Cell growth and protein formation on various microcarriers

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Abstract

A large number of microcarriers are commercially available. The capability of cells to successfully proliferate on microcarriers varies with cell lines and media. Choosing the right microcarrier for a particular cell line is more than a choice of a microcarrier. It is part of an integrated process design. A detailed picture of cell growth and product formation will not only be essential in identifying the kind of microcarrier, but also in determining other parts of the process, such as operation mode and media. Our initial screening on thirteen microcarriers showed that cultures on some microcarriers reached a low cell density but high cell-specific productivity, and high density microcarrier cultures have a low specific productivity. The result is a similar product output per unit volume and time for these two types of cultures. An ideal culture system shall have increased volumetric productivity at elevated cell density. This requires the process goal to be incorporated as early as cell line construction and screening. A high output process can then be realized through high density culture.

Introduction

Eukaryotic cells have long been used for the production of recombinant proteins that require exquisite and precise post-transcriptional assembly in order to sustain their biological functionality. Some cells like hybridomas and Namalwas can grow in suspension. Many others are anchorage-dependent or prefer attached growth. Since its introduction in 1967, microcarrier culture has been applied successfully in growing primary cell lines and established cell lines such as CHO and VERO for the production of recombinant proteins (Nilsson, 1988). Several advances have been noted since the onset of the microcarrier culture. Charge group capacity and bead size of microcarriers have been optimized to enhance cell growth. Coating materials like collagen/gelatin, glass, ProNectin and Poly-Lysine have been applied to microcarrier surfaces. More carrier matrix materials have been introduced, including DEAE dextran, collagen/gelatin, glass, polystyrene, polyacrylamide, and cellulose. Spherical microcarriers prevail largely due

to their ease of manufacturing. Recent introduction of porous microcarriers offers large surface-to-volume ratio and maximal achievable cell density (Nilsson, 1988; Miller et al., 1989).

For the production of recombinant proteins, the use of microcarrier culture can greatly simplify perfusion and cell retention, two key factors to attain high cell density and high volumetric productivity. The choice of a microcarrier has more intriguing effects on cell attachment, further proliferation and recombinant protein expression, which are still poorly characterized and understood. The effects also vary widely with cell lines. Initial screening for the best microcarriers is a vital step to identify a more efficient and economic process. Three major interrelated parameters to be considered are the performance of a cultural system, cells, and media, which can be translated into volumetric productivity, cell specific productivity, and product titer, respectively. Or simply the goal of a process is to maximize the amount of products generated per bioreactor volume, per cell, and per medium volume consumed.

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Table 1. A list of microcarriers, sources and properties

Microcarrier	Maker	Type	Matrix	Charge (meq/g)	Density (g/ml)	Size (μm)	Area (cm^2/g)	Load (g/L)	Cell count method
Biosilon	Nunc	solid	polystyrene	–	1.05	160–300	255	20	1
Cytodex 1	Pharmacia	solid	DEAE-dextran	+	1.03	147–248	4400	2.5	1
Cytodex 3	Pharmacia	solid	DEAE-dextran	+	1.04	141–211	2700	2.5	1
Collagen	SoloHill	solid	polystyrene	none	1.03	150–210	325	20	1
Plastic	SoloHill	solid	polystyrene	none	1.03	150–210	325	20	1
FACT	SoloHill	solid	polystyrene	none	1.03	150–210	325	20	1
ProNectin	SoloHill	solid	polystyrene	none	1.03	150–210	325	20	1
Plastic Plus	SoloHill	solid	polystyrene	+	1.03	150–210	325	20	1
Cultispher-G	Hyclone	porous	gelatin	none	1.04	170–270	unknown	1	2
Cultispher-GL	Hyclone	porous	gelatin	none	1.04	170–270	unknown	1	2
Cultispher-S	Hyclone	porous	gelatin	none	1.04	170–270	unknown	1	2
Cytopore 1	Pharmacia	porous	cellulose	+1.1	1.03	200–280	11000	1	3
Cytopore 2	Pharmacia	porous	cellulose	+1.8	1.03	200–280	11000	1	3

We present our initial screening of thirteen commercially available microcarriers. The microcarrier set contains solid and porous microcarriers, microcarriers with various surface coatings or properties, and made from different matrix materials. It has often been hard to differentiate publications on microcarrier cell cultures because of different cell lines, media and process employed. These conditions were kept constant for this microcarrier screening study. The focus has been directed to the potential of a microcarrier for an improved production process. In order to evaluate the process performance parameters, maximal cell density, attachment efficiency, cell growth rate, production rate and major metabolite metabolism have been analyzed. Autonomous cell transfer among microcarriers, an important scale-up issue, is studied, and additional questions raised related to integrated process design.

