**Optimization of Media Change Intervals Through Hydrogels Using Mathematical Models**

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**ABSTRACT**

Three-dimensional cell culture in engineered hydrogels is increasingly used in tissue engineering and regenerative medicine. The transfer of nutrients, gases, and waste materials through these hydrogels is of utmost importance for cell viability and response, yet the translation of diffusion coefficients into practical guidelines is not well established. Here, we combined mathematical modeling, fluorescent recovery after photobleaching, and hydrogel diffusion experiments on cell culture inserts to provide a multiscale practical approach for diffusion. We observed a dampening effect of the hydrogel that slowed the response to concentration changes and the creation of a diffusion gradient in the hydrogel by media refreshment. Our designed model combined with measurements, provides a practical point-of-reference for diffusion coefficients in real-world culture conditions, enabling more informed choices on hydrogel culture conditions. This model can be improved in the future to simulate more complicated intrinsic hydrogel properties and study the effects of secondary interactions on the diffusion of analytes through the hydrogel.

Keywords: Hydrogel, diffusion, mathematical models, parameter fitting, media change

**INTRODUCTION**

Hydrogels are of increasing interest in regenerative medicine due to their similarities to the extracellular matrix in network formation and water content 1, 2. When cells are encapsulated in hydrogels, diffusion of gases, growth factors, and metabolic waste is of great importance for cell viability and fate 3. In the biomaterials field, materials are usually characterized by diffusion coefficients obtained via fluorescent recovery after photobleaching (FRAP) measurements 4-10. However, these FRAP measurements focus on structural (mesh size) and local diffusion, while understanding diffusion patterns throughout a bulk hydrogel system is mainly overlooked 11. Furthermore, FRAP measurements require expensive and specialized equipment and software to measure the fluorescence intensity of the experiments.

There is a disconnect between the information on local diffusion and the practical translation to cell culture. Therefore, we set out to test the ability to couple fluorescence plate reader measurements with mathematical models to improve our understanding of growth factor availability and diffusion across a hydrogel. We were interested to see if the mathematical models could identify the diffusion coefficients of the hydrogel systems as a time- and cost-effective alternative to FRAP. Various mathematical models have been used to investigate the diffusion coefficients of hydrogels in culture as reviewed in 12-14, ranging from the traditional hydrodynamic radius defined by the Stokes-Einstein equation at the molecular scale 15 to multiscale diffusion models that combine the hydrodynamic, free volume and obstruction theoretical frameworks to capture solute diffusion in, for example, poly(ethylene glycol) (PEG) hydrogels 16. More specifically, the hydrodynamic theory considers the friction between the polymer chains of the hydrogel and solute 17 while the free volume theory assumes that the solute particles move via the dynamic empty spaces between the polymer chains 18 and the obstruction theory models the hindrance caused by the polymer chains 19. These frameworks, in combination with coarse grained simulations such as Brownian dynamics simulations, have been extended to also include electrostatic interactions 20, 21 and binding 22 to better capture the dynamic movement of solutes within hydrogel networks. Recently Amsden systematically compared the predictive quality of four models (i.e. a hydrodynamic model, an obstruction model, an obstruction-exclusion model, and a combined free volume/obstruction model) when either the correlation length or mesh radius were used as approximations for the mesh size 23. Next to these detailed, albeit complex, mathematical models, others have lumped the various interactions into a constant (measured) diffusivity coefficient and used Fick’s law to describe the macroscopic diffusion behavior 24, 25; most recently attempts at multi-scale models have started to provide a unified look at the total picture 16.

Here, we propose an alternative methodology that does not require specialized analytical systems. Instead, we measured the concentration of fluorescently-labeled dextran over time (with a multichannel fluorescence plate reader) that passed through a hydrogel of a specific thickness, and we developed a diffusion coefficient estimation model of the hydrogel. More specifically, we selected 3–5 and 70 kDa dextran for concentration versus time plots to feed into the model, as many growth factors and nutrients, such as albumin, EGF, FGF-2, insulin, and IGF-1, have molecular weights of a similar size 26-28. Using a cell culture insert, we collected experimental concentration versus time data points (up to 48 h) of 3–5 and 70 kDa dextran diffusing across a 2 mm alginate hydrogel cross-linked in two different ways (thiol-ene; Nor-Alg, 2wt %, 11.3 µmol norbornene units, cross-linked with 4-arm PEG-thiol and calcium; Ca2+-Alg, 2 wt%, 83 mM CaCl2, the experimental details in *Dextran concentration versus time data- diffusion sample preparation*) (**Figure 1a,** see *Dextran concentration versus time data- diffusion measurements* for more details). We designed a comprehensive mathematical model in Virtual Cell, an open-source software, to quantify the diffusion coefficients of 3–5 and 70 kDa dextran by a parameter-fitting algorithm (see *Diffusion coefficient estimation* for more details*)*. The fitted diffusion coefficients were compared with FRAP recovery experimental curves, the gold standard in the hydrogel field. We then developed a COMSOL Multiphysics 5.4 model with the estimated diffusion coefficient to investigate the diffusion of growth factors with a similar molecular weight of the 3–5 kDa dextran through the 2 mm hydrogels in relevant cell culture scenarios, such as media refreshments and growth factor degradation. We confirmed that the model reliably simulated cell culture scenarios and highlighted the importance of the growth factor diffusivity of the hydrogels. The controlled and quantified diffusion in hydrogels represents a potent approach to instruct biomimetic development, where controlled signaling gradients instruct cell fate and organoid development, but also to steer differentiation and test the effects of molecules or drugs, requiring immediate signals that can be obtained by controlling the refreshment times of the compartments.

In summary, this study provides proof of concept of a simple, time- and cost-effective alternative to FRAP where fluorescence plate reader measurements are combined with mathematical models to determine the diffusion coefficients in hydrogel systems. In addition, researchers can apply the physical properties of their molecule of interest to the developed models to gain an insight into the best media change practices or the media change intervals that will affect the molecule's availability in culture, which is particularly important when culturing highly sensitive cells on hydrogels. The proposed integrated approach allows for the quantification and characterization of local concentrations in a variety of cell culture systems, providing a connection between local concentration information and cell culture practice.

**EXPERIMENTAL**

**Dextran concentration versus time data- diffusion sample preparation**

***Ca2+ cross-linked hydrogels;*** A 2 wt% alginate (30 mg) stock solution of 1.5 mL was prepared. Cell culture insert (Thincert cell culture inserts, 8 µm pore size, Greiner Bio-One) were covered by the hydrogel solution to form a hydrogel of approximately 2 mm in height (226.2, µL) by gently spreading the solution evenly with a pipet after which 1 mL calcium chloride (9.27 g/L, 110.98 g/mol, 83 mM) was added below the cell culture insert left overnight at RT to ensure complete cross-linking. The hydrogel and cell culture insert were washed with PBS to remove any traces of CaCl2 before real-time diffusion measurements.

