Polymer (Korea) Vol. 15, No. 4, pp 474－480 (1991)

알긴을 함유한 혼합 겔 비드 내에서 동물 세포의 고정화

이 진 호 · 박 종 우 · 이 해 방
한국화학연구소 고분자제3연구실
(1991년 4월 29일 접수)

Immobilization of Animal Cells in the Gel Beads of Alginate-Functional Polymer Mixture

Jin Ho Lee, Jong Woo Park, and Hai Bang Lee
Polymer Research Laboratory 3, Korea Research Institute of Chemical Technology, P. O. Box 9, Daedeog Danji, Dae Jeon, Korea 305-606
(Received April 29, 1991)

요 약 : 알긴과 여러가지 관능기를 갖는 고분자들의 혼합 겔 비드 내에 동물세포인 CHO 세포와 BHK 세포를 고정화하여 이들 세포가 비드 내에서 살아있는 정도와 증식 경향을 고찰하였다. 혼합 겔 비드 제조를 위해 사용한 고분자들로서는 -OH기를 갖는 폴리비닐알코올, -COOH기를 갖는 폴리아크릴산, $-NH_2$기를 갖는 폴리아크릴 아미드 등이었다. 혼합 겔 비드 내에 고정화된 세포들은 37 ℃, 5 % CO_2 개스 분위기하에서 페트리 디쉬 내에서 배양되었다. 일정기간 배양후 비드 내에 살아있는 세포들의 밀도는 트리판블루 염색법과 헤모사이토메터를 사용하여 산출되었다. 비드 내부의 구조와 비드 내에서 자라고 있는 세포들은 SEM과 현미경에 의해 관찰되었다. 혼합 겔 비드들 중에서는 알긴과 폴리비닐알코올로 제조된 겔 비드가 세포 생존율과 증식성에 있어 다른 것들보다 우수한 것으로 판명되었다.

Abstract : Animal cells, Chinese Hamster Ovary (CHO) and Baby Hamster Kidney (BHK) cells, were immobilized in the gel beads of alginate-functional polymer mixture to investigate the effect of the mixtures of the alginate and the polymer with different functional group on the viability and growth of the cells. The polymers used were poly(vinyl alcohol) (PVA, -OH functional group), poly(acrylic acid) (PAA, -COOH functional group), and poly(acryl amide) (PAAM, $-NH_2$ functional group). The cells immobilized in the beads were cultured in Petri dishes at 37 ℃ under 5 % CO_2 atmosphere. The viable cell densities in the beads after given time culture were measured by the trypan blue dye exclusion method using a haemocytometer. Morphologies of the beads and the cells growing in the beads were examined with a scanning electron microscope (SEM) and an inverted microscope. It was observed that the beads prepared by the alginate-poly(vinyl alcohol) mixture are effective for the immobilization of both CHO and BHK cells in terms of cell viability and density in the beads after culturing in Petri dishes.

INTRODUCTION

Cell immobilization techniques, which permitted new advances in the production of natural or recombinant biological products, have been adapted for a variety of animal cells. Immobilization of the cells by entrapment in particles or beads makes separation of the culture medium and the cells easy, and still enables the cells to grow to high concentrations. It also protects cells from environments such as shear stress, transport, or contamination sources in large-scale culture equipments. Investigations of immobilized animal cells have dealt with the production of high value molecules secreted by the natural or genetically engineered cells. They include monoclonal antibodies, vaccines, interferons, interleukins, plasminogen activators, hormones like erythropoietin, proteins, etc.[1,2] The immobilization processes often require harsh conditions such as the use of non-aqueous solvents, extremes of pH, or high temperature. Such techniques are unsuitable for the immobilization of animal cells because the animal cells are highly sensitive to toxic compounds and changes in the environment caused by chemical reactions.[3] Ideally, the immobilization techniques for live animal cells should use mild gelation conditions and a gelling material which is inert and non-toxic to the cells. Also, the techniques should allow the cells to be immobilized in a semi-permeable membrane providing cells access to nutrients and other substances necessary for viability and growth but protecting cells from substances having a high molecular weight such as antibodies, toxins or bacteria.[4,5]

Entrapment in the beads of calcium alginate gel is one of the most widely used techniques for immobilizing live animal cells because this natural polymer fulfills above mentioned all criteria.[6] The gelation of this polymer occurs under mild conditions which do not cause the cell damage. Such gel beads can easily be made by allowing droplets of sodium alginate solution to fall into a bath of calcium chloride solution.

