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Superior Cell Delivery Features of Poly(ethylene glycol) Incorporated Alginate, Chitosan, and Poly-L-lysine Microcapsules

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Abstract: Microencapsulation is an emerging technology in the development of bioartificial organs for drug, protein, and delivery systems. One of the advancements in establishing an appropriate membrane material for live cell and tissue encapsulation is the incorporation of poly(ethylene glycol) (PEG) to the widely studied alginate microcapsules. The current study investigates the properties of integrating PEG to microcapsules coated with poly-L-lysine (PLL) and chitosan as well as a novel microcapsule membrane which combines both PLL and chitosan. Results show that microcapsules containing PEG can support cell viability and protein secretion. The addition of PEG to PLL and chitosan-coated microcapsules improves the stability of microcapsules when exposed to a hypotonic solution. We also compared the novel microcapsule with two other previously used microcapsules including alginate-chitosan-PEG and alginate-PLL-PEG-alginate. Results show that all three membranes are capable of providing immunoprotection to the cells and have the potential for long-term storage at −80 °C. The novel membrane containing PEG, chitosan, and PLL, however, revealed the highest cell viability and mechanical strength when exposed to external rotational force, but it was unable to sustain osmotic pressure. The study revealed the potential of using PEG-incorporated alginate, chitosan, and PLL microcapsules for encapsulating live cells producing proteins and hormones for therapy.

Keywords: Microencapsulation; alginate; poly(ethylene glycol); chitosan; transplantation; immunoisolation; cell delivery; cell therapy

Introduction

Microencapsulation of live cells and tissues within a protective membrane is being widely studied as a method of eliminating the problems associated with immune rejection during allogenic and xenogenic transplantation.[1−6] The microcapsules provide a large surface area to volume relationship, which allows the rapid diffusion and passage of oxygen, nutrients, metabolites, and waste products. However, the polymeric semipermeable membrane blocks the exchange of leukocytes, antibodies, and tryptic enzymes

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across the microcapsules, which prevents any direct contact between the entrapped cells and its surrounding environment. The concept of microencapsulation may therefore eliminate the requirement for immunosuppressants when used in transplantations.[4−9]

Several polymers have been studied for encapsulating biologically active materials, including synthetic and naturally occurring polymers. The preparation of synthetic polymers such as poly(HEMA-MMA) copolymer and acrylamide can require exposure to organic solvents, toxic monomers, high temperatures, and irradiation, which may be undesirable for some applications.[10] On the contrary, naturally occurring polymers such as alginate provide suitable biocompatibility and mild preparation procedures. Calcium alginate beads have been widely researched and utilized for the encapsulation of animal tissues. The mechanical strength of alginate microcapsules is often increased by the addition of a poly-L-lysine (PLL) coating to form alginate-PLL-alginate microcapsules (APA).[9,11−13] Although this membrane has been shown to support tissue growth, previous research has demonstrated that it may induce necrosis of encapsulated cells, and fibrotic tissue growth around the membrane surface is observed when transplanted directly in rat models.[5,14,15] Chitosan, a naturally occurring polysaccharide, is an alternative to the conventionally studied APA microcapsules and has been studied for the encapsulation of drugs.[16−19] More recently, the addition of poly(ethylene glycol) (PEG), a water soluble polymer, has been suggested as a means of increasing biocompatibility of microcapsules.[10,20−23] Improvements to the APA membrane as well as the design of new microcapsule membranes continue to be studied in order to implement the technology of microencapsulation. This study investigates the feasibility of using PEG integrated into PLL and chitosan-coated microcapsules for cell encapsulation therapy.

Material and Methods

Chemicals. Sodium alginate (viscosity 2%), PEG (MW 10000), MTT (thiazolyl blue), poly-L-lysine hydrobromide (MW 27400), sodium citrate, and Bradford reagent were purchased from Sigma Chemicals (St. Louis, MO). Chitosan 10 was obtained from Wako Chemicals, Japan.

