The optimal incubation time for *in vitro* hemocompatibility testing: Assessment using polymer reference materials under pulsatile flow with physiological wall shear stress conditions

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Abstract: During hemocompatibility testing, activation products may reach plateau values which can result in less distinction between hemocompatible and hemo-incompatible materials. Of concern is an underestimation of the blood activation caused by the biomaterial of interest, which may result in a false assessment of hemocompatibility. To elucidate the optimal incubation time for *in vitro* hemocompatibility testing, we used the Haemobile circulation model with human whole blood. Blood from healthy volunteers was *in vitro* incubated under pulsatile flow with physiological wall shear stress conditions at 37°C for 30, 60, 120, or 240 min. Test loops containing low-density polyethylene and polydimethylsiloxane served as low and high reference materials, that is, hemocompatible and hemo-incompatible biomaterials, respectively. In addition, empty loops served as a negative reference. Thrombogenicity, platelet function, inflammatory response, coagulation, and hemolysis between references and incubation times were compared. We found that thrombogenicity and platelet function were significantly affected by both the duration of incubation and the type of material. In particular, thrombogenicity and platelet function assessments were affected by incubation time. We found that an exposure time of 60 min was sufficient, and for almost all variables an optimal incubation time to discriminate between the low and high reference material. © 2019 The Authors. *journal Of Biomedical Materials Research Part B: Applied Biomaterials* Published By Wiley Periodicals, Inc. J Biomed Mater Res B Part B:2335–2342, 2019.

Key Words: biomaterials, blood–material interaction, cardiovascular device, hemocompatibility, hemocompatibility testing

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INTRODUCTION

The lack of standardized methods for hemocompatibility testing constitutes a major problem for the preclinical evaluation of blood contacting medical devices. Direct contact of biomaterials with blood results in the activation of platelets, white blood cells, the coagulation cascade, the complement system, and can lead to hemolysis.[1,2] Activation of these biological systems can occur throughout the entire cardiovascular system and can result in the formation of thrombi and (micro)embolisms which in turn can lead to life-threatening conditions.

The current ISO 10993-Part 4 standard for testing hemocompatibility states that "blood or plasma should first be exposed to the material or device under standardized conditions including time, temperature, and flow rate"; however, appropriate exposure times are not elaborated.[3] Literature shows that blood exposure times during hemocompatibility testing vary from 15 min to 5 h and is contradictory about the optimum exposure time.[1,4–7]

Biomaterials can result in rapid blood activation, adsorption of proteins, and the activation of platelets. The activation of platelets can occur in <0.2 s, after which other platelets can be activated as well and start adhering.[2,8] The activation of the coagulation cascade is somewhat slower and can take up to several minutes. Material-mediated or mechanically mediated hemolysis take longer exposure times to allow relevant results. This is clearly described by Henkelman et al., who reported that medical steel induced 188% more hemolysis after 24 h of incubation when compared to 4 h of incubation. Furthermore, they showed that 24 h of incubation resulted in better distinction between their negative and positive controls

