SECRETION OF MARKER PROTEINS FROM ALGINATE-POLY-L-LYSINE-ALGINATE MICROCAPSULES AND HYDROXYETHYL METHACRYLATE-METHYL METHACRYLATE CAPSULES

BY

MAY TSE,
B.Sc.

APA MICROCAPSULES & HEMA-MMA CAPSULES

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MAY TSE, B.Sc.

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AUTHOR: May Tse, B.Sc. (Queen's University)

SUPERVISOR: Dr. P.L. Chang, Professor, Pediatrics

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Abstract

The objective of this study was to encapsulate cell lines that secrete marker proteins that cover a large molecular weight range (M_r from 45,000 to 300,000) and monitor the secretion of the marker proteins from alginate-poly-L-lysine-alginate (APA) microcapsules and hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) thermoplastic capsules. Different parameters for the APA microcapsules, such as the duration of poly-L-lysine (PLL) and sodium citrate treatment, the initial cell density for encapsulation was studied, and their effects on secretion rate and cell proliferation were closely examined.

Cell lines used for encapsulation secreted human growth hormone (hGH) (M_r 45,000), ß-hexosaminidase (ß-hexo.) (M_r 120,000) and ß-glucuronidase (ß-gluc.) (M_r 300,000). Monitoring the secretion rates, as well as the distribution of the marker proteins within the microcapsules following encapsulation enabled the permeability of the membrane to be assessed over one month in culture.

Encapsulation of cell lines in both types of capsules was effective in producing viable cells capable of proliferating within a semi-permeable membrane. Encapsulating cells in single-coated APA microcapsules at 4°C, treated with 10 minutes PLL, 20 minutes sodium citrate and at a cell density of $2x10^6$ cells/ml alginate was found to provide the most optimal conditions for prolonged viability of and stable secretion by the recombinant cells.

Human growth hormone diffused readily across the capsule membrane into the culture media from both APA and HEMA-MMA capsules, at rates similar to the non-encapsulated cells. Human growth hormone did not accumulate in the intracapsular space in significant quantities.

ß-glucuronidase and ß-hexosaminidase could diffuse across APA capsule membrane, but not across HEMA-MMA capsule membrane into surrounding media. ß-glucuronidase secretion from APA microcapsules was 8-fold lower than non-encapsulated cells. ß-hexosaminidase secretion from APA microcapsules was 4.5-fold lower than non-encapsulated cells. Slight retention of both ß-glucuronidase and ß-hexosaminidase was observed in the intracapsular space of APA capsules. HEMA-MMA capsules completely blocked the secretion of both ß-glucuronidase and ß-hexosaminidase out of the capsule. Massive accumulation of both kinds of secretory enzymes was found in the intracapsular space of HEMA-MMA capsules. This indicated APA microcapsules have a molecular weight cut-off of >300,000 whereas HEMA-MMA microcapsules have a molecular weight cut-off of <120,000.

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4-MU	4-methyl-umbelliferone
4-MUG	4-methylumbelliferyl-ß-D-glucuronide
4-MUNG	4-methylumbelliferyl-NAcetyl-ß-D-glycosaminide
ß-gluc.	ß-glucuronidase
ß-hexo.	ß-hexosaminidase
APA	alginate-poly-L-lysine alginate
BSA	bovine serum albumin
$CaCl_2$	calcium choride
cDNA	complementary deoxyribonucleic acid
CHES	2-(n-cyclohexylamino)ethane sulfonic acid
CHO	chinese hamster ovary
CTP	cytosine triphosphate
$CuSO_4$	copper sulphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
g	gram
HCl	hydrochloric acid
HEMA-MMA	hydroxyethyl methacrylate-methyl methacrylate
hGH	human growth hormone
hr	hour
IgG	immunoglobulin G
kb	kilobase (1000 bases of nucleic acid)
kDa	kilodalton
L	litre
M	molarity (one mole per litre)
mg	milligram

min.	minutes
ml	millilitre
mM	millimolar
mm	millimetre
M_r	relative molecular weight
mμ	microns
N	normality (chemical equivalents per litre)
Na	sodium
Na_2CO_3	sodium carbonate
NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
PBS	phosphate buffered saline
PEG 200	polyethylene glycol 200
pH	negative logarithm of the concentration of hydrogen ions
PLL	poly-L-lysine
RGM	regular growth hormone
rpm	revolutions per minute
SD	standard deviation from the mean
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
U	unit of enzymatic activity
μg	microgram
μL	microlitre
μM	micromolar
UV	ultraviolet
V	voltage

1.0 Introduction

1.1 Current Status of Somatic Gene Therapy

1.1.1 Justification for Developing Non-Autologous Somatic Gene Therapy

With the application of the tools of molecular genetics, and with new approaches to the identification and characterization of disease-related defects, therapy through the correction of genetic defects has come within reach (Friedmann,1989; Anderson,1992). During the past decade, techniques have developed to introduce DNA into mammalian cells with great efficiency. Many of these techniques have involved the use of transducing viruses, such as retroviruses.

Retroviruses have the appropriate machinery to introduce their genes into vertebrate cells, to have those genes stably integrated into chromosomal DNA, and to have those genes expressed efficiently. Furthermore, retroviruses have a genetic organization that allows other genes to be inserted easily into them without preventing any step in the normal virus life cycle, and usual retrovirus replication does not kill infected cells (Temin,1986). Despite their popularity, there are several difficulties associated with retroviral vectors that complicate their use for therapeutic genetic modification of cells. Since retroviruses integrate in a quasi-random fashion into the target-cell genome, they have the potential for activation of adjacent genes or inactivate essential cellular elements (Shih et al,1988).

The integration of a replication-defective vector may trigger a preneoplastic cell to become neoplastic through the activation of a previously silent protooncogene or the inactivation of a tumor-suppressor gene. Replication-competent virus, or helper virus, can occasionally be generated by genetic recombination between the defective viral vector and cellular elements. Studies have shown that helper virus produced in large amount can lead to very high frequency of malignancies in immunosuppressed monkeys that have received grafts of bone-marrow stem cells infected with such vectors (Kolberg et al,1992).

Adenovirus type 2 and type 5 belong to a subclass of adenovirus that are not associated with human malignancies (Strauss, 1984). They are currently being developed for use in gene therapy. The ability to infect numerous different cell types and the absence of a requirement for dividing cells make adenovirus an attractive candidate for in vivo gene therapy (Kozarsky & Wilson, 1993). Several studies have shown that adenovirus can be used to infect brain cells in vivo. When a recombinant , replication-defective adenovirus encoding lac Z was introduced into the caudate putamen of mice, a variety of different cell types were infected, including neurons, oligodendrocytes, and myelinated axons. Expression of ß-galactosidase persisted through at least eight weeks (Davidson et al, 1993).

Although adenoviruses that have been developed for gene therapy contain deletions of the entire E1 region, which regulates adenoviral

transcription and is required for viral replication, the possibility remains that the recombinant virus retains the capacity to replicate at very low levels. The generation of adenovirus vectors with furthur deletions or mutations in additional adenovirus genes may eliminate such drawbacks (Kozarsky & Wilson, 1993).

An alternate procedure of transkaryotic implantation was developed in which cells for implantation were genetically altered through DNA-mediated transfer (Seldon et al,1987). It was demonstrated that human growth hormone can be delivered into rodents after implantation of either a syngeneic fibroblast cell line (Seldon et al,1987) or autologous primary fibroblasts (Chang et al,1990) that have been transfected with the human growth hormone gene, demonstrating the feasibility of non-viral-mediated gene transfer to somatic gene replacement. The main advantage of non-viral-mediated gene transfer is that there is no co-transfer of unwanted viral genetic material to the cell. One of the most popular non-viral-mediated gene transfer technique is receptor-mediated gene transfer, accomplished by the conjugation of DNA to a protein ligand via polylysine. It was demonstrated that when asialoglycoprotein-polylysine conjugate was mixed with DNA encoding human serum albumin in the proper proportions and was injected directly into the blood, the soluble DNA conjugate was targeted to

the liver via asialoglycoprotein receptors and then DNA was internalized. The targeted DNA produced human serum albumin for at least 4 weeks after injection (Wu et al, 1991).

1.1.2 Need for Immuno-Isolation

Although implantation of genetically altered autologous rodent fibroblasts has been shown to be a feasible approach to the delivery of new gene products in somatic gene therapy, the human growth hormone elicited intense immune response in their recipients that would have limited the effectiveness of the therapy.

Implantation of genetically modified fibroblasts in the thymus did not lead to tolerization toward human growth hormone secreted by these cells (Behara et al, 1992). High titer of anti-human growth hormone was detected in all animals regardless of the site of implantation. The failure of the thymus to offer immune protection for the foreign antigen was further confirmed when the animal was subsequently challenged with purified human growth hormone. Co-administration of immunosuppresive drugs during implantation provided suppression to the antibody response (Behara et al,1992). Unfortunately, immunosuppressive therapy has adverse side effects on the rapidly proliferating cells of the hematopoietic system and other organs, such as nephro- and hepato-toxicity and growth-inhibitory properties (Bennett et al,1991). Moreover, long-term immunosuppressive

therapy leads to an increased risk of malignant disease. There was a high incidence of lymphomas, karposi's sarcomas and renal carcinomas, which occured rapidly when cyclosporin A was co-administered during kidney transplantation (Luciani et al, 1993).

It is necessary to develop immunoprotection for genetically modified cells, to eliminate the need for expensive autologous tissue manipulations and to avoid the adverse side effects of immunosuppressive therapy. Moreover, immunoisolated cell lines, such as encapsulated human growth hormone secreting cells, can be fully characterized in vitro before implantation.

The rejection of transplanted tissues or cells is a cell-mediated immune response. Antigen presenting cells, such as macrophages and monocytes, engulf and process the transplanted tissue, which then present the antigenic determinant, or epitope, to helper T cells. Upon recognition of a foreign epitope, helper T cells elicit a cell-mediated immune response that involves cytotoxic T cells and natural killer cells to destroy the transplanted tissue. Helper T cells can also stimulate the proliferation of a clone of B cells that produce antibodies specific against the epitope (Golub & Green, 1991).

The theory behind encapsulation is to protect the transplanted cells or tissues from the host's immune system by means of perm-selective membranes. While preventing the passage of immune components, the

membrane must allow sufficient transport of nutrients to maintain cell viability and of the desired cell product in order for the implant to be pharmacologically effective. The membrane must also provide the mechanical strength to maintain the device configuration in the face of in vivo movement and pressures. Although antibodies of small sizes, such as IgG, may be able to diffuse through the membranes, antibody mediated rejection becomes important only in cases where the host has been previously exposed to the transplanted cells. This is due to sensitization of the host against the antigenic determinants of the previously transplanted cells, which leads to a more rapid and intense immune response, with mass production of specific IgG against the subsequently exposed transplant (Lysaght et al,1993).

1.2 Biomaterials Available for Immuno-Isolation

1.2.1 History of Alginate Capsules and Applications

Different types of microcapsular membranes have been reported to be effective in protecting the encapsulated cell mass against the host's immune system (O'shea et al,1984; Aebischer et al,1988; Sefton et al,1992). Among them, the alginate-poly-L-lysine-alginate (APA) microcapsules are most widely used.

In 1980, rat islets encapsulated in semi-permeable alginate-polylysine-polyethyleneimine membranes and implanted into the peritoneal cavity of diabetic rats restored normoglycemia for only two weeks (Lim & Sun,1980). The rapid failure of the encapsulated islets was found to be due to an inflammatory response induced by polyethyleneimine (Sun et al,1983). The polylysine-polyethyleneimine membrane was replaced by a more biocompatible polylysine-alginate membrane, with alginate being the outer membrane.

The construction of APA capsules involves suspending islets or cells in sodium alginate, which are extruded through a fine needle connected to concentric air flow from the peripheral channel. The microdroplets are stabilized by the formation of the cross-linked network of polymer chains with calcium chloride, crosslinked with poly-L-lysine and the excess charge

neutralized with a more diluted sodium alginate. The calcium alginate core is liquefied with sodium citrate, leaving islets or cells encapsulated in an APA semi-permeable membrane (Sun et al,1983).

The molecular weight of poly-L-lysine and its incubation time with the calcium alginate gel are important factors that determine the permeability of the membrane (King et al,1987). The permeability of the microcapsules to hemoglobin (M_r 68,000) released from encapsulated red blood cells was determined for different poly-L-lysine molecular weights (M_r 4,000 to M_r 400,000; Goosen et al, 1985). The poly-L-lysine molecular weight of M_r 17,000 and M_r 22,000, with an incubation time of 6 minutes were determined to be optimal for the construction of APA capsules (King et al,1987; Goosen et al,1985).

APA microcapsules have been shown effective in protecting islet allografts, which reversed the diabetic state in streptozotocin-induced diabetic rats for up to 1 year, from the host's immune system (O'shea et al, 1984). Subsequent reports indicated normoglycemia can be maintained for up to 21 months (Sun et al, 1986). Xenografts of rat islets encapsulated in APA microcapsules can prolong xenograft survival without immunosuppression for up to 308 days (Lum et al, 1992). Allogeneic mice implanted with microencapsulated hGH secreting cells demonstrated significant levels of circulating hGH for up to 3 months and the encapsulated

cells remained viable and proliferative during the course of the experiment (Chang et al, 1993). As such, microencapsulated xenografts and microencapsulated recombinant cell lines can be used to generate biologically active molecules in the presence or absence of immunosuppressive therapy.

The encapsulation of liposomes containing drugs has been demonstrated to be useful for the pulsed release of drugs or other substance both in vitro and in vivo (Kibet et al,1990). Pulsatile release of labelled BSA from APA microcapsules was demonstrated for one month in vitro (Kibat et al,1990). This approach overcomes the limitations of the liposomes due to uptake by the reticulo-endothelial system or destabilization as a result of adsorption of blood proteins to the lipid bilayer membrane.

The APA microcapsules have been used as a short-term in vivo assay for evaluating the antitumor activity of chemotherapeutic drugs. Microencapsulated human tumor cells were injected intraperitoneally into mice, anti-cancer drugs were administered and microcapsules were recovered at various intervals following treatment. This assay seemed attractive to use in new drug development because the sensitivity of tumor cells can be assessed following exposure to drugs at concentrations that are achievable in vivo, the effect of each drug injection can be quickly evaluated and the test is applicable to virtually all histological types of human tumor cells (Gorelik et al,1987).

1.2.2 History of Thermoplastic Capsules and Applications

Other methods of encapsulation involve loading of cells into hollow fibers (Aebischer et al,1990) or polyacrylate capsules (Sefton & Broughton, 1982).

Aebischer et al. described a technique of macroencapsulation that involves loading cells into poly(acrylonitrile vinyl chloride) and then closing the extremities. This procedure has been used to encapsulate a variety of mammalian tissue such as pituitary, islet of Langerhans, parathyroid, thymus and embryonic mesencephalon (Altman et al,1986; Aebischer et al,1986). Inconsistent results have arisen, however, due to the unreliable closure with conventional macro-encapsulation. A new technique has been developed by coextruding living cells in the core of forming hollow fibers and integrally sealing the partially coalescent organogel into discrete tubular macrocapsules (Aebischer et al, 1980).

Macroencapsulated PC12 cells and bovine chromaffin cells were able to deliver dopamine for at least four weeks in vitro. When implanted into the striatum, encapsulated PC12 cells were able to reduce lesion-induced rotational asymmetry in rats (Aebischer et al,1991; Jaeger et al, 1992). Recent studies indicated that macroencapsulated xenogeneic chromaffin cell implants, but not empty control capsules, could repeatly reduce pain sensitivity with nicotine stimulation for up to 3 months following implantation into the rat spinal subarachnoid space. In addition, in vitro

neurochemical studies of recultured capsules revealed sustained release of catecholamines from encapsulated cells 3 months following implantation into the spinal subarachnoid space, suggesting that immunologically isolated xenogeneic cells can provide a long-term source of neuroactive substances for the alleviation of pain (Sagen et al, 1993).

Sefton et al. reported an interfacial precipitation process for encapsulation of red blood cells in polyacrylate membranes (Sefton et al,1982). A spherical core of cell suspension was coated with a shell of polymer solution, after which the polymer solvent was extracted to precipitate the polymer membrane. A concentric needle assembly was used to form the initial liquid core-shell droplet with a co-axial air stream to blow droplets off the needle tip into a precipitation bath. Shearing of capsules was later achieved by vertical oscillation of the needle assembly at an hexadecane/air interface, which resulted in more reproducible capsule formation with improved capsule morphology (Crooks et al,1990).

Several types of highly differentiated mammalian cells, including rat pancreatic islets, human diploid fibroblasts and rat hepatocytes have been encapsulated. In vitro studies of encapsulated Chinese Hamster Ovary (CHO) fibroblasts have shown that the encapsulated cells survived the process of encapsulation and retained an active metabolic state afterwards. They also underwent rapid proliferation until an overall quiescent state was reached (Uludag & Sefton, 1991; Uludag et al, 1993).

Secretion of insulin by the encapsulated rat pancreatic islets were compared with un-encapsulated cells. It was found that entrapment within polyacrylate membranes did not alter the ability of rat islets to secrete insulin to any significant degree. Moreover, the secretion of insulin under high glucose conditions by both the control and encapsulated cells were significantly higher (Uludag & Sefton, 1992; Uludag et al, 1993).

1.3 Objective of Current Investigation

Characterization of in vitro performance of encapsulated cells is the present focus of research. Two different kinds of capsular membranes were characterized simultaneously with respect to the permeability of proteins of different molecular weights secreted from encapsulated recombinant cells. In this project, the permeability of APA microcapsular membranes and HEMA-MMA polyacrylate thermoplastic membranes to human growth hormone (hGH:M_r 45,000), ß-hexosaminidase (ß-hexo:M_r 120,000) and ß-glucuronidase (ß-gluc:M_r 300,000) was studied. The distribution of these marker proteins was monitored for one month. The secretion from the two types of capsules was determined and compared to non-encapsulated cells. A more precise molecular weight cut off for the two types of capsules and the feasibility of the encapsulated cell lines for long term gene therapy could be determined.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Sources of Chemicals and Reagents

Most chemicals used in this project were purchased from Sigma (St. Louis, MO), Gibco (Burlington, ON), Fisher Scientific Company (Fair Lawn, NJ), BDH (Bethesda, MD) and J.T. Baker Chemical Company (Phillipsburg, NJ). Sodium alginate (Keltone lot no.77373A) was a gift from Kelco, Division of Merck and Co. Inc. (Chicago, IL).

The plasmid pNMG-3 contains an open reading frame coding for human growth hormone fused to the murine metallothionein promoter and enhancer region (Chang et al, 1990). The cDNA coding for dihydrofolate reductase and mouse ß-glucuronidase was transfected into the cell line, L-MPR 2A50, MTX 3.2, which is deficient in mannose-6-phosphate receptor (a gift from Dr. W. S. Sly, St. Louis University, MO).

The hGH ELIZA kit was purchased from United Biotech Inc. (cat# HP-901, Mountain View, CA). The fluorometric substrates 4-methylumbelliferyl-ß-D-glucuronide (4-MUG) (cat#M9130, Sigma, St.Louis, MO) and 4-methylumbelliferyl-N-Acetyl-ß-D-glucosaminide (4-MUNG) (cat#M2133, Sigma, St.Louis, MO) were purchased for the detection of ß-glucuronidase and ß-hexosaminidase, respectively.

2.2 Methods

2.2.1 Tissue Culture and Cell Lines

Ltk$^-$, LhGH-1 and Rec$^-$ mouse fibroblasts were grown in regular growth media that contained alpha modified minimal essential medium supplemented with 10% newborn calf serum, 2mM glutamine, 100U/ml penicillin G and 100µg/ml streptomycin sulfate.

2A50 mouse fibroblasts were grown in regular growth media(RGM) that contained Dulbecco's modified minimal essential medium supplemented with 0.1g/L sodium pyruvate, 2.2g/L sodium bicarbonate, 100µg/ml penicillin G, 100µg/ml streptomycin sulfate, 1.2mM glutamine, 5% dialized FBS and 3.2µM methotrexate.

Cells were grown in the presence of 5% carbon dioxide and 100% humidity at 37°C in a water jacketed incubator.

Derived cell lines were frozen in liquid nitrogen for storage. Cell lines derived from Ltk$^-$ cell line (LhGH-1 cells) were frozen in RGM with 10% dimethyl sulphoxide (DMSO). LhGH-1 cells was provided by D. E. Awrey of McMaster University. It was constructed by transfecting the Ltk$^-$ cells by calcium phosphate precipitation (Graham & Van der Eb, 1973) with the plasmid pNMG-3 which contains an open reading frame coding for human growth hormone fused to the murine metallothionein promoter and enhancer region. The construction of pNMG-3 has been reported previously (Chang et al, 1990).

2.2.2 Assays for Recombinant Product Determination

The amount of hGH in cell culture media was quantified with an ELISA kit and protocol supplied by UBI. Briefly, 25μL of samples were incubated in a micro-titre well bound with anti-hGH antibody. One hundred μL of anti-hGH conjugate antibody, which were chemically conjugated with horseradish peroxidase, were added to each well and allowed to incubate at 25°C for one hour. The unbound samples were removed by rinsing the wells ten times with ddH_2O and drained on paper towels. The amount of bound peroxidase is proportional to the concentration of the hGH present in the samples. The intensity of color developed is proportional to the concentration of hGH in the media. Then, 200μL of chromogen substrate was added. After 30 minutes, the reaction was stopped with 50μL, 1N sulfuric acid and the absorbance was read on a Titermax ELISA plate reader (Titermax, Titertex multiskan plus).

ß-glucuronidase and ß-hexosaminidase activities in cell culture media were measured by fluorometric assays (Glaser & Sly, 1973). To detect for ß-glucuronidase activities, 10μL of sample were added to 40μL sodium acetate

buffer (0.2M glacial acetic acid, 0.2M sodium acetate, pH4.8) in a 10x75mm test tube. One hundred and fifty µL of substrate 4-MUG were added to each tube and allowed to incubate in a shaking water bath at 37°C for one hour. The reaction was stopped with 1ml glycine carbonate stopping buffer (24.02g/L glycine, 21.02g/L Na_2CO_3, pH10.0).

To detect for ß-hexosaminidase activities, 20µL of sample were added to 76µL citrate phosphate buffer (0.1M citric acid, 0.2M dibasic sodium phosphate, 3.75g/L human albumin fraction v, pH4.45) in a 10x75 mm test tube, with 4µL of 2.5% Triton. One hundred µL of substrate 4-MUNG was added to each tube and allowed to incubate in a shaking water bath at 37°C for one hour. The reaction was stopped with 1ml propanol stopping buffer (1.9ml/L 2-amino-2-methyl-1-propanol, adjust to pH10.8 with 1N NaOH).

Formation of 4-methyl-umbelliferone (4-MU) product was measured in a fluorometer using a primary corning filter No.7-60 with a peak transmittance at 370mµ for excitation, and two emission barrier filters, Kodak Wrattan No.2A that transmits light above 415mµ and Kodak Wrattan No.48 with a peak at 460mµ (Aminco fluoro-colorimeter, Technical marketing associates Limited). One unit of enzyme activities was defined as one nmole of 4-MUG hydrolyzed per hour (Glazer & Sly, 1973).

The amount of protein in the cell lysate was quantified with the well-documented Lowry assay (Lowry et al, 1951). The assay is based on the

reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein, which has a characteristic blue color with maximum absorbance at 750 nm. Ten µl of samples were added to 190µl of dH_2O. Then, 1ml of reagent C was added to each sample and allowed to incubate for 10 minutes. (Reagent C is composed of 50ml of 20g Na_2CO_3/L in 0.1M NaOH and 1ml of 0.5g $CuSO_4 \cdot 5H_2O$/100ml in 1% Na tartrate). One hundred µl of 1M Folin reagent (cat#F9252, Sigma, St.Louis, MO) was added to each sample and simultaneously vortexed. After 30 minutes, absorbance at 750nm was measured with a spectrophotometer (model#250, Gilford instrument, ON).

2.2.3 Microencapsulation Procedure (Alginate-Poly-L-lysine-Alginate Capsules)

The procedure for microencapsulation has been described (Goosen et al, 1985; King et al, 1987). Briefly, cells were suspended in 1.5% sodium alginate at a concentration of 2 million cells per ml for most experiments unless otherwise specified. The suspension was extruded through a 26 gauge needle at syringe pump rate 37.5 ml per hour and concentric airflow 3.0 litres per minute.

The cell suspension was sheared around the needle to form small spheres that fell into a $CaCl_2$ bath which stabilized the alginate. The spheres

were then washed with 0.55% and 0.28% $CaCl_2$ in saline. The spheres were crosslinked with poly-L-lysine, washed with saline and rinsed with dilute alginate to neutralize any excess surface charge. Then, the core of calcium alginate was dissolved with sodium citrate to create free floating cells inside a semi-permeable membrane. Alginate capsules containg 2A50 cells were constructed by Gonzalo Hortelano at McMaster University, Department of Pediatrics.

2.2.4 Characterization of Microencapsulated Cells

Viable cells have a characteristic of excluding a dye trypan blue after being released from the capsules. Viability of encapsulated cells was determined by placing 20 to 30 capsules on a microscope slide, with the addition of a drop of trypan blue and a cover slip over the capsules to allow the weight of the cover slip to rupture the capsules without destroying the cell membrane. Excess liquid was removed with a kimwipe. The number of viable and dead cells was counted under low power with a light microscope. Viable cells excluded trypan blue. Non-viable cells have sufficient membrane damage to allow the dye in, appeared blue in color.

The number of cells per capsule was determined by gently rupturing a known amount of capsules (50 to 100) with a pestle in an eppendorf tube

containing a known volume of phosphate buffer saline (PBS) (100 µL or 200 µL). Fifteen µL of ruptured cell suspension was tranferred onto a hemacytometer for cell count. The number of viable cells per capsule was calculated by multiplying the number of cells per capsule with the viability of the encapsulated cells.

