Cell Based Therapy Using Genetically Modified, Encapsulated Cells

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Introduction

Encapsulation of cells in polymers has many applications, both in biomedicine and biotechnology. The use of genetically modified cells further extends, refines and improves these uses (Hauser 2004). Thus, gene or cell-based therapies with encapsulated cells can be considered as an alternative to viral or physical gene delivery. As a basic requirement, the materials used for encapsulation must be biologically inert and biocompatible both with the host tissue and the cells that are encapsulated (Orive 2003). Although alginate-polylysine-alginate based capsules are far more often used for cell encapsulation, cellulose sulphate polymers offer certain advantages over alginate such as well characterised, chemically defined materials (Dautzenberg 1999**)**, better long-term stability of capsules and higher tolerability upon implantation into animals.

One medical application of encapsulated, genetically modified cells represents the improved treatment of solid tumours. In this scenario, encapsulated cells overexpressing a cytochrome P450 enzyme are used as a biocatalyst. The cell containing capsules are implanted near the primary tumour either by delivery through the vasculature leading to the tumour (Löhr 2003) or by direct injection. Cytochrome P450 enzymes convert non-toxic chemotherapeutic agents like ifosfamide or cyclophosphamide into their cytotoxic forms (Winiarczyk 2002). Thus, subsequent to capsule delivery, the chemotherapeutic agent is administered resulting in high local concentrations of the activated and thus tumour killing form.

This study focused on the optimisation of the production process for microcapsules containing immobilised *HEK293* cells, which are over expressing cytochrome P450 (NovaCaps®). Special emphasis was given on the encapsulation process, the proliferation of immobilised cells to high cell densities and the storage conditions for the capsules. Based on this study a GMP-compliant, industrial scale production has been established.

Material and Methods

Cell lines:

HEK293 cells (ATCC CRL-1573) stably transfected with expression vectors containing the cytochrome P450 2B1 gene or the luciferase gene (*293-Luc*), respectively, were cultured in serum-free medium in an humidified atmosphere at 37°C and 8% CO_2.

Encapsulation of cells:

Capsules have been produced with the vibrating fluid technology: Small beads are formed if a laminar sodium cellulose sulphate solution (SCS) jet breaks under controlled parameters. The negatively charged polymer beads drip into the hardening bath containing the positively charged polymer pDADMAC (poly-(diallyldimethylammonium-chloride)). For cell immobilisation, up to $3x10^6$ cells were mixed into 1 ml of a 1.8% SCS solution containing 1% NaCl.

Cultivation of encapsulated cells:

Encapsulated, stably transfected *HEK293* cells were cultivated in 300 ml of serum-free medium using a 1 l bioreactor (DASGIP, cellferm-pro®) at 37°C, 30% pO_2 and pH 7.2. The capsules were

maintained in culture until the cell density within the liquid part of the capsules reached approximately 80%.

AlamarBlue assay:
The alamarBlue™ assay (Serotech) was used to determine the viability of non-encapsulated and encapsulated cells. Briefly, quadruplicates of either $1x10^5$ suspension cells/well or 10 capsules/well were transferred into wells of a 96-well plate (Greiner) containing 100 µl cell culture medium. 10 µl of the alamarBlue reagent was added to each sample. After incubation for 4 hours at 37°C and 8% CO_2 the samples were measured with a fluorescence reader (Genios, Tecan; excitation λ=520 nm and emission λ=590 nm).

***In vivo* bio imaging:**
Female nude mice (Hsd: Athymic Nude (*Foxn1*^{nu})) were used for the *in vivo* imaging experiments. Capsules containing immobilised, luciferase expressing *293-Luc* cells have been implanted intraperitoneally (IP) or subcutaneously (SC) into the left shoulder. Each test group included five mice. Prior to imaging, 240 mg of luciferin (Xenogen) per kg of body weight was injected IP into anaesthetised mice (Isofluran). *In vivo* images were acquired with the IVIS50 (Xenogen Corp.) and analysed with the IGORpro software (Xenogen Corp.).

Results and Discussion

Production of encapsulated cells:
We have optimised the encapsulation process to produce capsules with a size deviation of less than 5%. Dependent on the encapsulation parameters the capsule diameter can now be adjusted within a range from 200 to 2000 µm. **Figure 1** exemplarily shows pictures of SCS-pDADMAC capsules and the typical Gaussian size distribution of the produced batches. For the following experiments, $2.5x10^6$ cells/ml SCS solution were immobilised leading to capsules of 700 µm in diameter containing approximately 450 cells per capsule directly after encapsulation.

