Morphological Assessment of HEMA-MMA Microcapsules for Liver Cell Transplantation

by

Julia Elizabeth Babensee

B.A.Sc., Chem. Eng., University of Toronto (1990)

A Thesis Submitted in Conformity with the Requirements for the Degree of Doctor of Philosophy in the University of Toronto

Department of Chemical Engineering and Applied Chemistry, University of Toronto

© copyright by Julia Elizabeth Babensee, 1996.
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
Morphological Assessment of HEMA-MMA Microcapsules for Liver Cell Transplantation

A Thesis Submitted in Conformity with the Requirements for the Degree of Doctor of Philosophy, 1996,
Julia Elizabeth Babensee,
Department of Chemical Engineering and Applied Chemistry,
University of Toronto

Abstract

Hepatoma cells were microencapsulated within a hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA; 75% HEMA) copolymer membrane. The in vitro morphology and viability of microencapsulated hepatoma cells were indicators of the cellular effects of the intracapsular microenvironment. The applicability of HEMA-MMA microcapsules as a cell delivery device was assessed by examining the morphology and viability of model hepatoma cells within microcapsules that were implanted into rats and the tissue response to these microcapsules.

Human hepatoma cells (HepG2) within the non-adherent environment of the HEMA-MMA microcapsule, formed aggregates with necrosis occurring at day 7 in large aggregates. Their co-encapsulation within Matrigel®, an extracellular matrix for cellular attachment, resulted in an initially uniform distribution of individual cells, and cellular viability and limited necrosis at day 14; a favourable cell microenvironment.
Small diameter microcapsules (~ 450 μm), containing an aggregate of rat hepatoma, H4IIEC3, cells after their implantation into an omental pouch in non-immunosuppressed Wistar rats (a model of allogeneic transplantation), contained viable cells after 7 days but not 14 days. However, similar transplantation of microencapsulated aggregates of human hepatoma, HepG2, cells (a model of xenogeneic transplantation), did not result in viable cells at 7 days. Prompt vascularization of the tissue surrounding microcapsules occurred after 4 days in vivo and was maintained for up to 14 days. Differences in the tissue reaction between hepatoma species were noted with thicknesses shifting toward higher values and containing significantly more eosinophils associated with microcapsules containing human hepatoma cells than with rat hepatoma cells. The results of these microcapsule implantation studies suggested a relationship between the viability of microencapsulated cells and the degree of vascularization of the surrounding tissue reaction.

Capsules were characterized by x-ray photoelectron spectroscopy (XPS) and immunoblotting of adsorbed proteins. XPS showed that the capsule surface was not pure HEMA-MMA but that the Pluronic surfactant, L101, had adsorbed from the microcapsule precipitation bath. Capsules, maintained in medium containing serum, showed a nitrogen signal consistent with adsorbed serum proteins which were not completely removed with phosphate buffered saline (PBS) washing. Almost all 24 human proteins probed for in immunoblots were associated with microcapsules following their in vitro incubation in
medium containing serum. Rat proteins, including fibrinogen, IgG, and complement C3, were detected in immunoblots of the eluate of implanted capsules.
Acknowledgments

I would like to thank my supervisor, Professor Michael V. Sefton, for inspiring me to study the subject of microencapsulation and biomaterials, for his supervision of my thesis, for his advice and patience during the writing of this document, for his generosity of time, knowledge, and wisdom, for the opportunities that he has made available to me, and for his continual support, encouragement and friendship. I would also like to thank Professor Umberto De Boni for taking me into his lab during my Master’s work, for his supervision, constructive criticism of my work, support and encouragement. Professor Bradley Saville, the other member of my reading committee, is acknowledged for his helpful comments and support. The patience and comments of my Ph.D. oral committee members, including Professor M.S. Shoichet, Professor K.A. Woodhouse, Dr E.L. Yeo, Professor M.J. Phillips and my external appraiser, Professor A.G. Mikos, are very gratefully acknowledged.

For technical assistance and advice, I would like to thank the following people: Trudy Franklin and Susan Wang for their enormous dedication of time and effort in preparing histological sections of the in vivo microcapsule samples; Cheun Lo for assisting in initiating rat surgery, performing surgeries in the early studies, and for teaching me the surgical techniques; Vlad Horwath, Hasan Uludag and Shahab Lahooti for microcapsule preparation; Dr. David Howarth, pathologist at Mount Sinai Hospital, Toronto, for his instruction in the morphological identification of immune and inflammatory cells in
histological sections of biomaterial implants; Battista Calveri, at the Electron Microscopy Facility in the Faculty of Medicine for his technical advice and instruction in SEM and TEM; Rana Sodhi, at the Surface Science lab at the Centre for Biomaterials, for his running of samples and his comments on XPS surface analysis of microcapsules; the staff at the Division of Comparative Medicine, Faculty of Medicine for technical assistance in animal handling. Finally, I would like to thank two groups, Rena Cornelius and Dr. John L. Brash at McMaster University in Hamilton, Ontario, for their collaboration on the protein adsorption studies and Dr. David Kaplan and Dr. Grace Picciolo, at the Food and Drug Administration, Centre for Devices and Radiological Health, Rockville MD, for their collaboration on the macrophage superoxide response to microcapsules, for being a pleasure to work with.

I would like to thank past and current members of Professor Sefton's lab, including Hasan Uludag, Michael May, Maud Berger-Gorbet, Elly Campioni, and Cynthia Gemmel, for their friendship, encouragement, and for sharing the experience of graduate school with me. I appreciate the members of Professor De Boni's lab, John Janevski, Jim Sahlas, Vivian Choh, Paul Park, Kwame Amankwah and Trudy Franklin, for making me laugh. Recently, during the writing phase of my Ph.D. I have been working out of the Toronto Hospital, Hematology, Oncology Research Department. I would like to thank everyone there for taking me in, making me feel a part of the group and for their friendship.
Finally, I would like to thank my parents and my sister for their love and support and for always being there for me to turn to. I am especially grateful to my mother for her dedication to me, her constant love, encouragement, and help.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>2</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>5</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>10</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>17</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>20</td>
</tr>
</tbody>
</table>

## CHAPTER 1

**BACKGROUND**

1.1 ENCAPSULATION FOR CELL TRANSPLANTATION - IDENTIFICATION OF ISSUES AND EXAMPLES

1.1.1. Basic Principles of Microencapsulation for Cell Transplantation

1.1.1.2. Cells as Delivery Systems

1.1.1.3. Implantations

1.1.1.4. The Microcapsule

1.1.1.5. Diffusion-Limited Microenvironment

1.1.1.6. Extracellular Matrix Effects

1.1.4. Alginate-Polylysine and Other Microcapsules

1.1.4.1. Alginate-Polylysine Microcapsules

1.1.4.2. Agarose Microcapsules

1.1.4.3. Polyelectrolyte Microcapsules

1.1.4.4. Thermoplastic Microcapsules

1.1.2. Immunoisolation and the Immunology of Cell Transplantation

1.1.3. Biocompatibility of Immunoisolation Devices

1.1.3.1. Microcapsule Biocompatibility

1.1.3.2. Immunoisolation and Immunology

1.1.5. Polycrylates for Cell Microencapsulation

1.1.5.1. HEMA-MMA Microcapsules

1.1.5.1.1. Microcapsule Preparation

1.1.5.1.2. Membrane Structure and Formation

1.1.5.1.3. Microcapsule Permeability

1.1.5.2. In Vitro Performance of HEMA-MMA Microencapsulated Cells

1.1.5.2.1. Cell Survival and Proliferation

1.1.5.2.2. Protein Release

1.1.5.2.3. Intracapsular Cell Behaviour

1.2 THESIS SCOPE AND OBJECTIVES

## CHAPTER 2

HEMA-MMA MICROENCAPSULATED CELL MORPHOLOGY IN VITRO

2.1. MORPHOLOGICAL ASSESSMENT OF HEPATOMA CELLS (HEPG2) MICROENCAPSULATED IN A HEMA-MMA COPOLYMER WITH AND WITHOUT MATRIGEL®
CHAPTER 3

HEMA-MMA MICROENCAPSULATED CELL MORPHOLOGY IN VIVO

3.1. TRANSPLANTATION OF HEPATOMA CELL LINES INTO RATS WITHIN SMALL DIAMETER HEMA-MMA MICROCAPSULES

ABSTRACT

CHAPTER 4

HEMA-MMA MICROCAPSULES CHARACTERIZATION

4.1. XPS SURFACE ANALYSIS OF POLYACRYLATE MICROCAPSULES FOR CELL TRANSPLANTATION

ABSTRACT

CHAPTER 5

CONCLUSIONS

CHAPTER 6

REFERENCES
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-MEM</td>
<td>α-Minimum Essential Medium</td>
</tr>
<tr>
<td>ACI</td>
<td>rat strain</td>
</tr>
<tr>
<td>AG</td>
<td>α₁-acid glycoprotein</td>
</tr>
<tr>
<td>AKR</td>
<td>C5-deficient mouse strain</td>
</tr>
<tr>
<td>Al Kα</td>
<td>aluminum Kα x-rays</td>
</tr>
<tr>
<td>AN69</td>
<td>polyacrylonitrile-sodium methylsulphonate</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>AT</td>
<td>antitrypsin</td>
</tr>
<tr>
<td>B7-1, -2</td>
<td>co-stimulatory molecule on APCs</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>barium chloride</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney cells</td>
</tr>
<tr>
<td>C 1s</td>
<td>carbon 1s orbital</td>
</tr>
<tr>
<td>C-C</td>
<td>carbon singly bonded to carbon</td>
</tr>
<tr>
<td>C-N</td>
<td>carbon singly bonded to nitrogen</td>
</tr>
<tr>
<td>C-O</td>
<td>carbon singly bonded to oxygen</td>
</tr>
<tr>
<td>C1</td>
<td>first complement component</td>
</tr>
<tr>
<td>C1q</td>
<td>first complement component, q subunit</td>
</tr>
<tr>
<td>C1r</td>
<td>first complement component, r subunit</td>
</tr>
<tr>
<td>C1s</td>
<td>first complement component, s subunit</td>
</tr>
<tr>
<td>C₃</td>
<td>third complement component</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>C3(H₂O)</td>
<td>fluid phase C3</td>
</tr>
<tr>
<td>C3a</td>
<td>third complement component, a subunit</td>
</tr>
<tr>
<td>C3b</td>
<td>third complement component, b subunit</td>
</tr>
<tr>
<td>C3c</td>
<td>third complement component, c subunit</td>
</tr>
<tr>
<td>C3d</td>
<td>third complement component, d subunit</td>
</tr>
<tr>
<td>C3dg</td>
<td>third complement component, dg subunit</td>
</tr>
<tr>
<td>C3f</td>
<td>third complement component, f subunit</td>
</tr>
<tr>
<td>C4b</td>
<td>fourth complement component, b subunit</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>mouse strain</td>
</tr>
<tr>
<td>C5a</td>
<td>fifth complement component, a subunit, anaphylatoxin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CD4</td>
<td>helper T cells, macrophages, eosinophils</td>
</tr>
<tr>
<td>CD8</td>
<td>cytotoxic T cells</td>
</tr>
<tr>
<td>CF1</td>
<td>mouse embryonic lung tissue</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CR1</td>
<td>complement receptor type 1</td>
</tr>
<tr>
<td>CR3</td>
<td>complement receptor type 3</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CTLA4-Ig</td>
<td>soluble recombinant fusin molecule</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>DMAEMA</td>
<td>polydimethylaminoethyl methacrylate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EHS</td>
<td>Engelbreth-Holm-Swarm</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>EMA</td>
<td>ethyl methacrylate</td>
</tr>
<tr>
<td>Eosin.</td>
<td>eosinophil</td>
</tr>
<tr>
<td>FB</td>
<td>factor B</td>
</tr>
<tr>
<td>Fbg</td>
<td>fibrinogen</td>
</tr>
<tr>
<td>FBGC</td>
<td>foreign body giant cell</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Fcγ</td>
<td>fragment, crystalline gamma portion of immunoglobulin</td>
</tr>
<tr>
<td>FcR</td>
<td>receptor for the Fc (fragment, crystalline) portion of immunoglobulin</td>
</tr>
<tr>
<td>Fib.</td>
<td>fibroblast</td>
</tr>
<tr>
<td>h.i.</td>
<td>heat inactivated</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>H4IIIEC3</td>
<td>rat hepatoma cell line</td>
</tr>
<tr>
<td>HEMA</td>
<td>hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>HEMA-MMA</td>
<td>hydroxyethyl methacrylate-methyl methacrylate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HepG2</td>
<td>human hepatoma cell line</td>
</tr>
<tr>
<td>hGH-1</td>
<td>human growth hormone-1</td>
</tr>
<tr>
<td>HMWK</td>
<td>high molecular weight kininogen</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>human serum</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>iC3b</td>
<td>inactivated third complement component</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin isotype A</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin isotype G</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin isotype M</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Imm.</td>
<td>Immune</td>
</tr>
<tr>
<td>Inflamm.</td>
<td>Inflammatory</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>L101</td>
<td>Pluronic surfactant, L101</td>
</tr>
<tr>
<td>LFA</td>
<td>lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LM</td>
<td>light microscopy</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Lym.</td>
<td>lymphocyte</td>
</tr>
<tr>
<td>Mφ</td>
<td>macrophage</td>
</tr>
</tbody>
</table>
MAA  methacrylic acid
MAC  membrane attack complex
Mg Kα  magnesium Kα x-rays
Mg^{2+}  magnesium ion
MHC  major histocompatibility complex
MMA  methyl methacrylate
MPEG  monomethoxy poly(ethylene glycol)
mRNA  messenger ribonucleic acid
MTT  3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MW  molecular weight
M_w  molecular weight
NA  not applicable
ND  not determined
Neut.  neutrophil
NGF  nerve growth factor
NK  natural killer cell
NO  nitrous oxide
NOD  non-obese diabetic
O-C=O  carboxyl group
O_2^-  superoxide free radical
P-buffer  0.1M Sorenson’s phosphate buffer
P.C.  plasma cell
PAGE polyacrylamide gel
PAGE-SDS sodium dodecyl sulphate - polyacrylamide gel electrophoresis
PAN-PVC polyacrylonitrile-polyvinyl chloride
PB polybrene
PBS phosphate buffered saline
PC12 rat pheochromcytoma cell line
PE50 polyethylene 50
PEEK polyetheretherketone
PEG polyethylene glycol
PEO polyethylene oxide
PEO-NH₂ polyethylene oxide-amine
pO₂ oxygen partial pressure
ppm parts per million
PPO polypropylene oxide
PS polystyrene
PSSa poly(styrene sulfonic acid)
PTFE polytetrafluoroethylene
PVA-SbQ polyvinyl alcohol-bearing styrylpyridinium group
PVP poly(vinyl pyrrolidone)
ROI reactive oxygen intermediates
rt-PCR reverse transcriptase polymerase chain reaction
S.D. standard deviation
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5b-9</td>
<td>soluble terminal membrane attack complex</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TBS</td>
<td>TRIS buffered saline</td>
</tr>
<tr>
<td>TEG</td>
<td>triethylene glycol</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris-(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>UV-B</td>
<td>untraviolet-B</td>
</tr>
<tr>
<td>XPS</td>
<td>x-ray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>
List of Figures

**Figure 1.1** Schematic of the concepts involved in an inflammatory response to microcapsules containing cells. 48

**Figure 1.2** Schematic of the concepts involved in the immune recognition of microencapsulated cells. 49

**Figure 1.3** SEM micrographs of HEMA-MMA microcapsules 75

**Figure 2.1** Representative SEM micrographs of microcapsules containing HepG2 cells without Matrigel® 100

**Figure 2.2** Representative light micrographs of sections of microcapsules containing HepG2 cells without Matrigel® 102

**Figure 2.3** Representative SEM micrographs of microcapsules containing HepG2 cells within Matrigel® 105

**Figure 2.4** Representative light micrographs of sections of microcapsules containing HepG2 cells within Matrigel® 107

**Figure 2.5** SEM micrograph of a HepG2 cell within a Matrigel® capsule. 109

**Figure 2.6** Intact cell number per histological section distribution curves, *in vitro*, for regular and Matrigel® capsules. 111

**Figure 3.1** Schematic of the omental pouch in the rat. 128

**Figure 3.2** Representative micrographs of microcapsules containing rat hepatoma, H4IIEC3, cells after 8 and 21 days, *in vitro*. 135

**Figure 3.3** Representative micrographs of microcapsules containing
human heptoma, HepG2, cells after 8 and 21 days, *in vitro.*

**Figure 3.4** Viable cell number per histological section distribution curves, *in vivo,* for microcapsules containing rat hepatoma, H4IIEC3, cells and human hepatoma, HepG2, cells.

**Figure 3.5** Representative micrographs of microencapsulated rat hepatoma, H4IIEC3, cells after their implantation into omental pouches in Wistar rats

**Figure 3.6** Representative micrographs of microencapsulated human hepatoma, HepG2, cells after their implantation into omental pouches in Wistar rats

**Figure 3.7** Tissue reaction thickness measurement distribution curves for microcapsules containing rat hepatoma, H4IIEC3, cells and human hepatoma, HepG2, cells.

**Figure 3.8** Representative micrographs of the tissue reaction to microcapsules containing rat hepatoma, H4IIEC3, cells after their implantation into omental pouches in Wistar rats.

**Figure 3.9** Representative micrographs of the tissue reaction to microcapsules containing human hepatoma, HepG2, cells after their implantation into omental pouches in Wistar rats.

**Figure 4.1.1** Survey spectrum of HEMA-MMA capsules maintained in medium with serum
**Figure 4.1.2** High resolution C1s spectra of HEMA-MMA capsules maintained in medium with or without serum after various PBS wash regimes

206

**Figure 4.1.3** High resolution C1s spectra of HEMA-MMA capsules maintained in PBS and freshly-made capsules.

208

**Figure 4.2.1** Immunoblot of human serum proteins associated with HEMA-MMA capsules following 1 week incubation in medium containing human serum

227

**Figure 4.2.2** Immunoblot of human C3 and Factor B associated with HEMA-MMA capsule eluates, day 0 medium samples, samples of medium in which the capsules were maintained and samples of medium to which capsules had not been added.

233

**Figure 4.2.3** Rat peritoneal fluid proteins associated with HEMA-MMA capsules after implantation for 1 day. Immunoblotts of fibrinogen, IgG, and Complement C3 of a rat serum sample obtained by cardiac puncture and of capsule eluate.

239
List of Tables

Table 1.1 Microcapsule Components 23

Table 1.2 Properties of Microencapsulated Cells - Advantages and Disadvantages 36

Table 1.3 Techniques for Microencapsulation of Mammalian Cells 45

Table 2.1 HepG2 Cell Viability In Vitro in Regular and Matrigel® Capsules 111

Table 3.1 Microencapsulated Hepatoma Cell Viability In Vitro And In Vivo 135

Table 3.2 Microencapsulated Hepatoma Cellular Aggregate Sizes 142

Table 3.3 Average Tissue Reaction Thicknesses and Average Closest
Blood Vessel Distance for Microcapsules Containing Rat
Hepatoma, H4IIEC3, Cells 161

Table 3.4 Average Tissue Reaction Thickness and Average Closest Blood
Vessel Distance for Microcapsules Containing Human Hepatoma,
HepG2, Cells 162

Table 3.5 Histological Scores for the Tissue Reaction to Microcapsules
Containing Rat Hepatoma, H4IIEC3, Cells 182

Table 3.6 Histological Scores for the Tissue Reaction to Microcapsules
Containing Human Hepatoma, HepG2, Cells 183

Table 3.7 Histological Scoring of the Inflammatory and Immune Cell
Profile of the Tissue Reaction to Microcapsules Containing
Rat Hepatoma, H4IIEC3, Cells 186

Table 3.8 Histological Scoring of the Inflammatory and Immune Cell
Profile of the Tissue Reaction to Microcapsules Containing
Human Hepatoma, HepG2, Cells

Table 4.1.1 Carbon Atom Bonding from C1s High Resolution Spectra
- HEMA-MMA Capsules

Table 4.1.2 Elemental Composition of HEMA-MMA Capsules

Table 4.1.3 Carbon Atom Bonding from C1s High Resolution Spectra
- HEMA-MMA Films

Table 4.2.1 Polyclonal Antibodies Used in Immunoblotting

Table 4.2.2 Molecular Weights, Fragment Assignment and Intensity of Major Bands in the Immunoblots of Capsule Eluates

Table 4.2.3 Molecular Weights, Fragment Assignment and Intensity of Major Bands in C3 and Factor B Immunoblots

Table 4.2.4 Rat Proteins Detected Using the Anti-Human Antibodies to the Corresponding Protein

Table 4.2.5 Classes of Proteins Probed for in Immunoblots
Chapter 1

Background

1.1 Encapsulation for Cell Transplantation - Identification of Issues and Examples

1.1.1. Basic Principles of Microencapsulation for Cell Transplantation

Microencapsulation is the process of placing a protective polymer membrane around a cellular core. Microcapsules are prepared by different methods based on the underlying mechanism of membrane formation and can be 300 μm to 1500 μm in diameter. Membrane formation by interfacial adsorption, interfacial polymerization, polyelectrolyte complexation, and simple coacervation or precipitation have been used to prepare cell containing capsules (Section 1.1.4.).

Microcapsules are comprised of three components; the polymer membrane enclosing mammalian cells which may be embedded in an immobilization matrix or a cell attachment matrix. The function of each of these components and common examples of each microcapsule component are listed in Table 1.1.
<table>
<thead>
<tr>
<th>Microcapsule Component</th>
<th>Function</th>
<th>Examples</th>
</tr>
</thead>
</table>
| Polymer membrane       | • Permeability  
                         • Permeoselectivity 
                         • Immunoisolation 
                         • Sequester cells  
                         • Prevent tumour formation  
                         • Prevent encapsulated cell contact with host cells  
                         • Encapsulated cell compatibility  
                         • Biocompatibility  
                         • Mechanical structure | HEMA-MMA polyacrylate  
                         Alginate-polylsyline  
                         Agarose |
| Living mammalian cells | • Renewable source of secreted therapeutic product in response to physiological stimuli or at a constant rate as long as the cells remain viable and differentiated | Primary cells - e.g., islets, hepatocytes  
                         Cell Lines - e.g., PC12, HepG2  
                         Genetically engineered cells  
                         (Isograft, Allograft, Xenograft) |
| Immobilization matrix  | • Distribute cells within capsule core  
                         • Prevent aggregation of cells and central necrosis due to diffusion limitations | Alginate  
                         Agarose  
                         Chitosan  
                         Synthetic Polymers  
                         (e.g. thermoresponsive hydrogels) |
| Cell attachment matrix | • Provide sites for attachment (differentiation, physiological environment) for anchorage-dependent cells  
                         • Immobilization matrix | Collagen  
                         Matrigel®  
                         Laminin |
1.1.1.1. Applications

Microencapsulation of mammalian cells within a synthetic polymer membrane is proposed as a means of using these cells as a source of therapeutic biomolecules, the absence or abnormality of which cause a disease state. Alternatively, they may replace diseased organ function. The polymer wall is to prevent contact between the encapsulated cells and the host immune system to allow for the transplantation of allogeneic or xenogeneic cells or cell lines without immunosuppressive therapy. The permeability of the polymer membrane allows the diffusion of nutrients into the capsule to sustain cells and the diffusion of the secreted therapeutic product out of the capsule. The need for immunosuppression therapy and the limited availability of suitable donor tissue are the primary limitations of cell transplantation. Immunoisolation is an alternative to immune suppression (1, 2, 3, 4) or induction of tolerance (5, 6, 7) of the host or immunomodulation of the graft (8, 9).

Microcapsules are one of a number of cell-based delivery systems which may be used for protein delivery but usually for cell transplantation. A variety of cell types have been encapsulated to demonstrate the success of the various techniques in maintaining encapsulated cell viability, product secretion, and applicability to a variety of diseases. Alginate-polylysine microcapsules have been prepared containing islets for diabetes treatment (reviewed in 10, 11), PC12 cells (12) and bovine chromaffin cells (13) for Parkinson's disease, hepatocytes for liver disease (14, 15, 16, 17), and parathyroid
tissue for hypoparathyroidism (18, 19). Microencapsulation of genetically-modified cells has added other potential applications including Factor IX secretion for the treatment of hemophilia B (20), growth hormone secretion for the treatment of pituitary dwarfism (21, 22, 23, 24), replacement of adenosine deaminase activity (25) and cytokine delivery for modulation of specific immune responses (26). Microencapsulation can also protect sensitive cells from the adverse effects of handling, cryopreservation, and long-term storage (27, 28).

Macrocapsules, prepared by filling preformed poly(acrylonitrile-co-vinyl chloride) hollow fibers with cells, have been used for the transplantation of islets (29, 30, 31), PC12 cells (32, 33, 34), rat fibroblasts genetically-modified to secrete nerve growth factor (NGF) (35) and baby hamster kidney cells genetically-modified to secrete human NGF for the treatment of Alzheimer’s disease (36, 37, 38) and Huntington’s disease (39), baby hamster kidney (BHK) cells genetically-modified to secrete glial cell line-derived neurotrophic factor (40, 41) and bovine adrenal chromaffin cells for the treatment of chronic pain (42, 43). Macrocapsules, in the shape of hollow fiber tubular structures, have also been prepared from other polymers such as regenerated cellulose, polysulphone, and polyvinylidine chloride for hepatocyte transplantation (44), and polyurethane (45), polyetheretherketone (PEEK 10a) (46) and polysulphone (46, 47), and mesh-reinforced polyvinyl alcohol (48, 49, 50) for islet transplantation. Planar chambers comprised of laminated membranes with an inner cell-impermeable PTFE Biopore membrane (pore size of 0.45 μm) for immunoisolation and an outer cell-
permeable expanded PTFE (Goretex) membrane (pore size typically 5 µm) of a microarchitecture which induces close vascular structures (51), have been used for the transplantation of various cells (52, 53) with a view towards gene therapy applications.

Intravascular devices that require anastomoses to the blood supply have also been used for the transplantation of islets with immunoisolation provided by the tubular membrane (54, 55). However, device failure due to loss of islet viability and thrombosis frequently occurred.

Microencapsulated cells have also been used, in vitro, in biotechnology applications such as antibody production by encapsulated hybridomas (56). The polymer membrane protects the encapsulated cells from high shear stresses created as the bioreactor contents are agitated. The secreted protein(s) are easily recovered from the bioreactor medium and separated from the cells by removing the microcapsules. As another example, alginate polylysine microencapsulation of human bone marrow combined with rapid medium exchange has been used as a method for clonal expansion of single hematopoietic progenitor cells in vitro (57).
1.1.1.2. **Cells as Delivery Systems**

Microencapsulated cells function as a renewable source of secreted therapeutic product in response to physiological stimuli as long as the cells remain viable and differentiated. By using cells in the protein delivery device, we are able to take advantage of the physiological control mechanism by which these cells function. Glucose stimulation of insulin secretion by islets of Langerhans is perhaps the best known mechanism (58). However, there are many other features of cells, that at least in principle, could be exploited in cell-based delivery systems. Cell-based biomolecule delivery can be modulated by factors which influence the phenotypical gene expression of the cells such as secreted paracrine (59) and autocrine growth factors or cytokines (60), positive/negative feed-back loops of these factors, hormones, neurotransmitters, extracellular matrix (61) and associated cytokine effects (62, 63). One of the main difficulties to be overcome in using living cells in microcapsule applications is their characteristic sensitivity to their external environment which restricts the insults that the cells will endure and still remain viable and functional. Therefore, the encapsulation process must be carefully designed since the cells cannot be exposed to pH, osmotic, thermal, shear or solvent “stresses”.

Cell-based protein delivery systems differ from conventional drug delivery systems in that the amount of protein available is not restricted by the finite amount pre-loaded into the device or delivered by a pump. The cells provide a continuous source of protein. Conventional drug delivery systems, in general, are not as responsive to physiological
stimuli as are cells, although there are a few exceptions (64). While most conventional drug delivery system secrete a single protein, cell-based protein delivery devices may supply more than one protein, depending on the cell type. For example, microencapsulated hepatocytes could secrete a variety of plasma proteins which may be deficient due to liver disease. It has been suggested that Parkinson's disease could effectively be treated by microcapsules containing two cell types, one genetically-modified to secrete a neurotrophic factor and one producing dopamine to treat the neurodegenerative component of the disease at the same time as supplying the depleted neurotransmitter (41). Fabrication of the protein delivery system in which cells are the source of the therapeutic protein is, in some sense, simplified over conventional protein delivery systems. Since the protein is produced by viable cells from within the capsules, protein degradation due to heat, enzymes, solvents, pH, and water during device fabrication is not likely an issue (65). Furthermore, there is no need to prepare the protein [e.g., using recombinant technology (66)] nor are protein separation, purification, concentration or sterilization steps required (67, 68).

1.1.1.3. Implantations

Microencapsulated cells may be implanted into a location which is dependent on the protein and its route of delivery. Microencapsulated cells, implanted into the orthotopic (physiological) location would deliver the biomolecule at the site of its action. Microencapsulated cells containing neurotrophic factor- or neurotransmitter-secreting cells, implanted into the brain would circumvent biomolecule passage across the blood
brain barrier and its activity would be regulated by other neurotrophic factors, cofactors or enzymes at the site. Lack of systemic administration of such molecules obviates their deleterious action on peripheral receptors of the nervous system. In other cases, microcapsules need not be placed in the orthotopic site, but there may be an indication for protein delivery via the physiological route (69). Insulin delivered to the peritoneal cavity would be absorbed by the liver (70) and insulin absorbed by omental tissue vasculature would be transported to the liver (71) both of which deliver insulin to the systemic circulation via its physiological route. If the route of protein administration is not important, placement of capsules in the subcutaneous site may be preferred because of the minimal invasive surgery (72), however, cell function in this site is not preserved (73), but has recently been improved by prevascularization with a polymer scaffold (74).

While a typical site for microcapsule implantation is intraperitoneally, as discussed above, other sites may better satisfy issues of physiological product delivery route, endogenous vascularizing capabilities [e.g., omentum (75)] or ease of implantation surgery (e.g., subcutaneously)]. Tissue implantation of capsules avoids chronic, direct contact with flowing blood, circumventing problems of coagulation and vascular anastomoses. Capsules of small enough size may be injected, eliminating the need for invasive surgery. The issues of retrievability, the number of cells required and the capsule size will influence the choice of implant site [e.g., kidney capsule, liver (76), omental pouch (77), epididymal fat pad (78)] and the mode of access. Microcapsules may also be seeded in a prevascularized implantation bed generated in response to a porous polymer
scaffold (44, 74, 79) or a vascularized planar device (80, 81). Problems in the retrievability of free floating capsules implanted intraperitoneally would suggest the need to localize the capsules in a receptacle such as a “tea bag” (82). The retrievability and localization of PAN-PVC macrocapsules was enhanced by attaching a tether at the end of the device to remove the implanted macrocapsule after localization with the aid of a radio-opaque marker (43).

1.1.1.4. The Microcapsule

To provide an environment which is favourable for the maintenance of cellular viability and function, the capsules must be permeable to small molecules (nutrients and metabolites: glucose, oxygen, lactate) and intermediate or large molecules [growth factors, (~ 13-30 kDa), transferrin (80 kDa)]. Yet following implantation, the capsules must be impermeable to components of the host immune system, specifically IgG (150 kDa) and IgM (950 kDa) antibodies, and complement components such as C3 (195 kDa), C1q (410 kDa) or C1 (900 kDa) and possibly also cytokines [e.g., IL-1β (17 kDa) or TNF-α (a trimer 3 X 17 kDa)] such that the encapsulated cells are not attacked nor their function affected by the host immune system on a molecular level. Furthermore, for a functioning cell based biomolecule delivery system, the polymer wall must be permeable to the therapeutic product: e.g., dopamine (153 Da), insulin (6000 Da, if a monomer), growth hormone (23 kDa).
Microcapsules have a high surface to (total) volume ratio, an optimal geometry for diffusion, and are thus expected to result in implants of lower total volume than is possible with hollow fibers with faster response times to physiological stimuli. The implant volume is strongly dependent on capsule size, on the number of capsules that need to be implanted and on how many cells are contained in each capsule. This calculation is particularly important for pancreatic islets since more than $3 \times 10^5$ islets are thought to be necessary for human application (83). With one islet per capsule, and a low $300,000$ islets per transplant, the transplant volume would need to be an unmanageable $1150$ mL for $900 \mu m$ capsules, a reasonable $30$ mL with $400 \mu m$ capsule and only $2.3$ mL with $170 \mu m$ "capsules". Loss of islet efficacy or viability due to transport limitations or cell damage increases the implant volume needed. This effect may be compensated by packing more than 1 islet/capsule. The geometric limitations of hollow fibre or planar geometries are worse, resulting in even higher implant volumes. Recent modifications to microencapsulation processes have added greater flexibility in the size of microcapsules by making possible the preparation of small diameter (84, 85) or conformally coated cellular aggregates (86, 87, 88).

An intact polymer membrane sequesters the transplanted cells to the capsule core and in the case of potentially tumourgenic cell lines such as PC12 cells, acts as a physical barrier limiting cell growth and preventing tumour formation. A mechanically strong polymer membrane is essential for the maintenance of capsule integrity and the tough thermoplastic HEMA-MMA microcapsules may be preferred in this context to the
mechanically weaker alginate-poly-L-lysine capsules. Microcapsules are tougher and mechanically more stable in vivo, without failures due to bending and breakage as observed with tubular structures (31). With certain cell lines and host combinations, tumour formation would not occur since the host’s immune system would recognize the leaked transplanted cells as being foreign (e.g., because of the presence of tumour-specific antigens) and resorb them. Microencapsulation may protect the transplanted cells from the original disease pathology, which in the case of diabetes, may be effected by autoantibodies against islet antigens (89, 90) or insulin (91). The transplantation of cells within microcapsules may protect them from non-immunologic mediated destruction of grafts as a consequence of the transplantation surgery due to ischemia, reperfusion, and surgical manipulation of the tissue (92).

The clinical applicability of microencapsulation of living cells for the treatment of disease or drug delivery brings up other issues in addition to those already discussed. Scale up of the microencapsulation techniques is needed to produce the required large number of capsules of reproducible quality. The isolation of enough cells from a suitable source (e.g., cadaver islets or porcine islets) on a large scale with a high degree of purity and sterility, remains an issue which is being addressed.
1.1.1.5. Diffusion-Limited Microenvironment

Once encapsulated, the cells are in a non-physiological environment in which the polymer wall acts as a diffusion barrier to nutrient transport into and secreted biomolecule and cellular waste transport out of the capsule. Furthermore, the intracapsular environment may not support the attachment of anchorage-dependent cells such that they may not proliferate or may form aggregates in the nonadherent intracapsular environment in the absence of a co-encapsulation cell immobilization or attachment matrix.

Minimization of the diffusion limitations associated with microencapsulation is critical to the success of encapsulation. For example, depletion of a particular nutrient (e.g., oxygen) may cause cells to grow at a lower, diffusion limited rate or the centre of a cell cluster may become necrotic. This has been observed (93, see Chapter 1.1.3.5.2 and Chapter 2) and is similar to what occurs with tumour spheroids (94). Because the cells in spheroids are at a distance from the surrounding medium, gradients of critical nutrients and growth factors and metabolites (and also pH) are set up (95). As a spheroid grows, the number of proliferating cells decreases, the proportion of quiescent cells increases and eventually, due to nutrient deprivation and waste product accumulation, necrosis develops at the centre of spheroids (95). The diffusion of larger molecular weight species (such as growth factors, hormones, and cytokines) into spheroid regions, their interaction with the receptors of cells in the outer few layers of an aggregate (96) and cell contact with the ECM are also important. Insulin secretion by encapsulated islets may be altered because of limitations in nutrient delivery to
those cells located at a distance from the nutrient source [e.g., insulin secretion by islets is sensitive to oxygen concentration (97)].

The microenvironment of spheroidal aggregates appears to maintain the in vivo differentiated characteristics of cells for longer times than monolayer cultures (98, 99), presumably, due to the three dimensional arrangement and the corresponding diffusion gradients and ECM-cell and high cell-cell contact. With the capsule wall controlling the aggregate size and strongly influencing the diffusion gradients, such differentiated cells with prolonged lifespans would presumably be ideally suited for microencapsulated cell applications. Microcapsules are also characterized by a locally high cell density and this too is expected to influence intracapsule cell behaviour (100, 101).

