BIOENCAPSULATED PEPTIDES, PROTEINS, ANIMAL CELLS: PREPARATION AND APPLICATIONS IN BIOMEDICAL FIELDS

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Presently bioencapsulation is widely employed in many biomedical fields. Bioencapsulated peptides and proteins are used as controlled drug delivery systems. Bioencapsulated genetically modified animal cells in immunoprotective microcapsules are considered as artificial organs. This approach, so called "somatic gene therapy", allows universal nonautologous cell lines to be implanted into any host and to produce the desired gene product (hormones, enzymes, recombinant proteins etc) without triggering graft rejection. Therefore, it can be used for any patient with the same disease. The list of diseases which could be treated using this approach in the future is rather large. We would like to mention several reports in the literature describing somatic gene therapy approach to treat diabetes, anemia and hemophilia, lysosomal storage disease, for instance deficient of beta–glucuronidase diseases of the central nervous system. The same approach may be proposed to treat cancer or multiple sclerosis.

Multicellular tumor spheroids are being widely used to study small size tumors in various aspects of tumor biology, including studies in the field of radiation biology and photodynamic therapy (Santini M.T., et al., 1999). Bioencapsulated multicellular sheroids can be generated by *in vitro* cultivation of tumor cells in microcapsules (Markvicheva et al 2003), and can be proposed as a novel *in vitro* model to study the effect of photodynamic therapy on small size tumors. 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. Cellular organization of spheroids allows to recreate in vivo small tumors much better than two–dimensional *in vitro* models. However, all these methods are time consuming and can not provide production of spheroids with narrow size ranges. Cell encapsulation method could be proposed as a novel technique to quickly and easily prepare a high number of spheroids with narrow size distribution within a determined diameter range. The method can be applied to all cell types (aggregating and non–aggregating in suspension culture).

The polymers used for bioencapsulation of proteins/peptides or animal cells have to be biocompatible, biodegradable and have to provide bioencapsulation under mild conditions. In our research we used both natural materials (alginate, chitosan) and synthetic polymer poly(d,l)lactide–co–glycolide 50–50 (PLGA).

MATERIALS AND METHODS

Chemicals. Sodium alginate (medium viscosity), calcium chloride were from Sigma, USA. Low molecuular weight chitosan (30 kDa, DD 98%) and oligochitosan (3.5 kDa, DD 89%) were kindly provided by Prof. G. Vikhoreva and by Prof. Bartkowiak, respectively. Peptide TRAP–6 (Ser–Phe–Leu–Leu–Arg–Asn–NH_2: SFLLRN) was synthesized at Shemyakin Institute. Poly(d,l)lactide–co–glycolide 50–50 (PLGA) was from Boehringer Ingelheim (Germany). Gelatin G–2500 and polyvinylalcohol, Mowiolâ, (PVA) 3–83, Mw 18000, were from Sigma and Hoechst (Belgium), respectively.

Cells. Human breast adenocarcinoma cells MCF–7 kindly provided by Centre Alexis Vautrin (Nancy, France); Endostastatin–transfected CHO cells; endostatin–transfected 293 cells (human kidney cells) were obtained by transfection with DNA vectors and kindly provided by Dr. A. Surovoy (Shemyakin Inst.).

Cultivation media. MCF–7 cells were cultivated in RPMI–1640, supplemented with 10% foetal calf serum

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(FCS), Gibco. DMEM, Ham's F–12, a–MEM, foetal calf serum, Gibco, were used for endostatin–transfected cells.

Cell encapsulation in alginate–chitosan microcapsules was carried out using two–step procedure using a coaxial air jet to get 800–1000 μm (Markvicheva et al, 2001) or an electrostatic bead generator (7.8 kV) as decribed earlier (Markvicheva E et al., 2003). The latter allowed to vary the bead size within 200–900 μm. Briefly, sodium alginate solution in 2 ml of 0.9% NaCl (1.5–2% w/v) with cells (final cell concentration 1–3x10^6 cells/ml of alginate solution) was added to a 0.5–1% (w/v) $CaCl_2$ solution dropwise using a peristaltic pump. Chitosan solution was obtained by dissolving chitosan sample in 0.9% NaCl at magnetic stirrer with adding several drops of glacial acetic acid. Oligochitosan was easily dissolved in 0.9% NaCl giving pH 3.8. The pH value of both solutions was then adjusted to pH 6.0–6.5 with 2M NaOH. After incubation in 0.2–0.4% (w/v) chitosan (oligochitosan) solution for 8–10 min at room temperature, the beads were washed twice with 0.9% NaCl, then treated with 50 mM EDTA (10 min). The obtained microcapsules were washed with cultivation medium and transferred to culture flasks with 20 ml of culture medium (the microcapsule volume was about 10% of total medium volume).

