published by Ural Federal University
eISSN 2411-1414; chimicatechnoacta.ru

ARTICLE
2022, vol. 9(2), No. 202292S13
DOI: 10.15826/chimtech.2022.9.2.S13

Phosphorous-containing copolymers loaded with silver nanoparticles for biomedical purposes

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This paper belongs to the MOSM2021 Special Issue.

Abstract

New silver nanocomposites (AgNCs) based on copolymers of tris(diethylamino)diallylaminophosphonium tetrafluoroborate (DAAP-BF_4) and chloride (DAAP-Cl) with N-vinylpyrrolidone (VP) were developed. The reduction of silver ions into silver nanoparticles was achieved using $NaBH_4$ as a reducing agent. UV-spectroscopy and scanning electron microscopy techniques were used to characterize the formation of silver nanoparticles in copolymers. The average silver particle size ranged from 10 to 20 nm, with the corresponding UV-vis absorption peak position at 395–405 nm. The AgNCs exhibited significant cytotoxic activity towards rhabdomyosarcoma and melanoma line cells and completely inhibited bacterial growth, including both Gram-positive and Gram-negative bacteria.

Keywords

phosphonium salts
radical polymerization
silver nanoparticles
biocides
cytotoxic activity

Received: 05.04.22
Revised: 19.07.22
Accepted: 20.07.22
Available online: 08.08.22

1. Introduction

Nowadays, the creation of nanomaterials from silver nanoparticles is the most promising, especially due to their outstanding significant chemical and biological properties [1–5].

Numerous nanoparticle stabilizers are known, among which special attention is paid to polymers. On the one hand, the inclusion of nanoparticles in traditional polymers leads to the formation of materials with fundamentally new functional properties. On the other hand, the polymer matrix acts as a stabilizing component, preventing aggregation of metal nanoparticles. These factors determine the unique properties of nanocomposites (biological, catalytic, optical, magnetic, semiconductor) and, ultimately, determine the breadth of their application spectrum.

It is known that the stability of particles is significantly affected by the chemical structure of the polymer [6]. A large number of studies were devoted to the use of (co)polymers of N-vinylpyrrolidone (VP) as a nanostabilizing matrices [7–12].

Phosphoric polymer derivatives are of significant interest because they are used as extractants and complexing agents [13]. Due to high selectivity, phosphorylated polyamines can be successfully employed in the isolation of cations which are in hardly separable combinations [14]. The ability of aminopolyphosphonic compounds to form complexes with various elements opened up the possibility for their use in the removal of metal from organisms [15]. That is why phosphorous-containing copolymers are promising for stabilizing silver particles, since they have a high sorption capacity with respect to metal ions due to the formation of chelate complexes.

Previously, by reducing silver nitrate with sodium borohydride, we obtained polymer nanocomposites that contained silver nanoparticles, stabilized with polysulfones based on tris (diethylamino)-diallylaminophosphonium salts [16]. It is important to continue and expand these studies aimed at the synthesis of phosphorous-containing copolymers used as stabilizing agents for silver nanoparticles.

The article is devoted to the incorporation of silver nanoparticles into the matrices of VP copolymers with DAAP-BF_4 and DAAP-Cl. Thorough structural and optical characterization of nanocomposites was performed using UV-spectroscopy, scanning electron microscopy, DLS and XRD. Antimicrobial activity and cytotoxic effect of nanocomposites were determined.

2. Experimental

2.1. Materials

Tris(diethylamino)diallylaminophosphonium tetrafluoroborate (DAAP-BF_4) and chloride (DAAP-Cl) were obtained as

described in our previous publications [13, 17]. The yield of DAAP-Cl was 86.5%. ($C_{18}H_{40}ClN_4P$) (378.5): Calcd. C 57.07, H 10.57, N 14.79. Found C 56.82, H 11.06, N 14.55. The yield of DAAP-BF_4 was 70.1%. ($C_{18}H_{40}F_4N_4PB$) (430): Calcd. C 50.23, H 9.30, N 13.02; Found C 49.72, H 10.03, N 12.83.