1.1.1.6. Extracellular Matrix Effects

It is well known that the extracellular matrix (ECM), through binding to cell surface integrins, has a strong influence on cell behaviour. Accordingly, adding ECM components to cells prior to encapsulation or otherwise altering the substrate on or within which the cells are grown provides a means to control the behaviour of the transplanted cells. For example, Matrigel grown hepatocytes have elevated mRNA synthesis as compared to cells grown on plastic or in collagen gel. (102). P-450 activities were also induced in Matrigel but not collagen I (103). Alteration of the ECM or other features of the cell microenvironment thus provides another means for controlling the performance of the encapsulated cell. The ECM is
a dynamic environment undergoing constant remodeling (e.g., through the indirect effect of cytokines and growth factors) (104). The capsule wall will likely affect the extent of remodelling, e.g., by impeding the inward transport of interstitial proteases that otherwise cause ECM turnover and consequently stabilizing the intracapsule ECM. On the other hand, necrotic cells may release significant amounts of lysosomal enzymes which can degrade biomolecules. The capsule wall will impair their outward diffusion and could cause a build-up of degrading enzymes or of the acidic lysosomal environment. Encapsulated cells may also produce their own extracellular matrix, consisting of proteins such as collagen, fibronectin or laminin (98, 105).

The features of microencapsulated cells and the advantages and disadvantages of each feature in the applicability of microcapsules for the delivery of cells is summarized in Table 1.2.
Table 1.2 Properties of Microencapsulated Cells - Advantages and Disadvantages

<table>
<thead>
<tr>
<th>Property</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
</table>
| Microcapsule preparation     | • Microcapsules produced already containing living cells so do not need to fill or seal pre-formed membranes  
• Most microencapsulation techniques are applicable to a variety of cell types, different polymer systems, incorporation of a cell immobilization or attachment matrix, core additives  
• Surface modifications possible to improve capsule biocompatibility | • Scale up for the production of a therapeutically useful number of capsules is complex and not yet realized  
• Poor reproducibility of capsule quality between encapsulation runs and between investigators  
• Capsule variability within a batch (e.g., cell #, eccentricity) |
| Cells source of biomolecules | • Cells are a continuous source of secreted therapeutic product in response to physiological stimuli or at a constant rate as long as cells remain viable and differentiated  
• Can secrete more than one protein (e.g., liver cells, genetically engineered cells)  
• Many sources of cells available - autologous, allogeneic, xenogeneic, cell lines, primary cells, genetically modified cells  
• Potential for long-term maintenance of cell viability and function in vitro and in vivo | • Sensitivity of cells to their environment - during encapsulation, and subsequently  
• Delivery dependent on the maintenance of encapsulated cell viability and differentiation  
• Delivery is not easily predicted since it is dependent on the activity of the cells  
• Limited availability of certain cell sources  
• Cell Lines - tumour potential if breach capsule membrane  
• Sterility, purity of cell preparation  
• Limited functional lifespan of capsule implant because of cell death, possibly because of dedifferentiation or else, in the case of genetically-modified cells, loss of transgene expression |
| Polymer Membrane             | • Good biocompatibility of pure polymer is achievable  
• Mechanically tough capsules are feasible  
• Permeable to nutrients/secrated products  
• Permeoselective against high molecular weight molecules (e.g., antibodies, complement components)  
• Sequesters cells to capsule core  
• Prevents tumor formation by cell lines  
• Compatible with microencapsulated cells (surface properties, permeability)  
• Gives capsule mechanical structure | • With cells or xenoantigens biocompatibility can be more limited  
• Capsules have thin, fragile walls |
<p>| High total surface/volume    | • Optimal mass transfer properties for nutrient, secreted product | • Retrievability of many small capsules is difficult |
| ratio                        |                                                                                                                                                                                                           |                                                                                                                                                                                  |</p>
<table>
<thead>
<tr>
<th>Delivery</th>
<th>Capsule Implantation</th>
<th>Immunoisolation</th>
</tr>
</thead>
</table>
| - Small total implant volume  
- Since need to implant a large number of capsules, success not dependent on the integrity of a single device implant (i.e., success of implant is not completely compromised by a few defective capsules) | - Ease of implantation surgery - precise site-specific implantation  
- many potential implantation sites  
- No direct vascular access required  
- Adaptable for incorporation into other devices such as polymer scaffolds or planar chambers with vascularizing membranes such that the benefits of both technologies may be realized  
- Since need to implant a large number of capsules, success not dependent on the integrity of a single device implant (i.e., success of implant is not completely compromised by a few defective capsules) | - No or minimal need for immunosuppression / immunomodulation therapy  
- polymer membrane prevents direct contact between implanted cells and the recipient's immune and inflammatory cells |
| | | - Immune reactions to secreted proteins or shed antigens |
| | - Migration of capsule in vivo  
- Need to localize implant  
- Transplantation of a sufficient number of cells for a therapeutic benefit requires a large number of small capsules |
1.1.4. Alginate-Polylysine and Other Microcapsules

1.1.4.1. Alginate-Polylysine Microcapsules

Based on the pioneering work of Chang (106) numerous biologically active species (e.g. enzymes and whole microbial or plant cells) have been encapsulated or immobilized in polymeric matrices. The more fragile mammalian cells have been encapsulated most frequently in polylysine stabilized calcium alginate. The use of microencapsulated islets of Langerhans for the treatment of diabetes was first proposed in 1980 by Lim and Sun (107). The alginate polylysine microencapsulation technique developed by Sun and coworkers is based on capsule membrane formation by interfacial adsorption of cationic polylysine onto the surface of an anionic alginate cell-containing bead. Microcapsules were prepared by forming droplets of cells suspended in 1.5% (w/v) sodium alginate which upon contact with a bath of 1.5% (w/v) CaCl₂ formed spherical gelled beads as the alginate complexed Ca²⁺ ions. Electrostatic interactions of the anionic alginate upon contact with an aqueous solution of polylysine resulted in their complexation to form a homogeneous outer membrane on the surface of the alginate bead. At first, these capsules were treated with polyethyleneimine to stabilize the membrane for a more durable capsule. The pronounced tissue response to capsules coated with polyethyleneimine (108), and the resultant early failure (2 to 3 weeks) of allogeneic microencapsulated islet transplantations in diabetic rats (107), prompted its later replacement with polylysine such that islet allografts then functioned in a streptozotocin-induced diabetic rat for up to 1 year (109). A uniform coating with alginate was required to cover the positively charged amine groups of the polylysine which would otherwise support inflammatory cell
adherence (110) and a tissue reaction (111, 112). The microcapsules were subsequently treated with a solution of the Ca\(^{2+}/\)Mg\(^{2+}\) chelator, sodium citrate, to liquify the alginate core and extract residual alginate leaving the alginate/polylysine/alginate hydrogel membrane of ~ 4 \(\mu\)m. This microencapsulation technique facilitated the gentle entrapment of sensitive mammalian cells in an aqueous environment and without the action of forces such as shear or pressure, such that they remained functional. This pioneering work demonstrated the conceptual feasibility of islet microencapsulation and therapeutic benefits of transplantation in diabetic animals. Further work has been necessary to refine the process and translate the concept into a practical reality.

Modifications to this alginate-polylysine microencapsulation technique have been aimed at enhancing the cytocompatibility of the encapsulation process (113) and at improving microcapsule properties such as the intracapsular environment for cellular viability and function (16, 114, 115), mechanical strength (116, 117), permeability (116, 118, 119), size (120, 121) and microcapsule biocompatibility (without encapsulated cells) (110, 122, 123, 124, 125). The use of the electrostatic droplet generator to produce small diameter and stronger alginate entrapped islets has given renewed impetus to this area (84, 126).

As noted in the introduction, the size of the microcapsule should be at a minimum in order to minimize diffusion and implant volume constraints. Therefore, Hubbell has described an interfacial photopolymerization technique to conformally coat islets with polyethylene glycol, which is based on the same principles as that described above to coat the surface of
alginate-polylysine capsules (87). The resultant PEG coating on the surface of the pancreatic islets was ~ 30 μm and the conformally coated islets were responsive to glucose with stimulated insulin secretion. In another method to conformally coat islets, islets suspended in an alginate solution were centrifuged through discontinuous density layers, passing through a BaCl$_2$-Ficoll solution in which entrained alginate surrounding the islet is gelled. Coated islets were collected in RPMI 1640 containing 20% (w/v) bovine serum albumin (88). Islets, conformally coated with 10 μm of alginate, exhibited a biphasic insulin response identical to control, uncoated islets, upon an increase in glucose concentration.

Other studies have demonstrated discordant xenograft survival in streptozotocin-induced diabetic mice using alginate microspheres without the polylysine membrane. These microspheres were 800 - 900 μm in diameter and were permeable to IgG, IgM and complement proteins (127). Furthermore, there was a dependency of porcine islet cell survival in vivo on the diameter of the Ca-alginate beads used for their encapsulation (128). Specifically, no viable porcine islets were found within uncoated alginate spheres of 800 μm or 1600 μm after 2 weeks in vivo. However, when islets were encapsulated within very large beads (e.g., 3000 μm), islets were partially or mostly viable.

1.1.4.2. 

*Agarose Microcapsules*

Instead of alginate, agarose-based microcapsules have been prepared by Dupuy and co-workers in which cells suspended in an agarose solution were extruded into a flowing
hydrophobic medium to form spherical droplets which solidified upon cooling (129). The potentially water soluble and enzyme sensitive agarose bead was subsequently coated with water insoluble polyacrylamide by interfacial polymerization (130). Islets encapsulated by this method secreted insulin in response to glucose stimulation (129); however, due to fibrosis, explanted islets were unresponsive to glucose increases (131). The level of cytotoxic free radical production during the photopolymerization of the high concentration monomer solution (~30% v/v), necessary to produce a membrane of sufficient mechanical integrity, was lowered by incorporating polyacrylamide microlatex beads (hydrodynamic diameter 320 nm) into a now 5% acrylamide, 0.25% bisacrylamide aqueous solution (132). Improved cytocompatibility of this modified microencapsulation process was indicated as an increased prolactin secretion by encapsulated pituitary cells.

Beads of agarose without an outer microcapsule membrane have also been prepared. Iwata et al. described a method in which agarose droplets are formed containing islets upon emulsification in paraffin oil and solidified as they are cooled in an ice bath to form beads (133). Longer survival times of allograft transplantations (134) as compared to xenograft transplantations (135) appeared to indicate that agarose provided immunoisolation to immune cells which mediated allograft rejection but were permeable to xenoantibodies and complement which mediate the rejection of xenografts. This antibody permeability may be due to the absence of an immunoprotective membrane on these agarose beads. The sensitivity of agarose to enzymes, combined with its instability at elevated temperatures and water solubility, raises concerns regarding the long-term in vivo
stability and immunoisolation ability of these beads. As an alternative, Iwata et al. proposed the use of a photocrosslinkable polyvinyl alcohol-bearing styrylpyridinium group (PVA-SbQ) as a material to encapsulate islets (136).

A new method has been described to prepare agarose beads with a microcapsule membrane which is less permeable to antibodies. A mixture of agarose (5 % (w/v)) and 10% (w/v) poly(styrene sulfonic acid) (PSSa) was used to prepare microcapsules which were then treated with Polybrene (PB) to stabilize the surface structure and minimize the leaching of PSSa (137, 138, 139). To enhance the biocompatibility of the microcapsules, the surface was modified by the treatment with chondroitin sulfate (137), carboxymethyl cellulose (138) or poly(acrylic acid) (139). Encapsulated hamster islet graft survival in NOD mice depended on the PSSa concentration and the type of polyanion.

Large diameter capsules have also been prepared using combinations of agarose, collagen and Gelfoam with agarose coating for islet transplantation (140). Intraperitoneal implantation of collagen-agarose or Gelfoam-agarose beads containing rat islets into streptozotocin-induced diabetic mice restored normoglycemia for 120 - 170 days, longer times than microcapsules made just of agarose-agarose.
1.1.4.3. Polyelectrolyte Microcapsules

Cell-containing microcapsules can also be prepared by adding droplets of cell suspension in an aqueous solution of one polyelectrolyte to an aqueous solution of an oppositely charged polyelectrolyte to form the capsule wall by polyelectrolyte complexation around the aqueous cellular core. Microcapsules have been prepared using combinations of synthetic polyacrylate-based polyelectrolyte polymers (141, 142, 143, 144) or natural polyelectrolyte combinations of collagen and chitosan and alginate (145), carboxymethyl cellulose and chitosan (146), Matrigel®/carboxymethyl cellulose/chondroitin sulfate A and chitosan/polygalacturonate (147), cellulose sulphate and poly(dimethylallylammoniumchloride) (148, 149), polyphosphazenes and polylysine (150). The first anionic polyelectrolyte is that of the cell containing core, the second, the corresponding cationic polyelectrolyte. The acidic polymer, whether it be synthetic or natural, is used as the interior member of the capsule forming pair due to the expected cytocompatibility of acidic polysaccharides such as alginate and carboxymethyl cellulose. Islets secreted insulin (148), hybridoma cells produced antibody (141, 146, 150) and hepatocytes demonstrated urea and protein synthesis and drug metabolism (147) and hepatocytes of transgenic mice, HbsAg secretion, even upon capsule transplantation into congenic mice for up to two weeks (145). However, the synthetic polyelectrolyte membranes, although highly permeable, were mechanically weak.
1.1.4.4. **Thermoplastic Microcapsules**

AN69, a copolymer of polyacrylonitrile-sodium methylsulphonate has been used to prepare microcapsules by phase inversion (151). AN69, dissolved in DMSO, was co-extruded with cell suspension to form capsule droplets which passed through a layer of ethanol (a moderate precipitant), and into a physiological saline precipitation bath. Insulinoma, RINm5F, cells survived encapsulation, remained viable and secreted insulin upon stimulation with Krebs buffer containing arginine and theophylline. Macrocapsules prepared by this process were permeable to albumin (69 kDa) and IgG (150 kDa) but not to IgA (170 kDa) or IgM (900 kDa) (152). Encapsulated hepatocytes showed improved viability and continued albumin secretion compared to control, unencapsulated hepatocytes, during a 10 day culture period. Hollow fiber encapsulated Lewis rat hepatocytes were 85% viable after syngeneic, intraperitoneal implantation for 45 days (152).

The various techniques for microencapsulation of mammalian cells and the membrane formation principle are listed in Table 1.3.
Table 1.3 Techniques for Microencapsulation of Mammalian Cells

<table>
<thead>
<tr>
<th>Microencapsulation Technique</th>
<th>Capsule Membrane Formation Principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate-Polylsine</td>
<td>Interfacial polyelectrolyte complexation of anionic alginate and cationic polylysine</td>
</tr>
<tr>
<td>Alginate</td>
<td>Ca(^{2+})-induced gelation</td>
</tr>
<tr>
<td>Alginate</td>
<td>Ba(^{2+})-induced gelation</td>
</tr>
<tr>
<td>Poly(ethylene glycol) surface modification of alginate polylysine capsules</td>
<td>Interfacial polyelectrolyte complexation of anionic alginate and cationic polylysine</td>
</tr>
<tr>
<td>Poly(ethylene glycol) surface modification of alginate polylysine capsules</td>
<td>Photopolymerization</td>
</tr>
<tr>
<td>Alginate-polylysine/ polyethyleneimine/protamine sulfate/heparin</td>
<td>Interfacial polyelectrolyte complexation of anionic alginate and cationic polyethyleneimine and protamine sulfate/heparin</td>
</tr>
<tr>
<td>Polyacrylate polyelectrolyte polymers [e.g., polydimethylaminoethyl methacrylate (DMAEMA) + methacrylic acid (MAA)]</td>
<td>Interfacial polyelectrolyte complexation</td>
</tr>
<tr>
<td>Collagen/Chitosan and Alginate</td>
<td>Interfacial polyelectrolyte complexation</td>
</tr>
<tr>
<td>Carboxymethyl Cellulose and Chitosan</td>
<td>Interfacial polyelectrolyte complexation</td>
</tr>
<tr>
<td>Matrigel/Carboxymethyl Cellulose/Chondratin sulfate A and Chitosan/polygalacturonate</td>
<td>Interfacial polyelectrolyte complexation</td>
</tr>
<tr>
<td>Cellulose sulfate and Poly(dimethyldiallylammonium chloride)</td>
<td>Interfacial polyelectrolyte complexation</td>
</tr>
<tr>
<td>Polyphosphazenes and Poly(L-lysine)</td>
<td>Interfacial polyelectrolyte complexation</td>
</tr>
<tr>
<td>Polyacrylonitrile-sodium methylsulphonate</td>
<td>Interfacial precipitation</td>
</tr>
<tr>
<td>Agarose/Polyacrylamide</td>
<td>Interfacial photopolymerization</td>
</tr>
<tr>
<td>Agarose</td>
<td>Thermally-induced gelation</td>
</tr>
<tr>
<td>Agarose [5% (w/v)] and Poly(styrene sulfonic acid) (PSSa) [10% (w/v)] and Polybrene + Chondratin sulfate A, Carboxymethyl Cellulose, poly(acrylic acid)</td>
<td>Thermally-induced gelation and polyionic complexation</td>
</tr>
<tr>
<td>Polyvinyl alcohol (PVA-SbQ)</td>
<td>Photopolymerization</td>
</tr>
<tr>
<td>Agarose/Agarose, Agarose/Collagen, Agarose/Gelfoam</td>
<td>Thermally-induced gelation and polyelectrolyte complexation</td>
</tr>
</tbody>
</table>
1.1.2. Immunoisolation and the Immunology of Cell Transplantation

The two main components of microencapsulated cells are the polymer membrane and the transplanted cells which may be of isogeneic, allogeneic, or xenogeneic origin. Therefore, the implantation of polymer encapsulated cells combines concepts of biomaterials and cell transplantation. The implantation of a biomaterial (without transplanted cells) initiates a sequence of events akin to a foreign body reaction starting with an acute inflammatory response and leading in some cases to a chronic inflammatory response and/or granulation tissue development. The duration and intensity of each of these is dependent upon the extent of injury created in the implantation and the physical and chemical characteristics of the biomaterial (153). Microcapsules containing living cells may be transplanted into a variety of tissue sites such as the peritoneal cavity, subcutaneously, or in the brain without vascular access. The biocompatibility of the microcapsule material in this soft tissue involves concepts of protein adsorption, complement activation, and macrophage and leukocyte adhesion and activation with the biomaterial as the agonist as in blood biocompatibility (Figure 1.1). A central consequence of the inflammatory response to the microcapsule material is the activation of macrophages resulting in the release of cytokines, growth factors, proteolytic enzymes, and reactive oxygen and nitrogen intermediates as mediators of the inflammatory response. The nature of the inflammatory response determines the degree of fibrosis and vascularization of the tissue reaction. A fibrous tissue reaction surrounding the implanted microcapsule may act as a barrier to nutrient and product diffusion (154, 155, 156), to an
extent depending on its thickness. A thin fibrous tissue reaction may have a negligible diffusion resistance relative to the capsule membrane itself. In contrast, a granular tissue reaction would include vascular structures to facilitate the delivery of nutrients and the absorption of cell-derived therapeutic products and has been induced with particular membrane architectures (46, 51, 157). The polymer capsule, by promoting a non-specific inflammatory reaction, recruiting antigen-presenting cells (APC’s) (e.g., dendritic cells or macrophages) and inducing their activation, may act as an adjuvant in the immune response to antigens originating from encapsulated cells. This may therefore intensify an immune response to shed antigens or shorten the time until its onset. The transplantation of allogeneic or xenogeneic cells (without immunosuppression of the host or immunomodulation of the graft) typically results in a graft rejection process (158, 159). Taken together, the host response to microcapsules containing live cells may be viewed as an inflammatory response to the biomaterial (Figure 1.1) and an immune response to the transplanted cells (Figure 1.2). The interconnections between the interaction of these two capsule components with the host are fundamental to understanding the in vivo response to encapsulated cells.

Originally, the idea of immonoisolation, using a polymer membrane, was to physically separate allogeneic and even xenogeneic tissue from the host immune system by preventing contact with immunoglobulin, complement components and immune and inflammatory cells. In this way, the presence of the polymer membrane would prevent an immune response originating from the outside of the capsule acting on cells inside the
The Host Response to Microcapsules Containing Cells

Inflammatory Response

- Foreign Body Reaction
- Acute Inflammatory Reaction
- Chronic Inflammatory Reaction
- Granulation Tissue
- Fibrosis
- Protein adsorption
- Complement Activation
- Leukocyte Adhesion and Activation
- Qualities of the Tissue Reaction (e.g., thickness, cell types)
- Vascularization
- Polymer Membrane
  - (Surface Permeability, Mechanical Properties)
- Macrophage Activation
  - (Release of Cytokines, Growth Factors, Proteolytic Enzymes, ROI)

Figure 1.1  Schematic of the concepts involved in an inflammatory response to microcapsules containing cells.
The Host Response to Microcapsules Containing Cells

**Immune Response**

- Antibodies (Preformed Elicited Class)
- Complement Activation
- Inflammatory/Immune Cells (Macrophages, T helper cells, Cytotoxic T cells, B cells, Plasma Cells, Eosinophiles)
- Cytokines Th1 or Th2 or Th0
- Free Radicals
- Indirect Antigen Presentation
- Polymer Membrane (Permeability, Molecular weight cut-off)
- Cells (Syngeneic, Allogeneic, Xenogeneic)
- Antigens (e.g., secreted proteins, cell surface molecules, cellular debris) type (e.g., alloantigen, xenoantigen) load

**Figure 1.2** Schematic of the concepts involved in the immune recognition of microencapsulated cells.
capsule. However, an immune response is potentially also possible towards antigens shed by encapsulated cells, such as foreign proteins secreted by the cells including the therapeutic agent, cell surface molecules, or cell components (e.g., proteins, phospholipids, DNA), released upon cell death, which then diffuse through the polymer membrane to be recognized as foreign by the host’s immune system. Degradation of biological capsule components such as an extracellular matrix for cell attachment could generate immunologic or proinflammatory products. Antigen shedding from microcapsules would sensitize the host to the transplanted cells and initiate a potentially humoral or molecular cytotoxic tissue response around the implant to enhance the tissue reaction to the implant which could directly affect encapsulated cell viability and function.

An immunological reaction against encapsulated cell surface antigens (e.g., donor MHC molecules) or cell-specific secreted proteins may be produced by the host if these soluble shed antigens permeate through the polymer wall. These shed antigens may be internalized, processed and presented in association with the host Class II Major Histocompatibility Complex (MHC) molecules, most effectively by macrophages or dendritic cells, to host CD4⁺ helper T cells: the indirect pathway of antigen recognition (160). This pathway may lead to the activation of T helper cells, which secrete cytokines and provide the necessary signals for the growth (autocrine, IL-2-mediated T cell proliferation), maturation and activation of effector CD8⁺ cytotoxic T cells, B cells, macrophages, inflammatory leukocytes and endothelial cells. In this way, they would promote and regulate humoral and cell-mediated immune responses and inflammation. Discussion is focused on the indirect
pathway of antigen presentation since the direct presentation of graft antigens by donor APC's (e.g., carrier leukocytes) to host helper or cytotoxic T cells (161), would not be expected because the polymer membrane should prevent the required cell contact.

Helper T cells activated by indirect antigen presentation may function in the graft rejection process in three ways. The helper T cells may cause the activation of cytotoxic T cells (162), which if they could contact encapsulated cells, would lyse them. Helper T cells may secrete cytokines (e.g., IL-2, IL-4, IL-5) to affect the proliferation, differentiation of B cells to plasma cells and the isotype of secreted antibody, leading to an encapsulated cell-specific antibody response (163). Macrophages, influenced by cytokines produced by the helper T cells, may also secrete cytokines including IL-1, TNF, and IL-6, which may be proliferation and differentiation factors for B cells in addition to their proinflammatory role (164). Effector cells (activated macrophages, T helper cells, cytotoxic T cells, eosinophils, mast cells) may mediate graft destruction by direct interaction with the graft, or more pertinently in this application, through non-specific inflammatory tissue damage through the release of cytokines or mediators (e.g., IL-4, IL-5, major basic protein, histamine, prostaglandins or leukotriens) (165). Immunologists term this type of cell-mediated immune response and the associated inflammation, a delay-type hypersensitivity reaction (166).

Generally, antibodies which permeate the immunoisolating polymer membrane may not on their own destroy antibody-targeted cells if the required immunocellular and
complement components are excluded. However, in certain situations, the binding of antibody alone to encapsulated cells could be sufficient to cause damage if bound to a ligand essential for cell survival or function (e.g., transferrin receptor). Antibodies, specific to encapsulated cells, would be those recently produced by the host in an immunological response to shed antigens, preformed antibodies due to an immunologically-based disease etiology (e.g., Type I diabetes) or present in discordant xenogeneic transplantation. Complement mediated lysis of encapsulated cells would be possible if the C1q component of the C1 molecule were to bind to an aggregate of IgG molecules or an individual IgM molecule on the surface of a cell (167). Activation of C1 would initiate the classical pathway of complement activation which, with the addition of the subsequent complement components, (of lower molecular weight) would potentially result in the assembly of the membrane attack complex (MAC) and cell lysis. Alternatively, complement activation by the alternative pathway may also occur. If the alternative pathway of complement activation was not inhibited by regulatory membrane proteins on the surface of the encapsulated mammalian cells, then the polymer capsule would need to exclude C3 (Mw 195 kDa) the alternative pathway component of highest molecular weight and a key component forming, the C3 convertase. The sequence of steps in the initiation of the classical complement pathway would determine the immunoisolating membrane molecular weight cut-off to prevent lysis of cells by this pathway. If the C1 molecule (900 kDa) could be assembled inside the capsule upon permeation of free C1q (410 kDa), binding of the tetramer C1r2C1s2 [C1s (87 kDa) C1r (190 kDa)] and C1 activation (168, 169) then a molecular weight cut-off of 410 kDa would be sufficient to exclude C1q. Alternatively, if the entire C1 molecule must bind to the cluster of antibody Fc
through the C1q subunit (170) then a molecular weight cutoff of 900 - 950 kDa would exclude C1 and also IgM. The relative concentrations of C1 and dissociated C1q and C1r2s2 in the tissue surrounding microcapsules may be factors in determining which mechanism would predominate. Macrophages (171, 172) and fibroblasts (173) produce the components of the C1 macromolecule - C1q, C1r, and C1s.

These same antibodies and complement components (IgG and C3b and iC3b) could coat (opsonify) encapsulated cells to be recognized by accessible macrophages through their receptors (FCγ receptors and complement receptor type 1 and type 3, respectively), activating the macrophages and phagocytosing the cells. Antibody-targeted encapsulated cells could be lysed by cell-specific cytotoxic T lymphocytes or natural killer cells if these could contact encapsulated cells. Obviously, the polymer membrane should provide a physical barrier preventing contact between encapsulated cells and immune and inflammatory cells. However, because of the ability of the immune and inflammatory cells to form cellular processes which may extend over large distances, it has been suggested that membrane spanning pores greater than 0.1 \( \mu \text{m} \) in size be avoided (10).

Non-specific lysis of encapsulated cells may be mediated by lysosomal enzymes or reactive oxygen (e.g., \( \text{O}_2^\cdot \), \( \text{H}_2\text{O}_2 \)) or nitrogen (e.g., NO) intermediates released by activated leukocytes. The free radicals are highly reactive and have a short lifetime until inactivation by chemical reaction, usually functioning locally to their site of secretion. A soluble nitrogen mediator (NO) has been shown to be involved in the morphological destruction of islet cells
which were co-cultured with syngeneic activated peritoneal macrophages (174). Islet cell lysis was not cell contact dependent since it was not prevented by alginate embedding of either islets or macrophages. Coencapsulation of islets with live erythrocytes, which are able to scavenge NO (175) and/or convert it to nitrate (176), protected islets from damage caused by the NO derived from activated macrophages (177). Although the half life of NO in H2O is ~ 3-50 sec (178), the reversible stabilization of NO for several minutes by cysteine and for several hours by proteins as S-nitroso-thiols, may allow for the diffusion of this macrophage-derived mediator through the capsule membrane to reach islets (177).

Cytokines, such as IL-1β, IFN-γ, IL-6, and TNF-α released by inflammatory and immune cells in the tissue reaction to polymer microcapsules containing cells (179), may be present in sufficiently high concentrations intracapsularly, to affect synergistically cell viability and function (180, 181, 182, 183, 184, 185, 186), possibly resulting in graft failure (187). The incubation of IL-1β with alginate-embedded whole islets resulted in cell lysis to an extent which was dependent on the concentration of IL-1β and which was slightly enhanced by the addition of TNF-α (188). Interestingly, some microcapsules appear to prevent IL-1β inhibition of insulin secretion (189), suggesting that the intracapsular microenvironment is such that the sensitivity of islets to IL-1β is lessened after encapsulation. As another example, the exposure of human hepatoma, HepG2, cells to medium containing cytokines such as IL-1β, TNF-α, IL-6, or IFN-β2 increased the secretion of acute phase plasma proteins to an extent dependent on the cytokine and combinations thereof (190, 191, 192, 193). The exposure of
encapsulated cells to any of these cytokines *in vivo*, could affect their viability or differentiated functions. Therefore, the polymer membrane should lessen the concentration of these cytokines within the capsule. In order to achieve this, a polymer membrane molecular weight cut-off of ~20 kDa would be necessary, but this but would likely compromise cell viability due to nutrient diffusion restrictions. Encapsulated cells may secrete cytokines or growth factors that influence the inflammatory and immune response to these capsules (194, 195) or induce angiogenesis surrounding the cell-containing implant (52, 196, 197).

1.1.3. Biocompatibility of Immunoisolation Devices

1.1.3.1. Microcapsule Biocompatibility

The biocompatibility of alginate-polylysine capsules has been questioned mainly due to the solubility of the individual polyelectrolytes in an aqueous environment. Calcium alginate gels are sensitive to chelating compounds such as phosphate, citrate, and lactate as well as anti-gelling cations such as Na\(^+\) and Mg\(^{2+}\) (198). Hence, the ionic environment surrounding implanted alginate polylysine capsules formed by Ca\(^{2+}\) complexation, may result in biodegradation of the microcapsule; dissolution has even been described during their *in vitro* culture (199). However, the complexation of the alginate with polylysine may stabilize the microcapsule membrane (198) such that biodegradation of the complexed membrane would be much less than that which would be expected based on the individual components. Dixit has noted degenerative changes in the microcapsule membrane of capsules containing hepatocytes after 2 months *in vivo* (200). Areas where the microcapsule membrane was broken,
exposed encapsulated hepatocytes, and permitted infiltration of inflammatory cells as well as foreign body giant cells engulfing the donor hepatocytes. *In vitro*, alginate poly-L-lysine microcapsule membranes degenerated rapidly with extreme pH variations. At physiological pH *in vitro*, however, the microcapsules containing hepatocytes remain intact for at least 2-3 weeks longer than *in vivo* following IP implantation. As microencapsulated hepatocytes completed their normal life cycle and died *in vitro*, the microcapsule membrane became weak and broke down with agitation. It was suggested that a similar process of alginate-poly-L-lysine capsule biodegradation occurs *in vivo* as hepatocyte lysis couples with mechanical shear forces generated by intestinal peristalsis (17). The issue of alginate-poly-L-lysine microcapsule biostability has been addressed by Federlin and coworkers who, by replacing the CaCl$_2$ gelation step with BaCl$_2$, prepared nonbiodegradable barium alginate beads for islet transplantation (122); barium has a higher affinity for alginate than calcium (198) but ionic barium is toxic.

Macrophages which had migrated out of alginate-poly-L-lysine-alginate encapsulated rat islets of Langerhans were found to contain gel particles from the inner capsule wall (194). Although the macrophage-like cells were able to ingest portions of the inner capsule wall, no breaches of the membrane were detected over the 99 day observation period. Phagocytosis of the alginate-poly-L-lysine-alginate capsules by inflammatory, tissue macrophages (194) or foreign body giant cells may also contribute to their biodegradation *in vivo* and could be a mode of clearing capsules from the body at the end of their functional lifetime (127).
Islets have been shown to lose their function *in vivo* even with immunoprotection provided by a polymer microcapsule membrane and this loss of function has been correlated with the capsule-associated tissue reaction (187). Several studies have addressed possible contributing factors to the microcapsule-associated tissue reaction. Commercial alginate (purified to remove lipopolysaccharides and polyphenols) stimulated human monocytes to release the cytokines IL-1 and TNF-α which are mediators of fibrosis acting by stimulating fibroblast proliferation (123). Mannuronic acid-rich alginate but not guluronic acid-rich alginate stimulated cytokine release (123) and capsules with an outer layer of high mannuronic content alginate elicited a tissue response upon implantation (111, 123). Tissue macrophages possess cell surface receptors for mannose (201) which may account for this observation. The uncomplexed alginate content within alginate-poly-L-lysine capsules can be quantified (202) and may affect the level of inflammatory response to these microcapsules. Cyclosporin (CsA) inhibited mannuronic acid induced cytokine release (203), facilitating the prolonged microencapsulated graft acceptance (204), possibly by modulating the tissue response. Reach and coworkers demonstrated cytokine (IL-1β) production and release by activated macrophages in direct contact with alginate-polylysine capsules (205). These macrophages were stimulated to release significantly more IL-1β, however, even then it was only 10% of that induced by endotoxin (LPS). Intracellular IL-1α and IL-1β also increased with capsule contact and was 20-30% the value caused by LPS. Agarose beads coated with polyacrylamide by interfacial polymerization activated complement in human serum (131) and caused IL-1 secretion by
phorbol-12-myristate-13-acetate differentiated U937 macrophage-like cells but only in the presence of LPS (206).

Macrophages and fibroblasts are the most prominent cell types in the foreign body reaction associated with some capsules. Destruction of islet cells has been demonstrated during their co-culture with syngeneic intraperitoneal-activated macrophages (174). The alginate embedding of islets or of macrophages did not prevent islet cell lysis, indicating that cell contact was not required and that a soluble mediator was responsible. The activated macrophage-derived soluble mediator of islet cell lysis was nitric oxide (NO) since lysis of alginate-embedded rat islet was inhibited in a dose-dependent manner by a specific nitric oxide-synthase inhibitor and was morphologically similar to that induced by nitric oxide generating compounds (177). Co-encapsulation of islets with live erythrocytes protected activated macrophage-derived NO-mediated islet damage to an extent which depended on the number of erythrocytes. Ferrous hemoglobin and erythrocytes are able to scavenge NO (207, 208) and/or convert it to nitrate (209).

An attempt to modify the surface of alginate-polylysine capsules for the enhancement of biocompatibility has been described by Hubbell and associates (110, 125). The surface of the microcapsule was modified by the polyelectrolyte complexation of the polyanionic alginate with a polycationic graft copolymer, lysine co-monomethoxy poly(ethylene glycol) (lysine-MPEG) (110). The inability of the MPEG treated surface to support fibroblast attachment was indicated in their rounded morphology and reduced attachment to MPEG treated capsules as
compared to the other two surfaces. A more stable surface modification was obtained by the photopolymerization of polyethylene glycol tetraacrylate onto the polylysine capsule surface (125); an example of interfacial polymerization for microcapsule preparation. Alginate polylysine capsules containing islets were coated with eosin Y and suspended in an aqueous solution of polyethylene glycol tetraacrylate (MW = 18500), triethanolamine and n-vinyl pyrolidinone. Photopolymerization was initiated by the exposure of capsules to the visible light of an argon laser, resulting in a capsule coating of ~ 5 - 10 μm. Hubbell found that cellular overgrowth occurred within 4 days on 60 - 80% of alginate polylysine capsules recovered from the peritoneal cavity of mice. In contrast, PEG-coated microcapsules were free floating and without attached cells, even up to 20 days post-implantation. Kung and colleagues have also modified the surface of double layer alginate/poly(L-lysine) microcapsules using tosyl chloride-activated poly(ethylene glycol), cyanuric chloride-activated poly(ethylene glycol) or tosyl chloride-activated poly(vinyl alcohol) (210).

