Combining magnetic nanoparticles and icosahedral boron clusters in biocompatible inorganic nanohybrids for cancer therapy

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Biocompatible Fully Inorganic Nanohybrids for Biomedical Applications: Combining Magnetic Nanoparticles and Icosahedral Boron Clusters.

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INTRODUCTION

In the past decade, the synthesis of magnetic nanoparticles (MNPs) has been intensively developed for many technological and medical applications. Typical MNPs obtained by the bottom-up synthesis consist of a magnetic core and an organic or inorganic shell that provides a barrier between the core and its environment dispersing them in water at a range of different pH values, among other tasks. While physical properties of MNPs are determined by their inorganic magnetic core, their surface properties also play an important role, especially in effective interfacing (e.g., ensuring biocompatibility and specific site) with biological systems. Superparamagnetic iron oxide nanoparticles (SPIONs or MNPs) have been extensively investigated for numerous in vivo and in vitro applications, such as magnetic resonance imaging (MRI) contrast enhancement, tissue repair, and detoxification of biological fluids, hyperthermia, drug delivery, immunoassays and cell separation techniques.

All these biomedical applications require that MNPs have high magnetization values, a size smaller than 100 nm, and a narrow particle size distribution. These applications also require a demanding surface coating of the MNPs, which has to be nontoxic and biocompatible. Such MNPs have been bound to drugs, proteins, enzymes, antibodies, or nucleotides and can be directed to an organ, tissue, or tumor using an external magnetic field.

The most studied carborane is the icosahedral 1,2-dicarba-closo-dodecaborane, 1,2-closo-C\(_2\)B\(_{10}\)H\(_{12}\), and its isomers (1,7 and 1,12-... that can be viewed as 3D aromatic systems whose volume approximates to that of one displayed by a benzene molecule rotating on one of its twofold axes. These carboranes exhibit an unusual combination of properties such as low nucleophilicity, chemical inertness, thermal stability, as well as stability and low toxicity in biological systems. The rigid geometry and the relative easy functionalization at the carbon vertexes of the carborane clusters allow the preparation of a wide number of compounds potentially useful as precursors of more complex materials.

Furthermore, the use of carboranes in supramolecular chemistry is a topic which raises great interest for their particular properties that may induce an unexpected behavior in the supramolecular structures in which they are inserted. Our vision of the carboranyl substituent, however, is that it is unique as a ligand because it is a rigid sphere appended to a metal coordinating site. This, along with its hydrophobicity and electron withdrawing properties through the carbon cluster, \(C_n\), suggests the possibility of inducing distinct geometrical behavior in boron rich macromolecules or particles of significance for Boron Neutron Capture Therapy (BNCT) an alternative radiotherapy used for aggressive and infiltrating types of cancer that can not be treated with surgery or standard radio- or chemio-therapy, and for drug delivery. Particularly in this work the carborane derivative utilized, is \(m\)-carboranylphosphinate and its acid form that is \(m\)-carboranyl phosphoric acid (Chart 1), which is recently reported. The ligand is subjected to the properties bestowed by the \(m\)-carborane, though the most notable properties in reference to the...
results obtained in this work are the reduced tendency to be oxidized of the coordinating group, the spherical nature of the boron and the hydrophobicity of the boronanyl unit.

In this paper, we have assessed the in vitro and in vivo properties of the boron cluster-MNPs nanohybrids coated with m-boranalanylphosphinate ([I]), which were prepared (1-MNPs)\textsuperscript{18} by the classic co-precipitation synthesis.

**Chart 1.** Schematic representation of m-boranalane, m-boranalanylphosphinic acid (H[I]), its sodium salt (Na[I]), and bidentate bridging mode of coordination of [I] onto the surface of MNPs.

