Title:

Designing optimal calcium phosphate scaffold-cell combinations using an integrative model based approach

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1. Introduction

It has been shown repeatedly that calcium phosphate (CaP) scaffolds induce bone formation, but at present still result in a wide range of *in vivo* outcomes. Despite the enormous research efforts that were devoted to optimize (CaP) bone tissue engineering scaffolds, the answer still remains undiscovered [1]. Bohner *et al*. nicely summarize the main difficulties in defining the optimal scaffold architecture and also propose a new strategy to tackle this multidisciplinary problem: an integrative approach in which mathematical modeling is used to explain a mechanism of biomaterial-cell interactions, combined with experimental research to provide data for the determination of the model parameters as well as the validation of the model [1]. This process requires both a careful design and extensive characterization of the scaffold. Moreover, it is inherently an iterative process in which new experimental results can be fed to the model and thorough model analysis can lead to new research hypotheses.

This study applies the proposed approach to further elucidate the *in vivo* bone formation capacity of CaP biomaterials, more specifically the influence of calcium ions on osteogenic cells. Therefore, we combined semi-quantitative mathematical modeling with dedicated experimental work. The mathematical tool that was built according to the proposed strategy, allows us to design and test possible culture strategies *in silico* before they are tested *in vitro* or *in vivo*, thereby reducing the trial and error in experimental work. Moreover, it is a first step towards predicting the *in vivo* bone formation capacity of CaP-scaffolds based on specific biomaterial characteristics.

Calcium phosphate (CaP) based scaffolds have proven to stimulate bone formation, however, the underlying mechanism is still largely unknown [2-8]. One possible hypothesis that was put forward is that a high, local concentration of soluble factors such as growth factors can be achieved by adsorption on the biomaterial substrate, thereby creating a favorable microenvironment for bone formation [3,7]. Another mechanism through which calcium phosphate scaffolds can contribute to osteoinduction is the effect of surface topography on osteoblastic guidance and attachment and the asymmetrical division of mesenchymal stem cells (MSCs) [6,9]. The chemical precipitation of a bioapatite layer, which is recognized by MSCs, is another mechanism proposed in the literature [6,10].

The release of calcium (Ca^{2+}) and phosphate (P_i) ions by dissolution is, however, believed to be the main origin of the bioactivity of CaP biomaterials [4,6,9,10]. Experimental evidence clearly indicates the key role of Ca^{2+} and P_i in osteoinduction. Firstly, more ectopic and orthotopic bone formation has been observed in scaffolds made up of biphasic CaP than of hydroxyapatite, the latter having a lower dissolution rate, after implantation without cells in dogs [3]. Secondly, Hanawa *et al*. investigated the effect of Ca^{2+} implantation in titanium on orthotopic bone formation in rat tibia and a larger amount of new bone was found on the Ca^{2+}-treated side than on the untreated side [5]. Thirdly, differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs) towards osteoblasts is accompanied by the expression of Ca^{2+} binding-proteins and the incorporation of Ca^{2+} into the extracellular matrix [11]. Furthermore, osteoblasts sense and respond to the extracellular Ca^{2+} concentration independently of systemic calciotropic factors in a concentration-dependent manner, as was shown by Dvorak *et al*. in an *in vitro* model system [12]. Finally, the extracellular Ca^{2+} concentration could control the frequency of the

intracellular calcium spiking [13], which encodes specific cellular information [14]. Based on the above information we hypothesize that the primary condition for inducing ectopic bone formation is a critical level of free extracellular Ca^{2+}.

Mathematical models can be applied to unravel the role of CaP biomaterials in bone formation, being a very complex physiological process, and use this knowledge to develop clinically relevant cell carriers. However, current computational models of bone formation and regeneration in general (reviewed in [15]) or even in (CaP) scaffolds specifically [16-18], do not include this influence of the local Ca^{2+} concentration on cellular activities and MSC-driven bone formation. Additionally, models [19] describing calcium phosphate scaffold resorption *in vivo* exclusively look at geometrical scaffold properties and do not include biological variables such as cells or matrix densities. In this study a semi-quantitative mathematical model was developed, incorporating the results of dedicated *in vitro* experiments, to investigate the role of Ca^{2+} on *in vivo* ectopic bone formation in CaP scaffolds. Overall performance was corroborated by means of comparison with experimental results reported in the literature for normal [20] and impaired [2] cases of bone formation in ectopically implanted CaP scaffolds. Finally, the design of the CaP scaffold (in terms of Ca^{2+} release rate) was studied *in silico* by investigating the interaction between initial cell seeding density and the required calcium release rate in order to customize cell-carriers and maximize the predicted amount of bone formation.

2. Materials and Methods

In order to optimize CaP scaffold selection and make their use in combination with cells more clinically relevant, this study used an integrative model based approached to study the effect of Ca^{2+} on MSC-driven ectopic bone formation. Since Ca^{2+} appears to be the most important dissolution product [21], as discussed above, this semi-quantitative model neglects the effects of P_i. Fig. 1A shows a conceptual overview of the research presented in this study. The development of the mathematical model and the execution of dedicated experiments are discussed in more detail below.

2.1 Mathematical framework

The presented semi-quantitative calcium model is inspired by the bioregulatory model of Geris *et al*. [22]. This starting point was chosen since the model of Geris *et al*. [22] is a mathematical framework of bone formation that was successfully applied to the set-up of fracture healing [22], peri-implant bone healing [23] and bone formation in a rabbit bone chamber [24]. The calcium model consists of six delay differential equations and describes the effect of CaP biomaterials on the activity of osteogenic cells as a temporal variation of six variables: free extracellular Ca^{2+} concentration (Ca), MSC density (c_m), osteoblast density (c_b), mineral matrix density (b), collagen matrix density (m) and a generic, osteogenic growth factor concentration (g_b). The sum of the mineral matrix and the collagen matrix represents the total bone density. Fig. 1B shows a schematic overview of the model, its variables and their interactions.

A short definition of all the model parameters, as well as the parameter values, is given in table 1. Due to space limitation a thorough discussion of the parameter values can be found in the supplementary material.

2.1.1 Calcium

The dissolution kinetics of the CaP scaffold are modeled by a general empirical equation:

$$\frac{dc}{dt} = k.s.\left(c_{\infty} - c\right)^{n} \quad (1)$$

where *dc/dt* is the rate of dissolution, *k* is the rate constant for dissolution, *s* represents the specific area, c_{∞} stands for the maximal concentration at which artificial precipitation does not yet occur (the solubility limit), *c* for the calcium concentration in the extracellular space and *n* is the effective order of the reaction [25]. For the specific case that is modeled here, the effective order of the reaction is considered to be 1 due to lack of experimental data. The parameters *k* and *s* are combined in a single model parameter σ. During dissolution, both the parameter *s* and the parameter *k* will change since the geometry and the microstructure respectively of the CaP will alter as the dissolution proceeds. The time-dependency of the parameter σ was however neglected due to the lack of experimental data. Remark that *c(t)* represents the time-dependent calcium concentration due to the dissolution of the CaP biomaterial. *Ca(t)* represents the time-dependent calcium concentration due to the dissolution of the CaP biomaterial and the uptake of calcium by the cells.

