Silver nanocomposites based on copolymers of N,N-diallyl-N’-acylhydrazines with N-vinylpyrrolidone

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Abstract. New nanocomposites based on Ag and copolymers of N-vinylpyrrolidone with N,N-diallyl-N’-acetylhydrazine [poly(VP-DAAH)], N,N-diallyl-N’-butanoylhydrazine [poly(VP-DABH)] and N,N-diallyl-N’-benzoylhydrazine [poly(VP-DABEH)] have been developed. The average silver particle size ranged from 10 to 20 nm for poly(VP-DAAH), from 13 to 21 nm for poly(VP-DABH) and from 30 to 42 nm for poly(VP-DABEH), with the corresponding UV-vis absorption peak position at 400-410 nm. New nanocomposites based on copolymers of N,N-diallyl-N’-acylhydrazines have cytotoxic activity against rhabdomyosarcoma RD and melanoma MS. This effect is comparable to the activity of camptothecin, an alkaloid with high antitumor activity.

1. Introduction
High-molecular compounds and their metal-containing nanocomposites are characterized by unique properties and are promising for medicine, optoelectronics, nanophotonics, and catalysis [1-4]. Silver nanoparticles have antimicrobial activity, which allows them to be used to obtain antibacterial drugs [3]. Silver nanoparticles are used as anti-cancer therapeutic agents for the treatment of leukemia, breast cancer, hepatocellular carcinoma, lung cancer, skin cancer, and oral cancer [5-9].

Recently, among the rapidly developing methods for producing nanomaterials, the greatest attention is paid to methods for producing composite materials based on organic polymer matrices and metal nanoparticles. The polymer matrix acts as a nanostabilizing component, preventing the aggregation of metal nanoparticles. Moreover, the polymer nature has a significant effect on the particle stability [10]. Polymers of both synthetic (polyethylene glycol, polyvinylpyrrolidone, polyethylene, etc.) and natural (cellulose, chitosan, arabinogalactan, etc.) origin are studied. The highly stable hydrosols with isolated silver nanoparticles stabilized by poly-N-vinylpyrrolidone were obtained by borohydride method [11]. Hydrophilic poly-N-vinylpyrrolidone is also used to stabilize sols and simultaneously restore silver ions to a molecular metallic state in water-alcohol solutions in the preparation of poviargol [12]. The nanocomposites based on silver nanoparticles and homo- and copolymers of 1-vinyl-1,2,4-triazole were obtained [13, 14] which are promising for the development of new water-soluble antiseptics and bactericidal preparations. We obtained biocidal silver nanocomposites based on copolymers of 2,2-diallyl-1,1,3,3-tetraethylguanidinium chloride with N-vinylpyrrolidone and vinyl acetate [15,16].

Our work is focused on the incorporation of silver nanoparticles into the matrices of N-vinylpyrrolidone copolymers with N,N-diallyl-N’-acylhydrazines (DAH). Thorough structural and optical characterization of nanocomposites was performed using UV-spectroscopy and scanning...
electron microscopy. Antimicrobial efficiency of new nanocomposites was tested against both Gram-positive and Gram-negative bacteria. Cytotoxic activity of nanocomposites towards lung carcinoma, rhabdomyosarcoma and melanoma was determined by the MTT method.

2. Experimental

2.1. Materials

N-vinylpyrrolidone (VP) was dried by potassium hydroxide and purified by vacuum distillation. A fraction with $T_b=97\,^\circ\mathrm{C}/13\,\text{mm Hg}$, $n_D^{20}=1.5117$ was used.

N,N-diallyl-N'-acylhydrazines (DAH) were obtained as a result of the interaction of the corresponding hydrazide and allyl chloride by the technique as described in our previous work [17].

$$R=\text{CH}_3(\text{DAAH}),\text{C}_3\text{H}_7(\text{DABH}),\text{C}_6\text{H}_5(\text{DABEH})$$

The fractions of DAAH with $T_b=120\,^\circ\mathrm{C}/5\,\text{mm Hg}$, $n_D^{20}=1.4771$, DABH with $T_b=145\,^\circ\mathrm{C}/8\,\text{mm Hg}$, $n_D^{20}=1.4740$, DABEH with $T_{\text{mel}}=108\,^\circ\mathrm{C}$ (acetone:water=1:1) were used in our experiments.

