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Microencapsulation of small intestinal neuroendocrine neoplasm cells for tumor model studies

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Basic cancer research is dependent on reliable *in vitro* and *in vivo* tumor models. The serotonin (5-HT) producing small intestinal neuroendocrine tumor cell line KRJ-1 has been used in *in vitro* proliferation and secretion studies, but its use in *in vivo* models has been hampered by problems related to the xeno-barrier and tumor formation. This may be overcome by the encapsulation of tumor cells into alginate microspheres, which can function as bioreactors and protect against the host immune system. We used alginate encapsulation of KRJ-1 cells to achieve long-term functionality, growth and survival. Different conditions, including capsule size, variations in M/G content, gelling ions (Ca^{2+}/Ba^{2+}) and microcapsule core properties, and variations in KRJ-1 cell condition (single cells/spheroids) were tested. Viability and cell growth was evaluated with MTT, and confocal laser scanner microscopy combined with LIVE/DEAD viability stains. 5-HT secretion was measured to determine functionality. Under all conditions, single cell encapsulation proved unfavorable due to gradual cell death, while encapsulation of aggregates/spheroids resulted in surviving, functional bioreactors. The most ideal spheroids for encapsulation were 200–350 μm. Long-term survival (>30 days) was seen with solid Ca^{2+}/Ba^{2+} microbeads and hollow microcapsules. Basal 5-HT secretion was increased (sixfold) after hollow microcapsule encapsulation, while Ca^{2+}/Ba^{2+} microbeads was associated with normal basal secretion and responsiveness to cAMP/PKA activation. In conclusion, encapsulation of KRJ-1 cells into hollow microcapsules produces a bioreactor with a high constitutively activate basal 5-HT secretion, while Ca^{2+}/Ba^{2+} microbeads provide a more stable bioreactor similar to non-encapsulated cells. Alginate microspheres technology can thus be used to tailor different functional bioreactors for both *in vitro* and *in vivo* studies. (*Cancer Sci* 2012; 103: 1230–1237)

Serotonin (5-HT) abnormalities are a key feature of a number of gastrointestinal disorders, including inflammatory bowel diseases[1,2] and gastrointestinal (GI) neuroendocrine neoplasms (NEN), particularly those derived from enterochromaffin (EC) cells.[3] The latter are increasing rapidly in incidence (approximately 6%/year).[4] The vast majority are malignant and have a considerable metastatic propensity.[5] No consistently effective therapy is available to inhibit NEN cell proliferation or metastasis and no reliable *in vivo* models are available to test treatment strategies.[6] Fundamental problems with establishing tumor models are related to the xeno-barrier. In addition, establishing tumor growth in the abdomen is made difficult in nude mice by the mobile environment. These challenges may be overcome by the encapsulation of tumor cells into alginate microspheres, which would provide a barrier against the hostile immunological environment and create a slow release system of cells, thus prolonging the time for tumor establishment in the surrounding tissue.

Alginate microspheres (microcapsules and microbeads) have been explored as bioreactors and protective devices for cells producing therapeutic proteins in relation to hormonal dysfunction[7–10] and in a variety of cancer models.[11–15] The properties of alginate microspheres can be designed to fulfill criteria for different applications, in relation to choice of alginate and protocols. The two sugar monomers in alginates, β-D-mannuronic acid (M) and α-L-guluronic acid (G) are binary linked with 1–4 linkages.[16] The gel forming ability is related to divalent cations (Ca^{2+} and Ba^{2+} most commonly used) with different affinities to the G, MG or M blocks in the anionic alginate chains.[17,18] This influences the gel matrix strength and elasticity.[19,20] Further variations in microsphere properties can be achieved in the formation of an outer membrane using polycations (commonly used poly-L-lysine)[21–23] and dissolving the alginate microcapsule core.[8,24]

Previous published studies have demonstrated variation in survival and growth abilities for different cell types after encapsulation,[25–29] and conditions must, therefore, be optimized for any new cell line to achieve high cell performance. A candidate cell line for establishing GI-NEN bioreactors is the human small intestinal NEN cell line, KRJ-1,[30,31] which is well-characterized (from an EC cell-derived tumor) and has been documented to have a functional NEN phenotype secreting hormonally active amines (e.g. 5-HT) and peptides (e.g. chromogranin A) *in vitro*.[31,32]

The establishment of an *in vivo* EC cell GI-NEN model would represent an important tool to test new drugs specifically developed for treatment of NEN growth and related fibrosis.[33] Good candidates (e.g. cytotoxic-labeled somatostatin analogs) with promising effects on KRJ-1 growth *in vitro* have been developed and elucidated.[34,35] Testing new agents and treatment strategies in an animal model would greatly enhance the opportunity to find efficacious drugs to treat GI-NEN that currently have no curative treatment option.

The present *in vitro* study aims to optimize the survival and growth of alginate microencapsulated KRJ-1 cells by evaluating various combinations of alginate microspheres and cell conditions. Functionality of the encapsulated NEN cells is tested within the most promising microspheres types.