Biological mineralization is a complex process resulting in the deposition of hydroxyapatite on the mature collagen matrix. There are two general approaches described in literature: (1) matrix vesicle-mediated mineral initiation and (2) heterogeneous nucleation of bone mineral crystals on collagen fibrils [26]. The formation of intracellular vesicles containing bioapatite is a metabolic process that requires the uptake of calcium [26]. The calcium flux from the extracellular space towards the cytosol is modeled as a flux, inspired by work from Maurya and Subramanian [27]:

$$J(t) = J_{in}.\frac{Ca(t)}{H_{Ca4} + Ca(t)}. \quad (2)$$

The calcium uptake for the metabolic activities of both osteoblasts and MSCs, and hence its removal from the extracellular space, is included in the mathematical model by a constant decay function (d_{Ca}).

In an *in vivo* situation there will be interstitial fluid flow, as well as blood flow in the scaffold. Moreover, calcium ions will diffuse in the microenvironment. Due to lack of experimental data, however, these additional sinks or sources of calcium ions were neglected.

2.1.2 Mesenchymal stem cells

The proliferation of MSCs is modeled by a logistic growth function where the proliferation rate depends on the surrounding matrix density (m) and Ca^{2+} concentration (Ca) [28-30]:

$$A_m(t) = \frac{A_{m0}.m(t)}{K_m^2 + m(t)^2}. \quad (3)$$

Specific experiments, described below, were designed and conducted to determine the effect of calcium on proliferation. The results of these dedicated experiments show that the proliferation depends on the calcium concentration in a Gaussian manner. A least-squares fit was done on the proliferation data to determine the parameter values of a_{cm}, b_{cm} and c_{cm} of the Gaussian function $\beta_{cm}(t)$ (see also section 3.2):

$$\beta_{cm}(t) = \frac{a_{cm}}{c_{cm}}.\exp\left(-\frac{1}{2}.\left(\frac{Ca(t) - b_{cm}}{c_{cm}}\right)^2\right). \quad (4)$$

The differentiation of MSCs towards osteoblasts is mediated by a generic, osteogenic growth factor. A minimal chemical concentration is included in the computational model by defining a

sixth order polynomial function. For high chemical concentrations a saturation effect was modeled to take place [22]. The calcium dependency of the differentiation follows a Gaussian distribution that was derived from experimental results described in the literature [31]. Since both growth factors and calcium are indispensable for the differentiation of MSCs to osteoblasts their respective influences are multiplied:

$$F_1(t) = \frac{Y_{11}.g_b(t)^6}{H_{11}^6 + g_b(t)^6}.F_{11}.\exp\left(-\frac{1}{2}.\left(Ca(t) - F_{12}\right)^2\right). \quad (5)$$

Upon production of mineral matrix by osteoblasts, the remaining MSCs gradually become entrapped. When a MSC is surrounded by bone matrix, it will differentiate or die (apoptosis), the latter being modeled by a decay term

$$d(t) = d_{cm}.b(t).c_m(t). \quad (6)$$

2.1.3 Osteoblasts

The proliferation of osteoblasts is modeled by a logistic growth function where the proliferation rate depends on the surrounding matrix density (*m*) and calcium concentration (*Ca*) [28-30]:

$$A_b(t) = \frac{A_{b0}.m(t)}{K_b^2 + m(t)^2}. \quad (7)$$

According to Maeno *et al*. [32] the calcium dependency of the proliferation follows a Gaussian distribution similar to the MSC but with a different optimal calcium concentration b_{cb}:

$$\beta_{cb}(t) = \frac{a_{cb}}{c_{cb}}.\exp\left(-\frac{1}{2}.\left(\frac{Ca(t) - b_{cb}}{c_{cb}}\right)^2\right). \quad (8)$$

A least-squares fit was done on the measured proliferation data to determine the parameter values of a_{cb}, b_{cb} and c_{cb} of the Gaussian function (see also section 3.2).

Upon production of mineral matrix, osteoblasts gradually become entrapped by the matrix they are producing. When an osteoblast is completely surrounded by bone matrix, it will either mature and become an osteocyte or die (apoptosis). In both cases, the osteoblast is removed from the active matrix producing population. Since both apoptosis and maturation of osteoblasts towards osteocytes are calcium dependent processes [11,32], this removal is modeled by a step function ($d_b.c_b(t)$). The mathematical model defines a threshold γ for the calcium concentration at which the value of the decay term d_b changes in a stepwise manner. Below the threshold γ, the parameter value of d_b is low, which models the minimal amount of osteoblast apoptosis and maturation.

2.1.4 Collagen matrix

The evolution of the collagen matrix density m (eqn. (13)) was modeled according to Geris *et al.* [22]. Collagen matrix production was assumed proportional to the cell density of the matrix producing cells (the osteoblasts) with a delay of three days (t_2). The production rate decreases as the surrounding collagen matrix density increases. The parameter value of the initial production rate P_{bs} was determined numerically. Yuan *et al*. [3] reported the initiation of ectopic bone formation at 20 days post implantation of biphasic calcium phosphate scaffolds in dogs, P_{bs} was determined such that the time frame of the modeled bone formation would match this experimentally observed time frame.

2.1.5 Mineral matrix

The temporal variation of the mineral matrix b (eqn. (14)) is modeled in a similar way as the collagen matrix. The mineral matrix production is assumed proportional to the cell density of the matrix producing cells (the osteoblasts). The production rate decreases as the surrounding mineral matrix density increases. A sixth order polynomial yielded the best fit of the experimental data [3] and was therefore used to model the production of mineral matrix.

The parameter value of the bone formation rate P_{bb} was determined using the same canine experimental data used to determine the collagen production rate P_{bs} as described above [3]. The amount of bone formation after 90 days is approximately 31% according to the experimental observations of Yuan *et al*. [3]. The parameter value of P_{bb} was chosen such that the modeled amount of bone formation would match this reported amount of bone formation.

The mineralization of the last fraction of (non-mineralized) collagen takes place after six months to several years [33,34]. This period is not encompassed in the timeframe of the mathematical model and is taken into account by introducing an offset δ to the maximal degree of mineralization that can be reached in the simulated period.

There are two conditions that need to be satisfied in order to have production of mineral matrix and they are incorporated in the model by conditional statements. Firstly, mineral matrix deposition occurs following the maturation of the collagen matrix during bone formation ($m > m_{thres}$) [9, 32]. Secondly, an equivalent amount of calcium deposited as mineral matrix should be taken up from the environment by the mineral matrix producing cells (the osteoblasts).

