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Short Technical Report

Calcium-Alginate Gel Bead Cross-Linked with Gelatin as Microcarrier for Anchorage-Dependent Cell Culture

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ABSTRACT

*Valuable products obtained from the cultivation of anchorage-dependent mammalian cells require large-scale processes to obtain commercially useful quantities. It is generally accepted that suspension culture is the ideal mode of operation. Because anchorage-dependent cells need surfaces to be able to attach and spread, the incorporation of microcarriers to suspension culture is indispensable. Since the dextran-based microcarrier was first introduced, many different types of microcarriers have been developed and commercialized. In this study, alginate-based microcarriers were made in the following order: (*i*) calcium-alginate gel beads prepared by dropping a blend of sodium alginate and propylene glycol alginate (PGA) into calcium chloride solution, (*ii*) the PGA section of gel beads cross-linked with gelatin in alkaline solution (i.e., via the transacylation reaction between the ester group of PGA and amino group of gelatin), and (*iii*) gelatin membrane around the beads further cross-linked by glutaraldehyde. The glutaraldehyde-treated gelatin-transacylated PGA/alginate microcarrier showed superior features in high stability under phosphate-containing solution, density close to that of culture medium, and transparency. Moreover, the Chinese hamster ovary CHO-K1 and amphotropic retrovirus producer PA317 cells cultivated on the newly synthesized microcarriers exhibited similar growth kinetics of these two types of cell lines cultured on commercial polystyrene microcarriers. However, cell morphology was easily monitored on the transparent microcarriers made in this study.*

INTRODUCTION

Large-scale culture of anchorage-dependent cells is important for the production of recombinant human proteins in biopharmaceutical industries. For example, recombinant human erythropoietin and tissue plasminogen activator are produced by cultivating genetically modified Chinese hamster ovary cells (2,18). In addition, most viral vectors used for gene therapy are currently produced from anchorage-dependent packaging cell lines (14). Because of the very low concentration of the products, high-density culture of anchorage-dependent cells is extremely desirable. Moreover, high concentration of the products promotes the efficiency of downstream processes such as recovery and purification, which determine the economic value of the products, by reducing the volume to be processed. Unlike the hollow fiber and roller bottle methods (4,10), culturing anchorage-dependent cells with microcarriers can be implemented into the suspension culture system, which is easy to characterize and control, as well as high production efficiency per reactor volume (20,21). Thus far, many different microcarriers made of cellulose (19), gelatin (22), dextran (21), and polystyrene (8) have been reported. Here we have developed a new type of microcarrier made of alginate and investigated its capability of supporting the growth of CHO-K1 and PA317 cells.

Alginate is biocompatible, relatively cheap, and able to encapsulate active enzyme or live cells simply by adding alginate droplets into solution containing bivalent cations (1). It is well known that mammalian cells are unable to interact with alginate polysaccharides because the hydrophilic alginic acid adsorbs less protein and promotes less cell attachment and spreading. Thus, there are very few studies on alginate as the substratum to support cell adhesion, spreading, and proliferation. For example, applying aqueous carbodiimide chemistry, Rowley et al. (16) have covalently modified alginate polysaccharides with RGD-containing cell adhesion ligands to promote cell attachment and spreading. By coating (via simply adsorption) the Ba^{2+}-alginate beads with collagen, Grohn et al. (7) demonstrated that the anchorage-dependent mammalian cells could attach and proliferate well. In large-scale culture systems, microcarriers must remain in suspension either by mechanically or pneumatically mixing. The adsorption force between collagen and Ba^{2+}-alginate may not be strong enough to prevent the detachment of cells because of the high level of shear forces.

It has been documented that the ester group of propylene glycol alginate (PGA) can form amide linkage with the amino group of proteins (12,13). Based on this unique feature, we first blended PGA with sodium alginate at different ratios, and the mixture was added dropwise into calcium chloride solution to form gel beads. On the surface of the

beads, gelatin was covalently bound with the region containing PGA through transacylation. In the end, gelatin was further cross-linked by glutaraldehyde to strengthen the rigidity of microcarriers. The covalent binding force generated between the ester group of PGA and the amino group of gelatin should be able to withstand the shear stresses. Moreover, since gelatin is denatured collagen by heat, it is more stable at room temperature and, more importantly, cheaper than native collagen. Our results showed that both CHO-K1 and PA317 cells attached, spread, and proliferated well on the newly formed alginate microcarriers.

