OPTIMIZATION OF ENZYME DISSOCIATION PROCESS BASED ON REACTION DIFFUSION MODEL TO PREDICT TIME OF TISSUE DIGESTION

DISSETATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Bhavya Mehta, B.E.

*****

The Ohio State University

2006

Dissertation Committee:

Dr. Jeffrey J. Chalmers

Dr. David L. Tomasko

Dr. Deborah M. Grzybowski

Approved By

-------------------------------------

Adviser

Chemical Engineering Graduate Program
ABSTRACT

With the advent and improvement of highly sensitive and accurate molecular analysis technologies, it is possible to study increasingly complex cellular processes. These technologies have played a significant role in cancer diagnosis. However, a common complaint of studies using these technologies is the heterogeneity of the original sample. The lack of homogeneity is a challenge when one works with non-cultured, three-dimensional tissue, which normally consists of a variety of different cell types, in different states. Therefore, the ability to analyze and separate a heterogeneous cell population based on cellular characteristics is a significant analytical and preparative resource for molecular analysis techniques. The sample preparation techniques in case of a tissue sample are rudimentary and opportunities are ample.

The cellular components of a tissue sample are bonded tightly together by extracellular material (ECM). The main components of ECM are proteins (collagen and elastin), which are broken down using enzymes such as collagenase, elastase, metalloproteinases etc. The enzyme dissociation process is an integral part of life sciences laboratory and is designed predominantly based on empirical experience of each research group. There is little or no fundamental understanding available about the enzyme dissociation process to produce single cell suspension from a tissue. We believe that enzyme dissociation technique can be predicted and improved by applying
fundamentals of enzyme kinetics of ECM degradation and diffusion of enzyme molecule through ECM. This research utilized breast tissue (normal and malignant) as a model, but the principles are applicable to other tissue digestion process as well.

This study focused on understanding each ECM component in breast tissue and concluded that collagen concentration and distribution are the rate controlling component in breast tissue digestion. Based on high collagen concentration we hypothesized that actual time of tissue digestion can be modeled based on digestion time of collagen. To model the digestion time of collagen in tissue; primary variables like collagen concentration in the tissue, the solution kinetics of collagen degradation and the effect of mass transfer of collagenase in the breast tissue was determined. It was quantified that average collagen concentration in breast tissue is $53 \pm 37 \, \mu g$ of collagen per mg of wet breast tissue weight. This concentration provided the basis for the substrate concentration in enzyme digestion process. The kinetic parameters of collagenase was quantified based on fluorescence measurement as $5.9 \pm 3.1 \, \mu g$ of collagen/U.min ($k_{cat}$) and $7.2 \pm 1.7 \, \mu g$ of collagen/ml ($K_M$). These kinetic parameters along with the collagen concentration were utilized to calculate the time of digestion based on analytical solution of Michaelis – Menten kinetics. This time was an order of magnitude less than observed by other researchers, which ranged from 1 hour to 16 hours for breast tissue. This discrepancy in the time of digestion was attributed to mass transfer of enzyme in the tissue matrix. The diffusion of enzyme was modeled based simple reaction diffusion model and the predictions were confirmed with empirical studies. Based on these results, it was confirmed that time of breast tissue digestion can be predicted based on time of collagen digestion.
Dedicated to my grandmother, my parents and my brother
ACKNOWLEDGMENTS

This exploratory study would not have been possible without the guidance, motivation and challenges presented by my adviser Dr. Jeff Chalmers. I would also like to thank my co-advisor Dr. Deb Grzybowski for her valuable insights and inspirational support. I also wish to express my gratitude to Dr. David Tomasko and Dr. S.T. Yang for being part of my committee and providing constructive criticism on my work.

I am indebted to Ms. Kristie Melnik (Research Associate, Dr. Chalmers Lab) and Dr. Xiaodong Tong (Post doc, Dr. Chalmers Lab) for their important recommendations. It was a privilege to work and discuss challenges with highly motivated colleagues like Mr. David Holman, Dr. Shubhaya Basu, Mr. Luis G. Velazquez-Vargas, Mr. Shunahshep Shukla, Dr. Mei Shao, Dr. Mike Mollet, Dr. Huading Zhang and Dr. Oscar Lara. Mr. Ruben Godoy, Mr. Weiwei Hu, Mr Burr Zimmerman and Dr. Supaporn Suwannakham have been constant source of help in and around our laboratory. I consider myself fortunate to enjoy memorable graduate research experience with all my lab mates and fellow graduate students. I am also grateful to Mr. Brian Kemmenoe and Ms. Kathy Wolkan at Campus Microscopy and Imaging Facility at The Ohio State University for their helpful nature and technical know how in matters of imaging. Also, it was an honor to mentor and work with Mr. Andrew Galusha during my research.
I also want to extend my gratefulness to Dr. Thomas Abraham, Ms Leena Uki, Ms Tahera Zabuawala and my circle of friends who have never let me miss my family. I also thank Mr. Suvankar Sengupta and all my other team members for helping me to pursue my love for cricket. I am indebted to Mr. Rakesh Jain and Ms Neha Jain for knowledgeable discussions on teachings in Jainism.

Lastly, I thank my grandmother, Ms. Pushapaben Mehta; my father, Mr. Chandrakantbhai Mehta; and my brothers, Mr. Somil Mehta and Mr. Nirav Vora and the rest of my family members for their unconditional love and immense faith in me.
VITA

April 18, 1979                  Born, Bombay, India
June 2000                      B.E. Chemical Engineering, 
                              University of Mumbai, India
October, 2000 – June, 2001     Trainee Engineer, NemOrganics  
                              Ltd., Bombay
September, 2001 – Present     Graduate Research Associate, The 
                              Ohio State University

PUBLICATIONS

Research Publication

cytoskeletal and junctional proteins expressed by cells cultured from human arachnoid 

FIELD OF STUDY

Major Field: Chemical Engineering

Minor Field: Bioseparation, Cell Culture and Immunological techniques
# TABLE OF CONTENTS

Abstract......................................................................................................................... ii  
Dedication....................................................................................................................... v  
Acknowledgments.......................................................................................................... vi  
Vita................................................................................................................................. viii  
List of Tables.................................................................................................................... xiii  
List of Figure.................................................................................................................... xvi  

Chapters:

1. Introduction................................................................................................................ 1  
   1.1 Research Motivation.............................................................................................. 1  
   1.2 Overview of sample preparation methods for molecular diagnosis.................. 3  
   1.3 Research Objective............................................................................................ 5  
   1.4 Contribution and impact of research................................................................. 6  
   1.5 Dissertation organization................................................................................... 8

2. Literature Review...................................................................................................... 12  
   2.1 Epidemiology of breast cancer.......................................................................... 12  
   2.2 Cancer detection and molecular diagnosis....................................................... 13  
   2.3 Sample preparation techniques for molecular analysis of tissue..................... 14  
       2.3.1 Freeze thaw, tissue pulverization and reagent based methods............... 14  
       2.3.2 Microdissection......................................................................................... 14  
       2.3.3 Single cell suspension method................................................................. 16  
           2.3.3.1 Mechanical dissociation................................................................. 17  
           2.3.3.2 Enzyme digestion............................................................................. 18
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4 Problem definition</td>
<td>20</td>
</tr>
<tr>
<td>2.5 Research plan</td>
<td>22</td>
</tr>
<tr>
<td>2.5.1 Description of extracellular matrix</td>
<td>22</td>
</tr>
<tr>
<td>2.5.2 Types of collagenase, enzyme kinetics and measurement of kinetic parameter</td>
<td>24</td>
</tr>
<tr>
<td>2.5.3 Influence of reaction rate and diffusion rate on enzyme digestion</td>
<td>28</td>
</tr>
<tr>
<td>2.6 Culture of arachnoidal cells in 3D matrix to study of cerebrospinal fluid (CSF) outflow</td>
<td>33</td>
</tr>
<tr>
<td>2.6.1 Background of Cerebrospinal fluid</td>
<td>33</td>
</tr>
<tr>
<td>2.6.2 Circulation of CSF around brain</td>
<td>34</td>
</tr>
<tr>
<td>2.6.3 Structure of Arachnoid granulation</td>
<td>35</td>
</tr>
<tr>
<td>2.6.4 In vitro model of CSF outflow</td>
<td>38</td>
</tr>
<tr>
<td>3. A Simple Method of Characterizing The Kinetics Of Bacterial Collagenase Using Dye Quenched Fluorescence Substrate</td>
<td>55</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>55</td>
</tr>
<tr>
<td>3.2 Materials and methods</td>
<td>60</td>
</tr>
<tr>
<td>3.2.1 Materials</td>
<td>60</td>
</tr>
<tr>
<td>3.2.2 Methods</td>
<td>60</td>
</tr>
<tr>
<td>3.2.2.1 Preparation of standard curve</td>
<td>60</td>
</tr>
<tr>
<td>3.2.2.2 Preparation of Collagen and enzyme solutions</td>
<td>61</td>
</tr>
<tr>
<td>3.2.2.3 Time course measurements</td>
<td>61</td>
</tr>
<tr>
<td>3.2.2.4 Data analysis and comparison</td>
<td>62</td>
</tr>
<tr>
<td>3.3 Results</td>
<td>63</td>
</tr>
<tr>
<td>3.3.1 Preparation of standard curve</td>
<td>63</td>
</tr>
<tr>
<td>3.3.2 Measurement of increase in fluorescence as a measure of reaction rate</td>
<td>63</td>
</tr>
<tr>
<td>3.3.3 Determination of kinetic parameters</td>
<td>64</td>
</tr>
<tr>
<td>3.3.4 Reproducibility of assay and determination Michaelis Menten kinetic parameter for $\text{DQ}^{\text{TM}}$ gelatin from porcine skin and $\text{DQ}^{\text{TM}}$ human collagen type IV</td>
<td>65</td>
</tr>
<tr>
<td>3.4 Discussion</td>
<td>65</td>
</tr>
<tr>
<td>4. Optimization of Enzyme Dissociation Protocol Based on Reaction Diffusion Model to Predict Time of Tissue Digestion</td>
<td>80</td>
</tr>
<tr>
<td>4.1 Introduction</td>
<td>80</td>
</tr>
<tr>
<td>4.2 Theory</td>
<td>83</td>
</tr>
<tr>
<td>4.3 Materials and methods</td>
<td>87</td>
</tr>
<tr>
<td>4.3.1 Breast tissue procurement</td>
<td>87</td>
</tr>
<tr>
<td>4.3.2 Qualitative analysis of breast tissue for collagen content</td>
<td>87</td>
</tr>
<tr>
<td>4.3.3 Quantification of collagen in breast tissue</td>
<td>88</td>
</tr>
</tbody>
</table>
6.2.3.2 Brightfield microscopy and scanning electron microscopy…. 150
6.2.3.3 Confocal laser scanning microscopy…………………….. 151
6.2.3.4 Protein analysis with Western blot……………………… 152
6.2.4 Functional Outflow Assessment………………………………… 153
   6.2.4.1 Filter seeding for functional assessment………………….. 153
   6.2.4.2 Lucifer Yellow (LY) permeability assay………………… 153
   6.2.4.3 Determination of permeability coefficient………………. 154
   6.2.4.4 Experimental hydraulic conductivity………………….. 155
   6.2.4.5 Hydraulic conductivity calculation………………… 156
   6.2.4.6 Post perfusion analysis …………………………… 158
6.3 Results................................................................................. 158
   6.3.1 Cell culture..................................................................... 158
   6.3.2 Identification of Phenotype........................................... 159
   6.3.3 Growth kinetics and seeding efficiency in 3D culture……… 159
   6.3.4 Brightfield and scanning electron microscopy study……… 160
   6.3.5 Confocal laser scanning microscopy study………………… 161
   6.3.6 Confirmation of phenotype based on immunoblot study… 161
   6.3.7 Characterization of AG barrier function………………….. 162
      6.3.7.1 LY permeability.................................................... 162
      6.3.7.2 Perfusion study.................................................... 163
      6.3.7.3 Live/Dead analysis............................................. 164
6.4 Discussion......................................................................... 164

7. Conclusions and Recommendations......................................... 182
   7.1 Conclusions..................................................................... 182
   7.2 Recommendations.......................................................... 185
      7.2.1 Measurement of diffusivity of solute in human tissue…… 185
      7.2.2 Fluorescent labeling of collagenase…………………… 186
      7.2.3 Determination of collagen concentration with time…….. 186
      7.2.4 Shrinking core model.............................................. 187
      7.2.5 End point of tissue digestion and applicability to other systems… 187

References................................................................................ 189

Appendix A: Morphology of normal breast tissue......................... 204

Appendix B: Protocols.................................................................. 221
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Enzyme activities of crude collagenases from commercial source (Bond MD 1984)</td>
<td>41</td>
</tr>
<tr>
<td>2.2</td>
<td>Physicochemical properties of different collagenase classes (Bond MD 1984)</td>
<td>43</td>
</tr>
<tr>
<td>2.3</td>
<td>Enzyme activities of different collagenase classes (Bond MD 1984)</td>
<td>44</td>
</tr>
<tr>
<td>2.4</td>
<td>Kinetic parameter for hydrolysis of radioactive rat type I, bovine type II and human type III collagen using different classes of collagenases (Mallya SK 1992)</td>
<td>45</td>
</tr>
<tr>
<td>2.5</td>
<td>Diffusion and kinetic data of hydrolysis of immobilized FALGPA by collagenase on quartz surface (Gaspers 1994; Gaspers 1995)</td>
<td>46</td>
</tr>
<tr>
<td>2.6</td>
<td>Power law parameters for different systems to determine diffusivity (Jain 1987)</td>
<td>47</td>
</tr>
<tr>
<td>2.7</td>
<td>Composition of plasma and CSF (Kandel ER 2000)</td>
<td>48</td>
</tr>
<tr>
<td>3.1</td>
<td>Physicochemical properties of different collagenase classes {Bond, 1984 #169}</td>
<td>69</td>
</tr>
<tr>
<td>3.2</td>
<td>Enzyme activities of crude collagenases from commercial source {Bond, 1984 #169}</td>
<td>70</td>
</tr>
<tr>
<td>3.3</td>
<td>Enzyme activities of different collagenase classes (Bond MD 1984)</td>
<td>72</td>
</tr>
<tr>
<td>3.4</td>
<td>Concentration and dilution ratios of stock solutions to perform time course study at constant substrate concentration and constant enzyme</td>
<td>xiii</td>
</tr>
</tbody>
</table>
3.5 Michaelis – Menten kinetic parameters ($k_{cat}$ and $K_M$) for digestion of DQ™ gelatin from porcine skin, DQ™ type I collagen bovine skin and DQ™ human type IV collagen from placenta using Control (~500 U), Sigma (~350 U) and Roche (~250 U)

4.1 Sample data set for preparation of standard curve for collagen quantification in tissue

4.2 Average Michaelis Menten kinetic parameter ($k_{cat}$ and $K_M$) for digestion of DQ™ type I collagen bovine skin and DQ™ human type IV collagen from placenta using Control (~500 U), Sigma (~350 U) and Roche (~250 U) collagenases {Mehta, 2006 #298}

4.3 Average time required for complete digestion of breast tissue minced in different sizes and subjected to different initial enzyme concentration. The digestion was carried out at 37°C under constant agitation (n=8)

5.1 Mean cost of selected blood components per unit purchased by hospitals in 1999 {Sullivan, 2005 #348}

5.2 Physical properties of percoll {#9}

5.3 Volumetric proportions for the preparation of standard isotonic percoll (final diluted percoll = 35 ml) using percoll (in ml) and 10 X PBS (in ml) based on equation (5.1)

5.4 Volumetric proportions for the preparation of pH adjusted PBS 1 X (final diluted percoll = 35 ml) using PBS 1 X (in ml) and 0.1 N HCl (in ml)

5.5 Physical properties of diluted percoll prepared using SIP (Table 5.3) and PBS 1 X (Table 5.4). The density was determined based on equation (5.2)

5.6 Density distributions of different cell types determined using continuous density gradient medium. The mean cell densities of these cell types were compared with each other using Tukey Kramer test. The last column presents the density reported in literature for different cell types
5.7 Recovery of CHO cells at different pH levels. Based on Tukey Kramer test, it was determined that the recoveries at different pH levels were not significantly different ..................................................... 138

5.8 Recovery of CHO cells at different initial cell loading density. Based on Tukey Kramer test, it was determined that the recoveries at different initial cell loading density were significantly different ……. 138

5.9 Average density of CHO cells at different pH levels. Based on Tukey Kramer test, it was determined that the average density at different pH levels was not significantly different ...................... 138

5.10 Average density of CHO cells at different initial cell loading density. Based on Tukey Kramer test, it was determined that the recoveries at different initial cell loading density were not significantly different .. 138

6.1 Comparison of permeability coefficients of cells from culture plates and scaffolds ........................................................................ 170

6.2 Summary of perfusion study results for AG cells cultured on culture plates (2D) and on scaffolds (3D) ........................................ 170

A.1 Markers to distinguish between luminal epithelial and myoepithelial cells (Ronnov-Jessen L 1996) ............................................. 213

A.2 Markers to distinguish breast cellular components ..................... 215

A.3 Markers for specific cells that come under category leukocytes in table A.2 ................................................................. 216

B.1 Preparation of loading buffer......................................................... 226

B.2 Recommended proportion of stock solutions to prepare 0.1M phosphate buffer with desired pH.................................................. 232

B.3 Sample data set for preparation of standard curve for collagen quantification in tissue .............................................................. 233
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Brief descriptions of current protocols for molecular analysis of normal and malignant tissue samples</td>
<td>10</td>
</tr>
<tr>
<td>1.2</td>
<td>Diagram showing possible different methods to prepare genetic material from tissue for molecular analysis</td>
<td>11</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic presentation of the tissue structure. The interstitial space is made up of collagen and elastin shown as fibers here. The spaces enclosed by these fibers represent the hydrophilic gel made up of proteoglycan and glycoprotein. The cells and vasculature is also shown (Truskey GA, 2004 #101)</td>
<td>49</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic figure showing different steps in enzyme digestion process</td>
<td>50</td>
</tr>
<tr>
<td>2.3</td>
<td>Tissue sample treated as a semi-infinite slab with thickness = 2L. The enzyme concentration is C₀ in the bulk and Cₘ at the surface of the tissue</td>
<td>51</td>
</tr>
<tr>
<td>2.4</td>
<td>CSF outflow around brain. (a) CSF is produced in choroid plexus and is circulated around brain via subarachnoid space. CSF is finally absorbed in venous blood via arachnoid granulation (b). Arachnoid granulations are microscopic projection of subarachnoid space in venous sinus via dura mater (b). (c) Subarachnoid space is created due to the anatomical structure of pia and arachnoid mater. This space is made of arachnoid cells, fibroblasts and CSF surrounded by in loose connective tissue. (<a href="http://www.arts.uwaterloo.ca/~bfleming/psych261/image22.gif">http://www.arts.uwaterloo.ca/~bfleming/psych261/image22.gif</a>)</td>
<td>52</td>
</tr>
<tr>
<td>2.5</td>
<td>Schematic diagram of human arachnoid granulation showing four different regions. The cap cell cluster has been shown in</td>
<td>xvi</td>
</tr>
</tbody>
</table>
green color here (Kida 1988) ................................................. 53

2.6 Schematic view of arachnoid granulation showing open channel structure. Abbreviations: e = sinus endothelium; SAS = subarachnoid space and SDS = subdural space (Upton 1985) ................................................. 54

3.1 Standard plot of Fluorescence unit vs. concentration of fluorescein in nmoles/ml. The stock solution of fluorescein was prepared in reaction buffer and diluted in two fold serial dilution. The working volume per reaction well was 200μL and the fluorescence was measured at 37°C using cytofluor® 4000 fluorescence multi-well plate reader at excitation of 485 nm and emission at 530 nm. The standard curve data can be fitted with $y = 55612.78(x)/(16.01 + x)$ ................................................. 75

3.2 Plot of concentration of fluorescein (nmoles of fluorescein/ml) vs. time (minutes) show increase in fluorescence due to fluorescein on degradation of substrate at constant substrate concentration. The substrate was DQ™ bovine collagen type I was digested using control (~ 500 U) collagenase provided by Molecular Probes Inc. (Eugene, OR). The reaction was carried out at 7.5 pH and 37°C ................................................. 76

3.3 Plot of concentration of fluorescein (nmoles of fluorescein/ml) vs. time (minutes) show increase in fluorescence due to fluorescein on degradation of substrate at constant enzyme concentration of 0.05 U/ml. The substrate was DQ™ bovine collagen type I was digested using control (~ 500 U) collagenase provided by Molecular Probes Inc. (Eugene, OR). The reaction was carried out at 7.5 pH and 37°C ................................................. 77

3.4 Plot of rate of reaction (v, nmoles/(ml.min)) vs. enzyme concentration ([E₀], U/ml) at constant substrate concentration of 100 µg/ml. The rate of reaction is obtained by applying initial rate method on the data utilized to plot figure 3.2. Here the substrate is DQ™ bovine type I collagen and the enzyme is control (~ 500 U) collagenase purchased from Molecular Probes Inc. The reaction was carried at pH 7.5 and temperature 37°C. This plot can be fitted using $v = 0.0069[E₀] - 3.92 \times 10^{-5}$ as shown in the figure ................................................. 78

3.5 Plot of rate of reaction (v, nmoles/(ml.min)) vs. substrate concentration ([S], µg/ml) at constant enzyme concentration of 0.05 U/ml. The rate of reaction is obtained by applying initial rate method on the data utilized to plot figure 3.3. Here the
substrate is DQ™ bovine type I collagen and the enzyme is control (~ 500 U) collagenase purchased from Molecular Probes Inc. The reaction was carried at pH 7.5 and temperature 37°C. This plot can be fitted using \( v = \frac{0.0004[S]}{(4.58 + [S])} \) as shown in the figure ……………………………………………………

4.1 Schematic diagram showing thin semi–infinite tissue slab in y–direction. The tissue slab is subjected to enzyme solution maintained at constant concentration (\( C_0 \)). For digestion the enzyme has to penetrate in the bulk of tissue slab (positive x–direction) by diffusion as there is no convection in the tissue slab. This process can be modeled by applying equation of conservation of mass in cartesian co-ordinate system ………

4.2 Masson trichrome staining of invasive ductal carcinoma (breast cancer) representing the distribution of collagen fibers (five point star) and nuclei (arrow). The blue fibers represent collagen fiber (A – D) and the black dots represent the nuclei (B – D). (A) represent high concentration of collagen in tissue section at 100 X. (B, C and D) represent distribution of cells and collagen at 100 X, 200 X and 400 X respectively ………

4.3 Verhoeff’s staining of invasive ductal carcinoma (breast cancer) representing the distribution of elastin fiber (four point star), collagen fibers (five point star) and nuclei (arrow). The black fibers represent the elastin (C, D), the pink fibers represent the collagen (A – D) and bluish purple dots represent nuclei (A, B). (A and B) represent high concentration of collagen in tissue section at 100 X and 200 X respectively. (C and D) represent very less concentration of elastin compared to collagen in invasive ductal carcinoma at 200 X, 100 X respectively ……………………………………………………

4.4 Standard curve of absorbance @ 557 nm vs. µg of hydroxyproline measured using Woessner’s protocol. (Absorbance = 0.0453×[hydroxyproline] + 0.0190) …………

4.5 Standard curve of absorbance @ 557 nm vs. µg of bovine type I collagen measured using Woessner’s protocol. (Absorbance = 0.0061× [collagen] + 0.0133) ………………………………….

4.6 Quantification of collagen in 17 breast tissue samples. The collagen was converted to hydroxyproline, which was further consumed to produce a chromophore detected at 557 nm. Based on 17 samples the concentration of collagen in breast …
tissue was 53 ± 37 µg of collagen per mg of wet tissue. Collagen concentration was determined based on standard plots represented in figure 4.4 and 4.5. The open circle, filled circle and filled triangles represent different runs performed on same tissue sample …………………………………………………………….. 109

4.7 Plot of rate of reaction (v, nmoles/(ml.min)) vs. enzyme concentration ([E₀], U/ml) at constant substrate concentration of 100 µg/ml. Here the substrate is DQ™ bovine type I collagen and the enzyme is control (~ 500 U) collagenase purchased from Molecular Probes Inc. The reaction was carried at pH 7.5 and temperature 37°C. This plot can be fitted using v = 0.0069[E₀] – 3.92 × 10⁻⁵ as shown in the figure ……………. 110

4.8 Plot of rate of reaction (v, nmoles/(ml.min)) vs. substrate concentration ([S], µg/ml) at constant enzyme concentration of 0.05 U/ml. Here the substrate is DQ™ bovine type I collagen and the enzyme is control (~ 500 U) collagenase purchased from Molecular Probes Inc. The reaction was carried at pH 7.5 and temperature 37°C. This plot can be fitted using v = 0.0004[S] / (4.58 + [S]) as shown in the figure ………………… 111

4.9 Time of collagen digestion determined based on kinetic parameters (Table 4.2) for bovine type I collagen at different initial enzyme concentration. (a) represent the digestion time when initial collagen concentration is 53 µg, (b) represent the digestion time when initial collagen concentration is 530 µg. In both figures filled dots represent the time for 25% conversion, open dots represent the time for 50% conversion, filled inverted triangles represent the time for 75% conversion and open triangle represent the time for 87.5% conversion ……………… 112

4.10 Variation in spatial distribution (x – axis) of enzyme concentration with respect to time in a semi – infinite tissue slab at different effective diffusivities (a = D_eff = 0.1 × 10⁻⁸ cm²/s, b = D_eff = 1 × 10⁻⁸ cm²/s and c = D_eff = 10 × 10⁻⁸ cm²/s) determined based on reaction diffusion model. The concentration of collagen, rate constant and Michaelis – Menten constant are determined based on analysis performed here ………………………………………………………………………………….. 113

4.11 Time required for complete digestion of breast tissue pieces of the size (0.69 mm and 5.38 mm) subjected to collagenase concentration of 2 mg/ml and 10 mg/ml respectively. It can be observed that the size effects (diffusion effects) are much more
pronounced than the enzyme concentration effects .................. 115

4.12 Brightfield microscopy images taken during the breast tissue digestion. Here a piece of breast tissue was subjected to 2 mg/ml collagenase concentration and 25 µg/ml of DNase concentration in medium at 37°C. The enzyme penetrates in breast tissue in similar manner as predicted by shrinking core model for coal combustion ............................................. 116

5.1 Density distribution of different cell types determined using continuous percoll density gradient medium and density marker beads. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the means of density distribution. The quantitative results are presented in table 5.6 ................................................................. 139

5.2 Recovery of CHO cells at different pH levels. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the means recoveries. Based on Tukey Kramer HSD test, it can be concluded that the recoveries are not significantly different. The quantitative results are presented in table 5.7 ................................................................. 140

5.3 Recovery of CHO cells at different initial cell loading density. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the means recoveries. Based on Tukey Kramer HSD test, it can be concluded that the recoveries are significantly different. The quantitative results are presented in table 5.8 ................................................................. 141

5.4 Average density of CHO cells at different pH levels. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the means cell density. Based on Tukey Kramer HSD test, it can be concluded that the recoveries are not significantly different. The quantitative results are presented in table 5.9 ................................................................. 142

5.5 Average density of CHO cells at different initial cell loading density. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the
means recoveries. Based on Tukey Kramer HSD test, it can be concluded that the cell density are not significantly different. The quantitative results are presented in table 5.10 .......... 143

6.1 Cerebrospinal fluid is produced by the choroid plexus in the lateral, 3rd, and 4th ventricles where it circulates to the subarachnoid space and eventually returns to the venous blood via the arachnoid granulations (Perkin et al., 1986) (A). AGs are projections of the arachnoid membrane into the dural venous sinuses and lateral lacunae (Warwick and Williams, 1973) (B) ......................................................... 171

6.2 Schematic diagram of a human arachnoid granulation showing four different regions. The arachnoid membrane and cap cell cluster are shown in green color here (Kida, 1988) ............... 172

6.3 AG cells were perfused in a modified Ussing chamber. Cells were seeded onto culture inserts and perfused in the physiological (Basal→Apical) direction (see inset). A hydrostatic pressure head was applied by adjusting the height of the fluid reservoir relative to the cell layer. An in-line strain gauge pressure transducer interfaced with a data acquisition computer and allowed for real-time monitoring of the pressure drop across the cell layer. Volumetric flow rate was measured by collecting the perfusate from a downstream sampling port ... 173

6.4 Human arachnoid granulations were collected at autopsy within 24 hours post-mortem (A). Individual AGs were excised with micro-surgical scissors and explanted to fibronectin coated culture dishes. Cell growth from the explant was seen within 7-10 days (B). AG cells in culture show polygonal cell morphology and when confluent, pack in densely to form a cobblestone-like appearance typical of epithelial cells in culture (C) ................................................................. 174

6.5 Immunocytochemical methods were used to identify arachnoidal cell phenotype. AG cells were grown on fibronectin coated coverslips and labeled with antibodies to broad-spectrum cytokeratins (A). AG cells were also labeled with antibodies to the desmosomal plaque protein desmoplakin 1&2 (B) and the tight junction protein ZO-1 (C). Positive expression Desmoplkin and ZO – 1 of these proteins was seen at cell-cell borders. Primary antibody expression was visualized by labeling the cells with a secondary antibody conjugated to a fluorescent molecule. Secondary antibodies
were either anti-IgG FITC (green) or anti-IgG Alexa Fluor 555 (red) .................................................................................. 175

(A, B) Brightfield microscopy images of cells cultured on scaffolds. The cells were seeded by agitated seeding and the images were taken after 5 days in culture at 100X magnification (A) and 320X magnification (B). The cells form tissue-like structure and the cells span across fibers. This was confirmed by scanning electron microscopy (C, D). SEM images were taken after 11 days in culture. The cells form multiple clusters of tissue-like structures (C). On close observation the cells are tightly attached to each other confirming epithelial-like morphology (D) ..................... 176

6.7 Confocal microscopy images of arachnoidal cells cultured on PET scaffold. The cells expressed vimentin (orange) intermediate filaments (A). The epithelial behavior was confirmed by expression of ZO 1 (green) ((B) at 400 X magnification and in (C) at 600 X magnification) and epithelial specific cadherin (E-cadherin) (green) membrane protein (D). The cells were also stained for nucleus (blue, A-D) and actin (orange, B-D) filaments. The bar represents 20 µm ............ 177

6.8 Western blot profiles of two-dimensional cells from two different tissue batches compared with three-dimensional culture for expression of ZO-1 (A) and ZO-2 (B). The cells cultured on PET matrix showed similar expression of zona occludens as observed in two-dimensional culture ............ 178

6.9 Plot of permeability coefficient of cells (in cm/sec) vs. duration of culture (in days). The cells were cultured in culture inserts and incubated with 100µM LY solution on the apical side of the insert. The permeability of LY across cell layer was measured after two hours by taking samples from the basal side. The permeability coefficient initially decreases with increase in duration of culture. The minimum values were observed around 21 – 23 days. Here open squares and filled squares represent the permeability coefficients of cells from culture plate and scaffolds respectively. There was no statistically significant difference between the permeability coefficients of cells from culture plates and scaffolds (Table 6.1) ................................................................. 179

6.10 Plot of average cellular hydraulic conductivity vs. average pressure for cells cultured on culture plate (open squares) and
on scaffolds (filled triangles). It can be observed that the average cellular hydraulic conductivity for both the cases are not significantly different (Table 6.2), thus confirming the results of permeability coefficient in LY permeability assay 

6.11 Calcein AM (red) and Ethidium homodimer -1 (green) staining of cells cultured on filter inserts from 3D scaffolds (A) and 2D culture plates (B). These cells were perfused with serum free media under physiological pressure. It was observed that few cells had compromised cell membrane (red nuclei) compared to the majority of viable cells showing diffused esterase activity (diffused green cytoplasm). These results confirmed that perfusion study did not cause significant damage or death of the cells 

A.1 Human breast is primarily composed of fat pad, connective tissue and breast tissue. The breast tissue consists of lobules and ducts embedded in fat pad and connective tissue as shown above. The lobules are milk-producing glands, and ducts carry this milk to the nipples during lactation. Breast also has other vascular and nervous components. The preliminary sites of breast cancer are ducts or nodules and it generally metastasizes via auxiliary lymph nodes {Wymelenberg, 2000 #38} 

A.2 Normal TDLU. Small duct (D) give rise to terminal D and a single lobule (L). Terminal D is composed of ETD and ITD, which are in continuity. ITD gives rise to blindly ending DTL that are often branched. ITD and DTL are covered with intralobular connective tissue (stripped) (Wellings SR 1975) 

A.3 Lobular structure of mammary gland along with microvasculature. Artery, veins, arterioles and venules are shown here. Intra- and inter-lobular stroma is not shown here 

A.4 Luminal and myoepithelial cells form the duct and lobule (A). It is believed that ductal cancer cells are progeny of one mutated luminal epithelial cell. When the cells are enclosed in basement membrane (B) then it is called as ductal carcinoma in situ (DCIS). This cancer is detected as small calcification in mammogram. In advance stages the cancer cells breach the basement membrane (C) and spread to other parts of breast and other parts of body via blood vessel and lymphatic system. This process is called metastasis and marks advance stage of cancer (Wymelenberg, 2000)
B.1 Schematic drawing of electroblotting cassette assembly for protein transfer to nitrocellulose membrane for probing 234

B.2 Standard curve of absorbance @ 557 nm vs. µg of hydroxyproline measured using Woessner’s protocol. (Absorbance = 0.0453× [hydroxyproline] + 0.0190) 235

B.3 Standard curve of absorbance @ 557 nm vs. µg of collagen measured using Woessner’s protocol. (Absorbance = 0.0061× [collagen] + 0.0133) 236
CHAPTER 1

INTRODUCTION

1.1 Research motivation

With the advent and improvement of highly sensitive and accurate molecular analysis technologies, it is possible to study increasingly complex cellular processes including cell differentiation and proliferation based on molecular markers from normal and malignant tissue source (St Croix et al., 2000). These technologies have played a significant role in diagnosis of cancer. However, a common question/complaint of studies using these technologies is the homogeneity of the original sample (Sieben NL, 2000; Tomlinson IP, 2002). The lack of it can affect the desired final outcome dramatically. For example, ovarian carcinoma samples from patient or cell samples taken from cell culture (HOC-1 or MCF-7) when analyzed in a microarray, express variety of RNA, and RNA levels suggesting various stages of the cell cycle, which may or may not be related to the true process studied (Mackillop et al., 1982; Podhajcer et al., 1986; Yang et al., 1977). Consequently, the overall expression pattern might not represent any one single cell, but a “mean” of the entire population (Ko et al., 2000). The lack of homogeneity is even more of a problem when one works with non-cultured, three-dimensional tissue, which normally consists of a variety of different cell types, and in different states. Therefore, the
ability to analyze and separate a heterogeneous cell population based on cellular characteristics is a significant analytical and preparative resource for molecular analysis techniques (Pantel et al., 1999).

The sample preparation techniques in case of a tissue sample are rudimentary and opportunities are available to improve these techniques. Currently, the majority of the samples for cancer diagnosis are prepared either by pulverizing whole frozen tissues (normal or malignant) or by preparing single cell suspension by liquefaction of tissue using enzyme dissociation or mechanical dissociation or by laser capture microdissection. These samples are then analyzed using techniques like immunohistochemistry, RT-PCR or PCR (Ghossein and Bhattacharya, 2000; Pantel et al., 1999; Wellings et al., 1975) without regard to its components.

A tumor sample consists of several different types of cells and connective tissue components (Alberts B, 2002). The cellular components such as fibroblast, epithelial cells, endothelial cells and so on are bonded tightly to each other by extracellular material (ECM) that consists of collagen, elastin, proteoglycans and glycoproteins (Alberts B, 2002; Hay, 1991). The proteins (collagen and elastin) are the main components of ECM and can be broken down using enzymes such as collagenase, elastase, pronase, dispase, trypsin (metalloproteinases) etc. (Allalunis-Turner MJ, 1986; Costa A, 1987; Curry MP, 2000; Eade OE, 1981; Engelholm SA, 1985; Hamburger et al., 1982; Haraldsen G, 1995; Keng PC, 1998; Lee TK, 1993; Penning JJ, 1981; Queral AE, 1984; Singh, 1998; Visscher DW, 1994; Wood GW, 1978; Zavros Y, 2000). The enzyme dissociation process is an integral part of life sciences laboratory and is designed predominantly based on empirical experience of each research group. There is little or no fundamental
understanding available about the enzyme dissociation process to produce single cell suspension from a tissue. For the analysis purposes, the solid tumor samples are liquefied using enzyme dissociation and further separated in different cells populations based on molecular marker (Hamburger et al., 1982). Significant cell sorting techniques are currently developed based on engineering principles outside the scope of pathology and molecular analysis of human tissue samples. These techniques separate/fractionate cells into fractions based on expression level of molecular markers (Shapiro, 2003).

The impact of highly sensitive molecular analysis techniques in cancer diagnosis will be only realized when the sample preparation techniques can be improved and are predictable based on first principles and experience. We believe that enzyme dissociation technique can be predicted and improved by applying fundamentals of enzyme kinetics of ECM degradation and diffusion of enzyme molecule through ECM thus improving single cell suspension preparation from a tissue. It is also important to introduce the concepts of cell recovery and purity while performing sample preparation for molecular analysis of tissue sample.

