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Modeling Bone Morphogenetic Protein Binding Peptide Action on Perfused Osteoblast Growth

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Author
Sun, Argus

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Los Angeles

Modeling Bone Morphogenetic Protein Binding Peptide

Action on Perfused Osteoblast Growth

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Biomedical Engineering

by

Argus M. Sun

2014
ABSTRACT OF THE DISSERTATION

Modeling Bone Morphogenetic Protein Binding Peptide Action on Perfused Osteoblast Growth

by

Argus M. Sun

Doctor of Philosophy in Biomedical Engineering

University of California, Los Angeles, 2014

Professor Samuel S. Murray, Chair

Bone Morphogenetic Protein-2 (BMP-2) is a growth factor in the TGF-β cytokine family, among other ubiquitous effects in development, it promotes bone mineralization by inducing osteoblast differentiation from precursor cells. This effect is the basis of its use as a therapeutic in procedures such as spinal fusion. However, because of its ubiquitous nature, side effects such as inflammation and swelling of surrounding tissues can be seen when the amount of BMP-2 implanted and released is not regulated. In the extracellular space, BMP-2 has numerous endogenous binding proteins. Among these is secreted phosphoprotein 24 (spp24). This protein can be modified in hopes of isolating the residues necessary to bind with BMP-2. This binding protein and its peptide
derivatives can be used to modulate the release of BMP-2. Using a collagen sponge as a delivery vehicle, the rate and pattern of release can be analyzed. Collagen sponges can serve as tissue engineering scaffolds that can host cell growth. An analysis of growth factor release from implantable collagen sponges cannot ignore the effect of ingrowth of osteoblast precursor cells. To study BMP-2 release modulation by spp24 with cellular response as an output, a perfusion bioreactor can be used. Perfusion bioreactors immerse a scaffold in growth media, delivering nutrients and gases permitting cell growth. In the bioreactor efflux, the release of BMP-2 from the sponge can be quantified. A BMP-2 scaffold-bioreactor system has the advantage of being sufficiently well-characterized in terms of parameters to the point where simulation is possible. Furthermore, the designed bioreactor can be fabricated to verify the predictions made by simulation. Finally, the BMP-2 SMAD pathway can be used to create a cellular model to predict the response generated by BMP-2 release.
The Dissertation of Argus M. Sun is approved.

Jeffrey C. Wang
Louis Bouchard
Guoping Fan

Samuel S. Murray, Committee Chair

University of California, Los Angeles

2014
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“We must constantly try to formulate and solve problems. ...[Our field] is a young subject. Our understanding of the subject is yet imperfect. Many needed pieces of information have not yet been obtained; many potentially important applications have not yet been made.”

- YC Fung
VITA

1996-1998 Major: Undeclared Biological Science
University of California, Irvine

1998-2001 Major: Organismic Biology and Evolutionary Ecology
Specialization: Business and Administration
Minor: English Literature
University of California, Los Angeles

1999-2001 Researcher
UCLA Surgery, Human Genetics

2002 Research Fellow- Combinatorial Chemistry Laboratory
UC Davis Cancer Center

2003-2004 Laboratory Assistant- Vestibular Science Laboratory
UCLA Head & Neck Surgery

2005-2006 Teacher – Integrated Science Program
Sylmar High School, Los Angeles Unified School District

2007 Teaching Assistant
Biomedical Engineering Interdepartmental Program
University of California, Los Angeles

2009 M. S. Biomedical Engineering
University of California, Los Angeles

2009-2013 Teaching Assistant
Chemistry & Biochemistry
University of California, Los Angeles
Presentations and Publications


Stochastic Simulation for analyzing Risk in a Biomedical Time Series, R^2 Risk and Regulation Workshop, National Univ Singapore, workshop proceedings, 2014.


Proteomic Methods for Orthopedic Biopharmaceuticals, J Biomol Tech. 2013 May; 24(Suppl): S64.


Society Memberships

Introduction

The skeletal system is important to humans and other vertebrates to provide mechanical support, to protect vital organs, to allow muscle attachment facilitating locomotion, to house hematopoietic cells, and also to act as a reservoir for storage of mineral ions. As shown in the accompanying figure, forces from the upper and lower extremity must balance each other and the weight of the body. Any route that force travels from the hands to the feet, must be transmitted through the vertebral column. This demonstrates how a particular set of bones must be structurally sound to handle repetitive loading. Bone’s strength is due to its nature as a composite structure composed of cellular elements, collagen fibers, and crystallized mineral. The skeleton undergoes a constant destruction-reformation cycle of remodeling which replaces tissue that has sustained stress-related microfracture damage, with new tissue. Injury to the skeleton prompts a defined sequence of repair; first a callus of collagen replaces clot formed at the injury, followed by repeated cycles of remodeling. At the cellular level, this process occurs with multinucleated osteoclast cells resorbing bone while new bone is deposited by osteoblast cells. The process is sensitive to mechanical loading and the resulting bone is formed in a shape that is optimized by strain patterns. However, there are many situations where this response is insufficient and a graft of bone tissue becomes necessary. Previously, the source of this tissue originated from other species in the form of xenografts or the same species in the form of allografts. Both these sources can be problematic.
due to rejection\textsuperscript{7} of the transplanted tissue in a host vs. graft reaction. This immunological problem can be avoided through the transplantation of the host’s autologous tissue, however autografts tend to be limited by the amount of tissue available at the donor site alongside morbidity caused by the extraction of that tissue.\textsuperscript{8} To address this problem, attempts to engineer skeletal tissue have been developed in recent years. Tissue engineering tends to follow a three component paradigm consisting of cells, scaffold, and signals. Cell sources span from pluripotent stem cells to lineage-committed osteoblast precursor cells. These cells then need a substrate for attachment and growth that fits the shape of the desired tissue graft. The scaffold usually has an open porous structure to allow cells to infiltrate and attach, and its material is resorbable synthetic polymer or a crosslinked protein or polysaccharide. To mediate cell growth, soluble growth factors signal cells to proliferate to the desired shape guided by the structure of the scaffold. Delivery of the growth factor is accomplished in several ways. One such strategy is to incorporate the growth factor into the scaffold and allow its release as the scaffold material is resorbed. BMP-2, a ubiquitous growth factor known to be implicated in many aspects of development such as body plan, increases bone deposition. A problem to address in incorporating BMP-2 into a scaffold is controlling its release and diffusion from the scaffold\textsuperscript{9}. The challenge of dealing with a scaffold is its unique characteristics stemming from its nature as a biomaterial. It must be able to mimic the physical properties of the original tissue, and also be biocompatible to allow cell growth. Furthermore, it must also be resorbable so that it can eventually be replaced by endogenous tissue. In the case of bone, the scaffold has to accommodate the requirements of osteoblastic cells—it has to mechanically allow fluid flow through so that gases and nutrients can permeate the whole volume. It also has to facilitate cell development by acting as a delivery vehicle releasing growth factor at the appropriate rate. To
approach this problem from an engineering standpoint, it is inherently complex with a large number of unknowns. To test the system iteratively and exhaustively is likely not a feasible solution, both in terms of material costs of testing and man-hours required of testing. One approach to complex problems is to examine components individually, then reassemble the component solutions in a model that can be analyzed computationally. In this case, the problem can be divided into the following components: a biochemical problem of binding protein-ligand interaction, a biomaterial and fluid mechanics problem of flowing fluid through a porous substrate, and a cell biology problem of osteoblast growth and differentiation in this system. In the following chapters, these components will each be encapsulated then interconnected by a single biointeraction—the binding between BMP-2 and a binding protein known as spp24. Hypothetically, this interaction can be employed to modulate bone growth. Fitting the binding to a kinetic model elucidates how differing sequence variation and intra-chain bonds influence binding affinity. The structural biology of the binding interaction explains the observed kinetics. One component of spp24’s structure that affects its growth factor binding is the existence of disulfide bonds. The proteomic method of peptide conjugation followed by mass spectrometry confirms the existence of disulfides within a peptide derived from the essential binding residues of spp24. This peptide, known as cyclic BMP-2 Binding Peptide or cBBP, has potential for use as a modulator of BMP-2 release. The remainder of the work will describe exploration of that possibility, including transcriptional response to growth factor release, characterization of peptide-functionalized biomaterials that sequester growth factor, fusion of the peptide to additional protein domains, design of a bioreactor to test release, simulation and validation of release as well as systems biology of cellular response. This series of experiments helps in finding a solution to a complex problem.
References


Chapter 1

Binding Kinetics Parameter Estimation

A key parameter in modeling growth factor release is the strength of specific binding between the factor and other proteins, such as the aforementioned spp24. Growth factors like BMP-2, when applied to collagen sponges, interact with collagen fibers non-specifically—BMP-2 adheres to collagen due to inherent affinity all proteins have for one another. As expected, rapid diffusion of BMP-2 away from the sponge occurs after implantation. Therapeutic BMP-2 is bolus-released as opposed to sustained release and infusion-like delivery that likely occurs when BMP-2 is released endogenously after fracture. A moderate release rate that lowers steady state concentration is thought to avert side effects. This type of release is seen in delivery of existing biopharmaceuticals. To modulate BMP-2 release, efforts have been made to incorporate specific binding proteins into collagen sponges. As opposed to relying on nonspecific interactions, attaching specific binding proteins allow more effective growth factor seeding onto the scaffold. The affinity of the binding protein for growth factor is amenable to bioengineering. Studies where the binding protein is modified, changing the binding affinity, will be described in this chapter and further elaborated in following chapters.

On the molecular scale, interesting phenomena occur not only in the bulk of aqueous solution, but also in the miniscule region immediately surrounding the surface of an interface between two phases. Receptor-binding is one such phenomenon, occurring in the immediate vicinity of the interface between the phospholipids of a cell membrane and the solution that composes extracellular fluid. Although spp24-BMP-2 binding likely occurs in free solution as well as near membranes, the kinetics can be treated as a receptor-ligand interaction, with either
receptor or ligand immobilized. Although portions of membranes can be isolated into nanoparticles\textsuperscript{7,8} and ion channels can be studied with microfluidics in lipid bilayer covered apertures,\textsuperscript{9,10} it is currently not readily feasible to immobilize a cell membrane laden with receptors to measure ligand concentrations over it. To study receptors, two methods have been widely used. The first of these methods is Enzyme-Linked ImmunoSorbent Assay (ELISA), while the other is Surface Plasmon Resonance (SPR). Serving as a substitute for the cell membrane, the polymer base of each well in an assay tray serves as a hydrophobic substitute for the lipophilic bilayer, allowing insertion of the membrane spanning portion of receptors. In SPR, molecules are bound to a matrix surface such as dextran, and the analyte molecule solution is released and flows over the matrix surface. Unfortunately dextran is hydrophilic, as a result only the truncated extracellular portion of a receptor is linked to it. A similar tactic can also be used in ELISA, though the surface is more hydrophobic. On ELISA plates, due to this hydrophobicity, either the extracellular portion or holoreceptor can be adsorbed, allowing for comparison if needed.

While ELISA is a powerful tool, it cannot be as reliably quantified as SPR. Quantification of binding kinetics is necessary because the parameters are used for modeling growth factor release. To model pathways in systems biology, SPR-generated-data can be employed. Reliability of such data determines a model’s ability to accurately predict the outcome of a biological process. In a multistep process, error is amplified and propagated emphasizing the importance of increasing reliability of the initial data. The most obvious way to raise precision is to increase the number of trials by repeating binding studies such as ELISA or SPR. Often, instrument availability and personnel time are limited, in these situations additional
assays or instrument runs are constrained. However, data reliability can be improved without repeating experiments.

Numerical methods can be used to improve the fit of the binding model to the data. This can be seen in a two parameter linear model shown below:

1.1 \[ y = p_0 x + p_1 \]

The independent variable in eqn 1.1 is \( x \), and \( y \) is the dependent or output variable, \( p_0 \) and \( p_1 \) are parameters that describe the relation between the two variables. In the two parameter linear model, experimental repetition reduces the spread of the data points by increasing the total number of points, whereas numerical methods adjust the slope \( p_0 \) and intercept \( p_1 \) of the best fit line until the distance between the data and the model is minimized. The linear model can be generalized to extend the variable and parameter set as follows:

1.2 \[ y = f(p_1 \ldots p_n, x_1 \ldots x_m) \]

In the general form, the output \( y \), is defined by a function \( f \) that demonstrates a relation between each individual parameter \( p_i \) and individual variable \( x_i \) with the total set \( n \) and \( m \) respectively.

With binding kinetics, the model is still more complex, but algorithms can be used to try parameter values iteratively until the best set of values are found, at which point the search concludes. Biological data such as the kinetic energy per unit volume of bloodflow, enzyme kinetics, saturable ligand-receptor binding or drug clearance\(^1\) all can demonstrate nonlinear relations. These nonlinear relations are often seen in the following exponential form:

1.3 \[ y = f(p_i \ldots p_j, e^{(p_{j+1} \ldots p_n} x)) \]
In 1.3 the function f now displays a parameter subset $p_j$ that is distinct from the remainder of the total parameter set $p_n$. Although not without their own analytic error, numerical methods can be used to fit data to a nonlinear model.

Least squares regression is a numerical technique where the weighted sum of squares of the residual for data points reflecting model behavior is minimized through varying the values of model-associated parameters. When the model is nonlinear, as in the case with binding kinetics, the method is termed nonlinear regression (NLR). In systems that exhibit biphasic behavior, two types of NLR are frequently used, sequential and simultaneous. They differ by whether they partition the data set and the parameter set prior to fitting. Data in a system displaying biphasic behavior and can be partitioned as follows:

$$f(x) = \begin{cases} g(x_1 \ldots x_{jct}) \\ h(x_{jct+1} \ldots x_{total}) \end{cases}$$

The piecewise function $f$ in 1.4 is split into separate functions $g$ and $h$, the complete data set $x_{total}$ is partitioned at the biphasic junction data point $x_{jct}$. Comparisons of performance and accuracy between the two methods are another area where they differ. SPR kinetics data typically has two distinct phases, making it particularly apt for these two types of NLR. This chapter will describe binding kinetics data of spp24 related peptides and will also describe procedural details of how shifting between NLR methods improved fit to a binding model.

**Table 1.1 NLR Methods overview**

<table>
<thead>
<tr>
<th></th>
<th>Sequential</th>
<th>Simultaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biphasic Data</td>
<td>Required</td>
<td>Not Required</td>
</tr>
<tr>
<td>Data Set Partition</td>
<td>Required</td>
<td>None</td>
</tr>
<tr>
<td>Parameter Set Partition</td>
<td>Sometimes</td>
<td>None</td>
</tr>
<tr>
<td>Fits</td>
<td>Two or more</td>
<td>One</td>
</tr>
<tr>
<td>Length of Computation</td>
<td>Fast</td>
<td>Variable</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Implementation Dependent</td>
<td>Accurate</td>
</tr>
</tbody>
</table>
Simultaneous and Sequential Non-linear Regression: Theory and Examples

For a model to be analyzed sequentially, its parameter set must be partitioned into two or more subsets. That is:

1.5 \[ y = f(p_i \ldots p_j, p_{j+1} \ldots p_n, x) \]

where \( y \) does not vary linearly with respect to \( x \).

The preceding equation partitions a parameter subset \( p_j \) that is mutually exclusive with the remainder of the total parameter set \( p_n \). In simultaneous NLR, \( p_j \) and the remainder of \( p_n \) are fit at the same time obviating the need for partitioning. However in sequential NLR the partitioning allows treatment of the portion of the model dependent on the \( j \) subset separately. The model can then take the form:

1.6 \[ f(x) = \begin{cases} g(p_n, x) \\ h(p_j, x) \end{cases} \]

Equation 1.6 is an extension of 1.4 with the parameter set partitioned to correspond to the data partition. Minimization then occurs in sequence first for \( h \), then expanding into the unexplored parameter space. The function \( g \) has its residual with the data minimized across the total parameter space with either \( p_j \) fixed in the \( j \) subspace or using \( p_j \) as initial points in search. Partitioning \( f \) into \( g \) and \( h \) should not affect equality with output variable \( y \). A pharmacokinetics-pharmacodynamics (PKPD) system can be partitioned as described, where pharmacokinetics are actions of the body on the drug to clear and sequester it, and pharmacodynamics are actions of the drug on the body producing physiological response. (The output variables for the PK and PD portions of the model are dimensionally distinct. The separate output variables \( y_1 \) and \( y_2 \) denote the PK drug concentration and PD physiological response, respectively and should be plotted on separate axes. It is arguable in analogy with structural modeling where \( y_1 \) is actually a hidden
variable and $y_2$ is the true output) The PKPD system lends itself to partitioning for sequential fitting. The function $h(x)$ can be fit first, and $g(x)$ fitted next in sequence. Receptor binding can be partitioned likewise with association as $g(x)$ and dissociation as $h(x)$. A brief derivation of $g(x)$ and $h(x)$ will be interesting and useful for describing subsequent binding models. A system such as receptor-ligand interaction can be described as a transformation of the receptor from an unbound state $x$ to a bound state $y$. The $x \rightarrow y$ transformation is appropriate if $y$ is readily measurable, allowing it to serve as the output variable. It is also reasonable to assume that the system is isolated in a fashion where total receptor amount is a constant $c_0$, shown as follows:

1.7a \[ x + y = c_0 \]

With the preceding constraint 1.7a in place, the rate of transformation can be described with the following first order differential equation relating the appearance of bound and disappearance of unbound receptor:

1.7b \[ -\frac{dy}{dt} = \frac{dx}{dt} = -c_1 x \]

The newly introduced rate constant $c_1$ can vary with implicit variables of the system, though unaffected by $x$ and $y$. 1.7b can be rearranged and integrated as follows:

1.7c \[ \int \frac{dx}{x} = - \int c_1 dt \]

1.7d \[ \ln x = -c_1 t + c_2 \]

1.7e \[ x = c_3 e^{-c_1 t} \]

Where $c_2$ in 1.7d is a constant of integration. The form seen in 1.7e is a standard function describing exponential decay processes. As a corollary to 1.7e, $c_3$ is also the initial value of $x$ and can be shown directly by setting $t$ to zero. Substitution of 1.7e into 1.7a yields the following:

1.7f \[ y(t) = c_0 - c_3 e^{-c_1 t} \]

Renaming the constants using the notation of 1.6, the binding for the association phase is written in the following form:
The saturation behavior of the system is seen if the association phase is allowed to equilibrate. When $t$ approaches infinity, 1.7g is described by the following limit:

$$\lim_{t \to \infty} p_0 - p_1 e^{-p_2 t} = p_0$$

This limit restates that if the association phase equilibrates, the transformation from $x$ to $y$ is total. It follows that the starting value is less than or equal to $p_0$ when the system transitions from association to dissociation phase. Proof of this statement is offered here:

1.7i

$$h(x) \leq p_0 \quad \forall x, p_n$$

1.7j

if for some $r$ and $t_\Delta$, $h(x) = h'(x) + r$

where $r > 0, t_\Delta > t_{sup(g)}$

1.7k

then from 1.7h, $h(x, t_{\Delta}+) = p_0 + r$

Where $r, h', t_\Delta, t_{sup}$ are a constant, unmodified $h$, time increment and end of time interval for the given phase, respectively. For $c_0 = p_0$ to hold, 1.7h violates 1.7a for nonnegative values of $y$, therefore 1.7i is proven by contradiction. It follows from 1.7b-e and 1.7i that the reverse transformation from $y$ to $x$ in the dissociation phase is written as follows:

$$h(x) = p_4 e^{-p_5 t} + p_6 \quad \text{where } p_4 \leq p_0$$

A limit analogous to 1.7h will show that the dissociation phase equilibrates to $p_6$. Furthermore, receptor binding can be condensed into one set of functions and mass transport to the binding site into another. Whether the system is PKPD or Receptor-Ligand, if partitioning is possible, then it can be subject to NLR fitting to increase reliability.

Since a model’s governing equations are fundamentally unaltered by either method, the decision between simultaneous and sequential NLR for use with a particular model should consider factors such as computational demands, level of accuracy and ease of implementation.
In examining these factors, it is first important to show current examples of NLR usage to frame the context of the argument. Two closely related studies demonstrate simultaneous and sequential NLR, the two regression methods which can be applied to binding kinetics data. Both studies examine a PKPD model for recombinant human erythropoietin (rHuEPO). Although recombinant erythropoietin acts on hematopoiesis, and not on osteogenesis like recombinant BMP-2, it is a sample system for effector protein delivery where NLR is used on biological data. For this reason, EPO is compared as an example to NLR treatment of spp24/cBBP-BMP-2 binding data. The first is an interspecies comparison, while the second is done solely on rats.

Erythropoietin is a protein growth factor produced endogenously in the kidneys and acts on hematopoietic stem cells to stimulate red blood cell (RBC) production. EPO is used to treat anemia resulting from kidney disease and cancer chemotherapy—it is an example of a biopharmaceutical with demonstrated efficacy. The pharmacokinetics portion of the model is a two compartment model (see figure 1.1) with an intravenous or subcutaneous injection of rHuEPO into the central compartment generating a concentration, $A_{EPO}$. Exchange with a tissue compartment occurs generating a concentration $A_T$ there. Elimination only occurs in the central compartment through a route characterized by saturable kinetics ($v_{max}$, $K_M$) or a linear route ($k_{el}$). The drug concentration $A_{EPO}$ acts on the pharmacodynamic compartment, also modeled compartmentally. Another set of compartments represent cellular pools of stages in the RBC lineage. These pools are progenitor 1 (P1), progenitor 2 (P2), reticulocytes (RET), mature RBCs ($RBC_M$) with lifespans $T_{P1}$, $T_{P2}$, $T_n$. Interaction between the two model components occur through regulation of entry into P1 and P2.
The rate and concentration data were then fit to model with equations generated from the conceptual framework described in the above diagram. From a large selection of programs that exist to generate those equations including SAAMII or Simulink, ADAPTII was chosen. Cross validation of ADAPTII and SAAMII show good agreement in calculation. A simultaneous fit is shown in figure 1.2. The top row is rat data, and the bottom row is human data, with the first column showing pharmacokinetic action through rHuEPO concentration and the second col-

![Diagram of Erythropoietin Pharmacokinetics-Pharmacodynamics compartment model](image)

**Figure 1.1** Erythropoietin Pharmacokinetics-Pharmacodynamics compartment model (from Woo, 2007)

**Figure 1.2** EPO PK/PD dosage and response curves (from Woo, 2007) a. rat rHuEPO concentration b. rat reticulocyte concentration c. human rHuEPO concentration d. human reticulocyte concentration
umn showing pharmacokinetic action through reticulocyte count. Although the rHuEPO plots are shown in log scale, the nonlinear nature is still evident in both.

Whether or not a sequential fit was tried for their data is unknown, though it seems that it would be possible to partition between and within the PK/PD portions of the data because biphasic behavior is seen in both, providing convenient independent variable values to partition the data set. However, the software-based simultaneous fitting seen in figure 1.2 already shows close fitting and further improvement from a sequential fit may not have been necessary in this case.

In a second study on erythropoietin (EPO), a receptor-based extension of the previous rHuEPO study’s model was used. Differences include a different choice of hematopoietic stem cell pool (in figure 1.3 stem cells are split into 3 pools as opposed to 2 in figure 1.2), some subtle changes in regulation of the PD model component, however the most significant change was the addition of receptor binding kinetics seen in R and RC pools (top middle section of figure 1.3). The combined PKPD model generated three groups of equations. These equations included initial conditions, ordinary differential equations (ODEs) for cell counts based on population lifetimes, for hemoglobin in various compartments and EPO concentration. The first of these three groups of ODEs is the PD portion of the model, while the remaining two are PK. To choose between competing statistical models, selection criteria such as the Akaike Information Criterion (AIC) for evaluating model complexity was used. An alternative model with a lower AIC (4156 vs. 4191.3) was considered, but the model of choice was based on practicalities such
as available data and ease of collecting measured quantities.

The four plots shown in figure 1.4 are analogous to a pulse-chase experiment, regularly scheduled EPO injections shown with arrows were abruptly stopped and absolute reticulocyte count, mean corpuscular hemoglobin (MCH), total RBC count, and hemoglobin concentration were examined. In all but absolute reticulocyte count a similar pattern was observed. This can be fitted piecewise with the saturable kinetic equation seen earlier:

\[ y(t) = p_0 - p_1 e^{-p_2 t} \]

Where \( p_n \) are the parameters to be fitted and \( t \) is the elapsed time since the first dose, followed by exponential decay (which is fit with \( 1.7l \)) after cessation of injections (where the output value \( y \) reaches a peak except in the case of absolute reticulocyte count). The abnormal result was attributed to the development of EPO tolerance (perhaps through receptor downregulation or endocytosis). In graphs not shown, baseline changes in hematopoiesis in placebo-injected control animals were examined. These plots were fitted to empirical Hill functions for cooperative biochemical binding. The baseline fitting was optimized sequentially in the package Scarabee, for MATLAB. Additional methods such as Runge-Kutta for numerical solution of differential equations were also used in their data analysis alongside model optimization.
Using the EPO PKPD data, a brief comparison of sequential and simultaneous fitting methods was performed. In order to evaluate the fit of the data to the relevant model, the data vectors were mapped onto a curvilinear coordinate system. The system resulting from this biject transformation has coordinate lines that curve with the model function. This change of coordinates is performed as follows:

\[ M(x) = A_{\text{new}}x - b \]

Where \( A \) is a diagonal matrix with entries \( \frac{1}{y_1}, \frac{1}{y_2}, \ldots, \frac{1}{y_n} \). In equation 1.8 the mapping transformation \( M \) is defined by a coordinate matrix \( A_{\text{new}} \), an offset vector \( b \) that has unit entries. The function that vector \( x \) is being mapped onto has output values \( y_1, y_2, \ldots, y_n \). After mapping, the data points are clustered close to the axis of the curvilinear coordinate system, allowing a linear regression of the points. The linear regression is analogous to autocorrelation or autoregression done for stochastic time series. When the data set is partitioned, the regression can be performed sequentially on each segment. The cessation of EPO injections at 36 days is chosen as the partition for the receptor-modeled datasets, for all other datasets the maximum or end of a plateau region is chosen as the partition. The results are shown in table 1.2, in columns 1 and 3. As a contrast, the unmapped data...
was also fitted to a cubic polynomial. This polynomial function was chosen because of its natural multiphasic behavior, effectively mimicking a data partition. In all cases, the sequential fit was an improvement compared to simultaneous fitting.

Table 1.2 EPO Coefficients of Determination (R²) for Simultaneous and Sequential Fits

<table>
<thead>
<tr>
<th></th>
<th>Simultaneous Curvilinear</th>
<th>Cubic Unmapped</th>
<th>Sequential Curvilinear</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK Rat</td>
<td>0.251</td>
<td>0.373</td>
<td>0.678</td>
</tr>
<tr>
<td>PD Ret (Rat)</td>
<td>0.080</td>
<td>0.533</td>
<td>0.526</td>
</tr>
<tr>
<td>PK Human</td>
<td>0.201</td>
<td>0.126</td>
<td>0.718</td>
</tr>
<tr>
<td>PD Ret (Human)</td>
<td>0.038</td>
<td>0.497</td>
<td>0.717</td>
</tr>
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<td>PD RET Receptor Model</td>
<td>0.329</td>
<td>0.809</td>
<td>0.911</td>
</tr>
<tr>
<td>PD RBC Receptor Model</td>
<td>0.015</td>
<td>0.878</td>
<td>0.680</td>
</tr>
<tr>
<td>PD MCHC Receptor Model</td>
<td>0.016</td>
<td>0.662</td>
<td>0.485</td>
</tr>
<tr>
<td>PD Hb Receptor Model</td>
<td>0.026</td>
<td>0.892</td>
<td>0.635</td>
</tr>
</tbody>
</table>

Computational Performance

After discussion of NLR techniques in a biological context, it is necessary to look at comparison of the two techniques. If the model is complex and the data set large, the less computationally demanding NLR technique triumphs. Although sensorgrams typically do not exceed several hundred data points making allocation of computational resources trivial, when examined in aggregate, computational speed might become prohibitive. In a population PKPD study, the performance of simultaneous NLR was looked at alongside several sequential NLR techniques. Data was simulated in study where w observations were made on v individuals. The PKPD data was fit using the following two equations:

1.9a \[ y_{vw} = g_{PK}(\theta_v, d_{vw}) + \varepsilon_{PK_{vw}} \]
1.9b \[ z_{vw} = h_{PD}(g_{PK_{vw}}, \varphi_v) + \varepsilon_{PD_{vw}} \]

Where parameter variables \( \theta \) and \( \varphi \) partition the PK and PD parameter space respectively where \( d_{vw} \) contains the independent variables such as dosage regimen and time, while output variables \( y \) and \( z \) were the model-predicted PK and PD responses, \( g_{PK} \) and \( h_{PD} \) respectively. \( \varepsilon_{PK_{vw}} \) and
εPD<sub>v,w</sub> are noise terms to account for stochastic deviation from a model being fitted. Inspection of both equations revealed that 1.9a is devoid of PD terms, while 1.9b contains both PK and PD terms. For sequential fitting, it is logical to condition z on y. This is a specific example of the partitioning seen in 1.5. To perform a simultaneous NLR fit, the likelihood function is integrated<sup>6</sup>, the likelihood function is given here in the general form:

\[ L(a_1, a_2, \ldots, a_w) = \prod_{v=1}^{N} P_v \]

In 1.10a, the probability P of a given pharmacokinetic or pharmacodynamic output x for individual v conditions on the parameter set a<sub>1</sub>, a<sub>2</sub>, ... a<sub>w</sub>. In 1.10b, product over v individuals is taken across the population/sample total, N. To perform sequential NLR, the likelihood was examined in sequence with 1.9a and 1.9b. Alternating the order as well as substituting a third Bayesian equation provided the strategy for devising the NLR methods. The Bayesian equation consisted of a prior probability distribution, used to infer a subsequent or posterior probability distribution. These equations were used to generate three distinct sequential NLR methods, which were then tested. These methods consisted of Population PK Parameters (PPP), Population PK Parameters and Data (PPP&D) and Individual PK Parameters (IPP). In the final method, the conditioning is done on a posterior bayesian estimate with a prior derived from PK data. The three methods were compared to simultaneous NLR in the boxplot shown in figure 1.5. The plot shows statistically significant savings in time for PPP and IPP sequential NLR methods.
However, PPP&D gave a large distribution (which in some cases saved no time at all), this was attributed to time savings being lost due to increased method complexity.

