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Structural surface changes and inflammatory responses against alginate-based microcapsules after exposure to human peritoneal fluid

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Abstract: Microencapsulation of cells is a promising approach to prevent rejection in the absence of immunosuppression. Clinical application, however, is hampered by insufficient insight in factors influencing biocompatibility of the capsules in humans. In the present study we exposed alginate-based capsules prepared of different types of alginate to human peritoneal fluid. Subsequently we studied the physicochemical changes of the capsule's surface by applying micro-Fourier Transform Infrared Spectroscopy. We did test alginate-beads and alginate-poly-L-lysine capsules prepared of different types of alginate. In all tested capsule formulations we found adsorption of components from human peritoneal fluid and clear physicochemical changes of the surface. These changes were alginate-dependent. The adsorption had no significant effects on the permselective properties of the capsule but we found a strong increase of TNFα production by human peripheral blood mononuclear cells when exposed to alginate-beads treated with human peritoneal fluid. This elevated responsiveness was not observed with alginate-PLL capsules. The results show that alginate-based capsule surfaces always undergo physicochemical changes of the surface when exposed to human peritoneal fluid. This adsorption may lead to enhancement of the inflammatory responses against the microcapsules. Our result implicate that biocompatibility measurements should not only been done with freshly prepared capsules but also with capsules that have been exposed to fluid from the implantation site in order to predict the *in vivo* responses. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 98A: 394–403, 2011.

Key Words: alginate, microencapsulation, inflammation, surface analysis

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INTRODUCTION

Cell transplantation is a growing therapeutic field for the treatment of metabolic disorders such as dwarfism,[1] Hemophilia B,[2] kidney[3] and liver[4] failure, pituitary[5] and central nervous system insufficiencies,[6] and diabetes mellitus.[7] Wide-spread application of cell transplantation, however, is limited due to the necessity to apply immunosuppression. To avoid the application of immunosuppression, many research groups are focusing on technologies to encapsulate hormone- or protein-secreting cells in permselective membranes to protect donor-cells against antibodies and cytotoxic cells of the host. This immunoprotection by encapsulation avoids application of permanent immunosuppression[7–9] and allows for successful grafting of cells from non-human origin, that is, xenografts, which overcomes the obstacle of limited supply of donor tissue.[1,2,4,5,10]

A commonly used procedure for immunoprotection is microencapsulation of tissues in alginate–poly-L-lysine (PLL)-based capsules, as originally described by Lim and Sun.[7] These capsules have been extensively tested for its efficacy in transplantation of pancreatic islets for the treatment of diabetes.[11,12] During recent years, many modified capsule types with improved biocompatibility have been described.[11,13–16] A critical issue that has been overlooked in these studies is that the properties associated with biocompatibility were studied on freshly prepared capsules without taking into account the physico-chemical changes that capsules undergo after grafting. In the recent past we have demonstrated that fluctuation in pH,[17] immunoglobulin[18] and other types of protein adsorption[19,20] in the transplantation site may lead to physico-chemical changes of the capsules which ultimately results in inflammatory responses.[20,21]

Fourier transforming-infrared spectroscopy (FT-IR) has been shown to be a relatively fast and a successful approach to study the structural features of intramolecular and

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intermolecular interactions at alginate-based capsule surfaces.[22–25] It has been shown that FT-IR allows for detection of small but essential modifications in the capsular composition.[22,24,25] In the present study we applied micro-FTIR to study the structural changes of capsules after exposure to human peritoneal fluid. The rational for choosing peritoneal fluid is that most capsule grafts are being transplanted into the peritoneal cavity. The structural changes were studied on alginate-based microcapsules in the presence and absence of a structural immunoprotective PLL membrane. The biological importance of the changes was tested by studying the permeability for relevant solutes and by assessing the inflammatory responses of peripheral blood mononuclear cells. We demonstrate that exposure to human peritoneal fluid changes the capsule surface with in some cases inflammatory responses as a consequence. This illustrates that commonly applied assays that measure the responses against fresh capsules are not predictive for what happens *in vivo*.

MATERIALS AND METHODS

Materials

Intermediate-G sodium alginate (Keltone® LVCR, International Specialty Products Corp, UK) containing 40% of guluronic acid (as specified by the manufacturer) and high-G sodium alginate (Manugel® DMB, International Specialty Products Corp, UK) containing 50% of guluronic acid (as specified by the manufacturer) were used for microcapsule production. The alginates were purified as previously described.[8] Briefly, crude sodium alginate was dissolved at 4°C in a 1 m*M* sodium EGTA solution to a 1% solution for intermediate-G alginate and to a 0.25 % solution for high-G alginate under constant stirring. Subsequently the solutions were filtered over, successively, 5.0, 1.2, 0.8, and 0.45 μm filters (Schleicher & Schüll, Germany). During this filtration step, all visible aggregates were removed.

Next, the pH of the solution was lowered to 3.5 by addition of 2 *N* HCL + 20 m*M* NaCl. The solution was kept on ice to prevent hydrolysis of alginate. The next step was slow lowering the pH from 3.5 to 1.5 and was associated with gradual precipitation of alginate as alginic acid.[26] Routinely, the solutions were brought at a pH of 2.0 and subsequently filtered over a Buchner funnel (pore size 1.5 mm) to wash out non-precipitated contaminants. To extend the wash-out of non-precipitated contaminants, the precipitate was brought in 0.01 *N* HCL + 20 m*M* NaCl, vigorously shaken, and filtered again over the Buchner funnel. This washing procedure was performed three times.