Materials and Methods

Reagents. The following ultrapure sodium alginates were used (FMC BioPolymer AS, NovaMatrix, Sandvika, Norway): Pronova UP-MVG (67% guluronic acid, intrinsic viscosity 1105 mL/g,

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endotoxin <43 EU/g), Pronova UP-LVG (67% guluronic acid, viscosity 1051 mL/g, endotoxin <23 EU/g) or Pronova UP 100 M (*Macrocystis pyrifera*, 56% mannuronic acid, intrinsic viscosity 908 mL/g, endotoxin <26 EU/g). Calcium chloride, barium chloride and sodium citrate of analytical grades were from Merck (Darmstadt, Germany), and poly-L-lysine (P2658) from Sigma-Aldrich (St. Louis, MO, USA). Mannitol (HPLC degree, BDH Anala R., VWR International, Pool, England), sterile saline (0.9% NaCl, non-pyrogenic, B. Braun, Melsungen, Germany), Quantum 263 Complete Medium for Tumor Cells (PAA, Pashing, Austria), Pen-strep (Sigma, Life Technology, USA), L-glutamine (G-7513) and MTT (3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromide) (Sigma-Aldrich Chemic, Steinheim, Germany), a LIVE/DEAD viability kit (Invitrogen, Molecular Probes, Eugene, Oregon, USA), and isoproterenol hydrochloride and forskolin (Sigma-Aldrich, St Louis, MO, USA) were used in the present study. Chromogranin A (Dako, Oslo, Norway) and tryptophan hydroxylase-1 (Abcam) antibodies were used for immunohistochemistry.

Alginate microspheres. Alginate (2% in 0.3 M Mannitol pH 7.4) was mixed with KRJ-1 cells to a final concentration of 1.8% alginate, as previously described,[25] using an electrostatic droplet generator (7 kV), 0.4 mm needles (unless otherwise stated) and a flow of 5–20 mL/h, depending on the alginate source. Gelling solutions were 1 mM $BaCl_2$/50mM$CaCl_2$/0.15 M mannitol or 50 mM $CaCl_2$/0.15 M. Alginate-poly-L-lysine-alginate (APA) microcapsules were made with 0.05% PLL and 0.1% M. *pyrifera* alginate.[25] Adding 20 mL citrate buffer (55 mM, pH 7.4), for 10 min, hollow microcapsules were made.

Preparation of cells. KRJ-1 cells (1×10^5/mL) grew in Quantum 263 (PAA Laboratories, Cölbe, Germany) with penicillin/streptomycin and glutamine in 5% CO_2 at 37°C, and re-cultured every 4–5 days, with media supply on day 3.

Encapsulation of KRJ-1. Single cells ($2–8 \times 10^6$/mL) or aggregates/spheroids were dispended in alginate. The size and shape of aggregates/spheroids depended on growth time and cell concentration. The ideal size (200–400 μm) and spheroid formation was achieved by seeding cells in a concentration of 2×10^5/mL for 5 days. Spheroids were carefully harvested by centrifugation (200*g*, 3 min), with the lower fraction taken out corresponding to a volume of 1/10 of the alginate, and finally carefully mixed.

MTT assay. The viability/growth was measured using a MTT assay.[26]

Confocal laser scanner microscopy. Cells were evaluated with LIVE/DEAD viability stain (Invitrogen Molecular Probes) and confocal laser scanner microscopy (CLSM) (Zeiss LSM 510; Carl Zeiss MicroImaging GmbH, Göttingen, Germany), as described previously.[26] 3-D data projections were made by overlapping sections through the microspheres using z-stacks.

Chromogranin A and tryptophan hydroxylase 1 immunohistochemical staining. Cells were stained with antibodies against Chromogranin A or tryptophan hydroxylase 1 (TPH1, the rate limiting enzyme in serotonin synthesis) using standard immunohistochemical protocols and visualized using DAB, as described previously.[36]

Microspheres allocation for functional studies. Before performing functional studies, the microspheres were allocated as equally as possible in a 24-vial plate, by the following procedure: washed and dispended microspheres (1 mL) in media (40 mL) were divided in four equal parts (4 × 10 mL). Each part was distributed between 6-vials in a 24-vial plate. After performing the functional studies, MTT was added[26] and the 24-vial plate scanned to count the number of microspheres, before estimating cell viability by extracting the MTT.[26]

Functional studies. Serotonin release was measured in medium harvested from encapsulated KRJ-1 cells and compared to non-encapsulated cells under the same conditions (basal, isoproterenol [β-adrenergic receptor agonist; 10 pM–0.1 μM] and forskolin [PKA/cAMP activator; 0.1nM–1μM]) stimulation using the Serotonin BAE-5900 ELISA kit (Labor Diagnostica Nord, Nordhorn, Germany), as described previously.[32,37]

Statistical analysis. Differences in functional data were analyzed using a two-tailed Mann–Whitney test.

Results

KRJ-1 characteristics. KRJ-1 cells aggregated upon culturing and formed rounded spheroids during a 5-day growth period (Fig. S1A,B). Pseudopodia were observed in the outer cell layers (Fig. S1C). Staining with anti-TPH1 and anti-CgA antibodies (Fig. S1D,E) confirmed NEN features and active 5-HT synthesis.

