Engineered cobalt oxide nanoparticles readily enter cells

Elena Papis\textsuperscript{a}, Federica Rossi\textsuperscript{a}, Mario Raspanti\textsuperscript{b}, Isabella Dalle-Donne\textsuperscript{c}, Graziano Colombo\textsuperscript{c}, Aldo Milzani\textsuperscript{c}, Giovanni Bernardini\textsuperscript{a,d}, Rosalba Gornati\textsuperscript{a,∗}

\textsuperscript{a}Department of Biotechnology and Molecular Science, Insubria University, Varese, Italy
\textsuperscript{b}Department of Human Morphology, Insubria University, Varese, Italy
\textsuperscript{c}Department of Biology, University of Milan, Milano, Italy
\textsuperscript{d}Centro di Ricerca Interuniversitario Politecnico di Milano e Università dell’Insubria “The Protein Factory”, Italy

A R T I C L E   I N F O

Article history:
Received 6 May 2009
Received in revised form 4 June 2009
Accepted 7 June 2009
Available online 16 June 2009

Keywords:
Magnetic nanoparticles
Cobalt
Nanotoxicity

A B S T R A C T

Magnetic nanoparticles (NPs) have great potential for applications not only as catalysts or energy storage devices, but also in biomedicine, as contrast enhancement agents for magnetic resonance imaging, or for drug delivery. The same characteristics that make cobalt-based NPs so attractive raise serious questions about their safety. In this context, we investigated Co\textsubscript{3}O\textsubscript{4}-NPs. Believing that the characterization of NPs is relevant for understanding their biological activity, we analyzed them by atomic force and electron microscopy to define size, shape, and aggregation. To clarify whether their biological effects could be due to a potential release of cobalt ions, we evaluated spontaneous dissolution in different media. To determine their potential toxicity to human cells, we measured cell viability and ROS formation in two human cell lines using CoCl\textsubscript{2} for comparison. Co\textsubscript{3}O\textsubscript{4}-NPs induced a concentration- and time-dependent impairment of cellular viability, although cobalt ions were more toxic. We also demonstrated that cobalt causes a rapid induction of ROS if supplied in the form of Co\textsubscript{3}O\textsubscript{4}-NPs rather than as ions. Moreover, we evaluated the cellular uptake of NPs. Interestingly, Co\textsubscript{3}O\textsubscript{4}-NPs are able to enter the cell very rapidly, remaining confined in vesicles inside the cytoplasm. They were found also inside the cell nuclei, though less frequently.

© 2009 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

In the last decade we witnessed a rapid growth in nanotechnology and as a result nanomaterials, in general, and nanoparticles (NPs), in particular, are being incorporated in several aspects of our lives (Mazzola, 2003; Paull et al., 2003). Engineered nanoparticles are already used in sporting goods, tires, sunscreens, cosmetics, and electronics and in the promising field of medical biotechnology (Nel et al., 2006). As a consequence of this widespread use, nanoparticles are likely to be ubiquitously present in the environment and possibly enter the human body by ingestion, inhalation, and skin adsorption (Ober dorster et al., 2005). In consideration of possible uses for biomedical applications, however, we should add to these classical routes, at least for metal NPs, intravenous injection.

Metal NPs exhibit unique properties in terms of optical, magnetic and electrical activity (Mandal et al., 2005). In particular, iron, nickel, and cobalt NPs are destined to find their place in medical biotechnology because of their magnetic properties. Magnetic NPs, in fact, have been proposed for drug delivery and hyperthermic cancer treatment (Pankhurst et al., 2003). Furthermore, they are emerging as a class of novel contrast agents for medical imaging (LaConte et al., 2005). In particular, when used for magnetic resonance imaging (MRI), magnetic NPs are very efficient as relaxation promoters, enhancing tissue contrast and helping to form sharper images of the area of interest (Ito et al., 2005). Iron oxide NPs are the most widely used contrast agent in MRI (Kim et al., 2001), but more recently cobalt NPs have been suggested as an alternative to iron due to their greater effects on proton relaxation (Parkes et al., 2008).

