Romanian Biotechnological Letters *Vol. 14, No. 5, 2009, pp. 4665-4672*

ORIGINAL PAPER

Investigations on Antimicrobial Activity of Collagen and Keratin Based Materials doped with Silver Nanoparticles

Received for publication, April 5, 2009
Accepted, September 25, 2009

CARMEN GAIDAU[a*], AURORA PETICA[b], CONSTANTIN CIOBANU[c] and TAMARA MARTINESCU[d]

[a*] *INCDTP-Division Leather and Footwear Research Institute, Bucharest, Romania*
[b] *INCDIE-ICPE CA – Advanced Research, Bucharest, Romania*
[c] *"Petru Poni" Institute of Macromolecular Chemistry, Iasi, Romania*
[d] *SC Nappa Com Ltd, Ploiesti, Romania*
[1*] *Corresponding author: 93 Ion Minulescu St, sector 3, 031215, Bucharest, Romania, carmen.gaidau@icpi.ro, tel./fax: 021 3235060/0213235280*

Abstract

Silver nanoparticles as ecological alternative for organic biocides, which are mainly volatile organic compounds (VOC), represent an innovative challenge for treatment of collagen based materials as are medical furskins. The design of new silver nanoparticles solution and polyurethanes doped with has had in view the complex structure of collagen cross linked with chromium compounds. The collagen and keratin structures, rich in chemical groups as are: -COOH, -OH, -NH_2, -SH have affinity with polyurethane isocyan groups, -N=C=O and allows obtaining a biocompatible support for reacting with nanosilver particles active against bacteria and fungi. The paper presents the main characteristics of new nanosilver colloidal solutions and nanosilver doped polyurethanes evaluated by using UV-vis, Zetasizer Nano equipment, TEM and the possibilities for interactions with collagen and keratin based materials. The correlation of silver concentration with antibacterial and antifungal resistance of new collagen and keratin supports doped with nanosilver particles was done by using AAS, antibiogram method for Aspergillus, Penicillium, Trichoderma,Candida species and inhibitory minimal concentration for Staphylococcus aureus (ATCC), Pseudomonas aeruginosa (ATTC), Escherichia coli (ATCC), Acinetobacter spp and E. Enterobacter spp.

Keywords: nanosilver particles; colloidal silver solutions; antibacterial and antifungal characteristics, polymers doped with nanosilver; collagen doped with nanosilver

Introduction

Nanoscale materials have received attention as novel antimicrobial agents due to their high surface area to volume ratio and the unique chemical and physical properties. The importance of bactericidal nanomaterials study is because of the increase in new resistant strains of bacteria against antibiotics. Silver ions have been demonstrated to be useful and effective in bactericidal applications, but due to the unique properties of nanoparticles technology present an alternative for developments of new bactericides [1, 2]. The bactericidal action of silver ions and SNPs with sizes up to 20 nm dispersed in different medium is well known [3, 4].

Multifunctional materials, containing silver nanoparticles (SNPs) in reactive or non-reactive polymer networks, are in top of research for applications as biocidal products, biomaterials, drug supports etc [5,6]. Ag salts containing Ag ions have been used for decades

as antimicrobial agents in various fields because of their growth-inhibitory capacity against microorganisms. However, Ag ions from salts have only limited usefulness as an antimicrobial agent in time because of the fast diminishing of the Ag ion concentration. In contrast, SNPs allowed growth of the contact surface of Ag with microorganisms and Ag ions are released gradually. Usually, the colloidal silver solutions (CSSs) contain about 70 % Ag^0 from the total silver content (the rest of 30 % consist of Ag^+ ions), which is released in its ionized form. The Ag^+ ions have a bactericidal activity at concentrations over 1 ppm while the colloidal Ag^0 has a bactericidal activity at concentrations over 20 ppm [7]. Although the SNPs kill a great number of microorganisms, like virus, fungus and bacterium, however it is known as non-toxic and does not cause skin irritation [8]. Moreover, silver nanoparticles have antifouling properties decomposing organic compounds from transpiration.

Chemistry in nanotechnology consists in synthesizing of some functional metal nanoparticles which can be dispersed in aqueous solvents, like water, or with which can be doped synthetic or natural polymers [9,10]. In our work we are using silver nanoparticles to create biocompatible multifunctional materials with antimicrobial and antifungal properties, which have large applications, including treatment of medical furskins.

