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Alginate microbeads are complement compatible, in contrast to polycation containing microcapsules, as revealed in a human whole blood model

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ABSTRACT

Alginate microbeads and microcapsules are presently under evaluation for future cell-based therapy. Defining their inflammatory properties with regard to humans is therefore essential. A lepirudine-based human whole blood model was used as an inflammation predictor by measuring complement and leukocyte stimulation. Alginate microbeads were complement-compatible since they did not activate complement as measured by the soluble terminal complement complex (sTCC), Bb or the anaphylatoxins C3a and C5a. In addition, alginate microbeads were free of surface adherent leukocytes. In contrast, microcapsules containing poly-L-lysine (PLL) induced elevated levels of sTCC, Bb, C3a and C5a, surface active C3 convertase and leukocyte adhesion. The soluble PLL induced elevated levels of sTCC and up-regulated leukocyte CD11b expression. PMCG microcapsules containing poly(methylene-co-guanidine) complexed with sodium alginate and cellulose sulfate triggered a fast sTCC response and C3 deposition. The PMCG microcapsules were still less activating than PLL-containing microcapsules as a function of time. The amounts of anaphylatoxins C3a and C5a were diminished by the PMCG microcapsules, whereas leukocyte adherence demonstrated surface activating properties. We propose the whole blood model as an important tool for measuring bioincompatibility of microcapsules and microbeads for future applications as well as determining the mechanisms leading to inflammatory reactions.

1. Introduction

Cell-based therapy using alginate containing microspheres has been suggested for the treatment of hormone deficiencies [1] as well as brain cancer [2]. The long-term function in experimental animals is, however, often hampered by overgrowth reactions leading to reduced graft performance [1]. The factors contributing to graft failure of the encapsulated cells are only partly understood. There is currently a need for experimental models relevant to humans reflecting the complexity of host factors that are encountered upon transplantation. Human blood contains most of the cells and effectors of the inflammatory machinery, thus it could be used as a source. The critical steps required to mimic the physiological in vivo situation using whole blood lie in the sampling, anticoagulation and incubation conditions, which need to be fully controlled.

Biomaterials in direct contact with blood induce immediate inflammatory responses through plasma cascades like the complement, coagulation and contact systems, with subsequent interplay with inflammatory cells [3]. The level of activation is closely related to the surface properties of the materials. The activation of complement may thus be a sensitive indicator of the ability of a biomaterial to trigger inflammatory reactions. In the present work we have used the novel whole blood model anti-coagulated with the hirudin analog lepirudin [4] to study different microcapsules containing alginate with a focus on complement and leukocyte activation. Lepirudin specifically inhibits thrombin in the coagulation cascade while not affecting the complement cascade and inflammatory cells [4]. In this way it has been possible to study the mutual interactions between complement and leukocytes, as well as between other branches of the inflammatory network.

Complement is a major pro-inflammatory system that acts upstream of the leucocyte and cytokine responses. The complement system consists of around 30 plasma and membrane-bound proteins. The central event of complement activation is cleavage

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of the C3 protein into the opsonin C3b and the anaphylatoxin C3a. C3 is cleaved by the C3 convertase from the classical/lectin pathway (C4bC2a) or from the alternative pathway (C3bBb), depending on the activators. The activators on a biomaterial surface may be adsorbed IgG inducing classical activation or various forms of C3b (e.g. conformationally changed C3 [5] and/or spontaneously hydrolyzed C3b analog C3(H_2O)) inducing alternative pathway activation. Irrespective of the initial event leading to C3b deposition, this essential step initiates amplification by the alternative pathway convertase, leading to escalated C3 activation. Surface bound C3b is further assembled with the C3 convertases to form the C5 convertases (C4bC2aC3b and C3bBbC3b), which cleave C5 to C5a and C5b. The anaphylatoxin C5a is the most potent inflammatory mediator of complement, whereas C5b is the staging point for formation of the terminal C5b-9 complement complex (TCC) on cell membranes or in solution, sC5b-9 (sTCC) [6]. The ability of biomaterials to trigger complement activation seems to be directly related to whether C3b is able to form covalent links to the surface hydroxyl or amino groups. This linkage may be formed directly on biomaterial surfaces [7] or through adsorbed proteins exposing hydroxyl or amino groups [8]. In addition, the amount of bound water to the surface polymers might be important for their complement activating abilities [9].

To our knowledge, complement reactions to alginate microbeads (Ca^{2+} and Ba^{2+} crosslinked alginate) and microcapsules (an alginate core with a polycation/polyanion complexed membrane) have not been addressed before. By using the lepirudin-based whole blood model the ability of various types of microspheres to activate complement could be studied under identical conditions. Alginate microbeads [10,11] and PMCG microcapsules [12–14] considered for future pancreatic islet transplantation were evaluated. In addition, poly-L-lysine (PLL) containing microcapsules were included to further elucidate the mechanisms behind their inflammatory reactivity [15–18].

Thus, the aim of the present study was to compare the inflammatory potential of different alginate microspheres, as their ability to activate complement and leukocytes using the human lepirudin anti-coagulated whole blood model.

2. Materials and methods

2.1. Study design

The study included five different types of microspheres: Ca/Ba beads (gelled in 1 mM $BaCl_2$/50 mM $CaCl_2$), Ba beads (gelled in 20 mM $BaCl_2$), APA microcapsules (Ca beads coated with PLL and alginate), AP microcapsules (Ca beads coated with PLL) or PMCG microcapsules, formed by polyelectrolyte complexation between sodium alginate (SA)/cellulose sulfate (CS) with polycation poly(methylene–co-guanidine) hydrochloride (PMCG) and calcium cations. All microspheres were made with ultrapure alginate (specified in Section 2.2). In addition, dissolved UP-MVG was evaluated as the alginate source for the microbeads.

2.2. Reagents and equipments

Ultrapure sodium alginates acquired from FMC BioPolymer AS (NovaMatrix, Sandvika, Norway) were used: Pronova UP-MVG (67% guluronic acid, intrinsic viscosity 1105 ml g^{-1}, endotoxin <43 EU g^{-1}), Pronova UP-LVG (66% guluronic acid, intrinsic viscosity 830 ml g^{-1}, endotoxin <100 EU g^{-1}) or Pronova UP100 M (*Macrocystis pyrifera*, 44% guluronic acid, intrinsic viscosity 908 ml g^{-1}, endotoxin <26 EU g^{-1}). The protein content was less than 0.3% for all alginates, as specified by the manufacturer. Cellulose sulfate (CS) sodium salt was from Acros Organics (Geel, Belgium), PMCG hydrochloride supplied as a 35% aqueous solution was from Scientific Polymer Products Inc. (Ontario, NY). Sodium chloride, calcium chloride, barium chloride and sodium citrate of analytical grade were from Merck (Darmstadt, Germany). Poly-L-lysine hydrochloride (P2658, lot No. 96H5502), Tween 20, zymosan, phosphate-buffered saline (PBS) with calcium and magnesium, ethylenediaminetetraacetic acid (EDTA), paraformaldehyde and LDS-751 were all purchased from Sigma–Aldrich (St Louis, MO). Other reagents, were mannitol (HPLC degree, BDH Analar, VWR International, Pool, UK), sterile saline (0.9% NaCl), non-pyrogenic (B. Braun, Melsungen, Germany), lepirudin (Celgene Europe, Boudry, Switzerland). Antibodies had the following specifications: fluorescein isothiocyanate (FITC) control antibody (BD555057), anti-CD14 FITC (BD347497) and anti-CD11b phycoerythrin (PE; BD347557), all from Becton Dickinson (San Jose, CA). In addition, FITC-conjugated rabbit anti-human C3c (F0201, Dako, Glostrup, Denmark), FITC-conjugated poly rabbit anti-mouse (F0261, Dako, Glostrup, Denmark), anti-human C5b-9 (clone aE11, Diatec, Oslo, Norway) and biotinylated 9C4 [19] were used. For blood sampling the following equipment was used: Nunc 1.8 and 4.5 ml polypropylene vials (Nunc, Roskilde, Denmark) and a BD vacutainer top (Becton Dickinson, Plymouth, UK).