In this study, the gel beads prepared by the mixtures of alginate and polymer with different functional group were investigated in terms of the viability and growth of the immobilized cells. The effectiveness of these gel beads was compared to that of the alginate gel.

EXPERIMENTAL

Cells and Culture

CHO (CHO-KI-BH_4, Oak Ridge National Laboratory, U. S. A.) and BHK (Green Cross Medical Co.) cell lines were used to be immobilized in the gel beads.

They were routinely cultured in tissue culture polystyrene flasks (Coring, U. S. A.) at 37 ℃ under 5 % CO_2 atmosphere. The culture media used for the CHO and BHK cells were Ham's F-12 nutrient mixture and minimum essential medium (GIBCO, U. S. A.), respectively. Both of them contained 10 % newborn calf serum, 100 unit/ml penicillin, and 100 μg/ml streptomycin. They were sterilized using a sterilization filter unit (pore size 0.2 μm, Nalge Co., U. S. A.). After monolayer culture in the culture flasks, the cells were harvested after trypsin treatment (0.05 % trypsin/0.02 % EDTA (GIBCO)) and used for the immobilization. The detailed procedures were reported in the previous papers.[7,8]

Polymers

The cells were immobilized in sodium alginate (ALG, also called algin or alginic acid sodium salt) and the mixtures of sodium alginate and polymer with different functional group. The sodium alginate (Sigma, U. S. A.) was dissolved in Dulbecco's phosphate buffered saline[7] (PBS, pH 7.2-7.3) to prepare 4 % solution.

The polymers used were poly(vinyl alcohol) (M. W. about 100,000, Aldrich, U. S. A.) with -OH functional group, poly(acrylic acid) (M. W. about 100,000, Aldrich) with -COOH functional group, and poly(acryl amide) (M. W. about 5,000,000, Aldrich) with $-NH_2$ functional group. They were also dissolved in the PBS to 4% solutions. The sodium alginate solution and each polymer solution were

uniformly mixed with the volumetric ratio of 1 : 1 and the pH's of the solutions were adjusted to 7.2-7.3 using 1 N HCl and 1 N NaOH.

Immobilization of Cells and Cultivation

The CHO and BHK cells harvested from the culture flasks were suspended in the Ham's F-12 nutrient mixture and the minimum essential medium at a concentration of 2×10^6 cells/ml, respectively. The sodium alginate solution diluted to 2 % in the PBS and the mixture solutions of 4 % sodium alginate and 4 % polymer with different functional group were sterilized in an autoclave at 121 ℃ for 15 min. Equal amount of the sodium alginate solution or the mixture solution was added to the cell suspension so as that the final alginate and the polymer concentrations in the mixture were 1 % each and the cell density was 1×10^6/ml.

Fig. 1 shows the schematic diagram of the cell immobilization and cultivation process. The mixture solution containing cells was packed in a syringe (22-gauge needle) and added dropwise into autoclave-sterilized 1.5 % $CaCl_2$ solution (in the culture medium). The gel beads produced (bead size, 2-3 mm), in which the cells were immobilized, were left for 10 min in the solution for hardening. After washing with the medium, the cell-immobilized gel beads were transferred to Petri dishes (Coring) containing the medium and then incubated at 37 ℃ under 5 % CO_2 atmosphere. During the culture, the medium was replaced by fresh medium every two days.

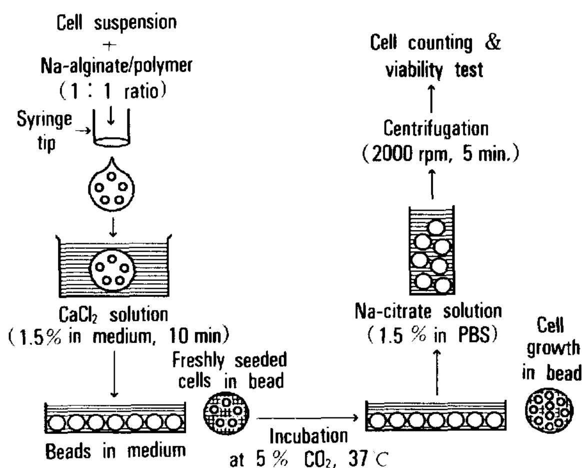


Fig. 1. Experimental scheme showing cell immobilization, cultivation, and analysis processes.

