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Reducing Stress on Cells with Apoferritin-Encapsulated Platinum Nanoparticles

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ABSTRACT The great potential for medical applications of inorganic nanoparticles in living organisms is severely restricted by the concern that nanoparticles can harmfully interact with biological systems, such as lipid membranes or cell proteins. To enable an uptake of such nanoparticles by cells without harming their membranes, platinum nanoparticles were synthesized within cavities of hollow protein nanospheres (apoferritin). In vitro, the protein-platinum nanoparticles show good catalytic efficiency and long-term stability. Subsequently the particles were tested after ferritin-receptor-mediated incorporation in human intestinal Caco-2 cells. Upon externally induced stress, for example, with hydrogen peroxide, the oxygen species in the cells decreased and the viability of the cells increased.

KEYWORDS Apoferririn, ferritin receptor, nanoparticle, platinum, ROS

A high surface-to-volume ratio and a high surface energy make noble metal nanoparticles attractive for various technological and scientific purposes.[1−3] A homogeneous dispersion of nanoparticles is expected to be applicable also for biological systems and in biomedicine. However, especially for those biomedical applications, the activity, the stability and the toxicological impact of nanoparticles need to be examined. The limited data indicate that manufactured nanoparticles can alter the integrity of biological membranes and the enzyme functions through interactions with lipid membranes, proteins, and DNA.[4−6] The ability of nanoparticles to produce reactive oxygen species (ROS) and induce oxidative stress can also feature as a mechanism of toxicity.[7] ROS are ions or small molecules that contain peroxide (e.g., hydrogen peroxide), free radicals, and oxygen ions (e.g., superoxide). These are highly reactive species formed during the normal metabolism of oxygen. Under normal physiological conditions ROS are important for cell signaling.[8,9] Cells are able to detoxify most of excess ROS through several antioxidant systems, including enzymes (e.g., catalase), as well as small molecules (e.g., vitamin C). However, the formation and accumulation of ROS will increase dramatically if the cell is exposed to oxidative stress. High levels of ROS have been linked to pathological conditions such as aging, asthma, diabetes, atherosclerosis, inflammatory arthritis, and neurodegenerative diseases.[10,11]

For reducing cellular oxidative stress with nanoparticles, platinum is of special interest since platinum nanoparticles are capable of quenching hydrogen peroxide (H_2O_2) and superoxide (O_2^-), which resemble two biological enzymes, catalase and superoxide dismutase (SOD).[12] To avoid the potential interactions with biological membranes, surface modifications are necessary. However, the modification should not passivate the surface of the nanoparticles or hinder the access of the oxidant to the particle surface. Here we show that stable platinum nanoparticles can be successfully synthesized within the cavity of apoferritin and show catalytic reactivity for scavenging H_2O_2 and O_2^-. After ferritin-receptor-mediated incorporation in human intestinal Caco-2 cells, apoferritin-encapsulated platinum nanoparticles decrease the amount of H_2O_2-induced intracellular reactive oxygen species (ROS) and have positive effects on the viability of the cells under various types of stress.

Ferritin is a protein complex consisting of 24 subunits forming a sphere with 12 nm outer diameter and an inner cavity of 7.6 nm. The junctions of the subunits provide 14 channels, 3−4 Å in diameter, which perforate the protein shell and serve as pathways between the exterior and interior.[13] As iron storage protein, ferritin is present in many organisms. Releasing the iron containing core from the cavity leads to a hollow sphere, the apoferritin, which has already been used as template for the preparation of various nanoparticles.[14−16] The activity of apoferritin with incorporated platinum has not yet been investigated in terms of its efficiency to reduce ROS and the potential to reduce oxidative stress on cells.

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SCHEME 1. Preparation of Apoferritin-Encapsulated Platinum Nanoparticles (Pt-apo)

