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Original Article

An Optimized Calcium Alginate Microcapsule for Mesenchymal Stem Cells Encapsulation

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Abstract:

Background: Recent studies suggest that the three dimensional (3D) cellular environment plays major roles in cell function. Hydrogel cell encapsulation is a method to provide a 3D culture system which controls cell fate and can be used as a drug delivery tool for continuous delivery of therapeutic products.

Methods and Materials: In order to provide an optimized three dimensional cellular environment for mesenchymal stem cell (MSC) encapsulation, various concentrations of alginate hydrogel (0.8, 1.2% and 1.6% w/v) and calcium ions (102 and 153 mM) were used. MSC viability was assessed through trypan blue staining, sub-G1 analysis, and fluorescent microscopy. Structural porosity and mechanical properties of hydrogel capsules were examined.

Results: Encapsulated MSCs within 1.2% concentration (w/v) of alginate showed the highest level (84.8% ± 1.77) of MSC viability. Samples with 1.2% w/v alginate in 102 and 153mM concentrations of calcium chloride bath as well as 1.6% alginate in 102 mM $CaCl_2$ yielded integrated cross link networks and interconnected porous structure which was confirmed by scanning electron microscopy.

Conclusion: Findings of this study indicate that the sample with 1.2% alginate in 153mM $CaCl_2$ is a suitable candidate for using in MSC encapsulation.

Key words: Cell encapsulation; Hydrogel; Alginate; Mesenchymal stem cell

Introduction

Transplantation of encapsulated cells is a promising strategy for treatment of a wide variety of diseases [1-4]. Cell immunoisolation method can protect transplanted cells from the host immune system [5-7]. This technology deals with two main aspects: a suitable cell source and a proper biomaterial as a selectively permeable barrier. The biomaterial permits the bidirectional diffusion of nutrients, oxygen,

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secreted bio-therapeutic productions, and wastes. In addition, they can also prevent the penetration of high molecular weight compounds such as antibodies, immunocytes and complement factors generated by the host immune system [5, 8-10]. One of the best candidate cells for cell therapy are mesenchymal stem cells (MSCs) which have the potential of differentiating into a different type of cells and their exert immunomodulatory function due to educating antigen-specific regulatory T-cells. In addition, compared to embryonic stem cells, there is no ethical concern, source limitation or tumor formation problem associated with using mesenchymal stem cells [1, 11-14].

On the other hand, hydrogels have been suggested to be multi-purpose materials which can provide three dimensional environment similar to the natural extracellular matrices of cells. The special properties significantly affect cellular processes such as differentiation, proliferation and migration [15-19]. Among different hydrogels, alginates have been suggested to be one of the most suitable polymers for capsule fabrication as a result of their abundance, easy gelling properties, suitable mechanical stability and apparent biocompatibility [8, 20-21]. Alginate is a linear natural polysaccharide consists of a family of unbranched binary copolymers of 1→4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G), derived from seaweed. Multivalent cations such as calcium form physical crosslinks in alginate through interacting with carboxylic acid groups of the G blocks of two neighboring alginate chains. As a consequence, a flexible gel network forms which can be used in cell encapsulation process [21-23]. Wang et al. [24] reported that alginate encapsulation provides a 3D environment for differentiation of mouse embryonic stem cells into pancreatic insulin producing cells. In this work, different concentrations of alginate solution and calcium bath for the encapsulation of human mesenchymal stem cells were evaluated. The effects of various concentrations of alginate and calcium ions on the cell viability, structural porosity and diffusivity as well as mechanical properties of alginate beads were studied.

Materials and Methods

Cell culture

MSCs were isolated from human adipose tissue (removed during liposuction) according to a previously described protocol [25]. Isolated MSCs were cultured in α-MEM (GIBCO, Invitrogen, UK) containing 1% penicillin-streptomycin (Gibco, Invitrogen, UK) and 10% fetal bovine serum (Gibco, Invitrogen, UK). MSCs were incubated at 37 ºC with 5% CO2. 3rd passaged cells were used for cell encapsulation studies.

Cell encapsulation

Alginate capsules were prepared under sterile conditions by extruding the MSC-alginate mixture (10^4 cells/ml) into calcium chloride solution containing 150 mM NaCl (Sigma-Aldrich, USA) through a plastic syringe (Soha, Iran) with a 30G needle. Samples were prepared using various concentrations of alginate (0.8, 1.2 and 1.6% w/v) and $CaCl_2$ (102 and 153 mM), which were labeled as Alg(0.8-102), Alg(0.8-153), Alg(1.2-102), Alg(1.2-153), Alg(1.6-102), and Alg(1.6-153).

