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DEVELOPMENT OF LONG-LASTING ANTIMICROBIAL AND POTENTIAL HEMOSTATIC NANOCOMPOSITES (pyrophyllite based) WITH PVP-coated COLLOIDAL SILVER NANOPARTICLES

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Abstract: Pyrophyllite clay, modified with PVP coated silver nanoparticles (PYRO-PVP/AgNPs), with recently proved antibacterial activity was prepared. Silver nanoparticles were synthesized by the chemical reduction method of $AgNO_3$ using $NaBH_4$ and poly(vinyl pyrrolidone) (PVP) as a stabilizer and excellent dispersant. This research aimed to elucidate the mechanisms and kinetics of AgNPs, along with the PVP protective mechanism responsible for antibacterial activity towards the microorganisms. Pioneering steps were made toward coagulation studies due to the potential of aluminosilicate layered clays to serve as alternatives to hemostatic agents currently in use. The isoelectric point of pyrophyllite samples with 5, 20, and 45 μm diameter particles and PYRO/PVP/AgNPs sample (Ag25mg/L) was evaluated to understand how the anticoagulant or procoagulant properties of the pyrophyllite varied according to the pH of the isoelectric point. Characterization of the PYRO-PVP/AgNPs samples was performed using FTIR spectroscopy, while the release mechanism and kinetics of silver ions were monitored using atomic absorption spectroscopy (AAS). Additionally, AAS was used for the evaluation of heavy metals content in pyrophyllite clay and a simple, cost-effective procedure was proposed for the extraction of heavy metals.

Keywords: pyrophyllite, nanocomposite, colloidal silver, release mechanism, kinetics.

1. INTRODUCTION

Clay is hydrated aluminosilicate sediment, mixed in nature with quartz sand, limestone and iron oxides. It is usually formed by slow mechanical and complex chemical processes of decomposition of rocks of volcanic origin. These processes take place due to the action of water, air and carboxylic acid. All types of clay have the following properties: absorption of large amounts of water (80%), stickiness, shrinkage on drying and retention of shape after annealing. In recent years, clay minerals proved to be very interesting for drug development, since they possess good chemical inertness and biocompatibility, low toxicity, and excellent physicochemical properties [1]. They are widely used as pharmaceutical excipients and active substances. There are some reports that clay

improves the stability of drugs, or even prevents or reduces side effects. More recently, the possibility of clay being used as a carrier of drugs or certain significant chemical elements was being investigated due to the high ion exchange capacity, large surface area, chemical inertness and low or zero toxicity [2-5]. The development of these systems requires a completely solid-state characterization followed by a biopharmaceutical evaluation. Aguzzi et al reported on the intercalation of tetracycline (TC) into a layered clay mineral, as a modified drug carrier. It was proved that the amount of drug retained and the drug release profile greatly depends on the TC/clay interaction. In the TC/clay complex, decomposition of the TC was delayed compared to the physical mixture and drug alone, due to a thermal protection effect resulting from drug intercalation in the clay interlayer [6]. The intercalation of timolol maleate (TM) into montmorillonite (MMT) interlayers at different pH values and initial concentrations was also performed. Timolol maleate was successfully intercalated into the interlayers of montmorillonites, due to the exchange of interlayer Na^+ ions and cations of TM molecules. During the experiment, a controlled release of timolol maleate from the MMT-TM hybrid complex was observed. The *in vitro* release properties of timolol maleate were tested in simulated gastric fluid (pH = 1.2) and simulated intestinal fluid (pH = 7.4) at 37 ± 0.5 ° C and it was shown that about 43% and 48% of TM was released from MMT–TM hybrid in simulated gastric fluid (pH 1.2) and intestinal fluid (pH 7.4), respectively. The conducted study proved that MMT can be used as the sustained release carrier of TM in oral administration [7]. Also, some layered inorganic materials such as kaolin, zeolite, smectite, bentonite and porous silica were used for accelerating blood coagulation [8].

