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Release characteristics of cellulose sulphate capsules and production of cytokines from encapsulated cells

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ABSTRACT

The size and speed of release of proteins of different sizes from standard cellulose sulphate capsules (Cell-in-a-Box®) was investigated. Proteins with molecular weights of up to around 70kD can be released. The conformation, charge and concentration of the protein being released play a role in the release kinetics. Small proteins such as cytokines can be easily released. The ability to produce cytokines at a sustained and predefined level from encapsulated cells genetically engineered to overexpress such cytokines and implanted into patients may aid immunotherapies of cancer as well as infectious and other diseases. It will also allow allogeneic rather than autologous cells to be used. We show that cells encapsulated in polymers of cellulose sulphate are able to release cytokines such as interleukin-2 (IL-2) in a stimulated fashion e.g. using phorbol 12-myristate 13-acetate (PMA) plus ionomycin. Given the excellent documented safety record of cellulose sulphate in patients, these data suggest that clinical usage of the technology may be warranted for cancer treatment and other diseases.

1. Introduction

The implantation of encapsulated cells overexpressing specific biomolecules offers a means to treat a variety of diseases (for reviews see (Acarregui et al., 2013; Gurruchaga et al., 2015; Gonzalez-Pujana et al., 2017), including cancer (Salmons and Gunzburg, 2010; Salmons et al., 2010). Encapsulated cells have been evaluated in clinical trials for the treatment of diabetes (Orlando et al., 2014; Salmons et al., 2014) as well as for the treatment of pancreatic (Löhr et al., 2014) and mammary cancer (Michalowska et al., 2014).

A number of different materials can be used for encapsulation of cells, such as alginate, agarose, polysulphone (for a review of materials see (Gasperini et al., 2014). We have focussed on using cellulose sulphate as an encapsulation material (Dautzenberg et al., 1999) since it has a number of advantages over other encapsulation materials (Dangerfield et al., 2013; Salmons et al., 2014).

Cellulose sulphate encapsulation of mammalian cells has been shown to provide a means to protect cells from rejection by the immune system, to localize the cells at the site of implantation, to provide a long term microenvironment for survival of the cells, and to allow release of biomolecules from the capsules, as well as entry of food and nutrients for the cells (Dangerfield et al., 2013). Moreover, cellulose sulphate encapsulated human cells have been shown to be safe and efficacious in 27 human patients in clinical trials (Löhr et al., 2014).

To date however, there has been no systematic characterization of the size of the molecules released from standard cellulose sulphate capsules (Cell-in-a-Box®). This would be important since the size of the molecules that can be released will dictate which molecules can be successfully used therapeutically without changing the chemistry of the capsules.

The ability to produce, for instance, cytokines at physiological concentrations in patients could be of benefit in at least two major areas: (i) boosting or improving immune responses as a result of vaccination (important for combatting infectious agents as well as of potential use in combination with therapeutic vaccines for the treatment of tumours) and (ii) to directly combat tumours. Immuno-stimulatory cytokines have been administered to boost the immunogenic potential of other agents such as immune checkpoint-blocking antibodies, anticancer vaccines, oncolytic viruses and immunogenic chemotherapeutics (Vacchelli et al., 2014).

A number of cytokines have been used for cancer treatment including interferon alpha (IFN-α), interferon beta (IFN-β), gamma-interferon (γ-IFN) and interleukin-1 (IL-1) as well as IL-2, and IL-12. These cytokines demonstrate their efficacy by inducing apoptosis and other anticancer functions in the tumour microenvironment and some of these mechanisms of action have been recently reviewed (Kumar and Chandra, 2014).

The first reproducibly effective human cancer immunotherapies

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have been obtained using the pro-inflammatory cytokine IL-2 as a means to expand tumour infiltrating, anti-tumour T cells. These anti-tumour T cells lead to durable, complete, and apparently curative regressions in patients with metastatic melanoma and renal cancer (Rosenberg, 2014).

IL-2 is currently manufactured using recombinant DNA technology and is marketed as a protein therapeutic called aldesleukin. It has been approved by the Food and Drug Administration (FDA) and in several European countries for the treatment of cancers (such as malignant melanoma and renal cell cancer) in large intermittent doses and has been extensively used in continuous doses (Bhatia et al., 2009). Different dosages of IL-2 are recommended for the treatment of patients, depending on the country, and the efficiency and side effects of different dosages is often a point of disagreement. In the U.S., patients are typically given high dosages for five consecutive days, three times a day, for fifteen minutes, with a 10 day recovery period between treatment dosages. IL-2 is delivered intravenously (i.v.) for this type of dosing and hospitalization and even intensive care is required throughout due to the side effects. Alternatively, a lower regimen can be given as an injection of IL-2 under the skin at home or in the doctor's office. It is also possible to receive this dosage by i.v. delivery in the hospital over 1–3 days, similar to and often including the delivery of chemotherapy (MedlinePlus, 2013). IL-2 has a narrow therapeutic window, and the level of dosing usually determines the severity of toxicity and the side effects, leading to the search for other means of delivery (Shaker and Younes, 2009).

Tumour necrosis factor-α (TNF-α) is also a potential anticancer cytokine however, systemic use in humans has been limited due to significant toxicities as well as a lack of efficacy. More recently, the use of TNF-α as a facilitator, rather than as a direct actor, is being explored and this has been reviewed by Roberts and colleagues (Roberts et al., 2011).

Immuno-protective encapsulation of cells is an ideal way to achieve delivery of cytokines at physiological concentrations in patients. Moreover, it obviates the need for individualized *ex vivo* processing of autologous cells, thus allowing well characterised, defined and pre-tested cytokine secreting human allogeneic cells to be implanted (Schwenter et al., 2011). The feasibility of this approach has been demonstrated by encapsulating granulocyte-macrophage colony-stimulating factor (GM-CSF) producing K562 cells in alginate (Schwenter et al., 2011). Side effects of using purified cytokines and targeting to the site of action also favour the use of encapsulated cells for the production of cytokines.

Interleukin-6 (IL-6) is a cytokine with pleiotropic effects that plays a central role in normal and abnormal hepatic function and response. Moran and colleagues (Moran et al., 2006) reported the encapsulation of genetically modified CHO cells in alginate followed by intraperitoneal (i.p.) implantation in a rat model of hepatocellular carcinoma (HCC). They observed a significant increase in the circulating and intra-hepatic levels of human IL-6 up to 4 days post-implantation but this was not accompanied by a significantly altered rate of overall tumour progression. Stat3 activity was however significantly increased in both normal liver and HCC tissue resected from animals implanted with the IL-6 producing CHO cells. These data demonstrate the short-term advantage of using cell encapsulation technology to generate high levels of active circulating and intra-hepatic cytokines as well as raising the possibility of modifying specific signal transduction cascades that have been identified as important during tumour progression (Moran et al., 2006).