Silver at nanometric scale, with diameter less than 10 nm is very reactive and it can be combined with macromolecules which contain –RN-CO- structures like polyvinyl pyrrolidone[11], polyurethanes [12] or proteins [13].

Materials and Methods

SNPs were obtained as CSSs, either by electrochemical way, or by chemicals methods. The electrochemical synthesis of CSSs was performed by so-called "sacrificial anode method", using a constant current pulse generator, with stirring and alternating polarity, electrodes of 99.999 Ag with sizes of 155 / 27 mm. To prepare stable and concentrated CSSs, a mix of stabilizer and co-stabilizer agents have been used, respectively poly (N-vinylpyrrolidone) (PVP) and Na-lauryl sulfate (Na-LS). The experiments were carried out using the following materials:

- deionized water with conductivity < 1μS, resistivity of 18 μΩ·cm and pH = 5– 7;
- poly [1-vinyl-2-pyrrolidone] $(C_6H_9NO)_n$ (PVP10) with M = 10,000 from Sigma – Aldrich);
- Na-lauryl sulfate, provided from Sigma – Aldrich.

CSSs were also chemically synthesized by reduction of silver nitrate solutions with sodium citrate solution. CSSs with different concentrations of SNPs were mixed with PHU solutions in different molar ratio with the purpose of obtaining microbiological resistant PHU doped with SNPs.

Polymeric solution presents following characteristics: aqueous, homogeny solution, light yellow color, 45-65% concentration, 10000cP viscosity.

The Ag concentration of the obtained CSSs was determined by UV-vis absorbance spectra recording using a JASCO V 500 spectrophotometer. The nanoparticles sizes were analyzed by DLS (Dynamic Light Scattering) technique using Zetasizer Nano equipment. The silver nanoparticles morphology was evidenced by TEM measurements. To evaluate the antifungal effect, the antibiogram method was used, with a fungi mix from the following species: *Aspergillus, Penicillium, Trichoderma* containing different micotoxines (e.g. aflatoxins). To evaluate the antimicrobial efficiency, the minimal inhibitorial concentration (MIC) upon the: *Staphylococcus aureus* (ATCC), *Pseudomonas aeruginosa* (ATTC), *Escherichia coli* (ATCC), *Acinetobacter spp* and *E. Enterobacter spp* using H.M. Ericsson and J.C. Sherris scheme[14] was determined.

The collagen and keratin based materials were the furskins originating from raw sheepskins that were processed with specific technologies for medical use [15]. The main kind of treatment applied for interaction with SNPs or PHU doped with SNPs was by 24 hours immersion, at 30^0C, followed by material drying. The characterization of collagen based materials was done by AAS for Ag concentration and AFM for surfaces modification. Antibacterial and antifungal properties of new collagen and keratin materials doped with nanosilver particles were assessed by using different methods as are difusimetric, MIC and antibiogram methods.

Results and Discussion

Characteristics of electrochemically and chemically obtained CSSs

Electrosynthesis of SNPs in aqueous media is an efficient and ecological process which offers the advantage of a high purity of final formed solution and a broad spectrum of antibacterial and antifungal activity [16]. A CSS containing 29.27 ppm Ag, obtaining in the presence of PVP 10 and Na-LS was used for treatment of medical furskins. The characteristics of this solution from grain size distribution, morphology and stability point of view are presented in Figures 1-4.

