# Microenvironmental restrictive effects on the growth of hybridoma cells inside various types of microcarriers

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## Introduction

Immobilized hybridoma cell systems for monoclonal antibodies (mAb) production are widely used in biomedicine (Bugarski et al., 1993, 1999)**.** Of the basic interest for various applications is the choice of material and procedure of micro-bead matrices preparing. Owing to the very gentle, simple and rapid procedure, the entrapment of cells in Ca-alginate microbeads, as well as in alginate-poly-L-lysine (alginate-PLL) microcapsules is still the most frequently used method for immobilization.

Bugarski et. al. (1999) optimized the internal nutrients transfer and performance of the alginate-PLL membrane for both microcapsules and Ca-alginate microbeads. However, after nutrient optimization it was found that only about 20% of the microcapsule volume was filled with hybridoma cells, indicating the existence of other factors acting restrictive on cells growth. Moreover, when the final hybridoma cell concentrations for microencapsulated cells and cells immobilized in Ca-alginate matrices were compared, three times higher cell concentrations were demonstrated for microencapsulated hybridoma cells (Bugarski et al., 1993, 1999), pointing to different microenvironmental conditions and growth limiting factors existing within different carriers. Since a number of other authors also reported early suppression of cell growth for different systems of immobilized cells (Bugarski et al., 1999, Pajic-Lijakovic et al., 2007a, 2007b), this seems to be rather common and serious problem for the development of cell immobilization techniques.

The contribution represents the comparable phenomenological analyze of impact of various microenvironmental restriction factors on the growth and monoclonal antibody (mAb) production kinetics of hybridoma cell immobilized within different microcarriers. Analyze was based on experimental data for cell and mAb concentrations obtained within two types of microcarriers, Ca-alginate microbead and alginate-poly-L-lysine (alginate-PLL) microcapsules, during air-lift bioreactor cultivation.

## Materials and methods

The cell line used in these experiments was a mouse/mouse-hybridoma for production of monoclonal IgG (kindly provided by INEP, Zemun, Serbia and Montenegro). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Sigma, St. Louis, MO, USA) in 75 ml tissue culture flasks (Fisher Scientific) and incubated at 37$^{o}$C in a humidified atmosphere with 5% $CO_2$ in air. Detail procedure of cell count is given in Bugarski et al. (1993). The content of a 75 ml subconfluent flask was collected by centrifugation (10 min at 1000 rpm). The cell pellet was resuspended in sterile Na-alginate solution (1.5 %, Kelco Gel LV).

Spherical droplets were formed by extrusion of Na-alginate/hybridoma cell suspension through a blunt stainless still needle into 1 % CaCl2 solution. The detail procedure is given in Bugarski et al., (1999). Diameters of microbeads were measured with accuracy of 10 μm using optical microscope. The average microbead diameter and standard deviations were then calculated from the measured

