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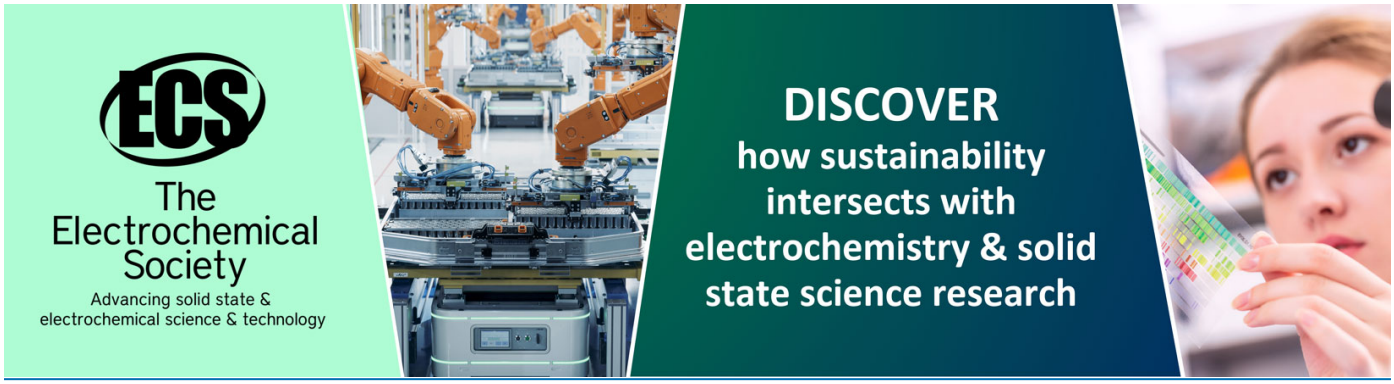

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The effect of particle size on the toxic action of silver nanoparticles

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Abstract. Silver nanoparticles in AOT reverse micelles were obtained by means of the biochemical synthesis. Synthesis of nanoparticles was carried out with variation of the three parameters of reverse-micellar systems: concentration of silver ions, concentration of the stabilizer (AOT) and hydration extent w = $[H_2O]/[AOT]$. The combinations of varied parameters have been found, allowing to prepare micellar solutions of spherical silver nanoparticles with average sizes 4.6 and 9.5 nm and narrow size distribution. From micellar solution the nanoparticles were transferred into the water phase; water solutions of the nanoparticles were used for testing their biological activity. Our assay is based on negative chemotaxis, a motile reaction of cells to an unfavorable chemical environment. Plasmodium of the slime mold *Physarum polycephalum* used as an object is a multinuclear amoeboid cell with unlimited growth and the auto-oscillatory mode of locomotion. In researches of chemotaxis on plasmodium it is learned that silver nanoparticles of smaller size exhibit a higher biological activity (behave as stronger repellent) and this correlates with the literary data obtained in studies of silver nanoparticles interaction with other biological objects.

1. Introduction

It is known that in the course of interaction with living organisms, metal nanoparticles can both render curative effects and cause various pathologies. Silver nanoparticles exhibit the widest spectrum of applications, the fact that stimulated intensive studies of their biological effects. The main goal of such researches is accumulation of the data on the toxicity of these nanoparticles for living organisms, that allows to define conditions of their safe usage. Therefore, the necessity arose to determine, first, the range of nanoparticle concentrations where their toxic action is dangerous for life and health and, second, to obtain as much information as possible about the effect of various nanoparticle parameters (size, form, structure, surface charge) on their biological activity.

In studies of the biological effects of silver nanoparticles experimental evidence was gained on their toxicity towards bacteria and viruses [1-7], algae [8], animal and human cultured cells [9-11], fish embryos [12], animal organisms [13, 14]. Apart from the determination of lethal concentrations

and threshold concentrations, which mark the appearance of the toxic effects, it was found also that the extent of toxicity depends of the particle size [3, 7] and shape [4].

