LJILJANA MOJOVIĆ[1]
GORAN JOVANOVIĆ[2]

[1]**Department of Biochemical Engineering and Biotechnologies, Faculty of Technology and Metallurgy, Belgrade, Serbia and Montenegro**

[2]**Department of Chemical Engineering, Oregon State University, Corvallis, Oregon, U.S.A.**

SCIENTIFIC PAPER

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IMMOBILIZATION OF FISH CHROMATOPHORES FOR USE AS A MICRO–BIOSENSOR FOR BIOLOGICAL TOXINS

Chromatophores isolated from the Siamese fighting fish, Betta splendens, represent a class of living cells that provide a vivid color response to microbial pathogens and environmental toxins. The selection of the most appropriate microcarrier and the development of the optimal technique for the chromatophore immobilization in order to enable directed transport of the sensor cells throughout microchannels of the biosensor, as well to preserve the cell survival and its functionality was studied. Microcarriers derived from glass, polystyrene and gelatin (collagen) were tested as substrates for chromatophore attachement. Gelatin microcarriers were found to be the most suitable, due to high attachment efficiency (95% of attached cells), preservation of the cell viability and enhanced cell sensitivity. The optimum conditions for fish cell immobilization on collagen microcarriers were determined based on the cell–to–microcarrier bead ratio and the pH of the solution. The rate of cell attachment to the gelatin microcarrier followed first–order kinetics. Pretreatment of the gelatin beads with fibronectin, known as a cell attachment–promoting agent, resulted in a 10% higher attachment rate constant (k).

Animal cell cultures offer a basis for a variety of toxicity and environmental pollutant tests [1–4]. Chromatophores are terminally differentiated, neuron–like cells containing pigmented granules that are responsible for the brilliant colors of fish, amphibians, reptiles and cephalopods, as well as for their unique color adaptations and camouflage capability [5,6]. Various biologically active substances act as signaling molecules interacting with receptors placed on the chromatophore cell surface. The information is then transferred into the cell through a specific signal transduction pathway that triggers a response to the signal. The most common signal transduction pathway observed is the activation of G–proteins, which then regulate second–messenger–generating enzymes or ion channels. As a result, pigment granules move through the cell microtubules [6–8]. Depending on the type of signaling molecule and the type of chromatophore cells (brown to black melanophores; orange to red erythrophores or yellow to orange xanthophores), different responses may be optically monitored and quantified, thus making chromatophores a very promising base for a real–time, optical biosensor.

Betta splendens erythrophores have been previously described as a potentially effective biosensor for the detection of various environmental toxins such as heavy metals and polynuclear aromatic hydrocarbons [9]. Chromatophore response to certain biologically active substances and specific pharmaceuticals holds promise for their use in medical screening and for pharmaceutical research [10]. The mode of the response may vary for different classes of agents, with some causing the hyperdispersion of pigment granules, others causing only partial aggregation, and some showing no visible effects. A very pronounced aggregation of pigment granules, presented in Fig. 1, occurs when chromatophores are exposed to the neurotoxin, nor–epinephrine.

In order to take advantage of the properties of fish chromatophores, a novel microchannel bioreactor system is being developed at Oregon State University for which immobilization technology is a key component. The appropriate immobilization method should preserve the cell viability and sensitivity, whilst enabling the directed motion and positioning of cells and samples within the three–dimensional architecture of the micro–device.