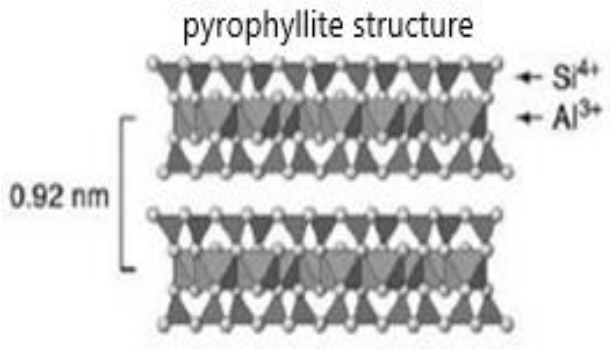

Figure 1. *The structure of pyrophyllite clay* [13]

Furthermore, very significant research goes toward the formulation of new antimicrobial composites based on metallic nanoparticles incurporated in some inorganic/polymeric matrices. Among metallic nanoparticles, silver nanoparticles proved to be a very good candidate for such development [9, 10]. One of the reasons is that silver is stable enough for use as an antibiotic because other cations are highly reactive and therefore short-lived. Silver nanoparticles have higher antibacterial efficacy than silver in the form of salts or complexes for two reasons: silver nanoparticles have a large surface area, so they can make better contact with the surface of bacteria and silver nanoparticles act as effective Ag^+ reservoirs. Gradual oxidation of silver atoms on the surface can continuously release biologically active species, providing a constant influx of silver cations that can effectively attack bacterial targets over a long period. Nevertheless, one should be very careful regarding the cytotoxic mechanism of action caused by Ag^+ ion on bacterial cells. Therefore, the kinetics release of silver proved to be very important. The silver mechanism for the elimination of bacteria could be described as follows:

1) The silver ion inhibits the absorption and exchange of phosphate causing the accumulation of this anion, favors the release of K^+ and the release of protons across the cytoplasmic membrane, leading to bacterial cell death

2) Ag^+ can bind to nucleic acids (instead of the phosphate part) in a very efficient way so that the processes of bacterial cell replication can be stopped

3) The silver ion coordinates the thiol groups of proteins and enzymes that are located on the cell surface, causing destabilization of the cell membrane and disruption of the synthetic processes of ATP [11].

In our previous papers, we reported on the excellent antimicrobial properties of pyrophyllite clay modified with silver NPs, namely PYRO/PVP/AgNPs [12]. Pyrophyllite itself is a type of clay, more precisely a monoclinic mineral from the group of phyllosilicates of the general formula Al_2 $[Si_4O_{10}](OH)_2$. It is formed by the hydrothermal metamorphosis of rocks rich in aluminum. The pyrophyllite structure consists of an octahedral -Al-O layer located between two tetrahedral -Si-O layers (Figure 1)[13]. In general, pyrophyllite and other aluminosilicates have wide applications in medicine due to their antiseptic, antitoxic and antibacterial properties.

This research aims to elucidate the mechanisms and kinetics of AgNPs intercalated onto the pyrophyllite surface and give a physical and chemical explanation of the silver ion migration mechanism. Overall, the agglomeration phenomenon of silver contributes to the weakness of antibacterial properties due to the growth of nanoparticles, so special attention was attributed to the PVP protective role.

2. MATERIALS AND METHODS

The research was conducted on the pyrophyllite sample, Konjic (Parsovići location), Bosnia and Herzegovina. The pyrophyllite samples were mechanically treated before research to obtain 5, 20 and 45 μm particle size.

2.1. Synthesis of a silver-pyrophyllite nanocomposite material

PYRO-PVP/AgNPs were synthesized by the chemical reduction, which consisted of two main steps.

First step - Synthesis of silver nanoparticles, AgNPs

A freshly prepared solution of $NaBH_4$ (c = 0.002M) was cooled for 20 minutes in an ice bath with constant stirring. Then solution of $AgNO_3$ (c = 0.001M) was added dropwise (1 drop/second) in $NaBH_4$ solution with constant stirring. Finally, a 0.3% solution of polyvinylpyrrolidone (PVP) was added.

