Co-microencapsulation Technology to Improve Cell Viability

Xiaolin Tang[1], Yuesheng Tong[1], Shibin Wang[1,2]*, Aizheng Chen[1,2], Wenguo Wu[1,2] and Yuangang Liu[1,2]*

[1]College of Chemical Engineering, Huaqiao University, Xiamen - 361 021, China.
[2]Institute of Biomaterials and Tissue Engineering, Huaqiao University, Xiamen - 361 021, China.

Microencapsulation technology is a useful tool for cell cultivation and metabolite production of bacteria. In this paper, Alg-Sr^{2+} microcapsules were used to co-culture two kinds of cells to enhance cell viability. Alginate and strontium chloride are introduced to prepare microcapsules for their resistance to immune rejection and perfect biocompatibility. Four groups including HepG2, co-cultured 3T3/HepG2, microencapsulated HepG2 and co-microencapsulated 3T3/HepG2 were prepared to determine the cell viability, albumin secretion and urea synthesis. MTT assay showed the relative growth rate (RGR) of co-cultured 3T3/HepG2 was the highest, co-microencapsulated group was the second and microencapsulated HepG2 was the lowest. Additionally, significant difference was shown on albumin secretion among the four groups. Co-microencapsulated 3T3/HepG2 had the notable albumin secretion. On the contrary, HepG2 group increased slowly and even decreased after five days. Besides, co-cultured or co-microencapsulated cells synthesize more urea than other two groups. The results indicate that 3T3 cells and Alg-Sr^{2+} microencapsulated environment can promote the viability of hepatocytes, which also provides an alternative way for the future bacterium cultivation to increase the bacterium yield or metabolite production.

Key words: Alg-Sr^{2+} microcapsules; co-culture; HepG2; 3T3

Microencapsulation technology has been used in bacterium encapsulation[1-3]. In this paper, the possibility of Alg-Sr^{2+} microcapsules for co-microencapsulation of two kinds of cells was tested, which were used for treating liver disease.

Liver disease is a severe health problem, which affects millions of people in the world. Nowadays, transplantation is the most effective treatment for acute liver failure (ALF) or end-stage chronic liver injury[4]. However, there are some restrictions of transplantation, such as high cost, well-matched donor livers. Liver tissue engineering may be the promising therapy for liver diseases, and hepatocytes are the only cell source for liver tissue engineering. But hepatocytes are apt to lose their biological characteristics and long-term culture abilities *in vivo*[5,6] which may be partially because cell and cell or cell and extracellular matrix (ECM) gradually lose connection[7,8]. Various ways have been used to overcome these drawbacks, including culturing cells in a sandwich configuration[9] or in a more suitable culture medium[10] which only extends the lifespan to 1 month, three-dimensional culturing which has limited applications, microencapsulated culturing[11] and co-culturing.

* To whom all correspondence should be addressed.
Tel.: +86-592-6162326, Fax: +86-592-6162326;
E-mail: sbwang@hqu.edu.cn; ygliu@hqu.edu.cn

In recent years, researchers found that co-culturing hepatocytes with non-parenchymal or non-liver cells could simulate the environment of liver cells *in vivo*. It could effectively extend cell life, promote their proliferation and differentiation, help maintain specific bile duct structure, facilitate the formation of gap junctions, enhance synthesis and secretion, and maintain the detoxification function of liver cells[5]. These non-parenchymal or non-liver cells include Kupffer cells, sinusoidal endothelial cells, stellate cells, bone marrow cells, islet cells, fibroblast cells, etc.[12].

