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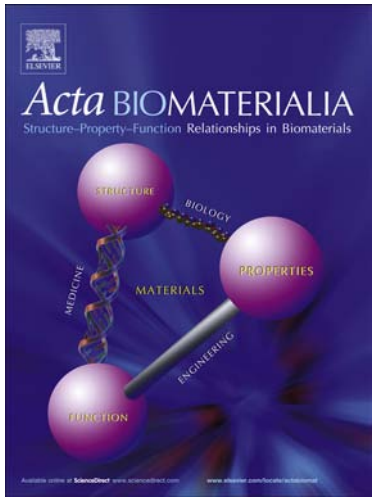


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# Sulfated alginate microspheres associate with factor H and dampen the inflammatory cytokine response

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## Abstract

Alginate microspheres show promise for cell-encapsulation therapy but encounter challenges related to biocompatibility. In the present work we designed novel microbeads and microcapsules based on sulfated polyalternating MG alginate (SMG) and explored their inflammatory properties using a human whole blood model. SMG was either incorporated within the alginate microbeads or used as a secondary coat on poly-L-lysine (PLL)-containing microcapsules, resulting in reduction of the inflammatory cytokines (IL-1β, TNF, IL-6, IL-8, MIP-1α). The sulfated alginate microbeads exhibited a complement inert nature with no induction of terminal complement complex (TCC) above the values in freshly drawn blood and low surface accumulation of C3/C3b/iC3b. Conversely, SMG as a coating material lead to substantial TCC amounts and surface C3/C3b/iC3b. A common thread was an increased association of the complement inhibitor factor H to the alginate microbeads and microcapsules containing sulfated alginates. Factor H was also found to associate to non-sulfated alginate microbeads in lower amounts, indicating factor H binding as an inherent property of alginate. We conclude that the dampening effect on the cytokine response and increased factor H association points to sulfated alginate as a promising strategy for improving the biocompatibility of alginate microspheres.



## Keywords

Alginate, complement, cytokines, factor H, sulfated alginate

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# 1 Introduction

Cell encapsulation therapy is a concept for transplantation of cells and sustainable delivery of therapeutic factors without immunosuppression. This concept could be particularly well suited in treatment of type I diabetes, currently extensively investigated with focus on device development and function. The concept encompasses the transplantation of immobilized cells in a matrix, providing a protective mechanical and semi-permeable barrier against immunological rejection. The most extensively explored encapsulation devices are microspheres of alginate hydrogels (diameter size of 0.5-1 mm). One of the major challenges is the host responses, resulting in fibrotic overgrowth depriving the free exchange of oxygen, nutrition and waste products required for a functional device [1]. New strategies to improve the alginate microsphere surface to avoid fibrotic overgrowth, and thus improve the biocompatibility, are critical toward constructing clinical suitable devices.

Alginates are a group of linear polysaccharides consisting of 1→4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). Consecutive G units form binding sites for divalent cations, cross-linking the alginate and forming a hydrated gel network suitable for cell immobilization [2]. This hydrated gel-network formed as a microbead can be sufficient to provide protection and ensure cell viability upon transplantation in small animal models [3]. An outer polycation layer, such as poly-L-lysine or poly- L-ornithine, has commonly been employed to reduce the permeability of host proteins and to stabilize the microspheres. The cationic surface does, however, promote cell attachment and stimulates an inflammatory response [4, 5]. Cellular overgrowth and fibrosis are reduced by introducing a secondary alginate coat [6], which also provides a slight reduction in the inflammatory potential [4, 7]. While the alginate microbeads can be free of fibrotic overgrowth and show long-term function of the implant in small animal models, they have been prone to fibrotic overgrowth in primate models and humans. In clinical studies alginate microbeads were surrounded with fibrotic tissue, and the encapsulated pancreatic islets failed to restore the blood glucose [8, 9]. Although the clinical setting is complicated with the causative relationship involving oxygen deprivation and secretion of inflammatory and immunogenic components by the encapsulated cells, these findings still point to the requirement of device modifications. The severity of a fibrotic response to microspheres depends among other factors on the molecular composition and surface properties of the implanted material [1]. Other important variables are the choice of animal model, site of introduction and the implantation procedure

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[10]. There is an initial connection between the ability to trigger inflammation and the fibrotic responses [11], and the inflammatory potential can be studied by the use of a human whole blood model with the possibilities of comparing a set of variables during identical conditions [12, 13]. The human whole blood model is therefore valuable as a first-screening tool of the inflammatory properties caused by the biomaterial surface in order to define the most promising candidate device for transplantation.

We have previously shown a close connection between the potential of alginate microspheres to activate complement and the induction of inflammation [7]. A general strategy of increasing biomaterial biocompatibility is by inhibiting the coagulation and complement cascades by heparin coating of the surfaces [14, 15]. This strategy potentially also involves the binding of inflammatory cytokines [16]. In addition to directly associating with complement factors, heparin as well as other glycosaminoglycans has been shown to bind factor H [17]. Factor H possesses two important functions in reducing the complement activation, by hindering establishment of the C3 convertase (decay-accelerating activity) as well as assisting factor I-mediated cleavage of C3 convertase products C3b and iC3b [18, 19]. Due to its potent inhibiting properties, direct conjugation of factor H to biomaterials has been proposed for increasing complement compatibility [20]. Sulfated alginates can be viewed as structural analogs to heparin, and we have previously shown that sulfated alginates inhibit formation of the terminal complement complex in human plasma [21].

Heparin coating may efficiently improve the biocompatibility in blood contact devices for short-time exposure [22]. The biocompatibility has also been improved when used as a coating on alginate microbeads over a four week transplantation period in the peritoneal cavity of mice [23]. However, the heparin coating was gradually lost, demonstrating a challenge to the long-term stability. Alginate forms a relatively strong complex with polycations and is slowly degraded *in vivo*, making it suitable for long-term applications [24, 25]. The structural similarities of sulfated alginate to heparin, as well as their previously demonstrated anti-complement activity, motivated exploring its potential to reduce the inflammatory properties of alginate microspheres.

In the present work we designed alginate microspheres using sulfated alginate, incorporated as a secondary coat on PLL-alginate microcapsules or mixed with the gel core of a non-coated alginate microbead. A human whole blood model was used to evaluate the inflammatory properties of the

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microspheres [12], as well as the impact of the modifications on the complement activating protein C3 and the complement-inhibitory factor H. This is to our knowledge the first biocompatibility evaluation of sulfated alginates microspheres.

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# 2 Materials and methods

## 2.1 Materials

Ultra-pure (UP) grade LVG ($F_G$=0.67, Mw = 199 kDa, endotoxin <43 EU/g) provided by FMC Biopolymer (Sandvika, Norway) was used as gelling alginate. The poly-M alginate ($F_M$=1.0) used for epimerization was ultra-pure (UP) grade (endotoxin < 100 EU/g) mannuronan produced by an AlgG$^-$ strain of *Pseudomonas fluorescens* [26]. Poly-L-lysine ($M_w$ = 15,000-30,000 Da) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chlorosulfonic acid (99%) provided by Sigma-Aldrich and formamide provided by Merck (Whitehouse Station, NJ, USA) were used for sulfation of alginates. Poly-MG and SMG alginates were filtered using Millistak+® CR40 activated carbon filters (Millipore, Billerica, MA, USA). PE α-CD14 and FITC α-CD11b were purchased from BD BioSciences (San Jose, CA, USA). For protein deposition analysis FITC-conjugated Polyclonal Rabbit anti-human C3c (F0201) was provided by Dako (Glostrup, Denmark) and the control antibody was a FITC-conjugated Rabbit polyclonal anti mouse Ig (F0261), both from Dako (Glostrup, Denmark). Sheep α-human Factor H was purchased from Binding Site (San Diego, CA, USA) and the secondary antibody CF633-conjugated α-sheep IgG was provided by Sigma Aldrich.