Accuracy Comparison

Another deciding factor to select between the two NLR methods is accuracy or the distance between fitted function and the true physical phenomenon. An analogy would be the comparison of a numerical solution to an analytical solution. Another study examined an application of NLR to binding kinetics. Monte Carlo simulation was used to generate experimental data with 3 different measures of error to test the accuracy of each of the two methods. As with the PKPD study, the software package NONMEM was used. The simultaneous fits showed the smallest interquartile range. Other measures of error looked at were Between Experiment Variability, and Non-Specific Binding. The results agree with the population PKPD study showing greater accuracy with Simultaneous NLR methods. However, as seen earlier with the EPO studies, particular implementations of sequential fitting can sometimes match data more closely than simultaneous fits.
Implementation for Binding Kinetics

The preceding discussion of simultaneous and sequential NLR allows further discussion of implementation of both NLR methods on an experimental data set. The experimental data is from protein-protein binding analysis using Surface Plasmon Resonance (SPR). In particular, the binding interaction is between spp24 variants and BMP-2, the binding protein and growth factor of interest. SPR functions through the detection of small changes in mass overlaying a gold film on the sensor “chip” surface. SPR relies on electron resonance (surface plasmons) from mass being in close proximity to the gold film, a prism on the other side of the film responds to surface plasmons with a small change in refractive index in the prism near the film. This in turn alters the angle of refraction of near infrared light shining through the prism. The angle change $\Delta \theta_{\text{SPR}}$ is detected by a light-detecting sensor and recorded as described by equation 1.11a and plotted as a sensorgram. Contained in the sensor chip on the side opposite the prism, is a $w_{\text{dextran}}$ (typically 100nm) thick layer of dextran serves as an anchoring matrix for binding biomolecules. In ligand-analyte binding reaction, one protein acts as ligand and is covalently bound to dextran. The other half of the protein-protein pair acts as the analyte. Analyte solution of varying concentration is flowed through the microfluidic cell encompassing the dextran. The output for a given SPR setup is shown in the following set of equations:

$$R = \sigma \Delta \theta_{\text{SPR}} = \zeta [C]$$

where $[C] = M_{\text{surf}} w_{\text{dex}}$

In equations 1.11a-b, $R$ is output measurement in Refractive Units $\text{Ru}$, $\Delta \theta_{\text{SPR}}$ is the raw refractive angle change in degrees $^\circ$, $C$ is the analyte concentration in mg/mL, $M_{\text{surf}}$ is the surface density of protein in ng/mm$^2$, $w_{\text{dex}}$ is the matrix thickness in nm, $\sigma$ and $\zeta$ are empirical constants with values of $10^4 \text{Ru/}^\circ$ and $10^{-3} \text{Ru/mL/mg}$, respectively. The interval when analyte flows in
through microfluidic channels is the association phase, characterized by the set of equations\textsuperscript{16} shown below:

\begin{equation}
R = R_{eq}(1 - e^{-(k_a C + k_d)(t-t_o)}) + R_I
\end{equation}

where \( R_{eq} = \frac{k_a C}{k_a C + k_d} \cdot R_{\text{max}} \)

In 1.11c, \( R_I \) is an empirical correction term that accounts for a linear offset where the association curve is elevated above the equilibrium refractive value \( R_{eq} \). \( R_{\text{max}} \) is the maximum refraction, \( C \) is the analyte concentration, \( t \) is the elapsed time and \( t_o \) is the initial time. Equation 1.11c is a specific case of equation 1.7g with \( p_0 \) as \( R_{eq} + R_I \), \( p_1 \) as \( R_{eq} \), and \( p_2 \) as the quantity \( k_a C + k_d \).

The binding kinetic constants \( k_a \) and \( k_d \) are the association and dissociation kinetic constants, respectively. When analyte stops flowing, the dissociation phase begins. An alternate fluid now flows through the microfluidic channels, with no analyte—effectively flushing the dextran matrix. Mathematically, this phase is characterized by exponential decay, as in the following equation\textsuperscript{16} shown below:

\begin{equation}
R = R_0 e^{-k_d(t-t_o)} + R_{\text{Disp}}
\end{equation}

In 1.11e, \( R_{\text{Disp}} \) is an empirical linear term that corrects for the offset of the dissociation phase curve from the association phase, which should be connected directly with one another if the system were behaving ideally. Although 1.11d does not have an as clearly defined format as 1.11c, both 1.11c and 1.11d along with 1.11e can be derived from mass action principles, shown as follows:

\begin{equation}
P + C \rightarrow PC
\end{equation}

\begin{equation}
K_A = \frac{[PC]}{[P][C]}
\end{equation}

In the binding reaction described by 1.11f, \( C \) represents the analyte in solution and \( P \) is the protein ligand covalently ligated to the matrix, in the biological receptor and ligand coupling is
often reversed. The equilibrium expression for association 1.11g equates the first order association constant $K_A$ (1/M) to a quotient of complex and binding components (M). For convenience, the reverse reaction is often used to describe the strength of a given binding reaction, shown as follows:

1.11h \hspace{1cm} PC \rightarrow P + C

1.11i \hspace{1cm} K_D = \frac{[P][C]}{[PC]} \text{, where } K_D = \frac{1}{K_A}

Equation 1.11i provides an equilibrium dissociation constant $K_D$ in concentration units (M), resulting in its use to characterize the strength of binding affinity. In order to connect the equilibrium expressions in 1.11f-i to the output measurements in 1.11a, an additional expression that describes the occupancy of binding sites should be presented:

1.11j \hspace{1cm} \Theta = \frac{[PC]}{[P]+[C]}

Equation 1.11j gives a dimensionless number $\Theta$, the fraction saturation of binding sites. $\Theta$ sufficiently describes first order binding with a single site, for higher order binding, additional dimensionless numbers must be considered. Up to this point, only equilibrium binding constants have been considered, to address the kinetics of a binding reaction, the rate of reaction must be quantified. So that rate laws may be written, the binding forward and back reactions in 1.11f and 1.11h must be modified to include kinetic rate constants in the following fashion:

1.11k \hspace{1cm} P + C \xrightleftharpoons[k_d]{k_a} PC

1.11l \hspace{1cm} \frac{d[P]}{dt} = k_d[PC] - k_a[P][C]

1.11m \hspace{1cm} \frac{d[PC]}{dt} = -k_d[PC] + k_a[P][C]

1.11n \hspace{1cm} \text{Where } [C] \sim \text{ constant and } [C] \gg [P]
The assumption 1.11n accounts for high analyte concentrations during the association phase, and is inherent to the design of the experiment. The coupled set of equations 1.11l and 1.11m can be written in the following matrix form, though transform methods can also be used to arrive at a solution:

\[
\begin{bmatrix}
\frac{d[P]}{dt} \\
\frac{d(PC)}{dt}
\end{bmatrix} =
\begin{bmatrix}
-k_a[C] & k_d \\
k_a[C] & -k_d
\end{bmatrix}
\begin{bmatrix}
[P] \\
(PC)
\end{bmatrix} = K_{\text{rate}}c
\]

The eigenvalues of the rate matrix \(K_{\text{rate}}\) can be found by setting \(\text{det}(K_{\text{rate}})\) to zero and solving the resulting polynomial. This generates a general solution shown below:

\[
\lambda_1 = 0, \lambda_2 = -(k_d + k_aC)
\]

\[
[P]_t = c_1 e^{\lambda_1 t} + c_2 e^{\lambda_2 t} = c_1 + c_2 e^{\lambda_2 t}
\]

\[
(PC)_t = c_3 + c_4 e^{\lambda_2 t}
\]

A unique solution can be found by setting the time to infinity and allowing the decay term to vanish. The constants \(c_1\) and \(c_3\) take the equilibrium concentration of ligand or complex given by the quotient of the forward or back rate and the sum of the forward and back rates. For the complex PC, this yields the ratio found in equation 1.11d as follows:

\[
c_3 = \frac{k_aC}{k_d + k_aC}
\]

The output of the association phase reflects binding sites becoming occupied towards the limit of saturation. This can be shown with the fraction term created by subtracting \(\Theta\) from unity. Shown as follows, this generates an upsloping function the asymptotically approaches saturation:

\[
R = R_{eq}(1 - \Theta) = R_{eq} \left(1 - \frac{PC}{P+C}\right)
\]

\[
\sim R_{eq} \left(1 - \frac{PC}{C}\right) = R_{eq} \left(1 - \frac{c_4}{C} e^{\lambda_2 t}\right)
\]
Using assumption 1.11n and substituting 1.11r into 1.11t yields 1.11c when the constants $c/a/C$ are absorbed into the $R_{\text{max}}$ component of $R_{\text{eq}}$ and when the intercept term $R_1$ is added. To derive equation 1.11e for the decay phase, the solution in 1.11r can be used directly as a decay function with need for the fraction saturation, however the analyte concentration value $C$ in $\lambda_2$ now drops to zero. Equation 1.11e closely matches the general form described in 1.7g.

Fits using equations from 1.11 are seen in figure 1.6 and 1.7. The two kinetic constants $k_a$ and $k_d$ are the adjusted parameters when data-fitting. The same algorithm was used in both simultaneous and sequential NLR of this data set. This was done using BIAevalulation and MATLAB respectively. Sometimes called the Levenberg-Marquardt algorithm; it is considered very robust, or relatively insensitive to small parameter fluctuations, though there can be some computational inefficiency.\textsuperscript{11,17-20}

**Materials and Methods**

SPR was performed at the UCLA Molecular Instrumentation Center (MIC)\textsuperscript{2} using a Biacore X SPR workstation (Biacore AB, Uppsala, Sweden) connected to a Dell Desktop. A CM5 Sensor Chip was prepared by activating the carboxylated dextran matrix with a 7-minute injection of 1:1 EDC/NHS solution allowing covalent binding of BMP-2 as the immobilized ligand. Excess activation was quenched with an injection of ethanolamine for 7 minutes. The analyte samples were diluted using HEPES-EP buffer as follows: 1:800; 1:400; 1:200; 1:100; 1:50; 1:25; 1:16; 1:8; 1:4; 1:2. The flow rate was set to 50uL/min and injection set on a 600s delay. 50uL of analyte solution was injected into the IFC microfluidics sample loop. Following injection, the sensor chip is regenerated using an injection of pH2.5 Glycine solution. Preinjection baseline, Peak Response Units and Post-regeneration baseline are flagged using the Biacore Control software.
Fitting

The original fit on SPR data gathered as described in the preceding section seen in figure 1.6 used the BIAevaluation software package, the fit was performed simultaneously using an alternate implementation of the Levenberg-Marquardt algorithm (see table 1.1). Table 1.1 shows the typical range of binding values obtained from spp24 with chain truncations and various other modifications. Reduced $\chi^2$ was calculated using the following equation:

$$\chi^2_L = \log \frac{\sum (r_f - r_x)^2}{n-p}$$

In eqn 1.12 $r_f$ is the fitted value and $r_x$ is the data value, $n$ is the degrees of freedom, $p$ is the number of parameters being fitted. The assumption $n>>p$ is made, making $\chi^2$ essentially the squared residual per data point. In table 1.1 $n = 175$. $\chi^2$ is used to measure goodness of fit rather than the correlation coefficient for sensitivity purposes. In this particular binding model and experimental setup, a $\chi^2$ value close to 1 or below is considered acceptable. It is interesting to note the similar piecewise shape of the curves to the receptor inclusive PKPD model in fig 1.4. The biphasic behavior of both plots corresponds to the cessation of injection, initiating a decay phase. While the $K_D$ ($k_d/k_a$) for N-ethyl-maleimide SPP24 protein conjugate had a value within ranges consistent with biological activity (10^{-6}–10^{-10}M), the upper and lower bounds of the 95%

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_a$ (1/M·s)</th>
<th>$k_d$(1/s)</th>
<th>$K_A$(1/M)</th>
<th>$K_D$(M)</th>
<th>$\chi^2_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>spp24 fl</td>
<td>3.11x10^5</td>
<td>5.5x10^{-3}</td>
<td>5.66x10^7</td>
<td>1.77x10^{-8}</td>
<td>1.01</td>
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<td>spp18</td>
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<td>1.26x10^{-3}</td>
<td>2.09x10^8</td>
<td>4.79x10^{-9}</td>
<td>0.28</td>
</tr>
<tr>
<td>NEM spp24 fl</td>
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<td>4.52x10^{-3}</td>
<td>4.62x10^6</td>
<td>2.16x10^{-7}</td>
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<tr>
<td>NEM spp18</td>
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<td>3.21x10^{-3}</td>
<td>9.46x10^6</td>
<td>1.06x10^{-7}</td>
<td>0.54</td>
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</table>
Figure 1.6 Simultaneous Fit plotted with SPR Data for NEM-modified full-length spp24  (Plotted in relative units vs. seconds) black lines are model fits, color lines are data points; purple, brown, indigo, cyan, green, magenta and red correspond to the model curves for dilutions 1:1, 1:2, 1:4, 1:8, 1:16, 1:25, 1:100, further dilutions were not evaluated as they were indistinguishable from baseline. (from Sun 2009) confidence interval stretched outside the biological range and even into physically impossible negative values. It was conjectured that a sequential NLR method would generate more plausible results than a simultaneous NLR method. A sequential fit of $k_d$ using the dissociation phase equations and the association phase equations in sequence is shown in table 1.2. (For MATLAB files, see appendix). Only the 4 highest concentrations (the top four curves a-d in figure 1.6) were used because the lower concentrations had resonance values that were not distinguishable from baseline and were therefore considered too dilute. This fit yielded a mean $k_d$ that was on the same order of magnitude as the simultaneous data. Fitting occurred without
much problem for the first 2 curves, converging at 20 or less iterations. However at the lower 2 concentrations, convergence did not occur until past 500 iterations, generating parameter estimates that were too large in magnitude to make physical sense. Furthermore, when exceeding 500 iterations, a periodic crescendo-decrescendo of step-size occurred, suggesting the algorithm was oscillating due to instability. As a result, the maximum iteration value was set manually which resulted in a fairly large reduction of residuals along with parameter estimates that were physically consistent.

Table 1.4 Sequential NLR fitted Binding Kinetics of NEM-spp24

<table>
<thead>
<tr>
<th></th>
<th>$k_a$ (1/M·s)</th>
<th>$k_d$ (1/s)</th>
<th>$K_D$ (M)</th>
<th>$\mu_w$</th>
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<tbody>
<tr>
<td>a 2uM</td>
<td>6282.3</td>
<td>0.019912</td>
<td>3.16949x10^{-06}</td>
<td></td>
</tr>
<tr>
<td>b 1uM</td>
<td>6315.703</td>
<td>0.022788</td>
<td>3.60813x10^{-06}</td>
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</tr>
<tr>
<td>c 500nM</td>
<td>52857.87</td>
<td>-0.00161</td>
<td>-3.04999x10^{-08}</td>
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</tr>
<tr>
<td>d 250nM</td>
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<td>$\sigma^2$</td>
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<td>$\sigma^2 n^{-1/2}$</td>
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</tbody>
</table>
Using the $k_d$ values generated from the first fit at each concentration was a critical step in sequential NLR. These values converged much more rapidly without any limit on maximum iterations. The new value for $K_D$ (equilibrium binding affinity) was less than 10% from the simultaneous values. The 95% CI upper bound was now on micromolar magnitude with rapid dissociation indicative of biological processes with very weak affinity generating momentary binding, such as leukocyte rolling. However, the lower bound was still negative. At this point, it was appropriate to impose the additional inequality constraint $K_D \geq 0$. It was assumed that the physical and non-physical $K_D$ values were sampled from separate distributions with variances $\sigma_p$ and $\sigma_{np}$ which were .817 and .999, respectively. The raw nx1 $K_D$ data vector was transformed with a nxn diagonal weighting matrix $D_w$ with unit trace. The individual entries for $D_w$ given as follows:

$$d_w = \frac{w_i}{\sum_{i=1}^{n} w_i}$$

**Figure 1.7** Sequential Fit Plot, binding data from spp14, a variant protein containing the C-Terminal residues of spp24. The association phase peak is matched more closely than the simultaneous fit, the dissociation initial dip and plateau also matches the slope more closely.
The transformed $K_D$ data vector was then multiplied by the transpose of the column vector $1_n^T$ ($n=4$) yielding the weighted mean $\mu_w$ as follows:

1.14a & 1.14b

\[ D_w K_{D,\text{raw}} = K_{D,\text{Transf}} \]
\[ 1_n^T K_{D,\text{Transf}} = \mu_w \]

The operations in 1.14a&b generated an error of the weighted mean $21^2$ ($\sigma_{\mu}^2$) of 0.200. The new confidence interval centered around $\mu_w$ (table 1.4) demarcated an interval that meant slightly weaker binding. This range still covered the mean from simultaneous NLR, satisfied the inequality constraint and therefore physically consistent. Furthermore, the interval was in the biological range.

**Conclusion**

In conclusion, this chapter examined two methods for NLR and discussed factors to consider when choosing between the two. These factors include computational expense, accuracy and ease of implementation. In terms of saving computational resources, sequential NLR is typically superior to simultaneous NLR, while the opposite is true of accuracy. Contrasting the two methods in terms of implementation, the simultaneous fit was done with equipment-specific software, so there was little difficulty in implementation. Without such precoded software, sequential NLR was readily implemented in a general-purpose technical computing environment, with a challenge hidden in adjusting optimization search variables. In this specific case, the sequential NLR generated parameters that were much more plausible. Both these methods examined existing data without needing to replicate experiments. The current gains were made by switching from simultaneous to sequential fitting using a langmuir binding model with a 1:1 receptor-ligand ratio. These gains allow for increased confidence in
the protein-growth factor-binding kinetic model, and can be used in subsequent binding studies and extrapolated to simulation. After this analysis of the adherence of a receptor-ligand binding model to kinetic data, some points of caution should be addressed prior to using the results for further modeling. It is possible that further refinement of the binding model, such as including cooperative binding effects or allowing ligand to bind in multiple layers would improve the fit. Being careful to avoid the danger of overfitting, such improvements could further increase the reliability and predictive capability of the generated models. Furthermore, in a biological system, it is possible that interactions between the ligand or receptor with other molecules could distort the receptor-ligand interaction causing the kinetic output to deviate from the binding model. To illustrate how the preceding discussion fits into the remainder of this dissertation, a compartmental diagram in figure 1.8 shows an in-vivo model of BMP-2 and cBBP. The release constant $k_{rel}$ is grouping of the binding affinities fitted in this chapter. Further discussion of additional effects that modulate this rate will be seen in chapters 5-8. The reverse process of rebinding $k_{rbd}$ is discussed in chapter 8. The absorption of BMP-2 by cellular targets, $k_{abs}$, is the topic of chapters 4 and 9. Although the elimination of cBBP $k_{disp}$ and $k_{elim}$ is not thoroughly discussed, studies show that cyclic peptide biopharmaceuticals$^{22-23}$ tend to have a lower release rate than their linear counterparts. The cyclic structure of BBP is the topic of chapter 2 and 3.

Figure 1.8 Compartmental Model for BMP-2
References


Chapter 2

Spp24 structural biology

Introduction

Protein structure, when known, can be used to predict important micro and macroscopic properties such as sedimentation rate or diffusivity.\(^1\) The error of those parameters are dependent upon the accuracy of the coordinates of each atom within the macromolecule is known. Qualitatively, valuable insight can also be obtained by searching for causes of experimental phenomena such as binding kinetics discussed previously in corresponding features in molecular structure. This approach adapts well to the problem of BMP-2 and spp24. Spp24 or secreted phosphoprotein-24 kilodaltons is a secreted protein produced in the liver. From there it travels as a plasma protein to the active site of interest, bone. It is thought to form serum complexes with proteins such as \(\alpha_2\) macroglobulin.\(^2\) The bovine protein is translated as a 203 amino acid precursor protein. Prior to secretion into the extracellular space, a 23 amino acid signal sequence is cleaved off the N terminus. In the extracellular space, it binds to BMP-2, slowing BMP-2’s binding to receptors and subsequent signal transduction. In fact, there is some evidence that spp24 was one of the original proteins purified by Marshall Urist using hydroxyapatite chromatography.\(^3\) A TGF-beta receptor II homology-1 (TRH1) domain close to the C-terminus is likely responsible for BMP-2 binding.\(^3\) As a TGF-beta family cytokine, BMP-2 has affinity to a contiguous 19 amino acid stretch (residues 110-128) found in the TRH1 domain.\(^4\)-\(^6\) Synthesized as a peptide, BMP Binding Peptide BBP, this sequence has been tested for affinity to BMP-2 using surface plasmon resonance as was done for the truncated variants for spp24 in the previous chapter. Affinity exists despite separation from the whole folded protein, suggesting the peptide
retains some of its conformation. As will be discussed in a following chapter, this is likely due to the cyclization of the molecule due to a disulfide bond between the terminal cysteines. The existence of disulfide bonds in untruncated spp24 has been shown to be critical for affinity to BMP-2, this was demonstrated by blocking the formation of disulfide bonds by conjugating an inert maleimide to the cysteine thiols. The modified protein had much less affinity\(^5\) for BMP-2, suggesting that crosslinked cysteines are an important determinant for proper folding and bioactivity of spp24. Disulfide bonds bring secondary structure elements into proximity and sufficiently lower the energy of the folded protein to allow a stable BMP-2 binding conformation.

**Structural Analysis**

The conformation of spp24 has been studied using circular dichroism CD spectroscopy. Proteins absorb primarily in the ultraviolet range (\(\lambda=100-400\)nm) due to the presences of aromatic side chains such as phenylalanine, tryptophan and tyrosine. Light absorption can be described using the Beer-Lambert law:

\[
A = \log \left( \frac{I_o}{I} \right) = \varepsilon c l
\]

Where \(A\) is the absorbance, \(I\) is the transmitted intensity, \(I_o\) is the incident light intensity, \(c\) is the protein concentration, \(l\) is the path length the light travels through the sample, and \(\varepsilon\) is the molar extinction coefficient. Chiral molecules such as proteins have different molar extinction coefficients for left and right circularly polarized light. CD spectroscopy relies on this difference as shown in the following equation.

\[
\Delta \varepsilon = \varepsilon_L - \varepsilon_R
\]
Where $\varepsilon_L$ and $\varepsilon_R$ are the left and right circularly polarized light, respectively. A CD plot then graphs the variation of $\Delta \varepsilon$ across $\lambda$ in the UV range. The resulting pattern gives a rough estimate of the type of secondary structure present in a given protein.\(^7\) CD spectroscopy was performed on spp24 showing that much of the protein is in $\alpha$-helix and some $\beta$-sheet.

Figure 2.1 a) Sialostatin L2, a tick protein,\(^8\) with CTTVIYRLQGEKSISSFEC highlighted in green b) Structure of cBBP constructed by simulated mutation, unchanged residues are in green, the inserted gly is in cyan and the disulfide is in yellow. Backbone atoms within proximity for H-bonding are shown with distances in Å c) sequence comparison with same coloring as b showing 31.5% identity within 1 residue. (figures were created with Chimera and PyMol)

Although at present there are no known NMR or X-ray crystallographic structures of spp24, such studies have been conducted for homologous proteins. The cystatin family of protease inhibitors share a significant amount of structural similarity to spp24. In the secreted cystatin from Ixodes Scapularis\(^8\) (Deer Tick) salivary gland, sialostatin L2, a stretch of 20 amino
acids CTTVIYRLQGEKSISSFEC shares greater than 30% sequence identity within one residue compared the 19-mer stretch of BBP CRSTVRMSAEQVQNVWVRC. In figure 2.1a, the CTTVI... sequence is shown in crystal structure, while figure 2.1c shows the sequence similarity. When a single glycine is inserted into BBP, the two chains can be aligned where three residues overlap exactly. In a 1.8Å resolution x-ray crystallographic structure of sialostatin L2, it is seen in figure 2.1a that the N-terminal and C-terminal cysteines of CTTVIYRLQGEKSISSFEC are linked together with a disulfide bond. This sequence is also the start of a beta sheet with residues doubling back on itself to span two periods of the sheet. A little greater than 50% of the surface of the sequence is exposed to solvent, while the remainder is loosely bent around an alpha-helix. In beta-sheets, the peptide backbone hydrogen-bonds with itself; CTTVIYRLQGEKSISSFEC also shows this behavior with many of the amide nitrogens and oxygens with the 2-3 Å proximity required for H-bonding. This makes it a great choice for a backbone configuration. When glycine-inserted BBP is bent into this backbone configuration, many of the non-identical side chains spatially overlap because they are structurally similar, one such example is the similarity between threonine and valine. Using this method of simulated mutagenesis in PyMol (Schrödinger llc), a possible three-dimensional structure for cyclic BBP is constructed in figure 2.1b. One problem that occurred during choice of sidechain rotamers (rotational isomers) is W16’s indole side chain has rotamers that all create significant Van derWaals radii overlap. When rotated independent of backbone conformation, one rotamer minimizes overlap, though W16 is still more sterically strained than any other residue. It is possible that this overlap strains the tryptophan side chain making it more prone to reaction. In examinations of the mass spectrometry of BBP (described in detail in a following chapter), often this residue is oxidized generating extra weight from oxygen atoms. This
oxidation could be the cause for the pinkish-purplish hue seen peptide in solid phase. The strain is evident in angle formed by the α-β carbon bond and the β-indole bond shown in figure 2.2. This bond angle is stretched to 114.7° greater than the 111.3° found in the unmutated serine 108 and much greater than the 109.5° found in tetrahedral sp3 hybrized carbon. If designing a synthetic peptide to mimic the effects of cBBP, this residue could be optimized with respect to bioactivity or ligand binding and varied with residues with progressively lower steric footpring.

![Figure 2.2](image)

**Figure 2.2** a) W16 in cBBP measuring 114.7° b) S108 of Sialostatin measuring 111.3°

Although the structure for cBBP generated from this method is plausible, it is also important to consider the protein-protein interaction that spp24 is competing with, the receptor-ligand interaction of BMP-2. A disulfide closed loop similar to the one on spp24 appears on the BMP-2 Receptor. In figure 2.3, a 2.9 Å resolution crystal structure (1REW) of BMP-2 binding as a dimer to a pair of Ia receptors (accession numbers PDB:3BMP_A and NP_004320.2, respectively) provides the necessary structural information for this analysis. BMP-2 is found extracellularly as a domain-swapped dimer where the knuckle epitope of one chain binds to the wrist epitope of another ligand. The knuckle epitope is essentially a C-terminal beta sheet, while the wrist epitope consists primarily of an N-terminal alpha-helix. The sequence most relevant to
cBBP is found on BMPRIα, in the form of a contiguous 15 amino acid stretch also flanked at the termini by cysteines that are bonded as a disulfide seen in figure 2.3a. BMPRIα residues 110-124 (86-101 in the mature peptide with signal sequence cleaved), just prior to the membrane spanning region of the receptor, is composed of the sequence CKDSPKAQLRRTIEC. This sequence is much less similar to BBP than the Silostatin L2’s CTTVIYRLQGEKSISSFEC, sharing little more than 10% sequence similarity. Structurally CKDSPKAQLRRTIEC also doubles back on itself with atoms in proximity for H-bonding as seen in figure 2.3b, however unlike CTTVIYRLQGEKSISSFEC, the H-bonding does not occur between backbone atoms in the context of a beta sheet, but instead between side chains whose increased reach can span the distance between the wider gap. In terms of secondary structure motifs, CKDSPKAQLRRTIEC is the turn connecting the beginning of a beta sheet and the end of an alpha helix. Although the 15mer cannot be subject to simulated mutation without a 20% truncation of BBP’s sequence, it’s

![Figure 2.3](image-url)
conformation may be more similar to cBBP in free solution than CTTVIYRLQGEKSISSFEC, because of its greater solvent accessible surface. Shaped like a bent oval in figure 2.4, one face is exposed to solvent, while the reverse binds to the BMP-2 dimer. The cystine looped motif

![Figure 2.4 BMPRIa](image)

(olive) in complex with BMP-2 Dimer (purple & green) with solvent exposed surface of CKDSPKAQLRRTIEC (in light blue)

covers about 30-40% of the contact area between BMPRIa and the dimerized ligands. The area covered spans both the knuckle domain of one chain and the wrist of the other.

In summary, a cystine closed loop such as cBBP, CTTVIYRLQGEKSISSFEC or CKDSPKAQLRRTIEC could have an ordered structure such as a beta-sheet or a more loosely defined structure. The looped of amino acids closed by cysteine residues is shown to exist in a protein homologous to spp24 with significant exposure to solution as would be expected for a motif involved directly in ligand binding. A similar looped motif is seen in BMP-2’s natural receptor and it has several contacts with the ligand molecule. This suggests that spp24 can use its cystine loop motif to competitively bind with BMP-2 occupying one of the same sites as the receptor, BMPRIa. This structural insight explains the reduced binding affinity seen when spp24 is oxidized compared to its reduced form (table 1.1).
Prediction of Chemical and Biophysical Parameters

Diffusivities can be predicted up to 10% accuracy\(^1\) with structural information. The shapes of protein components are used in subsequent chapters for subtle improvements in accuracy of diffusion coefficient. Using the rigid protein assumption\(^10\) and a partial specific volume of \(0.730 \text{ cm}^3/\text{g}\) (calculated from more than a dozen sample proteins), the homology modeled structure was used to generate the following hydrodynamic predictions:

Table 2.1 Hydrodynamics for cBBP\(^\text{gly}\)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Translation Diffusivity</th>
<th>Radius of Gyration</th>
<th>Rotat Diffusivity</th>
<th>Intrinsic Viscosity</th>
<th>Sedimentation Coeff</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C</td>
<td>(1.756 \times 10^{-6} \text{ cm}^2/\text{s})</td>
<td>(1.074 \times 10^{-7} \text{ cm})</td>
<td>(8.575 \times 10^{-7} \text{s}^{-1})</td>
<td>0.05351 dL/g</td>
<td>0.4494 S</td>
</tr>
<tr>
<td>37°C</td>
<td>(1.858 \times 10^{-6} \text{ cm}^2/\text{s})</td>
<td>(1.074 \times 10^{-7} \text{ cm})</td>
<td>(9.072 \times 10^{-7} \text{s}^{-1})</td>
<td>0.05351 dL/g</td>
<td>0.4494 S</td>
</tr>
</tbody>
</table>

The predictions in table 2.1 can be verified by methods such as dynamic light scattering and analytical ultracentrifugation, bearing in mind the rigid assumption. Molecular dynamics simulation show that peptides of this size can have \(R_g\) that vary by up to 30% of the calculated rigid value.\(^11\)

Using Pymol 1.3, the binding surface between BMP-2 and BMPR was analyzed and the results are shown in figure 2.5. Counting both its knuckle and wrist interactions, CKDSPKAQLRRTIEC occupies a significant portion (39%) of the BMP-2-BMPR interaction. The 5.57 pKD interaction is also particularly compact. (This is a fairly strong interaction with a \(\Delta G_{\text{Binding}}^\circ\) of \(-6.7\text{kJ/mol.}\) Totaling both wrist and knuckle interactions, it spans \(1353 \text{ Å}^2\) while a search of other structures\(^12\) in the 5 pKD range spans \(2097 \pm 240 \text{ Å}^2\).
Although it might be criticized as an overly reductionist view, increased resolution of molecular structure should improve many aspects of modeling in ways that likely have not been considered. Structural studies have shown that mesoscale properties can be directly affected by protein conformation. Examples include the formation of supramolecular complexes and vesicle formation which are thought to important factors in the secretion of proteins forming the extracellular matrix, upon which bone mineralizes.\textsuperscript{13-15}

\textbf{Figure 2.5 Surface Area of Individual and Interacting Proteins and Peptides} A) contact area between BMP-2 and various peptide and protein configurations (Å \(^2\)). The contact surface area is calculated from comparison of solvent exposed surface area of individual peptides extracted from atomic coordinates. The holoreceptor ligand interaction has a pKD 5.57 and \(\Delta G_{\text{Binding}}^\circ\) -6.7 kJ/mol. B) Total solvent exposed surface area (Å \(^2\)) of peptides and proteins.
References


2. Zhao KW, Murray SS, Murray EJ 2012. Secreted phosphoprotein-24 kDa (Spp24) attenuates BMP-2-stimulated Smad 1/5 phosphorylation and alkaline phosphatase induction and was purified in a protective complex with Alpha2-macroglobulins from serum. J Cell Biochem. [Epub ahead of print].


