

Hsp70 as an indicator of stress in the cells after contact with nanoparticles

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Abstract. In recent years, production of nanoparticles is increased and thus grows our contact with them too. Question of safety is closely related to the issue of use nanoparticles. There are a number of tests that monitor the viability, ROS production, the effect on the DNA and cell cycle, however, rarely encountered studies on stress in the cells after contact with nanoparticles. Heat shock proteins (HSP) are among the substances that can be used for monitoring stress in cells. HSP are structures with a chaperone activity. They are evolutionarily very old, conservative and they are found with a high degree of homology in prokaryotes and eukaryotes including humans. They exist at low concentrations under physiological conditions, while in the denaturing conditions e.g. high or low temperature, radiation, exposure to chemicals, heavy metals, or nanoparticles their expression is changed. HSPs are involved in maintaining homeostasis in the cell that the denatured protein conformations allow recovery to the original stage. One of the most common proteins from HSP family is Hsp70 - protein with a molecular weight of 70 kDa. The level of Hsp70 in a cell after exposure to the stress changes depending on the stress level to which the cell is exposed to and a time period during which lasted stressful conditions. Our research monitors stress levels of cells manifesting by Hsp70 production after contact with silver nanoparticles. Nanoparticles show different toxicity towards different types of target cells, which is reflected in the values of IC₅₀ – concentration that kills 50 % tested cells. Concentration of test substance toxic to one cell type may be innocuous to cells of another type. IC₅₀ obtained from the MTT assay provides a suitable default data and if multiples of IC₅₀ values are used, we can compare and generalize. Studies can be used to compare stress levels in cells that show different sensitivity to the tested nanoparticles compared with cells under optimal growth conditions. The study was done on two types of mouse fibroblasts NIH-3T3 and L929. While NIH-3T3 cells exhibit stress response proportional to the concentration of silver nanoparticles, for L929 cells this was not observed.



1. Introduction

Nanoparticles are widely used because they give us the advantages that are associated with their nano dimension. Higher utilization of nanomaterials, however, brings risks as well. Nanoparticles are toxicologically tested to determine the IC₅₀, ROS, membrane potential, it is monitored DNA damage, cell cycle and many other parameters. With the exception of a few studies [1-3] is not paid attention to the stress induced by contact with nanoparticles. Stress at the cellular level is manifested production of proteins known as stress proteins. The best known include heat shock proteins – Hsp. The heat shock proteins are group of proteins that provide the stress response of cells when exposed to high temperature, extreme pH, heavy metals or nanoparticles. Heat shock proteins are evolutionarily ancient structures, with a high degree of homology. In addition to the repair of damaged or improperly formed proteins participates in a number of immunological processes [4]. Hsp occur both in prokaryotes and in eukaryotes (including man). Hsp70 - protein with a molecular weight of 70 kDa is expressed in stressful situations and appears to be a suitable biomarker [5-9].

If the stress upon contact with nanoparticles is studied, *Drosophyla melanogaster* [1], *Japanese medaka* [10] or other aquatic organisms [11] are tested. Less frequent testing is performed on isolated cell lines [3, 12].

Silver nanoparticles are among the most widely used nanoparticles for their antibacterial properties. They are used whenever is possible to use their abilities to kill microbes. The widespread use of silver nanoparticles leads to intense contact with this kind of nanoparticles, so it is necessary to carefully consider their toxicity, even at the cellular level. The novelty of our study is that have not been tested the same concentration of the nanoparticles, but their multiples derived from IC₅₀ values. Our study explores the stress caused by contact with silver nanoparticles on two very similar lines of mouse fibroblasts, but they have a different toxicological response - their IC₅₀ is slightly different.

2. Materials and methods

Silver nanoparticles

Silver nanoparticles (NPs) prepared by modified Tollens method [13]. As the reducing sugar maltose was used. The average size of the particles was 26±2.5 nm.

Buffer

Lysis buffer - 3 ml 5M NaCl, 10 ml 10% Triton X, 5 ml Tris pH 8, 82 ml water, prior to use 1 tablet of protease inhibitors (cOmplete Protease Inhibitor Cocktail Tablets, Roche) per 10 mL of buffer was added. All other used chemicals were from Sigma and were used without further purification.

Viability

Viability of two cellular types (NIH-3T3 and L929) was evaluated using the MTT assay. Cells were seeded at a density 10⁴ cells per well in 96-well plates. After spreading (4 hours), cells were treated by increasing concentration (0.25 – 30 µg/mL) of the silver nanoparticles and incubated 24 hours. Subsequently, 20 µl of MTT solution (5 mg/mL) was added in the each well and kept in a CO₂ incubator (NIH-3T3 cells for 4 hours, L929 cells for 1.5 hours). Finally, the blue formazan crystals were dissolved by 100 µL of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a multiplate reader Infinite 200 PRO (Tecan). The dose-response curves were obtained and the inhibitory concentration (IC₅₀) determined. Values of IC₅₀ were used in following tests.