## 2.2 Alginate microspheres

### 2.2.1 Preparation of alginates

Poly-M alginate was epimerized by mannuronan C5 epimerase AlgE4. This enzyme acts processively introducing a strictly polyalternating sequence, poly-MG ($F_G$=0.47, $F_{GG}$=0) [27]. The poly-MG alginate was chemically sulfated to sulfation degrees (DS, sulfate/monosaccharide) of 0.30 and 0.75 by adding respectively 1.75% and 4% (v/v) chlorosulfonic acid in formamide to dried alginate for 2.5 h at 60 °C. The sulfation procedure and resulting substitution pattern have previously been described for alginates with homogeneous sequence patterns [21]. The poly-MG and SMG alginates were purified using activated charcoal filters, and were subsequently sterile filters along with the UP-LVG alginate. The endotoxin levels of the poly-MG, SMG 1 and SMG 2 were 35, 14 and 11 pg/mL, respectively, as determined by the LAL-assay (Lonza, NJ, USA).

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### 2.2.2 Microsphere formation

Six different microspheres were used in the present study (Table 1). For the microbead core a 1.8% solution alginate in 0.3 M mannitol was dripped into a solution of 50 mM $CaCl_2$ and 0.15 M mannitol, using a high-voltage electrostatic bead generator (7kV) [28]. For the A/MG and A/SMG microbeads, UP-LVG was mixed with poly-MG or SMG (DS=0.75) alginate in a ratio of 80/20% w/w, respectively, while the gelling solution contained additionally 1 mM $BaCl_2$. For primary coating the microbeads were transferred to a 0.1% solution of poly-L-lysine in 0.15 M mannitol (10 min), and the AP-MG and AP-SMG microcapsules were exposed to a secondary coating layer (10 min) of 0.1% poly-MG or SMG (DS=0.30) alginates, dissolved in 0.15 M mannitol. The microspheres were washed with 0.9% NaCl following microbead formation and each coating step, and finally stored in 0.9% NaCl. All steps above were carried out in sterile conditions. Microsphere diameter was measured as an average of 30 spheres using a light microscope with a stage micrometer, and is shown in Table 1. To visualize the sulfated alginate in the microsphere constructs, alginates were in a separate experiment labeled with fluoresceinamine and studied by confocal microscopy (Appendix A).

**Table 1:** Composition of alginate microspheres. The gel core was prepared from 1.8% solutions of ultra-pure (UP) grade LVG alginate. A subset of the microspheres was coated with poly-L-lysine (AP) and a secondary coat of either non-sulfated poly-MG alginate (AP-MG), or sulfated poly-MG (AP-SMG, DS=0.30). A/MG and A/SMG were uncoated microbeads created from UP-LVG containing 20% w/w MG or SMG (DS=0.75), respectively.

| | A | A/SMG | A/MG | AP | AP-MG | AP-SMG |
|---|---|---|---|---|---|---|
| **Core** | UP-LVG | UP-LVG/ SMG | UP-LVG/ MG | UP-LVG | UP-LVG | UP-LVG |
| **Primary coat** | X | X | X | PLL | PLL | PLL |
| **Secondary coat** | X | X | X | X | MG | SMG |
| **Avg. diameter (µm)** | 549 ± 39 | 437 ± 27 | 450 ± 17 | 546 ± 39 | 580 ± 35 | 539 ± 37 |

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## 2.3 Assessment of inflammatory properties in whole blood

### 2.3.1 Whole blood model

Fresh blood was drawn from five volunteers and the coagulation inhibited by lepirudin. PBS supplemented with $CaCl_2$ and $MgCl_2$ (100 µL) was added to microspheres (50 µL) suspended in 0.9% NaCl (50 µL), followed by addition of blood (500 µL) to each sample in 1.5 ml Nunc cryotubes. The procedure has previously been described in details [4]. LPS (100 ng/mL) and zymosan (10 µg/sample) were used as positive controls. The microspheres were incubated in blood at 37 °C under constant rotation using a unidirectional tube roller (4 h). EDTA was added to the blood to a concentration of 10 mM, followed by centrifugation (3000 rpm, 15 min) and harvesting of plasma. The plasma was stored frozen at -20 °C prior to further analysis.

### 2.3.2 Cytokine induction

Plasma samples were analyzed using a Bio-Plex Human cytokine 27-Plex Panel (Bio-Rad, Hercules, CA, USA) for the following cytokines: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8(CXCL8), IL-9, IL-10, IL-12, IL-13. IL-15, IL-17, Eotaxin-1 (CCL11), bFGF, G-CSF, GM-CSF, IFN-γ, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), PDGF-BB, RANTES (CCL5), TNF and VEGF. Analyses were carried out according to the provided protocol with half the recommended amount of magnetic bead-coupled capture antibodies.

### 2.3.3 Complement activation (TCC)

The level of soluble terminal complement complex (TCC) in the harvested plasma was measured using the Human Terminal Complement Complex ELISA kit (Hycult Biotech, Uden, Netherlands) in accordance with the provided protocol, at 1:200 dilution of the plasma incubated with coated microcapsules and 1:100 dilution of plasma from non-coated microbeads.

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#### 2.3.4 Leukocyte activation

After 1 h incubation, blood (25 µL) was fixed using a 0.5% solution of PFA (25 µL) at 37 °C (4 min). The samples were stained with FITC-conjugated mouse anti-human CD14 and PE-conjugated mouse anti-human CD11b (5 µg/mL) protected from light (20°C, 15 min). The monocytes and granulocytes were gated in an SSC/FITC-anti-CD14 dot plot using a BD FACSCanto™ II flow cytometer, and the CD11b and CD14 expression was given as median fluorescence intensity (MFI).

#### 2.3.5 Complement C3 and Factor H surface deposition

For each sample, microspheres (50 µL) in saline (200 µL) were incubated with human plasma (300 µL) for 4 h at 37 °C. The microspheres were washed in saline and incubated with either FITC-conjugated rabbit anti-human C3c (50 µg/mL) or its control FITC-conjugated rabbit anti-mouse IgG. The binding of Factor H was evaluated using Sheep anti-human Factor H (50 µg/mL) followed by secondary CF633-conjugated IgG (10 µg/mL) for 30 min under constant rotation (25 °C). As a control, staining was performed using the secondary CF633-conjugated IgG only. The microspheres were washed with 0.9% NaCl before deposition of C3c and factor H was visualized using a Zeiss LSM 510 confocal microscopy equipped with a 488 nm Argon and a 543 nm HeNe laser (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were captured by optical cross-sections through equator by laser and differential interference contrast. 3D projections were made from sections through the microspheres using z-stacks and ImageJ software (National Institutes of Health, Bethesda MD, USA). Surface intensity profiles were made from the bottom section of the microspheres using ImageJ.

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### 2.3.6 Statistical Methods

Statistical analyses were performed using repeated measures (RM) one-way ANOVA for multiple comparisons. Dunnett's multiple comparison tests were used for all treatments related to the baseline (T0), as well as comparison between microspheres of interest. Statistical significance was set to $p \leq 0.05$ in both cases. Due to the low number of replicates, we assumed that the data were not normally distributed, thus the analyses were done after log-transformation.

### 2.3.7 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 according to their guidelines.

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# 3 Results

## 3.1 Cytokine induction

The induction of selected inflammatory cytokines by the various alginate microspheres and controls is shown in Figure 1. Most treatments resulted in a significantly elevated cytokine induction from the baseline value (T0, freshly drawn blood), with the exception of the different alginate microbeads inducing a slight but often non-significant elevation. The alginate microbeads showed cytokine induction at a similar or lower level compared to the saline control. Sulfated alginate microbeads (A/SMG) induced lower cytokine levels compared to the other alginate microbeads (A and A/MG), with significant reductions of IL-8, MIP-1α and IL-6. The PLL-coated microcapsules (AP) induced a prominent cytokine response with elevated levels of all presented cytokines. By coating the microcapsules with non-sulfated poly-MG (AP-MG) a general lowering was observed with significant values for IL-8 and MIP-1α. Microcapsules coated with sulfated alginate (AP-SMG) resulted in a significant reduction of the inflammatory cytokines (IL-1β, IL-6, TNF, IL-8 and MIP-1α) compared to the AP microcapsules. Compared to the AP-MG microcapsules AP-SMG led to a significant reductions of IL-1β and IL-6. An exception to this general trend was IL-8 where the AP-SMG coat did not lead to any further lowering. Another exception was the MCP-1 induction which was increased by the poly-MG coat. This effect was counteracted by the sulfated coat resulting in equal induction of MCP-1 as for the AP microcapsules. The concentrations of the remaining cytokines are presented in Appendix B. IL-2, IL-5, IL-15 and IFN-γ concentrations were below the detectable range, and were thus excluded from the study.