Microcapsule Preparation. To prepare alginate microcapsules, 50 mL of a 1.5% sodium alginate solution in deionized H_2O was sterile filtered through a 0.22 μm filter. The solution was extruded through an INOTECH microencapsulator using a 60 mL syringe and 300 μm nozzle. The gelation process took place in a 0.1 M $CaCl_2$ solution for 10 min. APA microcapsules were prepared by immersing the alginate capsules in a 0.05% PLL solution dissolved in 0.45% NaCl for 10 min and then recoating with a layer of 0.1% alginate for 5 min after washing twice with physiological solution. PEG was incorporated into APA microcapsules to form alginate-PLL-PEG-alginate (APPA) microcapsules by subjecting the microcapsules in a 0.5% solution of PEG dissolved in 0.45% NaCl for 10 min after being coated with PLL and washed with physiological solution. After washing,

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a final layer of 0.1% alginate was added for 5 min. Alginate-chitosan (AC) microcapsules were prepared by coating the alginate beads with a 0.5% chitosan solution dissolved in dilute acetic acid at a pH of 5.2 for 30 min. The AC microcapsules were then exposed to a solution of PEG to form alginate-chitosan-PEG (ACP) microcapsules. The novel microcapsule ACPPA was prepared by coating the alginate capsules with a 0.5% chitosan solution. The capsules were washed twice with physiological solution and immersed in the 0.5% solution of PEG for 10 min. The microcapsules were then washed once and transferred to a solution of 0.05% PLL for 10 min. After being washed with physiological solution, the microcapsules were finally coated with 0.1% alginate for 5 min. All microcapsules were stored in physiological solution prior to being used for testing.

Microcapsule Stability Tests. The mechanical stability of the microcapsules was determined using an osmotic pressure test and a rotational stress test. The osmotic pressure test was performed by subjecting 100 ± 5 microcapsules which were previously stored in 0.85% saline solution to 2 mL of deionized water in 35 × 10 mL Petri dishes. The effect of osmotic pressure on the microcapsule membrane was observed microscopically, and the number of damaged capsules was recorded. To assess the microcapsules' ability to sustain the mechanical stress of rotation, cell-free microcapsules were treated with a 0.05 M sodium citrate solution for 4 min to dissolve the alginate core, and 200±10 capsules were placed in 25 mL volumetric flasks containing 3 mL of physiological solution. The flasks were rotated in an ENVIRON shaker at a speed of 150 rpm at 37 °C. The number of damaged capsules was observed and counted under a light microscope at various time intervals.

Cell Lines and Growth Conditions. Cell lines HepG2 and lymphocyte leukemia cells were purchased from ATCC and routinely subcultured in MEM (minimum Eagle's essential medium) supplemented with 10% FBS and 1% penicillin−streptomycin obtained from Sigma Aldrich. The cells were grown in 75 cm^2 tissue culture flasks and incubated at 37 °C with an air atmosphere of 5% CO_2 in a Sanyo MCO-18M multigas incubator. HepG2 were epithelial hepatocellular carcinoma tissues derived from human organisms. The cells were detached and subcultured every 10 days using Trypsin 0.53mM/EDTA (purchased from ATCC). Lymphocyte leukemia cells were derived from *Mus musculus* host, and its cellular products consist of interleukin. Fresh medium was added every 2 days for cell culturing.

Method for Cell Encapsulation. HepG2 cells were encapsulated in alginate microcapsules using previously established procedures.[1,24] Briefly, HepG2 cells were trypsinized and then centrifuged at 1000 rpm for 10 min at 20 °C. The medium was decanted, and the cells were mixed with 0.5 mL of fresh medium and 30 ± 10 mL of sterile filtered 1.5% alginate solution to attain a concentration of 1.5×10^6 cells/mL. The encapsulation process followed the same procedure as described for alginate microcapsule preparation. The alginate capsules were coated to form APPA, ACP, and ACPPA microcapsules, which were stored in complete growth media used for culturing free cells at 37 °C and 5% CO_2. The medium was changed once every 2 days. The entire procedure was performed under sterile conditions in a Microzone biological containment hood (Microzone Corporation, Ottawa, ON, Canada), and all solutions were autoclaved with the exception of chitosan, PEG, PLL, and the alginate solution, which were 0.22 μm sterile filtered prior to usage.

