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Comparison of microscopic methods for evaluating platelet adhesion to biomaterial surfaces

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Microscopic methods usable for sample surface imaging and subsequent qualitative and quantitative evaluation of platelet adhesion to the surface of the biomaterial studied were compared. It was shown, making use of the samples of medical steel (AISI 316L), that such tools as surface imaging with scanning electron microscopy (SEM), glutaraldehyde induced fluorescence technique (GIFT) and metallurgical microscopy (MM) are equivalent in evaluating surface platelet adhesion. The importance of biological variability of blood samples for a proper result assessment and the necessity of using internal standards were also considered.

Key words: *biomaterials, medical steel AISI 316L, platelet adhesion, platelet activation, thrombogenicity, surface imaging*

1. Introduction

Dynamic development in the area of both technique and medicine allows patients to recover from illness and to lead relatively active lifestyle thanks to the use of implants. Despite the fact that biomaterials comply with the conditions required, the implantation often causes serious complications. Because of an unavoidable contact of implant with blood, even for a short period of time, the issue of hemo- and thrombocompatibility of implants is especially vital.

Under physiological conditions platelets do not adhere to the endothelial cells lining the inner surface of the blood vessels. The first step of biomaterial contact with blood is adhesion of plasma proteins to the surface. Then the same happens with platelets, but the intensity of this process is dependent on the type of proteins adhered to the surface. Some proteins, e.g. fibrinogen, stimulate the platelet adhesion, while others inhibit this process [1]. Platelets adhered to the surface of implant become activated: their shape and size change, the content of granules is released and finally platelets aggregate [2]. This results in a hemostatic system activation and a blood clot formation. The above facts force us to assess the thrombogenicity of biomaterials.

European Standard EN ISO 10993-4, applied in biological assessment of medical devices in contact with blood, describes the types of tests for biomaterial interaction with blood. In vitro tests include platelet activation assessment (release of platelet factor-4, beta-thromboglobulin and serotonin, formation of microparticles, morphological changes of platelets), quantitative analysis of platelets, their adhesion and aggregation as well as bleeding time and platelet lifespan assessment [3].

Several methods for quantitative analysis of platelets adhered to the surface of biomaterials have been reported. The basic techniques are optical microscopy and scanning electron microscopy used mainly for qualita-

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tive assessment of platelet activation, rarely for their quantitative analysis. RESMI [4] and KRISHNAN [5] developed the procedure with the use of ^{125}I-labelled platelets in order to estimate their spatial distribution on the surface of biomaterials (a radio-scintigraphy technique). Another method is the application of fluorescence-labelled antibodies that recognize certain surface antigens, specific to platelets. The visualization of adhered platelets is then performed with the use of fluorescence microscope [6], [7]. Morphological changes are one of the most commonly used criteria for qualitative assessment of platelet activation. The platelets, which remain inactivated, are discoidal in shape and their diameter ranges from about 2 to 3 μm. The early stage of platelet activation is connected with the change of their shape into more spherical and the development of tiny pseudopodia. On the other hand, fully spread pseudopodia, up to 5 μm, and irregular shape of the cell are indicative of the further stage of platelet activation [8]. Based on the shape of cells, Goodman categorized platelets into five morphological forms, arranged according to the increasing stage of their activation [9]. These are, in the sequence of activation degree, discoid (round), dendritic (early pseudopodia), spread dendritic (intermediate pseudopodia), spread, and fully-spread platelets.

Another method, helpful in evaluating the platelet morphology, is a glutaraldehyde-induced fluorescence technique (GIFT) [10], [11]. It is based on the epifluorescence of glutaraldehyde-fixed platelets and is useful both for opaque and transparent materials. This method allows us to omit conventional and immunological staining and time-consuming procedures of sample preparation. Simple approach available with metallurgical microscope (MM) developed in our lab also seems promising in the case of surface imaging, especially for opaque samples. The aim of this study was to carry out a comparative analysis of morphological changes and quantity of platelets adhered to the surface of biomaterial, observed on the same samples. These examinations were made with the use of fluorescence, metallurgical and scanning electron microscopes, with regard to Goodman's classification.

2. Materials and methods

2.1. Materials

Disc-shaped samples of medical steel (AISI 316L), 8 mm in diameter and 3 mm thick, were used in this study.