2.1.6 Growth factor

The generic osteogenic growth factors are produced by osteoblasts, up to a certain saturation concentration, after which the production rate levels off. The production rate (E_{gb}) is not limited by the matrix density:

$$E_{gb}(t) = \frac{G_{gb}.g_b(t)}{H_{gb} + g_b(t)}. \quad (9)$$

The removal of growth factors is modeled by a rate constant (d_{gb}) representing denaturation and irreversible binding to matrix proteins [35]. The removal of growth factors from the osteogenic environment due to endocytosis of the growth factor-receptor complexes or irreversible binding of the growth factor to the receptor is modeled by the following function:

$$R(t) = \left|c_m(t) - c_m(t-t_3)\right|.\frac{G_{con}.g_b(t)}{H_{con} + g_b(t)} \quad (10)$$

where $c_m(t)$ represents the MSC density at time t and $c_m(t\text{-}t_3)$ the MSC density at time $t\text{-}t_3$. The difference between these two densities indicates the amount of cells that differentiated or proliferated during that time interval and, consequently, the amount of growth factor that was removed from the osteogenic environment.

2.1.7 Mathematical model equations

The biological processes described in detail above, result in the following set of equations:

$$\frac{\partial c_m(t)}{\partial t} = \overbrace{A_m(t).c_m(t).\left(1-\alpha_m.c_m(t)\right).\beta_{cm}(t)}^{proliferation} - \overbrace{F_1(t).c_m(t-t_1)}^{differentiation} - \overbrace{d(t)}^{removal} \quad (11)$$

$$\frac{\partial c_b(t)}{\partial t} = \overbrace{A_b(t).c_b(t).\left(1-\alpha_b.c_b(t)\right).\beta_{cb}(t)}^{proliferation} + \overbrace{F_1(t).c_m(t-t_1)}^{differentiation} - \overbrace{d_b.c_b(t)}^{removal} \quad (12)$$

$$\frac{\partial m(t)}{\partial t} = \overbrace{P_{bs}.\left(1-\kappa_b.m(t)\right).c_b(t-t_2)}^{production} \quad (13)$$

$$\frac{\partial b(t)}{\partial t} = \overbrace{P_{bb}.\left(1-\delta-\kappa_{bb}.b(t)\right)^{6}.c_b(t)}^{production} \tag{14}$$

$$\frac{\partial g_b(t)}{\partial t} = \overbrace{E_{gb}(t).c_b(t)}^{production} - \overbrace{d_{gb}g_b(t)}^{decay} - \overbrace{R(t)}^{consumption} \tag{15}$$

$$\frac{\partial Ca(t)}{\partial t} = \overbrace{\sigma.\left(Ca_{\infty}-Ca(t)\right)}^{release} - \overbrace{J(t).c_b(t)}^{consumption\ (HA)} - \overbrace{d_{Ca}.Ca(t).\left(c_b(t)+c_m(t)\right)}^{consumption\ (metabolism)} \tag{16}$$

2.1.7 Scaling and non-dimensionalization

The following scaling factors were chosen for the non-dimensionalization of the model variables:

$$\tilde{t} = \frac{t}{T}, \quad c_m = \frac{c_m}{c_0}, \quad c_b = \frac{c_b}{c_0}, \quad m = \frac{m}{m_0}, \quad \tilde{b} = \frac{b}{m_0}, \quad g_b = \frac{g_b}{g_0}, \quad Ca = \frac{Ca}{Ca_0}$$

The time $T = 1\ day$ was considered to be a representative unit time for the process under study (similar to fracture healing models e.g. Geris *et al*. [22]). Representative concentrations for the collagen content ($m_0 = 0.1\ g/ml$) and growth factors ($g_0 = 100\ ng/ml$) are adopted from Geris *et al*. [22]. A typical value for the cell density ($c_0 = 10^6\ cells/ml$) is derived from Bailón-Plaza and van der Meulen [35]. The scaling factor for the calcium concentration was assumed to be equal to the extracellular calcium concentration ($Ca_0 = 1\ mM$). An overview of the model parameter values is given in table 1 and further discussed in the supplementary material.

2.1.8 Simulation details

The set of non-linear differential equations was implemented in Matlab (The MathWorks, Inc.) using its built-in solver for delay differential equations. To complete the implementation of the

aforementioned delay differential equations (eqn. 11-16), the necessary time delays and initial conditions were specified.

The proposed mathematical model does not include the whole lineage of osteoprogenitor cells. Only two cell types, the undifferentiated MSCs and fully differentiated osteoblasts are modeled. To minimize the error due to this simplification, a time delay is adopted. Malaval *et al*. [36] report eight to ten population doublings in cultures of osteoprogenitors before the appearance of differentiated osteoblasts. This results in a time delay (t_1) of 10 days. The mature osteoblasts start producing collagen after three days (t_2). The exact value of t_2 was estimated due to the lack of experimental data. However, the total delay in the model ($t_1 + t_2$) is similar to the results of Malaval *et al.*, who report the appearance of osteoblasts after 14 days [36]. Moreover, differentiation towards the osteogenic lineage is not a discrete event in time but progresses through different stages that finally lead to mineralization [37]. From this point of view it is difficult to accurately define the time delay between a MSC and a fully differentiated, active osteoblast so that an intermediate value was chosen.

To model the removal of growth factors, the difference between two cell densities in a small time interval of 0.0001 days is calculated (t_3).

At the start of the simulation there are MSCs ($c_{m,ini} = 10^6$ *cells/ml*) and growth factors ($g_{b,ini}$ = *1,50 µg/ml*) present. The Ca^{2+} concentration is assumed equal to the normal extracellular concentration ($Ca_{ini} = 1$ *mM*). Only a very small amount of collagen matrix ($m_{ini} = 0.001$ *g/ml*) is present at the beginning of the simulation. All other variables are assumed to be zero initially.

2.1.9 Sensitivity analysis

To determine the most influential factors of the mathematical model, a sensitivity analysis by 'Design Of Experiments' (DOE) was performed [38]. DOE (or experimental design) is a

statistical tool that allows to determine an efficient design for (in this case) a multi-parameter sensitivity analysis. It will generate an array of combinations of different parameter values within a pre-defined parameter space in order to assess the influence of these parameters ('factors') (and their interactions) on the model behavior. All parameters included in the mathematical model were examined, except α_m, α_b κ_b and κ_{bb} since their ranges would not have any physiological meaning. The initial conditions were also included in the investigated set of parameters (see table 3). The chosen parameter space for each of the investigated model parameters was based on a literature survey (see table 1 and supplementary material).

In order to assess the results obtained from the parametric study, criteria that characterize the outcome of the system were determined. In this study the amount of bone formation at different time points (7, 21 and 42 days post implantation) was used to characterize the outcome of the system.

The large number of factors and potential interactions between the factors required a large number of runs in the design array. In the first stage, a uniform and a latin hypercube design (LHD), each with 80 runs, were used to determine the most influential factors. In the second stage the same designs (uniform and LHD) but with 150 runs were used to further investigate a smaller subset of important factors. The arrays were generated and analyzed with the statistical analysis software JMP (8.0.1 SAS Institute Inc.). The data of the two designs were analyzed in each stage using a screening analysis and a stepwise regression. The screening analysis estimates the importance of each factor and ranks them accordingly ($p < 0.1$). The stepwise regression was conducted in mixed mode (forward and backward steps) on the main effects only. In each step, a factor with a sufficiently high ($p < 0.25$) or low ($p > 0.25$) significance probability can respectively enter or be removed from the regression model. The sum of squares was considered

as a measure of importance for each factor. For additional information on the theory of DOE we refer the reader to [38, 39].