Method of Testing Membrane Cytotoxicity and Metabolic Activity of Encapsulated Cells. The microcapsule membranes were tested for cytotoxicity and suitability for live cell encapsulation using an MTT calorimetric assay. The MTT was also used to detect the metabolic activity of cells within the microcapsules. Previously established procedures as described by Uludag[25] were used with some modifications. The MTT assay is based on the ability of mitochondrial dehydrogenase enzymes present in viable cells to convert MTT into insoluble, purple formazan crystals. The amount of formazan formed can be quantified and used to detect the level of cellular activity.[26] Approximately 30 ± 2 capsules were incubated with 100 μL of medium and 25 μL of an MTT solution (1% MTT in PBS) for 24 h in 96 well plates. The medium and MTT solution were removed from the wells, and the microcapsules were washed once with physiological solution. The formazan crystals formed by the conversion of MTT were dissolved in 100 μL of DMSO. After 30 min of incubation, the absorbance was measured using a multiwell spectrophotometer at a wavelength of 570 nm. The cell number was obtained using a calibration curve correlating cell quantity with absorbance.

Protein Secreted by Encapsulated HepG2 Cells. The ability of the microcapsule membrane to permit the outflow of necessary proteins produced by liver cells was determined by monitoring albumin secretion using a Bradford assay. The Bradford assay is based on the ability of Brilliant Blue G dye to form complexes with proteins, which causes a shift in color, depending on the quantity of protein present. The amount of protein can then be quantified using an absorbance reader. Approximately 100 ± 5 microcapsules encapsulating HepG2 cells were washed 3 times with physiological solution to remove any traces of medium remaining on the membrane surfaces. The capsules were placed in 96 well plates, and 100 μL of serum-free medium was added. The serum-free medium contained no proteins in order to ensure that any protein detected in the medium was solely from the entrapped cells. The microcapsules were incubated for 48 h prior to

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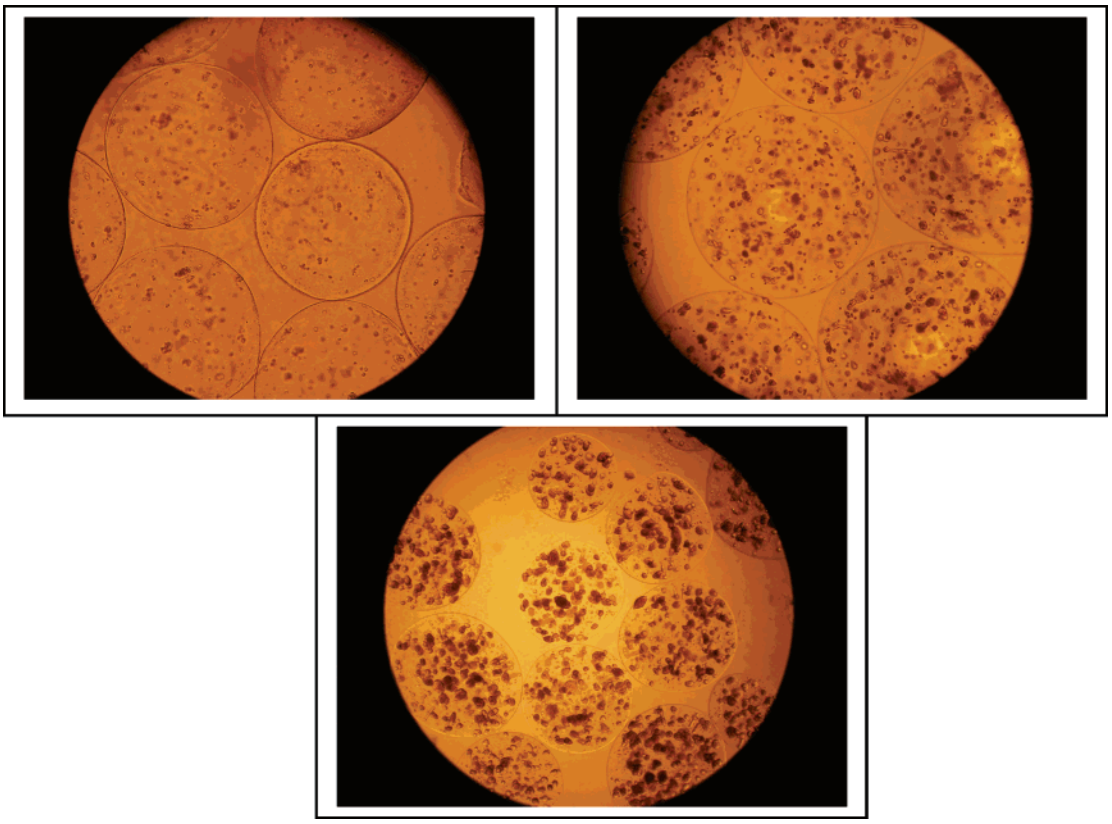

