THROMBORESISTANT SILICON PLATES MODIFIED WITH CHITOSAN AND HEPARIN BY THE LAYER-BY-LAYER ASSEMBLY METHOD

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Abstract

Fifteen samples of silicone plates (PlateSi, area=12 cm^2), with surfaces modified layer-by-layer with chitosan and unfractionated heparin, were obtained. The sample surfaces were pre-treated by cold oxygen plasma in a planar-type plasma chemical reactor with 50 W power before coating with layered polysaccharides. Pre-treatment was carried out in two alternative operation modes of the reactor, namely in the plasma etching mode and in the reactive-ion etching mode. Thromboresistance was assessed in vitro in contact with human blood. The thromboresistant silicon plates, modified layer-by-layer (3, 5, 7, and 9 bilayers) with chitosan, with molecular weights of 65 kDa, increased with the increase in the number of layers, up to 5. An increase in the duration of thromboresistance was observed in layer-by-layer modification of the surface of the plates with chitosan with a molecular weight of 200 kDa or with quaternized chitosan with a molecular weight of 200 kDa. Some samples of highly thromboresistant, modified PlateSi contributed to the adhesion of platelets and the haemolysis of red blood cells to a lesser extent than untreated silicon plates. The three most promising samples of modified PlateSi were selected.

Keywords: *chitosan, heparin, polyelectrolyte, layer-by-layer, cold plasma treatment, AFM, silicone rubber, thromboresistance*

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1. Introduction

As a result of the contact of some medical devices (drainage tubes, vascular stents and catheters, blood vessel prostheses, equipment for artificial or auxiliary blood circulation systems, artificial heart valves) with human blood, the activation of plasma and cellular haemostasis, as well as complement system and inflammation system are possible [1,2]. The development of such the protective reactions of the human body can lead to the appearance of blood clots on the surface of the medical device or thrombosis of blood vessels [3,4].

Modern materials for cardiovascular surgery may consist of metals, alloys, ceramics, single crystals, carbon materials, biological tissues, and polymers [5–8]. The latter group includes biostable polymers (acrylates, epoxysilane, fluorocarbons, polyamides, polycarbonates, polyimides, polysulfone, polyurethanes, polyorganosiloxanes), biodegradable polymers (polyanhydride, polycaprolactone, lactitol and glycolide copolymers, polyhydroxyalkanoate), and natural polymers (cross-linked albumin, cellulose acetate and cellulose hydrate, chitosan, collagen, elastin, hyaluronic acid, gelatin) [7,8].

Hemocompatibility is the required quality of biopolymers for medical devices that are used in contact with blood [9]. In the study of the hemocompatibility of materials, the influence on blood components and blood clot formation has been analysed at the initial stage of *in vitro* experiments, along with the assessment of reaction of the complement system [10]. In the first place, medical devices should not activate blood clotting, induce thrombosis, or promote haemolysis of red blood cells in contact with blood.

To improve hemocompatibility, researchers have modified polymer surfaces for medical devices in order to reduce the formation of thrombus and the development of inflammatory reactions. Blood-contacting biomaterials may consist of polyurethane copolymers [11,12] or silicone rubbers [13,14]. To improve thromboresistance of the surface of polyurethane artificial heart valves, P. Alves et al. 2-hydroxyethylmethacrylate was grafted to the surface of polyurethane artificial heart valves and treated with ultraviolet or plasma to improve thromboresistance [15].

The selection of materials that do not induce thrombosis remains crucial. Currently, considerable attention is paid to the design of thin multilayer films based on polysaccharides, particularly chitosan and heparin, which is due to the functional properties of the polysaccharides, including antimicrobial and anticoagulant properties, and their possible use as coatings for medical products [16,17].

Layer-by-layer (LbL) formation of polyelectrolyte multilayers is characterized as a simple and reproducible method for obtaining thin films deposited on a substrate [18]. The LbL method involves successively dipping the substrate into dilute solutions of oppositely charged polyelectrolytes alternately. This approach makes it possible to compensate the charged surface of the previous layer with the oppositely charged layer of the next precipitated polymer. This method is characterized by simplicity in its execution, universality, low operating costs, and the possibility of step control [19]. Silicone products are widely used as biomaterials in clinical practice [13,14].

A significant disadvantage of the silicon material is its tendency towards bacterial adhesion with the formation of biofilms, which can contribute to a patient's risk of infection. Chemical modification of the silicone surface is problematic due to the lack of reactive functional groups. Therefore, the silicone surface is activated before the first layer of polyelectrolyte is applied to it; for example, a negative charge is created on the surface using short-term oxygen plasma treatment.

Chitosan is a deacetylated chitin derivative obtained by alkaline treatment [20]. Chitosan molecules tend to interact with each other in solution, thus, forming aggregates.

Therefore, for the formation of multilayer coating we used dilute solutions of biopolymer with molecular weight (MW) 65 and 200 kDa. Chitosan is insoluble in most organic solvents and in aqueous solutions above its pKa value, but it is highly soluble in aqueous solutions of organic acids due to the protonated form of the amino groups, that is, below the pKa value, which ranges from 6.46 to 7.32 depending on the degree of deacetylation (DD) [21]. In this study, chitosan with a DD of 90–95% was used.

Heparin is a polyanion that is highly soluble in a wide range of pH values. It is used for surgical interventions, prevention, and therapy of thromboembolic diseases and their complications, as well as for maintaining the liquid state of blood in cardiopulmonary bypass [22]. Hydrogels and nanoparticles based on heparin exhibit anticoagulant activities, are able to bind to growth factors, and have antiangiogenic and apoptotic effects, which make them desirable materials to use [23].

The aim of our study was to define the thromboresistance (resistance to the appearance of blood clots) of polyelectrolyte multilayer films on a silicon substrate formed from chitosan and heparin.