2.3. Microsphere preparation

2.3.1. Alginate microbeads and microcapsules

Ca/Ba beads and Ba beads as well as APA and AP microcapsules were made as described previously using 1.8% UP-MVG alginate [20]. The gelling solution varied according to the type of microsphere: Ca/Ba beads, 1 mM $BaCl_2$/50 mM $CaCl_2$ in 0.15 M mannitol; Ba beads, 20 mM $BaCl_2$ in 0.15 M mannitol; APA and AP, 50 mM $CaCl_2$ in 0.15 M mannitol. PLL was used at a concentration of 0.05% and incubation was for 10 min. As the outer coating for the APA microcapsules a solution of 0.1% Pronova UP100 M in 0.15 M mannitol was used. The microspheres were made with sterile solutions and under strictly sterile conditions, using autoclaved equipment and a sterile hood for all steps.

2.3.2. PMCG microcapsules

Before the formation of PMCG microcapsules the polyelectrolytes were prepared as follows. PMCG was isolated by lyophilization, ending with a residual water content of less than 2%. CS was purified by treatment with activated charcoal, filtration and precipitation in acetone as described previously [21]. The residual water content of CS was about 10%.

PMCG microcapsules were prepared as described previously [14], except for the following concentration changes in the polymer solutions. The polyanion solution contained 0.90% UP-LVG, 0.90% CS (taking into account the residual water content) in 0.9% NaCl. The polycation solution contained 1.2% PMCG, 1% $CaCl_2$, 0.9% NaCl and 0.025% Tween 20. A multi-loop reactor [22] provided a continuous encapsulation process with a reaction time of about 40 s for polyelectrolyte complexation. Equilibration of membrane composition was obtained by treatment with 50 mM sodium citrate in 0.9% NaCl solution, pH 7.4 for 10 min. The additional coating layer was made with 0.1% CS in 0.9% NaCl solution for 10 min.

2.4. Whole blood model

2.4.1. Preparation of microspheres and controls for experiments

It is essential that the proportions by volume between blood and additives are equal in the whole blood experiments. The total volume of each additive was 200 µl. Of this, 100 µl consisted of saline containing either 50 µl microspheres, 900 µg UP-MVG alginate, 10 µg zymosan (positive control) or saline (negative control). The amount of soluble alginate corresponds to the alginate content

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within 50 μl of microbeads. To these additives was added 100 μl of PBS (with $CaCl_2/MgCl_2$) immediately before addition of 500 μl of whole blood.

2.4.2. Whole blood model performance

Single experiments for each blood donor were performed as previous described using lepirudin (50 μg ml^{-1}) as the anticoagulant [4]. Blood was withdrawn into low activating polypropylene Nunc tubes (4.5 ml). Immediately thereafter, 500 μl of whole blood were added to the various additives in low activating sterile Nunc tubes (1.8 ml). Avoiding blood contamination of the screw cap is essential to avoid biased activation. The samples were incubated for 30, 120 and 360 min in an incubator (37°C) under continuous rotation. Complement activation was stopped by adding EDTA (10 mM final concentration) and centrifugation (3000 r.p.m., 15 min). Aliquots of plasma were stored at −80 °C before analysis.

2.5. C3 deposition

After incubation of the microspheres in whole blood (Section 2.4) the complement cascade was stopped by addition of EDTA (10 mM final concentration). Microspheres were harvested and washed (3×) in a wash solution (0.1% bovine serum albumin, 2 mM $CaCl_2$, 0.02% sodium azide in saline). For each type of microsphere one fraction was added to 50 μg ml^{-1} FITC-conjugated poly rabbit anti-human C3c (C3 deposition) and the other FITC-conjugated poly rabbit anti-mouse (control) antibodies. The samples were protected from light and continuously agitated for 30 min. Thereafter the microspheres were washed (3×). The deposition of C3 was visualized by confocal laser scanning microscopy (CLSM) (Zeiss LSM 510, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with a 488 nm laser source (BP 505–530). Identical laser settings were used on all microspheres using PMCG and APA microcapsules incubated for 120 min as references.

2.6. Expression of CD11b

Expression of CD11b was measured after 15 min incubation in whole blood as described in Section 2.4. Whole blood was fixed with 0.5% PFA in an equal volume for 4 min at 37 °C, and then stained with PE anti-CD11b, FITC anti-CD14 and the nuclear dye LDS-751 and analyzed using a flow cytometer (Beckman Coulter Epics XL-MCL, Coulter Corp, FL). To exclude red cells and debris the threshold was set at FL-3. Granulocytes and monocytes were gated in a SSC/FITC anti-CD14 dot plot, and CD11b expression measured as mean fluorescence intensity (MFI).

2.7. Cell adherence

Microspheres were prepared as described in Section 2.4 and incubated for 180 min. Blood was removed and the microspheres fixed in 0.5% PFA for 20 min. In order to keep the cells attached the blood samples were prepared without addition of EDTA, as would otherwise have been used to stop the complement cascade. From each sample one fraction was stained with antibodies (7.5 μg ml^{-1} in PBS) against CD14 (FITC anti-CD14) and CD11b (PE anti-CD11b) or with control antibodies (FITC mouse IgG2b,κ/PE mouse IgG2a,κ). Both combinations were incubated for 30 min in the dark and under continuous agitation, thereafter carefully washed and finally 0.15% PFA was added. Evaluation of the microspheres was performed using CLSM (Zeiss LSM 510, Carl Zeiss MicroImaging GmbH, Göttingen, Germany), with 488 nm (BP 505–530) and 543 nm (LP 650) excitation and emission wavelengths, respectively.

2.8. Assay of complement activation

2.8.1. sTCC

The terminal sC5b-9 complex (sTCC) was quantified by electroimmunoassay using mAb aEll specific for C9 incorporated in the sC5b-9 complex and biotinylated 9C4 specific for C6 in the respective complex. The assay has been described in detail previously [6] and was performed according to a later modification [19].

2.8.2. Bb, C3a and C5a

C3a and Bb was analyzed by ELISA using kits from Quidel (San Diego, CA). C5a was analyzed using an ELISA kit from BD Bioscience (San Diego, CA).

2.9. Statistical methods

One-way repeated measurements ANOVA with Dunnet's multiple comparison test were used to define statistical differences between saline and the other additives at a given time point. Differences for the various additives over time were tested using a two-way ANOVA. The data was not normally distributed due to the low sample numbers ($n = 5$), therefore the data were log transformed before analysis. Differences were considered significant at $P < 0.05$.

2.10. Ethics

The use of human whole blood for basal experiments was approved by the Regional Ethic Committee at the Norwegian University of Science and Technology. The experiments were performed in accordance with their guidelines.

