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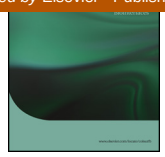

Blood coagulation and platelet adhesion on polyaniline films

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ABSTRACT

Polyaniline is a promising conducting polymer with still increasing application potential in biomedicine. Its surface modification can be an efficient way how to introduce desired functional groups and to control its properties while keeping the bulk characteristics of the material unchanged. The purpose of the study was to synthetize thin films of pristine conducting polyaniline hydrochloride, non-conducting polyaniline base and polyaniline modified with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA) and investigate chosen parameters of their hemocompatibility. The modification was performed either by introduction of PAMPSA during the synthesis or by reprotonation of polyaniline base. The polyaniline hydrochloride and polyaniline base had no impact on blood coagulation and platelet adhesion. By contrast, the polyaniline reprotonated with PAMPSA completely hindered coagulation thanks to its interaction with coagulation factors Xa, Va and IIa. The significantly lower platelets adhesion was also found on this surface. Moreover, this film maintains its conductivity at pH of 6, which is an improvement in comparison with standard polyaniline hydrochloride losing most of its conductivity at pH of 4. Polyaniline film with PAMPSA introduced during synthesis had an impact on platelet adhesion but not on coagulation. The combined conductivity, anticoagulation activity, low platelet adhesion and improved conductivity at pH closer to physiological, open up new possibilities for application of polyaniline reprotonated by PAMPSA in blood-contacting devices, such as catheters or blood vessel grafts.

1. Introduction

Despite remarkable progress in understanding the blood coagulation system, as well as in the development of blood-compatible biomaterials and blood-contacting devices, the problem of foreign-surface-induced thrombosis still remains unsolved [1]. In fact, the contact of any material with blood induces multiple defensive mechanisms, such as the activation of coagulation cascade, platelet adhesion, the triggering of complementary systems, and others [2]. The most common compound with known anticoagulant activity is heparin, and its efficacy is mainly ascribed to the simultaneous presence of sulfate, sulfamic, and carboxylic groups and their arrangement along the polysaccharide backbone of this polymer (Fig. 1a). It has already been reported that synthetic polymers and copolymers with heparin-like activity might be applicable to medical devices or surfaces coming into contact with blood.

Polyaniline (PANI), as a conducting polymer, has immense potential with regard to practical applications in the biomedical field. In particular, cardiomyocyte synchronization [3], myoblast differentiation [4], neuronal lineage differentiation, and cardiac tissue engineering [5] have been highlighted with respect to the use of conducting polymers. Commonly, the standard PANI, emeraldine salt is prepared *via* oxidative polymerization of aniline hydrochloride with ammonium peroxydisulfate [6]. Considering the biological properties of PANI, the number of papers is surprisingly limited. Humpolicek et al. [7] studied pristine PANI powders

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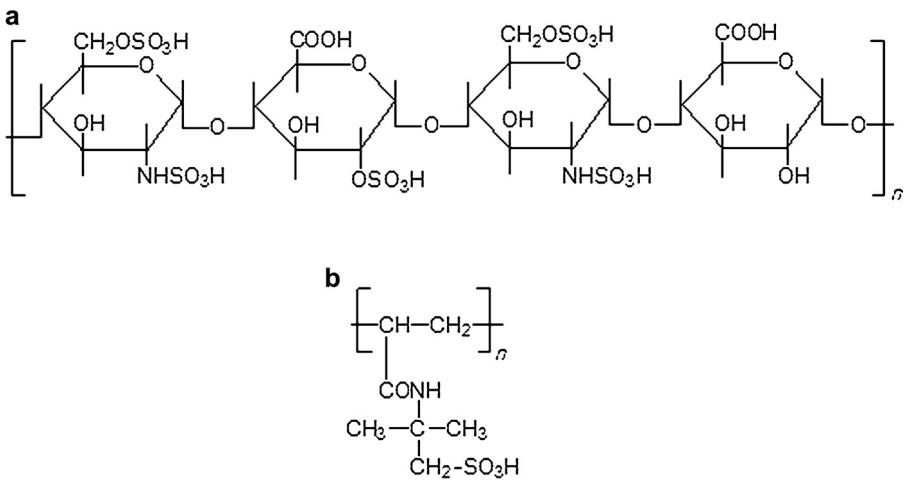

Fig. 1. Formulae of (a) heparin and (b) poly(2-acrylamido-2-methyl-1-propanesulfonic acid).

for their cytotoxicity, skin irritation and sensitization. Also cell proliferation on PANI films [8] and implantability in tissues was investigated by Kamalesh et al. [9] and Mattioli-Belmonte et al. [10]. To the best of authors' knowledge, no information about PANI hemocompatibility has previously been published.

Considerable advantage of PANI consists also in its ability to easily form thin films on substrates immersed in the reaction mixture used for PANI preparation [11]. The films can be further modified by using various dopant acids with the aim to change their surface properties [12]. This opens new possibilities for targeted surface modification of PANI by acids, or generally substances showing desired properties. Polyacids are good candidates for protonation of PANI, and some of them alone have been observed to exhibit notable anticoagulant effects. Methacrylic copolymers containing, similarly to heparin, the above-mentioned functional groups, can be listed as examples [13]. Poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA; Fig. 1b) was shown to act against blood clotting in a similar way to heparin, either alone [14] or incorporated in copolymers [15]. Setoyama et al. [16] also reported that PAMPSA is capable of inhibiting the activation of serum complement activity, which is another important factor with respect to the applicability of materials in biomedical engineering.

In the present study, PANI has a dual role. The first is represented by easy modification of surfaces by deposition of thin PANI films of sub-micrometre thickness. This is done *in situ* on substrates immersed in the reaction mixture used for the oxidation of aniline [11]. The second role then consists in the subsequent immobilization of PAMPSA onto PANI films by the formation of a salt (Fig. 2). The feasibility of a polyanion–polycation complex formation between PANI and PAMPSA has been demonstrated [17,18] and the resulting material was used in the electrodes of energy-storage devices [19,20]. PANI films with PAMPSA have also been tested for their biocompatibility [21], and normal cell adhesion, proliferation, and low cytotoxicity was observed. Polymer combining hemocompatibility and conductivity can be with advantage used for example in biosensors. These types of sensing materials can be safely placed *in vivo* while monitoring the desired parameters *via* conductivity. Moreover, inherently conducting PANI can easily be combined with other biocompatible materials and applied for example for artificial blood vessels where electrical performance can be used to monitor a range of medically important parameters, such as triacylglycerol/cholesterol concentration in blood or velocity of blood flow. A growing interest has been also in electrochemical sensors capable of *in vivo* monitoring of metabolites, for example glucose, hormones, neurotransmitters, antibodies and antigens [22,23].

