Characterization of Steady State Growth Factor Gradients in Engineered Tissues via Damkohler Number Analysis

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INTRODUCTION: Musculoskeletal tissue engineering (TE) is an emerging treatment strategy where cell-seeded tissue constructs are initially cultivated *in vitro* with an array of anabolic biomolecules, consisting of nutrients, vitamins, hormones, and growth factors (GFs), to accelerate tissue growth. In contrast to native developmental processes—whereby biomolecule transport is aided through delivery via dense vascular networks—in TE applications, biomolecules are supplemented in culture medium and expected to transport sizable diffusion distances through thick avascular constructs to reach their cell targets [1,2]. Consequentially, growth-stimulating biomolecules can encounter significant transport limitations in TE constructs, giving rise to steady-state spatial concentration gradients, which in turn, can lead to detrimental non-uniformity in the viability and composition of developing tissues [1,2]. On a theoretical basis, steady-state concentration gradients of biomolecules arise when the rate of cell-mediated chemical degradation/consumption of a biomolecule (via receptor-mediated endocytosis, etc.) exceeds its rate of diffusive transport through the construct [2,3]. Accordingly, more pronounced gradients are observed for a higher biomolecule consumption rate and/or lower biomolecule diffusivity. Generally, for small nutrients (e.g., glucose, oxygen) gradients can arise because of their characteristic high rates of cellular consumption but are also mitigated due to their high diffusivity. In contrast, large protein biomolecules (e.g., GFs) are expected to exhibit lower consumption rates but also possess far reduced diffusivities. As such, it remains challenging to predict how these parametric variations for different types of biomolecules manifest into differential concentration gradients in engineered tissues. In this work, we explore the utility of adopting the Damkohler number, which has classically been used to relate chemical reaction rates to mass transfer rates [4], to serve as a novel analytical tool for evaluating the steady-state gradients that arise for specific biomolecules in engineered tissues. Here, we utilize a cartilage TE model system, consisting of chondrocyte-seeded agarose constructs to examine the variation of Damkohler number and accompanied steady state concentration gradients in constructs for a range of different classes of growth-stimulating biomolecules: nutrients (glucose (Glu)), vitamins (ascorbic acid 2-phosphate (AAP)), protein hormones (insulin (Ins)), and GFs (TGF-β1, TGF-β3, EGF, IGF-1, BMP-2 and BMP-7).

METHODS: Reaction-diffusion analytical solution: The steady-state 1D reaction-diffusion equation appears as Eq. (1) where D: diffusivity, C: biomolecule concentration, R_i: consumption rate constant. Eq. (1) can be non-dimensionalized to Eq. (2) where $\bar{C} = C/C_S$; C_S: construct surface conc.; $\bar{x} = x/L$; L: tissue thickness; Damkohler number $(\pi) = R_i L^2/D$. Solving Eq. (2) for the boundary conditions (BCs), where **BC1:** $\bar{C} = 1$ when evaluated at $\bar{x} = 0$ and **BC2:** $d\bar{C}/d\bar{x} = 0$ when evaluated at $\bar{x} = 1$, yields Eq. (3) depicting steady-state gradients of biomolecules as a function of Damkohler number (π).

$$D\frac{d^2C}{dx^2} - R_iC = 0 \ (\mathbf{1}) \qquad \frac{d^2\bar{C}}{d\bar{x}^2} - \pi\bar{C} = 0 \ (\mathbf{2}) \qquad C = C_S\frac{1}{1+e^{2\sqrt{\pi}}}\left(e^{2\sqrt{\pi}-\sqrt{\pi}\frac{x}{L}} + e^{\sqrt{\pi}\frac{x}{L}}\right) \ (\mathbf{3})$$

Damkohler number measures (π): Computation of π requires knowledge of biomolecule diffusivity and cell consumption rate. Biomolecule diffusivity in 2% agarose scaffolds were calculated via interpolation of diffusivity measures on size-matched dextran molecules measured via FRAP (not shown). R_i values were experimentally measured for immature bovine and porcine chondrocytes where cells in monolayer (2×10^6 cells/mL) were exposed to chondrogenic medium (CM), consisting of glucose [4.5mg/mL], AAP [0.45mg/mL], and insulin [6μg/mL], and supplemented with 10 ng/mL TGF-β1, TGF-β3, IGF-1, EGF, BMP-2 or BMP-7. The transient concentration decrease of each biomolecule in medium over 24h was measured via ELISA and curve-fit for R_i assuming first order reaction kinetics and linearly scaled to the cell density in constructs (45×10^6 cells/mL). π was calculated for an L=3 mm construct thickness. **Model validation:** Constructs (∅6×3mm) were exposed to CM supplemented with TGF-β3 (10 ng/ml) for 5 days. Constructs were sub-punched, axially sectioned and biomolecules in each section were buffer extracted and subjected to glucose assay or TGF-β3 ELISA, yielding depth-dependent concentration profiles for these biomolecules. Experimental measures were compared to model generated gradients determined via Eq. (3).

