Appl Biochem Biotechnol (2010) 160:1600–1615
DOI 10.1007/s12010-009-8674-0

Rapid Screening of Serum-Free Media for the Growth of Adherent Vero Cells by Using a Small-Scale and Non-invasive Tool

Emma Petiot · Frantz Fournier · Cécile Gény · Hervé Pinton · Annie Marc

Received: 2 February 2009 / Accepted: 18 May 2009 /
Published online: 9 June 2009

Abstract The paper proposes a rapid screening method for a first step improvement of an animal component-free medium dedicated to the growth of the anchorage-dependent Vero cell line. A new, rapid, and non-invasive technique is presented to specifically monitor cultures of adherent cells in 96-well plates. The operating conditions of an image analyzer are adapted to take into account the decrease of cell size when the attached cell density increases. An experimental design is carried out to assess the influence of ten component groups in the original medium. Two groups including protein extracts, growth factor, insulin, glucose, and pyruvate show significant positive effects. The groups with vitamins and molecules related to nitrogenous bases display a less pronounced influence. The mixture of amino acids, B_1 vitamin, magnesium sulfate, and sodium phosphate as well as the couple sodium citrate and ferric chloride lead to a downward trend. The screening results are proved to be scalable in stirred cultures with cells on microcarriers. An improved serum-free medium, with some component groups being removed or added, can be rapidly formulated to reach respectively similar or 1.6 times higher cell density than in the original medium. The results from this global approach could be helpful to further focus experiments on identified medium components.

Keywords Adherent Vero cells · Serum-free medium · Small-scale screening · Non-invasive cell counting · Microcarriers

Nomenclature

a_x	Coefficients of the polynomial model (x=1 to 10)
C	Cell concentration (cell ml^{-1})
DoE	Design of experiments
M_R	Reference medium

E. Petiot · F. Fournier · A. Marc (✉)
Laboratoire des Sciences du Génie Chimique, UPR CNRS 6811, Nancy-Université,
2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France
e-mail: annie.marc@ensic.inpl-nancy.fr

C. Gény · H. Pinton
Sanofi pasteur, 1541 avenue Marcel Mérieux, 69280 Marcy L'Etoile, France

M_C Complete medium
M_x Screening media formulated for DoE (x=1 to 16)
PA "Cellscreen" device module for adherent cell analysis
PS "Cellscreen" device module for suspension cell analysis
G_x Groups of components present in M_C medium (x=1 to 10)
WS Percentage of well surface covered by Vero cells in the 96-well plates (%)

Introduction

Large-scale mammalian cell cultures are nowadays extensively used for biological productions such as monoclonal antibodies, proteins, or vaccines [1]. The various cell lines cultivated for all these production processes display specific needs in particular for the culture media composition [2]. On the other hand, due to considerations of media safety, variability, and cost, and despite the cell growth stimulation by animal sera or animal-derived substances, serum-free and animal-free culture media have been extensively developed and established for large-scale production for 20 years [1]. While these new medium formulations have been mainly proposed for cultures of suspension cells producing recombinant proteins and antibodies, the challenge seems more complicated for classical processes of vaccine production which involve anchorage-dependent cells such as Vero cells. Additional molecules could then be required to promote cell attachment, cell detachment, cell growth, or virus production [3–5].

Vero cells, derived from African green monkey kidney, are extensively used in the manufacture of human and veterinary vaccines such as rabies, polio, and Japanese encephalitis vaccines [6–9]. Indeed, they have been shown to be good substrates for the propagation of numerous viruses with frequently higher titers than that reached using other type of cells. Existing processes often involve the culture of Vero cells on solid microcarriers in large bioreactors up to 6,000 l. Several works in the literature have proposed the use of serum-free medium for these productions [7, 10, 11], but very few of them focused on fully animal-free process in stirred tank bioreactor [12, 13]. So, the validation of such completely defined media adapted to Vero cells issued from new serum-free master cell banks remains a challenge for running industrial processes or for the ones still under development.

The optimization of serum-free medium formulations is generally time-consuming, and incremental improvements could take several years if studying one-factor-at-a-time effect [13]. Most of the recent media optimization techniques are using design of experiments (DoE) and statistical analysis which provide easier and faster information on medium component effects [14–16]. Nevertheless, these methods often require high number of experiments and, consequently, the availability of easy-to-use, rapid, reproducible and non-invasive culture systems become essential for the optimization of animal cell culture media.

The cell growth data acquisition in a non-invasive way remains one of the main issues to the use of small-scale culture systems for easy and rapid media screening. Presently, most of the classical culture systems require sampling of the culture medium for further cell density evaluation by manual or automatic cell counting [17–19]. Besides, new screening devices have been recently proposed to permit the simultaneous test of numerous culture conditions with on-line cell density quantification by absorbance measurement [20]. But until now, only the "Cellscreen" system, based on cell counting in 96-well plates by optic image analysis, has been proposed to non-invasively monitor the cell growth of both suspension and adherent cells [21]. The only published work about its use to follow adherent cell growth concerns liver progenitor cells (LPC) for medical study purpose [22]. But in this study, the "Cellscreen" was not considered as a medium screening tool coupled to a DoE methodology.

This work aims at proposing a rapid, easy and small-scale protocol to improve a serum-free medium, without any animal products and with a very low protein content, for industrial Vero cell growth on microcarriers in stirred cultures. A procedure based on a screening DoE method is developed to evaluate the influence of various groups of the components which composed the original medium. The operating conditions for a valuable non-invasive monitoring of Vero cell growth by the "Cellscreen" device are carefully examined. Kinetics of Vero cell growth in various media formulations are investigated in well plates. Then, the critical component groups which affect cell growth are identified and the effect trends are used to propose first medium improvement and simplification in stirred culture conditions.