3. Results

3.1. Activation of the complement cascade detected as sTCC formation in human whole blood

The formation of sTCC indicates activation of the complement cascade and is suggested to be the most sensitive and specific marker of complement activation. Fig. 1A shows the time kinetics of sTCC formation after addition of saline, Ca/Ba beads and APA and PMCG microcapsules as well as zymosan. The kinetics of sTCC activation were significantly different ($P < 0.0001$) for each additive. Fig. 1B–D shows the data for the entire panel of additives at each time point. After 30 min incubation the amount of sTCC was similar for the Ca/Ba beads, Ba beads and saline control (Fig. 1B). Over time, the generation of sTCC was slower for Ca/Ba beads and Ba beads compared with the saline control (Fig. 1C and D). This resulted in significantly lower values ($P < 0.05$) for Ba beads after 120 and 360 min incubation and for Ca/Ba beads after 360 min (Fig. 1D). The polycation containing microcapsules, APA, AP and PMCG, resulted in significant increases ($P < 0.05$) in sTCC compared with saline at all time points (Fig. 1B–D). The APA and AP microcapsules showed a time-dependent increase in sTCC (Fig. 1B–D). The PMCG microcapsules induced a rapid initial increase in sTCC, detected after 30 min (Fig. 1B). This was followed by a slower increase compared with the APA and AP microcapsules at 120 (Fig. 1C) and 360 min (Fig. 1D). The dissolved UP-MVG alginate induced a small increase in sTCC after 30 min, although non-significant (Fig. 1B). After 120 and 360 min the sTCC amounts where slightly lower than for the saline control (Fig. 1C and D). The amount of UP-MVG alginate (900 μg) corresponded to the amount of alginate in the aliquots of microbeads. The data therefore indicate minor differences between dissolved and gelled alginate.

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3.2. Alternative pathway factor B activation

The amount of Bb, an activation product of the alternative pathway factor B, increased with time (Fig. 2A–D) in a pattern resembling the sTCC data. The increase in Bb was, however, slower compared with sTCC. The kinetics of Bb formation were significantly affected ($P < 0.0001$) by each additive (Fig. 2A). In samples containing Ca/Ba beads, Ba beads or dissolved UP-MVG alginate the amounts of Bb were lower than for the saline control over time (Fig. 2C and D), with a significant ($P < 0.05$) difference at 360 min (Fig. 2D). The highest concentration of Bb was found in samples containing APA and AP microcapsules. The increase was significant for AP at 120 and 360 min (Fig. 2C), and for APA at 360 min (Fig. 2D). A non-significant elevation of Bb was found in the samples incubated with PMCG microcapsules for 30 min (Fig. 2B). The early induction at 30 min was followed by an evident and significant ($P < 0.05$) increase after 120 min (Fig. 2C). Interestingly, the amount of Bb was not further increased by the PMCG microcapsules at 360 min (Fig. 2D). The saline control, however, showed a steady increase, which resulted in similar Bb amounts for the PMCG microcapsules and saline control after 360 min (Fig. 2D).

3.3. Anaphylatoxin C3a and C5a release

The anaphylatoxins C3a and C5a are potent pro-inflammatory molecules derived from the cleavage of C3 and C5. The kinetics of C3a production are shown in Fig. 3A after addition of Ca/Ba beads and APA and PMCG microcapsules and for the controls. The kinetics were significantly different ($P < 0.0001$) for each additive (Fig. 3A). At the specific time points only a few of the additives resulted in significantly different C3a amounts relative to the saline control. The trends in stimulation were still consistent with the sTCC and Bb findings, with the exception of the PMCG microcapsules. Briefly, addition of Ca/Ba beads and Ba beads resulted in lower concentrations of C3a after 120 and 360 min (Fig. 3C and D), while addition of APA and AP microcapsules increased C3a (Fig. 3B–D). The PMCG microcapsules initially gave a slight elevation of C3a (Fig. 3B). However, after 120 and 360 min the PMCG microcapsules resulted in significantly ($P < 0.05$) lower amounts of C3a than saline (Fig. 3A–D). The dissolved UP-MVG alginate induced a slight but significant ($P < 0.05$) increase in C3a after 30 min (Fig. 3B), but after 120 and 360 min the amount of C3a was lower than for the saline control (Fig. 3C and D). A particular finding for the saline solution was the pronounced increase in C3a over time. This may be due to activation by the plastic surface [23].

The kinetics of C5a production were also significantly different ($P < 0.0001$) in response to Ca/Ba beads and APA and PMCG microcapsules and the controls (Fig. 4A). After 30 min Ca/Ba beads and Ba beads resulted in non-significant, slight increases in C5a levels relative to the saline control (Fig. 4B). The C5a increase was slower for Ca/Ba beads and Ba beads (Fig. 4C and D) compared with saline, resulting in significantly ($P < 0.05$) lower amounts after 360 min (Fig. 4D). A rise in C5a was detected on addition of APA and AP microcapsules, giving significant increases ($P < 0.05$) for APA at all time points and for AP at 120 and 360 min (Fig. 4B–D). PMCG microcapsules initially gave a modest, but statistically significant ($P < 0.05$), increase in C5a (Fig. 4B). Over time the PMCG microcapsules resulted in lower C5a amounts compared with the saline control, with a significant ($P < 0.05$) difference after 360 min (Fig. 4D).

3.4. C3 deposition on the microsphere surface

Deposition of C3 on the different microsphere surfaces is shown in Fig. 5. The detected C3c fragment of the C3 molecule is present in both the native C3 molecule, the active C3b convertase and its analog C3(H_2O). Detected C3 may, therefore, represent either adsorbed native C3 molecule or the active C3 convertase (C3b or the analog C3(H_2O)) on the microsphere surface. The deposition of C3 on the surface of Ca/Ba beads and APA, AP and PMCG microcapsules is shown at different incubation times (Fig. 5A-L). After 30 min a slight C3 staining was detected for Ca/Ba beads (Fig. 5A) and APA (Fig. 5B) and AP microcapsules (Fig. 5C). In contrast, the PMCG microcapsules showed pronounced staining at 30 min (Fig. 5D). The C3 staining on the PMCG microcapsules revealed surface irregularities and ruptures (Fig. 5D). No further increase in C3 staining was observed for the PMCG microcapsules after 120 (Fig. 5H) and 360 min (Fig. 5L). The surfaces of the APA (Fig. 5F and J) and AP microcapsules (Fig. 5G and K) showed increased staining with time. C3 staining also increased with time for the Ca/Ba beads (Fig. 5E and I). However, Ca/Ba beads with low detectable staining were estimated ~~to form~~ approximately 70–90% of the microbead population at all time points. The C3 distribution pattern was smooth and evenly distributed on the Ca/Ba beads (Fig. 5E and I). For the APA (Fig. 5B, F and J) and AP (Fig. 5C, G and K) microcapsules, C3 accumulated at certain points, resulting in spotted patterns. Further, equatorial sections of the microspheres at 360 min showed C3 located on the surface of the microspheres (Fig. 5M–P). The depth of penetration of C3 was estimated by LSM510 software fluorescence intensity profile analysis, as shown in Fig. A1. This analysis indicated that the Ca/Ba beads were most permeable, since C3 was found to penetrate 10–125 μm into the microbeads (S1A–B). Shorter penetration depths was found for the microcapsules, estimated at 20–40 μm for APA (Fig. A1C and D), 10–20 μm for AP (Fig. A1E and F) and 20 μm for the PMCG microcapsules (Fig. A1G and H). The staining was specific for C3, as demonstrated by the negative controls with corresponding insets showing light transmission (Fig. 5Q–T).

3.5. Leukocyte activation as measured by CD11b expression and cell adherence to the different microspheres

CD11b is the receptor for iC3b and an early activation marker of leukocytes. Granulocyte and monocyte CD11b expression was analyzed by flow cytometry 15 min after addition of microspheres or controls (Fig. 6A and B). The PMCG microcapsules showed significantly ($P < 0.05$) higher granulocyte CD11b expression compared with the saline control (Fig. 6A). CD11b expression was also higher on monocytes, although not statistically significantly so (Fig. 6B). The APA and AP microcapsules also resulted in a slight increase in granulocyte CD11b, although not significantly (Fig. 6A). An overall, moderate increase in CD11b expression was found on addition of the different microcapsules compared with yeast zymosan.