All the other chemicals were obtained from commercial suppliers. The characteristics of applied initiator (2,2'-azobisisobutyronitrile (AIBN)) and solvents (DMSO, acetone, diethyl ether, methanol) conformed to the reference data after purification by conventional methods.

2.2. Copolymerization

Copolymerization of VP with DAH was conducted in bulk in the presence of $3.0 \cdot 10^{-2}\,\text{mol L}^{-1}$ AIBN. Copolymers were precipitated and purified by three-fold reprecipitation by diethyl ether from methanol solution. The purified copolymers were dried under vacuum at $50\,^\circ\mathrm{C}$ 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. Poly(VP-DAH), poly(VP-DABH) or poly(VP-DABEH) (10² mol) were dissolved in water or methanol. Then AgNO₃ (5 x 10⁻⁴ mol of 1% aqueous solution) was added and the reactive mixture was stirred for one hour at room temperature. Then NaBH₄ (10⁻³ mol, 0.038 g) 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 $50\,^\circ\mathrm{C}$ until constant weight was achieved.

2.4. Instruments

Fourier transform infrared spectra (FT-IR) were recorded using a IFS 66/S Bruker spectrometer at a resolution of 4 cm⁻¹. Samples were coated on KBr plates and dried. The measurements were performed in the range from 400 to 4000 cm⁻¹ at room temperature.

The $^1\text{H}$ and $^{13}\text{C}$ 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).

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

2.5. Microbiological test
Microbiological tests were performed by serial two-fold dilution of preparations in LB medium. Test cultures were *Staphylococcus aureus*, ATCC 25923; *Staphylococcus epidermidis* 33; *Micrococcus luteus*, NCIMB 196; *Escherichia coli*, ATCC 25922; *Bacillus subtilis* ATCC 6633; *Klebsiella pneumonia*, ATCC 700 603. Microbial loads were $10^6$ cells in 10 μl in LB medium. The measurements were made after incubation of bacteria for 20 h at 37°C. Experiments were repeated three times.

2.6. Cytotoxicity test

Cytotoxicity of compounds was performed 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). Cells were kept in DMEM medium (for A549 and RD) and in 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 CO₂ incubator. The 50% cell growth inhibitory concentration (IC$_{50}$) of the synthesized compounds was determined by the MTT method. The optical density was measured at 544 nm using a FLUOstar Optima microplate reader. 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 deviation. 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

Copolymers of N-vinylpyrrolidone with N,N-diallyl-N'-acetylhydrazine (poly(VP-DAAH)), N,N-diallyl-N'-butanoylhydrazine (poly(VP-DABH)), N,N-diallyl-N'-benzoylhydrazine (poly(VP-DABE)) were obtained by radical polymerization in the presence of AIBN.

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R = \text{CH}_3, \text{C}_2\text{H}_5, \text{C}_3\text{H}_7, \text{C}_6\text{H}_5
\]

The IR spectra of copolymers contain several characteristic bands that are useful to the structural characterization of the polymers. We observe characteristic aliphatic methylene stretches between 3100 and 2850 cm$^{-1}$ and the broadened carbonyl band at 1668 cm$^{-1}$ VP. This broadened band is the result of spectral overlap between the acylhydrazine C=O stretch and the pyrrolidone C=O stretch. By $^{13}$C NMR it was found that N,N-diallyl-N'-acylhydrazines copolymerize with VP, both double bonds participating with formation of five-membered pyrrolidine structures in a cyclolinar polymer chain. Analysis of $^{13}$C NMR spectra of copolymers indicates that block fragments of a vinyl monomer with inclusion of DAH units predominate in the copolymer structures. The DAH units consist of a mixture of cis-, trans-stereoisomers with a significant predominance of cis-heterocycles. The copolymers obtained are light powders. They are soluble in methanol, chloroform, DMSO, dimethylformamide. The poly(VP-DAAH) is also soluble in water. The intrinsic viscosity of poly(VP-DAAH) (80:20 mol%) is 0.11 dl/g (water, 25°C). Molecular weight of poly(VP-DAAH) (80:20 mol%), calculated by the Levy and Frank equation for PVP ([η] = 6.76 M$^{0.55}$ $\times$ 10$^{-4}$[18]), is equal to 10500.

