Hemocompatibility study of surface-attached antibiofouling polymer monolayers

Bidhari Pidhatika
Center for Leather, Rubber, and Plastics, Agency of Industrial Research and Development, Ministry of Industry, Republic of Indonesia, Jl. Sokonandi No. 9, Yogyakarta 55166, Indonesia.
Email: pidhatika@kemenperin.go.id, bpidhatika@gmail.com

Abstract. The interface between biomaterials and body fluid such as blood is a critical concern, because biomaterial-centered protein fouling and infection adversely affects the quality of life of many patients and cause major health care costs. Our research focused on polymer functionalization on surfaces to create antibiofouling interfaces (i.e. interfaces that repel the adhesion of proteins and microorganisms) in biomaterial-related applications, such as surgical instruments and blood-contacting biomedical devices. To this end, we have studied two hydrophilic polymers, namely poly(2-oxazoline) and poly(ethylene glycol), attached on metal oxide surfaces in brush configuration through a polyelectrolyte surface anchor, poly(L-lysine). Apart from antibiofouling properties, blood-contacting surfaces must also serve hemocompatibility. Here the hemocompatibility of antibiofouling monolayer on silicon oxide surfaces prepared from either poly(2-oxazoline) or poly(ethylene glycol) grafted on a main backbone poly(L-lysine), has been studied. The activation of C5a (complement system), TAT and kallikrein (coagulation cascade), PF4 and sP-selectin, (platelet activation) after incubation of the polymer-modified surfaces in whole blood was measured by means of ELISA kit. The results showed that in general, the contact between blood and polymer monolayer activated the complement system, but relatively did not activate the coagulation cascade and the platelet surface marker.

1. Introduction
There is an increasing demand on highly biocompatible biomaterials in this aged society. Referring to the definition of biomaterials described by the American National Institute of Health [1], biomaterials comprises any substances that augments or replaces partially or totally any tissue, organ, or function of the body, in order to maintain or improve the quality of life of the individual. Examples of biomaterials are contact lenses, urinary catheters, dental implants, heart stents, and surgical devices. During applications, biomaterials have direct contact with tissue and/or body fluid including blood.

Insertion of charged or hydrophobic biomaterials such as metals that carry high surface energy at solid-water interface into the tissue or body fluid spontaneously triggers non-specific protein adsorption, which can be followed by bacterial infection and biofilm formation, foreign body response, encapsulation, inflammation, and failure (disintegration) of the biomaterials from the body [2]. Further consequences may include cost burden, severe pains, morbidity, and in the worst case, mortality. A plethora of studies have been performed in combating such foreign body response and failure by, among many others, the development of antibiofouling surfaces that repels the adhesion of any biomolecules and microorganisms [3]. To this end, we have, since several years, been studying the
functionalization of biomaterials surfaces using hydrophilic polymers such as poly(ethylene glycol) (PEG) [4,5], poly(2-methyl-2-oxazoline) (PMOXA) [6-9], and poly(N,N-dimethylacrylamide) (PDMAA) [10]. The first two, PEG and PMOXA, have been grafted into a polycationic backbone poly(L-lysine) (PLL), resulting in PLL-g-PEG and PLL-g-PMOXA graft copolymers. It has been found that the PLL anchors the graft copolymers to strongly and stably attach as a monolayer on metal oxide surfaces such as SiO$_2$, TiO$_2$, and Nb$_2$O$_5$, while the PEG or PMOXA protrudes to the interface and conveys antibiofouling properties when brush configuration is achieved on the surface [7].

In the case of blood-contacting biomaterials, hemocompatibility is a crucial aspect apart from antibiofouling properties. Hemo-incompatibility may lead to stimulation of the immune system and lethal hypersensitivity. For example, contact between a heart stent (that may also contains pyrogenic materials) and the blood may activate the hemostatic and inflammatory systems that include complement (C) system, coagulation, platelets, and leukocytes, leading to complications [11]. Therefore, biomaterials for applications with blood contact should be routinely tested according to the guideline ISO 10993-4 ((ISO 10993: Biological evaluation of medical devices — Part 4: Selection of tests for interactions with blood) [11]. This paper presents the hemocompatibility study of surface-attached antibiofouling PLL-g-PEG and PLL-g-PMOXA brush monolayers on SiO$_2$ surfaces.

