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Alginate microsphere compositions dictate different mechanisms of complement activation with consequences for cytokine release and leukocyte activation

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Key words: alginate microcapsules, complement component 3, CR3 (CD11b/CD18), C5a, inflammation, polycation

Abstract

The inflammatory potential of 12 types of alginate-based microspheres was assessed in a human whole blood model. The inflammatory potential could be categorized from low to high based on the four main alginate microsphere types; alginate microbeads, liquefied core poly-L-ornithine (PLO)-containing microcapsules, liquefied core poly-L-lysine (PLL)-containing microcapsules, and solid core PLL-containing microcapsules. No complement or inflammatory cytokine activation was detected for the Ca/Ba alginate microbeads. Liquefied core PLO- and PLL-containing microcapsules induced significant fluid phase complement activation (TCC), but with low complement surface deposition (anti-C3c), and a low proinflammatory cytokine secretion, with exception of an elevated MCP-1(CCL2) secretion. The solid core PLL-containing microcapsules generated lower TCC but a marked complement surface deposition and significant induction of the proinflammatory cytokines interleukin (IL-1) β , TNF, IL-6, the chemokines IL-8 (CXCL8), and MIP-1 α (CCL3) and MCP-1(CCL2). Inhibition with compstatin (C3 inhibitor) completely abolished complement surface deposition, leukocyte adhesion and the proinflammatory cytokines. The C5 inhibitions partly lead to a reduction of the proinflammatory cytokines. The leukocyte adhesion was abolished by inhibitory antibodies against CD18 and partly reduced by CD11b, but not by CD11c. Anti-CD18 significantly reduced the (IL-1) β , TNF, IL-6 and MIP-1 α and anti-CD11b significantly reduced the IL-6 and VEGF secretion. MCP-1 was strongly activated by anti-CD18 and anti-CD11b. In conclusion the initial proinflammatory cytokine responses are driven by the microspheres potential to trigger complement C3 (C3b/iC3b) deposition, leukocyte activation and binding through complement receptor CR3 (CD11b/CD18). MCP-1 is one exception dependent on the fluid phase complement activation mediated through CR3.

1. Introduction

In the field of cell-encapsulation therapy, the host inflammatory responses to the transplanted devices remain a major challenge. In order to design functionally performing microspheres for cell-encapsulation therapies it is of crucial importance to understand the interplay between the biomaterial and the host defense system. The surface of the microspheres represents the interface between the material and the biological factors and is critical for the initial inflammatory reactions. Potentially, the early inflammatory reactions serve as the starting points for a chronic inflammatory state [1] and could determine the success or failure of a cell-encapsulation device upon transplantation. A functional microsphere containing encapsulated cells would require an adequate exchange of oxygen, nutrition and waste products at the transplantation site which is hampered by cellular overgrowth. In general, host proteins are immediately covering the biomaterials upon transplantation, and through conformational changes and activated proteins [2-4] serve as the starting point for inflammatory reactions and cellular adhesion. A connection between the physico-chemical properties of alginate-based microspheres and the host responses are recognized as important for the functional performance, nevertheless, the direct correlation and mechanisms leading to cellular adhesion are only partially understood [5]. Alginate is the collective term of unbranched polysaccharides consisting of the two sugar residues 1-4 β -D-mannuronic acid (M) and α -L-guluronic acid (G) in variable amounts of alternating or block structures with impact on its properties [6]. It is the most used material for cell encapsulation due to the ability to form gels under cell-friendly conditions [6], and in combination with a low ability to bind proteins, alginate microbeads has been regarded as suitable candidates for cell encapsulation even in the clinical setting [5]. However, the combinations of alginate with polycations and different gelling protocols lead to variability in the final physico-chemical properties with consequences for *in vivo* performance.

The complement system is easily activated upon contact with biomaterial surfaces [7]. C3 is the key protein of the complement cascade, and is abundantly present in plasma. In addition, C3 is found in the peritoneal fluid [8] as well as in subcutaneous and omental adipose tissue [9]. C3 is a contributor to the biomaterial-induced inflammation during blood contact [10], and recently C3 was demonstrated to be involved in the inflammatory host response against subcutaneous and intraperitoneally implanted

meshes [11]. The complement system consists of a cascade of serine proteases acting in sequence, initiated through three different pathways: the classical pathway, the lectin pathway, and the alternative pathway. The first two can be activated through antibody binding or by pattern recognition, whereas the alternative pathway can be activated through spontaneous activation of C3 to nearby surfaces. All three pathways converge upon the activation of C3, which mainly serves as an amplification loop. The subsequent events lead to the formation of the C3 and C5 convertases and the formation of activation products. One of the activation products C5a is a strong chemoattractant and a potent contributor to inflammation [12]. The C3 convertase's can be established on surfaces of biomaterials further accelerating the formation of C3 to C3b. C3b is rapidly converted to iC3b, which is the main ligand for the leukocyte adhesion and phagocytosis receptor CR3 (CD11b/CD18) and a ligand also for CR4 (CD11c/CD18).

The interplay between complement and the leukocytes can be studied using a whole blood model based on specific blockage of thrombin for anticoagulation [13]. This is an efficient physiologically relevant model to study the earliest inflammatory events and gives the possibility to evaluate a set of microspheres under identical conditions. Using the whole blood model, we previously demonstrated differences between alginate-based microcapsules, prepared in the presence of a polycation, and alginate microbeads, prepared by gelling alginate droplets with divalent cations, in the ability to activate complement [14] and inflammatory cytokines [15].

In the present study, the whole blood model was employed on microspheres to evaluate the inflammatory properties of 12 different alginate-based microspheres, divided into the following groups: alginate microbeads, liquefied core alginate–poly-L-ornithine (PLO) microcapsules, liquefied core alginate–poly-L-lysine (PLL) microcapsules and solid core alginate–PLL microcapsules. Evident differences in the complement activation patterns were determined for various microspheres, which significantly contributes to correlation between the potential of inflammatory cytokines and the biomaterial-induced complement activation.

2. Materials and Methods

2.1 Reagents and materials

The present study utilized ultrapure high G sodium alginate (*Laminaria hyperborea*, 67% G residues, UP-LVG) and intermediate G sodium alginate (*Macrocystis pyrifera*, 42% G residues, UP-100M) from FMC BioPolymer AS (Novamatrix, Norway) with further characteristics given in Table 1.

Table 1.

Chemical composition and sequence distribution obtained from ¹H-NMR spectra of alginates used in this work.

Source	Batch	F _G	F _{GG}	F _{MG/GM}	F _{MM}	F _{GGM/MGG}	F _{MGM}	F _{G₃G₃}	N _{G>1} ^{b)}	M _w ^{c)} (x10 ³) ^a	M _w /M _n ^a	[η] ^{d)} (ml/g) ^b	Endotoxin (EU/g) ^{e)}	Proteins (%) ^{e)}
<i>Laminaria hyperborea</i> , stipe	UPLVG FP-603- 04	0.67	0.55	0.12	0.21	0.05	0.09	0.5	12	187	1.99	1051	<100	<0.3
<i>Macrocystis pyrifera</i>	UP100M FP-209- 02	0.42	0.48	0.22	0.39	0.03	0.16	0.19	8	200	2.3		<100	<0.3

a) FG, molar fraction of guluronic acid, FGG and FG₃G₃ are dyad and triad frequencies respectively

b) N_{G>1} the average of number of consecutive units in the G blocks

c) The weight average molecular weight given in Dalton measured by SEC-MALLS

d) The intrinsic viscosity was measured at 20°C in 0.1M NaCl aqueous solution in a Micro Ubbelohde viscometer.

e) Content given by the manufacturer.

