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Cell-compatible covalent alginate beads obtained from a chemoenzymatically engineered alginate

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Introduction

Alginate capsules containing cells producing proteins with therapeutic potentials may be one way for treatment of diseases like diabetes or brain tumors. However, several issues need to be addressed to meet the demands for successful design of a microcapsule for cell therapy, including the long-term stability. Here we present a chemoenzymatic strategy resulting in covalently linked alginate beads with high stability, and compatible with cells having a low post-encapsulating proliferative ability.

Materials and methods

Mannuronan (alginate with 100% mannuronic acid (M)) was grafted with methacrylate moieties. This was followed by two enzymatic steps converting M to guluronic acid (G). First, the use of AlgE4 introduced alternating sequences (MG-blocks) and then the use of AlgE6 resulted in long stretches of G (G-blocks) as demonstrated in figure 1 and measured by NMR. Gel beads were made using an electrostatic bead generator and a gelling solution of 50mM $CaCl_2$. The beads were exposed to white light in combination with TEA (triethanol amine), EY (eosin Y) and VP (1-vinyl-pyrrilidinone) that initiated the polymerization of the methacrylate moieties and thus resulted in covalently linked beads with diameter of approximately 456±5 µm. The beads were named CEPC (chemoenzymatic photocrosslinked). The new chemoenzymatic alginate and beads were compared to native alginate and native alginate grafted with methacrylate moieties using Young`s modulus and saline stability assay. The stability of the cross-linked beads was tested using saline stability assay and EDTA treatment. The compatibility with cells was

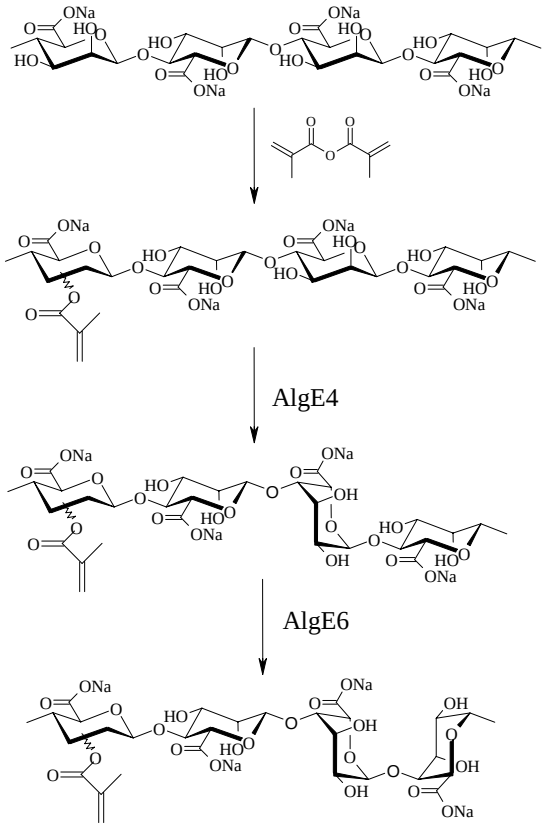


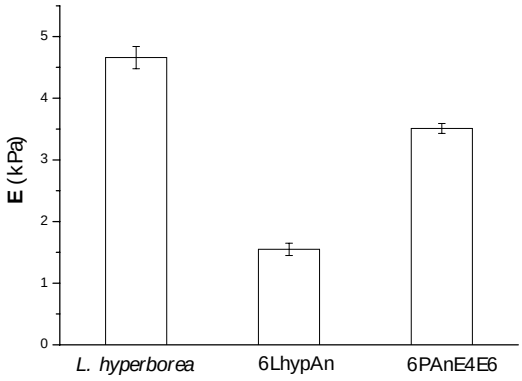
Figure 1. Chemoenzymatic approach for the production of alginate selectively modified on M residues.

tested using human 293 endo cells, mouse C2C12 myoblasts and human pancreatic islets. The assays to evaluate the growth/viability of the cells were MTT assay, live/dead viability staining using confocal imaging and insulin secretory response to glucose. The stability after cell encapsulation was evaluated with the saline stability assay. A complementary description of materials and methods are found in Rokstad *et al*. (2006). The chemoenzymatic approach was based upon a similar approach coupling galactose to alginate described in Donati *et al*. (2005).