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

Standard microencapsulation procedure of TRAP–6 into PLGA microparticles. A theoretical peptide loading of 5% (w/w; peptide/polyester) was aimed. The first emulsion was prepared as follows: in a polypropylene eppendorf tube of 1.5 ml, 30 μl of a gelatin solution (20% w/v) were placed at 40°C in the presence of 90 μl peptide solution/suspension (20 mg/ml). The mixture was stirred with a rotor/stator homogeniser (ultra–turrax IKA (T8, head 5 mm) for 30 s at 13500 rpm. Then this aqueous phase was dispersed in 0.3 ml of a PLGA oil phase (13.6% in methylene chloride or ethyl acetate) first equilibrated at 40°C. The emulsion was promoted by homogenisation with the ultra–turrax (20000 rpm, 1 min). The obtained w/o primary emulsion was gelled by cooling at 15°C for 2 min without stirring, and then was transferred into 10 ml of a PVA solution (2.5%) equilibrated at 15°C in a 25 ml beaker. A four–pitched blade impeller (rod diameter 25 mm, blades 4 x 10 mm pitched at 45°) was used for stirring (500 rpm, IKA motor RW20) at constant temperature for 15 min, and then the latter raised up to 30°C to facilitate solvent elimination (15 min). Solid microspheres were collected by filtration, washed with water and dried overnight by lyophilisation. Dry microspheres were stored at –20°C under silicagel.

ANALYTICAL METHODS

Morphological analysis of PLGA microparticles with bioencapsulated TRAP–6. The morphology of PLGA microparticles was analysed by scanning electron microscopy (Jeol JSM–840 A, Technics co., Ltd, Tokyo). The dried microparticles were cross–sectioned with a razor blade using a binocular microscope. The microcapsule surface cross–section were coated with gold–palladium for 120 s in an argon atmosphere.

PLGA microparticle size distribution. Microparticles were suspended in Isotonâll + Tween 80 (0.1%), and their size distribution was analysed with Coulter Counter Multisizer (Coulter, USA, orifice size 140 μm).

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

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

Determination of endostatin was carried out by ELISA method. Monoclonal antibodies to endostatin were obtained by previously developed DNA–immunization method.

RESULTS AND DISCUSSION

TRAP–6 bioencapsulated in PLGA microparticles for wound healing

Tissue repair is one of central problems one of the most complicated challenges in medicine. Playing a central role in blood coagulation mechanism, thrombin has a numerous biological functions related to inflammation, tissue remodeling and healing. However thrombin is rather expensive and very easily inactivated protein. Some synthetic peptides which can "do thrombin's job" in terms of wound healing are cheaper, more stable, and therefore may be more promising than thrombin for wound therapy. In our research we encapsulated peptide TRAP–6 (thrombin receptor agonist peptide) in biocompatible biodegradable PLGA microparticles. Earlier the effect of TRAP–6 encapsulated in polymer films on wound healing in a mouse model has been demonstrated (Strukova et al, 2001). To study the in vivo wound healing effect of TRAP–6 encapsulated in PLGA microparticles we estimated wound sizes in mouse model (Fig. 1). As can be seen, on day 7 the wound size under microparticles with bioencapsulated TRAP–6 was double less than that one

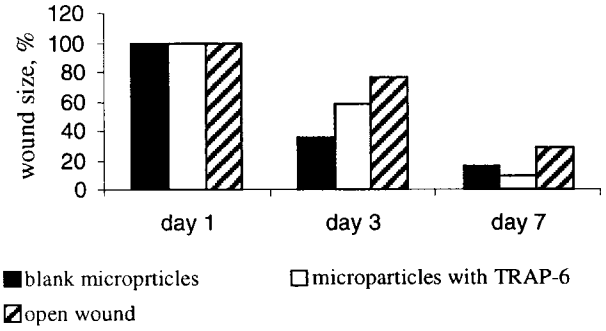

Figure 1. The effect of TRAP–6 encapsulated in PLGA microparticles on wound size. Initial wound size was taken as 100%. The data were means from 3 to 5 independent exepriments.

oligochitosan (3.5 kDa). The alginate–chitosan microcapsules were obtained in two steps: 1) by adding sodium alginate solution to $CaCl_2$ solution to get CaAlg beads which have been then coated with chitosan (oligochitosan) and 2) dissolving the alginate core by EDTA. The analyses of CaAlg beads demonstrated the size to be a function of several parameters, such as 1) concentration of alginate solution (1.5 and 2.0% w/v); 2) diameter of the needle used for the alginate solution dispersion (0.25 mm, 0.33 mm, 0.45mm, and 0.6 mm); 3) voltage of the electrostatic bead generator (5.0–8,5 kV); 4) a distance between a tip and gelling $CaCl_2$ solution; 5) the flow rate of alginate solution provided with peristaltic pump etc. One of the key parameters

