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Preparation of microcarriers based on zein and their application in cell culture

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ABSTRACT

Microcarriers with a large surface area have attracted much attention in the field of large-scale cell culture. The preparation of microcarriers is mainly based on methods such as emulsification, suspension polymerization, solvent evaporation, organic phase separation (cohesion) and spray drying. Many of these require the use of organic solvents. In general, organic solvents are considered to be toxic and not conducive to environmental protection. In this study, zein microcarriers were successfully prepared via a novel method in which only glycerol was used as the dispersing medium. The size of these microcarriers was controllable, and the preparation process was simple to perform. Vero cells, commonly used in the production of vaccines, were cultured on the surface of the zein microcarriers to evaluate the microcarriers' performance in cell culture. The cells could attach and spread on the surface, without any difference compared with cells on the surface of Cytodex 1. The cell density reached its maximum on the fourth day, which was approximately 10 times the initial density. This is the first report that zein can be used as a microcarrier for the mass production of a large-scale suspension cell culture, which shows the potential of zein as a replacement for commercial non-degradable materials.

1. Introduction

The technique of large-scale animal cell culture has developed since Capstick succeeded in establishing a large-scale suspension culture of baby hamster kidney 21 (BHK-21) cells in 1962 [1].This technique has a significant role in the manufacture of biological medicinal products. These biological products include vaccines [2], interferons [3], growth factors [4] and monoclonal antibodies [5], which have promoted the development of biology and medicine and have brought huge economic and social benefits to the healthcare industry.

As the animal cells used in production systems are mainly anchorage-dependent cells [6,7], mature practical technologies include hollow-fiber cell culture [8], microencapsulation [9], and microcarrier culture. Since its first use in adherent animal cell culture by Van Wezel et al. [10], the microcarrier technique has become an effective method for large-scale amplification of animal cells and has been successfully used for vaccine production and gene engineering [11]. Microcarriers offer many advantages over traditional culture technology: they have a large surface area, the process parameters can be effectively traced and controlled, the cost is low, and a higher cell density can be produced [12]. With the help of microcarriers, cells can not only proliferate rapidly to produce useful drugs in a biological reactor but can also be directly used as a cell carrier for the repair and regeneration of organs in the body [13]. Methods for preparing microcarriers are mainly based on emulsification, suspension polymerization, solvent evaporation, organic phase separation (condensation) and spray drying. However, most of these use large amounts of organic solvents. Organic solvents are considered to be toxic and not conducive to environmental protection in general. So far, dozens of types of microcarriers have been sold on the market. Among them, the most common are the Cytodex, Cytopore and Cytoline series, produced by GE Healthcare, and Hillex (dextran matrix with a treated surface), produced by SoloHill Engineering, Inc. Recently, certain microcarriers made of biodegradable materials became available, such as the CultiSpher series, composed of gelatin [14], and the Cellagen series, composed of highly cross-linked collagen [15]. Certain microcarriers using poly (lactic-co-glycolic acid) (PLGA) [16], poly (L-lactic acid) (PLLA) [17] and hydroxyapatite [18] as raw materials are under investigation. At present, work is focused on collagen [19], gelatin [20], cellulose [21], sodium alginate [22], and chitosan [23] among natural materials. The most used microcarriers are still those in the Cytodex series, whether in the laboratory or in industrial production. However, these types of carriers need chemical modification of the surface of the dextran matrix, so they are relatively expensive. Moreover, they are disposable.

Zein is the main protein in corn and is soluble in 70–92% ethanol solutions [24,25]. Above all, zein is easily available and has good biocompatibility [26]. Zein can easily be processed into microspheres of 0.1–5 μm in diameter through solvent evaporation and phase separation [27,28]. However, methods for preparing zein microspheres

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larger than 10 μm are rarely reported. Generally, it is requisite for microcarriers to be in the range of 50–200 μm in diameter. Microcarriers with lower diameters have been reported to support limited cell proliferation due to the small surface area [29]. Meanwhile, if the diameter is less than 10 μm, it cannot meet the needs of cell growth. The purpose of the present study was to establish a method for preparing size-controllable zein microcarriers larger than 50 μm.

The use of Vero cells, commonly used for vaccine production, is recommended by the World Health Organization (WHO) and the National Requirements for Biological Products. Compared with other cells used for vaccine production, the Vero cells' source is extensive, and they are sensitive to a variety of virus infections. Moreover, high biological safety and high virus titers make these cells an ideal matrix for many viruses, so these cells can be used in human and animal vaccine production [30,31]. Therefore, we chose Vero cells to evaluate cell proliferation on zein microcarriers. We hope that the prepared microcarriers can be used for large-scale culture of animal cells.

2. Materials and methods

2.1. Materials

Zein was obtained from Wujiang BaChe Pharmaceutical Necessities Industry (Jiangsu, China). Glycerol and acridine orange (AO) (analytical-grade reagents) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Calf serum was supplied by Genetimes Technology, Inc. (Shanghai, China). African green monkey kidney cells, or Vero (ATCC CCL-81), were purchased from Fuxiang Technology, Inc. (Shanghai, China). Cytodex 1 is a product of GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Cell culture medium-related reagents were purchased from Gibco (Invitrogen, Singapore).