Other reagents for microspheres formations were: D-mannitol BDH Anala R., VWR International (Ltd, Pool, England), analytical grade calcium chloride, barium chloride and Na-Citrate tribasic dehydrate were all from Merck, Darmstadt, Germany. Poly-L-lysine hydrochloride (P2658, lot nr.091K5120, MW 20900 Da), Poly-L-ornithine hydrobromide (P3530, lot nr. 48F503, MW 23000 Da) and HEPES (H4034), Zymosan A (Z-4250), LPS from *E.coli* strain 0111:B4 (Invivogen, San Diego, CA), PBS with calcium and magnesium, EDTA, paraformaldehyde, and BSA were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Non-pyrogenic sterile saline (0.9% NaCl) and endotoxin free, non-pyrogenic, water (50 ml) were from B. Braun (Melsungen, Germany).

Reagents for the whole blood assay and analysis were as follows: The anti-coagulant lepirudin was obtained from Celgene Europe, Boudry, Switzerland. The C3 inhibitor compstatin analog 1MEW and CP20 [16] with a corresponding control peptide described in [15] was synthesized and kindly provided from the laboratory of Prof. John D. Lambris. A monoclonal anti-C5 antibody eculizumab (Soliris®, Alexion Pharmaceuticals, Lausanne, Switzerland) was used to inhibit C5. For inhibition of the CR3 and CR4 receptors, the following antibodies were used; CD11b, ultra-leaf purified anti-mouse/human CD11b (Rat IgG2b clone M1/70) with the control ultra-leaf purified Rat IgG2b ; CD18, ultra-leaf purified anti-human CD18 (Mouse IgG1 clone TS1/18); CD11c, ultra-leaf purified anti-human CD11c (Mouse IgG1 clone 3.9) with the control ultra-leaf Mouse IgG1 clone MOPC-21 (all from Biolegend, San Diego, CA). Other antibodies were anti-CD11b PE (BD Biosciences, San Jose, Ca), anti-CD14 FITC (BD Biosciences, San Jose, CA), anti-human C5b-9 clone aE11 (Diatech, Oslo, Norway), biotinylated 9C4 (an in-house antibody described in [17]), FITC conjugated rabbit anti-human C3c (F0201), detection C3 and all its fragments except for C3a and C3d, and FITC conjugated poly- rabbit anti mouse (F0261) from Dako (Glostrup, Denmark). Streptavidin-PE was from BioLegend (San Diego, CA) and substrate reagent A and B from R&D Systems (Minneapolis, MN). Equipment for blood sampling included polypropylene vials (NUNC, Roskilde, Denmark) and BD vacutainer top (Belliver Industrial Estate, Plymouth, UK).

2.2 Microsphere preparation

The microspheres were made using filter-sterilized solutions under strictly sterile conditions, autoclaved equipment and sterile hood in all steps. Endotoxin was measured by Endpoint Chromogenic LAL assays (Lonza) according to producers manual. The endotoxin content for the polycation solutions and gelling solutions were below 20 pg/ml. In brief, the alginate microbeads were made of a low-viscosity high G alginate by the mixture of $\text{Ca}^{2+}/\text{Ba}^{2+}$ ions in the molar ratio 50/1 in three different sizes, the liquefied core alginate–PLO and alginate–PLL microcapsules were made of intermediate G alginate complexed with either PLO or PLL in two different concentrations and subsequently citrate-treated and the solid core alginate–PLL microcapsules were made of either intermediate G or high G alginate, complexed with PLL in two different concentrations and sizes.

More detailed, the core alginates were made as previously described [18] using 1.8% UP-LVG or 1.8% UP-100M alginate (Novamatrix) dissolved in 300 mM M mannitol. The gelling solutions used for Ca/Ba microbeads were 1 mM BaCl₂/50 mM CaCl₂/150 mM mannitol/10 mM HEPES, whereas for the microcapsules 50 mM CaCl₂/150 mM mannitol/10 mM HEPES gelling solution was used. For each microsphere type, 5 ml of alginate was dripped into the gelling bath using a high-voltage electrostatic bead generator operating at 7kV. The microcapsules were made using 4 needles of internal diameter (ID) size of 0.35 or 0.40 mm and flow of 10 ml/h per needle. The microbeads were made with single needle either of ID 0.25mm and with flow rate of alginate solution of 6ml/h, or of needle ID (mm) equal to 0.35mm or 0.4mm with the flow rate of alginate solution of 8ml/h. In all cases the microbeads were gelled for 10 min after the last droplet formation. Microcapsules were prepared by soaking the microbeads in polycation solutions (25 ml saline containing from 0.05 to 0.14 % PLL or 0.14% PLO) for 10 minutes, and further incubated in 0.1% UP-100M in saline for 10 min. Liquefied core microcapsules were made by an additional step incubating microcapsules in 10 ml Na-citrate solution (55 mM) for 10 minutes. Between each step the microspheres were washed in 30 ml saline. After the final step, microcapsules were dispersed in 12 ml of saline and 10 aliquots containing approximately 0.5 ml of microspheres. The endotoxin content of the microspheres storing solutions was all below 20 pg/ml. The microspheres diameters (N=30 for each type) were measured using a Nikon TMS microscope at resolution 10x and with an ocular (CFWE10xA/18 Nikon, Japan) containing an internal scale bar. Before each whole blood assay, 0.5 ml microspheres were additionally divided into 10 samples each containing approximately 50 µl of microspheres. The overview of microspheres composition and final sizes are given in Table 2.

Table 2. *Microspheres preparation conditions and final size.*

Microsphere type	Alginate type	F _G (%G)	Polycation	Polycation conc. (%)	Needle size (mm)	Gelling ions	Citrate	Microsphere Size±SD (µm)
APA Liquefied core	UP100M	42	PLL	0.10	0.40	Ca ²⁺	+	683 ±70
APA Liquefied core	UP100M	42	PLL	0.14	0.40	Ca ²⁺	+	645 ±57
APA Liquefied core	UP100M	42	PLO	0.14	0.40	Ca ²⁺	+	652 ±48
APA Solid core	UP100M	42	PLL	0.05	0.40	Ca ²⁺	-	647 ±85
APA Solid core	UP100M	42	PLL	0.10	0.40	Ca ²⁺	-	588 ±53
APA Solid core	UP-LVG	67	PLL	0.05	0.40	Ca ²⁺	-	566 ±45
APA Solid core	UP-LVG	67	PLL	0.10	0.40	Ca ²⁺	-	572 ±22
APA Solid core	UP-LVG	67	PLL	0.05	0.35	Ca ²⁺	-	573 ±40
APA Solid core	UP-LVG	67	PLL	0.10	0.35	Ca ²⁺	-	554 ±26
Ca/Ba microbeads	UP-LVG	67	-	-	0.40	Ca ²⁺ /Ba ²⁺	-	589 ±15
Ca/Ba microbeads	UP-LVG	67	-	-	0.35	Ca ²⁺ /Ba ²⁺	-	477 ±32
Ca/Ba microbeads	UP-LVG	67	-	-	0.25	Ca ²⁺ /Ba ²⁺	-	343 ±14

Alginate characteristics are given in Table 1. The gelling solution designated as Ca²⁺: 50 mM CaCl₂/150 mM mannitol/10 mM HEPES. The gelling solution designated as Ca²⁺/Ba²⁺: 1 mM BaCl₂/50 mM CaCl₂/150 mM mannitol/10 mM HEPES. PLL: Poly-L-lysine hydrochloride (P2658, MW 20900 Da), PLO: Poly-L-ornithine hydrobromide (P3530, MW 23000 Da). The polycation-containing microcapsules were made using four needles of different internal diameters, each with a flow of alginate solution of 10 ml/h. The Ca/Ba microbeads were made with a single needle of internal diameter 0.25 mm at the flow rate of alginate solution of 6 ml/h (needle) or at the flow rate of alginate solution of 8 ml/h using the needles of internal diameter 0.35 and 0.4 mm. The high voltage electrostatic bead generator was employed for all microsphere preparation operating at 7 kV.