2. Materials and Methods

The following materials were used in this study: silicon plates with thicknesses of 0.1 cm ("Euro chemicals SPb"), chitosan with a MW of 200 kDa and a DD of 95% ("Bioprogress", Russia), which was additionally purified by the deposition method at pH 9.0 from the polymer solution (pH 3.0), unfractionated heparin (“Belmedpreparaty”, Republic of Belarus), tris (hydroxymethyl) aminimethane (Bio-Rad Laboratories, USA), and calcium chloride (anhydrous, 93%) (Sigma-Aldrich).

2.1. Oxygen Plasma Treatment of Silicon Plates

Silicone plates, previously washed with ethyl alcohol before the deposition of polyelectrolytes, were used as substrates for the assembly of LbL..

Silicon plates were treated in a parallel-plate radiofrequency (13.56 MHz) plasma chemical reactor. Two operation modes of the reactor were tested: the plasma etching configuration (the electrode on which the workpieces are mounted is connected to the ground and the opposite electrode is connected to a radiofrequency power supply) and the reactive-ion etching configuration (the electrode on which the workpieces are mounted is connected to an RF power supply and the opposite electrode is grounded). Plasma treatment conditions were as follows: RF power, 50 W; gas pressure, 1.0 Torr; and plasma density, about 10^{10} cm^{-3}. Oxygen was used as a plasma generating medium. Treatment duration varied within the range of 2–15 minutes.

2.2. Contact Angle and Surface Free Energy (SFE) Measurements of Silicon samples (PlateSi)

The wettability of the silicon polymer before and after plasma treatment was characterized by static contact angles and surface free energy (SFE).

The contact angles (θ) with deionized water or diiodomethane (Sigma-Aldrich, Germany) were measured by the sessile drop method using an optical contact angle-measuring device (CAM 101, KSV Instruments, Finland). Measurements were carried out at room temperature and at a relative humidity of 45%. Liquid droplets of approximately 3 μL were placed on the substrate using a threaded plunger microsyringe (Hamilton, USA). The contact angles of the sessile drops were measured both on the left-hand and right-hand sides of the drop contour using an image analysis program with an accuracy of ±0.1 degrees. The contact angles were measured at intervals of 0.2–1 min, depending on the rate of change of droplet size and shape. Independent

measurements were carried out for 3 droplets on each plane substrate, and mean values of the contact angles were evaluated. The SFE and its polar (deionized water) and dispersive (diiodomethane) components were calculated by the Owens and Wendt method [24].

The angle and SFE were measured immediately after plasma treatment. To avoid the adsorption of organic impurities, each sample was kept in a separate clean glass container with a ground glass stopper and partially filled with silica gel. The experimental data was statistically analysed by the Student's test, and p-values smaller than 0.05 were considered reliable.

2.3. Preparation of Chitosan Derivatives

Low MW chitosan (CS), with MWs of 20 and 65 kDa, was obtained using nitric acid. Hydrolysis was carried out at a temperature of 70°C for 7 h [25]. Quaternized chitosan (QCS) derivatives with degrees of substitution of 98% and 92% were synthesized using glycidyltrimethylammonium chloride from chitosan with MWs of 20 and 200 kDa, accordingly. The ratio of reagent to chitosan was 4:1. The alkylation reaction was carried out for 5 h at 85°C [26].

2.4. Production of Modified Surfaces of Silicone Plates [PlateSi (CS/HEP)]

The process of obtaining the first polyelectrolyte bilayer on the activated silicone plate consisted of several successive steps. Initially, the plate was immersed in 0.1% chitosan solution containing 0.15 M NaCI, prepared in 1% acetic acid and kept at 23°C for 15 min. The plate was washed with distilled water (3 times) for 5 min.

The plate was then immersed in 0.1% heparin solution and prepared in 0.15 M NaCI at 23°C for 15 min. The treated plate was washed with distilled water 3 times for 5 min. The plate coated with the first polyelectrolyte bilayer was analysed using a microscope. The process of applying bilayers was repeated n=(2–9) times.

2.5. Control of Electrostatic Layer-by-Layer (LbL) Assembly of New Thin Multilayer Films Based on the Atomic Force Microscopy (AFM) Method

The surface images of chitosan–heparin films were obtained with an atomic force microscope (AFM) (NTEGRA Prima, NT MDT SI is a «Molecular Devices and Tools for Nanotechnology» Spectrum Instruments, Russian Federation). Scanning was performed in tapping mode in air using silicon cantilevers (Etalon HA_NC, NT MDT SI, Russian Federation) with tip curvature radii of less than 10 nm, a force constant of 3.5 N/m, and a resonant frequency of 235 kHz. Scanning was performed with a frequency of 0.8–1.4 Hz. Image processing was carried out with Nova and Image Analysis P9 (NT MDT SI, Russian Federation) software programs.

2.6. Obtaining Human Blood and Plasma for Research

Blood from the ulnar vein of donors (blood was taken to the mark in a plastic syringe S-Monovette 5ml 9NC; "Sarstedt", Germany; all donors gave written informed consent to the collection and use of blood), stabilized in 0.106 M trisodium citrate solution, platelet-rich human plasma and platelet-depleted human plasma were used in the study. The blood was centrifuged at 150 g at room temperature for 7 min in order to obtain platelet-rich human plasma. Platelet-depleted plasma was obtained after centrifugation of stabilized donor blood at 1300 g for 20 min.

2.7. Evaluation of the Influence of PlateSi Samples on the Formation of Surface Blood Clots

The influence of silicone plates {PlateSi (CS/HEP) see 2.4.} samples on the formation of blood clots on the surface was determined by Wang X. et al [27], with small modifications. PlateSi samples, each with an area of 0.25 cm^2, were cut from plates with an area of 12 cm^2 and placed in plastic tubes. Then, 0.5 mL of human blood stabilized in 0.106 M trisodium citrate solution was added, and coagulation was activated by adding $CaCl_2$ aqueous solution (the final concentration was 0.05 M). After 20, 40, and 60 min (at 37°C), the plates were removed, photographed, and then placed in a haemolysis solution for 1 h (37°C). Then, the optical density of the solution obtained after incubation with the plates was determined at 545 nm (against a cuvette with 0.05 M Tris-HCl buffer with 0.175 M NaCl, pH 7.4). The areas of clots in the images of the PlateSi surfaces (in pixels) were estimated using the program PhotoM 1.31 (© copyright A. Chernigovskii, 2000–2004). PlateSi thromboresistance (%) was calculated by the following formula:

$$100\% - (ODo \cdot 100\%/ODk),$$

Where: ODo is the optical density of the solution after incubation with the PlateSi sample and ODk is the optical density of the solution after incubation with the untreated control plate sample.