Although quite a lot work has been done on co-culturing hepatocytes with other cells, cells transplantation often causes immunologic rejection which could be avoided by cell microencapsulation. Cell microencapsulation allows nutrients and oxygen entering and waste or other products existing[13,14]. Alginate is the most widely used material on microencapsulation due to its good biocompatibility, suitable semi-permeability, strong mechanical stability and lower immunogenicity[15]. Microcapsule films have available cell attachment matrix which makes liver cells contact with each other and form the three-dimensional structure. Membrane of microcapsule is semipermeable which would stop macromolecules, antibodies and other immunologic moieties from entering and contacting the microencapsulated cells then destroying them as invaders[16,17]. There are various advantages of the liver cell microencapsulation technology, which showing great potential for the treatment of liver failure in transplanted liver cells[18]. The recent majority of microencapsulated hepatocytes based on bio-artificial liver has conducted many clinical trials[4], and achieved encouraging efficacy. In previous work, we[19] reported that among the four crosslinking agents (Ba^{2+}, Sr^{2+}, Ca^{2+}, Zn^{2+}) microcapsules, microcapsules prepared by crosslinking agent Sr^{2+} present smaller particle size, lower expansion rate, smaller film thickness, and more desired biocompatibility of cell and tissue with well optical morphology.

And three kinds of liver cells are used for microencapsulation to obtain bioartificial liver (BAL), such as human, other mammalian liver cells and immortalized liver cells[16,20]. Immortalized liver cells have the advantages of readily culturing, contacting cells with no inhibition, long-term cryopreservation, and so on. Currently, liver cell lines for BAL are C3A, HepG2, HH01, HH02, HHY41, OUMS-29, Hep Z and NKNT-3.

The purpose of this paper is to combine the advantages of co-culture and microencapsulation. In order to study the co-cultured hepatocytes with other cells in microcapsules, we selected Sr^{2+} as an ion crosslinking agent of microencapsulated cells, embedded HepG2 cells and BALB/3T3 (3T3) to obtain Alg-Sr^{2+} microcapsules. To provide experimental evidence for hepatocytes co-microencapsulation and its applications, we compared the viability, albumin secretion and urea synthesis content of HepG2, co-cultured 3T3/HepG2, microencapsulated HepG2 and co-microencapsulated 3T3/HepG2 cells.

MATERIALS AND METHODS

Materials

Sodium alginate was purchased from Sigma Co., Ltd. (USA). Chitosan was purchased from Golden-Shell Biochemical Co., Ltd. (Zhejiang, China). Anhydrous ethanol was bought from Chenghai Chemical Industry Co., Ltd. (Shanghai, China). Albumin detection kit and urea detection kit were purchased from BIOSINO (China). Sodium chloride was obtained from Xilong chemical plant (Shantou, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibico Co., Ltd. (USA). Fetal Bovine Serum and Penicillin-Streptomycin Solution were bought from Hyclone. Human liver hepatocellular carcinoma cell line HepG2 and 3T3 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences.

Cells culture and determination of cell growth curves of HepG2 and 3T3

HepG2 and 3T3 were cultured with medium (DMEM supplemented with $NaHCO_3$ 3.7 g/L; 100 U/ml penicillin and 100 μg/ml streptomycin; and 10% (v/v) FBS). Cells were digested with trypsin to obtain cell suspension when they were in exponential phase with 70-80% confluence, stained with trypan blue and counted. The cell suspension was diluted to a final concentration of 1×10^4 cells/mL, and then inoculated on 24-well plates, respectively. All cells and plates were cultured in humidified atmosphere with 5% CO_2 incubator at 37°C and medium was changed every

other day. Cell numbers were counted triplicate every 24h and lasted for ten days. Finally, the mean values were calculated and the corresponding cell growth curves were drawn. Population doubling time (PDT) was calculated using following formula (1), where t_2, t_1, x_2 and x_1 denote the final time, initial time, final cell number, and initial cell number, respectively.