![Schematic representation of m-boranalane, m-boranalanylphosphinic acid (H[I]), its sodium salt (Na[I]), and bidentate bridging mode of coordination of [I] onto the surface of MNPs.](image)

To illustrate their potential biomedical applications, we have assessed the cellular uptake of these 1-MNPs from culture media by a human cell line of capillary-derived human brain endothelial cells (hCMEC/D3). We show by TEM images that the 1-MNPs penetrate into these cells in membrane-vesicles and remain within the cell cytoplasm. Additionally, their potential ability to penetrate into malignant tumors as boron carriers for selective cancer treatment with BNCT also in the presence of infiltrating tumors was explored. The results showed that these 1-MNPs are taken up from culture media by the glioblastoma multiforme cell line A172. Due to the magnetic core of 1-MNPs, labeled cells have a reduced signal on T\textsubscript{2} weighted Magnetic Resonance Imaging (MRI) allowing the indirect quantitative determination of boron at the target site before and during neutron irradiation. This greatly enhances the chances of success of the treatment as it allows the determination of the optimal neutron irradiation time and a precise calculation of the delivered dose.\textsuperscript{18}RIF-A Moreover, BNCT was performed on A172 cells treated with 1-MNPs, demonstrating the eligibility of 1-MNPs as boron vectors for an efficient BNCT. Finally, it was assessed that the systemic administration of these 1-MNPs in adult mice is well tolerated at mid-term with no major signs of toxicity.

**EXPERIMENTAL SECTION**

**Methods.**

Dynamic Light Scattering (DLS) and Zeta Potential: The hydrodynamic diameter (θD) of 1-MNPs dispersed in water and in several biological media was investigated with a ZETASIZER NANO ZS (Malvern Instruments Ltd) equipped with a He-Ne 633 nm laser using 1 mL of particle dispersion in a disposable plastic cuvette. Measurements were run in triplicate at ambient temperature and at 37 °C for samples in biological media. Number of scans was set up in automatic mode. Laser Doppler Microelectrophoresis is the technique used to measure zeta potential. The zeta potential of a colloidal suspension in aqueous media was obtained by filling a disposable cell with 1 mL of the colloidal suspension. Measurements were run in triplicate at ambient temperature. Number of scans was set up in automatic mode. Magnetic characterization of 1-MNPs was carried out in a Superconductive Quantum Interference Device (SQUID) magnetometer (Quantum Design MPMS3XL). Magnetization vs magnetic field measurements were performed at 300 K and 5 K in a field 6 T. Zero-field cooling (ZFC) and field cooling (FC) temperature dependent magnetization measurements were carried in a field of 50 Oe. The samples were prepared using a polycarbonate capsule filled with 1 mg of 1-MNPs and compacted cotton. High resolution X-ray photoelectron spectroscopy (HRXPS) was performed with a Phoibos 150 analyzer (SPECS GmbH, Berlin, Germany) in ultra-high vacuum conditions (base pressure 4E-10 mbar) with a monochromatic aluminium K alpha X-ray source (1486.74 eV). The energy resolution as measured by the FWHM of the Ag 3d5/2 peak for a sputtered silver foil was 0.8 eV. Scanning Transmission Electron Microscopy (STEM) images were acquired at 200 keV on an FEI Tecnai G2 F20 microscope using a high angle annular dark field (HAADF) detector. Energy Dispersive X-Ray (EDX) spectra were obtained with an EDAX super ultra-thin window (SUTW) X-ray detector. Electron Energy-Loss Spectroscopy (EELS) and Energy Filtered TEM (EFTEM) experiments were performed using a Gatan Imaging Filter (GIF) Quantum SE 963 fitted with a 2k x 2k CCD camera. 1-MNPs and cell samples were prepared by dispersing a small amount of powder in Milli-Q water. Afterwards, the drop dispersion was dried onto a carbon coated TEM grid.

**TEM of cells:** The presence of cytoplasmatic and intracellular localization of 1-MNPs into human brain endothelia hCMEC/D3 and glioblastoma A172 cells was done by using a JEOL JEM-1400 microscope operating at 120 kV.

**Materials**

Starting compounds 1-OPH(OH)-1,7-closo-C\textsubscript{2}B\textsubscript{10}H\textsubscript{11} (I), Na[1-OPH(O)-1,7-closo-C\textsubscript{2}B\textsubscript{10}H\textsubscript{11}] (Na[I]) and 1-MNPs were synthesized as reported.\textsuperscript{17,18} 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide was purchased from Sigma-Aldrich. Milli-Q water was used to do all preparations and dilutions.

