Final Draft
of the original manuscript:

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In: Clinical Hemorheology and Microcirculation.
First published online by IOS Press: 14.12.2020

https://dx.doi.org/10.3233/CH-201029

The influence of different rewetting procedures on the thrombogenicity of nanoporous poly(ether imide) microparticles

S. Braune[a], J. Bäckemo[a], S. Lau[a], M. Heuchel[a], K. Kratz[a], F. Jung[a*], M. Reinthaler[a,c], A. Lendlein[a,b*]

a: Institute of Biomaterial Science and Berlin-Brandenburg Center for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Teltow, Germany

b: Institute of Chemistry, University of Potsdam, Potsdam, Germany

c: Department for Cardiology, Charité Universitätsmedizin, Berlin, Germany

Corresponding authors: Friedrich Jung and Andreas Lendlein, E-Mail: Andreas.lendlein@hzg.de

Keywords: biomaterial, polymer, microparticle, thrombogenicity, hemocompatibility, dynamic *in vitro* test, rewetting

Abstract

Nanoporous microparticles prepared from poly(ether imide) (PEI) are discussed as candidate adsorber materials for the removal of uremic toxins during apheresis. Polymers exhibiting such porosity can induce the formation of micro-gas/air pockets when exposed to fluids. Such air presenting material surfaces are reported to induce platelet activation and thrombus formation. Physical or chemical treatments prior to implantation are discussed to reduce the formation of such gas nuclei. Here, we report about the influence of different rewetting procedures – as chemical treatments with solvents – on the thrombogenicity of hydrophobic PEI microparticles and PEI microparticles hydrophilized by covalent attachment of poly(vinyl pyrrolidone) (PVP) of two different chain lengths.

Autoclaved dry PEI particles of all types with a diameter range of 200 – 250 µm and a porosity of about 84% ± 2% were either rewetted directly with phosphate buffered saline (24 h) or after immersion in an ethanol-series. Thrombogenicity of the particles was studied *in vitro* upon contact with human sodium citrated whole blood for 60 min at 5 rpm vertical rotation. Numbers of non-adherent platelets were quantified, and adhesion of blood cells was qualitatively analyzed by bright field microscopy. Platelet activation (percentage of CD62P positive platelets and amounts of soluble P-Selectin) and platelet function (PFA100 closure times) were analysed.

Retention of blood platelets on the particles was similar for all particle types and both rewetting procedures. Non-adherent platelets were less activated after contact with ethanol-treated particles of all types compared to those rewetted with phosphate buffered saline as assessed by a reduced number of CD62P-positive platelets and reduced amounts of secreted P-Selectin ($P<0.05$ each). Interestingly, the hydrophilic surfaces significantly increased the number of activated platelets compared to hydrophobic PEI regardless of the rewetting agent. This suggests that, apart from wettability, other material properties might be more important to regulate platelet activation. PFA100 closure times were reduced and within the reference ranges in the ethanol group, however, significantly increased in the saline group. No substantial difference was detected between the tested surface modifications. In summary, rewetting with ethanol resulted in a reduced thrombogenicity of all studied microparticles regardless of their wettability, most likely resulting from the evacuation of air from the nanoporous particles.

Introduction

A major drawback of current hemodialysis treatments are remaining uremic toxins, such as phenylacetic acid, indoxyl sulfate and *p*-cresyl sulfate, in the blood of chronic renal failure patients[1]. In a previous work, we have introduced poly(ether imide) microparticles (PEI particles) as candidate adsorber materials for the removal of such uremic toxins [2]. The particle surface is nanoporous with a pore size distribution of 350 nm – 500 nm and interconnected with larger inner pores. This allows an exchange of toxins from blood through the outer nano-size pores into the particle lumen, while blood cells can only interact with the surface. The particles adsorbed four tested toxins (phenylacetic acid, p-cresylsulfate, hydroxy hipuric acid and indoxyl sulfate) and revealed highest affinities for phenylacetic acid and p-cresylsulfate [3]. In principle, poly(ether imide) polymers are non-toxic, show adequate biocompatibility and can be excellently processed in a variety of forms, such as membranes, films and particles[3]. With regard to the adsorption of toxins from blood, one has to consider the hydrophobic character of PEI surfaces. This may result in an unwanted adsorption of blood proteins such as fibrinogen blocking the pore openings. Poly (vinyl pyrrolidone) (PVP) has attracted attention as surface modificator for preventing this adsorption effect by hydrophilization of the polymer surface [4-7]. However, the kind of processing can influence bulk and surface properties substantially, and thus, the biological performance [8,9]. Beyond the chemical composition of the polymer bulk and surface, also physical properties can influence the interactions with blood components and cells [10,11]. For example, high shear rates can activate platelets. This can occur when the flow around and between the microparticles becomes turbulent, which can occur above all for high perfusion pressure [12,13]. Surface roughness is another key characteristic, which can influence the performance of blood contacting devices via e.g. plasma protein adsorption and platelet adhesion [14,15]. Particularly hydrophobic porous polymers were shown to mediate the formation of gas pockets, even in lowest micrometer ranges [16,17]. Within these pockets, the gas bubbles are stable and are not displaced by the flowing blood [18]. Presented at the blood-material interphase, the trapped gas can induce severe thrombogenicity [19,20]. Madras et al. and others have shown that the removal of gas nuclei from the blood-material interface can reduce protein adsorption and platelet adhesion on gas permeable polymers and particularly vascular grafts [19,21-24]. Different processing steps have been suggested, which allow the dislodgement of air from porous implant materials. Physical techniques include ultrasound, vacuum or hydrostatic pressure. As chemical treatments, immersions in saline, acetone or ethanol, were successfully applied – partially in combination with above mentioned physical techniques [18,23,25]. For the denucleation of expanded poly(tetrafluoroethylene) (PTFE) arterial vascular grafts, ethanol treatments were reported to be more efficient (~94%) compared to those with saline solution (~87%) [18].