Then, proteins were removed by extraction with chloroform/butanol.[27] The alginic acid was suspended in 100 mL of 0.01 *N* HCl + 20 m*M* NaCl and supplemented with 20 mL chloroform and 5 mL 1-butanol. The mixture was vigorously shaken for 30 min and filtered over the Buchner funnel. This chloroform/butanol extraction was performed three times. Next, the alginic acid was brought in water and slowly dissolved by gradually raising the pH to 7.0 by slow addition of 0.5 *N* NaOH + 20 m*M* NaCl over a period of at least 1 h. The alginate solution obtained was subjected to a chloroform/butanol extraction to remove those proteins which can only be dissolved in chloroform/butanol at neutral pH.[27] The solution was vigorously shaken in a mixture of chloroform (20 mL at each 100 mL alginate solution) and 1-butanol (5 mL at each 100 mL alginate solution) for 30 min. The mixture was centrifuged for 3–5 min at 3000 rpm, which induced the formation of a separate chloroform/butanol phase which was removed by aspiration. The extraction was repeated once.

The last step was precipitation of the alginate with ethanol.[28,29] To each 100 mL of alginate solution we added 200 mL absolute ethanol. After an incubation period of 10 min all alginate had precipitated. The alginate was filtered over the Buchner funnel and washed two times with absolute ethanol. Subsequently, the alginate was washed three times with ethylether. Finally, the alginate was freeze-dried overnight. The endotoxin content of the purified alginate was always <0.006 ng mg^{-1} as measured by LAL.

PLL hydrochloride (Sigma-Aldrich, USA) having a molecular weight of 22,200 was used to form the microcapsule membrane.

Microencapsulation

For microcapsule production, the alginates were dissolved in a 220 mOsm Ca^{2+}-free Krebs-Ringer-Hepes (KRH) solution consisting of 90.0 m*M* NaCl, 4.7 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, and 25.0 m*M* Hepes. Alginates were dissolved at a concentration of 3.4% w/v for the purified intermediate-G alginate, 3.5% w/v for the purified intermediate-G alginate, and 1.9% w/v for each of the high-G alginates. These concentrations provided alginate solutions of appropriate viscosity for microcapsule fabrication. All alginate solutions were sterilized by filtration (0.22 μm, Schleicher & Schüll, Germany). The difference in alginate concentration does not influence the biological responses against the capsules.[30,31]

Microcapsules were produced using an air-driven droplet generator following previously described methods.[32] Alginate solutions were extruded from a 23-G needle using a syringe and a co-axial air stream to produce droplets. The alginate droplets were immersed in a 100 m*M* $CaCl_2$ solution and allowed to gel for 5 min after extrusion of the last droplet. The extrusion process never lasted longer than 4 min. Gelled calcium alginate beads were 650–675 μm in diameter. To form the microcapsule membrane, the calcium alginate beads were rinsed and then immersed for 10 min in a PLL solution that consisted of 0.1% w/v PLL dissolved in 310 mOsm Ca^{2+}-free KRH. The microcapsules were rinsed again and then immersed in a 0.1% diluted solution of alginate (i.e., 0.19, 0.34, or 0.35% w/v in Ca^{2+}-free KRH) for 5 min. In all cases, the same type of alginate was used for both the microcapsule gel core and the coating step. After final rinsing, the microcapsules were stored in KRH until analysis. Final alginate-poly-L-lysine-alginate (APA) microcapsules were 650–800 μm in diameter. All solutions used for microcapsule fabrication were sterilized by filtration (0.22 μm).

TABLE I. Major Protein Components in the Applied Peritoneal Fluid

Components	Concentration
Total protein	26.1 g L^{-1}
Transferrine	1.05 g L^{-1}
Albumine	14.2 g L^{-1}
Fibrinogeen	$<$0.3 g L^{-1}
C1q	0.062 g L^{-1}
C3	0.488 g L^{-1}
C4	0.065 g L^{-1}
IgG	2.2 g L^{-1}
IgM	0.80 g L^{-1}
α1-antitrypsine	1.78 g L^{-1}
β2 microglobuline	2.30 mg L^{-1}

Adsorption studies

For adsorption studies *in vitro*, human peritoneal fluid (PF) was obtained from two male donors within 12 h of decease. The PF was pooled from two male donors. Table I shows the composition of the applied PF. IgG, IgM, AAT, b2M, C1Q, C3, C4, and fibrinogen were quantified by nephelometry on a Nephelometer of Siemens. Totale protein, albumin, and transferrin were determined by applying turbidimetry. To this end we applied the P-module of the Modular of Roche. Complement screening was performed with Wielisa from Wieslab.

Samples of 30 calcium alginate beads or microcapsules were transferred to a polypropylene test tube. The supernatant (i.e., KRH) was removed by aspiration. Samples were incubated with 1 mL solution containing PF and KRH (ratio 1:1) in a warm water bath at 37°C with gentle agitation for 1 h. Afterward, the PF:KRH was removed and the microcapsules were rinsed five times with KRH before analysis.

