Published in final edited form as:

J Tissue Eng Regen Med. 2017 February ; 11(2): 481–488. doi:10.1002/term.1940.

Mathematical modelling of glycosaminoglycan production by stem cell aggregates incorporated with growth factor-releasing polymer microspheres

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Abstract

Systems composed of high density cells incorporated with growth factor-releasing polymer microspheres have recently been shown to promote chondrogenic differentiation and cartilage formation. Within these systems, the effects of spatial and temporal patterning of growth factor release on hyaline cartilage-specific extracellular matrix production have been examined. However, at present, it is unclear which microsphere densities and growth factor delivery profiles are optimal for inducing human mesenchymal stem cell differentiation and glycosaminoglycan production. A mathematical model to describe glycosaminoglycan production as a function of initial microsphere loading and microsphere degradation rate over a period of 3 weeks is presented. Based on predictions generated by this model, it may be feasible to design a bioactive microsphere system with specific spatiotemporal growth factor presentation characteristics to promote glycosaminoglycan production at controllable rates.

Keywords

cartilage tissue engineering; chondrogenic differentiation; gelatin hydrogel; mesenchymal stem cells; transforming growth factor-beta 1

1. Introduction

Owing to the limited ability of articular cartilage to repair itself after disease or injury and the variable efficacy of current treatment options, cartilage tissue engineering strategies have been developed with the goal of promoting cartilage repair and regeneration. Many of these approaches utilize bone marrow-derived mesenchymal stem cells (MSCs) because of their availability, ease of expansion in culture and capacity to differentiate into chondrocytes. Chondrogenic differentiation of MSCs can be induced in high-density culture in the

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Conflict of interest

The authors have declared that there is no conflict of interest.

Supporting information

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presence of growth factors such as transforming growth factor (TGF)- β 1 or TGF- β 3 (Yoo *et al.*, 1998). To improve control over the temporal and spatial presentation of growth factor within densely populated cellular constructs and avoid transport limitations that can occur with exogenous supplementation in the media, systems have been developed incorporating growth factor-releasing polymer microspheres within the constructs (Ferreira *et al.*, 2008; Carpenedo *et al.*, 2010; Solorio *et al.*, 2010, 2012a,b; Garcia Cruz *et al.*, 2013). Such approaches may also permit more rapid clinical therapeutic implementation without extensive in vitro culture.

Within these systems, factors including microsphere amount, initial growth factor concentration in the microspheres and polymer microsphere degradation time influence the growth factor release profiles and spatial distribution and in turn the rate and degree of chondrogenic differentiation (Solorio *et al.*, 2012a,b). While the effects of changes in some of these system parameters have been explored, it is unknown which combination of variables could result in faster chondrogenesis or increased total glycosaminoglycan (GAG) production. Owing to cost considerations, system design strategies that enable chondrogenic differentiation while using a minimal amount of growth factor are also important. A mathematical model that predicts the effects of different bioactive microsphere incorporation conditions, such as total microsphere loading and polymer degradation time, in high cell density cell systems would be useful as a predictive guide for the development of tailored experimental conditions designed to achieve specific temporal patterns of cell differentiation and GAG production and/or to minimize the amount of growth factor needed.

Mathematical modelling of tissue engineering systems is inherently difficult because of the complex interplay between the array of biological, chemical and mechanical factors that control the process of functional tissue formation. Simple empirical models have been developed to characterize such systems. For example, an empirical second-order polynomial equation was developed based on a least-square fit to experimental data on chondrocyte growth in polymers of different thicknesses (Freed *et al.*, 1994). While relatively easy to formulate and useful to characterize some systems, it can be challenging to use empirical models to gain insight into the key underlying mechanisms that control tissue formation.

Alternatively, mechanistic models based on cellular processes and transport and reaction phenomenon are exploited to maximize tissue formation via optimization of design parameters. Such models have been generated to characterize common tissue engineering systems such as growing cells in polymer scaffolds. When modelling chondrogenesis with cells seeded within a polymer matrix, Galban and Locke (1997) demonstrated a decrease in cell growth as the thickness of polymer increased. Using a similar framework, with MSCs seeded on a polymer scaffold, Byrne *et al.* (2007) showed that polymer properties such as porosity, Young's modulus and dissolution rate were important factors affecting bone formation. In addition to scaffold dimensions and polymer characteristics, factors such as oxygen and glucose concentrations were shown to be critical factors affecting scaffold-based heart and cartilage tissue engineering in nutrient-dependent population models (Chung *et al.*, 2006; Brown *et al.*, 2007).