Analyses

After given time culture in the Petri dishes, some beads were taken out, washed with the PBS, and aspirated. Then, the beads were solubilized in 1.5 % sodium citrate in the PBS and centrifuged at 2000 rpm for 5 min (Fig. 1). After removing the supernatant and resuspending the cell pellet with Hank's balanced salt solution (Sigma, U. S. A.), the cell viability and number were measured by the trypan blue dye (0.4 %, Sigma) exclusion method using a haemocytometer. The dead cells were stained blue and thus distinguished from the unstained viable cells. The average of three counts was used to determine the cell viability and number.

Some beads were also taken out from the Petri dishes, washed with water, and freeze-dried, then half-sectioned with a razor blade. The morphologies of the half-sectioned beads were observed with a scanning electron microscope (SEM, JSM-840A, Jeol Co., U. S. A.). The cells immobilized and growing in the beads were directly observed using an inverted microscope (Diaphot-TMD, Nikon, Japan).

RESULTS AND DISCUSSION

CHO and BHK cells have popularly been used as model systems because they exist as reasonably stable single cells and are not unreasonably fastidious in terms of culture requirements.[9] These cells, after genetical recombination, have been cultured to obtain biological products secreted by the cells, such as erythropoietin,[10~12] interleukins,[13] and interferons.[14,15]

In this study, the CHO and BHK cells were immobilized in the gel beads of alginate-functional polymer mixture to investigate the effect of the mixtures of the alginate and the polymer with different functional group (-OH, -COOH, and $-NH_2$

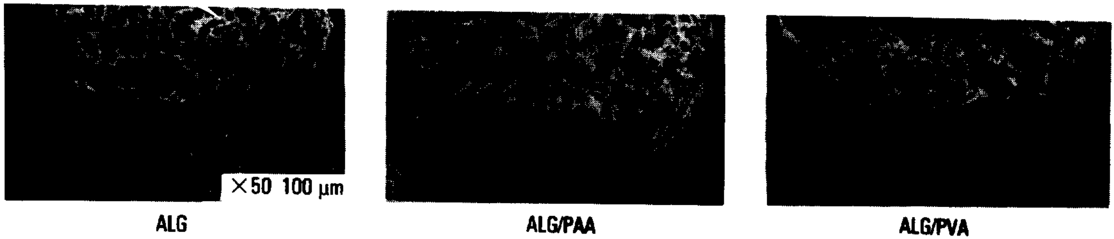


Fig. 2. SEM pictures of the gel beads prepared by alginate and alginate-functional polymer mixtures after freeze-drying and half-cutting (× 50).

functional groups) on the viability and growth of the cells. The cell-immobilized alginate beads were also used as controls.

The alginates are natural copolymers consisted of β-D-mannuronate and α-L-guluronate linked by 1→4 glycosidic linkage.[6] They have correspondingly high affinities for divalent ions such as Ca^{2+}, Sr^{2+}, and Ba^{2+}. In the solution of divalent ions, the alginates rapidly form cross-linkages and thus gels. The structure of the gels was well described elsewhere.[16] The gel surface produced has a semi-permeable characteristic.

Fig. 2 shows morphologies of the gel beads prepared by the alginate and the alginate-functional polymer mixtures. It was observed that the beads have highly porous structures. The beads prepared by the alginate-poly(vinyl alcohol) mixture showed a little different structure compared to others.

It is thought that the cells are immobilized and growing in the pores inside the bead, and the nutrients such as vitamins, minerals, amino acids, etc., oxygen, glucose as a carbon source and other substances necessary for the cell growth are supplied from the culture medium through the semi-permeable membrane of the bead surface (Fig. 3). The metabolites such as ammonia and lactate secreted by the cells immobilized in the bead, which inhibit the cell growth, are come out from the bead to the medium. The desired product produced by the cells can be obtained inside or outside the bead, depending on its molecular weight.