Chang and colleagues (Cirone et al., 2004) investigated a combined immunotherapy and angiostatic therapy in the B16-F0/neu melanoma mouse model using intraperitoneally implanted, alginate poly-L-lysine-alginate (APA) microencapsulated mouse myoblasts (C2C12), genetically modified to deliver angiostatin and an interleukin 2 fusion protein (sFvIL-2). The data that they obtained was promising in that the combination treatment improved survival of the mice. In addition, the treatment delayed tumour growth, and this was associated with increased histological indices indicative of apoptosis and necrosis. Moreover, the combination of immuno- and anti-angiogenic therapies delivered by immune-isolated cells was superior to individual treatments and gave rise to unexpected protection against the adverse side effects of the single treatments alone (Cirone et al., 2004).

Recently, Werner and colleagues (Werner et al., 2015) encapsulated a human leukemia T lymphocyte Jurkat cell line in cellulose sulphate capsules. The encapsulated cells divided more actively and showed less propensity to undergo programmed cell death (apoptosis).

It has been reported that stable transfection of a subclone of HEK293 cells with various cytokines and chemokine expression vectors, including GM-CSF, IFNγ and hIL-15 and alginate encapsulation resulted in the stable production of relevant cytokines and chemokines from the encapsulated cells for at least 2 weeks *in vitro* (Hamilton et al., 2006; Huang, 2005). Moreover, in anti-tumour therapy experiments in C57BL/6 mice with either B16 melanoma, EL4 or EG7 tumours, GM-CSF produced from alginate encapsulated cells was particularly potent in stimulating anti-tumour, cellular mediated, immune responses (Huang, 2005).

Currently the evaluation of encapsulated cells to boost anti-tumour immune responses is being tested in an ongoing clinical trial. In this immunotherapy study, patients with advanced stage solid tumours (ovary, pancreas, head and neck, colon, prostate) are being given encapsulated cells producing GM-CSF subcutaneously as a strong immune booster for the co-administered vaccine consisting of $4x10^6$ of the patients' own irradiated tumour cells. In this study, two "theracyte" macrocapsules made of polysulphone ester and each producing 20 ng GM-CSF/24hr are implanted in each patient. Data from 15 treated patients has recently been reported. As well as showing safety (no Serious Unexpected Suspected Adverse Reactions (SUSAR) nor Systemic suspected Adverse Drug Reactions (SADR) reported), 2 of the 15 patients treated to date showed partial responses and another 6 patients showed stable disease (Mach et al., 2016). The anecdotal results from the first successful immunotherapy treatment of patients with a chordoma were also recently published (Migliorini et al., 2017).

Here we show for the first time that cells encapsulated in cellulose sulphate are able to produce interleukin-2.

2. Methods

2.1. Fluorescent dextran study

Fluorescein isothiocyanate (FITC) coupled dextran of different molecular weights (4, 10, 20, 40 and 70 kDa) was encapsulated in cellulose sulphate essentially as outlined previously (Hauser et al., 2006; Ortner et al., 2012). The resulting capsules were placed in the wells of cell culture plates (5, 10 or 30 capsules per well) in 200 µl phosphate buffered saline (PBS) and analysed by fluorescence microscopy and photography at various time points.

In a separate experiment, 10 or 30 capsules per well were incubated in 200 µl of PBS and 24 h or 48 h after incubation (37 °C), 100 µl of supernatant of each well was transferred into another well and the fluorescence measured by spectrophotometry. The fluorescence is proportional to the amount of 10, 20 and 40 kDa Dextran-FITC that has left the capsules.

2.2. Protein release from capsules

A mix of proteins from SDS6H2 SIGMA, Molecular Weight Marker (M.W. 30,000–200,000) was encapsulated and 3000 capsules incubated in 2 ml PBS for periods between 2 h and 7 days at 37 °C. At fixed time points, the supernatant was removed from the capsules, lyophilized and taken up in non-denaturing loading buffer before being loaded onto a polyacrylamide gel and separated by electrophoresis. Proteins were visualized by silver staining. Separately, β-Galactosidase (116kD) and

chicken egg albumin (45kD) were made up to a calculated final concentration of 24 fmol each per capsule. The resulting mixture was encapsulated and 3400 capsules incubated in 2 ml PBS at 37 °C for either 6 h or overnight. At these time points, the supernatant was removed from the capsules, lyophilized and taken up in non-denaturing loading buffer before being loaded onto a polyacrylamide gel and separated electrophoretically. Proteins were visualized by silver staining.

2.3. Cells and cell culture

Hut-78 human T cell lymphoma cells were originally derived from the peripheral blood of a 53 year old human male with Sezary syndrome (Bunn and Foss, 1996). The cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) +20% foetal calf serum. They were encapsulated essentially as outlined previously (Hauser et al., 2006; Ortner et al., 2012).

2.4. Viability of encapsulated cells

The viability of the encapsulated cells was determined by a modified AlamarBlue® assay essentially as described by Hauser and colleagues (Hauser et al., 2006). Briefly the number of capsules to be assayed were washed in cell culture medium and 10 capsules placed per well of a 96 well plate in 100 µl of cell culture medium. For each sample quadruplicates were pipetted. 10 µl of Alamar Blue reagent (Serotech Cat. No. BUF012B) was added to each well and the assay incubated for 4 h at 37 °C. The Relative Fluorescent Units (RFU) were measured at an excitation wavelength of 520 nm and an emission wavelength of 590 nm with a gain of 60, and an integration time of 40 µs. 10 flashes were recorded for each sample. The RFU recorded with a blank (no capsules) was always subtracted from the RFU obtained with the capsule containing samples.

2.5. Elisa

3, 6,9, 11, 14 and 18 days after encapsulation and subsequently two times per week 500 capsules were transferred from roller bottles into 50 ml falcon tubes containing 5 ml of IMDM + 20% FCS (PAA, inactivated) + PMA (10 µl of a 1:100 dilution of 1 mg/ml) + Ionomycin (50 µl of a 1:100 dilution of 1 mg/ml) The capsules were incubated for 17–18 h at 37 °C, 5% CO_2 and > 90% rH. After 17–18 h supernatant from the capsules was frozen at −80 °C for ELISA. As negative control 500 capsules were transferred from roller bottles into 50 ml falcon tubes containing 5 ml of IMDM + 20% FCS (without stimulation). As control for IL-2 production, 1×10^6 HUT-78 cells were seeded in 25 cm^2 flasks containing 5 ml of IMDM + 20% FCS + PMA + Ionomycin. The cells were incubated for 17–18 h at 37 °C, 5% CO_2 and > 90% rH. After 17–18 h supernatant from the cells was frozen at −80 °C for ELISA. As negative control, cells were cultivated in IMDM + 20% FCS without stimulation agents.

