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ENCAPSULATED MULTICELLULAR TUMOR SPHEROIDS AS A NOVEL IN VITRO MODEL TO STUDY SMALL SIZE TUMORS

Presently multicellular tumor spheroids (MTS) are being widely used in various aspects of tumor biology, including studies in biology and photodynamic therapy. The cellular organization of spheroids allows the recreation of in vivo small tumors much better than all common two–dimensional in vitro models. The cell encapsulation method could be proposed as a novel technique to quickly and easily prepare a large number of spheroids with narrow size distribution within a desirable diameter range. Moreover, the proposed technique for spheroid generation using encapsulated growing tumor cells could provide entirely new avenues to develop a novel spheroid co–culture model (for instance, the in vitro co–cultvation of tumor cells and monocytes, or epithelial cells, or fibroblasts etc). The current research was aimed at developing a simple and reliable method to encapsulate tumor cells and to cultivate them in vitro. In order to generate spheroids, MCF–7 cells were encapsulated and cultivated in 200 ml T–flasks in a 5% CO_2 atmosphere at 37°C for 4–5 weeks. The cell proliferation was easily observed using a light microscope. The cells grew in aggregates increasing in size with time. The cell growth resulted in the formation of large cell clusters (spheroids) which filled the whole microcapsule volume in 4–5 weeks.

Presently multicellular tumor spheroids are being widely used in various aspects of tumor biology, including studies in the field of radiation biology and photodynamic therapy (Santini, M.T., et al., 1999). Multicellular spheroids are three–dimensional structures. They are formed from monolayer tumor cells grown by various *in vitro* methods, such as liquid–overlay, spinner flask and gyratory rotation systems. The cellular organization of spheroids allows the recreation of in vivo small tumors much better than two–dimensional *in vitro* models. However, all these methods are time consuming and cannot provide the production of spheroids with narrow size ranges. Moreover, some tumor cells (HT29, EMT6, KB3) cannot form spheroids in suspension at all. The cell encapsulation method could then be proposed as a novel technique to quickly and easily prepare a large number of spheroids with narrow size distribution within a desired diameter range. The method can be applied to all cell types (aggregating and non–aggregating in suspension culture). The technique for spheroid generation using encapsulated growing tumor cells could provide entirely new avenues to develop a novel spheroid co–culture model (for instance, the *in vitro* co–cultvation of tumor cells and monocytes, or epithelial cells, or fibroblasts etc). The preparation of multicellular spheroids consisting of several cell types is very difficult or in some cases an even impossible process when using common methods. The proposed approach based on the *in vitro* co–culture model could allow the better mimicing of *in vivo* small size tumors where, as is well known, several kinds of cells are engaged in the tumor growth process.

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MATERIALS AND METHODS

Chemicals. Sodium alginate (medium viscosity), and calcium chloride were from Sigma, USA.

The samples of low molecuular weight chitosan (30 kDa, deacetylation degree 98%) and oligochitosan (3.5 kDa, DD 89%) were kindly provided by Prof. G. Vikhoreva and by Prof.. Bartkowiak, respectively.

Cells. In our study we used human breast adenocarcinoma cells MCF–7 kindly provided by the Centre Alexis Vautrin (Vandeuvre–les–Nancy, France).

Cultivation media. For cell cultivation we used RPMI–1640, supplemented with 10% foetal calf serum (FCS), Gibco.

Cell encapsulation in alginate–chitosan microcapsules was carried out using a two–step procedure. A sodium alginate solution in 0.9% NaCl (2 ml, 1.5–2% (w/v) with cells (final cell concentration $3x10^6$ cells/ml of alginate solution) was added dropwise to a 1% (w/v) $CaCl_2$ solution using a special sprayer and peristaltic pump. An electrostatic bead generator (7.8 kV) was designed at the Laboratoire des Sciences du Génie Chimique – UPR 6811 of CNRS (ENSAIA, Vandoeuvre–les–Nancy, France) to provide the formation of small calcium alginate (CaAlg) beads. The bead size could be varied from 200–900 μm. The chitosan solution was obtained by dissolving the chitosan sample in 0.9% NaCl using a magnetic stirrer with the addition of several drops of glacial acetic acid. The oligochitosan sample was easily dissolved in a physiological solution giving pH 3.8. The pH value of both polymer solutions was then adjusted to pH 6.0–6.5 by adding several drops of

2M NaOH. After incubation in 0.2 –0.4% (w/v) chitosan (or oligochitosan) solution for 8–10 min at room temperature, the beads were washed twice with physiological solution. The alginate core was then dissolved by treatment of the beads with 50 mM EDTA in physiological solution (10 min). The obtained alginate–chitosan microcapsules were subsequently washed with RPMI–1640 medium and transferred to culture flasks containing 20 ml of culture medium. The microcapsule volume was about 10% of the total medium volume in the culture flask.