High-G alginate microspheres. KRJ-1 cells were encapsulated in APA microcapsules as single cells (Fig. 1a–d) or spheroids (Fig. 1e–h) using high-G alginate (UP-MVG). CLSM demonstrated that the KRJ-1 cells survived the encapsulation both as single cells (Fig. 1a,b) and as spheroids (Fig. 1e,f). Extensive cell death was found among single cells at days 8 and 14 (Fig. 1C,D). Viability was prolonged in spheroids seen at day 14 (Fig. 1g), but decreased at day 34 (Fig. 1h). Unsuccessful encapsulations were shown for Ca^{2+} microbeads (UP-MVG alginate) with disruption (Fig. S2A,B) or by failing in encapsulation of spheroids larger than 400–500 μm (Fig. S2C).

Intermediate-G microspheres. Alginate-poly-L-lysine-alginate microcapsules with intermediate G-alginate (*Macrocystis pyrifera*, UP 100 M) were made. The microcapsules' core was kept solid or made hollow. MTT uptake was higher for single cells within hollow microcapsules compared to APA (Fig. 2a). CLSM demonstrated that single cells in hollow microcapsules aggregated 1 day after encapsulation, and by days 14 and 26, the cell aggregates had increased in size, with viable cells detected (Fig. 2b–d). In the solid APA microcapsules, the majority of the cells were viable at day 1, but only a few at day 14, with only dead cells observed on day 26 (Fig. 2e–g). Spheroids in solid APA microcapsules contained mostly viable cells from days 1 to 14 (Fig. 2i,j); after 34 days, however, most had died (Fig. 2k). The spheroid MTT uptake declined gradually until day 35; thereafter, a slight increase was detected, indicating re-growth within the spheroids (Fig. 2h).

Hollow microcapsules with variations in size and cell concentration. First, standard sized microcapsules (0.4 mm needle) were compared to smaller microcapsules (0.35 mm needle) containing 4×10^6/mL cells. The MTT uptake was higher in the standard sized microcapsules (Fig. S3A). The smaller microcapsules also contained smaller cell aggregates at day 6 (Fig. S3B,C). Furthermore, survival was shorter in small microcapsules; however, a minor population (<10%) of the cells in the small microcapsules behaved differently and had developed large spheroids at day 28 (Fig. S3E,F). In contrast, surviving cell aggregates were found in all examined standard sized microcapsules (Fig. S3F).

Second, doubling of the standard cell amount (8×10^6/mL alginate) within standard sized microcapsules resulted in an initial increase in MTT uptake. The difference in MTT uptake decreased with time, and with slightly larger cell aggregates with doubled cell amount (Fig. S3A,D,G).

Spheroids in hollow microcapsules and Ca^{2+}/Ba^{2+} microbeads. Three and 5-day cultures of KRJ-1 cells (2×10^5/mL) were encapsulated in Ca^{2+}/Ba^{2+} microbeads or hollow microcapsules. The initial MTT uptake by 3-day cultured KRJ-1 spheroids was high in both microspheres (Fig. 3a). The MTT uptake oscillated over time for hollow microcapsules, while gradually declining for Ca^{2+}/Ba^{2+} microbeads. CLSM images show spheroid-shaped aggregates with high viability in hollow