For the studies of biological effects of silver nanoparticles it is necessary that they were stable on air in solution, and homogeneous in sizes. The method of biochemical synthesis in reverse micelles [15, 16] allows to prepare silver nanoparticles, stable on air in solution for a long time. In the present work the biochemical synthesis has been used for preparation of silver nanoparticles of different average diameter with narrow size distribution. This allows to study the dependence of their biological effects on the particle size. It seemed important to investigate the area of small sizes (less than 20 nm), where the so-called "size effects" are most clearly expressed [e.g.17]. To obtain micellar solutions achieve our goal we varied three parameters of the reverse-micellar system: the concentration of stabilizer (AOT), that of silver ions and the hydration extent $w = [H_2O]/[AOT]$. As reported earlier in a series of works devoted to the studies of metal nanoparticles formation in reverse micelles (e.g. [18-20]), these parameters affect the sizes of water micelles and (or) nanoparticles. As shown earlier [21], we managed to find the combinations of parameters providing the formation of at least two populations of spherical silver nanoparticles with rather narrow distribution and large enough difference in average sizes, the maximum average size not exceeding 10.5 nm.

From their micellar solutions, water dispersions of silver nanoparticles were prepared by the specially developed procedure [22]. These water dispersions may be used, in particular, for the studies of the nanoparticles' interaction with biological objects. In this work, two water dispersions obtained from the nearly monodispersed micellar solutions of silver nanoparticles were tested on plasmodium of the acellular slime mold Physarum polycephalum.

Physarum is a classical object for studying cell motility, and characteristic features of its chemotactic behavior are well established [24–26]. In particular, substances causing negative taxis (repellents) were shown to increase the period of contractile oscillations and to decrease the area of spreading when applied at spatially uniform concentrations [25, 26]. Due to this characteristic features, our experiments with *Physarum* allowed to register not only the lethal events, as with the majority of other objects, but also the changes in its behavior taking place in the mild conditions, at sublethal nanoparticle concentrations. Hence, this chemotaxis – based assay may be regarded as a highly sensitive tool for the studies of the biological action of silver nanoparticles.

The experiments performed with pairs of *Physarum* preparations on agar plates [23] showed that the chemotactic tests were sensitive enough for recognizing the difference in their efficiency, the fact that allowed to use such tests for the estimation of the particle size effect in the repellent action of silver nanoparticles.

2. Experimental

Silver Nanoparticles (SNP) were obtained by the biochemical synthesis.
Silver nitrate (analytical grade) and deionized water were used to prepare the $AgNO_3$ aqueous solution. The $[Ag(NH_3)_2]NO_3$ solution was obtained by adding 27 % aqueous ammonium hydroxide until silver hydroxide sediment was fully dissolved with creation of $[Ag(NH_3)_2]^+$-ions. To obtain reverse micelles, AOT (sodium bis(2-dioctyl)sulphosuccinate) was used as surfactant, and isooctane (analytical grade), as solvent. The natural flavonoid quercetin (Qr or 3,5,7,3',4'-pentahydroxyflavone) was applied as reducing agent.

The standard procedure for preparation of Qr micellar solution with the known concentration of the flavonoid is described in detail elsewhere [16, 22, 27].

For the synthesis of silver nanoparticles, $[Ag(NH_3)_2]NO_3$ water solution was added to the quercetin micellar solution to the given silver salt concentration (C_{Ag}) and hydration extent. The AOT concentration (C_{AOT}) in micellar solution did not exceed 0.135 M. The concentration of silver nanoparticles (C_{SNP}) in micellar solution was determined from the measured optical densities in

absorption band maximum and the extinction coefficient (ε = 1.03 *10^4 l/mol *sm) found by us as described in [27]. The C_{SNP} in solution is estimated as equivalent concentration either of silver salt (usually in millimols/liter) or as weight concentration of metallic silver (most often in μg/ml).

The absorption spectra of micellar or water solutions were recorded on spectrophotometer Helios-α (Thermo Electronics, GB) in 1 mm quartz cell at room temperature. Either isooctane or distilled water was used as reference solution. Particle sizes in micellar solutions were determined by transmission electron microscopy on LEO912 AB OMEGA microscope (production of Carl Zeiss, Germany) at 120 kV accelerating voltage. From electron micrographs particle size distributions were found for no less than 350 particles. Average sizes and standard deviations were determined using the Gauss approximation.