Fish chromatophores are anchorage–dependent cells that require a compatible surface for attachment and subsequent spreading and growth. Generally, immobilization on the surface of small beads or microcarriers is a suitable method for cultivating anchorage dependent cells. Van Wezel [11] first reported the immobilization of mammalian cells on diethylaminoethyl (DEAE)–Sephadex–A50. Subsequently, this type of carrier was improved by optimising the surface electrostatic charge allowing cells to grow to higher densities [12–15]. These findings led to the development of the first commercial microcarriers, which were based on derivatized sephadex (Cytodex I, II, III, Pharmacia) and are utilized extensively for the commercial production of human vaccines. [16]. Various other microcarriers, such as polyacrylamide [16,17], polyurethane foam [18], polystyrene [19,20], glass [14,15] etc., are reported to be suitable for various cell lines. However, a microcarrier of pure gelatin developed by Nilsson and Mosbach [16], and later commercialised as Cultisphere (Percell, Biolytica, Sweden), has attracted a great deal of attention from researchers in the cell culture area [17,21]. The main advantages of this type of carrier are efficient cell attachment due to biospecific

Author address: Lj. Mojović, Department of Biochemical Engineering and Biotechnologies, Faculty of Technology and Metallurgy, Karnegijeva 4, Belgrade, E–mail: lmojovic@tmf.bg.ac.yu
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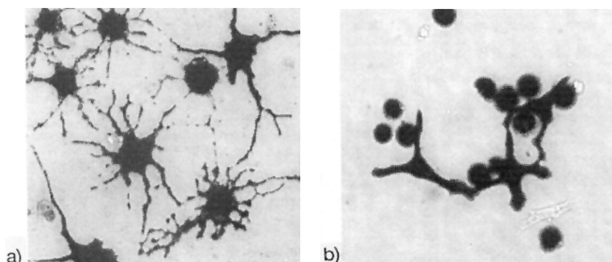

Figure 1. Fish chromatophores. a) before exposure to nor–epinephrine and b) 5 minutes after exposure to nor–epinephrine.

binding, a high surface area and good environmental protection that can be achieved [22]. In addition, cells can be recovered by dissolving the microcarriers with proteolytic enzyme.

In this paper we tested three different types of microcarriers – glass, polystyrene and gelatin beads, for their efficiency in binding fish chromatophores. The kinetics of cell attachment onto gelatin beads were also investigated, and the optimal conditions for cell binding were determined. A crucial factor for the integration of microcapsules of immobilized cells in the biosensor system is the preservation of cell sensitivity and this parameter was thoroughly characterized during the immobilization process.

MATERIALS AND METHODS

Isolation of primary cell culture

Fish chromatophores were isolated from the tails and fins of *Betta splendens* fish. Only Red *Betta splendens* fish which consisted of erytrophores (red pigmented cells) were used in this study. The Tissue was washed a minimum of six times with skinning solution (1mM NaEDTA – Sigma; 5.6 mM glucose – Sigma; penicillin/streptomycin mixture 1/100 w/v – Sigma; in calcium and magnesium free phosphate–buffered saline – Gibco) and then treated with an enzyme solution (Collagenase type 1, Worthington Biochemical Co, 178 U/mg., 20 mg, and Hyaluronidase, Worthington Biochemical Co. 348 USP/NF, 1 mg, in 7 ml of PBS, Gibco) for 20 min, with gentle agitation (n=80 rpm). After 20 minutes, the enzyme solution with digested tissue was separated from the undigested tissue pieces, and subsequently centrifuged for 2 minutes at 3250xg in a clinical centrifuge. The supernatant was removed using a sterile transfer pipette and reapplied to the tissue. The first pellet, which contained mostly epithelial cells, was discarded. After an additional 30 min of shaking, the above procedure was repeated, but the cell pellet, containing fish chromatophores, was suspended in L–15 (Leibovitz, Hyclone Lab) media, and centrifuged again. After removing the supernatant, the fish chromatophores were resuspended in a desired volume of L–15 media and used as an inoculum for microcarriers.