Materials and Methods

Cell Line and Culture Conditions

The industrial Vero cell line used in this study was provided by Sanofi pasteur (Marcy L'Etoile, France) at the passage 140 from a serum-free master cell bank. The confidential serum-free culture medium used was entitled complete medium, M_C. The Vero cells were cultivated at 37°C and 5% CO_2 for either static or agitated cultures, without exceeding the passage 148 to avoid cell variability. Cells were expanded in static culture T-flasks (Fisher Bioblock Scientific, France) with a seeding density of 3.2×10^5 cell ml^{-1}. Culture experiments for "Cellscreen" assays were performed in 96-well plates (Nunc, Rochester NY, USA) seeded with cell densities ranging from 0.08 to 6×10^5 cell ml^{-1} in a volume of 200 µl per well. Cells were all seeded from the exponential growth state of the amplification culture. Each tested condition was performed in eight parallel wells of the same 96-well plate and each experiment (plate) was duplicated. The stirred cultures were performed in spinner flasks (Techne, UK) with a seeding cell density of 2×10^5 cell ml^{-1} and 2.5 g l^{-1} Cytodex 1 microcarriers (GE Healthcare Bioscience-AB, Sweden) in 250 ml working volume. Stirring speed was maintained at 45 rpm by the use of a magnetic stirrer (Techne). Twice a day, 4-ml samples were taken for further analyses. Since the Vero cell adherence, either on well surface or on microcarriers, takes about 4 h at 37°C, 5% CO_2, image acquisition in 96-well plates of attached cells or sampling in spinner flasks were both performed after at least 4 h of culture. Different formulations of serum-free media were used as further described.

Cell Counting

Manual Method

After cell dissociation by trypsinization in T-flasks or well plates, the viable cell density was determined by using Thoma hemacytometer (Preciss, France) and Trypan blue dye exclusion method with 10% accuracy [23]. For the numeration of viable cells attached on microcarriers, samples were washed twice with PBS after microcarrier settling and then treated with Crystal violet (Sigma, France) for at least 1 h at 37°C prior to cell numeration of the released nucleus on Fush-Rosenthal hemacytometer (Preciss, France) with 15% accuracy.

Automatic Cell Counting in 96-Well Plates

The "Cellscreen" system (Innovatis, Germany) was employed for numeration of cell growing in 96-well plates. This device used a digital image analyzer with pattern recognition strategies to

quantify cell density. It was composed of a microscope coupled to a CCD camera recording images of the wells and of a motorized tray on which 96-well plates were automatically moved under the microscope. Images were further analyzed by an image recognition software to detect cells on the well surface [21]. This device could be used either with a PS module to evaluate the concentration of suspension single cells, or with a PA module to determine the percentage of well surface covered by adherent cells (Fig. 1). To remove eventual failure of the software image analysis, "Cellscreen" automatic determination was visually checked by controlling the suspension cell selection or the adherent cell surrounding on recorded images, and inaccurately treated images were further suppressed. The relative standard deviation of the well replicates for each condition (eight wells) always represented less than 10%. In this study, the PS module was used to numerate suspension cells just after seeding time or after well trypsinization, while the PA module was used to monitor the adherent cell growth all over the cultures.

Screening of Serum-Free Medium Components

Reference Medium and Component Groups for Experimental Design

In this work, the improvement of the complete medium, M_C, was based on the study of its composition effect on Vero cell growth. The original complete medium M_C was developed several years ago by Sanofi pasteur to specifically cultivate Vero cells in vaccine production processes, but its formulation was not obtained by a similar screening method. Because of the high number of components in this medium, it was decomposed in ten groups called G_1 to G_{10}, on the basis of component nature or solubility. Due to patent right, the G_{10} group can not be indicated. The composition of other groups was as follows: G_1 (20 free amino acids, vitamin B_1, magnesium sulfate, sodium phosphate); G_2 (vitamins H, B_2, B_9, and molecules related to nitrogenous bases: thymidine, uracil, xanthin, hypoxanthine); G_3 (vitamins B_{12}, B_3, B_7, choline chloride, pyridoxal); G_4 (vitamins B_3, B_6, B_X, putrescin); G_5 (vitamins A, D_2, K_3, linoleic and lipoic acids); G_6 (2 deoxyribose, adenine, adenosine, ethanolamine); G_7 (plant and yeast extracts, recombinant Epidermal Growth Factor (rEGF),

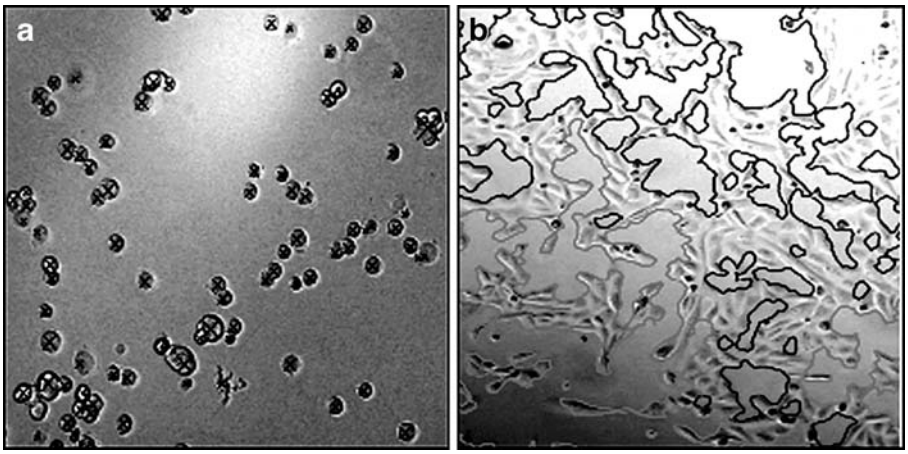

Fig. 1 Cell concentration evaluation by "Cellscreen" analyzer with PS (**a**) or PA (**b**) modules. With the PS module, cell recognition was based on size range and counted cells were marked with *black cross marks*. With the PA module, the percentage of surface covered by cells was visualized with *gray* (occupied zones) and *black lines* (cell-free zones)