3. Results

IL-2 has a molecular weight of 14–15.5 kDa (Waldmann et al., 1998). We have previously published that molecules larger than this can readily escape from encapsulated cells (Dangerfield et al., 2013). However, to better characterise this as well as to examine the kinetics of release of molecules of various sizes, fluorescent dextran of defined, discrete sizes was encapsulated and release of the dextran was visualised as well as quantified. Fig. 1A shows visually the release of FITC labelled dextran from the capsules. Very small dextran of 4 kDa is immediately released from the capsules and no fluorescence is visible. Larger dextran of 10 kDa is mainly released by 20 h although there is some fluorescence visible after 40 h and even still at 120 h, There is little difference in the fluorescence from 70 kDa dextran even after 120 h, suggesting that the most of the dextran of this size is retained in

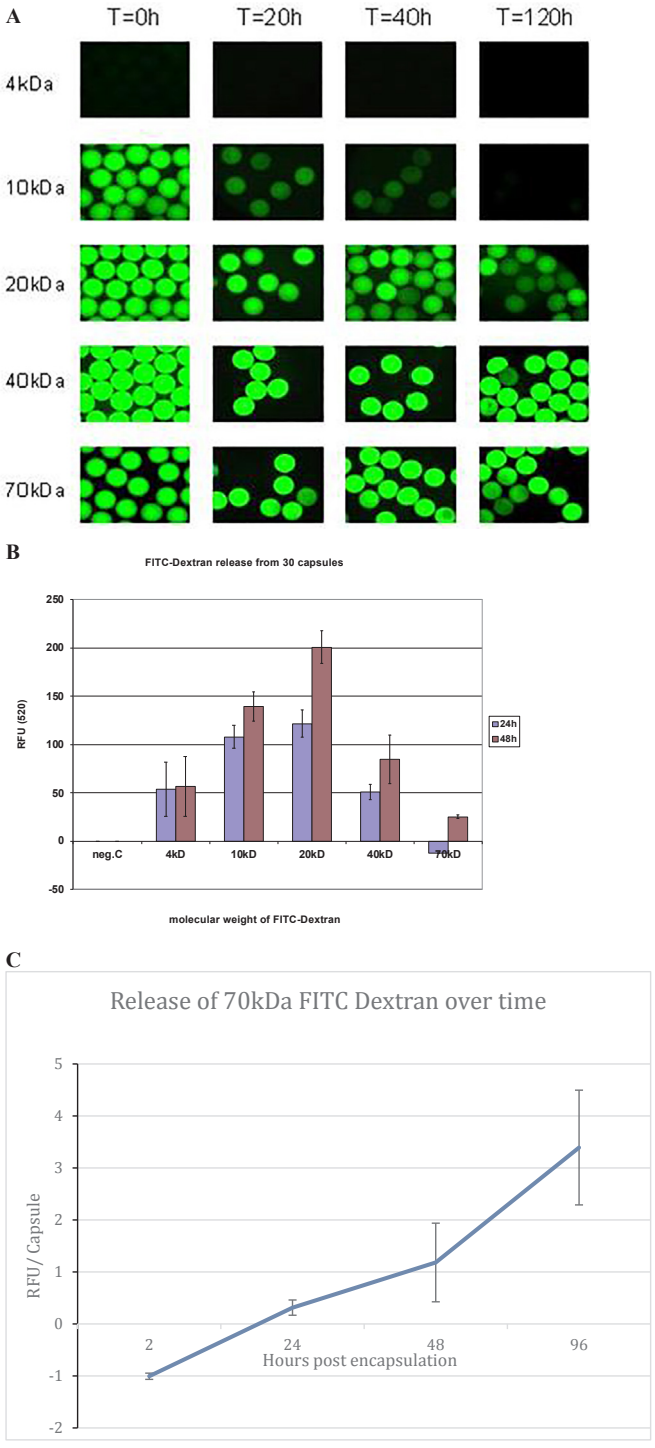


Fig. 1. Escape of FITC-labelled dextran of different molecular weights from cellulose sulphate capsules. Dextran-FITC of different molecular weight was encapsulated and the fluorescence of the dextran-FITC containing capsules visualized photographically at different times post encapsulation (Fig. 1A). 30 capsules per well were incubated in 200 µl of PBS. After 24 or 48 h incubation at 37 °C, 100 µl of supernatant of each well was transferred into another well and the fluorescence was measured by spectrophotometer. The fluorescence after subtraction of the background from the negative control was plotted and is proportional to the amount of 4, 10, 20, 40 and 70 kDa Dextran-FITC that had escaped the capsules. Data shown is from 4 replicates with the S.D. indicated (Fig. 1B). The rate of release of the 70 kDa Dextran-FITC was followed over a 4 day period. Measurements were made after 2 h, 1 day, 2 days and 4 days in quadruplicate. The number of capsules in each sample was also counted. The results are plotted as the fluorescence (RFU) per capsule and the mean and S.D. were plotted (Fig. 1C).

the capsules. We did not quantitate the release of the FITC labelled dextran from the capsules based upon the reduction in fluorescence intensity of the capsules themselves, because, although the imaging parameters and the post processing parameters were kept the same, the level of fluorescence was so high in most of the capsules that it masked small changes. Therefore, in order to quantitate these results, we measured the increase in fluorescence of PBS incubated with 30 capsules loaded with each size of fluorescent dextran after removal of the capsules after 24 h or 48 h (Fig. 1B). Similar low levels of fluorescence (around 50 RFU) were measured after 24 and 48 h from capsules that contained 4 kDa dextran, confirming that release of the beads had most probably already occurred during the encapsulation process. Proportional fluorescence levels between 10 and 30 capsules were measured for both the 10 kDa and 20 kDa dextran containing capsules at 24 and 48 h, suggesting similar kinetics of release. However, proportional fluorescence levels between 10 and 30 capsules were only measured for the 40 kDa dextran after 48 h, confirming a longer time to release. There is also release, albeit slower, of the 70 kDa dextran. Since the release of the 70kD dextran was fairly slow we evaluated the rate of release over a longer period (4 days) and calculated the release per capsule (Fig. 1C). The data shows a steady increase in the measured fluorescence indicating a constant, albeit slow, rate of release over time.

Dextran is a complex branched glucan which forms chains. The length of the chains determines its molecular weight. The lower the molecular weight, the less branching, with molecular weights of 20 kDa almost being more coil-like and having a narrower range of "real" molecular weight distribution than higher molecular weight dextrans. On the other hand, the molecules attain greater symmetry as their molecular weight increases. The diffusion properties of other molecules such as IL-2 which are more globular in structure might be different. For this reason a second experiment was conducted where a mixture of proteins of different structures and of different molecular weights were used. A protein mixture (SIGMA. Cat. SDS6H2) consisting of myosin (200kD), β-Galactosidase (116kD), phosphorylase b (97.4kD), bovine albumin (66kD), chicken egg albumin (45kD) and carbonic anhydrase (29kD) was encapsulated and then 3000 capsules were incubated in PBS for varying lengths of time at 37 °C. After these fixed times, the supernatants from the capsules were applied to a polyacrylamide gel, separated by electrophoresis, and the released proteins visualized by silver staining.

Fig. 2 shows the released proteins in the supernatant. As expected, the smallest protein, carbonic anhydrase (29kD) can already be detected by 6 h (Fig. 2A, lane 5). However interestingly, the larger bovine albumin (66kD) can already be detected after 4 hours (Fig. 2A, lane 4). By 24 h (Fig. 2A lane 8) bovine albumin (66kD), chicken egg albumin (45kD) and carbonic anhydrase (29kD) are all clearly detectable, however Myosin (200kD), β-Galactosidase (116kD) are still not detectable even after 7 days incubation (Fig. 2B, lane 6). This suggests that the molecular weight cut-off for the capsules lies somewhere between 66kD and 97kD in line with the results previously obtained with dextran.

