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Running Title: Inflammatory reactions at surface bound actin

Abstract

Actin exists as globular (G) monomers or polymeric filaments (F) in the cytoplasm of eukaryotic cells and mediates cell morphological changes and motility. Large amounts of this protein may be released out to the extracellular compartment during tissue injury, but little is known about its role in biomaterial-related inflammation. We immobilized actin to methylated glass, methylated- and aminated silicon and gold model surfaces and studied subsequent blood serum deposition and complement activation, generation of reactive oxygen species (ROS), and adhesion and aggregation of neutrophils and platelets. Null-ellipsometry shows that approximately one monolayer of G-actin can be immobilized onto the model surfaces and actin in buffer polymerizes on top of this by the addition of K^+ and Mg^{2+} ions to form a thicker layer of firmly bound F-actin. F-actin bound after serum incubation low amounts of anti-complement factor 1q (anti-C1q). The analysis of cell responses upon contact with actin-coated surfaces by luminol-amplified chemiluminescence, lumi-aggregometry and fluorescence microscopy show that surface-triggered aggregation, spreading, and generation of ROS were down-regulated and comparable to the response by adsorbed albumin. However, F-actin on gold surfaces recruited platelets in a C1q-dependent manner. We conclude that adsorbed actin *in vitro* is a weak complement, platelet and neutrophil activator, although F-actin associates with both C1q and platelets.

INTRODUCTION

Protein adsorption precedes the adhesion of cells to artificial surfaces. The composition and thickness of the adsorbed protein layer may vary over time depending on the surface characteristics,[1] but is believed to play an important role for the behavior of early appearing inflammatory cells such as platelets and neutrophil granulocytes. It is well known that the surface deposition of fibrin/fibrinogen, coagulation proteins, and the activation of the complement system may amplify the acute inflammatory response (reviewed by Tang and Eaton[2]). Complement is a humoral part of the defense against infection and acts by perforation of bacterial cell walls, recruitment of leukocytes and facilitation of phagocytosis through opsonization of natural as well as artificial surfaces.[3] There exist in principle two routes to activate complement at artificial surfaces: *i*) a rapid[4] activation induced by surface deposited immunoglobulins and complement facor 1q (C1q) (classical activation) or *ii*) a slower[4] activation on nucleophilic surface groups that form ester or amide bonds with complement factor 3 (C3) (alternative activation). The latter mechanism has been described as the main activation mechanism at artificial surfaces,[5-7] although recent studies reveal that also the classical pathway is indeed relevant for biomaterials.[8-10]

The cytoskeletal protein actin (42 kDa) is among the most abundant proteins in the human body and is a key protein in cell morphological changes and motility. Actin represents 10-30% of the intracellular protein content of eukaryotic cells and in the neutrophil peripheral cytoplasm the concentration may locally be approximately 20 mg/mL.[11] It is also found in the extracellular compartment, especially after tissue injury, and micromolar concentrations have then been reported.[12-14] Despite this, biocompatibility and protein adsorption studies have been performed almost exclusively using plasma proteins and until date little is known about the effects of adsorbed actin. This protein exists in the form of globular monomers (G-actin) or filamentous polymers (F-actin). G-actin can be polymerized to F-actin in the presence of adenosine triphosphate (ATP) and cations such as Mg^{2+} and K^+. Others and we have demonstrated that the adhesion of neutrophils and platelets to artificial surfaces is associated with a rapid and extensive actin polymerization,[15-17] leading to spreading and possibly also frustrated phagocytosis and necrosis. The osmotic conditions in the extracellular compartment favor F- before G-actin. However, in order to avoid the harmful effects of free actin in circulation, blood plasma contains a regulatory system of

specific actin binding proteins. Plasma gelsolin shortens actin filaments and vitamin D binding protein (Gc-globulin) sequesters G-actin under physiological calcium concentrations.[18] The synthesis rate of the regulatory proteins increases after tissue injury.[19] Actin may adsorb to various surfaces,[20] and Renault *et al.* demonstrated *in vitro* surface polymerization of actin.[21] However, little is known about how the early inflammatory response is affected by surface immobilized actin.

Several reports suggest a direct interaction between actin and humoral systems that affect cell behavior. Serum spiked with G-actin was shown to be toxic to pulmonary endothelial cells, possibly due to complement activation,[22] and vitamin D-binding protein enhanced the chemotactic effects of C5a (reviewed by Haddad[23]). A direct binding of actin to C1q,[24] immunoglobulins (IgG and IgM),[25] and fibrin clots[26] (and between fibrin clots and gelsolin[27]) have been reported. Furthermore, it was shown in cell-free systems that actin enhances ROS generation from the isolated NADPH oxidase system[28] that is abundant in leukocytes[29] and possibly also in platelets.[30] F-actin is suggested to be able to activate platelets, probably by presenting adenosine diphosphate (ADP) to ADP-receptors leading to intracellular actin polymerization.[31]

In the present study we utilized an *in vitro* model system to elucidate serum plasma and complement binding properties, and platelet and neutrophil granulocyte responses to surface bound actin.

MATERIALS AND METHODS

Reagents, antibodies, sera and plasma

All chemicals were from Sigma Chemical Co. (St. Louis, MO) and of analytical grade if not otherwise stated. The polyclonal rabbit antibodies used during ellipsometry studies were IgG-fractions of polyclonal anti-human A 0062 (anti-C3c), A 0136 (anti-C1q), A 0063 (anti-C3d), A 424 (anti-IgG, specific for γ-Chains), A 0001 (anti-albumin) and A 0080 (anti-fibrinogen, that also reacts against fragments D and E(1)) from Dakopatts (Glostrup, Denmark). The polyclonal sheep anti-human PC115 (anti-high molecular weight kininogen, anti-HMWK) and PC116 (anti-properdin) were delivered by The Binding Site Ltd. (Birmingham, U.K.). Blood was drawn from non-medicated apparently healthy volunteers, and pooled

normal human serum (NHS) and heparinized plasma were prepared according to standard protocols and stored below -70°C until use. C1q-depleted serum and purified C1q were from Quidel Corp. (San Diego, CA, USA). The C1q-depleted serum was reconstituted with 180 μg/mL C1q.[32]

Model surfaces

Flat silicon wafers from Wacker-Chemie GmbH (München, Germany) cleaved in the (100) crystal direction into 5 x 10 mm pieces, or 40 x 8 mm glass (approximately 75% SiO_2) test tubes (Assistent KHG, Sondheim-Rhön, Germany), were cleaned from organic contaminations during 5 min at 80°C in 5:1:1 parts of distilled water, hydrogen peroxide (30% v/v) and NH_4OH (25% v/v). After ten rinsings in distilled water, the inorganic contaminations on substrates were removed by 5 min at 80°C in 6:1:1 parts of distilled water, hydrogen peroxide (30% v/v) and hydrochloric acid (37% v/v). This yields a thin hydrated surface layer of SiO_2[33, 34] (water/surface static contact angle, θ_w, <10°, when measured with a Ramé-Hart NRL Model 100 goniometer, USA).

The hydrophilic substrates were excessively washed in water, 99.5% (v/v) ethanol and xylene (Merck KGaA, Darmstadt, Germany), and methylated during 5 min in 1% (v/v) dichlorodimethylsilane ($Cl_2(CH_3)_2Si$) in xylene at room temperature. Silane contamination traces were eliminated by rinsings in xylene, ethanol, and finally sonicated for 1 min in xylene (Sondrex RK100 (Bandelin Electronics, Berlin, Germany). The methylated SiO_2-surfaces were hydrophobic (θ_w>90°) and stored in xylene for less than one week. The silicon surfaces were used primarily in ellipsometry studies and glass surfaces in blood cell experiments.

Cleaned hydrophilic silicon wafers were dried and placed in a chamber containing 200 μl amino-propyl-triethoxysilane (APTES, Merck KGaA). The surfaces were then vapor aminated during 10 min at 60°C in low vacuum (6 mtorr), followed by extended baking during 60 min at 150°C in low vacuum. The APTES-coated surfaces were rinsed in xylene, ethanol, and sonicated for 1 min in xylene. The layer thickness was approximately 20 Ångströms ($1\text{Å} = 1 \times 10^{-10}$m) and the surfaces were stored in xylene until use in ellipsometry experiments. Similarly aminated (amino-propyl-trimethoxysilane, APTMS) silicon particles with average diameter of 20 μm (non-phagocytosable size for neutrophils) were purchased from

Gerlinde Kisker GbR (Steinfurt, Germany) for use in lumi-aggregometry experiments. Aminated particles or flat silicon were incubated for 30 min in 6% glutaraldehyde (GA; Merck KGaA) at room temperature in phosphate buffered saline (PBS, buffer composition as stated below but pH 9).[35, 36] For particles, each rinsing step was followed by a 5 min centrifugation at 300 x g.

Glass slides (9 x 9 mm, Biacore AB, Uppsala, Sweden) were sputter coated with 2000Å gold, and cleaned during 5 min in ozone and ultraviolet light in a UVO-cleaner (Model 42-220, Jelight Company Inc., Irvine, CA). The clean gold surfaces were then immediately transferred to a protein containing buffer (37°C). The protein coated gold surfaces were subsequently used in both ellipsometry and blood cell studies.