2.3 Whole blood model

Whole blood from voluntary donors (N=3-7 donors per experiment as stated in the figures) was collected in polypropylene vials containing lepirudin (50 µg/ml). Polypropylene vials (NUNC 1.8 ml) were utilized for various microcapsules and controls following the previously established protocol [13, 14], which includes a washing step in sterile saline (medical quality) followed by an aliquot step. Briefly, samples of 100 µl saline containing microspheres (approximately 50 µl), zymosan (10 µg) or LPS (7 ng) were added to the polypropylene vials. Thereafter 100 µl PBS (with Ca²⁺/Mg²⁺) was added followed by the addition of 500 µl of blood. Samples were incubated for 60 and 240 minutes prior to complement inhibition by EDTA of final concentration equal to 10 mM. Aliquots of EDTA inactivated plasma were stored at -20°C prior to analysis. In the inhibitory experiments utilizing C3 inhibitor compstatin, the C5 inhibitor eculizumab, the ultra-leaf anti-CD11b, anti-CD18 or anti-CD11c or its controls, blood was pre-incubated with the inhibitors or PBS control prior to the addition to the stimuli in the ratio blood: inhibitors or control equal to 5:1. After pre-incubation for 7 min, 600 µl of pre-

incubated blood was added to the microspheres samples. The final concentration of compstatin was 10-25 μ M, eculizumab was 100 μ g/ml and the ultra-leaf antibodies were 40 μ g/ml.

2.4 Complement

Activation of complement in the fluid-phase was measured by the terminal soluble C5b-9 complement complex (TCC) by an ELISA using TCC specific capture Ab (aE11 reacting with a C9 neoepitope exposed only when C9 is incorporated in the C5b-9 complex) and detection Ab (biotinylated anti-human C6) as described previously [19]. C5a was measured using Human C5a OptEIA Kit II (BD Biosciences). Optical density was measured using the Bio-Rad plate reader (Bio-Rad Laboratories, CA, USA) and analyzed further using Microplate Manager software (version 6.1).

2.5 Cytokines

Cytokines, including interleukins, chemokines and growth factors, were analyzed by a multiplex cytokine assay (Bio-Plex Human cytokine 15-Plex Panel using a Bio-Plex 200 system and Bio-Plex Pro Wash station; all from Bio-Rad Laboratories, Hercules, CA) containing the following analytcs: IL-1 beta (IL-1 β), IL-1 receptor antagonist (IL-1RA), IL-6, IL-8 (CXCL8), IL-10, interferon gamma (INF- γ), chemokine (C-X-C motif) ligand 10 (IP-10 or CXCL10), monocyte chemoattractant protein-1 (MCP-1, or CCL2), macrophage inflammatory protein-1-alfa (MIP-1 α , or CCL3), platelet derived growth factor-BB (PDGF-BB), regulated upon activation T-cell expressed and secreted (RANTES, or CCL5), tumor necrosis factor alfa (TNF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and macrophage migration inhibitory factor (MIF). The multiplex analyzes were performed as recommended by the producer using half amounts of beads. In addition, IL-8 and TNF were also analyzed by ELISA (Duoset, R&D systems) following recommended protocols.

2.6 Leukocyte CD11b expression

Expression of monocyte and granulocyte CD11b was determined after 60 minutes incubation in whole blood. Blood (50 μ l) was fixed in 0.05% PFA, and further stained by PE anti-CD11b (BD Biosciences) and FITC anti-CD14 (BD Biosciences) for 15 minutes. Thereafter, the samples were transferred to 500 μ l EasyLyse erythrocyte lysis buffer following the 15 minutes incubation. Monocytes and granulocytes were analyzed in Epics XL-MCL Flow cytometer (Beckman Coulter, Inc., Brea, CA, USA), gated in a SSC/CD14 plot and the CD11b activation was measured as the median fluorescence intensity (MFI).

2.7 C3c deposition

Selected alginate microspheres were incubated in human plasma (300 μ l) or in whole blood (500 μ l) for various times. In addition, microspheres incubated either in plasma, serum or whole blood for four hours was evaluated. Then the surface was stained for C3c using FITC-conjugated rabbit anti-human C3c (50 μ g/ml) FITC conjugated poly-rabbit anti-mouse (50 μ g/ml) used as a control. The surface of the microspheres was evaluated using CLSM (Zeiss LSM 510 Meta, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) at the CMIC core facility at NTNU with the following settings: excitation wavelength 488 nm, emission wavelength BP 505-530. Optical cross-sections through the equator were taken using 488 nm laser and differential interference contrast (DIC), and 3D projections were made from z-stacks through the entire microspheres using the 488 nm laser only. The employed objective for imaging was C-Apochromat 10x/0.45W.

2.8 Statistical methods

The comparison of various microspheres with saline control was done by a one-way analysis of variance (ANOVA) with Dunnett's post-test for comparison with the saline control, and with Tukey's post-test for comparison between the various groups of microspheres (PLL liquefied, PLO liquefied, PLL solid, Beads) or individual microspheres. The data was log-transformed since N was too small to assume normality. The non-parametric Mann-Whitney test ($N \leq 6$) or Wilcoxon matched pairs signed

rank test ($N \geq 7$) was used for comparison of various inhibitors with their respective controls. Both tests were performed using Graph Pad Prism 5 for Windows version 5.03. Data were considered statistically significant with $P < 0.05$.

2.9 Ethics

The use of human whole blood for basal experiments was approved by the Regional Ethic Committee at NTNU in Norway. The experiments were performed in accordance with their guidelines.

3 Results

3.1 Fluid-phase complement activation (TCC)

TCC was variably induced and was dependent on the microsphere compositions (Fig. 1). The liquefied core PLL microcapsules induced a prominent and significant activation of TCC as compared to the other microspheres categories (PLO liquefied, PLO solid or Beads) after four hours incubation (Fig. 1).

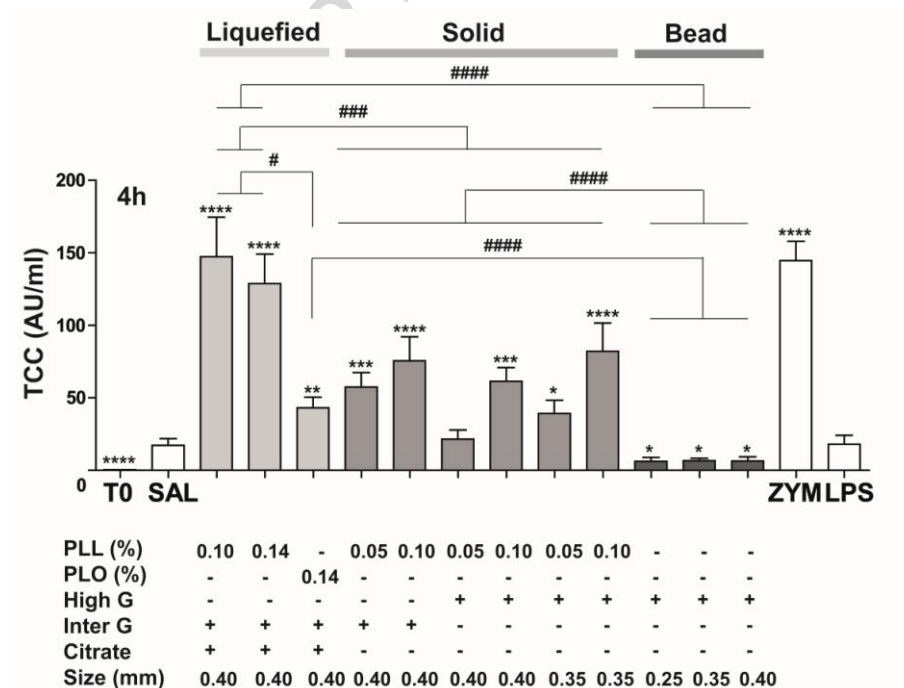


Figure 1. Fluid phase C5b-9 (TCC) after incubation of microspheres in human whole blood for four hours. T0: baseline value at blood withdrawal, SAL: background activation from saline, ZYM and

*LPS are the positive controls. Bars are means \pm SEM, N = 5 (N=4 for microbeads made with needle ID 0.25 and 0.4 mm). * compared to the saline control, # comparison between microspheres categories. #P \leq 0.05, ##P \leq 0.01, ###P \leq 0.001, ####P \leq 0.0001 (*correspondingly).*

