Microsupport with two-dimensional geometry (2D-MS)

In situ determination of the growth kinetics of anchorage-dependent cells by Laser Diffraction Particle Sizing (LDPS)

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Abstract

The procedure used to establish *in situ* (without cell trypsinization) the growth kinetics characteristics of anchorage-dependent cells propagated on microcarriers by Aperture Impedance Pulse Spectroscopy can be replaced by a novel method based on the time-dependent shifts of the size distribution histograms of cell-laden microcarriers. This we have named Laser Diffraction Particle Sizing.

Abbreviations: ADC, Anchorage-dependent cells; AIPS, Aperture Impedance Pulse Spectroscopy; LDPS, Laser Diffraction Particle Sizing; M, Mode; PCN, Peak Channel Number; ρ , cell density; rpm, revolutions per minute; 2D-MS, microcarrier with two-dimensional geometry (flake); 3D-MS, microcarrier with three-dimensional geometry (bead).

Introduction

Till now, Aperture Impedance Pulse Spectroscopy (AIPS) is the only established method to determine *in situ* (without cell trypsinization) the cell density ρ , i.e. the number of cells attached per individual microcarrier particle with two-(flakes) or three-(beads) dimensional geometry (2D-MS or 3D-MS). With this method, growth kinetics of anchorage-dependent cells (ADCs) propagated on microcarriers is established by recording the time-dependent shift towards higher values of the Peak Channel Number (PCN). Several technological difficulties hamper the development of a biomass probe based on AIPS. To bypass these, other approaches have been evaluated.

The results presented in this article show that Laser Diffraction Particle Sizing (LDPS) offers an alternative to AIPS.

Materials and methods

The physico-chemical characteristics of MicroHexa-

gon-shaped MicroHex (i.e. tissue culture grade microsupport with 2D-geometry manufactured by Nunc A/S) (Gatot et al., 1998), the method to determine the cell density ρ (number of cells per microsupport particle) and the PCN (Peak Channel Number) together with the rationale underlying AIPS, have been already described (Degouys et al., 1996a, b, c).

Laser Diffraction Particle Sizing (LDPS) using Malvern's 'Mastersizer 2000' granulometer and the Hydro S dispersion unit is performed according to the manufacturer after a ten fold dilution of the sample with PBS (Phosphate Buffered Saline). Inoculation of a culture is as follows: 1.32 g of MicroHex (950 cm² g⁻¹) and 3×10^7 anchorage-dependent recombinant CHO cells are incubated in 75 ml DMEM-F12 containing 10% newborn calf serum (v/v) in 250 ml nominal capacity Techne spinner vessel gassed with a mixture of 5% CO₂ in air (T₀). To allow cell attachment, the suspension is kept agitated at 18 rpm during 17 hr. To ensure exponential growth throughout the experiment, growth medium is renewed 48 and 72 hr after inoculation. At T₁₇, 75 ml of fresh prewarmed complete growth medium is added (final volume =

Figure 1. Influence of pump/stirrer speed (rpm) on the apparent size of naked MicroHex. Hundred ml of suspension containing 0.088 g of MicroHex ($950 \text{ cm}^2 \text{ g}^{-1}$) is submitted to various pump/stirrer speeds and the mode of the corresponding size distribution histograms is measured.

Figure 2. Influence of the concentration of naked MicroHex (g ml^{-1}) on the measured percent obscuration.

Figure 3. Growth kinetics of CHO cells on MicroHex ($950 \text{ cm}^2 \text{ g}^{-1}$). A culture is inoculated as described in the section Materials and Methods. At preset times, the time-dependent shifts of ρ are measured by AIPS (PCN) and LDPS (Mode).

150 ml). From 17 hr onwards, agitation is increased from 18 to 28 rpm and kept at that value. Twenty ml of the culture are sampled daily for the simultaneous determination of the particle size distribution by AIPS (PCN), by LDPS (Mode) and determination of the cell density ρ by crystal violet staining. Experiments are terminated when confluence is reached i.e. when seven 20 ml samples have been taken from the initial 150 ml culture. Notwithstanding the progressive decrease of culture volume (only 10 ml remains after the seven samples) no significant deviation is observed in the first order reaction kinetics characteristic of exponential growth.

Results

Figure 1 shows the influence of pump/stirrer speed (operating between 1000 and 3000 rpm) on the size distribution histograms of naked MicroHex analysed with a laser diffraction granulometer.

Irrespective of the pump/stirrer speed used to keep naked MicroHex in suspension, all size distributions are gaussians, the mode (M) of which decreases progressively between 1000 and 3000 rpm (Figure 1).

To minimise cell detachment during the time needed to establish the size distribution histogram, whatever the age of the culture (cells from 65 hr-old culture begin to detach more readily than those from a 41 hr-old one), all samples in this work have been analysed at 1250 rpm during 30 sec only. (Cells from 65-hr-old cultures resist 3–4 min agitation in PBS at 1250 rpm).