1.2 Overview of sample preparation methods for molecular diagnosis

Currently, the great majority of samples for molecular analyses are prepared by pulverizing whole frozen tissue (normal or malignant), without regard to its cellular components, for extraction of nucleic acids. Frozen samples yield higher quality of DNA and RNA and are technically easier to work with. But no attention is paid to separate the heterogeneous cell populations. When archived paraffin-embedded tissue is used, more
often than not, the entire section is scraped off for extraction of nucleic acids without regard to the cellular components of the tissue.

Laser capture microdissection (LCM) was invented to selectively microdissect single cells or groups of cells to overcome challenge associated with heterogeneity of cells in a tissue. This system utilizes a transparent thermoplastic film applied on the surface of the thick tissue section (10 microns instead of the 3-5 microns used in diagnostic pathology) on standard histopathology slides. The target cells to be microdissected are identified and targeted through a microscope, and a narrow (~ 15 µM) carbon dioxide laser-beam pulse is used to melt the film around these cells. The resulting strong focal adhesion allows selective procurement of only the targeted cells (Emmert-Buck MR, 1996). This technique clearly relies on direct visual observation and histomorphology to distinguish target from non-target cells and is labor intensive. It is straightforward enough to distinguish the surrounding stromal cells from epithelial cells in carcinoma in situ (refer Appendix A for details) but it is not so easy to do especially for invasive carcinoma (refer Appendix A for details) with stained thick sections. Figure 1.1 summarizes the typically used techniques to obtain human tumor samples for molecular analysis.

Significant sorting techniques are currently developed independently outside the scope of pathology and molecular analysis of human tissue samples. These techniques separate/fractionate cells into subfractions based on physical properties of cells and/or molecular marker expression levels (e.g. protein expression level). However, these techniques can be only applied to cell suspensions like blood (Martin et al., 1998; Shapiro, 2003; Sun et al., 1998). Therefore, in the case of a solid tissue, the cells from the
tissue sample have to be separated from the ECM without affecting molecular profile to prepare a single cell suspension. This is routinely done by catabolically degrading the ECM protein with enzymes or mechanically dissociating the ECM proteins. But there is no fundamental understanding applied to this process and all the protocols depend on empirical knowledge (Allalunis-Turner MJ, 1986; Costa A, 1987; Curry MP, 2000; Eade OE, 1981; Engelholm SA, 1985; Hamburger et al., 1982; Haraldsen G, 1995; Keng PC, 1998; Lee TK, 1993; Penning JJ, 1981; Queral AE, 1984; Singh, 1998; Visscher DW, 1994; Wood GW, 1978; Zavros Y, 2000).

The single cell suspension prepared in above fashion is then sorted in different fractions based on size, density or expression of specific markers. Size and density based separation provide crude separation as there is overlap in size and density among different cell types. Partially purified cells could be then sorted into different fractions based on the different immunologic properties of different cell types (like Epithelial, Stromal, Endothelial, etc.; refer table A.1, A.2 and A.3 in appendix A for more details) contained within the tissue sample. Figure 1.2 summarizes all the methods used to obtain a single cell suspension for analysis.

1.3 Research objective

The overall objective of this study is to develop the fundamental understanding of enzyme dissociation of tissue to improve the single cell suspension preparation technique. To achieve this goal following particular objectives will be fulfilled:

a) To develop a substantial understanding of tissue architecture in terms of cellular composition and ECM composition (Appendix A).
b) To set up basis for understanding enzyme dissociation process based on principles of enzyme kinetics and diffusion of molecules through a porous tissue media. The physicochemical properties of tissue will be at a center stage while developing an approach for the problem.

c) To set up a protocol for quantifying the collagen concentration of normal as well as malignant tissue samples.

d) To determine the kinetic parameters for collagen digestion using *Clostridium histolyticum* collagenase samples.

e) Model the process based on reaction diffusion model.

f) Confirm model prediction based on experiments.

Fulfilling above milestones will make it possible to predict the behavior of enzyme dissociation technique for preparation of single cells suspension.

1.4 Contribution and impact of research

Cancer is second leading cause of death in U.S. and breast cancer is the leading cause of death in women. Currently, the most important methods to diagnose and determine modality for cancer treatment are based on pathologic examination of solid tumor and confirmation by molecular analysis. In last two decades, significant methods are developed and improved for molecular characterization of cancer. The knowledge of cancer was never more than what we have now and it is possible to learn more about cancer if we can make better use of molecular detection techniques available. One of the bottlenecks to achieve this goal is lack of good sample preparation techniques. National Cancer Institute has recognized this challenge and has addressed it by issuing a RFA-CA-
This study focuses on developing fundamental understanding of enzyme dissociation of tissue for preparation of single cell suspension, which is one of the most commonly used techniques in life sciences laboratories for sample preparation.

Currently, for the purpose of genetic analyses, the samples are prepared by freeze thaw method, tissue pulverization method or by microdissection based on the morphology of sample under microscope. This method yield less amount of genetic material (Chacko, 2005) and there are higher possibilities of false negative results (Ko et al., 2000). Above all these drawbacks, these techniques are developed in ad hoc fashion depending on requirement of a researcher’s lab and no standardization of technique is performed. Based on these facts the main contribution of this study will be standardization of enzyme dissociation technique for single cell suspension preparation from tissue, which will lead to improvement in yield of genetic material from a tissue sample, thus, better utilizing the valuable human tissue material.

The freeze thaw method and tissue pulverization method completely disregards the heterogeneity of tissue sample while recovering genetic material. LCM does address this issue but the separation of cells is achieved based on morphological observations. This methodology requires skillful pathologist and is a labor intensive process. It does not utilize the available molecular marker information of cancer cells for sample preparation and involves observer’s bias in preparing the samples. The successful application of this study will make it possible to implement immunologic separation of desired cell populations using immunomagnetic separation or flow cytometry, thus avoiding observer’s bias and utilizing the available molecular information of cancer cells.
Finally, this study will achieve technological advancement by implementing engineering principles on a life science protocol. This will provide a common basis to compare methodology from different laboratories and will provide reasons to justify a particular decision. This development will also make it possible to understand the fundamental variables in enzyme dissociation process and therefore the same methodology can be applied for various applications. Few of the common applications envisioned are improvement in genetic analyses of other cancers like head and neck squamous carcinoma (Pereira, 2005; Quon et al., 2001), better yield of primary cells for cell line development (Freshney and Freshney, 2002; Sacks, 1996) and improvement in recovery of Islet of Langerhans for islet transplantation and islet culture as a cure of diabetes (Alberts B, 2002; London et al., 1998; Murdoch et al., 2004; Ricordi et al., 1988).

1.5 Dissertation organization

Chapter 2 presents an overview of study set up and literature research to support the assumptions made for the set up. Here different techniques and protocol development are also discussed and their importance in the project is emphasized. This chapter provides a basis for rest of the chapters to follow. Here, we have also, briefly discussed the motivation for 3 D culture of arachnoid cells from arachnoid granulation as an *in vitro* model to study outflow pathway of cerebrospinal fluid (CSF) and motivation for improvement of percoll density gradient medium for better fractionation of cells based on density.
Determination of kinetic parameter for degradation of collagen based on Michaelis Menten kinetics is discussed in chapter 3. First, the need to set up a novel fluorescence based approach for kinetics is discussed and then the results of the new approach are discussed in light of old data.

Chapter 4 discusses the quantification of collagen concentration in normal as well as malignant tissue samples. Also, the basic equation based on reaction diffusion model is set up here and solved based on kinetic parameters from chapter 3 and diffusion data published in literature.

Chapter 5 reiterates the importance of accurate and reproducible density gradient separation method. Percoll is a commonly used density gradient medium, which is optimized to determine the effect of pH and initial cell loading on density and recovery of primary cells and immortalized cell line.

The 3 D primary culture of arachnoid cell culture from arachnoid granulation is discussed in chapter 6. Here the results on successful 3 D culture of cells are presented along with biochemical characterization of these cells.

Chapter 7 includes the recommendations and directions for future work. This will include implementation of enzyme digestion technique to other systems.

Appendix A briefly introduces the morphology of normal and malignant breast and provides details knowledge of cellular and ECM components found in breast. This information is relevant for all the chapters except chapter 5 and 6. Appendix B discusses important protocols utilized for performing this research.
Figure 1.1 Brief descriptions of current protocols for molecular analysis of normal and malignant tissue samples.
Figure 1.2 Diagram showing possible different methods to prepare genetic material from tissue for molecular analysis.
CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of breast cancer

Breast cancers are a major health burden worldwide. It is the principal cause of death in women. It is estimated that one million out of 10 million cases of neoplasm detected in both sexes are breast cancer. In 2001, 375,000 women died because of the breast cancer. Breast cancer is the second most common tumor after lung cancer in both sexes (Bray F, 2004). USA is one of the high-risk areas of breast cancer. As per the GLOBOCAN 2000 survey 184,000 new cases of breast cancer were detected in US in year 2000. In the same year 45,500 women died because of the breast cancer in US (Bray F, 2001).

Based on the epidemiologic studies the risk factors can be attributed to the dietary fat consumption and family history. It is difficult to control these two variables, thus in turn difficult to control the high incidence rate of breast cancer. It is observed that in last decade the incidence rate of breast cancer is steadily increasing, but the mortality rate is remaining constant. This can be attributed to early detection of breast cancer. Mammographic screening is helping in detecting the breast carcinoma early and is the
principal factor that contribute to reduced mortality due to breast cancer (Tannock IF, 1998) (For more information on breast tissue architecture refer Appendix A).

2.2 Cancer detection and molecular diagnosis

Cancer diagnosis can be divided in two parts. First, the primary detection of cancer is routinely performed with non invasive techniques like mammography, ultrasound detection or magnetic resonance imaging (MRI). If there are some signs of malignant growth then the second part of diagnosis is performed. Here a small biopsy is performed on the patient and the sample is analyzed by performing routine pathology, immunohistochemistry (IHC) and genetic analysis. The final diagnosis is performed based on a complementary report of non invasive and invasive diagnosis.

Pathology, IHC and genetic analysis play an important role in staging the cancer, which is necessary for finalizing the therapy for the patient. Pathology and IHC are highly mature techniques providing qualitative results for the cancer diagnosis. During last two decades significant improvement has been made in genetic analysis due to improvement and invention of new techniques (Ghossein and Bhattacharya, 2000; Pantel et al., 1999). The success of highly quantitative genetic analysis relies on sample preparation techniques. Unfortunately, the sample preparation techniques have not improved at the same pace as genetic analysis techniques. Moreover, there is clear lack of standard sample preparation technique, which will enable comparison of results from one laboratory to other laboratory.
2.3 Sample preparation techniques for molecular analysis of tissue

Genetic material from tissue is routinely recovered by freeze thaw method, reagent based method (Chomczynski and Sacchi, 1987; Latham, 2005), tissue pulverization method, microdissection (Rekhter and Chen, 2001) and single cell suspension method.

2.3.1 Freeze thaw, tissue pulverization and reagent based methods

In freeze thaw method, the tissue is frozen and thawed in quick succession to disrupt the cellular and ECM architecture, thus recovering the genetic material (refer figure 1.1). Tissue pulverization method also employs similar principle in which the tissue is frozen and pulverized using pestle. The pulverized tissue is then subjected to genetic material extraction buffer to recover the genetic material (refer figure 1.1). The reagent based approach utilizes different enzymatic reagents (Latham, 2005) or organic extraction reagents (Chomczynski and Sacchi, 1987) to extract the genetic material. These methods can be performed in very time efficient manner but they disregard the heterogeneity of tissue sample. Therefore the genetic material obtained by such methods is significantly adulterated with genetic material from surrounding normal tissue.

2.3.2 Microdissection

To overcome this challenge, laser capture microscopy (LCM) is developed by national institute of health (NIH). It is a widely used technique for recovery of normal and neoplastic cells for extraction of genetic material for analysis (Maitra et al., 2001). This is a microscopy-based technique in which cells are directly isolated from the tissue
by use of a laser beam and a polymer membrane. The samples are placed on a slide and covered with a special polymer (100 µm thick ethyl vinyl acetate (EVA)) that absorbs the energy of laser in the near infrared region. This entire setup is then placed under a microscope, where the desired cells are located under microscope and recovered with focused laser beam (carbon dioxide laser) that melts the polymer around the required cell, therefore forming a tight binding with cells. The cells are removed with polymer when it is removed from the slide. In this fashion 3000-5000 cells can be isolated per slide, which will be then treated for extraction of desired genetic material.

In LCM, cells are recovered based on their appearance under microscope. Therefore it is necessary to process the sample before LCM. Based on sample preparation methods, there are two types of LCM: routine and immuno LCM. In routine LCM the samples are sectioned from either fixed and paraffin embedded tissue or frozen tissue, which is then stained with standard histological dyes to improve morphology of the cells. In immuno LCM the samples are stained using IHC protocols to improve detection of the cells (refer figure 1.2).

The cells stained in this fashion are isolated using infrared laser in case of LCM. There are other available techniques like ‘selective ultraviolet radiation fractionation (SURF), selective laser-ablation, laser microbeam microdissection coupled with laser pressure catapulting (LMM/LPC)’ that make use of UV laser beam for the separation of the cells. The basic principle of the working of these equipments is more or less similar to LCM (Bonner et al., 1997; Emmert-Buck MR, 1996; Fend and Raffeld, 2000; Maitra et al., 2001; Rekhter and Chen, 2001; Simone et al., 1998).
LCM requires knowledge of pathology of tissue and skillful labor to achieve consistent and reproducible results. The laser beam system is accurate, thus it is possible to make use of the same sample for further isolation of the different types of cell and can yield up to 95% cell purity if performed properly (Emmert-Buck et al., 2000; Emmert-Buck et al., 2000; Leethanakul et al., 2000; Ornstein et al., 2000). These cells are directly separated on a polymer film and are readily accessible for genetic material extraction for further analysis. The genetic material separated by this technique is of good quality and can be used as control for comparing the result from other techniques.

However there are some disadvantages associated with this technique. Due to significant preparatory steps, sensitive molecules like mRNA will undergo significant degradation. Also, the quality of results depends on sample preparation technique and observer’s bias. LCM yields low number of cells compared to enzymatic or mechanical dissociation methods and requires amplification techniques like RT-PCR or PCR. The operating cost of LCM is significant. Some of these disadvantages like observer’s bias can be compensated with the immuno-LCM technique and automatic LCM. Overall LCM is a useful tool and can provide a good standard or control for comparison of results with enzyme dissociation technique.

2.3.3 Single cell suspension method

Significant sorting techniques are currently developed independently outside the scope of pathology and molecular analysis of human tissue samples. These techniques separate/fractionate cells into sub-fractions based on molecular marker expression levels. However, these techniques can be only applied to cell suspensions like blood (Martin et
al., 1998; Shapiro, 2003; Sun et al., 1998). Therefore, in the case of a solid tissue, the cells in that tissue sample need to be separated from the ECM without affecting molecular profile to prepare what is called a single cell suspension. Ideally, these cells could be then sorted into different fractions based on the different immunologic properties shown by different types of cells (like Epithelial, Stromal, Endothelial, etc.) contained within the tissue sample. There are two routinely used methods for preparation of single cell suspension.

2.3.3.1 Mechanical dissociation

This is the simplest and quickest of all the techniques available for tumor dissociation. In this technique the tissue in question is minced mechanically using different methods like scrapping, mechanical mincing and grating of tissue. Mechanical dissociation methods can be carried out rapidly. They do not require any preparatory steps and can be applied directly to fresh samples as well as frozen samples. Although mechanical dissociation yields higher amount of cells compared to any other method cell viability and cloning efficiency are lower than cells obtained by enzyme dissociated tissue. This method also does not give due importance to the heterogeneity of tissue sample. Thus the genetic material is adulterated substantially, which might make further molecular analysis difficult. This method might not work very efficiently in fat rich and collagen rich tissues like breast tissue and alternative strategy might become necessary (Curry MP, 2000; Eade OE, 1981; Engelholm SA, 1985; Hamburger et al., 1982; Lee TK, 1993; Singh, 1998; Van Dam PA, 1992; Visscher DW, 1994).
2.3.3.2 Enzyme digestion

Enzyme digestion is the other method for preparation of single cell suspension from tissue. As stated in appendix A, a tissue sample consists of several different types of cells and connective tissue components (Alberts B 2002). Cells such as fibroblast, epithelial cells, endothelial cells and so on are bonded tightly to each other by extracellular material (ECM) that consists of collagen, elastin, proteoglycan and glycoproteins (Hay 1991; Alberts B 2002). The proteins (collagen and elastin) are the main component of ECM and can be broken down using catabolic enzymes such as collagenase, elastase, pronase, dispase, trypsin (metalloproteinases) etc. (Wood GW 1978; Eade OE 1981; Penning JJ 1981; Hamburger AW 1982; Queral AE 1984; Engelholm SA 1985; Allalunis-Turner MJ 1986; Costa A 1987; Lee TK 1993; Visscher DW 1994; Haraldsen G 1995; Keng PC 1998; Singh 1998; Curry MP 2000; Zavros Y 2000). Typically, the sample of interest is minced into small pieces and then exposed to one or a mixture of enzymes. This solution is constantly stirred using bacterial shaker or magnetic mixer. The mincing increases the mass transfer rate for achieving faster dissociation of the tissue. The time taken for dissociation is highly variable and lacks a standard based on tissue parameters.

According to Hamburger et al (1982) enzymatic dissociation yields a lower number of cells compared to mechanical dissociation. However in 93 % of cases the cell viability and cloning efficiency (ability to grow in vitro) is found to be higher in enzymatic dissociation. Also, the histology of cells observed before and after dissociation and cells obtained by different methods do not differ significantly. Enzymatic dissociation theoretically does not change the functional activity of the cells. However,
some authors are skeptical about the specificity of different enzymes and they do comment that “enzymatic disassociation techniques may cause subtle, hard-to-control changes in the cells, such as membrane alterations, protein loss, altered metabolism, and changes in antigenicity and drug sensitivity” (Hamburger AW 1982). Queral et al (1984) have reported that the source and method of enzyme preparation is more important compared to any other factor in the isolation of viable hepatocytes from liver. As per their finding, collagenase from different sources may contain different proteolytic enzymes that can harm cells and cell surface markers. Curry et al (2000) have reported that the viability of the lymphocytes obtained from human liver sample depend on how much time the sample is exposed to the enzyme mixture. As per their result the longer the exposure time the lower the viability of the cells. Others (Eade OE 1981; Penning JJ 1981; Queral AE 1984; Engelholm SA 1985; Allalunis-Turner MJ 1986; Lee TK 1993; Haraldsen G 1995; Curry MP 2000) have also reported similar results and concerns. They have tried different combinations for increasing yield, viability, cloning efficiency and DNA yield but effectiveness of the method depends on the tissue type.

Enzyme digestion has immense potential to improve. This technique, unlike others discussed above depends on processes like enzyme kinetics and diffusion, which can be modeled mathematically and can be further improved. According to different researchers the single cell suspension obtained by enzyme digestion is of better quality than mechanical dissociation and has higher cell yield compared to LCM. Single cell suspension prepared from this technique can be further subjected to cell sorting techniques based on physical parameter dependent separation or immunologic separation to obtain homogeneous cell populations. Homogeneous cell populations can answer
queries related to false positive results and dilution effects observed while performing molecular analysis techniques. Enzyme digestion technique will be discussed in more detail in further sections.

2.4 Problem definition

The overall goal of this study is to develop a better understanding of the sample preparation technique. The end point of this technique is to achieve molecularly homogeneous cell populations from a tumor, which is a heterogeneous entity with significant structural differences compared to normal tissue. Currently, for purposes of somatic genetic analyses, there are clear limitations in the manner in which we can selectively procure specific cellular compartments from any solid tumor sample. We currently separate the neoplastic epithelium occurring in immediate vicinity of stromal fibroblasts from non-neoplastic cells on the basis of direct visual observation through a microscope (LCM). While this is relatively straightforward with stained thin (5 µm) sections normally used for pathological diagnosis, this becomes more challenging when thick (~10 µm) sections are stained and subjected to LCM. As a result, samples with a certain histological characteristic are selected. For example, samples where single neoplastic epithelial cells are intermixed with stroma have not been used in preliminary analyses because of technical difficulty in recognizing and then microdissecting the selected cells. Even if the stroma were clearly demarcated from the epithelial component, the stroma is not a uniform entity. Thus, it would also be desirable if the various component cells in stroma can be routinely distinguished. Using thick stained sections, no finer histological or cellular detail apart from neoplastic versus non-
neoplastic and epithelium versus stroma can be distinguished. Therefore, a better methodology, not entirely dependent on the human eye is needed to sort neoplastic epithelium from surrounding stroma from non-neoplastic cells systematically and with high specificity.

These goals can be achieved by sorting the cells based on their immunological characteristics. To sort cells from a tissue it is necessary to prepare a single cell suspension of the tissue. Single cell suspension from tissue is routinely prepared using enzyme digestion technique in life sciences laboratories and is one of the most widely used protocols. This process has been widely studied empirically for different tissue systems in different laboratories and significant discrepancies are observed in the recovery and purity of the desired cells. *Despite such widespread use of enzyme digestion as a sample preparation technique, there has been no attempt till date to the author’s knowledge to understand this technique based on principles of enzyme kinetics and diffusion and/or convection of enzymes through a porous tissue block.* This study proposes to study enzyme digestion of normal as well as malignant human breast biopsies (Appendix A). Significant emphasis will be put on developing theoretical understanding of the diffusion of enzymes through the extracellular compartment and enzyme kinetics of tissue digestion and interplay between these two processes. The end goal of this study would be to make it possible to model and reduce the time of preparation of single cell suspension from tissue.
2.5 Research plan

As stated previously, the overall goal of the project is: Development of methodologies/technologies to separate/fractionate cells within a solid tumor (or tissue sample containing a solid tumor) for further molecular analysis. To achieve this overall goal, one has to optimize preparation of single cell suspension using enzymatic digestion of tissue. The study discussed here attempts to present a possible way of understanding this process based on principles of enzyme kinetics and diffusion of molecules through a porous media (here normal and malignant breast tissue in different stages of carcinogenesis). As discussed above, breast carcinoma is the leading cause of cancer related death in women. Therefore, we propose to study the enzyme digestion technique for normal as well as malignant breast tissue samples. Fresh breast tissue samples will be procured from Tissue Procurement Shared Resource (TPSR) at The Ohio State University as per the guidelines of Office of responsible research.

2.5.1 Description of extracellular matrix

The stromal compartment of any tissue is made up of extracellular matrix (ECM) component and cellular component as shown in figure 2.1. The functions, composition and distribution of ECM are not clear and are active areas of study. ECM is mainly made up of proteins secreted by fibroblast cells but there is evidence that other cell types also participate in ECM synthesis. ECM proteins can be divided into four sub-groups. They are hydrophobic collagen and elastin matrix and hydrophilic proteoglycan and glycoprotein gels. Collagen and elastin provide the mesh structure that is filled with the hydrophilic gel and cellular compartments (Hay, 1991 #49; Truskey GA, 2004 #101).
Among ECM proteins the collagen concentration is highest and it plays major role in tissue architecture (Jain, 1987 #176).

A decade ago ECM was considered as an amorphous mass but now the notion of the role of ECM has changed significantly. It is a dynamic system that is remodeled continuously and performs task of mesoderm in adult tissue. This remodeling process necessitates local destruction and then rebuilding of the matrix. The destruction task is performed by matrix metalloproteinases (MMP) like collagenase, gelatinase etc. and are strictly regulated by the body. To understand the enzyme digestion technique we begin with understanding the structural protein collagen. Collagen is made up of 3 \( \alpha \)-helices. These \( \alpha \)-helices are in turn made up of repeated units of Gly-X-Y residues. The X and Y can be any amino acid residues but in 20 % or more cases, they are proline and hydroxyproline. The \( \alpha \)-helix is wound in a left-handed poly-L-proline type helix. Three of such helices come together to form rope like tropocollagen structure. This superstructure in turn has super twist in right-handed manner. This entire structure is stabilized by hydrogen bonds. The tropocollagen molecule with globular protein type domain at N and C- termini form procollagen. The cells secrete procollagen which then undergoes proteolysis at N- and C- termini to release tropocollagen polypeptide chain. These chains then form collagen fibril. Many of such fibrils participate to form collagen fibers (Seifter 1970; Hay 1991).

The \( \alpha \)-helices of tropocollagen can be similar or they can exhibit significant variations in their polypeptide chain. Based on variation in \( \alpha \)-helix structure, the collagen fibers are classified into 14 different know types. This is still an active area of research, where more types of collagen are being discovered and the functional role and tissue
distribution of known types is being determined. For example, collagen type I (two similar $\alpha_1$ helices and one $\alpha_2$ helix) is distributed mainly in fibrous matrix, collagen type IV (two similar $\alpha_1$ helices and one $\alpha_2$ helix, but different subunits as compared with collagen type I) is mainly distributed in basement membrane (Hay, 1991 #49; van der Rest, 1991 #191). In case of breast tissue collagen type I and type III are the major collagen components (Barsky et al., 1982; Kauppila et al., 1998; Marotta et al., 1985; Minafra et al., 1984; Okano, 1985; Pucci Minafra et al., 1985). It has been shown by Jain et al that collagen content in tissue control the diffusion of drug in the tissue (Jain, 1987). Based on this fact we hypothesized that collagen digestion in a tissue governs the time of enzyme digestion. To validate this hypothesis, we assume that the ECM of breast predominantly contains collagen. This assumption will be validated using IHC and collagen concentration will be quantified based on determination of hydroxyproline found in collagen molecule (Bancroft, 1996 #212; Woessner, 1961 #213).

2.5.2 Types of collagenase, enzyme kinetics and measurement of kinetic parameter

As discussed above procollagen structure can be divided into two components: tropocollagen and globular protein at N and C termini. The globular regions are susceptible to the action of simple enzymes like proteases, trypsin, chymotrypsin etc. But these enzymes cannot degrade tropocollagen. The enzyme capable of causing hydrolytic scission of peptide bonds located in characteristic poly-L-proline type of helical regions when the substrate is in the undenatured state is defined as collagenase (Seifter 1970).
There are approximately 20 enzymes that have been classified as collagenase. They can be divided into two subgroups depending on the source of production. First source is from mammalian tissue. Here collagenase is employed for the process of rebuilding the animal tissue. Currently matrix metalloproteinases (MMP) functions are studied in detail as a relationship has been observed between MMP production by cancer cells and metastasis. Tissue collagenase attacks the helical structure at a single site. The other source is microbial collagenase that attacks the helical tropocollagen structure at multiple sites simultaneously. Collagenase is produced in commercial scale from pathogenic bacteria like *Clostridium histolyticum* that employ it for invading the host organism. The commercially produced collagenase is routinely used to digest tissue from different sources (like human, animal etc.) for various studies like tissue engineering, cell line development, genetic analysis of tissue for cancer detection.

Crude collagenase produced from *C. histolyticum* contains a mixture of proteinases. This includes elastase and non-specific proteinases. It is essential to separate elastase and non-specific proteinases before using the collagenase. The commercial sources do a good job of removing elastase but the non-specific proteinases are still present. Table 2.1 presents information on some of the commercially available collagenases and their specific activities. Here the column labeled mg of protein / mg of crude powder represents the ratio of mg of collagenase present / mg of crude powder. Specific activity due to collagenase was measured using \(^{14}\text{CH}_3\)-collagen and a synthetic polypeptide 2-furancryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA) Whereas any activity due to non-specific proteinases was measured using N-\(\alpha\)-benzoyl-L-arginine ethylester (BAEE) and \(^{14}\text{CH}_3\)-casein (Bond MD 1984).
Bacterial collagenase can be further separated into two classes: class I collagenase ($\alpha$, $\beta$, $\gamma$) and class II collagenase ($\delta$, $\epsilon$, $\zeta$). Each of these classes can be further divided into three subclasses (shown in parenthesis) based on chromatographic separation (Bond MD 1984). Table 2.2 shows the physical properties of individual subclasses. Table 2.3 presents enzyme activities of individual subclasses against ($^{14}$CH$_3$)-collagen, FALGPA, 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-proline (FALGPP), ($^{14}$CH$_3$)-gelatin (denatured collagen) and PZ peptide. The activity is measured in terms of nkat/mg (nkat = nanoketals = nanomoles per second) or nkat / nm. It can be observed from table 2.3 that class I collagenase has high specific activity against collagen whereas class II collagenase has high specific activity against FALGPA (Bond MD 1984). Van Wart et al have shown previously that FALGPA is a good synthetic substrate to understand and monitor the kinetics of collagen hydrolysis. But, there is no linear correlation between the specific activity determined by FALGPA as a substrate and collagen as a substrate. On the other hand, the kinetics of collagen hydrolysis determined using collagen as a substrate involves precipitation of undigested collagen. This step is a labor intensive and error prone process (Van Wart HE 1981; Van Wart HE 1982).

To overcome precipitation step, Mallaya et al have determined the kinetic parameters of class I and class II collagenase components using radioactive rat type I collagen, bovine type II collagen and human type III collagen. They observed that collagenases follow Michaelis Menten kinetics when substrate is in solution (Mallya SK
Thus, the enzyme kinetics can be expressed as

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P
\]

\[
ES \xrightarrow{k_{-1}} E + S
\]

where, \(E = \text{enzyme}; S = \text{substrate}; P = \text{product}; ES = \text{complex}\)

\[
v = \frac{v_{\text{max}} \cdot S}{K_M + S}
\]  \hspace{1cm} (2.1)

where,

\[
v_{\text{max}} = k_2(E_0)
\]  \hspace{1cm} (2.2)

\[
K_M = \frac{k_1 + k_{-1}}{k_2}
\]  \hspace{1cm} (2.3)

\((E_0) = \text{initial enzyme concentration and } k_2 = k_{\text{cat}} \text{ (in biological literature)}\)

Table 2.4 shows kinetic parameters for classes of collagenase for the hydrolysis of radioactive substrate mentioned above (Mallya SK 1992). When this data was compared with tissue collagenase (data not shown), it was apparent that the specific activities of tissue collagenase was not significantly different than that of *C. histolyticum* collagenases (CHC). But the tissue collagenase is much more specific compared to CHC. Also, tissue collagenase cleaves the collagen fiber only once and then it is degraded by gelatinase. But CHC continues to attack collagen fiber repeatedly to reduce it to small size peptide.

*It can be seen from table 2.4 that CHC does not have specificity for type of collagen but the specific activity \((k_{\text{cat}}/K_M)\) does vary with the type of collagen. Thus the time of enzyme digestion will vary depending on collagen composition and distribution in different tissue. The information about the kinetic parameter is determined for the*
components of collagen which does not predict behavior of crude collagenase. Also, the exact component of crude collagenase changes from lot to lot and company to company, therefore the above information can not be used without determining the exact composition of crude collagenase. We propose here to develop a novel assay based on increase in fluorescence to quantify the kinetic parameters of crude collagenase. The kinetic parameter obtained by the new assay will be compared with table 2.1, 2.3 and 2.4. Using the collagen concentration determined in previous step and kinetic parameters here, the time of enzyme dissociation of tissue can be determined without considering diffusion effects.

2.5.3 Influence of reaction rate and diffusion rate on enzyme digestion

Protein interaction (here enzyme) on interface is not a well understood phenomenon. There are several processes that depend on the interfacial properties of enzyme like tissue dissociation, biosensors, bioremediation of insoluble waste, food processing etc. This is complex phenomenon occurring in the following stages. First the protein has to get adsorbed on the interface from the bulk. Then it has to diffuse around the interface to perform desired reaction and finally the protein has to react with its substrate. Figure 2.2 shows a simple schematic of this process with respect to tissue digestion process (Gaspers 1994; Gaspers 1995).

Several studies have been done to understand and model this phenomenon with different proteins like collagenase, amylase, bovine serum albumin etc. Gast et al have developed a system where they covalently immobilized FALGPA on quartz surface. This surface was then subjected to a buffer containing collagenase which was analyzed for the
three rate processes described above (Gaspers 1994). They have observed that the hydrolysis of FALGPA was dependent on lateral diffusion and surface reaction rate. The lateral diffusion of enzyme was measured using a technique combining fluorescence recovery after photobleaching (FRAP) with total internal reflection fluorescence (TIRF) pioneered by Tilton et al (Tilton 1990) and the reaction rate was monitored using a multifold cell device (similar to the plate and frame filter unit). Table 2.5 summarizes the diffusion and kinetic data obtained by Gaspers et al (Gaspers 1994).

The preliminary parameters obtained here were used to model the consumption of FALGPA on quartz surface with respect to time. This phenomenon was modeled based on two separate hypothesis. In first hypothesis the lateral diffusion of enzyme can be modeled using modified Smoluchowski’s theory in two dimensions. Here the enzymes were hypothesized as fixed sink with mobile substrate that is undergoing reaction when it enters the enzyme sink. This theory analyzes diffusion and reaction rate simultaneously and yields an analytical solution (Gaspers 1995). The second hypothesis emphasizes an active complex formation phenomenon in Michaelis-Menten kinetics. Here the substrate is covalently bounded to the surface and the enzyme is diffusing and forming complex non-specifically. Thus here the reaction depends on two steps. First, the chance of active complex formation by proper orientation of enzyme with respect to substrate molecule and the second step is the hydrolysis reaction itself (Trigiante G 1999). The theory models the experimental data well.

*This approach can be taken as a starting point for determining the digestion time of collagen in tissue dissociation process. The anticipated challenges are 3D nature of
tissue sample, the unknown initial concentration of collagen in the tissue and an unknown diffusivity of collagenase in tissue.

The concentration of collagen in tissue can be determined as discussed in section 2.5.1 and appendix B.5 by Woessner’s protocol (Woessner, 1961 #213). The challenge associated with 3D nature of tissue can be overcome if tissue structure is considered as a porous material as shown in figure 2.1. This problem is similar to solving general case of non catalytic gas solid reaction. Enzyme digestion can be studied by analogy to coal combustion problem. The tissue can be treated as an infinite slab with diffusion of enzyme occurring perpendicular to the y axis as shown in figure 2.3. For simplification of the problem, it is assumed that the resistance due to boundary layer around the tissue is negligible and the concentration of collagen is spatially uniform and constant for small time scale. The second assumption has to be modified to obtain more accurate predictions. Now, applying equation of continuity for species A (enzyme) we get following simplified equation (2.4 – 2.5) depicting the system described by figure 2.3 (Bird, 2002 #144; Truskey GA, 2004 #101).

\[
\frac{\partial C_A}{\partial t} = D_{\text{eff}} \frac{\partial^2 C_A}{\partial x^2} - \frac{k_2 C_A C_S}{K_M + C_S} \quad (2.4)
\]

\[
\frac{\partial C_A}{\partial t} = D_{\text{eff}} \frac{\partial^2 C_A}{\partial x^2} - k' C_A \quad (2.5)
\]

Initial condition \( t = 0, C_A = 0 \) for all \( x = 0 \) \quad (2.6)

Boundary condition \( t > 0, C = C_0 \) for all \( x = 0 \) \quad (2.7)

In the above set of equations (2.4 – 2.7), \( C_A \) represents enzyme concentration in tissue, \( C_0 \) is bulk initial concentration of enzyme, which remains constant through out the
digestion due to excess supply, \( C_S \) is concentration of collagen in the tissue, \( C_{S0} \) is initial concentration of the collagen, \( k_2 \) and \( K_M \) are Michaelis Menten kinetic parameters, \( K_{AV} \) is available volume fraction and \( D_{eff} \) is effective diffusivity of the enzyme in tissue (Bird, 2002 #144; Truskey GA, 2004 #101). \( K_{AV} \) and \( D_{eff} \) both depend on the molecular weight of the solute (enzyme) and the porosity of the tissue. In this problem the porosity of the tissue changes with respect to time as tissue is digested, but for simplification it is assumed that the porosity of the tissue remains constant for small time, therefore \( K_{AV} \) and \( D_{eff} \) depend on molecular weight of the enzyme. Thus, to solve this equations (2.4 – 2.7), a good estimate of \( K_{AV} \) and \( D_{eff} \) is required.

A similar challenge is faced for blood- borne drug delivery in any disease (Jain 1996; Jain 2001). Jain et al and others have analyzed this challenge with respect to drug delivery in solid tumors. Due to lack of lymphatic system in solid tumors (Appendix A), a high interstitial pressure is experienced in solid tumors. The increased pressure flushes out all the drug molecules. Hence there is no covective transport of drug molecules in tumor interstitium (Jain 1996; Jain 2001). Thus all the drug molecules have to diffuse through ECM. There are conflicting views in literature about predominant resistance to the diffusion of molecules in ECM. Swabb et al have shown that hydrophilic glycosaminoglycan offer maximum resistance to diffusivity of molecule in ECM (Swabb EA 1974). Recently Jain et al have demonstrated that collagen offers the major resistance compared to any other ECM molecule (Netti PA 2000; Pluen A 2001; Ramanujan S 2002). The diffusivity of any molecule in porous tissue type matrix depends on physicochemical (e.g. size, charge, structure and composition) and physiological (e.g. pressure) properties of extracellular compartment and physicochemical properties of
solute molecule (Jain 2001). Table 2.6 presents power law constants for measuring diffusivity using following equation (2.8) (Jain 1987) for dextran particles in different tissues.

\[ D = A \cdot (\text{Molecular Weight})^{-B} \]  

(2.8)

Jain et al have analyzed movement of dextran and BSA particles conjugated to fluorochrome molecule using fluorescence recovery after photobleaching. In this method a well-defined concentration gradient of fluorochrome is artificially imposed in ECM by photobleaching with a laser beam. The relaxation of gradient is monitored and utilized to determine the diffusivity and the convective velocity (Jain 1987).