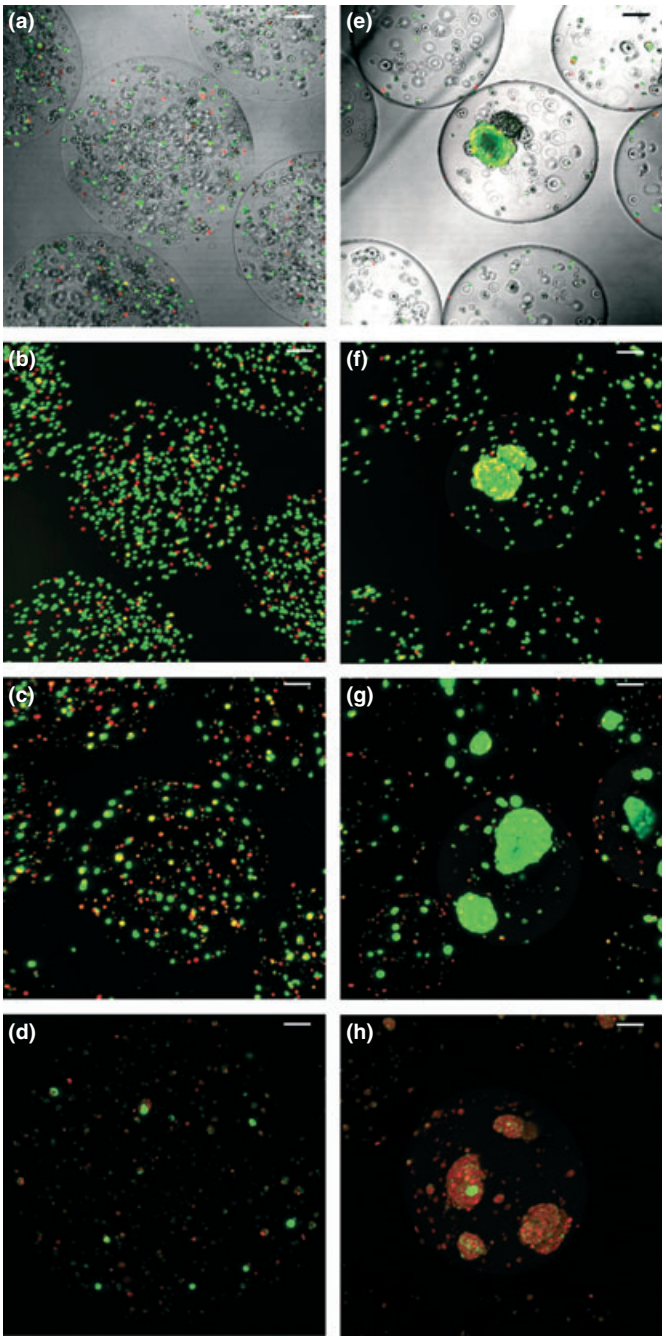

Fig. 1. Alginate microspheres using high-G alginate (UP-MVG). Confocal laser scanner microscopy images of encapsulated KRJ-1 cells with the following conditions. Single cells in alginate-poly-L-lysine-alginate (APA) microcapsules are shown: (a) 1 day after encapsulation as cross-section with transmitted light, (b) day 1 as a 3-D projection, (c) day 8 as a 3-D projection and (d) day 14 as a 3-D projection. Spheroids (from a 1 day culture of 1 $\times$ 10^6/mL cells) encapsulated in APA microcapsules are shown: (e) 1 day after encapsulation by transmitted light, (f) day 1 as a 3-D image, (g) day 14 as a 3-D image and (h) day 34 as a 3-D image. Live cells are green and dead cells red. Bars = 100 μm.

microcapsules at days 16 and 29 (Fig. 3b–d). The spheroids in Ca^{2+}/Ba^{2+} microbeads were small, with gradually decreased amounts of live cells from days 3 to 29 (Fig. 3e–g).

By pre-culturing KRJ-1 cells for 5 days, large spheroids with a fluctuating uptake of MTT in Ca^{2+}/Ba^{2+} microbeads were achieved. Spheroids concentrated from two culture bottles ((5d-sph)$\times$2) showed an initial 1.7 times higher MTT-uptake than from one (5-d sph), but the difference diminished over time (Fig. 3h). Whereas cells within spheroids remained alive up to 27 days, single cells were all dead at day 16 (Fig. 3i–n).

Different KRJ-1 encapsulation conditions and basal serotonin release. At days 2–3 after encapsulation, a sixfold increase in secretion was found from the hollow microcapsules (Fig. 4a). A steady secretion level was detected from the Ca^{2+}/Ba^{2+} microbeads. For the non-encapsulated cells, a more than two-fold increase was seen by day 2, followed by a reduction to the initial level by day 3. The basal 5-HT release was normalized to MTT-uptake (as a measure of cell number), as shown in Figure 4b. KRJ-1 cells in hollow microcapsules released significantly more 5-HT, while KRJ-1 cells in Ca^{2+}/Ba^{2+} microbeads released significantly less than non-encapsulated cells (after normalization to MTT uptake). The hollow microcapsules released approximately twice as much 5-HT per microsphere compared to the Ca^{2+}/Ba^{2+} microbeads (Fig. 4c).

Effect on serotonin release through stimulation with forskolin and isoproterenol. Forskolin and isoproterenol are agents that activate cAMP/PKA signaling and β-adrenergic receptors: both key regulators of 5-HT synthesis and release.[31] Both non-encapsulated cells and cells within Ca^{2+}/Ba^{2+} microbeads released significantly more 5-HT upon forskolin (1 μM, and additionally tested at 10 and 0.1 nM) stimulation, whereas no response was seen from cells encapsulated in the hollow microcapsules (Fig. 5a). Ca^{2+}/Ba^{2+} microbeads released significantly more ($P < 0.05$) 5-HT compared to the control at all concentrations of isoproterenol (10 pM, 1.0 nM and 0.1 μM) (Fig. 5b).

Discussion

The goal of the present study was to generate functional bioreactors with the EC cell-derived GI-NEN cell line, KRJ-1, in alginate microspheres. The approach was to identify the ideal encapsulation protocols with variations in M/G content, gelling ions (Ca^{2+}, Ba^{2+}), microcapsules with solid versus hollow core, as well as variations in the KRJ-1 cell condition (single cells or spheroids). The progression of cell growth, viability and functionality (5-HT secretion) was dependent on the type of microsphere used and whether the cells were encapsulated as single cells or as spheroids.

