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Bioactive Polymer-apatite Coatings with Antimicrobial Properties on Model Titanium Implants

L.F. Sukhodub[1,*], L.B. Sukhodub[1], M.O. Kumeda[1], A.P. Denysenko[1], M.I. Kravchenko[2]

[1] *Sumy State University, 2, Rymsky-Korsakov St., 40007 Sumy, Ukraine*
[2] *Sumy National Agrarian University, 160, Gerasim Kondratiev St., 40000 Sumy, Ukraine*

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Bioactive hydroxyapatite (HA) coatings containing zinc sulfide (ZnS) and alginate (Alg) were obtained on model titanium substrates by thermal substrate deposition (TSD) method. HA-based coatings provide implants for bioactivity and ZnS acts as an antimicrobial agent. It is proved that the introduction of Alg into the ZnS structure reduces the particle size, which contributes to the increased antimicrobial activity of the coatings. The antimicrobial activity of ZnS and ZnS/Alg layers was investigated by agar diffusion microbiological method. ZnS and ZnS/Alg provide the growth inhibition zones for E. coli ATCC No. 25922-5 and 11 mm, for S. aureus ATCC No. 25923-5 and 10 mm, for K. pneumonia No. 93-7 and 12 mm, respectively. The morphology and structure of the coatings were determined by X-ray diffraction (XRD), X-ray fluorescence analysis (RFA) and scanning electron microscopy (SEM). The obtained data confirmed the formation of a single-phase ZnS layer on the HA surface. The results presented underline the formation of biocomposite coatings with the functions of biocompatibility and antimicrobial activity.

Keywords: Zinc sulfide, Alginate, Hydroxyapatite, Antibacterial properties, Thermal substrate deposition.

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1. INTRODUCTION

Modification of the metal implants surface is one of the promising methods for improving the physicomechanical and biological properties of medical materials. To this aim, bioactive coatings of various compositions, in particular, based on calcium phosphates and biomolecules, are applied to the surface of metal implants. Among the calcium orthophosphates ceramics, hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$ is the most promising for metal implants coating due to chemical similarity to bone mineral [1]. Recently, hybrid nanocomposites based on natural polymers have attracted considerable attention due to their properties and successful application in biomedicine. Improvement of biocompatibility and bioactivity of the polymer based materials by their modification by metal nanoparticles has been reported [2].

Considering the serious risk of bacteria to human health, as well as their increasing resistance to antibiotics, particles and ions of inorganic origin are investigated as antibacterial agents [3, 4]. Among them, Zn^{2+} ions and zinc oxide (ZnO) particles are promising candidates for this problem because they do not exhibit excessive toxicity [5]. ZnS nanoparticles are characterized by their attractive properties and have a limited scope, especially in the pharmaceutical field [6].

Due to the fact that the mechanisms of antibacterial action of ZnS have not been studied sufficiently, and some contradictions of the obtained experimental data are present, there is a need for a more detailed study of the mechanisms of antibacterial action of these nanoparticles [7-9]. This study focused on the investigation of the ZnS nanoparticles contribution to the physical and biological properties of obtained coatings on titanium substrates. The antimicrobial activity of pure ZnS and ZnS deposited in the presence of sodium alginate (ZnS-Alg) was compared, and conclusions regarding the mechanism of their antimicrobial action were drawn.

2. MATERIALS AND METHODS

2.1 Materials

The following reagents were used in the experiment: zinc nitrate $Zn(NO_3)_2.6H_2O$, thiourea NH_2CSNH_2, calcium anhydrous chloride $CaCl_2$, orthophosphoric acid H_3PO_4, ammonia solution NH_4OH, sodium hydroxide NaOH, sodium fluoride NaF, sodium alginate (Alg) – (E401 dietary supplement, manufactured by China). The experiment used Gramm-positive bacteria *Staphylococcus aureus* (ATSC No. 25923 (F-49), and Gramm-negative microorganisms *Escherichia coli* (ATCC No. 25922 (F-80), *Klebsiella neumonia* (No. 93). The cell suspension was prepared from a 24-hour agar bacterial culture in meat-peptone broth (MPB). All reagents were of analytical grade.

2.2 Deposition of Bioactive Coatings of HA and ZnS on a Titanium Substrates

The bioactive coatings were deposited on a titanium substrate with a surface area of 0.02 dm^2, which was previously anodized at room temperature in an electrolyte of the following composition: sulfuric acid H_2SO_4 – 20 wt. %; sodium fluoride NaF – 0.5 wt. %. The anodizing process lasted for1 h at a given current density of 0.2 A/dm^2. A lead plate and a titanium substrate were used as the cathode and the anode, respectively.

