A novel multilayer immunoisolating encapsulation system overcoming protrusion of cells

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Application of alginate-microencapsulated therapeutic cells is a promising approach for diseases that require a local and constant supply of therapeutic molecules. However most conventional alginate microencapsulation systems are associated with low mechanical stability and protrusion of cells which is associated with higher surface roughness and limits their clinical application. Here we have developed a novel multilayer encapsulation system that prevents cells from protruding from capsules. The system was tested using a therapeutic protein with anti-tumor activity overexpressed in mammalian cells. The cell containing core of the multilayer capsule was formed by flexible alginate, creating a cell sustaining environment. Surrounded by a poly-L-lysine layer the flexible core was enveloped in a high-G alginate matrix that is less flexible and has higher mechanical stability, which does not support cell survival. The cells in the core of the multilayer capsule did not show growth impairment and protein production was normal for periods up to 70 days in vitro. The additional alginate layer also lowered the surface roughness compared to conventional cell containing alginate-PLL capsules. Our system provides a solution for two important, often overlooked phenomena in cell encapsulation: preventing cell protrusion and improving surface roughness.

Immunoisolation of cells for implantation purposes is based on enveloping the cells in a biocompatible and semipermeable membrane that allows diffusion of essential nutrients and therapeutic molecules but prevents deleterious effects of the humoral and cellular part of the host immune system 1,2. The technique is proposed for the delivery of cell based therapeutics where local and minute-to-minute release of the active molecule is desired for effective treatment. Encapsulation of living cells for the release of biological therapeutics is under investigation for a variety of diseases such as diabetes3, neurological diseases4-10, and cancer4-10. 

A commonly applied technology of immunoisolation is microencapsulation of therapeutic cells in spherical beads made of alginate polymers12 using different encapsulation techniques like electrostatic bead generator, jet cutter, vibrating nozzle, and coaxial air driven droplet generator12. Spherical beads are often preferred over discs or tube like structure because of the favorable surface-to-volume ratio which facilitates nutrition of the cells and the release of therapeutic agents11. Another argument to favor microcapsules is the relative small size that allows for transplantation in many sites without interference with the receiving organ11. The latter is an important consideration for example in the treatment of brain tumors, where implantation of microencapsulated cells producing anti-tumor agents has been proposed as an effective approach to delete remnants of malignant cells13,14. Invasive brain tumor cells are impossible to recognize and eliminate during surgery and are a major cause of morbidity.

Microcapsules that are being proposed for diseases such as for the treatment of brain tumors should meet a number of requirements. A key feature in designing immunoisolating microcapsules is avoiding protrusion of cells from capsules. Most conventional alginate encapsulation systems do not meet this prerequisite, as protrusion of cells is more the rule than an exception (figure 1). Protrusion of cells is associated with rejection and fibrotic responses followed by necrosis of the therapeutic cells, which ultimately leads to graft failure15. Another danger is that the therapeutic cells based on cell lines may form tumors themselves when they leak out of the capsules. This is a major safety hurdle preventing the application of cell encapsulation technology in the clinic. Therefore cell based therapy requires a system in which protrusion of cells is prevented.
Alginates and their capsules are used in the encapsulation of cells for therapeutic purposes. The chemical properties of the capsules, which affect cell function, are determined by the molecular weight of alginate and the position of G and M residues. Alginate is classified based on the distribution of these residues along the polymer chain. The aim of the study was to develop a novel multilayer capsule with improved mechanical stability and cell survival. The results showed that multilayer capsules prevent protrusion of cells and improve cell survival compared to conventional alginate capsules.

**Results**

To prevent cell protrusion from alginate capsules, we applied different types of alginate to produce gels with different mechanical stability. The 3.4% intermediate-G alginate beads gelled in 100 mM CaCl$_2$ solution had a rigidity of 9.47 ± 1.61 g and an elasticity of 7.99 ± 0.76 s. The 2% high-G alginate beads were more rigid (11.05 ± 0.77 g) and less elastic (9.12 ± 0.57 s) than 3.4% intermediate-G alginate beads. A too rigid environment would lead to deregulation of cellular metabolism and protein synthesis, eventually leading to cell death. The tests were performed with a mammalian cell line (BHK cells), as these cells are often applied in preclinical models for the release of therapeutic agents.