Chapter 3

Mass Spectrometry Analysis of BMP-2 Binding Peptide Cyclization

This chapter examines the cyclization of BMP binding peptide (BBP), a derivative of spp24. It is hypothesized that a structural determinant of BBP’s affinity to BMP-2 is the cyclization by a disulfide linkage. Binding affinity influences release kinetics of BMP-2, having important implications for BBP’s use as an implantable biologic. (In chapter 8, this influence on release from collagen sponges\(^1\) will be the subject of modeling and simulation.) The affinity of cyclic BMP-2 Binding Peptide (cBBP) for its ligand\(^{2,3}\) can be used to modulate BMP-2’s release from absorbable collagen sponges. Spp24 demonstrated reduction of binding affinity when its cysteines were blocked from disulfide oxidation.\(^{2,3}\) The necessity of disulfide bonds in spp24 suggested that they are also necessary in BBP. This points toward a disulfide bond bridging the amino and carboxy terminal cysteine residues, generating the linkage responsible for ring closure of cyclic BBP (cBBP). To conclusively show derivative generation, a vinylpyridine\(^4\) moiety was conjugated to the cysteines (see figure 3.1) in the presence and absence of a reducing agent, β-mercaptoethanol. As an aside, addition to the cysteines also demonstrate that these residues can serve as points for modification of the peptide. This reaction was performed with cBBP and alanine-substituted variants. Alanine substitution at the fifth (A5), twelfth (A12) and disubstitution at both fifth and twelfth (A5A12) residues has shown altered

![Figure 3.1](image)

**Figure 3.1** Vinyl pyridine-cysteine reaction, the pyridyl group has a 106.144 amu calculated mass
SPR binding kinetics. Mass spectrometry is a technique ubiquitous in analytical biochemistry and often applied to examine cysteine and disulfide bond structure. The peptides derivatives were subjected to Matrix Assisted Laser Desorption Ionization– Time of Flight Mass Spectrometry MALDI-TOF MS as well as Electrospray Ionization Mass Spectrometry ESI-MS. These two soft ionization methods were chosen because the peptide is only a few residues in length, smaller than most enzyme digests. Fragmentation is a problem for short peptides using hard ionization methods such as electron ionization EI or chemical ionization CI by collision with a reagent gas such as methane or isobutane. Despite using soft ionization, some fragmentation still occurs (the pattern is shown in Figure 3.2) MALDI instead uses a UV laser to vaporize the surface of a solid formed by encapsulation of the peptide or protein of interest in a more volatile small molecule. ESI uses small electric fields to create a fine aerosol of charged droplets from which solvent is further evaporated.

**Materials and Methods**

The vinylpyridine conjugation of BBP was performed in sealable pyrex tubes with Teflon-lined caps. The tubes were thoroughly cleaned and dried prior to use. Each peptide was dissolved at a concentration of 5µg/mL in guanidinium chloride buffer. 1mL of this solution was placed in three reaction vessels, the first with peptide only, the second with 2µL of 4-vinylpyridine (Sigma-Aldrich) and 5µL of β-mercaptoethanol, and the third with 2µL of 4-vinylpyridine with no β-mercaptoethanol. The tubes were shaken, then flushed with Nitrogen gas and allowed to react for 30 minutes at 37°C in the dark. In the β-mercaptoethanol containing
tube an additional aliquot of 5µL of 4-vinylpyridine was added and the reaction mixture was allowed to incubate for another 30 minutes at room temperature in the dark. The samples were then desalted by adsorbing to C18 Supel-Tips (Thermo-Fisher) and then eluted from the tips using 0.1% TFA in 60% acetonitrile. MALDI-TOF mass spectrometry was then performed on the samples using an Applied Biosystems Voyager DE-STR mass spectrometer located at the UCLA Molecular Instrumentation Core. The samples were mixed with α-cyano-4-hydroxycinnamic acid and spotted onto the test plate then treated with a vacuum dessicator. Samples where the solvent did not evaporate after an extended amount of time were discarded after an aliquot was saved for Electrospray Ionization Mass Spectrometry (ESI-MS), and the

![Figure 3.3 MALDI MS of cBBP without terminus modification, magenta – BME & 4VP, yellow – 4VP only, red – control.](image-url)
reaction was repeated. The matrix encapsulated sample was laser-desorbed and the time of flight through the instrument was recorded.

Electrospray Ionization was performed on an Applied Biosystems Q-STAR elite Quad-TOF mass spectrometer in positive mode.

The MALDI-TOF data was exported from the instrument as delimited txt files and imported into R x64 2.15.0 statistical software and analyzed using the MALDIquant package\textsuperscript{16} to label and quantitate peaks after filtering by signal to noise ratio. Peptide fragments masses were generated using ProsightPTM\textsuperscript{17} and compiled into a list consisting of possible variations in addition and combination of fragments via disulfide linkage. The list was then searched for peak assignment. In addition four databases of protein/peptide masses were searched using the MASCOT search engine. This was done to anticipate alternative explanations for difficult to assign peaks.

**Results**

In previous experiments, the reaction was attempted with a nearly identical protocol on the double alanine-substituted peptide A5A12; this peptide generated an intense peak at a mass of 2236 in the unreduced condition whether or not vinylpyridine was added. When the disulfide was reduced with β-mercaptoethanol, one intense peak was generated at 2448.26 indicative of modification of both cysteines with pyridylethyl moieties. In the unsubstituted cBBP with valines intact, the reduced peptide (table 3.2, figure 3.3) generated a single intense peak at 2503.226 Da, consistent with the molecular weight of a double pyridylethlated peptide. This molecular weight follows from the amino acid sequence and includes N-terminal acetylation and C-terminal amidation performed during synthesis for aminopeptidase protection and synthetase
blocking. The weight also increased from nucleophilic attack by 4-vinylpyridine with protonation at the pyridine nitrogen, generating a 212.2958 Da addition in total.

**Table 3.1** cBBP control

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>Δ</th>
<th>Sequence</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>568.14</td>
<td>567.2016</td>
<td>0.938414</td>
<td>zC-CRSTc</td>
<td></td>
</tr>
<tr>
<td>2291.051</td>
<td>2291.64</td>
<td>0.589</td>
<td>C[ac] RSTVRMSAEQVQNVWVRC [am]</td>
<td></td>
</tr>
<tr>
<td>2307.055</td>
<td>2291.64</td>
<td>15.415</td>
<td>C[ac] RSTVRMSAEQVQNVWVRC [am]</td>
<td>O</td>
</tr>
<tr>
<td>2323.042</td>
<td>2291.64</td>
<td>31.402</td>
<td>C[ac] RSTVRMSAEQVQNVWVRC [am]</td>
<td>2O</td>
</tr>
</tbody>
</table>

Unfortunately the two samples where no pyridylethylation reaction should have occurred (tables 3.1 and 3.3) generated multiple peaks though none of them occurred in the range expected if 4-vinyl pyridine adds. The discrepancy in table 3.1 at 2307.055 and 2323.042 are 15.415 and 31.402 respectively, suggesting addition of one to the first and two oxygens to the second. A similar situation occurs in table 3.3 where the mass is nearly replicated at a value of 2307.071. Structurally this might occur from hydration followed by deprotonation elsewhere.

**Table 3.2** cBBP with β-Mercaptoethanol and 4-Vinylpyridine

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>Δ</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2503.226</td>
<td>2503.936</td>
<td>0.7098</td>
<td>C[ac] [pyrH]RSTVRMSAEQVQNVWVRC [am][pyrH]</td>
</tr>
</tbody>
</table>

A set of peaks that occur in both tables 3.1 and 3.3 are the ones at 568. An intact disulfide bond after fragmentation explains many of the masses seen in later sets. As shown in figure 3.3, fractionation can occur in locations on the peptide backbone; these breakpoints are in order from N-terminus to C-terminus: a-x (rarer), occurring before the carbonyl, b-y, the amide bond, and c-z after the amide N (figure 3.2). A convenient molecular weight of 567.2016 results
from c and z fragment with disulfide intact. Alternatively a multiple of 568 is 2272, the weight of the intact peptide after losing a molecule of water. It is possible that a dehydration reaction generates a charge that distorts the m/z ratio to 568.

**Table 3.3** cBBP with 4-Vinylpyridine

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>Δ</th>
<th>Sequence</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>568.149</td>
<td>567.2016</td>
<td>0.947414</td>
<td>zC-CRStc</td>
<td></td>
</tr>
<tr>
<td>2291.07</td>
<td>2291.64</td>
<td>0.57</td>
<td>C[ac] RSTVRMSAEQVQNVWVRC [am]</td>
<td></td>
</tr>
<tr>
<td>2307.071</td>
<td>2291.64</td>
<td>15.431</td>
<td>C[ac] RSTVRMSAEQVQNVWVRC [am]</td>
<td>O</td>
</tr>
</tbody>
</table>

A peculiar situation arises when the peptide is synthesized without acetylation or amidation. In this case (table 3.4) the peaks are all singular without a discrepancy fitting an oxygen. This suggests that pyridylethylation occurred as expected when the disulfide is reduced and does not occur in the oxidized state despite addition of 4-vinylpyridine in excess. Furthermore, it also shows that the addition of oxygen only occurs to a significant extent in peptide with acetylation and amidation, suggesting that hydration likely occurs on the acetyl group.

**Table 3.4** cBBP without terminus modification

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>Δ</th>
<th>Sequence</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2096.873</td>
<td>2097.4</td>
<td>0.527</td>
<td>CRSTVRMSAEQVQNVWVRC</td>
<td>Control</td>
</tr>
<tr>
<td>2309.008</td>
<td>2309.696</td>
<td>0.6878</td>
<td>C[pyrH]RSTVRMSAEQVQNVWVRC[pyrH]</td>
<td>BME 4VP</td>
</tr>
<tr>
<td>2096.913</td>
<td>2097.4</td>
<td>0.487</td>
<td>CRSTVRMSAEQVQNVWVRC</td>
<td>4VP</td>
</tr>
</tbody>
</table>

When the reaction was performed on an alanine substituted peptide on the twelfth residue (tables 3.5, 3.6, and 3.7) oxygen addition is again seen. In this case, no peptide was synthesized.
without acetylation and amidation. In table 3.5 a set of double peaks occur with one at the expected molecular weight of the intact peptide and another with the extra weight of an oxygen. Interestingly, there is no peak at 568.

**Table 3.5** A12 control

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>Δ</th>
<th>Sequence</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2263.07</td>
<td>2263.59</td>
<td>0.52</td>
<td>C[ac]RSTVRMSAEQAQNVWVRC [am]</td>
<td></td>
</tr>
<tr>
<td>2279.051</td>
<td>2263.59</td>
<td>15.461</td>
<td>C[ac]RSTVRMSAEQAQNVWVRC [am]</td>
<td>O</td>
</tr>
</tbody>
</table>

When the cysteines are reduced (table 3.6), the set of peaks at 2475.196 and 2491.191 indicate that pyridylethylation occurred at both ends of the peptide. The peak at 2491.191 again indicates the likely addition of oxygen. However, this is in contrast to the unsubstituted peptide where the pyridinylethylated peak is singular. This shows that pyridylethylation is not enough of a steric hindrance to block the addition of oxygen or vice versa. Again in this case, there is no peak found at 568.

**Table 3.6** A12 with β-Mercaptoethanol and 4-Vinylpyridine

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>Δ</th>
<th>Sequence</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2475.196</td>
<td>2475.886</td>
<td>0.6898</td>
<td>C[ac][pyrH]RSTVRMSAEQAQNVWVRC [am][pyrH]</td>
<td></td>
</tr>
<tr>
<td>2491.191</td>
<td>2475.886</td>
<td>15.3052</td>
<td>C[ac][pyrH]RSTVRMSAEQAQNVWVRC [am][pyrH]</td>
<td>O</td>
</tr>
</tbody>
</table>

When the sample is treated with 4-Vinylpyridine without first reducing the disulfide bonds (table 3.7), the peaks mirror the ones in table 3.6 at 2263.041 and 2279.037. This is evidence pointing towards the cyclized state of peptide A12. This reaction also adds oxygen as shown by the peak weighing 2279.037. Overall, the A12 reactions occurred as expected for a disulfide cyclized peptide.
When alanine substitution is performed at the twelfth valine as opposed to the fifth valine, the peptide reacts in an unexpected fashion with 4-vinylpyridine. The control sample with no added reagent shows that this unpredicted reaction was not the result of nitrogen flushing, temperature change, or the chromatographic purification steps. Two peaks at 2264 and 2280.1 indicate the peptide is intact and wholly unmodified other than the addition of oxygen. Taken together with the controls for A12 and unsubstituted, the 2280.1 peak shows that the addition of oxygen requires N and C-terminus modification, yet can also occur in the absence of reducing and alkylating agents, thus oxidation might be occurring shortly after peptide synthesis.

When β-mercaptoethanol and 4-vinylpyridine are added, the number of significant peaks increases by a factor of eight. It is interesting to note that for peaks with masses below 2000 Da, while the peptide does fragment, it retains an intact disulfide bond. Interchain disulfide bonds are a common occurrence in many proteins, one example is insulin propeptide cleavage and another is LTBP growth factor binding complex.

### Table 3.7 A12 with 4-Vinylpyridine

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>Δ</th>
<th>Sequence</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2263.041</td>
<td>2263.59</td>
<td>0.549</td>
<td>C[ac] RSTVRMSAEQAQNVWVRC [am]</td>
<td></td>
</tr>
<tr>
<td>2279.037</td>
<td>2263.59</td>
<td>15.447</td>
<td>C[ac] RSTVRMSAEQAQNVWVRC [am]</td>
<td>O</td>
</tr>
</tbody>
</table>

### Table 3.8 A5 Control

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>Δ</th>
<th>Sequence</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2264</td>
<td>2263.63</td>
<td>0.37</td>
<td>C[ac] RSTARMSAEQVQNVWVRC [am]</td>
<td></td>
</tr>
<tr>
<td>2280.1</td>
<td>2263.63</td>
<td>16.47</td>
<td>C[ac] RSTARMSAEQVQNVWVRC [am]</td>
<td>O</td>
</tr>
</tbody>
</table>
Contamination of the sample was searched against databases containing common MS contaminants as well as protein databases, these searches yielded results in the molecular weight range above 2000 Da only. While the mouse and bacterial proteins in the search results cannot be ruled out, the fragments in table 3.9 are more convincing closer matches. It is also interesting that the pyridine functional group becomes protonated when the peptide length nears 19 residues. For the peptides weighing 2439 and 2492, the mass discrepancy that fits water could have

| Table 3.9 A5 with β-Mercaptoethanol and 4-Vinylpyridine |
|---|---|---|---|---|
| **Observed** | **Expected** | **Δ** | **Sequence** | **Explanation** |
| 1046.5 | 1046.516 | 0.01622 | y-VWVRC[am]-C[ac]RS-b | |
| 1060.6 | 1060.684 | 0.08437 | [pyr][ac]CRSTARM[pyr]-c | |
| 1296.7 | 1296.622 | 0.07803 | y-RC[ac]-C[am]RSTARMSA-c | |
| 1311.3 | 1311.652 | 0.35153 | CRSTARM[pyr]SAEQ-b | |
| 1311.708 | | 0.4075 | y-VRC[am]-C[ac]RSTARM[pyr]-b | |
| 1347.7 | 1347.745 | 0.045256 | z-EQVQNVVRC[am][pyr] | |
| 1363.8 | 1363.764 | 0.03602 | y-EQVQNVVRC[am][pyr] | |
| 1672.9 | 1673.788 | 0.88762 | y-AEQVQNVWVRC[am]-CRS-b | |
| 1673.788 | | 0.88762 | y-SEQVQNVWVRC[am]-CR-b | |
| 2439.4* | 2421.453 | 17.9471 | y-RSTARM[pyr]SAEQVQNVW[pyr]VRC[pyr] | H₂O |
| 2466.2 | 2466.513 | 0.313 | C[ac][pyrH]RSTARM[pyrH]SAEQVQNVW[pyrH]VRC-c | |
| 2477.2 | 2476.395 | 0.8052 | C[ac][pyrH]RSTARMSAEQVQNVWVRC[pyrH][am]-y | |
| 2492.2 | 2456.368 | 35.83192 | C[ac][pyrH]RSTARMSAEQVQNVWVRC[pyrH][am] | 2 H₂O |
| 2543.1* | 2547.481 | 4.38058 | C[ac][pyrH]RSTARM[pyrH]SAEQVQNVWVRC[pyrH][am]-b | |
| 2559.1 | 2541.49 | 17.6103 | C[pyrH]RSTARM[pyrH]SAEQVQNVWVRC[pyrH]-b | H₂O |
| 2648.2 | 2632.602 | 15.5979 | C[pyrH]RSTARM[pyrH]SAEQVQNVWVRC[pyrH]-b | O |

*Largest intensity peak of multiplet spanning ~3 Da
Figure 3.4 MALDI MS of A5, green – BME & 4VP, cyan – 4VP only, gray – control.
hydrogens bound to pyridine as protons (figure 3.6) with the oxygen adding at a separate site. Peaks 2439 and 2492 were found as sets of closely overlapping peaks (figure 3.4). Both of these peaks contain pyridinylethyl modification, so the closely spaced series are likely the same peptide fragment in various states of pyridynyl protonation (the pyridine group has a 5.25 pKa).

Although the preferred substrate of 4-Vinylpyridine is the cysteine sulfhydryl, it can also react with methionine and tryptophan when reactant concentrations are high enough. These side reactions are seen in the peaks weighing 1060, 1311, 2439, 2466, 2543, 2559 and 2648 Da.

**Table 3.10 A5 with 4-Vinylpyridine**

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>Δ</th>
<th>Sequence</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>625.4</td>
<td>625.2571</td>
<td>0.14289</td>
<td>y-C-C[ac]RST-c</td>
<td></td>
</tr>
<tr>
<td>644</td>
<td>644.3342</td>
<td>0.334166</td>
<td>z-VWVRC[pyr]</td>
<td></td>
</tr>
<tr>
<td>719.1</td>
<td>719.3317</td>
<td>0.2317</td>
<td>y-VRC[am]-CRS-b</td>
<td></td>
</tr>
<tr>
<td>735.1</td>
<td>736.3581</td>
<td>1.25805</td>
<td>y-VRC[am]-CRS-c</td>
<td></td>
</tr>
<tr>
<td>1046.5</td>
<td>1046.516</td>
<td>0.01622</td>
<td>y-VWVRC[am]-C[ac]RS-b</td>
<td></td>
</tr>
<tr>
<td>1296.7</td>
<td>1296.622</td>
<td>0.07803</td>
<td>y-RC[ac]-C[am]RSTARMSA-c</td>
<td></td>
</tr>
<tr>
<td>1311.3</td>
<td>1311.652</td>
<td>0.35153</td>
<td>CRSTARM[pyr]SAEQ-b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1311.708</td>
<td>0.4075</td>
<td>y-VRC[am]-C[ac]RSTARMSA-pyr-b</td>
<td></td>
</tr>
<tr>
<td>1347.7</td>
<td>1347.745</td>
<td>0.04526</td>
<td>z-EQVQNVVRC[am][pyr]</td>
<td></td>
</tr>
<tr>
<td>1363.8</td>
<td>1363.764</td>
<td>0.03602</td>
<td>y-EQVQNVVRC[am][pyr]</td>
<td></td>
</tr>
<tr>
<td>1672.9</td>
<td>1672.795</td>
<td>0.105114</td>
<td>z-QAQNVVRC-C[ac]RSTA-b</td>
<td></td>
</tr>
<tr>
<td>2264.1</td>
<td>2263.63</td>
<td>0.47</td>
<td>C[ac] RSTARMSAEQVQNVVRC [am]</td>
<td></td>
</tr>
<tr>
<td>2280.1</td>
<td>2263.63</td>
<td>16.47</td>
<td>C[ac] RSTARMSAEQVQNVVRC [am]</td>
<td>O</td>
</tr>
<tr>
<td>2466.2</td>
<td>2466.23</td>
<td>0.03</td>
<td>C[ac][pyrH]RSTARMS[pyrH]SAEQVQNVW[pyrH]VR-c</td>
<td></td>
</tr>
</tbody>
</table>
When the disulfide was not treated with a reducing agent, the reaction with 4-vinylpyridine still generated a large spread of peaks in the fifth residue alanine-substituted peptide (table 3.10). Similar to the reduced reaction pyridylethlylation occurred at cysteines as well as methionines and tryptophans as seen on peaks 1311 and 2466. Likewise, an oxygen addition product is also seen in peak 2280. One difference not seen in the reduced reaction is the low molecular weight products below 1000 Da, however such low molecular weight products were seen in the unsubstituted cBBP. In this case second multiples of peaks 625, 644, 719 and 735 all yield fragments within 1-2 Da. A likely alternative explanation for these peaks is that fragmentation generates a charged molecule throwing off the m/z ratio. Another interesting phenomenon is that pyridylethyl groups do not tend to be protonated below 2000 Da. This is consistent with the reduced reaction where protonation does not occur unless the fragment has 18 residues.

Electrospray Ionization

Electrospray ionization was performed on 4 reaction conditions, unreduced A5 with and without 4-vinylpyridine, unreduced A12 as well as unreduced cBBP. These samples were reacted independent of the samples used for MALDI, however conditions were identical with minor differences during desalting into acetonitrile TFA buffer. On the whole, ions fragmented to a much smaller size and showed more charge as is typical of the electrospray method. Data for unreduced A5 with 4-Vinylpyridine (table 3.11) indicated that the ionization method yielded fragments that broke at points not easily correlated with the MALDI fragments (table 3.10). This suggested that fragmentation is random. To account for multiple charges on a single fragment, the molecular weight of the whole peptide was scaled by the reciprocal of the total charge. Peaks at 366 Da and 667 Da generated masses that were only possible had the cysteines been
<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>$\Delta$</th>
<th>Sequence</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>375.2724</td>
<td>375.2052</td>
<td>0.06723</td>
<td>y-VRC</td>
<td>-</td>
</tr>
<tr>
<td>391.2825</td>
<td>392.188</td>
<td>0.9055</td>
<td>y-VWVRC[am]-CRSTA-b</td>
<td>.333</td>
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<tr>
<td>377.2749</td>
<td>377.1302</td>
<td>0.14475</td>
<td>y-RC[am]-C-b</td>
<td>-</td>
</tr>
<tr>
<td>377.1302</td>
<td>0.14475</td>
<td>y-C[am]-RC-b</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>350.2425</td>
<td>350.1623</td>
<td>0.080167</td>
<td>z-WVRC-CRST[ac]-c</td>
<td>.333</td>
</tr>
<tr>
<td>358.2456</td>
<td>359.1864</td>
<td>0.940846</td>
<td>z-RC[pyr]</td>
<td>-</td>
</tr>
<tr>
<td>430.8984</td>
<td>430.7117</td>
<td>0.186697</td>
<td>z-VRC[am]-C[ac]R-c</td>
<td>0.5</td>
</tr>
<tr>
<td>430.6999</td>
<td>0.19846</td>
<td>y-WVRC-C[ac]R-b</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>439.2846</td>
<td>439.2131</td>
<td>0.071485</td>
<td>y-WVRC-C[ac]R-c</td>
<td>0.5</td>
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<tr>
<td>519.1449</td>
<td>519.243</td>
<td>0.098086</td>
<td>z-VRC[am]-C[ac]-c</td>
<td>-</td>
</tr>
<tr>
<td>519.2195</td>
<td>0.07456</td>
<td>y-VRC-C[ac]-b</td>
<td></td>
<td>-</td>
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<tr>
<td>667.4907</td>
<td>667.4086</td>
<td>0.08212</td>
<td>y-WVRC[pyr]</td>
<td>-</td>
</tr>
<tr>
<td>697.4814</td>
<td>697.8452</td>
<td>0.36379</td>
<td>y-VRC[am]-C[ac]RSTARMSA-c</td>
<td>0.5</td>
</tr>
<tr>
<td>800.5937</td>
<td>800.8789</td>
<td>0.285188</td>
<td>z-QVQNVVWVRC-C[ac]RST-b</td>
<td>0.5</td>
</tr>
<tr>
<td>800.8845</td>
<td>0.290803</td>
<td>z-QNVVWVRC-C[ac]RSTAR-b</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>884.6597</td>
<td>884.414</td>
<td>0.245705</td>
<td>y-VWVRC-CRSTARMSAE</td>
<td>0.5</td>
</tr>
<tr>
<td>1007.631</td>
<td>1007.594</td>
<td>0.0361</td>
<td>y-QNVVWVRC[pyr]</td>
<td>-</td>
</tr>
<tr>
<td>1007.466</td>
<td>0.164184</td>
<td>z-WVRC[am]-CRST-c</td>
<td>-</td>
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<tr>
<td>1007.512</td>
<td>0.11816</td>
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<td>-</td>
<td></td>
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<tr>
<td>1145.749</td>
<td>1145.561</td>
<td>0.187974</td>
<td>z-QVQNVVWVRC[am]-C[ac]-c</td>
<td>-</td>
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<tr>
<td>1145.537</td>
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<tr>
<td>1145.525</td>
<td>0.224074</td>
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<tr>
<td>1268.969</td>
<td>1269.563</td>
<td>0.59425</td>
<td>y-C[am]-C[ac]RSTARMSAE-c</td>
<td>-</td>
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<tr>
<td>1383.389</td>
<td>1383.628</td>
<td>0.23906</td>
<td>y-RC[am]-CRSTARMSAE-c</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.5 ESI-MS of A5 with 4VP only

pyridylethylated with delta from the expected value of less than 0.1 Da. Similarly in the peak seen at 1007 Da, pyridylethylation could also have occurred here, and has a lower delta than any of the alternative fragments. However in figure 3.5, the spectra shows that these peaks were
relatively low in intensity, suggesting that the extent of the pyridylethylation reaction was incomplete and that the peptide is at least partially cyclized.

**Discussion and Conclusion**

One phenomenon that was observed was the presence of disulfide linked fragments despite treatment with β-Mercaptoethanol. This peculiar observation is explained with a closer look at the equilibrium constant of the reduction.

\[ \text{BME}_{\text{red}} + \text{RSSR} \xrightleftharpoons[k_{\text{eq}}]{pH7.4} 2 \text{RSH} + \text{BME}_{\text{ox}} \]

Although BME is a stronger reducing agent than many of its counterparts such as TCEP or DTT, the reaction has an equilibrium constant \( K_{\text{EQ}} = 3.6 \). Using the following reaction quotient:

\[ \frac{[\text{RSH}]^2[\text{BME}_{\text{ox}}]}{[\text{RSSR}][\text{BME}_{\text{red}}]} \]

and an equimolar reactant concentration of 1M, at equilibrium 44.07% of the disulfides remain oxidized. With a large proportion of disulfides intact, many will survive fragmentation. While fragmentation can also occur across the disulfide bond, generating a negatively charged fragment, this did not occur to a measurable extent with any of the peptides.

The mass of 4-vinylpyridine fluctuated from 105.14 to 106.14 due to protonation at the nitrogen to pyridinium(figure 3.6). The pyridine group without ethylation has a pKa of 5.2,
while a solution of 0.1% TFA typically has a pH of 2.0-2.2. This generates a ratio of 1000:1 protonated to deprotonated. The pyridinium is particularly stable because the heterocycle can distribute the positive charge. Furthermore, protonation can also occur during ionization. Pyridylethylation of the peptide beyond the two cysteine residues required alternate lower affinity sites. These two sites are found in the form of methionine and tryptophan. Methionine is attacked by the electrophilic vinyl group and in the reaction loses the methyl group bound to sulfur, generating 4-pyridylethylhomocysteine\(^7\) (figure 3.6). Tryptophan is attacked at the indole nitrogen generating 4-pyridylethyltryptophan. One strategy to prevent side reactions such as pyridylethylation at ectopic sites is to protect methionines and tryptophans using phenol.\(^{18}\)

Another mass discrepancy that occurred in nearly every reaction was the addition of oxygen. This addition occurred with any loss of mass, not easily accomplished when all bonds are exhausted by the 4 covalent bonds attaching to carbon, while sulfur can use one of its two lone pairs to form an additional two bonds. This allows oxygen to double-bond to sulfur while leaving the disulfide intact forming a thiosulfinate (figure 3.6). In the case seen in cBBP control, this can occur on both sulfurs generating thiosulfonite. In the reduced A5 sample, oxygen adds

![Figure 3.7 Acid Catalyzed Gem-Diol Formation, figure created in Chemdraw.](image)

with two hydrogens, giving a total increase consistent with a water molecule. This occurs in a reaction seen typically in carbonyls—the formation of acetals and hemiacetals. In an acid catalyzed reaction (figure 3.7) water attacks the carbonyl carbon and the resulting gem-diol has two hydroxyl groups accounting for the entire water molecule.
Taken in total, these data show that A12, A5A12 and unmodified cBBP, all spp24 derivatives, are completely cyclized, blocking pyridylethylation in the unreduced form. A5 however is not fully cyclized shown by the complex group of peaks seen in tables 3.10 & 3.11.
References


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19. Koskinen VR, Emery PA, Creasy DM, Cottrell JS. 2011, Hierarchical clustering of
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Biomol Tech. 24(Suppl): S64.
Chapter 4

Osteoblast Mineralization Marker Expression

The overall goal of this work is to sufficiently characterize a growth factor releasing system harboring osteogenic cells so that the system can be computationally modeled. An important system component is the cells and cellular physiology can be determined by marker expression. Bone mineralization in osteoblasts is characterized by three stages, early, intermediate and late. This study examined one early marker, alkaline phosphatase\(^1\)-\(^4\) ALP, an intermediate marker, osteocalcin\(^5\)-\(^7\) OCN, and one late marker collagen I \(\alpha_1\)\(^8\),\(^9\) ColI\(\alpha_1\). Functionally, Osteocalcin along with Osteonectin mediates nucleation of hydroxyapatite crystals from calcium and phosphates available to the osteogenic cell, while Collagen forms fibers in bone around which Hydroxyapatite mineralizes giving form and reinforcing strength. These markers were examined in static culture to serve as a baseline to gauge cell behavior in the perfused scaffold growth. It was expected that there will be stage specific marker expression showing a differential rate of differentiation and mineralization with varying growth conditions. Conditions that affect mineralization marker expression include addition of cytokines as well the presence of pro-differentiation factors such as glucocorticoids,\(^10\),\(^11\) ascorbate\(^1\) or glycerolphosphates.\(^12\) Although it is possible to assay the actual protein product of each marker, analyzing the mRNA that is required for translation of those proteins achieves the same effect and is more easily quantified and is more straightforward for comparison.
Materials and methods

Cell Culture

MC3T3E1 clone 4 preosteoblasts were seeded onto six-well plates at a density of 3 x 10^5 cells in 3 mL of α-minimal essential media (α-MEM) with 10% fetal bovine serum and supplemented with penicillin-streptomycin and glutamine. Following 4 hours to allow cells to attach, the media alone were in control wells, but were replaced with mineralization media (MinM) in experimental groups (see table 4.1). MinM consists of α-MEM modified as previously described, but also supplemented with 400 ng/mL dexamethasone, 50 μg/mL ascorbate, and 1.08 mg/mL β-glycerophosphate. The MinM wells were subdivided into 3 groups which received 1.25 μg/mL BMP-2, 0.4 ng/mL TGF-β or no treatment. The protocol called for media change every three days. The cells were harvested using trypsin at 7 and 14 days.