N-vinylpyrrolidone (VP) (Lancaster, reagent grade) was dried by potassium hydroxide and purified by vacuum distillation. A fraction with T_b = 97 °C/13 mm Hg, n_D^{20} = 1.5117 was used.

2,2'-Azobisisobutyronitrile (AIBN) was recrystallized three times from methanol and dried in vacuum at room temperature until a constant weight; the melting temperature was T_m = 103 °C (decomposition).

All the other chemicals were obtained from commercial suppliers. The characteristics of applied solvents conformed to the reference data after purification by conventional methods.

2.2. Copolymerization

Copolymerization of DAAP-BF_4 and DAAP-Cl with VP was conducted in bulk in the presence of AIBN. Copolymers were precipitated and purified by three-fold reprecipitation by diethyl ether from methanol solution. The purified copolymers were dried under vacuum at 50 °C until constant weight was achieved. The copolymer composition was calculated from the elemental analysis data.

2.3. Synthesis of nanocomposites

Synthesis of silver nanocomposites was conducted as follows. The copolymer (10^{-2} mol) was dissolved in water (50 ml). Then $AgNO_3$ (10^{-4} mol of 1% aqueous solution) was added and the reactive mixture was stirred for one hour at room temperature. Then $NaBH_4$ ($2{\cdot}10^{-4}$ mol) was added dropwise with the constant intensive stirring, and the reactive solution was stirred for ten hours at room temperature. Nanocomposites were separated by dialysis. The purified nanocomposites were dried under vacuum at 50 °C until constant weight was achieved.

2.4. Characterization

Fourier transform infrared spectra (FT-IR) were recorded using an IFS 66/S Bruker spectrometer at a resolution of 4 cm^{-1}.

The ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II spectrometer operating at 400 and 100 MHz, respectively, using a broad-band proton decoupling and in a JMOD (J-modulated) mode. DMSO-d_6 was used as a solvent; tetramethylsilane was used as an internal standard.

The optical properties of the nanocomposites were measured using a CF-2000 spectrophotometer in a wavelength range of 200–600 nm.

Concentration of Ag in aqueous solutions was determined with using of atomic-absorption spectrometer iCE 3500 («Thermo Fisher Scientific», USA).

The particle size was determined from the dynamic light scattering measurements using a ZetaPALS analyzer (Brookhaven, USA).

The samples of nanocomposites were studied by means of an Evex Mini-SEM HR-3000 microscope.

The structure of produced nanocomposites was explored by X-ray phase analysis on the XRD-7000 difractometer (Shimadzu, Japan) using the Cu Kα radiations (λ = 1.54062 Å) in the angular interval 2θ = 10–80°.

2.4.1. Microbiological tests

Microbiological tests were performed by serial two-fold dilution. The test cultures were *Staphylococcus aureus,* ATCC 25923; *Staphylococcus epidermidis* 33 GIS; *Micrococcus luteus*, NCIMB 196; *Escherichia coli,* ATCC 25922. Thee bacterial strains used in the work were obtained from the FSBI «Scientific Centre for Expert Evaluation of Medicinal Products» of the Ministry of Health of the Russian Federation (Moscow). The microbial loads were 10^6 cells in 1 ml in LB medium. A bacterial culture was put into 96-well polystyrene microtiter plate for 24 h at 37 °C. The concentration of planktonic cells was evaluated by measuring the optical density at 570 nm (OD^{570}). Duplicate sets of plates were prepared each time, and each experiment was repeated three times to obtain accurate results.