**Multilayer capsules secrete therapeutic proteins for prolong periods of time.** We quantified the secretion of therapeutic sLrig1 from 50 multilayer capsules, cultured in 96 well plates with 100 μl of culture medium. The culture supernatant was collected and replaced with fresh culture medium twice a week from day 14 till day 70. The secretion of sLrig1 was detected by Western blot and quantified as percentage of secretion of sLrig1 with respect to day 14 (figure 4c). The secretion of sLrig1 increased exponentially till day 42, where the highest secretion was observed. After day 42 the secretion was relatively stable with slight fluctuations in secretion of sLrig1 until day 70. As mentioned above traditional 3.4% intermediate-G alginate APA capsules showed protrusion and growth of cells in the culture flask, therefore the secretion of sLrig1 from the APA capsules cannot be reliably quantified. Therefore we restricted quantification of sLrig1 from multilayer capsules.

**Cell load and cell growth increases surface roughness of capsules.** Surface roughness of the capsules as a consequence of cell growth is correlated with host responses. We therefore questioned how protrusion of cells influences surface roughness. To this end we studied surface roughness of cell containing APA capsules and multilayer capsules; APA capsules without cells were used as control. Analysis was done on day 30 post encapsulation. As shown in figure 5, APA capsules with cells (Rq=20.84 ± 15.13) had a significant higher surface roughness compared to empty APA capsules (Rq=1.65 ± 0.35) (p < 0.05). Although there was a tendency that surface roughness of multilayer capsules with cells (Rq=11.53 ± 2.82) had lower surface roughness compared to APA capsules with cells, the difference was not statistically significant. There was also no significant difference between surface roughness of empty APA capsules and cell containing...
multilayer capsules. In conclusion we find that cell growth and protrusion increase the surface roughness of capsules, and this may be ameliorated in multilayer capsules.

Discussion

The fact that mechanical strength of alginate influences cell behavior is based on the principle of mechanotransduction. Mechanotransduction is a process by which mechanical forces acting on cells influence biochemical cell behaviour and viability. This principle of mechanotransduction was applied to design a multilayer capsule with a minimal risk of protrusion of therapeutic cells. The system does not involve the inclusion of toxic components or any other molecules that might interfere with the survival of the encapsulated cell or with the host tissue. It is simply based on applying a rigidity on the outside of the capsule that is not compatible with cell survival. To our best knowledge we are the first to demonstrate this principle in microencapsulation systems. Different cells require different circumstances for optimal survival. For every cell type it may be necessary to adapt the alginate with cell facilitating and inhibitory properties. Here we used calcium-alginate because the rigidity to kill BHK cells was already reached with this alginate-divalent cation combination. We have also tested the efficacy of multilayer capsules in preventing protrusion of human embryonic kidney cells (supplementary figure 1).

Similarly different cells may require different conditions for optimal survival in the capsule core. The advantage of the multilayer system is that the inner capsule is not in direct contact with the microenvironment in the host. This implies that within the multilayer system the balance between optimal biocompatibility in the host and optimal cell-survival environment is less strict. The multilayer system also may improve the surface roughness as shown in figure 5. Another issue that is overcome by the novel system is the reported variations in PLL binding and the associated host responses. PLL is often applied to the alginate beads in order to reduce the pore size. Immunoprotective systems should protect the encapsulated cells against high molecular weight effector molecules of the adaptive immune system such as immunoglobulins and complement factors. The PLL capsules usually have a permeability that allows for diffusion of molecules below 160 kDa. Binding of poly-aminoacids to alginate beads is not straightforward. Alginate should form a superhelical core around the PLL and the PLL itself should be forced into β-sheets. This requires an ion exchange process which if not correctly done leads to host responses against
Polyamino-acid encapsulation is less important for the initial cell load used for encapsulation and/or number of beads implanted can be increased to achieve higher efficacy. It might be argued that the rigidity of 11.05 g for the 2% high-G gel is also interfering with host-compatibility or biocompatibility by forming a too stiff layer for host tissues in the vicinity of the capsules. In this study however we applied only alginate-types and concentrations that have already been tested in vivo in different implantation sites including the brain and did not show any toxicity or severe response in the host. It seems that intracapsular rigidity is a different issue than surface rigidity, which previously has been reported to be a critical issue for biocompatibility.

