Braune, S.; Ruesten-Lange, M.v.; Mrowietz, C.; Luetzow, K.; Roch, T.; Neffe, A.T.; Lendlein, A.; Jung, F.:

Dynamic in vitro hemocompatibility testing of poly(ether imide) membranes functionalized with linear, methylated oligoglycerol and oligo(ethylene glycol)

In: Clinical Hemorheology and Microcirculation (2013) IOS Press

DOI: 10.3233/CH-131729

Dynamic *in vitro* hemocompatibility testing of poly(ether imide) membranes functionalized with linear, methylated oligoglycerol and oligo(ethylene glycol)

Steffen Braune[1], Maik von Ruesten-Lange[1], Christof Mrowietz[1], Karola Lützow[1,2], Toralf Roch[1,2], Axel T. Neffe[1,2], Andreas Lendlein[1,2], Friedrich Jung[1,2,*]

[1] Institute of Biomaterial Science and Berlin-Brandenburg Centre for Regenerative Therapies, Helmholtz-Zentrum Geesthacht , Kantstrasse 55, 14513 Teltow, Germany

[2] Helmholtz Virtual Institute − Multifunctional Biomaterials for Medicine, Teltow and Berlin, Germany

Corresponding author:

Prof. Dr. F. Jung

email: friedrich.jung@hzg.de

tel. ++49-(0)3328 - 352 269

fax ++49-(0)3328 - 352 452

Keywords:

Oligoglycerol, protein resistance, hemocompatibility, surface functionalization, membranes, biomaterial

Abstract

Linear, side-chain methylated oligoglycerols (OGMe) were recently reported as potential surface passivating molecules for improving the protein resistance of cardiovascular application relevant poly(ether imide) (PEI) membranes. A previously reported *in vitro* screening under static test conditions allowed an end-point evaluation of the adhesion and activation of adherent thrombocytes performed on the material surfaces and revealed similar levels of thrombogenicity on PEI membranes, functionalized with OGMe and oligo(ethylene glycol) (OEG) of similar molecular weight (M_n = 1,300 g·mol^{-1} - 1,800 g·mol^{-1}). In the present study, we investigated the hemocompatibility of these materials in a dynamic closed loop system, in order to study time-dependent thrombocyte material interactions also of the circulating thrombocytes by mimicking *in vivo* relevant flow conditions in a dynamic test system with multiple material contacts. Activation and aggregation of circulating thrombocytes as well as complement activation and plasmatic coagulation were evaluated after 40 circulations of thrombocyte rich plasma in the closed loop system. The results of the dynamic tests revealed no differences between the OGMe and OEG functionalized PEI membranes. Furthermore, no differences were observed between the latter and a PEI membrane treated under the conditions of functionalization at pH 11 (PEI-pH11) without an oligoether being present. Blood plasma protein adsorption, as well as activation, and adherence of circulating thrombocytes occurred in a comparable, but minor manner on all investigated PEI membranes. From this we conclude that the OGMe and OEG surface functionalization did not lead to an improvement of the already good hemocompatibility of the PEI-pH11 membrane.

1. Introduction

Thrombotic processes, prompted by the adsorption of blood plasma proteins, are one of the major causes for failure of cardiovascular implants [19,34]. Thrombocyte adhesion, activation, and further activation of circulating thrombocytes can lead to the formation of thrombi, which may occlude the vessel in the vicinity of the implant or detach and form emboli that may induce major adverse events in more distal regions of the blood vessel [28,29,45]. Surface functionalization of biomaterials is one strategy to improve their hemocompatibility [34,36]. Recently, glycerols grafted on very smooth model surfaces like gold or glass, were shown to lead to a significantly diminished protein adsorption, while side chain methylated oligoglycerols (OGMe) revealed best surface shielding properties. These results have raised increasing attention and designated oligoglycerols as possible oligoethyleneglycol (OEG) alternatives [30,31]. Furthermore, OGMe was shown to be more stable to oxidative degradation than OEG [33], which is prerequisite for long-term cardiovascular implants.

It is unclear, if data obtained for planar and smooth model surfaces can be transferred to application relevant membrane surfaces. This was investigated in a recent study, in which poly (ether imide) (PEI) membranes were functionalized with OEG (M_n = 1320 g·mol^{-1}) and OGMes (M_n = 1120 g·mol^{-1}, 1800 g·mol^{-1}, or 2270 g·mol^{-1}) [33]. Physicochemical surface characterizations proved the successful grafting of OEG and OGMe on a PEI membrane and confirmed a consistent membrane morphology, which was not affected by the grafting procedure [33]. The functionalized membranes were compared with PEI membranes treated under the conditions of functionalization at pH 11 (PEI-pH11) without an oligoether being present (which induces the formation of free carboxylic acid groups on the surface). For PEI-pH11, highest amounts of adsorbed bovine serum albumin (BSA) and bovine fibrinogen (BFIB) were observed, while BSA adsorption was reduced on OEG and OGMe functionalized

PEI membranes. However, BFIB adsorption was not changed on any of the functionalized PEI membranes. This was discussed to result from an incomplete coverage of oligoether molecules on the porous PEI membrane surface as it was reported for other polymers [25,32]. Additionally is was shown that BSA adsorption can be reduced on non-charged, hydrophilic polymer surfaces, while fibrinogen adsorption is not influenced by these material surface characteristics [57,59]. In a first *in vitro* screening test under static conditions, no differences were observed between OEG or OGMe functionalization in respect to number and activation status of adherent thrombocytes. Neither thrombocyte aggregates nor small thrombi were found on the functionalized PEI membranes. However, a non-significant trend for improved hemocompatibility was reported for higher molecular weight OGMe [33].

In the present study, the hemocompatibility of PEI membranes functionalized with OGMe and OEG of similar molecular weights (M_n = 1.8 kDa vs. 1.3 kDa) were compared with non-functionalized PEI-pH11 membranes under dynamic conditions *in vitro*. The *in vitro* dynamic test system was chosen because in closed-loop systems ongoing contacts of thrombocytes can occur with the material surface studied. These contacts can occur since the tube system exhibits a parabolic velocity profile with thrombocytes preferentially located in the cell free plasma layer, which is in close vicinity to the tube wall. Therefore, thrombocytes can be activated and adhere to the material surface, which is placed directly onto the tube wall. This experimental setup mimics very well the *in vivo* situation at the site of cardiovascular implants. In the static test system, only one contact between thrombocyte and material surface occurs after sedimentation. In contrast, the ongoing circulation of the thrombocyte rich plasma in the dynamic test system allows - subsequent to an initial but reversible thrombocyte/material contact - the adhesion of activated circulating thrombocytes. Activated circulating thrombocytes may - by the release of soluble mediators, e.g. serotonin or adenosine diphosphate - further activate neighbored but non-activated thrombocytes. This

increases the probability of activation and adherence of circulating thrombocytes on thrombogenic material surfaces. In our closed loop system, shear rates are far below values required for shear rate induced activation of thrombocytes (less than 50 $N \cdot m^{-2}$). Consequently, activation of the thrombocytes in the dynamic test system can be attributed to thrombocyte/material interactions, so that the hemocompatibility/thrombogenicity of polymers can be evaluated. Therefore, the present work comprises the characterization of the activation and aggregation of circulating thrombocytes as well as complement and coagulation activation, before and after 40 circulations of thrombocyte rich plasma in a closed loop system.