$$PDT = (t_2\text{-}t_1)/[3.322 \times (lgx_2\text{-}lgx_1)] \quad ...(1)$$

Preparation of microencapsulated HepG2 and co-microencapsulated 3T3/HepG2 cells

Cell-encapsulated microcapsules were prepared using an electrostatic droplet generation method as described previously[21, 22]. Briefly, Cells in exponential phase were digested by trypsin. HepG2 cell concentration was adjusted to 1×10^6 cells/mL or 8.89×10^5 cells/mL and 1.11×10^5 cells/mL for 3T3 as needed, respectively. For co-microencapsulation, the two kinds of cell suspensions were mixed in equal proportion to guarantee the amount of HepG2: 3T3 was 8:1. A suspension of HepG2 cells or a mixture of 3T3/HepG2 cells: 1.5% (w/v) sterile filtered alginate solution was 1:4. Then the suspension was injected to a plate with 10mM strontium chloride with a syringe. And microcapsules were formed through the combination of alginate and Sr^{2+} [19]. A membrane around microcapsules was shaped by adding 0.5% chitosan (w/v). After being liquefied by 55 mM sodium citrate, the microcapsules were cultured in 5% CO_2 incubator at 37°C.

The morphology and MTT assay of cells.

The microencapsulated cells were placed under an inverted microscope to observe the cells morphology of microcapsules after they were prepared. MTT assay was used to assess the cell viability or metabolic activity. The experiment was divided into four groups: HepG2, co-cultured 3T3/HepG2, microencapsulated HepG2 and co-microencapsulated 3T3/HepG2 group. Cells were seeded in 96-well plates at 2.5×10^4 cells/mL in 200 µL/well and medium was set as control. Medium was removed, another 100 µL/well medium and 20 µL/well MTT was added after incubation for 4, 24, 48, and 72 h, respectively. Then cells were incubated for a further 4 h following by adding DMSO 150 µL/well for 15 min to dissolve the formazan crystals. The absorbance value at 550 (670) nm of each well was measured by the enzyme-linked immunosorbent assay (ELISA) reader[23, 24].

The Relative growth rate of cells was determined using the following formula.

$$RGR = (OD_{55}0\text{-}OD_{670})sample/(OD_{550}\text{-}OD_{670})_{control} \times 100\,(\%) \quad ....(2)$$

Determination of albumin and urea in microencapsulated cells.

Metabolic activities of the four groups including albumin and urea content were characterized according to the protocols of the Albumin Detection Kit and Urea Detection Kit supplied by the companies.

Statistical analysis

The results are given as means ± standard deviation (SD) values (n = 3).

RESULTS AND DISCUSSION

Growth curves of HepG2 and 3T3 cells

HepG2 and 3T3 both experienced a typical lag phase, exponential phase and stationary phase after being inoculated (Fig. 1a and b). Cells went into exponential phase after culturing for 24 h, and the duration time was 2-6 d which was the appropriate time for sampling. The PDT of HepG2 and 3T3 were 24.4h and 36.2 h, respectively, which showed that HepG2 grew more quickly than 3T3. The concentration of HepG2 cells was up to 1.9×10^5/mL when it entered plateau phase, while the concentration of 3T3 cells only reached 1.6×10^5/mL.

Morphology of cells and microcapsules

Figure 2 revealed that majority of HepG2 cells showed triangle or spindle morphology. Moreover, small cytoplasm morphology and aggregation growth without obvious borders were also observed (Fig. 2a). However, most 3T3 cells were polygon and their cytoplasm form was abundant. Also, the nucleus was clearly visible, and the cells could fully start to expand on culture plates (Fig. 2b). Once the two cells cultured together, HepG2 cells exhibited a more distinct cell borders which just agree with the finding of the reference [6] (Fig. 2c).