insulin); G_8 (sodium citrate, ferric chloride); and G_9 (glucose, pyruvate). Due to their very low concentrations in the M_C medium, trace elements were not included in these groups. To easier screen the effects of the G_x groups on Vero cell growth, a reference medium, M_R, was defined as the lowest concentration of the complete medium, M_C, which permitted cell growth. It was determined by several dilutions (from 1/3 to 1/20) of M_C in a dilution buffer. This dilution buffer was formulated from Hepes, salts (D-calcium pantothenate, $NaHCO_3$, NaCl, $CaCl_2$, KCl, and $MgCl_2$), and vitamin C, at concentrations equivalent to those found in the complete medium. Then, a 1/20 dilution of M_C, which allowed three times lower cell-specific growth rate than in M_C medium without cell detachment, was chosen as the M_R medium.

Fractional Factorial Design

Fractional factorial designs are experimental designs generally dedicated to the first step of the culture medium optimization [24]. They are very useful and efficient in identifying the most important nutrients and interactions between two or more nutrients with relatively few experiments in comparison with the one-factor-at-a-time technique. In our case, a two-level fractional factorial design was implemented as experimental design to study the impact of the G_x groups on the cell growth at high (+1) and low (−1) levels. The low levels corresponded to their concentration in the reference medium, M_R, while the high levels referred to concentrations equivalent to the complete medium, M_C. More precisely, the high levels were attained by enriching the reference medium, M_R, with G_x component groups as defined by the DoE. As a result, the fractional factorial design proposed the formulation of 16 M_x screening media (Table 1).

Table 1 Fractional factorial design for the formulation of the screening M_x media.

Screening media M_x	Groups of medium components, G_x									
	G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_8	G_9	G_{10}
M_1	−	−	−	−	+	+	+	+	+	+
M_2	+	−	−	−	−	−	−	+	+	+
M_3	−	+	−	−	−	+	+	−	−	+
M_4	+	+	−	−	+	−	−	−	−	+
M_5	−	−	+	−	+	−	+	−	+	−
M_6	+	−	+	−	−	+	−	−	+	−
M_7	−	+	+	−	−	−	+	+	−	−
M_8	+	+	+	−	+	+	−	+	−	−
M_9	−	−	−	+	+	+	−	+	−	−
M_{10}	+	−	−	+	−	−	+	+	−	−
M_{11}	−	+	−	+	−	+	−	−	+	−
M_{12}	+	+	−	+	+	−	+	−	+	−
M_{13}	−	−	+	+	+	−	−	−	−	+
M_{14}	+	−	+	+	−	+	+	−	−	+
M_{15}	−	+	+	+	−	−	−	+	+	+
M_{16}	+	+	+	+	+	+	+	+	+	+

High (+) and low (–) levels for serum-free medium component groups, G_x, correspond respectively to concentration in M_C or M_R media

Analysis of the DoE Results

Experimental results obtained by "Cellscreen" monitoring during the serum-free media screening experiments were treated in two steps. In the first step, for each medium and each culture time, the percentage of well surface covered by the cells (WS, percent) and the cell concentration (C, cell per milliliter) were calculated from the average of data from eight wells. In the second step, these growth parameters were analyzed by the specific experimental design software, MODDE7 (Umetrics, New Jersey, USA). The mathematical model corresponding to this fractional factorial design was a linear combination of the factors, G_x, pondered by their respective coefficients, a_x, which represented their effects on Vero cell growth (Eq. 1). The MODDE7 software also allowed the evaluation of the quality of the mathematical model by the calculation of the regression coefficient of the correlation between calculated and experimental values, r^2.

$$\mathrm{WS} = [G_1] \times a_1 + [G_2] \times a_2 + [G_3] \times a_3 + [G_4] \times a_4 + \cdots\cdots + [G_{10}] \times a_{10} \quad (1)$$

Results and Discussion

Monitoring of Adherent Vero Cell Growth in Serum-Free Media with "Cellscreen" Device

Correlation Between Manual and "Cellscreen" Numeration of Non-attached Vero Cells

The single cell concentrations obtained by automatic measurement with "Cellscreen" PS module were first compared to concentrations obtained by the Trypan blue numeration method. Manual cell numeration was performed just after cell trypsinization from culture flasks, while the "Cellscreen" analysis was realized 10 min after seeding the 96-well plates to allow for complete cell settling. The range of tested cell concentration was from 0.06×10^5 to 8×10^5 cell ml^{-1}. The results presented in Fig. 2 indicated that a linear and accurate correlation could be found between the two numeration methods up to 5×10^5 cell ml^{-1}. Above this value, the cell concentration was underestimated by the image analysis software, when

