**Cell uptake and intracellular fate of phospholipidic manganese-based nanoparticles**

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Manuela Costanzo[a], Lucia Scolaro[b], Gloria Berlier[c], Alessandro Marengo[b], Sabrina Grecchi[a], Carlo Zancanaro[a], Manuela Malatesta[a*], Silvia Arpicco[b*]

[*]*co-corresponding authors*

[a]Dipartimento di Neuroscienze, Biomedicina e Movimento, Sezione di Anatomia e Istologia, Università di Verona, Strada Le Grazie 8, 37134 Verona, Italy.
[b]Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Via P. Giuria 9, 10125 Torino, Italy.
[c]Dipartmento di Chimica and NIS (Nanostructured Interfaces and Surfaces) Centre, Università di Torino, Via P. Giuria 7, 10125 Torino, Italy.

*Co-corresponding authors:

Manuela Malatesta
Dipartimento di Neuroscienze, Biomedicina e Movimento, Sezione di Anatomia e Istologia, Università di Verona
Strada Le Grazie 8, 37134 Verona, Italy
Tel.: +39.045.8027157
Fax: +39.045.8027163
E-mail address: manuela.malatesta@univr.it

Silvia Arpicco
Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino
Via P. Giuria 9, 10125 Torino, Italy
Tel: +39.011.6706668
Fax: +39.011.6706663
E-mail address: silvia.arpicco@unito.it

**Abstract**

During the last decades, several studies have proposed manganese (Mn) complexes as alternative contrast agents for magnetic resonance imaging (MRI). With the nanotechnology surge in recent years, different types of Mn-based nanoparticles (Nps) have been developed. However, to design effective and safe administration procedures, preliminary studies on target cells, aimed at verify their full biocompatibility and biodegradability, are mandatory.

In this study, MnO containing-Nps encapsulated in a phospholipidic shell (PL-MnO Nps) were tested in cultured cells and flow cytometry; confocal and transmission electron microscopy were combined to understand the Nps uptake mechanism, intracellular distribution and degradation pathways, as well as possible organelle alterations.

The results demonstrated that PL-MnO Nps undergo rapid and massive cell internalization, and persist free in the cytoplasm before undergoing lysosomal degradation without being cytotoxic or inducing subcellular damage. Based on the results with this cell model *in vitro*, PL-MnO Nps thus proved to be suitably biocompatible, and may be envisaged as very promising tools for therapeutic and diagnostic applications, as drug carriers or contrast agent for MRI.

**Keywords:** manganese, nanoparticles, cell uptake, fluorescent microscopy, transmission electron microscopy.

## 1. Introduction

Manganese (Mn) is known as an essential trace element for many cellular processes; it is required for proper bone growth, reproduction, blood coagulation and hemostasis, immune function, regulation of blood sugar, protection against reactive oxygen species, and it is also necessary for normal brain and nerve function (Wedler and Denman, 1984; Patchett et al., 1991; Zwingmann et al., 2004; Miao and St Clair, 2009; Horning et al., 2015). Interestingly, $Mn^{2+}$ is paramagnetic, and Mn complexes have been proposed as contrast agents for magnetic resonance imaging (MRI) (Mendonça-Dias et al., 1983; Fornasiero et al., 1987).

MRI is a powerful diagnostic methodology in clinical medicine that produces accurate images *in vivo* (Brown and Semelka, 2010); however, its inherent low sensitivity often requires the use of contrast agents. $Mn^{2+}$ is able to reduce $T_1$ relaxation times of water, thus resulting in positive MRI contrast enhancement (Nordhoy et al., 2004; Lelyveld et al., 2011). Unfortunately, Mn complexes easily dissociate after administration with the formation of free $Mn^{2+}$, resulting in Mn poisoning (Santamaria, 2008).