Leukocyte adherence on microspheres was evaluated after incubation in whole blood for 3 h (Fig. 6C–J). Leukocytes did not adhere to the Ca/Ba beads (Fig. 6C) or Ba beads (Fig. 6D). However, adherent leukocytes were found on the surface of APA (Fig. 6E and F), AP (Fig. 6G and H) and PMCG microcapsules (Fig. 6I and J). Cells appeared as small circular dots on the surface of the microcapsules and in the surrounding area, as seen by transmitted light (Fig. 6F, H and J). The larger fraction of the adherent cells stained positive for CD11b, while a smaller fraction stained positive for CD14 (Fig. 6E, G and I). CD11b is present on both granulocytes and monocytes in only slightly different amounts, while CD14 is expressed in higher amounts on monocytes. The monocytes in the present study displayed 23–50 times higher CD14 expression compared with the granulocytic population (data not shown). This indicates that the adherent cells were mainly granulocytes, with fewer numbers of monocytes.

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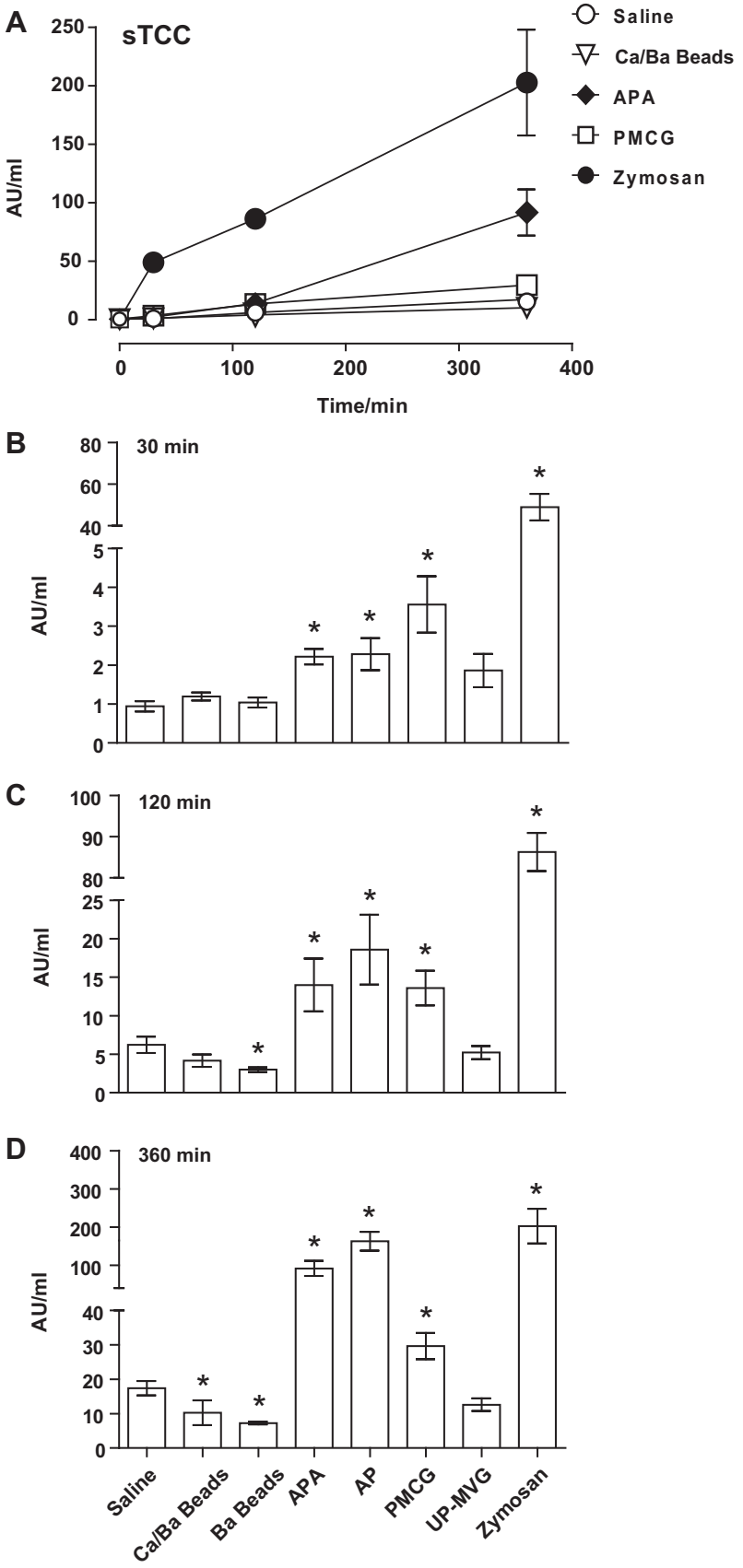

Fig. 1. sTCC concentration in plasma after incubation with various microspheres, alginate and controls in human lepirudin anti-coagulated whole blood. (A) Time-dependent sTCC amounts after addition of Ca/Ba Beads, APA, PMCG microcapsules, saline and zymosan. The additives significantly ($P < 0.0001$) affect sTCC formation over time. In the lower three panels each time point is shown (B) 30, (C)120 and (D) 360 min with additional Ba beads, AP microcapsules and dissolved UP-MVG alginate included. (B–D) $*P < 0.05$. The baseline sTCC value measured in the sample at the start of the experiment was 0.55 ± 0.16 AU ml^{-1}. Data are expressed as means ± SEM, $n = 5$.

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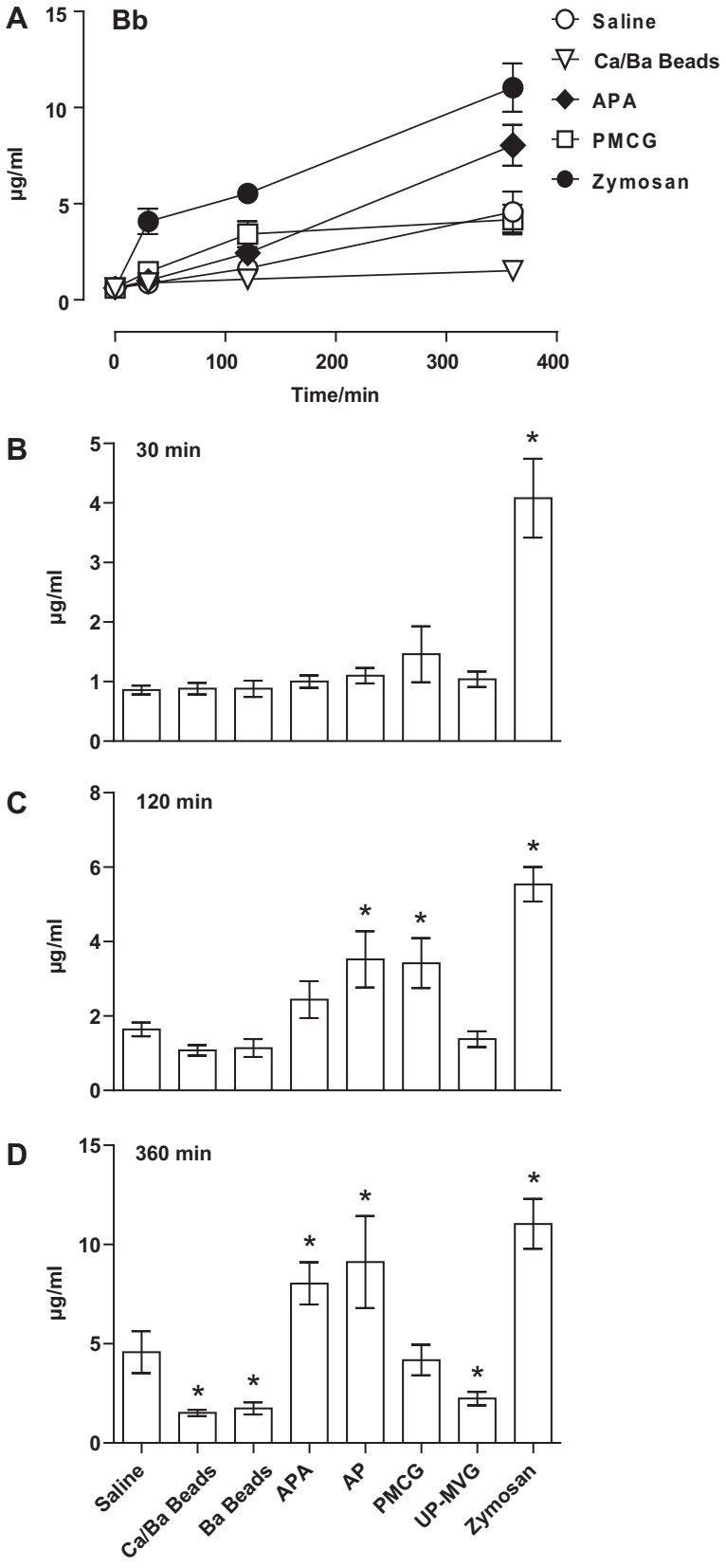

