Polyelectrolyte Microcapsules with Entrapped Multicellular Spheroids as a Novel Tool to Study the Effects of Photodynamic Therapy

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Abstract: In the current study, semi-permeable alginate-oligochitosan microcapsules for multicellular tumor spheroids (MTS) generation were elaborated and tested, in order to estimate a response of the microencapsulated MTS (MMTS) to photodynamic therapy (PDT). The microcapsules (mean diameter 600 μm) with entrapped human breast adenocarcinoma MCF-7 cells were obtained using an electrostatic bead generator, and MMTS were generated by *in vitro* long-term cell cultivation. The formed MMTS were incubated in Chlorin e6 photosensitizer solution and then irradiated using 650 nm laser light. The cell viability was measured by MTT-assay in 24 h after irradiation, and histological analysis was performed. The proposed MTS-based model was found to be more resistant to the PDT than the 2-dimensional monolayer cell culture model. Thus, MMTS could be considered as a promising 3-dimesional *in vitro* model to estimate the doses of drugs or parameters for PDT *in vitro* before carrying out preclinical tests.

Keywords: cell encapsulation, microcapsule, multicellular tumor spheroid, oligochitosan, photodynamic therapy

INTRODUCTION

Recent fast development of photodynamic therapy (PDT) and new photosensitizer (PS) delivery systems (for example, liposomes, nanoemulsions, etc.[1-3]) requires an efficient and high-throughput PS screening and testing system which could be able to faithfully mimic light-accessible solid-tumors *in vivo*. PDT is a clinical treatment that combines effects of visible light irradiation with subsequent biochemical events arising from the presence of photosensitizing drug (possessing no dark toxicity) to cause the destruction of selected tumor cells.[4,5] PSs being introduced into the body, are accumulated in rapidly dividing cells, and the photodynamic effect is accomplished mainly through the PS activation by visible light and following formation of singlet oxygen in the quenching of the sensitizer triplet state by ambient molecular oxygen.[6] The resulting photodamage originates from singlet oxygen and other reactive oxygen species.[7]

One of the key advantages of PDT as a therapy is the concept of dual selectivity. Collateral damage to a normal tissue can be minimized by increasing the selective PS accumulation in the tumor or other desired tissues, and by delivering the light in a spatially confined and focused manner.[8]

Therefore, main constrains of PDT deals with delivery, low selectivity, efficacy, non-toxicity, photoactivation, photodamage, problems of sustained skin photosensitivity and inconveniently long drug-to-light intervals.[4,5] Evidently, most of the challenges related to PDT could be avoided by a discovery of a novel "ideal" PS. Therefore, in order to examine all new promising PS forms development of a simple and reliable *in vitro* test system is needed.

Use of 3-dimensional (3D) spherical cell aggregates in biomedical research is known since beginning of the 70th when Sutherland RM proposed a multicellular tumor spheroid (MTS) model, in order to mimic the 3D structure of small size solid tumors.[9] Over two decades of research have demonstrated that, with respect to traditional 2-dimensional (2D) cell culture

systems, 3D cell models have a potential to improve a physiological relevance of cell based assays and to advance quantitative modeling biological systems based both on cells and living organisms.[10] Furthermore, several studies of PS photochemical consumption based on MTS have been performed.[11-14]

A simple and reproducible technique to generate MTS is a prerequisite for spheroid-based applications. The general criteria for selecting MTS production methods include efficiency, MTS unimodality, convenience and suitability for subsequent applications.[15]

Microencapsulation seems to be one of the simplest methods to generate spheroids. Presently, biocompatible polyelectrolyte microcapsules are widely used for cultivation of animal cells producing monoclonal antibodies, insulin, recombinant proteins and peptides used as therapeutics. However, microencapsulation could be considered also as a useful tool to form MTS representing 3D *in vitro* model of small size solid tumors.

Tumor cell microencapsulation provides several advantages over all classical techniques commonly used, such as generation of significant spheroid quantities, production of MTS with desired sizes, generation of MTS based on tumor and non-tumor cells which normally can't form aggregates in suspension culture, and finally, co-cultivation of tumor and normal cells in one microcapsule.

Careful selection of materials and an appropriate device design are of great importance for the development of encapsulation technology for spheroid fabrication. Since 1980 when Lim F and Sun AM proposed an original technique to entrap animal cells in calcium alginate-poly-L-lysine (PLL) microcapsules,[16] some new methods as well as many modifications of this technique have been proposed.[17,18] Among them there is a replacement of PLL with other polycations, such as poly-L-arginine, poly-L-ornithine etc.,[19] but these materials are rather expensive and toxic even at small concentrations (more than 0.02%).[20] Chitosan could be considered as an alternative to these polycations. In fact, most commercial chitosans are soluble only at acidic pH value while cell encapsulation procedure should be carried out

under physiological conditions (pH 6-8). Oligochitosans (MW below 20 kDa) are soluble at physiological pH values and can be used to form polyelectrolyte microcapsules.[21]

In the present study we aimed to elaborate biocompatible alginate-oligochitosan microcapsules with entrapped multicellular tumor spheroids and to estimate a response of the microencapsulated MTS (MMTS) to PDT.

