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Full Length Research Paper

Effect of ferric oxide nanoparticles on microtubules organization

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Nanoparticles (NPs) are widely used in several manufactured products. The small size of nanoparticles facilitates their uptake into cells as well as transcytosis across epithelial cells into blood and lymph circulation to reach different sites, such as the central nervous system. Studies have shown different risks of Fe_2O_3NPs in the neuronal system and other organs. They are membrane-bound layer aggregates or single particles that could not enter only cells, but also in mitochondria and nuclei. Therefore, these particles can interact with cytoplasmic proteins such as microtubules (MTs). MTs are cytoskeleton proteins that are essential in eukaryotic cells for a variety of functions, such as cellular transport, cell motility and mitosis. MTs play an important role in neurons and to act as a substances transport such as neurotransmitters. Single Fe_2O_3NPs in cytoplasm can interact with these proteins and affect their crucial functions in different tissues. In this study, we showed the effects of Fe_2O_3NPs on MTs organization and structure using ultraviolet spectrophotometer and fluorometry. The fluorescent spectroscopy showed a significant tubulin conformational change in the presence of Fe_2O_3NPs and the ultraviolet spectroscopy results showed that Fe_2O_3NPs causes MTs depolymerization and decrease turbidity intensity as well as increase spectra emission. The aim of this study was to find the potential risks that Fe_2O_3NPs pose to human organs and cells for cancer treatment.

Key words: Ferric oxide, tubulin, microtubule, protein interaction, nanoparticle.

INTRODUCTION

Ferric oxide nanoparticles (Fe_2O_3NPs) are used in numerous manufactured products, including cosmetics, sunscreen, toothpaste and medicines. However, there is insufficient knowledge about the potential risks they posses (Baalousha et al., 2008). The effect of nanoparticles (NPs) on the human body is increasing concern as these particles are found in an ever-expanding variety of goods. Humans are exposed to NPs through inhalation, ingestion, dermal contact and injection (Baalousha, 2008; Frazer, 2001) . The small size of NPs facilitates their uptake into cells as well as transcytosis across epithelial cells into blood and lymph circulation to reach sensitive target sites where they then persist (Lomer et al., 2004). Previous studies have observed the translocation of NPs along axons and dendrites of neurons as well as access to the central nervous system and ganglia (Stearns et al., 2001). Therefore, the study of the potential risks presented by NPs is immediately needed.

The lungs are continuously exposed to environmental particles (Rutishauser et al., 2007). Several studies have reported phagocytosis of particles by lung epithelial cells (Stearns et al., 2001). Fe_2O_3NPs do not clear from the cells; rather, they persist there, and their concentration is increases. Analytical transmission electron microscopy of different cell culture types showed single Fe_2O_3 particles, small aggregates free and membrane-bound layer aggregates in cytoplasm after exposure to Fe_2O_3 NPs. In another study, Fe_2O_3 (20 - 30 nm) was detected as free single particles in cytoplasm. Exposure cells to Fe_2O_3NPs leads to their accumulation in different organs where

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there are some vital proteins, such as microtubule, that can interact with them (Oberdorster et al., 2004). Although many studies have been done on Fe_2O_3 toxicity in animal models and cell cultures (Oberdorster, 2004; Muhlfeld, 2007), there is little data on the interaction of Fe_2O_3NPs with sub cellular structures.

MTs are cytoskeletal proteins that are essential in eukaryotic cells for a variety of functions, such as cellular transport, cell motility and mitosis. They are crucial in the development and maintenance of cell shape, the transport of vesicles, organelles and other components throughout cells, cell signaling, cell division and mitosis. MTs in neurons are used to transport substances such as neurotransmitters. MTs are formed from polymers of tubulin heterodimers (α- and β-tubulin) that polymerize from end to end (Lowe, 2001; Desai, 1997). Agents that interfere with MTs assembly also interfere with their dynamics and function, and inhibit all MTs functions such as cell division and neurotransmitter transportation (Downing, 2000; Jordan, 2004). Evidence of oxidative stress responses after NPs endocytosis indicates that research on possible cellular interactions between such particles and vital proteins, such as microtubules (MTs), is urgently needed. By studying NPs' biological and toxicological effects, its interactions with sub cellular structures, such as MTs, may be clarified.