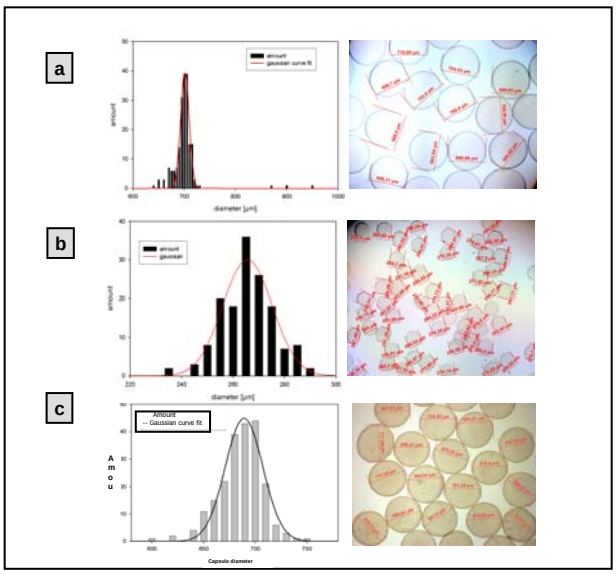

Figure 1: Empty SCS-pDADMAC capsules with a mean diameter of (a) 700 µm and (b) 200 µm; (c) 700 µm capsules containing $2x10^6$ cells/ml SCS.

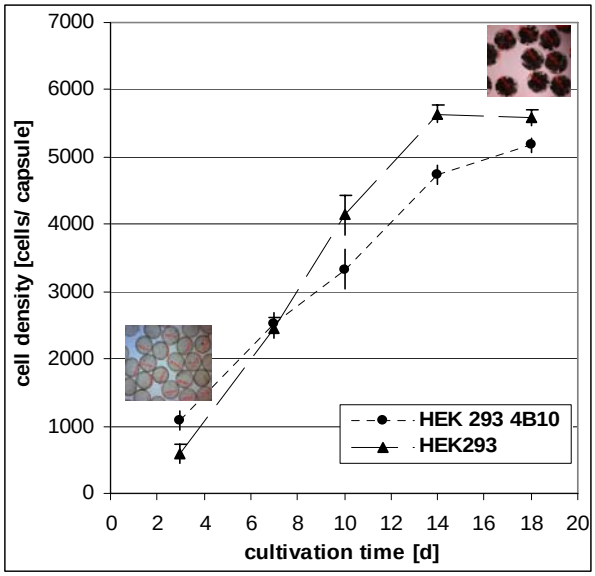

Figure 2: Growth curve of encapsulated *HEK293* cells in 700 µm capsules. Capsules were cultivated for 19 days using a DASGIP Bioreactor with a 1 l spinner flask.

Maturation of encapsulated cells:
After production, encapsulated cells were cultivated under controlled conditions within a bioreactor to increase the cell density per capsule. Monitoring cell growth of immobilised cells revealed a typical sigmoid course (**Figure 2**). A minimum doubling-time (t_D) of 110 hours was calculated for encapsulated *HEK293* cells in the logarithmic growth phase compared to t_D=30 hours of non-encapsulated cells kept in suspension culture. The stationary phase was reached 14 days after encapsulation. At this time the inner liquid part of the capsule has been mostly overgrown by the cells resulting in a cell concentration of ~$6x10^3$ cells per capsule (or ~$1x10^8$ cells/ml SCS). The determined increased doubling time of encapsulated cells may be caused by a limited oxygen supply mediated through capsule membrane.

Cryo-preservation of encapsulated cells:
In order to ensure stable batches for clinical use, long-term storage such as freezing at -80°C is a prerequisite. Since the generated capsules contain viable cells, these have to be protected within the freezing process. Evaluation of different cryo-protection protocols finally facilitated freezing of cell containing capsules at reproducible conditions and with a high recovery rate. Furthermore, the process was designed to be automated and up-scaleable. SCS-capsules showed no physical damaging of the capsule membrane upon thawing. The relative viability of thawed encapsulated cells decreased less than 20% when compared to non-frozen samples from the same batch (**Figure 3 3).**

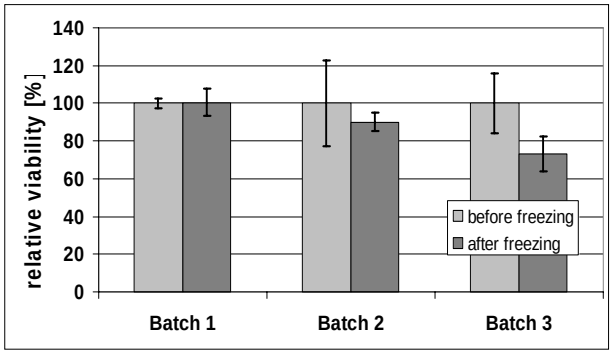

Figure 3: Relative cell viability (alamarBlue assay) of encapsulated cells before and after freezing. Viability prior to freezing was set to 100%.