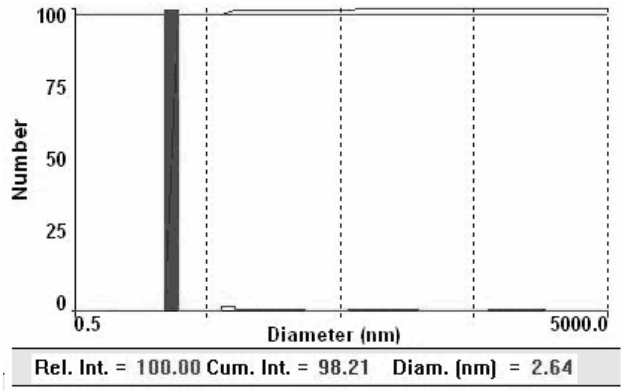


Figure 1. Grain size distribution of SNP

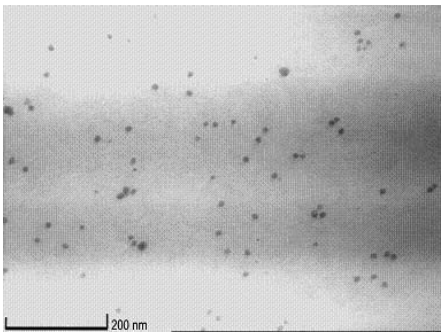


Figure 2. TEM micrograph of SNP

It was observed that the major of SNPs (98.21%) have a diameter up to 2.64 nm and a spherical shape. The zeta potential value of – 50. 25 mV indicates that the solution is very stable in time.

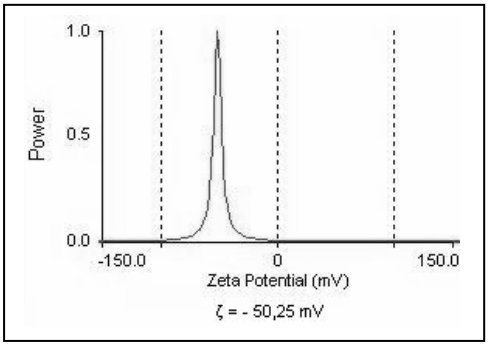


Figure 3. Zeta potential distribution of SNP

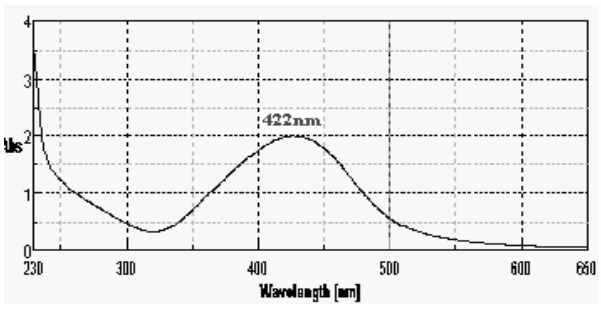


Figure 4. UV-vis spectra of CSS with 29.27 ppm Ag

The chemically obtained CSS has a concentration of 143 ppm SNPs, a size distribution of particles under 10 nm (fig. 5 and 6) and the Zeta potential of 24.9 mV.

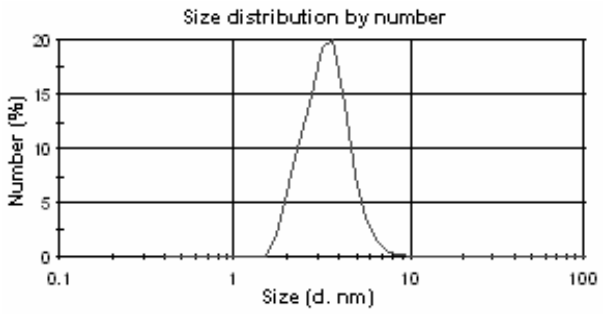


Figure 5. Size distribution of chemically obtained SNPs

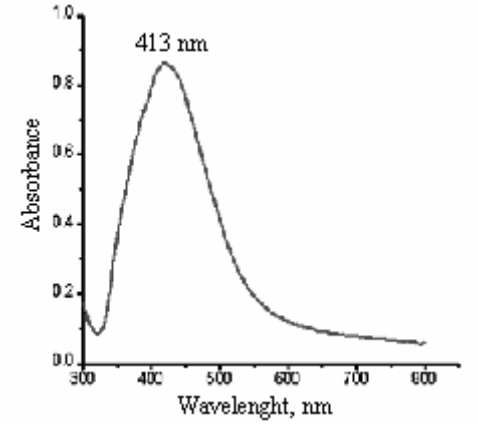


Figure 6. UV-vis spectra for CSS with 143 ppm Ag

Antibacterial effect of SNPs and PHU doped with SNPs

Bacteriostatic and bactericidal effects of electrochemically obtained CSSs was evaluated by using minimal concentration with bacteriostatic effect – MCBs and minimal concentration with bactericidal effect – MCBc against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Table 1).