Plasmodium ***Physarum polycehallum*** is capable of unlimited growth as a single multinuclear cell. The migrating plasmodium looks like a fan-shaped protoplasmic film, which is smooth and continuous at the front and transforms into a tree-shaped network of individual protoplasmic strands in more caudal regions. Migration of plasmodium occurs due to the more intensive or more prolonged protoplasm streaming toward the leading edge. Any fragment of plasmodium restores the integrity of the plasma membrane [28] and resumes the contractile and motile activities. By this property, strands excised from a plasmodium can be used for force measurements, and film fragments of standard size and shape can be used in chemotactic assays based on the sensitivity of plasmodium growth to its physiological state and external influence.

For the studies of size effects the agar strips have been used, containing the nanoparticles added as water solution. The plasmodium film fragment was placed on the boundary between the two agar strips with the SNP of different size introduced to the equal total silver concentration. The strips were incubated in standard conditions and the difference in the direction of plasmodium growth was recorded, showing the preference of Physarum to the nanoparticles of one of the two sizes studied.

3. Results and discussion

3.1. Synthesis of Silver nanoparticles with different average size

As shown earlier [21], we managed to find the conditions which allow to receive solutions of silver nanoparticles with the average size in the range from 4.6 to 9.3 nm and with small degree of polydispersion. The nanoparticles are approximately spherical, stable in the solution for a long time (for several years).

From these micellar solutions water dispersions were obtained for the studies of biological effects. It is known that, during their transfer to the water phase, the sizes of nanoparticles do not change, and their concentration appears to be equal or close to that in micellar solution [22, 29]. Typical change of the optical spectra at the transfer of silver nanoparticles from micellar solution to water phase in the process of preparation of AgNPw water solution is shown in Figure 1.

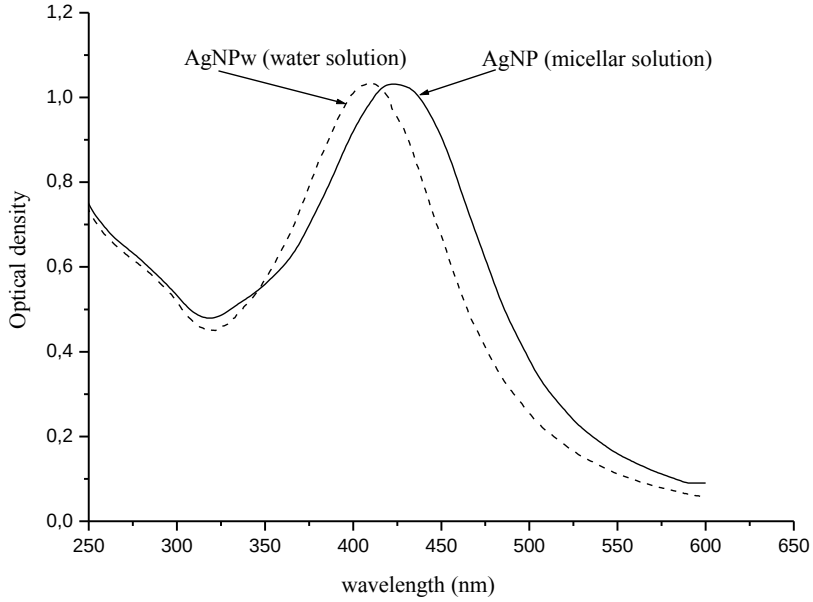

Figure 1. Typical change of the optical spectra at the transfer of silver nanoparticles from organic (micellar solution) to water phase in the process of preparation of SNP water solution.