2.4.2. MTT tests

Cytotoxicity of compounds was measured as follows. Cell lines of human lung carcinoma (A549), human rhabdomyosarcoma (RD TE32) and human melanoma (MS) were obtained from the Research Institute of Experimental Tumor Diagnostics and Therapy, N.N. Blokhin Cancer Research Center, Russian Academy of Medical Sciences (Moscow). The cells were kept in the DMEM medium (for A549 and RD) and in the RPMI 1640 medium (for MS) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% gentamicin at 37 °C in the Isotemp Barnstead CO_2 incubator. The 50% cell growth inhibitory concentration (IC_{50}) of the synthesized compounds was determined by the MTT method. A549, RD and MS cells were inoculated at $1.0{\cdot}10^4$ cells/200 mL in 96-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 24 h incubation, various concentrations of the tested compounds (1.00–1.56 μM) were added into each well, and these cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 72 h. All compounds were dissolved in DMSO. The final DMSO concentration in each well did not exceed 0.1% and was not toxic for the cells. The wells with a specific cell culture containing 0.1% DMSO solution in the medium were monitored as control. After incubation, 20 μM MTT (3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl tetrazolium bromide), at a final concentration of 5 mg/mL, was added into each well, and the cells were incubated for another 4 h. The medium was removed and 60 μL DMSO was added to each well. The optical density was measured at 544 nm using a FLUOstar Optima microplate reader. The concentrations (IC_{50}) were calculated according to the dose-

dependent inhibition curves. All experiments were performed for three times and the data were presented as means ± standard deviations (SD). To test the significance of observed differences between the study groups, Student's t-test was applied. A value of $p<0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Synthesis of copolymers

The study of the activity of diallylaminophosphonium salts showed that these compounds do not enter the homopolymerization reaction by the radical mechanism. Diallylaminophosphonium salts are also inactive during copolymerization with vinyl monomers (acrylonitrile, methyl methacrylate, acrylamide, N-vinylpyrrolidone, etc.) with low reaction rates and their insignificant entry into the polymer chain. In particular, during the copolymerization of DAAF-BF_4 with VP (70 mol.%) in DMSO in the presence of AIBN (3 wt.%) at 80 °C for 10 h, the VP content in copolymer is 93 mol.%. An analysis of the above results showed that the homo- and copolymerization of DAAF-BF_4 and DAAP-Cl is difficult, and a significant contribution to this process is made by the degradation chain transfer to the monomer. The activity of diallylaminophosphonium salts can be increased by changing the reaction medium. It is known that the nature of the solvent has a great influence on the VP polymerization [18]. In the VP molecule, the vinyl group through the nitrogen atom is conjugated with the C=O group, which is capable of forming bonds with the molecules of the proton-donor solvent. For this reason, the electronic state of the vinyl group and, consequently, the VP reactivity largely depends on the nature of the solvent. It was found that during the polymerization of DAAF-BF_4 with VP, the activity of the diallyl monomer in a proton-donor solvent (methanol) increases as compared to the polymerization in DMSO. In particular, upon copolymerization of DAAF-BF_4 with VP (70 mol.%) in methanol under similar conditions, the VP content in copolymer is 85 mol.%. The higher activity of DAAF-BF_4 in methanol, as compared with polymerization in DMSO, is associated with the formation of hydrogen bonds between comonomers and the solvent, while the formation of bonds between VP and methanol is more significant, which leads to the displacement of VP from the solvation layer and to a decrease in VP activity.

The dependence of the composition of the DAAF-BF_4 with VP copolymer on the composition of the initial monomer mixture in methanol and DMSO is shown in Figure 1. The studies showed that during copolymerization the resulting products have a statistical distribution of comonomer units in the macromolecule. The values of the relative reactivities presented in Table 1 indicate that, as expected, VP is characterized by a higher reactivity than the diallyl monomer.

The structure of the copolymers obtained was investigated using both 1H and 13C NMR. The spectral parameters of copolymers are presented in Table 1 in Supporting information file. It was established that the copolymerization of diallylaminophosphonium salts with N-vinylpyrrolidone proceeds, both double bonds participating, with formation of cis-, trans-stereoisomeric pyrrolidine structures in a cyclolinear polymer chain. Block fragments of the VP with inclusions of DAAP units prevail in the structure of the copolymer.