2.8. Evaluation of the Effect of PlateSi Samples on the Time of Fibrin Clot Appearance in the Activated Partial Thromboplastin Time (APTT) Test

The plates were placed in plastic 1.5-mL microtubes to assess the effect of PlateSi samples (0.25 cm^2). Then, 0.2 mL of platelet-poor human plasma and 0.2 mL of a mixture of ellagic acid and phospholipids were added (NPO "RENAM" Russia). Next, 0.2 mL of 0.025 M $CaCl_2$ solution was added at 37°C after 3 min of incubation, and the time of appearance of the clot on the programmable semi-automatic coagulometer (APG2-01, minilab-701M, LLC "EMKO" Russia) was recorded [28].

2.9. Evaluation of the Influence of PlateSi Samples on Erythrocyte Haemolysis

The influence of PlateSi samples (0.25 cm^2) on erythrocyte haemolysis was determined by the modified method described by Dash B. et al [29]. Stabilized blood was centrifuged at 250 g for 15 min, and erythrocytes were washed 3 times with 10 mM phosphate buffer at pH 7.4. Erythrocytes were then suspended in 10 mM phosphate buffer. Furthermore, plate samples were put in plastic test tubes and 0.5 mL of a suspension of red blood cells was added. After incubating the samples in a thermostat for 2 h (37°C), the samples were centrifuged at 250 g for 15 min. Then, the optical density of the supernatant was measured at 540 nm. The following formula was used to calculate the degree of haemolysis (%):

$$[OD_o - OD(k-)/ OD(k+) - OD(k-)] \times 100\%,$$

where OD_o is the optical density of the solution after incubation with a prototype plate, OD(k–) is the optical density of the solution after incubation with 10 mM phosphate buffer, and OD(k+) is the optical density of the solution after incubation with distilled water.

2.10. Evaluation of the Impact of PlateSi Samples on the Adhesion of Platelets

The effect of untreated and treated PlateSi plate samples (0.25 cm^2) on platelet adhesion was determined by modifying and combining methods Kolodziejczyk-Czepas J. et al [30] and Walkowiak B. et al [31]. Therefore, platelet-rich human plasma was diluted with 10 mM phosphate buffer 100 times, and the plates were added to it. The optical density of the diluted plasma was measured at 800 nm at 37°C after 2 h of incubation.

The results are presented by arithmetic means with standard arithmetic mean errors from 4–12 independent determinations. The significance of the differences between the readings was evaluated according to the criteria of Kruskal–Wallis and by Student's t tests. Statistical processing of the results and the reliability of the correlation coefficient were performed using the Primer of Biostatistics software program Version 4.03.

3. Results and Discussion

3.1. Oxygen Plasma Processing of Silicon Plates

In simple terms, macromolecules of a one-component silicone polymer can be described as chains of alternating oxygen and silicon atoms. The silicone polymer is characterized by inertia, but it may contain fillers to improve its properties.

In this study, silicone plates, which were treated with oxygen plasma in order to become activated, creating a negative charge on the surface, were used as substrates for the assembly of a multilayer film by the LbL method.

The second version was preferable since the workpiece surface is subject to intensive fluxes of energetic ions. In addition, the ion energy can be controlled during reactive-ion etching by either adjusting the relative surface area of the electrodes or by DC biasing the powered electrode with respect to the ground. The benefits of reactive-ion etching mode are supported by the observed significant increase in silicon rubber wettability caused by plasma treatment at atmospheric pressure. Under these conditions, the fast ions are responsible for the mentioned effect.

3.2. Hydrophilic Properties of the PlateSi

Contact angles (θ_W and θ_{DM}) were measured with water and diiodomethane, respectively, and the calculated values of the total surface energy (γ_{tot}), as well as its polar (γ_{pol}) and dispersive (γ_{disp}) components are presented in Table 1 and Fig. 1. The significant decrease in water contact angles was found after plasma modification for 5 min (**Table 1**). The surface free energy also increased as a result of plasma treatment. The increase in the SFE from 17.52 mJ/m^2 to 39.23mJ/m^2 was obtained after plasma modification for 5 min. The polar component becomes more effective, while the contribution of γ_{disp} decreases, suggesting the formation of oxygen-containing polar chemical groups at the plasma-modified polymeric surfaces (C=O, −COOH, −OH, etc.).

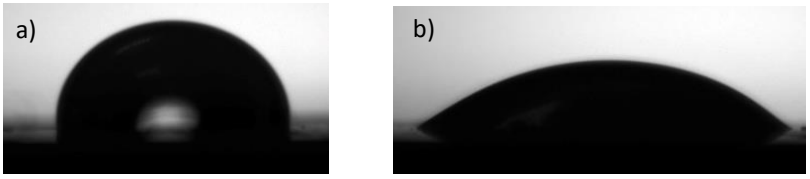

Figure 1. Modification of the hydrophilic properties of the silicon polymer as a result of plasma treatment. (a) Original sample and (b) sample treated with oxygen RF discharge for 5 min.

The longer treatment duration did not result in further considerable θ_W and SFE changes. Moreover, prolongation of the treatment time to 15 min resulted in some decreases in θ_W. This phenomenon could be due to the ageing effect of the polymeric surface [32,33], stimulated by active oxygen and nitrogen radicals produced in the plasma.

