

Original article

Changes in the acid resistance of erythrocytes upon contact with TiC, TiN, Ti-Nb, Ti-Zr and Ti-Ta compositions

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Abstract: *Background* — There is a high demand for new implant materials in traumatology, orthopedics and dentistry. Biodegradation of titanium alloys leads to undesirable effects that reduce the quality of life in patients with implants. Metal ceramics titanium carbide and titanium nitride, as well as binary alloys (titanium-niobium, titanium-tantalum and titanium-zirconium) applied to implants by electrospark deposition (ESD), improve their performance, while their biosafety has not been fully confirmed.

Objective — To study the effect of ESD coatings of titanium nitride, titanium carbide, titanium-niobium, titanium-tantalum and titanium-zirconium on the course of acid hemolysis induced by HCl.

Methods — To test biocompatibility, coating samples on Ti6Al4V alloy substrates were incubated with a suspension of erythrocytes. After incubation, the suspension was subjected to a test for HCl-induced hemolysis with photometric recording of hemolysis dynamics parameters and calculation of erythrocyte subpopulation volumes.

Results — HCl-induced hemolysis was more forceful after contact of erythrocytes with uncoated Ti6Al4V alloy, titanium carbide and titanium nitride coatings. The maximum hemolysis rate increased, and the onset and end points of hemolysis shifted. Titanium-niobium, titanium-tantalum and titanium-zirconium coatings were not completely inert, but the demonstrated reactivity did not exceed the parameters of uncoated Ti6Al4V.

Conclusion — Based on the obtained results, a pronounced membranotropic effect of ESD coatings made of titanium carbide and titanium nitride was determined. The effect of coatings made of titanium-niobium, titanium-tantalum and titanium-zirconium alloys is limited and allows them to be considered as hemocompatible and biocompatible coatings.

Keywords: erythrocytes, hemolysis, biocompatibility, alloys, implants.

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Introduction

Titanium alloys are widely used in medical devices, particularly in traumatology, orthopedics and dentistry. For surgical purposes, patients may be implanted with pins, plates, screws, distraction implants, joint prostheses, dental abutments and bridges and many other devices. The suitability of implants is determined by the balance of technological qualities and biocompatibility. Insufficiently biocompatible implant materials may provoke direct and indirect toxic effects, oxidative stress, apoptosis, necrosis, tumor transformation, and cell differentiation disorders. Possible causes include tissue infiltration with diffusion and oxidation products, inflammation, hyper- or hypotrophy, formation of fibrous conglomerates (capsules), osteolysis, secondary fractures (bone fracture by the implant), aseptic instability, peri-implantitis, and allergic reactions [1-3]. Signs of insufficient biocompatibility may manifest as individual effects or specific symptom complexes.

These phenomena include titanium metallosis (yellow nail syndrome), which is the accumulation of titanium metabolism products in the skin, nail plates and hair. It is also worth noting the potential risk of neurotoxicity of titanium dioxide nanoparticles released from the alloy during operational wear and tear [4-6].

In recent years, protective coatings of medical devices were used to protect the body from the adverse effects of implants. It is assumed that a layer of inert and/or biocompatible material isolates the patient's body from a foreign body. However, coatings are not free from the initial problem of wear and tear, which over time leads to exposure of the alloy surface and its inclusion in the development of pathological processes. This occurs due to abrasion, peeling, or cracking of coatings. It seems reasonable to improve the coatings per se in these parameters.

Coatings made of binary titanium alloys applied by electrospark deposition (ESD) represent a solution to the problem,

since they are both wear-resistant and technologically advanced. They have specific composition and distribution of elements in binary compositions, and also form a developed surface relief. These factors are important for cell survival, adhesion, proliferation and differentiation. It is important that the coatings are biocompatible. In our experiment, we tested coatings made of binary alloys such as titanium-niobium (Ti-Nb), titanium-zirconium (Ti-Zr), titanium-tantalum (Ti-Ta), as well as of metal ceramics such as titanium carbide (TiC) and titanium nitride (TiN), since their components individually have a satisfactory degree of biocompatibility.