**Figure 1:** Plasma concentrations (pg/ml) of cytokines IL-1β, IL-6, TNF, IL-8, MIP-1α and MCP-1 following 4 h incubation of alginate/sulfated alginate microspheres in whole blood anti-coagulated with lepirudin. Significant values are given as $p<0.05$ compared to the baseline value (*) and from internal comparison of selected microspheres (#). Data are expressed as the mean value of 5 donors ± SEM.

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## 3.2 Complement activation (TCC)

The plasma concentrations of soluble terminal complement complex (TCC) are shown in Figure 2. All conditions significantly increased TCC after four hours incubation compared to the baseline level (T0), with the exception of the sulfated alginate microbeads (A/SMG). The TCC induction by the saline control represents the background activation by the polypropylene tubes, and the alginate microbeads all displayed lower TCC than the saline control. Sulfated alginate microbeads (A/SMG) induced significantly lower TCC than the alginate microbeads controls (A and A/MG), to approximately the same amounts found in freshly drawn blood (T0). The results indicate that the alginate microbeads in general reduce the complement response, with an additional inhibitory effect provided by the inclusion of sulfated alginate. The AP microcapsules caused a prominent induction of TCC at a similar level as the *E.coli* control. Coating the AP microcapsules with the non-sulfated poly-MG alginate (AP-MG) resulted in a significant reduction by approximately 40%. Conversely, coating with sulfated MG alginate (AP-SMG) led to a significant induction compared to the AP-MG microcapsules and a slight elevation compared to the AP microcapsules.

**Figure 2:** Concentration of soluble terminal complement complex (TCC) in plasma following incubation of alginate/sulfated alginate microspheres in lepirudin anti-coagulated whole blood. Microspheres were incubated in blood for 4 h followed by inactivation with EDTA and collection of plasma. Significant values are given as $p<0.05$ compared to the baseline value (*) and from internal comparison of selected microspheres (#). Data are expressed as the mean value of 5 donors ± SEM.

## 3.3 Leukocyte activation

The expression of CD11b on granulocytes and monocytes is shown in Figure 3. CD11b is a subunit of the complement receptor CR3 (CD11b/CD18) and is involved in leukocyte adhesion and migration, by binding to the complement activated product of iC3b [29], as well as to fibrinogen [30]. For the monocytes, all conditions except for the sulfated alginate microbeads led to a significant increase in CD11b from the baseline. The CD11b on granulocytes showed a similar trend with significantly increased expression from the AP-MG and AP-SMG microcapsules. There was observed a generally low CD11b expression in samples treated with alginate microbeads, whereas the sulfated alginate

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microbeads displayed a significant reduction compared to the saline and the alginate controls, indicating an inhibitory effect. The CD14 expression was less distinguished for the various treatments, where the lowest expression was observed in samples incubated with A/SMG microbeads (Appendix A3).

**Figure 3:** Expression of CD11b on monocytes (top) and granulocytes (bottom) in lepirudin anti-coagulated whole blood following 1 h incubation with alginate/sulfated alginate microspheres. Samples were analyzed by flow cytometry, expressed as the median fluorescence intensity (MFI). Significant values are given as $p<0.05$ compared to the baseline value (*) and from internal comparison of selected microspheres (#). Data are expressed as the mean value of 5 donors ± SEM.

## 3.4 Complement C3 surface deposition

Alginate microspheres were incubated with plasma, followed by fluorescence staining of C3c. The microspheres were imaged using confocal microscopy, revealing the presence of C3 or its activation products (C3b/iC3b). Figure 4 shows the surface deposition of C3c in an equatorial cross-section (i) and by 3D-projection of the microspheres (ii). The A and A/MG microbeads displayed an overall low degree of C3c deposition, where weak fluorescence was observed in approximately one of twenty microbeads, devoid of the deposition pattern observed on the AP microcapsules. There was observed a weak and homogeneous C3c deposition on the A/SMG microbeads and was, in contrast to the non-sulfated controls, found on all microbeads in the sample. On the AP microcapsules C3c was deposited in a spotted pattern, indicating propagation sites for additional factor recruitment and assembly of the C3 convertase, consistent with previous findings [4]. Interestingly, no C3c was observed on the surface of the AP-MG microcapsules in the current experiment or from repeated incubations in various plasma samples using the same batch of microspheres. Conversely, AP-SMG microcapsules displayed excessive C3c deposition oversaturating the 3D projections and complicating distinction of deposition patterns. No fluorescence was observed on or around the microspheres using control antibodies, excluding contribution from non-specific staining. Measurement of the fluorescence intensity on the surface of microspheres showed relatively narrow intensity profiles for the A/SMG and AP-SMG microspheres, indicating a more homogeneous deposition contrasting the spotted pattern observed on AP microcapsules (Figure 5). The surface

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intensity was not measured for the alginate controls (A and A/MG) as the majority of the microbeads did not display any C3c deposition.

**Figure 4:** Deposition of FITC-stained C3c on alginate/sulfated alginate microspheres following 4h incubation in human plasma, shown in the equatorial cross-section (i) and by 3D projection of the entire sphere (ii). White bars represent 200 µm.

**Figure 5:** Fluorescence intensity of FITC-stained C3c on the surface of alginate/sulfated alginate microspheres following 4h incubation in human plasma.

## 3.5 Factor H surface deposition

The alginate microbeads displayed homogenous distribution of factor H with the most prominent fluorescence on the sulfated alginate microbeads (A/SMG), indicating a larger amount of associated factor H (Figure 6). For the AP microcapsules a low-degree, spotted pattern of factor H deposition was found, similar as observed for C3c. Also consistent with the C3c deposition, no stained factor H was observed on the AP-MG microcapsules. The AP-SMG microcapsules displayed a substantial amount of surface-associated factor H, whereas the microcapsules appeared damaged potentially due to swelling of the microcapsules from repeated saline washes during staining. Cross-sections of the microspheres revealed that factor H was localized on the surface of the microcapsules, while a graded penetration into the bead core was observed for the alginate microbeads. No fluorescence was observed in the control samples, excluding non-specific labeling and retention of unbound antibodies in the alginate microspheres. The surface of the A/SMG and AP-SMG microcapsules showed the greatest fluorescence intensity, with a narrow intensity distribution indicating homogeneous protein deposition. The alginate controls (A and A/MG) displayed a similar surface deposition as the sulfated microspheres, but with a slightly lower intensity. There was measured an overall low intensity on the AP microcapsule surface, with some high-intensity aggregates as observed when staining C3c (Figure 7).

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**Figure 6:** Deposition of CF633-stained factor H on alginate/sulfated alginate microspheres following 4h incubation in human plasma, shown in the equatorial cross-section (i) and by 3D projection of the entire microsphere (ii). White bars represent 200 µm.

**Figure 7:** Fluorescence intensity of CF633-stained factor H on the surface of alginate/sulfated alginate microspheres following 4h incubation in human plasma.

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# 4 Discussion

In this paper we introduced sulfated alginates as a strategy to modify alginate microspheres for improved biocompatibility, and assessed their inflammatory properties using a human whole blood model. Briefly, the sulfated polyalternating MG alginate, designated SMG, was mixed with alginate to form sulfated alginate microbeads (A/SMG) or incorporated as a secondary coat on alginate-poly-L-lysine (AP) microcapsules. Sulfated alginate microbeads induced the overall lowest cytokine responses of all tested treatments including the non-sulfated microbeads. Notably, the amounts of fluid phase complement (TCC) were after four hours incubation comparable to the amounts in freshly drawn blood, indicating a true complement inert nature of the sulfated alginate microbeads. The sulfated alginate coated microcapsules demonstrated the ability to dampen the cytokine response in comparison to the AP microcapsules, thus also pointing to an anti-inflammatory effect. Overall sulfated alginate demonstrates interesting anti-inflammatory properties warranting further exploration in cell-encapsulation therapy.