When encapsulated as single dispersed cells, gradual cell death was observed in the different types of solid microspheres. It is well known that several different cell types are growth-inhibited[26,38] or die in matrixes that possess a high gel strength.[29] Alginates rich in M moieties possess lower gel strength in contrast to the G-rich alginates.[20,39] Irrespective of M-rich or G-rich alginate microspheres with solid cores, the outcome of single cells was associated with cell death. In contrast, KRJ-I cells grew well in hollow microcapsules. This indicates that a reduction in matrix tension (caused by dissolving the microcapsules core) allowed single KRJ-1 cells to form cell aggregates, a condition that predisposes them to growth.[32] As shown in the present study using KRJ-1 cells, encapsulation in microcapsules with liquefied/hollow cores can also be beneficial for growth of fibroblasts,[28] βTC3 cells,[27] feline kidney cells,[40] mouse embryonic stem cells[40] and hybridoma cells.[41] However, this is not universal, because other cells, like myoblasts and MDCK epithelial cells, do not benefit from a liquefied core.[28] Even if cumbersome, every single cell line must, therefore, be tested specifically to find its optimal encapsulation conditions. Our results suggest that the KRJ-1 cells are sensitive to matrix resistance and are dependent upon cell–cell contact for growth. These findings are consistent with the nature of the KRJ-1 cells, because they form aggregates upon

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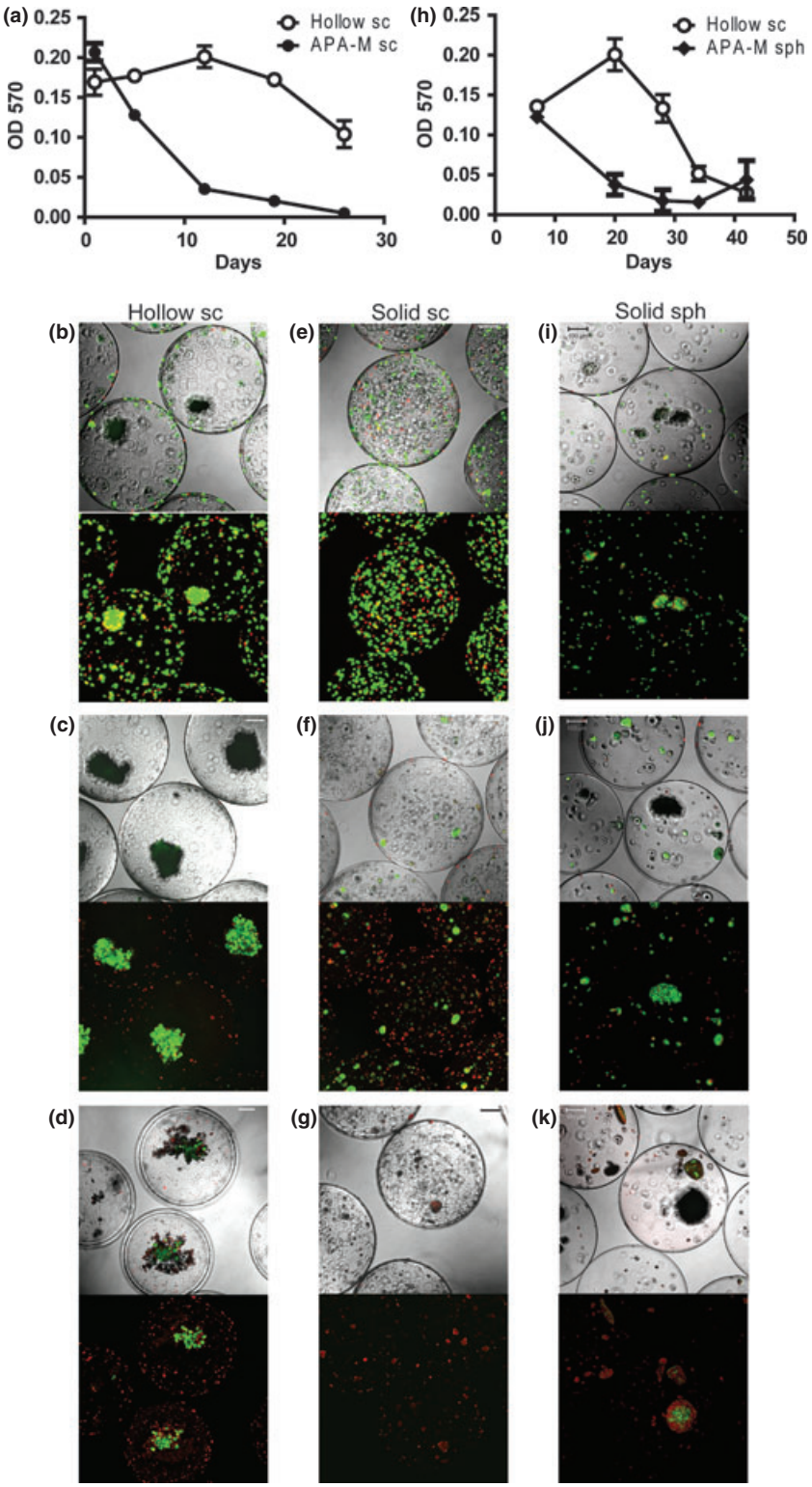

Fig. 2. Solid or hollow alginate-poly-L-lysine-alginate (APA) microcapsules with intermediate-G alginate (*Macrocystis pyrifera*). (a) MTT uptake in KRJ-1 cells after encapsulation as single cells (sc, 5.5×10^6/mL). (b) Transmitted light sections (upper) or a 3-D projection (lower) of hollow microcapsules with single cells 1 day after encapsulation, (c) hollow microcapsules with single cells by day 14, (d) hollow microcapsules with single cells by day 26, (e) solid microcapsules with single cells 1 day after encapsulation, (f) solid microcapsules with single cells by day 14, (g) solid microcapsules with single cells by day 34. Live cells are green and dead cells red. Bars = 100 μm. Graphs: mean ± SD, $N = 4$. (h) MTT uptake in KRJ-1 cells after encapsulation as spheroids (from a 2-day culture of 1×10^6/mL cells). (i) Transmitted light sections (upper) or a 3-D projection (lower) of solid microcapsules day 1 after encapsulation of spheroids (sph) with transmitted light and as a 3-D projection, (j) solid microcapsules with spheroids by day 14, (k) solid microcapsules with spheroids by day 26. Live cells are green and dead cells red. Bars = 100 μm. Graphs: mean ± SD, $N = 4$.