Capsules were stored at weekly interval in 10% formalin at 4°C until pictures were taken with an Olympus OM-10 camera using Kodak TMAX film. The photographs of the microencapsulated 2A50 cells were taken by Gonzalo Hortelano and reproduced with permission.

2.2.5 Macroencapsulation Procedure

The procedure for macroencapsulation was reported earlier (Crooks et al, 1990; Uludag & Sefton, 1992). Briefly, a cell suspension and polymer solution were coextruded through a coaxial needle assembly and the droplets were sheared at the hexadecane/air interface. Cells were suspended either at 2 or $5x10^6$ cells/ml. The cell : polymer extrusion ratio was 21:56 µL/min respectively.

The polymer used was 10% (w/v) hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) (75% HEMA synthesized according to Stevenson et al, 1987) dissolved in polyethylene glycol (PEG 200). The droplets passed

into a precipitation bath containing PBS with Pluronic L101 surfactant and cured for 30 minutes while suspended by a magnetic stirrer. The curing process was repeated with fresh PBS for another 30 minutes.

Regular capsules were prepared by suspending cells in 20% Ficoll 400 solution in RGM supplemented with 10% FBS. For matrigel capsules, cells were suspended in 1:1 mixture of RGM supplemented with 10% FBS and 10% matrigel (Collaborative Research Inc., Bedford, MA). To facilitate the encapsulation of the matrigel-cell suspension which is gelatinous at room temperature, the polyethylene tubing delivering this suspension was submerged in ice water.

All HEMA-MMA capsules were made in Dr. M. V. Sefton's Laboratory, Department of Chemical Engineering, at University of Toronto by Hasan Uludag.

2.2.6 Characterization of Macroencapsulated Cells

To determine the number of cells per capsule, a known amount of capsules (20 to 30) was dissected in 30 mm dishes and incubated with trypsin for 15 minutes, to release the cells from the capsules. The dissociated cells and ruptured capsules were transferred to centrifuge tubes, vortexed and the capsule shells allowed to settle. The supernatant was transferred to a new centrifuge tube, centrifuged and suspended in a known amount of PBS (200 μL). The cell number was determined with a hemocytometer.

Cell viability was determined by placing a drop of cell suspension on a microscope slide with an addition of a drop of trypan blue and a cover slip. Capsules on day 1 and day 22 postencapsulation were washed with PBS and then fixed with 3.5% glutaraldehyde for freeze fracture and SEM examination (De Boni, 1988). The SEM for HEMA-MMA capsules (Fig 8G) was performed by Hasan Uludag of University of Toronto. Light microscopy photograph of non-dissected blank capsules (Fig 8H) was a courtesy of Dr. M. V. Sefton of University of Toronto. Light microscopy photographs of dissected HEMA-MMA capsules (Fig 8I, Fig 9F) were taken in Dr. Sefton's laboratory with the assistance of Julie Barbansee, Michael May and Mr. Cheun Lo. Capsules were fixed by Hasan Uludag in 1% osmium tetroxide, then frozen-embedded and stained with aqueous toluidine blue.

2.2.7 Secretion Rate Determination from Non-Encapsulated Cells

Secretion rates of gene product from non-encapsulated cells were determined in triplicate from cells which were actively growing 72 hr after passaging, usually at a ratio of 1:10. Cells were washed and re-fed with 10 ml of pre-equilibrated RGM. Four samples of 0.5 ml were taken at one hour, two hour and four hour intervals. The cells were then harvested with trypsin and the cell number determined with a coulter counter.

A cell lysate was prepared by ultrasonication (Kontes Micro Ultrasonic

Cell Disruptor) of harvested cells suspended in ddH_2O. Subsequent centrifugation of lysed cells 14,000 rpm (Eppendorf 5415) for 10 min. at 4°C removed cell debris. Intracellular concentration of gene product was determined with appropriate assay.

2.2.8 Secretion Rate Determination from Encapsulated Cells

Secretion rates of gene products from encapsulated cells were determined in triplicate. Three independent aliquots of 100 µL of packed capsules were placed in microcentrifuge tubes. Capsules were washed twice and re-fed with 1 ml of pre-equilibrated RGM. The capsules were transferred to a sterile 24 well plate and incubated under regular growth conditions. Four samples of 100 µL were taken at one hour, two hour and four hour intervals. Alginate capsules were stained with trypan blue and counted with a dissecting microscope (Bausch and Lomb). HEMA-MMA capsules were counted under a dissecting microscope without staining.

Alginate capsules were washed with PBS and ruptured with a pestle in 200 µL PBS. HEMA-MMA capsules were dissected with a sharp razor blade in 1.0 ml PBS. The supernatant representative of the intracapsular compartment was collected after centrifugation for 10 minutes at 4°C at 14000 rpm. Two hundred µL of distilled water was added to the cell pellet and was ultrasonicated to release the intracellular content. The cell debris

were removed by centrifugation for 10 minutes at 4°C at 14000 rpm (Eppendorf 5415). The appropriate assay was used to determine the secretion rate, intracapsular and intracellular distribution of the gene product.

2.2.9. Statistical Analysis

The formula applied for statistical analysis of our data was developed by Dr. G. Norman of the Department of Biostatistics, McMaster University.

From basic definitions:

$$\text{Standard Deviation}=\sqrt{\frac{\Sigma(\bar{x}_{ij}-\bar{x}_j)^2}{n-1}}$$

$$\text{Therefore, S. D.}^2=\frac{\Sigma(\bar{x}_{ij}-\bar{x}_j)^2}{n-1}$$

x_j is the mean of the group j and n is the sample size of a group. In our experiments, the sample size is three and therefore n=3 for all the groups.

The standard error is equal to S. D./$\sqrt{n}$:

$$\text{Therefore, S. E.}^2=\frac{\Sigma(\bar{x}_{ij}-\bar{x}_j)^2}{n(n-1)}$$

From the theory of ANOVA, the Sum of Squares(within) equals:

$$SS(within)=n\Sigma(x_{ij}-\bar{x}_j)^2$$

which in turn equals:

$$SS(within)=n^2(n-1)\Sigma S.E._j^2$$

Since the Mean Square within is the Sum of Squares within, divided by the appropriate degrees of freedom k(n-1), the Mean Square(within) equals:

$$MS(within)=\frac{S.S.(within)}{k(n-1)}=\frac{n^2}{k}\Sigma S.E._j^2$$

where k is the number of groups, which is always equal to three in our experiment.

Then, Tukey's Honestly Significant Difference (HSD) (Norman & Streiner, 1993) was applied in association with our derived formula. As such,

$$q=\frac{|\bar{x}_1-\bar{x}_2|}{\sqrt{MS_{within}/n}}=\frac{|\bar{x}_1-\bar{x}_2|}{\sqrt{\frac{n}{k}\Sigma S.E._j^2}}$$

where x_1 is the mean for all the data in group 1 and x_2 is the mean for all the data in group 2. MS_{within} is calculated for all three groups even when only two are compared. Then, the calculated q value is compared to $q_{n,0.05}$ where 5% is the cut-off for significant differences between between two groups. In this thesis, the sample size for all groups is 3 and $q_{3,0.05}$ is 2.506. As such, if the calculated q value is larger than 2.506, there is less than 5% probability that the difference between two groups was due to chance and therefore the difference is significant, and vice versa.

3.0 RESULTS

3.1 Characteristics of Original Cell Lines

Secretion rate of ß-gluc., ß-hexo. and hGH was measured from original cell lines. Data obtained can serve as a control to ensure the proteins detected from transfected cell lines were due to the vectors that express the products, and not solely due to the 'background' secretion from original cell lines.

3.1.1 ß-Glucuronidase and ß-Hexosaminidase Secretion from Rec$^-$ Cells

The average±S.D. secretion rate of ß-glucuronidase (ß-gluc.) from Rec$^-$ cells from 4 determinations was 2.27±0.03 unit of ß-gluc. per hour per million viable cells (U/hr/10^6 cells), or 15.0±1.6 unit of ß-gluc. per hour per milligram protein (U/hr/mg protein). The amount of intracellular ß-gluc. was 4.21±0.01 unit of ß-gluc. per million viable cells (U/10^6 cells), or 27.8±2.5 U/mg protein, 1.9-fold higher than the amount secreted from the same number of cells per hour (Table1).

The average±S.D. secretion rate of ß-hexosaminidase (ß-hexo.) from Rec$^-$ cells from 4 determinations was 425±2.02 U/hr/10^6 cells, or 1400±62.5 U/hr/mg protein. The amount of intracellular ß-hexo. was 854±15 U/10^6 cells, or 2810±510 U/mg protein, two fold higher than the amount secreted from the same number of cells per hour (Table1).

3.1.2 HGH Secretion from Ltk⁻ Cells

There was no hGH secretion from Ltk⁻ cells nor could hGH be detected intracellularly. This was because Ltk⁻ cells was not transfected with a vector that expresses hGH.

3.2 Characteristics of Recombinant Cell Lines

Secretion rate of ß-gluc., ß-hexo. and hGH was measured from transfected cell lines before encapsulation. These data can be compared to the secretion rate of the same products from encapsulated cells so that the amount of recombinant gene products actually delivered outside the capsules can be determined.

3.2.1 ß-Glucuronidase and ß-Hexosaminidase Secretion from 2A50 cells

The average±S.D. secretion rate of ß-gluc. from 2A50 cells from 8 determinations was 43.6±4.8 U/hr/10^6 cells, or 126±11 U/hr/mg protein. The amount of intracellular ß-gluc. was 1046±16 U/10^6 cells, or 3010±73 U/mg protein, 24-fold higher than the amount secreted from the same number of cells per hour. Secretion rate of ß-gluc. from 2A50 cells was 20-fold higher than from Rec^- cells since these cells were transfected with a vector that allows high expression of ß-gluc.

The average±S.D. secretion rate of ß-hexo. from 2A50 cells from 8 determinations was 424±35 U/hr/10^6 cells, or 1220±55 U/hr/mg protein. The amount of intracellular ß-hexo. was 954±30 U/10^6 cells, or 2743±24 U/mg protein, 2.3-fold higher than the amount secreted from the same number of cells per hour (Table1). Secretion rate of ß-hexo. from 2A50 cells

was the same as compared to Rec$^-$ cells, indicating the endogenous production of ß-hexo., and not due to the tranfected vector.

3.2.2 HGH Secretion from LhGH-1 Cells

The average±S.D. secretion rate of hGH from 10 determinations was 18.9±0.9 ng/hr/10^6 cells, or 106±6.9 ng/hr/mg protein. The amount of intracellular hGH was 0.81±0.04 ng/10^6 cells, or 4.40±0.53 ng/mg protein, 23-fold lower than the amount secreted from the same number of cells per hour, 24-fold lower than the amount secreted from the same amount of protein per hour (Table1).

Table 1: The secretion rates of recombinant gene products from non-encapsulated cells. Gene products under studied were ß-glucuronidase, ß-hexosaminidase and human growth hormone (hGH). Secretion rates of ß-glucuronidase and ß-hexosaminidase were measured from Rec^- cells and 2A50 cells. Samples were kept in triplicate dishes and an aliquot was removed from each dish and assayed for ß-gluc. and ß-hexo. for 4 times from Rec^- cells, 8 times from 2A50 cells. HGH was assayed for 10 times from Ltk^- and LhGH-1 cells.

Rec^- and Ltk^- cells were non-tranfected original cell lines whereas 2A50 cells were transfected with a vector that codes for high expression of ß-glucuronidase and LhGH-1 cells were transfected with a vector that codes for high expression of hGH. Data following the $\pm$ sign are standard deviations.

Table 1: Secretion rates of recombinant gene products from original and transfected cell lines

Secretion of ß-gluc. and ß-hexo. from Rec$^-$ derived cell lines

cell lines	Rec$^-$		2A50	
gene products	ß-gluc.	ß-hexo.	ß-gluc.	ß-hexo.
secretion rate (U/hr/10^6 cells)	2.27±0.03	425±2.02	43.6±4.8	424±35
secretion rate (U/hr/mg protein)	15.0±1.6	1400±62.5	126±11	1220±55
activities in cell lysate (U/10^6 cells)	4.21±0.01	854±15	1046±16	954±30
activities in cell lysate (U/mg protein)	27.8±2.5	2810±510	3010±73	2743±24

Secretion of hGH from Ltk$^-$ derived cell lines

Cell lines	Ltk$^-$	LhGH-1
gene product	hGH	hGH
secretion rate (ng/hr/10^6 cells)	0	18.9±0.9
secretion rate (ng/hr/mg protein)	0	106±7
activities in cell lysate (ng/10^6 cells)	0	0.81±0.04
activities in cell lysate (ng/mg protein)	0	4.40±0.53

3.3 Microencapsulation of 2A50 Cells and LhGH-1 Cells with Alginate using previously published protocol

APA capsules were constructed by crosslinking the extruded alginate droplets with poly-L-lysine (PLL) for 6 minutes and then dissolving the core of calcium-alginate with sodium citrate for 6 minutes to create single coated capsules. Double coated capsules were constructed with a 6 min. PLL wash and a 4 min. diluted alginate treatment followed by another 6 min. PLL treatment and a 4 min. diluted alginate wash. Then, a final 6 min. sodium citrate wash was used to remove the core alginate. Cells were encapsulated at densities of $1x10^6$ cells/ml or $2x10^6$ cells/ml alginate in single or double coated capsules. Secretion rates of recombinant gene products were measured so one has a better understanding as to the effects of initial 'seeding' densities and the presence of a double coat might have on cell viability and secretion rates.

3.3.1 Morphology of APA Capsules and Cell Proliferation

Most of the APA capsules (estimated 75% of each batch of experiment by visual judgement through a light microscope) were perfectly spherical. Since only the capsules with normal morphology were chosen for photography, the malformed capsules e.g. capsules have 'tails', non-spherical, etc. were not observed on our photographs. Both 2A50 and LhGH-1 cells were attachment-

dependent and formed clusters that were easily observable on day 15 with light microscopy.

3.3.2 Distribution of ß-Gluc. and ß-Hexo. in Single and Double Coated Capsules

2A50 cells were encapsulated at a density of $2x10^6$ cells/ml alginate using the above washing conditions, into single or double coated capsules. The distribution of ß-gluc. and ß-hexo. from these capsules has been summarized (Table 2).

Short term secretion assay from single coated capsules indicated a ß-gluc. secretion rate of 5.38 ± 1.1 U/hr/10^6 cells one day postencapsulation, which was 8-fold lower than non-encapsulated cells (Table 1). After one week, ß-gluc. secretion into the media increased 4-fold, to 21.0 ± 2.6 U/hr/10^6 cells and remained steady two weeks after encapsulation, at 22.5 ± 1.3 U/hr/10^6 cells. Double coated capsules exhibited a depressed ß-gluc. secretion on Day 1 but quickly increased to a rate similar to that of single coated capsules one week later (Fig 1A).

Intracapsular retention of ß-gluc. activities was in the same order of magnitude in both types of capsules. Intracellular ß-gluc. in both single and double coated capsules was 4 times less than non-encapsulated cells (Fig 1C).

ß-hexo. secretion from 2A50 cells entrapped in single coated capsules

was 5 times higher than the double coated capsules (Fig 1D). There was no dramatic change in ß-hexo. secretion rate in both types of capsules throughout the duration of the entire experiment. ß-hexo. secretion from single coated capsules (94.2±10.7 U/hr/10^6 cells, day 1) was 4.5-fold lower than from non-encapsulated cells (424±35 U/hr/10^6 cells), whereas ß-hexo. secretion from double coated capsules (18.5±4.1 U/hr/10^6 cells, day 1) was 24-fold lower than non-encapsulated cells. There were no significant differences between single and double coated capsules in terms of intracapsular and intracellular ß-hexo. activities throughout the experiment (Fig 1E, Fig 1F).

On day 15, a significant difference in viability was observed (Fig 1H). Cells in single coated capsules increased from 70.0±0.6% viability on day 1 to an 82.2±2.5% while those cells in double coated capsules decreased from 66.6±3.0% to 55.0±3.3%. Total cells per capsule at various time point indicated rapid cell proliferation and a doubling in cell number every 7 days (Fig 1G).

Table 2: Enzyme distribution and proliferation of 2A50 cells encapsulated in APA capsules using previously published protocol. Single and double coated capsules were made. All capsules were treated with 6 min. poly-L-lysine and 6 min. sodium citrate. All cells were encapsulated at a density of $2x10^6$ cells/ml. Intracapsular enzymes were released from crushed capsules. Intracellular enzymes were obtained by sonication of released cell pellets. Triplicate samplings were done and the standard devaitions were calculated.

Table 2: Enzymes distribution and cell proliferation of 2A50 cells encapsulated in APA capsules with previously published protocol

	ß-glucuronidase		
Days	secretion rate ($U/hr/10^6$ cells)	Intracap. ($U/10^6$ cells)	Intracell. ($U/10^6$ cells)
single coated			
1	5.38±1.10	286±27	222±31
8	21.0±2.6	76.0±4.5	269±15
15	22.5±1.3	130±8	274±14
double coated			
1	1.01±0.80	264±30	239±27
8	18.0±1.4	87.9±6.3	227±16
15	20.1±1.9	269±23	269±23

	ß-hexosaminidase		
Days	secretion rate ($U/hr/10^6$ cells)	Intracap. ($U/10^6$ cells)	Intracell. ($U/10^6$ cells)
single coated			
1	91.2±8.8	1830±249	1800±204
8	94.9±5.6	1790±91	1810±92
15	90.3±5.2	1740±99	1790±97
double coated			
1	18.1±4.1	1720±210	1700±204
8	19.0±1.5	1690±119	1710±120
15	19.1±1.9	1800±170	1820±159

Days	viability(%)	no.of cells/ capsule	no. of viable cells/capsule
single coated			
1	70.0±0.6	361±13	253±11
8	73.4±0.3	759±33	557±27
15	82.2±2.5	1450±29	1190±60
double coated			
1	66.6±3.0	360±21	240±24
8	69.3±1.7	732±31	507±34
15	55.0±3.3	1420±24	781±60

Fig 1: Enzyme distribution and cell proliferation of encapsulated 2A50 cells in APA capsules constructed with previously published protocol. 2A50 cells were encapsulated at 4°C, single and double coated, treated with 6 min. polylysine and 6 min. sodium citrate. All cells were encapsulated at a density of $2x10^6$ cells/ml. Single and double coated capsules were compared under the parameters below:

A: Secretion rate of ß-glucuronidase
B: Intracapsular concentration of ß-glucuronidase
C: Intracellular concentration of ß-glucuronidase
D: Secretion rate of ß-hexosaminidase
E: Intracapsular concentration of ß-hexosaminidase
F: Intracellular concentration of ß-hexosaminidase
G: Number of viable 2A50 cells/capsule
H: Viability of encapsulated 2A50 cells

Triplicate samplings were done and the standard deviation was shown as error bars.

Fig 1A:B-gluc.secretion
single vs double coats:APA capsules

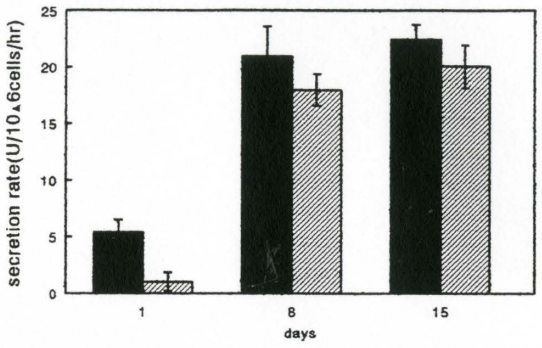


Fig 1B:Intracap. B-glucuronidase
single vs double coats:APA capsules

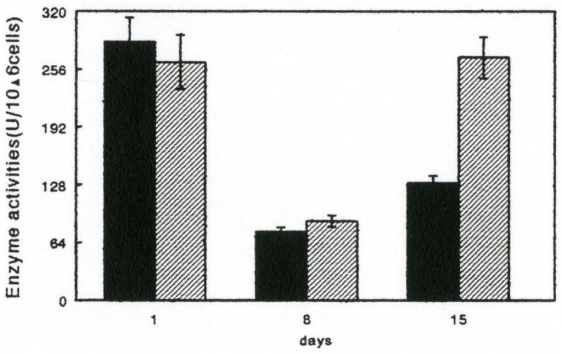


Fig 1C:Intracell. B-glucuronidase
single vs double coats:APA capsules

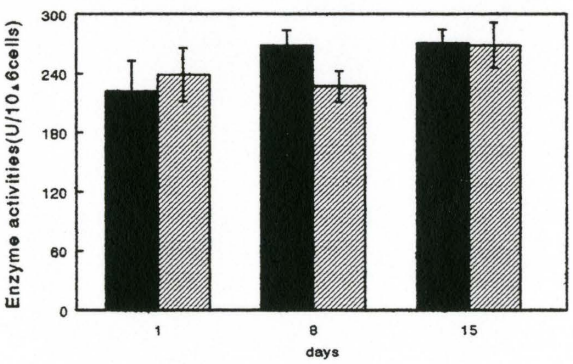


Fig 1D:B-hexo.secretion

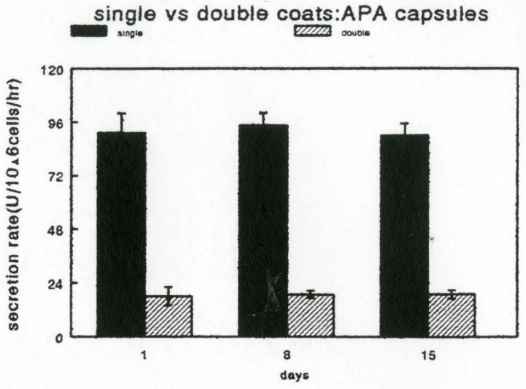


Fig 1E:Intracap. B-hexosaminidase

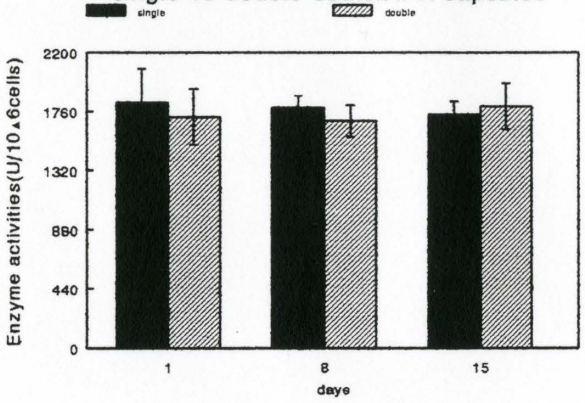


Fig 1F:Intracell. B-hexosaminidase

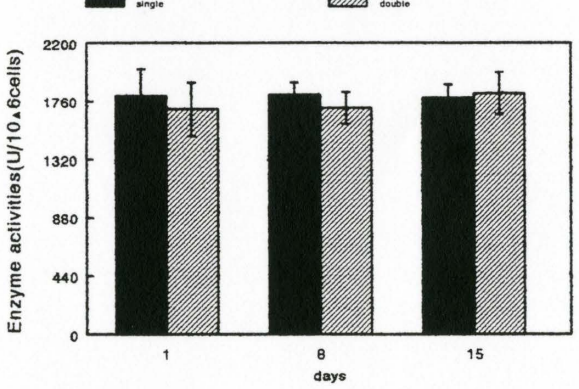


Fig 1G:no.of viable cells/capsule

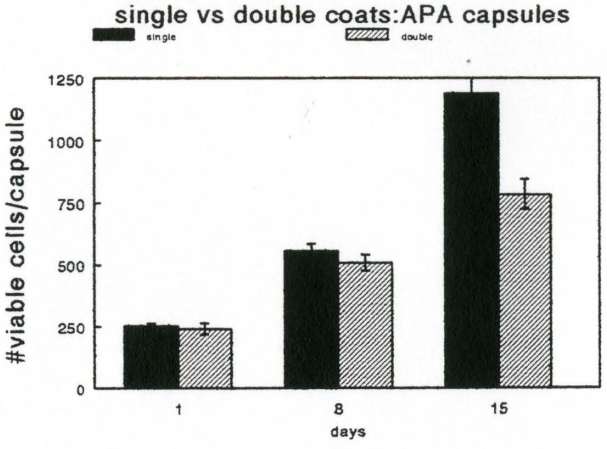
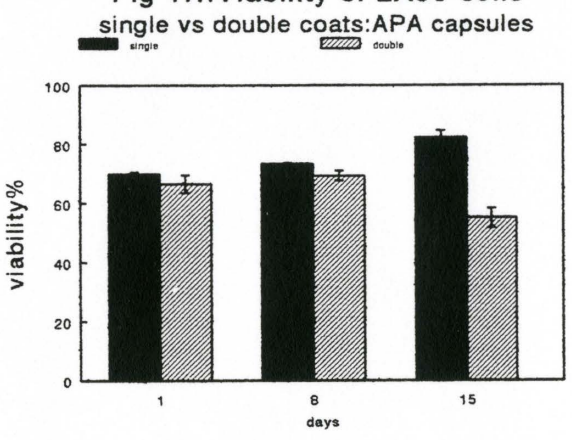


Fig 1H:Viability of 2A50 cells



3.3.3 Distribution of hGH in Single and Double Coated Capsules of Same Cell Density ($2x10^6$ cells/ml Alginate)

LhGH-1 cells were encapsulated at a cell density of $2x10^6$ cells/ml alginate into single or double coated capsules using the washing conditions described in the beginning of this section. The distribution of hGH was tabulated (Table 3).

There was no significant difference between single and double coated capsules in hGH distribution and cell proliferation. hGH secretion (Fig 2A) from the encapsulated cells on day 1 was depressed and secretion rates determined on all subsequent days were similar to the secretion rate from non-encapsulated cells. The depressed hGH secretion just after encapsulation indicated the inability of the line to recover from the process of encapsulation within 24 hours of the encapsulation experiment.

Intracapsular retention of hGH (Fig 2B) was 19% the amount secreted from encapsulated cells into the media, from the same number of viable cells assayed in one hour. This indicated hGH can freely diffuse across the capsular membrane. Intracellular hGH (Fig 2C) was 60% the amount secreted from encapsulated cells into the media per hour per million viable cells and 40% the amount secreted from non-encapsulated cells per hour per million viable cells.