MATERIALS AND METHODS

Reagents

Sodium alginate (medium viscosity, approx. 3,500 cps at 25 °C), EDTA and $CaCl_2x2H_2O$ were from Sigma, Germany. Oligochitosan (MM 3.5 kDa, deacetylation degree 98%) was prepared by previously described radical degradation,[21] where chitosan of MM 1200 kDa was used as the starting material (chitosan HMW, Aldrich, Germany). All solutions for cell microencapsulation were prepared using 0.9% NaCl.

Chlorin e6 (Ce6) was supplied by Porphyrin Products (Logan, UT, USA). A Ce6 stock solution (2 mM) was prepared in dimethylsulfoxide (DMSO) and stored at -20 °C. Before being added into cell cultures, the Ce6 stock solution was previously diluted with culture medium.

Cells and Culture Conditions

In our study human breast adenocarcinoma cell line MCF-7 was cultured in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) purchased at BioClot, at 37 °C in a 5% CO_2 humidified atmosphere and were reseeded every 2-3 days.

Microencapsulation of Tumor Cells

Cell precipitate ($6x10^6$ cells) obtained by trypsinization of monolayer cell culture was mixed with 2 ml of a sterilized sodium alginate solution (1.3% w/v), and the mixture was added into $CaCl_2$ solution (0.5% w/v) using an electrostatic bead generator. The obtained hydrogel Ca alginate microbeads were incubated with oligochitosan solution (0.2% w/v) for 10 min, in

order to form an alginate-oligochitosan membrane on the microbead surface. Then the microbeads were washed 3 times with physiological solution. To get hollow microcapsules, the microbeads were incubated in a 50 mM EDTA solution for 10 min, then washed again with physiological solution, and finally transferred to the culture medium. Empty microcapsules (without cells) were prepared in the same way as described above. The microcapsule size distribution, the membrane thickness and the cell overall morphology were characterized using optical microscopy (Reichert Microstar 1820E, Germany). A non-specific sorption of Ce6 by microcapsules was analyzed using a computer-controlled Perkin-Elmer LS50B luminescence spectrofluorimeter. The excitation wavelength was 410 nm, and spectra were collected for emission wavelength ranged between 600 – 800 nm.

Generation of MTS in Microcapsules

To form MTS based on MCF-7 cells within alginate-oligochitosan microcapsules the previously encapsulated cells were cultivated in RPMI-1640 medium supplemented with 10% FBS using 75 cm^2 flasks (Corning Inc.) at 37 °C in 5% CO_2 for 2-4 weeks. Aliquots of microcapsules with entrapped cells (100 μL slurry) were collected every 3rd day within 20 days of the cultivation. The microcapsules were destroyed by suspending them through a needle (26G, d=0.3 mm), in order to release entrapped cells, and Trypan Blue assay was used to calculate alive cells.

Study of Ce6 dark cytotoxicity

An aliquot of MMTS (100 μL) was incubated with Ce6 (0 - 34 μM) solution in 24-well plates for 24 h in a darkness. Each well contained RPMI-1640 medium (0.5 mL) supplemented with FBS (2%). Monolayer cell culture (10^5 cells per well) was used as a control. Cell viability was assessed using the MTT colorimetric assay (where MTT is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenil-2H-tetrazolium bromide, PanEco). Briefly, 50 μl of a MTT solution (125 μM) was added into each well for 3 h at 37 °C. After incubation, formazan crystals were solubilized by adding a of DMSO solution (50 μL) into each well. Absorbance was measured

at 540 nm using a Multiscan plate reader (Flow Laboratories). The relative viability of the cells related to the control wells was calculated as [test viability]/[control viability] × 100. Results were expressed as mean ± standard deviation for three replicates.

Study of Ce6 Phototoxicity

The MMTS aliquots (100 μL) and the monolayer culture (10^5 cells) were incubated with the Ce6 solution (8.4 μM and 1.7 μM, respectively) in 24-well plates for 24 h. Then, MMTS and the cells were washed 3 times with PBS, then 0.5 mL of the culture medium was added to each well, and the samples were irradiated at 650 nm diode laser light (light power density was 30 mW cm^{-2}, and light energy densities were varied within a range of 0.5 to 70 J cm^{-2}). In order to measure the irradiation fluence the MMTS aliqoutes and the monolayer culture without Ce6 were irradiated at various energy densities within a range of 0.5 to 70 J cm^{-2} and were used as a an additional control. The cell viability was measured by MTT-assay in 24 h after irradiation.