Blood was withdrawn from healthy volunteers who have abstained from taking any antiplatelet drugs for last two weeks. The blood was anticoagualated with 3.8% sodium citrate in the proportion of 9:1 (v/v). The Department of Biophysics of Technical University of Łódź has the consent from Bioethical Committee, RNN/46/06/KB, from 21st February 2006 to conduct such investigations.

All chemicals were purchased from SIGMA-ALDRICH, Poland.

2.2. Methods

The samples of polished medical steel were washed in an ultrasonic bath. Then they were put into Eppendorf test-tubes' caps and incubated with 2 cm^3 of citrated blood for one hour at room temperature. The test-tubes were softly moved end-to-end to imitate blood circulation. After incubation the samples were washed three times for five minutes in 10 mM phosphate buffer containing 140 mM NaCl, pH 7.4, and fixed for an hour at 4 °C in 2.5% solution of glutaraldehyde prepared in the phosphate buffer. Next the samples were washed three times in the phosphate buffer and dehydrated with the use of ethyl alcohol whose concentration was increasing (60–96%), ten minutes in each. Thereafter the samples were dried at room temperature. Finally, their surface was examined with the use of fluorescence and metallurgical microscope (Olympus GX71). The fluorescence was excited by the light of 450–490 nm wavelength emitted from a mercury vapour tube. Before application of SEM method the samples were additionally covered with a thin film of gold (20–30 nm). Ten regions of examination were chosen randomly, according to the procedure described by YI LIU et al. [12].

3. Results

Platelets adhered to the medical steel surface were found as single cells or platelet aggregates. Pictures obtained with SEM allow distinguishing all morphological forms of activated platelets and platelet aggregates, with the exception of round form corresponding to the very early phase of platelet activation (figure 1).

The pictures obtained with optical microscope involving fluorescence and metallurgic approaches also allow distinguishing the above mentioned morphological forms of activated platelets (figures 2 and 3).

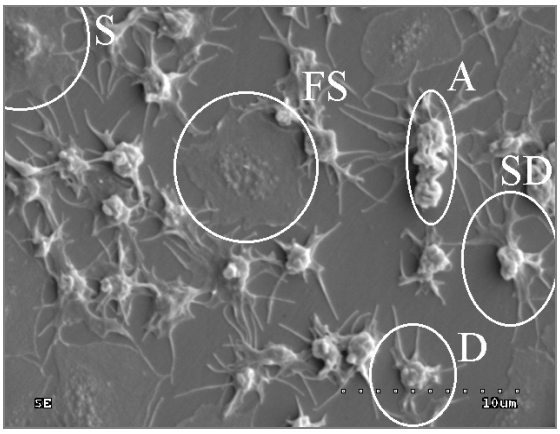

Fig. 1. SEM image of platelets adhered to the surface of polished medical steel. Different morphological forms of activated platelets are indicated: D – dendritic; SD – spread dendritic; S – spread; FS – fully-spread. Additionally a small platelet aggregate is labelled as A

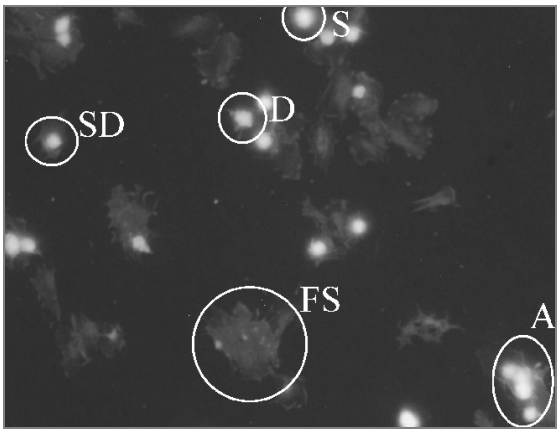

Fig. 2. Platelets adhered to the surface of polished medical steel were examined with the use of GIFT technique. Different morphological forms of activated platelets are indicated: D – dendritic; SD – spread dendritic; S – spread; FS – fully-spread. Additionally a small platelet aggregate is labelled as A