Second step - Synthesis of Ag-pyrophyllite nanocomposites, PYRO/PVP/AgNPs

100 mg of a sample of pyrophyllite was added to the solution of the prepared silver nanoparticles with constant stirring for 2 h. Thereafter, the solution was centrifuged (10 minutes at 5000 rpm) and the synthesized composite was washed with deionized water, centrifuged again, and then dried for 24 h at 50 °C. The following equation shows the process of chemical reduction:

$$AgNO_3 + NaBH_4 \rightarrow Ag + 1/2H_2 + 1/2B_2H_6 + NaNO_3$$

Thus PYRO/PVP/AgNPs samples were prepared in the following concentration: 5 mg/L (Ag_5), 25 mg/L (Ag_{25}), 50 mg/L (Ag_{50}) and 100 mg/L (Ag_{100}).

2.2. Characterization of pyrophyllite samples

The Fourier-transform infrared spectroscopy (FTIR), Shimadzu was used to characterize the synthesized Ag-pyrophyllite composite. Spectrum recording was performed on wave numbers from 4000 to 400 cm^{-1}.

2.3. Kinetics and migration of silver from pyrophyllite

Migration of silver ions was tested in phosphate buffer (pH = 6) which imitates biological conditions. Namely, the samples modified with silver with the following mass concentrations: 5, 15 and 25 mg / L, were dissolved in phosphate buffer and constantly shaken for five days. A portion of the liquid phase was taken each day and the amount of silver released was determined using an atomic absorption spectrometer (AAS). A Perkin Elmer AAnalyst 400 atomic absorption spectrophotometer (air/acetylene flame, appropriate hollow cathode lamp for absorption technique) was used to measure the metal content.

2.4. Determination of isoelectric point of original pyrophyllite of different granulations and synthesized Ag-pyrophyllite composite

The potentiometric method of salt addition was used for the prediction of the isoelectric point (point of zero charge) of pure (5, 20, 45 μm) and modified pyrophyllite PYRO-PVP/AgNPs (Ag_{25}). Hydrochloric acid solutions (c = 0.1 mol/L) and sodium hydroxide solution (c = 0.1 mol/L) were used for adjustment of the pH. The solutions were mixed on a magnetic stirrer and 0.05 g of pyrophyllite samples were added to the solutions thus prepared. The prepared samples in parafilm-protected beakers were allowed to stand for 24 h, after which they were filtered and an additional measurement of pH was performed.

2.5. Extraction of heavy metals from pyrophyllite samples

Hydrochloride acid (c=0,1 mol/L) was added to 2 g of pyrophyllite and stirred on a magnetic stirrer for 30 to 60 minutes at room temperature. Upon stirring, samples were centrifuged. The

supernatant was saved for further analysis and the precipitate was dried in an oven for 24h at 50°C. 0.1 g was taken from the dried samples and subjected to the additional extraction process using EDTA. The obtained samples were treated as the previous ones. The extracts obtained after described procedures were stored at 4°C and afterwards analyzed for Cu, Pb and Zn. A Perkin Elmer AAnalyst 400 atomic absorption spectrophotometer (air/acetylene flame, appropriate hollow cathode lamp for absorption technique) was used to measure the metal contents (Table 1). Standard solutions for quantification were made using individual 1000 mg/L standards for AAS (Perkin Elmer, USA) and proper extractant for dilution. In all cases, standard solutions and blank were treated in the same way as the real samples to minimize the interferences during analysis. To assure a correct calibration of the instrument, at least one standard sample has been run every 10 test samples. Concentrations have been reported as mean values of three replicates. All analytical procedures in the laboratory were triplicated and were made with acid-prewashed (10% HNO_3) glassware and plastic bottles for extracts storage.