With the nanotechnology surge in recent years, different types of Mn-based nanoparticles (Nps) have been developed to be used as MRI contrast agent. In particular, MnO Nps obtained by thermal decomposition of Mn-oleate complex and then encapsulated in a biocompatible shell proved to be very promising as both MRI contrast agents and drug carriers (Na et al., 2007; Shin et al., 2009; Howell et al., 2013; Lee et al., 2014).

However, to design effective and safe administration procedures of these interesting Mn-based Nps for therapeutic and diagnostic applications, preliminary studies on target cells are required. The studies are aimed at clarifying Nps uptake mechanism(s), intracellular distribution, interactions with cell organelles and intracellular persistence (with special attention to their degradation pathways). It is also necessary to investigate the possible occurrence of structural and functional alterations related to the permanence of Nps in the intracellular milieu.

In this work, we focused our attention on Mn-based Nps encapsulated in a phospholipidic shell (PL-MnO Nps), which proved to efficiently act as MRI contrast agents (Howell et al., 2013), with the aim to explore, in an *in vitro* model, their biocompatibility at short and long term, as well as their internalization kinetics and intracellular distribution and stability by combining flow cytometry, confocal and transmission electron microscopy.

## 2. Materials and methods

### *2.1. Materials*

The phospholipids were provided by Avanti Polar-Lipids (distributed by Spectra 2000, Rome, Italy). The chemicals, Trypan blue, Hoechst 33258 and PKH67 Green Fluorescent Cell Linker were obtained from Sigma-Aldrich (Milan, Italy). All the solvents used were of analytical grade, purchased from Carlo Erba Reagenti (Milan, Italy). Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-DHPE) were from Invitrogen (Life Technologies, Monza, Italy). Dulbecco Modified Eagles Medium (DMEM), fetal calf serum, glutamine, penicillin, streptomycin and trypsin were purchased from Euroclone (Milan, Italy). Osmium tetroxide, potassium ferrocyanide, Epon resin components and grids were purchased from Electron Microscopy Sciences (Società Italiana Chimici, Rome, Italy). The human autoimmune serum recognizing lysosomal proteins was a kind gift from Dr. C. Alpini (Alpini et al., 2012); the Alexa 488-conjugated anti-human IgG secondary antibody was provided by Molecular Probes (Invitrogen, Milan, Italy).

### *2.2 Preparation of MnO nanoparticles encapsulated in a phospholipidic shell (PL-MnO Nps)*

MnO nanoparticles were prepared following the methods described previously with minor modifications (Howell et al., 2013; Park et al., 2004). Initially Mn-oleate complex was prepared heating at 70°C overnight 2 g (13 mmol) of manganese sulfate and 6.1 g (20 mmol) of sodium oleate dissolved in a mixture of 7.5 ml of ethanol, 10 ml of distilled water and 17 ml of n-hexane. At the end of the reaction, the solution was washed three times with distilled water. The upper organic layer was dried over anhydrous magnesium sulphate and evaporated under reduced pressure giving the Mn-oleate complex as a red waxy solid.

To prepare MnO nanoparticles 1.24 g of the previously prepared Mn-oleate complex (2 mmol) was dissolved in 14 ml of 1-octadecene and the mixture was degassed at 70 °C for 1 h under vacuum with vigorous stirring. The solution was then heated to 300°C and maintained at this temperature for 90 min under nitrogen atmosphere. During the reaction, as the temperature reached 300°C, the red solution became transparent and then turned

to pale green. The solution was then cooled to room temperature, and 15 ml of a mixture of dichloromethane and acetone (2:4 v/v) was added and the obtained precipitate was centrifuged at 4000 rpm at 4°C for 10 min. Supernatants were eliminated and precipitation and centrifugation procedure was repeated several times.

Finally, to encapsulate MnO nanoparticles in a lipidic shell, a mixture of 2.9 mg of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 0.1 mg of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-DSPE) was dissolved in 1 ml of chloroform and added to 1.5 mg of the previously prepared MnO nanoparticles. The solvent was then evaporated and the resulting lipid film was dried under vacuum overnight, then hydrated with 2 ml of water: this suspension was vortexed and bath sonicated for 120 min. The MnO nanoparticles encapsulated in a phospholipidic shell (PL-MnO Nps) were then filtered through a 0.45 micron syringe filter.