Protein adsorption

The model surfaces were washed at least three times in respective coating buffer and a monolayer of normal human plasma IgG, albumin (both from Pharmacia & UpJohn, Stockholm, Sweden) or rabbit muscle G-actin (Molecular Probes, Inc., Eugene, OR, product number A-12375) was adsorbed during ≥1 hour of incubation at room temperature (gold surfaces at 37°C) in 0.1 mg/mL of protein in a suitable buffer, followed by duplicate rinsings in buffer. Phosphate buffered saline (PBS; 10 mM sodium hydrogen phosphate, 10 mM potassium dihydrogen phosphate and 0.15 M sodium chloride, pH 7.3) was used as the buffer during albumin and IgG depositions, and 2.0 mM Tris (pH 8.0) for G-actin. After the protein coating step, all experiments were performed at 37°C. In order to discriminate between a spontaneous protein adsorption and covalent immobilization in the control experiments, the samples were incubated for 30 min at 37°C in sodium dodecyl sulfate (SDS, 10.4 mM) in PBS.[37] In some experiments, Ca^{2+} and/or Mg^{2+} were chelated in NHS by 10 mM ethylene-diamine-tetraacetic acid (EDTA) and ethylene-glycol-tetraacetic acid (EGTA; both from Merck KGaA). In F-actin surface control experiments, the actin polymerization stabilizer jasplakinolide (Molecular Probes) was used. Plasma protein coated APTMS-particles were endotoxin negative (*Limulus* Amebocyte Lysate Coatest, Charles River Endosafe, Charleston, SC). Polyclonal anti-albumin antibodies did not bind to actin-coated surfaces, indicating that the purified actin

contained low amounts of albumin (important since albumin-coated surfaces were used as a reference for actin-coated surfaces).

Ellipsometry

Flat silicon and gold surfaces were illuminated at a 70° angle of incidence with elliptically polarized light (HeNe laser, λ=6328Å) in an AutoEl III ellipsometer (Rudolf Research, NJ). By recording the ellipsometric angles Δ and ψ before adsorption and by finding new ψ and Δ after adsorption, the thickness of deposited surface films could be iterated according to the McCrackin evaluation algorithm.[38] The protein refractive index (N) was assumed to be N_{film} = 1.465. The adsorbed surface film thickness was converted to deposited mass per unit area according to de Feijter *et al.*[39] Experiments with methylated or aminated silicon surfaces and NHS or heparinized plasma were performed at 37°C in veronal buffered saline (VBS^{2+}; 5 mM 5,5'-dietylbarbituric acid, the corresponding sodium salt, 145 mM sodium chloride, 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.4) and analyzed in air. The ellipsometric experiments on gold were performed *in situ* in a cuvette with assumed buffer refractive index N_{buffer} = 1.335.[8, 40] Generally, the IgG-, albumin- and actin-coated model surfaces were incubated in 10% NHS or plasma for both 5 and 90 min. A short interaction allows detection of surface deposited complement components of the classical activation pathway, whereas an alternative pathway activation is primarily recognized after a long interaction.[4, 8, 41] The protein solutions were exchanged for polyclonal antibodies in PBS at 1/50 dilutions for 30 min for the detection of selected proteins in the adsorbed NHS or plasma films.[4, 8, 41]

Blood cells

Peripheral blood was drawn from apparently healthy non-medicated blood donors at the Linköping University Hospital. The blood was anti-coagulated by 5 IU/mL heparin. Calcium was excluded from all buffers during the cell separations, but reconstituted through the addition of 1.1 mM $CaCl_2$ before the experiments. Neutrophil granulocytes (with <0.1 platelet per neutrophil) were prepared according to the

methodology of Böyum and others.[42-44] One part of fresh whole blood was layered on one part of Lymphoprep over four parts of Polymorphprep (Nycomed Pharma AS, Oslo, Norway) in 50 mL test tubes, which were immediately centrifuged at room temperature for 40 min at 480 x *g*. The resulting middle band containing neutrophils was harvested. The neutrophil fraction was washed in room tempered PBS for 10 min at 480 x *g*, and the remaining red cells were eliminated by brief hypotonic lysis at 4°C followed by washing (200 x *g* at 4°C) in Krebs-Ringer phosphate buffer supplemented with 10 mM glucose and 1.5 mM $MgSO_4$, pH 7.3 (KRG). The neutrophils were kept at 4°C until pre-warming to 37°C, 5 min before the experiments. The number of neutrophils per unit volume and the purity of the preparations were analyzed in a Coulter Counter ZM Channelyser 256 (Coulter-Electronics Ltd., Luton, UK).

The platelets were prepared at room temperature by collection of platelet rich plasma after 20 min of centrifugation (220 x *g*) of whole blood mixed with 17% (v/v) of an acid-citrate-dextrose solution (85 mM sodium citrate, 71 mM citric acid and 111 mM glucose). The platelets were then isolated through centrifugation of the platelet rich plasma (480 x *g*) for 20 min, followed by removal of the plasma and gentle washing and resuspension of the cells in KRG.[45] The platelet count was determined with a Bürker chamber, and the cells displayed no apparent aggregation or morphological changes due to the preparation procedure.

Chemiluminescence and aggregation

The generation of ROS during cellular interaction with the model surfaces was studied by luminol-amplified chemiluminescence (CL; reviewed by Dahlgren *et al.*[46]). The CL-response in methylated glass test tubes was analyzed in 4 mL cuvettes in a six-channel Biolumat (LB 9505 C, Berthold Co., Wildbaden, Germany), as previously described.[47, 48] The tubes contained pre-warmed solutions of platelets and/or neutrophils, 4 U/mL horseradish peroxidase (Boehringer Mannheim GmbH, Mannheim, Germany) and 50 μM luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) in KRG. The ROS-production induced by APTMS-particles was detected essentially as above, but under stirring conditions in a two-sample Lumi-Aggregometer model 560 (Chrono-Log Corp., Havertown, PA) according to a recent protocol.[49] The Lumi-Aggregometer records, simultaneously with CL, increase in light transmission, as

occurs when cells and particles aggregate. Reagents and cells were equilibrated for 5 min in polystyrene cuvettes (#367) at 37°C under stirring (700 rpm; stir bar #370). The optical channels were immediately set to baseline after the addition of 1 x 10^5/mL APTMS-particles. The CL data were exported and integrated by the KaleidaGraph 3.5 software (Synergy Software, Reading, PA).

Cell adhesion and morphology

Neutrophils and/or platelets were allowed to interact with protein-coated gold surfaces with or without the presence of NHS. The cells were then fixed for 30 min in 4% ice-cold paraformaldehyde in PBS. The samples were washed twice in PBS and the cells permeabilized and stained for F-actin through incubation in 100 μg/mL lysophosphatidylcholine and 0.6 μg/mL bodipy-phallacidin (Molecular Probes) in PBS for 30 min at room temperature in darkness. The staining solution was removed by duplicate washings in PBS. The samples were mounted in Dako fluorescence mounting medium (Dakopatts) and the cell morphology and adhesion was studied in a Zeiss Axioscope (Carl Zeiss, Oberkochen, Germany) at 630 x original magnification. The documentation was made with a Carl Zeiss ZVS-47E digital camera, or by manual counting of the number of adhered cells in at least 25 microscopic views per sample. Key samples were evaluated independently by two persons. Original pictures were generated at a constant gain using the Easy Image Measurement 2000 software (version 2.3, Bergström Instrument AB, Solna, Sweden). Sparse editing of pictures (all images treated similarly) was performed with Canvas 5.0 software (Deneba Systems, Inc., Miami, FL). Importantly, G- or F-actin coated surfaces did not show enhanced fluorescence after staining with bodipyphallacidin (compared to non-coated gold surfaces), probably since metal films quench fluorescence near the surface.[50]

Statistics

The data were assumed Gaussian distributed and are presented as mean ± SEM. *n* denotes the number of independent experiments, and in blood cell experiments the number of individual blood donors. The statistical evaluation was performed using the two-tailed paired Student's *t*-test. The significance notations in illustrations represent the conventional significance levels * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and NS (not significant).

RESULTS

Actin immobilization

Surfaces were incubated in 0.1 mg/mL actin and the ellipsometric thickness was approximately 9Å on methylated surfaces, 5Å on aminated silicon and 16Å on gold. Upon addition of 100 mM K^+ and 2 mM Mg^{2+} to the actin solution, a rapid additional deposition of actin was induced at gold surfaces, reaching saturation at approximately 40Å during one hour of incubation (Fig. 1). When the same procedure was applied on G-actin on aminated silicon, a similar protein layer thickening process was observed (not shown). The rapid deposition indicated a polymerization process on top of the initial actin film and formation of F-actin. Only a minor part ($\leq$10Å) of this F-actin layer could be removed by *in situ* rinsings in SDS solution or by extended incubations in the buffer (without K^+ and Mg^{2+}). In cases where the F-actin layer was first stabilized by jasplakinolide (0.1 μM for 5 min), it became completely unaffected by subsequent rinsings. The thickness of the F-actin layer could not be affected by elutions in SDS, or addition of 10 mM EDTA or extra adenosine triphosphate (ATP). The adsorbed G-actin on methylated surfaces was easily removed by SDS, whereas the G-actin layer remained intact on aminated silicon and gold, indicating a covalent (or equivalent to covalent) protein immobilization in the latter cases. The thickness of the G-actin layer was comparable to the thickness of albumin films, but thinner than IgG films (see Fig. 2-3).