After being immersed in the $CaCl_2$ bath for 5 min, the prepared beads formed gels (Fig. 1). The $CaCl_2$ solution was removed and the beads were washed twice with 5ml of saline solution. After removal of saline solution, cell culture medium was added. The medium was changed every two days.

Viability analysis

For investigating the effect of encapsulation process on MSC survival, MSCs were encapsulated according to pervious section and the viability was examined via trypan blue staining within 1, 5 and 10 days and sub-G1 analysis at day 7. In order to release encapsulated MSCs, alginate beads were incubated in a solution of 55 mM sodium citrate (Sigma-Aldrich, USA) and NaCl (150 mM) for 10 min.

In order to perform sub-G1 test, after liquefaction and subsequent centrifuging, the supernatant was removed. Thereafter, 100µL of cold sterile phosphate buffered saline (PBS) and 4mL of cold ethanol were added and agitated gently. MSCs were incubated overnight at 4 ºC. MSC pellets were then centrifuged at 800 rev/min for 10 min at 4 ºC. Afterwards, pellets were re-suspended in 250 µL of PBS and supplemented with 250µL of phosphate buffer for 5 min in room temperature. Thereafter, MSCs were incubated with 50µL of Propidium Iodide (1µg/mL, Sigma-Aldrich, USA) and 5µL of RNAse (Thermo Fisher scientific, UK) in the dark. Samples were analyzed using flow cytometery (Becton Dickinson, USA, FACSCcalibur).

Diffusivity of alginate beads

Diffusional properties of alginate capsules were investigated by testing two model molecules with different molecular weights: Glucose (Merck, Germany, M_w=180Da) and Albumin (Sigma-Aldrich, USA, M_w=67000Da). Alginate beads without MSCs were incubated with saline solution in 24-well tissue culture plates. The solute concentrations were as follows: glucose (3 mg/mL), and BSA (1 mg/mL, Sigma-Aldrich, USA). Penetration of solutes into the gel beads was determined at intervals using spectroscopy (Pharmacia Biotech, Sweden).

Morphological Study

The structure of alginate network in prepared samples with different alginate and $CaCl_2$ concentrations was studied using scanning electron microscopy (SEM, Philips-XL30, USA). Capsules with saline as core solution were quenched in liquid nitrogen and lyophilized at -80 ºC. The beads were cut manually using a knife blade and mounted on metal stubs and sprayed with gold before being observed under SEM. Electron micrographs were scanned at 100X magnification.

Evaluation of Mechanical Stability

The mechanical stability of alginate capsules was assessed using a shear-force rupture assay [26]. Approximately 100 capsules were placed in 6-well plates containing saline solution. The plates were then agitated at 250 rev/min on an orbital shaker at 37 ºC. The number of intact capsules at different time points was determined using an inverted microscope with phase contrast optics (IX71 Olympus, Japan).

Fluorescent microscopy of AO/PI stained MSC

The viability of encapsulated MSC for the selected sample was assessed at days 0, 3 and 7 using acridine orange-propidium iodide (AO/PI) staining to confirm the outputs of the two previous viability tests. Briefly, the stock solution of 1 mL of AO (670 mmol/L, Sigma Aldrich, USA) and 100 µL of PI (750 mmol/L, Sigma Aldrich, USA) in the medium was prepared. Precisely before using, 0.01 ml of AO and 1.0 ml of PI were mixed and then diluted 10 times using PBS. The solution was then filtered through a 0.2 mm filter. Encapsulation samples were incubated in AO/PI solution at 37 ºC for 10 min. Following incubation, capsules were washed twice with PBS. Live and dead MSCs were stained with acridine orange and

propidium iodide, respectively. Phase contrast converted microscope (IX71 Olympus, Japan) was used to observe the samples.

Statistical analysis

Data are presented as a mean value ± standard deviation (n=4). Results were statistically analyzed using independent sample t-test and one way ANOVA, via SPSS software 16.0. Statistical significance was set at $p < 0.05$.