For the *in vitro* studies, fluorescent labelled PL-MnO Nps were prepared as described previously with the addition of 0.2 mg of rhodamine-DHPE to the lipid mixture in chloroform before lipid film preparation.

*2.3 Characterization of nanoparticles*

Fourier transform infrared spectra (FTIR) were recorded on a Bruker IFS 28 spectrophotometer equipped with a DTGS detector, working with 4 $cm^{-1}$ resolution over 128 scans in the Mid IR (MIR) interval (4000-400 $cm^{-1}$). Measurements were carried out on MnO nanoparticles before encapsulation in the lipidic shell, working in ATR (Attenuated Total Reflectance) mode. A MKII Golden Gate (Specac) equipped with diamond crystal at 45° was employed for the measurements.

High Resolution Transmission Electron Microscopy (HRTEM) analyses were performed by means of a JEM 3010-UHR microscope (JEOL Ltd.) operating at 300 kV. For the measurements, MnO nanoparticles and PL-MnO Nps were dispersed on a copper grid coated with a perforated carbon film. The size distribution of the samples was elaborated by employing the software Comptage de Particules v 2.0. In the case of MnO nanoparticles a statistically representative number of particles (ca. 400 particles) was measured, and the results are indicated as mean particle diameter ± standard deviation (dm ± STD). Size measured in the case of PL-MnO Nps is not statistically relevant due to small number of objects measured.

Thermogravimetric analysis (TGA) was carried out on PL-MnO Nps on a TAQ600 (TA instruments) by heating the samples, after equilibration, from 30 to 700°C at a rate of 10°C/min. Once the target temperature was reached, an isotherm was run for 5 min in air in order to burn carbonaceous residues from pyrolysis reactions.

The mean particle size and polydispersity index of the PL-MnO Nps were determined at 20°C by quasi-elastic light scattering (QELS) using a nanosizer Coulter® N4MD (Coulter Electronics, Inc., Hialeah, FL). The selected angle was 90°, and the measurement was performed after dilution of the samples in MilliQ® water. Each measurement was carried out in triplicate. Zeta potential was determined using a Zetasizer (Zeta Potential Analyzer Ver. 2.17, Brookhaven Inst. Corp., Holtsville, NY).

The nanoparticles were also analyzed for physical stability in the storage conditions at 4°C evaluating at different interval times the diameter value.

*2.4 In vitro cell culture*

HeLa cells ($5x10^4$) were grown in DMEM supplemented with 10% (v/v) fetal calf serum, 1% (w/v) glutamine, 100 U of penicillin and 100 µg/mL streptomycin, at 37°C in a 5% $CO_2$ humidified atmosphere. Cells were trypsinized when subconfluent and seeded on glass coverslips in 6 multiwell dishes for fluorescence and transmission electron microscopy.

Two days after seeding, the initial medium was replaced with fresh medium plus fluorescent PL-MnO Nps to obtain different Mn-oleate concentrations (see below). The incubation time with PL-MnO Nps varied from 10 min to 24 h. For long-term studies, the cells were kept in the medium containing PL-MnO Nps for 24 h, and then this medium was replaced with fresh medium without nanoparticles (retrieval). After retrieval, the cells were further grown for 24, 48, and 72 h.

*2.5 Cell viability assay*

Based on previous studies (Na et al., 2007; Howell et al., 2013) three concentrations of PL-MnO Nps (0.006, 0.06 and 0.6 µg/ml) were tested in cultured cells. Cell viability was estimated at both short (1 and 2 h) and long term (24 and 48 h).

At the end of each incubation time, HeLa cells were detached by mild trypsinization (0.25% trypsin in PBS containing 0.05% EDTA) and stained in suspension for 2 min with 0.1% Trypan blue in the culture medium: cells that were permeable to Trypan blue were

considered as non-viable and their percentage was estimated by microscope counting on a Burker hemocytometer; cell samples not exposed to nanoparticles were considered as controls. Results were expressed as the mean ± standard deviation (STD) of three independent experiments.