Microcarriers

Three types of microcarriers were used in this study – glass (Sigma, d=150–212 μm), polystyrene (BangsLabs, Inc., d=186 μm), and macroporous gelatin (Cultisphere–S, Percell Biolytica, Sweden, d=130–380 μm). All three types of microcarriers were hydrated in phosphate–buffered saline (PBS, Sigma) without Ca^{+2} and Mg^{+2}, washed extensively, and then resuspended in PBS at concentration of 5g/l. The glass and gelatin microcarriers were autoclaved for 20–min at 121 ^{o}C, while the polystyrene microcarriers were sterilized by incubating at 70 ^{o}C for 2h, as recommended by the manufacturer. After sterilization, the microcarriers were kept in solution at room temperature. In preparation for the microcarrier culture, the appropriate amount of microcarrier stock suspension was transferred to a 50–ml sterile conical centrifuge tube (Corning Glass) and the beads were allowed to settle by gravity. After withdrawing the supernatant, the microcarriers were washed twice with growth medium (L–15) and then transferred to an Erlenmeyer flasks, where the cells were attached to the beads. If the effect of cell attachment promoting agents such as fibronectin (Sigma) was studied, the appropriate amount of microcarrier was kept for 2 hours prior to use in PBS (20 ml) with added 100 μl of fibronectin stock solution (Sigma).

The number of Cultisphere S per gram was determined in order to the optimize cell/bead ratio (λ). The beads were counted in a standard volume on a haemocytometer grid. The value for Cultisphere S was found to be $0.9x10^6$ beads/g.

Measurement of Cell Attachment

Attachment of the cells to the beads was performed in siliconized Erlenmeyer flasks in L–15 medium under very gentle stirring (30–50 rpm). The L–15 medium was enriched with 5% of fetal bovine serum, FBS (Hyclone, Lab)

The rate of disappearance of free cells from the inoculated microcarrier cultures was determined as an indication of cell attachment to the microcarriers. Culture samples (200μl) were taken at 20 minute time intervals and allowed to settle for 1 min in an Eppendorf tube. The microcarrier–free supernatant was introduced into the haemocytometer for cell counting. At least 100 cells were counted from each sample to allow a statistically significant determination of the free cell concentration.

The culture samples were also examined microscopically to determine cell viability and toxin–sensitivity. The chromatophores that responded to the addition of nor–epinephrine (Sigma) in the way presented in Fig. 1 were considered to be alive and toxin–sensitive.

Analysis of Attachment Kinetics

The rate of disappearance of free cells was followed by an exponential decay curve:

$$C_t = C_0 \cdot 10^{kt} \quad (1)$$

where C_t is the concentration of free cells (cells/ml) at time t, C_0 the original cell concentration (cells/ml), and k the rate constant. This equation can be expressed logarithmically as:

$$\log C_t = \log C_0 - kt \quad (2)$$

Thus, a straight line would represent a first–order kinetic rate from a plot of log C_t vs. time with a gradient (k). The value of k was interpreted at the specific attachment rate (min^{-1}).

RESULTS AND DISCUSSION

Attachment of Cells to Microcarriers

Fig 2. shows the percent of attached cells to gelatin, glass and polystyrene microcarriers 3 hours after cell inoculation. The best results were obtained with gelatine beads (95 % of attached cells). Attachment the to glass microcarrier resulted in a significantly lower percent of cells attached (62%), while the fish chromatophores showed the lowest affinity towards the polystyrene microcarrier (17% of attached cells).

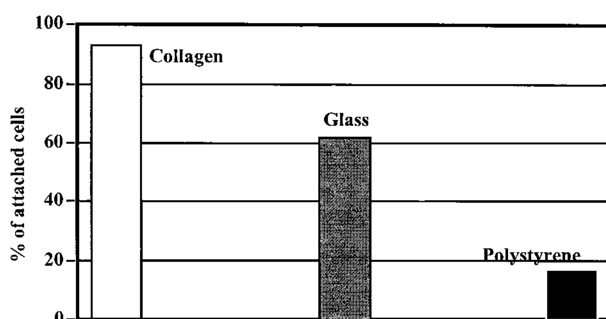

Figure 2. Attachment of fish chromatophores to microcarriers. Reaction conditions: L–15 medium supplemented with 5% FBS, pH=7.4, time τ=3 hours; stirring rate ν=40 rpm