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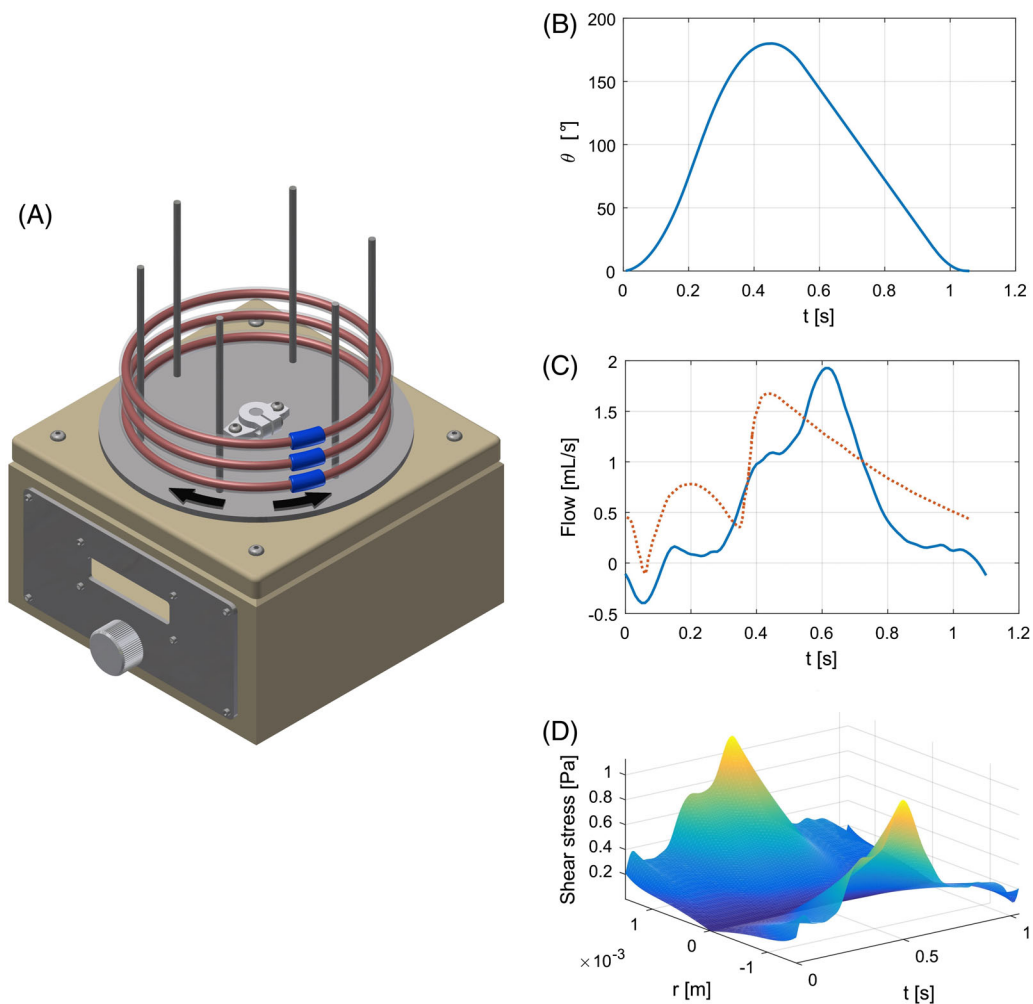

FIGURE 1. (a) Three-dimensional model of the Haemobile. The black arrows indicate the back and forth movement of the round plateau carrying the stacked test loops. The test loops have a radius of 72.5 mm. The blue components represent the unidirectional check valves. (b) The angle of the round plateau in time, with the Haemobile programmed to the following settings: angle of rotation = 180°; clockwise angular velocity = 720°/s; anticlockwise angular velocity = 360°/s; angular acceleration/deceleration = 3600°/s² (fixed setting). (c) Ensemble average of the measured flow (solid blue line) and typical coronary blood flow (dashed red line). (d) Calculated shear stress across the internal diameter of the test loop in time.

as compared to 4 h of incubation.[9] To obtain an overall impression of hemocompatibility, the exposure time for testing must be chosen so that activation of the different biological systems can be measured and that reference materials can be discriminated from each other.

To elucidate the optimal incubation time for *in vitro* hemocompatibility testing by means of the Haemobile model,[10] we analyzed thrombogenicity, platelet function, inflammatory response, coagulation, and hemolysis of human whole blood which had been incubated *in vitro* under pulsatile flow with physiological wall shear stress conditions at 37°C for 30, 60, 120, or 240 min. Low-density polyethylene (LDPE) and polydimethylsiloxane (PDMS) are well known reference materials used during hemocompatibility testing[2,11,12] and, in our study, served as low and high activating reference materials, that is, hemocompatible and hemo-incompatible biomaterials, respectively. In addition, empty test loops served as a negative reference and represented the background activation of the model. Incubation times with the greatest distinction between low and high references were identified.

MATERIALS AND METHODS
Blood collection
Fresh human blood was gently collected in a syringe (Omnifix Solo, B. Braun, Melsungen, Germany) by venipuncture with a 19 G butterfly needle from nine apparently healthy adult volunteers (age range: 23–26, male/female: 4/5) who received no medication within 2 weeks prior to blood withdrawal. Anticoagulation was achieved with a clinical dose of heparin (1.5 IU/mL, Leo Pharmaceutical Products BV, Weesp, The Netherlands).

In vitro incubation
In vitro incubations were performed at 37°C using the Haemobile (HaemoScan BV, Groningen, The Netherlands)[10] with an angle of 180°, a clockwise angular velocity of 720°/s, an anticlockwise angular velocity of 360°/s, and no delay between motions (Figure 1). Test loops consisted of polyvinyl chloride tubing (180 Clear PVC Tubing USP class VI, Nalgene, New York, NY) with an internal diameter of 3 mm, were fitted with a hemocompatible unidirectional check valve (HaemoScan BV,

Groningen, The Netherlands), and contained ~3 mL of blood. These conditions resulted in a unidirectional and pulsatile flow with an average flow of 19.4 mL/min and an average wall shear stress of 0.50 Pa (5 dyne/cm^2). The average flow and (wall) shear stress was calculated based on Doppler measurements of fluid velocity.[10] The calculated values were close to typical average wall shear stresses observed in coronary arteries [0.68 Pa (6.8 dyne/cm^2)].[10,13]