Micro-FTIR analysis

The structural features of the microcapsule's surface was measured with micro-FTIR by using a Jasco MFT-2000 apparatus (Tokyo, Japan), supported by Jasco FT software. The spectra were collected at room temperature in the 4000–600 cm^{-1} wave-number range in transmittance mode. The spot size was 10 μm. Calcium alginate beads or microcapsules were placed on the top of KBr disks, previously obtained by compressing a KBr powder by a hydraulic press. The microscope of the micro-FTIR was concentrated on the edge of the bead instead of on the core. This avoided disturbed transmissions that are associated with the high density of the Ca-alginate in the core. Micro-FTIR spectra of sodium alginate, polylysine, and of peritoneal fluid were collected as controls.

Permeability measurements

Microcapsules were incubated in a 12 mL solution containing albumin (0.61 m*M* Sigma), insulin (15 mU L^{-1}; acrapid), or glucose (7.5 m*M*; Sigma). Samples were taken after 0, 2, 5, 15, 30, and 60 min to determine the permeability of the capsules. Insulin concentrations were quantified with an Ultrasensitive human insulin radioimmumoassay (Linco Research, USA), albumin analyte was determined using the nefelometric immunoassay with the Behring technique with the Hitachi 917 analyzer. Glucose was measured with a Gluco-quant kit from Roche using and Hitachi 917 analyzer.

Co-incubations with human peripheral blood mononuclear cells (PBMCs)

Samples of 120 calcium alginate beads or microcapsules were transferred to a polypropylene test tube. The supernatant (i.e., KRH) was removed by aspiration. PF was diluted 1:1 in KRH and 300 μL of the diluted PF was added to each test tube. Samples were incubated in a warm water bath at 37°C with gentle agitation for 1 h. Afterward, the PF was removed and the calcium alginate-beads or microcapsules were washed five times with KRH. Next, PF-treated calcium alginate beads or microcapsules were incubated in complete RPMI 1640 medium (GIBCO BRL, Breda, The Netherlands) supplemented with 60 μg mL^{-1} gentamycin.

Human peripheral blood mononuclear cells (PBMCs) were applied for testing the immune responses after exposure of alginate beads and APA capsules to peritoneal fluid. We used this model to mimic the immediate early response against microencapsulated tissues which is associated with a significant loss of functional tissue.[9,20] To this end we applied short time incubation periods of not more than 1 h. To find adequate biomarkers for responses we first applied a broad screening for immunemarkers (IL-1, TNF-α, IL-6, IL-8, IL10, IL-12, IL-17, ICAM, CD14, and CD11b). We selected the two most prominent responders on the challenge with APA capsules exposed to peritoneal fluid which were TNF-α and IL-6 in our experimental set-up. In subsequent experiments only these cytokines were taken as a measure to monitor differences in responses by PBMCs.

PBMCs were isolated from peripheral blood by centrifuging over a gradient of hypertonic Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). The 120 alginate beads or APA capsules with or without treatment were cultured in a six-well plate with 10×10^6 PBMCs in RPMI medium. Monensin (3 μ*M*) was added to facilitate accumulation of TNFα and IL-6 in the Golgi complex by interrupting intracellular transport processes. The calcium alginate beads or microcapsules were incubated with the PBMCs for 4 h at 37°C and 5% CO_2.

Intracellular cytokine production was measured as described before with minor modifications.[33,34] Before staining, 0.3×10^6 cells per well were placed into a round bottom 96-well plate. After centrifugation at 600*g* for 5 min at 4°C the pellet was resuspended and fixed for 5 min in 100 μL of 0.5% paraformaldehyde in PBS. After centrifugation at 600*g* for 5 min at 4°C, the pellet was resuspended in 40 μL phosphate-buffered saline with 0.5% bovine serum albumin and 0.1% NaN_3 containing a saturating dilution of CD14 (PerCP/Cy5 anti human CD14: Biolegend, San Diego, CA) for monocyt staining, while another well was incubated with the isotype control at the same dilution. After incubation for 60 min, cells were centrifuged at 600*g* for 5 min at 4°C, the pellet was subsequently resuspended in saponin solution (phosphate-buffered saline with 0.5% bovine serum albumin and 0.1% NaN_3 and 0.1% saponin) to permeabilize the

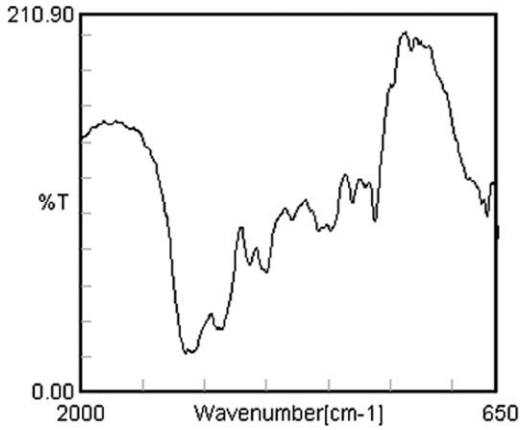

FIGURE 1. MicroFTIR spectrum of peritoneal fluid.

PBMCs for intracellular staining. After 5-min incubation, the plate was centrifuged and aspirated. Then, all the wells were incubated with 40-μL PE labeled mouse anti human TNFα- and APC-labeled mouse anti human Il-6 (both from Biolegend, San Diego, CA) at a saturating dilution, while the other wells were incubated with the isotype control at the same dilution. After incubation for 60 min, cells were washed with saponin buffer and then fixed with 0.5% paraformaldehyde in PBS. The cells were kept in dark at 4°C until analysis by flow cytometry within 24 h.