Moreover, cobalt-based NPs, in general, and cobalt oxide NPs (Co\textsubscript{3}O\textsubscript{4}-NPs), in particular, are currently attracting enormous interest owing to their unique size- and shape-dependent properties and potential applications in, for example, pigments, catalysis, sensors, electrochemistry, magnetism, energy storage, etc. (Liu et al., 2005). For example, ordering assemblies of Co\textsubscript{3}O\textsubscript{4}-NPs along multiwalled carbon nanotubes have been proposed for fabricating high-performance electronic devices (Fu et al., 2005), while Au–Co\textsubscript{3}O\textsubscript{4} composite films were proposed as highly sensitive CO and H\textsubscript{2} sensors (Ando et al., 1997).

Considering, on the one hand, the future developments of these cobalt-based NPs and, on the other, the generally claimed adverse effects of nanomaterials, accurate studies regarding the possible interactions of NPs with cells or tissues and the consequences of...
these interactions are desirable. Moreover, it is evident that NP biocompatibility is crucial for any clinical application.

Therefore, in the present paper, we have focused our interest on the study of Co3O4-NPs. Believing that the characterization of the physical–chemical properties of NP is relevant for the study of their biological activity, we analyzed Co3O4-NP morphology by atomic force and electron microscopy in order to define aggregation, size, shape, and surface texture. These properties may have a significant influence on their biological effects. Surface area rather than mass accounts for the fact that nanoparticles can interact only by contact of their surface. Then, the surface area should be a better alternative to mass or number as a measure of dose, although, surface area per se may be an incomplete reflection of the ‘biologically active’ surface area which may vary from one circumstance to another. On the other hand, several reports agree that mass remains a substantially better metric than surface area (Pauluhn, 2009). At the moment, the issues regarding the most appropriate unifying metric of dose are still unresolved, and there are no sufficient evidences to preferentially select one exposure metric over another (Maynard, 2007; Wittmaack, 2007); therefore, in this paper, we have chosen to use the most conventional mass as exposure metric.

Moreover, to clarify whether Co3O4-NPs release cobalt ions once they are in solution, we measured the spontaneous dissolution of these NPs in different media. In addition, since one of the aims of the present work was to provide a general evaluation of Co3O4-NPs toxicity on human cells, we measured cellular viability and oxidative stress induction in endothelial-like cells (EVC-304) and hepatoma cells (HepG2). We employed the EVC-304 cell line as a model for endothelial cells, notwithstanding some indication of a genetic similarity with a human cancer cell line (Brown et al., 2000). However, evidence has accumulated that EVC-304 cells are a valuable biomedical research tool and can be used to study endothelial-related processes (Halleli et al., 2007, 2008). HepG2 cells, in contrast, are commonly used as a surrogate for human hepatocytes and have already found application in the study of nanoparticle cytotoxicity (Jan et al., 2008). We also employed transmission electron microscopy to evaluate the cellular uptake of NPs with the aim to obtain new insights regarding the mechanisms of entry and distribution into the cellular environment.

2. Materials and methods

2.1. Nanoparticle characterization

Nanoparticle shape and morphology were investigated by TEM, SEM, and AFM. TEM characterization was performed on a Jeol 1010 electron microscope (Jeol, Tokyo, Japan) operated at 90 kV. Co3O4-NPs (Sigma–Aldrich) were examined after depositing powder onto formvar/carbon-coated TEM grids.

For SEM analysis, Co3O4-NPs were deposited on cover glasses spread with Sylgard (Dow Corning). Specimens were then gold-coated in an Emitech K225 sputter-coater and observed on a Philips (Eindhoven, The Netherlands) XL30 field-emission scanning electron microscope (FESEM) operated at 7 kV or 10 kV. Images were obtained in digital form as R-heap grayscale TIFF files.

For AFM analysis, Co3O4-NPs were spun down at 300,000 × g in a centrifuge tube (Ultra-Clear™, Beckman Coulter). Tubes were then cut into pieces which were mounted onto the AFM stub. Specimens were observed with a Nanoscope IIIa controller fitted with Nanosensors TESP silicon probes (C = 40 Nm⁻¹ and F = 300 KHz) and operated in tapping-mode atomic force microscopy (TMAFM). Observations were carried out in air at a scan rate of ≈1.5 Hz.