In the present work, we studied the influence of two rewetting agents on the thrombogenic potential of nanoporous poly(ether imide) microparticles (PEI) and PEI particles surface-modified with PVP to render them hydrophilic. We hypothesized that the different wetting properties of phosphate buffered saline and ethanol as well as hydrophilic surface modifications of PEI particles do not lead to different wetting transitions and replacement of trapped air with the liquid. This *in vitro* study majorly focused on the interaction of human platelets from fresh whole blood with the rewetted particles.

Materials and methods

Preparation of poly(ether imide) (PEI) microparticles

The PEI microparticles were prepared from ultra-purified Ultem®1000 (SABIC Deutschland GmbH, Düsseldorf, Germany) by an improved spraying/coagulation process, as published earlier [3,26]. Mean particle diameters were about 226 µm ± 14 µm with a relatively narrow size distribution (Figure 1, Table 1). The accessible porosity was 84% ± 2% [27] and particle surfaces revealed pore sizes of around 110 nm ± 40 nm (determined from SEM pictures of particle surfaces). These external pores were connected to the internal and larger core pores (pore size distribution: 350 nm – 500 nm) [28,29]. Dynamic water-air contact angle measurements revealed hydrophobic surface properties with θ_{adv} = 100 ° ± 6 ° [29].

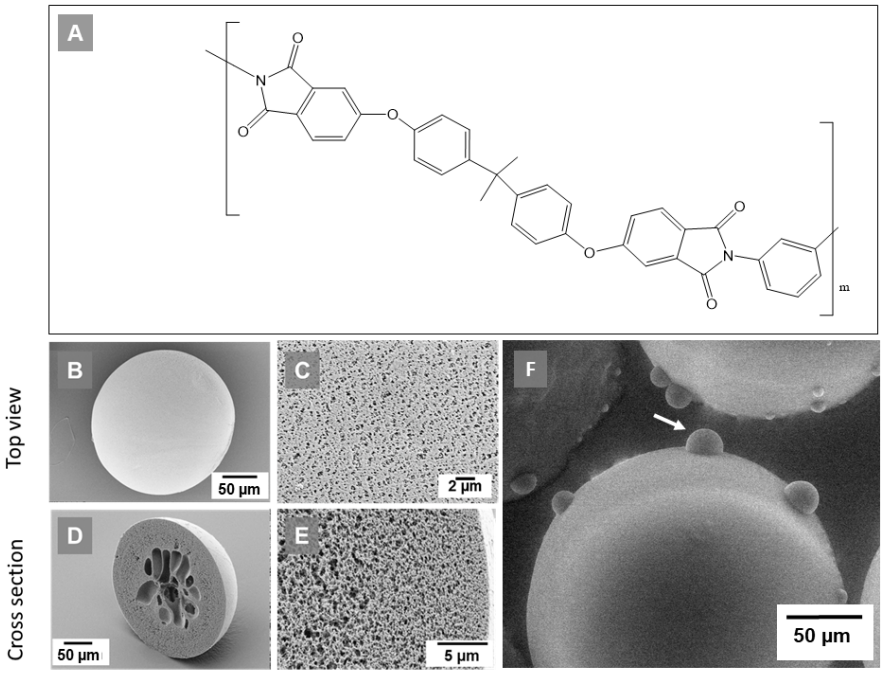

Figure 1. Representative scanning electron images of poly(ether imide) (PEI) microparticles. (A) Structural formula of the PEI polymer. (B) and (D) show overview images in the top-view (B) and cross-section-view (D); (C) and (E) show detail images of the nanoporous particle surfaces and the

interconnected inner pores, respectively. (F) shows an environmental scanning electron microscopic (ESEM) image. Such images were used to determine water-wettability on the particle surfaces. The arrow indicates the water droplet on the particle surface illustrating the hydrophobicity of PEI particles.

Surface modification of PEI microparticles

The hydrophilic surface modification of PEI microparticles with synthesized amino-terminated poly (vinyl pyrrolidone) (PVP-NH_2) was described in detail in a recent publication [29]. First, the modifier PVP-NH_2 was synthesized by free radical polymerization of vinyl pyrrolidone (VP) under nitrogen atmosphere in 1,4-dioxane in the presence of 2,2´-azobis-(2-methyl-butyronitril) (AIBN) for 2 h at 70 °C. As chain transferring and terminating agent, 2-aminoethanthiol (cysteamin) was added, and the mixture was stirred for another 1.5 h. The reaction product, named PVP-NH_2, was obtained with 92% yield after precipitating the reaction mixture in cooled diethyl ether. Gel-Permeation Chromatography (GPC) resulted in PVP-NH_2 chains with an average molecular weight of M_n = 5,400 g·mol^{-1} and a polydispersity index of PDI = 2.8. To obtain shorter PVP-NH_2 chains in a narrower molecular weight range from the same amounts of starting material, the reaction procedure was changed. At room temperature, AIBN and cysteamin were immediately added to VP in dioxane. This mixture was heated to 60 °C and stirred at this temperature for 24 h. Instead of precipitating, the product mixture was filled in two dialysis tubes with a small molecular weight cut off of 2,000 g·mol^{-1} (Spectra Por 6 Dialysemembran MWCO 2kD, Spectrum Laboratories, Carl Roth, Karlsruhe, Germany). To remove monomers, short oligomers, and the solvents, the tubes were rinsed one after the other for 3 h, 22 h, and 24 h in three different water/ethanol baths (vol. ratios of 60%/40%, 80%/20%, and 100%/0%). The remaining product was cooled in liquid nitrogen, freeze dried, and stored in vacuum for two days. According to this altered experimental procedure, significantly shorter PVP-NH_2 chains with M_n = 2,900 g·mol^{-1} and PDI = 1.4 were obtained in a lower yield of 43%.