Preparation of Histological Samples and Histological Assay

To estimate cell morphology in MMST before and after PDT at various Ce6 concentrations, spheroid samples were collected after all experiments. After incubation with the Ce6 solution (0-34 μM) for 24 h and for 24 h after PDT (light energy densities 1-70 J cm^{-2}), MMTS samples were fixed in formaldehyde solution (4% w/v). To obtain cross-sections, the microcapsules were previously embedded in an alginate gel by suspending them in sodium alginate solution (2% w/v) and then cross-linking alginate with formaldehyde (4% w/v). Then, the samples were embedded in paraffin, and 5 μm thickness sections were prepared. The cross-sections were stained with hematoxylin and eosin (H&E) according to a standard protocol.[22] The samples were examined under a light microscope (Reichert Microstar 1820E).

RESULTS

Optimization of the Method for Microcapsule Preparation

The formation of the microcapsule membrane is based on the reaction between two oppositely charged polyelectrolytes: alginate (negatively charged) and oligochitosan (positively charged). Microcapsules with polyelectrolyte alginate-chitosan membrane are stable enough and were described earlier.[21]

To encapsulate cells, we used an electrostatic bead generator originally developed by Bugarski B et al.[23] A scheme of the generator as well as a microcapsules preparation layout are demonstrated in **Figure 1**. The evaluation of the prepared calcium-alginate microbeads demonstrated that their size distribution was in function of several parameters, such as follows:

1. Voltage of the electrostatic bead generator (5.0 – 8.5 kV);
2. Concentration of the alginate solution (1.0 - 2.0% w/v);
3. Diameter of the needle for dispersion of the alginate solution (0.3 – 0.6 mm).

The best calcium-alginate microbeads of an ideal spherical shape and with narrow bead size distribution within the range of 350 ± 50 μm were prepared using the voltage of 7.8 kV (**Table1**).

The microbeads were coated with oligochitosan to get polyelectrolyte alginate-oligochitosan membrane on the bead surface, and then the calcium-alginate core was easily dissolved with an EDTA solution, in order to form hollow microcapsules (Figure 1). The size distribution for the calcium alginate beads and microcapsules prepared from these beads is demonstrated in Figure 2. All microcapsules swelled and increased in their sizes up to 600 ± 50 μm in the culture medium.

Microcapsules stability both in a physiological solution and in the culture medium was estimated. The obtained microcapsules had a nice spherical shape and were stable both in the physiological solution and culture medium RPMI-1640 (pH 6.5 – 8.5) at storage for 4 weeks. This suggested that the incubation time was long enough to form mechanically stable membranes in the culture medium during long-term cell cultivation. An average membrane

thickness easily observed by optical microscope was 80 μm. The non-specific Ce6 sorption by empty microcapsules was less than 5%. Therefore, the obtained alginate-oligochitosan microcapsules were further used for cell microencapsulation and MMTS generation by cell long-term cultivation.

Generation of MMTS by Long-Term Cell Cultivation

To get MMTS, MCF-7 cells were encapsulated and cultivated for 2 – 4 weeks. Cell proliferation has been easily observed by light microscope (Figure 3). The cells were growing in aggregates, which have been increasing in their sizes with the cultivation time. The obtained cell clusters completely filled the microcapsule volume (Figure 3D). The growth curve of the cells within microcapsules demonstrated that after 20 days of cultivation cell density in the generated MMTS was at least 30 times bigger compared to initial one (Figure 4).

Ce6 dark cytotoxicity for MMTS and the Monolayer Culture

Ce6 was chosen as a model PS, since it possess a low dark cytotoxicity, fast and sufficiently selective accumulation in a target tissue, and a well-balanced combination of diagnostic properties and tumoricidal ability.[24] The dark cytotoxicity was measured after incubation of MMTS and monolayer culture with Ce6 solution for 24 h. The results obtained for MCF-7 monolayer were in a good agreement with the data previously reported.[25] Maximal non-toxic concentrations were 8.4 μM and 1.7 μM for the encapsulated spheroids and the monolayer culture, respectively (Figure 5). As a result, we revealed that difference between 2D monolayer and 3D MMTS is remarkable even for the PS cytotoxicity test.

Photodynamic Therapy Effect on MMTS and the Monolayer Culture

After the preliminary incubation of spheroids with Ce6 for 24 h, MMTS and the monolayer culture were irradiated at light power density of 30 mW cm^{-2} by varying light energy densities within a range of 0.5 – 70 J cm^{-2}. The Ce6 photocytotoxicity was determined by MTT-test in 24 h after irradiation. As can be seen in Figure 6, Ce6 phototoxicity increased

with light energy density enhancement for both MMTS and the monolayer culture. However, the cell viability in MMTS was higher than that one in case of the monolayer culture, in spite of the fifth-fold non-toxic Ce6 concentration used in the case of MMTS. For instance, a percentage of viable cells in MMTS was tree times bigger compared to monolayer culture at light energy density of 10 J cm^{-2}.