Table 1. . Contact angles, SFE and its components of PlateSi before and after plasma modification*

Sample	θ_W (deg)	θ_{DM} (deg)	γ_{pol} (mJ/m^2)	γ_{disp} (mJ/m^2)	γ_{tot} (mJ/m^2)
PlateSi, Control	97.9 ± 0.2	87.2 ± 0.5	14.16	3.36	17.52
PlateSi, τ=2 min	86.8 ± 0.2	72.3 ± 0.1	22.03	6.09	28.12
PlateSi, τ=5 min	52.5 ± 0.1	68.8 ± 0.3	21.69	17.54	39.23
PlateSi, τ=15 min	61.8 ± 0.4	72.3 ± 0.2	23.8	12.26	36.06

The data for contact angles are expressed as mean ± S.D.
*All data are reliable with respect to the control sample ($p < 0.05$ *vs.* original silicon polymer).

3.3. Preparation of Chitosan Derivatives

Chitosan with MWs of 65 and 200 kDa and quaternized derivatives obtained with chitosan with MWs of 20 kDa and 200 kDa were used to form positively-charged monolayers on the surface of the silicate plates (**Table 2**). Depolymerization of high molecular weight chitosan (HMWC) by inorganic acids under the chosen conditions contributed to the production of low molecular weight chitosan (LMWC) with a high DD of 90–98%.

The advantage of using LMWC instead of HMWC is the greater availability of protonated amino groups at pH values <6.0–6.5 and the lower solution viscosity in acetic acid. The use of a quaternized derivative of LMWC with a DD of 98% is associated with a high density of positively-charged ammonium groups and its solubility in a wide range of pH values.

3.4. Production of Modified Silicone Wafer Surfaces and Study of the Morphology of Deposited Monolayers and Bilayers of Polyelectrolytes

Diluted solutions (0.1% w/v) of chitosan in acetic acid (pH 3.2) were used to form polyelectrolyte layers on the surface of the PlateSi. All primary amino groups were fully protonated under these conditions. Therefore, the first chitosan monolayer was formed on the surface of the activated PlateSi mainly due to the electrostatic interaction with negatively charged oxygen ions. Approximately 50% of the amino groups (pH 67) retained a total positive charge of 32–35 mV after intermediate washing of the chitosan monolayer with water. As a result of functional charged groups, heparin solutions (0.1% and 0.5% w/v) in 0.15 M NaCl were characterized by a negative charge of 63–65 mV, which contributed to the formation of a heparin monolayer on the chitosan surface and the conservation of a negative charge to create the next bilayer.

The LbL deposition technique with the formation of new thin multilayer films was controlled using the atomic force microscopy (AFM) method (Fig. 2).

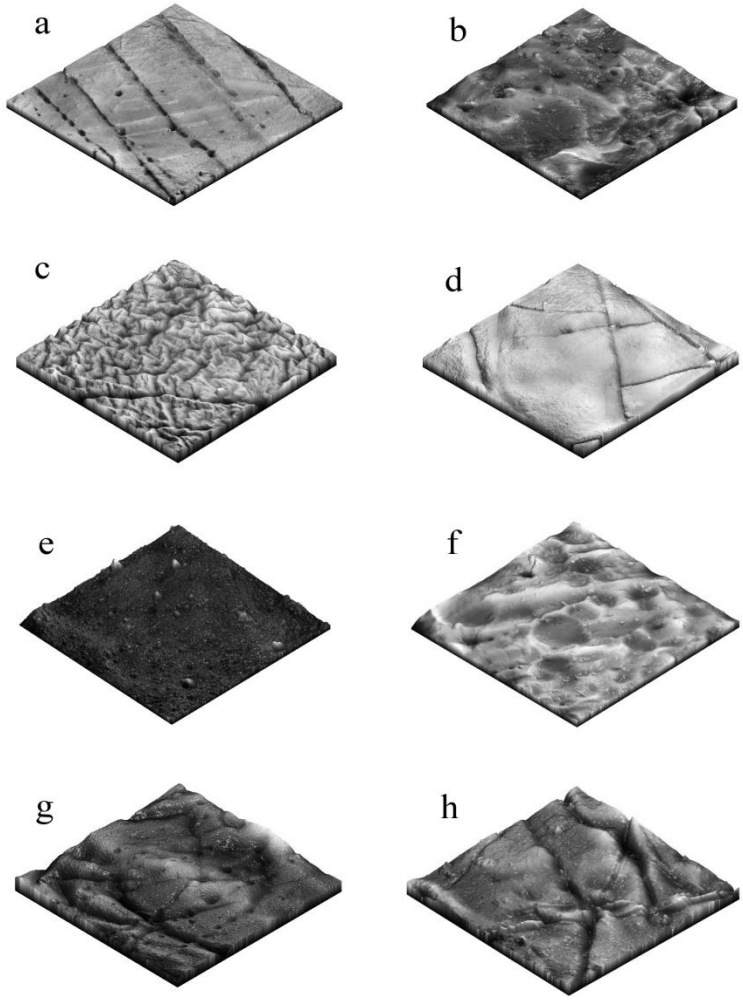

Figure 2. 3D AFM images of silicone substrates. a) PlateSi before plasma treatment, b) PlateSi after plasma treatment, c) PlateSi QCS, d) PlateSi $(QCS/HEP)_5$, e) PlateSi CS, f) PlateSi $(CS/HEP)_1$, g) PlateSi $(CS/HEP)_3$, and h) PlateSi $(CS/HEP)_9$. Scan area size is about 20×20 μm.

Fig. 2 shows 3D images obtained through AFM analysis. The thickness of the polymer coating was determined by constructing a cross-section perpendicular to the X- and Y-axes and passing through the relief modifications in the 2D image. This cross section is the profile of the sample surface along the Z-axis. An example of constructing a cross section for a film from chitosan and heparin (CS/HEP)9 is illustrated in Fig. 3

AFM images of PlateSi before and after plasma treatment are shown in Fig. 2a and Fig. 2b, respectively. PlateSi is characterized by the presence of the relief on the surface in the form of parallel recesses, with a depth of more than 500 nm. This relief was almost indistinguishable on PlateSi after plasma treatment. This is due to the physical modification of the plasma surface and the appearance of a negative charge on it, which, in turn, could have an impact on the research process.