data. The averaged bead diameter was 1 mm with 3% dissipation. Ca-alginate microbeads were optimized for the internal nutrients transfer, while the product, mAb, passed through and accumulated in the solution.
The concentration of mAb produced during bioreactor studies was measured in culture medium and expressed per bioreactor volume.
The alginate-PLL microcapsules were formed by reacting one part of previously prepared Ca-alginate beads for 6 min with aqueous 0.1% w/V poly-L-lysine (MW=22 000, Sigma, St. Louis, MO, USA), and then for further 5 min with 0.04% Na-alginate, as described by Bugarski et al. (1993). The interior of the microcapsules was liquefied by treatment with 0.05M Na-citrate for 4 min. The averaged capsule diameter was also about 1 mm with 5% dissipation. The alginate-PLL membrane permeability (cutoff about 70 kD) enabled the nutrients and oxygen to diffuse freely to the outer solution, while the product, mAb were kept within the microcapsule (Bugarski et al., 1999).
The concentration of mAb produced was measured in volume/microcapsule and then calculated for the bioreactor volume for further comparison analysis. In the cell culture studies, 30 ml of microcarriers (Ca-alginate microbead or alginate-PLL microcapsules) containing $C_0=1x10^6$ hybridoma cells/ml were loaded into the air-lift bioreactor with 1 l of culture medium (Bugarski et al., 1999).
In the cell culture studies, 30 ml of microcarriers (Ca-alginate microbead or alginate-PLL microcapsules) containing $C_0=1x10^6$ hybridoma cells/ml were loaded into the air-lift bioreactor with 1 l of culture medium (Pajic-Lijakovic et al., 2007a). The low microcarrier quantity per bioreactor volume (up to 5% vol.) enabled the optimal hydrodynamic conditions on macro-level (Bugarski, 1993). The cells were then batch cultured for up to 25 days. Samples of both the medium (1 ml) and the immobilized cells (0.2 ml) were withdrawn every one or two days. The medium was renewed once a day, to eliminate nutrient limitations and product inhibition of cell growth.
Cell counts were enumerated using hemocytometer as an average from 10 microcapsules that were previously mechanically ruptured, while the cell viability was assessed using Trypan blue exclusion test. To determine the concentration of mAb produced an enzyme-linked immune-adsorbent assay (ELISA) was used. The sensitivity of the assay enabled detection of mAb levels as low as 2.5 ng/mL.

**Results and discussion**

For the phenomenological analyze the growth of hybridoma cells and corresponding mAb production within two types of microcarriers, Ca-alginate microbeads and alginate-PLL microcapsules, during the air-lift bioreactor cultivation, were considered and compared. The experimental results concerning the hybridoma cells growth demonstrated higher cell concentration within the alginate-PLL microcapsules than within the Ca-alginate microbeads (Figure 1), which is in accordance with our previous observations (Bugarski et al., 1999). Within the microbeads hybridoma cells reached the equilibrium state after 11 days of culture, with the equilibrium cell concentration of $3.5x10^7$ cells/ml. On the other side, hybridoma cells grown within the microcapsules reached the equilibrium state after 21 days of culture, with the equilibrium cell concentration of $9x10^7$ cells/ml, i.e. 2.6 times higher than in the microbeads.
These results pointed to the existence of quiet different microenvironmental restrictive factors affecting the cell growth dynamics within these microcarriers. One way to explain the complex nature of microenvironmental resistance to cell growth is to take into consideration the tendency of cells to make enough free space inside the microcarrier by pressing the surrounding matrix. This pressure might result in generation of the additional force within the matrix that can have the feedback restrictive action to the cells growth. The tension created within the microcarrier matrix can further initiates particular mechanisms in the cell responses affecting their growth and mAb production. The matrix response to the cell growth can cause various limiting factors. As we have

previously shown, the major limiting factor for hybridoma cell growth inside the alginate-PLL microcapsule was the rapid increase of the osmotic pressure (Pajic-Lijakovic et al., 2007a). On the other side, for the cells growing within the Ca-alginate microbeads, the main restriction to their growth arose as a consequence of elastic expansion and partial disintegration of polymer network (Pajic-Lijakovic et al., 2007b, 2007c). In both cases the hybridoma cells grown within the microcarriers were subjected to the increased stress, either osmotic or biomechanical, as well as to the increased rate of stress changes.

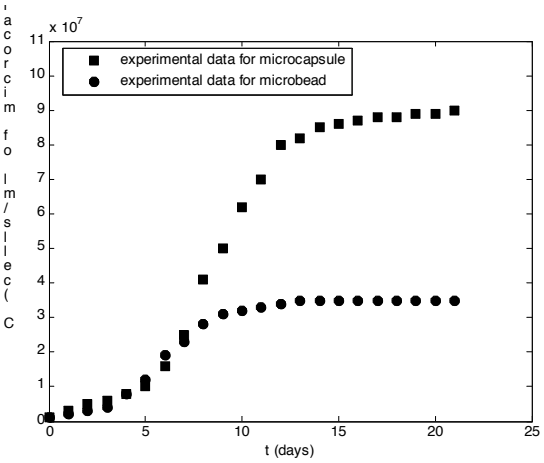


Figure 1. Experimental data for hybridoma cell concentration per microcarrier as a function of time