In the actual surface modification step, PEI particles were stirred in an aqueous solution (4 wt%) of the synthesized PVP-NH_2 for 30 min at 90 °C. NH_2-endgroups of the amino terminated PVP modifier opens imide rings of the PEI macromolecules (Figure 1A) by creation of an amide bond. After washing and drying, the modified PEI-PVP particles (see Figure 6A) were obtained. The unmodified “PEI” and modified “PEI-PVP” particles of Ref. [29] will be used here for the hemocompatibility experiments under the abbreviations “PEI” and “PEI-PVP_5k”. The similarly prepared surface modified PEI particles with the shorter PVP-NH_2 chains of M_n = 2,900 g·mol^{-1} are also included in the study under the name “PEI-PVP_3k”. Basic geometric properties of all three different particle types, such as particle diameter and size of pore openings on the particle surface, are presented in Table 1. It can be seen that the surface modification has not changed the particle size. In contrast, the surface hydrophilicity was increased as indicated by the lower (mean) advanced water contact angles θ_{adv} of 75° (PEI-PVP_5k)

and 79° (PEI-PVP_3k) compared to 100° for unmodified PEI. The contact angles were determined in environmental scanning electron microscopy (ESEM) experiments. A detailed description of all characterization experiments can be found in Ref. [29].

Table 1. Geometric properties of (modified) PEI particles and microwetting behavior determined by ESEM experiments.

Sample ID[a]	Modifier		Mean particle diameter[d]	Mean diameter of pore openings[e]	Contact angles
	name[b]	M_n ($g \cdot mol^{-1}$)	(µm)	(µm)	θ_{adv}[f] (°)
PEI	-	-	226 ± 14	112 ± 67	100 ± 6
PEI-PVP_3k	PVP-NH_2	2,900[c]	220 ± 8	102 ± 46	79 ± 5
PEI-PVP_5k	PVP-NH_2	5,400[c]	227 ± 11	110 ± 40	75 ± 5

[a] Sample ID of (modified) PEI particles
[b] Short cut name of the amino-terminated oligomeric modifier
[c] Number averaged molecular weight *M*n determined by gel permeation chromatography (GPC)
[d] Mean particle diameter of particles determined from raster electron microscopy (REM) images
[e] Mean diameter of pore openings on the particle surface determined from REM images
[f] Advanced wetting (θ_{adv}) contact angles for water determined from environmental scanning electron microscopy (ESEM) images

The prepared PEI microparticles were steam sterilized. The rewetting process was carried out in sterile syringes (Injekt, B.Braun Melsungen AG, Melsungen, Germany) and under vertical rotation (5 rpm). Two different rewetting protocols were applied. (1) Treatment with phosphate buffered saline (without calcium and magnesium, ThermoFisher Scientific, Life Technologies, Paisley, UK) for 24 hours at 37 °C (PEI/PBS) and (2) with ethanol (PEI/EtOH). The second protocol included different steps. Initially, samples were immersed for 1 hour (37 °C) in 50% ethanol solution (100% ultra-pure ethanol diluted in water for injection purposes). In the second step particles were immersed in a 100% ethanol solution. The solution was exchanged after 24 hours and particles were allowed to equilibrate for 24 hours in saline solution. Prior to the incubation with fresh human whole blood, phosphate buffered saline was exchanged with fresh solution and equilibrated for another hour.

In vitro study design

This study was designed according to the guidelines of the International Society on Thrombosis and Haemostasis and the British Committee for Standards in Haematology [30,31]. The study protocol was approved by the ethics committee of the Charité University Medicine Berlin (EA2-018-16). Included

blood donors were apparently healthy (according to Nordkem-Workshop) and had not taken any medication that could influence platelet function (at least ten days) [32]. Candidates with lipid metabolism disorder, hypertension and diabetes mellitus were excluded. Blood was withdrawn (cubital vein) following an atraumatic protocol and collected in 0.106 $mol \cdot L^{-1}$ tri-sodium citrate or EDTA 1.6 $mg \cdot mL^{-1}$, respectively (S-Monovettes, Sarstedt, Nümbrecht, Germany). Collection containers were agitated during the blood collection to ensure instant mixing of blood and anticoagulant solution. The initial 4 mL of collected whole blood were discarded. The same applied to samples that showed unexpected clotting. Age, sex, size, weight and body mass index were collected as demographic data of the donors. Further donor characterization included blood pressure and heart rate as well as whole blood cell count, haematocrit, platelet indices and C-reactive protein levels (EDTA-anticoagulated blood). Function of platelets was tested by flow cytometry (anti human CD42a/CD62P antibody positive events) and the platelet function analyser (PFA-100, Siemens Healthcare Diagnostics, Marburg, Germany) [33,34]. Test subjects were included in the study in case test results were within reference ranges for apparently healthy humans. The blood was allowed to rest for 15 minutes after donation before it was used in the experiment.

Hemocompatibility testing of particles

For the hemocompatibility test, the equilibration solution was exchanged with fresh human whole blood. Particles were contacted with blood for 1 hour at 37 °C under continuous vertical rotation (5 rpm). The particles were separated from the blood afterwards using nylon meshes (Falcon Cell strainer, 40 µm, Corning Incorporated, New York, USA). Cells adherent on the particle surfaces were fixed with glutardialdehyde (2%, 30 minutes, room temperature) and analysed with a bright field microscope (Axio-Imager.Z2 m, ZEISS, Zeiss, Jena, Germany)[35]. Non adherent blood cells were quantified by complete blood cell counts (Sysmex XS-800i, SYSMEX Deutschland, Norderstedt, Germany). For flow cytometry measurements (MACSQuant® analyzer, Miltenyi Biotec, Bergisch Gladbach, Germany), the whole blood was fixed with Thrombofix platelet stabilizer (Beckman Coulter, Marseille, France). Anti-human-CD42a (FITC-conjugated, Becton Dickinson Bioscience, San José, USA) and anti-human-CD62P (PE-conjugated, Immunotech, Beckman Coulter, Marseille, France) antibodies were used for labelling platelet membrane glycoproteins GPIb/IX (identification marker) and P-Selectin (activation marker), respectively[33]. Determination of platelet function with the PFA-100 was performed using COLLAGEN/ADP-cartridges (Siemens Healthcare, Erlangen, Germany). Blood samples were centrifuged at 1,500 g for 20 minutes to obtain cell free plasma. Plasma samples were utilized for measurements of soluble P-Selectin and tested within a maximum of 6-month storage at -80 °C. Plasma P-Selectin concentrations were determined by an enzyme-linked immunosorbent assay (R&D systems, Wiesbaden-Nordenstadt Germany).