The viability of the encapsulated cells was the highest one week after encapsulation (90%) and gradually decreased at subsequent time points (Fig 2E) . Cell proliferation was rapid during the first week (Fig 2D).

Table 3 & Table 4: Distribution of hGH from LhGH-1 cells encapsulated in APA capsules using previously published protocol. All capsules were treated with 6 min. poly-L-lysine and 6 min. sodium citrate. Intracapsular hGH was released from from crushed capsules. Intracellular hGH was obtained by sonication of released cell pellets. Triplicate samplings were done and the standard deviations were calculated.

Table 3: LhGH-1 cells were encapsulated at a density of $2x10^6$ cells/ml. Single and double coated capsules were made.

Table 4: LhGH-1 cells were encapsulated at densities of $1x10^6$ and $2x10^6$ cells/ml. All capsules were single coated.

Table 3: Distribution of hGH in APA capsules where LhGH-1 cells were encapuslated at a density of $2x10^6$ cells/ml alginate

Days	secretion (ng/hr/ 10^6 cells)	Intracap. (ng/10^6 cells)	intracell. (ng/10^6 cells)	viability(%)	no. of cells/ capsule	no.of viable cells/ capsule
single						
1	0.55±0.27	0.10±0.05	0.35±0.14	78.3±5.8	340±55	266±62
8	12.9±4.5	2.44±0.19	7.50±2.53	89.2±3.7	1100±87	981±118
15	13.3±3.6	2.50±1.03	7.98±3.76	80.0±1.5	1750±122	1400±124
22	13.7±4.0	2.52±1.10	7.95±4.08	72.3±1.2	1860±152	1345±132
29	13.5±3.1	2.43±1.20	7.62±2.30	58.3±2.6	1910±113	1114±116
double						
1	0.31±0.15	0.06±0.03	0.18±0.03	77.5±3.2	327±38	253±40
8	10.5±5.2	2.00±0.55	6.20±3.37	90.2±2.8	1030±18	929±45
15	13.8±3.2	2.60±1.31	6.88±2.74	82.3±1.3	1780±150	1465±147
22	13.5±4.1	2.53±1.12	7.35±3.25	70.5±0.8	1850±180	1304±142
29	13.4±4.5	2.58±1.42	7.54±2.58	55.8±1.5	1890±193	1055±136

Table 4: Distribution of hGH in single coated APA capsules where LhGH-1 cells were encapsulated at different densities

Days	secretion (ng/hr/ 10^6 cells)	Intracap. (ng/10^6 cells)	Intracell. (ng/10^6 cells)	viability (%)	no. of cells/ capsule	no. of viable cells/ capsule
$1x10^6$						
1	0.44±0.21	0.07±0.02	0.26±0.05	81.8±1.2	181±90	148±76
8	3.17±0.62	0.63±0.23	1.90±0.80	94.8±1.4	474±180	449±177
15	9.68±0.89	1.80±0.58	5.80±3.73	88.9±0.8	1120±200	996±187
22	9.85±1.58	1.85±0.62	6.23±3.52	83.0±2.1	1170±150	971±149
29	1.47±0.62	0.28±0.15	0.88±0.23	55.0±3.4	1170±122	644±107
$2x10^6$						
1	0.21±0.12	0.04±0.03	0.13±0.03	69.5±1.4	341±120	237±88
8	8.52±1.82	1.60±0.60	5.22±2.79	89.9±4.8	1170±43	1052±95
15	17.6±4.8	3.33±1.58	8.26±6.37	70.9±2.3	1640±200	1163±180
22	13.1±1.3	2.50±1.50	6.38±4.97	65.3±0.2	1860±250	1215±167
29	13.7±3.3	2.60±0.85	6.52±4.80	51.6±3.7	1900±111	980±128

Fig 2: Distribution of hGH and cell proliferation of encapsulated LhGH-1 cells in APA capsules constructed with previously published protocol. 2A50 cells were washed at 4°C, single and double coated, treated with 6 min. polylysine and 6 min. sodium citrate. All cells were encapsulated at a density of $2x10^6$ cells/ml. Single and double coated capsules were compared under the parameters below:

A: Secretion rate of hGH
B: Intracapsular concentration of hGH
C: Intracellular concentration of hGH
D: Number of viable LhGH-1 cells/capsule
E: Viability of encapsulated LhGH-1 cells

Triplicate samplings were done and the standard deviation was shown as error bars

Fig 2A:hGH secretion

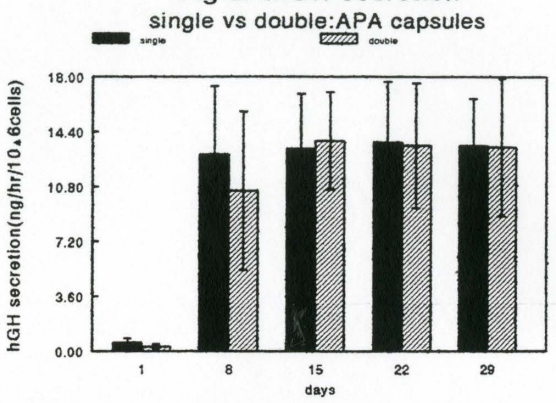


Fig 2B:Intracap. hGH

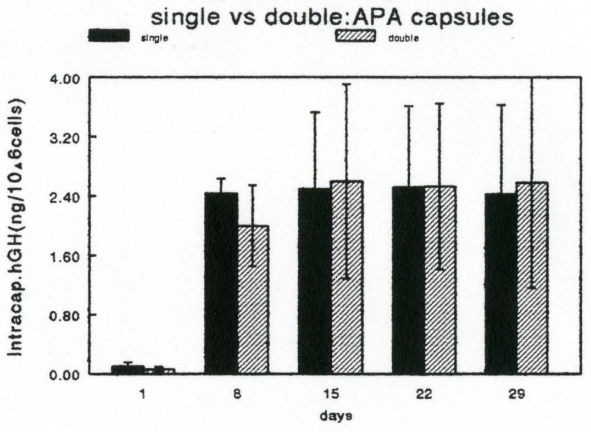


Fig 2C:Intracell. hGH

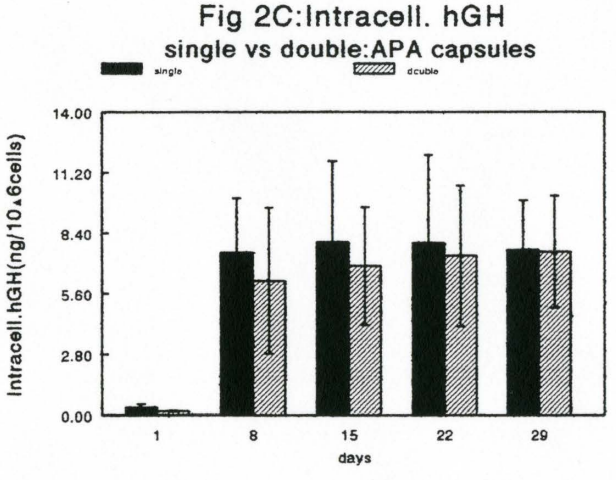


Fig 2D:no.of viable cells/capsule

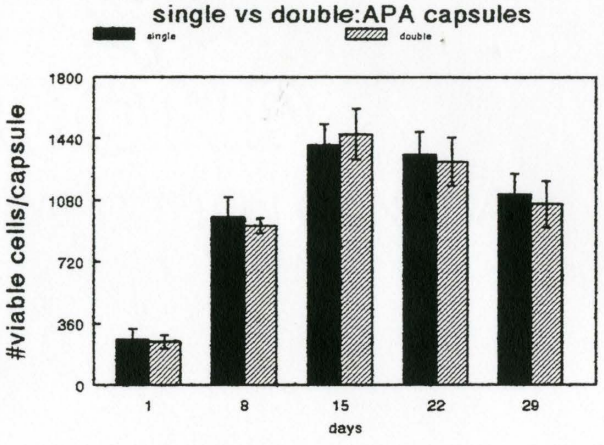
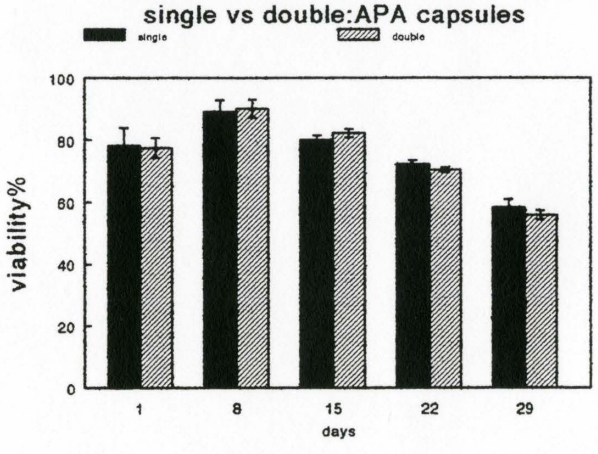


Fig 2E:Viability of LhGH-1 cells



3.3.4 Distribution of hGH in Single Coated Capsules Encapsulated at Different Densities

LhGH-1 cells were encapsulated at $1x10^6$ cells/ml alginate (low density) or $2x10^6$ cells/ml alginate (high density). The hGH distribution from both batches of capsules was tabulated (Table 4).

Cells encapsulated at low density exhibit a depressed hGH secretion on day 1 (Fig 3A), similar to the high density capsules. The low density capsules needed two weeks to attain a secretion rate similar to those from non-encapsulated cells whereas the hGH secretion rate determined from high density capsules from day 8 onwards was similar to the secretion rate from non-encapsulated cells assayed at the same time (15.5 ± 5.8 ng/hr/10^6 cells). A sharp decrease in hGH secretion was observed on day 29 from the low density cells. A similar profile was observed with cell proliferation (Fig 3D). Cells proliferate rapidly in the first week of the experiment (Fig 3F). Then the total number of cells per capsule remained constant from day 15 onwards. The maximum cell number per capsule determined from the low density capsules was less than 1200, which was 63% the maximum number of cells/capsule obtained from the high density capsules (Fig 3F).

Intracapsular hGH retention (Fig 3B) was 20% the amount secreted from encapsulated cells into the media, per hour per 10^6 viable cells, indicating hGH can freely diffuse across the capsular membrane. Intracellular hGH (Fig 3C) was 60% the amount secreted from encapsulated cells per hour per 10^6

viable cells, 40% the amount secreted from non-encapsulated cells. Cells encapsulated at low density maintained a viability of >80% in the first three weeks of the experiment and then decreased sharply to 55% on day 29, indicating its limitation to long term cell survival and proliferation in vitro (Fig 3E).

Fig 3: Distribution of hGH and cell proliferation of LhGH-1 cells encapsulated at different densities in APA capsules using the previously published protocol. Capsules were washed at 4°C, single coated, treated with 6 min. polylysine and 6 min. sodium citrate. Cells encapsulated at densities of $1x10^6$ and $2x10^6$ cells /ml were compared under the parameters below:

A: Secretion rate of hGH
B: Intracapsular concentration of hGH
C: Intracellular concentration of hGH
D: Number of viable LhGH-1 cells/capsule
E: Viability of encapsulated LhGH-1 cells

Triplicate samplings were done and the standard deviation was shown as error bars

Fig 3A:hGH secretion
Various densities:APA capsules

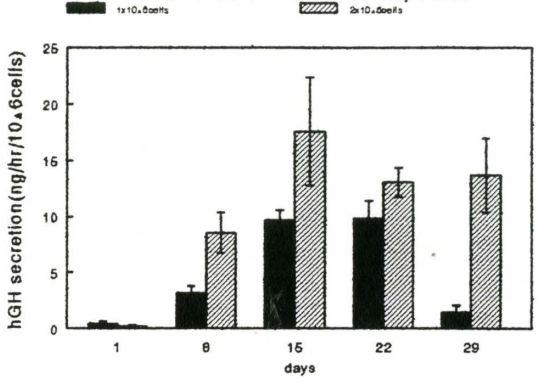


Fig 3B:Intracap. hGH
Various densities:APA capsules

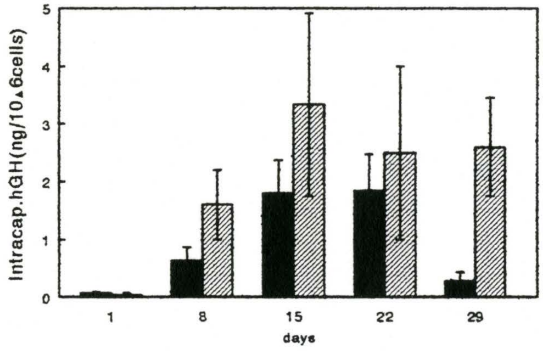


Fig 3C:Intracell. hGH
Various densities:APA capsules

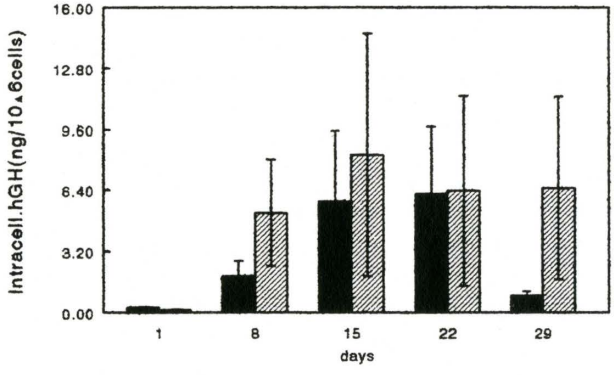


Fig 3D:no.of viable cells/capsule

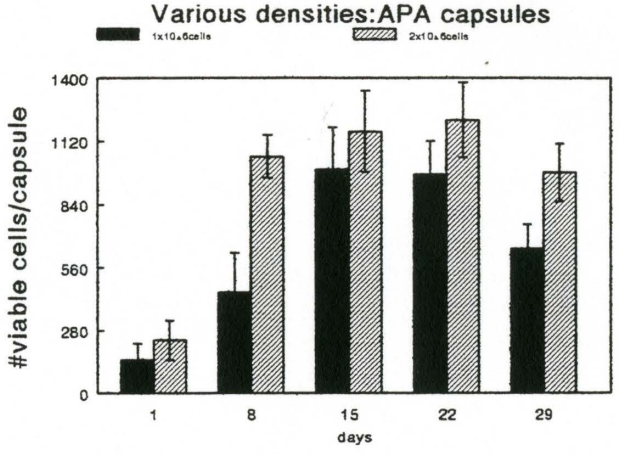
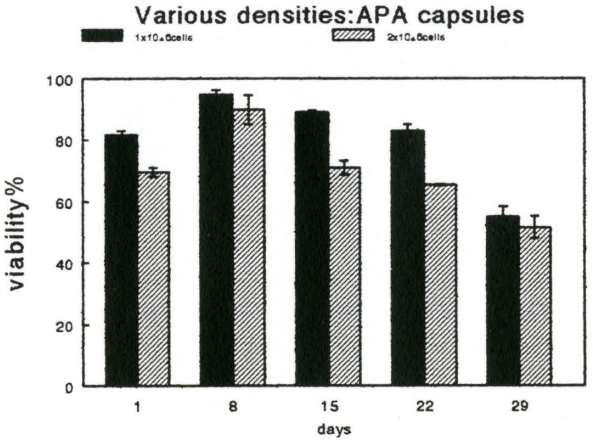
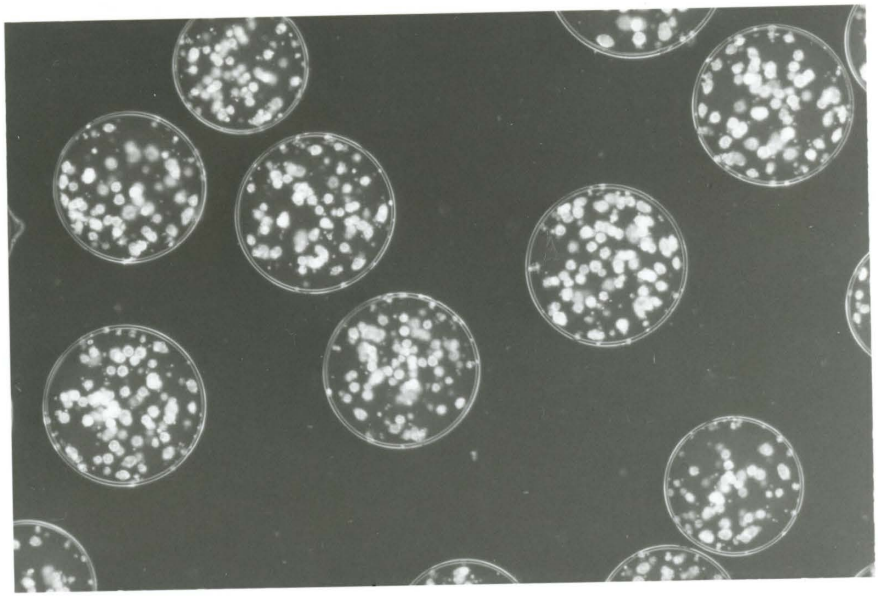


Fig 3E:Viability of LhGH-1 cells



400µm

A



B

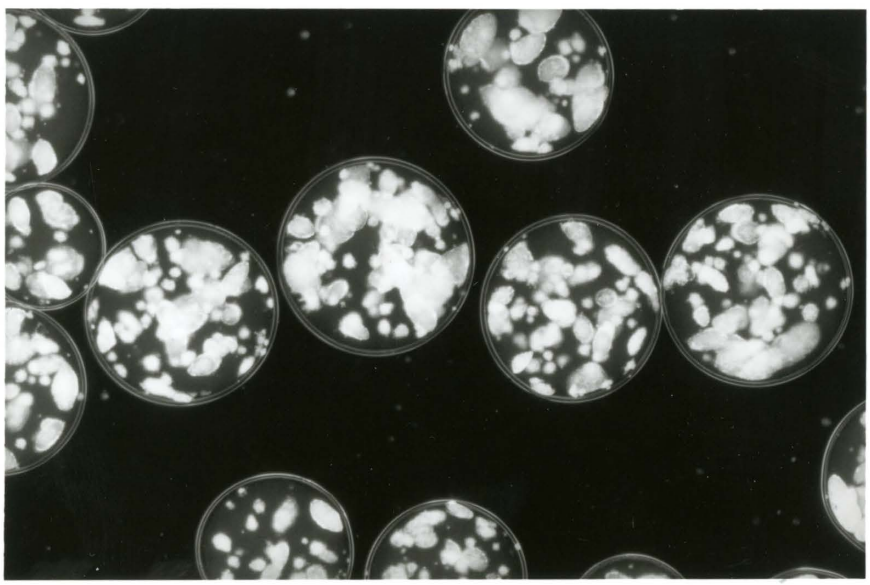


Fig 3F: Photomicrographs of encapsulated LhGH-1 cells in APA capsules. Cells were encapsulated at a density of $2x10^6$ cells/ml, single coated, treated with 6 min. polylysine and 6 min. sodium citrate. Photograph A illustrated LhGH-1 cells started to form clusters inside the capsules on day 8. Photograph B showed cell colonies had expanded by day 29 and started to fill up the capsules.

3.4 Microencapsulation of 2A50 Cells and Lh6H-1 Cells with Modified Protocol

A sensitive mouse fibroblasts cell line, 2A50, was chosen to define the conditions necessary to maximize the survival of the cells during encapsulation. The effects of temperature, duration of PLL treatment, duration of citrate treatment, cell density at encapsulation and different coatings at encapsulation on cell proliferation and membrane integrity were examined. 10 min. poly-L-lysine and 20 min. sodium citrate treatment were chosen to be optimal conditions for our modified protocol for the experiments on encapsulation at different cell densities and different coatings. Post-hoc pairwise comparison was applied to analyse the statistical significance of our data because raw data for sections 3.4.2 and 3.4.3 were not available, due to my failure to realize the importance of keeping all the raw data and repeating the relevant experiments should any of the raw data be lost.

3.4.1 Effects of Temperature on Cell Viability

2A50 cells were encapsulated with 6 min. PLL and 6 min. citrate treatment at 4°C, 10°C or room temperature. Viability decreased as the temperature of processing increased. Cells encapsulated at 4°C retained a viability that was significantly higher than that of cells encapsulated at room

temperature (post-hoc pairwise comparison: q=3.14, >95% significance). The viability of cells processed at 10°C was 35.6% and not significantly different from those processed at either 4°C or room temperature (post-hoc pairwise comparison: q=1.28, <95% significance) (Table 5A, 5B). As such, all washing solutions were kept at 4°C for subsequent experiments to ensure good viability for encapsulation cells.

Table 5A: Viability and the number of viable cells/capsule of encapsulated 2A50 cells treated with different temperature. During the process of encapsulation, APA capsules were either washed at 4°C, 10°C and 25°C. All capsules were treated with 6 min. poly-L-lysine and 6 min. sodium citrate. Triplicate samplings were done and the standard deviations were calculated.

Table 5B: Analysis at the significance of encapsulated 2A50 cells treated with different temperature. Post-hoc pairwise comparison was applied in association with equations derived by Dr. Norman for such analysis, where q value at 95% confidence interval=2.506. Data shown in this table were q values. If $q>2.506$, difference between groups was significant, noted as (s) after the q values. If $q< 2.506$, difference between groups was insignificant, noted as (ins) after the q values.

Table 5A: Viability and viable cells/capsule of APA encapsulated 2A50 cells treated with different washing temperatures

Temperature (celsius)	viability(%)	no. of viable cells/capsule
4°C	42.5±3.1	48±2
10°C	35.6±2.2	42±4
25°C	32.0±1.6	36±4

Table 5B: Analysis of the significance of 2A50 capsules treated with different washing temperatures

Groups compared	Viability	no. of viable cells/capsule
4°C vs 10°C	2.06(ins)	1.28(ins)
4°C vs 25°C	3.14(s)	2.56(s)
10°C vs 25°C	1.06(ins)	1.28(ins)

3.4.2 Effects of the Duration of Polylysine Treatment

2A50 cells were encapsulated at 4°C with 6, 10 or 20 min. PLL treatment and then treated with 6 min. sodium citrate. In the first two weeks, ß-gluc. secretion (Fig 4A) from the 6 and 10 min. PLL treated capsules was 8-fold lower than that of non-encapsulated cells. ß-hexo. secretion (Fig 4D) from all capsules was 4.5-fold lower than that of non-encapsulated cells. From day 15 onwards, the secretion rates and intracellular activity of both enzymes decreased when capsules were treated with 20 min. PLL. No significant differences on the viability and the proliferation of encapsulated 2A50 cells were observed for the first two weeks in culture (post-hoc pairwise comparison: viability, $q<0.76$, <95% significance; no. of viable cells/ capsule, $q<2.00$, <95% significance) (Table 6C).

From day 15 until day 29, viability (Fig 4H) and number of viable cells (Fig 4G) decreased rapidly in the samples treated with 20 min. PLL. On day 22, viability decreased to 42%. On day 29, the number of viable cells from the 20 min. sample was only 50% of those treated with 6 or 10 min. PLL. Viability of those treated with 6 or 10min. PLL declined more gradually to 70% at the end of the experiment (Table 6B). For subsequent experiments, 10 min. PLL was chosen since the best viability was maintained for one month.

Table 6A: Enzyme distribution from encapsulated 2A50 cells treated with polylysine of different durations. APA capsules were treated with either 6 min., 10 min. or 20 min. polylysine. All capsules were washed at 4°C and treated with 6min. sodium citrate. Intracapsular enzymes were obtained from crushed capsules. Intracellular enzymes were obtained by sonication of released cell pellets. Triplicate samplings were done and the standard deviation was calculated.

Table 6A: Enzymes distribution of APA encapsulated 2A50 cells treated with different durations of poly-L-lysine

	ß-glucuronidase			ß-hexosaminidase		
PLL duration (min.)	secretion (U/hr/ 10^6 cells)	Intracap. ($U/10^6$ cells)	Intracell. ($U/10^6$ cells)	secretion (U/hr/ 10^6 cells)	Intracap. ($U/10^6$ cells)	Intracell. ($U/10^6$ cells)
(day1) 6	5.28±0.1	294±20	224±6	95.8±1.7	1796±120	1806±82
10	5.12±0.1	286±18	224±8	95.0±0.2	1802±56	1813±94
20	4.95±0.12	275±36	222±5	95.0±0.1	1804±33	1814±63
(day8) 6	5.30±0.07	292±25	228±8	95.3±1.3	1802±46	1810±85
10	5.23±0.11	303±28	225±14	95.4±1.1	1800±82	1812±87
20	5.17±0.13	311±36	224±2	95.2±1.1	1802±31	1814±43
(day15)6	5.73±0.11	272±33	223±2	94.9±2.0	1806±28	1811±80
10	5.24±0.06	356±52	225±3	95.5±1.3	1803±48	1815±86
20	4.60±0.15	318±22	201±5	78.3±1.5	1804±68	1451±58
(day22)6	5.56±0.15	270±42	228±2	95.3±1.6	1807±77	1813±78
10	5.47±0.17	343±38	222±4	95.1±2.1	1803±98	1817±72
20	3.83±0.09	320±32	186±5	53.2±1.3	1801±62	1208±65
(day29)6	4.76±0.10	265±30	198±10	89.7±1.2	1800±104	1765±85
10	5.03±0.12	338±17	211±8	90.2±2.2	1798±48	1783±95
20	3.56±0.13	313±25	137±3	47.6±1.5	1788±53	1023±111

Table 6B: Proliferation of encapsulated 2A50 cells treated with different durations of polylysine. APA capsules were treated with either 6 min., 10 min. or 20 min. polylysine. All capsules were washed at 4°C and treated with 6 min. sodium citrate. Triplicate samplings were done and the standard deviation was calculated.