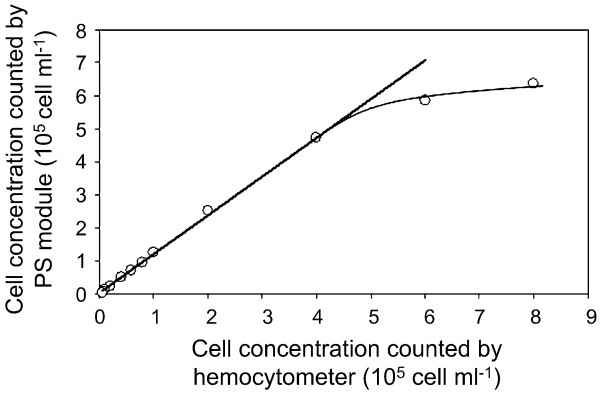

Fig. 2 Correlation between non-attached Vero cell concentrations measured with "Cellscreen" PS module or with manual Trypan blue method. Cells were trypsinized from culture flasks and counted just before (manual) or 10 min after (Cellscreen) seeding in 96-well plates. Each point is the average of eight wells repeated two times

compared to the hemacytometer data. Similar results have been observed with other adherent cells, such as LPC cells [22], and with different suspension cells, such as CHO and K562 human leukemia cells [21]. This effect is most likely due to the superposition of cell layers on the well surface, which are not detectable by the image analyzer. This upper limit of cell density is cell line dependent, while it is two times lower for Vero cells (5×10^5 cell ml^{-1}) than for CHO cells (11×10^5 cell ml^{-1}). Thus, the "Cellscreen" PS module was proved to give similar results than a reference manual method for numeration of non-attached Vero cells below 5×10^5 cell ml^{-1}. It could be used as a reference numeration method for further experiments performed with the PA module dedicated to attached cells.

Relation Between Vero Cell Concentration and Well Surface Covered by the Attached Cells

The concentration of adherent cells growing on surfaces is generally more problematic to acquire directly by image analysis than for suspension cells. The previous correlation between manual and PS module data only characterized the concentration of single suspended cell. Then, to assess the influence of the cell adhesion on the "Cellscreen" performances, cell counting of attached cells, determined with the percentage of the well surface covered by cells, WS, were performed using the "Cellscreen" PA module. A comparison between results from both "Cellscreen" modules was proposed on Fig. 3. In the first experiment (Fig. 3a), cells previously trypsinized from static flasks were seeded in 96-well plates at various concentrations ranging from 0.18 to 5×10^5 cell ml^{-1}. The cell numeration in the well plates was performed with the PS module (*C*, cell per milliliter) just after the cell seeding, and with the PA module (WS, percent) after 4 h of culture necessary to reach the complete cell

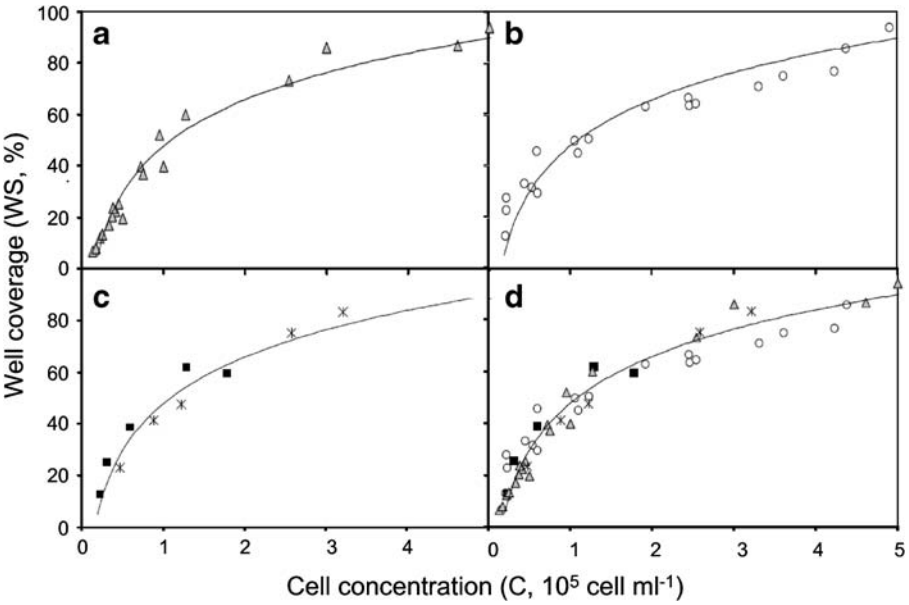

Fig. 3 Correlations between results from PA module (WS) and PS module (cell concentration, just after cell seeding or cell trypsinization). **a** (*gray triangles*) Measurements at seeding time with initial cell density from 0.18 to 5×10^5 cell ml^{-1}; **b** (*white circles*) measurements after 4 days of cultures in well plate seeded at concentrations from 0.08 to 0.75×10^5 cell ml^{-1}; **c** measurements all over two cultures seeded with 0.25 (*black squares*) and 0.5 (*asterisks*)$\times10^5$ cell ml^{-1}; **d** comparison of the three sets of data

adhesion assessed by image checking. Our results pointed out a good logarithmic correlation between well surface covered by cells WS and cell numeration C (r^2=0.98). This correlation suggested that the size of the attached Vero cells was dependent on the cell-seeding concentration. This observation was comparable to the decrease of cell size classically observed in T-culture flasks when the cell density reached confluence. By comparison with LPC cells, Vero cells appeared to be larger as 2×10^5 cell ml^{-1}, respectively, covers 50% and 70% of well surface. Contrary to the logarithmic correlation observed with Vero cells, in the case of LPC cells, a linear correlation was proposed from numerations realized after 12 h of culture [22]. However, the images of LPC cells acquired at that culture time clearly showed a non-complete cell adhesion that could alter the correlation.