Fig. 2. Bb concentration in plasma after incubation with various microspheres, alginate or controls. The various additives and time parameters in (A)–(D) are as described in Fig. 1. The time-dependent Bb concentration was significantly ($P < 0.0001$) affected by each additive. (B–D) $*P < 0.05$. The baseline value of Bb was 0.62 ± 0.12 µg ml^{-1}. Data are expressed as means ± SEM, $n = 5$.

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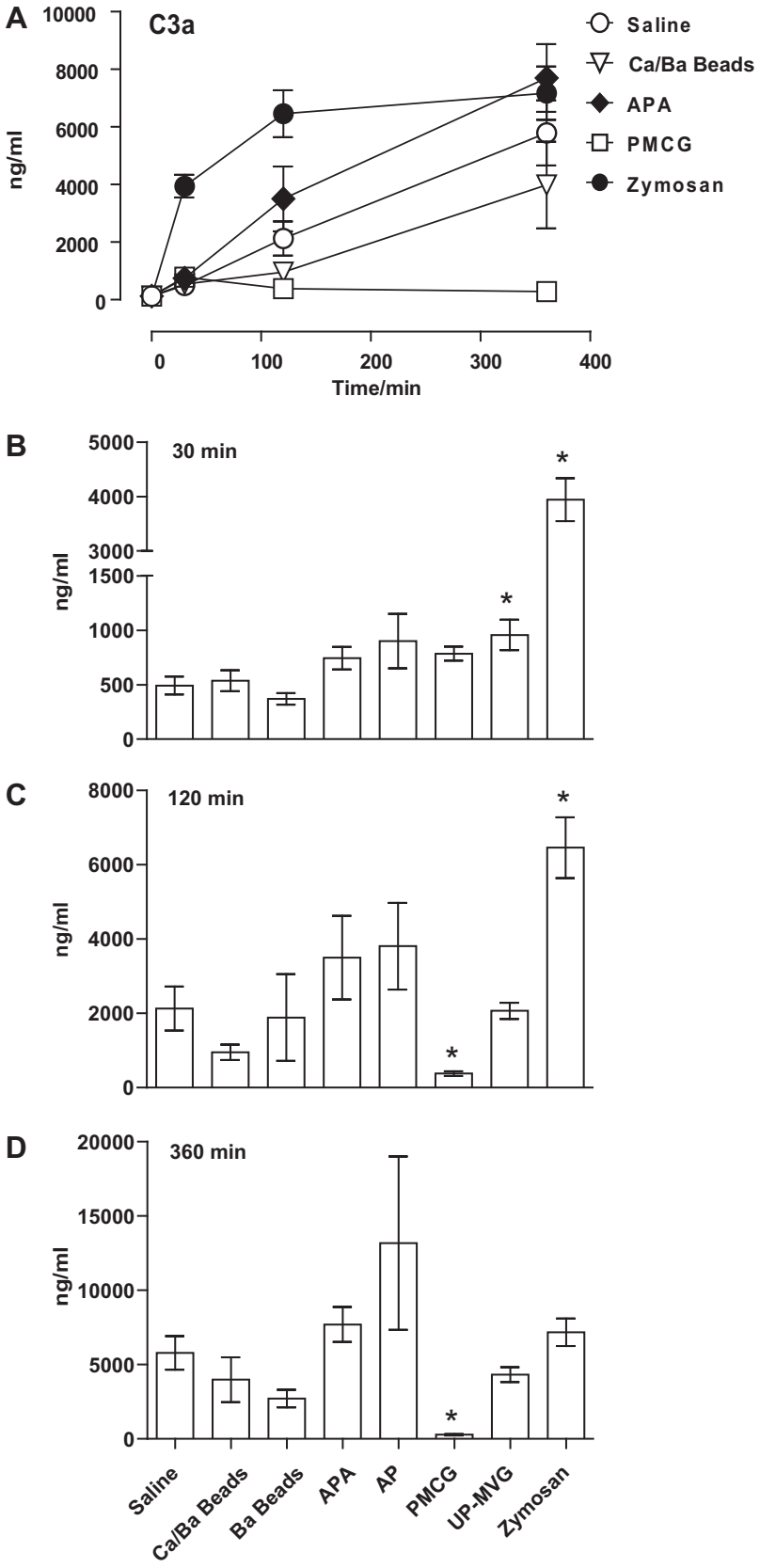

Fig. 3. C3a concentration in plasma after incubation with various microspheres, alginate or controls. The various additives and time parameters in (A)–(D) are as described in Fig. 1. The time-dependent C3a concentration was significantly ($P < 0.0001$) affected by the additives. (B–D) $*P < 0.05$. The baseline value of C3a was 120 ± 28.8 ng ml^{-1}. Data are expressed as means ± SEM, $n = 5$.

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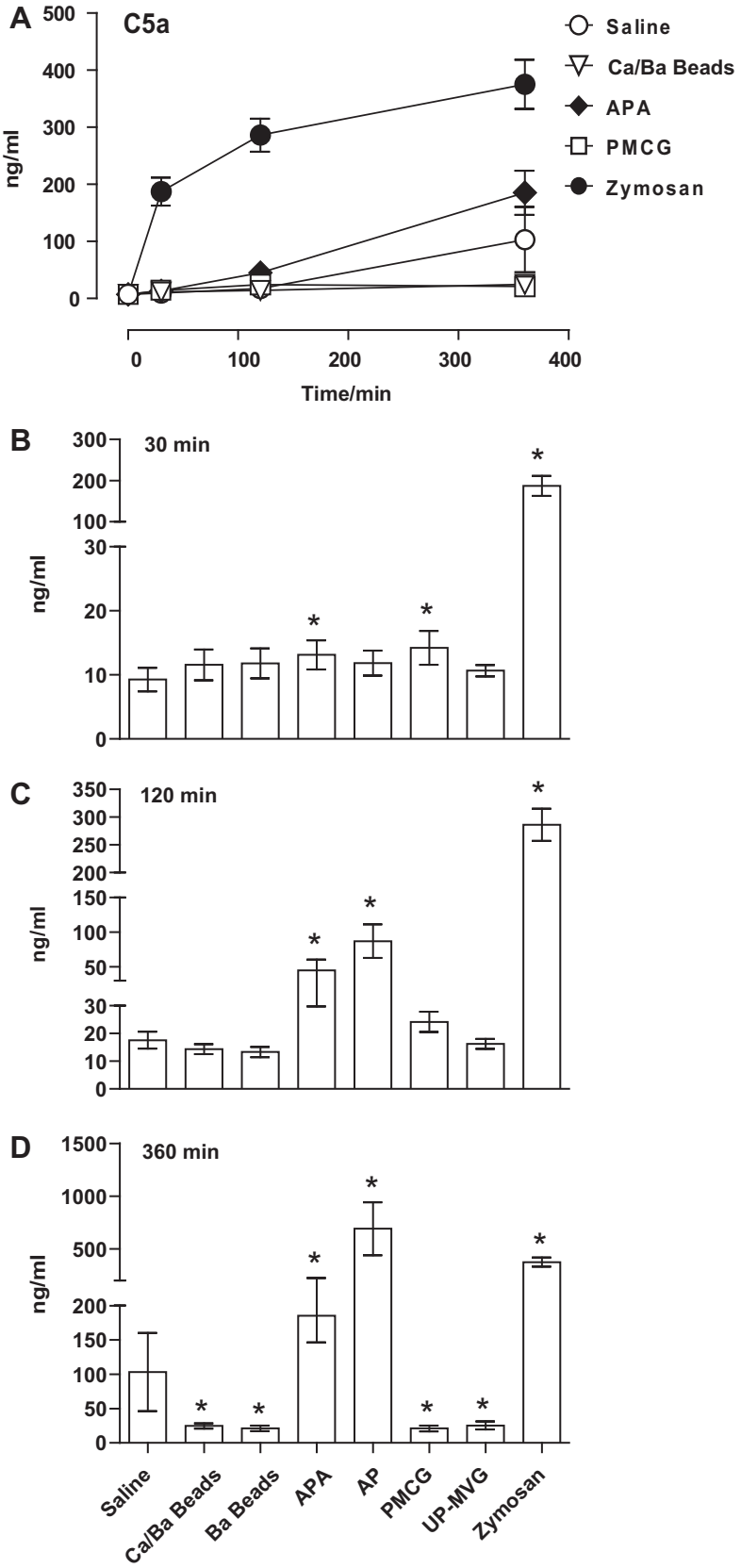