MATERIALS AND METHODS

Cells

The Chinese hamster ovary CHO-K1 cell line was obtained from the ATCC (Rockville, MD, USA), and the amphotropic retrovirus producer PA317 cell line was provided by Dr. W. French Anderson (Gene Therapy Laboratories, University of Southern California). CHO-K1 cells were proliferated in 1:1 mixture of DMEM and F12 medium supplemented with 2 mM L-glutamine, 10% FBS, 100 U penicillin, and 100 µL streptomycin (all from Irvine Scientific, Santa Ana, CA, USA) under 100% humidity and 5% CO_2. PA317 cells were cultured in DMEM supplemented with the same concentration of FBS, glutamine, and antibiotics mentioned above. At 80% confluence, the cells were detached from the tissue culture dish (BD Biosciences, San Jose, CA, USA) with 1× EDTA-trypsin solution for inoculation.

Preparation of PGA/Alginate Microcarriers

Aqueous solution containing 1% sodium alginate (Sigma, St. Louis, MO, USA) and 4% PGA (Kelcoloid® D; ISP Alginate, San Diego, CA, USA) was prepared. It should be noted that because of its highly viscous property, 4% PGA and 1% alginate solution were prepared indirectly by evaporating half the amount of water from the 2% PGA and 0.5% alginate solution. This solution was then filled into a 1-mL syringe attached with a 25-gauge needle and added dropwise into a gently agitated 1.5% (0.135 M) calcium chloride solution. The gel beads that formed were kept in the solution for 30 min to be fully cured. A stainless-steel mesh screen (Tyler, Mentor, OH, USA) was used to select beads with sizes 150–300 µm. The resulting beads were rinsed several times with deionized water. Over 80% of the beads observed under the microscope exhibited spherical shapes.

Formation of Glutaraldehyde-Treated Gelatin-Transacylated PGA/Alginate Microcarriers

Fifteen milliliters of the 1% gelatin solution were added to 15 mL suspension of PGA/alginate beads with gentle mixing. The transacylation was started by slowly pouring 30 mL 0.2 M NaOH to the suspension of gelatin-adsorbed beads. The suspension was stirred for 15 min and then neutralized with 1 M HCl, under magnetic stirring for another 15 min. The resulting microcarriers were rinsed and resuspended with deionized water. To increase the mechanical strength of the microcarriers, the gelatin covalently bound on the alginate beads was cross-linked with 0.4% glutaraldehyde (Sigma) for 30 min. Finally, the glutaraldehyde-treated gelatin-transacylated PGA/alginate microcarriers (abbreviated as GPA microcarriers) were washed thoroughly, resuspended in 50 mL growth media (DMEM + 10% FBS), and stored under 4°C for further use. The size of the GPA microcarriers was determined with a light microscope using a calibrated grid. The average radius of the GPA microcarriers was determined as 94.0 ± 27.5 µm. [Fifty microcarriers were sampled and analyzed using NIH Image software (National Institutes of Health, Bethesda, MD, USA).] To determine the number of

beads per milliliter, several aliquots of suspended GPA microcarriers were counted using a light microscope.

Microcarrier Spinner Culture

The microcarrier spinner culture was performed by inoculating 7×10^5 cells and 5×10^3 GPA microcarriers in a siliconized 100-mL spinner flask (Bellco, Vineland, NJ, USA) containing 40 mL growth media. The cells were first allowed to attach to GPA microcarriers for 2 h without spinning in the incubator. The suspension was then stirred every 2 h at 30 rpm for 5 min. After 12 h intermittent mixing, the culture was mixed continuously at 30 rpm. For the purpose of comparison, the cells were also cultured on 5×10^3 polystyrene microcarriers (Biosilon®; Nalge Nunc International, Rochester, NY, USA) with the average radius determined as 116.1 ± 16.1 µm..

Cell Harvesting

Suspension from the spinner culture (1 mL) was sampled on a daily basis. After centrifugation at 200× *g* for 10 min, the microcarriers were recovered and resuspended in Dulbecco's PBS (Irvine Scientific). Cells were then trypsinized from the microcarriers with moderate pipetting. Viable cell number was determined using a hemocytometer (Hausser Scientific, Horsham, PA, USA) with the Trypan Blue exclusion method. Triplicate data were plotted by $\bar{x} \pm SD$ based on the Student's *t* test, with significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Less Swelling of Alginate Microcarriers in PBS