Flow cytometry

Cells were analyzed with the Calibur (Becton Dickinson, USA). Ten thousand monocytes were acquired whilst gating on CD14 cells (i.e., monocytes). Analysis was performed using Win list 32 (Verity Software House, Topsham, ME). During analysis, a gate was set on $CD14^+$ cells. A single-parameter fluorescence histogram was defined for evaluation of intracellular cytokine production. Using the unstimulated control sample, a linear gate was set in the histogram so that ±99% of the unstimulated cells were negative for cytokine production. This gate was then copied to the stimulated sample. Results are expressed as the percentage of positive cells in the stimulated blood sample.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). Normal distribution of the data was confirmed using the Kolmogorov-Smirnov test. When data were normally distributed, statistical comparisons within the groups were performed by using the paired Student t test. p-values < 0.05 were considered to be statistically significant.

RESULTS

Surface analysis

Microcapsules are mostly transplanted into the peritoneal cavity. To gain insight in the structural changes at the surface of alginate-beads and alginate-PLL capsules after implantation we performed micro-FTIR analyses before and after exposure of alginate beads and alginate-PLL capsules to human peritoneal fluid. This micro-FTIR approach allows for studying intact capsules in the absence of procedures that interferes with the integrity and chemical structure of the capsules.

First the spectrum of peritoneal fluid as such was analyzed in order to identify characteristic peaks. Micro-FTIR does not allow for attribution of the adsorption bands to individual components due to the complexity of the composition of peritoneal fluid. However, as shown in Figure 1, human peritoneal fluid showed some characteristic peaks. Two forks were identified, the first one at 1666 and 1556 cm^{-1}, while the second at 1454 and 1400 cm^{-1}. Moreover, two peaks at 1232 and 1178 cm^{-1} were observed. Next, the spectrum of alginate bead and of alginate PLL capsules before and after incubation with human peritoneal fluid was analyzed. The spectra were compared with those obtained from the individual components of the alginate beads and APA capsules (data not shown). To study the effect of the alginate matrix on adsorption from human body fluids we studied the spectra of peritoneal fluid-exposed calcium alginate beads produced from intermediate-G alginate and high-G alginate.

We first analyzed fresh alginate beads in the absence of a previous incubation in peritoneal fluid. Micro-FTIR spectra of alginate beads prepared of both intermediate-G alginate and of high-G alginate show a broad absorption band in the 3370 cm^{-1} region, which is characteristic for the —OH groups, and a small peak at about 2937 and 2965 cm^{-1}, corresponding to asymmetric $—CH_2$ stretching. It also shows two peaks at 1614 and 1419 cm^{-1}, which are associated with the $—COO^-$ groups of alginate, and an absorption band, similar to a fork with a shoulder and multiple peaks, between 1200 and 1000 cm^{-1}, corresponding to the vibration of C—O bonds [Fig. 2(a,c)]. After incubation with peritoneal fluid the spectra of the alginate beads show some differences. Alginate beads prepared of intermediate-G alginate [Fig. 2(b)] showed after exposure to peritoneal fluid a shift of the peak at 1317 to 1311 cm^{-1}. This related to C—H deformation vibration. Also, we observed a shift of the peak at 1192–1174 cm^{-1} which is associated with changes in the vibration of C—O bonds. This was different with alginate-beads prepared of high-G alginate. Here we observed a broadening of the shoulder in the 1200 cm^{-1} region, which is associated with vibration of C—O bonds [Fig. 2(d)]. Moreover the small peak, associated with the C—H deformation vibration, is observed at 1261 cm^{-1}. Our results demonstrate that molecules in peritoneal fluid seem to modify and influence the vibrational modes of the C—H and C—O bonds in an alginate dependent fashion.

Next the spectra of PLL and of alginate PLL capsules prepared of intermediate-G and high-G alginate were analyzed. From the analysis of the PLL spectrum (data not shown), it is possible to distinguish an absorption band at 2020 cm^{-1}, corresponding to the NH_3^+ group of poly-L-lysine, and a peak at 1625 cm^{-1}, corresponding to the C=O stretching. These characteristic bands could be found back in the spectra of alginate-PLL capsules but was dependent on the type of alginate applied. With alginate-PLL capsules prepared from intermediate-G alginate the absorption band,