Table 1 Physico-chemical characterization of OGMe and OEG functionalized PEI membranes. Data summarized from Lange et al. [33]. © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

		PEI-pH11	OEG-1.3k	OGMe-1.8k
Modificator		-	Oligo(ethylene glycol)	Oligoglycerol
M_n modificator	[$g \cdot mol^{-1}$]	-	1,300	1,800
θ_{adv} [1]	[°]	28 ± 7	65 ± 2	66 ± 3
θ_{rec} [1]	[°]	27 ± 2	30 ± 9	29 ± 8
Hysteresis [1]	[°]	2	35	37
R_a [2]	[nm]	9 ± 41	19 ± 9	12 ± 7
R_q [2]	[nm]	14 ± 7	34 ± 19	18 ± 9
pH at IEP [3]	[$\zeta_u = 0$]	2.4	2.5	3.5
ζ_u at pH 7.4 [4]	[mV]	- 31	- 33	- 35
BSA adsorption	[$\mu g \cdot cm^{-2}$]	8 ± 1	3 ± 1	-1 ± 1 [5]
BFIB adsorption	[$\mu g \cdot cm^{-2}$]	16 ± 1	15 ± 1	15 ± 1

1: Contact angle measurements (captive bubble method).
2: Atomic force microscope scan size = 15 µm (wet state, PBS, pH 7.4).
3: pH at IEP (determined in the run from pH 9 to pH 3).
4: ζ_u at pH 7.4 (determined after equilibrium).
5: Values below detection limit.

2. Materials and Methods

2.1 Study design

This *in vitro* study was designed to examine the interaction between fresh human thrombocytes and PEI membranes, functionalized with OGMe and OEG, in a dynamic closed loop test system. The PEI membrane treated under the conditions of functionalization at pH 11 (PEI-pH11) without an oligoether being present was further included in the study, which was performed as a three-armed explorative study. The study design conforms the criteria of the declaration of Helsinki [64] and the current guidelines of the British committee for standards in haematology [23]. It was approved by the institutional review committee of the Charité Berlin before conduction of the work.

2.2 Membrane fabrication and functionalization

All solvents were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) or Merck KGaA (Darmstadt, Germany) and were used as received. Linear, side-chain methylated α-amino-ω-methoxy oligo(ethylene glycol) (M_n = 1,300 g·mol^{-1}) (OEG-1.3k) and α-amino-ω-hydroxy oligoglycerol (M_n = 1,800 g·mol^{-1}) (OGMe-1.8k) was provided by the Institute of Chemistry and Biochemistry, Freie Universität Berlin [63]. PEI Ultem ®; GE-Plastics, Fairfield, USA; M_w = 30,000 ± 10,000 g·mol^{-1}, M_n = 12,000 ± 4,000 g·mol^{-1}) flat sheet membranes were prepared on a polyester nonwoven support by continuous non-solvent induced phase separation using a solution of 17.5 wt.% PEI in 30 wt.% γ-butyrolactone and 52.5 wt.% dimethylacetamide and water as a coagulant. The PEI membranes were functionalized with a 2 wt.% aq. solutions of OEG or OGMe (pH = 11 adjusted) at 70 °C for 17 h. PEI-pH11 membranes were treated under the same conditions without an oligoether being present in the solution. The functionalized membranes were washed with deionized water to remove unreacted OEG and OGMe and stored in sodium azide solution (0.02 wt.%

sodium azide in Millipore water) at 4 °C. The full physicochemical characterization of the starting materials and functionalized membranes was reported before and summarized here in Table 1 [33]. To prepare samples for the *in vitro* testing, membranes were washed 72 h (15 washing steps) with Ampuwa® water (Ampuwa® Spüllösung, Fresenius-Kabi, Bad Homburg, Germany).

2.3 Study participants

According to the criteria described by Berg et al. for reference values in laboratory medicine, blood was collected from apparently healthy subjects (n = 6) [6]. Subjects receiving thrombocyte function inhibitors were excluded, since, e.g. acetylsalicylic acid (aspirin) medication can irreversibly inhibit thrombocyte cyclooxygenase activity and decreases the release of thromboxane B2, which may lead to an underestimation of the material's thrombogenicity [17,42,43]. The same applies to NO donor compounds, which affect not only thrombocyte aggregation but also thrombocyte adherence [27]. Preliminary analysis were carried out concerning the activation of the thrombocytic and coagulation system [29]. Haemogram values were obtained with a SYSMEX XS-800i hematolyzer (SYSMEX, Norderstedt, Germany) and tested to be within reference ranges. An adequate thrombocyte function was proven with the Dade® PFA-100 thrombocyte function analyzer (Siemens Healthcare Diagnostics, Marburg, Germany) using equine type I collagen and epinephrine bitartrate as well as equine type I collagen and adenosine-5'diphosphate (ADP) as activators [14].

2.4 Blood sampling and preparation of thrombocyte rich plasma

Blood was taken from the uncongested arm after puncture of the cubital vein. S-Monovettes® (Sarstedt, Nümbrecht, Germany) were used for blood withdrawal, each containing 1 ml citrated solution (0.106 mol/l final concentration) according to clinical routine thrombocyte

testings as anticoagulant to prevent spontaneous coagulation [3,4]. In addition to a slowly aspiration, the monovettes were further slowly agitated in order to prevent thrombus formation within the syringe.

To prepare thrombocyte rich plasma (TRP), citrate anticoagulated blood was centrifuged at 120 g for 30 minutes (Megafuge, Heraeus, Thermo Scientific, Langenselbold, Germany) [10]. The supernatant (TRP) was carefully removed and transferred to plastic test tubes. The TRP was allowed to rest for at least 30 minutes in order to prevent thrombus formation as a consequence of the centrifugation procedure. To ensure an adequate thrombocyte function, all following live cell experiments were carried out within 3 hours.

2.5 Dynamic test system - closed loop system

The closed-loop system (Figure 1) was operated in a temperature controlled housing (37 °C, Certomat HK, Sartorius, Göttingen, Germany) and a new tube system was used for each experiment. Medical grade Masterflex® platinum cured silicone (Masterflex® Tygon 3350, tube diameter 3 mm, Ismatec, Wertheim-Mondfeld, Germany) was used as tubing material for the two parts of the closed loop system, which were connected with three-way medical valves [26]. The PEI membranes were inserted into the lower tube with a total area of 4.5 cm^2. The solely silicone tubing was used as control (Blank system). The tube system was filled with approximately 6 ml of TRP via the lower valve and exhausted from air via the upper valve. After filling, 1 ml of TRP was extracted in order to determine the baseline values (t = 00). The extracted volume was refilled and the tube system was connected to a Masterflex® L/S pump equipped with a Masterflex® Easy-Load 3® pump head (Cole-Parmer Instrument Company, Ilinois, U.S.A). The TRP flow rate was 1,000 $ml \bullet h^{-1}$, corresponding a shear rate of 0.15 $N \bullet m^{-2}$, which is far below the shear stress required to activate thrombocytes (~ 50 $N \bullet m^{-2}$) [51]. Obtainable thrombocyte activation is, consequently, attributed to the interaction of material-thrombocyte interaction. Furthermore, the parabolic velocity profile, which is a consequence

of the round shape of the tube system, results in a thrombocyte flow next to the tube wall [7]. This ensures an interaction between thrombocytes and the material surface during the circulation, which may be reduced in flat reactor systems [46]. Final TRP samples were harvested after 40 circulations (t = 40).