Gelatin beads have already been reported to be appropriate for various cell types, such as human fibroblast cells [23], pancreatic islet cells [25], Chinese hamster ovary (CHO) cells, [24], green monkey kidney cells (Vero) [26] and human hepatocytes [27]. Cell attachments close to 100%, as well as high cell densities, have been reported for Cultisphere microcarriers. These were attributed to a microcarrier chemical structure that enables the biospecific binding of cells, as well as to a high surface area deriving from a porous structure. Thus, cells may also populate the interior of the cavities and withstand higher agitation rates than those on solid microcarriers [24].

Although glass carriers are widely used in cell culture studies [20], fish chromatophores showed only moderate affinity, lower than some other mammalian cell lines reported in the literature. Improvements in cell attachment might be achieved by either pretreatment with cell attachment promoting agents, or by using special types of aluminium borosilicate glass with controlled pore size [22].

Lee at al. [29] immobilized BHK cells on a sulphonated polystyrene microcarrier (S80), and on a surface modified microporous polystyrene (Polyhipe). The performances of these carriers were reported to be equivalent to these of Cytodex (dextran microcarrier) and Cultisphere (gelatin microcarrier). Maroudas' study [30] showed that sulphonated polystyrene with 2–5 negatively charged groups/nm^2 promotes maximum cell attachment and spreading. Despite the fact that many cell types adhere better to polystyrene than to glass [29], chromatophores demonstrated poor attachment. This result may be a consequence of the inappropriate surface charges present on the commercial support that was used. The gelatin beads gave the best results and were selected for further studies.

Kinetics of Cell Attachment to Gelatin Microcarriers

After inoculation of the microcarriers with 2.4 x 10^4 cells/ml, the rate of disappearance of free cells was determined by haemocytometer counting at 20 min time intervals. In order to promote cell adhesion, gelatin beads were pretreated with fibronectin. The results from two of the experiments are shown in Figure 3. The final attachment efficiency achieved after 140 minutes was more then 95% of attached cells for both experiments. Semi–logarithmic plots of unattached cell concentration with respect to time yielded straight lines indicating the first order kinetics, mathematically described in Eq (2). Such a first order kinetics have previously been reported by Himes and Hu [15] and Hu et al. [13] using DEAE–derivatized sephadex as a support for anchorage–dependent cells. Their study also indicated that the attachment rate increased with increasing exchange capacity of the microcarriers [13]. Generally, the kinetics of cell binding to charged microcarriers, such as derivatized sephadex or similar commercial Cytodex (Pharmacia) carrier are rather fast, with the rate attachment constant at least one order of magnitude

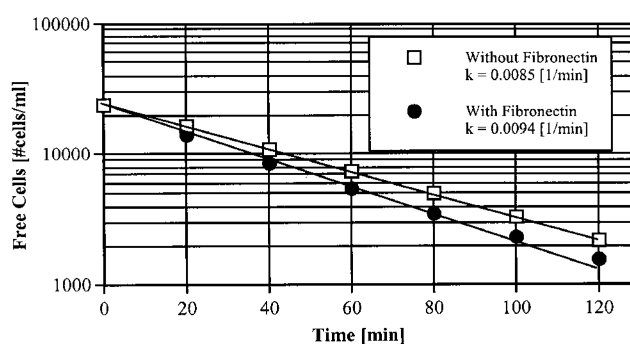

Figure 3. The kinetics of attachment of fish chromatophores on gelatin microcarrier. Reaction conditions: L–15 medium supplemented with 5% serum, pH=7.4, time τ=140 min; stirring rate ν=40 rpm; cell/:bead ratio, λ =50;

higher than that of attachment to biospecific macroporous gelatin carrier [24,26]. However, the final attachment efficiency was reported to be high, and in the range of 90–100% for both types of carriers.

