# Co-culture tumor spheroids in microcapsules to study tumor-normal cells interactions

**R. Akasov, D. Zaytseva-Zotova, S. Burov[1], E. Markvicheva***
Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow, Russia, zoolcat@yandex.ru

## INTRODUCTION

Three-dimensional tumor models *in vitro,* such as multicellular tumor spheroids (MTS), can represent quite realistically the organization of small size solid tumors and allow studying the effectiveness of anticancer drug delivery systems. They can be also used to explore biological mechanisms of cell proliferation and differentiation, apoptosis, invasion, angiogenesis, etc. However, tumors *in vivo* are highly complex structures that consist not only from tumor cells, but also from fibroblasts, endothelial cells, pericytes, and inflammatory cells embedded in an extracellular matrix (ECM). For example, it has been shown that cancer-associated fibroblasts stimulate prostate tumor progression and promote breast and colon cancer in animal models (Orimo 2006). Therefore development of methods for co-cultivation of tumor and non-tumor cells *in vitro* model is of great importance.

Microencapsulation is a promising technique to generate MTS by cultivation of cells in semi-permeable polymer microcapsules. It allows to generate spheroids with a narrow size distribution. More over, spheroids based on cells which are not capable to form aggregates in suspension culture can be also generated by this technique. The objective of our study was to develop a new approach to formation of microencapsulated co-culture tumor spheroids using RGD-dependent cell aggregation.

## MATHERIALS AND METHODS

***Reagents*** Sodium alginate (3.5 cP at 25°C, Sigma-Aldrich), $CaCl_2$ (Sigma, USA), EDTA (analytical grade), Angarsk plant of chemical reagents, Russia lipophilic dyes DiO and DiI (Sigma, USA) were used in this study for M3 and L929. Oligochitosan (MM 3410 Da, DD 87%) was kindly provided by Prof. A. Bartkowiak, Poland. All polymer solutions for cell immobilization were prepared in 0.9% NaCl solution.

***The synthesis of RGD-peptides*** Several peptides containing arginine - glycine - aspartic acid (RGD) sequence of both linear (RGDfK) and cyclic (cyclo-RGDfK) structures were synthesized. The synthesis was carried out by solid phase method on Wang resin using Fmoc/But strategy. Cleavage from the resin was carried out with 95% w/v solution of trifluoroacetic acid containing 2.5% triisopropylsilane and water, followed by precipitation with tert-butyl methyl ether. Cyclization was carried out in the presence of HCTU in DMF. Final peptide purification was performed by preparative RP-HPLC with a gradient of 0.1% acetic acid in acetonitrile-water system. Cyclo-RGDfK was modified with triphenylphosphonuim (TPP) to get more charged molecule.

***Cell lines*** Human breast adenocarcinoma (MCF-7 and MCF-7/DXR), human melanoma (A375), human keratinocytes (HaCaT), human colon colorectal carcinoma (HCT116), .murine melanoma (M3) and murine fibroblasts (L929) were used in this research. All cell lines were cultured in medium DMEM (PanEko, Russia) supplemented with 10% fetal calf serum (HyClone, USA) in a $CO_2$ incubator atmosphere containing 5% $CO_2$ at 37°C.

***MTS formation*** MTS formation by RGD-dependent cell aggregation was carried out by the following procedure. Cell suspension (100 ml, $5*10^5$ cells/ml) were seeded in 96-well plates. After 2-3 hours supernatant was removed and the solutions (100 ml) of the RGD-peptides (40 μM) DMEM supplemented with 10% fetal bovine serum were added to cells. MTS were observed in 2-3 days after treatment with RGD-peptides.

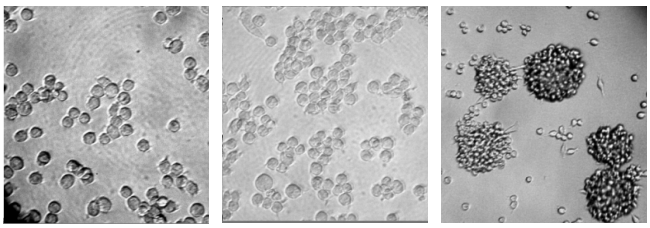
***Cell encapsulation and cultivation***
Microencapsulation of cells was performed using an electrostatic bead generator as described previously (Zaytseva-Zotova 2011). Briefly, the MTS based on M3 cells stained with DiO (green) and L929 single cells stained with DiI (red) were mixed in the sodium alginate solution (1.5% w/v), and this mixture was added into $CaCl_2$ solution (0.5% w/v). The obtained Ca-alginate microbeads were incubated with oligochitosan solution (0.2% w/v) for 10 min to form an alginate-oligochitosan membrane on the microbead surface. Then the microbeads were incubated in a 50 mM EDTA solution for 10 min to dissolve the inner core, and finally transferred to the culture medium. The cultivation of encapsulated cells was carried out in flasks of 25 or 75 $cm^2$ (SPL inc.) in a $CO_2$ incubator atmosphere containing 5% $CO_2$ at 37°C for 1-3 weeks.

***Microscopy study*** Microscopic observations of cell growth were performed with an inverted light microscope Reichert Microstar 1820E (Germany) and a confocal microscope Nikon TE-2000 C1 (Japan).