***Thiol-ene cross-linked alginate hydrogels;*** Norbornene functionalized alginate (71 mg, 11.3 µmol norbornene units) was dissolved in 2.3 ml PBS overnight at RT. The 4-arm 10 kDa PEG thiol (5 mg, 0.02 mmol SH units, Creative PEGWorks) and LAP UV initiator (3.3 mg, 11.2 µmol, 3.2 mM, Sigma-Aldrich) were dissolved separately in 1.2 ml PBS. The two solutions were added together to form a 2 wt% norbornene functionalized alginate solution. The dissolve norbornene-alginate and 4-arm 5 kDa PEG solution were added to obtain a 2 mm hydrogel on the cell culture inserts. These cell culture inserts with hydrogel solutions were exposed to 365 nm light (10 mW/cm2, UVP CL-1000 ultraviolet cross-linker) for 30 sec. After cross-linking, cell culture inserts with hydrogels were incubated with 500 µL PBS in the bottom compartment overnight at 4°C to ensure complete swelling before diffusion measurements

**Diffusion measurements of Dextran concentration versus time data**

Cell culture inserts with 2 mm thickness Ca2+- or thiol-ene cross-linked alginate hydrogels were transferred into a 12-well plate and 1 mL of 0.1 mg/mL 70 kDa or 1 mg/mL 3–5 kDa dextran was added in the bottom compartment. The cell culture insert top compartment was topped with PBS (773.8 µL) to obtain a total volume of 1 mL (hydrogel+PBS, 226.2+773.8 µL, respectively). Samples (20 µL) from the top and bottom compartments were taken after 5, 15, 30, 45, 60, 120, 240, 260, 1500, and 2940 min and were diluted in 30 µL PBS. The fluorescence intensity was measured at 495 nm excitation and 519 nm emission and the dextran concentration was calculated from a standard curve (Figure S1f).

**A computational model of diffusion through a hydrogel**

***Diffusion coefficient estimation:*** A well-mixed compartmental estimation model was developed in the Virtual Cell V7.4.0 software 29, 30 to estimate the diffusion coefficient of the two hydrogels. The estimation model can be found in the VCell Database as "*Hydrogel Diffusion Coefficient Estimation*” by “aureliecarlier” and can be accessed within the VCell software (available at https://vcell.org). The estimation model used the real-time data points to fit best the diffusion coefficient of 70 and 3–5 kDa dextran described in the **Diffusion measurements of Dextran concentration versus time data** section based on the general first-order Fickian Diffusion equation (equation 1).

|  |  |  |
| --- | --- | --- |
|  |  | eq. 1 |

Where is the flux of the dextran across the hydrogel in µM µm s-1, = unknown diffusion coefficient of dextran through the hydrogel in µm2 s-1, = difference between dextran concentration on either side of the hydrogel in µM, and = thickness of the hydrogel in µm.

The 12-well plate transwell inserts parameters were applied to define the domain geometry (Figure 2a) with a bottom compartment volume of 1 mL, top compartment volume of 0.77 mL, hydrogel thickness of 2E3 µm, and area of 1.13E8 µm2. Here, the hydrogel was modeled as a boundary condition applied over the transwell area with a specified thickness, resulting in a flux boundary condition dependent on the unknown diffusion coefficient of the hydrogel. The initial concentration of dextran in the bottom and top compartments was 3.2 and 0 µM, respectively.

An evolutionary programming algorithm was used to fit the real-time data to the model to find the best fitting diffusion coefficients ( in equation 1) of 3–5 and 70 kDa using the settings specified in **Table 2**.

**Table 2**. COPASI parameter fitting settings for the evolutionary programming solver chosen to fit the diffusion coefficients

|  |  |  |  |
| --- | --- | --- | --- |
| COPASI parameter fitting settings for the evolutionary programming solver | | | |
| Number of generations | 200 | Number of generations the population evolves | |
| Population size | 20 | Number of individuals that survive | |
| Seed | 1 | Random number generator | |
| Number of runs | 10 | Increased number of runs to check if increasing the number of runs alters the fitted value | |
| COPASI parameter fitting settings for guessing fitted values for the diffusion coefficient estimation | | | |
| Parameter | **Initial Guess** | **Lower Limit** | **Upper Limit** |
| [µm2 s-1] | 12 | 1 | 100 |

The root means square error (RMSE) was calculated as shown in equation 2, to evaluate how good the fit was between the average concentration of 3–5 kDa dextran predicted by the estimated diffusion coefficient model and the real-time experimental data at 5, 15, 30, 45, 60, 120, 240, 260, 1500, and 2940 min (Table 2), which correspond to the sampling times of the real-time experimental data points.

|  |  |  |
| --- | --- | --- |
|  |  | eq.2 |

Where, is the average dextran concentration in the top compartment at time of the real-time experiment in µM, is the average dextran concentration in the top compartment at time of the simulation in µM, is the total number of data points, is the index of the data points.

More details on developing the computational models of various cell culture scenarios can be found in the supporting information under, ***Simulating the diffusion effects in various cell culture scenarios***.