Table 1. *Working conditions of atomic absorption spectrophotometry*

Element	Technique	λ [nm]	Calibration standards concentration [mg/L]
Cu	FAAS	324.75	0.2, 0.4, 0.8, 1.0
Pb	FAAS	283.00	1, 2, 4, 8
Zn	FAAS+BG	213.70	0.1, 0.3, 0.6, 0.7

FAAS – flame atomic absorption spectrophotometry
BG – background correction of the signal with deuterium lamp

3. RESULTS AND DISCUSSIONS

Figure 2. presents freshly synthesized pyrophyllite samples containing silver PYRO/PVP/AgNPs in the following ratio: 5, 25, 50 and 100 mg/L. As one can see, the color of the sample was changeable due to the silver concentration increase. In our previous study, which focused on the antimicrobial properties of modified pyrophyllite PYRO/PVP/AgNPs, all samples containing silver in concentration range Ag_{25} to Ag_{100} mg/l proved to provide sterile conditions in the first 24 hours, while Ag_5 and Ag_{15} proved to be very selective and specific for *gram-positive* or *gram-negative bacteria*. Šmitran et al reported that the minimum inhibition concentration of pyrophyllite nanocomposite modified with silver was 25 mg/L [12]. Therefore, the selected samples were chosen for characterization using the FTIR technique to confirm the successful modification of pure pyrophyllite. Typify neat pyrophyllite (Figure 3a) and modified pyrophyllite containing 5 mg/L (Figure 3b) and 25 mg/L (Figure 3c) of silver were subjected to FTIR analysis and subsequently were subjected to kinetics analysis.

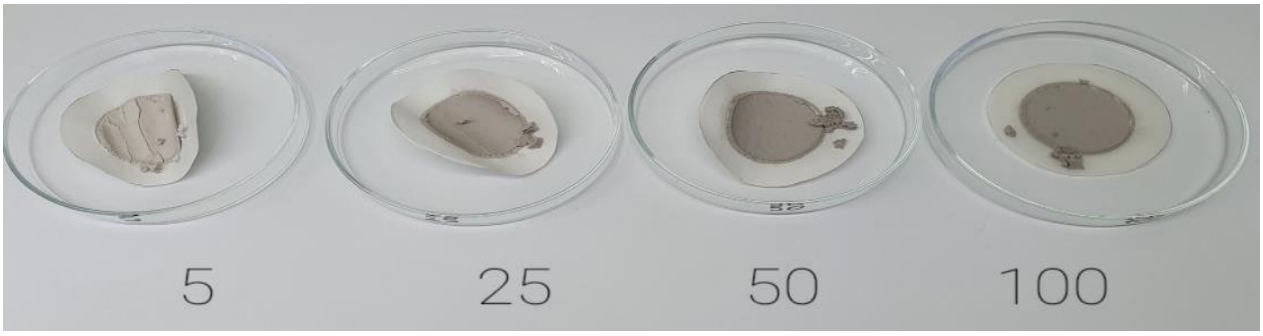

Figure 2*. Modified pyrophyllite samples with 5, 25, 50 and 100 mg/L of silver solution*

The bands that occur at 451.39 and 462.33cm^{-1} are attributed to vibrations due to the bending of –Si-O and –Al-O bonds. Another confirmation of bending - Si-O is the strip at 537.89 cm^{-1} and 526.96 cm^{-1}. The presence of -Si-OH was determined by the appearance of peaks at 930,623 and 963,43 cm^{-1}. Also, peaks close to 1000.0 cm^{-1} are a consequence of the asymmetric vibration of stretching - Si-O-Si groups. The peak at 1442.66 and 1453.60 cm^{-1} is the result of -OH bending. The signal at a wave number of approximately 3600.00 cm^{-1} indicates vibrations due to stretching of the -Al_2-OH group [6]. The Ag-Ag bonds are formed below 400 cm^{-1} but since the FTIR instrument records in the wavelength range of 4000–400 cm^{-1}, it cannot produce enough energy to vibrate the metallic bonds [14]. Therefore, the spectra cannot display any absorption peaks of Ag-Ag bonds. The peaks at 1029, 1126 and 1453 in pure pyrophyllite are shifted in PYRO/PVP/AgNPs samples, which could be attributed to an interaction between PVP and silver NPs. These shifts could indicate that pyrrolidinyl nitrogen electrons are involved in the formation of silver nanoparticles by donation of electrons from N to Ag or coordination between these atoms [15].

