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Factors influencing the mechanical stability of alginate beads applicable for immunoisolation of mammalian cells

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ABSTRACT

Transplantation of microencapsulated cells has been proposed as a cure for many types of endocrine disorders. Alginate-based microcapsules have been used in many of the feasibility studied addressing cure of the endocrine disorders, and different cancer types. Despite years of intensive research it is still not completely understood which factors have to be controlled and documented for achieving adequate mechanical stability. Here we studied the strength and elasticity of microcapsules of different composition with and without cell load. We compared strength (force) versus elasticity (time) required to compress individual microcapsule to 60% deformation. It is demonstrated that the alginate viscosity, the size of the beads, the alginate type, the gelling time, the storage solution and the cell load are dominant factors in determining the final strength of alginate-based microcapsules while the type of gelling ion, the polyamino acid incubation time, the type of polyamino acid and the culturing time determines the elasticity of the alginate-based microcapsules.

Our data underpin the essence of documenting the above mentioned factors in studies on encapsulated cells as mechanical stability is an essential factor in the success and failure of encapsulated grafts.

1. Introduction

Microencapsulation of cells is a commonly applied procedure to protect cells from the host immune system in the absence of immunosuppression. The technology of microencapsulation is proposed as therapeutic option for diseases where a minute-to-minute regulation of metabolic processes is required and where pharmaceutical intervention is not precise enough

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(Orive et al., 2003). Alginate is the most commonly applied molecule for the core of the microcapsules. Alginate is a linear binary polysaccharide with blocks of (1–4)-linked β-D-mannuronic (M) and α-G-guluronic (G) residues of widely varying composition and sequence (Andersen et al., 2012). Based on the G-content alginates are classified as high-G alginate, intermediate-G alginate, and low-G alginate. Usually the cells are entrapped in a gel of alginate that is crosslinked with divalent cations such as Ca^{2+}, Ba^{2+}, and Sr^{2+}, with uronic acid residues in alginate (Mørch et al., 2006). After crosslinking with cations the matrix is referred to as bead. When the surface is crosslinked with a polyamino acid the system is usually named a capsule (Bunger et al., 2003; Ponce et al., 2006; De Vos et al., 2012).

The mechanical stability of the microcapsules is an essential factor in the success and failure of encapsulated cells. This already starts before implantation. Beads or capsules should be strong enough to withstand the shear forces associated with the implantation procedure (Thanos et al., 2007). Also they should be able to withstand the forces and changes in the microenvironment when brought into transport fluid. In this fluid but also after implantation beads are exposed to all types of substances such as phosphate, sodium and potassium ions that might destabilize the capsules (Mørch, 2008; De Vos et al., 2009). Also, the capsules may undergo serious damage by shear forces they are exposed at the transplantation site (Thanos et al., 2007). In spite of this knowledge quantification and documentation of the mechanical stability of capsules has gained not more than minor attention in publications in the field (Zhao and Zhang, 2004; Zhang et al., 1999).

The mechanical stability of capsules is determined by the alginate type, the alginate concentration, and the type of applied gelling cation (Mørch et al., 2006). For example, alginate with a high guluronic acid content has a higher affinity for cations than alginates with a high mannuronic acid content. In many applications the mechanical stability is reinforced by applying a polycation layer around the alginate core (Thu et al., 1996b, 1996a). Commonly applied examples are poly-L-lysine (PLL) (Thu et al., 1996a; De Vos et al., 2002), poly-D-lysine (PDL) (Strand et al., 2002), poly-L-arginine (PLA), and poly-L-ornithine (PLO) (Leung et al., 2008; Darrabie et al., 2005). These polyamino acids form stabilizing membranes on the surface and simultaneously decrease the pore size of the alginate beads which is mandatory for providing immunoprotection. Also factors such as the cell load and culture conditions can influence the mechanical stability of beads or capsules (Shoichet et al., 1996; Hunt et al., 2010; Rokstad et al., 2002). Surprisingly this has not been studied up to now in a systematic fashion.

The present study was undertaken to investigate and document the effect of commonly applied variations in the encapsulation procedures on the mechanical stability of capsules. To this end we defined two parameters we wished to distinguish. This is (i) the strength and (ii) the elasticity of beads or capsules. The strength (i) is measured by quantifying the force required to compress the bead or capsule. The elasticity (ii) is assessed by measuring the time required to compress the bead or capsules to a predefined value. Combined these values determine the success of beads or capsules in vivo.

2. Materials and methods

2.1. Alginates purification procedure

Crude alginates containing varying amounts of guluronic acid (G)-chains and of mannuronic acid (M)-chains-intermediate-G (44% G+56% M) (Keltone LV) and high-G (67% G+33% M), (Manugel) sodium alginates were obtained from ISP Alginates Ltd UK. The method of alginate purification has been described in detail elsewhere (De Vos et al., 1997). After purification both intermediate-G and high-G alginates were dissolved in 220 mOsm Ca^{2+}-free Krebs–Ringer–Hepes (KRH) solution consisting of 90.0 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25.0 mM Hepes.

2.2. Polyamino acid

Poly-L-lysine hydrochloride (PLL) (product no. P2658), poly-D-lysine hydrobromide (PDL) (product no. P4408), poly-L-arginine hydrochloride (PLA) (product no. P7762), poly-L-ornithine hydrobromide (PLO) (product no. P0421) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A solution at 0.05% (w/v) of each polyamino acid solution was prepared in Ca^{2+}-free KRH 310 mOsm (135.0 mM NaCl, 4.7 mM KCl, 25.0 mM Hepes, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$).

2.3. Encapsulation procedure

A 4% viscosity alginate concentration was used as stock and further diluted to a desired concentration in Ca^{2+}-free KRH 310 mOsm/L. Beads were produced using an air driven droplet generator as previously described (De Vos et al., 1997) using a 23 g needle. We routinely apply in our lab 3.4% intermediate-G and 2% high-G alginate to produce beads. The reason is that with these concentrations a viscosity of 4 cps is reached, which is required for the formation of spherical beads (Klokk and Melvik, 2002). This is the upper limit at which 0.2 μm filtration for sterilization is still possible. To study the effect of the type of gelling ions we used 100 mM $CaCl_2$, 10 mM $BaCl_2$, 50 mM $SrCl_2$ as gelling solution. Beads were gelled for 5 min after the last drop of alginate extruded into the gelation bath. To study effects of the gelling time we used 100 mM $CaCl_2$ as gelling solution. Beads were incubated in the gelling solution for 5, 10, 15, and 20 min. All beads were washed with KRH buffer (132.0 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2 \cdot 6H_2O$, 25 mM Hepes, and 2.52 mM $CaCl_2 \cdot 2H_2O$) containing 2.5 mM/L $CaCl_2$ and stored in KRH solution (133.0 mM NaCl, 4.69 mM KCl, 25 mM Hepes, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, and 2.52 mM $CaCl_2 \cdot 2H_2O$) containing 2.5 mM/L $CaCl_2$ till further use. To study the effect of polyamino acid coating we used 100 mM $CaCl_2$ as gelling solution. Subsequently beads were gelled for 5 min, washed with KRH buffer containing 2.5 mM/L $CaCl_2$, and incubated with 0.05% of PLL, PDL, PLA, or PLO at room temperature for 5 min. To study the effect of polyamino acid coating time we used 100 mM $CaCl_2$ as gelling solution. Beads were gelled for 5 min, washed with KRH buffer containing 2.5 mM/L $CaCl_2$ and then incubated with PLL for 5 min and 10 min at room temperature. Non bounded polyamino acid was removed by washing with Ca^{2+}-free KRH

310 mOsm/L. Polyamino acid coated beads of intermediate-G alginate and high-G alginate were further immersed in 10 × diluted solution of 3.4% intermediate-G alginate and 2% high-G alginate respectively in Ca^{2+}-free KRH 310 mOsm/L, for 5 min to form alginate–polyamino acid–alginate capsule. All capsules were stored in KRH solution till further use. Images were taken with Leica DM IL inverted contrasting microscope with a S 90/0.23 condenser, free working distance of 90 mm and a numerical aperture of 0.23 (Leica microsystems, Wetzlar, Germany).