Figure 1. Photomicrograph of encapsulated HepG2 cells in ACP, APPA, and ACPPA microcapsules: (a, top left) APPA microcapsules 1 day after encapsulating; magnification 10×, size 450 μm; (b, top right) ACP microcapsules 1 week after encapsulating; magnification10×, size 450 μm; (c, bottom) ACPPA microcapsules 2 weeks after encapsulating; magnification 6.5×, size 450 μm.

being tested. The quantity of proteins secreted by the cells was measured by taking 15 μL of the medium in which the microcapsules were stored and adding 0.3 mL of Bradford reagent into 96 well plates. The absorbance was measured at 595 nm after 20 min of incubation. The protein concentration was attained using a calibration between albumin concentration and absorbance.

Method for Testing Immunogenicity of Cells. To investigate the ability of the PEG-integrated membranes to provide cell-required immunoprotection, approximately 200 ± 5 APPA, ACP, and ACPPA microcapsules containing HepG2 cells were grown in 1 mL of medium consisting of $9 \times 10^4 \pm 500$ cells/mL of lymphocytes in 24 well plates. Samples were withdrawn every 48 h for a period of 7 days. The volume in each well was kept constant by adding fresh medium after taking the sample. The viability of the HepG2 cells was determined using an MTT assay. The microcapsule membrane was analyzed microscopically using a light microscope.

Cryopreservation Studies. The possibility of long-term storage of encapsulated HepG2 cells in the membranes was tested. Approximately 150 ± 10 capsules were washed with medium and placed in a 2 mL cryovial containing 1 mL of complete growth medium supplemented with 0.10 mL of DMSO. The vials were placed at −20 °C for 1 h prior to being stored at −80 °C. After 30 days of storing, the microcapsules were thawed by immersing the vials in a 37 °C water bath and recultured in medium. The MTT assay was used to obtain the quantity of viable cells remaining.

Results

Morphological Studies. APPA, ACP, and ACPPA microcapsules were prepared. The capsule diameter was in the range 450 ± 30 μm for each membrane. Optical microscopy of encapsulated HepG2 cells revealed the hepatocytes to grow in clumps distributed within the membrane (Figure 1).

Mechanical Stability Test. The microcapsule membrane's ability to maintain integrity after being subjected to the mechanical impact of rotation was tested using a rotational stress test on sham capsules. The microcapsules were treated with citrate to dissolve the alginate core, and the membrane integrity was monitored for all five membranes. Results are shown in Figure 2. The AC and ACP membranes were unable to sustain the mechanical impact. Microscopic analysis revealed that the citrate treatment itself caused the membrane to weaken and lose its spherical shape. After 2 h of rotation at 150 rpm, a total of 85 ± 1% and 40 ± 2% had ruptured for AC and ACP microcapsules, respectively. After 10 h of treatment, a total of 100% and 96 ± 1% of the AC and ACP membranes had ruptured. The addition of PEG to the chitosan coating, therefore, seemed to slightly improve the membrane stability. The addition of PEG to the PLL

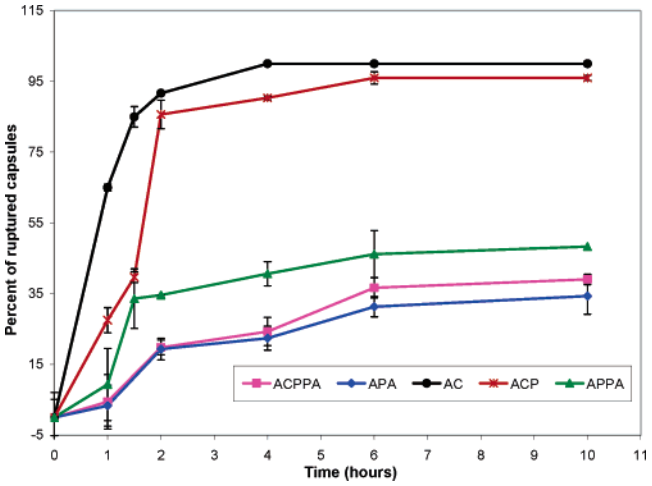

Figure 2. The effect of mechanical impact of rotation at 150 rpm and 37 °C on microcapsule integrity.

