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Chapter 2

ASSESSMENT OF THE CYTOTOXICITY OF SILVER NANOPARTICLES WITH DIFFERENT SURFACE CHARGE

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ABSTRACT

Evidence on the toxic effects of silver nanoparticles (AgNPs) to date is well documented and should be taken into account in working out their applications. Nevertheless, there is a lack of systematized information concerning the nanoparticles' parameters affecting the extent of their toxicity towards living organisms. In particular, it is important to elucidate the role of nanoparticles' surface charge in their interaction with biological cells, both *in vitro* and *in vivo*. Our study was aimed to estimate the influence of surface charge on the toxic effects of Ag NPs on endothelial cells *in vitro*. The nanoparticles were obtained by means of the biochemical synthesis based on the reduction of metal ions with natural flavonoid quercetin. To prepare nanoparticles with different surface charges, two different stabilizers were used during synthesis – aerosol-OT (AOT) and β-cyclodextrin (β-CD). The nanoparticles were characterized by optical absorption spectra, transmission electron microscopy and photon correlation spectroscopy technique; the latter was used in measurements of nanoparticles' size and zeta potential in water solution. Both kinds of AgNPs were negatively charged but their surface charge differed in magnitude, as followed from the measured electrophoretic mobility. Attention was paid to the fact that, according to the modern theories of electrophoresis, in case of nanoparticles true zeta potential (ζ) may differ from that found from the measured mobility through the Smoluchowski equation (ζ_{sm}). Therefore the mobility measurements have been carried out in the conditions when difference in ζ_{sm} values for the two kinds of AgNPs really reflects the difference in their surface charge. The effect of Ag NPs on viability of normal endothelial cells (EA.hy926 line) was estimated by MTT assay, also the apoptosis/necrosis detection in cell culture was performed by flow cytofluorimetry. It has been found that AOT-stabilized AgNPs bearing greater negative surface charge were more toxic than β-CD-stabilized Ag NPs. Our data testify to the connection between negative surface charge magnitude and

toxicity of Ag NPs towards normal human cells *in vitro*, as well as to the important role of stabilizer in biological activity of nanoparticles

Keywords: silver nanoparticles; zeta potential, endothelial cells; cell viability; apoptosis

INTRODUCTION

Silver nanoparticles (AgNPs) are one of the most widely used nanotechnological products, first of all because of their antibacterial properties (e.g., [1-3]). Nanosized silver is coming into use as a component of various consumer products such as disinfectants, textiles, varnish-paint materials and other industrial goods [4]. Among biomedical applications, the most known are wound dressings, coatings of surgical equipment such as catheters, implants etc. with composites containing AgNPs, and also biosensors used in diagnostic procedures [5-8]. At the same time, studies on the toxicity of silver nanoparticles (required for their safe applications) revealed their negative influence on the biological objects observed in experiments both in vitro and in vivo [9-12]. Thus as actual problem now is regarded the accurate knowledge of the conditions providing safe use of the nanoparticles and modified materials and elucidation of the mechanism of their toxic action on human organism.

The corresponding investigations include chiefly determination of the toxicity dependence on the nanoparticles' concentration as well as on their main characteristics – size, shape and surface properties. The latter usually have two constituents: chemical properties determined by composition of a capping layer and surface charge caused by the dissociation of ionizable groups present in the capping layer. More rarely used are uncapped nanoparticles bearing positive charge because of the release of electrons from the particles surface. The majority of studies was performed on mammalian cells in vitro; the results achieved in this field are considered in reviews and original papers published mainly in the last decade (e.g., [12-14]).

Probably the most obscure today is the role of nanoparticles' surface charge in their action on cell viability expressed as changes in proliferation rate and/or various functions of cells' internal structures. It is assumed that surface charge density of the nanoparticles can be estimated by measuring their zeta potential. Judging from the data obtained with differently charged metal nanoparticles (i.e., with nanoparticles which possess zeta potential different in sign or magnitude), both on animal and bacterial cells [15-21], there is no doubt that electrostatic interactions may contribute to the effects observed. However the evidence is still insufficient for the definite conclusion about the connection between the charge sign and/or magnitude and the amplitude of cell responses after exposure to nanoparticles. For example, it is unclear why in some cases the toxicity increases with the increase of negative zeta potential [11]; one should expect the opposite effect, taking into account that cell membrane is always negatively charged and hence the increase of the nanoparticles negative charge should result in the increase of electrostatic repulsion which prevents the nanoparticles from adsorption on cell membrane surface.

One possible reason may be simply that the data on AgNPs zeta potentials obtained for today are not numerous enough to make the reliable conclusions. There is, however, another explanation. As mentioned in our recent publications [22, 23], there are some methodological problems which should be solved in order to find the real effect of nanoparticles' surface

charge on cells in culture. One point is that, strictly speaking, the experiment should satisfy the obvious requirement, namely, that comparison of the effect of two nanoparticles preparations with different surface charge should be performed in conditions providing the equality of the other three nanoparticles parameters affecting their biological activity – size, shape and composition of the stabilizing shell. Taking into account that (1) in reality it is not so easy to provide different charges for the same stabilizing shells [22] and (2) AgNPs in cytotoxicity studies usually have spherical shape, the only necessary requirement is the equality of particle sizes. As follows from the literature available on cell viability changes after incubation with AgNPs with known zeta potential, this requirement is not fulfilled except for the papers by Liu et al. and Suresh et al. [18, 19]. In all other cases, for a given cell line, either particle sizes are different or size distribution is too wide so that it is impossible to make the correct conclusion on the effect of surface charge. These points are discussed in more details later in this paper (see Discussion).

Another problem is connected with invalidity of the Smoluchowski equation used conventionally for the determination of zeta potential for the charged particles in solution by photon correlation spectroscopy (PCS) or dynamic light scattering technique. The point is that zeta potential itself is not measured, but calculated from the measured electrophoretic mobility by means of the Smoluchowski equation. And, in the general case, this equation gives incorrect value of zeta potential because it does not take into account the relaxation effect which plays a role in electrophoresis process. In other words, zeta potential obtained from the Smoluchowski equation (ζ_{sm}) may be less than true zeta potential (ζ) considered in the classical electrical double layer theory. The error is significant for small particles with noticeable surface charge in water solutions of low ionic strength (below 10^{-2} M) [24-26]. More general expressions for the mobility — zeta potential relation were suggested by several authors studying the electrophoretic behavior of colloid particles [27-30]. As shown in our previous measurements on liposomes made from anionic lipids, the most reliable results are obtained by application of the Dukhin theory of electrophoresis; a detailed discussion of this point conformably to charged lipid membranes is given in [26, 31-33].

Since ζ_{sm} for silver nanoparticles used in toxicity studies is found for the particles of different charge and size, in solutions of different ionic strength, the error in zeta potential differs in magnitude. The biggest error can be supposed for the small particles (with $d<20$ nm) in deionized or distilled water (i.e., at very low ionic strength); this is the case, for example, in some studies with AgNPs synthesized by reduction in biological extracts (e.g., [34-38]). It is clear that, if the error is unknown, one cannot operate with true zeta potentials; hence it is impossible neither to make the correct comparison of ζ_{sm} values obtained for the particles of different sizes in solutions of different composition, nor to find the correct value of surface charge density using the appropriate equations of Gouy-Chapman-Stern double layer theory (e.g., [33]).