Study of the Culture Phase Impact on WS/C Correlation in Well-Plate Culture

Our following objective was to verify if the physiological state of the cells during culture inside the well plates influenced the WS/C correlation previously found (Fig. 3a). That is why, we compared the correlation for (1) just seeded cells (Fig. 3a), (2) cells cultivated for 4 days (Fig. 3b), or (3) cells monitored during complete culture kinetics (Fig. 3c). Cells were all seeded from the exponential growth phase of the amplification culture. As already mentioned, in Fig. 3a, the measurements were made just 4 h after cell seeding with different cell concentrations ranging between 0.18 and 5×10^5 cell ml^{-1}. In Fig. 3b, the measurements were made after 4 days of cell cultures performed in 96-well plates at various cell-seeding concentrations, with a range lower than in Fig. 3a from 0.08 to 0.75×10^5 cell ml^{-1}. WS was measured with PA module, while cell numeration C was assessed with PS module after trypsinization of the entire 96-well plate after 96 h. So, the observed values in Fig. 3b corresponded to cells which have grown in wells during 4 days. The obtained curve was very similar to the one with just seeded cells suggesting that the evolution of the cell size only depended on the cell concentration and not of the cell state at the end of the cultures. To complete this last observation, we followed two culture kinetics using initial cell concentrations of 0.25 and 0.5×10^5 cell ml^{-1}, from the initial time until 4 days (Fig. 3c). WS was monitored by the PA module, while C was numbered each day with PS module on selected wells previously trypsinized. Each culture kinetics contained five experimental points measured at 4, 24, 48, 72, and 96 h. Again, despite the different phases of the cultures, including lag, exponential and stationary ones, the correlation presented a similar profile than in Fig. 3a, b. This was confirmed in Fig. 3d which gathered Fig. 3a–c. Clearly, the percentage of well surface covered by the attached Vero cells only depended on the cell concentration and was not influenced by the cell culture phase. Based on the non-linear observed correlation, it could be assumed that the attached cell size decreased when the cell concentration increased, whatever the culture phase inside the well, while the covered surface per cell decreased. For further screening experiments, a single exponential law, described by Eq. 2, could be used to directly calculate the cell concentration from the measured well surface covered by cells WS (r^2=0.97):

$$C = 0.16 \times e^{(0.038 \times WS)} \quad (2)$$

Influence of the Cell-Seeding Concentration on the Cell Growth Kinetics in Well Plates

For media screening experiments, it was necessary to set the appropriate seeding cell concentration which allowed an observable cell growth while avoiding a rapid saturation of

the well surface. Several cultures in M_C medium were performed in 96-well plates seeded in a range from 0.19 to 6×10^5 cell ml^{-1} (Fig. 4). The kinetic results showed a rapid saturation of the wells for seeding concentrations higher than 0.74×10^5 cell ml^{-1}. Oppositely, a growth lag phase of 24 h was observed with 0.18×10^5 cell ml^{-1} seeding. The best growth curves were obtained between 0.25 and 0.49×10^5 cell ml^{-1} seeding concentrations, and the last one was chosen for further media screening experiments.

Screening of Serum-Free Medium Components Affecting Vero Cell Growth

From the previous results, and particularly the correlation between data of both "Cellscreen" modules, further non-invasive growth monitoring of adherent Vero cells cultivated in 96-well plates in various serum-free media could be realized by only using the PA module. Different G_x groups of components present in the M_C medium were screened, and the experimental data were modeled to evaluate their effects on Vero cell growth.

Experimental Results of the Screening Assays

To be reliable, the procedure of DoE medium optimization should first include a screening of a large number of media component groups, followed by the use of a response surface methodology to evaluate the effects of the most interesting component groups [14]. In this study, the aim was only to propose a rapid pre-screening step by using the "Cellscreen" device to evaluate which main components of the serum-free medium could affect the Vero cell growth. According to the fractional factorial design, 16 M_x media were formulated (Table 1). Cultures were then performed in 96-well plates, in the M_x, M_C, and M_R media, and monitored until 4 days of culture with PA module (Fig. 5, Table 2). Results clearly attested various effects of the media formulations on Vero cell growth. Firstly, trace elements of M_C medium presented an important positive influence, particularly in the last days of culture. Indeed, M_{16} formulation, which contained all the G_x solutions, only differed from the M_C medium composition by the absence of the trace elements. After 4 days of

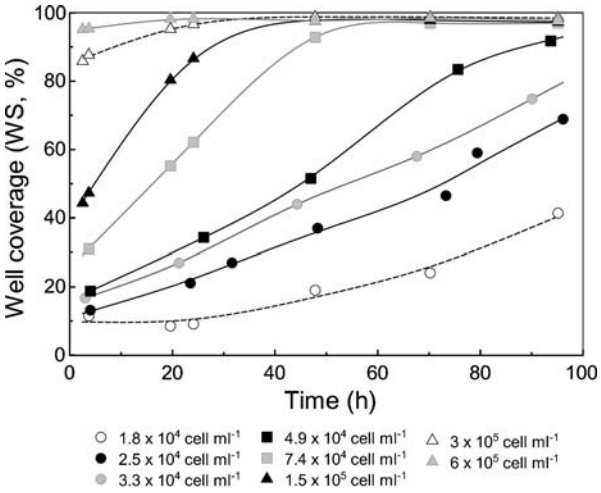

Fig. 4 Vero cell growth kinetics determined with PA module for cultures performed in 96-well plates at different cell-seeding concentrations