*2.6 Flow cytometry*

For these experiments, the cells were exposed to PL-MnO Nps (0.6 µg/ml concentration) for 15 to 240 min. At each time-point, cell samples were detached by mild trypsinization, centrifuged and re-suspended in fresh complete medium at the concentration of $2.5x10^5$ cells per ml. Measurements were taken with a Partec PAS III flow cytometer using an argon laser excitation at 488 nm (power 200 mW) and a 550-600 band-pass filter for rhodamine fluorescence detection. The cells were identified based on their forward and side scatters, and at least 20,000 cells were measured for their fluorescence signal in the histogram region used for calculations. As a control for possible cell autofluorescence and thresholding, untreated HeLa cell samples were processed and measured, in parallel with the PL-MnO Np-treated cell specimens.

*2.7 Analysis of nanoparticles intracellular distribution*

At each time, HeLa cells were fixed for fluorescence microscopy with 4% (v/v) paraformaldehyde in PBS, pH 7.4 (30 min at 4°C).

The samples were stained for DNA with Hoechst 33258 (1 µg/ml in PBS for 5 min), counterstained with 0.1% Trypan blue in PBS for 2 min, rinsed in PBS, and mounted in a 1:1 mixture of glycerol:PBS. This allowed to visualize the intracellular presence of fluorescent PL-MnO Nps and verify their possible intranuclear location.

In order to investigate whether the PL-MnO Nps enter the cells via endocytosis, some cell samples were pre-incubated with PKH67 Green Fluorescent Cell Linker to stain the plasma membrane (and the resulting endocytotic vesicles) prior to being briefly (5 min) incubated with the PL-MnO Nps; the cell samples were finally fixed and processed for fluorescence microscopy as described above. This procedure allowed to detect possible co-localization of the green (endocytotic vesicles) and red (Nps) fluorescence signals.

To elucidate whether internalized PL-MnO Nps undergo degradation in lysosomes, some cell samples were immunolabelled with a human autoimmune serum recognizing

lysosomal proteins, diluted 1:500 in PBS. After 60 min incubation at room temperature, immunolabelling was revealed with an Alexa 488-conjugated anti-human IgG antibody diluted 1:200 in PBS. The DNA was counterstained with 0.1 μM Hoechst 33258 in PBS for 5 min, and cells were mounted in a 1:1 mixture of glycerol:PBS as above.

An Olympus BX51 microscope (Olympus Italia Srl, Milan, Italy) equipped with a 100W mercury lamp was used under the following conditions: 540 nm excitation filter (excf), 580 nm dichroic mirror (dm), and 620 nm barrier filter (bf) for rhodamine; 450–480 nm excf, 500 nm dm, and 515 nm bf, for Alexa 488 and PKH67; 330–385 nm excf, 400-nm dm, and 420 nm bf, for Hoechst 33258. Images were recorded with an Olympus Magnifire digital camera system (Olympus Italia Srl, Milan, Italy), and stored on a PC by the Olympus software for processing and printing.

For confocal laser scanning microscopy, a Leica TCS-SP system mounted on a Leica DMIRBE inverted microscope (Leica Microsystems Italia, Milan, Italy) was used; for fluorescence excitation, an Ar/Vis laser at 488 nm for FITC, a He/Ne laser at 543 nm for Alexa 594, and an Ar/UV laser at 364 nm for Hoechst 33258 were used. Spaced (0.5 μm) optical sections were recorded using a 63x oil immersion objective. Bright-field images were also taken. Images were collected in the 1024x1024 pixel format, stored on a magnetic mass memory and processed by the Leica confocal software.