Fig. 4. C5a concentration in plasma after incubation with various microspheres, alginate or controls. The various additives and time parameters in (A)–(D) are as described in Fig. 1. The time-dependent C5a concentration was significantly ($P < 0.0001$) affected by the additives. (B–D) $*P < 0.05$. The baseline value for C5a was 7.27 ± 0.99 ng ml^{-1}. Data are expressed as means ± SEM, $n = 5$.

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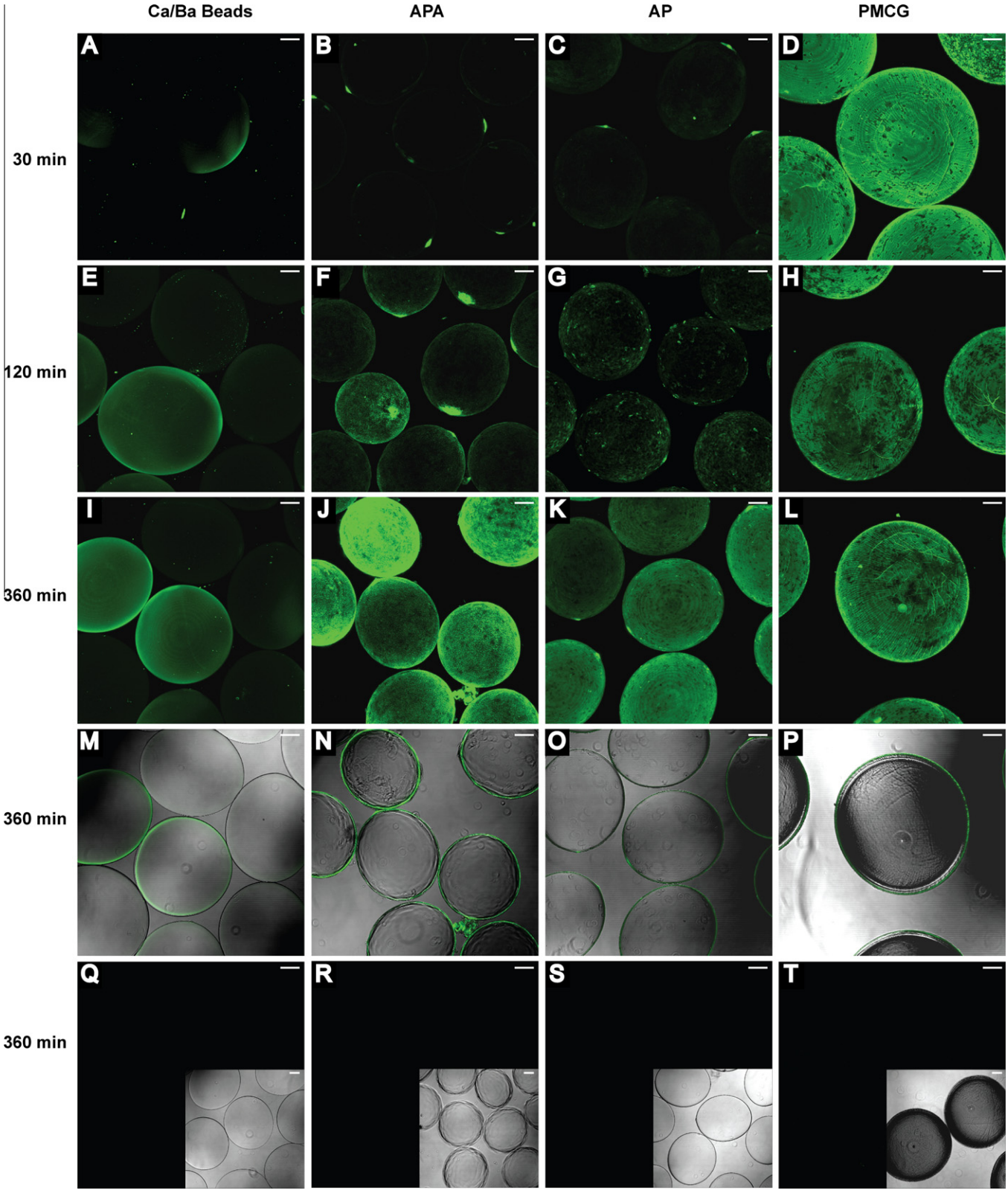

Fig. 5. Deposition of C3 on the microsphere surface after incubation in human lepirudin anti-coagulated whole blood. (A–L) 3D projections made by sectioning entire microspheres after incubation for 30, 120 and 360 min. (M–P) Projections through the equator overlaid with transmitted light images after 360 min. (Q–T) Controls are given in the lower panels as projections (black pictures). The inserts show transmitted light equatorial sections for visualization. Bars are 100 μm.

3.6. Effect of soluble PLL, CS and PMCG

The activation potential of dissolved PLL, CS and PMCG was evaluated by measuring sTCC production and CD11b expression. A clear dose-dependent induction of sTCC was found by addition of PLL (Fig. 7A). The polycation PMCG resulted in increased sTCC formation at the highest dose (1000 μg ml^{-1}), whereas addition of CS showed a weak inhibitory effect (Fig. 7A). In contrast, CS gave