The objective of our research was to study the effect of ESD coatings made of titanium nitride, titanium carbide, titanium-niobium, titanium-tantalum and titanium-zirconium on the course of acid hemolysis induced by HCl.

Material and Methods

Making coatings

For biocompatibility testing, samples were made using an original modification of the ESD technology [7]. The samples were Ti6Al4V alloy washers ($d=12$ mm, $h=2-4$ mm) coated on the outside with TiN, TiC, Ti-Nb, Ti-Ta and Ti-Zr compositions, 5 pieces of each type, that is 30 in total.

Groups

Based on the coating composition, study groups were formed as follows: uncoated (Ti6Al4V), coated (TiN, TiC, Ti-Nb, Ti-Ta, Ti-Zr), comparison (CG), and control (CL).

Preparation of erythrocyte suspension

The membranotropic effect of the materials was assessed in the Terskov-Gitelson hemolytic test [8, 9]. For testing, blood was aseptically collected from the tail veins of six white Wistar rats

(0.75 mL from each). A suspension of erythrocytes was prepared from the blood by diluting with 0.9% NaCl until an optical density of 0.700 units at $\lambda = 670$ nm was achieved. For the test with one type of material, a suspension obtained from one animal was used. To exclude the influence of individual characteristics of animals, the suspension was divided into 20 portions of 2 mL as follows: 5 portions of the intact suspension were immediately sent for hemolysis as a control material (CL); the remaining suspension was divided into 5 parallel incubation series of 3 portions each, where 2 portions were incubated with samples (Ti6Al4V/TiC/TiN/Ti-Nb/Ti-Zr/Ti-Ta), and one portion was incubated without samples (CG). Incubation lasted 180 min at a temperature of 24 ± 0.1 °C. The incubation duration was limited by the maximum shelf life of the suspension. The incubation temperature was standard for this method, which allowed balancing the reaction rate with the possibility of collecting detailed data [9]. Resuspension was carried out before hemolysis. The procedure was repeated for each new animal-material combination. A total of 120 reactions were carried out in this way.

Induced hemolysis test

The hemolytic agent was 0.004 N HCl solution in 0.9% NaCl. Hemolysis was carried out in 4 mL photometric cuvettes, where equal volumes of suspension and hemolytic agent were taken. Kinetic photometry was performed with 15 s sampling at $\lambda=670$ nm and $L_{opt.dens.}=10$ mm on a UV-1100 spectrophotometer (Ecoview, China).

We used the obtained data to plot hemolysis rate curves (Figure 1), from which the starting (t_{start}) and ending (t_{end}) times of hemolysis were determined, the hemolysis rate (V_{max}) was estimated, and the proportions of erythrocyte subpopulations were calculated. The V_{max} values were selected from the V_t values. V_t was calculated as the percentage of optical density change for every 15 seconds.