We propose to measure in vitro collagen concentration of human normal and malignant breast tissues (Appendix A and B.5) using Woessner’s protocol.

We propose here to develop a novel assay based on increase in fluorescence to quantify the kinetic parameters of crude collagenase. The kinetic parameter obtained by the new assay will be compared with table 2.1, 2.3 and 2.4. Using the collagen concentration determined in previous step and kinetic parameters here, the time of enzyme dissociation of tissue can be determined without considering diffusion effects.

The above parameters and diffusion prediction will be then used to predict the time of enzyme digestion using reaction diffusion model developed in section 2.5.3. This model will be solved using analytical solution and the predictions will be compared with actual experimental time in the lab set up for digestion of breast tissue with collagenase.
2.6 Culture of arachnoidal cells in 3D matrix to study of cerebrospinal fluid (CSF) outflow

This research project is a collaborative effort with Dr. Deborah Grzybowski in Biomedical Engineering Center, The Ohio State University, Columbus, Ohio. The overall goal of project is to improve understanding of 3D culture of arachnoidal cells \textit{in vitro} and characterize the cells to study the outflow pattern of cerebrospinal fluid (CSF) around brain. This understanding will help in treating diseases like pseudotumor cerebri (PTC), hydrocephalus, subarachnoid hemorrhage etc.

2.6.1 Background of Cerebrospinal fluid

CSF is a very important component of brain and is necessary for proper functioning of the brain. It provides an ideal extracellular milieu to glial cells for proper functioning as well as protects brain against mechanical shocks. CSF is a clear colorless fluid with a specific gravity of 1.004-1.007 gm/cc and is slightly heavier than water. The physical properties of CSF are fairly similar to plasma but the composition is different than plasma. The physical properties and composition of CSF and plasma is compared in table 2.7.

In human the total volume of CSF surrounding brain is estimated to be 80-150 ml with majority of fluid situated in subarachnoid space (SAS). The choroid plexus (site of major production of CSF) produces CSF at a rate of 0.35 ml/min. or 500 ml/day. At this rate the total volume of CSF is replaced 2-3 times a day. Due to rigid walls of cranium and continuous production of CSF from different sites in brain, a positive pressure is maintained on the brain. In infant the normal pressure varies from 25-70 mm of H$_2$O and
in adults it varies from 65-195 mm of H₂O. This pressure controls the rate of drainage of CSF. For some reason if the drainage of CSF is affected by increased resistance to the CSF outflow then the pressure builds up in the subarachnoid space and leads the pathologic condition like PTC (http://www.umanitoba.ca/faculties/medicine/anatomy/csf-form.htm; Davson 1996; Bergsneider 2001).

2.6.2 Circulation of CSF around brain

Choroids plexus of lateral ventricle produces the majority of CSF via active transport of the blood constituents and secretion by choroid epithelial cells (Davson 1996). CSF then flows (as shown in figure 2.4 (a)) through interventricular foramina of Monro in to 3rd ventricle. 3rd ventricle communicates to 4th ventricle via aqueduct of Sylvius and CSF follows the same route to reach 4th ventricle. From 4th ventricle it flows to subarachnoid space and around the spinal cord (Davson 1996).

Brain is covered with three layers. They are (1) Fibrous dura mater (2) arachnoid mater and (3) pia mater. The pia layer follows the contours of nervous tissue accurately where as arachnoid layer bridges the sulci (deep fissure of brain) of the brain and cord. This results in empty space known as subarachnoid space (SAS) (Figure 2.4 (c)). SAS contains loosely attached connective tissue matrix containing arachnoid cells and fibroblast cells. CSF fills the rest of the empty space and thus forming a protective layer around brain (Figure 2.4 (a, b)) (Davson 1996; Johanson 1998).

This aspect for CSF outflow is fairly well understood. But the absorption CSF in venous blood is still a topic of debate. There are two mechanisms proposed for CSF absorption. The first model puts an emphasis on role of arachnoid granulations ((Figure
2.4 (c)) in CSF outflow from SAS to venous blood (Davson 1996; Johanson 1998; Kapoor K In preparation) whereas second model emphasis on role of extracranial lymphatics in absorption process (Johnston 2000; Kapoor K In preparation). Johnston et al have shown that in sheep 40-48% of CSF is absorbed via extracranial lymphatics (Johnston 2000) but no conclusive results are obtained for humans. The role of extracranial lymphatics is not undermined here but the focus of this study is to develop 3D culture model to understand the role of arachnoid granulations in the process of CSF absorption.

2.6.3 Structure of Arachnoid granulation

Arachnoid granulations (AG) are projections of the arachnoid mater through the dura into venous sinuses (superior sagittal sinus) as shown in figure 2.4 (b). It provides interface between CSF and venous blood. AG plays an important role as one-way valve to control the absorption of CSF in venous blood (Weed 1914; Davson 1996). This specialized task of AG is governed by a specialized structure. Most of the studies on AG have been performed on animal model, but Yamashima, Upton et al have shown significant difference between animal arachnoid villi and human arachnoid granulation. Thus only structure of human AG is discussed here. Based on immunohistochemistry (IHC), electron microscopy and immunocytochemistry (ICC) studies performed on frozen and paraffin embedded sections from leptomeningial (pia and arachnoid layer) layer, it was concluded that AG has four main compartments as shown in figure 2.5. They are cap cell cluster, fibrous capsule or fibrous dura, arachnoid cell layer and central core (Upton 1985; Kida 1988).
The central core is continuation of subarachnoid space (SAS) in arachnoid granulation as shown in figure 2.5. The composition of central core is similar to SAS. It consists of gel like connective tissue containing fibroblasts, arachnoid cells and CSF (Kida 1988). The presence of fibroblast cells can be confirmed by different markers like CD90 (Thy 1), anti-fibroblast antigen or by presence by intermediate filaments like vimentin but absence of cytokeratin filaments. The arachnoid cells are of mesodermal and neural ectodermal origin and therefore share both mesenchymal and epithelial characteristics (O'Rahilly R 1986; Rutka JT 1986; Rutka JT 1986; Schnitt SJ 1986; Winek RR 1989). They produce extracellular matrix and basement components like collagen, fibronectin and laminin. They also show positive expression of CD90, anti-fibroblast marker and vimentin and thus cannot be characterized as well as separated based on expression of these markers (Rutka JT 1986; Rutka JT 1986). But they form intercellular junctions like desmosomes, tight junctions and gap junctions (Hasegawa M 1997). They also express surface proteins like E-cadherins, EMA (epithelial membrane antigen), zona occludens, connexins and desmoplakin (Hasegawa M 1997; Arishima H 2002). These are all characteristics of epithelial cells. All of these studies were performed on either frozen or paraffin embedded sections (Kartenbeck J 1984; Ng HK 1987; Kida 1988; Winek RR 1989, Kasper M, 1991 #96) or on cell cultured from leptomeningial layer or meningiomas (tumors of arachnoid cell layer) (Rutka JT 1986; Rutka JT 1986; Murphy M 1991; Motohashi O 1994; Grafstein B 1999).

Arachnoid cell layer is continuation of arachnoid mater as shown in figure 2.5. This layer predominantly consists of arachnoid cells, which are similar in characteristics as arachnoid cells in central core (Wolpow ER 1972; Upton 1985; Kida 1988). In animal
arachnoid villi, this layer is completely surrounded by fibrous dura layer, which is continuation of dura mater (Wolpow ER 1972). The dura mater is strongest membrane covering the brain and is made up of fibrous connective tissue invested with endothelial cells. These cells are characterized by factor VIII related antigen and VE-cadherin (CD144) (Kida 1988; Lampugnani MG 1992). In human arachnoid granulation the arachnoid cell layer is not completely covered by dura layer as shown in figure 2.5 (Kida 1988).

The apical portion of arachnoid granulation in human is made up of arachnoidal cell layer. This layer is made up of two separate zones of cells. The outer zone is electron lucent and shows lesser expression of cytoplasmic filaments and desmosomes. The inner zone is electron rich and has higher expression of cytoplasmic filament and desmosomes. This specialized structure implies that it may have some role in CSF outflow. It also confirms that the animal models are simple and cannot give correct picture of the human arachnoid granulations (Wolpow ER 1972; Yamashima 1986; Kida 1988; Yamashima 1988).

Researchers do have some disagreement on these four compartments of AG. Upton et al and Wolpow et al commented that some arachnoid granulations are similar to animal arachnoid villi where the arachnoid cell layer is completely covered by endothelial cells (Wolpow ER 1972; Upton 1985). Based on these structural differences there are two proposed model of CSF transportation across CSF-blood barrier. One model proposes open channels across AG as shown in figure 2.6 (Tripathi 1977; d'Avella 1980; d'Avella D 1983; Upton 1985). The CSF outflow follows pressure gradient which is high on CSF side to low on venous blood side. The other model proposes close channel
approach where micropinocytotic vesicles, intracytoplasmic vacuoles and extracellular cisterns facilitate transportation of the CSF (Kida 1988; Yamashima 1988).

This study focuses on the study of close channel model. In this model it is proposed that arachnoidal cells as well as dural sinus endothelial cells play role in CSF transport. The mechanism of transportation is of different types depending on the cell types. Yamashima et al observed that the micropinocytotic vesicles and intracytoplasmic vacuoles are predominant modes of CSF outflow via sinus endothelial cells. Where as extracellular cisterns are responsible for CSF outflow across arachnoidal cells. Thus arachnoidal cell layer are responsible for bulk of CSF outflow. This observation was confirmed in a pathologic condition known as subarachnoid hemorrhage, where erythrocytes are transported across arachnoidal cells in form of extracellular cisterns (Yamashima 1986; Kida 1988; Yamashima 1988).

2.6.4 In vitro model of CSF outflow

Dr. Grzybowski et al have developed an in vitro flow perfusion technique to study the arachnoidal cells and various pathological conditions related to CSF outflow. This is one of the first attempts to perform in vitro studies of human arachnoidal cells from AG. The motivation behind this study is due to high cost associated with animal model and lack of similarity between the arachnoid villi from animal compared to AG from human (Rivera 1998; Holman DW 2004). For success of this flow perfusion model it is necessary to grow the arachnoidal cell in vitro on filter support free of other contaminating cell type to perform various studies.
The specialized task of CSF egress in venous blood is governed by AG due to specialized tissue structure. AG has four main compartments as shown in figure 2.5. They are cap cell cluster, fibrous capsule or fibrous dura, arachnoid cell layer and central core (Kida, 1988; Upton, 1985). The central core is continuation of subarachnoid space (SAS) in arachnoid granulation as shown in figure 2.5 and is made up of connective tissue containing fibroblasts, arachnoid cells and CSF (Kida, 1988). Arachnoid cell layer is continuation of arachnoid mater as shown in figure 2.5. This layer predominantly consists of arachnoid cells, which are similar in characteristics as arachnoid cells in central core (Kida, 1988; Upton, 1985; Wolpow ER, 1972). In animal arachnoid villi, this layer is completely surrounded by fibrous dura layer, which is continuation of dura mater (Wolpow ER, 1972). In human AG the arachnoid cell layer is not completely covered by dura layer as shown in figure 2.5 (Kida, 1988). The apical portion of arachnoid granulation in humans is made up of arachnoidal cap cell layer and is an important controller of CSF egress in venous blood. The success of in vitro model to study CSF egress relies on primary culture of functional arachnoidal cells.

The differentiated state of arachnoidal cells is governed by the microenvironment of the tissue. In recent years natural and synthetic polymers have been used to provide suitable in vitro extracellular environment for functional tissue like structure formation (Lanza, 2000; Sittinger et al., 1996). Multiple cell types have been used along with polymers to produce functional blood vessels, bladder and cornea (Griffith et al., 1999; Niklason et al., 1999; Oberpenning et al., 1999). The success of in vitro tissue formation relies on suitable polymer selection and suitable culture system responsible for simulating in vivo environment. Both these parameters have equally important role in development
of tissue structure similar to in vivo structure. The purpose of this study is to culture primary arachnoidal cells on suitable polymer substrate.

The central core of AG is made up of connective tissue and extracellular matrix that supports the function of arachnoid cells. To simulate this condition non-woven poly(ethylene) terephthalate (PET) matrix was selected. PET matrix has been already used for successful culture of human trophoblast cells, mesenchymal embryonic stem cells, astrocytes, hybridoma cells, NIH 3T3 cells (Basu and Yang, 2005; Li Y, 2001; Ma et al., 1999; Ma T, 2000; Takahashi and Tabata, 2004). This system is well characterized in terms of physical properties (fiber density: 1.35 gm/cm³, fiber diameter ~ 20 µm and porosity ~ 93%) (Basu and Yang, 2005; Bhat, 1995) and chemical properties (Phaneuf MD, 1997). In this study we utilized agitated seeding to culture primary arachnoidal cells on PET matrix. The cultured cells were then characterized based on morphology and immunocytochemistry. These results were compared with existing 2D culture method (Holman et al., 2005). These cells were then seeded on a filter support to characterize their permeability characteristics and were compared with the existing permeability characteristics (Grzybowski, 2005).
<table>
<thead>
<tr>
<th>Supplier</th>
<th>Designation</th>
<th>Lot #</th>
<th>mg of protein/mg of crude powder&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific activity (nkat/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Crude preparation</td>
<td></td>
</tr>
<tr>
<td>Sigma Chemical Co.</td>
<td>Type I</td>
<td>12F-516</td>
<td>0.37</td>
<td>0.0029</td>
</tr>
<tr>
<td></td>
<td>Type IA</td>
<td>101F-6831</td>
<td>0.46</td>
<td>0.0013</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>91F-6812</td>
<td>0.26</td>
<td>0.00045</td>
</tr>
<tr>
<td></td>
<td>Type IV</td>
<td>42F-6838</td>
<td>0.43</td>
<td>0.0023</td>
</tr>
<tr>
<td></td>
<td>Type V</td>
<td>11F-6805</td>
<td>0.43</td>
<td>0.0033</td>
</tr>
<tr>
<td>Z-9999</td>
<td></td>
<td>81F-6819</td>
<td>0.37</td>
<td>0.0038</td>
</tr>
<tr>
<td>Worthington Biochemical Co.</td>
<td>Type I</td>
<td>42C008</td>
<td>0.46</td>
<td>0.0022</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>W2H209</td>
<td>0.33</td>
<td>0.0023</td>
</tr>
<tr>
<td></td>
<td>Type III</td>
<td>41S130</td>
<td>0.36</td>
<td>0.0023</td>
</tr>
<tr>
<td></td>
<td>Type IV</td>
<td>41S123</td>
<td>0.74</td>
<td>0.0024</td>
</tr>
<tr>
<td>Roche-Manheim</td>
<td>None</td>
<td>1451142</td>
<td>0.39</td>
<td>0.0023</td>
</tr>
</tbody>
</table>

Table 2.1 Enzyme activities of crude collagenases from commercial source (Bond MD 1984):

<sup>a</sup>The milligrams of crude powder was determined by weighing the preparation, as received, while the milligram of protein was determined by the dye binding assay. <sup>b</sup>sp. Act. is less than 0.0010 nkat/mg.
Table 2.1 continued

<table>
<thead>
<tr>
<th>Company</th>
<th>Sample</th>
<th>Code</th>
<th>Concentration</th>
<th>Titer</th>
<th>Activity</th>
<th>% Activity</th>
<th>Recovery</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced Biofactures Corp.</td>
<td>ABC-TD</td>
<td>111-051082</td>
<td>0.23</td>
<td>4.9</td>
<td>43</td>
<td>0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABC-I</td>
<td>P-78R</td>
<td></td>
<td>0.20</td>
<td>0.0060</td>
<td>0.65</td>
<td>100</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>ABC-II</td>
<td>P-79-02R</td>
<td></td>
<td>0.17</td>
<td>0.0010</td>
<td>0.76</td>
<td>120</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>234,222</td>
<td>103310</td>
<td></td>
<td>0.12</td>
<td>0.0065</td>
<td>1.2</td>
<td>130</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>23,415</td>
<td>102364</td>
<td></td>
<td>0.20</td>
<td>0.0013</td>
<td>2.9</td>
<td>44</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Partially purified preparation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sigma Chemical Co.</td>
<td>Type III</td>
<td>61D-0566</td>
<td>0.90</td>
<td>0.0077</td>
<td>7.8</td>
<td>720</td>
<td>0.087</td>
<td></td>
</tr>
<tr>
<td>Worthington Biochemical Co.</td>
<td>Type VII</td>
<td>111F-6831</td>
<td>0.77</td>
<td>0.0017</td>
<td>78</td>
<td>5.5</td>
<td>0.0011</td>
<td></td>
</tr>
<tr>
<td>Calbiochem-Behring Corp.</td>
<td>ABC-III</td>
<td>186E</td>
<td>0.11</td>
<td>0.020</td>
<td>19</td>
<td>3.0</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>Calbiochem-Behring Corp.</td>
<td>234,136</td>
<td>130075</td>
<td>0.15</td>
<td>0.0092</td>
<td>8.0</td>
<td>250</td>
<td>0.0075</td>
<td></td>
</tr>
<tr>
<td>Class</td>
<td>Subclass</td>
<td>Molecular weight</td>
<td>Isoelectric point</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>------------------</td>
<td>-------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>I</td>
<td>α₁</td>
<td>68,000</td>
<td>5.85</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>α₂</td>
<td>68,000</td>
<td>5.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td>β</td>
<td>115,000</td>
<td>5.55, 5.60, 5.75</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>I</td>
<td>γ₁</td>
<td>79,000</td>
<td>6.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td>γ₂</td>
<td>79,000</td>
<td>6.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>II</td>
<td>δ</td>
<td>100,000</td>
<td>5.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>II</td>
<td>ε</td>
<td>110,000</td>
<td>5.90, 5.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>II</td>
<td>ζ</td>
<td>125,000</td>
<td>5.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 Physicochemical properties of different collagenase classes (Bond MD 1984)
<table>
<thead>
<tr>
<th>[14CH₃] collagen</th>
<th>[14CH₃] gelatin</th>
<th>FALGPP</th>
<th>FALGPA</th>
<th>PZ peptide⁵⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nkat/mg</td>
<td>nkat/nm</td>
<td>nkat/mg</td>
<td>nkat/nm</td>
</tr>
<tr>
<td>A₁</td>
<td>0.023</td>
<td>0.0016</td>
<td>0.21</td>
<td>0.014</td>
</tr>
<tr>
<td>A₂</td>
<td>0.022</td>
<td>0.0015</td>
<td>0.20</td>
<td>0.013</td>
</tr>
<tr>
<td>β</td>
<td>0.017</td>
<td>0.0019</td>
<td>0.095</td>
<td>0.011</td>
</tr>
<tr>
<td>γ₁</td>
<td>0.02</td>
<td>0.0015</td>
<td>0.12</td>
<td>0.00095</td>
</tr>
<tr>
<td>γ₂</td>
<td>0.021</td>
<td>0.0017</td>
<td>0.19</td>
<td>0.015</td>
</tr>
<tr>
<td>δ</td>
<td>0.0069</td>
<td>0.00069</td>
<td>0.048</td>
<td>0.0048</td>
</tr>
<tr>
<td>ε</td>
<td>0.0054</td>
<td>0.00060</td>
<td>0.026</td>
<td>0.0029</td>
</tr>
<tr>
<td>ζ</td>
<td>0.0059</td>
<td>0.00073</td>
<td>0.027</td>
<td>0.0034</td>
</tr>
</tbody>
</table>

**Table 2.3 Enzyme activities of different collagenase classes (Bond MD 1984)**

⁵⁴Activities for both collagen and gelatin were calculated from a substrate Mr of 300,000. ⁵⁵p-Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg
<table>
<thead>
<tr>
<th>Collagenase class</th>
<th>Collagen</th>
<th>Collagenase subclass</th>
<th>$k_{\text{cat}}$ (hr$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{\text{cat}}/K_M$ (µM$^{-1}$hr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Rat type I</td>
<td>β</td>
<td>2100</td>
<td>4.0</td>
<td>530</td>
</tr>
<tr>
<td>I</td>
<td>Rat type I</td>
<td>γ</td>
<td>1000</td>
<td>3.5</td>
<td>290</td>
</tr>
<tr>
<td>I</td>
<td>Bovine type II</td>
<td>β</td>
<td>1100</td>
<td>4.0</td>
<td>280</td>
</tr>
<tr>
<td>I</td>
<td>Bovine type II</td>
<td>γ</td>
<td>660</td>
<td>3.3</td>
<td>200</td>
</tr>
<tr>
<td>I</td>
<td>Human type III</td>
<td>β</td>
<td>1900</td>
<td>5.0</td>
<td>380</td>
</tr>
<tr>
<td>I</td>
<td>Human type III</td>
<td>γ</td>
<td>840</td>
<td>5.0</td>
<td>170</td>
</tr>
<tr>
<td>II</td>
<td>Rat type I</td>
<td>ε</td>
<td>900</td>
<td>3.1</td>
<td>290</td>
</tr>
<tr>
<td>II</td>
<td>Rat type I</td>
<td>ζ</td>
<td>1100</td>
<td>5.5</td>
<td>200</td>
</tr>
<tr>
<td>II</td>
<td>Bovine type II</td>
<td>ε</td>
<td>340</td>
<td>3.3</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>Bovine type II</td>
<td>ζ</td>
<td>300</td>
<td>2.8</td>
<td>110</td>
</tr>
<tr>
<td>II</td>
<td>Human type III</td>
<td>ε</td>
<td>190</td>
<td>2.0</td>
<td>95</td>
</tr>
<tr>
<td>II</td>
<td>Human type III</td>
<td>ζ</td>
<td>410</td>
<td>3.3</td>
<td>120</td>
</tr>
</tbody>
</table>

Table 2.4 Kinetic parameter for hydrolysis of radioactive rat type I, bovine type II and human type III collagen using different classes of collagenases (Mallya SK 1992)
<table>
<thead>
<tr>
<th>Buffer</th>
<th>$V_{\text{max}} \times 10^5$ (µmol/s)</th>
<th>$K_M$ (mM)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$D \times 10^{10}$ (cm$^2$/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricine, pH 7.5</td>
<td>42</td>
<td>1.4</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>MES, pH 6.4</td>
<td>10</td>
<td>0.14</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>MES, pH 6.1</td>
<td>5.2</td>
<td>0.12</td>
<td>1.4</td>
<td>-</td>
</tr>
<tr>
<td>Tricine with EDTA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 2.5 Diffusion and kinetic data of hydrolysis of immobilized FALGPA by collagenase on quartz surface (Gaspers 1994; Gaspers 1995)
<table>
<thead>
<tr>
<th>Medium</th>
<th>Solute (mol. Wt.)</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Dextran (10,000-147,000)</td>
<td>1.26*10^{-4}</td>
<td>0.478</td>
</tr>
<tr>
<td>Human articular cartilage (in vitro)</td>
<td>Dextran (5000-40,000)</td>
<td>6.17*10^{-2}</td>
<td>1.34</td>
</tr>
<tr>
<td>Various normal tissues (in vitro)</td>
<td>Various solutes (32-69,000)</td>
<td>1.778*10^{-4}</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td><strong>Mesentery (in vivo)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td>Dextran (3,400-393,000)</td>
<td>2.75*10^{-3}</td>
<td>0.758</td>
</tr>
<tr>
<td>Rat</td>
<td>Dextran (3,450-41,200)</td>
<td>5.5*10^{-3}</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td><strong>Rabbit ear (in vivo)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mature granulation tissue</td>
<td>Dextran (19,400-150,000)</td>
<td>10^{-6}</td>
<td>2.96</td>
</tr>
<tr>
<td>VX2 carcinoma</td>
<td>Dextran (19,400-150,000)</td>
<td>2.51*10^{-2}</td>
<td>1.14</td>
</tr>
</tbody>
</table>

Table 2.6 Power law parameters for different systems to determine diffusivity (Jain 1987)
<table>
<thead>
<tr>
<th>Component</th>
<th>CSF</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water content (%)</td>
<td>93</td>
<td>99</td>
</tr>
<tr>
<td>Protein (mg/dl)</td>
<td>35</td>
<td>7000</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>Osmolarity (mOsm/liter)</td>
<td>295</td>
<td>295</td>
</tr>
<tr>
<td>Na⁺ (meq/liter)</td>
<td>138</td>
<td>138</td>
</tr>
<tr>
<td>K⁺ (meq/liter)</td>
<td>2.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Ca⁺ (meq/liter)</td>
<td>2.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Mg⁺ (meq/liter)</td>
<td>0.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Cl⁻ (meq/liter)</td>
<td>119</td>
<td>102</td>
</tr>
<tr>
<td>pH</td>
<td>7.33</td>
<td>7.41</td>
</tr>
</tbody>
</table>

Table 2.7 Composition of plasma and CSF (Kandel ER 2000)
Figure 2.1 Schematic presentation of the tissue structure. The interstitial space is made up of collagen and elastin shown as fibers here. The spaces enclosed by these fibers represent the hydrophilic gel made up of proteoglycan and glycoprotein. The cells and vasculature is also shown (Truskey GA, 2004 #101)
Figure 2.2 Schematic figure showing different steps in enzyme digestion process
Figure 2.3 Tissue sample treated as a semi-infinite slab with thickness = 2L. The enzyme concentration is $C_0$ in the bulk and $C_{As}$ at the surface of the tissue.
Figure 2.4 CSF outflow around brain. (a) CSF is produced in choroid plexus and is circulated around brain via subarachnoid space. CSF is finally absorbed in venous blood via arachnoid granulation (b). Arachnoid granulations are microscopic projection of subarachnoid space in venous sinus via dura mater (b). (c) Subarachnoid space is created due to the anatomical structure of pia and arachnoid mater. This space is made of arachnoid cells, fibroblasts and CSF surrounded by in loose connective tissue. (http://www.arts.uwaterloo.ca/~bfleming/psych261/image22.gif)
Figure 2.5 Schematic diagram of human arachnoid granulation showing four different regions. The cap cell cluster has been shown in green color here (Kida 1988)
Figure 2.6 Schematic view of arachnoid granulation showing open channel structure. Abbreviations: e = sinus endothelium; SAS = subarachnoid space and SDS = subdural space (Upton 1985)
CHAPTER 3

A SIMPLE METHOD OF CHARACTERIZING THE KINETICS OF BACTERIAL COLLAGENASE USING DYE QUENCHED FLUORESCENCE SUBSTRATE

The content of this chapter is prepared for publication in Analytical Biochemistry: Mehta, BC; Chalmers, JJC; A Simple Method of Characterizing the Kinetics of Bacterial Collagenase Using Dye Quenched Fluorescence Substrate.

3.1 Introduction

With the advent and improvements of highly sensitive and accurate molecular analysis technology, it is possible to study increasingly complex cellular processes including cell differentiation and proliferation based on molecular markers from normal and malignant tissue source (St Croix et al., 2000). However, a common question/complaint of studies using these technologies is the homogeneity of the original sample (Sieben NL, 2000; Tomlinson IP, 2002). This lack of homogeneity can affect the desired final outcome significantly. For example, ovarian carcinoma samples from patient or cell samples taken from cell culture (HOC-1 or MCF-7) when analyzed in a microarray, express variety of RNA, and RNA levels suggesting various stages of the cell
cycle, which may or may not be related to the true process studied (Mackillop et al., 1982; Podhajcer et al., 1986; Yang et al., 1977). Consequently, the overall expression pattern might not represent any one single cell, but a “mean” of the entire population. The lack of homogeneity is even more of a problem when one works with non-cultured, three-dimensional tissue, which normally consists of a variety of different cell types, and in different states. In contrast, when cells are in suspension, a number of technologies exist to separate a heterogeneous cell population based on cellular characteristics and these technologies are a significant analytical and preparative resource (Pantel et al., 1999).

To address this challenge of separating a specific cell type from a heterogeneous tissue, number techniques/technologies are used depending on the end use of the cells which are separated. These techniques/technologies include enzymatic digestion, mechanical dissociation and Laser Capture microscopy (Emmert-Buck MR, 1996; Ghossein and Bhattacharya, 2000; Ricordi et al., 1988; Singh, 1998; Stingl et al., 2005). Among these dissociation techniques, the enzyme dissociation is the most widely applied. The principle of enzyme dissociation is simple; the extracellular matrix proteins of the tissue are subjected to matrix metalloproteases that selectively digest these proteins. For routine applications, like tumor dissociation or islet isolation, these enzymes are derived from either bacterial source or animal source (Seifter, 1970). Currently, to best of our knowledge, there is no generic approach available to dissociate the various types of tissues digested (i.e. the various variables involved in the process must be optimized for each process). Moreover all the current approaches are developed based on substantial empirical knowledge and there is no underlying first principle or theory behind this
process. This study attempts to understand and formulate this process based on enzyme kinetics of extracellular matrix (ECM) digestion.

ECM is made up of mainly four components: a) collagen, b) elastin, c) glycoproteins and d) proteoglycans (Hay, 1991). Each of these components can be further divided into different subtypes and concentration, which vary from tissue type to tissue type (Hay, 1991; van der Rest and Garrone, 1991). To simplify the complexity of characterization of the kinetics of this enzymatic process, we have focused on enzyme digestion of collagen using *Clostridium histolyticum* collagenase.

Crude collagenase produced from *C. histolyticum* contains a mixture of proteinases (Table 3.1) including elastase and non-specific proteinases (Bond and Van Wart, 1984). It is essential to separate elastase and non-specific proteinases before using the collagenase. The commercial sources do a good job of removing elastase but the non-specific proteinases are still present. Table 3.2 presents information on some of the commercially available collagenases and their specific activities. Here the column labeled mg of protein / mg of crude powder represents the ratio of mg of collagenase present / mg of crude powder. Specific activity due to collagenase was measured using (14CH3)-collagen and a synthetic polypeptide 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA) Whereas any activity due to non-specific proteinases was measured using N-\(\alpha\)-benzoyl-L-arginine ethylester (BAEE) and (14CH3)-casein (Bond and Van Wart, 1984).

Bond *et al* have shown that bacterial collagenase can be separated into multiple components as shown in Table 3.3 and each of these components have different activities towards different collagen substrate and synthetic peptides (Bond and Van Wart, 1984). Also, the concentration of each component varies from lot to lot (designated as different
types of collagenases in table 3.2) and thus the activities vary as well. Thus, the kinetic parameters for different collagenases procured from different lots from a source will give different results. The same will hold true for enzymes from different sources (Peterkofsky, 1982). To overcome this problem, we have developed a simplified assay to evaluate kinetic parameters of crude collagenase as a single entity compared to its individual components as performed by Bond et al (Bond MD, 1984). This assay is based on fluorescence enhancement due to degradation of dye quench substrate, which can be measured directly using fluorescence plate reader.

There are many other assays available to detect the activity of collagenase. Synthetic substrates such as N-[3-(2-Furyl)acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine (FALGPA) (Van Wart and Steinbrink, 1981) and 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-α-Arg (Pz-PLGPR) (Wuensch and Heidrich, 1963) are routinely used for reporting the unit of activity of collagenase. These units are not suitable to directly correlate with protein substrate derived from human tissue samples like breast tumor or porcine pancreas. Mookhtiar et al have reported preparation of radiolabeled collagen to determine the kinetic parameter of collagenase (Mookhtiar et al., 1986). This assay is very sensitive and can determine the kinetic data that can be correlated with tissue dissociation. However radioactivity is inherently hazardous, labor intensive and expensive tool (Grubb, 1994). Another way to characterize the activity is to analyze the activity using insoluble tendon collagen. This method will provide the closest results for tissue dissociation; however this method involves precipitation of undigested collagen, which is an error prone step. Apart from precipitation, sampling at multiple time points and careful control of sample volume are challenging and may lead to unforced errors.
O’Grady et al have reported preparation of collagen substrate with a fluorescent compound 2-methoxy-2, 4-diphenyl-3(2H)-furanone (O'Grady et al., 1984). This substrate fluoresces over a wide range of pH and looses its fluorescence when subjected to metalloproteases as 2-methoxy-2, 4-diphenyl-3(2H)-furanone is not fluorescent in unbound form. This method overcomes most of the challenges discussed above and multiple samples can be process simultaneously. However this assay is not as sensitive as radioactivity assay as very small change in fluorescence is observed when minute quantity enzyme is to be detected (Grubb, 1994).

The above discussed challenges can be overcome by employing a dye quenched fluorescent substrate. In this study we have employed fluorescein-5- isothiocyanate (FITC) conjugated to porcine gelatin, bovine collagen type I and human collagen type IV to characterize the enzyme kinetics from different sources of enzymes. These substrates are excessively labeled with FITC, which results in total internal absorbance of fluorescence by fluorescent dyes to quench the autofluorescence of dye labeled substrate (Haugland, 1996). The background fluorescence does not change much over long period of incubation time during the assay. The pH of the assay utilized is physiological range as it mimics the tissue degradation protocol performed with tumor and pancreas samples. A similar approach to analyze the activity of serine, acid, sulfhydryl and metalloproteases has been reported using 4,4-Difluoro-5,7-dimethyl-4-bora- 3a,4a-diaza- sindacene-3-propionic acid (BODIPY) labeled casein substrate (Jones et al., 1997). The aim of this study is to show and generate Michaelis – Menten kinetic parameters for degradation of porcine gelatin, bovine collagen type I and human collagen type IV using different collagenases from different commercial sources. This is a simplified approach to compare
different commercial sources of enzymes for the purpose of tissue degradation and provides a basis for selecting a particular enzyme for a particular system. These data will be utilized in future to characterize degradation of tissue based on first principle.

3.2 Materials and methods

3.2.1 Materials

Dye Quenched (DQ™) porcine gelatin type A, DQ™ bovine collagen type I, DQ™ human collagen type IV conjugated to FITC and EnzChek® Gelatinase/Collagenase Assay Kit was purchased from Molecular Probes, Inc. (Eugene, OR). Collagenase control (500 Mandl U) was purchased from Molecular Probes, Inc. (Eugene, OR). Collagenase samples (Cat # C9263) were purchased from Sigma Chemical Co. (St. Louis, MO) and Roche Applied Science (Cat # 1088785) (Indianapolis, IN). Fluorescein and Sodium hydroxide were purchased from Fisher Scientific Inc. (Hampton, NH). Tris, hydrochloric acid, sodium chloride, calcium chloride, sodium azide and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

3.2.2 Methods

3.2.2.1 Preparation of standard curve

Fluorescein stock solution was prepared by dissolving fluorescein in 1 X reaction buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, 0.2 mM sodium azide, pH 7.6). To facilitate solubilization of fluorescein, 0.1 N NaOH was added to the solution. The stock solution was diluted using a two fold serial dilution strategy for standard curve. The standard curve was prepared by measuring fluorescence of the diluted samples at
excitation of 485 nm and emission at 530 nm using CytoFluor® 4000 Fluorescence Multi-Well Plate Reader from Applied Biosystems (Foster City, CA).

3.2.2.2 Preparation of Collagen and enzyme solutions

DQ™ Porcein gelatin A, DQ™ bovine collagen type I and DQ™ human collagen type IV conjugated to FITC was supplied in 1 mg vial in lyophilized form. This powder was dissolved in 1 ml of distilled water containing 2 mM sodium azide. To facilitate dissolution; all the substrates were ultrasonicated for 5 minutes. If the substrate is still in powder form then the temperature can be raised to 50º C for 5 minute with ultrasonication. This stock solution of substrate was preserved at 4º C and was further diluted as per the requirement.

The reaction buffer was prepared in 10 X concentration. The concentration of constituents was 0.5 M tris, 1.5 M NaCl, 50 mM CaCl₂ and 2 mM sodium azide. The pH was adjusted to 7.6 using 0.1 N HCl. For preparing the enzyme stock solution 1 mg of enzyme was dissolved in 1 ml of 1 X reaction buffer. This stock solution was preserved at -20º C to avoid loss of activity. It is advisable to prepare fresh stock solution to avoid any source of error due to extended preservation of stock solution. The working dilution (1:1000) was prepared freshly just before the experiment as shown in table 3.4.

3.2.2.3 Time course measurements

Time course measurement of substrate degradation was conducted under two different conditions. In one of the conditions the substrate concentration was kept constant and in the other set of conditions the enzyme concentration was kept constant.
All the kinetic measurements were performed in clear bottom and top and black side polystyrene 96 well cell culture treated microwell plates (Corning Life Sciences Inc., Corning, NY). The reaction volume in each well was 200 µL. Table 3.4 shows the concentration of substrate and enzyme utilized to perform the time course study.

For each experiment, 200 µL of standards were added in first row each time. In each of the reaction mixture, 80 µL of 1 X reaction buffer or media was added to each well. To the reaction buffer 20 µL of diluted substrate (as per table 3.4) was added. To avoid any loss of fluorescence most of the steps associated with substrate were carried out in dark. The plate was then adjusted in the cytofluor plate reader, which was ready at 37º C. At time t = 0, the diluted enzyme (as per table 3.4) were added to each well and increase in fluorescence was measured every 2 minutes or 4 minutes depending on the substrate. The reaction mixture was incubated in plate reader for 200 minutes or 1000 minutes depending on the type of substrate.

3.2.2.4 Data analysis and comparison

The data was analyzed using Sigmaplot 9.0 Systat Software, Inc. (Point Richmond, CA). The kinetic measurements along with standards were used to determine the rate constant k_{cat} or k_2 and Michaelis - Menten constant K_M. These values were compared with the published values (Bond and Van Wart, 1984).
3.3 Results

3.3.1 Preparation of standard curve

To determine the relationship between the fluorescence intensity output of the CytoFlour 4000 (excitation of 488nm, emission of 530 nm) as a function of fluorescein concentration, a standard curve was prepared and is plotted in Figure 3.1. To obtain a range of concentrations of fluorescein, the 1µmole/ml stock solution was serially diluted. Using a Sigmaplot 9.0 Systat Software, a single rectangular hyperbola best fit the data and the relationship, and corresponding constants, are also presented in Figure 3.1.

