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Internalization and cytotoxicity effects of carbon-encapsulated iron nanoparticles in murine endothelial cells: Studies on internal dosages due to loaded mass agglomerates

Monika A. Cywinska [a], Michal Bystrzejewski [b], Magdalena Poplawska [c], Anita Kosmider [a], Robert Zdanowski [d], Slawomir Lewicki [d], Zbigniew Fijalek [e], Agnieszka Ostrowska [f], Magdalena Bamburowicz [a], Andrzej Cieszanowski [g], Ireneusz P. Grudzinski [a,*]

[a] Department of Toxicology, Faculty of Pharmacy, Medical University of Warsaw, ul. S. Banacha 1, 02-097 Warsaw, Poland
[b] Department of Physical Chemistry, Faculty of Chemistry, University of Warsaw, ul. L. Pasteura 1, 02-093 Warsaw, Poland
[c] Department of Organic Chemistry, Faculty of Chemistry, Warsaw University of Technology, ul. S. Noakowskiego 3, 00-664 Warsaw, Poland
[d] Department of Regenerative Medicine, Military Institute of Hygiene and Epidemiology, ul. Kozielska 4, 01-163 Warsaw, Poland
[e] Department of Bioanalysis, Faculty of Pharmacy, Medical University of Warsaw, ul. S. Banacha 1, 02-097 Warsaw, Poland
[f] Analytic Centre, University of Life Sciences, ul. J. Ciszewskiego 8, 02-786 Warsaw, Poland
[g] Department of Clinical Radiology, Faculty of Medicine, Medical University of Warsaw, ul. S. Banacha 1a, 02-097 Warsaw, Poland

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ABSTRACT

Carbon-encapsulated iron nanoparticles (CEINs) qualified as metal-inorganic hybrid nanomaterials offer a potential scope for an increasing number of biomedical applications. In this study, we have focused on the investigation of cellular fate and resulting cytotoxic effects of CEINs synthesized using a carbon arc route and studied in murine endothelial (HECa-10) cells. The CEIN samples were characterized as pristine (the mean diameter between 47 and 56 nm) and hydrodynamic (the mean diameter between 270 and 460 nm) forms and tested using a battery of methods to determine the cell internalization extent and cytotoxicity effects upon to the exposures (0.0001–100 μg/ml) in HECa-10 cells. Our studies evidenced that the incubation with CEINs for 24 h is accompanied with substantial changes of Zeta potential in cells which can be considered as a key factor for affecting the membrane transport, cellular distribution and cytotoxicity of these nanoparticles. The results demonstrate that CEINs have entered the endothelial cell through the endocytic pathway rather than by passive diffusion and they were mainly loaded as agglomerates on the cell membrane and throughout the cytoplasm, mitochondria and nucleus. The studies show that CEINs induce the mitochondrial and cell membrane cytotoxicities in a dose-dependent manner resulting from the internal dosages due to CEIN agglomerates. Our results highlight the importance of the physicochemical characterization of CEINs in studying the magnetic nanoparticle–endothelial cell interactions because the CEIN mass agglomerates can sediment more or less rapidly in culture models.

1. Introduction

Endothelial cells (ECs) are the primary cell type that form a semi-selective permeability barrier between the vascular wall and the blood, thus playing an important role in maintaining vascular functions and homeostasis. In healthy endothelium surface layer, the ECs synthesize and secrete various biological mediators, such as prostacyclin, prostaglandin, nitric oxide (NO) and endothelin-1 to maintain the vascular tone, barrier integrity, and control the processes of inflammation, thrombogenesis and fibrinolysis (Cines et al., 1998; Gibbons, 1997). Additionally, the organ-specific structural and functional properties of the dynamic interface of the endothelium play an important role in many physiological and pathophysiological processes, including protection of nonvascular tissues, such as brain, from detrimental substances through the restriction of their diffusion into cerebrospinal fluid, vasculogenesis, angiogenesis and tumor angiogenesis, crucial in tumor growth and its subsequent metastatic potential (Pries and Kuebler, 2006; Saunders et al., 2008). According to the latter, endothelial cells can be considered as an important target for drug delivery nanosystem (DDS) in modern cancer therapy (Dass and Su, 2000; Munn, 2003). Note that nanoparticles can translocate from the entry sites to the blood stream through the capillaries and thus come into a direct contact with endothelial cells. Considering the small openings of inter-endothelial junctions with an average size of 3 nm and the restrictiveness of endothelium deep in solid tumors, with the gaps of less than 12 nm, the endothelial monolayer presents yet a real obstacle for the transport of anti-cancer based DDS (Mehta and Malik, 2006; Sarin