Statistics

For all samples, arithmetic means and standard deviations are presented. Normal distribution of the data was tested using the d'Agostino & Pearson omnibus normality test. Differences between rewetted poly(ether imide) particles and control were analysed by repeated measure one-way ANOVA with Geisser-Greenhouse correction. The Tukey test was performed as posttest for multiple pairwise comparisons. P-values < 0.05 were considered significant. For all data analyses, GraphPad Prism version 6.00 was used (GraphPad Software, La Jolla, California, USA).

Results and discussion

In the present study, the influence of two rewetting procedures on the thrombogenicity of nanoporous, hydrophobic PEI particles and nanoporous, hydrophilic PEI-PVP particles was analyzed. While the PBS-treated particles were solely rewetted with phosphate buffered saline, EtOH-treated samples were treated with ethanol prior to washing with the saline solution. In a first step of the study, the effect of PBS and ethanol was tested on hydrophobic PEI particles. The choice of solvent had an influence on the rewetting of the microparticles. After the saline treatment, microparticles were solely floating at the water-air interface (Figure 2A). This indicates that air was still trapped in the particle pores and the weight of the polymer bulk was not sufficient to let the particles sink to the bottom of the vial. The opposite was observed for the particles first rewetted with the ethanol protocol. These samples were obviously heavier and appeared at the bottom of the vial (Figure 2B). The sinking of the particles most likely is a consequence of the evacuation of the trapped air and its replacement with the rewetting liquid. Capillarity is majorly involved in the penetration of fluids into porous structures [36]. On hydrophobic surfaces - such as the tested PEI particles - capillary rise is little when the polymers are immersed in water or saline solutions. The organic solvent ethanol reduces surface tension between the liquid and the air phase, which results in a high capillary rise. This very likely led to a displacement of the trapped air and to an infiltration of the rewetting liquid into the pores. Bensen et al. have shown that immersion in organic solvents is superior in its efficiency to denucleate air from expanded PTFE grafts compared to pressure or vacuum treatments [18]. These findings are very well in line with our observations.

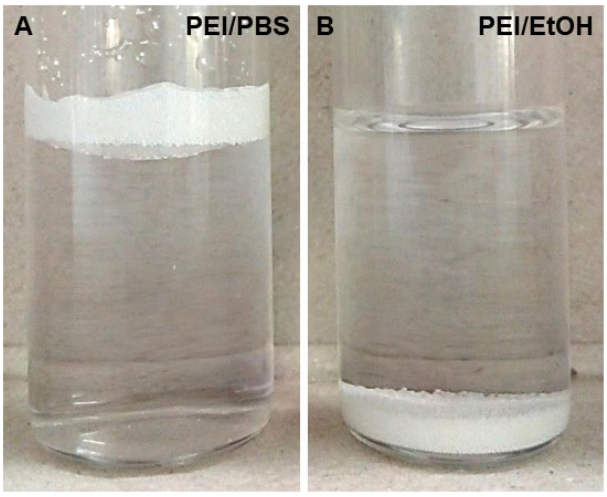

Figure 2. Images of the poly(ether imide) microparticles rewetted according to (A) the phosphate buffered saline and (B) the ethanol protocol. The pictures show the particles after the rewetting procedure, stored in phosphate buffered saline. While particles in (A) are floating at the liquid-air interface, particles in (B) are submerged in the solution.

In previous studies, it was further shown that the evacuation of air resulted in an improved hemocompatibility of porous implants, e.g. of vascular grafts [23,37]. In principle, this is also supported by the data of the present study. Though adherence of erythrocytes and platelets was comparable between the differently rewetted PEI particles (Figure 3, 4A and 4B), the data clearly revealed a reduced influence of the ethanol treated samples on platelet function/activation. For instance, mean platelet volumes were significantly smaller after contact with PEI/PBS (9.36 fL ± 0.23 fL) than with PEI/EtOH samples (9.71 fL ± 0.40 fL) (Figure 4C). Likewise, after the saline solution treatment, mean platelet volumes were also lower compared to the control blood (9.61 fL ± 0.58 fL), however, not in a statistically significant manner. This indicates an activation of the platelets after contact with PBS-treated PEI particles and formation of platelet aggregates. Activated platelets and aggregates were either adherent on the particle surface or circulating (see microscopic data in Figure 3). As shown in other studies, strong activation and aggregation is associated with the formation of smaller platelet fragments (macro- and microvesicles) [38,39]. The circulating larger aggregates are not detected as platelets by the applied hematolyzer, due to the size-restricted measurement window (12 fL – 40 fL, for the Sysmex XS-800 device). An increased detection of relatively small platelets and platelet fragments was the consequence and thus, a decrease of the parameter [40]. Values between the control blood and the ethanol rewetted samples did not differ, emphasizing that the ethanol procedure did not lead to an activation of the circulating platelets.