The protective bioactive HA coating was deposited on the surface of the titanium substrate as the bottom layer in a 2-layer HA/ZnS coating by the method of thermal substrate deposition from aqueous solution according to the method described in our previous work [10].

* l.sukhodub@gmail.com

The formation of an antimicrobial ZnS coating consists in the deposition of a layer of zinc sulfide from an alkaline (*pH* about 11) colloidal solution containing ZnS nanoparticles. ZnS was deposited on the HA coating as the top layer. For this purpose, after completion of HA deposition stage, the HA-coated plate without detachment from the electrodes was thoroughly washed with deionized water and covered with zinc sulfide. The colloidal solution of ZnS nanoparticles was prepared as follows: 100 ml of 0.2 M thiourea solution was added to 100 ml of 0.2 M aqueous zinc nitrate solution $Zn(NO_3)_2.6H_2O$; 30 ml of aqueous 25 wt. % solution of ammonia (NH_4OH) was added to the resulting mixture. The titanium plate was immersed in this solution, through which an alternating electric current was passed for 30 min, whereby the titanium plate was heated to a predetermined temperature of 80-100 °C, which promoted the deposition of ZnS layer on the coating plate.

ZnS-Alg coatings were precipitated from the colloidal solution described above to which sodium alginate was added. To this, 2 ml of a 1 % solution of sodium alginate in 0.01M NaOH solution was added to 100 ml of a 0.2 M aqueous solution of zinc nitrate $Zn(NO_3)_2.6H_2O$. Subsequently, the technological conditions were similar to the ZnS coating formation conditions.

2.3 Instrumental Investigation Methods

The X-ray diffraction studies of the sample crystallographic structure were performed on the automatized diffractometer DRON 3 (LTD «Burevestnik», www.bourevestnik.ru). CuKα-radiation (wavelength 0.154 nm) with 2θ Bragg-Brentano geometry (2θ is the Bragg angle) was used. The values of current and voltage on the X-ray tube were 20 mA and 40 kV, respectively. The microelement composition of the samples was determined using an ElvaX Light SDD X-ray fluorescence spectrometer (Ukraine, Kiev). The spectrometer allows to determine the content of elements in the range from Na ($Z = 11$) to U ($Z = 92$).

The surface morphology of the samples was examined using a scanning electron microscope (SEM, REMMA-102) produced by SELMI (Ukraine). Images were made in the secondary electron mode with an accelerating voltage of 20 kV and a beam current of 1-10 A.

The antibacterial activity of the experimental samples was tested against gram-positive bacteria *S. aureus* (ATCC No. 25923) and gram-negative bacteria *E. coli* (ATCC No. 25922), *K. pneumonia* (No. 93) by modified diffusion in agar. The method involves placing the experimental samples on the surface of the agar inoculated with bacterial cultures. A daily culture with microorganism concentration of 1.5×108 CFU/ml (colony forming units) that equals to 0.5 units on the scale of Mc. Farland was used. Petri dishes with samples were incubated in a thermostat at 37 °C. The antibacterial properties were evaluated by measuring the growth inhibition zone of the microorganisms after 24 and 48 hours. The growth inhibition zone (in mm) reflected the antimicrobial susceptibility of the samples – the greater the inhibition zone, the greater the antimicrobial activity.

3. RESULTS AND DISCUSSION

The thermal substrate deposition method from aqueous solutions is based on the properties of zinc sulfide to reduce its solubility and to form a precipitate when the temperature of the solution increases [11]. Schematic representation of the processes of deposition of HA, ZnS and ZnS-Alg coatings is shown in Fig. 1.

Electron-microscopic images of HA, HA-ZnS and ZnS coatings are presented in Fig. 2. The images show the consolidation of the HA coating structure upon deposition of the top layer of zinc sulfide.