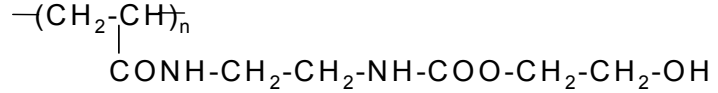
Table 1. Minimal concentration with bacteriostatic effect and minimal concentration with bactericidal effect of the tested CSSs

Initial CSS concentration (ppm)	*Staphylococcus aureus*		*Escherichia coli*		*Pseudomonas aeruginosa*	
	MCBs, (ppm)	MCBc, (ppm)	MCBs, (ppm)	MCBc, (ppm)	MCBs, (ppm)	MCBc, (ppm)
29.27	14.635 – 7.317	29.27	7.317 – 3.658	14.635	7.317 – 3.658	14.635

From table 1 it can be seen that in the case of *Staphylococcus aureus*, the MIC is higher than the one for the other two bacteria. This is due to its membrane which consists of a thick peptidoglycan layer and creates biofilms; the results are in agreement with literature data [17]. Bacteriostatic effect is achieved at a dilution of 1/2 from the initial CSS concentration, in the case of *S. aureus* and 1/4-1/8 for *E. coli* and *P. aeruginosa*, while the best bactericidal effect is at 1/2 from the initial CSS concentration, in the case of *E. coli* and *P. aeruginosa.*

The design of chemical CSSs and polyurethanes doped with has had in view the complex structure of collagen based materials [18,19, 20] crosslinked with chromium compounds, as are medical furskins. The collagen and keratin structures, rich in chemical groups with hydrogen as are: -COOH, -OH, $-NH_2$, -SH have affinity with silver nanoparticles and polyurethane isocyan groups, -N=C=O and allow obtaining of a biocompatible support, reactive against bacteria and fungi.

Polyhydroxiurethane synthesized [21] to interact with SNPs, collagen and keratin based materials has following structure:



Based on reported scientific observations [10, 13] we have doped PHU and medical furskins with different concentrations of SNPs with diameter less than 10 nm with the final aim to obtain antibacterial and antifungal resistant biomaterials for medical use.

The antibacterial characteristics of PHU doped with chemical synthesized SNPs were assessed for different molar ratio of SNPs in PHU solution (1-4 samples in Table 2) in interaction with different bacterial species.

Table 2. Minimal inhibitory concentration (MIC) of PHU doped solutions (1-3 samples) and control sample against: **A.** *Staphylococcus aureus,* **B**. *Escherichia coli,* **C.** *Pseudomonas aeruginosa,* **D.** *Enterobacter spp.,* **E.** *Pseudomonas aeruginosa and* **F.** *Acinetobacter spp.*

PHU doped with NSPs sample	Concentration of Ag^0 in doped PHU, ppm	MIC (ppm)					
		A	**B**	**C**	**D**	**E**	**F**
1	75.6	75.6	37.8	37.8	37.8	37.8	37.8
2	51.06	-	51.06	12.51	51.06	51.06	51.06
3	28.27	-	14.13	14.13	14.13	28.27	28.27
Control	0	-	-	-	-	-	-

"-"without bacteriostatic effect

The best bacteriostatic effect was obtained for PHU doped with 75.6 ppm SNP in the case of *Staphylococcus aureus* and for PHU doped with 37.8 ppm SNP (the 1/2 dilution from the PHU solution doped with 75.6 ppm SNP) in the case of the other bacteria strains (table 2, figure 8). The bacteriostatic effect for all tested bacteria strains have disappeared at 1/8 dilution of PHU doped with 75.6 ppm SNP (figure 9).

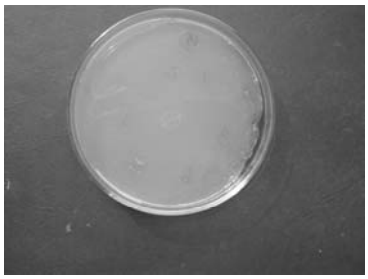


Figure 8. MIC test for PHU solution doped with 75.6 ppm SNP at 1/2 dilution

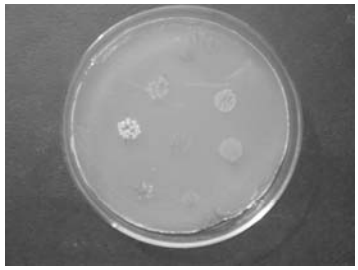


Figure 9. MIC test for PHU solution doped with 75.6 ppm SNP at 1/8 dilution

Antifungal effect of electrochemically obtained CSSs

In the case of CSSs, according to antibiogram method, the fungistatic properties are expressed by the presence and magnitude of inhibition area for mould growth around the filter paper padded with CSSs. The used test fungi contained various mico-toxines, as aflatoxins. Figure 10 displays photographic images of the inhibition area produced by the tested CSSs on a mould colony containing *Aspergillus, Penicillium* and *Trichoderma*, after 7 and 14 days of exposure. It can be seen than even after 14 days of exposure the inhibition area around the filter paper is present.