2.4. *Cell culture and encapsulation*

Human Embryonic Kidney (HEK) cells were grown to confluence in 75 cm^2 culture flasks containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum and 1% (v/v) of antibiotic–antimycotic (Invitrogen, product no. 15240096). When the cells were confluent they were harvested, counted, and bought to the desired concentration of 1 million, 5 million, and 10 million cells per milliliter of sterile (0.2 μm filtered) 3.4% intermediate-G alginate. Air driven droplet generator was used for encapsulation, using a 23 g needle and 100 mM $CaCl_2$ as gelling solution. A portion of the alginate beads containing cells were applied to manufacture alginate-poly-L-lysine-alginate (APA) capsules as described above. Both beads and capsules were washed with growth medium before culturing. The encapsulated cells were cultured in 25 cm^2 culture flasks containing 5 milliliter growth medium and kept in a standard tissue culture incubator at 37 °C, 100% humidity, 95% air, and 5% CO_2. Media was changed three times per week.

2.5. *Mechanical properties of beads*

The mechanical properties of beads and capsules were quantified with a Texture Analyzer XT plus (Stable Micro Systems, Godalming, UK) equipped with a force transducer with a resolution of 1 mN. Texture Exponent software version 6.0 was used for recording and analyzing the data. The equipment consisted of a mobile probe (P/25L) moving vertically at a constant velocity. The mechanical stability of beads/capsules was measured by compressing the individual bead/capsule ($n=10$). Individual beads/capsules were carefully inspected and sorted by using a dissection microscope (Leica MZ75 microsystems, Heerbrugg, Switzerland) equipped with an ocular micrometer with an accuracy of 25 μm. Individual bead/capsule was placed on a plate. Storage solution was carefully removed. Subsequently the probe was moved towards the capsule with a pretest speed of 0.5 mm/s, a test speed of 0.01 mm/s, and a posttest speed of 2 mm/s. The trigger force was set to 2 g. The uniaxial compression test was initiated; the probe triggered on the surface of the sample and the force (expressed in grams) was quantified at a compression of the sample to 60%. The force exerted by the probe to compress the bead/capsule was recorded as function of time (Fig. 1). A high-speed camera was used to capture the event during the test. The probe was set to return to the original position immediately after compression.

2.6. *Statistical analysis*

Anova and Tukey tests were carried out using *R* software package, version 3.0.0. All values were expressed as mean ± standard deviation (SD), differences were considered significant if $p<0.05$.

3. Results

3.1. *Definition of capsule stability*

Our analysis of the beads and capsules was performed in two fashions. We quantified the force to reduce the size of the bead and the capsule with 60%. A second measure was the

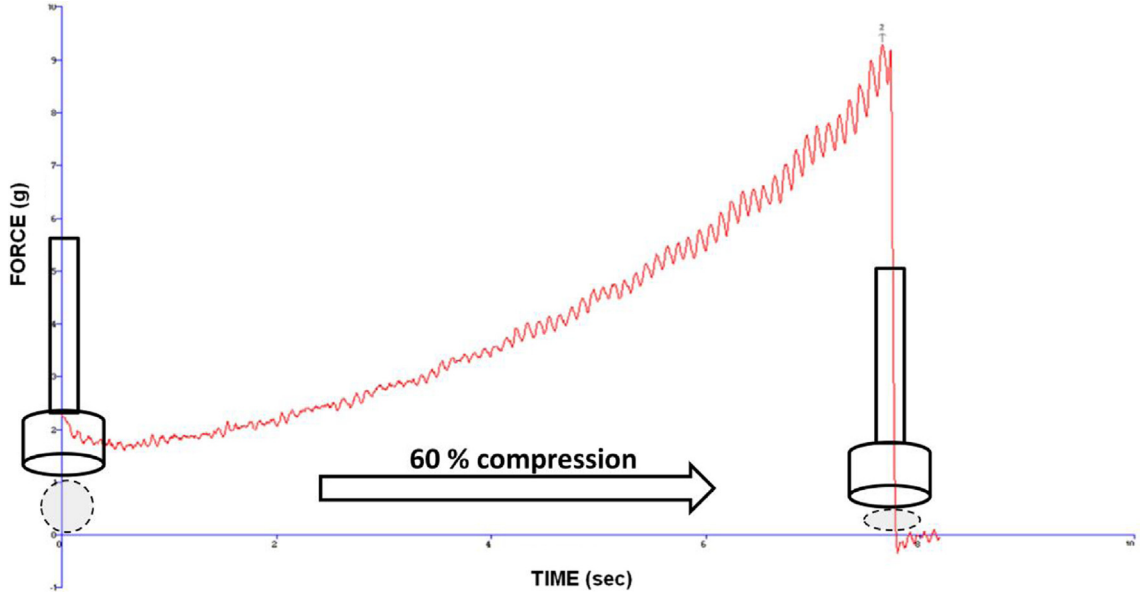

Fig. 1 – Schematic representation of work plan for measuring stability of alginate based microcapsules with Texture Analyzer XT plus using P/25L mobile probe.

time required to reach this value. The required force represents the strength, while time to reach the 60% compression is a value for the elasticity of the bead or capsule. Together these values determine the stability of the bead or capsule (Fig. 1).

3.2. Viscosity determines the shape and size of bead

Beads and capsules were only tested in a perfect spherical shape as this is the form defined as optimal for transplantation (King, 2001; Kizilel et al., 2005). Irregular shapes are associated with protrusion of cells which is undesirable when proposing cell encapsulation for immunoprotection (De Vos et al., 1996a). The viscosity of an alginate solution increases with higher concentrations of alginate. Intermediate-G alginates at low concentrations and with low viscosities are associated with irregular fragments of alginate of around 100 μm which are called satellites (Chan et al., 2009; Gautier et al., 2011). Also with low viscosities we observe many ruptured beads which lack immunoprotective properties. This is somewhat different when high-G alginates are applied instead of intermediate-G alginates. High-G alginates at high concentrations and with high viscosities are associated with undesired shapes such as with tail formation (Fig. 2). With application of higher viscosities and unchanged settings of the droplet generator we observed an increase in size. This increase was alginate type dependent and smaller with intermediate-G alginate than with high-G alginate. Satellite formation was mainly found with 2% intermediate-G alginate, and not with high-G alginates at the same concentration. Tailed capsules were only formed in 3.4 and 4% high-G alginate.

3.3. Size increases the stability of beads in an alginate type dependent fashion

To determine the effect of bead size on stability of beads we applied 3.4% intermediate-G alginate gelled for 5 min in 100 mM $CaCl_2$. As size increases stability increases. The force and time required to reach 60% compression increases are shown in Fig. 3. Both force and time required to compress beads increased significantly ($p<0.001$) and shows a linear relationship with size.

3.4. Increasing gelling time decreases the strength of beads

To study the effect of gelling time on the stability of beads we used 100 mM $CaCl_2$ as gelling solution. Beads of 3.4% intermediate-G alginate were incubated for 5, 10, 15, and 20 min in the gelling solution. Increasing the gelling time decreased the stability of 3.4% intermediate-G alginate beads (Fig. 4). Gelling times from 5 to 15 min did not have any significant impact on both force and time required for compression and thus did not influence the stability of the beads. Gelling time of more than 15 min decreased the required force to compress the beads ($p<0.001$), whereas the time required for compression did not have a significant impact on stability. A 15% decrease in required force and a 8% decrease in time was observed by increasing the gelling time from 5 to 20 min.

