# BIOSENSOR FOR DETECTION OF ENVIRONMENTAL AND BIOLOGICAL TOXINS BASED ON FISH CHROMATOPHORES

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Chromatophores isolated from the Siamese fighting fish, Betta splendens, represent a class of colored living cells that provide a vivid color response to environmental and biological toxins, such as those present in water and food. The effect of color change that the chromatophores undergo in response to exposure to toxins is illustrated in Figure 1, which shows chromatophores before and after exposure to neurotoxin clonidine, known as adrenergic agonist.

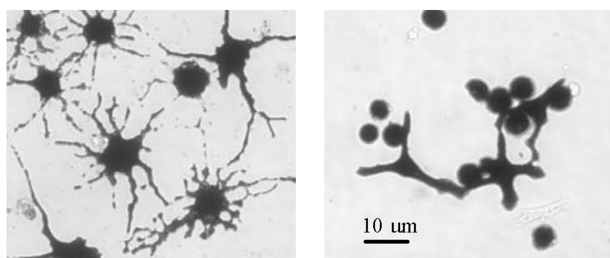


*Figure 1. Fish chromatophores; (left) before exposure to clonidine and (right) after exposure to clonidine.*

In order to take advantage of the properties of the fish chromatophores, a novel biosensor design based on microchannel reactor presented in Fig. 2 is being developed for which immobilization technology is a key component. The appropriate immobilization method should preserve cell viability and sensitivity whilst enabling the directed motion and positioning of sensor cells and samples within the microchannel. The transport and positioning of immobilized cells is facilitated by fluid flow and by magnetic field generated by micro solenoid embedded in channel wall (called "capture–dot").

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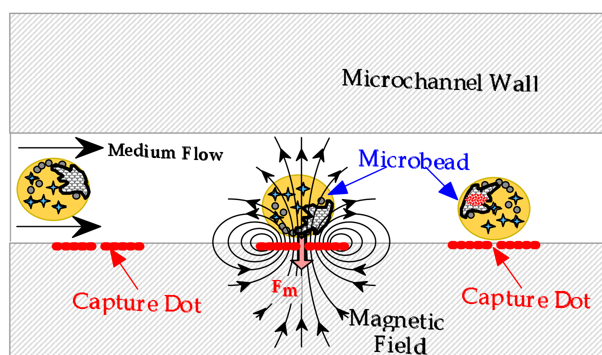


*Figure 2. Microchannel type biosensor. Transport and capturing of microcapsules containing immobilized chromatophores facilitated by the magnetic field generated by "capture dot".*

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 cells attachment onto gelatin beads with various amounts of embedded ferromagnetic powder was investigated, and the optimal conditions for cell attachment 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.

## MATERIAL AND METHODS

*Primary Cell Culture*. Fish chromatophores were isolated from the tails and fins of Betta splendens fish according to the procedure described by Mojovic et al. (2003).

*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 (d=130–380 μm) with various amounts (5%, 10%, 15%, 20% and 25% w/w on gelatin powder) of embedded ferromagnetic powder (iron (II, III)

oxide, powder < 5 μm, Aldrich). Gelatin beads were prepared according to the procedure described by Nilsson et al (1991). All types of microcarriers were hydrated in phosphate–buffered saline (PBS) without $Ca^{+2}$ and $Mg^{+2}$, washed extensively, and then resuspended in PBS at concentration of 5 g/l. Glass and gelatin microcarriers were autoclaved for 20–min at 121°C, while the polystyrene microcarriers were sterilized by incubating at 70°C for 2 h, as recommended by the manufacturer. After sterilization, microcarriers were kept in solution at room temperature.

*Kinetics of cell attachment*. The attachment of the cells to the microcarriers was performed in siliconized (by Sigmacote, Sigma) Erlenmeyer flasks in Leibovitz (L–15) media with very gentle stirring (30–50 rpm). L–15 media was enriched with 5% of fetal bovine serum, FBS (Hyclone, Lab). Microcarriers from stock solution were washed twice with growth medium (L–15), transferred to Erlenmeyer flasks and inoculated with chromatophores isolated from the fish tissue. The rate of attachment of cells to microcarriers was determined by counting cells remaining in the solution. Culture samples (200 μl) were taken at 20–minute intervals and allowed to settle for 1 min in an Ependorf tube. The microcarrier–free supernatant was introduced into a haemocytometer for cell counting.