To overcome this problem using the devices programmed for the calculation of ζ_{sm}, it is necessary, first, to estimate possible error using, for example, the simple way suggested in [26]; if the error is found to be significant, it is possible to calculate true zeta potential from the measured mobility using one of the theoretical approaches mentioned above. However, there is a possibility to avoid the necessity to make these calculations, if the aim of the study is to clear out, just qualitatively, the effect of surface charge on the nanoparticles cytotoxicity (or on the other kind of their biological activity). This is the case, for instance, when measured changes in cells viability are compared after incubation with two (or more)

differently charged nanoparticles' preparations (e.g., [18, 19]). Then accurate knowledge of the surface charge magnitude is not required, and the task may be solved by means of comparison of the toxicity of nanoparticles' preparations with ζ_{sm} values found in conditions when they reflect only the difference in the nanoparticles' surface charge. An example of such kind is suggested in the present work.

In this paper we report the results of our study of the toxic effects of AgNPs bearing different surface charge, on the normal endothelial cells in culture. The nanoparticles were synthesized using different stabilizers: (1) synthetic anionic surfactant (AOT) and (2) β-cyclodextrin – a cyclic oligosaccharide with hydrophilic external surface bearing hydroxyl groups. It was found that, for AOT-stabilized nanoparticles the ζ_{sm} was significantly more negative that that found for those stabilized with β-cyclodextrin. Our aim was to elucidate the effect of surface charge magnitude using ζ_{sm} obtained from the PCS measurements; to avoid the error arising from the difference in particle size and ionic strength, mobility was measured for the particles equal in size in water solutions of equal conductivity. In these conditions, the difference in ζ_{sm} values between the two nanoparticles preparations reflects the real difference in their surface charge density.

Endothelial cells have been chosen for investigation as they belong to the class of highly significant cells with broad spectrum of functional activities; at the same time, they are permanently subjected to various compounds present in the blood flow, including foreign agents. Once introduced into a bloodstream, Ag NPs are able to affect endothelium which, in its turn, can impair a normal vessel functioning and exert general negative influence on cardiovascular system [39]. Cytotoxicity of the AgNPs was estimated by measuring changes in cell viability manifested (1) in the decrease of mitochondrial activity reflected by the well-known MTT assay, and (2) by the appearance of apoptotic and necrotic cells detected by flow cytofluorimetry. The results are compared with those available from literature for the other cell lines with nanoparticles of various sizes and ζ_{sm} values.

2. MATERIALS AND METHODS

2.1. Reagents

For the nanoparticles' preparation silver nitrate was used (chemical purity) and 27% ammonium hydroxide obtained from Labtech (Moscow, Russia), quercetin (3, 5, 7, 3', 4'-pentahydroxyflavon) obtained from Merck, AOT (aerosol-OT, bis-(2-ethylhexyl) sulphosuccinate, sodium salt) purchased from Sigma-Aldrich and β-cyclodextrin (m.w. 1135) placed at our disposal by the All-Russia Research and Development Institute of Starch Products (Moscow region, Russia). Silver nitrate solutions were prepared in deionized water ($R > 10$ MΩ) obtained from the device "Vodoley" (production of "Chimelectronika," Moscow).

For the treatment of cells we used Dulbecco's modified Eagle's medium (DMEM), supplements (non-essential amino acids (NEAA) solution, HAT supplement, L-glutamine), and bovine serum albumin (BSA) were purchased from Thermo Fisher Scientific (USA). Trypsin solution (0.25% of trypsin in 0.53 mM EDTA solution) and phosphate-buffered saline were obtained from PanEco (Russia).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was supplied by Amresco (USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (USA). An Annexin V-FITC Apoptosis Detection Kit I was purchased from BD Biosciences (USA).

2.2. Synthesis and Characterization of AgNPs

The AOT-stabilized Ag NPs (or AOT-Ag NPs) were obtained first in micellar solution (in organic solvent) by means of the original method (biochemical synthesis) based on the reduction of silver ions in reverse micelles by the natural flavonoid quercetin (Qr); principles of the method and details of Ag NPs preparation are given elsewhere [40, 41]. The reverse micelles are formed from AOT – synthetic anionic surface active substance [42, 43]. Water solution of Ag NPs is obtained by their transfer from micellar solution into the water phase by means of the specially developed procedure described in [40, 44]. In water solution the nanoparticles are stabilized by the AOT bilayer shell bearing negative surface charge due to the dissociation of its sulpho groups. The AOT concentration in solution is found by means of the standard procedure used for the determination of anionic surface active substances in water (see e.g., ISO 7875-1:1996). In the water solution used in this work the AOT concentration was 3.5 mM.

The β-cyclodextrin stabilized AgNPs (referred to as (β-CD-AgNPs) were prepared directly in water solution by means of the appropriate modification of biochemical synthesis. Briefly, 1 mM solution of β-CD in distilled water was first prepared at 50-60^{o} C. Then Qr solution in ethanol was added to the concentration of 0.1 mM; the ethanol contents did not exceed 1% by volume. To obtain the nanoparticles, diamine nitrate ($Ag(NH_3)_2\ NO_3$) solution was prepared by adding 27% NH_4OH to 100 mM $AgNO_3$ solution in deionized water; then $Ag(NH_3)_2\ NO_3$ was added to the Qr/β-CD solution under stirring to the silver concentration of 1 mM. Immediately after the addition of silver salt the light-yellow Qr/β-CD solution acquires reddish-brown color, showing to the AgNPs formation. Details of the synthesis procedure are described in [22].

Optical absorption spectra of both Ag NPs preparations were measured by spectrophotometer Shimadzu UV-2600 (Shimadzu, Japan). The nanoparticles concentration in water solution was determined from the optical density of the corresponding absorption bands using the extinction coefficient ($\varepsilon=1.14\cdot10^4$ l/mol · cm) obtained in independent measurements; details may be found in [22, 45, 46]. In the stock AOT-AgNPs and β-CD-AgNPs water solutions used in this work the Ag NPs concentration (equivalent to metallic silver) was 0.4 and 1.316 mM, respectively.

Size distribution and zeta potential of the nanoparticles in water solution were found by PCS technique on ZetaPALS (Brookhaven Instruments, USA). Note that the same technique often appears in literature as dynamic light scattering (DLS). Morphology, structure, and size distribution were determined by transmission electron microscopy (TEM) on LEO912 AB OMEGA microscope (Carl Zeiss, Germany). Samples for microscopy were prepared by placing 3 μl of the AgNPs water solution on the formvar coated copper grid with subsequent drying for 30 minutes on air. From the data given by electron micrographs, histogram was created for no less than 500 particles.

2.3. Cell Line

As a model of endothelial cells, EA.hy926 cell line was used. It was obtained from the American Type Culture Collection (ATCC; USA). Cells were maintained in DMEM supplemented with 10% (v/v) BSA, 1% (v/v) NEAA solution, 2 mM glutamine and 2% (v/v) HAT supplement in a 95% (v/v) humidified atmosphere and 5% (v/v) CO_2 at 37^0C. Prior to an experiment, cells were seeded into appropriate plate and grown for three days to reach confluence.

2.4. Cell Viability Test by MTT Assay

Cells were seeded in a 96-well plate ($5 \cdot 10^3$ cells in 200 μL of medium per well) and grown in conditions described in subsection 2.3. After cell monolayer formation, AOT-AgNPs and β-CD-AgNPs were added to the medium in wells to the final concentrations 0.5; 1.0; 1.5; 2.0; and 2.5 μg/mL. To consider potential toxic effects of stabilizers of Ag NPs, cells were also incubated with AOT and β-CD water solutions in dilutions corresponding to those of Ag NPs in wells. Medium in each well was gently mixed by pipetting and after that, cells were incubated for 24 hours. After incubation, a plate was centrifuged at 500*g* for 5 min to prevent a loss of detached cells, medium was removed and cells were washed with PBS (pH 7.4, 37°C) and centrifuged again. Cells were placed into fresh medium without BSA and supplements, and then MTT solution was added into each well to a concentration of 1 mg/mL. Cells were incubated with MTT for 4 hours, after that medium was gently removed and 100 μL of DMSO was added to dissolve formazan crystals. This solution in each well was diluted with 100 μL of deionized water, after that optical density was measured at 450 nm using Chameleon V microplate reader (Hidex, Finland). Cell viability for each concentration point was calculated as the ratio of the mean optical density of replicated wells to that of the negative control. As negative control we used cells incubated with medium containing the same volume of deionized water as that added with nanoparticles.