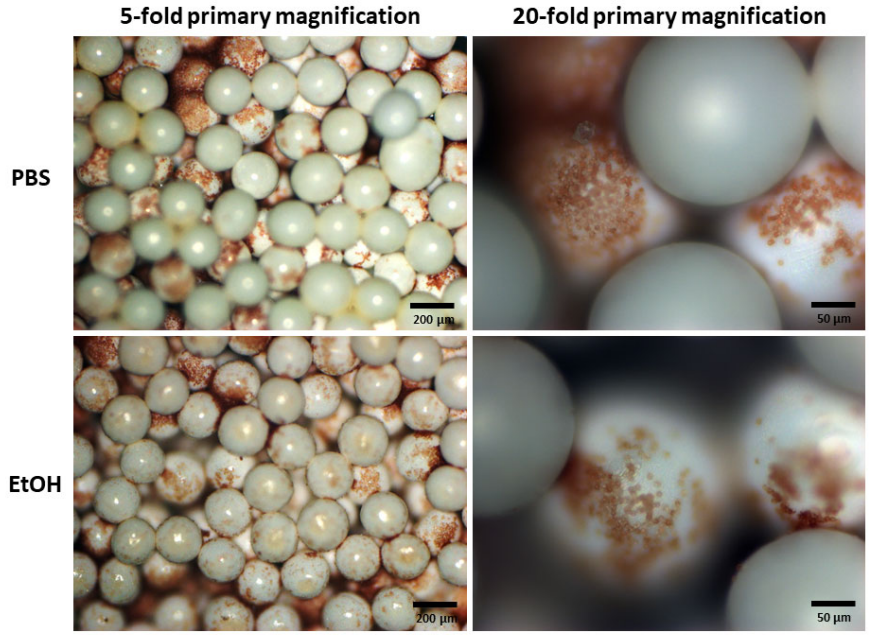

Figure 3. Adherent blood cells. Representative bright-field-microscopy images of the microparticles after 1 hour of incubation with fresh human whole blood. The upper row represents samples rewetted with phosphate buffered saline (PBS) and the lower row represents those rewetted with ethanol prior to storage in PBS in 5-fold and 20-fold primary magnification.

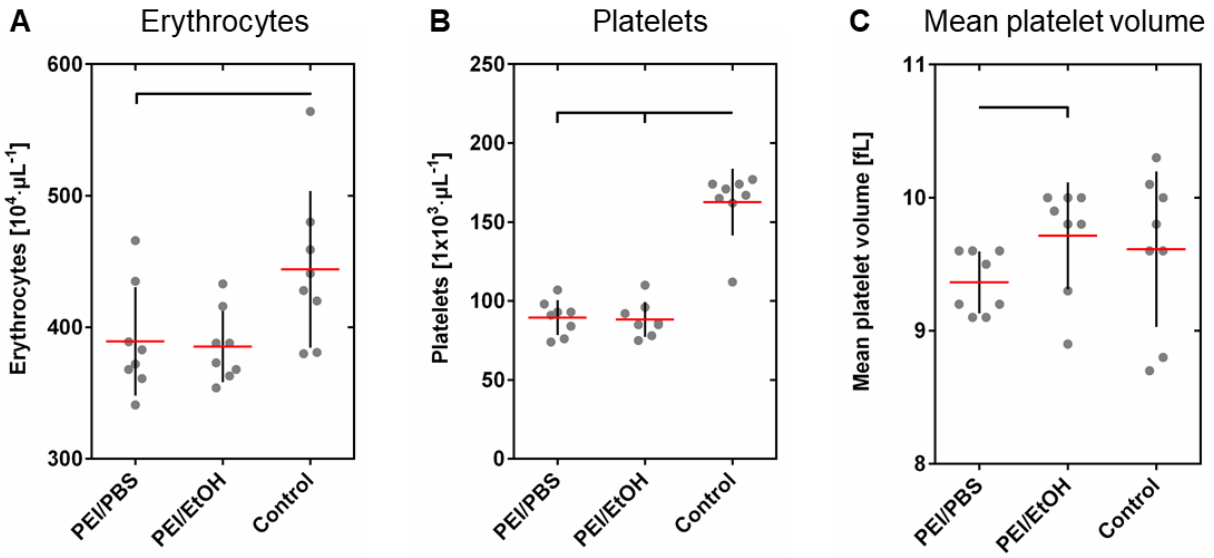

Figure 4. Non adherent blood cells. Numbers of (A) erythrocytes and (B) platelets in whole blood sampled after 1 hour of incubation with the poly(ether imide) microparticles. (C) shows data on platelet volume as morphometrical indicator of platelet activation. Data are shown as single data points, mean (horizontal red bar) ± standard deviation (vertical black bar) with n=4 donors. For each donor, measurements were performed in duplicate. Horizontal black bars indicate $P<0.05$ of an ANOVA analysis.

Similarly, analysis concerning platelet activation in whole blood revealed substantial differences between the two rewetting procedures (Figure 5A and 5B). The saline treated samples showed more than two times higher numbers of CD62P-positive/activated platelets compared to the ethanol treated ones and almost four times higher numbers of CD62P-positive platelets compared to the control (CD42 and P-Selectin positive events: PEI/PBS = 46.0% ± 4.4%; PEI/EtOH = 19.5% ± 5.6%; Control = 12.2% ± 2.1%). For the activation-induced soluble P-Selectin secretion, values for PEI/PBS were about six-times higher compared to ethanol-treated samples and four-times higher compared to the control (sP-Selectin: PEI/PBS = 195 ng·mL^{-1} ± 13 ng·mL^{-1}; PEI/EtOH = 32 ng·mL^{-1} ± 9 ng·mL^{-1}; Control = 47 ng·mL^{-1} ± 40 ng·mL^{-1}). Thus, the difference of PBS-treated particles to the control was much larger than with ethanol-treated samples. In particular, flow cytometry data revealed only slightly but significantly higher P-Selectin values for the ethanol treated microparticles compared to the control blood.