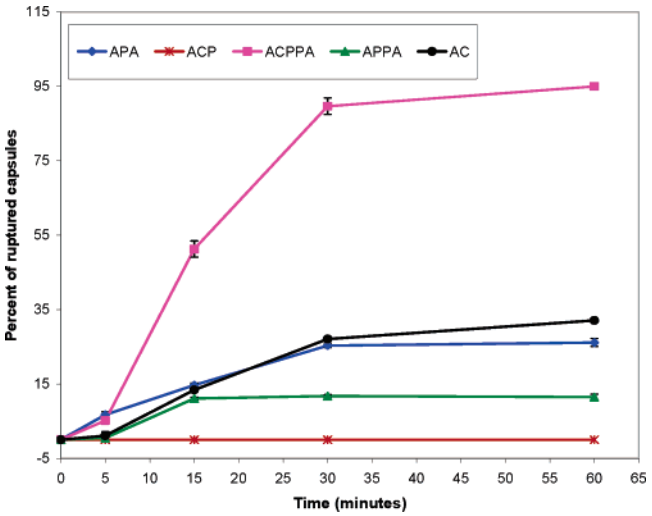

Figure 3. The effect of osmotic pressure on microcapsule integrity when exposed to hypotonic solution.

coating revealed contrary results. Within 2 h, 34 ± 2% of capsules were broken. At the completion of the 10 h study, 48 ± 7% were found broken. The membrane integrity was strongest for the APA and ACPPA membranes. Both membranes retained their spherical and uniform shape after citrate treatment. After 2 h of exposure to rotational impact, 19 ± 3% of APA microcapsules had ruptured while a total of 20 ± 2% of the ACPPA membranes had broken. Within 10 h, the number of broken capsules of APA and ACPPA membranes was 34 ± 4% and 39 ± 1% respectively.

Results for the membrane integrity after exposure to osmotic pressure were significantly different from the results for the mechanical rotation test. The addition of PEG to PLL and chitosan coatings greatly improved the stability of microcapsules in hypotonic solutions. Results are presented in Figure 3. Within the 60 min analysis, it was observed that over 32 ± 0.3% of chitosan coated microcapsules were broken. APA microcapsules displayed a higher stability with a total of 26 ± 1% of broken capsules. In both cases, the addition of PEG greatly enhanced mechanical stability. PEG incorporated in the chitosan membrane resulted in all of the capsules remaining intact. Within 60 min, a total of 11 ± 0.7% of APPA microcapsules were ruptured, which is a reduction of over 50% of broken capsules observed for the PLL membrane without PEG. The ACPPA membranes were unable to sustain prolonged osmotic pressure. Within 5 min of exposure, 5 ± 0.3% of microcapsules had ruptured. At the completion of the 60 min analysis, 95 ± 0.0001% had broken.

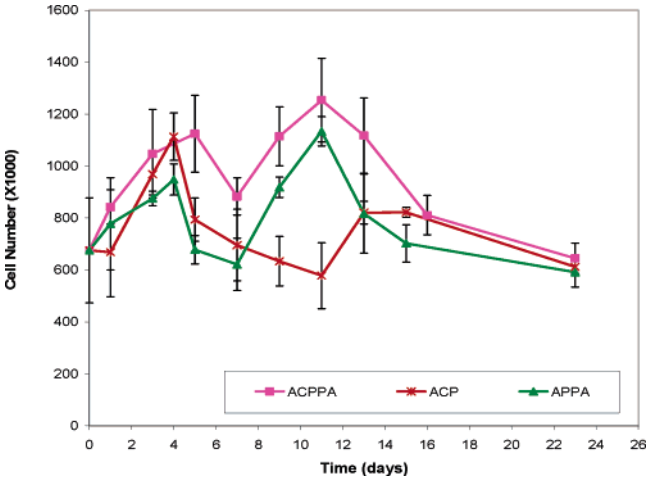

Figure 4. Cell viability of HepG2 cells encapsulated in APPA, ACP, and ACPPA microcapsules.