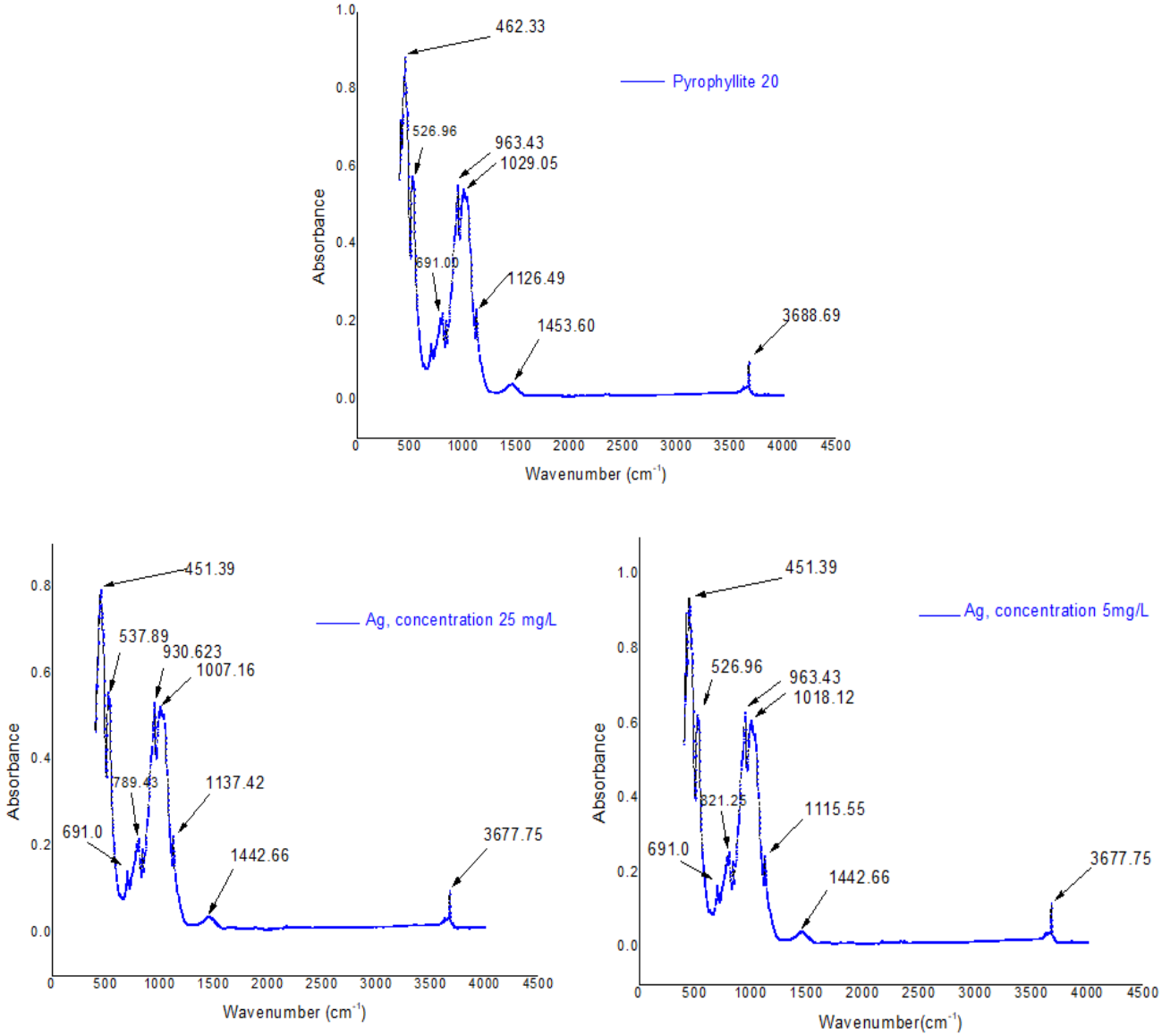

Figure 3a, 3b and 3c. *FTIR spectra of pure pyrophyllite (3a) and modified pyrophyllite Ag_5 (3b) and Ag_{25} (3c)*