Electrostatic droplet generation method was used to produce microencapsulated HepG2 and co-microencapsulated 3T3/HepG2. HepG2 and 3T3/HepG2 cells were successfully encapsulated (Fig. 3a and b) and distributed evenly in microcapsules.
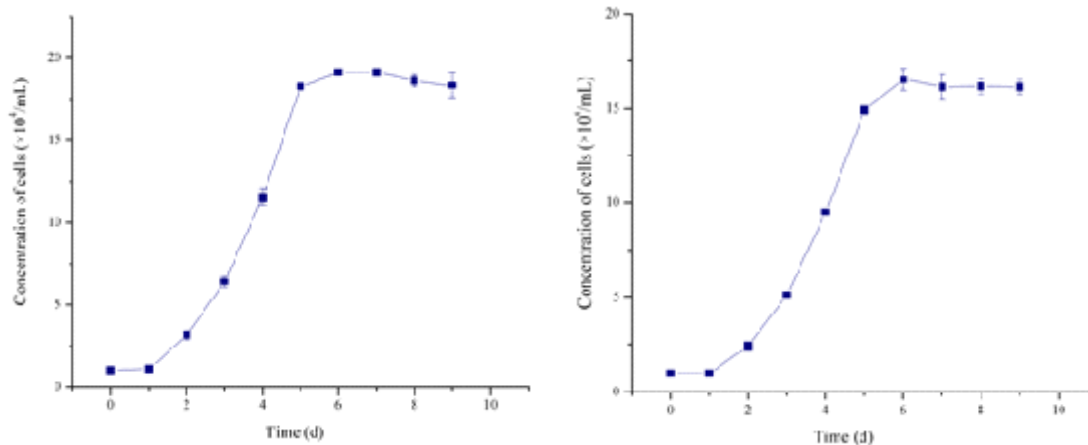
Fig. 1. Growth curves of HepG2 (a) and 3T3 (b)

Evaluation of cell viability using MTT assay

Fig. 4 showed MTT assay results of HepG2, 3T3/HepG2, microencapsulated HepG2 and microencapsulated 3T3/HepG2. As shown in Fig. 4, cells in four groups had the similar OD at 4 h which demonstrated cells in microcapsules were not affected by microcapsule preparation process. The cell viability of 3T3/HepG2 was greater than HepG2 group at 24 h. And co-microencapsulated 3T3/HepG2 showed higher activity than

microencapsulated HepG2 while was lower than co-cultured 3T3/HepG2. The viability of co-microencapsulated 3T3/HepG2 and co-cultured 3T3/HepG2 cells were increased more quickly than the other two groups in the following two days. These results showed that the internal environment from microcapsules would affect the activity of cells in the first two days and cells in co-microencapsulated became to proliferate quickly in the third day. Cells from co-cultured and co-
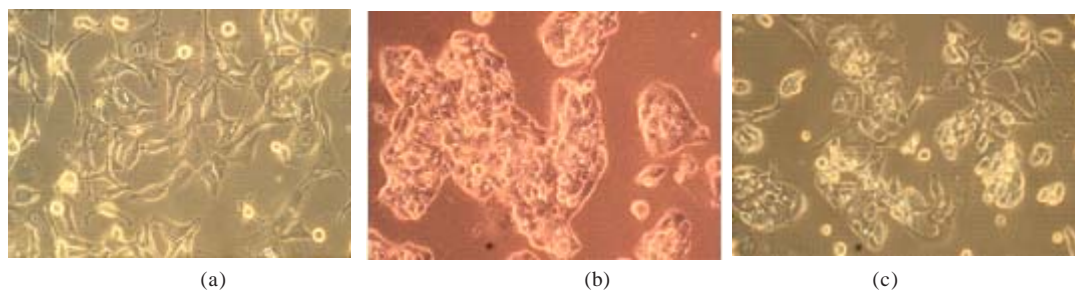

Fig. 2. Light micrograph of (a) 3T3 cells; (b) HepG2 cells and (c) 3T3/HepG2 cells cultured for 24 h ($\times$ 200 magnifications)
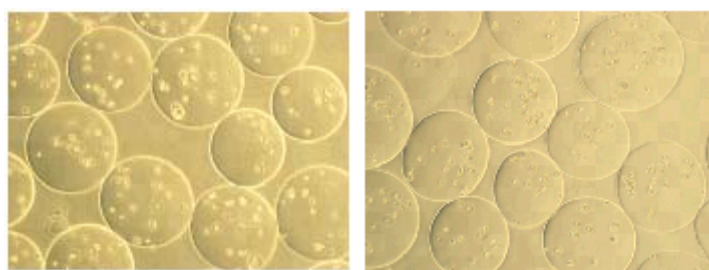

Fig. 3. Light micrograph of microencapsulated (a) HepG2 and (b) 3T3/HepG2 cells cultured for 24 h

microencapsulated 3T3/HepG2 had higher viability than HepG2 and microencapsulated HepG2, respectively, which indicated that co-cultured groups could more fully imitate liver cells in vivo survival environment and promote the proliferation and differentiation of liver cells in accordance with the finding of the reference[13].