AFM images of the surface of activated silicone with one applied layer of the quaternized chitosan (PlateSi QCS) and the initial chitosan (PlateSi CS) are shown in Fig. 2c and Fig. 2e, respectively. Unmodified CS formed a coating in the form of globular particles, about 50 nm in size, and their agglomerates formed on the PlateSi. QCS formed a structured homogeneous film on the 50- to 100-nm thick silicon. This may be due to the lower viscosity of the QCS solution.

A bilayer resulting from chitosan and heparin [PlateSi $(CS/HEP)_1$] is shown in Fig. 2f. The polymer coating is represented by lighter areas, whereas dark gaps could account for the surface of the substrate. Thus, applying a single polymer bilayer is not enough to completely cover the surface of the silicone and form a uniform film.

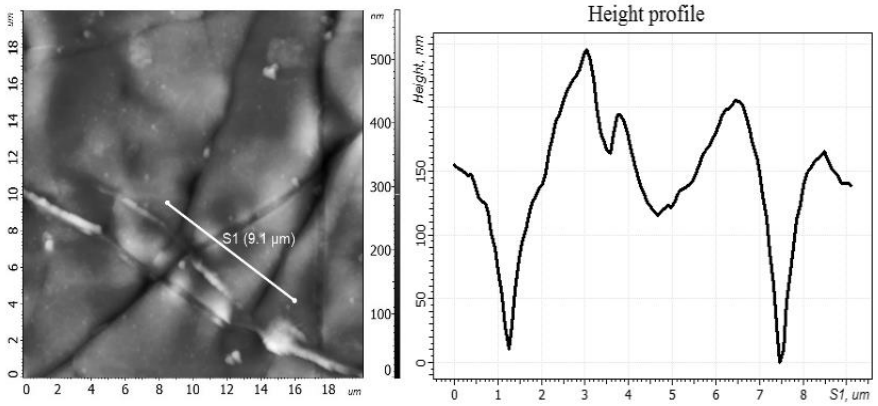

Figure 3. AFM image of the film surface profile.

Furthermore, the morphology of multilayer coatings consisting of 3 (Fig. 2g) and 9 chitosan–heparin bilayers (Fig. 2h) was investigated. The film thickness of the 3 bilayers was approximately 150 nm, while the thickness of the 9 bilayers was 250±50 nm. In both cases, a uniform film was formed on the surface, with a rough surface and large grooves located independently of the relief of a clean substrate. Unlike chitosan–heparin films (CS/HEP), multilayer coatings formed on the basis of quaternized chitosan and heparin (QCS/HEP) had a more uniform relief (Fig. 2d) and thickness of 200±30 nm; however, deep furrows were also present on the surface. It, therefore, can be concluded that the formation of such cracks does not depend on the sample composition, but on the preparation characteristics and drying of the films, as well as the number of polymer layers.

The AFM results show that the surface treatment of PlateSi with a solution of polyelectrolyte for 5 min was insufficient, especially during the formation of the first bilayer. The optimal number of CS/HEP bilayers was determined. When applying from 3 to 9 bilayers of chitosan and heparin (CS/HEP), a stable coating was formed that completely covered the surface of the silicon wafer (Fig. 2g and Fig. 2h). In addition, it was found that increasing the concentration of the heparin solution from 1 to 5 mg/mL

was not essential as it did not result in a significant effect on the morphology and thickness of the multilayer films (no data provided).

Table 2. PlateSi Modifications Chitosan and Heparin

Nr (N)	Samples	Chitosan		Time of polysaccharide (monolayer) processing (min)	Number of bilayers (n)	Time of plasma processing (min)
		MW (kDa)	DD, DQ* (%)			
C	Control (PlateSi)	–	–	–	–	–
1	PlateSi$(CS/HEP)_3$	65	90	5	3	15
2	PlateSi $(CS/HEP)_3$	65	90	15	3	15
3	PlateSi $(CS/HEP)_5$	65	90	5	5	15
4	PlateSi $(CS/HEP)_5$	65	90	15	5	15
5	PlateSi $(QCS/HEP)_3$	20	98*	15	3	15
6	PlateSi $(QCS/HEP)_5$	20	98*	15	5	15
7	PlateSi $(CS/HEP)_3$	65	90	15	3	2
8	PlateSi $(CS/HEP)_3$	65	90	15	3	5
9	PlateSi $(CS/HEP)_7$	65	90	15	7	15
10	PlateSi $(CS/HEP)_9$	65	90	15	9	15
11	PlateSi $(CS/HEP)_3$	200	95	15	3	15
12	PlateSi $(CS/HEP)_5$	200	95	15	5	15
13**	PlateSi $(CS/HEP)_5$	200	95	15	5	15
14	PlateSi $(QCS/HEP)_3$	200	92*	15	3	15
15	PlateSi $(QCS/HEP)_5$	200	92*	15	5	15

**Heparin concentration used for sample 13 was 5 mg/mL. For all other samples (1–12, 14, 15), a heparin concentration of 1 mg/mL was used.

3.5. Relationship Between the Area of Blood Clots in the Plate Surface Images and the Optical Density of the Solutions Obtained after Incubation with the Plates

Surfaces completely covered with blood clots of different sizes were observed after incubation of control PlateSi (number C, Table 2) samples with human blood (stabilized by sodium citrate) for 20 and 40 min. The coverage density increased with increasing incubation time (Fig. 4a and Fig. 4d). Blood clot areas were smaller on treated with chitosan and heparin surfaces PlateSi (CS/HEP)3 (Fig. 4b,c,e,f).