Table 6B: Proliferation of APA encapsulated 2A50 cells treated with different durations of poly-L-lysine

PLL duration (minutes)	viability(%)	no. of viable cells/capsule
(day1) 6	42.5±4.0	43±3
10	40.7±3.4	42±2
20	45.2±4.9	49±4
(day8) 6	74.3±2.3	96±6
10	73.8±7.6	98±5
20	69.6±1.2	101±5
(day15)6	82.6±3.1	125±1
10	81.6±2.0	131±2
20	66.1±3.4	112±2
(day22)6	72.4±1.3	131±5
10	70.4±2.1	142±2
20	42.1±1.0	80±4
(day29)6	52.4±0.5	121±10
10	63.6±0.4	133±7
20	36.2±0.4	72±7

Table 6C: The analysis of the significance of encapsulated 2A50 cells treated with different durations of polylysine. Post-hoc pairwise comparison was used in association with equations derived by Dr. Norman for such analysis, where q values at 95% confidence interval=2.506. Data shown in this table were q values. If q> 2.506, then differences between two groups were significant, designated as (s). If q< 2.506, differences between two groups were insignificant, designated as (ins).

Table 6C:Analysis of the significance of 2A50 cells treated with different polylysine duration

	β-glucuronidase			β-hexosaminidase				
PLL duration (minutes)	secretion	intracap.	intracell.	secretion	intracap.	intracell.	viability	viable cells/ capsule
Day1								
6 vs 10	1.13(ins)	0.18(ins)	0(ins)	0.56(ins)	0.04(ins)	0.05(ins)	0.31(ins)	0.23(ins)
6 vs 20	2.33(ins)	0.42(ins)	0.18(ins)	0.56(ins)	0.06(ins)	0.06(ins)	0.46(ins)	1.41(ins)
10 vs 20	1.20(ins)	0.24(ins)	0.18(ins)	0(ins)	0.01(ins)	0.01(ins)	0.76(ins)	1.64(ins)
Day8								
6 vs 10	0.49(ins)	0.25(ins)	0.18(ins)	0.06(ins)	0.02(ins)	0.01(ins)	0.08(ins)	0.26(ins)
6 vs 20	0.92(ins)	0.43(ins)	0.25(ins)	0.12(ins)	0(ins)	0.02(ins)	0.72(ins)	0.66(ins)
10 vs 20	0.42(ins)	0.18(ins)	0.06(ins)	0.06(ins)	0.02(ins)	0.01(ins)	0.64(ins)	0.40(ins)
Day15								
6 vs 10	3.10(s)	1.28(ins)	0.32(ins)	0.26(ins)	0.03(ins)	0.03(ins)	0.24(ins)	2.00(ins)
6 vs 20	7.15(s)	0.70(ins)	3.57(s)	7.27(s)	0.02(ins)	2.75(s)	4.03(s)	4.33(s)
10 vs 20	4.05(s)	0.58(ins)	3.89(s)	7.54(s)	0.01(ins)	2.78(s)	3.79(s)	6.33(s)
Day22								
6 vs 10	0.46(ins)	1.12(ins)	0.89(ins)	0.08(ins)	0.03(ins)	0.03(ins)	0.92(ins)	2.04(ins)
6 vs 20	8.87(s)	0.77(ins)	6.26(s)	17.5(s)	0.04(ins)	4.86(s)	13.9(s)	9.47(s)
10 vs 20	8.41(s)	0.35(ins)	5.37(s)	17.4(s)	0.01(ins)	4.89(s)	13.0(s)	11.5(s)
Day29								
6 vs 10	0.05(ins)	1.71(ins)	0.99(ins)	0.21(ins)	0.02(ins)	0.11(ins)	19.2(s)	1.03(ins)
6 vs 20	7.08(s)	1.13(ins)	4.64(s)	17.7(s)	0.10(ins)	4.39(s)	27.8(s)	4.20(s)
10 vs 20	8.67(s)	0.59(ins)	5.63(s)	17.9(s)	0.08(ins)	4.50(s)	47.0(s)	5.23(s)

Fig 4: Enzyme distribution and cell proliferation of encapsulated 2A50 cells treated with different durations of polylysine. 2A50 cells were encapsulated at a density of $2x10^6$ cells/ml. Capsules were washed at 4°C, single coated and treated with 6 min. sodium citrate. They were treated with 6 min, 10 min. or 20 min. polylysine. The parameters below were examined:

A: Secretion rate of ß-glucuronidase
B: Intracapsular concentration of ß-glucuronidase
C: Intracellular concentration of ß-glucuronidase
D: Secretion rate ß-hexosaminidase
E: Intracapsular concentration of ß-hexosaminidase
F: Intracellular concentration of ß-hexosaminidase
G: Number of viable 2A50 cells/capsule
H: Viability of encapsulated 2A50 cells

Triplicate samplings were done and the standard deviation was shown as error bars

Fig 4A:B-gluc.secretion

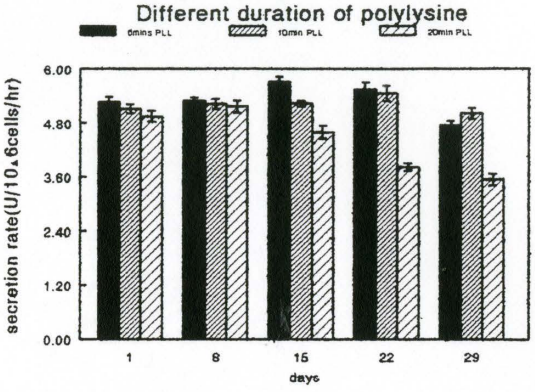


Fig 4B:Intracap. B-glucuronidase

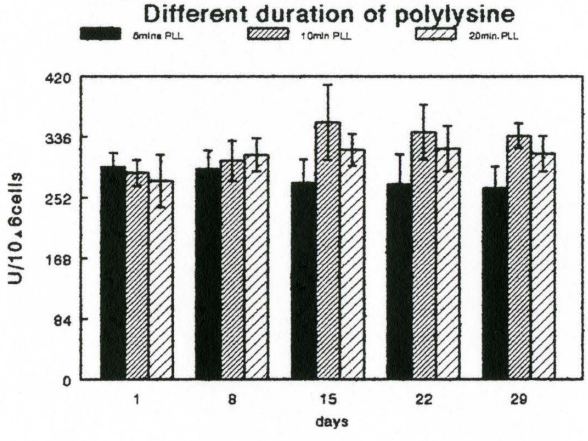


Fig 4C:Intracell. B-glucuronidase

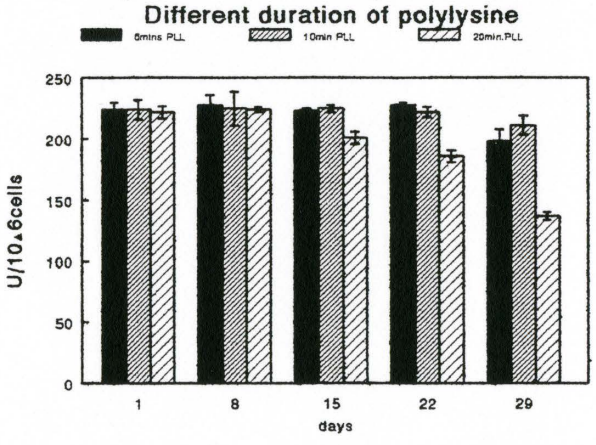


Fig 4D:B-hexo.secretion

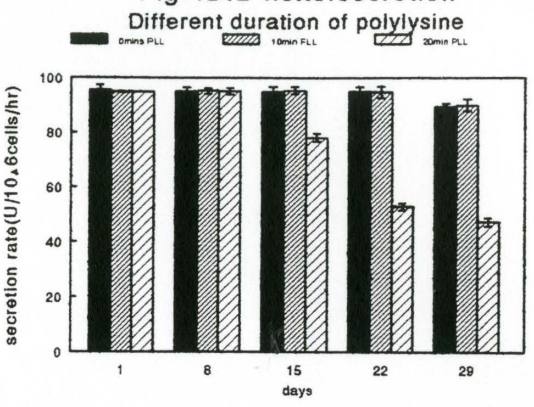


Fig 4E:Intracap. B-hexosaminidase

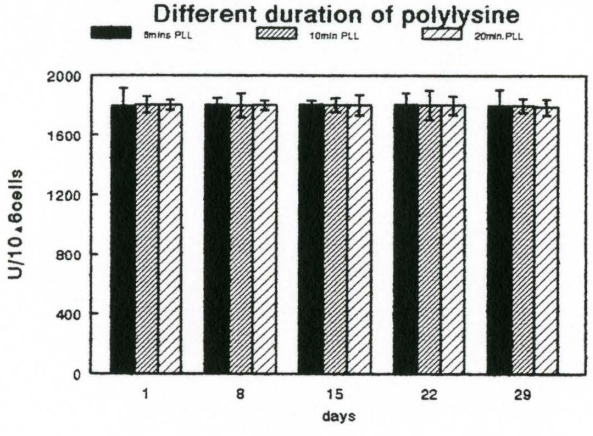


Fig 4F:Intracell. B-hexosaminidase

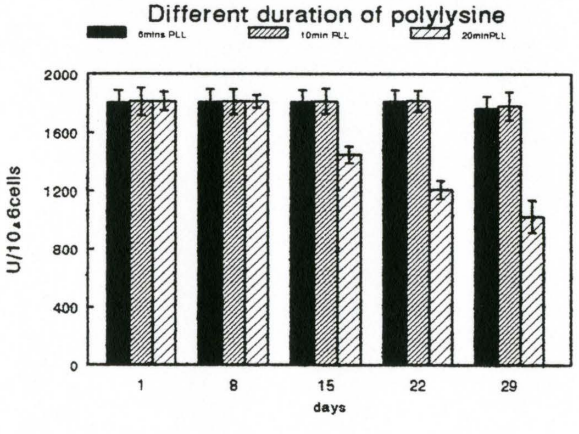


Fig 4G:no.of viable cells/capsule

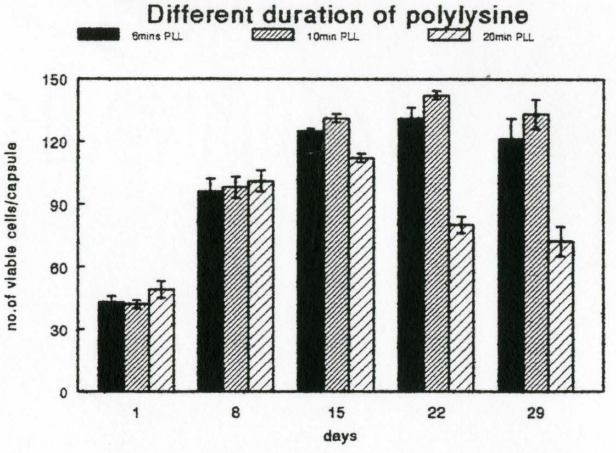
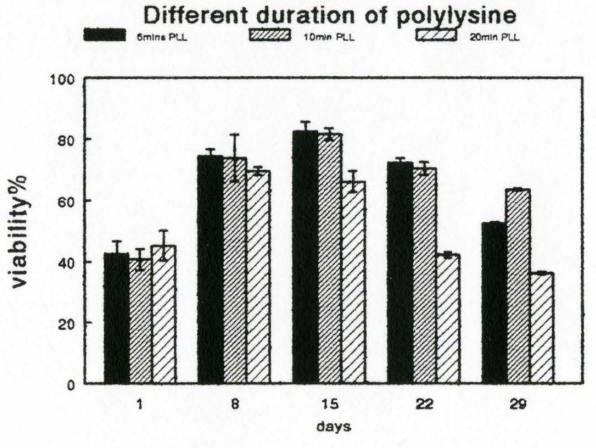


Fig 4H:Viability of 2A50 cells



3.4.3 Effect of the Duration of Sodium Citrate Treatment

Sodium citrate liquefied the gelled core alginate and create an intra-capsular space. Six minutes of treatment as suggested in published articles appeared inadequate to liquefy the core. The effects of prolonged sodium citrate treatment on cell proliferation was studied in this experiment. 2A50 cells was encapsulated at 4°C with 6 min. PLL treatment and liquefied with either 6, 10 or 20 min. sodium citrate.

The viability (Fig 5B) and cell proliferation (Fig 5A) of all samples were similar in the first two weeks of the experiment. On day 22, viability of the samples treated with 6 min. citrate decreased from 95% to 80% whereas that of samples treated with 20 min. citrate was maintained at over 90%. On day 29, significant differences were observed with samples treated with 20 min. citrate (87%), as compared to those treated with 10 min. citrate (71%) or 6min. citrate (62%) (Table 7A).

A similar trend could be observed for cell proliferation. On day 22, cell number in samples treated with 20 min. citrate was two fold higher than those treated with 6min. citrate. By day 29, the samples treated with 20 min. citrate (Fig 5C) contained 1300 viable cell per capsule whereas those treated with 10 min. citrate (Fig 5C), about 960 viable cells per capsule and only 600 cells per capsule for the 6 min. citrate (Fig 5C) (Table 7A). Hence, 20 min. citrate treatment was chosen as the washing condition for subsequent experiments so that the encapsulated cells have more space for proliferation.

Table 7A: Proliferation of encapsulated 2A50 cells treated with different durations of sodium citrate. APA capsules were treated with either 6 min., 10 min. or 20 min. sodium citrate. All capsules were washed at 4°C and treated with 6min. polylysine. Triplicate samplings were done and the standard deviation was calculated.

Table 7B: The analysis of the significance of encapsulated 2A50 cells treated with different durations of sodium citrate. Post-hoc pairwise comparison was applied in association with equations derived by Dr. Norman for such analysis, where q values at 95% confidence intervals=2.506. Data shown in this table were q values. If $q > 2.506$, differences between two groups were significant, designated as (s). If $q < 2.506$, differences between two groups were insignificant, designated as (ins).

Table 7A: Proliferation of APA encapsulated 2A50 cells treated with different durations of sodium citrate

	viability(%)			no. of viable cells/capsule		
Days	6min.	10min.	20min.	6min.	10min.	20min.
1	41.8±0.1	43.1±0.9	43.0±0.1	58±6	53±7	57±5
8	82.2±3.8	85.2±2.3	84.3±1.3	122±12	125±9	124±10
15	95.2±2.7	95.9±0.1	96.9±1.0	524±10	500±10	550±50
22	80.4±3.8	88.9±1.0	93.7±0.4	829±36	1156±34	1683±29
29	61.9±0.7	71.4±0.6	86.5±0.5	598±9	959±15	1333±21

Table 7B: Analysis of significance of 2A50 capsules treated with different durations of sodium citrate

Groups compared	viability(%)	no. of viable cells/capsule
Day1		
6min. vs 10min.	1.82(ins)	0.57(ins)
6min. vs 20min.	1.68(ins)	0.11(ins)
10min. vs 20min.	0.14(ins)	0.46(ins)
Day8		
6min. vs 10min.	0.79(ins)	0.21(ins)
6min. vs 20min.	0.55(ins)	0.14(ins)
10min. vs 20min.	0.24(ins)	0.07(ins)
Day15		
6min. vs 10min.	0.30(ins)	0.56(ins)
6min. vs 20min.	0.73(ins)	0.61(ins)
10min. vs 20min.	0.43(ins)	1.18(ins)
Day22		
6min. vs 10min.	2.64(s)	6.97(s)
6min. vs 20min.	4.14(s)	18.2(s)
10min. vs 20min.	1.49(ins)	11.2(s)
Day29		
6min. vs 10min.	10.8(s)	16.4(s)
6min. vs 20min.	28.0(s)	33.5(s)
10min. vs 20min.	17.2(s)	17.0(s)

Fig 5: Cell proliferation of encapsulated 2A50 cells treated with different durations of sodium citrate in APA capsules. 2A50 cells were encapsulated at 4°C, single coated, at a density of $2x10^6$ cells/ml and treated with 6 min. polylysine. Capsules were washed with either 6 min., 10 min. or 20 min. sodium citrate. The parameters below were examined:

A: Number of viable 2A50 cells/capsule
B: Viability of encapsulated 2A50 cells

Triplicate samplings were done and the standard deviation was shown as error bars

Fig 5A:no.of viable cells/capsule

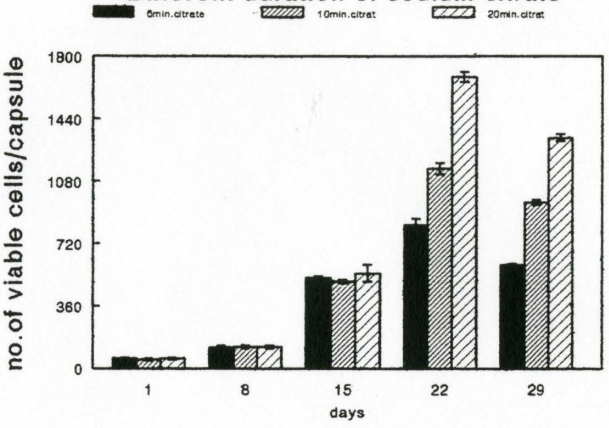
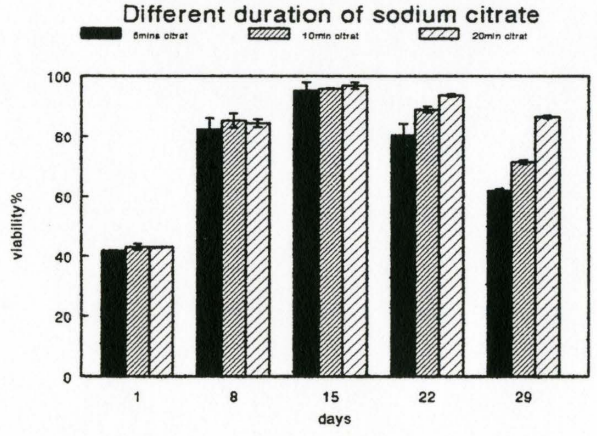
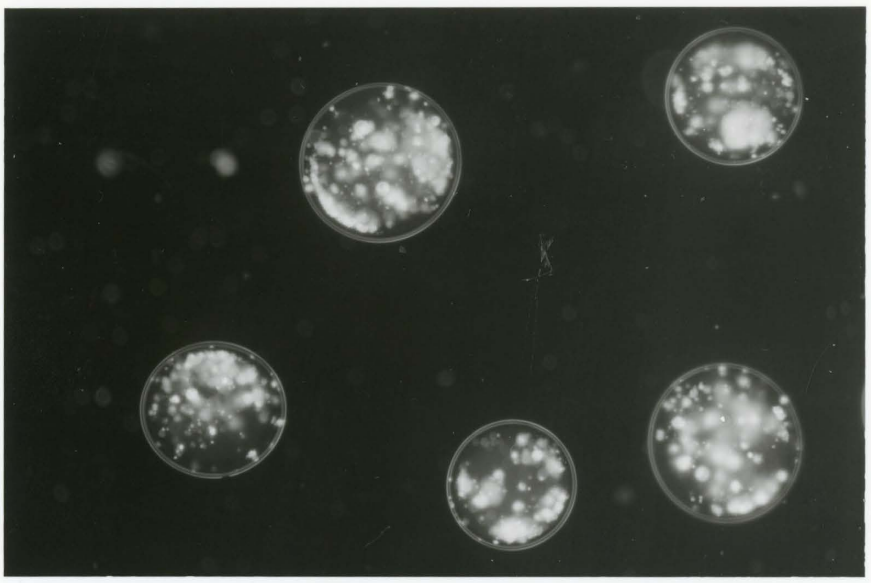


Fig 5B:Viability of 2A50 cells



400µm

A



B

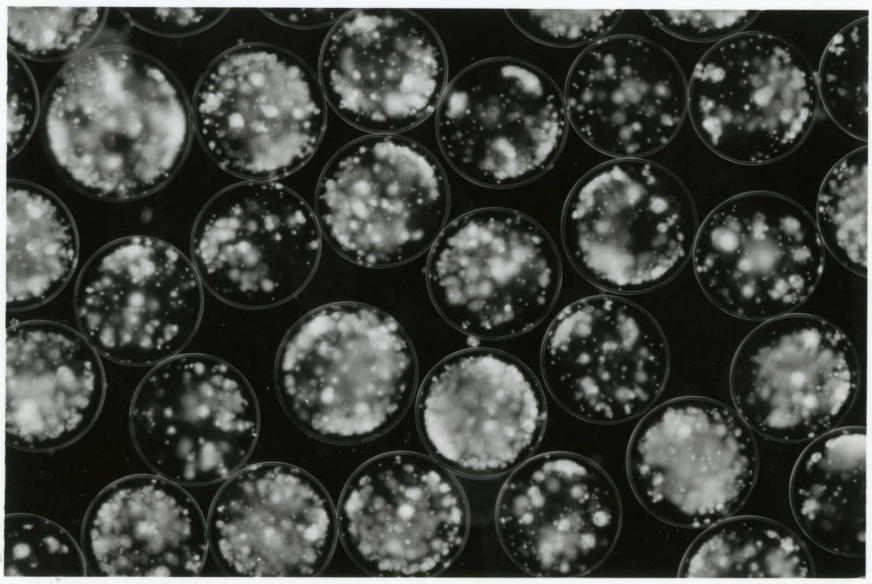
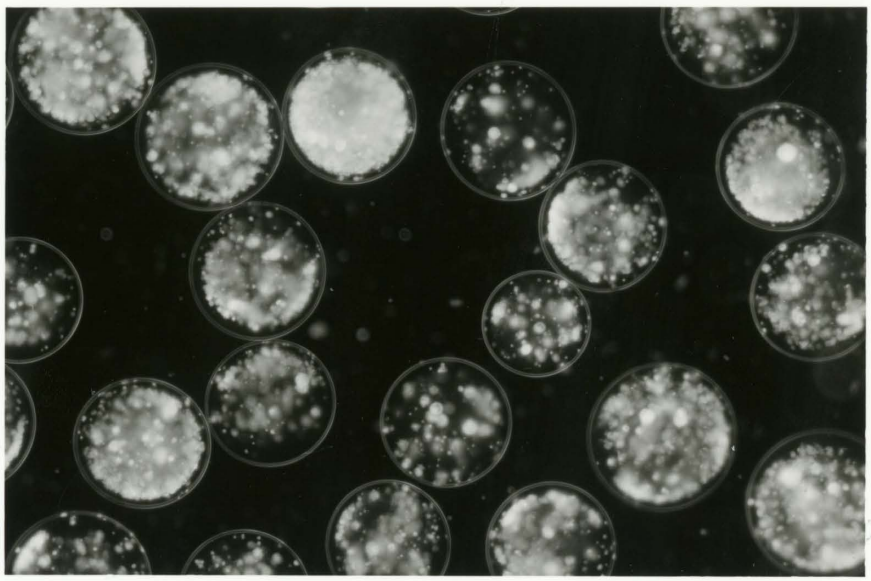


Fig 5C: Photomicrographs of encapsulated 2A50 cells treated by different durations of sodium citrate on day 29 of the experiment. 2A50 cells were encapsulated at a density of $2x10^6$ cells/ml, single coated and treated with 6 min. polylysine. Photograph A demonstrated the low cell density per capsule when treated with 6 min. sodium citrate. Photograph C demonstrated much higher cell density per capsule, with some of the capsules almost completely filled up with cells, when treated with 20 min. citrate. Photograph B showed intermediate cell density when treated with 10 min. citrate.

C

400µm



3.4.4 Distribution of ß-Gluc. and ß-Hexo. in Single Coated Microcapsules with Different Cell Densities

The effects of cell density at encapsulation on enzyme secretion and cell proliferation was examined. 2A50 cells were encapsulated at 4°C, treated with 10 min. polylysine, 20 min. sodium citrate, at a density of either $1x10^6$ cells/ml, $2x10^6$ cells/ml or $4x10^6$ cells/ml alginate.

Significant differences in ß-gluc. secretion (Fig 6A) among all samples encapsulated at various densities was observed (Table 8A). Cells encapsulated at $1x10^6$ cells/ml secreted twice the amount of ß-gluc. as compared to those encapsulated at $4x10^6$ cells/ml, per hour per 10^6 viable cells. Intracapsular ß-gluc. (Fig 6B) accumulate with time only when cells were encapsulated at $1x10^6$ cells/ml, where the retention of ß-gluc. activities increased more than three fold from 200U/10^6 cells on day1 to 655U/10^6 cells on day 29. Regardless of cell densities, ß-hexo. secretion remained the same throughout the experiment (Fig 6D) and intracapsular ß-hexo. did not accumulate with time (Fig 6E), indicating ß-hexo. diffused freely across the capsule membrane.

Viability (Fig 6H) of cells encapsulated at $1x10^6$ cells/ml and $4x10^6$ cells /ml never exceeded 60% whereas those encapsulated at $2x10^6$ cells/ml increased from less than 50% on day 1 to 87.4% on day 15. Data on the number of viable cells/capsule (Fig 6G) indicated only those encapsulated at $2x10^6$ cells/ml proliferated readily with time (Fig 6I). The most ideal density for encapsulation of 2A50 cells seemed to be $2x10^6$, in terms of proliferation and long term survival of the cells (Table 8B).

Table 8A: Enzyme distribution in APA capsules where 2A50 cells were encapsulated at different cell densities. The "seeding" densities were $1x10^6$, $2x10^6$ and $4x10^6$ cells/ml alginate. All APA capsules were washed at 4°C, treated with 10 min. polylysine and 20 min. sodium citrate. Intracapsular enzymes were released from crushed capsules. Intracellular enzymes were obtained by sonication of released cell pellets. Triplicate samplings were done and the standard deviation was calculated.

