P-100	**A novel technique for the generation of multiple alginate layers**	

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INTRODUCTION AND OBJECTIVES

Immobilized cells are not only in use for technical and biotechnological aspects but can fulfill their purpose as artificial organs or drug producers in the human body. One example is the use of Langerhans' Islets for diabetes therapy (Zimmermann 2007). We use massive capsules made of ultra high viscous, biocompatible barium alginate extracted from *Lessonia nigrescens* and *L. trabeculata*. The spherical capsules are homogenously cross-linked and long-term stable (more than a year in mice, Schneider 2005) without need of stabilizing outer membranes.

Due to the capsule production process, cells and tissue fragments remain randomly distributed in the capsule. A homogenous alginate layer is preferable to provide equal diffusion ranges. We observed that tissue, which lies at the outer rim of a capsule is often attacked by the immune system (unpublished data), so a minimum hydrogel layer for immunoprotection is desirable.

This problem of centralization has, to our knowledge, never been solved for alginate capsules. One possibility is, to cover the capsule with polyelectrolyte shells but these are possibly not safe to fibrotic overgrowth.

The objective of this work is to present a technique to re-encapsulate alginate beads stably with an extra outer layer of alginate without use of Poly-L-Lysine or any other polycationic agents. The second alginate layer is generated to cover the polymerized core capsule using a layer of $BaSO_4$ crystals. We explain this effect by the hardly soluble $BaSO_4$ acting as a Ba^{2+} donor for adhesion of free, soluble alginate molecules provided in a second preparation step. First experiments, presented here shows also the biocompatibility of this technique.

With this technique we hope to do a step towards the clinical use of immunoisolated small endocrine tissue. The alginate double layer could solve the problem of immune response to miss-encapsulated tissue.

MATERIAL AND METHODS

Alginate preparation Ultra high viscous alginate was prepared from *L. nigrescens* and *L. trabeculata* stipes as described elsewhere (Zimmermann et al. 2003). Purity and sterility was proven using several physical and biological test systems (Storz et al. 2009). Alginate solution is prepared by dissolving dry alginate granulate in sterile 0.9% NaCl. Alginate concentration was 0.3 – 0.7%.

Capsule preparation A well established droplet generator, including crystal gun device for improved homogenous polymerization as described elsewhere (Zimmermann et al. 2003), was used for preparation of the core capsule. Alginate concentration of the core capsule was always 0.65%. If cells are used, the cell pellet was suspended in this alginate before transfer in a 1ml syringe and proceeded in the crystal gun device. The capsules were allowed to polymerize 15min in 20mM $BaCl_2$ solution (isotonic with NaCl, buffered with 5mM Histidine) (figure 1 A).

Alginate shell After 15min incubation time, the same amount of 6mM Na_2SO_4 solution (isotonic with NaCl) was added (figure 1 B). The $BaSO_4$ crystals fall out immediately and were allowed to settle on the capsules for about 10min. Washing with isotonic NaCl solution was performed to remove unbound crystals (figure 1 C). The now crystal covered capsules were transferred into 2ml fresh alginate solution of different concentrations (0.3 – 0.7%) and were kept there under gentle shaking for 5 or 10min. Unbound alginate was washed away using isotonic NaCl solution and capsules were transferred into polymerization solution for about 10min (figure 1 D).

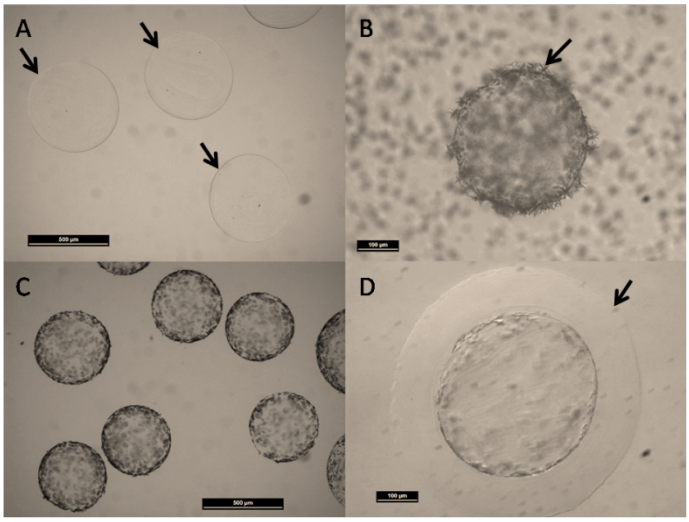

Figure 1: Preparation of alginate layer on massive alginate beads. A) freshly prepared alginate „core", B) alginate core capsule after treatment with Na_2SO_4, note the dark crystal layer, C) alginate core capsules after washing, D) double layered capsule

