

Improvement of the insulin secretion from beta cells encapsulated in alginate/poly-L-histidine/alginate microbeads by platelet-rich plasma

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Abstract: Type 1 diabetes is clinically characterized as the loss of control of glucose homeostasis due to the reduced number of insulin-producing cells. Long-term glycemic control after implantation could be maintained by preserving the cell viability and tissue-specific functions during the process of microencapsulation. In this study, alginate solution was supplemented with platelet-rich plasma (PRP) to improve the viability and preserve the cell functions during the encapsulation of a beta cell line (BRIN-BD11). Cell viability was assessed and insulin secretion and insulin stimulation index were evaluated. The polymerization of alginate with PRP enhanced the viability up to 61% in the alginate microbeads. PRP supplementation to the alginate composition not only increased the number of viable cells by 1.95-fold, but the insulin secretion also improved by about 66%. The stimulation index, however, was not affected by the PRP supplementation.

Key words: Calcium alginate microbeads, diabetes, droplet method, pancreatic beta cells, insulin

1. Introduction

The transplantation of insulin-producing beta cells or pancreatic islets promises a cure for insulin-dependent (type 1) diabetes. Due to the constant attacks of the immune system, the protection of these cells is essential. Microencapsulation in alginate is the longest and most commonly applied technology for immunoisolation of pancreatic islets/beta cells (Zimmermann et al., 2001; Bhujbal et al., 2014). So far, various encapsulation techniques have been developed, but biocompatibility, stability, and permeability of the polymers are the main factors for a successful clinical application (Prüsse et al., 2008; de Vos et al., 2009). The initially insufficient beta cell mass or the compromised survival of implants is considered to play a critical role in the low efficiency of treatment (Jacobs-Tulleneers-Thevissen et al., 2013). Due to the processing method of alginate microcapsules, viability could be significantly decreased and even become unsuitable for transplantation.

The materials supplemented into microbeads during their generation might change the stability, permeability, and cellular events. The type of cationic agents in the polymerization of alginate, for example, determines the physiological properties of the polymer, like rigidity and

stability. Poly-L-histidine (PLH) is a homo-amine cationic polymer that shows pH-dependent amphoteric properties. The ionization of PLH below a specific pH level was reported to change its characteristics from hydrophobic to hydrophilic (Lee et al., 2003a, 2003b). This polymer has been known as the pH-sensitive part of pH-responsive nanoparticles. At physiological pH, this polymer is in its hydrophobic state, but its physiochemical properties change below this pH level (Wu et al., 2013; Coue and Engbersen et al., 2015; Bilalis et al., 2016).

The physiochemical properties of the microcapsules might be improved, but it is not always sufficient for the survival of cells. Platelet-rich plasma (PRP) is a blood product that contains high concentrations of diverse growth factors, such as TGF- β 1, VEGF, and PDGF, which can stimulate cell proliferation, migration, differentiation, and angiogenesis so that tissue regeneration could be improved (Kakudo et al., 2014; Kushida et al., 2014). Applications with PRP have become popular in recent years in the fields of neurosurgery and general surgery (Dohan Ehrenfest et al., 2014).

In the present study, we compared the effect of PRP supplementation to alginate-encapsulated pancreatic beta cell (BRIN-BD11) preparations on the viability and the

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glucose-responsive character of the cells. Three different commercially available alginate powders were tested for stable structure, and PLH was used as a cationic agent in the encapsulation during the process. The aim was to improve the microbeads for functional implants to be used in the treatment of diabetes by utilizing the supportive effect of PRP. The released insulin level to the medium was analyzed for different glucose concentrations to determine the beta cell function.

2. Materials and methods

2.1. Cell culture

A glucose-responsive rat cell line, BRIN-BD11, a hybrid cell line of a primary culture of NEDH rat pancreatic islets and RINm5F, was used in the encapsulation. The cells were cultured in RPMI 1640 culture medium (GIBCO, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; GIBCO) and 1% Pen-Strep (GIBCO). For the passage, cells were washed with phosphate-buffered saline (PBS; GIBCO) prior to detachment from tissue culture flasks with 0.25% (w/v) trypsin-EDTA (GIBCO) and seeded at 2.0×10^5 cells per T75 culture flask.

2.2. PRP preparation

PRP was obtained from blood of Fischer 344 (F344) inbred rats ($n = 9$) by the 2-step centrifugation method (Nagata, 2010). The blood samples were collected in a vacuum tube (BD Vacutainer; BD, Plymouth, UK) containing sodium citrate buffer (0.1 M). The blood cell component was removed from the medium by centrifugation at $160 \times g$ for 20 min at room temperature. The upper fraction was transferred into a new tube, where it was centrifuged at $400 \times g$ for 15 min at 4°C to separate PRP from the serum component. The final fraction contained 6.2×10^6 platelets/mL.

2.3. Production of microcapsules

Three different alginate powders were used for the alginate formulation: alginate of low viscosity (4–12 cP, 1% in H_2O ; Sigma-Aldrich, St. Louis, MO, USA; C.N.: A1112), alginate of high viscosity (≥ 2000 cP, 2% in H_2O ; Sigma-Aldrich; C.N.: A2033), and alginate of medium viscosity (Sigma-Aldrich, C.N.: 71238). Alginate solution (1.5%) was prepared in low-glucose basal medium (DMEM with 5.5 mM glucose) supplied with 1% Pen-Strep. PRP was mixed with alginate solution (1:20, v/v) prior to the addition of cells (2.0×10^6 cells/mL). The mixed solution was drawn using a syringe with a 30-G needle (BD, Franklin Lakes, NJ, USA) and gradually dropped into a cross-linking bath containing 100 mM CaCl_2 at the rate of 1 mL/min. After 5 min of reaction, the microbeads were collected in a cell strainer (100 μm ; BD) and then washed with PBS.