Samples of immobilized cells were examined microscopically to determine cell viability and toxin–sensitivity. Chromatophores that responded to addition of neurotoxin were considered alive and toxin–sensitive. When the effect of cell attachment–promoting agent fibronectin was studied, the appropriate amount of microcarrier was kept for 2 hours prior to its use in PBS (20–ml) with 100 μl of fibronectin stock solution (Sigma).

The number of microbeads per gram of beads is determined in order to optimize cell/bead ratio (λ).

## RESULTS AND DISCUSSION

### Attachment of Cells to Microcarriers

Figure 3 shows the percent of attached cells to gelatin, glass, and polystyrene microcarriers 3 hours after cell inoculation. The best results were obtained with gelatin beads (95% of attached cells). Attachment to glass microcarrier resulted in significantly lower percent of cells attached (62%), while fish chromatophores showed the lowest affinity towards polystyrene microcarrier (17% of attached cells). Gelatin beads have already been reported to be appropriate for various cell types, such as human fibroblast cells (Himes, et al., 1987), pancreatic islet cells (Malaisse, et al., 1999), Chinese hamster ovary (CHO) cells (Nikolai, et al., 1992), green monkey kidney cells (Vero) (Ng, et al., 1986), and human hepatocytes (Warner, et al., 2000). High cell densities that have been reported could be

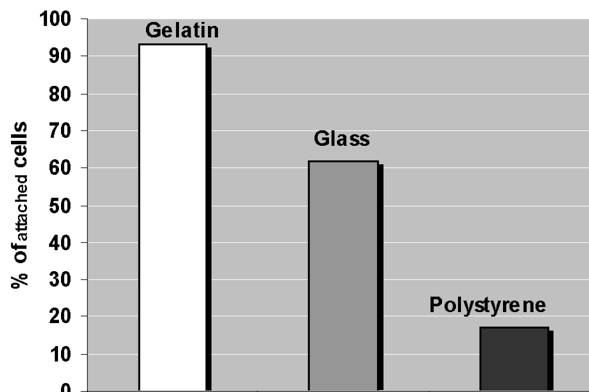


*Figure 3. Attachment of fish chromatophores to microcarriers. Reaction conditions: L–15 media supplemented with 5% of FBS, pH=7.4 time τ=3 hours; stirring rate ν=40 rpm.*

attributed to suitable chemical structure which enables biospecific binding of cells, as well as to a high surface area deriving from a porous structure.

Although glass carriers are widely used in cell culture studies, the chromatophores showed only moderate affinity. Improvements in cell attachment might be achieved by either pretreatment with cell attachment promoting agents, or by using special types of aluminum borosilicate glass with controlled pore size (Koller, et al., 1995). Despite the fact that many cell types adhere better to polystyrene than to glass (Zuhlke, et al., 1993), fish chromatophores demonstrated poor attachment. Chromatophores exhibited the largest affinity towards gelatin support that is why this microcarrier was selected for further studies.

The kinetics of the attachment to gelatin beads containing 10% of ferromagnetic material is presented in Figure 4. To promote cell adhesion, gelatin beads were pre–treated with fibronectin. After 140 minutes 95% of all cells present in the solution were attached on the

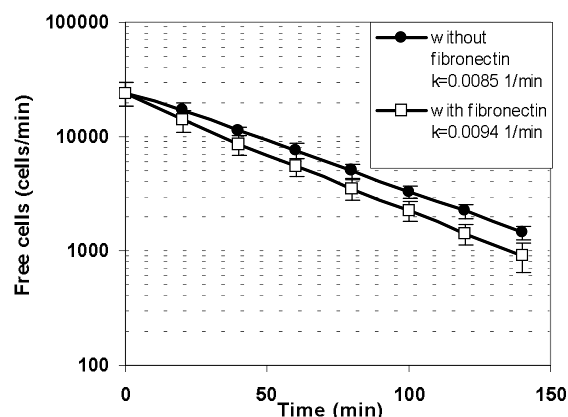


*Figure 4. The kinetics of attachment of fish chromatophores on gelatin microcarriers with 10% (w/w) of iron (II, III) oxide. Reaction conditions: L–15 media supplemented with 5% of FBS, pH=7.4 time τ=140 min; stirring rate ν=40 rpm; cell/bead ratio, λ=70.*