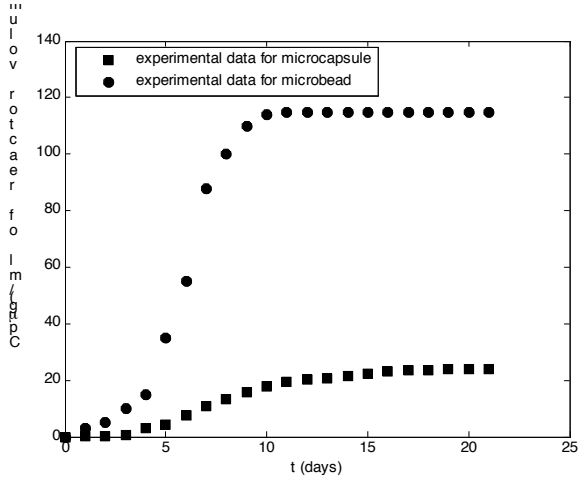


Figure 2. Experimental data for the mAb concentration per bioreactor volume as a function of time.

At the same time, when the mAb concentrations, expressed per bioreactor volume, were compared for the microcarriers studied, mAb production was much higher for hybridoma cells grown within the microbeads than within the microcapsules, as shown in Figure 2. The mAb production reached the equilibrium state almost at the same time when the hybridoma cells growth reached the equilibrium state within the same type of microcarrier. The equilibrium concentration of mAb for hybridoma cells grown within the microbeads was 115 μg/ml and was obtained on day 11 of cultivation, while the equilibrium of mAb concentration produced by hybridoma cells grown within the microcapsules was 24 μg/ml and was obtained on day 21 of cultivation. From the experimental data for cell concentration $C(t)$, and mAb concentration $C_p(t)$ expressed as functions of time, the hybridoma cells growth rate (i.e. $\Delta C(t)/\Delta t$) and the mAb production rate (i.e. $\Delta C_p(t)/\Delta t$) were estimated. When these rates were compared, the hybridoma cells growth rate and the mAb production rate were parallel in our experiments.

When the hybridoma cells growth rate increased, the corresponding mAb production rate also increased within the same type of microcarrier. At the same time, when the hybridoma cells growth rate dropped to zero, the mAb production rate also dropped to zero. In both types of microcarriers, cells growth rate and mAb production rate were lower in the early stage of cultivation, up to 5 days, than in the further stages of cultivation.

## Conclusions

In summary, the results of this study pointed out that the comparison of various limiting factors influencing the dynamics of hybridoma cell growth and mAb production within different

microcarriers might be useful for further optimization of microcarrier design in order to maximize the mAb production rates**.**

The results presented here are generally in line with various other reports regarding the growth and production kinetics of hybridoma cells. Namely, hybridoma cells were already shown to be highly sensitive to the osmotic stress, as well as to the rate of the stress changes, since a number of authors reported their suppressed growth as the response to hyperosmotic pressure (Oh et al., 1993; Young and Min, 1995; Sue and Min, 2000)**.** However, hyper-stimulation by osmotic stress was found to be generally effective in enhancement of mAb production (Oh et al., 1993; Young and Min, 1995; Sue and Min, 2000). The mechanisms of the changes that occur in hybridoma cells in response to hyperosmotic stress are not yet understood, but it was demonstrated that hyperosmotic stress initiates changes in the intracellular biochemical signals that are crucial for the cell responses to the extracellular stimuli. Recently, it was also shown that mechanical stress, i.e. physical stimuli can also be sensed by the cells and transmitted through the intracellular biochemical signals to the nucleus resulting in altered cell responses (Li and Xu, 2007). Since the results obtained in our study pointed that the biomechanical stress in murine hybridoma systems can also increase specific mAb production by suppressing cell growth, having even more profound effect then the hyperosmotic stress, the activation of stress-initiated intracellular multiple signals might be involved in the mechanisms underlying this phenomenon.

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