The microbeads were incubated in PLH solution (100 mM; Santa Cruz Biotechnology, Heidelberg, Germany)

for 10 min at room temperature. PLH-coated spheres were then washed with PBS before an outer alginate layer was coated [0.4% (w/v) in DMEM] for 10 min. The microbeads with cells were maintained in low-glucose (5.5 mM) DMEM supplemented with 10% FBS and 1% Pen-Strep.

2.4. Swelling ratio of microcapsules

After the microbeads were generated, they were air-dried and the weight was recorded. Then the beads were soaked in PBS buffer (pH 7.4; Thermo, GIBCO) at 37°C . At different time points, the wet weight of microcapsules was measured after removing the excess fluid and the swelling ratio was calculated according to the formulation given by Sarker et al. (2014).

2.5. pH response of poly-L-histidine coated microcapsules

The effect of pH on protein release from the microcapsules was analyzed by mixing bovine serum albumin (BSA; Sigma) at a final concentration of 1 mg/mL into the alginate composition. After coating the microcapsules with poly-L-histidine, the microcapsules were soaked in 0.1 M phosphate buffer with different pH values varying from 5.0 to 8.0. The released protein level was measured by bicinchoninic acid (BCA) assay (Duruksu et al. 2018).

2.6. Assessment of cell viability

The cell viability was assessed by WST1 assay and Calcein AM staining. Following the culture, the culture medium was replaced with basal medium (DMEM with 5.5 mM glucose) including 10% WST1 reagent (Roche, Mannheim, Germany). After incubation for 1 h, the absorbance was measured at 450 nm.

The cell viability in microbeads was determined by staining with Calcein AM (4 mM) and ethidium homodimer-1 (2 mM), according to the instructions for the live-dead staining kit (Invitrogen, Eugene, OR, USA). Cells containing microbeads were incubated for 30 minutes in 2 mL of serum-free medium containing 1 mL of Calcein AM and 2 mL of ethidium homodimer-1. Subsequently, capsules were washed and visualized under a fluorescence microscope (Leica DMI 4000B, Wetzlar, Germany). Dead cells exhibited red fluorescence and viable cells green.

2.7. Insulin assay and response to glucose

Insulin level in the medium was estimated by rat insulin ELISA kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The minimum detectable dose of the ELISA kit was reported as 0.2 ng/mL. All experiments were repeated at least three times. Total protein in the medium was measured by the BCA assay using fresh culture medium as a blank.

To determine the cell response to varying glucose concentrations, the insulin levels were determined in the same basal medium with two different glucose concentrations. The microbeads were incubated for 24 h either in low-glucose DMEM (5.5 mM) or in high-glucose

DMEM (25 mM). The sample from each medium was collected to estimate the insulin levels by ELISA.

2.8. Statistical analysis

Statistical differences in the groups were analyzed with two-tailed, nonpaired Student t-tests assuming unequal variance. Results are expressed as mean $\pm$ standard deviation (SD). The validity of the data was evaluated by the Fisher exact test. All statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). The results were considered statistically significant when $P < 0.05$ and highly significant when $P < 0.01$.

3. Results

Three different alginate powders with different viscosity degrees were used for the preparation of the encapsulation, which provides diverse properties to the spheres after their polymerization. The cell-free polymerization studies showed that the formulation with low viscosity was not suitable for encapsulation: the structures were neither uniform nor spherical in shape (Figure 1a). The medium and highly viscous alginate formulations gave solid, uniform, and spherical structures, and the addition of PRP did not change their morphology significantly (Figures 1b and 1c). The coating of spheres with PLH was successfully employed and the thickness of the coating could be regulated by increasing the incubation time (Figures 2a–2d). Both types of the alginate microbeads kept their physical structure intact during the study. PRP addition or coating with PLH was not observed to change the spheres. During the studies with the encapsulated cells, the structure of microbeads produced from medium and highly viscous alginates was not disrupted (Figures 2c and 2d). The sizes of the spheres were measured and the size frequency was evaluated (Figure 3). The size of the alginate spheres fabricated from the medium viscous alginate (Med Alg) preparation presented a peak of 23.44% at approximately 1600 μm (Figure 3a). The spheres made of highly viscous alginate (High Alg) gave a peak of 24.05% at approximately 1400 μm (Figure 3b).

The effect of PLH coating and the PRP on the cell viability and beta cell function were evaluated separately. The soaking rates of spheres were evaluated to reveal the microcapsules' ability to keep and release the buffer at physiological conditions (pH 7.4, 37 °C), which is related to the transfer of cell nutrients and metabolites in and out of the alginate spheres. Overall, the Med Alg group had a higher soaking rate than the High Alg group (Figure 4). The rate in the initial period was slow, but it accelerated with time. The effect of PLH on swelling characteristics was limited. Although a decrease in swelling rate could be observed in both alginate preparations, statistically the effect was insignificant. On the other hand, the addition of PRP to the formulations considerably affected the swelling.