2.5. Detection of Apoptosis and Necrosis by Flow Cytofluorimetry

An Annexin V-FITC Apoptosis Detection Kit I was used, and procedure was performed according to the manufacturer's protocol. Cells were seeded into 12-well plate ($1 \cdot 10^5$ cells in 1000 μL of medium per well) and grown in conditions described in subsection 2.3. After cell monolayer formation, AOT-AgNPs and β-CD-AgNPs were added to the medium in wells to the final concentrations 0.5; 1.0; 1.5; 2.0; and 2.5 μg/mL. Medium in each well was gently mixed by pipetting, and after that, cells were incubated for 24 h. After incubation, cells were transferred into cytometry tubes in two steps. At the first step, medium from plate was transferred to avoid a loss of self-detached cells present in the medium. At the second step, to detach cells from wells, they were treated with trypsin solution for 3 minutes at 37°C, then cell suspensions obtained were transferred into the same tubes. Cells were washed twice in cold (4°C) PBS by centrifugation at 500*g* for 7 minutes, and resuspended in annexin binding buffer. Annexin and propidium iodide (PI) were added into each tube with Ag NPs-treated

cells. After incubation with these reagents, cells were diluted with binding buffer and then analysed in FACSCalibur flow cytometer (BD Biosciences, USA). Fluorescence levels of FITC and PI were measured.

2.6. Data Analysis

All experiments were performed in triplicate. The data from MTT assay are presented as means ± standard deviation. Statistical significance was determined by Student's t-test. Data from cytofluorimetry were analysed using CellQuest Pro software (BD Biosciences). Results were represented as percents of normal, early- and late-apototic, and necrotic cells.

3. RESULTS

3.1. Optical Spectra, Sizes and Zeta Potentials of Silver Nanoparticles

Figure 1 shows the absorption spectra of the AOT-AgNPs and β-CD-AgNPs solutions used for incubation with endothelium cells. As seen from the figure, both preparations exhibit almost identical positions of absorption band. To minimize also possible influence of the difference in nanoparticles concentration issuing from the difference in dilution extent required for the experiment with cells, stock β-CD-AgNPs solution was diluted so as to obtain the optical density equal to that of the AOT-AgNPs solution.

Electron micrographs, the corresponding diffraction patterns and hystograms of the two nanoparticles preparations are presented in Figures 2 - 4. Both kinds of nanoparticles are spherical, and have crystalline face centered cubic structure inherent to the massive gold specimen (as required for the silver crystal). As seen from the hystograms, analysis in Gauss approximation gives for AOT- and β-CD-stabilized nanoparticles 13.2 ± 4.72 nm and 13.3 ± 7.97 nm, respectively. Thus the nanoparticles have equal mean sizes, but for β-CD-AgNPs size distribution is wider.

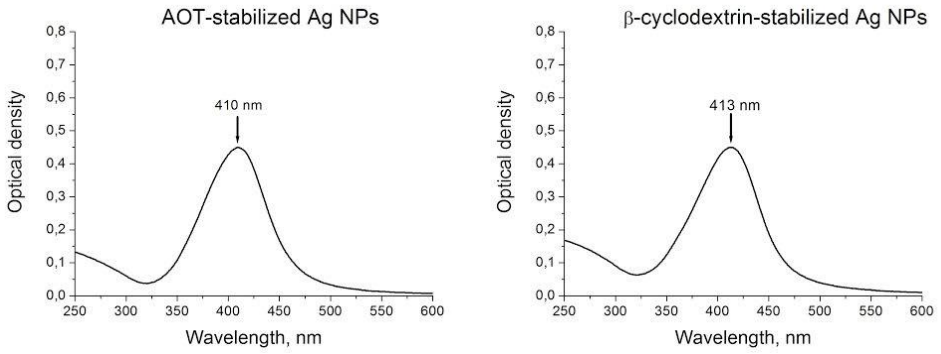

Figure 1. Absorbance spectra of Ag NPs solutions used in this study. The stock solution of β-CD-stabilized Ag NPs was diluted to obtain optical density D= 0.45, equal to that of AOT-stabilized Ag NPs.

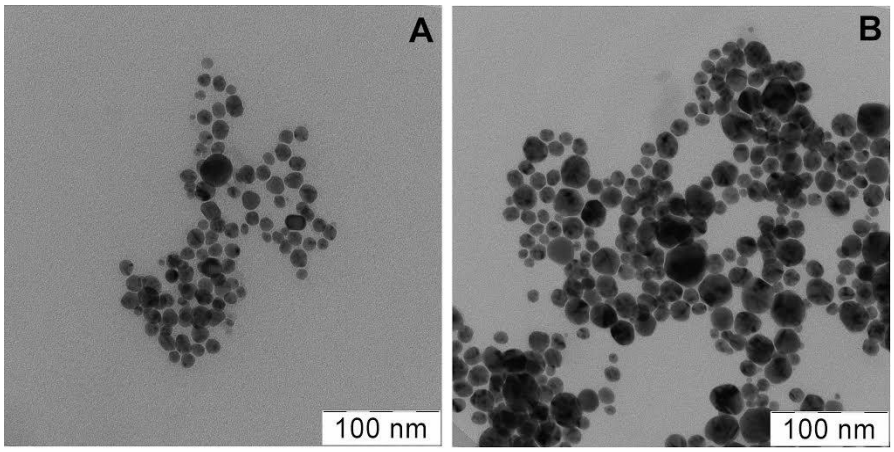

Figure 2. TEM micrographs of AOT-stabilized (A) and β-CD-stabilized (B) Ag NPs.

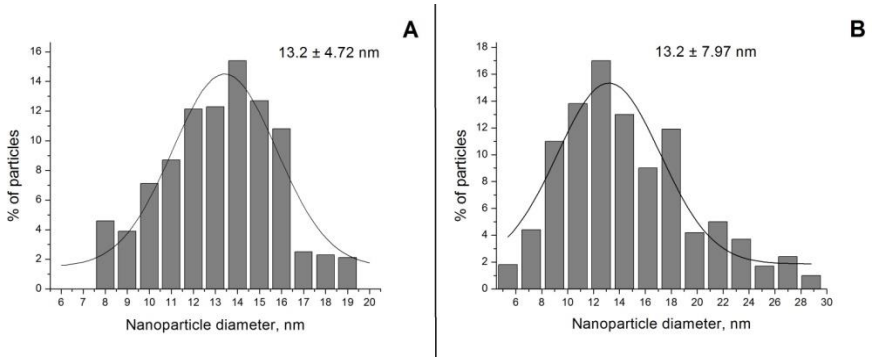

Figure 3. Size distribution of AOT-stabilized (A) and β-CD-stabilized (B) Ag NPs obtained from TEM data. Gauss approximation curves and nanoparticle diameters (mean ± SD) are also presented.