LDPE flat sheets (Goodfellow Cambridge Ltd., Huntingdon, England) and PDMS flat sheets (RX Silicone, ERIKS BV, Alkmaar, The Netherlands) served as low and high references, respectively. Reference materials were cut into 5×0.3 cm pieces (3 cm^2) and cleaned by sonication in 70% ethanol for 15 min. After sonication, reference materials were air-dried and placed into the test loops, resulting in blood/surface ratios of ~1 mL/cm^2. In addition, empty loops were used as negative reference. In vitro incubations were performed in duplicate for exposure times of 30, 60, 120, or 240 min, resulting in 24 test loops per donor.

Heparinized whole blood was transferred from the syringe to the test loop by connecting the syringe to the hemocompatible unidirectional check valve and by gently filling the test loop and displacing all remaining air in the test loop. As described previously, storage of whole blood affects key determinants of hemocompatibility within 4 h of blood collection.[1,3,4,11] Therefore, the time between blood collection and initiation of in vitro incubations was kept to a minimum, never exceeding 30 min.

After incubation, reference materials were gently rinsed three times with 50 mM Tris-buffered saline, pH 7.6 using tweezers and dipping in three 100 mL beakers (to prevent washing away any of the adhered components), photographed, cut to pieces of 10×3 mm (60 mm^2), transferred to polystyrene tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and stored at 2°C–8°C until further use. Incubated blood was collected in polystyrene tubes (Greiner Bio-One GmbH) and immediately split in two aliquots, of which one aliquot was centrifuged at 79g for 5 min to obtain platelet-rich plasma (PRP) and one aliquot was anticoagulated with 10% (v/v) ethylenediaminetetraacetic acid (EDTA) to a final concentration of 5 mM (preventing any further blood activation).

Thrombogenicity

Thrombi adhered to LDPE and PDMS were visualized by means of macroscopy and scanning electron microscopy. For scanning electron microscopy, pieces of reference materials were fixated with 2% (v/v) glutaraldehyde/0.1 M cacodylate buffer and dehydrated with an ethanol series and solutions containing tetramethylsilane.[14] Materials were sputter-coated with gold/palladium (SC7620 Mini Sputter Coater, Quorum Technologies Ltd., UK) before visualization with a tabletop scanning electron microscope (Phenom-World BV, Eindhoven, The Netherlands).

In addition to the visualization of adhered thrombi, platelet adhesion was also quantified as an indicator for thrombogenicity. Platelet adhesion was quantified based on the presence of acid phosphatase[15] in platelet granules using exposure of ~1 cm^2 of material to a citrate buffer containing 4-nitrophenylphosphatase and Triton X-100 (acid phosphatase substrate). The optical density was measured before and after addition of sodium hydroxide

(PowerWave 200, Bio-Tek Instruments, Inc., Winooski, VT). Counted platelets in PRP (cell counter Medonic CA 530, Sweden) served as a standard curve, whereby the amount of adhered platelets could be determined.

Fibrin binding was quantified on 1 cm^2 of material by means of an enzyme immunoassay (EIA), based on a biotin-conjugated mouse anti fibrin bèta chain IgG antibody (American Diagnostica GmbH, Pfungstadt, Germany) followed by horseradish peroxidase (HRP)-conjugated streptavidin (Thermo Fisher Scientific Inc., Waltham, MA) and a citrate–phosphate buffer containing o-phenylenediamine and hydrogen peroxide (OPD substrate).

Platelet function

Cell count was performed (Medonic CA 530) on whole blood containing 5 mM EDTA, and counted cells were corrected for the EDTA dilution. The remaining EDTA containing whole blood was centrifuged at 13,400g for 1 min, and the platelet poor plasma (PPP) was stored at −80°C until further analysis.

In addition to quantifying platelet adhesion as an indicator for thrombogenicity, the remaining capacity of platelets to adhere to collagen was used to assess platelet function. The capacity of platelets to adhere to collagen was analyzed in collagen-coated microtiter plates as we described previously.[1] Briefly, platelet adhesion was achieved by incubating PRP in a collagen-coated microplate, unattached platelets were removed by washing with phosphate-buffered saline, pH 7.4, and adhered platelets were analyzed using acid phosphatase substrate similar to the quantification of platelet adhesion, and the percentage of adhered platelets was calculated.