Results and conclusion

Cell viability

As an indicative parameter of biological performance of biomaterials, biocompatibility assessment is usually performed in most cell encapsulation studies. Given the direct contact of encapsulated cells with the surrounding matrix, cells viability and function are influenced by biocompatibility of the polymeric gel; otherwise, the excessive growth of fibrous tissues around the materials leads to incomplete permeation of nutrients and hence necrosis of encapsulated cells and eventually transplantation failure [27].

On the first day post encapsulation, for all samples (0.8%, 1.2% and 1.6%, w/v), almost 90% of MSCs were recovered from the prepared bead. After 10 days, cell viability of 1.2% alginate capsules (84.8 ± 1.77) was significantly ($p<0.05$) higher than that of 0.8% capsules (76.13±1.88) (Fig. 2A).

Furthermore, according to the results of Sub-G1 analysis after 7 days encapsulation, cell viability of 1.2% and 1.6% beads remained at a relatively high level (80%). These outputs were obtained using the total number of living cells in other phases after G1 peak in PI histogram (Fig. 2B), and are listed in Table 1.

Morphological study of hydrogels

Internal morphology of alginate beads was studied using a scanning electron microscope. Fig. 3 shows that 0.8% alginate samples do not result in a dense structure. They are associated with large pores which could allow the permeation of albumin molecules. Therefore, they suffer from a weak and brittle structure which is not practically suitable for encapsulation purposes. On the other hand, compared to 1.2% samples, 1.6% alginate beads yields more packed structures. This is evidently visible in the case of Alg(1.6-153) sample. Among the SEM images for the three samples Alg(1.2-102), Alg(1.2-153) and Alg(1.6-102), the second one showed a more integrated structure with smaller pores comparing the two other samples.

Diffusion study

The membrane used in cell encapsulation needs an interconnected porous structure so as to be capable of transporting nutrients and therapeutic agents secreted by cells as well as excreted waste materials. Hydrogels with high amount of water, create suitable porous structures [5, 8, 9].

Table 1. Mean viability percent at seven days post encapsulation based on Sub-G1 analysis

Sample code	Control	Alginate(0.8%)	Alginate(1.2%)	Alginate(1.6%)
% Viability	92.47	63.21	86.77	82.99

Table 2. Percentage of capsules remained intact

Sample code	Alg(0.8-1.2)	Alg(0.8-153)	Alg(1.2-102)	Alg(1.2-153)	Alg(1.6-102)	Alg(1.6-153)
% Intact microcapsules	**71±3.46	90±1.15	100	100	100	100

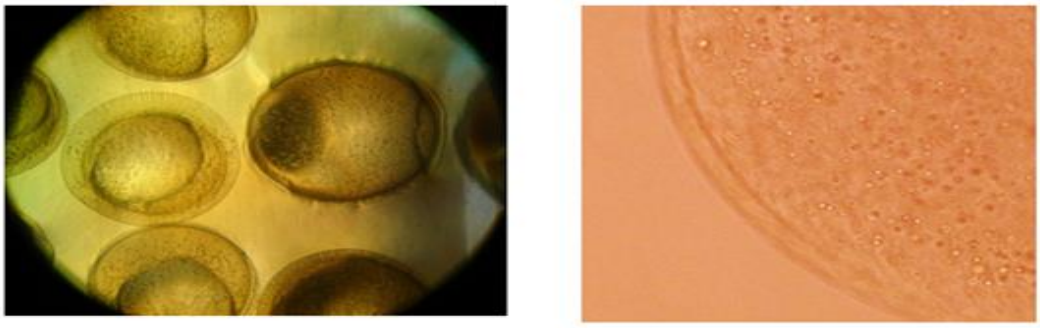

Fig. 1. Mesenchymal stem cells encapsulated in Alg(1.2-153) sample with 1.2% w/v alginate and 153 mM $CaCl_2$ captured by inverted microscope (Left), and fluorescent microscope (Right).