Metabolic Activity and Cell Viability of Encapsulated HepG2 Cells. To test if the PEG incorporated membranes can support liver cell proliferation and activity, HepG2 cells were encapsulated in ACP, APPA, and ACPPA membranes, and their cell viability was monitored for a period of 25 days using an MTT assay. Figure 4 represents the cell number for 30 ± 2 capsules at various time intervals. All microcapsules revealed an increase in cell number and thereby metabolic activity within the first 5 days after encapsulating. Following the 5 days, a decrease in cell number is observed. After 1 week of encapsulating, the ACPPA and APPA membranes once again display an increase in HepG2 activity resulting in a maximum cell number of $1.26 \times 10^6 \pm 1.27 \times 10^5$ and $1.13 \times 10^6 \pm 5.65 \times 10^4$, respectively, at 9 days. After the ninth day of encapsulating, a gradual decrease in cell activity is observed for the remainder of the 25 day study. The ACP membrane continues to result in a decrease in cell number and HepG2 activity until the 11th day after encapsulating, where a slight increase in cell number is observed. A maximum cell number of $1.11 \times 10^6 \pm 9.16 \times 10^4$ is attained after the fourth day of encapsulation. All of the membranes, however, express viability for the complete 25 days studied.

Protein Secretion by Cells. The amount of protein produced and secreted by the encapsulated cells to the surrounding medium was tested using the Bradford assay. Results are presented in Figure 5, which presents the protein secreted by 100 ± 5 microcapsules at various time intervals. No significant difference is observed for the microcapsules

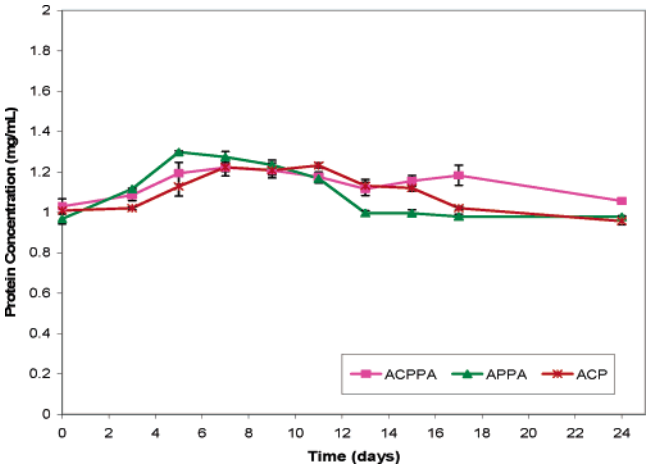

Figure 5. Protein released from encapsulated HepG2 cells in APPA, ACP, and ACPPA membranes.

studied. A peak in protein secretion is apparent between the fifth and the 11th day, after which a decrease is observed implying reduced functionality of the HepG2 cells. A maximum concentration of 1.30 ± 0.006 mg/mL is attained for the APPA microcapsules. The protein secreted by the ACP-encapsulated cells was slightly lower at 1.28 ± 0.05 mg/mL. HepG2 cells encapsulated in the ACPPA membranes showed a different pattern of protein expression. Although the total concentration of protein secreted was slightly lower, the quantity remained consistent for a longer period of time ranging from the fifth to the 17th day. A maximum protein concentration of 1.22 ± 0.04 mg/mL was attained.

Immunogenic Properties of Membranes. The ability of the membranes to provide immunogenic protection to the encapsulated cells was tested by growing HepG2 microcapsules in media containing lymphocytes derived from mouse origin. Results are presented in Figure 6. The APPA and ACPPA show similar behavior. No change between the cells grown with lymphocytes and those grown without is apparent within the first 3 days. Following the third day, a slight decrease in HepG2 activity is noted. There were no significant changes in the cell count for encapsulated HepG2 cells grown without lymphocytes. Throughout the 7 day analysis, the cell count for APPA-encapsulated HepG2 cells showed a decrease in cell number from $7.51 \times 10^5 \pm 8.18 \times 10^4$ to $6.01 \times 10^5 \pm 6.50 \times 10^4$. ACPPA encapsulated HepG2 cells decreased from $9.29 \times 10^5 \pm 7.13 \times 10^4$ to $6.73 \times 10^5 \pm 5.30 \times 10^4$. The ACP membranes revealed different results. Encapsulated HepG2 cells in ACP membranes grown in the presence of lymphocytes showed no significant changes in cell viability.

Cryopreservation Studies. The possibility of the membranes to be used for long-term storage of liver cells was analyzed at −80 °C. The viability of the cells was only slightly reduced for the APPA, ACP, and ACPPA membranes. All of the membranes reveal positive metabolic activity after the freeze−thaw procedure (Figure 7).

