a conditional distribution for the function value $y_B = N(\mu_{B|A}, \Sigma_{B|A})$, where

$$\mu_{B|A} = \mu_B + \Sigma_{BA} \Sigma_A^{-1} (y_A - \mu_A) \quad (15)$$

$$\Sigma_{B|A} = \Sigma_B - \Sigma_{BA} \Sigma_A^{-1} \Sigma_{AB} \quad (16)$$

In order to determine the best next location to be sampled, we need to make a trade-off between exploration (we are not very certain about the objective function values) and exploitation (choosing values for sampling where the objective function is expected to be high for maximization, low/small for minimization). Bayesian optimization uses an acquisition function that is built based on the mean and variance of the previous samples to maintain exploration and exploitation. Common examples of acquisitions functions are the probability of improvement (Kushner 1964) and the expected improvement (Moćkus 1975). The next location for evaluating the objective function is chosen by maximizing the acquisition function.

In order to apply Bayesian optimization into our problem, we used the Pybo package, which is a modular approach to Bayesian optimization written in Python (Hoffman and Shahriari 2014). In our study, two parameters were varied, namely the time between medium refreshments (between 1 and 96 hours) and the fraction of the medium that should be refreshed at each refreshment step (between 0 and 100%).

Experimental set-up

Calibration and testing of the presented models was performed with the help of experimental data that was generated specifically for the purpose of model validation. The basic experimental set-up used is similar to that of Sonnaert et al. (2017) where cells are seeded on a titanium scaffold and cultured for 28 days in an in-house built perfusion bioreactor. A schematic representation is shown in Fig. 1A.

The cells used in this experiment are human Periosteum Derived Cells (hPDCs), chosen for their pluripotency and their bone forming capacity (De Bari et al. 2006). hPDCs were expanded in Dulbecco's modified Eagle's medium with high-glucose medium (Invitrogen) containing 10% fetal bovine serum (BioWhittaker), 1% sodium

pyruvate (Invitrogen) and 1% antibiotic-antimycotic (100 units/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL amphotericin B; Invitrogen). hPDCs were passaged 6 times at 80%-90% confluence prior to their use in this study. 3D additively manufactured open porous Ti6Al4V scaffolds with triply period inner structure (G10) has been produced using a non-commercial, in-house developed selective laser melting machine (Van Bael et al. 2012) and were used in this experiment. The outer dimensions of the cylindrical scaffolds were 6mm diameter and 6mm height. Prior to the start of the experiment, cells were trypsinized with Tryple Express (Invitrogen) and seeded on the scaffolds. A static drop-seeding protocol was used for seeding cells onto pre-wetted 3D Ti scaffolds. 0.2 million cells per scaffold were seeded and left to attach overnight ($t=24h$). For dynamic culturing, TE constructs were placed in an in-house developed perfusion bioreactor equipped with parallel perfusion circuits. Each perfusion chamber (length=26 mm, diameter=6mm), holding a single scaffold, was connected to an individual medium reservoir containing 10 ml of cell culture medium via a Tygon (Cole Parmer) tubing and a two stop tubing (BPT, Cole Parmer) connected to a peristaltic pump (IPC 24, Ismatec SA). Scaffolds were positioned in the perfusion chambers with a random orientation (top-bottom) with respect to the direction of drop seeding (Impens et al. 2010) and cultured for 28 days. Basic growth medium in the reservoir was fully refreshed (100%) every three days for the entire culture period, not taking into account the volume of medium sitting in the tubing and bioreactor chamber. Filling of the scaffold with neotissue was quantified by means of Contrast-Enhanced nanoComputed Tomography (CE-nCT) as described in Sannaert et al. (2015a).