3.3.2 Measurement of increase in fluorescence as a measure of reaction rate

DQTM substrates are over labeled with fluorescein molecules, thus they have very little or no inherent fluorescence. On exposure to enzyme, the substrate is broken down in fragments containing significantly less amount fluorescein molecules compared to original substrate. These fragments have significantly higher fluorescence compared to the original substrate as the dye quenching phenomena does not take place. Therefore, with time course measurement of increase in fluorescence, it is possible to follow the substrate degradation and determine the kinetic parameters of this reaction. Here the degradation was conducted under two different conditions. In one of the conditions the substrate concentration was kept constant and in the other set of conditions the enzyme concentration was kept constant as shown in table 3.4. All the kinetic measurements were performed in clear bottom and top and black side polystyrene 96 well cell culture treated microwell plates from Corning Life Sciences Inc. (Corning, NY). Figure 3.2 and 3.3 show a characteristic plot of increase in fluorescence (in nmoles/ml) vs. time (minutes)
for reaction at constant substrate concentration and reaction at constant enzyme concentration respectively.

### 3.3.3 Determination of kinetic parameters

Figure 3.2 and 3.3 were used to measure the rate of reaction using initial rate method. In this method only the linear portion or the initial phase of the reaction was considered to determine the rate of reaction at different enzyme concentration and substrate concentration respectively. In our analysis we employed Sigmaplot (single rectangular, 2 parameter model) to curve fit to each plot and then the initial slope was determined at time $t = 0$ using the fitted curve. This slope was then utilized to obtain Michaelis – Menten kinetic parameter for collagen degradation reaction using collagenase. Figure 3.4 shows the change in initial rate of reaction (nmoles of fluorescein/(ml.min)) vs. change in enzyme concentration (U/ml) at constant substrate concentration (100µg/ml). This plot is obtained by employing initial rate method for analysis of data used for figure 3.2. It can be observed that the rate of reaction varies linearly with increase in enzyme concentration. Figure 3.5 shows the change in initial rate of reaction (nmoles of fluorescein/(ml.min)) vs. change in substrate concentration (µg/ml) at constant enzyme concentration (0.05 U/ml). This plot is obtained by employing initial rate method for analysis of data used for figure 3.3.
3.3.4 Reproducibility of assay and determination Michaelis Menten kinetic parameter for DQ™ gelatin from porcine skin and DQ™ human collagen type IV

The equation obtained by fitting curve for figure 3.5 can be further utilized to determine the $k_{cat}$ and $K_M$. These parameters can be further utilized to predict the time requirement of enzyme dissociation of tissue to obtain single cell suspension. This liquefaction of tissue is an important step to obtain homogeneous cells populations for genetic diagnosis of cancer. To check the reproducibility of above protocol, the same assay was performed multiple times with collagenase purchased from Molecular Probes Inc. (~ 500 U), from Sigma Chemical Co. (St. Louis, MO) (~ 350 U) and from Roche Applied Science (Indianapolis, IN) (~ 250 U). These enzymes are designated at Control (~ 500 U), Sigma (~ 350 U) and Roche (~ 250 U) for the data presented below. Apart from different source of enzyme, we used different substrates like DQ™ gelatin from porcine skin and DQ™ human type IV collagen from placenta. The $k_{cat}$ and $K_M$ values for these enzymes and substrates are reported in table 3.5.

3.4 Discussion

Collagen is an important extracellular component of cancer. Jain et al have shown that collagen content of the solid cancer controls to distribution of drug molecules in the tumor (Jain, 1987). We have hypothesized that the degradation of collagen using collagenase is the rate limiting step in the preparation of single cell suspension process from solid tumor. We have attempted here to develop a simplified approach based on fluorescence to quantify the Michaelis – Menten kinetic parameters of collagen
degradation reaction. There are many other assays utilized to detect the activity of collagenase. Synthetic substrates like N-[3-(2-Furyl)acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine (FALGPA) (Van Wart and Steinbrink, 1981) and 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-d-Arg (Pz-PLGPR) (Wuensch and Heidrich, 1963) are routinely used for reporting the unit of activity of collagenase. These units are not suitable to directly correlate the kinetic parameter with collagen content of the human tissue samples like breast tumor or porcine pancreas. Mookhtiar et al have reported preparation of radiolabeled collagen to determine the kinetic parameter of collagenase (Mookhtiar et al., 1986). This assay is very sensitive and can determine the kinetic data that can be correlated with tissue dissociation. However radioactivity is inherently hazardous, labor intensive and expensive tool (Grubb, 1994). Another way to characterize the activity is to analyze the activity using insoluble tendon collagen. This method will provide most accurate kinetic parameters due to no modification of collagen; however this method involves sampling at multiple time points, careful control of sample volume and separation of undigested protein by precipitation. All these steps may lead to unforced errors. O’Grady et al have reported preparation of collagen substrate with a fluorescent compound 2-methoxy-2, 4-diphenyl-3(2H)-furanone (O'Grady et al., 1984). This substrate is fluoresces over a wide range of pH and looses its fluorescence when subjected to metalloproteases as 2-methoxy-2, 4-diphenyl-3(2H)-furanone is not fluorescent in unbound form. This method overcomes most of the challenges discussed above and multiple samples can be processed simultaneously. However this assay is not as sensitive as radioactivity assay as very small change in fluorescence are difficult to observe when minute quantity enzyme is to be detected (Grubb, 1994).
The above disadvantages associated with synthetic substrates and radiolabeled substrate was overcome by using dye quench gelatin or collagen substrate for quantification of Michaelis – Menten kinetic parameters for *C. histolyticum* collagenases (Haugland, 1996). Here the dye labeled collagen has significantly less fluorescence due to fluorescence quenching. This was confirmed by blank run for 16 hours (data not shown). The fluorescence increases on degradation of protein substrate, which was measured with respect to time to quantify the kinetic parameters. The fluorescence units were converted to molar unit by comparing the fluorescence units with standard plot shown in figure 3.1. The rates of fluorescence increase were measured from figure 3.2 and figure 3.3 using initial rate method. These measurements were plotted as shown in figure 3.4 and figure 3.5 to determine the kinetic parameters shown in table 3.5. Thompson, Jones and Voss *et al* have used similar approach to quantify the activity of different proteases using BODIPY-FL labeled casein and FITC labeled BSA (Jones et al., 1997; Thompson et al., 2000; Voss et al., 1996).

Bacterial collagenases are commonly utilized in life sciences laboratory for digestion of various tissues. The single cell suspension product is widely used for genetic analysis, cell line development work and transplantation of various cellular components (Ghossein and Bhattacharya, 2000; Ricordi et al., 1988; Stingl et al., 2005). There are multiple companies producing these enzymes and majority of them report the activity in terms of synthetic peptides. This mode of measurements does not facilitate comparison among different products as different enzyme components have different activities towards synthetic substrate as shown in table 3.2 and 3.3. Also, the composition of individual components of enzyme varies from lot to lot and is generally unknown. Thus,
the kinetic parameter reported by Bond *et al* for different components can not be utilized unless weight percentage of each component is known in crude enzyme. The approach proposed here considers both these short comings. We have shown here comparison among three crude enzyme products from different manufacturers and compared the kinetic parameters for three different substrates as shown in table 3.5. This kind of comparison assists researchers to make a quantitative decision about the crude enzyme selection. Such simple approach is very useful to quantify highly empirical enzyme digestion process based on simple enzyme kinetics and reaction diffusion model.
<table>
<thead>
<tr>
<th></th>
<th>Class</th>
<th>Subclass</th>
<th>Molecular weight</th>
<th>Isoelectric point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>α₁</td>
<td>68,000</td>
<td>5.85</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>α₂</td>
<td>68,000</td>
<td>5.9</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td>β</td>
<td>115,000</td>
<td>5.55, 5.60, 5.75</td>
</tr>
<tr>
<td>4</td>
<td>I</td>
<td>γ₁</td>
<td>79,000</td>
<td>6.10</td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td>γ₂</td>
<td>79,000</td>
<td>6.20</td>
</tr>
<tr>
<td>6</td>
<td>II</td>
<td>δ</td>
<td>100,000</td>
<td>5.80</td>
</tr>
<tr>
<td>7</td>
<td>II</td>
<td>ε</td>
<td>110,000</td>
<td>5.90, 5.95</td>
</tr>
<tr>
<td>8</td>
<td>II</td>
<td>ζ</td>
<td>125,000</td>
<td>5.35</td>
</tr>
</tbody>
</table>

Table 3.1 Physicochemical properties of different collagenase classes (Bond and Van Wart, 1984)
<table>
<thead>
<tr>
<th>Supplier</th>
<th>Designation</th>
<th>Lot #</th>
<th>mg of protein/mg of crude powder&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Crude preparation</th>
<th>[&lt;sup&gt;14&lt;/sup&gt;CH₃]-collagen</th>
<th>FALGPA</th>
<th>BAEE</th>
<th>[&lt;sup&gt;14&lt;/sup&gt;CH₃]-casein&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma Chemical Co.</td>
<td>Type I</td>
<td>121F-516</td>
<td>0.37</td>
<td></td>
<td>0.0029</td>
<td>2.8</td>
<td>540</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Type IA</td>
<td>101F-6831</td>
<td>0.46</td>
<td></td>
<td>0.0013</td>
<td>1.0</td>
<td>25</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>91F-6812</td>
<td>0.26</td>
<td></td>
<td>0.00045</td>
<td>0.30</td>
<td>58</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Type IV</td>
<td>42F-6838</td>
<td>0.43</td>
<td></td>
<td>0.0023</td>
<td>3.0</td>
<td>77</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Type V</td>
<td>11F-6805</td>
<td>0.43</td>
<td></td>
<td>0.0033</td>
<td>2.7</td>
<td>160</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>Z-9999</td>
<td>81F-6819</td>
<td>0.37</td>
<td></td>
<td>0.0038</td>
<td>4.8</td>
<td>98</td>
<td>0.80</td>
</tr>
<tr>
<td>Worthington Biochemical Co.</td>
<td>Type I</td>
<td>42C008</td>
<td>0.46</td>
<td></td>
<td>0.0022</td>
<td>2.6</td>
<td>250</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>W2H209</td>
<td>0.33</td>
<td></td>
<td>0.0023</td>
<td>3.2</td>
<td>650</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Type III</td>
<td>41S130</td>
<td>0.36</td>
<td></td>
<td>0.0023</td>
<td>2.7</td>
<td>36</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Type IV</td>
<td>41S123</td>
<td>0.74</td>
<td></td>
<td>0.0024</td>
<td>2.4</td>
<td>490</td>
<td>0.27</td>
</tr>
<tr>
<td>Roche-Manheim</td>
<td>None</td>
<td>1451142</td>
<td>0.39</td>
<td></td>
<td>0.0023</td>
<td>2.3</td>
<td>440</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 3.2 Enzyme activities of crude collagenases from commercial source (Bond MD 1984):

<sup>a</sup>The milligrams of crude powder was determined by weighing the preparation, as received, while the milligram of protein was determined by the dye binding assay. <sup>b</sup>sp. Act. is less than 0.0010 nkat/mg.
Table 3.2 continued

<table>
<thead>
<tr>
<th>Advanced Biofactures Corp.</th>
<th>ABC-TD</th>
<th>111-051082</th>
<th>0.23</th>
<th>0.0020</th>
<th>4.9</th>
<th>43</th>
<th>0.17</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC-I</td>
<td>P-78R</td>
<td>0.20</td>
<td>0.00060</td>
<td>0.65</td>
<td>100</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>ABC-II</td>
<td>P-79-02R</td>
<td>0.17</td>
<td>0.0010</td>
<td>0.76</td>
<td>120</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>234,222</td>
<td>103310</td>
<td>0.12</td>
<td>0.00065</td>
<td>1.2</td>
<td>130</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>23,415</td>
<td>102364</td>
<td>0.20</td>
<td>0.0013</td>
<td>2.9</td>
<td>44</td>
<td>0.19</td>
<td></td>
</tr>
</tbody>
</table>

**Partially purified preparation**

<table>
<thead>
<tr>
<th>Sigma Chemical Co.</th>
<th>Type III</th>
<th>61D-0566</th>
<th>0.90</th>
<th>0.0077</th>
<th>7.8</th>
<th>720</th>
<th>0.087</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worthington Biochemical Co.</td>
<td>Type VII</td>
<td>111F-6831</td>
<td>0.77</td>
<td>0.0017</td>
<td>78</td>
<td>5.5</td>
<td>0.0011</td>
</tr>
<tr>
<td></td>
<td>CLOSSA</td>
<td>51H319</td>
<td>0.49</td>
<td>0.00065</td>
<td>38</td>
<td>1.3</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>CLSPA</td>
<td>W2J452</td>
<td>0.72</td>
<td>0.012</td>
<td>11</td>
<td>72</td>
<td>b</td>
</tr>
<tr>
<td>Advanced Biofactures Corp.</td>
<td>ABC-III</td>
<td>186E</td>
<td>0.11</td>
<td>0.020</td>
<td>19</td>
<td>3.0</td>
<td>b</td>
</tr>
<tr>
<td>Calbiochem-Behring Corp.</td>
<td>234,136</td>
<td>130075</td>
<td>0.15</td>
<td>0.0092</td>
<td>8.0</td>
<td>250</td>
<td>0.0075</td>
</tr>
<tr>
<td></td>
<td>$[^14]CH_3$ collagen$^a$</td>
<td>$[^14]CH_3$ gelatin$^a$</td>
<td>FALGPP</td>
<td>FALGPA</td>
<td>PZ peptide$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td>---------</td>
<td>---------</td>
<td>------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nkat/mg</td>
<td>nkat/nm</td>
<td>nkat/mg</td>
<td>nkat/nm</td>
<td>nkat/mg</td>
<td>nkat/nm</td>
<td>nkat/mg</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>0.023</td>
<td>0.0016</td>
<td>0.21</td>
<td>0.014</td>
<td>46</td>
<td>3.1</td>
<td>7.3</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>0.022</td>
<td>0.0015</td>
<td>0.20</td>
<td>0.013</td>
<td>46</td>
<td>3.1</td>
<td>7.3</td>
</tr>
<tr>
<td>$\beta$</td>
<td>0.017</td>
<td>0.0019</td>
<td>0.095</td>
<td>0.011</td>
<td>32</td>
<td>3.7</td>
<td>5.1</td>
</tr>
<tr>
<td>$\gamma_1$</td>
<td>0.02</td>
<td>0.0015</td>
<td>0.12</td>
<td>0.0095</td>
<td>43</td>
<td>3.4</td>
<td>6.0</td>
</tr>
<tr>
<td>$\gamma_2$</td>
<td>0.021</td>
<td>0.0017</td>
<td>0.19</td>
<td>0.015</td>
<td>42</td>
<td>3.3</td>
<td>5.2</td>
</tr>
<tr>
<td>$\delta$</td>
<td>0.0069</td>
<td>0.00069</td>
<td>0.048</td>
<td>0.0048</td>
<td>100</td>
<td>10</td>
<td>48</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>0.0054</td>
<td>0.00060</td>
<td>0.026</td>
<td>0.0029</td>
<td>140</td>
<td>15</td>
<td>71</td>
</tr>
<tr>
<td>$\zeta$</td>
<td>0.0059</td>
<td>0.00073</td>
<td>0.027</td>
<td>0.0034</td>
<td>77</td>
<td>9.7</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 3.3 Enzyme activities of different collagenase classes (Bond MD 1984)

$^a$Activities for both collagen and gelatin were calculated from a substrate Mr of 300,000. $^b$p-Phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg
Table 3.4 Concentration and dilution ratios of stock solutions to perform time course study at constant substrate concentration and constant enzyme concentration

<table>
<thead>
<tr>
<th>Serial #</th>
<th>[Collagen] or [Gelatin] (in µg/ml)</th>
<th>[Collagenase] (in U/ml)</th>
<th>Volume of collagen or gelatin stock solution (in µL)</th>
<th>Volume of reaction buffer (in µL)</th>
<th>Volume of collagenase working reagent (in µL)</th>
<th>Volume of reaction buffer (in µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>0.0125</td>
<td>20</td>
<td>0</td>
<td>2.5</td>
<td>97.5</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>0.025</td>
<td>20</td>
<td>0</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>0.05</td>
<td>20</td>
<td>0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>0.1</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>0.2</td>
<td>20</td>
<td>0</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serial #</th>
<th>[Collagen] or [Gelatin] (in µg/ml)</th>
<th>[Collagenase] (in U/ml)</th>
<th>Volume of collagen or gelatin stock solution (in µL)</th>
<th>Volume of reaction buffer (in µL)</th>
<th>Volume of collagenase working reagent (in µL)</th>
<th>Volume of reaction buffer (in µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0.05</td>
<td>20</td>
<td>0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>0.05</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>0.05</td>
<td>5</td>
<td>15</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>12.5</td>
<td>0.05</td>
<td>2.5</td>
<td>17.5</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>6.25</td>
<td>0.05</td>
<td>1.25</td>
<td>18.75</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>3.125</td>
<td>0.05</td>
<td>5 µL of 12.5 µg/ml dilution</td>
<td>15</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>1.5625</td>
<td>0.05</td>
<td>2.5 µL of 12.5 µg/ml dilution</td>
<td>17.5</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Substrate</td>
<td>Control (~500 U)</td>
<td>Sigma (~350 U)</td>
<td>Roche (~250 U)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------------</td>
<td>----------------</td>
<td>----------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Units</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>k&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>K&lt;sub&gt;M&lt;/sub&gt;</td>
<td>Slope of v vs. E&lt;sub&gt;0&lt;/sub&gt;</td>
<td>k&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>K&lt;sub&gt;M&lt;/sub&gt;</td>
<td>Slope of v vs. E&lt;sub&gt;0&lt;/sub&gt;</td>
</tr>
<tr>
<td>porcine gelatin</td>
<td>Mean</td>
<td>22</td>
<td>4.5</td>
<td>0.2</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>σ</td>
<td>7</td>
<td>0.1</td>
<td>0.1</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>bovine collagen type I</td>
<td>Mean</td>
<td>0.4</td>
<td>6</td>
<td>0.007</td>
<td>0.9</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>σ</td>
<td>0.1</td>
<td>1</td>
<td>0.002</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>human collagen type IV</td>
<td>Mean</td>
<td>0.3</td>
<td>24</td>
<td>0.004</td>
<td>0.60</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>σ</td>
<td>0.1</td>
<td>4</td>
<td>0.001</td>
<td>0.1</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 3.5 Michaelis – Menten kinetic parameters (k<sub>cat</sub> and K<sub>M</sub>) for digestion of DQ<sup>™</sup> gelatin from porcine skin, DQ<sup>™</sup> type I collagen bovine skin and DQ<sup>™</sup> human type IV collagen from placenta using Control (~ 500 U), Sigma (~ 350 U) and Roche (~ 250 U)
Figure 3.1 Standard plot of Fluorescence unit vs. concentration of fluorescein in nmoles/ml. The stock solution of fluorescein was prepared in reaction buffer and diluted in two fold serial dilution. The working volume per reaction well was 200µL and the fluorescence was measured at 37°C using cytofluor® 4000 fluorescence multi-well plate reader at excitation of 485 nm and emission at 530 nm. The standard curve data can be fitted with $y = \frac{55612.78x}{16.01 + x}$
Figure 3.2 Plot of concentration of fluorescein (nmoles of fluorescein/ml) vs. time (minutes) show increase in fluorescence due to fluorescein on degradation of substrate at constant substrate concentration. The substrate was DQ™ bovine collagen type I was digested using control (~ 500 U) collagenase provided by Molecular Probes Inc. (Eugene, OR). The reaction was carried out at 7.5 pH and 37°C
Figure 3.3 Plot of concentration of fluorescein (nmoles of fluorescein/ml) vs. time (minutes) show increase in fluorescence due to fluorescein on degradation of substrate at constant enzyme concentration of 0.05 U/ml. The substrate was DQ™ bovine collagen type I was digested using control (~ 500 U) collagenase provided by Molecular Probes Inc. (Eugene, OR). The reaction was carried out at 7.5 pH and 37°C.
Figure 3.4 Plot of rate of reaction (v, nmoles/(ml.min)) vs. enzyme concentration ([E₀], U/ml) at constant substrate concentration of 100 µg/ml. The rate of reaction is obtained by applying initial rate method on the data utilized to plot figure 3.2. Here the substrate is DQ® bovine type I collagen and the enzyme is control (~ 500 U) collagenase purchased from Molecular Probes Inc. The reaction was carried at pH 7.5 and temperature 37°C. This plot can be fitted using $v = 0.0069[E₀] - 3.92 \times 10^{-5}$ as shown in the figure.
Figure 3.5 Plot of rate of reaction (v, nmol/(ml.min)) vs. substrate concentration ([S], µg/ml) at constant enzyme concentration of 0.05 U/ml. The rate of reaction is obtained by applying initial rate method on the data utilized to plot figure 3.3. Here the substrate is DQ™ bovine type I collagen and the enzyme is control (~ 500 U) collagenase purchased from Molecular Probes Inc. The reaction was carried at pH 7.5 and temperature 37°C. This plot can be fitted using $v = 0.0004[S] / (4.58 + [S])$ as shown in the figure.
CHAPTER 4

OPTIMIZATION OF ENZYME DISSOCIATION PROTOCOL BASED ON REACTION DIFFUSION MODEL TO PREDICT TIME OF TISSUE DIGESTION

The content of this chapter is prepared for publication: Mehta, BC; Chalmers, JJC; Optimization of Enzyme Dissociation Protocol Based on Reaction Diffusion Model to Predict Time of Tissue Digestion.

4.1 Introduction

Breast cancers are a major health burden worldwide. It is the principal cause of death in women. It is estimated that one million out of 10 million cases of neoplasm detected in both sexes are breast cancer. In 2001, 375,000 women died because of the breast cancer. Breast cancer is the second most common tumor after lung cancer in both sexes (Bray F, 2004). USA is one of the high-risk areas of breast cancer. As per the GLOBOCAN 2000 survey 184,000 new cases of breast cancer were detected in US in year 2000. In the same year 45,500 women died because of breast cancer in US (Bray F, 2001).

With the advent and improvement of highly sensitive and accurate molecular analysis technologies, it is possible to study increasingly complex cellular processes
including cell differentiation and proliferation based on molecular markers from normal and malignant tissue source (St Croix et al., 2000). These technologies have played a significant role in diagnosis of cancer. However, a common question/complaint of studies using these technologies is the homogeneity of the original sample (Sieben NL, 2000; Tomlinson IP, 2002). The lack of it can affect the desired final outcome dramatically. For example, ovarian carcinoma samples from patient or cell samples taken from cell culture (HOC-1 or MCF-7) when analyzed in a microarray, express variety of RNA, and RNA levels suggesting various stages of the cell cycle, which may or may not be related to the true process studied (Mackillop et al., 1982; Podhajcer et al., 1986; Yang et al., 1977). Consequently, the overall expression pattern might not represent any one single cell, but a “mean” of the entire population (Ko et al., 2000). The lack of homogeneity is even more of a problem when one works with non-cultured, three-dimensional tissue, which normally consists of a variety of different cell types, and in different states. Therefore, the ability to analyze and separate a heterogeneous cell population based on cellular characteristics is a significant analytical and preparative resource for molecular analysis techniques (Pantel et al., 1999).

Currently, the majority of the breast cancer samples for cancer diagnosis are prepared either by pulverizing whole frozen tumors or by laser capture microdissection (Emmert-Buck MR, 1996). These samples are then analyzed using immunohistochemistry, RT-PCR or PCR (Ghossein and Bhattacharya, 2000; Pantel et al., 1999; Wellings et al., 1975). With pulverization and laser capture microscopy, it is not possible to separate intact, viable cell populations from tissue. But it is possible to separate cell populations based on molecular profiles of the cells, from single cell
suspension prepared from tissue, using cell sorting techniques like flow sorting or magnetic sorting. (Chalmers et al., 1998; Martin et al., 1998; Shapiro, 2003; Zborowski et al., 1995). Such sorting techniques are routinely applied to recover epithelial or stromal components from solid breast tumor for genetic analysis or to isolate islets of Langerhan from pancreas for allogenic transplantation (Ghossein and Bhattacharya, 2000; Ricordi et al., 1988). It is necessary to dissociate these tissues to single cell suspension to recover the cells of interest. Multiple dissociation techniques like enzyme dissociation, mechanical dissociation or both combined are commonly employed for these applications (Stingl et al., 2005). Among these dissociation techniques, enzyme dissociation is the most widely applied technique. The principle of enzyme dissociation is simple; the extracellular matrix (ECM) proteins of the tissue are subjected to matrix metalloproteases that selectively digest these proteins. For applications, like tumor dissociation or islet isolation, these enzymes are derived from either bacterial source or from animal source (Seifter, 1970). Currently, there is no standard approach available to dissociate all the tissue samples. Moreover, all the current approaches are developed based on substantial empirical knowledge and there is no underlying first principle or theory behind this process (Allalunis-Turner MJ, 1986; Costa A, 1987; Curry MP, 2000; Dairkee et al., 1997; Eade OE, 1981; Engelholm SA, 1985; Gomm et al., 1995; Hamburger et al., 1982; Haraldsen G, 1995; Keng PC, 1998; Lee TK, 1993; Pechoux et al., 1999; Penning JJ, 1981; Queral AE, 1984; Visscher DW, 1994; Zavros Y, 2000). This study attempts to understand and formulate ECM digestion of breast tissue based on enzyme kinetics and reaction diffusion model.
There are a number of enzymes available specific for different ECM components to perform the task of tissue digestion. The goal here is to model the process based on the most important component of ECM that will be rate limiting step in tissue digestion and will govern the time of digestion. The ECM of breast tissue is predominantly made up of collagen, elastin, glycoproteins and proteoglycans (Hay, 1991; Ronnov-Jessen L, 1996; Wellings et al., 1975). Apart from ECM components, significant fat depositions also participate in breast tissue architecture. Among all these components, collagen is vital for providing the structural stability to breast tissue (Pucci Minafra et al., 1985). Based on these observations, we have hypothesized that digestion of collagen using bacterial collagenase is the rate limiting step and we have attempted to model collagen digestion to predict the overall time of tissue digestion.

4.2 Theory

Breast tissue is made up of a heterogeneous mixture of cells and ECM. The cellular components are suspended in ECM. In simple terms, a tissue can be considered as a porous matrix (Truskey GA, 2004). The structure of this porous matrix is made up of collagen and elastin fibers, where as the intricacies are filled with gel material made up of proteoglycans and glycoproteins. This gel binds collagen molecules with other components of ECM (Hay, 1991). Therefore, the success of enzyme digestion process will depend on how efficiently the enzyme can degrade the structural components of tissue i.e. the collagen molecules. It is necessary for the enzyme to diffuse in the bulk tissue to attack the collagen matrix and degrade it. There are two separate phenomena involved in this process: first is a transport phenomenon where enzyme has to overcome
the resistances offered by the tissue structure and second is a reaction rate dependent on the activity of enzyme.

This process is similar to reaction between a fluid (Enzyme) and a solid (Tissue) or a component of solid (Collagen) (For example coal gasification or ore processing). Therefore, enzyme digestion process can be modeled in similar fashion as Gas – Solid reactions. This reaction system is essentially non steady state in nature as the solid (collagen from tissue) is also consumed during the process. Such system can be quantitatively evaluated using different models like heterogeneous shrinking core model, general model, truly homogeneous model or grain model depending on the rate of fluid (enzyme) transport and rate of solid (collagen) consumption (Froment GF, 1990). In all these models the information about fluid and solid components are utilized. Due to unknown nature of solid components (cancer samples), we have simplified this model by making simplifying assumptions. Here we have assumed that for a small change in time the concentration of solid (collagen) does not change substantially, the diffusivity of the solid does not change substantially over a period of time. Based on these assumptions, the system was modeled on simple reaction diffusion model.

For this problem, geometry of tissue will depend on how the tissue is minced before subjecting it to enzyme dissociation process. In this study the tissue is minced in thin semi – infinite slabs as shown in figure 4.1. Therefore, the enzyme (A) is diffusing in positive x – direction as shown in figure 4.1. The diffusion of enzyme in the tissue can be modeled by applying an equation of conservation of mass on the enzyme (Truskey GA, 2004)
\[
\frac{\partial C}{\partial t} = -\nabla \cdot N + (\Phi_B - \Phi_L) + R_{rxn}
\]  \hspace{1cm} (4.1)

where \( C \) is the concentration of enzyme based on tissue volume, \( N \) is the enzyme flux in tissue due to convection and diffusion, \( \Phi_B \) is the rate of enzyme transport across a blood vessel per unit volume of tissue, \( \Phi_L \) is the rate of enzyme transport across a lymph vessel per unit volume of tissue and \( R_{rxn} \) is the rate of collagen degradation using collagenase.

Tissue digestion process is an in vitro process; therefore \( \Phi_B, \Phi_L \) and convective flux term in \( N \) are zero. To simplify equation (4.1), it was also assumed that the tissue is an isotropic and uniform material. Applying these simplifications on equation (4.1)

\[
\frac{\partial C}{\partial t} = D_{sij} \nabla^2 C + R_{rxn}
\]  \hspace{1cm} (4.2)

The reaction term here represents the consumption of collagen, which can be assumed to follow Michaelis – Menten kinetics,

\[
R_{rxn} = \frac{k_{cat} \cdot C \cdot C_T}{K_M + C_S}
\]  \hspace{1cm} (4.3)

where \( k_{cat} \) and \( K_M \) are rate constant and Michaelis – Menten constants respectively and \( C_S \) is concentration of collagen in the tissue. For small time at the beginning of process, it can be assumed that \( C_S \) is constant, thus equation (4.3) can be simplified as,

\[
R_{rxn} = k' C
\]  \hspace{1cm} (4.4)

where, \( k' = \frac{k_{cat} \cdot C_S}{K_M + C_S} = \text{constant} \)  \hspace{1cm} (4.5)

Substituting equation (4.5) in equation (4.2);
\[
\frac{\partial C}{\partial t} = D_{\text{eff}} \nabla^2 C - k'C
\]  \hspace{1cm} (4.6)

The negative sign is due to consumption of enzyme as it penetrates through the tissue matrix. The boundary conditions for this reaction diffusion model are

I.C. \hspace{0.5cm} t = 0, \ C = 0 \text{ for all } x = 0 \hspace{1cm} (4.7)

B.C. \hspace{0.5cm} t > 0, \ C = C_0 \text{ for all } x = 0 \hspace{1cm} (4.8)

Equation (4.6) can be solved analytically with initial conditions and boundary conditions given in equation (4.7) and (4.8). The analytical solution is (Crank, 1975)

\[
C(x,t) = k' \int_0^t C_1 \text{erfc} \left( \frac{x}{2\sqrt{D_{\text{eff}}t'}} \right) e^{-k't'} dt' + C_0 \text{erfc} \left( \frac{x}{2\sqrt{Dt}} \right) e^{-k't} \hspace{1cm} (4.9)
\]

Where \( C_1 \) is analytical solution (4.11) of simple diffusion equation (4.10) for the same initial and boundary conditions given by equation (4.7) and (4.8)

\[
\frac{\partial C}{\partial t} = D_{\text{eff}} \frac{\partial^2 C}{\partial x^2} \hspace{1cm} (4.10)
\]

\[
C_1(x,t) = C_0 \text{erfc} \left( \frac{x}{2\sqrt{Dt}} \right) \hspace{1cm} (4.11)
\]

The solution of equation (4.6) can be obtained by substituting equation (4.11) in equation (4.9). This equation can be plotted analytically to obtain the spatial distribution of enzyme concentration in tissue slab with respect to time.
4.3 Materials and methods

4.3.1 Breast tissue procurement

Fresh and frozen human normal and malignant breast tissue were procured from Tissue Procurement Shared Resource (TPSR) at The Ohio State University. Tissue donors ranged from 17 to 85 years old. Breast tissue was collected as per the guidelines and regulations set forth by the Office of Responsible Research Practices Institutional Review Board for human subjects at The Ohio State University (IRB# 2000C0330). The frozen tissue was snap frozen in liquid nitrogen. The fresh tissue was supplied Dulbecco’s Modified Eagle Medium/Ham’s F-12 media with penicillin/streptomycin, amphotericin B and 10% fetal bovine serum (all from Gibco, Invitrogen, Carlsbad, CA). Prior to tissue digestion, a small piece of tissue was frozen for collagen quantification. The tissue was washed 3X in sterile Dulbecco’s phosphate buffered saline (D-PBS) (Gibco, Invitrogen, Carlsbad, CA) before tissue digestion.

4.3.2 Qualitative analysis of breast tissue for collagen content

The collagen and elastin distribution in the breast tissue was analyzed using histological methods. The collagen distribution was analyzed using Masson trichrome staining and elastin distribution was analyzed using Verhoeff’s staining (Bancroft and Stevens, 1996). These two techniques are standard histological techniques and were performed at Pathology core facility at The Ohio State University. Briefly, for Masson trichrome staining, the tissue was first embedded in paraffin block and 4 μm sections were cut. These sections were then dewaxed and brought in water. The nuclei were then stained with Celestin blue-haemalum method; muscles, cytoplasm, fibrin etc. were
stained with acid fuchsin solution A and collagen fibers were stained with methyl blue solution C. The stained slide was dehydrated through alcohol series, cleared in xylene and mounted in DPX. For Verhoeff’s staining, the tissue sections were brought in water and were stained with alcoholic haematoxylin in ferric chloride solution. The expression of elastin was differentiated using 2% ferric chloride solution. The slide was then counter stained with Van Gieson stain. The stained slide was dehydrated through alcohol series, cleared in xylene and mounted in DPX. These slides were observed under Zeiss Axioskop widefield light microscope (Bancroft and Stevens, 1996).

4.3.3 Quantification of collagen in breast tissue

The concentration of collagen in breast tissue based on wet tissue weight was determined by converting collagen to hydroxyproline (Woessner, 1961). This is a colorimetric assay, which is widely used for quantification of collagen in tissue samples and urine samples. Briefly, a small amount of wet tissue (~1-3 mg) was weighed and digested completely in 16 hours at 37°C with 100 µl of 2 mg/ml of collagenase dissolved in reaction buffer (solution containing 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂ and 0.2 mM sodium azide, pH 7.6). This digested tissue was then hydrolyzed using 6 N HCl at 130°C for 3 hours. The content was then allowed to cool down at room temperature and acid was neutralized using 2.5 N NaOH with 0.02 % methyl red as an indicator. The reaction mixture was then diluted with distilled water (~ 4 ml). The hydroxyproline in reaction mixture was oxidized using 1 ml chloramine T solution for 20 minutes at room temperature. The oxidation reaction was stopped using 1 ml of 70 % perchloric acid for 5 minutes at room temperature. This reaction mixture was then further treated with p-
diaminobenzaldehyde to produce chromophore at 60°C for 20 minutes (all chemicals from Sigma, St Louis, MU). The reaction mixture was cooled down to room temperature under tap water. The developed color was measured using Shimadzu UV – visible spectrophotometer (Shimadzu UV – 1601, Columbia, MD). The tissue collagen concentration of 17 tissue samples (6 invasive ductal carcinoma samples, 2 breast carcinoma samples, 7 macromastia samples, 1 lobular carcinoma sample and 1 adenocarcinoma sample) was determined by comparing the absorbance at 557 nm with collagen and hydroxyproline standard as shown in table 4.1 (Woessner, 1961).

**4.3.4 Enzyme kinetics of collagenase**

Bacterial collagenase is an active enzyme used for collagen degradation to liquefy a solid tumor. Collagenase consists of number of components with different activity towards collagen (Bond MD, 1984; Bond MD, 1984; Mallya et al., 1986). The kinetic parameters reported by Mallaya *et al* for individual collagenase components could not be used here due to unknown weight fraction of these components in enzyme provided by suppliers. Also the enzyme activity information provided by suppliers is not useful as it is measured in terms of synthetic substrates and the conversion in term of collagen digestion units is not straight forward (Van Wart and Steinbrink, 1981; Wuensch and Heidrich, 1963). To overcome these challenges, we have designed a novel technique to quantify the Michaelis – Menten kinetics in solution based on Dye Quenched (DQ™) bovine collagen type I and human collagen type IV substrates (Mehta, 2006).

Briefly, the substrate DQ™ collagen (Molecular Probes, Eugene, OR) was dispensed (20 µl) in individual wells of 96 well plate in concentration range from 1.5625
µg/ml to 100 µg/ml. This substrate was further diluted using 80 µl reaction buffer (solution containing 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂ and 0.2 mM sodium azide, pH 7.6, all chemicals from Sigma, St Louis, MU). The reaction was started at time \( t = 0 \), by adding 100 µl of collagenase (Sigma, St Louis, MU) in concentration range from 0 to 0.2 U/ml. On degradation, the collagen substrate released small polypeptides bound to fluorescein isothiocyanate (FITC), which increased the fluorescence level of the solution. This increase in fluorescence was quantified using FITC standard with respect to time, which was utilized to quantify the rate of reaction based on method of initial rate (Fogler, 2006). The reaction rate information was utilized to determine the Michaelis–Menten kinetics parameters for collagen digestion in solution, which allowed comparison of enzymes from different suppliers (Mehta, 2006).

