Photodynamic effects of chlorine e6 on bioencapsulated tumour spheroids

D. Zaytseva-Zotova[1], O. Udartseva[2], E. Andreeva[2], L. Bolotine[3], JL. Goergen[4], E. Markvicheva[1] *
[1]Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences Miklukho-Maklaya str., 16/10, 117997 Moscow, Russia
*lemark@mw.ibch.ru [2]Institute of experimental cardiology – Moscow, Russia
[3]Unite de Recherche en Therapie Photodynamique, CAV – Vandoeuvre-les-Nancy, France [4]Laboratoire des Sciences du Génie Chimique, UPR CNRS 6811, ENSAIA-INPL – Vandoeuvre-lès-Nancy, France

Introduction

Since Sutherland developed the multicellular tumor spheroid (MTS) model to mimic the 3D-structure of small size solid tumors (Sutherland 1970), MTS have been found to be useful in several aspects of tumor biology, including studies in the field of radiation biology and photodynamic therapy (PDT). Cellular organization of MTS allows to imitate *in vivo* small size tumors much better than 2D *in vitro* models (Hamilton 1998). MTS were demonstrated to represent quite realistically the 3D growth and organization of solid tumors, and consequently to simulate well the cell-cell interactions and microenvironmental conditions found in tumor tissue. This similarities to a tumor xenograft let us apply MTS as a more rapid and valid *in vitro* model for anticancer drug screening compared to a monolayer culture.

MTS could be formed from monolayer tumor cells grown by various *in vitro* classical methods, such as liquid-overlay, spinner flask and gyratory rotation systems. At the same time all classical methods are time consuming and can not provide the production of MTS with narrow spheroid size distribution within a range of 300 – 900 µm. More over, some tumor cells cannot form spheroids in suspension. The method proposed by (Markvicheva 2003) for microencapsulated MTS production provides several advantages over all classical techniques, such as generation of significant spheroid quantities, production of MTS of desired sizes, generation of MTS based on tumor and non-tumor cells which normally can't form aggregates in suspension culture.

The objective of this research was to estimate the response of a novel *in vitro* model based on encapsulated MTS to PDT. Chlorine e6 was chosen as a model photosensitizer.

Materials and Methods

<u>Chemicals:</u> Sodium alginate (medium viscosity), EDTA and $CaCl_2$ were from Sigma. All solutions for cell immobilization were prepared using 0.9% NaCl. Oligochitosan (MM 3500 Da, DD 98 %) was kindly provided by prof. A.Bartkowiak (Poland). Chlorine e6 (Ce6) was supplied by Porphyrin Products (Logan, UT, USA). Ce6 stock solution (2 mM) was prepared in dimethilsulphoxide (DMSO) and stored at -20°C. Before being added into the cell cultures, Ce6 was further diluted in the culture medium. All solutions for cell immobilization were prepared using 0.9% NaCl.

<u>Cells and cell cultivation media:</u> In our study MCF-7 human adenocarcinoma cell line was used. The cells were cultured as a monolayer in DMEM medium supplemented with 10mg/l human insulin, 10 % fetal calf serum (FCS) BioClot at 37^{0}C in a 5 % CO2 humidified atmosphere and were reseeded into fresh medium every 2-3 days.

Bioencapsulation of tumor cells in microcapsules: Cell precipitate (10^7 cells), obtained by trypsinization of monolayer culture was mixed with 2 ml of a sterilized sodium alginate solution, and the mixture was extruded using an electrostatic bead generator into 0.5 % $CaCl_2$ by peristaltic pump. The obtained hydrogel microbeads were incubated with 0.2 % oligochitosan solution for 10 min, in order to form alginate-oligochitosan membrane on microbeads surface. Then microbeads were washed 3 times with physiological saline. In order to get hollow microcapsules, the microbeads were incubated in 50 mM EDTA solution for 10 min and they were again washed and transferred into cultivation medium. Empty microcapsules were prepared as mentioned above.

Cultivation of bioencapsulated cells to generate MTS: The resulted alginate-oligochitosan microcapsules with MCF-7 cells were cultivated in 150 cm^2 (Corning Inc.) flasks at 37^0C in 5 % CO_2 in RPMI medium supplemented with 10 % FBS for 2-4 weeks until MTS were formed.

Ce6 non-specific adsorption on microcapsule surface: Microcapsules were incubated in Ce6 solution in the darkness at various final Ce6 concentrations for 24 h. Then supernatants and microcapsules were washed in the physiological solution, and were analysed using a computer-controlled luminescence spectrofluorimeter (Perkin-Elmer LS50B). The excitation wavelength was 410 nm, and spectra were collected at emission wavelength ranged between 600 – 800 nm. Ce6 solution in the same conditions but without microcapsules was used as a control.

Ce6 cytotoxicity: Encapsulated MTS (100 µl) were coincubated with Ce6 (0 - 34 nM) in 24-well plates for 24h. Each well contained 0.5 ml of RPMI medium supplemented with 2 % FBS. Cell viability was measured using MTT-assay. MCF-7 cells growing in monolayer were used as control. The cytotoxicity was expressed in the form of the viability using the following formula: Viability (%) = (Viable cells concentration in experiment / Viable cells concentration in control) x 100. The experiments were repeated three times.

Ce6 photoxicity: Encapsulated MTS (100 µl) were coincubated with Ce6 (8.4 nM) in 24-well plates for 24h. Then MTS were washed in PBS 3 times and 0.5 ml of RPMI was added in each well. The cells were irradiated by 650 nm diode laser (Coherent, France). Light energy densities were 0.5 – 70 J cm^{-2} at power density 30 mW cm^{-2}. The cell viability was measured using MTT-assay 24 h after irradiation. To study the structure of MTS before and after irradiation at various Ce6 concentrations, a set of MTS samples was selected. The samples were fixed in a 2 % (w/v) formaldehyde solution and embedded to the paraffin to prepare thin sectioned on slides.