The present paper is focused on the changes in blood coagulation and platelet adhesion induced by the surfaces of different PANI films, namely PANI hydrochloride, PANI base and PANI salts with PAMPSA. The films with PAMPSA were prepared using two procedures. In the first, reprotonation of PANI base using PAMPSA solution was performed giving rise to films with acid deposited onto the PANI surface. In the second procedure, a standard oxidative polymerization of aniline was conducted, with PAMPSA added directly into the reaction mixture of aniline hydrochloride and ammonium peroxydisulfate. While the PANI film reprotonated with PAMPSA contains exclusively polymeric counter-ions, the second PANI is protonated in part with sulfuric acid produced in the course of aniline oxidation from peroxydisulfate.

2. Materials and methods

2.1. Deposition of polyaniline films

The surfaces of blood collection tubes (Vacuette, Austria) were coated with conducting films of PANI hydrochloride. Aniline

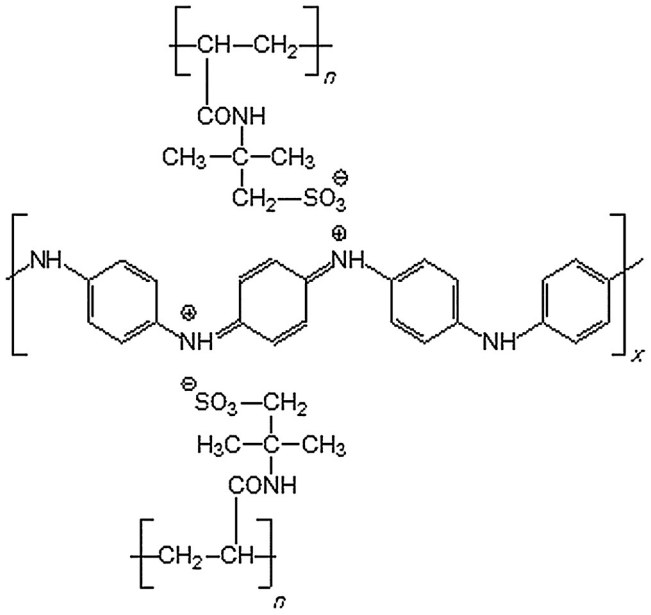

Fig. 2. Polyaniline salt with poly(2-acrylamido-2-methyl-1-propanesulfonic acid).

hydrochloride (2.59 g; Sigma-Aldrich) was dissolved in water to yield 50 mL of solution; ammonium peroxydisulfate (5.71 g; Sigma-Aldrich) was similarly dissolved to 50 mL of solution. Subsequently, both solutions were mixed at room temperature and immediately poured into tubes [11]. The concentrations of reactants were thus 0.2 M aniline hydrochloride and 0.25 M ammonium peroxydisulfate [6]. After 1 h, the tubes were emptied and the films of green conducting PANI hydrochloride deposited on the walls were rinsed with 0.2 M hydrochloric acid, followed by methanol, and left to dry in air for 5 days.

Some films were deprotonated by immersion in 1 M ammonium hydroxide for 12 h and thus converted to blue, non-conducting films of PANI base.

In order to prepare first type of PANI film with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PANI–PAMPSA), the reprotonation of PANI base with a 7.5% (v/v) aqueous solution of PAMPSA (molecular weight $M = 2 \times 10^6$ g mol^{-1}, Sigma-Aldrich) was performed by exposing the film to the PAMPSA solution. The neutralization reaction was left to proceed for 24 h; then the residual PAMPSA solution was poured out, the film was rinsed with methanol and left to dry in air.

The second type of film combining PANI and PAMPSA was prepared with PAMPSA present in the reaction mixture of aniline hydrochloride and ammonium peroxydisulfate. For this purpose, modified procedures published by Stejskal et al. [24], Yoo et al. [25], and Bayer et al. [21] were employed. First, an aqueous solution of PAMPSA was prepared with a target concentration corresponding to 0.028 mol (5.8 g) of its constitutional unit, (2-acrylamido-2-methyl-1-propanesulfonic acid). In practice, 38.5 mL of 15% PAMPSA solution was diluted by water to 375 mL. Aniline hydrochloride (0.028 mol, 3.6 g) was then added to the PAMPSA solution and stirred at room temperature for 1 h. The mole ratio of aniline hydrochloride to PAMPSA units was adjusted to 1:1 (PANI-1:1). Then the oxidant, ammonium peroxydisulfate (0.025 mol, 5.8 g), at a 1:0.9 aniline hydrochloride to oxidant mole ratio, was dissolved separately in 25 mL water and added to this solution. The polymerization was completed within 60 min. The films were rinsed with water to remove the adhering precipitate and left to dry in air. In the contrast to PANI–PAMPSA, the PANI-1:1 film contains also sulfate or hydrogen sulfate counter-ions produced by the decomposition of peroxydisulfate in addition to PAMPSA. For that reason, the molecular structure of PANI-1:1 may be structurally closer to PANI hydrochloride films than to PANI–PAMPSA.

2.2. Spectroscopic characterization

The UV–Vis spectra of films were recorded in the presence of buffer solutions with pH ranging from 2 to 12 using the UV/VIS Spectrometer (Lambda 25, Perkin Elmer, UK). The inner surfaces of standard polystyrene cuvettes were coated by PANI–PAMPSA films according to procedure described above. The buffer solutions consisted of 0.0225 M solutions of citric acid (monohydrate) (≥99.5%), Tris (p.a. ≥ 99.8%), KCl (p.a. ≥ 99.5%), obtained from Fluka, KH_2PO_4 and $Na_2B_4O_7{\cdot}10H_2O$ (Merck), and the pH was adjusted in the individual buffer solutions by adding either HCl or NaOH solutions. In the UV–Vis measurements, a syringe was used to manually fill the sample cuvettes with buffer solution and to remove the previous solution. UV–Vis spectra were recorded within the wavelength range 360–1100 nm after 1 h at each pH.