The earlier detection of the release of the larger bovine albumin (66kD, Fig. 2A, lane 4) compared to the carbonic anhydrase (29kD, Fig. 2A, lane 5) could be a result of the relatively more bovine albumin in the protein mixture compared to carbonic anhydrase (compare in Fig. 2A, lane 1), although visually a similar quantity of chicken egg albumin (45kD) was encapsulated and this was not detected as early as the larger bovine albumin. Thus individual mixtures of proteins were prepared at equal concentrations to give 24 fmol each protein/ capsule.

An equimolar mix of β-Galactosidase (116kD) and chicken egg albumin (45kD), was encapsulated and 3400 capsules incubated for either 6 h or for 24 h in PBS at 37 °C. After this time the supernatant from the capsules was removed, separated electrophoretically on a polyacrylamide gel and the proteins visualized by silver staining. Fig. 2C shows that after overnight incubation of the β-Galactosidase (116kD) and chicken egg albumin (45kD) only the chicken egg albumin protein could be detected in the supernatant (Fig. 2C, lane 4). No proteins could be detected after 6 h incubation (lane 3) although both proteins were present in the original mix (lane 1) and the system is capable of detecting protein concentrations as low as 13fmol (lane 2). These results suggest that a fairly small protein like IL-2 (14–15.5 kD) with a more globular structure, should easily be released from the capsules.

Hut-78 cells are a T cell lymphoma cell line that have the properties of mature human T cells with helper/inducer activity. These cells secrete interleukin-2 (IL-2), as well as express receptors for IL-2. The growth rate of Hut-78 cells is stimulated by IL-2. TNF-alpha is an autocrine growth factor for Hut-78 cells.

To determine whether this commonly used T cell line could be used as a continuous source of IL-2, the Hut-78 cells were encapsulated and subsequently examined for their ability to grow within the capsule as well as for their ability to produce and secrete IL-2 from the capsules.

After encapsulation of Hut-78 cells in cellulose sulphate, the encapsulated cells were cultivated in cell culture medium in roller bottles. The number of cells within the capsule increased over time and the cells filled out the available space within the capsule by day 17 (Fig. 3). Importantly, the cells did not breach the outer confines of the capsules and thus they were not released from the capsules. This data is similar to data obtained after encapsulating HEK293 cells (Hauser et al., 2006; Salmons et al., 2007).

In a second experiment, two further independent encapsulations of Hut-78 cells were performed (V133 and V134). The V133 and V134 Hut-78 encapsulated cells showed slightly different growth kinetics within the cellulose sulphate beads, with the second (V134) growing slightly faster (Fig. 4). The viability of the encapsulated Hut-78 cells over time from these two encapsulations was assessed using a modified alamarBlue® Assay (Fig. 4). The data confirms the accelerated growth of the V134 encapsulated cells, but also confirms the steady increase in metabolic activity, mirroring the increase in cell number, over time after encapsulation.

At various days after encapsulation, 500 capsules containing Hut-78 cells were transferred into falcon tubes containing 5 ml of IMDM and 20% FBS and either stimulated or not with PMA plus ionomycin for 18 h. PMA activates the signal transduction enzyme protein kinase C (PKC) and ionomycin is an ionophore used in conjunction with PMA to stimulate the intracellular production of IL-2 as well as other cytokines such as interferon and IL-4. An ELISA of the medium was then performed to estimate the IL-2 release from the capsules (Fig. 5).

Stimulation with PMA and ionomycin (PMA + I) of Hut-78 cells encapsulated in two independent experiments (V133 and V134) induces detectable IL-2 production as early as 3 days after the cells are encapsulated, with a modest increase in IL-2 levels being observed beyond this i.e. at 6 and 9 days respectively. In the absence of PMA + I (non-stim.), no IL-2 induction could be measured in the medium surrounding the capsules (Fig. 5).

To better relate the amount of IL-2 produced to cell or capsule number, the amount of IL-2 produced from encapsulated Hut-78 cells on day 9 after encapsulation was calculated per encapsulated cell, using the numbers of cells per capsule that had been calculated using the alamarBlue® assay in Fig. 4B. At day 9 after encapsulation there are between 430 (V133) and 900 (V134) cells per capsule. Thus 500 capsules contain between 2.15×10^5 (V133) and 4.5×10^5 (V134) cells. Table 1 shows the amount of IL-2 produced from these cells numbers compared to the amount produced from 1×10^6 non-encapsulated Hut-78 cells.

The experiments were repeated a further two times using a standard 100 capsules each containing on average 6.6×10^4 encapsulated Hut-78 cells. The encapsulated Hut-78 cells could be shown to produce between 620 and 3250 pg IL-2/million encapsulated cells (data not shown). This is consistent with the previously obtained results Table 1. In one case we were able to detect a background IL-2 activity in the absence of stimulation (data not shown).

