Chapter

Some Methodological Aspects in Studies of Metal Nanoparticles' Toxicity towards Cultured Cells

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Abstract

Some actual questions arising in studies of the toxic effects of metal nanoparticle water solutions on cultured cells are considered. *First*, basic conditions required for the correct determination of nanoparticle size effect; the arguments are adduced in favor of the use of number nanoparticle concentration instead of the conventional mass one. *Second*, the problem of invalidity of the Smoluchowski equation; for charged nanoparticles the error in zeta potential value calculated from the measured electrophoretic mobility by the Smoluchowski equation cannot be neglected. *Third,* for the nanoparticles stabilized with surfactants, elucidation of the mechanism of cytotoxicity should include the determination of separate contributions of surfactant molecules and micelles into the total effect on cell viability.

Keywords: metal nanoparticles, cytotoxicity, methodological aspects, particle size effect, zeta potential, toxicity of surfactants

1. Introduction

In the last decades, the intensive development of medical applications of silver, gold and other metal nanoparticles brings into light the problem of their toxicity for a human organism. Therefore, studies on the biological activity of these nanoparticles are oriented mostly on elucidation of the mechanisms of their toxic effects on living organisms and determination of the conditions providing their safe usage. These studies lie within the scope of new branch in toxicology – nanotoxicology; general information about this direction may be found in several reviews [1–3].

One of the main lines of studies in nanotoxicology is focused on the toxicity of silver nanoparticles (AgNPs) used as water solutions. The reason lies in the widespread applications of these nanoparticles for medical purposes due mostly to their expressed antimicrobial activity; some recent results in this field are summarized in [4–6]. Studies are fulfilled mainly on the three objects: microorganisms, cultured cells and animals (mice and rats). The results obtained in the last two decades are presented in a great number of original papers, reviews and books; several examples are given in [7–9]. Nevertheless, one can conclude that there is no clearness in the main questions important for the estimation of AgNPs safety for a human organism.

Analysis of the literature as well as our long-term experience in studies of AgNPs biological effects allow to infer that the main reason lies in the field of methodology. ***First***, there is a wide variety of factors affecting the main toxicity criteria, one

faces therefore the significant dispersion of their values for a given type of bacterial or mammalian cells, or for a given animal organism. ***Second***, it is not rarely met in literature that the main nanoparticle characteristics are determined incorrectly, hence the results reported are not reliable. ***Third***, the non-coordination in similar studies conducted by different laboratories takes place, so it becomes impossible, for one and the same bacterial species, cell or animal line, to obtain a sufficient pool of independent results for the nanoparticles with the same parameters, so that the reliable mean toxicity criteria values could be obtained and the corresponding safe ranges of nanoparticle concentrations established. The methodological problems under question are minutely described in our review of the cytotoxicity studies of AgNPs prepared by the biological reduction [10].

In the present chapter some methodological questions arising in studies of the AgNPs toxic effects on cultured cells are considered. Here belong (1) basic conditions required for the correct determination of nanoparticle size effect; the arguments are adduced in favor of the use of number nanoparticle concentration instead of the conventional mass one, (2) the problem with invalidity of the Smoluchowski equation used for zeta potential calculation from the measured electrophoretic mobility (EPM) of nanoparticles and (3) for the nanoparticles stabilized with surfactants, the expediency to determine separate contributions of surfactant molecules and micelles to the total effect on cell viability.

These questions are expounded in the three sections presented below. In the first two, apart from purely methodological aspects the necessary calculations are included and illustrations on examples taken from literature which make clear the significance of the corrections discussed. In the last section we briefly describe our original approach suggested for gaining a more detailed information about the mechanism of cytotoxicity in case when nanoparticles are stabilized with surfactant. The approach is illustrated on example of anionic surfactant–stabilized AgNPs interaction with Jurkat cells.

2. Basic requirements for the studies of particle size effect on living cells

Studies on the biological activity of metal nanoparticles are devoted mostly to estimation of the influence of one or another nanoparticle characteristic on functional activity of bacterial or mammalian cells and multicellular organisms. Up till now the majority of works were carried out with AgNPs. This was conditioned, *first*, by the relatively simple ways of preparation and nanoparticle stability and *second*, by the wide possibilities of their application as antimicrobial means, both as water solutions and as small additives to various liquid-phase or solid materials.

The main characteristics of nanoparticle parameters are size, form, surface charge and the nature of stabilizer; the latter two are often referred to as surface properties. In studies of the biological activity the nanoparticles are used as their water solutions; the solutions should satisfy to the general conditions considered in our monograph [11]; here we described also the criteria used for the quantitative estimation of the influence of nanoparticle parameters on viability of the biological objects, as well as results of the relevant investigations on microorganisms, including some methodological problems.

In the more recent textbook [12] we considered the basic methodological requirements for experimental design in studies on the influence of the main nanoparticle parameters mentioned above. This point deserves attention because the nanoparticles as biologically active agents are principally different from molecular solution of an individual substance, namely, a nanoparticle solution is a complex object, composed of several components, including those capable of exerting their own

effect. This distinction should be particularly emphasized, since, for the time being, its importance is not always realized by the researchers (mostly biologists) using ready-made nanoparticle solutions either supplied by the nanotechnological companies or synthesized by the other laboratories. Estimation of the nanoparticle effects is carried out often according to the standard protocols accepted for the effects of individual substances in water solutions. It is clear however that, to provide the correct experimental procedure in studies of the nanoparticle cytotoxicity, it is necessary to take into account the properties of the nanoparticles as special system so as to obtain the results which really allow to achieve the goal pursued in experiment.

This is true even in case of the simplest task – a study of the nanoparticle concentration effect on various properties of cells. The more so it is justified for the special requirements in studies of the influence of basic nanoparticle parameters on the characteristics of living cells' state. As seems evident from methodological point of view, to determine the influence of one main nanoparticle parameter, the three others should remain constant. Here the importance of this rule is illustrated on example of the nanoparticle size effect on cell viability.

To estimate in experiment the toxic effect of particle size, one should eliminate the toxic action of the three other nanoparticle parameters, that is, to create the conditions where a cell response registered is not sensitive to the changes of particle form, surface charge or surface composition. These conditions are formulated below:

1. **Nanoparticles of different (no less than two) sizes in the range 1–100 nm should have the same form, surface charge and (for those obtained with stabilizer) the same composition of a stabilizing shell;**

2. **In case if the nanoparticles of different sizes are prepared by means of synthesis in the water medium, it is desirable that they were synthesized by the same procedure, since this provides the minimal difference in the composition of nanoparticle solutions (and hence, in the possible side-effects);**

3. **Size distribution of nanoparticles with different mean size should be narrow enough to exclude overlapping;**

4. **The action of nanoparticles with different sizes should be studied by the same method, on the same cells and at the same cell concentrations**.

If these conditions are satisfied, cell viability dependence on the concentration of nanoparticles with different sizes allows to estimate the difference in viability criteria and thus to clear out, which size of the nanoparticles under question is more toxic for the cells studied. It is important to stress here that, in studies of the size effects the necessity arises to change the conventional way of expressing the nanoparticle concentration and hence the experimental design. This statement was briefly substantiated earlier in our monograph ([11], p. 187). In view of its significance for the correct determination of the connection between nanoparticle size and cytotoxicity and for the corresponding applications of nanomaterials we thought it reasonable to dwell upon this question in more detail.

In all the relevant publications available, the biological activity of differently sized nanoparticles is compared by determination of the dose-effect dependence, where under "dose" one means the mass nanoparticle concentration, expressed in mg/L or μg/mL. Meanwhile, for the correct solution of the question about the influence of particle size a cell viability dependence should be measured not on the mass, but on the number and (or) "surface" nanoparticle concentration, that

is, on the number of nanoparticles or on their total surface per unit volume of solution under study. This becomes clear if attention is payed to the fact that the influence of nanoparticle size proper manifests itself after nanoparticle adsorption on a cell surface. Then, by analogy with the well-known regularities of molecular adsorption on solid surfaces, it is natural to believe that the adsorption density (the number of adsorbed nanoparticles per unit of cell membrane surface) will be proportional to their number nanoparticle concentration in solution (in a cell medium used in experiment). In other words, **the effect of nanoparticles' size depends on their number concentration in cell surrounding medium**. Since at the same mass concentration, the nanoparticles of different sizes have different number concentrations, estimation of the size effects should be carried out by comparison of a cell response (e.g. viability) for the same *number* nanoparticle concentrations.

Besides, when the nanoparticles are introduced into solution (cell medium), metal ions are released from the nanoparticle surface, the ions concentration in solution volume being proportional to the "surface" nanoparticle concentration defined above. Thus, investigation of the biological activity of nanoparticles having different sizes allows to find the contributions of the two different mechanisms of their action – *first*, the effect of size proper and, *second*, the effect of metal ions released from the nanoparticle surface. However, when a cell response on the *mass* nanoparticle concentration is measured, both mechanisms act simultaneously, so there is no possibility to separate the size effect from that of metal ions. To separate the two mechanisms, one should measure the dependences of cell response either on number nanoparticle concentration (the effect of nanoparticle size) or on their "surface" concentration (the effect of metal ions).