The culture media used for cell growing and treatments were EGM-2 (Clonetics, Lonza) and DMEM (Thermo Fisher), with 2% and 10% FBS respectively. Phosphate buffer saline (PBS) was prepared from 10x concentrated solution (Sigma-Aldrich) in sterile Milli-Q water. Cultured cells hCMEC/D3 (as human cerebral microvascular endothelial cells),\textsuperscript{19} and A172 (as human glioblastoma cell line) were purchased at ATCC (Manassas, VA, USA). Collagen I coating was purchased at BD Biosciences and was used for hCMEC/D3 cultures.

**Preparation of 1-MNPs aqueous suspension at the physiological pH:** A stable 1-MNPs (0.5 mg/mL) colloidal aqueous dispersion was prepared as follows. 1-MNPs (5 mg) were re-dispersed in deionized H\textsubscript{2}O (10 mL) containing 1 μL of [NMe\textsubscript{3}]OH by using ultrasound radiation. The as-prepared dispersion has a pH of about 5.5-6.5, and then pH was adjusted to 7.3-7.5 by using additional [NMe\textsubscript{3}]OH aqueous solution. The corresponding vehicle media used in control conditions consisted of equal content of m-boranalanylphosphinic acid (I) and [NMe\textsubscript{3}]OH as in a 1-MNPs colloidal suspension.

**Cellular uptake of 1-MNPs:** 1-MNPs (first sonicated for 10 minutes) were tested at concentrations varying from 0 to 100 μg/mL of total iron Fe\textsuperscript{2+}\textsuperscript{3+} or with vehicle solution, and incubated at 37 °C in a CO\textsubscript{2} incubator and as follows: Brain endothelial cells (hCMEC/D3, 1x10\textsuperscript{4} cells) were cultured in 24-well plates, and after 48 hours, cells were washed with PBS twice and treated with 1-MNPs or vehicle at the concentrations described above. Finally, glioblastoma A172 cells were seeded in 24-well plates (15x10\textsuperscript{4} cells/well) and grown for 24 hours. Afterwards cells were washed with PBS twice and treated with the indicated concentrations of 1-MNPs in DMEM-1% FBS. A172 and hCMEC/D3 cells were also treated with Na[I] salt from 0 to 7.5 mM in each corresponding basal media.
All cells were incubated for 24 hours with corresponding medium containing 1-MNPs or the Na[1] salt in duplicate. Afterwards, cells were used to assess cell viability or to determine the intracellular uptake of the 1-MNPs.

**Cell viability assays:** MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a yellow compound that turns into a purple formazan after its reduction by mitochondrial enzymes, which are only present in metabolically active live cells, but not in dead cells. Formazan can be photometrically quantified at 590 nm.

After incubation either with 1-MNPs, Na[1] or vehicle solution, cells were washed with PBS and incubated with 50 μl of MTT in 300 μl of each treatment media (final concentration of MTT at 0.5 mg/mL). After 90 minutes, in which the MTT reduction took place, the cell medium was discarded and 200 μl of dimethylsulfoxide was added to each well plate. Absorbance by the isolated supernatant was measured at 590 nm in the duplicate. Final data is expressed as percentage of viable cells vs the control media condition. Differences versus the control condition were subjected to analyses of variance followed by Dunnett post hoc tests (statistical significance was considered when p < 0.05).

**Cell count:** This assay uses a proprietary mix of two DNA intercalating fluorescent dyes in a single reagent (the Count & Viability Assay Kit, Millipore). One of the dyes permeates the membrane and stains all the cell nuclei. The second dye only stains cells with compromised membranes of dying or dead cells. This combination allows for the determination of the total number of viable cells in a given sample using a Muse™ Cell Analyzer (Millipore). Briefly, hCMEC/D3 and A172 cells were seeded in flasks/dishes of similar surface, grown until sub-confluence and the number of viable cells after 24 h treatment with 1-MNPs were quantified using the above-mentioned method in a small portion of the cell suspension (100 μL). Data expressed as percentage of viable cells was used to calculate the amount of iron/cell.