Fourier-transform infrared (FTIR) spectra of the films deposited on silicon windows were recorded with a Thermo Nicolet NEXUS 870 FTIR Spectrometer with a DTGS TEC detector in the 400–4000 cm^{-1} wavenumber region.

2.3. Contact angle measurements

Contact angle measurements were conducted with the aid of the "SEE system" (surface energy evaluation system) (Advex Instruments, Czech Republic) with deionized water as a testing liquid. The droplet volume was set to 2 μL in all experiments.

2.4. Anticoagulation test

In all tests, venous blood was collected from healthy donors by venipuncture using the vacuum blood collection system into the 5 mL collecting tubes (VACUETTE, Greiner Bio-One) after obtaining informed consent. All tests were conducted in accordance with the Helsinki Declaration. Plasma was prepared by centrifugation (15 min, 3000 × *g*). The following coagulation parameters in human blood plasma treated with citric acid (0.109 mol/L) have been studied: (1) thrombin clotting time (TCT), (2) activated partial thromboplastin time (aPTT), and (3) prothrombin time (PT). The tests were performed using a SYSMEX CA-1500 (Siemens, Germany). Each of the samples was assessed three times. To determine the interaction of PANI films on the surface with blood plasma, the coagulation factors Xa (Stuart-Prower factor), Va (proaccelerin), IIa (prothrombin), factor I (fibrinogen), antithrombin III (AT) and D-dimer were selected and the following methods were employed for their determination. The Clauss assay was used to detect the impact on factor I using a SYSMEX CA-1500 (Siemens, Germany). The same equipment with INNOVANCE® Antithrombin and INNOVANCE D-Dimer reagents was used to determine the AT and D-dimer. The activities of factors Xa, Va and IIa in the plasma have been determined by a modified prothrombin time test using an ACL ELITE Pro (IL-Instruments, Italy). Tested plasma was diluted and added to commercial plasma which was deficient always in one of the individual factors (HemosIL™, Instrumentation Laboratory, USA). The clotting time of the deficient plasma is proportional to the concentration (% activity) of a factor in the tested plasma, interpolated from a calibration curve.

2.5. Platelet adhesion

Additional information about the interaction of films with human blood was obtained by the modified TOX 6 assay (Sigma-Aldrich, USA). For this type of experiment, the PANI films were deposited on discs of 10 mm diameter. The discs were subsequently incubated in the presence of 1 mL of human blood. Sample incubation was performed in a 24-well microtiter plate at 37 °C under shaking at 200 rpm for 15 min. After incubation, the samples were rinsed with phosphate buffered saline in order to remove all unattached blood constituents and fixed with 1 mL 50% trichloroacetic acid (Sigma-Aldrich) at 4 °C. After 1 h the platelets adhered on the surface were stained with the Sulforhodamine B. The incorporated dye was liberated from the platelets using Tris base solution. Difference in the number of platelets on different surfaces results in the amount of dye incorporated by the platelets. The incubation of all samples without blood was performed to reveal if used dye did not interact with tested surfaces. The intensity of released colour by the means of absorbance was measured with a Lambda 1050 UV/VIS/NIR spectrophotometer (Perkin Elmer, USA) at a wavelength of 565 nm. The assay was performed on three discs in triplicates.

3. Results and discussion

3.1. Blood plasma coagulation

Standard films of PANI hydrochloride, PANI base and PANI-1:1 did not have any significant impact on the coagulation parameters

Table 1
Impact of PANI surfaces on selected coagulation parameters expressed as times (s) to the coagulation start.

	Reference[a]	PANI base	PANI hydrochloride	PANI–PAMPSA	PANI-1:1
PT	12.1 ± 0.1	11.9 ± 0.2	12.2 ± 0.1	NC[b]	12.0 ± 0.1
aPPT	26.4 ± 0.1	25.6 ± 1.1	26.4 ± 0.0	NC[b]	29.9 ± 0.2
TCT	18.6 ± 0.1	16.7 ± 0.1	17.56 ± 0.2	NC[b]	18.8 ± 0.1

[a] The surface of blood collection tube.
[b] No coagulation. Normal ranges for a healthy person are: PT 11.0–13.5, aPTT 25–32, TCT > 20. The values are expressed as mean value ± standard deviation, $n = 3$.

(Table 1). The coagulation on the PANI–PAMPSA surface, however, was prevented. The fact that neither standard PANI hydrochloride nor PANI base induce any changes in blood clotting suggests that the anticoagulant activity of PANI–PAMPSA is a surface effect of the used polymeric acid and is not inherently caused by PANI as such. The role of PANI thus consists in the immobilization of PAMPSA. This conclusion can be supported by the fact that PANI-1:1, in which the PAMPSA is incorporated directly under polymerization into the PANI film and not attached on its surface, does not show the above-described anticoagulation effect. In this case, however, also the fraction of PAMPSA on the surface may be lower due to the presence of sulfate counter-ions that compete with PAMPSA for the interaction with PANI.

In principle, three important variables influencing coagulation of blood in the contact with foreign matter can be identified: (1) the acidity (pH), (2) the surface charge, and (3) the interaction with coagulation factors. As acidity is one of the above mentioned factors, the pH was measured on freshly taken blood and after blood addition to Vacuette tubes coated with the PANI films. The pH of blood was not notably influenced by any of the tested samples and remained within the range of values for which no effect on blood clotting is expected (PANI hydrochloride: pH = 7.02 ± 0.01; PANI base: pH = 7.06 ± 0.02; PANI–PAMPSA: pH = 6.95 ± 0.01). This is understandable because the specific mass of thin PANI films coated on the tubes is low, of the order of 10 $\mu g\,cm^{-2}$. Such small quantities cannot significantly affect the bulk acidity of the blood in the tube. There are several studies about the impact of pH on the blood coagulation [26], but it is generally accepted that a reduction in thrombus formation starts at a pH below 6.8 and thus coagulation is reduced [27]. Moreover, the pH values of all tested samples were almost equal and impact on coagulation was observed only in case of PANI–PAMPSA. Based on this we can conclude, that reported effect of PANI–PAMPSA on blood plasma coagulation is not related to change of pH value.

As to the second factor, blood coagulation on foreign materials is activated by negative charge present on the hydrophilic surfaces [28,29]. Considering the surface properties of all the tested samples, it can be concluded that their behaviour with respect to coagulation is not influenced by surface charge, because negative charges on PAMPSA are balanced by the positively charged PANI backbone.