2.2. Atomic absorption spectroscopy (AAS)

AAS was used to determine the spontaneous dissolution of Co3O4-NPs in different media. Co3O4-NPs received in a dry powder form were suspended in deionized water at a concentration of 1.6 mg/ml and then sonicated for 15 min in a water bath sonicator to aid their dispersion. This stock solution was then diluted to a final working concentration of 120 µg/ml in deionized water, PBS, or cell culture media. Samples were kept at 37 °C for 72 h in the presence or absence of a 5% CO2 atmosphere. After the incubation period, samples were ultracentrifuged at 300,000 × g for 2 h at 4 °C to separate cobalt nanoparticles from ions, and the supernatants were collected and filtered using a 0.22 µm pore size membrane. Prior to the analysis, 1% HNO3 and 10% H2O2 were added to each sample.

The analysis was performed using a graphite furnace atomic absorption spectrometer (SOLAR AR M6, Thermo Electron Corporation). The pretreatment and atomization temperatures recommended by the manufacturer were used in the furnace programmer to ensure that standard linear curves were obtained. Calibration was performed using 0, 1, 4, 12, and 24 µg/L as calibration points in triplicate. The sample solutions were introduced into the AAS and cobalt concentrations were calculated using SolarA AA System 1013 software. Three readings were recorded for each sample and three experiments were performed for each medium tested. The obtained values were tabulated and the differences between the means in the groups were compared using ANOVA, followed by a post hoc comparison (Scheffe’s test).

2.3. Cell culture

HepG2 and EVC-304 cell lines were maintained as adherent cell lines in MEM and Medium 199, respectively, at 37 °C in a humidified 5% CO2 atmosphere. Both media were supplemented with 10% fetal bovine serum and l-glutamine (4 mM for HepG2 and 1 mM for EVC-304). Cells were passaged as needed using 0.5% trypsin-EDTA. All reagents were obtained from Sigma–Aldrich.

2.4. Cell viability

Cell viability was determined by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega Italia, Milan, Italy) according to the manufacturer’s instructions. Briefly, 200 µl of cell suspension at a concentration of 2 × 104 (HepG2) and 104 cells/ml (EVC-304) were seeded into 96-well assay plates and cultivated for 24 h at 37 °C in 5% CO2 to equilibrate and become attached prior to the treatment. Then, cells were exposed to increasing concentrations of CoCl2 and Co3O4-NPs for 24, 48, and 72 h. CoCl2 and Co3O4-NP solutions were prepared as reported in Section 2.2. Following the treatment, assay plates were equilibrated at room temperature and 100 µl of CellTiter-Glo reagent was added to each well. Plates were shaken for 2 min and left at room temperature for 15 min prior to recording the luminescent signals. Luminescence was determined using the Infinite F200 (Tecan Group, Switzerland) plate reader. For both cell lines, three experiments were carried out for each exposure time and three wells per concentration were used in each experiment. Relative cell viability, normalized against control values, was taken for the analysis.

2.5. Nanoparticle uptake

To determine NP cellular uptake and localization, HepG2 and EVC-304 cells were exposed to Co3O4-NPs for 30 min and 3, 24, and 72 h and analyzed by electron microscopy (TEM and SEM). For TEM studies, exposed cells were harvested, fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 2 h at room temperature, washed in the same buffer, and postfixed for 1 h with 1% osmium tetroxide in 0.1 M Na-cacodylate buffer (pH 7.2) at room temperature. After standard steps of serial ethanol dehydration, samples were embedded in an Epon-Araldite 812 mixture. Thin sections (80 nm) were obtained with a Reichert Ultracut S Ultratome (Leica, Nussloch, Germany), stained by standard methods with uranyl acetate and lead citrate, and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan) operated at 90 kV.

For SEM and X-ray spectroscopy, cells, fixed as above, were dehydrated in graded ethanol and in hexamethyldisilazane (Sigma Aldrich), gold-coated in an Emitech K225 apparatus, and observed on a Philips (Eindhoven, The Netherlands) XL30 FESEM fitted with probes for secondary and back-scattered electrons and connected to an EDAX Sirion 200/400 dispersive X-ray spectroscopy apparatus.

2.6. ROS production

The oxidation of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to estimate the production of reactive oxygen species (ROS) in EVC-304 and HepG2 cells after exposure to CoCl2 and Co3O4-NP. Hydrogen peroxide (10 mM) was used as a positive control for the assay.