When placed in an aqueous medium containing phosphate, citrate, or Mg^{2+}, calcium-alginate gel beads swell and release the encapsulated active agents. The swelling can be prevented by coating the beads with a polycationic material such as poly-L-lysine or chitosan, which forms a stable membrane (15). A higher ratio of guluronic acid to mannuronic acid in alginate also decreases the swelling of the alginate bead (17). One of the major tasks of manufacturing the GPA microcarriers was to make sure that the swelling of the microcarriers in the PBS solution was negligible. To achieve this, the stable membrane was formed around the PGA/alginate gel bead using the transacylation reaction between the ester group of PGA and the amino group of gelatin. To strengthen the microcarrier's endurance in the PBS solution further, the gelatin-transacylated PGA/alginate microcarrier was cross-linked by glutaraldehyde. No significant swelling of the alginate beads during the production (i.e., transacylation, fixation with glutaraldehyde, and all washing steps with deionized water after Ca^{2+} cross-linking) was noticed. We examined the swelling of PGA/alginate beads, gelatin-transacylated PGA/alginate microcarriers, and GPA microcarriers in PBS solution. As a result, the swelling speed of gelatin-transacylated PGA/alginate microcarriers in PBS solution decreased. Moreover, the degree of the GPA microcarrier's swelling dropped further after the cross-linking of gelatin by glutaraldehyde (data not shown). The reason behind this observation is probably the dense gelatin macromolecules covalently binding with the PGA fraction of the gel bead, resulting in a strong binding force that holds the microcarrier's shape, even though the Ca^{2+}-alginate bridge has been broken down. The intactness of the microcarrier's shape is further enhanced through the glutaraldehyde cross-linking of gelatin macromolecules. Compared with glutaraldehyde, it is surmised that the cross-linking reagent 1-ethyl-3-(dimethyl aminopropyl) carbodiimide (EDC) can be used as an alternative to generate much more highly cross-linked gelatin matrix because of the formation of a zero-length cross-linking spacer (6). In addition, for certain fastidious cells, the potential toxicity effects might be caused by using glutaraldehyde as the cross-linking reagent. The relatively mild cross-linking reagent EDC can be used to avoid this problem.

Unity Specific Gravity of GPA Microcarriers

The density of GPA microcarriers was estimated by using the Stokes equation. The terminal velocity of a spherical particle with a given radius in aqueous solution can be easily determined by

$$u_t = \frac{2R^2(\rho_s - \rho_f)g}{9\eta}$$

where η, R, ρ_s, ρ_f, g, and u_t and are viscosity of fluid, radius of the particle, density of the particle, density of fluid, gravitational acceleration, and terminal velocity, respectively. Here we added the same number of GPA microcarriers and polystyrene microcarriers separately into two identical tubes containing PBS solution with the height of 5 cm. The time needed for the polystyrene microcarriers to fully settle down was 1 min, while it took 2 h for the GPA microcarriers to reach the bottom of the tubes. The terminal velocity was therefore determined to be 0.042 cm/min for the GPA microcarriers and 5 cm/min for the polystyrene microcarriers. According to the data provided by the company, the density of polystyrene microcarriers is 1.05 g/cm^3. The density of PBS is assumed to be the same as water (i.e., 1 g/mL). Applying all of these numerical values into the equation given above, the density difference between GPA microcarriers and PBS solution is close to zero. Because microcarrier-based cell cultures are commonly performed in fluidized-bed, fixed-bed, and stirred-tank reactors (3,5,11), power is needed to make the culture medium circulate or impellers rotate. If the density of the microcarriers is close to culture medium, then a low power level or intermittent mixing is enough to suspend them. As a result, cell detachment and necrosis resulting from the mixing mode used can be mitigated.

Cell Growth on Polystyrene and GPA Microcarriers

We compared the cell growth kinetics of CHO-K1 and PA317 cells on our laboratory-made GPA microcarriers and commercial polystyrene beads and obtained a similar maximum specific growth rate for the CHO-K1 and PA317 cells cultured on the GPA microcarriers (Figure 1). In addition, the initial cell attachment (at Day 1) on GPA microcarriers seems to be higher than the attachment on polystyrene microcarriers (data not shown). This implies that GPA mi-