**Dried cells preparation for magnetization measurements, XPS, HRSTEM, EELS and EFTEM studies:** Total cells collected from the cell count cultures were centrifuged at 1500 rpm for 5 minutes, then the cell pellet was resuspended in 50 μl of each cell culture medium, and transferred into a polycarbonate capsule to be dried at 60 °C using a speed vacuum centrifuge (1500 rpm for 1 hour).

**Cytoplastmatic 1-MNPs detection by Prussian blue and 1-MNPs visualization by Transmission Electron Microscopy:** Growing hCMEC/D3 and A172 cells were treated with increasing doses of 1-MNPs as described above. Afterwards, Prussian blue stain was performed to prove the iron uptake by the cells as described.

For verifying the presence of cytoplasmatic MNPs core by TEM, cells were prepared as follows: cells were seeded in 25 cm² flasks, grown, treated with 1-MNPs for 24 h (for the hCMEC/D3 cells, [Fe] = 25 μg/mL; for the A172 cells, [Fe] = 50 μg/mL), trypsinized, and collected by standard centrifugation. Then 1.5 mL of 2% glutaraldehyde in cacodylate buffer was added to the remaining pellets and cells were quickly incubated in the fixation solution at 4 °C for 1 h, post-fixed in 1% OsO₄, dehydrated in 12 steps by using 50-100 % acetone and embedded in Epon resin. Finally, ultrathin sections (70 nm) were transferred onto copper grids and analyzed by TEM at 120 kV.

In parallel, to assess the presence of the m-carboranoyl cluster surrounding the observed MNPs core present in the cytoplasm, Electron Energy Loss Spectroscopy (EELS) was performed using the same cells’ samples as used for subcellular 1-MNPs localization.

**MRI:** 5 x 10⁵ A172 cells were seeded in 6 cm diameter dishes. After 24h, cells were incubated for 6 and 24 h with 25 and 50 μg/ml Fe of 1-MNPs (determined by ICP-MS, see below) in DMEM-1% FBS. At the end of the incubation, cells were washed three times with PBS and detached with trypsin/EDTA (ethylendiaminetetraacetic acid). A172 cells were then transferred into glass capillaries inside an agar phantom and a T₂-weighted MRI image was acquired using a RARE sequence protocol (TR/TE/NEX=5000:13.5:12 FOV=1.13 cm). The MRI scanner used for this study was a Bruker Avance 300 spectrometer (7T) provided with a Micro 2.5 microimaging probe (Bruker BioSpin, Ettlingen, Germany).

**Boron and Iron determination by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis:** 5 x 10⁵ A172 cells were seeded in 6 cm diameter dishes. After 24h, cells were incubated for 24h with 20 μg/ml Boron of 1-MNPs in DMEM-1% FBS. At the end of the incubation, cells were washed three times with PBS and detached with trypsin/EDTA. Then A172 cells were suspended in 200ul of PBS, sonicated for 30° at 30% power in ice and their protein concentration was measured by the Bradford method (Bio-Rad, Hercules, CA, USA) using BSA (bovine serum albumin) as standard. Boron content in the cell samples was determined by ICP-MS (Element 2, Thermo-Finnigan, Rodano (MI), Italy) and values were normalized to the protein content of each cell sample that was correlated to the number of cells by means of a calibration curve. The amounts of Boron [μg/g tissue] were thus calculated considering that 1 g of tissue contains 1x10⁹ cells. Digestion was carried out by heating under microwave (Milestone Ethos Up) 0.2 mL of cell suspensions or 10μL of 0.5mg/mL 1-MNPs for 6’ at 150 °C, after the addition of concentrated HNO₃ (70%) until reaching a final volume of 0.5 mL. After digestion, 3 ml of ultrapure water were added to the remaining sample volumes for ICP-MS analysis.