We seeded 104 cells/well into 96-well assay plates and cultivated them for 24 h at 37 °C in 5% CO2 atmosphere to equilibrate and become attached prior to the treatment. Then, cells were washed with PBS and incubated with 10 µM DCFH-DA for 30 min at 37 °C. To remove non-incorporated DCFH-DA, cells were washed with PBS and then incubated with CoCl2 (50–400 µg/ml) or Co3O4-NPs (20–120 µg/ml) for 1 h in PBS. The fluorescence intensity of DCF was recorded at λ emission = 485 (band width 20 nm) and at λ excitation = 535 (band width 25 nm) using the Infinite F200 (Tecan Group, Switzerland) plate reader. The data were normalized and expressed as fold increase in fluorescence intensity with respect to the controls (cells not treated).

3. Results

3.1. Nanoparticle characterization and dissolution

The morphological characterization of Co3O4-NPs was assessed by AFM and EM. Representative TEM and SEM images of these
NPs are shown in Fig. 1 (panels a and b, respectively). EM analysis revealed that Co3O4-NPs exhibit a spherical structure. Consistent with TEM, AFM images showed round particles of approximately 45 nm in size (Fig. 1, panel c). Due to their magnetic properties, Co3O4-NPs tend to form agglomerates of different shape and size as shown in Fig. 1b.

Stability of Co3O4-NPs was assessed in terms of quantity of cobalt released into the solution. To quantify the cobalt, graphite furnace-AAS was used. Metal concentrations in the ppb range can be measured using this technique. Cobalt dissolution, i.e., release of cobalt by Co3O4-NPs, was assessed in different media, i.e., PBS, distilled H2O, culture medium with or without cells and under 5% CO2 atmosphere or not. The pristine quantity of Co3O4-NPs (120 mg/L) used for sample preparation was equivalent to an amount of 88.1 ppm (mg/L) of cobalt. After 72 h all the samples were processed as reported in Section 2.2 to remove the Co3O4-NPs. Our results showed that the release of cobalt ions depended on the medium (Fig. 2) and ranged from the lowest value of 4 ppb (μg/L) to the highest value of 780 ppb (μg/L). Therefore, NP dissolution was very low and remained under 1% in all the samples tested. Moreover, the presence of a layer of cells did not seem to influence NP dissolution since no statistically significant differences between media with or without cells were observed.

3.2. Cytotoxic effects of Co3O4-NPs and CoCl2

Co3O4-NP cytotoxicity on ECV-304 and HepG2 cells was evaluated by measuring cellular ATP content after exposure for 24, 48, and 72 h in culture medium.

To compare the effects of NPs to cobalt ions, CoCl2 was also assayed. As reported in Fig. 3, a dose- and time-dependent reduction in cell viability was observed for both cobalt forms, although CoCl2 showed a higher cytotoxic effect than the NPs. Moreover, hepatoma cells were less sensitive to cobalt exposure than ECV-304 cells.

3.3. Nanoparticle uptake

When ECV-304 and HepG2 cells were exposed to Co3O4-NPs, a massive internalization of particles was observed. Thin-section TEM images showed that Co3O4-NPs were taken up very readily (Fig. 4). After 30 min of incubation, agglomerates of Co3O4-NPs were already inside the cells, while others were about to enter, as shown in panels b and d of Fig. 4. The total amount of NPs within the cells increases with the length of exposure time (Figs. 4 and 5).

Although the mechanism of uptake was not investigated, it is more likely that the NPs were internalized in an agglomerated form than taken up as individual particles. As shown in Figs. 4a and 5, objects with high electron density were mostly stored in vesicular structures, which may also contain amorphous cellular material.
These objects were identified as Co$_3$O$_4$-NPs since particles maintained the characteristic morphology observed in the cell-free environment. Moreover, although less frequently, Co$_3$O$_4$-NPs were also found very readily within cell nuclei. Interestingly, some of these nuclear agglomerates consisted of NPs lined up as a chain (Fig. 4c).