For transmission electron microscopy, HeLa cells were exposed to rhodamine-labelled PL-MnO Nps as above, and processed for 3,3' diaminobenzidine (DAB) photo-oxidation: this is a histochemical technique based on the capability of an excited fluorophore to induce DAB oxidation into a stable electron-opaque precipitate (Maranto, 1982). Briefly, the cells were fixed with 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 1h, washed and incubated with 3,3' diaminobenzidine (DAB) (20 mg/10 ml in Tris HCl 0.05 M, pH 7.6) under irradiation with two 8W Osram Blacklite 350 lamps for 2 h at room temperature (these lamps emits with high intensity in the spectral range between 430 and 470 nm, thus being suitable for rhodamine excitation); the cells were then post-fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide at room temperature for 1 h, dehydrated with acetone and embedded in Epon. Samples processed as described above, but omitting either DAB incubation or exposure to the excitation light were used as control.

Ultrathin sections were weakly stained with 2.5% uranyl acetate in water, and observed in a Philips Morgagni transmission electron microscope (FEI Company Italia Srl, Milan, Italy) operating at 80kV and equipped with a Megaview II camera for digital image acquisition.

## 3. Results

### *3.1 Preparation of PL-MnO Nps*

Nanoparticles composed of MnO core surrounded by a hydrophobic surface layer of oleic acid were synthesized by the thermal decomposition of the previously prepared Mn-oleate complex. The purified MnO nanoparticles were dispersible in many organic solvents such as n-hexane and chloroform. In order to increase their hydrophilicity and biocompatibility, a hydrophilic shell was created by adding the MnO nanoparticles to a mixture of DSPC and mPEG-DSPE dissolved in chloroform. The suspension was evaporated under vacuum and the dry lipid film was hydrated, sonicated and filtered to give the corresponding PL-MnO Np.

### *3.2 Characterization of nanoparticles*

MnO nanoparticles were characterized by FTIR spectroscopy and HRTEM techniques. The FTIR spectrum of MnO nanoparticles is reported in Figure 1a. It is characterized by intense bands in the spectral range of C-H stretching modes ($\nu$CH), with a weaker band in the region of C-H bending ones ($\delta$CH). More in detail, bands at 2955 (shoulder), 2922 and 2852 $cm^{-1}$ are related to asymmetric $\nu CH_3$, asymmetric and symmetric $\nu CH_2$ vibrations, respectively. The weak band at 1461 $cm^{-1}$ is the corresponding $\delta CH_2$ (scissor vibration). These bands (typical of saturated hydrocarbons) confirm the presence of a hydrophobic surface layer on MnO nanoparticles.

HRTEM images acquired on MnO nanoparticles are reported in Figure 1b-c. These consist of well dispersed crystalline nanoparticles, with prismatic shape and well defined edges. A bimodal size distribution was measured, centered at 6.5 and 13 nm, with average size of $11 \pm 4$ nm. The magnification reported in Figure 1d allows to appreciate the presence of lattice planes. Fourier Transform analysis of the high resolution images allowed to measure the crystal lattice distances typical of tetragonal $Mn_3O_4$ phase.

The same technique was employed to analyze PL-MnO Nps (Figure 2), showing that after inclusion in phospholipidic shells the same primary MnO nanoparticles are present (as seen in the high magnification image, panel c), but they form larger aggregates, around

120 nm in diameter. TGA on the same material showed a weight loss around 64%, assigned to the thermal decomposition of the hydrophobic layer and phospholipidic shell.
As found by QELS analysis, PL-MnO Nps dispersed in water showed a mean diameter of about 120 nm (in very good agreement with TEM analysis). The surface charge was evaluated by Z potential analysis showing a slightly negative value of -6.8 mV. After 4 weeks of storage at 4°C no appreciable size change of PL-MnO Nps (< 2%) was detected by QELS.

*3.3 Cell viability*

The incubation of cells with PL-MnO Nps for 10 min up to 48 h did not increase cell mortality: the Trypan blue exclusion test showed that dead cells were always less than 1% of the total cell population for each nanoparticle suspension at no variance with control samples not exposed to nanoparticles.