Owing to differences in system design, models of cartilage formation based on seeding cells on a polymer scaffold may not be generalized to describe microsphere-incorporated cell aggregates. The key mechanisms and processes in the model presented here include differentiation of human MSCs (hMSCs), release of growth factor from polymer microspheres, and uptake of growth factor by hMSCs. Parameters associated with these processes, which are difficult to measure directly via experiments, were optimally estimated by comparison of model simulations with experimental data. This model shows how GAG production could be controlled via combination of various growth factor loading and polymer degradation conditions.

2. Materials and methods

2.1. Isolation and culture of hMSCs

A human bone marrow aspirate was obtained under a protocol approved by the University Hospitals of Cleveland Institutional Review Board and processed as previously described (Haynesworth *et al.*, 1992). Following isolation, hMSCs were cultured as previously described (Lennon *et al.*, 1996) and used at passage 3.

2.2. Gelatin microsphere synthesis

Gelatin microspheres were produced according to a protocol previously described (Solorio *et al.*, 2012a). Briefly, 11.1 wt% acidic gelatin (Sigma-Aldrich, St. Louis, MO, USA) was emulsified in warm olive oil (Gia Russa, Coitsville, OH, USA) and the temperature was reduced to enable gelation. The resultant microspheres were then collected via filtration, washed with acetone to remove residual oil, and air dried. Dry microspheres were crosslinked at room temperature in a 1 wt% genipin (Wako USA, Richmond, VA, USA) solution in ultrapure deionized water (diH_2O) for 2 h ('fast degrading') or 21 h ('slow degrading') to create microspheres that were $28.3 \pm 7.2\%$ or $67.6 \pm 4.5\%$ crosslinked, respectively, as determined by ninhydrin assay (Solorio *et al.*, 2012). Two levels of crosslinking were used to enable different polymer degradation rates, with a higher percentage of crosslinking resulting in slower enzymatic degradation of the microspheres (Solorio *et al.*, 2012). Crosslinked microspheres were collected by filtration, washed with ultrapure diH_2O and lyophilized. Following ultraviolet (UV) sterilization for 10 min, growth factor-loaded microspheres were prepared by soaking dry microspheres in a solution of TGF- β 1 (Peprotech, Rocky Hill, NJ, USA) in phosphate buffered saline (PBS) at pH 7.4 for 2 h at 37°C. At this pH, negatively charged gelatin forms a complex with positively charged TGF- β 1, enabling growth factor to be retained within the hydrogel matrix until proteolytic degradation occurs (Yamamoto *et al.*, 2001). The volume of TGF- β 1 solution added was much less than the equilibrium swelling volume of the microspheres, ensuring complete growth factor absorption during loading (Park *et al.*, 2005).

2.3. Microsphere-incorporated hMSC aggregate production

Sterile microspheres were loaded as described previously with 400 ng TGF- β 1 per mg microspheres (Solorio *et al.*, 2012). The hMSCs were suspended at a concentration of 1.25×10^6 cells per ml in a chemically defined medium [DMEM-HG with 1% ITS + Premix (BD Biosciences, Sparks, MD, USA), 37.5 $\mu\text{g/ml}$ ascorbate-2-phosphate (Wako USA), 10^{-7} M

dexamethasone (MP Biomedicals, Solon, OH, USA), 1% nonessential amino acids (HyClone, Logan, UT, USA) and 1% sodium pyruvate (HyClone)] and slow-degrading or fast-degrading microspheres with loaded growth factor were added at a concentration of 0.05 mg microspheres per aggregate ('low loading') or 0.15 mg microspheres per aggregate ('high loading') according to the conditions described in Table 1. The cell and microsphere suspensions were added in 200 μ l aliquots to the wells of sterile 96-well V-bottom polypropylene plates (Phenix, Candler, NC, USA), centrifuged for 5 min at 500 g and cultured in a humidified incubator at 37°C with 5% CO₂ (Solorio *et al.*, 2010). The medium was changed every other day. Aggregates were harvested for analysis after 7, 10, 14 and 21 days.

