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Comparative cytotoxicity studies of carbon-encapsulated iron nanoparticles in murine glioma cells

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ABSTRACT

Carbon-encapsulated iron nanoparticles (CEINs) have recently emerged as a new class of magnetic nanomaterials with a great potential for an increasing number of biomedical applications. To address the current deficient knowledge of cellular responses due to CEIN exposures, we focused on the investigation of internalization profile and resulting cytotoxic effects of CEINs (0.0001–100 μg/ml) in murine glioma cells (GL261) *in vitro*. The studied CEIN samples were characterized (TEM, FT-IR, Zeta potential, Boehm titration) and examined as raw and purified nanomaterials with various surface chemistry composition. Of the four type CEINs (the mean diameter 47–56 nm) studied here, the as-synthesized raw nanoparticles (Fe@C/Fe) exhibited high cytotoxic effects on the plasma cell membrane (LDH, Calcein AM/PI) and mitochondria (MTT, JC-1) causing some pro-apoptotic evens (Annexin V/PI) in glioma cells. The effects of the purified (Fe@C) and surface-modified (Fe@C-COOH and Fe@C-$(CH_2)_2$COOH) CEINs were found in quite similar patterns; however, most of these cytotoxic events were slightly diminished compared to those induced by Fe@C/Fe. The study showed that the surface-functionalized CEINs affected the cell cycle progression in both S and G2/M phases to a greater extent compared to that of the rest of nanoparticles studied to data. Taken all together, the present results highlight the importance of the rational design of CEINs as their physicochemical features such as morphology, hydrodynamic size, impurity profiles, and especially surface characteristics are critical determinants of different cytotoxic responses.

1. Introduction

The interest in using of magnetic nanoparticles (MNPs) has gained a great deal of attention in the last few years following numerous preclinical studies showing that MNPs capable of being imaged using magnetic resonance and loaded with anticancer drugs have offered attractive possibilities to start an extensive revolution in various diagnostic and treatment procedures [1–4]. It should be emphasized that the hybrid MNPs-carbon/graphene composites have recently become a hot spot of research in modern nanomedicine, as they incorporate some functions for multimodal bioimaging and guided cancer therapy [5] comprising also tissue engineering [6,7], biosensing [8] and controlled drug or gene delivery nanosystems [9,10]. This innovative medical concept, based on the idea of diagnostic and therapy combined in one "smart" nanoscale platform is still evolving, yet considered to become particularly important in the therapy of malignant cancers [5,11,12].

Amongst the most promising novel tools being developed for (nano)theranostic drug candidates are carbon-encapsulated iron nanoparticles (CEINs). These core/shell type nanoparticles, qualified as metal-carbon hybrid nanomaterials are composed of the iron or iron-metal core, generally of spherical shape, and of the shell (i.e., carbon coating), which is built from curved graphene monolayers [13]. The carbon coating is relatively thin (a few nm in thickness) and completely covers the encapsulated metal nanoparticle. Note, that the magnetic core is also protected from the

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surrounding environment including biological fluids and does not undergo any unwanted degradation processes, e.g., corrosion and oxidation. Thus, the encapsulated magnetic nanoparticles preserve their inherent physical and chemical properties upon the exposure to different organic and inorganic media [14]. The carbon coating in CEINs is generally hydrophobic; however, one can apply both covalent and non-covalent functionalization for controlled modification its surface properties [15,16]. Especially, the introduction of new surface biological functionalities opens a novel possibility to construct the so-called "smart" hybrid magnetic CEINs with predicted biological properties in experimental models [17,18].

The cellular interaction of magnetic nanoparticles is one of the big challenges in recent nanotoxicology programs [19–21]. Although a variety of preclinical studies dealing with the cytotoxicity of MNPs exist in recent literatures, the final conclusions on particular magnetic nanoparticle-type toxicity should be viewed very carefully due to the complexity of the mechanism determining the potent interactions at the nano-cell and/or nano-extracellular matrix interfaces [19]. To our knowledge, there are number of different physicochemical metrics that are really important to address in nanoparticle-induced cytotoxicity effects. These include nanoparticle size and size distribution, shape, topology, molecular weight, aggregation or agglomeration state, purity and impurity profiles, chemical composition, stability, and especially surface characteristics [22–25]. Recent observations in different biological systems have also elucidated the importance of nanoparticle structure and textural properties in modulating some cytotoxic effects [26–28]. Some of the key factors that can plausibly affect the nanoparticles internalization and intracellular distribution, and thus contribute tremendously to their toxicity are particle geometry, surface chemistry and the degree of nanomaterial agglomeration or aggregation under experimental conditions [15,29,30]. These physicochemical features seem to be very important metrics for such magnetic nanomaterials as carbon-encapsulated iron nanoparticles composed of the iron core and of the carbon coating, which is also suspected to affect the cell behavior in contact with CEINs [31]. Recent studies evidence that CEINs physicochemical properties, such as surface charge, diameter distribution, phase composition, magnetic properties, can be controlled by synthesis processes employed and thus may have significant impact on their biological activity [32–34]. Having considered the diverse biomedical applications of CEINs, the systematic evaluation of their toxicity to mammalian cell types that are most likely involved in the future biomedical use becomes critically important.