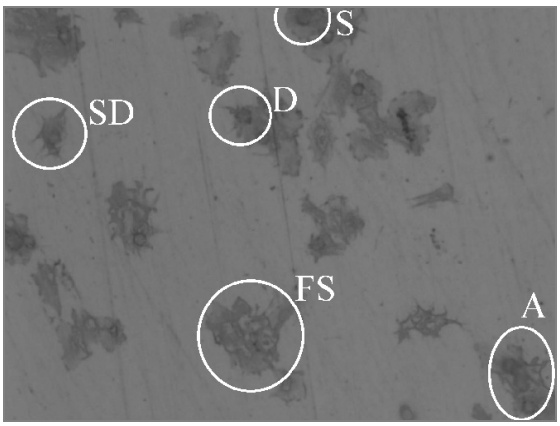

Fig. 3. Platelets adhered to the surface of polished medical steel were examined with the use of MM technique. Different morphological forms of activated platelets are indicated: D – dendritic; SD – spread dendritic; S – spread; FS – fully-spread. Additionally a small platelet aggregate is labelled as A

Although pseudo-spatial image of the surface obtained with SEM technique seems to be more clearer, even shortly trained operator can easily identify the same objects by means of GIFT and/or MM methods. To prove this thesis a small surface area was examined and documented by three techniques involved in the investigation. Large objects visible on the left-hand side of the pictures correspond to adsorbed leucocytes. Dense platelets, mainly in their dendritic and spread dendritic forms, are well seen in the whole area of examination. In the middle of the right-hand side of the area, the platelet aggregate is present.

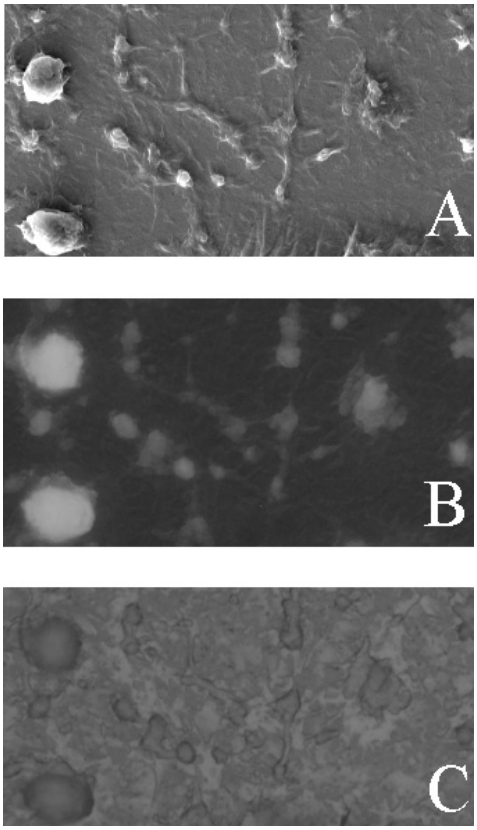

Fig. 4. Selected surface area of polished medical steel was used for imaging with SEM (A), GIFT (B) and MM (C) methods

Quantitative analysis was performed for ten randomly selected areas of the samples examined. The areas were photographed and the pictures obtained were used for the count of adhered platelets. Two separate samples of polished medical steel came into contact with blood collected from two different donors (the table). The counts of adhered platelets on the same surface determined by means of three different methods proved to be comparable, but there was also found a substantial difference in the platelet count for different donors.

Material	Medical steel 316 L	
Technique	Platelet count per 100 μm^2	
	Sample I	Sample II
MM	1.04±0.37	2.59±0.88
GIFT	1.03±0.32	3.28±1.02
SEM	0.84±0.42	3.27±0.46

The platelet adhesion to the surfaces of medical steel samples incubated with blood obtained from two different donors was assessed. The results are presented as a mean and standard deviation of the mean. No statistically significant difference was found within the same sample, but huge difference was found between the samples.

4. Discussion of results

There is no doubts that the scanning electron microscopy provides the most reliable and readable data on adhesion of cells to the surfaces of biomaterials. The interpretation of the image obtained with this pseudo-spatial method is very easy. On the other hand, this is a time- and money-consuming technique and requires direct access to an expensive instrument. Thus, it is worth considering alternative methods of sample surface imaging, especially for the evaluation of the results of biomaterial contact with tissues. One of the alternative and simple methods suitable for visualization of biological objects is glutaraldehyde induced fluorescence technique (GIFT). High quality images obtained with GIFT method allow both qualitative and quantitative analyses of platelets and other cells, their adhesion to the surfaces of biomaterials, with comparable and reliable effects. Our experience with metallurgic microscope (MM) examinations has assured us that this method is usable also for the analysis of the biomaterial surfaces with adhered cells, and the results obtained are very similar.