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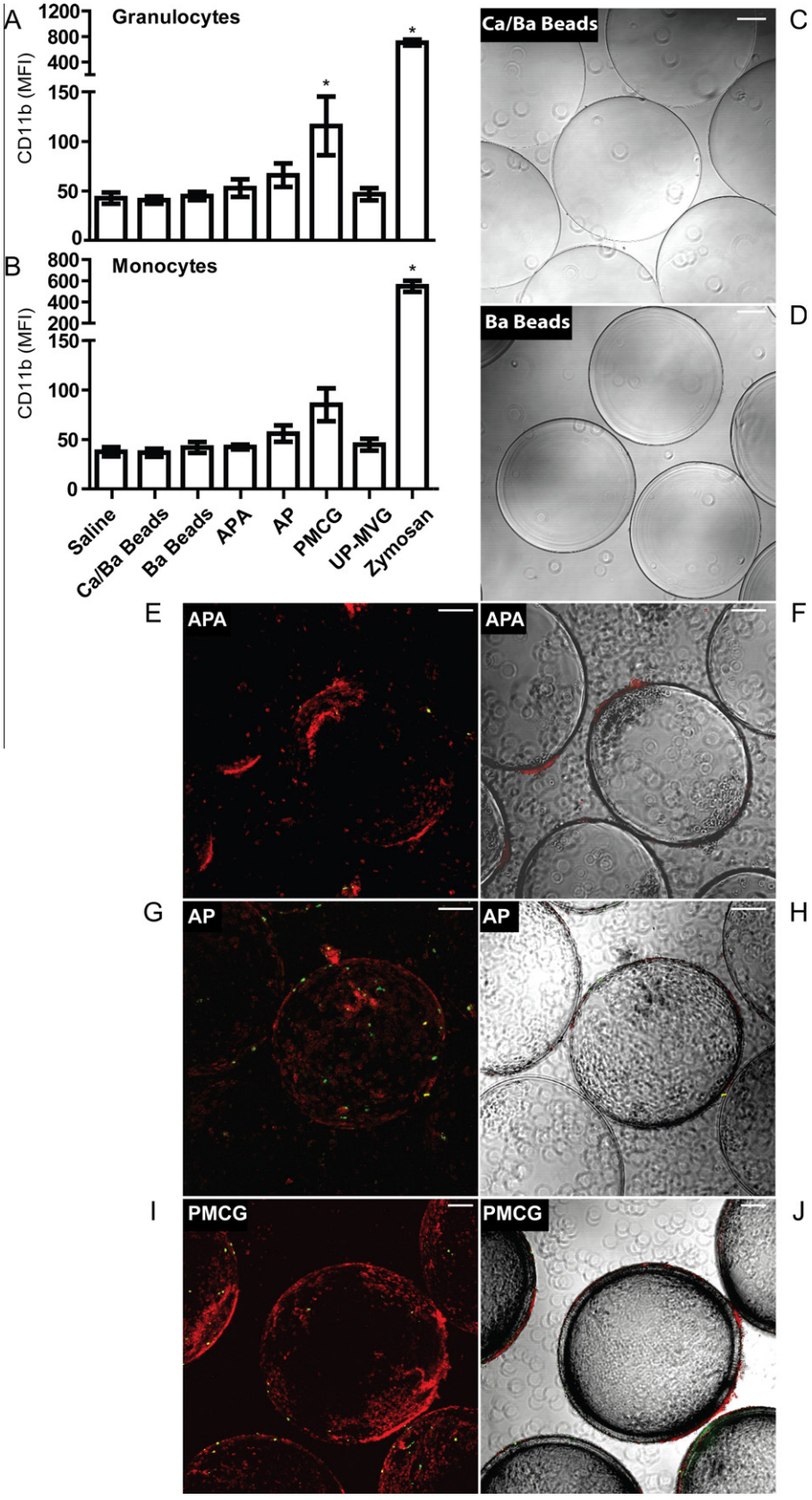

Fig. 6. Leukocyte CD11b expression and surface adhesion after the addition of various microspheres to lepirudin anti-coagulated whole blood. Leukocyte CD11b expression detected by flow cytometry 15 min after addition of microspheres, UP-MVG alginate and controls. (A) Granulocytes CD11b expression and (B) monocytes CD11b expression. Results are given as means ± SEM, $n = 5$. $*P < 0.05$. (C–J) Leukocyte adhesion on microspheres after 3 h incubation. Images taken by CLSM are presented as optical sections at the equator and 3D projections were produced from several sections through the microspheres. (C) Ca/Ba beads section, (D) Ba beads section, (E) APA projection, (F) APA section, (G) AP projection, (H) AP section, (I) PMCG projection, (J) PMCG section. CD11b is shown in red and CD14 in green. Bars are 100 μm.

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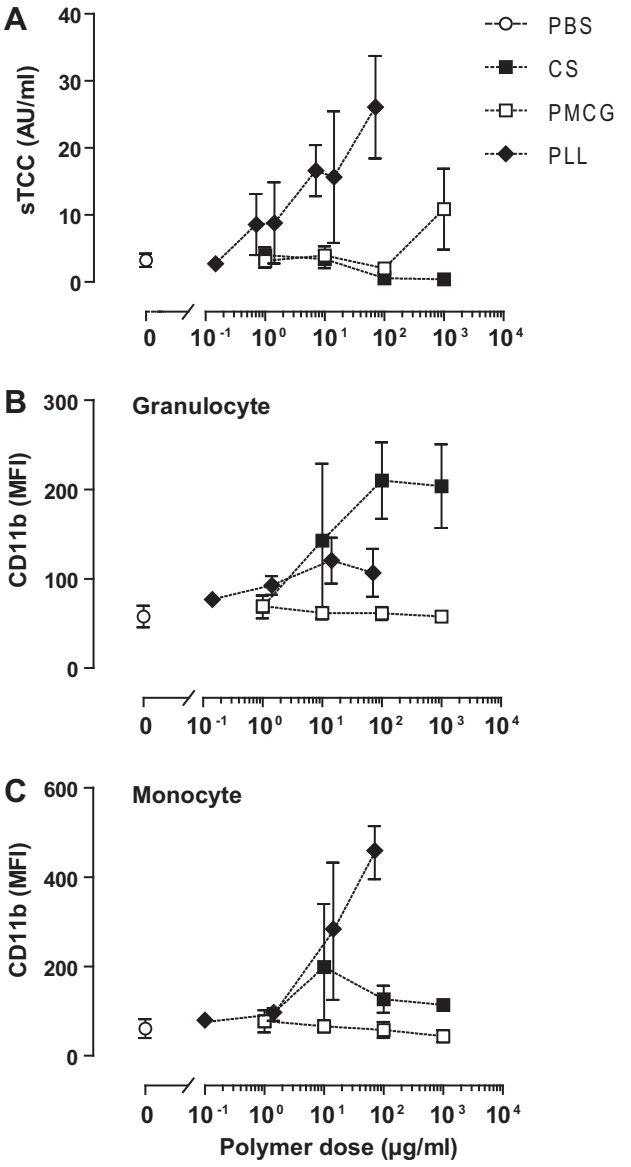

Fig. 7. sTCC concentrations and leukocyte CD11b expression after addition of PLL, CS and PMCG in solution to lepirudin anti-coagulated human whole blood. (A) sTCC after 120 min incubation, (B) granulocyte CD11b expression 15 min after addition, (C) monocyte CD11b expression 15 min after addition. Data are given as means ± SEM, $n = 3$ for sTCC and $n = 2$ for CD11b expression.

a clear dose-dependent elevation of CD11b expression in granulocytes (Fig. 7B). Increased granulocyte CD11b expression was also found after addition of PLL (Fig. 7B), although with lower potency than CS. In contrast, dissolved PMCG showed a slight dose-dependent reduction in CD11b expression in both granulocytes (Fig. 7B) and monocytes (Fig. 7C). PLL was a more potent inducer of CD11b expression than CS in monocytes (Fig. 7C). It is important to stress the time difference between the sTCC data from 120 min incubation and the CD11b expression data measured after 15 min incubation.

4. Discussion

The ability of different types of microspheres to provoke complement and leukocyte activation was evaluated using a whole blood model. The whole blood model made it possible to unmask differences in complement and leukocyte activation between the various types of microspheres. The three types of microcapsules containing polycations activated complement, while the two types of alginate microbeads did not induce activation. The activation was mainly through the alternative pathway, as the patterns of product Bb specific for this pathway corresponded well with the end product sTCC. Also, the level of C4d, formed by activation of the classical or lectin pathway, remained low (data not shown), confirming that activation occurred mainly through the alternative pathway.