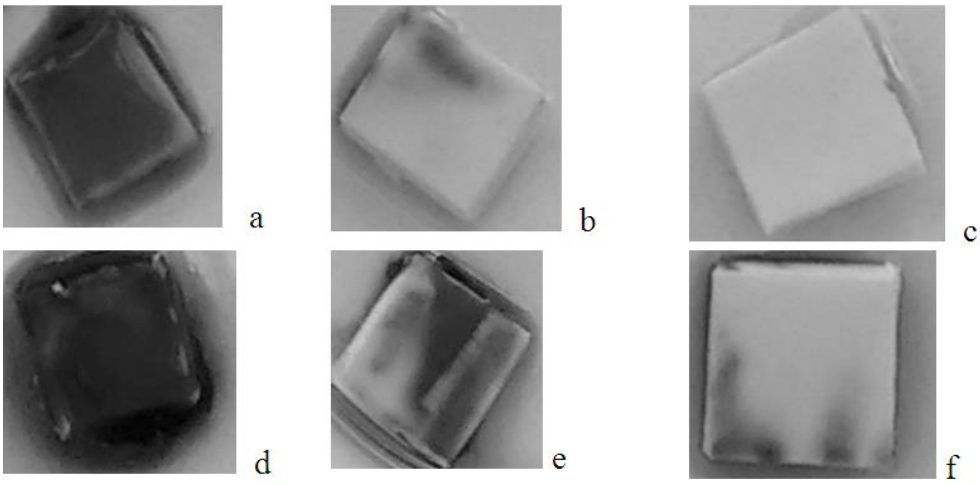

Figure 4. Control PlateSi (it's the samples number C in Table 2) – a, d; CS – chitosan; Hep – unfractionated heparin; Number 1 in Table 2 - b, e; Number 2 in Table 2 – c, f; the incubation time in blood: a, b, c – 20 min и d, e, f – 40 min.

Table 3. The blood clot areas in PlateSi images after incubation with human blood and the optical density of the haemolysis solution after incubation of PlateSi in it with clots.

Nr	The incubation time in blood (min)			
	20		40	
	Area (pixel)	OD (relative unit)	Area (pixel)	OD (relative unit)
C	0.5422±0.0441	0.235±0.047	0.9368±0.0459	0.394±0.043
1	0.3223±0.0921	0.064±0.014	0.8148±0.0431	0.246±0.021
2	0.1620±0.0839	0.037±0.010	0.4983±0.0728	0.141±0.019
3	0.0747±0.0178	0.034±0.010	0.5477±0.0880	0.176±0.018
4	0.0407±0.0068	0.016±0.005	0.0914±0.0290	0.054±0.014
5	0.2995±0.0576	0.064±0.014	0.6880±0.0751	0.250±0.026
6	0.1575±0.0336	0.044±0.013	0.5508±0.1054	0.238±0.044
7	0.1253±0.0671	0.016±0.010	0.5770±0.0808	0.120±0.045
8	0.0674±0.0110	0.017±0.007	0.5610±0.1557	0.135±0.045

Note: OD is the optical density of the solution.

To confirm the visual assessment of the stability of the studied materials to the appearance of blood clots on the surface, the blood clot areas shown on the images of PlateSi after incubation in the human blood were compared with the optical density of the solution in which PlateSi samples were placed with clots already appearing during incubation with blood (Table 3).

We observed a close positive correlation between the results obtained by the optical density of the PlateSi images after incubation with human blood and the optical density of the haemolysis solution after incubation of PlateSi in it with blood clots ($r=0.9341$, $p=0$, $y=0.3642\ x–0.0161$).

3.6. Relationship Between PlateSi Thromboresistance and the Surface Modification Conditions and the Incubation Time in Blood

The resistance of the surfaces to the appearance of blood clots (thromboresistance) after incubation of PlateSi with and without blood clots was calculated (see section 2.0) as a percentage, based on the optical densities of the haemolysis solution.

PlateSi samples 1–4, 9, and 10 (chitosan with a MW of 65 kDa was used for LbL modification) demonstrated high thromboresistance from 68.08±8.46% to 93.27±2.72% after 20 min of incubation with human blood and from 34.2±7.64% to 86.00±1.67% after 40 min of incubation. The thromboresistance of silicone plates without coating was 0±0% (Table 4). An increase in incubation from 20 to 40 min led to a significant decrease in thromboresistance ($p=0.006 – 0.037$), the exception was the most effective PlateSi sample number 4 PlateSi (CS/HEP)5 sample in Table 2.

After 40 min of incubation with blood, the values of thromboresistance plates with different time of polysaccharide (monolayer) processing 5 min for number 1 in Table 2 and 15 min for number 2 in Table 2 were 34.20±7.64% and 61.80±5.86%, respectively (Table 4); the values of thromboresistance plates with different time of polysaccharide (monolayer) processing 5 min for number 3 in Table 2 and 15 min for number 4 in Table 2 were 50.88±8.33% and 83.81±6.43% respectively. The significance of the differences of the readings for different time of polysaccharide (monolayer) processing samples reached $p = 0.025$ and $p = 0.018$, respectively. Hence, an increase in the processing time with polysaccharides led to an increase in thromboresistance.

An increase in the number of layers to 5 led to a significant increase in thromboresistance when comparing N2 PlateSi $(CS/HEP)_3$ and N4 PlateSi $(CS/HEP)_5$ samples during 40 min of incubation with blood ($p=0.045$) (Table 4) when the same treatment time with polysaccharides (15 min) was applied. A further increase to 7 and 9 heparin layers (samples N9 and N10, respectively) did not lead to a significant increase in thromboresistance. Thromboresistance N10 PlateSi $(CS/HEP)_9$ plates remained high at 20, 40, and 60 min of incubation with blood and did not decrease significantly (84.04±2.03%, 86.00±1.67%, and 75.31±6.67%, respectively).

Table 4. Thromboresistance (%) of PlateSi samples with different numbers of chitosan (MW of 65 kDa)/heparin layers at different incubation times with human blood.

