ZnO@Gd₂O₃ core/shell nanoparticles for biomedical applications: Physicochemical, in vitro and in vivo characterization

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The chemical composition of nanoparticles (NPs) may be so designed as to provide measurability for numerous imaging techniques in order to achieve synergistic advantages. Innovative and unique structure of the core/shell ZnO@Gd₂O₃ NPs possesses luminescent and magnetic properties, and is expected that they will become a new generation of contrast agents for Magnetic Resonance Imaging (MRI) and nanocarriers for theranostics. Thus, by surface biofunctionalization, it is possible to indicate particular nanoparticle compositions which provide efficient imaging, targeted drug delivery, and biocompatibility.

Novel ZnO@Gd₂O₃ NPs were synthesized and biofunctionalized by folic acid (FA) and doxorubicin (Doxo) to provide target and anticancer functions. Physicochemical analyses of the nanoparticles were performed. The biological study included a cytotoxicity in vitro, cellular distribution evaluation, as well as toxicity analyses, performed for the first time, on the in vivo zebrafish (Danio rerio) model. Nanoparticles were found to be effective double-function biomarkers (MRI T₂ contrast agents, fluorescent imaging). The biological study showed that ZnO@Gd₂O₃ and ZnO@Gd₂O₃@OA-polySi@FA NPs are biocompatible in a particular concentration ranges. Conjugation with folic acid and/or doxorubicin resulted in effective drug delivery targeting. The in vivo results described the toxicology profile toward the zebrafish embryo/larvae, including new data concerning the survival, hatching ratio, and developmental malformations.

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1. Introduction

Recently, the design, synthesis and characterization of new multifunctional inorganic nanosystems aroused an interest of scientists in the field of biomedicine [1–5]. One of the main directions is to develop material, which combines detection and therapy in a single nanocarrier. The significant advantage of this theranostic approach includes the potential for target drug delivery, the reduction of side effects, possibility to use sparingly soluble and rapidly degradable drugs, the biodistribution and drug efficacy control, as well as early detection of cancer and monitoring the progress of the therapy.

The nanocarriers used in theranostics must meet some special requirements. First of all, such carrier should have optical and/or magnetic properties and also be biocompatible and non-toxic. Furthermore, the drugs should be effectively delivered ensured by means of target specific ligand attachment, and released into the cells. Therefore, finding a novel nanocarrier complying all these requirements became a challenging topic of the last decade.

Inorganic and organic materials (hybrid materials - materials with core-shell structures, inorganic-organic or inorganic-organic composites, materials with high specific surface area – porous materials, nanomaterials) due to their unique properties are upcoming materials for potential application in drug and gene delivery/release, photodynamic therapy, bioimaging, sensors, etc. [6–9]. Almost all well-known nanomaterials – metallic (Au), carbon-based materials, semiconductors nanoparticles (quantum dots), up-conversional NPs, polymeric and others materials already used for biomedical purposes [9,10–12]. The future lays on synthesis modification strategy (bioinspired, bioassisted) [8,13,14] as well as developing multi-functional, real-time controlled and an externally triggered delivery systems [15,16,17].

Although theranostic delivery nanosystems have been proposed before, in present work we decided to develop new nanocarriers based on the core/shell nanoparticles, due to the dual or multifaceted nature of their components. These structures represent a group of nanomaterials...
with a significant number of potential applications in biomedicine [18, 19]. Obtaining nanomaterials which combine two or more functions (e.g. multimodal imaging, targeted drug delivery) into one system is still a complex and difficult task. Therefore, there are some main approaches to solving this issue. Firstly, the search for single-phase multi-functional nanomaterials which already exhibit desirable properties; the second approach focuses on the formation of nanomaterials, doped with impurities, where impurities equip the material with specific properties; and the third approach aims at designing some materials with core/shell structures [20,21]. Varying the core and shell parameters (core shape and size, and shell thickness), as well as their properties (e.g. magnetic, optical), allow to develop of a new multimodal imaging nanosystem for an extensive, both scientific and biomedical, application [22,23].

Consequently, for the purpose of this study, a luminescent-magnetic nanocomposite was synthesized, based on two independent oxide phases – biocompatible, resistant to photo-bleaching and non-toxic ZnO matrix with green emissions and Gd2O3, which possesses magnetic properties. This approach generates significant advantages. The emission of ZnO nanocrystals at two different wavelengths is very important for integrated biological fluorescence labelling. Gadolinium properties resulting in an efficient shortening of the longitudinal relaxation time and in the increasing magnetic resonance signal intensity [24]. To reduce observed toxicity of the gadolinium ions in chelates [25], Gd ions were designed in the form of a shell, which is enough to obtain a better contrast in the MRI experiment.

Neither ZnO nor Gd2O3 are stable in water. At the same time, their surface can be easily modified by several functional groups, like COO, NH2 [26]. Surface functionalization may provide new chemical and physical properties, or change those already known; it also reduces the nanoparticles agglomeration, increases their stability in aqueous, saline or medium solutions, and most importantly for the biomedicine purposes, allows targeting agents to be attached. Various strategies, using organic ligands, an inorganic shell or polymer coupling agents, have been developed to stabilize and disperse nanoparticles in an organic or aqueous media. Oleic acid is a well-known coupling agent, which prevents the nanoparticle agglomeration in organic media [27]. One of the popular methods of stabilizing nanoparticles in water is the method based on SiO2 compounds [28]. We proposed functionalization by organosiloxanes as a good alternative to well-known SiO2-based methods for nanoparticle stabilization. The stepwise functionalization of the nanoparticles’ surface improves the compatibility between inorganic nanoparticles and the organic matrix. Functionalized oxide nanoparticles (including ZnO and Gd2O3), stable in aqueous media were obtained by the other approaches [26,29,30].