3.5. Stability of beads is dependent on type of gelling ion

Three types of commonly applied gelling solutions were tested and compared, i.e. 100 mM $CaCl_2$, 10 mM $BaCl_2$, and 50 mM $SrCl_2$. In case of intermediate-G alginate, alginate

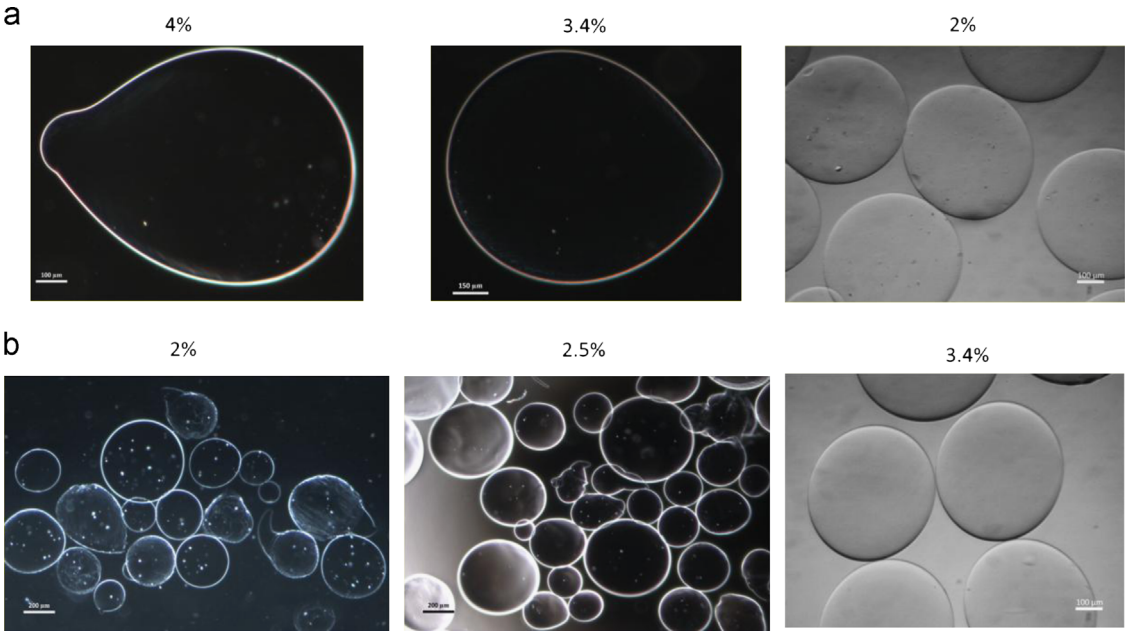

Fig. 2 – Alginate viscosity determines size and shape of beads. (a) High-G alginate at high-concentration and high-viscosity are associated with beads with tails. (b) Intermediate-G alginate at low concentration and with low viscosities are associated with satellite formation.

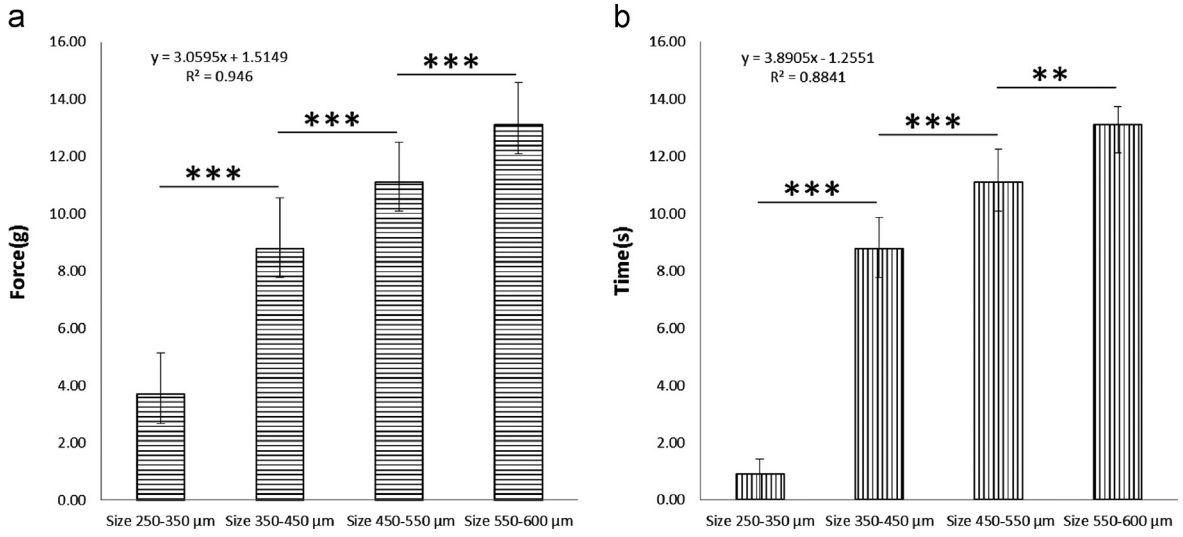

Fig. 3 – Alginate bead size determines the strength of the beads. Effect of size on stability (a) force, and (b) time required to compress intermediate-G alginate beads to 60% deformation. Time for gelling with 100 mM $CaCl_2$ was kept constant for 5 min. Both force and time required to compress beads ($n=15$) increases significantly ($p<0.001$) and shows a linear relationship. Values are expressed as mean ± SD, $^{***}p<0.001$, $^{**}p<0.01$, $n=15$.

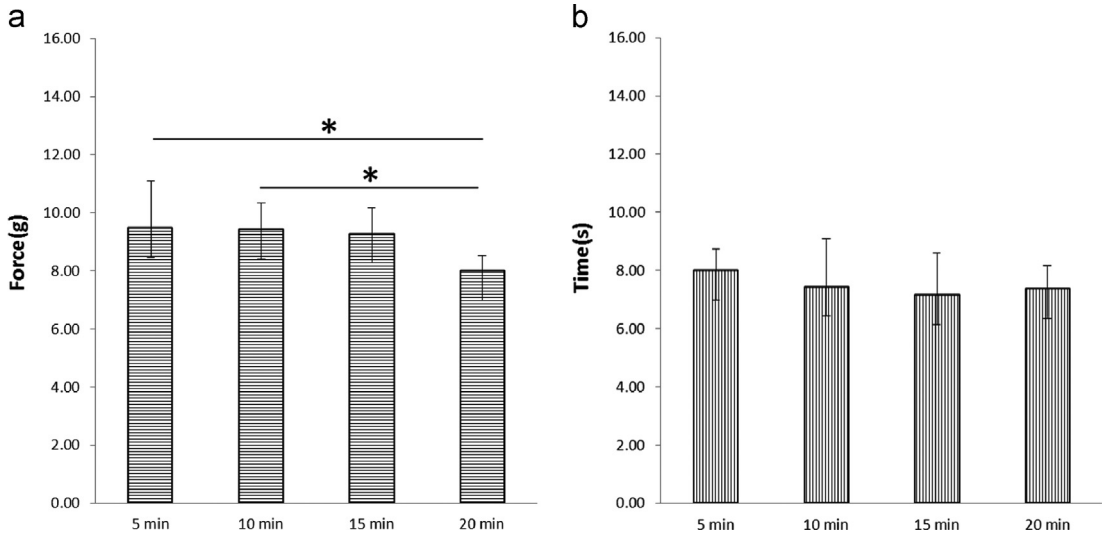

Fig. 4 – Prolong gelling time reduces strength of beads. Effect of prolonged gelling time with 100 mM $CaCl_2$ on 3.4% intermediate-G alginate. (a) Force and (b) time required to compress intermediate-G alginate beads to 60% deformation. Size of the beads was kept constant at 500 µm. Values are expressed as mean ± SD, $^{*}p<0.05$, $n=10$.