Complement on silicon

Immobilized G- and F-actin layers on aminated silicon adsorbed proteins during incubations in 10% NHS or heparinized plasma, and the layer thickening was larger than 10Å after 5 min of incubation (Table 1, Fig. 2A-B data group I and Fig. 3). Spontanously adsorbed G-actin on methylated silicon bound 13Å of proteins after one hour in 10% plasma (not shown). Anti-C3c (11Å), anti-C1q (7Å), anti-C3d (9Å), anti-fibrinogen (8Å) and anti-HMWK (5Å) bound onto this plasma layer (not shown). The reference

plasma layer on non-coated methylated silicon bound close to identical amounts of anti-fibrinogen (10Å) and anti-HMWK (4Å) compared to the G-actin surface, but the binding of anti-C3c, anti-C1q and anti–C3d were reduced by approximately 46%, 57% and 33%, respectively (not shown). For this reason, further analysis was focused on the evaluation of complement binding to immobilized actin on aminated silicon and pure gold surfaces.

Covalently immobilized G-actin on aminated silicon bound approximately 22Å and 45Å of proteins after 5 and 90 min incubations in 10% NHS, respectively (Fig. 2A, data groups I and II, and Table 1). A significant increase in layer thicknesses was observed upon subsequent incubations in anti-C1q (13Å after 5 min and 7Å after 90 min incubation in NHS, respectively), anti-C3c (18Å and 56Å) and anti-C3d (5Å and 14Å). However, anti-properdin did not bind into the protein layer, indicating that the complement protein deposition was not due to a sustained classical activation since properdin stabilizes C3- and C5-convertases and serves as a marker of prolonged surface activation of complement. Thus, the half-life time of the convertases was too short to present any detectable amounts of properdin in the serum layer. Alternatively, sterical hindrances made it impossible for the antibodies to bind to properdin.

F-actin on aminated silicon surfaces bound 11Å and 18Å of serum after 5 and 90 min incubations, respectively (Fig. 2B, group I and II, and Table 1). C1q, C3c and C3d could be identified by polyclonal antibodies in this film (significant depositions of 9Å and 5Å anti-C1q, 7Å and 46Å anti-C3c, and 6Å and 12Å anti-C3d after 5 and 90 min NHS incubation, respectively). The serum incubated F-actin surface did not bind anti-properdin.

The serum deposition to immobilized albumin (recognized as a low activator in the literature) on aminated silicon was comparable to that for immobilized G-actin, 19Å after 5 min and 36Å after 90 min of incubation (Fig. 2C, group I and II, Table 1). Furthermore, the subsequent depositions of anti-C1q (13Å and 7Å, after 5 and 90 min in NHS, respectively), anti-C3c (11Å and 43Å) and anti-C3d (3Å and 14Å), with no binding of anti-properdin, suggests that covalently immobilized albumin as well as actin on aminated silicon are weak or moderate activators of the complement system. Studies on serum protein deposition to IgG on aminated silicon, 9Å and 40Å after 5 and 90 min incubation, respectively (Fig. 2D and

Table 1), show that the antibody binding was higher but not very different from that observed on albumin- and actin-coated surfaces, anti-C1q (12Å and 9Å), anti-C3c (19Å and 62Å), and, anti-C3d (4Å and 19Å) after 5 and 90 min incubations in NHS, respectively. However, the IgG-surfaces showed the largest serum binding after the prolonged incubation and the highest subsequent binding of anti-C3c and anti-C3d. Furthermore, only IgG-surfaces bound appreciable amounts of anti-properdin, 7Å after the 90 min NHS incubation. Since bare aminated glutaraldehyde treated surfaces also have the capacity to bind C1q, C3c and C3d after 90 min of serum incubation (Table 1 and Fig. 2E), the analysis of interactions between C1q and actin required yet another base surface. Actin was therefore spontaneously adsorbed to gold.

Complement on gold

The serum layer thickness at G-actin on gold was approximately 33Å after 5 min of incubation (Fig. 3, data group I). The corresponding F-actin layer thickness was 25Å (Fig. 3, group II), albumin on gold, 26Å (Fig. 3, group III) and IgG on gold, 39Å (Fig. 3, group IV). Non-coated gold deposited approximately 40Å serum upon incubation in a similar way (not shown). No significant amounts of anti-C1q bound to the serum layers on G-actin (Fig. 3, group I), albumin (group III) or IgG (group IV). However, small amounts of anti-C1q bound to serum incubated G-actin, and when G-actin was replaced for F-actin a significant binding of approximately 9Å anti-C1q occurred (Fig. 3, group III). In order to confirm that the anti-C1q binding was not a result of a non-specific binding to F-actin, control experiments with C1q-depleted serum were performed. No anti-C1q bound to the protein layers when actin-, albumin-, IgG-, or non-coated surfaces were incubated for 5 min in 10% C1q-depleted serum (Table 1). When the depleted serum was reconstituted with C1q approximately three times more anti-C1q bound to serum incubated F-actin surfaces (27Å), compared to the other three surfaces ($\leq$11Å, Table 1). Is C1q and the classical activation pathway then necessary for the deposition of C3? Approximately 13Å anti-C3c bound to F-actin after a 5 min NHS incubation, and this deposition disappeared completely upon the addition of EGTA to the serum. The anti-C1q binding remained intact on F-actin surfaces after incubations in EGTA-serum. This is in accordance with earlier studies showing that the assembly of the C1-complex is Ca^{2+}-dependent, but the surface binding of C1q is not. The results show that C1q is important for the deposition of C3 on F-actin. Despite this, it is important to note that the anti-C3c binding to NHS films on F-actin surfaces was

relatively low. In comparison, the NHS protein layer on IgG and albumin bound approximately 63Å and 32Å of anti-C3c, respectively, and the depositions disappeared after addition of EGTA to the serum. In summary, actin surfaces are poor complement system activators, although F-actin sequesters C1q. Double diffusion Ouchterloney test of soluble actin and C1q in agarose showed no precipitation line (not shown).

Chemiluminescence

No direct or primed ROS generation was observed upon stimulation of the neutrophils with 10 μg/mL soluble actin (measured as luminol-amplified chemiluminescence (CL) with or without a second stimulation of 0.1 μM formyl-Met-Leu-Phe). G-actin precoated hydrophobic glass surfaces reduced the neutrophil CL by approximately 43% (Fig 4), and the response was further decreased by 81% when NHS (5%) was added to the cell solution (not shown). This is similar to the ROS generation on low-activator surfaces such as spontaneously adsorbed albumin, but very different from the potent neutrophil ROS generation triggered e.g. by immobilized IgG during NHS incubations.[47] In contrast to the previously reported potentiating effects of activated platelets on the neutrophil ROS-production on IgG-coated surfaces,[49, 51, 52] the introduction of platelets to neutrophils that contacted G-actin induced no amplification of the total CL-response, quite similar to albumin surfaces (Fig. 4).

The ROS generation was studied after stimulation of the cells with protein-coated aminated silicon particles under stirring conditions in a Lumi-Aggregometer. The results correlate well with similar results on methylated surfaces. The neutrophil CL-response remained low on G-actin on aminated silicon particles. Reference IgG-coated particles on the other hand activated the neutrophil respiratory burst via $F_{c\gamma}$-receptors (Fig. 5A).[49] In general, the neutrophil CL-response on G-actin coated particles was not significantly different from that of albumin-coated particles (Fig. 5A-C), and the response became almost totally quenched with the introduction of 5% NHS to the cell solution (Fig. 5B). This indicates that the observed complement deposition was not a result of a true or prolonged complement activation since the actin- and

albumin-coated surfaces gave rise to low neutrophil respiratory burst responses in serum containing media. Furthermore, the introduction of platelets to the neutrophil test system showed no effect on the total ROS generation in response to G-actin or albumin particles (Fig. 5C), providing further support for the hypothesis that surface bound actin is a poor activator of the inflammatory response.

Cell-particle aggregation

The platelet is a specialized wound healing and hemostasis cell, but possess also potent inflammatory capabilities. Platelets aggregate upon activation and this can serve as a marker for the degree of activation. For example, surface bound IgG is a moderate activator of aggregation (via the $F_{c\gamma}$-receptor) and shows approximately one third of the aggregation response that is induced by collagen.[49] The aggregation by G-actin particles was approximately one third of that of IgG-coated particles (Fig. 6A), and did not differ significantly from that of albumin coated particles (Fig. 6A-B). Hence, the platelet binding to G-actin was low. Similar findings were observed for neutrophils or mixtures (at a physiological ratio) of neutrophils and platelets (Fig. 6B). Since the particle-based measurement system was not a useful tool for the study of responses by F-actin coated particles (the particles aggregated during the polymerization stage), the influence of F-actin on the platelet and neutrophil behavior was primarily evaluated by the use of morphological methods.