The liquefied core PLL microcapsules also induced a prominent and significant activation as compared to the other microspheres already after one hour incubation (Suppl. S1). The liquefied core PLO microcapsules induced a lower amount of TCC, but statistically significant more than the saline control and the Ca/Ba Beads (Fig. 1 and Suppl. Fig. S1). The corresponding PLL microcapsules with a solid core induced significant amount of TCC but still substantially lower amount than the liquefied core counterparts (Fig. 1). The amount of TCC induced by the Ca/Ba microbeads was significant lower than the saline control (Fig. 1). The other variables induced smaller and mostly non-significant differences (Supplementary Table 1) that can be summarized in the following; For the solid core PLL microcapsules the highest concentration of PLL (0.1%) induced more TCC than the lower concentration (0.05%), while the opposite tendency was found for the liquefied core microcapsules (Fig. 1). The intermediate G solid core PLL microcapsules showed a slight elevation compared to microcapsules made of high G alginate (Fig. 1). The solid core PLL microcapsules containing high G alginate were made with either needle sizes of 0.35 or 0.4 mm. This resulted in no differences in the mean diameters, but with a slightly elevated TCC response by the microcapsules made by the 0.35 mm needles between otherwise comparable conditions. The alginate microbeads made with needles of 0.25, 0.35 or 0.4 mm resulted in mean diameters of 343, 477, 589 μ m respectively, but did not show any difference in the TCC response.

3.2 Complement C3c deposition on the surface of microspheres

The deposition of C3c on the surface of microspheres was detected after incubating in human lepirudin anticoagulated plasma. A massive deposition of C3c with accumulation between the incubation times of six and 24 hours was found on the solid core microcapsules (Fig. 2). The citrate treatment resulted in a lower deposition with only minor deposition after six hours and with some accumulation after 24 hours on the liquefied core microcapsules. A lower deposition was observed on the PLO compared to PLL containing microcapsule after 24 hours incubation. For the solid core PLL microcapsules, a

significant C3c deposition was detected on microcapsules made of both high G and intermediate G. The C3c deposition was not detected on Ca/Ba beads, which is consistent with previously published data [15], and therefore these data are not shown. Additionally we compared the C3c deposition between whole blood, lepirudin anti-coagulated plasma and serum (same donor), which in all cases showed to be deposited, but with some variability in the patterns of deposition (Supplementary S2).

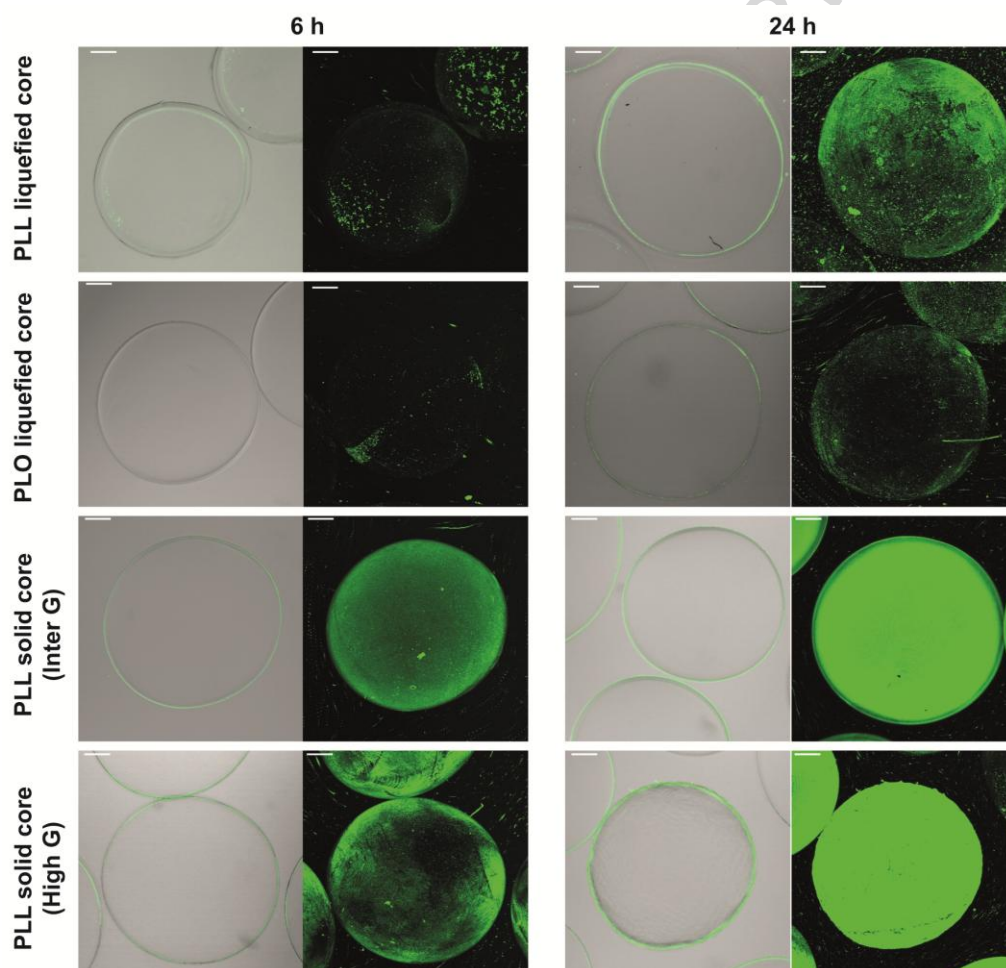


Figure 2. C3c deposition on the surface of selected microcapsules after incubation in lepirudin plasma (human) for 6 and 24 hours. The microcapsule composition of the selected types: APA using intermediate G alginate and 0.14 % PLL with liquefied core, APA using intermediate G alginate and 0.14 % PLO with liquefied core, APA using intermediate G alginate and 0.1% PLL with solid core, and APA using high G alginate and 0.1% PLL with solid core. Bars are equal to 100 μm .

3.3 Cytokines

The secretion profiles of selected proinflammatory cytokines, including chemokines and growth factors, are shown in Fig. 3 and Suppl. Fig. S3. The most potent inducers of the inflammatory cytokines were the solid core PLL microcapsules (all six microcapsule types prepared using different conditions as described in Table 2), showing statistically significant increase of TNF, IL-6, IL-8, MIP-1 α and VEGF as compared to the saline control. The liquefied core microcapsules generally induced lower amounts of the cytokines, with significant lower amounts of TNF, IL-1 β , IL-8 and MIP-1 α as compared to the solid microcapsules. One exception to the low cytokine induction, was a strong and significant elevation of MCP-1 by the PLL containing liquefied core microcapsules, and a small but significant elevation of IL-8 as compared to the saline control. No clear differences in the cytokine response patterns could be detected between the solid core PLL microcapsules prepared using either high G or intermediate G alginate. The Ca/Ba Beads did not induce statistically significant amount of cytokines compared to the saline control, and induced statistically lower amounts of all the inflammatory cytokines as compared to the solid microcapsules, and lower MCP-1 and IL-8 as compared to the liquefied PLL microcapsules. The different size of the Ca/Ba Beads did neither have any impact on the cytokine responses (Fig. 3 and Suppl. Fig. S2).