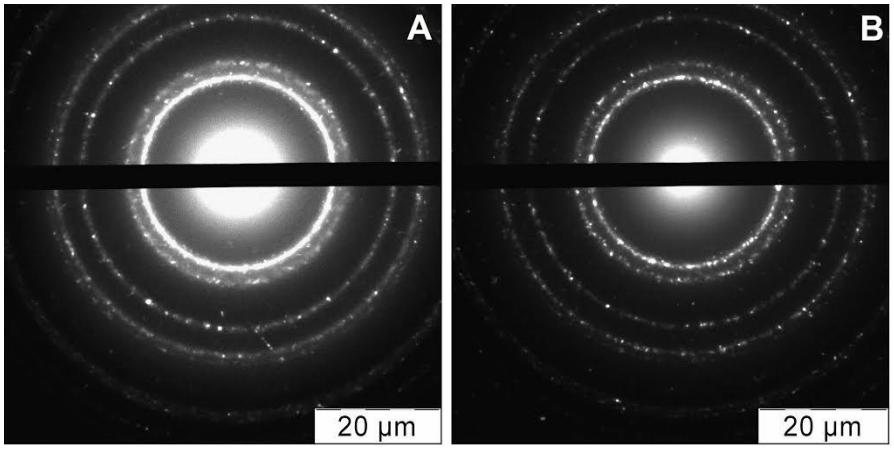

Figure 4. TEM diffraction patterns of AOT-stabilized (A) and β-CD-stabilized (B) Ag NPs.

Results of the size measurements in nanoparticles' solutions by DLS (PCS) technique are shown in Figure 5. For AOT-AgNPs and β-CD-AgNPs we obtain 49,44 ± 0,80 nm and

38,75 ± 0,59 nm, respectively. The larger sizes given by DLS technique in comparison with those found from TEM measurments are not surprising; similar results for silver and gold nanoparticles were reported in literature [37, 47 - 50] and in our previous publications [40, 46, 51]. As discussed already in [22, 51], apart from the presence in solution of a small portion of the larger particles which contribute to the light scattering (see e.g., [52, 53]), such an enhancement may originate also from non-spherical particle shape and (at least for gold nanoparticles), closeness of the nanoparticles absorption band position to that of the laser wavelength; more details on this point may be found in [22, 40, 51].

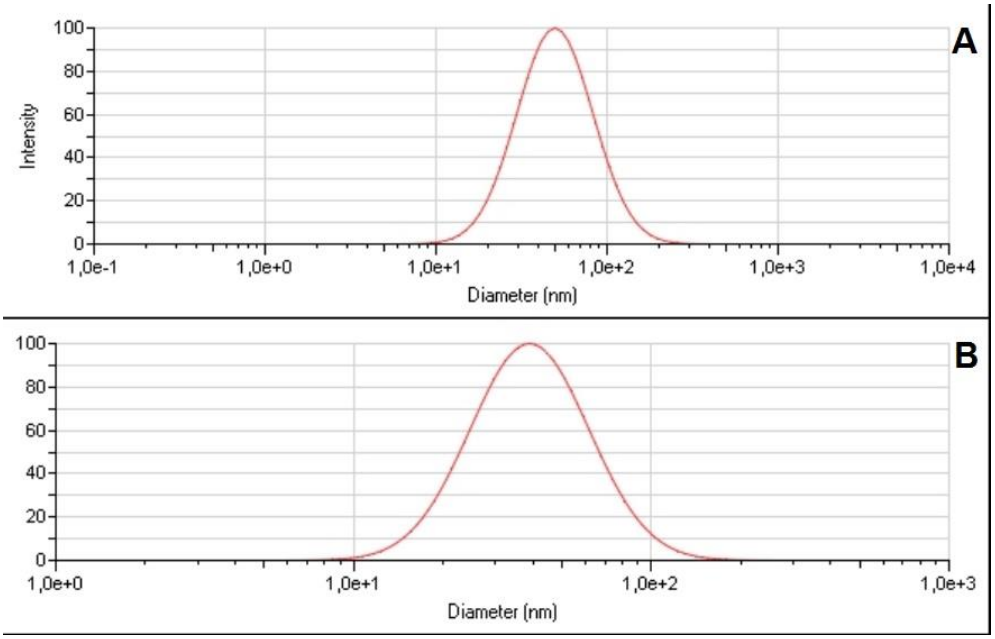

Figure 5. Particle sizes given by ZetaPals for AOT-stabilized (A) and β-CD-stabilized (B) Ag NPs in stock solutions.

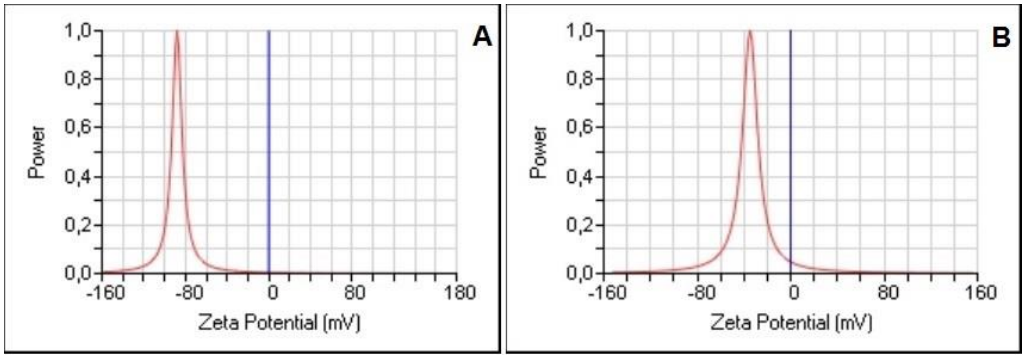

Figure 6. Zeta potentials obtained from ZetaPals for AOT-stabilized (A) and β-CD-stabilized (B) Ag NPs. Measurements were carried out in stock solutions reduced to the equal ionic strength.

Table 1. Characteristics of Ag NPs used in this work

	AOT-stabilized Ag NPs	β-CD-stabilized Ag NPs
Absorbtion band maximum, nm	410	413
Optical density (in stock solution)	0.45	1.5
Size (TEM), nm	13.2 ± 4.72	13.3 ± 7.97
Size (DLS), nm	49.44 ± 0.80	38.75 ± 0.59
Zeta potential (ζ_{sm}), mV	–89.42 ± 4.32	–35.93 ± 2.52

Electrophoretic mobilities for AOT-AgNPs and β-CD-AgNPs were measured in their water solutions shown in Figure 1; zeta potentials were calculated by means of the Smoluchowski equation and designated as ζ_{sm}. As shown in Figure 6, for AOT-AgNPs and β-CD-AgNPs the ζ_{sm} values were found as –89.42 ± 4.32 mV and –35.93 ± 2.52 mV, respectively. To avoid the error in ζ_{sm} connected with the difference in ionic strength, care was taken for the control of conductivity; in both solutions the measured conductivity lied within 440 ± 40 μS/cm. Since both kinds of nanoparticles are equal also in size and shape, the difference in ζ_{sm} may be attributed to the difference in their surface charge density. All measured parameters of the two nanoparticles preparations are summarized in Table 1.

3.2. Cell Viability Changes after Incubation with AgNPs

Results of the viability measurements by MTT assay observed after incubation with 0.5 – 2.5 μg/ml AgNPs are presented in Figure 7. The data for the AgNPs are combined with those obtained independently for the AOT and β-CD water solutions added to the same concentrations as those introduced with the nanoparticles. First, it is seen that there is the marked difference between the actions of the two kinds of nanoparticles. With β-CD-AgNPs cell viability does not suffer noticeable changes and remains close to 80% of control in the whole concentration range studied, while with AOT-AgNPs a decrease in viability to 70% of control is observed already at 1 μg/ml and significant fall to 20% of control at higher AgNPs concentrations. Hence follows that AOT-AgNPs are obviously more toxic than β-CD-AgNPs. Accordingly, the half-inhibitory concentration for AOT-AgNPs, $IC_{50} \approx 1.3$ μg/ml, while that for β-CD-AgNPs is greater than 2.5 μg/ml.