P-selectin (CD62P), stored in α-granules of platelets, is rapidly expressed on the surface of activated platelets, functioning as an adhesion receptor for white blood cells to mediate adhesion to endothelial cells.[16] P-selectin can be used as an indicator for platelet activation. Surface-bound P-selectin expression was quantified by means of an EIA, based on a mouse anti-human P-selectin antibody (clone 9E1, R&D Systems, Inc., Minneapolis, MN) followed by an HRP-conjugated rabbit anti mouse IgG antibody (Sigma-Aldrich Co. LLC., St. Louis, MO) and OPD substrate.

In addition to expression of P-selectin on platelet surfaces, platelet activation leads to activation of the arachidonic acid synthesis pathway to produce thromboxane A2 (TXA2) which is highly unstable and rapidly converted to thromboxane B2 (TXB2).[1] Thromboxane B2 in PPP was analyzed by means of an EIA (Cayman Chemical Company, Ann Arbor, MI), based on the competition between labeled TXB2 and sample TXB2.

Inflammatory response, coagulation, and hemolysis

White blood cells were counted (Medonic CA 530) and elastase in PPP was analyzed as indicators for white blood cell activation. Elastase was measured by means of an enzyme-linked immunosorbent assay (ELISA) based on a capture antibody against human elastase and a labeled antibody against alpha 1 anti-trypsin (Affinity Biologicals Inc., Ancaster, Canada).

Complement complex C5b-9 in PPP was analyzed as an indicator for complement activation by means of ELISA based on a mouse anti human C5b-9 capture antibody (DAKO,

FIGURE 2. Macroscopic image of low-density polyethylene (LDPE) and polydimethylsiloxane (PDMS) flat sheets after *in vitro* incubation under pulsatile flow with physiological wall shear stress conditions with human whole blood at 37°C for 1 h. LDPE barely showing thrombus formation; PDMS clearly showing vast red-thrombus formation.

Glostrup, Denmark) and a goat anti C5 detection antibody (Quidel, San Diego, CA).

Thrombin–antithrombin III (TAT III) complex in PPP was analyzed to determine thrombin formation, as an indicator for coagulation activity. Thrombin–antithrombin III complex was analyzed by means of ELISA, using a capture antibody against human thrombin and a labeled antibody against antithrombin III (Cedarlane Laboratories Ltd., Hornby, Canada).

Free hemoglobin in PPP was used as an indicator for hemolysis and was measured as described by Harboe.[17] Percentage of hemolysis was determined by comparison with a 100% hemolysis sample.

Statistical analysis

Normally distributed variables were reported as mean + one standard deviation. Baseline concentrations of elastase and complement complex C5b-9 showed great interindividual differences. These variations were eliminated for elastase and complement complex C5b-9 by normalizing the data to a percentage of the baseline (% baseline). Paired samples *t* test was used for all parameters to assess any significant differences between the baseline and 30, 60, 120, or 240 min of incubation. Independent samples *t* test was used for all parameters to assess differences between reference materials. All tests performed to test the (null-) hypothesis of no difference were two-sided. A *p* value <0.05 was considered statistically significant. Statistical analyses were performed with SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL).
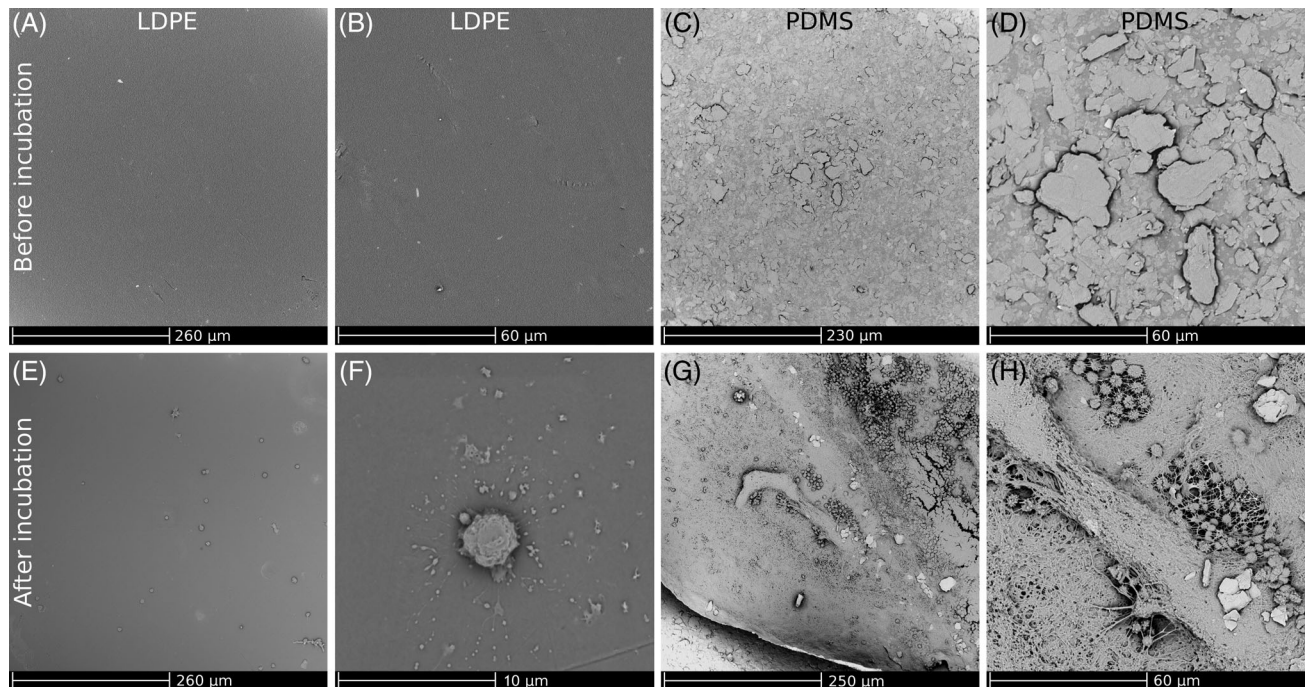