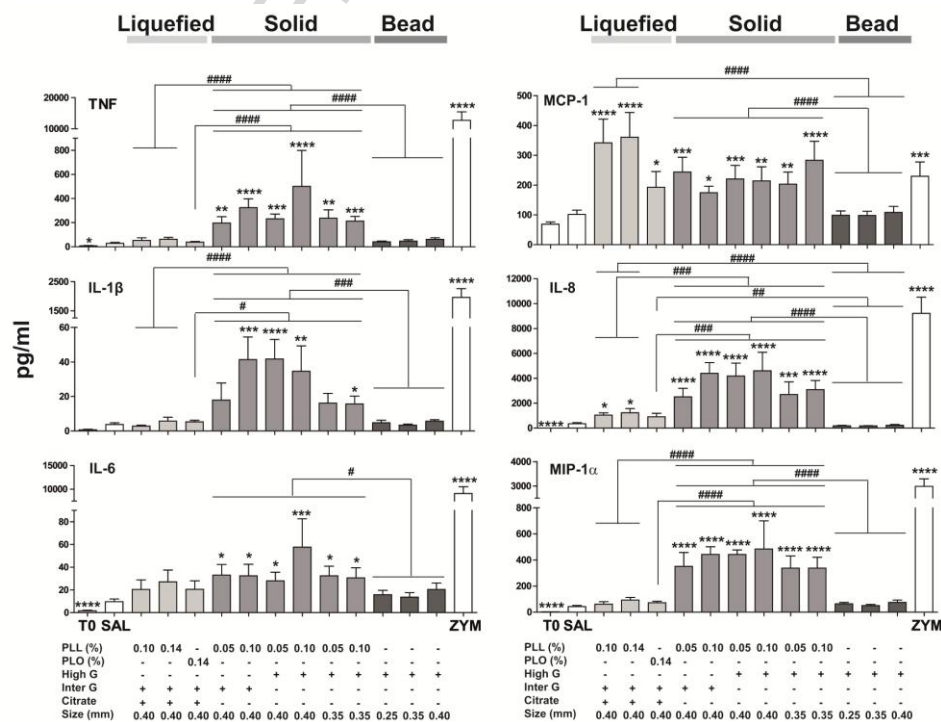


Figure 3. Inflammatory mediators (TNF, IL-1 β , IL-6, MCP-1(CCL2), IL-8 (CXCL8) and MIP-1 α (CCL3)) secreted after four hours incubation of microspheres in human whole blood. T0: baseline value at blood withdrawal, SAL: background activation from saline, ZYM and LPS are the positive controls. Bars are means \pm SEM, N = 5 or N=4 for the microbeads prepared using needle ID 0.25 mm and 0.4 mm, respectively. * compared to the saline control, # compared to microspheres categories. #P \leq 0.05, ##P \leq 0.01, ###P \leq 0.001, ####P \leq 0.0001 (*correspondingly).

3.4 CD11b expression

The monocyte and granulocyte CD11b expression was determined after one hour incubation of microspheres in whole blood (Fig. 4). The Ca/Ba microbeads did not induce CD11b expression above the saline control, and were inducing statistically lower CD11b expression compared to the other microspheres with exception of the monocytes population given PLO liquefied microcapsules. The polycation containing microcapsules all induced increased CD11b expression reaching statistical significance in five out of nine microcapsule types compared to the saline. The liquefied core PLL-containing microcapsules revealed statistical significance, while non-significant elevated values were determined for PLO-containing liquefied core microcapsules. The solid core PLL-containing microcapsules made of high G alginate exhibited statistically significant enhanced CD11b expression in case of the highest concentration of PLL (0.1%). In contrast, the opposite trend was observed for solid core PLL-containing microcapsules made of intermediate G alginate.

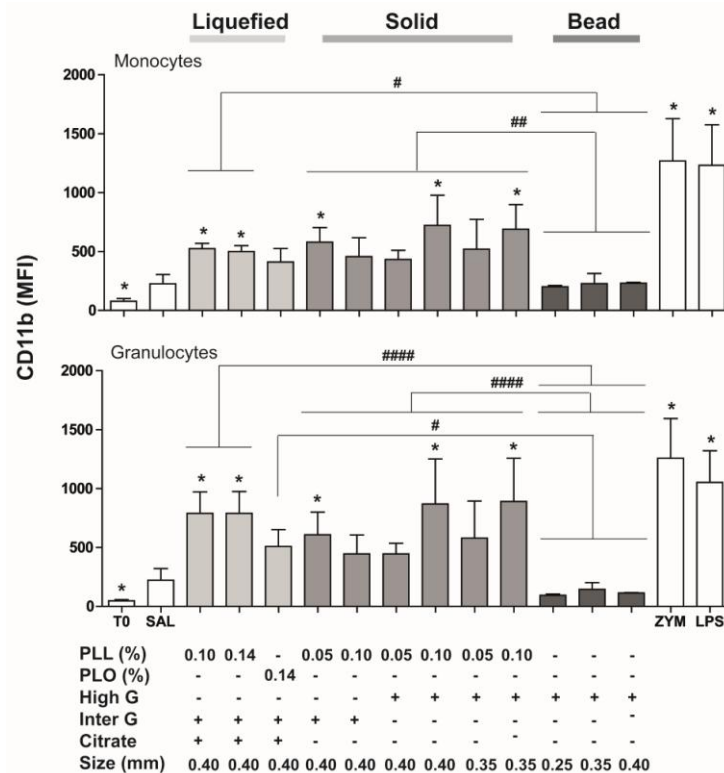


Figure 4. Monocyte and granulocyte CD11b expression after one hour exposure of various microspheres to human whole blood. T0: baseline value at blood withdrawal, SAL: background activation from saline, ZYM and LPS are the positive controls. Bars are means \pm SEM, N = 3. * compared to the saline control, # compared to microspheres categories. # $P \leq 0.05$, ## $P \leq 0.01$, ### $P \leq 0.001$, #### $P \leq 0.0001$ and * $P \leq 0.05$.

3.5 Complement inhibition

The observed differences in complement fluid and solid phase activation and cytokine responses between the solid and liquefied core PLL-containing microcapsules made us to proceed with complement inhibition to further elucidate the underlying mechanisms behind the inflammatory response caused by these microspheres. The experiments for liquefied and solid core PLL-containing microcapsules with inhibition of C3 are shown in Fig. 5. The inhibition of C3 resulted in statistically significant reduction of TCC and C5a thus confirming that the liquefied core microcapsules activated a strong complement response. The data also demonstrated that the activation mechanism was through the C3 activation with subsequent formation of C5a and TCC, and not caused by a direct cleavage of C5 which could have been the alternative. IL-8 showed an opposite activation profile than TCC with the solid core microcapsules as the most stimulating ones. Notably, the C3 inhibition reduced the IL-8

secretion statistically significant only for the solid core microcapsules while the liquefied core microcapsules resulted in less pronounced response.

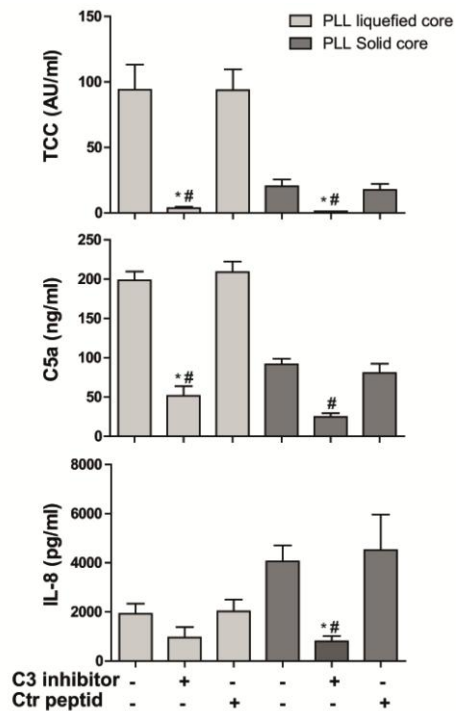


Figure 5. The impact of C3 inhibition on TCC, C5a and IL-8(CXCL8) after incubation with liquefied core or solid core PLL microcapsules in human whole blood for four hours. Bars are means \pm SEM, N=6 for TCC and IL-8, and N=4 for C5a. * $P < 0.05$ compared to corresponding native condition, # $P < 0.05$ compared to corresponding control (Ctr) peptide.

The solid core PLL (0.1%) microcapsule made of high G alginate was studied in whole blood by inhibition of complement either at the level of C3 or C5 (Fig. 6). A strong C3c deposition was found on the solid core microcapsules under native conditions, with added control peptide or C5 inhibitor (Fig. 6). Under these conditions, the cell adhesion to the surface of microcapsules was also observed. In contrast, the inhibition of C3 resulted in complete absence of the C3c deposition as well as the cell adhesion. This pointed to a strong connection between the complement surface activation and the cell adhesion. The inflammatory cytokines IL-1 β , TNF, IL-6 and IL-8 were statistically significantly inhibited by the C3 blockage but not by the C5 blockage, still with a partly reduced secretion (Fig. 6).