The surface roughness of microcapsules also plays a crucial role in host responses. A high Rq is associated with enhanced adsorption of proteins and inflammatory cells. Although the Rq value of APA capsules with and without cells was not statistically significant from the multilayer capsules with cells, we postulate that providing an additional alginate layer may reduce the variations induced by cell inclusions. In the present study we show that loading the system with cells leads to increased surface roughness as cell free APA capsules had a lower surface roughness than cell containing APA capsules and multilayer capsules. This suggests that inclusion of cells affects the gellification process. Increasing the cell load, hinders the cross linking of gelling ion and as a consequence decreases mechanical stability. This further increases protrusion and surface roughness. As protrusion is a random phenomenon, protrusion or disruption of the surface structure can occur anywhere on the surface and in different degrees as shown by large standard deviations values in this study. We found that APA-capsules sometimes had Rq values varying in range between 3.34 nm and 40.3 nm. Previous AFM studies have been conducted on cell free capsules which did not report large variations on APA capsules. We suggest that the variation in surface in cell containing capsules observed in our study may contribute to the reported variations in host responses (biocompatibility) against APA-encapsulated cells. Cell load and surface roughness are interrelated and do matter for in vivo success of implanted capsules. Moreover it has been shown that the Rq of alginate beads increases with increasing affinity of the divalent cations for alginate.

Using divalent cations with a higher affinity for alginate the surface roughness of alginate capsules can be gradually increased. Therefore, to keep the surface roughness low on the multilayer system we recommend to use calcium ions as long as high-G alginate is applied.

**Conclusion**

A system in which protrusion of cells is prevented is mandatory for cell based therapies. In particular with encapsulated therapeutic cell lines, outgrowth of these cells from the capsules may lead to tumor formation. Here we report on a novel microencapsulation system that strongly reduces cell protrusion of cells. Different types of highly biocompatible alginates were applied to facilitate growth and survival of therapeutic cells in the core of the capsules while destroying cells escaping from the core of multilayer capsule. Studies are ongoing to determine the efficacy of this system in pre-clinical models in vivo.

**Methods**

Alginate purification. Alginates of different compositions were obtained from ISP Alginates Ltd UK. Two types of alginates have been purified: 1) intermediate-G alginate (containing 44% G-chain residues, 56% M-chain residues, 23% MG-chain residues, 21% MG-chain residues, 37% MM-chain residues) and 2) high-G alginate (containing 67% G-chain residues, 33% M-chain residues, 54% GG-chain residues, 13% MG-chain residues, 21% MM-chain residues). Alginates were purified in-house as described in detail elsewhere. Briefly 12–15 μm of crude alginate was dissolved in ice cold 1 mM Na-EGTA (ethylene glycol tetraacetic acid), under constant stirring. The dissolved alginate was filtered through 5 μm, 1.2 μm, 0.8 μm, 0.45 μm filter (Whatman®, Dassel, Germany), to remove visible aggregates. Subsequently the pH of the solution was carefully lowered to 2 with 2 N HCl to 20 mM NaCl on ice. Lowering of pH causes alginate to precipitate as alginic acid. Alginic acid precipitate was isolated by centrifugation.

| 3.4% intermediate-G alginate APA capsule | 2% high-G alginate beads |
|-----------------------------------------|------------------------|
| Day 1                                   |                        |
| Day 7                                   |                        |
| Day 14                                  |                        |
| Day 21                                  |                        |
| Day 28                                  |                        |