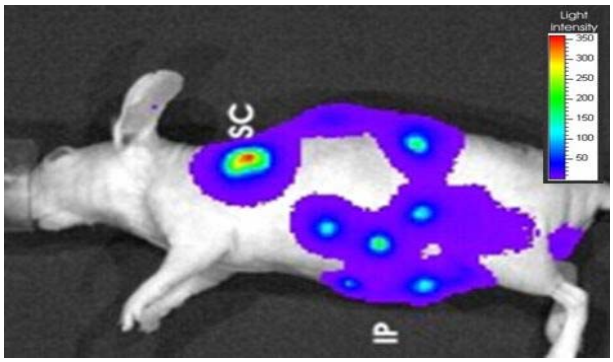

Figure 4: Detection of IP and SC implanted capsules containing *293-Luc* cells. Red colours indicate high concentrations of encapsulated cells.

Long term survival of encapsulated cells *in vivo*:
In order to investigate the viability of encapsulated cells *in vivo,* SCS-capsules containing luciferase expressing *293-Luc* cells were implanted into nude mice. *In vivo* bio imaging of animals after application of luciferin revealed (i) that capsules implanted subcutaneously into the left shoulder stayed localised, whereas intraperitoneally implanted capsules spread abdominally (**Figure 4**), and (ii) that the cells within the implanted capsules stayed enzymatically active and thus alive for at least 102 days (**Figure 5**). These results indicate that the capsule membrane remains structural intact promoting long term survival of immobilised cells *in vivo*.

Encapsulated cell therapies:
In a clinical phase I/II trial, involving the implantation of encapsulated cytochrome P450 2B1 expressing cells in 14 patients with advanced, non-resectable pancreatic cancer, tumour reductions or stable diseases were obtained showing a clear clinical benefit. The median survival was doubled

and the one year survival rate was three times better compared to a historic control, which included the best standard treatment (Löhr 2001). Based on these data, a pivotal phase III clinical trial has been planned with the European Medicines Evaluation Agency (EMEA) and is now in preparation. According to the improved encapsulation process described above the industrial scale production of encapsulated cells was established to fulfil the criteria of a GMP compliant large-scale production (**Figure 6**). Therefore, we generated a clonal, genetically stable and optimised cell line expressing cytochrome P450 2B1. A fully characterised and tested master cell bank of the produced cell clone was laid down under cGMP conditions. In addition, we identified qualified sources of the required raw materials enabling a reproducible, large-scale cell encapsulation and production process.

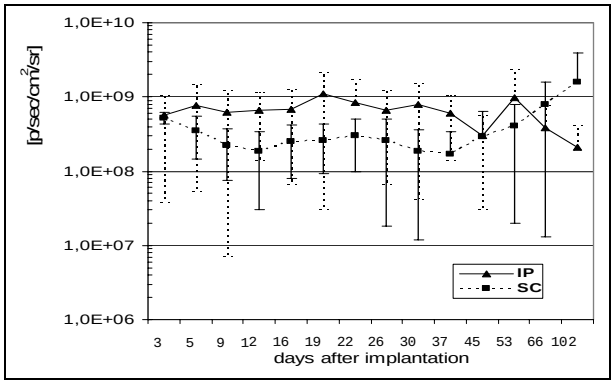

Figure 5: Monitoring enzymatic activity of intraperitoneally (IP) and subcutaneously (SC) implanted capsules containing immobilised *293-Luc* cells.

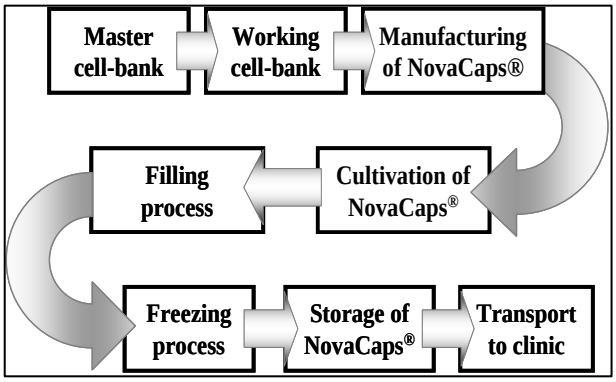

Figure 6: Flow chart describing the main working steps of the NovaCaps® GMP manufacturing process.

CONCLUSIONS

Micro-hollow sphere capsules containing allogeneic cells as bio-catalysers are a promising universally applicable tool for *in vivo* therapies. The semi-permeable membrane sufficiently protects the immobilised cells. The mechanical strength of these capsules supports cultivation, freezing, thawing and implantation. Encapsulated cells expressing cytochrome P450 (NovaCaps®) are currently being produced under GMP conditions. A pivotal phase III clinical trial has been planned with the EMEA and will commence by the end of this year.

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