Comparison with the results obtained with the two stabilizers shows that AOT solutions possess the toxicity, though it is less than that of the AOT-AgNPs. This indicates to the possible contribution of the stabilizer into the toxic action of AgNPs. To obtain more visual impression about the pure effect of nanoparticles, we presented this contribution as ΔV_c - C_{AgNPs} plot (Figure 8), where $\Delta V_c = V_c$ (AOT-AgNPs) – V_c (AOT) is the difference in cell viabilities, given in Figure 7 for AOT-AgNPs and AOT; C_{AgNPs} is concentration of AgNPs. From the non-monotonous change of ΔV_c in the concentration range studied it is possible to infer that the nanoparticles are more toxic than AOT at smaller concentrations (in the range 0.5-1.5 μg/mL), with most noticeable difference (70% in cell viability) and minimal AOT contribution at 1.5 μg/mL. At the two higher nanoparticles concentrations the difference falls sharply, showing to the corresponding increase of the effect of the stabilizer. As for the β-CD

solutions, they do not provoke any significant changes in cell viability, since its level is not lower than 85% of control even at the highest β-CD concentration.

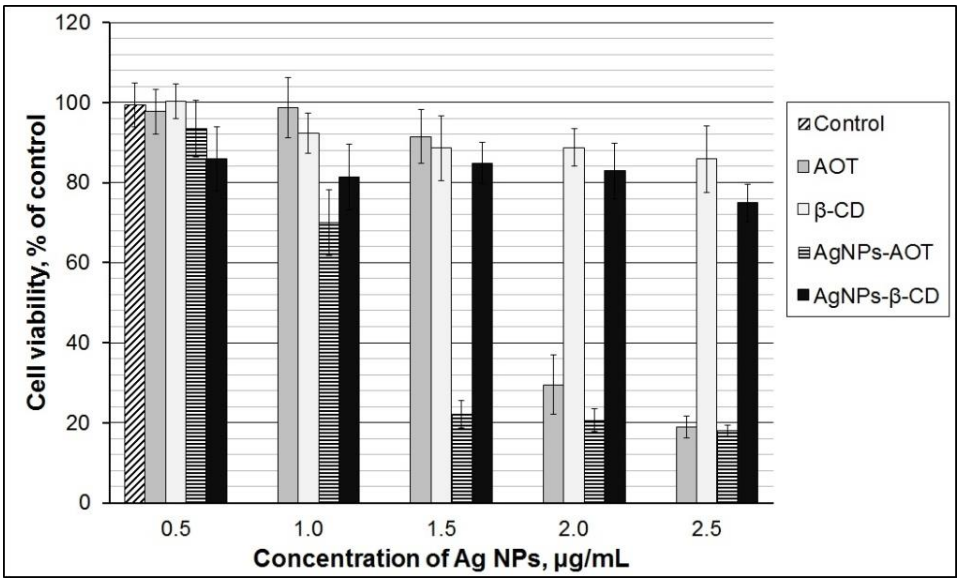

Figure 7. Viability of EA.hy926 cells after 24 h incubation with Ag NPs at various concentrations. MTT assay. Viability values were calculated as a percentage of cell viability in control.

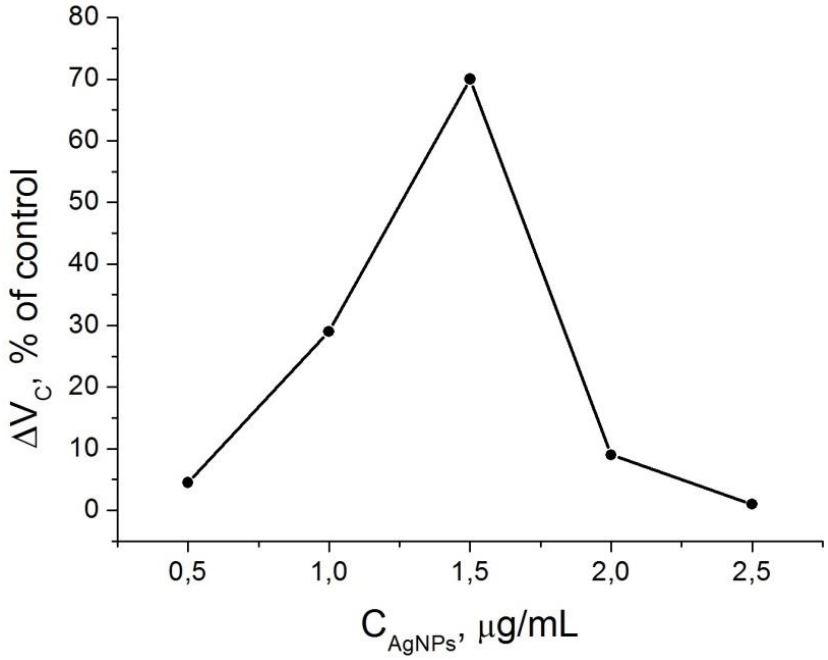

Figure 8. The difference between viabilities of cells treated with AOT-AgNPs and those treated with AOT as a function of AOT-AgNPs concentration. At each nanoparticles concentration, the AOT concentration is equal to that introduced with AgNPs solution.