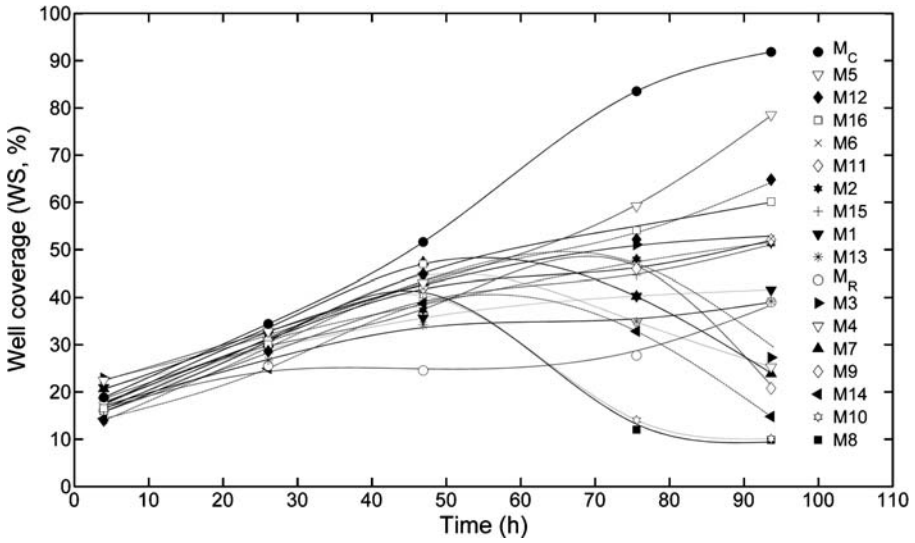

Fig. 5 Growth kinetics of Vero cells monitored with PA module, all over cultures performed in 16 M_x screening media and in M_C and M_R media

Table 2 Experimental design results in well surface occupation (WS, percent) and r^2 correlation coefficient between experimental values and model prediction for different culture times.

	Culture time (h)			
Screening media M_x	4	47	76	94
M_1	20.9	35.6	40.2	41.5
M_2	16.4	37.2	47.9	51.2
M_3	22.9	42.6	51.0	27.2
M_4	18.1	44.4	34.8	25.2
M_5	22.4	42.9	59.2	78.5
M_6	16.7	42.0	51.5	52.3
M_7	20.6	47.3	40.2	23.8
M_8	15.9	42.3	12.0	9.8
M_9	18.4	36.7	47.7	20.7
M_{10}	17.0	40.4	14.0	10.0
M_{11}	17.7	42.0	45.9	52.0
M_{12}	13.9	44.8	52.1	64.8
M_{13}	16.9	34.1	34.9	39.2
M_{14}	14.3	38.7	32.8	14.7
M_{15}	15.8	39.4	44.6	51.1
M_{16}	16.5	46.9	54.0	60.1
M_C	18.8	51.6	83.5	91.8
M_R	16.0	24.5	27.7	39.0
r^2	**0.81**	**0.72**	**0.60**	**0.93**

culture, the percentage of well surface covered by cells reached in M_{16} medium was only 60% of the one observed in M_C medium. This lower performance can be only attributed to the lack of trace elements. Furthermore, the cell growth kinetics seemed to be very similar until 48 h of culture whatever the medium composition. Neither nutrient limitation nor metabolite inhibition may be suspected even in very poor formulations on the basis of growth curve observation. Nevertheless, after 48 h of cell growth, an important influence of the concentration level of some groups of components could be observed on the cell concentration. For example, a decrease of cell concentration appeared in M_8 and M_{10} media, while the cell growth seemed higher in M_5 medium than in the richest M_{16} medium.

Evaluation of the Growth Effects of Various Component Groups of the Serum-Free Medium

To identify the effects on cell growth of the ten G_x groups of medium components, the experimental results obtained at 4, 47, 76, and 94 h of culture were analyzed with MODDE7 software (Table 2). The regression coefficients of the correlation between model and experimental values, r^2, calculated at 4, 47, and 94 h of culture were all above 0.7, a value which corresponded to a strong correlation as specified by Shallamer [25]. The value of 0.6 obtained after 76 h represents a moderate but usable correlation to catch trends of effects. Table 3 gives the a_x coefficients issued from the data modeling (Eq. 1). Each coefficient characterized the respective influence of the level increase of G_x component groups in the M_R reference medium. Significant effects could be pointed out when the absolute values of a_x coefficients were higher than their standard error. Positive a_x value suggests a favorable effect of the G_x group, while a negative value identifies a limiting or inhibiting influence. The effects of the component groups on Vero cells could then be classified with respect to the culture phases. The results at 4 h of culture corresponded to a major impact on cell adhesion and viability. The coefficients calculated at 47 h of culture mainly provided information on the impact on the cell growth. And finally, the effects identified at 76 and 94 h indicated the importance of M_x composition when nutrient limitation appears.

Only the component group G_7 seemed to have a positive effect on cell adhesion during the first hours of culture, while G_1 and G_4 could negatively affect the cell attachment (Table 3). Furthermore, the G_7 and G_4 groups also displayed their respective positive and negative influences on cell growth during the exponential growth phase until 47 h of culture. Furthermore, G_2 group exhibited a high cell growth enhancer effect during this culture phase. After 47 h of culture, some of the cultures appeared to be limited or inhibited. Coefficients a_x calculated during the last culture phase pointed out a negative effect on cell viability of G_1 and G_8 groups from 76 h and of G_6 from 94 h. In contrast, the G_9 and G_5 groups greatly promoted the cell growth during the same period. Obviously, these positive

Table 3 Model coefficients, a_x, and their standard errors for different culture times.