Characterization of MMTS after PDT

To reveal a difference in cell morphology before and after PDT, histological analysis of MMTS exposed to various irradiation doses has been carried out (Figure 7). Most of the cells in a control sample (MMTS without PDT) were found to have typical epithelioid morphology with homogenously stained cytoplasm and elongated or rounded light nuclei (Figure 7A). Besides, it was possible to observe cells in a mitotic phase as well as cells of smaller size with condensed dark nuclei, supposedly apoptotic.

Unlike the cells in the control (MMTS without PDT), the cells exposed to 5 J cm^{-2} irradiation lost their epithelioid phenotype, their cytoplasm became non-uniform with the increased acidity while cell nuclei decreased in size, and chromatin condensation was observed (Figure 7B). A number of small size cells with dense nuclei, rarely seen in the control, was raised. Nevertheless, most of the cells were found to represent an initial morphology with small changes in cytoplasm acidity, evidenced by more intensive pink eosin staining. The further increase in irradiation fluence up to 30 J cm^{-2} led to the significant cell destruction and the loss in cells integrity (Figure 7C). The number of small dense cells increased, and only a few cells showed their initial morphology. After 70 J cm^{-2} exposition almost all cells lost their entirety, their cytoplasm looked like being ruptured (Figure 7D), and small dense cells became predominant in the cell population. The features of cell apoptosis such as cytoplasmic vacuolization and chromatin fragmentation could be easily observed.

DISCUSSION

The choice of the most relevant cancer model to test PS is a complex and multi-factorial task, and very often it is not rationally justified. Apart from obvious economic and ethical considerations, *in vivo* animal models are in fact progressively showing their limits: although they can mirror some aspects of human responses, they fail to reproduce others.[26]

On the other hand, in the pharmaceutical industry, cell based assays are now routinely used for screening drug safety and efficacy.[27] Data accumulated over the past 30 years have demonstrated significant limitations of traditional 2D cell monolayer cultures in predicting cell behavior in living organisms. Unlike the monolayer culture MTS were demonstrated to represent quite realistically the 3D cell growth and cell organization of small size solid tumors, and consequently to better mimic cell-cell interactions and microenvironmental conditions in tumor tissue.[10] Since MTS were shown to capture the complexity of solid tumors, a spheroid model has been strongly promoted as a highly useful model to replace the traditional 2D monolayer culture.

In the present study, human breast adenocarcinoma cells (MCF-7) cultivated as a monolayer culture, were chosen to form MTS. Unfortunately, all methods traditionally used for spheroid formation can not provide generation of MCF-7 spheroids with narrow size distribution within a desired diameter range, although these cells are anchorage-dependent.[28] While hanging-drop culture is rather effective to obtain MCF-7 homogeneous spheroids, a large-scale production is still a difficult challenge.[29]

Compared to the MTS formation methods mentioned above, microencapsulation is a promising technique for an efficient generation of MCF-7 spheroids with well-defined spherical geometry, which allows to reach the correlation of structure with function and even heterogeneity. At the same time, cell microencapsulation in semi-permeable microcapsules allows a free exchange of nutrients, oxygen as well as cell products between the entrapped cells and the culture medium. Moreover, microencapsulated tumor cells have been found recently to grow in 3D pattern providing microenvironment similar to their *in vivo*

geometry.[30] The idea to form MTS based on human tumor cells was proposed by us previously,[31] and later on, polyelectrolyte microcapsules with the entrapped MCF-7 cells were used for drug screening.[32,33] In the current study we propose to evaluate MMTS generated in polyelectrolyte microcapsules as a tool to study the effect of PDT.

At the same time, we consider that the replacement of PLL with oligochitosan could be a good solution for MMTS generation, since oligochitosans possess low cytotoxicity.[21] In the current study biocompatible microcapsules with polyelectrolyte semi-permeable membrane were obtained using two natural polysaccharides, namely alginate and oligochitosan. The proposed biomaterials and procedure allowed to encapsulate MCF-7 cells in physiological conditions for 30 min. The proposed alginate-oligochitosan microcapsules were shown to be stable during long-term cell cultivation and enabled cell growth and proliferation resulted in MTS formation.

In order to reduce a diffusion limitation to the MTS core an electrostatic bead generator for microcapsules formation was used. As a result, the microcapsule size and a spheroid diameter was decreased (mean MTS size 300 μm). Nevertheless, the results obtained by Trypan Blue assay and histological analysis of cross-sections revealed a few amount of dead cells in the untreated MCF-7 spheroids. This could be explained by the necrotic core formation in the center of MTS. This observation is in agreement with the appropriate data, and indicate that spheroids being analogous to avascular tumor mass have a diffusion limitation of about 150-200 μm to many molecules, in particular O_2.[34]

Our results also demonstrated that Ce6 phototoxicity has a positive relationship with the Ce6 concentration and irradiation fluence to cells both in the monolayer culture and MMTS. The inhibition rate of cell viability differed, and spheroids were generally more resistant to PDT than the monolayer culture. This can be explained by the fact that MTS are dense cell aggregates, and the cells in the core of these aggregates are better protected compared to the cells which are on the surface of these cell aggregates. Therefore, damage of cells in the 3D