2.2 Experiments

2.2.1 Preparation of Ca^{2+} supplemented growth media

100x Ca^{2+} stock solutions of 200, 400, 600, 800 and 1000 mM were prepared by dissolving appropriate amounts of calcium chloride ($CaCl_2.6H_2O$, Applichem) in Hepes Buffer Solution (HBS: 25 mM Hepes (sigma) + 140 mM NaCl + 390 ml demineralized water) [40] and sterilized by a syringe filter (0.2 μm). The Ca^{2+} supplemented growth media were prepared by diluting stock solution to 1% (v/v) in growth media (dulbecco's modified eagle medium (DMEM + GlutaMax^{TM}-1, Gibco) + 10% FBS (FBS, Gibco) + 1% antibiotics + 1% sodium pyruvate) yielding final concentrations of 2, 4, 6, 8 and 10 mM. Fluctuations in pH of the growth medium were not observed following Ca^{2+} addition. For nomenclature purpose, Ca^{2+} is denoted as "C", followed by a numerical number indicating the final concentration in millimolar (mM), e.g. C2 means that the media contained a final concentration of 2 mM.

2.2.3 Isolation of the human periosteum-derived cells (hPDCs)

Human periosteum derived cells (hPDCs) were isolated from periosteal biopsies of different donors as described by Eyckmans *et al.* [2]. In short, the periosteal biopsies were harvested from the medial side of the proximal tibia of male and female patients during total knee replacement surgery or distraction osteogenesis. A periosteal lifter was used to strip the periosteum from the tibia. The biopsies were finely minced and digested. The periosteal cells were collected by centrifugation.

2.2.3 Cell culture and growth of human periosteum-derived cells (hPDCs)

Cells were expanded in monolayer using growth medium. Upon confluence, hPDCs were trypsin released and replated with a seeding density of 4500 cells/cm^2. After plating the cells were cultured for 2 days and synchronized for 24 hours in serum free medium (DMEM + 0.01% bovine serum albumin (BSA, BD Biosciences)). Freshly prepared Ca^{2+} supplemented growth media was then added to the cell cultures and incubated for 1, 3, 7, 14, 21 and 28 days (n=4) before harvested for analysis. At each time point, the cell cultures were harvested and lysed in 0.05% Triton-X100 (in PBS). The DNA content was quantified by using Quanti-iT^{TM} dsDNA HS Assay Kit (Invitrogen). Cells treated with growth medium were used as negative control (GM), growth medium containing 1% (v/v) HBS as vehicle control (Vh) and osteogenic medium (growth medium supplemented with 100 nM dexamethasone, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate) as positive control (OM). The culture medium was refreshed twice a week.

2.2.4 Statistical analysis

Data are expressed as mean ± standard deviation (S.D.). An unpaired two-sided student-t test was performed to evaluate the statistical difference between two sample means with unequal variance, statistical significance was established at $p < 0.05$.

3. Results

3.1 Enhancement of hPDCs growth by Ca^{2+} supplemented growth media in a time- and dose-dependent manner

The growth of hPDCs after treatment was evaluated by comparing the total DNA content of each sample to that of all other culture treatments for each time point (Fig. 2). At day one significantly

higher DNA content was obtained for C2 with respect to C4 and C6 (the numerical number indicates the final Ca^{2+} concentration in mM). After three and seven days C2 and C4 caused a significant increase in DNA content with respect to C6, C8, C10 and GM, C8, C10 respectively. At day 14, C4-C6 produced significantly higher DNA content with respect to GM, C2, C8 and C10. Cultures treated with C8 and C10 induced the highest DNA content at day 21, statistically significant from all the other treatments. At day 28 C4-C6 caused a significant increase in DNA content with respect to all other culture conditions. In summary, low Ca^{2+} (i.e. C2-C4) induced significant cell growth at early time points ($\leq$ 7 days), whereas at later time points this effect shifted towards higher Ca^{2+} concentrations. The effect of vehicle control (Vh) treatment on cell growth was minimal as no significant increase in DNA content was observed for all time points except at day 21, indicating this growth effect is induced specifically by Ca^{2+} (results not shown).

3.2 Experiment-based derivation of proliferation parameters

The data measured at 7 days were assumed to be representative of MSC proliferation, whereas the data measured at 28 days were assumed to be representative of cells further down the osteoblastic lineage. Consequently, the proliferation is assumed to be time-independent, which is likely to be a simplification of the underlying biology.

The data were normalized with respect to the total DNA content measured at day 1. Since the 0 mM calcium concentration means that no exogenous calcium was added to the culture medium, which originally contained 1.8 mM of calcium, the normalized DNA content, measured at 0 mM supplemented calcium concentration was subtracted from the data to compensate for this effect. However, the proliferation is not zero at 0 mM. Therefore, the values of $\beta_{cb}(t)$ and $\beta_{cm}(t)$ are set

to zero in the simulation code when the calcium concentration decreases below a certain threshold (Ca < 0.001 mM). A least-square fitting through the experimental data determined the parameters a_{cm}, b_{cm}, c_{cm}, a_{cb}, b_{cb} and c_{cb} of the Gaussian distributions. Note that the absolute value of the proliferation term is not only determined by the value of $\beta_{cm}(t)$ or $\beta_{cb}(t)$ but also by the density of the extracellular matrix and the proliferation rates A_{m0} and A_{b0} yielding an overall higher proliferation for MSCs than for osteoblasts.

3.3 Simulation of normal and impaired bone formation in CaP scaffolds

The mathematical model predicts 31% bone formation at 90 days post implantation (Fig. 3). The calcium concentration initially increases, due to the dissolution of calcium phosphates. This increase, together with the presence of osteogenic growth factors triggers the differentiation of the MSCs towards osteoblasts. The remaining stem cells proliferate until they reach maximal cell density. As the total cell density (MSCs + osteoblasts) increases, the overall metabolic need for calcium rises and the calcium concentration decreases. The osteoblast density reaches a maximal value and then declines since the environment becomes less viable due to the decrease in calcium concentration. After three days the differentiated osteoblasts start to produce collagen matrix. This process continues and at a collagen matrix density of 85% (m_{thres}) it triggers the mineralization of the osteoid. Gradually the scaffold fills up with bone, and the MSCs that are trapped in the bone matrix differentiate or die.

At 90 days most of the variables seem to be in equilibrium except the mineralized matrix. Although it is hardly visible in Fig. 3, the variables are still slowly changing. To investigate possible steady states, the results of the normal bone formation were calculated up to 900 days.

After about 600 days a steady state was reached for all the variables except for the mineralized matrix ($m = 1$; $c_m = 0$; $g_b = 1.91$; $c_b = 0.62$; b = -; $Ca = 0.56$).