### 4.3.5 Experimental design to determine the effect of enzyme concentration and diffusion on tissue digestion

The determination of above parameters assisted in predicting the diffusion of enzyme in breast tissue. It was compared with experiments by procuring breast tissue samples from Tissue Procurement Shared Resource (TPSR) at The Ohio State University. These tissue samples were then minced in a tissue mincer designed in our lab to obtain uniform thin slabs of thickness 0.690 mm and 5.38 mm respectively. These tissue fragments were then subjected to two different enzyme concentrations of 2 mg/ml and 10 mg/ml in 10 ml of DMEM/F12 medium with 10% FBS and 25 µg/ml of DNAse to digest stray DNA. The tissue fragments in enzyme solution were digested in T25 flask, which was agitated on a shaker. The end point of this digestion was the amount of time it took...
to digest the tissue completely. During digestion, images were taken using Zeiss Axiocam inverted microscope to observe the digestion of the tissue.

4.4 Results

4.4.1 Qualitative analysis of breast tissue for collagen content

Masson trichrome staining of breast tissue sample is showed in figure 4.2 (A – D), where collagen is labeled blue and nuclei black. Figure 4.2 (A) showed considerable amount of collagen in tissue section. The collagen was well interspersed with cells as shown in figure 4.2 (B – D). This tissue sample was procured from patient suffering invasion ductal carcinoma. This was confirmed based on random distribution of cells in ECM (figure 4.2 (B – D)). The collagen concentration is the breast tissue was qualitatively compared with elastin concentration. Elastin is another component of ECM responsible for tissue architecture. The black fibers (four point star) in figure 4.3 (C, D) represented the elastin concentration and the pink fibers (five point star) represented the collagen concentration. It was observed qualitatively that collagen concentration is substantially higher than the elastin concentration. Figure 4.3 (A, B) also confirmed the random distribution of cells (nuclei – bluish purple) as observed in figure 4.2 (B – D). These qualitative results supported the hypothesis that in breast cancer collagen digestion will be the important parameter to predict the time of tissue digestion.

4.4.2 Quantification of collagen in breast tissue

To prove the hypothesis it was necessary to quantify the collagen concentration in the tissue and to determine the collagen degradation kinetics. These values would be
utilized to predict the time of tissue digestion. The collagen concentration was quantified by hydrolyzing collagen to hydroxyproline and then converting hydroxyproline to chromophore, which was detected at 557 nm. Figure 4.4 and 4.5 represented the standard plot prepared for hydroxyproline and bovine type I collagen respectively. Based on these two linear plots, it was determined that 7.4 µg of hydroxyproline is present per µg of bovine type I collagen. This ratio is in accordance to 6.8 µg of hydroxyproline present per µg of bovine type I collagen determined by Netti et al (Netti PA, 2000). Figure 4.4 and 4.5 was then utilized to quantify the collagen concentration based on wet tissue weight. Figure 4.6 showed distribution of ratio of collagen to wet tissue weight for 17 breast tissue samples. The average concentration of collagen was 53 ± 37 µg of collagen per mg of wet tissue. This concentration was then utilized along with enzyme kinetics parameters to quantify the time of collagen digestion.

4.4.3 Enzyme kinetics of collagenase

Kinetics of collagen degradation was determined by real time measurement of increase in fluorescence DQ™ collagen substrate. The fluorescence of FITC molecule increased with time when DQ™ collagen was digested using collagenase and polypeptide fragments containing the FITC molecule was released in solution. This method was used to obtain profiles of increase in fluorescence at varying enzyme concentration for constant substrate concentration and at varying substrate concentration for constant enzyme concentration (Mehta, 2006). These profiles were then employed to determine the rate of reaction by applying method of initial rates. Figure 4.7 showed the linear increase in reaction rate with increase in enzyme concentration at constant substrate
concentration and figure 4.8 showed single rectangular hyperbola (2 parameters) profile of change in reaction rate with substrate concentration at constant enzyme concentration. This behavior can be explained by Michaelis – Menten kinetics. Table 4.2 represented the average kinetic parameters for bovine type I collagen degradation and human type IV collagen degradation with three different collagen enzyme source from Molecular probes (500 U), Sigma (~350 U) and Roche (~250 U). It can be observed that $k_{\text{cat}}$ did not changed significantly with change in type of collagen and with change in source of collagenase. Also, the $K_M$ did not change with source of enzyme but did changed significantly with type of collagen. We have used average $k_{\text{cat}}$ and $K_M$ values determined for type I collagen using all source of enzyme to calculate the time of collagen digestion in solution.

### 4.4.4 Calculation of time of collagen digestion in solution

Based on solution kinetics determined for collagenase, time of collagen digestion was calculated using analytical solution of Michaelis – Menten kinetics. Figure 4.9 (a and b) represented the time of digestion when initial collagen concentration is 53 µg and 530 µg respectively for different initial collagenase concentration ranging from 0.1 mg/ml to 10 mg/ml. The different plots showed change in time of collagen digestion for different conversions (filled dots = 25%, open dots = 50%, filled inverted triangle = 75% and open triangle = 87.5%). It was observed that increase in concentration above 2 mg/ml did not have significant effect on time of collagen digestion for both the cases. At 2 mg/ml the time of collagen digestion for 53 µg and 530 µg cases at 87.5 % conversion was 11.13 minutes and 111.38 minutes respectively. These time of digestion were much less than
the actual time observed for mammary tissue digestion, which ranged anywhere from 1 hour to 16 hour (Costa et al., 1987; Dairkee et al., 1997; Gomm et al., 1995; Pechoux et al., 1999). This discrepancy can be explained based on either the mass transfer limitation in the tissue or due to different components of ECM in breast tissue. Here we have analyzed effects due to mass transfer limitation of enzymes in the tissue.

### 4.4.5 Reaction diffusion model prediction

The mass transfer limitation of enzyme in the tissue was confirmed by plotting the profiles of equation (4.9) in Matlab with collagen concentration in tissue, the rate constant and Michaelis–Menten constant terms determined here. The diffusivity was predicted based on power law expression given by Jain et al (Jain, 1987) as

$$D = a \left( M_r \right)^b \text{cm}^2/\text{s}$$

(4.12)

for range of molecular weight from 10 kDa to 393 kDa. Figure 4.10 showed the profile determined by reaction diffusion model for three different effective diffusivities ($a = D_{\text{eff}} = 0.1 \times 10^{-8} \text{ cm}^2/\text{s}$, $b = D_{\text{eff}} = 1 \times 10^{-8} \text{ cm}^2/\text{s}$ and $c = D_{\text{eff}} = 10 \times 10^{-8} \text{ cm}^2/\text{s}$). It can be observed at low diffusivity the enzyme penetration is substantially hindered despite long incubation time. The enzyme distribution changed dramatically with increase in one order of magnitude of diffusivity (figure 4.10 (b and c)). It required 5 order of magnitude change in reaction term to observe similar effect as observed in figure 4.10 (a-c) (data not shown here). These profiles clearly indicated mass transfer as rate limiting step in enzyme digestion of tissue.
4.4.6 Experimental analysis of effect of diffusion and enzyme concentration

The concentration profiles determined based on reaction diffusion model implied that larger size tissue particle would require longer time for digestion and time of digestion would not substantially changed by increasing the rate of reaction. Also, increase in enzyme concentration in bulk solution \( (C_0) \) will increase enzyme concentration proportionately at any point inside the tissue bulk, but it did not affect the concentration profile in the tissue with respect to time. These implications were tested by subjecting tissue pieces of different sizes (0.690 mm and 5.38 mm) at different enzyme concentrations (2 mg/ml and 10 mg/ml) to observe the time required for complete digestion. Figure 4.11 showed the results of time required for complete digestion of tissue with respect to enzyme concentration. Table 4.3 summarized the results shown in figure 4.11. It can be clearly observed in figure 4.11 that the size did have significant effect on the time of tissue digestion at both the enzyme concentrations (p-values for size effects in table 4.3). But it can be observed that enzyme concentration also had significant effect on the time of tissue digestion at both the sizes of minced tissue (p-values for enzyme concentration effects in table 4.3).

4.5 Discussion

Collagen is one of the major components of ECM in breast tissue. Breast tissue mainly consists of type I and type III collagen (Barsky et al., 1982; Kauppila et al., 1998; Marotta et al., 1985; Minafra et al., 1984; Okano, 1985; Pucci Minafra et al., 1985). The concentration of type I collagen is substantially more than other types (Marotta et al., 1985; Okano T, 1993) and increases during the process of carcinogenesis (Pucci Minafra
et al., 1986). This increase in collagen concentration facilitates the random distribution of malignant cells (Kauppila et al., 1998). Figure 4.2 and 4.3 confirmed these observations based on qualitative histological staining and figure 4.6 confirmed these observations based on quantitative analysis. This random distribution and higher concentration of collagen has a significant impact in terms of enzyme digestion of tissue as enzyme molecules have to penetrate through the matrix to release the cells in suspension. This task can be controlled by enzyme kinetics and/or mass transfer of enzyme in the breast tissue matrix.

There are a number of enzymes like trypsin, chymotrypsin, collagenase, elastase DNAse and so on are available to perform the task of enzyme digestion (Allalunis-Turner MJ, 1986; Costa A, 1987; Curry MP, 2000; Dairkee et al., 1997; Eade OE, 1981; Engelholm SA, 1985; Gomm et al., 1995; Hamburger et al., 1982; Haraldsen G, 1995; Keng PC, 1998; Lee TK, 1993; Pechoux et al., 1999; Penning JJ, 1981; Queral AE, 1984; Visscher DW, 1994; Zavros Y, 2000). Sometimes these enzymes are used together and sometimes independently. However, not all of these enzymes are controlling the rate of tissue digestion process. For example, DNAse enhances the rate of single cells suspension preparation by digesting DNA released from membrane compromised cells, but DNAse alone can not digest the tissue. Thus, it is necessary to decide which enzyme controls the overall rate of enzyme digestion process. Based on high collagen concentration and its prevalent random distribution we hypothesized that collagen digestion using collagenase is the rate controlling step in tissue digestion process and it would be possible to predict the time of tissue digestion based on collagen digestion time
using collagenase. To prove this hypothesis we have designed a simplified approach based on fluorescence to quantify the kinetic parameters of collagen degradation.

There are many assays available to quantify the activity of collagenase. Synthetic substrates like N-[3-(2-Furyl)acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine (FALGPA) (Van Wart and Steinbrink, 1981) and 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-\(\rho\)-Arg (Pz-PLGPR) (Wuensch and Heidrich, 1963) are routinely used for reporting the unit of activity of collagenase. These units are not suitable to directly correlate the kinetic parameter with collagen content of breast tissue samples. Mookhtiar et al have reported preparation of radiolabeled collagen to determine the kinetic parameter of collagenase (Mookhtiar et al., 1986). This assay is very sensitive and can determine the kinetic data that can be correlated with tissue dissociation. However radioactivity is inherently hazardous, labor intensive and expensive tool (Grubb, 1994). Another way to characterize the activity is to analyze the activity using insoluble tendon collagen. This method will provide most accurate kinetic parameters due to no modification of collagen; however this method involves precipitation of undigested collagen from solution, which can lead to significant unforced error. O’Grady et al have reported preparation of collagen substrate with a fluorescent compound 2-methoxy-2, 4-diphenyl-3(2H)-furanone (O'Grady et al., 1984). This substrate fluoresces over a wide range of pH and looses its fluorescence when subjected to metalloproteases as 2-methoxy-2, 4-diphenyl-3(2H)-furanone is not fluorescent in unbound form. This method overcomes most of the challenges discussed above and multiple samples can be processed simultaneously. However this assay is not as sensitive as radioactivity assay as very small changes in
fluorescence are difficult to observe when minute quantity of enzyme is to be detected (Grubb, 1994).

The above disadvantages associated with synthetic substrates and radiolabeled substrate were overcome by using dye quench collagen substrate for quantification of kinetic parameters for *C. histolyticum* collagenases (Haugland, 1996). Thompson, Jones and Voss *et al.* have used similar approach to quantify the activity of different proteases using BODIPY-FL labeled casein and FITC labeled BSA (Jones et al., 1997; Thompson et al., 2000; Voss et al., 1996). Here the dye labeled collagen has virtually no fluorescence due to fluorescence quenching. This was confirmed by blank run for 16 hours (data not shown). The fluorescence increases on degradation of collagen substrate, which was measured with respect to time to quantify the kinetic parameters (Figure 4.7 and 4.8). The average kinetic parameters based on above assay are reported in Table 4.2. It can be observed that $k_{cat}$ and $K_M$ for bovine type I and human type IV collagen did not changed substantially with change in enzyme source. Thus, we used average kinetic parameters for bovine type I collagen to quantify the time of collagen digestion in solution, due to high concentration of type I collagen in breast tissue samples.

The time required for collagen digestion is showed in figure 4.9. These plots showed breast tissue digestion time much less than the observed values (Costa et al., 1987; Dairkee et al., 1997; Gomm et al., 1995; Pechoux et al., 1999). The cause of this discrepancy was due to analytical solution of Michaelis – Menten kinetics, which predicted the substrate consumption in solution and did not consider the mass transfer effects associated with enzyme transport in solid breast tissue. This challenge was overcome by assuming breast tissue slices (minced tissue) as a semi – infinite slabs and
equation of conservation of mass was applied for enzyme transport in the tissue. During the movement of enzyme it reacted with collagen molecule and thus did not move further. Therefore, it is necessary for other enzyme molecules to penetrate in the tissue for continuing the tissue digestion. Figure 4.10 showed the spatial profile of enzyme concentration in tissue with respect to time. These plots were based on the analytical solution of reaction diffusion model (equation 4.6 and 4.9). These concentration profiles were encouraging as the time of enzyme transport is of the same order of magnitude as observed by other researchers for breast tissue digestion (Costa et al., 1987; Dairkee et al., 1997; Gomm et al., 1995; Pechoux et al., 1999). These concentration profiles showed that time of enzyme digestion in the tissue was strong function of diffusivity. This implied that diffusion of enzyme was the rate controlling step in the tissue digestion. The model also predicted that change in enzyme concentration of the bulk enzyme solution would change the enzyme concentration at a particular point in the tissue matrix proportionately. The implication of this effect on time of tissue digestion was not clear.

The effect of diffusion and enzyme concentration was empirically analyzed (figure 4.11 and table 4.3) to support the model predictions. These results showed that the diffusion of enzyme in tissue had significant effect on time of tissue digestion as predicted by the reaction diffusion model. But, the time of breast tissue digestion was less than that predicted by the reaction diffusion model. It was actually in between the time predicted by analytical solution of Michaelis – Menten kinetics and analytical solution of reaction diffusion model. Other interesting result was enzyme concentration had a significant effect on the time of digestion. These observations hinted towards more complex model compared to simple Michaelis – Menten kinetics and reaction diffusion
model. This can be explained based on brightfield microscopy images (figure 4.12) of breast tissue taken at different time points during tissue digestion. It was observed that at time $t = 0$ minutes, the tissue sample appeared dark at the center and it had sharp dark boundaries around it. As the time progresses ($t = 30$ minutes and $50$ minutes) the boundary appears more blurry and fibrous due to penetration of enzyme in tissue matrix and consumption of collagen. This is also confirmed by appearance of fat globule at the periphery of the breast tissue, which was not apparent at time $t = 0$. And in the final figure at time $t = 70$ minutes, the core also appeared blurry and fibrous compared to initial dark core. The phenomenon is similar to shrinking core model for coal combustion, where a well defined reaction boundary moves in coal particle with respect to time. The overall time of tissue digestion is controlled by mass transfer of reactants or rate of reaction depending on the thiele modulus. Based on figure 4.12 and other results discussed above, we propose that initially the rate of breast tissue digestion is limited by diffusion of enzyme in the tissue bulk, thus mass transfer is the rate limiting step. But after certain time, when the porosity of tissue increases due to consumption of collagen, the rate of reaction becomes an important factor as well. These effects will be studied based on shrinking core model to further improve the breast tissue digestion using catabolic enzymes.
<table>
<thead>
<tr>
<th>#</th>
<th>µg of Collagen or hydroxyproline</th>
<th>Volume of stock</th>
<th>Volume of HCl</th>
<th>Volume of 2.5 N NaOH</th>
<th>Volume of H₂O</th>
<th>Absorbance @ 557 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4 ml</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10-collagen</td>
<td>10 µl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 µl of 12 N and 80 µl of 6N HCl</td>
<td>240 µl</td>
<td>4 ml</td>
<td>0.048</td>
</tr>
<tr>
<td>3</td>
<td>25-collagen</td>
<td>25 µl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 µl of 12 N and 50 µl of 6N HCl</td>
<td>240 µl</td>
<td>4 ml</td>
<td>0.150</td>
</tr>
<tr>
<td>4</td>
<td>50-collagen</td>
<td>50 µl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50 µl of 12 N</td>
<td>360 µl</td>
<td>4 ml</td>
<td>0.347</td>
</tr>
<tr>
<td>5</td>
<td>100-collagen</td>
<td>100 µl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100 µl of 12 N</td>
<td>480 µl</td>
<td>4 ml</td>
<td>0.659</td>
</tr>
<tr>
<td>6</td>
<td>2 – hydroxyproline</td>
<td>20 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.98 ml</td>
<td>0.1362</td>
</tr>
<tr>
<td>7</td>
<td>4 – hydroxyproline</td>
<td>40 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.96 ml</td>
<td>0.179</td>
</tr>
<tr>
<td>8</td>
<td>6 – hydroxyproline</td>
<td>60 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.94 ml</td>
<td>0.282</td>
</tr>
<tr>
<td>9</td>
<td>10 – hydroxyproline</td>
<td>100 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.9 ml</td>
<td>0.490</td>
</tr>
<tr>
<td>10</td>
<td>15 – hydroxyproline</td>
<td>150 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.85 ml</td>
<td>0.673</td>
</tr>
<tr>
<td>11</td>
<td>20 – hydroxyproline</td>
<td>200 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.8 ml</td>
<td>0.885</td>
</tr>
<tr>
<td>12</td>
<td>Collagenase control</td>
<td>100 µl of 2 mg/ml solution</td>
<td>100 µl of 12 N</td>
<td>480 µl</td>
<td>4 ml</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Table 4.1 Sample data set for preparation of standard curve for collagen quantification in tissue

<sup>a</sup> 1 mg/ml collagen stock solution
<sup>b</sup> 100 µg/ml of hydroxyproline stock solution
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control (~500 U)</th>
<th>Sigma (~350 U)</th>
<th>Roche (~250 U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_M$</td>
<td>Slope of v vs. E0</td>
</tr>
<tr>
<td></td>
<td>Units</td>
<td>µg/ml</td>
<td>nmol/U.min</td>
</tr>
<tr>
<td>Bovine collagen type I</td>
<td>Mean 0.4</td>
<td>6</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>σ 0.1</td>
<td>1</td>
<td>0.002</td>
</tr>
<tr>
<td>Human collagen type IV</td>
<td>Mean 0.3</td>
<td>24</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>σ 0.1</td>
<td>4</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 4.2 Average Michaelis Menten kinetic parameter ($k_{cat}$ and $K_M$) for digestion of DQ<sup>TM</sup> type I collagen bovine skin and DQ<sup>TM</sup> human type IV collagen from placenta using Control (~ 500 U), Sigma (~ 350 U) and Roche (~ 250 U) collagenases (Mehta, 2006)
<table>
<thead>
<tr>
<th></th>
<th>0.690 mm</th>
<th>5.38 mm</th>
<th>p value based on t test analyzing the size effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mg/ml</td>
<td>79 ± 20</td>
<td>311 ± 106</td>
<td>0.0003</td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>59 ± 8</td>
<td>215 ± 80</td>
<td>0.0006</td>
</tr>
<tr>
<td>p value based on t test analyzing the enzyme concentration effects</td>
<td>0.0014</td>
<td>0.0035</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3 Average time required for complete digestion of breast tissue minced in different sizes and subjected to different initial enzyme concentration. The digestion was carried out at 37°C under constant agitation (n=8)
Figure 4.1 Schematic diagram showing thin semi-infinite tissue slab in y-direction. The tissue slab is subjected to enzyme solution maintained at constant concentration ($C_0$). For digestion the enzyme has to penetrate in the bulk of tissue slab (positive x-direction) by diffusion as there is no convection in the tissue slab. This process can be modeled by applying equation of conservation of mass in cartesian co-ordinate system.
Figure 4.2 Masson trichrome staining of invasive ductal carcinoma (breast cancer) representing the distribution of collagen fibers (five point star) and nuclei (arrow). The blue fibers represent collagen fiber (A – D) and the black dots represent the nuclei (B – D). (A) represent high concentration of collagen in tissue section at 100 X. (B, C and D) represent distribution of cells and collagen at 100 X, 200 X and 400 X respectively
Figure 4.3 Verhoeff’s staining of invasive ductal carcinoma (breast cancer) representing the distribution of elastin fiber (four point star), collagen fibers (five point star) and nuclei (arrow). The black fibers represent the elastin (C, D), the pink fibers represent the collagen (A – D) and bluish purple dots represent nuclei (A, B). (A and B) represent high concentration of collagen in tissue section at 100 X and 200 X respectively. (C and D) represent very less concentration of elastin compared to collagen in invasive ductal carcinoma at 200 X, 100 X respectively
Figure 4.4 Standard curve of absorbance @ 557 nm vs. µg of hydroxyproline measured using Woessner’s protocol. (Absorbance = 0.0453×[hydroxyproline] + 0.0190)
Figure 4.5 Standard curve of absorbance @ 557 nm vs. µg of bovine type I collagen measured using Woessner’s protocol. (Absorbance = 0.0061 × [collagen] + 0.0133)
Figure 4.6 Quantification of collagen in 17 breast tissue samples. The collagen was converted to hydroxyproline, which was further consumed to produce a chromophore detected at 557 nm. Based on 17 samples the concentration of collagen in breast tissue was $53 \pm 37 \mu g$ of collagen per mg of wet tissue. Collagen concentration was determined based on standard plots represented in figure 4.4 and 4.5. The open circle, filled circle and filled triangles represent different runs performed on same tissue sample.
Figure 4.7 Plot of rate of reaction ($v$, nmoles/(ml.min)) vs. enzyme concentration ([E$_0$, U/ml]) at constant substrate concentration of 100 µg/ml. Here the substrate is DQ$^{TM}$ bovine type I collagen and the enzyme is control (~ 500 U) collagenase purchased from Molecular Probes Inc. The reaction was carried at pH 7.5 and temperature 37°C. This plot can be fitted using $v = 0.0069[E_0] − 3.92 \times 10^{-5}$ as shown in the figure.
Figure 4.8 Plot of rate of reaction (v, nmoles/(ml.min)) vs. substrate concentration ([S], µg/ml) at constant enzyme concentration of 0.05 U/ml. Here the substrate is DQ\textsuperscript{TM} bovine type I collagen and the enzyme is control (~ 500 U) collagenase purchased from Molecular Probes Inc. The reaction was carried at pH 7.5 and temperature 37°C. This plot can be fitted using $v = 0.0004[S] / (4.58 + [S])$ as shown in the figure.
Figure 4.9 Time of collagen digestion determined based on kinetic parameters (Table 4.2) for bovine type I collagen at different initial enzyme concentration. (a) represent the digestion time when initial collagen concentration is 53 µg, (b) represent the digestion time when initial collagen concentration is 530 µg. In both figures filled dots represent the time for 25% conversion, open dots represent the time for 50% conversion, filled inverted triangles represent the time for 75% conversion and open triangle represent the time for 87.5% conversion.
Figure 4.10 Variation in spatial distribution (x – axis) of enzyme concentration with respect to time in a semi – infinite tissue slab at different effective diffusivities (a = $D_{eff} = 0.1 \times 10^{-8}$ cm$^2$/s, b = $D_{eff} = 1 \times 10^{-8}$ cm$^2$/s and c = $D_{eff} = 10 \times 10^{-8}$ cm$^2$/s) determined based on reaction diffusion model. The concentration of collagen, rate constant and Michaelis – Menten constant are determined based on analysis performed here.
Figure 4.10 continued

(c) \( D_{\text{eff}} = 10 \times 10^{-8} \text{ cm}^2/\text{s} \)
Figure 4.11 Time required for complete digestion of breast tissue pieces of the size (0.69 mm and 5.38 mm) subjected to collagenase concentration of 2 mg/ml and 10 mg/ml respectively. It can be observed that the size effects (diffusion effects) are much more pronounced than the enzyme concentration effects.
Figure 4.12 Brightfield microscopy images taken during the breast tissue digestion. Here a piece of breast tissue was subjected to 2 mg/ml collagenase concentration and 25 µg/ml of DNAse concentration in medium at 37°C. The enzyme penetrates in breast tissue in similar manner as predicted by shrinking core model for coal combustion.
CHAPTER 5

EFFECT OF pH ON CELL DENSITY DURING ISOPYCNIC SEPARATION OF CELLS USING PERCOLL DENSITY GRADIENT

The content of this chapter is prepared for publication: Mehta, BC; Galusha, AD; Chalmers, JJC; Effect of pH on Cell Density during Isopycnic Separation of Cells Using Percoll Density Gradient.

5.1 Introduction

With the advent and improvements of highly sensitive and accurate molecular analysis technology, it is possible to study increasingly complex cellular processes including cell differentiation and proliferation (St Croix et al., 2000). One of the factors affecting the reliability of data obtained from these new techniques depend on how accurately and precisely cell samples are prepared from heterogeneous cell sources like normal tissue, tumor, blood or a simple tissue culture flask. The ability to analyze and separate a heterogeneous cell population based on cellular characteristics is a significant analytical and preparative resource (Pantel et al., 1999). These cellular characteristics can be classified into physical attributes and molecular attributes. Examples of physical properties include cell size, density and morphology while molecular properties include
presence and number of cell surface markers as well as internal reporters such as green fluorescence protein, GFP (Pretlow et al., 1982; Shapiro, 2003).

The purpose of this study is to focus on one of these physical attributes; cell density. Separation based on the density of the cell is one of the fastest ways to separate population of cells. Some of the common applications of density based separation are fractionation of specific cell types from blood, detection of disseminated cancer cells in blood and fractionation of heterogeneous tissue (Fehm et al., 2002; Gertler et al., 2003; Kruger et al., 2000; Lara et al., 2004; Mackillop et al., 1982; Resnicoff et al., 1987; Rosenberg et al., 2002; Van Vlasselaer, 1998).

Each year worldwide 70 million units (1 unit = 450 ml) of blood is collected. As per the Nationwide Blood Collection and Utilization Survey (NBCUS) in 1999, 13.87 million units of blood were collected in U.S. alone (Sullivan and Wallace, 2005). This blood was utilized for transfusion. Majority of the collected blood was allogeneic, thus it is critical to separate the red blood component and platelets for suitable transfusion. This separation is routinely done using centrifugation and density gradient separation. Orbisac system™, COBE® Spectra and COBE® 2991 Cell Processor (Gambro BCT Inc.) are some of the commonly used instruments for large scale blood processing. Based on market survey at Gambro, the global market for blood bank technology is approximately U.S. $ 1.4 billion and is growing at 5 % a year. The blood component collection and separation account for U.S. $ 578 million. The costs of blood component units are listed in table 5.1 as reported by NBCUS survey (Sullivan and Wallace, 2005). For appropriate control of above processes it is necessary to characterize the physical attributes, especially density as accurately as possible.
There are numerous methods available to characterize the physical properties of cells including cell size using electrical conducting technology (i.e. Coulter Counter), cell size and density using centrifugal elutriation and solely density using gravity sedimentation and centrifugal enhanced sedimentation (i.e. Ficoll and Percoll) (Bertoncello, 1987). All of these methods have implicit assumption and/or limitations. Centrifugal elutriation is a complex method to determine cell size and density. There are various challenges associated with elutriation system like controlling many factors, for example, temperature, choice of media, elutriation chamber and variation of flow rate, variation of rotor speed, loading system and fraction collector. Apart from all these factors, the recovery of cells at low cell loading density decreases significantly (Meistrich, 1983). In unit gravity sedimentation, the heterogeneous cell suspension is allowed to separate based on its sedimentation velocity, which is a function of cell size and density difference between cells and media. This method has several advantages like simplicity and gentleness on cells, but one of the major limitations is slowness of the method (Bertoncello, 1987).

Several density gradient media were developed to utilize the advantages of unit sedimentation technique to determine the density of cells. With the aid of centrifugation, the processing time can be reduced significantly. Some of the commonly used density gradient media include sucrose, polysucrose, polydextran, iodinated compound, mixed gradient and colloidal silica (Pertoft H, 2000; Rickwood, 1984). Sucrose, polysucrose and polydextran are not suitable for density gradient separation due to variation in osmolality of these gradients; this directly affects the density of cells due to osmotic imbalance across the semi-permeable plasma membrane. Iodinated compounds and mixed gradients
(Ficoll Paque) overcome this problem, but these are low molecular weight compounds and can easily penetrate the cell membrane. It is observed that these compounds are cytotoxic to the cells. Colloidal silica (Ludox HS) also has similar challenges, which were overcome by coating colloidal silica with polyvinylpyrrolidone (PVP). This led to the development of percoll, which has following physical properties shown in table 5.2.

Percoll is not suitable for cell characterization and separation under the condition in which it is supplied. The physical properties like osmolality and pH of percoll should be adjusted to physiological conditions. Substantial efforts have been spent in understanding the effects of percoll osmolality on the change in cell density (Pertoft, 1982; Vincent R, 1984). The osmolality of percoll can be modified by diluting the percoll with phosphate buffer saline (PBS) (for cell separation) or sucrose (for subcellular particle separation). These methods are explained in detail in handbook published by Amersham Biosciences on percoll. These handbook and other published reports do not discuss the effect of basic pH of percoll on the cell density. This study focuses on developing a simple protocol for adjusting pH and osmolality of percoll simultaneously. This modified percoll will be then utilized to quantify density of multiple cell lines (MCF – 7, A549, CHO) and primary cultures (Human breast fibroblast cells, lymphocytes from buffy coat). Also, the effect of pH and cell loading density on percoll gradient was evaluated for CHO cell density and CHO cell recovery from the gradient.
5.2 Materials and methods

5.2.1 Reagents

Percoll was purchased from Amersham biosciences, New Jersey, USA and was used as density gradient medium. Percoll was diluted with phosphate buffer saline (PBS) to adjust the pH, osmolality and density. The osmolality and density of percoll were roughly adjusted with 10 X PBS (Invitrogen Corporation, USA) to produce standard isotonic percoll (SIP). The fine adjustment of pH, osmolality and density was done with 1X PBS (Invitrogen Corporation, USA) mixed with appropriate amount of 0.1 N HCl (Sigma-Aldrich, St. Louis, MO, USA) to produce diluted percoll solution. Diluted percoll solution was then used to produce continuous density gradient using ultracentrifuge (Beckman Coulter, USA) at 17000 rpm for 30 minutes.

5.2.2 Density measurement

The density of the continuous density gradient was measured using density marker beads® (Amersham biosciences, NJ, USA).

5.2.3 Osmolality measurement

Osmolality was measured in a freezing point osmometer from Advance Instrument Inc., Newton Highlands, MA.

5.2.4 pH measurement

The pH of different solutions was measured using Accumet pH meter 910 from Fisher Scientific, Pittsburg, PA.
5.2.5 Preparation of dilute percoll solution

For the purpose of cell separation, the percoll was diluted with PBS. Here two step procedures were utilized for preparation of diluted percoll of desired density, osmolality and pH. In the first step the osmolality and pH were coarsely adjusted by diluting the percoll with 10 X PBS. This percoll was designated as stock isotonic percoll (SIP). The volume of percoll and 10 X PBS was determined using equation (5.1) based on desired osmolality for particular type of cell separation,

\[
V_p = V_c \cdot \frac{O_c - O_f}{R \cdot (O_f - O_p)}
\]  

(5.1)

Where \(V_p\) and \(V_c\) were volume of percoll and 10 X PBS respectively, \(O_p\) and \(O_c\) were osmolality of percoll (~20 mOsm) and 10 X PBS (2880 mOsm) respectively, \(R\) is the ratio of aqueous volume to total volume of percoll (0.85 for PBS) and \(O_f\) was the desired osmolality. Equation (5.1) was utilized to prepare two different SIP solutions with 10 X PBS to percoll ratio of 1:10 and 1:11 respectively (Table 5.3). These solutions were further diluted using 1 X PBS premixed with desired quantity of 0.1 N HCl (Table 5.4) based on required density as per equation (5.2),

\[
V_y = V_i \cdot \frac{\rho_i - \rho}{\rho - \rho_y}
\]

(5.2)

Where \(V_y\) and \(V_i\) were volume of 1 X PBS and SIP respectively, \(\rho_y\) and \(\rho_i\) were density of 1 X PBS (1.0046 g/ml) and SIP respectively and \(\rho\) is density of diluted solution produced in g/ml.

The pH of the diluted percoll was adjusted in the physiological range by adding 0.1 N HCl in 1 X PBS (Table 5.5). The acid neutralized the alkaline SIP solution and did
not significantly affect the osmolality of the diluted percoll (Table 5.5). This diluted percoll was then ultracentrifuged at 17000 rpm for 30 minutes to prepare the continuous density gradient medium. This density gradient medium was utilized for quantification of buoyant cell density and cell recovery after cell separation. The cells were layered carefully on the top of the density gradient medium in minimum amount of cell culture medium. The tubes with cells and density gradient medium were then centrifuged at 1400 rpm for 20 minutes at 4°C with break off to facilitate the sedimentation of cells depending on their buoyant density. After sedimentation, the cell density was measured by comparing it with the sedimentation of density gradient beads under exactly same conditions. The cells were then recovered from top using pipette based on visual observation of cell band in the density gradient medium. The recovered cells were washed with 1 X PBS and centrifuged at 1400 rpm for 8 minutes at 4°C. The cell pellet was resuspended in appropriate amount of medium and cell concentration was quantified using hemacytometer. Following different cell lines were utilized to determine the cell density in diluted percoll medium with adjusted pH.

5.2.6 Cell culture

A breast carcinoma cell line (MCF-7) and Chinese Hamster ovary (CHO) were cultured to confluence in Minimal Eagle medium (Invitrogen Inc., Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS, JRH Biosciences, Lenexam, KS) in a 75 cm² tissue culture flask (Corning, USA) and incubated at 37°C in 5 % CO₂ incubator. MCF-7 and CHO cells were harvested using Accutase™ (Innovative Cell Technologies, San Diego, CA).
A Lung carcinoma cell line (A-549) was cultured to confluence in F 12K medium (Mediatech, Inc., Herndon, VA) supplemented with 10 % FBS in a 75 cm² tissue culture flask and incubated at 37°C in 5 % CO₂ incubator. A-549 cells were harvested using Trypsin (Invitrogen Inc., Carlsbad, CA). For harvesting 1 ml of trypsin was used per tissue culture flask and was incubated for 5 minutes at 37°C in 5 % CO₂ incubator.

A primary cell culture of fibroblast cell was recovered from the breast tissue sample using enzyme digestion. This primary cell culture of fibroblast was cultured to confluence for 10 passages in Minimal Eagle medium/F 12 medium (Invitrogen, Inc., Carlsbad, CA) supplemented with 10 % FBS in a 75 cm² tissue culture flask and incubated at 37°C in 5 % CO₂ incubator. Fibroblast cells were harvested using Trypsin. For harvesting 1 ml of trypsin was used per tissue culture flask and was incubated for 5 minutes at 37°C in 5 % CO₂ incubator. After 10 passages the growth rate of cells decreased significantly and new primary cell culture was prepared with a new breast tissue sample.

Peripheral blood buffy coats from apparently healthy donors were purchased from the American Red Cross, Central Ohio Region. The leukocytes and erythrocytes were separated using Ficoll-Hypaque (Accurate Chemical and Scientific Corporation, Westbury, NY) density gradient separation. Cell concentration was measured using hemacytometer for leukocytes.

Cell concentration was assessed using hemacytometer and the cell viability was measured using a tryphan blue (Fisher Scientific, Pittsburg, PA) dye exclusion method. Viability results showed at least 90 % healthy cells after detachment from culture flask.
The viability test was also performed after subjecting the cells to density gradient separation using diluted percoll solutions.

5.2.7 Statistical analysis

Different cell culture suspensions discussed above were layered carefully on top of density gradient medium prepared using method described above. The mean density of the cells were measured based on comparison with density marker beads. The mean density of different cell lines discussed above was compared using Tukey Kramer HSD test. Also, the effects of cell loading density and pH of percoll density gradient medium on CHO cell density and recovery was analyzed. A randomized $3 \times 2$ factorial design with two replicates (total 12 experiments) were performed for this purpose and Tukey Kramer HSD test was perform to quantify the effect of independent variables. All the analyses were performed using JMP 5.1.2 statistical software.

5.3 Results

5.3.1 Preparation of density gradient medium with physiological properties

Percoll is a suitable density gradient medium for various cell separation and sub – cellular component separation process due to appropriate properties described in table 5.2. Percoll is not suitable for direct use as it has high initial density and low osmolality. These two properties provide an opportunity for a user to customize percoll as per the need of the experiment. There are two common additives (PBS or sucrose) used for preparation percoll density gradient medium. PBS is commonly used for cell separation application. Up to best of author’s knowledge, basic nature of percoll is not considered
while preparing the density gradient medium. Here we have discussed a protocol based on volumetric balance to prepare the percoll density gradient medium. Table 5.2 and 5.3 present some representative dilutions commonly utilized. It can be observed in table 5.5 that any desired physiological pH and osmolality can be achieved. For the further experiments to determine the cell density and analyze cell recovery after percoll separation, the density gradient medium was prepared with same conditions as media utilized for cell culture.