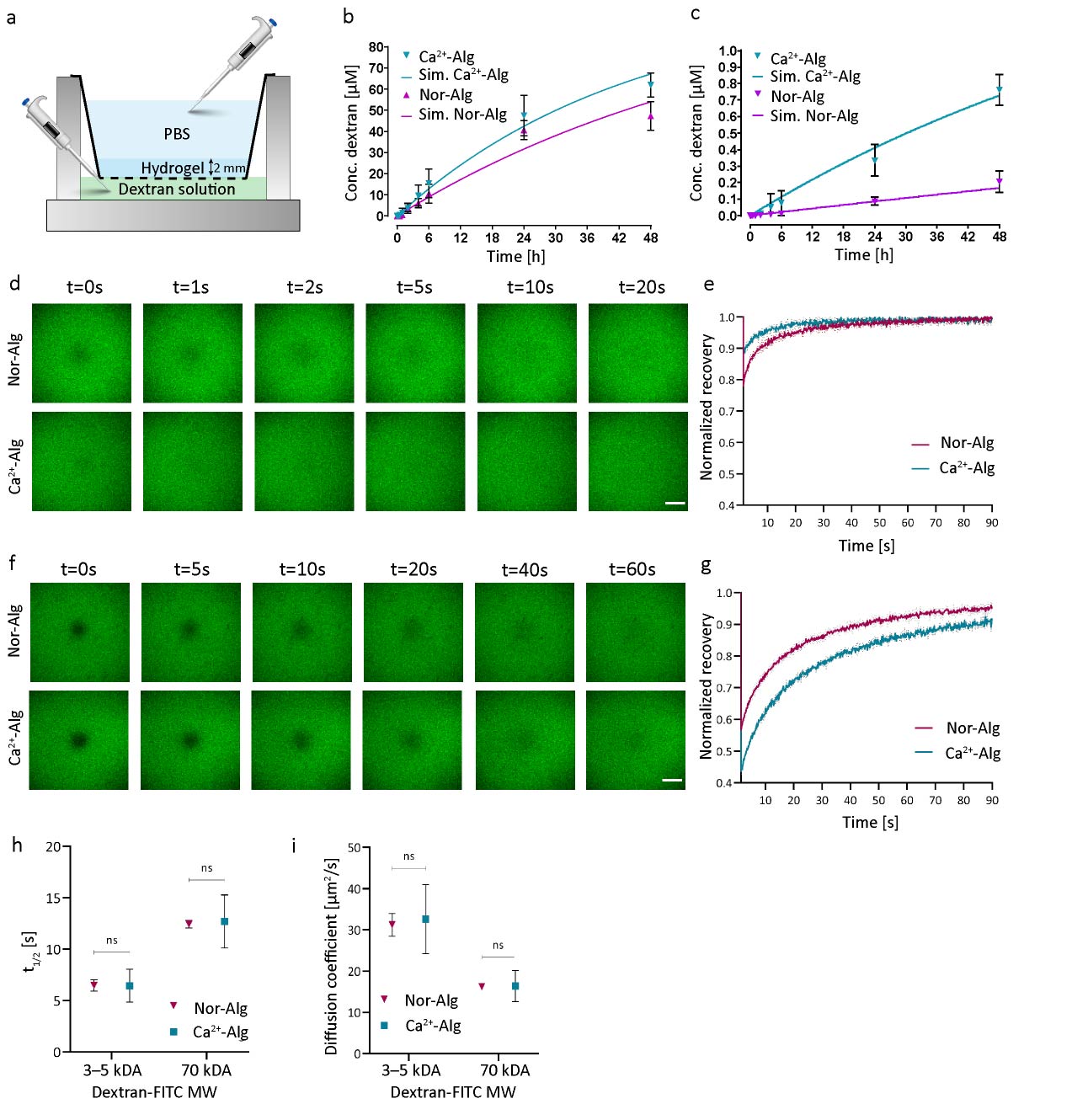
**RESULTS AND DISCUSSION**

We started with a diffusion coefficient (first-order Fickian diffusion) estimation using the obtained time-series data of dextran diffusion through the hydrogel (**Figure 1a-c**). Then, we compared the diffusion through two different cross-linked alginate hydrogel systems: based on 1) a covalent cross-linking via thiol-ene chemistry (Nor-alg) or 2) an ionic cross-linking of alginate with calcium (Ca2+-alg). The resultant diffusion coefficient estimations from the dextran concentration versus time data were 31 and 15 µm2/s for the 3–5 and 70 kDa dextrans in the Nor-alg hydrogel, respectively, while the Ca2+-alg hydrogel had diffusion coefficients of 48 and 23 µm2/s for the 3–5 and 70 kDa dextrans, respectively (**Table 1**). The estimated diffusion coefficients were tested against the time-series data by calculating the root mean square error (RMSE) between the experimental and predicted data **(Figure 1b and c and Table 2).** The simulated time-series data were observed to fit well against the experimental data (**Figure 1b–c,** solid line) as the calculated RMSE for both fitted diffusion coefficients was less than 15% of the fitted value.

Additionally, the simulated concentration versus time curves were within the standard deviation of their corresponding experimental data points (**Figure 1b-c**). The accuracy of the *diffusion coefficient estimation model* was highly sensitive to the initial dextran concentration set in both the top and bottom compartments since this was when the steepest concentration gradient existed in the model (0 µM in the top versus 3.2 µM in the bottom compartment).

**Table 1.** Fitted diffusion coefficient from dextran concentration versus time data vs. FRAP data for 3–5 kDa and 70 kDa in the two different hydrogel systems (Thiol-ene cross-linked: Nor-Alg and Calcium cross-linked: Ca2+-Alg). The transwell experiments were done for two different transwell pore sizes resulting in different fitted diffusion coefficients for 70 kDa, whereas the FRAP data was performed in N=3 individual bleaching areas within the dextran-containing hydrogel.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Thiol-ene Cross-linked Alginate** | | | **Calcium Cross-linked Alginate** | | | |
| **Dextran Size [kDa]** | 3–5 | 70 | 70 | 3–5 | 70 | | 70 |
| **FRAP Diffusion Coefficient (SD) [µm2/s]** | 31.23  (± 2.75) | 16.72  (± 0.48) | 16.72  (± 0.48) | 32.63  (± 8.37) | 16.38  (±3.75) | | 16.38  (±3.75) |
| **Transwell pore size [µm]** | 0.2 | 0.2 | 8 | 0.2 | 0.2 | 8 | |
| **Fitted Diffusion Coefficient in Transwell (RMSE) [µm2/s]** | 31.30  (3.80) | 4.36  (7.8E-4) | 15.85  (9.00E-3) | 48.39  (2.70) | 1.13  (2.20E-2) | 23.05  (1.5E-3) | |

**Figure 1.** a) Diffusion time-series data schematic of the experimental setup. Approximately 2 mm-thick hydrogels were cross-linked on a Cell culture insert filter and the diffusion of 3–5 kDa and 70 kDa dextran from the bottom to the top was determined by measuring the concentration in the top compartment for 48 h (N=6 Cell culture inserts with hydrogels). Diffusion time-series data, the concentration of b) 3–5 kDa and c) 70 kDa dextran in the top compartment after diffusion through the hydrogels (Nor-Alg and Ca2+-Alg). A comparison of the experimentally measured diffusion of b) 3–5 kDa and c) 70 kDa dextran through Nor-Alg or Ca2+-Alg compared to the fitted diffusion coefficient estimation model (solid lines). d) Fluorescence recovery after photobleaching (FRAP) images of 3–5 kDa dextran from 0–60 s in Nor-Alg (top) and Ca2+-Alg (bottom) hydrogels, and e) the corresponding recovery curves (N=3 difference bleaching areas, scale bar:100 µm). f) FRAP images of 70 kDa dextran from 0–20 s in Nor-Alg (top) and Ca2+-Alg (bottom) hydrogels, and g) the corresponding recovery curves (n=3 difference bleaching areas, scale bar=100 µm). h) The half-time recovery (t1/2) and i) diffusion coefficients were not significantly different (ns, one-way ANOVA) for either 3–5 or 70 kDa dextran in Nor-Alg and Ca2+-Alg.

With data from the model in hand, we wanted to utilize FRAP measurements to compare the experimental data of the two hydrogels. Therefore, the FRAP recovery curves for 3–5 and 70 kDa were generated (**Figure 1 d–g**), the τ1/2 were extracted (**Figure 1h**), and diffusion coefficients were calculated by the Soumpasis equation (eq. S1, supporting information; **Figure 1i**). There was no significant difference in the FRAP diffusion coefficients from Nor-Alg compared to Ca2+-Alg hydrogels (one-way ANOVA, *p*=0.4633). We did notice that the Ca2+ alginate samples had slightly different diffusion values between the FRAP and experimental model; this is likely attributed to the known inhomogeneity of CaCl2 crosslinked alginates on large scales. While the FRAP data accurately captured the local diffusion coefficients, the large thickness and inhomogeneity of crosslinking across the sample was likely better represented in the transwell experiment.