The fact that the PANI–PAMPSA film is conducting, and thus antistatic, may also play some role. The conductivity alone, however, cannot be the reason for anticoagulation effect. Typical conductivities of PANI hydrochloride and PANI base are of the orders 10^0 and $10^{-11}\,S\,cm^{-1}$, respectively [6]. Irrespective of these significantly different values, the coagulation properties of both films are comparable. A conductivity of $\approx 10^{-2}\,S\,cm^{-1}$ was reported for PANI–PAMPSA [17], which is a value within the above interval. Also the conductivity of PANI-1:1 with anticoagulation activity absent does not deviate from the values typically reported for conducting PANI and is of $1.7\,S\,cm^{-1}$ [21].

Although it is known that both extrinsic (tissue factor) and intrinsic (contact activation) coagulation pathways are interconnected *in vivo* [30], the plasma-coagulation cascade is usually divided into the two pathways for the convenience of discussion and coagulopathy testing. It is beyond the scope of this article to provide a comprehensive explanation of coagulation cascade and platelet adhesion in its complexity; this can be found, for example, in the paper by Vogler and Siedlecki [31]. Generally, PT detects the defects or deficits in extrinsic and common coagulation pathways, aPTT similarly in intrinsic and common coagulation pathways, and TCT is used for discriminating between problems in thrombin generation (normal TCT) and the inhibition of thrombin activity (abnormal TCT). Considering the fact that PANI–PAMPSA influences all studied coagulation variables (Table 2), it can be concluded that it affects the common pathway as a consequence of interference with coagulation factors. Thus, the effect of films on the main factors of the common pathway, factor Xa, factor Va, factor IIa, and factor I, was studied in more detail (Table 2). The results clearly show that, in the contrast to all other tested films, PANI–PAMPSA interacts with factor Xa, factor Va and factor IIa. To verify that the surfaces do not induce fibrin production, its amount was measured. The results confirm that none of tested surfaces increase the concentration of fibrin (Table 2). Such effect might be regarded as parallel to that of heparin sulfate, which binds to the enzyme inhibitor antithrombin III (AT), thus causing its activation. The activated AT then inactivates thrombin and other proteases involved in blood clotting, most notably factor Xa formation found in the common pathway. PANI–PAMPSA, therefore, might follow a similar pathway, as it further influences factors in a common way, with the exception of factor I. To test this theory, the activity of AT after contact with tested surfaces was performed. The results clearly show (Table 2), that the activity of AT is physiological after contact with all tested surfaces. The above given conclusion indicates that although PAMPSA acts against blood clotting in a similar way to heparin, which confirms the results of previous studies [14,15], the mechanism must be different. The similarity relies on the fact that heparin and PAMPSA exhibit some common features, which probably play a role in their anticoagulation activity. The fact, that both substances are polymers, although the molecular weight

Table 2
Impact of PANI surfaces on selected coagulation factors.

Factor	Reference[a]	PANI base	PANI hydrochloride	PANI–PAMPSA	PANI-1:1
Xa (%)	100	100	98.4	25.8	94.5
Va (%)	100	89.8	70.5	0.7	86.2
IIa (%)	100	92.7	86.7	0.4	83.6
I (g/L)	3.6[b]	3.7	3.5	2.3	3.3
AT (%)	101.4[b]	107.2	110.0	101.6	104.5
D-dimer (ng/mL FEU)	430[b]	470	450	480	460

[a] Uncoated surfaces of blood collection tube. The factors Xa, Va and IIa are expressed as percentage of values determined for reference.
[b] Normal range for a healthy person are I = 2–4 g/L; AT = 80–120% and D-dimer = 0–500 ng/mL FEU.

Table 3
Platelets adhesion on PANI surfaces determined as absorbance at 565 nm.

	Reference[a]	PANI base	PANI hydrochloride	PANI–PAMPSA	PANI-1:1
Platelet adhesion	2.5 ± 0.2	2.0 ± 0.3	2.5 ± 0.5	0.4 ± 0.0	1.0 ± 0.0

[a] Polystyrene was used as a reference. The values are expressed as mean value ± standard deviation, $n = 3$.

of heparin is much lower compared to PAMPSA, and the polysaccharide backbone of heparin is rigid in contrast to the flexible chain observed in PAMPSA [18], must also be taken into account. Moreover, both polymers are polyanions and both contain nitrogen- and sulfur-containing ionizable groups. This is illustrated by the covalent bond –NH–SO_3^- H^+ in heparin (Fig. 1a) and the ionic bond –NH^+ SO_3^- in PANI–PAMPSA (Fig. 2). On the other hand, the heparin effect is complex and depends not only on mentioned factors but also on certain pentasaccharide sequence in the polymer. The above results again indicate that the role of PANI is limited to the immobilization of PAMPSA at its surface.

Formation of thrombus can be also initiated by protein adsorption on polymer surfaces, which can be minimized when surface energy is on the minimum [32]. This situation occurs on highly hydrophilic surfaces. On the other hand, surfaces of higher hydrophobicity are able to more absorb the protein from plasma, as well as to induce more conformation changes of the adsorbed proteins [33,34]. More comments on this topic is provided in Section 3.5.

3.2. Platelet adhesion on polyaniline surfaces

Using sulforhodamine B colorimetric assay, the total mass of platelets adhered to the surfaces was determined, after the staining of their intracellular proteins. The platelet adhesion significantly decreased on both PANI films modified with PAMPSA irrespective of whether the PAMPSA was deposited on PANI film or added to the polymerization mixture. Of all the tested samples PANI–PAMPSA exhibited the lowest platelet adhesion compared to PANI hydrochloride, and PANI base (Table 3). The behaviour of samples with PAMSA anchored to the surface or incorporated directly into the polymer is hence similar with respect to platelet adhesion, but dissimilar with respect to the behaviour during the blood plasma coagulation.