To target nanoparticles toward cancer cells, many ligands were investigated and of these, folic acid is the most common one. FA is essential in living organisms during the processes of cell division and growth, as well as in DNA repair processes. Many cancer cells overexpressed the folic acid receptor on the cell membrane surface and were used as starting materials. For ZnO@Gd2O3 NPs surface modification, oleic acid (OA, Sigma-Aldrich), tetramethylammonium hydroxide (TMAL, Sigma-Aldrich), N-(2-aminoethyl)aminopropyltrimethoxysilane (AEAPS, Sigma-Aldrich), toluene, ethanol, methanol, N-hydroxy succinimide (NHS, 98%), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC, 98%), folic acid (≥97%) and dimethyl sulfoxide (DMSO, 99.2%) were used. All chemicals were of analytical grade and were used without any further purification.

2. Materials and methods
2.1. Materials
To synthesize ZnO@Gd2O3 core/shell nanoparticles, zinc acetate dihydrate (Zn(CH3COO)2 × 2H2O, Sigma-Aldrich), sodium hydroxide (NaOH, Stanlab), gadolinium(III) nitrate hexahydrate (Gd(NO3)3 × 6H2O, 99.9%, Sigma-Aldrich) and methanol were used as starting materials. For ZnO@Gd2O3 NPs surface modification, oleic acid (OA, Sigma-Aldrich), sodium hydroxide (NaOH, Stanlab) and TMAH (99%) were used.

2.2. Synthesis of core/shell ZnO@Gd2O3 nanoparticles

The synthesis of ZnO@Gd2O3 nanoparticles consists of two stages. The core preparation involves adapted synthesis of ZnO NPs via the simple low-temperature sol-gel route based on the study of W. Beek et al. [33]. Zn(CH3COO)2 × 2H2O (13.4 mmol) was dissolved in 125 ml methanol at a constant temperature of 60 °C. Next, a solution of NaOH (23 mmol) in 65 ml of methanol was added dropwise to zinc acetate dihydrate in methanol under vigorous stirring. After approx. 3 h, a white precipitate of ZnO nanoparticles was separated from the mother liquor, washed with methanol twice and dried at room temperature (RT).

The Gd2O3 shell was prepared by the “seed deposition method” described by Y. D. Han et al. [34]. A 300 mg of the ZnO@Gd2O3 nanoparticles were immersed in a 0.1 mol/l Gd(NO3)3 water solution for 96 h. The core-shell nanostructures obtained were separated, washed with deionized water (18 MΩ·cm) and dried in an excitor. To reach gadolinium oxide phase, (Gd2O3), nanoparticles were annealed at 900 °C in air for 1 h.

2.3. Functionalization of ZnO@Gd2O3 core/shell nanoparticles by oleic acid and aminosiloxanes

The ZnO@Gd2O3 core/shell nanoparticles were fractionated by centrifugation to obtain particles with a size up to 50 nm. For functionalization of ZnO@Gd2O3 NPs by OA, OA (0.22 mmol) was added to ZnO@Gd2O3 NPs in ethanol (20 ml) under vigorous stirring. The mixture was heated to reflux, then TMAH was dissolved in refluxing ethanol (5 ml) and rapidly injected into a flask containing ZnO@Gd2O3 core/shell nanoparticles and OA. After several minutes, the mixture was diluted with ethanol (50 ml) and cooled down to 0 °C in an ice bath. The resulting solution was centrifuged, and washed several times with ethanol. Since the oleate-capped ZnO@Gd2O3 NPs obtained are not dissolvable in ethanol, the precipitate was finally dispersed in 10 ml of toluene, leading to a clear oleate-capped ZnO@Gd2O3 core/shell NPs dispersion. Then 1 ml of AEAPS in toluene (0.1 M) was mixed with ZnO@Gd2O3 core/shell NPs in toluene (10 ml) under stirring at RT. After 5 min, 1 ml of TMAH in ethanol (0.2 M) was added and the temperature was set at 85 °C for 45 min. After removal of the supernatant, the precipitate was washed with toluene, acetone and distilled water, and subsequently dried at 60 °C.
2.4. Biofunctionalization of ZnO@Gd₂O₃@OA-polySi core/shell nanoparticles by folic acid and doxorubicin

For biofunctionalization of ZnO@Gd₂O₃@OA-polySi core/shell nanoparticles by folic acid, 10 mg of FA were dissolved in 2 ml DMSO under vigorous stirring at RT. After full dissolution, 10 mg of NHS and 10 mg of EDS were added to the FA solution. After stirring for 15 min, ZnO@Gd₂O₃@OA-polySi NPs (10 mg) were added to the mixture and maintained overnight. After that, the precipitate was washed with DMSO and distilled water, and then dried at 60 °C.

For biofunctionalization of ZnO@Gd₂O₃@OA-polySi nanoparticles by doxorubicin, 5 ml of doxorubicin in water (0.7 mg/ml) were added to 10 mg ZnO@Gd₂O₃ core/shell NPs on a magnetic stirrer and maintained at RT overnight. For double biofunctionalization by both FA and doxorubicin, NPs with FA were immersed in the solution with doxorubicin and were prepared as described above.