HepG2 cells are prone to lose their functions during *in vitro* culture, which could be maintained by co-culturing HepG2 with non-parenchymal cells[25]. Obviously, co-cultured 3T3/HepG2 and co-microencapsulated 3T3/HepG2 had

notable relative growth rate (RGR) among groups (Fig. 5). And co-cultured 3T3/HepG2 was remarkable higher than the HepG2 group. Also RGR of co-microencapsulated 3T3/HepG2 was enhanced as time went and was slightly lower than co-cultured one while the microencapsulated HepG2 group was reduced. However, cells are often confronted with graft rejection for immune mechanisms. And cell encapsulation becomes the main solution. So model of co-microencapsulated 3T3/HepG2 has great potential for liver transplantation.
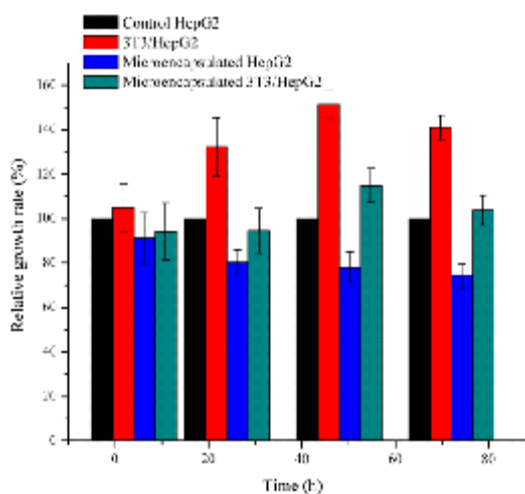

Fig. 4. MTT assay

Fig. 5. Relative growth rate (RGR) of cells.

Detection the content of albumin secretion and urea synthesis

The metabolic properties of hepatocytes were also investigated by detecting the albumin and urea content. On the first day, content of albumin was approximately at the same level which was 0.8 mg/mL (Fig. 6). Co-microencapsulated and co-cultured 3T3/HepG2 showed markedly increasing in albumin secretion especially the former one during 2-5 days. After 6 days, the amount of albumin in HepG2 group began to decrease, while the other three groups almost maintained the original level. Co-microencapsulated 3T3/HepG2 had the maximum albumin secretion in the seven days which was much higher than other groups.

Trends in the urea synthesis were slightly different. The co-cultured 3T3/HepG2 was similar with co-microencapsulated 3T3/HepG2 and the