Cell cultures for nanoparticles and heat shock testing

Cell (NIH-3T3 and L929) were seeded in 6 well plate at a density 2.5 x 10⁵ cells per well and cultivated in DMEM (Dulbecco's Modified Eagle medium,), at 37°C in a moisturized 5% CO₂ incubator. After 24 hours, the silver nanoparticles were added and incubate 24 hours. Following this period, cells were washed 3 times with PBS, trypsinised and centrifuged 10 min at 4°C in speed 6 000 g. Supernatant was removed and pellet resuspended in 0.5 ml chilled lysis buffer, subsequently sonicated for 30 s. Each sonication was repeated 5 times and samples were kept on ice between each step. Supernatant containing proteins was obtained by centrifugation 20 min at 4°C in speed 10 000 g. Positive controls were prepared by the same protocol – cells exposed to a temperature 43°C for 1, 2

and 3 hours. After 24 hours of recovery and spreading, cells were lysed as described above.

Total protein content

Total protein content was determined by Bradford method using BSA as a standard. The absorbance was measured at 595 nm using a multiplate reader Infinite 200 PRO (Tecan).

SDS-Page and Western blotting

Supernatants were diluted 1:1 with SDS sample buffer (2x) and loaded on 10% polyacrylamide gel. Then proteins were transferred on a PVDF membrane and incubated with anti-Hsp70 (1:1000, Sigma H5147) overnight. The Hsp70 complex was detected using HRP-conjugated secondary antibody (Sigma A9917) and ECL (Amersham). Quantification of Hsp70 was performed by densitometry analysis using free software ImageJ.

Characterization techniques

Transmission electron microscopy (TEM) images were obtained using a transmission electron microscope (JEOL JEM-2010) operating at 160 kV with a point-to-point resolution of 1.9 Å. The samples dispersed in deionized water were sonicated for \approx 1 min. Then, a drop of very diluted dispersion was placed on a holey carbon-coated copper grid and allowed to dry at ambient temperature. Hydrodynamic size of silver nanoparticles was performed on Malvern Instrument Zetasizer Nano-ZS instrument

Statistical analysis

All measurements were performed at least three times and the results are given in the form: average value \pm standard deviation.

3. Results

Silver nanoparticles used in our experiments were characterised by TEM (see figure 1) and dynamic light scattering (DLS). The particle sizes obtained from the TEM and DLS were well correlated; the average particle size was 26 ± 2.5 nm.

MTT assay provides IC_{50} - the concentration of test compound that kills 50% tested cells (see figure 2). IC_{50} was 12 μ g/mL for NIH-3T3 cells and 15 μ g/mL for L929 cells. It means that silver nanoparticles are more toxic for NIH-3T3. To test the stress response caused by silver nanoparticles were chosen two concentrations - one equals IC_{50} and second half of IC_{50} (6 and 7.5 μ g/mL).

First positive controls were prepared by incubating the cells in temperature higher than its optimum (figure 3). Total protein was measured by Bradford method, Hsp70 quantification was done by densitometry analysis. Subsequently Hsp70 content in control and test samples were calculated. Content of Hsp70 under physiological conditions was defined as 100%; the other values were related to this value. Figure 4 shows relative changes in Hsp70 level after 24 hours contact with silver nanoparticles. As is evident from Figure 3 and 4, both cell lines respond differently to heat shock, and on contact with the nanoparticles. The reaction of NIH-3T3 cells to a higher temperature is rapid. After one hour incubation at 43°C is seen increase the level of Hsp70 to 300%. A maximum (385%) is reached during the three hour incubation. In contrast, the reaction of L929 cells is gradual, the maximum (419%) is reached at two hours incubation and then decreases to 253% after three hours.

Cells react differently on contact with nanoparticles too. Here it is possible to observe an increase of the Hsp70 content, although not as high as that induced by heat stress. For NIH-3T3 cells is evident increase to 196 and then to 318%, while for L929 cells is observed to rise to 128% at a dose of 1/2 IC_{50} and then drop to 113%. In the case of NIH-3T3 cells, both tested doses were significantly different from the controls and there is noticeable concentration dependence. L929 cells respond differently, there is no concentration dependence. A dose equivalent to half the IC_{50} is significantly different from controls, while dose equal to the IC_{50} is no different.