beads cross linked in either $CaCl_2$ or $BaCl_2$ required the same force for compression, whereas in case of 2% high-G alginate, $BaCl_2$ cross liked beads required less force than $CaCl_2$ cross linked beads. The $SrCl_2$ cross linked beads however were much weaker in both 2% high-G alginate and 3.4% intermediate-G alginate ($p<0.001$) (Fig. 5). However calcium-beads were more elastic as the time required to compress barium beads was lower than for calcium beads in both 2% high-G alginate and 3.4% intermediate-G alginate. Time required to compress strontium beads was significantly higher compared to calcium and barium beads in 2% high-G alginate. However there was no significant difference in time required to compress strontium beads and calcium beads in 3.4% intermediate-G alginate. Thus, barium ions decrease elasticity, while strontium ions increase the elasticity at the cost of strength.

3.6. *Stability of beads is dependent on alginate type*

Next we studied whether stability of beads is dependent on alginate type. To study the effect of the alginate type we compared the results with 2% high-G alginate gelled for 5 min in 100 mM $CaCl_2$, 10 mM $BaCl_2$, and 50 mM $SrCl_2$. The 2% high-G alginate beads were much more stable than 3.4% intermediate-G beads (Fig. 6). There was no significant difference in force required to compress 10 mM $BaCl_2$ beads of intermediate-G alginate and high-G alginate. But the time required to compress 10 mM $BaCl_2$ high-G alginate beads was

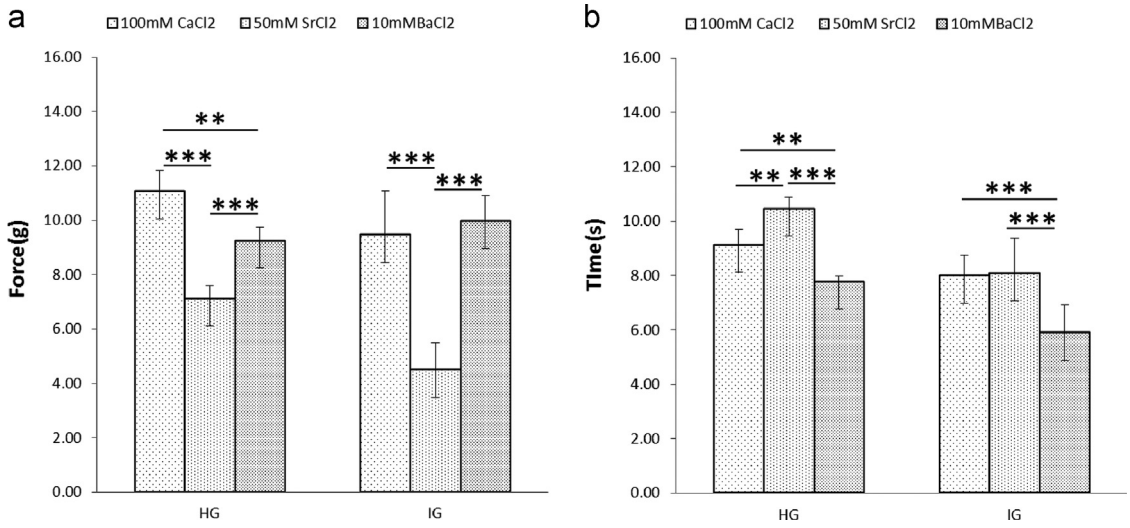

Fig. 5 – Stability of beads is dependent on the type of gelling ion applied. Effect of gelling solution 100 mM $CaCl_2$, 10 mM $BaCl_2$ and 50 mM $SrCl_2$ on 3.4% intermediate-G alginate (IG) and 2% high-G alginate (HG). (a) Force and (b) time required to compress intermediate-G alginate and high-G alginate beads to 60% deformation. Time for gelling and size of bead was kept constant for 5 min and 500 μm, respectively. Barium ions decreased elasticity, while strontium ions increased the elasticity at the cost of strength. Values are expressed as mean ± SD, $^{***}p<0.001$, $^{**}p<0.01$, $n=10$.

significantly higher than time required to compress 10 mM $BaCl_2$ intermediate-G alginate beads.

3.7. *Increasing the coating time with polyamino-acids makes the capsule less stable*

To test the effect of PLL coating time on the stability, we applied beads prepared of 3.4% intermediate-G alginate and 2% high-G alginate cross linked with 100 mM $CaCl_2$ for 5 min. Beads were incubated in PLL for 5 and 10 min. We avoided longer coating times, as this is associated in our hands with decrease in viability of cells.

PLL coating for both high-G alginate and intermediate-G alginate decreases the force required for compression ($p<0.001$) as shown in Fig. 7. In case of intermediate-G alginate, PLL coating for 10 min was associated with an increase in force required for compression, which was not statistically significant. Decrease in time required to compress PLL coated beads of both high-G and intermediate-G alginate was just a trend ($p<0.1$), illustrating that increasing coating time causes capsules to become less strong.

3.8. *Capsule stability is dependent on the type of polyamino acid applied for coating*

To study the effect of the type of polyamino acid coating on stability of capsules we applied poly-L-lysine (PLL), poly-D-lysine (PDL), poly-L-arginine (PLA), or Poly-L-ornithine hydrobromide (PLO). The effects of these polyamino acids were tested on beads prepared of 3.4% intermediate-G alginate cross linked in 100 mM $CaCl_2$ for 5 min. We always applied 0.05% (w/v) polyamino acid solutions.

Intermediate-G alginate, PLO and PLA coating were associated with a higher stability of capsules than coating with PLL or PDL (Fig. 8). PLA and PLO capsules required a higher force and a longer time ($p<0.001$) to compress capsules when compared to PLL. PDL capsules required a lower force ($p<0.05$) and lower time ($p<0.001$) to compress compared to PLA and PLO. The difference in stability between PLA and PLO and between PLL and PDL was just a trend ($p<0.1$).

3.9. *Storage solution affects stability of capsule*

Many different types of media are applied to store beads before transplantation or during transport (Lee et al., 2010). We questioned what is the effect of the storage solution on the stability of the beads. This was studied with 3.4% intermediate-G alginate. Both beads and APA capsules were investigated. We applied 5 min gelling in $CaCl_2$ and 5 min incubation with PLL (Vos et al., 1997). As storage solution we applied KRH solution containing 2.5 mM/L $CaCl_2$ and DMEM (i.e. tissue culture medium, containing 1.3 mM/L $CaCl_2$) for 1 day. As shown in Fig. 9, DMEM decreased the stability of both beads and APA capsules. The beads stored in DMEM showed a significant decrease in both required force ($p<0.001$) and time ($p<0.05$) for compression compared to beads stored in KRH. Thus beads become less stable in storage and DMEM culture medium. However, the type of storage medium has an influence on bead strength. This was different with capsules with a polyamino acid membrane. The loss in force required for compression of capsules stored in DMEM was only a trend ($p<0.1$) but the time required for this was lower ($p<0.05$) suggesting that the capsule became more elastic. This effect on elasticity was even more pronounced in capsules stored in DMEM, again illustrating an effect of the type of storage medium.