Surface properties can be considered as an important factor in PANI/platelet interactions and therefore the water contact angle, as a measure of surface hydrophilicity/hydrophobicity was determined. The PANI hydrochloride and PANI–PAMPSA surfaces (Table 4) were the most hydrophilic of all surfaces tested and showed similar behaviour in contact with water. The lowest value (34.24 ± 1.46°) was, however observed for film composed of PANI–PAMPSA. Significant change was then observed for PANI base and PANI 1:1, both showing increase in contact angle to 66.33 ± 2.45° and 69.95 ± 3.42°, respectively. It is generally accepted that hydrophobic surfaces adsorb more plasma proteins than those with hydrophilic character. This corresponds to the lowest platelet adhesion observed on the most hydrophilic surface of PANI–PAMPSA. Nevertheless, the differences in surface hydrophilicity alone, measured on the tested surfaces, cannot unambiguously explain the observed changes in either platelet adhesion or blood coagulation.

Table 4
Contact angle (°) of individual surfaces.

	PANI base	PANI hydrochloride	PANI–PAMPSA	PANI-1:1
Water	63.33 ± 2.47	44.97 ± 3.53	34.21 ± 1.46	69.95 ± 3.42

3.3. pH stability of PANI–PAMPSA films

The UV–Vis spectra of the PANI–PAMPSA films under different pH were recorded with the aim to evaluate the pH of the transition between conducting PANI–PAMSA salt and its non-conducting, deprotonated base. From the spectra (Fig. 3) it is seen that, at pH 2, PANI–PAMPSA has two absorption maxima typical for the conducting PANI form, namely at 410 nm and at approximately 840 nm, which are assigned to the π–π* transition of the benzenoid rings and the polaron band transitions, respectively [35]. The figure also illustrates that at pH higher than 5 the conversion from the conducting salt to non-conducting base occurs. The evidence of this transition is a gradual shift of the spectrum maxima from 840 nm towards lower values. At the same time the shift of the maximum is not abrupt but gradual and, at pH > 8, the maxima are located at 605–650 nm, which corresponds to the n–π* transition of quinonoid rings. Under these conditions the film is fully deprotonated and non-conducting PANI base is formed. The results obtained thus indicate that surface deposition of PANI with PAMPSA improved the pH stability of the film, which was able to retain at least part of its conductivity at pH 6. In the comparison with standard PANI, emeraldine hydrochloride, with transition from salt to base occurring at pH ≈ 4 [36], this is clear improvement. The enhanced stability under physiological conditions might be of importance for PANI applications in biomedicine.

3.4. Spectroscopic investigation

The FTIR spectroscopic analysis has been performed (Fig. 4) to prove the presence of PAMPSA in the films and its interaction with PANI. The infrared spectrum of PANI–PAMPSA strongly differs from the spectrum of the original PANI base, and it is very close to the spectrum of PANI hydrochloride, which exhibits the typical bands of the emeraldine salt [37,38]. This confirms the protonation of the PANI base with PAMPSA.

Absorption bands of the secondary amine –NH– and protonated imine –NH^+= are detected in the region 3400–2800 cm^{-1} (Fig. 4b).

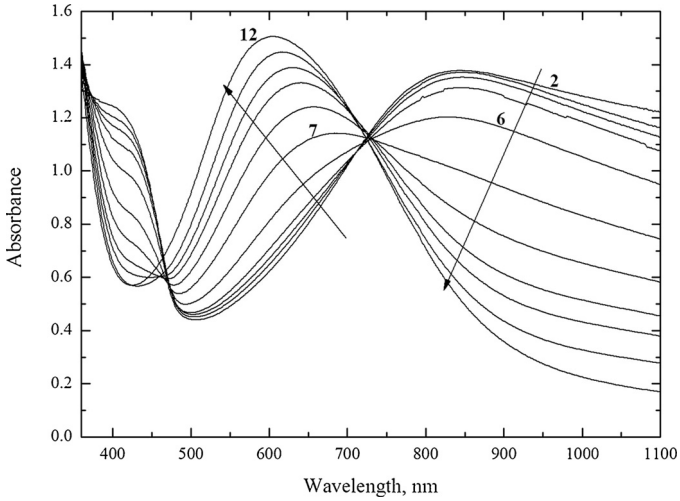

Fig. 3. UV–Vis spectra of the PANI–PAMPSA films measured in buffer solutions between pH 2 and 12.

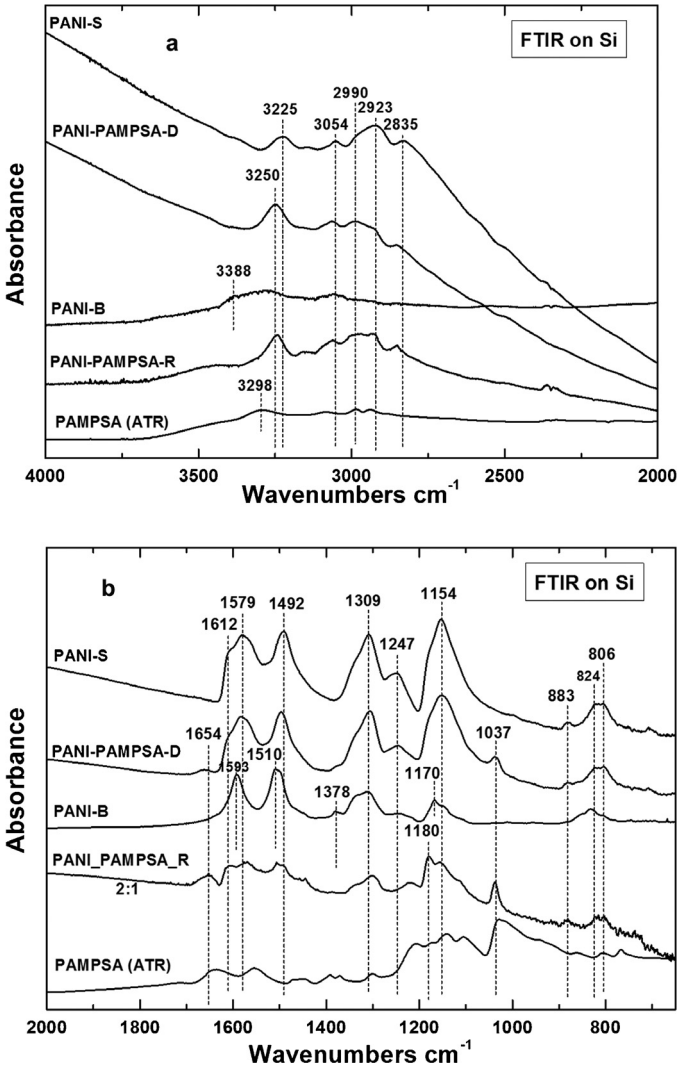

Fig. 4. FTIR spectra of the *in situ* deposited standard PANI hydrochloride (S), PANI base (B), PANI–PAMPSA (D) and PANI-1:1(R) on silicon window in (a) high and (b) low wavenumber regions. Spectrum of neat PAMPSA is shown for comparison.

