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Cell uptake and intracellular fate of phospholipidic manganese-based nanoparticles

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

[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

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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 verifying 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.

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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

* Corresponding author.
** Corresponding author.
E-mail addresses: manuela.malatesta@univr.it (M. Malatesta), silvia.arpicco@unito.it (S. Arpicco).

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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 Nps 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 Nps 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 Nps 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 μm 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 Nps 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 Nps 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 (5×10^4) 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

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