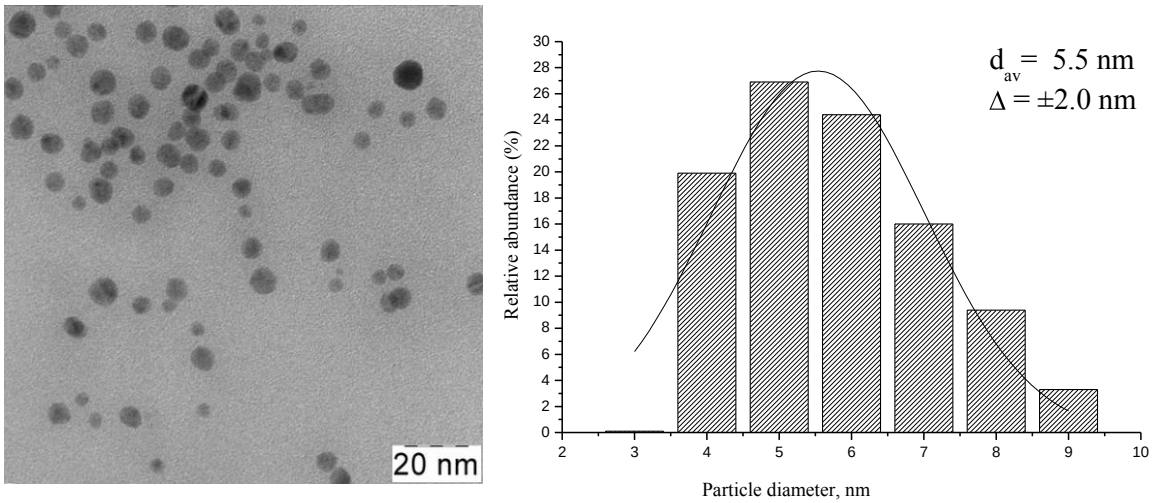

Figure 2. Electron micrograph (a) and histograms of the sizes (b) of silver nanoparticles in water solution, average diameter 5.5 nm.

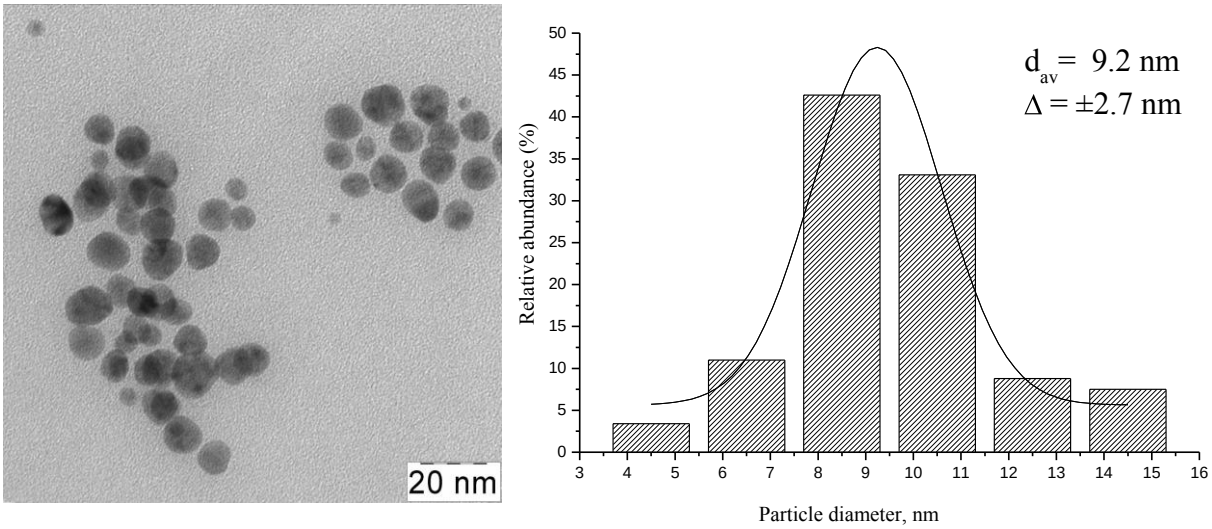

Figure 3. Electron micrograph (a) and histograms of the sizes (b) of silver nanoparticles in water solution, average diameter 9.2 nm.

Comparison of the TEM micrographs and hystograms for water solution (Figures 2, 3) and the corresponding micellar solution [21] shows that there is almost no change both of average size and size distribution.