They reflect the organization of PANI chains within the film by hydrogen bonding involving these groups. A relatively sharp peak with a maximum at 3225 cm^{-1} is attributed to the secondary amine $N–H^+$ stretching vibrations hydrogen-bonded with hydrogen sulfate HSO_4^- and sulfate SO_4^{2-} counter-ions which are present due to the protonation of PANI with sulfuric acid which is a by-product of the aniline oxidation (spectrum PANI-1:1). In the case of reprotonation of PANI base with PAMPSA this peak is stronger and it is shifted to higher wavenumber 3250 cm^{-1} (spectrum PANI–PAMPSA). The maximum of the broad band observed at 2990 cm^{-1} corresponds to the stretching vibrations of $–NH^+=$ in protonated imine groups bonded by a hydrogen bond with hydrogen sulfate HSO_4^- or sulfate SO_4^{2-} counter-ions. This supports the interaction of PANI with PAMPSA by stronger hydrogen bonding [12] and explains the fact that PANI–PAMPSA has improved pH stability in the transition from conducting salt to non-conducting base.

The band typical of the conducting form of PANI is observed at wavenumbers higher than 2000 cm^{-1} (Fig. 4b). Two main bands with maxima situated at 1579 and 1492 cm^{-1}, assigned to quinonoid and benzenoid ring-stretching vibrations, respectively, dominate the spectrum of protonated film in the region bellow 2000 cm^{-1}. The absorption band corresponding to π-electron delocalization induced in the polymer by protonation is situated at 1309 cm^{-1}, the band of $C{\sim}N^{+\bullet}$ stretching vibrations is observed at 1247 cm^{-1}, and the prominent band situated at 1154 cm^{-1}, has been assigned to the vibrations of the $–NH^+=$ structure. The region 900–700 cm^{-1} corresponds to the aromatic ring out-of-plane deformation vibrations. The presence of PAMPSA on the surface of PANI film is reflected in the bands at 1654 and 1037 cm^{-1} observed also in the spectrum of PAMPSA (Fig. 4b).

The shift of the sharp peak present at 3225 cm^{-1} and the maximum of the broad band at 2990 cm^{-1} in the spectrum of PANI salt is lower than in the case of the film PANI–PAMPSA. This signifies that the hydrogen bonding of PAMPSA is weaker in the film of PANI-1:1. The broad polaron band above 2000 cm^{-1} disappeared, as it is observed after deprotonation of the PANI salt to PANI base (Fig. 4). The bands of quinonoid and benzenoid-ring vibrations shift to higher wavenumbers, 1593 cm^{-1} and 1510 cm^{-1}. The band of the C–N stretching vibrations in the neighbourhood of a quinonoid ring at 1378 cm^{-1} appeared in the spectrum of PANI-1:1 and of PANI base. The 1309 cm^{-1} band of the C–N stretching of a secondary aromatic amine and of the aromatic C–H in-plane bending modes are observed in the region at about 1170 cm^{-1} and they are not enhanced in the spectrum of PANI-1:1 and PANI base. Out-of-plane deformations of C–H on 1,4-disubstituted rings are located in the region of 800–880 cm^{-1}. The presence of PAMPSA in the film is also reflected by the bands present at 1654 and 1037 cm^{-1} observed also in the spectrum of PAMPSA (Fig. 4b).

3.5. General discussion

In blood-contacting devices, various polymers can be applied. Just briefly listed, catheters are made of poly(vinylchloride), poly(tetrafluorethylene), polyethylene, polyurethanes and polysiloxane; hemodialysis membranes of polyethersulfone and regenerated cellulose, and oxygenator membranes of polysiloxane or polypropylene [39]. These polymers, however, do not possess an electrical conductivity which can be important in some applications where both conductivity and contact with blood are expected, *e.g. in vivo* sensing materials.

Considering the complexity of material/blood interaction, some general rules have to be considered regarding hemocompatibility of the polymer materials. It is well known, that uncharged hydrophilic surfaces exhibit low interaction with proteins and blood. Nevertheless, this is not a general rule, as for example plasma oxidation of polyethylene substrate was reported to increase wettability with increased protein adsorption while the platelet adhesion was reduced [40]. With respect to hydrophilicity, hydrogels for application in blood contacting devices were shown to be efficient to cause steric repulsion of blood proteins and were prepared for example of poly(vinyl alcohol), poly(*N*-vinylpyrrolidone), poly(ethylene oxide) or cellulose [41]. The poor mechanical properties of hydrogels can be avoided by their grafting on the surfaces of standard polymers. However, highly hydrophobic polytetreafluorethylene or fluorinated surfaces also show high hemocompatibility [42,43]. Surface charge plays also an important role. Positive surface charges stimulate platelet adhesion and activation [44] and negative surface charges activate the plasmatic coagulation systems [29].

In general, there are several approaches to prepare modified materials with improved hemocompatibility. The first one, passivation of the surface can be performed with the aim to achieve minimal interaction with blood proteins and cells. The next approach relies in immobilization of active molecules interacting with blood proteins and cells; and finally, promotion of the growth of endothelial cells is also a possible way of modification. In this context, the surface fluorination [45,46], immobilization of heparin (*e.g.* [29]) as well PEGylation (immobilization of polyethylene glycol) [47] can serve as examples of modified surfaces. Also, the

prevention of non-specific adsorption while selective adsorption of proteins can lead to the desired bioactive function. In this context, the main advantages of PANI relies not only in its conductivity, but also in its ability to be modified by another biological active substances or to act as a surface modifier itself.

The mentioned protein adsorption can be also affected by surface topography of a substrate. Rough surfaces adsorb more proteins than relatively smooth ones [48]. Moreover, the nanoscale surface topography [49] can play an important role. As the polymer chain of PAMPSA modifies the surface topography of PANI, it can also contribute to a different anticoagulation activity on its surface.