Results

Stability data showed that alginate beads made of the chemoenzymatic modified alginate regained stability compared with native alginate substituted with methacrylate (Figure 2). The chemoenzymatic approach thus made it possible to make beads of the alginate after methacrylate grafting. After cross-linking for 1 and 5 minutes the stability of the beads was evaluated by the saline assay by measurement of diameter increase. After cross-linking for 5 minutes there was no change in diameter with 12 saline changes (Figure 3a). Cross-linked beads were stabile after addition of EDTA, while uncrosslinked beads were dissolved (Figure 3b).

a)



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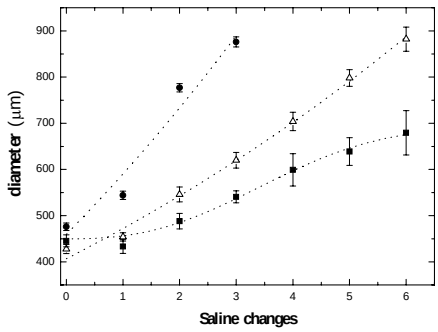
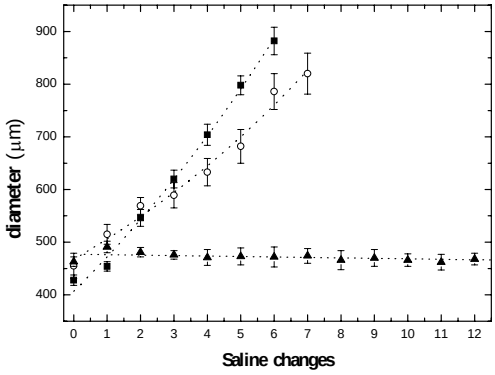


Figure 2. Comparison of stability between native alginate (■, L.hyperborea), native alginate grafted with methacrylate (●, 6LhypAn) and chemoenzymatic modified alginate (Δ, 6PAnE4E6) measured as Young`s modulus (a) or diameter increase in saline assay (b).

a)



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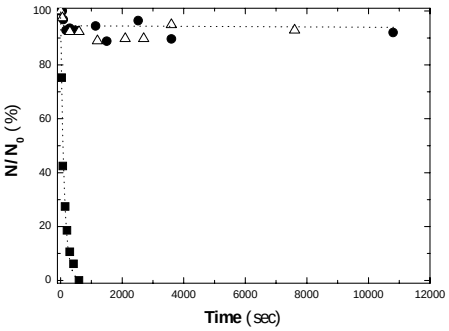

Figure 3. Stability of cross-linked beads measured as diameter change in saline (a) (■) uncrosslinked, crosslinked 1 (○) and 5 (▲) minutes. (b) Stability after EDTA treatment . (■) uncrosslinked, crosslinked (●, solution 1) and (Δ, solution III) .

The cross-linking conditions (including TEA, EY and PV solutions and white light) were compatible with cell-survival after preliminary adjustments of the concentrations. Both encapsulated 293-endo cells, C2C12 cells and pancreatic islets survived the encapsulation conditions (Figure 4 and 5). However, the 293-endo cells died between day 6 and 13 post-encapsulation, while C2C12 myoblasts and islets stayed alive for the measured period of 119 and 33 days, respectively. Measurement of the insulin response ratio showed that the encapsulated islets produced insulin (Figure 5b) and staining with dithizone showed that approximately 83% of the encapsulated islets were positive 33 days post encapsulation.

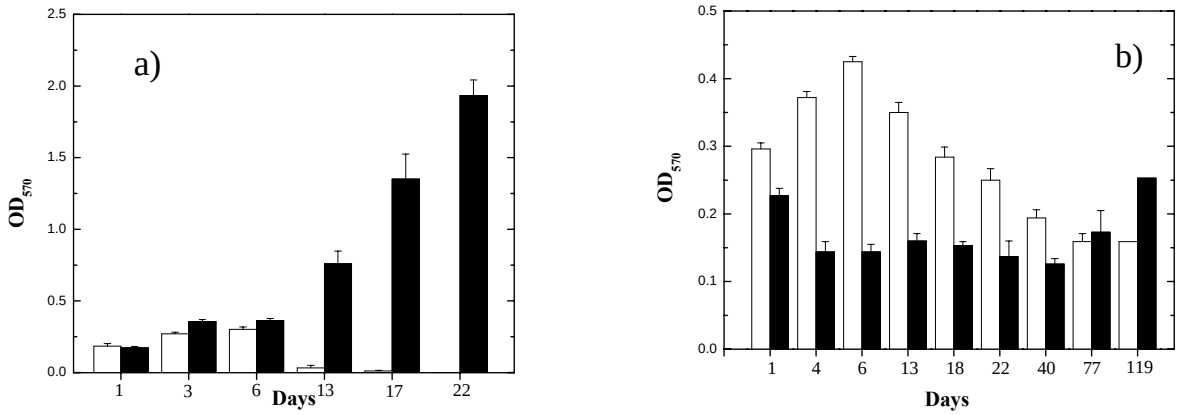

Figure 4. Survival and growth of 293-endo cells (a) and C2C12 myoblasts (b) after encapsulation in CEPC beads (white bars) and compared to encapsulation in Ba Beads measured by the MTT assay.