Materials and methods

Cell line, media and cell cultures

An anchorage-dependent CHO clone, CHO.M-CIF.46, expressing monocyte-colony inhibition factor (M-CIF) was used in the microcarrier comparison. Routine T-flask passage was carried out every 3–5 days at 1:2–1:4 split ratio in MEM alpha medium supplemented with 5% dialyzed fetal bovine serum (DiFBS) and 100 μM methotrexate (MTX). An average of 1.5 T-225 flasks was used to inoculate

each 100 ml culture in HGS-CHO-3 medium, a D-MEM/F12 based medium, containing 1% regular fetal bovine serum and supplemented with glucose and glutamine.

Microcarriers were prepared according to manufacturers' recommended procedures. Before use they were conditioned in the HGS-CHO-3 medium. Microcarriers and their loads for each type of microcarrier are listed in Table 1. Because some of the microcarriers require intermittent stirring during initial attachment stage, all spinner cultures underwent 30 sec stirring followed by 5 min of settling. The process was repeated three times. 70 to 80% medium exchanges were done twice on every the other day following the batch culture. For cultures reaching at least 5×10^5 cells/ml, a 1:5 split was done with respective microcarriers.

Nutrients, product and cell density analysis

Glucose and lactate were analyzed by YSI 2700 Select Biochemistry Analyzer (YSI Inc., Yellow Springs, OH). Ammonia was measured by Kodak IBI Biolyzer (New Haven, CT). M-CIF concentration was analyzed by reverse phase HPLC (HP1100, Hewlett Packard, Wilmington, DE) and ELISA. Product quality was checked with Western Blot gel using day 4 samples in batch cultures. Floating cells and viability were estimated by running 1 ml of culture sample through a 75 μm cell strainer and counting on a haemocytometer using trypan blue stain. Methods for total cell enumeration varied with microcarriers and are listed

Figure 1. Product titers, volumetric productivities and cell specific productivities.

Figure 2. Western blot analysis of various microcarrier batch culture samples taken on day 4. Lanes: 1, 9, 11 and 19, pre-stained marker proteins; 2, Biosilon; 3, Cytodex 3; 4, Collagen; 5, Plastic; 6, FACT; 7, ProNectin; 8, Plastic Plus; 12, Cytodex 1; 13, Cytodex 3; 14, Cultispher-G; 15, Cultispher-GL; 16, Cultispher-S; 17, Cytopore 1; 18, Cytopore 2; 10 and 20, purified recombinant M-CIF standard. The apparent molecular masses of the marker proteins are given on the right.

Table 2. Cell attachment, growth and productivity

Microcarrier	Attachment		Cell growth		Floating Cells %	Batch		Media exchanges		
	1 hr %	1 day %	Maximal 10 ⁶ /ml	Batch folds		HPLC mg/L	ELISA mg/L	mg/L	mg/L/day	mg/billion cells/day
Biosilon	19.9	22.2	0.829	2.69	6.1	11.72	13.43	14.88	5.08	7.02
Cytodex 1	86.5	99.0	1.73	4.15	0.82	13.64	14.36	15.35	5.21	3.31
Cytodex 3	43.9	97.2	2.32	4.08	1.88	14.19	16.01	16.39	5.54	2.87
Collagen	30.9	27.2	0.496	1.22	20.2	11.19	10.98	11.96	3.38	7.93
Plastic	26.9	17.2	0.386	1.60	90.0	11.70	12.02	9.443	2.34	12.60
FACT	12.6	69.1	0.815	2.93	14.1	11.32	14.35	13.54	3.89	5.76
ProNectin	37.6	15.2	0.385	2.01	32.6	10.54	11.80	9.04	2.31	8.88
Plastic Plus	18.1	51.5	0.346	1.77	13.6	10.65	11.69	7.57	2.18	6.99
Cultispher-G	4.5	13.9	0.698	1.59	37.4	n/a	8.12	5.80	2.50	6.03
Cultispher-GL	0	39.1	0.475	1.22	51.0	n/a	6.81	3.11	1.02	3.21
Cultispher-S	35.6	47.3	1.16	1.83	15.3	n/a	10.23	14.74	6.74	7.73
Cytopore 1	73.5	100.0	2.00	2.31	0	9.87	n/a	10.99	4.22	2.27
Cytopore 2	84.0	99.4	2.08	2.57	0.32	10.26	14.03	13.56	5.40	3.60

in Table 1. Cell count method #1 involved spinning down 1 ml well-mixed microcarrier containing culture sample and counting cells with a haemocytometer using crystal violet stain. Cell count method #2 involved spinning down cells and microcarriers, and incubating with collagenase. After the digestion of microcarriers, cells were counted using trypan blue stain method. Cell count method #3 was almost the same as cell count method #1, except that before loading onto a haemocytometer, the sample was vigorously mixed through a 30 gauge needle and syringe.