To confirm the previously reported facts and to elucidate the mechanism of silver NPs migration, as well as take into consideration the toxic effect of silver ions, the kinetics of silver release were monitored. Based on the obtained antimicrobial data we could hypothesize that silver NPs migrations from Ag_5, Ag_{15} and Ag_{25} are governed by a different mechanism. Note that the migration profile of Ag^+ must be taken into account due to the cytotoxicity of silver ions in human cells. In its metallic form, silver is inert and cannot kill bacteria. For silver to manifest its bactericidal activity, AgNPs must lose an electron and become positively charged silver ions (Ag^+). The ionization of the metallic silver nanoparticles present on the surface of the nanocomposite is subjected to an oxidation reaction during its exposure to an air/humid medium, while that of the nanoparticles incorporated within the matrix would occur only after penetration of water molecules into the matrix [16]. Recently, it was suggested that the migration mechanism of silver nanoparticles takes place in three main steps as follows: water penetration into the nanocomposite, the reaction of water molecules with Ag NPs forming Ag+ ions and finally migration of the Ag+ ions formed from the inside of the matrix to the external medium [17 – 20]. Since the majority of silver ions must appear on the surface to be released, we believe that the last stage (migration of silver ions) is dominated by the diffusion process.

Figure 4. displays the release of silver from pyrophyllite samples Ag_5, Ag_{15} and Ag_{25} for six days. As can be seen, investigated samples showed quite different kinetic behavior. The samples Ag_5 and Ag_{15} proved to increase the migration of silver ions each day for six days, while sample Ag_{25} showed a typical kinetic behavior, i.e., concentration was decreasing with time. Note that the concentration value of released silver ions in the Ag_{25} sample in the first 24h is equal to 446 ppm, while in samples Ag_5 and Ag_{15} this value was reached after six days. The kinetics of Ag nanoparticles depends on their concentration and various parameters such as size, morphology, stability, chemical composition, media etc. [21]. So, this information on different kinetic behavior could be valuable for a better understanding of human exposure levels, application of such materials, as well as safety aspects. We potentially believe that reason for this different kinetic behavior is in the absorption of silver ions into pyrophyllite structure, its possibility to aggregate and diffusion of Ag^+ ions linked to the intercalated/exfoliated sheets of pyrophyllite by ionic bonds, towards the external medium. The role of PVP is also important since PVP ensure the stability of silver NPS for a longer period. This is a