seeding and 3-D structured spheroids upon growth in culture (Fig. S1).[30–32]

Improvement in KRJ-1 survival time was achieved by encapsulating spheroids instead of single cells, and, in addition, our data identifies a connection between the size of the spheroids and the survival time. The most ideal spheroids for encapsulation were achieved by a 5-day culture period, with spheroid sizes up to 350 μm. Examples are shown in Figure 3i and j. The MTT uptake demonstrated a fluctuating pattern during cultivation, which we interpret to indicate the dynamics of cell proliferation and death. As single cells were not observed to divide after encapsulation, proliferation was probably restricted to spheroids. Our data demonstrate the potential to achieve long-term survival of the KRJ-1 cells within the solid Ca^{2+}/Ba^{2+} microbeads by encapsulating large sized spheroids (200–350 μm).

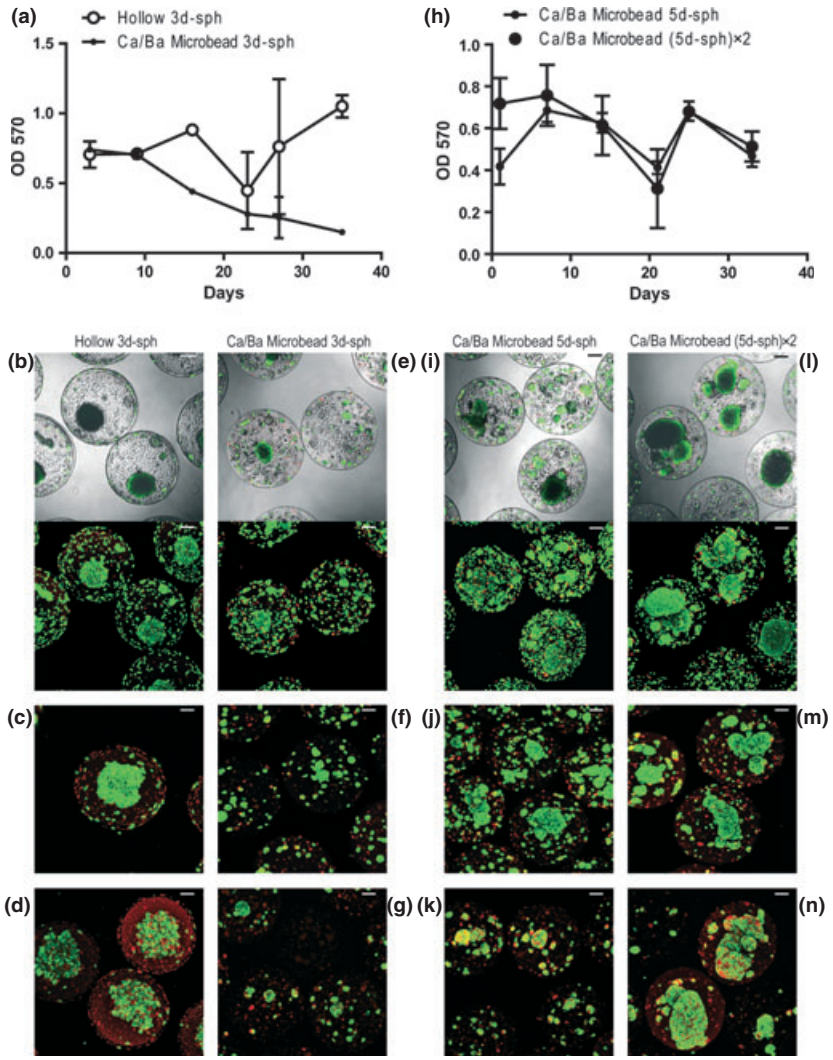

Fig. 3. Hollow alginate-poly-L-lysine-alginate (APA) microcapsules or Ca^{2+}/Ba^{2+} microbeads with KRJ-1 spheroids (sph). Spheroids were grown for 3 days (3d-sph) or 5 days (5d-sph) from initial cultures of 2×10^5/mL. Ca^{2+}/Ba^{2+} microbeads were made with UP-LVG alginate (high-G) while hollow microcapsules were made with *Macrocystis pyrifera* alginate (intermediate G). (a) Growth/survival of 3-day spheroids in hollow microcapsules or Ca^{2+}/Ba^{2+} microbeads measured as MTT uptake. (b–g) Confocal laser scanner microscopy images through equator overlaid transmitted light with respective 3-D projections of the respective capsules. (b) 3 days after encapsulation in hollow microcapsules with 3d-sph, (c) hollow day 16, (d) hollow day 29, (e) Ca^{2+}/Ba^{2+} microbead with 3-D spheroids 3 days after encapsulation, (f) day 16, (g) day 29. (h) Growth/survival of 5-day spheroids in Ca^{2+}/Ba^{2+} microbeads measured as MTT uptake. (i–n) Confocal laser scanner microscopy images through equator overlaid transmitted light with 3-D projections of the respective capsules. (i) Ca^{2+}/Ba^{2+} microbead with 5-d spheroids taken day 1 after encapsulation, (j) Ca^{2+}/Ba^{2+} microbead 5d sph day 14 after encapsulation, (k) Ca^{2+}/Ba^{2+} microbead 5d sph day 27 after encapsulation, (l) Ca^{2+}/Ba^{2+} microbead with (5-d spheroids $\times$ 2) taken day 1 after encapsulation, (m) Ca^{2+}/Ba^{2+} microbead (5d sph x 2) day 14 after encapsulation, (n) Ca^{2+}/Ba^{2+} microbead (5d sph $\times$ 2) day 27. Bars = 100 μm. Graphs: mean ± SD, $N = 4$.