3.10. *Cell load decreases stability of capsules*

To study the effect of cell load on capsule stability we compared empty capsules with capsules containing 1, 5, or 10 million HEK cells per milliliter of alginate on day 1. Both

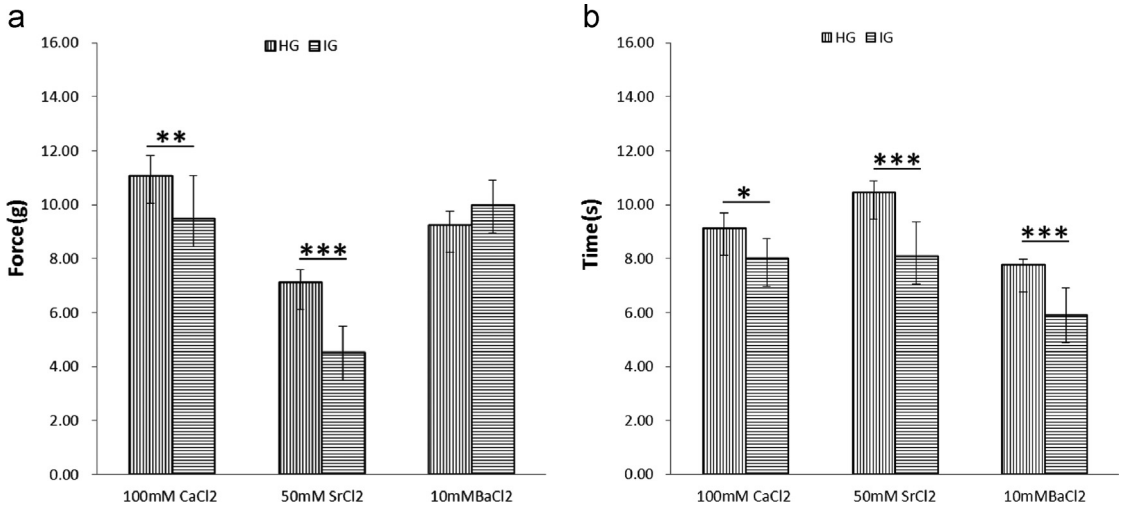

Fig. 6 – Stability of beads is dependent on alginate type and type of gelling ion. Effect of 3.4% intermediate-G alginate (IG) beads and 2% high-G alginate (HG) beads in different gelling solution 100 mM $CaCl_2$, 10 mM $BaCl_2$ and 50 mM $SrCl_2$. Time for gelling and size of bead was kept constant for 5 min and 500 μm, respectively. The 2% high-G alginate beads are more stable than 3.4% intermediate-G beads ($p<0.001$) irrespective of the applied gelling solution. Values are expressed as mean ± SD, $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, $n=10$.

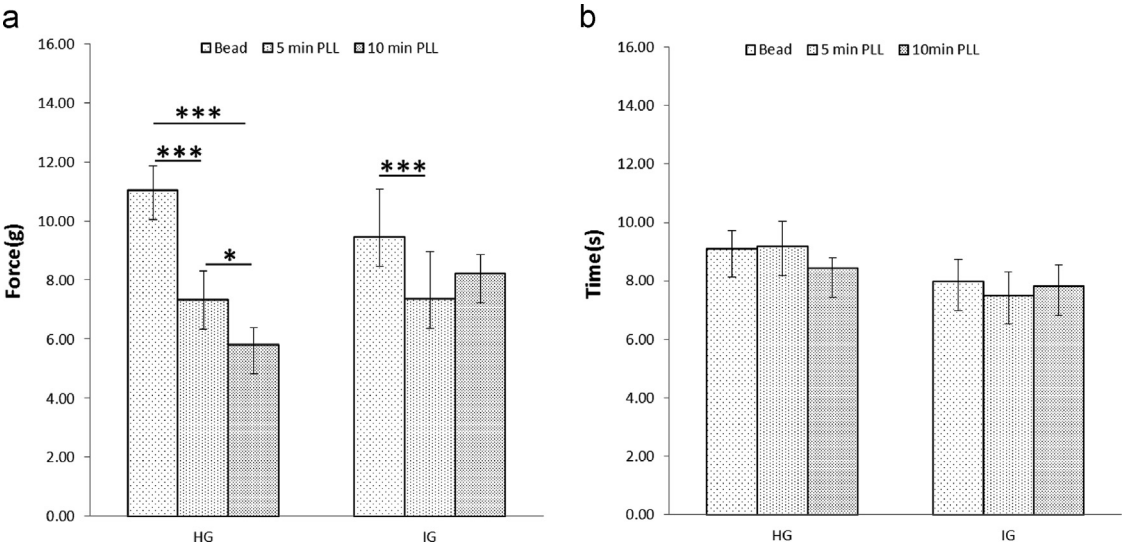

Fig. 7 – Increasing coating time decreases the stability of bead. Effect of poly-L-lysine (PLL) incubation time on 3.4% intermediate-G alginate (IG) and 2% high-G alginate (HG). (a) Force and (b) time required to compress intermediate-G alginate and high-G alginate microcapsules to 60% deformation. Gelling time was 5 min in 100 mM $CaCl_2$. PLL incubation time was 5 and 10 min. For analysis, size was kept constant at 500 μm. Values are expressed as mean ± SD, $^{***}p<0.001$, $^{*}p<0.05$, $n=10$.

beads and APA capsules were studied. As shown in Fig. 10, increasing the cell load decreases the stability of the beads and the APA capsules. Empty beads and APA capsules are more stable than cell-containing beads and APA capsules. On day 1, both force ($p<0.001$) and time ($p<0.05$) required to compress empty beads and APA capsules is higher than cell-containing beads and APA capsules. However, with the highest cell-load, i.e. 10 million HEK cells per milliliter of alginate, a statistical significant higher force was required to compress the beads and APA capsules than with a cell load with 1 and 5 million HEK cells per milliliter of alginate ($p<0.001$). The time required to compress the 10 million cell containing beads and APA capsules was significantly lower than with beads and APA capsules with 1 and 5 million HEK cells per milliliter of alginate ($p<0.001$). There was no significant difference in time required to compress empty APA capsules with APA capsules with 1 and 5 million HEK cells per milliliter of alginate ($p<0.1$).

3.11. *Cell growth increases stability of capsules*

Next we studied whether cell growth in the capsule had an effect on the stability of the capsules. To this end we compared the stability of the highest cell load, i.e. 10 million