Table 8A: Enzymes distribution of 2A50 cells encapsulated in APA capsules at different cell densities

	ß-glucuronidase			ß-hexosaminidase		
Density (10^6 cells/ml)	secretion (U/hr/ 10^6 cells)	Intracap. (U/10^6 cells)	Intracell. (U/10^6 cells)	secretion (U/hr/ 10^6 cells)	Intracap (U/10^6 cells)	Intracell. (U/10^6 cells)
(day1) 1	3.67±0.06	200±18	181±20	95.0±0.2	1813±38	1819±24
2	5.27±0.02	285±14	226±27	94.9±0.1	1803±32	1813±17
4	7.70±0.06	180±12	240±42	95.1±0.3	1793±48	1810±33
(day8) 1	3.70±0.04	313±15	225±28	94.8±0.2	1800±33	1813±23
2	5.28±0.05	287±13	225±18	94.9±0.3	1807±42	1817±21
4	7.70±0.01	180±14	229±10	94.9±0.1	1804±31	1815±25
(day15)1	3.65±0.06	425±22	225±23	95.2±0.2	1800±62	1812±26
2	5.25±0.08	284±13	239±17	95.1±0.4	1803±48	1826±64
4	7.68±0.04	180±21	225±34	95.3±0.3	1805±32	1818±35
(day22)1	3.76±0.09	543±22	225±17	95.1±0.4	1821±52	1786±26
2	5.26±0.04	286±15	231±18	94.8±0.3	1806±39	1819±40
4	7.73±0.05	175±27	225±10	94.7±2.2	1805±32	1818±35
(day29)1	3.73±0.09	655±53	225±17	95.3±0.7	1850±120	1850±48
2	5.29±0.03	287±12	221±13	94.3±0.5	1798±86	1820±99
4	7.69±0.05	173±21	221±13	94.0±1.1	1808±85	1827±56

Table 8B: Proliferation of 2A50 cells encapsulated at different cell densities. The "seeding" densities were $1x10^6$, $2x10^6$ and $4x10^6$ cells/ml alginate. All APA capsules were washed at 4°C, treated with 10 min. polylysine and 20 min. sodium citrate. Triplicate samplings were done and the standard deviation was calculated.

Table 8B: Proliferation of 2A50 cells encapsulated in APA capsules at different cell densities

density (10^6 cells/ ml)	viability(%)	no.of viable cells/ capsule
(day1) 1	46.9±1.4	24±3
2	48.3±0.7	46±3
4	48.6±0.4	91±5
(day8) 1	52.7±3.0	48±5
2	59.5±3.3	98±5
4	57.3±3.1	180±11
(day15)1	54.0±2.7	58±2
2	87.4±1.0	213±23
4	52.6±1.4	188±0
(day22)1	52.1±0.8	64±9
2	74.1±2.0	408±7
4	51.2±0.2	203±12
(day29)1	47.2±2.6	57±0
2	73.5±3.0	716±6
4	45.0±1.5	188±5

Table 8C: The analysis of the significance of 2A50 cells encapsulated at different cell densities. Post-hoc pairwise comparison was applied in association with equations derived by Dr. Norman for such analysis, where q values at 95% confidence intervals=2.506. Data shown in this table were q values. If q> 2.506, differences between two groups were significant, designated as (s). If q< 2.506, differences between two groups were insignificant, designated as (ins).

Table 8C:Analysis of the significance of 2A50 cells encapsulated at different densities

	B-glucuronidase			B-hexosaminidase				
Density (10^6cells/ml)	secretion	intracap.	intracell.	secretion	intracap.	intracell.	viability	#viable cells/ capsule
Day1								
1 vs 2	18.4(s)	3.30(s)	0.84(ins)	0.27(ins)	0.14(ins)	0.14(ins)	0.87(ins)	3.35(s)
1 vs 4	46.2(s)	0.78(ins)	1.10(ins)	0.27(ins)	0.29(ins)	0.20(ins)	1.05(ins)	10.2(s)
2 vs 4	27.9(s)	4.07(s)	0.26(ins)	0.53(ins)	0.14(ins)	0.07(ins)	0.19(ins)	6.86(s)
Day8								
1 vs 2	24.4(s)	1.07(ins)	0(ins)	0.27(ins)	0.11(ins)	0.10(ins)	1.25(ins)	3.82(s)
1 vs 4	61.7(s)	5.48(s)	0.12(ins)	0.27(ins)	0.06(ins)	0.05(ins)	0.85(ins)	10.1(s)
2 vs 4	37.3(s)	4.41(s)	0.12(ins)	0(ins)	0.05(ins)	0.05(ins)	0.41(ins)	6.27(s)
Day15								
1 vs 2	14.9(s)	4.26(s)	0.32(ins)	0.19(ins)	0.03(ins)	0.16(ins)	10.4(s)	6.71(s)
1 vs 4	34.7(s)	7.40(s)	0(ins)	0.19(ins)	0(ins)	0.03(ins)	0.44(ins)	5.63(s)
2 vs 4	22.6(s)	3.14(s)	0.32(ins)	0.37(ins)	0.03(ins)	0.13(ins)	10.9(s)	1.08(ins)
Day22								
1 vs 2	13.9(s)	6.78(s)	0.22(ins)	0.13(ins)	0.21(ins)	0.56(ins)	10.2(s)	20.8(s)
1 vs 4	35.9(s)	9.70(s)	0(ins)	0.18(ins)	0.22(ins)	0.54(ins)	0.42(ins)	8.40(s)
2 vs 4	22.4(s)	2.93(s)	0.22(ins)	0.04(ins)	0.01(ins)	0.02(ins)	10.6(s)	12.4(s)
Day29								
1 vs 2	14.5(s)	6.32(s)	0(ins)	0.72(ins)	0.31(ins)	0.19(ins)	6.20(s)	84.4(s)
1 vs 4	36.9(s)	8.27(s)	0.11(ins)	0.93(ins)	0.25(ins)	0.15(ins)	0.52(ins)	16.8(s)
2 vs 4	22.4(s)	1.96(ins)	0.11(ins)	0.21(ins)	0.06(ins)	0.04(ins)	6.72(s)	67.6(s)

Fig 6: Enzyme distribution and cell proliferation of 2A50 cells encapsulated at different densities. Capsules were washed at 4°C, single coated, treated with 10 min. polylysine and 20 min. sodium citrate. 2A50 cells were "seeded" at densities of $1x10^6$, $2x10^6$ or $4x10^6$ cells/ml. The parameters below were examined:

A: Secretion rate of ß-glucuronidase
B: Intracapsular concentration of ß-glucuronidase
C: Intracellular concentration of ß-glucuronidase
D: Secretion rate of ß-hexosaminidase
E: Intracapsular concentration of ß-hexosaminidase
F: Intracellular concentration of ß-hexosaminidase
G: Number of viable 2A50 cells/capsule
H: Viability of encapsulated 2A50 cells

Triplicate samplings were done and the standard deviation was shown as error bars

Fig 6A:B-gluc.secretion
Different cell densities:APA capsules

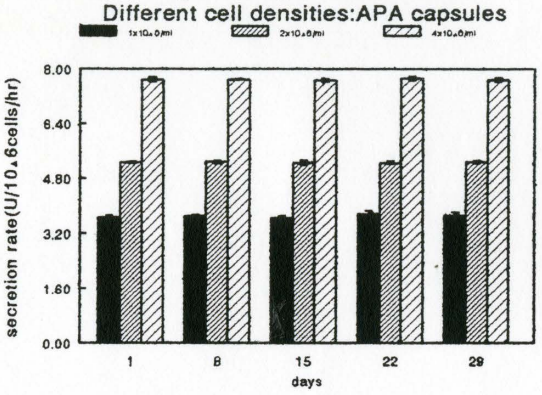


Fig 6B:Intracap. B-glucuronidase
Different cell densities:APA capsules

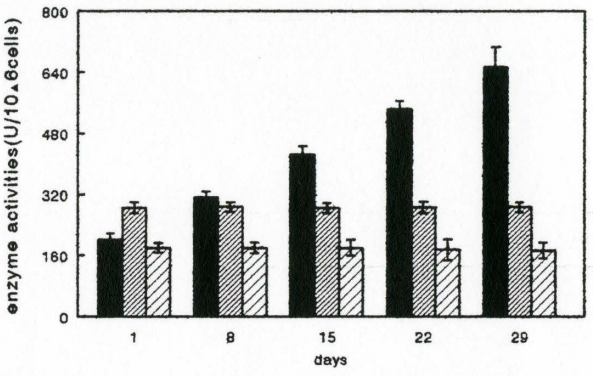


Fig 6C:Intracell. B-glucuronidase
Different cell densities:APA capsules

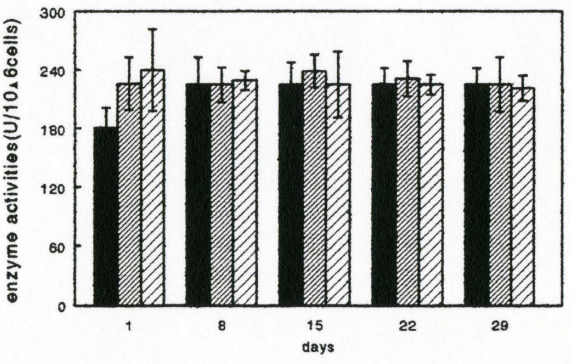


Fig 6D:B-hexo.secretion

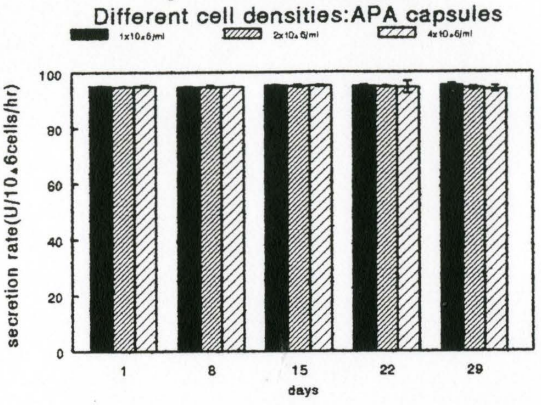


Fig 6E:Intracap. B-hexosaminidase

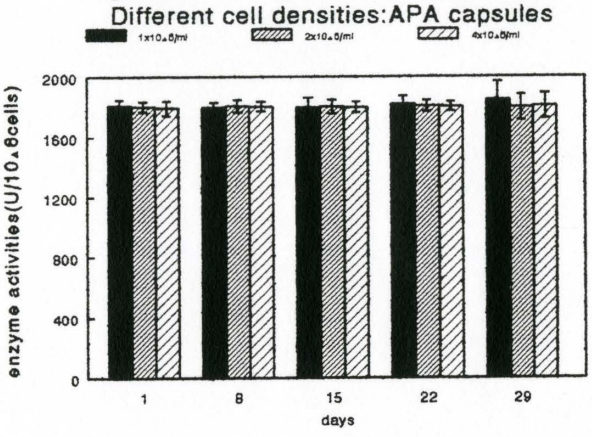


Fig 6F:Intracell. B-hexosaminidase

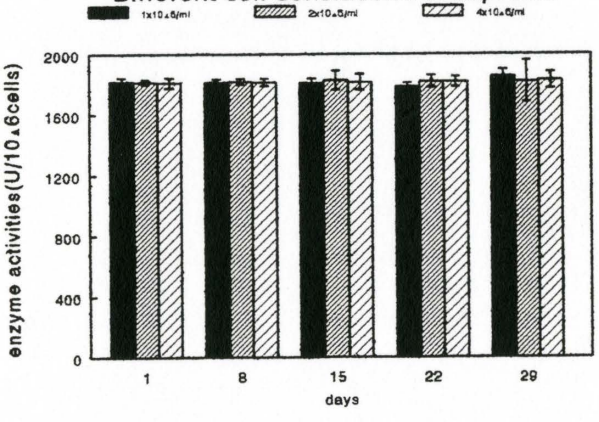


Fig 6G:no.of viable cells/capsule

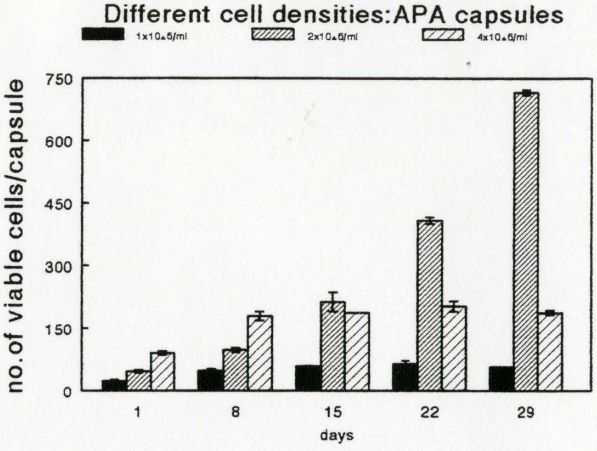
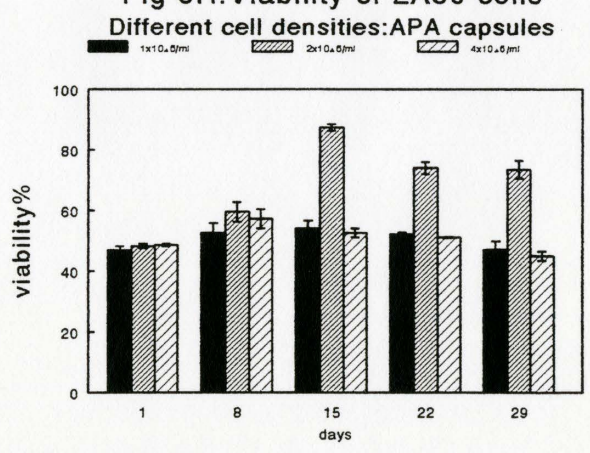
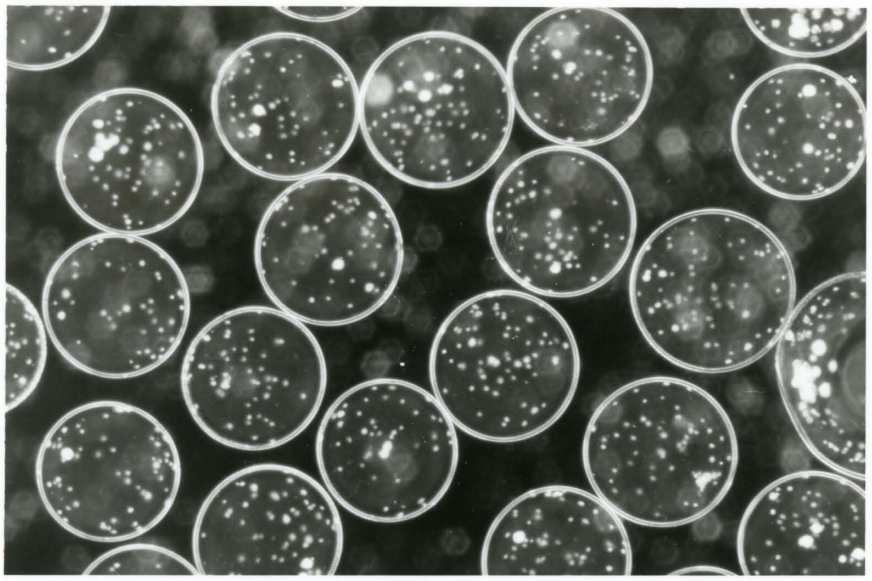


Fig 6H:Viability of 2A50 cells



400µm

A



B

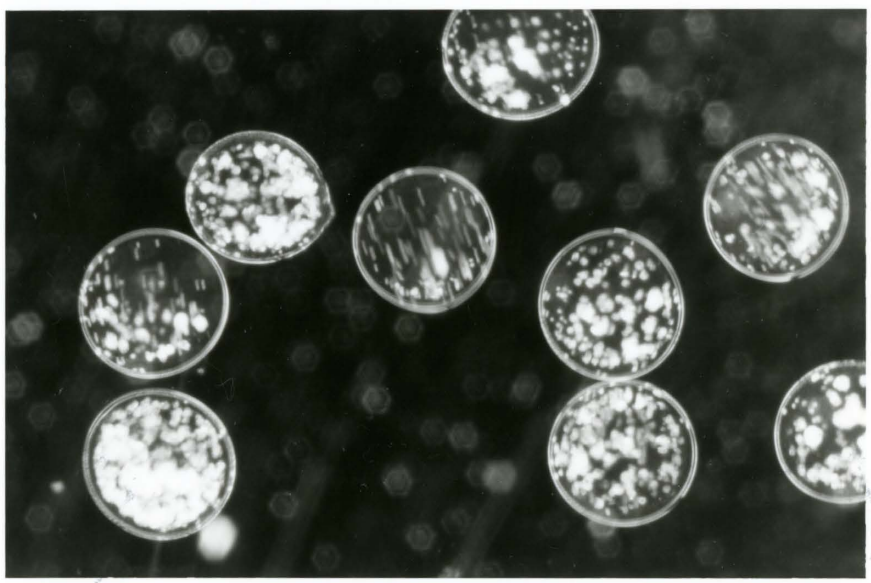
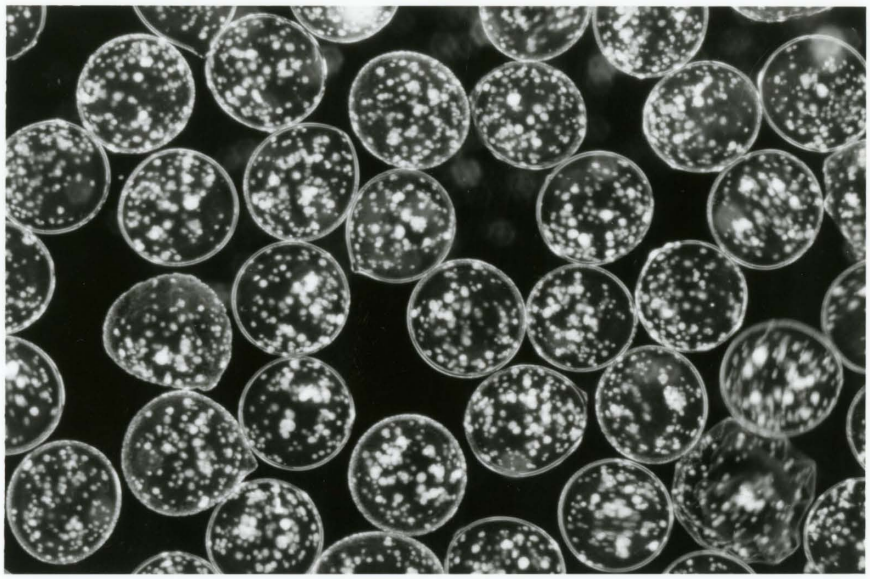


Fig 6I: Photomicrographs of 2A50 cells encapsulated at different cell densities in APA capsules on day 16 of the experiment. Capsules were single coated, treated with 10 min. polylysine and 20 min. sodium citrate. Photograph A illustrated cells encapsulated at $1x10^6$ cells/ml, photograph B illustrated cells encapsulated at $2x10^6$ cells/ml while photograph C demonstrated cells encapsulated at $4x10^6$ cells/ml. More cells were found embedded in the capsule membrane when encapsulated at $4x10^6$ cells/ml than that encapsulated at $2x10^6$ cells/ml and $1x10^6$ cells/ml.

400µm

C



3.4.5 Distribution of ß-Gluc. and ß-Hexo. in Single and Double Coated Microcapsules of Same Cell Densities

The effects of double coated capsules on cell proliferation and their ability to retain recombinant gene product within the intra-capsular space were examined. 2A50 cells were encapsulated at 4°C, treated with 10 min. PLL, 20 min. citrate and at a density of $2x10^6$ cells/ ml.

In the first two weeks of the experiment, ß-gluc. secretion (Fig 7A) from single coated capsules was four fold higher than the double coated capsules. Intracapsular accumulation (Fig 7B) of ß-gluc. in double coated capsules increased three fold from 406 U/10^6 cells on day1 to 1306 U/ 10^6 cells on day 15 (Table 9A). Secretion of ß-hexo. from single coated capsules was 1.1-fold higher than the double coated capsules (Fig 7D). Intracapsular accumulation of ß-hexo. in the double coated capsules was 1.2-fold higher than the single coated capsules (Fig 7E). There were no significant differences in viability and cell growth in the first two weeks (Post-hoc pairwise comparison: viability, $q<1.01$, <95% significance; no. of viable cells/ capsule, $q<0.73$, <95% significance) (Table 9C).

Viability of cells (Fig 7H) encapsulated in both single and double coated capsules recovered from less than 50% on day 1 to over 85% on day 15. After two weeks, viability of double coated capsules decreased to 55% on day 29 whereas single coated capsules remained over 70% on day 29 (Table 9B).

Similarly, the ability of the double coated capsules to retain ß-gluc. and ß-hexo. in the intracapsular space was largely reduced to values comparable to single coated capsules and no significant difference in secretion rate was found between the single and the double coated capsules from day 22 to day 29 (Table 9D).

Table 9A: Enzyme distribution from 2A50 cells encapsulated in single and double coated APA capsules. The cell density used for encapsulation was $2x10^6$ cells/ml alginate. All capsules were washed at 4°C, treated with 10 min. polylysine and 20 min. sodium citrate. Intracapsular enzymes were released from crushed capsules. Intracellular enzymes were obtained by sonication of released cell pellets. Triplicate samplings were done and the standard deviation was calculated.

Table 9A: Enzymes distribution of 2A50 cells encapsulated in single and double coated APA capsules

	ß-glucuronidase			ß-hexosaminidase		
	secretion (U/hr/ 10^6 cells)	intracap. (U/10^6 cells)	intracell. (U/10^6 cells)	secretion (U/hr/ 10^6 cells)	intracap. (U/10^6 cells)	intracell. (U/10^6 cells)
(day1)						
single	5.27±0.02	285±14	226±27	94.9±0.1	1803±32	1813±17
double	1.40±0.02	406±22	224±19	76.3±0.3	2250±42	1813±19
(day8)						
single	5.28±0.05	287±13	225±18	94.9±0.3	1807±42	1817±21
double	1.43±0.02	852±32	226±22	76.2±0.1	2252±28	1816±22
(day15)						
single	5.25±0.08	284±13	239±17	95.1±0.4	1803±48	1826±64
double	1.47±0.03	1306±86	239±25	76.0±0.0	3500±117	1825±78
(day22)						
single	5.26±0.04	286±15	231±18	94.8±0.3	1806±39	1819±40
double	5.03±0.05	300±18	226±13	92.8±0.3	1868±68	1816±27
(day29)						
single	5.29±0.03	287±12	225±28	94.3±0.5	1798±86	1820±138
double	5.22±0.30	295±18	225±26	94.5±0.0	1800±55	1825±73

Table 9B: Proliferation of 2A50 cells encapsulated in single and double coated APA capsules. The initial "seeding" density was $2x10^6$ cells/ml alginate. All capsules were washed at 4°C, treated with 10 min. polylysine and 20 min. sodium citrate. Triplicate samplings were done and the standard deviation was calculated.

Table 9B: Proliferation of 2A50 cells encapsulated in single and double coated APA capsules

	Viability(%)	no. of viable cells/capsule
(day1)		
single	48.3±0.7	46±3
double	49.2±0.2	46±3
(day8)		
single	59.5±3.3	98±5
double	59.9±3.2	102±11
(day15)		
single	87.4±1.0	213±23
double	85.4±1.5	187±18
(day22)		
single	74.1±2.0	408±7
double	56.5±2.1	156±10
(day29)		
single	73.5±3.0	716±6
double	55.5±1.4	167±24

Table 9C: The analysis of the significance of 2A50 cells encapsulated in single and double coated APA capsules. Post-hoc pairwise comparison was applied in association with equations derived by Dr. Norman for such analysis, where q values at 95% confidence interval=2.506. Data shown in this table were q values. If q> 2.506, differences between two groups were significant, designated as (s). If q< 2.506, differences between two groups were insignificant, designated as (ins).