Cultivation of encapsulated tumor cells. The MCF–7 cells encapsulated in alginate–chitosan microcapsules were cultivated in 200 ml flasks in a 5% CO_2 atmosphere at 37°C for 4–5 weeks. The medium was completely replaced every 2nd or 3rd day starting with the 3rd day after cell encapsulation.

The Microcapsule size distribution was studied using robotized automatic image analysis (Centre Alexis Vautrin Vandoeuvre–les–Nancy, France). To provide this analysis a completely automatized integrated device, combining an automatic microscope with image analysis software and a robotic arm (Techlab, St. Julien–les–Metz, France) was designed. This device was constructed to analyse spheroids and was then successfully used by us to also measure the microcapsule size. The results were reported as size distribution histograms.

Cell growth in transparent microcapsules was observed using a light microscope (Leitz, Germany) and monitored by counting viable cells after staining them with Trypan Blue (Gibco) in a hemocytometer.

RESULTS AND DISCUSSION

Alginate–chitosan beads and microcapsules, as promising candidates for long term cultivation of animal cells, have attracted considerable research attention over recent decades. Several reports were focused on the quantitative study of the interaction between alginate and chitosan (Gaserod, 1998), the mechanical resistance and permeability of the membrane (Bartokwiak and Hunkeler, 2000). Chitosan is widely employed in wound healing, the reduction of blood cholesterol levels, and immune system stimulation. The disadvantage of the "chitosan–based" method for the encapsulation of animal cells deals with poor chitosan solubility at physiological pH in contrast to poly–L–lysine, which can be easily used at pH 7. However, taking into account that the polyelectrolyte alginate–chitosan complex can be considered as stronger than alginate–poly–L–lysine (PLL) (Thu, 1997), and that PLL may provoke inflammatory responses (Strand et al., 2001), we suppose that "improved" modified alginate–chitosan microcapsules will be widely used for the encapsulation of various animal cell lines already in the nearest future. A promising approach, namely the preparation of alginate–oligochitosan microcapsules has been recently demonstrated (Bartkowiak and Hunkeler, 1999). In this paper oligochitosan (MM 2900), obtained by the radical degradation method from chitosan 50 kDa, was proposed for the preparation of a strong membrane at pH 7. In our research we used low molecular weight chitosan (30 kDa) and oligochitosan (3.5 kDa). The alginate–chitosan microcapsules were obtained in two steps : 1) by adding sodium alginate solution to $CaCl_2$ solution to obtain CaAlg beads which were then coated with chitosan (oligochitosan) and 2) by dissolving the alginate core by EDTA.

Analysis of the CaAlg beads demonstrated that the size was a function of several investigated parameters, such as: 1) the concentration of the alginate solution (1.5 and 2.0% w/v); 2) the diameter of the needle used for the alginate solution dispersion (0.25 mm, 0.33 mm, 0.45 mm, and 0.6 mm); 3) the voltage of the electrostatic bead generator (5.0–8.5 kV); 4) the distance between the tip and the gelling calcium chloride solution; 5) the flow rate of alginate solution provided by a peristaltic pump etc.

Typical histograms for microcapsule size distribution are shown in Fig. 1 (Fig. 1a and Fig. 1b), while a picture of microcapsules with entrapped cells is shown in Fig. 2. As can be seen, after incubation in the cultivation medium for 2h, the microcapsules swelled, and their mean size increased from 367.7 μm to 489.4