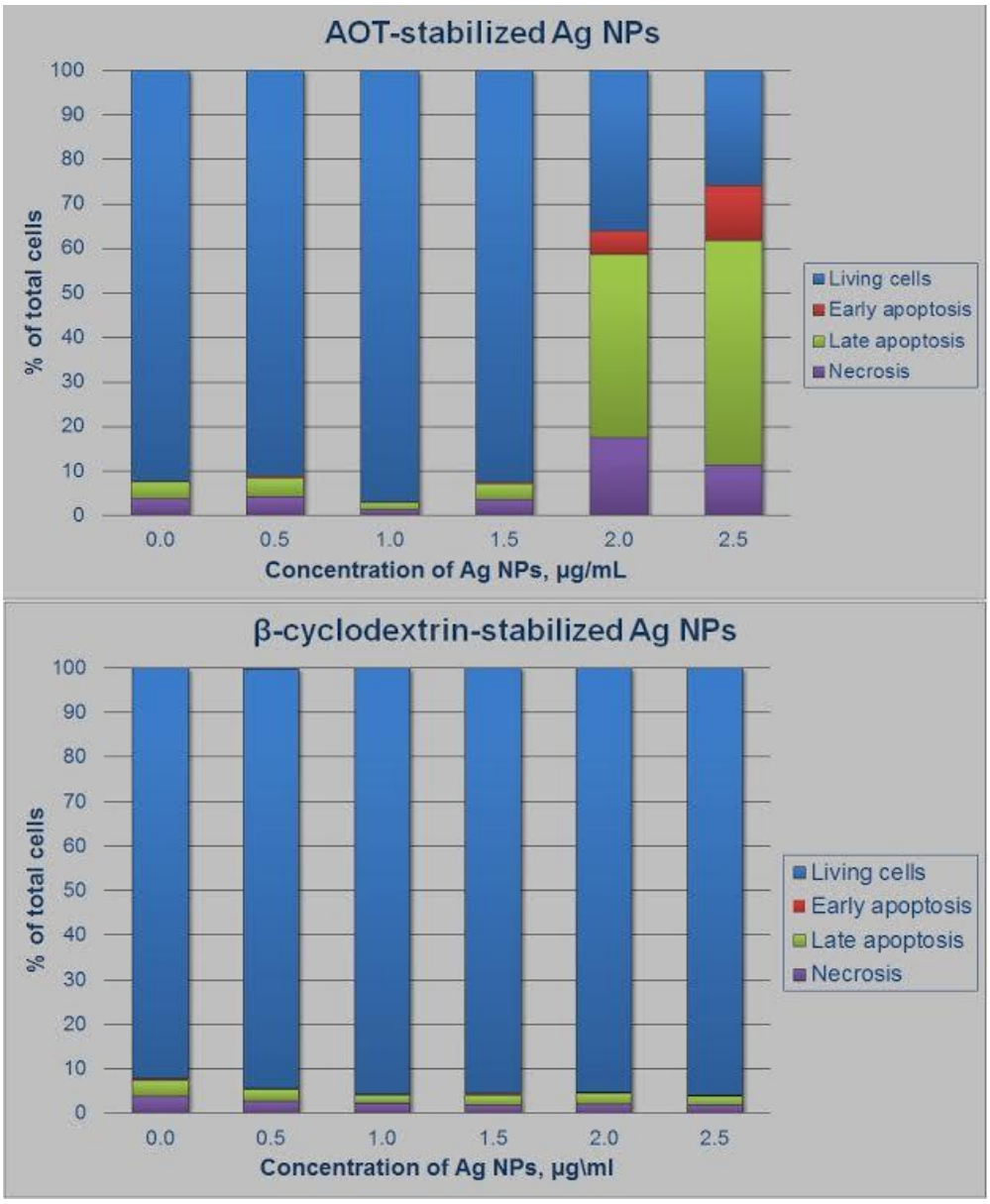

Figure 9. Apoptotic and necrotic events in EA.hy926 cells after 24 h incubation with various concentrations of AgNPs. FITC Annexin V binding and propidium iodide staining detected by flow cytofluorimetry. Events expressed in percentages (total amount of cells = 100% of events, corresponding to 15000 cells).

This difference in the nanoparticles toxicity observed in MTT test is further supported by the results of flow cytofluorimetry, allowing to detalize cell viability changes by estimation of the percent of apoptotic and necrotic cells at each nanoparticles concentration. As seen from Figure 9, with AOT-AgNPs obvious decrease in the percent of viable cells is observed at the two highest nanoparticles concentrations; here the viability decrease begins from the higher

AgNPs concentration (2.0 μg/mL) than that found by MTT assay (1.5 μg/mL). This means probably that changes in mitochondrial activity registered by MTT test (at 1.5 μg/mL) take place earlier than cells fall into the apoptotic state. That is, mitochondrial activity appears to be more sensitive to the toxic agent than proapoptotic functional elements within the cell. Such sensitivity, in turn, may be connected with the fact that triggering of apoptotic machinery takes more time than *functional alterations* in mitochondria, especially in case when apoptosis *naturally* results from mitochondrial injury and generation of reactive oxygen species [54-56]. With β-CD-AgNPs no significant changes in the contribution of viable cells are detected in the whole concentration range.

Also changes in cell morphology under the influence of AgNPs were investigated by means of phase contrast microscopy. It was found that incubation with AOT-AgNPs, beginning from 2.0 μg/mL, leads to the formation of apoptotic bodies and clearly expressed destruction of cells, in agreement with the results obtained in cytofluorimetric measurements. At the same time, incubation with β-CD-AgNPs exerts practically no influence on cell morphology (data not shown).

Taken as a whole, these data lead to the conclusion, that the AOT-AgNPs demonstrate a higher toxicity towards endothelium cells than the β-CD-AgNPs. Taking into account that (1) the only difference in the nanoparticles parameters is the difference in ζ_{sm} values and hence, in surface charge density and (2) incubation with AOT decreases cell viability and β-CD shows no such effect, the difference in the nanoparticles toxicity may be attributed partly to difference in the nanoparticles' surface charge density and partly to the difference in the biological activity of stabilizer. In other words, for the nanoparticles with equal size and shape, their biological activity depends on the composition and surface charge of the stabilizing shell. More precisely, the toxicity of nanoparticles towards the endothelial cells increases with the increase of the AgNPs negative surface charge. As seen from Figure 8, in spite of the toxicity detected for the AOT solutions, the effect of surface charge is clearly visible at the nanoparticles concentration in the middle of the whole concentration range.

DISCUSSION

So we found that AgNPs with bigger negative charge are more toxic than those with smaller negative charge. Since it is known that cell membrane is also negatively charged, this result disagrees with reasoning based on the assumption that electrostatic repulsion should lead to the decrease of nanoparticles adsorption on a cell surface and hence, of their toxicity. Also, this repulsion should be the more intensive, the bigger is negative charge of the particle surface.

The role of electrostatic interactions in bactericidal action of metal and metal oxide nanoparticles was considered in literature [10-12, 57]. In particular, a disagreement between the nanoparticles' negative zeta potential and their quite measurable toxic action was discussed conformably to the bactericidal action of negatively charged TiO_2 nanoparticles [11]. By contrast, obvious difference in bactericidal activity (on Gram positive bacillus species) was reported for negatively and positively charged AgNPs [21], namely, positively charged nanoparticles were essentially more toxic than those negatively charged. Similar conclusion was made from experiments with AgNPs on normal and malignant cells [18, 19].

Table 2. Cytotoxicity of Ag NPs obtained by chemical or biological reduction. Cell viability was estimated by MTT assay

Method of Nanoparticles preparation	Size, nm	Stabilizing shell	ζ_{sm}, mV	Cell line	IC_{50} µg/ml (incubation time)	Reference
a) Biogenic synthesis (bacterial extract)	4.0 ± 1.5 (TEM) 43.4 (DLS)(*)	Proteins in the Bacterial extract	-12 ± 2	Macrophages Lung epithelium	0.1 (4h) 0.45	[19]
b) Chemical reduction with $NaBH_4$	4.5 ± 1.5 (TEM) 82.5 (DLS)	Poly(DDAC)	+45 ± 3.1	Macrophages Lung epithelium	0.125 0.7	
c) Chemical reduction with $NaBH_4$	9.0 ± 2 (TEM) 62.6 (DLS)	No stabilizer (?)	-42.5 ± 5.2	Macrophages Lung epithelium	4.9 6.3	
d) Chemical reduction with $NaBH_4$	4.0 ± 1.0 (TEM) 46.3 (DLS)	Sodium oleate	-45.8 ± 4.4	Macrophages Lung epithelium	1.1 1.6	
Chemical reduction with $NaBH_4$	d_{av}= 8 nm (DLS) d_{av}= 8 nm (DLS)	PVP PVP+polypeptide (TAT)	-9.9 (Ag NP) +23.3 (Ag NP-TAT)	MCF-7 HeLa B16 MCF-7 HeLa B16	11.6nM 42 nM 7.5 nM 1.9 nM 1.8 nM 1.3 nM (24 h)	[18]
Biological reduction with *Annona Squamosa* leaves extract	20 - 100 (TEM)	Phytochemicals (tannins, polyphenols et al)	-37	HBL 100 MCF-7	80 (24 h) 60(48 h) 50 (24 h) 30 (48 h)	[36]

Method of Nanoparticles preparation	**Size, nm**	**Stabilizing shell**	**ζ_{sm}, mV**	**Cell line**	**IC_{50} µg/ml (incubation time)**	**Reference**
Biological reduction with Olive leaves extract	90 nm (av.) (DLS)	Not specified	-25,3	MCF-7	50 (Ag NPs in water) 0,024 (Ag NPs in extract) (4 h) No comparison with toxicity of pure extract	[35]
Melia azedarach leaves extract	78 nm (DLS)	Phenol compounds	−24.9	HeLa HBL 100	300 750 (48 – 72 h)	[38]
Albizia Adianthifolia leaves extract	10 (TEM) 27 – 80 (DLS)	Saponins, proteins, sugars	-24.7	A549	43 (6 h)	[48, 49]
Biological reduction with *Agrimoniae herba* extract Chemical reduction (citrate method)	11,22 (TEM) 30,34± 5,9 (DLS) 26,42± 3,2 (DLS)	Phytochemicals (flavonoids, phenols) Citrate	- 36,8 -28.3 ± 4.7	A549	38,13 11470 (extract) (24 h) 184.87	[50]
Origanum vulgare extract	d_{av} = 136±10.09 nm (DLS)	Phytochemicals (not specified)	-26 ± 0.77	A549	100 (36 h)	[34]
Cell free extract of *Saccharomyces boulardii*	d_{av} = 3 -10 nm (TEM)	Proteins in the yeast extract	-31	MCF-7	<10 (24 h)	[58]