Figure 1. TEM image of silver nanoparticles

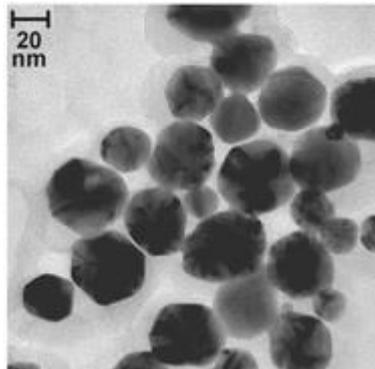


Figure 2. MTT tests

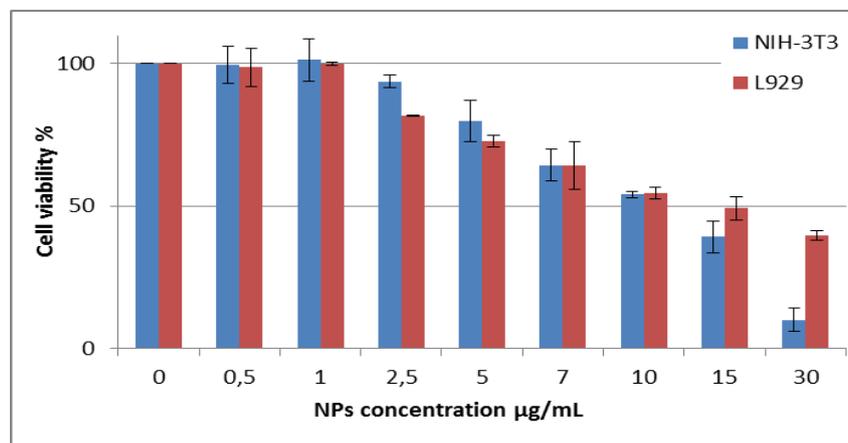
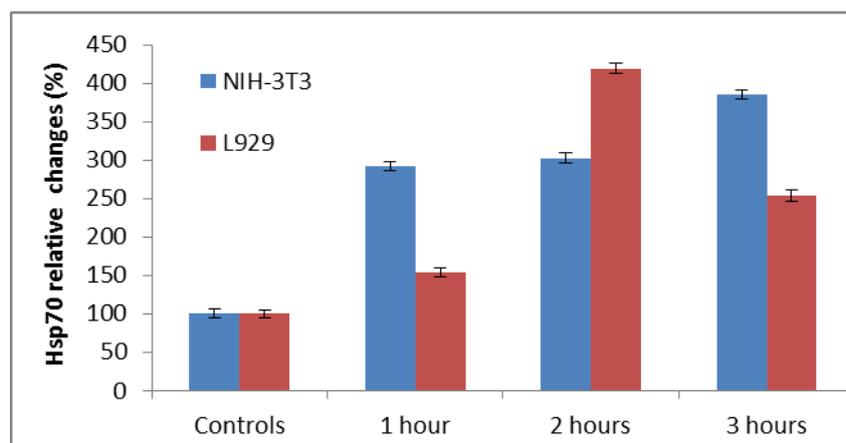
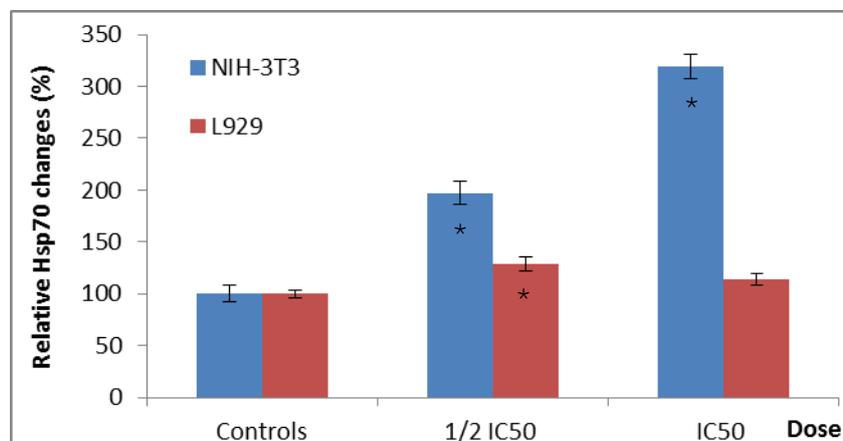


Figure 3. Relative Hsp70 changes – heat shock



Controls – physiological condition, tested cells – 43°C for 1, 2 or 3 hours. All tested samples are significantly different from controls, ($P < 0.05$).

Figure 4. Relative Hsp70 changes – contact with silver nanoparticles



* Significantly different from controls ($P < 0.05$).

4. Discussion

As already mentioned, increased use of nanoparticles also entails some risks. Commonly performed tests monitor viability, ROS production and other parameters. Negligible number of studies follows the stress at the cellular level. The importance of stress monitoring just mentions in his work Mukhopadhyay et al. [9] especially with regard to the environment.