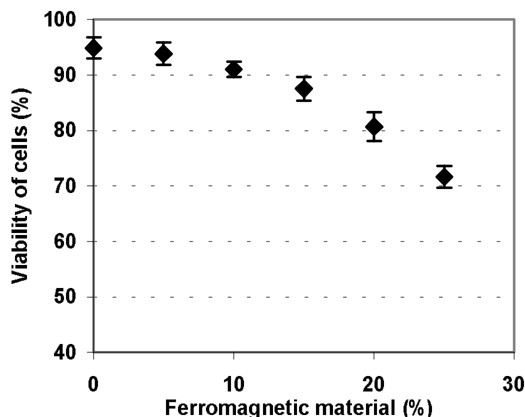


*Figure 5. Effect of the amount of ferromagnetic material in gelatin beads on chromatophore viability. React. conditions are the same as in Fig. 4.*

microcarrier. Semi–logarithmic plots of unattached cell concentration as a function of time yielded straight lines, indicating first order kinetics (Figure 4). The first order attachment kinetics has been reported previously for immobilization of anchorage–dependent cells on DEAE–derivatized sephadex (Himes, et al., 1987). However, the authors used charged microcarriers. Apparently, the kinetics of cell binding to charged microcarriers and the attachment rate constant are at least one order of magnitude higher than the one reported for the attachment to the biospecific macroporous gelatin carrier (Himes, at al., 1987; Hu, et al., 1985). However, for both types of carriers the final attachment efficiency is reported as high as 90–100%. Figure 4 shows that the attachment rate constant for fibronectin–pretreated beads ($k=0.94x10^{-2}$/min) is approximately 10% higher than for beads without pretreatment ($k=0.85x10^{-2}$/min). This result could be expected since proteins like fibronectin; vibronectin, laminin and collagen make up the extracellular matrix between cells or between cells and substratum, and mediate cell attachment and spreading (Ruoslahti, et al., 1987).

No significant effect of ferromagnetic material on the cell attachment rate constant was noticed in the range 0% to 25% of iron (II, III) oxide concentrations used in this study (data not presented). Attachment rates for samples containing different amount of ferromagnetic material were found to be statistically indistinguishable from rates reported for gelatin beads without ferromagnetic material (Mojović, et al., 2001). However, as shown in Figure 5, a significant effect of the concentration of ferromagnetic material on the cell viability is found. Cell viability is seriously affected on beads with 25% iron (II, III) oxide (Figure 5). The 10% concentration of iron (II, III) oxide may be considered appropriate for use in this biosensor study because it

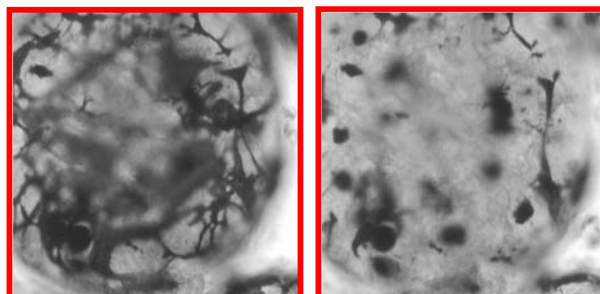

*Figure 6. Video image frames of immobilized gelatin beads with 10% of ferromagnetic powder after exposure to clonidine (c=50nM)*

does not compromise cell viability and it supports complete cell functionality and toxin sensitivity.

### Testing of immobilized chromatophores with clonidine

Figure 6 presents experimental video–image output at time intervals t=0s; and t=360s after addition of neurotoxin to fish chromatophores immobilized on gelatin microcarrier with 10% of ferromagnetic material The aggregation of pigment granules within cells induced by toxin is obvious. It confirms that immobilized chromatophores on gelatin microcarrier with 10% of ferromagnetic powder retained toxin sensitivity and could be employed as a basic sensor element of the biosensor device.

## CONCLUSIONS

Development of a micro–biosensor based on immobilized living chromatophores of Siamese fighting fish, *Betta splendens*, for detection of biological and environmental toxins was investigated in this study. Several microcarriers such as glass, gelatin and polystyrene were tested for efficiency to immobilize chromatophores. The best results were obtained with gelatin microcarrier. The rate of cell attachment to the gelatin microbeads followed first–order kinetics with attachment efficiency of more than 95%. Pretreatment of beads with fibronectin, known as a cell promoting agent, results in a 10% increase of the attachment rate constant ($k=0.94x10^{-2}$/min) when compared to the attachment rate constant obtained without fibronectin ($k=0.85x10^{-2}$/min). A detrimental effect on cell viability was observed when more than 10% of ferromagnetic material is added to the beads. Operation of micro–biosensor was tested with the neurotoxin analog Clonidine as a model toxin.

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