TEM observations were further supported by SEM analysis. In particular, important information was obtained by using back-scattered electrons (BSE) and energy dispersive X-rays (EDX). BSE are often used in analytical SEM along with the spectra derived from the characteristic X-rays to identify the composition and the distribution of elements in the sample. Fig. 6 shows a SEM micrograph of a cell with a large internal content of electro dense materials (bright spots obtained with BSE electrons). X-ray spectroscopy of cells exposed to Co$_3$O$_4$-NPs indicates that cobalt was the main component of this hard material (Fig. 7).

![Fig. 3. ECV-304 and HepG2 viability after exposure to Co$_3$O$_4$-NPs (b and d respectively) and CoCl$_2$ (a and c respectively) for 24, 48, and 72 h. Viability values were normalized against control samples and plotted against cobalt content ($\mu$g/ml) of Co$_3$O$_4$-NP and CoCl$_2$ test solutions.]

![Fig. 4. TEM images of ECV-304 (a–c) and HepG2 (d) cells exposed to Co$_3$O$_4$-NPs for 24 h (a) and 30 min (b–d). Interestingly, Co$_3$O$_4$-NPs are taken up very readily and NPs are present inside cytoplasmic vesicles and cell nuclei.]

3.4. ROS production

Co$_3$O$_4$-NPs were investigated for their ability to induce ROS formation in ECV-304 and HepG2 cells. Prior to ROS detection, cell viability was assessed as reported in Section 2.4. Co$_3$O$_4$-NPs did not seriously impair viability since the percentages of living cells were above 90% for all the NPs concentrations tested (Fig. 8).

One-hour exposure of DCFH-DA-preincubated cells to Co$_3$O$_4$-NPs resulted in a dose-dependent increase in ROS production in both cell lines, although hepatoma cells were less sensitive. As shown in Fig. 8, ROS levels in treated samples were up to an order of magnitude higher than in controls. To compare the NP-derived effects with a corresponding bulk material, the same amount of cobalt in an ionic form (supplied as CoCl$_2$) was used. In the case of CoCl$_2$ no statistically significant increase in ROS formation could be observed as compared to untreated cultures, at any tested concentration.

4. Discussion

Nano- and biotechnologies are expected to generate innovative and highly promising biomedical products and to revolutionize bioengineering and clinical and diagnostic medicine. In the last decade, major breakthroughs were made in developing and using magnetic (iron, nickel, and cobalt) NPs for in vivo applications, such as drug delivery, hyperthermic cancer treatment, MRI contrast enhancement, and tissue repair. Cobalt-based NPs are also exploited for different types of technological products such as sensors, catalysts, or energy storage devices (Liu et al., 2005). The overall benefit that nanotechnology, in general, and cobalt-based nanoproducts, in particular, have to offer must be balanced with the potential health risks associated with their use. The issue of potential cobalt-NP toxicity becomes even more serious for intravenously injected nanoparticles, such as those used for MRI.

In spite of the potential environmental or biomedical exposure of humans to Co$_3$O$_4$-NPs, information regarding their potential health impact is still lacking. Moreover, although data are available about the toxicology of cobalt ions, it is generally accepted that the biological activity and potential toxicity of metallic NPs cannot simply be derived from previous data on ionic forms, indicating the importance of testing the effects of chemicals in nanostructural form (Roco, 2005). In this context, we studied Co$_3$O$_4$-NPs. In our experiments, Co$_3$O$_4$-NPs induced a concentration- and time-dependent impairment of cellular viability, although cobalt ions proved to be more cytotoxic. Our results disagree with those obtained for dermal microvascular endothelial cells (HDMEC), which showed that Co-NPs exerted a higher toxic effect than the corresponding ion (Peters et al., 2007). These contrasting results are due to a low sensitivity of HDMEC to cobalt chloride rather than a greater toxicity of Co-NPs.

Sabbioni et al. (2006) have reported that Co-NPs gradually dissolve in culture medium and that after 72 h almost half the quantity of cobalt was present in ionic form. To clarify whether Co$_3$O$_4$-NP toxicity was due to a similar release of cobalt ions within the solution, we measured the spontaneous dissolution of these NPs in culture medium. Interestingly, we found that the amount of cobalt ions released by Co$_3$O$_4$-NPs in the culture medium was very low and did not reach any effective concentrations. Nevertheless, we cannot exclude that, once inside the cells, Co$_3$O$_4$-NPs could dissolve more efficiently, acting as a reservoir of ions that in turn could cause the toxic effect. This hypothesis is further supported by the finding that Co-NPs altered the expression of the same set of genes that were modified by cobalt ion exposure (Papis et al., 2007a, 2007b).