3.2. Synthesis of silver nanocomposites

New silver nanocomposites based on new copolymers were obtained by the reduction of $AgNO_3$ with $NaBH_4$ in the copolymer solution. The reaction proceeds via formation of the stable dark brown sols, from which silver nanocomposites are separated.

In the IR spectra of nanocomposites, except for the signals of initial copolymers, there are no signals. This proves that the formation of silver polymer nanocomposites is not accompanied by a change in the chemical structure of the polymer matrix.

The content of silver in the composites was found to be 8 and 11% for DAAP-Cl-VP and DAAP-BF_4-VP, respectively.

UV-spectroscopy and scanning electron microscopy techniques were used to characterize the formation of silver nanoparticles in copolymers. The average silver particle size ranged from 10 to 20 nm, with the corresponding UV-vis absorption peak position at 395–405 nm (Figure 2).

SEM results prove the obtaining of nanocomposites with regular narrow-dispersed distribution of silver nanoparticles of spheric and elliptic forms in the polymer matrix (Figure 3).

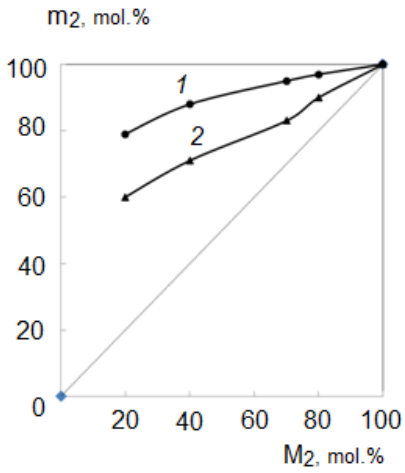

Figure 1 Composition of DAAP-BF4 with VP (M2) copolymers vs. the monomer ratio: [AIBN] = 3 wt.%, T = 80 °C. 1 – DMSO, 2 – methanol.

Table 1 Relative reactivities of DAAP-BF_4 and VP (M_2) at bulk copolymerization (AIBN, 80 °C).

Medium	r_1	r_2	$1/r_2$	$r_1 r_2$
methanol	0.023±0.001	1.928±0.411	0.52	0.04
DMSO	0.072±0.022	7.638±0.135	0.13	0.54

To obtain size distributions of the silver particles, approximately 100–200 particles were counted and then combined into histograms. The average particle sizes of silver nanoparticles ranged from 10 to 20 nm. This experimental result is consistent with DLS measurements (Figure 4). The average particle size of the silver nanoparticles was determined to be in the range of 14–16 nm for both copolymers. Moreover, as it turned out, the colloidal solutions are stable at least further six months due to high stabilizing effect of DAAP copolymers.

The XRD measurements of nanocomposites (Figure 5) were also performed. The peaks at 2θ values of 38°, 44°, 65° and 78° are indexed in, respectively, (111), (200), (220) and (311) planes, signifying a face-centered-cubic (fcc) phase of metallic silver.

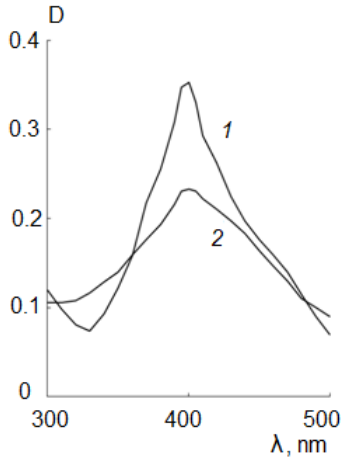

Figure 2 UV extinction spectra of nanocomposite: 1 – DAAP-Cl-VP, water solution, $C = 7.5 \cdot 10^{-3}$ mol/l; 2 – DAAP-BF_4-VP, alcoholic solution, $C = 5 \cdot 10^{-4}$ mol/l.