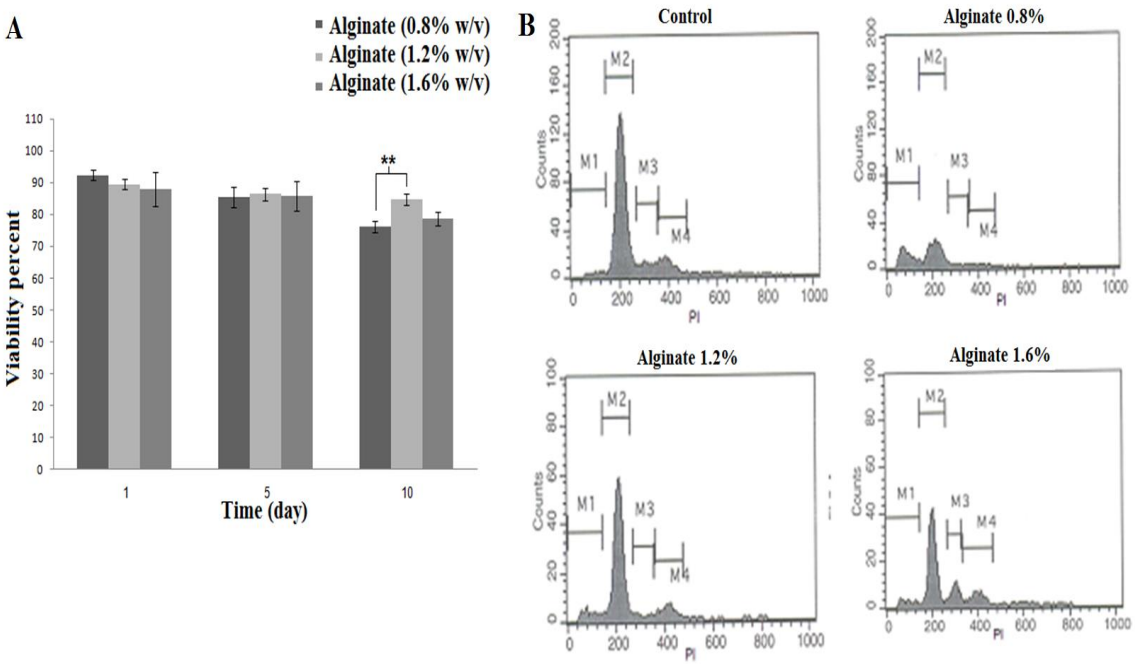

Fig. 2. Viability of encapsulated cells measured by Hemocytometer assay (A), Sub-G1 analysis results (B) for various alginate concentrations (0.8, 1.2, and 1.6% w/v).

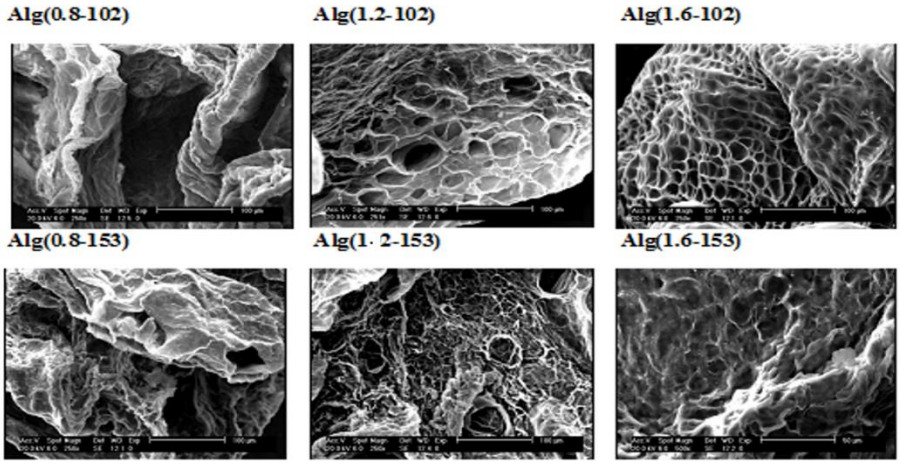

Fig. 3. SEM photographs of alginate beads

Fig. 4A, B demonstrates the temporal diffusion profiles of glucose and albumin at 37°C for beads with 0.8%, 1.2% and 1.6% (w/v) alginate in 102 and 153 mM calcium bath. C_t and C_0 respectively represent the solute concentration of medium at times t and 0. The Glucose concentration of solution for the 0.8%(w/v) alginate beads decreased rapidly, compared to 1.2% and 1.6% samples, however all samples reached their equilibrium value in about 150 min. Furthermore, one could see the regular diffusion profile for Alg(1.2-102), (1.2-153) and (1.6-102) capsules among which, the two samples with 1.2% initial alginate concentration showed about 0.87-0.90% diffusivity for glucose, may indicate appropriate formed cross link network in these specimens. For albumin molecule diffusion as a large molecule model, decrease in BSA concentration in the supernatant was not detected after 200 min, except for the 0.8% alginate beads. Considering SEM images, the observed fluctuations in albumin concentrations for Alg (0.8%) specimens are due to the presence of very large pores and lack of well integrated holes.