*3.4 Flow cytometry*

In Figure 3 the cytometric results of a typical experiment are shown: consistent with the microscopic evidence (Figure 3, upper row), PL-MnO Nps proved to be rapidly internalized by HeLa cells and a progressive increase in the mean fluorescence intensity was observed from 15 to 240 min of treatment (Figure 3, lower row).

*3.5 Intracellular distribution of PL-MnO Nps*

Fluorescence microscopy confirmed that, after 15 min incubation, PL-MnO Nps already entered the cells and their amount progressively increased with the incubation time (Figure 3). PL-MnO Nps were found throughout the cytoplasm, progressively accumulating in perinuclear position; however, they were never observed inside the cell nucleus (Figure 4). When the cell cultures were pre-incubated with PHK67 to stain the plasma membrane before being incubated with rhodamine-labelled PL-MnO Nps, no red-fluorescing nanoparticles were found to co-locate with green-fluorescing intracellular membranes (Figure 4b), thus suggesting that endocytosis does not represent the preferential internalization mechanism.

From 24 h to 72 h after PL-MnO Np retrieval, similar intracellular distribution was observed: they were exclusively found throughout the cytoplasm in both interphasic and mitotic cells (not shown); accordingly, rodamine-labelled PL-MnO Nps were never found to co-localize with the nuclear or chromosomal fluorescent signal for DNA.

The fluorescence signals of PL-MnO Nps and lysosomes were only occasionally found to co-localize at the different incubation times, indicating that most of the internalized Nps escape lysosomal degradation (Figure 4b, c).

At transmission electron microscopy, PL-MnO Nps appeared as roundish, moderately electron-dense structures evidently labelled with the fine electron dense granular reaction product of DAB photo-oxidation (Figure 5). At all times, free-ranging PL-MnO Nps were found in the cytosol (Figure 5); they were ubiquitously distributed in the cytoplasm, mostly occurring in the perinuclear region, but were never found in contact with any organelle (Figure 5a). After 24h, a few PL-MnO Nps were observed inside dual-membrane-bounded autophagosomes (Figure 5c), and some Nps remnants still showing the photo-oxidation product were found inside residual bodies (Figure 5d).

The ultrastructural analysis demonstrated that no morphological alteration or damage of cell organelles ever occurred.

## 4. Discussion

MnO-based Nps encapsulated in lipidic shells previously proved to be able to enhance $T_1$ MRI contrast and deliver DNA and/or drugs to target cells *in vitro* and *in vivo* (Howell et al., 2013). These Nps are particularly interesting because the Mn oxide core is very hydrophobic and must be coated for *in vivo* use; to this aim Mn containing nanocomposite were encapsulated in phospholipids containing also a small amount of PEG, thus significantly improving their water solubility, stability (no change in mean particle size was found after one month of storage), biocompatibility and bioavailability. Accordingly, the viability assay carried out in our *in vitro* system revealed that PL-MnO Nps administration did not increase cell mortality after both short (1-2 h) and long (1-2 days) incubation times, even at the highest Nps concentration tested. Checking cell viability after short exposure time is essential to avoid underestimation of possible Nps negative effects: it is, in fact, possible that some cells undergo damage/death immediately after Nps exposure, but the surviving population will continue to expand so that, at long term, no effect will be detectable by viability tests. Aiming at an *in vivo* administration of PL-MnO Nps for

diagnostic purposes, it is mandatory to avoid cell damage and/or death since this may trigger an inflammatory response in tissues and organs of the patient receiving the nanoparticulated MRI contrast agent (Rock and Kono, 2008; Kono et al., 2014).

Our analyses by flow cytometry provide the first experimental evidence of the internalization kinetics of PL-MnO Nps: they are able to enter the cells very rapidly since already after a 15 min exposure many cells showed a detectable fluorescent signal, and after 1 h the signal reached its maximum. These data explains the efficient PL-MnO Np uptake and accumulation at the target sites previously reported *in vivo*, essential to produce MRI contrast enhancement (Na et al., 2007; Howell et al., 2013).