2. Materials and Methods

2.1. Graft copolymers and blood
The following (co)polymers were used:

a) PLL-g-PMOXA4
b) PLL-g-PEG2
c) PLL-g-PEG5
d) PLL

The number next to the polymer name indicates the molecular mass of the polymer in kDa. For example, PLL-g-PMOXA4 refers to graft copolymer that contains PMOXA 4 kDa grafted onto PLL-HBr 20 kDa, with approximately 0.35 of grafting density [4]. PLL-g-PMOXA4 was synthesized following previously published protocol [4,12,13]. Both PLL-g-PEG2 and PLL-g-PEG5 were purchased from Surface Solution AG (Switzerland). PLL (PLL-HBr) 20 kDa was purchased from Alamanda Polymers, Inc. (Alabama). The copolymers are dissolved in aquadest with a concentration of 1 mg/ml, and stored in a refrigerator (4°C) until use.

Whole blood was obtained from the blood banks in Zürich and in Yogyakarta, with EDTA as the anticoagulant.

2.2. Preparation of surface-attached monolayer
SiO$_2$ (glass) microscope cover slide slips were cleaned using piranha cleaning solution and blow-dried using N$_2$ gas. The slips were then incubated in 1 mg/ml copolymer solution at room temperature for approximately 2 hours, to allow for copolymer attachment and monolayer formation on the SiO$_2$ surface. The slips were rinsed carefully with aquadest followed by blow-dried using N$_2$ gas.

2.3. Testing of antibiofouling properties of the surface-attached monolayer
SiO$_2$ (glass) microscope cover slide slips (both un-modified and modified ones) were then incubated in whole blood, as shown in figure 1.
After 30 minutes of incubation, the slips were rinsed thoroughly using PBS buffer, followed by fixation using methanol, dyeing using Giemsa, cleaning of the Giemsa dye using aquadest, and blow-drying. Optical microscopy was then used to observe the adsorption of any biomolecules on the (modified) SiO$_2$ slips.

### 2.4. Testing of hemocompatibility of the surface-attached monolayer

The hemocompatibility study was performed using ELISA kits. The activation of five components were monitored, *i.e.* C5a, TAT, PF4, sP selectin, and kallikrein. The kits used were C5a ELISA (from IBL, No. IB 79153), human sP-Selectin/CD62P immunoassay (from R&D Systems No. BBE6), AssayMax Human Thrombin-antithrombin (TAT) Complexes ELISA Kit (from Gentaur No. ET1020d), and plasma kallikrein-like activity (DIAPHARMA, No S-2302).

### 3. Results and Discussions

The surface-attached polymer monolayer in a brush configuration is depicted in figure 2. The PLL plays role as surface-anchor for the attachment of PMOXA or PEG chains on the surface. The protruding hydrophilic PEG and PMOXA chains convey antibiofouling properties, following a mechanism that has been explained in literature [14,7].

![Figure 2. Surface-attached PLL-g-PEG or PLL-g-PMOXA monolayer on SiO$_2$ surface.](image)

**Figure 1.** Incubation of surface-attached monolayers on SiO$_2$ in whole blood.
3.1. Antibiofouling properties of the surface-attached monolayer: interaction with whole blood.

The antibiofouling properties of the surfaces were investigated by exposing the surfaces to whole blood. Figure 3 shows the exemplary microscopy images of the surfaces after being exposed to whole blood.

![Microscopy images](image)

**Figure 3.** Microscopy images of (a) un-modified, (b) PLL-modified, (c) PLL-g-PMOXA4-modified, (d) PLL-g-PEG2-modified, and (e) PLL-g-PEG5-modified SiO\textsubscript{2} surfaces after incubation in whole blood for 30 minutes. Attached blood cells are visualized as circles.

Surface biofouling is observed on both control surfaces, \textit{i.e.} un-modified and PLL-modified SiO\textsubscript{2} surfaces, shown by the high amount of proteins and blood cells attachment. The lack of steric repulsion on un-modified SiO\textsubscript{2} and high amount of positive charges on PLL-modified SiO\textsubscript{2} surface are responsible for the observed biofouling on surfaces shown in figure 3a and 3b, respectively.