Mechanical stability

Mechanical stability is an important factor in preparation and injection of cell-carrier capsules, their contact with somatic tissues and their therapeutic efficiency [5, 28]. Based on results obtained from mechanical stability tests on the alginate capsules, it can be concluded that except for the Alg (0.8-102), the remaining samples showed appropriate stabilities against applied shear forces. Alg (0.8-153) sample was slightly weaker than other samples (containing 1.2% and 1.6% alginate); however, it still showed an acceptable stability where about 90% of capsules in this sample maintained their initial spherical shape after 30 hours examination (Table 2). In summary, a ccording to the results obtained from viability, permeability, morphology and mechanical analyses, it can be concluded that the sample with 1.2% alginate in 153 mM $CaCl_2$ bath is the best candidate for being used in MSCs encapsulation; therefore, the viability of MSCs inside the hydrogel matrix was confirmed by fluorescent microscopy images for Alg(1.2-153).

Fluorescent microscopy of AO/PI stained MSCs

We observed the viability of encapsulated cells within the alginate beads after 3 and 7 days encapsulation using fluorescent dyes (propidium iodide and acridine orange). Dead cells were stained red by propidium iodide that bound to exposed DNA where the integrity of cell membrane was interrupted. On the other hand, live cells were identified by the fluorescence of acridine orange which was able to enter the cells and bind to the DNA[29, 30].

Fig. 5 shows the 3D fluorescent images of the encapsulated MSCs within the alginate capsules just after encapsulation (A), after 3 (B) and 7 (C) days encapsulation. The majority of cells was alive and appeared as green spots in each image. In addition, there were not significant differences in the distribution of live and dead cells during this span. These results demonstrate that the viability remained constant and nutrient transport throughout this selected capsule was adequate.

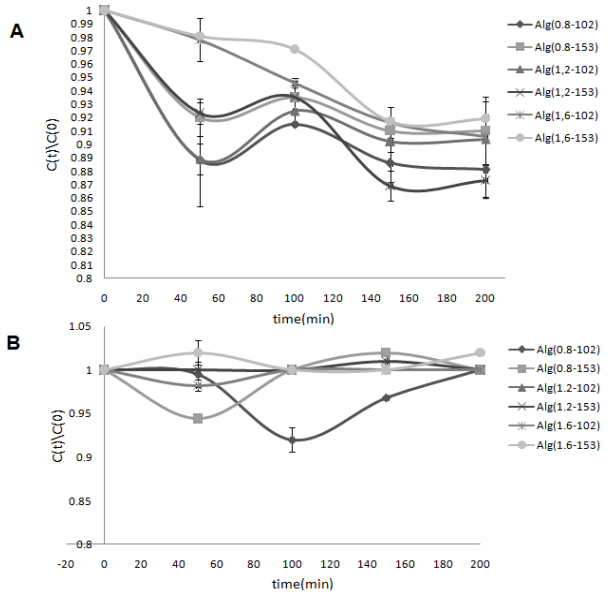

Fig.4. Diffusion profile of the capsules. (A) Glucose diffusion profile; (B) Albumin diffusion profile.

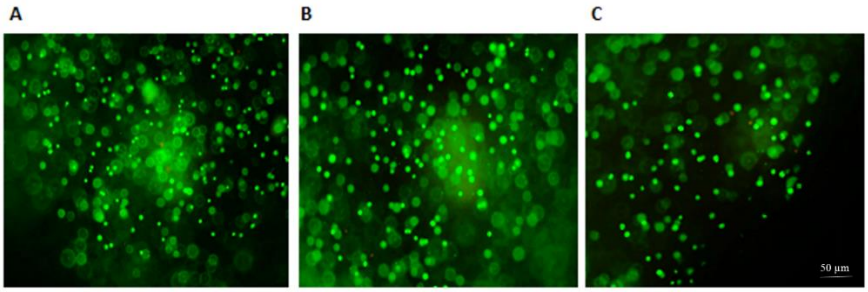

Fig. 5. Fluorescent microscopy of dead and live cells encapsulated in Alg(1.2-153) sample with 1.2% w/v alginate and 153 mM $CaCl_2$ at day 0 (A), day 3 (B) and day 7 (C) (Original magnification ×10).