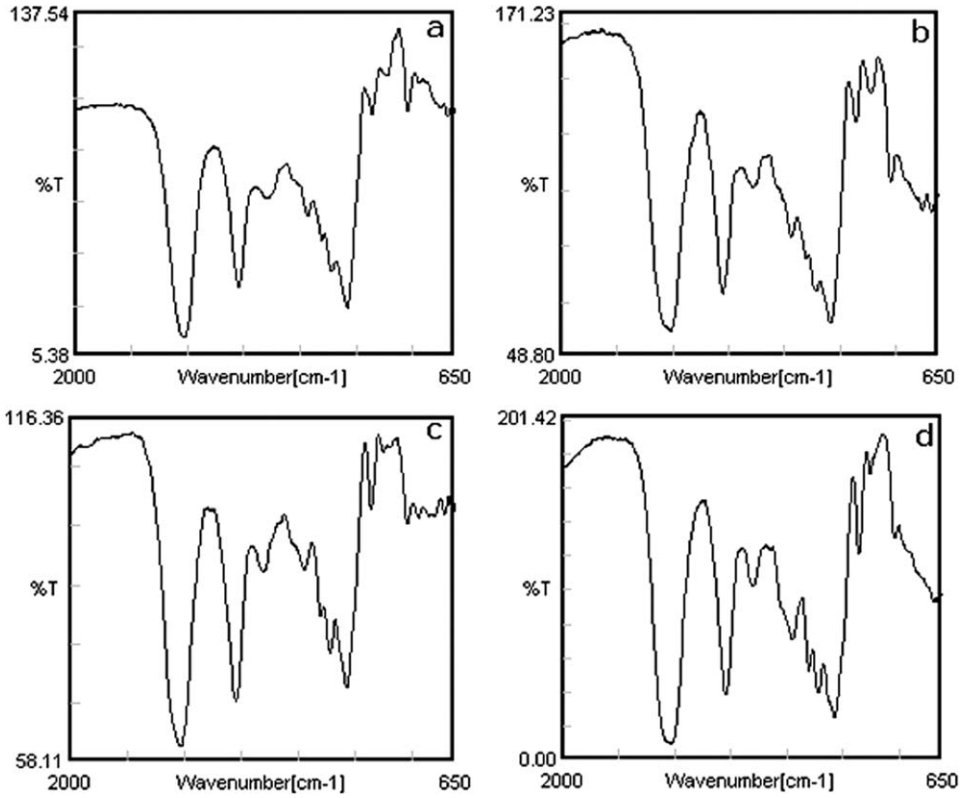

FIGURE 2. MicroFTIR spectra of intermediate-G alginate beads (a), of intermediate-G alginate beads after exposure to peritoneal fluid (b), of high-G alginate beads (c) and high-G alginate beads after exposure to peritoneal fluid (d).

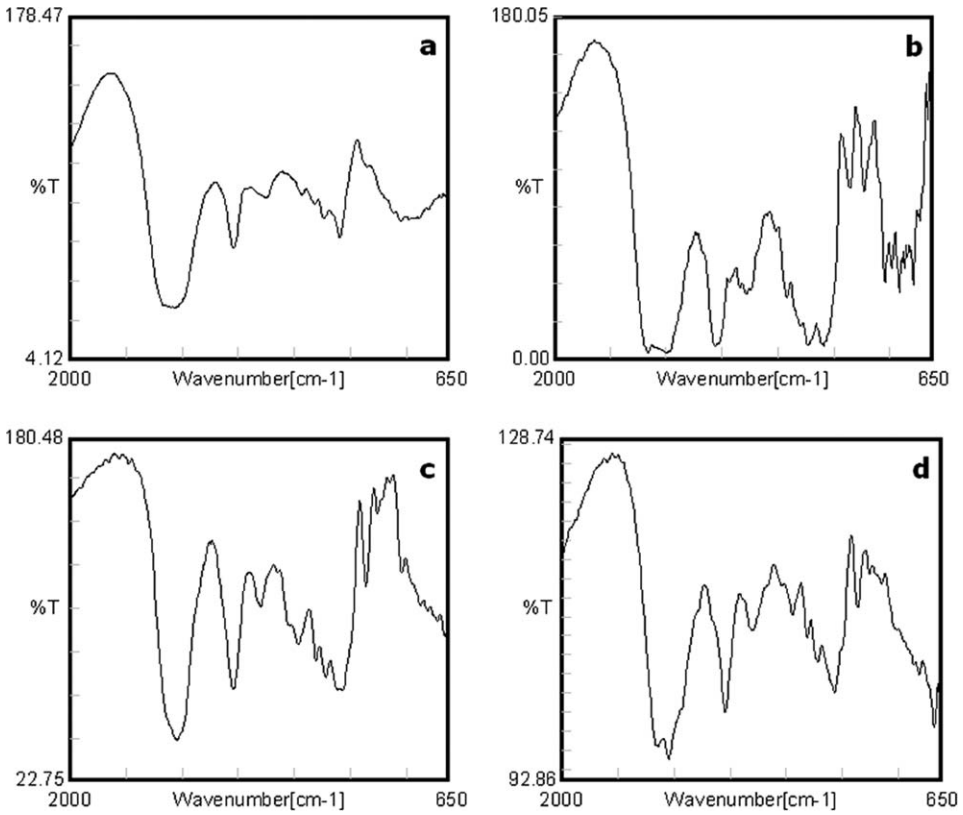

FIGURE 3. MicroFTIR spectra of intermediate-G alginate-PLL-alginate (APA) capsules (a), of intermediate-G APA capsules after exposure to peritoneal fluid (b), of high-G APA capsules (c) and high-G APA capsules after exposure to peritoneal fluid (d).