Number (N)	Incubation time with blood (min)		
	20	40	60
C	0±0	0±0	0±0
1	68.08±8.46	34.20±7.64	nd
2	79.63±6.38	61.80±5.86	nd
3	85.13±4.00	50.88±8.33	nd
4	93.27±2.72	83.81±6.43	nd
9	80.42±3.55	77.53±3.31	66.13±7.48
10	84.04±2.03	86.00±1.67	75.31±6.67

Note: nd=not defined. N – samples numbers in Table 2.

The incubation of N9 PlateSi-$(CS/HEP)_7$ plates resulted in a slight but significant ($p=0.045$) reduction of thromboresistance from 80.42±3.55% (20 min) to 66.13±7.48% (60 min). Finally, after 40 min of incubation, sample N10 (9 layers, $p=0.025$) was found to be more beneficial in comparison with the N9 sample (9 layers) (Table 4).

Table 5 demonstrates that additional pre-treatment of the silicon rubber plates in reactive-ion etching mode of the plasma chemical reactor (a-technique in Table 5) did not result in the reduction of thromboresistance for N7 and N8 samples produced when the reactor operated in plasma etching mode (b-technique in Table 5). Peculiarities of these modes are given in Section 2.1. In addition, experiments on contact angle measurements showed that the increase in the wettability depends on the plasma treatment duration in a threshold manner. At the beginning (approximately the initial 2–3 min of the treatment), the wettability did not change and remained equal to the wettability of the untreated samples. It then sharply increased, but after the increasing stage the wettability increase stopped. Thus, optimal conditions for time-saving processes of plasma treatment are found to obtain sufficiently high adhesion of a silicon rubber substrate with the upper layers of thrombus-resistant PlateSi compositions.

Table 5. Influence of cold plasma treatment on thromboresistance (%) in three-layer modification of chitosan (65 kDa) and heparin on PlateSi surface.

Number (N)	**Action time of plasma to plate (min)**	**Incubation time with blood (min)**	
		20	**40**
C	0	0±0	0±0
7	2[a]	90.07±7.30	72.82±9.45
8	5[a]	89.53±4.43	67.88±10.66
2	15[b]	79.63±6.38	61.80±5.86

Note: [a]a technique, n=4. [b]b technique, n=6. N – samples numbers in **Table 2.**

Use of chitosan with a MW of 200 kDa for LbL modification showed a significant increase in thromboresistance after 40 and 60 min compared with 20 min of incubation. p_{40}=0.004 and p_{60}=0.01 for N11 PlateSi $(CS/HEP)_3$ plates, p_{40}=0.016 and p_{60}=0.004 for N12 PlateSi $(CS/HEP)_5$ plates, and p_{40}=0.01 and p_{60}=0.004 for N13 PlateSi $(CS/HEP)_5$ plates (Table 6). This increase in thromboresistance can be explained by a lower rate of growth of blood clots on the surface of the plates, in comparison with the plates, the surface of which included chitosan with MW 65 kDa. No significant effect of the number of layers or the amount of heparin on thromboresistance was found.

Table 6. Thromboresistance (%) of PlateSi samples with three or five chitosan (MW of 200 kDa) - heparin layers at different incubation times with human blood

Number (N)	**Incubation time with blood, min**		
	20	**40**	**60**
C	0±0	0±0	0±0
11	45.67±11.37	82.75±2.07	87.30±3.31
12	66.75±2.56	80.68±3.61	90.18±1.34
13	56.48±6.00	80.93±2.66	88.40±1.00

Note: N – samples numbers in Table 2.

The treatment of plates with QCS was important due to the increase in the positive charge of such compounds (see Section 3.3.). The stronger bond between the polysaccharide layers in LbL modification was expected. Table 7 shows the thromboresistance values of plates with QCS (MWs of 20 and 200 kDa) with LbL

modification. It should be noted that the thromboresistance value of N14 PlateSi $(QCS200/HEP)_3$ and N15 PlateSi $(QCS200/HEP)_5$ samples reached 66.28±7.26% and 86.48±4.73%, respectively, after 40 min of incubation with blood and significantly exceeded the thromboresistance value of N5 PlateSi $(QCS20/HEP)_3$ and N6 PlateSi $(QCS20/HEP)_5$ plates (chitosan with a MW of 20 kDa was used for LbL modification). In addition, high thromboresistance values of N14 and N15 samples after 40 and 60 min of incubation with blood did not differ from the thromboresistance after 20 min of incubation. The use of LbL modification of PlateSi plates of chitosan of 200 kDa instead of chitosan of 20 kDa led to an increase in the thromboresistance duration.

Table 7. Influence of MW of QCS (20 kDa and 200 kDa) on thromboresistance (%) of PlateSi with 3- and 5-layer surface modification.

Number (N)	Incubation time with blood (min)		
	20	**40**	**60**
C	0±0	0±0	0±0
5	71.42±5.42	42.64±2.46	nd
6	77.68±8.35	47.56±9.46	nd
14	72.47±8.58	66.28±7.26*	70.20±6.20
15	71.10±8.60	86.48±4.73*	81.50±5.75

Note: *$p_{5–14}$<0.05, $p_{6–15}$<0.05. nd=not defined. N – samples numbers in Table 2.

3.7. Clotting Time of Platelet-Poor Human Plasma After *In Vitro* Incubation with PlateSi in the APTT Test

To increase the biocompatibility and reduce the thrombogenic potential of the steel stent surface, Li et al. immobilized microspheres based on heparin/poly-L-lysine conjugates [34]. Incubation of such stents with human plasma resulted in increased coagulation time in activated partial thromboplastin time (APTT) and thrombin time tests. This may indicate that the microspheres with anticoagulant activity "disappeared" from the surface of the stent and the time of fibrin plasma clot appearance increased accordingly.