1.1.3.2. Immunoisolation and Immunology

The immunoisolating ability of various devices has been tested in vitro with successful results. The alginate polylysine membrane was able to prevent the cell contact necessary for cytotoxic T-lymphocyte and natural killer cell-mediated lysis of encapsulated cells (211). Furthermore, in vitro studies showed that the alginate polylysine membrane excluded IgG antibody binding to encapsulated cells (212). Alginate-polylysine encapsulation prevented complement mediated lysis of rat insulinoma cells (213).
indicating that the alginate polylysine membrane functioned to provide humoural immunoisolation in vitro. Another study showed that the alginate polylysine membrane could protect porcine islets from antibody-dependent complement mediated lysis using human serum (214). The human serum used in these experiments was from a non-diabetic donor. Therefore the antibodies involved in the complement-mediated lysis of the porcine islets were preformed anti-porcine antibodies and not specific anti-islet antibodies. This experiment was an in vitro simulation of a discordant xenograft transplantation with immunoisolation provided by the alginate-polylysine membrane. However, the immunoprotective property of the alginate polylysine capsules was not consistently reproducible but was maintained by microcapsules of various compositions (high mannuronic acid alginate-polylysine, high guluronic acid-polylysine, high mannuronic acid alginate-poly-L-ornithine, and high guluronic acid-polyornithine) (215).

In vivo studies, have demonstrated the actual applicability of immunoisolation devices in preventing graft rejection, which has not always been as positive. To elucidate the mechanisms of immune-recognition of encapsulated cells, different animal models and implant recipient immunomodulation have been used.

Several studies with immunisolation devices have shown allograft survival for longer times than xenografts. Agarose beads supported allograft survival for longer times (134) as compared to xenograft transplantations (133) which appeared to indicate that agarose provided immunoisoation to immune cells which mediated allograft rejection but
were permeable to xenoantibodies and complement which mediate the rejection of xenografts. Agarose beads prepared from a 7.5% (w/v) agarose solution, instead of 5% (w/v), were more dense, thus less antibody and complement permeable to result in an increase in the length of xenograft survival time from 32.2 ± 17.8 days to 85.3 ± 23.1 days (135). Capsules prepared from a mixture of agarose and poly(styrene sulfonic acid) (PSSa) with polyionic complexation of the surface with Polybrene (PB), surface modified with chondroitin sulfate facilitated hamster islet xenograft survival for >77.4 ± 21.7 days as compared to that provided by agarose microcapsules of >31.5 ± 42.4 days, respectively, in streptozotocin-induced diabetic mice (137). The immunoisolation ability of these laminated vascularizing membranes in diffusion chambers was assessed by comparing the viability of allogeneic and xenogeneic tissue (216). Allograft tissue survived for longer than 9 months within chambers with intact membranes but not if the membrane was punctured to allow cell entry, in which case they survived for only 21 days. In contrast to allografts, xenografts survived for less than 21 days even when the membrane was intact.

Weber et al. found that xenogeneic implantation of microencapsulated dog (discordant) or rat (concordant) islets survived longer in streptozotocin-induced diabetic C57BL/6J mice (100 and 51 ± 8 days, respectively) than in spontaneously diabetic NOD mice (10 ± 2 and 11.5 ± 3 days, respectively) (217). NOD mice are the best available model of human insulin-dependent diabetes. The tissue reaction to capsules containing dog islets transplanted into pre-diabetic NOD mice may have been directed at
xenoantigens, as a more extensive tissue reaction was observed in diabetic NOD mice than in diabetic C57BL/6J mice; islet-specific antibodies may have been involved. Subsequent studies were focused on elucidating the reasons for this result.

The tissue reaction to microencapsulated islet xenografts was characterized by immunohistochemical staining for inflammatory cell markers, cytokines, and antibodies. The predominant cells involved in the tissue response to microcapsules containing xenografts of porcine, canine or rat islets in NOD mice were macrophage and lymphocytes (217). Microencapsulated xenografts which were undergoing rejection in NOD mice, had associated with the capsule surface and interior, mouse IgG antibodies and with the surface only, mouse IgM antibodies (218). The presence of IgG antibodies within the capsule, indicated that this molecule is not excluded by the alginate polylysine membrane in vivo and that it may play a role in the rejection of xenografts. However, complement components would presumably need to enter the capsule as well to result in complement-mediated lysis of xenogeneic encapsulated cells. No immunolabelling for complement components such as C3 or C1q was performed. Flow cytometric analysis of the cellular infiltrate surrounding alginate polylysine capsules containing rat islets in NOD mice, as compared to empty capsules, showed that the percentage of CD11b+ and Gran-1+ cells recovered by peritoneal lavage were increased in the samples from cell-containing functioning (from normoglycemic animals) and rejecting (from hyperglycemic animals) microcapsules as compared to empty capsules (179). Furthermore, the percentage of CD4+ cells was higher with functioning capsules as compared to controls. The percentage
of B cells fell with the onset of graft rejection. Few inflammatory cells were associated with the capsules before rejection. Immunocytochemical staining of the tissue reaction identified the inflammatory cell profile in which 5% of the cells were CD4\(^+\), 10% were CD8\(^+\), and 40 - 75% were CD11b\(^-\). Upon rejection, a thicker tissue reaction (8 - 15 layers) was associated with the capsules which was mainly comprised of macrophages (90%) with few CD4\(^+\) and CD8\(^+\) cells. Immunohistochemistry indicated the intracellular presence of the macrophage-derived cytokines IL-1 and TNF within the tissue reaction to the capsules. The results indicated the involvement of macrophages during the rejection of rat islets in NOD mice possibly by presenting donor antigens to T cells.

CD4\(^+\) cells (T helper cells, and also macrophages, the main cells in the tissue reaction) were found to be important in prolonging xenograft survival in NOD mice since their \textit{in vivo} depletion with anti-CD4 monoclonal antibody depletion prolonged graft survival (217). Encapsulated rat islet xenograft survival was found to depend on the MHC molecule, presumably expressed on antigen presenting cells presenting xenoantigens (219). The mechanism of destruction of microencapsulated discordant islet xenografts in NOD mice was further elucidated by examining the involvement of antigen presenting cells in the immune response towards xenoantigens shed from microcapsules (220). The co-stimulatory interaction of the T-cell surface antigen, CD28, with its ligands B7-1 and B7-2, on APCs was blocked \textit{in vivo} by administering CTLA4-Ig to NOD mice with microcapsule implants, thereby inhibiting indirect presentation of xenoantigens and a host T cell response. Treatment of NOD mice with CTLA4-Ig significantly prolonged survival
(up to 92 days) of intraperitoneal poly-L-lysine-alginate microencapsulated rabbit islet xenografts when compared to either islet microencapsulation (up to 28 days). Anti-CD8 monoclonal antibody therapy and cyclosporin A immunosuppression failed to prevent the rejection of encapsulated xenografts in NOD mice.

Several studies have assessed the role of antigens shed from encapsulated cells on the tissue reaction and graft survival. Alginate-polylysine microencapsulated rat islets, depleted of antigen presenting cells by ultraviolet-B irradiation or pre-encapsulation culture at 24 °C prior to their encapsulation and transplanted into diabetic NOD mice, a concordant xenograft, survived for 68.2 ± 19.1 days while untreated microencapsulated xenografts survived for only 12.6 ± 1.2 days (221). Furthermore, the effect of antigen shed from encapsulated islets was demonstrated by an intense tissue reaction towards capsules containing rat islets, but a milder response towards microcapsules containing UV-B treated islets. The authors hypothesized that that antigen(s) released from encapsulated islets were responsible, at least in part, for the NOD cellular reaction to islet xenograft, and that these antigens were sensitive to UV-B. Since UV-B treatment depletes antigen presenting cells, the UV-B sensitive antigens may be MHC class II molecules on antigen presenting cells which are highly immunogenic (222, 223). Syngeneic (Lewis rat) or allogeneic cells (Wistar rat) were embedded in barium-alginate microcapsules (molecular weight cut-off > 100 kDa) and the effect of their respective released antigens, on the associated tissue reaction was assessed (224). Microcapsules containing syngeneic islets exhibited a slightly greater tissue reaction as compared to that
towards empty microcapsules. However, most microcapsules containing syngeneic islets were recovered as free floating by peritoneal lavage and contained viable islets with some central necrosis. Microcapsules, containing allogeneic islets, recovered from normoglycemic rats were mostly free of a tissue reaction and contained viable islets. In contrast, microcapsules, recovered from hyperglycemic rats exhibited severe tissue reactions and contained nonviable allogeneic islet remnants. These results appeared to indicate that products of allogeneic cellular necrosis elicited a tissue reaction or conversely that the tissue reaction caused cell death. However, viable allogeneic cells in microcapsules without a tissue reaction appeared to indicate that there was not an induction of tissue reaction by alloantigens. Other studies have also shown that encapsulated cells influence the tissue reaction to the capsules through antigens which are shed from the encapsulated cells. There were more inflammatory immune cells associated with alginate polylsine capsules containing tumourogenic or non-tumourogenic cells as compared to empty capsules (225). Furthermore, tumourogenic cells elicited an enhanced tissue response potentially indicating that tumour specific antigens are released by these cells.

Antigens shed from microencapsulated and macroencapsulated rat islets into the surrounding culture media have been shown to activate host lymphocytes, in vitro (226). Barium-alginate microencapsulated rat islets induced a lymphocyte proliferation which was similar to that of unencapsulated islets; both much less than that induced by IL-2 treatment. A portion of the activation in response to the microencapsulated islets was due
to the microcapsules alone. Polysulphone macroencapsulated islets induced a lymphoid reaction which was lower than that of non encapsulated islets but significantly different from that induced by empty hollow fibers. Neither micro- nor macroencapsulation provided immunoisolation since shed islet antigens shed activated lymphocytes. Macrocapsules were slightly more effective in immunoisolating islets presumably because of a lower molecular weight cut-off of macrocapsules (< 100 kDa) as compared to microcapsules (150 - 250 kDa) and/or geometric considerations (e.g., diffusion distances and the surface-to-volume ratios). Xenoantibodies against porcine or canine cellular antigens shed from PAN-PVC macrocapsules have been detected in streptozotocin-induced diabetic rats (227). However, pre-sensitization of the recipient with allogeneic islets did not affect the ability of barium-alginate beads to protect subsequent islet transplants from destruction in streptozotocin-induced diabetic Lewis rat with the same functional performance in naive and preimmunized rats (228).

Recently, uncoated alginate microspheres, without a synthetic poly(L-lysine) membrane, which were 800 - 900 µm in diameter and permeable to IgG, IgM and complement proteins supported survival of discordant porcine or bovine islet xenografts in streptozotocin-induced diabetic mice (127). In fact, Ca-alginate enclosed islets implanted intraperitoneally were immunopositive for IgG and C3 inside the capsule. Apparently, in spite of this apparent lack of humoral immunisolation, intraperitoneal implants of xenogeneic (bovine or porcine) islets into streptozotocin-induced diabetic mice reversed diabetes in all but one recipient for at least 1 month. The majority of alginate encapsulated
islets were recovered as free floating and some capsules were attached to mesentery which exhibited mild reactive hyperplasia consisting of fibrofatty tissue. The external surfaces of the beads were generally free of fibrosis and host cell adherence, although occasionally, the beads were encapsulated by a monolayer of fibroblasts and/or host mononuclear blood cells. It was proposed that the role of natural xenogeneic antibodies in the complement mediated destruction of pig and cow islet grafts in mice was not a significant mechanism. Furthermore, the viability or function of islets encapsulated within Ca-alginate were also not affected by cytokines, nitric oxide or other cytotoxic moieties which were small enough to diffuse into the gel matrix. The immunoprotection offered by the Ca-alginate gel appeared to be in preventing cell-mediated cytotoxicity. Furthermore, it was hypothesized that the charge of the cytotoxic proteins (e.g., complement components, IgG) may be such as to be affected by electrostatic interactions with the Ca-alginate membrane.

The mechanism of rejection of xenogeneic tissue within planar chambers comprised of laminated cell impermeable membrane and vascularizing membrane was investigated to determine if the death of the tissue was due to specific immune destruction or to non-specific lysis due to the local inflammation (229). When fetal bovine islets and fetal lewis rat lung were combined and implanted into adult Lewis rats, the islets were destroyed but the lung tissue survived. This result indicated that sufficient nutrients were present for cell survival and that non-specific immunity alone was not responsible for xenograft death. Xenograft destruction was accelerated by the pre-immunization of the
recipient with the graft tissues. Immunostaining of these implants showed a positive reaction for IgG, IgM and the complement component C3, which correlated with the destruction of the tissues. Encapsulated xenografts were rejected in AKR (C5-deficient) mice indicating that complement was not required for destruction. The heavy binding of antibodies to the xenograft cell surface suggested that antibody binding alone was responsible for cell death. Survival of xenogeneic islet tissue transplants in diffusion chambers was extended in diabetic NOD mice by immunosuppression with either anti-CD4 antibodies or Cyclosporin A from 3 weeks in untreated animals to 10 or 8 weeks, respectively (230).

The local response to implanted xenografts and allografts in comparison to isografts in membrane-bound diffusion chambers with 0.4-μm pore membranes was examined after their implantation into the epididymal fat pad of rats (231). The membranes prevented host cell entry into the device but did not prevent passage of large molecules such as IgG and IgM. Allogeneic rat embryonic lung tissue implanted into Lewis rats survived for 1 year when implanted in intact devices, but similar tissues were destroyed within 3 weeks when implanted within devices with holes poked in the membrane to allow host cell contact. In contrast, xenografts (CF1 mouse embryonic lung implanted into Lewis rats) were destroyed within 3 weeks even in devices with intact membranes. Xenografts in devices implanted into athymic rats survived indicating that the tissue death was caused by immunological factors in immunocompetent animals and not by species-dependent physiological incompatibility. The death of xenogeneic tissues was
accompanied by a severe local accumulation of inflammatory cells and a decrease in local vascularization. The absence of close vascular structures in xenografts correlated with both high host reaction and low tissue survival. When isogeneic tissues (Lewis rat embryonic lung implanted into Lewis rats) were mixed with xenogeneic tissues, a local inflammatory response occurred which was sufficiently hostile to kill both isogeneic tissue and xenogeneic tissue within 5 weeks. In the absence of xenogeneic tissue, isografts would have survived. It was hypothesized that xenogeneic antigens which diffuse through the membrane are taken up by antigen presenting cells which orchestrate a local cellular response that inhibits vascularization around the implant resulting in the death of the immunoisolated cells. The observation that the local response to xenografts is sufficient to kill isografts complicates issues of immunoprotection, suggesting that successful immunoisolation will require membranes that not only provide protection of the encapsulated tissues from the host immune system but also have properties that diminish the release of xenogeneic antigens. Other macroporous systems which have been successfully used by Lacy et al (29) and Lanza et al. (232) for transplantation of islet xenografts, had molecular weight cutoffs of 50 and 50-80 kDa, receptively, consistent with ultrafiltration rather than the microporous type membranes used in the diffusion chambers. Around these macrocapsules there was no significant local cell response suggesting the possibility that the more restrictive pores may prevent the molecules that initiate the reaction from exiting the device (233).
Membrane enclosed COS (monkey kidney) xenografts survived in CD4⁺ T cell depleted mice but not in CD8⁺ T cell depleted or nondepleted control mice (234). Encapsulated xenografts survived when implanted into either athymic or SCID mice but were destroyed in athymic and SCID mice reconstituted with normal leukocytes and normal mice. Athymic mice, reconstituted with the CD8⁺ cell depleted preparation resulted in death of encapsulated monkey cells whereas the injection of CD4⁺ cell depleted preparation did not. The study highlighted the critical role of CD4⁺ T cells, in the absence of CD8⁺ cells and B cells, in the processes leading to the ultimate destruction of encapsulated xenografts. Encapsulated xenografts did not survive in NK cell deficient mice and normal mice suggesting that NK cells are not crucial for rejection of encapsulated xenografts. Circulating anti-xenograft antibodies produced by preimmunization had no effect on the survival of the encapsulated xenografts implanted into anti-CD4-treated mice. Because of the cell-impermeable membranes used, it was hypothesized that the most likely involvement of CD4⁺ T cells is in the indirect antigen recognition by these cells and subsequent stimulation of inflammatory cells.

1.1.5. Polyacrylates for Cell Microencapsulation

The suitability of a polymer for microencapsulation of cells is determined by its processability (e.g., its viscosity and solubility especially in solvents that are tolerated by the cells), its permselectivity in the form of the microcapsule wall and its biocompatibility. The capsule wall must have a high permeability to nutrients and cell-derived products, but
provide immunoisolation. Since the surface chemistry has a large role in defining the capsule biocompatibility, it too is significant.

Sefton and his co-workers have based much of their research in microencapsulation of living cells on the use of a thermoplastic polyacrylate copolymer, hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA; ~75 mole % HEMA). HEMA-MMA is prepared by solution polymerization after careful monomer purification to reduce the crosslinker content (235). This copolymer is hydrophilic with a ~25 - 30 % (w/w) water uptake (236) consistent with the poly(HEMA) content, but has mechanical strength, toughness and elasticity imparted by the poly(MMA) component. These properties lead to adequate permeability of the polymer capsules to aqueous solutes for cellular sustenance (237) and sufficient mechanical durability to tolerate normal handling and stresses in vivo. The critical requirement of microcapsule biocompatibility was considered likely due to the common use of the homopolymers poly(MMA) (238) and poly(HEMA) (239) in biomedical applications such as bone cement and intraocular and contact lenses, respectively. The water insolubility of the HEMA-MMA polymer provides stability in the aqueous physiological environment but necessitated the use of an organic solvent to prepare the polymer solution. The ultimate success of this material depended on the ability to select a tolerable solvent and to design a gentle encapsulation process.
Polyacrylate microcapsules were prepared using two other water-insoluble polymers, namely, commercially available EUDRAGIT RL™ (240, 241, 242) and copolymers of dimethylaminoethyl methacrylate and methyl methacrylate (DMAEMA-MMA, 243), before the more often used, HEMA-MMA. Common to all these polymers is the use of co-axial extrusion of cell suspension and polymer solution, shearing of the capsule droplet, and polymer wall formation by interfacial precipitation upon nonsolvent contact. However, each polymer has different properties such as its solubility in solvents which would be suitable for microencapsulation with cells (diethyl phthalate or polyethylene glycol), the solution viscosity and the precipitation characteristics in an appropriate nonsolvent. Therefore, their application to microencapsulation required appropriately selected polymer solvents and nonsolvents and droplet shearing conditions. The early microencapsulation studies using EUDRAGIT RL™ and DMAEMA-MMA [reviewed in (244)] indicated that encapsulation of living cells within water insoluble polyacrylate polymers was feasible but that improvement of polymer properties were necessary in the areas of biocompatibility and permeability. The expected properties of HEMA-MMA, suggested its suitability for microencapsulation purposes. Modifications to the co-axial extrusion, interfacial precipitation process used for EUDRAGIT RL™ were
required to accommodate HEMA-MMA (237, 245). Microcapsule preparation using HEMA-MMA has been described in more detail elsewhere (246).

Large diameter microcapsules were prepared using a co-axial extrusion submerged jet, interfacial precipitation process (237). Microcapsules were produced as capsule droplets, consisting of the cellular core surrounded by the polymer solution, by pumping the HEMA-MMA/PEG-200 polymer solution and the mammalian cell suspension in their complete tissue culture medium, to the tip of a co-axial needle assembly. The polymer solution flowed through the outer needle and the cell suspension through the inner needle. Each capsule droplet was sheared from the needle assembly as its tip was withdrawn from the hexadecane overlayer and it then passed through the hexadecane overlayer into the PBS precipitation bath which contained 100 ppm of the Pluronic surfactant L101. The latter was added to facilitate the passage of the droplet through the hexadecane/PBS interface. In this precipitation bath, the polymer solvent was extracted, leaving behind a polymer wall surrounding the cellular core. Microcapsules produced by this process were spherical, opaque and uniform in their diameters of 750 - 900 μm.

A more recent development has been the submerged nozzle-liquid jet extrusion process, to produce HEMA-MMA microcapsules of even smaller diameter (300 - 600 μm) (85). In this process, the polymer solution and cell suspension are delivered to the tip of a co-axial needle assembly as above. However, the needle assembly remains stationary while the hexadecane (or dodecane) flows uniformly, co-axially to the needle assembly to
shear off each capsule droplet. It is this inherently higher capsule shearing force which produces the smaller capsules. The flow rate of hexadecane/dodecane affected both the diameter of the resultant capsules as well as their production rate.

Following the trend towards diminishing even further the stagnant water layer by reducing the microcapsule diameter (and at the same time, the membrane thickness), a conformal coating process that results in cellular aggregates (e.g., HepG2 cells of a diameter of < 500 µm) or islets of Langerhans that are coated with a very thin layer of HEMA-MMA (< 10 µm) is currently under development (86). Cellular aggregates in suspension are introduced at one end of the coating chamber and under the influence of centrifugal force, pass through three liquid layers of different densities (a polymer solution containing the coating polymer, a solvent layer and a non-solvent, polymer-precipitating layer), to produce conformally coated cellular aggregates at the other end.

A consequence of the biocompatibility of HEMA-MMA is its failure to support the attachment, spreading and growth of anchorage dependent cells. The non-adherent HEMA-MMA intracapsule environment was modified by coencapsulating cell attachment and growth substrates such as the commercially available Matrigel® (Collaborative Research, Bedford, MA) (93, 247) or hydrated cell-preloaded Cytodex beads (248) for anchorage-dependent cells or cell immobilization matrices such as agarose or chitosan (249). Modifications to the microencapsulation process described above were made to
accommodate these additives in the capsule core solution as noted in corresponding references.

1.1.5.1.2. Membrane Structure and Formation

The structures of small and large diameter HEMA-MMA capsules, prepared by either submerged jet process, were similar [Figure 1.3(a), 1.3(b), respectively]. The polymer walls were of an asymmetric morphology similar to ultrafiltration membranes (250, 251), but in a spherical geometry, instead of the typical hollow fibre or flat sheet geometry of the latter. The wall consisted of a thin outer skin, a macroporous sublayer, a thick seemingly dense layer and an inner skin [Figure 1.3(c)]. The capsule walls were ~150 and ~50 μm thick for large and small diameter capsules, respectively. Capsules were frequently eccentric, although this was diminished by the addition of the viscosity and density enhancer, Ficoll-400, to the capsule core solution (252).

Figure 1.3 (over) SEM micrographs of HEMA-MMA microcapsules: (a) large-diameter capsules (o.d., 660 μm); (b) small-diameter capsule (o.d., 450 μm); (c) wall structure of a large capsule. Magnification bars: (a) 100 μm, (b) 100 μm, (c) 25 μm. (from ref. 246)
Each region of the HEMA-MMA capsule wall has a particular function. The skin layers are expected to provide the selectivity to molecule permeation. The inner skin as well as an outer skin may provide a permeselective barrier even if mechanical or chemical change occurs at the outer skin upon implantation. The macroporous region is effective for the permeation of nutrients, cellular waste products and cell-derived biomolecules through the capsule polymer wall while it and the “dense” layer provide mechanical support. Capsules with well centered cores are expected to be mechanically more stable since weak spots can be avoided. These capsules would withstand any internal pressure exerted by proliferating cells or by a swollen immobilization matrix and protect cells more completely, upon their implantation, from cellular infiltration. Furthermore, these capsules would be tolerant of normal handling and stresses in vivo.

1.1.5.1.3. Microcapsule Permeability

Early measurements of HEMA-MMA capsule permeability, estimated by determining the time-dependent release of one of several molecules of different molecular weights into an extracapsular PBS sink from an aliquot of capsules, suggested a molecular weight cutoff for the membrane which was on the order of 100 kDa (237). Capsule permeability could be altered by changing the normal polymer precipitation conditions (e.g. precipitation bath, polymer solution composition) to modify the degree of macroporosity of the polymer wall (244). The primary resistance to diffusion was the skin layer. Permeability increased with the diameter of small capsules, presumably reflecting a
difference in rate of precipitation associated with the respective differences in diameter affecting wall structure, thickness and capsule eccentricity (253).

More recent capsule permeability studies examined the permeability of individual capsules, because of the observation of significant fibrinogen release from a subset of presumably defective capsules containing HepG2 cells (for details see below, 254). The broad distribution in fibrinogen release was consistent with the distribution in the permeability of similar capsules (without cells) to the model protein, horseradish peroxidase (HRP; $M_w$ 40 kDa). It is therefore probable that heterogeneity of capsules in the delivery of cell derived proteins was partly due to variability in the permeability of the capsules to proteins of similar molecular weight.

Control over microcapsule permeability was extended by replacing the polymer solvent, PEG-200, with triethylene glycol (TEG; $M_w$ 150 Da) which has a higher diffusivity to enhance the rate of solvent removal upon precipitation. Microcapsules prepared from a 9% (w/v) HEMA-MMA solution in TEG had a higher permeability than those prepared from a 10% solution (255). An increase in capsule permeability was also noted upon the addition of a pore forming additive poly(vinyl pyrrolidone) (PVP; $M_w$ 10000) to the 10% (w/v) HEMA-MMA in TEG solution at a ratio of 0.3 parts PVP to 1 part HEMA-MMA on a weight basis. This improvement in permeability was attributed to a significant increase in the apparent surface pore density in the presence of PVP. It remains to be seen how these changes affect the molecular weight cutoff.
1.1.5.2. In Vitro Performance of HEMA-MMA Microencapsulated Cells

A variety of cell types, including the Chinese Hamster Ovary (CHO) fibroblast (252), rat pheochromocytoma, PC12 (256), human hepatoma, HepG2 (93, 247), murine fibroblast, L929 (257) cell lines, primary rat hepatocytes (258) and primary rat islet tissue (259) and mouse fibroblasts (2A-50), genetically-engineered to secrete human growth hormone and β-glucuronidase (260), have been encapsulated within HEMA-MMA microcapsules. These studies have shown that the cells survive the encapsulation procedure, despite the exposure to shear forces and organic solvents/nonsolvents, and grow or function afterwards in vitro for periods from 2 to at least 6 weeks.

1.1.5.2.1. Cell Survival and Proliferation

The success of encapsulation is initially dependent on the number of viable cells that are entrapped within the capsule on an absolute basis or compared to the theoretical number of cells fed to each capsule - the encapsulation efficiency. It may be desirable in certain situations, such as when few cells are encapsulated, that cell proliferation occur within the microcapsule. Such proliferation also indicates a reasonable microenvironment within the capsule core for the cells. The rate of proliferation may, however, be lower than under normal tissue conditions in part since the environment inside the capsule and
the normal tissue culture dish are different. On the other hand, cells in an unproliferative state, may function in a more differentiated manner with enhanced production of the desired product. For other cell types, such as pancreatic islets, which do not proliferate in culture, the initial encapsulation efficiency must be high to provide adequate cell mass within the capsule.

Generally, only a fraction of the cells delivered to the needle assembly actually become enclosed by the polymer wall. For example, the encapsulation efficiency for HepG2 cells, suspended in medium augmented with 20% Ficoll-400, was ~20-25% (247) which increased to 50% with the co-encapsulation of Matrigel®. The encapsulation efficiency was also dependent on the sensitivity of the cells to the encapsulation process and the quality of the encapsulation run with better centered capsules having higher efficiencies as more cells were completely enclosed by the polymer wall (261).

Cellular proliferation within microcapsules has been examined directly by counting the number of cells from an aliquot of capsules and indirectly by examining the metabolic activity of cells within a single capsule using the MTT assay (262). PC12 (256) and HepG2 (247) cells proliferated within HEMA-MMA microcapsules to an extent which was dependent on the initial encapsulation density, hence, the number of cells per capsule. The results suggested that the intracapsular environment (without a matrix) could only maintain cellular proliferation and metabolic activity up to a certain extent (approximately 500 cells/capsule or 17 x 10⁶ cells/ml internal volume). Intracapsular space limitations
may control the number of viable cells per capsule. Alternatively, the arrangement of cells within the capsule core (e.g., spheroids in the nonadherent capsule environment, see below) may limit diffusion of nutrients and metabolites, such that more cells per capsule would have a lower metabolic activity per cell. In contrast, HepG2 cells, distributed in Matrigel®, an attachment substrate, within large and small diameter capsules, resulted in cellular proliferation to reach higher cell number values per capsule than in its absence but without a parallel increase in the cellular metabolic activity per capsule, over a 2-3 week period (85, 247). The relatively constant metabolic activity may indicate that the cells are correspondingly more differentiated while within Matrigel®.

The picture that is emerging is that cells grow to fill the capsule space but to the extent limited by the nutrient supply and intracapsule and intercellular diffusion gradients (see below). They then may maintain a roughly constant cell number and cell activity level thereafter, provided the intracapsule environment is suitable: e.g., anchorage dependent cells have the necessary attachment substrate. The rate of proliferation is likely less than that in normal tissue culture as is the rate of metabolic activity on a per cell basis (as exemplified by MTT conversion). How long this steady cell number/activity is maintained is not known but MTT conversion has been observed for several months after encapsulation. Interestingly, cells are not found outside of the capsule, except on rare occasions, suggesting the capsule wall is strong enough to limit proliferation to the intracapsular space. Cells that do not receive adequate nutrients or which are apoptotic, will die within the capsule. These products of necrosis remain proximal to other cells and
may affect their viability. Eventually, proteases released upon cell death will have sufficiently degraded the cellular debris such that it could be removed from the capsule core by diffusive transport. However, the cell cycle and cell death due to an inadequate nutrient supply are dynamic processes, implying that the capsule core would presumably never be free of cellular debris. The implications of this on cell viability and function in vitro and as shed antigens eliciting an immune and inflammatory response in vivo are not yet understood.

1.1.5.2.2. Protein Release

For the ultimate application of encapsulated cells as a bioartificial organ or physiologically controlled biomolecule delivery system, the cells must not only survive the encapsulation process and remain viable, but they must also express their differentiated functions. The ability of the HEMA-MMA microcapsule to support the functional state of the cells has been assessed by the quantification of encapsulated cell-derived biomolecule release into the extracapsular milieu.

As a protein release example, human hepatoma, HepG2, cells were used as a model for hepatocytes (247). Four plasma proteins, of various molecular weights, were released into the surrounding medium by HepG2 cells from an aliquot of large diameter Matrigel capsules, at a rate proportional to the increase in the number of cells per capsule. The amount of fibrinogen (Fbg) released, relative to \( \alpha_1 \)-acid glycoprotein (AG), was lower
for cells in large and small capsules, as compared to control unencapsulated cells within Matrigel® in tissue culture, consistent with a sieving effect of the polymer membrane on the higher molecular weight protein, Fbg, relative to the smaller AG (85, 247). However, more acid glycoprotein was secreted by HepG2 cells in small microcapsules than from large ones when compared on an equivalent core volume basis. This was attributed to a more efficient use of internal capsule volume and a lower degree of cellular necrosis at the centre of the core of the smaller capsules.

The release of fibrinogen, whose molecular weight was higher than that of molecules which would be expected to permeate the polymer membrane of capsules with a molecular weight cutoff of ~100 kDa, into the medium surrounding capsules containing HepG2 cells, was thought to have originated from a subset of these capsules which were defective. Defective capsules cause are those with poor capsule wall integrity due to thin areas, pinholes or tears. To test this hypothesis, protein secretion was quantified from individual capsules (254). Both AT and Fbg were secreted in variable amounts from capsules with a significant proportion of capsules from which no protein release was detected. This proportion of non-protein-releasing capsules decreased with time, presumably as cellular proliferation occurred within the capsules and a detectable level of protein was secreted by these cells. Furthermore, the proportion of non-protein-releasing capsules was higher for proteins of higher molecular weight. The variability in the amount of protein released from capsules containing HepG2 cells may be only partly explained by the permeability to proteins of that molecular weight. There may also be contributions to
this protein delivery variability due to a variability in the number of protein-secreting cells per capsule.

Glucose enhancement of insulin secretion, expressed as a stimulation index (the ratio of insulin secretion for high to low glucose), was similar for both encapsulated and unencapsulated control islets (259). However, the rates of insulin release were significantly lower (3.5 - 5 X) for encapsulated islets as compared to the control unencapsulated islets. This reduction was attributed to the capsule wall acting as a barrier to insulin diffusion since islets freed from within the capsule secreted insulin at rates similar to that of control islets. A high intracapsular insulin concentration was expected to downregulate insulin secretion by the encapsulated islets, exacerbating the diffusion barrier effect of the polymer wall. By reducing the stagnant water layer surrounding the islets, it is expected significant part of the diffusion resistance would be reduced with smaller capsules (85) or the conformally coated islets (86), both with thinner polymer membranes.

Mouse fibroblasts, engineered to secrete human growth hormone (Mw 48 kD, cell line LhGH-I) and β-glucuronidase (Mw~300 kD, cell line 2A-50) survived microencapsulation and proliferated within capsules (260). Protein release studies showed that neither β-glucuronidase (Mw ~ 300 kD) nor hexosaminidase (a constitutive product molecular weight, Mw 120 kD) could diffuse out of the capsule while human growth hormone (Mw 48 kD) was freely diffusible. Therefore, the capsules appeared to have a molecular weight cut off of <120 kD. The limitation to β-glucuronidase diffusion from
these capsules is in contrast to the release of fibrinogen (247) of a similar molecular weight, from capsules containing HepG2 cells. All proteins were present in the intracapsule (extracellular) space at similar levels as determined by breaking open the capsules and assaying the extracellular supernatant. A significant amount of growth hormone was retained in the intracapsular but extracellular space when Matrigel® was included in the encapsulation. The level of expression of the gene product accumulating in the intracellular fractions was not affected by the presence of Matrigel®. The presence of this extracellular matrix material did not appear to pose a physical barrier to the exit of the recombinant gene product as the rates of human growth hormone secretion from the microcapsules were similar in the presence and absence of Matrigel®.

1.1.5.2.3. Intracapsular Cell Behaviour

Diffusion limitations associated with the capsule (because of its wall and related to its size) affect not only the transport of products such as insulin but also the transport of metabolites (nutrients and waste products). The corresponding changes in the intracapsular environment (e.g., oxygen, nutrient or metabolite concentrations), and the presence of products from dying or dead cells further influence cell behaviour. These are expressed both through their morphology or three dimensional arrangement and protein secretion. The three dimensional arrangement further influences the transport limitations as the intracapsular or intercellular space may be rate-limiting. The changes in cell arrangement and morphology are illustrated by anchorage-dependent cells such as CHO.
cells (261) and HepG2 cells (93) and anchorage-independent PC12 (256) cells placed into the nonadherent environment of the HEMA-MMA capsule. These have been found to grow as spherical or elliptical aggregates, of varying sizes, within intracapsular polymer pockets. This was consistent with several reports on unencapsulated cell (e.g. hepatocyte) culture under non-adherent conditions (263). In the absence of a substrate for the cells to attach and grow as in a normal tissue culture situation, cells attach to each other or to cell-secreted extracellular matrix components such as laminin, fibronectin and collagen to arrange into a spheroidal shape (98). Large aggregates of HepG2 cells or PC12 cells (without coencapsulated Matrigel®) showed an outer shell, 2 to 5 cells in thickness (~30 μm) of viable cells and an inner necrotic core (see Chapter 2 for more details).

One consequence of the intracapsule microenvironment and cell morphology is the sensitivity of the cells to exogenous influences such as cytokines. Murine connective tissue L929 cells, when encapsulated, were unaffected by the cytotoxic effects (264) of tumour necrosis factor (TNF-α) (257). TNF-α (Mₘ - 51 kDa as a trimer), presumably permeated the capsule but the lack of a response by the normally TNF-sensitive L929 cells probably relates to effects of the capsule microenvironment such as the three dimensional arrangement of the cells. In the non-adherent environment of the HEMA-MMA capsule, the cells possess a different morphology from the elongated fibroblast morphology of the TNF-sensitive monolayer (265). Furthermore, the interaction of cellular receptors with extracellular matrix components is key to their response to cytokines (266). The TNF resistance of “tight” L929 colonies has been attributed to their inability to incorporate
secreted fibronectin in their extracellular matrix unlike TNF-sensitive "loose" colonies (267). It is also possible that environmental stresses of the capsule interior (e.g. nonadherence) affect L929 cells to the extent that they produce heat shock protein, hsp70 (268), which confers protection against the cytotoxic effects of TNF (269). In vitro tests of the effects of cytokines on encapsulated cells are especially useful since they may predict the response in vivo upon possible contact with these and other cytokines during an inflammatory reaction. Furthermore, the lack of sensitivity of L929 cells to TNF may reflect the impact of the high cell density and or low pO2 (270).

