Journal of Microbiology and Biotechnology
Vol. 5, No. 3, 163-166, 1995

Chemical Modification of Macroporous Gelatin Microcarriers and Characterization of Cell Growth and Attachment

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Chemical modification of gelatin-based macroporous microcarrier beads was achieved by increasing the charge density through incorporation of (diethylamino)ethylchloride-hydrochloride (DEAE:Cl-HCl) or lysine, and this significantly improved the attachment and growth of HepG2 cells. When microcarriers were modified by the addition of 2% lysine, positive charge density was 0.95 meq/g-carriers. In case of modification of microcarriers with DEAE:Cl-HCl, positive charge density was 0.6 meq/g-carriers. An increase in charge density of the microcarriers to improve cell attachment has facilitated the growth of the cells on macroporous gelatin microcarriers. Also, final HepG2 cell concentration cultivated on modified beads with DEAE:Cl-HCl was increased up to 10^7 cells/ml. This was 2-3 times higher than that obtained with unmodified macroporous gelatin microcarriers.

Macroporous gelatin microcarriers have been used for the culture of various cell lines (6, 7). However, some cell lines, especially HepG2, do not show active growth and show a relatively low concentration of cells on macroporous gelatin microcarriers. A reasonable cause of this was thought to be the slow initial attachment of cells on gelatin based macroporous microcarriers.

The use of microcarriers for animal cell cultures has proved to be an efficient technology. These microcarriers have been progressively developed to put a positive charge which is the most suitable matrix for attachment of cells (3, 4). The presence of a positivly charged group is essential for cell attachment, but a specific degree of substitution of the beads with diethylaminoethyl groups is needed for optimal cell growth (3, 4). However, the charged groups of inner part of beads are not essential for the attachment and growth of cells because these catch the medium component and cell products (2). Therefore, we report the result of our investigation of optimal surface charge density of macroporous gelatin microcarriers based on chemical modification to enhance the attachment and growth of cells.

MATERIALS AND METHODS

Cells and Cell Maintenance

HepG2 cells were maintained in T-150 flasks at 37℃ in a 5% CO_2 incubator. Dulbecco's modified Eagle's media (DMEM) supplemented with 5% fetal calf serum was used. The medium was also supplemented with penicillinG (100 units/ml) and streptomycin (100 μg/ml).

Culture Conditions

3 g/l of microcarriers were used for each culture. The 100ml microcarrier cultures were carried out in 5% CO_2 incubator at 37℃ using 250 ml spinner bottles (Wilber Scintific, Inc., USA). The bottles were equipped with two 45° pitched blades-impellers placed approximately 1 cm above the bottom. The stirring speed was fixed at 60 rpm. The cells in the late exponential phase were trypsinized from T-150 flasks using 0.2% trypsin in PBS with 0.02% ethylenediamine tetraacetic acid (EDTA) and inoculated into the spinner bottles. Cells of the same batch were used as inoculum for each spinner culture at a concentration of 3.5×10^5 cells/ml and during cultivation, 75 ml of medium was exchanged when the color of culture broth became yellow due to the decrease of pH.

Monitoring Cell Attachment

The agitation was temporarily stopped and the microcarriers were allowed to settle. The supernatant was removed from the microcarrier-free region. And the unattached cells were counted under a microscope.

Cell Counting

A 1 ml sample was removed from a well-mixed spinner bottle and placed into a 15 ml centrifuge tube. The microcarriers were allowed to settle and the supernatant was removed with a Pasteur pipette by suction. The same

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Key words: macroporous gelatin microcarrier, charge density, chemical modification

amount of counting solution (0.1% crystal violet in 0.1 M citric acid) was then added. The suspension was incubated for 1 hour at 37℃ and sheared with a Pasteur pipette. Then the stained nuclei were counted on a hemacytometer.

Chemical Modification of Macroporous Gelatin Microcarriers

Modification with lysine ; 1 g of gelatin and 0.2 g of lysine were dissolved in 100 ml of distilled water at 40℃ and then mixed with 12% (w/v) calcium carbonate particles (size : av. 25 μm). This suspension was dispersed in 100 ml of the mixture of toluene and chloroform (73:27, v/v) containing 1% (v/v) tween 80 at room temperature under vigorous stirring. Then the formed small beads entrapping the particles were sieved between 125~500 mm and washed with tap water and then resuspended in 0.5 M HCl solution in order to dissolve out the entrapped calcium carbonate particles. The beads were further washed with distilled water and crosslinked with 2.5% glutardialdehyde solution for 30 min in order to increase the strength of the beads. The collected beads were washed again with distilled water and then freeze-dried.