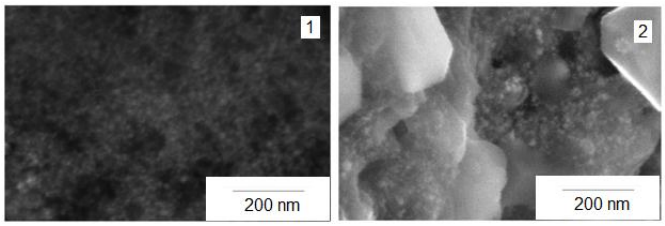

Figure 3 SEM images of silver nanoparticles in DAAP-Cl-VP (1) and DAAP-BF_4-VP (2).

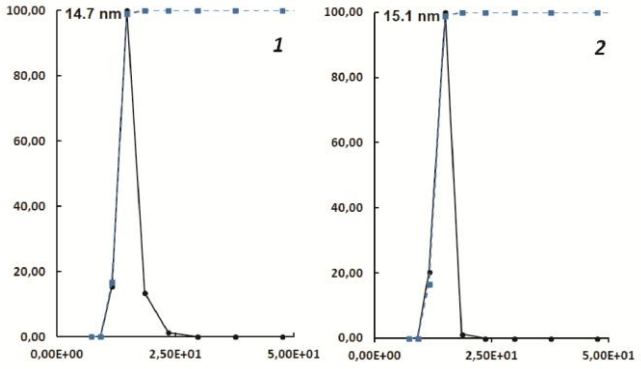

Figure 4 The particle size distribution histograms of AgNCs: 1 – DAAP-Cl-VP, 2 – DAAP-BF_4-VP.

This is in agreement with the data reported for the PVP–Ag nanocomposite [19]. The average crystallite sizes of silver nanoparticles were estimated using the Scherrer equation and were found to be 16.5 and 17.4 nm for DAAP-Cl-VP and DAAP-BF_4-VP, respectively. It should be noted that the XRD data concerning the size of silver nanoparticles are in good agreement with the results of SEM and DLS analysis.

3.3. Antimicrobial effect

Among biocide polymers, the polymeric phosphonium salts have gained importance as medical agents and antiseptics [16, 20–23]. They are believed to be effective in inhibiting the growth of bacteria.

Our studies of antimicrobial activity showed that new copolymers exhibit bactericidal effect (Table 2). The copolymer concentration of 31.2–62.5 μg/mL ensured 100% reduction of *Staphylococcus aureus* and *Micrococcus luteus*. The DAAP-Cl-VP at concentration of 125 μg/mL and DAAP-BF_4-VP at concentration of 250 μg/mL inhibited 100 % *Escherichia coli*.

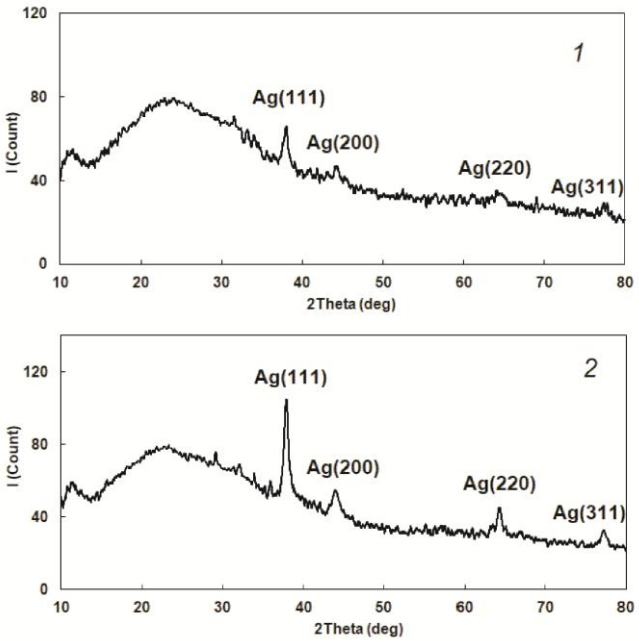

Figure 5 X-ray powder diffractograms of AgNCs based on DAAP-Cl-VP (1), DAAP-BF4-VP (2).