The detailed schematic diagram of functionalized and biofunctionalized (example - FA-capped NPs) ZnO@Gd₂O₃ NPs is presented in Fig. 1.

Due to the silanization process, Zn²⁺ added to the trimethoxysilane group of AEAPS, where the Zn−O−Si groups are covalent bonded with ZnO surfaces. During the silanization process, TMAH was added to initiate nanoparticle surface functionalization. Then the γ-carboxyl group of FA was activated by EDC and conjugated onto a ZnO surface through reactions between FA and amine groups (from AEAPS) to produce the final stable ZnO@Gd₂O₃@OA-polySi@FA nanostructures.

For the sake of convenience, we refer throughout this article to the nanoparticles studied as: sample A (as-obtained ZnO NPs, synthesis at 60 °C), sample B (ZnO NPs, modified by Gd₂O₃ for 96 h and annealed at 900 °C for 1 h), sample C (ZnO NPs, modified by Gd₂O₃ for 96 h and annealed at 900 °C for 1 h and capped by OA and polysiloxanes), sample D (ZnO NPs, modified by Gd₂O₃ for 96 h and annealed at 900 °C for 1 h and capped by OA and polysiloxanes and FA), sample E (ZnO NPs, modified by Gd₂O₃ for 96 h and annealed at 900 °C for 1 h and capped by OA and polysiloxanes and Doxo), and sample F (ZnO NPs, modified by Gd₂O₃ for 96 h and annealed at 900 °C for 1 h and capped by OA and polysiloxanes, FA and Doxo).

2.5. Physicochemical characterization

Various instruments were employed to characterize the functionalized ZnO@Gd₂O₃ NPs. The size of the nanoparticles obtained was studied with the method of Small Angle X-ray Scattering (SAXS) using an Empyrean PANalytical diffractometer. Powder X-ray diffraction (XRD) studies of the powder samples were carried out on an Empyrean (PANalytical) diffractometer using Cu Kα radiation (λ = 1.54 Å), a reflection-transmission spinner (the sample stage) and a PIXcel 3D detector operating in Bragg−Brentano geometry. Scans were recorded at RT.
(300 K) at angles ranging from 20 to 80° (2Theta) with a step size of 0.006, and in the continuous mode. In order to examine the stability of nanoparticles, the relationship of the hydrogenic size of nanoparticles to their electrokinetic potential was measured by Dynamic Light Scattering (DLS). The molecular size and zeta (ζ) potential of the nanoparticles were determined using a Zetasizer Nano (Malvern). All measurements were performed at 25 °C and were done in triplicate. The Gd-layer formation, and FA and Doxo bonding were recognized by means of the Fourier transform infrared (FT-IR) spectra on a Tensor 27 spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a global source and an MCT detector in the range of 4500 – 400 cm⁻¹, using the KBr pellet method. The UV–Vis absorption data were recorded using a UV–Vis spectrometer and by dispersing the nanoparticles in water.

### 2.6. Magnetic Resonance Imaging

**T₂**-weighted magnetic resonance images (MRI) were generated using a 9.4 Tesla MRI horizontal scanner (Agilent) with the volume RF Millipede coil (40 mm). The resolution was 0.112 × 0.112 × 2 mm³. Prior to imaging, the experiment samples with different concentrations of nanoparticles were placed in a specially designed holder. Moreover, a reference specimen with solvent (water) was placed in the central part of holder. The measurements were performed at 16 °C. In the experiment, a spin echo CPMG sequence was used with 

### 2.7. Cell culture

The examinations were conducted on HeLa (cervical cancer, ATCC CCL-2), KB (epidermal carcinoma ATCC® CCL-17™) and MSU-1.1 (normal human fibroblasts, kindly provided by Prof. C. Kieda - CBM, CNRS, Orléans, France) cell lines. The cells were cultured in a complete medium composed of the basal medium (DMEM - Dulbecco’s Modified Eagle’s Medium, Sigma-Aldrich - for KB cells, 10% foetal bovine serum (FBS, Sigma-Aldrich) and 1% antibiotics (penicillin 100 μg/ml, streptomycin 100 μg/ml, Sigma-Aldrich). The cells were maintained under sterile conditions and incubated at 37 °C in a humidified atmosphere supplemented with 5% CO₂. The cell morphology and confluence were evaluated using an inverted microscope (Leica DMIL LED).

### 2.8. Cytotoxicity assays

To assess cell viability in response to sample B (ZnO@Gd₂O₃), sample D (ZnO@Gd₂O₃@OA-polySi@FA), sample E (ZnO@Gd₂O₃@OA-polySi@Doxo) and sample F (ZnO@Gd₂O₃@OA-polySi@FA@Doxo) nanoparticles, the cytotoxicity in various cell types was determined by the WST-1 Cell Proliferation Assay (Clontech). This test is based on the enzymatic cleavage of the tetrazolium salt WST-1 to a water-soluble formazan dye, which may be quantified by absorbance (wavelength of λ ~ 420–480 nm) by living cells. When the cell cultures reached the appropriate density (2.5 × 10⁴ cells per well), the cells were trypsinized and seeded in microtiter plates (tissue culture grade, 96 wells, flat bottom). All cell lines were seeded at a density of 2.5 × 10⁴ cells per well. After 24 h, the cell culture medium was removed, fresh media containing the increased concentrations of samples B, D, E, F were added to each well (1, 6, 8, 16, 32, 100, 300 μg/ml in complete medium), and then the cells were incubated for another 24 and 48 h. After incubation, 10 μl of the WST-1 Cell Proliferation Reagent was added to each well and incubated for 2–4 h. Then, absorbance at wavelength of λ ~ 450 nm (the reference λ ~ 620 nm) was recorded against the background control with the use of a multiwell plate reader (Zenyth, Biochrom). All experiments were carried out in duplicate. For each experiment, the untreated cells served as a negative control, and the cells incubated with 10% dimethyl sulfoxide – DMSO (Sigma-Aldrich) served as a positive control. Data are presented as the mean ± standard deviation (SD).