2.2. Preparation of the microcarriers

Zein was grinded using a disintegrator (Q-100A2, Shanghai Bingdu Electronic Co., Ltd., China) and then sieved through a standard screen (Shanghai Yichang Instrument Factory, China). The prepared zein particles were added to 99.9% glycerin in a 500 ml broad-necked flask. The mixture was shaken well and heated to 120 °C for 5 min. The glycerin was removed by suction filtration after being cooled to approximately 60 °C. The particles were then repeatedly washed with pure water (Ultra pure UF, Hitech Instruments Co., Ltd., China) until the glycerin was undetectable. The washed zein microspheres were air dried at 40 °C. In this method, glycerin is used as a supporting and heating matrix and can be used repeatedly. The diameter of the zein microcarriers can be controlled by controlling the size of the zein particles.

2.3. Characterization of the microcarriers

Scanning electron microscopy (SEM) (Stereoscan 260, Cambridge, and JSM-6700F, JEOL Ltd., Tokyo, Japan) was employed to observe the morphology of the microcarriers. A fluorescence microscope (IX71 Olympus Optical Co., Ltd., Japan) was used to observe and calculate the diameter of the zein microcarriers due to the spontaneous fluorescence of zein. We selected a microscope field at 20× and performed a statistical analysis of the diameters of 60 microcarriers.

2.4. Attachment and proliferation of Vero cells on the microcarriers

Vero cells were cultured in minimum essential medium (MEM; Gibco, Invitrogen, Singapore) supplemented with 10% calf serum, 100 units/ml penicillin and 100 g/ml streptomycin. The cells were seeded in a T-75 flask with 25 ml culture medium and incubated in a 5% CO_2 incubator at 37 °C. At 80–90% confluence, cell passaging was carried out at a 1:4–1:5 ratio with 0.25% trypsin. The cells were counted using a hemocytometer (XB-K-25, Shanghai Anxin Optical Instrument Manufacturing Co., Ltd., Shanghai) before inoculation. Cytodex 1 was used as the control. The dry microcarriers were soaked in Ca^{2+}, Mg^{2+}-free PBS (100 ml/g Cytodex 1 or 10 ml/g zein microcarriers, pH 7.2) at room temperature with occasional gentle agitation for 3 h, followed by replacement of the buffer with fresh PBS at the same concentration as used before.

Attachment assays were carried out in 48-well plates. Cells (2×10^5/ml) were added to wells containing zein microcarriers or Cytodex 1 and incubated in a humidified incubator (Series II Water-Jacketed CO_2 Incubator Model 3111, Thermo Electron Corporation, USA) with 5% CO_2 at 37 °C. After 3 h, the number of unattached cells in the plates was determined by counting with a hemocytometer. The microcarriers were then removed, and the plates were washed in PBS twice, with slight movement. After the PBS was removed, the cells were separated from the plates with 500 μl 0.25% trypsin solution and counted.

The percentage of attached cells was calculated according to the following formula:

$$1-\frac{\textit{The unattached cell count in the plates}+\textit{The atteched cell count in the plates}}{\textit{The total cell count}}\times 100\%$$

The dry microcarriers, consisting of 0.2 g Cytodex 1 or 2.0 g zein microcarriers (with the same surface area), were added to 500 ml spinner bottles (Bellco) and swelled in 50 ml PBS at 37 °C for 6 h, then were autoclaved (HVE-50, HIRAYAMA, Japan) at 121 °C for 40 min. PBS was removed, and 50 ml of fresh MEM containing 10% calf serum was added into the bottles and stayed for 6 h, then Vero cells were seeded. After 24 h incubation, fresh medium was added to reach 200 ml volume (the final cell density was 1 × 105/ml), stirring continuously at a speed just sufficient to keep the microcarriers in suspension (60 rpm) for 5 days. A 1 ml sample containing microcarriers was taken and transferred to a 96-well plate to examine the attached cells. The cell morphology was first observed by staining with AO fluorescent dye in PBS (pH 7.4) for 5 min (Cytodex 1) or 12 h (zein microcarriers). The fine morphology of the Vero cells on the surface of the zein microcarriers was observed by SEM.

2.5. Effect of trypsin treatment on the microcarriers

The sterilized microcarriers were dipped into 0.25% trypsin (0.25 g trypsin powder dissolved in 100 ml PBS, pH 7.4) at 37 °C for 30 min or 2 h. The degradation rate could be calculated by calculating the decrease in the weight of the dried microcarriers.

To evaluate the effect of the enzymatic degradation on cell culture, the attachment and proliferation of the cells on the microcarriers after treatment with trypsin for different times were assessed according to the protocol in Section 2.4, except that the proliferation was carried out in 10 ml glass bottles with 5 ml MEM containing 10% calf serum.

3. Results and discussion

3.1. Preparation of the microcarriers

A variety of solvents, including water, ethanol, glycerol and polyvinyl alcohol, were tried. Glycerol showed the best performance, so glycerol was chosen as the heating medium. When zein particles were heated in glycerol, they became soft, and their rigidity declined. The surface of the particles accepted homogeneous pressure from the surrounding glycerol, and the zein particles easily assumed a round shape. When the temperature rose to 80 °C, the particles began to swell, and all of the particles turned into microspheres when the temperature rose to 120 °C. The microspheres shrank into spherical microcarriers with low porosity during the following cooling process, as shown in Scheme 1.