The Application of a New Type of Sintered Glass Carriers for the Cultivation of Anchorage-Dependent Mammalian Cells

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Summary

Cylinder-shaped sintered glass carriers were successfully used for immobilizing and cultivating cells of murine line L929. The process has been performed in a fixed-bed bioreactor (working volume of 150 ml) with perfusion and aeration of the medium (total volume of 3.5 l). The maximum cell density of \(1.23 \times 10^7\) cells/ml was achieved when the basal medium (MEM + 10% bovine serum) was enriched with glucose (up to 2 g/l) and L-glutamine (up to 4 mM). The carriers have proved to be nontoxic, mechanically stable and reusable.

Introduction

Generally, animal cells grow in two completely different modes as anchorage-independent and as anchorage-dependent. Anchorage-dependent cells (ADC) by definition grow when attached to a substrate; therefore, to obtain a high cell density, an appropriate surface area must be provided. For this purpose, numerous methods have been developed, including roller bottles, multi-tray units systems, membrane systems, glass beads, microcarriers, and porous matrix particles [1-9]. Among these techniques, the microcarrier culture seems to be the most widely used. There is a wide range of microcarriers commercially available, e.g. Cytodex (PHARMACIA), Biosilon (NUNC) and Bioglas (SOLOHILL Eng.), which allow to obtain a cell density up to \(3 \times 10^7\) cells/ml [1, 2, 5, 8]. The method which gives great promise at present is the use of porous matrix particles. These substrates provide a large surface area (0.4 m\(^2\)/g) for cell growth and require a simple construction of the bioreactor and an easy process operation (e.g. fluidized-bed and fixed-bed system) [4, 5, 10]. The porous carriers commercially available are produced from gelatin (Cultispher-G, PORCELL BIOLYTICA), collagen (Microsphere, VERAX Corp.) and glass (Siran, SCHOTT GLASWERKE) [4].

In this study we examined a new type of the sintered glass carrier made in our University for immobilizing and cultivating the murine line L929 as ADC model.
Materials and Methods

Carriers

The sintered glass carriers were designed in the Department of Chemical Physics (A. L. DAWIDOWICZ, A. KSIĘŻYCKI, Pat. R.P. no. 153 105, 1990 and A. L. DAWIDOWICZ, patent pending no. 272 278, 1988). Their physical properties are shown in Tab. 1. Carriers before use were treated with concentrated HNO₃ for 24 h and then washed in distilled water and stabilized in 0.85% NaCl to equilibrate the pH. After that carriers were packed into the bioreactor, and were steam-sterilized (2 atm, 30 min).

| Shape | Cylinder |
| Size | 5/5 – 8/5 mm |
| Void volume | 53% |
| Pore diameter | 180 – 480 μm |
| Surface | 0.4 m²/g |

Cell Line

Murine L-929 cell line obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, was used in this work.

Formulation of Media

The basal medium consisted of MEM (FLOW LABORATORIES) supplemented with 10% bovine serum (prepared in our laboratory from bovine blood), gentumycin 80 units/ml and nystatin 50 units/ml. Four medium formulations used during the experiment were as follows:

- *Medium I*
  - basal medium

- *Medium II*
  - basal medium with increased glucose concentration to 2 g/l

- *Medium III*
  - basal medium with increased L-glutamine concentration to 4 mM

- *Medium IV*
  - basal medium with increased concentration of glucose (2 g/l) and L-glutamine (4 mM).

Bioreactor System

A scheme of the bioreactor system is shown in Fig. 1. The system consisted of a 200-ml fixed-bed bioreactor (fixed-bed volume of 150 ml), a medium reservoir bottle (5 l) and a recirculating pump. It was operated as a perfusion culture system.

Culture Conditions

The bioreactor system was placed in a water bath (37 °C) and equilibrated for 4 h by circulating the culture medium (3.5 l). Inoculum was prepared from actively growing cultures on Roux bottles. The cells were suspended in an appropriate culture medium (150 ml), introduced to the bioreactor and allowed to attach to carriers for 5 h. After this time the perfusion with the flow rate of 0.8 linear cm per minute was initiated. The flow rate during cultivating was increased up to 3.2 linear cm per minute. The culture was oxygenated by introducing a mixture of air + 5% CO₂.
Culture Monitoring

Every 24 hours a sample of the culture medium was collected. The pH was controlled (pH electrode), ammonia production (NH₃-gas electrode) and oxygen concentration (O₂ electrode) were measured. Cell growth was determined indirectly by measuring the glucose consumption [11].