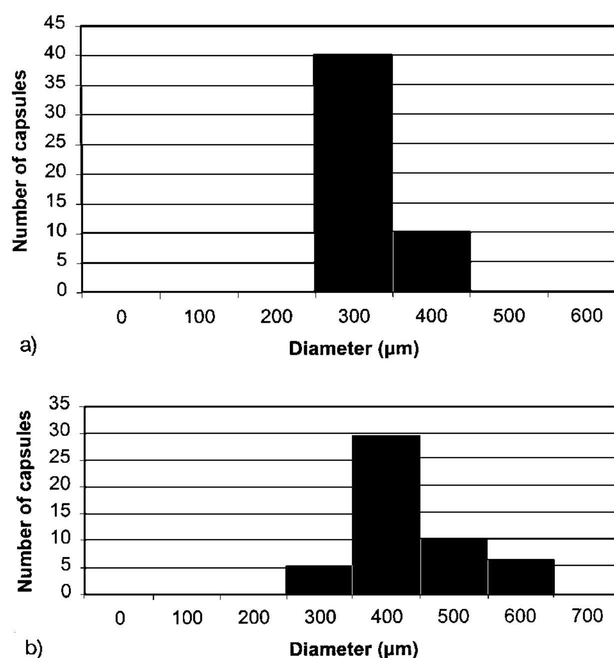

Figure 1. Microcapsule size distribution in physiological solution (a) and in cultivation medium after 2h storage (b). The capsules were prepared using 1.5% alginate solution and a needle diameter of 33 mm.

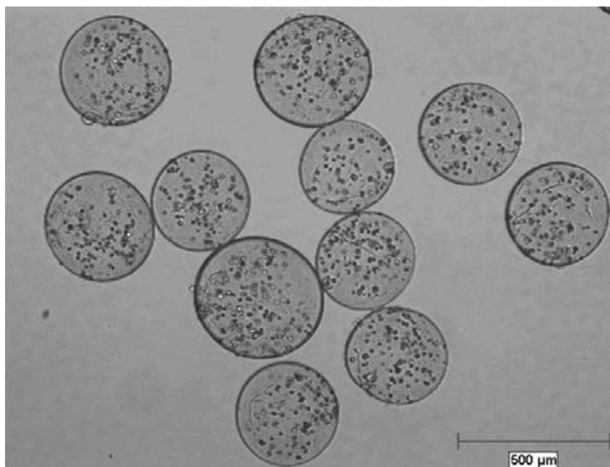

Figure 2. Microcapsules with encapsulated MCF-7 cells immediately after the encapsulation procedure.

Table 1. The microcapsule size as function of alginate solution concentration and needle diameter

Concentration of sodium alginate solution, %	Needle diameter, mm	Mean microcapsule size in physiological solution, μm
2.0	0.60	791.8
2.0	0.33	425.4
1.5	0.45	512.0
1.5	0.33	367.7
1.5	0.25	289.5

μm. To decrease swelling in the cultivation medium, as well as to increase alginate–chitosan membrane stability, the microcapsules were additionally coated with alginate (0.2% (w/v), 10 min).

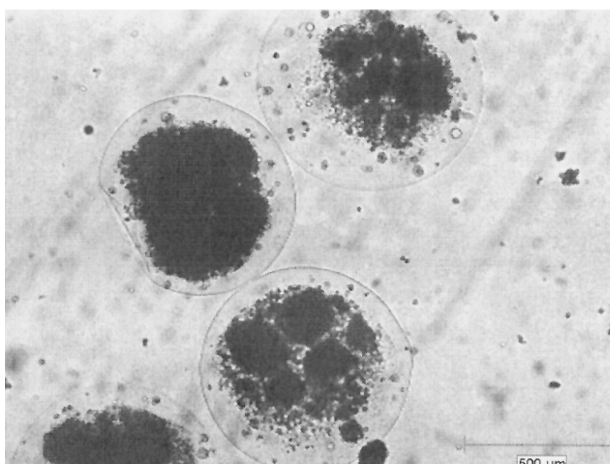

Figure 3. The growth of MCF-7 cells entrapped in alginate-chitosan microcapsules on the 23rd day after encapsulation. Medium RPMI, 10% FCS

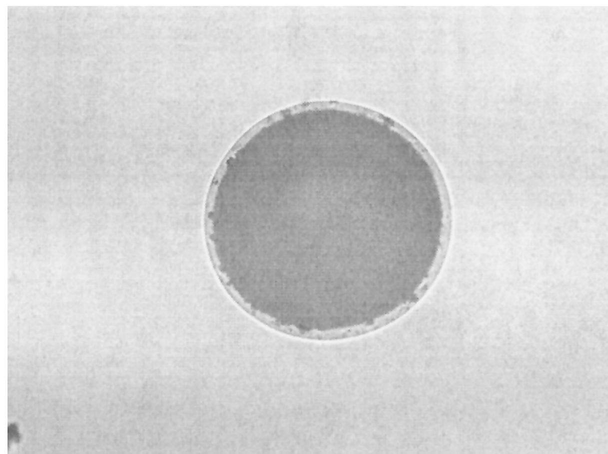

Figure 4. The formed spheroid of MCF-7 cells within the alginate-chitosan microcapsule. Medium RPMI, 10% FCS. Time: 28 days after encapsulation.