Results and Discussion

Cell encapsulation was carried out using a special device, namely an electrostatic bead generator provided by Prof. JL. Goergen. The best calcium-alginate microbeads with a narrow bead size distribution (within the range of 300 – 600 µm, a mean size 340 ± 40 µm) were prepared using the voltage of 7.8 kV (Tabl.1). At the same time alginate-oligochitosan microcapsules mean diameter was much higher – 608 ± 50 µm that that one of microbeads (Fig. 1).

In order to understand the Ce6 non-specific adsorption on microcapsule surface microcapsules were coincubated with Ce6 solution for 24 h. The non-specific Ce6 sorption by empty microcapsules was 30 nmol per 1 ml of microcapsule slurry. Therefore the Ce6 sorption on the microcapsule surface could be ignored, and there was no necessity to remove the microcapsule membrane at photodynamic treatment of encapsulated MTS.

To get MTS model, tumor cells (MCF-7) were microencapsulated and cultivated in 150 ml T-flasks

in a 5 % CO_2 atmosphere at 37 °C for 2 – 4 weeks. The cell proliferation has been easily observed by light microscope (Leitz, Germany). The cells grew in aggregates which have been increasing in their sizes with the cultivation time. The cell concentration in obtained encapsulated MTS was 5 x 10^6 cells/ml slurry.

The cytotoxicity of Ce6 was estimated as an inhibition rate in cell viability. The cytotoxicity increased with increasing of photosensitizer concentration in both encapsulated MTS and monolayer culture, but the viability in encapsulated MTS was higher than that one in monolayer culture (Fig. 2). Results obtained for MCF-7 monolayer were in agreement with data previously reported (Merlin 2003). Maximal non-toxic concentrations were 8.4 µM and 1.7 µM for encapsulated spheroids and for monolayer culture, respectively. These results revealed that the difference between monolayer culture and MTS was rather remarkable even for these previous experiments. These two concentrations were chosen for the next experiments.

Parameter	Value
Voltage (Electrostatic bead generator)	7.8 kV
Flow rate (peristaltic pump)	0.5 ml/min
Tube diameter (peristaltic pump)	1.3 mm
Needle diameter	0.3 mm
Sodium alginate solution concentration	1.3 % (w/v)

Table 1: Optimized conditions for microcapsule preparation technique using an electrostatic bead generator

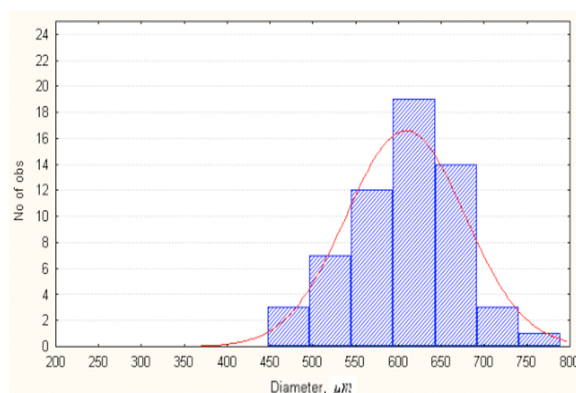

Figure 1: Microcapsule size distribution in RPMI medium (mean diameter 608±50 µm; membrane thickness 70 ± 5 µm)

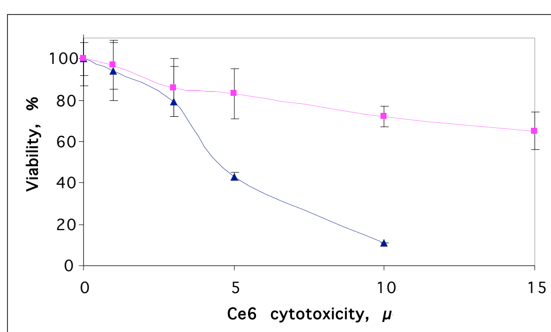

Figure 2: Cell viability in monolayer model (▲) and MTS model (■) after incubation with Ce6 for 24 h

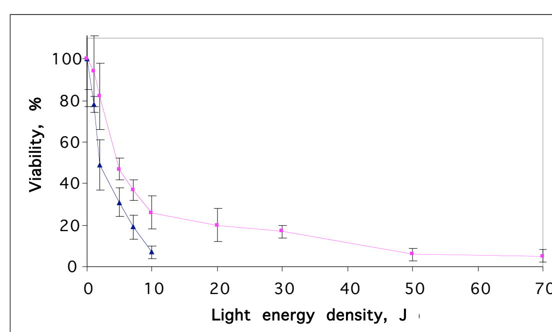

Figure 3: Cell viability in monolayer model (▲) and MTS model (■) 24h after PDT at light energy densities 1-70 J cm^{-2}

As can be seen in Fig. 3, phototoxicity increased with light energy density enhance both for spheroids and monolayer culture. However, the cell viability of the encapsulated MTS was higher than that one of monolayer culture, in spite of the fifthfold Ce6 concentration taken for MTS. For instance, a percentage of viable cells in MTS was tree times bigger compared to monolayer culture at light energy density 10 J cm^{-2}.

Conclusions

Biocompatible polyelectrolyte microcapsules were used to generate MCF-7 cell based encapsulated MTS. Our results demonstrated that the proposed MTS model was much more resistant to the photodynamic treatment than monolayer model. We concluded that the encapsulated MTS model could mimic small size solid tumors more precisely, than commonly used classical monolayer model.

Acknowledgments

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