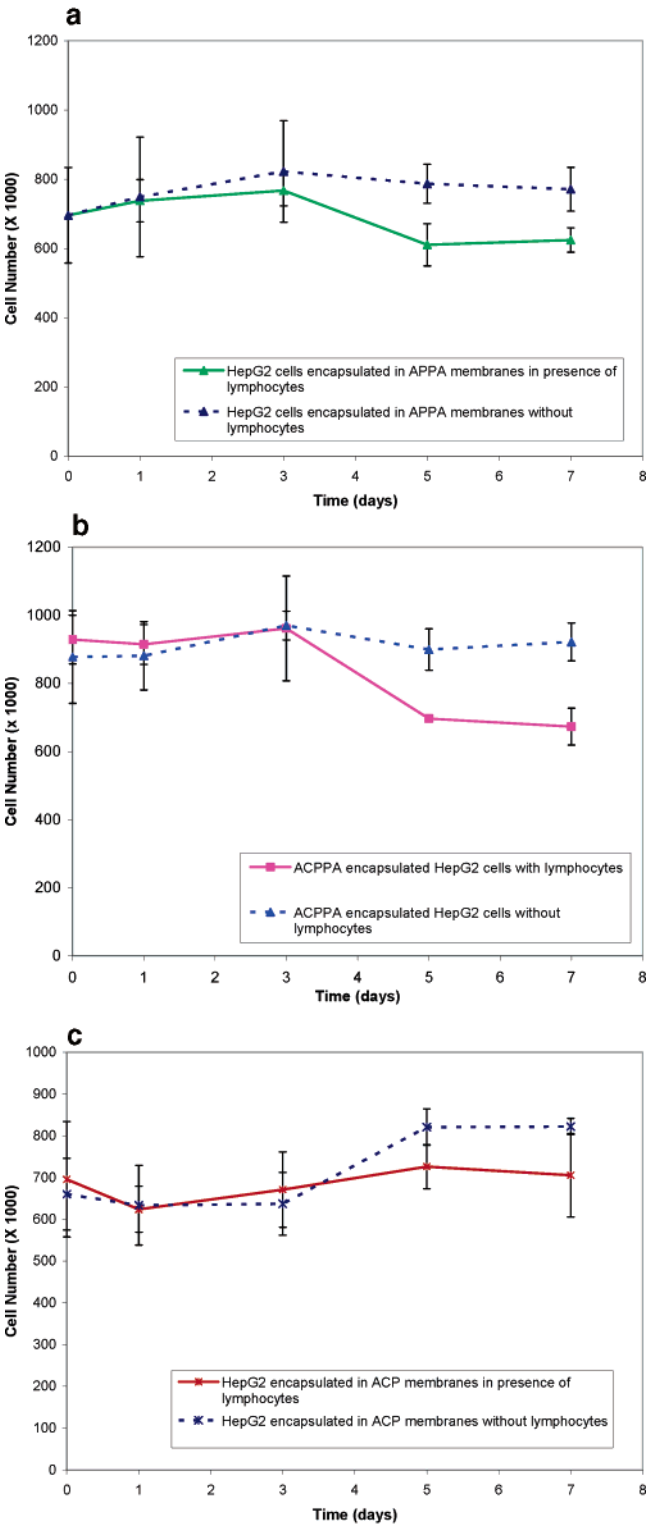

Figure 6. (a) Viability of encapsulated HepG2 cells in APPA microcapsules grown in the presence and absence of lymphocytes. (b) Viability of encapsulated HepG2 cells in ACPPA microcapsules grown in the presence and absence of lymphocytes. (c) Viability of encapsulated HepG2 cells in ACP microcapsules grown in the presence and absence of lymphocytes.

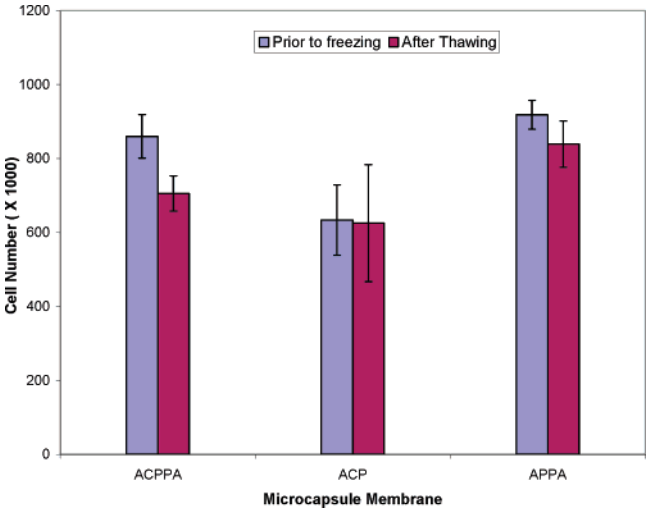

Figure 7. Cryopreservation study: cell number before and after storing at −80 °C for 30 days.