The CHO and BHK cells immobilized in the gel beads of the alginate or the alginate-functional po-

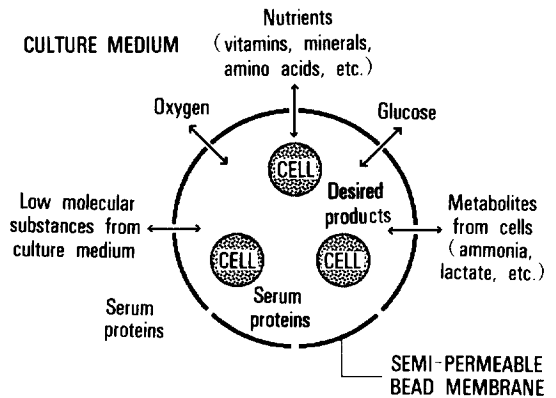


Fig. 3. Schematic diagram showing the transport of the low molecular substances between the cells in the gel bead of alginate or alginate-functional polymer mixture and culture medium.

lymer mixture were cultured in the Petri dishes. Fig. 4 shows the appearance of the BHK cells immobilized and growing in the gel beads of the alginate and the alginate-functional polymer mixture after 3 days culture in the Petri dishes. The beads in the culture medium were directly photograghed using a camera-attached inverted microscope. We could see that the cells are uniformly distributed and growing in the beads.

The viabilities of the CHO and BHK cells in the beads after given time culture were examined using a haemocytometer after the trypan blue staining (Fig. 5). Since the dead cells were stained blue, the live cells were easily distinguished from the dead ones. The viability was determined by the percentage of the number of live cells to the

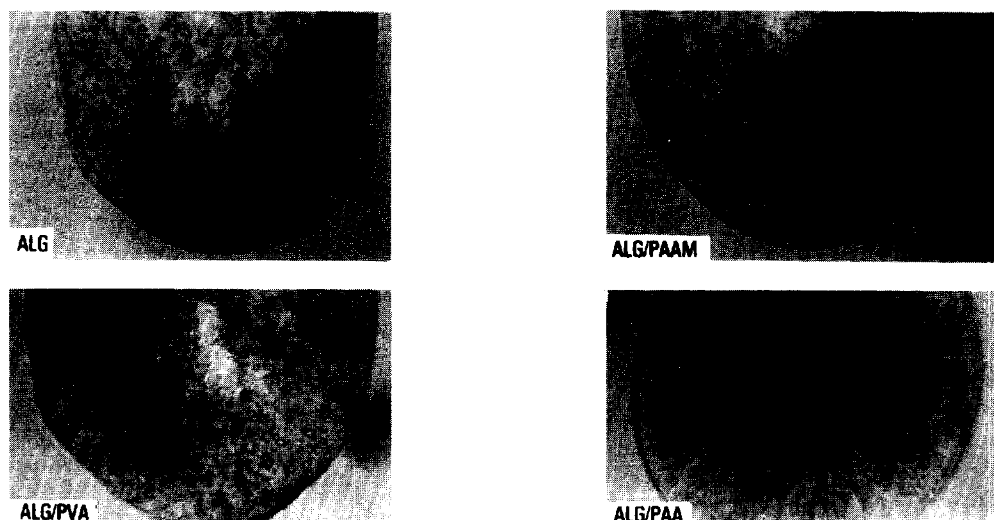


Fig. 4. Appearance of BHK cells immobilized and growing in the gel beads of alginate and alginate-functional polymer mixture after 3 days culture in Petri dishes (inverted microscope, × 40).

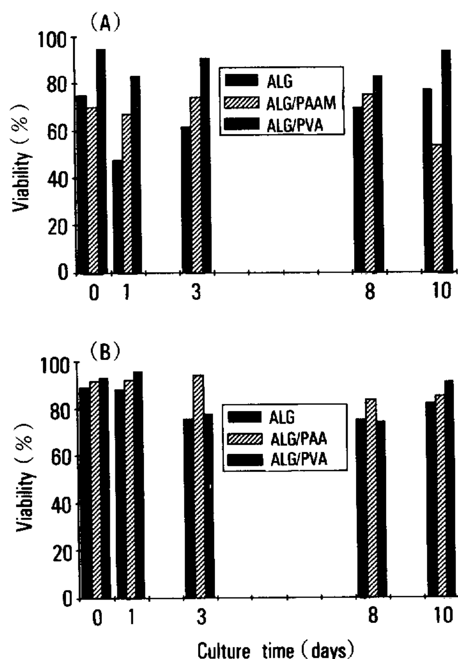


Fig. 5. Viabilities of CHO and BHK cells immobilized in the gel beads of alginate and alginate-functional polymer mixture as a function of culture time, (A) CHO cells and (B) BHK cells.