The mathematical model predicted the absence of bone formation ($\tilde{b} = 0$) in the case of scaffold decalcification (Fig. 4, solid line) and insufficient cell seeding (Fig. 4, dashed line). For low initial cell seeding, exponentially decreasing the calcium release rate (σ) of the CaP scaffold *in silico* created a more favorable environment for cell proliferation. Following this 'culture strategy', the model predicted bone formation to start at 40 days post implantation (solid line in Fig. 5). The amount of bone formation is predicted to be almost 22% at 90 days, whereas the same initial cell density without reduction of the calcium release rate would lead to absence of bone formation as shown in Fig. 4E (dashed line). The amount of bone formation can be further increased by injecting a bolus of osteogenic growth factors. Fig. 5 (dashed line) shows the effect of osteogenic growth factor bolus administration at 50 days post implantation, causing the differentiation of MSCs and, consequently, a rapid increase in the osteoblast concentration and bone formation. Osteogenic growth factor administration at any time point after the reduction of calcium release yields similar results. However, the injection of growth factors alone (at any time point), without the reduction in calcium release, is not able to induce bone formation in the case of an insufficient cell seeding density. In situations with a sufficient initial cell density, bolus administration of osteogenic growth factors leads to an increase in bone formation similar to the one observed in Fig. 5 (results not shown).

3.4 Sensitivity analysis by DOE

The first stage analysis showed (for both designs: uniform and LHD) that the model was robust with respect to changes in the parameter values for P_{bs}, A_{m0}, H_{11}, d_{cm}, G_{gb}, H_{gb}, d_{gb}, d_b, c_{cb}, d_{ca}, F_{12}, m_0 and $g_{b0.}$ These parameters were hence omitted from the second stage analysis. The first stage analysis furthermore showed that the value of the initial bone density in the scaffold b_0 had a substantial influence on the final amount of bone predicted by the model. This is a logical result but does not contribute to the final goal of the model, being its application in designing optimal CaP scaffold-cell combinations. Therefore the second stage experiment (150 runs) was started with all the parameters shown to be important in the first stage experiment but without b_0. The screening analysis and the stepwise regression gave similar results for the data of both designs (uniform and LHD) in the second stage analysis at days 21 and 42. It appears that the bone formation rate P_{bb}, the initial MSC density c_{m0} and the initial osteoblast density c_{b0} are the most important factors influencing the amount of bone formation at day 21 and 42 (see table 2).

3.5 In silico design of optimal CaP biomaterial-cell combinations

Based on the results obtained in the sensitivity analyses and the modeling of impaired bone formation cases, the model was applied to design conditions leading to maximal bone formation by altering those variables that were shown to be important and can be altered experimentally. Optimal calcium release rates were investigated for various initial MSC concentrations. For the lower initial MSC concentrations, the window of the optimal calcium release rate is quite narrow (Fig. 6) whereas for higher initial MSC concentrations the actual value of the calcium release rate becomes less critical but a maximal release rate still exists. Maximal bone formation for any initial MSC concentration is obtained at the high end of the spectrum of allowable calcium release rates.

4. Discussion

In this integrative study, a semi-quantitative model was developed, as part of a design and manufacturing strategy for customized cell-carrier combinations, using delay differential equations to describe the influence of the calcium ions released from CaP scaffolds on the *in vivo* bone formation observed after implantation of cellularized CaP scaffolds. A number of model parameter values were determined from dedicated *in vitro* experiments whereas other parameter values were derived from experimental results described in the literature. Bone formation was predicted by the model in accordance to experimental reports. Previously reported cases of impaired bone formation due to scaffold decalcification or insufficient initial MSC seeding densities were successfully recapitulated by the model. A thorough multi-parameter two-stage sensitivity analysis was carried out to determine the most influential parameters and subsequently different culture strategies were designed *in silico* with the aim of maximizing the amount of bone formation.

The effect of extracellular Ca^{2+} on *in vitro* osteoblast behavior has been investigated with reports of enhanced cell proliferation, upregulation of osteogenic markers and mineralization [12,32]. However, these investigations were mainly carried out on differentiated osteoblasts and for a limited amount of Ca^{2+} concentrations. In this study we have performed *in vitro* experiments on hPDCs which allowed us to investigate the effect of various Ca^{2+} concentrations on the proliferation of MSCs (at the early time points) and osteoblasts (at the later time points). The experiments were carried out on hPDCs since they are clinically relevant mesenchymal stem cells and have been shown to be multipotent [41]. Moreover, it is a critical cell type for bone defect regeneration [42, 43] and was reported to be physiologically responsive to a fluctuating

environment of calcium ions. Several studies in literature were within a similar range of concentrations and clearly support the data on Ca^{2+}-enhanced growth [12, 32, 44].

The Gaussian distributions fitted on the *in vitro* experiments correspond well to experimental observations reported in the literature. The Gaussian distribution predicts an optimal proliferation of MSCs in a range of 2-4 mM Ca^{2+}. This is similar to the findings by Liu *et al*. [31] who report an optimal concentration of 1.8 mM. The parameter values for osteoblast proliferation used in this study were derived from experiments conducted on MSCs. After 28 days in culture, upregulation of a number of osteogenic markers was observed [45], suggestive of the progression of these cells along the osteogenic lineage. Maeno *et al*. [32] measured maximal proliferation of fully differentiated murine osteoblasts at 5 mM Ca^{2+}, which corresponds to the fitted Gaussian function used in this study.

The model is able to reproduce the sequential events observed experimentally during intramembranous healing: (1) proliferation, (2) differentiation, (3) collagen production and (4) mineralization [46,47]. Hartman *et al*. determined experimentally the amount of bone formation at day 7, 21 and 42 post implantation. The percentage was calculated as the ratio of the surface area of newly formed bone to the surface area of the CaP-implant [20]. These values are represented by the bars in Fig. 3E. The results of the simulation and the experiment correspond qualitatively. However, bone formation is observed to start at a later time point in the simulation compared to experimental observations. The amount of bone measured at day 21 is larger than the amount predicted. It is worth noting, however, that this time point has a large standard deviation. At the same time, it should be mentioned that the experimental results were measured

from scaffolds that were subcutaneously implanted in rats [20] whereas the parameter values of the model presented in this study were derived from *in vitro* and *in vivo* experiments using different species such as cell experiments using hPDCs and canine ectopic bone formation experiments [3].

Remark as well that Hartman *et al.* [20] seeded $5x10^6$ cells per scaffold whereas the simulation considers an initial seeding density of 10^6 cells/ml. We chose this specific initial seeding density based on the results of Eyckmans *et al.* [2] who seeded different quantities of cells in CaP scaffolds ($1x10^6$ and $5x10^6$ cells) and did not find a statistical difference between the amounts of bone formation in these cellularized constructs. This led to the hypothesis that there may be cell death occurring when the seeding densities are too high. Since there are no quantitative data available on the relation between the initial seeding density and the amount of cells that are still alive and contributing to the *in vivo* bone formation, the same density was chosen as the one that was used to fit the data of Yuan *et al.* [3].