**Cell irradiation and proliferation assay:** four flasks, two containing A172 cells previously incubated for 24 h in the presence of 1-MNPs (20 μg/mL Boron concentration in DMEM-1% FBS) and two with non-treated control cells, were irradiated in the thermal column of the TRIGA Mark II reactor at the University of Pavia, Italy. These irradiated group of cells were compared with the respective non-irradiated analogous. The cells incubated with 1-MNPs were washed with cold PBS before irradiation. At the end of the irradiation, the medium was removed, it was replaced with DMEM-10% FBS and flasks were placed at 37 °C in a humidified atmosphere of 5% CO₂. The irradiation position had been previously characterized from the point of view of neutron flux distribution by means of thin activation foils. At a reactor power of 250 kW the thermal neutron flux in air at that position is (1.17 ± 0.03) x 10¹⁰ cm⁻² s⁻¹, while the epithermal and fast components are at least two orders of magnitude lower. The flux is roughly constant (less than 1%) along the vertical direction, thus the flasks were superposed and irradiated at the same time. The irradiation time has been fixed at 15 minutes at a reactor power of 30 kW, corresponding to a thermal neutron fluence of 1.26 x 10¹² cm⁻². The radiation dose absorbed by cells treated was 4.7 Gy and the dose absorbed by control cells was 0.6 Gy. The day after irradiation cells were detached with trypsin/EDTA and their viability was evaluated by trypsin blue exclusion test. Then, 1.5 x 10⁶ A172 cells from each differently treated flasks were seeded in 10 cm diameter culture dishes. After 2, 4, 6 and 8 days, cells were washed with PBS, detached with trypsin/EDTA and transferred into falcon tubes.
Finally, cells were sonicated for 30” at 30% power in ice and the protein content (mg) from cell lysates was determined by the Bradford method.

**In vivo 1-MNPs administration:** To explore potential “in vivo” toxicity of the new 1-MNP nanohybrids, adult C57BL/6 mice (males, 11-12 weeks-old) received one intravenous administration of 80 µL of 1-MNPs at218 µg/mL iron concentration or corresponding vehicle solution. The mice were briefly anesthetized with 5 % isoflurane in Medicinal Air (from Air Liquide), weighed and treated with 1-MNPs or vehicle solution by retroorbital injection as described. After recovery from anesthesia, the mice were returned to their housing boxes and supervised daily looking for sudden death, seizures, dystonia, dehydration or restrictions in mobility. Additionally, body weight was registered before administration (baseline), at day 1, 2, 3 and 10 in both the treated and the naïve mice. Importantly, the amount of iron administered with the 1-MNPs was close to the approved dose for Feridex® in humans (0.56 mg/Kg of body weight). Differences in weight between groups were subjected to analyses of variance and statistical significance was considered when p < 0.05.

**RESULTS AND DISCUSSION.**

It has been recently reported that pH produces an effect on the hydrodynamic radius of the aqueous 1-MNPs suspensions. In addition, there are many studies revealing that MNPs behave differently in biological media than in water at physiological pH (7.45) because of the presence of inorganic salts, proteins, amino acids or polysaccharides in biological media. This is why studies of colloidal stability of the 1-MNPs suspension at different culture media and temperatures (room temperature (r.t) and 37 °C) have been studied in this paper.

Prior to testing the in vitro toxicity of 1-MNPs, it was important to perform colloidal stability assays of 1-MNPs in commonly used biological media for cell culturing (DMEM-F12-1% FBS, DMEM-1% FBS, EGM2-2%FBS and RPMI) as well as with the well-known phosphate buffered saline (PBS) solution that contains inorganic salts (NaCl, NaHPO₄, KH₂PO₄, KCl). So, the stability of colloidal dispersions of 1-MNPs (50 µg 1-MNPs/mL) in PBS and culture media was studied at different time intervals (10 min, and 24 h), and temperatures (r.t. and 37 °C). The results are on display in Table 1. In all the culture media no precipitation was observed neither after 10 min nor after incubating 24 h at r.t. or at 37 °C while in PBS 1-MNPs sedimented within 24 h. In the case of DMEM F12–1% FBS, 1% non-essential amino acids and 1% antibiotics, and in RPMI the size of detected particles was close to the mean particle diameters determined by TEM, ØTEM = 7.6 ± 0.6 nm. In EGM-2 medium with 2%FBS and in DMEM-1% FBS 1-MNPs rapidly formed aggregates with hydrodynamic diameters in the range of 50-140 nm and 60-170 nm respectively, maintaining an invariable size for 24 h. Comparing results at r.t. and 37 °C, a slight increase in hydrodynamic diameters was observed in all culture media as displayed in Table 1.