The number of MicroHex particles per gram of dry microcarrier is a constant (a function of the geometry and of the size of the microcarrier), in our case: 1.19×10^6 particles g^{-1} . For a given experiment, N, the total output of ADCs is obtained by multiplying the number of MicroHex added to the system (i.e. the number of MicroHex particles per dry weight, $1.19 \times 10^6 \times W$, where W is the weight of dry microsupport used) by the cell density ρ (number of cells attached per MicroHexagonal particle):

$$N = W \times 1.19 \times 10^6 \times \rho . \quad (1)$$

W, the weight of MicroHex added is known at the onset of a culture. However due to losses of microcarrier (in the pipets, on glass walls, etc.), the actual value of W can be smaller. Nevertheless, this value can be accurately determined by calibrating beforehand the granulometer ran at a constant stirrer/pump speed of

1250 rpm with naked MicroHex suspensions of different concentrations, simultaneously measuring the corresponding obscuration in % (i.e. laser light lost due to the introduction of MicroHex particles within the laser beam).

Under these conditions for MicroHex concentrations between 0.008 and 0.015 g ml^{-1} , all size distribution histograms overlap one another (figure not shown). In addition the percent obscuration varies linearly with the concentration of naked MicroHex particles (Figure 2).

The first data suggesting that the mode value, M, which characterises the size distribution histogram, as determined by LDPS, can be used as an alternative to AIPS to measure cell density, stems from the almost perfect coincidence observed between the time-dependent shifts of M and PCN (Figure 3).

For the recombinant CHO cells used in this study, Figure 4a confirms the linear relationship between the cell density ρ and the PCN established earlier. Figure 4b shows the same relationship to hold between ρ and M (Figure 4).

The minimal distance observable between the modes of two size distribution histograms is 2 microns. Resolution, defined here as the culture time elapsed between two mode values separated by 2 microns, varies depending on the age of the culture. Indeed, because of the progressive reduction of growth rate characteristic of cells approaching confluency, resolution will be less than that calculated from a 48-hr-old culture, when cells are multiplying exponentially. Based on this criterion, resolution afforded by LDPS is 4 hr, as calculated from a '48-hr-old' culture used as standard.

Discussions

Establishing *in situ* (without detaching the cells) the growth curve of anchorage-dependent cells can be achieved by recording the time-dependent shift of PCN as obtained by Aperture Impedance Pulse Spectroscopy (AIPS) (Degouys et al., 1996a, b, c; Miller et al., 1986).

For eight established cell lines, electronic sizing by AIPS of cell-laden microbeads (microsupport characterized by a three-dimensional geometry: 3D-MS) has been shown to be an accurate descriptor of ρ , the cell density (number of cells attached per microsupport particle) (Degouys et al., 1996c). This observation makes it highly likely that, the results obtained with

Figure 4. ρ/PCN and ρ/Mode double plots of the growth kinetics for CHO cells on MicroHex ($950 \text{ cm}^2 \text{ g}^{-1}$). The PCN, M and cell density of the culture whose growth kinetics are described in Figure 3 are plotted as ρ/PCN (a) and ρ/Mode (b).

AIPS and microbeads can be extended and generalized to all existing anchorage-dependent cells.

Because of the centrifugal pump and type of helix characteristic of the dispersion chamber of Malvern's granulometer used to keep particles in suspension during the time needed to perform a measurement, it came to us as a total surprise that cell-laden MicroHex effectively resist this type of treatment.

Before investing in depth the potentials offered by LDPS, conditions have been sought to keep cell detachment to a minimum. This has been obtained

by performing the measurements at 1250 rpm during 30 sec only.

Transfer of microsupport from a sterile stock suspension into a bioreactor can result in extensive losses of MicroHex along the walls of the pipettes and/or glass vessels being used. The actual amounts of microsupport present in the bioreactor which may therefore differ considerably from the calculated ones, can nevertheless be accurately determined, before addition of the cells, following calibration of the granulometer with MicroHex suspensions of known concentration.

With the actual value of W in Equation (1) being now determined, the only parameter needed to calculate the bioreactor's cells output is the cell density ρ .

Our results show that for each time point during a growth experiment, when the cell density ρ (as determined by counting under the microscope the nuclei from cells stained with crystal violet) increases exponentially, so do the PCN and M .

The perfect match between the time-dependent exponential shifts of PCN and M makes the latter a descriptor of ρ as accurate as the PCN. This is further reflected by the linear relationship observed between ρ and M .

For all these reasons, it is assumed that the use of M as a surrogate for the measurement of ρ , can be generalized to all existing anchorage-dependent cell lines whether these are grown on 3D-MS (microbeads) or MicroHex (microsupport with two-dimensional geometry).

Resolution afforded by LDPS is defined here as the time (hours) separating the modes of two size distribution histograms 2 microns apart, taking the 48-hr-old culture as reference. Our results show that, based on this criteria, 48- and 52-hr-old cultures can just be discriminated one from the other. This 4-hr resolution is comparable to that afforded by AIPS (Miller et al., 1986).

As of today, evaluation of ρ by LDPS is as cumbersome and time-consuming as that which relies on AIPS.

The choice between these two methods will depend upon which instrument will allow the construction of an *in situ* biomass probe which would also be

steam-sterilizable and cleanable in place, able to perform on-line, non invasively, and to provide real time estimations of the biomass during extended periods of time.

Such a probe will open a wealth of new opportunities in the field of mammalian cell culture automation.

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