2.6 Number of single circulating thrombocytes and thrombocyte reactivity index

After sampling, TRP was either diluted in ethylenediaminetetraacetic acid phosphate buffer solution (EDTA) (E-Monovette®, Rheomed GmBH, Hürth, Germany) to dissolve reversibly bound thrombocyte aggregates, or were fixed in formaldehyde enriched EDTA solution (F-Monovette®, Rheomed GmBH, Hürth, Germany). The number of single circulating thrombocytes was determined using a SYSMEX XS-800i hematolyzer (SYSMEX, Norderstedt, Germany) and the thrombocyte aggregation index (TAI) was calculated for each measurement time point according to Grotemeyer [22,65].

2.7 Flow cytometry

Formaldehyde fixed thrombocytes were processed for flow cytometric analysis, which was carried out on a Cytomics FC 500, equipped with the CXP software (Beckman Coulter®, Krefeld, Germany). A calibration of the device was performed prior to all analyses including the Immuno-Brite™ standard kit (Beckman Coulter®, Krefeld, Germany) and FluoroSpheres calibration beads (Dako, Hamburg, Germany). The optimal antibody concentration was determined as previously described [20]. Mean thrombocyte receptor densities were calculated from the mean equivalent fluorochrome amounts using the FluoroSpheres calibration beads (Dako, Hamburg, Germany) as standards. The ratio of the fluorochrome molecules to the number of antibody molecules (F/P ratio) was calculated batch-specific, according to the information of the supplier. To identify thrombocytes, samples were stained for the GPIb/IX thrombocyte membrane glycoprotein (anti-CD42b-PE (Clone SZ2),

Immunotech, Beckman Coulter, Marseille, France; anti-CD42a-FITC (Clone Beb1), Becton Dickinson Bioscience, San José, U.S.A). The lysosomal membrane associated glycoprotein 3 (anti-CD63-FITC (Clone CLBGran/12), Immunotech, Beckman Coulter, Marseille, France) and the activated gpIIbIIIa receptor (anti-PAC-1-FITC, Becton Dickinson Bioscience, San José, U.S.A) were analyzed, as markers for the activation of thrombocytes [50,52].

2.8 Thrombin generation and coagulation activation

TRP samples were centrifuged at 2000 g for 20 minutes (Eppendorf Centrifuge 5804 R, Hamburg, Germany) to obtain thrombocyte poor plasma (TPP), which was used for all enzyme-linked immunosorbent assays (ELISA). Plasma concentrations of prothrombin fragments F1+2 and the thrombin–antithrombin III complexes were determined using commercially available ELISA (Enzygnost® F1+2 and Enzygnost® TAT micro, Siemens Healthcare Diagnostics, Marburg, Germany) [9,44,61].

2.9 Complement activation

According to a previously reported protocol [49], C5a fragments were quantified in order to analyze the membrane induced complement activation (human complement component C5a DuoSet ELISA, R&D, Minneapolis, MI, USA) [37,47]. The ELISA kits were performed according to manufacturer instructions. Data acquisition was carried out at an Infinite 200 PRO microplate reader (TECAN, Salzburg, Austria) for all above mentioned ELISA.

2.10 Microscopy

Subsequent to the TRP incubation, PEI membranes were explanted from the closed loop system and treated with glutardialdehyde (1 wt.% in Millipore water, Sigma Aldrich, Steinheim, Germany) for 60 minutes to fix and visualize adherent thrombocytes and proteins (Glutardialdehyde Induced Fluorescence Technique - GIFT) [15]. Membrane samples were

embedded (Mowiol® 4-88, Polyscience Inc, Eppelheim, Germany) and qualitatively examined with a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss MicroImaging, Jena, Germany). The epifluorescence of glutardialdehyde was excited with an argon laser at 488 nm wavelength. The emission was detected using a bandpass filter between 505 nm and 530 nm wavelength.

2.11 Statistics

For all samples, arithmetic mean values and standard deviations are given (mean ± standard deviation). Gaussian distributions were tested for all samples using Kolmogorov and Smirnov test. Repeated measures ANOVA were performed to evaluate statistical differences between the four study groups. In addition for the two sample comparisons, a *post hoc* analysis was performed using the Newman Keuls test. P values less than 0.05 were considered as significant differences.

3. Results and Discussion

3.1 Number of single circulating thrombocytes and thrombocyte reactivity index

Differences in the number of single circulating thrombocytes before and after 40 circulations have been studied and were significant for all PEI membranes compared to the blank closed loop system (Figure 1), tested without material sample ($SCT_{\Delta t00\text{-}t40}$: $P < 0.01$ for the whole dataset, $P < 0.5$ for pairwise comparisons). This indicates a stronger decrease of circulating thrombocytes for the PEI membranes than for the blank closed loop system. Similar observations were made for the thrombocyte aggregation indexes, which describe the amount of circulating thrombocyte aggregates in the TRP. Compared to the blank closed loop system, the OGMe and OEG functionalized membranes showed a significant increase in the amount of circulating aggregates after 40 circulations ($TAI_{\Delta t00\text{-}t40}$: $P = 0.04$, $p < 0.05$). Values for the

PEI-pH11 membrane were not significantly different, compared to the blank closed loop system ($TAI_{\Delta t00\text{-}t40}$: $p > 0.05$).

Table 2 Results obtained in the blank closed loop system and for PEI membranes treated under the conditions of functionalization at pH 11 (PEI-pH11) without an oligoether being present as well as for PEI membranes functionalized with OGMe and OEG. Following parameters were quantified: Number of single circulating thrombocytes (SCT) in blood plasma, thrombocyte aggregation indexes (TAI), mean receptor density of CD42/CD63 and CD42/PAC1 positive thrombocytes (RPT = number of receptors per thrombocyte), thrombin-antithrombin complex III (TAT), prothrombin fragments F1+2 (F1+2) and complement fragment C5a generation (Mean ± standard deviation, n = 6 donors).