total number of cells. From the Figure, we can see that the viability was not reduced with the culture time for both CHO and BHK cells. The gel beads prepared by the alginate-poly(vinyl alcohol) mixture showed best result for the viability of the CHO cells, i. e. more than 80 % during the given culture periods. The viability of the BHK cells were not much dependent on the kind of the beads used.

Fig. 6 shows the viable cell densities for the CHO and BHK cells grown in 1 gram of the gel beads of the alginate and the alginate-functional polymer mixture. The cells were grown in the beads with the culture time. The gel beads of the alginate-poly(vinyl alcohol) mixture also showed best result for the CHO cells, i.e. about 3-fold increase in the cell number after 10 days culture. The BHK cells were not grown well until 3 days culture, but the cells immobilized in the gel beads of the alginate-poly(vinyl alcohol) mixture were also grown about 3-fold after 10 days culture.

It is not clear yet why the beads prepared by the alginate-poly(vinyl alcohol) mixture show better cell viability and growth than others, but it may be

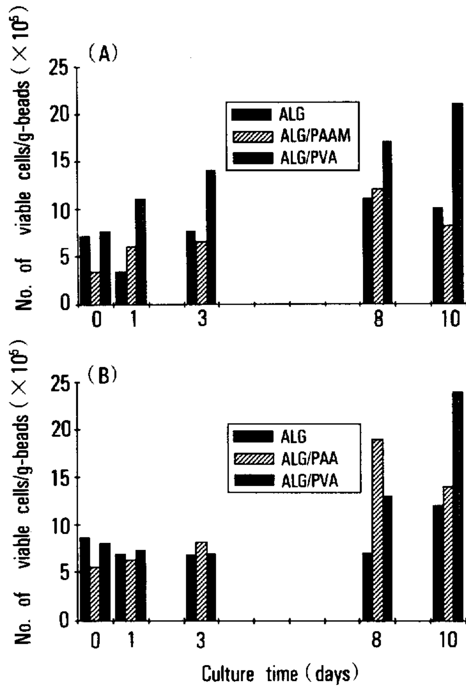
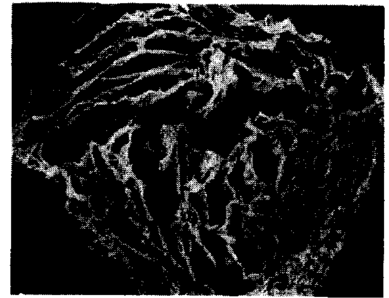
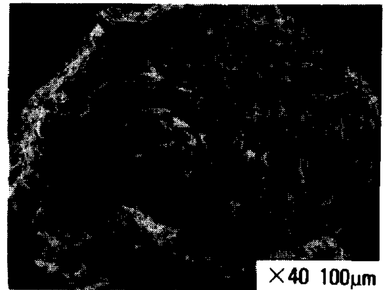


Fig. 6. Viable cell densities for CHO and BHK cells in the gel beads of alginate and alginate-functional polymer mixture as a function of culture time, (A) CHO cells and (B) BHK cells.