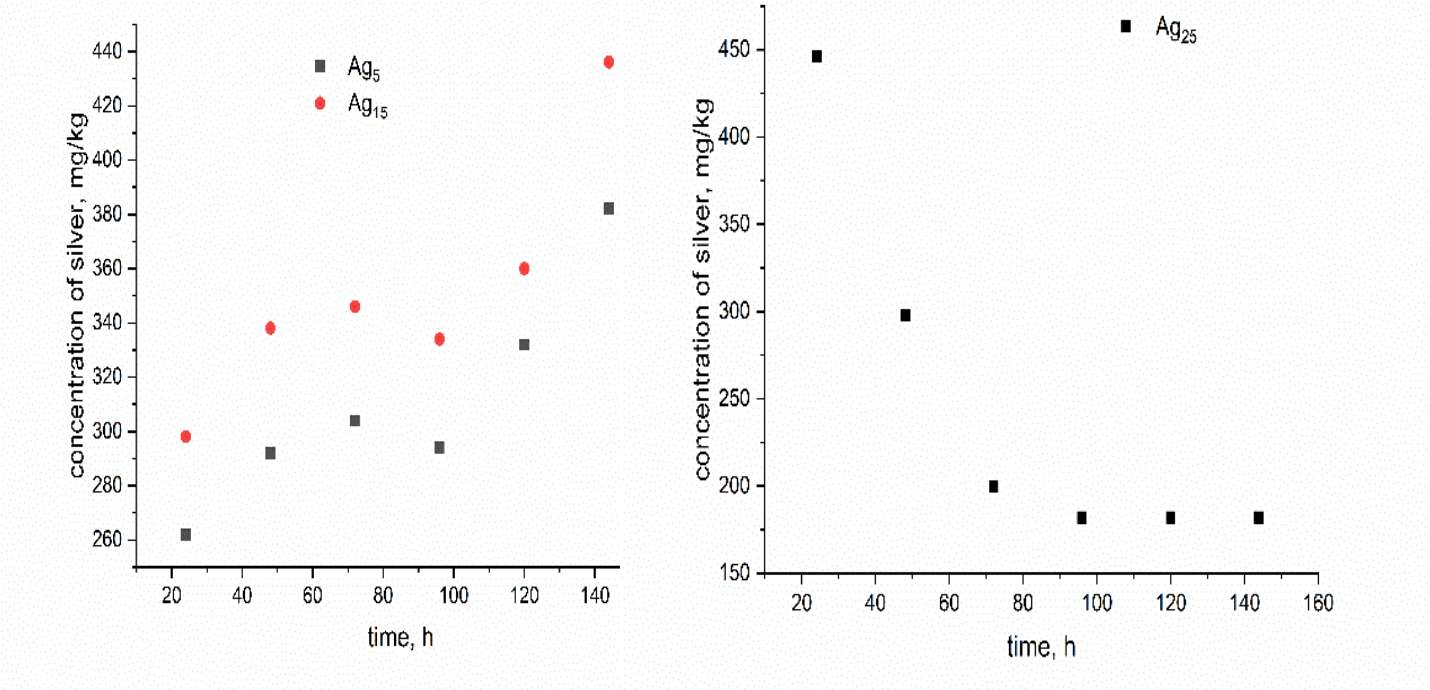

Figure 4. *Kinetic mechanism of releasement of silver ions in Ag_5, Ag_{15} and Ag_{25}*

consequence of the coordination of nitrogen from PVP with silver, forming a protective layer. We potentially believe that PVP ensured better stability for Ag_5 and Ag_{15} samples and the diffusion process was postponed due to stabilizing effect of PVP. Nevertheless, regardless of the mechanism employed, silver NPs once released could interact with negatively charged membrane surfaces of bacteria, causing a destabilization of the bacterial cell wall as well as a disturbance of the metabolism. This results in a significant decrease in the nutrient exchange between the bacterial cells and the external environment, leading to the radical death of bacteria [22].

Additionally, pioneering steps were made toward coagulation studies due to the potential of aluminosilicate layered clays to serve as alternatives to hemostatic agents currently in use. It was reported that the negative charge surface of clays provides the key to surface chemistry, which can rapidly activate the coagulation process. The hemostatic activities observed for the inorganic materials identify the isoelectric point as an important parameter that strongly affects the biological response of blood during contact activation of the clotting cascade of reactions. Ostomel et al reported that the most negatively charged metal oxides are associated with the fastest coagulation, while the most positively charged surfaces are associated with the slowest coagulation. Furthermore, negatively charged metal oxides accelerate the rate of coagulation and positively charged metal oxides decelerate the rate of coagulation [23].

To get insight into the hemostatic properties of the pyrophyllite surface, pure and modified, we determined the isoelectric point of pyrophyllite samples with 5, 20, 45 μm diameter particles and PYRO/PVP/AgNPs sample (Ag_{25}). Table 2 contains the value of isoelectric points for pure and modified pyrophyllite and it seems that the isoelectric point of neat pyrophyllite containing different particles size showed rather different isoelectric point values. To do a preliminary study toward hemostatic potential ability, we have chosen the one which had an isoelectric point value below the blood pH value (7.35-7.45). The pyrophyllite with a particle size of 20 μm was modified with silver, and the isoelectric point value proved to be lowered and amounted to 7.12. This information could be of special interest to future experiments concerning the hemostatic potential and surface chemistry of pure and modified pyrophyllite. The surface under pH of isoelectric value should be positively charged properties, and negatively charged above. Note that all hazardous species, such as toxins, are positively charged and their elimination via adsorption and electrostatic interaction between their positive and negative surface could contribute to their elimination. It is worth mentioning that the pI value of Ag_{25} is below the pH value of the blood.