As a result, the necessity arises to supplement the four conditions mentioned above with the fifth one, which should be fulfilled in order to obtain the exact answer to the question: "How the nanoparticle size affects its biological activity?" This additional condition can be formulated as follows:

5. **To estimate the influence of nanoparticle size one should obtain the dependence of cell reaction on the number, but not mass concentration of differently sized nanoparticles in cell incubation medium.**

As far as we know, for the time being, this condition was not met in studies of the nanoparticle size effects, simply because nobody was aware of the importance of these considerations. Unfortunately, almost the same is true for the necessity to keep to the equality of the three other nanoparticle parameters (***condition 1***), since one of them – particle surface charge – in principle cannot be estimated correctly from zeta potentials obtained using the devices based on photon correlation spectroscopy (PCS) technique. The problem here is that, to our knowledge, in most cases the software installed in these instruments uses the Smoluchowski equation for zeta potential calculation from the measured EPM. However, as shown by the more general theories of electrophoresis developed in the past century [13–20], there are practically significant combinations of conditions (small particle size, not very small charge, and low ionic strength of the medium) where the assumptions used in the Smoluchowski theory do not work. As shown in the review [21] and explained also in our monograph ([11], p. 191) the Smoluchowski theory is not valid for the charged nanoparticles in the whole nanodimensional range (1–100 nm). Therefore, zeta potential values found by the Smoluchowski equation cannot be regarded as correct measure of the nanoparticle charge and the more general theories should be applied. A detailed discussion of this point is given in the next section.

Apart from this, in some cases one faces the impossibility to obtain the nanoparticle samples with narrow enough size distribution at the small difference of mean

particle sizes, hence the overlap of standard deviations takes place, i.e., the ***condition 3*** is also violated. Consequently, even if the other conditions are satisfied, the overlap of standard deviations leads to the non-reliable conclusions. As an example, we consider the work by Panacek et al. [22]. The authors studied the effect of AgNPs of different sizes on several bacterial species. As follows from experimental section, the nanoparticles were synthesized by the same technique; 4 nanoparticle samples were used, of the same (approximately spherical) form with mean sizes of 25, 35, 44 and 50 nm and narrow distribution. The antibacterial activity was determined by the same method on the same cells. So, the conditions 1–4 were satisfied and even the equality of surface charge seems to be fulfilled. The authors concluded that "the 25 nm-sized silver particles synthesized via reduction by maltose showed the highest activity, comparable even with ionic silver for certain strains" ([22], p.16252). Thus, one can infer that, the smaller are the nanoparticles, the higher is their biological activity. However, examination of the mean particle sizes and standard deviations shows that all the nanoparticle samples have too wide distributions and the standard deviations overlap, so that it is hardly possible to regard the samples studied as different in sizes and hence, to claim that the difference in particle sizes is responsible for the difference in their antibacterial activity.

In the publication discussed above, as well as in some other *in vitro* studies of AgNPs interaction with cells (e.g. [23–30]) the authors came to the conclusion that, among various sizes explored, the most toxic are small nanoparticles (less than 25 nm in size). However, there are grounds to doubt the reliability of such conclusions, because in these studies both the condition of equality of the three other nanoparticle parameters (in what concerns the surface charges), and that of the equality of number nanoparticles concentration (***condition 5***) are sure to be violated. The importance of the last condition may be elucidated using the results of calculations of number and surface concentrations for AgNPs in a chosen size range at the same mass concentration.

For a monodisperse suspension of AgNPs at their mass concentration 10^{-3} mol/L (108 μg/mL) the number concentration of particles in 1 L of solution (N_{tot}) may be found from the general formula (see for details Appendix in [31]):

$$N_{tot} = N_A \cdot 10^{-3} / N_{Ag} = 6.023 \cdot 10^{23} \cdot 10^{-3} / 41.84 \cdot \left(d_{NP}\right)^3 \qquad (1)$$

Where N_A is the Avogadro number, N_{Ag} is the number of atoms in a single silver nanoparticle, d_{NP} is particle diameter (nm). Since the nanoparticles are introduced to a cell culture usually to the mass concentrations in the range 1–100 μg/mL, the corresponding number concentrations are expressed in the number of particles per 1 mL. For example, for the size of 5 nm and mass concentration, C_M = 1 μg/mL, the number concentration, C_N will be found as:

$$C_N = \left[6.023 \cdot 10^{23} \cdot 10^{-3} / 41.84 \cdot (5)^3 \right] \cdot 10^{-3} / 108 = 1.066 \cdot 10^{12} \text{ particles / mL.} \qquad (2)$$

The surface concentration or the total surface area (C_S) for the nanoparticles of diameter d_{NP} is found as the surface area of a single particle (S_{NP}) multiplied by the number of particles in 1 mL of suspension:

$$C_S = S_{NP} \cdot C_N\left(d_{NP}\right) = \pi \cdot \left(d_{NP}\right)^2 \cdot C_N\left(m^2/mL\right) \qquad (3)$$

As follows from **Table 1**, at the equal mass nanoparticle concentration, the increase of particle diameter from 5 to 100 nm leads to the decrease of their number concentration, C_N, by 4 orders. This may be illustrated by the C_N (d_{NP}) dependence

Particle size, nm	Number concentration, C_N (particles/mL)	Surface concentration, C_S, m^2/mL
5	$1.064 \cdot 10^{12}$	$8.4 \cdot 10^{-5}$
10	$1.33 \cdot 10^{11}$	$4.18 \cdot 10^{-5}$
15	$3.94 \cdot 10^{10}$	$2.785 \cdot 10^{-5}$
20	$1.066 \cdot 10^{10}$	$2.09 \cdot 10^{-5}$
25	$8.512 \cdot 10^{9}$	$1.67 \cdot 10^{-5}$
30	$4.9 \cdot 10^{9}$	$1.38 \cdot 10^{-5}$
50	$1.064 \cdot 10^{9}$	$8.36 \cdot 10^{-6}$
70	$3.9 \cdot 10^{8}$	$6.0 \cdot 10^{-6}$
100	$1.33 \cdot 10^{8}$	$4.18 \cdot 10^{-6}$

Table 1.
Number and surface concentrations of 5–100 nm AgNPs in solutions with nanoparticle concentration 1 μg/mL.

in the range 5–30 nm (**Figure 1**). It is seen that the sharpest fall of number concentration takes place at small sizes (below 10 nm). Hence it is clear that comparison of the biological activity of nanoparticles with sizes of, say, 5 and 20 nm, at such a significant difference in their number concentrations does not allow to obtain the reliable data on the influence of the size proper. Similarly looks the corresponding dependence of surface concentrations, $C_S(d_{NP})$ (**Figure 2**). Here the decrease of specific particle surface in the same size range is more smooth than in the case of number concentrations and remains within one order of magnitude; still it should be taken into account if it is important to know the contribution of Ag^+ ions released from the nanoparticle surface into the nanoparticles action upon cells.

The effect of nanoparticle size at the equal number concentration can be demonstrated using some literary experimental data obtained in studies of the action of differently sized nanoparticles on viability or other cell parameters. In case of viability, the measured viability values, V_c, after incubation with nanoparticles should be

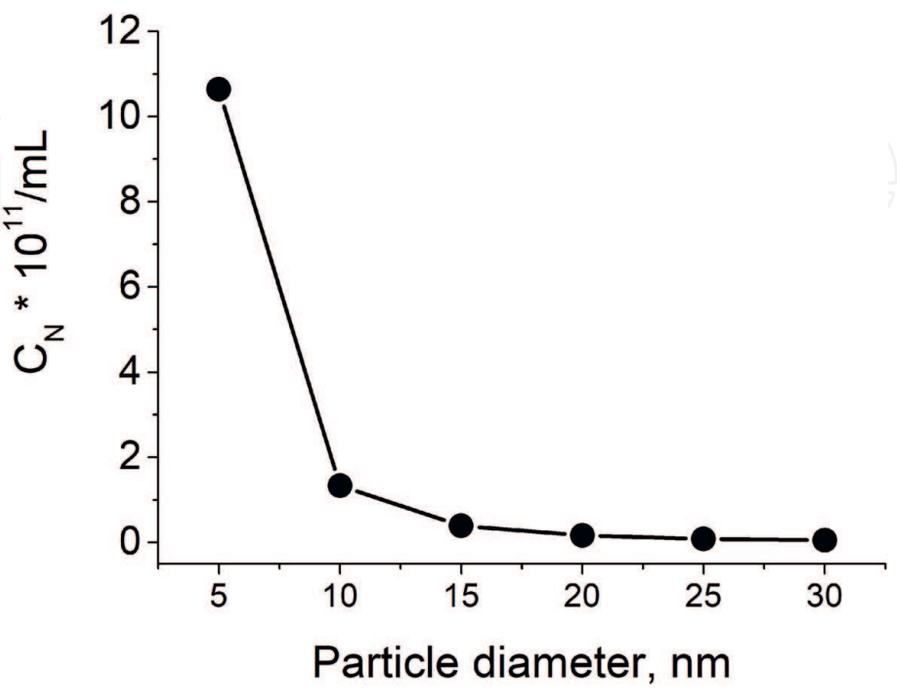

Figure 1.
*Dependence of AgNPs number concentration (C_N) on the nanoparticle size in the range 5–30 nm (data from **Table 1**).*