A novel finding in the present study was the association of factor H, a negative regulator of complement, to the alginate microbeads and even more pronounced to the sulfated alginate microbeads. The lack of complement inhibitors on a biomaterial surface may lead to a continuous assembly of the C3 convertases leading to activation [31]. As demonstrated in the present study, as well in previous work [4], alginate microbeads demonstrate a slight anti-complement activity as the resulting TCC formation is consistently lower than the background activation by the saline control. The low presence and smooth distribution of non-aggregated C3c indicates adsorption of C3 in its native state or an inactivation to its iC3b state, and thus that the C3 convertase is either not formed or rapidly inactivated. The presence of factor H on the alginate microbeads may indicate an inherent binding to alginate that is further strengthened by the addition of sulfate groups, and provide the explanation for the lack of C3 convertase formation. Factor H has three polyanion binding sites on the protein surface, and has been demonstrated to associate with sulfated glycosaminoglycans [32, 33]. Sulfated alginate has a similar polysaccharide backbone to glycosaminoglycans, and have previously been demonstrated to have protein-binding properties analogous to heparin and heparan sulfate [21, 34]. The sulfated alginate in the present study (polyalternating MG alginate) was designed to mimic heparan sulfate in structure and approximately in charge density, potentially improving association

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with factor H. Since we also detected factor H on the ordinary alginate microbeads, alginate may have an inherent ability to bind factor H through its polyanion binding sites which can also provide an explanation for the complement compatible nature of alginate.

Our data may further indicate a connection between factor H binding and the cytokine dampening potential. A/SMG microbeads with a higher amount of associated factor H induced slightly lower cytokine levels than the non-sulfated alginate microbeads. Furthermore, the SMG-coated PLL microcapsules bound more factor H than its respective controls. We have previously found that complement inhibition completely blocks the inflammatory cytokine induction by alginate microcapsules, indicating a strong connection [7]. It has also been observed that the building of the C3 convertase on the microsphere surface rather than the fluid phase activation is of importance to the cytokine induction [35]. In the present study microcapsules with a secondary sulfated coat showed very high amounts of deposited C3, but nonetheless a dampening of the cytokine response, which we propose is related to the elevated factor H association. Notably, the detected TCC concentration was not remarkably higher compared to the AP microcapsules, indicating that a significant proportion of the deposited C3 was not associated with an active convertase, but rather in the form of native C3 or inactivated iC3b. This was further indicated by the surface fluorescence intensity, showing a relatively homogeneous C3c deposition on AP-SMG with a narrow intensity distribution, contrasting the spotted deposition on AP microcapsules. The high negative charge of sulfated alginates presumably facilitates electrostatic interactions with complement factors directly, and indirectly through additional plasma proteins as previously described for heparin [36]. While the cationic surface of AP microcapsules may be more susceptible for convertase formation by covalent binding to C3b [37], increased recruitment of native C3 to the surface of sulfated alginate coated microcapsules may accelerate activation resulting in the strong TCC induction observed in the present study. On the AP microcapsules factor H was limited to the surface in similar patterns to the C3 deposition. This potentially represents deregulation of the convertases by binding of factor H to C3b and inactivating C3b as a secondary effect to C3 cleavage, contrasting the factor H binding on the microbeads presumably due to direct interaction with alginate.

An observation that further supports the impact of factor H on the cytokine response was a lower factor H association accompanied with an increased cytokine induction using a SMG coat with

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sulfation degree of 0.75 (Appendix D). This might further indicate that there exists an optimum between the degree of sulfation (as presented here of 0.3) on the alginate and the factor H binding. Variance in sulfation degree affects the net surface charge and has a strong influence on complexation with PLL, both relevant properties for the inflammatory response to alginate microspheres. The immunological response to biomaterials is additionally influenced by surface topography which is expected to vary depending on the coating material, whereas sulfated alginate in the microbeads do not form stable cross-links, causing a decrease in the stiffness of the gels and potentially an altered network structure [38].

Surprisingly, there was found no C3 or factor H deposition on the microcapsules coated with non-sulfated poly-MG alginate (AP-MG). It has previously been shown that poly-MG alginate forms a denser coat on alginate-PLL microcapsules compared to other alginate sequences, presumably due to higher chain flexibility [39, 40]. Furthermore, the interaction strength between alginates and PLL has been found to increase with the mannuronic acid content of the alginates [25]. Calcium ions disassociate more readily from poly-MG alginates than alginates containing G-blocks, increasing PLL binding and potentially improving charge neutralization. Upon sulfation the poly-MG alginate adopts a more rigid conformation due to steric effects and intramolecular charge repulsion by the sulfate groups, and may hinder optimal coverage of the polycation layer. We observed in addition to the presented data that microspheres retrieved immediately following blood incubation showed cell aggregates on the surface of AP and AP-SMG microcapsules, but not on the AP-MG microcapsules. This may indicate a less effective coating by the sulfated SMG alginates, exposing PLL susceptible to cell binding. Alternatively, cells may associate directly with sulfated alginate, as proposed for heparan sulfate binding L-selectins during leukocyte extravasation over the endothelium [41]. While the present results suggest an inert surface on the AP-MG microcapsules, there was observed a significant induction of TCC and the strongest induction of MCP-1 was from the AP-MG microcapsules. In a recent study we observed that MCP-1 was primarily influenced by complement-activating products of the soluble phase (TCC, C5a), rather than the C3 deposition patterns that are more critical for the majority of the inflammatory cytokines [35]. From the present complement data (TCC, and C3 and factor H deposition) we cannot provide a complete explanation of the cytokine findings by the AP-MG microcapsules. We can, however, not exclude the possibility of formation and

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release of associated coating materials, which together with the fluid phase products of complement may explain the observed results.

The CD11b expression on monocytes and granulocytes mirrored the TCC response, implicating complement as an initiating event in leukocyte activation. C5a is a critical stimulant of CD11b expression on leukocytes [12, 42], and is formed when C5 is cleaved by the C3 convertase upon formation of TCC. CD11b is a part of the complement CR3 receptor that mediates leukocyte adhesion to the inactivated iC3b [43] as well as to the RGD sequence presented within fibrin [44]. Our data indicate that the sulfated coat triggers the CD11b expression, preparing the leukocytes for cell-attachment while the sulfated alginate microbeads are doing the opposite by rendering the CD11b expression approximately at the baseline level.

Heparin exerts inhibitory effects on the complement system, and is currently used as a coating material to reduce thrombosis, fibrosis and the inflammatory response to cardiovascular devices [45]. Heparin coating has been shown to reduce C3 activation products and increase the conversion of C3b to the inactive form iC3b [46]. Notably, heparin coating of alginate microcapsules has not been successful due to low surface stability, making it less eligible for cell encapsulation purposes. Based on the results of the present work and previous studies demonstrating complement-dampening effects analogous to heparin [21], sulfated alginates may provide a promising alternative for a more stable system, both physically due to alginate-sulfated alginate interaction and chemically due to the lack of alginate-degrading enzymes in humans.

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## 5 Conclusions

We present for the first time sulfated alginate microspheres with evaluation of their inflammatory properties. Inclusion of sulfated alginate in the gel core resulted in microbeads that were practically inert toward complement and cytokines in our model. We propose an inherent association of factor H with sulfated and non-sulfated alginate, contributing to its biocompatible nature. As a coating material, the sulfated alginate showed a complexity of responses dependent on the sulfation degree. Using alginate with a low sulfation degree resulted in a generally low cytokine response, which may be related to an observed high degree of surface-bound factor H. In conclusion sulfated alginates provide anti-inflammatory properties and thus show promise in microbeads and microcapsules modifications, warranting further exploration in animal models.

## 6 Acknowledgements

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## 7 Disclosures

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

## 8 Appendices

Appendix A shows the presence of sulfated alginate in the core of microbeads, or as a coat on microcapsules, visualized by confocal microscopy. Cytokines not included in the results section are found in Appendix B, and Appendix C shows the CD14 expression on monocytes and granulocytes measured by flow cytometry. Appendix D compares the effect of SMG coat sulfation degree on protein deposition and cytokine induction.