Scheme 1 shows the preparation of Pt-nanoparticles (Pt-nps) in apoferritin (Pt-apo). The chemical reduction of Pt(II) and the formation of Pt-nps inside the apoferritin cavity were confirmed by transmisstion electron microscopy (TEM) and X-ray photoelectron spectroscopy (XPS) (Figure 1). The diameter of approximately 2 nm was averaged from 100 randomly selected particles. The sample was negatively stained with uranyl acetate to ensure the intact protein shell of apoferritin (Figure 1B and Supporting Information S1). After reduction of Pt(II) to Pt(0), the inner cavity of apoferritin was clearly observable, which was not the case when the pure protein without Pt was stained. There were no observable Pt particles outside the protein spheres. It should be noted that some apoferritins showed less enhanced contrast of the cavity. This indicates that some of the spheres show less capability for accumulation of the platinum precursor. Figure 1C shows XPS spectra of the Pt 4f region in which the signal is not influenced by the protein. Pt $4f_{7/2}$ and Pt $4f_{5/2}$ peaks (77.9 and 81.3 eV) shifted to 73.1 and 76.5 eV after the reduction, respectively. This approximate 5 eV energy shift confirmed a chemical reduction. However, the binding energy of Pt 4f in Pt-apo is almost 2 eV higher than expected for bulk Pt ($4f_{7/2}$: 71.2 eV),[17] but still 1.5 eV lower than 74.6 eV, which would be expected for PtO or PtO_2.[18] The reason for this higher binding energy could be derived from an extremely small size of the Pt-nps.[19] Another explanation is potential interactions between Pt-nanoparticles and the protein shell. A similar effect has been observed when small Pt-nps produced by chemical reduction were used to modify carbon nanotubes.[20]

The quenching activity of platinum nanoparticles against H_2O_2 and superoxide ions has been described recently.[12] Measured with a SOD-Activity Assay Kit, apoferritin, and Pt-

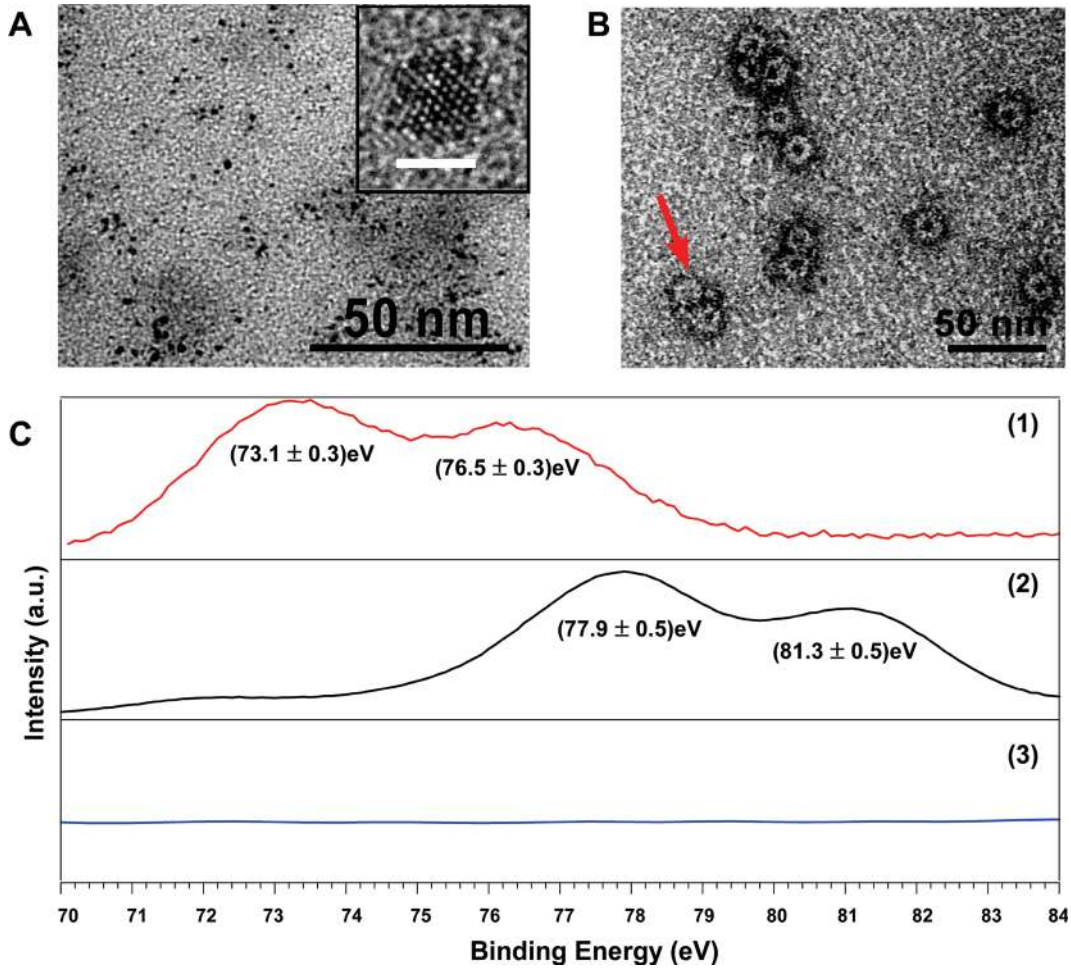

FIGURE 1. (A) TEM image of Pt-apo prepared from K_2PtCl_4/apoferritin with a molar ratio of 24 000:1. The concentration of K_2PtCl_4 in the mixture was 312 mM. (Inset: High resolution electron micrograph of a platinum nanoparticle; scale bar, 2 nm). (B) TEM image of Pt-apo after negative staining with 1.5% uranyl acetate. The red arrow points toward an apoferritin without or with very small Pt-nps in the cavity. (C) XPS-spectra of Pt 4f region of (1) Pt-apo; (2) apoferritin and K_2PtCl_4; and (3) apoferritin.