### 2.9. Confocal bioimaging

The cellular distribution, targeting of the nanoparticles and the doxorubicin release in the cells were all performed using fluorescent staining with a cell membrane dye concanavalin A (Molecular Probes, 100 μg/ml), the luminescence of core/shell nanoparticles and doxorubicin autofluorescence. All the cell lines were seeded at a density of 10⁵ cells/ml in a Lab-Tek™ 8 Chamber Slide. After one day, samples B, D, E, F (concentration of 300 μg/ml) were added. After 24, 48 and 72 h of incubation, the medium with nanoparticles was discarded and the cells were washed, fixed (4% paraformaldehyde, 0.2% Triton X-100, Sigma-Aldrich) and stained with concanavalin A. This dye shows the emission maximum at a wavelength of λ ~ 668 nm, whereas the nanoparticle luminescence/fluorescence and the doxorubicin autofluorescence show the emission at wavelength of λ ~ 520 nm and λ ~ 560 nm, respectively. Confocal imaging was performed with a Leica TCS SP5 system.

### 2.10. Toxicity in vivo

The in vitro analyses were performed on the embryonic zebrafish (Danio rerio) animal model. For the provision of embryos, the adult zebrafish (the AbxTL wild type strain) were kept in aquaria with a light/dark cycle of 14 h/10 h at the Institute of Molecular and Cell Biology in Warsaw. The fish were allowed to breed naturally and the eggs were collected in plastic egg chambers, approximately 1 h post fertilization (hpf). Any unfertilized embryos were removed, the eggs were then cleaned, sorted after the microscopic evaluation and distributed (20 embryos per dish) into 3.5 cm culture dishes using a plastic pipette. At all stages, the developing embryos were maintained at 28 °C in an aqueous E3 medium. The zebrafish embryos were then exposed for 96 h to three different concentrations (10, 50, 100 μg/ml) of the nanoparticle samples being analysed (B, D, E). During the exposure period (after 24 h and 48 h), photographs of the embryos were captured using a stereomicroscope in order to estimate the percentage of surviving embryos, as well as their morphological changes. The embryos were also observed to estimate the hatching rate every 24 h.

### 3. Results and discussion

#### 3.1. Morphology and structure characterization

ZnO@Gd₂O₃ (non-functionalized) core/shell nanoparticles were obtained and characterized earlier by Babayevska et al. [31]. Previously obtained results showed that the core, as-obtained ZnO NPs (sample A) are highly crystalline and have a hexagonal wurtzite ZnO structure. Following ZnO surface modification by the Gd₂O₃ shell (sample B), the diffraction pattern consists of two sets of lines – the hexagonal wurtzite ZnO structure and new peaks corresponding to (222) and (400) planes of the cubic Gd₂O₃ phase (Fig. S1). ZnO NPs have a uniform spherical shape, are highly crystalline and monodispersed with a mean size of about 7 nm. After covering ZnO NPs with a Gd₂O₃ layer, the resulting ZnO@Gd₂O₃ NPs are polydispersed with the core/shell structure and have a mean size range of 30–100 nm. It was shown that the Gd₂O₃ shell thickness of these nanoparticles (with the core size of approx. 70 nm) is about 4 nm (sample B) [31]. These ZnO@Gd₂O₃ core/shell NPs are too large for in vivo/in vitro vivo measurements. Therefore for any future functionalization, the core/shell NPs were fractionated by centrifugation.

The luminescent characteristics of these ZnO@Gd₂O₃ core/shell NPs were studied earlier by Babayevska et al. [31]. ZnO NPs (core) have intensive green luminescence. After covering the ZnO NPs surface with a
Gd$_2$O$_3$ shell, luminescence intensity decreases. The relative quantum yield (QY) was calculated according to Sun et al. [35]. The QY of the as-obtained ZnO NPs is about 20%, after Gd$_2$O$_3$ ZnO (NPs surface modification 96 h) QY in green emissions is about 8%. The organic shell does not affect the luminescent efficiency of the whole system.

Despite the fact that the QY is not high, it is enough to use ZnO NPs as fluorescent markers and we can see this in our experiments, presented below.

The average sizes of the ZnO NPs; ZnO NPs, modified by Gd$_2$O$_3$; ZnO NPs, modified by Gd$_2$O$_3$ and capped by OA and polysiloxanes; ZnO NPs, modified by Gd$_2$O$_3$ and capped by OA, polysiloxanes and FA; ZnO NPs, modified by Gd$_2$O$_3$ and capped by OA, polysiloxanes and Doxo; ZnO NPs, modified by Gd$_2$O$_3$ and capped by OA, polysiloxanes, FA and Doxo, based on small angle X-ray scattering (SAXS), are given in Table 1. From these results it is clear that the particle size increased upon coating with (poly)aminosiloxanes, oleic and folic acids.