<table>
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<tr>
<th>Media</th>
<th>T, °C</th>
<th>ØHYD, (nm)</th>
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<tr>
<td>PBS</td>
<td>r.t</td>
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<td>4.90</td>
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<td>RPMI</td>
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<td>11.0 ± 12.9</td>
<td>4.09</td>
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<tr>
<td>DMEM F12–1% FBS</td>
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<td>FBS</td>
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<td>4.31</td>
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<td>with 2%FBS</td>
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**Figure 1.** Cell viability was tested after exposing brain endothelial cells (A), and Glioblastoma cells (B) to increasing doses of 1-MNPs and corresponding vehicle solutions for 24 hours. Data is expressed as mean ±SD of n=3/4 per condition; * p<0.05 indicates differences vs. control media (dashed line). Right panels correspond to images of each cell line (a) and to iron deposits observed after Prussian Blue Stain (b, treatment dose 10 µg/mL).
The presence of cytoplasmatic endocitosis of the iron core of the 1-MNPs. Cell viability assay shows that brain endothelial (hCMEC/D3) cells were more sensitive to 1-MNP toxicity than glioblastoma A172 cells (Figure 1A and 1B) since doses of 25 μg Fe/$^{2+/3+}$/mL significantly reduced endothelial cell viability. Previous investigations to this regard have reported safety of other iron oxide compounds at similar concentrations in both endothelial and other cancer cells. The reduction in hCMEC/D3 viability could be only partially explained by the vehicle solution, but certainly the 1-MNPs nano-hybrid induced cell toxicity starting at 25 μg/mL. Under the same administered doses of 1-MNPs, glioblastoma A172 cells presented full viability as observed in Figure 1. However, higher doses induce cell toxicity to the cancer cells, maybe due to the acidity of $m$-carboranylphosphinic acid present in the vehicle solution.

Quantification of 1-MNPs uptake by cells: To confirm the uptake of MNPs core by the A172 and hCMEC/D3 cells after 6 or 24 h of incubation in the presence of 1-MNPs, the cells were dried, as described in the experimental section and the magnetism measurements were run (see S. I.). The uptake of 1-MNPs was measured through the MNPs core and calculated as follows: first, dividing the MR value of the treated cells by the total number of cells which provides the magnetization per cell (emu/cell), then further dividing this value by the remanent magnetization of the 1-MNPs (emu/g 1-MNPs) at 5 K to obtain the amount of iron per cell. The results determine the amount of iron per cell and show a clear time and dose-dependent relationship with both endothelial and glioblastoma cell lines as shown in Figure 2A. Moreover at the same dose of 1-MNPs (25 μg/mL) A172 cells presented larger higher cellular iron content than endothelial cells (a 6-fold and 4-fold increase after 6 and 24 hours, respectively) as shown in Figure 2B, indicating a higher capacity for cell labeling with iron oxides without toxic effects (Figure 2). These are interesting results suggesting that by using low doses of MNPs, glioblastoma cancer cells may be largely labelled with the 1-MNPs compound compared to other neighbouring cells in the tissue.