We used our novel VP copolymers as stabilizing agents in the synthesis of silver nanoparticles. The synthesis of silver nanocomposites was carried out by the reduction of silver nitrate with sodium...
borohydride in an aqueous (DAAH) or alcohol (DABH and DABEH) copolymer solution. Reaction proceeds via formation of the stable dark brown sols, from which soluble nanocomposites were separated by dialysis. The silver content depends on the copolymer used and is from 4 to 19%. The ratio of silver nitrate, reducing agent and copolymer also affects the concentration of silver in nanocomposites.

In the IR spectra of the obtained nanocomposites, except for the signals of the initial copolymers, there are no other signals, this fact indicates the constancy of copolymer structure. Nevertheless, in the IR spectra of nanocomposites based on DAH copolymers, one can observe an increase in the intensity of the absorption bands of NH group stretching vibrations at 1553 cm⁻¹ and CN group stretching vibrations at 1282 cm⁻¹. We can notice a decrease in the intensity of the absorption band of stretching vibrations of carboxyl groups at 1668 cm⁻¹. These changes indicate the involvement of O and N atoms of VP and DAH in the interaction with silver nanoparticles.

It should be noted that due to the coordination binding of silver ions with the functional groups of macromolecules, a favorable microenvironment is created for the reduction of these ions [19]. Consequently, the obtained silver nanoparticles are incorporated into the macromolecules and are retained therein mainly through numerous specific electrostatic or covalent bonds between the functional groups of the copolymers and silver atoms on the surfaces of the nanoparticles. In addition, physical adsorption (dipole interactions, van der Waals forces) also makes a certain contribution, but the effect of the latter is much smaller than the effect of chemisorption.

In the UV spectra of nanocomposite solutions, a characteristic plasmon absorption band with a maximum in the region of 400–410 nm is observed (Fig. 1). The shape of the absorption spectra and the half-width of the maximum indicate the formation of sols with isolated silver nanoparticles [20].

![Figure 1. UV extinction spectra of nanocomposite solution: 1 – poly(VP-DAAH), water, C=7.5·10⁻³ mol/l; 2 – poly(VP-DABH), methanol, C=10⁻³ mol/l; 3 – poly(VP-DABEH), methanol, C=5·10⁻⁴ mol/l.](image-url)

The results of SEM microscopy confirm the preparation of nanocomposites with a uniformly narrow distribution of silver nanoparticles in a polymer matrix. SEM micrographs and the size distribution of nanoparticles are shown in Fig. 2. It was established that the nanoparticles of spherical and elliptical shapes are formed.
Figure 2. SEM images of silver nanoparticles and particle size distribution histograms of AgNCs based on: poly(VP-DAAH) (a,d), poly(VP-DABH) (b), poly(VP-DABEH) (c).

The influence of molecular weight of copolymer, the copolymer concentration and Ag$^+$ concentration on the size of Ag nanoparticles was investigated (Table 1, 2, 3). It was determined that with increasing molecular weight of copolymer, the copolymer concentration and Ag$^+$ concentration the average particle size decreased.

Table 1. The influence of M_n on the size of Ag nanoparticles.

| Copolymer          | M_n  | Size of Ag nanoparticles, nm |
|--------------------|------|------------------------------|
| poly(VP-DAAH)      | 13000| 16                           |
| C(copolymer) = 2.0 wt. % |      |                              |
| C(Ag$^+$) = 0.05 mol/L   | 15900| 13                           |
|                     | 21000| 12                           |
| poly(VP-DABEH)     | 15700| 20                           |
| C(copolymer) = 2.0 wt. % |      |                              |
| C(Ag$^+$) = 0.05 mol/L   | 17500| 18                           |
|                     | 28000| 17                           |

Table 2. The influence of the copolymer concentration on the size of Ag nanoparticles.

| Copolymer          | Concentration, wt. % | Size of Ag nanoparticles, nm |
|--------------------|----------------------|------------------------------|
| poly(VP-DAAH) Mn = 15900 | 1.0                 | 14                           |
| C(Ag$^+$) = 0.05 mol/L   |                     |                              |
|                       | 2.0                 | 13                           |
| poly(VP-DABEH) Mn = 17500 | 1.0                 | 22                           |
| C(Ag$^+$) = 0.05 mol/L   |                     |                              |
|                       | 2.0                 | 18                           |
Table 3. The influence of the Ag+ concentration on the size of Ag nanoparticles.