		Blank system	OGMe-1.8k	OEG-1.3k	PEI-pH11	P
SCT_{t40}[1]	($10^3 \cdot \mu l^{-1}$)	45 ± 10	45 ± 10	44 ± 11	44 ± 11	0.67
$SCT_{\Delta t00\text{-}40}$[1]	($10^3 \cdot \mu l^{-1}$)	0.5 ± 1.9$^{*+\#}$	4.8 ± 2.8*	4.7 ± 2.7^{+}	4.2 ± 1.9$^{\#}$	<0.01
TAI_{t40}[2]	(-)	1.09 ± 0.08	1.14 ± 0.09	1.16 ± 0.11	1.11 ± 0.07	0.24
$TAI_{\Delta t00\text{-}t40}$[2]	(-)	0.00 ± 0.02^{*+}	-0.10 ± 0.10*	-0.11 ± 0.08^{+}	-0.07 ± 0.04	0.04
$CD63_{t40}$	(RPT)	4,565 ± 1,653	2,601 ± 953	2,842 ± 999	3,420 ± 1,817	0.06
$CD63_{\Delta t00\text{-}t40}$	(RPT)	-273 ± 185	-301 ± 470	-111 ± 176	107 ± 124	0.53
$PAC1_{t40}$	(RPT)	268 ± 68	248 ± 59	260 ± 75	265 ± 88	0.08
$PAC1_{\Delta t00\text{-}t40}$	(RPT)	12 ± 7	10 ± 12	9 ± 31	1 ± 9	0.70
TAT_{t40}	($\mu g \cdot l^{-1}$)	3.91 ± 2.27	3.67 ± 1.24	4.25 ± 1.75	3.80 ± 1.52	0.63
$TAT_{\Delta t00\text{-}t40}$	($\mu g \cdot l^{-1}$)	0.32 ± 0.75	0.13 ± 0.41	-0.16 ± 1,78	0.42 ± 1.25	0.80
$F1{+}2_{t40}$	($pmol \cdot l^{-1}$)	170 ± 95	185 ± 124	177 ± 110	169 ± 115	0.22
$F1{+}2_{\Delta t00\text{-}t40}$	($pmol \cdot l^{-1}$)	-4.8 ± 7.5	-19.1 ± 27.2	-10.4 ± 4.2	4.1 ± 7.4	0.09
$C5a_{t40}$	($ng \cdot ml^{-1}$)	55.7 ± 23.5	56.4 ± 22.9	55.8 ± 22.9	53.0 ± 22.6	0.25
$C5a_{\Delta t00\text{-}t40}$	($ng \cdot ml^{-1}$)	3.5 ± 3.9	3.6 ± 3.5	4.3 ± 3.2	3.0 ± 3.1	0.93

1: SCT: Number of single circulating thrombocytes
2: TAI: Thrombocyte activation index
*+#: Symbol pairs describe significant differences between values ($P < 0.05$, repeated measures ANOVA)

From both datasets we conclude that a certain interaction of thrombocytes with the surface of all PEI membranes occurred, which was clearly distinguishable from the blank closed loop system. Activated adherent thrombocytes release components from lysosomes, dense- and α-granules (degranulation of e.g. ADP, Ca^{2+}, serotonin, proteins and growth factors or cytokines and enzymes) that amplify the adhesion process by autocrine and paracrine mechanisms. The activation process of adherent thrombocytes is thereby reinforced (autocrine). Non-activated thrombocytes are recruited from the circulation (paracrine) and accelerated to form aggregates with adherent or still circulating ones [41]. Therefore, time-dependent quantitative changes of single circulating thrombocytes and circulating aggregates mark a certain material induced thrombocyte activation [11,16]. However, the observed decreased numbers of single circulating thrombocytes were rather moderate and the levels of thrombocyte aggregation were suspicious but not pathologic for all PEI membranes (normal TAI: < 1.05, suspicious TAI: 1.05 – 1.20, pathological TAI: >1.2) [22]. No differences were observable between functionalized PEI membranes and PEI-pH11, nor between the OGMe and OEG functionalized ones. This shows that the samples influenced the above mentioned parameters similarly and independently from functionalization or modificator.

3.2 Flow cytometry

The activation of circulating thrombocytes was further analyzed using flow cytometry (Figure 2). Therefore, the lysosomal membrane associated glycoprotein 3 (anti-CD63) and the activated gpIIbIIIa receptor (anti-PAC-1) have been chosen as molecular markers [1,2,21,24,39,67]. Activated circulating thrombocytes expose increased numbers of integral membrane proteins following lysosomal degranulation, which can be determined by the quantification of anti-CD63-antibody binding.

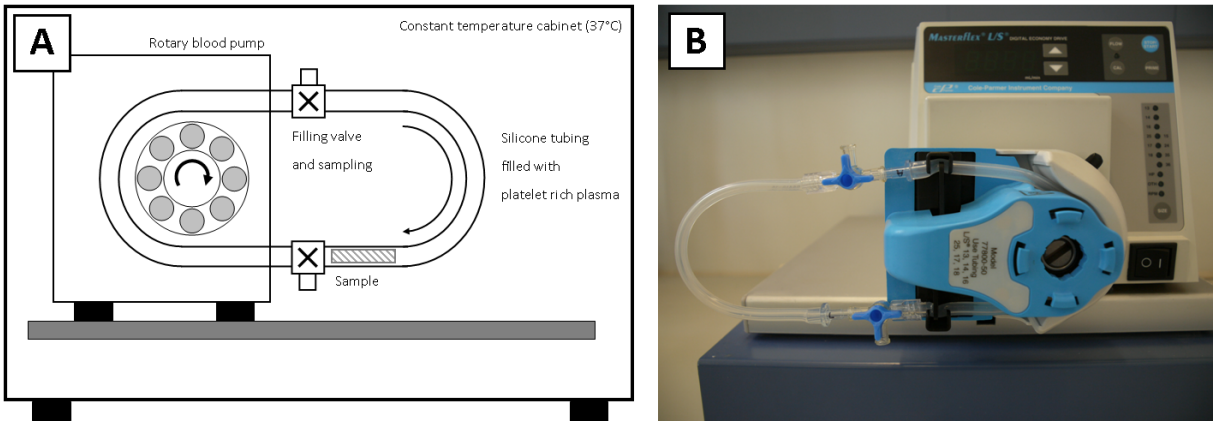

Figure 1: Scheme (A) and photograph (B) of the closed loop test system for the evaluation of material mediated hemocompatibility under dynamic conditions. (A) adapted from [28] (Reprinted from Clinical hemorheology and microcirculation, 53, F. Jung et al., Haemocompatibility testing of biomaterials using human platelets, 97-115, Copyright (2013), with permission from IOS Press.)

About 650 molecules per cell are reported for unstimulated thrombocytes and up to 12,600 molecules have been detected on the surface of thrombin activated thrombocytes [38]. Considering these levels, the here reported values of CD63 positive molecules per thrombocyte (2,601 to 4,565) indicate an overall low activation of the circulating thrombocytes for all analyzed samples. Compared to the blank system, the surface density of CD63 positive molecules per thrombocyte was lower after 40 circulations ($CD63_{t40}$) for all analyzed PEI membranes, which might evidence a slightly reduced degree of activation. The surface density increased during the incubation for all analyzed samples, except for the PEI-pH11 membrane. Differences for OGMe-1.8k were more pronounced, compared to OEG-1.3k. However, the statistical analysis revealed no significant differences for both datasets ($CD63_{t40}$: $P = 0.06$, $CD63_{\Delta t00\text{-}t40}$: $P = 0.53$), confirming a rather similar degree of activation for all analyzed PEI membranes.