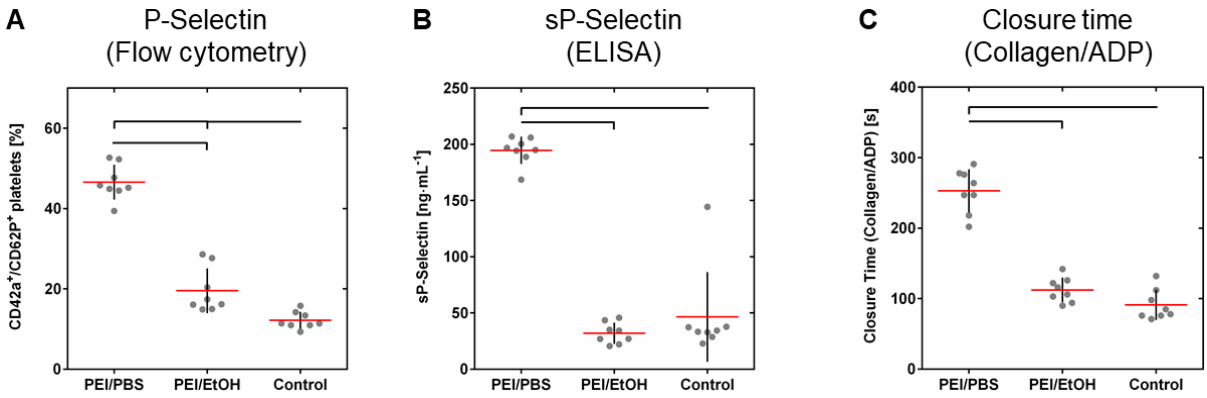

Figure 5. Platelet activation and function. Data on the (A) expression (flow cytometry data) and (B) secretion (ELISA data) of platelet P-Selectin (CD62P) after 1 hour of incubation with the poly(ether imide) microparticles. (C) shows data on closure times subsequent to stimulation of the whole blood samples in the COLLAGEN/ADP cartridge of the Platelet Function Analyser (PFA100). Data are shown as single data points, mean (horizontal red bar) ± standard deviation (vertical black bar) with n=4 donors. For each donor, measurements were performed in duplicate. Horizontal black bars indicate $P<0.05$ of an ANOVA analysis.

However, soluble P-Selectin values did not differ between these two samples. This indicates that rewetting with ethanol hardly activated platelets. The same applied for the closure times (Collagen/ADP cartridge) determined by the PFA100 (Figure 5C). In good agreement to the previous two parameters, the closure times differed substantially between the saline and ethanol treatments. While the closure time after contact to ethanol-treated PEI particles was comparable to the control (PEI/EtOH = 112 s ± 18 s; Control = 91 s ± 22 s), the closure time after contact with PBS-treated particles was significantly higher (PEI/PBS = 253 s ± 31 s) indicating an impaired platelet function. Thus, these data revealed that the rewetting procedure with ethanol highly reduced the thrombogenicity of the hydrophobic PEI particles compared to the treatment with phosphate buffered saline. Considering the evacuation of trapped gas nuclei by this treatment, the remaining thrombogenic potential is very likely

a direct consequence of the chemical and physical properties of the surface. Here, particularly roughness and the hydrophobic character of the PEI particle surface might play a critical role. Thus, in a second step of the study, PEI particles 'modified with PVP to render them hydrophilic' were analysed regarding their thrombogenicity after rewetting with either PBS or ethanol. As described in the Materials and Methods section, unmodified PEI and surface modified PEI-PVP particles have practically the same geometrical shape (see Table 1) but differ in their surface hydrophilicity. For unmodified PEI, small water droplets were formed on the particle surface in the microwetting experiment as detected by ESEM (see Figure 1F). In contrast, for modified PEI-PVP particles, water bridges were formed between the particles (see Figure 6B and 6C) as a result of the higher hydrophilicity and the reduced advanced contact angles by 20° and 25°, respectively (see Table 1). From a chemical perspective, differences between PEI-PVP_3k and PEI-PVP_5k concerning the surface chemistry are small. The covalently attached PVP chains have the same methyl end group. In PEI-PVP_3k, the chains of M_n = 2,900 g·mol^{-1} are roughly only half as long as in PEI-PVP_5k (M_n = 5,400 g·mol^{-1}). As a consequence, one could expect a larger influence of the formed amide (-CO-NH-) groups on the surface of PEI-PVP_3k. For PEI-PVP_5k, the larger PVP chain is likely to stronger shield the amide group.