2.4. Biochemical analysis

Aggregates designated for DNA and GAG quantification ($n = 4$) were digested in a papain solution (Sigma) (Ponticello *et al.*, 2000) at 65°C for 2 h, and the digests were assayed for DNA and GAG content with PicoGreen (Invitrogen, Carlsbad, CA, USA) (McGowan *et al.*, 2002) and dimethylmethylene blue (DMMB) dye (Sigma-Aldrich) (Farndale *et al.*, 1986), respectively. Data is reported as average $\pm$ standard deviation.

2.5. Model development and simulation methods

2.5.1. hMSC differentiation dynamics—We assume that hMSC differentiation undergoes a discrete number of stages in which the number of cells is $N_i(t)$ at stage i . The total number of cells N_T is assumed to be constant:

$$N_T = \sum_{i=1}^q N_i(t) \quad (1)$$

The number of cells in each stage changes by differentiation according to

$$\frac{dN_1}{dt} = -kN_1 \quad (2)$$

$$\frac{dN_i}{dt} = k(N_{i-1} - N_i) \quad (i = 2, 3, \dots, q-1) \quad (3)$$

$$\frac{dN_q}{dt} = kN_{q-1} \quad (4)$$

where k is a differentiation rate coefficient assumed to be stage-independent (Lemon *et al.*, 2007), but proportional to growth factor concentration C in the extracellular space, $k = \gamma C$, with proportionality factor γ . Although cell division and cell death are potentially important processes that influence N , our model excludes both of these processes because the net change in N caused by cell division and cell death is assumed to be negligible based on DNA quantification conducted during the study (see the Supporting Information, Figure S1).

In terms of cell fractions $F_i(t) = N_i(t)/N_T$, Eqs (2–4) are expressed as:

$$\frac{dF_1}{dt} = -\gamma CF_1 \quad (5)$$

$$\frac{dF_i}{dt} = \gamma C(F_{i-1} - F_i) \quad (i = 2, 3, \dots, q-1) \quad (6)$$

$$\frac{dF_q}{dt} = \gamma CF_{q-1} \quad (7)$$

The initial conditions for cell fractions are

$$t = 0 : F_1 = 1; F_i = 0 \quad (i = 2, 3, \dots, q)$$

2.5.2. GAG dynamics and aggregate volume—Within this model, the extent of cell differentiation is characterized by the amount of GAG-containing matrix produced by the cells (Obradovic *et al.*, 2000). Some type II collagen, the other primary component of cartilage extracellular matrix (ECM), is also expected to be present within the GAG-containing matrix, but this model does not take the amount of collagen into account separately (Freed *et al.*, 1998). A greater mass of GAG is assumed to be produced by cells at later stages of differentiation as indicated by:

$$G(t) = \varepsilon \sum_{i=2}^q iF_i^i \quad (8)$$

where ε is a scale factor used to convert GAG-producing cell fraction into mass of GAG, while keeping units consistent. The initial condition for GAG is $G(0) = 0$. Alternative weighted functions for $G(t)$, including iF and F^i , were also considered, but neither functions was sufficient at predicting the initial delay of GAG production evident in Figure 1.

The total volume of the cell-microsphere aggregate is

$$V_T(t) = V_{cell} + V_{ms} + V_{ex}(t) \quad (9)$$

where V_{cell} is considered constant because the number of cells per aggregate remains nearly the same throughout the experiment as verified by DNA quantification (Figure S1). In addition, V_{ms} is considered constant because the initial volume occupied by the microspheres is maintained even as the microspheres degrade as the space is filled with remodelled GAG-containing ECM (Solorio *et al.*, 2012a,b). The volume external to both cells and microspheres, $V_{ex}(t)$, is expressed as the sum of GAG-containing matrix and interstitial volume:

$$V_{ex}(t) = V_{GAG}(t) + V_{in} \quad (10)$$

Initially, $V_{GAG}(0)=0$ so that $V_{ex}(0) = V_{in}$. As the cells differentiate and produce GAG, $V_{in} \ll V_{GAG}(t)$ so that $V_{ex} \ll V_{GAG}(t)$.