The simulation was also run for longer time periods to analyze possible steady states. There are more rigorous methods available to determine steady states analytically but they were found to be very complicated due to the nature of the mathematical model which consists of a set of coupled and highly non-linear delay differential equations. After about 600 days a steady state was reached. However, this steady state lies outside the timeframe of the biology that was considered in the mathematical model which only looks at the first stages of bone formation.

It is suggested that CaP needs to be present in sufficient quantities to induce ectopic ossification when using cell-carrier combinations without the addition of growth factors. Experimental

evidence of this hypothesis is provided by the observations reported in a recent study showing that the bone spicules were only present on the CaP granules of cellularized CaP scaffolds (Collagraft^{TM}) implanted ectopically in mice, and that no bone or fibrous tissue was found eight weeks after implantation of cellularized decalcified scaffolds [2]. The model was able to qualitatively predict the impaired healing situations of scaffold decalcification and insufficient cell seeding. Additionally, for the case of low initial cell seeding densities the effect of reducing the calcium release after implantation was tested *in silico* and found to lead to successful bone formation. The controlled ad hoc reduction of calcium release after implantation is not feasible in reality. However, due to cellular attachment and protein adsorption the theoretical release rates which are generally determined in acellular dissolution experiments most likely do not correspond to the actual release rates present *in vivo*. This quantification of actual release rates has not yet been reported in the literature to the best of our knowledge.

Sensitivity analyses such as the one carried out in this study are only able to identify the most important factors within the chosen parameter space for the defined outcome criteria. If the parameter ranges or the responses would be altered, a different result would be obtained. With respect to the sensitivity analyses carried out in the framework of this study, both designs (uniform and LHD) and both analysis techniques (screening and stepwise regression) indicate the importance of the initial conditions (initial seeding density of MSCs and osteoblasts) and the bone formation rate (see table 2). It is interesting to notice that the significant model parameters are design criteria that can be engineered and controlled in a tissue engineering context, i.e. the cell seeding conditions and the release rate of calcium ions. The same factors appear to be

significant in all designs and time points but their ranking in terms of importance may differ between the two designs (uniform and LHD).

The factors that were indicated to be of key importance by the sensitivity analysis were the bone formation rate P_{bb}, the initial MSC seeding density c_{m0} and the initial osteoblast seeding density c_{b0}. It appears that the model outcome largely depends on initial conditions which consequently should be realistically defined. Based on experimental results the initial MSC seeding density was chosen to be 10^6 cells/ml [2]. However, there is an uncertainty between the number of cells that is seeded on a scaffold and the amount that will survive and actively contribute to the bone formation process. To make the current model more realistic a more thorough quantification of the *in vivo* bone forming cell population is necessary. Since there are no osteoblasts seeded on the scaffold, this initial condition was chosen to be 0 cells/ml.

The sensitivity analysis also pinpoints some factors that were initially estimated due to lack of quantitative experimental data. It is necessary to further investigate these parameters and to design specific experiments so that their parameter values can be established. Currently, we are setting up *in vitro* and *in vivo* experiments to determine the calcium release rate σ and relate it to the *in vivo* bone forming capacity of different CaP scaffolds.

A sensitivity analysis does not only indicate the most important factors, but the least important factors are pointed out as well. As such, the results of the sensitivity analysis can be used to reduce the complexity of the model, which still remains quite large although the spatial characteristics are neglected in the current 1D mathematical model. We have investigated the

effect of some model terms (G_{con}, d_{cm}, d_b, d_{gb}) on the simulation results. Since the goal of this study was to model the effects of Ca^{2+} in detail, model terms that are not directly related to the calcium dynamics were chosen (see supplementary material S4 for more information and additional figures). The consumption of growth factors (G_{con}) can be removed from the set of differential equations without altering the results (data not shown). The removal of the term $d_{gb}.g_b$, which represents the removal of growth factors by denaturation or irreversible binding to matrix proteins, alters the result significantly (see Fig. S4.1). The growth factor concentration (see Fig. S4.1C) is a lot higher since there is no longer a removal term present, which causes an oversimplification of the biological reality. Moreover, this simplified system would not allow simulating the effect of growth factor injection on the bone formation process since the growth factor concentration is inadequately modeled. Consequently, the removal of the term $d_{gb}.g_b$ reduces the predictive potential of the mathematical model. Remark that the amount of bone formation is little influenced which was also found by the first sensitivity analysis. However, the sensitivity analysis was conducted for specific outcome criteria, i.e. the amount of bone formation at day 7, 21 and 42. As such, the outcome criteria do not take the effects on all biological processes into account. Hence, the sensitivity analysis correctly concluded that the effect of d_{gb} on the amount bone formation is not significant, but misses the fact that the growth factor concentrations become unrealistically large. Similarly, the amount of bone formation in the impaired cases (see Fig. S4.2, S4.3) is also not influenced, but the high growth factor concentration alters the kinetics of the other variables. Analogous conclusions can be drawn for the terms d_{cm} and d_b (data not shown). From these results we can conclude that little more simplifications can be done to the model without significant loss of accuracy in the description of the underlying biological processes.

Although the release rate of calcium was not the most important parameter, it still has a significant influence on the outcome and can be engineered for practical applications [21]. Therefore, the interaction of calcium release with one of the most important model parameters, the initial mesenchymal stem cell seeding density, was further investigated (see Fig. 6). The model shows that a low initial MSC density requires a low calcium release rate, while a high initial MSC density requires a high calcium release rate in order to maximize the amount of bone formation. Moreover, the optimization is critical for low initial MSC concentrations since the margin is very small. On the other hand, the high MSC concentrations allow for more optimization and potentially higher benefits. Roberts *et al*. [8] studied the *in vivo* bone formation capacity of different CaP scaffolds seeded with a fixed concentration of hPDCs. The calcium release rate was found to be a strong determinant in discriminating bone forming scaffolds from scaffolds that did not lead to any bone formation, whereby the absence of bone formation was observed for those scaffolds with the highest calcium release rate. Although the high MSC concentrations might yield higher benefits at first sight, from an economical perspective it is important to investigate further the possibilities of low initial MSC concentrations. A tissue engineering construct with a small amount of initial cell seeding can be advantageous in cases where there are limited disease-free autologous cells that can be harvested and more generally to reduce the costs of the expansion and culture techniques [48].

It might be possible, for example, to stimulate the proliferation of the low MSC concentration with growth factors so that the optimization of CaP scaffolds for low MSC seeding densities becomes less critical. For the moment it is not possible to simulate this effect in the model, since the proliferation terms A_b and A_m are growth factor independent, but this extension can be implemented in future versions of the model.