**Figure 3** | Mechanical stability of alginate influences cell growth. Phase contrast microscopic images of BHK-cell growth in 3.4% intermediate-G alginate-poly-L-lysine (APA) capsules, and 2% high-G alginate beads. APA capsules facilitate cell growth while 2% high-G alginate inhibited cell growth.
Figure 4 | Cell growth and survival in intermediate-G alginate APA capsules and in the inner core of multilayer capsules. (a) Confocal microscope images and quantification (n=3) of live-dead encapsulated BHK cells in APA capsules and multilayer capsule after live-dead staining at different time points. Live cells emit green fluorescence, dead cells emit red fluorescence. (b) Proof of concept of multilayer capsule: localization of live (green) and dead (red) BHK cells in a multilayer capsule. Protruding cells are effectively killed in multilayer capsules. (c) Western blot (n=3) analysis of secreted sLrig1 protein (upper band) from BHK-sLrig1 cells encapsulated in multilayer capsules. Multilayer capsules secrete sLrig1 for prolonged periods of time. Bovine serum albumin is visualized by nonspecific binding of secondary antibody (lower band).
was filtered through a Buchner funnel of pore size 1.5 mm and washed with 1 liter of 0.01N HCl to remove non precipitated contaminants. Next the proteins from the aggregate alginate acid were removed by chloroform: butanol (4:1 ratio) extraction. The mixture was vigorously shaken for 20 minutes. The suspended mixture was filtered over Buchner funnel. Chloroform: butanol extraction step was repeated twice. Subsequently the alginic acid was then brought into water by slowly and carefully increasing the pH to 7 with 0.5 N NaOH over a period of at least 1 hr. The alginic acid solution obtained was further treated with chloroform: butanol (4:1 ratio) to remove proteins which can only be dissolved in at neutral pH. The mixture was centrifuged for 3 minutes at 1800 rpm, which induced the formation of separate chloroform: butanol phase, which was removed by aspiration. This step was repeated one more time. The last step of purification is precipitation of alginate from alginic acid using ethanol. To each 100 ml of alginate solution we added 200 ml absolute ethanol. Constant stirring for 10 minutes in ethanol led to precipitate alginate. The alginate was filtered over the Buchner funnel and washed two times with absolute ethanol. Later, the alginate was washed three times with diethyl ether and was freeze-dried overnight. After purification 3.4% (w/v) intermediate-G alginate and 2% (w/v) high-G alginate were dissolved at 4°C in Krebs-Ringer-Hepes (KRH) with an appropriate osmolarity and further sterilized by 0.2 μm filtration.

Mechanical stability of empty beads. Empty beads of 3.4% intermediate-G alginate and 2% high-G alginate were made using air driven droplet generator using 23 g needle, gelled in 100 mM CaCl2 for 5 minutes as previously described by us. Mechanical stability of alginate capsule can be quantified using different methods like burst pressure, ultrasound, Young's modulus, however we prefer to calculate the mechanical stability based on force and time required to resist compression. The mechanical stability was quantified with a Texture Analyzer XT plus (Stable Micro Systems, Godalming, UK) equipped with a force transducer with a resolution of 1 mN as previously described by us. Texture Exponent software version 6.0 was used for analyzing the data. Briefly individual beads of size 500 μm micrometer were carefully sorted using a dissection microscope (Leica MZ75 microsystems) equipped with an ocular micrometer with an accuracy of 25 μm. Individual beads were carefully placed on plate, storage solution was carefully removed. The mechanical stability of beads was measured by compressing individual microcapsules to 60% using P/25L mobile probe with a pretest speed of 0.5 mm/sec, a test speed of 0.01 mm/sec, and a posttest speed of 2 mm/sec. The trigger force was set to 2 grams. The force exerted by the probe to compress the bead was recorded as function of time.

Cell microencapsulation. Baby hamster kidney (BHK) cells expressing anti-tumor protein sLrig1 (soluble leucine rich repeats and immunoglobulin like domain 1) a negative regulator of growth factor signaling were grown in DMEM medium supplemented with 4.5 g/L glucose, 10% (v/v) fetal bovine serum (complement deactivation), 1% (v/v) Antibiotic-Antimycotic (100X) (Sigma-Aldrich,). BHK control and BHK-sLrig1 expressing cells at concentrations of 6.3 x 10^6 cells per milliliter of sterile 3.4% intermediate-G alginate or 2% high-G alginate were carefully mixed and were transferred into droplets with an electrostatic bead generator, using a 27 g needle. The droplets were collected in 100 mM CaCl2 as gelling solution for 5 minutes. Subsequently, intermediate-G alginate beads were incubated with sterile (0.2 μm filtered) 0.05% poly-L-lysine (PLL) (poly-L-lysine-HCl, Mw 22 kDa, Sigma-Aldrich, The Netherlands) for a period of 3 minutes on ice and 4 minutes at room temperature. PLL coated capsules were subsequently incubated with 0.34% intermediate-G alginate in calcium-free KRH solution with an osmolarity of 310 to form alginate-poly-L-lysine alginate (APA) capsule. Next the cell-containing
intermediate-G alginate-PLL membrane was suspended in 2% high-G alginate beads and APA capsules had diameter of 250–350 μm while the final multilayer capsules had a diameter of 550–650 μm.