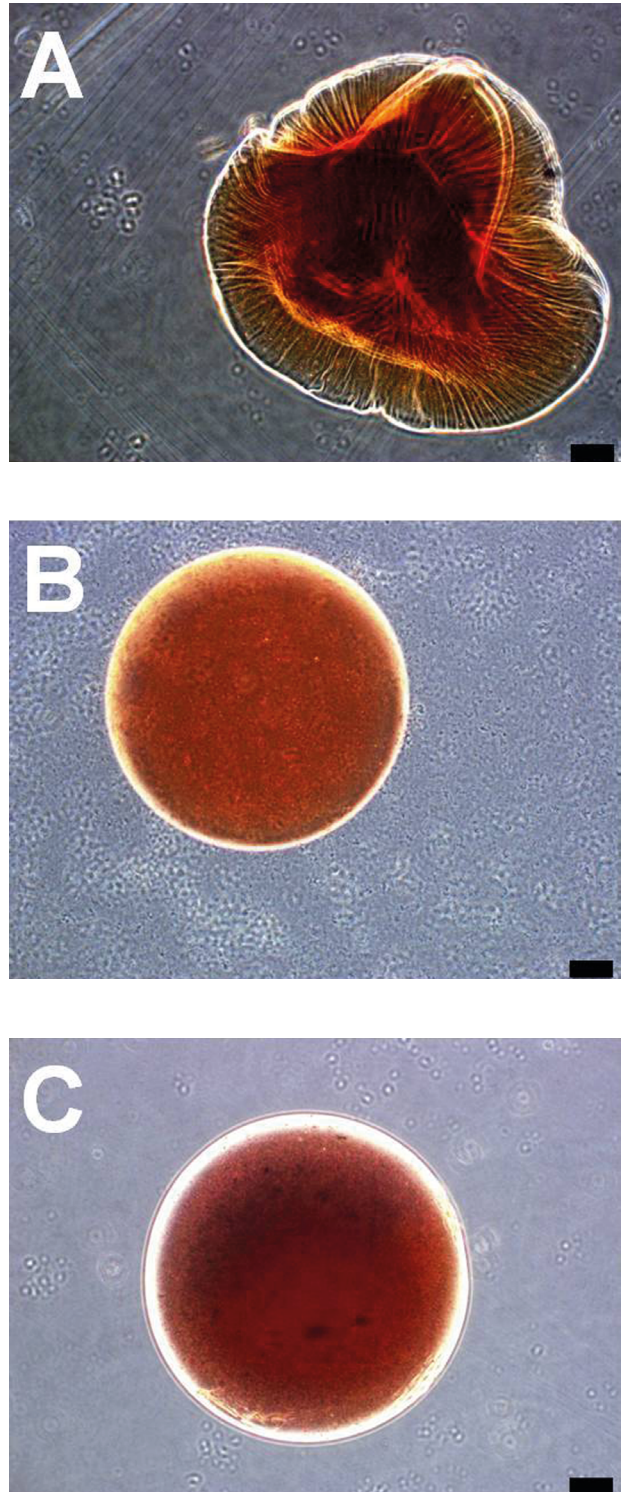


Figure 1. The cell-free polymerization of alginate solutions with different viscosity values. Three different alginate products were used to prepare the microcapsules: a) low viscosity alginate (4–12 cP); b) medium viscosity alginate; and c) high viscosity alginate (≥ 2000 cP). Alginate solutions (1.5%) were prepared in low-glucose (5.5 mM) DMEM. The microcapsules were stained with Congo Red (0.15%; Sigma-Aldrich). Scale bars: 200 μm .