Table 2. *pH values of isoelectric point*

Sample	isoelectric point pH
5 μm	7.44
20 μm	7.23
45 μm	7.00
Ag_{25} (20μm)	7.12

In addition to the experiments, extraction of heavy metals from pyrophyllite samples was performed. According to US pharmacopoeia, alumosilicate minerals should not contain more than 100 mg/kg taking into account all heavy metals present. Therefore, the elimination of heavy metals was performed using successive extraction with hydrochloride acid and EDTA. Elimination of heavy metals proved to be rather successful and the elimination was 75% and 100% depending on the samples employed (Table 3).

***Table 3**. Heavy metal content in pyrophyllite samples*

Sample	Phase of the extraction	Pb, mg/kg	Cu, mg/kg	Zn, mg/kg
0-75 μm	Untreated sample	58.60	24.56	132.69
0-75 μm	Precipitation with HCl extraction	29.32	18.91	162.68
0-75 μm	Precipitation with EDTA extraction	8.73	<0.01	65.45
100 μm	Untreated sample	15.17	2.48	112.07
100 μm	Precipitation with HCl extraction	12.49	0.25	38.90
100 μm	Precipitation after EDTA extraction	<0.01	<0.01	39.97

4. CONCLUSION

The research gave insight into kinetics of silver ions incorporated in PYRO-PVP/AgNPs. The kinetics proved to be dependent on silver ions concentration and amount of PVP which was used as a stabilizer. The different PYRO/PVP/AgNPs nanocomposites showed to undergo different mechanisms of silver ion release. Based on the zero-point charge, we can conclude that PYRO-PVP/AgNPs have favorable hemostatic properties. Successive extraction with hydrochloride acid and EDTA proved to be a very useful, cost-effective and simple method for purification of alumosilicate minerals for their further application.

5. ACKNOWLEDGEMENTS

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РАЗВОЈ ДУГОТРАЈНИХ АНТИМИКРОБНИХ И ПОТЕНЦИЈАЛНИХ ХЕМОСТАТИЧКИХ НАНОКОМПОЗИТА (НА БАЗИ ПИРОФИЛИТА) СА КОЛОИДНИМ НАНОЧЕСТИЦАМА СРЕБРА ОБЛОЖЕНИМ ПОЛИВИНИЛ-ПИРОЛИДОНОМ

Сажетак: Пирофилитна глина модификована је наночестицама сребра пресвученим ПВП-ом (ПИРО-ПВП/Ag наночестице), са недавно доказаном антибактеријском активношћу. Наночестице сребра синтетизоване су методом хемијске редукције $AgNO_3$ користећи $NaBH_4$ и поли (винил пиролидон) (ПВП) као стабилизатор и одлично средство за распршивање. Ово истраживање има за циљ да разјасни механизме и кинетику наночестица сребра, заједно са ПВП-ом, које су одговорне за антибактеријско дјеловање према микроорганизмима. Учињени су пионирски кораци ка студијама коагулације због потенцијала слојевитих алуминосиликатних глина да послуже као алтернатива хемостатским агенсима који су тренутно у употреби. Одређивана је изоелектрична тачка узорака пирофилита са честицама пречника 5, 20, 45 μm и узорка ПИРО-ПВП/AgНП (Ag25mg/L) да би се схватило како се антикоагулантна или проко-агулантна својства пирофилита разликују у складу са pH изоелектричне тачке. Карактеризација узорака ПИРО-ПВП/AgНПс изведена је помоћу ФТИР спектроскопије, а механизам ослобађања и кинетика јона сребра праћени су атомском апсорпционом спектроскопијом (ААС). Додатно, ААС је коришћен за процјену садржаја тешких метала у пирофилитној глини и предложен је једноставан и исплатив поступак за пречишћавање пирофилита.

Кључне ријечи: пирофилит, нанокомпозит, колоидно сребро, механизам ослобађања, кинетика.

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