5.3.2 Comparison of density from different cells after pH adjustment

Densities of different cell lines were compared using Tukey Kramer test as shown in the figure 5.1. This analysis included cells that constitute the tissue structure. It can be observed that normal cells like lymphocytes, monocytes and fibroblast have tighter cell density distribution compared to cancer cell lines like MCF – 7 or A549 or genetically modified cells like CHO cells. The mean densities of all these cells are summarized in table 5.6. For the success of density gradient based separation it is necessary that there is statistically significant difference in the densities of the different cell types. Table 5.6 shows the results of Tukey Kramer test. Here the mean cell densities represented by same letter are not significantly different from each other. For example, MCF – 7, monocytes and fibroblast did not have statistically different cell densities compared to each other (represented by letter ‘D’ in Table 5.6).
5.3.3 Effect of pH and cell loading on CHO cells

The effect of basic nature of diluted percoll gradient medium was analyzed on CHO cells. Here 3 × 2 (three cell loading densities, 2.5 × 10^6, 5 × 10^6 and 7.5 × 10^6 and two pH levels, 7.63 and 7.94) factorial design was performed with buoyant CHO cell density and cell recovery as response variables. Based on JMP 5.1.2 analysis, it was observed that the pH (at all the cell loading density 2.5 × 10^6, 5 × 10^6 and 7.5 × 10^6) of diluted percoll did not have significant effect on the cell recovery from percoll, whereas cell loading density (at all pH, 7.63 and 7.94) had significantly effect on the cell recovery from percoll. These observations were made based on the comparison of mean recoveries at different pH levels (figure 5.2 and table 5.7) and at different cell loading densities (figure 5.3 and table 5.8). The mean recovery of cells at both the pH levels was approximately 82 % (Table 5.7), whereas the recovery of cells increased with increase in initial cell loading density (Table 5.8). Again, the mean recoveries represented by same letter in table 5.8 are not statistically significantly different from each other based on Tukey Kramer test. It implies that the mean recovery at cell loading density of 5 million is not statistically significantly different than the mean recovery at cell loading density of 7.5 million cells (represented by letter ‘B’ in table 5.8). It can be also observed from figure 5.3 and table 5.8 that the cell recovery followed saturation kinetics with respect to increase in initial cell loading density.

The effect of cell loading density and pH on CHO cell density was interesting. It was observed that the pH (at all the cell loading density 2.5 × 10^6, 5 × 10^6 and 7.5 × 10^6) and cell loading density (at all pH 7.63 and 7.94) did not have significant effect on the cell density measured by percoll. Table 5.9 and 5.10 showed the average cell density at
different pH levels and at different initial cell loading densities. Figure 5.4 and 5.5 showed the Tukey Kramer test for comparison of cell density at different pH levels and at different initial cell loading densities. It was anticipated that the basic environment pH will have some effect on buoyant density of CHO cells and will result in shift from physiological value.

5.4 Discussion

Biological systems (like blood or solid tissue) are composed of heterogeneous cell types. It is necessary to separate these cell types from each other for various applications like basic research, genetic analysis or for transplantation in patients. Different cell types have shown differences in physical properties like size and density as well as molecular properties like protein marker expression or mRNA profiling. The focus of this study was to analyze the separation of cells based on physical properties. The differences in physical properties are employed to separate cells by performing velocity sedimentation (based on size and density differences of cells, principle of centrifugal elutriation) or by performing isopycnic separation (based on density) (Pertoft, 1982; Pretlow et al., 1982). Here we have focused on understanding the differences in density determined by isopycnic separation using percoll as a density gradient medium.

Percoll is a polydisperse silica colloid coated with polyvinylpyrrolidone (PVP). PVP coating renders colloidal silica non-toxic and suitable for density gradient separation. The properties of percoll solution available from manufacturer are listed in table 5.2 (Pertoft H, 2000). It is clear based on these properties that percoll is only suitable for cell separation after modification of osmolality and pH. The osmolality of
percoll is adjusted by diluting it with PBS to prepare diluted percoll density gradient medium as per manufacturer’s recommendations. Despite adjusting the osmolality of the percoll, it was observed that the pH of the percoll is still basic (Table 5.3). Therefore, to neutralize the basic nature of SIP, we adjusted the pH of PBS (1X) (Table 5.4) utilized for finer adjustments of diluted percoll density gradient medium (Table 5.5). These modifications were performed based on volumetric quantity of percoll, PBS (10X), PBS (1X) and 0.1 N HCl and it was possible to prepare diluted percoll density gradient medium of any desired density, osmolality and pH. The protocol discussed here was then utilized for the quantification of cell density of different cell types.

Buoyant density of different cell types was determined using continuous density gradient medium. The density determined here (Table 5.6) is in comparable range of the reported values in literature (Pertoft, 1982). This confirmed the suitability of percoll density gradient medium for rapid determination of cell density compared to centrifugal elutriation and unit gravity sedimentation. The implication of this comparison is important for the metastatic cancer cell detection in blood for cancer diagnosis as well as for isolation of cells from tissue, like separation of luminal epithelial cells from breast tissue or like separation of islets of langerhans from pancreas (Lara et al., 2004; Martin et al., 1998; Ricordi et al., 1988; Ronnov-Jessen L, 1992).

It has been well documented that the tumor mass consists of heterogeneous cell population showing variation in physical and molecular attributes. This is also observed in disseminated epithelial cells. Resnicoff et al observed that the breast cancer cell line (MCF 7) can be fractionated in to six different fractions when subjected to discontinuous percoll density gradient (Podhajcer et al., 1986; Resnicoff et al., 1987). Other researchers...
have also observed the same behavior in terms of density by ovarian cancer cell line (HOC-7) (Grunt et al., 1991; Mackillop et al., 1982), melanoma cell line (Guerra et al., 1989), head and neck cancer cells (King and Parsons, 1988). One of the common challenges in epithelial cell culture is high infestation by fibroblast cells. Epithelial cells are routinely separated from tissue by enzyme digestion of tissue and then density gradient separation of single cell suspension. Based on figure 5.1 it can be observed that fibroblast cells have density in the same range as MCF – 7 and A549 cells (epithelial cells). Therefore, during density gradient separation fibroblast cells will separate with the epithelial cells. Similar challenges have been reported by Pretlow et al with isopycnic separation of various cell types like hepatocytes, cardiac myocytes and pancreas acinar cells from their respective organs (Pretlow et al., 1982). To overcome this challenge, it is necessary to perform immunological separation of cell of interest from other cells.

Another example of density separation is the detection and separation of disseminated epithelial cells from blood. Detection of such epithelial cells is related to the metastasis of primary cancer to distant sites. While not commonly used, it has been suggested that detection of such cells in either peripheral blood or bone marrow can have significant prognostic importance (Braun et al., 2005; Cristofanilli et al., 2004). Cancer cells have been reported to have densities in the range of 1.04 – 1.08 gm/cc and are usually separated with mononuclear cells (1.0762 ± 0.0008 gm/cc, Table 5.6) after Ficoll-paque separation (1.077 gm/cc) of diluted blood (Kruger et al., 2000; Lara et al., 2004; Resnicoff et al., 1987). This might not be completely true as cancer cells have wide range of cell densities. Our lab have used MCF – 7 commonly for simulating cancer cell depletion from blood in past. It can be observed in figure 5.1 that MCF – 7 has a wide
range of density of 1.025 – 1.0575 gm/cc. These results are in accordance with results published by Resnicoff et al (Resnicoff et al., 1987). The reason behind loss of MCF – 7 cells during ficoll separation can be explained based on stokes law. The sedimentation coefficient (\( \mu \)) can be expressed as:

\[
\mu = \frac{2 \cdot r^2 \cdot (d - d_0) \cdot G}{9 \cdot \eta}
\]  

(5.3)

Where \( r \) is the radius of the particle (cell), \( d \) is the density of cell, \( d_0 \) is the density of fluid, \( G \) is the gravitational or centrifugal acceleration and \( \eta \) is the viscosity of the fluid.

In the case of ficoll separation, the blood (density ~ 1.060 gm/cc) with cancer cells is layered on the ficoll. After centrifugation the erythrocytes form a pellet in the tube and majority of granulocytes are found in ficoll media. The mononuclear cells form a band at the interface of ficoll and plasma (density 1.025 gm/cc) containing platelets (1.03 – 1.06 gm/cc) (Hinghofer-Szalkay and Greenleaf, 1987; Hinghofer-Szalkay et al., 1995; Pertoft, 1982). Due to presence of platelets in plasma the average density of plasma increases resulting in no driving force (equation (5.3)) for low density cancer cells to sediment with mononuclear cells. This phenomenon can significantly affect the diagnostic capabilities of immunological separation techniques (Kruger et al., 2000; Lara et al., 2004; Martin et al., 1998).

Apart from density measurements, this study also focused on analyzing the effect of basic pH on CHO cells. It was hypothesized that basic extracellular pH will adversely effect the cell volume and in turn the cell density. Mammalian cells are fragile in comparison to plant cells or any prokaryotic cells. They undergo significant volume change under external stimuli like osmolar perturbation that results in water flux across
the semi-permeable cell membrane. To avoid shrinking under hyper-osmolar conditions and bursting under hypo-osmolar conditions, mammalian cells have developed significant volume regulatory mechanisms. There are two main types of volume regulatory mechanisms. In one of the mechanisms the cell adjusts its osmolality by adjusting the ion concentration in cell by releasing or accumulating ions through respective channels or transport system across the cell membrane (e.g. Na$^+$/K$^+$ channel). The second mechanisms involves adjustment of osmolality by formation or accumulation of organic osmolytes (organic molecules like sorbitol, inositol etc.). Apart from extracellular osmolality there are many other factors that affect the water flux across the cell membrane. These includes nutrient uptake, activation of ion channels and transport systems at the cell membrane, formation or cleavage of osmotically more active compounds like proteins and cleavage of organic molecule to CO$_2$ and H$_2$O (Lang F, 1998).

A number of studies have reported change in function of ion channels under external stimuli of pH. Gros et al have reported change in shape of erythrocytes under acidic pH that eventually results in formation of vesicles from erythrocytes at pH 5.4 (Gros M, 1996). Austin et al have reported that when rat mesenteric vessels are subjected to change in pH from 7.4 to 7.9, it induces increase in intracellular pH that results in increase in Ca$^{2+}$ in the cell (Austin C, 1996). Based on these studies, it was expected to observe change in buoyant density of CHO cells with basic extracellular pH. Here two response variables, recovery and density were analyzed with the change in cell loading density and percoll pH.
The recovery of CHO cells was significantly affected by initial cell loading density but it did not change much with change in percoll pH. This result with respect to initial cell loading density was in accordance with similar observation by Lara et al for MCF – 7 cells in ficoll. The cause of this phenomenon is due to poor visibility of cell layer in the centrifuge tube at low cell density (Figure 5.3). This challenge becomes particularly evident when one is attempting to separation rare cancer cells from blood for cancer detection or separation of primary cells from tissue. It was observed by Mackillop et al that ovarian carcinoma cells from a tissue sample have much wider range of density compared to HOC – 1 cells (ovarian carcinoma cell line) cultured in vitro. Thus, the combined effect of less cell loading and wider density range makes it more challenging to recover the primary cancer cells from a tissue source (Lara et al., 2004; Mackillop et al., 1982). On the other hand, external pH did not affect the recovery of cells significantly (figure 5.2), the reason being, extracellular pH did affect the performance of volume regulatory ion channels as discussed above initially. But over a period of time, (~ 10 – 15 minutes) the cells return to their normal physiological density by adjustment of cell volume (cell ion and water content). Only criterion for this to happen is that the extracellular stimulus should not be too drastic to induce the cells to undergo apoptosis or necrosis (Boyum et al., 2002).

The buoyant density of CHO cells were not affected significantly by either pH or initial cell loading density (figure 5.4 and 5.5). It was expected that basic pH of extracellular environment will have effect on salt and water transportation across cell membrane and the buoyant cell density will be affected. This was not the case due to the percoll density gradient separation took approximately 30 - 45 minutes to perform.
Boyum et al observed that during this period the cell density was adjusted to its original buoyant cell density despite being affected by external stimuli (Boyum et al., 2002). It was expected that cell loading density will not affect the cell density as it is not a physiological parameter of cells. Thus, it should not affect the buoyant density of cells. These results confirmed that cell loading density on the percoll gradient did not have any effect on cell density determined using percoll.

This study discussed the density determination capability of percoll and analyzed effect of cell loading density and basic pH of percoll on buoyant density of CHO cells and recovery of CHO cells. It is proposed that similar studies should be performed with primary cells and cancer cell lines (MCF – 7 or HOC – 1) as they are more susceptible to external environment compared to immortalized cell lines.
<table>
<thead>
<tr>
<th>Blood component</th>
<th>Mean</th>
<th>Mean</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
<td>Mean</td>
</tr>
<tr>
<td>RBC’s, O+, Leukofilterd, not irradiated, not CMV(^{**})-negative</td>
<td>$117.78</td>
<td>$125.95</td>
<td>$121.98</td>
</tr>
<tr>
<td>Pjeresis, PLT(^{\text{0}})s with 3-4×10^{11} PLTs, leukoreduced</td>
<td>$472.26</td>
<td>$497.95</td>
<td>$487.00</td>
</tr>
<tr>
<td>Whole blood-derived PLT concentrate, not leukoreduced, not irradiated, 3 days</td>
<td>$49.13</td>
<td>$52.83</td>
<td>$52.34</td>
</tr>
<tr>
<td>remaining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFP(^{\text{00}}), Group AB, ~250 mL</td>
<td>$47.87</td>
<td>$52.38</td>
<td>$50.38</td>
</tr>
</tbody>
</table>

Table 5.1 Mean cost of selected blood components per unit purchased by hospitals in 1999 (Sullivan and Wallace, 2005)

\(^{*}\)RBC = Red blood cells, \(^{**}\)CMV = cytomegalovirus, \(^{\text{0}}\)PLT = Platelets, \(^{\text{00}}\)FFP = Fresh frozen plasma
Density of percoll: 1.130 ± 0.005 gm/ml
Viscosity of solution: 10 ± 5 cP at 20°C
pH: 8.9 ± 0.3 at 20°C
Osmolality: 20 mOsm/kg of H₂O
Refractive index: 1.3540 ± 0.005 at 20°C

Table 5.2 Physical properties of percoll

<table>
<thead>
<tr>
<th>Solution ID</th>
<th>Volume of 10 X PBS</th>
<th>Volume of percoll</th>
<th>Theoretical osmolality</th>
<th>Measured osmolality</th>
<th>pH of SIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIP 2 (1:10)</td>
<td>1.2</td>
<td>12</td>
<td>321</td>
<td>327 ± 1</td>
<td>7.85 ± 0.02</td>
</tr>
<tr>
<td>SIP 3 (1:11)</td>
<td>1.2</td>
<td>13.2</td>
<td>296</td>
<td>304 ± 0</td>
<td>7.87 ± 0.01</td>
</tr>
</tbody>
</table>

Table 5.3 Volumetric proportions for the preparation of standard isotonic percoll (final diluted percoll = 35 ml) using percoll (in ml) and 10 X PBS (in ml) based on equation (5.1)

<table>
<thead>
<tr>
<th>Solution ID</th>
<th>Volume of 1 X PBS</th>
<th>Volume of 0.1 N HCl</th>
<th>pH of PBS</th>
<th>Osmolality of PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS 4</td>
<td>9.6</td>
<td>0.4</td>
<td>6.23 ± 0.01</td>
<td>281 ± 1</td>
</tr>
<tr>
<td>PBS 5</td>
<td>9.5</td>
<td>0.5</td>
<td>5.99 ± 0.01</td>
<td>279 ± 1</td>
</tr>
<tr>
<td>PBS 6</td>
<td>9.4</td>
<td>0.6</td>
<td>5.63 ± 0.01</td>
<td>277 ± 1</td>
</tr>
<tr>
<td>PBS 7</td>
<td>9.3</td>
<td>0.7</td>
<td>4.5 ± 0.1</td>
<td>276 ± 1</td>
</tr>
<tr>
<td>PBS 8</td>
<td>9.2</td>
<td>0.8</td>
<td>3.04 ± 0.01</td>
<td>274 ± 2</td>
</tr>
</tbody>
</table>

Table 5.4 Volumetric proportions for the preparation of pH adjusted PBS 1 X (final diluted percoll = 35 ml) using PBS 1 X (in ml) and 0.1 N HCl (in ml)

<table>
<thead>
<tr>
<th>Solution ID</th>
<th>Volume of 1 X PBS</th>
<th>Volume of 0.1 N HCl</th>
<th>pH of PBS</th>
<th>Osmolality of PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS 4</td>
<td>9.6</td>
<td>0.4</td>
<td>6.23 ± 0.01</td>
<td>281 ± 1</td>
</tr>
<tr>
<td>PBS 5</td>
<td>9.5</td>
<td>0.5</td>
<td>5.99 ± 0.01</td>
<td>279 ± 1</td>
</tr>
<tr>
<td>PBS 6</td>
<td>9.4</td>
<td>0.6</td>
<td>5.63 ± 0.01</td>
<td>277 ± 1</td>
</tr>
<tr>
<td>PBS 7</td>
<td>9.3</td>
<td>0.7</td>
<td>4.5 ± 0.1</td>
<td>276 ± 1</td>
</tr>
<tr>
<td>PBS 8</td>
<td>9.2</td>
<td>0.8</td>
<td>3.04 ± 0.01</td>
<td>274 ± 2</td>
</tr>
</tbody>
</table>

a Determined based on equation (5.1), b Average ± SD for n = 3

a Average ± SD (n = 3)
Table 5.5 Physical properties of diluted percoll prepared using SIP (Table 5.3) and PBS 1 X (Table 5.4). The density was determined based on equation (5.2)

\[ a \text{Average} \pm \text{SD (n = 3)} \]

<table>
<thead>
<tr>
<th>Solution ID</th>
<th>pH of diluted percoll(^a)</th>
<th>Osmolality of diluted percoll(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIP 2 + PBS 4</td>
<td>7.34 ± 0.02</td>
<td>305 ± 4</td>
</tr>
<tr>
<td>SIP 2 + PBS 5</td>
<td>7.28 ± 0.03</td>
<td>302 ± 3</td>
</tr>
<tr>
<td>SIP 2 + PBS 6</td>
<td>7.2 ± 0.01</td>
<td>302 ± 2</td>
</tr>
<tr>
<td>SIP 2 + PBS 7</td>
<td>7.13 ± 0.02</td>
<td>301 ± 2</td>
</tr>
<tr>
<td>SIP 2 + PBS 8</td>
<td>7.05 ± 0.02</td>
<td>300 ± 3</td>
</tr>
<tr>
<td>SIP 3 + PBS 4</td>
<td>7.36 ± 0.01</td>
<td>292 ± 3</td>
</tr>
<tr>
<td>SIP 3 + PBS 5</td>
<td>7.29 ± 0.01</td>
<td>291 ± 3</td>
</tr>
<tr>
<td>SIP 3 + PBS 6</td>
<td>7.2 ± 0.02</td>
<td>291 ± 2</td>
</tr>
<tr>
<td>SIP 3 + PBS 7</td>
<td>7.12 ± 0.02</td>
<td>290 ± 2</td>
</tr>
<tr>
<td>SIP 3 + PBS 8</td>
<td>7.05 ± 0.03</td>
<td>290 ± 3</td>
</tr>
</tbody>
</table>

Table 5.6 Density distributions of different cell types determined using continuous density gradient medium. The mean cell densities of these cell types were compared with each other using Tukey Kramer test. The last column presents the density reported in literature for different cell types

\[ a \] (Pertoft, 1982)
Table 5.7 Recovery of CHO cells at different pH levels. Based on Tukey Kramer test, it was determined that the recoveries at different pH levels were not significantly different.

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean ± Std Dev</th>
<th>Tukey Kramer HSD test</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.63</td>
<td>81 ± 15</td>
<td>A</td>
</tr>
<tr>
<td>7.94</td>
<td>82 ± 23</td>
<td>A</td>
</tr>
</tbody>
</table>

Table 5.8 Recovery of CHO cells at different initial cell loading density. Based on Tukey Kramer test, it was determined that the recoveries at different initial cell loading density were significantly different.

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean ± Std Dev</th>
<th>Tukey Kramer HSD test</th>
</tr>
</thead>
<tbody>
<tr>
<td>2500000</td>
<td>63 ± 15</td>
<td>A</td>
</tr>
<tr>
<td>5000000</td>
<td>87 ± 13</td>
<td>A</td>
</tr>
<tr>
<td>7500000</td>
<td>93 ± 14</td>
<td>B</td>
</tr>
</tbody>
</table>

Table 5.9 Average density of CHO cells at different pH levels. Based on Tukey Kramer test, it was determined that the average density at different pH levels was not significantly different.

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean ± Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.63</td>
<td>1.057 ± 0.004</td>
</tr>
<tr>
<td>7.94</td>
<td>1.056 ± 0.004</td>
</tr>
</tbody>
</table>

Table 5.10 Average density of CHO cells at different initial cell loading density. Based on Tukey Kramer test, it was determined that the recoveries at different initial cell loading density were not significantly different.

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean ± Std Dev</th>
<th>Tukey Kramer HSD test</th>
</tr>
</thead>
<tbody>
<tr>
<td>2500000</td>
<td>1.058 ± 0.006</td>
<td>A</td>
</tr>
<tr>
<td>5000000</td>
<td>1.056 ± 0.001</td>
<td>A</td>
</tr>
<tr>
<td>7500000</td>
<td>1.056 ± 0.004</td>
<td>A</td>
</tr>
</tbody>
</table>
Figure 5.1 Density distribution of different cell types determined using continuous percoll density gradient medium and density marker beads. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the means of density distribution. The quantitative results are presented in table 5.6.
Figure 5.2 Recovery of CHO cells at different pH levels. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the means recoveries. Based on Tukey Kramer HSD test, it can be concluded that the recoveries are not significantly different. The quantitative results are presented in table 5.7.
Figure 5.3 Recovery of CHO cells at different initial cell loading density. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the means recoveries. Based on Tukey Kramer HSD test, it can be concluded that the recoveries are significantly different. The quantitative results are presented in table 5.8.
Figure 5.4 Average density of CHO cells at different pH levels. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the means cell density. Based on Tukey Kramer HSD test, it can be concluded that the recoveries are not significantly different. The quantitative results are presented in table 5.9.
Figure 5.5 Average density of CHO cells at different initial cell loading density. Three lines along with data points represent the mean and standard deviation. The circles on right of the plot represent the result of Tukey Kramer HSD test comparing the means recoveries. Based on Tukey Kramer HSD test, it can be concluded that the cell density are not significantly different. The quantitative results are presented in table 5.10
CHAPTER 6

CHARACTERIZATION OF ARACHNOIDAL CELLS CULTURED ON THREE-DIMENSIONAL NON-WOVEN PET MATRIX

This research project is a collaborative effort with Dr. Deborah Grzybowski in Biomedical Engineering Center, The Ohio State University, Columbus, Ohio. The content of this chapter is prepared for publication: Mehta, BC; Holman, DW; Grzybowski, DM; Chalmers, JJC; Characterization of Arachnoidal Cells Cultured on Three-Dimensional Non-Woven Pet Matrix.

6.1 Introduction

The arachnoid granulations (AGs) are projections of the arachnoid membrane through the dura mater into the venous sinuses and lacunae (Figure 6.1) (Perkin et al., 1986). They represent the interface between the cerebrospinal fluid (CSF) and venous blood and provide a pathway for the return of CSF to the systemic circulation (Warwick and Williams, 1973; Weed, 1914; Weed, 1923). Our understanding of the function of human AGs is limited due to the complex tissue structure of human AGs, the lack of an in vitro human model and the primitiveness of the animal models. One of the pathological conditions associated with increased intracranial pressure (ICP) is known as
idiopathic intracranial hypertension (IIH), which may lead to papilledema. Papilledema can lead to progressive optic atrophy and irreversible visual loss. One of the causes of IIH is a disruption of normal CSF dynamics which may be due to an increased resistance to CSF egress at the AGs (Johnston, 1975; Johnston et al., 1991; Levine, 2000; Martins, 1973). To study the function of human AGs we have developed an *in vitro* model using cells cultured from human AG tissue.

The specialized task of AGs is to regulate the outflow of CSF and is governed by a specialized tissue structure. An AG has four main components as shown in Figure 6.2. They are the cap cell cluster, fibrous capsule, arachnoid cell layer and central core (Kida, 1988; Upton, 1985). The central core of the AG is a continuation of the subarachnoid space (SAS) through which the CSF normally circulates. It is made up of a connective tissue matrix containing fibroblasts interspersed with arachnoid cells and arachnoid trabeculae(Kida, 1988). The arachnoid cell layer is a continuation of the arachnoid mater and consists of arachnoidal cells, 2 – 7 cell layers in thickness (Kida, 1988; Upton, 1985; Wolpow ER, 1972). The fibrous capsule represents where the arachnoid granulation projects through the dura and into the sinus/lacunae lumen (Wolpow ER, 1972). In human AG the arachnoidal cell layer is not completely covering the fibrous capsule as shown in Figure 6.2 (Kida, 1988). The apical portion of arachnoid granulation in humans is made up of arachnoidal cap cell layer and may be important in regulating CSF egress.

The successful implementation of an *in vitro* model to study CSF egress relies on primary cultures of functional arachnoidal cells. The differentiated state of arachnoidal cells is governed by the microenvironment of the tissue. In recent years natural and synthetic polymers have been used to provide a suitable extracellular environment for
functional tissue structure formation \textit{in vitro} (Lanza, 2000; Sittinger et al., 1996). Multiple cell types have been used along with polymers to produce functional blood vessels as well as bladder and corneal constructs (Griffith et al., 1999; Niklason et al., 1999; Oberpenning et al., 1999). The success of \textit{in vitro} tissue formation depends upon a suitable polymer selection and a suitable culture system that properly simulates the \textit{in vivo} environment. The purpose of this study is to culture primary arachnoidal cells on a suitable polymer substrate.

The central core of an AG is composed of connective tissue and extracellular matrix proteins that provides structural support to the overlying arachnoidal cells. To simulate this condition, a non-woven poly(ethylene) terephthalate (PET) matrix was selected. PET matrices have been used successfully to culture human trophoblast cells, mesenchymal embryonic stem cells, astrocytes, hybridoma cells and NIH 3T3 cells (Basu and Yang, 2005; Li Y, 2001; Ma et al., 1999; Ma T, 2000; Takahashi and Tabata, 2004). This system is well characterized in terms of physical properties (fiber density: 1.35 gm/cm$^3$, fiber diameter $\sim$ 20 $\mu$m and porosity $\sim$ 93%) (Basu and Yang, 2005; Bhat, 1995) and chemical properties (Phaneuf MD, 1997). In this study we utilized agitated seeding to culture primary arachnoidal cells on a PET matrix. The cultured cells were then characterized based on morphology, growth kinetics and immunocytochemistry. These results were compared with our existing 2D culture method (Holman et al., 2005). Cells grown on 3D scaffolds were then seeded onto permeable cell culture inserts for functional analysis. To assess barrier function, cells were characterized by their permeability to the hydrophilic macromolecule Lucifer yellow. Permeability coefficients were compared to existing data for cell grown in 2D culture method (Grzybowski, 2006).
6.2 Materials and methods

6.2.1 Primary culture of Arachnoidal cells and identification of cell phenotype

Arachnoidal cells were cultured from brain tissue obtained from human cadavers. The detailed methodology of the culture technique is discussed by Holman et al (Holman et al., 2005). Briefly, brain tissue was obtained within 24 hours post-mortem from the Ohio State University Regional Autopsy Center. At autopsy, AGs were collected from the superior sagittal sinus and adjacent tissue. Samples were placed into sterile phosphate buffered saline with penicillin and streptomycin. The tissue was washed 3X in sterile Dulbecco’s phosphate buffered saline (D-PBS) (Cellgro Mediatech, Herndon, VA) prior to explantation. The washed tissue was then placed into fresh culture medium containing Dulbecco’s Modified Eagle Medium/Ham’s F-12 Nutrient medium (50:50 v/v), with L-glutamine, penicillin/streptomycin, amphotericin B (all Cellgro Mediatech), and 10 % newborn calf serum (Invitrogen Gibco, Carlsbad, CA). The explantation was performed under a dissecting microscope, where individual granulations were secured adjacent to the apical cap cell portion of the granulation and cut just below the cap of the granulation. The AG cap was removed, washed again in medium, and placed into a 24-well culture plate coated with fibronectin solution (30 µg human fibronectin (Sigma, St. Louis, MO) per ml M 199 culture medium (Cellgro Mediatech)). Culture medium was added to each well, and the explants were incubated undisturbed for three to four days. The medium was changed every three to four days. When confluent, cells were washed with D-PBS, removed from their wells with 0.05% Trypsin EDTA in Hanks buffered saline (Cellgro
Mediatech), and spun down at 1300 rpm. The cell pellet was resuspended and plated to a T-25 cm² culture flask.

These cells were qualitatively analyzed using brightfield microscopy. Brightfield images were taken using a Zeiss Axiocam inverted microscope. Arachnoidal cells were also characterized using immunocytochemistry. For this purpose the cells were cultured on fibronectin coated coverslips by following the same harvesting protocol as discussed above. The cells were analyzed after 1 – 1.5 weeks post confluency. The following monoclonal antibodies were used at the dilutions indicated: mouse anti-human cytokeratin antibody clones AE1/AE3 (1:50, DakoCytomation, Carpinteria, CA), mouse anti-human desmoplakin 1&2 antibody (1:40, Chemicon International, Temecula, CA), and FITC conjugated mouse anti ZO-1 antibody (1:50, Zymed). The secondary antibody used was an Alexa Fluor 555 conjugated donkey anti-mouse IgG1 antibody (Molecular Probes, Eugene, OR) at a 1:50 dilution for 45 minutes at 37°C. All primary and secondary antibodies were diluted in 10% calf serum in D-PBS.

6.2.2 Scaffold preparation, cell seeding method and seeding efficiency

Needle punched non-woven poly(ethylene terephthalate) (PET) fabrics (fiber density: 1.35 gm/cm³, fiber diameter ~ 20 µm and porosity ~ 93%) were used as cell culture scaffolds. PET fabrics were first cut into small circular patches (diameter 5mm) and washed with scouring solution (10 gm Na₂CO₃, 10 ml Tween 20 in 1L deionized water) for 30 minutes at 60°C followed by a rinse in 100 ml deionized water for 30 minutes at 60°C. These scaffolds were then treated with 1% NaOH solution at the boiling temperature for 60 minutes to create carboxyl and hydroxyl groups on the fiber
surface by partially hydrolyzing PET in order to increase biocompatibility (Bhat, 1995; Phaneuf MD, 1997). Treated scaffolds were then rinsed with deionized water to remove traces of NaOH and autoclaved at 121°C and 15 psig pressure for 1 hour. For cell seeding, individual scaffolds were placed in 24-well culture plate. Each scaffold was then incubated in 500 µl of 30 µg/ml fibronectin solution (prepared as discussed above) for 30 minutes at 37°C.

The primary cultures were utilized for cell seeding. Second passage arachnoidal cells from a T-25 cm² flask were harvested by incubating in 2 ml of Accutase™ for 5 minutes (Innovative Cell Technologies, USA). The harvested cells were mixed with media and spun down at 1300 rpm. The pellet was resuspended in appropriate amount of media and the cell concentration was determined using a hemacytometer. Two seeding methods were employed to culture the arachnoidal cells. The first method was based on static seeding. Here each well containing one PET scaffold was inoculated with ~1 × 10⁵ cells/ml at the center of the matrix using a pipette. After incubation in a CO₂ incubator for ~1 hour to allow for cell attachment to the matrix, the seeded matrix was transferred to a new culture plate (each well containing 1 ml of the medium) to ensure that all growth in the well was attributed to cells attached to the PET matrix and not the surface of the tissue culture plate. The second method was based on agitated seeding. Here each well containing one PET scaffold was inoculated with ~1 × 10⁵ cells/ml. The scaffold was then agitated using a plate shaker for ~24 hours in CO₂ incubator. After incubation, the seeded matrix was transferred to a new culture plate (each well containing 1 ml of the medium) to ensure that all growth in the well was attributed to cells attached to the PET matrix and not the surface of the tissue culture plate. The seeding efficiency was calculated based on
the remaining cells in the suspension and attached to the initial culture plate. The cell culture medium was changed initially every 3-4 days and later on every 2 days.

6.2.3 Biochemical Characterization

6.2.3.1 Determination of Glucose and Lactate concentration and growth kinetics

The metabolic rates of cells cultured on scaffolds and on culture plate were measured in terms of glucose consumption and lactate production. Glucose and lactate concentration of the medium samples taken every 24 hours were analyzed using YSI Biochemistry Select Analyzer (Yellow Spring, OH). Growth kinetics was measured by quantifying the number of cells on the scaffold. For quantification, the cells from the scaffold were harvested by incubating the scaffold in 1 ml Accutase™ for 1 hour. The cells were recovered from scaffolds by repeated pipeting. The harvested cells were washed with medium and spun down at 1300 rpm. The pellet was resuspended in appropriate amount of medium and cell concentration was determined using hemacytometer. The viability of cells was determined by trypan blue assay.

6.2.3.2 Brightfield microscopy and scanning electron microscopy(SEM)

The cells cultured on scaffold were qualitatively analyzed using brightfield microscopy and SEM. Brightfield images were taken using a Zeiss Axiocam inverted microscope. For SEM visualization, the fibrous scaffold samples were fixed overnight with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). They were then washed with the phosphate buffer for 30 minutes. After fixation, the samples were dehydrated in
a graded series of ethanol and then Hexamethyldisilazane (HMDS). The samples were then sputter coated with gold/palladium and were examined using a SEM (Philips XL30).

### 6.2.3.3 Confocal laser scanning microscopy

Arachnoidal cells cultured on scaffold were also characterized using immunocytochemistry. The following monoclonal antibodies were used at the dilutions indicated: Cy3 conjugated mouse anti-vimentin antibody (1:100, Sigma), FITC conjugated mouse anti E-cadherin antibody (1:40, Becton Dickinson, Franklin Lakes, NJ), FITC conjugated mouse anti ZO-1 antibody (1:50, Zymed), Alexa Fluor 546 conjugated phalloidin (1:40, Molecular Probes, Eugene, OR) and DRAQ5 (1:400, Biostatus, Shepshed, UK). All antibodies were diluted in 10% calf serum in D-PBS.

The immunocytochemistry was performed on second passage cells on scaffold, which were grown to confluency. Cell cultures were tested at 1 month post-confluency for the presence of vimentin, actin, E-cadherin and ZO-1. The cells were washed 3X with sterile D-PBS and fixed with 3.7% paraformaldehyde for 10 minutes, then permeabilized with 0.2% Triton X-100 (Sigma) in PBS at 37°C for 5 minutes. The cells were incubated for 30 minutes in 10% calf serum in D-PBS to block non-specific binding of the antibody, then incubated with the primary antibodies at the dilutions indicated above for 60 minutes at 37°C. Cells were then washed in D-PBS and mounted with Tissue Tek® OCT (Fisher Scientific, Houston, TX) onto slides for visualization. The scaffolds were visualized using a Zeiss 510META confocal microscope equipped with Red Helium-Neon, Green Helium-Neon and Argon lasers.
6.2.3.4 Protein analysis with Western blot

Arachnoidal cells cultured on scaffolds were also analyzed for proteins using western blot to confirm the microscopy based results. Confluent cells from 2D and 3D cultures were lysed using 50 µl of mammalian protein extraction reagent (M-PER, Pierce, Rockford, IL) at 37°C for 15 minutes. All further steps were performed at 4°C. The lysate was spun down at 14,000 rpm for 10 minutes and the supernatant was separated as sample of interest. The supernatant at this stage can be stored for long term at -80°C. The supernatant was probed with mouse anti-ZO-1, 2 antibodies (1:1000, Zymed).

An appropriate amount of supernatant was taken in microcentrifuge tubes so that the protein concentration was 10 – 50 µg. The supernatant was denatured by boiling the content with loading buffer (containing sodium dodecyl sulfate, β-mercaptoethanol and bromophenol blue dye) for 5 minutes. The supernatant was then loaded on 10% Tris-HCl gel and run at 120 V for 90 minutes. The protein from the gel was transferred to a nitrocellulose membrane 90V for 60 minutes. The membrane was then blocked for 60 minutes with 5% milk, followed by overnight primary antibody labeling. This membrane was probed with secondary antibody and horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (1:5000 dilution, Santa Cruz Biotech, Santa Cruz, CA) for 45 minutes. The protein of interest was detected by reacting Chemiluminescence substrate for 2 to 3 minutes (PerkinElmer, Boston, MA) and exposing film to the nitrocellulose membrane.
6.2.4 Functional Outflow Assessment

6.2.4.1 Filter seeding for functional assessment

Above discussed methods were utilized for biochemical characterization of the cultured AG cells. These cells were further characterized in vitro for their function of controlling the outflow of fluid by seeding them on polycarbonate cell culture inserts (Millipore PCF inserts, 12 mm diameter, 0.6 cm² effective growth area, 0.4 μm pore size). For cell seeding, individual inserts were placed in 12-well culture plates. Each insert was then incubated in 200 μl of 30 μg/ml fibronectin solution (prepared as discussed above) for 30 minutes at 37°C. Cells from primary cultures as well as from the scaffolds were utilized for the cell seeding. The cells were harvested and cell concentration was determined in the same manner as discussed above for the T-25 cm² flask and scaffold. For each culture insert the cells were seeded at a density of approximately 3.5 × 10⁵ cells. These culture inserts were then incubated in a 5 % CO₂ incubator at 37°C.