Conclusion

In the present study, we used alginate capsules to provide three dimensional hydrogel networks for encapsulation of mesenchymal stem cells. Various concentrations of alginate and calcium chloride bath were examined to obtain the optimized membrane characteristics in terms of cell viability, matrix permeability and mechanical stability.

Viability tests including trypan blue staining and Sub-G1 analysis showed that Alg(1.2-153) sample exhibited the best results, which was also confirmed by fluorescent microscopy. Diffusion experiment and SEM images indicated using a proper selection of alginate concentration and $CaCl_2$ solution, an interconnected structure can be produced and these two factors play important roles in membrane porosity. Overall, it can be suggested that the sample with 1.2% alginate in 153mM $CaCl_2$ provides suitable structural integrity, appropriate cross link density, proper diffusion of oxygen and growth factors, preventing interference of the immune system molecules as well as sufficient mechanical strength for cell encapsulation purposes.

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Conflict of interest

There is no conflict of interest.

References

1.Vija, L., D. Farge, J.F. Gautier, P. Vexiau, C. Dumitrache, A. Bourgarit, F. Verrecchia, and J. Larghero. (2009) Mesenchymal stem cells: Stem cell therapy perspectives for type 1 diabetes. *Diabetes Metab.*35: 85-93.

2. Robertson, R.P. (2004) Islet Transplantation as a Treatment for Diabetes - A Work in Progress. *N. Engl. J. Med.* 350: 694-705.

3. Black, S.P., I. Constantinidis, H. Cui, C. Tucker-Burden, C.J. Weber, and S.A. Safley (2006) Immune responses to an encapsulated allogeneic islet β-cell line in diabetic NOD mice. *Biochem.Biophys. Res. Commun.* 340: 236–243.

4. Jones, P.M., M.L. Courtney, C.J. Burns, and S.J. Persaud (2008) Cell-based treatments for diabetes. *Drug Discov.Today* 13: 888-893.

5. Hernández, R.M., G. Orive, A. Murua, and J.L. Pedraz (2010) Microcapsules and microcarriers for in situ cell delivery.*Adv. Drug Deliv.Rev*. 62: 711-730.

6. Wang, T., I. Lacík, M. Brissová, A.V. Anilkumar, A. Prokop, D. Hunkeler, R. Green, K. Shahrokhi, and A.C. Powers (1997) An encapsulation system for the immunoisolation of pancreatic islets. *Nat. Biotechnol.* 15: 358 – 362.

7. Teramura, Y., H. Iwata (2009) Islet encapsulation with living cells for improvement of biocompatibility. *Biomaterials* 30: 2270–2275.

8. Orive, G., A.R. Gascón, R.M. Hernández, M.Igartua, and J.L. Pedraz (2003) Cell microencapsulation technology for biomedical purposes: novel insights and challenges. *Trends Pharmacol. Sci.*24: 207-210.

9. Lu, H.F., E.D. Targonsky, M.B. Wheeler, and Y.L. Cheng (2007) Thermally Induced Gelable Polymer Networks for Living Cell Encapsulation. *Biotechnol.Bioeng*. 96: 146-155.

10. Nafea, E.H., A. Marson, L.A. Poole-Warren, and P.J. Martens (2011) Immunoisolating semi-permeable membranes for cell encapsulation: Focus on hydrogels.*J. Control Release* V. 154: 110-122.

11. Limbert, C., and J. Seufert (2009) *In vitro* (re)programming of human bone marrow stromal cells toward insulin-producing phenotypes. *Pediatr. Diabetes* 10: 413–419.

12. Ben-Ami, E., S. Berrih-Aknin, and A. Miller (2011) Mesenchymal stem cells as an immunomodulatory therapeutic strategy for autoimmune diseases. *Autoimmunity Rev.* 10: 410–415.

13. Krampera, M., A. Pasini, G. Pizzolo, L. Cosmi, S.Romagnani, and F. Annunziato(2006) Regenerative and immunomodulatory potential of mesenchymal stem cells.*Curr.Opin.Pharmacol*. 6: 435-441.