Results and discussion

Cell attachment, maximal density and growth

Initial attachment of anchorage-dependent cells to a substrate affects later proliferation and protein expression. In Table 2, the percentages of attached viable cells to the total number of viable cells are listed for 1 hr and 1 day after inoculation to represent the rate and the equilibrium of attachment. Cytodex 1, Cytodex 3, Cytopore 1, and Cytopore 2 had very good initial attachment. Within a day, more than 97% of the viable cells attached onto the four microcarriers. While these four microcarriers were either solid or porous and made of DEAE-dextran or cellulose, they shared one common characteristic in that they all possess a positive charge. This charge affect can also be seen from 1 hr attachment percentages (Table 2).

High charged Cytodex 1 and Cytopore 2 microcarriers have a high attached cell percentage, thus a faster attachment rate than their low charged counterparts, Cytodex 3 and Cytopore 1 microcarriers. The effect of the charge was also shown in the rest of the microcarrier set. Besides these four microcarriers, the only positive charged microcarriers were FACT and plastic plus microcarriers. They had better attachment than the rest of the microcarriers with more than 50% viable cells attached. For this particular CHO cell line and the experimental conditions, the surface charge density had a significant influence on cell attachment. Non-charged collagen and ProNectin coated microcarriers as well as gelatin-made CultiSpher microcarriers had less than 50% cells attached by day one. Various coatings on microcarriers did not seem to improve attachment. Given a large number of references where mostly primary cells had been satisfactorily grown on these microcarriers (Mignot et al., 1990; Reiter et al., 1990; Nikolai and Hu, 1992; Varani et al., 1992; Schrimpf and Friedl, 1993; Varani et al., 1993), we considered the coatings to be less important for an established cell line such as the CHO cells in the study. Overall, initial attachment and cell growth were good on DEAE-dextran and cellulose microcarriers, but not on gelatin and polystyrene.

The initial attachment has significant impact on further cell proliferation. Table 2 shows the maximal cell densities reached during repeated medium exchanges and the folds of cell density increased from

Figure 3. Specific glucose consumption and lactate generation.

Table 3. Summary of microcarrier-to-microcarrier cell transfer

Microcarrier	Percentage of cell-containing microcarriers		
	1 hour	1 day	4 day
Biosilon	19	29	59
Cytodex 1	28	100	100
Cytodex 3	26	100	100
Collagen	20	32	38
FACT	17	29	42
Cultispher-G	24	27	36
Cultispher-GL	18	31	35
Cultispher-S	25	37	68
Cytopore 1	24	75	100
Cytopore 2	28	84	100

day one to day four during batch cultures. The reason for selecting cell density increase from day one was to eliminate potential differences in lag time of various microcarriers, so that the increase could reflect cell growth rate. Cytodex and Cytopore microcarriers

that had good initial attachment reached the highest maximal cell densities among all the microcarriers. From day one to day four in the batch culture, cell densities increased more than 4 times on Cytodex 1 and 3, and more than 2 times on Cytopore 1 and 2. At the end of medium exchanges, these four microcarriers reached similar cell densities around 2×10^6 cells/ml. This indicates that cells grew faster during batch culture on Cytodex microcarriers, and during subsequent media exchanges growth rates were fast on Cytopore microcarriers since they reached a similar cell density during media exchange. Other microcarriers failed to attain high cell densities. Some of these microcarriers had nearly 3 fold cell density increase. However, most of the viable cells were presented in floating form, as can be seen from the percentage of viable cells in the form of floating cells during the medium exchange (Table 2). Having a large fraction of floating cells is a disadvantage in perfusion culture. As perfusion is a necessity to high density cell culture, floating cells are difficult for cell retention and selective removal of dead cells.