By combining spheroid encapsulation with hollow microcapsules, a high cell load and larger cell aggregates could be formed. This was reflected by substantially increased MTT uptake compared to the single cell studies, suggesting accommodation to the hollow microcapsules environment. The differences in cell debris accumulation between the hollow microcapsules versus Ca^{2+}/Ba^{2+} microbeads may be explained by differences in porosity and cell growth, the hollow microcapsules having lower porosity and higher cell growth.

The two most promising bioreactors, the Ca^{2+}/Ba^{2+} microbeads and the hollow microcapsules, were tested for functionality by 5-HT secretion. The basal secretion of 5-HT from KRJ-1 cells was increased after encapsulation into hollow microcapsules as compared to non-encapsulated cells. In contrast, encapsulation into Ca^{2+}/Ba^{2+} microbeads lowered the basal secretion compared to the hollow microcapsules to levels similar to non-encapsulated (or control) cells. Stimulation by the PKA activator forskolin or the β-adrenergic receptor antagonist isoproterenol resulted in 5-HT secretion from the cells within the Ca^{2+}/Ba^{2+} microbeads, demonstrating that these encapsulated cells (and their microenvironment) was functional. In contrast, the encapsulated KRJ-1 cells within hollow microcapsules did not respond upon stimulation to either forskolin or isoproterenol. The low molecular weights of forskolin (410 Da) and isoproterenol (248 Da) mean that our findings are unlikely to be a result of impermeability of the microspheres as molecules as large as insulin (5.8 kDa) easily diffuse into APA microcapsules coated with PLL.[42] The lack of stimulation of KRJ-1 cells in the hollow microcapsules likely reflects that the cells were constitutively activated with a high basal 5-HT secretion, and, therefore, not able to respond further.[32] Irrespective, cells encapsulated using these two methods were functional, with the Ca^{2+}/Ba^{2+} microbeads probably most similar to non-encapsulated conditions.

An analysis of the non-functional bioreactors identified a number of additional observations highlighting potential issues with some of the encapsulation approaches. First, Ca^{2+} microbeads exhibited ruptures in the alginate matrix a short time (approximately 24 h) after formation. This might be a consequence of calcium uptake by the KRJ-1 cells (a prerequisite for secretion),[32] which will lead to gel destabilization in the absence of barium. This has also been observed for the cancer cell line JJN3.[26] Second, large-sized spheroids (above 400 μm) resulted in a low encapsulation ratio. Third, a reduction of the hollow microcapsule size lowered cell survival, and, fourth, increased cell numbers of single cells rendered minimal growth profit, with a potential risk of increased loss

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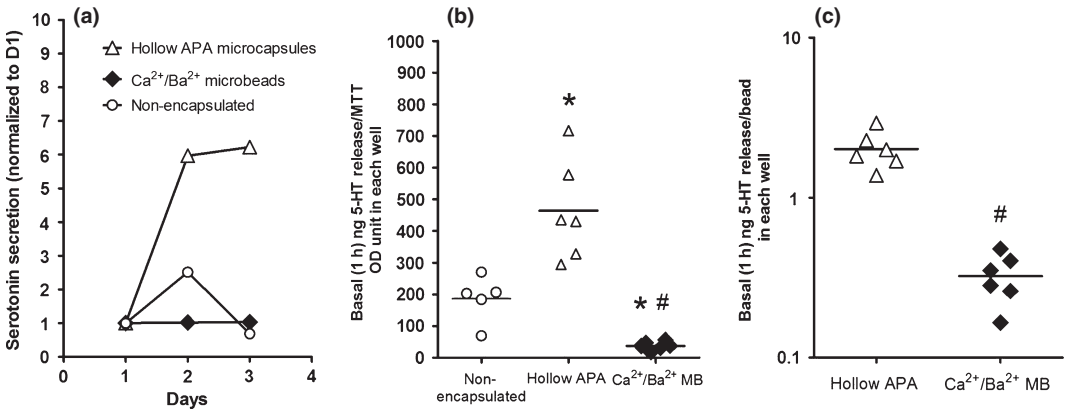