Modification with DEAE:Cl-HCl ; 5 ml of 3.0 M 2-(diethylamino) ethylchloride-hydrochloride (DEAE:Cl-HCl) was added to 0.3 g of macroporous gelatin microcarriers and the mixture was heated to 60℃ in a water bath. Then, 5 ml of 3.0 M NaOH was added and mixed well. The reaction was carried out for 12 h. The reacted microcarriers were washed with 200 ml of distilled water, 150 ml of 0.1 M HCl, and 250 ml of 0.1 mM HCl, in due order. Then the modified microcarriers were washed with 500 ml of distilled water and 1000 ml of magnesium- and calcium-free phosphate-buffered saline (PBS). Finally, washed microcarriers were suspended in PBS (5).

Determination of Positive Charge Density

1 g of each unmodified and modified microcarriers in PBS was washed with 200 ml of 1.0 M NaOH for the deprotonization and 200 ml of 0.1 M NaCl. The deprotonized microcarriers were then resuspended in 100 ml of 0.15 M NaCl. At this fixed ionic strength, the deprotonized microcarriers were protonized by adding 0.3 M HCl dropwise. After each dropwise addion of 0.3 M HCl, the suspension was stirred well and its pH was allowed to stabilize. Then the pH of the microcarrier suspension was measured with a pH meter. Hence, the degree of protonation, which was referred to as positive charge density, was determined. Also the same procedure was carried out for 0.15 M NaCl solution without microcarriers.

RESULTS AND DISCUSSION

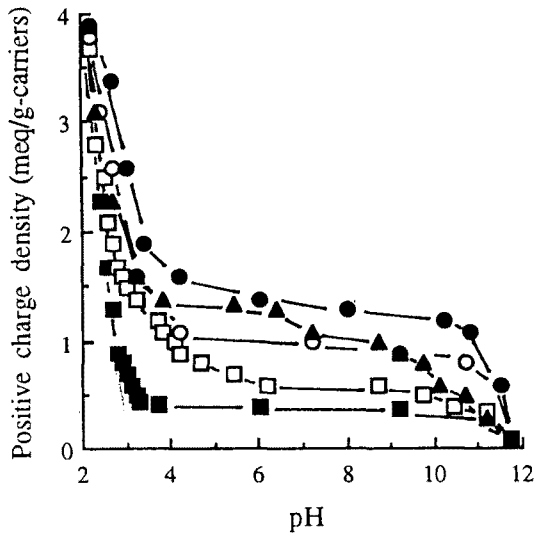

Fig. 1. Positive charge density of beads according to various pH.
■: reference, □ unmodified macroporous beads, ●: modified beads with 2% lysine, ○: modified beads with DEAE, ▲: Cytodex-1.

Many investigations have studied how various surface modifications affect cell attachment to microcarriers. Among them, the important factors are charge density (5), and the length of the carbon backbone which carries the charged molecules (8, 9). Also, charge density is affected by the pH and ionic strength of the solution. To obtain controlled charge density of microcarriers, macroporous gelatin microcarriers were modified by incorporation of lysine and DEAE:Cl-HCl.

Figure 1 shows the positive charge density of microcarriers as a function of pH. Positive charge density (0.6 meq/g and 0.95 meq/g-carriers for microcarriers modified with DEAE:Cl-HCl and with 2% lysine, respectively) of modified microcarriers were more than that (0.2 meq/g-carrier) of unmodified microcarriers at pH 7.2. Therefore, by coupling the positively charged groups (DEAE:Cl-HCl or lysine), we increased the positive charge density about to the level (0.8 meq/g-carrier) of Cytodex-1. The low positive charge density of macroporous gelatin microcarriers seems to be due to the Schiff's base (imine) formation reaction between amino groups of gelatin material and aldehyde groups of glutardialdehyde occuring in the process of strengthening microcarriers.

Attachment and Growth Kinetics of HepG2 Cells on Microcarriers

Attachment and growth kinetics of HepG2 cells on unmodified and modified macroporous gelatin microcarriers are shown in Fig. 2A and 2B, and the related kinetic parameters which were determined from Fig. 2A and 2B are summarized in Table 1. HepG2 cells attached poorly to unmodified macroporous gelatin microcarriers.