As was shown above, the model is able to define the optimal scaffold properties (in terms of Ca^{2+} release rate) for different initial cell seeding conditions. Moreover, the model can be used determine the optimal scaffold properties for different cell types (e.g. hPDCs, hBMSCs) since these cell types are characterized by different proliferation and differentiation parameters (amongst others). Besides the optimization of scaffold properties eventually resulting in more robust clinical outcomes, the model allows for an *in silico* screening of novel biomaterials based on biomaterial characteristics encompassed in the model (such as calcium release rate), thereby increasing the initial quality of the biomaterials selected for *in vitro* experimentation and reducing the number of false positive or negative results.

In the course of this study, several simplifying assumptions were made. Due to the lack of experimental evidence demonstrating true chondrogenic differentiation of the cells inside CaP scaffolds, chondrocytes were not considered in this study [2]. Also osteoclasts were neglected in the modeling framework since only the first stages of bone formation are modeled and bone remodeling is not taken into account. However, the nature of the model and its implementation allow for easy extension of the model to include additional cell types and tissues. Another important aspect that is not included in the mathematical model is angiogenesis. The invasion of blood vessels will present an additional source of calcium ions and growth factors which needs to be taken into account.

Although this study focuses on the role of calcium on cellular activity and bone formation, phosphate ions have been shown to considerably influence cellular processes such as proliferation, differentiation, apoptosis and matrix production [4,31,49] as well. Ultimately, these influences will need to be encompassed by the model.

A major simplification, and the first one to be dealt with in future versions of this model, lies in the fact that the model only looks at temporal variations thereby neglecting any spatial aspects that might influence bone formation in CaP scaffolds such as the macropore size and macropore size distribution as well as the biomaterial's micro-architecture. With respect to the latter however, although the micro-architecture of bioceramic scaffolds has recently been shown to influence bone formation in CaP scaffolds, its effect was found to be significantly less important than the rate of calcium release by the scaffold [21]. An extension of the DDE model to a PDE (partial differential equations) framework would allow more accurate modeling of the local Ca^{2+} release during dissolution, the change in scaffold permeability and the influence of cellular migration on bone formation.

This study is an illustration of the integrative approach proposed by Bohner *et al.* [1] to further elucidate the interactions between (CaP) biomaterials and osteogenic cells. Even though some dedicated experiments were designed and conducted to determine specific model parameter values, the mathematical model still contains a lot of estimated parameter values due to the lack of accurate, quantitative data in literature and the difficulty to make quantitative *in vivo* measurements. Therefore, it is important to stress the semi-quantitative nature of the proposed mathematical model. The simulation results that were obtained and discussed in this study should be interpreted in a relative way. But, despite the simplifications and estimations, the model is able to recapitulate some experimental findings qualitatively. The current tool, however, would have much more potential if it could be made more quantitative. Extensive quantification of *in vivo* measurements would not only allow a more accurate determination of the model parameter values, but also a more complete validation and enhancement of the model predictions. A precise

quantification of *in vivo* measurements is therefore imperative to the success of integrative approaches as the one discussed in this study. From this perspective, the vision of Bohner *et al.* [1] should be extended: not only careful design and extensive characterization of the scaffold is necessary for an integrative approach to (bone) tissue engineering, but also an accurate quantification of *in vivo* measurements.

5. Conclusion

In this study, an integrative approach was used to investigate the influence of the calcium released from CaP scaffolds on the *in vivo* bone formation observed after implantation of cellularized CaP scaffolds. Bone formation was predicted by the semi-quantitative model in accordance to experimental reports in the literature. Previously reported cases of impaired bone formation due to scaffold decalcification or insufficient initial MSC seeding densities were successfully recapitulated by the model. Subsequently, the model was applied to design optimal biomaterial and culture condition combinations (i.e. calcium release rates and initial cell seeding densities) with the aim of maximizing the amount of bone formation. The results obtained in this study suggest that this model can be used as a tool to design and optimize CaP scaffolds in tissue engineering applications. In the future the model could be refined with additional cell and tissue types, allowing for an *in silico* triage of cell-customized biomaterials.

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Figure 1: (A) Conceptual overview of the general approach in tissue engineering. (B) Schematic overview of the calcium model. W = maximum tissue density for proliferation, X = minimum calcium concentration for proliferation of MSCs, Z = maximum calcium concentration for proliferation of MSCs, U = minimum calcium concentration for proliferation of osteoblasts, V = maximum calcium concentration for proliferation of osteoblasts, ca = Ca^{2+}, gb = growth factor, m = osteoid, b = mineral matrix. The participation of a variable in a subprocess is indicated by showing the name of that variable next to the arrow representing that subprocess, e.g. calcium modulates differentiation and bone formation.

Figure 2: hPDCs growth in Ca^{2+} supplemented growth media after 28 days compared to all other culture treatments. The treatment conditions indicated by a bracket are statistically significantly different from the treatments indicated by a star. *p<0.05. Mean + S.D. (n= 4). GM = growth medium. Cx: x indicates the final Ca^{2+} concentration in mM.

Figure 3: Temporal evolution (days post implantation) of (A) collagen matrix density *m*, (B) mesenchymal stem cell density c_m, (C) growth factor concentration g_b, (D) osteoblast cell density c_b, (E) bone matrix density *b* and (F) calcium concentration *Ca* during normal bone formation. Represented by bars are the experimental results of Hartman *et al*. [20], obtained under experimental conditions similar to the modeled ones. The asterisk on the left side of each subfigure indicates the model initial conditions.

Figure 4: Temporal evolution (days post implantation) of (A) collagen matrix density *m*, (B) mesenchymal stem cell density c_m, (C) growth factor concentration g_b, (D) osteoblast cell density c_b, (E) bone matrix density *b* and (F) calcium concentration *Ca*. Solid lines represent the predicted evolution for a decalcified scaffold. Dashed lines represent the evolution in a scaffold with low initial cell seeding (c_{m0} = 0.3x10^6 cells/ml). The asterisk and the rectangle on the left side of each subfigure indicate the model initial conditions.

Figure 5: Temporal evolution (days post implantation) of (A) collagen matrix density *m*, (B) mesenchymal stem cell density c_m, (C) growth factor concentration g_b, (D) osteoblast cell density c_b, (E) bone matrix density *b* and (F) calcium concentration *Ca* in a CaP scaffold with an initial cell density c_{m0} of 0.3x10^6 cells/ml. After ten days the calcium release is exponentially reduced (solid line). Dashed lines show the effect of administrating a bolus of osteogenic growth factors at day 50 post implantation. The asterisk and the rectangle on the left side of each subfigure indicate the initial conditions. In subfigures (A) and (F) hardly any effect of the growth factor administration can be observed.

Figure 6: Amount of bone formation at day 90 as a function of the calcium release rate (σ) and initial MSC concentration (c_{m0}) according to the mathematical model.

Figure S4.1: Temporal evolution (days post implantation) of (A) collagen matrix density m, (B) mesenchymal stem cell density c_m, (C) growth factor concentration g_b, (D) osteoblast cell density c_b, (E) bone matrix density b and (F) calcium concentration Ca in a CaP scaffold. The asterisk and the rectangle on the left side of each subfigure indicate the initial conditions. The removal of growth factors due to denaturation or irreversible binding to matrix proteins ($d_{gb} = 0$) is excluded from the set of equations and this condition represented by the dashed lines. Remark that the amount of bone formation is only slightly influenced but that the growth factor concentration becomes unrealistically large.