The low complement activation from the alginate microbeads suggests a high degree of complement biocompatibility. The slow increase in complement activation in the saline control is consistent with activation of complement induced by the surface of the polypropylene vials used. The lower formation of complement activation products over time by alginate microbeads shows that they are less activating than the polypropylene vials and clearly supports their low activation property. The lower activity of the alginate microbeads compared with the control could be explained by absorption of complement components in the open structured gel matrix, as these alginate microbeads are estimated to be permeable for IgG (150 kDa) [24] and proteins of up to 350 kDa [11]. The C3 protein in its native form has a molecular weight of 185 kDa, thus it should be able to diffuse into the alginate matrix. Penetration of C3 was found in the alginate microbeads to variable depths of 10–125 µm (Fig. A1). Despite this absorption, we emphasize that accessible amounts of C3 should be available for formation of C3 convertase since C3 is abundant in plasma (0.7–1.8 g l^{-1}).

It is suggested that the ability of materials to bind proteins and to induce conformational changes in the proteins is essential for their complement activating capability [8]. Alginate is rich in carboxylic groups, thus negatively charged plasma proteins such as albumin (PI 4.7–5.1), C3 (PI 5.75) and C5 (PI 4.10) will have low affinities for the alginate matrix. C3 will, upon binding to the surface and undergoing a conformational change, expose highly reactive thioester [25] groups, leading to an amplification loop of the alternative pathway [5]. From the present study we could not verify whether the deposited C3 was in its native C3 form or in one of its activated or inactivated forms, as the anti-C3c antibody detects any part of C3 containing the C3c fragment. However, given the low sTCC and Bb concentrations found after incubation with alginate microbeads we suggest that this staining is mainly due to C3 present in its native form, absorbed in the permeable alginate matrix. This is also deduced from the smooth distribution pattern that indicates freely diffusive molecules rather than a process involving active convertases (discussed below). The low complement activation property of the alginate microbeads was also confirmed by a lack of adherent leukocytes, which is consistent with complement being the most likely candidate for leukocyte activation in this model.

In contrast to the alginate microbeads, the APA and AP microcapsules induced elevated levels of sTCC, Bb and anaphylatoxins C3a and C5a (Figs. 1–4), collectively demonstrating substantial complement activation. The anaphylatoxins C3a and C5a are potent inflammatory mediators which may play an active role in the initiation of inflammatory reactions against the APA microcapsules. The present data also demonstrate deposition of C3 fragments on the APA surface, starting as local spots and increasing with time, with prominent deposition after 360 min (Fig. 5). While the detected C3c fragment is present in both native C3 and active C3b, the present data indicate active C3b on the APA surface since: (1) deposition started at specific points giving a spotted pattern, indicating activation loop formation of active convertases; (2) elevated levels of C3a, which is produced in equimolar amounts to activated C3b, were found; (3) elevated levels of C5a indicated C5 convertase formation on the capsule membrane; (4) elevated levels of sTCC indicated formation of active convertases.

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Complement activation is shown to be initiated when C3b forms amide bonds with exposed amino groups [7]. Amino groups are abundant within the polypeptide PLL and are likely candidates for C3b surface binding. Soluble PLL also induced an increase in sTCC, demonstrating the complement stimulatory property of PLL.

Ideally the PLL should be neutralized by alginate, as previously demonstrated for soluble polyelectrolytes [17]. However, in APA microcapsules PLL and alginate interact at the surface [20] and PLL is found in relatively high amounts within the 100 Å thick outermost layer of [26]. Although the complex of alginate and PLL has been shown to be evenly distributed in the outermost surface, the degree of interaction may vary, resulting in exposure of PLL in a stimulatory state [26]. Insufficient neutralization of PLL could therefore explain both the stimulatory properties of PLL present in the APA microcapsules as well as the small difference between the AP and APA microcapsules in the present study.

Surface bound iC3b is an important ligand for CD11b/CD18 (CR3 receptor) on leukocytes [27]. CD11b positive cells were found to attach to the surface of APA microcapsules, thus iC3b may have been involved in the observed cell attachment. However, we cannot exclude involvement of other ligands, including surface bound fibrinogen, since this is also a known ligand for the CD11b receptor [28]. The adherent cells on APA microcapsules were mainly granulocytes with a scattered distribution of monocytes. These findings are consistent with normal inflammatory reactions, the early stages of which are dominated by neutrophilic granulocytes. The anapylatoxin C5a up-regulates neutrophil CD11b expression [29] and may thus contribute to the observed cell attachment.

The PMCG microcapsules initiated the fastest complement activation, as reflected in the early detectable levels of sTCC and Bb and deposition of C3. In combination with the sTCC data this may reflect active convertase formation. However, no further increase in C3 staining was seen after 30 min incubation, indicating reduced convertase formation with time. This was also reflected in the sTCC response, which only doubled between 120 and 360 min incubation, while the APA microcapsules induced an increase of 6–10-fold over the same time period. The explanation for these differences may be found in the ability of the various polyelectrolytes to stimulate or inhibit complement activity, affect surface properties and their ability to form stable complexes. When forming PMCG microcapsules poly(methylene–co-guanidine) diffuses into the CS/alginate microbead and complexes predominantly with CS. Leakage of polyelectrolytes may occur as a result of loose complexation or excess polymer. In the present study PMCG in solution was found to induce an increase in sTCC. Moreover, the soluble CS induced rapid up-regulation of leukocyte CD11b. Thus, rapid activation by the PMCG microcapsules might be explained by the release of polymer, with CS a likely candidate for early leukocyte CD11b up-regulation and PMCG for rapid complement activation. In contrast, soluble CS gave a slight concentration-dependent decrease in sTCC response compared with the control. This finding points towards the reported complement inhibiting activity of cellulose sulfate [30–32] and may thus explain the lower complement stimulatory abilities of the PMCG microcapsules with time.

A particularly interesting finding with the PMCG microcapsules was the low amounts of the anaphylatoxins C3a and C5a detected after the longer incubation times. The increased amount of Bb and particularly sTCC indicates that the anaphylatoxins had been formed. The most plausible explanation is therefore adsorption of the highly positively charged C3a (PI 9.7) and C5a (PI 8.6) to the negatively charged CS on the surface of PMCG microcapsules. This implies that the fluid phase concentrations do not necessarily reflect the activation potential of a surface. Anaphylatoxins present on the surface may still be biologically active and contribute to the leukocyte adherence on the PMCG microcapsule, in addition to the opsonic effect of bound iC3b, as previously discussed.

The complement activation profiles of alginate microbeads and APA microcapsules in the present study correspond well with previous biocompatibility studies showing polycation containing microcapsules to be less biocompatible [15–18,33,34]. In such studies bioincompatibility has been measured as overgrowth reactions that might have been caused by inflammatory reactions. The complement system is a primary inductor of inflammation were its protein effectors reacts upstream of leukocytes and cytokines [4,23,35–37]. Complement activity may therefore be a useful parameter for revealing bioincompatibility. The lepirudin whole blood model could be used as a rapid "screening" assay to unmask reactive surfaces, as in the case of the APA microcapsules in the present study. The presence of platelets and coagulation proteins are likely to provide a tougher environment in the whole blood model compared with, for example, the peritoneal cavity, which is a common implantation site for microspheres. Also, platelets may contribute to enhanced complement reactions [38]. However, complement and leukocytes are present in body fluids [39] and blood may come into direct contact with the capsule material during implantation, thus the same inflammatory mechanisms must be anticipated, but perhaps at lower intensities. A sensitive model like the one used in the present study is advantageous for safety evaluations, since it reveals the immune incompatibility of the implanted material in a human model.

5. Conclusion

The present study has demonstrated the effectiveness of the lepirudin-based whole blood model to reveal reactive surfaces by triggering complement and activating leukocytes. Polycation containing APA and PMCG microcapsules triggered complement and leukocyte activation, while alginate microbeads consisting of only alginate and divalent cations did not provoke complement reactions. The human whole blood model seems to be a sensitive and efficient method of revealing bioincompatibility. The method could therefore be used to determine the safety of different microcapsules for transplantation purposes.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 5 and 6, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:doi:10.1016/j.actbio.2011.03.011.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2011.03.011.

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