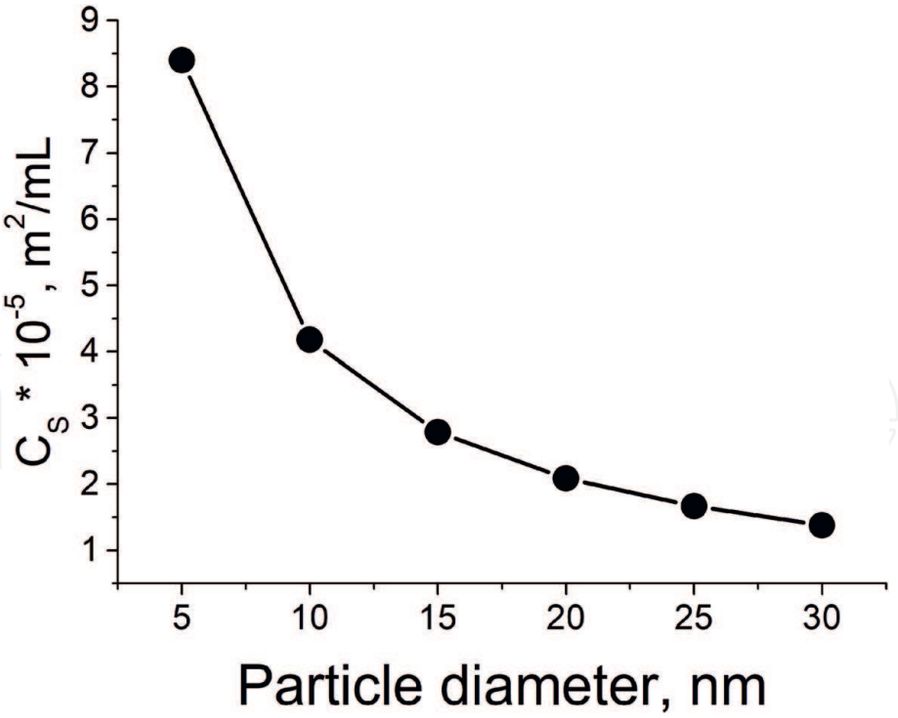

Figure 2.
*Dependence of AgNPs surface concentration (C_S) on the nanoparticle size in the range 5–30 nm (data from **Table 1**).*

compared at the equal number nanoparticle concentration. Such comparisons were found possible, for example, for the data reported in [25], where the V_c dependences on mass concentration for AgNPs of different sizes were measured in the wide range of mass concentrations so that one could find the suitable pairs of V_c values.

The authors used the citrate-stabilized AgNPs, 10, 20, 50 and 100 nm in size, produced by nanoComposix (San Diego, USA). The particle sizes were found by PCS and TEM, electron micrographs of good quality **(Figure 3),** mean diameters were close to those claimed by the manufacturer, standard deviations narrow enough, so the nanoparticle sizes are quite reliable. It is clear also that all the nanoparticles are

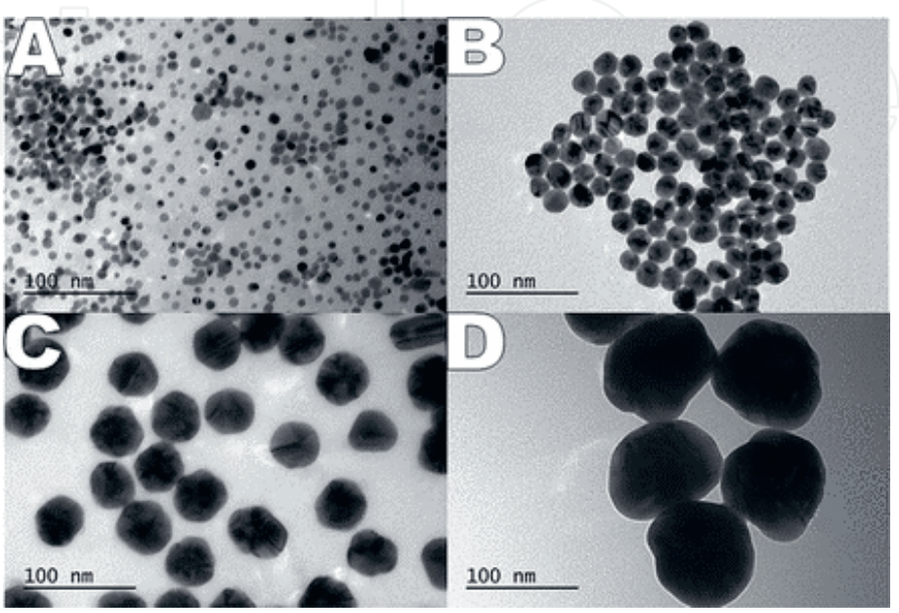

Figure 3.
TEM micrographs of AgNPs used in all experiments discussed: A – 10 nm; B – 20 nm; C – 50 nm; D – 100 nm. Reproduced from [25].

spherical in form and negatively charged, as follows from their negative zeta potentials. Apart from the other experimental data the authors present the cell viability dependences on mass nanoparticle concentration; the latter was no higher than 50 μg/mL (**Figure 4**). The Jurkat and THP-cells taken in equal concentrations ($2{\cdot}10^5$/mL) were incubated with nanoparticles for 24 hours. In both cases the viability was estimated from the mitochondrial activity by means of MTS test. Thus, the experimental design satisfied the ***conditions 1–4***, except for the equality of surface charge values; this condition was probably violated due to the unreliable zeta potential values, the defect common to practically all studies performed with nanoparticles in the last decades.

To compare the toxic effect for the nanoparticles of different sizes we calculated the number concentrations of 10 and 20 nm AgNPs at various mass concentrations (**Table 2**) and so obtained the mass 20 nm nanoparticles' concentrations equal or nearly equal to those of 10 nm nanoparticles. As seen from the table, for 20 nm AgNPs there are 4 C_N values corresponding to those of 10 nm nanoparticles at their concentrations in the range 1–6 μg/mL. For these C_N values from **Figure 4** the V_c values have been found at the four mass concentrations (8, 30, 40 and 50 μg/mL)

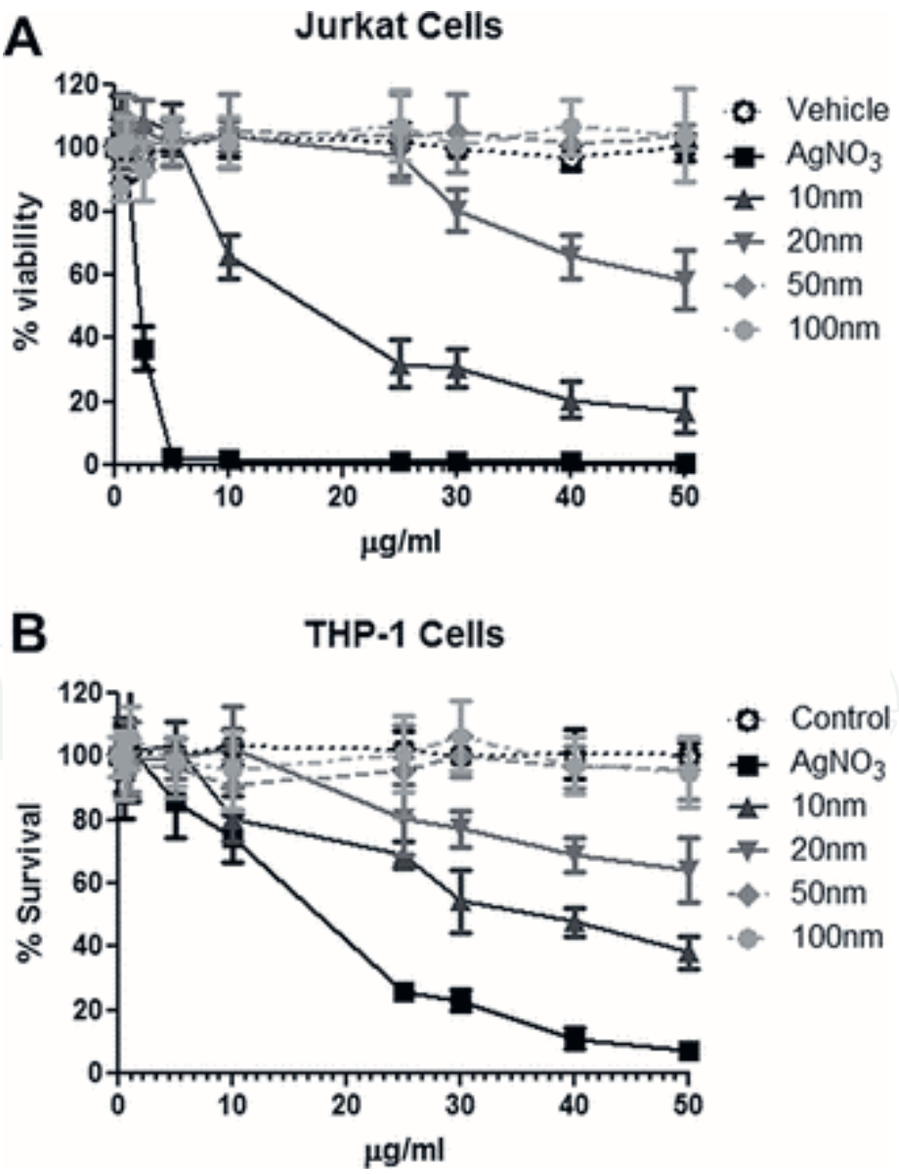

Figure 4.
The mitochondria activity of Jurkat (A) and THP-1 (B) cells after 24 h incubation with various concentrations of 10 nm, 20 nm, 50 nm, 100 nm AgNPs and with the corresponding $AgNO_3$ concentrations. Reproduced from [25].