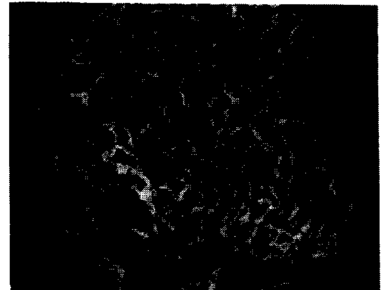
related to the morphology and chemical structure. First, the morphology inside the gel beads of the alginate-poly(vinyl alcohol) mixture was much different from those of other beads, as seen in Fig. 7 (also refer to Fig. 2). It has the channel-like pore structure extended towards the bead center, while the others have the random honey comb-like structures. We do not know why these structural differences happened but this probably affect the viability and growth of the cells immobilized in the beads. Second, we already have some evidence from the previous work[7,8] that the polymer surfaces with -OH functional groups show good cell adhesion and growth properties. Although the situation is not exactly same here, i.e. the substrates used in this study are porous beads while those in the previous work were flat sheets, we may say that the -OH functional groups probably affect positively for the cell viability and growth in



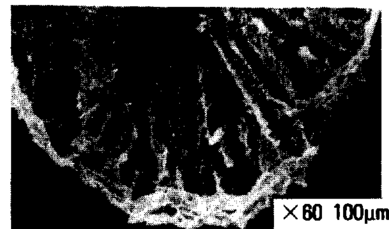
ALG



ALG/PAAM



ALG/PAA



ALG/PVA

Fig. 7. SEM pictures of the cross-sectioned gel beads of alginate and alginate-functional polymer mixture after freeze-drying (× 40 for top three pictures and × 60 for bottom picture).

the beads. It will be further studied to elucidate this phenomenon in detail.

CONCLUSIONS

The animal cells, CHO and BHK cells, were immobilized in the gel beads of the alginate and the mixtures of the alginate-polymer with different functional group (-OH, -COOH, and -NH_2). The morphology inside the gel beads prepared by the alginate-poly(vinyl alcohol) mixture was different from those of other gel beads. The gel beads of the alginate-poly(vinyl alcohol) mixture were more effective for the immobilization of both CHO and BHK cells compared to other beads in terms of the cell viability and density after culturing in the Petri dishes.

The continuous cell culture under flowing culture medium system is on progress using the gel beads of the alginate-poly(vinyl alcohol) mixture to obtain the mass production of animal cells.

Acknowledgement : This work was supported by the Korea Ministry of Science and Technology.

REFERENCES

1. K. Nilsson, *Methods in Enzymology,* **135,** 387 (1987).
2. C. Tamponnet, S. Boisseau, P. N. Lirsac, J. N. Barbotin, C. Poujeol, M. Lievremont, and M. Simonneau, *Appl. Microbiol. Biotechnol.,* **33,** 442 (1990).
3. Y. Shirai, K. Hashimoto, H. Yamaji, and M. Tokashiki, *Appl. Microbiol. Biotechnol.,* **26,** 495 (1987).
4. F. Lim, *U. S. Patent,* 4,409,331 (1983).
5. S. C. Nigam and H. Y. Wang, *PCT Int. Appl.,* WO 89/01034 (1989).
6. C. Bucke, *Methods in Enzymology,* **135,** 175 (1987).
7. J. H. Lee, J. W. Park, and H. B. Lee, *Biomaterials,* in press (1991).
8. J. H. Lee, G. S. Khang, K. H. Park, H. B. Lee, and J. D. Andrade, *J. of KOSOMBE,* **10,** 195 (1989).
9. W. B. Jakoby and I. H. Pastan, Eds., "Methods in Enzymology. Vol. LVIII. Cell Culture," Academic Press, New York, 1979.
10. Y. Shirai, R. Sasaki, K. Hashimoto, H. Kawahara, K. Hitomi, and H. Chiba, *Appl. Microbiol. Biotechnol.,* **29,** 544 (1988).
11. H. Y. Lee and J. K. Kang, *Hwahak Konghak,* **27,** 286 (1989).
12. M. Goto, K. Akai, A. Murakami, C. Hashimoto, E. Tsuda, M. Ueda, G. Kawanishi, N. Takahashi, A. Ishimoto, H. Chiba, and R. Sasaki, *Bio/Technology,* **6,** 67 (1988).
13. T. Ryll, M. L. Lange, V. Jager, and R. Wagner, *J. of Biotechnol.,* **14,** 377 (1990).
14. M. S. Croughan and D. I. C. Wang, *Biotechnol. Bioeng.,* **36,** 316 (1990).
15. A. L. Smiley, W. S. Hu, and D. I. C. Wnag, *Biotechnol. Bioeng.,* **33,** 1182 (1989).
16. R. Kohn, *Pure & Appl. Chem.,* **42,** 371 (1975).