Cytodex and Cytopore microcarriers reached a cell density of about 2×10^6 cells/ml and contained

less than 3% of viable cells in floating form. They are suitable for high cell density culture. Because Cytopore microcarriers have more available surface areas, low floating cells, and high cell growth during medium exchanges, it is expected that Cytopore microcarriers can support high cell density culture better than Cytodex microcarriers. Cell growth was limited by available surface in Cytodex microcarriers. The Cytopore microcarriers are porous and have more surface areas. Microscopic inspection showed Cytodex microcarriers were almost confluent at the end of repeated media exchanges. However, cells only sparsely occupied Cytopore microcarriers. Considering cell growth in spinner culture is also limited by nutrients, by-products and oxygen, Cytopore microcarriers would yield a high cell densities to fully utilize the available microcarrier space if these conditions are met in bioreactor operation. Further differentiation of the four microcarriers will have to be evaluated in bioreactors. The achievable maximal cell density is one of the considerations in selecting a microcarrier. If a bioreactor contains a high number of cells as miniature producing units, volumetric productivity would thus be improved, which means a high system performance and a fully utilized bioreactor as a major capital investment. A good system performance also relies on bioreactor design, process optimization and medium formulation, which also affect system performance.

Productivity and product formation

Cell density and growth are not the final goal. For the production of recombinant proteins, the ultimate goal is an economic and effective process, which maximizes volumetric productivity, cell specific productivity and product titer. Cell line construction, bioreactor operation and media all have impact on the goal. The choice of a microcarrier is no exception.

Despite the cell densities varying as much as 5 folds in the four day batch cultures, most of the accumulated product titers during the period were close to each other, in the range of 10.23–16.01 mg/L, with the exception of CultiSpher-G and CultiSpher-GL microcarriers, which were under 8.2 mg/L (Figure 1A). A high titer will facilitate purification and also represents efficient use of media. During subsequent medium exchanges, Biosilon, Collagen, Cytodex 1, Cytodex 3, FACT, CultiSpher-S, Cytopore 1 and Cytopore 2 reached titers equivalent or higher than those of the batch cultures. The other microcarriers all had reduced titers. Volumetric productivities were dropping

during the batch culture (Figure 1B), even though cell densities were increasing. In repeated medium exchanges, most of microcarriers showed higher volumetric productivity. Specific productivities were also dropping during the batch culture (Figure 1C). The titers, volumetric productivity and cell specific productivity at the end of second medium exchange are listed in Table 2. Results from both reverse phase HPLC and ELISA were shown to be consistent (Table 2). During medium changes, the microcarrier cultures with low cell densities, like plastic, collagen and Biosilon microcarriers, exhibited high specific productivity. The reason for running separate ELISA was that materials released from all three CultiSpher microcarriers interfere with HPLC analysis. There were huge peaks covering product peaks. These peaks were present only in microcarrier-conditioned media and were removable by three media washes. However, the peak came up again after 1 hr incubation. Incubation with collagenase eliminated the peaks, but introduced many collagenase peaks, which also interfered with HPLC assay. Quantification of CultiSpher microcarrier product levels were performed with ELISA assays. The CultiSpher-S, being tightly cross-linked, released much less such material than the other two.

To examine any significant changes in product formation using different microcarriers, crude cell culture supernatants were analyzed by Western-blot assays. Affinity-purified polyclonal rabbit antibody against M-CIF was used in the assays. The non-reduced Western-blot gel results for day 4 batch culture samples are shown in Figure 2. No band location and antibody-binding specificity changes were noticed compared to M-CIF purified from a previous batch, which has shown biological activity in various cell-based and animal model studies.

The question remains as to which microcarriers are best suited for the production of recombinant proteins. As stated before, the goal of a process is to increase volumetric productivity, cell specific productivity and product titer. A high cell density culture is not the final goal, but one of the vehicles to attain the goal. Among the productivities, the volumetric productivity is a more direct measure of how fast products can be made. From the maximal cell density and the productivity data at the end of medium exchanges in Table 2, Cytodex and Cytopore microcarriers reached cell densities of $1.73\text{--}2.32 \times 10^6$ cells/ml and volumetric productivities of 4.22–5.54 mg/L/day. Biosilon, CultiSpher-S and FACT had low cell densities, only around half of those of Cytodex and Cytopore microcarriers, and they

had similar volumetric productivities of 5.08, 6.74 and 3.89 mg/L/day, respectively. The final titers after the medium exchanges were also similar in the range of 10.99–16.39 mg/L. For the low cell density Biosilon, CultiSpher-S and FACT cultures, cell specific productivities were high around 5.76–7.73 mg/billion cells/day. The high cell density Cytodex and Cytopore microcarriers showed low specific productivities of 2.27–3.60 mg/billion cells/day.