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Figure 1

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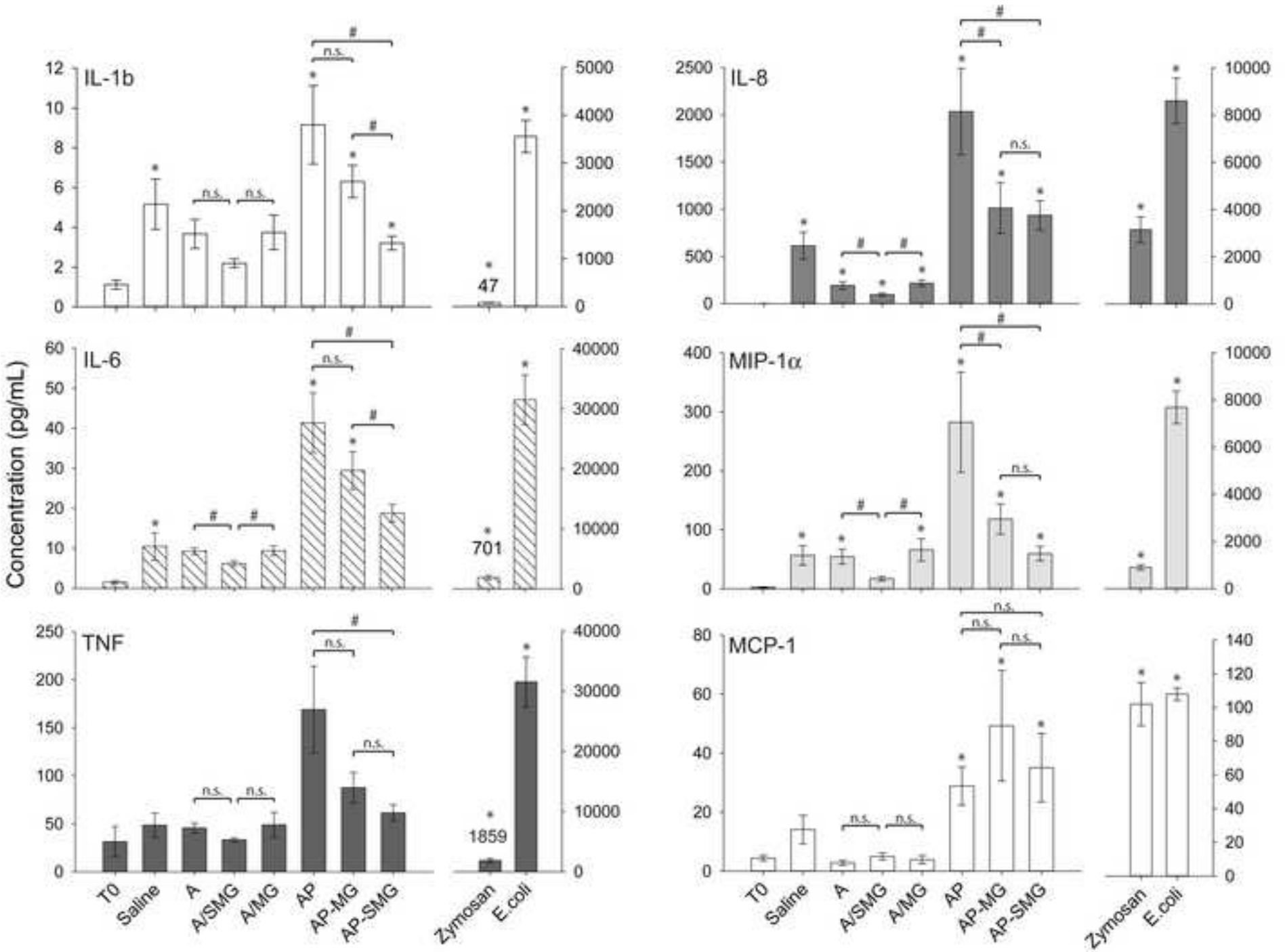

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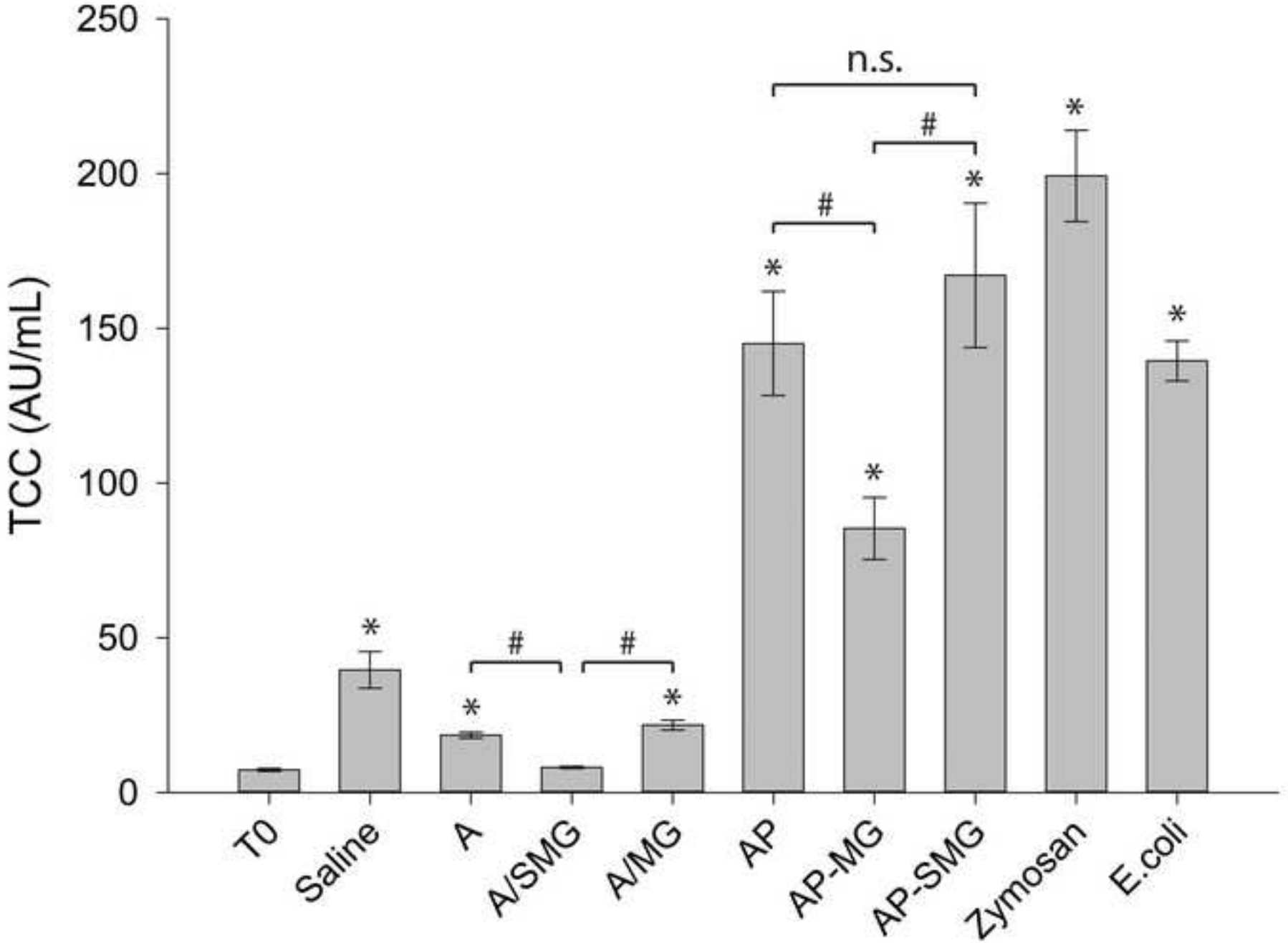