The environment within the HEMA-MMA capsule core may be altered by the coencapsulation of extracellular components such as a cell attachment substrate, Matrigel, or the coencapsulation of an immobilization matrix, such as agarose or chitosan. Whereas the former provides a substrate for cell attachment (which HEMA-MMA does not) the latter two may simply improve the distribution of cells within the capsule, and minimize intercellular diffusion limitations. Matrigel® has been shown to support hepatocyte and other cell differentiation to a degree beyond that reported for individual extracellular matrix components (102, 103, 271) and has been shown to support the growth and sustained NGF transgene expression by PAN-PVC macroencapsulated rat fibroblasts (35). Agarose has been used to enclose islets for their use in a bioartificial pancreas (134), as a means of preserving islet structure, mass, function for use in transplantation (272), preventing clumping of encapsulated islets (273) and has enhanced the growth and phenotypical expression of immobilized chondrocytes (274). From the perspective of cell
attachment, agarose is relatively inert. It remains to be seen what happens with anchorage
dependent cells or whether addition of extracellular matrix components such as laminin
can enhance beneficial effects of agarose even for anchorage dependent cells. Precipitated
chitosan, a cationic hydrogel, in the lumen of polyacrylonitrile-vinyl chloride (PAN-PVC)
macrocapsules provided a matrix for the attachment and spreading of anchorage-
dependent fibroblasts and of PC12 cells, and the neurite extension of the latter upon
exposure to NGF (275). On the other hand, microcapsules of triphosphate crosslinked
chitosan were unable to support PC12 cell or fibroblast viability and growth (275).
Presumably, the tightly crosslinked nature of the matrix may have mechanically inhibited
cell attachment and spreading and limited nutrient transport to encapsulated cells.

Both anchorage-dependent HepG2 cells (See Chapter 2, 247) or anchorage-
independent PC12 cells (249) encapsulated within a Matrigel® core, one day after
encapsulation, were viable and uniformly distributed throughout the capsule as individual
cells or small cellular aggregates (see Chapter 2 for more details). Within agarose
containing capsules, many more individual and small aggregates of viable PC12 cells were
distributed and even more uniformly than in Matrigel® containing capsules and a high cell
density was maintained for up to 21 days (249). Presumably because of the acidity of
the chitosan core solution, initially, chitosan-filled capsules contained primarily non-viable
PC12 cells and few viable PC12, uniformly distributed cells within the immobilization
matrix (249). Cellular proliferation was evident within these capsules from day 1 to day 7,
as cellular aggregates were observed at day 7 which originated from the viable cells
present at day 1. By 21 days, chitosan-filled capsules appeared to support the highest cell number as compared to Matrigel® or agarose filled capsules. The subsequent constant cell number and a latter increase in cell number may be attributed to a transient state of cellular adaptation and regeneration followed by a delayed enhanced growth period. This delayed cell growth response was attributed to biochemical interactions between chitosan and cell surface receptors.
1.2 Thesis Scope and Objectives

The primary focus of this thesis was to examine the in vivo behaviour of hydroxyethyl methacrylate-methylmethacrylate (HEMA-MMA) microcapsules for the transplantation of liver cells. Human hepatoma (HepG2) cells and rat hepatoma (H4IIEC3) cell lines were used as models for hepatocytes.

The HEMA-MMA capsule microenvironment is one that does not support the attachment of anchorage-dependent HepG2 cells but this was altered by the co-encapsulation of Matrigel®, a commercially available reconstituted extracellular matrix, to provide sites for the attachment. In the study presented in Chapter 2, the effects on HepG2 cells in these two capsule microenvironments, during in vitro culture, was assessed by examining their spatial arrangement, morphology and viability using cryosectioning and scanning electron microscopy techniques that preserved the polymer capsule.

The microcapsule implantation studies into rats, the main focus of the thesis, collectively examined the host response to the HEMA-MMA microcapsules, the effect of capsule implantation site, the effect of device components (pre-adsorbed serum proteins, and Matrigel®) and the effect of polyacrylate composition, on the tissue reaction to microcapsules. The most recent study to which all the other implantation studies lead, focused on the transplantation of rat (H4IIEC3) and human (HepG2) hepatoma cells in Wistar rats as models of allogeneic and xenogeneic transplantations and is the one
presented in this thesis document as Chapter 3. The transplanted encapsulated cell viability and morphology and the tissue reactions to these cell-containing capsules were examined in cryostat sections.

The characterization of HEMA-MMA microcapsules, which in the past has included membrane morphology and permeability, was furthered by examining aspects which are expected to affect the biocompatibility of these capsules \textit{in vivo}. The surface of microcapsules was characterized using x-ray photoelectron spectroscopy, presented in Chapter 4.1. Proteins, associated with microcapsules following their \textit{in vitro} incubation in medium containing human serum and implantation into the rat peritoneal cavity were examined using SDS-PAGE and immunoblotting (Chapter 4.2).

The work reported in this thesis has lead to an understanding of the issues of biocompatibility and the immunology of cell transplantation with and without immunoisolation as presented in Sections 1.1.2. Immunoisolation and the Immunology of Cell Transplantation.

While the specific aims of this thesis were to examine the host response to HEMA-MMA microcapsules and whether their implantation would facilitate cell transplantation, the objective was to initiate \textit{in vivo} studies with HEMA-MMA microcapsules and to identify issues for further study for the advancement of this and other immunoisolation technologies.
Chapter 2

HEMA-MMA Microencapsulated Cell Morphology In Vitro

2.1. Morphological Assessment Of Hepatoma Cells (HepG2) Microencapsulated In A HEMA-MMA Copolymer With And Without Matrigel®

ABSTRACT

Hepatoma cells (HepG2), an anchorage-dependent cell line, were microencapsulated in a HEMA-MMA polyacrylate membrane to which the cells do not adhere. This environment was altered by the co-encapsulation of Matrigel, a reconstituted extracellular matrix derived from the Engelbreth-Holm-Swarm (EHS) mouse tumour basement membrane, to provide sites for cell attachment. The effect on the cells of these two capsule microenvironments during a 2 week in vitro culture period was assessed by examining the spatial arrangement, morphology, and viability of the cells using light microscopy and scanning electron microscopy (SEM). In preparation for microscopy, dissolution of the polymer was prevented by the use of frozen sections embedded in a water soluble compound. Similarly, freeze cleavage of conductively stained capsules permitted SEM observation of the capsule interior along with ultrastructural detail of the cells. In the absence of Matrigel, cells in HEMA-MMA capsules were found to form

---

1 This work received the Student Research Award in the Undergraduate, Master Candidate, or Health Science Degree Candidate Category, and was presented at the Fourth World Biomaterials Congress (18th Annual Meeting of the Society for Biomaterials), in Berlin, Germany, April, 24-28, 1992. It was published in the Journal for Biomedical Materials, Vol. 26, 1401-1418 (1992).
aggregates in intracapsular pockets with central necrosis occurring at day 7 in large aggregates. The co-encapsulation of HepG2 cells with Matrigel, resulted in an initially uniform distribution of essentially individual cells with aggregates appearing later within the Matrigel. Many cells within these capsules had remained viable when examined up to day 14 with only limited cellular necrosis, implying a favourable environment for microencapsulated HepG2 cells.

INTRODUCTION

Microencapsulation of living secretory cells within a polymer membrane isolates them from the immune system and enables their use as an artificial organ upon transplantation. The most common method, which entraps cells within an alginate-polylysine capsule (107, 108), has been used to encapsulate Islets of Langerhans (276) and, with collagen added to the cell suspension, hepatocytes (15,16, 277). As an alternative to the alginate-polylysine system a HEMA-MMA copolymer has been used. This polymer was chosen for its expected biocompatibility, its water insolubility which ensures stability in an aqueous/physiological environment, and its high water content which favourably affects diffusion of aqueous solutes. Mammalian cells such as CHO fibroblasts, PC12 cells, hepatocytes, and aggregates thereof, and Islets of Langerhans have been encapsulated by a coaxial extrusion-interfacial precipitation process (237, 256,258, 278). The cells survive encapsulation even though they are exposed to organic solvent/non-solvents and shear forces (262).
For a functioning hybrid artificial organ, cells must, for a significant length of time, remain viable, maintain normal physiological functions, and express their differentiated characteristics while in contact with a biomaterial. The properties of the polymer play a key role in defining the environment of the cells. Due to the characteristic high water content of the HEMA-MMA capsule membrane, it is not expected that the polymer wall would act as a diffusion barrier to critical nutrients such as glucose and oxygen (237). However, HEMA-MMA and similar hydrogels are unable to support cellular attachment, primarily due to their hydrophilicity and low mechanical stiffness (279, 280, 281).

To study the effects of the HEMA-MMA capsule microenvironment on the behaviour of attachment-dependent cells, HepG2 cells were encapsulated as a model. HepG2 cells represent a human hepatoma cell line, display a cellular morphology similar to that of hepatocytes (282), are able to proliferate in culture and continue to express differentiated hepatocyte functions including synthesis of major plasma proteins (282, 283). They therefore may be employed as a model for hepatocytes, the latter being of interest for their use in the potential application of a bioartificial liver. For the purpose of altering the non-adherent environment of the HEMA-MMA capsule, Matrigel was co-encapsulated as an attachment substrate for HepG2 cells. Matrigel, a reconstituted basement membrane consisting of the components associated with the in vivo basement membrane including laminin, collagen type IV, and heparin sulfate proteoglycan (284), has been shown to support hepatocyte differentiation to a degree beyond that reported for individual extracellular matrix components (102, 103, 271, 285). DNA synthesis by hepatocytes on Matrigel® is comparatively lower.
than on collagen (103, 285). Its presence in the capsule creates a more suitable environment for cytotypic function of the microencapsulated cells.

MATERIALS AND METHODS

Cell Culture

The human hepatoma cell line, HepG2, (American Type Culture Collection, Rockville, MD) was routinely maintained in α-MEM (powdered medium, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 ng/ml streptomycin, at 37 C in a humidified atmosphere of 5% CO₂/95% air. The medium was replaced twice a week. For weekly subculture (10-fold dilution) as well as for preparation of cell suspension for microencapsulation, cells were recovered by trypsinization (0.25% trypsin-EDTA solution; Gibco; 10 min.).

Microencapsulation

HepG2 cells were microencapsulated using the apparatus and procedure previously reported (237). Briefly, microcapsules were prepared by pumping cell suspension and polymer solution through a coaxial needle assembly at 21 μl/min and 56 μl/min, respectively. The cell suspension consisted of HepG2 cells at a density of 5 x 10⁶ cells/ml in complete tissue culture medium augmented for encapsulation with 20% (w/v) Ficoll 400 (Sigma Chemical Company, St. Louis, MD) or in complete tissue culture medium containing Matrigel (50% v/v, 14.2 mg protein/ml initial Matrigel concentration; Collaborative Research Incorporated,
Bedford, MA). These capsule types will be referred to as regular and Matrigel capsules respectively. To facilitate the encapsulation of the Matrigel-cell suspension which is gelatinous at room temperature, the polyethylene 50 (PE50) tubing delivering this suspension was submerged in ice water. In this manner, a uniform distribution of the Matrigel-cell suspension among capsules was obtained (247). The polymer used for the preparation of capsules was a poly(hydroxyethyl methacrylate-methyl methacrylate) (poly(HEMA-MMA), 75% HEMA) synthesised as previously described (235), dissolved in polyethylene glycol 200(PEG-200) (10% w/v, BDH Chemicals, Toronto, Ontario).

The capsules were sheared at a hexadecane air interface and passed into a 250 ml flask containing a precipitation bath of calcium-containing (0.1 g/l CaCl$_2$) phosphate buffered saline (PBS) with Pluronic L101 surfactant [100 ppm (v/v); BASF Chemicals, Wyandotte, MI] which facilitated the passage of the microcapsules through the hexadecane-aqueous interface. After completion of coextrusion, the hexadecane overlayer was removed and the capsules were kept suspended by use of a magnetic stirrer in the precipitation bath for 30 min and then in fresh PBS for an additional 30 min. This permitted more complete curing of the polymer membrane. The capsules were then transferred into 100 mm diameter petri dishes (Corning, Corning, NY) and incubated in α-MEM. The capsule medium was exchanged twice a week.
Scanning Electron Microscopy (SEM)

Regular and Matrigel capsules were prepared for freeze cleavage and SEM examination following a modification of the procedure of De Boni (286). Briefly, microcapsules, washed with PBS (2 X 15 min) to remove serum proteins, were fixed [3.5% glutaraldehyde, EM grade; Polysciences, Warrington, PA; containing 2% (w/v) tannic acid in 0.1M Sorenson's phosphate buffer (P-buffer)] at various times after encapsulation. For processing, capsules were first washed (2 X 15 min.) with P-buffer and, to impart partial conductivity, were post-fixed for 1 h with osmium tetroxide (1% in P-buffer; JBS, St. Laurent, Quebec). Afterward, washed microcapsules were exposed to 2% (w/v) aqueous tannic acid for 1 hour. Tannic acid preserves fine structure of cells and aids in subsequent osmium reduction at its binding sites (287). Following another washing, the microcapsules were again post-fixed with 1% osmium tetroxide for 1 h and washed. They were then cryoprotected with 7.5% and 15% aqueous glycerol solutions, respectively (20 min each). For cleavage, individual microcapsules were placed into liquid nitrogen and fractured by use of a microscalpel under a dissecting microscope. Fractured microcapsules were then transferred to 15% and then to 7.5% aqueous glycerol solutions, respectively (20 min each), and washed with distilled water (2 X 15 min.). They were placed on dry filter paper and permitted to air dry overnight. Alternatively, a supplementary batch of Matrigel capsules were prepared without cryoprotection, distilled water washes being substituted, and freeze dried (48 h). Capsule fragments were mounted on aluminum stubs and sputter-coated with a minimal layer of gold, in an argon atmosphere at 100 mTorr. Samples were examined using a Hitachi S-570 scanning
electron microscope at an accelerating voltage of 20 kV. Three separately prepared batches each of regular and of Matrigel capsules respectively were examined at each time point post encapsulation.

Light Microscopy (LM)

For light microscopy, 10 to 12 regular or Matrigel capsules per post encapsulation time were fixed and cryoprotected as described above, but not cleaved. They were then washed with P-buffer (2 X 15 min) and 5 or 6 capsules frozen-embedded in OCT compound (10.24% w/w polyvinyl alcohol, 4.26% w/w polyethylene glycol, 85.5% non reactive ingredients; Miles Inc., Diagnostics Division, Elkhart, IN) within a plastic mold (Miles Inc., Diagnostics Division, Elkhart, IN) and stored on dry ice. Cryostat sections were cut at a 10 µm thickness, at -20 C, and placed on gelatin-chrome alum (0.1% (w/v) gelatin, 0.01% (w/v) chromic potassium sulphate)-treated slides. Sections were cleared of OCT embedding compound in water and then stained with aqueous toluidine blue (0.1% (w/v) and 0.05% (w/v) for regular and Matrigel capsules, respectively). Stained, air dried sections were mounted with Permount (Fisher Scientific Company, Fair Lawn, NJ) to improve cellular resolution. Sections of three separately prepared batches of regular and of Matrigel capsules were examined by LM at each post encapsulation time. The LM and SEM micrographs shown were obtained from the same, single batch of regular or Matrigel capsules. Cellular aggregate dimension ranges are given for aggregates of one batch of regular or Matrigel capsules as the diameter, for those of circular outline, and as the major and minor axes, for those of elliptical outline.
Cell Number per Histological Section

The number of intact cells per histological section was estimated by counting under a light microscope in sections prepared from 10 of both regular and Matrigel capsules 1 (i.e., ~24 h), 4, 7, and 14 days after encapsulation. The results were plotted as percentage of sections with a certain number of cells per section or less against the cell number per section.

RESULTS

Microcapsules

The present microencapsulation process resulted in microcapsules of ~700-800 μm diameter at day 7 postencapsulation. The capsule wall consisted of a thick inner microporous region, a thinner outer macroporous region (the former is four to five times as thick as the latter) and a dense skin on the outer and possibly as well, the inner capsule surface. Adding a density/viscosity enhancer (here 20% w/v Ficoll-400) to the cell suspending medium resulted in a well defined capsule core and a reduced eccentricity (wall thickness ranged from 50 to 150 μm) relative to that observed when tissue culture medium alone is used (252). This result may be attributed to less mixing of the more viscous core solution with the polymer solution during capsule formation. Part of the core (~20-50%) may be subdivided with thin (~10-60 μm)
polymer partitions resulting in separate intracapsular pockets, in addition to the main capsule core.

Microencapsulation with Matrigel® as the cell suspending medium resulted in capsules of the same sizes as regular capsules, with well defined but eccentric cores and wall of the same morphology as regular capsules. Matrigel® gels as the capsule forms, hence very little mixing with the polymer solution occurs and only a single core, without pockets of polymer, was observed.

A variation of this method may be used to produce smaller diameter capsules (300-400 μm) (85).

Regular Capsules

Microcapsules without Matrigel, 1 day in vitro, typically showed the presence of individual, spherically shaped cells, approximately 7 μm in diameter [Fig. 2.1(a), Fig. 2.2(a)] and small clusters of cells [Fig. 2.1(a)] lying close to the inner capsule wall in SEM and some

Figure 2.1 (over) Representative SEM micrographs of microcapsules containing HepG2 cells without Matrigel® at 1 day (a), 4 day (b,c), 7 day (d), and 14 day (e,f) after encapsulation. (a), (b), (e), and (f) show entire capsules whereas (c), and (d) show detail of cellular aggregates within capsules. Solid arrows identify cells within the capsule. Curved solid arrows identify the polymer capsule. (c) is a higher magnification of the aggregate shown in (b). (d) shows a cellular aggregate with an outer viable layer (solid arrow) and an inner necrotic region (open arrow). Magnification: bar=150 μm (a,b,e,f); bar=50 μm (c,d).
LM micrographs. In other LM micrographs, cells were found within intracapsular pockets, the latter consisting of polymer partitions inside the capsule core [Fig. 2.2(a)]. Some cell debris was visible in histological sections [Fig. 2.2(a)].

SEM and LM examination of regular microcapsules, 4 days in vitro, revealed the presence of numerous cellular aggregates, not completely filling their inner capsule space [Fig. 2.1(b), Fig. 2.2(b)]. SEM examination showed these aggregates to be spherical or elliptical in outline. The outer surface of such aggregates was composed of cells of spherical morphology [Fig. 2.1(c)], while cells within aggregates appeared tightly packed [Fig. 2.1(c), Fig. 2.2(b)]; all cells appeared viable. Spherical aggregate diameters ranged from approximately 40 μm to 190 μm while elliptically shaped aggregate axes ranged from approximately 30 μm X 20 μm to 200 μm X 160 μm.

At day 7 in vitro, numerous sites of cellular foci were observed within capsules with large aggregates contained within their own polymer pockets [Fig. 2.2(c)]. Large aggregates had an outer rim of viable cells and an inner necrotic core [Fig. 2.1(d), Fig. 2.2(d)]. The outer shell of viable cells, 2 to 5 cells in thickness (~30 μm), was composed of tightly-packed,

**Figure 2.2** (over) Representative light micrographs of sections of microcapsules containing HepG2 cells without Matrigel® at 1 day (a), 4 days (b), 7 days (c,d), and 14 days (e,f) after encapsulation. (c), and (e) show entire capsules whereas (a), (b), (d), and (f) show detail of cells within capsules. Large arrowhead in (c) identifies the capsule outline. Small closed arrow in (c) identifies cellular aggregate within capsule. (d) and (f) show cellular aggregates in (c) and (e) respectively at a higher magnification. In (d), a viable cell layer (open arrows), surrounds a necrotic core with pyknotic nuclei (solid arrow). Magnification: bar=150 μm (c,e); bar=50 μm (a,b,d,e).
polyhedral cells which were characterized in histological sections by differentially-staining nuclei and cytoplasm. An inner necrotic core was clearly distinguished by dark-staining, pyknotic nuclei and lightly-staining cytoplasmic debris. The diameter of spherical aggregates ranged from approximately 16 \( \mu \text{m} \) to 70 \( \mu \text{m} \); axes of elliptical aggregates ranged from 25 \( \mu \text{m} \times 15 \mu \text{m} \) to 300 \( \mu \text{m} \times 100 \mu \text{m} \).

Microcapsules without Matrigel, 14 days in vitro, showed two types of cellular arrangement. There were aggregates which were similar to those seen at day 7, composed of intact cells of a similar morphology, not completely filling their respective intracapsular space [Fig 2.1(e.')] and ranging in size from approximately 30 \( \mu \text{m} \times 20 \mu \text{m} \) to 250 \( \mu \text{m} \times 220 \mu \text{m} \). Often, in histological sections, a solid staining material was observed appearing to attach such aggregates to the inner polymer wall. Alternatively, and more frequently, cellular material was found in regions between polymer folds which were more extensive than seen previously [as shown in both Fig. 2.1(f), Fig. 2.2(e)]; these regions forming an irregular pattern. Detailed examination of such regions of cells [Fig. 2.2(f)], showed them to contain many pyknotic nuclei and some faintly-stained cells of a fibrous morphology. This fibrous cell morphology was typical of cells which completely filled an intracapsular pocket.
Matrigel Capsules

The core of Matrigel capsules consisted of a discrete mass of Matrigel in which cells were dispersed; no cells were observed outside the Matrigel [Fig. 2.3(a)-(c), Fig. 2.4(a)-(f)]. Matrigel was found to assume the shape of the inner capsule wall [Fig. 2.4(a)-(f)]. Matrigel capsules, 1 day in vitro, showed a uniform cell suspension within the Matrigel of many oval-shaped, viable cells with an average dimension of 13 μm X 9 μm as well as some small clusters with typical axes dimensions ranging from approximately 35 μm X 30 μm to 140 μm X 60 μm [Fig. 2.4(a)]. Very little cell debris was present at day 1 [Fig. 2.4(a)]. At day 4, cells were distributed throughout the Matrigel as small clusters which consisted of intact, and viable cells surrounded by cell-free Matrigel [Fig. 2.3(a), Fig. 2.4(b)]. Such cellular aggregates, often of elliptical outline, ranged in size from approximately 40 μm X 25 μm to 120 μm X 80 μm.

Figure 2.3 (over) Representative SEM micrographs of microcapsules containing HepG2 cells within Matrigel at 4 days (a), 7 days (b), and 14 days (c) after encapsulation. (a), (b), and (c) show the entire capsule containing uniformly distributed cells (closed arrows) within Matrigel (open arrows). Inserts show a higher magnification of areas within respective micrographs. Note, few necrotic cells at day 14 [closed arrows, (c) insert]. Magnification: bar=150 μm (a,b,c); bar=50 μm [(a), (b) inserts]; bar=25 μm ((c) insert).
Differences in cellular and Matrigel morphology were noted in some day 7 capsules. At this time, aggregates of polyhedral-shaped cells were contained within "solid" Matrigel while other regions were composed of fibrous strands of Matrigel and loosely-arranged cells [Fig. 2.4(c)]. In other capsules, many small aggregates of polyhedral- and oval-shaped cells were distributed throughout the Matrigel core [Fig. 2.3(b), Fig. 2.4(d)]. Axes of aggregates in Matrigel capsules 7 days in vitro, ranged from 30 μm X 25 μm to 110 μm X 80 μm.

Matrigel capsules, 14 days in vitro, typically showed Matrigel in close contact with the inner polymer wall [Fig. 2.4(e)], containing regions of many viable cells [Fig. 2.4(f)] of oval and polyhedral morphology. Although cellular regions and multiple aggregates were observed, Matrigel without cells was also noted [Fig. 2.3(c), Fig. 2.4(e)]. At day 14, small foci of necrotic cells were present, generally near the centre of the capsule [Fig. 2.4(e)]. Capsules containing areas with fibrous strands of Matrigel and loosely-arranged polyhedral cells, similar to those at day 7, were observed in histological sections of some capsules at day 14. Small to medium aggregates displayed axes dimensions ranging from approximately 25 μm X 20 μm to 145 μm X 115 μm.

Figure 2.4 (over) Representative light micrographs of sections of microcapsules containing HepG2 cells within Matrigel microcapsules at 1 day (a), 4 days (b), 7 days (c,d), 14 days (e,f) after encapsulation. (a), (b), and (c) show the entire capsule containing the Matrigel and cells. (d) and (e) show high magnification of the inner core of capsules. Large arrow in (a) identifies capsule. Cells and aggregates are indicated with a small solid arrow within the Matrigel (closed arrows). Viable and necrotic regions in (e) are indicated with curved open and closed arrows respectively. (f) shows a higher magnification of a portion of the viable region in (e). Magnification: bar=150 μm (a,b,c); bar=75 μm (d,e); bar=50 μm (f).
It was considered that air drying of capsule samples may have resulted in some shrinkage of the Matrigel. Hence, for one batch, freeze drying was used as an alternative method. The apparent volume occupied by Matrigel within capsules prepared by this method did not differ from air-dried Matrigel capsules, as determined by SEM examination, although, there was some decrease in the outer capsule diameter. Cryoprotection could not be used for freeze-dried samples (see Discussion) and hence unavoidable ice crystal damage was observed in Matrigel regions and in many cells. Typically however, cells close to the polymer wall were structurally intact [Fig. 2.5] and ultrastructural detail was readily observable: a round nucleus, the nucleolus, and cytoplasm free of vacuoles and containing several mitochondria with resolvable cristae.

**Cell Numbers per Histological Section**

Not surprisingly, there was a broad distribution of the number of cells per section for both capsule types. The average number of intact cells per histological of regular and Matrigel® capsules and a qualitative assessment of the amount of necrosis is shown in Table 2.1 for the various times examined. There is a general increase in cell number for both regular and Matrigel® capsules with time. The average number of cells per section is higher at day 14 in Matrigel® than regular capsules as a higher degree of necrosis is observed in the latter.

**Figure 2.5** (over) SEM micrograph of HepG2 cell within a Matrigel capsule, fractured through the centre of the cell. Note, nucleus (solid white arrow) with 2 nucleoli (open arrows), mitochondria (small solid arrows) with cristae. bar=4 μm.
Table 2.1 HepG2 Cell Viability In Vitro in Regular and Matrigel® Capsules

<table>
<thead>
<tr>
<th>Day</th>
<th>Regular Capsules</th>
<th>Matrigel® Capsules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average Number of Viable Cells/Section</td>
<td>Cell Debris */Necrosis</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>+ *</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>58</td>
<td>++</td>
</tr>
<tr>
<td>14</td>
<td>74</td>
<td>+++</td>
</tr>
</tbody>
</table>

Cell debris/necrosis based on their relative areas: (-) none, 0 %; (+) < 20 %; (++) 20 - 50 %; (+++) 50 - 100 %.

n values same as in Figure 2.6

The distribution curves of cell number per section for section for regular capsules [Fig. 2.6(a)], showed that capsules 1 day in vitro had a narrow distribution with only 10% of sections containing 26 to 78 cells per section while 50% of the sections containing more than 6 cells. By day 4 in vitro, the distribution curve broadened to include higher cell numbers. A slight shift in the median cell number per section towards higher values occurred subsequently with similar distribution curves. For days 7 and 14 in vitro, 10% of sections contained between 175 to 367 and 188 to 449 cells per section, respectively. Values were similar despite the increased degree of necrosis in capsules 14 days in vitro.

Figure 2.6 (over) Intact cell number per histological section distribution curves; days 1, 4, 7, and 14 in vitro for regular (a) and Matrigel (b) capsules. Vertical axis represents the percentage of sections with a certain number of cells/section or less. Data sets for days 1, 4, 7, and 14 are comprised of n=218, 255, 328, and 426 and of n=87, 72, 88, and 191 histological sections from regular and Matrigel capsules respectively.
The distributions curves of cell number per section for Matrigel capsules [Fig. 2.6(b)], showed that at day 4, the values of the cell number/section were similar to those at day 1, indicating that significant changes in cell number had not occurred. At day 7 and again at day 14, higher cell numbers per section were observed relative to Matrigel capsules day 4 in vitro as well as to regular capsules at days 7, and 14 in vitro. Specifically, at day 14, 50% of the sections contained more than 181 cells; 10% of sections contained between 369 and 578 cells per section.

DISCUSSION

Microscopy Methodological Aspects

The microscopic techniques applied herein to fulfilled two key criteria: the cells were well preserved and the polymer capsule remained intact. Conventionally, graded ethanol dehydrations are used to displace water from biological samples prior to Epoxy embedding or critical point drying; paraffin embedding requires the use of xylene. Ethanol and xylene dissolve the polymer. For Epoxy embedding, infiltration with Epoxy miscible chemicals such as propylene oxide is necessary which was expected to disturb the capsule structure (288 ). It was therefore necessary to use cryosectioning of OCT (water soluble) embedded specimens for light microscopy and freeze cleavage of conductively stained capsules for electron microscopy.

For preservation of cellular morphology during sample preparation, cryoprotection was necessary to prevent ice crystal damage. Aqueous glycerol was chosen, rather than other
standard cryoprotectants such as DMSO, which itself is known to dissolve the polymer. The minimal effect of aqueous glycerol such as swelling of the polymer due to changed ionic strength, was considered tolerable. Air drying was employed for SEM preparation with the associated negative impact of surface tension effects being tolerated since freeze drying without a cryoprotectant damaged the cells. During freeze drying, a cryoprotectant would have been retained by the specimen because of its low vapour pressure, thus obscuring surface details and contaminating the column of the microscope (289) and hence could not be used.

For SEM sample preparation, osmium tetroxide treatment was chosen as it is particularly effective when used in conjunction with tannic acid; the latter provides more reducing agent for osmium reduction and deposition. Osmium stained material is partially conductive, diminishing charging effects during SEM analysis and thus requiring only a thin layer of sputtered gold, allowing high resolution of ultrastructural detail (eg. Fig. 2.4). For light microscopy, aqueous toluidine blue provided staining of nucleic acids and some proteins, permitting a rapid and effective method to localize cells and to generally assess capsule and cell morphology.

Cells within the HEMA-MMA Microcapsule Environment

The encapsulation of HepG2 cells within a HEMA-MMA microcapsule in the absence of Matrigel places anchorage-dependent cells within an environment unsuitable for cellular attachment. This resulted in the formation of cellular aggregates (eg. [Fig. 2.2(d)]), as
graphically represented in cell number per section distribution curves [Fig. 2.6(a)]. Although, in some sections high cell numbers are observed, the median is low, indicating localized aggregation and limited proliferation. This is supported by observations which show that neonate rat liver cells form spheroidal aggregates when cultured on poly-(HEMA) coated dishes (98, 290).

The observation of an outer viable cell region and an inner necrotic core in large aggregates of HepG2 cells in 7 day microcapsules without Matrigel, may reflect the presence of gradients of critical nutrients such as glucose and oxygen, growth factors, and hormones in one direction and gradients of metabolic wastes such as lactate and carbon dioxide and products of necrosis in the opposite direction. This has been described for tumour spheroids (95). In tumour spheroids, the viable cell layer is comprised of both proliferating and quiescent populations of cells (95). At a critical size the flux of growth and viability promoters into the centre of the spheroid becomes restricted and central necrosis develops (95). Neonate (98) and adult (290) rat liver cells, cultured as unencapsulated spheroids, have been shown to reach diameters ranging from 150 to 175 µm and 50 to 200 µm, respectively, not showing central necrosis within the 14-day culture period examined. On the other hand, thyroid epithelial cells, grown as multicellular spheroids, exhibited a central necrotic core in all cell spheroids which grew to exceed a diameter of 250-300 µm (291). These results are presumably a reflection of cell-related differences in metabolism and/or transport kinetics.
In a recent study by Takabatake et al. (292), preformed hepatocyte multicellular spheroids, approximately 120 μm in diameter, were encapsulated using alginate-polylysine system without added collagen. However, no necrotic cores were observed in these aggregates, only cell debris on their outer surface after 28 days of stationary culture or after 64 h of circulating culture. Sections of encapsulated spheroids in stationary culture, however, were not shown. SEM examination of alginate polylysine encapsulated hepatocytes revealed round or elongated essentially individual cells, as seen in this study in day-1 regular capsules, which were in close association with the coencapsulated collagen (15).

The encapsulation of HepG2 cells with Matrigel, within the HEMA-MMA capsule, provided for favourable attachment of the cells such that they remained viable, up to 14 days. Cells also maintained over time, the oval and polyhedral morphology observed at day 1. This oval cell morphology in Matrigel capsules, in contrast to a spherical cell morphology, at day 1 in regular capsules, may indicate the benefits of Matrigel. There appears to be a greater number of cells per section in Matrigel® as compared to regular capsules (Table 2.1), however, direct counting of total capsule cell number in these capsules 1 day after encapsulation has shown no statistical difference between the two (247). The cell numbers per capsule obtained by the direct counting of a pool of 20 capsules, shows a statistically significant increase from day 1 to day 14 (p<0.05) (247). Similarly, the cell number per section distribution curves show higher values at day 14 than day 1. It is not possible, however, to translate cell number per section values to a total cell number per capsule, since complete serial sections were not obtained. This suggests, nonetheless, that within at least some capsules, proliferation has
occurred. A comparison of the cell number distribution curves of regular and Matrigel capsules shows higher values in the latter at least at days 7 and 14, implying a preference for the Matrigel environment.

The increased cell viability in the presence of Matrigel may be related to the manner of cellular arrangement and capsule size. The uniform distribution of individual cells and aggregates thereof throughout the Matrigel during the culture time as opposed to a few larger aggregates in regular capsules may aid in maintaining viability. The local consumption of nutrients and production of waste products would likely be lower in these smaller aggregates. It is noteworthy that even though cellular aggregates were observed within Matrigel capsules, central necrosis was not observed, even within the larger aggregates at day 7, at which time aggregates in capsules without Matrigel, showed incidence of central necrosis. Rather, only a limited amount of cellular necrosis was found centrally within the capsule. Presumably Matrigel, by providing alternate sites for attachment, may limit cell-cell association, affecting the local cell density of the aggregates, as well as affect the metabolic state of the cells. The necrosis observed at the centre of the Matrigel capsules may be a result of the distance required for the diffusion of solutes to the centre of the capsule. A decrease in capsule size, to 300 - 400 μm from the 650 - 750 μm used in this study, would be expected to diminish the small amount of remaining necrosis.

These two types of microenvironments, with and without Matrigel, have also been examined for the in vitro maintenance of hepatocytes (98, 103, 271, 284, 285, 290, 293).
While hepatocyte differentiation in culture has been maintained by contact with Matrigel (271, 284, 285, 290), culture of hepatocytes in a nonadherent environment results in a three dimensional spheroidal arrangement resembling the *in vivo* situation (98, 290, 293). In this arrangement, cells proliferated less but exhibited high albumin secretion (290, 293). HepG2 cells, serving as a model for hepatocytes, may be used further to examine similar effects of the manner of cell encapsulation on the differentiated state of encapsulated cells by examining liver-specific characteristics such as protein synthesis.

CONCLUSION

The microscopic techniques described here permitted the observation, *in situ*, of preserved cells in structurally intact HEMA-MMA microcapsules as a function of time. The non-adherent environment of the HEMA-MMA microcapsule resulted in the formation of cellular aggregates by the anchorage-dependent HepG2 cells. The larger aggregates displayed a necrotic core at day 7, probably resulting from diffusional limitations implicit in such aggregates. The co-encapsulation of Matrigel with cells sufficiently altered the microenvironment to provide sites for cellular attachment, and a larger fraction of viable HepG2 cells up to 14 days *in vitro*. This indicates that the latter may be the preferred manner for their encapsulation.

These microscopic techniques, which permit *in situ* observation of cell morphology without disturbing the polymer, will enable further study of the detailed arrangement and
interaction between cells and biomaterial substrates. Such morphological studies may help in understanding how the local microenvironment in the capsule and near the biomaterial wall affects cell behaviour.
Chapter 3

HEMA-MMA Microencapsulated Cell Morphology in Vivo

3.1. Transplantation of Hepatoma Cell Lines into Rats Within Small Diameter HEMA-MMA Microcapsules

ABSTRACT

Small diameter hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA; 75% HEMA) microcapsules, containing an aggregate of viable rat hepatoma, H4IIEC3, cells within each core, after implantation into an omental pouch in non-immunosuppressed Wistar rats (a model of allogeneic transplantation), contained viable cells at 7 days but not 14 days post implantation. However, a similar transplantation of microencapsulated aggregates of human hepatoma, HepG2, cells (a model of xenogeneic transplantation) did not result in viable cells even at 7 days. Death of encapsulated cells, as aggregates in the absence of exogenous extracellular matrix, occurred in vitro, as a baseline to changes observed upon implantation, although, viable cells were observed after 21 days (controls for 14 days in vivo). The tissue reactions to microcapsules containing rat or human hepatoma cells, at day 1, was one cell layer thick and avascular (within omental tissue). At later times, the tissue reactions were comprised of three regions: macrophages, fibroblasts and some foreign body giant cells apposed to the polymer membrane, a fibrous

---

2 This work will be presented at The Cell Transplantation Society Third International Congress, Miami Beach, USA, Sept. 29 - Oct. 2, 1996.
region containing immune and inflammatory cells and a region of vascularized granulation tissue. Prompt vascularization of the tissue surrounding microcapsules occurred after 4 days \textit{in vivo} and was maintained for up to 14 days. Furthermore, for capsules embedded in omental tissue, the closest distance of blood vessels to the polymer/tissue interface did not change with time past day 4. The cellular profile of the tissue reactions were similar around microcapsules containing rat or human hepatoma cells, days 1 and 4 \textit{in vivo}. Even at 14 days, immune cells such as lymphocytes, plasma cells, eosinophils and mast cells were observed, presumably indicating that an immune response was still in progress towards antigens shed from the encapsulated cells. Differences in the tissue reaction associated with microcapsules containing human hepatoma as compared to rat hepatoma cells were a shift to include larger thickness values and significantly more eosinophils, in the former case. Correspondingly, the ongoing, intense immune/inflammatory response to microcapsules containing human hepatoma, HepG2, cells, resulted in the maintenance of a granulomatous tissue response and a higher degree of vascularization. The results of these microcapsule implantation studies suggested a relationship between the viability of microencapsulated cells and the degree of vascularization of the surrounding tissue reaction.