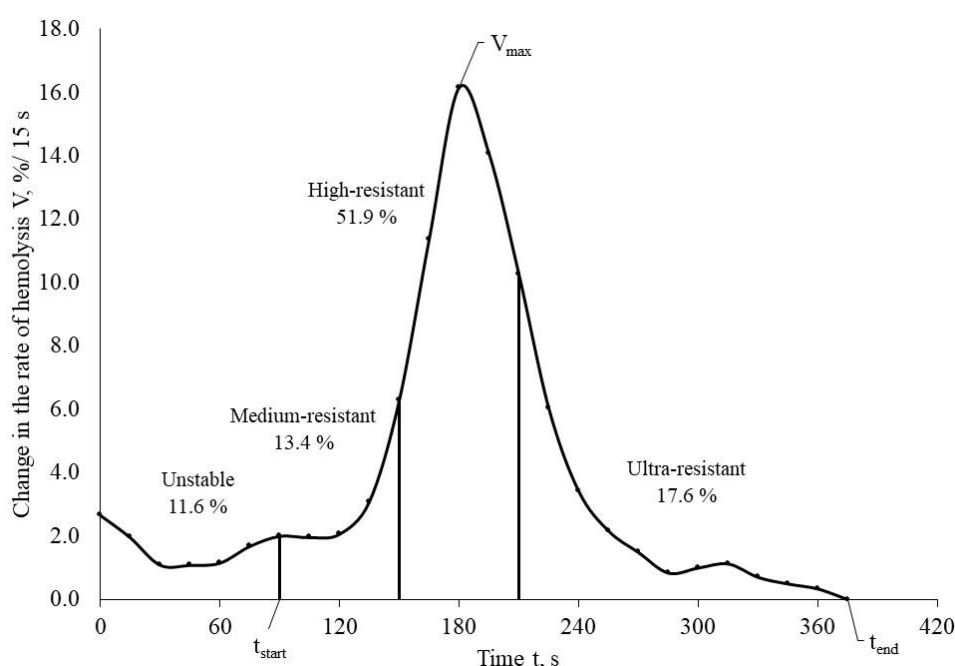


Figure 1. Typical acid hemolysis rate change curve with subpopulation time boundaries.

Table 1. Parameters of acid hemolysis in the study groups

	t_{start}, s		t_{end}, s		$V_{max}, \%/15 s$	
	Me	[Q ₂₅ ; Q ₇₅]	Me	[Q ₂₅ ; Q ₇₅]	Me	[Q ₂₅ ; Q ₇₅]
Control group	90.0	[90.0; 105.0]	315.0	[300.0; 345.0]	16.9	[16.2; 18.9]
Comparison group	60.0	[45.0; 75.0]	225.0	[210.0; 300.0]	28.7	[23.2; 30.3]
*		0.000017		0.000005		0.000003
Ti6Al4V	90.0	[78.8; 90.0]	300.0	[273.8; 315.0]	25.8	[25.0; 26.1]
*		0.075015		0.054740		0.000005
**		0.006580		0.006897		0.125893
TiN	52.5	[45.0; 60.0]	225.0	[183.8; 255.0]	28.0	[24.6; 36.2]
*		0.000468		0.000004		0.000004
**		0.628288		0.684705		0.416733
***		0.007285		0.001706		0.198766
TiC	120.0	[108.8; 120.0]	285.0	[285.0; 296.3]	24.2	[23.3; 25.4]
*		0.049093		0.000178		0.000006
**		0.000215		0.142096		0.125893
***		0.015565		0.256840		0.082100
Ti-Nb	90.0	[67.5; 90.0]	300.0	[285.0; 315.0]	18.3	[16.2; 18.8]
*		0.072496		0.035002		0.381807
**		0.045607		0.028785		0.000122
***		0.969850		0.879829		0.003197
Ti-Ta	90.0	[78.8; 101.3]	330.0	[273.8; 345.0]	17.3	[16.2; 26.2]
*		0.390366		0.742938		0.472512
**		0.008307		0.000983		0.019150
***		0.520523		0.289919		0.150928
Ti-Zr	90.0	[90.0; 90.0]	300.0	[300.0; 300.0]	17.9	[17.8; 18.5]
*		0.260823		0.011406		0.159855
**		0.004937		0.019966		0.000011
***		0.405680		0.939743		0.000157

* p-value for comparison with the control group; ** p-value for comparison with the comparison group; *** p-value for comparison with the Ti6Al4V group.

The erythrocyte subpopulation values were determined by the ratio of the optical density change over the time allotted to the subpopulation to the total change in optical density. A total of 4 erythrocyte subpopulations were identified: nonresistant (0-90 s), moderately resistant (105-150 s), highly resistant (165-210 s), and ultra-resistant cells (more than 210 s). The subpopulation size was determined by the sum of optical density changes over the subpopulation existence interval.

Statistical data processing

Statistical analysis using Statistica 10.0 (StatSoft, USA) software was performed to determine the statistical significance of the detected differences. The distribution was checked for normality by the Shapiro-Wilk test. The significance of differences between groups was determined by the Mann-Whitney test. Differences were considered significant at $p < 0.05$. Values of t_{start} , t_{end} , and V_{max} are presented as medians and quartiles (Me [Q₁; Q₃]), and subpopulation shares are presented as percentages.