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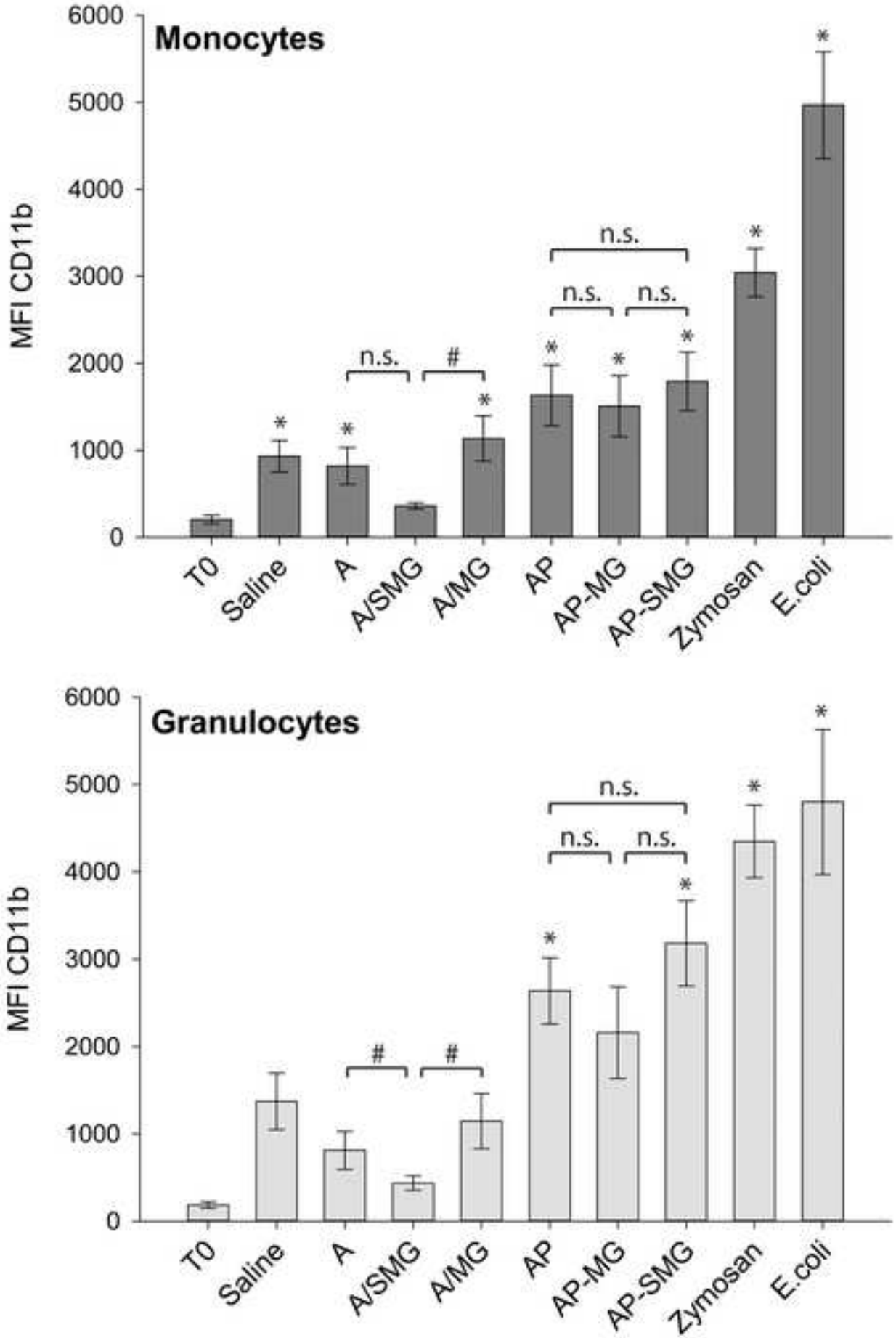

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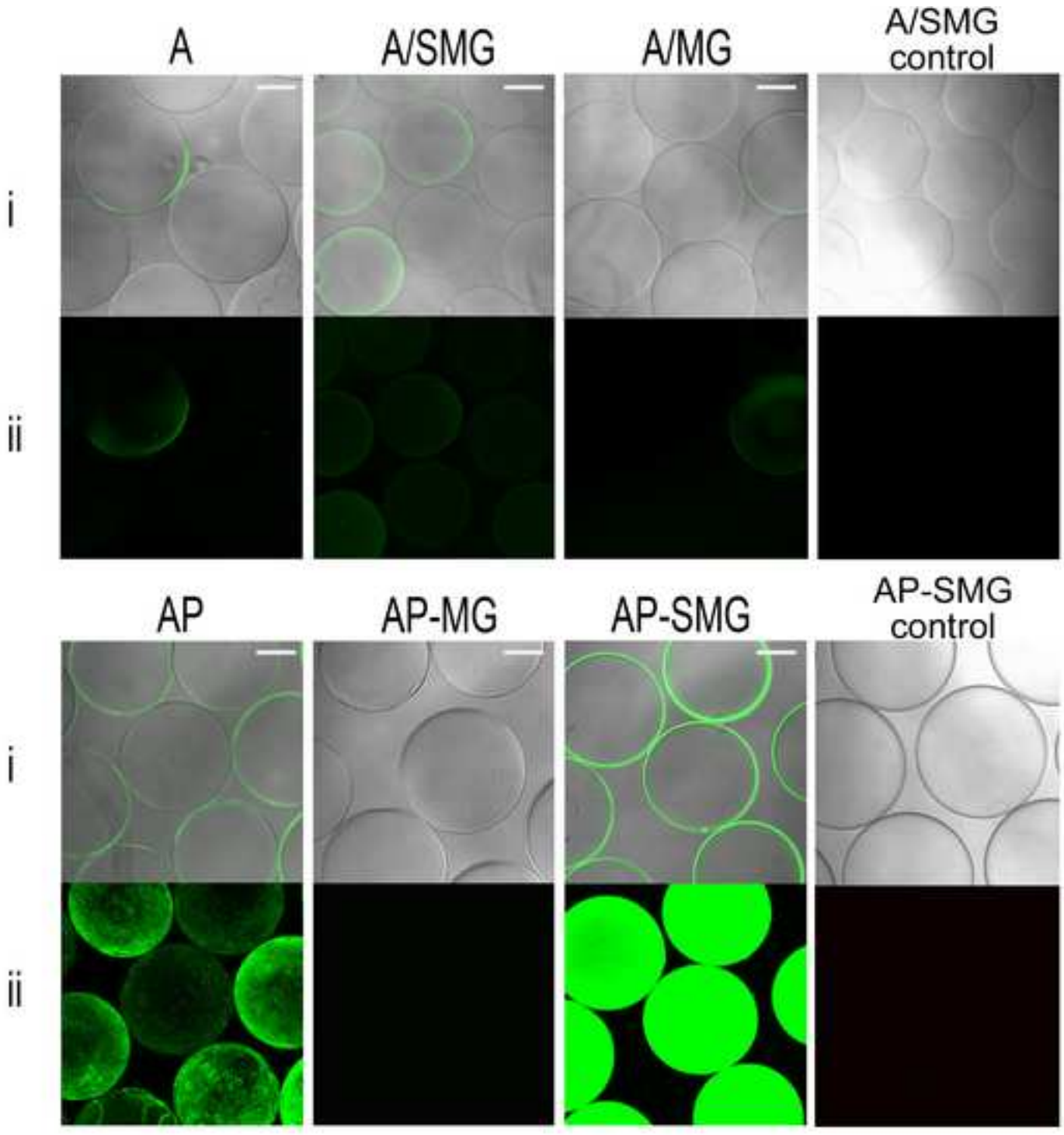

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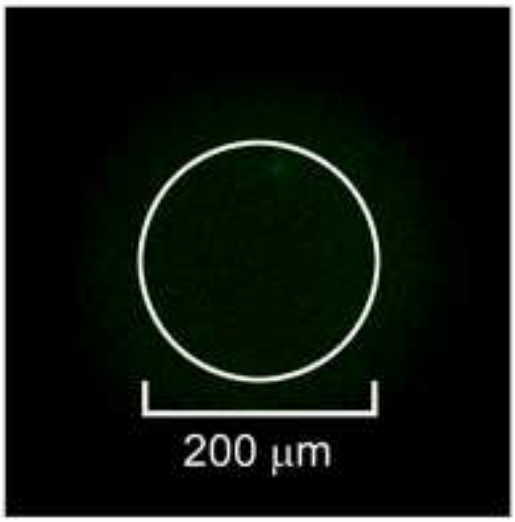