Visualization of 1-MNPs uptake by cells: The presence of cytoplasmatic MNPs core and its intracellular localization into hCMEC/D3 and A172 cells were visualized by TEM analysis in membrane-bound compartments matching with endosomal or lysosomal organelles (Figure 3) at least 24 hours after labeling, as described for other iron oxide compounds. However, the presence of the $m$-carboranyl cluster surrounding the observed MNPs core present in the glioblastoma A172 cytoplasm could not be confirmed by means of EELS and EFTEM elemental maps, probably because of the low levels of boron, thus only Fe was clearly detected (see S. I.). To overcome this drawback and to unambiguously prove the presence of the $m$-carboranyl cluster coordinated at the MNPs core, high resolution XPS and EELS
spectra on the A172 dried-cells sample were done. Peaks at 189 and 133 eV in the XPS analysis, which are characteristic of B-B\textsuperscript{27} and P-O bonding, were observed and that clearly confirmed the presence of \( m \)-carboranyl phosphinate coordinated to the MNP core (Figure 4). EELS analysis on the A172 dried-cells sample also shows the B-K and PL2.3 edges present in the sample proving that the carboranylphosphinate coordinates to MNPs surface. Finally, the toxicity of the ligand shell coating the MNPs core (Na\textsuperscript{1} salt) was determined in both A172 and hCMEC/D3 cells in a dose-response cell viability assay. As observed for the whole 1-MNPs, compound endothelial cells were more sensitive to the Na\textsuperscript{1} salt than the glioblastoma cells since Letal Dose 50 was around 1 mM compared to the 7.5 mM observed in A172 cells; see Figure 5A and 5B. Those doses correspond to 230 \( \mu g/\text{Na}[1]/\text{mL} \) and 1725 \( \mu g/\text{Na}[1]/\text{mL} \) of Na\textsuperscript{1}, respectively.

Figure 4. a) High resolution spectra XPS of 1-MNPs and A172 cells treated with 1-MNPs. High resolution spectra XPS of 1-MNPs and A172 cells treated with 1-MNPs in the B 1s and P 2p regions, (b) and (c) respectively

Figure 5. Cell viability was tested after exposing brain endothelial cells (hCMEC/D3 cells) and glioblastoma cells (A172) to increasing concentrations of the Na\textsuperscript{1} salt and compared to control treatment (vehicle). A) bar graph representing cell viability after 24 h treatment (mean ±SD). B) Representative images of cells after MTT reduction

MRI: in order to assess whether the amount of Fe internalized in A172 cells was enough to allow MRI visualization, a \( T_2 \) weighted image of cell pellets in glass capillaries was acquired at 7 T. Cell labeling protocol is based on an incubation in the presence of 1-MNPs (25 and 50 \( \mu g/\text{Fe/mL} \)) for 6 or 24h. As shown in Figure 6 the acquired signal intensity (SI) is dramatically lower in all the treated samples with respect to non-treated control cells. This open the opportunity to use MRI to carry out a non invasive quantification of Fe and consequently Boron taken up by target cells.

Figure 6. \( T_2 \)-weighted MRI image acquired at 7T of glass capillaries containing cell pellets of untreated A172 cells (1) or A172 cells incubated for 6h with 25 or 50 \( \mu g \text{Fe/mL} \) 1-MNPs (2 and 3
respectively) or for 24h with 25 or 50 µg Fe/mL NPs (4 and 5 respectively).

**Cell neutron irradiation:** BNCT, studies were carried out incubating A172 cells for 24h with 1-MNPs (20 µg/mL Boron). The amount of internalized Boron measured by ICP-MS was of 133±25 µg/g corresponding to a ^10^B concentration of 26±5 µg/g that exceeds the minimum amount necessary to perform BNCT^REF^-C. Using the above mentioned condition, two groups of A172 cells were irradiated for 15 min in the thermal column of the TRIGA Mark II reactor at the University of Pavia (Reactor Power 30 kW): untreated control cells and 1-MNPs-treated cells. These were compared with the respective non-irradiated analogous. The proliferation rate (Figure 7) of irradiated and 1-MNPs treated cells re-plated the day after BNCT is significantly lower than both control cells thus demonstrating the efficacy of 1-MNPs as boron carriers for this therapy.

![Figure 7](image_url) - Proliferation curves of A172 cells re-plated one day after BNCT treatment. Data are the mean±SD of two different experiments.