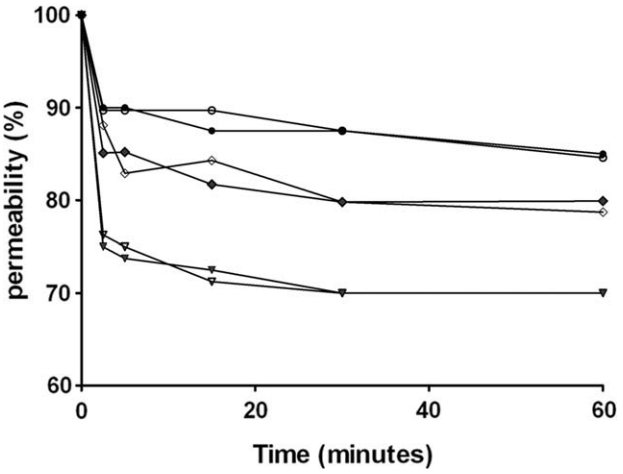

FIGURE 4. Diffusion of glucose (triangles), albumin (circles) and insuline (squares) in freshly prepared intermediate-G capsules (open symbols) or capsules exposed to peritoneal fluid (closed symbols).

corresponding to the NH_3^+ group of poly-L-lysine, shifted from 2020 to 2084 cm^{-1} [Fig. 3(a)]. This shift should be explained by the interaction of NH_3^+ of poly-L-lysine with COO^- of sodium alginate. The peak related to the C=O stretching shifts to 1641 cm^{-1}. This band is reinforced and broadened by both the amidic C=O in poly-L-lysine and the carboxylic C=O in sodium alginate. In the case of alginate-PLL capsules prepared of high-G alginate the absorption band, corresponding to the NH_3^+ group of poly-L-lysine, shifted from 2020 to 2065 cm^{-1} [Fig. 3(b)]. The peak related to the C=O stretching shifts to 1619 cm^{-1}. In both the spectra of intermediate-G and high-G alginate APA-capsules, a slight broadening and indentation of the absorption band in the 3800–2800 cm^{-1} region was observed.

When alginate-PLL capsules were exposed to human peritoneal fluid we found characteristic changes in the spectra which again was dependent on the type of alginate applied. With intermediate-G alginate PLL capsules exposed to peritoneal fluid we observed shifts of the band at 2138 and the peaks at 1671, 1425, 1313, and 1043 cm^{-1} [Fig. 3(c)]. Moreover, the peak at about 1556 cm^{-1}, only observed in peritoneal fluid, shifts to 1587 cm^{-1} on intermediate-G alginate-PLL capsules. With high-G PLL capsules we found the shift of the band at 2107 and the peaks at 1658, 1178, and 804 cm^{-1} [Fig. 3(d)]. The most significant changes are associated with the NH_3^+ group and the C=O stretching of poly-L-lysine and to the vibration mode of C=O and C—H groups of alginate.

Effects of adsorption on permeability

To determine whether adsorption of peritoneal fluid factors is influencing the functional properties of the capsules we studied the diffusion of a number of solutes into the alginate beads and APA capsules before and after exposure to human peritoneal fluid. We determined the permeability of the capsules for glucose, albumin, and insulin. We found decreases in the first minutes after start of the test ($p < 0.05$) but never found any difference in diffusion in the presence or absence of a previous incubation in peritoneal fluid (Fig. 4, only data shown for intermediate-G alginate-PLL capsules). We therefore conclude that the adsorption of peritoneal fluid components had no influence on the permeability diffusion properties of the capsules for the tested solutes.

Effects on immune activation

Another important functional property of capsules, which should not be influenced by adsorption of body fluid components is their compatibility with the immunological environment. We therefore determined the activation of human PBMCs after contact with alginate beads and capsules exposed to peritoneal fluid. To this end, alginate beads or capsules were incubated with peripheral blood PBMCs for a period of 4 h, harvested, and stained for the activation markers TNFα and IL-6.

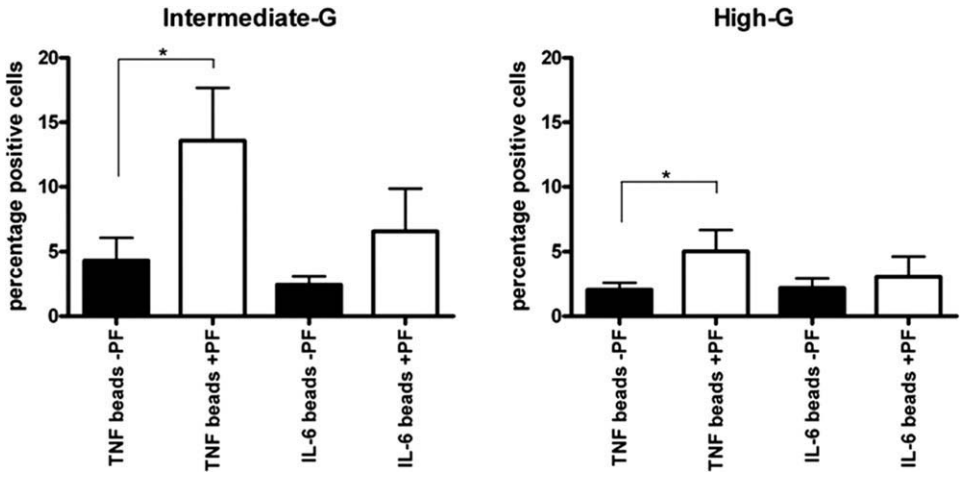

FIGURE 5. Number of TNFα and IL-6-positive PBMC after incubation with intermediate-G and high-G alginate beads in the presence and absence of a previous exposure to human peritoneal fluid (PF). *$p < 0.05$.