MTS is less than in the 2D monolayer culture. These data were in good agreement with those reported previously.[12] The results on Ce6 phototoxicity obtained by MTT-test (Figure 6) can be used to characterize the activity of cell mitochondria but they do not describe an overall cell state. To get an additional information on cell viability after PDT, the analysis of the histological cross-sections was carried out (Figure 7). It revealed the evidence of cell apoptosis, in particular enhancement of cytoplasm vacuolization and chromatin fragmentation when the irradiation fluence increased from 30 to 70 J cm^{-2} (Figure 7C and D). Moreover, the obtained results were in a good agreement with many other data previously reported, namely related to the fact that apoptosis is a prominent form of cell death caused by PDT.[35] However, the mechanism of cell death caused by PDT, complexity of a survival network and death pathways are still not completely clear, and the appropriate studies are in progress.

In conclusion, biocompatible polyelectrolyte microcapsules (mean size 600 μm) were obtained using the electrostatic bead generator. These microcapsules were used to generate multicellular tumor spheroids based on MCF-7 and to study the effects of PDT compared to the monolayer culture. Our results demonstrated that the proposed model based on MMTS was much more resistant to the photodynamic treatment than the monolayer model. The study of cell morphology in spheroids before and after PDT revealed that most of the cells in MTS were apoptotic with characteristic condensed nuclei after PDT while the cells before incubation with Ce6 and irradiation had typical epithelioid morphology with homogenously stained cytoplasm and light rounded or elongated nuclei.

We concluded that 3D model based on MMTS could mimic small size solid tumors more precisely than commonly used classical 2D monolayer model. We suggest that biocompatible polymeric microcapsules with entrapped multicellular tumor spheroids can be considered as a novel useful tool to study the mechanism of PDT.

The proposed technique for MTS generation could also provide entirely new avenues for developing a novel co-culture *in vitro* model (for instance, based on co-cultivation of

endothelial and tumor cells). More over, it could be proposed to study novel drug delivery systems based both on common and new photosensitizers for PDT before preclinical tests.

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REFERENCES

[1] Hu FQ, Jiang XH, Huang X, Wu XL, Yuan H, Wei XH, Du YZ. Enhanced cellular uptake of chlorine e6 mediated by stearic acid-grafted chitosan oligosaccharide micelles. JDrugTarget 2009;17:384-391.

[2] Isakau HA, Parkhats MV, Knyukshto VN, Dzhagarov BM, Petrov EP, Petrov PT. Toward understanding the high PDT efficacy of chlorin e6-polyvinylpyrrolidone formulations: photophysical and molecular aspects of photosensitizer-polymer interaction *in vitro*. JPhotochemPhotobiolB 2008;92:165-174.

[3] Qian HS, Guo HC, Ho PCL, Mahendran R, Zhang Y. Mesoporous-Silica-Coated Up-Conversion Fluorescent Nanoparticles for Photodynamic Therapy. Small 2009;5:2285-2290.

[4] Donnelly RF, McCarron PA, Woolfson D. Drug delivery systems for photodynamic therapy. RecentPatDrugDelivFormul 2009;3:1-7.

[5] Pervaiz S, Olivo M. Art and science of photodynamic therapy. ClinExpPharmacolPhysiol 2006;33:551-556.

[6] Henderson BW, Dougherty TJ. How does photodynamic therapy work? PhotochemPhotobiol 1992;55:45-157.

[7] Pass HI. Photodynamic therapy in oncology: mechanisms and clinical use. JNatlCancerInst 1993;85:443-456.

[8] Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy. PhotodiagnosisPhotodynTher 2004;1:279—293.

[9] Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 1988;240:177-184.

[10] Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. NatRevMolCellBiol 2007;8:839-845.

[11] Mathews MS, Angell-Petersen E, Sanchez R, Sun CH, Vo V, Hirschberg H, Madsen SJ. The effects of ultra low fluence rate single and repetitive photodynamic therapy on glioma spheroids. LasersSurgMed 2009;41:578-584.

[12] Marchal S, Fadloun A, Maugain E, D'Hallewin MA, Guillemin F, Bezdetnaya L. Necrotic and apoptotic features of cell death in response to Foscan photosensitization of HT29 monolayer and multicell spheroids. BiochemPharmacol 2005;69:1167-1176.

[13] Bressenot A, Marchal S, Bezdetnaya L, Garrier J, Guillemin F, Plénat F. Assessment of apoptosis by immunohistochemistry to active caspase-3, active caspase-7, or cleaved PARP in monolayer cells and spheroid and subcutaneous xenografts of human carcinoma. JHistochemCytochem 2009;57:289-300.

[14] Kamuhabwa AA, Huygens A, De Witte PA. Photodynamic therapy of transitional cell carcinoma multicellular tumor spheroids with hypericin. IntJOncol 2003;23:1445-1450.

[15] Lin RZ, Chang HY. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. BiotechnolJ 2008;3:1172-1184.