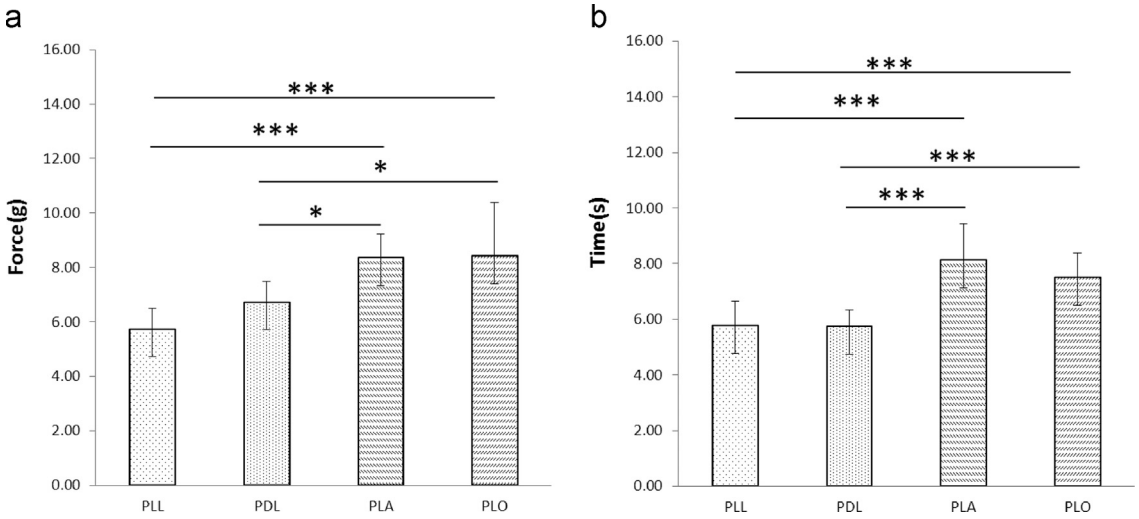

Fig. 8 – Capsule strength is dependent on the type of coating. Effect of poly-L-lysine hydrochloride (PLL), poly-D-lysine hydrobromide (PDL), poly-L-arginine hydrochloride (PLA), poly-L-ornithine hydrobromide (PLO) coating on 3.4% intermediate-G alginate. (a) Force and (b) time required to compress intermediate-G alginate microcapsules to 60% deformation. Time for gelling, was kept at 5 min gelling in 100 mM $CaCl_2$. PLL incubation time was 5 min. Values are expressed as mean ± SD, $^{***}p<0.001$, $^{*}p<0.05$, $n=10$.

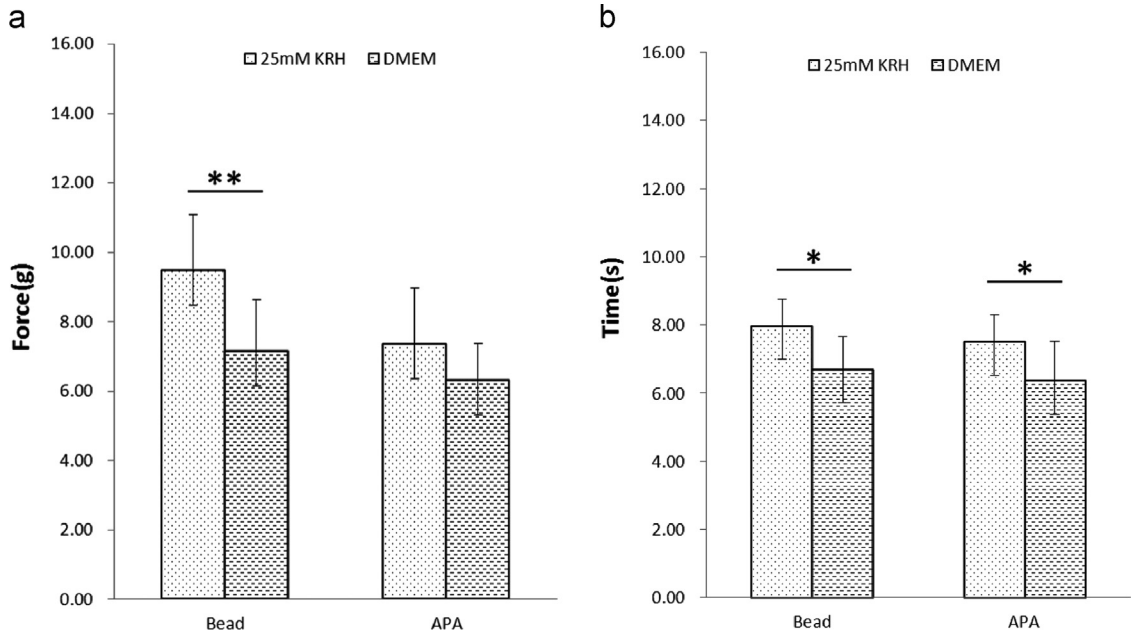

Fig. 9 – Storage solution type and storage in general has a negative impact on the strength of microcapsules. As storage solution we applied KRH solution containing 2.5 mM/L $CaCl_2$ and DMEM (i.e. tissue culture medium) for 1 day. (a) Force and (b) time required to compress intermediate-G alginate microcapsules to 60% deformation. Time for gelling, was kept at 5 min gelling in 100 mM $CaCl_2$. PLL incubation time was 5 min. DMEM decreases stability of microcapsules. Values are expressed as mean ± SD, $^{**}p<0.01$, $^{*}p<0.05$, $n=10$.

HEK cells per milliliter of alginate on day 1, 7, or 14. As shown in Fig. 11 both force and time required to compress beads and APA capsules increases with cell growth. There was no significant difference ($p<0.1$) in both force and time required to compress beads and APA capsules till day 7. On day 14 the force required for compressing beads and APA capsules increased by 8 and 11.8 fold when compared to beads and APA capsules on day 1 ($p<0.001$), respectively. Similarly the time required for compressing beads and APA capsules on day 14 increased by 1.24 and 1.62 fold with respect to beads and APA capsules day 1 ($p<0.001$), respectively.

4. Discussion

Our study shows that the size of the beads, the alginate type, the gelling time, the storage solution, are dominant factors in determining the final strength of alginate-based capsules

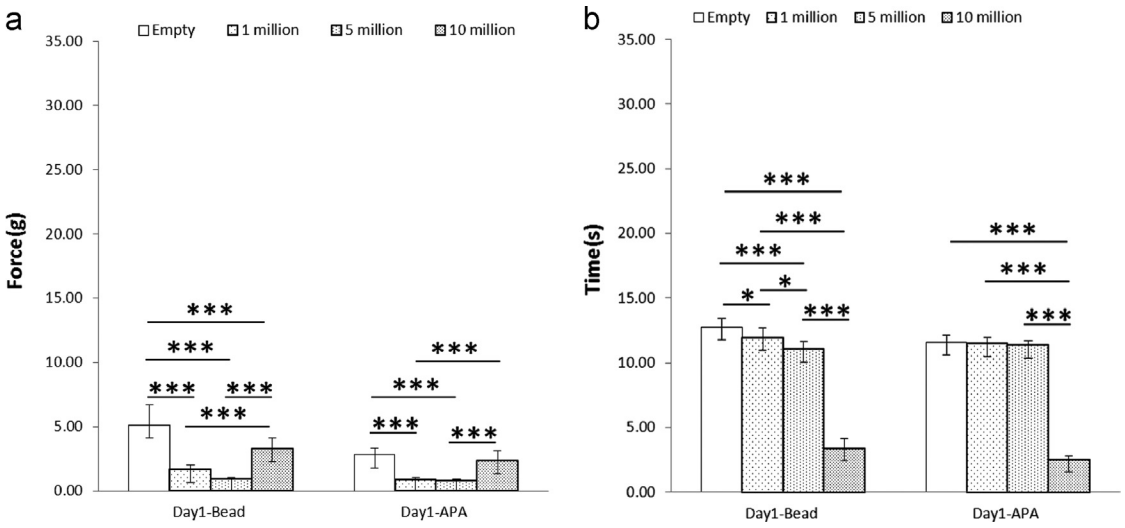

Fig. 10 – Effect of cell load and microcapsule type. (a) Force and (b) time required to compress intermediate-G alginate microcapsules to 60% deformation with and without cell load on day 1 of culture. Time for gelling, was kept at 5 min gelling in 100 mM $CaCl_2$. PLL incubation time was 5 min. Cell load decreases stability of microcapsule. Values are expressed as mean ± SD, $^{***}p<0.001$, $^{*}p<0.05$, $n=10$.