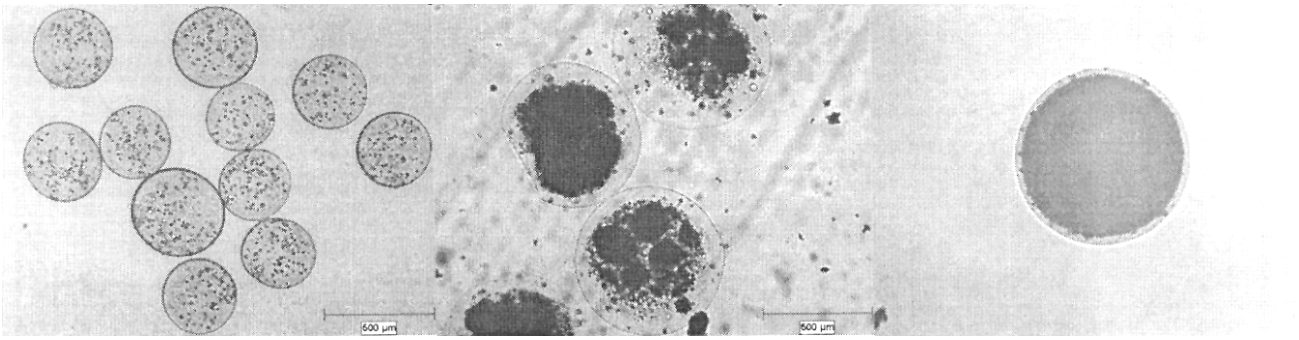

Figure 2. The growth of MCF–7 cells in alginate–chitosan microcapsules : a) after encapsulation; b) on day 23; c) on day 28. Medium RPMI, 10% FCS.

in the case of empty microparticles (8.5 and 15.6% relatively) while the open wound was 28%.

Thus, we suppose that development of new formulations based on the proposed bioencapsulated peptide can contribute to a new generation of effective dressings with controlled drug release for wound therapy.

Bioencapsulated animal cells

Alginate–chitosan microcapsules for long term cultivation of animal cells have attracted considerable attention of researches over the recent decades. Chitosan is widely employed in wound healing, the reduction of blood cholesterol levels, and immune system stimulation. The disadvantage of "chitosan–based" method for encapsulation of animal cells deals with chitosan poor solubility at physiological pH in contrast to poly–L–lysine, which can be easily used at pH 7. The promising approach using oligochitosan–based microcapsules has been recently demonstrated (Bartkowiak and Hunkeler, 1999). Oligochitosan (MM 2900) obtained by a radical degradation method from chitosan 50 kDa was proposed for preparation of a strong membrane at pH 7. We used low molecular weight chitosan (30 kDa) and affecting membrane formation process, membrane thickness and its stability was molecular weight of chitosan. The use of oligochitosan (3.5 kDa) allowed us to prepare stable microcapsules. In its turn, for every polysaccharide sample (chitosan or oligochitosan), the membrane thickness depended upon 2 parameters: 1) pH value of the chitosan solution; 2) the incubation time of CaAlg beads in the chitosan solution. Stability of the microcapsules enhanced with increasing the incubation time due to the increase of the membrane thickness of microcapsules.

To generate encapsulated multicellular spheroids, MCF–7 cells were encapsulated and cultivated for 4–5 weeks. The cell proliferation has been easily observed using a light microscope (Fig. 2). The tumor cells grew in aggregates increasing in their sizes with time which resulted in formation of large cell clusters (spheroids) completely filling the microcapsule volume (Fig. 2 c).

To demonstrate the capability of encapsulated genetically engineered cells for endostatin production, CHO and 293 cells were transfected and encapsulated. Endostatin was chosen as a promising therapeutic agent for cancer therapy. Endostatin is an inhibitor of angeogenesis (vessel formation). In particular, it inhibits migration and proliferation of endothelial cells. As shown

earlier, systemic therapy with recombinant mouse endostatin resulted in tumor regression in experimental mouse models. Endostatin is a 20 kDa fragment of collagen XIII, it was shown to release from microcapsules, accumulating in cultivation medium (Markvicheva, et al., 2002).

Thus, the developed alginate–chitosan microcapsules were successfully used for a long term cultivation of several cell lines, including tumor cells (MCF–7) and 2 genetically modified cell lines.

The proposed technique for spheroid generation within alginate–chitosan microcapsules could provide entirely new avenues for developing a novel co–culture *in vitro* model to study effects of radio– or chemo– or photodynamic therapy on small size tumors.

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