Cell adhesion and morphology

Neutrophils and/or platelets were fixed and F-actin stained after interactions with gold surfaces and evaluated by fluorescence microscopy. Adhered neutrophils (Fig. 7) and platelets (Fig. 8) demonstrated an extensive spreading that was reduced by coating the surfaces with G- or F-actin. The presence of 5% NHS did not cause any quantitative or morphological changes in the neutrophil adhesion on G- or F-actin,

but inhibited the binding and spreading on non-coated surfaces (Fig. 7). Neutrophils that interacted with immobilized IgG displayed the opposite, i.e. an enhanced binding and spreading in serum containing buffer (Fig. 7). When NHS was exchanged for C1q-depleted serum, no difference regarding the neutrophil adhesion to actin-coated surfaces was observed (not shown). Platelets reacted, in contrast to neutrophils, with a reduced adhesion to G-actin, F-actin and non-coated gold when NHS was introduced (not shown, essentially like the reconstituted serum in Fig. 8). Furthermore, the number of adhered platelets on NHS-opsonized F-actin was reduced by 37% in C1q depleted serum (Fig. 8-9). Interestingly, the F-actin surface deposited significantly higher amounts of anti-C1q after the NHS incubation (Fig. 3; data group III), an observation that disappeared in C1q-depleted serum. Although the platelet morphology showed no apparent signs of activation, the cell count was higher on F-actin than on G-actin surfaces in NHS (Fig. 9). This support the observation by Vasconcellos and Lind[31] who reported some platelet activation capacity by F-actin.

DISCUSSION

Free actin in the extracellular space may have harmful and even toxic effects on cells.[22] The present study suggests that surface bound actin is a relatively poor activator of the coagulation and complement systems and interacts moderately with neutrophils and platelets *in vitro*. However, we observed that C1q binds to immobilized F-actin, with a subsequent adhesion of platelets. The binding decreased dramatically in C1q-depleted serum. F-actin may be localized to interfaces between biomaterials and body fluids since: *i*) the insertion of a device induces tissue injury, *ii*) conditions that give rise to cell lysis are reported in biomaterial applications, *iii*) platelets and leukocytes adhere to surfaces during the acute phase of inflammation and form large amounts of intracellular F-actin,[15-17] *iv*) foreign body giant cells that appear during a chronic inflammatory phase display distinct F-actin formations[53] and *v*) fibroblasts use the actin cytoskeleton for orientation during the regenerative phase at biomaterial interfaces.[54] Nishioka *et al.* observed a precipitation in a C1q-actin system,[24] and the present study

supports their observation in that association occurred when actin was surface bound, and was stronger for polymerized actin. The C1q-actin association was observed during typical extracellular osmotic conditions and may take part in the regulation of platelet adhesion.

C1q may bind to extracellular actin and act as a marker for C1q-receptor expressing cells. It was recently discovered that complement receptor 1 (CR1 or CD35, receptor for C3b, iC3b and C4b) also binds C1q.[55] However, at least three other C1q-receptors have been identified: *i*) cC1qR or collectin receptor (60 kDa), *ii*) C1qRq (126 kDa) and *iii*) gC1qR (33 kDa), as reviewed by Peerschke and Ghebrehiwet.[56-58] Neutrophils express the four types of C1q receptors and platelets display gC1qR (33 kDa) and cC1qR (60 kDa). Platelets have been shown to utilize their C1q-receptors during the adhesion to C1q-coated surfaces.[59] The 33 kDa receptor is unique in recognizing the globular heads of C1q, and the other three receptors are specific for the collagen-like region of the molecule. Platelet but not neutrophil adhesion to the present actin surfaces was sensitive to the presence of C1q. Perhaps this is a reflection of different utilization or expression of the receptors in response to C1q-epitopes exposed after surface binding. Furthermore, platelets likely adhere more rapidly than neutrophils, and if surface localized C1q is only transiently available for cell interactions, neutrophils may appear too late to become affected. The neutrophils that interacted with F-actin in the presence of NHS showed no apparent signs of activation and were not affected when C1q was depleted from serum. This is in sharp contrast to the neutrophil behavior on potent classical activator surfaces such as IgG-coated methylated glass[47, 48] in serum containing media. The binding of other specific complement antibodies was moderate in the F-actin/NHS system. Hence, unlike IgG, F-actin likely binds C1q by the collagen-like region and present the globular heads to the cells. However, more studies need to be performed to ascertain whether the platelet 33kDa receptor is in fact involved in the interaction with C1q on F-actin, or not. Alternatively, the C1q-supported platelet adhesion may be due to binding to other surface active serum proteins, for instance other complement fragments and IgG.

F-actin but not IgG on gold bound significant amounts of anti-C1q after the NHS incubations. This is in agreement with earlier findings that IgG is a much more efficient classical activator on

hydrophobic than on hydrophilic surfaces. On hydrophobic surfaces (such as the methylated silicon in the present study) IgG is not easily desorbed and may be denatured in a fashion that facilitates C1q-binding. IgG can also directly bind C3[60] and perhaps this explains why hydrophilic IgG-coated surfaces activate the complement without the initial transient antibody-detection of C1q. The low-activating albumin surface acquired sparse, but far from negligible, complement (anti-C3c) deposition after the serum incubation. It is important in this context to remember that protein-resistant surfaces are rare and not many surfaces withstand deposition of complement during prolonged static interactions with blood *in vitro*. It seems therefore more appropriate to divide materials into low, moderate and potent binders of complement and ascertain whether these are true activators or not through detection of late appearing complement cascade factors, such as the alternative C3-convertase stabilizer properdin, or C3-degradation fragments like C3d. Also, the antibody binding to adsorbed NHS layers on surfaces is not necessarily proportional to the amount of adsorbed antigen, and protein size, charge and flexibility largely determine the avidity of the antibody.

In conclusion, this study reveals that surface bound actin binds low amounts of complement proteins from serum and induces a relatively low activation of neutrophils and platelets. However, immobilized actin filaments recruit platelets and associates with C1q in a way that facilitates platelet adhesion. The extracellular immunoregulatory capacity of actin is relevant for a better understanding of the basic inflammatory mechanisms involved in biomaterial-tissue interactions.

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CAPTIONS

Table 1.

Net serum and concomitant antibody depositions to pre-adsorbed protein layers on aminated silicon and gold. See Fig. 2-3 for experimental conditions. NHS: normal human serum. Å: Ångström ($1x10^{-10}$m). In the right column, the C1q-depleted serum was reconstituted with 180 μg/mL C1q.

Fig. 1.

Rapid actin polymerization onto an immobilized monolayer of G-actin on gold. The surfaces were mounted in a cuvette at 37°C and the surface-deposited mass was measured *in situ* by null ellipsometry. Clean gold surfaces were incubated for one hour in 0.1 mg/mL G-actin in 2.0 mM Tris, pH 8.0 (non-filled squares). After one hour, the osmotic conditions were changed by addition of 100 mM KCl and 2mM $MgCl_2$, resulting in a near threefold increase in the deposited mass (filled squares). The formed F-actin layer could not be removed by a 10.4 mM SDS-elution. Representative experiment.

Fig. 2.

Complement deposition from normal human serum (NHS) onto covalently immobilized G-actin (A), F-actin (B), albumin (C) and IgG (D) on amino-propyl-triethoxysilane + glutaraldehyde (APTES + GA, figure E) on silicon. The surfaces were incubated in 10% NHS in veronal buffered saline supplemented with Mg^{2+} and Ca^{2+} (VBS^{2+}) for 5 (data group I) or 90 (group II) min, at 37°C. The protein depositions were studied by null-ellipsometry. The samples were gently rinsed 4 times before each measurement. The subsequent binding from 1/50 dilutions of anti-C1q, anti-C3c, anti-C3d or anti-properdin, respectively, onto the adsorbed protein layers was followed. Bars indicates the SEM, $n = 6$-10.

Fig. 3.

F-actin surfaces bind C1q from normal human serum (NHS). Hydrophilic gold surfaces were coated with G-actin (data group I), F-actin (group II), albumin (group III) or IgG (group IV) and subsequently exposed to 10% NHS at 37°C in VBS^{2+} *in situ* during 5 min. Ellipsometric detection after the subsequent incubation in 1/50 dilutions of polyclonal anti-C1q showed a significant (9Å) deposition of the antibody to the NHS layer on F-actin. Bars indicate the SEM, $n = 6$.

Fig. 4.

A monolayer of spontaneously adsorbed actin does not amplify the respiratory burst in neutrophils and platelets. Luminol-amplified chemiluminescence (CL) was measured in neutrophils (2 x 10^6/mL) and/or platelets (1 x 10^8/mL) in KRG in the presence of horseradish peroxidase (4 U/mL) under non-stirring conditions at 37°C during 30 min in G-actin or non-coated hydrophobic glass tubes. CPM: counts per minute. Representative experiment.

Fig. 5.

The luminol-amplified chemiluminescence (CL) response from cells that contact G-actin coated particles does not differ from the response triggered by albumin-coated particles. Pre-warmed cells were stimulated with non-phagocytosable aminated silicon particles (1 x 10^5/mL) with various glutaraldehyde-coupled protein coatings. Data from 15 min of CL under stirring conditions (700 rpm) in a two-sample Lumi-Aggregometer in KRG in the presence of horseradish peroxidase (4 U/mL). Bars represent the SEM, a.u.: arbitrary unit.

(A) Representative CL-signal in the neutrophil system (2 x 10^6/mL). IgG-coated particles induced a semi-potent ROS-generation, but the responses induced by G-actin and albumin, respectively, were close to baseline levels.

(B) As in (A), but integrated values. No significant difference in the CL-response was found between G-actin and albumin-coated particles in a buffer system (first data set, $n=9$) or with 10% normal human serum added to the test system (second data set, $n=3$).

(C) Experiments performed as in (B), but with or without washed platelets (1 x 10^8/mL) added. No significant differences were found between the G-actin and albumin surface induced CL-responses ($n=3$).