3.2. Toxic Effects of Silver nanoparticles

Water dispersions presented in Figures 2, 3 have been used were tested on the Physarum plasmodium. Testing was made on the agar strips containing 3 μg-ion/l of SNP1 (d_{av}=5.5±2.0 nm) and SNP2 (d_{av}=9.2±2.7 nm). The result of testing is shown in Figure 4. It is seen that almost all plasmodia are oriented towards SNP2, and consequently, the SNP1 behave as more strong repellent. This correlates with the data reported in literature on the more strong toxicity of smaller nanoparticles [3, 7]. It seems probable that the effect of particle sizes is connected mainly with the difference in their number per unit volume: for a given overall concentration of silver, the number of smaller nanoparticles will exceed that of the nanoparticles of larger size. This shows probably to the significance of the numerical concentration of nanoparticles per unit volume of the medium: at the equal silver concentration, numerical concentration of the smaller particles will be higher, thus providing the possibility of the more intensive adsorption onto the cell surface.

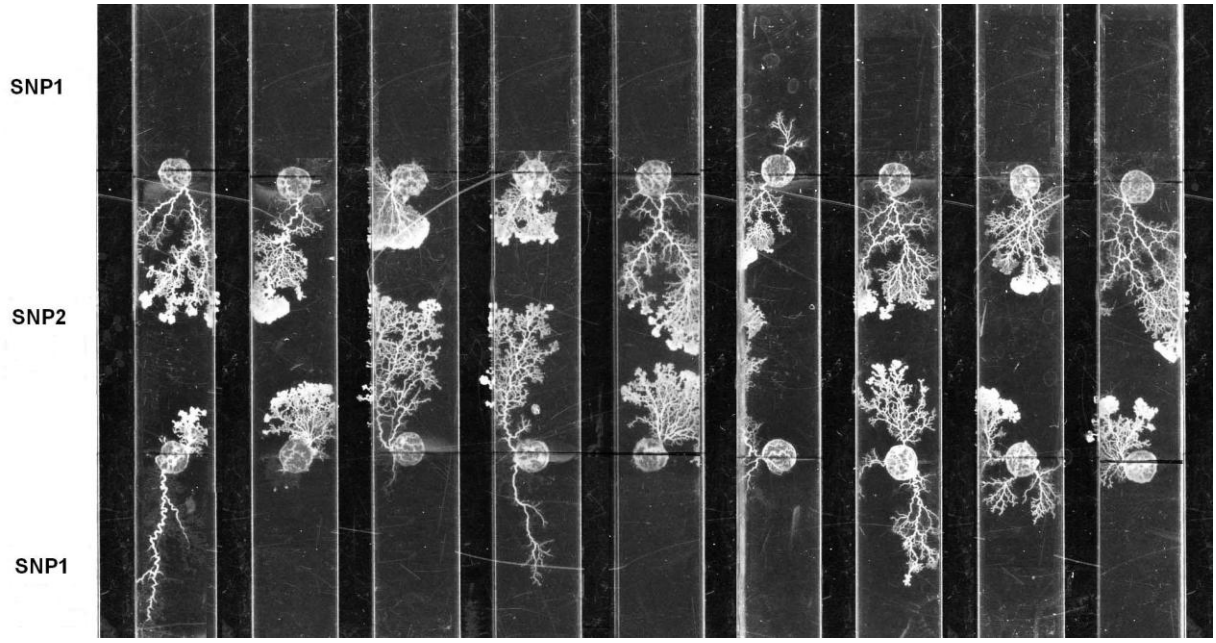

Figure 4. Comparison of the repellent action of SNP1 (d_{av}=5.5±2 nm) and SNP2 (d_{av}=9.2±2.7 nm) at equal concentration of 3 μg-ion/l. Directional movement of all plasmodia toward SNP2 containing strips (in the center of chamber) proves the higher repellent efficiency of SNP1. Scale bar, 1 cm.

4. Conclusions

The conditions are found allowing to obtain stable silver nanoparticles of average size in the range 4.6 - 9.3 nm with narrow size distribution. The nanoparticles are approximately spherical, stable in solution for a long time (for several years). For the studies of the nanoparticles interaction with living organisms, water dispersions of silver nanoparticles different in size were obtained from their micellar solutions. Investigation of the effect of water dispersions on chemotactic behavior of plasmodium of the slime mold *Physarum polycephalum* showed that nanoparticles of smaller size demonstrate a higher biological activity (are stronger as repellent). This observation agrees with the literary data obtained in studies of the interaction of SNP with some other biological objects.

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