NP binding to the plasma membrane and cellular uptake are probably a necessary prerequisite for NPs to exert their toxicity. On the other hand, this can be exploited in several biomedical
applications, such as hyperthermic treatment and gene therapy. We have shown that Co$_3$O$_4$-NPs were taken up rapidly by human cell cultures and that the amount of NPs inside the cells continued to increase with time. We have not investigated the mechanism through which Co$_3$O$_4$-NPs enter the cells, but the most probable pathway involves endocytosis of NP agglomerates. As observed for Co-NPs (Peters et al., 2007) and Fe$_2$O$_3$-NPs (Gojova et al., 2007), Co$_3$O$_4$-NPs were mainly taken up and stored in cytoplasmatic vesicles that contain NP agglomerates associated with amorphous material. This may be of relevance since the adsorption of proteins present in the culture medium on the Co$_3$O$_4$-NP surface could facilitate their cellular uptake, as already demonstrated for gold-NPs covered with albumin (Geoffroy and Becker, 1984) or LDL (Handley et al., 1983). Moreover, adsorption of protein onto the magnetic NP surface represents a fundamental topic in immunology and pharmaceutical science for designing specific drug carriers or optimizing medical devices (Berry, 2005).

Less frequently, but consistently, Co$_3$O$_4$-NPs were found inside the cell nuclei, usually arranged as a chain, as they probably had to line up in a row to pass through the nuclear pores. In fact, since nuclear pores are known to contain a large channel with a diameter close to 50 nm (Peters, 2009), Co$_3$O$_4$-NP passage via this route is feasible. Moreover, SiO$_2$-NPs, with a size between 40 and 70 nm, were shown to enter cell nuclei and to influence nuclear structure and function (Chen and von Mikecz, 2005). Alternatively, NP agglomerates could enter cell nuclei by taking advantage of the breakdown and reassembly of the nuclear envelope that occurs during cell mitosis.

In vivo and in vitro studies on manufactured NPs have indicated that there is a close link between ROS production, oxidative stress, and nanotoxicity (Nel et al., 2006; Xia et al., 2006). In agreement with this hypothesis, we have shown that Co$_3$O$_4$-NPs were able to induce a dose-dependent increase in ROS production, although the extent of this induction is considerably lower than that observed by Limbach et al. (2007) in lung epithelial cells. This, however, may be attributed to a different sensitivity of the cell lines. Other authors indirectly support the oxidative stress hypothesis of Co-NPs by evaluating their genotoxicity (Colognato et al., 2008) and pro-inflammatory effects (Peters et al., 2007).

Exposing the cells to the same amount of cobalt, but in ionic form, we did not observe an increase in ROS production. The debate as to whether cobalt ions can induce oxidative stress is still wide open. Cobalt has been reported to enhance ROS formation (Zou et al., 2001), oxidize proteins (Petit et al., 2005), and cause oxidative DNA damage (De Boeck et al., 1998), but it has also been shown to exert a neuroprotective effect on hypoxia-induced oxidative stress.
(Shrivastava et al., 2008). Although the reason for these contradictory results is unknown, it is possible that they might be caused by differences in cellular behaviors or experimental conditions (doses, exposure length, etc.). Cell membrane offers an excellent barrier for most ions and it has been shown that cobalt, in ionic form, is taken up by the cells with lower efficiency (Colognato et al., 2008). In this study, we exposed the cells to cobalt for a relatively short period of time, demonstrating that cobalt was able to cause a rapid induction of ROS if supplied in the form of Co$_3$O$_4$-NPs rather than as cobalt ions. This could be linked to the rapid and massive uptake of NPs by the cells.

In conclusion, we have seen that Co$_3$O$_4$-NPs are readily taken up by cells. This could be the basis of their toxicity, which therefore could represent a disadvantage for some applications, such as diagnosis by MRI. Toxicity, however, can probably be modulated by means of a suitable coating with tailored characteristics. Conversely, NP toxicity can be exploited for other applications, such as cancer treatment.

Conflict of interest

None.

References