Viability and live-dead quantification of encapsulated cells. Viability of BHK-sLrig1 cells encapsulated in APA capsules and multilayer capsules was studied with a live-dead staining kit of Invitrogen (Calcium AM (4 mM), Ethidium homodimer-1 (2 mM)). Cell containing capsules were washed three times with KRH solution containing 2.5 mM CaCl2. Capsules were incubated in the dark for 30 minutes in 2 ml of serum free medium containing 1 μl of calcine AM and 2 μl of ethidium homodimer-1. Subsequently capsules were washed five times with KRH solution containing 2.5 mM CaCl2. Stained cells in capsules were visualized by a Leica TCS SP2 AOBIS confocal microscope and objective HC PL, APO CS 10/x0.3 dry, 0.11 mm. An Argon laser was used for excitation of calcine AM and the emission was filtered at 505 nm and 530 nm was measured for live cells. A Helium Neon 543 nm laser was used for excitation of ethidium homodimer-1 and the emission light over 650 nm detected for dead cells. The pictures of sections were taken through entire Z-axis through the capsules. Imaris (R) 64 version 7.6.4 software was used to process the raw data to construct 3D images and quantify live-dead cells. Viability and live-dead quantification of cells in capsules was determined on day 1, 7, 14, 21, 28, 35, 49, 56, 63, and 70.

Quantification and detection of sLrig1. To quantify the relative secretion of sLrig1 protein from multilayer capsules we carefully sorted 50 multilayer capsules using a dissection microscope (Leica MZ75 microsystems) The 50 individually sorted capsules were placed in 96 well plates and cultured in 100 μl of growth medium. Culture supernatants were collected twice a week between day 14 - day 70. Culture supernatants from day 21 to day 70 from encapsulated cells were used to construct 3D images and quantify live-dead cells. Viability and live-dead quantification of cells in capsules was determined on day 1, 7, 14, 21, 28, 35, 49, 56, 63, and 70.

Viability of cell encapsulated in APA capsules was determined by atomic force microscopy (AFM). APA capsules without cells were used as control. Surface roughness of empty APA capsules (n=4), APA capsules (n=4) and multilayer capsules (n=4) with cells was studied on day 30 post-encapsulation using a Bruker dimensions 3100 atomic force microscopy. Topographic imaging of capsules was performed at room temperature using the tapping mode. Briefly single capsules were carefully sorted using a dissection microscope (Leica MZ75 microsystems), washed with demineralized water and placed on microscopic slides. The excess of water was carefully aspirated. The surface of the microcapsules was scanned by the tip of a silicon nitride cantilever (Nanoprobes GmbH, Darmstadt, Germany), spring constant k was set to 0.05 N/m, which corresponds to an oscillating (z-oscillating) near the tapping frequency (x-y raster scanning) less than 1 Hz. Surface roughness (Rq) was evaluated by using the root mean square average of height deviations taken from the mean image data plane at a 5 μm scan. NanoScope V software was used to analyze the raw data of AFM.
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Author contributions
S.V.B., B.D.H. and P.D.V. designed the experiments. S.V.B. performed and analyzed the experiments with B.D.H. and P.D.V. S.B.V. and S.P.N. wrote the main manuscript. All authors reviewed the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Bhuhal, S.V., de Haan, B., Niclou, S.P. & de Vos, P. A novel multilayer immunoisolating encapsulation system overcoming protrusion of cells. Sci. Rep. 4, 6856; DOI:10.1038/srep06856 (2014).

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