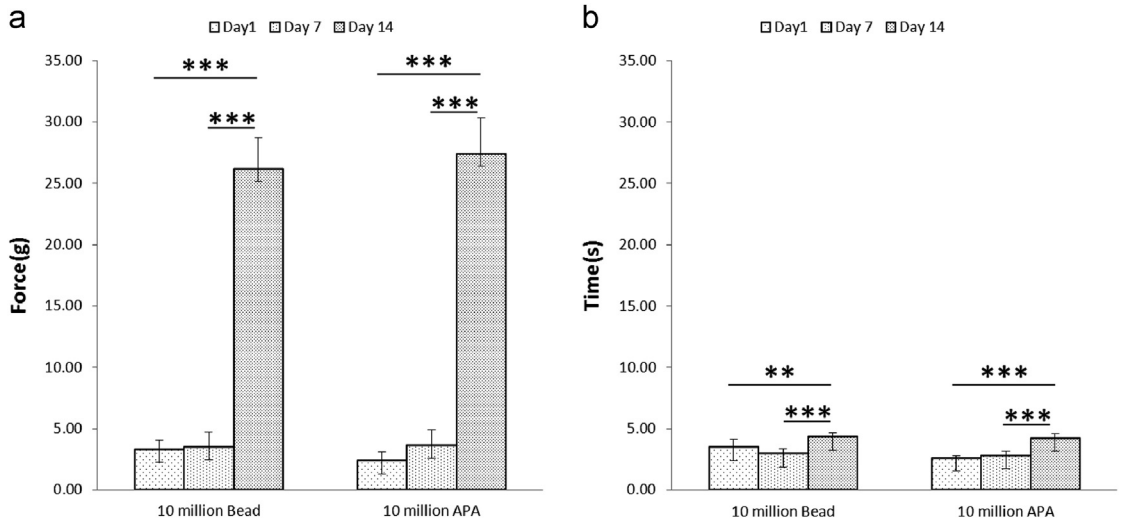

Fig. 11 – Effect of cell growth in microcapsule with 10 million HEK cells per milliliter of alginate. (a) Force and (b) time required to compress 3.4% intermediate-G alginate microcapsules to 60% deformation with 10 million HEK cells per milliliter of alginate on day 1, 7 and 14. Time for gelling, was kept at 5 min gelling in 100 mM $CaCl_2$. PLL incubation time was 5 min. Cell load and cell growth increases stability of microcapsule. Values are expressed as mean ± SD, $^{***}p<0.001$, $^{**}p<0.01$, $n=10$.

while the type of gelling ion, the polyamino acid incubation time, and the type of polyamino acid determines the elasticity of the alginate-based capsules. To our best knowledge there are no studies available in which all these factors have been adequately documented and measured. For in vivo application both strength and elasticity are essential factors for the functional survival of cells (Stabler et al., 2001) as will be outlined in the following sections.

The bead shape is an essential factor in functional survival of encapsulated cells (King, 2001; Kizilel et al., 2005). Broken beads or capsules with many satellites are associated with protrusion of cells (Lewińska et al., 2004) and inflammatory responses (De Vos et al., 1996a). Alginate beads should therefore be produced from alginate solutions with an optimal viscosity. The viscosity of the alginate is determined by the concentration as well as by the type of alginate (the ratio of mannuronic acid/guluronic acid content) and the average molecular weight of alginate. As illustrated in Fig. 2 this should all be in balance to produce beads with an optimal shape and geometry.

To guarantee an optimal spherical shape we apply for transplantation studies 2% high-G and 3.4% intermediate-G alginate. After droplet formation we usually end up with 100% spherical beads with these alginate concentrations. Satellite formation was especially observed with low concentrations and lower viscosity intermediate-G alginate

solutions, while tail formation was an issue with high-G alginate. The satellite formation is due to the fact that the coaxial air-flow of the system cuts the alginate droplet even before it reaches a critical volume to form a droplet (Chan et al., 2009). A different process is responsible for tail formation with high-G alginate from high viscosity solutions. With high viscosity solutions the coaxial air flow of the system requires high flow rates to cut the droplet. This leads to an increase in size of the bead. For these kinds of beads the conventional distance between the needle tip to the gelling bath is too short to form a perfect sphere with tailed beads as a consequence (Chan et al., 2009).

With increasing the size also the stability of the bead increases. The volume of a bead is a function of radius to the power of three, and therefore if the diameter of the bead is halved, the volume will be decreased to an eighth. Hence enhancing the size of the bead increases the overall force and time required for compression. As size increases the force and time required for 60% compression increases linearly (Fig. 3). This can be explained by the fact that if the size increases more divalent ions crosslink the alginate bead core (Thu et al., 1996b) increasing the overall stability of bead.

Our finding that increasing the gelling time decreases the strength of beads (Fig. 4), corroborate the findings of Vaithilingam et al. (2011) who studied and compared barium beads gelled for 2 min and for 20 min. These findings should be explained as follows. During gel formation uronic acid blocks in alginate binds to cations, like in an egg box model (Grant et al., 1973; Smidsrod, 1974). The constitutive uronic molecules in alginate create junction zones within the gel (Thu et al., 1996b). As the gelling time increases the number of junction zone in the gel will increase until saturation of the gel is reached. As binding of cation is a cooperative process also unzipping the junction zones is a cooperative process. When gelling time increases, more cations participate in the formation of junction zones, forming more and larger junction zones. Increasing the junction zones also increases the susceptibility for uncoupling in the junction zones. A junction carrying the highest stress, normally the shortest one in length fractures first and the energy released from the fractured junction is transferred to the neighboring chains, accelerating the uncoupling of their junctions (Zhang et al., 2007). If saturation has not been reached such as with 5 min gelling time, there will be less junction zones in the core of the bead than with longer gelling times. This implies that the 5 min gelled beads are more elastic than their counterparts gelled for longer times. This is the reason for significant decrease in force and not in time, as gelling time increases, suggesting that increasing the gelling time causes beads to become weak.

For gellification of alginate-droplets different types of gelling solutions are applied. Typically for formation of alginate-PLL capsules, alginate droplets are collected in 100 mM $CaCl_2$ (Vos et al., 1997; De Vos et al., 2002). For formation of barium-beads droplets are collected in 10 mM $BaCl_2$ (Tam et al., 2011) and in some recent studies $SrCl_2$ was used (Ludwig et al., 2012; Mørch et al., 2006). The stability of beads is dependent on the type of cation applied and the alginate type applied. This confirms the findings of Mørch et al. (2006) and Chan et al. (2011). This effect is caused by the chemical properties of the applied cations such as atomic number, ionic radius, ionic strength, association constant, and chemical affinity towards alginate (Chan et al., 2011). It has been reported that the minimum length of G–G blocks required for cross-linking decreases with increasing affinity of ions for the alginate chain (Stokke et al., 1991). The binding of ions is highly selective and the affinity strongly depends on the alginate composition and sequence (Smidsrod, 1974). More specifically, Ba^{2+} binds to G–G and M–M blocks, Ca^{2+} binds to G–G and M–G blocks, and Sr^{2+} binds to G–G blocks solely, not to M–G and M–M blocks (Mørch et al., 2006). This specificity and probability of binding pattern causes Ba^{2+} and Ca^{2+} crosslinked alginate beads to form two times more junction zones than Sr^{2+}. This can be explained by the fact that Ba^{2+} and Ca^{2+} ions have two options to bind during alginate gelation, while Sr^{2+} ions has only one option to bind, i.e. G–G blocks. Therefore the force to compress Sr^{2+} is smaller than with Ba^{2+} and Ca^{2+} beads (Fig. 5a), but the time required to compress Sr^{2+} beads was similar to Ca^{2+} beads in intermediate-G alginate, and higher in high-G alginate (Fig. 5b). This explains the elastic nature of Sr^{2+} beads and the brittle nature of Ba^{2+} beads.