| Copolymer                        | Concentration Ag+, mol/L | Size of Ag nanoparticles, nm |
|----------------------------------|--------------------------|------------------------------|
| poly(VP-DAAH) Mₙ = 15900         | 0.025                    | 15                           |
| C(copolymer) = 2.0 wt. %         |                          |                              |
| poly(VP-DABEH) Mₙ = 17500        | 0.025                    | 22                           |
| C(copolymer) = 2.0 wt. %         | 0.050                    | 18                           |

Studies of antimicrobial activity of nanocomposites obtained showed (Table 4) that our nanocomposites have a moderate activity both against Gram positive and Gram negative microflora. The biocide effect of new nanocomposites is higher compared to initial copolymers. It should be kept in mind that the nanosilver is about one twentieth of the total composite concentration.

Table 4. Antimicrobial activity of copolymers and their nanocomposites

| Test cultures                  | Minimal bacteriostatic concentration (MBsC), μg/ml |
|--------------------------------|---------------------------------------------------|
|                                | poly(VP-DAAH) | Silver nanocomposite of poly(VP-DAAH) | poly(VP-DABEH) | Silver nanocomposite of poly(VP-DABEH) |
| Staphylococcus aureus ATCC 25923 | >1000        | 125                                    | 500            | 125                                    |
| Staphylococcus epidermidis 33   | >1000        | 250                                    | 500            | 62.5                                    |
| Micrococcus luteus NCIMB 196    | >1000        | 250                                    | 250            | 62.5                                    |
| Bacillus subtilis ATCC 6633     | >1000        | 500                                    | 500            | 125                                    |
| Escherichia coli ATCC 25922     | >1000        | 500                                    | >1000          | 62.5                                    |
| Klebsiella pneumonia ATCC 700 603| >1000        | 500                                    | >1000          | 62.5                                    |

New nanocomposites based on DAH copolymers have cytotoxic activity against rhabdomyosarcoma RD and melanoma MS cell lines (Table 5). Nanocomposites at a concentration of 0.36 and 2.59 μM, for poly(VP-DAAH) and poly(VP-DABEH) respectively, inhibit 50% of MS melanoma cells. The death of 50% RD rhabdomyosarcoma cells is observed at nanocomposites concentrations of 10.14 and 11.28 μM, for poly(VP-DAAH) and poly(VP-DABEH), respectively. The cytotoxic activity of the new nanocomposites is comparable to the activity of camptothecin, an alkaloid with high antitumor activity.

Table 5. Cytotoxic activity of AgNCs based on poly(VP-DAAH) and poly(VP-DABEH).

| Culture                | Camptothecin Poly(VP-DAAH) | Silver nanocomposite of poly(VP-DAAH) | Poly(VP-DABEH) | Silver nanocomposite of poly(VP-DABEH) |
|------------------------|---------------------------|---------------------------------------|----------------|---------------------------------------|
| Rhabdomyosarcoma RD     | 1.72±0.37                 | 28.16±1.34                           | 32.18±2.00     | 11.28±0.26                           |
| Bronchial carcinoma A549| 1.31±0.03                 | no effect                             | no effect      | 85.49±0.084                          |
| Melanoma MS             | 0.77±0.34                 | 72.34±2.86                           | 65.48±1.44     | 2.59±0.42                            |

The discovered biological activity of DAH-based nanocomposites makes them promising for the development of new medicines and biocides.
4. Conclusion
Thus, new water-soluble nanocomposites based on Ag and copolymers of N-vinylpyrrrolidone with N,N-diallyl-N’-acylhydrazines have been developed. These nanocomposites have cytotoxic activity against rhabdomyosarcoma RD and melanoma MS. Following these results, the newly developed nanocomposite scaffold may be considered for the development of new drugs for the complex treatment of malignant neoplasms.

Acknowledgment
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.

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. Darya Eroshenko, ITCh UB RAS, for providing cytotoxic experiments and Dr. Larisa Lemkina, IEGM UB RAS, for microbiological tests. We also wish to thank Dr. Dmitriy Kisel’kov, ITCh UB RAS, for his generous advices for SEM analysis.

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