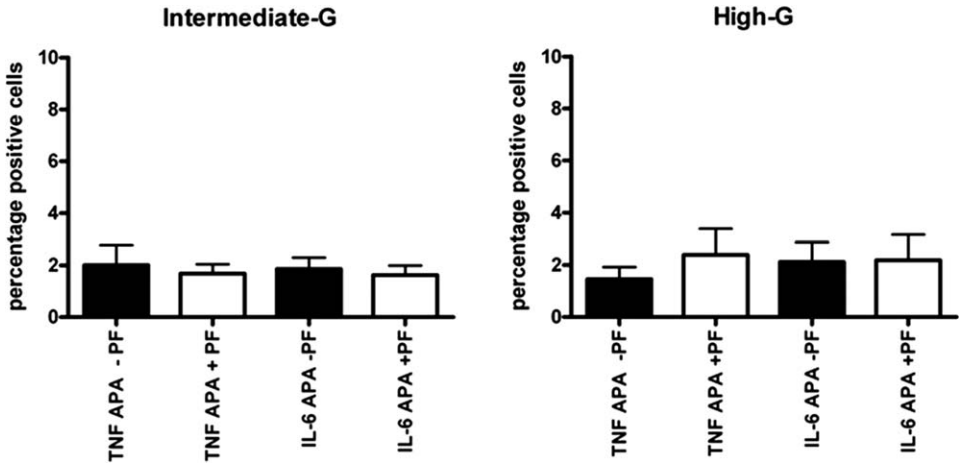

FIGURE 6. Number of TNFα and IL-6-positive PBMC after incubation with intermediate-G and high-G alginate alginate PLL capsules (APA) in the presence and absence of a previous exposure to human peritoneal fluid (PF).

PBMCs exposed to intermediate-G alginate beads showed an increase in TNF-α and IL-6 expression after peritoneal fluid exposure which reached statistical significant differences for TNF-α ($p < 0.05$) [Fig. 5(a)]. Also with high-G alginate beads we observed an increase in TNF-α ($p < 0.05$) and IL-6 after peritoneal fluid exposure [Fig. 5(b)]. The increase was alginate-dependent as exposure to peritoneal fluid showed less pronounced effects on high-G beads than on intermediate-G beads [$p < 0.05$, Fig. 5(a) vs. 5(b)].

Next, we determined the activation of PBMCs after exposure to alginate-PLL capsules. A first observation was that PLL binding to the membrane reduced the body fluid-induced activation of the PBMCs for both types of alginates (see Figs. 5 vs. 6). The PLL binding on the alginate matrix abandoned the increased inflammatory response of PBMCs after exposure to peritoneal fluid. We found no statistical significant increases or decreases for TNF-α and IL-6 production from PBMCs.

DISCUSSION

In the present study we applied micro-FTIR instead of the more commonly applied FT-IR spectrometry.[22,23] Micro-FTIR has some advantages over other FT-IR technologies since it allows for studying of focalized sections of non-freeze dried, intact capsule membranes. To assure that we were only studying the surface, we placed the infrared reflectance beam on the outer edges of the APA-capsules or alginate-beads instead of on the core. This was done microscopically which makes it reasonable to assume that only the surface of the alginate beads or microcapsules was analyzed. Because of the size of the studied alginate beads and APA capsules, the penetration depth of the radiation from this infrared reflectance technique (4.4 μm at 1000 cm^{-1}) is many times smaller than the diameter of the alginate beads and APA capsules. It is reasonable to assume together with positioning of the beam that in this study the surface of the alginate beads and APA capsules was analyzed. Although, in contrast to FT-IR analyses of freeze dried samples some H_2O may be present in the micro-FTIR spectra, the technology is sensitive enough to detect the small but pertinent differences on the capsule surface after exposure to human peritoneal fluid.

A striking observation is that alginate beads and APA capsules showed a unique change in intramolecular and intermolecular interactions at the surface of alginate beads and APA capsules after exposure to human peritoneal fluid. This suggests that adsorption and the consequent structural surface changes are highly dependent of the materials applied in capsule fabrication. This corroborates the findings of others with other biomaterials demonstrating unique adsorption patterns with different materials but also with different body fluids such as serum.[35–39] Also our findings show that adsorption of various substances present in peritoneal fluid always occurs. The surface changes were highly alginate-type dependent. With both alginate beads prepared of intermediate-G alginate we found changes in the spectra associated with C—O bonds and C—H groups deformation vibration but the frequency and shifts were different for alginate beads prepared of intermediate-G alginate and high-G alginate.

The adsorption on alginate beads was quantitatively not high enough to cause interference with the diffusion of the tested solutes but it did influence the responses of human PBMCs. PBMCs have been applied in many studies to predict the human immunological responses against biomaterials.[40–44] PBMCs did not respond with elevated TNFα and IL-6 production when exposed to fresh capsules. A minority of less than 4% of the cells (equal to medium control) expressed TNFα and IL-6. This changed when the alginate beads were exposed to peritoneal fluid. Especially the number of TNFα expressing cells increased significantly. With both intermediate-G and high-G beads we found a two- to threefold increase in TNFα expressing cells in the PBMCs.