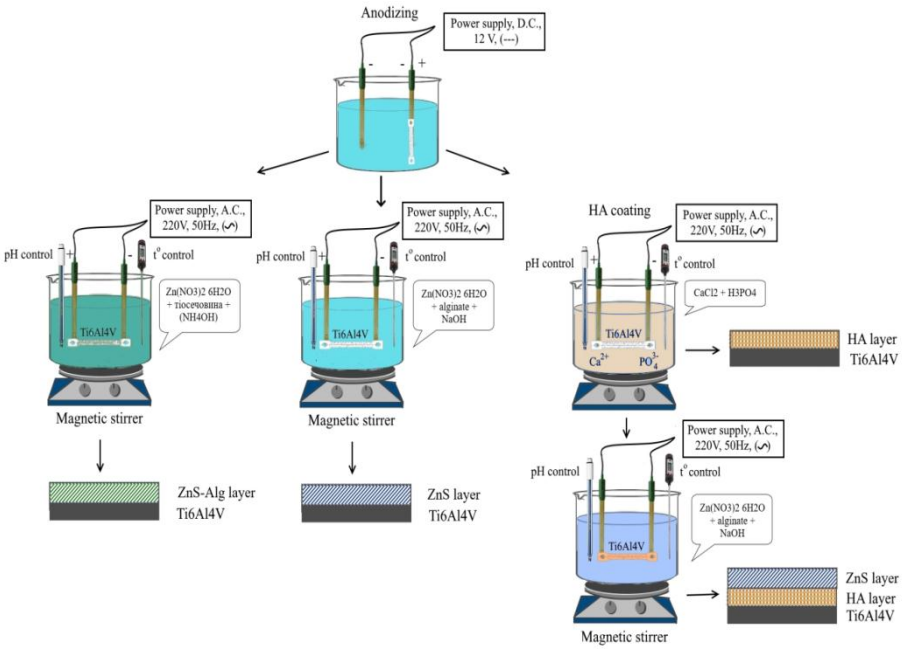

Fig. 1 – Scheme of obtaining of bifunctional HA, ZnS and ZnS-Alg coatings on model titanium implants by thermal deposition method

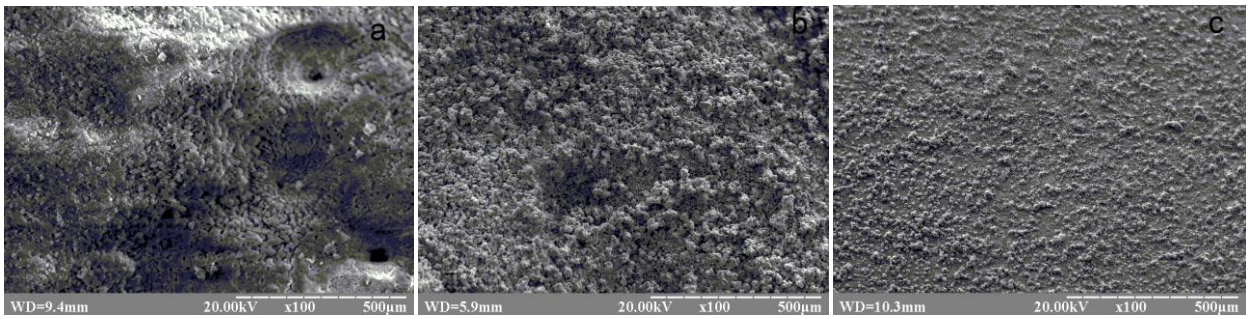

Fig. 2 – Surface morphology of bioactive coatings: a) hydroxyapatite; b) a 2-layer coating of hydroxyapatite with a top layer of zinc sulfide; c) zinc sulfide coatings at different magnifications

Structural features and elemental composition of the obtained coatings were determined by X-ray diffraction and X-ray fluorescence analysis (RFA) (Fig. 3 and Fig. 4, respectively). In the HA coating, 2 phases were identified: HA (JCPDS 86-740) and monetite (JCPDS 89-5969). In the 2-layers HA-ZnS sample, the following phases are identified: HA (JCPDS 86-740), monetite (JCPDS 89-5969) and crystalline ZnS modification – wurtzite (12-688). The ZnS coating demonstrates crystalline ZnS modification – wurtzite (12-688) [12].

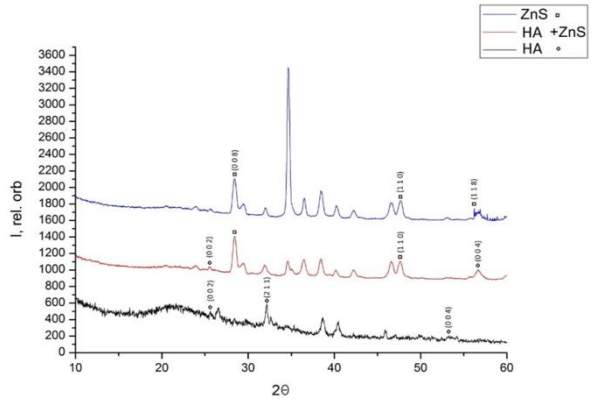

Fig. 3 – Diffraction patterns of coatings: (bottom up) hydroxyapatite (◊); two-layer hydroxyapatite with zinc sulfide; zinc sulfide (□). The corresponding peaks in the HA-ZnS diffraction pattern are indicated by □ and ◊

The diffraction patterns recorded characteristic reflections from the crystallographic planes of HA: 002 (25.62°), 211 (32.16°), 004 (53.28°) and ZnS: 008 (28.42°), 110 (4.68°), 118 (56.24°).