6.2.4.2 Lucifer Yellow (LY) permeability assay

On days 7, 14, 21, 28, 35, 42 after seeding culture inserts, cells were tested for LY permeability. For the permeability assay, a stock solution was made by dissolving lucifer yellow (Sigma, lucifer yellow dipotassium salt) in basal media (in this case DMEM/F12 50:50) to a concentration of 4 mM. For each experiment, the stock solution was diluted to a working solution of 200 μM. The working solution was used to make the dilutions for the standard curve as well as the 100 μM solution that was used for the permeability experiments. Normal media was removed from inside the insert, hereafter referred to as
the apical side, and replaced with a 100 μM solution of LY in basal media. Normal cell culture media was also removed from the well surrounding the insert, the basal side, and replaced with basal media. To determine the permeability characteristics of the culture insert alone; a blank insert without cells was tested as well. Next, inserts were incubated for two hours at 37 °C on a plate shaker to ensure uniform concentration of Lucifer yellow in both apical and basal sides. At the end of the incubation period, the plate with the inserts was removed from the incubator and 3×100μl aliquots were sampled from the basal side of each culture insert. Samples were measured using CytoFluor® 4000 Fluorescence Multi-Well Plate Reader from Applied Biosystems (Foster City, CA) with 485nm excitation wavelength and 530nm emission. To correlate the fluorescent units read from the aliquots taken from the basal chamber of the inserts to concentration values, a standard curve was prepared. This was made from a series of dilutions from 200, 150, 100, 50, 25, 10, 5, 1, 0.5, 0.1, 0.01 μM solutions of LY. Additionally, the intrinsic fluorescence in basal media was measured and subtracted from all fluorescence values to approximate the true fluorescence from LY alone.

6.2.4.3 Determination of permeability coefficient

The permeability of tracer molecules across a cell layer is often reported as permeability coefficients, $P_{cells}$ (in cm/sec). The method used to calculate the permeability coefficient has been used extensively to characterize blood brain barrier endothelial permeability and is described in detail elsewhere (Deli et al., 2005). In brief, the volume of LY solution cleared through the apical to basal side is calculated by
Clearance Volume = \([\text{concentration}]_{\text{basal}} \times \text{volume}_{\text{apical}}\) \quad (6.1)

If the clearance volume is divided by the time of the experiments, the result is the permeability surface area product (in cm³/sec) of the cells plus the insert, PS\(_{\text{cells+insert}}\). The permeability surface area product of the cells alone, PS\(_{\text{cells}}\), was calculated from the formula:

\[
\frac{1}{\text{PS}_{\text{cells}}} = \frac{1}{\text{PS}_{\text{cells+insert}}} - \frac{1}{\text{PS}_{\text{insert}}} \quad (6.2)
\]

The PS\(_{\text{cells}}\) was calculated according to the fact that the total resistance of the system towards passage of LY is composed additively of the two parallel resistances of the cell layer and the filter insert. To determine the permeability coefficient of the cells, \(P_{\text{cells}}\) (in cm/sec), the PS\(_{\text{cells}}\) was divided by the cell culture insert growth area (0.6 cm²).

**6.2.4.4 Experimental hydraulic conductivity**

The culture inserts with cells were further analyzed for functional study using a perfusion system (Grzybowski, 2006) to understand the behavior of cells under pressure. Cells were seeded at near confluent densities (~3.5 × 10⁵) and grown on the filter support for at least 21 days to ensure the formation of tight junctions. After 21 days, cells on filters were inserted into the perfusion system shown in Figure 6.3. The cell perfusion system consisted of a modified Ussing chamber, connected through tubing to a pair of fluid reservoirs, one for each side of the perfusion chamber. The reservoirs held culture media and provided the hydrostatic pressure gradient. To maintain viability of cells over the 4-6hrs of perfusion, the system was placed in an incubator at 37° C.
The pressure drop across the cell layer was continuously monitored by an in-line pressure transducer (Transpac IV, Abbott Critical Care Systems, North Chicago, IL), interfaced with a data acquisition computer equipped with Labview software to record pressure in real time. In addition to monitoring the cell layer pressure, a second pressure transducer monitored ambient pressure during perfusion. The ambient pressure was subtracted from the cell layer pressure measurements for the true pressure drop across the cell layer.

In order to determine the volumetric flow rate across the cell layer, the perfusate was collected from a sampling port on the downstream side of the cell layer. At the conclusion of the perfusion, this volume was measured and then divided by the time of the run to get an average volumetric flow rate. To measure evaporation, a known amount of media (2ml) was placed in an open tube, next to and identical to the perfusate collection tube. Following the run, this volume was re-measured and the evaporation rate calculated. This value was then added to the average volumetric flow rate to correct for evaporation. This corrected volumetric flow rate along with true pressure drop across cell layer was utilized to determine the hydraulic conductivity through the cell layer. The following section briefly discusses the hydraulic conductivity calculations, more details have been described by Grzybowski et al (Grzybowski, 2006).

6.2.4.5 Hydraulic conductivity calculation

The hydraulic conductivity ($L_p$) is a measure of the amount of fluid that will pass through the cell layer or membrane, and depends on the surface area, volumetric flow rate and pressure drop. The resistance of the cell layer to flow is inversely related to the
cellular hydraulic conductivity; as the resistance across the cell layer increases the cellular hydraulic conductivity decreases. The resistance of the cell layer is also inversely related to the area across which the fluid flows.

\[ \frac{1}{L_p} = \frac{\Delta P A}{Q} \]  

(6.3)

To accurately calculate the \( L_p \) of the cell layer alone, the resistance of the filter membrane must be accounted for. Although more permeable than the cell layer, it is possible that the filter membrane still has a significant resistance to fluid flow. To calculate how the hydraulic conductivity of the filter affects the total resistance, the cells and filters can be considered a pair of resistors in series;

\[ R_{\text{cells+filter}} = R_{\text{cells}} + R_{\text{filter}} \]  

(6.4)

And

\[ \frac{1}{L_{p,\text{cells+filter}}} = \frac{1}{L_{p,\text{cells}}} + \frac{1}{L_{p,\text{filter}}} \]  

(6.5)

\[ L_{p,\text{cells}} = \frac{Q_{\text{cells+filter}}}{\Delta P A (1 - \frac{Q_{\text{cells+filter}}}{Q_{\text{filter}}})} \]  

(6.6)

From this equation, the cellular hydraulic conductivity can be calculated since the surface area (A) for the filters is known; the pressure drop can be calculated based on the pressure at the cell layer and ambient pressure \( \Delta P = P_{\text{cell layer}} - P_{\text{ambient}} \); the flow rate across the cells and filter, \( Q_{\text{cells+filter}} \), is calculated, and the flow rate across the filter, \( Q_{\text{filter}} \), is measured by perfusing empty filters without cells and calculating the average volumetric flow rate across the filter alone.
6.2.4.6 Post perfusion analysis

Perfused cell layers were stained with a cell viability assay to determine if the perfusion conditions caused cell damage or death. Cell viability was assessed with an assay (Live/Dead® viability/cytotoxicity kit, Molecular Probes) of intracellular esterase activity and membrane integrity. Diffuse cytoplasmic green fluorescence indicated live cells, while localized nuclear red fluorescence identified dead or dying cells. After perfusion, cells on filter were removed from the perfusion chamber and incubated with a solution of 2μM calcein and 4μM ethidium homodimer in PBS for 40 minutes. The filter membrane was then excised from its casing and the cells on the filter were inverted onto a microscope slide, coverslipped, and viewed on a Zeiss Axiocam inverted microscope equipped with FITC and rhodamine filters.

6.3 Results

6.3.1 Cell culture

AGs were collected at autopsy from the superior sagittal sinus and lateral lacunae (Figure 6.4A) and explanted into culture plates. Cell migration from human AG explants was seen within 7-10 days (Figure 6.4B). The AG cap cells in culture grew in monolayers, exhibited a polygonal morphology, and packed densely to form a cobblestone-like appearance characteristic of epithelial cell types (Figure 6.4C).
6.3.2 Identification of Phenotype

These cells in 2D culture were characterized in second passage cultures, which were immunoreactive to the anti-human cytokeratin antibody (AE1/AE3), which recognizes a wide range of human cytokeratins (Moll’s designation 1-8, 10, 13-16, 19). A subpopulation of cells expressed cytokeratin intermediate filaments in a perinuclear pattern, with long filaments surrounding the nucleus in a basket-like structure (Figure 6.5A). AG cells expressed the desmosomal junctional proteins desmoplakin 1&2 at cell borders (Figure 6.5B), where desmosomes mediate cell-cell attachments. Finally, human AG cells were immunoreactive to the anti-ZO-1 antibody expressed at tight junctions (Figure 6.5C).

6.3.3 Growth kinetics and seeding efficiency in 3D culture

The arachnoidal cells characterized above were harvested at second passage from 2D culture and were seeded to NaOH treated scaffolds. The seeding efficiency of static seeding was less than 50% and a significant number of cells were growing on the plate compared to the scaffolds. The seeding efficiency of agitated seeding was 89% and the cells grew reproducibly in the treated scaffold based on cell counting using a hemacytometer. Following analysis of these results, all further studies were performed using agitated seeding. The behavior of cells cultured on scaffolds was compared with cells cultured in 12 well culture plates from the same tissue donor. It was observed that the doubling time of the cells cultured on 3D scaffolds was 28 hours whereas the doubling time of cells cultured on a 2D plate was 20 hours. The glucose consumption rate
of cells cultured on scaffolds was 0.0029 gm/(L.hr) compared to 0.002 gm/(L.hr) for cells cultured on the plates. The lactate production rate of cells cultured on scaffolds was 0.0056 gm/(L.hr) compared to 0.0019 gm/(L.hr). These results show a similarity between the growth kinetics of cells cultured on a 3D scaffold compared to cells cultured on a 2D culture plate. For further biochemical characterization of the cells, the scaffolds were analyzed using various microscopy methods.

6.3.4 Brightfield and scanning electron microscopy study

Figure 6.6 shows the morphology of cells cultured on scaffolds after five days. It can be clearly seen (Figure 6.6 A and B) that the cells were distributed evenly and were forming tissue-like structures. The cells often formed sheets that bridged between the adjacent filaments. It is observed under higher magnification (Figure 6.6 (B)) that the cells were closely attached to each other as seen in Figure 6.4 (C). The brightfield microscopy results were further confirmed with SEM studies on the same scaffold after 11 days in culture. It was observed that a substantial number of cells was lost during SEM sample processing; even so, under low (Figure 6.6 (C)) magnification, it was shown that the cells were stretched and bridged between the adjacent filaments. The cells covered a significant portion of the top surface of the scaffold and were forming tissue-like structures. On higher magnification (Figure 6.6 (D)), it was clearly observed that cells formed sheets with no gaps in between. This behavior is similar to Figure 6.4 (C) and the cap cell region of arachnoid granulations that is believed to be responsible for controlling the cerebrospinal fluid outflow. This was confirmed by immunocytochemistry.
with the formation of tight junctions between the cells using laser scanning confocal microscopy studies.

### 6.3.5 Confocal laser scanning microscopy study

Arachnoidal cells cultured on scaffolds were analyzed after 1 month post–confluency for the expression of surface proteins (ZO-1 (participate in tight junctions) and E cadherin (participate in cell adhesion)) to confirm the formation of tight (non–leaky) tissue–like structures observed in brightfield and SEM images. Figure 6.7 (white arrows) show expression of ZO-1 (green, B and C) and E cadherin (green, D) respectively. Similar results were observed by Holman *et al* in primary cultures of arachnoidal cells from AG tissue sectioned from the superior sagittal sinus and lateral lacunae of the human brain (Holman *et al.*, 2005). ZO-1 protein forms tight junctions and were visualized as finger–like structures in cells cultured on plates. Similar phenotype was observed at higher magnification (white arrow in Figure 6.7, C). These cells were also labeled for intermediate actin filaments (orange) and nucleus (blue). Arachnoidal cells were also analyzed for the expression of vimentin intermediate filaments (orange) shown in Figure 6.7 (A). The epithelial proteins (E–cadherin and ZO–1) expressed by the cells were further confirmed by performing western immunoblot assays to confirm epithelial phenotypic behavior as discussed below.

### 6.3.6 Confirmation of phenotype based on immunoblot study

The cells cultured on scaffolds were compared with cells cultured on culture plates for the expression of ZO-1 and ZO-2 proteins as shown in Figure 6.8 (A and B)
respectively. Multiple bands are observed in Figure 6.8 (A and B) for expression of ZO proteins. This is a common phenomenon in all of our samples. We believe the reason for this break down of ZO protein is due to usage of mammalian protein extract reagent and proteolysis. This reagent is a mild detergent solution which dissolves the cell membrane to extract protein. This methodology might damage the occludin protein, also involved in tight junction formation, spanning across the cell membrane as well. These results further confirm the epithelial nature of AG cells on scaffolds. These cells were further studied for their function of controlling CSF outflow based on the permeability measurements of fluid flow across the cell layer.

6.3.7 Characterization of AG barrier function

6.3.7.1 LY permeability

The functional characterization of AG cells was performed based on simple permeability measurements of LY solution across the cell layer in culture inserts. These measurements were utilized to determine the permeability coefficient $P_{cells}$ (in cm/sec) based on equations (6.1 and 6.2) discussed in the section on permeability coefficient determination. Here, the permeability coefficients of AG cells cultured in culture plates (open squares) and scaffolds (filled squares) were compared as shown in Figure 6.9. It can be observed that initially the permeability coefficients decreased with increase in duration of culture in inserts (in days). But, after reaching a minimum value, the permeability coefficients increased again. For the culture inserts utilized in this study the minimum value was observed around 21 – 23 days. Based on these results the perfusion study was performed after the culture stabilized for 21 days after seeding. Also, the
permeability coefficients from cells from culture plates were compared with cells from scaffolds (Table 6.1) using two sample unequal variance t test. Based on the results of the t – test, it was observed that the permeability coefficients were not significantly different (p > 0.05) for both the culture conditions on 14, 21, 28 and 35th day. The permeability coefficient of cells from the culture plate was lower than that of cells from scaffolds on day 7. This observation might be due to a higher growth rate of cells in 2D culture compared to 3D culture, therefore making faster tight junctions. The comparison could not be made for day 42 due to lack of permeability measurements for cells cultured in a culture plate.

6.3.7.2 Perfusion study

Culture inserts similar to ones used for LY permeability were used for the perfusion study. Here the cells were subjected to a physiologic hydrostatic pressure of ~3 mm Hg in a Ussing chamber set up as shown in Figure 6.3. The cells allowed passage of media under pressure. Figure 6.10 shows the average cellular hydraulic conductivity plotted versus the average pressure drop across the cell layer. The open squares represent perfusion runs performed on cells cultured in culture plates where as filled triangles represent perfusion runs performed on cells cultured on scaffolds. It can be observed that the average cellular hydraulic conductivity for both the cases is in the same range. Table 6.2 provides a summary of the average pressure, average volumetric flow rate, and average cellular hydraulic conductivity for the perfusion runs shown in Figure 6.10. It can be observed that based on a two sample unequal variance t – test, the average pressure, average volumetric flow rate, and average cellular hydraulic conductivity for
both the cases are not statistically different (p < 0.05). These results are in accordance with those observed for LY permeability measurements. After the perfusion runs, the cells in the culture inserts were analyzed for viability to ensure that significant damage or death had not occurred during the perfusion runs.

6.3.7.3 Live/Dead analysis

The perfused cells from Ussing chamber were incubated in diluted solution of Calcein AM and Ethidium homodimer – 1. It was observed (Figure 6.11) that the majority of the cells had diffused esterase activity and intact membranes. Thus, the perfusion study did not affect the viability of the cells cultured on the filter inserts.

6.4 Discussion

AGs as a pathway for CSF egress have been studied since the works of Weed (Weed, 1914; Weed, 1923). Substantial studies have been performed using light and electron microscopy to understand the ultrastructure of the AGs and arachnoid membrane (Hasegawa M, 1997; Kida, 1988; Tripathi, 1974; Tripathi, 1977; Tripathi, 1973; Yamashima, 1986; Yamashima, 1988; Yamashima et al., 1988). However functional studies have been restricted to animal models due to ethical issues associated with human \textit{in vivo} functional studies and the inaccessible location of the AGs in the cranial cavity. The knowledge available from animal models does not necessarily predict human conditions appropriately due to the complex tissue structure of human AG compared to primate and canine (Kida, 1988; Ohta et al., 2002). In one of the animal models, dura and arachnoid villi from both monkey and canine were excised and perfused with colloid
gold, erythrocytes and polystyrene microspheres. However, a significant proportion of the preparations developed leaks (Pollay and Welch, 1962; Welch and Pollay, 1961). Therefore, to understand the role of AGs in CSF egress in humans under more controlled conditions, we have developed an in vitro model (Grzybowski, 2006). This model will help in understanding normal CSF outflow and pathological conditions associated with increased intracranial pressure.

Arachnoidal cells situated in the cap cell region of AG may play an important role in CSF egress (Kida, 1988; Yamashima, 1986; Yamashima, 1988; Yamashima et al., 1988). We have developed the first protocol for successfully growing primary cultures of these cells from human AG tissue. The cultures were shown to be free from contaminating fibroblast and endothelial cells based on immunological characterization (Holman et al., 2005). This study attempted to improve the proliferation and differentiation of arachnoidal cells. Functional studies performed to produce various tissue structures like blood vessel, bladder and cornea have shown that microenvironment, suitable polymer selection and suitable culture system are responsible for controlling and modifying the differentiated state of the cells (Griffith et al., 1999; Niklason et al., 1999; Oberpenning et al., 1999).

Tissue engineering utilizes a natural or synthetic polymer scaffold to provide suitable three dimensional environments for growing cells. Two – dimensional cultures are usually inadequate to model complex tissues like AG. Three – dimensional tissue culture scaffolds provides more surface area per unit volume, promotes high cell – cell interaction and are better representative of the actual tissue. The physical as well as chemical properties of the tissue scaffold have tremendous impacts on the seeding of the
scaffolds, cell morphology, spatial organization, proliferation, differentiation and function (Lanza, 2000). To simulate AG structure in vitro, a high cell number in a small volume is desired. This condition promotes the formation of tight junctions necessary for controlling the outflow of CSF.

In this study, static and dynamic (agitated) seeding was utilized. In static seeding the cells were not distributed uniformly (data not shown) and majority of the cells grew on the periphery of the matrix. Also, a significant amount of cells were not seeded on the scaffold at all due to high porosity and lower cell residence time in the scaffold. Agitated seeding improved the seeding efficiency (89%) and distribution of cells due to agitation and higher residence time of the cells in the scaffold. These results are in accordance with the observation that dynamic seeding methods such as mixing and agitation improved cell spatial distribution in small scaffolds with one type of pore structure (Burg et al., 2000; Vunjak-Novakovic et al., 1998). The improved spatial distribution of cells due to agitated seeding resulted in uniform tissue – like structure formation in the entire scaffold (Brightfield and SEM images in Figure 6.6). The cells formed sheets that spanned multiple fibers and filled the majority of the porous scaffold. They were in close proximity to each other thus enhancing the possibility of the formation of cell – cell junctions.

Figure 6.7 (B, C, D) shows confocal microscopy images of the positive labeling of Z0-1 and E – cadherin proteins by cells cultured on PET matrix. Zonula occludins (ZO proteins) are peripheral membrane proteins that participate in association with tight junction protein (occludins) and intermediate filament actin (Alberts B, 2002). ZO proteins are commonly found in epithelial and endothelial tissue. Kida et al have shown...
the presence of tight junctions in arachnoid cells lining the AG (Kida, 1988; Yamashima, 1986; Yamashima, 1988). E-cadherins are Ca$^{2+}$ dependent cell-cell adhesion molecules and play a critical role in tissue differentiation (Alberts B, 2002). Over 40 types of cadherins are known; among these E-, P- and N-cadherins are most widely expressed, particularly during early differentiation (Alberts B, 2002). The expression of E-cadherin in the arachnoid membrane, AG and meningioma has been confirmed by immunohistochemistry (Figarella-Branger et al., 1994; Tohma et al., 1992; Yamashima et al., 1992). The expression of ZO-1 and E-cadherin protein (Figure 6.7) is also comparable to our previous study on the culture of arachnoidal cells in 2D culture (Holman et al., 2005).

The expression of ZO-1 and ZO-2 (Figure 6.8 A and B) was further confirmed using Western blot. It can be observed that the ZO-1 and ZO-2 proteins are broken down in multiple bands. This was believed to be due to the detergent extraction protocol and proteolysis. We have taken utmost care to perform all the steps at 4°C to avoid proteolysis. Stevenson et al have also observed similar phenomenon in their study and have attributed the protein breakdown to detergent extraction protocol and proteolysis (Stevenson et al., 1986). Despite proteolysis, it can still be observed that a band of ZO-1 is present around 225 kDa and a band of ZO-2 is present around 130 kDa confirming the confocal microscopy results (Alberts B, 2002). These results are in accordance with arachnoidal cells cultured in culture plates (Holman et al., 2005).

The above discussed results confirmed the biochemical characterization of AG cells cultured on scaffolds. Human AG cells *in vitro* expressed many of the intercellular junctional proteins necessary to regulate fluid flow (Holman et al., 2005), it was also
necessary to demonstrate that these cells could mimic some of the physiological function of the AG cells \textit{in vivo}. This was confirmed by characterizing the cells functionally based on LY permeability and \textit{in vitro} perfusion studies. As a functional means of assessing tight junction formation, cells were monitored with time for their permeability to the hydrophilic macromolecule lucifer yellow (LY). Since this tracer molecule is too large (522 Da) and polar to passively diffuse through the cell membrane, it can only pass through a cell layer via paracellular routes (Wise, 2002). The ability of AG cells to prevent the paracellular passage of LY is therefore a useful means of determining the formation of tight junctions and therefore barrier integrity. The paracellular flux of LY across AG cell layers was tested by calculating the permeability coefficient of LY across AG cells seeded to permeable cell culture inserts. LY permeability is a common method utilized extensively to characterize the endothelial cultures responsible for formation of the blood brain barrier (BBB) and epithelial culture (Caco – 2) to study drug transport across intestinal epithelial barriers. Deli \textit{et al} have reported values of permeability coefficients (for sucrose (342 Da)) of $1 – 30 \times 10^{-6}$ for \textit{in vitro} brain endothelial cultures from different species (Deli \textit{et al}., 2005). The permeability value determined here for AG cells (Table 6.1) was in this range. This comparison confirmed the formation of intact AG cells multilayer (qualitatively confirmed by fluorescence microscopy, data not shown here) on the culture inserts. The permeability assay is suitable only for confirming the integrity of cell layers. The physiological function of AG cells was further confirmed by perfusion studies by applying a physiological hydrostatic pressure across the AG cells.

Under \textit{in vivo} conditions in a normal human, the CSF pressure is approximately 3 – 5 mm of Hg greater than the pressure in the venous sinuses, creating a driving force for
the outflow of CSF from the subarachnoid space, across the basal membrane of the cells lining the AGs, and finally across the apical membrane into the venous sinuses (Blomquist et al., 1986; Friden and Ekstedt, 1983). Normal flow of CSF across the AG cells is basal to apical (B→A). In order to test the behavior of the cells under similar conditions, cells cultured on culture inserts from culture plates and scaffolds were perfused with serum free DMEM/F12 medium at pressure drops of 3 mm of Hg in the physiological (B→A) directions. Table 6.2 compared the average hydraulic conductivities of cells from culture plates and scaffolds and it was concluded that there was not a significant difference between both types of cells. Also, both types of cells were viable (Figure 6.11) after performing the functional analysis using the perfusion study.

Based on the biochemical and physiological analysis, it was concluded that the cells cultured on PET scaffolds represented similar properties as cells in culture plates. To expand this study it is proposed to culture AG explants directly onto the PET scaffolds as well as to improve culture condition to simulate the hydrostatic pressure environment experienced by AG cells in vivo. Also, the average hydraulic conductivity measured in vitro can be compared with average CSF outflow across brain, if the surface area of AG participating in CSF outflow is known. The overall goal of the study is to develop an in vitro model to test drugs for pathological conditions like pseudotumor cerebri or hydrocephalus.
<table>
<thead>
<tr>
<th>Duration in culture (in days)</th>
<th>( P_{\text{cells}} \text{culture plates} \times 10^{-6} ) cm/sec</th>
<th>( P_{\text{cells}} \text{scaffolds} \times 10^{-6} ) cm/sec</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 7</td>
<td>9 ± 1</td>
<td>22 ± 3</td>
<td>0.002</td>
</tr>
<tr>
<td>2 14</td>
<td>17 ± 3</td>
<td>13 ± 2</td>
<td>0.304</td>
</tr>
<tr>
<td>3 21</td>
<td>8 ± 1</td>
<td>11 ± 2</td>
<td>0.292</td>
</tr>
<tr>
<td>4 28</td>
<td>10 ± 1</td>
<td>23 ± 5</td>
<td>0.078</td>
</tr>
<tr>
<td>5 35</td>
<td>13 ± 1</td>
<td>22 ± 5</td>
<td>0.155</td>
</tr>
<tr>
<td>6 42</td>
<td>Culture not extended to 42 days</td>
<td>13 ± 2</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.1 Comparison of permeability coefficients of cells from culture plates and scaffolds

<table>
<thead>
<tr>
<th></th>
<th>Average Pressure</th>
<th>Average Flow Rate</th>
<th>Average Cellular ( L_p )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mmHg)</td>
<td>(ml/min)</td>
<td>ml/min/mmHg/cm²</td>
</tr>
<tr>
<td>2D</td>
<td>2.917078785</td>
<td>113.1765404</td>
<td>81.24611096</td>
</tr>
<tr>
<td>3D</td>
<td>2.568244644</td>
<td>111.9427234</td>
<td>95.22298192</td>
</tr>
<tr>
<td>p-Value</td>
<td>0.223610296</td>
<td>0.943153425</td>
<td>0.399167105</td>
</tr>
</tbody>
</table>

Table 6.2 Summary of perfusion study results for AG cells cultured on culture plates (2D) and on scaffolds (3D)
Figure 6.1 Cerebrospinal fluid is produced by the choroid plexus in the lateral, 3rd, and 4th ventricles where it circulates to the subarachnoid space and eventually returns to the venous blood via the arachnoid granulations (Perkin et al., 1986) (A). AGs are projections of the arachnoid membrane into the dural venous sinuses and lateral lacunae (Warwick and Williams, 1973) (B)
Figure 6.2 Schematic diagram of a human arachnoid granulation showing four different regions. The arachnoid membrane and cap cell cluster are shown in green color here (Kida, 1988)
Figure 6.3 AG cells were perfused in a modified Ussing chamber. Cells were seeded onto culture inserts and perfused in the physiological (Basal→Apical) direction (see inset). A hydrostatic pressure head was applied by adjusting the height of the fluid reservoir relative to the cell layer. An in-line strain gauge pressure transducer interfaced with a data acquisition computer and allowed for real-time monitoring of the pressure drop across the cell layer. Volumetric flow rate was measured by collecting the perfusate from a downstream sampling port.
Figure 6.4 Human arachnoid granulations were collected at autopsy within 24 hours post-mortem (A). Individual AGs were excised with micro-surgical scissors and explanted to fibronectin coated culture dishes. Cell growth from the explant was seen within 7-10 days (B). AG cells in culture show polygonal cell morphology and when confluent, pack in densely to form a cobblestone-like appearance typical of epithelial cells in culture (C)
Figure 6.5 Immunocytochemical methods were used to identify arachnoidal cell phenotype. AG cells were grown on fibronectin coated coverslips and labeled with antibodies to broad-spectrum cytokeratins (A). AG cells were also labeled with antibodies to the desmosomal plaque protein desmoplakin 1&2 (B) and the tight junction protein ZO-1 (C). Positive expression Desmoplakin and ZO – 1 of these proteins was seen at cell-cell borders. Primary antibody expression was visualized by labeling the cells with a secondary antibody conjugated to a fluorescent molecule. Secondary antibodies were either anti-IgG FITC (green) or anti-IgG Alexa Fluor 555 (red)
Figure 6.6 (A, B) Brightfield microscopy images of cells cultured on scaffolds. The cells were seeded by agitated seeding and the images were taken after 5 days in culture at 100X magnification (A) and 320X magnification (B). The cells form tissue-like structure and the cells span across fibers. This was confirmed by scanning electron microscopy (C, D). SEM images were taken after 11 days in culture. The cells form multiple clusters of tissue-like structures (C). On close observation the cells are tightly attached to each other confirming epithelial-like morphology (D)
Figure 6.7 Confocal microscopy images of arachnoidal cells cultured on PET scaffold. The cells expressed vimentin (orange) intermediate filaments (A). The epithelial behavior was confirmed by expression of ZO 1 (green) ((B) at 400 X magnification and in (C) at 600 X magnification) and epithelial specific cadherin (E – cadherin) (green) membrane protein (D). The cells were also stained for nucleus (blue, A-D) and actin (orange, B-D) filaments. The bar represents 20 µm
Figure 6.8 Western blot profiles of two-dimensional cells from two different tissue batches compared with three-dimensional culture for expression of ZO-1 (A) and ZO-2 (B). The cells cultured on PET matrix showed similar expression of zona occludens as observed in two-dimensional culture.
Figure 6.9 Plot of permeability coefficient of cells (in cm/sec) vs. duration of culture (in days). The cells were cultured in culture inserts and incubated with 100µM LY solution on the apical side of the insert. The permeability of LY across cell layer was measured after two hours by taking samples from the basal side. The permeability coefficient initially decreases with increase in duration of culture. The minimum values were observed around 21 – 23 days. Here open squares and filled squares represent the permeability coefficients of cells from culture plate and scaffolds respectively. There was no statistically significant difference between the permeability coefficients of cells from culture plates and scaffolds (Table 6.1)
Figure 6.10 Plot of average cellular hydraulic conductivity vs. average pressure for cells cultured on culture plate (open squares) and on scaffolds (filled triangles). It can be observed that the average cellular hydraulic conductivity for both the cases are not significantly different (Table 6.2), thus confirming the results of permeability coefficient in LY permeability assay.
Figure 6.11 Calcein AM (red) and Ethidium homodimer -1 (green) staining of cells cultured on filter inserts from 3D scaffolds (A) and 2D culture plates (B). These cells were perfused with serum free media under physiological pressure. It was observed that few cells had compromised cell membrane (red nuclei) compared to the majority of viable cells showing diffused esterase activity (diffused green cytoplasm). These results confirmed that perfusion study did not cause significant damage or death of the cells.
7.1 Conclusions

The present study was focused on understanding the parameters that control the tissue digestion process using catabolic enzymes, which is a common life sciences methodology for various applications like recovery of genetic material for diagnosis, recovery of cells for in vitro research, recovery of cells for transplantation and so on. This research utilized breast tissue as an example to conduct this study, but the principles are applicable to other tissue digestion process as well. There are multiple catabolic enzymes available to digest different ECM components of the breast tissue and current knowledge about which enzymes to be used is strictly empirical and varies from one laboratory to other. This study focused on understanding the importance of each ECM component in breast tissue and concluded that collagen concentration and distribution are the rate controlling component in breast tissue digestion.

This conclusion was supported based on the results showing the distribution of collagen and elastin in breast tissue performed by standard histological techniques. The collagen content was much higher compared to elastin and the cells were well interspersed within the collagen matrix. Thus, to separate cells in viable and unmodified
state from the breast tissue it was imperative to digest the collagen matrix surrounding the cells as fast as possible to release the cells in suspension. Based on high collagen concentration we hypothesized that actual time of tissue digestion can be modeled based on digestion time of collagen. To model the digestion time of collagen in tissue; this study focused on determining collagen concentration in the tissue, determining the solution kinetics of collagen degradation and determining the effect of mass transfer of collagenase in the breast tissue as primary variables.

To model the tissue digestion process, the collagen concentration was quantified by converting the collagen to an amino acid hydroxyproline unique to collagen, which was further quantified by converting it to a chromophore. Based on this quantification, it was determined that average collagen concentration in breast tissue is $53 \pm 37 \, \mu g$ of collagen per mg of wet breast tissue weight. This concentration provided the basis for the substrate concentration in enzyme digestion process. Collagenase is a specific enzyme responsible for the digestion of collagen. Bacterial collagenase consists of multiple components with different kinetic parameters. Thus, we quantified the kinetic parameters of crude collagenase (containing all the individual components) based on a novel approach designed in this study. The principle of this approach is simple where bovine type I collagen substrate, which is highly labeled with FITC molecule is subjected to collagenase. Due to high concentration of FITC on collagen molecule the fluorescence is quenched up to virtually zero value. When such substrate is degraded the FITC molecules are released in solution, thus increasing the fluorescence of the solution. The kinetic parameters of collagenase was quantified based on this increase in fluorescence as $5.9 \pm 3.1 \, \mu g \, \text{of collagen/}U\cdot\text{min} \, (k_{cat})$ and $7.2 \pm 1.7 \, \mu g \, \text{of collagen/ml} \, (K_M)$. These kinetic
parameters along with the collagen concentration were utilized to calculate the time of
digestion based on analytical solution of Michaelis – Menten kinetics. The time of
digestion was calculated to be in the range of 10 minutes to 120 minutes for 87.5%
consumption of collagen from starting concentration range of 53 µg to 530 µg of collagen
respectively using 2 mg/ml collagenase concentration. This time was an order of
magnitude less than observations of other researchers, which ranged from 1 hour to 16
hours for breast tissue (Costa et al., 1987; Dairkee et al., 1997; Gomm et al., 1995;
Pechoux et al., 1999).

This discrepancy in the time of digestion was attributed to mass transfer of
enzyme in the tissue matrix. The diffusion of enzyme was modeled based simple reaction
diffusion model (Equation 4.6 and 4.9). Here it was assumed that the effective diffusivity
was constant, collagen concentration of the tissue was constant and the reaction rate was
same as Michaelis – Menten kinetics parameters determined for collagen degradation
based on protocol designed in this study. The diffusivity values were predicted based on
power law relationship reported by Jain et al between diffusivity and molecular weight of
solute for mouse model (Jain, 1987). Based on reaction diffusion model the time
prediction of enzyme diffusion in tissue and time of tissue digestion were in the same
range as observed by other researchers (Costa et al., 1987; Dairkee et al., 1997; Gomm et
al., 1995; Pechoux et al., 1999).

The reaction diffusion model predicted that the enzyme diffusion is the rate
limiting step in the tissue digestion. This prediction was confirmed by subjecting
different sizes (0.69 mm and 5.38 mm) of breast tissue samples at different enzyme (2
mg/ml and 10 mg/ml) concentrations. These experiments also showed that the time
predicted by reaction diffusion model for enzyme digestion is an overestimation of actual situation. This overestimation can be attributed to change in diffusivity and collagen concentration in the tissue with respect to time and spatial position in tissue.

The contribution of this study is the design of engineering analysis of tissue digestion process to optimize the time of digestion based on collagen degradation. This analysis relied on determination of primary variables of collagen digestion that do not change from one system (breast tissue) to other system (head and neck squamous cell carcinoma). This design will also ensure standardization of protocols based on primary variables of collagen digestion and will allow transferring technology from one system to other system. Thus, it will be possible to compare optimization and improvement from one system to other system.

7.2 Recommendations

7.2.1 Measurement of diffusivity of solute in human tissue

The model prediction discussed here utilized the diffusivity determined by the mouse window model developed by Jain et al (Jain, 1987). Berk et al have developed a method based on principle of fluorescence recovery after photobleaching (FRAP) to quantify different parameters in vivo (Alexandrakis et al., 2004; Berk et al., 1993; Brown et al., 2003). FRAP has been utilized regularly to quantify diffusivity of molecules like BSA, dextran particles and IgG molecules in the human xenograft models (Fox and Wayland, 1979; Nugent and Jain, 1984; Nugent and Jain, 1984; Pluen A, 2001; Yuan et al., 1995). Recently, this technique has been applied to quantify the diffusivity of BSA and IgM in human tumors (Brown et al., 2004). A similar approach can be applied here to
 quantify the diffusivity of enzyme in the breast tissue. Primary estimates of diffusivity in breast tissue can be obtained by utilizing proteins like BSA or IgG conjugated to FITC, which are of similar molecular weight as collagenase (68 kDa to 125 kDa).

7.2.2 Fluorescent labeling of collagenase

The diffusivity measured by BSA and IgG will not be able to predict the behavior of collagenase accurately due to differences in protein structure and charge effects (Creighton, 1993). This challenge can be overcome by labeling the collagenase enzyme with fluorescent molecules like FITC based amine chemistry or any other chemistry depending on the specificity desired (Garman, 1997). Fluorescent molecule labeled collagenase can be then utilized in similar fashion as BSA or IgG discussed by Jain et al. One of the other challenges in diffusivity measurement with collagenase is interference due to reaction between collagen and collagenase that will change the tissue structure. This interference can be overcome by measuring diffusivity at 4°C, where enzyme activity is almost zero and then extrapolating the diffusivity at 37°C using Stokes Einstein relationship (Cussler, 1997). The other alternative would be to use inhibitor to prevent the enzyme activity (Seifter, 1970).

7.2.3 Determination of collagen concentration with time

During the tissue digestion process the collagen concentration is continuously decreasing due to digestion by collagenase enzyme. This fact has not been taken in to consideration for simple reaction diffusion model. The accuracy of the model will be improved substantial by determining the change in collagen concentration in the tissue
with respect to time. This can be obtained by stopping the reaction at various time intervals and quantifying the average concentration of collagen by converting it to hydroxyproline and then to chromophore (Woessner, 1961).