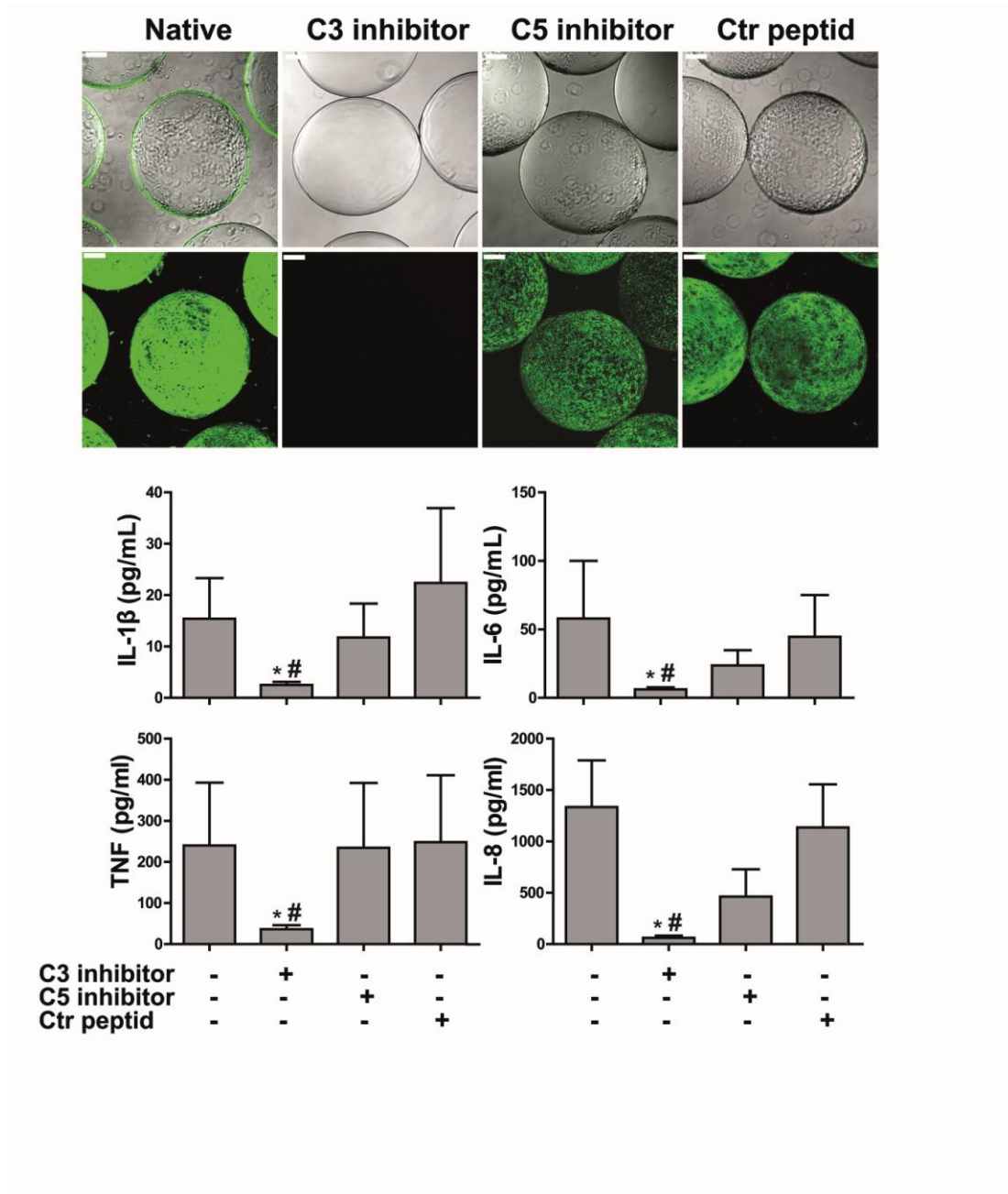


Figure 6. Effect of C3 and C5 inhibition on C3c deposition, cell adhesion and inflammatory cytokines after four hours incubation with solid core PLL microcapsules in whole blood. Bars graphs are means \pm SEM, N=5. * $P < 0.05$ compared to the native condition, # $P < 0.05$ to the control (Ctr) peptide. Scale bars are 100 μ m.

3.6 CD11b, CD18 or CD11c blockage

The integrin CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are receptors for iC3b, which is the inactivated C3b. By blocking CD18 by an inhibitory monoclonal antibody, the cell adhesion to the

microcapsule surface was abolished (Fig. 7). A reduced cell-adhesion was observed by blocking CD11b while no apparent reduction could be observed when blocking CD11c. We proceeded also with measuring the cytokine response after CD18, CD11b and CD11c blockage (Fig. 7). CD18 blockage reduced the secreted amounts of TNF, IL-1 β , IL-6 and MIP-1 α and increased the secretion of MCP-1 significantly. The CD11b blockages lead to significant reduction of IL-6 and VEGF, and a strong induction of MCP-1. The CD11c blockages induced a slight significant reduction of IL-6 and partly TNF, although less pronounced (Suppl. S4). No effect on the MCP-1 induction was detected by the CD11c blockage (Suppl. S4). Despite a reduction of IL-8 with the C3 and C5 inhibition, no effect could be found on IL-8 by the antibody blockage (Suppl. S5). However, upon a combined blockage of CD11b or CD18 with C5 inhibition, the IL-8 secretion was significantly reduced. The controls (native or IgG isotype control) were also reduced by the C5 inhibition, but a small but significant additional reduction was observed with the CD11b as compared to the controls (Suppl. S5). MCP-1 was also significant and substantially reduced by the C5 inhibition, although the chemokine was not fully taken down by the combined inhibition of CD11b or CD18 with C5 (Suppl. S5). A slight additional effect of C5 inhibition on the IL-6 secretion after CD18 blockage was also found, whereas this effect was not apparent for other cytokines (not shown).