### 3.2. Functionalization and biofunctionalization of ZnO@Gd$_2$O$_3$ nanoparticles

To provide water stability and to follow drug targeting, ZnO@Gd$_2$O$_3$ nanoparticles were firstly functionalized by oleic acid and aminosiloxanes, and then biofunctionalized by folic acid and doxorubicin. Confirmation of the particle surface modification was achieved via FT-IR. Fig. 2 presents the FT-IR spectra of samples A, B, C.

In Fig. 2, the broad band centred at about 3315 cm$^{-1}$ is assigned to the O–H stretching mode of the surface hydroxyl groups for all the samples studied. These peaks may also be attributed to the HO–ZnO hydroxyl species formed via the dissociative adsorption of water with ZnO NPs, and probably to the water molecules adsorbed on the surface of nanoparticles. The IR absorption peaks found at 457 cm$^{-1}$ and 680 cm$^{-1}$ are attributed to Zn–O. Two sharp peaks are located at 1585 cm$^{-1}$ and 1400 cm$^{-1}$, which are characteristic of the COO and COO–Zn stretching vibrations (samples A, B).

The new small additional peak at 547 cm$^{-1}$ corresponds to a typical Gd–O vibration from the Gd$_2$O$_3$ layer (sample B). The Zn–O stretching mode vibration shifted to a lower frequency (peak at 420 cm$^{-1}$), which indicates the existence of the Gd$_2$O$_3$ shell on ZnO NPs’ surface.

Following ZnO@Gd$_2$O$_3$ NPs surface modification by oleic acid and (poly)aminosiloxanes – TMAH and AEAPS (sample C), the spectrum at 3702–3047 cm$^{-1}$ range is broader than that presented on the graph for samples A, B. This broad adsorption band besides HO–ZnO and adsorbed H$_2$O may consist of the N–H stretching vibration of amino groups presented in the ligand. The two peaks located at 2932 and 2877 cm$^{-1}$ are due to the symmetric and asymmetric C–H stretching vibrations of the CH$_2$ groups from oleic acid. Two small peaks, located at 1585 cm$^{-1}$ and 1400 cm$^{-1}$ and corresponding to the COO and COO–Zn stretching vibrations, are smaller. The sharp peak at 1483 cm$^{-1}$ is also attributed to the N–H stretching vibration from the amino groups. It was also found that the FT-IR spectra of ZnO exhibit absorptions at 1007 cm$^{-1}$ and 948, which could be assigned respectively to the characteristic peaks of the Si–O–Si and Zn–O–Si symmetric stretching vibrations. These peaks indicate that ZnO is bonded to the aminosiloxanes ligands through covalent chemical bonds.

Following ZnO@Gd$_2$O$_3$@OA@polySi NPs surface modification by folic acid, the white powder of ZnO changes colour to yellow. In Fig. 3 (sample D), there are small peaks at 1616 cm$^{-1}$ and 1520 cm$^{-1}$ corresponding to the C=O groups from folic acid. The Doxo modified nanoparticles were also confirmed by FT-IR spectroscopy (Fig. 4). Despite the fact that the nanoparticles were intensely purple following functionalization by doxorubicin, the FT-IR peaks are weak. The bands at 1724 cm$^{-1}$, 1611 cm$^{-1}$, and a small peak at 761 cm$^{-1}$ are referred to as the stretching vibration of two carbonyl groups of the anthracene ring of doxorubicin. Following double functionalization by FA and Doxo, we may see peaks from the COO$^-$ and COO–Zn stretching vibrations.

The Doxo loading is due to electrostatic forces. In order to investigate the nature of these electrostatic interactions, zeta potential measurements were performed (Table 2). The zeta potential measurements for pure Doxo at pH around 7 were also performed to analyse the influence of electrostatic interactions between Doxo molecules and ZnO@Gd$_2$O$_3$@OA@polySi core/shell NPs surface at RT.
The stability of nanoparticles is one of the critical aspects in ensuring safety of nanomaterials. It should be pointed out that formation of aggregates with a size larger than 5 μm may lead to capillary blockade and embolism [38]. Thus, the particle size, size distribution and electrokinetic potential of nanomaterials should be carefully monitored to evaluate the physical stability of nanoparticles. The core/shell ZnO@Gd2O3, ZnO@Gd2O3@OA-polySi@FA, ZnO@Gd2O3@OA-polySi@Doxo and ZnO@Gd2O3@OA-polySi@FA@Doxo nanoparticles were characterized by DLS in terms of zeta potential, particles size and polydispersity index (PDI). The nanoparticles dimensions monitored by DLS over time showed that all core/shell nanoparticles are in the nanoscale range in the first 24 h (Fig. 5a). A significant aggregation of unmodified ZnO@Gd2O3 NPs, ZnO@Gd2O3@OA-polySi and ZnO@Gd2O3@OA-polySi@FA@Doxo nanoparticles were characterized by DLS in terms of zeta potential, particles size and polydispersity index (PDI). The nanoparticles dimensions monitored by DLS over time showed that all core/shell nanoparticles are in the nanoscale range in the first 24 h. After 72 h, all nanostructures formed large clusters with particle size reaching micrometer scale, probably due to their lower value of zeta potential. Fig. 5b shows the zeta potential profile of nanoparticles versus time. Except for ZnO@Gd2O3@OA-polySi@FA@Doxo NPs, the zeta potential of all nanoparticles decreased with the time of experiments, resulting in a high sticking efficiency between nanosurfaces (aggregation). This is in agreement with the general literature that a decrease in zeta potential leads to a decrease in the physical stability of nanomaterials [39]. From this result it can be seen that the highest stability of nanoparticles was observed in the first 24 h. It could be concluded that the dispersion stability of loaded by drug nanoparticles remains a very challenging issue. It depends on various parameters, such as surface properties of nanomaterials, their concentration in aqueous media, pH and adsorption of molecules on their surface.