Table 1 presents data on the crystallite sizes of HA, ZnS and ZnS-Alg and the parameters of their crystalline lattice. The average crystallite size (L) along [002] direction can be estimated from the corresponding peak broadening by the Scherrer equation [13].

The parameters of the hexagonal lattice a and c were calculated using the length of the X-ray and h, k, l Miller indices.

X-ray diffraction analysis revealed the decrease of ZnS crystallites synthesized in the presence of Alg. It is known that Alg plays the role of dispersant in solution during synthesis and reduces the degree of formed ZnS particles aggregation and therefore their size. Smaller particles have a larger radius of the surface curvature, as well as greater surface energy that determines particles activity [14].

Table 1 – Structural parameters of the coating material deposited on titanium substrates

Sample	2θ	The crystallite size L, nm	Crystal lattice parameters	
			a	c
HA	25.62	29.67	0.949	0.684
	32.16	27.91	0.944	0.679
	53.28	9.56	0.948	0.686
ZnS	28.42	18.35	0.381	2.50
	47.68	16.72	0.380	2.48
	56.24	28.99	0.382	2.48
ZnS-Alg	28.42	16.12	0.378	2.52
	47.68	13.89	0.380	2.48
	56.24	23.11	0.371	2.46

Further studies of antimicrobial activity confirm this fact. The typical characteristic RFA spectra of the samples presented in Fig. 4 confirm the presence of HA and ZnS in the coating material. The titanium peak belongs to the substrate material.

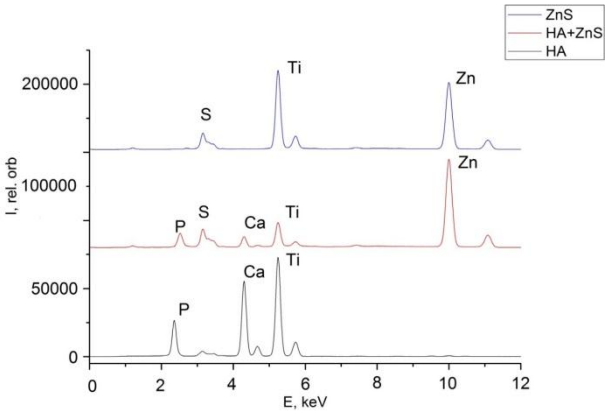

Fig. 4 – X-ray fluorescence spectra of the coating material: the lower spectra is HA; the middle is HA-ZnS; the upper one is ZnS

ZnS and ZnS-Alg coatings were taken to study and compare their antimicrobial activity. The results of the study are shown in Fig. 5 and Table 2.

The analysis of the experimental data showed that higher antimicrobial activity is exhibited by ZnS-Alg coating. It is known that the antimicrobial properties of zinc nanoparticles are provided primarily by their high reactivity, which is determined by the size of the nanoparticles (less than 100 nm) [15].

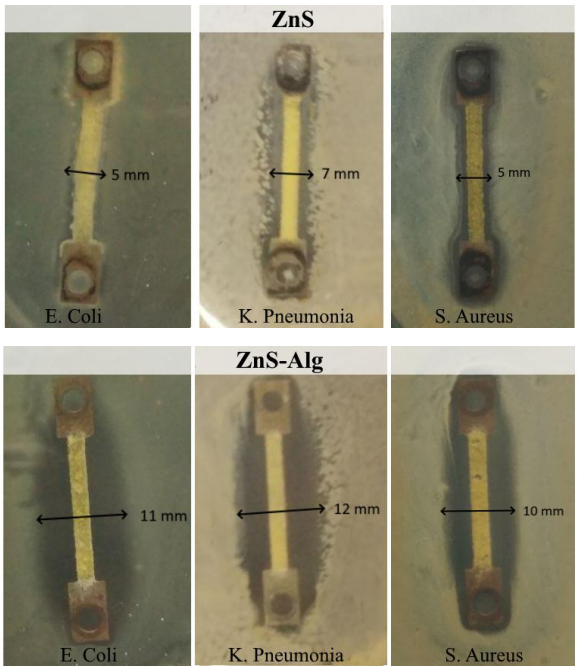

Fig. 5 – Antimicrobial activity of ZnS and ZnS-Alg coatings against microorganisms *E. coli, K. pneumonia, S. aureus*