Results

The measurements carried out during the experiments revealed various phenomena. The Ti6Al4V alloy without coatings brought the red blood cells to significantly later boundaries of the onset and end of the hemolysis process than the parameters of the CG (33.3% higher t_{start} and t_{end} values), and mediated a higher hemolysis rate compared with the intact suspension of CL group (52.7% higher V_{max}).

Cells incubated in the presence of TiN-coated samples were lysed without significant differences from CG, although the differences with CL group were greater (t_{start} occurred earlier by 41.7%, t_{end} occurred earlier by 28.6%, and V_{max} was 52.7% higher).

There were also differences in the hemolysis time relative to the Ti6Al4V group (uncoated), with hemolysis starting and ending earlier by 41.7% and 33.3%, respectively.

Differences in the course of hemolysis caused by the presence of TiC during the incubation of the erythrocyte suspension were also noticeable vs. CL group. A 33.3% later t_{start} , 9.5% earlier t_{end} , and 43.2% higher hemolysis rate were discovered. Only t_{start} changed relative to CG and Ti6Al4V by 50% and 33.3%, respectively.

Ti-Nb alloy coating resulted in an earlier cessation of hemolysis, t_{end} occurred 4.8% earlier compared with the same indicator in CL group. Much more pronounced differences were shown compared with comparison group: t_{start} and t_{end} increased by 33.3%, and the hemolysis rate V_{max} declined by 63.8%. The Ti-Nb and Ti6Al4V groups did not differ from each other in hemolysis timing, but the maximum hemolysis rate for Ti6Al4V was 29.1% higher.

Addition of Ti-Ta coated samples to the incubation medium did not result in significant differences in subsequent HCl-induced hemolysis vs. the intact group (CL). However, the differences in t_{start} , t_{end} , and V_{max} relative to CG were large. Induced hemolysis started 33.3% later, ended 46.7% later, and the rate was 60.3% lower. There were no significant differences in hemolysis dynamics in the Ti6Al4V group.

Ti-Zr coatings affected the hemolysis ending time when comparing the t_{end} value with that of the CL group: the difference was -4.8%. More pronounced differences were observed relative to CG: t_{start} and t_{end} were 33.3% later, and V_{max} was 37.6% lower. Ti-Zr coatings differed from uncoated Ti6Al4V by -30.6% in their effect on hemolysis V_{max} .

The acid hemolysis parameters by groups are presented in [Table 1](#).

Based on the constructed curves of acid erythrograms, the proportions of erythrocyte subpopulations were calculated in terms of their resistance to hemolytic action. We discovered that incubation of the erythrocyte suspension for 3 hours more than doubled the subpopulation of nonresistant erythrocytes and reduced the moderately resistant subpopulation. Introduction of uncoated Ti6Al4V samples into the suspension leads to an increase in the proportion of nonresistant cells and a decrease in the ultra-resistant subpopulation, while the moderately resistant subpopulation remains virtually unchanged. The proportion of moderately resistant cells was higher compared with the CG. Incubation of the erythrocyte suspension with TiN coatings led to the formation of the largest proportion of nonresistant cells. The ultra-resistant subpopulation did not disappear, but increased compared with CL group and CG, but not with Ti6Al4V. The structure of erythrocyte subpopulations in the TiC group was similar to that of CL group, but an increase in the proportion of moderately resistant cells was observed due to a decrease in the highly resistant subpopulation. There were no ultra-resistant cells. The highly resistant subpopulation predominated relative to the Ti6Al4V group. A similar result was characteristic of the Ti-Zr group. Similarity in the ratios of subpopulations was observed in the Ti-Nb and Ti-Ta groups. A higher proportion of nonresistant cells and a reduced proportion of highly resistant cells were noted compared with CL group. There were no ultra-resistant cells. The structure of erythrocyte subpopulations is shown in [Figure 2](#).

Discussion

The study assessed and compared the parameters of induced hemolysis after incubation of erythrocyte suspension with ESD coating samples.