We also looked at IL-2 production from encapsulated HUT-78 cells

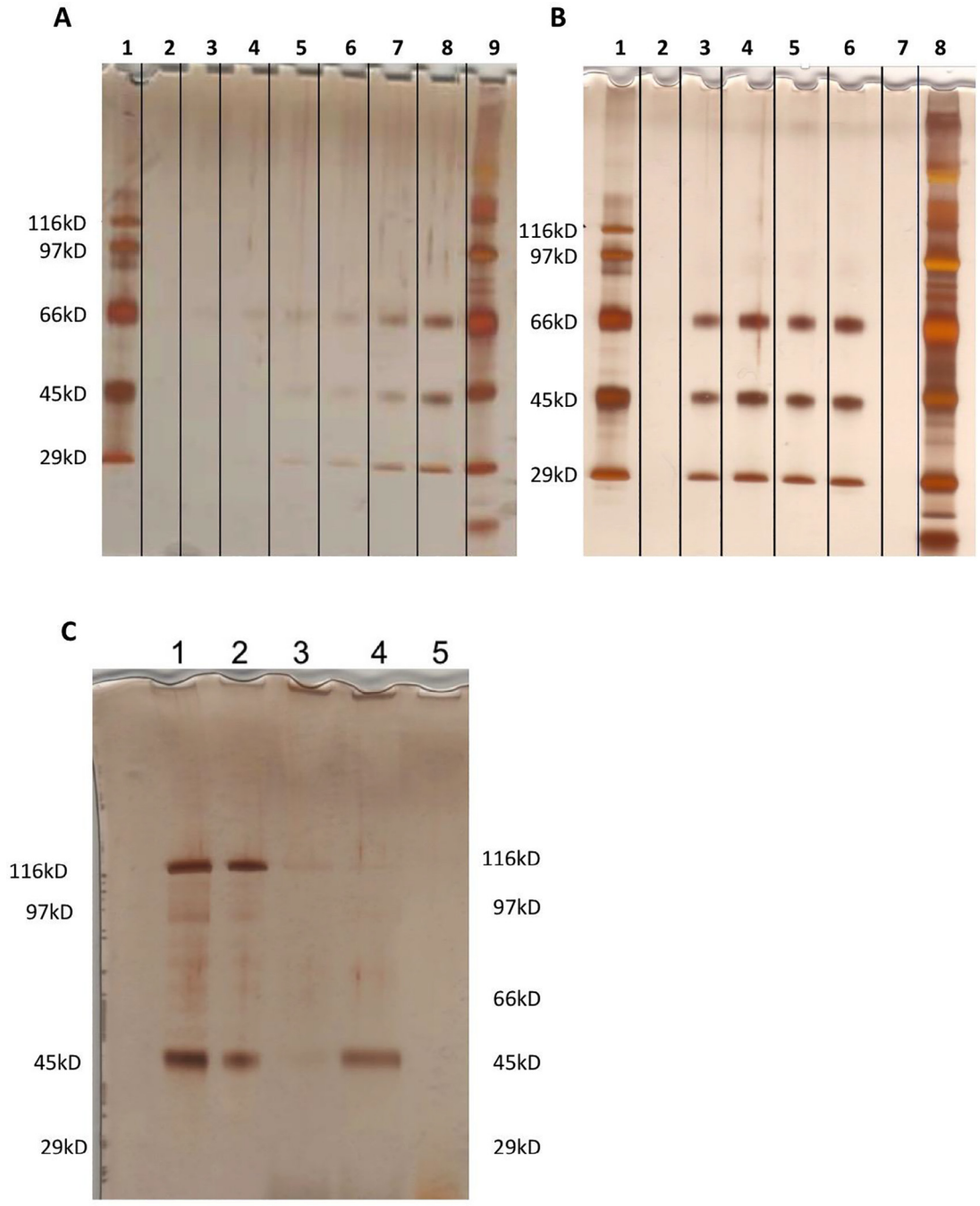


Fig. 2. Release of specific encapsulated proteins over time. A and B: A mix of proteins from SDS6H2 SIGMA, Molecular Weight Marker (M.W. 30,000–200,000) consisting of myosin (200kD), β-Galactosidase (116kD), phosphorylase b (97.4kD), bovine albumin (66kD), chicken egg albumin (45kD) and carbonic anhydrase (29kD), was encapsulated and 3000 capsules incubated in 2 ml PBS for periods between 2 h and 7 days at 37 °C. The supernatant from the encapsulated cells was removed after 2 (A lane 3), 4 (A lane 4), 6 (A lane 5), 8 (A lane 6), 16 (A lane 7), and 24 h (A lane 8, B lane 3), or after 3, (B lane 4), 4 (B lane 5) or 7 days (B lane 6), lyophilized and resuspended in non-denaturing buffer before being separated by electrophoresis and silver stained. The supernatant from empty capsules was loaded in A lane 2 and B lane 2, whilst the complete mixture of encapsulated proteins was loaded in A lane 1 and B lane 1. A rainbow marker was loaded in A lane 9 and B lane 7. C: An equimolar mix of β-Galactosidase (116kD) and chicken egg albumin (45kD), was prepared at equal concentrations and encapsulated to give a final concentration of 24 fmol each protein/ capsule. 3400 capsules were incubated for either 6 h or for 24 h in 2 ml of PBS at 37 °C. After this time the supernatant from the capsules was removed, separated electrophoretically on a polyacrylamide gel and the proteins visualized by silver staining. 26 fmol (C lane 1) or 13 fmol (C lane 2) of each protein was loaded and compared to the 6 h (C lane 3) and overnight (C lane 4) supernatant from the capsules.

over time. The peak level of IL-2 produced from 500 capsules of encapsulated HUT-78 cells was between 400 and 480 pg/ml and this level was maintained until at least day 9 post encapsulation (Fig. 5). Surprisingly, already at day 11 post encapsulation the level of IL-2 production from encapsulated cells was slightly lower and by day 14 and beyond it was even lower at less than 80 pg/ml, even though the total number of viable cells was still increasing (Fig. 4).

4. Discussion

This study provides insight about the influence of the size of molecules on the kinetics of molecule release from cellulose sulphate

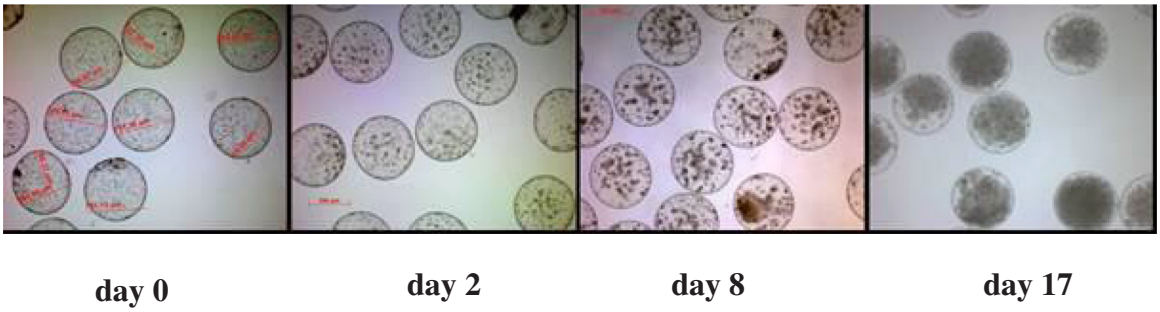
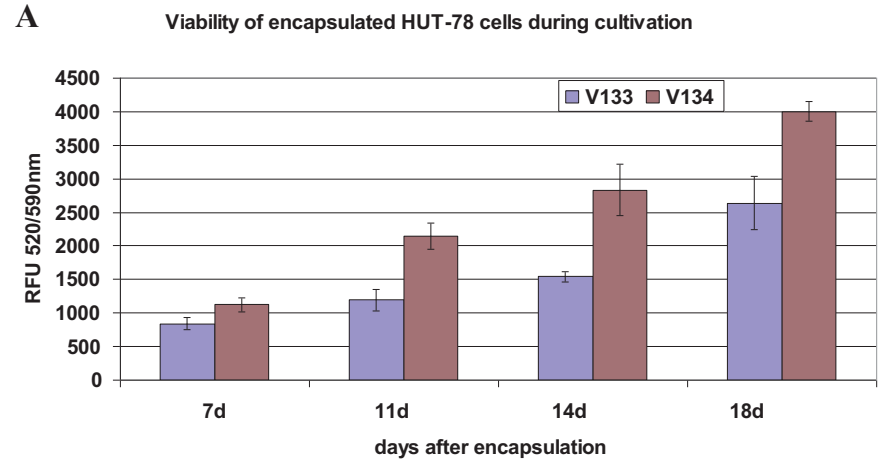


Fig. 3. Growth of Hut-78 cells within cellulose sulphate capsules over time. Between 1.5×10^6 and 2.5×10^6 viable Hut-78 cells were encapsulated per ml of cellulose sulphate and the capsules then incubated in cell culture medium at 37 °C for up to 17 days. Photomicrographs taken on day 0 (day of encapsulation), day 2, day 8 and day 17 demonstrate continual growth of the cells within the capsules and good filling of the capsules by day 17. The capsules are in all cases regular with a measured diameter of 0.7 mm.

encapsulated cells. We know that the membrane of the capsule is not like a lipid bilayer and does not have lipid bilayer like pores. Rather the membrane is a gelated area and the "pores" are a maze like structure through which molecules have to pass to exit the capsules. Thus size, structure, flexibility and charge play a role in determining the rate of release. Very small molecules are small enough to pass out rapidly based entirely on their size characteristics, However the larger the molecule the more important are the other characteristics. Thus the larger molecules will generally exit much more slowly, however, with the right flexibility or structure, they could exit faster.