As GAG consists of mostly water, we can assume that

$$V_{ex}(t) = V_{GAG}(t) = \frac{G(t)}{\rho} \quad (11)$$

where ρ is the density of water. From Eqs (8) and (11), the extracellular volume changes according to

$$\frac{dV_{ex}}{dt} = \frac{1}{\rho} \frac{dG(t)}{dt} = \alpha \sum_{i=2}^q t^2 F_i^{i-1} \frac{dF_i}{dt} \quad (12)$$

where $\alpha = \frac{\varepsilon}{\rho}$.

2.5.3. Growth factor concentration dynamics—The mass of growth factor in the extracellular volume changes according to

$$\begin{aligned} \frac{d(V_{ex}C)}{dt} &= V_{ex} \frac{dC}{dt} + C \frac{dV_{ex}}{dt} = R - U \\ \Rightarrow \frac{dC}{dt} &= \frac{1}{V_{ex}} \left(R - U - C \frac{dV_{ex}}{dt} \right) \end{aligned} \quad (13)$$

where C is the average growth factor concentration in the extracellular volume. R and U are the release and uptake rates of growth factor, respectively.

Assuming that the growth factor uptake increases with higher growth factor concentrations (Wakefield *et al.*, 1987) and is hindered by expanding volume occupied by GAG, we propose that U is proportional to C and increasing V_{ex} causes an exponential decrease in growth factor uptake. The uptake rate can be expressed as:

$$U = \lambda \exp(-\sigma V_{ex}) C \quad (14)$$

where λ and σ represent growth factor uptake rate coefficient and uptake hindrance coefficient, respectively. The rationale behind Eq. (14) is that as C increases, more growth factor becomes available in the cellular microenvironment, which effectively increases the rate of uptake. In contrast, rate of growth factor uptake is expected to decrease as V_{ex} expands, which likely hinders the transport of growth factors and limits their availability for uptake.

2.5.4. Microsphere degradation and growth factor release—If the microspheres degrade exponentially (Mladenovska *et al.*, 2002), then the mass of microspheres can be expressed as:

$$M_{ms}(t) = M_{ms}^0 e^{-\beta t} \quad (15)$$

where M_{ms}^0 is the initial mass of microspheres and β is the degradation rate coefficient.

The rate of growth factor release is expected to be proportional to polymer degradation (Yamamoto *et al.*, 2001):

$$R = \phi M_{ms}^0 e^{-\beta t} \quad (16)$$

where ϕ is a rate constant. If all the growth factor is eventually released, then the total mass of growth factor released over time is

$$M_g = \int_0^\infty R dt = -\frac{\phi}{\beta} M_{ms}^0 e^{-\beta t} \Big|_0^\infty = \frac{\phi M_{ms}^0}{\beta} \quad (17)$$

where $\phi = \beta M_{gf}/M_{ms}^0$.

By combining Eqs (16) and (17), the rate of release is

$$R = \beta M_{gf} e^{-\beta t} \quad (18)$$

Substituting Eqs (14) and (18) into Eq. (13), growth factor concentration changes according to

$$\frac{dC}{dt} = \frac{1}{V_{ex}} \left(M_{gf} \beta e^{-\beta t} \lambda e^{-\sigma V_{ex}} C - C \frac{dV_{ex}}{dt} \right) \quad (19)$$

Initially, $C(0) = 0$.

2.5.5. Model simulation and parameter estimation—The governing equations for model simulation are Eqs (5–7) and (19) with known and unknown parameters listed in Tables 2 and 3, respectively. Values of M_{gf} and β were determined based on experimental conditions. We calculated β for fast- and slow-degrading microspheres based on an exponential decay model with half-lives of 7 days and 18 days, respectively, as estimated from previous histological observations of microsphere degradation (Solorio *et al.*, 2012a,b).