The literature provides information on surface and intracellular hemolytic targets that mediate the lysis algorithm and limit it at different stages. Hemolysis kinetics is determined by the

morphological and biochemical features of the erythrocyte cytolemma, which acts as a target for organic and mineral toxicants. Deviations in hemolysis parameters indicate a change in the course of the processes described below. E.g., saturation of the extracellular environment with H^+ ions due to the introduction of hydrochloric acid causes changes in the structure of membrane proteins in the direction from the outside to the inside [10, 11]. In the context of enhanced hemolysis, denaturation of ion pumps (Na^+/K^+ -ATPase), water channels (aquaporin-1), glycophorin B, band III protein, and ankyrin-1 is of interest. This triggers an uncontrolled flow of hydrogen ions into the cell without compensation by Na^+ , K^+ and Ca^{2+} flows, as well as the development of a functional overload of proton pumps (V-ATPases) [12]. Denaturation of the glycophorin B – band 3 anion transport protein – ankyrin-1 complex mediates the destruction of the actin-spectrin skeleton bond with the cytolemma and the erythrocyte swelling under the action of osmotic pressure. Saturation of the cytoplasm with H^+ is associated with the effect of hemoglobin protonation, which serves as a buffer factor prolonging the hemolysis and providing short-term (30–90 s) pH compensation [13]. As a result, the erythrocyte swells, retains a spherical shape for some time, and is destroyed during decompensation.

Oxidative stress is a separate acting factor in the erythrocyte – solution system. The model erythrocyte suspension is not oxygenated in vitro in the same way as whole blood when passing through the lungs. Under such hypoxic conditions, the effect of oxyhemoglobin autooxidation becomes pronounced, which leads to the release of superoxide anions and the development of oxidative stress. The consequence of oxidative stress is the activation of p38 kinase, protein kinase C and phosphorylation of nonselective ion channels, as well as destabilization of the cytolemma [14].

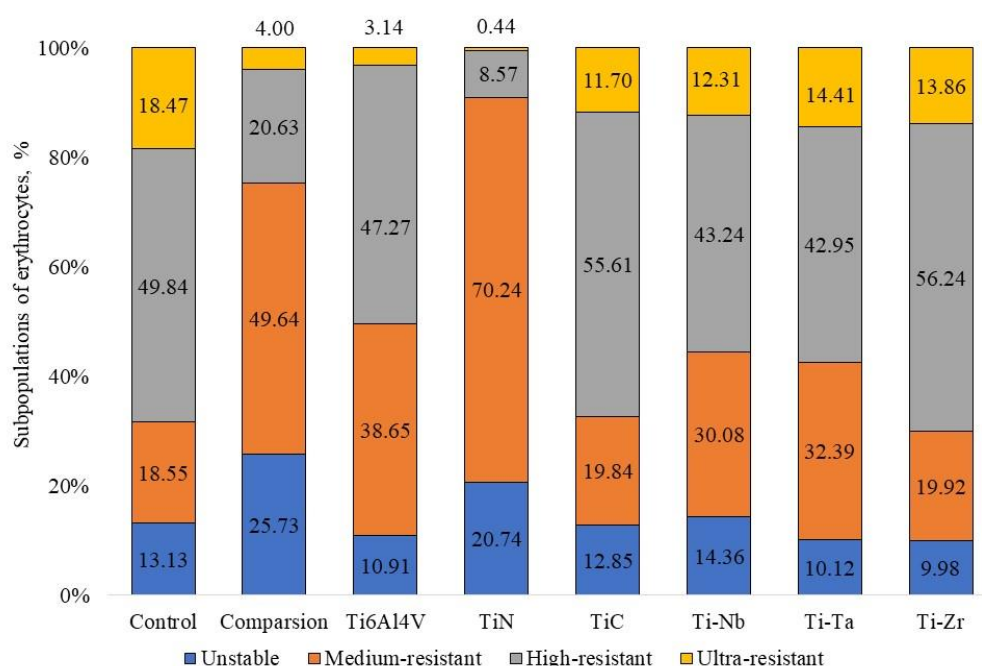


Figure 2. Distribution of erythrocytes within groups according to the degree of resistance to hemolysis.

An increase in the maximum hemolysis rate by 11.8% in the group subjected to 3-hour incubation without samples indicates the processes described above leading to the destabilization of erythrocyte membranes. The increase in the hemolysis rate is consistent with an increase in the subpopulations of nonresistant (+12.6%) and moderately resistant erythrocytes (+31.09%) due to a reduction in the proportion of cells with increased resistance (-29.24%).