Table 9C: Analysis of the significance of 2A50 cells encapsulated in single and double coated APA capsules

single vs double	day1	day8	day15	day22	day29
ß-gluc. secretion	112(s)	58.4(s)	36.1(s)	2.93(s)	0.19(ins)
intracap. ß-gluc.	3.79(s)	13.4(s)	9.59(s)	0.49(ins)	0.30(ins)
intracell. ß-gluc.	0.05(ins)	0.03(ins)	0(ins)	0.18(ins)	0(ins)
ß-hexo. secretion	48.0(s)	48.3(s)	39.0(s)	3.85(s)	0.33(ins)
intracap. ß-hexo.	6.91(s)	7.20(s)	11.0(s)	0.65(ins)	0.02(ins)
intracell. ß-hexo.	0(ins)	0.03(ins)	0.01(ins)	0.05(ins)	0.03(ins)
viability	1.01(ins)	0.07(ins)	0.91(ins)	4.96(s)	4.44(s)
viable cells/ capsule	0(ins)	0.27(ins)	0.73(ins)	16.9(s)	18.1(s)

Fig 7: Enzyme distribution and cell proliferation of encapsulated 2A50 cells in single and double coated APA capsules. 2A50 cells were washed at 4°C, encapsulated at a density of $2x10^6$ cells/ml, treated with 10 min. polylysine and 20 min. sodium citrate. Single and double coated capsules were compared under the parameters below:

A: Secretion of ß-glucuronidase
B: Intracapsular concentration of ß-glucuronidase
C: Intracellular concentration of ß-glucuronidase
D: Secretion of ß-hexosaminidase
E: Intracapsular concentration of ß-hexosaminidase
F: Intracellular concentration of ß-hexosaminidase
G: Number of viable 2A50 cells/capsule
H: Viability of encapsulated 2A50 cells

Triplicate samplings were done and the standard deviation was shown as error bars

Fig 7A:B-gluc.secretion
single vs double coats:APA capsules

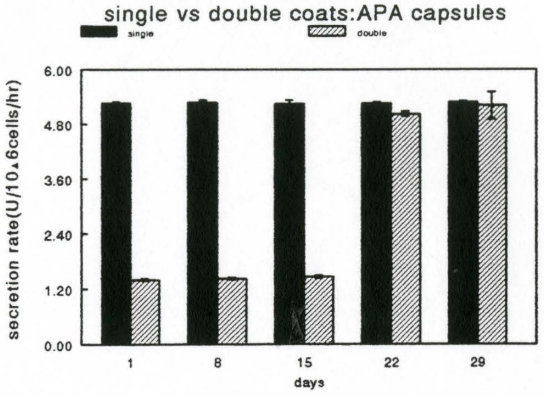


Fig 7B:Intracap. B-glucuronidase
single vs double coats:APA capsules

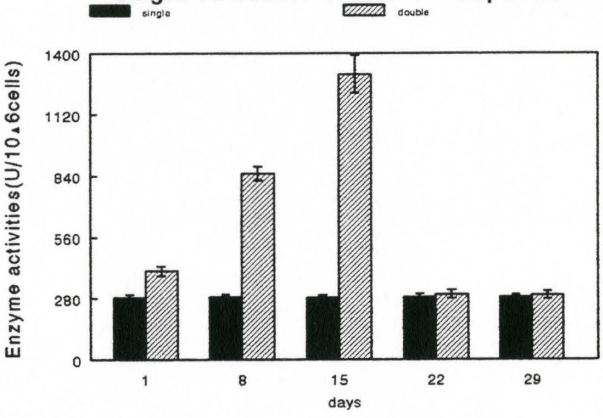


Fig 7C:Intracell. B-glucuronidase
single vs double coats:APA capsules

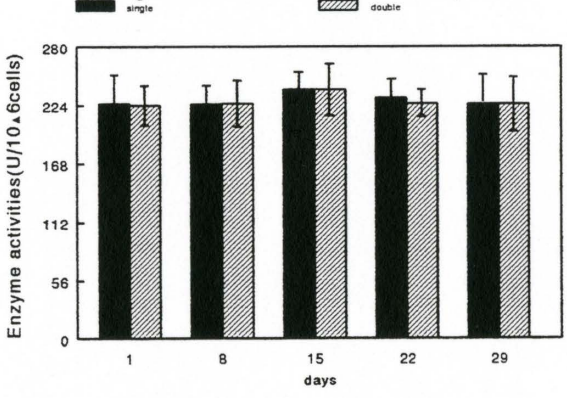


Fig 7D:B-hexo.secretion
single vs double coats:APA capsules

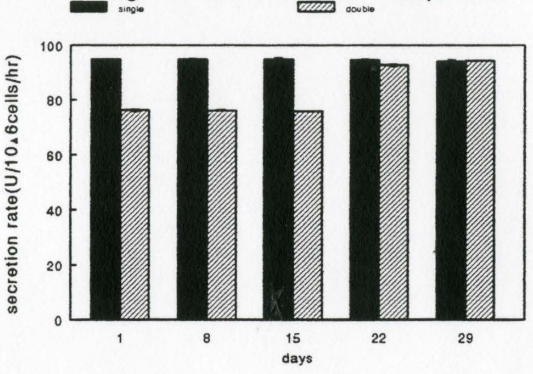


Fig 7E:Intracap. B-hexosaminidase
single vs double coats:APA capsules

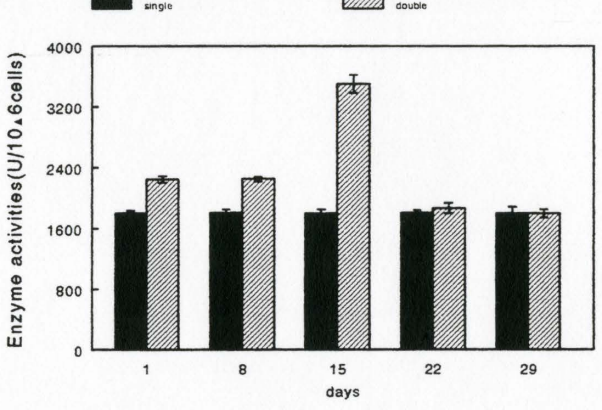


Fig 7F:Intracell. B-hexosaminidase
single vs double coats:APA capsules

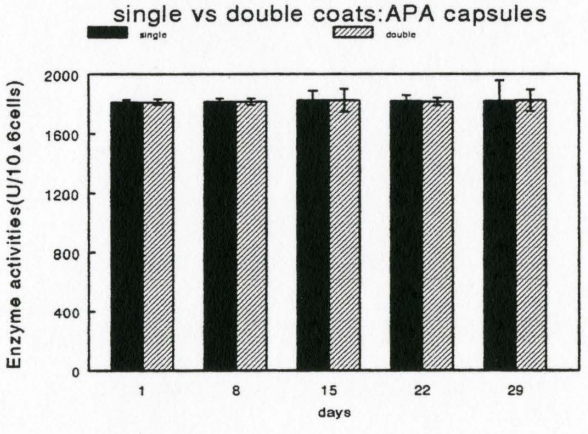


Fig 7G:no.of viable cells/capsule

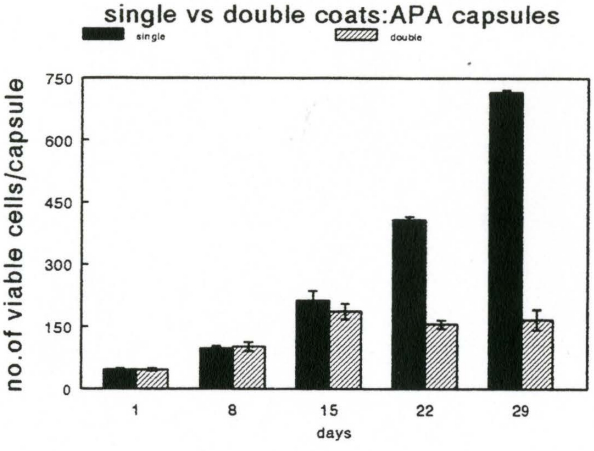
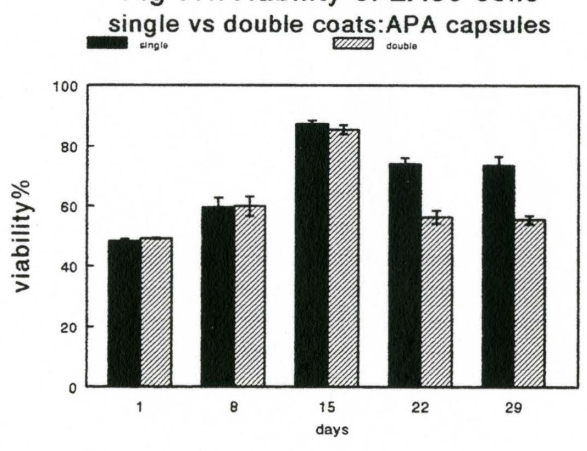


Fig 7H:Viability of 2A50 cells



3.5 Encapsulation of 2A50 Cells and LhGH-1 Cells in Thermoplastic Capsules

The properties of thermoplastic capsules, such as their permeability to various recombinant gene products and their biocompatibility to different cell lines were examined. 2A50 cells and LhGH-1 cells were encapsulated at 4°C into HEMA-MMA capsules, in the presence (refer to as matrigel capsules) or absence (refer to as regular capsules) of matrigel, a substance which formed a continous layer in the interior of the capsular membrane and is thought to support cell proliferation (Uludag et al, 1993).

3.5.1 Distribution of ß-Gluc. and ß-Hexo. in Thermoplastic Capsules

2A50 cells were encapsulated at a density of $5x10^6$ cells/ml polymer solution (HEMA-MMA), in the presence or absence of matrigel. Characteristics such as ß-gluc. distribution, ß-hexo. distribution and cell proliferation were very similar between regular and matrigel capsules.

ß-gluc. and ß-hexo. were not detectable in the surrounding media when enzyme assays were performed with regular or matrigel capsules, indicating both enzymes were not secreted out of the capsules (Table 10). Instead, enzymes accumulation in the intracapsular space increased rapidly. ß-gluc. retention (Fig 8A) on day 1 was about 436 U/10^6 cells, which increased 8-fold by day 8 to more than 3400 U/10^6 cells. By day 22, ß-gluc. retention

was about 8900 U/10^6 cells, 21-fold higher than samples taken on day 1. ß-hexo. retention (Fig 8C) on day 8 was 2.4-fold higher than those on day 1 while samples taken on day 22 exhibited a retention of more than 10000 U/10^6 cells, 5-fold higher than samples examined on day 1 (Table 10).

Viability of cells in both regular and matrigel capsules recovered rapidly from 52% on day 1 to 72% on day 8 (Fig 8F). On day 15, both types of capsules had viability of more than 80% although it decreased to 72% on day 22. Cells proliferated readily in both regular and matrigel capsules. On day 15, the number of viable cells per capsule exceeded 1300 and decreased to 1150 on day 22, due to a drop in viability (Fig 8E). Total number of cells per capsule on both days remained the same, about 1600, indicating cell proliferation had reached its plateau (Table 10).

Unlike the APA capsules, HEMA-MMA capsules tend to aggregate together and can be pulled apart from one another with a forcep. The presence of matrigel did not seem to enhance cell proliferation since there was no difference in viability and cell number between regular and matrigel capsules. The molecular weight cut-off seemed to be less than 120,000 because ß-hexo. could not diffuse through the capsule membrane. Scanning electron microscopy showed that matrigel capsules were acentric. Matrigel was washed out of the capsule during the preparation of the scanning electron photomicrograph and therefore was not found in the core of the

capsules (Fig 8G). Cross-linking patterns was observed between the interior and the exterior surface of the capsules. However, the interior surface of the capsules was the determinant of the molecular weight cut-off of the capsules. Light microscopy indicated HEMA-MMA capsules was not transparent (Fig 8H) and therefore it was necessary to dissect the capsules with razor blade to examine cell proliferation inside the capsules. On day 22, cells within regular capsules appeared as dark "dots" scattered all over the interior core of the capsules. In contrast, cells within matrigel capsules was localized near the interior membrane and appeared as spindle- shaped fibroblasts (Fig 8I).

Table 10: Enzyme distribution and cell proliferation of 2A50 cells encapsulated in HEMA-MMA capsules. The initial "seeding" density was $5x10^6$ cells/ml. Intracapsular enzymes were released from dissected capsules. Intracellular enzymes were obtained by sonication of released cell pellets. Triplicate samplings were done and the standard deviation was calculated.

Table 10: Enzymes distrbution and cell proliferation of 2A50 cells encapsulated in HEMA-MMA capsules

Enzymes distribution

	ß-glucuronidase			ß-hexosaminidase		
days	secretion (U/hr/ 10^6 cells)	intracap. (U/10^6 cells)	intracell. (U/10^6 cells)	secretion (U/hr/ 10^6 cells)	intracap. (U/10^6 cells)	intracell. (U/10^6 cells)
regular						
1	0±0	43±24	226±2	0±0	2153±61	1819±24
8	0±0	3439±23	225±5	0±0	5185±23	1799±46
15	0±0	6440±131	224±3	0±0	8712±133	1810±13
22	0±0	8889±192	225±4	0±0	10444±195	1822±39
matrigel						
1	0±0	436±6	226±4	0±0	2159±114	1810±13
8	0±0	4317±36	229±8	0±0	5167±36	1817±7
15	0±0	6301±58	225±4	0±0	8838±579	1793±44
22	0±0	8910±73	224±6	0±0	10096±481	1811±5

Cell Proliferation

Days	viability(%)	no. of viable cells/capsule
regular		
1	52.0±2.2	257±3
8	71.3±8.4	646±15
15	83.3±0.5	1342±16
22	72.1±0.7	1149±40
matrigel		
1	52.5±2.3	253±9
8	72.3±2.0	663±13
15	82.0±1.2	1316±33
22	71.8±1.4	1153±20

Fig 8: Enzyme distribution and cell proliferation of 2A50 cells encapsulated in HEMA-MMA capsules. 2A50 cells were encapsulated at a density of $5x10^6$ cells/ml. Regular and matrigel capsules were compared under the following parameters:

A: Intracapsular ß-glucuronidase
B: Intracellular ß-glucuronidase
C: Intracapsular ß-hexosaminidase
D: Intracellular ß-hexosaminidase
E: Number of viable 2A50 cells/capsule
F: Viability of encapsulated 2A50 cells

Triplicate samplings were done and the standard deviation was shown as error bars

Fig 8A:Intracap. B-glucuronidase
regular vs matrigel:HEMA-MMA capsules

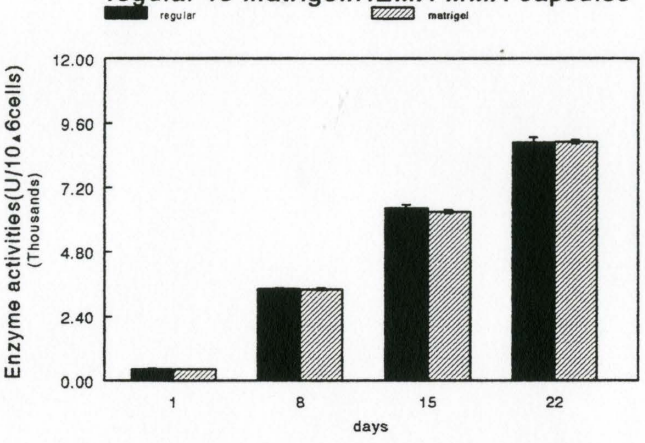


Fig 8B:Intracell. B-glucuronidase
regular vs matrigel:HEMA-MMA capsules

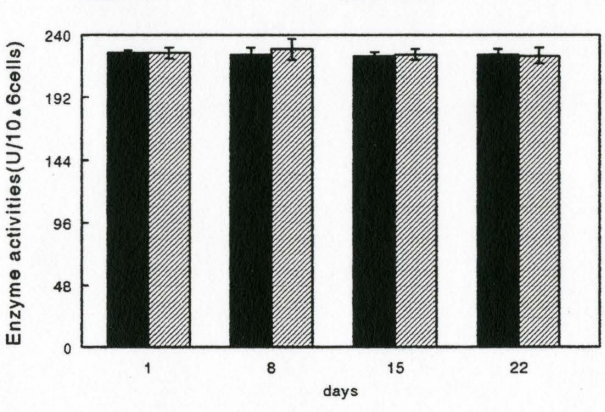


Fig 8C:Intracap. B-hexosaminidase
regular vs matrigel:HEMA-MMA capsules

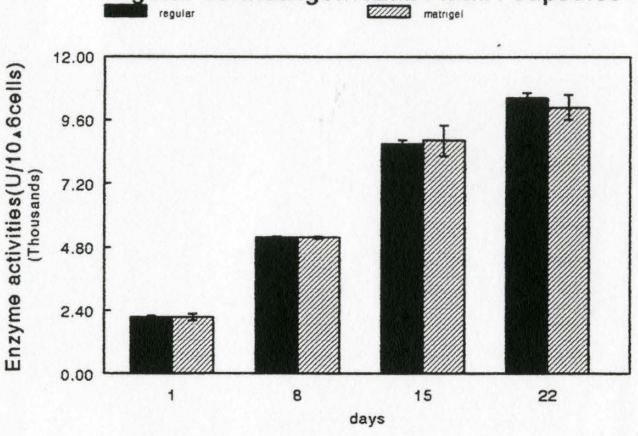


Fig 8D:Intracell. B-hexosaminidase
regular vs matrigel:HEMA-MMA capsules

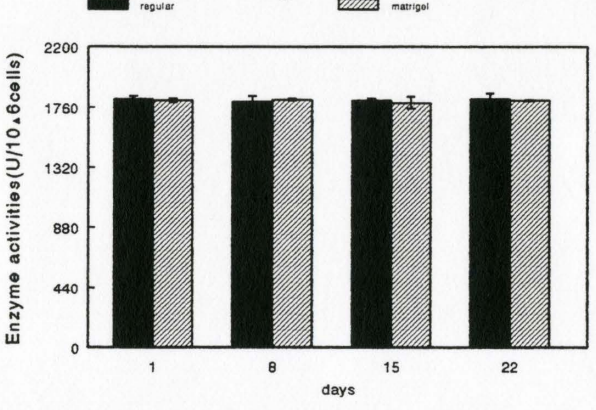


Fig 8E:no. of viable cells/cap.

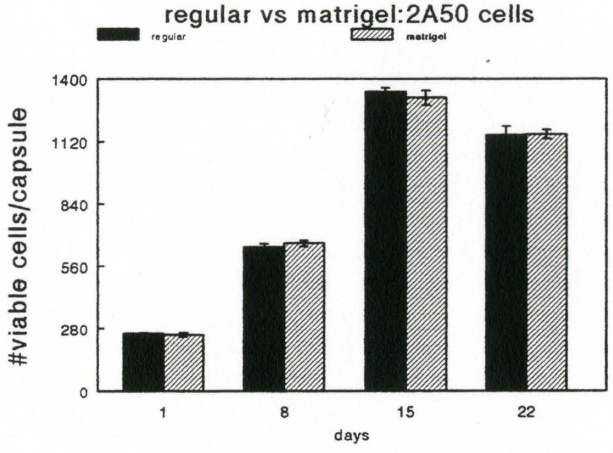


Fig 8F:Viability of 2A50 cells

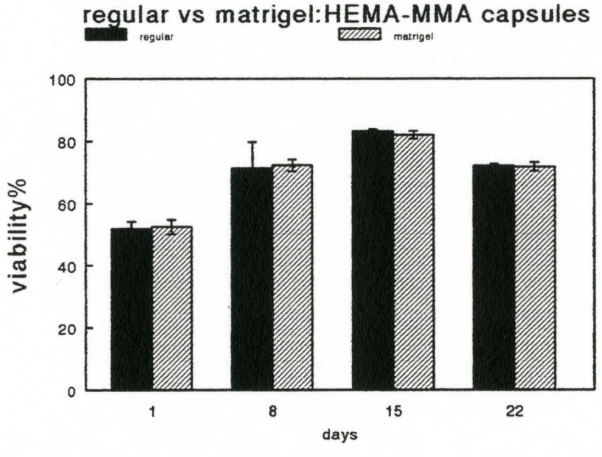


Fig 8G: Scanning electron photomicrograph of encapsulated 2A50 cells in HEMA-MMA matrigel capsules. The cell density for encapsulation was $5x10^6$ cells/ml polymer. On day 22, the dissected capsule appeared acentric. Matrigel was washed out of the capsule during the preparation of the scanning electron photomicrograph and therefore was not found in the core of the capsule. Cross-linking patterns was observed between the interior and the exterior surface of the capsules.

200µm

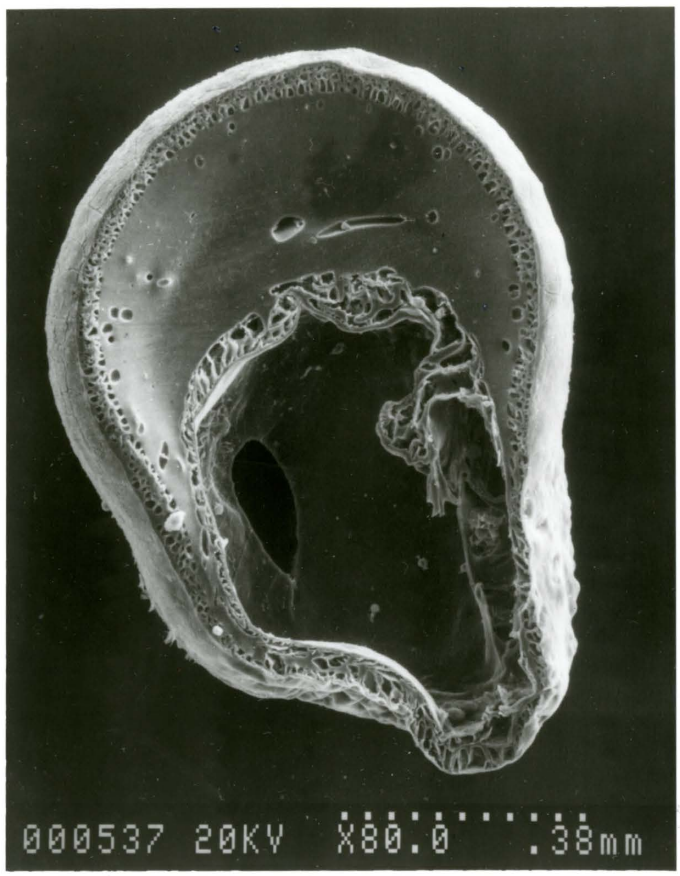


Fig 8H: Light photomicrograph of non-dissected blank HEMA-MMA capsules (courtesy of Dr. M. V. Sefton). Unlike the alginate capsules, the HEMA-MMA capsules were not transparent. Cell proliferation cannot be visualized unless capsules were dissected at different stages of the experiments. The diameter of these capsules was about 800μm.

800µm

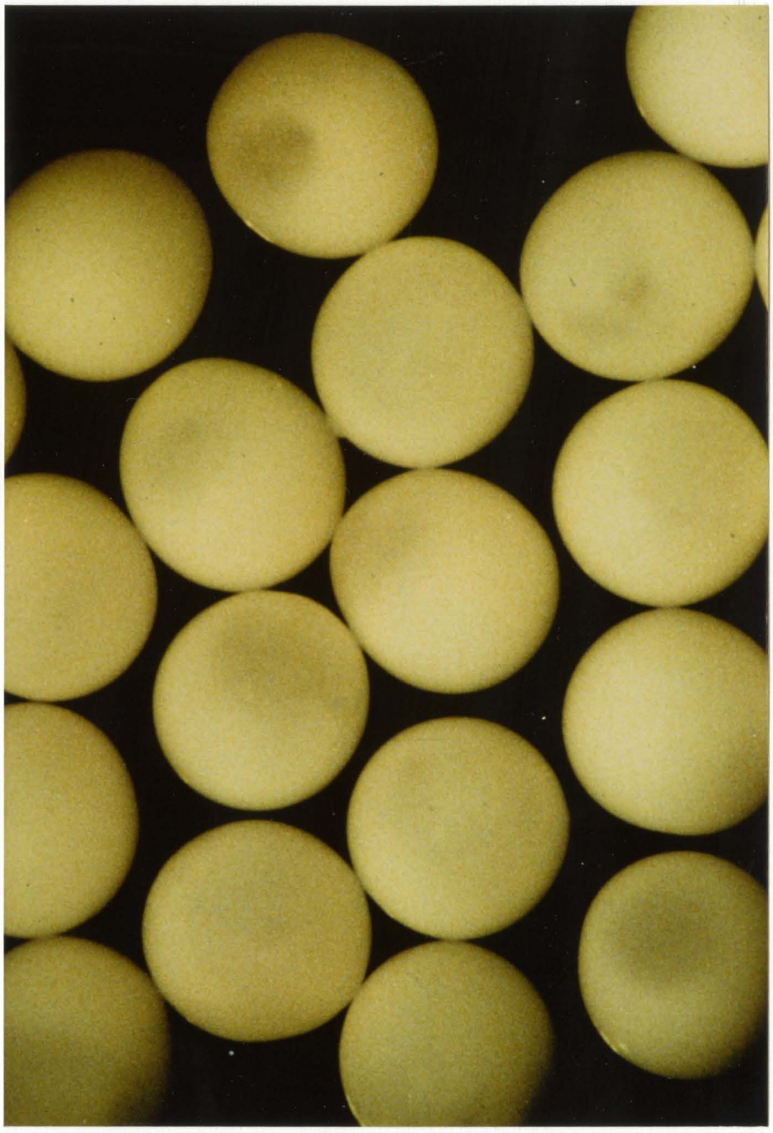
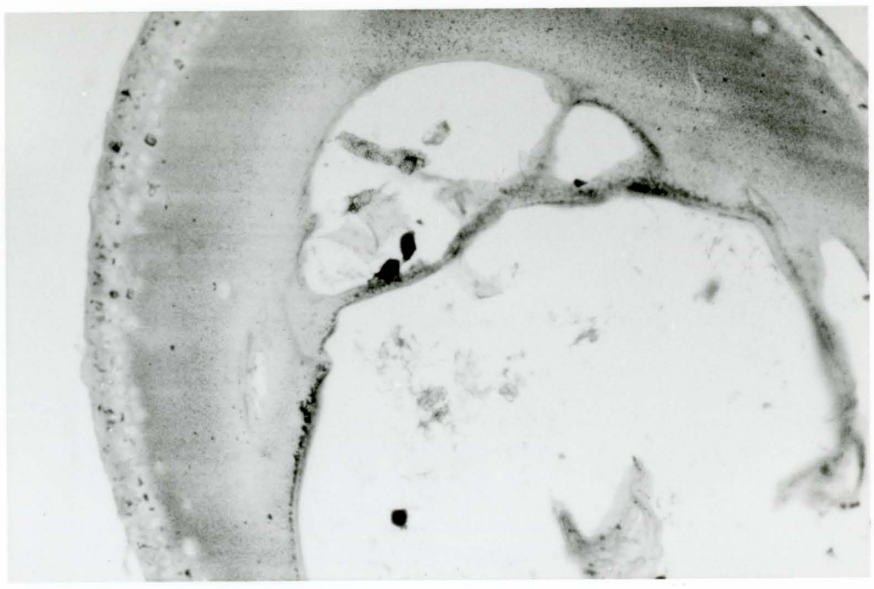


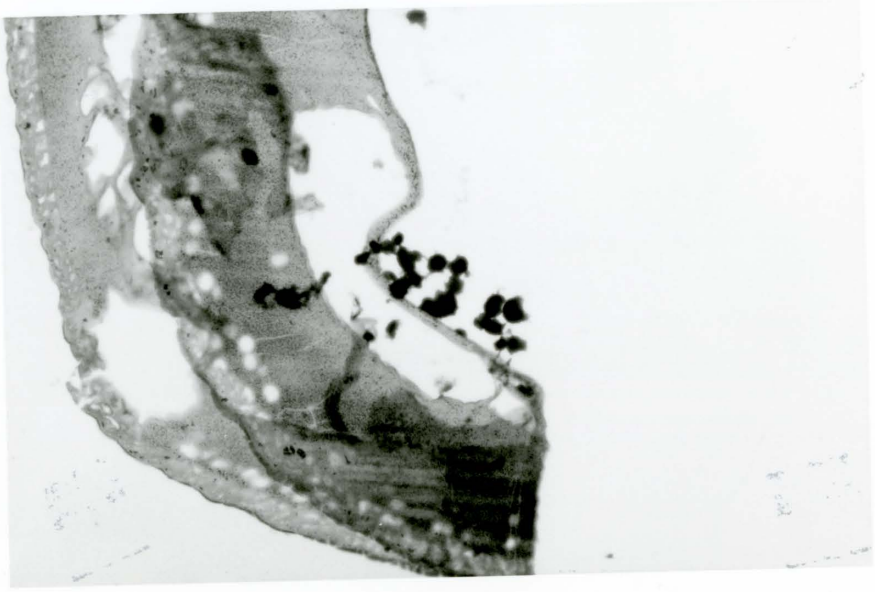
Fig 8I: Light photomicrographs of 2A50 cells encapsulated in HEMA-MMA capsules at a density of $5x10^6$ cells/ml. Photographs A and B illustrated 2A50 cells encapsulated in regular capsules on day 1 and day 22 respectively. Cells were scattered as dark "dots" in the interior core of the capsules. Photographs C and D illustrated 2A50 cells encapsulated in matrigel capsules on day 1 and day 22 respectively. Cells in matrigel capsules was localized near the interior membrane and appeared as spindle-shaped fibroblasts. More fibroblasts can be found on day 22 than on day 1.