Results and Discussion

Simulation results of reduced vs 3D model and comparison to experimental results

Fig. 3 shows the comparison of the results of the reduced model with those of the 3D model for the G7 structure for the concentrations of the model species oxygen, glucose, lactate and neotissue filling percentage over 21 days of culture by using the parameters of Table 2. The culture medium in this simulation is refreshed every 3 days by 100%. As shown in Fig. 3, the increasing neotissue volume filling percentage in the scaffold over time (Fig. 3D) results in a drop in the oxygen (Fig. 3A) and glucose (Fig. 3B) concentrations as the increasing amount of cells in the neotissue will continuously consume oxygen and glucose. Concomitantly, the concentration of lactate, a waste product (Fig. 3C) increases over time with increasing neotissue volume filling.

The results of the reduced model are quite similar to those of the 3D model for the different model variables as can be appreciated from Fig. 3, even though we have removed all references to spatial heterogeneity in the reduced version of the model.

Figure 3: Concentrations of different model species over 21 days of culture with medium refreshment every 3 days for the G7 scaffold (A) oxygen concentration, (B) glucose concentration, (C) lactate concentration and (D) Neotissue filling (%). Results obtained with the reduced model are shown in blue, and those obtained with the 3D model of Guyot (2015) in green.

The results from the GSA performed on the parameters added to the model during the reduction phase ($\alpha, \beta, \lambda, \gamma$) are shown in Table 3 (for G7 structure). They are expressed by the value S_{ij} where $0 \leq S_{ij} \leq 10 \leq S_{ij} \leq 1$ and $S_{ij} = 1$ means the combination of the i^{th} and j^{th} input plays a decisive role in the output. $i=j$ corresponds to a single parameter SA.

Table 3: Quantification of the contribution S_{ij} of the combinations of each input parameter to the total variance of the output for single parameter (diagonal elements) and global sensitivity analysis. The values in bold relate to the parameters the model is most sensitive to.

GSA	$S(\alpha)$	$S(\beta)$	$S(\lambda)$	$S(\gamma)$	$S(A)$
$S(\alpha)$	0.0015	0.0370	0.1243	0.0012	0.6533
$S(\beta)$	0.0370	0.0369	0.1686	0.0370	0.7155
$S(\lambda)$	0.1243	0.1686	0.1243	0.1242	0.9208

S (γ)	0.0012	0.0370	0.1242	0.0012	0.6533
S (A)	0.6533	0.7155	0.9208	0.6533	0.6533

For the single SA (diagonal cells), the model is most sensitive to the value of parameter A whereas for the global SA the results show that the fitting is most sensitive for any combination of parameter A and in particular with parameter λ (0.9208). As both parameters appear in Eq. (1) and therefore have a direct effect on the neotissue volume (V_n), this is a rather straightforward result. Yet it also implies that oxygen and glucose, the variables to which parameters β , λ and γ refer, have a smaller influence (within their tested range) on the overall model outcome. Furthermore, whereas the parameter A affects the neotissue volume in a direct linear way, parameter λ affects the neotissue growth in a non-linear way. The latter seems to imply that neotissue growth mainly depends on presence of neotissue – more so than the changes in levels of oxygen and glucose. The physical interpretation hereof is that the presence of cells and their ECM provides a necessary template for further cell proliferation and ECM production, limited by the available space in the scaffold. GSA could be used as a measure of robustness of the system (Kent et al. 2013), being the opposite of sensitivity. In this study, we have used GSA for determining the values of all the parameters (A , α , β , γ , λ), making sure those values were chosen such that the resulting model predictions were most robust.

Fig. 4 shows the comparison between experimental data and computational results for neotissue filling in a G7 structure over 28 days of culture. The 3D model was calibrated minimizing the RMSE between the experimental read-out and the model result for each measurement time point. The reduced model captures the same neotissue volume evolution leading to a filling of around 22% at day 28.

Figure 4: Percentage of predicted scaffold filling over time for the G10 scaffold. Continuous curve depicts the results of the reduced and 3D model (full overlap), dots depict the experimental results (average and standard deviation).