We chose to test two cell culture insert pore sizes, namely 8 µm pore size, as these have large pores that do not interfere with the diffusion measurements, and 0.2 µm pore size, as these are often used in cell culture experiments. The estimated diffusion coefficients from the mathematical model were comparable to those from the FRAP measurements (**Table 1**) for the 3–5 and 70 kDa dextran performed on 8 µm pore size cell culture inserts (**Figure 1h-i and S1c)**. The result showed that the fluorescence plate reader measurements of dextran concentration versus time (taking into account the appropriately sized membrane pore size) could be used to calculate the diffusion coefficients accurately. However, we observed that the 70 kDa diffusion experiments performed on 0.2 µm pore size cell culture inserts differed significantly from the corresponding FRAP diffusion coefficients (**Table 1**). We showed that the 0.2 µm pore size resulted in an extra barrier to the diffusion of large nutrients (70 kDa dextrans). Indeed, both pore size and dextran molecular weights need to be considered as distributions, resulting in a proportion of dextrans to have a hydrodynamic diameter of more than 30 nm, and the pores to have sizes around 100 nm. At these values, free diffusion is replaced by pore diffusion, which confounds the measurements. While piecing out this subtlety remains challenging, we can show that the pore size should be carefully considered when designing an experiment with cell culture inserts. Therefore, we used the data obtained from cell culture inserts with the 8 µm pore size in the case studies to follow.

With a calibrated mathematical model in hand, we then sought to quantify the diffusion of growth factors throughout a hydrogel in four cases relevant for cell culture: 1) changing culture media in the bottom cell culture insert compartment, 2) changing media in the bottom cell culture insert compartment and PBS in the top compartment, 3) increasing the media growth factor concentration with a bolus injection of growth factor in the bottom compartment after 12 h, and, 4) changing culture media in the bottom compartment including a growth factor decay rate. We designed a mathematical model in COMSOL Multiphysics 5.4 to simulate growth factor diffusion through the 2 mm Nor-alg (**Figure 2 and S5**) and Ca2+-alg hydrogel (**Figure S3-4**) using the estimated diffusion coefficients in 8 µm pore size cell culture insert displayed in Table 1. The diffusion coefficients of the dextran molecules in the media were assumed to be the same as those measured in PBS by FRAP (**see data Figure S2**).

R:\Carlier group\Shared folders\Sangita\Hydrogel paper figures_FP edit_reduced size\Figure 2_hyrogel paper_FP edit_Reduced.tif**Figure 2. a)** A computational model of growth factor transport in a hydrogel system was designed based on the dextran concentration versus time experimental setup. The height of the bottom compartment is 8.85 mm, the height of the hydrogel is 2 mm and the height of the top compartment is 6.85 mm. Four case studies were simulated in the Nor-alg hydrogel system using the diffusion coefficient for 3–5 kDa dextran of 31.3 µm2/s. The top and hydrogel compartments had initial concentrations of 0 µM dextran, and the bottom compartment had an initial concentration of 3.2 µM dextran for all cases. **b)** Case 1: the concentration (µM) of 3–5 kDa dextran at the middle of a 2 mm–thick Nor-alg hydrogel was determined over time when the bottom compartment was refreshed with the starting dextran concentration of the bottom compartment (i.e. 3.2 µM) at **i)** 12, **ii)** 24, and **iii)** 48 h. **c)** Case 2: the concentration (µM) of 3–5 kDa dextran at the middle of a 2 mm–thick hydrogel was determined over time when the top and bottom compartments were refreshed with the starting dextran concentration of the bottom compartment (i.e. 3.2 µM) at **i)** 12, **ii)** 24, and **iii)** 48 h. **d)** Case 3: the concentration (µM) of 3–5 kDa dextran was determined overtime when the bottom compartment was refreshed with concentration bursts (1.6, 6.4, and 9.6 µM) of 3–5 kDa dextran after 12 h of 3.2 µM concentration in the **i)** the middle of the 2 mm–thick hydrogel (red dot) and **ii)** at the top of the 2 mm–thick hydrogel (blue dot). e) Case 4: the concentration (µM) of IGF-1 with a halftime of 17h and FGF2 with a halftime of 27h at the **i)** middle (red), and **ii)** top (blue) of a 2 mm–thick hydrogel as determined over time when the bottom compartment was refreshed at 12, 24 and 48 h with 3.2 µM. Grey vertical striped lines represent media refreshments points. The colors of the lines represent the sampling position. Figure S5 provides additional information on the concentration data for the Nor-alg simulations at the top and bottom of the hydrogel. Similar results, but for the Ca2+-alg hydrogel can be found in the supplement (Figures S3 and S4).

*Case 1. Media change in bottom compartment.* We modeled the scenario where the media was changed below the hydrogel (bottom compartment) at different time intervals (12, 24, and 48 h.) while leaving the top of the hydrogel (top compartment) without refreshment resulting in the curves presented in **Figure 2b**. Here we assumed that growth factors and waste of ±3–5 kDa sizes, such as EGF, FGF-2, insulin, and IGF-1, would diffuse at a similar rate as the dextran diffusion coefficient data obtained (for the results with other diffusion coefficients, see Figure S6). The 3–5 kDa dextran concentration gradient into the hydrogel was observed to decrease at each subsequent media change interval. Here, it was shown that Case 2 achieved a steady-state after two media changes of both 12 h and 24 h intervals. This was considerably faster than Case 1, meaning that a researcher should change both the top and bottom compartments if they want to develop a steady-state concentration of growth factors across the hydrogel as fast as possible. This can be needed in systems where immediate signals are relevant, for example for directed differentiation and the testing of drugs or screening of molecules. More specifically, in the first case, when specific signaling pathways need to be induced for specific durations in a cell culture system without a hydrogel, the cells receive these signals immediately as a pulse (upon adding them to the culture medium) 31. When translating such a system to a hydrogel set-up, the diffusion alters the dynamics in which the signals are provided, and in order to be comparable and provide the required pulsed stimuli the steady state concentration needs to be established as fast as possible in a hydrogel set-up by changing the medium in both top and bottom compartment (case 2). Similarly, when testing or screening drugs or molecules for toxicity in, for example, organoids or aggregates of cells (which are often encapsulated in a hydrogel to improve how well they mimic the tissue), it is important to understand how quickly these drugs/molecules reached the cells and at what dose 32.

The concentration of growth factors above the hydrogel remained steady, and the final concentration after 96 h in the top compartment was similar for all the media change intervals, approximately 1.3–1.6 µM for 3–5 kDa dextran (**Figure S5a**). Calculations from the middle of the hydrogel showed increased growth factor concentration immediately after refreshing the media at all time intervals. However, the hydrogel, acting as a diffusion barrier, reduced the concentration gradient, resulting in a smooth concentration curve measured in the top compartment of the cell culture insert (**Figure S5a**). The observed plateaus in the concentration curves within the hydrogels in Case 1 indicated a dampening capacity of the hydrogel (**Figure 2b and video S1**). We use the term "dampening capacity" to define the hydrogel's ability to dissipate significant disturbances from the compartment below when the media is changed. Due to the dampening capacity of the hydrogel, the cells cultured on the top of the hydrogel would sense gradual changes in concentration, despite repeated refreshment or bolus injections.