To investigate the effects of Fe_2O_3NPs on intracellular interactions, we studied the effects of Fe_2O_3NPs on tubulin organization and MTs protein function by ultraviolet (UV) spectrophotometer and fluorescent spectroscopy. To understand the mechanism of these reactions, repolymerization tests were carried out. Fe_2O_3NPs were added after tubulin polymerization to discover whether Fe2O3NPs disrupted the structure of MTs. Fluorescent spectroscopy was used to study conformational changes in tubulin that altered its function.

MATERIALS AND METHODS

Materials

Ultra fine Fe_2O_3, with particles averaging 100 nm, was a gift from Dr. mohhamadi (engineering faculty, University of Tehran, Tehran, Iran). The particles were suspended in piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) buffer (Merck, Darmstadt, Germany) to obtain an 8-mg/ml final concentration. Before use, KOH was added to adjust the pH of the colloidal solution to 6.9. Particles were then sonicated with a Bandelin sonicator (Bandelin, Berlin, Germany) for 3 min and immediately added to the protein solution. EGTA, guanosine-5′-triphosphate (GTP), ATP, glycerol and MgSO4 were acquired from Sigma (Dorset, England). Phosphocellulose P11 was obtained from Whatman (Florham Park, USA). All other chemicals (NaCl, KOH and ANS, Merck) were from analytical grade and used without further purification. All solutions were prepared with double distilled water and were kept at 4°C before use.

Purification of tubulin

After homogenization in PEM buffer (100 mM PIPES, pH 6.9, 1 mM EGTA, 2 mM MgSO4) and 1 mM MgATP, followed by two cycles of temperature dependent assembly and disassembly, MTs proteins were prepared from sheep brains. PMG (100 mM PIPES, pH 6.9, 2 mM MgSO4, 1 mM EGTA and 3.4 M glycerol) was used as polymerization buffer. Microtubule-associated proteins tubulin was prepared by chromatography on phosphocellulose P11 with a slight modification of the method used by Weingarten et al. (1974) . Eluted tubulin fractions were stored at 70°C for further study. The protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, USA) with bovine serum albumin as standard (Marshal and William, 1993).

UV spectroscopy

Turbidimetric assay of MTs and tubulin was carried out by incubating the protein in PIPES buffer (with the final concentration of 2 mg/ml) in cuvettes at 37°C in a thermostatically controlled UV spectrophotometer (Varian, Melbourne, Australia). Turbidity change was measured at 350 nm. To examine the effect of NPs on polymerization, the MTS and tubulin proteins were pre-incubated with NPs at 4°C for 30 min, and polymerization was initiated with the addition of 1 mM GTP. The mixture was warmed to 37°C.

Fluorescence spectroscopy

All fluorescence experiments were carried out using a Varian eclipse spectrofluorometer equipped with a computer to add and subtract spectra. Denaturation of tubulin was measured as tryptophan emission after excitation at 295 nm. Interactions in the presence of increasing concentrations of Fe_2O_3NPs were carried out at 25°C. To test conformational changes, 8-anilino-1-naphthalenesulfonic acid (ANS) was used to detect whether the Fe_2O_3-treated tubulin had an exposed hydrophobic surface area. The excitation wavelength was 380 nm, and emission was monitored between 450 - 550 nm. All measurements used 2 µM tubulin and all experiments were carried out at 25°C.

Electronic microscopy

Exposure of tubulin samples to 37°C for MTs polymerization was studied by electronic Microscopy. Tubulin polymerization was occurring in suitable temperature as a control. Inhibitory effect of Fe_2O_3 on MTs polymerization process was concentration dependent manner.