* Corresponding author.
E-mail address: ireneusz.grudzinski@wum.edu.pl (I.P. Grudzinski).

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et al., 2009). The above raises a deep concern over the safety application of some nanoparticles used as the DDS medicines that may have a direct contact with endothelial cells and very likely interact in a detrimental manner (Zhu et al., 2011).

Recent advances in nanomedicine provide a novel promising strategy to improve the prevention, diagnosis and treatment of some fatal diseases at the cellular and molecular levels (Prabhu and Patravale, 2012). This innovative medical concept, the so-called theranostics, based on the idea of diagnostic and therapy combined in one "smart" nanoscale system is still evolving, yet considered to become particularly important in the therapy of malignant tumors, lung and cardiovascular diseases as well as neurodegenerative disorders (Hosseinkhani et al., 2013; Singh et al., 2012; Tang et al., 2012; Vij, 2011; Wadajkar et al., 2013). Among the most promising tools being developed for theranostics are carbon-encapsulated iron nanoparticles (CEINs) (Grudzinski et al., 2013; Grudzinski et al., 2014; Kai et al., 2011; Poplawska et al. 2014; Wadajkar et al., 2013). These core/shell type magnetic nanoparticles, qualified as metal-inorganic hybrid nanomaterials are composed of the iron or iron-metal core, generally of spherical shape, and of the carbon coating built of graphene few layers, which protects the core against any oxidation processes and thus preserving its specific physical and chemical properties (Bystrzejewski et al., 2007). With respect to the recent reports indicating that both magnetic and carbon-based nanomaterials are shown to elucidate some cytotoxic effects on human aortic endothelial cells (Ge et al., 2013; Gutiérrez-Praena et al., 2011; Vesterdal et al., 2012), a more in-depth investigations dealing with the cytotoxicity assessment of CEINs are warrant to be considered using endothelial cells as a model (in vitro). In the present work, we have focused on examining the selected cytotoxic endpoints related to the membrane integrity and metabolic activity of murine endothelial (HECa-10) cells. To data, a battery of assays (SEM, TEM, Zeta potential, ICP-MS, MTT, LDH leakage, calcein AM/propidium iodide, annexin V-APC/propidium iodide) was used with special emphasis on the evaluation of time- and dose-dependent cytotoxic effects due to CEIN loaded mass agglomerates. The comprehensive physicochemical characterization of CEINs was performed to develop a fundamental understanding of the interface between these magnetic nanomaterials and murine endothelial cells.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin, DMEM (without phenol red and sodium pyruvate), 0.05% Trypsin-EDTA, Phosphate-buffered saline (Ca^{2+}, Mg^{2+} free; PBS) and HEPES buffer were purchased from Gibco-Invitrogen (Carlsbad, CA, USA). DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hydrochloric acid, absolute isopropanol, 5-fluorouracil, Ribonuclease A from bovine pancreas and propidium iodide were purchased from Sigma-Aldrich Chemie Inc. (Steinheim, Germany). The Fluo Cell Double Staining Kit containing propidium iodide and acetoxymethyl ester of calcein (calcein-AM) was purchased from MoBiTec GmbH (Gottingen, Germany). The APC Annexin V Apoptosis Detection Kit containing annexin V and propidium iodide was purchased from Becton Dickinson (Heidelberg, Germany). The Cytotoxicity Detection Kit (LDH) and Triton X-100 were purchased from Roche Applied Science (Berlin, Germany). Glutaraldehyde, osmium tetroxide, Epon-812, uranyl acetate and lead citrate were purchased from Serva (Heidelberg, Germany). Iron powder was purchased from Inorganic Ventures (Christiansburg, USA). Indium powder was purchased from Merck (Darmstadt, Germany). The nitric acid was obtained locally (POCH, Gliwice, Poland) and was of analytical reagent grade.