*Case Study 2. Media and PBS change in the bottom and top compartments*. To further explore the dampening capacity of the hydrogels, a new model was simulated to include analyte removal in the top compartment. When Case 2 was compared with Case 1, it was observed that the dampening property in the middle of the hydrogel (**Figure 2b, iv-vi, and Figure S3c**) occurred in a shorter time frame. Figure 2b iv-vi shows the concentration difference between the time intervals was factually zero after the second refreshment in Case 2 for all simulated intervals, while Case 1 was still establishing its steady-state by the last refreshment simulated. This finding shows the potential to create a diffusion gradient in a hydrogel system by simply changing the media or PBS in the bottom and top compartments. Furthermore, this stable diffusion gradient is noticeable when visualizing Cases 1 and 2 in two-dimensional space (**video S1**), where the dextran concentration in the hydrogel is gradually affected by the sudden media refreshments. Similarly, solute removal (reflecting for example metabolite removal), results in concentration fluctuations in the top and bottom part of the hydrogel whereas the concentration within the hydrogel reaches a steady state level (see figure S7). Note that Case 2 represents a means to control diffusion gradients with an in vitro hydrogel culture, which is important to instruct biomimetic development 33. Indeed, the gradients of growth factors and signaling molecules are extremely important in the culture of more advanced multi-cellular models like organoids since development is carefully controlled by signaling gradients that control and regulate cell fate in a concentration dependent manner.

*Case Study 3. Concentration burst/bolus injection.* We then simulated how changes in the concentration of the 3–5 kDa growth factors (bolus injections of 1.6, 3.2, 6.4, or 9.4 µM) in the bottom compartment after 12 h would affect the simulated growth factor gradient. As expected, the overall concentration of 3–5 kDa dextran at 24 h post-refreshment (36 h time-point) increased in the top compartments from 1.1 to 3.2 and 4.7 µM in the states of 6.4 or 9.4 µM bolus respectively (**Figure 2d and S3d**), and decreased to 0.9 and 0.5 in the middle and top compartment respectively for 1.6 µM bolus. In addition, a slight delay in increasing growth factors of 1 h was observed in the middle and top compartment. These results show the possibility of changing the growth factor concentration within the hydrogel without a significant burst by gradually increasing or decreasing the growth factor concentration.

*Case Study 4. Growth factor decay.* We modeled the diffusion profiles of FGF-2 and IGF-1 as they decay based on their half-life and estimated diffusion coefficient. Since cells are typically cultured on or within the hydrogels, we explored the effect of the growth factors concentration in the middle and top of the hydrogels. **Figure 2e and S2e** (red and blue curve, respectively) show that upon refreshing the media in the bottom compartment at 12 and 24 h intervals, IGF-1 (Figure 2e i-iii) reached a minimum concentration of 0.38 and 0.25 µM in the top of the hydrogel, respectively, and therefore was available to the cells. However, if the media is changed every 48 h, IGF-1 concentration decreased to 0.12 µM before refreshment and is less than 0.2 µM for the last 13 hours of culture at the top of the hydrogel. It was seen that cells had significantly slower cell doubling times when treated with 5 ng.mL-1 (0.7 µM) IGF-1 compared to 20 ng/mL (2.7 µM) 34. Therefore, IGF-1 availability is insufficient for the cells over an extended culture period when media is changed every 48 h, suggesting more frequent media changes should be performed when using 2 mm thick hydrogels. Similarly, FGF-2 (Figure 1e iv-vi) had a minimum concentration of 0.57, 0.36, and 0.26 µM available to the cells on top of the hydrogel at 12, 24, and 48-hour intervals, respectively. These results showed that the frequency of media refreshments should be carefully considered in the experimental design, as each growth factor's half-life directly affects growth factor diffusion and availability in culture over time. This is exemplified with IGF-1, which has a shorter decay time compared to FGF-2, resulting in less availability in the middle and top of the hydrogel.

Our results show that the dextran concentration versus time data through the hydrogel can be used to approximate the diffusion coefficient of macromolecules diffusing through a hydrogel with a mathematical model. Moreover, the results show that this methodology is a reliable alternative to FRAP experiments, and may better take into account gel spatial inhomogeneity. This is especially interesting for research groups without reliable access to confocal microscopy but starting with standard wet lab equipment and reagents.

In our studies, we noticed a profound dampening capacity of the hydrogel system. This result indicates that the hydrogel system can accommodate a limited but stable growth factor concentration in the cell environment compared to a conventional monolayer culture in which the growth factor concentration fluctuates due to refreshment intervals. Moreover, this model showed the potential of maintaining a diffusion gradient throughout a hydrogel by simply refreshing the solution around it. However, over time, the degradation of growth factors needs to be considered when deciding on media change frequency, as there might be too few growth factors available for an extended period before the next media refreshment. Therefore, the frequency of media change or the growth factor concentration of interest can be increased to ensure sufficient growth factor availability in the hydrogel. Mehrian et al. similarly demonstrated that neotissue growth supported by a 3D scaffold in a perfused bioreactor was maximized with a high frequency of media refreshments and increased concentration 35. Similarly, this model can be used to optimize scenarios where less frequent media changes are preferred, as the cultured cells rely on the secreted products to proliferate or self-organize.

The observed dampening capacity of the hydrogels can be both an advantage and disadvantage for cell culture. Firstly, the dampening prevents the cells from experiencing a "growth factor shock/stress" with cell culture media changes 35. Instead, the concentration gradually increases to a steady-state, which can be maintained when carefully considering the correct media change interval and growth factor decay patterns (simulated in our model). Secondly, growth factor levels can be increased, without the initial burst observed when cells are cultured in media only by applying a higher concentration of growth factors during media changes. Moreover, we observed a steady gradient within the hydrogel system by simply refreshing the compartments above and below the hydrogel. This can be of interest for research focused on the influence of gradients within a biological system to guide cell behavior such as migration, which to date are obtained through microfluidic devices that establish growth factor gradients by flow 36 or via porosity changes to facilitate diffusion coefficient gradients within the hydrogel 37. Although this gradual concentration change and dampening capacity have their advantages, in some cases, a short burst of specific growth factors is required, for example, in the various differentiation protocols of pluripotent stem cells 38.

There were assumptions made in designing the mathematical models for this study, leading to potential improvements to be made in the future. In particular, as described in the *diffusion coefficient estimation model*, the hydrogel was simplified as a flux boundary condition dependent on the thickness and diffusion coefficient of the hydrogel. The spatial components of the hydrogel were simplified to generalized diffusive flux to make the model fitting possible. It is known that, next to diffusing through, proteins can bind to the hydrogel or to the surface receptors of cells present within the hydrogel. For example, Limasale et al. used a combined experimental-computational approach, to understand and predict the local concentration of free and bound vascular endothelial growth factor 165 (VEGF165) in glycosaminoglycan (GAG) networks, including both protein diffusion and GAG-binding 25. Similar approaches could be taken in the future to account for (un)binding reactions that will affect local concentrations and potential gradients. Considering that an increase in complexity will also increase the calculation time for finding the optimal media refreshments settings, it would be interesting to derive statistical relations that directly link the required (input) constraints to the optimal settings, see for example 39. Finally, it would be interesting to explore other hydrogel systems to explore whether the proposed methodology is generic.