\textbf{INTRODUCTION}

Microencapsulation of mammalian cells is proposed as a means for cell delivery to provide a source of therapeutic biomolecules, the absence of which cause a disease state.
The success of cell transplantation is determined by the prolonged maintenance of cell viability and expression of differentiated functions within the implanted capsule. This functional success of a cell-containing implant is dependent in part on the absence or minimization of tissue reaction and fibrous capsule formation upon implantation. Alternatively, vascularization of the tissue reaction to these microcapsules would provide for enhanced delivery of nutrients to sustain cells, the systemic delivery of the secreted product(s) and the removal of cellular metabolic waste products.

The goal of microencapsulation is that the transplanted cells be ignored by the immune system of the host. Originally, immunoisolation was to physically separate allogeneic or xenogeneic tissue from the host immune system by preventing contact with immunoglobulin, complement components and immune and inflammatory cells. In this way, the membrane would prevent an immune response originating from the outside of the capsule acting on cells inside the capsule. However, an immune response is also possible towards antigens shed by encapsulated cells may be alloantigens, xenoantigens, tumour specific antigens such as foreign proteins secreted by the cells including the therapeutic agent, cell surface molecules (e.g., MHC molecules), or cell components released upon cell death that then diffuse through the polymer membrane to be recognized as foreign by the host’s immune system. Degradation of biological capsule components such as an extracellular matrix for cell attachment could also generate immunologic or proinflammatory products. An immunological reaction against antigens shed from
encapsulated cell surface may be produced via indirect antigen presentation if these soluble shed antigens permeate through the polymer wall.

To explore the feasibility of cell transplantation within HEMA-MMA microcapsules and any differences between allogeneic and xenogeneic model cells, encapsulated cell viability, morphology and the tissue response to cell-containing capsules was assessed after their implantation into the omental pouch site in Wistar rats. HepG2 cells, used here as the model cell for xenogeneic transplantation, are a human hepatoma cell line, which display a cellular morphology similar to that of hepatocytes (282). They are able to proliferate in culture and continue to express differentiated hepatocyte functions including the synthesis of major plasma proteins (282, 283). Previously, it was demonstrated that HepG2 cells survived entrapment within large (93, 247) and small diameter (85) HEMA-MMA capsules, and were viable for up to 3 weeks when coencapsulated with the cell attachment substrate, Matrigel® (85, 93, 247), during which time they secreted plasma proteins (85, 247), and were responsive to a physiological protein release stimulus (247). H4IIEC3 cells are a well differentiated rat hepatoma cell line (294, 295, 296, 297, 298, 299), derived from ACI rats (300, 301). H4IIEC3 cells are a suitable model cell for allograft transplantation since their major histocompatibility complex allele is RT1k which is different from the RT1k allele of the Wistar rat recipients. However, since these hepatomas are transformed, cultured cell lines, they are used as model cells to assess the encapsulation device in vivo for cell transplantation and are not true allografts and xenografts. Earlier implantation studies of
HepG2 cells within large diameter HEMA-MMA capsules into Wistar rats showed that the tissue reaction to capsules was thin and well vascularized in the omental tissue, indicating the omental pouch as the preferred site for capsule implantation (302). Because of differences in the rejection mechanism of allogeneic and xenogeneic cells, it was expected that microencapsulated model allogeneic cells would remain viable longer than would model xenogeneic cells and that rejection mechanism differences would be apparent in the tissue reaction to these microcapsules (e.g., thickness and the immune and inflammatory cell profile).

**MATERIALS AND METHODS**

**Cell Culture**

The human hepatoma cell line, HepG2 [American Type Culture Collection (ATCC), Rockville, MD], was routinely maintained in α-MEM (powdered medium, Gibco, Grand Island, NY, prepared at the Tissue Culture Medium Preparation Facility, University of Toronto, Toronto, Ontario) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 ng/ml streptomycin. The rat hepatoma cell line, H4IEC3, (ATCC) was routinely maintained in Dulbecco’s Minimum Essential medium (Gibco, prepared at the Tissue Culture Medium Preparation Facility, University of Toronto, Toronto, Ontario) supplemented with 10% heat inactivated horse serum (Gibco) and 5% heat inactivated fetal bovine serum (56 °C, 30 min.), 100 U/ml penicillin and 100 ng/ml
streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂/95% air and 10% CO₂/90% air for HepG2 and H4IIEC3 cells, respectively. The medium was replaced twice a week for Hep G2 cells, and every other day for H4IIEC3 cells. For weekly subculture (1:10 dilution and 1:7 dilution for HepG2 and H4IIEC3 cells, respectively), as well as for preparation of cell suspension for microencapsulation, cells were recovered by trypsinization (0.25% trypsin-EDTA solution; Gibco; 10 min. and 5 min. for HepG2 and H4IIEC3 cells, respectively) Furthermore, to minimize serum transfer with the cell suspension for microencapsulation, the centrifuged cell pellet was suspended in Hormonally Defined Medium [PC-1™ Low Protein Serum-Free Liquid base medium containing PC-1™ Sterile Supplement (Hycor Biomedical Inc., Irvine, CA, USA), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 ng/ml streptomycin (Gibco)] before being suspended in the core solution.

Animals

Male Wistar rats (175-200 g) (Charles River Canada Inc., St. Constant, Quebec) were used as the recipients. Animals were housed and cared for at the Division of Comparative Medicine, University of Toronto, under conditions of controlled temperature (25 °C) and 12 h light-dark schedule. Food and water were available ad libitum. The experimental protocol was approved by the Animal Care Committee, University of Toronto.
Microencapsulation

HEMA-MMA microcapsules, ~ 400 μm in diameter, were prepared by the submerged nozzle - liquid jet extrusion process as described elsewhere (85). The polymer solution was 10% (w/v) poly(hydroxethyl methacrylate-methyl methacrylate) [poly(HEMA-MMA), 75% HEMA] copolymer, dissolved in polyethylene glycol 200 (PEG-200) (BDH Chemicals, Toronto, Ontario). The capsule core solutions consisted of HepG2 cells or H4IIEC3 cells suspended at 5 X 10^6 cells/mL in 10% (w/v) Ficoll-400 (Sigma Chemical Company, St. Louis, MO) dissolved in Hormonally Defined Medium. The cell suspension flowrate was 5.6 μL/min and the polymer solution flowrate was 12.5 μL/min. The hexadecane flowrate of 140 mL/min produced capsules at a rate of 60-64 capsules/min. The liquid capsule droplets were extruded for 1 hour into a precipitation bath of phosphate buffered saline (PBS Ca^{2+}/Mg^{2+}-free: 0.2 g/L KCl; 0.2 g/L KH_2PO_4; 8 g/L NaCl; 2.16 g/L Na_2HPO_4·H_2O; Gibco) containing 100 ppm of Pluronic® L101 nonionic surfactant (BASF Chemicals, Wyandotte, MI) at room temperature. The capsules were then washed twice with fresh PBS for 20 minutes while being suspended by a magnetic stirrer. Capsules were transferred with sterile plastic transfer pipettes to petri dishes and 20 mL of Hormonally Defined Medium was added and encapsulated cells maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 8 days before implantation. Medium was changed on the day following capsule preparation and again on day 4. On the day before capsule implantation, the metabolic activity of cells within
representative capsules was assessed using the MTT assay using the method previously described (262).

**Microcapsule Implantation**

For implantation, capsules containing cells were selected using a dissecting microscope in a sterile flow hood. Flawed capsules with cells protruding through the capsule wall, very thin polymer walls or visible tears or holes were discarded. For implantation, an aliquot of 200 capsules were placed into a petri dish containing fresh Hormonally Defined Medium and washed with PBS (3 times for a total time of 15 min.) and implanted into recipient rats in omental pouches (200 capsules/pouch/rat) for 1, 4, 7, and 14 days (3 rats per day per cell type).

In preparation for surgery, each rat was placed into a bell jar containing Ethrane USP (Abbott Laboratories Limited, Montreal, Quebec) vapour until unconscious. Anaesthetic was maintained by inhalation of Ethrane [4-5% (v/v) in air, i.e., 5 setting on vaporizer tank with an O₂ flowrate of 500 ccs/min.] through a mask, and the ventral area was shaved and wiped with a 70% isopropanol solution (Regal Pharmaceuticals, Burlington, Ontario). The omental pouch [Fig. 3.1] was prepared by folding the omentum up towards the stomach and suturing (7'o' silk sutures; black braided, laser-drilled needle,

![Figure 3.1](image-url) (over) Schematic of omental pouch in the rat.
Omental Pouch

Pancreas
Stomach
Omental Pouch
taper BV-1, Ethalloy, 45 cm, Ethicon, Johnson & Johnson Medical Products, Somerville, NJ) along the left and right edges of the omentum and along the top of the pouch but leaving an opening for the placement of the capsules. Individual capsules, suspended in PBS, were placed into the omental pouch using a sterile custom made glass pasture pipette with a flame-polished tip. Aggregates of capsules were lifted out of the petri dish with tweezers and placed into the pouch. After capsule implantation, the opening was sutured closed to completely enclose the implanted capsules in omental tissue. The muscle layer of the surgical incision was closed with 6'o' silk sutures (black braided, TE-1, 0.7 m, Davis + Geck, Markham, Ontario), the skin layer sutured with 4'o' silk sutures (cardiopoint, black braided, CV-301, 1.5 m, Davis + Geck) and the incision coated with flexible colloidon (Mallinckrodt, U.S.P. UN 2059). Similar capsules from the same batches were maintained in vitro for the duration of these implantations and fixed for light microscopy.

Microcapsule Recovery

At the end of the implantation period, the rats were anaesthetized with injections of Atravet acepromazine maliate (0.1 ml/100 g of rat; 1mg/ml Ayerst Laboratories, Montreal, Quebec) and Rogarsetic ketamine hydrochloride (0.1 mg/100 g of rat; 115.4 mg/ml, Vetrepharm Canada Inc., London, Ontario). Rats typically weighed ~300 g at the end of a 2 week implantation period. The ventral area was shaved and wiped with isopropanol solution. In a sterile laminar flow hood, the peritoneal cavity was exposed.
The capsules were recovered within the omental tissue into which they had been placed. To retrieve any capsules which had fallen out of the omental pouch and were free floating or attached to other tissue within the peritoneal cavity, the entire peritoneal cavity was gently examined, removing capsules along with any associated tissue or free floating capsules. The location of these capsules was noted. Capsules and associated tissue were washed with PBS and then fixed [3.5% (v/v) glutaraldehyde, EM grade; Polysciences, Warrington, PA; containing 2% (w/v) tannic acid in 0.1M Sorenson's phosphate buffer (P-buffer)].

**Light Microscopy (LM)**

Tissue samples containing capsules and capsules maintained *in vitro* were prepared for light microscopy as previously described (Chapter 2.1) with modifications as noted. Briefly, fixed samples were washed with PBS (2 x 15 min.) and *in vivo* samples were cryoprotected with 30% (w/v) sucrose in PBS (overnight at 4 °C) or *in vitro* samples were cryoprotected with 7.5% and 15% aqueous glycerol solutions (20 min. each, respectively). Samples were then frozen embedded in OCT compound (Miles Inc., Diagnostics Division, Elkhart, IN) within a plastic mold (Miles Inc., Diagnostics Division). Serial cryostat sections (5-8 μm in thickness), cut at -20 °C, were cleared of OCT in water and then stained with either aqueous toluidine blue [0.1 % (w/v) in distilled water, BDH Chemicals, Toronto, Ontario], Masson trichrome (Sigma Chemical Company) or Harris haemotoxylin and alcoholic eosin (Sigma Chemical Company).
The Ca\(^{2+}\) ion of calcium salt deposits (eg. phosphate, carbonate, sulphate, urate) were stained according to the von Kossa method (303) in two representative tissue sections containing capsules, for each implant. Cell nuclei were counterstained with 0.1% (w/v) nuclear fast red (BDH Chemicals) in 5% (w/v) aluminum sulfate in distilled water.

Morphometric Analysis

Cryostat sections of implanted capsules and capsules maintained in vitro were examined on a Zeiss Axiovert 135 inverted epifluorescent microscope (Zeiss, Oberkochen, Germany). Morphometric analysis of the tissue reaction to capsules and the encapsulated cell morphology was performed with Image 1 image analysis software (Version 4.0, Universal Imaging Corporation, West Chester, PN) connected to the Axiovert microscope via a 3CCD Video Camera (Model DXC-930, Sony). Photomicrographs were taken using a Contax 167MT camera (Kyocera Corporation, Tokyo, Japan). The number of capsules forming each sample set was estimated by counting the number of different capsules from which, serial sections had been cut of the entire sample. The thickness of the tissue reaction was measured, using the image analysis point-to-point measurement function, at four equi-distant points around the capsule at points where the sections were intact and for capsule sections as close to the equator of the capsule (largest capsule diameter); the average was recorded for a cell type and implant duration for all rats. The number of blood vessels, within 250 \(\mu m\) of the polymer/tissue interface in the associated tissue
reaction and surrounding endogenous tissue, were visually counted (40X objective) and the degree of vascularization graded accordingly. The distances of the closest blood vessels were measured and the average and standard deviation determined therefrom. The percent contribution of fibroblasts, macrophages, lymphocytes, plasma cells, eosinophils, mast cells, foreign body giant cells, and neutrophils to the total cellular profile of the capsule tissue reaction was assessed using a pathology grading scheme. The degree of fibrosis of the tissue reaction was graded according to the percentage of the tissue reaction area occupied by the fibrous tissue comprised primarily of fibroblasts and collagen. The degree of inflammatory and immune response of the tissue reaction was graded according to the percentage of the tissue reaction made up of macrophages, lymphocytes, plasma cells, foreign body giant cells, eosinophils, mast cells and neutrophils, according to the cellular profile.

Cellular aggregate dimension ranges are given for aggregates within capsules as the major and minor axes, for those of elliptical outline. In these same samples, the number of viable cells within each aggregate of each capsule was counted and averaged for all capsules containing cells. The encapsulated cell viability was graded according to the overall percentage of viable cells within the core of all capsules of the sample.
RESULTS

Microencapsulated Hepatoma Cells *In Vitro*

The viability of encapsulated hepatoma cells before microcapsule implantation was confirmed by the MTT assay as a measure of cellular metabolic activity. Encapsulated H4IIEC3 cells gave a formazan absorbance per capsule (mean ± S.D.) of 35 ± 11 (n=8 capsules) and encapsulated HepG2 cells gave a formazan absorbance per capsule of 47 ± 28 (n=8 capsules); blank capsules gave a significantly lower formazan absorbance per capsule of 3 ± 2 (n=8 capsules).

LM micrographs of microcapsules, 8 days *in vitro*, (prior to implantation) similar to those implanted, contained viable rat hepatoma [Fig. 3.2(a)] or human hepatoma [Fig. 3.3(a)] cells arranged as cellular aggregates within the capsule core. At 21 days *in vitro* (corresponding to 14 days *in vivo*), microencapsulated aggregates (100 - 300 μm) of rat hepatoma [Fig. 3.2(b)] and human hepatoma [Fig. 3.3(b)] cells were comprised of viable cells of oval and polyhedral morphology with prominent nuclei and lightly-staining cytoplasm within well-defined cell membranes, although some cellular degeneration and necrosis was observed at the core of larger aggregates [Fig. 3.3(a), Fig. 3.2(b), Fig. 3.3(c)]. Encapsulated cell death, in the absence of exogenous extracellular matrix, occurred *in vitro*, as a baseline to changes observed upon capsule implantation.
Figure 3.2 (over) Representative micrographs of microcapsules containing rat heptoma, H4IIEC3, cells (a) after 8 days and (b) after 21 days, in vitro. Between large arrows in (a) is the polymer membrane forming the capsule outline. Large arrow head identifies cellular aggregate in (a). Note that viable cells are identified in (a) and (b) with a large solid arrow in capsules after 21 days in vitro. Necrotic rat hepatoma cells are identified in (b) with a small solid arrow. Magnification: bar=50 μm (a, b). Toluidine blue staining.

Figure 3.3 (over) Representative micrographs of microcapsules containing human heptoma, HepG2, cells (a) after 8 days and (b) after 21 days, in vitro. Large solid arrows in (a) and (b) identify viable cells within the cellular aggregate (arrow head); small solid arrows in (a) and (c) identify necrotic cells. Note that viable cells are identified after 21 days in vitro (b). Also note fibrous and degenerating cellular morphology in (c). Magnification: bar=50 μm (a, b, c). Staining: Toluidine blue.

Table 3.1 Microencapsulated Hepatoma Cell Viability In Vitro And In Vivo

<table>
<thead>
<tr>
<th>Day</th>
<th>Rat H4IIEC3 Cells</th>
<th>Human HepG2 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In Vitro In Vivo</td>
<td>In Vitro In Vivo</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>+++</td>
</tr>
<tr>
<td>14</td>
<td>7</td>
<td>+++</td>
</tr>
<tr>
<td>21</td>
<td>14</td>
<td>++</td>
</tr>
</tbody>
</table>

Cell viability scored based on the percentage of encapsulated cells that are viable: (-) none; (+) 0-25%; (+++) 25-50%; (++++) 50-100%

m=3 rats per sample time per cell type.

* m=2 rats
Microencapsulated rat hepatoma, H4IIIEC3, and human hepatoma, HepG2, cell viability in vitro is summarized in Table 3.1 for the various times examined.

Hepatoma cells were microencapsulated without an extracellular matrix, such as Matrigel® which was shown previously to beneficially affect cell viability (93), since coencapsulated Matrigel elicited a thicker tissue reaction in the rat than capsules without a Matrigel® core (304), presumably due to the "release" of mouse proteins (305). While there is no equivalent rat-derived reconstituted basement membrane formulation commercially available, rat Type I collagen is available. However, the co-encapsulation of cells with collagen as the attachment substrate was initially unsuccessful and has been achieved only recently (306).

Encapsulated hepatoma cells remained viable with cytotypic morphology in the hormonally defined medium to which they adjusted while encapsulated and exhibited the same level of metabolic activity as capsules maintained in serum-containing culture medium. Hepatoma cells were routinely cultured in serum-containing medium before encapsulation and cells were not weaned off serum for maintenance in hormonally-defined medium and encapsulation. Serum-containing medium was avoided because serum proteins, including cell adhesive fibronectin and vitronectin and complement C3 fragments, become associated with capsules during a pre-incubation in medium containing serum (see Chapter 4.2). Capsules, with associated serum proteins, implanted into Wistar rats,
elicited a thicker tissue reaction, with a thicker layer of macrophages apposed to the capsule surface, as compared to similar capsules maintained without FBS (307).

**Implantation and Recovery of Microcapsules**

The small diameter microcapsules were implanted into a pre-sutured omental pouch. Care was taken during implantation not to wash away capsules already in the pouch with the PBS in which individual capsules were suspended. This was not a problem when small capsule aggregates were placed into the pouch with tweezers. Suturing the omental pouch completely enclosed the implanted capsules which were clearly visible through thin, delicate regions of the tissue. Suturing was confined to the white milky spots, which are fatty, vascularized regions of the omentum, being careful not to pierce the other thin fascia-like regions.

At the time of capsule recovery, an intact omental pouch was found which contained numerous capsules. In two out of twenty-two rats, the omental pouch adhered to the liver. Typically, no inflammation was observed elsewhere in the peritoneal cavity, except for two animals that showed liver abscesses. The entire peritoneal cavity was examined for free floating capsules (not attached to other tissues) and for capsules which had attached to other tissue. In general, at most, 2 out of the 200 capsules implanted were found free floating in the peritoneal cavity and up to 13 capsules were associated with fascia or embedded at the suture line. The number of capsules in the omental pouch at the
time of recovery was not determinable since the tissue was thick (~ 1 - 2 cm), opaque and completely surrounded the small white capsules. The histomorphological results are based on roughly 30-40 capsules (actual range: 5 to 46 % of what was implanted) as inferred from histological sections. The actual number of capsules recovered within the omental pouch at explantation may actually be higher since capsules may be lost during cryosectioning and staining and a complete set of serial sections is impossible given the difficulty in cryosectioning omental tissue.

**Microencapsulated Hepatoma Cells In Vivo**

Cryosectioning of OCT (water soluble) embedded specimens and histological staining with aqueous toluidine blue or nuclear fast red allowed for the observation of microencapsulated hepatoma cells within an intact polymer membrane and the tissue reaction apposed to this membrane. Alcoholic reagents, used in the Masson trichrome and haemotoxylin and eosin staining techniques, dissolved the polymer capsule, but the features of the tissue reaction were well differentiated by their morphological staining characteristics.

Microencapsulated rat hepatoma cells, implanted into omental pouches in Wistar rats, were viable for up to and including 7 days, but not 14 days (Table 3.1). The morphological features of implanted, microencapsulated human hepatoma, HepG2, cells
were similar to rat hepatoma cells but viable cells were only found up to day 4 (Table 3.1). Hepatoma cells were arranged as aggregates of the sizes listed in Table 3.2.

Distribution curves of the number of viable rat hepatoma, H4IIEC3, cells within implanted microcapsules [Fig. 3.4(a)] showed a broad distribution at day 1 with 50% of those sections containing cells (median value), containing 34 viable cells per section; highest value 163 viable cells/section. With time, the distribution curves shifted to the left, as more cellular necrosis and fewer viable cells were observed within the capsules, until at day 14, at most 27 viable H4IIEC3 cells were observed within a capsule section. Not surprisingly, there was a similar broad distribution of the number of viable human hepatoma, HepG2, cells within implanted microcapsules [Fig. 3.4(b)]. However, there was a dramatic drop in the median values from 102 to 5, at days 1 and 4, respectively, although there was little change in the upper decile range with 10% of those sections containing cells containing between 220 and 317 and 203 and 264 viable cells per section, at these times. By days 7 and 14, no viable cells were observed in any sections.

No hepatoma cells were located outside of the capsule membrane and less than 5% of implanted capsule exhibited host inflammatory or immune cell infiltration. Therefore, the polymer membrane provided a physical barrier, sequestering transplanted cells within the capsule core and preventing direct physical contact between the implanted hepatoma cells and recipient’s immune and inflammatory cells.
Table 3.2 Microencapsulated Hepatoma Cellular Aggregate Sizes

<table>
<thead>
<tr>
<th>Day</th>
<th>Range of Aggregate Sizes (μm X μm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>In Vivo</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat H4IIEC3 Cells</td>
<td>Human HepG2 Cells</td>
</tr>
<tr>
<td>1</td>
<td>30 X 60 -</td>
<td>30 X 130 -</td>
</tr>
<tr>
<td></td>
<td>130 X 200</td>
<td>70 X 200</td>
</tr>
<tr>
<td>4</td>
<td>6 X 50 -</td>
<td>30 X 80 -</td>
</tr>
<tr>
<td></td>
<td>60 X 220</td>
<td>175 X 250</td>
</tr>
<tr>
<td>7</td>
<td>30 X 180 -</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>60 X 300</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

n and m values as given in Figure 3.4
N.D. = not determined

Figure 3.4 (over) Viable cell number per histological section distribution curves; days 1, 4, 7, and 14 in vivo for microcapsules containing (a) rat hepatoma, H4IIEC3, cells and (b) human hepatoma, HepG2, cells. Vertical axis represents the percentage of sections with a certain number of viable cells/section or less. Data sets for days 1, 4, 7, and 14 are comprised of n = 36, 41, 9, and 14 and of n = 14, 37, 16, and 5 histological capsule sections (one section/capsules). Each sample time is the pooled data from m = 3 rats per sample time, except for microcapsules containing HepG2 cells (b) for days 1 and 14, for which the sample set is pooled from m = 2 rats.
Number of Viable Microencapsulated H4IIEC3 Cells In Vivo Distribution Curve

Percentage of Sections

Number of Viable Cells/Section

Day 1
Day 4
Day 7
Day 14
Microcapsules, 1 day in vivo, showed an aggregate of H4IIEC3 cells [Fig. 3.5(a)] within the capsule core. The aggregate was comprised of tightly-packed, viable cells of polyhedral morphology with prominent oval nuclei, darkly-staining nucleoli and lightly-staining abundant cytoplasm within well defined nuclear and cellular membranes, respectively. An outer single layer of cellular debris surrounded some aggregates [Fig. 3.5(a)]. In other microcapsules, large aggregates had an outer rim of viable cells and an inner necrotic core [Fig. 3.5(b)]. The outer shell of viable cells, 2 to 5 cells in thickness (~20 μm), was comprised of distinct, tightly-packed, polyhedral cells which were characterized in histological sections by differentially staining nuclei and cytoplasm. The inner necrotic core was clearly distinguished by lightly-staining cytoplasmic and nuclear debris. In other micrographs, 1 day in vivo, both viable and necrotic rat hepatoma cells were scattered throughout the cellular aggregate located within the capsule core (not shown). By examining the sections of all capsules implanted for 1 day, 42% of capsules contained rat hepatoma cells [n=86 capsules, (1 section/capsule), m=3 rats].

Figure 3.5 (over) Representative micrographs of microencapsulated rat hepatoma, H4IIEC3, cells after their implantation into omental pouches in Wistar rats for 1 day (a, b), 4 days (c - e), 7 days (f, g), and 14 days (h). All micrographs show detail of cells within the polymer capsules surrounded by the tissue reaction (curved solid arrows). The outline of the polymer membrane, indicated in (c). After 4 days, the microcapsule membrane [large solid arrow in (d)] exhibited calcium salt deposits (slanted solid arrow) at the outer surface and the macroporous sublayer the outer. Viable cells were observed within such capsules (d) and a.e indicated in all micrographs with small solid arrows. Regions of cellular debris are identified with curved open arrows in (e, g, h) Magnification: bar=50 μm. Staining: Toluidine blue (c, f); hemotoxylin and eosin (a); von Kassa and Nuclear fast red (d); Masson trichrome (a, c, d).
At 4 days in vivo, microcapsules showed three types of rat hepatoma cell morphology and viability as progressive cell degeneration was observed. There were aggregates composed of loosely-associated viable cells of polyhedral morphology which filled the entire capsule core [Fig. 3.5(c)]. In similar capsules, for the first time, von Kossa staining identified calcium phosphate deposits in the macroporous layer, just below the skin layer of the polymer membrane, and on the outer surface of the capsule [Fig. 3.5(d)]. In these same capsules, nuclear fast red staining distinguished viable H4IIEC3 cells in aggregates [Fig. 3.5(d)]. In other capsules, viable and necrotic hepatoma cells were distributed throughout the cellular aggregate [Fig. 3.5(e)] or there were regions of cellular material, similar in size and shape the above aggregates, which were comprised of lightly-staining cellular debris, and no viable cells (not shown).

Microcapsules, 7 days in vivo, contained either loosely-associated cellular aggregates comprised completely of viable rat hepatoma cells [Fig. 3.5(f)] or viable and necrotic cells distributed throughout the aggregate [Fig. 3.5(g)]. Some microcapsules also showed large regions of cellular debris (not shown).

By day 14 [Fig. 3.5(h)], there were virtually no viable cells only regions of cellular matter (lightly-staining cytoplasmic debris and dark-staining pyknotic nuclei) similar in size to cellular aggregates.
NOTE TO USERS

Page(s) missing in number only; text follows. Microfilmed as received.

151

UMI
**Human Hepatoma (HepG2) Cells**

After 1 day *in vivo*, microcapsules contained an aggregate of HepG2 cells of characteristic polyhedral or cuboidal morphology, clearly defined by intact cell membranes with lightly-staining cytoplasm containing distinct secretory granules and prominent nuclei with a dark nucleolus [Fig. 3.6(a)]. In a minority of microcapsules, there were regions of lightly-staining cellular debris and few viable cells (not shown). By examining the sections of all capsules recovered after implantation for 1 day, 39% of capsules contained human hepatoma cells \( n=36 \) capsules, \( 1 \) section/capsule, \( m=2 \) rats.

Microcapsules containing human hepatoma cells, 4 days *in vivo*, exhibited three types of cell viability and morphology as progressive cell degeneration was observed. LM micrographs showed aggregates comprised of viable hepatoma cells, polyhedral in morphology, with intact, lightly-staining cytoplasm and prominent nuclei [Fig. 3.6(b)]. In other micrographs, large cellular aggregates were comprised of viable cells at the periphery and an inner necrotic core [Fig. 3.6(c)].

**Figure 3.6** (over) Representative micrographs of microencapsulated human hepatoma, HepG2, cells after their implantation into omental pouches in Wistar rats for 1 day (a), 4 days (b - e), 7 days (f), and 14 days (g). All micrographs show detail of encapsulated cellular aggregates and the apposed tissue reaction. An intact capsule membrane is observed in (b) and (g), indicated with a large solid arrow and the apposed tissue reaction with a curved solid arrow. In all micrographs, viable cells in aggregates are indicated with small solid arrows. Necrotic cells or regions of cellular debris are indicated with curved open arrows in (c), (d), (f), and (g). Blood vessels in the apposed tissue reaction or surrounding omental tissue are indicated with long, thin solid arrows. Magnification: bar=50 \( \mu m \). Staining: Toluidine blue (b, g); hemotoxylin and eosin (e, f); Masson trichrome (a, c, d).
Yet, in other micrographs, microcapsules contained cellular material which was comprised of darkly-staining pyknotic nuclei and cytolasmic debris; no viable cells [Fig. 3.6(d)]. Microcapsules, retrieved outside of the omental pouch, in the healing muscle at the suture line, also contained an aggregate of viable cells of characteristic morphology [Fig. 3.6(e)]. At this time, as with HEMA-MMA microcapsules containing H4IIEC3 cells, von Kossa staining identified calcium deposits associated with the outer regions of the polymer membrane (not shown).

Microcapsules, 7 days [Fig. 3.6(f)] and 14 days [Fig. 3.6(g)] in vivo, contained regions of cellular debris, similar in size to cellular aggregates, which were comprised of dark, pyknotic nuclei and cytoplasmic debris. No viable HepG2 cells were observed within microcapsules after 7 or 14 days in vivo.
Tissue Reaction to Encapsulated Hepatoma Cells

The average tissue reaction thicknesses and average closest blood vessel distances are presented in Table 3.3 for rat hepatoma, H4IIEC3, cells and in Table 3.4 for human hepatoma, HepG2, cells.

Distribution curves of tissue reaction thicknesses associated with microcapsules containing rat hepatoma, H4IIEC3, cells [Fig. 3.7(a)] showed a narrow distribution at day 1 with 50% of tissue reaction measurements (median value) were less than 9 μm. At day 4, 7, and 14 the distribution of tissue reaction thicknesses were similar and spread to include higher values such that 50% of the measurements were less than 39 μm, 55 μm, and 65 μm. The distribution curves of the tissue reaction thicknesses associated with microcapsules containing human hepatoma, HepG2, cells [Fig. 3.7(b)] showed a narrow distribution at day 1 similar to that toward microcapsules containing H4IIEC3 cells; 50% of all tissue reaction thickness measurements were less than 6 μm. However, by day 4, the distribution of tissue reaction thicknesses had spread such that the median shifted to 62 μm, shifting further at day 7 to a median value of 119 μm with a slight drop at day 14 to 61 μm. The distribution curves of the tissue reaction measurements associated with microcapsules containing human hepatoma, HepG2, cells were shifted towards higher thickness values at days 4, 7, and 14 as compared to tissue reactions associated with microcapsules containing H4IIEC3 cells. For example, at day 4, the upper decile (10%) of tissue reaction measurements tissue reactions were 214 μm to 520 μm, associated with
microcapsules containing HepG2 cells, while tissue reactions associated with H4IIEC3 cells were 130 μm to 342 μm in thickness.

To assess whether the observed tissue reaction surrounding capsules was due to the presence of the microcapsules containing hepatoma cells, the tissue response to omental pouch sham surgery and to HEMA-MMA beads were examined. Omental pouch sham surgery (pouches sutured without the implantation of capsules) showed tissue far from the sutured edges which was highly vascularized, containing fibroblasts and regions of abundant associated collagen and numerous inflammatory and immune cells, primarily, immature macrophages and lymphocytes. Omental tissue collected from rats without any sham surgery or capsule implants is characteristically comprised of adipose tissue with milky spots which are highly vascularized, containing immature macrophages, lymphocytes (T and B cells) and few mast cells in long tissue pedicules.

HEMA-MMA beads, without a core of co-encapsulated cells, not embedded directly in omental tissue after 2 weeks in vivo, were surrounded by either one cell layer of macrophages and fibroblasts. Beads associated with omental tissue for 2 weeks were surrounded by a poorly vascularized tissue reaction that was comprised of a layer of macrophages against the polymer membrane and a dense layer of elongated fibroblasts and their abundant collagen and few macrophages, lymphocytes and eosinophils. The tissue reactions to HEMA-MMA beads, 12 weeks in vivo, were similar in thickness,
<table>
<thead>
<tr>
<th>Day</th>
<th>Tissue Site</th>
<th># of Capsule Sections</th>
<th>Average TR Thickness, (Range) (mm)</th>
<th># of Capsule Sections</th>
<th>Average Closest BV Distance, (Range) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>omentum</td>
<td>n=70</td>
<td>12, (0 - 61)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>omentum</td>
<td>n=190</td>
<td>57, (0 - 342)</td>
<td>n=154</td>
<td>33, (1 - 235)</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>n=23, s=1</td>
<td>68, (7 - 294)</td>
<td>n=11, s=1</td>
<td>179</td>
</tr>
<tr>
<td>7</td>
<td>omentum</td>
<td>81</td>
<td>66, (7 - 284)</td>
<td>n=71</td>
<td>36, (3 - 177)</td>
</tr>
<tr>
<td>14</td>
<td>omentum</td>
<td>n=67</td>
<td>74, (7 - 294)</td>
<td>n=59</td>
<td>32, (7 - 294)</td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>n=19, s=2</td>
<td>42</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>n=31, s=1</td>
<td>111, (14 - 353)</td>
<td>n=27, s=1</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>free</td>
<td>n=2</td>
<td>32</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

m=3 rats

Tissue reaction data for tissue sites other than the omentum [fascia, suture line and free (no tissue attachment)] are for capsules which fell out of the omental pouch (not all rats had capsules outside of the omental pouch)

s= number rats with capsules in other sites
Table 3.4 Average Tissue Reaction (TR) Thickness and Average Closest Blood Vessel (BV) Distance for Microcapsules Containing Human Hepatoma (HepG2) Cells

<table>
<thead>
<tr>
<th>Day</th>
<th>Tissue Site</th>
<th># of Capsule Sections (n), (s)</th>
<th>Average TR Thickness, (Range) (mm)</th>
<th># of Capsule Sections (n), (s)</th>
<th>Average Closest BV Distance, (Range) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>omentum</td>
<td>n=31</td>
<td>11, (0 - 105)</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>omentum</td>
<td>n=50</td>
<td>90, (0 - 343)</td>
<td>n=46</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>n=31, s=2</td>
<td>116</td>
<td>n=15, s=2</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>n=6, s=1</td>
<td>47</td>
<td>n=1, s=1</td>
<td>34</td>
</tr>
<tr>
<td>7</td>
<td>omentum</td>
<td>n=64</td>
<td>125</td>
<td>n=58</td>
<td>50</td>
</tr>
<tr>
<td>14</td>
<td>omentum</td>
<td>n=40</td>
<td>90</td>
<td>n=40</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>n=2, s=1</td>
<td>151</td>
<td>n=2, s=1</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>n=5, s=1</td>
<td>211</td>
<td>n=5, s=1</td>
<td>39</td>
</tr>
</tbody>
</table>

For legend see Table 3.3

m=3 rats for days 4 and 7

m=2 rats at other times

Figure 3.7 (over) Tissue reaction thickness measurement distribution curves; days 1, 4, 7, and 14 *in vivo* for microcapsules containing (a) rat hepatoma, H4IIEC3, cells and (b) human hepatoma, HepG2, cells. Vertical axis represents the percentage of measurements with a certain tissue reaction thickness or less. Data sets for days 1, 4, 7, and 14 are comprised of *n* = 70, 190, 81, and 67 and of *n* = 31, 50, 64, and 40 capsules (one section/capsule, 4 measurements per capsule), for rat hepatoma and human hepatoma cells, respectively. Each sample time is the pooled data from *m* = 3 rats per sample time, except for microcapsules containing HepG2 cells (b) for days 1 and 14, for which the sample set is pooled from *m* = 2 rats.
Tissue Reaction Thickness Distribution Curve - H4IIEC3 Cells

Percentage of Measurements

Thickness (micro meters)

Day 1
Day 4
Day 7
Day 14
Tissue Reaction Thickness Distribution Curve - HepG2 Cells

Percentage of Measurements

Thickness (micro meters)

Day 1
Day 4
Day 7
Day 14
morphology, cellular profile and degree of vascularization. This was the tissue response to the HEMA-MMA polymer only.