FIGURE 3. Scanning electron microscopy images of low-density polyethylene (LDPE) and polydimethylsiloxane (PDMS) surfaces before and after *in vitro* incubation under pulsatile flow with physiological wall shear stress conditions with human whole blood at 37°C for 1 h. (a, b) 375× and 1500× magnification of LDPE before incubation, respectively. A clean, smooth surface is visible. (c, d) 420× and 1500× magnification of PDMS before incubation, respectively. A clean, rough surface is visible. (e, f) 375× and 6000× magnification of LDPE after incubation, respectively. An almost clean surface, showing only few adhered platelets is visible. (g, h) 385× and 1500× magnification of PDMS after incubation, respectively. The bare polymer surface is almost completely covered by vast thrombus layers, mostly consisting of fibrin and red blood cells.
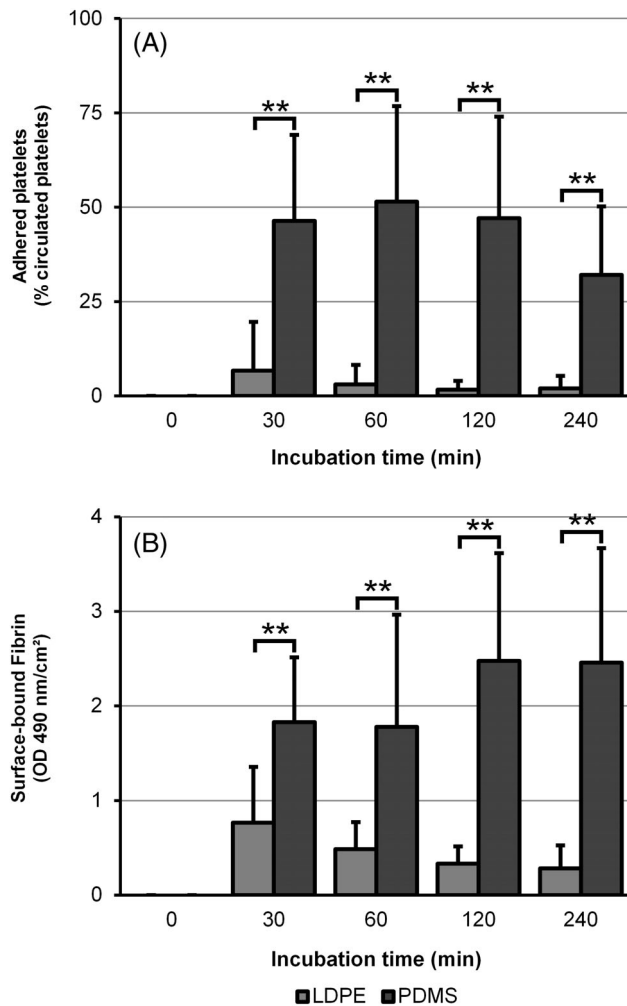
FIGURE 4. (a) Platelet adhesion and (b) fibrin binding on low-density polyethylene (LDPE) and polydimethylsiloxane (PDMS) after *in vitro* incubation under pulsatile flow with physiological wall shear stress conditions with human whole blood at 37°C for various incubation times. **$p < 0.05$ between indicated references, using an independent samples t test.