Our microscopy analyses also provide original information about the internalization mechanisms and intracellular fate of PL-MnO Nps; in fact, combining fluorescence and transmission electron microscopy allowed us to monitor PL-MnO Nps from their uptake to degradation. In particular, DAB photo-oxidation (Maranto, 1982) was confirmed to be an especially suitable method to unambiguously visualize fluorescently-labeled nanoparticles at transmission electron microscopy (Malatesta et al., 2012, 2014). The application of this technique in our experimental model allowed to overcome the limit due to the moderate electron density of native PL-MnO Nps, which makes them hardly distinguishable from the cytosolic milieu, and to unequivocally identify the Nps even inside residual bodies i.e., after they underwent lytic degradation and had therefore lost their typical morphology.

Our observations demonstrated that PL-MnO Nps rapidly enter the cells and occur in the cytoplasm as single units, thus proving that PEG coating proficiently prevents Nps aggregation in the culture medium and at the cell surface. The cellular uptake probably takes place by fusion with the plasma membrane, as demonstrated by both fluorescence microscopy (the signals of nanoparticles and endosomal membrane never overlapped) and transmission electron microscopy (the nanoparticles were never observed inside endosomes). It is known that cellular uptake of anionic, neutral and hydrophobic Nps may occur by lipid raft-mediated endocytosis, which includes hydrophobic internalization *via* lipid membrane fusion as well as uptake of lipophilic and anionic groups via scavenger receptor-mediated membrane fusion: both mechanisms bypass the endo-lysosomal route thus facilitating the intracellular permanence of Nps (Lanza et al., 2011). Since lipid rafts are characteristics of many human cancer cells (Mollinedo and Gajate, 2015; Nicolson, 2015), the use of PL-MnO Nps could represent an advantage for both diagnostic and therapeutic purposes.

Once inside the cell, PL-MnO Nps occur free (i.e., devoid of a surrounding membrane) in the cytosol preserving their original morphology and without establishing spatial relationships with any particular cell organelle or structure. PL-MnO Nps migrate from the cell periphery and accumulate in the perinuclear region, where they persist for long time without showing alterations of their structure, thus indicating that cytosolic enzymes are unable to attack these nanoconstructs. This also implies that MnO and/or Mn ions are not released by the PL-MnO Nps, thus explaining the absence of ultrastructural alterations of cell organelles (e.g. Klimis-Zacas, 1993; Villalobos et al., 2009; Mertz, 2013) despite the long persistence of free Nps in the cytosol. The absence of any intracellular injury also proves the high biocompatibility of the organic shell.

By avoiding the endocytic pathways, most of PL-MnO Nps likely escape from a rapid degradation due to lysosomal enzymes, as confirmed by the rare co-location of Nps and lysosome signals at fluorescence microscopy. However, PL-MnO Nps may undergo the autophagic process: as demonstrated by electron microscopy, the Nps are surrounded by dual-membrane structures and then enter the lysosomal pathways, being finally found as DAB-positive remnants inside secondary lysosomes or residual bodies. Therefore, although the PL-MnO Nps can persist inside the cells for some days, they are finally degraded through physiological pathways without perturbing cell metabolism, thus further demonstrating their high biocompatibility and safety. However, the long-lasting intracellular permanence of PL-MnO Nps may represent a risk in the case of multiple administrations; therefore, this must be taken into account when using these Nps for both diagnostic and therapeutic purposes. On the other hand, the capability of PL-MnO Nps to maintain for long time their structural integrity in the intracellular milieu makes them especially suitable for sustained drug release.