**Rat Hepatoma (H4IIEC3) Cells**

At day 1, microcapsules, were loosely associated with omental tissue [Fig. 3.8(a)] and there was either no or only a very thin tissue reaction which was either one cell layer thick or was comprised of a lightly staining material [Fig. 3.5(a)], possibly fibrin: fibrinogen was adsorbed soon after the capsule contacted the peritoneal/interstitial fluid (see Chapter 4.2). Thicker tissue reactions were comprised of macrophages, fibroblasts, lymphocytes and some neutrophils and eosinophils. Many of these cells were immature, precursor leukocytes of omental tissue. The tissue reaction directly apposed to capsules was not vascularized although there were blood vessels in the surrounding omental tissue.

**Figure 3.8** (over) Representative micrographs of the tissue reaction to microcapsules containing rat hepatoma, H4IIEC3, cells after their implantation into omental pouches in Wistar rats for 1 day (a), 4 days (b), 7 days (c,d), and 14 days (e - f) or embedded in fascia (g) and suture line (h) at day 14. In all micrographs, medium-sized solid arrows identify transplanted cells. Capsules were observed as aggregates within omental tissue (a, c). Surrounding implanted capsules was a tissue reaction that was minimal at day 1 [curved solid arrows in (a)] but developing after 4 days (b). After 14 days *in vivo*, the three layers of the capsule-associated tissue reaction are numbered with arrows (e): (1) macrophages or FBGCs; (2) dense fibrous capsule; (3) highly vascularized region containing inflammatory and immune cells [close up in (f)]; similar morphology in (h). Blood vessels, indicated in the tissue reaction apposed to microcapsules with long thin arrows (b, d, f, h), were observed promptly at day 4 (b). Eosinophils are indicated in (b, d) with small black and white arrows, lymphocytes with small black arrows (b) and curved arrows (d). Magnification: bar=200 μm (a, c); bar=100 μm (e); bar=50 μm (b, d, f, g, h). Staining: hemotoxylin and eosin (h); Masson trichrome (a-g).
By 4 days, in omental tissue, the microcapsule tissue reaction comprised of loose fibrovascular tissue containing disorganized mature fibroblasts with elongated nuclei and immature fibroblasts with abundant cytoplasm and their associated collagen, and macrophages, lymphocytes, plasma cells, eosinophils, mast cells and neutrophils [Fig. 3.8(b)]. While qualities of the tissue reaction changed with time, the thicknesses of the tissue reaction did not change from that at day 4. At the surface of the polymer membrane were macrophages, fibroblasts, scattered foreign body giant cells, other inflammatory or immune cells and around some capsules, a dense layer of fibroblasts. Interestingly, at day 4, it was noticed that the tissue reaction was well vascularized with proximal vascular structures which numbered up to 100 within a 250 μm radial region. The tissue reactions to capsules embedded in the healing muscle at the suture line, was similar in thicknesses and cellular profile but with a thicker layer of fibroblasts and fewer, more distant blood vessels from the polymer tissue interface (not shown).

By day 7, microcapsules were still arranged as capsule aggregates, but there was a partial alignment of fibroblasts along the capsule curvature even at a distance from the polymer surface to from a thin fibrous capsule [Fig. 3.8(c)]. The tissue reaction to these rat hepatoma cell-containing capsules was comprised of primarily of disorganized fibrovascular tissue containing macrophages, fibroblasts, lymphocytes, plasma cells, and some eosinophils [Fig. 3.8(d)]. Adjacent to the capsule wall was an interrupted layer of macrophages, fibroblasts and some foreign body giant cells. The tissue reactions with blood vessels as close to the polymer/tissue interface as they were at day 4.
After 14 days *in vivo*, capsules were arranged as aggregates within omental tissue with each capsule surrounded by its own tissue reaction. The capsule-associated tissue reaction had three layers [Fig. 3.8(e)]; macrophages or very large FBGC against the polymer wall, a dense fibrous capsule up to 100 µm comprised of fibroblasts and associated collagen and some inflammatory and immune cells and then a highly vascularized region comprised of fibroblasts and collagen, macrophages, lymphocytes, plasma cells, eosinophils and mast cells [Fig. 3.8(f)]. In spite of the high degree of fibrosis in the middle layer, blood vessels were localized in the dense fibrous tissue adjacent to the polymer capsule so that their closest distance was unchanged from days 4 and 7. Capsules were also found in fascia between the stomach and spleen [Fig. 3.8(g)] and at the suture line [Fig. 3.8(h)]. Those embedded in fascia were surrounded by a dense fibrous, poorly vascularized tissue reaction comprised primarily of fibroblasts and few inflammatory or immune cells except for the outside of the fibrous capsule and areas of macrophages and foreign body giant cells apposed to the polymer membrane [Fig. 3.8(g)]. Other capsules associated with the healing abdominal muscle of the suture line, were surrounded by a tissue reaction of disorganized granulation tissue which was well vascularized and contained macrophages, lymphocytes, plasma cells and eosinophils [Fig. 3.8(h)]. Directly apposed to the capsule was a thick layer of macrophages and numerous FBGC and a dense fibrous capsule. Two free floating capsules (not attached to tissue) exhibited thin tissue reactions.
**Human Hepatoma (HepG2) Cells**

Microcapsules containing human HepG2 cells, similar to those containing rat cells, 1 day *in vivo*, were arranged as aggregates each exhibiting a very thin tissue reaction of comprised primarily of lightly staining fibrin with associated macrophages, fibroblasts, lymphocytes and some eosinophils and neutrophils [Fig. 3.9(a)]. These thin tissue reactions were not vascularized but there were blood vessels, proximal to the capsules, in the surrounding omental tissue [Fig. 3.6(a) and Fig. 3.9(a)].

**Figure 3.9** Representative micrographs of the tissue reaction to microcapsules containing human hepatoma, HepG2, cells after their implantation into omental pouches in Wistar rats for 1 day (a), 4 days (b, c, d), 7 days (e, f), and 14 days (g) or embedded at the suture line (h) at day 14. In all micrographs, medium-sized solid arrows identify transplanted cells. Similar to microcapsules containing rat hepatoma cells, microcapsules were arranged as aggregates within the omental tissue (e). At day 1, the tissue reaction was comprised of fibrin and scattered inflammatory cells [small arrows in (a)]. Vascular structure were observed in the tissue reaction at day 4 (long thin arrows) and maintained at days 7 (f) and 14 (h). At day 7, three distinct regions of the tissue reaction are identified (e). Numerous eosinophils (kidney shaped nucleus and eosinophilic cytoplasm), indicated with small arrows, were observed in the tissue reaction to microcapsules containing HepG2 cells at day 4 (d), day 7 (f) and day 14 (g, h). Cells of the tissue reaction after 14 days (g) included, a FBGC against the polymer membrane (large arrow), fibroblasts (curved arrow), macrophages, smallest arrows, plasma cells (small curved arrows and eosinophils (medium-sized arrows). Magnification: bar=200 μm (a, c); bar=100 μm (e); bar=50 μm (b, d, f, g, h). Staining: Toluidine blue (b); hemotoxylin and eosin (d - h); Masson trichrome (a, c).
Microcapsules containing human hepatoma, HepG2, cells, 4 days *in vivo*, were typically associated with a tissue reaction comprised of macrophages and scattered foreign body giant cells against the polymer wall, followed by a thin region of mature fibroblasts and associated collagen oriented along the curvature of the capsule and then a highly vascularized region comprised of immature fibroblasts and large areas of associated collagen. As with H4IIEC3 cells, vascularization was first noted on day 4 with HepG2 cell-containing capsules, however, the vascularization of the tissue reaction was greater for microcapsules containing human hepatoma cells. Numerous macrophages, lymphocytes, plasma cells, eosinophils, and some mast cells were distributed throughout all three regions [Fig. 3.9(c)]; eosinophils were especially noticeable and significantly higher in number than at this time for rat hepatoma transplants [Fig. 3.9(d)]. In some capsule sections, the dense fibrous layer was absent and the vascularized region containing the same type of immune and inflammatory cells was apposed to the polymer capsule [Fig. 3.9(b)]. Other capsules embedded in omental tissue, such as that associated with the capsule with necrotic cells shown in Fig. 3.6(d), exhibited a poorly vascularized tissue reaction which was comprised similar regions and cell types. Capsules embedded in the healing muscle of the suture line exhibited a tissue reaction of fibroblasts, macrophages, and some foreign body giant cells apposed to the capsule wall, and a dense fibrous region within surrounding tissue which was comprised primarily of disorganized fibroblasts and associated collagen also containing macrophages, lymphocytes, plasma cells, and eosinophils (not shown). Other capsules, associated with fascia tissue between the stomach and the spleen, exhibited a thin unvascularized tissue reaction comprised of
macrophages, eosinophils, and some fibroblasts, lymphocytes, plasma cells, mast cells and against the capsule wall, foreign body giant cells (not shown).

Microcapsules, 7 days \textit{in vivo}, were arranged as capsule aggregates embedded within omentum, were surrounded by a tissue reaction which was comprised of three regions [Fig. 3.9(e)]; some macrophages and a dense layer of foreign body giant cells against the entire capsule, a dense region of fibroblasts and numerous macrophages, eosinophils, lymphocytes, plasma cells and some mast cells which was vascularized and a then a region of highly vascularized fibrous tissue with a high content of mature collagen and the same cellular profile as the apposed region [Fig. 3.9(f)].

Microcapsules, 14 days \textit{in vivo}, embedded in omental tissue, were surrounded by a layer of macrophages and foreign body giant cells and a thick, dense layer of elongated fibroblasts (more mature fibroblasts than at day 7) (and some immune/inflammatory cells) and a region of highly vascularized fibrous tissue which contained numerous macrophages, lymphocytes, plasma cells, and eosinophils [Fig. 3.9(g)]. Blood vessels were found even in the dense fibrotic layer, such that the closest blood vessel distance had not changed from that at day 4. Microcapsules, 14 days \textit{in vivo}, embedded at the suture line, were surrounded by a thick layer of foreign body giant cells and a dense fibrous capsule comprised of elongated fibroblasts and abundant associated collagen with some macrophages, lymphocytes and eosinophils [Fig. 3.9(h)]. The area between capsules was
well vascularized fibrous tissue which also contained the same inflammatory/immune cells.

Even within the dense fibrous tissue layer, blood vessels were found.
DISCUSSION

Microcapsule Implantation and Recovery

The microcapsules used in this study were those of a small diameter, ~ 450 μm. There has been a trend towards using capsules with as small a diameter as possible because of advantages in mass transport characteristics such as a better use of the internal volume of the capsule and a higher surface to volume ratio for nutrient diffusion into and cell-derived biomolecule and waste removal out of the capsule. Small diameter capsules for implantation are preferred because of the smaller total volume of the implant, the higher number of capsules that can be implanted into a tissue site and the greater flexibility in the choice of implant site. In the omental pouch, the number of small diameter capsules that could be accommodated is approximately ten fold higher than the number of large diameter capsules (~ 750 μm).

However, there were some problems associated with the implantation of small diameter capsules, primarily because of the small size. Because a given site can hold more capsules, the smaller their diameter, more pipette loads of a given number of capsules, suspended in PBS, were delivered. Microcapsules, already in the site were easily washed out of the omental pouch with PBS delivering more capsules. Furthermore, small diameter capsules could easily slip between the sutures of the omental pouch and become free in the peritoneal cavity. Complete recovery of small diameter microcapsules from the peritoneal cavity is particularly difficult because of their size and morphological changes.
with time. At later times, past day 7 in vivo, their diameter decreases as the polymer wall becomes more dense with a corresponding change in their optical properties from opaque spheres at early times to more translucent at later times. They become more difficult to discern against the tissue background with time. Small diameter capsules, free in the peritoneal cavity, are able to become hidden in tissue crevasses and folds.

**Tissue Reaction to Microencapsulated Cells**

The tissue reactions to HEMA-MMA capsules containing rat (Table 3.5) or human (Table 3.6) hepatoma cells into Wistar rats exhibited some common trends which were independent of the species differences. Initially (day 1), the microcapsules were arranged as loosely-associated capsule aggregates typically with thin tissue reactions that shifted towards higher thickness values after 4 days in vivo and did not change their distribution of thicknesses thereafter in any of the tissue sites including the implant site (the omentum) or for those that were found in fascia or at the suture line. At day 1, the tissue reactions were not vascularized and any blood vessels around the capsules were from the surrounding omental tissue. Prompt vascularization occurred after 4 days in vivo, after which time, the tissue reactions could contain up to 120 blood vessels within a 250 µm radial region of the tissue reaction; vascularization was maintained for up to 14 days. Typically, the highest degree of vascularization of the tissue reaction to capsules was observed for capsules embedded in omental tissue as compared to those found in other tissue sites, consistent with what was observed in a separate study (302).
Table 3.5 Histological Scores for the Tissue Reaction to Microcapsules Containing Rat Hepatoma (H4IIEC3) Cells

<table>
<thead>
<tr>
<th>Day</th>
<th>Tissue Site</th>
<th>Degree of Vascularization</th>
<th>Degree of Fibrosis</th>
<th>Degree of Inflamm/Immune Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>(c)</td>
</tr>
<tr>
<td>1</td>
<td>omentum</td>
<td>1, 1, 1</td>
<td>0, 0, 0</td>
<td>1, 1, 1</td>
</tr>
<tr>
<td>4</td>
<td>omentum</td>
<td>3, 4, 3</td>
<td>1, 2, 1</td>
<td>3, 3, 3</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>omentum</td>
<td>3, 3, 3</td>
<td>2, 2, 3</td>
<td>3, 3, 2</td>
</tr>
<tr>
<td>14</td>
<td>omentum</td>
<td>4, 3, 3</td>
<td>4, 4, 2</td>
<td>2, 2, 3</td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>1, 1</td>
<td>1, 4</td>
<td>3, 1</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>free</td>
<td>NA</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

(a) score based on the number of vascular structures within a 250 mm of the polymer/tissue interface: (1) 0-15; (2) 0-30; (3) 0-85; (4) 0-120.

(b) score based on the percentage of the total tissue reaction area which is comprised of fibroblasts and collagen and with the morphological characteristic of fibrosis: (0) no fibrous tissue; (1) 1-5% (disorganized fibrous tissue reaction); (2) 5-20% (thin fibrous tissue reaction); (3) 20-50% (dense fibrous tissue reaction but not a quality of the entire tissue reaction); (4) 50-100% (dense fibrous tissue reaction).

(c) score based on the percent contribution of macrophages, lymphocytes, plasma cells, foreign body giant cells, eosinophils, mast cells, and neutrophils to the cell profile: (0) none; (1) 1-5%; (2) 5-20%; (3) 20-50%; (4) 50-100%.

Histological scores for each rat in each sample set (m=3 rats) are separated with commas. Histological scores in tissue sites other than the omentum (fascia, suture line and free (no tissue attachment)) are for capsules which fell out of the omental pouch (not all rats had capsules outside of the omental pouch).

Inflamm = Inflammatory
Imm = Immune
NA = not applicable
Table 3.6 Histological Scores for the Tissue Reaction to Microcapsules Containing Human Hepatoma (HepG2) Cells

<table>
<thead>
<tr>
<th>Day</th>
<th>Tissue Site</th>
<th>Degree of Vascular.</th>
<th>Degree of Fibrosis</th>
<th>Degree of Inflam/Imm Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>omentum</td>
<td>1, 1</td>
<td>0, 0</td>
<td>1, 1</td>
</tr>
<tr>
<td>4</td>
<td>omentum</td>
<td>4, 1, 4</td>
<td>2, 3, 2</td>
<td>4, 4, 4</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>2, 3</td>
<td>3, 3</td>
<td>3, 3</td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>omentum</td>
<td>4, 3, 3</td>
<td>3, 3, 3</td>
<td>3, 3, 3</td>
</tr>
<tr>
<td>14</td>
<td>omentum</td>
<td>3, 4</td>
<td>4, 4</td>
<td>2, 2</td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

For legend see Table 3.5

m=3 at day 4 and 7, m=2 at other times
Furthermore, for capsules embedded in omental tissue, the closest distance of blood vessels to the polymer/tissue interface did not change with time past day 4.

The tissue reactions to microcapsules in omental tissue, 4 days in vivo, was comprised of disorganized, loose fibrous vascularized tissue. After 7 days in vivo, in omental tissue, the degree of fibrosis of the tissue reactions increased as a layer of fibroblasts and collagen were apposed to the polymer capsule oriented along the curvature of the capsule. After 14 days in vivo, in omental tissue, capsules were surrounded by dense fibrous tissue up to 100 μm in thickness accounting for the degree of fibrosis grades of 4. Although this high degree of fibrosis was observed, 14 days in vivo, as compared to at day 7, there were some blood vessels in this dense fibrous tissue such that the average blood vessel distance remained the same as at day 7. However, in the case of capsules embedded in fascia which also exhibited a dense fibrous tissue reaction, this tissue was poorly vascularized, indicating one of the benefits of capsule association with omental tissue. Corresponding with the high degree of fibrosis was a low degree of inflammatory/immune response as indicated by the cellular profile of the tissue reaction at day 14, consistent with fibrotic tissue formation being the resolution of a chronic granulomatous tissue or delayed hypersensitivity reaction. Conversely, a low degree of fibrosis (e.g., day 4) accompanied a high degree of inflammatory/immune response, with most of the inflammatory and immune cell types identified.
There were also changes with time in the inflammatory and immune cellular profile of the tissue reaction to microencapsulated rat hepatoma (Table 3.7) and human hepatoma (Table 3.8) cells that were independent of the species of cell transplanted. The cellular profile of the tissue reactions were similar around rat and human microencapsulated hepatoma cells, days 1 and 4 in vivo (compare Table 3.4 and Table 3.5). Neutrophils were observed only up to 4 days in vivo. At later times (days 7 and 14), as the degree of fibrosis of the tissue reaction was higher, more fibroblasts were observed than macrophages. Furthermore, a high surface density of FBGC was observed between days 7 and 14, presumably, as macrophages fused (308) on the polymer capsule surface. However, even at 14 days, immune cells such as lymphocytes, plasma cells, eosinophils and mast cells were observed, potentially indicating that an immune response was still in progress, presumably towards antigens shed from the encapsulated cells.

Despite the overall similarities, there were several distinguishing characteristics of the tissue reactions to microcapsules containing the two types of cells. With human hepatoma cells tissue reaction thicknesses shifted to higher values than with the rat hepatoma cells. Furthermore, there appeared to be more eosinophils associated with microcapsules containing human cells than with rat cells (compare cellular profiles for transplanted microencapsulated rat and human hepatomas in Tables 3.4 and 3.5, respectively). The greater numbers of eosinophils and lymphocytes albeit, a similar number of plasma cells, which were associated with day 4 human hepatoma, as compared to rat hepatoma, microcapsule implants, accounted for the higher degree of
Table 3.7 Histological Scoring of the Inflammatory and Immune Cell Profile of the Tissue Reaction to Microcapsules Containing Rat Hepatoma (H4IIEC3) Cells

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>omentum</td>
<td>+ (3)</td>
<td>+ (2)</td>
<td>+ (2)</td>
<td>- (3)</td>
<td>-/+ (3)</td>
<td>- (3)</td>
<td>- (3)</td>
<td>-/+ (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- (1)</td>
</tr>
<tr>
<td>4</td>
<td>omentum</td>
<td>++ (3)</td>
<td>++ (3)</td>
<td>+ (3)</td>
<td>+ (2)</td>
<td>+ (2)</td>
<td>+ (1)</td>
<td>-/+ (2)</td>
<td>+ (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td>-/+ (1)</td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td>/+ (1)</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>+++ (1)</td>
<td>++ (1)</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>- (1)</td>
<td>-/+ (1)</td>
</tr>
<tr>
<td>7</td>
<td>omentum</td>
<td>++ (2)</td>
<td>++ (3)</td>
<td>++ (1)</td>
<td>+ (2)</td>
<td>+ (3)</td>
<td>- (3)</td>
<td>-/+ (3)</td>
<td>- (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>omentum</td>
<td>+++ (2)</td>
<td>++ (3)</td>
<td>+ (2)</td>
<td>+ (1)</td>
<td>+ (3)</td>
<td>+ (2)</td>
<td>++ (2)</td>
<td>- (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td></td>
<td></td>
<td>/+ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>+++ (1)</td>
<td>+ (1)</td>
<td>/+ (1)</td>
<td>- (2)</td>
<td>-/+ (1)</td>
<td>- (2)</td>
<td>-/+ (1)</td>
<td>- (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ (1)</td>
<td>+++ (1)</td>
<td>/+ (1)</td>
<td>/+ (1)</td>
<td></td>
<td></td>
<td>++ (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>+++ (1)</td>
<td>++ (1)</td>
<td>+ (1)</td>
<td>/+ (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>++ (1)</td>
<td>- (1)</td>
</tr>
<tr>
<td></td>
<td>free</td>
<td>++ (1)</td>
<td>/+ (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td></td>
</tr>
</tbody>
</table>

Inflammatory and immune cell profile of the tissue reaction was evaluated based on the percentage of each cell type in the tissue reaction: (-) none; (-/+ 1-5%; (+) 5-20%; (+++) 20-50%; (++++) 50-100%. The number of rats whose tissue reaction contained the indicated score for the cell type is in brackets; n=3 total number of rats per sample time. Cellular profiles in tissue sites other than the omentum [fascia, suture line and free (no tissue attachment) are for capsules which fell out of the omental pouch; the number rats with capsules in these tissues is in brackets (s).

Fib. = fibroblasts, Mφ = macrophages, Lym. = lymphocytes, P.C. = plasma cells, Eosin. = eosinophils, Mast = mast cells, FBGC = foreign body giant cells, Neut. = neutrophils
Table 3.8 Histological Scoring of the Inflammatory and Immune Cell Profile of the Tissue Reaction to Microcapsules Containing Human Hepatoma (HepG2) Cells

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>omentum</td>
<td>+ (2)</td>
<td>+ (2)</td>
<td>+ (2)</td>
<td>- (2)</td>
<td>-/+ (1)</td>
<td>+ (1)</td>
<td>- (2)</td>
<td>-/+ (2)</td>
</tr>
<tr>
<td>4</td>
<td>omentum</td>
<td>++ (3)</td>
<td>+ (3)</td>
<td>++ (3)</td>
<td>++ (3)</td>
<td>+ (2)</td>
<td>-/+ (1)</td>
<td>- (2)</td>
<td>-/+ (1)</td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>+++ (2)</td>
<td>+ (2)</td>
<td>-/+ (1)</td>
<td>-/+ (1)</td>
<td>- (2)</td>
<td>-/+ (1)</td>
<td>- (2)</td>
<td>- (2)</td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>+ (1)</td>
<td>++ (1)</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td>++ (1)</td>
<td>+ (1)</td>
<td>-/+ (1)</td>
<td>- (1)</td>
</tr>
<tr>
<td>7</td>
<td>omentum</td>
<td>+++ (1)</td>
<td>++ (1)</td>
<td>++ (1)</td>
<td>++ (1)</td>
<td>-/+ (1)</td>
<td>-/+ (1)</td>
<td>- (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>+++ (2)</td>
<td>+ (1)</td>
<td>-/+ (1)</td>
<td>-/+ (1)</td>
<td>- (2)</td>
<td>-/+ (1)</td>
<td>++ (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>+++ (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>++ (1)</td>
<td>- (1)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>omentum</td>
<td>+++ (2)</td>
<td>+ (1)</td>
<td>-/+ (1)</td>
<td>-/+ (1)</td>
<td>- (2)</td>
<td>++ (1)</td>
<td>- (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>suture line</td>
<td>+++ (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>++ (1)</td>
<td>- (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fascia</td>
<td>+++ (1)</td>
<td>+ (1)</td>
<td>-/+ (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>++ (1)</td>
<td>- (1)</td>
<td></td>
</tr>
</tbody>
</table>

For legend see Table 3.7
m=3 rats at days 4 and 7
m=2 at other times
immune/inflammatory cell scoring. Correspondingly, the ongoing, higher level of cellular immune/inflammatory response to microcapsules containing human hepatoma cells, resulted in the maintenance of a granulomatous tissue response and a higher degree of vascularization (Table 3.2). The presence of eosinophils in the tissue reaction to microcapsules containing rat hepatoma cells and especially human hepatoma cells may suggest mechanisms of the host’s cytokine response with a bias to a Th2 cytokine profile including IL-3, IL-4, and IL-5 all of which promote eosinophilia (309). Eosinophils, effector cells in an immune response, can adhere to bound IgG through their Fc receptors and damage graft tissue by releasing cytokines (major basic protein), hydrolytic enzymes, and reactive oxygen intermediates (310). Furthermore, since eosinophils are CD4+, they could function as APC’s bearing xenoantigens in stimulating T cell responses (311). Reverse transcriptase polymerase chain reaction mRNA analysis of the tissue reaction would confirm this hypothesis.

The morphology of the tissue in omental pouch shams (surgery without implanted microcapsules) was different from the gross morphology of endogeneous omental tissue but similar to the tissue reaction around capsules farthest from the capsule wall, the third region of vascularized granulomatous tissue. However, the shams exhibited a lower density of inflammatory and immune cells but similar numbers of blood vessels. The tissue reaction adjacent to microcapsules because of its gross morphology and cellular composition was clearly distinguishable from the surrounding omental tissue of the pouch.
Therefore, the tissue reaction characterized in this study was that towards HEMA-MMA microcapsules containing rat or liver cells and not just a result of the omental pouch surgery.

Each capsule was surrounded by its own tissue reaction that at the periphery, if there were not other capsules around it, blended into tissue morphologically similar to that of omental sham tissue. The manner of capsule arrangement within the omental pouch tissue was arbitrary and presumably set early after their implantation. The thickness of the tissue reaction associated with microcapsules was partially dependent on their arrangement within the omental pouch tissue in relation to each other. For two adjacent microcapsules, the tissue reaction thickness between the two was measured and half of this thickness assigned as a measurement for each microcapsule. The stopping point of the tissue reaction to capsules, isolated from others with tissue reactions blending into the surrounding omental tissue, was determined based on changes in gross morphology. The tissue reaction thickness measurements plotted in the distribution curves in Fig 3.7(a) and Fig 3.7(b) are the raw data (4 measurements per capsule) so that extremes in thicknesses are not averaged out.

Omental tissue has some unique characteristics which may be beneficial for the transplantation of microencapsulated cells. Omental tissue possesses unique vascularizing capabilities presumably because of the presence of basic fibroblast growth factor (312) and endothelial cell growth factor (313), the great source of endothelial cells from which
new capillaries sprout, and the presence of macrophages, B cells, T cells and mast cells
\( (314, 315) \) which can release cytokine mediators of angiogenesis \( (316, 317) \). The
omentum functions as an intestinal thymus \( (71) \) and may aid in the induction of tolerance
to antigens shed from encapsulated cells. Others have used the omental tissue site for cell
transplantation \( (77) \) and as transplanted pedicles for enhanced revascularization and
healing of tracheal transplants \( (318) \). Capsule implantation into an omental pouch
localizes capsules in a tissue site unlike microcapsule implanted freely in the peritoneal
cavity \( (319) \) to become or not become attached to tissue. Microcapsule association with
essentially one tissue type was expected to decrease variability in the tissue response and
allow for a localized assessment of the effect of microcapsulated cells on the tissue
response. Another site for microcapsule implantation is the epididymal fat pad in rats
which was chosen because of the an expected high microcapsule recovery \( (78) \) and the
localization of the implant \( (231) \).

Few of the capsules implanted into the omental pouch formed the sample set for
examination and analysis of microencapsulated cell viability and the tissue reaction. This
lead to concerns as to whether the results were biased because only a certain small
percentage of the implanted capsules were analyzed. It is not know whether the non-
recoverable capsules exhibited a thinner or thicker tissue reaction or one with different
morphological characteristics or whether the cells within these capsules would be of a
different morphology or viability.
Microcapsules for Cell Transplantation

Microencapsulated rat hepatoma cells remained viable longer *in vivo* than did human hepatoma cells, although microencapsulated cell viability was limited in both cases. HEMA-MMA microcapsules with an aggregate of rat hepatoma, H4IIEC3, cells, after implantation into an omental pouch in immunocompetent Wistar rats, a model of allogeneic transplantation, contained viable cells at 7 days [Fig. 3.5(f), 3.5(g)] but not 14 days [Fig. 3.5(h)] post implantation. A similar model of xenogeneic transplantation of microencapsulated human hepatoma, HepG2, cells resulted in viable cells up to 4 days [Fig 3.6(b, c, e)] but not 7 days [Fig. 3.6(f)]. The cells decreased from an initial percent viability of 50-100% to 20-50% after 7 days of microencapsulated rat hepatoma cell implantation into rats and after 4 days of microencapsulated human hepatoma cell implantation into rats (Table 3.1). Death of encapsulated cells, as aggregates in the absence of exogenous extracellular matrix, occurred *in vitro*, as a baseline to changes observed upon implantation, although, some viable cells were observed after 21 days (controls for 14 days *in vivo*) [rat hepatoma, Fig 3.2(b); human hepatoma, Fig. 3.3(b, c)]. Large aggregates of microencapsulated hepatoma cells *in vitro* [Fig. 3.2(b), Fig 3.3(c), (93)] and *in vivo* [Fig. 3.5(b), Fig 3.6(c)] exhibited an outer rim of viable cells and an inner necrotic core which may reflect the presence of gradients of critical nutrients such as glucose and oxygen, growth factors, and hormones in one direction and gradients of metabolic wastes such as lactate and carbon dioxide and products of necrosis in the opposite direction. For more detail about the temporal morphology and viability of
microencapsulated HepG2 cells as cellular aggregates and distributed within an exogeneous cell attachment substrate, Matrigel®, see Chapter 2.1. *In vivo*, there are additional factors which would influence the viability of cells such as the inflammatory and immunological response to encapsulated cells, and nutrient delivery to the cells.

The results of these microcapsule implantation studies suggested a relationship between the viability of microencapsulated cells and the degree of vascularization of the surrounding tissue reaction. Human hepatoma cell viability and morphology in microcapsules, 4 days *in vivo*, correlated with the degree of vascularization of the tissue reaction in omental tissue. Microcapsules, within omental tissue, with a well vascularized tissue reactions, contained viable human hepatoma cells [Fig. 3.6(b), Fig. 3.6(c)] while those with poorly vascularized tissue reactions did not [Fig. 3.6(d)]. However, it was not clear from this study that the perfusion of the implant site would be sufficient to sustain microencapsulated cell beyond day 4. In other studies, the degree of vascularization of the tissue reaction correlated with encapsulated islet cell viability and insulin secretion *ex vivo* (320). Furthermore, prevascularization of the subcutaneous site in response to polyglycolic acid polymer foams facilitated syngeneic islet cell transplantation and normoglycemia in streptozocin-diabetic mice which was otherwise unsuccessful in this site (74). It is expected that vascularization of the tissue surrounding implanted capsules would provide a system for nutrient delivery to sustain encapsulated cells as well as a route for the systemic delivery of the secreted product. However, the blood stream also carries leukocytes that would attach to and migrate between endothelial cells to
participate in the tissue reaction to the microcapsules. There is however a condition under which a vascularized tissue surrounding microcapsules would unequivocally be favourable. That would be if the endothelial cells were numerous and stable, that is, not activated by inflammatory cytokines to a state that promotes endothelial cell-leukocyte interaction (321, 322).

Antigens, shed from microencapsulated cells could induce an inflammatory/immune response which would cause an activation of inflammatory cells surrounding the capsule with concomitant release of inflammatory/immune mediators to mediate the inflammatory response and the influx of leukocytes into the area of the capsule. As the intensity of the tissue reaction surrounding the capsule increases, the microencapsulated cells would begin to die, because of a lack of nutrients, specific immune responses or non-specific immune/inflammatory effects such as cytotoxic cytokines or free radicals. There would presumably be a modulation of the antigen load (e.g., type or dose) associated with microcapsules as products of necrosis were released to further stimulate an immune response. A vicious cycle proceeds: cell loss causes a tissue reaction and a tissue reaction causes further cell loss. The capsule membrane, by activating macrophages, could act as an adjuvant to increase the intensity of an immune response to shed antigens or decrease the time until its onset. Furthermore, an inflammatory response to the microcapsule would recruit macrophages to the vicinity of the microcapsule which could then function as antigen presenting cells for shed antigens.
For this reason, the biocompatibility of the polymer membrane of microcapsules for cell delivery may be of central importance.

It is presumed that antigen shedding is a major mechanism accounting for loss of encapsulated cell viability. Alloantigens, xenoantigens and tumour specific antigens such as foreign proteins secreted by the cells including the therapeutic agent, cell surface molecules, or cell components released upon cell death which diffuse through the polymer membrane to be recognized as foreign by the host’s immune system, could elicit an immune response. An immunological reaction against encapsulated cell surface antigens (e.g., donor MHC molecules) or cell-specific secreted proteins may be produced via indirect antigen presentation if these soluble shed antigens permeate through the polymer wall. These shed antigens may be internalized, processed and presented in association with the host Class II Major Histocompatibility Complex (MHC) molecules, most effectively by macrophages or dendritic cells, to host CD4+ helper T cells whose activation would promote and regulate humoral and cell-mediated immune responses and inflammation. Antigen shedding from microcapsules would sensitize the host to the transplanted cells and initiate a potentially deleterious humoral or molecular cytotoxic tissue response in the tissue surrounding the implant that could directly or indirectly cause the death of encapsulated cells.
HEMA-MMA microencapsulated cell viability is limited in non-immunosuppressed Wistar rats. Microencapsulated rat hepatoma cells, H4IIEC3, a model of allogeneic transplantation, arranged as a cellular aggregate were viable after 7 days but not 14 days of implantation. A similar model of xenogeneic transplantation of microencapsulated human hepatoma cells, HepG2, resulted in viable cells at 4 days but not 7 days. Vascularization of the tissue reaction to both types of microencapsulated cells occurred promptly after 4 days in vivo and was maintained for up to 14 days. With HepG2 cells at day 4 in vivo, there appeared to be a relationship between the viability of microencapsulated cells and the degree of vascularization of the surrounding tissue reaction. Calcification of the polymer membrane, containing both types of cells, first identified using von Kossa staining at day 4 presumably reduced the permeability of the membrane and could compromise nutrient diffusion and cell viability.