Table 2 Antimicrobial activity of copolymers and their nanocomposites.

Test cultures	MIC, μg/ml			
	DAAP-Cl-VP	Nano DAAP-Cl-VP	DAAP-BF_4-VP	Nano DAAP-BF_4-VP
Staphylococcus aureus	62.5	7.8	62.5	15.6
Staphylococcus epidermidis	62.5	7.8	31.2	7.8
Micrococcus luteus	62.5	15.6	62.5	15.6
Escherichia coli	125	31.2	250	31.2

It is seen that our nanocomposites have a significant activity against both Gram-positive and Gram-negative bacteria. The biocide effect of new nanocomposites is higher as compared to initial copolymers. It should be kept in mind that the fraction of nanosilver is about one-tenth of the total composite concentration.

3.4. Cytotoxic activity

It is well-known that metal-polymer nanocomposites are considered as promising drugs for the treatment of cancer tumors [24]. Cytotoxicity of AgNCs was evaluated *in vitro* by MTT-test using three cell lines, namely the Bronchial carcinoma (A549), Rhabdomyosarcoma (RD) and Melanoma (MS) (Table 3). The nanocomposites have a significant activity against both RD and MS line cells. Moreover, cytotoxic effects of new nanocomposites with respect to RD and MS line cells are comparable to activity of camptothecin – an alkaloid with significant antitumor activity. With the initial copolymers exhibiting low cytotoxic activity, one may say of a decisive contribution of silver nanoparticles to the cytotoxic activity of the composites.

4. Conclusions

In summary, new silver nanocomposites based on copolymers of tris(diethylamino)diallylaminophosphonium tetrafluoroborate and chloride with N-vinylpyrrolidone have been developed. The copolymers provide a very good covering for silver particles and prevent particle growth or formation of large aggregates. The developed silver nanocomposites are well characterized by using different techniques to confirm the formation of silver nanoparticles with size of 10–20 nm. New silver nanocomposites possess significant antimicrobial properties against both Gram positive and Gram-negative microflora. Also, they have a significant cytotoxic activity towards RD and MS line cells, and these cytotoxic effects are not inferior to activity of camptothecin. Thus, the use of phosphorus-containing copolymers in the synthesis of silver nanoparticles opens up a number of opportunities for development of non-toxic functional materials for biomedical applications.

Supplementary materials

Spectral parameters of copolymers are provided in the supporting information file.

Funding

The study was funded by Russian Foundation for Basic Research and Government of the Perm Region according to the research project № 19-43-590019 r_a. https://www.rfbr.ru/rffi/eng; http://minobr.permkrai.ru.

Acknowledgments

Analytical, spectroscopic, and biological studies were performed at the "Research of materials and substances" collective Center of PFRC UB RAS.

The authors gratefully acknowledge Dr. Dmitriy Kiselkov, ITCh UB RAS, for his generous advice on SEM analysis.

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Supervision: M.N.G.
Validation: M.N.G., Y.A.B.
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Conflict of interest

The authors declare no conflict of interest.

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Table 3 Cytotoxic activity of AgNCs.

Culture	IC_{50}, μM				
	Camptothecin	DAAP-Cl-VP	Nano DAAP-Cl-VP	DAAP-BF_4-VP	Nano DAAP-BF_4-VP
Rhabdomyosarcoma RD	2.79±0.08	101.26±0.36	25.83±11.26	97.16±2.26	5.73±0.15
Bronchial carcinoma A549	2.26±0.01	no effect	no effect	81.51±1.47	31.19±0.63
Melanoma MS	1.63±0.00	91.25±0.03	1.27±0.00	no effect	1.87±0.02

Institute of Ecology and Genetics of Microorganisms of the UB RAS, http://www.iegm.ru/.

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