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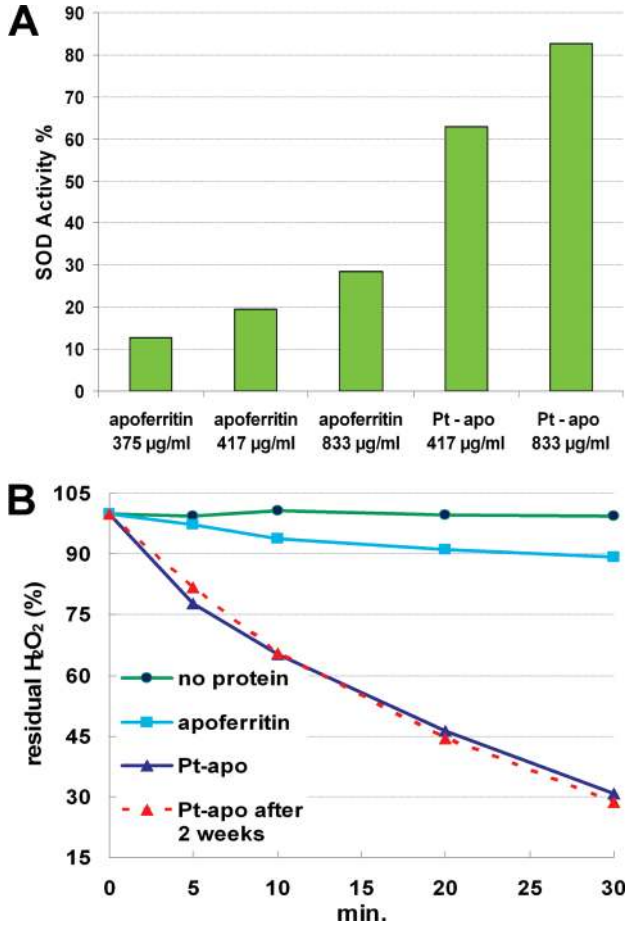

FIGURE 2. (A) SOD Activity of apoferritin and Pt-apo prepared with a molar ratio of 15 000:1. The end concentration of K_2PtCl_4 was 250 mM. The concentration of Pt-apo is given by the protein concentration of the composite. (B) Activities of apoferritin and Pt-apo for the H_2O_2-decomposition and activity change of Pt-apo after two weeks storage in water at 4 °C. The protein and hydrogen peroxide concentration for the reaction were 0.8 mg/mL and 330 μM, respectively. The temperature was 30 °C and pH was 7.4.

apo quenched superoxide ions in a dose-dependent manner (Figure 2A). However, when platinum nanoparticles were present inside the protein cavity, the activity increased almost 3 fold with the same protein concentration. Figure 2B shows the H_2O_2 decomposition activities of apoferritin and Pt-apo in vitro. For the assay, the concentration of the protein in our solution was kept constant at 0.8 mg/mL. Without apoferritin or Pt-apo the hydrogen peroxide was stable in the assay conditions. When apoferritin was present in the solution only 10% of hydrogen peroxide was eliminated after 30 min. However, approximately 70% H_2O_2 could be decomposed with Pt-apo. The enhancement of these two activities clearly shows that the surface of the Pt-nps prepared inside the apoferritin was not seriously passivated by the protein shells and possessed a high activity for scavenging superoxide ions and hydrogen peroxide.

Because of their high surface energy, noble metal nanoparticles tend to agglomerate in solution, forming energetically more stable units. The H_2O_2 decomposing activity of Pt-apo exhibited almost no change after 2 weeks storage at 4 °C in water (Figure 2B), indicating that the protein shell of apoferritin stabilized the Pt-nps in its cavity, which is an advantage for both the storage and potential long-term applications.