RESULTS: Damkohler number & biomolecule gradient: The normalized biomolecule concentration, $\bar{C}$, versus normalized tissue depth, $\bar{x}$, in a TE construct was plotted for a wide range of Damkohler numbers (π =0.01-1000; Fig 1). Steep state gradient severity increases with π. For $\pi \leq 0.2$ gradients were largely absent. For $\pi > 10$, severe gradients were observed where the concentration at the construct bottom decays to a near zero value.

Damkohler number (π): Measured R_i for biomolecules varied by an order of magnitude, ranging from 2×10^{-5} s^{-1} for insulin to 5×10^{-4} s^{-1} for TGF-β3 (Fig 2A&B). Calculated Damkohler numbers varied by two orders of magnitude, ranging from π =0.6 (glucose) to π =57 (TGF-β3) (Fig 2B). Predicted steady-state gradients reflected these Damkohler numbers—modest gradients were observed for Ins, Glu and AAP while severe gradients for GFs (Fig 3A).

Analytical model validation: Analytical model predictions of steady-state gradients of glucose and TGF-β3 exhibited strong agreement with the experiment measures (R^2=0.67, R^2=0.89 respectively, Fig. 3B).

DISCUSSION: This work advances the use of the nondimensional Damkohler number as a novel tool for estimating biomolecule concentration gradients in engineered tissues. Here we demonstrate that Damkohler number analysis can accurately predict the steady-state distribution of biomolecules in TE cartilage, allowing for a parametric analysis of concentration gradients of different classes of growth-stimulating biomolecules. An intriguing outcome of this analysis is the substantially elevated Damkohler number observed for GFs (π>10) relative to other biomolecules (π<2) for both immature bovine and porcine cells (Fig. 2C). Physically, this disparity arises from the combination of the significantly higher consumption rates and lower diffusivity of GFs relative to glucose and AAP—ultimately, these GFs are consumed by cells at a faster rate and replenished from the medium bath more slowly. This observation suggests that GF transport limitations may constitute the dominant contributor to heterogeneities of developing engineered tissues. This represents an unanticipated outcome given the primary focus of small nutrients in prior transport analysis investigations [3,5].

Damkohler number analysis can serve as an easy-to-implement analytical framework for identifying the dominant transport-limited biomolecules in a wide range of TE applications. This analysis requires measures of two easy-to-measure biomolecule parameters: D and R_i. D can be readily determined via transport characterizations of sized-matched fluorescent tracers or estimated from prior literature characterizations in TE scaffolds. R_i can be determined by monitoring biomolecule depletion in culture media for cell monolayer as performed in this study.

SIGNIFICANCE: This work demonstrates the utility of Damkohler number analysis in TE applications which might guide the development of novel TE cultivation systems to optimize growth-stimulating biomolecule delivery, leading to control of engineered tissue composition and mechanics to improve their performance after clinical implantation.

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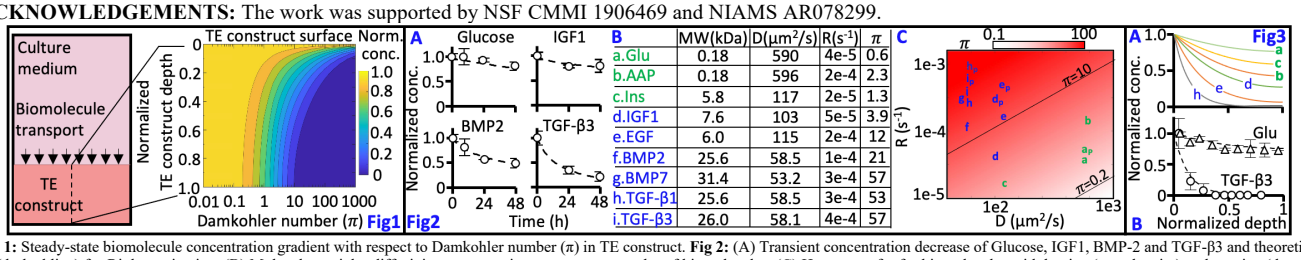

Fig 1: Steady-state biomolecule concentration gradient with respect to Damkohler number (π) in TE construct. **Fig 2:** (A) Transient concentration decrease of Glucose, IGF1, BMP-2 and TGF-β3 and theoretical fit (dashed line) for Ri determination. (B) Molecular weight, diffusivity, consumption rate constant, and π of biomolecules. (C) Heat map of π for biomolecules with bovine (no subscript) and porcine (denoted by 'p' subscript) chondrocytes. Letters denote biomolecules listed in Fig.2B. **Fig 3:** (A) Spatial gradient of different biomolecules in TE cartilage constructs. (B) Experimentally measured (circle&triangle) and analytically predicted (dashed line) steady-state spatial gradient of glucose and TGF-β3 in TE construct after 5 days.