To date, bioreactor operating conditions have been, and are being determined based on trial and error (dos Santos et al. 2014; Egger et al. 2017; Eibes et al. 2010), which results in wasting time and consumables while there is a high probability of missing global optima in terms of operating conditions. There is therefore a clear need to optimize the process of cell expansion and neotissue growth to improve the efficiency of cell and tissue products (Panchalingam et al. 2015). One of the main reasons for developing computer models is to increase cost efficiency in terms of money and time by minimizing or even replacing wet-lab screening experiments with their *in silico* counterparts prior to the execution of now more targeted experiments. Additionally, these models can be incorporated into control loops for automated control of the bioreactor process. Both applications require the computer models to be calculated in a reasonable time frame to almost real-time for the control loop, a requirement that is met by the reduced model presented in this study.

Model optimization results

Bayesian optimization was applied to both the reduced and the 3D model, the results of which are shown in Fig. 5. Taking into account the computational cost of the 3D model and the observation that in almost all cases the calculated tissue amount at day 7 was an indication of the amount after 21 days of culture (supplementary material), in a first step, we ran the optimization routine for the day 7 time point (Fig. 5 A,B) before running it for the day 21 time point (Fig. 5 C,D).

Figure 5: Neotissue filling for different combinations of medium refreshment time and medium refreshment amount. A-B: results of the Bayesian optimization on the reduced (A) and 3D model (B) at 7 days. C-D: results of the Bayesian optimization on the reduced (C) and 3D model (D) at 21 days. The dots show the combinations of inputs that were used to calculate neotissue formation in the Bayesian optimization. The colors indicate the (extrapolated) scaffold filling in % with the red area corresponding to a higher degree of scaffold filling (color bars on the right are valid for both panels on the row). The black encircled areas represent the highest neotissue filling obtained with a higher flow rate, compensating for lower refreshment times or higher refreshment amounts. The yellow circle groups the data points that were manually added after the optimization procedure.

For the reduced model, optimized both at day 7 (Fig. 5A) and day 21 (Fig. 5C), higher neotissue filling (above 85%) is obtained with decreasing the medium refreshment time or increasing the amount of medium being refreshed. The region with optimal filling for the 21 days time point (see top left corner of Fig. 5C) is narrower than that of the day 7 time point as the longer the culture continues, the higher the glucose consumption and lactate production and thus the stronger the need for frequent and complete medium refreshment. Important to remember regarding Bayesian optimization is that it chooses the next point for function evaluation based on previous data points. This can be appreciated by the low amount of function evaluations in regions giving lower neotissue filling (blue area). This leads to an identification of the optimal settings with minimum computational effort. To further compare the behavior of the reduced model developed in this study and the 3D model by Guyot (2015), we also ran the Bayesian optimization on the 3D model using not only refreshment frequency and amount but also perfusion flow rate as a variable in the optimization. The area representing high neotissue filling (Fig. 5B, in red) is different from that obtained in the other simulations due to the undersampling of high-refreshment-