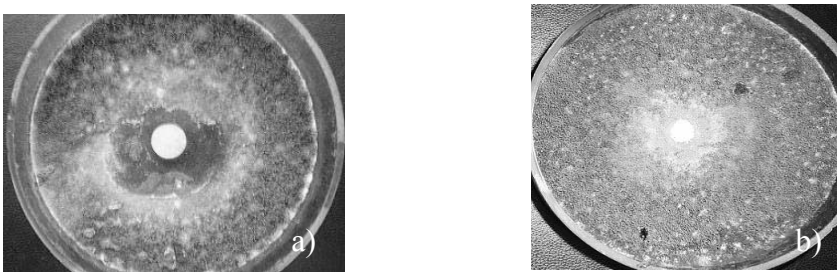


Figure 10. Inhibition zone produced by a CSS with 29.27 ppm Ag after: a) 7 days b) 14 days.

Morphological characteristics of collagen and keratin based materials treated with electrochemically obtained CSSs and PHU doped with SNPs

The AFM images of furskin hair and derma treated with electrochemically obtained CSSs in comparison with untreated hair and derma have indicated a specific surface structure modification at micrometric scale in term of roughness aspect (figure 11).

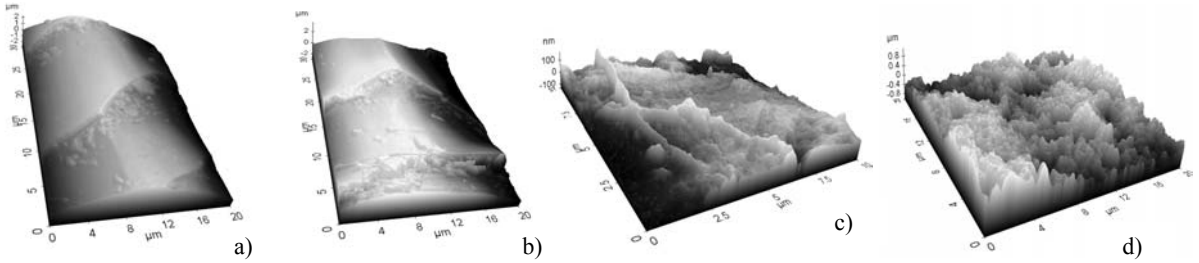


Figure 11. The AFM images of medical furskin surfaces: a) hair untreated, b) hair treated with electrochemically obtained CSSs, c) derma untreated, d) derma treated with electrochemically obtained CSSs

The interaction of PHU doped with SNPs was evidenced at macroscopic level by specific color change of hair cover and derma of medical furskins (figure 12).

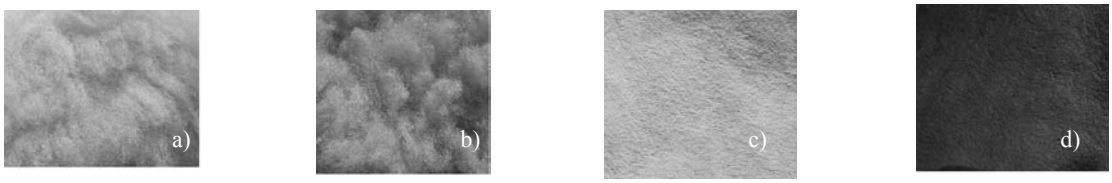


Figure 12. Macroscopic imagines of medical furskin surfaces before interaction with PHU doped solutions: a) hair; c) derma and after treatment: b) hair; d) derma.

Antibacterial and antifungal resistance of collagen and keratin based materials treated with electrochemically obtained SNPs and PHU doped with chemically obtained SNPs

The medical furskins treated by immersion in electrochemically obtained CSSs, with a content of 490 ppm Ag in dermal surface have displayed resistance against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* (Table 3).