On the other hand, only small amount of blood cell attachment was observed on PLL-g-PEG2-modified surface, while negligible amount was observed on PLL-g-PMOXA4- and PLL-g-PEG5-modified surfaces. These observable phenomena validate the antibiofouling properties of PEG and PMOXA are previously reported [4].

3.2. Hemocompatibility study of the surface-attached monolayer

Hemocompatibility study of the surface-attached monolayers was tested by observing the concentration of C5a, TAT, PF4, sP selectin, and kallikrein concentration in blood after being exposed to un-modified SiO\textsubscript{2} and to the surface-attached monolayers for 2 hours. The concentrations of the mentioned substances in fresh blood were also measured as controls. **Figures 4, 5, 6, 7, and 8** show the concentration of C5a, TAT, PF4, sP selectin, and kallikrein, respectively, after the hemocompatibility experiments.
Figure 4. Complement 5a (C5a) concentration in blood after being exposed to un-modified and different monolayer surfaces.

The complement-activated product, C5a, displays powerful biological activities that lead to inflammatory reactions. It may play roles in the cellular and molecular mechanisms of inflammatory disorders, sepsis, acute lung injury, ischemia-reperfusion injury, and asthma [15]. Figure 4 shows that exposure of blood to un-modified SiO$_2$ activates C5a to some extent. This activation is more pronounced in the case of copolymer monolayer-modified surfaces, with the lowest and highest activation observed on PLL-g-PMOXA4 and PLL-g-PEG2 monolayer, respectively.

Figure 5. Thrombin antithrombin (TAT) concentration in blood after being exposed to un-modified and different monolayer surfaces.

The presence of TAT indicates the activation of coagulation cascades in blood [16], in this context, due to the presence of foreign compound(s). It is seen in figure 5 that exposure of blood to different surfaces does not significantly increase the TAT concentration relative to that in the fresh blood as the reference value.
PF4 is the most abundant protein contained in platelet alpha-granules [17]. Thus, PF4 activation means platelet activation, which also means that the immune response in the body is activated. Very often, platelet makes important contribution in host inflammatory response. Upon uncontrolled pathological conditions, platelet activation may lead to atherosclerosis and cardiovascular diseases, uncontrolled inflammation, tumor metastasis, and neurodegenerative diseases including Alzheimer's disease. The basic function of platelet is rapidly binding to damaged blood vessels [17]. It is seen in figure 6 that exposure to the different surfaces does not significantly increase the PF4 concentration in blood, relative to the value of fresh blood as the reference.

sP-selectin is the soluble form of P-selectin that circulates in plasma, is expressed on activated platelets, plays an important role in atherosclerosis, and contributes to lesion development [18]. It is seen in figure 7 that exposure to the different surfaces does not significantly increase the sP-selectin concentration in blood, relative to the value of fresh blood as the reference.
Figure 8. Kallikrein concentration in blood after being exposed to un-modified and different monolayer surfaces.

The plasma kallikrein-kinin system was first recognized as a surface-activated coagulation system that is activated when blood or plasma interacts with artificial surfaces. It is activated in vivo in the case of tissue destruction or developing thrombus [19]. Figure 8 shows that the kallikrein concentration slightly increases in blood after being exposed to un-modified and all monolayer surfaces, interestingly, with exception on PLL-g-PEG2 monolayer.

4. Conclusions

In the quest of development of high quality blood-contacting biomaterials, the antibiofouling properties and hemocompatibility of 3 (three) different (co)polymer monolayers in brush configuration on SiO$_2$ surfaces have been studied. It was shown that all monolayers, prepared using PMOXA and PEG to provide the functionality, convey the antibiofouling properties. Furthermore, 5 (five) parameters, i.e. C5a, TAT, PF4, sP-selectin, and kallikrein, were measured in order to study the hemocompatibility of the prepared surfaces. In general, it was found that C5a, related to complement system, was activated upon the contact between blood and the artificial surfaces. The activation varied between studied surfaces, with the most significant increase was observed on PLL-g-PEG2 monolayer surface. Interestingly, this monolayer surface showed the lowest value of kallikrein concentration. The kallikrein concentration value was even lower than the value of the fresh blood. The explanation for this phenomenon is not yet understood and further study is needed to get more insights. Moreover, PLL-g-PMOXA4 and PLL-g-PEG5 monolayers showed, in general, similar parameter values as shown in fresh blood. This observed phenomenon indicates good hemocompatibility of the two monolayers.

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