During the activation process, the functional state of the glycoprotein IIb-IIIa receptor complex (the fibrinogen receptor) on the thrombocyte membrane changes from the resting to the activated and later to the ligand-occupied state [18]. The activated fibrinogen receptor can

be characterized by the binding of PAC1 antibodies with approximately 45,000 receptors that can appear upon thrombocyte activation [53]. PAC1 surface densities range from 248 to 268 molecules ($PAC1_{t40}$) and, similar to the results for CD63 expression, reflect an overall low activation of circulating thrombocytes compared to the blank system. Variations during the incubation time ($PAC1_{\Delta t00\text{-}t40}$) were beneath 4.5 % and, in both data sets, the bare and functionalized PEI membranes were not significantly different ($PAC1_{t40}$: P = 0.08, $PAC1_{\Delta t00\text{-}t40}$: P = 0.70).

3.3 Thrombin generation and coagulation activation

Plasma concentrations of prothrombin fragments F1+2 (F1+2) and the thrombin–antithrombin III complexes (TAT) have been studied as molecular markers for the thrombin generation and activation of the coagulation, which is closely linked to the activation of thrombocytes [5,9]. The TAT levels of all analyzed PEI membranes did not significantly differ and decreased with incubation time, except for the OEG functionalized membrane ($TAT_{\Delta t40}$: P = 0.63, $TAT_{\Delta t00\text{-}t40}$: P = 0.80). Generated prothrombin fragments F1+2 increased similarly with incubation time, except for the PEI-pH11 membrane ($F1{+}2_{t40}$: P = 0.22, $F1{+}2_{\Delta t00\text{-}t40}$: P = 0.09).

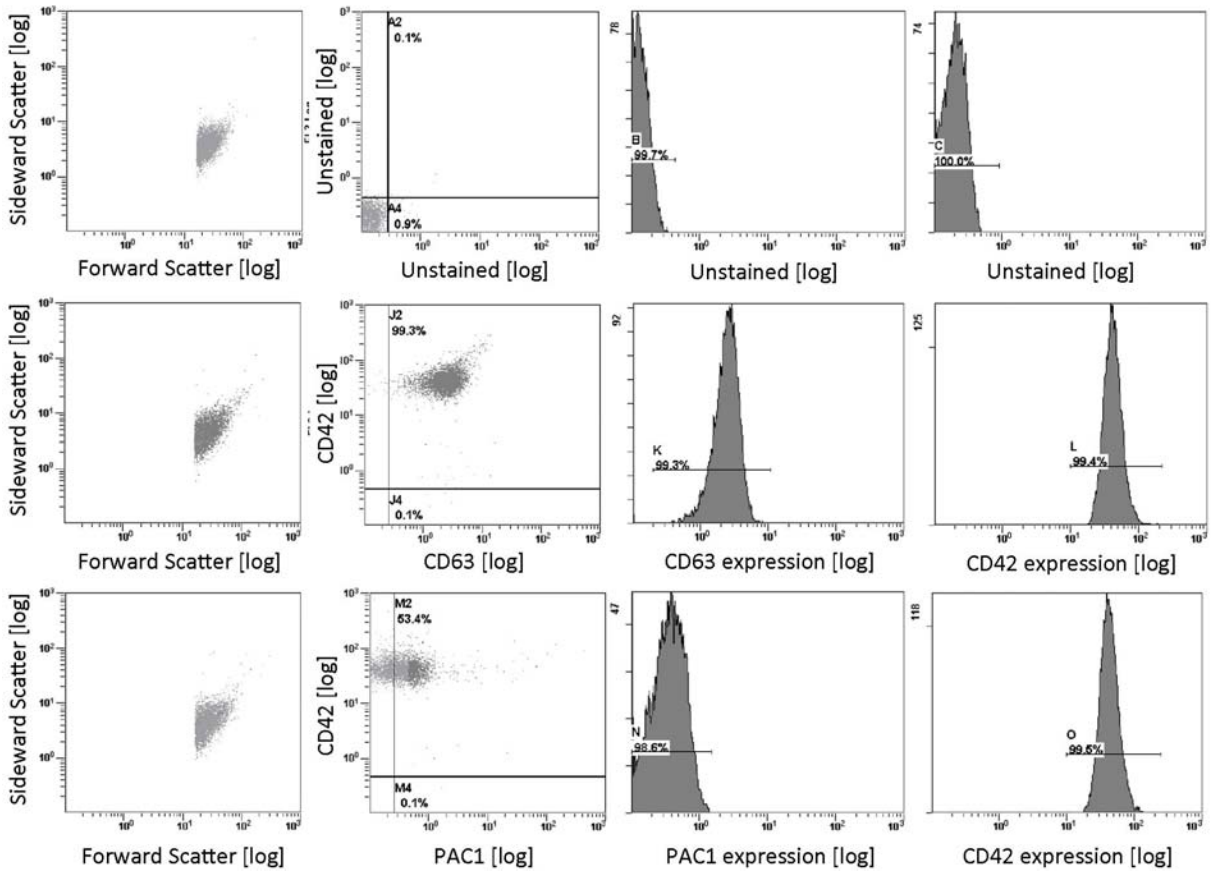

Figure 2: Representative surface and activation marker expression of thrombocytes after 40 circulations in the closed loop system in contact with a material surface. Unstained and CD42/CD63, CD42/PAC1 co-stained thrombocytes were investigated on a Cytomics FC 500 flow cytometer, equipped with the CXP software (Beckman Coulter®, Krefeld, Germany).

Both parameters were within reference ranges reported for citrate anticoagulated plasma of apparently healthy adults (TAT: 2.0 µg · l^{-1} – 4.2 µg · l^{-1}, n = 196; F1+2.69 pmol · l^{-1} – 229 pmol · l^{-1}, n = 137, Siemens Healthcare Diagnostics, Marburg, Germany). They reflect an initial cell material interaction, which might promote a certain generation of prothrombin fragments and TAT complexes, but did not further increase with incubation time.

3.4 Complement activation

To assess complement activation, C5a generation was determined before and after 40 circulations of thrombocyte rich plasma in the closed loop system. For all analyzed samples

and measurement time points, the concentrations of C5a fragments were not significantly different ($C5a_{t40}$: P = 0.25, $C5a_{\Delta t00\text{-}t40}$: P = 0.93). Biomaterial induced complement activation is thought to be induced by the initial binding of C3b molecules to adherent plasma proteins including albumin, fibrinogen and immunoglobulins [19,40]. The subsequent release of C5a protein fragments from the complement component C5 recruits and activates circulating thrombocytes [48]. Thrombocyte activation and adhesion to biomaterial surfaces is therefore closely related to the generation of C5a complement fragments and the observed values in good agreement with all previously discussed data [19,40,48]. Similarly to the results for PAC1 surface densities and TAT complex concentrations, values decreased after 40 circulations, which might indicate an initial thrombocyte/material interaction that did not result in a continued recruitment and activation of thrombocytes by the generation of complement fragments.