Mass concentration C_M, μg/mL	**C_N, particles/mL d = 10 nm**	**C_N, particles/mL d = 20 nm**
1	**$1.33 \cdot 10^{11}$**	$1.66 \cdot 10^{10}$
2	$2.66 \cdot 10^{11}$	$3.32 \cdot 10^{10}$
3	$4 \cdot 10^{11}$	$5 \cdot 10^{10}$
4	**$5.32 \cdot 10^{11}$**	$6.64 \cdot 10^{10}$
5	**$6.65 \cdot 10^{11}$**	$8.3 \cdot 10^{10}$
6	**$8 \cdot 10^{11}$**	$9.96 \cdot 10^{10}$
7	$9.31 \cdot 10^{11}$	$1.16 \cdot 10^{11}$
8	$1.06 \cdot 10^{12}$	**$1.33 \cdot 10^{11}$**
10	$1.33 \cdot 10^{12}$	$1.66 \cdot 10^{11}$
20	$2.66 \cdot 10^{12}$	$3.32 \cdot 10^{11}$
30	$4 \cdot 10^{12}$	**$5 \cdot 10^{11}$**
40	$5.32 \cdot 10^{12}$	**$6.64 \cdot 10^{11}$**
50	$6.65 \cdot 10^{12}$	**$8.3 \cdot 10^{11}$**

The equal or similar C_N values at different nanoparticle sizes are marked in bold.

Table 2.
Number concentrations (C_N) of 10 and 20 nm AgNPs at various mass concentrations.

for the 20 nm nanoparticles. The results are shown in **Figure 5**. It turned out that, for the two cell types studied, the V_c values for 20 nm nanoparticles were less than 100% and decreased with the increase of C_N. At the same time, the corresponding V_c values for 10 nm particles remained at the 100% level, i.e., demonstrated the absence of toxicity. Hence follows that, at the equal number concentration the bigger nanoparticles possessed a higher toxicity than the smaller ones.

Similar conclusion can be drawn from the results reported in several other studies on the size dependence of AgNPs toxicity conducted on various cell types [26–30]. **Table 3** presents the experimental data that could be extracted from the plots of cell response vs. mass nanoparticle concentration. Similarly to what has been done with the data reported by Butler et al. [25], we have chosen the pairs of experimental values obtained for the same number concentration of differently sized nanoparticles at the relevant mass concentrations within the range studied. As seen from the table, for different combinations of sizes and cell lines, the bigger nanoparticles exert a stronger toxic influence at least on the two kinds of cell response: V_c and RCD (relative cell population doubling). It should be noted that, at present, there is a very limited number of data that can be used for the comparison under question. The main restriction here is that, in the majority of publications available, mass concentration of the bigger nanoparticles required for their number concentration to be equal to that of the smaller ones exceeds the upper limit of mass concentration in the range studied.

Basing on our calculations and analysis of the published results presented above one may suppose that, at the equal number nanoparticle concentration, the increase of particle size leads to the enhancement of its biological activity. If this supposition is confirmed in future experiments (at least for AgNPs), this will lead to the conclusion opposite to that present in the majority of the known studies of the nanoparticle size effects on animal or bacterial cells. Certainly, for the final statement the transfer from mass to number concentrations is not sufficient. The true zeta potential values should also be known, found from the measured electrophoretic

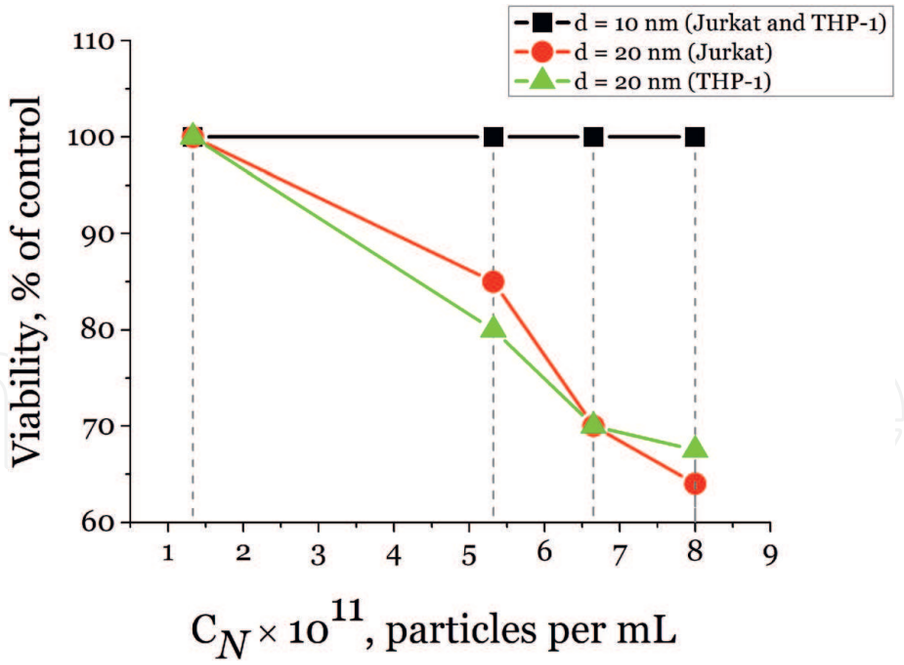

Figure 5.
Viabilities of Jurkat and THP-1 cells at the four relevant number concentrations of 10 nm and 20 nm AgNPs taken from the ***Table 2****. The viability values are elicited from the data shown in* ***Figure 4****. See text for details.*

D_{NP} (1), nm	D_{NP} (2), nm	C_N, particles/mL	C_M (1–2) μg/mL	Cell type/ Stab	Tox (1), %	Tox (2), %	Ref.
10	30	$1.33 \cdot 10^{11}$	1–27	Jurkat/ /PEI /PEG /citrate	V_c 100	V_c 10 30 45	[26]
20	50	$\approx 3.3 \cdot 10^{10}$	2–30	MLC	RPD 85	RPD 30	[27]
20	40	$4.17 \cdot 10^{11}$	25–200	THP-1/ tripeptide (Lys-Lys-Cys)	83	50	[28]
20	50	$1.67 \cdot 10^{11}$	10–150	RAW 264.7/ bovine serum albumin	60	10	[29]
5	50	$1.07 \cdot 10^{11}$	0.1–100	HaCaT/ PVP	100	60	[30]

Abbreviations: C_N – number concentration, C_M – mass concentration, V_c – cell viability.
Stab – stabilizer, PEI – polyethylene imine, PEG – polyethylene glycol, MLC – mouse lymphoma cells, PVP – polyvinyl pyrrolidone, RAW 264.7 - mouse monocyte macrophage cell line, Tox – parameter characterizing the AgNPs toxicity; RPD – relative population doubling.

Table 3.
AgNPs toxicity for cells obtained from experimental data reported in literature for the two different nanoparticle diameters (D_{NP}) at the equal number concentrations. Incubation for 24 h.

mobilities (EPM) by the equations considering the relaxation effect. The problem with invalidity of the Smoluchowski equation in studies of metal nanoparticles is discussed in the next section.

3. The problem with invalidity of the Smoluchowski equation in determination of particle surface charge using zeta potential calculated from the measured electrophoretic mobilities

As mentioned above, particle surface charge is one of the basic parameters used in studies of the biological action of metal nanoparticles. The sign and magnitude of surface charge may be found from electrokinetic (or zeta, ζ) potential calculated from the measured EPM of nanoparticles. For this purpose, the well-known relations of the classical electrical double-layer (EDL) theory may be used, modified conformably to the studies of membranes charged due to the presence of ionized groups (e.g. liposomes or biological vesicles) [32, 33]. Here belong also metal nanoparticles coated with surfactants or polymers bearing ionizable groups.

In studies of the biological activity of nanoparticles, their surface charge is characterized almost exclusively by ζ-potential values and not by those of surface charge density, and this is considered to be sufficient for the estimation of nanoparticle charge effects on functional activity of living cells or other objects explored. Therefore, we dwell here upon the methodological questions essential for determination just of ζ-potential; for the readers interested in estimation of surface charge the above references may be recommended, as well as some works where the nanoparticle surface charges are reported (e.g. [34]).

In this section we give a brief account of theoretical considerations necessary, to our view, for the correction of possible errors in determination of ζ-potential magnitude. The necessity of such an account issues from the fact that, as far as we know, at present the majority of researchers dealing with metal or metal oxide nanoparticles operate with ζ-potentials obtained by PCS technique on the devices which do not allow to obtain the correct ζ-potential values. As we noted earlier ([11], p. 190)," the problem is that standard software incorporated in the corresponding instruments (manufactured by Malvern, Coulter Electronics, or Brookhaven) usually calculates ζ-potential from the EPM measured by PCS without the relaxation correction, applicable for the measurements of nanoparticles. At the same time, the correction is essential in view of the small particle size, because a substantial error in the ζ-potential value is otherwise inevitable, as was shown, for instance, in [35–37]. Such errors are likely to occur in the relevant studies since the ζ-potential is usually calculated from the Smoluchowski equation, and the resulting values (ζ_{sm}) may considerably differ from the true ζ-potential".

"The relaxation correction" under question appears as a consequence of invalidity of the Smoluchowski equation in experimental conditions which do not satisfy the two basic assumptions of the Smoluchowski theory: (1) the width of EDL is small compared to the particle radius and (2) the double layer near the charged surface is in equilibrium state. The first assumption is determined by the inequation:

$$\kappa\alpha >> 1 \tag{4}$$

where κ is reciprocal Debye length, α is particle radius. The second assumption allows interpretation of the ζ_{sm} values within the frames of classical double-layer theory. These assumptions issue from the notion that the force applied to a particle by the external field is equilibrated by (a) viscous resistance of the fluid and (b) the electroosmotic motion of the fluid along the particle surface. Thus, here the electrophoretic retardation is considered, similar to that taking place at the movement of ion surrounded by the ionic atmosphere, but the influence of ionic atmosphere deformation at the particle movement (known as the relaxation effect) is neglected (e.g. [16]). The neglection of the ionic atmosphere deformation follows from the assumed equilibrium of the EDL. However, this assumption can be invalid even for

the thin double layer determined by the expression (4). This can manifest itself in experiment as EPM dependence on particle size, the possibility which is not considered by the Smoluchowski theory (e.g. [16, 20, 38]).