Table 4.1 Cell Culture

<table>
<thead>
<tr>
<th>MinM</th>
<th>BMP-2 (μg/mL)</th>
<th>TGF-β (ng/mL)</th>
<th>n 7d</th>
<th>n 14d</th>
<th>ECM at 7d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>-</td>
<td>5</td>
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<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>1.25</td>
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<td>+++</td>
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<td>4</td>
<td>Yes</td>
<td>0.4</td>
<td>3</td>
<td>3</td>
<td>+</td>
</tr>
</tbody>
</table>

RNA Extraction

RNA was extracted using the Qiagen RNEasy miniprep kit. The extracted RNA was stored at -80°C. The UV absorbance was measured at 260 and 280nm (data not shown). A MOPS-formaldehyde agarose gel confirmed extraction of total RNA with 2 bands with little smearing indicative of 18s and 28s rRNA largely intact and not cleaved by RNAses.
qPCR

Samples were diluted 1:25 with RNAse free water and assayed using Qiagen Quantitect SYBR green qPCR kit and and were ran on an Opticon Monitor thermocycler. The quantitation was done according to the -ΔΔC(t) method. Threshold was arbitrarily set at 0.0107 ng because initial trials showed that this value was appropriate. The internal control gene used was 18s rRNA and calibrated to untreated ALP at the initial time point. Day 0 RNA was later extracted from untreated cells and was used for calibration and validation.

Alizarin Red Staining

Cells were fixed in 10% neutral buffered formalin for 30-60 minutes, then washed in dH2O. The cells were then stained in a 1% Alizarin red in 0.1% NH4OH. The stain was rinsed 2x with water, immersed for 15 min, then rinsed several times with dH2O. The stained cells were allowed to air dry for 24-48 hours, then examined with light microscopy.

After obtaining micrographs, the cells were destained using a 10% glacial acetic acid extraction solution. Each tissue culture plate well was immersed in 1mL of the extraction solution. The extracted stain was then quantified with an absorbance reading at 450 nm, then compared to a standard curve made from dilutions of a 1 μg/mL Alizarin red solution.

Results

The qPCR method was validated using day 0 ALP expression normalized to 18s rRNA expression. The cycles to threshold values (Ct) were shown to be in the linear range (n=3) (Fig. 4.1). The primers used for amplification are shown in table 4.2.
ALP mRNA showed a modest increase in untreated controls, but remained below levels of cells growing in mineralization media. At fourteen days ALP mRNA levels in all treatment wells were at equivalent levels, the most significant increase from 7 to 14 days was seen in the growth factor free cells, followed by TGF-β, with no significant change seen in BMP-2 treated

![qPCR Validation](image)

**Figure 4.1** Method Validation for qPCR

### Table 4.2 PCR primers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s</td>
<td>5’-CGGGTCATAAGCT-3’</td>
<td>5’-CCGCAGGTTCACCTACGG-3’</td>
</tr>
<tr>
<td>ALP</td>
<td>5’-GCAGGATTTGACCACGGACATCATG-3’</td>
<td>5’-TTCTGCTCATGGACGCGCCTACCG-3’</td>
</tr>
<tr>
<td>OCN</td>
<td>5’-CTGACCTACAGATCCCAAGC-3’</td>
<td>5’-TGCTCTGATAGCTCGTCACAAG-3’</td>
</tr>
<tr>
<td>ColIa1</td>
<td>5’-GCTCCTCTTACGGGCGCCT-3’</td>
<td>5’-ATTGGGGACCCCTTAGGCCAT-3’</td>
</tr>
</tbody>
</table>
Figure 4.2 qPCR Expression Plots

A) ALP mRNA Expression Time Course

B) OCN mRNA Expression Time Course

C) Relative Marker Expression - Semilog plot.
cells (Figure 4.2 a & c). BMP-2 treatment shows an increase in ALP expression at seven days, and reaches a plateau at 14 days, while mineralization media and TGF-β have lower 7 day expression, but continue to climb and reach the same level where BMP-2 plateaus.

Figure 4.3 A) Collagen I a1 expression time course  B) Mineralization Assay
OCN mRNA levels showed more intergroup variation than ALP levels. In untreated controls, OCN expression increased more than twelve-fold, while BMP-2 remained relatively unchanged at levels equivalent to the 14 d untreated controls (Figure 4.3 b & c). In the MinM only group, OCN showed a slight decrease from moderate levels (being lower than both growth factors, but higher than αMEM without mineralization supplements) while TGF-β showed a large decline from levels between BMP-2 and mineralization media without growth factor 7 day values, to a level significantly below all other conditions.

Compared to other markers examined, ColIa1 transcripts were present in very high copy number, but modest temporal changes within each condition and compared between conditions (figure 4.3a). Unlike OCN (figure 4.4) and ALP, ColIa1 shows decline instead of increase. Mineralization, measured by Alizarin red staining did not show such mild results, in figure 4.3b untreated control was over 200 fold higher than MinM and BMP-2 groups, while TGF-β treated cells were over 400 fold higher than the two said groups. In figure 4.4, mineralization only increases with increase in Osteocalcin expression in BMP-2 treated cells, where in mineralization media only and TGF-β media (4.4C,D), there is a decline in OCN expression that counters the trend of mineralization increase. This suggests that in those conditions mineralization is not occurring through the osteogenic deposition of hydroxyapatite, but possibly an alternate process of calcification such as necrosis. The lack of mineralization increase seen in figure 4.4a despite OCN presence is likely due to insufficient hydroxyapatite substrate.
Figure 4.4 A) OCN in αMEM   B) OCN with BMP-2   C) OCN in MinM   D) OCN in TGFβ

Error bars for mineralization assay are seen in figure 21B.

A  
Osteocalcin Expression and Mineralization in αMEM

B  
BMP-2 Treated Cells Osteocalcin Expression and Mineralization
In summary, the results indicate that untreated controls (αMEM) saw an increase in OCN and high expression of Col1a1, while MinM saw an increase in ALP, BMP-2 had stable moderate to high levels of expression of all three markers and TGF-β saw marginal increase of ALP and decrease in OCN with high levels of Col1a1 expression. Across all conditions, Col1a1 had high levels of expression that fluctuated slightly and in general declined.
**Discussion and Conclusion**

Untreated control showed an increase in the intermediate marker, OCN suggesting the cells were entering an intermediate stage of differentiation without any treatment, furthermore, the high level of expression of late marker ColIa1 indicates that differentiation had progressed to a certain extent prior to any treatment. Clone 4 MC3T3E1 cells are a mineralizing subclone, a certain level of differentiation has already occurred during cell line creation\(^{15}\) making them more susceptible to osteoblast differentiation than primary cells. A relevant observation is that during trypsinization, the untreated controls were relatively easy to remove suggesting that little extracellular matrix had been synthesized by these cells. This suggests that the level of ColIa1 mRNA, though high compared to other markers, did not result in substantial extracellular matrix accumulation. In fact, collagen biosynthesis does not correspond temporally to extracellular matrix accumulation, collagen accumulation and deposition can occur in full force when collagen biosynthesis is on the decline.\(^ {16}\) Down regulation of collagen expression suggests exiting late stage differentiation, but the culture can be regressing back to an earlier stage or fully differentiating into an osteoblastic phenotype and accumulating ECM. Without evidence of ECM accumulation, regression into an earlier stage in the untreated control culture is more likely. Intermediate stage of differentiation is signaled by downregulation of markers from other stages and upregulation of an intermediate stage marker, Osteocalcin was used as that marker. It has been demonstrated that mineralizing subclones can be induced by ascorbate to express a luciferase reporter gene when it is transfected under an osteocalcin promoter,\(^ {15}\) showing one mechanism of inducing of osteocalcin transcription. Increase in OCN expression with mineralizing media containing ascorbate verifies this result.
In the TGF-β treated group, similarly high levels of ColIa1 expression was attained, but unlike the other conditions, there was a rebound in ColIa1 mRNA at 14 d. This is consistent with experiments in serum containing media where TGF-β can suppress collagen biosynthesis at high doses, but slightly increase it at low doses, where the increase in cell number at day 14 was equivalent to lowering dosage. The mineralization profile is also lower than other conditions in mineralizing media, suggesting TGF-β is antagonizing osteoblastic differentiation. Supporting antagonism, at 14 d, OCN has declined beneath all other conditions including αMEM. Osteoblastic antagonism suggests chondrocyte differentiation. The only inconsistent element is the ALP mRNA—it climbs, where in serum containing media, it is expected to fall. However in other work, the inhibition of ALP is seen already at 72 hours. ALP downregulation may have also already occurred in the TGF-β cultures and the ALP level seen at 7 days is the result of a subsequent round of transcription induction caused by mineralizing supplements.

Stable levels of all three markers for BMP-2 treated cells suggests that these cells have differentiated early due to the potency of the growth factor, and have already passed late stage and the abundant ColIa1 transcripts decline to signaling biosynthesis of ECM. This is evidenced by the difficulty trypsinizing these cells suggesting copious collagenous ECM deposition (table 4.1).

The mineralization media only treatment group shows somewhat contradictory results. These cells are expected to be in a stage intermediate to untreated controls and BMP-2 supplemented group, and while there is upregulation of ALP intermediate to both groups as well as decline of ColIa1 sugessting collagen biosynthesis, OCN drops to levels comparable to the TGF-β group. This is inconsistent with evidence of extensive ECM production seen from extended trypsinization times as well as intermediate levels of mineralization as expected. One
explanation would be to consider the mechanism of differentiation induction in these cells. In morphological examination of the TGF-β treated cells, they tend to form nodules, seen in later stage post-confluent MC3T3E1 cells. Since differentiation in this group is not triggered uniformly across the well by a diffusible growth factor supplemented in the media, differentiation likely occurs slower and to a less complete extent. The presence of mineralization cofactors and substrates do allow differentiation to a mineralization-conducive state as evidenced by nodule formation, not seen in the α-MEM control. This type of substrate-driven differentiation might occur without expression Osteocalcin to mediate formation of hydroxyapatite, yielding the slower mineralization rate. Another explanation for the cellular behavior seen in the MinM cultures is the antagonistic action of Dexamethasone, it has been shown that while a potent inducer of osteoblastic differentiation in bone marrow stromal cells, it can repress osteoblast differentiation and induce apoptosis in later stage preosteoblastics cells, at the same time it has also been shown that in these cells it induces expression of BMP-2 causing autocrine triggering of the BMP-2 pathway. Both effects could be occurring simultaneously, with antagonistic results, causing the slight decrease in OCN expression.

While stage specific expression was not perfectly delineated, clear and distinct effects were seen in each of the treatment conditions. It is possible to explain the 7 and 14 day interval data based on literature examples of osteoblast marker expression. Differentiation rate seems to be in this order, from slowest to fastest: untreated control, MinM, followed by BMP-2, with TGF-β acting as an antagonist to differentiation. The rate of differentiation based on the bioavailability of BMP-2 allows prediction of cellular behavior. This bioavailability can be modeled computationally and predicted from implantable system release of BMP-2 modulated by proteins such as spp24 and its various derivatives.
References


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Miner Res.4:1, 37-45.

and calcification in a new clonal osteogenic cell line derived from newborn mouse 

19. Martinovic S, Borovecki F, Miljavac V, Ksic V, Maticic D, Francetic I, Vukicevic S. 
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Chapter 5

Scaffold Characterization

This chapter will describe important properties of biomaterials that can be used to deliver growth factors in bone tissue engineering. These properties, used in bioreactor simulation and testing, will allow the selection of an appropriate scaffold to mediate bone growth. The choice of scaffold in tissue engineering is an important decision that involves many different factors. Some important factors include the difficulty of synthesis, material properties, biocompatibility, and bioresorbability. To create the construct tested in the simulation, a number of different scaffolds were considered. These included crosslinked collagen sponge, solvent-casted/particulate-leached PLGA and chitosan. One reason collagen sponges are often chosen as a biomaterial for delivery of growth factors is because of cell compatibility stemming from its natural origins. As a composite material, collagen has been shown to increase initial $^{125}$I-labelled BMP-2 retention at the cost of poorer retention past four days.\(^1\) The decrease in prolonged retention is tolerable if osteoblast-targeted BMP-2 action occurs in the first 96 hours but the window of action is not known exactly, therefore sustained delivery of growth factor over an extended period ensures desired cell response. This shows the need for engineering a collagen-based delivery system. Composed of collagen I fibers cross-linked by formaldehyde,\(^2\) Helistat has been used to examine release kinetics of BMP-2 modulated by binding proteins. As mentioned previously, excessive BMP-2 release creates inflammation and soft tissue edema in vivo.\(^3\) Thus, the scaffold containing BMP-2 should be closely examined to determine its effect on BMP-2 release. To characterize Helistat, volume displacement densitometry was used to ascertain its density and porosity. A form of rate dialysis was used to determine how helistat attenuates diffusion compared to free solution. These methods however did not elucidate
significant content regarding the microstructure. Two microstructural parameters of interest are pore diameter and shape. To reveal initial estimates for these parameters, paraffin embedding was used followed by treatment with hematoxylin and eosin. While collagen readily takes up the stain, the method does distort the original geometry of the tissue—furthermore histological sections are by nature two-dimensional.

To more accurately analyze helistat microstructure, a three dimensional image was needed. Standard methods for three-dimensional examination of scaffolds include scanning electron microscopy SEM and microcomputed tomography µCT. While SEM resolution is high, the depth of section scanned is thin. When used to examine a scaffold interior, the assessment of morphology is primarily qualitative. Another method sometimes used for the analysis of collagen is optical coherence tomography OCT, this technique is limited because the depth of penetration of unscattered light is thin and penetrated light is further complicated by collagen’s birefringence. Using X-ray radiation, µCT is not affected by scattering and limited depth of penetration, however collagen’s translucency to X-rays creates another problem. Images generated tend to be too light and difficult to discern from background noise. To address this problem, collagen was stained with a contrast agent to increase its opacity. SEM staining agents such as osmium tetroxide (OsO₄) and uranyl acetate (UO₂(OAc)₂) have also been used as contrasts to varying levels of effectiveness. Silver nitrate (AgNO₃), commonly used in protein gel electrophoresis was chosen as a reagent because of its ability to resolve histological details as fine as neuronal processes. The utility of this method was evident through processing the generated three-dimensional images for computational fluid dynamics simulation. Knowledge of the microarchitecture gave the dimensions for further simulation with helistat on an even smaller scale. Modeling a material sample with thickness similar to a pore wall, compression and
bending under fluid flow were determined for helistat and compared to PLGA. Finally, a bioassay using MC3T3 cells determined scaffold receptivity to cell growth.

**Materials and Methods**

Helistat (Integra, Plainsboro, New Jersey) was purchased from the manufacturer and cut into cylindrical plugs using an 8 mm diameter biopsy punch in a biosafety cabinet then stored in sterile petri dishes until use. The average thickness of a helistat sheet was 6.4 mm.

PLGA or Poly-Lactide-co-Glycolic Acid (Lactel Absorbable Polymers, Pelham, Alabama) was purchased as 85% Lactide and 15% Glycolic Acid (85:15 PLGA). The polymer was dissolved overnight in a 15% w/w chloroform:water solution. 1.8 g of this solution was mixed with methanol. Sugar (6.48 g) was added as a porogen and thoroughly mixed. The resulting paste was molded into ultrasonically cleaned Teflon molds (3.5 cm diam x 0.3 cm thickness). The scaffolds were immersed in purified H<sub>2</sub>O for several hours to ensure removal of porogen.\textsuperscript{11} The polymer scaffolds were stored in sterile petri dishes until ready for use.

The collagen plugs were also subjected to volume displacement densitometry using purified H<sub>2</sub>O and standard glassware and an electronic balance. Acquiring wet and dry weights allowed calculation of porosity as well as density. Porosity was calculated as follows:

\[
\varepsilon = \frac{v_{\text{calc}} + v_{o} - v_{f}}{v_{\text{calc}}}
\]

Where \(v_{f}\) is final volume with sponge, \(v_{o}\) is the volume prior to sponge placement, and \(v_{\text{calc}}\) is the volume of a typical sponge calculated from measured dimensions.

Rate dialysis was performed on a Beckman DU-65 spectrophotometer with a sipper attachment. A helistat plug was placed in a 24 well tissue culture plate with Falcon cell culture
inserts (BD Biosciences, San Jose, CA). The helistat was carefully placed to cover the entire interface between the well and the insert creating two chambers. The membrane on the inserts was removed to prevent interference with measurements (figure 5.1B). Peristaltic pump tubing was used to connect both the inflow and outflow of the sipper. Continuous operation of the sipper ensured a well-mixed chamber with a total volume of 5.1 mL. The spectrophotometer was programmed to take a UV$_{280nm}$ measurement once a minute for the 180 minute duration of the experiment. At the start of the experiment, 400μL of a 5mg/mL casein solution in phenol red-free α-MEM, was placed in compartment one or the insert and allowed to diffuse to compartment two throughout the course of the experiment.

Helistat scaffolds were fixed using 10% formalin for 24-72 h, followed by storage in 100% ethanol. Histological examination by paraffin embedding followed by hemotoxylin & eosin staining, embedding and staining were performed at the Translational Pathology Core Laboratory at UCLA (TPCL).

Silver staining followed the same fixation procedure as above. Glassware for the stain was cleaned with nitric acid to prevent contaminants from interfering with the stain. The sponges were washed for 5 min with a 2.5% potassium dichromate ($K_2Cr_2O_7$) – 4% formaldehyde solution. They were then incubated in the dark for 7 days at 25°C in a 2.5% potassium dichromate, then blotted with filter paper and incubated for 48 h in 0.75% silver nitrate ($AgNO_3$). The sponges were then rinsed with water to remove excess silver nitrate. The potassium dichromate and silver nitrate steps were repeated three times. The treated sponges were stored in 70% ethanol in the dark until they were imaged. For comparison, a protocol using the Silver Stain Plus kit for protein electrophoresis gels (Biorad, Hercules, California) was applied to control sponges. The sponges were scanned using a microCT 35 system (ScanCo, Basserdorf, Switzerland). 600 slices
per sponge were taken over the course of 5 hours. The image slices were post-processed on an OpenVMS workstation using ScanCo’s proprietary software written in IPL. The slices were then imported into ScanIP (Simpleware, London, United Kingdom) as grayscale files. Further image processing and reconstruction was done on an Intel i7 with 8 gigabytes of memory. The images were cropped and resampled, then segmented and a mask was applied. Several filters were then applied including morphological close, gaussian smooth and island removal. Reconstruction yielded a 31.25 micron resolution three-dimensional image. Volume meshes were then created using the +FE module. The resulting meshes were exported as finite element and lithography files. The meshes were imported into Comsol Multiphysics (Comsol, Stockholm, Sweden) and fluid flow was modeled through using the laminar flow module. The module employs the incompressible Navier-Stokes Equations as follows:

\[
\mathbf{u} = -\nabla p + \eta \nabla^2 \mathbf{u} \\
\nabla \cdot \mathbf{u} = 0
\]

With boundary conditions: \( \mathbf{u} \cdot \mathbf{n} = u_0 \), inlet; \( \mathbf{u} = 0 \), walls; \( p = 0 \), outlet where \( \mathbf{u} \), \( p \), \( \eta \), \( \mathbf{I} \) and \( \mathbf{n} \) are velocity, pressure, viscosity, identity matrix and normal vector respectively. Fluid flow was simulated for 60s with a set of inlet velocities ranging from 400 to 800 \( \mu \text{m/s} \). The solver used was flexible generalized minimization of residuals (FGMRES) preconditioned with a Vanka smoother. Shear stress was calculated from the velocity field during post-processing.

In order to determine if compression and deformation occur due to fluid flow, the wall thickness of pores was measured from 2D sections taken from the reconstructed \( \mu \)CT image. The thickness was used to create a simulation of thin structure of equal thickness to the wall, the structure was placed in a flow channel and subject to flow velocities expected in a perfusion system. The technique used to track a continuously deforming structure in Comsol is the
arbitrary Lagrangian-Eulerian (ALE) technique. The 2D simulation used laminar flow at the channel entrance with characteristic parabolic velocity profile described as follows:

\[
5.3 \quad u_{in} = \frac{U \cdot t^2}{\sqrt{(0.04 - t^2)^2 + (0.1t)^2}}
\]

Where \( U \) is the steady state x-velocity amplitude, \( u_{in} \) is the centerline velocity in the x direction and \( t \) is in seconds. The structured is fixed to bottom of the channel at its base, while the remainder is free to be deformed by fluid load as described by the following equation:

\[
5.4 \quad F_T = -n \cdot (-p + \eta \nabla^2 u)
\]

Where \( n \) is the vector normal to the channel boundary, \( u \) is the velocity vector, \( p \) is pressure and \( \eta \) is the viscosity. Structure width was varied from 20-100\( \mu \)m with a fluid entrance velocity from 400-1800\( \mu \)m/s. Average solution time to sweep through all structure widths and flow velocities was 3-5 hrs.

MC3T3E1 cells were cultured as described in the previous chapter using αMEM in 10% FBS, supplemented with glutamine and antibiotics, but without mineralizing supplements. Cells were seeded after trypsinization from culture wells using a 3.2x10^6 cells/mL suspension. 125 \( \mu \)L of this cell suspension was dripped onto each scaffold and incubated at 37°C for 4 hours. Each scaffold was then rinsed with warm PBS and placed in cell culture inserts. The wells surrounding the inserts were filled with approximately 5.75 mL of culture media. The plates were then placed on a tilt shaker set at the minimal setting housed within an incubator (n=6). In parallel, a sponge was also cultured in the bioreactor described in following chapters (n=3). This bioreactor loaded scaffold was perfused with culture media using a peristaltic pump. The scaffolds were grown for 48 or 96 hrs, at which point they were fixed for histology or subject to a cell viability assay. The cells were assayed in triplicate for 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide (MTT) metabolism, using a Vybrant MTT cell proliferation kit (Molecular Probes, Eugene, Oregon).

Results

Porosity Measurement

The porosity was determined using 2 cm x 2 cm squares of helistat immersed in purified H₂O. There was some variability in weighing the wet helistat because some water was lost during transfer. In addition, the shape of helistat changes upon wetting, distorting the volume measurement. To correct for this, the porosity data vector for each sponge was multiplied by a 2x2 unit trace diagonal weighting matrix μ, μ₂₂ was .75 and .25 was used for μ₁₁. The resulting vector was multiplied by ln⁡T (n=2) to generate εₚ. This gave a measured porosity εₚ of 0.8834, a value consistent with the porosities of other scaffold materials. The results shown in table 5.1 agree well with the accepted value of 90% porosity for tissue engineering scaffolds.

Table 5.1 Porosity Measurements

<table>
<thead>
<tr>
<th>Wet wt (g) (±0.0001)</th>
<th>Dry wt (g) (±0.0001)</th>
<th>H₂O wt (g) (±2x10⁻⁵)</th>
<th>Dim vol (mL) (±7x10⁻⁵)</th>
<th>H₂O vol (mL) (±22x10⁻⁵)</th>
<th>Displ vol (mL) (±5x10⁻⁵)</th>
<th>εᵱ (±5x10⁻⁴)</th>
<th>εᵳ (±5x10⁻²)</th>
<th>εₚ (±5x10⁻²)</th>
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<td>0.9599</td>
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<td>0.10</td>
<td>0.5396</td>
<td>0.96</td>
<td>0.86</td>
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</tbody>
</table>

Rate Dialysis

Plot of concentration change in the well-mixed sipper compartment gave a linear fit at shown in figure 22A. The slope per unit area of the interface gives a flux of 3.758 nmol/m²·s. Using the first diffusion law (J = D∇C), the diffusivity of casein across a 5mg/mL concentration gradient was 11.53 x 10⁻⁷ cm²/s .
Casein was chosen because of its close molecular weight to BMP-2. The diffusivity of BMP-2 in free solution is $13 \times 10^{-7} \text{cm}^2/\text{s}$, scaling that diffusivity by the scaffold porosity gives a diffusivity of $11.7 \times 10^{-7} \text{cm}^2/\text{s}$, a very close match to the measured value using the casein rate dialysis (figure 5.1).

**Microtomography**

The kit-based silver stain scanned much lighter than the extended incubation. After scanning, the sponges lost their burgundy color from potassium dichromate oxidation. From a 50 slice reconstruction using ScanIP seen in figure 5.3, the scaffold had an average pore size 50.6 μm and a porosity of .905. The porosity agreed well with volume displacement, the pore size was significantly smaller than measured in histological sections, the discrepancy could be due to artifact generated by paraffin embedding and sectioning. Although it was possible to mesh a quarter of the entire cylindrical section, the large size of the mesh file was unwieldy to manipu-
**Figure 5.2** Microtomography and Reconstruction (a) Grayscale microCT slice, arrow denotes the wall of the imaging solution container. Black dots are areas of particularly low signal intensity, likely large pores that captured air bubbles. (b) Quarter scaffold on “cut” side. There is some evidence of anisotropy--pore size shrinks towards the center of the scaffold. Meshing this section required 814,710 elements. (c) Section used for simulation, the box-shaped rectangular prism had a volume of 2.75mm$^3$, this size was tractable for the finite element analysis. (d) Histological section of Helistat for comparison, embedded in paraffin with Hemotoxylin and Eosin used as stain, scale bar ~300μm (e) Inversion of the original mask allowed creation of a fluid domain that was flush against the scaffold domain, both domains were meshed simultaneously.
Figure 5.3 Histology and Computational Fluid Dynamics CFD simulation (a) 5 slice reconstruction, closeup view shows disc-like indentations floored with thin membranes. (b) Low and high power histological sections of MC3T3 osteoblast cells growing on helistat. Around the periphery, cell growth follows the contour of the scaffold, yet some processes extend outward into pore space suggesting supporting substrate is present, but missed by the plane of section. The central mass is composed of cells clumping to occlude a pore, scale bar ~10μm (c) plot of shear stress along the fluid-scaffold boundary, separation into component subplots show magnitude is concentrated in the x direction.
late. Instead we chose to use a pocketbook-shaped square prism, measuring 1.78 x 2.06 x 0.75 mm (figure 5.2C&E), in a region that was fairly representative of the microstructure found throughout the scaffold. While only 0.85% of the entire scaffold volume, 814,710 tetrahedral elements were needed to mesh the section, generating a corresponding number of degrees of freedom. Average solution time for a single condition in a velocity sweep was ~1 hour. The velocity profile showed low magnitudes with a characteristic fluid shear stress at the fluid-scaffold boundary of 0.2dyn/cm$^2$. A three dimensional component plot of viscous stress showed magnitude to be concentrated in the direction of fluid flow as expected.

![Plot of Von Mises Stress and Velocity](image)

**Figure 5.4** Velocity Profile and Stress Plot of Flow Channel and Collagen Structure. Streamlines in red across the bottom of the flow channel show the path of fluid around the material sample.

**Fluid Structure Interaction**

The thickness of a wall taken from sections of the reconstructed image range from 200-300 μm, with the thinnest section spanning around 100 μm, thus if a material were flow channel-tested with a thickness 100 μm or less, its elastic deformation would describe how a scaffold of that material would likely behave globally. Although helistat has considerable variability in
microstructure, the tomographs show that it can be separated into a fluid/void domain and a solid domain. However the solid domain takes up more volume than accounted for by the porosity. One explanation is that collagen is in a gel phase, retaining water molecules and swelling. A reasonable analogue material would be a collagen gel. A 17.5% gel accounts for the porosity discrepancy and gels in that range of percentage collagen composition have been material-tested thoroughly,\textsuperscript{12} giving a good starting point for simulation parameters. Beams of this composition deform very little, even at the thinnest width of 20μm and challenged with 1800μm/s flow rates (figure 5.5). This simulation showed that at peak amplitude, the displacement was only 7.5 μm, less than half the width of a beam, likely creating very little distortion in pore shape. Although fairly stiff, collagen is considerably more flexible when compared to polymers such as PLGA. The same simulation yielded sub-nanometer displacements in a PLGA structure. In both

![Point Graph of Total Displacement](image)

\textbf{Figure 5.5} Fluid-Structure Interaction with Collagen. Starting at the bottom is 100 μm thickness structure subject to 400 μm/s flow rates, to the top with 20 μm thickness with 1800 μm/s flow rate. A characteristic pattern of initial bending, recovery and equilibration at a slightly bent position is observed.
materials, stress was concentrated around the base of the structure near the edges (figure 5.4). Although simulations were not done with this model for chitosan (deacetylated chitin), the material’s rigidity will likely generate similar results to PLGA.

**Cell Viability Assay**

![Cell Viability Assay Chart](image)

**Figure 5.6** Cell Viability Assay, comparing scaffold material and culture method

The MTT tetrazolium dye assay (figure 5.6) showed that collagen is a better cell substrate than PLGA, though the situation may be complicated by experimental procedures, such as insufficient prewetting or flushing of loosely attached cells using PBS. Cell attachment could be the deciding factor between Helistat and PLGA. PLGA, as a polyester, lacks cell-friendly peptide attachment sites, which are abundantly found on proteinaceous collagen fibers. Comparing cells grown on collagen, perfused cells fared much more poorly than open shaker cells. Oxygenation could be the determining factor for cell viability, however without measurement of inlet oxygen for the perfused cells, it is difficult to conclusively make such a statement. Cell morphology after 48h growth in the bioreactor (figure 5.7 b & c) is not significantly different from growth in shaker. This demonstrates that perfusion does not wash
cells off the sponge or mechanically injures the cells, at least not to an extent that is noticeably different from shaker-grown cells. In addition, cell growth is equally, if not more prevalent, 100µm into the scaffold (figure 5.7c). Furthermore, in the perfused scaffold, cell growth is seen up to the edge where the scaffold was divided in half (the true macroscopic center of the scaffold), though it is possible that compression effects there may have distorted the pore size.