3.5 Microscopy

In order to assess the adhesion of thrombocytes to the PEI membrane surfaces, material samples were explanted from the closed loop system after 40 circulations and microscopically examined. Figure 3 shows representative confocal laser scanning microscopic images of the bare and functionalized PEI membranes after 40 circulations and subsequent GIFT staining. A small number of adherent thrombocytes in different stages of activation, ranging from dendritic and early pseudopodial to fully spread and aggregated, could be visualized on all membrane surfaces. These findings correspond to the reduced number of circulating thrombocytes that was observed for all PEI membranes compared to the blank system. However, numbers of adherent thrombocytes as well as their covered area on the material surface was relatively low confirming certain, but low thrombogenicity of all samples. In addition to cellular structures, also protein networks were prominent on all samples. Morphologically, these structures correspond to fibrinogen networks as they have been

reported e.g. by Yaseen on silica-poly(urethane) copolymers [66]. However, since the staining procedure is unspecific, it cannot be excluded that the images show already polymerized fibrin fibers [8,39]. The visual proof of a sparse protein deposition on the material surface is in good agreement with our observations for F1+2, TAT and C5a concentrations, which confirmed a very moderate interaction of plasma proteins and thrombocytes with all PEI membrane surfaces. Also protein adsorption data, obtained for single protein solutions of BSA and BFIB, are in good agreement with these findings. Whereas albumin adsorption was clearly reduced on the OEG and OGMe functionalized membranes, fibrinogen adsorption was similar for all analyzed membranes [33]. These results do not consider the very dynamic and time-dependent adsorption and desorption processes, which occur in complex protein solutions like blood and are described as the Vroman effect [62]. The latter describes the sequential adsorption of blood plasma to material surfaces and the replacement of initially adsorbed small proteins (e.g. albumin, ~68 kDa) by larger and higher-affinity proteins (e.g. fibrinogen, ~340 kDa) over time [12,58,59].

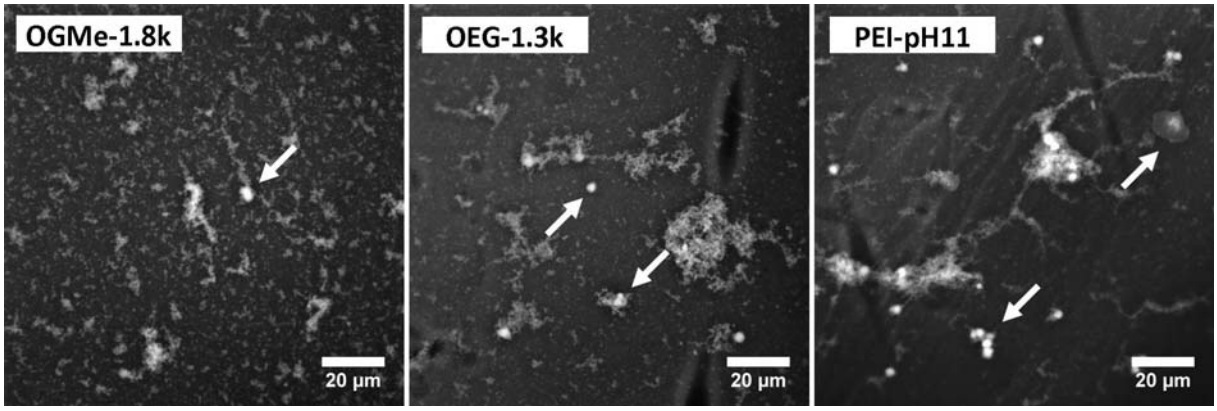

Figure 3: Representative confocal laser scanning microscopic images of a PEI membrane treated under the conditions of functionalization at pH 11 (PEI-pH11) without an oligoether being present as well as PEI membranes functionalized with OGMe and OEG displaying adherent proteins and thrombocytes on the membrane surfaces. Thrombocytes in different stages of activation are marked by arrows exemplarily (100 fold primary magnification, GIFT staining).

Nonetheless, the data presented here provide reasonable evidence that fibrinogen adsorption can occur on functionalized and non-functionalized PEI membrane surfaces, potentially mediating the observed thrombogenic effects. Conformational changes of the immobilized albumin or fibrinogen molecules, which can occur during or after the process of adsorption, may also influence the PEI membrane thrombogenicity. Sivaraman, Latour and others [55,56,60] reported for albumin and fibrinogen, as the most abundant and hemostatically relevant blood plasma proteins, that thrombocyte adhesion is strongly correlated with the degree of adsorption induced unfolding and not with the amount of adsorbed proteins. Therefore, it seems reasonable that the observed interaction of thrombocytes with all PEI membranes may be mediated by adsorbed fibrinogen molecules, which underwent conformational changes resulting in the exposure of cryptic thrombocyte receptor recognition domains [13,35,54].

4. Conclusion

The hemocompatibility of PEI membranes functionalized with OGMe and OEG was investigated in comparison to a PEI-pH11 without an oligoether being present. PEI membranes are a candidate material for cardiovascular applications and extracorporeal supporting systems. In a previous study, we confirmed the successful covalent surface functionalization of PEI membranes with the above mentioned oligoethers [33]. We further proved the higher stability of OGMe compared to OEG under oxidative conditions, which marks them as a promising alternative to OEG. In the present work, hemocompatibility was studied in a dynamic closed loop system with the aim to investigate material/thrombocyte interactions after ongoing contacts of thrombocytes with the PEI membrane surfaces, in an experimental setup that mimics very well the *in vivo* situation at the site of cardiovascular implants. The study under dynamic conditions, which, beyond the analysis under static

conditions [33], comprises the investigation of an activation and aggregation of circulating thrombocytes as well as coagulation and complement activation, confirms a comparable hemocompatibility for both functionalized membranes, which was not different from the PEI-pH11 membrane. Although, the dynamic test setup allows much more *in vivo* relevant statements about the hemocomopatibility/thrombogenicity of polymer samples, the negligible differences observed between the dynamic and static test systems may be attributed to an already good hemocompatibility of the PEI membrane after the functionalization treatment at pH 11. In view of our results, it remains to be studied whether other architectures of the functionalized glycerol structures, e.g hyperbranched polyglycerols, or mixtures of different glycerol architectures may lead to an improved hemocompatibility through a better surface shielding.

Acknowledgement

The authors thank Prof. Dr. Rainer Haag and Dr. Marie Weinhart from the Institute of Chemistry and Biochemistry of the Freie Universität Berlin for providing the linear, side-chain methylated oligoglycerol and oligo(ethylene glycol) used in this work. We thank the Bundesministerium für Bildung und Forschung (BMBF) for funding within project number 0315696A “Poly4bio BB".

References

[1] C.S. Abrams, N. Ellison, A.Z. Budzynski, and S.J. Shattil, Direct detection of activated platelets and platelet-derived microparticles in humans, *Blood* **75** (1990), 128–38.

[2] C. Abrams and S.J. Shattil, Immunological detection of activated platelets in clinical disorders, *Thromb Haemost* **65** (1991), 467–73.

[3] American National Standard, *Biological Evaluation - Part 4: Selection of tests for interaction with blood*, Association for the Advancement of Medical Instrumentation, Arlington, 2009

[4] O.K. Baskurt, M. Boynard, G.C. Cokelet, P. Connes, B.M. Cooke, S. Forconi, F. Liao, et al., New guidelines for hemorheological laboratory techniques, *Clin Hemorheol Microcirc* **42** (2009), 75–97.