[16] Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980;210:908-910.

[17] Sakai S, Ito S, Ogushi Y, Hashimoto I, Hosoda N, Sawae Y, Kawakami K. Enzymatically fabricated and degradable microcapsules for production of multicellular

spheroids with well-defined diameters of less than 150 microm. Biomaterials 2009;30:5937-5942.

[18] Sakai S, Ito S, Kawakami K. Calcium alginate microcapsules with spherical liquid cores templated by gelatin microparticles for mass production of multicellular spheroids. ActaBiomater 2010;6:3132-3137.

[19] Murua A, Portero A, Orive G, Hernández RM, de Castro M, Pedraz JL. Cell microencapsulation technology: towards clinical application. JControlRelease 2008;132:76-83.

[20] Strand BL, Skjak-Braek G, Gaserod O. Microcapsule formulation and formation. Willaert R, Nedovich V, editors. Fundamentals of cell immobilization biotechnology, Series Focus on Biotechnology, Dordrecht–Boston–London: Kluwer Academic Publishers; 2004:165–185.

[21] Orive G, Bartkowiak A, Lisiecki S, De Castro M, Hernández RM, Gascòn AR, Pedraz JL. Biocompatible Oligochitosans as Cationic Modifiers of Alginate/Ca Microcapsules. JBiomedMaterResBApplBiomater 2005;74B:429 - 439.

[22] Kiernan JA. Methods for connective tissue. Kiernan JA, editors. Histological and histochemical methods, theory and practice, Oxford: Butterworth-Heinemann; 1999:144–164.

[23] Bugarski B, Li Q, Goosen MFA, Poncelet D, Neufeld RJ, Vunjak G. Electrostatic droplet generation: Mechanism of polymer droplet formation. AIChE 1994;40:1026–1031.

[24] Kostenich GA, Zhuravkin IN, Zhavrid EA. Experimental grounds for using chlorin e6 in the photodynamic therapy of malignant tumors. JPhotochemPhotobiol 1994;22:211–217.

[25] Merlin JL, Gautier H, Barberi-Heyob M, Teiten MH, Guillemin F. The multidrug resistance modulator SDZ-PSC 833 potentiates the photodynamic activity of chlorine e6 independently on P-glycoprotein in multidrug resistant human breast adenocarcinoma cells. IntJOncol 2003;22:733-739.

[26] Mazzoleni G, Di Lorenzo D, Steimberg N. Modelling tissues in 3D: the next future of pharmaco-toxicology and food research? GenesNutr 2009;4:13-22.

[27] Griffith LG, Swartz MA. Capturing complex 3D tissue physiology *in vitro*. NatRevMolCellBiol 2006;7:211-224.

[28] Yuhas JM, Tarleton AE, Molzen KB. Multicellular tumor spheroid formation by breast cancer cells isolated from different sites. CancerRes 1978;38:2486-2491.

[29] Kelm JM, Timmins NE, Brown CJ, Fussenegger M, Nielsen LK. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. BiotechnolBioeng 2003;83:173-180.

[30] Wang Q, Li S, Xie Y, Yu W, Xiong Y, Ma X, Yuan Q. Cytoskeletal reorganization and repolarization of hepatocarcinoma cells in APA microcapsule to mimic native tumor characteristics. HepatolRes 2006;35:96-103.

[31] Markvicheva E, Bezdetnaya L, Bartkowiak A, Marc A, Goergen JL, Guillemin F, Poncelet D. Encapsulated multicellular tumor spheroids as a novel model to study small size tumors. ChemInd 2003;57:585-588.

[32] Zhang X, Wang W, Yu W, Xie Y, Zhang X, Zhang Y, Ma X. Development of an *in vitro* multicellular tumor spheroid model using microencapsulation and its application in anticancer drug screening and testing. BiotechnolProg 2005;21:1289-1296.

[33] Tsoy AM, Zaytseva Zotova DS, Edelweiss EF, Bartkowiak A, Goergen JL, Vodovozova EL, Markvicheva EA. Microencapsulated multicellular tumor spheroids as a novel *in vitro* model for drug screening. Biochem(Mosc)SupplSeriesBBiomedChem 2010;4:243–250.

[34] Curcio E, Salerno S, Barbieri G, De Bartolo L, Drioli E, Bader A. Mass transfer and metabolic reactions in hepatocyte spheroids cultured in rotating wall gas-permeable membrane system. Biomaterials 2007;28:5487-5497.

[35] Agostinis P, Buytaert E, Breyssens H, Hendrickx N. Regulatory pathways in photodynamic therapy induced apoptosis. PhotochemPhotobiol.Sci 2004;3:721-729.