RESULTS

Thrombogenicity

Following incubation, visual inspection of the reference materials already revealed large differences, where LDPE barely showed thrombus formation and PDMS showed vast red-thrombus deposition (Figure 2). Electron microscopy of LDPE mainly revealed separate adhered platelets [Figure 3 (e,f)], while PDMS revealed vast thrombus deposition covering the entire surface. The thrombi clearly consisted of fibrin, red blood cells, and platelets [Figure 3(g,h)]. The extent of thrombus formation on LDPE and PDMS did not seem to change over time.

Platelet adhesion on LDPE reached a plateau value after 30 min, while on PDMS, it plateaued after 60 min [Figure 4(a)]. The largest difference in platelet adhesion between the reference materials was observed after 60 min ($p \leq 0.001$).

Fibrin binding on LDPE reached a plateau value after 30 min, while on PDMS, it plateaued after 120 min [Figure 4 (b)]. The largest difference in fibrin binding between the reference materials was observed after 240 min ($p \leq 0.001$).

Platelet function

Mean platelet volume did not change over time or after exposure to LDPE or PDMS. The number of circulating platelets decreased over time and the largest difference between the reference materials was observed after 60 min [Figure 5 (a), $p = 0.020$]. Platelet–collagen adhesion did not change after exposure to LDPE or PDMS and no clear changes were observed over time [Figure 5(b)].

During the measured timeframe (0–240 min), surface-bound P-selectin expression continued to increase after exposure to LDPE, while PDMS seemed to reach a plateau value after 60 min [Figure 5(c)]. The largest difference in surface-bound P-selectin expression between the reference materials was observed after 60 min ($p = 0.023$).

Thromboxane B2 release in plasma increased during incubation, and at most time points, PDMS resulted in significantly higher TXB2 concentrations than LDPE and/or the negative reference [Figure 5(d)]. The largest difference between LDPE and PDMS was observed after 60 min ($p = 0.012$).

Inflammatory response, coagulation, and hemolysis

The number of circulating white blood cells did not change over time or after exposure to LDPE or PDMS. Elastase release and complement complex C5b-9 formation in plasma increased over time [Figure 6(a,b)]. There were, however, no differences between the reference materials.

Similar to the inflammatory markers, TAT III complex formation in plasma increased over time. However, there was no difference between the reference materials [Figure 6(c)].

Free hemoglobin in plasma increased over time, and again there was no difference between the reference materials [Figure 6(d)].

DISCUSSION

The aim of this study was to elucidate the optimal incubation time for *in vitro* hemocompatibility testing by means of the Haemobile model. We found that an exposure time of 60 min was sufficient and for most variables an optimal incubation time to discriminate between the low and high reference materials. Another important observation was that the activation markers measured in plasma were not as effective in discriminating the reference materials from each other or from the negative reference as the surface-bound markers, which resulted in very clear differences.