The tissue reactions to microcapsules, once developed, were comprised of three regions: macrophages, fibroblasts and some foreign body giant cells apposed to the polymer membrane, a fibrous region containing immune and inflammatory cells and a region of vascularized granulation tissue. For capsules embedded in omental tissue, the closest blood vessel distance to the polymer/tissue interface did not change past day 4, neither did the typical tissue reaction thicknesses. There were also noticeable differences in the tissue reaction between microencapsulated implants of hepatoma cells of allogeneic and xenogeneic origin in terms of the thickness of the tissue reaction and the immune cell

195
profile. Microcapsules containing human hepatoma, HepG2, cells typically exhibited thicker tissue reactions containing more eosinophils than towards microcapsules containing rat hepatoma cells after 4, 7, and 14 days in vivo. Cellular antigens, of allo or xeno origin, shed from microencapsulated cells, could stimulate an immune response via the indirect pathway of antigen presentation, to initiate a humoral or molecular cytotoxic tissue response surrounding the implant which could directly or indirectly compromise the viability of encapsulated cells.
Chapter 4

HEMA-MMA Microcapsules Characterization

4.1 XPS Surface Analysis of Polyacrylate Microcapsules for Cell Transplantation

ABSTRACT

High resolution carbon, C 1s, x-ray photoelectron spectroscopy (XPS) spectra of the surface of hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA; 75 mole % HEMA) capsules maintained in PBS for 1 week showed that the surface was not pure HEMA-MMA. In these spectra, more carbon was bonded in the C-O form than in the C-C form indicating the presence of the Pluronic surfactant, L101, adsorbed from the precipitation bath to the surface during microcapsule preparation. Capsules maintained in medium containing fetal bovine serum for 1 week showed a nitrogen signal consistent with the presence of adsorbed serum proteins. There was a decrease in the amount of nitrogen on the surface after phosphate buffered saline (PBS) washing, however this did not decrease to zero. There was no detectable calcium on freshly-made capsules or capsules maintained in PBS for 1 week but calcium was detected on capsules maintained in medium containing serum.

---

INTRODUCTION

Microencapsulation of living cells within a polymer membrane isolates them from the host immune system, facilitating their use as a bioartificial organ. The success of cell transplantation is determined by the maintenance of cell viability and expression of differentiated functions within the implanted capsule, which in turn is dependent on the tissue reaction upon implantation. The functional success of a cell-containing implant is therefore partially dependent on the absence or minimization of the tissue reaction and fibrous capsule formation upon implantation. In addition to the influence of cell-derived products, the surface chemistry of the capsules is presumed to influence the interaction of the material with host cells, thus affecting the host tissue response. The surface chemistry of HEMA-MMA capsules was characterized by x-ray photoelectron spectroscopy (XPS). XPS surface analysis was used to examine the effects of the microencapsulation process, capsule incubation conditions and wash regimes on the surface chemistry of HEMA-MMA capsules. Surface characterization is an initial step towards the understanding of capsule biocompatibility and will ultimately lead to the development of capsules with an improved host response.
MATERIALS AND METHODS

HEMA-MMA Capsule and Film Preparation

Hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA; 75% HEMA) copolymer, prepared as before (235), was dissolved in polyethylene glycol 200 (PEG 200) [10% (w/v); BDH Chemicals, Toronto, Ontario] for the polymer solution used for the preparation of microcapsules and films. Microcapsules were prepared by the submerged jet process with polymer precipitation in Ca\(^{2+}/\)Mg\(^{2+}\)-free phosphate buffered saline (PBS: 0.2 g/L KCl; 0.2 g/L KH\(_2\)PO\(_4\); 8 g/L NaCl; 2.16 g/L Na\(_2\)HPO\(_4\)/H\(_2\)O; Gibco) containing Pluronic L101 surfactant [100 ppm (v/v), BASF Chemicals, Wyandotte, MI] (237). The core solution consisted of a 1:1 (v/v) mixture of α-MEM containing 100 U/mL penicillin 100 ng/mL streptomycin (tissue culture medium) and 10% fetal bovine serum (FBS) (complete tissue culture medium) and the cell attachment substrate, Matrigel® (Collaborative Research Incorporated, Bedford, MA). Pluronic L101 surfactant is a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer with a core of 54 propylene oxide groups and 7 ethylene oxide end groups. Capsules prepared in this way are identical to those used for studies of transplantation of attachment-dependent cells except that here, the cells have been omitted (304). Capsules were maintained (1 week) in either complete tissue culture medium, in tissue culture medium without added FBS or in PBS alone.
Films of HEMA-MMA were prepared by carefully placing a sterile glass slide with 0.2 ml of the polymer solution into a Petri dish containing PBS (45 mL) with or without the Pluronic surfactant, L101 (100 ppm). Upon contact with water, a polymer film formed by precipitation. After 30 minutes, the films were removed from the slides, washed in fresh PBS without added L101 for 30 minutes and then washed with PBS (3 times for a total time between 10 and 15 minutes). Films were maintained in either complete tissue culture medium, or in PBS, similar to the maintenance of capsules. A solvent cast film of polymer was prepared by overnight evaporation of 0.2 mL of 10% (w/v) HEMA-MMA polymer solution in ethanol placed on a glass cover slip in a sterile hood.

Capsules and precipitated films were PBS washed (3 times for a total time between 10 and 15 minutes; similar to what was done before implantation) or not PBS washed and prepared for XPS analysis by freeze drying for 72 hours.

XPS Surface Analysis

XPS spectra were obtained on a Leybold MAX200 x-ray photoelectron spectrometer (Centre for Biomaterials, University of Toronto). For XPS analysis, ~100 capsules or a piece of film were mounted on sticky-backed copper tape and placed normal to the electron analyzer. For capsules, the depth probed varied slightly due to surface curvature. Staggered placement of the capsules, ensured that any gaps between the
spherical capsules were shadowed from the x-ray source (55° from the normal), hence all the signal came from the sample. Unmonochromatized Al Kα x-ray radiation, run at 15 kV and 30 mA or unmonochromatized Mg Kα x-ray radiation run at 12 kV and 25 mA, was used as the excitation source gathering a signal from a large sample area (4 mm X 7 mm) in order to minimize collection time and hence, possible sample damage. Atomic percentages of elements were derived from low resolution spectra typically obtained using the Mg Kα X-ray source (pass energy 192 eV) which were corrected for the transmission function of the electron spectrometer by means of a routine provided by the manufacturer (323) and using the following sensitivity factors appropriate for these spectra. High resolution C 1s spectra were also obtained using the Al Kα or Mg Kα source (pass energy 48 eV) and resolved using curve fitting routines ESCATOOLS (Surface/Interface, Mountain View, CA). For almost all samples, differential charging was not apparent and the energy scale was adjusted to place the hydrocarbon peak at 285 eV.

RESULTS

A low resolution survey scan of a HEMA-MMA ethanol-cast film indicated an elemental composition of 68% carbon, 31% oxygen with traces of nitrogen and chlorine. A small amount of silicon contamination (1 - 2%), presumably from the laboratory environment (e.g., tissue culture bench) was present on all capsule and film samples. The relative amounts of C-C, C-O, and O-C=O carbon bonds (Table 4.1.1) agreed reasonably well with the stoichiometric values.
Table 4.1.1 Carbon Atom Bonding (% ± S.D.) from C1s High Resolution Spectra - HEMA-MMA Capsules

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Theory</th>
<th>Ethanol Cast Film</th>
<th>Incubation for 1 Week (^a)</th>
<th>Fresh (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS Wash</td>
<td>2 *</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td># of Samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C (285 eV)</td>
<td>52</td>
<td>48 ± 1</td>
<td>52 ± 6</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>C-O (286.6 eV)</td>
<td>30</td>
<td>37 ± 1</td>
<td>33 ± 4</td>
<td>49 ± 4</td>
</tr>
<tr>
<td>O=C=O (289 eV)</td>
<td>17</td>
<td>15 ± 0</td>
<td>14 ± 2</td>
<td>9 ± 0.5</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± Range/2
\(^b\) Capsules were incubated for 1 week in indicated incubation medium, washed with PBS or not
\(^\) freshly-made capsules
The elemental surface compositions of HEMA-MMA capsules maintained in α-MEM with or without serum proteins or in PBS and then washed are shown in Table 4.1.2. A survey spectrum is shown in Figure 4.1.1 for capsules incubated in medium containing serum. Without washing, capsules incubated in medium containing serum had a high nitrogen content (6.2 %) which decreased with washing to 1.2 %. There was no nitrogen on freshly-made capsules. There was a small amount of nitrogen on capsules incubated in α-MEM without added serum proteins, presumably due to the presence of amino acids in the base medium. Washing these capsules with PBS resulted in a reduction in the nitrogen content from 0.9 % to 0.2 %. There was no detectable Ca on the surface of capsules maintained in PBS for 1 week or on freshly prepared capsules. Low levels of calcium were detected, however, on capsules maintained in medium containing serum, even after washing with PBS.

The high resolution carbon, C 1s, spectrum of unwashed capsules maintained in medium with serum is shown in Fig. 4.1.2a. The C 1s envelope was fitted with three peaks corresponding to C-C (285 eV), C-O (286.6 eV) and O=C-O (289 eV). The highest peak corresponded to C-C. An intermediate peak at a binding energy between C-O and O=C-O, due to C-N bonding (from serum protein), was not resolvable. After washing, the C 1s spectrum of the capsules maintained in medium with serum (Fig. 4.1.2b) appeared to approach that of the capsules which had been maintained in medium without added serum proteins (Fig. 4.1.2c and Fig. 4.1.2d). The high resolution spectrum, for
Table 4.1.2 Elemental Composition (% ± S.D.) of HEMA-MMA Capsules

<table>
<thead>
<tr>
<th>Incubation</th>
<th>α-MEM + 10% FBS</th>
<th>α-MEM</th>
<th>PBS</th>
<th>Fresh</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS Wash</td>
<td>Not Washed</td>
<td>Washed</td>
<td>Not Washed</td>
<td>Washed</td>
</tr>
<tr>
<td># of Samples</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C 1s</td>
<td>69 ± 0.8</td>
<td>62 ± 2.6</td>
<td>71</td>
<td>69</td>
</tr>
<tr>
<td>O 1s</td>
<td>21 ± 0.5</td>
<td>26 ± 0.4</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>N 1s</td>
<td>6.2 ± 0.7</td>
<td>1.2 ± 0.2</td>
<td>0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Na KLL</td>
<td>1.7 ± 0.2</td>
<td>5.4 ± 1.7</td>
<td>1.7</td>
<td>2.8</td>
</tr>
<tr>
<td>Cl 2p</td>
<td>0.9 ± 0.3</td>
<td>3.3 ± 1</td>
<td>0.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Si 2p</td>
<td>0.9 ± 0.1</td>
<td>2.1 ± 0.3</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>P 2p</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.4</td>
<td>n.d</td>
<td>0.2</td>
</tr>
<tr>
<td>Ca 2p</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.03</td>
<td>n.d</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. = not detectable
* Mean ± range/2
* Capsules were incubated for 1 week in indicated incubation medium, washed with PBS or not
b freshly-made capsules
Figure 4.1.1  Survey spectrum of HEMA-MMA capsules maintained in α-MEM with 10% FBS for 1 week before washing with PBS. Note the relatively large nitrogen peak attributed to adsorbed protein.
Figure 4.1.2 High resolution C1s spectra of HEMA-MMA capsules maintained in α-MEM with 10% FBS (1 week), then (a) not washed, (b) PBS washed, maintained in α-MEM without FBS (1 week), then (c) not washed, (d) PBS washed. Symbols: o, experimental data; smooth curves represent best fits of the experimental data and C-C, C-O, and O-C=O peak fits.
HEMA-MMA capsules maintained in α-MEM without added serum proteins (Fig. 4.1.2c and Fig. 4.1.2d) showed more carbon bonded in the C-O form than in the C-C form and was similar to that for capsules which had been maintained in PBS for 1 week (Fig. 4.1.3a). The high resolution spectrum for freshly-made capsules (Fig. 4.1.3b) shows C-C and C-O peaks which were resolved with slightly more carbon being bonded in the C-O form. Carbon bondings for capsule samples are given in Table 4.1.1.

Since the Pluronic surfactant (a polyether) was presumed to be the source of the high C-O content, films of HEMA-MMA were precipitated in the presence and absence of the Pluronic L101 surfactant. HEMA-MMA film, precipitated in PBS without added L101 and incubated in PBS had a surface which was similar to that of the ethanol cast film (Table 4.1.3). However, the intensity of the C-O peak was higher with respect to the C-C peak for films made with L101 in the PBS precipitation bath (Table 4.1.3). High resolution carbon spectra of films resembled those of the corresponding capsules maintained in PBS (Fig. 4.1.3a) or in serum-containing medium without (Fig. 4.1.2a) and with PBS washing (Fig. 4.1.2b). Furthermore, the carbon bonding compositions were similar (compare Table 4.1.1 and 4.1.3). As the adsorbed protein layer was removed with PBS washings, the underlying adsorbed surfactant was exposed, resulting in a decrease in the ratio of C-C to C-O.
Figure 4.1.3  High resolution C1s spectra of HEMA-MMA capsules (a) maintained in PBS (1 week) then PBS washed, and (b) freshly-made capsules which were PBS washed. Symbols: o, experimental data; smooth curves represent best fits of the experimental data and C-C, C-O, and O-C=O peak fits.
Table 4.1.3 Carbon Atom Bonding (% ± S.D.) from C1s High Resolution Spectra - HEMA-MMA Films

<table>
<thead>
<tr>
<th>Film Type</th>
<th>EtOH Cast</th>
<th>PBS-Precipitated</th>
<th>PBS + L101-Precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-MEM + 10 % FBS</td>
<td>PBS α-MEM + 10 % FBS</td>
</tr>
<tr>
<td>Incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS Wash</td>
<td>Not Washed</td>
<td>Washed</td>
<td>Washed</td>
</tr>
<tr>
<td># of Samples</td>
<td>2 *</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C</td>
<td>48 ± 1</td>
<td>47 ± 3.2</td>
<td>45 ± 3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-O</td>
<td>37 ± 1</td>
<td>39 ± 4.4</td>
<td>42 ± 4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O=C-O</td>
<td>15 ± 0</td>
<td>13 ± 1.5</td>
<td>13 ± 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± Range/2
DISCUSSION

Adsorbed Pluronic L101 Surfactant

High resolution XPS analysis of HEMA-MMA films prepared by casting from an ethanol solution or by precipitation in PBS gave a composition which agreed well with that expected from the stoichiometry: most carbon was singly bonded to other carbons (48 % C-C). On the other hand, high resolution analysis of freshly-made capsules or capsules maintained in PBS or α-MEM for 1 week showed that the surface was not that of pure HEMA-MMA. In these spectra, more carbon was bonded in the C-O form than in the C-C form at amounts much higher than would be expected for pure HEMA-MMA. This indicated the presence of Pluronic surfactant, L101, that was adsorbed from the precipitation bath. Films precipitated in PBS containing L101 also showed more carbon bonded in the C-O form than in the C-C form, confirming that L101 had adsorbed to the surface of the films. Similar percentages of carbon bound in the three forms (C-C, C-O, O=C-O) were obtained whether the temperature of the capsule precipitation bath (100 ppm L101 in PBS) was above (23 °C) or below (14 °C) the solution cloud point [15 °C for a 1 % (w/v) aqueous solution (324)] indicating that the adsorption of the Pluronic surfactant did not depend on whether the surfactant was in solution or precipitated (data not shown).
Polymer surfaces have been deliberately modified with poly(ethylene oxide) (PEO) groups either by the direct adsorption of Pluronic surfactants (325) or by chemical techniques (326). PEO-modified surfaces are characteristically resistant to protein adsorption (325, 326), presumably due to its neutral charge and its high hydrophilicity which can repel other macromolecules by steric repulsion or excluded volume mechanisms (327, 328, 329, 330). The protein resistant properties imparted to a polymer coated with a Pluronic surfactant has been attributed to the PEO units but the PPO units, even though they are less hydrophilic than PEO, could also contribute to the protein resistant characteristic of the surfactant coating. Because of the reduced amount of adhesive proteins (and others), these surfaces do not support cell adhesion and hence are considered more biocompatible (330). A PEO layer hinders the approach and attachment of inflammatory cells such as phagocytic macrophages (331), fibroblasts (326), platelets (332) and bacteria (333) to the biomaterial surface.

These features of PEO have been exploited in the immunoisolation literature. Alginate-polylysine microcapsules were modified by the use of a monomethoxy poly(ethylene glycol) (MPEG) modified polylysine (110). This treatment reduced fibrinogen, albumin, and iC3b adsorption, and the MPEG treated capsules were unable to support fibroblast attachment. Microcapsules coated with a more stable layer of PEG by photopolymerization were free floating without attached cells in the peritoneal cavity of mice up to 20 days postimplantation, unlike uncoated microcapsules, which were recovered with cellular overgrowth within 4 days (125). Poly(acrylonitrile-co-vinyl
chloride) (PAN/VC) hollow fiber membranes have also been chemically-modified with PEO, resulting in an implant which was resistant to bovine serum albumin (BSA) adsorption and with slightly improved biocompatibility in the rat brain compared to the unmodified controls (334). The membrane transport properties and mechanical properties were unaffected by the chemistry involved. While chemical grafting of PEO onto the polymer surface should result in a more stable surface modification, adsorbed PEO-NH2 (5k) was as effective as grafted PEO-NH2 at reducing BSA adsorption on PAN/VC hollow fiber membranes (334). Another method for grafting PEO chains on microcapsules made from polyacrylamide-co-methyl methacrylate-co-vinyl amine is currently being developed (335). In addition to improving the biocompatibility of cell-containing polymer implants, and reducing the amount of protein adsorbed in vivo (336), PEO modification is expected to also decrease the amount of protein adsorbed in vitro (337) during a pre-implantation incubation in serum-containing medium. Proteins adsorbed to the surface of cell-containing polymer implants during an incubation in serum-containing medium have been shown to increase the tissue response to these implants (304, 334, 338).

The amount of surfactant adsorbed and the stability of its association with HEMA-MMA will affect whether adsorbed L101 can have a significant and sustained effect on protein adsorption, in vitro and in vivo, and on capsule biocompatibility after implantation. In vitro, its stability appears to be sufficient since the surface of capsules maintained in PBS for 1 week was similar to that of freshly-made capsules. However, it is not known if
adsorbed L101 would be retained and/or be effective *in vivo*. Adsorbed Pluronic surfactants may be displaced by plasma proteins (339). Similarly, a reduction in the phagocytic uptake of particles coated with Pluronics was observed *in vitro* but the adsorbed Pluronic was not protective against immunological recognition of the coated particle *in vivo* (340).

On the other hand, the ability of adsorbed Pluronic surfactants to reduce protein adsorption and prevent platelet adhesion and activation has been found to be dependent on their composition, being more strongly dependent on the number of PPO units than on the number of PEO units (332). Pluronics which were well anchored to the particle surface with at least 39 PPO units, reduced particle phagocytosis to an extent that increased proportionally with the number of PEO units (341). Steric stabilization increases with PEO length (342). Efficient reductions in particle phagocytosis (341), fibrinogen adsorption and platelet adhesion and activation (332), *in vitro*, could be achieved with very short chains of 7 or 21 EO units if using Pluronics with a central PO blocks ranging from 47 to 67 units. Pluronic F127, with 67 PO units forming the hydrophobic anchor, combined with 98 EO units at each end, was most efficient at preventing particle phagocytosis (341). The strength of the PPO anchor determined whether the adsorbed surfactant would be susceptible to displacement by large-molecular-weight proteins (337). In apparent contrast to these results and explanations, in another study, the amount of human serum albumin (HSA) adsorbed to polystyrene microparticles coated with Pluronic or Tetronic surfactant (as compared to control PS) decreased with increasing PEO content.
for the same number of PPO units but not with increasing PPO content (343). In vivo, the amount of peritoneal proteins adsorbed onto PS microparticles coated with Pluronic surfactants was reduced (but the types of proteins types unchanged), which unlike the in vitro situation, was independent of surfactant PPO or PEO chain lengths (336).

Pluronic surfactant, L101 was added to the precipitation bath to lower the interfacial tension between the hexadecane overlayer and the PBS precipitation bath to facilitate the passage of capsule droplets into the bath. It was not chosen with consideration for the biological effects it might have. L101 is comprised of 54 PPO units, flanked on either side by only 7 PEO units. Larger PEO blocks would be preferred because of the known effect of PEO chain length on the steric stabilization effect (332, 341, 342). A large PPO block would presumably be useful in anchoring the Pluronic to the surface and enhancing the stability of its association (332, 341). Based on these considerations, the Pluronic surfactant, F127 (PEO$_{98}$-PPO$_{67}$-PEO$_{98}$), was selected as an alternative to L101 for microencapsulation. The reported interfacial tension value was still low enough for its use in the encapsulation process; 7.5 dynes/cm for F127 as compared to 3.5 dynes/cm for L101 (324). Microcapsules prepared using a PBS precipitation bath containing 100 ppm of Pluronic F127 also had a higher percentage of carbon bonded as C-O form (42 % ± 3 %, C-C; 46 % ± 3 %, C-O; 12 % ± 3 % O=C-O: n=3 samples) (344). The effect of the longer PEO and PPO blocks of F127 on capsule biocompatibility and protein adsorption however, remains to be demonstrated. Alternatively, the surface chemistry of HEMA-MMA beads could be further modified by a post treatment with a
PBS solution containing 1000 ppm of Pluronic L101, Tetronic T908 \([(PEO_{120})_2-(PPO_{16})_2-NCH_2CH_2N-(PPO_{16})_2-(PEO_{120})_2]\), and Pluronic F127, but the Pluronics P105 (PEO_{38}-PPO_{54}-PEO_{38}) and F108 (PEO_{128}-PPO_{54}-PEO_{128}) were not effective (344). Hence, several techniques are available for modifying the surface of HEMA-MMA capsules using other surfactants that may have beneficial effects on biocompatibility.

It was not possible to determine how much Pluronic surfactant was adsorbed to the capsule surface since there was no elemental or structural distinguishing feature in XPS spectra to allow for its quantification. Also, the surface composition analyzed in this study was that of freeze dried capsules and would presumably be different from the actual hydrated form of the capsule. For example, the conformation of the adsorbed surfactant and the extent to which the PEO block extends into the aqueous medium can not be measured.

**Adsorbed Protein and Other Components**

The presence of nitrogen in the low resolution spectrum (Figure 4.1.1) was attributed to serum protein adsorbed from the incubation medium to the capsule surface. Washing capsules with PBS reduced the nitrogen content but not to zero, as much of the protein was removed but some remained. These residual serum proteins, which are typically xenogeneic to the implant recipient, have been shown to increase the tissue response to these implants (304, 334, 338) resulting in thicker tissue reactions with a
thicker layer of macrophages apposed to the implant surface (304, 338). The composition of the protein adsorbate associated with HEMA-MMA capsules has been determined in part using immunoblotting (see Chapter 4.2).

Low levels of calcium were found on capsules maintained in medium containing serum, even after washing with PBS. In these same samples, phosphorous was also found. Calcium binding to surface-bound proteins, which in solution do not bind calcium, has been reported by others (345). While calcium phosphate could act as nucleation sites for calcification of HEMA-MMA capsules in vivo, other mechanisms could apply (346, 347). Calcium phosphate precipitated at nearly undetectable levels during in vitro maintenance of microcapsules in medium containing serum, as assessed by von Kossa staining of histological sections (304). After microcapsule implantation for a least 4 days, there was evidence of calcium salt deposits, specifically identified by von Kossa staining and EDAX microanalysis [scanning electron microscope (SEM) equipped with x-ray diffraction analysis and a backscatter detector] (348), within HEMA-MMA capsules which had been maintained in PBS before implantation and had no XPS-detectable calcium before implantation.
CONCLUSIONS

X-ray photoelectron spectroscopy permitted the determination of the elemental composition and the carbon bonding chemistry of the surface of HEMA-MMA copolymer microcapsules. The surface of microcapsules was modified during their preparation from that of pure HEMA-MMA as Pluronic surfactant, L101, adsorbed from the precipitation bath. Proteins were adsorbed to the microcapsule surface from a solution of medium containing fetal bovine serum during a one week incubation. Adsorbed proteins were not completely removed by washing capsule with PBS. Calcium was detectable only on capsules with adsorbed serum proteins.
4.2 Immunoblot Analysis of Proteins Associated With HEMA-MMA Microcapsules: Human Serum Proteins In Vitro and Rat Proteins Following Implantation

ABSTRACT

Human serum proteins and their activation fragments were associated with hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) copolymer microcapsules following their 1 week in vitro incubation in medium containing human serum (HS), were characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. Almost all proteins probed for in the immunoblots were found in the capsule eluates. This included fibronectin, plasminogen, IgG, vitronectin, Factor B, Factor H, Factor I, C3, but not β-lipoprotein, fibrinogen, HMWK, or IgM. Complement activation fragments were also detected in the immunoblots of capsule eluates and in the medium containing serum without capsules. Thus, the adsorption of these fragments, formed independent of capsule presence, may be partially or completely responsible for the complement fragments found with capsules. The prevention of complement activation by the addition of 5.8 mM EDTA resulted in fewer low molecular weight C3 fragments associated with capsules. Similarly, rat proteins were detected in

---

4 This work was performed in collaboration with Rena M. Cornelius and John L. Brash at the Department of Chemical Engineering, McMaster University, Hamilton, Ontario. Protein sample PAGE-SDS and Immunoblot analysis was performed by Rena Cornelius. JEB designed the experiments, prepared the samples for PAGE-SDS, wrote this test, and presented the work at the 21st Annual Meeting of the Society for Biomaterials, San Francisco, USA, March 18-22, 1995. The data analysis in this text was also that of this author.
immunoblots of the eluate of “free floating” capsules from the rat peritoneal cavity following implantation for 1 day using human antibodies which cross-reacted with rat HMWK, fibrinogen, antithrombin III, transferrin, α1-antitrypsin, fibronectin, albumin, α2-macroglobulin, vitronectin, β2-microglobulin, Factor B, and Factor I. Rat fibrinogen, IgG, and complement C3 fragments were also detected, in these immunoblots with monoclonal antibodies against the rat proteins.

INTRODUCTION

Polymer microcapsules containing living cells are being investigated as a means of providing a host with an adequate mass of cells to ameliorate a disease caused by the deficiency of a particular biomolecule or as a means of replacing the function of a diseased organ. The polymer membrane prevents or restricts the passage of cytotoxic components of the host’s immune system which could be deleterious to the encapsulated cells.

Prior to the implantation of cell-containing microcapsules, they may be maintained in tissue culture medium containing serum. The serum is typically of a different species (xenogeneic) [e.g., fetal bovine serum (FBS)] from the implant host, at least in pre-clinical implantation studies. For hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA; 75 mole % HEMA) capsules, this time period (typically 1 week), allows for the capsule wall to fully form with a consequent reduction of the permeability of the capsules to high molecular weight components of the immune system such as the potentially cell-lysing
complement components (244). Moreover, cells can become accustomed to their intracapsular environment and depending on the cell type, proliferate to increase the transplanted cell number. A time lag between encapsulation of living cells and device implantation may also be required because of the limited availability of donor tissue, the time of implantation, and to facilitate storage or transportation. X-ray photoelectron spectroscopy analysis of the surface of HEMA-MMA microcapsules maintained in medium containing FBS demonstrated a nitrogen (N1s) signal, indicating the presence of adsorbed serum proteins which were not fully eliminated by washing with PBS (307). These adsorbed serum proteins influenced the biocompatibility of the HEMA-MMA capsule upon implantation: a thicker tissue reaction with a thicker layer of macrophages apposed to the capsules was observed with FBS incubated capsules relative to capsules incubated without serum (304, 338)

In this study, proteins associated with HEMA-MMA capsules were characterized using PAGE-SDS and immunoblotting with specific antibodies for proteins after in vitro incubation in medium containing human serum and after in vivo implantation into rats. Capsules were incubated in medium containing human serum, and not with fetal bovine serum, because anti-human monoclonal antibodies were readily available. The human serum proteins which were specifically probed for included cell adhesive proteins, complement components, coagulation proteins, as well as abundant serum proteins such as albumin and IgG. The effect of serum heat inactivation and calcium chelation with EDTA
on complement components in particular was also investigated. Finally, the proteins associated with capsules implanted into rats were characterized.

MATERIALS AND METHODS

Serum Preparation

Venous blood was collected from two healthy human donors using a butterfly needle and sterile 50 mL syringe. The blood was centrifuged (3000 rpm, 15 min.) and the plasma supernatant was collected, allowed to clot overnight at 4 °C and centrifuged again. The sera were pooled. Human serum was heat inactivated in a water bath at 56 °C for 30 min. For capsule incubation in vitro, three media were prepared by supplementing α-MEM (prepared from powdered medium, Gibco, Grand Island, NY, at the Tissue Culture Medium Preparation Facility at the University of Toronto, Toronto, Ontario) containing 100 U/ml penicillin and 100 ng/mL streptomycin with human serum (HS), with or without EDTA (5.8 mM), or with heat inactivated human serum (h.i. HS) to a concentration of 10 % (v/v). Each medium was filter sterilized (Corning 200 mL Filter System, 0.22 μm Cellulose Acetate sterilizing, low protein binding membrane, Corning, NY, USA) before being used.

Rat blood was collected by sterile cardiac puncture. Rat sera were prepared from three male Wistar rats (250 - 350 g) (Charles River Canada Inc., St. Constant, Quebec,
Canada) using the same technique as described above for human serum, except that sera were pooled.

**HEMA-MMA Microcapsule Preparation**

Empty HEMA-MMA (75 mole % HEMA) copolymer microcapsules (~ 750 μm in diameter) were prepared using the submerged jet interfacial precipitation method, as previously described (237); the capsule core contained 20% (w/v) Ficoll-400 in α-MEM without serum.

**In Vitro Incubation of Capsules With Human Serum**

Freshly-prepared HEMA-MMA capsules (~ 200-250 capsules) were incubated (1 wk) in each medium type (10-15 mL) under sterile tissue culture conditions at 37 °C in a humidified atmosphere of 5% CO₂/95% air (medium pH 7.8 ± 0.4 during incubation). Capsule incubation in α-MEM with 10% HS was similar to the regime followed before implantation of microcapsules, except that fetal bovine serum was used then (304). As controls, incubation media were maintained similarly but without added capsules. Medium was changed on the day following capsule preparation and again on day 4. On day 7, capsules (~ 200-250 capsules) were washed with phosphate buffered saline (PBS Ca²⁺/Mg²⁺-free: 0.2 g/L KCl; 0.2 g/L KH₂PO₄; 8 g/L NaCl; 2.16 g/L Na₂HPO₄·H₂O;
Gibco) (3 times for a total time of 15 min) or alternatively, not washed and then placed into a sterile eppendorf tube.

**In Vivo Capsule “Incubation”**

Freshly-made microcapsules were maintained in PBS for 1 week and rinsed with PBS (3 times for a total time of 15 min.) before implantation into the peritoneal cavity of male Wistar rats (250-350 g) using a sterile custom-made Pasture pipette with a flame-polished tip (Glass Blower, Department of Chemical Engineering and Applied Chemistry, University of Toronto) through a small ventral incision at the midline. A suspension of capsules in PBS (175 - 200 capsules per rat) was implanted for 1 day or 10 minutes. For 10 minute implants, the incision was held closed with tweezers while the abdomen was gently massaged to distribute the capsules and expose them to the peritoneal fluid. At explantation, approximately half of the recovered capsules (35 - 69 capsules) were placed into a sterile eppendorf tube and not washed with PBS; the remainder of the capsules (60 - 83 capsules) were placed in a second eppendorf tube and washed with PBS (1 mL; 3 times for a total time of 15 min.).

**Protein Solubilization**

Ethanol (100%, 1 mL, 4 °C) was added to the eppendorf tubes containing capsules to dissolve the capsules and precipitate the proteins. *In vivo* samples were kept on ice
until most of the capsules had dissolved with periodic vigorous vortexing. All samples were ultracentrifuged (2 times 30 min. each, 13,000 g, 4 °C) and the ethanol precipitated protein pellet was dissolved in sterile 2% (w/v) sodium dodecyl sulfate (SDS) (Ultra Pure) in Tris Buffered Saline [25 mM TRIS base (Tris-(hydroxymethyl)-aminomethane), 8 g/L NaCl, 0.2 g/L KCl, pH = 7.4] overnight on a shaker. Protein solutions in SDS, and relevant control mixtures were aliquoted and stored at -70 °C until analysis.

**Polycrylamide Gel Electrophoresis and Immunoblotting**

Polyclonal antibodies to human and rat plasma proteins and alkaline phosphatase conjugated secondary antibodies used in immunoblotting are listed in Table 4.2.1.

SDS-PAGE and immunoblots were run on the protein samples using the method described elsewhere (349). Briefly, SDS-protein solutions (10-80 μL; protein concentration 0.4 - 0.5 mg/mL) were loaded onto an acrylamide gel consisting of a 12% separating gel and a 4% stacking gel. The separated proteins were then electrophoretically blotted onto Immobilon PVDF membrane (Millipore Co., Bedford, MA). The ‘blot’ was blocked with a 5% solution of nonfat dry milk in 50 mM Tris buffered saline (50 mM TRIS base, 150 mM NaCl; pH 7.4), cut into strips, and the strips were incubated with primary antibodies to the proteins listed in Table 4.2.1, diluted 1/1000, in 1 % (w/v) nonfat dry milk and 0.05 % (v/v) Tween 20 in Tris buffered saline.
(50 mM TRIS base, 150 mM NaCl; pH 7.4). Antibody binding was detected with an alkaline phosphatase conjugated secondary antibody, diluted 1/1000 in 1 % (w/v) nonfat dry milk and 0.05 % (v/v) Tween 20 in Tris buffered saline (50 mM TRIS base, 150 mM NaCl; pH 7.4), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (both from Bio-Rad) as substrates. Prestained markers (Bio-Rad) were run concurrently.

PAGE gels were washed twice with PBS (pH 7.4) and unbound membrane sites were blocked with 0.3 % (v/v) Tween 20 in PBS at 37 °C for 1 hour. The membrane was then stained overnight with Protogold solution (Biocell Research Laboratories, Cardiff, UK, distributed by Cedarlane Laboratories, Hornby, Ontario, Canada).

**Total Protein Content**

An aliquot of ~ 20 - 30 capsules was dissolved and associated proteins precipitated in 100% ethanol as above. The resultant protein pellet was dissolved in Lowry’s solution (1 mL, 20 min with vigorous vortexing) (Protein Assay Kit No. 5656, Sigma Diagnostics, St. Louis, MO, USA), to which was added 1 mL of distilled, deionized water and 0.5 mL of Folin & Ciocalteu’s phenol reagent working solution (Sigma Diagnostics) with vortexing. The solution colour was developed for 30 minutes and the absorbance measured at 750 nm. The protein concentration was determined with a calibration curve based on ethanol-precipitated bovine serum albumin, fraction V (standard solutions of
<table>
<thead>
<tr>
<th>Source of Antibody</th>
<th>Protein</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CedarLane Laboratories Inc., Hornby, Ontario, Canada</td>
<td>Preeallkirein</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>HMWK</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>Antithrombin III</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>Albumin</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>Prothrombin</td>
<td>goat</td>
</tr>
<tr>
<td>Sigma Chemical Company, St. Louis, MO, USA</td>
<td>Fibrinogen</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>Plasminogen</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>Transferrin</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>b-Lipoprotein</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>a2-Macroglobulin</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>b2-Microglobulin</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td>Haemoglobin</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td>Haptoglobin</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td>Complement C3c</td>
<td>rabbit</td>
</tr>
<tr>
<td>Calbiochem Corporation, La Jolla, CA, USA</td>
<td>Fibronectin</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td>Protein C</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td>Vitronectin</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td>Complement C3</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>Factor B</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>Factor H</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>Factor I</td>
<td>goat</td>
</tr>
<tr>
<td>Cappel Laboratories, Cochraneville, PA, USA</td>
<td>a1-Antitrypsin</td>
<td>goat</td>
</tr>
<tr>
<td><strong>Rat Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organon Teknika Inc., Scarborough, Ontario, Canada</td>
<td>Complement C3</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen</td>
<td>goat</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>goat</td>
</tr>
<tr>
<td><strong>Alkaline Phosphatase Conjugated Secondary Antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sigma Chemical Company, St. Louis, MO, USA</td>
<td>goat IgG</td>
<td>rabbit</td>
</tr>
<tr>
<td>Bio-Rad, Richmond, CA, USA</td>
<td>rabbit IgG</td>
<td>goat</td>
</tr>
</tbody>
</table>
concentrations 2 to 160 µg/mL). Similar total protein results were obtained by dissolving capsules directly in Lowry's Solution (1 mL, 20 min.) without ethanol precipitation.

RESULTS

Total Protein Content of HEMA-MMA Capsules

The protein content of unwashed capsule samples following a one week incubation in α-MEM containing 10% human serum with and without 5.8 mM EDTA was 5.26 µg/capsule and 6.08 µg/capsule, respectively. This included the protein in the medium transferred along with the capsules. After washing with PBS, the protein content was reduced to 2.21 µg/capsule or 2.28 µg/capsule (with or without EDTA), respectively. The amount of protein in the PBS washes decreased with each subsequent wash step.