According to the views developed in the electrophoresis theory, the relaxation effect is conditioned by the two main causes: (1) by the existence of surface conductivity, since the ionic surface currents change the potential distribution in the vicinity of a particle and (2) by double-layer deformation (polarization) under the influence of external field [16, 20]. Both phenomena are interconnected and represent a complicated combination of processes proceeding in the double layer of a moving particle. In the past century several theoretical approaches were suggested for the description of double layer relaxation; the most widely known are those developed by Overbeek-Booth-Wiersema [14, 15], O'Brien and White [18, 19] and S.S.Dukhin and co-workers [16, 17].

To illustrate the necessity of correction for the relaxation effect, we give here one example from the results of our PCS measurements of ζ- potential dependences on ionic strength of salt solution obtained for liposomes made from negatively charged phospholipids (**Figure 6**). The measurements were conducted in monovalent electrolytes in the range 10^{-3} – 1 M. It was established that, at the low ionic strengths, ζ-potentials calculated from the measured EPMs by the Smoluchowski equation (ζ_{sm}) differ significantly from the true zeta potential (ζ) calculated from the classical EDL theory equations [35–39]. It is clear that the deviation can be essential even for the particles with diameter of 400–600 nm (0.4–0.6 μm), especially at low salt concentrations; for example, at C = 10^{-3} M the difference $\zeta - \zeta_{sm}$ exceeds 100 mV. It is seen also that much more satisfactory agreement with experimental ζ_{sm} values is achieved with the use of the equation suggested by the Dukhin theory [34, 35, 40] which allows for the relaxation effect.

The results of such studies brought us to the conclusion that, for the correct determination of zeta potential for liposomes in the wide range of the meaningful parameters – particle size, charge, and ionic strength of water solution – for the calculation

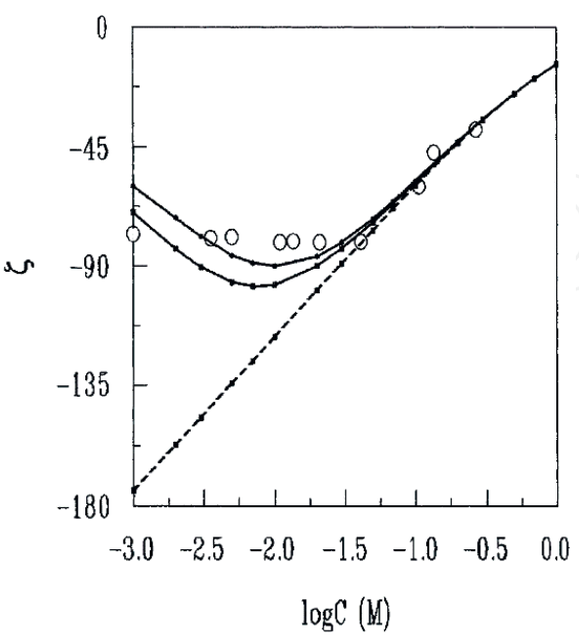

Figure 6.
ζ_{sm} as a function of electrolyte concentration for phosphatidylserine liposomes in NaCl. Open circles – experimental data. Solid lines – theoretical predictions for ζ_{sm} values calculated from one of the equations suggested by the Dukhin theory [35, 36] for the known membrane charge and liposome size 0.4 μm (upper curve) and 0.6 μm (lower curve). Dashed line – the corresponding change of true ζ-potential predicted by the classical EDL theory. Reproduced from [39, 41].

of true ζ-potential from the measured EPM one should apply not the Smoluchowski formula, but the more general relations between EPM and ζ-potential suggested by the Dukhin theory. It became clear also that correction for the relaxation effect is important for the estimation of ζ-potential (and hence, of particle surface charge) not only for lipid membranes, but for the wide range of objects studied both in colloid chemistry (e.g. latexes) and in membrane biophysics (biological vesicles, small cells et al) and in any other fields where small charged particles are explored. At the same time, since the due regard for the relaxation effect is necessary not in each case, but only for the definite combinations of the three meaningful parameters, it was of practical importance for the researchers to clear out, first of all, whether the Smoluchowski formula was applicable in the given experimental conditions. That is, whether it is reasonable to use for ζ-potential calculation any of the more complicated relations absent in the instruments applied for the EPM measurements under consideration.

To facilitate the solution of this question in studies of lipid membranes, we suggested a simple approach based on the calculation of criterion Rel[1] used in the S.S.Dukhin theory for the estimation of significance of the relaxation effect [37]:

$$\mathrm{Rel} = \left[\exp\left(\bar{e}|\psi_d|/2kT\right) - 1\right]/\kappa\alpha \tag{5}$$

where ψ_d is Stern potential here assumed as equal to the surface potential ψ_s (see e.g. [32]), κ is reciprocal Debye length, α is particle radius. For the high charges and low ionic strengths this equation is reduced to

$$\mathrm{Rel} = \left[\exp\left(\bar{e}|\psi_d|/2kT\right)\right]/\kappa\alpha \tag{6}$$

If electrophoresis is not complicated with surface conductivity and is not sensitive to the form of particles, that is, the Smoluchowski formula is valid, then Rel should satisfy the condition:

$$\mathrm{Rel} << 1 \tag{7}$$

As shown in [37], for the sizes, surface charges and ionic strengths taking place in lipid membrane studies, condition (7) is satisfied when 0.04 < Rel < 0.06. As for the nanoparticles, it is easy to guess that, for the corresponding particle sizes this condition cannot be fulfilled, and therefore the Smoluchowski equation is, in principle, invalid. The same conclusion was made recently in the review of the methods used for zeta potential determination [21]. As an illustration we give here an example of the calculation of criterion Rel for the nanoparticles stabilized with anionic or cationic surfactant bearing dissociating groups fully ionized at the neutral pH in the diluted salt solution; the relevant expressions for the calculation of κ and φ_s may be found elsewhere [37, 39, 41]. In this case surface charge density of the particles is maximal: $\sigma = \sigma^{max}$. For the area per surfactant molecule in the dense monolayer on the particle surface equal to $l\bar{e}/60$ Å^2, $\sigma^{max} = 26.67$ μCoul/cm^2, $\varphi_s = \varphi_d = -252.7$ mV. For the mean particle diameter 20 nm (i.e. radius = 10 nm = 100 Å) and ionic strength of solution, C = 10^{-3} M, we obtain: 1/κ = 96.34 Å, κ = 0.0104 1/Å, κα = 1.04. Then Rel = [exp (252.7/50.86)] /1.04 = **138.2 > > 1.**

For the nanoparticles with diameter of 100 nm Rel = **27.64 > > 1**. For C = 10^{-2} M and particle size 100 nm we obtain $\varphi_s = -194.2$, 1/κ ≈ 30 Å, κα = 16.7, Rel = [exp

[1]This criterion has also the other name (Du, abbreviation from Dukhin), suggested by Lyklema [41]. Here we use the initial name Rel, accepted in the fundamental works of the author, S.S.Dukhin and in the subsequent publications of his followers.

(194.2/50.86)] /16.7 = **2.73** > **1**. Thus, even for the particle size on the upper boundary of nanometer range at ionic strengths of the order of millimoles the calculation of zeta potential by the Smoluchowski equation leads inevitably to the erroneous results. It is clear that, the higher is particle charge and the smaller its diameter and the lower is ionic strength, the bigger is the error.

In summary one can conclude that zeta potential values calculated from the measured EPMs according to the Smoluchowski equation used in the PCS devices mentioned above are not equal to the true zeta potential (ζ) determined within the frames of classical EDL theory and therefore cannot be applied for the estimation of particle surface charge density. Actually, a researcher obtains here only the equivalent of EPM calculated by the Smoluchowski equation and expressed in potential units (ζ_{sm},); this value may be essentially smaller than the true zeta potential, that is, $\zeta_{sm} < \zeta$ in a wide range of particle sizes and charges as well as of salt concentrations in the nanoparticle solution. Therefore, the correct zeta potential values for nanoparticles can only be obtained if one applies either the more general analytical expressions developed in the theory of electrophoresis [13, 14, 16, 17, 41] or the numerical methods [15, 18–20], According to our experience in the field, for highly charged particles an optimal way is suggested by one of the equations of the Dukhin theory of electrophoresis [36, 40, 41].

4. On the mechanism of cytotoxicity for the nanoparticles stabilized with surfactant

As may be deduced from the literature on the biological activity of metal or metal oxide nanoparticles, in the last decade the data has been reported, testifying to the significance of one more meaningful parameter, namely, of the activity manifested by the nanoparticle stabilizer present in its free state in the nanoparticle solution. As shows both the analysis of literature and our own experience obtained in experiments on cell cultures, the contribution of stabilizer, at least for stabilization with surface active substances (SAS), can be essential. This conclusion issues from the results of control experiments with SAS solutions introduced to the same concentrations as in the synthesis of gold and silver nanoparticles, as well as from the studies on cytotoxicity of SAS solutions, including those used as nanoparticle stabilizers [42–47].

It is worth noting that, as shown in the special investigation [46], the toxic action of free SAS can manifest itself not only if the nanoparticle solution is used directly as obtained after the synthesis, but even if the nanoparticles had been removed from the initial solution, washed 3 times and resuspended in distilled water, i.e., when SAS concentration in the nanoparticle solution does not exceed 100 μmol/L. It is not surprising, since the presence of free SAS in the nanoparticle solution results from the equilibrium between stabilizer molecules in the nanoparticle shell and those in solution. Hence, in principle, it is impossible to maintain the nanoparticles stable in water solution at the free surfactant concentration lower that its equilibrium value at a given temperature. Therefore, in studies of cytotoxicity of the SAS-stabilized nanoparticle solution it is necessary to carry out the control measurements of SAS water solution toxicity. This is justified also for the nanoparticles washed from the initial solution and resuspended in water, but in this case, before the control measurement on stabilizer solution one should determine first the residual stabilizer concentration in thus prepared nanoparticle suspension. Correspondingly, at the elucidation of the mechanism of nanoparticle action on cells the literary data become actual on the

toxicity of the relevant surfactants found in experiments with SAS water solutions on microbial or animal cells.