Figure S4.2: Temporal evolution (days post implantation) of (A) collagen matrix density m, (B) mesenchymal stem cell density c_m, (C) growth factor concentration g_b, (D) osteoblast cell density c_b, (E) bone matrix density b and (F) calcium concentration Ca in a decalcified CaP scaffold. The asterisk and the rectangle on the left side of each subfigure indicate the initial conditions. The removal of growth factors due to denaturation or irreversible binding to matrix proteins ($d_{gb} = 0$) is removed from the set of equations and represented by the dashed lines. Remark that the amount of bone formation is only slightly influenced. The growth factor concentration becomes however unrealistically large, causing differentiation and collagen matrix formation.

Figure S4.3: Temporal evolution (days post implantation) of (A) collagen matrix density m, (B) mesenchymal stem cell density c_m, (C) growth factor concentration g_b, (D) osteoblast cell density c_b, (E) bone matrix density b and (F) calcium concentration Ca in a CaP scaffold with an initial cell density c_{m0} of 0.3×10^6 cells/ml. The asterisk and the rectangle on the left side of each subfigure indicate the initial conditions. The removal of growth factors due to denaturation or irreversible binding to matrix proteins ($d_{gb} = 0$) is removed from the set of equations and represented by the dashed lines. Remark that the amount of bone formation is only slightly influenced. The growth factor concentration becomes however unrealistically large.

Parameter	*Definition*	*Nominal value*	*Range*	*Source*
MSCs				
α_m	reciprocal of maximum MSC density constant	1	/	[35]
A_{m0}	MSC proliferation rate coefficient	0.85	0.1-1.01	[35]
K_m	ECM density constant related to MSC proliferation	0.1	0.01-0.1	[35]
a_{cm}	concentration constant related to proliferation (calcium)	5.98	4.72-6.97	measured
b_{cm}	optimal calcium concentration for MSC proliferation	1.67	3.05-3.73	measured
c_{cm}	concentration constant related to proliferation (calcium)	3.33	1.55-1.70	measured
Y_{11}	differentiation rate coefficient (growth factor)	10	10-100	[35]
H_{11}	concentration constant related to differentiation	14	10-20	[35]
F_{11}	differentiation rate coefficient (calcium)	8	6.5-10	[12]
F_{12}	optimal calcium concentration for MSC differentiation	1.5	1.2-1.8	[12]
d_{cm}	MSC removal rate coefficient	1.5	0.001-3.5	estimated
Osteoblasts				
A_{b0}	osteoblast proliferation rate coefficient	0.202	0.2-2.02	[35]
K_b	ECM density constant related to osteoblast proliferation	0.1	0.01-0.1	[35]
α_b	reciprocal of maximum osteoblast density constant	1	/	[35]
a_{cb}	concentration constant related to proliferation (calcium)	41.82	35.2-46.94	measured
b_{cb}	optimal calcium concentration for osteoblast proliferation	5.06	3.32-5.41	measured
c_{cb}	concentration constant related to proliferation (calcium)	1.9	1.9-2.28	measured
d_b	osteoblast removal rate coefficient	0.1	0.05-0.2	[35]
γ	threshold for osteoblast removal rate coefficient	7	/	estimated
Collagen matrix				
P_{bs}	collagen matrix production rate coefficient	0.18	0.1-2	[3]
κ_b	reciprocal of maximum of collagen matrix constant	1	/	[35]
Mineral matrix				
δ	1-maximal degree of mineralization	0.05	/	estimated
P_{bb}	mineralized matrix production rate coefficient	0.0398	0.0298-0.0731	[3]
κ_{bb}	reciprocal of maximum of mineralized matrix constant	1	/	[35]
m_{thres}	threshold for maturation of collagen matrix	0.85	/	estimated
Calcium				
σ	calcium release rate	10	0-22	estimated
Ca_∞	maximal calcium concentration (solubility limit)	50	/	[32]
J_{in}	calcium removal rate coefficient (HA production)	750	400-800	estimated
H_{Ca4}	concentration constant related to calcium uptake	0.01	0.01-0.1	estimated
d_{ca}	calcium removal rate coefficient (metabolic activities)	100	10-500	estimated

Growth factors				
G_{gb}	growth factor production rate coefficient	350	200-1000	[22]
H_{gb}	concentration constant related to growth factor production	1	0.1-2	[22]
G_{con}	growth factor consumption rate coefficient	1	1-75	estimated
H_{con}	concentration constant related to growth factor consumption	0.001	0.0001-0.1	estimated
d_{gb}	growth factor decay rate coefficient	75	33-1000	[22]

Table 1: Non-dimensionalized parameter values of the presented model. Using the equations in the supplementary material the dimensionalized values can be obtained.

		LHD	uniform
amount of bone formation at day 21	screening analysis	c_{b0} P_{bb} c_{m0}	P_{bb} c_{m0} c_{b0}
	stepwise regression	b_{cm} (SS = 0.003) G_{con} (SS = 0.006) A_{b0} (SS = 0.008) b_{cb} (SS = 0.006) **P_{bb} (SS = 0.067)** σ (SS = 0.007) **c_{m0} (SS= 0.024)** **c_{b0} (SS = 0.071)**	A_{b0} (SS = 0.005) K_b (SS = 0.002) **P_{bb} (SS = 0.091)** J_{in} (SS = 0.003) σ (SS = 0.004) **c_{m0} (SS = 0.024)** **c_{b0} (SS = 0.078)**
amount of bone formation at day 42	screening analysis	c_{b0} c_{m0} P_{bb}	P_{bb} c_{b0} c_{m0}
	stepwise regression	b_{cm} (SS = 0.010) G_{con} (SS = 0.011) A_{b0} (SS = 0.009) **P_{bb} (SS = 0.081)** **c_{m0} (SS= 0.069)** **c_{b0} (SS = 0.106)**	G_{con} (SS = 0.006) A_{b0} (SS = 0.006) K_b (SS = 0.007) **P_{bb} (SS = 0.133)** J_{in} (SS = 0.012) σ (SS = 0.031) **c_{m0} (SS= 0.081)** **c_{b0} (SS = 0.108)**

Table 2: Overview of the results of the sensitivity analysis (150 runs, all parameters of the first stage are included except b_0). For the screening analysis only the most important factors are indicated without a measure of their importance. The most important factors of the stepwise regression are indicated in bold (LHD = latin hypercube design, SS = sum of squares).

Variable	*Nominal value*	*Range*	*Source*
c_{m0}	1	0-1	[2]
c_{b0}	0	0-1	estimated
m_0	0.01	0.01-0.1	[31]
b_0	0	0-1	estimated
g_{b0}	15	10-100	[31]
ca_0	1	1-50	estimated

Table 3: Non-dimensionalized initial variable values of the presented model. Using the equations in the supplementary material the dimensionalized values can be obtained.