200µm

A

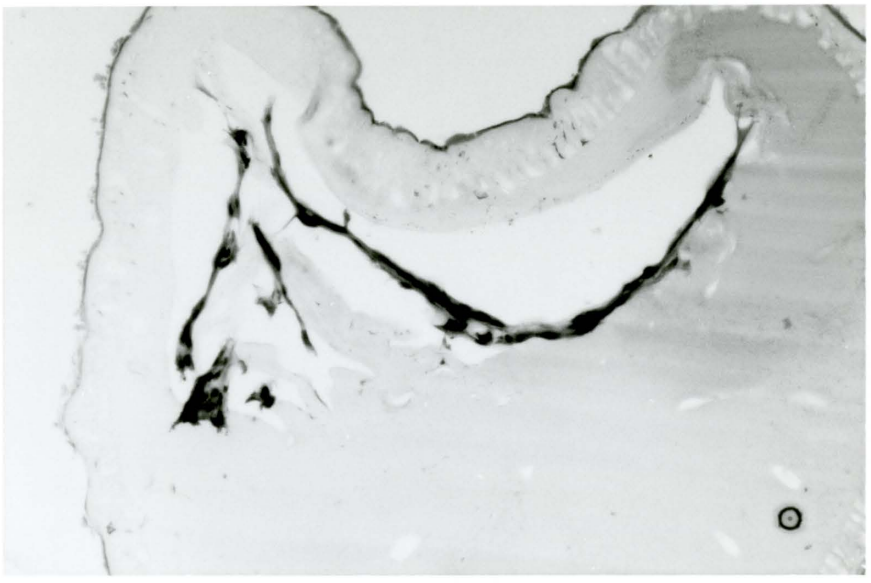


B



200µm

C



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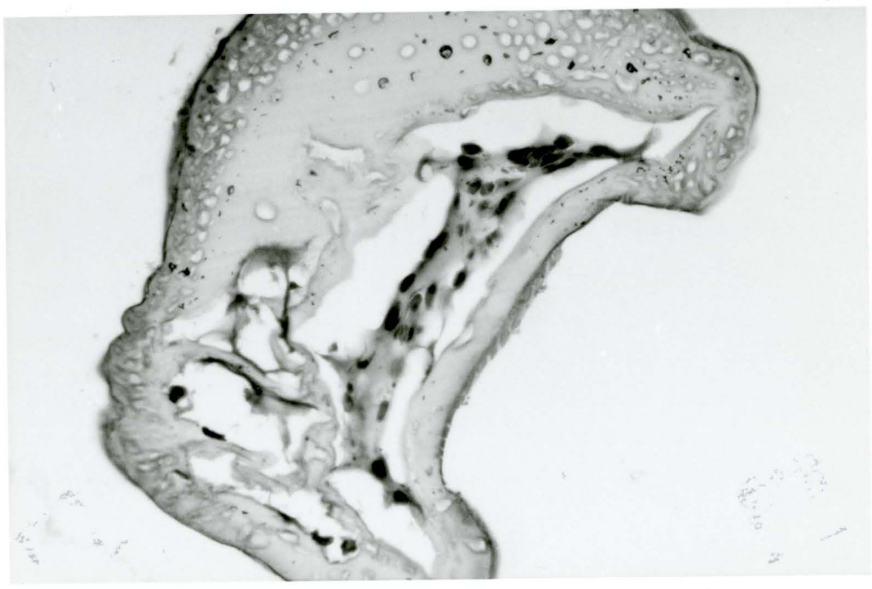


3.5.2 Distribution of HGH in Thermoplastic Capsules

LhGH-1 cells were encapsulated at a density of $2x10^6$ cells/ml polymer solution, in the presence or absence of matrigel. hGH was secreted readily from regular and matrigel capsules. hGH secretion rate (Fig 9A) from regular capsules was 11.2 ng/hr/10^6 cells, which was very similar to those secreted from matrigel capsules, 10.8 ng/hr/10^6 cells.

Intracapsular hGH retention (Fig 9B) was 1.35 ng/10^6 cells on day 1 from regular capsules, which increased 6-fold to 8.50 ng/10^6 cells on day 8 and had remained steady until day 22. Matrigel capsules were more capable of retaining hGH within their intracapsular space than regular capsules. On day 1, hGH retention in matrigel capsules was 5.87 ng/10^6 cells, 4.3-fold higher than those of regular capsules. On day 8, hGH retention increased 9 fold to 54.4 ng/10^6 cells, which was 6.4-times higher than that retained inside regular capsules taken on the same day. hGH accumulation in matrigel capsules retained the same order of magnitude at 54.4 ng/10^6 cells until day 22, indicating the establishment of an equilibrium at the trafficking of hGH between the interior of the capsules and their surrounding media (Table 11).

Viability of cells (Fig 9E) in both regular and matrigel capsules was 72% on day 1, indicating the LhGH-1 cell line survived the process of encapsulation better than 2A50 cells. The viability for both types of capsules

increased to >80% on day 8 and has remained steady until day 22. Cells proliferated readily in both regular and matrigel capsules. The number of viable cells per capsule (Fig 9D) on day 22 has not reached a maximum (>700 cells/capsule), probably due to the lower cell density at encapsulation (Table 11). The LhGH-1 cells appeared as spindle-shaped fibroblasts located near the interior surface of the capsule membrane. Some regular capsules contained nearly no cells on day 1, which was probably due to the inefficiency of encapsulation with low seeding densities (Fig 9F).

Table 11: hGH distribution and cell proliferation of LhGH-1 cells encapsulated in HEMA-MMA capsules. The initial "seeding" density was $2x10^6$ cells/ml. Intracapsular hGH was released from dissected capsules. Intracellular hGH was obtained by sonication of released cell pellets. Triplicate samplings were done and the standard deviation was calculated.

Table 11: The distribution of hGH and proliferation of LhGH-1 cells encapsulated in HEMA-MMA capsules

days	hGH secretion (ng/hr/ 10^6 cells)	intracap. hGH (ng/10^6 cells)	intracell. hGH (ng/10^6 cells)	viability(%)	no. of viable cells/capsule
regular					
1	8.59±3.87	1.59±1.24	3.65±0.76	72.2±4.8	130±9
8	11.0±4.5	8.58±0.14	4.49±0.46	83.0±0.5	161±12
15	12.4±1.6	9.48±1.88	3.41±0.07	80.1±2.9	256±9
22	9.42±6.94	6.49±0.71	4.50±0.52	78.2±1.6	641±15
matrigel					
1	8.97±2.34	6.38±2.28	4.82±0.70	69.8±3.0	123±8
8	9.26±1.50	56.7±12.6	4.27±0.61	84.2±1.7	171±13
15	11.0±2.6	54.7±5.8	4.75±0.62	76.8±3.7	336±7
22	14.3±1.6	48.9±7.7	5.14±0.39	79.5±5.5	568±14

Fig 9: HGH distribution and cell proliferation of LhGH-1 cells encapsulated in HEMA-MMA capsules. LhGH-1 cells were encapsulated at a density of $2x10^6$ cells/ml. Regular and matrigel capsules were compared under the following parameters:

A: Secretion rate of hGH
B: Intracapsular concentration of hGH
C: Intracellular concentration of hGH
D: Number of viable LhGH-1 cells/capsule
E: Viability of encapsulated LhGH-1 cells

Triplicate samplings were done and the standard deviation was shown as error bars

Fig 9A:hGH secretion

regular vs matrigel:HEMA-MMA capsules

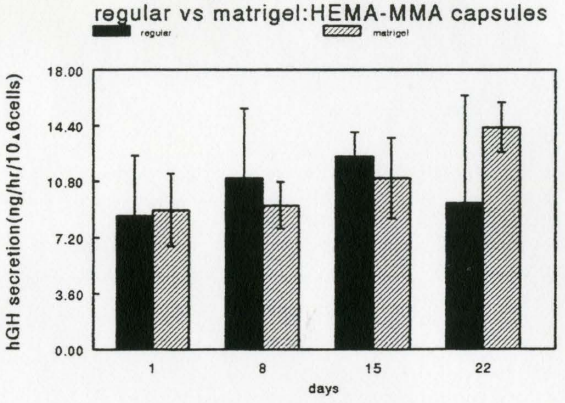


Fig 9B:Intracap. hGH

regular vs matrigel:HEMA-MMA capsules

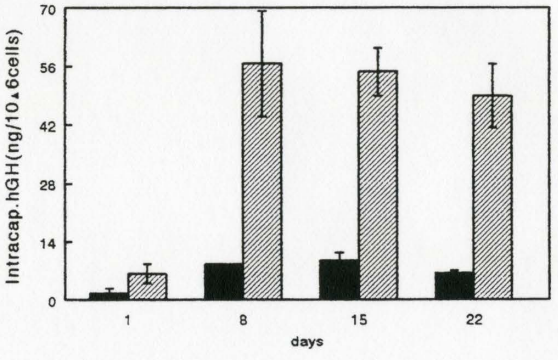


Fig 9C:Intracell. hGH

regular vs matrigel:HEMA-MMA capsules

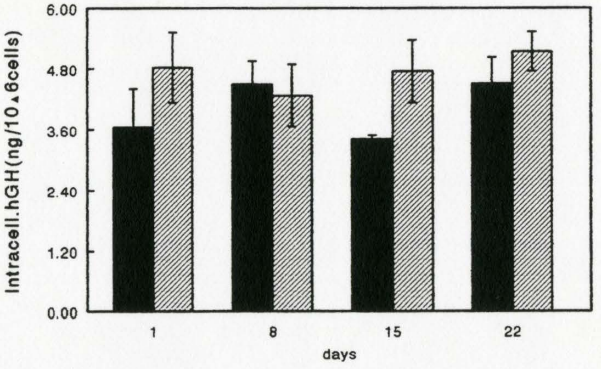


Fig 9D:no.of viable cells/capsule

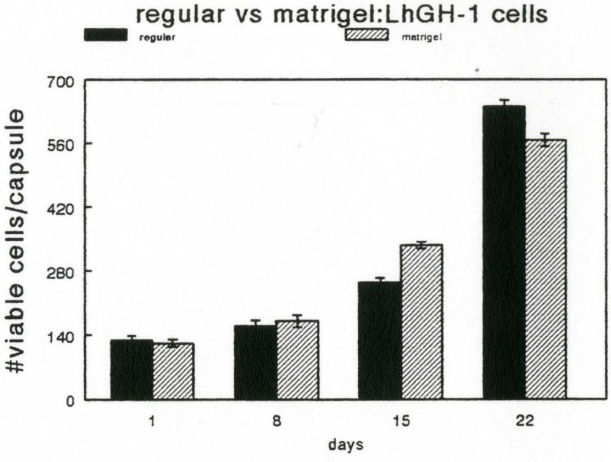
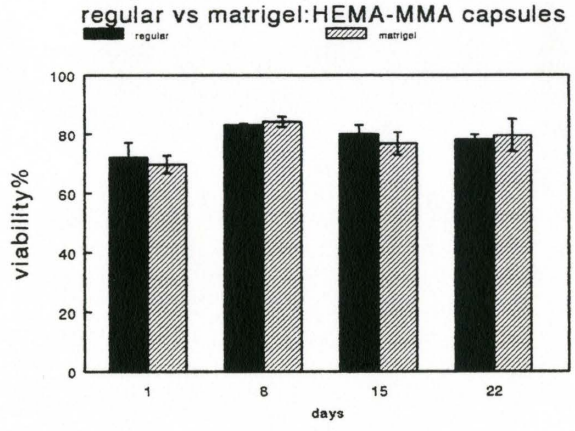
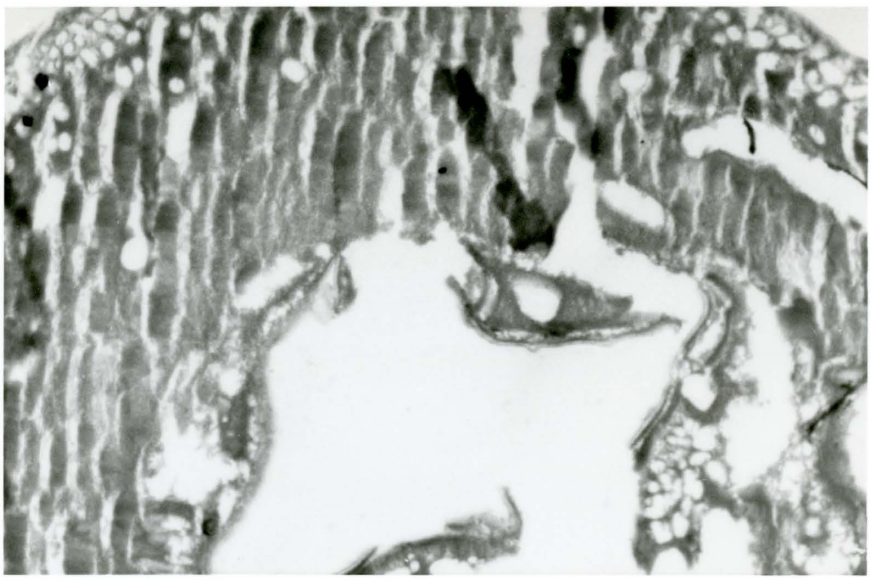


Fig 9E:Viability of LhGH-1 cells



200µm

A



B

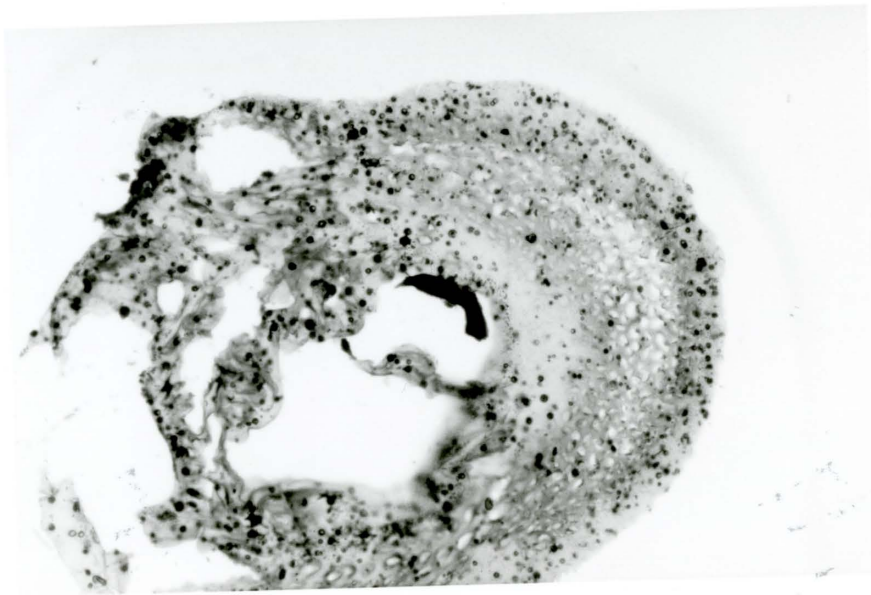
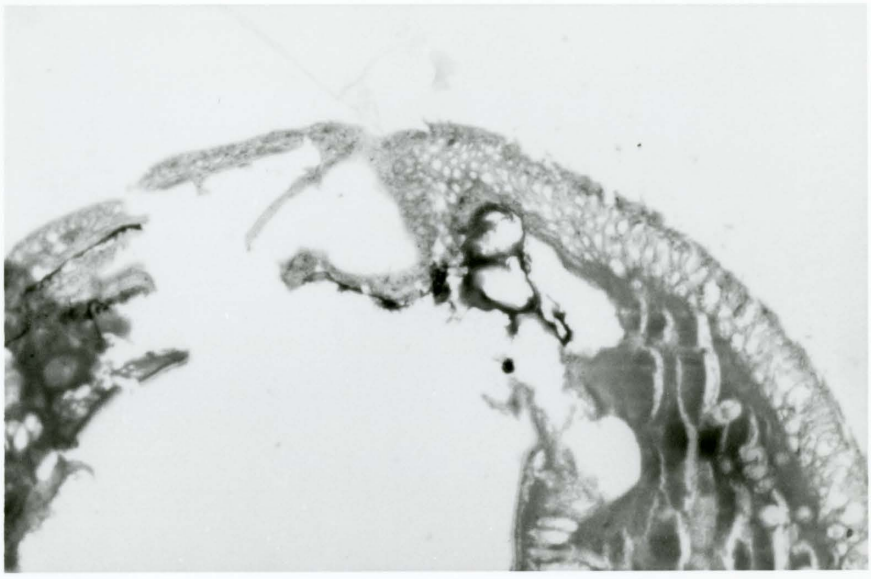


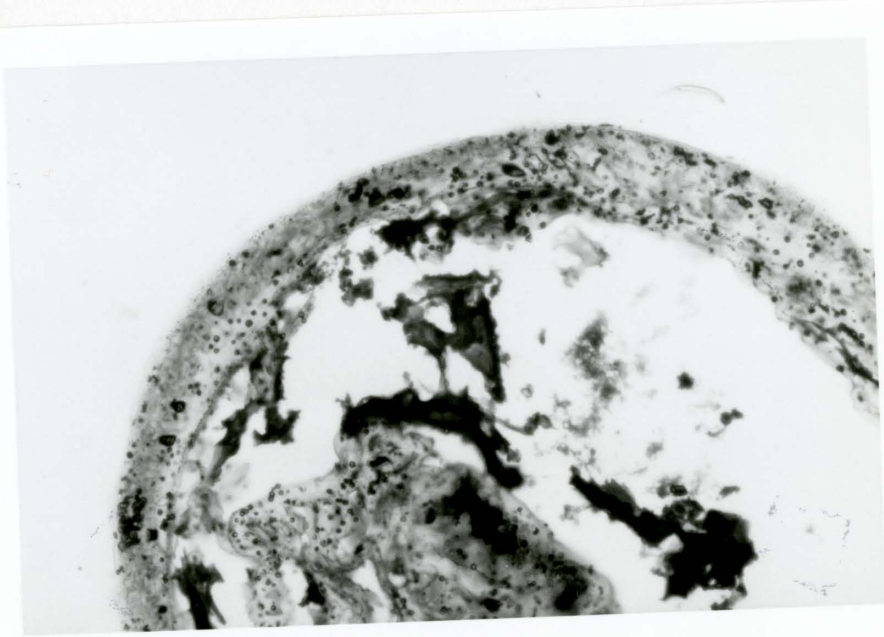
Fig 9F: Light photomicrographs of LhGH-1 cells encapsulated in HEMA-MMA capsules at a density of $2x10^6$ cells/ml. Photographs A and B illustrated LhGH-1 cells encapsulated in regular capsules on day 1 and day 22 respectively. Some regular capsules contained very few cells on day 1 due to the low efficiency of encapsulation. Photographs C and D illustrated LhGH-1 cells encapsulated in matrigel capsules on day 1 and day 22 respectively. In the presence of matrigel, cells appeared as spindle-shaped fibroblasts. More fibroblasts can be found on day 22 than on day 1.

200µm

C



D



4.0 DISCUSSION

4.1 Cell Proliferation in APA Microcapsules

4.1.1 Proliferation of Encapsulated LhGH-1 Cells

LhGH-1 cells proliferate readily in the capsules in early stages of the experiment (<8 days). The growth was attachment-dependent, consistent with the adherent nature of the cell line. In later stages of growth (>15 days), clusters of cells were observed. Total number of cells per capsule did not exceed two thousand even though the capsules did not appear to be filled up with cells. This phenomenon reflected the insufficiency in the sodium citrate wash (6 minute in duration) to liquefy the remnants of alginate in the core of microcapsules to create space for cell proliferation (Goosen et al, 1985).

4.1.2 Proliferation of Encapsulated 2A50 Cells

Although 2A50 cells survived the process of encapsulation, viability of encapsulated 2A50 cells was only 50% one day after encapsulation, indicating 2A50 cells were more fragile compared to LhGH-1 cells. Cold temperature (4-10°C) were preferable to room temperature during microencapsulation. Cells were able to survive with about 10% higher viability when processed at lower temperatures. The lowering of metabolic activity at reduced temperatures during the stress of encapsulation probably

played a protective role to ensure greater survival. 2A50 cells were attachment-dependent. When treated with 6 minute sodium citrate, encapsulated 2A50 cells exhibited the same growth pattern as encapsulated LhGH-1 cells, forming cell clusters after 15 days. Goosen et al suggested six to nine minute citrate treatment as the most ideal parameter for encapsulation (Goosen et al, 1985). However, encapsulated cells treated with 20 min. citrate contained more than 1600 viable cells/capsule on day 22 and retained a viability of more than 95% on day 15 as compared to capsules treated with 6 min. citrate (Table7), where the maximum cell number per capsule never exceed 1000. As such, prolonged treatment of sodium citrate during encapsulation seemed to be effective in dissolving the core alginate, leaving more intracapsular space for cell proliferation.

4.2 Secretion of Recombinant Gene Products from APA Microcapsules

4.2.1 Secretion of hGH from APA Capsules

HGH was secreted from APA microcapsules containing LhGH-1 cells at rates similar to the secretion rate per 10^6 cells from non-encapsulated cells, except day 1 after encapsulation. The low rate of secretion of hGH for encapsulated cells on day 1 was due to the inability of the cells to recover from the process of encapsulation within one day (Awrey, 1993). The sudden decrease of hGH secretion on day 29 (Fig 3A) when LhGH-1 cells was encapsulated at $1x10^6$/ml was due to a reduction in hGH gene expression as reflected by a decrease in intracellular hGH on day 29 (Fig 3C). It was not known why hGH expression was reduced when the viability of the encapsulated cells was still more than 50%.

HGH did not accumulate in the intracapsular space of single and double coated capsules, indicating that APA membrane was freely permeable to hGH (45,000). This finding is consistent with the published molecular weight cut-off for APA capsules, which was 67,000. As such, molecules less than 67,000 can be secreted out of the capsules without any obstacle (King et al, 1987; Goosen et al, 1985).

4.2.2 Secretion of ß-Gluc. and ß-Hexo. from APA Microcapsules

ß-gluc. (M_r 300,000) and ß-hexo. (M_r 120,000) were secreted outside the capsules, at lower rates compared to non-encapsulated cells. The published

molecular rate cut-off was 67,000, which was not consistent with our findings (Goosen et al, 1985). Awrey et al reported the detection of IgG (M_r 145,000) in the surrounding media from encapsulated HIgG cells, which was also beyond the published cut-off value (Awrey, 1993). When 6 min. polylysine and 6 min. sodium citrate was applied for encapsulation, the double coated capsules seemed to retain ß-hexo. but secreted ß-gluc. into the media (Fig 1A, Fig 1D). Since ß-gluc. has a higher molecular weight than ß-hexo., it is possible that ß-gluc. was secreted as a monomer of M_r 75,000, or a dimer of M_r 150,000, instead of its original tetrameric form of M_r 300,000. ß-hexo. may have remained as a dimer of M_r 120,000 or oligomerised into a trimer of M_r 180,000 and not secreted as readily as ß-gluc. If this hypothesis is true, then the microcapsules constructed using this protocol appeared to have a molecular weight cut-off in the range of 75,000 to 150,000.

Prolonged poly-L-lysine treatment during encapsulation strengthens the integrity of capsule membrane by allowing more crosslinking between alginate and poly-L-lysine (Goosen et al, 1985). Encapsulated cells treated with 20 min. poly-L-lysine exhibited a significantly lower secretion for both enzymes, a lack of enzymes retention and a poor viability (<40%), as compared to those treated with 6 or 10 min. poly-L-lysine. As such, an increase in poly-L-lysine treatment seemed to strengthen the integrity of the capsule membrane because the stronger the membrane, the lower the

diffusivity (King et al, 1987; Goosen et al, 1985). Although there is a possibility that ß-gluc. was secreted as a monomer of M_r 75,000, the decreased diffusivity may have prevented even the ß-gluc. monomer to diffuse freely across the capsule membrane at rates compared to capsules treated with only 6 min. poly-L-lysine (Fig 7A, Fig 7D). Moreover, a decrease in diffusivity also implied that nutrients cannot diffuse into the capsules to nourish the encapsulated cells, as indicated by the decrease in intracellular enzyme activitiy, which explained the poor viability and a lack of gene expression. It seemed the upper limit for poly-L-lysine treatment was 10 min. The 20 min. poly-L-lysine treatment was not chosen to be one of the washing conditions for our subsequent experiments since poor viability is most undesirable for long term gene therapy.

More cells were found embedded in the capsule membrane in the early stages of encapsulation when cells were encapsulated at a higher density (e.g. $4x10^6$ cells/ml), which later on (day 8 and onwards) were found colonizing the bottom of the petri dish. The fact that cells encapsulated at higher density allowed secretion of enzymes at higher rates and retention of enzymes to a lesser degree was probably due to larger pore size or more pores created by more embedded cells which fell off the membrane (Wong & Chang, 1992).

Double coated capsules effectively reduced enzyme secretion and allowed enzyme accumulation only in the first two weeks of the experiment,

after which the secretion rate per unit cell was the same as the single coated capsules (Table 9A). This is probably due to some microscopic deterioration in the outer coat of the capsules, a phenomenon that cannot be reflected from simply looking through a simple light microscope. If scanning electron microscopy were available to diagnose the configuration of capsules with time, it would be better to verify that the capsule coats had degraded with time.