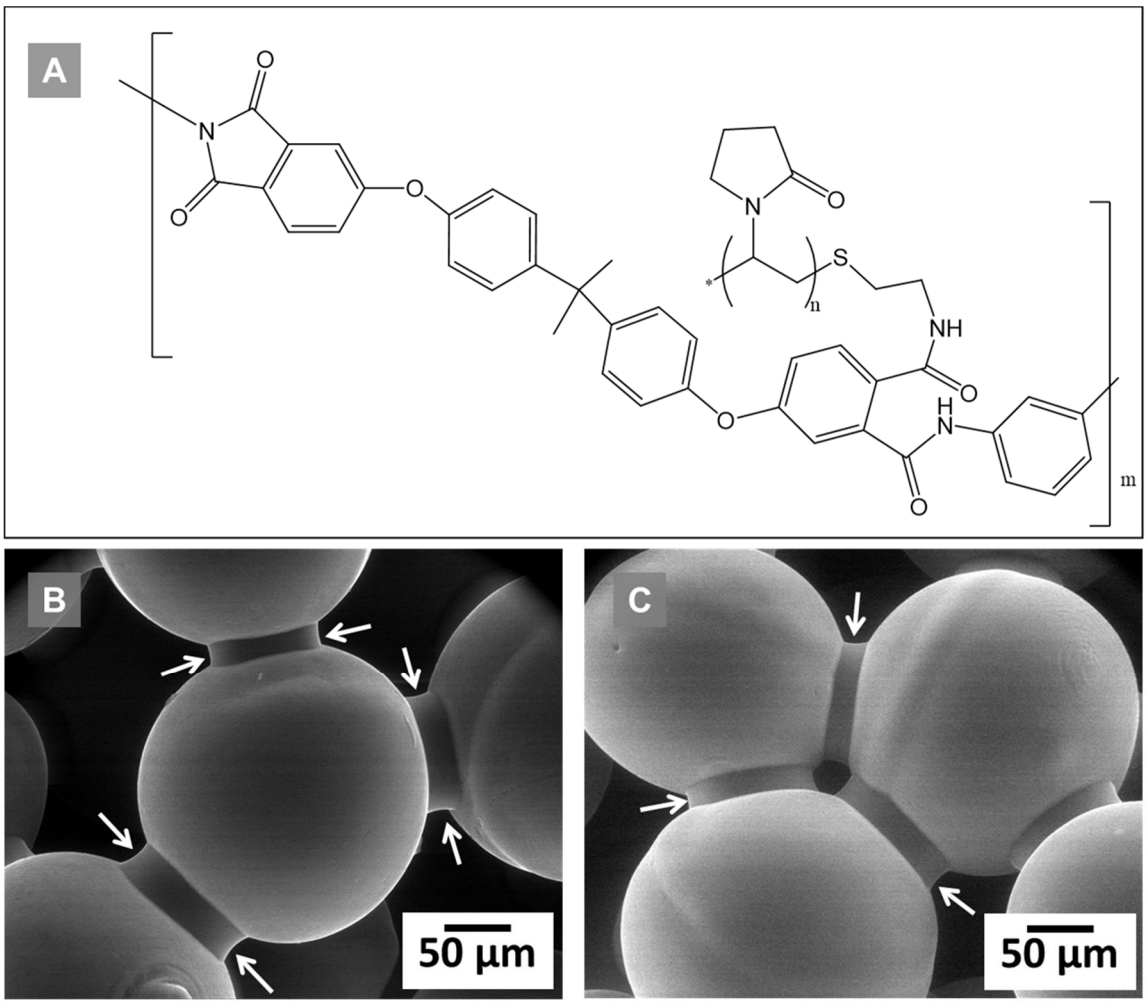

Figure 6. Hydrophilic modification of poly(ether imide) (PEI) microparticles with poly(vinyl pyrrolidone) (PVP). (A) Structural formula of the PEI-PVP polymers and environmental scanning electron microscopy images visualizing condensed water bridges in microwetting experiments for the two surface modified PEI microparticles: (B) PEI-PVP_3k, and (C) PEI-PVP_5k. The magnification was 500-fold. The formation of water bridges (indicated by white arrows) stands in contrast to the water droplets formed on unmodified PEI particles (see Figure 1F).

The adherence of erythrocytes to modified particles was similar for both rewetting procedures and comparable to unmodified particles as shown in Figure 3. Likewise, adhesion of platelets was similar for unmodified and modified particles treated with both PBS and ethanol (Figure 7A). In terms of ethanol treated particles, the number of platelets was significantly lower after contact with PEI-PVP_5k compared to PEI indicating that more platelets adhered to PEI-PVP_5k (PEI-PVP_5k = $65 \cdot 10^3 \cdot \mu L^{-1} \pm 10 \cdot 10^3 \cdot \mu L^{-1}$; PEI = $88 \cdot 10^3 \cdot \mu L^{-1} \pm 11 \cdot 10^3 \cdot \mu L^{-1}$). However this difference was small and most likely not biologically relevant. Platelet activation was assessed by the expression of the surface molecule P-Selectin (CD62P) and soluble P-Selectin secreted in the environment by activated platelets. Numbers of CD62P-expressing platelets were significantly higher for all types of PEI particles compared to the control regardless of the rewetting procedure (Figure 7B). A direct comparison of the rewetting agents revealed that PBS induced a stronger platelet activation than ethanol (PBS = 46.6% ± 4.4% to 53.4% ± 4.3%; EtOH = 19.5% ± 5.6% to 33.5% ± 5.1%). Interestingly, the hydrophilic particles PEI-PVP_3k and PEI-PVP_5k further increased the number of CD62P-positive platelets compared to unmodified PEI particles regardless of the rewetting agent. Soluble P-Selectin was secreted in significantly larger amounts by all tested particles treated with PBS (195 $ng \cdot mL^{-1}$ ± 12 $ng \cdot mL^{-1}$ to 287 $ng \cdot mL^{-1}$ ± 125 $ng \cdot mL^{-1}$) compared to the control (46 $ng \cdot mL^{-1}$ ± 40 $ng \cdot mL^{-1}$). However, in this case, the hydrophilic modifications did not further support platelet activation compared to hydrophobic particles. With regard to ethanol-treated particles, no influence of any particle type was detected compared to the control (Figure 7C). Thus, it can be speculated that during platelet activation, the expression of CD62P on the platelet surface comes prior to the secretion of soluble P-Selectin. Incubation times exceeding one hour might also induce an increase in soluble P-Selectin after contact with particles rewetted with ethanol. Platelet function was evaluated by the time needed to close a hole in a membrane coated with the platelet activators collagen and ADP (closure time; Figure 7D). In agreement with the aforementioned parameters, PBS-rewetted particles of all types showed significantly increased closure times (260 s ± 27 s to 264 s ± 30 s) compared to the control (90 s ± 23 s). In contrast, closure times obtained with EtOH-rewetted particles were similar to the control except for PEI-PVP_3k, which showed a slightly, but significantly increased closure time (149 s ± 46 s) compared to the control (91 s ± 21 s). Taken together, it becomes obvious that the rewetting procedure with ethanol is mainly responsible for the reduced thrombogenicity of the tested particles while the hydrophilic modifications do not play a role. The hydrophobic character of materials was intensely discussed to influence blood