Along with the progression in nanotoxicological research it becomes clear that some critical determinants of the magnetic core–shell type nanoparticle biological behavior can be found both at their surface and at the core features, as well. One should be aware that the surface properties related to different functional components (e.g., aliphatic, aromatic or biological ligands) attached to the carbon surface, may influence CEINs uptake in biological systems and plausibly trigger or modulate some cytotoxic effects, as it has been previously reported in normal and cancer mammalian cells [15,31,35]. Especially the surface chemistry and functional group density can be considered as a major factor in promoting cellular toxicities [36–38]. Therefore, the proper *in vitro* toxicity assessment of CEINs thus requires a comprehensive physicochemical characterization of the tested nanomaterials in consort with multiparametric biological studies that cover the broad spectrum of nanoparticle-cell interactions. In the present work, we looked into the cytotoxic effects of non-modified and surface-functionalized CEINs on murine glioma (GL261) cells. To date, gliomas are the most aggressive tumors of the central nervous system, in part, due to their unique ability to rapidly invade the neighboring tissues, thus making anticancer therapy much more difficult in humans [39,40]. In order to present a comprehensive cytotoxic profile of CEINs in glioma cells (*in vitro*), the mitochondrial metabolic activity, cellular membrane toxicity, and pro-apoptotic events triggered by both extrinsic and intrinsic pathways were examined. The mitochondrial membrane potential and glioma cell cycle progression were also studied to data. To find more details on the cellular uptake due to CEIN exposures, the internalization profile of both non-functionalized and surface-functionalized CEINs were also examined. The most important question raised in our studies, in general, is whether there is a minimum set of physicochemical characterization data for CEINs that is really required for their characterization in assessing cytotoxicity effects. In the preset study, therefore, a complete evaluation of the size, shape, zeta potential, hydrodynamic diameter distribution, and the surface characteristics of CEINs were evaluated to support the cytotoxicity findings in glioma cells.

2. Experimental

2.1. Synthesis and surface modification of CEINs

Carbon-encapsulated iron nanoparticles (CEINs) were synthesized using a carbon arc route. The procedure was described in details elsewhere [41,42]. The raw product nominated as Fe@C/Fe contains (i) Fe nanoparticles encapsulated in carbon shells and (ii) uncoated Fe nanoparticles. The uncoated Fe particles were removed by 24 h refluxing of the raw product in boiled 3 M HCl with further washing in excess of water and ethanol, and drying in air at 70 °C. The corresponding purified nanoproduct is noted as Fe@C. The surface acidic groups were introduced onto the surface of CEINs by sonicating the raw product in H_2SO_4 and HNO_3 acid (3:1, volume fractions) to obtain the final product Fe@C-COOH. The surface-functionalized CEINs with acidic groups were also obtained via the radical attack of $^*(CH_2)_2COOH$ species, which were formed via thermal decomposition of the organic acyl peroxides of dicarboxylic acids, e.g., $HOOC(CH_2)_nC(O)OO(O)C(CH_2)_nCOOH$ ($n = 2, 3$) [43]. The radical assisted route resulted in higher functionalization efficiency and yielded higher surface acidity. In the present study, a purified water solution of carboxymethyl cellulose (CMC) in concentration of 0.1 mg/ml was used as a surfactant for carbon-encapsulated iron nanoparticles. The water suspensions of CEINs were prepared using an ultrasonic homogenizer (30 kHz, 80 W). All reagents used to data were from Sigma–Aldrich.

2.2. CEINs characterization

The size and morphology of CEINs were determined by JEM 1220 TEM (JEOL) transmission electron microscopy. The diameter distribution was obtained by analyzing at least 300 objects on TEM images. The zeta potential and dynamic light scattering measurements were conducted on a Malvern Zetasizer ZSP. Fourier transform infrared (FT-IR) spectra were recorded in a transmission mode with a Perkin Elmer System 2000 spectrophotometer. The amount of the surface acidic groups in CEINs was determined by Boehm titration [44].

2.3. Cell culture and treatment

The murine glioma cell line (GL261) was obtained from the Institute of Oncology (Gliwice, Poland). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco-Invitrogen). The incubation was carried out at 37 °C in a humidified atmosphere with 5% CO_2. To perform the experiments, the cells were seeded in sterile 24-well plates (NUNC) at a density of 4×10^4 cells per well and grown to ca. 80% of confluence. Then, the culture medium was