other two groups increased almost the same (Fig. 7). In addition, synthesis rate of microencapsulated HepG2 was basically unchanged and HepG2 started to decline on day five. It is notable that co-cultured 3T3/HepG2 maintained the higher urea synthesis in the first five days and stayed lower in the next two days than co-microencapsulated group. It appears that 3T3 could promote the proliferation and enhance the metabolism of HepG2 to improve albumin and urea synthesis. Moreover, microcapsules prepared by alginate and Sr^{2+} have outstanding biocompatibility. So microcapsules can fully simulate the 3D survival environment of liver cells *in vivo* and improve albumin synthesis and secretion of hepatocytes while urea synthesis of microencapsulated hepatocytes was less affected.
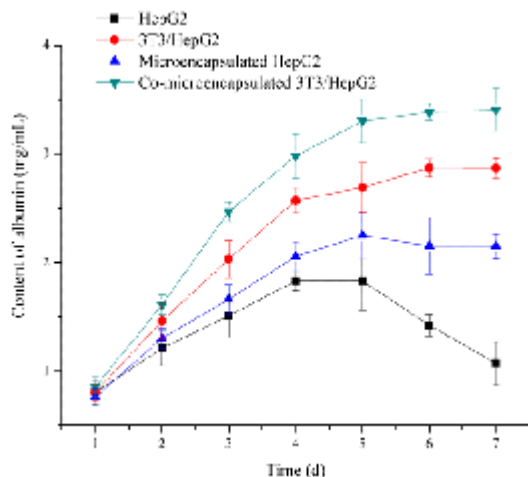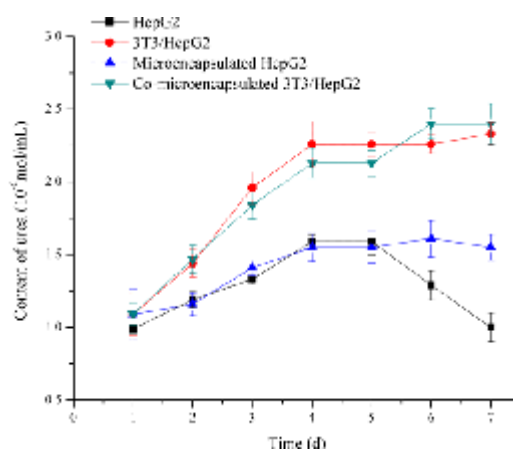
Fig. 6. Albumin secretion

Fig. 7. Urea synthesis

CONCLUSIONS

A widely used material alginate was used to prepare microcapsules with strontium chloride. And 3T3 cells were chosen to co-microencapsulate with HepG2 cells. Our results show that HepG2 cells have better RGR when co-culture or co-microencapsulate with 3T3 cells. Also, the albumin and urea synthesis are greater than the other two groups. The present result suggests that 3T3 cells can enhance the proliferation and differentiation of HepG2 cells. Moreover, the microencapsulated environment fully simulates the 3D survival environment of liver cells *in vivo* and improves albumin synthesis and secretion of hepatocytes while urea synthesis of microencapsulated hepatocytes was less affected. However, the effect mechanism of co-cultured or co-microencapsulated should be further investigated so that liver engineering have the potential for broad successful application. Our research also suggests that co-microencapsulation may be a useful tool for the future bacterium cultivation to improve the bacterium yield or metabolite production.

ACKNOWLEDGMENTS

Conflict of Interest

The authors declare no conflict of interest.

REFERENCES

1. Park, J.W., Lee, S.W., Balaraju, K., et al. Chili-Pepper Protection from Phytophthora capsici and Pectobacterium carotovorum SCC1 by Encapsulated Paromomycin Derived from Streptomyces sp AMG-P1. *J. Pure Appl. Microbio.,* 2012; **6**(4): 1517-1522.

2. Semyonov, D., Ramon, O., Kaplun, Z., et al. Microencapsulation of Lactobacillus paracasei by spray freeze drying. *Food Res. Int.*, 2010; **43**(1): 193-202.

3. Yu, C., Han, D., Wang, W. Microencapsulation of Gram-Negative Bacteria by Spray Drying. *Int. J. Food Eng.,* 2010; **6**(2): 1-13.

4. Demetrio,u A.A., Brown, R.S., Busuttil, R.W., et al. Prospective, Randomized, Multicenter, Controlled Trial of a Bioartificial Liver in Treating Acute Liver Failure. *Ann. Surg.*, 2004; **239**(5): 660-670.

5. Zhou, M., Zhao, F., Li, J., et al. Long-term maintenance of human fetal hepatocytes and prolonged susceptibility to HBV infection by

co-culture with non-parenchymal cells. *J. Virol. Methods,* 2014; **195**: 185-193.

6. Kim, K., Ohashi, K., Utoh, R., et al. Preserved liver-specific functions of hepatocytes in 3D co-culture with endothelial cell sheets. *Biomaterials*, 2012; **33**(5): 1406-1413.

7. Ben-Ze'ev, A., Robinson, G.S., Bucher, N., et al. Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. *Proc. Natl. Acad. Sci.*, 1988; **85**(7): 2161-2165.