To prepare a series of microcapsule samples with narrow size distribution for every sample, the concentration of the sodium alginate solution and the needle diameter were varied (Table 1). The stability of the microcapsules depended upon the inner structure (density) and the thickness of the polyelectrolyte membrane. One of the key parameters affecting the membrane formation process, the membrane thickness and its stability was the molecular weight of chitosan. The use of oligochitosan (3.5 kDa) allowed us to prepare stable microcapsules the membrane of which was 15–20 μm thicker compared to those made of chitosan 30 kDa. Regardless of polysaccharide sample (chitosan or oligochitosan), the membrane thickness depended upon two parameters: 1) the pH value of the chitosan solution; 2) the incubation time of CaAlg beads in the chitosan solution. The incubation time of CaAlg beads in chitosan solution had a profound influence on the process of microcapsule formation. The stability of the microcapsules increased with time due to an increase of the microcapsule membrane thickness.

In order to generate spheroids, the MCF–7 cells were encapsulated and cultivated in 200 ml T–flasks in a 5% CO_2 atmosphere at 37°C for 4–5 weeks. The cell proliferation was easily observed using a light microscope (Fig. 3). The cells grew in aggregates increasing in size with time and forming large cell clusters (spheroids) which filled the whole microcapsule volume (Fig. 4).

Thus, the proposed encapsulation method allowed us to obtain multicellular tumor spheroids within alginate–chitosan microcapsules. The spheroid morphology will be characterized by light and electron microscopy, and the effect of photodynamic therapy on tumor cells will be evaluated. The technique for spheroid generation could provide entirely new avenues for developing a novel co–culture *in vitro* model.

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IZVOD

INKAPSULIRANI SFEROIDI TUMORSKIH ĆELIJA KAO NOV *IN VITRO* MODEL ZA ISPITIVANJA MALIH TUMORA

(Naučni rad)

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Višećelijski sferoidi tumora (VST) se danas koriste u izučavanju tumora, za razna ispitivanja u biologijii i fotodinamičkoj terapiji. U sferoidima se ćelije mogu organizovati gotovo identično kao u malim tumorima *in vivo*, pa je ovo mnogo pogodniji model tumora nego postojeći dvodimenzioni *in vitro* modeli. Opisani metod inkapsulacije ćelija može se predložiti kao nova tehnika za jednostavnu i brzu pripremu velikog broja sferoida sa prilično ravnomernom distribucijom željenog opsega prečnika. Pored toga, predložena tehnika za pravljenje sferoida na bazi rastućih tumorskih ćelija pruža mogućnosti za razvoj novog sferoidnog modela kulture tkiva (na pr. za *in vitro* kultivaciju tumorskih ćelija i monocita, ili ćelija epitela, ili fibroplasta itd.) Cilj ovog istraživanja je bio razvoj jednostavnog i pouzdanog metoda za inkapsulaciju tumorskih ćelija i za njihovu *in vitro* kultivaciju. Da bi se napravili sferoidi, MCF–7 ćelije su inkapsulirane i kultivisane u T–posudama za gajenje u atmosferi sa 5% CO_2 na 37°C u toku 4–5 nedelja. Proliferacija ćelija je praćena korišćenjem svetlosnog mikroskopa. Ćelije su rasle u agregatima koji su se uvećavali tokom vremena, što je rezultiralo formiranjem velikih nakupina (sferoida) koji su za 4–5 nedelja ispunili celokupnu zapreminu mikrokapsule.

Ključne reči: Višećelijski sferoidi • Inkapsulacija ćelija • Alginat–hitozan mikrokapsule •
Key words: Multicellular spheroids • Cell encapsulation • Alginate–chitosan microcapsules •