Fig. 6.

The aggregation of neutrophils (2 x 10^6/mL) and/or platelets (1 x 10^8/mL) in KRG after stimulation with G-actin- or albumin coated silicon particles (1 x 10^5/mL). The experiments were performed at 37°C in a Lumi-Aggegometer under stirring (700 rpm).

(A) Representative trace of platelet aggregation. G-actin and albumin surfaces triggered very low aggregation in comparison to IgG-coated particles.

(B) Averaged maximum aggregation after 15 min. No significant differences were found between albumin and G-actin coated particles in platelet, neutrophil or mixed neutrophil-platelet suspensions. Bars indicate the SEM, $n=3$.

Fig. 7.

Actin coatings reduce the neutrophil spreading at surfaces. G- or F-actin were immobilized to gold and some of the surfaces were exposed to 5% NHS prior to the addition of 1 x 10^6/mL neutrophils. The samples were incubated for 20 min at 37°C in KRG, fixed during 30 min in 4% paraformaldehyde, and permeabilized and stained for F-actin in lysophosphatidylcholine and bodipyphallacidin. The cell appearance was then documented by fluorescence microscopy. The presence of NHS showed no effect on the neutrophil morphology on G- or F-actin, but reduced the spreading on pure gold. The opposite occurred on the relatively potent complement activator surface IgG. No fluorescence was noted from bodipyphallacidin-stained cell-free actin-coated surfaces. Bar: 10 μm.

Fig. 8.

Actin coatings reduce platelet spreading at surfaces. Platelets (1 x 10^8/mL) were allowed to interact during 20 min with non-, G- or F-actin coated gold surfaces with or without 5% C1q-depleted serum present at 37°C in KRG. The cells were then fixed, permeabilized, stained for intracellular F-actin with bodipyphallacidin and documented by fluorescence microscopy. The platelet adhesion was quantitatively lower after the addition of NHS (morphology as shown for the reconstituted depleted serum in figures to the right). Platelet adhesion to F-actin in diluted serum depended on C1q. When C1q-depleted serum was reconstituted by the addition of 180 μg/mL C1q prior to interaction with surfaces, the F-actin surfaces recruited more platelets than when C1q was missing. This result was similar for mixed neutrophil-platelet suspensions. Bar: 10 μm.

Fig. 9.

C1q supports platelet adhesion to F-actin surfaces and more platelets adhere to F- than G-actin in serum containing solutions. The samples were prepared as in Fig. 8 and the platelets were exposed to C1q-depleted serum that was reconstituted, or not, with 180 μg/mL C1q. The number of adhered cells per view was counted using fluorescence microscopy at 630 x magnification. At least 25 views per sample were counted for each donor. The platelet count was higher on the reconstituted serum layers on F-actin than on C1q-depleted serum layers (for all donors, p=0.16). Bars indicate the SEM, *n*=4.

Table 1

| | Aminated silicon (Å) | | Gold (Å) | |
	NHS *(5 min)*	*NHS* *(90 min)*	*C1q-depleted* *(5 min)*	*C1q-depleted + C1q* *(5 min)*
non-coated				
serum	24	43	51	41
anti-C1q	10	11	-5	9
anti-C3c	11	50		
anti-C3d	2	18		
anti-properdin	0	3		
IgG				
serum	9	40	3	5
anti-C1q	12	9	-5	1
anti-C3c	19	62		
anti-C3d	4	19		
anti-properdin	1	7		
albumin				
serum	19	36	22	20
anti-C1q	13	7	-5	10
anti-C3c	11	43		
anti-C3d	3	14		
anti-properdin	0	2		
G-actin				
serum	22	45	18	28
anti-C1q	13	7	2	11
anti-C3c	18	56		
anti-C3d	5	14		
anti-properdin	-1	0		
F-actin				
serum	11	18	6	18
anti-C1q	9	5	2	27
anti-C3c	7	46		
anti-C3d	6	12		
anti-properdin	0	0		