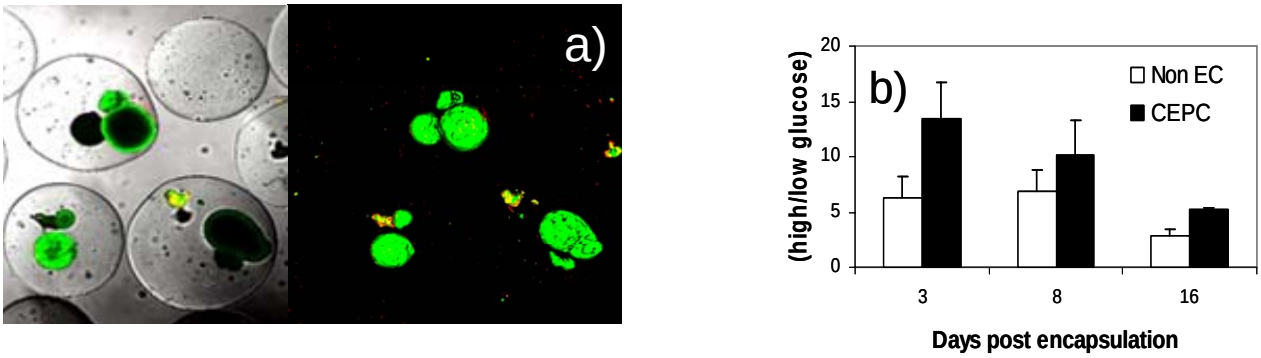

Figure 5. CEPC beads containing human pancreatic islets of Langerhans as shown by confocal imaging using live/dead staining (a). Left picture is cross-section through the equator of the bead, while the right picture is a 3D construction made from several cross-sections. (b) Islets produced insulin after encapsulation in CEPC beads (black bars) in higher amounts than free islets (white bars).

Relative swelling data (bead diameter compared with the diameter day 1 post-encapsulation) from CEPC beads containing encapsulated cells showed only slight increase in diameter with time (1,015 for 293-endo cells 22 days after encapsulation and 1,046 for C2C12 myoblasts 83 days post-encapsulation). In contrast, the relative swelling of the Ba beads were high (1,250 for beads containing 293-endo cells at day 22 and 1,304 for beads containing C2C12 myoblasts at day 83).

Discussion

Here we present a new strategy of obtaining alginate beads with a notable achievement in stability due to the introduction of covalent links. This was obtained by a chemical coupling of methacrylate moieties to mannuronan followed by two epimerization steps that introduced both strictly alternating MG sequences and G blocks. This strategy leads to a selective modification of M residues, in contrast to the native alginate that are randomly substituted on both M and G residues. We demonstrated a notable regain in physico chemical properties compared to the natural sample. This allowed for the encapsulation of live cells and tissue within alginate beads that next could be covalently cross-linked. By modifying the cross-linking conditions, we succeeded in finding conditions compatible with cell-survival and at the same time preserving the bead stability. Since the stability issues have been one of the main obstacles for using alginate beads in cell therapy, the achievement of cell survival within extremely stabile alginate beads is highly encouraging and represents an advance in cell microencapsulation. However, the long-term survival of the encapsulated cells was affected by the cell properties. Our findings demonstrated that the proliferative ability of the cells post-encapsulation provided the outcome of cell-survival over time. The 293-endo cells are highly proliferative post encapsulation as demonstrated in this study and in two other studies (Rokstad *et al*. 2002, Rokstad *et al*., 2003). The rigidity of the CEPC beads did not allow these cells to proliferate more than to small spheroids of cells, and probably induced the death of 293 endo cells within the CEPC beads. In contrast, C2C12 myoblasts are reported to have a low post-encapsulation dividing ability (Peirone *et al*., 1998) and this cell-line were surviving up till the end of the study at day 119. Also human pancreatic islets which have a low proliferation survived for prolonged time within the CEPC beads. The choice of cells within the beads seems therefore to be an important parameter of succeeding. The high stability of the CEPC beads makes the PLL coating redundant according to stability. This is also a substantially achievement as PLL is highly involved in the inflammatory reactions against alginate capsules (Strand *et al*., 2001). However, the PLL also reduces the porosity of the beads. At the moment we have no data on porosity of the CEPC beads. Work is in progress to get porosity data of the CEPC beads as well as evaluation of inflammatory reactions against these new beads. Further studies on the long-term survival of C2C12 myoblasts and islets will also be performed.

Conclusion

The new CEPC beads may be interesting for future applications using encapsulated cells producing therapeutic proteins in therapy. Further studies on permeability, immunocompatibilty and long-time survival of cells will reveal their potential.

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