Culture time (h)	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8	a_9	a_{10}	Coefficient standard error
4	**−1.7**	−0.1	−0.4	**−1.5**	0.2	0.1	**0.8**	−0.1	−0.2	−0.1	0.5
47	0.6	**2.2**	0.3	**−1**	0.4	0	**1.6**	−0.2	0.4	−0.8	1
76	**−4.2**	0.4	−0.4	−0.6	0.3	0.4	1.4	**−4.3**	**8.2**	1.1	3.7
94	**−2.9**	0.3	2.3	0.2	**3.6**	**−4**	1	**−5.4**	**17.7**	0.1	2.4

Bolded values correspond to significant positive or negative effects of G_x component groups on VERO cell growth

influences observed during the last culture phase could be explained by the remove of nutrient limitations, while the negative effects were essentially related to nutrient limitations or inhibiting factors which accelerated cell death. Some of these observed effects were expected, as for example the positive influence of the group G_9 which contained glucose and pyruvate. Nevertheless, unexpected effects of other component groups, such as G_4 (vitamins B_3, B_6, B_X, putrescin) or G_1 (20 free amino acids, vitamin B_1, magnesium sulfate, sodium phosphate), could be advantageously detected.

Thus, this easy and rapid pre-screening method allowed to early identify five of the G_x mixtures of components present in M_C medium which displayed a major significant effect on Vero cells. The groups G_7 and G_9 both enhanced the cell adhesion and growth. This result confirmed the importance of non-limiting concentrations of rEGF, insulin, protein extracts, and carbohydrate source in the culture medium. Beside, the groups G_1, G_4, and G_8 either inhibited the cell adhesion and growth or increased cell death. It could be assessed, in the first global approach, that the level in the M_C medium of amino acids, magnesium sulfate, sodium phosphate, vitamins B_1, B_3, B_6, B_X, putrescin, sodium citrate, and ferric chloride was overestimated and that diluted concentrations would have to be further considered. Furthermore, the addition of G_3 and G_{10} groups in M_R reference medium did not lead to significant effects during the whole cell growth. Then, the removal of vitamins B_{12}, B_3, B_7, choline chloride, or pyridoxal from the M_C medium formulation could be tested. The other groups of components (G_2, G_5, and G_6) mainly composed of vitamins, molecules related to nitrogenous bases, and fatty acids displayed a low influence on Vero cell growth and could be neglected in further optimization studies.

Interestingly, this screening procedure gave satisfactory qualitative results to catch trends of the G_x groups influence on Vero cell growth and to propose early improvements of the original serum-free medium. Despite the global approach proposed in this work that did not allow to clearly understand the effect of each component, the results also represented a reliable basis for further medium optimization focused on individual selected components of G_x mixtures, based on an integrative application of experimental designs and complementary screening steps [14, 24, 26]. The rapidity and easiness of this medium screening method has to be highlighted in comparison with usual one-factor-at-a-time procedure [27, 28]. Furthermore, the operating conditions required for the use of the "Cellscreen" device to monitor the growth of anchorage-dependent cells in small volumes without any sampling have been precisely studied and validated. Obviously, one of the main advantages of this equipment remained in its capacity to report adherent cell growth data, contrary to the other automatic devices such as, for example, Simcell™ (Bioprocessors Corporation), Hexascreen (Hexascreen Culture Technologies), or Cellstation™ (Fluorometrix Corporation) [27, 29]. For the first time, a rapid first screening of media component effects was proposed with adherent cells cultivated in small-scale culture systems.

Improvement of Serum-Free Medium Formulation for Vero Cells Cultivated on Microcarriers

After the kinetic studies realized in small-scale static culture systems, supplementary cultures were performed in stirred conditions closer to industrial cell processes. Culture kinetics were monitored in 250 ml spinner flasks with cells attached on Cytodex 1 microcarriers with a cell-seeding concentration 5.5 times higher than in 96-well plate experiments. Based on the previous screening results, the further target was to evaluate the impact of the highlighted component groups by increasing their concentration in the

original serum-free medium M_C. With regard to the higher seeding cell density in spinner flask than in well plate, these groups were added at a concentration 5.5 times higher than the one used for the screening tests. For their respective effects as growth enhancer or inhibitor, the groups G_7 and G_1 were chosen for addition in M_C medium. Figure 6 reports the growth kinetics of cultures performed respectively in M_C and in M_C enriched with G_7 or G_1. The addition of G_7 allowed a 1.6 times higher maximal cell concentration than in the initial serum-free medium which confirmed the positive effect of protein extracts, EGF, and insulin in stirred culture conditions with cells attached on microcarriers. The concentration increase of these components in a further optimized medium would benefit from studies based on each independent component. The inhibiting effect of G_1 was also checked as the culture performed in M_C added with G_1 reached a maximal cell concentration two times lower than the one obtained in M_C. Clearly, a deeper understanding of the effect of this group of components has to be completed in future studies dedicated to specific molecules. Moreover, the previous screening experiment demonstrated that the increase of the level of G_3 and G_{10} from M_R to M_C had no consequence on the Vero cell behavior. That is why the suppression of these groups from initial serum-free medium M_C could be envisaged to simplify the medium. Figure 7 gives the results observed with duplicated cultures performed in parallel either in M_C medium or in M_C medium depleted of the G_{10} group. The cell growth on microcarriers was observed to be similar with or without the presence of G_{10} all over the culture.