Before estimating the unknown parameters in Table 3, simulations were performed to investigate the effects of different parameter values and to determine a reasonable range for the upper and lower bounds of parameter values for optimal estimation. Using ‘fmincon’ in MATLAB® (R2007a, The MathWorks, Natick, MA, USA) with these bounds as constraints,

optimal estimates of the parameters were obtained to provide the best least-square fit of the model output to the experiment data. For each of the four experimental conditions, GAG concentrations were empirically measured at five time-points. The standard deviations associated with the estimated parameter values were also computed.

3. Results and discussion

3.1. Estimates of model parameters

Optimal estimates of parameters were obtained to allow the model to closely simulate the experimental data as shown in Figure 1. The computed standard deviations were all within 6% of the estimated parameter values. Owing to the limited number of experimental data points, the experimental GAG productions appeared to resemble stepwise functions. However, the simulated GAG results, based on continuous functions, were continuous by nature. As a result, the predicted GAG values at day 7 for three experimental conditions were higher than those determined empirically. Compared with the predicted results at day 7, GAG productions at days 10, 14 and 21 more closely resemble experimental data. It is useful to have accurate predictions of GAG production at later time-points because one goal of the model analysis was to determine how to maximize GAG production at the end of experiments.

3.2. Predicting the effects of experimental parameters

After substituting the previously unknown parameters with ones estimated in Table 3, the model could predict the effects of growth factor loading and microsphere degradation rate on GAG production in the system. In Figure 2A, B, GAG vs. time plots were simulated using either fast- or slow-degrading microspheres and a variety of growth factor loading, ranging from 5 to 200 mg. Regardless of the microsphere degradation condition, 5 mg and 20 mg growth factor loading resulted in substantially less GAG output than the higher loading conditions. Similarly, with both degradation conditions, when the experiment was terminated at day 15 or later, diminishing return was evident with high growth factor loading (e.g., 200 mg). A similar analysis could be performed to examine the effect of microsphere degradation on GAG. In Figure 2C and 2D, GAG vs. time plots were simulated using either high or low growth factor loading and a variety of microsphere degradation rates, ranging from 0.1–0.01/day. With very slow degradation (e.g. 0.01/day), GAG production was low because of limited growth factor release. There was also little increase in additional GAG production for degradation rates faster than 0.064/day.

Previous studies have shown that the level of chondrogenic differentiation within microsphere-incorporated cell systems is controlled by system parameters that include microsphere amount, initial growth factor loading and polymer degradation rate (Solorio *et al.*, 2012a,b). These factors influence both the growth factor release profile and the spatial distribution of growth factor, directing spatiotemporal GAG production and chondrogenesis within the cellular constructs. However, it is unclear which specific combinations of variables could result in optimally enhanced GAG production rate or amount. Figure 2A–D can serve as useful guidelines for maximizing GAG, selecting the microsphere type and

reducing experimental costs (e.g. less growth factor loading and early termination of experiments).

According to the experimental findings and model simulations, growth factor concentration critically drives GAG production in our system of microsphere-incorporated hMSC aggregates. This can be compared with a model developed by Catt *et al.* (2011) describing GAG production in a system of dedifferentiated chondrocytes that were redifferentiated in high density culture on collagen-coated membranes. In their model, GAG production was dependent on cellular processes that included the state of differentiation as well as the cell differentiation rate, while in the system used in the present study it was directly affected by growth factor presentation. Although Catt's filter culture setup is inherently different from the microsphere-incorporated aggregate model used in the present study, their model, which is very similar, also suggested that increasing potential 'drivers,' such as cell seeding density, does not always lead to more GAG production. The implication is that ECM production by tissue engineered cartilage constructs has innate limits that can derive from either system design limitations or 'saturated' cellular processes such as cell differentiation.

3.3. Model assumptions and limitations

Numerous assumptions were made in the mathematical model to make the experimental studies and simulations tractable. First, the model assumed that TGF- β 1 concentration was the dominant factor driving hMSC differentiation. If sufficient data were available, then it would be worthwhile developing a more complete mathematical model. Such a model could incorporate the diffusion and reaction of other nutrients (e.g. oxygen and glucose) in regulating hMSC differentiation. Second, the model assumed that the increase in ECM volume is solely a result of GAG formation. However, there are other important components of cartilage ECM such as type II collagen, and with additional experimental studies, the model framework can be expanded to include additional ECM molecules.