Improper hemocompatibility assessments may result in unjustly use of blood contacting medical devices that can lead to extensive inflammation and hemolysis, or even a direct risk for life-threatening conditions as a result of the formation of blood clots, thrombi, and/or (micro)embolisms.[18] Although the thrombogenicity of medical devices may be attenuated by the administration of platelet inhibitors and/or anticoagulants, higher doses are needed for
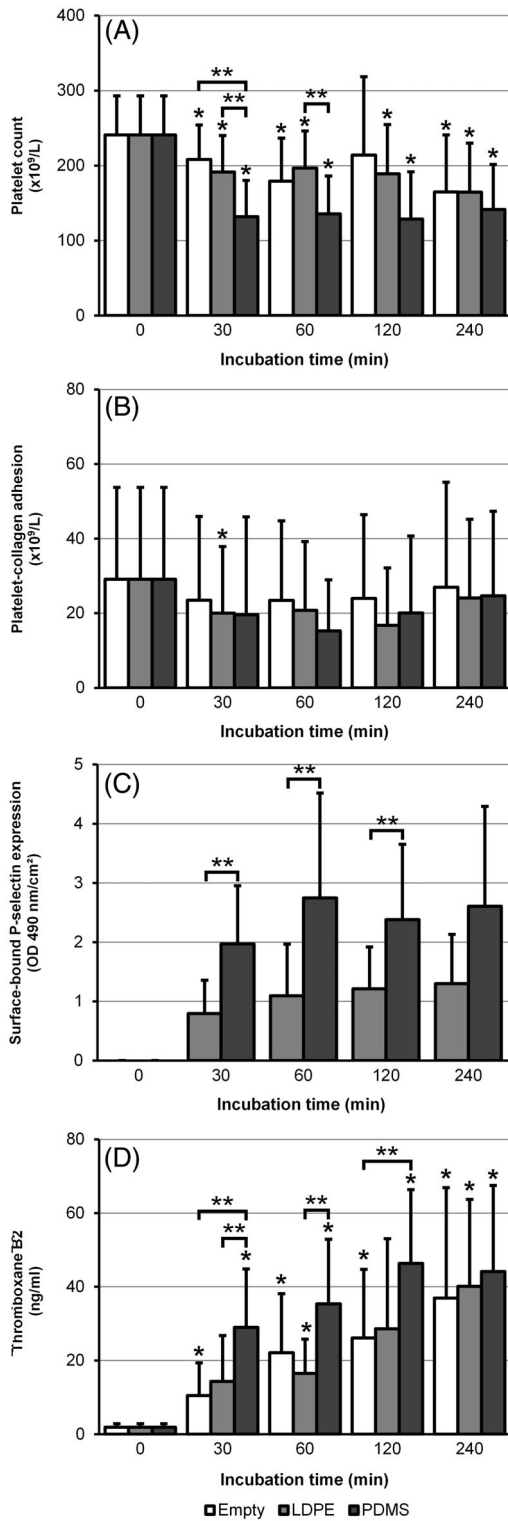
FIGURE 5. (a) Whole blood platelet count, (b) platelet adhesion to collagen, (c) surface-bound P-selectin expression, and (d) thromboxane B2 release in plasma after *in vitro* incubation under pulsatile flow with physiological wall shear stress conditions with human whole blood at 37°C for various incubation times using empty loops (Empty) as negative reference, low-density polyethylene (LDPE) as low reference, and polydimethylsiloxane (PDMS) as high reference. *$p < 0.05$ compared with the baseline (0 min), using a paired samples t test. **$p < 0.05$ between indicated references, using an independent samples t test.
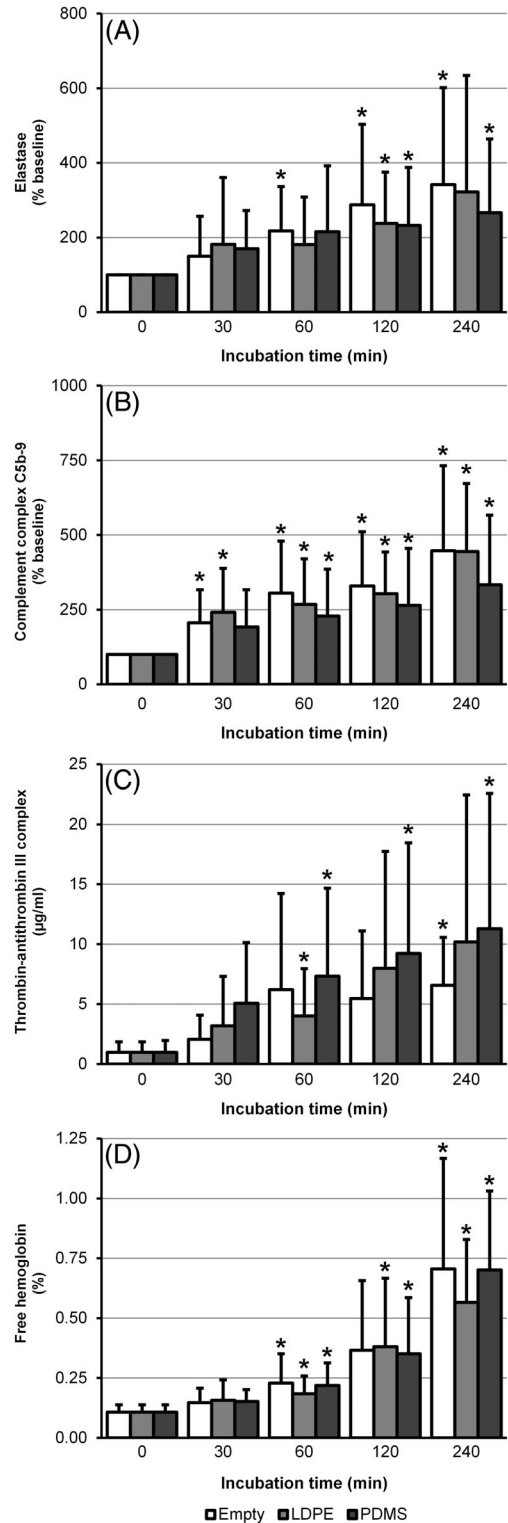