[5] K.A. Bauer, Activation markers of coagulation, *Baillieres Best Pract Res Clin* **12** (1999), 387–406.

[6] B. Berg, H. Solberg, J. Nilsson, and N. Tryding, Practical experience in the selection and preparation of reference individuals: empirical testing of the provisional Scandinavian recommendations, in: *Reference values in laboratory medicine*, H. Solberg, R. Grasbeck, T. Alstrom, ed., John Wiley & Sons, Chichester (UK), 1981, 55–64.

[7] P.L. Blackshear, R.J. Forstrom, F.D. Dorman, and G.O. Voss, Effect of flow on cells near walls, *Fed Proc* **30** (1971), 1600–11.

[8] M. Blombäck, Molecular aspects in clinical hemostasis research at Karolinska Institutet, *Biochem Biophys Res Commun* **396** (2010), 131–4.

[9] B. Boneu, G. Bes, H. Pelzer, P. Sié, and H. Boccalon, D-Dimers, thrombin antithrombin III complexes and prothrombin fragments 1+2: diagnostic value in clinically suspected deep vein thrombosis, *Thromb Haemost* **65** (1991), 28–31.

[10] K. Breddin, M. Ziemen, O. Bauer, W. Herrmann, L. Schaudinn, U. Schlosser, A. Winterhagen, et al., Time and temperature dependent changes of ADP- and collagen-induced and “spontaneous” aggregation, *Thromb Res* **19** (1980), 621–638.

[11] K. Breddin, In-vitro-Methoden zur Beurteilung der Plättchenfunktion, *Blut* **18** (1968), 84–89.

[12] K. C. Dee, D. A. Puleo, and R. Bizios, Chapter 3. Protein-Surface Interactions, in: *An Introduction To Tissue-Biomaterial Interactions*, John Wiley & Sons, Inc., New York, USA, 2002

[13] K.M. Evans-Nguyen, L.R. Tolles, O. V Gorkun, S.T. Lord, and M.H. Schoenfisch, Interactions of thrombin with fibrinogen adsorbed on methyl-, hydroxyl-, amine-, and carboxyl-terminated self-assembled monolayers, *Biochemistry* **44** (2005), 15561–8.

[14] E.J. Favaloro, Clinical utility of the PFA-100, *Semin Thromb Hemost* **34** (2008), 709–33.

[15] R.D. Frank, H. Dresbach, H. Thelen, and H.-G. Sieberth, Glutardialdehyde induced fluorescence technique (GIFT): A new method for the imaging of platelet adhesion on biomaterials, *J Biomed Mater Res* **52** (2000), 374–381.

[16] C. Frere, T. Cuisset, J. Quilici, L. Camoin, J. Carvajal, P.E. Morange, M. Lambert, et al., ADP-induced platelet aggregation and platelet reactivity index VASP are good predictive markers for clinical outcomes in non-ST elevation acute coronary syndrome, *Thromb Haemost* **98** (2007), 838–43.

[17] G. de Gaetano, C. Cerletti, E. Dejana, and R. Latini, Pharmacology of platelet inhibition in humans: implications of the salicylate-aspirin interaction, *Circulation* **72** (1985), 1185–93.

[18] M. Gawaz, F. Neumann, and A. Schomig, Evaluation of platelet membrane glycoproteins in coronary artery disease: consequences for diagnosis and therapy, *Circulation* **99** (1999), E1–E11.

[19] M.B. Gorbet and M. V Sefton, Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes, *Biomaterials* **25** (2004), 5681–703.

[20] S. Gordz, C. Mrowietz, G. Pindur, J.W. Park, and F. Jung, Effect of desmopressin (DDAVP) on platelet membrane glycoprotein expression in patients with von Willebrand's disease, *Clin Hemorheol Microcirc* **32** (2005), 83–7.

[21] J. Graff, U. Klinkhardt, V.B. Schini-Kerth, S. Harder, N. Franz, S. Bassus, and C.M. Kirchmaier, Close relationship between the platelet activation marker CD62 and the granular release of platelet-derived growth factor, *J Pharmacol Exp Ther* **300** (2002), 952–7.

[22] K.H. Grotemeyer, The platelet-reactivity-test--a useful "by-product" of the blood-sampling procedure?, *Thrombosis research* **61** (1991), 423–31.

[23] P. Harrison, I. Mackie, A. Mumford, C. Briggs, R. Liesner, M. Winter, and S. Machin, Guidelines for the laboratory investigation of heritable disorders of platelet function, *Br J Haematol* **155** (2011), 30–44.

[24] S. Hsu-Lin, C.L. Berman, B.C. Furie, D. August, and B. Furie, A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets, *J Biol Chem* **259** (1984), 9121–6.

[25] S. Jo and K. Park, Surface modification using silanated poly(ethylene glycol)s, *Biomaterials* **21** (2000), 605–16.

[26] F. Jung, R. Bach, C. Mrowietz, U. Seyfert, and R.P. Franke, Haemocompatibility of endovascular coronary stents: Wiktor GX., *Biomed Tech (Berl)* **46** (2001), 200–6.

[27] F. Jung, C. Mrowietz, U.T. Seyfert, R. Grewe, and R.P. Franke, Influence of the direct NO-donor SIN-1 on the interaction between platelets and stainless steel stents under dynamic conditions, *Clin Hemorheol Microcirc* **28** (2003), 189–99.

[28] F. Jung, S. Braune, and A. Lendlein, Haemocompatibility testing of biomaterials using human platelets, *Clin Hemorheol Microcirc* **53** (2013), 97–115.

[29] F. Jung, C. Wischke, and A. Lendlein, Degradable, Multifunctional Cardiovascular Implants: Challenges and Hurdles, *MRS Bull* **35** (2010), 607–613.

[30] R.K. Kainthan and D.E. Brooks, In vivo biological evaluation of high molecular weight hyperbranched polyglycerols, *Biomaterials* **28** (2007), 4779–87.

[31] R.K. Kainthan, S.R. Hester, E. Levin, D. V Devine, and D.E. Brooks, In vitro biological evaluation of high molecular weight hyperbranched polyglycerols, *Biomaterials* **28** (2007), 4581–90.

[32] P. Kingshott, H. Thissen, and H.J. Griesser, Effects of cloud-point grafting, chain length, and density of PEG layers on competitive adsorption of ocular proteins, *Biomaterials* **23** (2002), 2043–56.

[33] M. Lange, S. Braune, K. Luetzow, K. Richau, N. Scharnagl, M. Weinhart, A.T. Neffe, et al., Surface functionalization of poly(ether imide) membranes with linear, methylated oligoglycerols for reducing thrombogenicity, *Macromol Rapid Commun* **33** (2012), 1487–92.

[34] S. Li and J.J.D. Henry, Nonthrombogenic approaches to cardiovascular bioengineering, *Annu Rev Biomed Eng* **13** (2011), 451–75.