14. Tyndall, A., U.A. Walker, A. Cope, F. Dazzi, C. De Bari, W. Fibbe, S.Guiducci, C. Jorgensen, K. Le Blanc, F. Luyten, D. McGonagle, I. Martin, C. Bocelli-Tyndall, G. Pennesi, V. Pistoia, A. Uccelli, N. Wulffraat, and M. Feldmann (2007) Immunomodulatory properties of mesenchymal stem cells: a review based on an interdisciplinary meeting held at the Kennedy Institute of Rheumatology Division, London, UK, 31 October 2005. *Arthritis. Res. Ther*. 9: 301-310.

15. Batorsky, A., J. Liao, A.W. Lund, G.E. Plopper, and P.J. Stegemann (2005) Encapsulation of Adult Human Mesenchymal Stem Cells Within Collagen-Agarose Microenvironments.*Biotechnol.Bioeng.* 92: 492-500.

16. Jongpaiboonkit, L., W.J. King, G.E. Lyons, A.L. Paguirigan, J.W. Warrick, D.J. Beebe, and W.L. Murphy (2008) An adaptable hydrogel array format for 3-dimensional cell culture and analysis. *Biomaterials.* 29: 3346-3356.

17. Hunt, N.C., and L.M. Grover (2010) Cell encapsulation using biopolymer gels for regenerative medicine .*Biotechnol.Lett.* 32: 733-742.

18. Zhang, X., Y. Xie, C.G. Koh, and L.J. Lee (2009) A novel 3-D model for cell culture and tissue engineering. *Biomed.Microdevices*11: 795–799.

19. Orive, G., S.K. Tam, J.L. Pedraz, and J.P. Halle´ (2006) Biocompatibility of alginate–poly-L-lysine microcapsules for cell therapy. *Biomaterials* 27: 3691–3700.

20. Huang, K.S., M.K. Liu, C.H. Wu, Y.T. Yen, and Y.C. Lin (2007) Calcium alginate microcapsule generation on a microfluidic system fabricated using the optical disk process .*J. Micromech. Microeng.*17: 1428-1434.

21. Nunamaker, E.A., K.E. Purcell, R.D. Kipke (2007) In vivo stability and biocompatibility of implanted calcium alginate disks. *J. Biomed. Mater. Res. A.* 83: 1128-1137.

22. Tu, J., S. Bolla, J. Barr, J. Miedema, X. Li, and B. Jasti (2005) Alginate microparticles prepared by spray–coagulation method: Preparation, drug loading and release characterization". *Int. J. Pharm.* 303: 171–181.

23. LeRoux, M.A., F. Guilak, and L.A. Setton (1999) Compressive and shear properties of alginate gel: Effects of sodium ions and alginate concentration. *J. Biomed. Mater. Res.* 47: 46-53.

24. Wang, N., G. Adams, L. Buttery, F.H. Falcone, and S. Stolnik (2009) Alginate encapsulation technology supports embryonic stem cells differentiation into insulin-producing cells. J. Biotechnol. 144: 304-312.

25. Bernacki SH, Wall ME, Loboa EG (2008) Isolation of human mesenchymal stem cells from bone and adipose tissue. *Methods Cell Biol.* 86: 257–258.

26. Zhu, J.H., X.W. Wang, S. Ng, C.H. Quek, H.T. Ho, X.J. Lao, and H. Yu (2005) Encapsulating live cells with water-soluble chitosan in physiological conditions. *J. Biotechnol.* V. 117: 355-365.

27. Nafea, E.H., A. Marson, L.A. Poole-Warren, and P.J. Martens (2011) Immunoisolating semi-permeable membranes for cell encapsulation: Focus on hydrogels.*J. Control Release.* V. 154: 110-122.

28. Banerjee, A., M. Arha, S. Choudhary, R.S. Ashton , S.R. Bhatia , D.V. Schaffer , and R.S. Kane (2009) The influence of hydrogel modulus on the proliferation and differentiation of encapsulated neural stem cells". *Biomaterials.* 30: 4695-4699.

29. Cheah, P.S., K.H. Ling , K.A. Crouse , and R. Rosli (2007) Characterization of the S-benzyldithiocarbazate effects on cell proliferation and oncogene expression in human breast cancer cells. *J. Med. Biol. Sci.* 1: 1-7.

30. Hao, T., N. Wen, J.K. Cao, H.B. Wang, S.H. Lü, T. Liu, Q.X. Lin, C.M. Duan, and C.Y. Wang. (2010) The support of matrix accumulation and the promotion of sheep articular cartilage defects repair in vivo by chitosan hydrogels. *Osteoarthritis Cartilage.*18: 257-265.