Figure Legends:

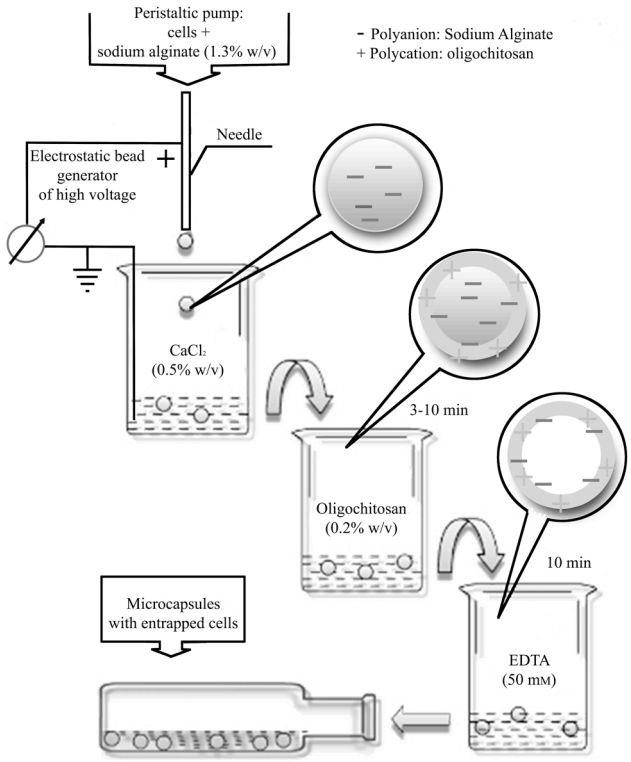

Figure 1. Scheme of alginate-oligochitosan microcapsule preparation procedure.

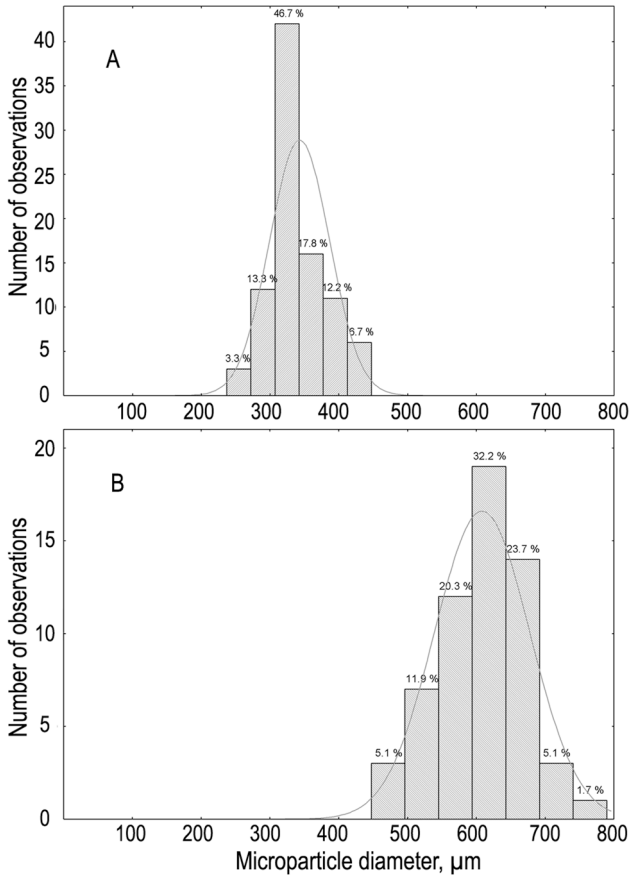

Figure 2. Size distribution of Ca-alginate microbeads in physiological solution (A) and alginate-oligochitosan microcapsules in RPMI-1640 medium (B). Microbeads mean size 340 ± 40 μm, Microcapsule mean size 600 ± 50 μm; membrane thickness 70 ± 5 μm.

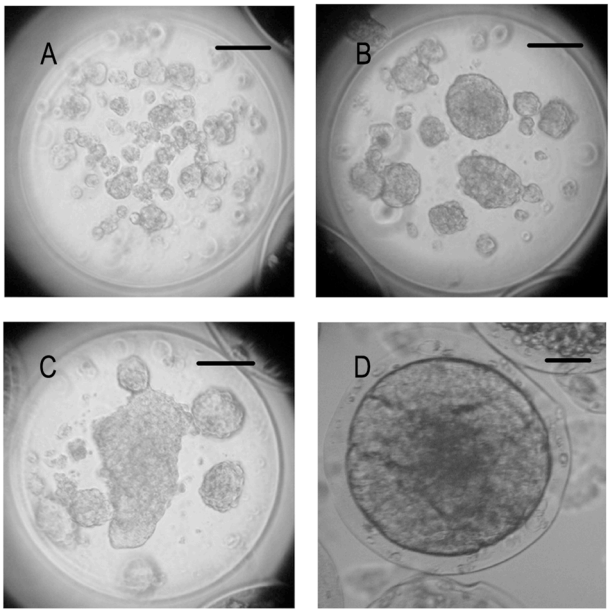

Figure 3. Generation of multicellular spheroids in alginate-oligochitosan microcapsules. Time after encapsulation: 1 day (A), 4 days (B), 7 days (C), 18 days (D). Size bar shows 100 μm.