plasma protein adsorption and conformational changes on metallic and polymer-based implant surfaces [41,42]. Particularly, hydrophobic polymeric materials are considered to induce exposure of cryptic epitopes, which are recognized by platelet receptors (e.g. GPIb and GPIIbIIIa) and can induce adhesion as well as activation [43-46]. Nevertheless, hydrophilic modification of PEI membranes by molecules such as oligoglycerols and polyglycerols was shown to be hardly effective in terms of reducing thrombogenicity. While adsorption of bovine serum albumin was reduced compared to hydrophobic PEI membranes, the amount of fibrinogen remained the same. Likewise, the number of adherent platelets was similar on hydrophobic und hydrophilic PEI membranes indicating that the tested glycerols did not reduce thrombogenicity [47,48]. In the present study, hydrophilic modification of particles with poly(vinyl pyrrolidone) did also not contribute to a reduced thrombogenicity. In contrast, data on the expression of CD62P show that hydrophilic particles led to an increased platelet activation compared to hydrophobic PEI particles (Figure 7B). Surface modification with PVP to influence thrombogenicity led to contradicting results in other studies. While early investigations by Kamath et al. showed that hydrophilization of dimethyldichlorosilane-treated glass with PVP did not prevent platelet adhesion [49], a current study by Kuzminska et al. proves that PVP hydrogel coating of polyurethane scaffolds reduced both fibrinogen adsorption and platelet adhesion [50]. These contradictory findings suggest that other material properties, e.g. porosity, might be more important for rendering materials less thrombogenic. Earlier studies revealed a stronger thrombogenic potential for porous PEI membranes compared to smooth films, which were processed from the same polymer batch and showed similar water-air contact angles [8,33]. Thus, surface roughness might play a crucial role for the observed platelet adhesion. Also, covalent binding of bioactive substances such as heparin was previously applied e.g. for improving the hemocompatibility of polysulfone adsorber membranes for selective removal of low-density lipoproteins [51,52]. Regional application of sodium citrate instead of systemic anticoagulation with heparin may be another strategy to improve the survival time of blood filtration devices, as shown e.g. for critically ill patients suffering from acute renal failure [53,54]. Altogether, the data of the present study clearly depicted that the local adhesion to ethanol-rewetted microparticles did not induce a systemic platelet activation in whole blood samples.

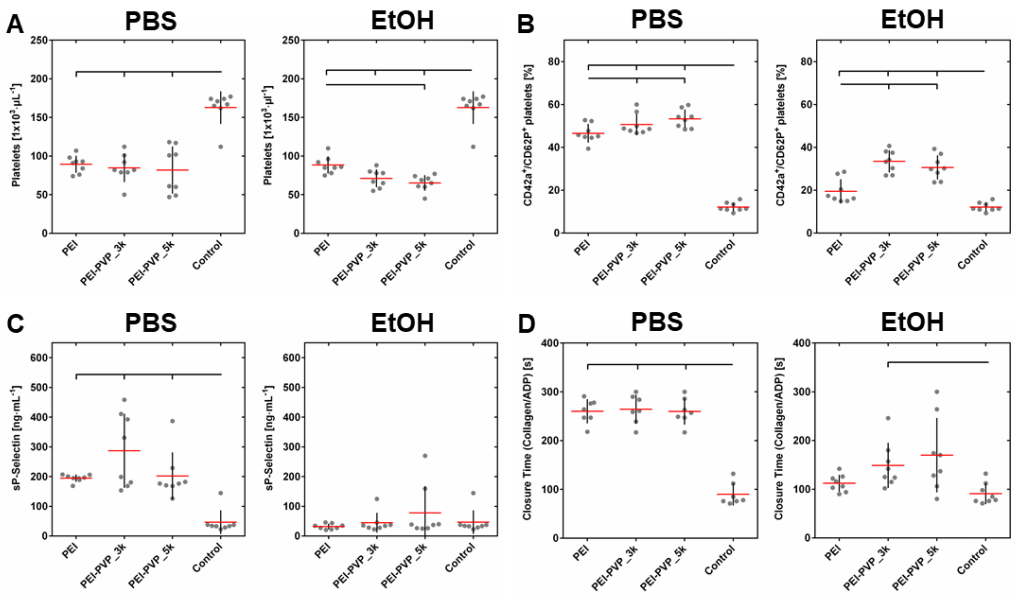

Figure 7. Adherence, activation and function of platelets. Poly(ether imide) microparticles (PEI) and hydrophilically modified particles with poly(vinyl pyrrolidone) of two different chain lengths (PEI-PVP_3k and PEI-PVP_5k) were either rewetted with phosphate buffered saline or ethanol prior to incubation for 1 hour in fresh human whole blood. After incubation, (A) the number of adherent platelets, (B) the expression of the activation marker P-Selectin/CD62P, (C) the secretion of soluble P-Selectin and (D) the closure time were analysed. Data are shown as single data points, mean (horizontal red bar) ± standard deviation (vertical black bar) with n=4 donors. For each donor, measurements were performed in duplicate. Horizontal black bars indicate $P<0.05$ of an ANOVA analysis.

Summary and conclusion

Quantitative data for the retention of platelets and erythrocytes on the PEI particle surfaces of all types were comparable for both particle treatment groups. However, circulating platelets were substantially less activated in the ethanol treated group compared to the PBS group. All platelet activation and function parameters were comparable between the ethanol group and the resting control platelets while rewetting with PBS only led to platelet activation and compromised platelet function. Hydrophobic modifications of PEI particles with PVP did not further reduce the thrombogenicity. Thus, it can be concluded that a rewetting procedure with ethanol - but not with phosphate buffered saline alone- reduces the thrombogenic potential of hydrophobic poly(ether imide) microparticles. This phenomenon is most likely a consequence of a reduced gas pocket formation at the nanoporous surface of the particles.

Acknowledgments

The authors acknowledge technical support by Mr. Mario Rettschlag, Mrs. Daniela Radzik, and Mrs. Yvonne Pieper. This work was financially supported by the Helmholtz Association through programme-oriented funding. Indo-German Science and Technology Centre (Grant No. IGST/NPORE/SDT/2012) and German Federal Ministry for Education and Research (BMBF) (Grant No. 01DQ13006B) are acknowledged for financial support.

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