The FITC-labelled dextran used here has a labelling of 0.003–0.020 mol FITC per mol glucose (SIGMA-Aldrich). Thus even



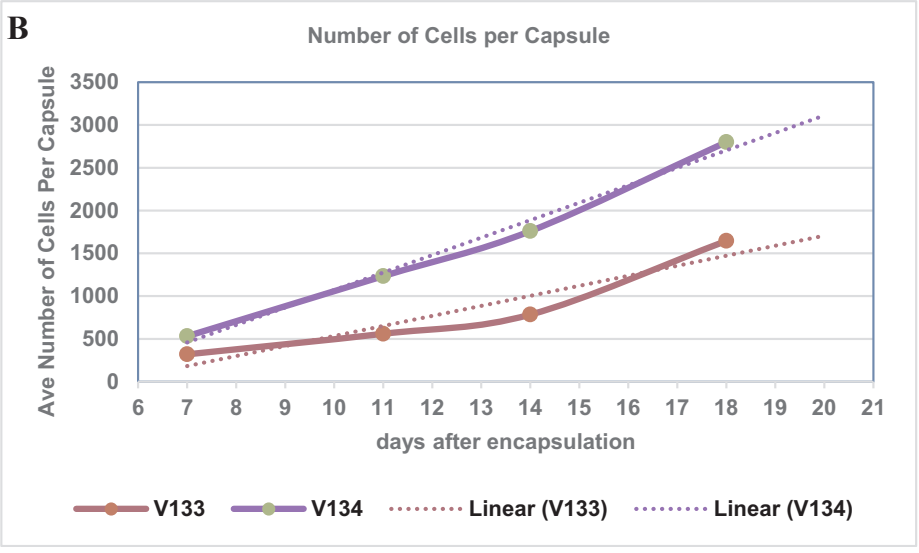


Fig. 4. Metabolic activity and number of encapsulated Hut-78 cells over time after encapsulation. Between 1.5×10^6 and 2.5×10^6 viable Hut-78 cells were encapsulated per ml of cellulose sulphate capsules in two separate experiments (V133 and V134) and then incubated in cell culture medium at 30 °C for up to 18 days. Quadruplicate samples for metabolic activity of 50 capsules were measured at days 7, 11, 14 and 18 post encapsulation using a modified alamarBlue® Assay (A). Using a standard curve for alamarBlue® activity versus number of cells, the number of cells per capsule was calculated and a line of best fit (linear – dotted) calculated for each experiment (B).

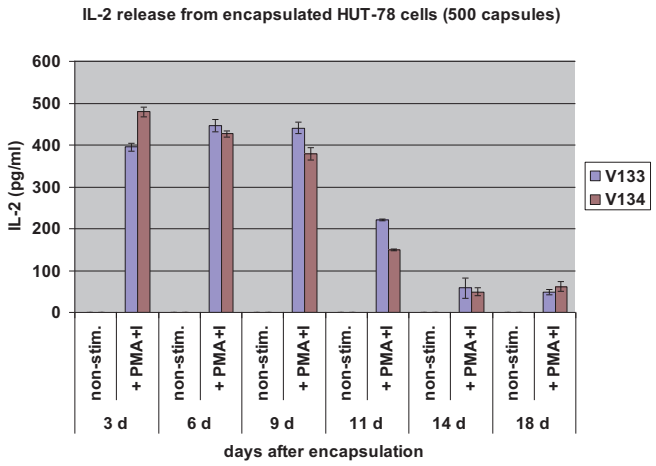


Fig. 5. IL-2 secreted into the medium by encapsulated Hut-78 cells. Three days, 6, 9, 11, 14 or 18 days post encapsulation, 500 capsules of Hut-78 cells from encapsulation V133 (purple bars) or V134 (red bars) were stimulated with phorbol 12-myristate 13-acetate (PMA) and Ionomycin (I) for 18 h (+PMA + I) or not (non-stim.) and IL-2 measured by ELISA.

though theoretically the larger the dextran molecule, the more FITC, this can vary in the range of almost 10 fold. Generally, however, these experiments show that molecules with a molecular weight of up to 70kD can be released from standard Cell-in-a-Box® cellulose sulphate capsules. Moreover the data reported here with various different encapsulated proteins, as well as previously published work showing the release of antibodies from encapsulated hybridoma cells (Pelegrin et al., 1998; Pelegrin et al., 1998) would confirm that release will be influenced not only by the size of the molecule to be released, but also strongly by its form (globular or linear chain like) and/or its charge. Previous studies (Tanaka et al., 1984) using alginate capsules have also seen a predominant effect of the size of the molecule, however Yuet et al. (1993) have speculated that carbonic anhydrase is a non-charged molecule, whilst albumin is negatively charged, opening the possibility that residual negative charges on the cellulose sulphate may actively repel bovine albumin, forcing it out, whilst carbonic anhydrase just diffuses out. This may account for the earlier appearance of the larger bovine albumin in the medium surrounding the capsules compared to the smaller carbonic anhydrase (Fig. 2A). More recently Nafea and colleagues have similarly described the effect of conformation and charge on release of molecules from capsules made from various hydrogels (Nafea and Poole-Warren, 2011).

The concentration in the capsule of the molecule to be released will also play a role, making it possible to release molecules of > 70 kDa in the right circumstances. Indeed, for a biologically synthesized molecule within the capsule (where the concentration of the biologically synthesized molecule is continuously increasing and thus could become very high compared to the concentration outside the capsule) we would expect a very efficient release of molecules. The effect of concentration on the passive diffusion of molecules over membranes has been known for a long time (Craig and Chen, 1972). Small proteins like IL-2 should however easily be able to be released from encapsulated cells.

This study also shows that Hut-78 cells survive and grow after encapsulation in cellulose sulphate. This is in line with previous observations for other cell types like HEK293 cells (Hauser et al., 2006; Salmons et al., 2007), as well as for some stem cell lines and various hybridomas (Dangerfield et al., 2013). In addition, the Hut-78 cells produce IL-2 which is released from the capsules and can be readily detected. Interestingly, the amount of released IL-2 decreased with increasing cell densities within the capsule (cf. Figs. 2, 3 and 4). A reason for this observation could be limited diffusion within the capsule caused by high cell density, however we consider this unlikely since IL-2 is a very small protein and other small molecules such as nutrients have no difficulty in diffusing through the capsule. More likely is that high local IL-2 concentration within the capsule might cause a negative feedback mechanism leading to a down-regulation of IL-2 expression from the encapsulated cells, a phenomenon that has previously been described (Popmihajlov and Smith, 2008). This would argue for using capsules with a relatively low cell density in future clinical scenarios, possibly coupled with irradiation or mitomycin C treatment of the encapsulated cells to keep them metabolically active but not dividing.