Factors, such as cell type, cell density, cell to bead ratio, as well as the presence of biospecific agents that could help cell attachment, may influence the cell attachment kinetics. The addition of fibronectin in our study resulted in a 10% higher kinetic rate constant (k=0.94x10^{-2}/min) compared to the value without fibronectin (k=0.85x10^{-2}/min). Proteins that make up the extracellular matrix between cells, or cells and substrate are capable of mediating cell attachment and spreading; these include fibronectin, vibronectin, laminin and collagen [22]. All these proteins may also be present in the medium serum. It has been confirmed that they all possess the tripeptide sequence Arg–Gly–Asp (RGD) that binds to cell receptors [31]. The mechanism of cell attachment to the gelatin microcarrier mediated by a biospecific binding of fibronectin, produced either by cells or added from outside into the medium is described in the literature [11].

In order to improve cell binding and viability, certain studies suggest the preincubation of microcarriers with serum–supplemented medium and subsequent inoculation in serum–free medium [32,33]. These studies also showed that some growth factors were more active in the immobilized state, than in the free state.

Effect of Cell to Bead Ratio

The effect of cell/bead ratio (λ = number of cells/number of beads) on the cell attachment rate was determined for Cultisphere S cultures (Table 1).

It is important to insure a large initial cell to bead ratio, which would not affect cell viability and also minimize the proportion of unoccupied beads during the immobilization process. Fish chromatophores are terminally differentiated cells and do not replicate in tissue culture, thus the initial cell/bead ratio will not increase with time, as reported for some other proliferating animal cells like Vero cells [26]. By microscopic examination, we observed that the immobilized fish chromatophores stayed functional, e.g. responsive to nor epinephrine for two to four weeks, although a small decrease in the cell/bead ratio occured due to apoptosis or cell death.

*Table 1. Effect of the cell/bead ratio on the fish chromatophore attachment rate constant k and on the cell viability of immobilized chromatophores**

Initial cell/bead ratio (λ)	Attachment rate constant k (x10^{-2}/min)	Immobilized cell viability (%)	Viable immobilized cell/bead ratio
15	0.74	96	14.4
30	0.78	95	28.5
50	0.84	94	47.0
70	0.87	91	63.7
80	0.85	78	66.3

*Reaction conditions: Attachment the performed on gelatin microcarrier, d=250 μm, in L–15 medium supplemented with 5% serum at pH=7.4 for τ=140 min at the stirring rate ν=40 rpm. The viability of immobilized cells was measured after 24 hours. The data presented are mean values of triplicate experiments

According to the results presented in Table 1, we found λ=50 as the optimum to apply for fish chromatophore immobilization. With higher cell: bead ratio, λ=70 and 80, a higher attachment rate was achieved, but lower cell viability. At λ=50, microscopic examination did not show the presence of unoccupied beads

Effect of pH

The effect of pH on cell attachment was determined from cultures supplemented with 25 mM N–(–2 hydroxy–ethyl) piperazine–N'–2–ethanesulfonic acid (HEPES, Sigma) to maintain a constant pH over the measurement period (140 min). The results (Table 2) indicated that pH had no significant effect on the cell attachment rates to gelatin microcarrier over the pH range 6.8–8.0. In contrast, studies performed with sephadex microcarrier (Cytodex–1) showed a much higher influence of pH [26], which is most probably related to the nature of the electrostatic interaction of the cells with the carrier.