FIGURE 6. (a) Elastase release in plasma, (b) complement complex C5b-9 formation in plasma, (c) thrombin–antithrombin III complex formation in plasma, and (d) free hemoglobin in plasma after *in vitro* incubation under pulsatile flow with physiological wall shear stress conditions with human whole blood at 37°C for various incubation times using empty loops (Empty) as negative reference, low-density polyethylene (LDPE) as low reference, and polydimethylsiloxane (PDMS) as high reference. *$p < 0.05$ compared with the baseline (0 min), using a paired samples t test. **$p < 0.05$ between indicated references, using an independent samples t test.

hemo-incompatible medical devices as compared to more hemocompatible medical devices.[19]

The exposure time during *in vitro* testing has been debated for a long time. Some suggest that the incubation time should last for many hours, when implants or long-contact duration devices are involved. However, most reports show a benefit of shorter exposure times. Seyfert et al. already suggested that the exposure time of a biomaterial to blood should be between 15 and 240 min.[4] Braune et al. stated that the total test duration should not exceed 4 h to ensure an appropriate function of blood cells and blood plasma proteins.[11] Furthermore, Hiebl et al. concluded that if hemocompatibility tests are focused on the coagulation parameters prothrombin time and fibrinogen, a test period of 30 min is advantageous to a prolonged test period of 90 min.[7]

Our findings indicate that the optimal incubation time for *in vitro* hemocompatibility assessments is 60 min for quantification of platelet adhesion, counting of platelets in whole blood, quantification of surface-bound P-selectin expression, and measurement of TXB2 release in plasma, and 240 min for quantification of fibrin binding. As quantification of fibrin binding also resulted in very clear differences after 60 min of incubation, our findings advocate an exposure time of 60 min for *in vitro* hemocompatibility assessments when incubating under pulsatile flow with physiological wall shear stress conditions.

However, this study has several limitations. First, the results obtained with the Haemobile model may not be generalized to other models, as other models may incorporate an air bubble in the test loop, do not use pulsatile flow, or do not describe the shear stress profile at all. All *in vitro* models have activation due to the tubing of the test loop. Therefore, it is important to minimize that background noise by using a hemocompatible tubing, such as USP class VI medical grade PVC. Second, the optimal incubation time found in this study was based on hemocompatibility testing of polymers; by changing the characteristics of the test samples (e.g., when testing metals instead of polymers, or when testing polymers with different surface roughness), the optimal incubation time may be different due to the more hemo-incompatible nature of metals as compared to polymers. Third, we used test loops containing ~3 mL of blood and samples of 3 cm^2, resulting in blood/surface ratios of ~1 mL/cm^2; altering the volume-to-surface ratio can have an influence on the rate of formation of blood activation products, changing the time until these activation products are exhausted, possibly changing the distinction between different biomaterials. Fourth, the incubation time of this study was based on blood which was anticoagulated with a clinical dose of heparin (1.5 IU/mL); by administrating a different concentration of heparin or a different anticoagulant (e.g., citrate), the optimal incubation time may change due to the altered coagulation activity. And finally, only one pulsatile waveform shape and shear stress level was evaluated in this study. This means that for different pulsatile waveform shapes and corresponding shear stress

levels (these may vary within different arteries), the optimal exposure time may be different.

Although we followed the recommendations of the current ISO 10993-Part 4,[3] not all activation markers measured in plasma were influenced by the type of biomaterial after exposure to blood. This is in agreement with previous reports.[7,9,20,21] However, decreasing the blood/surface ratio (e.g., using greater surface areas per ml) may result in greater distinction between different biomaterials, and thus significant changes in plasma activation markers. For complement activation, coagulation activity, white blood cell activation, and hemolysis in plasma, no clear differences were observed between reference materials. Even more disturbing, complement complex C5b-9 in plasma after exposure to PDMS was lower when compared to LDPE and empty test loops. This suggests that complement complex C5b-9 was either bound by PDMS and/or that the formation of complement complex C5b-9 in plasma was inhibited. Hamad et al. have shown that certain complement factors (C1q, C4, C3, and C9) can bind to the surface of activated platelets.[22] This might explain that soluble complement complex C5b-9 in plasma exposed to PDMS was lower as compared to LDPE and empty test loops, as PDMS showed extensive adhesion of activated platelets.

CONCLUSION

This study was set out to determine the effect of exposure time on the distinction between low and high reference materials during hemocompatibility testing by means of the Haemobile model. We observed that an exposure time of 60 min was sufficient, and for most variables an optimal incubation time to discriminate between the low and high reference material. In particular, thrombogenicity determined by deposition of thrombotic components on the material surface and platelet function assessments were affected by incubation time. Especially for these variables, it is important to use the optimal incubation time. As our findings are in agreement with suggested incubation periods based on other models,[4,7,11] we endorse that *in vitro* incubations for hemocompatibility assessments which use circulation models with human whole blood at 37°C should last for 60 min.

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