Figure 1
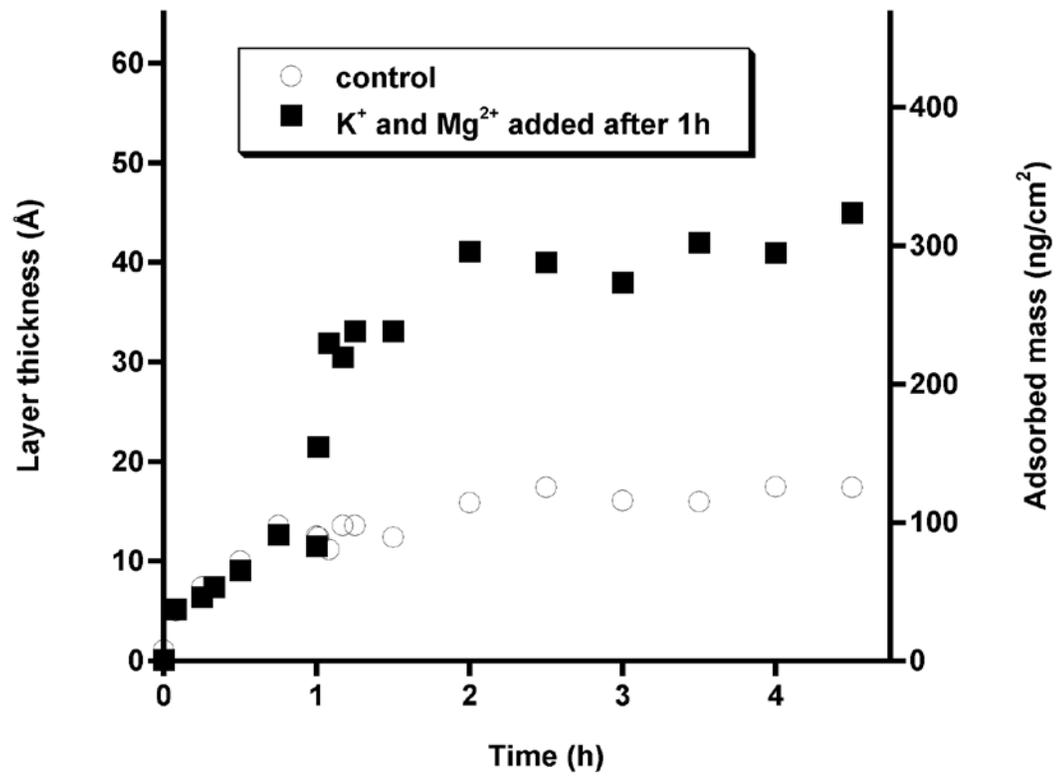
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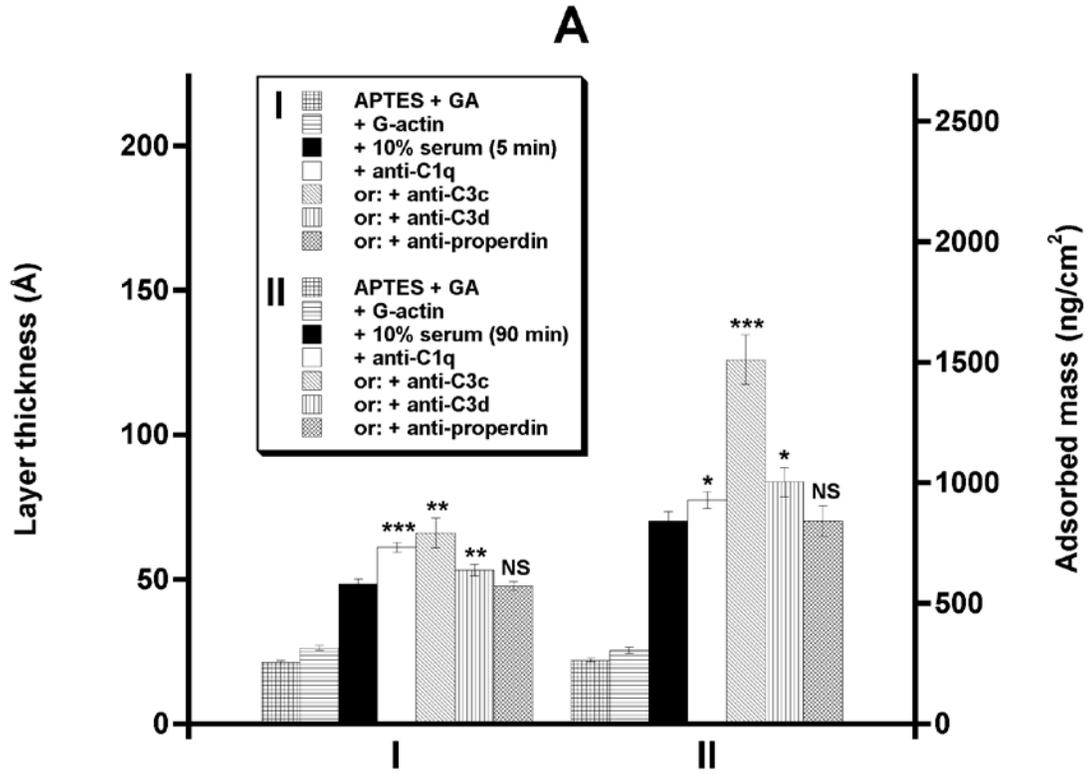
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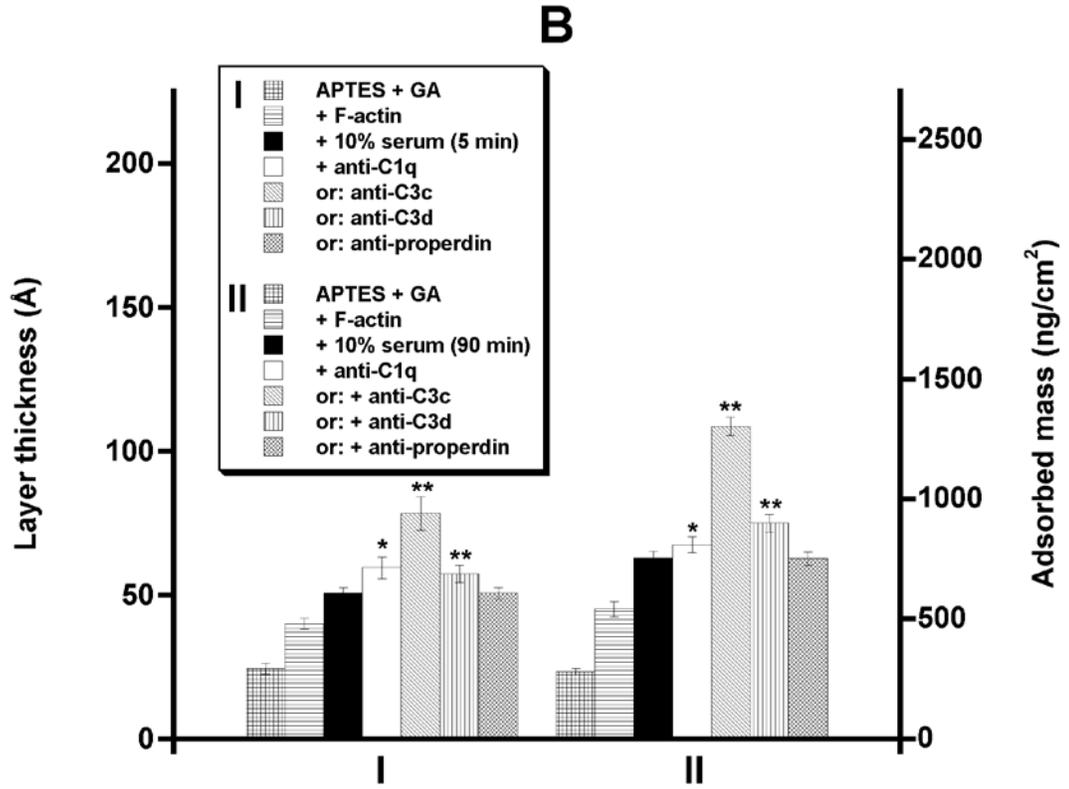
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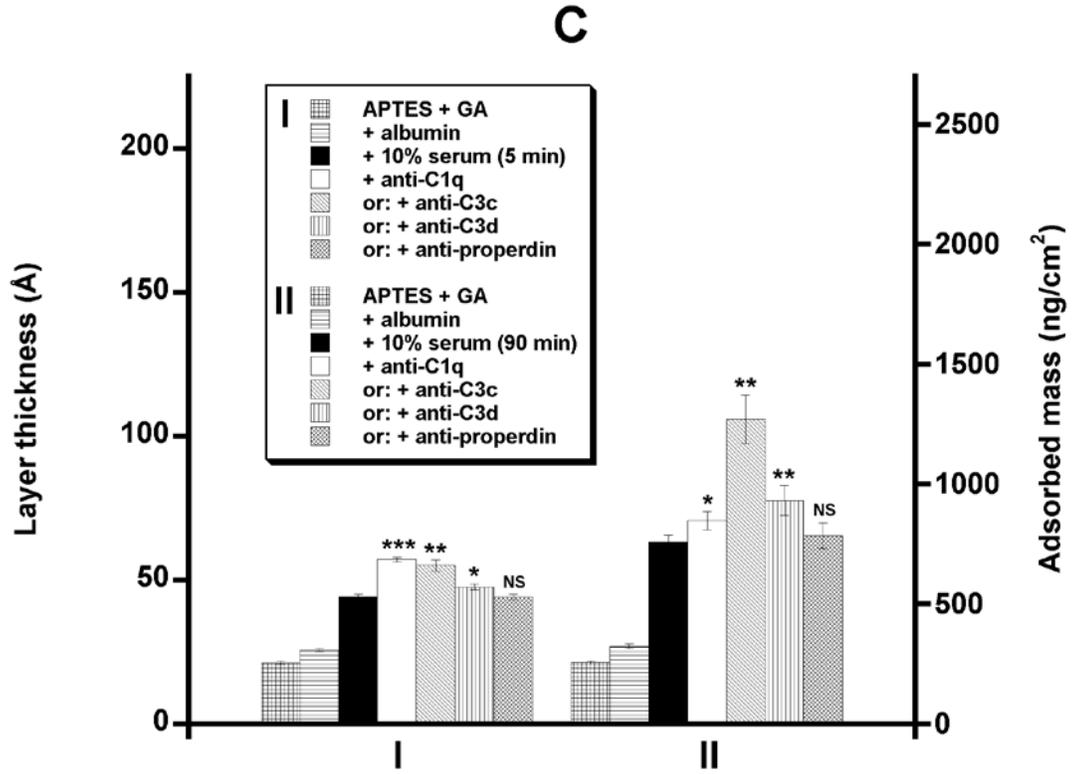
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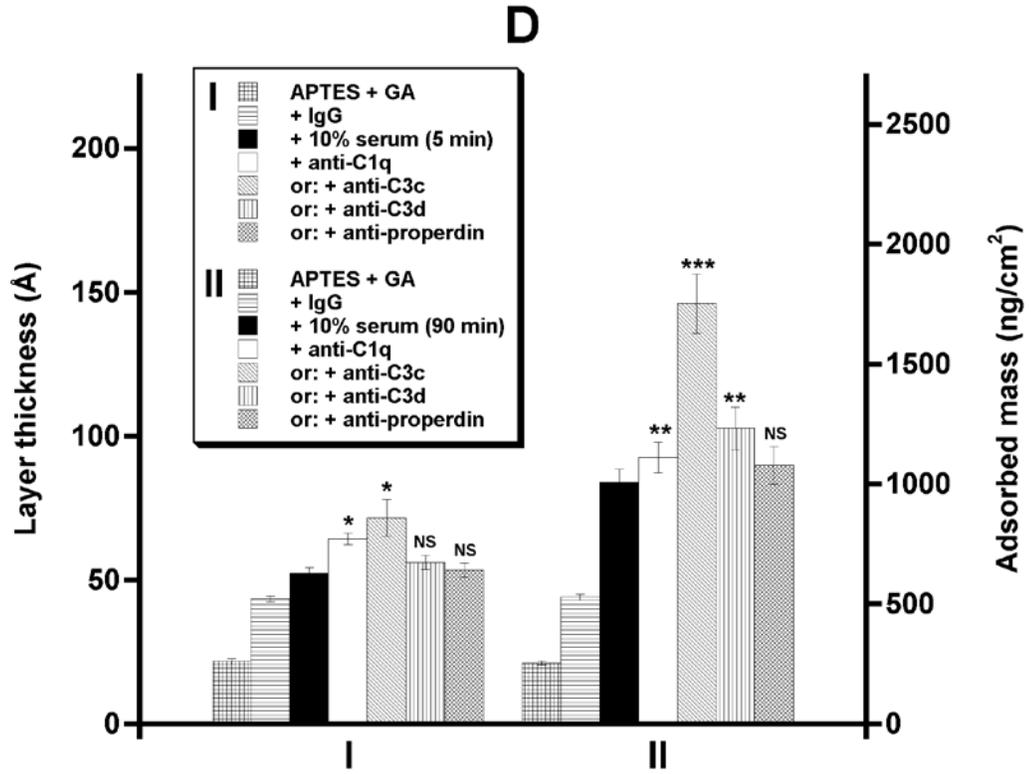