RESULTS

Inhibition of MTs polymerization by Fe_2O_3NPs

The effect of Fe_2O_3NPs on tubulin polymerization was measured as shown in Figure 1. MTs assembly was clearly inhibited by different concentrations of Fe_2O_3NPs in 2 mg/ml, MTs solution comparison with control sample. Specifically, Fe_2O_3NPs inhibited both the rate and extent of MTs assembly, and it had influence on the rate of MTs nucleation by increasing the time.

MTs repolymerization assay

MTs organization (assembled and disassembled) was

Figure 1. Structure of γ-Nano Fe2O3 powder, 99% magnetic. APS: 20 - 30 nm, SSA: >50 m2/g - Color: red brown, Morphology: nearly spherical, Bulk Density: 1.20 g/cm3, True density: 5.24 g/cm3,
APS = Average Particle Size, SSA = Special surface area.

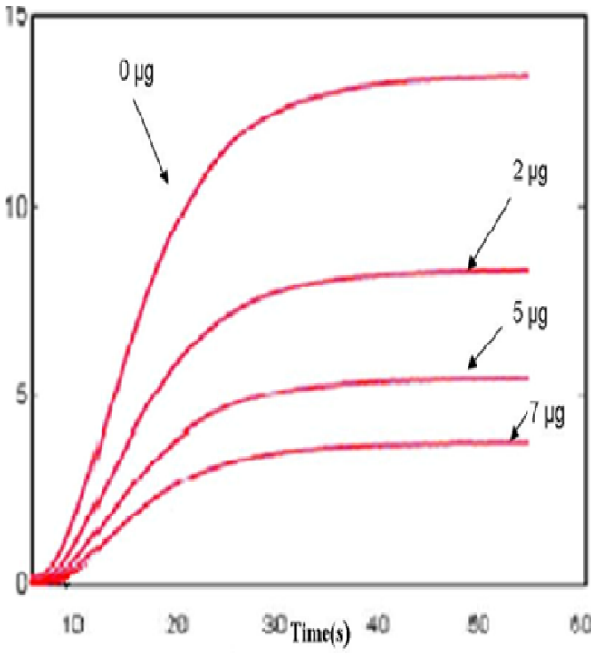

Figure 2. Effect of Fe_2O_3 on microtubule organization. Proteins (2 mg/ml) were preincubated at 4°C in piperazine-1,4-bis (2- ethanesulfonic acid) buffer with different concentration of Fe_2O_3. Polymerization was initiated by adding 1 mM guanosine-5′-triphosphate. The turbidity was monitored at 350 nm at 37°C. 0 µg/ml Fe_2O_3; 2 µg/ml Fe_2O_3; 5 µg/ml Fe_2O_3; 7 µg/ml Fe_2O_3.

cooling to 4°C, re-warming the solution to 37°C which induced assembly, as a control. Repolymerization assays showed that polymerized MTs made depolymers again after 30 min incubation in 4°C (Figure 2).

Effect of Fe_2O_3NPs on MTs dynamics at steady state

By adding Fe_2O_3 to a MTs solution at equilibrium point (Figure 3, vertical arrow), a decrease in turbidity was observed and the reaction reached a new equilibrium. The new equilibrium was the same as the equilibrium observed for MTs treated with Fe_2O_3NPs at zero time (Figure 1).

Intrinsic fluorescence spectra

To obtain structural information at the tertiary level, intrinsic (tryptophan) fluorescence spectrum of tubulin in the presence of different concentrations of Fe_2O_3NPs was measured. Fluorescence analysis indicated that the interaction of Fe_2O_3NPs with tubulin resulted in fluorescence quenching of surface-exposed tryptophans in tubulin. Figure 4 shows that the fluorescence intensity decreases with increasing amount of Fe_2O_3NPs.