The UV–Vis spectroscopy was used as an additional method which confirms surface functionalization and determines the amount of FA and Doxo adsorbed on the core/shell nanosurface. Fig. 4 presents the adsorption of FA and Doxo on the ZnO@Gd2O3@OA-polySi NPs surface at RT.

As can be seen after approximately 15 min of the functionalization process, >16% (1.62 mg on 10 mg NPs) of FA was adsorbed. In the case of Doxo adsorption, the adsorption efficiency was around 42% (74.1 mg on 10 mg NPs) after 15 min. of the functionalization. The adsorbed amount of FA and Doxo during the 24 h period was virtually unchanged. The adsorption of folic acid and doxorubicine on ZnO@Gd2O3@OA-polySi NPs surface is related to the conjugation of the carboxylic groups of FA and Doxo to the amine groups on the nanoparticle surface modified by (poly)aminosiloxanes.

### 3.3. Magnetic Resonance Imaging

To validate our assumption about the efficiency of our particles as MRI contrast agents, magnetic resonance images were collected. The six samples of different nanoparticles concentration in water were located: 0 mM – tube 1, 0.35 mM – tube 2, 0.17 mM – tube 3, 0.08 mM – tube 4, 0.04 mM – tube 5, 0.02 mM – tube 6. The suspension in the central tube (1) is brighter in all T2-weighted images (TE = 20 ms, TE = 180 ms, TE = 320 ms) than in the remaining tubes (2–6). The NMR signal disappears faster in the tubes with a higher concentration of nanoparticles (Video Still 1). This property corresponds with the T2 time value, which is shortest for tube 6. These results confirm the efficiency of the nanoparticles which were prepared in enhancing the negative contrast in the Magnetic Resonance Imaging experiments (Fig. 6).

### 3.4. In vitro cytotoxicity

The cellular toxicity analysis was based on the WST-1 assay dedicated to assessing the viability of the cells being examined. Obtained OD results at wavelength of λ = 450 nm may be evaluated as a percentage of the cell viability compared to the control [40]. To assess the effect of nanoparticles on cell viability, a wide spectrum of particle concentrations was examined at two time intervals. After 24 h of HeLa cell incubation with sample B, the average cell viability was concentration-dependent with the lowest value at 300 μg/ml. The incubation with sample D resulted in an average survival rate of 64% (Fig. S2a). Cell viability after incubation with sample E was stable, but decreased dramatically at the highest concentration (300 μg/ml – 1.6%). During incubation with sample F, the cells expressed an increased cytotoxic effect in the three highest concentrations (64, 100 and 300 μg/ml) (Fig. 7a). The HeLa cells showed a very high viability profile after...
48 h of incubation with samples B and D, except for the highest concentration (300 μg/ml) (Fig. S2b). A weak ability to reduce WST-1 salt was observed after incubation with sample E in concentrations of 100 and 300 μg/ml. After incubation with sample F, cytotoxicity was observed at a dose as low as 32 μg/ml (Fig. 7b).

The KB cells after 24 h showed an average viability 107% after sample B exposure. During incubation with sample D, the cells expressed an increased cytotoxic effect in the two highest concentrations (100 and 300 μg/ml) (Fig. S3a). After 48 h incubation, the same cellular reaction was detected (Fig. S3b). The lowest viability was 2.89% and was observed as a result of the incubation with 300 μg/ml of sample E. A similar response was observed after incubation with sample F. The trend for the cell viability described above was also detected after 48 h of incubation with the nanoparticles analysed (Fig. 8a, b).

During the 24 h incubation of samples B, D (Fig. S4a), and E, F (Fig. 9a), in the case of the MSU-1.1 cells, the viability was stable with increasing cytotoxicity in a concentration of 300 μg/ml. A lethal effect at this dose was observed after 48 h (Figs. S4b, 9b).

The in vitro study showed the cytotoxic profile of the B, D, E, F samples. To assess the cytotoxicity of the new materials, it is important to start with the basic ZnO@Gd2O3 core/shell NPs. This in vitro study confirmed that these nanoparticles (10–50 nm) are highly biocompatible in the range of concentrations (1–300 μg/ml) analysed, and thus may be used as a basic nanostructure for multimodal nanocarriers. Our investigations are in agreement with the observations of other authors that Gd-doped ZnO nanoparticles used for labelling HeLa cells have no cytotoxic effect, even at high concentrations as high as 1 mM [41].

The ZnO@Gd2O3 core/shell structure was biofunctionalized with folic acid in order to target the tested nanoparticles to the cancer cells analysed. HeLa and KB cells overexpress the folic acid receptor at a very high level, but an enhanced nanoparticle cellular uptake caused no reduction in viability within the 1–100 μg/ml range of concentrations. Other data also suggest that folate-coated ZnO nanoparticles doped with rare metals (i.e. Gd, Yb) have no effect on cell viability [42].