Although we have shown that encapsulated human HEK293 cells are safe and well tolerated in 27 human with pancreatic cancer (Löhr et al., 2014), the current study uses Hut78 cells, the safety of which have yet to be tested in patients. One potentially safe solution would be to stably transfect the HEK293 cells with an expression vector harbouring IL-2 cDNA (or other cytokines) and to use this cell line for encapsulation and later clinical trials. In this respect, the study of Yung and colleagues (Yung et al., 2010), where gelatin hydrogels enzymatically crosslinked by microbial transglutaminase (mTG-gels) were used to enmesh HEK293 cells genetically engineered to secrete hIL2, is of interest. They also showed that the enzymatically crosslinked hydrogels are cytocompatible and have suitable transport properties that will facilitate the design of sustained drug release devices for the potential treatment of cancer (Yung et al., 2010).

A number of other researchers have also taken the approach of delivering cytokines to stimulate an immune response against tumours from encapsulated cells. In another study (Hao et al., 2005); an engineered tumour cell line constructed to secrete functional tumour necrosis factor-alpha (TNF-alpha) was encapsulated and tested *in vitro* and *in vivo*. In cell culture, the microencapsulated cells could be shown to release 1.2 ng/ml TNF-alpha, which is functional since it had a clear cytotoxic effect on L929 indicator cells. Upon intra-tumoral implantation in athymic nude mice bearing the human breast cancer cell line MCF-7, the encapsulated cells induced extensive tumour cell apoptosis and necrosis leading to significant tumour regression and slower tumour growth than in the control groups.

The data presented here, together with the referenced studies, suggest that encapsulated cells could be used to deliver multiple anti-tumor recombinant molecules to improve the efficacy of the anti-tumour response. Moreover, this kind of concerted, multi-prong treatment, where encapsulated cells deliver a cocktail of anti-tumor molecules, can be tailor made to gain the maximum benefit for each individual patient while reducing side effects. Cellulose sulphate is an ideal encapsulation agent allowing good delivery efficiency of molecules smaller than 70 kDa from encapsulated cells.

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Table 1
Interleukin-2 production from encapsulated and non-encapsulated Hut-78 cells.

	Non-Encapsulated Hut-78	Encapsulated Hut-78 cells (V133)		Encapsulated Hut-78 cells (V134)	
Number of cells	1×10^6	2.15×10^5	1×10^6	4.5×10^5	1×10^6
Non-stimulated cells IL-2 (pg)	0	0	0	0	0
Stimulated cells IL-2 (pg)	2500	440	2050	380	850

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References

Acarregui, A., Orive, G., Pedraz, J.L., Hernández, R.M., 2013. Therapeutic applications of encapsulated cells. Methods Mol. Biol. Clifton NJ. 1051, 349–364. http://dx.doi.org/10.1007/978-1-62703-550-7_23.

Bhatia, S., Tykodi, S.S., Thompson, J.A., 2009. Treatment of metastatic melanoma: an overview. Oncol. Williston Park N. 23, 488–496.

Bunn, P.A., Foss, F.M., 1996. T-cell lymphoma cell lines (HUT102 and HUT78) established at the National Cancer Institute: history and importance to understanding the biology, clinical features, and therapy of cutaneous T-cell lymphomas (CTCL) and adult T-cell leukemia-lymphomas (ATLL). J. Cell. Biochem. Suppl. 24, 12–23.

Cirone, P., Bourgeois, J.M., Shen, F., Chang, P.L., 2004. Combined immunotherapy and antiangiogenic therapy of cancer with microencapsulated cells. Hum. Gene Ther. 15, 945–959. http://dx.doi.org/10.1089/hum.2004.15.945.

Craig, L.C., Chen, H.C., 1972. On a theory for the passive transport of solute through semipermeable membranes. Proc. Natl. Acad. Sci. USA 69, 702–705.

Dangerfield, J.A., Salmons, B., Corteling, R., Abastado, J.P., Sinden, J., Gunzburg, W.H., Brandtner, E.M., 2013. The Diversity of Uses for Cellulose Sulphate Encapsulation. Bioencapsulation Living Cells Diverse Med. Appl., Bentham Science Publishers, pp. 70–92.

Dautzenberg, H., Schuldt, U., Grasnick, G., Karle, P., Müller, P., Löhr, M., Pelegrin, M., Piechaczyk, M., Rombs, K.V., Günzburg, W.H., Salmons, B., Saller, R.M., 1999. Development of cellulose sulfate-based polyelectrolyte complex microcapsules for medical applications. Ann. N. Y. Acad. Sci. 875, 46–63.

Gasperini, L., Mano, J.F., Reis, R.L., 2014. Natural polymers for the microencapsulation of cells. J. R. Soc. Interface 11, 20140817. http://dx.doi.org/10.1098/rsif.2014.0817.

Gonzalez-Pujana, A., Santos, E., Orive, G., Pedraz, J.L., Hernandez, R.M., 2017. Cell microencapsulation technology: current vision of its therapeutic potential through the administration routes. J. Drug Deliv. Sci. Technol. 42, 49–62. http://dx.doi.org/10.1016/j.jddst.2017.03.028.

Gurruchaga, H., Saenz del Burgo, L., Ciriza, J., Orive, G., Hernández, R.M., Pedraz, J.L., 2015. Advances in cell encapsulation technology and its application in drug delivery. Expert Opin. Drug Deliv. 12, 1251–1267. http://dx.doi.org/10.1517/17425247.2015.1001362.

Hamilton, M.J., Huang, Q.X., Li, C.L., Ellem, K.A.O., 2006. The development and characterization of an HEK293-derived cell line for use in an intratumoral cytokine delivery system. Cell Transplant. 15, 343–350.

Hao, S., Su, L., Guo, X., Moyana, T., Xiang, J., 2005. A novel approach to tumor suppression using microencapsulated engineered J558/TNF-alpha cells. Exp. Oncol. 27, 56–60.

Hauser, O., Schwab, S., Tabotta, W., Gunzburg, W.H., Salmons, B., Holzmuller, H., 2006. Cell based therapy using genetically modified, encapsulated cells. Proc. 14th Int. Workshop Bioencapsulation, pp. 41–44.

Huang, X.Q., 2005. Depot Cytokines and Chemokines for Antitumour Therapy in a Mouse Model. The University of Queensland.

Kumar, S., Chandra, D., 2014. A therapeutic perspective of cytokines in tumor management. Inflamm. Cell Signal. 1. http://dx.doi.org/10.14800/ics.159.

Löhr, J.M., Haas, S.L., Kröger, J.C., Friess, H.M., Höft, R., Goretzki, P.E., Peschel, C., Schweigert, M., Salmons, B., Gunzburg, W.H., 2014. Encapsulated cells expressing a chemotherapeutic activating enzyme allow the targeting of subtoxic chemotherapy and are safe and efficacious: data from two clinical trials in pancreatic cancer. Pharmaceutics 6, 447–466. http://dx.doi.org/10.3390/pharmaceutics6030447.

Mach, N., Vernet, R., Belkouch, M.-C., Luy, P., Ancrenaz, V., Teta, P., Blazek, N., Grandjean, N., Wasem, J., Grogg, J., Perez, T., Migliorini, D., 2016. MVX-ONCO-1 phase 1 final results of the first personalized cell-based immunotherapy using cell encapsulation technology. Ann. Oncol. 27. http://dx.doi.org/10.1093/annonc/mdw378.12.