The clotting time of platelet-poor human plasma after incubation with N2 and N11 samples was estimated to proof the strength of applying layers of chitosan/heparin polysaccharides to the surface of silicone plates, which included chitosans with different MWs (65 and 200 kDa respectively). No differences between the control and treated plates in the analysis of plasma coagulation time after incubation for 20, 40, and 60 min (Table 8) was observed. According to the test results, even after 60 min of incubation, heparin from the surface of the treated plates did not enter the plasma, regardless of the MW of the chitosan used.

Table 8. The effect of *in vitro* incubation of the PlateSi (chitosan with a MW of 65–200 kDa, with 3-bilayer modifications) on the plasma clotting time in the APTT test (s).

Number (N)	Incubation time with plasma (min)		
	20	**40**	**60**
C	24.99±1.52	23.23±1.42	30.48±2.18, P vs 20 min=0.016
2	23.63±1.67	21.98±1.08	28.62±2.05
11	24.91±1.00	21.72±1.07	30.98±2.05, P vs 20 min=0.01

Note: N – samples numbers in Table 2.

3.8. Effect of *In Vitro* Incubation of PlateSi with a Suspension of Human Erythrocytes on Haemolysis of These Cells

Evaluation of the influence of materials on the stability of the erythrocyte membrane (haemolysis) is an important component of their hemocompatibility [9,10].

The most promising PlateSi samples with high thromboresistance were chosen for the analysis. The optical density of the solutions obtained after centrifugation of a suspension of washed erythrocytes, previously incubated with the N4 PlateSi $(CS/HEP)_5$ (65 kDa chitosan; thromboresistance, 20 min, 93.27±2.72%), N10 PlateSi $(CS/HEP)_9$ (65 kDa chitosan; thromboresistance, 20 min, 84.04±2.03%) and N11 (200 kDa chitosan; thromboresistance, 60 min, 87.30±3.31%) samples, are given in **Table 9**. The incubation of the erythrocyte suspension with N10 and N11 samples resulted in a significant decrease in the optical density of the solution ($p=0.004$ and $p=0.025$, respectively) in comparison with the optical density of the solution obtained as a result of incubation with the control sample (C). Sample N10 contributed to erythrocyte haemolysis (6.71±1.42%, 8.52±1.58%, and 13.00±0.54%, respectively) to a lesser extent than sample N11 and the control samples (C). It should be noted that the increase in the polysaccharide layers (65 kDa chitosan - heparin) for LbL modification from 5 to 9 leads to a decrease in the destroying effect of red blood cells.

Table 9. Optical density of the solution obtained after centrifugation of the suspension of washed human erythrocytes pre-incubated with PlateSi samples (chitosan of 65 kDa and 200 kDa for 3-, 5-, and 9-bilayer modifications).

Number (N)	Optical density of the solution (545 nm) (relative unit)
C	0.5981±0.0228
4	0.4767±0.0660
10	0.3522±0.0540, P vs C=0.004
11	0.4228±0.0500, P vs C=0.025
K "-"	0.0897±0.0053, P vs C=0.004
K "+"	4±0, P vs C=0.004

Note: K "-"– negative control,10 mM phosphate buffer was added to the erythrocyte suspension. K "+" – positive control, distilled water was added to the erythrocyte suspension. N – samples numbers in Table 2.

3.9. Effect of PlateSi Samples on the Adhesion of Human Platelets *In Vitro*

The contact between the blood and the surface of a biomaterial can lead to activation, adhesion, platelet aggregation, and subsequent appearance of blood clots. Therefore, analysis of the effect of biomaterials on platelet adhesion is also an important part of hemocompatibility tests [1–3].

PlateSi samples with QCS (20 and 200 kDa) modified by the LbL method were selected for analysis. **Table 10** shows the results of the influence of N6 PlateSi $(QCS20/HEP)_5$ (thromboresistance, 20 min, 77.68±8.35%) and N15 PlateSi $(QCS200/HEP)_5$ (thromboresistance, 20 min, 71.10±8.60%)$_5$ samples on the optical density of the solution containing human platelets after 120 min of incubation. Incubation with sample N15 led to a significant ($p=0.009$) increase in the optical density of the solution containing platelets in comparison with the control (C). Such an increase in the optical density of the analysed solution accounted for the fixation of a smaller

number of platelets on the surface of the material. Consequently, plates treated with QCS with a higher MW showed lower adhesive activity against platelets.

Table 10. The effect of PlateSi on the optical density of a solution containing human platelets (n=12).

Number (N)	Optical density of the solution (800 nm) (relative unit)
C	0.1803±0.0129
6	0.1988±0.0099
15	0.2276±0.0104; P vs C=0.009

Note: N – samples numbers in Table 2.

4. Conclusions

Sequential application (after 15 min of cold plasma treatment of the material surface) on the surface of the silicone plates of 3, 5, 7, and 9 bilayers of variously charged chitosan (MW of 65 kDa) and unfractionated heparin led to an increase in the resistance of the material compared to the control, with respect to the appearance of blood clots (thromboresistance) *in vitro*. The use of LbL modification of the surface of chitosan plates with a MW of 200 kDa or QCS chitosan with a MW of 200 kDa led to an increase in the time of appearance of blood clots on the surface. Samples of the treated material with high thromboresistance contributed to platelet adhesion and erythrocyte haemolysis to a lesser extent than control plates. The following samples were the most promising: N10 (65 kDa chitosan; the influence on haemolysis of red blood cells decreased by increasing the number of layers), N11 (200 kDa chitosan; the time of thromboresistance increased despite the smallest number of layers), and N15 (QCS of 200 kDa; the reduction in the adhesion of platelets was shown).

Plasma chemical pre-treatment of the original silicon plates by cold plasma of the radiofrequency discharge in oxygen significantly improved adhesion between substrate and upper coating polysaccharide layers due to the improvement in substrate surface wettability. Plasma chemical reactor in both reactive-ion etching configuration and the plasma-etching configuration can be applied for the pre-treatment; however, the former configuration is preferred.Pre-treatment regimes, including the duration, can be optimized taking into account the characteristics of the plasma-stimulated surface modification kinetics.

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