## RESULTS AND DISCUSSION

RGD sequence is a common element in cellular recognition. This is the cell attachment site of a large number of adhesive extracellular matrix, blood, and cell surface proteins, such as vitronectin, fibronectin, fibrinogen, etc. Both natural and synthetic RGD-

peptides are widely used in tissue engineering and drug targeting. We found that addition of cyclic synthetic RGD-peptides (cyclo-RGDfK and cyclo-RGDfK-TPP to cells (M3, L929, MCF-7 and HCT116) resulted in formation of dense aggregates of a regular spherical form (Fig. 1). The diameter of the MTS was in the range of 100 – 140 µm. The mechanism of this phenomenon could be obviously explained by the presence of integrins which are able to interact with RGD-peptides on the cell surface. Thus, αVβ5 and αVβ3 are the most common integrin receptors for RGD. We suppose that αVβ5 is responsible for this aggregation effect because HCT116 cells were shown to express αVβ5 but not αVβ3 integrin (Monnier 2008).



**24 hours** **48 hours** **72 hours**

**Fig. 1: Formation of spheroids from murine melanoma cells M3 after treatment with cyclo-RGDfK (100 µM).**

Cyclo-RGDfK-TPP peptide was found to be more efficient for inducing cell aggregation compared to Cyclo-RGDfK (Table 1). This fact could be explained by the lipophilic nature of TPP providing RGD-peptides accumulation in the cells. HaCaT, A375 and MCF7/DXR cells were not able to form MTS under these conditions.

**Table 1: Minimum peptide concentrations inducing spheroid formation**

| Cells | Peptide concentration, µM | |
|---|---|---|
| | Cyclo-RGDfK | Cyclo-RGDfK-TPP |
| M3 | 50 | 10 |
| HCT116 | 50 | 10 |
| L929 | 50 | 25 |
| MCF-7 | 100 | 50 |

The formed MTS may be used as a 3D *in vitro* model themselves, but we proposed to improve this system using microencapsulation technique. MTS generated from one cell type (cell line) were mixed with single cells of another cell type and encapsulated in semi-permeable microcapsules as described previously (Zaytseva-Zotova 2011). Cells of different types were stained with different dyes. The cells actively grew and proliferated without significant lost of dye fluorescence within 2 weeks.

This approach allowed to observe interactions of different cell types and to study their invasion. As seen in Fig.2, M3 spheroids maintained their structure even in the RGD-peptide absence while L929 cells grew both on the inner surface of chitosan-membrane and M3 spheroids. There was no significant invasion observed in these microcapsules.

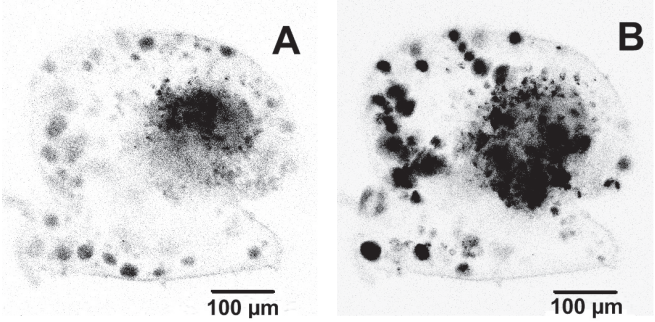



**Fig. 2: Confocal images of the microcapsule with the entrapped M3-based spheroid (a) and single L929 cells (b) after 10 days of cultivation. M3 and L929 were stained with DiO (green) and DiI (red) respectively. M3-based spheroid was formed by treatment of cyclo-RGD-TPP (40 µM).**

When the spheroid was generated from L929 cells, we observed the invasion of single M3 cells into this spheroid. It confirmed the higher invasion potential of M3 melanoma cells compared to L929 fibroblasts.

## CONCLUSIONS

Thus, we proposed a new approach to obtain MTS which can be used as 3D models by simple treatment of cell monolayers with RGD-peptides. To generate co-culture tumor spheroids, the obtained spheroids may be entrapped together with cells of another type in semi-permeable microcapsules. The formed microencapsulated co-culture MTS could be proposed as a 3D *in vitro* model for screening of new drug delivery systems (liposomes, polymeric micelles, nanoemulsions, etc.). More over, they can be promising to study mechanisms of cell proliferation, differentiation, apoptosis and invasion.

## REFERENCES

• Orimo A., Weinberg R.A. (2006) *Stromal fibroblasts in cancer: a novel tumor-promoting cell type*. Cell Cycle 5:1597–1601.

• Monnier Y. et al. (2008) *CYR61 and αVβ5 Integrin Cooperate to Promote Invasion and Metastasis of Tumors Growing in Preirradiated Stroma* Cancer Res 68:7323-7331

• Zaytseva-Zotova D. et al. (2011) *Polyelectrolyte microcapsules with entrapped multicellular tumor spheroids as a novel tool to study the effects of photodynamic therapy.* Journal of Biomedical Materials Research: Part B - Applied Biomaterials 97B 255–262

This research was supported by RFBR, research project № 12-04-31687

[1] Inst. Macromolecular Compounds RAS, St-Petersburg.