[35] J.N. Lindon, G. McManama, L. Kushner, E.W. Merrill, and E.W. Salzman, Does the conformation of adsorbed fibrinogen dictate platelet interactions with artificial surfaces?, *Blood* **68** (1986), 355–62.

[36] P. Menu, J.F. Stoltz, and H. Kerdjoudj, Progress in vascular graft substitute, *Clin Hemorheol Microcirc* **53** (2013), 117–29.

[37] S. Nagata and M.M. Glovsky, Activation of human serum complement with allergens. I. Generation of C3a, C4a, and C5a and induction of human neutrophil aggregation, *J Allergy Clin Immunol* **80** (1987), 24–32.

[38] H.K. Nieuwenhuis, J.J. van Oosterhout, E. Rozemuller, F. van Iwaarden, and J.J. Sixma, Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation, *Blood* **70** (1987), 838–45.

[39] S. Niewiarowski and E. Rogoeczi, Platelet interaction with polymerizing fibrin, *J Clin Invest* **51** (1972), 685–700.

[40] B. Nilsson, K.N. Ekdahl, T.E. Mollnes, and J.D. Lambris, The role of complement in biomaterial-induced inflammation, *Mol Immunol* **44** (2007), 82–94.

[41] A.T. Nurden, Platelets, inflammation and tissue regeneration, *Thromb Haemost* **105 Suppl** (2011), S13–33.

[42] J. Papp, B. Sandor, Z. Vamos, D. Botor, A. Toth, M. Rabai, P. Kenyeres, et al., Antiplatelet effect of acetylsalicylic acid, metamizole and their combination - in vitro and in vivo comparisons, *Clin Hemorheol Microcirc* (2012), DOI 10.3233/CH-2012-1636.

[43] J. Papp, P. Kenyeres, and K. Toth, Clinical importance of antiplatelet drugs in cardiovascular diseases, *Clin Hemorheol Microcirc* **53** (2013), 81–96.

[44] D. Poli, E. Antonucci, G. Ciuti, R. Abbate, and D. Prisco, Combination of D-dimer, F1+2 and residual vein obstruction as predictors of VTE recurrence in patients with first VTE episode after OAT withdrawal, *J Thromb Haemost* **6** (2008), 708–10.

[45] W.H. Reinhart, Platelets in vascular disease, *Clin Hemorheol Microcirc* **53** (2013), 71–9.

[46] A.J. Reininger, Coagulation activity of platelets, *Hämostaseologie* **27** (2007), 247–50.

[47] D. Ricklin, G. Hajishengallis, K. Yang, and J.D. Lambris, Complement: a key system for immune surveillance and homeostasis, *Nat Immunol* **11** (2010), 785–97.

[48] C.S. Rinder, H.M. Rinder, B.R. Smith, J.C. Fitch, M.J. Smith, J.B. Tracey, L.A. Matis, et al., Blockade of C5a and C5b-9 generation inhibits leukocyte and platelet activation during extracorporeal circulation, *J Clin Invest* **96** (1995), 1564–72.

[49] T. Roch, J. Cui, K. Kratz, A. Lendlein, and F. Jung, Immuno-compatibility of soft hydrophobic poly (n-butyl acrylate) networks with elastic moduli for regeneration of functional tissues, *Clin Hemorheol Microcirc* **50** (2012), 131–42.

[50] A. Ruf and H. Patscheke, Flow cytometric detection of activated platelets: comparison of determining shape change, fibrinogen binding, and P-selectin expression, *Semin Thromb Hemost* **21** (1995), 146–51.

[51] H. Schmid-Schonbein, H. Rieger, and T. Fischer, Blood Fluidity as a Consequence of Red Cell Fluidity: Flow Properties of Blood and Flow Behavior of Blood in Vascular Diseases, *Angiology* **31** (1980), 301–319.

[52] S.J. Shattil, M. Cunningham, and J.A. Hoxie, Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry, *Blood* **70** (1987), 307–15.

[53] S.J. Shattil, J.A. Hoxie, M. Cunningham, and L.F. Brass, Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation, *J Biol Chem* **260** (1985), 11107–14.

[54] E. Shiba, J.N. Lindon, L. Kushner, G.R. Matsueda, J. Hawiger, M. Kloczewiak, B. Kudryk, et al., Antibody-detectable changes in fibrinogen adsorption affecting platelet activation on polymer surfaces, *Am J Physiol* **260** (1991), C965–74.

[55] B. Sivaraman and R. Latour, The adherence of platelets to adsorbed albumin by receptor-mediated recognition of binding sites exposed by adsorption-induced unfolding, *Biomaterials* **31** (2010), 1036–44.

[56] B. Sivaraman and R. Latour, The relationship between platelet adhesion on surfaces and the structure versus the amount of adsorbed fibrinogen, *Biomaterials* **31** (2010), 832–9.

[57] C. Sperling, M. Fischer, M.F. Maitz, and C. Werner, Biomaterials Blood coagulation on biomaterials requires the combination of distinct activation processes, *Biomaterials* **30** (2009), 4447–4456.

[58] S. Sun, Y. Yue, X. Huang, and D. Meng, Protein adsorption on blood-contact membranes, *J Memb Sci* **222** (2003), 3–18.

[59] P. Thevenot, W. Hu, and L. Tang, Surface chemistry influences implant biocompatibility, *Curr Top Med Chem* **8** (2008), 270–80.

[60] W.-B. Tsai, J.M. Grunkemeier, and T. Horbett, Variations in the ability of adsorbed fibrinogen to mediate platelet adhesion to polystyrene-based materials: a multivariate statistical analysis of antibody binding to the platelet binding sites of fibrinogen, *Biomed Mater Res A* **67** (2003), 1255–68.

[61] N. Vene, A. Mavri, K. Kosmelj, and M. Stegnar, High D-dimer levels predict cardiovascular events in patients with chronic atrial fibrillation during oral anticoagulant therapy, *Thromb Haemost* **90** (2003), 1163–72.

[62] L. Vroman, The life of an artificial device in contact with blood: initial events and their effect on its final state, *Bull N Y Acad Med* **64** (1988), 352–7.

[63] M. Weinhart, I. Grunwald, M. Wyszogrodzka, L. Gaetjen, A. Hartwig, and R. Haag, Linear poly(methyl glycerol) and linear polyglycerol as potent protein and cell resistant alternatives to poly(ethylene glycol), *Chem Asian J* **5** (2010), 1992–2000.

[64] World Medical Association, Declaration of Helsinki: ethical principles for medical research involving human subjects, *J Int Bioethique* **15** (2004), 124–9.

[65] K.K. Wu and J.C. Hoak, A new method for the quantitative determination of platelet aggregates in patients with arterial insufficiency, *The Lancet* **304** (1974), 924–926.

[66] M. Yaseen, X. Zhao, A. Freund, A.M. Seifalian, and J.R. Lu, Surface structural conformations of fibrinogen polypeptides for improved biocompatibility, *Biomaterials* **31** (2010), 3781–92.

[67] P.M. van der Zee, E. Biró, Y. Ko, R.J. de Winter, C.E. Hack, A. Sturk, and R. Nieuwland, P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction, *Clin chem* **52** (2006), 657–64.