Table 3- Antibacterial action of medical furskin treated with electrochemically obtained CSSs

Ag conc from medical furskin sample, ppm	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*
490	+	+	+

"+" – with antibacterial activity determined by diffusimetric method

The exposure of the same medical furskins according to antibiogram method to a mix of fungi (*Aspergillus niger*, *Paecilomyces variotii*, *Trichoderma viride*, *Scopulariopsis brevicaulis*, *Penicillium glaucum*) used for inoculation has showed a good resistance after 7 days (figures 13 and 14). In figure 14 it could be seen the resistance of medical furskin samples to invasion of *Trichoderma viride.*

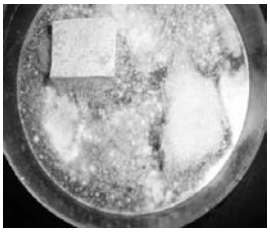


Figure 13-Fungitoxic resistance of medical furskins after 7 days

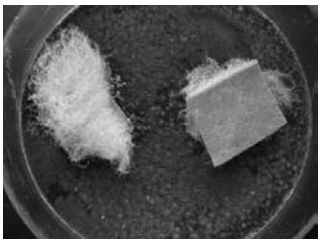


Figure 14-Fungitoxic resistance of medical furskins after 7 days, *Trichoderma viride*

The assessment of antibacterial and antifungal resistance by using MIC method for medical furkins treated with PHU doped with different concentrations of SNP is presented in table 4.

Table 4. The influence of SNP's concentration against S. *aureus, E. coli and C. albicans*

Strain	SNP conc. from medical furskin, ppm	Strain conc., (ml)		
		0 hours	24 hours	48 hours
Staphylococcus aureus	0	$1.7.10^5$	$5.3.10^6$	$2.0.10^7$
	8.98	$1.7.10^5$	$6.2.10^4$	$6.7.10^3$
	17.80	$1.7.10^5$	$5.1.10^4$	$2.4.10^3$
	33.82	$1.7.10^5$	$6.8.10^3$	$8.5.10^2$
	4200	$1.7.10^5$	$5.6.10^2$	<10
	53.98	$1.7.10^5$	0	0
Escherichia coli	0	$1.2.10^5$	$6.2.10^6$	$4.3.10^7$
	8.98	$1.2.10^5$	$4.5.10^3$	$7.2.10^2$
	17.80	$1.2.10^5$	$5.5.10^2$	7.4.10
	33.82	$1.2.10^5$	$3.4.10^2$	<10
	42.00	$1.2.10^5$	<10	0
	53.98	$1.2.10^5$	0	0
Candida albicans	0	$4.2.10^5$	$6.5.10^6$	$5.7.10^7$
	10.90	$4.2.10^5$	$4.7.10^4$	$3.8.10^3$
	15.96	$4.2.10^5$	$5.8.10^3$	$3.2.10^2$
	31.92	$4.2.10^5$	$7.5.10^2$	<10
	39.50	$4.2.10^5$	0	0
	56.88	$4.2.10^5$	0	0

From the Table 4 it can be seen that a concentration of minimum 31.92 ppm SNP in furskins structure assures the resistance to *Staphylococcus aureus, Escherichia coli and Candida albicans* and the values of strains concentrations decrease in time, indicating SNP antimicrobial activity.

Conclusions

The silver nanoparticles obtained by electrochemical or chemical route and the PHU doped with chemical nanoparticles have antimicrobial or antifungal activity and can be used for treatment of very useful biomaterials as are medical furskins in order to avoid the use of organic antibacterial materials, from the VOC product class. The direct use of CSSs obtained

by electrochemical method assures a concentration of silver in medical furskin structure of 490 ppm and resistance to the most important bacteria and fungi. The most efficient concentration of nanosilver particles in PHU and collagen based materials against S. *aureus, E. coli and C. albicans* are the concentration about 40 ppm Ag^0.
The use of silver nanoparticles for treatment of collagen and keratin based materials as are medical furskins supposes a complex research in designing new organo-metallic materials with complex affinity and biologic reactivity. This investigation represents a new approach in treatment of collagen and keratin based biomaterial for medical use and obtaining results are promising for further researches.

Acknowledgement
This work was supported by the Romanian National Management Centre for Research Programs; Research project PN 1 no. 71-146.

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