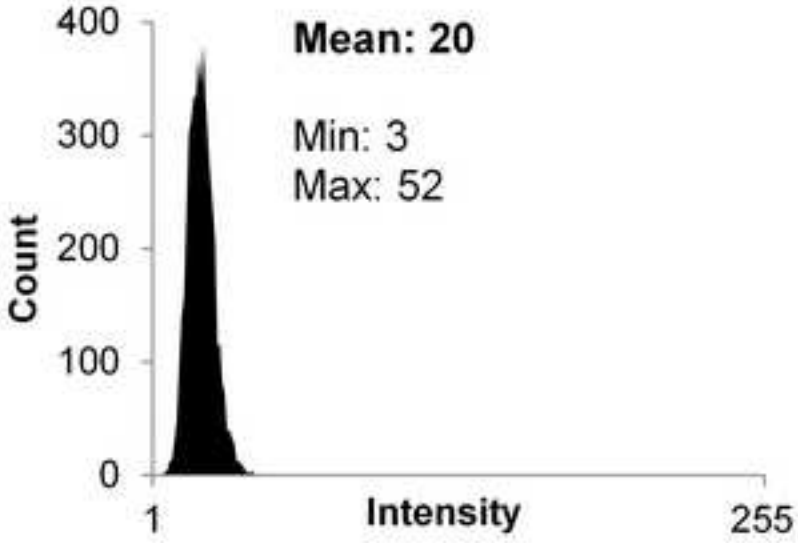


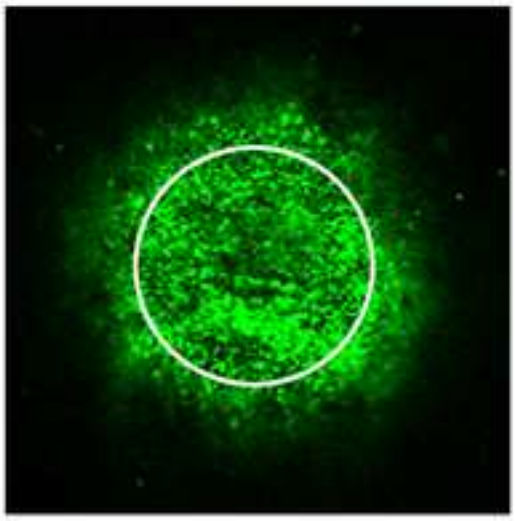

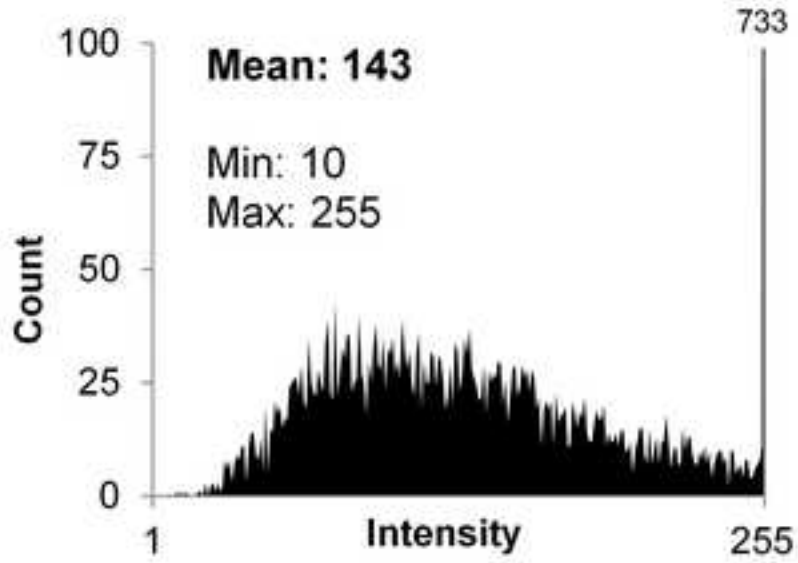


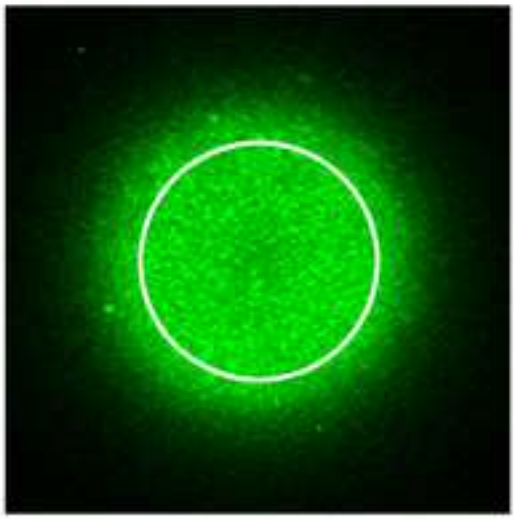

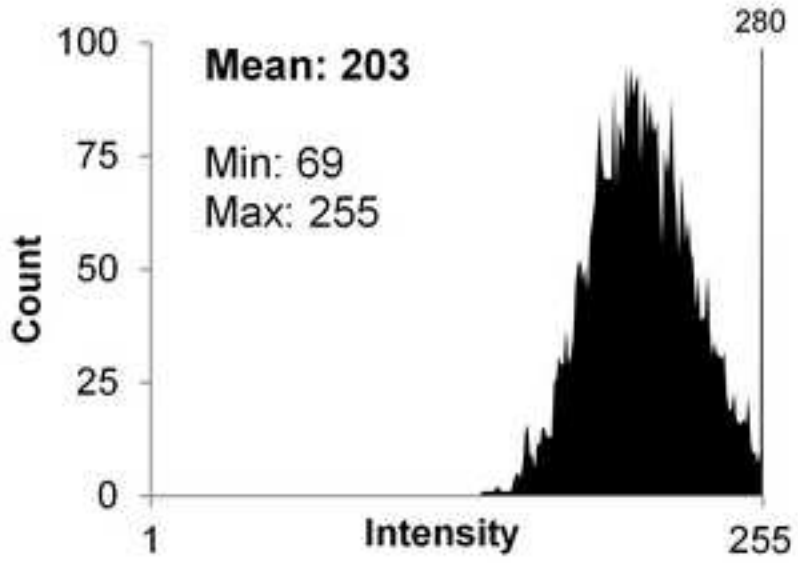

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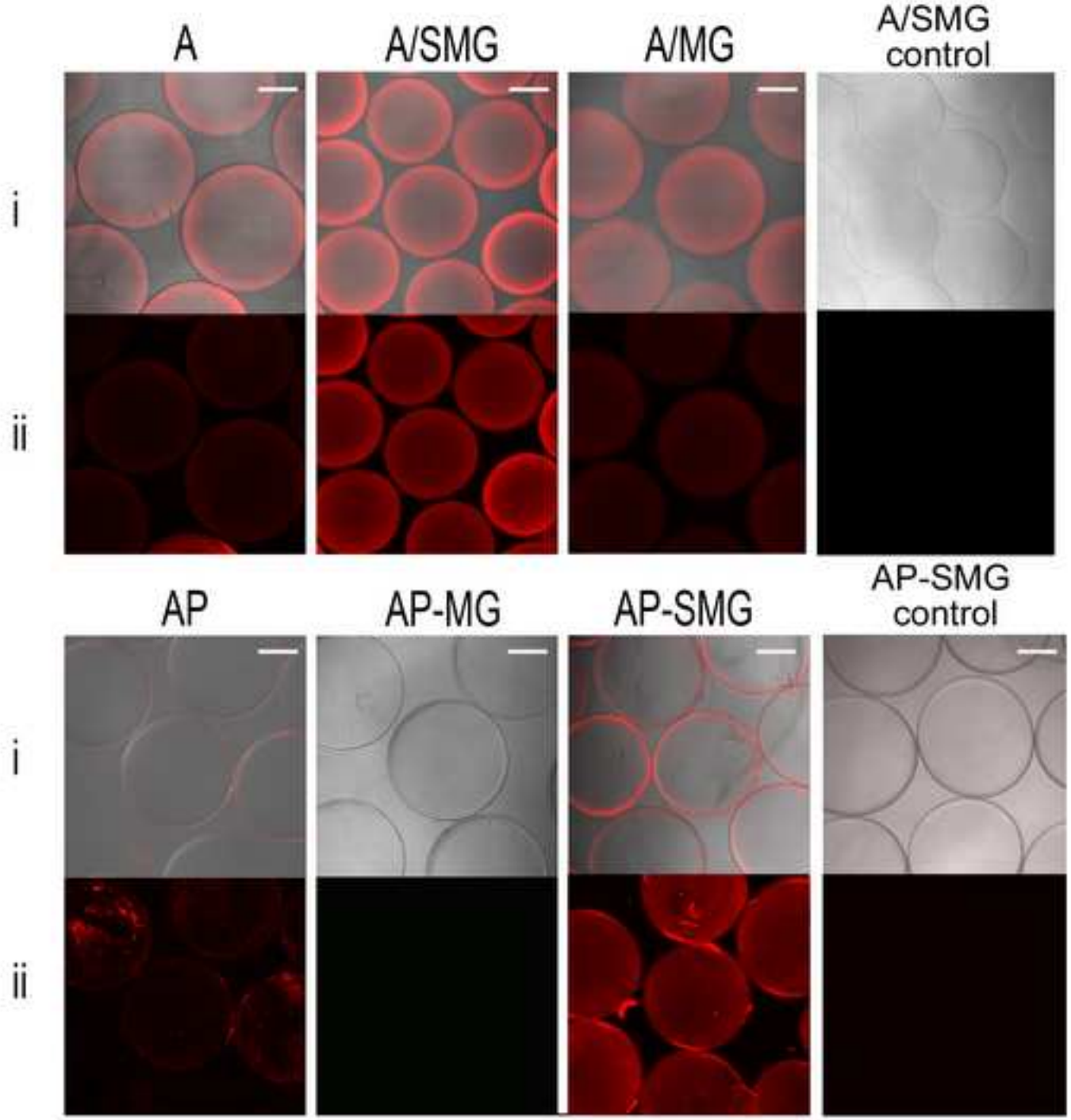