Increasing of tubulin-bis-ANS fluorescence by Fe_2O_3NPs

There are several low affinity sites and one high affinity site for the polar molecule ANS in tubulin. Tubulin-ANS complex has a strong fluorescence and is extremely environmentally sensitive. Therefore, it is a useful tool for probing the conformational state of the tubulin dimer. Tubulin-ANS fluorescence has been used to determine the nature of interactions. Tubulin (2 µM) was incubated in the presence of various concentrations of Fe_2O_3NPs for 10 min at 4°C. ANS (50 µM final concentration) was added to the tubulin Fe_2O_3NPs solution and incubated again for 7 min. Figure 5 show that Fe_2O_3NPs made a concentration-dependent increase in tubulin- ANS fluorescence. Furthermore, incubation of tubulin with ANS before the addition of Fe_2O_3 generated similar results (data not shown).

Effect of Fe_2O_3 on MTs organization

In agreement the behavior of Fe_2O_3 on MTs organization will down electronics microscopy experiments. Exposure of tubulin samples to 37°C for MTs polymerization was inhibited by Fe_2O_3. Tubulin assembling occurs in suitable temperature as a control. Inhibitory effect of Fe_2O_3 on MTs polymerization process was concentration dependent manner (Figure 5).

Discussion

Although Fe_2O_3NPs are widely used in various commercial products, there is insufficient knowledge about their side effects (Brannon-Peppas, 2004; Brunner,

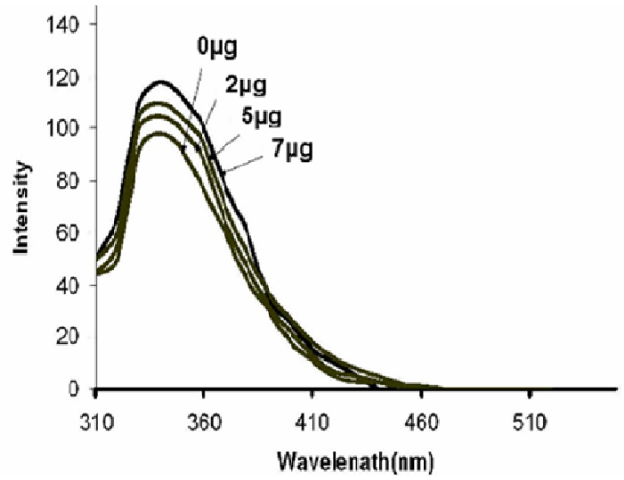

Figure 3. Emission spectra (excited at 295 nm) of 2 µM tubulin in the presence of increasing concentrations of Fe2O3. The solution conditions were 1 M piperazine-1,4-bis (2-ethanesulfonic acid), pH 6.9, containing 1 mM EGTA and 2 mM MgSO4.The excitation and emission band passes were 5 nm. 1) 0 µg/ml Fe_2O_3; 2 µg/ml Fe_2O_3; 5 µg/ml Fe_2O_3; 7 µg/ml Fe_2O_3.

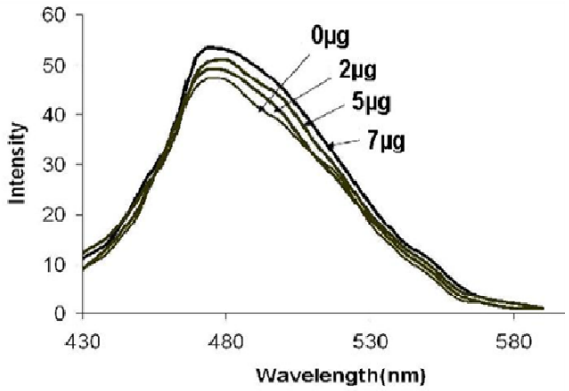

Figure 4. Emission spectra (excited at 380 nm) of 2 µM tubulin in the presence of increasing concentrations of Fe_2O_3.
Tubulin was mixed with 0 µg/ml Fe_2O_3 (curve 1), 2 µg/ml Fe_2O_3 (curve 2), 5 µ g/ml Fe_2O_3 (curve 3) and 7 µg/ml Fe_2O_3 (curve 4) for 10 min at 4°C.The 8-anilino- 1-naphthalenesulfonic acid (50 µM final concentration) was added and after 7 min fluorescence was measured. The solution conditions were 1 M piperazine-1,4-bis (2-ethanesulfonic acid), pH 6.9, containing 1 mM EGTA and 2 mM MgSO4. The excitation and emission band passes were 5 nm.