4. Conclusions

The PANI film reprotonated with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) had a significant impact on blood coagulation, which was hindered by the interaction with three coagulation factors, Xa, Va and IIa. Such modified polymer film also significantly reduced platelet adhesion, when compared with standard PANI films or uncoated reference surface, which exhibited neither of the mentioned effects. Anticoagulation activity and reduction of platelet adhesion was attributed to presence of PAMPSA polyanion immobilized on PANI polycationic surface. Reprotonation of PANI with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) also yielded other interesting results, namely improved pH stability of the PANI–PAMPSA polymer. While the standard PANI shows transition from non-conducting to conducting form at pH below 4–6, PANI modified with PAMPSA exhibited this transition at increased pH of 6. This is a notable improvement, shifting the conductivity of this polymer closer to physiological pH. The combination of conductivity, anticoagulation activity, low platelet adhesion capacity as well as improved pH stability of PANI coated with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) opens up new possibilities for application of this polymer as a biomaterial, for example in blood-contacting or collecting devices or for *in vivo* sensing materials requiring mentioned properties.

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References

[1] L. Xu, J.W. Bauer, C.A. Siedlecki, Proteins, platelets, and blood coagulation at biomaterial interfaces, Colloids Surf. B: Biointerf. 124 (2014) 49–68.
[2] F. Jung, S. Braune, A. Lendlein, Haemocompatibility testing of biomaterials using human platelets, Clin. Hemorheol. Microcirc. 53 (2013) 97–115.
[3] C. Hsiao, M. Bai, Y. Chang, M. Chung, T. Lee, C. Wu, B. Maiti, Z. Liao, R. Li, H. Sung, Electrical coupling of isolated cardiomyocyte clusters grown on aligned conductive nanofibrous meshes for their synchronized beating, Biomaterials 34 (2013) 1063–1072.
[4] S.H. Ku, S.H. Lee, C.B. Park, Synergic effects of nanofiber alignment and electroactivity on myoblast differentiation, Biomaterials 33 (2012) 6098–6104.
[5] R.M. Moura, A.A. Alencar de Queiroz, Dendronized polyaniline nanotubes for cardiac tissue engineering, Artif. Organs 35 (2011) 471–477.
[6] J. Stejskal, R.G. Gilbert, Polyaniline preparation of a conducting polymer (IUPAC technical report), Pure Appl. Chem. 74 (2002) 857–867.
[7] P. Humpolicek, V. Kasparkova, P. Saha, J. Stejskal, Biocompatibility of polyaniline, Synth. Met. 162 (2012) 722–727.
[8] P. Humpolicek, V. Kasparkova, J. Stejskal, Z. Kucekova, P. Sevcikova, Cell proliferation on a conducting polymer (polyaniline), Chem. Listy. 106 (2012) 380–383.
[9] S. Kamalesh, P.C. Tan, J.J. Wang, T. Lee, E.T. Kang, C.H. Wang, Biocompatibility of electroactive polymers in tissues, J. Biomed. Mater. Res. 52 (2000) 467–478.
[10] M. Mattioli-Belmonte, G. Giavaresi, G. Biagini, L. Virgili, M. Giacomini, M. Fini, F. Giantomassi, D. Natali, P. Torricelli, R. Giardino, Tailoring biomaterial compatibility: in vivo tissue response versus in vitro cell behavior, Int. J. Artif. Organs 26 (2003) 1077–1085.
[11] J. Stejskal, I. Sapurina, Polyaniline thin films and colloidal dispersions (IUPAC technical report), Pure Appl. Chem. 77 (2005) 815–826.
[12] J. Stejskal, J. Prokeš, M. Trchová, Reprotonated polyanilines: the stability of conductivity at elevated temperature, Polym. Degrad. Stab. 102 (2014) 67–73.
[13] M. Sorm, S. Nespurek, L. Mrkvickova, J. Kalal, Z. Vorlova, Anticoagulation activity of some sulfate-containing polymers of the methacrylate type, J. Polymer Sci. Part C-Polymer Symp. (1979) 349–356.
[14] D. Paneva, O. Stoilova, N. Manolova, D. Danchev, Z. Lazarov, I. Rashkov, Copolymers of 2-acryloylamido-2-methylpropanesulfonic acid and acrylic acid with anticoagulant activity, E-Polymers (2003) 52.
[15] E. Yancheva, D. Paneva, D. Danchev, L. Mespouille, P. Dubois, N. Manolova, I. Rashkov, Polyelectrolyte complexes based on (quaternized) poly[(2-dimethylamino)ethyl methacrylate]: behavior in contact with blood, Macromol. Biosci. 7 (2007) 940–954.
[16] H. Setoyama, Y. Murakami, K. Inoue, H. Iwata, H. Kitamura, T. Shimada, H. Kaji, Y. Ikada, M. Imamura, Extracorporeal circulation with an anticomplement synthetic polymer prolongs guinea pig-to-rat cardiac xenograft survival, Transpl. Proc. 31 (1999) 2818–2822.
[17] O.L. Gribkova, A.A. Nekrasov, V.F. Ivanov, O.A. Kozarenko, O.Yu. Posudievsky, A.V. Vannikov, V.G. Koshechko, V.D. Pokhodenko, Mechanochemical synthesis of polyaniline in the presence of polymeric sulfonic acids of different structure, Synth. Met. 180 (2013) 64–72.
[18] O.L. Gribkova, O.D. Omelchenko, M. Trchová, A.A. Nekrasov, V.F. Ivanov, V.A. Tverskoy, A.V. Vannikov, Preparation of polyaniline in the presence of polymeric sulfonic acids mixtures: the role of intermolecular interactions between polyacids, Chem. Papers 67 (2013) 952–960.
[19] J. Jeon, Y. Ma, J.F. Mike, L. Shao, P.B. Balbuena, J.L. Lutkenhaus, Oxidatively stable polyaniline:polyacid electrodes for electrochemical energy storage, Phys. Chem. Chem. Phys. 15 (2013) 9654–9662.
[20] J. Jeon, J. O'Neal, L. Shao, J.L. Lutkenhaus, Charge storage in polymer acid-doped polyaniline-based layer-by-layer electrodes, ACS Appl. Mater. Interf. 5 (2013) 10127–10136.
[21] C.L. Bayer, I.J. Trenchard, N.A. Peppas, Analyzing polyaniline-poly(2-acrylamido-2-methylpropane sulfonic acid) biocompatibility with 3T3 fibroblasts, J. Biomater. Sci.: Polymer Ed. 21 (2010) 623–634.
[22] C. Mao, A. Zhu, Q. Wu, X. Chen, J. Kim, J. Shen, New biocompatible polypyrrole-based films with good blood compatibility and high electrical conductivity, Colloids Surf. B: Biointerf. 67 (2008) 41–45.
[23] S. Singh, A. Chaubey, B.D. Malhotra, Amperometric cholesterol biosensor based on immobilized cholesterol esterase and cholesterol oxidase on conducting polypyrrole films, Anal. Chim. Acta 502 (2004) 229–234.
[24] J. Stejskal, I. Sapurina, J. Prokeš, J. Zemek, In-situ polymerized polyaniline films, Synth. Met. 105 (1999) 195–202.
[25] J.E. Yoo, J.L. Cross, T.L. Bucholz, K.S. Lee, M.P. Espe, Y. Loo, Improving the electrical conductivity of polymer acid-doped polyaniline by controlling the template molecular weight, J. Mater. Chem. 17 (2007) 1268–1275.
[26] M. Engstrom, U. Schott, B. Romner, P. Reinstrup, Acidosis impairs the coagulation: a thromhoelastographic study, J. Trauma-Injury Infect. Crit. Care 61 (2006) 624–628.
[27] G. Scharbert, G. Franta, L. Wetzel, S. Kozek-Langenecker, Effect of pH levels on platelet aggregation and coagulation: a whole blood in vitro study, Crit. Care 15 (2011) 1–190.
[28] Y. Marikovs, D. Danon, A. Katchals, Agglutination by polylysine of young and old red blood cells, Biochim. Biophys. Acta 124 (1966), 154.
[29] M.B. Gorbet, M.V. Sefton, Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes, Biomaterials 25 (2004) 5681–5703.
[30] I.M. Sainz, R.A. Pixley, R.W. Colman, Fifty years of research on the plasma kallikrein-kinin system: from protein structure and function to cell biology and in-vivo pathophysiology, Thromb. Haemost. 98 (2007) 77–83.
[31] E.A. Vogler, C.A. Siedlecki, Contact activation of blood–plasma coagulation, Biomaterials 30 (2009) 1857–1869.
[32] Y. Ikada, M. Suzuki, Y. Tamada, Polymer surfaces possessing minimal interaction with blood components, in: Polymers as Biomaterials, Springer, Heidelberg, 1984, pp. 135–147.
[33] T. Klose, P.B. Welzel, C. Werner, Protein adsorption from flowing solutions on pure and maleic acid copolymer modified glass particles, Colloid Surf. B: Biointerf. 51 (2006) 1–9.
[34] M. Okubo, H. Hattori, Competitive adsorption of fibrinogen and albumin onto polymer microspheres having hydrophilic/hydrophobic heterogeneous surface structures, Colloid Polym. Sci. 271 (1993) 1157–1164.
[35] A.P. Monkman, P. Adams, Structural characterization of polyaniline free standing films, Synth. Met. 41 (1991) 891–896.
[36] T. Lindfors, L. Harju, Determination of the protonation constants of electrochemically polymerized poly(aniline) and poly(*o*-methylaniline) films, Synth. Met. 158 (2008) 233–241.
[37] I. Šeděnková, J. Prokeš, M. Trchová, J. Stejskal, Conformational transition in polyaniline films – spectroscopic conductivity studies of ageing, Polym. Degrad. Stab. 93 (2008) 428–435.
[38] M. Trchová, Z. Morávková, I. Šeděnková, J. Stejskal, Spectroscopy of thin polyaniline films deposited during chemical oxidation of aniline, Chem. Papers 66 (2012) 415–445.