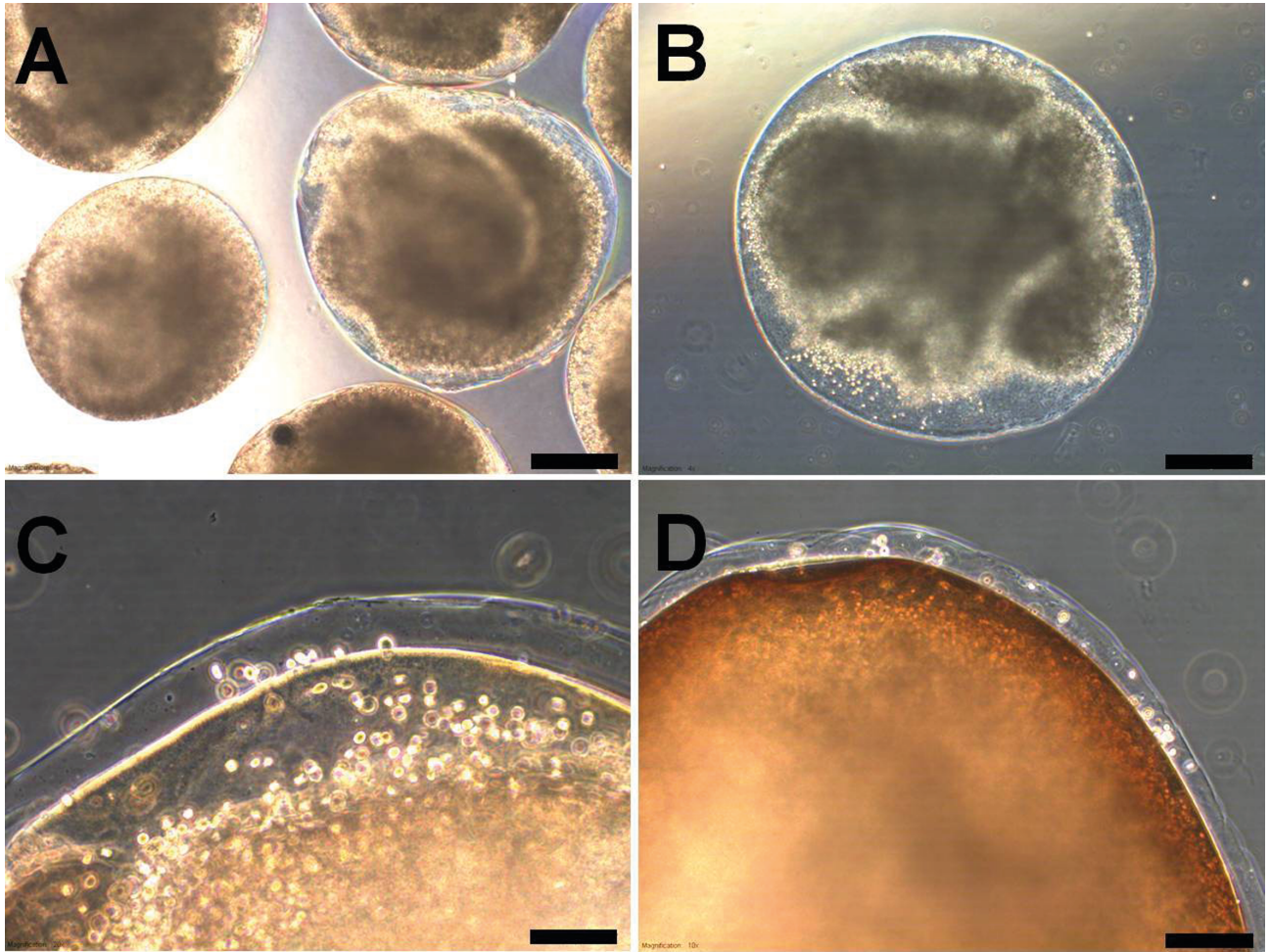


Figure 2. Encapsulated rat pancreatic beta cells (BRIN-BD11) in alginate hydrogels. The cells were encapsulated in medium viscosity (a) and high viscosity (b) alginate solutions and coated with PLH (c, d), respectively. Scale bars: 500 μm (a, b), 100 μm (c), and 200 μm (d).

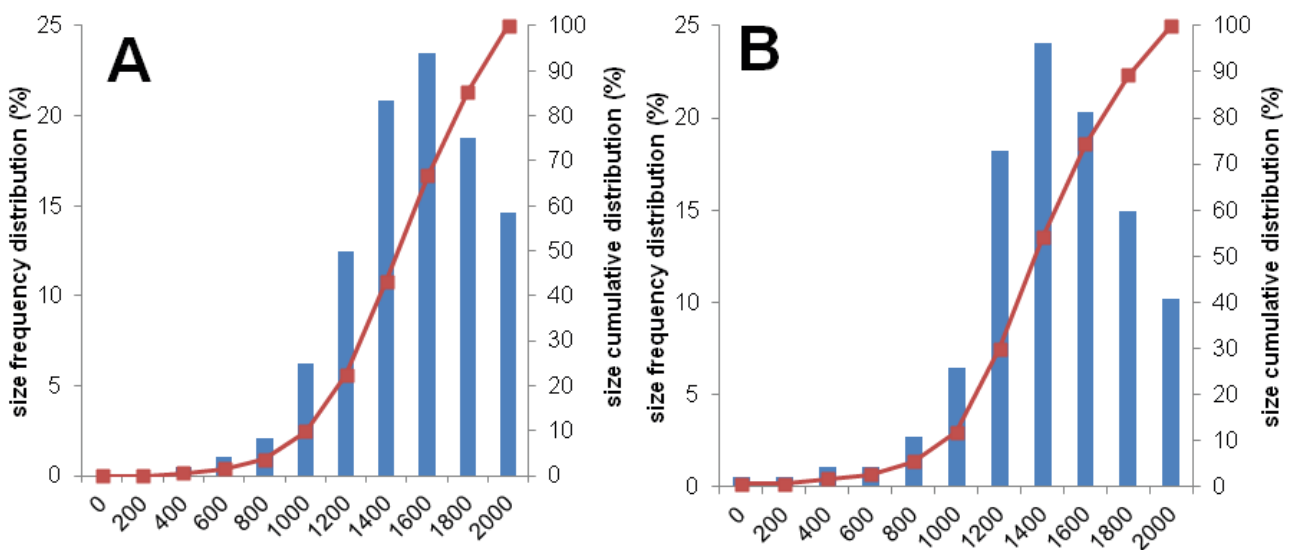


Figure 3. Histograms of particle size distribution frequency of medium and highly viscous alginate formulations. The size distribution frequency (%) is presented in bars and the size cumulative distribution (%) in continuous line diagram for Med Alg (a) and High Alg (b).