2006). Studies have shown that some neurons exposed to Fe_2O_3 initiate a cellular process that can ultimately lead to cell death, and according to Brannon et al, ultrafine Fe_2O_3 induces apoptosis (Brannon et al., 2004). There are few reports about the cytotoxic and genotoxic effects of Fe_2O_3NPs. Some studies have shown an interaction between Fe_2O_3NPs and some proteins, such as human plasma fibrinogen, but the toxic effect of Fe_2O_3NPs on MTs protein has not yet been elucidate (Fortina, 2004; Ferrari, 2005).

In this study, 0.50 µg/ml Fe_2O_3NPs was used. We showed that Fe_2O_3NPs inhibited tubulin polymerization and decrease MTs nucleation rate. The inhibition of tubulin polymerization depended on the concentration of Fe_2O_3NPs (Figure 1). Assembled MTs were disassembled by cooling and then reassembled by re-warming; repolymerization was observed (Figure. 2). These results show that Fe_2O_3NPs inhibited MTs repolymerization. Also, Fe_2O_3 did not induce formation of MTs aggregate even at high concentrations, while some agents at high concentrations induced irregular aggregates of MTs at that cooling state and did not appear to depolymerize these aggregates (Valiron and Caudron, 2001).

Fe_2O_3NPs decreased tubulin polymerization, and data showed that Fe_2O_3NPs also induce MTs depolymerization and change the MTs steady state equilibrium to a new equilibrium (Figure 3). Results show approximately 35 mg/ml IC_{50} for Fe_2O_3NPs. In the presence of 20 mg/ml Fe_2O_3NPs, the normal activity was 66%, and in 50 mg/ml Fe_2O_3NPs, activity decreased to 33%. Results indicated that Fe_2O_3 affected both soluble tubulin and tubulin in MTs structure, suggesting that tubulin conformational change led to decreased tubulin polymerization ability.

Using intrinsic fluorescent spectroscopy, we identified changes in protein conformation involved in protein function alteration. We used excitation wavelength at 295 nm so changing in emission wavelength showed tryptophan environment changes (Bhattacharya et al., 1996). Fe_2O_3NPs modified the polarity in the vicinity of tryptophan residues, and we therefore observed fluorescence quenching and the maximum blue shift of the emission wavelength (Figure 4). Fluorescence experiments with ANS demonstrated that Fe_2O_3NPs induce increases in fluorescence emission (Figure 5). The tubulin ANS complex's increase in fluorescence may result from exposing some of intrinstic tubulin's hydro-phobic pockets for ANS binding (Sarkar et al., 1995). Alternatively, binding may induce a conformational change in tubulin leading to increased ANS binding or tubulin-ANS fluorescence.

These results indicated that Fe_2O_3NPs induce conformational changes in tubulin that cause changes in tryptophan position, moving them towards GTP binding sites in protein structures. GTP has fluorescent quenching ability (Solomaha and Palfrey, 2005), so intrinsic fluorescence was decreased and blue shift was observed. Conformational changes in protein allowed some hydrophobic pockets to be reached, increasing tubulin-ANS fluorescence. Both GTP and its binding site in tubulin have a crucial role in tubulin polymerization. GTP should be hydrolyzed to guanosine-5′-diphosphate when tubulin wants to polymerize to MTs form (Desai and Mitchison, 1997). Fluorescence data showed conformational changes in