2.2. Preparation of CEIN suspensions for cytotoxicity studies

Carbon-encapsulated iron nanoparticles (CEINs) were synthesized as previously described (Borysiuk et al., 2008; Bystrzejewski et al., 2005; Grudzinski et al., 2013; Peng et al., 2003). Briefly, the synthesis method is based on sublimation of a heterogeneous anode containing iron (Fe) and graphite (C) powders. These materials are transformed to the vapor phase congruently due to the very high temperature in the carbon arc (5000–6000 K). Next, the as-formed metal-carbon gas undergoes rapid cooling, which leads to nucleation and solidification of iron nanoparticles encapsulated in carbon shells. The as-obtained raw product containing carbon-encapsulated iron nanoparticles and non-encapsulated iron particles were further subjected to a purification process in order to remove the non-encapsulated Fe particles. The purification procedure included 24 h refluxing in boiling 3 M HCl with further washing in excess of water and ethanol, and drying in air at 70 °C. The as-purified product was suspended in a purified water solution containing a surfactant, carboxymethyl cellulose (0.1 mg/ml). The as-prepared stock suspensions were sonicated to homogenize the nanoparticles, then diluted in PBS buffer (pH 7.4) to achieve the desired testing concentrations and used throughout experiments.

2.3. Cell culture and treatments

The HECa-10 cell line was originally obtained as a kind gift from Prof. Claudine Kieda (Molecular Biophysic Center, Orleans, France), who is the inventor of this line (Bizouarne et al., 1993). The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Incubation was carried out at 37 °C in a humidified atmosphere with 5% CO_2. The medium was renewed every 3 days. To perform the experiments the cells were seeded in sterile 24-well plates at a density of 4×10^4 cells/well and grown to ca. 80% of confluence.

Attributable to previously reported evidence of phenol red and sodium pyruvate interference with MTT and LDH absorbance readings, inhibition of LDH reaction (in case of sodium pyruvate) or even contribution to the toxicity of carbon nanoparticles in cells (in case of phenol red) (Zhu et al., 2011), to perform the experiments medium was replaced with the fresh DMEM (deprived of phenol red and sodium pyruvate) supplemented with 2% heat-inactivated FBS and the cells were exposed to defined concentrations of CEINs or left untreated for a negative control. After 24 h incubation with 5% CO_2 at 37 °C various cellular toxicity endpoints were evaluated.

2.4. MTT reduction assay

To perform the experiment the endothelial cells were treated with different concentrations of purified CEINs (0.0001–100 μg/ml) for 24 h. After the exposure, the medium containing nanoparticle suspension was removed, and the cells were washed twice with pre-warmed PBS and phenol red- and sodium pyruvate-free DMEM containing 0.5 mg/ml of MTT was added. The endothelial cells were incubated for 2 h to allow the formazan formation. Subsequently, the MTT solution was carefully aspirated and the resulting water-insoluble formazan crystals were dissolved with acidic isopropanol (0.1 N HCl in absolute isopropanol). The absorbance was measured with a spectrophotometer (UVmini-1240, Shimadzu) at 570 nm. The relative cell viability (%) was calculated as [B/A] × 100, where [A] is the absorbance of the control sample containing the untreated cells and [B] is the absorbance of the test sample.

2.5. Lactate dehydrogenase (LDH) release assay

The lactate dehydrogenase leakage in murine endothelial cells was measured after the exposure in reference to different concentrations