The potential of ferritin and apoferritin as antioxidants and for protection of cells from oxidant-mediated cytolysis and lipid peroxidation has already been investigated.[21−23] Those effects are related to the iron-sequestering ability and ferroxidase activity of the protein. Here we first demonstrate that apoferritin itself has peroxide- and superoxide-scavenging activities, which were greatly enhanced in a cell-free solution by incorporation of Pt-nps into the protein. When those bioinorganic nanoparticles are internalized into the cell, we expect that under oxidative stress, especially Pt-apo, will support the cells in the elimination of H_2O_2 and superoxide and the cells will become more resistant to oxidative stress. To confirm the supportive effect of the Pt-apo, tests were performed with the mammalian cell line Caco-2. It should be noted that Caco-2 cells were selected since they express constitutively ferritin receptors.[24] These receptors are expected to allow the internalization of the Pt-apo into the cell.

Prior to the determination of the potential antioxidant effect of Pt-apo in vivo, it had to be confirmed that Pt-nps encapsulated in apoferritin have no significant cytotoxicity in the applied experimental conditions. The Caco-2 cells were cultured as described earlier.[25] The viability of the cells treated with up to 2.5 mg/mL apoferritin or Pt-apo was measured (Supporting Information Figure S2), showing no concentration dependency of the toxic effect in the given range.

The cellular oxidative stress was determined by quantifying intracellular ROS with a dichlorofluorescein (DCF) assay.[26] Without H_2O_2 stimulation, the intracellular ROS of the preincubated cells remained at the same level, except in cells treated with 0.5 mg/mL Pt-apo. After 2 mM H_2O_2-induced oxidative stimulation, cells preincubated with apoferritin and Pt-apo showed much lower fluorescence intensity of DCF with respect to the control cells (Figure 3A). The reduction of ROS showed also concentration dependence: the higher the concentration of the nanoparticles, the lower the amount of intracellular ROS. This decrease of ROS accumulation indicated that Pt-apo could indeed reduce ROS inside the cell, even encapsulated in apoferritin, thus the surface of the Pt inside the apoferritin is accessible and catalytically active.

In the next step, the influence of the ROS-reducing effect of apoferritin and Pt-apo on the viability of treated cells was investigated. Figure 3B shows the viability of Caco-2 cells after treatment with 5 mM H_2O_2 for 1 h. Cells, without preincubation with apoferritin or Pt-apo, underwent a 40% cell death arising from H_2O_2-induced oxidative stress. On the other hand, cells were protected from the toxic effects of hydrogen peroxide by apoferritin and Pt-apo in a dose-dependent manner. Preincubation with 0.5 and 1 mg/mL

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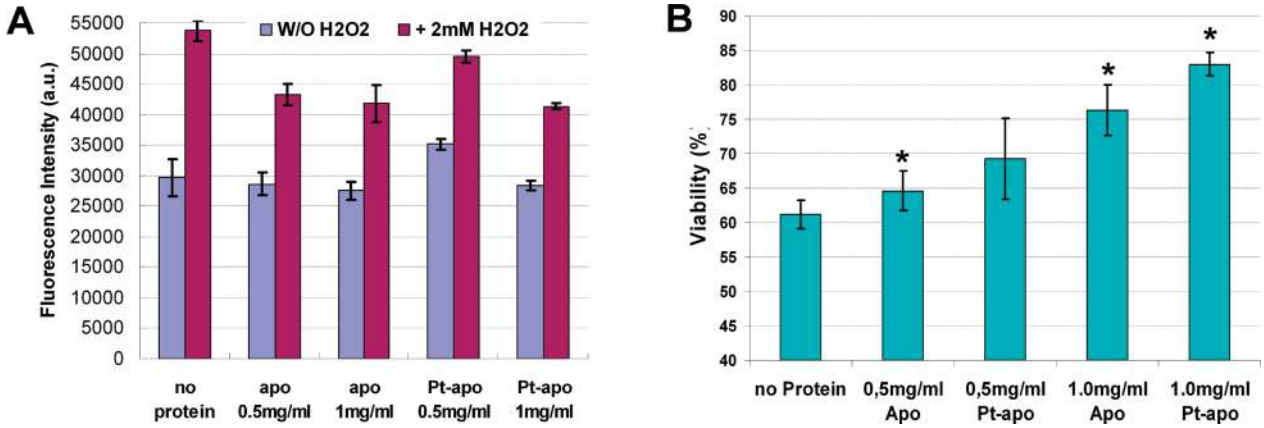