As evident in Figure 1, the model is capable of accounting for the delayed production of GAG as a result of low growth factor loading or slow-degrading microsphere degradation. However, with high growth factor loading and fast-degrading microspheres, the delay predicted by the model could be as short as 12 day (Figure 2A), which might not be sufficient time for cells to differentiate and start producing GAG. This potentially inaccurate prediction results from the assumption that GAG is produced at all stages of differentiation. A more sophisticated model would incorporate a time-delay parameter that would be estimated based on experimental data. Although the model predicts the possibility of having GAG production plateau at day 7 with a combination of high growth factor loading and fast-degrading microspheres (Figure 2A), it also remains to be tested if the GAG produced that reaches a plateau at day 7 is structurally and functionally the same as GAG that reaches a plateau at day 21.

Previous work by Mahoney and Saltzman (1996) had demonstrated that microsphere spacing could play a role in determining spatial distribution of growth factor after release and uptake is restricted to 1–2 mm from the surface of the microspheres. However, for the sake of simplicity, the effect of microsphere spacing was not taken into account in this model. The present model assumes that the microspheres were evenly distributed and that cells will have

equal growth factor exposure and opportunity for uptake. To obtain a complex model that may describe the system more accurately, microsphere spacing should be incorporated into the model along with its effects on growth factor diffusion and uptake.

4. Conclusions

A hybrid model, consisting of both mechanistic and empirical components, was developed to characterize chondrogenic differentiation of hMSCs in high-density cell aggregates with microspheres that release growth factor. Based on different growth factor loading and polymer degradation conditions, key parameters associated with cell differentiation and growth factor uptake were estimated. According to simulated results, GAG formation could reach a plateau at a high level (~50 µg) within 7–10 days with specific combinations of growth factor loading and polymer degradation rates. An important finding from model simulation is that an increase of growth factor loading beyond a certain amount yields diminishing returns. This information can potentially be used to reduce the cost of regenerating cartilage tissue using high-density cell aggregate systems. In a mutually iterative framework of this model analysis with additional experimental studies, further information about chondrogenic differentiation and GAG formation could be provided with fewer experiments and at a reduced cost.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors acknowledge funding for this work from the National Institutes of Health (AR007505 for LDS training support and AR063194 to EA).

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Figure 1.

Model output fitted to experimental data for glycosaminoglycan (GAG) production over 21 days with optimal estimates of parameters. Conditions examined are fast-degrading microspheres and high microsphere loading (fast deg & high loading), slow-degrading microspheres and high microsphere loading (slow deg & high loading), fast-degrading microspheres and low microsphere loading (fast deg & low loading), and slow-degrading microspheres and low microsphere loading (slow deg & low loading)

Figure 2.

Model outputs of glycosaminoglycan (GAG) production over 21 days with varied growth factor-containing microsphere loading with fast-degrading (A) or slow-degrading (B) microspheres, or varied microsphere degradation rates with high (C) or low (D) microsphere loading levels

Table 1.

Experimental conditions of microsphere-incorporated aggregates

Microsphere type	Mass of microspheres (mg/aggregate)	Total TGF- β 1 (ng/aggregate)	Remarks
Fast degrading	0.05	20	Low loading
Slow degrading	0.05	20	Low loading
Fast degrading	0.15	60	High loading
Slow degrading	0.15	60	High loading

TGF, transforming growth factor.

Table 2.

Parameters with known values

Symbol	Parameters	Value	Remarks
M_{gf} (ng)	Mass of growth factor	60	High loading
		20	Low loading
β (day)	Microsphere degradation rate coefficient	9.90×10^{-2}	Fast degrading
		3.85×10^{-2}	Slow degrading
ϵ (ng)	Scale factor	8^a	

^aEstimated based on experimental data.

Table 3.

Parameters from optimal estimation by model fitting to experimental data

Model symbol	Parameters	Value
γ (ng/day.mm ³)	Cell (fraction) differentiation coefficient	$(6.76 \pm 0.04) \times 10^{-3}$
λ (day/mm ³)	Growth factor uptake rate coefficient	$(2.24 \pm 0.01) \times 10^{-3}$
σ (mm ⁻³)	Uptake hindrance coefficient	2.70 ± 0.01