PLL coating is applied on alginate beads to provide immunoprotection.[7] Binding of PLL induced a characteristic broad absorption band at 1625 cm^{-1}, corresponding to the C=O stretching and by the amidic C=O in poly-L-lysine and the carboxylic C=O in sodium alginate. Also we could clearly

characterize the binding of NH_3^+ groups of poly-L-lysine with COO^- of sodium alginate by the shift from 2020 to 2183 cm^{-1}. These characteristic changes in the transition from Ca-bead to alginate-PLL could not be as clearly visualized with conventional FT-IR.[22] This illustrates the strength of micro-FTIR in characterizing the surface of microcapsules.

After exposure of the alginate-PLL capsules to peritoneal fluid, we found characteristic changes in the spectra. These changes were associated to the vibration of NH_3, C=O, and C—H groups. We found no significant changes in the C—O bond region that was characteristic for alginate-beads exposed to peritoneal fluid. These changes in the NH_3, C=O, and C—H groups were, however, not associated with increased responses in PBMCs. PBMCs exposed to alginate-PLL capsules showed in less than 3% of the cells expression of either TNFα or IL-6. This percentage was not increased after exposure to peritoneal fluid and was not alginate-type dependent.

It may be argued that our findings do not corroborate the results of others[45–50] who have demonstrated that PLL enhances the inflammatory responses both *in vitro* and *in vivo* in animal models. During recent years, however many advances have been made in understanding the complex binding of PLL onto alginate-based capsule surfaces.[19,21–23,31,51,52] Although, this is not the subject of the present study we have shown that when sufficient interactions are possible between PLL and alginate molecules, PLL will form random coil formations[31] that is associated with the formation of a stable, biocompatible membranes. The biological relevance of this finding was illustrated in a recent study showing that alginate-PLL capsules can stay intact and provide optimal biocompatibility up to two years in rats.[19]

Capsules prepared of high-G alginates have some advantages over capsules prepared of intermediate-G alginates. In *in vitro* studies it has been shown that capsules prepared of high-G alginates have a higher mechanical stability than capsules prepared of intermediate-G alginate.[21,30,53,54] Also they contain much lower numbers of incompletely and therefore inadequately encapsulated cells.[55,56] However, in experimental animals we consistently found a more pronounced inflammatory response to alginate-PLL capsules prepared of high-G alginate when compared to capsules prepared of intermediate-G alginate. This was not observed in the present study with human PBMCs. This might be interpreted as a suggestion that responses in humans and experimental animals are different. However, it cannot be excluded that in long term *in vivo* studies in humans other observations will be done.

Responses against microcapsules have an immediate phase that is mainly driven by innate immune responses[20,57] and a secondary, later response driven by leakage or digestion of capsule components.[12,50,58–61] The PBMCs responses against capsules exposed to peritoneal fluid is a model for the prediction of the immediate response against alginate-beads or APA-capsules. This immediate response has been shown to be responsible for loss of a significant portion of the encapsulated tissue.[9,20,62] With the combined technology of micro-FTIR and human PBMC responses we show for the first time in a human setting with peritoneal fluid (i.e., the fluid in the transplantation site) that surface changes as observed in experimental animals also occur in human settings and that this may results in inflammatory responses.[9,20] The technology may even serve for high through-put screening of new capsule formulations for adsorption and for testing functional consequences (i.e., immunological activation) of new capsule components.

This response to alginate-PLL capsules seems to be very modest in humans but strong against Ca-alginate beads. The strong responses against Ca-alginate-beads was associated with shift in the C—O region which suggests that interactions between components in the peritoneal fluid and C—O should be hold responsible for the increased inflammatory responses. This suggests that in contact with human PBMCs free C—O groups should be avoided in order to prevent undesired adsorption from human peritoneal fluid.

Our study confirms our previous findings that measuring responses against fresh microcapsules is not sufficient to predict the biocompatibility of alginate beads and APA capsules *in vivo*. This also seems to hold true for the human situation. Upon exposure to body fluids both alginate beads and APA capsules will adsorb bioactive components which will change the properties of the capsule surface and the responses against the capsules. In recent studies we showed that fluctuation in pH in body fluids changes the properties and charge density of capsules making capsules more susceptible for adsorption of body proteins and inflammatory responses.[17,18,20,21] Other factors that may influence the biocompatibility is adsorption of immunoglobulin's[18] and adsorption of other types of proteins.[61] Also this has been repeatedly shown to occur on alginate-beads and APA-capsules.[17,18,20,21]

Ultimately, biocompatible capsules should be created that are not susceptible to adsorption of body fluid components. However, up to now nobody has been able to design such a capsule. Therefore, it is advisable to test capsules not only under standard laboratory conditions but also to test them under the pathophysiological conditions mimicking the microenvironment in the transplantation site.[17] Our presented test system in which micro-FTIR is applied to study structural changes on the capsule surface in combination with measuring PBMC responses after exposure to human body fluids may be an effective screening tool to select capsule systems with the potential to be applied in humans. Such a readily available testing system is to our opinion a pertinent step to predict the clinical success or failure of the capsules. Our data suggest that these human responses might be different than observed in experimental animals.

CONCLUSIONS

We demonstrate that adsorption and physicochemical changes of the surface always occur when alginate beads or alginate-PLL capsules are exposed to human peritoneal fluid. The structural surface changes are highly dependent on the chemical composition of the capsules. Even

seemingly minor changes in guluronic acid content of alginate beads give different micro-FTIR profiles of the surface and different PBMC responses.

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