The works in the last direction are carried out with various kinds of SAS, including those applied as nanoparticle stabilizers (SDS, CTAB et al) and with cell types used in studies of the biological effects of nanoparticles. Thus, in studies on the cytotoxicity mechanisms of nanoparticle solutions, apart from the action of only nanoparticles the task appears to elucidate the mechanism of free SAS action on cells. A brief account of our considerations essential for the development of correct methodology in studies of the SAS cytotoxicity is given below.

4.1 *In vitro* studies on the mechanism of SAS action on cells: general remarks

Judging from the literature available, manifestations of SAS cytotoxicity (which underlie their toxic effects towards living organisms) are interpreted as the result of action of surfactant molecules on the structure and functions of cell membrane. To our view, the explanation of cell responses on the level of solely molecular interactions does not exhaust the question about the mechanism of SAS action because, as a rule, in studies of cytotoxicity the initial water SAS solutions are used, with concentrations higher than their critical micelle concentration (CMC), that is, containing both surfactant molecules (monomers) and micelles. These two forms can act by different mechanisms, the contribution of each form being dependent on the object under study and details of experimental design.

That is why in the general case elucidation of the mechanism of SAS effect on cells implies the work in three main directions: (1) separation of monomers' and micelles' contributions and determination of their relation, (2) determination of the monomers' mode of action and (3) determination of the micelles' mode of action. In some cases the cell responses observed may result from the action of only molecules or only micelles; then the study is limited to the two directions. Thus the question whether only monomers or both monomers and micelles are present in a cell medium after the introduction of initial (stock) SAS solution and hence, whether the necessity exists to determine first the separate contribution of each form into the cytotoxicity, cannot be solved otherwise than in the course of experiment allowing to register separately the effects of monomers and micelles.

Taking into account the considerations stated above, as well as the results of our earlier studies on the liposomes' interaction with planar bilayer (as a model of biological vesicle-cell interaction) [48, 49] where it was shown that liposomes and lipid monomers present in liposome suspension manifest different modes of action, it is possible to suggest the version of SAS cytotoxicity mechanism including the two parallel processes: (1) incorporation of SAS molecules into the lipid bilayer (presumably into the external monolayer) of cell membrane and (2) adsorption of SAS micelles on a cell surface. At SAS concentration below its CMC only the monomer incorporation takes place; at its concentration near or higher than CMC both processes are realized.

However, unlike the model system liposomes – planar lipid membrane, for surfactant micelles on a cell surface may take place also the other ways of interaction with cell membrane, depending on the SAS nature, micelle parameters (size, form, surface charge), individual cell properties and experimental conditions. Since at the increase of total SAS concentration above the CMC the monomer concentration remains constant and that of micelles increases, one can suggest that here the increase of toxicity is caused by the increase of micelle contribution into the total toxic effect of surfactant.

The whole picture of interactions between SAS solutions and biological cell described above is confirmed by the experiments fulfilled by us for clearing out the

origin of the difference in toxicity of silver nanoparticles stabilized with anionic SAS (AOT) at the concentrations below and above its CMC. Here we present a brief account of the strategy of research and the main results allowing to conclude about the trustworthiness of the toxicity mechanism suggested for the chosen SAS and two cell types and, correspondingly, about the reliability of our experimental approach. A detailed description of the methodology and experimental design may be found in our recent publications [31, 50].

4.2 *In vitro* studies on the toxicity mechanism of anionic SAS (AOT) used as stabilizer of silver nanoparticles

The toxic action of stabilizer has been repeatedly recorded in our studies of the biological effects of AOT-stabilized silver nanoparticles, including the experiments on cultured human cells [11, 51, 52]; for a long time, however, mechanism of the surfactant toxicity remained unclear. It was just several years ago that the favorable conditions formed for the progress in this direction. *First*, the new version of biochemical synthesis was created, allowing the nanoparticle preparation directly in water solution at the low stabilizer concentration [53]. *Second*, basing on this version of synthesis the data were obtained on the cytotoxicity of nanoparticle solutions at AOT concentration around its CMC in water. As a direct impact for the start of our work on the mechanism of AOT toxicity served the results of Inácio et al. [54] where the sharp fall of cell viability had been registered for the anionic surfactant (SDS) at the concentration around or exceeding its CMC (**Figure 7**). These findings gave grounds to suggest that the biological activity of surfactant molecules and micelles may be essentially different (i.e. micelles can be more toxic than monomers) and hence it is possible, in principle, to determine the effect of each form of surfactant present in solution.

Therefore, we have developed a strategy allowing the separation of monomer and micelle contribution using the results of cell viability measurements and estimation of the toxicity changes of each form at the changes of total surfactant concentration in the nanoparticle solution stabilized by this surfactant. This strategy is applicable at the most often used incubation times (24 hours and longer), for the ionic SAS and "native" nanoparticle solutions obtained by the chemical synthesis, which were not subjected to the procedures of nanoparticle separation and resuspending in the other medium (e.g. in water). Below we give the recommended sequence of steps with some illustrations reproduced from our recent article [31] where our approach was applied in studies of the AOT-stabilized AgNPs interaction with malignant Jurkat cells.

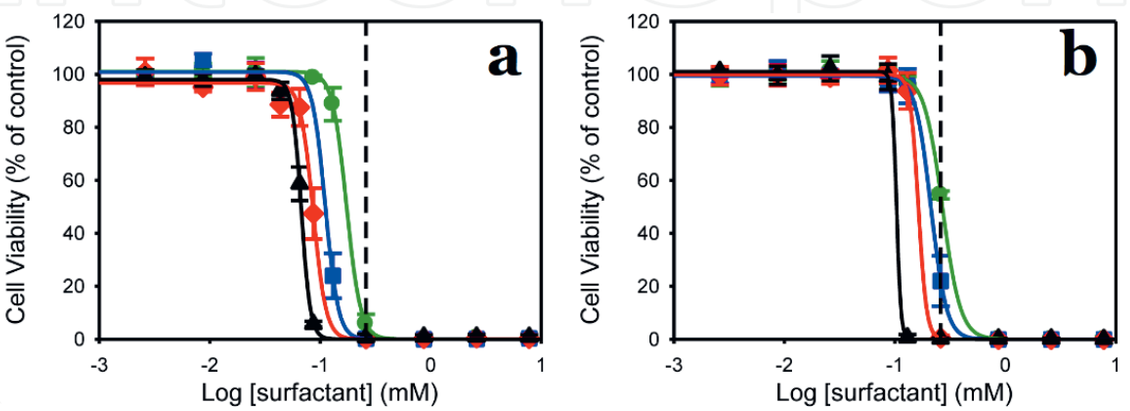

Figure 7.
Effect of SDS on viability of HeLa (a) and FDSC (b) cells. The viability was evaluated using MTT assay 24 h after treatment with SDS for 20 min (green circles), 60 min (blue squares), 180 min (red rhombi), and 540 min (black triangles). Dashed line is the SDS CMC value in serum-free medium. Reproduced from [54].

1. Determination of the CMC value for a stabilizer in distilled water; it may be taken from handbooks (e.g. [55]) or measured using a standard method available (e.g. [56, 57]). In our case the CMC for AOT was 2.81 mM as found from the conductivity measurements.

2. Synthesis of SAS-stabilized nanoparticles by the same method at surfactant concentrations higher and lower than its CMC in distilled water; in our work these concentrations were, respectively, 3 mM (3-AgNPs) and 1 mM (1-AgNPs). Mind that in both solutions the nanoparticle mean size, form and concentration should be the same, and also equal or similar should be the values of zeta potential. These conditions are necessary for the measured toxic effects on cell viability to be caused only by the difference in stabilizer concentration.

3. Measurements of cell viability (V) after the incubation during the same time at the same dilutions of the two named nanoparticle solutions and the respective stock stabilizer solutions. Determination of the difference in viability changes in the same range of dilutions; here the increase of toxicity should be observed for the nanoparticle and stabilizer solutions with concentration exceeding its CMC because of the appearance of surfactant micelles. Indeed, the expected result was observed in our viability measurements (**Figure 8**).

4. To verify the supposed role of surfactant micelles in this increase of nanoparticle toxicity one should find the surfactant CMC values in both solutions. The direct CMC measurement in nanoparticle solutions may be hampered because of the presence of stabilizer in the concentrations near its CMC. It is possible however, to substitute the nanoparticle solutions for the suitable model solutions, so that the CMC can be measured in conditions at most close to those in the nanoparticle solutions with respect to the parameters affecting the CMC value. Since the CMC for the ionic surfactants depends on ionic strength and ionic composition of solution [56, 57], these two parameters should be equal to those which exist in the nanoparticle solution. In our example, considering the measured conductivities and ionic composition of 1-AgNPs and 3-AgNPs, as the most suitable model solutions were used, respectively, 5 mM and 8 mM KNO_3. The relevant CMC values for AOT determined by the conductivity method were found to be, respectively, 2.14 and 1.7 mM (for more details see [31]).