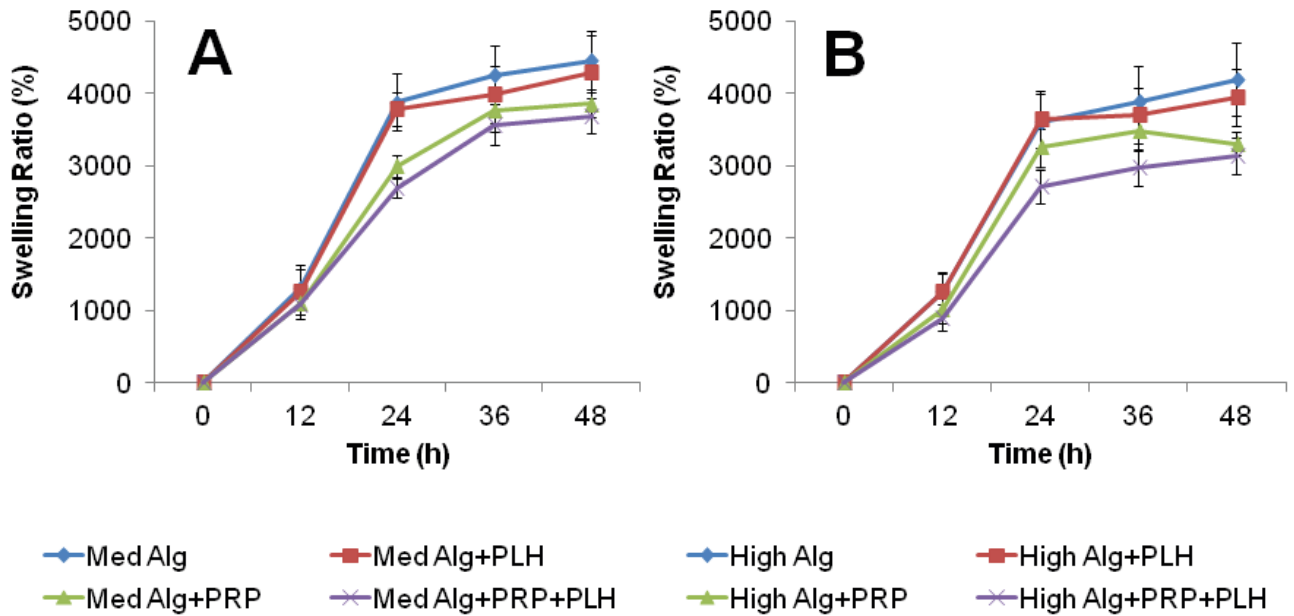


Figure 4. Swelling ratio in PBS at pH 7.4 as a function of time of the microbeads fabricated from medium (a) and highly (b) viscous alginate.

The PLH coating of PRP-added microcapsules further decreased the swelling ratio, which could be related to the diffusion rate of metabolites. In High Alg microspheres, the plateau was reached more rapidly than in Med Alg (Figures 4a and 4b).

As beta cells under normal physiological conditions do not proliferate substantially in humans, the liquefaction step in the alginate-polymer-alginate (APA) encapsulation procedure was not applied to keep the cells in their nonproliferative state. After the encapsulation process of beta cells, the viable cell number was determined after 48 h of incubation in the low-glucose DMEM culture medium. Staining with Calcein AM, which demonstrated the live/dead cell distribution in the microbeads, showed a significant number of dead cells (stained in red) after 48 h of incubation in medium alginate formulation with PLH + PRP compared to the alginate microbeads with PRP supplementation without the PLH coating (Figures 5a and 5b). The highly viscous alginate microbeads contained fewer dead cells compared to the medium alginate groups (Figures 5c and 5d). The metabolically active cells were quantified by WST1 assay (Figures 5e and 5f). Statistically significant differences between the microbeads prepared by medium and highly viscous alginate formulations (without PRP or PLH) were not observed. Compared to the alginate-only microcapsules, the application of PLH improved viability by 27% and 61% in the medium and highly viscous alginate microbeads. The effect of PLH coating addition substantially improved the viable cell numbers in the microbeads, but the

supplementation of alginate mixture with PRP showed the highest supportive effect in the groups. In the highly viscous alginate microbeads with PRP, the number of viable cells was increased almost 2-fold compared to the control microbeads without PRP (Figure 5f). Surprising results were obtained from the microbeads when both PLH coating and PRP addition were performed together. In the highly viscous alginate microbeads, the number of metabolically active cells decreased to the level observed in the single alginate formulation, and the decrease was even lower in the medium viscous alginate microbeads with PLH + PRP.

The insulin secretion from the microbeads was estimated by ELISA after 24 h of incubation of the microbeads in the low-glucose fresh basal medium (no serum) following 48 h of incubation under the same conditions. The level of insulin was normalized by the total protein in the medium (Figures 6a and 6b). In the culture with low-glucose medium, an increase in secreted insulin level was observed in the medium viscous agar microbeads with PRP and PRP + PLH, at 19.19 ± 1.51 ng/mg protein and 17.06 ± 1.07 ng/mg protein, respectively. The insulin secretion level of PLH (14.36 ± 1.48 ng/mg protein) was not significantly different from the alginate-only microbeads (Med Alg) at 12.27 ± 1.05 ng/mg protein (Figure 6a). The insulin secretion from the highly viscous alginate group (no PRP or PLH) was higher than that of the medium viscous alginate microbeads (Figure 6b). The insulin secretion levels between the groups of the highly viscous alginate preparations were almost the same,

