Supplementary Information

Dynamic *in vitro* hemocompatibility of oligoproline self-assembled monolayer surfaces

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Materials and methods

1. Polyurethane control

Polyurethane (PU) was purchased from the DSM Biomedical (The Netherlands). Bionate 80A was chosen based on our previous experience with the standardization of hemocompatibility assays.[1, 2] The polymer was cut out as disks with a diameter of 14.4 mm and a thickness of 2 mm.

2. Preparation of oligoproline-based self-assembled monolayers (Pro-SAMs)

The SAMs immobilized with oligoprolines were deposited on gold (Au)-sputtered glass coverslips (diameter: 13 mm, thickness: 0.17–0.25 μm; Matsunami Glass Ind., Ltd., Osaka, Japan) via Au-thiol binding according to a previous procedure[3]. Briefly, oligoproline peptides, Ac-Cys-$(Pro)_6$-$CONH_2$ (Pro6) and Ac-Cys-$(Pro)_9$-$CONH_2$ (Pro9), were synthesized by the Fmoc solid phase procedure. Au-sputtered glass coverslips were immersed in aqueous solutions of Pro6 or Pro9 (10 mM) for 2 h. After rinsing with ultrapure water, the immobilized SAMs of Pro6 (Pro6-SAM) and Pro9 (Pro9-SAM) were dried *in vacuo* for subsequent experiments.

3. Material surface characterization

Atomic force microscopy measurements were performed on an Innova microscope (Bruker, Poland). Topography images were taken using the tapping mode with MLCT C silicon nitride tips (Bruker, Poland). All measurements were conducted at room temperature. Data processing and presentation were conducted by Nanoscope 1.40 analysis software.

A static water contact angle was measured by the sessile drop method with a contact angle Drop Shape Analyzer DSA 100 goniometer (Krüss, Germany) equipped with video capture. An image was taken within 5 s of the placement of the drop on the surface. Contact

angle measurements were analyzed by the circle fitting profile available with the imaging software. Six separate measurements were made on each surface at different locations.

4. Cone-and-plate hemocompatibility assay

4.1. Blood–surface interactions under dynamic conditions

All experiments used human blood were performed in accordance with the Guidelines of International Organization for Standardization (ISO) on testing medical materials that have contact with circulating blood (ISO 10933-4), and approved by the polish center for accreditation (certificate of accreditation of the research laboratory No. AB 120).

Blood–surface interactions were evaluated under shear stress conditions in the cone-and-plate analyzer (Impact-R; DiaMed AG, Switzerland), as shown in Figure S1. The human whole blood was obtained from Regional Donation Centre (Cracow, Poland) and contained sodium citrate anticoagulant. Prior to experiments, the control for platelet activation was prepared by blood activation with adenosine diphosphate (ADP, 20 μM final concentration) for 5 min. For each experiment, 130 μL of blood was used at a shear rate of 1800 s^{-1} for 300 s. After the test, the conical rotor was carefully removed, and blood cells were stained with proper antibodies and analyzed by flow cytometry. The blood that was sampled after the test was performed in the absence of the investigated materials (only the polystyrene testing vessel) served as the baseline control (BASE) for dynamic conditions. The tested materials, i.e., PU, Pro6-SAM, Pro9-SAM, and Au-coated coverslips, were prepared for the confocal laser scanning microscopy evaluation of surface coverage by morphotic blood elements.

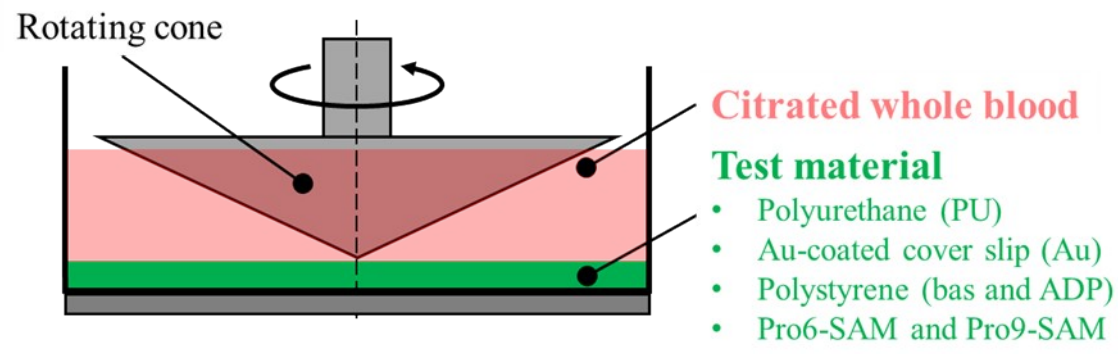

Figure S1. Schematic diagram of the cone-cylinder configuration with flow domain.

4.2. Observation of blood cells adhered to test materials with immunostaining

Samples after the test were rinsed with phosphate-buffered saline to remove all residual blood, fixed with 1% formalin, and stained with anti-CD62P-PE (platelets marker), anti-vWF-AlexaFluor555 (a marker of von Willebrand factor), and anti-CD45-FITC (leukocytes marker) monoclonal antibodies (Thermo Fisher Scientific, Poland). The surfaces of coatings were visualized by confocal laser scanning microscopy (CLSM Exciter5 AxioImager, Zeiss). The contribution of fluorescent single/aggregate blood cellular components to surface coverage was evaluated using the colocalization mode of the CLSM Zen 2008 and Fiji software.

4.3. Quantitative analyses of the activation of blood cells

The level of platelet activation and the number of circulating platelets, as well as monocyte-platelet aggregates, were evaluated in the blood after contact with the tested coatings. The level of platelet activation markers was indicated based on whole-blood staining with fluorochrome-conjugated monoclonal antibodies, such as PerCP-CD61 (GPIIb/IIIa complex: platelet marker), FITC-PAC-1 (conformational change of glycoprotein IIb/IIIa), and PE-CD62P (P-selectin) (BD Biosciences, Poland). The contribution of aggregates was evaluated

after immunostaining with PerCP-CD14 and FITC-CD61 monoclonal antibodies (BD Biosciences, Poland). Samples were analyzed using an EPICS XL flow cytometer (Beckman Coulter Inc., Brea, CA, USA). From the remaining blood, plasma was separated by centrifugation at 2000 g for 5 min and stored at −80°C for further analysis of thrombotic activity. The thrombogenic potential of blood plasma was evaluated with the ZymuphenMP-activity ELISA kit (Hyphen Biomed, Eragny, France).

5. Statistical analysis

The ANOVA statistical analysis and Tukey post hoc test (*p*-value smaller than 0.05 was considered significant) were performed on six replicates of each sample type using OriginPro 2018 software (OriginLab Corporation, MA, USA).

References

1. A. Mzyk, G. Imbir, K. Trembecka-Wójciga, J. M. Lackner, H. Plutecka, E. Jasek-Gajda, J. Kawałko and R. Major, ACS Biomater. Sci. Eng., 2020, 6, 898–911.
2. R. Major, M. Kopernik, A. Kuźmińska, G. Imbir, H. Plutecka, M. Pomorska, T. Ciach and J. M. Lackner, Colloids Surf. B Biointerfaces, 2021, 199, 111562.
3. Y. Noguchi, Y. Iwasaki, M. Ueda and S. Kakinoki, J. Mater. Chem. B, 2020, 8, 2233–2237.