Table 2. Effect of pH on the attachment rate constant k of fish chromatophores in Cultispher S cultures

pH	Attachment rate constant k (x10^{-2}/min)
6.8	0.80
7.1	0.82
7.4	0.84
8.0	0.83

*Reaction conditions: L–15 media supplemented with 5% serum, time τ=140 min; stirring rate ν=40 rpm; cell: bead ratio, λ=50

Sensitivity of the Immobilized Chromatophores

Fish chromatophores immobilized on gelatin microcarriers were microscopically observed in order to test their viability and toxin–sensitivity. This immobilization approach had no measurable detrimental effect on cell viability or sensitivity to added agents. Fig 4. presents an outlook of fish chromatophores immobilized on a gelatin microcarrier in the normal state without the addition of the model toxin noradrenaline (a), and after its addition (b). A visible colour change of the immobilized cells, comparable to that of free cells after exposure to nor adrenaline (Figure 1) indicated that the

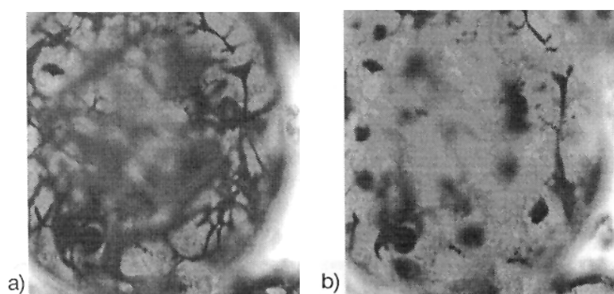

Figure 4. Immobilized fish chromatophores: a) before the addition of nor epinephrine, and b) 5 minutes after the addition of nor–epinephrine.

immobilized cells may serve as indicators of environmental toxicity.

CONCLUSIONS

Gelatin based microcarriers were shown to be the most appropriate for the immobilization of fish chromatophores among three tested carriers (gelatin, glass and polystyrene). The kinetics of chromatophore attachment were first–order with the constant rate reaching 0.94×10^{-2}/min. After 140 min, more than 95% of the fish chromatophores were attached to the gelatin support. Addition of fibronectin as an attachment promoting agent resulted in a 10% higher attachment rate. Viability and toxin–sensitivity were preserved on the microcarrier, which was the main precaution for further incorporation in the biosensor system.

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IZVOD

IMOBILIZACIJA HROMATOFORA RIBA RADI KORIŠĆENJA KAO BIOSENZOR ZA BIOLOŠKE TOKSINE

(Naučni rad)

Ljiljana Mojović[1], Goran Jovanović[2]
[1]Tehnološko–metalurški fakultet, Univerzitet u Beogradu, Beograd, Srbija
[2]Oregon Stete University, Corvallis, OR, USA

Hromatofore izolovane iz ribe *Sijamski borac* vrste *Betta splendens* predstavljaju vrstu živih ćelija koja reaguje vidjivom promenom boje i oblika na dejstvo mikrobnih toksina i zagađivača okoline. U radu je ispitivan izbor najpogodnijeg nosača i razvoj optimalne tehnike za imobilizaciju hromatofora u cilju omogućavanja usmerenog transporta senzorskih ćelija kroz predviđenu strukturu mikrokanala biosenzora. Odgovarajuća imobilizaciona tehnika je potrebno da ostvari dobru vijabilnost i funkcionalnost ćelija. Testirani su mikronosači na bazi stakla, polistirena i želatina (kolagena). Želatinski mikronosač se pokazao najpogodnijim u pogledu efikasnosti vezivanja (95 % ćelija je vezano) i očuvanja vijabilnosti i senzitivnosti ćelija na toksine. Optimalni uslovi za imobilizaciju ćelija na želatinski nosač su utvrđeni i to: odnos broja ćelija i broja mikronosača, i pH imobilizacije. Takođe je praćena kinetika vezivanja ćelija na želatinski nosač, koja je bila kinetika prvog reda. Pretretman želatinskih nosača sa fibronektinom, koji je poznat kao agens koji pomaže vezivanje ćelija, uticao je na povećanje kinetičke konstante prvog reda za 10%.

Ključne reči: Hromofore riba • *Betta splendens* • Biosenzor • Imobilizacija ćelija • Želatinski nosač •
Key words: Fish chromatophores • *Betta splendens* • Biosensor • Cell immobilisation • Gelatin carrier •