When whole organisms or cell cultures are exposed to elevated temperatures, they respond by synthesizing a small number of highly conserved proteins called heat shock proteins, because first observation was connected with heat temperature induction [14, 15]. This response is universal. Hsp are involved in a wide range of processes in cells. Maintain homeostasis, participating in de novo folding of nascent proteins and repair of damaged ones [16], play role in a number of events associated with the immune system [4]. One of the most frequently studied Hsp is Hsp70. Hsp70 occur across species [15] with high degree of homology. It is often described as a suitable biomarker of stress [5]. This stress can be caused by high or low temperature [17], by treatment with radiation, certain drugs, heavy metals [9] and nanoparticles [1, 5, 10]. The stress response to silver nanoparticles was chosen for our research.

The first part of our research was to prepare the positive controls. Positive control (cells exposed to a higher temperature) served as loading controls for SDS-PAGE and Western blots. When comparing the stress response of both cell lines to thermal shock, it is obvious that the reaction is different (see figure 3). For NIH-3T3 cells it is seen the increase of Hsp70 after one hour incubation. Longer incubation at elevated temperature leads to an increase of Hsp70. Increased production of protein indicates a higher level of damage. L929 cells respond to thermal shock by slower increase in Hsp70 after one hour exposure. This may mean that L929 cells have better tolerance to temperature rise, if it not takes too long. If cell is exposed to higher temperatures for a longer period (2 hours), it is observed a dramatic increase in Hsp70. Then there is a decrease, probably due to cell death. Two hours exposure at 43°C is the limit, after which the cell is unable to repair denatured proteins and other stay at this temperature is lethal. Ability to cope with fluctuations in temperature allowed survival of the species, an organism with better tolerance survived. If the temperature rise is slow, isolated cells or whole organism have time to prepare (stress proteins induction). These stress proteins act protectively towards different stressors than the original was [18]. The next step of our experiment was to investigate the stress response after contact with silver nanoparticles. The silver nanoparticles prepared by a modified Tollens process are toxic for both NIH-3T3 and L929 cells; IC₅₀ is 12 µg/mL and 15 µg/mL respectively. Contact with these nanoparticles induces stress which can be monitored via Hsp70 in both cell lines. The response of cell lines upon contact with silver nanoparticles was different. NIH-3T3 cells responded production of Hsp70 proportional to the concentration of

nanoparticles, L929 cells generate maximum at a dose equal to half the IC_{50} , and a higher dose of nanoparticles did not lead to a further increase. Conversely, a decrease from 128% to 113% relative to controls was observed. Kaur and Tikka [3] investigated the effect of surface charge on the cytotoxicity of silver nanoparticles using Hsp70 as just one of biomarkers. They performed tests on cancer line A431 (human epithelial carcinoma), A549 (human lung carcinoma) and murine RAW264.7 macrophages. They used relatively high doses of silver nanoparticles (up to 100 $\mu\text{g}/\text{mL}$). Maximum levels of rise Hsp70 they observed in A431 (11-fold increase), in the remaining two lines increase was about 3-fold. This finding is consistent with what we observed. Like us on L929 cells, they also observed a higher production of Hsp70 on the lower dose and explain to the fact that higher concentrations are less stressful for cells. We believe that the explanation is rather that high doses are so toxic that it exceeds cell's reparative capabilities and cells die. Only the live cell can produce proteins. Low numbers of cells produce low levels of Hsp70. Lim et al [12] tested a low dose of 5 nm silver nanoparticles on human macrophage cell line (U937) and they observed increase in the range of 1.2 to 2.3-fold.

5. Conclusion

The stress response caused by heat shock and exposure to silver nanoparticles was tested. Murine fibroblasts exposed to a temperature higher than their optimum react increased production of Hsp70. Compared to control cells cultured in ideal conditions, level of Hsp70 is increased until 4-fold. Our results show that the silver nanoparticles (size 26 nm, modified Tollens method) show signs of toxicity to murine fibroblasts L929 and NIH-3T3. IC_{50} values obtained by MTT assay was 15 and 12 $\mu\text{g}/\text{mL}$. Denaturation of proteins due to the contact with silver nanoparticles was found. Presence of denatured proteins is outwardly manifested by expression of Hsp70, which is involved in the repair in order to restore physiological state of the cell. NIH-3T3 cells exhibited a clear concentration dependency, while L929 responded markedly to lower concentrations of silver nanoparticles. It is possible to say that Hsp70 seems suitable biomarker for stress testing in cells upon contact with silver nanoparticles. The question is whether it can be used for other types of nanoparticles. This will be the subject of our further research.

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