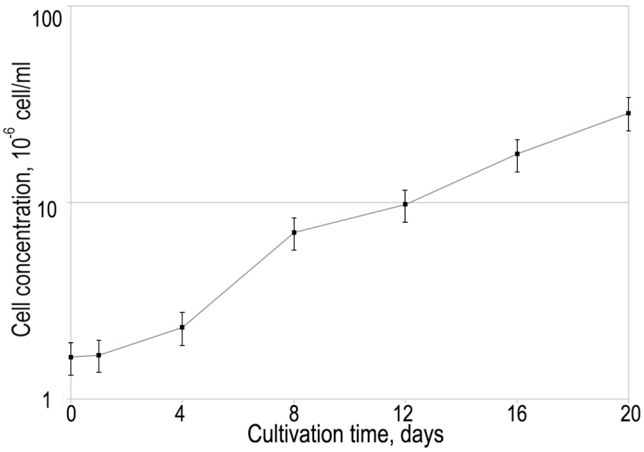

Figure 4. Growth curve of MCF-7 cells inside microcapsules based on alginate and oligochitosan.

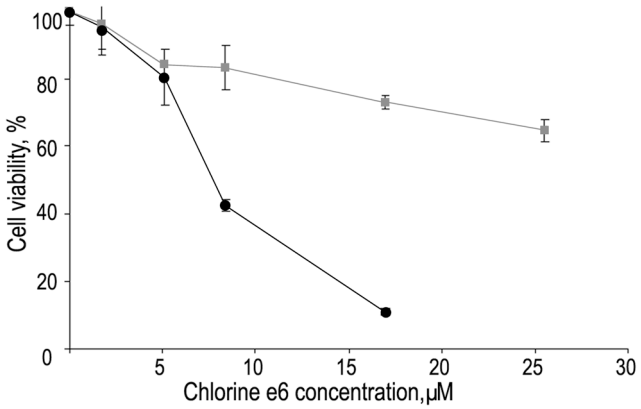

Figure 5. Cell viability in the monolayer model (●) and MMTS (■) after incubation with Ce6 for 24 h.

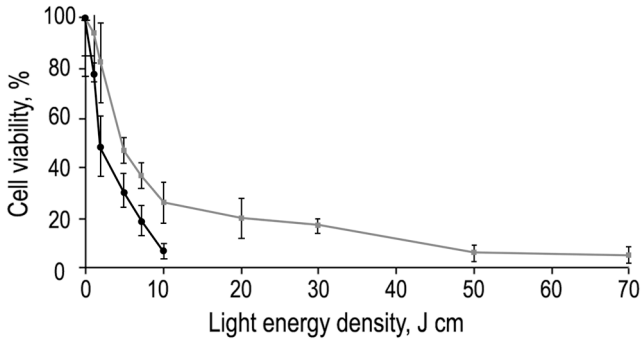

Figure 6. Cell viability in the monolayer model (●) and MMTS (■) 24h after PDT at light energy densities 1-70 J/cm2. Chlorin e6 concentrations were 1.7 μM and 8.4 μM for monolayer culture and MMTS, respectively.

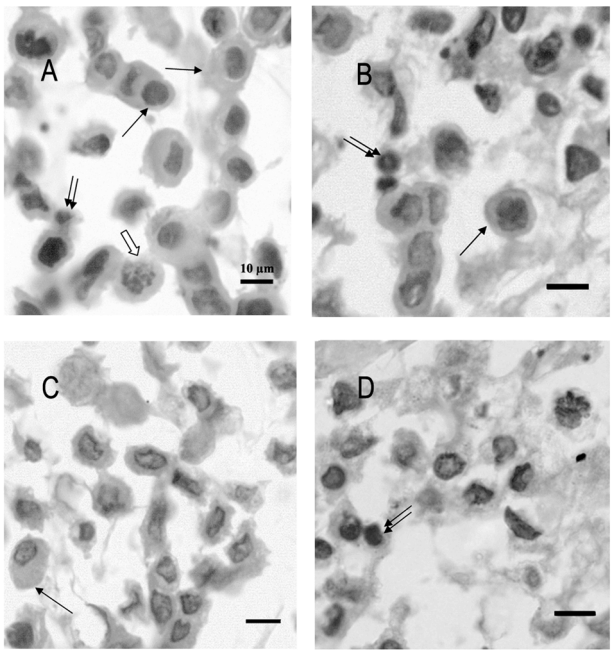

Figure 7. High magnification images of cross-sections of multicellular tumor spheroids based on MCF-7 cells in microcapsules on day 20 after cell encapsulation. Control sample, without photodynamic treatment (A). Samples after photodynamic treatment at light energy density 5 J cm^{-2} (B), 30 J cm^{-2} (C), 70 J cm^{-2} (D) Scale bar = 10 µm.). Typical epithelioid cells indicated with black arrows, mitotic figure – with white arrow, small cells with condensed nuclei – with double arrow.