Initial attachment rate constant and attachment yield were found to be 0.189 h^{-1} and 42.9%, respectively. However, their attachment kinetics to the two modified microcarriers, especially to the microcarriers modified with DEAE:Cl-HCl (Fig. 2A), were markedly improved, e.g. initial attachment rate constant was 0.678 h^{-1} and attachment yield was 98.6%. Improvement of attachment kinetics of HepG2 cells to the two modified microcarriers subsequently resulted in a satisfactory improvement of growth kinetics on these microcarriers (Fig. 2B). On the unmodified macroporous gelatin microcarrier culture, a long lag phase appeared and the final cell concentration was limited (2.3×10^6 cells/ml). However, on the microcarriers modified with DEAE:Cl-HCl, HepG2 cells grew to a concentration (1.2×10^7 cells/ml) 30 times higher than the inoculated cell concentration (3.5×10^5 cells/ml). The specific growth rate did not show a significant difference among three different microcarriers.

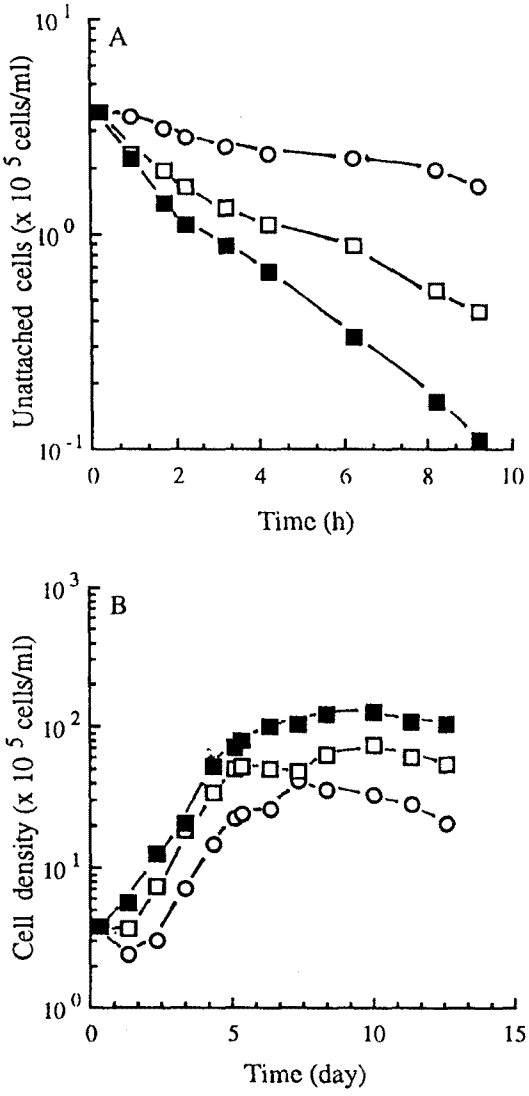

Fig. 2. Attachment (A) and growth (B) kinetics of HepG2 cells on beads.
○: unmodified macroporous beads, □: modified beads with 2% lysine, ■: modified beads with DEAE.

The reason why the attachment and subsquent growth kinetics of HepG2 cells on the modified microcarriers are improved markedly, is not clearly understood. However, based on the results of the positive charge density of the microcarriers, the electrostatic attractive force between positively charged groups on the microcarriers and negatively charged cell membranes should facilitate the cell attachment to microcarriers. The specific interaction between integrins of the cell membrane and the RGD (Arg-GLy-Asp) sequence of the extracellular adhesive protein-fibronectin, vitronectin, osteopontin, collagen, thrombospondin, fibrinogen, von Willebrand factor, etc. has been reported to be a major factor in the attachment of cells to their environments (1).

Therefore, a plausible explanation can be proposed that in the process of cell attachment to microcarriers, like many other biological process, first, the electrostatic attractive force is a major driving force for cells to approach microcarriers and then the specific binding between integrins of cells and extracellular molecules on microcarriers plays an important role for cells to attach to microcarriers. In spite of the lower positive charge density, better results are observed for attachment and growth kinetics on microcarriers modified with DEAE: Cl-HCl than on the microcarriers modified with lysine. Because lysine is a amino acid, it has both a negative and positive charge. So, the above reason may be related with electrostatic repulsion when considering the fact that the microcarriers modified with DEAE:Cl-HCl have a lower negative charge density than the microcarriers modified with lysine (data not shown).

Table 1. Summary of attachment and growth kinetics of HepG2 cells on beads.

	Initial attachment rate constant (h^{-1})	Attachment yield at 8 h (%)	Specific growth rate (h^{-1})	Final cell density at 8 days (10^6cells/ml)
Unmodified beads	0.189	42.9	0.032	2.3
Modified beads with 2% lysine	0.423	88.0	0.028	5.6
Modified beads with DEAE:HCl	0.678	98.6	0.027	12

Acknowledgement

This work was supported by a grant for the Bioprocess Engineering Research Center, KAIST, from the Korea Science and Engineering Foundation.

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(Received April 29, 1995)