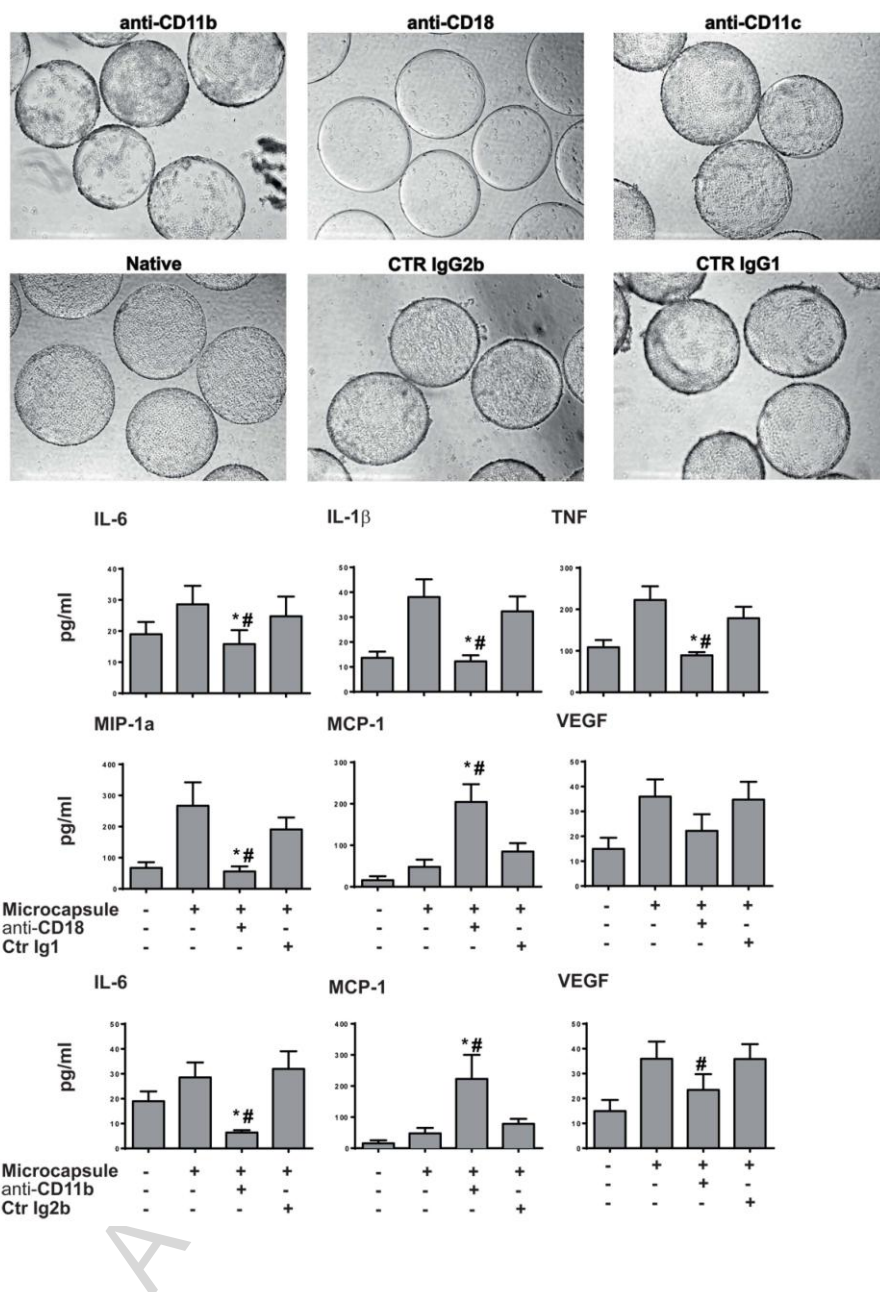


Figure 7. Effect of inhibitory antibodies against CD18, CD11b and CD11c on the leukocyte adhesion on solid core PLL microcapsules and the inflammatory cytokine secretion after four hours incubation in human whole blood. Bars are means \pm SEM, N=7. * $P < 0.05$ blockage compared with the native condition, # $P < 0.05$ blockage compared with the control Ig

4 Discussion

The present study revealed that the inflammatory potential of alginate-based microspheres assessed in a human whole blood model varied due to the microsphere preparation conditions, and can be categorized as inert for the Ca/Ba microbeads, low for the liquefied core PLO-containing microcapsules, intermediate for the liquefied core PLL-containing microcapsules and high for the solid core PLL-containing microcapsules. Differences in the PLL concentration, type of alginate (high G versus intermediate G) or needle size tested for solid core PLL-containing microcapsules showed minor effect on the inflammatory potential. The inflammatory properties of the Ca/Ba microbeads were not influenced by the size ranging from 343 to 589 μm . The change of alginate type from previously used high viscosity (UP-MVG) [14, 15] to the low viscosity alginate (UP-LVG) did not change the inflammatory properties of the Ca/Ba microbeads. In conclusions, three major variabilities seemed to be of importance for the inflammatory potential in the present study; 1) alginate Ca/Ba microbeads vs. polycation-containing microcapsules; 2) liquefied vs. solid core of microcapsules; 3) PLO vs. PLL used as a polycation for preparation of microcapsules. In summary, the microspheres surface reactivity to complement is suggested as the major reason for the observed differences among microspheres tested in this study. The surface activation of complement subsequently leads to the cell adhesion mainly through CD11b/CD18 with consequence for the cytokine release. The underlying relationship between the preparation conditions of microspheres and biological mechanisms are discussed in the next sections.

The alginate-based polycation-containing microcapsules in the present study influenced the complement activation patterns either by inducing substantial complement activation at the solid phase (microcapsule surface) or in the fluid phase. The liquefied core microcapsules activated complement in the fluid phase (TCC) but with low activation at the solid phase (surface deposition of C3b/iC3b). The opposite pattern was presented by the solid core microcapsules with a massive surface deposition of C3b/iC3b. Our data highlights the difference of complement potency when activated at the solid phase as compared to the fluid phase, and emphasizes the importance of examining both phases when using complement activation as readout for biocompatibility. The reason for the distinct difference between the liquefied and core microcapsules can possibly be explained by differences related to

physico-chemical properties of microcapsules discussed in the next sections.

The complement C3 convertase is commonly initiated through the covalent binding of C3b or C4b to hydroxyl groups but can also be initiated by covalent binding to amino groups [20]. Since we previously did not find any activation of the classical or lectin pathway by poly-L-lysine microcapsules, but elevated levels of the activation product Bb from the alternative pathway [14], we suggest that the C3 convertase activity is due to a direct binding through C3 tick-over activation to the poly amine groups of the alginate microcapsules. The analysis of the membrane of alginate-PLL microcapsules has shown that PLL is exposed in the outermost surface of microcapsules [18, 21] either as a complex with alginate or as a random coil exposing free amino groups [21]. Our data demonstrate a trend of increased complement reactivity with increased concentration of PLL for the solid core PLL-containing microcapsules using high G alginate, which probably reflected an increased amount of free amino groups to react with C3. Accordingly, the lower C3c deposition on the liquefied core microcapsules could be the result of a more complete complexation between the PLL and the alginate due to increased availability of the alginate chains when presented in a soluble (non-gelled) form.

The fast and strong TCC generated by the liquefied core microcapsules can be proposed to be related to the microcapsule stability. Liquefied core microcapsules are less stable than solid microcapsules [22]. We observed small fragments stained with C3c from the surface of the liquefied core microcapsules containing PLL possibly resulting from decomposed microcapsule membranes, and indicating a low stability. In comparison, the PLO coated microcapsules gave significantly less TCC. The use of PLO instead of PLL is shown to increase the stability of alginate-polycation microcapsules [23-25]. PLO differs from PLL by containing three methyl groups instead of four in the pendant moiety, which is responsible for more stable complexes with a negatively charged biopolymer as DNA [26]. The lower complement activation by the liquefied core PLO-containing microcapsules may be a consequence of stronger complexation with the negatively charged alginate resulting in improved stability and lower exposure of free amino groups compared to PLL-containing microcapsules. The TCC formation was only slightly elevated by the microcapsules containing intermediate G instead of high G alginate. Although these differences should be interpreted with great caution, they could be related to the increased swelling behavior and lower stability as previously demonstrated for PLL-containing

microcapsules made of intermediate G alginate [23]. In addition, the ability to bind more PLL but less alginate in the outer coating by intermediate G alginate [22] can further explain our data. Overall, the complement reactivity patterns for various alginate-based microcapsules may be explained by the existing knowledge on their physico-chemical and functional properties. We show here that the complement activation *per se* could be a supplementary method to study the surface properties of the microspheres.

The complement deposition on the microsphere surfaces was measured as C3c deposition, which is the larger part of C3. C3c is present both in its native (C3) and activated states (C3b and iC3b), thus it is not possible to distinguish between a non-activated and activated C3c deposition. However, upon the formation of the C3 convertases (C4b2a and C3bBb) or the C5 convertases (C4b2a3b or C3bBbC3b) on the microspheres surfaces, more C3 will be converted to C3b/iC3b, which will be manifested as accumulated C3c. Massive deposition, as in the case of the solid core PLL microcapsules (Fig. 2), thus indicate C3 activation products. The deposited C3b is further cleaved to iC3b [7], which serves as the main ligand for the complement receptor CR3 present on the plasma membrane of monocyte and granulocyte. The accumulated iC3b could therefore serve as the starting point for cell adhesion. The C3 deposition on the solid core PLL-containing microcapsule surface was completely abolished by blocking C3 using compstatin. Importantly, this also resulted in the lack of adhering cells that demonstrates the link between the surface complement reactivity and the cell adhesion. Further, the secretion of the inflammatory cytokines was blocked by the C3 inhibition, which is consistent with our previous data showing that a broad panel of inflammatory mediators were dependent on complement activation [15]. Whether the cytokine induction was due to fluid- or surface-deposited complement activating products was, however, not previously elucidated. By blocking the β 2 integrin chain, CD18, which forms complex with CD11b (CR3 receptor) or CD11c (CR4 receptor), the cell adhesion was fully inhibited. Further on, a partly inhibition of the cell-adhesion was found by CD11b blockage, while no apparent inhibition was observed with the CD11c blockage. This confirms that the CR3 receptor is responsible for the leukocyte adhesion to the solid PLL microcapsule surface. Moreover, the blockage of either CD18 or CD11b led to a selective reduction of TNF, IL-1 β , MIP-1 α , IL-6, and VEGF, and an induction of the MCP-1. While our data indicated also that CD18 was the most important receptor for induction of these cytokines, CD11b selectively contributed to IL-6 and VEGF secretion. The direct induction of MCP-1 by addition of the antibodies against CD18 or CD11b might