**Figure 5.7** Cell Growth – Optical images taken of histological sections, the sponges were sectioned in half with the remaining half used for the MTT assay (fig 5.6) A) Shaker 5d 100µm depth B) Shaker 3d 100µm depth C) Bioreactor 5d 100µm depth D) PLGA 3d E) Unseeded Collagen

**Discussion and Conclusion**

In this study, we characterized the microstructure of a commonly used collagen scaffold. We then demonstrated that the resulting three-dimensional reconstruction was useful in modeling physical phenomenon such as fluid flow. Without microstructural information, fluid flow can be
approximated using bulk porosity and permeability parameters. However with known microstructure, simulations can be refined to include the immediate microenvironment the cell experiences. The level of shear stress was found to be in a range that generates unique cellular responses. For example, with osteoblasts, 0.25-26 dyn/cm² is experimentally found to be indicative of nitric oxide (NO) release¹³, a common injury response. Although the simulation values show shear stress to be slightly below this range and experimental growth in the bioreactor showed no morphologically visible signs of injury, such responses cannot be conclusively ruled out. In examination of regular architectures such a gyroid or hexagonal architectures, a similar shear stress range is associated with chondrocyte or fibroblast differentiation in undetermined cells.¹⁴ Without doubt, this model is limited by the number of parameters we examined, cellular response may well be affected by a multitude of different factors. Furthermore, due to the heterogenous architecture of Helistat, it may be difficult to approximate different or larger sections with sufficient precision. In future studies and simulations, it would be important to better quantify pore structure¹⁵,¹⁶ and connectedness¹⁷ as well as to examine the fluid-structure interaction on the scaffold. If additional force vectors are applied to substrate, cell response could be altered. Stretching, bending and compression of the substrate could trigger additional mechanotransduction events not anticipated in a model looking primarily at fluid flowing above the cells. This type of real-time elastographic analysis¹⁸,¹⁹ may not be possible with microCT due to scan time. From inspection of the microstructure, there appears to be a porosity gradient directed outward from the center of the sponge. Such anisotropy in porosity could impair nutrient transport and hamper cell growth, playing an important role in limiting cell growth to the surface of scaffolds housed in continuously stirred bioreactors.²⁰ Similar effects need to be considered for the design and operation of a perfusion system.
References


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Chapter 6

Evaluation of Collagen Binding Domains in Fusion Protein Design

The adhesion of a specific binding protein to an implantable collagen sponge is another parameter that is required for design and modeling of a growth factor delivery system for bone tissue engineering. To construct a fusion peptide for the purpose of slowing diffusion of BMP-2 from an implantable scaffold, several factors must be considered. These include ease of synthesis, effect on bioactivity of the Collagen-binding Domain (CBD) on BMP-2 or analogous growth factor, and any biological effects created by the CBD or the whole fusion peptide outside from the intended effects of BMP-2. In considering ease of synthesis, two important concerns arise—the length of the CBD and the amino acid composition of the CBD. Peptides longer than 40 residues tend to be difficult to synthesize. Furthermore with longer chains, the backbone is vulnerable to proteolysis. Using Structural Inducing Probe (SIP), the backbone can be protected from proteolysis through induction of α-helical secondary structure.¹ This process can yield peptides up to 70 residues in length, but the commercial availability is limited. Certain side chains, such as tryptophan are difficult to incorporate and can react prior to use. To overcome the length limitation, an option is to link the two peptides using an EDC/NHS ester reaction with the N terminus for one peptide blocked by (acetylation) and the C terminus of the other blocked by (amidylation). For longer peptides, an alternative to peptide synthesis is to produce the fusion peptide recombinantly with an optimal size of 100 residues or less in E coli and a practically unlimited plasmid size of up to 2MB in mammalian cells.² Our endpoint of bioactivity is BMP-2 induced bone marker expression and mineralization. Comparison of BMP-2 based studies with other growth factor based studies presents an element of difficulty (which is further complicated when the second study is not performed on bone). Since each study uses their own internal
controls, only largely qualitative comparisons are possible. Similar comparison of each study’s unwanted biological effects is only possible as well.

Regardless of the system studied, one of the common goals of creating a fusion peptide is to retain a growth factor by binding it to extracellular matrix. However in considering CBDs, integrins are important because of their cell-attaching function. While integrins bind cells to collagen, a problem lies in that they are multimeric proteins—stymieing production of a single contiguous CBD. (Snake venoms, known as disintegrins mimic the collagen binding site and bind to integrins thereby dissociating cells from the ECM.) High peptide concentrations may block integrin binding, in turn having an effect on cell growth. To avoid interfering with cell attachment, care must be taken to choose binding sites that overlap minimally with integrin-collagen interaction. Fortunately, many of these sites have been characterized including Arg-Gly-Asp RGD and the triple helical GFOGER sequence, Gly-Phe-Hyp-Gly-Glu-Arg (O representing hydroxyproline), found on both collagens I and IV as well as on other collagens as sequence variants. Furthermore, if it is known what collagen residues the CBD recognizes, that sequence can be synthesized and the CBD binding affinity can be characterized on SPR. With such binding kinetic data, simulation of an implantable scaffold system can be further refined.

Aside from that consideration, most of the CBDs examined were easily classified. They were divided into four categories with a fifth for ones not categorized. The first category was von Willebrand’s Factor derived, the second collagenase derived, the third fibronectin derived and the fourth osteopontin derived.
Von Willebrand Factor

Von Willebrand Factor is a 308.9 kDa protein with 2803 residues; it is physiologically involved in hemostasis by promoting platelet-wall interactions at the site of vessel injury. Platelet adhesion is not known to be an important factor for osteogenesis in a collagen matrix, thus making vWF a good choice to avoid blocking needed attachment sites on the scaffold. A truncated protein variant (residues 922-1110) and decapeptide variants (WREPSFxFALS, where x can be Met or Cys) have been joined as fusions to growth factors. All residues of the peptide can be found in hvWF sequence as short contiguous fragments within and up to 300 residues upstream of the truncated protein stretch. The decapeptide sequence shows that M7C substitution that seems to have minimal effect on bioactivity. However, the study using rhBMP-2 retains methionine. This would be consistent synthetically, because side chain methylation will avoid regioselective disulfide oxidation during synthesis.

Figure 6.1 Von Willebrand Factor Protein Interaction The contact surface between vWF (in gray) and protein (botrocetin, in black) is 936.3 Å²
Collagenase

ColH is a 1021 residue protein from gram positive, spore-forming anaerobic rod, *Clostridium histolyticum*. Collagenase activity is isolated to the C-terminal portion of the enzyme (residues 767-981),\(^8,9\) however a minimal binding heptapeptide sequence\(^10,11\) is found in a 30 residue region within the C-terminal CBD. This region was found to have 91% of the binding of the entire 214 residue C-Terminal CBD.\(^9\) The heptapeptide has also been fused to BMP-2 producing an increase in ALP activity.\(^11\)

Fibronectin

Fibronectin is a 273 kDa glycoprotein with 2477 residues typically found in dimers localized to ECM, plasma and other bodily fluids.\(^4\) Fibronectin has been fused a truncated protein fragment\(^5,12\) and as a decapptide.\(^13\) The protein fragment has been used on reendothelialization\(^12\) and collagen fibril aggregation,\(^5\) while the peptide has been linked to biotin.\(^13\) The protein fragment was shown to have similar activity to the protein fragment of vWF.\(^5\)

Osteopontin

Osteopontin is an abundant bone sialoprotein also found in placenta, distal tubules of the kidney and CNS. It is highly phosphorylated with 12 phospho-serines and 1 phospho-threonine. Compared to the other proteins described, it is relatively smaller with 314 residues and a molecular weight of 35 kDa.\(^4\) A peptide CBM (collagen-binding motif) consisting of residues 150-177 has been shown to possess binding activity.\(^14,15\) Furthermore, the 28 residue CBM can induce Hydroxyapatite nucleation when implanted on a collagen scaffold.\(^15\)
Other Sources

In similar size range as osteopontin is Bone Sialoprotein, with 34.9 kDa and 317 residues. BSP is a single chain protein found exclusively in bone. A stretch of 27 hydrophobic residues found downstream of the signal sequence is shown to nucleate hydroxyapatite with increased strength in the presence of collagen. A sequence that also localizes to calcified tissues is polyaspartate Asp₆, this was shown by conjugation to fluorescent marker FITC.

Table 6.1 Collagen Binding Domain Surface Analysis

<table>
<thead>
<tr>
<th></th>
<th>Solvent Exposed (Å²)</th>
<th>Average Contact (Å²)</th>
<th>Percent Contact</th>
<th>pKD/Contact*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase</td>
<td>20268</td>
<td>877 ± 207</td>
<td>4.32</td>
<td>27.98 ± 8.15</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>16248</td>
<td>1291.5 ± 587</td>
<td>7.95</td>
<td>6.55 ± 3.73</td>
</tr>
<tr>
<td>vWF</td>
<td>22205</td>
<td>2231 ± 1051</td>
<td>10.05</td>
<td>6.08 ± 1.30</td>
</tr>
</tbody>
</table>

*relative surface area (contact surface/10⁶ Å²)

Binding Surface Structural Analysis

Using the structure-correlated kinetic database, structural information regarding protein-protein interactions between collagen binding domains and other proteins was comprehensively searched. Using Pymol 1.3, contact surfaces between the binding pairs were mapped out and compared with the total solvent-exposed surface areas of each protein. The results of the analysis are shown in table 6.1 above. Although fibronectin and vWF are similar in strength of association, collagenase is several times stronger.
Conclusion

In order to modulate release of BMP-2 from collagen, the fusion peptides in table 6.2 were designed. When the studies conducted on CBDs were weighed, each category had advantages. Collagenase and vWF have the advantage of existing testing with BMP-2 as well as short size, 7 and 10 amino acids respectively. Fibronectin also has a decapeptide, but with no BMP-2 testing. Osteopontin and BSP are slightly larger at 27 and 28 residues, but can nucleate hydroxyapatite with no growth factor fused. BSP is also found exclusively in bone. The HA nucleation might enhance BMP-2 induced mineralization. Finally Asp$_6$ is simple and likely easy to synthesize. Unfortunately the osteopontin, BSP and Asp$_6$ all lack BMP-2 testing. For ease of construction and evaluation of the collagen binding and release kinetics, vWF was selected as the CBD of choice for simulation purposes. With known binding kinetics, it provided parameters that could be used in finite element simulation of a BMP-2 release from a fusion-protein and growth factor loaded sponge. The resulting kinetics can be verified because vWF is produced by a number of biotechnology suppliers and relatively widely available.

Table 6.2 Fusion Peptide Sequence

<table>
<thead>
<tr>
<th>CBD</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF</td>
<td>WREPSFMA$\ldots$GCRSTVRMSAEQVQNVWVRC</td>
</tr>
<tr>
<td>ASP$_6$</td>
<td>DDDDDDGCRSTVRMSAEQVQNVWVRC</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>GLRSK$\ldots$KFRPPQPDADTEDGTMGCRSTVRMSAEQVQNVWVRC</td>
</tr>
<tr>
<td>BSP</td>
<td>SMKNLHRV$\ldots$GKRYPRYLYGCRSTVRMSAEQVQNVWVRC</td>
</tr>
<tr>
<td>Collagenase</td>
<td>TKKLRTGCRSTVRMSAEQVQNVWVRC</td>
</tr>
</tbody>
</table>
References


Chapter 7

Bioreactor Design

Design and iterative testing of therapeutic biomolecules can be removed from costly and often controversial in vivo animal studies. Ultimately, the goal is for all but final testing to be done by computer modeling. Initially, in vitro testing should be used to define parameters used in modeling. Once sufficient parameters have been characterized, this intermediate in vitro step may no longer be necessary. Computer modeling can be used to test large numbers of potential materials. Candidate materials can be tested in bioreactors and only end products will need to be tested in vivo. In this particular case, to measure the release of BMP-2 from the porous scaffold phase, it was necessary to construct a bioreactor. Bioreactors are reaction chambers that are typically multiphase and can accommodate cell growth. Although this definition encompasses a lot of design variations, bioreactors can be categorized into a few different groupings, based on how culture media is exchanged and agitated. While combinations that include aspects from two or more different categories also exist, these categories are simple batch, spinner flasks, rotating wall, compression and perfusion. The last, perfusion, was the design of choice, but each type was evaluated for suitability.

Bioreactor Categories

Simple batch culture is the method of cell culture many cell biologists are familiar with. It consists of growing cells in dishes or multiwell plates. Media is unagitated and mass transport occurs through diffusion. As a result media amounts are typically small, and must be changed in sterile conditions as nutrients are consumed and waste products build up. The passive mass transport issue is problematic when growing cells in a three-dimensional scaffold. This problem
can be addressed by placing the culture vessels on a tilt shaker small enough to be housed in an incubator. However, fluid flow is unpredictable on the shaker and difficult to model.

Spinner flasks are the benchscale offshoot of continuously stirred tank reactors seen in industrial use. The usually transparent flask has two side arms that can be opened to inject media and scaffolds are suspended in the media by strings tied to the top of the flask. The spinner flask contains a magnetic stir bar responsible for its namesake. Human mesenchymal stem cells have been cultured in spinner flasks for time periods from 5 weeks up to 84 days. In cells with osteoblast commitment or differentiation potential, Spinner flasks demonstrate improved marker expression and mineralization over static culture and rotating wall bioreactors. Unfortunately cell growth is limited to the outer 0.5-1.0 mm because the stirring is insufficient for media to consistently reach into the center of the scaffold. This limits the size of the scaffold, and becomes unsuitable for scaffolds greater than 2 mm in diameter.

Rotating wall bioreactors are two concentric cylinders with scaffolds immersed in media floating between the enclosed walls. Rotation around the long axis of the cylinder creates a microgravity environment for cell growth. As mentioned earlier, marker expression is poorer than spinner flasks. Although marker expression is better than static culture, cell proliferation shows no improvement. This is thought to be due to trauma to cells caused by scaffolds bumping into one another during wall rotation. Mineralization and proliferation are improved when scaffolds are fixed to the wall. Unfortunately, the problem of mass transport is not addressed and poorer marker expression favors other designs.

Compression can be incorporated into reactor design, to induce mechanical stimuli to cells responsive to it. In a study using preseeded HEMA (hydroxyethylmethacrylate)-Lactate-
Dextran Cryogels, the gels were placed in the compression bioreactor and subject to compression by a piston for 1 hr/day at 1 Hz frequency. The compressed cells showed improved alkaline phosphatase activity over uncompressed controls. However there is evidence that global compression induces endochondral ossification, whereas little or no compression favors intramembranous ossification. Since MC3T3 cells are from calvaria, a tissue that undergoes such ossification with nominal loading, the use of compression is not thoroughly justified.

The final design category is perfusion bioreactors. This was also the category that covers the design I will discuss later. In this category, the reactor contains a scaffold that is analogous to the porous phase of a packed bed chemical reactor. Fluid flows in the inlet, through the porous phase, and is collected at the outlet. In a bioreactor, the flow media contains nutrients necessary for metabolism in cells attached to the scaffold. One example of this design is the hollow fiber membrane bioreactor. The porous phase in this reactor setup is a parallel bundle of hollow membranes where cells grow in the interior and around the exterior. This design is used for growth factor production in mammalian cells. It has also been suggested that this design be used for bone tissue engineering because the bundled capillary architecture is structurally analogous to the Haversian canals of mature bone. However like other scaffolds, this construct lacks true circulatory architecture and must rely on perfusion from the bioreactor system to delivery nutrients and remove waste products. Tracer experiments have been done using a dextran dye allowing analysis of flow indices such as the Reynolds Number Re \( \left( \frac{\rho u L}{\mu} \right) \) and Peclet Number Pe \( \left( \frac{Lu}{D} \right) \) as well as the Thiele Modulus \( \varphi^2 \left( k a L ^2 C^{n-1}/D \right) \), a mass transport-reaction dimensionless grouping. Tracer use require bioreactors to be made from transparent material. When compared to spinner flasks and rotating wall bioreactors, perfusion systems tend to have more effective mass transport due to permeation of the entire scaffold by directed flow.
Although perfusion systems have this advantage, introducing fluid flow also generates a set of new variables in the system. These variables include flow velocity, the reactor geometry’s effect on fluid flow and the scaffold’s effect on fluid flow. Fortunately, the first and last considerations are conducive to modeling and simulation using the finite element method, and are given their due attention in following and preceding chapters, respectively. However, while the effects of geometry can be simulated and existing geometries can be altered by shape optimization, the initial geometry requires some effort to engineer a functional design that satisfies the design constraints.

**Analysis of Perfusion Bioreactors**

In order to create a working design, it was important to examine existing perfusion bioreactors. One example was the microfluidic perfusion bioreactor created at the Technische Universitat Braunshweig. Intended for the yeast *Saccharomyces cerevisiae*, this design was the result of topology optimization.\(^{10}\) This reactor was fabricated using photolithography of polydimethylsiloxane PDMS on glass coated with SU-8, an epoxy based material for photoresists. The entire apparatus measured 12 mm by 19 mm, with a channel height of .23 mm. This size of reaction volume is useful for high throughput screening, saving resources by avoiding extensive cell culture and avoiding consumption of potentially expensive substrate or growth factors. Furthermore the isolation of a small cell quantity may be useful for studying interactions that are missed in bulk cell and tissue culture.\(^{11}\) Microfluidic bioreactors are relatively newer incarnations of bioreactor technology. Typically, reactor sizes range from industrial microbial catalysis, spanning several liters, to more delicate animal cell bioreactors, spanning the mililiter range. While microfluidic bioreactors deal with a unique set of problems, their design bases follow their larger cousins. A larger reactor that is referred to in comparison is
the plug flow reactor; used industrially, it is chemical reactor that, like the perfusion bioreactor, relies on fluid flow to deliver and transport reactants. The plug flow reactor also operates with a continuous feed; the entering substrate flows in from a pipe the same diameter as the reaction chamber, in fact, PFRs are also known as tubular reactors. Plug flow refers to the non-laminar, non-parabolic velocity profile. The velocity profile is flat and typically well-mixed.

Further, the TUB microreactor occupant, *S. cerevisae* is a hardy yeast that is often used as an example system for methods usually suitable for generic eukaryotic cells. With further finetuning the microreactor design could easily adapted for the mammalian cells, lending the TUB design for further analysis. In the TUB microreactor system, a polymeric organosilicon (PDMS) compound was deposited on a glass substrate using a photoresist method. This choice of material is significant for mass transport considerations. The total reaction volume of the reactor chamber was 8μL. The inlet shape was optimized using numerical simulation. The resulting geometry was a main channel forking into 14 smaller channels (Figure 7.1). The reaction chamber facing the main channel was a triangle with a vertex pointing towards the main channel. The smaller channels then make inlets with the reaction chamber evenly spaced along the base with the final two placed just shy of meeting at the vertex. This inlet design allowed for homogenous distribution of concentration all along the entire reaction volume. The sensor cavity was at the opposite end of the reaction chamber next to the outlet. The sensor allows for online monitoring of oxygen content via a fluorescent dye system. Oxygen reaching the yeast cells was an important factor facilitating their survival and growth. Because the PDMS layer has limited gas permeability, its thickness had to be balanced with structural rigidity demands. The optimal thickness was found to be 300 microns.
The TUB group also performed computational fluid dynamics simulation of their design. They verified their CFD results with microparticle image velocitometry. The equation for residence time is shown as follows:

$$\bar{\tau} = \frac{v_{\text{reactor}}}{\nu} = \frac{1}{r}$$

In the preceding equation $\bar{\tau}$ is the residence time (s), $v$ is reactor volume ($\mu$L), $\nu$ is volumetric inflow rate($\mu$L/s), and $r$ is the dilution rate (1/s). The residence time distribution was found to be similar to that of a plug flow reactor. Residence time will be discussed later for my bioreactor design. Other important equations used were ones for the analysis of oxygen supply. In the following equation for overall volumetric oxygen transfer rate ($dc/dt$), $K_L$ is the overall liquid-sided oxygen transfer coefficient and $a$ is the specific gas permeable surface area per volume for PDMS, while $C_L^*$ is the oxygen concentration outside the PDMS membrane and $C_L$ is the concentration inside:

$$\frac{dc}{dt} = K_L a (C_L^* - C_L)$$

This equation should also prove useful in constructing a microfluidic bioreactor model. The oxygen mass transfer resistance $1/K_L$ was then equated to an expression consisting of reciprocal diffusivities, $D_{\text{PDMS}}, D_{\text{medium}}$, height $h_{\text{reactor}}$, membrane thickness $d_{\text{mem}}$, mass transfer coefficients $k_i$ and Henry’s law coefficients for the air/PDMS ($H_{G}$) and PDMS/growth media ($H_{L}$):

$$\frac{1}{K_L} = \frac{1}{k_{\text{air}} H_G} + \frac{1}{k_{\text{PDMS}} H_L} + \frac{1}{k_{\text{medium}}} = \frac{d_{\text{mem}}}{D_{\text{PDMS}} H_L} + \frac{h_{\text{reactor}}}{D_{\text{medium}}}$$

While the previous two equations require the use of PDMS, they can easily be adapted to another gas-permeable material should use of another material be necessary. Unfortunately for many materials, as structural rigidity increases, gas permeability tends to decrease.
Based on their results and schematics, their design was used to evaluate fluid flow with the added constraint of porous phase inclusion. Simulating this bioreactor design had a set of unique challenges. The first is the altered geometry. In preliminary simulations, a 2D axisymmetric coordinate system was used with a symmetry boundary condition. Use of a 3D coordinate system to illustrate scaffold-in-reaction chamber is computationally expensive. Solution times for a benchmark 2D pipe model bordered on excessive, especially with higher mesh sizes and solver settings. Reusing the symmetry boundary condition helps with this problem. Another strategy is to divide the geometry into segments and solve them individually with outputs from one portion as inputs to another. This requires the assumption of no backflow, however such an assumption is frequently made in CFD simulation made for the microfluidic bioreactor at TUB. An alternate method would be not to divide the geometry, but to reduce computational demands by solving each of the physics separately using a segregated solver.

The second obstacle is the existence of a porous cell support phase. In bioreactor simulations where such a phase exists, the complexity of the model increases several fold. In work done at the Indian Institute of Science\textsuperscript{13} cell growth was modeled on polyurethane foams. To model the reaction occurring in the bioreactor, the IIS reactor’s reaction volume was divided into two separate phases: a liquid cellular suspension phase and an immobilized phase with cells attached to foam. Metabolism or consumption of substrate was modeled as a first order reaction occurring in both phases. Cell growth was another process occurring in both phases. Finally, a cellular equilibrium existed between the two phases consisting of two half-reactions of cell attachment and detachment from the foam. Modeling these reactions can be done with the mass transport and convection-diffusion modules in Comsol/FEMLAB. Although heat generated from biochemical reactions can be significant when biomass is large, a simplifying isothermal
assumption for microscale reactors is typically used. The following equation is used to describe the occurrence of chemical reactions within a system:

\[ R_i = \sum_j v_{ij} r_j \]

Where \( R \) is the rate of reaction for product \( i \) formed from \( j \) reactants each having a concentration \( r \) and consumption rate \( v \), in many of the binding reactions discussed, dissociation is modeled as consumption of the various sources of bound complex \( r_j \) and formation of free ligand at production rate \( R_i \). If nonisothermal system considerations are necessary, a standard form of the Arrhenius equation (7.5a) can be used to model rate changes with temperature along with enthalpy of reaction (7.5b):

\[ \begin{align*}
(a) \quad k &= A \cdot \exp \left( -\frac{E}{RT} \right) \\
(b) \quad Q &= -\sum_j H_j n_j
\end{align*} \]

Where \( k \) is the rate constant, \( A \) is an empirical preexponential factor, \( E \) is an activation energy, \( R \) is the gas constant, \( T \) is the temperature, \( Q \) is the heat of reaction, and \( H_j \) and \( n_j \) the enthalpy and stoichiometry of reactant \( j \). While this accounts for the mass balance and energy balance of the cellular component of the reaction system, it does not account for the momentum balance or inertial flux describing the fluid behavior in the system. In practical terms, the model discussed later deals primarily with signaling molecules such as BMP-2 present at relatively low amounts, however heat buildup is more significant when considering cellular consumption of carbohydrates and lipids from growth media and subsequent energy production from glycolysis and cell respiration. On larger scales, heat buildup can affect product yield, however in microscale mammalian systems, thermal considerations are addressed by a physiological temperature incubator whose larger volume makes it robust to perturbations caused by fluctuations from within the relatively smaller culture system.
Flow through porous medium, where a portion of the system is the porous phase, can be described with Darcy’s law and a combination of Darcy’s law and the continuity equation:

\[ u = -\frac{\kappa}{\eta} \nabla p \]

\[ \frac{\partial}{\partial t} (\rho \epsilon) + \nabla \cdot (\rho u) = F \]

In equation 7.6, \( u \) is velocity, \( \kappa \) is permeability, \( \eta \) is viscosity, \( \epsilon \) is porosity, \( p \) is pressure, \( \rho \) is fluid density, \( t \) is time, \( F \) is a source term for external forces. Darcy’s law is appropriate in single phase systems where the entire system is contained in the same porous phase. In multiphase designs such as the basket housing polyurethane foam in the IIS bioreactor, the porous phase was contained in a polyethylene basket with openings to facilitate media flow. In the case of such reactor designs, flow into, out of and around the porous phase is possible. In those situations, matching solutions at the interface of the porous phase and the free solution becomes complex. Rather than solving the domains separately using Darcy and Generalized Momentum (Navier-Stokes) equations, the Brinkman equation is used and tends to be a good numerical fit for the porosity range of biological scaffolds.\textsuperscript{14-16} The generalized momentum equation describes fluid flow as follows:

\[ \rho \frac{Du}{Dt} = -\nabla p + \eta \nabla^2 u + \rho g \quad \text{where } \frac{D}{Dt} = \frac{\partial}{\partial t} + u \cdot \nabla \]

In 7.7, the LHS describes the inertia of the fluid using the substantial derivative of velocity \( u \) and the RHS contains the sum of the pressure gradient, a viscous force term and a gravitational term considering the fluid weight with gravitational acceleration \( g \). Additional constraints to equation 7.7 can include incompressibility and irrotation, though it has no constraints for velocity or Reynold’s number.

\[ \text{Re} = \frac{\text{Inertial Forces}}{\text{Viscous Forces}} = \frac{\rho u^2}{\eta u/L} \]
The Reynold’s number (Re), mentioned earlier, is a dimensionless quantity describing the ratio of inertial to viscous force at a characteristic length L that reflects the scale of the system. The Brinkman or momentum equation at low Reynold’s number is suitable for porous flow in biological systems where \( \rho u^2 \ll \eta u / L \) is typical. In such situations the viscous term dominates the equation, causing the LHS in 7.7 to become vanishingly small, allowing equation 7.7 to be rewritten as follows:

\[
7.9 \quad 0 = -\nabla p + \eta \nabla^2 u
\]

The preceding equation can be modified with an additional term to account for the dissipation of momentum by absorption into the porous phase. It is also notable that the gravitational term is ignored, this is justified because the effect is nominal at the length scales considered.

\[
7.10 \quad \eta \nabla^2 u - \frac{\eta}{\kappa} u - \nabla p = 0
\]

This modification by the \( \eta/\kappa \) (hydraulic conductivity) term yields the common form of the Brinkman equation. 7.10 simplifies to 7.6a when the Laplacian of velocity is removed. The hydraulic conductivity can be related to porous phase architecture for cylindrical and non-cylindrical porous structure using the Kozeny and Kozeny-Carman equations respectively. Although 7.6a can be derived from 7.7, reducing 7.10 to 7.6a results in loss of information.\(^{15, 16}\)

A vector form of 7.10 that retains the inertial term, scaling it with the porosity of the porous phase and adding the source term of 7.6b as a coefficient, is suitable for finite element solver adaption. This form is used in Comsol 3.5 for multiphase systems.

Using equations 7.6 and 7.10, models of milliliter-scale bioreactors containing tissue-engineering scaffolds exist.\(^ {15} \) Residence time distributions and velocity profiles were calculated using Comsol 3.5. These methods are also applicable to a microfluidic system. In these studies shear stress and pressure drop were evaluated across a variety of pore sizes. The rationale was
that cellular growth in a scaffold would be seen as the effect of pore occlusion. This was one reason for analysis of pore size variation—differing rates of occlusion should occur. Pore occlusion was used to model scaffold cell growth following reactor design was completion on my perfusion system, that analysis is discussed further in chapter 8.

The 2D geometry of the TUB design was drawn in Autocad and imported into Comsol for extrusion and meshing. The resulting geometries were run unmodified, with a cylindrical scaffold or rectangular parallelepiped which occluded the entire cross section area. The scaffolds were placed in the uniform flow culture chamber. The PDMS shell and glass floor were

**Table 7.1**

<table>
<thead>
<tr>
<th>Simulation Parameters</th>
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<tbody>
<tr>
<td>Total Reactor Volume</td>
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<tr>
<td>Inlet Velocity</td>
<td>3 mm·s⁻¹</td>
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<tr>
<td>Outlet Pressure</td>
<td>0 Pa</td>
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<tr>
<td>Scaffold Phase Porosity</td>
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<tr>
<td>Scaffold Phase Permeability</td>
<td>46 μm²</td>
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<td>Cylindrical Volume Fraction</td>
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<tr>
<td>Full Width Volume Fraction</td>
<td>.3206</td>
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</table>

**Figure 7.1** CAD drawing for TUB reactor a) after and b) prior to FEM meshing
included in the geometry and extrusion shown in figure 7.1, and functioned as inflexible no-slip boundaries in both Brinkman and Navier-Stokes modes. Among other simulation parameters in table 7.1, the porosity and permeability were chosen match chitosan with a 50 micron pore size. Although the scaffold-ladden geometries were only run in 2D, the data for unmodified chambers shows the 2D approximation to be very close to the 3D values.

**Table 7.2 Bioreactor Flow and Clearance**

<table>
<thead>
<tr>
<th>Simulation</th>
<th>Outlet Flow Rate (µL·s⁻¹)</th>
<th>Dilution Rate (s⁻¹)</th>
<th>Residence Time (s)</th>
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<tbody>
<tr>
<td>2D</td>
<td>.43717</td>
<td>.051312</td>
<td>17.369</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\tau_{\text{full width}})</td>
</tr>
<tr>
<td>3D</td>
<td>.38964</td>
<td>.057571</td>
<td>19.488</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\tau_{\text{cylindrical}})</td>
</tr>
</tbody>
</table>

Reactor residence times shown in table 7.2 were extracted from integration of the outlet velocity across the surface of the outlet boundary and calculated using equation 7.1. The scaffold residence times were calculated from the FEM generated velocity field along the efflux boundary of the scaffold. The resulting values for residence time show a slight decrease in residence time for the cylindrical scaffold model. The flow profile showed that flow went around the scaffold accelerating in the narrow channel between the scaffold and the PDMS wall creating a dead zone within the scaffold. This effect is not seen when porosity is increased to >95%. If a scaffold is placed in the culture chamber to avoid biofilm-like growth along the reactor walls, the simulation data suggests using a high porosity scaffold or a full cross-section occluding scaffold is ideal. Both characteristics can be incorporated into the same scaffold.