**CONCLUSION**

This study provides proof of concept of a simple, time- and cost-effective alternative to FRAP where fluorescence plate reader measurements are combined with mathematical models to determine the diffusion coefficients in hydrogel systems. We presented a mathematical model for fitting the diffusion coefficients of 3–5 and 70 kDa dextran through two different hydrogels with open-source software (Virtual Cell). The obtained diffusion coefficients were comparable to those measured through traditional methods (FRAP). Furthermore, we developed four computational scenarios relevant to typical cell culture practices and could visualize the hydrogels' dampening effect, where the dampening concentration was tunable based on the refreshment method. We showed the importance of considering the decay rate of essential growth factors, which affects their availability for the cells cultured on top of the hydrogels. We also provided evidence that scientists need to be careful when selecting their hydrogels and cell culture methods, for which our proposed integrated approach can be a valuable tool to quantify and characterize the local nutrient and growth factor concentrations, providing a connection between local concentration information and cell culture practice.

**SUPPORTING INFORMATION**

**Methodological details:** alginate purification, norbornene-alginate synthesis, FRAP sample preparation, FRAP measurements, statistics, computational modeling information

**Supporting figures**

**ACKNOWLEDGMENTS**

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**REFERENCES**

1. A.J. Feliciano, C. van Blitterswijk, L. Moroni and M.B. Baker, *Realizing tissue integration with supramolecular hydrogels.* Acta Biomater., 2021. **124**: p. 1-14.

2. N. Mitrousis, A. Fokina and M.S. Shoichet, *Biomaterials for cell transplantation.* Nat. Rev. Mater., 2018. **3**(11): p. 441-456.

3. R.J. McMurtrey, *Roles of Diffusion Dynamics in Stem Cell Signaling and Three-Dimensional Tissue Development.* Stem Cells Dev., 2017. **26**(18): p. 1293-1303.

4. M. Kang, C.A. Day, A.K. Kenworthy and E. DiBenedetto, *Simplified Equation to Extract Diffusion Coefficients from Confocal FRAP Data.* Traffic, 2012. **13**(12): p. 1589-1600.

5. N. Lorén, M. Nydén and A.-M. Hermansson, *Determination of local diffusion properties in heterogeneous biomaterials.* Advances in Colloid and Interface Science, 2009. **150**(1): p. 5-15.

6. R. Kohze, C.E.J. Dieteren, W.J.H. Koopman, R. Brock and S. Schmidt, *Frapbot: An open-source application for FRAP data.* Cytometry Part A, 2017. **91**(8): p. 810-814.

7. J. Karvinen, T.O. Ihalainen, M.T. Calejo, I. Jönkkäri and M. Kellomäki, *Characterization of the microstructure of hydrazone crosslinked polysaccharide-based hydrogels through rheological and diffusion studies.* Mater. Sci. Eng., C, 2019. **94**: p. 1056-1066.

8. S.T. Lust, D. Hoogland, M.D.A. Norman, C. Kerins, J. Omar, G.M. Jowett, T.T.L. Yu, Z. Yan, J.Z. Xu, and D. Marciano, *Selectively cross-linked tetra-PEG hydrogels provide control over mechanical strength with minimal impact on diffusivity.* ACS Biomater. Sci. Eng., 2021. **7**(9): p. 4293-4304.

9. A.M. Alexander and S.D. Lawley, *Inferences from FRAP data are model dependent: A subdiffusive analysis.* Biophys. J., 2022. **121**(20): p. 3795-3810.

10. N.R. Richbourg and N.A. Peppas, *High-Throughput FRAP Analysis of Solute Diffusion in Hydrogels.* Macromolecules, 2021. **54**(22): p. 10477-10486.

11. M.H. Hettiaratchi, A. Schudel, T. Rouse, A.J. García, S.N. Thomas, R.E. Guldberg and T.C. McDevitt, *A rapid method for determining protein diffusion through hydrogels for regenerative medicine applications.* APL bioeng., 2018. **2**(2): p. 026110.

12. M. Quesada-Pérez and A. Martín-Molina, *Solute diffusion in gels: Thirty years of simulations.* Adv. Colloid Interface Sci., 2021. **287**: p. 102320.

13. B. Amsden, *Solute Diffusion within Hydrogels. Mechanisms and Models.* Macromolecules, 1998. **31**(23): p. 8382-8395.

14. D. Caccavo, S. Cascone, G. Lamberti and A.A. Barba, *Hydrogels: experimental characterization and mathematical modelling of their mechanical and diffusive behaviour.* Chem. Soc. Rev., 2018. **47**(7): p. 2357-2373.

15. C.C. Miller, *The Stokes-Einstein law for diffusion in solution.* Proc. R. Soc. London, Ser. A, Containing Papers of a Mathematical and Physical Character, 1924. **106**(740): p. 724-749.

16. E. Axpe, D. Chan, G.S. Offeddu, Y. Chang, D. Merida, H.L. Hernandez and E.A. Appel, *A multiscale model for solute diffusion in hydrogels.* Macromolecules, 2019. **52**(18): p. 6889-6897.

17. R.I. Cukier, *Diffusion of Brownian spheres in semidilute polymer solutions.* Macromolecules, 1984. **17**(2): p. 252-255.

18. M.H. Cohen and D. Turnbull, *Molecular transport in liquids and glasses.* J. Chem. Phys., 1959. **31**(5): p. 1164-1169.

19. J.S. Mackie and P. Meares, *The diffusion of electrolytes in a cation-exchange resin membrane I. Theoretical.* Proc. R. Soc. London, Ser. A, 1955. **232**(1191): p. 498-509.

20. M.J. Grill, J.F. Eichinger, J. Koban, C. Meier, O. Lieleg and W.A. Wall, *A novel modelling and simulation approach for the hindered mobility of charged particles in biological hydrogels.* Proc. R. Soc. London, Ser. A, 2021. **477**(2249): p. 20210039.

21. L. Johansson, U. Skantze and J.E. Loefroth, *Diffusion and interaction in gels and solutions. 6. Charged systems.* J. Phys. Chem., 1993. **97**(38): p. 9817-9824.

22. S.K. Ghosh, A.G. Cherstvy and R. Metzler, *Non-universal tracer diffusion in crowded media of non-inert obstacles.* Phys. Chem. Chem. Phys., 2015. **17**(3): p. 1847-1858.

23. B.G. Amsden, *Hydrogel Mesh Size and Its Impact on Predictions of Mathematical Models of the Solute Diffusion Coefficient.* Macromolecules, 2022. **55**(18): p. 8399-8408.

24. H.-H. Hsu, J.-K. Kracht, L.E. Harder, K. Rudnik, G. Lindner, K. Schimek, U. Marx and R. Pörtner, *A method for determination and simulation of permeability and diffusion in a 3D tissue model in a membrane insert system for multi-well plates.* J. Visualized Exp., 2018(132): p. e56412.

25. Y.D.P. Limasale, P. Atallah, C. Werner, U. Freudenberg and R. Zimmermann, *Tuning the Local Availability of VEGF within Glycosaminoglycan-Based Hydrogels to Modulate Vascular Endothelial Cell Morphogenesis.* Adv. Funct. Mater., 2020. **30**(44): p. 2000068.