**Evaluation of in vivo toxicity of the 1-MNP compound in mice:** Before testing a drug or compound on humans we must find out their potential harmful effects in experimental models, and rodents have been widely used for this purpose. We aimed at proving that for the first time, the 1-MNPs were well tolerated and did not induce major acute toxicity signs such as death, seizures or convulsions but also acute pain, distress, decreased/increased motor activity or dehydration by monitoring body weight before and after treatment. Briefly, mice received 80 µL of 1-MNPs intravenously which corresponds to 0.58±0.03 mg/kg of body weight, very close to the approved dose for Feridex® in humans (0.56 mg/Kg of body weight) and previously tested in other “in vivo” studies.²⁸ Importantly, all mice survived the study-period (10 days) with no major signs of toxicity. In particular we found that the individual body weight of the 2 treated groups were comparable with the control group (naïve mice) with day to day fluctuations but without showing a clear trend of increase or decrease in the body weight. (Figure 8A).

![Figure 8](image_url) - Mouse weight after in vivo administration of 1-MNPs or vehicle in mice. (A) Mice were weighted before 1-MNPs (n=6) or Vehicle (n=7) intravenous administration, and followed-up at 1, 2, 3 and 10 days of injection. A group of naïve mice who did not receive any treatment were also weighted at the same days (n=3). (B) The Individual weight increase or decrease at day 10 was calculated for each mouse and represented, showing no differences between treatment groups.

**CONCLUSIONS.**

The newly synthesized boron nanohybrids 1-MNPs showed colloidal stability at different culture media and temperatures (room temperature and 37°C). Biological studies confirmed the uptake of 1-MNPs by the cultured cells (hCMEC/D3 and A172) that was visualized via Prussian blue staining identifying the presence of intracellular iron after 24 h treatment with 1-MNPs. These experiments suggested that there has been cytoplasmatic endocitosis of the iron core of the 1-MNPs that was further confirmed by TEM done on both types of studied cells treated with 1-MNPs. High resolution XPS and EELS spectra on the A172 dried-cells sample unambiguously proved the presence of the m-carboranylphosphinate; peaks at 189 and 133 eV, which are characteristic of B-B and P-O bonding as they were observed clearly, thus, confirming the presence of boron cluster ligands. Quantification of 1-MNPs uptake by cells displayed that glioblastoma A172
cells presented larger cellular iron contents than brain endothelial (hCMEC/D3) cells, suggesting that by using low doses of MNPs, glioblastoma cancer cells might be largely labeled with the 1-MNPs compound compared to other neighboring cells in the tissue. These newly synthesized boron nanohybrids have significant biocompatible properties at certain administered doses allowing cell labeling with potential applications to penetrate into malignant tumor cells as drug carriers or for Boron Neutron Capture Therapy. The amount of both Fe and Boron internalized by A172 tumor cells are sufficient to allow a successful MRI guided BNCT treatment. Moreover, nanoparticles can be prepared using 10B enriched carboranes to improve the toxic effect of neutrons. Importantly, in terms of drug safety we have shown that the systemic administration of the 1-MNPs nanohybrids does not show major signs of toxicity in mice, supporting its potential translation into the biomedical setting. We believe that these new boron cluster-MNPs nanohybrids, 1-MNPs might offer a broad scope for exciting research and future biomedical applications.

ASSOCIATED CONTENT

Supporting Information. Prussian Blue Iron Stain Protocol in hCMEC/D3 cells, STEM images and EELS analysis spectra on the square area of A172 cells; HAADF STEM images and EDX spectra on the square area of A172 cells; EFTEM elemental maps of A172 square area of A172 cells; HAADF STEM image and EELS spectra on the square area of A172 cells. Energy loss peaks with onsets at 188 eV and at 133 eV corresponding respectively to B and PL2 containing in the sample; DLS studies of 1-MNPs in different biological media. “This material is available free of charge via the Internet at http://pubs.acs.org.”

REFERENCES


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Author Contributions

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Dehydration with acetone in 12 steps: 50% acetone, 5 min, x1; 70% acetone, 5 min, x2; 90% acetone, 5 min, x3; 95% acetone, 5 min, x3; 100% acetone, 15 min, x3.


Uptake quantification of 1-MNPs content /cell = MR_{capu}d/(Ntatal cells - MR_{LMPN}).