[39] C. Werner, M.F. Maitz, C. Sperling, Current strategies towards hemocompatible coatings, J. Mater. Chem. 17 (2007) 3376–3384.
[40] J.H. Lee, H.B. Lee, Platelet adhesion onto wettability gradient surfaces in the absence and presence of plasma proteins, J. Biomed. Mater. Res. 41 (1998) 304–311.
[41] N.A. Peppas, Y. Huang, M. Torres-Lugo, J.H. Ward, J. Zhang, Physicochemical, foundations and structural design of hydrogels in medicine and biology, Ann. Rev. Biomed. Eng. 2 (2000) 9–29.
[42] A.R. Jahangir, W.G. McClung, R.M. Cornelius, C.B. McCloskey, J.L. Brash, J.P. Santerre, Fluorinated surface-modifying macromolecules: modulating adhesive protein and platelet interactions on a polyether-urethane, J. Biomed. Mater. Res. 60 (2002) 135–147.
[43] J.Y. Ho, T. Matsuura, J.P. Santerre, The effect of fluorinated surface modifying macromolecules on the surface morphology of polyethersulfone membranes, J. Biomater. Sci. Polym. Ed. 11 (2000) 1085–1104.
[44] S. Sagnella, K. Mai-Ngam, Chitosan based surfactant polymers designed to improve blood compatibility on biomaterials, Colloid Surf. B: Biointerf. 42 (2005) 147–155.
[45] K. Tokuda, T. Ogino, M. Kotera, T. Nishino, Simple method for lowering poly(methyl methacrylate) surface energy with fluorination, Polym. J. 47 (2015) 66–70.
[46] T. Hasebe, A. Shimada, T. Suzuki, Y. Matsuoka, T. Saito, S. Yohena, A. Kamijo, N. Shiraga, M. Higuchi, K. Kimura, H. Yoshimura, S. Kuribayashi, Fluorinated diamond-like carbon as antithrombogenic coating for blood-contacting devices, J. Biomed. Mater. Res. Part A 76A (2006) 86–94.
[47] B. Balakrishnan, D. Kumar, Y. Yoshida, A. Jayakrishnan, Chemical modification of poly (vinyl chloride) resin using poly (ethylene glycol) to improve blood compatibility, Biomaterials 26 (2005) 3495–3502.
[48] B. Walivaara, B.O. Aronsson, M. Rodahl, J. Lausmaa, P. Tengvall, Titanium with different oxides – in-vitro studies of protein adsorption and contact activation, Biomaterials 15 (1994) 827–835.
[49] C.C. Mohan, K.P. Chennazhi, D. Menon, In vitro hemocompatibility and vascular endothelial cell functionality on titania nanostructures under static and dynamic conditions for improved coronary stenting applications, Acta Biomater. 9 (2013) 9568–9577.