(*) Here and below DLS represent the results of particle size measurements in distilled or deionized water

Abbreviations of cell lines: A431, human epidermoid carcinoma cells; **A549**, human lung carcinoma cells; **B16**, murine melanoma cells; **C-10**, mouse lung epithelial cells; **CHO**, chinese hamster ovarian cells (non-cancerous); **HBL**, human blood lymphocytes; **H9C2**, rat cardiomyoblasts (non-cancerous); **HeLa**, human cervical adenocarcinoma cells; **HUVEC**, human umbilical vein endothelial cells (non-cancerous); **MCF-7**, human breast carcinoma cells derived from metastatic site: pleural effusion**.**

In view of the data presented in our work, it seemed reasonable to compare our results with those available from literature on *in vitro* studies of the toxic effects on mammalian cells observed for silver nanoparticles with known zeta potentials (ζ_{sm}). Our main purpose was to clear out, whether the relation between the surface charge and toxicity of AgNPs found in this work agreed with observations made in similar experiments on the other cell lines.

Besides, it was useful to consider the contribution of stabilizer in the toxicity of AgNPs prepared by various chemical or biological reduction methods. Since we failed to find enough data for the normal cells, several examples are included also from the studies on malignant cells. The relevant data are collected in Table 2. As a measure of nanoparticles' cytotoxicity we used the half-inhibitory concentrations (IC_{50}) estimated from the viability vs AgNPs concentration dependencies obtained by MTT test. In all papers present in the table the nanoparticles were reported to be spherical, as those used in our work; therefore we included here only three meaningful particle parameters – size, ζ_{sm} and capping shell (stabilizer).

Comparison of the IC_{50} values reported in literature for the AgNPs with those found in this work shows that the AOT-AgNPs belong to the group of highly toxic nanoparticles with IC_{50}<10 μg/ml [19, 58], but not to the other group with 30<IC_{50} < 1000 μg/ml [34-36, 38, 48-50]. As for the β-CD-AgNPs, they are obviously less toxic, but, because of the low upper boundary of the nanoparticles concentration (2.5 μg/ml), there is no possibility at present to attribute them to one of the two groups under consideration.

Prior to the further analysis of experimental data special comments are needed concerning the correct choice of the particles sizes used for the calculation of true zeta potential from electrophoretic measurements. As noted already above in this work, sizes of metal nanoparticles obtained from TEM may differ significantly from those measured by DLS in solution. It is clear that actual particle sizes are given by TEM; at the same time, electrophoretic mobility is measured in water solution where average size given by DLS is usually greater than that obtained by TEM. As noted above, the origin of this enlargement is not clear at present, and it is not necessarily connected with real changes of nanoparticles' sizes because of the presence of nanoparticles' and/or stabilizer aggregates, the latter being either differently charged or neutral, depending on their nature. Hence the average size may be essentially bigger than that obtained by electron microscopy, though it does not necessarily correspond to the nanoparticles population involved in electrophoretic measurements. This is probably the case with the results of DLS measurements in water solution reported in several papers cited in Table 2 [19, 34, 38, 48]. As only exception may be regarded the data by Liu et al. [18], where the DLS measurements resulted in small average size. Considering the other cases we used the nanoparticles' sizes obtained from TEM measurements since they are more reliable, as we believe, than those obtained by DLS technique.

The most suitable for our purpose are the results reported by Suresh et al. [19] for normal cells and by Liu et al. [18] for malignant cell lines. In both cases differently charged AgNPs were investigated, obtained by chemical reduction [18] or by biological and chemical reduction methods [19]. Almost all of the nanoparticles were small and equal in size (2 preparations in [18] and 3 of 4 preparations in [19]); in the latter paper we consider only the sizes obtained by TEM for the reasons explained above. The difference in the charge sign was provided by the use of different stabilizers; in the paper [19] also the nanoparticles preparations had negative ζ_{sm} different in magnitude. As seen from the comparison of IC_{50} values for negatively and positively charged nanoparticles, the latter are substantially more toxic towards both normal and malignant cells, in agreement with the assumption that, due to

the electrostatic attraction, positively charged nanoparticles should be able to adsorb easily on a cell surface and provoke further toxic effects on cell functions.

Comparison of the toxicity of nanoparticles with different negative surface charge can be made correctly only for the two nanoparticles' preparations with equal sizes (4 nm) investigated by Suresh et al. [19], on the assumption that the mobility measurements were fulfilled in solutions of equal ionic strength. As seen from Table 2, for AgNPs with ζ_{sm} = -12 ± 2 mV the IC_{50} for the two cell lines studied were found to be substantially smaller than for the AgNPs with ζ_{sm} = -45.8 ± 4.4 mV. This means that the nanoparticles with bigger negative charge are less toxic, in contrast to the results obtained in our study. The question remains, however, about the role of stabilizer in the high toxicity of the biogenic AgNPs used in [19]. As shown in our recent review on cytotoxicity of biogenic AgNPs [23], the nanoparticles obtained by the reduction in bacterial extracts and stabilized with bacterial proteins demonstrate a higher toxicity than those obtained with plant extracts and stabilized with polyphenolic compounds. Therefore, for the data by [19] under question the possibility exists that the high toxicity of biogenic AgNPs is conditioned not only by the small negative charge, which facilitates the nanoparticles contact with cell membrane, but also with specific action of stabilizing proteins. This emphasizes once more the necessity to consider the contribution of both electrostatic forces and chemical properties of a stabilizing shell into the nanoparticles interaction with cells.

As for the other AgNPs with mean negative ζ_{sm} = -42.5± 5.2 mV studied in [19], their toxicity cannot be compared with that obtained for the AgNPs with ζ_{sm} = -12 ± 2 mV, even on the assumption that ζ_{sm} values were measured in solutions of equal ionic strength, since the nanoparticles have different sizes. As explained above in our paper, in such conditions zeta potential calculated from the Smoluchowski equation is not a direct measure of surface charge and therefore, the smaller value of ζ_{sm} does not necessarily correspond to the smaller surface charge. Basing on equations of the Dukhin theory for the small charged particles at low ionic strength [24, 26] one may predict only that the error in zeta potential is large and, even for the equal ionic strength, this error will be greater for the smaller nanoparticles; hence here the difference in measured ζ_{sm} does not reflect the difference in the particles surface charge.

The same reason makes it impossible to estimate surface charges for the nanoparticles with almost equal ζ_{sm} but of different size. In the paper by Suresh et al. [19] there are two AgNPs preparations obtained by chemical reduction – uncapped (mean ζ_{sm} = -42.5 mV) and coated with sodium oleate (mean ζ_{sm} = -45.8 mV), with size 9.0 and 4.0 nm, respectively. Again, for 4.0 nm nanoparticles the difference between ζ_{sm} and true zeta potential will be greater than for those which are 9.0 nm in size, therefore the closeness of ζ_{sm} values does not testify to the closeness of surface charge densities.