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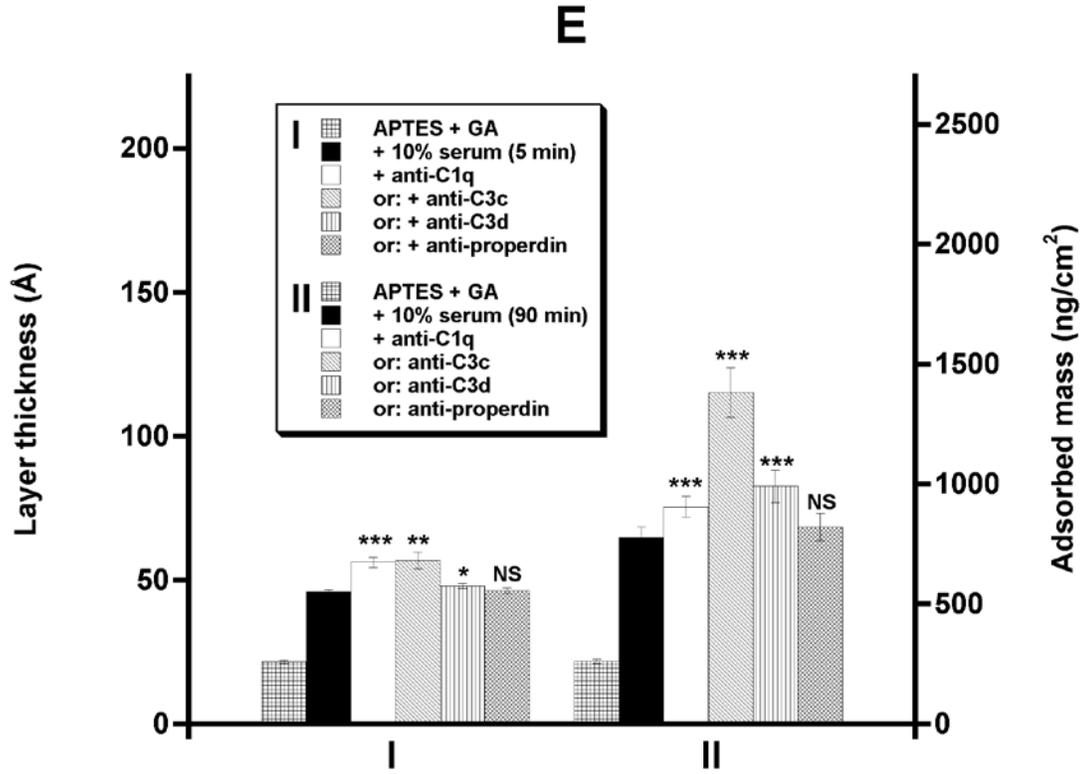
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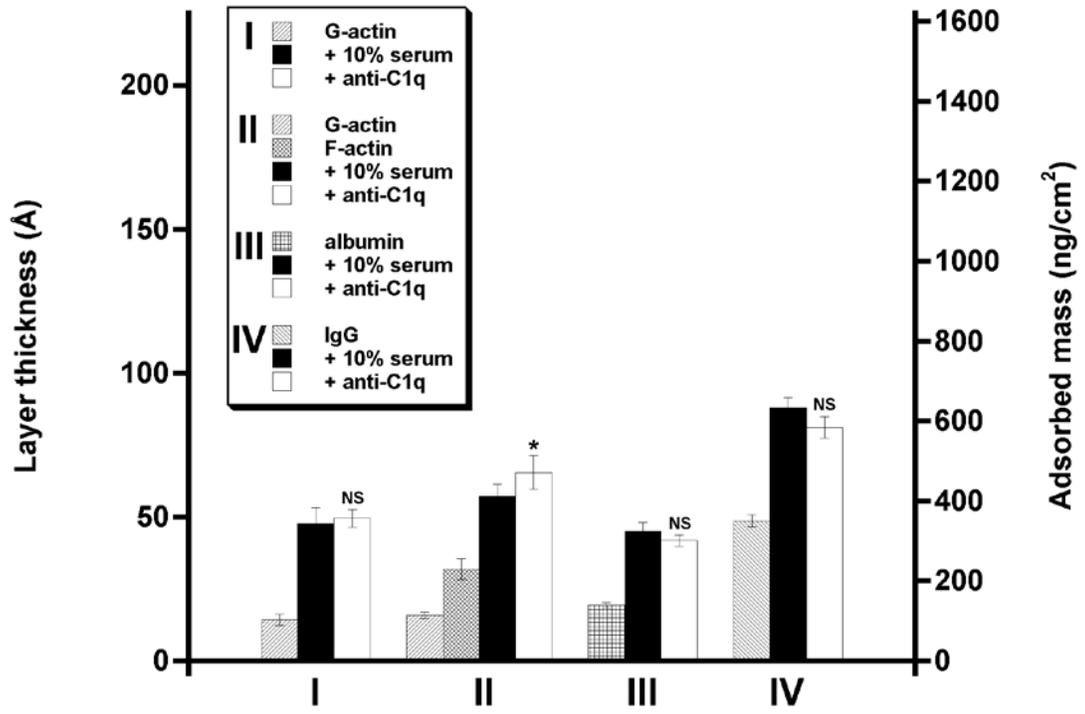
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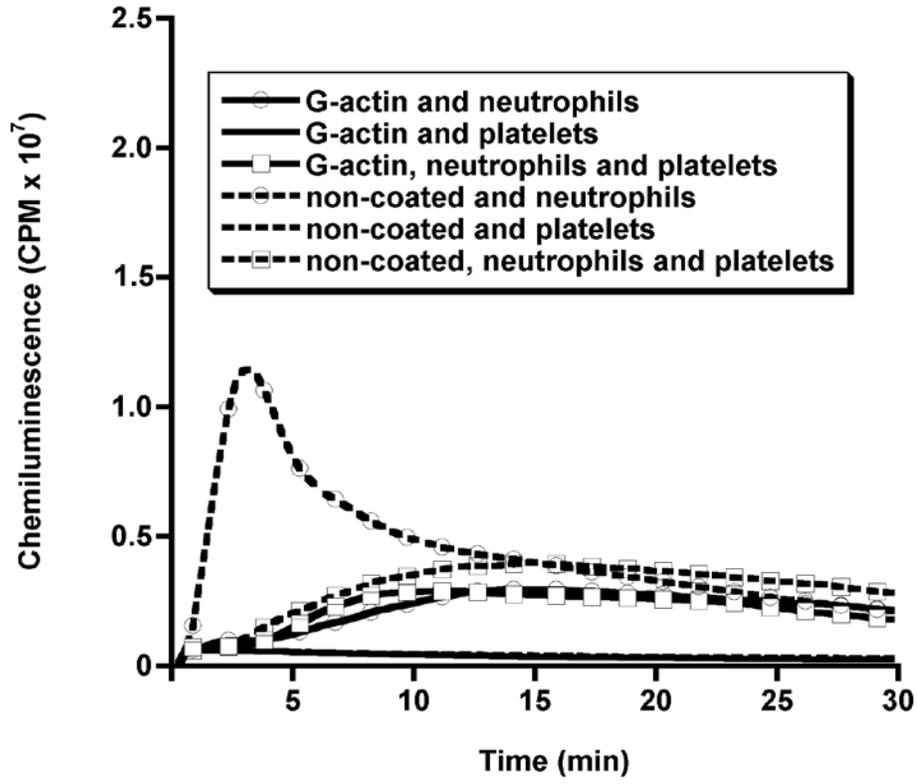
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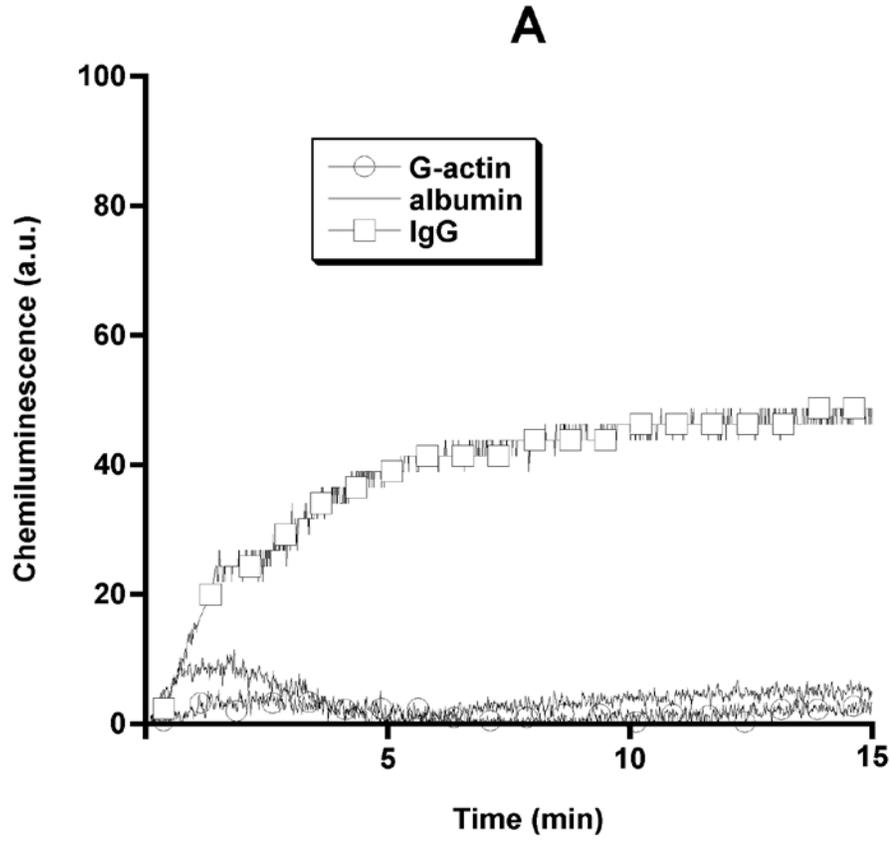