A correlation analysis of the absorption and the amount of doxorubicin showed that concentrations of 100 and 300 μg/ml should be the most efficient, since they contain an effective dose of doxorubicin at the in vitro level. Its effect is reflected in the cellular response observed (Figs. 7, 8). These data correspond to our previous results concerning cytotoxicity and the drug-loading profile of magnetite@polydopamine@doxorubicin NPs [43]. The differences between the cellular responses of cancer cells were connected with their different sensitivity to the chemotherapeutic agent [44].

The most visible effect of the improved cellular uptake was observed after the incubation of sample F with the HeLa cells. The reduction in cell viability was four times greater compared with the effect of sample E. These study results indicate that the nanoparticles tested are efficient in the targeted drug delivery system.

3.5. Confocal bioimaging

The incubation of the cells with sample B resulted in the fluorescence of the nanoparticles being observed within the cell interior. The microscopic study of sample D confirmed the targeting process in these cells, observed with a very high intensity in the cancer HeLa and KB cells, due to the preferential binding of folic acid to the overexpressed surface receptors (Fig. S5). The lowest nanoparticle specific fluorescence signals

Fig. 6. MRI T2-weighted images in tubes 1–6 with different concentrations of sample B (ZnO@Gd2O3) in water.
were detected in the case of sample D following incubation with the MSU-1.1 cells.

Confocal observations of sample E established an effective drug delivery using nanoparticles. The nucleus/cytoplasm doxorubicin release process was observed as early as the first day. Microscopic analyses of sample F confirmed these results (Fig. 10). Please note, that on Fig. 10b and c the red autofluorescence of doxorubicin and artificial green colour of membrane 647 nm dye are presented. This approach has allowed us to show indirect evidence for samples E and F cellular distribution. It was not our intention to distinguish fluorescence signal from the NPs and cell membrane.

The Z-stack mode performed in all cases confirmed the internalization process (Video Still 2).

The literature on the subject includes many scientific reports concerning cytotoxicity and biological imaging based on ZnO nanocrystals doped with Co, Cu, Ni, Au, Gd, the Fe₃O₄ core/shell, ZnO nanowires targeted to integrin αvβ₃, ZnO QDs coated with folate-conjugated chitosan loaded with doxorubicin, ZnO@Gd@Doxo and folate-coated ZnO doped with rare metals [41,42,45–50]. This report is assumed to be the first describing the cytotoxicity profile and cellular imaging of core/shell ZnO@Gd₂O₃, ZnO@Gd₂O₃@OA-polySi@FA, ZnO@Gd₂O₃@OA-polySi@Doxo and ZnO@Gd₂O₃@OA-polySi@FA@Doxo NPs toward HeLa, KB, MSU-1.1 cells.

3.6. In vivo toxicity

At present, the zebrafish appears to be a promising in vivo vertebrate model for nanotoxicological studies. The most important characteristics of this laboratory animal include its small size, rapid development, short life cycle, optical transparency, high level of genetic homology to humans and low culture cost [51]. A wide spectrum of nanomaterials (metallic, magnetic, polymeric, carbon and QD) has been examined in terms of in vivo toxicity using the Danio rerio model [52–57]. Our studies included both embryo survival and hatching rates, as well as the malformation analyses of zebrafish embryos after incubation with samples B, D and E. The embryos were not exposed to full range of concentrations (1–300 μg/ml). A close study of literature revealed that nanoparticles concentrations of 50 and 100 mg/l were the lethal doses for zebrafish embryos [58] and we didn’t want to exceed these values. Based on the results from the in vitro study, we have shortlisted 3 samples (B, D, E) and used these in in vivo tests. Samples E and F were similar in their action on cancer cells but sample E was slightly more toxic to normal cells

![Fig. 8. KB cell viability after (a) 24 h and (b) 48 h incubation with sample E (ZnO@Gd₂O₃@OA-polySi@Doxo) and sample F (ZnO@Gd₂O₃@OA-polySi@FA@doo).](image)

![Fig. 9. MSU-1.1 cells viability after (a) 24 h and (b) 48 h incubation with sample E (ZnO@Gd₂O₃@OA-polySi@Doxo) and sample F (ZnO@Gd₂O₃@OA-polySi@FA@doo).](image)
than sample F. In order to cover full range with least number of samples we have decided to omit sample F from further analysis. Although toxicity testing on zebrafish younger than 120 hpf is not considered as an animal testing (Directive 2010/63/UE and Polish ACT of 15 January 2015 on protection of animals used for scientific or educational purposes) we feel obliged to limit the number of embryos/larvae that need to be sacrificed in experiments.