time / low-refreshment-amount area and due to the inclusion of flow rate given that flow-induced shear stress can have a positive impact on neotissue growth (each point in Fig. 5B has a different flow rate). The optimization results for the day 7 data showed that the flow rate leading to maximum neotissue filling is 1.83 ml/min and all the samples yielding high filling have flow rates between 1.5 to 2 ml/min. The yellow area in the middle of Fig. 5B corresponds to an input with lower flow rate (1 ml/min). Although the influence of flow rate is overall relatively limited, it does provide an opportunity to compensate for either low refreshment amount or refreshment frequency by an increased flow rate. This will result in identifying the bottom left zone and top right zone in Fig. 5B as quite optimized areas whereas these identified as such for the reduced model (Fig. 5A). The proposed evaluation points chosen by the optimization procedure are not covering the whole space (Fig. 5B), especially the top-left corner which we are interested in. Due to the high computational costs of the 3D model, we stopped the optimization process after around 30 evaluations, representing about 2 months of computation time. For comparison: the whole optimization procedure for the reduced model takes around 1 minute. After stopping the optimization process for the 3D model, we manually added additional samples in underexplored areas where we expected to find good results (yellow encircled area in top-left of Fig. 5B). Based on the results obtained for the day 7 time point as well as the reduced model, we manually selected 10 samples to run the model for a complete 21 days simulation (Fig. 5D). Consistent with the other results, maximum neotissue volume was obtained for decreasing the refreshment time and increasing the refreshment amount. Again, compensation of the effect of lower refreshment times or amounts by and increased flow rate explains the larger optimal area of Fig. 5D compared to Fig. 5C. The optimization results are consistent with experimental reports in literature. For example, in Chen et al. (2015), the authors compared different medium exchange regimes such as 50% medium exchange every two days, daily medium exchange and a full medium feed based on glucose consumption under different microcarrier concentration for expansion of MSCs. They showed that the medium exchange with higher frequency (daily medium exchange) yields a higher MSCs production. In addition, in Eibes et al. (2010), the authors used two different strategies for cell expansion in which the medium exchange with higher frequency (25% everyday) resulted in larger cell numbers compared to the other regime (25% every two days).

Increasing neotissue filling by increasing the refreshment frequency and the refreshment amount leads to a dramatic increase of the total cost of the culture as this optimal regime proposed by the optimization procedure (determined in red in Fig. 5) requires substantially more medium to be used during a given culture time. Future

objective functions for optimizing the bioreactor set-up should therefore also consider the economic cost of the culture. In a follow up study, all the associated economic costs related to refreshing the medium in the laboratory such as labor costs and medium consumption amount will be added to the problem, and the multi-objective optimization will be solved.

Homogenizing the spatial variables reduces the mechanistic character of the model and does not address a number of issues for which the spatial character is important. For instance, in Guyot (2015), an intelligent method was proposed for medium refreshment based on the local pH level in the bioreactor. The authors used a criterion stating that when the local pH dropped below a certain point for 20% of the neotissue volume, medium refreshment needs to be carried out. This led to a dynamic refreshment regime starting with once a week at the beginning of the culture and gradually increasing to more than once a day towards the end of the culture due to the increased neotissue volume and associated lactate production / glucose consumption.

To date, in the field of tissue engineering, most of *in vitro* bioreactor cultures settings are determined in a suboptimal manner based on trial and error whereas computational models could be used for determining the best refreshment strategy before conducting laboratory experiments. In this study, we have developed a computational model that could be used for finding the best refreshment regime leading to maximum neotissue growth in a perfusion bioreactor saving a great amount of time and money by replacing *in vitro* screening by an *in silico* model.

Using computational models and *in silico* optimization of the process prior to performing laboratory experiments is a well-established procedure by pharmaceutical companies for production of therapeutic drugs (Elliott and Yuan 2011; Ginai et al. 2013; Kirouac and Zandstra 2008). For example, Jolliffe and Gerogiorgis (2015), used process modeling for pharmaceutical manufacturing of ibuprofen, yielding in a low environmental factor (E-factor=25.4), indicating a green design in pharmaceutical manufacturing as many process configurations could be quickly evaluated and further optimized by the availability of a computational model of the process. Furthermore, Misener et al. (2014) have shown how applying rigorous numerical optimization on a model describing the production of red blood cells in a hollow fiber bioreactor can lead to a reduction of the use of expensive growth factors by an order of magnitude. Optimization tools could be used in cell expansion and downstream processing technologies, e.g. Hassan et al. (2015) developed a decisional tool in order to obtain the most cost effective method for allogenic cell therapy investigating the impact of several factors such as

commercial doses, demands and lot sizes. Therefore, optimization of the process of neotissue growth inside a perfusion bioreactor, as performed in this study, is a step forward for production of tissue engineered bone grafts.