FIGURE 3. (A) Effect of apoferritin (apo) and Pt-apoferritin (Pt-apo) on H_2O_2-induced intracellular reactive oxygen species (ROS) generation in Caco-2 cells. After the H_2O_2-induction, the ROS levels of apo or Pt-apo treated cells are significantly lower than that of the control cells. (B) Effects of treatment with apo and Pt-apo on the viability of Caco-2 cells stressed with 5 mM H_2O_2. The viability was determined with the Cell Counting Kit-8. The viability of untreated (without both protein and H_2O_2) cells was set 100%. Values marked with an asterisk are significantly different from each other. The results are represented by the mean ±SD. Statistical analysis was done with the nonparametric two-tailed U-test, and a p-value of less than 0.05 was considered statistically significant.

apoferritin resulted in a cell survival rate of 65 and 76%, respectively. Enhanced protection with 69 and 83% cell viability was observed after preincubation with Pt-apo with concentrations of 0.5 and 1 mg/mL, respectively. At first sight, it seems odd that, although the intracellular accumulation of ROS was higher at 0.5 mg/mL Pt-apo (see Figure 3A), more cells survive the H_2O_2 treatment (see Figure 3B). The explanation is, however, simple. Considering the viability, the average ROS level in a single cell treated with Pt-apo is lower than in a cell treated with apoferritin. This promoted protective effect from Pt-apo gives an indirect evidence for the internalization of Pt-apo into the cells. The dose-dependent response of the effect shows that Pt-nps inside apoferritin are accessible to the intracellular ROS and can act as antioxidants. An increase of more than 20% of the cell viability after incorporation of Pt-apo demonstrates the high efficiency of Pt-apo as antioxidant.

Because of the nonlinear correlation between the viability and the intracellular ROS level, apart from ROS scavenging activities of Pt nanoparticles, additional protective effects of Pt-apo in the cell may occur. To evaluate such potential effects, we also induced nonoxidative stress to the cells, again resulting in an increased viability (see Supporting Information Figure S3). The protective effect of Pt-apo under the nonoxidative stress suggested that the origin of protective effects of Pt-apo is not solely based on the antioxidant potential of the Pt-nps. Together with apoferritin, Pt-apo potentially stabilizes the cell upon various types of externally induced stress.

Receptor-mediated internalization prevents direct interactions of Pt nanoparticles with the cell membrane and resulting membrane damages, which frequently occurs when small particles enter cells by diffusion through cell membranes.[5,27−29] Inside the cells, the protein cage will prevent interactions of platinum nanoparticles with further cellular proteins and random diffusion of nanoparticles. Both factors increase the biocompatibility of the encapsulated platinum nanoparticles. Small channels in the protein cage additionally provide substrate selectivity for Pt nanoparticles.[30] Since the presence of ferritin-receptors has been described on many cell lines,[24,31] the potential applications of Pt-apo is not restricted to intestinal cells. The cellular internalization of apoferritin-encapsulated nanoparticles via ferritin-receptors demonstrated a strategy to reduce possible toxicity, which will enable biological applications of such nanomaterials in general. There are still several unresolved issues concerning Pt-apo as antioxidant in cells. For example, we did not analyze the time dependent cytotoxicity of Pt-apo. Since ROS in physiologic conditions is involved in intracellular signaling, a prolonged incubation with Pt-apo or incubation with a high dosage can deplete ROS to an excessive extent, which may harm cells. Another issue is that the exact location of the Pt-apo and the metabolism of the apoferritin protein and the Pt-nps after incorporation into the cells are not yet known. These remain future challenges for a thorough understanding of detailed functions and for in vivo usage of protein encapsulated metal nanoparticles.

In summary, stable platinum nanoparticles were synthesized within apoferritin protein cages that served as nanotemplates and nanoreactors. The apoferritin-encapsulated Pt nanoparticles were shown to be capable of scavenging hydrogen peroxide and superoxide. The receptor-mediated internalization of ferritin allows an uptake of the encapsulated nanoparticles into the cell without compromising the cell membrane integrity and avoids the interaction with cellular proteins, as it would be the case if bare metal nanoparticles penetrate the cell by diffusion. After incorporation into Caco-2 cells, Pt-apo can decrease the H_2O_2-induced intracellular ROS level and improve the viability of the cells against oxidative stress and stress induced by treatment with PBS. Therefore, apoferritin-encapsulated platinum nanoparticles may function as new potential an-

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tioxidants for applications under pathophysiological conditions, such as ROS-mediated diseases. The results show a good example where bioscience and nanoscience can be combined beneficially.

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Supporting Information Available. Text describing detailed description of the materials and methods, and figures showing the viability of the cells without H_2O_2-treatment and after the incubation with phosphate buffered saline. This material is available free of charge via the Internet at http://pubs.acs.org.

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