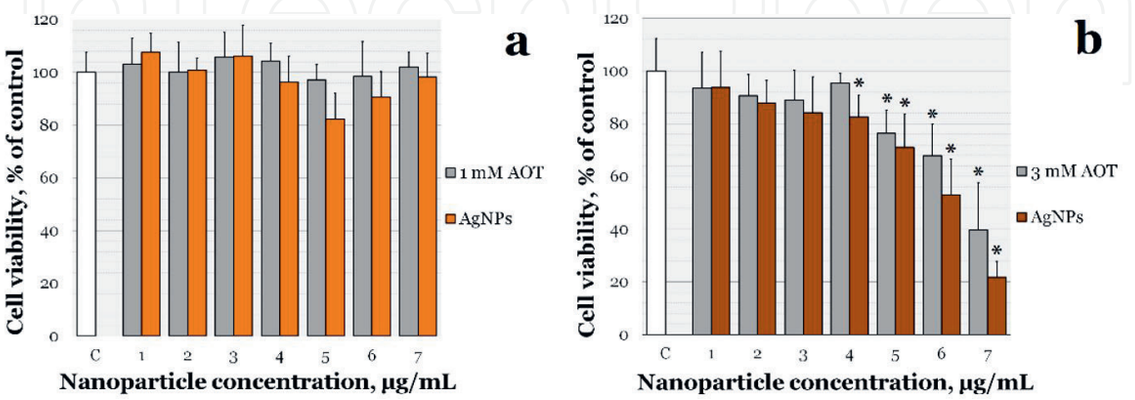

Figure 8.
*Jurkat cell viability dependence on AgNPs concentration. The nanoparticles were synthesized in water solution containing 1 mM AOT (a) and 3 mM AOT (b) and introduced from 108 μg/mL stock AgNPs solution in standard dilutions (108, 54, 36, 27, 21.6, 18, and 15.4) related to AgNPs concentrations in the range 1–7 μg/mL. The corresponding figures are given under the columns. The results obtained with 1 mM and 3 mM AOT water solutions in standard dilutions are also presented. Control – distilled water. * – here and in the* ***Figures 10*** *and* ***11****, the viability values different from control with statistical significance ($p < 0.05$). Reproduced from [31].*

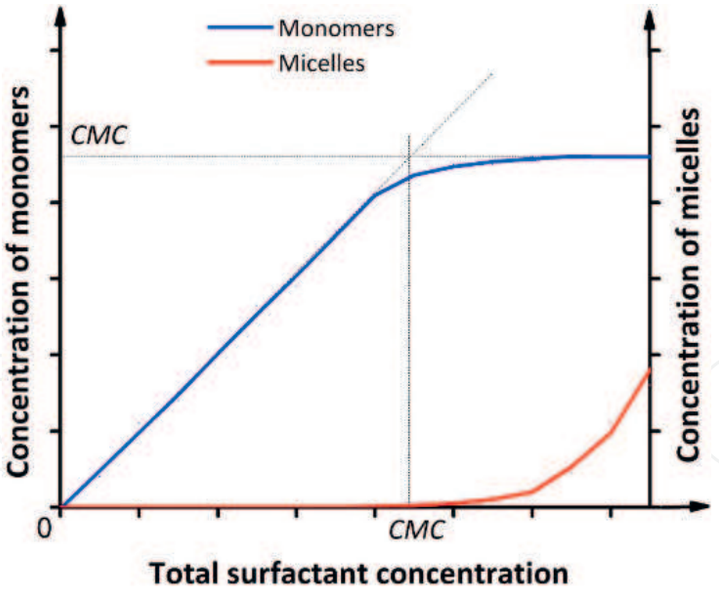

Figure 9.
Changes of monomer and micelle concentrations at low total surfactant concentrations (qualitative presentation). Reproduced from [31].

5. As follows from **Figure 9** which represents the general character of concentration changes for monomers and micelles in the region of small total surfactant concentration (C^{tot} near CMC), the monomer concentration, C^{mon}, is equal to CMC while that of micelles, C^{mic}, at C^{tot} > CMC is equal to the difference:

$$C^{mic} = C^{tot} - C^{mon} = C^{tot} - CMC \quad (8)$$

Relation (8) allows the estimation of micelle concentration in water and (assuming that CMC values in the model solutions are equal to those in the relevant AgNPs) also in the nanoparticle solutions under study. In our research, thus found monomer and micelle concentrations in 1-AgNPs and 3-AgNPs are shown in **Table 4**. As issues from the table, in 1 mM AOT containing solutions (both in distilled water and in 1-AgNPs) the stabilizer exists only in molecular form, while in 3 mM AOT containing solutions this surfactant is present in the two forms – molecules and micelles; however, in its water solution the per cent of micelles is considerably lower than in the 3-AgNPs. Hence follows that, to discover the effect of AOT micelles, in experiment with 3-AgNPs the correct control for the stabilizer toxicity should be carried out not with its water solution, but with the model

System	CMC, mM	Monomers, mM	Micelles, mM	% of total AOT concentration	
				Monomers	Micelles
Water, 1 mM AOT	2.81	1.0	0.0	100.0	0.0
Water, 3 mM AOT	2.81	2.81	0.19	93.7	6.3
AgNPs +1 mM AOT	2.14	1.0	0.0	100.0	0.0
AgNPs +3 mM AOT	1.7	1.7	1.3	56.7	43.3

Table 4.
Measured AOT CMC values, calculated concentrations of AOT monomers and micelles in water and AgNPs solutions, and their contributions to the total AOT concentration.

solution with the same CMC of the surfactant, that is, with the same concentration of its micelles. In our case the correct control was fulfilled with 8 mM KNO_3 solution where the AOT CMC was found to be 1.7 mM, presumably equal to that in 3-AgNPs.

6. Further, the monomer contribution (ΔV^{mon}) can be determined for the nanoparticle and AOT solutions with surfactant concentration higher than CMC. For this purpose, cell viability is measured after the incubation with dilutions of the stock AOT solution in the model salt solution with $C^{tot} = C^{mon} = CMC$, where AOT exists only as monomers. At each dilution (n) the decrease of cell viability is estimated as $\Delta V^{mon} = 100\% - V^n$. In our measurements (**Figure 10**) an example is shown for AOT concentration corresponding to n = 5 in the range of standard dilutions (see legend to **Figure 8**).

7. Then the micelle contribution (ΔV^{mic}) into the toxicity of nanoparticle and SAS solution with surfactant concentration $C^{tot} > CMC$ is determined from the results of the repeated measurements of nanoparticle and SAS toxicity with the use of surfactant dilutions not in water, but in the relevant model salt solution. At each dilution, the summary decrease of cell viability after incubation with SAS (ΔV^{tot}) is obtained, including the monomer and micelle contributions. The micelle contribution is found as $\Delta V^{mic} = \Delta V^{tot} - \Delta V^{mon}$, where ΔV^{mon} is that found as described in p.6. In our example this is illustrated in **Figure 11** for the same dilution as that shown in **Figure 10**. The results obtained for the AOT-stabilized AgNPs on Jurkat cells are summarized in **Table 5.**

8. Finally, the dependences of separate monomer and micelle contributions into the changes of cell viability are obtained for the chosen range of nanoparticle or SAS concentrations. These dependences reflect the difference in the toxic action of these two surfactant forms towards a cell line under study. The corresponding plots created according to the data in **Table 5** are presented in **Figure 12**. It is seen that the micelle contribution into the toxicity of 3 mM

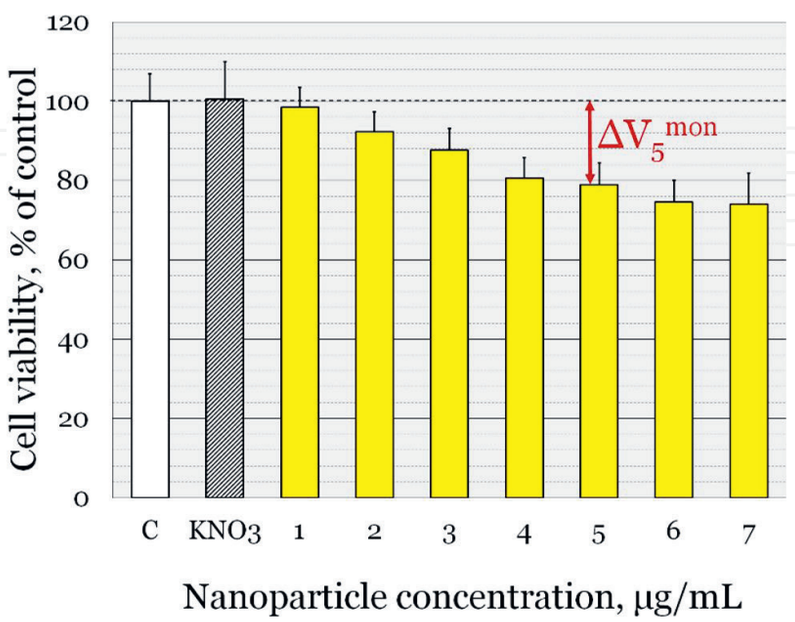

Figure 10.
Viability changes of Jurkat cells treated with AOT monomers. Cells were incubated with 1.7 mM AOT in 8 mM KNO_3 stock solution introduced in the standard dilutions. As an example, cell viability decrease at the dilution corresponding to 5 μg/mL of AgNPs (ΔV_5^{mon}) is shown. Control – distilled water (C) and 8 mM KNO_3 (KN). Adapted from [31].