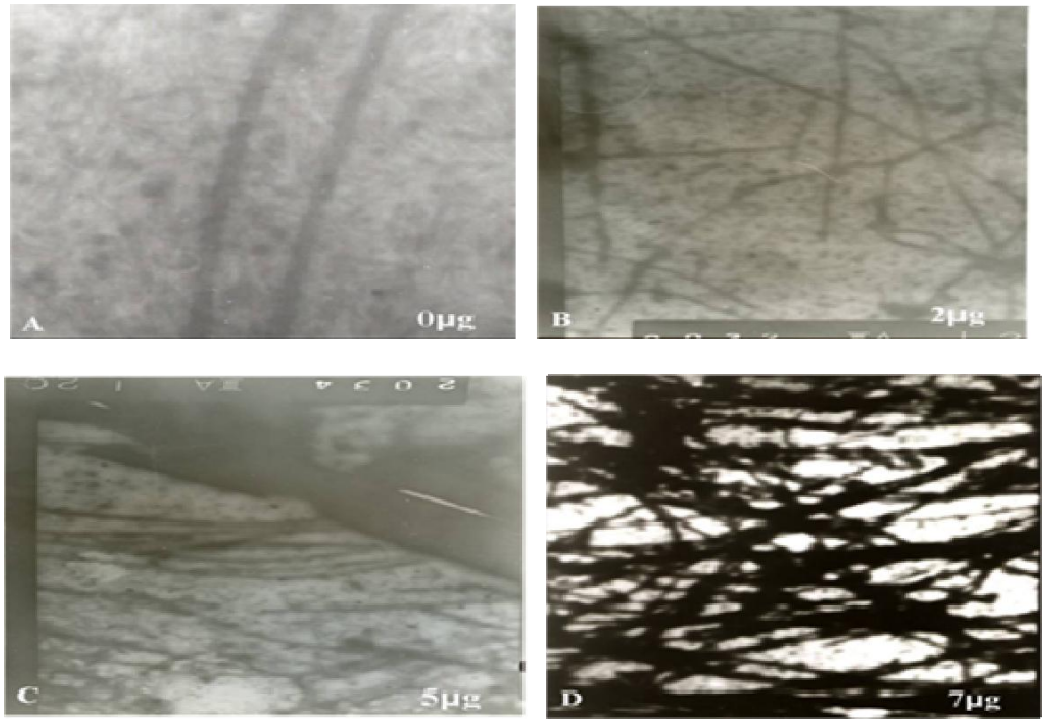

Figure 5. Effect of Fe2O3 on MTs organization.
Exposure of Tubulin samples to 37°C for MTs polymerization was studied by electronic Microscopy. (A) Tubulin assembling occurs in suitable temperature as a control. Inhibitory effect of Fe2O3 on MTs polymerization process was concentration dependent manner. Tubulin was mixed with 2 µg/ml Fe_2O_3 (B) 5 µg/ml Fe_2O_3 (C) 7 µg/ml Fe_2O_3 and (D) for 10 min.

GTP binding sites, and turbidimetric assays demonstrated that these changes result in the suppression of tubulin polymerization (Sparreboom et al., 2005).

In conclusion, our research has shown that Fe_2O_3NPs have an inhibitory effect on tubulin polymerization. Experiments demonstrated that 2 mg/ml tubulin protein activity fell to 50% in the presence of 35 µg/ml Fe_2O_3NPs. Furthermore, the same concentrations of Fe_2O_3NPs that disrupted MTs performance inhibited tubulin polymerization. Fe_2O_3NPs interact with both tubulin and MTs protein, which change their folding and results in function alteration. The GTP binding site in tubulin is also affected by Fe_2O_3NPs, leading to tubulin function changes. Finally In this study we find the potential risks that Fe_2O_3NPs pose to human organs and cells for cancer treatment. Ultimately, long-term exposure to NPs can be dangerous, and companies should further research on the hazards associated with NPs.

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