Cell culture L929 mouse fibroblast cells were obtained from DSMZ (Braunschweig, Germany) and cultured in DMEM supplemented with 10% FCS and Gentamycin. Cell culture media were obtained from Gibco (Invitrogen, Darmstadt, Germany). Spheroids were produces using hanging droplet technique as described elsewhere (Ehrhart 2009).

RESULTS AND DISCUSSION

To our first observation, thickness of the alginate shell depends on different effects: alginate concentration, incubation time and mechanical treatment. Higher alginate concentrations and longer incubation times lead to thicker alginate layers. Multiple alginate layers are also possible. A paper with detailed experiments to the mechanics of layer generation is currently in preparation.

L929 cell spheroids were viable over at least 5 days (figure 2). Other cell systems were tested and no harmful effects of the extra alginate layer on the viability of cells were found (paper in preparation).

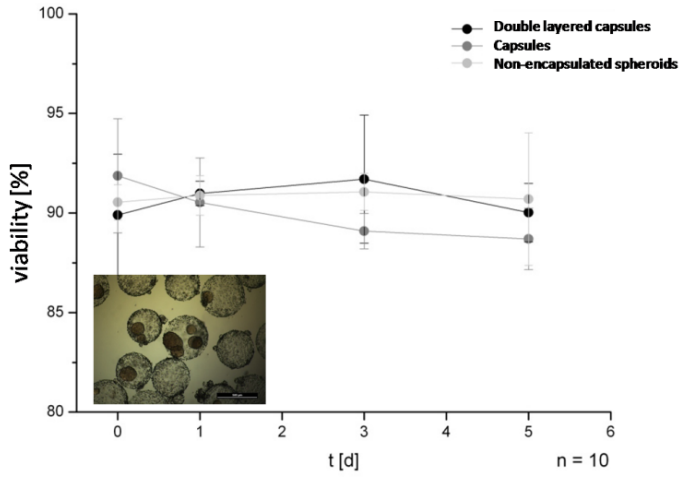

Figure 2: Viability of L929 spheroids in alginate capsules with or without double layer and non-encapsulated control. Over five days, cell viability was constantly high.

The alginate shell was furthermore effective in keeping growing cells inside the capsule. During encapsulation of cells and spheroids, some of them were immobilized directly at the outer rim. These cells crack the thin hydrogel layer quickly during cell growth and spread through the culture (figure 3).

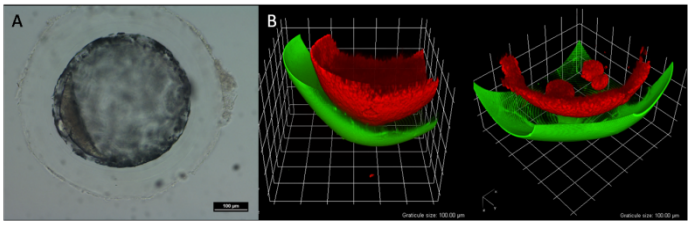

Figure 3: Growth and development of L929 fibroblasts inside the alginate double layer during 7 days. A frontier near spheroid broke out of the inner capsule and started to overgrow the capsule. A) light microscopy of the capsule. B) Cells were stained red using rhodamine, the outer alginate layer was stained with FITC-labelled Poly-L-Lysine. As the CLSM pictures show, the inner cell layer is still well beneath the outer alginate shell.

The second alginate shell prevents this effectively and forces the cells to grow between the layers. After 7 days of culture, an almost complete overgrowth of the inner capsule could be observed. Various possibilities derive from this technique, including not only safe immunoisolation but also the creation of hollow cell spheroids and other tissue engineered cell-matrix systems.

We present here a new technique for production of alginate double layers without use of polycationic agents. This technique will be backed up soon with detailed qualitative data about shell formation (paper in preparation) as well as the application in different cell models.

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