Cell Harvest

Cells were harvested by multiple trypsinization from the whole bed and then counted in a THOMA counting chamber. The cell attachment was investigated by staining the carrier with crystal violet and observing through a binocular as well as through a scanning electron microscope. Cell viability was determined by using the trypan blue exclusion test.

Results

The external structure of the carrier and the cells L₉₂₄₀ growing on its surface are shown in Fig. 2. Direct observation through a binocular has shown that the carriers were easily
customized, and the cells have grown spreading on the surface and in the large external pores. The growth of L929 cells was evaluated at four various medium formulations. Results given in Tab. 2 indicate that the most efficient growth occurred when the level of glucose and L-glutamine in the medium was enriched. The highest cell yield \(1.85 \times 10^9\) and a 17.8-fold increase in the cell number were achieved in the culture when the formula IV of the medium was used (Fig. 3). The intensity of cell growth was determined by measuring

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time [h]</th>
<th>Cell density* (\times 10^7/\text{ml})</th>
<th>DT** [h]</th>
<th>Final cell viability [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>126</td>
<td>0.64</td>
<td>37.5</td>
<td>90</td>
</tr>
<tr>
<td>II</td>
<td>174</td>
<td>0.90</td>
<td>43.4</td>
<td>88</td>
</tr>
<tr>
<td>III</td>
<td>168</td>
<td>0.84</td>
<td>42.2</td>
<td>90</td>
</tr>
<tr>
<td>IV</td>
<td>216</td>
<td>1.23</td>
<td>52.0</td>
<td>89</td>
</tr>
</tbody>
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* - cell density is expressed per unit fixed bed volume
** - population doubling time
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Fig. 3. Inoculum and cell harvest in different culture media formulations

the consumption of glucose. Fig. 4 shows an average glucose consumption rate, which reached the highest value of 216.6 μg/ml/24 h with the medium in formula IV. An accumulation of ammonia as a result of the cellular utilization of glutamine reached concentration values of 1.1 mM (medium I), 0.43 mM (medium II), 0.50 mM (medium III), and 0.70 mM (medium IV), and did not inhibit the cell growth. The ammonia production profile is presented in Fig. 5. During the cultivation a gradual decrease in the pH value was

Fig. 4. Average glucose consumption rate in different culture media formulations
Fig. 5. Ammonia production profile in different culture media formulations

observed, probably caused by the accumulation of lactic acid. To overcome this problem, the percentage of CO\textsubscript{2} in the gas mixture was decreased and the pH was maintained in the range of 7.0–7.5. To avoid the problem of oxygen deficiency, all the experimental cultures were aerated with a gas flow rate of 50 cm\textsuperscript{3}/s, and the oxygen concentration was maintained at a high level between 0.24 mM and 0.12 mM.

Discussion

The need to provide a large surface area for ADC growth gave rise to designing various types of porous materials [3, 4, 9]. It is very important that the substrate for the cell growth should have a great potential to be able to be scaled-up: unit cell density and volumetric size. The porous beads such as Verax and Siran seem to fulfill these criteria. By using these beads, a cell density of 4 \times 10\textsuperscript{7} cells/ml in scales of 200 l and 2000 l has been achieved [4]. The additional advantage of these carriers is that they can be used for cultivating anchorage-independent cells because of their ability to entrap the cells inside internal pores [5, 10]. Our preliminary experiments were mainly focused on the immobilization and ability for growth of the L\textsubscript{229} cells as an ADC model. Nevertheless, the results which have been obtained indicate quite clearly that the investigated carrier was nontoxic (cell viability of 90\%) and suitable for immobilization and cell growth. The growth of the cells to a density above 1 \times 10\textsuperscript{7} cells/ml could be achieved on these carriers. It should be emphasized that the carriers investigated were mechanically stable and reusable, which is very important in the production process and from the commercial point of view. In this study we showed that an appropriate oxygen and nutrient (glucose and L-glutamine) supply is critical to cell growth and can increase the process efficacy significantly. The cell growth also depends on the accumulation of products of the cell metabolism such as lactic acid and ammonia, so it is necessary to control these parameters during the cultivation [12, 13].

The fixed-bed cultivation system designed in our laboratory (similar to that used by LOOBY and GRIFFITHS) seems to be optimal for the sintered glass carriers due to a good transfer of nutrients, metabolism products and gas diffusion throughout the bed [10]. This construction is cheap, simple for operation and also good for scaling-up the culture. We plan to use this method to cultivate other anchorage-dependent mammalian cells and develop this system especially for the further production of substances with different biological potentials.
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References