Fig. 4. Basal 5-HT release after encapsulation. (a) Accumulated 5-HT in the culture media from hollow alginate-poly-L-lysine-alginate (APA) microcapsules or Ca^{2+}/Ba^{2+} microbeads 1, 2 and 3 days after encapsulation compared to single cells. 5-HT levels were normalized to day 1 values. (b) Basal 5-HT release over 1 h as a function of MTT uptake measured 4 days after encapsulation. Significant stimulation was noted for cells encapsulated in hollow microcapsules compared to single cells or cells encapsulated in Ca^{2+}/Ba^{2+} microbeads. (c) Basal 5-HT release/microsphere over 1 h measured 4 days after encapsulation. Hollow microcapsules generated significantly more basal 5-HT release than cells encapsulated in Ca^{2+}/Ba^{2+} microbeads. *P < 0.005 versus non-encapsulated cells, #P < 0.003 versus hollow APA microcapsules. OD, optical density.

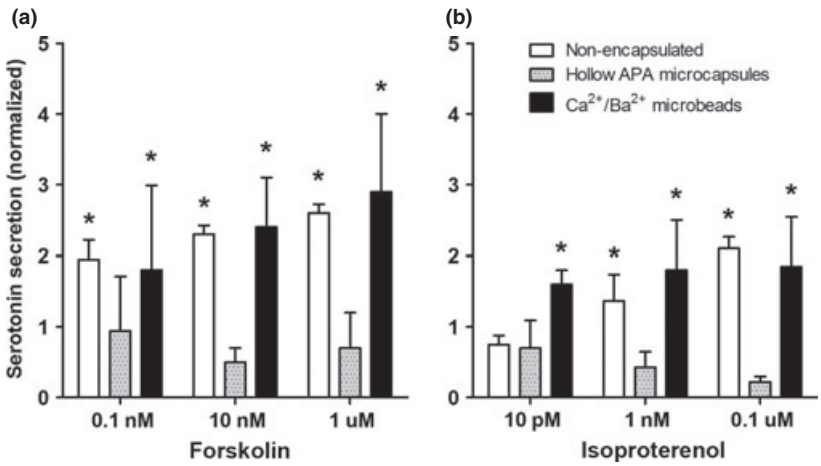

Fig. 5. Functional studies after addition of forskolin (PKA activator) or isoproterenol (β-adrenergenic receptor agonist) in non-encapsulated, hollow alginate-poly-L-lysine-alginate (APA) microcapsules or Ca^{2+}/Ba^{2+} microbeads. Values are normalized to control (unstimulated cells in each condition). (a) Forskolin stimulated both non-encapsulated and KRJ-I cells encapsulated in Ca^{2+}/Ba^{2+} beads but had no effect on hollow APA microcapsulated cells. (b) Isoproterenol dose-dependently stimulated 5-HT release from non-encapsulated and Ca^{2+}/Ba^{2+} microbeads but had no effect on hollow APA microcapsulated cells. *P < 0.05 versus non-stimulated cells. Data given as mean ± SD, N = 6 (capsules) and N = 4 (non-encapsulated cells).

of viability**.** Clearly, the Ca^{2+}/Ba^{2+} microbeads and the hollow microcapsules provide the most appropriate models for KRJ-I culture.

Overall, the present study demonstrates that functional 5-HT secreting bioreactors can be generated using the GI-NEN cell-line, KRJ-1. The functional properties of the KRJ-1 cells are clearly dependent on the environmental conditions provided by the alginate matrix. A high constitutive 5-HT secretion can be achieved using hollow microcapsules. High constitutive secretion of 5-HT is also a hallmark of EC cell-derived NEN.[31] Because of their low stability, the hollow microcapsules theoretically have the potential to establish tumor growth (immune-deficient animals) when they burst.[24] Bursting of hollow microcapsules with high cell load was registered during cell growth *in vitro*, and has also been found previously.[25] The disadvantage with the hollow microcapsule might be fibrosis in response to the polycation containing membrane.[43–45] However, this problem may be overcome by choosing an appropriate mouse model because variable responses occur due to different backgrounds. The advantage with the Ca^{2+}/Ba^{2+} microbeads is their higher biocompatibility related to lack of polycation.[10,44–46] Their low constitutive secretion of 5-HT coupled to normal responsiveness to forskolin and isoproterenol make this a useful model for testing physiological responses and may be applied to a range of 5-HT-associated diseases (e.g. Crohn's disease).[47] Irrespective of 5-HT regulatory control, growing tumor cells within a 3-D network might provide a relevant model for the evaluation of drug responses.[48] The use of different types of alginate microspheres has some potential for culturing tumor cells in 3-D with variability in matrix tension. Cancer growth may be regulated by the extracellular matrix,[49] while the angiogenic capability of cancer cells appears to be regulated by its 3-D growth.[50] Alginate microspheres technology might, therefore, have potential for future tumor studies both *in vitro* and *in vivo*.

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Disclosure statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. KRJ-1 cells during culturing.

Fig. S2. Encapsulation insufficiency in Ca^{2+} microbeads or alginate-poly-L-lysine-alginate microcapsules.

Fig. S3. Hollow APA microcapsules using intermediate G-alginate (*Macrocystis pyrifera*) and single cells (sc).

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