indicate that MCP-1 is induced by another mechanism not dependent on the cell-adhesion. We have previously shown that the MCP-1 was completely blocked by C3 inhibition [15], and here we also show that the C5 inhibition is reducing MCP-1, thus still pointing to a solely complement dependent effect. Together with the consistence between TCC, C5a and MCP-1 profiles seen from the microspheres screening, a possible explanation could be that MCP-1 is induced by activated iC3b fragments of the fluid phase released from the microspheres membranes of liquefied microcapsules with subsequent binding to the CR3 receptor. The present study exemplifies that complement activation at the surface induces a different cytokine pattern from the fluid state activation, and further emphasizes that the surface adhesion is highly important in understanding the interplay between implanted biomaterials and biological factors.

The strong TCC response induced by the liquefied core microcapsules corresponded with elevated C5a levels. Since both these activating products can be blocked by C3 inhibition, we confirmed that the activation was due to an initial activation by C3 and not a direct activation of C5, which could be an alternative mechanism [27]. The difference between the liquefied and solid core microcapsules to induce cytokines, and their opposite effects to trigger TCC and C5a show that the cytokine secretion was not caused by TCC or C5a under the current conditions. Complement products as C5a and toll-like receptors (TLRs) have been shown to act in synergy [28, 29]. Thus, the inflammatory reactions caused by the alginate microspheres could potentially be a result of cross-talking between these systems. Soluble alginate rich in mannuronic acid (>86%) is able to induce TNF in monocytes through mechanisms involving CD14 [30] TLR2 and TLR4 [31]. Recently it was demonstrated that purified alginate rich in guluronic acid as used currently, did not activate TLRs [32]. Soluble PLL is able to stimulate TNF in human monocytes through CD14 [33], but crosslinked with alginate as in PLL microcapsules the CD14 is not involved [15]. Our present and previous data show that blockage of C3 completely abolishes the inflammatory cytokine responses by the alginate microspheres, pointing to a solely complement mediated mechanism.

The role of C5a is multiple, and shown to be a potent chemoattractant with pleiotropic roles in inflammation [34], to induce IL-8 by endothelial cells [35] and NF- κ B activation in peripheral blood leukocytes [36]. Further on, C5a is a potent activator of CR3 (CD11b/CD18) on blood granulocytes and monocytes [13, 37] and therefore in the present study could be responsible for the observed

increase in leukocyte CD11b (CR3) expression. Increased CD11b expression could secondly impact the ability of leukocytes to adhere to the complement-activated surface. The effect action of C5a on the inflammatory cytokines by the alginate microspheres could therefore be an indirect effect through cell-adhesion as a consequence of CD11b activation increasing the binding of CR3 to C3b/iC3b at the microspheres surface. On the other hand, there might be a possibility for C5a to act in synergy with the integrin receptors in the cytokine induction for at least some of the cytokines, such as IL-8. One finding that may point in this direction was the combined blockage of C5 and CD11b leading to a significant reduction of the IL-8. The reduced IL-8 response by direct inhibition of complement C3 or C5 show that IL-8 is connected to the complement activation, and the combination of CD11b with C5 inhibitor resulted in an slight reduction of IL-8. A direct blockage did however not give any reduction of IL-8 in the present experiments. These discrepancies could be related to the high sensitivity of IL-8 to complement activation.

One should be careful to directly transfer our data from whole blood to an *in vivo* situation upon transplantation. Nevertheless, some interesting similarities and discrepancies between the findings in the whole blood and after implantation of empty microspheres should be mentioned. In mice studies using various strains, a stronger host cell adhesion has been observed to polycation containing microcapsules as compared to alginate microbeads [5, 38-40]. Further, the PLO-containing microcapsules exhibited lower cell adhesion than PLL-containing microcapsules after short-time (7 days) transplantation into the peritoneal cavity of C57BL6 mice [39]. In addition, identical types of microspheres either transplanted intraperitoneally in Wistar rats or evaluated in human whole blood showed consistency between host cell adhesion *in vivo* and the inflammatory response *in vitro* (Vaithilingam et al., unpublished). Alginate microbeads tested under *in vivo* conditions also exhibit the cell adhesion dependent on the choice of animal model [5] or mice strains [38-40]. The lack of ability to predict the cell adhesion for Ca/Ba microbeads indicates that other mechanisms could in addition be involved in inducing the cell adhesion. By inhibiting complement C3 or C5aR, the amount of immune cell infiltrated into the implanted meshes was half of that observed for wild-type littermates [11] pointing to complement as an important contributor to the observed immune response. Since the lepirudin whole blood model uniquely allows the interplay between the complement system and the

leukocytes, this model is an efficient tool to elucidate the inflammatory potential of the alginate-based microspheres as well as to understand the mechanisms behind the complex host reactions involved upon implantation of biomaterials.

Conclusion

The whole blood model is efficient for the study of the initial inflammatory responses to different types of alginate microspheres. The solid PLL microcapsules were the most inflammatory due to the massive binding of C3 to the surface with subsequent cell-adhesion and induction of inflammatory cytokines. The liquefied microcapsules caused an intermediate inflammatory response. The inflammatory potential of the liquefied PLO microcapsules was lower than the liquefied PLL microcapsule. The alginate microbeads were the prototype of an inert material, not inducing inflammatory readouts above the saline background. Our studies revealed a surprising distinguish between the complement reactivity at the microcapsules surface and the fluid-phase activation which had considerable impact on the cytokine activating potential. The complement C3 activation at the surface with subsequent cell-adhesion through the binding of CR3 (CD11b/CD18) receptor, seems to be the crucial mechanism for the cytokine release. The exception from this is the monocyte chemoattractant protein-1, MCP-1, which seems to be induced by complement activating products of the fluid phase and the CR3 receptor.

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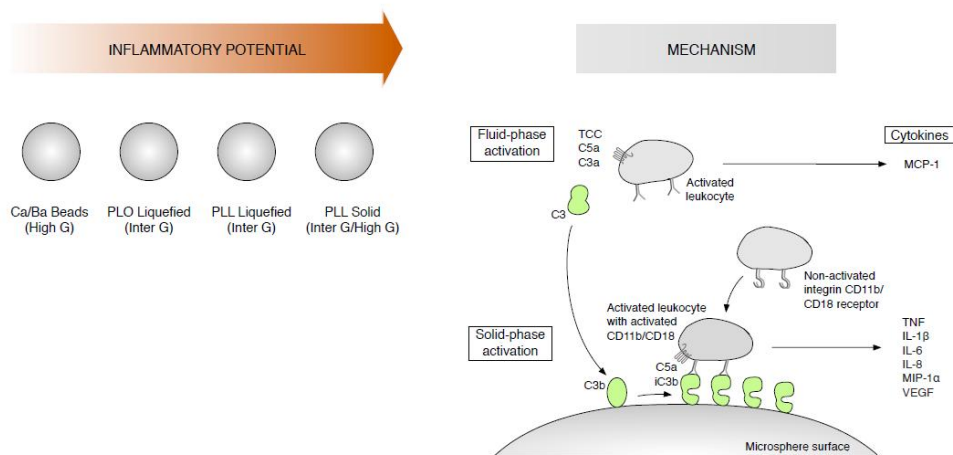
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