Although the analysis of a microfluidic perfusion bioreactor focused mainly on steady state behavior using stationary solver parameters, transient behavior was also briefly considered. The microfluidic system was compared to a same geometry modeled and 1000:1 scale. The
macro-scale reactor (data not shown) did not reach steady state after simulation time ran out, where as the microfluidic system reaches steady state at the second time point (figure 7.2) with reactants residing in the system for a total of just under 17 s prior to exiting through the efflux. This design showed that in 2 dimensions, a geometry with an inlet and outlet much smaller than the culture chamber is feasible. I used these findings to design the chamber geometry of the perfusion bioreactor.

**Design Process**

The topology optimized 2D TUB bioreactor used a series of branched microchannels to create a plug like flow profile as the fluid entered culture area. Translating two-dimensional geometries to three dimensions can involve extrusion or revolution. In the case of the TUB design, extrusion would create a cubic central chamber, where the corners could become stagnant to flow. If the geometry were revolved, a concentric series of cones would result that would prove difficult to fabricate, a possible variation with microchannels is shown in figure 7.3d. The TUB design relied on microchannels to create an evenly flat fluid front approaching the central chamber, with porous phase inclusion, the demand for this type of fluid front is
unaltered. Through iterative design guided by simulation, I found that a smoothened conical-funnel shape (figure 7.3b and appendix) creates a sloping increase in cross-sectional area as it leads to the central chamber housing the porous phase. This slows the fluid velocity, producing the same evenly distributed fluid front as seen with the 2D microchannel design. Furthermore, an analog of microchannels is used in the membrane frit discussed later. To make this funnel design, a rectangle with width of the inlet diameter and another rectangle with the width of the culture chamber were drawn and connected using splines and the same geometry was repeated at the outlet. This shape was then revolved around the inlet-outlet axis to create the 3D geometry. This interior chamber geometry was used to perform computational fluid dynamics simulation, described in the following chapter. In order to verify simulation results, it was necessary to create a working prototype. One design constraint that proved to be a challenge was access to

![Images of bioreactor design iterations](image)

**Figure 7.3** Bioreactor Design Iterations a) Design Iteration of Bioreactor Assembly b) Central Chamber - final design c) Reactor interior d) interior with microchannels leading to reactor chamber and quarter scaffold section e) photograph of functional prototype.
the culture chamber. In order to insert and remove the porous phase the culture chamber needed
to be opened and resealed. Another was porous phase placement, to prevent slippage of the
scaffold and resultant clogging of the outlet, the scaffold needs to be held in place. To address
these two problems the bioreactor was created as an assembly of five parts, the central
chamber, one flow adaptor containing the sloping conical funnel for each end, and two gaskets
to connect the central chamber and each flow adaptor (figure 7.3 and appendix). The
gasket and flow adaptor connect with a snap-joint, this snap joint holds a 100 μm porous
membrane in place. These two membranes act as frits to fix the scaffold position in the
central chamber. The gaskets connect to the central chamber with threaded screw joints. Each
flow adaptor has a notched insert shown in figure 7.3a that fits into peristaltic pump tubing.

A problem encountered was leaks occurring in various parts of the assembly. This
problem was solved by changing the material used on all the parts between the flow adaptors.
Originally, all the parts are fabricated using a rigid ABS-like plastic. A disadvantage of this
material is its relative opacity, though there have been recent developments in fluidic tracking
that omit tracer use.17 Aside from the leaks from improper sealing, once an ABS-like gasket
snapped onto a flow adaptor it could not be removed aside from breaking either part. Fabricating
selected parts from elastomer solved these problems. A hybrid plastic-elastomer assembly
sealed tightly when compressed slightly with a mounting assembly. To support the gaskets
against deformation from compression, the diameter of the intervening section of the central
chamber (figure 7.3b) was expanded to create a strut to brace the gaskets.

Iteratively changing materials did potentially influence one of the principle design
considerations in bioreactor design, cell viability. In static tissue culture flasks or batch
bioreactors, the material used is polystyrene. In the thicknesses typically used, the polymer is
permeable to oxygen. To facilitate cell attachment, growth surfaces are coated with polylysine. Fortunately with a porous phase, surface coating was not a concern allowing greater freedom in selecting design materials. However, without gas permeability testing, reactor walls could not be assumed gas-permeable as is the case with polystyrene and PDMS. The simulations performed in the subsequent chapters, reactor walls are assumed to be gas-impermeable.

**Conclusion**

This chapter discussed the parameters that need to be considered in bioreactor construction. The basic design features of my bioreactor were discussed in light of these considerations as well as many design challenges that had to be navigated. The bioreactor stemming from this design will be discussed further in subsequent chapters. When constructing bioreactor systems to tissue engineer larger sections of bone, these same design challenges should be encountered along with additional challenges in mass transport.
References


Chapter 8

Bioreactor Simulation

This chapter describes studies that were undertaken in my bioreactor in an effort to correlate simulation parameters with measurements from an in vitro device. However, the goal of biomaterial design completely by computational means is currently not reached and not the subject of this dissertation. In current spinal fusion procedures, absorbable collagen sponges are soaked with a solution containing recombinant human bone morphogenetic protein 2 (rhBMP-2) prior to implantation. While simple soaking of sponges is effective in mediating bone mineralization around the two or more vertebrae being joined, the unwanted side effect of ectopic bone growth\(^1\) can occur at distant sites. This is thought to be due to diffusion of BMP-2 away from the sponge, post-implantation\(^2\). In this chapter, a model of BMP-2 diffusion from a collagen sponge in a perfusion bioreactor\(^3\) is created using the finite element method. To model cellular infiltration, growth is tracked on the sponge through changes in physical characteristics due to function as a scaffold for cell growth. Consistent with previous chapters, MC3T3 E1 mouse calvarial cells were used.\(^4\) One strategy used to slow release is to model covalent linkages to the sponge with proteins and peptides. These proteins and peptides then bind with different affinities\(^5\) to the growth factor of interest, BMP-2. As described in chapter 6, a proposed fusion protein was also evaluated for delivery purposes.

The goals of simulating the perfusion bioreactor were to determine how well porous collagen scaffolds function as BMP-2 delivery devices, to determine if cells were viable for prolonged growth in the system, and finally to determine the optimal operating conditions that maximize cell growth and cell differentiation toward an osteoblast phenotype. For BMP-2
delivery, this involved modeling binding reactions alongside mass transport. The problem of cell viability was approached by modeling oxygen transport and cell crowding effects. Optimal operating conditions involved simulations of cellular microenvironment as determined by operator adjustable parameters such as inlet flow velocity. Once the governing equations were determined for the model, it was necessary to estimate simulation parameters with as much accuracy as possible. Where parameters were available from the literature, the most applicable value was chosen. When conflicting values were found, typically the parameter values were averaged, and weighted where appropriate. In cases where a parameter was not readily available, it was measured empirically. In some cases, questionable values were verified with experiment.

**Simulation of BMP-2 Delivery: Model & Governing Equations**

Fluid flow through the reactor is governed by Equation 8.1, a version of the Brinkman equations with terms included to describe porous media:

8.1a \[ \frac{n}{\varepsilon} \nabla^2 \mathbf{u} - \frac{n}{\kappa} \mathbf{u} - \nabla p = 0 \]

8.1b \[ \nabla \cdot \mathbf{u} = 0 \]

With boundary conditions:

8.1c \[ \mathbf{u} \cdot \mathbf{n} = u_o \text{, reactor inlet} \]

![Image](image_url)
The scalar coefficients $p$, $\eta$, $\kappa$, $\varepsilon$ are the pressure, viscosity, permeability and the porosity, with respect to order. The porosity is a dimensionless ratio between the void volume and the total volume. To avoid reactor washout\(^6\) prior to cell attachment, initial velocities were chosen on the basis of the residence time $\tau_{\text{res}}$ generated, as follows:

\begin{equation}
\tau_{\text{res}} = \frac{au_0}{V}
\end{equation}

Where variable $a$ is the inlet cross-sectional area, $u_0$ the initial velocity at the inlet and $V$ the total volume of the reactor. This prediction follows from restriction on fluid compression seen previously in equation 1b.

The effect of cell growth is described by pore shrinkage from crowding. The following equation characterizes the resulting decrease in porosity:

\begin{equation}
\varepsilon(t) = \varepsilon_0 - \frac{mE_{S\text{cell}}e^{\mu t}}{\rho_c V_S}
\end{equation}

The terms $\varepsilon_0$, $m$, $E_S$, $\mu$, $\rho_c$, $V_S$ are acellular porosity, mass per cell, seeding efficiency, cell growth rate, density of cellular material, and the volume of the scaffold, respectively. A constant $\mu$ was used because fluctuations should be negligible during time scales on the same order of magnitude as the doubling time. The accumulation of cellular matter on the sponge decreases $\varepsilon$ through the negative term in the preceding equation. This decrease in porosity can be treated as porous phase deposition\(^7\) described by the following power law:
8.4 \[ \kappa \approx \kappa_0 \left( \frac{\varepsilon(t)}{\varepsilon_0} \right)^{3.55} \]

The release of growth factor bound to the sponge is dependent on the affinity of the binding molecule-growth factor interaction. Binding kinetics governing the behavior of bound and free protein growth factor are modeled as two separate chemical species to facilitate independent treatment for mass transport. The rate equations are mass action rate laws and are shown as follows:

\[ \frac{dC_{\text{BMP-2,free}}}{dt} = R_1 = k_d C_{\text{BMP-2,bound}} - k_a C_{\text{BMP-2,free}} (C_0 - C_{\text{BMP-2,bound}}) \]

\[ \frac{dC_{\text{BMP-2,bound}}}{dt} = R_2 = k_a C_{\text{BMP-2,free}} (C_0 - C_{\text{BMP-2,bound}}) - k_d C_{\text{BMP-2,bound}} \]

Where \( R_i \) is the rate of reaction, \( k_a \) and \( k_d \) are the association and dissociation binding affinities, \( C_0 \) is the initial sponge loading concentration, \( C_{\text{BMP-2,free}} \) and \( C_{\text{BMP-2,bound}} \) are the instantaneous concentration of BMP-2 in free solution and bound to the scaffold respectively.

Finally, mass transport in the system is described by the convection-diffusion equations:

\[ \frac{\partial c_i}{\partial t} + \nabla \cdot (-D_{\text{BMP-2,bound}} \nabla c_i + \xi c_i u) = R_i \]

The diffusivity \( D \) has values particular to the species in question and the media phase they diffuse through. The convection term has velocity \( u \) from equation 1 and coefficient \( \xi \), a unit step function for binding to the porous phase. While \( i \) denotes the reactant of interest (scaled by domain for diffusivity), 1 for bound BMP-2 and 2 for free BMP-2. The reaction term \( R \) is from Equations 8.5. The comsol api recognized 8.5 and 8.6 as follows:

```plaintext
equ.D = {'D1','D2'};
equ.init = {'C1','C2'};
equ.R = {0,'kd*c3-ka*c*(Conc3-c3)'};
equ.D = {1,'D3'};
equ.init = {0,'Conc3'};
equ.R = {0,'-kd*c3+ka*c*(Conc3-c3)'};
```
In the preceding code, some parameter names were changed, such as Conc3 for the initial value of BMP-2 concentration while c3 is used by the solver as the variable for BMP-2 concentration. The complete file can be found in the appendix.

**Geometry**

The geometry (figure 7.3c) was designed to house the collagen sponge with dimensions identical to bioassay\(^2\) implants. The inlet and outlet were designed to fit peristaltic pump tubing. To prevent eddy formation from sharp corners a spline was drawn from inlet to the bulk of the reactor. The cross section was then revolved to form the final geometry. To facilitate insertion or removal of the sponge, a sealable notches run axially along the perimeter of the reactor chamber, these liquid-tight seals do not affect the interior geometry. Low flow rates necessary to generate long residence times make gas permeability a consideration for material choice.

**Parameters**

The parameters used in simulation are shown in table 8.1 and were derived from literature or obtained experimentally. The viscosity of flow media was based on a range of culture media with additives such as thickeners.\(^8,9\) Covalently cross-linked collagen I sponges were purchased from Integra (Plainsboro, New Jersey) and characterized by fluid displacement to determine porosity as described in chapter 5. The sponges were subject to H&E staining, paraffin embedding and histological sectioning for pore size determination, though not directly involved in simulation this value served as a guide for comparison with other scaffold materials. The permeability was obtained by multiplying the hydraulic conductivity\(^10\) with the viscosity\(^11\) of glutaraldehyde cross-linked collagen-I extrapolated from rheological data gathered under analogous conditions. MC3T3E1 clone 4 cells (figure 8.1) were originally obtained from
Table 8.1 Parameter List

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \eta )</td>
<td>Viscosity perfusion media</td>
<td>( 1.0 \times 10^{-7} \text{ Pa}\cdot\text{s} ) ± ( 2 \times 10^{-4} \text{ Pa}\cdot\text{s} )</td>
</tr>
<tr>
<td>( A )</td>
<td>Inlet Area</td>
<td>( 1.9567 \text{ mm}^2 ) ± ( 5 \times 10^{-4} \text{ mm}^2 )</td>
</tr>
<tr>
<td>( u_o )</td>
<td>Inlet Velocity*</td>
<td>( 4.36 \ \mu\text{m/s} ) ± ( 2 \times 10^{-2} \ \mu\text{m/s} )</td>
</tr>
<tr>
<td>( \varepsilon_o )</td>
<td>Porosity -sponge only</td>
<td>( 0.88 ) ± ( 0.04 )</td>
</tr>
<tr>
<td>( E_S )</td>
<td>Cell Seeding Eff</td>
<td>70.1% ± 5.0%</td>
</tr>
<tr>
<td>( M )</td>
<td>Mass per cell</td>
<td>24.6 ng ± 17.7 ng</td>
</tr>
<tr>
<td>( n_{\text{cell,i}} )</td>
<td>Cell Quantity</td>
<td>( 1.5 \times 10^4 ) ± ( 7.5 \times 10^2 )</td>
</tr>
<tr>
<td>( \mu )</td>
<td>Cell Growth Rate</td>
<td>( 1.07 \times 10^{-3} \text{ s}^{-1} ) ± ( 6 \times 10^{-7} \text{ s}^{-1} )</td>
</tr>
<tr>
<td>( \rho_c )</td>
<td>Cell Mass Density</td>
<td>1.004 g/cm(^3) ± ( 2 \times 10^{-3} ) g/cm(^3)</td>
</tr>
<tr>
<td>( V_s )</td>
<td>Scaffold Volume -quarter</td>
<td>( 0.07453 \text{ cm}^3 ) ± ( 5 \times 10^{-5} \text{ cm}^3 )</td>
</tr>
<tr>
<td>( \kappa_o )</td>
<td>Permeability -sponge</td>
<td>( 9.8 \times 10^{-4} \text{ m}^2 ) ± ( 1.2 \times 10^{-4} \text{ m}^2 )</td>
</tr>
<tr>
<td>( D_{1,1} )</td>
<td>Diffusivity of free BMP-2</td>
<td>( 13 \times 10^{-7} \text{ cm}^2/\text{s} ) ± ( 1.3 \times 10^{-7} \text{ cm}^2/\text{s} )</td>
</tr>
<tr>
<td>( D_{1,2} )</td>
<td>Diffusivity of free BMP-2 (Porous Phase)</td>
<td>( 11 \times 10^{-7} \text{ cm}^2/\text{s} ) ± ( 1.1 \times 10^{-7} \text{ cm}^2/\text{s} )</td>
</tr>
<tr>
<td>( D_{2,2} )</td>
<td>Diffusivity of bound BMP-2</td>
<td>( 0.66 \times 10^{-7} \text{ cm}^2/\text{s} ) ± ( 6.6 \times 10^{-8} \text{ cm}^2/\text{s} )</td>
</tr>
<tr>
<td>( C_0 )</td>
<td>Initial Bound BMP-2</td>
<td>( 8.0 \ \mu\text{M} ) ± ( 1.6 \ \mu\text{M} )</td>
</tr>
</tbody>
</table>

*using a syringe pump it is possible to achieve volume flow rate error orders of magnitude lower than the inlet area measurement error

ATCC (Manassas, Virginia) and grown starting from passage 8, cell mass measurements were taken at 24, 48 and 72 hours and confirmed by amount of total protein through BCA Protein assay. Cell volume was also determined through examination of day 4 confluent culture micrographs. The seeding efficiency \( E_S \) was estimated based on a scaffold seeding protocol with a similar cell type and seed time. Inlet velocity required optimization—the resulting velocity was chosen to match residence time in the reactor to cell doubling time (18 hrs). The doubling time was based on characterization of the cell line and used to find the growth rate.

The diffusion constant for cytochrome C was used because its molecular weight is nearly identical to monomeric rhBMP-2 (12.31 kDa for Cytochrome C vs. 12.53 kDa for BMP-2). Although there are various ways to determine diffusivity including methods described in chapter 2 as well as lattice-type simulation methods to determine diffusivity through porous materials, rate dialysis in chapter 5 demonstrated that for cross-linked collagen scaffolds, scaling a diffusion coefficient as a product of porosity is a sufficiently accurate approximation. The tortuosity and microstructure of the sponge impedes diffusion, so diffusivity was multiplied...
by a coefficient slightly lower than the porosity to account for these effects in calculating \( D_{1,2} \).

Rather than treat the scaffold bound BMP-2 as linked to a large inert crosslinked macromolecule, it was assumed that the crosslinks allow relatively free range of motion by acting as a tether. To reflect local concentration fluctuations the collagen bound complex of BMP-2 and binding protein was treated as a single entity, and because the aspect ratio of collagen I dominates, the porosity limited diffusivity of a collagen I monomer\(^{15}\) was used. Initial concentration of BMP-2 was based on the effective dose\(^2\) from bioassay.

Three molecules spanning a range of binding affinities\(^2,5\) to BMP-2 were used to test release rates of the growth factor from the scaffold. The kinetic constants discussed in chapters 1-3 are shown above in table 8.2.

Simulation of oxygen transport was performed using a similar model to that used for BMP-2 release. Inlet velocity was based on previous studies\(^{16,17}\) and covered the range between 4 and 800 \( \mu \text{m/s} \). Relevant simulation parameters are listed in the following table:

**Table 8.2 Binding Affinities**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Affinity</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>bsp24 (full length)</td>
<td>( k_d )</td>
<td>2.29x10^{-3} s^{-1}</td>
</tr>
<tr>
<td></td>
<td>( k_a )</td>
<td>3.86x10^{3} L/mol·s</td>
</tr>
<tr>
<td>spp14.5 (truncated)</td>
<td>( k_d )</td>
<td>3.60x10^{3} s^{-1}</td>
</tr>
<tr>
<td></td>
<td>( k_a )</td>
<td>1.53x10^{4} L/mol·s</td>
</tr>
<tr>
<td>cBBP (peptide)</td>
<td>( k_d )</td>
<td>0.72x10^{3} s^{-1}</td>
</tr>
<tr>
<td></td>
<td>( k_a )</td>
<td>1.35x10^{5} L/mol·s</td>
</tr>
</tbody>
</table>

**Table 8.3 \( \text{O}_2 \) Transport**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( U )</td>
<td>Inlet Velocity</td>
<td>4-800 ( \mu \text{m/s} )</td>
</tr>
<tr>
<td>( C_{\text{Oxygen}} )</td>
<td>Inlet ( \text{O}_2 ) concentration</td>
<td>202.48 ( \mu \text{mol/L} )</td>
</tr>
<tr>
<td>( D_{\text{O2}} )</td>
<td>Diffusivity ( \text{O}_2 )</td>
<td>3.29x10^{-5} ( \text{cm}^2/\text{s} )</td>
</tr>
</tbody>
</table>
Method Validation

In order to compare the error involved in using the finite element approximation for the bioreactor system, a problem with the same length as the center-line of the reactor (19.8 mm) from inlet to outlet was solved analytically and the resulting solution was compared to the finite element solution. Although the finite element method divided the 1D geometry into three equal-length intervals with the central interval representing the porous phase, the analytical problem was approximated as a single phase for simplicity. The diffusivities used in other simulations for scaffold-phase BMP-2 differ by ~0.2 cm²/s when compared to free solution BMP-2. However, the diffusivity used in the single phase was the average of the scaffold and free solution diffusivity. The convection-diffusion equation 8.6 was solved assuming the geometry can be represented as a tubular pipe with equal cross-sectional area across its length and continuing indefinitely in each direction at the ends of the interval. The initial concentration is designated with the differential equation 8.7a where M is the solute mass in an infinitesimal thickness slice (dx) and A is the pipe cross-sectional area. Solving the equation analytically, one arrives at the following solution 8.7b for a point source:

\[ C_{x=0} = \frac{M}{A} \int dx \]

\[ c(x, t) = \frac{1}{\sqrt{4\pi Dt}} \exp \left( -\frac{(x-x_0+u)^2}{4Dt} \right) \]

The resulting equation (8.7b) is a classical result used in environmental fluid dynamics, where u is the convection velocity, x₀ is the initial position, and D is the pipe diameter. The gaussian spread of a single point source is due to dissipation while traveling along the x-coordinate due to advection or convection (bulk flow). The initial protein loaded onto the sponge is assumed to be spread evenly throughout the length of the sponge, consequently the interval of the sponge can
be considered the sum of multiple point sources. For each time point, the shape of the protein distribution curve along the length of the geometry can be found by summation of point source

![Graph showing protein concentration over time and distance](image)

**Figure 8.2** Analytical Solution Comparison

**a)** One dimensional Finite Element Solution to Protein Transport. **b)** Comparison of Finite Element to Analytical Solution, Black – FEM, Blue – Analytical, Red – Error.

Unfortunately, the one-dimensional system washes out very quickly and the analytical solution has physically unrealistic transient behavior seen in initial increments prior to 3s. However a non-trivial comparison can be made between the analytic solution and the finite
element solution (by itself in figure 8.2a) in the time interval from 3 to 28 s. A plot of the comparison is shown in figure 8.2b.

The sum of the residuals shows the error to be regular and concentrated in the immediate upwind and downwind areas surrounding each concentration pulse. This demonstrates that calculation of the pulse’s spread from diffusion is markedly different in the finite element solution, had the two methods been similar, the error would not increase periodically corresponding to climb towards the plateau regions. Further, this mesh (simplifying to a grid in 1 dimension) has not been optimized for hydrodynamics as done in the following section. Without element weighting or mesh optimization, the solver algorithm is biased toward downstream elements, which can be seen in figure 8.2b as a positive bulge in the Finite Element Method solution above the analytical concentration maxima in the downstream portion of an individual concentration distribution and a corresponding upstream dip below zero.

Simulation

In order to show the release of growth factor out of the porous phase, I used finite element modules to simulate different aspects of the physical phenomenon. The convection-diffusion (chcd), diffusion (chdi) and Brinkman (chns) equation modules were used. The flow module was stationary while the other two were transient. Prior to running time-dependent simulation, a parameter sweep at a single time point was performed to find the optimal inlet velocity. Time-dependent runs stretching 18 hours with time steps of 30 minutes were then performed with each binding protein. Simulations would converge consistently up to 14 days. To speed simulation, the geometry was quartered and two symmetry boundary conditions applied. Coarser meshes of 10,790 elements proved sufficient. The iterative solver used was
Biconjugate Gradient Stability. Incomplete LU was used as a preconditioner to factorize the stiffness matrix, with a tolerance of 0.15 and a fill-in of $1 \times 10^{-5}$. Simulation was performed on a Dual 2.394 Ghz Intel Xeon workstation with 50 GB RAM and on a Intel Pentium i7 Ideapad with 8GB of RAM. Versions 3.5a, 4.2 and 4.3 of Comsol multiphysics were used. Simulations required on average 71 minutes per condition and up to 72h for parameter sweeps.

**Results and Discussion**

The simulation data shown in figure 8.3 verified oxygen concentrations levels that could sustain cells\textsuperscript{16,17} starting at 400 $\mu$m/s. Although prolonged simulations at higher flow velocities experienced convergence problems, simulation times reaching two minutes (fig 8.3) showed that after one minute of perfusion, oxygenated media had penetrated into the scaffold boundaries (10-16mm). Based on previous studies,\textsuperscript{17} 52.6 $\mu$mol/L dissolved O$_2$ concentration permits cell
metabolism. In the simulated time points, portions of the scaffold have oxygen concentrations that well-exceed this threshold level. At two minutes, oxygen permeation continued to deepen into the scaffold space. If oxygenated continued to permeate the scaffold at this rate, the entire scaffold is oxygenated for cell growth in 6-7 minutes.

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Value</th>
<th>Rel Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial U} )</td>
<td>2.24x10^{-21} mol/s/μm</td>
<td>9.76x10^{-13}</td>
</tr>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial k_a} )</td>
<td>6.38x10^{-30} mol^2/s/L</td>
<td>1.06x10^{-12}</td>
</tr>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial k_d} )</td>
<td>3.62x10^{-24} mol/s</td>
<td>1.04x10^{-12}</td>
</tr>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial m_{\text{cells}}} )</td>
<td>8.13x10^{-16} mol/ng</td>
<td>8.46x10^{-13}</td>
</tr>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial \mu} )</td>
<td>1.03x10^{-15} mol/s</td>
<td>1.10x10^{-12}</td>
</tr>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial \rho_{\text{cells}}} )</td>
<td>8.91x10^{-30} mol cm^3/g</td>
<td>9.12x10^{-13}</td>
</tr>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial \xi_0} )</td>
<td>9.51x10^{-24} mol</td>
<td>1.38x10^{-12}</td>
</tr>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial D_1} )</td>
<td>3.24x10^{-5} mol/s cm^2</td>
<td>4.21x10^{-01}</td>
</tr>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial D_2} )</td>
<td>1.21x10^{-5} mol/s cm^2</td>
<td>1.33x10^{-01}</td>
</tr>
<tr>
<td>( \frac{\partial N_{\text{eff}}}{\partial c_0} )</td>
<td>1.31x10^{-24} mol/μM</td>
<td>1.04x10^{-12}</td>
</tr>
</tbody>
</table>

**Table 8.4 Parameter Sensitivity**

The total flux regardless of direction was integrated across the cross-sectional area of the reactor outlet, \( N_{\text{eff}} \) is mol, while the unit for raw sensitivity value is the quotient of mol and the parameter in question found in table 8.1. The relative values are scaled by the parameter value used in the original simulation and flux value similar in magnitude to the mean flux. It is noteworthy that the model is particularly sensitive to \( D_1 \) and \( D_2 \).

Cell growth did not affect porosity significantly within the doubling time of 18 hrs (6.48x10^4 s), however when continued to 7 days (6.02x10^5 s), \( \varepsilon(t) \) drops to zero, clogging the scaffold and forcing fluid around it. (figure 8.4a & 8.4b). However the change in permeability
was not dramatic enough to create a significant alteration in flow pattern at either 18 hours or 7 days, though the streamline profile did shift nominally. One reason for this is likely because the sloping increase of cross-sectional area within the geometry slows flow to an almost stagnant velocity, stifling all but the largest velocity shifts. An analogy seen in physiology would be slowing in blood flow during transition from arteriole to capillary. In earlier simulations done

**Figure 8.4** Bioreactor Simulation Plots
with lower porosity scaffolds such as chitosan, flow is channeled around the porous phase and speeds up significantly in the small gap between the porous phase and the wall.

In evaluating different binding proteins effect on BMP-2 release, the trend of fastest release was headed by spp14.5 followed by full length bovine spp24 with cBBP peptide closely behind (figure 8.4c). It should be noted that all binding molecules are significantly slower than sponge alone. At the end of 18 hr simulation, concentration of BMP-2 in the sponge-only system have nearly equilibrated throughout the entire reactor volume, while systems with binding peptide or proteins have retained a reservoir of BMP-2 within the sponge compartment. In the distribution slice and boundary plot (figure 8.4d), ends of the sponge are exposed to the larger volumes of the longitudinal ends of the flow chamber have released much more BMP-2 compared to a radial distribution, where the space between the sponge and the chamber wall is a much smaller volume and equilibrates rapidly with the local portion of the sponge.

The simulations up to this point have demonstrated potential for this bioreactor design to be used to evaluate the kinetics of osteobiologic delivery with collagen sponges modified by binding proteins. The model is limited in the length of simulation by the accurate depiction of biological response. As currently modeled, cell growth rate is constant, while MC3T3E1 cells in tissue culture display marked changes in growth rate during differentiation to an osteoblast phenotype. The rate of that differentiation is in turn responsive to BMP-2 concentration. These concerns have not been fully addressed, and will be considered further in Chapter 9 when dealing with cellular modeling of BMP-2 signal transduction.
Fusion Protein Evaluation

In the previous simulations of BMP-2 release, the binding protein is covalently linked to the scaffold. To conjugate the binding protein to collagen, reagents such as those described in Chapter 1 used to link BMP-2 to dextran could be used. Variants of ethylenediamine carbodiimide N-hydroxysuccinimide EDC/NHS can be used to form amide bonds between peptides. Still other strategies include aldehyde crosslinking as done to crosslink collagen fibers in the porous phase. However, covalent conjugation can be avoided, bypassing potentially laborious and unnecessary bioconjugation steps. Using a fusion protein constructed from domains of two different proteins is one way to circumvent covalent linkages. As described in Chapter 6, this fusion protein would have two binding sites, one to bind to BMP-2, the other to bind to collagen. To perform BMP-2 release studies, a sample fusion protein needed to be designed. The chosen domains were the Collagen binding Domain (CBD) of von Willebrand Factor vWF, a factor found in the clotting cascade, and the growth factor binding domain sel-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{a,vWF}$</td>
<td>Affinity of vWF for Collagen</td>
<td>$1 \times 10^5$ L/mol·s</td>
</tr>
<tr>
<td>$k_{d,vWF}$</td>
<td>Dissociation of vWF-Collagen</td>
<td>$7.3 \times 10^{-4}$ s$^{-1}$</td>
</tr>
<tr>
<td>$D_{Cmplx,1}$</td>
<td>Diffusivity Complex (H$_2$O)</td>
<td>$1.19965 \times 10^6$ cm$^2$/s</td>
</tr>
<tr>
<td>$D_{Cmplx,2}$</td>
<td>Diffusivity Complex (Scaffold)</td>
<td>$1.01509 \times 10^6$ cm$^2$/s</td>
</tr>
<tr>
<td>$D_{FusP,1}$</td>
<td>Diffusivity FusP (H$_2$O)</td>
<td>$1.9606 \times 10^6$ cm$^2$/s</td>
</tr>
<tr>
<td>$D_{FusP,2}$</td>
<td>Diffusivity FusP (Scaffold)</td>
<td>$1.6590 \times 10^6$ cm$^2$/s</td>
</tr>
<tr>
<td>$C_{FusP,bnd}$</td>
<td>Concentration Bound FusP</td>
<td>$1.1894$ mmol/L</td>
</tr>
</tbody>
</table>

Table 8.5 Fusion Protein Simulation Parameters
ected was the nineteen residue cyclic BMP-2 binding peptide cBBP. (As a practical concern, this peptide has proven somewhat difficult to synthesize.) While in principle BMP-2 release mediated by a fusion protein is similar to the previous problem, because there are now two binding-dissociation reactions occurring, one of which is no longer surface contained, the simulation becomes more complex with additional rate laws needed to model the binding and release kinetics.