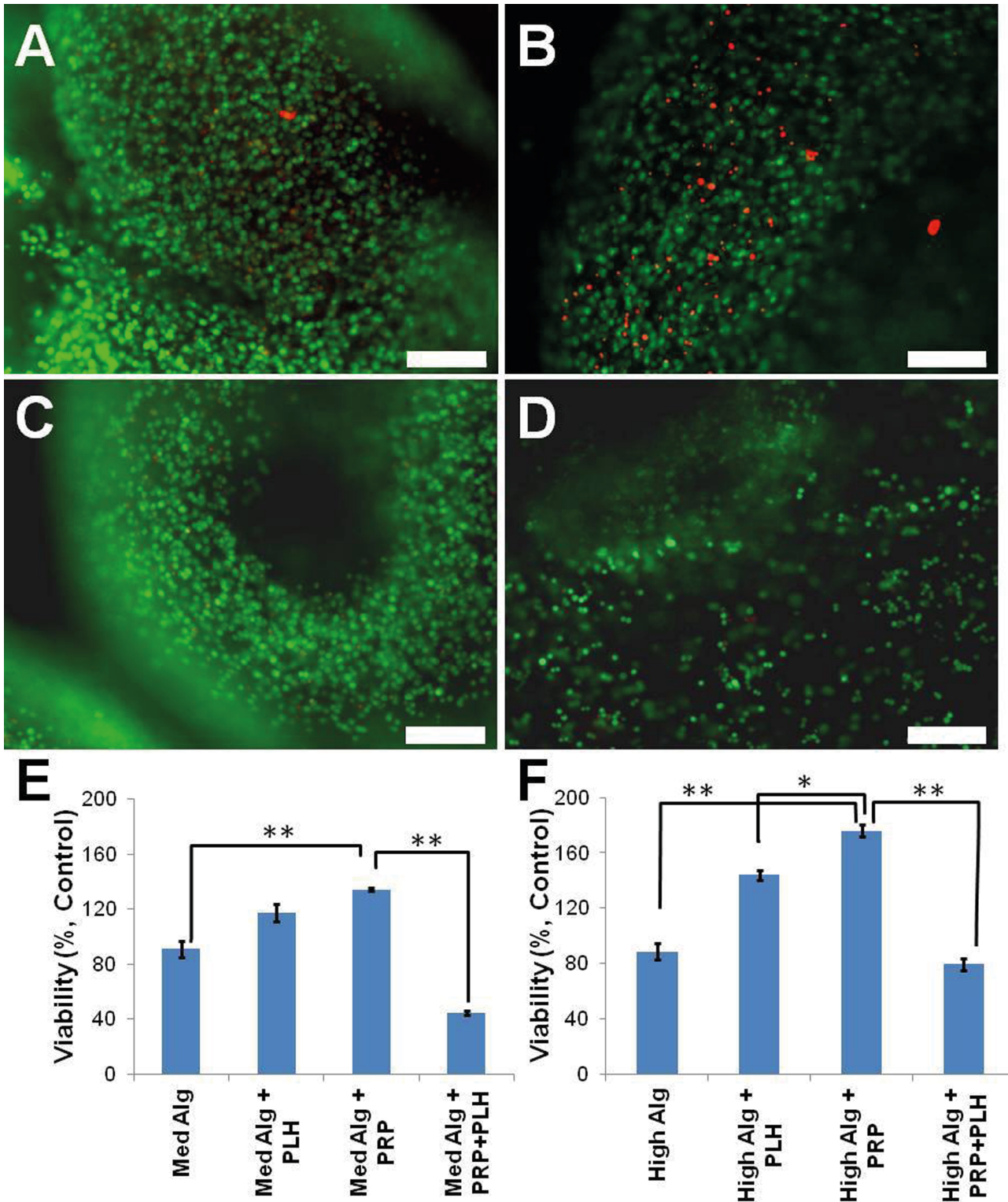


Figure 5. Cell viability in the microcapsules. The medium viscous alginate microcapsules (a, b) were compared with highly viscous alginate microcapsules (c, d). The microcapsules supplemented with PRP (a, c) showed higher viability (green) in both types of alginate compared to alginate with PRP + PLH groups (b, d). Dead cells were stained by ethidium homodimer (red). Scale bars: 200 μ m. The cell viability was quantified by WST1 assay in medium viscous alginate groups (e) and in highly viscous alginate groups (f). Statistically significant ($P < 0.05$) and highly significant ($P < 0.01$) differences are shown by * and ** on the graph, respectively.

ranging between 16.37 and 17.35 ng/mg protein, but the PLH-coated highly viscous alginate preparation secreted significantly less insulin into the medium (11.88 ± 3.59 ng/mg protein).

The stimulation index, which was defined as the fold increase in the secretion level of insulin in response to the high glucose level, was determined (Figures 6c and 6d). Beta cell functions were found to be preserved after the encapsulation with small variations. The addition of PRP improved the response to the glucose, but the difference was not statistically significant compared to the other groups with medium alginate formulations. The PLH coating in these microbeads slightly decreased the response to the stimulus, but opposite results were obtained in the PLH-coated group with the highly viscous alginate formulation (Figure 6d). The coating improved the response to the stimulus in these microbeads, but the stimulation was

insignificant in the PLH-coated microbeads with PRP supplementation.

To analyze the effect of pH on the polypeptide-coated microspheres and their secretion capacity, all PLH-coated groups were incubated in phosphate buffer with different pH levels (Figure 7). At high pH levels, the secretion capacity of the High Alg formulation increased, and it was significantly higher than that of the Med Alg formulation. The results showed that pH is effective on protein diffusion: the mass transfer declines at lower (acidic) pH and increases at higher (basic) pH (Figure 7).

4. Discussion

For a long-term treatment of insulin-dependent diabetes mellitus, it is important to develop a functional system that supports insulin secretion in response to varying glucose levels while protecting against cell-mediated immune