The strength of the alginate gel is influencing the growth characteristics of encapsulated cells (Simpson et al., 2004). Too rigid structures are not compatible with growth and proliferation of cells (Stabler et al., 2001). The strength of alginate beads is considered to depend on the number of interactions with cations (Stokke et al., 1991). The number of bindings depends on the alginate composition (Stokke et al., 1993a, 1993b, 1991). G–G blocks have a higher affinity for divalent cations than M–M blocks. As a consequence alginates with a high G–G block content bind cations more efficiently and stronger. This is caused by the fact that cations are bound in the G–G polymers in an egg-box model (Smidsrod, 1974). In this egg-box sequential polymers of G–G blocks will be close to each other after binding of the first cations which makes the binding of the other molecules more efficient and faster. This is called an autocooperative process (Stokke et al., 1991; Smidsrod, 1974). This occurs less in alginates with higher amounts of M–M block or M–G block. Therefore high-G alginate binds more cations in the same amount of gelling time than intermediate-G alginate (Smidsrod, 1974). This causes the high-G alginate to form a dense network of cation-alginate, which hinders its compression and significantly increases the force and time required for compression, thus improving stability (Fig. 6). Also the degree of binding is different. Alginate contains glycosidic linkage in four forms: the diequatorial (M–M), the diaxial (G–G), the equatorial–axial (M–G), and the axial equatorial (G–M) conformation. The diaxial linkage in G–G blocks results in a large hindered rotation around the glycosidic linkage which may account for the stiff, less flexible and extended nature of this alginate chain. M–G blocks contain equatorial–axial and axial–equatorial linkages, but with different degrees of freedom of the two residues which gives greater overall flexibility (Smidsrød et al., 1973; Stokke et al., 1993a, 1993b). This structural configuration also contributes to increased stability of high-G alginate networks and explains our results on the far higher stability of high-G beads and capsules when compared to intermediate-G beads.

The polyamino acids poly-L-lysine hydrochloride, poly-D-lysine hydrobromide, poly-L-arginine hydrochloride and poly-L-ornithine hydrobromide have all been applied by

researchers to modulate the permeability of alginate beads to provide immunoprotection (Ponce et al., 2006; De Vos et al., 2002; Kulseng et al., 1997; Strand et al., 2002; Darrabie et al., 2006; Leung et al., 2008). The effect of these polyamino acids on stability of capsules have not been compared and documented up to now. Increasing the polyamino acid coating time decreases the stability of the capsules. Alginate beads are often coated with polyamino acid such as poly-L-lysine (PLL) to reduce the porosity of the alginate network and to provide immune protection as well as to provide mechanical stability (Orive et al., 2006; De Vos et al., 2002). However, increasing the poly-L-lysine coating time decreases the stability of capsules (Fig. 7). This seems to contradict the findings of Gugerli et al. (2002). This discrepancy should be explained by differences in swelling and shrinkage behavior during polyamino acid coating (De Vos et al., 1996b).

During poly-L-lysine coating for 5 or 10 min, capsules are incubated in KRH buffer containing 2.5 mM/L $CaCl_2$ inducing partial displacement of Ca^{2+} by Na^{+}. This is an essential step in allowing binding of the PLL (De Vos et al., 2012; Vos et al., 1997). Only if this step is undertaken the PLL is forced in superhelical cores with alginate and in beta-sheets (Van Hoogmoed et al., 2003). This is required to avoid inflammatory responses against the capsules (De Vos et al., 2007). A less desirable consequence of this displacement of Ca^{2+} by Na^{+} is that the bead increase in size and becomes more fragile. Increasing the coating time causes a dysbalance in loss of gelling ions from the inner core of capsule and the shrinkage induced by the PLL. The net result is a loss in strength. However, the thickness of poly-L-lysine layer increases with increasing exposure time (Strand et al., 2003; Thu et al., 1996a) making the capsules more elastic.

The type of polyamino acid applied for coating determines the stability of the capsules. The strength of interaction and the stability of the alginate–polyamino acid complexes strongly depend on molecular parameters of both the alginate and the polyamino acid such as chemical composition, sequential structure, conformations, and molecular size. We analyzed four different polyamino acids—poly-L-lysine (PLL), poly-D-lysine (PDL), poly-L-arginine (PLA), and poly-L-ornithine hydrobromide (PLO) (Fig. 8). Polyamino acid binds to alginate via electrostatic interaction between side chain NH_3 functional group of polyamino acid and carboxyl group of alginate. Arginine (Molecular Formula: $C_6H_{14}N_4O_2$) has two extra N atoms than lysine (Molecular Formula: $C_6H_{14}N_2O_2$) and ornithine (Molecular Formula: $C_5H_{12}N_2O_2$). Due to presence of two more N atoms in arginine, it binds more firmly to alginate. This increases the overall force and time required to compress the capsule. Ornithine on other hand has one C atom less than both lysine and arginine, which decreases its atomic size. This might facilitate binding of more ornithine than lysine and arginine (Thu et al., 1996a). The additional interaction/binding of arginine and ornithine increases the overall stability with respect to lysine isomers. Both PLL and PDL capsules had lower values in stability compared to PLA and PLO capsules. But PDL capsules required more force to compress capsules compared to PLL capsules. This may be due to stereo configuration of L and D isomer of lysine, which may form a different pattern of electrostatic interaction with alginate (Strand et al., 2002; Kulseng et al., 1997).

The type of storage solution may decrease the stability of microcapsules. In our lab we store empty beads and microcapsules in KRH buffer containing 2.5 mM calcium and beads with cells are stored in physiological medium such as DMEM (Shen et al., 2009). To our best knowledge, the role of storage solution has never been studied in relation to stability. DMEM media, a conventionally applied storage solution, decreases the stability of microcapsules (Fig. 9). Under physiological conditions microcapsules are exposed to a combination of destabilizing forces comprising of osmotic swelling of the core, slow dissociation of the alginate polycation complex, and shear forces (Mørch, 2008). The decrease in stability of microcapsules stored in DMEM is due to presence of calcium chelators such as phosphate, monovalent ions such as Na^{+}, and non-cross linking divalent ions like Mg^{2+} that are present in DMEM (Mørch, 2008; De Vos et al., 2009) and not in KRH solution containing 2.5 mM/L $CaCl_2$. Our data illustrate the importance of selecting storage and transport media that preserve the functional survival of the cells in combination with stability of the capsules. Beads and APA capsules should always be stored in medium containing 2.5 mM/L $CaCl_2$.

Cells in the capsules decrease the stability of beads and microcapsules in a cell-load dependent fashion (Fig. 10). This can be explained by destabilization of the calcium-alginate junction zones by the cells. However this destabilizing effect was not observed with the 10 million cell-load. This can be explained by the stabilizing effect of the mechanical properties of the cells that at a certain threshold dominates the strength of the calcium alginate network. This same argumentation explains that cell growth in the capsules is associated with an increase in the stability (Fig. 11). This latter argumentation should receive some further argumentation as it implies that for fast proliferating cells like HEK cells a very strong matrix should be applied to avoid that the cell-mass dominates the mechanical properties of alginate matrix.

5. Conclusion

By applying a quantification of both strength (force) and elasticity (time) a novel manner is introduced to quantify mechanical stability of beads and microcapsules. Stability is considered to be an important parameter that requires documentation (Ponce et al., 2006) when addressing efficacy of encapsulated cells. Our study illustrates that many factors influence the stability of capsules and underpins the essence of documenting this critical parameter. It is demonstrated that the size of the beads, the alginate type, the gelling time, the storage solution, are dominant factors in determining the final strength of alginate-based capsules while the type of gelling ion, the polyamino acid incubation time, and the type of polyamino acid determine the elasticity of the alginate-based capsules.

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