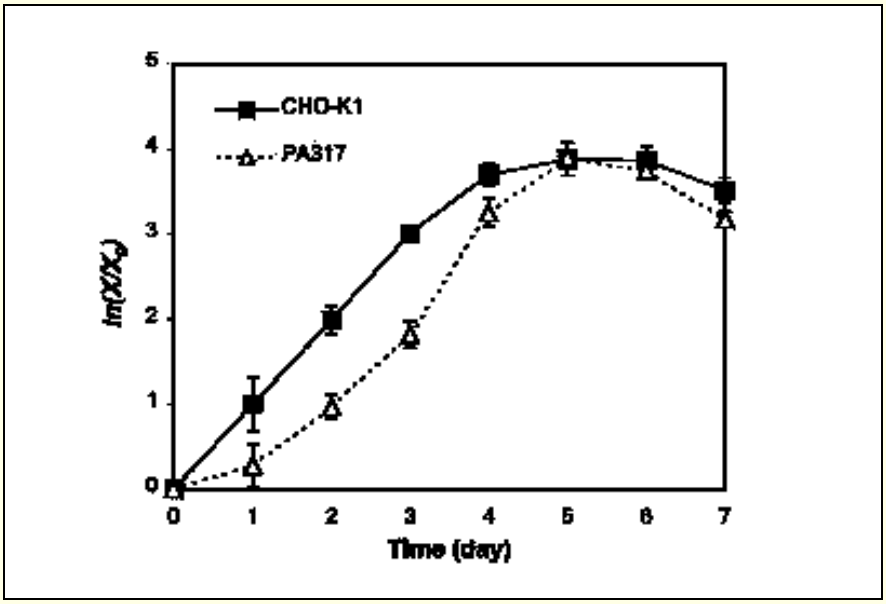

Figure 1. Growth kinetics of CHO-K1 and PA317 cells, respectively, on GPA microcarriers. Both cells showed the maximum specific growth rate for first four days, followed by decelerated and decreased cell growth. Triplicate data were used with a significance level of $P < 0.05$. Here X is the cell number harvested after a given period of culture, and X_0 is the inoculation cell number.

crocarriers provide more inductive surface for cell attachment and spreading. This is probably because the gelatin macromolecule has a higher binding affinity to the serum-containing fibronectin, which is one of the most important extracellular matrix molecules involved in cell attachment and spreading (9). Our results also showed that none of the CHO-K1 cells were observed to have fully spread on the GPA microcarriers made of 2% PGA and 1% alginate (data not shown). Only a small fraction of cells exhibited the attachment and spreading behavior on the surfaces of the GPA microcarriers composed of 3% PGA and 1% alginate. This is probably due to the low density of gelatin-PGA conjugates that formed on the GPA microcarrier. However, for the microcarriers with the components of 4% PGA and 1% alginate, the cells attached, spread, and proliferated very well. Therefore, the threshold of gelatin con-

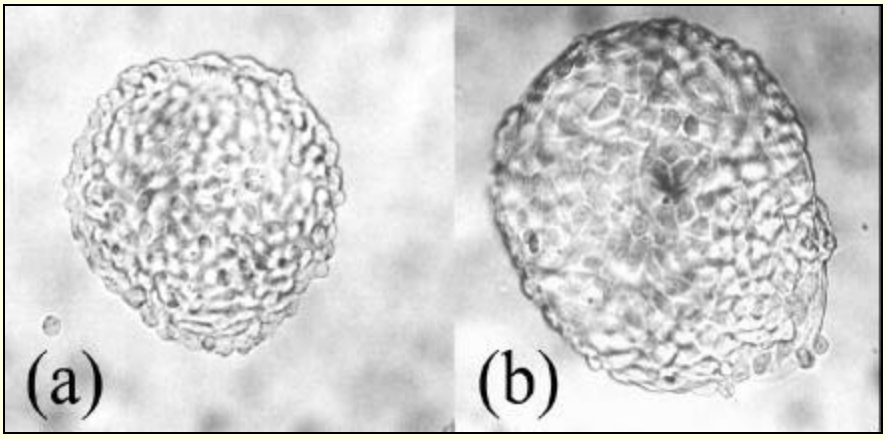

Figure 2. Morphology of (a) PA317 and (b) CHO-K1 cells on GPA microcarriers on the fourth day of spinner culture. These photomicrographic images show the GPA microcarrier covered by fully spread cells. The transparency of the GPA microcarrier provides fairly easy monitoring of cell morphology (magnification: 250×).

centration density required for cell anchorage has been reached with this composition. Apparently, the degree of cross-linking between the gelatin and PGA via the transacylation reaction plays a vital role in the efficacy of the microcarrier spinner culture studied here. To maximize the attachment, spreading, and proliferation of cells on GPA microcarriers, it is worthwhile to examine certain operating parameters. For transacylation reaction done by the alkalinization of the PGA/alginate gel beads with absorbed gelatin, a higher concentration of NaOH than is used here (i.e., 0.1 M) can presumably result in a higher degree of cross-linking caused by a greater number of amino groups of gelatin being incorporated in the nucleophilic substitution of ester groups. The time executed for the transacylation reaction (15 min was used here) may also influence the degree of cross-linking. An optimal cross-linking condition can be defined if a series of these tests were done.

The morphology of cell attachment and spreading on GPA microcarriers was taken with bright field illumination at Day 4 (Figure 2). Since the GPA microcarrier is transparent, cell morphology on its surface can be observed simply by light microscopy. The results showed that the cells on the GPA microcarriers in the spinner culture exhibited the same cell morphologies as were normally observed when the cells were cultured on a 2-D tissue culture-treated polystyrene surface. Obviously, an advantage of using the GPA microcarrier over the polystyrene bead is the ability to monitor cell morphology because of its transparency.

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