Parameters

The following parameters\textsuperscript{21} in table 8.5 were used in simulation along with relevant parameters from the covalently linked binding protein simulation. The fusion protein diffusivity was based off a protein of similar molecular weight and scaled by the porosity for transport in the scaffold phase.

Model and Governing Equations

The reaction kinetics were modeled using a series of ordinary differential equations. In principle, the model is quite similar to the previous one, however as mentioned earlier there are a few important distinguishing features. Previously, all the reactions occurred as surface reactions where truncated spp24 variants are covalently bound to the pore surface in the scaffold, and BMP-2 acts as ligand. This is less demanding to model, and therefore less demanding to simulate. However, when the scaffold is loaded with BMP-2 bound to a fusion protein, the protein complex can dissociate from the pore surface with intra-complex bonds intact. This complex can then diffuse away from the pore surface and subsequently dissociate in either the bulk solution found in the pore void volume or dissociate later in the bulk of free solution if it is
carried out of the scaffold by diffusion and convective fluid flow. This binding reaction is shown in equation form as follows:

8.8 \[
\begin{align*}
\frac{\text{d}C_{\mathrm{BMP}}}{\text{d}t} &= k_{d,\text{BBP}}C_{\text{Cmplx}} - k_{a,\text{BBP}}C_{\text{BMP}}C_{\text{FusP}} \\
\frac{\text{d}C_{\text{Cmplx}}}{\text{d}t} &= k_{a,\text{BBP}}C_{\text{BMP}}C_{\text{FusP}} - k_{d,\text{BBP}}C_{\text{Cmplx}} \\
\frac{\text{d}C_{\text{FusP}}}{\text{d}t} &= k_{d,\text{BBP}}C_{\text{Cmplx}} - k_{a,\text{BBP}}C_{\text{BMP}}C_{\text{FusP}}
\end{align*}
\]

To approximate the kinetics in 8.8, the following series of equations are needed:

8.9a \[
\frac{\text{d}C_{\text{BMP}}}{\text{d}t} = k_{d,\text{BBP}}C_{\text{Cmplx}} - k_{a,\text{BBP}}C_{\text{BMP}}C_{\text{FusP}}
\]

8.9b \[
\frac{\text{d}C_{\text{Cmplx}}}{\text{d}t} = k_{a,\text{BBP}}C_{\text{BMP}}C_{\text{FusP}} - k_{d,\text{BBP}}C_{\text{Cmplx}}
\]

8.9c \[
\frac{\text{d}C_{\text{FusP}}}{\text{d}t} = k_{d,\text{BBP}}C_{\text{Cmplx}} - k_{a,\text{BBP}}C_{\text{BMP}}C_{\text{FusP}}
\]

In the kinetic equations comprising 8.9, \(C_{\text{BMP}}, \ C_{\text{Cmplx}}, \ C_{\text{FusP}}\) denote the concentrations of BMP-2, BMP-Fusion Protein complex and Fusion Protein respectively, where \(k_{a,\text{BBP}}\) and \(k_{d,\text{BBP}}\) are the association and dissociation affinity constants for BMP-2 with the BBP domain of the fusion protein. There are three distinct molecules and molecular complexes in solution, BMP-2, modeled with equation 8.9a, the Fusion protein-BMP-2 complex with 8.9b, and unbound fusion protein shown with 8.9c. The release of each of the three molecules can occur individually, as shown in the following set of equations:

8.10a \[
\frac{\text{d}C_{\text{BMP}}}{\text{d}t} = k_{d,\text{BF}}C_{\text{bCmplx}} - k_{a,\text{BF}}C_{\text{BMP}}C_{\text{BFusP}}
\]

8.10b \[
\frac{\text{d}C_{\text{Cmplx}}}{\text{d}t} = k_{d,\text{vWF}}C_{\text{bCmplx}} - k_{a,\text{vWF}}C_{\text{Cmplx}}((C_{\text{FusP,bnd,o}}+C_{o}) - (C_{\text{Cmplx}}+C_{\text{FusP}}))
\]

8.10c \[
\frac{\text{d}C_{\text{FusP}}}{\text{d}t} = k_{d,\text{vWF}}C_{\text{bFusP}} - k_{a,\text{vWF}}C_{\text{FusP}}((C_{\text{FusP,bnd,o}}+C_{o}) - (C_{\text{Cmplx}}+C_{\text{FusP}})) - (C_{\text{Cmplx}}+C_{\text{FusP}}))
\]
In 8.10, the new terms introduced are \( k_{d,vWF}, k_{a,vWF}, C_o, C_{bFusP}, C_{FusP,bnd,o} \) \( C_{bCmplx} \) which represent the dissociation and association constants for the von Willebrand Factor domain of the Fusion Protein, the initial BMP-2 concentration, the instantaneous and initial bound Fusion Protein concentrations, and the instantaneous bound Fusion Protein-BMP complex concentration respectively. The bound complex \( bCmplx \) dissociates with the fusion protein still bound to the scaffold, while 8.10b primarily shows the entire complex releasing from the scaffold, and in 8.10c, unbound fusion protein is released from the scaffold. In 8.10b and c, the terms in parentheses are the availability of binding sites on the collagen scaffold, assuming the scaffold is saturated initially.

Another set of equations is needed to track the amount of complex on the scaffold, as well as the amount of unbound fusion protein retained on the scaffold. The equations for binding retained by the scaffold are listed as follows:

\[
8.11a \quad \frac{dC_{bCmplx}}{dt} = -k_{d,BBP}C_{bCmplx} + k_{a,BBM}C_{bFusP} - k_{d,vWF}C_{bFusP} - k_{a,vWF}((C_{FusP,bnd,o} + C_o) - (C_{bCmplx} + C_{FusP})) - (C_{bCmplx} + C_{FusP})
\]

\[
8.11b \quad \frac{dC_{bFusP}}{dt} = k_{d,BBP}C_{bCmplx} - k_{a,BBM}C_{bFusP} - k_{d,vWF}C_{bFusP} - k_{a,vWF}((C_{FusP,bnd,o} + C_o) - (C_{bCmplx} + C_{FusP})) - (C_{bCmplx} + C_{FusP})
\]

As in 8.10b & c, the terms in parentheses in 8.11 indicate the available binding sites on the scaffold. Finally all the equations required a delta function to prevent reactant concentrations from declining below zero when completely consumed.

**Geometry and Mesh**

Although the shape of the reactor chamber and the shape of the porous scaffold are unchanged from the previous simulation, the mesh discretizing the geometry had to be changed to accommodate differing simulation requirements. In the fusion protein model, the reaction kinetics were more complex, requiring more computational resources. Furthermore, the model
also became unstable with the solver generating oscillatory solutions. To stabilize solutions and reduce simulation times, the mesh size had to be optimized for hydrodynamics. One tactic to accomplish this was to find the optimal element size along the length of the geometry. The optimum was to minimize the finite element-cell Peclet number $P_{e_{cell}}$ in the entire geometry. $P_{e_{cell}}$ is defined in the following equation:

$$8.12 \quad P_{e} = \frac{u l}{D}$$

Where $u$ is the fluid velocity, $l$ is the cell or element length and $D$ is the diffusivity. The mesh

![Figure 8.5 BMP-2 Concentration Distribution at 20 minutes](image)
size was chosen to match the reduction in $u$ that occurs when the fluid slows as the geometry widens. By shrinking the range of the Peclet number magnitude throughout the length of the reactor, the solution is stabilized to oscillations. Also to reduce simulation time, the fluid velocity was assumed to reach steady state fairly quickly, so was therefore solved as a stationary problem, and the resulting velocity field was mapped onto the convection-diffusion problem, conserving computational resources.

**Results**

The pattern of release for all three species of interest was a rapid burst of initial transient behavior seconds after simulation begins, leveling off to a plateau of steady state release (figure [Figure 8.6](#Figure-8.6) Simulated Concentration of Fusion Protein Complexes over 7 days).
8.6) for the duration of the simulation. Although the a steady rate of release is ideal for a delivery system, the steady state concentration of BMP-2 36.94 pmol/L might be too low to constitutively activate signal transduction in the SMAD pathway. This can be remedied by changing the binding affinity of BBP for BMP-2. Another way to increase the amount of BMP-2 is to increase the amount of another free solution species that can act as a BMP-2 source, the Fusion Protein-BMP-2 complex. With a steady state concentration in the range of 48 pmol/L, it accounts for over 56% of BMP-2 that is not bound to scaffold. By choosing a collagen binding domain that has less collagen affinity that vWF will speed ligand release, increasing concentration in the relevant time interval. Lowering the affinity of the complex for substrate compared to intracomplex affinity will increase the steady state amounts available to dissociate for biological action, but the exact ratio of component affinities between the two sites is unknown. Another tactic is to manipulate the ratio of initial concentrations of complex compon-
Figure 8.7 Spatiotemporal Diffusion-Rebinding Profile  a) The Thiele modulus ($\Phi$), a ratio of diffusion to reaction (rebinding in this case, see eqn 8.13) plotted across the scaffold length (L) given in mm. b) $\Phi$ across Scaffold width, time index is magnified near beginning to show transient phenomenon with intervals increasing along the time axis.

-bents. The unbound Fus-P concentration is greater than both BMP-2 species. It is present at more than two orders of magnitude greater, as the result of excess while loading the scaffold. This tactic employs mass action kinetics to ensure that a large initial amount of BMP-2 is bound to the scaffold. The high level of species bound to the scaffold ensures that the system is in no danger of depletion, further increase will likely raise the steady-state solution concentration. Both fusion protein and complex, as seen in figure 8.6, are present at five orders of magnitude greater than their free solution counterparts. This amount of retention of the complex and fusion
protein by the scaffold suggests that rebinding of released species must play a significant role in maintaining steady state concentrations. One important dimensionless quantity useful in analyzing reactants in free solution interacting with a functionalized surface is the Thiele modulus described by the following equation:

\[
\Phi = \sqrt{\frac{k_n L^2 C^{n-1}}{D}} = \frac{\text{diffusion time}}{\text{reaction time}}
\]

Where \(k_n\) is the order dependent rate constant, for binding reactions, it is also written as \(k_a\), the association affinity constant, \(L\) is the length scaling factor, this is the distance needed to diffuse to the center of a porous pellet. Typically, the pellet is considered spherical, and the radius is used. In the situation of interest, the porous phase is cylindrical and the diameter and height differ by more than 25%. For this reason, it is useful to consider the width and the length of the scaffold separately when comparing diffusion and reaction times. For both plots in figure 8.7, the time index interval increases, giving greater resolution initially to show transient behavior. This time scale was chosen because the simulation algorithm acts deterministically, where a high amount of precision is needed at early time points. Evident from figure 8.7, much of the interesting transient behavior occurs during the first minute and quickly localizes to the front and center of the scaffold, there the rebinding occurs rapidly generating the peaks seen, this saturates rebinding sites creating valleys surrounding the peaks where reaction rate is slowed compared ligand diffusing into proximity. This transient behavior is smooths out and by one minute, the system reaches steady state where diffusion is slightly faster than rebinding with \(\Phi \sim 0.8\).

**Parameter Sensitivity**

It is important to consider the sensitivity of the model to changes in parameter values. With a sizeable number of parameters, it is only practical to examine a limited subset of
parameters. Two important parameters to consider are scaffold porosity and inlet velocity because they are easily controlled through scaffold choice and pump operation. Certainly other important parameters exist, such as the affinity constants of the involved proteins, but they are not as easily controlled and therefore are less practical to analyze for sensitivity. The sensitivity was calculated using a parameter sweep through a narrow range of values surrounding the parameter values used in the original simulation. The objective value was the total flux of BMP-2 leaving the system at the outlet. The resulting sensitivities are shown in the following table:

**Table 8.6**

<table>
<thead>
<tr>
<th>Parameter Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{dN}{dU=5.9E^{-4}} = 2.0675 \times 10^{-20} \text{ mol/s} )</td>
</tr>
<tr>
<td>( \frac{dN}{dU=0.91} = 3.51 \times 10^{-14} \frac{\text{mol/s}}{\text{m/s}} )</td>
</tr>
</tbody>
</table>

The system is fairly robust to porosity changes, so effect on growth factor release due to fluid flow should not be a concern when selecting scaffolds. The system is more sensitive to inlet velocity, consequently pump speed should be a concern for release calculations. Furthermore the oscillatory nature of peristaltic pumps also warrants consideration.

**Simulation Verification**

To experimentally validate these BMP-2 release studies, I performed a series of bioreactor trials on a prototype fabricated using the design from Chapter 7. These runs included testing release of two generic proteins with similar molecular weight to BMP-2 and spp24.
Casein and serum albumin were chosen as these two proteins. Casein and BSA were prepared using PBS solutions of the appropriate concentration and dripped onto each sponge, then dried overnight, after which they were lyophilized and chemically sterilized. The treated sponges were inserted into the central chamber of the bioreactor and PBS was perfused through the system at a flow rate of 2-3 mL/hr. For casein, the experiment was conducted at two different temperatures to gauge the effect of physiological temperature compared to room temperature. The elutions were collected by a Foxy Pro Jr fraction collector programmed to collect hourly fractions of the bioreactor efflux. The experiments were either conducted in a laminar flow hood or in a CO₂ regulated incubator. After collection the casein and BSA elutions were assayed using BCA protein assay, Pierce, Rockford, IL. This assay detects total protein concentration through binding to amino acid side chains. The BMP-2 elutions were assayed using the Quantikine Immunoassay, R&D Mineapolis, MN.

**Results & Discussion**

Casein release at 37°C peaks immediately (figure 8.8a) and then declines to a low level where it oscillates slightly. When eluted at 25°C, Casein concentration peaks later at 2 to 3 hrs. An explanation for this is that at lower temperatures, the binding affinities act according to Arrhenius’ law \( k = A e^{(-Ea/RT)} \) and decrease correspondingly. However the shape of the two elution curves are similar with the lower temperature curve shifted to the right. BSA showed a quicker decline with a lower peak, though otherwise very similar in shape to the 37°C casein curve. These two proteins are similar in molecular weight to BMP-2, so it is likely they will experience very similar mass transport behavior as well as similar non-specific protein-protein interactions. The upper limit of detection on the BMP-2 assay (15000 pg/mL), is several orders
of magnitude lower compared to the working ranges of the BCA Assay. However, there is some overlap at time points ranging from 10-24 hours with the protein elution and with the BMP-2 release.

**Figure 8.8 Bioreactor Elution Profiles**

A. Casein and Bovine Serum Albumin Release  B. BMP-2 Release modulated by spp24 elution (figure 8.8b). In this interval, BMP-2 concentrations are beneath $1.5 \times 10^{-5}$ mg/mL and BSA/casein concentrations are above $1 \times 10^{-3}$ mg/mL. This occurs both with and without spp24.
present, confirming that BMP-2 has natural affinity to collagen\textsuperscript{19,21} that slows its release over casein and BSA.

In the BMP-2 release profile from experiment compared to simulation (figure 8.8b) the simulation curve without spp24 had a \textasciitilde15 h lag with the experimental curves, the covalently bound spp24 lag was similar in magnitude. One reason for this might be the physical gap between elution and collection. The dead volume between the reactor outlet and sample collector tube, consisting of the fraction collector and all the associated connecting tubing, is not accounted for in the simulation. Although the dead volume likely does make a contribution, there is a kinetic explanation that could contribute at least equally. As mentioned in chapter 1, BMP-2 has some affinity for collagen that can distort the 1:1 receptor-ligand model of binding. This binding interaction is reported to be several orders of magnitude weaker\textsuperscript{22} than the spp24-BMP-2 binding interaction ($K_D$ range $10^{-3}$-$10^{-4}$), the porous nature of the scaffold creates a large surface area that exposes many collagen sites to ligands traversing the solvent. This can create a mass action effect that pulls the equilibrium towards a scaffold-bound phase. Such a phenomenon accounts for the absence of lag in the non-covalent binding model where BMP-2 can bind to collagen when complexed with spp24. The greater retention times likely comes from using the spp24-collagen binding affinity which is stronger than the non-complexed BMP-2-collagen affinity. To correct for these discrepancies, the plot for the simulation curves without spp24 and with spp24 covalently bond has been shifted forward by 15 hrs, correspondingly this was not done with the simulation curve for noncovalent spp24 binding. After this shift, the experiment without spp24 matches simulation well, but the covalent spp24 binding curve still seems to have faster depletion than seen in experiment, however an oscillating concentration of the $(+)$spp24 covalent curve is also seen in experiment (likely stemming from rebinding and subsequent burst.
release), though with lower peaks and shallower troughs. However the global shape of the curve parallels noncovalent spp24. When spp24 is simulated using the fusion protein model, where spp24 and BMP-2 can dissociate while on the scaffold or in solution, thus the release of free BMP-2. The exaggeration of this effect could be another reason it is slower than the experimental curve. At present, the binding affinity of spp24 to collagen is not known, but was assumed to be in the micromolar range, roughly equivalent to transient interactions such as leukocyte rolling. Correcting the spp24-collagen affinity and refining the contribution of collagen-BMP-2 affinity will yield a better fit, paying for the increased model complexity with computational expense. As it stands, the experiments confirm that spp24 slows the release of BMP-2 on collagen, and this is likely due to dissociation of the BMP-2-spp24 interaction with spp24 either still attached to the sponge or in free solution as a complex. The experimental results are in alignment with previous studies\textsuperscript{20,23} for BMP-2 alone on collagen. Furthermore, retention of BMP-2 with spp24 is improved to a similar extent as seen in some polymer substrates,\textsuperscript{20} however in polymers with the most retention, steady state is not reached until day 5, where in the fusion protein simulation in previous sections, steady state is reached relatively quickly.

The bioreactor system can provide insight into the implantable sponge system it seeks to model. In rodents, an implant loaded with BMP-2 generates peak inflammation when first measured at 3 hours if implanted subcutaneously and at 2 days if implanted intramuscularly but with a two-fold increase in volume of inflamed tissue.\textsuperscript{23} A sterile collagen sponge loaded with radiolabelled BMP-2 implanted subcutaneously retains greater than 20% of protein at 5 hrs, but when implanted intramuscularly less than 5% is retained.\textsuperscript{20} In the perfused bioreactor system, I found that peak release occurs in the first twenty hours, regardless of whether or not a binding
protein is used. (figure 8.8b) However, when a binding protein such as spp24 is included, the concentration of BMP-2 being released into flow media perfusing through the sponge increases several thousand fold at two days, suggesting much more is retained from slower release. When analyzing inflammation, the curves for subcutaneous and intramuscular implantation are nearly identical when BBP is included. Essentially a binding protein counters the washout effect from perfused fluid flow due to tighter binding to the sponge.

In this chapter, I showed that the pattern of release of growth factor can be modeled using finite element simulation and I verified the simulation by testing a prototype bioreactor. The simulation was validated and used to test various different conditions and outputs including the inclusion of binding proteins modulating release.
References


Chapter 9

Cell Response Modeling

In the previous chapter, cell growth on the scaffold was modeled as an increase in volume, filling the void within the pores of the scaffold, gradually causing impedance to fluid flow. With some refinement, it is likely that this is an accurate representation of what occurs up to a week of bioreactor culture. At this time point, based on the growth rate extrapolated from static 2D culture, media flow is completely obstructed, clogging the scaffold. Unfortunately, this black-box approach does not consider the mechanism of how the cells are growing and differentiating. By looking at cell-signalling, biological subtleties behind the more macroscopically quantifiable output of bone growth can be understood.

The BMP-2 SMAD signal transduction pathway is a junction of six different cellular signaling pathways and processes. Shown in figure 9.1, these pathways are MAPkinase pathway

Figure 9.1 SMAD
Protein-Protein Interaction Network

Node and edge size are proportional to number of paths in which each component is involved. A band of three central nodes connect the TGF-β, Activin BMP pathways. Cellular response modeling involved the lower portion primarily focused around the BMP pathway. This graph was created using Cytoscape 3.0.1 with relevant pathways from the KEGG database.
shown in magenta, pitx2 homeobox gene transcription shown in green, the TGF-β pathway shown in Orange, the Activin/Nodal pathway shown in Cyan, Extracellular regulation of BMP by Chordin/Noggin in the indigo nodes, and the BMP-2 SMAD pathway itself shown in yellow. Visualized as a protein-protein interaction network, these pathways/processes can be partitioned into 51 nodes connected by 57 undirected edges. Without isolated nodes or self-loops, there are 2550 unique paths between all possible node pairs in the network with path length averaging 4.896. Characteristic of biochemical networks, each node has an average of 2.235 neighbors, indicating a linear flow of products to reactants in much of the network. The graph in figure 9.1 emphasizes portions of the network experiencing the highest traffic in terms of possible routes. A set of 3 interconnected central nodes emerges in the graph corresponding to TGF-β receptor2, SMAD4 and SMAD1,5,8 nodes. Since only BMP-2 is used in the delivery system with little or no TGF-β present in the extracellular space, the cell response model will focus on the bottom half of the network, specifically consisting of BMP-2 SMAD pathway, and more peripherally the MAPkinase pathway.

Ideally, a model should include how external signals are transduced from the membrane through the cytoplasm and into the nucleus, leading to regulation of transcription, thereby eventually altering the phenotype of the cell. In cells that are lineage-determined towards an osteoblastic phenotype, two pathways shown in figure 9.2 are triggered by BMP-2—the SMAD (SMAll mothers against decapentaplegic) pathway and the MAPK (Mitogen Activated Protein Kinase) pathway. Both pathways are triggered by the same receptors, clustering into differently configured complexes on the membrane. The receptors are categorized into two types, I and II, with type I being further subdivided into receptors Ia and Ib. Dimers consisting of a single molecules of type I and type II, in either the configuration IaII or the configuration IbII will
trigger the SMAD pathway, while larger complexes of 4 receptors are thought to trigger the MAP kinase pathway.\textsuperscript{1} Although there is some evidence that markers for osteoblastic differentiation considered in Chapter 4, such as alkaline phosphatase, have their expression affected by SMAD independent pathways,\textsuperscript{2,3} the SMAD pathway is important in upregulating several osteoblastic markers. For this reason, the SMAD pathway is the primary focus of this model, however the MAP kinase pathway is also considered to a limited extent. There are a significant amount of interesting biological phenomena that occur with the co-receptors of BMP receptor, the TGF-β superfamily of receptors, and the co-receptors of other associated pathways\textsuperscript{4,5} which must be ignored in order to construct the model described earlier.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{bmp2_smad_pathway.png}
\caption{BMP-2 SMAD Pathway adapted from Canalis et al\textsuperscript{1} using ChemBioDraw.}
\end{figure}

As shown in figure 9.2, a dimer of Type I and II receptors are phosphorylated upon BMP-2 binding (although pseudoreceptors are known which can interfere competitively with ligand
binding). The intracellular domain of the receptor complex can then act as a kinase to phosphorylate SMAD 1, 5 and 8, collectively termed receptor SMADs (RSMADs). The RSMADs are similar in properties so are modeled collectively as a single entity. Three important regulation points exist for the BMP-2 SMAD pathway in the cytoplasm and they center around the formation of a SMAD complex that can subsequently translocate into the nucleus acting as transcription factors to various genetic elements. The phosphorylated RSMADs dimerize, this process is the first regulation point. This step can be inhibited by SMADs 6 and 7, SMAD 7 can be activated by YAP, a transcription factor coregulator. The phospho-RSMAD dimer can bind to SMAD4 to form a protein-protein complex. The phospho-RSMAD dimer is regulated by ubiquitin tagging for proteasome degradation. Factors that can mediate this are SMURF 1 and 2, SMURF1 is an E3 Ubiquitin ligase. The SMURF2 factor is further regulated by a pseudokinase, Tribbles 3 Trb3. From a systems biology perspective, these regulation points are interesting because they control flux through the SMAD pathway separate from extracellular BMP-2 ligand concentration and cell surface receptor concentrations.

What occurs after the SMAD4-RSMAD complex enters the nucleus is less well understood. Although it is known that the SMAD4 MH1 domain binds to the sequence 5’-CAGAC-3’, this sequence occurs approximately every 1200bps, show the necessity of co-transcription factors such as the RSMADs. As discussed previously, up regulation of osteoblast differentiation markers is one endpoint for gauging cellular state. From qPCR studies, the most pronounced effect at seven days is seen in alkaline phosphatase ALP, while at fourteen days, the clearest effect is with osteocalcin. Although induction of ALP is rapid, protein synthesis is still required prior to transcription suggesting BMP-2 activation is indirect. As described in the previous chapter, the doubling time of MC3T3 cells is approximately 18 hours, and while
osteoblast differentiation does slow growth and proliferation, it is likely that at seven days, several mitotic divisions will have occurred for the majority of cells. Mitotic division becomes very difficult to model for a spatially resolved eukaryotic system because the position of the nucleus and its division is structurally complex. Furthermore, there is some evidence of interaction between SMADs and tubulin, an essential mitotic factor. Therefore, without modifying the model to account for mitosis, the simulation is only realistic up to the doubling. It is not known how much differentiation slows proliferation, furthermore that process itself could be dependent on BMP-2 signaling. Although the simulation is extended to 60 hours post-BMP-2 exposure, it is worth noting that there is potential for mitotic activity past 18 hours.

In addition to the three markers mentioned earlier, ALP, OCN, and COLIA, some studies have also looked at markers such as MMP13 as well as two transcription factors, RUNX2 and FOXO1. While MMP13, a matrix metalloproteinase, has an inferable role of extracellular matrix digestion in a cell undergoing osteoblast differentiation and performing mineralization, the transcription factors are more elusive to understand. FOXO1 is a member of the Forkhead family of transcription factors (so called because a mutation in this gene, leads to a developmental defect in head formation in mice). In an RNAi study of FOXO1, its depletion led to a drop in ALP, OCN, COLIA and MMP13. There was evidence that FOXO1 is required to bind to promoter regions to mediate the appropriate level of transcription. In a kinetic analysis of forkhead transcription factor expression in T-cells stimulated by TGF-β, the critical time for activation is 2-3 days. To examine the presence of a SMAD pathway components in the nucleus over the course of 60 hours is likely to avoid mitotic events, but also gauge SMAD presence to a initiate a transcription factor cascade.
Model

In previous chapters, the cell line of choice was MC3T3E1 Clone 4 cells because of their ready ability to differentiate into osteoblasts and under appropriate conditions, form nodules and mineralize. It has been shown that ascorbate found in mineralizing media can increase respiration rate fourfold, ATP production threefold, and ATP content fivefold in primary

Table 9.1 Cell Simulation Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{\text{BMP-2}}$</td>
<td>BMP-2 Diffusivity</td>
<td>93$\mu$m$^2$/s</td>
</tr>
<tr>
<td>$D_{\text{BMPRII}}$</td>
<td>BMPRII Mb Assoc Diffusivity</td>
<td>240$\mu$m$^2$/s</td>
</tr>
<tr>
<td>$D_{\text{BMPRIa}}$</td>
<td>BMPRIa Mb Assoc Diffusivity</td>
<td>200$\mu$m$^2$/s</td>
</tr>
<tr>
<td>$D_{\text{BMPRIb}}$</td>
<td>BMPRIb Mb Assoc Diffusivity</td>
<td>200$\mu$m$^2$/s</td>
</tr>
<tr>
<td>$D_{R,\text{SMAD}}$</td>
<td>Receptor SMAD Diffusivity</td>
<td>63.5$\mu$m$^2$/s</td>
</tr>
<tr>
<td>$D_{\text{SMAD4}}$</td>
<td>SMAD4 Diffusivity</td>
<td>63$\mu$m$^2$/s</td>
</tr>
<tr>
<td>$K_{D,\text{BMP2-II}}$</td>
<td>Affinity BMP-2 to BMPRII</td>
<td>1.5x10$^6$ M</td>
</tr>
<tr>
<td>$K_{D,\text{BMP2-la}}$</td>
<td>Affinity BMP-2 to BMPRIa</td>
<td>8x10$^{-10}$ M</td>
</tr>
<tr>
<td>$K_{D,\text{BMP2-ib}}$</td>
<td>Affinity BMP-2 to BMPRIb</td>
<td>8x10$^{-10}$ M</td>
</tr>
<tr>
<td>$K_{D,\text{la-II}}$</td>
<td>Affinity BMPRIa to BMPRII</td>
<td>1.0334x10$^{-6}$ M</td>
</tr>
<tr>
<td>$K_{D,\text{lb-II}}$</td>
<td>Affinity BMPRIb to BMPRII</td>
<td>1.0334x10$^{-6}$ M</td>
</tr>
<tr>
<td>$K_{D,\text{R-SMAD-SMAD4}}$</td>
<td>Affinity RSMAD to SMAD4</td>
<td>3.5898x10$^{-10}$ M</td>
</tr>
<tr>
<td>$K_{M,\text{receptor kinase}}$</td>
<td>Enz kin const Receptor Kinase</td>
<td>5.848x10$^{-13}$ M</td>
</tr>
<tr>
<td>$K_{M,\text{nuclear phosphatase}}$</td>
<td>Enz kin const Nucl Phosph</td>
<td>5.234x10$^{-9}$ M</td>
</tr>
<tr>
<td>$V_{\text{cell}}$</td>
<td>Cell Volume</td>
<td>2839pL</td>
</tr>
<tr>
<td>$V_{\text{nucl}}$</td>
<td>Nuclear Volume</td>
<td>887pL</td>
</tr>
</tbody>
</table>
osteoblasts.\textsuperscript{13} MC3T3E1 cells have a doubling time of 18 hours in non-mineralizing conditions, the total simulation time was set to 60 h, while media composition can significantly influence cell physiology, it is possible that osteoblast differentiation could slow the mitotic rate to this length or greater (though some researchers claim that mitosis is rapid for the first 9 days of culture).\textsuperscript{14}

Therefore, monitoring cellular behavior in the current simulation to extended time points ensured that a true steady state was reached. Systems simulation of the TGF-β SMAD pathway based on ordinary differential equations (ODEs) were used\textsuperscript{15} as an initial starting point for the BMP-SMAD pathway. The approach used was a finite volume method to generate spatially resolved concentration data over the course of the simulation.\textsuperscript{16} In table 9.1, diffusivities for pathway components and binding affinities between components were extracted from literature where available\textsuperscript{12} and approximated from biophysically similar situations where not available.\textsuperscript{17} The cell and nuclear volumes were determined by reconstructing light and scanning electron micrographs.\textsuperscript{18} Initial concentrations of BMP-SMAD pathway components were obtained from similar cells where the appropriate measurements had been made.\textsuperscript{15}

\textbf{Software}

The model was created using Simmune 2.1.2085 a finite volume software written by the NIAID systems biology laboratory. It uses a rule-based\textsuperscript{16} approach to handling ODEs for reaction kinetics. The models are composed in custom built sqLite (structured query language lite) database editor. Although it does allow for cell shape changes, this aspect of simulation can only be done for prokaryotic cells in this version.
Cell Volume Measurements

The cell volume parameter was used to build the three dimensional cell geometry seen in figure 9.3—it served as a constraint to determine variable elements not directly extractable from histology. For an accurate measurement of cell volume, a microfluidic cell counter was used.19

Table 9.2 Initial Concentrations

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear Phosphatase (nuclear concentration)</td>
<td>1.93304x10⁻⁶ mol/L</td>
</tr>
<tr>
<td>R-SMAD2:SMAD4 Complex (nuclear importing pore mb conc)</td>
<td>3 molecules/µm²</td>
</tr>
<tr>
<td>BMPRII</td>
<td>10.31 molecules/µm²</td>
</tr>
<tr>
<td>SMAD4 (nuclear exporting pore)</td>
<td>1 molecule/µm²</td>
</tr>
<tr>
<td>SMAD4 (nuclear concentration)</td>
<td>5.616x10⁻⁸ mol/L</td>
</tr>
<tr>
<td>BMPR1a</td>
<td>10.31 molecules/µm²</td>
</tr>
<tr>
<td>RSMAD (nucleus concentration)</td>
<td>3.3698x10⁻⁸ mol/L</td>
</tr>
<tr>
<td>RSMAD (nuclear exporting pore mb conc)</td>
<td>1 molecule/µm²</td>
</tr>
<tr>
<td>BMPR1b</td>
<td>10.31 molecules/µm²</td>
</tr>
<tr>
<td>RSMAD</td>
<td>5.994x10⁻⁸ mol/L</td>
</tr>
<tr>
<td>SMAD4</td>
<td>6.7839x10⁻⁸ mol/L</td>
</tr>
</tbody>
</table>

Figure 9.3 Finite Volume Cell Geometry
From these measurements, an average cell volume of 2839 pL was obtained. Based on morphometry, the nuclear volume was calculated as 887pL.