Similar problem arises for the comparison of the toxic action towards one and the same cell line reported in various publications presented in Table 2, for the nanoparticles with different negative ζ_{sm}. On normal human blood lymphocytes the toxicity was determined for two AgNPs preparations obtained by biological reduction with plant extracts, bearing negative charge, with ζ_{sm} = -37 mV [36] and -24.9 mV [38]; the IC_{50} after 48 h incubation were estimated as 60 and 750, respectively. This result could testify to the higher toxicity of the nanoparticles with greater negative charge, in accordance with our results with AOT-AgNPs and β-CD-AgNPs. Unfortunately, this inference is not justified, because of the uncertainty in particle sizes: AgNPs analyzed by TEM have too wide size distribution [36]

and there is no possibility to choose the mean particle diameter for the comparison with size obtained by DLS in [38]; besides, such a comparison is, in principle, incorrect, as discussed above. For malignant MCF-7 cells, the uncertainty in particle sizes, though obtained by TEM, prevents from the correct comparison of the toxicity of AgNPs studied in [36] and [58]. The additional problem is that the error in zeta potential determination is sure to be much more significant for the smaller particles used in [58]; therefore, the difference in reported ζ_{sm} values does not correlate with the difference in their surface charge.

As a single possible example we can use here the data obtained with malignant cells chosen as the most suitable for comparison because of the closeness of their sizes determined by TEM. For A549 (human lung carcinoma) cells with negatively charged AgNPs obtained by reduction with plant extracts, for ζ_{sm} = -24.7 mV (d = 10 nm) [48] and ζ_{sm} = -36.8 mV (d = 11.22 nm) [50] the IC_{50} values were found to be 43 and 38.13, respectively. So with different ζ_{sm} values the toxicity appeared to be almost the same. Taking into account that the toxicity increases with increase of the incubation time (see e.g., [36]) one can suppose that in [48] the IC_{50} would be even less (and hence more close to that obtained in [50]) if the incubation time was 24 h instead of 6 h. Thus one can see that for the AgNPs with different negative surface charge their toxicity towards one and the same cell line is nearly equal. Since both AgNPs preparations were obtained by biological reduction with plant extracts, such result probably shows that, in this case, the nature of stabilizing shell plays more important role than electrostatic interactions.

In summary, our attempt to compare the results obtained by us on the cytotoxicity of silver nanoparticles bearing different surface charge with those present in the publications devoted to the studies of charged AgNPs shows that (1) positively charged nanoparticles demonstrate the higher toxicity than those negatively charged, (2) for the nanoparticles with negative charge different in magnitude the correct comparison cannot be made, except for one case reported in [19] and (3) the effect of particle surface charge can hardly be considered separately from that of the nature of a capping layer. The main problem with comparison of the cytotoxicity for negatively charged nanoparticles lies in the difference or wide dispersion of their sizes, which leads to the difference in deviation of ζ_{sm} from the true zeta potential; as a result, ζ_{sm} cannot be regarded as parameter which reflects the difference in particles surface charge density. The necessity to pay attention to the effect of nanoparticles stabilizer or (for biogenic nanoparticles) of the biological extract was realized by various investigators; the corresponding controls were performed in some works on cytotoxicity [23], including those cited in Table 2 [19, 50] as well as in studies on bactericidal activity of AgNPs [21, 59]. The role of stabilizer was studied and discussed also in our previous publications [22, 40, 46, 60]; it was shown that the influence of a stabilizing shell may be revealed in the course of cells incubation with concentrations of pure stabilizer corresponding to those introduced with nanoparticles solution.

However, for the charged capped nanoparticles, we are unaware of the distinct evidence on the contribution of stabilizer separated from that of the particle surface charge. Meanwhile, this task seems to be important because, first, a noticeable part of AgNPs used in studies of their biological effects is covered with charged stabilizing shell and second, usually it is unknown a priory, whether a stabilizer used for the nanoparticles synthesis is toxic for a biological object studied. It is worth noting also that, isolation of nanoparticles from the "native" solution by means of centrifugation and/or filtration (e.g., [61-63]) does not exclude

the effect of stabilizer since it remains in the capping shell which interacts with cell membrane.

Hence follows that, for differently charged nanoparticles, it is necessary to estimate the influence of each stabilizer separately in the whole range of nanoparticles concentrations and, for the stabilizer which manifests toxicity, to determine the nanoparticles concentration(s) where this toxicity is negligible compared to that of the nanoparticles. As seen from our results obtained with AOT-AgNPs (Figure 8), such a way may be quite successful for finding the nanoparticles concentration which corresponds to almost pure effect of the particles surface charge on cell viability.

CONCLUSION

In this work we described the results of our studies on the toxicity of differently charged silver nanoparticles towards normal cultured endothelial cells. Difference in surface charge of the nanoparticles was achieved by the use of different stabilizers in the course of synthesis – anionic surface active substance (AOT) and starch-like product - cyclic oligosaccharide (β-cyclodextrine). Both were negatively charged because of the dissociation of surface ionizable groups, the nanoparticles stabilized with AOT bearing bigger charge than those stabilized with β-cyclodextrine. The relation between the nanoparticles charges was determined from zeta potentials calculated from the measured electrophoretic mobility by the Smoluchowski equation; these potentials were designated here as ζ_{sm}. In view of the inevitable deviation of ζ_{sm} from true zeta potential, predicted by the modern theories of electrophoresis, the mobility measurements were fulfilled on the nanoparticles with equal mean size in solutions of equal ionic strength, i.e., in the conditions providing that the relation of ζ_{sm} values for the two nanoparticles preparations really reflects the relation of their surface charges.

The toxicity of nanoparticles was estimated from the changes in mitochondrial activity (MTT assay) and appearance of apoptotic and necrotic cells in the nanoparticles concentration range 0.5 – 2.5 μg/ml. It was found that, beginning from 2 μg/ml, more strongly negatively charged AOT-AgNPs manifest a high toxicity, while less negatively β-CD-AgNPs remain practically non-dangerous for cells at all the nanoparticles concentrations. It was shown also that the higher toxicity of the AOT-AgNPs was conditioned by both the greater surface charge and toxicity of the stabilizer. Comparison of the cell viabilities after incubation with AOT-AgNPs and AOT solutions allowed determination of the nanoparticles concentration corresponding to the minimal contribution of the AOT toxicity, where the effect of nanoparticles prevails. This concentration was found to be 1.5 μg/ml, i.e., placed in the middle of the concentration range studied. At this concentration, as we believe, the effect of the particles charge was separated from that of stabilizer. Taking into account that the AOT-AgNPs and β-CD-AgNPs were equal in size and form, we may infer that, at least at this intermediate concentration, the difference in nanoparticles toxicity results from the difference in their surface charge.

Analysis of the results of cytotoxicity studies of charged AgNPs available from literature showed that the correct comparison with our data is possible only for the data reported in [19], for the nanoparticles of equal size with different negative ζ_{sm}. Contrary to our results, here it was found that more strongly negatively charged nanoparticles were less toxic than

those less negatively charged. However, since in [19] the two nanoparticles preparations were obtained with different stabilizers, the possibility exists that the difference in their toxicity issues also from the different effects of stabilizers. As for the other publications dealing with negatively charged nanoparticles, conclusion about the connection between the charge magnitude and cytotoxicity of nanoparticles towards one and the same cell line could not be made mainly because of the difference or wide dispersion of particle sizes. As additional hindrance the difference in composition of the capping layers may be considered, caused by the different methods of nanoparticles preparation. As a result, the question still remains unsolved about the connection between the negative charge magnitude and the toxic effect of silver nanoparticles.

To sum up, it is clear that, for the future progress in studies of the role of surface charge of silver nanoparticles in their biological activity, including cytotoxicity, it is important to be able, first, to estimate correctly the particles charge or zeta potential from electrophoretic measurements and second, to eliminate the effect of stabilizer from that of nanoparticles. These requirements we intend to fulfill in our subsequent studies.

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