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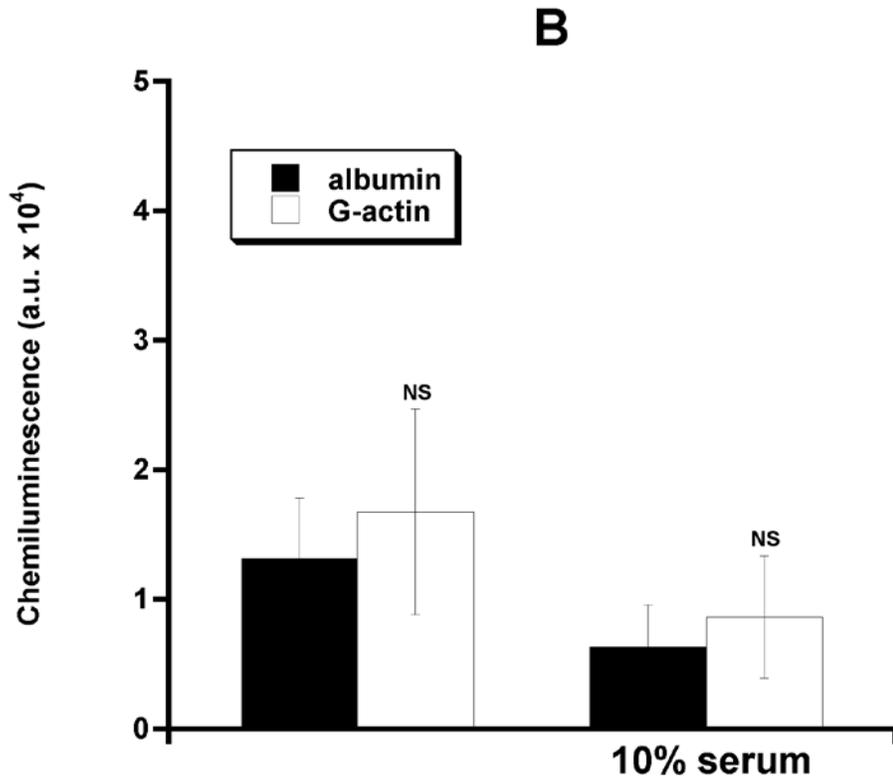
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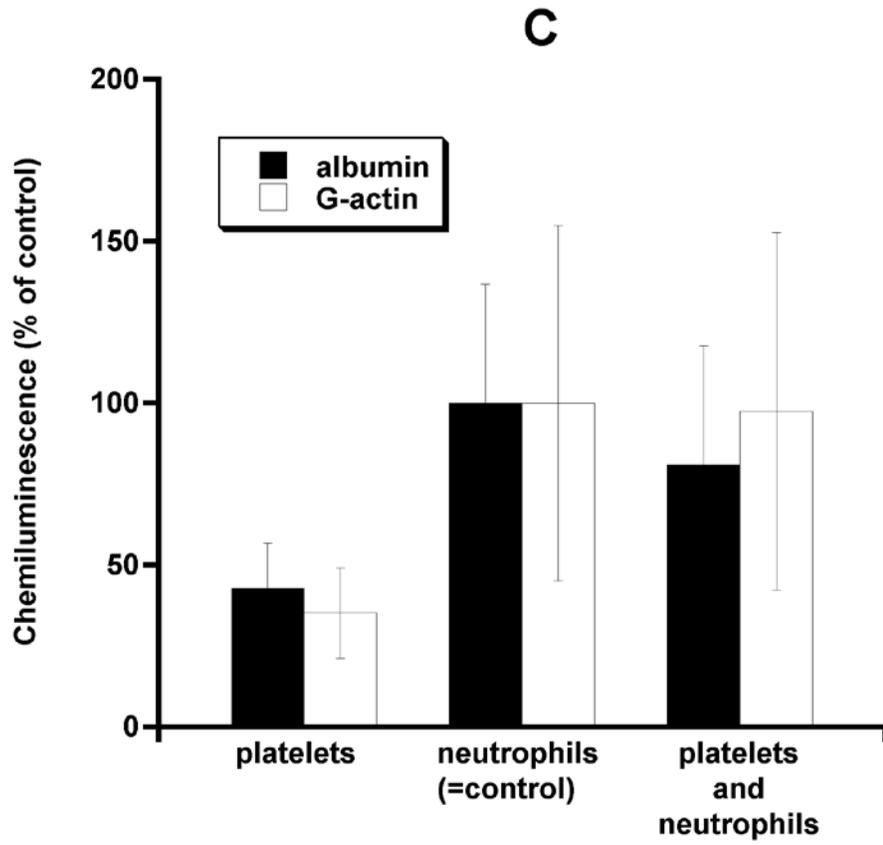
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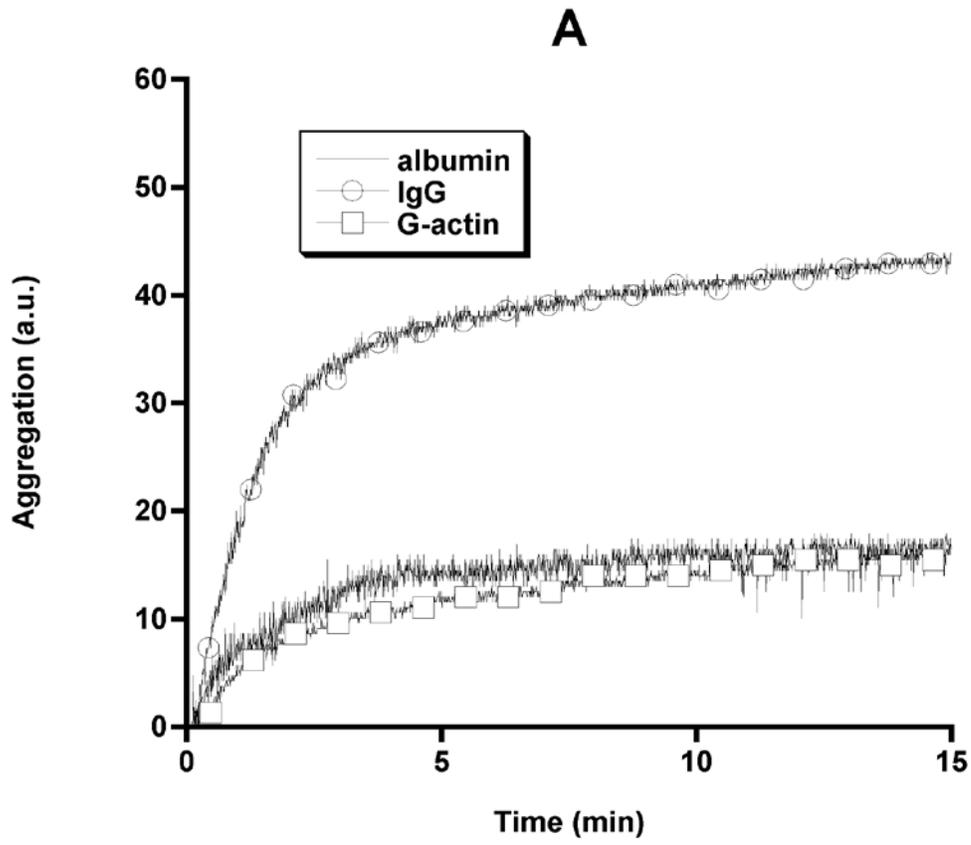
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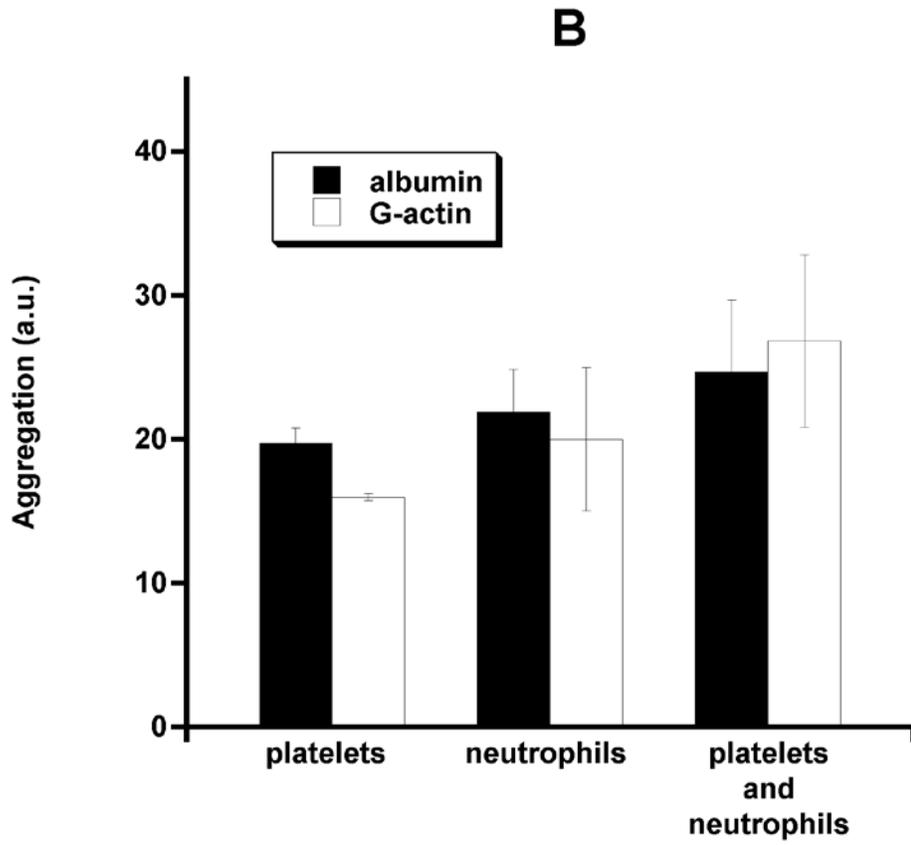
Figure 7

Figure 8

Figure 9