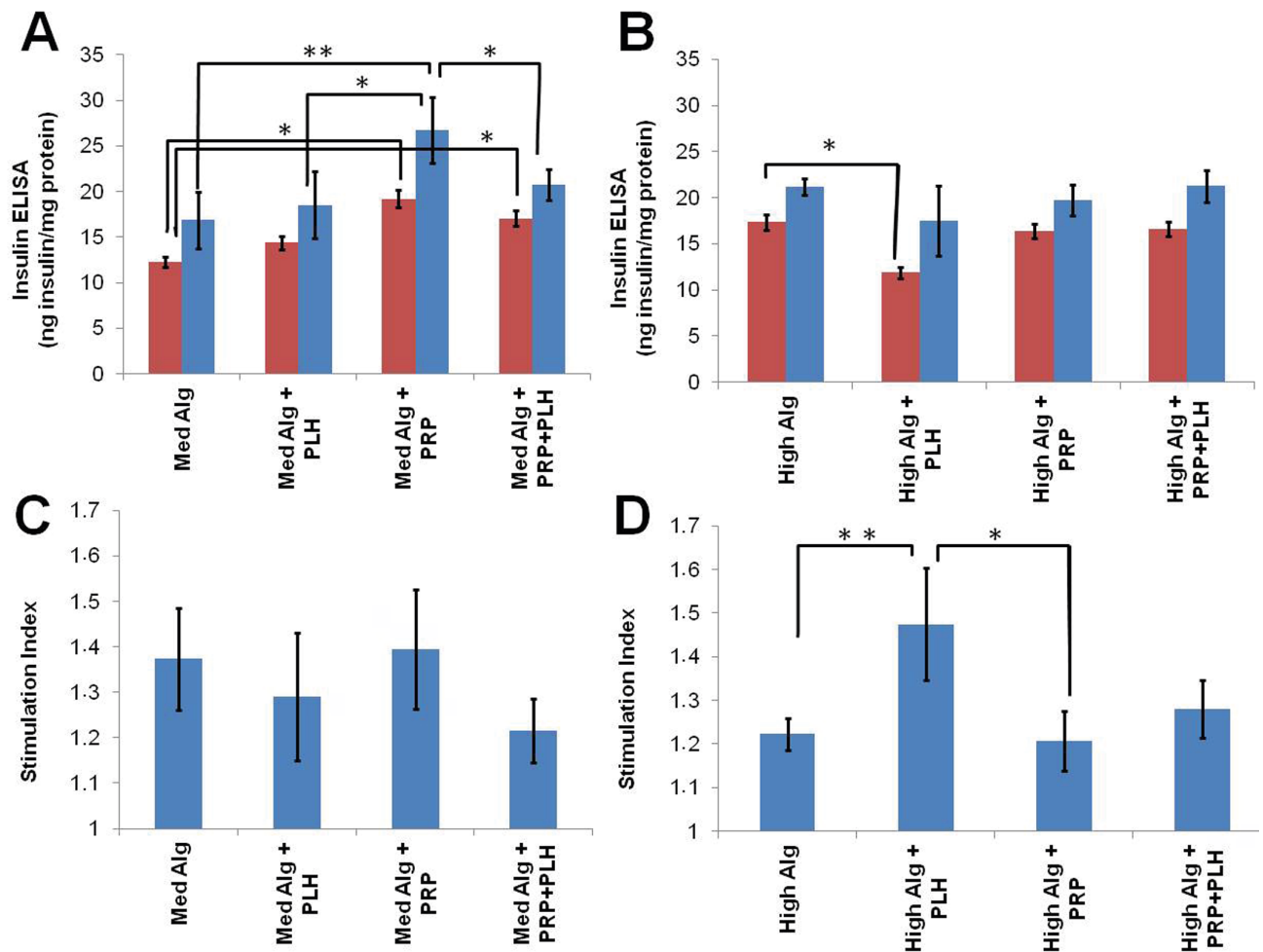


Figure 6. Insulin secretion levels and glucose stimulation index. The secreted insulin levels in the low-glucose (5.5 mM; red bar) and in the high-glucose (25 mM; blue bar) media were determined by ELISA (a, b). The fold increase in secreted insulin levels in response to the increased concentrations of glucose in media was determined for medium viscous (c) and highly viscous (d) alginate microcapsules. Statistically significant differences were indicated by * ($P < 0.05$) and highly significant differences by ** ($P < 0.01$).

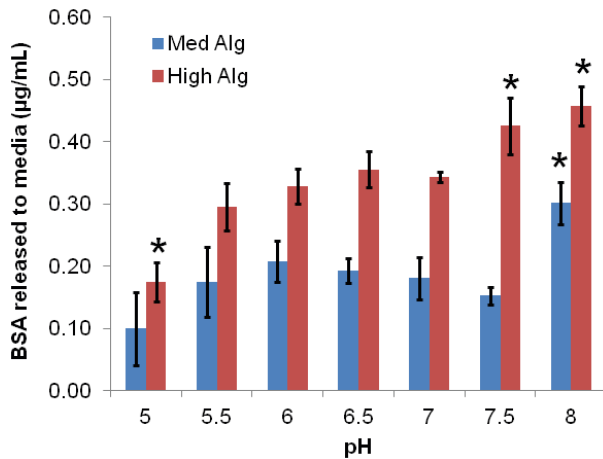


Figure 7. BSA release from PLH-coated microcapsules was evaluated for Med Alg and High Alg formulations at 37 °C. After 24 h, the protein level secreted to the media was measured. Asterisk indicates significance difference ($P < 0.05$) from the measurement at pH 7.0 in the same alginate formulation.

attacks. The transplantation of encapsulated islets for the treatment of patients diagnosed with type 1 diabetes has been successfully practiced for a long time, but the shortage in islet donor numbers limits its widespread application (Calafiore et al., 2006; Desai and Shea, 2017). The improvement in cell viability is not sufficient, and tissue function should be preserved during the manipulations to maintain the glucose homeostasis capacity of the islets. Alginate hydrogels are widely used in microencapsulation of pancreatic islets with different protocols (Robles et al., 2014).