26. I. Ding and A.M. Peterson, *Half-life modeling of basic fibroblast growth factor released from growth factor-eluting polyelectrolyte multilayers.* Sci. Rep., 2021. **11**(1): p. 1-13.

27. N. Mizuno, Y. Kato, M. Iwamoto, A. Urae, T. Amamoto, T. Niwa and Y. Sugiyama, *Kinetic analysis of the disposition of insulin-like growth factor 1 in healthy volunteers.* Pharm. Res., 2001. **18**(8): p. 1203-1209.

28. K. Chlebová, V. Bryja, P. Dvorak, A. Kozubík, W.R. Wilcox and P. Krejci, *High molecular weight FGF2: the biology of a nuclear growth factor.* Cell. Mol. Life Sci., 2009. **66**(2): p. 225-235.

29. A.E. Cowan, I.I. Moraru, J.C. Schaff, B.M. Slepchenko and L.M. Loew, *Spatial modeling of cell signaling networks*, in *Methods in cell biology*. 2012, Elsevier. p. 195-221.

30. J. Schaff, C.C. Fink, B. Slepchenko, J.H. Carson and L.M. Loew, *A general computational framework for modeling cellular structure and function.* Biophys. J., 1997. **73**(3): p. 1135-1146.

31. Jeremy E. Purvis and G. Lahav, *Encoding and Decoding Cellular Information through Signaling Dynamics.* Cell, 2013. **152**(5): p. 945-956.

32. O. Degerstedt, J. Gråsjö, A. Norberg, E. Sjögren, P. Hansson and H. Lennernäs, *Drug diffusion in biomimetic hydrogels: importance for drug transport and delivery in non-vascular tumor tissue.* Eur. J. Pharm. Sci., 2022. **172**: p. 106150.

33. C.R. Marti-Figueroa and R.S. Ashton, *The case for applying tissue engineering methodologies to instruct human organoid morphogenesis.* Acta Biomater., 2017. **54**: p. 35-44.

34. Y.-l. Huang, R.-f. Qiu, W.-y. Mai, J. Kuang, X.-y. Cai, Y.-g. Dong, Y.-z. Hu, Y.-b. Song, A.-p. Cai, and Z.-g. Jiang, *Effects of insulin-like growth factor-1 on the properties of mesenchymal stem cells in vitro.* J. Zhejiang Univ., Sci., B, 2012. **13**(1): p. 20-28.

35. M. Mehrian, Y. Guyot, I. Papantoniou, S. Olofsson, M. Sonnaert, R. Misener and L. Geris, *Maximizing neotissue growth kinetics in a perfusion bioreactor: an in silico strategy using model reduction and Bayesian optimization.* Biotechnol. Bioeng., 2018. **115**(3): p. 617-629.

36. J. Rosser, B. Bachmann, C. Jordan, I. Ribitsch, E. Haltmayer, S. Gueltekin, S. Junttila, B. Galik, A. Gyenesei, and B. Haddadi, *Microfluidic nutrient gradient–based three-dimensional chondrocyte culture-on-a-chip as an in vitro equine arthritis model.* Mater. Today Bio, 2019. **4**: p. 100023.

37. S. Sant, M.J. Hancock, J.P. Donnelly, D. Iyer and A. Khademhosseini, *Biomimetic gradient hydrogels for tissue engineering.* Can. J. Chem. Eng., 2010. **88**(6): p. 899-911.

38. M. Takasato, P.X. Er, H.S. Chiu, B. Maier, G.J. Baillie, C. Ferguson, R.G. Parton, E.J. Wolvetang, M.S. Roost, and S.M. Chuva de Sousa Lopes, *Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis.* Nature, 2015. **526**(7574): p. 564-568.

39. M. Mehrian and L. Geris, *Optimizing neotissue growth inside perfusion bioreactors with respect to culture and labor cost: a multi-objective optimization study using evolutionary algorithms.* Comput. Methods Biomech. Biomed. Eng., 2020. **23**(7): p. 285-294.

C:\Users\a.carlier\DATA\publications\under construction\Jasia-Floor hydrogel diffusion\Submitted to BioMacromolecules\revisions\0Hydrogel Paper\Graphic_update.tif

1. A.J. Feliciano, C. van Blitterswijk, L. Moroni and M.B. Baker, *Realizing tissue integration with supramolecular hydrogels.* Acta Biomaterialia, 2021. **124**: p. 1-14.

2. N. Mitrousis, A. Fokina and M.S. Shoichet, *Biomaterials for cell transplantation.* Nature Reviews Materials, 2018. **3**(11): p. 441-456.

3. R.J. McMurtrey, *Roles of Diffusion Dynamics in Stem Cell Signaling and Three-Dimensional Tissue Development.* Stem Cells and Development, 2017. **26**(18): p. 1293-1303.

4. M. Kang, C.A. Day, A.K. Kenworthy and E. DiBenedetto, *Simplified Equation to Extract Diffusion Coefficients from Confocal FRAP Data.* Traffic, 2012. **13**(12): p. 1589-1600.

5. N. Lorén, M. Nydén and A.-M. Hermansson, *Determination of local diffusion properties in heterogeneous biomaterials.* Advances in Colloid and Interface Science, 2009. **150**(1): p. 5-15.

6. R. Kohze, C.E.J. Dieteren, W.J.H. Koopman, R. Brock and S. Schmidt, *Frapbot: An open-source application for FRAP data.* Cytometry Part A, 2017. **91**(8): p. 810-814.

7. J. Karvinen, T.O. Ihalainen, M.T. Calejo, I. Jönkkäri and M. Kellomäki, *Characterization of the microstructure of hydrazone crosslinked polysaccharide-based hydrogels through rheological and diffusion studies.* Materials Science and Engineering: C, 2019. **94**: p. 1056-1066.

8. S.T. Lust, D. Hoogland, M.D.A. Norman, C. Kerins, J. Omar, G.M. Jowett, T.T.L. Yu, Z. Yan, J.Z. Xu, and D. Marciano, *Selectively cross-linked tetra-PEG hydrogels provide control over mechanical strength with minimal impact on diffusivity.* ACS biomaterials science & engineering, 2021. **7**(9): p. 4293-4304.

9. A.M. Alexander and S.D. Lawley, *Inferences from FRAP data are model dependent: A subdiffusive analysis.* Biophysical Journal, 2022. **121**(20): p. 3795-3810.

10. N.R. Richbourg and N.A. Peppas, *High-Throughput FRAP Analysis of Solute Diffusion in Hydrogels.* Macromolecules, 2021. **54**(22): p. 10477-10486.

11. M.H. Hettiaratchi, A. Schudel, T. Rouse, A.J. García, S.N. Thomas, R.E. Guldberg and T.C. McDevitt, *A rapid method for determining protein diffusion through hydrogels for regenerative medicine applications.* APL bioengineering, 2018. **2**(2): p. 026110.

12. M. Quesada-Pérez and A. Martín-Molina, *Solute diffusion in gels: Thirty years of simulations.* Advances in Colloid and Interface Science, 2021. **287**: p. 102320.