Human Serum Proteins Associated With HEMA-MMA Capsules In Vitro

The majority of human serum proteins probed for in the immunoblot were found to have been associated with the HEMA-MMA capsules following a one week incubation in α-MEM containing 10% human serum (Figure 4.2.1, Table 4.2.2).

Figure 4.2.1 Immunoblot of human serum proteins associated with HEMA-MMA capsules following 1 week incubation in α-MEM + 10% human serum. Prestained markers, of the molecular weights indicated at the left hand side, were used to estimate the molecular weight of the bands. Molecular weights, fragment assignment, where possible, and intensity of major bands are listed in Table 2.
<table>
<thead>
<tr>
<th>Capsule Incubation Media</th>
<th>Protein</th>
<th>MW (kDa)</th>
<th>Assignment</th>
<th>α-MEM + 10% Human Serum</th>
<th>α-MEM + 10% Human Serum + 5.8 mM EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsule Incubation Media</td>
<td>Prekallikrein</td>
<td>110</td>
<td>intact</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>88</td>
<td>intact</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52</td>
<td>heavy chain</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>light chain</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HMWK</td>
<td>110</td>
<td>intact</td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>heavy chain degradation fragment</td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>light chain degradation fragment</td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>50</td>
<td>intact</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Plasminogen</td>
<td>98</td>
<td>intact</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>97</td>
<td></td>
<td>+</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td></td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>88</td>
<td></td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>66-55</td>
<td></td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>119</td>
<td>C3 (α)</td>
<td>+</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>C3b (α')</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>97</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>β</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>66</td>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>64</td>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>intact fragment</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>-------</td>
<td>----</td>
<td>----------------</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Factor B</td>
<td>90</td>
<td>intact</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Bb</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>Ba</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Factor H</td>
<td>150</td>
<td>intact</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Factor I</td>
<td>50</td>
<td>intact</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IgM</td>
<td>70</td>
<td>heavy chain</td>
<td>++</td>
<td>ND</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>heavy chain</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>light chain</td>
<td>-</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

++; dark band: +; faint band: -; not determined
* corresponds to bands in Figure 1
Fibrinogen, β₂-lipoprotein, and β₂-microglobulin were not found in Run 1, while in Run 2, HMWK, β₂-lipoprotein, and IgM were not found. Except for C3 and Factor B, as described below, EDTA at a concentration of 5.8 mM did not affect the proteins identified (Table 4.2.2).

In the results shown in Figure 1 and tabulated in Table 4.2.2, it is noteworthy that cell adhesive proteins fibronectin and vitronectin, the coagulation proteins prekallikrein, HMWK, and plasminogen and the antibodies IgG and IgM were detected. Fibrinogen was resolved into only faint low molecular weight bands since most of the fibrinogen had been converted to fibrin during serum preparation. Factor B was detected in its intact form as well as the Bb fragment and the Ba fragment. The complement activation regulators Factor H and Factor I were also identified. Immunodetection of C3 (lane 6) indicated a faint band corresponding to intact α and β chains and activation fragments C3b (α' chain), C3f + iC3b, C3dg, iC3b, and C3d. Immunodetection of C3c (lane 20) identified a band at ~70 kDa, presumably due to cross-reactivity with the β chain and C3c. These low molecular weight fragments indicated the presence of activated complement in the medium in which the capsules had been maintained.

Further insight into the presence of activated complement in the medium was obtained by examining the C3 bands of the eluates from capsules maintained in the presence or absence of human serum (heat inactivated or not), or EDTA and the various control media (Figure 4.2.2a, Table 4.2.3). C3 immunoblotting of the eluate from
capsules maintained in medium containing 10% human serum (lane 1) showed the same C3 fragments as in lane 6 in Figure 1 and in the medium in which these capsules had been maintained (lane 7) (except that in the medium, no α chain was detectable). While the C3 α chain was detected in the medium with human serum that was maintained for 7 days in the absence of capsules (lane 10), the presence of low molecular weight fragments indicated that complement activation occurred during this incubation in the absence of capsules. EDTA decreased the number of low molecular weight fragments and their intensity: lower iC3b, no C3d and no 41 kDa bands were found in the capsule eluate (lane 2). However, the number and intensity of low molecular weight fragments was not affected in the corresponding medium with EDTA in which these capsules had been maintained (lane 8). Medium containing serum that was not held at 37 °C for 1 week (day 0 medium) (lane 4) showed a higher intensity band at ~ 119 kDa, (α chain), the β chain at ~ 74 kDa and faint low molecular weight bands, indicating some initial complement activation. The day 0 heat inactivated serum sample also showed the presence of low molecular weight fragments (lane 6). Although heat inactivation was expected to prevent the formation of active C3 convertase, low molecular weight fragments were detected with a similar

Figure 4.2.2 Immunoblot of human C3 (A) and Factor B (B) associated with HEMA-MMA capsule eluates (Lanes 1-3), day 0 medium samples (Lanes 4-6), samples of medium in which the capsules in Lanes 1 - 3 were maintained (Lanes 7 - 9) and samples of medium to which capsules had not been added (Lanes 10 - 12). The three medium samples are indicated by the letters (a) α-MEM + 10 % human serum, (b) α-MEM + 10% human serum + 5.8 mM EDTA, and (c) α-MEM + 10 % heat inactivated human serum. Molecular weights, fragment assignment and intensity of major bands are listed in Table 3.
### Table 4 Molecular Weights (kDa), Fragment Assignment and Intensity of Major Bands in C3 and Factor B Immunoblots

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
<th>Assignment</th>
<th>Capsules*</th>
<th>Day 0 Medium</th>
<th>Medium With Capsule Contact</th>
<th>Medium Without Capsule Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HS</td>
<td>HS+ EDTA</td>
<td>h.i. HS</td>
<td>HS+ EDTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HS</td>
<td>HS+ EDTA</td>
<td>h.i. HS</td>
<td>HS+ EDTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HS</td>
<td>HS+ EDTA</td>
<td>h.i. HS</td>
<td>HS+ EDTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HS</td>
<td>HS+ EDTA</td>
<td>h.i. HS</td>
<td>HS+ EDTA</td>
</tr>
<tr>
<td>C3</td>
<td>119</td>
<td>C3 (α)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>C3b (α')</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>β</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>C3f+iC3b</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>C3dg</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>iC3b</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>C3d</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Factor B</td>
<td>90</td>
<td>FB</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Bb</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>Ba</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

++: dark band; +: faint band; -: not detectable

Table corresponds to immunoblots in Figures 2a and 2b
- capsule incubated in medium for 1 week
- medium in which capsules were maintained from days 4 - 7
- medium maintained without capsule contact for days 4 - 7
intensity to that in capsule eluates with heat inactivated serum (lane 3) or with normal serum (lane 1). These fragments were presumably generated during the heat inactivation step.

Factor B immunoblotting (Figure 4.2.2b, Table 4.2.3) of the eluate from capsules maintained in medium containing 10% human serum (lane 1), the medium in which the capsules were maintained (lane 7), and the medium maintained for 7 days in the absence of capsules (lane 10) showed bands corresponding to intact Factor B (~98 kDa) and the cleavage products, fragment Bb and Ba. The same Factor B and fragment bands were found with heat inactivated serum. EDTA decreased the intensity of the Bb and Ba fragment bands: they were absent in the capsule eluate (lane 2), of low intensity in lane 8 (corresponding medium in which these capsules had been maintained), and lane 11 (medium without capsules). There was a greater effect of EDTA on Factor B cleavage fragments than on C3 activation fragments. The day 0 serum sample showed the presence of predominantly intact factor B with faint bands corresponding to the Bb and Ba fragments (lane 4); this band profile was not affected by EDTA (lane 5). Heat inactivation of the serum resulted in darker bands corresponding to Bb and Ba fragments and a slightly less intense band corresponding to the intact Factor B (lane 6) as compared to the normal serum (lane 4).
Rat Proteins Associated With HEMA-MMA Capsules Following Implantation

The proteins associated with HEMA-MMA capsules following their implantation into the rat peritoneal cavity for 1 day were identified using the anti-human antibodies and compared to bands obtained with rat serum obtained by cardiac puncture. The results are presented in Table 4.2.4. Plasminogen, C3, IgG, C3c, Factor H (although detected in the rat serum) were not found associated with the capsule eluates while they had been found after in vitro incubation in human serum. HMWK, antithrombin III, transferrin, α1-antitrypsin, fibronectin, albumin, Factor B, and Factor I were identified by the anti-human antibodies in both the rat serum and capsule eluate. Interestingly, fibrinogen, α2-macroglobulin, vitronectin, and β2-microglobulin were identified in the capsule eluates but antibodies did not cross react with rat serum from cardiac puncture. The remainder of the proteins [prekallikrein, β2-lipoprotein, protein C, prothrombin, haemoglobin, and haptoglobin] were not identified in either the rat serum or capsule eluate, presumably because of the lack of cross-reactivity, although they had been found after in vitro incubation in human serum.

C3, IgG, and fibrinogen were also probed with anti-rat antibodies. Fibrinogen (Figure 4.2.3a) was resolved into three bands of ~ 60, ~ 43, and ~ 28 kDa, corresponding to the α, β, and γ chains, respectively, in the eluate of capsules implanted for 1 day; intact fibrinogen was not detected in the rat serum, as expected, but it was detected in the eluate from capsules implanted for 10 minutes (results not shown). Only a very faint IgG band
was detected at ~ 55 kDa (heavy chain) in the capsule eluate after 1 day (Figure 4.2.3b) and 10 minutes of implantation, while both heavy and light (~ 28 kDa) chains were found in the serum. C3 was resolved into (Figure 4.2.3c) two bands; ~ 66 kDa, (the β chain or albumin due to cross-reactivity) and ~ 43 kDa (probably the C3f + iC3b or C3dg chains) in the eluate of capsules implanted for 1 day. No C3b (α' chain) was detected. In the serum sample, bands corresponding to both intact C3 (α,β chains), C3b and low molecular weight C3 fragments were obtained. In the capsule eluates of 10 minute implants, bands corresponding to intact C3 and low molecular weight C3 fragments were obtained (results not shown).

**Figure 4.2.3** Rat peritoneal fluid proteins associated with HEMA-MMA capsules after implantation for 1 day. Immunoblots of (A) fibrinogen, (B) IgG, and (C) Complement C3 of a rat serum sample obtained by cardiac puncture (Lanes 1 and 2) and of capsule eluate (Lanes 3 and 4).
FIBRINOGEN

kD  Serum  Serum  Capsule  Capsule
97.4  —  —  —  —
66  —  —  —  —
45  —  —  —  —
31  —  —  —  —
21.5  —  —  —  —
14.5  —  —  —  —

COMPLEMENT C3

kD  Serum  Serum  Capsule  Capsule
97.4  —  —  —  —
66  —  —  —  —
45  —  —  —  —
31  —  —  —  —
21.5  —  —  —  —
14.5  —  —  —  —

IgG

kD  Serum  Serum  Capsule  Capsule
97.4  —  —  —  —
66  —  —  —  —
45  —  —  —  —
31  —  —  —  —
21.6  —  —  —  —
14.5  —  —  —  —
Table 4.2.4 Rat Proteins Detected Using the Anti-Human Antibodies to the Corresponding Protein

<table>
<thead>
<tr>
<th>Protein</th>
<th>Rat Serum</th>
<th>HEMA-MMA Capsules - 1 Day in Rat Peritoneum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prekallikrein</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HMWK</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Transferrin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>a1-Antitrypsin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibroectin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Albumin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IgG</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>β2-Lipoprotein</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Protein C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β2-Microglobulin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3c</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Factor B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Factor I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Factor H</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IgM</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+; protein band(s) detected; -; no band(s) detected
DISCUSSION

Methodological Aspects of Protein Characterization

Immunoblotting of SDS-PAGE gels of protein adsorbates enabled the identification of specific capsule-adsorbed proteins with high sensitivity and provided indication of degradation. The list of proteins which were specifically probed for in this study has evolved with other protein adsorption studies in Dr. J.L. Brash’s laboratory as they were chosen to address issues of contact phase coagulation, activation of the kinin system, complement activation, and fibrinolysis upon contact with dialyser membranes or other biomaterials. The proteins identified as associated with capsules in this study are only a subset of those actually present since testing for all possible proteins is unrealistic.

The adsorbed proteins were expected to be desorbed using eluants such as 2% SDS in TBS without disturbing the material surface or structure. In preliminary studies, EMA-MMA capsules were found to dissolve in 2% SDS in TBS (350). Consequently, PAGE gels of such eluates did not stack properly and the proteins did not resolve into discrete bands. Thus, the protein desorption process was modified to dissolve the polymer capsules by using 100% ethanol, simultaneously precipitating the proteins and removing the polymer in the ethanol supernatant. The precipitated proteins (and any residual polymer) were then re-dissolved in 2% SDS in TBS. Identical banding patterns were obtained in PAGE gels, for all proteins in both ethanol precipitated and unprecipitated
serum. This indicated that ethanol precipitation did not irreversibly alter the conformation of the proteins or change their cleavage patterns.

The dissolution of capsules prior to protein solubilization in 2% SDS means that the proteins analyzed in this study were those associated with the entire capsule and were not only surface adsorbed proteins; hence we have used the term “capsule-associated proteins”. Furthermore, this protein solution included proteins that had irreversibly bound to the capsule and not just those elutable with SDS. The capsule-associated proteins may thus be different from those whose presence was detected using x-ray photoelectron spectroscopy (Chapter 4.1). The total amount of protein found on the capsule (~ 2 μg/capsule or ~ 125 mg/cm², if confined to the outside surface, based on a capsule diameter of 750 μm) is several orders of magnitude larger than the maximum surface concentration for unperturbed globular proteins in a monolayer (side-on orientation, 0.1 to 0.5 μg/cm², 351). It is most likely that serum proteins had permeated the capsule to load the capsule core and/or adsorb to the surfaces of pores within the capsule wall.

Significance of Proteins Associated With HEMA-MMA Capsules

A variety of serum proteins were associated with HEMA-MMA capsules following their 1 week incubation in medium containing human serum or after implantation intraperitoneally in rats. Cell adhesive proteins, proteins of the blood coagulation and
fibrinolytic system, complement components, and other abundant serum proteins were found (Table 4.2.5). Inflammatory and immune cells, mediating the host response to biomaterials, possess cell-surface integrin receptors to the identified cell adhesive proteins (352, 353, 354, 355). Therefore, it is expected that leukocyte attachment and activation in response to cell adhesive proteins adsorbed on the surface of polymers, would be mediated through these same integrins (373, 375, 356). The complement system proteins are involved directly in regulating cellular inflammatory and immune responses (357). The blood coagulation and fibrinolytic proteins determine the biocompatibility and functional integrity of blood contacting materials (358) and are presumably also involved in the soft tissue biocompatibility of materials.

The range of proteins associated with HEMA-MMA capsules is consistent with studies of protein adsorption on hydroxyethyl methacrylate-ethyl methacrylate (HEMA-EMA) graft copolymers in which the amount of adsorbed fibrinogen, immunoglobulin G, albumin and hemoglobin was at a minimum on copolymers containing 25% EMA in the monomer mixture (351). Adsorbed fibronectin, a cell adhesive protein, mediated the initial spreading of 3T3 cells on various copolymers of HEMA and EMA to an extent depending on the amount adsorbed (359). There was little cell spreading on polymers made with less than 30% EMA. The conformation of the adsorbed fibronectin, presumably by exposing sites for cellular attachment, was as important as the amount of protein adsorbed (360). Even though pure HEMA adsorbed fibrinogen, immunoglobulin, albumin and hemoglobin (351) and fibronectin (359, 360), it was unable to support 3T3
### Table 4.2.5 Classes of Proteins Probed for in Immunoblots

<table>
<thead>
<tr>
<th>Cell Adhesive Proteins</th>
<th>Coagulation Factors</th>
<th>Fibrinolytic Factors</th>
<th>Complement Proteins</th>
<th>Other Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>Prekallikrein</td>
<td>Plasminogen</td>
<td>C3</td>
<td>Transferrin</td>
</tr>
<tr>
<td>Vitronecin</td>
<td>HMWK</td>
<td>α₁-Antitrypsin</td>
<td>C3c</td>
<td>Albumin</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Fibrinogen</td>
<td>α₂-Macroglobulin</td>
<td>Factor B</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Factor H</td>
<td>β-Lipoprotein</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>Protein C</td>
<td></td>
<td>Factor I</td>
<td>β₂-Microglobulin</td>
</tr>
<tr>
<td>α₂-Macroglobulin</td>
<td></td>
<td></td>
<td></td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>Protein C</td>
<td></td>
<td>α₂-Macroglobulin</td>
<td></td>
<td>Haptoglobin</td>
</tr>
<tr>
<td>Prothrombin</td>
<td></td>
<td></td>
<td></td>
<td>IgM</td>
</tr>
</tbody>
</table>
fibroblast spreading (359) or the adhesion of bovine aortic endothelial cells (360), presumably due to HEMA's hydrophilicity and low mechanical stiffness (279, 280, 281)

Complement activation, identified by the presence of C3 fragments such as C3b, C3f + iC3b, C3dg, iC3b, C3d, and Factor B fragments, Ba and Bb, was detected in the immunoblots both of capsule eluates and of control media that had not been exposed to capsules. These fragments, which formed independent of capsule presence during the 1-week in vitro culture period, may be partially or completely responsible for the complement fragments found associated with capsules. [C3a (9 kDa) is of too low a molecular weight to be detected in these gels and the C3 antibody does not bind to this fragment.] Adding EDTA to prevent or reduce the extent of divalent cation-dependent complement activation decreased the number of C3 fragments and their intensity in the capsule eluate. However, there was little effect of EDTA on complement fragments in the medium in the presence and absence of capsules. The requirement for Mg²⁺ in the formation of the alternative pathway C3 convertase is not essential (361). Indeed, complement activation by biomaterials in contact with serum in the presence of EDTA did occur, albeit to a lower extent; only 30% of the complement activation observed in its absence (362). It was hypothesized that the noncovalent interaction of C3(H₂O) or C3b with the artificial surface may induce such a conformation which does not require Mg²⁺ for the formation of the functional C3 convertase. In the study presented here, effects of Ca²⁺ chelation on the extent of complement activation was greater for Factor B than for C3. This indicated that Factor B fragments may be a more reliable indicator of complement
activation, as shown previously (363). Activated complement fragments were present in the starting serum and served as a baseline for subsequent complement activation. Heat inactivation cleaved C3 and factor B to its fragments, precluding the formation of an active C3 convertase and hence prevents further complement activation during the 1 week incubation period. However, by heat inactivation, all the fragments of activated complement are formed (as if the serum had been in contact with a highly activating agonist) and these fragments could become associated with the capsules during their incubation with heat inactivated serum.

This study did not however specifically address whether HEMA-MMA capsules can activate complement, although in a separate study, HEMA/EMA copolymers have been shown to activate complement resulting in bulk C3a generation (364). Other studies have shown that HEMA-MMA (75% HEMA) activates complement with the generation of the terminal membrane attack complex (SC5b-9) and Factor Bb at levels similar to that seen with the moderately complement activating cellulose coil (365, 366).

The protein adsorbate from capsules which had been implanted intraperitoneally in rats was comprised of similar proteins to those found after in vitro incubation in human serum. There is no evidence to suggest that there are proteins adsorbed in vivo which are not adsorbed in vitro. There was no difference observed in the rat proteins associated with capsules which had been implanted for 1 day or 10 minutes, indicating no transient variation in the proteins adsorbed (at least within this time frame). Shorter times may be
required to discern the rapid dynamics of protein adsorption. Since the total protein, albumin, and transferrin concentrations of rat peritoneal fluid are lower than that of plasma (367), implanted capsules were exposed to a protein-lean fluid which was further diluted with the PBS of the capsule suspension. This would reduce the absolute amount of adsorbed protein if the plateau region of the adsorption isotherm was not reached. An extension of the study presented here would be to implant capsules with preadsorbed human proteins into rats and analyze the capsule eluate with antibodies against human and rat proteins (e.g., albumin, fibrinogen, C3, IgG). It is hypothesized that upon implantation of a protein-preadsorbed capsule, rat proteins can displace (at least partially) the foreign pre-adsorbed protein or can adsorb alongside the foreign proteins, although, not all foreign proteins would be displaced. Thus, foreign serum proteins would still be present and may be biologically active for example mediating inflammatory cell adhesion. Furthermore, depending on the species combination of adsorbed proteins and implant host, adsorbed foreign C3 convertase may cross-react with host C3, cleaving it, generating C3a and C3b and amplifying complement activation by the host (368).

There are only a few other studies that have compared protein adsorption from biological fluids in vivo and in vitro (336, 369, 370). The same proteins were adsorbed to polystyrene microspheres in vitro from peritoneal fluid as in vivo in the peritoneal cavity but there were differences in the amounts of proteins adsorbed (336). However, different proteins were adsorbed after in vitro incubation with peritoneal fluids and plasma (336). Differences in the composition of the protein layers adsorbed to bone-substituting
material have been noted also after implantation in the intramuscular site and after incubations *in vitro* with serum (370).

The presence and composition of the protein adsorbate is consistent with the observed tissue reaction to HEMA-MMA capsules which were pre-incubated for 1 week in bovine serum-containing medium before implantation into Wistar rats. The tissue reaction to these capsules was thicker with a thicker layer of macrophages directly apposed to the capsule surface than to similar capsules maintained without FBS (304). Presumably, the serum proteins adsorbed to the capsule surface as well as those released from within the capsule (capsule membrane and core is loaded with serum proteins during incubation), contributed to this enhanced response. PAN/VC macrocapsules immersed in medium containing serum of a xenogeneic source evoked a host-tissue reaction in the brain and other sites but did not if they were immersed in medium which is chemically defined (334). An immune response to the adsorbed proteins is possible because of their xenogeneity (166) or because of their antigenicity due to conformational changes upon adsorption (371, 372). Foreign and/or host cell-adhesive proteins such as fibronectin, fibrinogen and vitronectin, adsorbed to the capsule surface, may mediate inflammatory cell (e.g., macrophages, neutrophils, lymphocytes, and fibroblast) attachment (373, 374) and activation in response to biomaterial contact (375, 376, 377) through specific integrin receptors on the surface of leukocytes (373, 378, 379). These steps of protein adsorption, adhesion and activation of leukocytes with their concomitant release of
cytokines, reactive oxygen intermediates and proteolytic enzymes interact, through interconnected pathways, to determine the tissue response to the implanted material.

Complement activation would further affect the inflammatory response to microencapsulated cells. The biological responses elicited by complement proteins result from ligand-receptor-mediated cellular activation. For example, release of the anaphylatoxin, C5a, around implanted capsules would mediate granulocyte chemotaxis, enzyme release, and neutrophil oxidative metabolism, activate endothelial cells, upregulate leukocyte expression of complement receptors 1, 3, and 4, LFA-1, and FcR, and monocyte production of cytokines (IL-1, IL-6, IL-8, and TNF-α) (380, 381, 382). Even if complement was not activated by the capsule (leading to C5a release) the presence of complement fragments adsorbed or bound to the capsule membrane or in the tissue surrounding the capsule could affect the adhesion and activation of the inflammatory cells associated with the capsules. Adsorbed Factor B would be able to promote macrophage spreading and enhance cytotoxicity of macrophages (383). Furthermore, the capsule surface coated with C3 fragments C3b and iC3b could be identified by neutrophils and macrophages both expressing the corresponding receptors complement receptor 1 (CR1) and complement receptor 3 (CR3), respectively. On neutrophils and macrophages, an appropriately activated CR1 would facilitate the phagocytosis of C3b- and C4b-coated particles or result in frustrated phagocytosis for non-injectable particles such as the microcapsule surface and activate the respiratory burst of neutrophils (384).
Complement fragments associated with various biomaterials (363, 385) and the mechanism of biomaterial-induced complement activation is being investigated (362, 385, 386, 387). *In vitro* studies support the role of complement C3 fragments in mediating monocyte and neutrophil adhesion and activation by biomaterials. For example, monocyte adhesion to surfaces of various chemistries was reduced when surface adsorbed C3 was absent (388). Monocyte adhesion to these surfaces appeared to be mediated by the interaction of the complement type receptor type 3 (CR3, CD11b/CD18) with adsorbed iC3b since adhesion was inhibited by an antibody against iC3b but not C3b. When adsorbed fibrinogen with other components was present, the apparent iC3b-mediated adhesion was diminished, suggesting alternative interactions between adsorbed fibrinogen and CD11/CD18 molecules. Similarly, neutrophil adherence to cuprophan was mediated through the interaction of complement type receptor type 3 (CR3, CD11b/CD18) with iC3b deposited on the membrane surface (389).

On the other hand, in a series of *in vivo* studies, complement adsorption or activation did not appear to be crucial to inflammatory cell recruitment to biomaterial surfaces. In decomplemented mice, normal accumulations of macrophages were associated with PET disks implanted intraperitoneally (390). Only in afibrinogenemic mice was there no accumulation of phagocytes, unless these disks were precoated with fibrinogen (391). Phagocytes appeared to mediate their interactions with fibrinogen-coated implants via the interaction of the cell integrin CD11b/CD18 (Mac-1) with the fibrinogen domain, gamma 190-202 (P1) (392).
To avoid the effects of preadsorbed serum proteins on the tissue response to microcapsule, freshly-made capsules may be implanted or capsules maintained in hormonally defined medium (334, see Chapter 3.1) that does not contain serum proteins such as complement or cell adhesive proteins before implantation. However, in hormonally defined medium, the proteins (typically of human origin) may be xenogeneic to the recipient, at least in preclinical studies. Alternatively, autologous or allogeneic serum could be used i.e., medium containing rat serum for capsule implantations into rats or human serum for capsule implantation into humans. Surface modification, for example to increase the surface PEG content (307, 334) should also reduce the amount of adsorbed protein and its impact, with a resultant improvement in capsule biocompatibility.

CONCLUSIONS

Human serum proteins and their activation fragments were associated with hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) copolymer microcapsules following their 1 week in vitro incubation in medium containing human serum (HS). The proteins associated with capsules included fibronectin, plasminogen, IgG, vitronectin, Factor B, Factor H, Factor I. Complement activation fragments formed independent of capsule presence, may be partially or completely responsible for the complement fragments found with capsules. The prevention of complement activation by the addition of 5.8 mM EDTA resulted in fewer low molecular weight C3 fragments associated with
capsules. Rat fibrinogen, IgG, and complement C3 fragments were detected following an capsule implantation intraperitoneally for 10 minutes and 1 day. By characterizing the proteins associated with HEMA-MMA capsules, this study begins to address the role of adsorbed foreign proteins from a pre-implantation incubation and of host proteins upon implantation, on the host response to HEMA-MMA polymer capsules containing living cells.
NOTE TO USERS

Page(s) missing in number only; text follows. Microfilmed as received.

254

UMI
Chapter 5
Conclusions

The nonadherent microenvironment of the HEMA-MMA polymer capsule does not support attachment of anchorage-dependent cells. In the absence of a substrate for the cells to attach and grow as in a normal tissue situation, cells attach to each other or to cell-secreted extracellular matrix components such as laminin, fibronectin, and collagen into a spheroidal shape. This was observed with HEMA-MMA microencapsulated human hepatoma (HepG2) cells, used as a model for hepatocytes. Because diffusion limitations, implicit in this cellular arrangement, affected nutrient delivery and metabolic waste removal, once aggregates reached a certain size (150 μm X 300 μm), for a certain length of time (1 week, in vitro), encapsulated cellular aggregates developed necrotic cores. This is similar to what has been observed with tumour spheroids.

The environment within the HEMA-MMA capsule core was altered by the co-encapsulation of extracellular matrix components (Matrigel®). Matrigel® co-encapsulation provided a favourable cellular environment as evidenced by the retention of cell viability for up to 14 days, and the maintenance of the initial cellular morphology at 14 days. The co-encapsulation of HepG2 cells with Matrigel®, resulted in an initially uniform distribution of essentially individual cells with aggregates appearing later within the Matrigel®. The uniform distribution of individual cells and aggregates thereof throughout Matrigel® during the culture time presumably caused the local consumption of nutrients
and production of waste products to be such that cellular necrosis did not occur at the 
centre of large aggregates but at the centre of the large diameter capsule (~ 750 μm).

The viability of HEMA-MMA microencapsulated cells is limited in non-
immunosuppressed Wistar rats. Microencapsulated rat hepatoma, H4IIEC3, cells (a 
model for allogeneic cell transplantation), arranged as a cellular aggregate, were viable 
after 7 days but not 14 days of implantation. A similar model of xenogeneic 
transplantation of microencapsulated human hepatoma, HepG2, cells, resulted in viable 
cells at 4 days but not 7 days. Vascularization of the tissue reaction to both types of 
microencapsulated cells occurred promptly after 4 days in vivo and was maintained for up 
to 14 days. With HepG2 cells at day 4 in vivo, there appeared to be a relationship 
between the viability of microencapsulated cells and the degree of vascularization of the 
surrounding tissue reaction. However, it was not clear that the perfusion of the implant 
site would be sufficient to sustain microencapsulated cells beyond 4 days. The omental 
pouch is the preferred site for small diameter capsules implantation because of thin, 
vascularized tissue reactions and its immunological properties. Calcification of the 
polymer membrane, containing both types of cells, first identified using von Kossa staining 
at day 4, presumably reduced the permeability of the membrane and could compromise 
nutrient diffusion and cell viability.

The tissue reactions to microcapsules, once developed, were comprised of three 
regions: macrophages, fibroblasts and some foreign body giant cells apposed to the
polymer membrane, a fibrous region containing immune and inflammatory cells and a region of vascularized granulation tissue. For capsules embedded in omental tissue, the closest blood vessel distance to the polymer/tissue interface did not change past day 4, neither did the distribution of the tissue reaction thicknesses. There were also noticeable differences in the tissue reaction between transplanted, microencapsulated cell species in terms of the thicknesses and the immune cell profile. Cellular antigens (alloantigens, xenoantigens, or tumour specific antigens) shed from microencapsulated cells, could stimulate an immune response via the indirect pathway of antigen presentation, to initiate a humoral or molecular cytotoxic tissue response surrounding the implant which could directly or indirectly compromise the viability of encapsulated cells. Microencapsulation may isolate transplanted cells to an extent that is dependent on the cell-type and the capsule permeability and biocompatibility.

High resolution carbon, C 1s, x-ray photoelectron spectroscopy (XPS) analysis of the surface of HEMA-MMA microcapsules showed that the surface was not pure HEMA-MMA. More carbon bonded in the C-O form than in the C-C form indicated the presence of the Pluronic surfactant, L101, adsorbed from the precipitation bath to the surface during microcapsule preparation. Capsule surface modification with adsorbed Pluronic, a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer, may favorably influence aspects of capsule biocompatibility including protein adsorption in vitro and in vivo and the attachment of inflammatory cells. However, the amount adsorbed and the stability of its association remain important issues. Capsules maintained
in medium containing serum showed a nitrogen signal consistent with the presence of adsorbed serum proteins. Washing capsules with phosphate buffered saline, as is done before capsule implantation, reduced the nitrogen signal but not to zero. Xenogeneic serum proteins, adsorbed during a pre-implantation incubation in medium containing serum, affected the tissue reaction to these implants resulting in thicker tissue reactions with a thicker layer of macrophages apposed to the implant surface. Calcium, detected by XPS, was presumably associated with calcium-binding proteins as it was only found on capsules which had been maintained in medium containing serum. Calcification of HEMA-MMA capsules, without pre-adsorbed serum proteins and associated calcium deposits, was detected by von Kossa staining of histological sections and EDAX microanalysis after their implantation for at least 4 days in Wistar rats. Therefore, mechanisms other than or in addition to calcium salt deposits formed in vitro acting as nucleation sites presumably apply.

A variety of serum proteins were associated with microcapsules following their in vitro incubation in medium containing serum or after implantation intraperitoneally in rats. Characterization of proteins using sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis identified almost all proteins probed for in the immunoblots. This included cell adhesive proteins (e.g., fibronectin and vitronectin), complement components (C3), proteins of the blood coagulation and fibrinolytic system (e.g., prekallikrien, HMWK) and other abundant serum proteins (e.g., albumin, IgG). Similar rat proteins were associated with HEMA-MMA capsules which
had been implanted intraperitoneal in rats as with capsules incubated *in vitro* in human serum. The presence and composition of the protein adsorbate is consistent with the observed tissue reaction to HEMA-MMA capsules which were pre-incubated in bovine serum-containing medium before implantation into Wistar rats. Presumably, the serum proteins adsorbed to the capsule surface as well as those released from within the capsule contributed to the enhanced response. Preadsorbed allogeneic or xenogeneic serum proteins and those adsorbed upon capsule implantation may mediate the adhesion and activation of leukocytes which, with their concomitant release of cytokines, reactive oxygen intermediates and proteolytic enzymes would interact, through interconnecting pathways, to determine the tissue response to the implanted capsule. To avoid the effects of preadsorbed serum proteins on the tissue response to microcapsules, freshly-made capsules may be implanted or capsules may be maintained in hormonally defined medium that does not contain serum proteins such as complement or cell adhesive proteins before their implantation.
Chapter 6

References


56 A. Jarvis and T. Grdina, Production of biologicals from microencapsulated living cells, Biotechniques, 1, 22-27 (1983).


P. Johnson, personal communication.


M. H. May, M.V. Sefton, unpublished observations.


Bissell, D.M.; Choun, M.O.; The role of extracellular matrix in normal liver, Scand. J. Gastroentrol. 23(Suppl 151): 1-7 (1988).


169 D. Isenman, personal communication.


V. Ablamunits, F. Baranova, T. Mandrup-Pulsen, J. Nerup, *In vitro* inhibition of insulin release by blood mononuclear cells from insulin-dependent diabetic and


function of porcine islets and single cells embedded in barium-alginate matrix,


H. Shirwan, M. Leamer, H.K. Wang, L. Makowka, D.V. Cramer, Peptides derived from α-helices of allogeneic class I major histocompatibility complex antigens are potent inducers of CD4+ and CD8+ T cell and B cell responses after cardiac allograft rejection, Transplantation, 59: 401-410 (1995).


T. Loudovaris, D. Maryanov, S. Young, S. Jacobs, L. Martinson, S. Neuenfeldt, J. Brauker, R. Johnson, Correction of diabetic NOD mice implanted with rat islets in


De Castro, A.; HEMA-MMA microencapsulated agarose, chitosan or Matrigel® as a PC12 cell immobilization matrix, Bachelor of Applied Science Thesis, Department of Chemical Engineering and Applied Chemistry, University of Toronto, 1994.


M. Baranyi, A. hever-Szabo, A. Venetianer, Heat-shock response of rat hepatoma

M. Takano, N. Itoh, K. Yayama, M. Yamano, R. Ohtani, H. Okamoto,
Interleukin-6 as a mediator responsible for inflammation-induced increase in

E.B. Thompson, G.M. Tomkins, J.F. Curran, Induction of tyrosine α-ketoglutarate
transaminase by steroid hormones in a newly established tissue culture cell line,

M.D. Reuber, A transplantable bile-secreting hepatocellular carcinoma in the rat, J.

J.E. Babensee, U. De Boni, M.V. Sefton, HEMA-MMA Microencapsulated
Human Hepatoma, HepG2, Cells - Cell Viability and Tissue Response After
Implantation into Various Sites in the Rat, manuscript in preparation.

Institute of Pathology, American Registry of Pathology, McGraw-Hill Book

J.E. Babensee, U. De Boni, M.V. Sefton, Tissue reaction to HEMA-MMA
capsules: Effect of device components, manuscript in preparation.

E. De Heer, A. Davidoff, A. Van Der Wal, M. Van Geest, L.C. Paul, Chronic
renal allograft rejection in the rat Transplantation-induced antibodies against

S. Lahooti, M.V. Sefton, unpublished observations.


Technical data on Pluronic ® polyols, Performance Chemical Division, BASF Corporation, Parsippany, NJ, USA.


Y. Yamamoto, and M.V. Sefton, unpublished observations.


D. Fernandes, SEM and EDAX studies of HEMA-MMA microcapsule calcification in vitro and in vivo, B.A.Sc., Department of Chemical Engineering and Applied Chemistry, University of Toronto, 1994.


J.P. Black, unpublished observations.

J.P. Black, Material-Induced Complement Activation: A New Method of Analysis, M. A. Sc. Department of Chemical Engineering and Applied Chemistry, University of Toronto, 1996.


D. Isenman, Personal communication.