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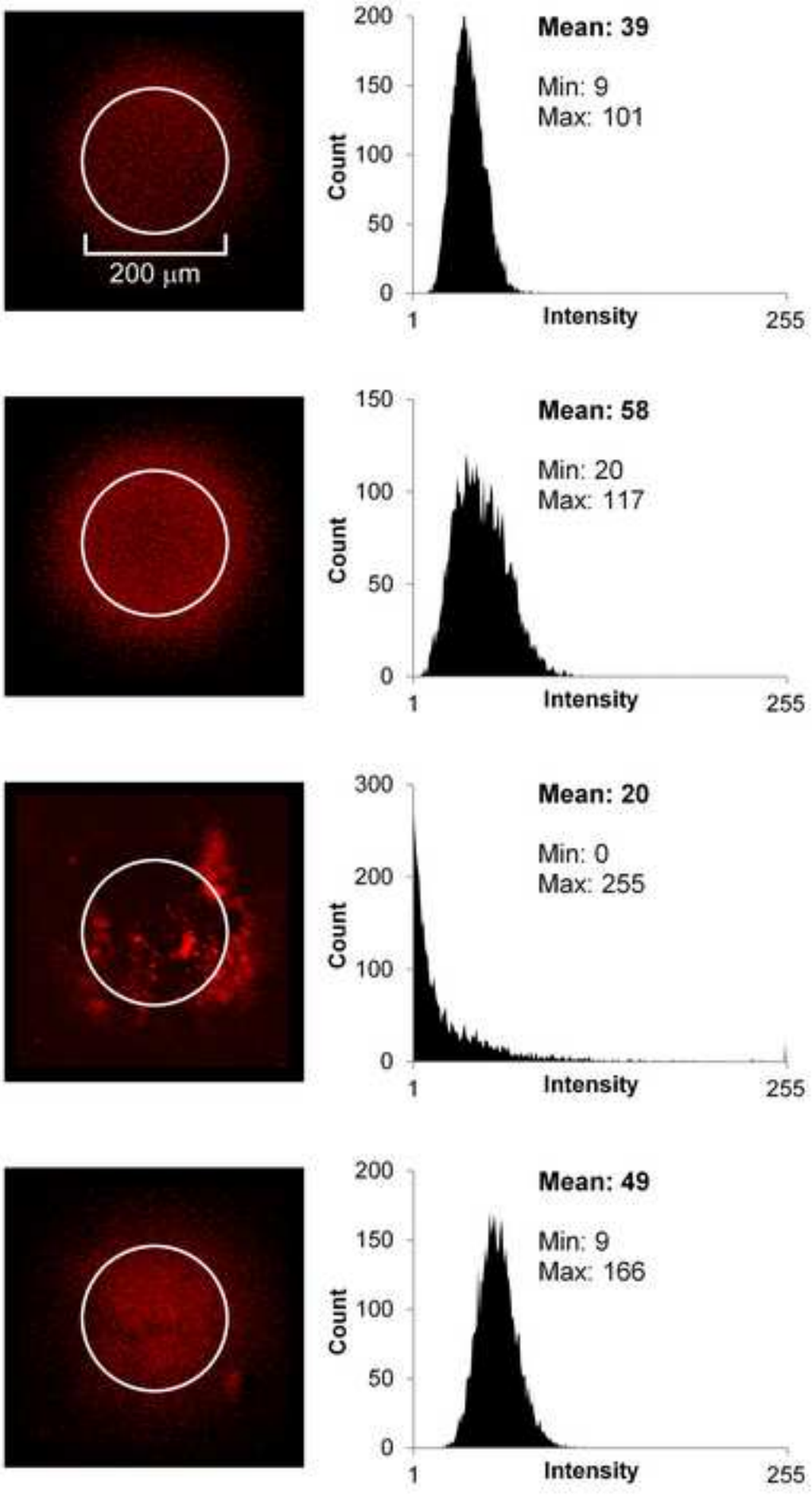

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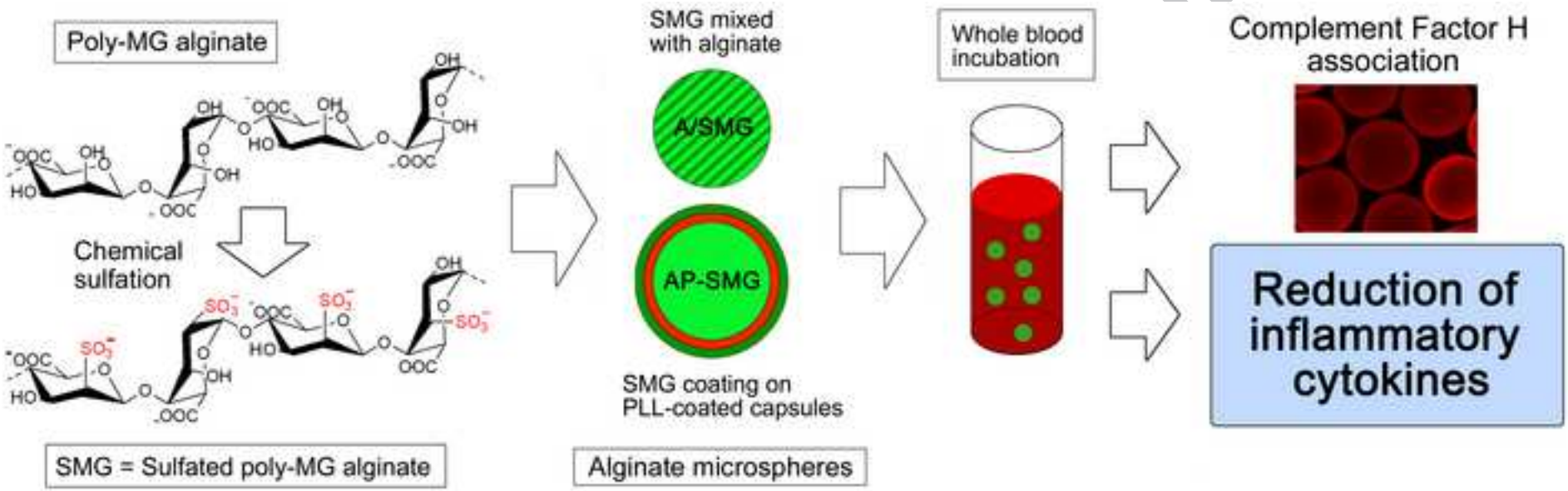


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## Statement of significance

Alginate microspheres are candidate devices for cell encapsulation therapy. The concept is challenged by the inflammatory host response, and modification strategies for improved biocompatibility are urgently needed. One potential strategy is using sulfated alginates, acting as versatile heparin analogues with similar anti-inflammatory properties. We designed novel alginate microspheres using sulfated alginate with an alternating sequence mimicking glycosominoglycans. Evaluation in a physiologically relevant human whole blood model revealed a reduction of inflammatory cytokines by a sulfated alginate coating, and sulfated alginate microbeads were complement inert. These effects were correlated with a strong factor H association, which may represent the mechanistic explanation. This novel approach could improve the biocompatibility of alginate microspheres *in vivo* and present a new strategy toward clinical use.

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