Discussion

APA microcapsules are the most commonly studied membrane for live cell encapsulation. They provide sufficient immunogenicity and support cell viability. However, due to the problems associated with direct transplantation resulting in cell necrosis, an alternate membrane continues to be studied.[14,27,28]

Results from the current study investigated the potential of adding PEG to APA and AC membranes. The osmosis test showed that the addition of PEG can greatly improve the integrity of both the APA and the AC microcapsules. These results are counter to expectations since PEG tends to increase the hydration and swelling characteristics of microcapsules due to hydrophilic segments in PEG which interact with water. As a result, PEG generally would lead to greater swelling of the microcapsules and hence eventual rupture.[20] The observed increased stability may be a result of the interpenetrating network formed by PEG near the microcapsule's membrane surface. It is also suggested that PEG incorporates directly into the alginate matrix, which may be a possible reason for the improvement in integrity observed for microcapsules exposed to hypotonic solutions.[10,22] The novel membrane ACPPA, however, was unable to sustain osmotic pressure. Although not investigated in the current study, this may be due to the interaction between the PLL and chitosan. Incorporating PLL to chitosan, which is generally hydrophobic, increases the hydrophilicity of the polymer.[29] The osmotic pressure causes rapid swelling of the membrane, resulting in rupture.

The ACPPA membrane, in terms of strength, was similar to the APA in the rotational stress test. The addition of PEG to the chitosan membrane slightly improved the strength of the AC membrane; however, it reduced the strength of the APA membrane. The integration of PEG may therefore lead to improvements of the membrane to sustain exterior physiological changes; however, it does not improve the mechanical strength of the microcapsules. The molecular weight and concentration of chitosan affect the mechanical properties of chitosan microcapsules.[18,20,29] Therefore, varying the concentration of chitosan used to coat the microcapsules may increase its strength.

The ACPPA membrane revealed the highest cell viability as apparent from the MTT study. The behavior of APPA and ACPPA was similar, while that of ACP differed. The slight fluctuations in cell viability may be a result of mass transfer either of nutrients or of the MTT itself. Since hepatocytes are anchorage dependent cells, the clusters of cells within the microcapsules can cause a delay or decrease in the rate of diffusion.[6,25] All of the microcapsules, however, retained metabolic activity and viability of cells for a prolonged period of time.

The three membranes allowed the outflow of secreted proteins from the cells to their surroundings. Despite the differences observed between the membranes in cell viability, the amount of protein secreted did not differ significantly. The reason for this observation is not evident from the current study. The novel membrane ACPPA resulted in the least amount of protein secretion; however, the duration was longer, which may be advantageous for cell transplantation. The reduction may be due to slower diffusion as a result of the additional layers.[6,30] This will require further investigation. On the other hand, the use of chitosan and PEG may lead to improved permeability, better cell attachment, increased strength, and reinforced immunoprotection in comparison to the widely investigated APA microcapsules. This, in turn, would lead to a better microcapsule for cell encapsulation techonology.

It has been previously shown in the literature that PEG can reduce protein adhesion and cell adhesion. As a result, the addition of PEG may minimize the interaction between the surrounding lymphocytes with the microcapsules, leading to better immunoprotection.[10,21] Results show that the chitosan-containing membrane provides the greatest protection against foreign cells. The hydrophobic nature of chitosan may be what causes the HepG2 cells within the microcapsules to be unaffected by the surrounding lymphocytes.[10,20]

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Conclusion

Results from the current study reveal that the incorporation of PEG may be a potential improvement to microcapsule membranes for use in cell encapsulation. PEG incorporated into alginate microcapsules coated with either or both PLL and chitosan provides a new direction for live cell encapsulation which can be used for therapy. The novel membrane ACPPA, in comparison to the ACP and APPA microcapsules, resulted in the highest cell viability. In order to test its full potential, however, further detailed studies on the biocompatibility and cell delivery features of the membrane in vivo remain to be tested.

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