8. Oda, H., Yoshida, Y., Kawamura, A., et al. Cell shape, cell-cell contact, cell-extracellular matrix contact and cell polarity are all required for the maximum induction of CYP2B1 and CYP2B2 gene expression by phenobarbital in adult rat cultured hepatocytes. *Biochem. Pharmacol.,* 2008; **75**(5): 1209-1217.

9. Dunn, J., Yarmush, M., Koebe, H., et al. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *The FASEB journal,* 1989; **3**(2): 174-177.

10. Ijima, H., Kakeya, Y., Yokonuma, T., et al. Composition of culture medium is more important than co-culture with hepatic non-parenchymal cells in albumin production activity of primary rat hepatocytes, and the effect was enhanced by hepatocytes spheroid culture in collagen gel. *Biochem. Eng. J.*, 2009; **45**(3): 226-231.

11. Jang, J.H., Castano, O., Kim, H.W. Electrospun materials as potential platforms for bone tissue engineering. *Adv. Drug Deliv. Rev.*, 2009; **61**(12): 1065-1083.

12. Malik, R., Selden, C., Hodgson, H. The role of non-parenchymal cells in liver growth, *Semin. Cell Dev. Biol.,* 2002; **13**: 425-431.

13. Haque T., Chen, H., Ouyang, W., et al. *In vitro* study of alginate-chitosan microcapsules: an alternative to liver cell transplants for the treatment of liver failure. *Biotechnol. Lett.,* 2005; **27**(5): 317-322.

14. Ming Swi Chang, T. Artificial cells, encapsulation, and immobilization. Ann. N. Y. Acad. Sci., 1999; **875**(1): 71-83.

15. Sun, J., Tan, H. Alginate-Based Biomaterials for Regenerative Medicine Applications. Materials (Basel), 2013; **6**(4): 1285-1309.

16. Orive, G., Maria, Hernández, R., Rodr1guez Gascón, A., et al. History, challenges and perspectives of cell microencapsulation. *Trends Biotechnol.,* 2004; **22**(2): 87-92.

17. Xie, J., Wang, C.H. Electrospray in the dripping mode for cell microencapsulation. *J. Colloid Interface Sci*, 2007; **312**(2): 247-255.

18. Dhawan, A., Puppi, J., Hughes, R.D., et al. Human hepatocyte transplantation: current experience and future challenges. *Nat. Rev. Gastroenterol Hepatol.,* 2010; **7**(5): 288-298.

19. Liu, Y., Tong, Y., Wang, S., et al. Influence of different divalent metal ions on the properties of alginate microcapsules and microencapsulated cells. *J. Sol-gel Sci. Techn.,* 2013; **67**(1): 66-76.

20. Sauer, I., Kardassis, D., Zeillinger, K., et al. Clinical extracorporeal hybrid liver support– phase I study with primary porcine liver cells. *Xenotransplantation*, 2003; **10**(5): 460-469.

21. Liu Y., He P., Wang S., et al. Preparation of embolic NEMs loading capecitabine. *J. Mater. Sci. Mater. Med.*, 2013; **24**(1): 155-160.

22. Fukui, Y., Maruyama, T., Iwamatsu, Y., et al. Preparation of monodispersed polyelectrolyte microcapsules with high encapsulation efficiency by an electrospray technique. Colloids Surf. A. *Physicochem. Eng. Asp.*, 2010; **370**(1-3): 28-34.

23. Stockert, J.C., Blazquez-Castro, A., Canete, M., et al. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta Histochem.,* 2012; **114**(8): 785-796.

24. Holder, A.L., Goth-Goldstein, R., Lucas, D., et al. Particle-induced artifacts in the MTT and LDH viability assays. Chem. Res. Toxicol., 2012; 25(9): 1885-1892.

25. Yagi, K., Sumiyoshi, N., Yamada, C., et al. *In vitro* maintenance of liver function in hierarchical co-culture of hepatocytes and non-parenchymal liver cells. *J. Fire Sci.*, 1995; **80**(6): 575-579.