### 7.2.4 Shrinking core model

Measurement of diffusivity and change in collagen concentration described here will address the assumptions made with respect to constant effective diffusivity of collagenase and constant collagen concentration in the tissue for implementing reaction diffusion model. Quantitative analysis to improve on these assumptions will improve the quality of the model. A better model to predict the breast tissue digestion will be shrinking core model (Froment GF, 1990) where tissue will be assumed as porous structure (Truskey GA, 2004). This model will deal with both the reacting components i.e. collagen and collagenase. The diffusivity will be a function of porosity of the tissue and porosity will be function of collagen concentration and spatial position. During the enzyme digestion process, as the collagenase will penetrate in tissue sample, the collagen concentration will decrease and the porosity of tissue will increase resulting in increase in diffusivity of subsequent collagenase molecules in the tissue. This is an unsteady state problem that can be solved using finite difference methods (Anderson, 1994).

### 7.2.5 End point of tissue digestion and applicability to other systems

Along with application of above discussed engineering principles, it is necessary to check for improvement in end point results. These results can be either recovery of carcinogenic cell populations using magnetapheresis or quadrupole magnetic sorter (Sun
et al., 1998; Zborowski et al., 1995) for diagnosis of cancer using genetic analysis or transplantation of cells in vivo. A number of epithelial specific cell markers like epithelial membrane antigen or HER-2/neu can be utilized for cell sorting from breast tissue (Ronnov-Jessen L, 1996).

The present work is also the basis for future work related to digestion of pancreas for Islet separation to transplant in diabetes patients (Ricordi et al., 1988) and digestion of head and neck squamous cell carcinoma for separation of squamous carcinoma cells to (Harrison et al., 1999) perform genetic analysis of carcinogenic cells.

Essentially this dissertation has focused on developing engineering analysis of tissue digestion process using catabolic enzymes for the first time. The proof of concepts will solve problems within the medical community associated with genetic diagnosis of cancer, cell line development for basic research and cells from transplantation. Along with these applications, a better understanding of the advantages, disadvantages and limitations of one system compared to other system can be also achieved.
REFERENCES

Percoll methodology and application © Amersham biosciences.


Okano F. 1985. [Study on stromal component of mastopathy--content and type of collagen]. Hokkaido Igaku Zasshi 60(4):555-70.


200


A.1 The normal mammary gland:

Mammary gland development is probably one of the most fascinating and complex biological phenomena. At the birth, mammary gland is the only organ that is not fully developed. In comparison to other organs it undergoes significant changes in size, shape and function during different stages of life like puberty, pregnancy and lactation. Apart from the growth, the breast regression (involution of breast) is also an important process as this is the period when development of cancer is manifested. Thus, it is necessary to remember that the development of this complex organ is dynamic phenomena compared to a simple static picture. These dynamic phenomena are results of complex interaction between two distinct tissue types, parenchyma and stroma of the breast. Therefore it is important to study these tissue types in conjunction rather than separately to understand histology, morphology, ultrastructure and physiology of normal and neoplastic breast tissue (Neville MC 1987).

In human the mammary gland is a modified sweat gland. It is derived from the epidermal ridge and appears first in 6-week embryo. This mammary gland remains rudimentary in male and female till puberty. At puberty, the male mammary gland
remains rudimentary with some involution. But in female it undergoes significant changes beginning with deposition of intra- and inter-lobular connective tissue. This deposition is followed by proliferation of epithelial compartment of the tissue. The epithelial compartment of mammary gland is made up of luminal epithelial cells and myoepithelial cells. These epithelial cells form grape like structure on branching ducts as shown in figure A.1. These structures are known as alveoli or lobuloalveoli or acini or terminal ductules (DTLs). A mature lobule consists of multiple DTLs. A DTL opens into an intralobular terminal duct (ITD) and intralobular connective tissue. ITD continues into extralobular terminal duct (ETD). Many of these ETDs join to form an interlobular duct (ILD), which lies in interlobular connective tissue. DTL, ITD and ETD are collectively referred as terminal duct lobular unit (TDLU) (Fig. A.2, A.3). The development of breast stops here at the puberty stage.

The cells of the TDLU and ILD undergo significant growth during pregnancy. The above-mentioned structures grow four times compared to the puberty structure. They also undergo significant differentiation during this stage. The mammary gland regress post pregnancy and its level of structural organization is almost indistinguishable as compared to nulliparous women. The growth and regression of the mammary gland is restricted up to the fat pad of the breast (stroma controls the extent of growth of mammary gland in normal breast tissue). The phenomenon of growth of epithelial compartment is very similar to the metastasis of cancer cells in surrounding stroma and thus study of mammary gland is very interesting. The growth of epithelial compartment in normal mammary gland is strictly regulated by the hormonal control and the stroma surrounding the tree structure (Wellings SR 1975; Ronnov-Jessen L 1996).
The stroma performs the same function as the mesenchymal cells do in organogenesis of embryo. It is made up of connective tissue that is separated from the epithelial compartment by an intact basement membrane. In breast the interstitial stroma accounts for (in greater than) 80 % of the breast volume. During the menstrual cycle, the breast volume changes up to 20 %. Stroma in breast can be divided in four main types:

1. Fat tissue
2. Interstitial/interlobular dense connective tissue
3. Intralobular loose connective tissue
4. Blood vessels

The fat tissue mainly comprises fat-containing cells surrounded by basement membrane and a capillary network. The interlobular and intralobular stroma contains mainly connective tissue with interspersed fibroblast cells. They are separated in two compartments based on the composition of connective tissue. The total amount of stroma and the ratio of fibrous interstitial stroma to fat tissue in the human breast are highly unique to an individual. The blood vessels or microvasculature is made up of small arteries, veins, arterioles and venules that nourish the surrounding fat tissue and epithelial tissue. Arteries show numerous concentric layers of highly myodifferentiated smooth muscle cells. Veins do not show this property and are thinner in wall size. The arteries and veins give rise to numerous fine structures like precapillary arterioles, precapillary venules and capillary network. All these finer structures are lined by pericytes. The entire structure of mammary gland is represented in figure A.3 (Wellings SR 1975; Ronnov-Jessen L 1996).
A.2 Cellular composition of breast tissue:

A.2.1 Luminal and myoepithelial cells:

The parenchyma of breast includes mainly two types of epithelial cells. They are luminal epithelial cells and myoepithelial cells. Luminal epithelial cells are true epithelial cells showing characteristics, like formation of tight junction that separate apical and basolateral domains and formation of highly polarized cell layer. They line the TDLUs and ducts in mammary gland. The myoepithelial cells show some characteristics of epithelial cells and some characteristics of smooth muscle cells. They are also known as basal cells as they are lined between luminal epithelial cells and basement membrane. Myoepithelial cells form a continuous layer in ducts of mammary gland but are less abundant and more flattened in TDLUs. Almost all breast carcinomas originate from ductal luminal epithelial cells. Thus these cells are of primary interest. Table A.1 lists a number of different markers used for characterizing these two epithelial cell types (Ronnov-Jessen L 1996; Petersen OW 2003).

A.2.2 Fibroblast:

Breast stroma plays a vital role in development of breast at different stages of life and in carcinomas. Fibroblast cells form the cellular component of stroma and are responsible for secreting the extracellular matrix (ECM) of breast stroma. Fibroblast cells have received least attention due to following two reasons:

1. Epithelial cells seem to do all the work and become cancerous.
2. There are no specific markers for fibroblast.
Thus, the characterization of fibroblast is based on spatial organization, morphology and the fact that they do not express cytokeratins and smooth muscle characteristics. Fibroblasts overgrow epithelial cells in culture and are necessary to be separated to achieve culture of epithelial cells. Recently, markers like 1B10 (Singer KH 1989; Ronnov-Jessen L 1992), CD26 (Dipeptidyl peptidase IV) (Ronnov-Jessen L 1996), and CD90 (Thy-1) (http://www.ncbi.nlm.nih.gov/PROW/ ) have been evaluated for fibroblast cells. These antigens show reactivity preferentially against fibroblast cells but are not specific for only fibroblast cells. Dipeptidyl peptidase IV (DPP4) can be used to differentiate between intralobular and interlobular fibroblasts. The prior does not show expression of DPP4 whereas the later is positive for it. This fact suggests that ECM environment for both these cells are different thus inducing different expression profile of antigens. When the same cells are cultured in vitro intralobular fibroblasts induce DPP4 expression over a period of time and thus are indistinguishable from interlobular fibroblasts.

A.2.3 Vascular smooth muscle cells (VSMC):

VSMC are important constituents of the microvasculature of breast. They form wall of arteries with endothelial cells. These cells show similar characteristics as myoepithelial cells as discussed above and are characterized by the contractile proteins similar to myoepithelial cells. Intracellular proteins like $\alpha$-smooth muscle actin, myosin and desmin are used to characterize these cells. VSMC undergo differentiation like other cells when cultured in vitro and down regulates expression of $\alpha$-smooth muscle actin. Thus it is difficult to characterize these cells. VSMCs have not received much attention
as they form lining of arteries and not veins. The veins play an important role in angiogenesis and are studied in detail compared to arteries (Ronnov-Jessen L 1996).

A.2.4 Endothelial cells:

Endothelial cells form the lining of arteries and veins. They are supported by VSMCs in arteries but are not supported by any cell type in veins. These cells are specialized epithelial cells showing unique characteristics to maintain regular function and flow of blood. The endothelial cells found in breast tissue do not express factor VIII related proteins. But endothelial cells can be distinguished from other cells by the expression of markers like VE-cadherin (CD144) or endocam (CD31) (http://www.ncbi.nlm.nih.gov/PROW/). These cells do not express the intracellular proteins found in VSMC. Thus it is possible to differentiate endothelial cells from VSMC by simple immunohistochemistry (IHC) (Ronnov-Jessen L 1996).

Like epithelial and fibroblast cells, endothelial cells also show heterogeneity among microvasculature segment. It has been observed that endothelial cells from veins and capillaries are stained with monoclonal antibodies Pathologische Anatomie Leiden-Endothelium (PAL-E) where as arteries and large veins do not stain with this antibody (Ronnov-Jessen L 1996). This fact further substantiates that various cell types present in vivo are in highly differentiated form. Endothelial cells show cobblestone morphology when cultured in vitro. They might undergo myodifferentiation in vitro. This shows that cells do loose their differentiated form when cultured in vitro (Ronnov-Jessen L 1996).
A.2.5 Pericytes:

The origin and function of pericytes are unknown and debatable. Pericytes are found in capillaries of all the organs but their role in capillary is unclear. There are mixed reviews about myogenic status of the pericytes and expression of $\alpha$-smooth muscle actin by these cells. Ronnov-Jessen L et al have established that pericytes found in breast tissue do expression $\alpha_1$-integrin and are negative from $\alpha$-smooth muscle actin. $\alpha_1$-integrin can be used to separate them from other breast cell constituents. Like their characterization it is very difficult to separate and culture these cells (Ronnov-Jessen L 1996).

A.2.6 Adipocytes and macrophages:

Adipocytes are fat containing cells. Due to high level of fat content these cells are fragile and are difficult to isolate in vitro. These cells are disrupted during collagenase treatment or during the centrifugation of dissociated cells. The fat released from these cells float on the air-supernatant interface (Ronnov-Jessen L 1996).

Macrophages are occasionally found in the dissociated cells and are responsible for removal of debris and dead cells from tissue environment. These cells are easily recognized by CD14 or CD45 marker (Ronnov-Jessen L 1996). Table A.2 and A.3 represent the summary of markers of all the cell types discussed above.

The cellular distribution discussed here represents a normal breast. The heterogeneity and differentiation level in the carcinogenic cells can be significantly different compared to normal cells. The above listed markers might not be useful for carcinogenic cells or other tumor associate antigens (TAAs) like HER-2/neu might be
useful for identification and separation of cancer cells. Most of the breast cancer originates in the ducts carrying milk and not in lobules as shown in figure A.4. It is thought that luminal epithelial cells are the cause of the cancer but there are still debates about it. It is important to understand the types of cancer before focusing on techniques for separation as not all the breast cancers originate in ductal epithelial cells.

A cancer that is contained, and thus does not yet has the ability to spread beyond the immediate area, is called in situ. Ductal carcinoma in situ (DCIS) is confined to the ducts of the milk-producing glands and has not invaded the surrounding tissues of the breast. It doesn't have access to the blood vessels or lymphatic channels that exist in the fatty tissue outside the gland, and it cannot spread to other organs in the body. DCIS is a breast cancer at its earliest stage (Stage 0), still confined to the ducts (Figure A.4). Nearly 100% of women with cancer at this stage can be cured. In screening centers, 20–30% of the new breast cancers diagnosed is DCIS and 70–80% is invasive. On a mammogram, DCIS in the majority of patients looks like a cluster of calcifications (tiny white spots or little grains of sand) without a mass. In about 15% of patients, there's also a lump, and in the remaining 10% of women, a mass is found without calcifications. When DCIS becomes metastatic it is known as Invasive (infiltrating) ductal carcinoma (IDC). IDC is the most common form of breast cancer and 70–80% of breast carcinomas are detected at IDC stage. This cancer begins in a duct, breaks through the duct wall, and invades the fatty tissue of the breast (Figure A.4). From there it can spread to other parts of the body via the lymphatic channels or bloodstream. Both these cancers originate in the ducts carrying the milk from lobule to the nipple (Wymelenberg 2000).
Invasive (infiltrating) lobular carcinoma is the cancer of lobule. 10–15% of invasive breast cancers are this type. The cancer cells have grown through the wall of the lobule and can spread to other parts of the body via the lymphatic channels or bloodstream. These three types of cancer are the most common type of breast cancers and account for 90% of the cases (Wymelenberg 2000).

Medullary carcinoma is an invasive cancer. The tumor shows pink color similar to brain tissue (the medulla) and hence the name. This cancer is a relatively well-defined, distinct boundary between tumor and normal breast tissue. About 6% of all breast cancers are of this type, and its prognosis is better than for invasive lobular or invasive ductal carcinomas. It can exhibit different degrees of aggressiveness. Another type of breast cancer is Tubular carcinoma. This accounts for 1–2% of breast cancers, and the cells look like little tubes (hence the name). It is an invasive cancer but it spreads beyond the breast only infrequently, and therefore has a better prognosis than invasive ductal or lobular carcinomas. Other less common breast cancers are Colloid or mucinous carcinoma, papillary carcinoma and inflammatory breast cancer (Wymelenberg 2000).
<table>
<thead>
<tr>
<th>Number</th>
<th>Marker</th>
<th>Luminal epithelial cells</th>
<th>Myoepithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Estrogen receptor</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Cytokeratin (CK) 7</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>3</td>
<td>CK 8</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>CK 18</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>CK 19</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>CK 5</td>
<td>-/+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>CK 13</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td>8</td>
<td>CK 14</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>CK 17</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>10</td>
<td>BG3C8</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Vimentin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>GFA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>α-Smooth muscle actin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Fodrin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>Smooth muscle-MHC</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>Calponin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>Episialin (EMA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>Secretory component</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>Luminal epithelial antigen 92</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>Calla (CD10)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>C1Br17</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>LICR-LON-59.2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>H type 2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>E-cadherin</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>25</td>
<td>P-cadherin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>α1-Integrin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td>α2-Integrin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>α3-Integrin</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>α4-Integrin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>α5-Integrin</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table A.1 Markers to distinguish between luminal epithelial and myoepithelial cells (Ronnov-Jessen L 1996).

+ Positive expression, - negative expression, (+) may be positive expression
Table A.1 continued

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>α6-Integrin</td>
<td>-/+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>β4-Integrin</td>
<td>(+)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>33</td>
<td>Connexin-43</td>
<td>-</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>34</td>
<td>BFGF</td>
<td>-</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>35</td>
<td>EGF</td>
<td>-/+</td>
<td></td>
<td>+/−</td>
</tr>
<tr>
<td>36</td>
<td>TGF-α</td>
<td>(+)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>37</td>
<td>Type IV collagen</td>
<td>-/(+)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>38</td>
<td>Laminin</td>
<td>-</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>39</td>
<td>Type IV collagenase</td>
<td>-/+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>40</td>
<td>Maspin</td>
<td>-</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>41</td>
<td>Methallothionein</td>
<td>-</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Serial #</td>
<td>Cell type</td>
<td>Marker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------</td>
<td>---------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Luminal Epithelial cell</td>
<td>Episialin (EMA), cytokeratin 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Myoepithelial cell</td>
<td>CALLA (CD10), connexin 43, α-smooth muscle actin, calponin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fibroblast</td>
<td>1B10, CD26, CD90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Vascular smooth muscle cells</td>
<td>α-smooth muscle actin, myosin, desmin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Endothelial cell</td>
<td>CE-6, PAL E, CD31, CD62P CD144</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Pericytes</td>
<td>α1-integrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Adipocytes</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Macrophages</td>
<td>CD14, CD45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Leukocytes</td>
<td>CD45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Erythrocytes</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table A.2 Markers to distinguish breast cellular components.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Marker</th>
<th>Common marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte</td>
<td>CD 2, 3, 5</td>
<td></td>
</tr>
<tr>
<td>Macrophage</td>
<td>CD 14, 64, 206</td>
<td>CD 11c, 18, 45</td>
</tr>
<tr>
<td>NK cell</td>
<td>CD 56</td>
<td></td>
</tr>
</tbody>
</table>

Table A.3 Markers for specific cells that come under category leukocytes in table A.2.
Figure A.1 Human breast is primarily composed of fat pad, connective tissue and breast tissue. The breast tissue consists of lobules and ducts embedded in fat pad and connective tissue as shown above. The lobules are milk-producing glands, and ducts carry this milk to the nipples during lactation. Breast also has other vascular and nervous components. The preliminary sites of breast cancer are ducts or nodules and it generally metastasizes via auxiliary lymph nodes (Wymelenberg, 2000).
Figure A.2 Normal TDLU. Small duct (D) give rise to terminal D and a single lobule (L). Terminal D is composed of ETD and ITD, which are in continuity. ITD gives rise to blindly ending DTL that are often branched. ITD and DTL are covered with intralobular connective tissue (stripped) (Wellings SR 1975).
Figure A.3 Lobular structure of mammary gland along with microvasculature. Artery, veins, arterioles and venules are shown here. Intra- and inter-lobular stroma is not shown here.
Figure A.4 Luminal and myoepithelial cells form the duct and lobule (A). It is believed that ductal cancer cells are progeny of one mutated luminal epithelial cell. When the cells are enclosed in basement membrane (B) then it is called as ductal carcinoma in situ (DCIS). This cancer is detected as small calcification in mammogram. In advance stages the cancer cells breach the basement membrane (C) and spread to other parts of breast and other parts of body via blood vessel and lymphatic system. This process is called metastasis and marks advance stage of cancer (Wymelenberg, 2000)
APPENDIX B

PROTOCOLS

B.1 Immunofluorescence staining

a) Wash cells on coverslip thrice with prewarmed phosphate-buffered saline, pH 7.4 (PBS).

b) Fix the sample in methanol free 3.7% formaldehyde solution in PBS for 10 minutes at room temperature.

c) Wash the cells three times with PBS.

d) Place each coverslip in a petri dish and permeabilize it with 0.1% Triton X-100 in PBS at room temperature for 3 to 5 minutes.

e) Wash the cells three times with PBS.

f) Pre-incubate fixed cells with PBS containing 10% serum from the same animal in which the secondary was raised for 30 minutes at room temperature to minimize non-specific binding of secondary antibody.

g) Wash the cells three times with PBS.

h) For staining with primary antibody, dilute 1 µL of primary antibody stock solutions in 29 µL of PBS for each coverslip to be stained. Incubate the coverslip in primary antibody at room temperature for 60 minutes.
i) Wash the cells three times with PBS.

j) For staining with secondary antibody, dilute 1 µL of secondary antibody stock solutions in 29 µL of PBS for each coverslip to be stained. Incubate the coverslip in secondary antibody at room temperature for 45 minutes.

k) Wash the cells three times with PBS.

l) For long-term storage, the cells should be air dried and then mounted in a permanent mountant such as ProLong® Gold or Cytoseal. Specimens prepared in this manner retain fluorescent staining for at least six months when stored in the dark at 2–6°C. Seal the coverslip with nail polish to prevent air bubble formation or evaporation loss of ProLong® or Cytoseal.

**B.2 Flow cytometry staining**

a) Harvest or prepare the cells from the source and measure the cell concentration using hemacytometer or automatic particle counter.

b) Take samples of known concentration (recommended concentration ~ $1 \times 10^6$ cells/ml) from cell suspension depending on experiment requirement and place it in $12 \times 75$ mm polystyrene tube(s).

c) If the sample is too dilute (i.e. if sample volume is greater than 100 µl), then centrifuge the sample for 5 min at 1400 rpm and 4°C. Discard excess supernatant.

d) Add the required volume of primary antibody solution according to the amount of antibody needed for labeling and resuspend the cell pellet.

e) Incubate for 30 min in the dark at 4°C.
f) Add PBS + 0.5 % BSA + 2mM EDTA (labeling buffer) to wash off the excess antibody. Add at least 10x the staining volume and mix well.

g) Centrifuge for 8 minutes at 1400 rpm at 4°C.

h) Count cells and dilute cells to the desired cell concentration (Optional, it depends on the type of experiment).

i) Depending on the experiment, either fix the cells using 2 % p-formaldehyde or resuspend the cells in labeling buffer or in media such that the final concentration is $1 \times 10^6$ cells/ml.

j) Perform the flow cytometry as per the operator’s instructions and past experience.

B.3 Western blot

B.3.1 Protocol

a) Wash the cell samples (from 2D culture) 3 times with PBS and lyse it with Mammalian Protein Extraction Reagent as per the manufacturer’s recommendations (M-PER, Catalog # 78501, Pierce, Rockford, IL). The cells in 3D culture were first harvested with accutase and then lysed with M-PER.

b) The lysed samples were spun down at 14000 rpm for 10 minutes to separate the cell debris from the protein extract. Recover the supernatant from the cell debris pellet and preserve both the samples for later use at - 80°C. It is recommended to perform most of the steps at 4°C to avoid excessive loss of protein.

c) Thaw the sample at 4°C and take an aliquot of sample in microcentrifuge tube such that the total protein content is 10-50 µg. Dilute the sample with loading
buffer* (2X or 4X) in such a manner that the total volume is less than 45 µl. Boil
the sample at 95°C for 5 minutes and then put it on ice till loaded on the gel.

d) Place the gel (either 5 %, 10 % or 4 – 20 % gradient gel depending on the protein
of interest) in the electrophoresis chamber and fill up the chamber with 1 X
running buffer+ making sure that gel does not dry out.

e) Carefully remove the comb from the wells making sure that no air bubbles are left
in the wells. Load the Precision plus protein standards as per the manufacturer’s
instruction (Catalog # 161-0374, BioRad Laboratories, Hercules, CA) and the
denatured protein from previous step. It is recommended not to exceed the
capacity of well (35 – 50 µl) while loading the sample.

f) Connect the electrophoresis chamber to the power supply and apply 120 V for 1.5
to 2 hours depending on the speed of protein migration in the gel based on the
protein standard and loading dye.

g) Remove the gel from the electrophoresis chamber and soak in distilled water to
remove SDS and then wash it 3 times for 10 minutes each with transfer buffer++ to
remove remaining SDS. Prepare the cassette for the transfer as shown in figure
B.1 for transferring the protein on nitrocellulose membrane for probing.

h) Put the cassette in transfer chamber containing chilled 1 X transfer buffer and an
ice block. Add a magnetic stirrer to improve the heat transfer. Transfer the
proteins on the nitrocellulose membrane at 90 V for 1 hour or at 180 mA for 16
hours.

i) The efficiency of transfer is confirmed based on the transfer of the protein
standards and Ponceau S temporary staining of protein on membrane. The
Ponceau S stain can be removed by washing the membrane with transfer buffer till the traces of red dye is removed.

j) Wash the membrane 2 times for 5 minute each with 1 X TBST buffer**.

k) Block the membrane with 5 % milk in TBST at room temperature with constant shaking for 1 hour.

l) Wash the membrane 3 times for 10 minutes each in TBST with constant shaking.

m) Incubate the membrane in primary antibody (1:1000 dilution, prepared in 5 % milk in TBST) for 1.5 hours at room temperature with constant shaking or overnight at 4°C with constant shaking.

n) Wash the membrane 3 times for 10 minutes each in TBST with constant shaking.

o) Incubate the membrane in secondary antibody (1:5000 dilution, prepared in 5 % milk in TBST) for 1 hour at room temperature with constant shaking.

p) Wash the membrane 3 times for 10 minutes each in TBST with constant shaking.

q) Incubate 1 ml of chemiluminescence substrate for 2 to 3 minutes for detection purpose. Wrap the membrane in cellophane and place the film in film exposure cassette.

r) In dark room, lay an unexposed film on the membrane in film exposure cassette for desired amount of time depending on the experimental requirement. Develop the film in machine and analyze it using image J software.

B.3.2 Buffer preparation

†10 X stock of running buffer
Add 30.3 gm (0.25 M) of Tris, 144.1 gm (1.92 M) of glycine and 10 gm (1 %) of sodium dodecyl sulfate (SDS) in 800 ml of distilled water. Adjust the pH to 7.6 with 0.1 N HCl and adjust the final volume to 1 L by adding distilled water. This 10 X running buffer should be diluted 10 times just before use.

++ 10 X stock of transfer buffer

Prepare in the same manner as above except addition of SDS. Dilute the transfer buffer 10 times in water to prepare 1 X transfer buffer.

* Loading buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>2X</th>
<th>4X</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris, pH 7.0</td>
<td>0.2 ml = 20 mM</td>
<td>0.4 ml = 40 mM</td>
</tr>
<tr>
<td>β– mercaptoethanol</td>
<td>1 ml = 10 %</td>
<td>2 ml = 20 %</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>4 ml = 4 %</td>
<td>8 ml = 8 %</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>100 µl = 5 mM</td>
<td>200 µl = 10 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2 ml = 20 %</td>
<td>4 ml = 40 %</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>5 mg = 0.5 mg/ml</td>
<td>10 mg = 1 mg/ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.7 ml</td>
<td>3.4 ml</td>
</tr>
</tbody>
</table>

Table B.1 Preparation of loading buffer

** 10 X TBST buffer

Add 12.1 gm of Tris, 87.5 gm of NaCl in 750 ml of distilled water. Adjust the pH to 7.6 by adding 0.1 N HCl and adjust the final volume to 1 L by adding distilled water. This is 10X TBS buffer. The final 1X TBST buffer is prepared by diluting TBS buffer 10 times and adding 0.1 % Tween 20 (1 ml in 1 L of 1X TBST buffer).

B.4 Scanning electron microscopy sample preparation

a) Remove the media from the scaffold containing the cells and wash it 3 times with PBS.
b) Fix the cells overnight in freshly prepared 2.5 % gluteraldehyde in 0.1 M phosphate buffer pH 7.4. Note that the cells can be kept in fixative for 2 or 3 days as well. The recipe for preparation 0.1 M phosphate buffer is as follows:

   a. 0.2 M Monobasic stock: Dissolve 13.9 gm of sodium phosphate monobasic in 500 ml distilled water.

   b. 0.2 M Dibasic stock: Dissolve 53.65 gm of sodium phosphate dibasic heptahydrate (or 28.4 gm of the anhydrous form) in 1 L distilled water.

   c. The above stock can be prepared in advance. They should be mixed in proportion shown in table B.2 to achieve the desired pH:

   d. Prepare 0.1 M phosphate buffer by mixing appropriate volume or stocks to achieve pH 7.4. Add appropriate amount of 30 % gluteraldehyde to prepare final fixative solution with 2.5 % gluteraldehyde based on weight percent.

   c) The fixed samples were then dried using increasing series of ethanol starting from 50 % ethanol in distilled water and then 65, 75, 80, 90 and 100 % ethanol. The samples were incubated for 20 minutes at room temperature in each ethanol dilution. To increase the efficiency of drying, the sample can be dried two times in each dilution, but this might cause loss of cells from sample due to excessive washing. This decision should be made based on the experimental requirement.

   d) Further dry the fixed sample using increasing series of hexamethyldisilazane (HMDS) starting from 25 % HMDS in ethanol and then 50, 75 and 100 % HMDS. The samples were incubated at room temperature for 20 minutes in each HMDS dilution. To improve the drying process, it is recommended to dry the
sample three times for 20 minutes in 100 % HMDS, but this might cause loss of cell from the sample due to excessive washing. This decision should be made based on the experimental requirement.

e) Remove as much HMDS as possible and evaporate rest of the HMDS overnight in a chemical hood.

f) Sputter coat the sample with gold alloy for observation under scanning electron microscope. Use the microscope as per the operator’s instruction and past experience.

B.5 Quantification of collagen (Woessner’s protocol)

B.5.1 Protocol

a) Prepare the blank, collagenase control and collagen standard as shown in table B.3. Sample standard curves are shown in figure B.2 and B.3 at the end of the protocol.

b) Measure the weight of wet tissue sample accurately depending on type of tissue. It is recommended to start with low tissue weight (1 – 10 mg) and then increase or decrease the amount of tissue depending on color development.

c) Digest the tissue for 16 hours at 37°C with 100 µl of 2 mg/ml of collagenase dissolved in reaction buffer (solution containing 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂ and 0.2 mM sodium azide, pH 7.6).

d) Hydrolyze the tissue using 6 N HCl at 130°C for 3 hours.

e) Let the content cool down at room temperature and add 10 µl of 0.02 % methyl red indicator.
f) Add the theoretical amount of 2.5 N NaOH so that the pH of the solution is in 6-7 range (pale yellow color). It is necessary to perform the neutralization properly to achieve accurate results.

g) Dilute the samples with distilled water so that final NaCl concentration is less than 0.4 M. For this procedure, 4 ml of distilled water per tube is sufficient. Make sure that the final volume of solution in all tubes has same volume.

h) Oxidize the hydroxyproline (released from collagen hydrolysis) by adding 1 ml of chloramine T solution to each tube and incubate at room temperature for 20 minutes.

i) Stop the oxidation reaction by destroying the chloramines T solution by adding 1 ml of perchloric acid in same order as addition of chloramine T solution. Incubate the reaction mixture for 5 minutes.

j) Finally add 1 ml of p-dimethylaminobenzaldehyde and heat the reaction mixture at 60°C for 20 minutes.

k) Cool down the reaction mixture under tap water for 5 minutes.

l) The developed color is stable for at least 1 hour. Measure the color by colorimetry at 557 nm.

m) For accurate and reproducible results, it is necessary to freshly prepare the chloramine T reagent and p-dimethylaminobenzaldehyde reagents. The recipes are explained below.
B.5.2 Solution preparation

Citrate buffer

Add 50 gm of citric acid monohydrate, 120 gm of sodium acetate, 34 gm of sodium hydroxide and 12 ml of glacial acetic acid. Dilute this solution with 750 ml of water and adjust the pH to 6. After pH adjustment, make the final volume of solution 1 L by adding more distilled water. Store the buffer under 10-15 drop of toluene at 4°C to avoid evaporation losses.

Perchloric acid solution (3.15 M solution)

Mix 27.0 ml of 70 % perchloric acid in distilled water to make final volume of 100 ml

Hydroxyproline stock solution

Dissolve 25 mg of hydroxyproline in 250 ml of 0.001 N HCl

Chloramine T solution

Prepare this solution fresh every time while performing experiment. Dissolve 1.41 gm of chloramine T in 20 ml of distilled water, 30 ml of methyl cellosolve and 50 ml of citrate buffer. Keep the solution in glass stoppered bottle.

p-dimethylaminobenzaldehyde solution

Prepare this solution fresh every time while performing the experiment. Dissolve 20 gm of p-dimethylaminobenzaldehyde in methyl cellosolve to make the final volume 100 ml. Warm the solution to 60°C to facilitate dissolution.

B.5.3 standard curve and Sample data table

a) Refer figure B.2 for standard curve of absorbance @ 557 nm vs. hydroxyproline concentration.

b) Refer figure B.3 for standard curve of absorbance @ 557 nm vs. collagen concentration.
c) Refer to Table B.3 for sample data set for preparation of standard curve for collagen quantification in tissue.
<table>
<thead>
<tr>
<th>Monobasic stock (ml)</th>
<th>Dibasic stock (ml)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>280.5</td>
<td>19.5</td>
<td>5.7</td>
</tr>
<tr>
<td>276</td>
<td>24</td>
<td>5.8</td>
</tr>
<tr>
<td>270</td>
<td>30</td>
<td>5.9</td>
</tr>
<tr>
<td>263.1</td>
<td>36.9</td>
<td>6.0</td>
</tr>
<tr>
<td>255</td>
<td>45</td>
<td>6.1</td>
</tr>
<tr>
<td>244.5</td>
<td>55.5</td>
<td>6.2</td>
</tr>
<tr>
<td>232.5</td>
<td>67.5</td>
<td>6.3</td>
</tr>
<tr>
<td>220.5</td>
<td>79.5</td>
<td>6.4</td>
</tr>
<tr>
<td>205.5</td>
<td>94.5</td>
<td>6.5</td>
</tr>
<tr>
<td>187.5</td>
<td>112.5</td>
<td>6.6</td>
</tr>
<tr>
<td>169.5</td>
<td>130.5</td>
<td>6.7</td>
</tr>
<tr>
<td>153</td>
<td>147</td>
<td>6.8</td>
</tr>
<tr>
<td>135</td>
<td>165</td>
<td>6.9</td>
</tr>
<tr>
<td>117</td>
<td>183</td>
<td>7.0</td>
</tr>
<tr>
<td>99</td>
<td>201</td>
<td>7.1</td>
</tr>
<tr>
<td>84</td>
<td>216</td>
<td>7.2</td>
</tr>
<tr>
<td>69</td>
<td>231</td>
<td>7.3</td>
</tr>
<tr>
<td>57</td>
<td>243</td>
<td>7.4</td>
</tr>
<tr>
<td>48</td>
<td>252</td>
<td>7.5</td>
</tr>
<tr>
<td>39</td>
<td>261</td>
<td>7.6</td>
</tr>
<tr>
<td>31.5</td>
<td>271.5</td>
<td>7.7</td>
</tr>
<tr>
<td>25.5</td>
<td>274.5</td>
<td>7.8</td>
</tr>
<tr>
<td>21</td>
<td>279</td>
<td>7.9</td>
</tr>
<tr>
<td>15.9</td>
<td>284.1</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Table B.2 Recommended proportion of stock solutions to prepare 0.1M phosphate buffer with desired pH
<table>
<thead>
<tr>
<th>#</th>
<th>µg of Collagen or hydroxyproline</th>
<th>Volume of stock</th>
<th>Volume of HCl</th>
<th>Volume of 2.5 N NaOH</th>
<th>Volume of H₂O</th>
<th>Absorbance @ 557 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4 ml</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10-collagen</td>
<td>10 µl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 µl of 12 N and 80 µl of 6N HCl</td>
<td>240 µl</td>
<td>4 ml</td>
<td>0.048</td>
</tr>
<tr>
<td>3</td>
<td>25-collagen</td>
<td>25 µl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 µl of 12 N and 50 µl of 6N HCl</td>
<td>240 µl</td>
<td>4 ml</td>
<td>0.150</td>
</tr>
<tr>
<td>4</td>
<td>50-collagen</td>
<td>50 µl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50 µl of 12 N</td>
<td>360 µl</td>
<td>4 ml</td>
<td>0.347</td>
</tr>
<tr>
<td>5</td>
<td>100-collagen</td>
<td>100 µl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100 µl of 12 N</td>
<td>480 µl</td>
<td>4 ml</td>
<td>0.659</td>
</tr>
<tr>
<td>6</td>
<td>2 – hydroxyproline</td>
<td>20 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.98 ml</td>
<td>0.1362</td>
</tr>
<tr>
<td>7</td>
<td>4 – hydroxyproline</td>
<td>40 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.96 ml</td>
<td>0.179</td>
</tr>
<tr>
<td>8</td>
<td>6 – hydroxyproline</td>
<td>60 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.94 ml</td>
<td>0.282</td>
</tr>
<tr>
<td>9</td>
<td>10 – hydroxyproline</td>
<td>100 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.9 ml</td>
<td>0.490</td>
</tr>
<tr>
<td>10</td>
<td>15 – hydroxyproline</td>
<td>150 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.85 ml</td>
<td>0.673</td>
</tr>
<tr>
<td>11</td>
<td>20 – hydroxyproline</td>
<td>200 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>3.8 ml</td>
<td>0.885</td>
</tr>
<tr>
<td>12</td>
<td>Collagenase control</td>
<td>100 µl of 2 mg/ml solution</td>
<td>100 µl of 12 N</td>
<td>480 µl</td>
<td>4 ml</td>
<td>0.035</td>
</tr>
</tbody>
</table>

<sup>a</sup> 1 mg/ml collagen stock solution  
<sup>b</sup> 100 µg/ml of hydroxyproline stock solution

Table B.3 Sample data set for preparation of standard curve for collagen quantification in tissue
Figure B.1 Schematic drawing of electroblotting cassette assembly for protein transfer to nitrocellulose membrane for probing.
Figure B.2 Standard curve of absorbance @ 557 nm vs. µg of hydroxyproline measured using Woessner’s protocol. (Absorbance = 0.0453× [hydroxyproline] + 0.0190)
Figure B.3 Standard curve of absorbance @ 557 nm vs. µg of collagen measured using Woessner’s protocol. (Absorbance = 0.0061 × [collagen] + 0.0133)