Table 2 – Growth inhibition zones of bacterial cultures under the influence of experimental coatings

Microorganism	Growth inhibition zones, mm	
	ZnS	ZnS-Alg
E. coli	5	11
K. pneumonia	7	12
S. aureus	5	10

In our experiment, the Alg introduction into the coating structure leads to a decrease in ZnS crystallite size, which can affect the degree of antimicrobial action. Also, upon ZnS dissolution, a sulfide anion is formed in which the sulfur atom has an undivided electron pair. Thus, this anion can form donor-acceptor bonds with functional groups of components of the cell wall of bacteria, disrupting their metabolism. Scientific sources provide two main mechanisms for the antimicrobial action of zinc particles: a) toxic effect of zinc ions on the bacteria cell membrane; b) toxicity of ROS (reactive oxygen particles) formed with the participation of ZnO and ZnS to the components of the bacterial cell.

The main mechanism of antibacterial action of synthesized coatings based on ZnS-Alg nanoparticles is associated with the development of oxidative stress, which is caused by the interaction of ZnS nanocrystals with cells of microorganisms. The antibacterial activity is the result of the formation of ROS, such as hydrogen peroxide (H_2O_2), peroxide anion (O^{2-}), hydroxyl radicals (OH–). These particles damage cellular components such as DNA, lipids and proteins [16]. Positively charged zinc ions can also interact directly with negatively charged components of the bacterial wall [17]. It is also known that composites with ZnO content disturb the integrity of the cell membrane, which leads to damage to the membrane proteins and lipid layer [18].

4. CONCLUSIONS

HA, ZnS and ZnS-Alg coatings with antimicrobial properties on model titanium substrates were obtained by thermal deposition. The X-ray diffraction method proved that the use of sodium alginate as a dispersant in the synthesis of coatings reduces the size of zinc sulphide crystallites due to the significantly larger number of ZnS particles crystallization centers in the presence of alginate macromolecules. The reduction in the size of ZnS particles has a positive effect on the antimicrobial activity of the coatings. ZnS-Alg coatings demonstrate a higher degree of antimicrobial activity due to specific electrostatic interactions with cell membrane components. The results presented underline the possible use of ZnS nanoparticles in various biological materials.

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Біоактивні полімер-апатитні покриття з протимікробними властивостями на модельних титанових субстратах

Л.Ф. Суходуб[1], Л.Б. Суходуб[1], М.О. Кумеда[1], А.П. Денисенко[1], М.І. Кравченко[2]

[1] *Сумський державний університет, вул. Римського-Корсакова, 2, 40007 Суми, Україна*
[2] *Сумський Національний аграрний університет, вул. Герасима Кондратьєва, 160, 40000 Суми, Україна*

Біоактивні покриття на основі гідроксиапатиту (HA), що містять сульфід цинку (ZnS) і альгінат (Alg), були отримані методом термічного осадження (TSD) на модельних титанових імплантатах. Покриття на основі HA забезпечують імпланти біологічною активністю, а ZnS діє як антимікробний агент. Доведено, що введення Alg в структуру покриття зменшує розмір ZnS частинок, що сприяє підвищенню антимікробної активності покриттів. Антимікробна активність шарів ZnS і ZnS/Alg була досліджень мікробіологічними методом дифузії в агар. Зони затримки росту мікроорганізмів *E. coli* ATCC № 25922 склали 5 і 11 мм, *S. aureus* ATCC 25923 – 5 і 10 мм, *K. pneumonia* № 93 – 7 і 12 мм, відповідно. Морфологію і структуру матеріалу покриття визначали методами рентгенівської дифракції (XRD), рентгено-флуоресцентного аналізу (RFA) і скануючої електронної мікроскопії (SEM). Отримані дані підтвердили утворення однофазного шару ZnS на поверхні HA. Представлені результати підкреслюють формування біокомпозитних покриттів з функціями біосумісності і протимікробної активності.

Ключові слова: Цинк сульфід, Альгінат, Гідроксиапатит, Антибактеріальні властивості, Метод термічної депозиції на субстрат.