4.3 Cell Proliferation in HEMA-MMA Thermoplastic Capsules

4.3.1 Proliferation of LhGH-1 Cells in HEMA-MMA Capsules

LhGH-1 cells survived equally well in both APA capsules and HEMA-MMA capsules. Viability of 70% to 90% was observed when LhGH-1 cells were encapsulated in HEMA-MMA capsules. The diameter of HEMA-MMA capsules was 800 μm whereas APA capsules was 400 μm. Although HEMA-MMA capsules were twice as big as APA capsules in diameter, the number of viable cells/capsule at day 1 post-encapsulation was 130 in HEMA-MMA capsules, only 50% in number as those encapsulated in APA capsule, which was 260, even though the initial cell density for encapsulation was at $2x10^6$ cells/ml for both types of capsules.

It was not fully understood why the HEMA-MMA capsules would contain fewer cells than the APA capsules when they were larger in size than the APA capsules and that space limitations in the HEMA-MMA capsules were not a factor for cell growth since there was a large void volume in the capsule core (Uludag et al, 1993).

4.3.2 Proliferation of 2A50 Cells in HEMA-MMA Capsules

Viability of encapsulated 2A50 cells in HEMA-MMA capsules was 70% except for day 1, which was only 50%. This phenomenon was also observed when this cell line was encapsulated in APA capsules, confirming the

fragile nature of this particular cell line. Encapsulated at a density of $5x10^6$ cells/ml, the number of viable cells/capsule on day 1 was 260 in HEMA-MMA capsules and 125 in APA capsules if 2A50 cells were encapulated at such density. This observation was very different from encapsulated LhGH-1 cells as described in the previous section. The explanation for such difference was due to the initial cell denstiy used for encapsulation in the HEMA-MMA capsules (Uludag et al, 1993).

Uludag et al reported that the initial "seeding" density for HEMA-MMA capsules affected the efficiency of encapsulation. When Chinese hamster ovary cells were encapsulated at low density (e.g. $4x10^5$ cells/ml), there was only a small increase in the metabolic activity of the cells. The most ideal "seeding" density for cell proliferation was $4x10^6$ and $5x10^6$ (Uludag & Sefton, 1992). As such, the lower cell number/capsule for encapsulated LhGH-1 cells in HEMA-MMA capsules was most likely due to the lower "seeding" density which in turn affected the efficiency of the encapsulation.

4.3.3 The Effects of Matrigel on Cell Proliferation

Matrigel was described as a poorly defined, solubilized basement membrane that contains laminin, collagen type IV, heparan sulfate proteoglycans and entactin (Kleinman et al, 1982). Hadley et al reported matrigel as being effective for the attachment and differentiation of both

normal and transformed anchorage dependent epitheloid and other cell types (Hadley et al, 1986). It was found that Chinese hamster ovary cells encapsulated in matrigel capsules contained twice the number of viable cells/capsule compared to regular capsules encapsulated at the same cell density and monitored at the same time point. In general, Chinese hamster ovary cells proliferated more rapidly in matrigel capsules than in regular capsules (Uludag et al, 1993).

Our findings from the encapsulation of both LhGH-1 cells and 2A50 cells in regular and matrigel capsules were not consistent with published results. There was no significant difference in viability and cell proliferation when the cell lines under studied were encapsulated in regular and matrigel capsules. Matrigel did not enhance the growth of LhGH-1 cells and 2A50 cells. Since both cell lines were anchorage dependent , it was not understood why matrigel did not enhance the proliferation of our cell lines. Perhaps more research needs to be done to study the nature of matrigel and the mechanism behind its interaction with different cell lines so that we can understand why it supports the growth of some, but not all types of cells.

4.4 Secretion of Recombinant Gene Products from HEMA-MMA Thermoplastic Capsules

4.4.1 Secretion of hGH from HEMA-MMA Thermoplastic Capsules

HGH secretion rate from matrigel capsules was the same as regular capsules, indicating the presence of matrigel did not affect secretion of hGH. This is consistent with published results since matrigel was reported as enhancing cell proliferation but not affecting the secretion of proteins (Uludag, 1992). The hGH secretion rate from HEMA-MMA capsules was the same as from APA capsules. HGH secretion rate from HEMA-MMA capsules was the same as from non-encapsulated LhGH-1 cells, indicating a molecular weight cut-off of HEMA-MMA to be over 45,000, which is consistent with the published cut-off of 100,000 (Sefton et al, 1992).

Matrigel capsules retained more hGH than regular capsules did. One possible explanation could be an interaction between matrigel and the hGH assay system, leading to some false positive results. However, empty capsules coated with matrigel were found to have no interaction with the hGH assay (unpublished data), indicating matrigel may have a binding capacity with hGH, causing its retention in the intracapsular space. This hypothesis cannot be confirmed at this moment since the nature of matrigel is still poorly defined. Moreover, other research groups usually did not monitor the intracapsular protein activities and therefore no references can be found to support our hypothesis.

4.4.2 Secretion of ß-Gluc. and ß-Hexo. from HEMA-MMA Capsules

HEMA-MMA capsules did not allow the secretion of ß-gluc. and ß-hexo. outside the capsules, indicating a molecular weight cut-off of smaller than 120,000, which was consistent with the published cut-off value of 100,000 (Sefton et al, 1992). Even if ß-gluc. was secreted as a dimer of M_r 150,000, it would be retained inside the intracapsular space. As such, the accumulation of enzymes in the intracapsular space was due to their large molecular size (M_r ß-gluc.=300,000; M_r ß-hexo.=120,000). Empty capsules coated with matrigel were found to have no interaction with the ß-gluc. and ß-hexo. assay systems, indicating our data were not false positive and that matrigel did not interfere with the assay systems.

4.5 Conclusions and Future Considerations

The secretion of marker proteins from encapsulated cells for 29 days has been used to determine the molecular weight permeability limit of APA capsules and HEMA-MMA capsules. ß-gluc. and ß-hexo. appeared to be able to diffuse across the APA membrane. The results presented here are inconsistent with the previous reported results, with respect to the apparent molecular weight cut-off of APA capsules (King et al, 1987; Goosen et al, 1985). Media surrounding microencapsulated 2A50 cells can be analysed by the method of non-denaturing polyacrylamide gel electrophoresis which separates molecules with respect to their molecular weight. If ß-gluc. was indeed secreted as a monomer of M_r 75,000, or a dimer of M_r 150,000, then the molecular weight cut-off of our capsule was in the range of 75,000 to 150,000, which was close to the published cut-off.

To solve the problem of cells embedding in APA capsule membrane, three to five small capsules can be encapsulated into a bigger microcapsule and then liquefy the alginate coat of the smaller microcapsules with sodium citrate. The end product will be cells encapsulated in a bigger microcapsule, but not embedded in the capsule membrane.

To minimize cells falling out of APA capsule membrane and form colonies on their own, APA capsules can be transferred to a new petri dish one week after encapsulation to ensure no more cells are getting out of the membrane before implantation.

More experiments need to be done to study the properties of matrigel, such as its mechanism of enhancing cell proliferation and whether it binds to certain secretory products. The "sticky" properties of the HEMA-MMA capsules, due to the nature of the polymer, may obstruct the diffusion of nutrients and secretory products. Since the nature of the polymer is not fully known, such "sticky" phenomena cannot be avoided. Future experiments should aim at analyzing the structural properties of the polymer in order to minimize capsule aggregation.

Future design should aim for smaller HEMA-MMA capsules to increase the diffusion of nutrients and secretory products and decrease the space required for implantation. Currently, a new design of the encapsulation system where the needle assembly is kept stationary and a co-axial hexadecane stream shears the liquid droplets enables production of capsules 300-600um in diameter (Uludag et al, 1993).

Despite the imperfections of our encapsulation systems, microencapsulated cells hold considerable potential as controlled release devices for local or systemic delivery of bioactive molecules. Cells remained viable through the process of encapsulation as well as during subsequent in vitro culture (Uludag & Sefton, 1992; Uludag et al, 1993; Sefton et al, 1992). Fibroblasts engineered to secrete recombinant human FIX were shown to survive the encapsulation procedure with about 70-90% viability,

proliferated within the microcapsules to twice their original number within 2 weeks, and continued to secrete FIX (M_r 72,000) into the culture medium at similar rates as the non-encapsulated cells (Liu et al, 1993).

Alternatively, allogeneic mice implanted with hGH secreting cells encapsulated in alginate microcapsules demonstrated hGH in their circulation within the first 2 weeks. The persistent expression of the transgene and survival of the transfected cells were verified when the microcapsules were retrieved periodically to demonstrate that the encapuslated cells remained viable, proliferative, and productive of hGH even by 78-111 days, indicating the feasibility for delivering gene products with genetically modified allogeneic cells in vivo for prolonged periods (Chang et al, 1993).

Recent studies have shown the transplantation of genetically modified autologous bone marrow cell or skin fibroblasts into mice lacking ß-glucuronidase activity. The human ß-glucuronidase cDNA was introduced with a retroviral vector into mutant mice bone marrow cells or skin fibroblasts. ß-glucuronidase enzyme activity was detectable in various organs, including the brain, and disappearance of lysosomal storage was obvious in the liver and spleen (Marechal et al, 1993; Moullier et al, 1993; Wolfe et al, 1992; Birkenmeier, 1991). Amniotic epithelial cells has been used for transplantation in patients with lysosomal storage diseases as an enzyme replacement therapy. However, there were no clinical beneficial

effects in the patients with Tay-Sach's disease and juvenile metachromatic leukodystrophy (Sakuragawa et al, 1992). As such, the technique of encapsulation may provide an alternative to somatic gene therapy and in treatment of somatic diseases such as mucopolysaccharidosis VII, which is due to the deficiency of ß-glucuronidase, or Tay-Sach's disease, due to the deficiency of ß-hexosaminidase A and Sandhoff disease, due to the deficiency of ß-hexosaminidase B. (Watts & Gibbs, 1986). Encapsulation of cells engineered to secrete ß-glucuronidase or ß-hexosaminidase may be implanted into patients in early stages of the diseases to reverse some of the symptoms, such as mental retardation and dysostosis multiplex. Encapsulation is indeed a very useful technique for long term gene therapy.

APPENDIH

Encapsulation of Cells in APA Microcapsules

A solution of 1.5% sodium alginate (Keltone lot no. 77373A, Kelco, Merck, Chicago, IL.) in 0.85% NaCl was prepared by 0.22 µm filter sterilization and stored at 4°C. The cells were harvested, counted and centrifuged at 1000 rpm (Sorcal RT 6000B) for 10 mins. at 10°C. The supernatant was aspirated and the cells were resuspended in ice cold 0.85% NaCl. $20x10^6$ cells were centrifuged under identical conditions and resuspended in 50 µl of cold 0.85% NaCl. 10 ml of 1.5% sodium alginate in 0.85% NaCl was added to the cells without the generation of air bubbles and transferred to a 10 ml syringe. The cells were then extruded through a 26 gauge needle housed in an air jacket which supplied concentric air flow at approximately 3 litres per minute. The cell suspension was extruded at 37.5 ml/hr into 40 ml of an ice cold 1.1% $CaCl_2$ in 0.85% NaCl bath which was approximately 7 cm below the extrusion housing. Gentle swirling was perfromed to prevent the alginate droplets from aggregating or colliding immediately after entry into the bath.

The crosslinked alginate spheres were evenly divided into two 50 ml conical centrifuge tubes and the remaining spheres in the beaker which contained the bath were rinsed out with ice cold 1.1% $CaCl_2$. The spheres were allowed to settle to the bottom of the conical centrifuge tube and the

solution aspirated, leaving approximately 5 ml of the packed spheres in 1.1% $CaCl_2$. 30 ml of ice cold 0.55% $CaCl_2$ in 0.85% NaCl was added and the spheres were resuspended and allow to settle. The 0.55% $CaCl_2$ in 0.85% NaCl was aspirated and the spheres were resuspended in 30 ml of ice cold 0.28% $CaCl_2$ in 0.85% NaCl. The spheres were allowed to settle and the 0.28% $CaCl_2$ in 0.85% NaCl was removed by aspiration. 30 ml of ice cold 0.1% CHES (0.1% 2-(n-cyclohexylamino)ethane sulfonic acid, 0.03% NaCl, 1.1% $CaCl_2$, pH8.2) was added and the suspension was allowed to gently agitate for 2 minutes. After settling for 1 minute the 0.1% CHES was aspirated and 30 ml of ice cold $CaCl_2$ was added and the spheres were resuspended. The spheres were allowed to settle and the 1.1% $CaCl_2$ was aspirated. 30 ml of ice cold 0.05% poly-L-lysine in 0.85% NaCl was added and the cells were allowed to gently agitate for 6 to 20 minutes. After settling for 2 minutes the 0.05% poly-L-lysine in 0.85% NaCl was aspirated and 30 ml of ice cold 0.1% CHES was added. The spheres were resuspended and allowed to settle. The 0.1% CHES was aspirated and 30 ml of 1.1% $CaCl_2$ was added. The spheres were allowed to settle and the 1.1% $CaCl_2$ was aspirated. 30 ml of ice cold 0.85% NaCl was added and the spheres were resuspended. The spheres were allowed to settle and the 0.85% NaCl was aspirated. 30 ml of ice cold 0.03% sodium alginate in 0.85% NaCl was added and allowed to gently agitate for 3 minutes. After settling for 1 minute the 0.03% sodium alginate was aspirated and 30 ml of ice cold 0.85%

NaCl was added and the spheres were resuspended. After settling the 0.85% NaCl was aspirated and 30 ml of ice cold 55 mM sodium citrate (55 mM sodium citrate, 0.85% NaCl) was added. The sphere were allowed to gently agitate for 6 to 20 minutes and then allowed to settle for 2 minutes. The 55 mM sodium citrate was aspirated and 30 ml of ice cold 0.85% NaCl was added. The capsules were resuspended and allowed to settle. The 0.85% NaCl was aspirated and 30 ml of ice cold 0.85% NaCl was added again. The capsules were resuspended and allowed to settle. The 0.85% NaCl was aspirated and 30 ml of regular growth media (RGM) was added. The capsules were allowed to settle and the RGM was aspirated. The capsules were resuspended in 30 ml of RGM and placed into sterile tissue culture containers and placed under regular growth conditions.

REFERENCES

Aebischer, P., Russell, P. C., Christenson, L., Panol , G., Monchik, J. M. and Galletti, P. M. (1986). A bioartificial parathyroid. Trans. Am. Soc. Artif. Intern. Organs., 32: 134-137

Aebischer, P., Wahlberg, L., Tresco, P. A. and Winn, S. R. (1990). Macroencapsulation of dopamine secreting cells by coextrusion with an organic polymer solution. Biomaterials, 12: 50-56

Aebischer, P., Winn, S. R. and Galletti, P. M. (1988). Transplantation of neural tissue in polymer capsules. Brain Res., 448: 364-368

Aebischer, P., Winn, S. R., Tresco, P. A., Jaeger, C. B. and Greene, L. A. (1991). Transplantation of polymer encapsulated neurotransmitter secreting cells: effect of the encapsulation technique. J. Biomech. Eng., 113: 178-183

Altman, J. J., Houlbert, D., Callard, P., McMillan, P., Solomon, B. A., Rosen, J. and Galletti, P. M. (1986). Long-term plasma glucose normalization in experimental diabetic rats with macroencapsulated implants of benign human insulinomas. Diabetes, 35: 625-633

Anderson, W. F. (1992). Human Gene Therapy. Science, 256: 808-813

Awrey, D. E. (1993). Secretion of marker protein from alginate-poly-L-lysine-alginate microcapsules. M. Sc. Thesis, Dept. of Medical Sciences, McMaster University, Ontario

Behara, A. M. P., Westcott, A. J. and Chang, P. L. (1992). Intrathymic implants of genetically modified fibroblasts. Faseb J., 6: 2853-2858

Bennett, V. J., Detmar, J. and Chang, P. L. (1991). Growth inhibition by cyclosporine A in vivo and in vitro. Life Sci., 48: 1455-1461

Birkenmeier, E. H. (1991). Correction of Murine Mucopolysaccharidosis Type VII (MPS VII) by Bone Marrow Transplantation and Gene Transfer Therapy. Hum. Gene Ther., 2: 113

Chang, P. L., Capone, J. C. and Brown, G. M. (1990). Autologous Fibroblast Implantation Feasibility and Potential Problems in Gene Replacement Therapy. Mol. Biol. Med., 7: 461-470

Chang, P. L., Shen, N. and Westcott, A. J. (1993). Delivery of Recombinant Gene Products with Microencapsulated Cells In Vivo. Hum. Gene Ther., 4: 433-440

Crooks, C. A., Douglas, J. A., Broughton, R. L. and Sefton, M. V. (1990). Microencapsulation of mammalian cells in a HEMA-MMA copolymer: Effects on capsule morphology and permeability. Biomed. Mat. Res., 24: 1241-1262

Davidson, B. L., Allen, E. D., Kozarsky, K. F., Wilson, J. M. and Roessler, B. J. (1993). A Model System for In Vivo Gene Transfer into the Central Nervous System Using an Adenoviral Vector. Nat. Genet., 3: 219-223

De Boni, U. (1988). Chromatin and nuclear envelope of freeze-fractured, neuronal interphase nuclei, resolved by scanning microscopy. Biol. Cell, 63: 1-8

Friedmann, T. (1989). Progress Toward Human Gene Therapy. Science, 244: 1275-1281

Glaser, J. H. and Sly, W. S. (1973). ß-Glucuronidase deficiency mucopolysaccharidosis: methods for enzymatic diagnosis. J. Lab. Clin. Med., 82: 969-977

Golub, E. S. and Green, D. R. (1991). In: Immunology a synthesis. 2nd edition, Sinauer Associates Inc. publishers, Sunderland, Massachusetts

Goosen, M. F. A., O'Shea, G. M., Gharapetian, H. M., Chou, S. and Sun, A. M. (1985). Optimization of Microencapsulation Parameters: Semipermeable Microcapsules as a Bioartificial Pancreas. Biotech. Bioeng., 27: 146-150

Gorelik, E., Ovejera, A., Shoemaker, R., Jarvis, A., Alley, M., Duff, R., Mayo, J., Herberman, R. and Boyd, M. (1987). Microencapsulated Tumor Assay: New Short-Term Assay for in vivo Evaluation of Effects of Anticancer Drugs on Human Tumor Cell Lines. Cancer Res., 46: 5739-5747

Graham, F. L. and Van der Eb, A. J. (1973). A New Technique for the Assay of Infectivity of Human Adenovirus 5 DNA. Virology, 52: 456-467

Hadley, M. A., Byers, S. W., Suarez-Quian, C. A., Kleinman, H. K. and Dym, M. (1985). Extracellular Matrix Regulates Sertoli Cell Differentiation, Testicular Cord Formation, and Germ Cell Development In Vitro. J. Cell. Biol., 101: 1511

Jaeger, C. B., Aebischer, P., Tresco, P. A., Winn, S. R. and Greene, L. A. (1992). Growth of tumour cell lines in polymer capsules : ultrastructure of encapsulated PC12 cells. J. Neurocyto., 21: 469-480

Kibat, P. G., Igari, Y., Wheatley, M. A., Eisen, H. N. and Langer, R. (1990). Enzymatically activated microencapsulated liposomes can provide pulsatile drug release. Faseb J., 4: 2533-2538

King, G. A., Daugulis, A. J., Faulkner, P. and Goosen, M. F. A. (1987). Alginate-Polylysine Microcapsules of Controlled Membrane Molecular Weight Cutoff for Mammalian Cell Culture Engineering. Biotech. Prog., 3: 231-240

Kirby, K. S. (1957). A new method for the isolation of deoxyribonucleic acids: evidence on the nature of bonds between deoxyribonucleic acid and protein. Biochem. J., 66: 495-504

Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K. and Martin, G. R. (1982). Isolation and characterization of Type IV Procollagen, Laminin, and Heparan Sulfate Proteoglycan from the EHS Satcoma. Biochemistry, 21: 6188-6193

Kozarsky, K. F. and Wilson, J. M. (1993). Gene therapy: adenovirus vectors. Curr. Opi. Gen. Dev. 3: 499-503

Lim, F. and Sun, A. M. (1980). Microencapsulated Islets as Bioartificial Endocrine Pancreas. Science, 210: 908-910

Liu, H. W., Ofosu, F. A. and Chang P. L. (1993). Expression of Human Factor IX by Microencapsulated Recombinant Fibroblasts. Hum. Gene Ther., 4: 291-301

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 256-265

Luciani, J., Benalia, M., Bitker, M. O., Mouquet, C., Chartier-Kastler, E., Ourhama, S., Jacobs, C., Chatelain, C. and Viars, P. (1993). Influence of Antilymphocyte Globulin Upon the Incidence of Malignancies After Kidney Transplantation. Trans. Proceed., 25: 2302

Lum, Z. P., Krestow, M., Tai, I. T., Vacek, I. and Sun, A. M. (1992). Xenografts of Rat Islets into Diabetic Mice. Transplantation, 53: 1180-1183

Lysaght, M. J., Christenson, L. and Dionne, K. E. (1993). Biomedical Applications of Immobilized Cells. In: Fundamentals of Animal Cell Encapsulation and Immobilization. CRC Press, Inc. pp. 7-41

Marechal, V., Naffakh, N., Danos, O. and Heard, J. M. (1993). Disappearance of Lysosomal Storage in Spleen and Liver of Mucopolysaccharidosis VII Mice After Tranplantation of Genetically Modified Bone Marrow Cells. Blood, 82: 1358-1365

Moullier, P., Bohl, D., Heard, J. M. and Danos, O. (1993). Correction of lysosomal storage in the liver and spleen of MPS VII mice by implantation of genetically modified skin fibroblasts. Nat. Genet., 4: 154-159

Norman, G. R. and Streiner, D. L. (1994). Post-hoc Comparison. In: Biostatistics: The Bare Essentials. Mosby-Year Book, Inc. pp. 67-68

O'Shea, G. M., Goosen, M. F. A. and Sun, A. M. (1984). Prolonged Survival of Transplanted Islets of Langerhans Encapsulated in a Biocompatible Membrane. Biochim. Biophys. Acta., 804: 133-136

O'Shea, G. M. and Sun, A. M. (1986). Encapsulation of Rat Islets fo Langerhans Prolongs Xenograft Survival in Diabetic Mice. Diabetes, 35: 943-946

Sagen, J., Wang, H., Tresco, P. A. and Aebischer, P. (1993). Transplants of Immunologically Isolated Xenogeneic chromaffin Cells Provide a Long-Term Source of Pain-reducing Neuroactive Substances. J. Neurosci., 13: 2415-2423

Sakuragawa, N., Yoshikawa, H. and Sasaki, M. (1992). Amniotic Tissue Transplantation: Clinical and Biochemical Evaluations for Some Lysosomal Storage Diseases. Brain Dev., 14: 7-11

Sefton, M. V. and Broughton, R. L. (1982). Microencapsulation of erythrocytes. Biochim. Biophys. Acta. 717: 473-477

Sefton, M. V., Kharlip, L., Horvath, V. and Roberts, T. (1992). Controlled release using microencapsulated mammalian cells. J. Cont. Release. 19: 289-298

Selden, R. F., Skoskiwicz, M. J., Howie, K. B., Russel, B. S. and Goodman, H. M. (1987). Implantation of Genetically Engineered Fibroblasts into Mice: Implications for Gene Therapy. Science, 236: 714-718

Shih, C. C., Stoye, J. P. and coffin, J. M. (1988). Highly preferred Targets for Retrovirus Integration. Cell, 53: 531-537

Southern, E. M. (1975). Detection of specific sequences among DNA fragments seperated by gel electrophoresis. J. Mol. Biol., 98: 503-517

Stevenson, W. T. K., Evangelista, R. A., Broughton, R. L. and Sefton, M. V. (1987). Preparation and characterization of thermoplastic polymers from hydroxyethyl methacylates. J. Appl. Poly. Sci., 34: 65-83

Strauss, S. E. (1984). Adenovirus Infection in Humans. In: The Adenoviruses. Ginsberg H. S. New York. Plenum Press. pp 451-496

Sun, A. M., O'Shea, G. M. and Gharapetian, H. (1986). Artificial cells containing islets as bioartificial pancreas. Prog. Artif. Organs, 1985: 601-609

Sun, A. M., O'Shea, G. M. and Goosen, M. F. A. (1983). Injectable, biocompatible islet microcapsules as a bioartificial pancreas. Prog. Artif. Organs. 1983: 769-776

Temin, H. M. (1986). Retrovirus vectors for gene transfer: Efficient integration into and expression of exogenous DNA in vertebrate cell genomes. In: Gene Transfer. Plenum press, New York and London. pp. 149-186

Uludag, H., Kharlip, L. and Sefton, M. V. (1993). Protein delivery by microencapsulated cells. Adv. Drug Delivery Rev., 10: 1-16

Uludag, H., and Sefton, M. V. (1992). Metabolic Activity and Proliferation of CHO cells in HEMA-MMA Microcapsules. Cell Transplant, 35: 1-15

Uludag, H., and Sefton, M. V. (1991). Metabolic Activity of CHO Fibroblasts in HEMA-MMA Microcapsules. Biotechn. Bioeng., 39: 672-678

Watts, R. W. E. and Gibbs, D. A. (1986). In: Lysosomal Storage Diseases: Biochemical and Clinical Aspects. Taylor and Francis Inc., London and Philadelphia. pp. 119-169.

Wolfe, J. H., Sands, M. S., Barker, J. E., Gwynn, B., Rowe, L. B., Vogler, C. A. and Birkenmeier, E. H. (1992). Reversal of pathology in murine mucopolysaccharidosis type VII by somatic cell gene transfer. Nature, 360: 749-753

Wong, H. and Chang, T. M. S. (1992). The microencapsulation of cells within alginate poly-L-lysine microcapsules prepared with the standard single step drop technique: histologically identified membrane imperfections and the associated graft rejection. Biomater. Artif. Cells Immob. Biotechnol., 19(4): 675-86.

Wu, G. Y., Wilson, J. M., Shalaby, F., Grossman, M., Shafritz, D. A. and Wu, C. H. (1991). Receptor-mediated Gene Delivery In Vivo. J. Biol. Chem. 266: 14338-14342