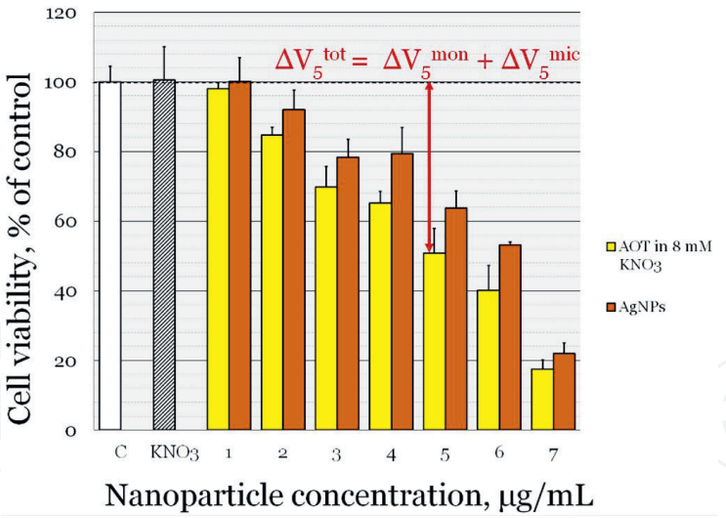

Figure 11.
Correction of the AOT toxicity in accordance with change of its CMC in the 3-AgNPs solution. Jurkat cells were incubated for 24 h with the standard dilutions of 3-AgNPs and 3 mM AOT in 8 mM KNO_3. At the dilution corresponding to 5 µg/mL of AgNPs, the total AOT contribution to viability decrease (ΔV_5^{tot}) is represented as sum of the corresponding contributions of AOT monomers (ΔV_5^{mon}) and micelles (ΔV_5^{mic}). Controls are the same as in ***Figure 10****. Adapted from [31].*

Viability changes	Nanoparticle concentrations (n), µg/mL						
	1	2	3	4	5	6	7
V_n^{mon}	98.5 ± 5.1	92.2 ± 5.1	87.6 ± 5.5	80.6 ± 5.2	78.9 ± 5.5	74.6 ± 5.4	74.0 ± 7.8
ΔV_n^{mon}	1.5	8.8	12.4	19.4	21.1	25.4	26.0
V_n^{tot}	98.0 ± 1.9	84.8 ± 2.1	69.8 ± 5.9	65.2 ± 3.3	50.8 ± 7.2	40.2 ± 7.1	17.4 ± 2.7
ΔV_n^{tot}	2	15.2	30.2	34.8	49.2	59.8	82.6
ΔV_n^{mic}	0.5	6.4	17.8	15.4	28.1	34.4	56.6

Data taken from ***Figures 9*** *and* ***10.***
Abbreviations: V_n^{mon} and ΔV_n^{mon}, respectively, measured cell viabilities and calculated contributions of monomers at standard dilutions (see ***Figure 10****); V_n^{tot} and ΔV_n^{tot}, respectively, measured cell viabilities and calculated changes in viabilities after treatment with 3 mM AOT at standard dilutions (see* ***Figure 11****); ΔV_n^{mic}, contributions of AOT micelles to cell viability changes calculated from the data of* ***Figure 11****. Adapted from [31].*

Table 5.
Contributions of monomers and micelles into the total toxic effect of AOT solutions.

AOT solutions (or of the AgNPs with the same stabilizer concentration) is more expressed than that of monomers at the AOT concentrations exceeding those introduced at n = 5. It is clear also that, contrary to the monomer contribution, that of the micelles demonstrates the tendency to exponential growth, the fact which indicates to the different mechanism of action of the two surfactant forms in water solution.

Qualitatively similar changes of monomer and micelle toxicity with changes of the total AOT concentration have been observed on the other cell type – on normal endothelium human cells (EA.hy926 line). In this case also the micelle toxicity changes with surfactant concentration reveal the tendency to exponential growth, while the monomer toxicity demonstrates the smooth increase which passes on plateau. Within the frames of the research strategy suggested, such a result was expected, since this approach supposes a qualitative difference in the mechanism of action between monomers and micelles. At the same time, the absolute values of ΔV^{mic} and ΔV^{mon} at the equal total nanoparticle concentrations differ from those

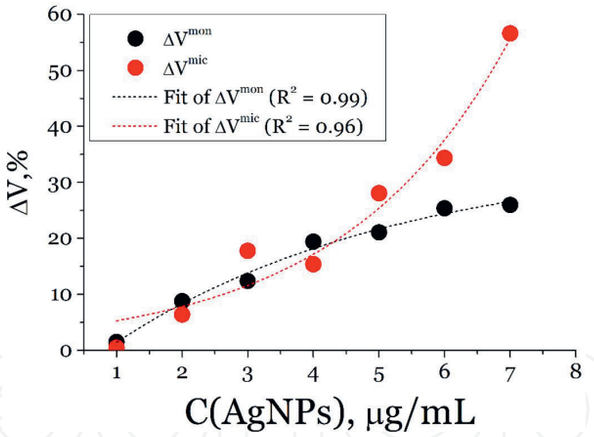

Figure 12.
Contributions of monomers and micelles to the toxic effect of AOT on Jurkat cells at various AgNPs concentrations. The 3-AgNPs solution was used. Abbreviations: ΔV, cell viability changes; ΔV^{mon} and ΔV^{mic}, cell viability changes resulted from the effect of, respectively, AOT monomers and micelles; C(AgNPs), concentration of Ag nanoparticles. Reproduced from [31].

obtained on Jurkat cells; this indicates to the dependence of monomer and micelle action on the individual properties of cells, the fact which may allow to obtain more detailed information about the cytotoxicity mechanism of surfactants.

The approach to studies of the mechanism of SAS cytotoxicity described in this section may be useful for the nanoparticles stabilized with various surfactants. It is important, as we believe, to draw attention to the active role of stabilizer micelles, because this opens the opportunity, *first*, to make the reliable conclusions concerning the mechanism of the nanoparticle action and, *second*, to specify the surfactant concentrations used in experiments on the nanoparticle cytotoxicity as well as in the surfactants' applications in the other fields. If either surfactant solution or that of surfactant-stabilized nanoparticles is introduced into a cell medium by means of dilution of the stock solution with surfactant concentration higher than CMC, it contains both surfactant monomers and micelles and both forms are present in a cell medium after the dilution, in the first moment in the same relation as in the stock solution. Then this relation changes in the process of establishment of the new equilibrium; however, it is unknown how great is the final micelle concentration and how will they affect the measured viabilities or other cell characteristics. Therefore, if it is desirable to determine the toxicity of only molecular surfactant, its concentration in the stock solution must not exceed CMC.

In the last years participation of surfactants in nanoparticle – cell interactions draws attention of many researchers (e.g. [43, 46, 47, 58, 59]). Hence there is hope that the considerations expounded above will favor the progress in the understanding of the mechanism of biological activity of surfactants applied both as nanoparticle stabilizers (in nanotoxicology) and as water solutions in medical practice.

5. Conclusion

In the end we consider it reasonable to emphasize the importance of the three essential points in methodology of studies of the metal nanoparticles' action on biological cells examined in this review.

First, in studies of the particle size effect on the biological activity it is strongly recommended to change the mode of expression of the nanoparticle concentration, namely to pass from the mass to number concentration with the corresponding change of the measured cell parameter dependence on the particle concentration.

As seen from our estimates of the toxic action of differently sized nanoparticles at the same number concentration, accumulation of similar data in the further studies may lead to the revision of the wide-spread opinion about the increase of toxicity with the decrease of nanoparticle size, with the positive consequences for their various applications in medicine.

Second, it is important to make corrections for the relaxation effect in calculations of zeta potentials from the measured electrophoretic mobilities of small particles in water solutions. It seems necessary to understand, as we believe, that there is the problem of invalidity of the Smoluchowski equation for zeta potential determination from the EPM measurements for the small charged particles of different nature, at the definite relations of their size, charge and ionic strength of solution. The error in zeta potential values calculated from the Smoluchowski equation is the more significant, the less is particle size, hence the necessity to take into account the relaxation effect is the most evident in studies of nanoparticles. We believe, however, that the corrections under question are actual not only for the correct estimation of nanoparticle surface charge in a wide variety of experiments, including the elucidation of nanoparticle cytotoxicity, but also probably for the revision of certain estimates of biological effects observed for the other charged particles used in medical and biological researches, e.g. for liposomes, biological vesicles, polymer containers for the delivery of medicines et al. Since, as a rule, the corresponding corrections are not provided by the software installed in devices used for the EPM measurements, we suggest applying for this purpose the equations proposed by the Dukhin theory.

Third, it is possible to change the conventional approach in studies of the mechanism of cytotoxicity both of nanoparticles stabilized with surfactants and of the surfactant solutions used in medical practice for the suppression of various infections. More precisely, the question is about the widening of the range of purposes, because it is suggested to include here the determination of the toxicity effects not only of molecular, but also of micellar form of surfactant, the latter being capable to exert its own contribution to cell responses.

It should be added that, certainly, the recommended changes in methodology do not exhaust the problems faced by researchers in studies of the biological effects of nanoparticles on cultured cells. They only indicate to some actual questions ripe, as we believe, in this direction of nanobiology. The main stimulus for the discussion of the questions raised in this review was our desire to draw attention to these questions and to suggest the ways for their solution, in order to achieve the better, the more reliable results in studies of the biological effects of nanoparticles for the aims of practical medicine.

Conflict of interest

The authors declare no conflict of interest.

Appendices and nomenclature

AgNPs	silver nanoparticles
AOT	aerosol-OT (bis –(2-ethylhexyl) sulphosuccinate, sodium salt)
CMC	critical micelle concentration
CTAB	cethyl trimethyl ammonium bromide
EDL	electrical double layer
EPM	electrophoretic mobility
PCS	photon correlation spectroscopy

SAS	surface active substances
SDS	sodium dodecyl sulphate
TEM	transmission electron microscopy

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