It sounds as though production at a certain rate can be attained by high density cultures in which specific productivity is relatively low. It can also be achieved by low density cultures where cell density specific productivity is high. The situation is not ideal. An ideal case will be a cell line that remains or has increased specific productivity when cell density is high and growth is slow, so that volumetric productivity will increase with cell density. The consideration should be taken into account at the time of cell line selection, transfection vector design, and clone screening. For example, at the stage of clone screening, screening criteria should not just measure accumulative product levels during initial growth, by which the resulting clones are more likely to have growth-dependent expression. Looking at expression of the clones at the stationary stage early on will help identify clones ideal for large-scale production.

Also from the specific productivity data, Figure 1C, specific productivities decreased during batch cultures when cell density increased. It suggests product synthesis was affected by the combination of cell growth rate, cell density, cell cycle, product and by-products or other interchangeable molecules among cells. During repeated medium exchanges, specific productivity all increased from the fourth day of batch cultures. Materials secreted by cells did have some effect on product synthesis. Further productivity gain can be obtained from removal of these materials, such as in a perfusion culture.

Metabolism

Like volumetric productivity and cell specific productivity, specific glucose uptake and lactate generation also exhibited similar patterns where consumption and generation were gradually decreasing during batch cultures (Figure 3). The profile of glucose and lactate specific rates appeared almost duplicates of each other. It indicates that the majority of consumed glucose went to lactate. The efficiency of glucose would be much higher if it did not go through a lactate

metabolic pathway. Media design based on metabolic consumption and controlled delivery of key nutrients can improve the efficiency of nutrient usage and reduce by-product formation, thereby providing space for additional productivity gain.

Cell transfer

Large-scale protein production from any eukaryotic cells requires a series of scale-up steps. Autonomous cell transfer from microcarriers to microcarriers is a desired feature to facilitate the scale-up of microcarrier cell culture. Because of the different extent cells attached to a microcarrier due to microcarrier types, matrix material and surface property, cell transfer will also be affected. For microcarrier cultures reaching at least 5×10^5 cells/ml at the end of repeated media exchanges, a 1:5 split was carried out. Quantification of cell transfers was done by counting bare microcarriers in 100 random selected microcarriers under petri dishes. Table 3 shows the percentage of cell carrying microcarriers at hour one, day one and day four, after the split. Cytodex 1 and Cytodex 3 had the fastest cell transfer. By day one, all fresh microcarriers contained transferred cells. Cytopore 1 and Cytopore 2 had a slow startup, about 80% of total microcarrier occupied by cells after a day. By day four, all Cytopore microcarriers contained cells. Since most of the cells resided inside the porous Cytopore microcarriers, fewer cells presented on the surface might slow down the cell transfer. All the other microcarriers still could transfer cells to fresh microcarriers, but failed to grow on all microcarriers, as none of them could utilize 70% of total microcarriers. Even from the seed culture after two medium exchanges, bare microcarriers were present in these microcarrier cultures. There might be subtle differences on the microcarriers, introduced either during manufacturing or initial microcarrier preparation. The failure of cells to grow on all CultiSpher-G and CultiSpher-GL microcarriers might be due to the fact that released gelatin promotes the growth of cells in suspension. All the cultures where microcarriers failed to be fully utilized by cells contained high floating cell counts. From Table 2, Cytodex and Cytopore microcarrier cultures contained very few viable cells in floating form. It is considered that cell transfer among microcarriers is not through floating cells, rather through transitional microcarrier contact. This assumption will also explain why Cytodex and Cytopore microcarriers

showed a difference in cell transfer rate while having essentially the same initial attachment.

Conclusions

This initial microcarrier screening laid the ground work for cell growth and cell transfer on microcarriers, which is cell line and medium-specific and will be applicable to other clones derived from the same host cell line. With the goal of a process identified, the choices of microcarriers are largely dependent on cell growth and product synthesis. To increase volumetric productivity is to increase the product of cell density and specific productivity. The largest variation of specific productivity increase can be expected from different clones. Additional specific productivity gain can be attained by feeding mode design to reduce by-product inhibition and increase nutrient usage efficiency. Overall, to lock specific productivity in a certain stage is not as easy as reaching a high cell density. The recommended procedures would be to identify microcarriers supporting high cell density, look for clones that have an increase (or less of a decline) in specific productivity, and then optimize the high cell density culture for additional productivity gain.

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