3.6.1. Survival and hatching rate

The embryo survival rates were evaluated after 24 and 48 h of incubation with the nanoparticles being tested. Microscopic observations showed embryo survival rates approx. 90–100% after incubation with samples B, D and E in concentrations of 10, 50, 100 μg/ml and at time intervals (24, 48 h) (Fig. 11a). This may be connected with the function of chorion, i.e. the three-layered outer membrane surrounding and protecting the embryo [59]. On the other hand, some research papers describe the ability of very small Ag nanoparticles (11.3 nm) to pass through the chorion pores [60]. Bai et al. described the toxicity of ZnO nanoparticles in concentrations of 50 and 100 mg/L as the lethal dose [58]. For Fe₂O₃ the survival rate was dose- and time-dependent [61]. The nanoparticles affected the hatching rate of the zebrafish embryos. The hatching time for the control began between 48 and 72 hpf. Exposure of embryos to sample B at 100 μg/ml resulted in no hatching being observed even at 96 hpf. The same type of nanoparticles at 50 μg/ml reduced the hatching rate at 96 hpf, whereas at a lower concentration (10 μg/ml), the hatching occurred earlier than in the control. During incubation with sample D, the dose of 10 μg/ml has no negative effect on the hatching ratio, whereas the concentration of 50 μg/ml reduced the rate after 72 and 96 hpf. The concentration of 100 μg/ml after 72 and 96 hpf inhibited totally the hatching process. Sample E
reduced the hatching after 96 h for the concentrations of 50 and 100 μg/ml (Fig. 11b) and as was the case with sample B, stimulated hatching at 10 μg/ml. Retarded embryo hatching after exposure to ZnO, Ag, Au, Pt, TiO₂ and Si nanoparticles was also described by other authors [60,62,63]. On the other hand, Samaee et al. showed the premature hatching time and decrease in time required for normal hatching after incubation with TiO₂ NPs in a dose-dependent manner [64]. Zhou et al. investigated the effect of surface chemical properties on the toxicity of 17 types of ZnO nanoparticles to the embryonic zebrafish and declared that it may increase the toxic impact [65].

3.6.2. Developmental abnormalities
As shown in Figs. 12 and 13, as a result of 48 h exposure to samples B, D and E, embryos failed to develop the normal morphology. The most typical malformations at 48 hpf initiated after exposure to sample B were a bent trunk and tail malformation. Contact of the embryos with sample D resulted in yolk sac oedema and pericardial oedema. Pericardial oedema, yolk sac oedema and bent trunk were the most frequent abnormalities of embryos following incubation with sample E. Toxic actions of sample E were expected due to the interactions with the DNA double helix and confirmed other authors’ findings concerning the cardiotoxicity of drug analysed [66,67]. All types of malformations were dose-dependent. No correlation between the premature hatching observed for samples B, D and E at a low concentration and malformations was detected.

The results of the present study remain in agreement with the observations reported by other authors on the effect of bare nanoparticles on the malformation development. The data concerning the toxicity of SiNPs described such abnormalities as pericardial oedema, yolk sac oedema, as well as tail and head malformations [68]. The toxicity assessments of the near-infrared up conversion luminescent LaF₃:Yb,Er injected through the chorion into the early developed zebrafish embryos showed some abnormalities, including a non-depleted or malformed yolk, spinal and tail malformations, pericardial sac or yolk formations, stunted body or eye growth, as well as pericardial or yolk sac oedema [51].

The results obtained in this paper indicate that all three types of nanoparticles tested have a slight effect on the survival rates, and influence the hatching rate, as well as being the cause of the embryo malformations. Literature sources describe three mechanisms for the toxic effect of different nanoparticle types (Fe₂O₃, carbon nanotubes, TiO₂, ZnO). The first mechanism involved aggregation and sedimentation on the chorion surface and changes in the mechanical properties of the membrane, as well as the effect on chorionic hatching enzymes [69]. The second approach concerned interference with the nutrient exchange between the embryos and the environment [70]. The third strategy was related to the toxic effect of the metal ions released from the nanoparticles and the induction of DNA damage, as well as the generation of reactive oxidative species ROS [71,72].

As far as the authors of the present study are aware, there are no data concerning the influence of the core/shell ZnO@Gd₂O₃, ZnO@Gd₂O₃@OA-polySi@Doxo nanoparticles on the zebrafish embryo development. In vivo nanotoxicity studies provided the survival and hatching rates, as well as the developmental abnormalities. These data are regarded as highly informative, since zebrafish share many physiological characteristics with higher vertebrates; thus, the toxicological results obtained in this study may be compared with those of studies on the developmental toxicity in mammals [61]. The in vitro-in vivo correlation of the recorded results is moderate. Due to the much more complex animal response (biodistribution, degradability), in vivo nanotoxicity cannot be properly addressed using in vitro experimental systems [64].

4. Conclusions
Physicochemical analyses showed the excellent potential applicability of the newly produced core/shell ZnO@Gd₂O₃ nanoparticles as effective and efficient novel double-function T₂ contrast agents for the
magnetic resonance, as well as for fluorescent imaging. Biological studies based on the in vitro cytotoxicity profile lead to the conclusion that samples B and D are bio compatible within a particular concentration ranges. ZnO@Gd2O3 nanoparticles with bio functionalized agents (folic acid, doxorubicin) exhibit sufficient biological activity and are effective nanocarriers for targeted drug delivery systems. In vivo results of this study, will broaden the current knowledge on the potential toxicological effects of ZnO@Gd2O3 based nanoparticles and support sustainable expansion of nanotechnology.

In summary, physicochemical and biological biological analyses indicate that ZnO@Gd2O3 core/shell nanocarriers are promising options for multimodal imaging and targeted drug delivery. The results meet modern medicine expectations. The presented solution may result in early diagnosis, monitoring of therapy results, while reducing drug doses, thus providing very important health, social and economic effects.

The future lies in optimizing the results obtained and moving toward the development of personalized therapy. Using molecular biology and genetic diagnostics tools, it is possible to establish the overexpression of surface protein which is characteristic and specific only to a given patient, thus creating some targeted and personalized therapy.

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