13. B. Amsden, *Solute Diffusion within Hydrogels. Mechanisms and Models.* Macromolecules, 1998. **31**(23): p. 8382-8395.

14. D. Caccavo, S. Cascone, G. Lamberti and A.A. Barba, *Hydrogels: experimental characterization and mathematical modelling of their mechanical and diffusive behaviour.* Chem. Soc. Rev., 2018. **47**(7): p. 2357-2373.

15. C.C. Miller, *The Stokes-Einstein law for diffusion in solution.* Proc. R. Soc. London Series A, Containing Papers of a Mathematical and Physical Character, 1924. **106**(740): p. 724-749.

16. E. Axpe, D. Chan, G.S. Offeddu, Y. Chang, D. Merida, H.L. Hernandez and E.A. Appel, *A multiscale model for solute diffusion in hydrogels.* Macromolecules, 2019. **52**(18): p. 6889-6897.

17. R.I. Cukier, *Diffusion of Brownian spheres in semidilute polymer solutions.* Macromolecules, 1984. **17**(2): p. 252-255.

18. M.H. Cohen and D. Turnbull, *Molecular transport in liquids and glasses.* The Journal of Chemical Physics, 1959. **31**(5): p. 1164-1169.

19. J.S. Mackie and P. Meares, *The diffusion of electrolytes in a cation-exchange resin membrane I. Theoretical.* Proc. R. Soc. London Series A. Mathematical and Physical Sciences, 1955. **232**(1191): p. 498-509.

20. M.J. Grill, J.F. Eichinger, J. Koban, C. Meier, O. Lieleg and W.A. Wall, *A novel modelling and simulation approach for the hindered mobility of charged particles in biological hydrogels.* Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences, 2021. **477**(2249): p. 20210039.

21. L. Johansson, U. Skantze and J.E. Loefroth, *Diffusion and interaction in gels and solutions. 6. Charged systems.* J. Phys. Chem., 1993. **97**(38): p. 9817-9824.

22. S.K. Ghosh, A.G. Cherstvy and R. Metzler, *Non-universal tracer diffusion in crowded media of non-inert obstacles.* Phys. Chem. Chem. Phys., 2015. **17**(3): p. 1847-1858.

23. B.G. Amsden, *Hydrogel Mesh Size and Its Impact on Predictions of Mathematical Models of the Solute Diffusion Coefficient.* Macromolecules, 2022. **55**(18): p. 8399-8408.

24. H.-H. Hsu, J.-K. Kracht, L.E. Harder, K. Rudnik, G. Lindner, K. Schimek, U. Marx and R. Pörtner, *A method for determination and simulation of permeability and diffusion in a 3D tissue model in a membrane insert system for multi-well plates.* JoVE (Journal of Visualized Experiments), 2018(132): p. e56412.

25. Y.D.P. Limasale, P. Atallah, C. Werner, U. Freudenberg and R. Zimmermann, *Tuning the Local Availability of VEGF within Glycosaminoglycan-Based Hydrogels to Modulate Vascular Endothelial Cell Morphogenesis.* Adv. Funct. Mater., 2020. **30**(44): p. 2000068.

26. I. Ding and A.M. Peterson, *Half-life modeling of basic fibroblast growth factor released from growth factor-eluting polyelectrolyte multilayers.* Sci. Rep., 2021. **11**(1): p. 1-13.

27. N. Mizuno, Y. Kato, M. Iwamoto, A. Urae, T. Amamoto, T. Niwa and Y. Sugiyama, *Kinetic analysis of the disposition of insulin-like growth factor 1 in healthy volunteers.* Pharm. Res., 2001. **18**(8): p. 1203-1209.

28. K. Chlebová, V. Bryja, P. Dvorak, A. Kozubík, W.R. Wilcox and P. Krejci, *High molecular weight FGF2: the biology of a nuclear growth factor.* Cell. Mol. Life Sci., 2009. **66**(2): p. 225-235.

29. A.E. Cowan, I.I. Moraru, J.C. Schaff, B.M. Slepchenko and L.M. Loew, *Spatial modeling of cell signaling networks*, in *Methods in cell biology*. 2012, Elsevier. p. 195-221.

30. J. Schaff, C.C. Fink, B. Slepchenko, J.H. Carson and L.M. Loew, *A general computational framework for modeling cellular structure and function.* Biophysical journal, 1997. **73**(3): p. 1135-1146.

31. Jeremy E. Purvis and G. Lahav, *Encoding and Decoding Cellular Information through Signaling Dynamics.* Cell, 2013. **152**(5): p. 945-956.

32. O. Degerstedt, J. Gråsjö, A. Norberg, E. Sjögren, P. Hansson and H. Lennernäs, *Drug diffusion in biomimetic hydrogels: importance for drug transport and delivery in non-vascular tumor tissue.* Eur. J. Pharm. Sci., 2022. **172**: p. 106150.

33. C.R. Marti-Figueroa and R.S. Ashton, *The case for applying tissue engineering methodologies to instruct human organoid morphogenesis.* Acta Biomaterialia, 2017. **54**: p. 35-44.

34. Y.-l. Huang, R.-f. Qiu, W.-y. Mai, J. Kuang, X.-y. Cai, Y.-g. Dong, Y.-z. Hu, Y.-b. Song, A.-p. Cai, and Z.-g. Jiang, *Effects of insulin-like growth factor-1 on the properties of mesenchymal stem cells in vitro.* J. Zhejiang Univ., Sci., B, 2012. **13**(1): p. 20-28.

35. M. Mehrian, Y. Guyot, I. Papantoniou, S. Olofsson, M. Sonnaert, R. Misener and L. Geris, *Maximizing neotissue growth kinetics in a perfusion bioreactor: an in silico strategy using model reduction and Bayesian optimization.* Biotechnol. Bioeng., 2018. **115**(3): p. 617-629.

36. J. Rosser, B. Bachmann, C. Jordan, I. Ribitsch, E. Haltmayer, S. Gueltekin, S. Junttila, B. Galik, A. Gyenesei, and B. Haddadi, *Microfluidic nutrient gradient–based three-dimensional chondrocyte culture-on-a-chip as an in vitro equine arthritis model.* Materials Today Bio, 2019. **4**: p. 100023.

37. S. Sant, M.J. Hancock, J.P. Donnelly, D. Iyer and A. Khademhosseini, *Biomimetic gradient hydrogels for tissue engineering.* The Canadian journal of chemical engineering, 2010. **88**(6): p. 899-911.

38. M. Takasato, P.X. Er, H.S. Chiu, B. Maier, G.J. Baillie, C. Ferguson, R.G. Parton, E.J. Wolvetang, M.S. Roost, and S.M. Chuva de Sousa Lopes, *Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis.* Nature, 2015. **526**(7574): p. 564-568.

39. M. Mehrian and L. Geris, *Optimizing neotissue growth inside perfusion bioreactors with respect to culture and labor cost: a multi-objective optimization study using evolutionary algorithms.* Computer Methods in Biomechanics and Biomedical Engineering, 2020. **23**(7): p. 285-294.