Stochasticity

When volumes become small making reacting molecules have totals of a few hundred or less in the entire volume, traditional mass action laws must be modified to account for stochasticity. In the finite volume single cell environment, a few signaling components reach that level. Randomness is introduced as noise terms, as was done in equations 1.9a-b. The general format is shown below:

9.1  \[ y_i = f(p_0, p_1 \ldots p_n) + \varepsilon_i \]
9.2  \[ \sum_i y_i = \sum_h j_h + \sum_k r_k \]

Equation 9.1 describes the cell output \( y \), to be a parameter dependent function, \( f(p_n) \), additive with a noise term \( \varepsilon \). Equation 9.2 constrains the system by equating the amount of output to the flux \( j \), and the consumption of components through reaction \( r \).

Simulation Results

The simulation assumed that the cell of interest was growing on the center of scaffold close to the downstream edge. In this region the concentration gradient could be established as the difference between the concentration in the scaffold domain and the free solution domain. The concentration gradient was synchronized to match the finite element results described in
**Figure 9.4** Nuclear Phosphatase, blue is nuclear phosphatase, green is phospho-RSMAD-enzyme complex and dephosphorylated RSMAD-enzyme complex in red (right ordinate) is hardly visible along the abscissa due to low concentration.

Chapter 8 every hour of simulated cell time. Although the concentrations for nuclear phosphatase were recorded across the entire cell, it cannot be transported outside of the nucleus, so the values (figure 9.4) can be adjusted by 3.2—the ratio between the nuclear volume and cell total volume. The plot shows a rapid influx of SMAD into the nucleus during the first 10,000 s (2.7h) that forms an enzyme-substrate complex. The dephosphorylated SMAD rapidly dissociates from nuclear phosphatase, yielding a negligible concentration. On the membrane, the simulation showed that there are initial spikes of the kinase-capable complexes BMPRIa-BMPRII and BMPRIb-BMPRII followed by low steady state concentrations, most prevalent on the membrane are the individual Ia and Ib receptors binding to BMP-2. This initially seemed to be a fault of the model. In order to save computation time, receptor recruitment by a ligand-
bound single receptor was not allowed and instead relied upon dissociation and rebinding to transfer a ligand molecule from a single receptor to a complex. However when this was corrected in a truncated simulation, the single receptor-ligand complex still dominated the membrane. What seems to occur is a burst of SMAD phosphorylation, corresponding to high initial BMP-2 concentrations then tapering off at extended time points as a response to the fall in BMP-2.

The SMAD concentration plot (Figure 9.5) is consistent with this hypothesis. There is an initial burst of the triple SMAD complex that equilibrates to steady state concentrations. For the RSMAD-SMAD4-RSMAD complex this concentration is only $6 \times 10^{-11}$ M, though an order of magnitude increase would occur if this amount was entirely found in the nucleus. Without knowing precisely what DNA sequences the complex binds to and with what affinity, it is difficult to predict more than presence of the complex at low concentration throughout the length of the 60 hr simulation.

In contrast to simulations with a dynamic ligand concentration, a static gradient shows a different receptor complex pattern on the membrane. In this scenario, at extended time points
most receptors on the membrane are found in quaternary complexes. This suggests that in the absence of any of the inhibitors of the BMP-2 pathway, a high concentration of BMP-2 can switch from activating the SMAD pathway to activating the MAPK pathway. This would be consistent with evidence showing that ALP activation can occur independent of the BMP-SMAD pathway.\textsuperscript{2,3}

Conclusion

In summary, the cell simulations give some mechanistic insight on scaffold cell growth when perfused with a variable BMP-2 ligand concentration. It suggests that BMP-2 is either needed early as a burst to initiate a chain of events leading to marker upregulation or that it can accomplish its tasks with a low concentration of SMAD complex concentration. This is plausible if a multi-step cascade of transcription factors such as RUNX2 and FOXO1 are needed. In a signal transduction cascade with multiple components, amplification can occur at each step necessitating a low initial ligand concentration. Alternatively, the extracellular concentration of BMP-2 might not be indicative of the concentration that is activating receptor-SMADs. There is recent evidence that endocytosis of receptors plays a significant role in delaying the kinetics of the BMP-SMAD pathway.\textsuperscript{2, 21-22} A concurrent sequestration of BMP-2 in endocytic vesicles could lead to SMAD activation on the cytosolic face of the vesicular membrane additive with the phosphorylation occurring on the plasma membrane. The vesicular concentration of ligand would be significantly higher than extracellular concentrations. Such a situation, though interesting, is difficult to model, as endocytosis can only be easily modeled as temporary receptor deactivation, giving no insight on more complex phenomena.
Additional understanding of BMP-2 action on osteoblastic cells will allow better design of therapeutic systems that maximize the growth factor’s pharmacodynamic effect on target cells and allow systems that maximize the duration of the growth factor’s pharmacokinetic effectiveness. This would minimize the needed dosage, avoiding unwanted side effects.
References


Conclusion of the Dissertation

In this dissertation, I have explored bone biology focused around the growth factor, BMP-2, and an extracellular binding protein, spp24. It was hypothesized that spp24 can be used to modulate BMP-2 release for the purpose of enhancing bone growth. Using the method of surface plasmon resonance, I examined binding kinetics to extract an accurate quantification of the binding affinity between variants of BBP, a spp24-derived peptide, and BMP-2. The binding affinity was relatively strong and able to mediate biological effects. To explain the causes of this level of affinity in molecular structure, I examined the structural biology of the receptor and ligand. I then further explored the structure with mass spectrometry and bioconjugate peptide modification. It was shown that the peptide exists as a loop closed by cysteines and this loop occupies a large portion of the surface interfacing spp24 and BMP-2. This binding interaction was examined in detail because it can be used as the chemical basis for modulating release of BMP-2 from a tissue engineering scaffold.

Osteoblastic cells were examined as the cellular component of this tissue engineering system. Marker expression was examined using qPCR, showing that temporal course of differentiation of osteoblastic cells when exposed to differing growth conditions including BMP-2 infusion. It was confirmed that at fourteen days, growth media with BMP-2, ascorbate, dexamethasone and β-Glycerophosphate produces that greatest level of late-stage differentiation along with mineralization. The pattern of marker expression also gave some insight into the physiology of BMP-2 induced mineralization. Furthermore, cellular growth was also tested on collagen and other scaffold materials under various conditions including perfusion. It was shown that collagen scaffolds produces the largest amount of live cells at extended time points. Sufficient oxygenation is another factor in mediating cell growth. Using simulation, it was
determined that nominal deformation occurs to the scaffold under fluid flow. After collagen sponge was selected as the scaffold material, methods of modulating BMP-2 release from it were examined.

To accomplish release modulation, fusion peptides with collagen-binding domains were designed. Simulation of release from a collagen scaffold using simple adsorption, fusion peptide, and covalent conjugation of the peptide to the scaffold. Simulation showed that a fusion peptide-based delivery system achieves the most sustained release profile. Verification of simulation required design and fabrication of a bioreactor, housing the flow chamber used in simulation. The necessity of a continuous level of BMP-2 presence was examined using systems biology simulation of the BMP-2–SMAD signal transduction pathway. This simulation showed that the pathway reaches steady state relatively rapidly and a potential regulatory point is the desphosphorylation of activated transcription factor in the nucleus. This suggests that to maintain transcription of BMP-induced genes, continuous activation of the receptor is needed. Although comprehensive verification was done, it is possible many elements of the cellular and bioreactor simulation can benefit from added verification and should be targets for further work. In summary, this is evidence that a peptide-based release modulation of BMP-2 will allow improved bone growth. The ability to engineer tissues provides new methods for therapeutic treatment of illness and disability through tissue replacement. It also provides a route where engineering of entire organs and organ systems could be possible.
**Appendix**

**MATLAB code for kinetics fitting**

```matlab
function [param, chisq, fit, ka, kd, KD, KA] = fitter(data, x0, t1, conc)
% fitter fits SPR data to binding model via sequential least sq
% data = two column matrix, data(1)=time data(2)=RU
% x0 = initial guess vector 4x1
% x0(1) = Rmax, x0(2)=kd, x0(3)=t0, x0(4)= offset
% t1 = length of association phase (contact time) (s)
% conc = Analyte conc (M)
% options1 = optimset('MaxIter',100000,'Algorithm', {'levenberg-marquardt' [0.0050]});
% trim vectors to tr and init tr
s = size(data);
i = 1;
if x0(1) > max(data(:,2))
    x0(1) = max(data(:,2));
end

% counts the length of association phase
while i<= s(1);
    if isequal(data(i,1), 0)
        zero = i;
        c = 0;
    elseif data(i,1) > t1
        i = s(1);
    elseif data(i,1) > 0
        c = c + 1;
    end
    i = i + 1;
end

dataintr = data(zero:zero+c-1,1:2) ;
datatr = data(zero+c:s(1),1:2) ;

% lsq fit tr
[ptr, resnorm] = lsqcurvefit(@Kdissociation, x0, datatr(:,1), datatr(:,2));
kd = ptr(2);

% lsq fit intr
x0a = [1/kd ; 1];
ub1 = [1e11 * kd; 1.1];
lb1 = [1e7 * kd; 0.9];
Rm = max(dataintr(:,2));
f = @(x,xdata)((x(1)*conc)/(x(1)*conc + kd))*Rm*(1-exp(-(x(1)*conc + ...
     kd)*xdata))+ x(2) - x(2); %+x(3);
[pintr] = lsqcurvefit(f,x0a,dataintr(:,1),dataintr(:,2),lb1,ub1);
ka = pintr(1);
```
pintr(2) = 0;

% fitted data curve for tr
[yfittr] = Kdissociation(ptr, datatr(:,1));
[fittr] = [ datatr(:,1) yfittr];

% fitted data curve for intr
[yfitintr] = Kassociation(pintr, conc, kd, Rm, dataintr(:,1));
[fitintr] = [ dataintr(:,1) yfitintr];

% concatenate tr and intr
fit = [fitintr ; fittr];

plot(data(:,1),data(:,2))
hold on;
plot(fit(:,1),fit(:,2),'k')
hold off;

% calculate chisq
alpha = size(fit) %;
beta = size(pintr);
gamma = size(ptr);
chisq = sum((dataintr(:,2); datatr(:,2)]-fit(:,2)).^2)/(alpha(1)-
(beta(1)+gamma(1)));
% calculate KD & KA
param = [pintr; ptr];
kd= kd
ka= pintr(1)
chisq = chisq
KD = kd/ka
KA = ka/kd

function F = Kassociation(x, conc, kd, Rm, xdata)
%Kassociation fitting function for ka
% c = analyte concentration
% k = kd
%c = 1e-6;
%k = 0.022787874741581;
F = ((x(1)*conc)/(x(1)*conc +kd))* Rm *(1-exp(-((x(1)* conc + kd)... 
* xdata)))+x(2);

function F = Kdissociation( x , xdata)
%Kdissociation fitting function for kd
% fit w/ a 4x1 parameter vector
F = x(1) * exp(-x(2) *( xdata - x(3))) + x(4);
function [ M ] = Curvmap( P, C )
% Curvmap 2d Coordinate transform
%   Maps a set of points P onto a curvilinear coordinate system
%   generated from the curve C
cl = length(C);
pl = length(P);
M = zeros(pl,2);
for r = 1:pl
    for r2 = 1:cl-1
        if P(r,1) > C(r2,1)
            if P(r,1) < C(r2 + 1,1)
                M(r,1) = P(r,1);
                M(r,2) = (P(r,2)/((C(r2,2)+C(r2 + 1,2))/2))-1;
            end
        end
    end
for r2 = 1
    if P(r,1) < C(r2,1)
        M(r,1) = P(r,1)
        M(r,2) = (P(r,2)/C(r2,2))-1
        sprintf('P starts before C')
    end
end
for r2 = cl
    if P(r,1) > C(r2,1)
        M(r,1) = P(r,1);
        M(r,2) = (P(r,2)/C(r2,2))-1;
        sprintf('C ends before P')
    end
end
end
% COMSOL Multiphysics Model M-file
% Some geometry objects are stored in a separate file.
% The name of this file is given by the variable 'flbinaryfile'.

flclear fem

% Constants
fem.const = {'rho1','1.002265 [g/cm^3]', ... 
  'rho2','1.1839[kg/m^3]', ... 
  'rho3','965[kg/m^3]', ... 
  'rho4','2500[kg/m^3]', ... 
  'eta1','1e-3[Pa*s]', ... 
  'eta2','1.963e-5[Pa*s]', ... 
  'U','4.364528995e-6[m/s]', ... 
  'eta3','33.33[kPa*s]', ... 
  'eta4','1e-19[Pa*s]', ... 
  'rho5','0.3[g/mL]', ... 
  'eta5','0.007[Pa*s]', ... 
  'epsilon0','0.9', ... 
  'kappa0','9.8249e-4[m^2]', ... 
  'Q','100[uL/min]', ... 
  'A','7.827174e-6[m^2]', ... 
  'D1','13e-7[cm^2/s]', ... 
  'D2','11e-7[cm^2/s]', ... 
  'C1','0[mol/m^3]', ... 
  'Conc3','0.0039913786[mol/m^3]', ... 
  'C2','0[mol/m^3]', ... 
  'rho6','0.392 [g/cm^3]', ... 
  'eta7','12.844 [Pa*s]', ... 
  'ka','2.88e-3 [L/mol/s]', ... 
  'kd','1.70e6[1/s]', ... 
  'mcells','0.0361686714[ug]', ... 
  'mu','1.069671575e-5[1/s]', ... 
  'rhocells','1.023116[g/cm^3]', ... 
  'Vtotal','7.4539991e-8[m^3]', ... 
  'D3','0.66e-7[m^2/s]'};

% (Default values are not included)

% Application mode 1
clear appl
appl.mode.class = 'ConvDiff';
appl.module = 'CHEM';
appl.sshape = 2;
appl.assignsuffix = '_chcd';
clear prop
clear weakconstr
weakconstr.value = 'off';
weakconstr.dim = ('lm3');
prop.weakconstr = weakconstr;
appl.prop = prop;
clear bnd
bnd.type = {'Nc','N0','cont'};
bnd.ind = [1,2,2,2,2,2,3,2,2,3,3,2,3,3,2,2,2,2,1];
appl.bnd = bnd;
clear equ
equ.D = {'D1','D2'};
equ.init = {'C1','C2'};
equ.R = {0,'kd*c3-ka*c*(Conc3-c3)'};
equ.ind = [1,2];
appl.equ = equ;
fem.appl{1} = appl;

% Application mode 2
clear appl
appl.mode.class = 'NavierStokes';
appl.dim = {'u2','v2','w2','p2','logk','logd','logw','phi','psi','nxw', ...
'nyw','nzw'};
appl.module = 'CHEM';
appl.gporder = {4,2};
appl.cporder = {2,1};
appl.assignsuffix = '_chns';
clear prop
prop.analysis='static';
prop.brinkmandef='On';
clear weakconstr
weakconstr.value = 'off';
weakconstr.dim = {'lm4','lm5','lm6','lm7','lm8','lm9','lm10','lm11','lm12'};
prop.weakconstr = weakconstr;
appl.prop = prop;
clear bnd
bnd.type = {'inlet','sym','walltype','int','outlet'};
bnd.U0in = {'U',1,1,1,1};
bnd.ind = [1,2,2,3,3,3,4,2,2,4,4,3,4,3,3,3,5];
appl.bnd = bnd;
clear equ
equ.eta = 'etal';
equ.gporder = {{1,1,1,2}};
equ.epslonp = {1,'por'};
equ.rho = 'rho1';
equ.kappadv = {0,'kappa'};
equ.cporder = {{1,1,1,2}};
equ.brinkmanegns = {0,1};
equ.k = {1,'kappa'};
equ.ind = [1,2];
appl.equ = equ;
fem.appl{2} = appl;

% Application mode 3
clear appl
appl.mode.class = 'Diffusion';
appl.dim = {'c3'};
appl.module = 'CHEM';
appl.sshape = 2;
appl.assignsuffix = '_chdi';
clear prop
prop.analysis='static';
clear weakconstr
weakconstr.value = 'off';
weakconstr.dim = {'lm2'};
prop.weakconstr = weakconstr;
appl.prop = prop;
clear bnd
bnd.type = {'cont','N0'};
bnd.ind = [1,1,1,1,1,1,2,2,2,2,2,2,1,1,1,1,1,1,1];
appl.bnd = bnd;
clear equ
equ.D = {'D3'};
equ.init = {0,'Conc3'};
equ.R = {[0,'-kd*c3+ka*c*(Conc3-c3)']};
equ.usage = {0,1};
equ.ind = [1,2];
appl.equ = equ;
fem.appl{3} = appl;
fem.frame = {'ref'};
fem.border = 1;
clear units;
units.basesystem = 'SI';
fem.units = units;

% Scalar expressions
fem.expr = {'por','epsilon0+(epsilon0*mcells*exp(mu*t))/(rhocells*Vtotal)', ...
'kappa','kappa0*(por/epsilon0)^3.55'};

% Descriptions
clear descr
descr.expr= {'kappa','Permeability'};
fem.descr = descr;

% Descriptions
descr = fem.descr;
descr.const= {'A','Inlet Area','rhocells','Cell Density','U','Inlet Velocity','eta1','Viscosity of Water','rho6','Density of collagen','rho5','Density of Chitosan','rho4','Density of Glass','Q','inlet flow rate','rho3','Density of PDMS','rho2','Density of Air','kd','affinity constant (dissoc)','rho1','Density of alpha-MEM ribo(+)', 'ka','affinity constant (assoc)', 'C1','Init BMP2 Conc - compartment 1','C2','Init free BMP2 Conc compartment 2','epsilon0','scaffold Porosity (collagen)','mcells','Mass per cell','Conc3','Init bnd BMP2 Conc compartment 2','eta7','Viscosity of Collagen','mu','Cell Growth Rate','eta6','Viscosity of DMEM','eta3','Viscosity of PDMS','eta2','Viscosity of Air','eta5','viscosity of Chitosan','eta4','Viscosity of Glass','kappa0','Scaffold Permeability (collagen)','D2','Diffusion of BMP2 in Porous Phase','D3','Diffusion -bound BMP2','D1','Diffusion of BMP2 in Water (from Cytochr C)', 'Vtotal','Total Scaffold Volume (qtr)'};
fem.descr = descr;

% ODE Settings
clear ode
clear units;
units.basesystem = 'SI';
od.e.units = units;
fem.ode=ode;
% Multiphysics
fem=multiphysics(fem);

% Extend mesh
fem.xmesh=meshextend(fem);

% Solve problem
fem.sol=femtime(fem,
    'solcomp',{'u2','p2','c','v2','c3','w2'}, ...
    'outcomp',{'u2','c','p2','v2','c3','w2'}, ...
    'blocksize','auto', ...
    'tlist',[colon(0,1800,7200)], ...
    'tout','tlist', ...
    'linsolver','bicgstab', ...
    'itol',0.15, ...
    'maxlinit',20000, ...
    'prefuntype','right', ...
    'prepar',{'droptol',1E-5});

% Save current fem structure for restart purposes
fem0=fem;

% Plot solution
postplot(fem,
    'slicedata',{'c','cont','internal','unit','mol/m^3'}, ...
    'slicexspacing',10, ...
    'sliceysspacing',[0.002399, 2e-3], ...
    'slicezspacing',[2.661535e-18 2.661535e-18], ...
    'slicemap','Rainbow', ...
    'tetdata',{'c3','cont','internal','unit','mol/m^3'}, ...
    'tetedgestyle','interp', ...
    'tetfacestyle','none', ...
    'tetmap','TrafficLight', ...
    'tetkeep',1, ...
    'tetkeeptype','random', ...
    'solnum','end', ...
    'title','Time=7200    Slice: Concentration, c [mol/m^3]   Subdomain: Concentration, c3 [mol/m^3]', ...
    'grid','on', ...
    'scenelight','on', ...
    'campos',[0.09245517309178675,0.032899142624752804,0.012667385526792394], ...
    'camtarget',[0.012967148330628,0.004448747844435275,0.00205000010058284], ...
    'camup',[0.028004070113512414,-0.4172341323686524,0.9083674646549406], ...
    'camva',10.642510072935238);

% Plot solution
postplot(fem,
    'slicedata',{'c','cont','internal','unit','mol/m^3'}, ...
    'slicexspacing',10, ...
    'sliceysspacing',[0.002399, 2e-3], ...
    'slicezspacing',[2.661535e-18 2.661535e-18], ...
'slicemap','Rainbow', ...
'tetdata',{c3,'cont','internal','unit','mol/m^3'}, ...
'tetedgestyle','interp', ...
'tetfacestyle','none', ...
'tetmap','TrafficLight', ...
'tetkeep',1, ...
'tetkeeptype','random', ...
'solnum',1, ...
'title','Time=0    Slice: Concentration, c [mol/m^3]    Subdomain: Concentration, c3 [mol/m^3]', ...
'grid','on', ...
'scenelight','on', ...
'campos',[0.015664159161980228,0.08692317127303249,0.02281524818050638], ...
'camtarget',[0.012967148330062628,0.004448747844435275,0.002050000010058284], ...
'camup',[-0.10131891099626994,0.23978464406119054,0.9655246256553883], ...
'camva',10.642510072935245);

% Plot solution
postplot(fem, ...
'slicedata',{'c','cont','internal','unit','mol/m^3'}, ...
'slicexspacing',10, ...
'sliceyspacing',[0.002399, 2e-3], ...
'slicezspacing',[2.661535e-18 2.661535e-18], ...
'slicemap','Rainbow', ...
'tetdata',{c3,'cont','internal','unit','mol/m^3'}, ...
'tetedgestyle','interp', ...
'tetfacestyle','none', ...
'tetmap','TrafficLight', ...
'tetkeep',1, ...
'tetkeeptype','random', ...
'solnum',2, ...
'title','Time=1800    Slice: Concentration, c [mol/m^3]    Subdomain: Concentration, c3 [mol/m^3]', ...
'grid','on', ...
'scenelight','on', ...
'campos',[0.007223283844569101,0.08265706159278871,0.035079798681844285], ...
'camtarget',[0.012967148330062628,0.004448747844435275,0.002050000010058284], ...
'camup',[-0.28322510144480156,0.3907044856633466,0.8758616025343968], ...
'camva',10.642510072935227);

% Plot solution
postplot(fem, ...
'slicedata',{'c','cont','internal','unit','mol/m^3'}, ...
'slicexspacing',10, ...
'sliceyspacing',[0.002399, 2e-3], ...
'slicezspacing',[2.661535e-18 2.661535e-18], ...
'slicemap','Rainbow', ...
'tetdata',{c3,'cont','internal','unit','mol/m^3'}, ...
'tetedgestyle','interp', ...
'tetfacestyle','none', ...
'tetmap','TrafficLight', ...
'tetkeep',1, ...
'tetkeeptype','random', ...
'solnum',3, ...
'title','Time=3600  Slice: Concentration, c [mol/m^3]  Subdomain:
Concentration, c3 [mol/m^3]', ...
'grid','on', ...
'scenelight','on', ...
'campos',[0.007223283844569101,0.08265706159278871,0.035079798681844285], ...
'camtarget',[0.012967148330062628,0.004448747844435275,0.0020500000010058284], ...
  'camup',[-0.28322510144480156,-0.3907044856633466,0.8758616025343968], ...
  'camva',10.642510072935227);

% Plot solution
postplot(fem, ...
'slicedata',{c', 'cont', 'internal', 'unit', 'mol/m^3'}, ...
slicexspacing',10, ...
sliceysspacing',[0.002399, 2e-3], ...
slicezspacing',[2.661535e-18 2.661535e-18], ...
slicemap','Rainbow', ...
tetdata',{c3', 'cont', 'internal', 'unit', 'mol/m^3'}, ...
tetedgestyle','interp', ...
tetfacestyle','none', ...
tetmap','TrafficLight', ...
tetkeep',1, ...
tetkeeptype','random', ...
solnum',4, ...
'title','Time=5400  Slice: Concentration, c [mol/m^3]  Subdomain:
Concentration, c3 [mol/m^3]', ...
'grid','on', ...
'scenelight','on', ...
'campos',[0.007223283844569101,0.08265706159278871,0.035079798681844285], ...
'camtarget',[0.012967148330062628,0.004448747844435275,0.0020500000010058284], ...
  'camup',[-0.28322510144480156,-0.3907044856633466,0.8758616025343968], ...
  'camva',10.642510072935293);

% Plot solution
postplot(fem, ...
'slicedata',{c', 'cont', 'internal', 'unit', 'mol/m^3'}, ...
slicexspacing',10, ...
sliceysspacing',[0.002399, 2e-3], ...
slicezspacing',[2.661535e-18 2.661535e-18], ...
slicemap','Rainbow', ...
tetdata',{c3', 'cont', 'internal', 'unit', 'mol/m^3'}, ...
tetedgestyle','interp', ...
tetfacestyle','none', ...
['tetmap', 'TrafficLight', ...]
['tetkeep', 1, ...]
['tetkeeptype', 'random', ...]
['solnum', 'end', ...]
['title', 'Time=7200    Slice: Concentration, c [mol/m^3]   Subdomain: Concentration, c3 [mol/m^3]', ...]
['grid', 'on', ...]
['scenelight', 'on', ...]
['campos', [0.007223283844569101, 0.08265706159278871, 0.035079798681844285], ...]
['camtarget', [0.012967148330062628, 0.004448747844435275, 0.002050000010058284], ...]
['camup', [-0.2832251044480156, -0.3907044856633466, 0.8758616025343968], ...]
['camva', 10.642510072935293];

% Integrate
I1=postint(fem,'c3', ...
    'unit','mol', ...]
    'recover','off', ...]
    'dl', 2, ...]
    'solnum','end');

% Plot solution
postplot(fem, ...
    'slicedata',{'c','cont','internal','unit','mol/m^3'}, ...
    'slicexspacing',10, ...]
    'sliceyspacing',[0.002399, 2e-3], ...]
    'sliceyspacing',[2.661535e-18 2.661535e-18], ...]
    'slicemap','Rainbow', ...]
    'tetdata',{'c3','cont','internal','unit','mol/m^3'}, ...]
    'tetbedestyle','interp', ...]
    'tetfacestyle','none', ...]
    'tetmap','TrafficLight', ...]
    'tetkeep',1, ...]
    'tetkeeptype','random', ...]
    'solnum','end', ...]
    'title', 'Time=7200    Slice: Concentration, c [mol/m^3]   Subdomain: Concentration, c3 [mol/m^3]', ...]
    'grid', 'on', ...]
    'scenelight', 'on', ...]
    'campos',[5.028073913508958E-4, 0.08080066823640829, 0.037483742075541476], ...]
    'camtarget',[0.01295574667029016, 0.004514805723997972, 0.0019043744175852174], ...]
    'camup', [0.07112097074116559, -0.41205408803271687, 0.9083794559854161], ...]
    'camva', 10.64251007293522);
'slicezspacing',[2.661535e-18 2.661535e-18], ...
'slicemap','Rainbow', ...
'tetdata',{['c','cont','internal','unit','mol/m^3'], ...}
'tetedgestyle','interp', ...
'tetfacestyle','none', ...
'tetmap','TrafficLight', ...
'tetkeep',1, ...
'tetkeeptype','random', ...
'solnum','end', ...
'title','Time=7200 Slice: Concentration, c [mol/m^3] Subdomain:
Concentration, c [mol/m^3]', ...
'grid','on', ...
'scenelight','on', ...
'campos',[5.028073913508958E-4,0.08080066823640829,0.037483742075541476], ...
'camtarget',[0.01295574667029016,0.004514805723997972,0.001904374175852174], ...
'camup',[0.07112097074116559,-0.41205408803271687,0.9083794559854161], ...
'camva',10.642510072935417);

% Plot solution
postplot(fem, ...
'slicedata',{['c','cont','internal','unit','mol/m^3'], ...}
'slicexspacing',10, ...
'sliceyspacing',[0.002399, 2e-3], ...
'slicezspacing',[2.661535e-18 2.661535e-18], ...
'slicemap','Rainbow', ...
'tetdata',{['c','cont','internal','unit','mol/m^3'], ...}
'tetedgestyle','interp', ...
'tetfacestyle','none', ...
'tetmap','TrafficLight', ...
'tetkeep',1, ...
'tetkeeptype','random', ...
'solnum','end', ...
'title','Time=7200 Slice: Concentration, c [mol/m^3] Subdomain:
Concentration, c [mol/m^3]', ...
'grid','on', ...
'scenelight','on', ...
'campos',[5.028073913508958E-4,0.08080066823640829,0.037483742075541476], ...
'camtarget',[0.01295574667029016,0.004514805723997972,0.001904374175852174], ...
'camup',[0.07112097074116559,-0.41205408803271687,0.9083794559854161], ...
'camva',10.642510072935417);
Bioreactor Schematic

**Gasket**

![Gasket Diagram](image)

**Flow Adapter**

![Flow Adapter Diagram](image)

**Central Chamber**

![Central Chamber Diagram](image)