It is also important to point out that the culture medium modifications did not modify the cell adhesion characteristics, as neither cell detachment nor aggregates were detected as reported by Butler et al. [13]. The maximal specific growth rates observed in M_C or in M_C enriched with the G_7 group were respectively of 0.021 and 0.033 h^{-1}, which are similar or better than literature results [13, 30]. Besides, other studies on serum-free cultures of Vero cells utilized various commercial or home-made media, but did not present detailed screening studies which succeeded in the improvement of their media [11, 12, 31, 32]. The medium used for the master cell bank of our Vero cell line was completely defined and animal component-free and had a very low protein content. The results obtained in this work by only one screening step allowed to propose a medium simplification by removing or diluting some components and to strengthen it by the level increase of some key

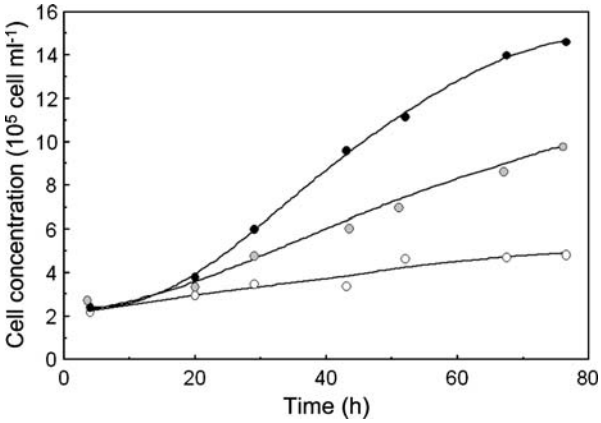

Fig. 6 Growth kinetics of Vero cells cultivated on microcarriers in spinner flasks, in M_C medium (*gray circles*) and in M_C medium supplemented with G_7 (*black circles*) or G_1 groups (*white circles*), 5.5 times concentrated to represent the cell-seeding concentration variation between well plates and spinner flasks

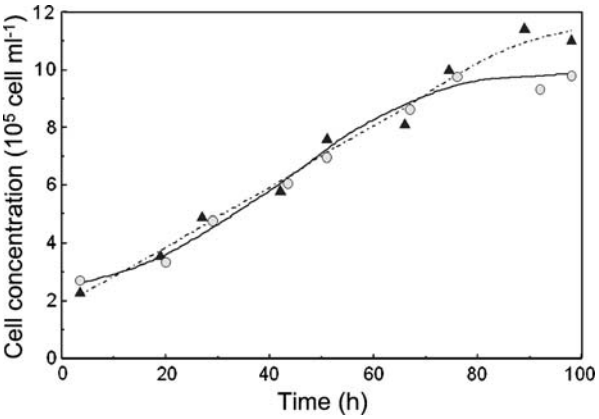

Fig. 7 Growth kinetics of VERO cells cultivated in spinner flasks on microcarriers, in M_C medium (*gray circles*) and in M_C medium without G_{10} component group (*black triangles*)

components. Complementary modifications based on our first step screening results remain to be evaluated, and further medium optimization could then be expected. More widely, the adequacy between "Cellscreen" results and Vero cell growth in stirred cultures was a very promising point for further development of vaccine production processes. The good scalability of the results led to the conclusion that the non-invasive monitoring of adherent Vero cells growth in 96-well plates by using the "Cellscreen" device represents an interesting tool to accelerate and facilitate the formulation and the improvement of a serum-free culture medium for industrial large-scale purposes. The medium decomposition in different component groups was also an interesting technique to rapidly evaluate some medium component impacts on Vero cell growth.

Conclusion

The objective of this work was to validate a method allowing to rapidly observe some global trends of component groups of a serum-free and animal-free culture medium on the growth of the anchorage-dependent Vero industrial cell line. The interest was to propose a first step of simplification and improvement of the medium allowing to increase cell growth. The results could also be useful to suggest further experiments focused on specific components. To achieve the screening experiments in small-scale cultures, the monitoring capabilities of the "Cellscreen" device in the case of adherent cells were first evaluated. Various operating conditions were checked and adapted to successfully non-invasively monitor the Vero cell growth in 96-well plates. A first linear correlation was proposed between manual and automatic counting of the non-attached cells. Then, a logarithmic relation was determined between data from both "Cellscreen" modules respectively dedicated to suspension or adherent cell density. This correlation was found to be reliable for the different phases of the Vero cell culture. The growth monitoring technique was then applied to perform an experimental design experiment to assess the influence of ten groups of components issued from the initial serum-free medium. Interesting results were obtained which pointed out the enhancer, inhibitor, or neutral effect of some component groups on Vero cell growth. The screening results were then successfully extrapolated in stirred cultures with cells attached on microcarriers. The improvement of the initial culture

medium was achieved either by removing some components or increasing the concentration level of others. In conclusion, the combination of an easy-to-use and appropriate screening procedure dedicated to adherent cells led to identify by only one step some medium component groups which have an expected or unexpected effect on adherent Vero cell growth. The composition of the initial serum-free medium could be rapidly improved and additional medium optimization further considered from complementary optimization steps focused on few identified components.

Acknowledgment The authors are grateful to Dr. Isabelle Chevalot (LSGC-CNRS, Nancy) for the help she provided on the use of the "Cellscreen" device.

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