During the generation of alginate beads, the viscous solution of alginate was mixed with cells and then hydrogels were stabilized by treatment with polycationic polymers. In the polymerization of the outer layer of alginate microcapsules, the cationic polymers poly-L-lysine and poly-L-ornithine have been frequently used. However, the study by Strand et al. (2001) demonstrated that TNF secretion and necrosis were induced by poly-L-lysine in alginate capsules. Although the results proposed that the necrosis- or fibrosis-inducing effect was reduced by mixing the cationic polymer with the alginate and also reducing its concentration, the toxic effects were not completely eliminated. It was shown that toxicity of cationic polymers depends on the polymer composition and the cell lines (Prokop et al., 1998). In our study, PLH was used instead. According to our results, the coating of the microbeads with PLH did not adversely affect the cell viability. The viability was even increased in the coated microbeads with PLH compared to the noncoated alginate microbeads. The cell viability was decreased only in the microbeads processed with both PLH and PRP.

The decrease in the viability might be explained by the stiffness of the microcapsules that originated due to the PRP. This characteristic of PRP was reported previously (Horimizu et al., 2013): the thickening of the outer layer by polycationic polymer might also cause insufficient nutrient and oxygen intake and lead to decrease in viability at small scale. However, despite decreased cell viability, the insulin secretion level from microbeads was not reduced. This might indicate the effect of the PLH on the controlled drug release. The swelling of Med Alg was greater than that of High Alg, which means greater fluid flow in and out of the microbeads. However, other structural components, like PRP and PLH, also influenced the diffusion rate of metabolites. PLH had a very limited effect on the swelling character, but the change of pH was one of the critical factors. In the regulation of insulin secretion, PLH will limit the produced insulin secretion out of the microcapsules at pH 7.0 and will release at physiological normal blood pH (about pH 7.4). PLH in the structure made the microbeads pH-sensitive polymeric carriers. Gao et al. (2005) and Lee et al. (2007) showed in their studies that pH-sensitive micelle formulation caused the release of the drug by reduced pH in the tumor niche, even with a small change in pH (pH 7.2 to 6.5). This effect might be very useful because ketoacidosis is frequently observed in type I diabetes and causes decreases in blood pH levels below 7.3. Furthermore, the pH of tissues does not fall below 7.35 in cases of alcohol abuse or heavy exercise (Ausländer et al., 2014). For that reason, this behavior of the PLH makes it ideal for any diabetes treatment. However, there is no significant alteration in the insulin release between the groups in medium buffered at pH 6.5 and at pH 7.4 (unpublished result). Different from in vivo settings, the in vitro culture conditions allow to accumulate the metabolites, which cause uncontrolled acidification. The culture model should be modified and the controlled release effect by PLH should be validated.

The high number of viable cells in the medium viscous alginate microbeads could also lead to high insulin secretion. The cell viability and insulin level demonstrated almost the same pattern in these microbeads. A major increase in insulin secretion was observed in the PRP-supplemented groups. The addition of PRP to the alginate composition supported both cell viability and beta cell function at the same time due to the rich content of PRP. Insulin secretion was increased in the alginate with medium viscosity. Similar results have also been obtained in other studies with PRP-added alginates. In a study on chronic periodontitis, PRP was mixed with alginate to form gels instead of mixing with fibrin, and a clinically significant improvement was achieved in the PRP-alginate group (Okuda et al., 2005). Combining different types of bioactive molecules from the PRP with the biomaterial

scaffold improves their regenerative capacity (Orive et al., 2014). Addition of PRP into the alginate microbead composition was reported to promote both angiogenesis and regeneration characteristics of the scaffold (Man et al., 2012). Although PRP improved secretion levels, the main factor affecting insulin secretion was the viscosity of the alginate used in the production. The alginate with medium viscosity supported a higher level of insulin secretion compared to the highly viscous alginate. The glucuronic acid/mannuronic acid ratio in the alginate composition mainly determines its physicochemical characteristics. By using highly viscous alginate, structural impairments can be reduced and microcapsules with better morphological characteristics can be generated (van Schilfgaarde and de Vos, 1999). The highly viscous alginate solutions could also produce dense microbeads, which block diffusion of molecules, like insulin. The results demonstrated that microbeads generated from highly viscous alginate solution had less capacity to secrete insulin and weakened glucose stimulation-response coupling.

Beta cell function was evaluated with the stimulus-secretion coupling assay. The response of beta cells to high glucose level was decreased in the highly viscous alginate microbeads compared to medium viscous alginate microbeads. The PLH-coated highly viscous alginate microcapsules without PRP showed the highest response compared to other highly viscous alginate groups, but this response was not distinct when comparing medium viscous alginate microbeads with or without PRP. The stimulation index directly indicates the beta cell functionality. Any

disruption in cell functionality will affect the insulin sensing and the secretion. Therefore, the cells can continue to secrete insulin, but they can degenerate due to environmental factors, like PRP or poly-L-histidine. PRP addition did not improve the beta cell function in medium viscous alginate groups, but stimulation-response coupling was preserved. Notably, the PLH-coated microbeads showed decreased beta cell stimulation-response coupling due to the diffusion blockage.

In conclusion, the most promising results were obtained from medium viscous alginate microbeads. The PLH coating, however, did not improve cell viability and secretion level in the medium. Quite the reverse, the blocking of diffusion by the copolymer caused decreased insulin levels in the medium. On the other hand, the PRP addition within the matrix structure substantially improved cell viability and consequently the insulin level in media, but the glucose stimulation-response coupling of beta cells was not affected. PRP applications have been shown to support regeneration processes in many cases. We also demonstrated that PRP supplementation into microbeads improved cell viability and preserved the cell function. These results might facilitate generation of more functional implants with primary beta cells or pancreatic islets for the treatment of diabetes.

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