Statistical analysis of differences with non-parametric ANOVA test unequivocally indicates no significant differences between the methods employed in this study; however, biological variability of platelets derived from different donors may impede proper comparison of the results obtained in separate assays. Different platelets reactivity significantly influences the results of their adhesion. Thus, both surface thrombogenicity and variable platelet reactivity can affect the results of examination. Additionally, for highly thrombogenic surfaces, where the density of adhered blood platelets is very high, distinguishing separate platelets becomes extremely difficult and the uncertainty of the result increases. We face this difficulty in all imaging techniques.

The above mentioned biological variation in the reactivity of platelets taken from different donors is often underestimated and can cause an incorrect assessment of biomaterial surface thrombocompatibility. In our laboratory, the samples of polished medical steel serve as internal standard, and are involved in any assay run for new biomaterial. In this way, we can compare the results obtained with those of blood derived from different donors.

Summing up, one can say that three methods of surface imaging, in respect of assessing the platelet adhesion, are equivalent in their productivity and can be used alternatively. This proposition is important when we use metallurgic microscope usually available in most materials science laboratories. It is also important to concentrate our attention on the variability of biological material and the possibility of reducing uncertainty by the use of the internal standard.

References

[1] JONES M.J., MCCOLL J.R., GRANT D.M., PARKER K.G., PARKER T.L., *Protein adsorption and platelet attachment and activation on TiN, TiC, and DLC coatings on titanium for cardiovascular applications*, Journal of Biomedical Material Research, 2000, 52, 413–421.

[2] KAMATH S., BLANN A.D., LIP G.Y.H., *Platelet activation: assessment and quantification*, European Heart Journal, 2001, 22, 1561–1571.

[3] EN ISO 10993 Biological evaluation of medical devices. Part 4: Selection of tests for interaction with blood.

[4] RESMI K.R., VARGHESE N., KRISHNAN L.K., *Procedure for quantification of platelet adhesion to biomaterials by radioscintigraphy*, Thrombosis Research, 2004, 114, 121–128.

[5] KRISHNAN L.K., VARGHESE N., MURALEEDHARAN C.V., BHUVANESHWAR G.S., DERANGERE F., SAMPEUR Y., SURYANARAYANAN R., *Quantitation of platelet adhesion to Ti and DLC-coated Ti in vitro using ^{125}I-labeled platelets*, Biomolecular Engineering, 2002, 19, 251–253.

[6] NYGREN H., *Initial reactions of whole blood with hydrophilic and hydrophopic titanium surfaces*, Colloids and Surfaces B: Biointerfaces, 1996, 6, 329–333.

[7] KANAGARAJA S., LUNDSTROM I., NYGREN H., TENGVALL P., *Platelet binding and protein adsorption to titanium and gold after short time exposure to heparinized plasma and whole blood*, Biomaterials, 1996, 17, 2225–2232.

[8] YANG P., HUANG N., LENG Y.X., CHEN J.Y., FU R.K.Y., KWOK S.C.H., LENG Y., CHU P.K., *Activation of platelets adhered on amorphous hydrogenated carbon (a-C:H) films synthesized by plasma immersion ion implantation–deposition (PIII-D)*, Biomaterials, 2003, 24, 2821–2829.

[9] GOODMAN S.L., *Sheep, pig, and human platelet–material interactions with model cardiovascular biomaterials*, Journal of Biomedical Material Research, 1999, 45 (3), 240–250.

[10] FRANK R.D., DRESBACH H., THELEN H., SIEBERTH H.G., *Glutardialdehyde induced fluorescence technique (GIFT): A new method for imaging of platelet adhesion on biomaterials*, Journal of Biomedical Material Research, 2000, 52 (2), 374–381.

[11] HASEBE T., ISHIMARU T., KAMIJO A., YOSHIMOTO Y., YOSHIMURA T., YOHENA S., KODAMA H., HOTTA A., TAKAHASHI K., SUZUKI T., *Effects of surface roughness on anti-thrombogenicity of diamond-like carbon films*, Diamond & Related Materials, 2007, 16, 1343–1348.

[12] YI LIU, ZHEYI LI, ZHENHUI HE, DIHU CHEN, SCHIRONG PAN, *Structure and blood compatibility of tetrahedral amorphous hydrogenated carbon formed by a magnetic-field-filter plasma stream*, Surface and Coatings Technology, 2007, 201, 6851–6856.