Triply period scaffolds

The five different scaffold internal structures used in this study all belong to the family of triply periodic designs. The use of triply periodic scaffold design in the field of tissue engineering is on the rise as they enable control of the structural properties of the scaffold such as pore size and porosity, which are key factors in guiding cells to form neotissue inside the scaffold (Yoo 2012). There are a few requirements that are considered in designing 3D scaffolds in the field of tissue engineering. Optimal scaffolds have (i) high porosity and proper pore size, (ii) interconnected pores within the scaffolds, and (iii) high permeability, which facilitates the inflow of nutrients and removal of metabolic waste. The triple period family meets all of the aforementioned criteria. Another important property of triply periodic scaffolds is that the mean curvature of these structures is zero, which is similar to the mean curvature of the trabecular bone making them suitable to be used in bone tissue regeneration (Bobbert et al. 2017). These scaffolds are already being used in *in vivo* studies to facilitate bone formation (Van der Stok et al. 2013).

In order to define the value of the five model parameters (A , α , β , γ , λ) for each of the scaffold designs, GA was used with the aim to minimize the trapped area between the curves depicting neotissue growth for the reduced model and the 3D model, as described in the *Methods* section. The results of the fits for individual designs yielded a good correspondence between the results of the reduced and the 3D model (Fig. 3) and between the results from simulations and experiments (Fig. 4). Given that the values of the five model parameters are very similar, we investigated the possibility of defining a single set of parameter values for all structures in the triply periodic family.

Fig. 6A-C shows the comparison of the results between the reduced model and the 3D model for G7 using averaged values of the five aforementioned model parameters (Table 2, final row).

Figure 6: Concentrations of oxygen and glucose with neotissue volume over 21 days of culture for the G7 design with medium refreshment every 3 days. Results are shown for the reduced model using averaged parameter values (blue) and the 3D model (green). (A) Oxygen concentration, (B) glucose concentration and (C) neotissue volume. (D) The result of the Bayesian optimization using averaged values for the model parameters.

In comparison to the results obtained using specific parameter values for G7 (Fig. 3, neotissue volume reaching 81%), the neotissue volume reaches 75% filling at 21 days while in the 3D model, the scaffold fills up to 82%. The reason for this difference can be explained to a large extent by the lower value for parameter A , which has an important effect on the goodness of fit as shown in the sensitivity analysis section. Additionally, Bayesian optimization was performed on the reduced model with averaged parameter values (Fig. 6D), the results of which are very similar to those obtained with the specific parameter values derived for the G7 structure alone. The biggest difference between Fig. 5C and Fig. 6D is that the use of the averaged parameter values will result in a lower neotissue filling percentage in the optimal area (maximum value = 83%) in comparison to the filling obtained using specific parameter values (maximum value = 88%).

The aforementioned single set of parameters is valid for all triply periodic structures, but they can be tested in a follow up study to investigate the possibility of using them for regular non-triply periodic structures or heterogeneous structures.

Conclusion

In this work, we developed a computationally inexpensive model describing the neotissue growth in regular 3D scaffolds in a perfusion bioreactor, starting from a mechanistic 3D model. In this reduced model, all spatial heterogeneity was homogenized, and a fitting procedure was carried out to reparametrize the equations. Comparisons between the reduced model and the 3D model as well as between both models and experimental

data showed the reduced model's capacity to capture neotissue growth as simulated by the 3D model and as occurring in experiments. Subsequently, Bayesian optimization was carried out on the reduced and 3D model for the 7-day time point and the 21-day time point leading to similar results for optimal neotissue formation with a high refreshment frequency (less than 40 hours) and a high medium amount (more than 70 percent). In summary, this study proposes a model of neotissue growth in perfusion bioreactors systems that is specifically designed to allow for rigorous optimization of scaffold based stem cell culture through the definition of optimal bioreactor operating conditions.

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