The measurements confirm the presence of biological activity in the Ti6Al4V alloy without coatings. No differences in the time frames of hemolysis were shown, but the difference in V_{max} with CL group (52.6%) confirms toxicity [15]. At the same time, the combined effect of 3-hour incubation and contact of erythrocytes with any of the studied materials, except TiN, was expressed weaker than incubation alone. A single mechanism explaining such results was not described, but for some forms of titanium carbide, the ability to limit the development of oxidative stress was noted, and tropism for membranes with a positive charge was described [16]. It was previously shown that nanoforms of titanium nitride are active inducers of oxidative stress [17]. There is no information revealing the mechanism of interaction of binary alloys with erythrocyte membranes. However, the hemolytic effects for Ti-Nb, Ti-Ta and Ti-Zr were observed at the level of ending time effect [18-20]. In all cases, the intensity of hemolysis was at the detection limit, and in some cases, the hemolysis parameters were lower than those of Ti6Al4V taken as a comparison material. Thus, the reason for the observed cytoprotective effect remains unclear.

For TiN and TiC metal ceramics, there is information in the literature on their favorable biocompatibility [21, 22]. Increased cell adhesion, low cytotoxicity (by the ratio of live and dead cells), and high osteoconductivity in vitro were noted. Adverse phenomena of predominantly biomechanical rather than chemical/biological nature were previously described. However, the studied TiN and TiC coatings were not sufficiently inert. The TiN group exhibited the strongest membranotropic effect among all the studied compositions. Membranotropic effects, also observed in the TiC group, were of a clear nature. There are data on the weak hemolytic effect of titanium carbide powder [23]. The powder form is close to ESD coatings in terms of the developed surface area available for contact with the solution. Titanium nitride applied to a titanium sample in a similar experiment did not demonstrate a hemolytic effect [22]. The observed enhancement of induced hemolysis by ESD-applied materials is apparently associated not with their composition, but with the features of the ESD technology of the protective coating, which creates a developed relief of the treated surface with a large specific area.

The magnitude of the effects created by Ti-Nb, Ti-Ta and Ti-Zr coatings in the erythrocyte – solution system did not exceed the effect of incubation alone and the combined effect of Ti6Al4V with incubation, which allows asserting biological compatibility and lower relative reactivity. After contact with the Ti-Ta alloy, erythrocytes did not exhibit statistically significant differences in hemolysis dynamics from those of the intact group (CL). Based on this, we can assume a limited redistribution of erythrocytes in subpopulations. Consequently, we suggest that the cells in contact with binary alloy coatings are less damaged by reactive oxygen species and experience weaker oxidative stress.

Conclusion

In summary, we conclude that the HCl-induced acid hemolysis test allows detecting early changes in the plasmalemma structure that occur under the influence of oxidative stress and exogenous factors. The revealed changes in the duration and end of hemolysis, as well as an increase in the maximum hemolysis rate caused by the incubation of erythrocytes with metal-ceramic TiN and TiC ESD coatings, are clearly destructive in nature and do not allow considering such coatings biocompatible. The change in the hemolysis dynamics under the influence of TiN is consistent with the change in the volumes of erythrocyte subpopulations. ESD coatings with binary Ti-Nb, Ti-Ta and Ti-Zr alloys do not have a more pronounced membranotropic effect than Ti6Al4V, while having better performance properties. Further consideration of Ti-Nb, Ti-Ta and Ti-Zr ESR coatings as materials for biomedical application is recommended.

Study limitations

We acknowledge that our study has some limitations. Although the erythrocyte cell membrane model is part of the first stage of the screening study, it has limitations in extrapolation. This model properly reflects short-term changes in the cytolemma, but caution should be exercised when transferring the results to other cell types and tissues over a longer period of time.

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Compliance with ethical standards

This study was approved by the Ethics Committee of Far Eastern State Medical University (No. 2 of 22 March 2023).

Conflict of interest

The authors declare no conflicts of interest.

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