The intracellular presence of PL-MnO Nps 72 h after they had been removed from the medium demonstrates that, similarly to other Nps (Guan et al., 2012; Malatesta et al., 2015), they are able to pass from mother to daughter cells through mitotic cycles. However, PL-MnO Nps were never found inside the nucleus, even after long time incubation, demonstrating not only that these Nps are unable to pass through the nuclear envelope (as expected due to their size being incompatible with their passage through the nuclear pore complex), but also that they are excluded from the newly formed nuclei at the end of mitosis (contrary to what observed for some polymeric Nps, see e.g. Guan et al., 2012; Malatesta et al., 2015). It should be underlined that entering the cell nucleus may

represent a risk because of the possible interactions of Nps with nucleic acids and/or nuclear factors that, in a cascade effect, could unpredictably impair multiple cell functions.

In conclusion, our study demonstrated that PL-MnO Nps undergo rapid and massive cell internalization, and persist for long times inside the cytoplasm, without being cytotoxic or inducing subcellular damage: based on the results with this cell model *in vitro*, PL-MnO Nps thus proved to be suitably biocompatible, and may be envisaged as very promising for therapeutic and diagnostic applications *in vivo*, as drug carriers or contrast agent for MRI.

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## Figure captions

**Figure 1.** Characterization of MnO nanoparticles. a) FTIR spectrum, b-d) HRTEM images acquired at b) 60000 X (bar 20 nm); c) 100000 X (bar 20 nm) and d) 400000 X (bar 5 nm).

**Figure 2.** Characterization of PL-MnO Nps. a)-c) HRTEM images acquired at a) 30000 X (bar 50 nm); b) 80000 X (bar 20 nm) and c) 400000 X (bar 5 nm). d) Thermogravimetric profile.

**Figure 3.** Internalization kinetics of PL-MnO Nps. HeLa cells after treatment with PL-MnO Nps for 15 to 240 min. The micrographs taken at conventional fluorescence microscopy (upper row) show that the intracellular fluorescent nanoparticles apparently increase in number for increasing treatment times. Bar 20 μm. This is consistent with the cytometric histograms (lower row), where the reference histograms (black line) corresponded to the negligible background fluorescence of control cell samples in the absence of nanoparticles.

**Figure 4.** Fluorescence microscopy analysis of PL-MnO Nps intracellular distribution. Micrographs of HeLa cells taken at confocal (a ,b) and conventional (c, d) fluorescence microscopy. a) After 3 h incubation, red fluorescent PL-MnO Nps are distributed in the whole cytoplasm, especially in the perinuclear region, but are absent from nuclei (N). The red fluorescent signal of nanoparticles has been merged with the brighfield image. b) Cells pre-incubated with the PKH67 green-fluorescing dye to label the plasma membrane and endocytotic vesicles, then incubated for 5 min with red fluorescent PL-MnO Nps (arrows): red fluorescence never co-locates with green fluorescent endosomes. DNA is stained with Hoechst 33258 (blue fluorescence). c, d) Cells incubated with red fluorescent PL-MnO Nps for 2 h (c) and 72 h after retrieval (d); immunolabelling for lysosomes (green fluorescence): the green and red fluorescence signals rarely overlap (thin arrow and inset), demonstrating that most of the internalized Nps escape enzymatic degradation. DNA is stained with Hoechst 33258 (blue fluorescence). Bars 20 μm.

**Figure 5.** Transmission electron microscopy analysis of PL-MnO Nps intracellular distribution. Micrographs of HeLa cells incubated with fluorescent PL-MnO Nps and submitted to DAB photo-oxidation; note the fine electron dense granular reaction product

in all nanoparticles. a) Six PL-MnO Nps (arrows) occur free in the cytoplasm without making contact with any organelle. N, nucleus; m, mitochondria; g, Golgi apparatus; r, ribosomes. b) A PL-MnO Np (arrow) where the phospholipidic shell is recognizable as electron dense concentric lines at the nanoparticle periphery. c) A PL-MnO Np (arrow) is partially surrounded by an autophagic dual membrane. d) An autophagic residual body containing heterogeneous material: a PL-MnO Np remnant (arrow) is still detectable thanks to the DAB photo-oxidation precipitates. Bars 200 nm.