MedlinePlus, Aldesleukin, (2013). https://medlineplus.gov/druginfo/meds/a692009.html.

Michalowska, M., Winiarczyk, S., Adaszek, Ł., Łopuszyński, W., Grądzki, Z., Salmons, B., Günzburg, W.H., 2014. Phase I/II clinical trial of encapsulated, cytochrome P450 expressing cells as local activators of cyclophosphamide to treat spontaneous canine tumours. PLoS One 9, e102061. http://dx.doi.org/10.1371/journal.pone.0102061.

Migliorini, D., Mach, N., Aguiar, D., Vernet, R., Landis, B.N., Becker, M., McKee, T., Dutoit, V., Dietrich, P.-Y., 2017. First report of clinical responses to immunotherapy in 3 relapsing cases of chordoma after failure of standard therapies. Oncoimmunology 6, e1338235. http://dx.doi.org/10.1080/2162402X.2017.1338235.

Moran, D.M., Koniaris, L.G., Jablonski, E.M., Cahill, P.A., Halberstadt, C.R., McKillop, I.H., 2006. Microencapsulation of engineered cells to deliver sustained high circulating levels of interleukin-6 to study hepatocellular carcinoma progression. Cell Transplant. 15, 785–798.

Nafea, E.H., Marson, A., Poole-Warren, L.A., Martens, P.J., 2011. Immunoisolating semipermeable membranes for cell encapsulation: focus on hydrogels. J. Control. Release 154, 110–122. http://dx.doi.org/10.1016/j.jconrel.2011.04.022.

Orlando, G., Gianello, P., Salvatori, M., Stratta, R.J., Soker, S., Ricordi, C., Domínguez-Bendala, J., 2014. Cell replacement strategies aimed at reconstitution of the β-cell compartment in type 1 diabetes. Diabetes. 63, 1433–1444. http://dx.doi.org/10.2337/db13-1742.

Ortner, V., Kaspar, C., Halter, C., Töllner, L., Mykhaylyk, O., Walzer, J., Günzburg, W.H., Dangerfield, J.A., Hohenadl, C., Czerny, T., 2012. Magnetic field-controlled gene expression in encapsulated cells. J. Control. Release Off. J. Control. Release Soc. 158, 424–432. http://dx.doi.org/10.1016/j.jconrel.2011.12.006.

Pelegrin, M., Marin, M., Noel, D., Del Rio, M., Saller, R., Stange, J., Mitzner, S., Günzburg, W.H., Piechaczyk, M., 1998. Systemic long-term delivery of antibodies in immunocompetent animals using cellulose sulphate capsules containing antibody-producing cells. Gene Ther. 5, 828–834.

Pelegrin, M., Noel, D., Marin, M., Bachrach, E., Saller, R.M., Salmons, B., Piechaczyk, M., 1998. In vivo production of therapeutic antibodies by engineered cells for immunotherapy of cancer and viral diseases. Gene Ther Mol Biol. 3, 1–9.

Popmihajlov, Z., Smith, K.A., 2008. Negative feedback regulation of T cells via interleukin-2 and FOXP3 reciprocity. PLoS One 3, e1581. http://dx.doi.org/10.1371/journal.pone.0001581.

Roberts, N.J., Zhou, S., Diaz, L.A., Holdhoff, M., 2011. Systemic use of tumor necrosis factor alpha as an anticancer agent. Oncotarget. 2, 739–751. http://dx.doi.org/10.18632/oncotarget.344.

Rosenberg, S.A., 2014. IL-2: the first effective immunotherapy for human cancer. J. Immunol. Baltim. Md 1950 (192), 5451–5458. http://dx.doi.org/10.4049/jimmunol.1490019.

Salmons, B., Brandtner, E.M., Hettrich, K., Wagenknecht, W., Volkert, B., Fischer, S., Dangerfield, J.A., Gunzburg, W.H., 2010. Encapsulated cells to focus the metabolic activation of anticancer drugs. Curr. Opin. Mol. Ther. 12, 450–460.

Salmons, B., Brandtner, E.M., Dangerfield, J.A., Gunzburg, W.H., 2014. Encapsulation of insulin producing cells for diabetes treatment using alginate and cellulose sulphate as bioencapsulation polymers: current status. Diabetes Res. Treat. 1, 1–7.

Salmons, B., Gunzburg, W.H., 2010. Therapeutic application of cell microencapsulation in cancer. Adv. Exp. Med. Biol. 670, 92–103.

Salmons, B., Hauser, O., Gunzburg, W.H., Tabotta, W., 2007. GMP production of an encapsulated cell therapy product: issues and considerations. Bioprocess. J. 4, 36–43.

Schwenter, F., Zarei, S., Luy, P., Padrun, V., Bouche, N., Lee, J.S., Mulligan, R.C., Morel, P., Mach, N., 2011. Cell encapsulation technology as a novel strategy for human antitumor immunotherapy. Cancer Gene Ther. 18, 553–562. http://dx.doi.org/10.1038/cgt.2011.22.

Shaker, M.A., Younes, H.M., 2009. Interleukin-2: evaluation of routes of administration and current delivery systems in cancer therapy. J. Pharm. Sci. 98, 2268–2298. http://dx.doi.org/10.1002/jps.21596.

Tanaka, H., Matsumura, M., Veliky, I.A., 1984. Diffusion characteristics of substrates in Ca-alginate gel beads. Biotechnol. Bioeng. 26, 53–58. http://dx.doi.org/10.1002/bit.260260111.

Vacchelli, E., Aranda, F., Obrist, F., Eggermont, A., Galon, J., Cremer, I., Zitvogel, L., Kroemer, G., Galluzzi, L., 2014. Trial watch: immunostimulatory cytokines in cancer therapy. Oncoimmunology 3, e29030. http://dx.doi.org/10.4161/onci.29030.

Waldmann, T., Tagaya, Y., Bamford, R., 1998. Interleukin-2, interleukin-15, and their receptors. Int. Rev. Immunol. 16, 205–226. http://dx.doi.org/10.3109/08830189809042995.

Werner, M., Schmoldt, D., Hilbrig, F., Jérôme, V., Raup, A., Zambrano, K., Hübner, H., Buchholz, R., Freitag, R., 2015. High cell density cultivation of human leukemia T cells (Jurkat cells) in semipermeable polyelectrolyte microcapsules. Eng. Life Sci. 15, 357–367. http://dx.doi.org/10.1002/elsc.201400186.

Yuet, P., Kwok, W., Harris, T.J., Goosen, M.F.A., 1993. Mathematical modeling of protein diffusion and cell growth in microcapsules. In: Fundam. Anim. Cell Encapsulation Immobil. CRC Press, pp. 79–111.

Yung, C.W., Bentley, W.E., Barbari, T.A., 2010. Diffusion of interleukin-2 from cells overlaid with cytocompatible enzyme-crosslinked gelatin hydrogels. J. Biomed. Mater. Res. A. 95, 25–32. http://dx.doi.org/10.1002/jbm.a.32740.