Excretable, ultrasmall hexagonal NaGdF4:Yb50% Nanoparticles for bimodal imaging and radiosensitization

Jossana Abcede Damasco
University of Texas MD Anderson Cancer Center

Tymish Y Ohulchanskyy
University at Buffalo - The State University of New York

Supriya D Mahajan
University at Buffalo - The State University of New York

Guanying Chen
University at Buffalo - The State University of New York

Ajay Singh
University at Buffalo - The State University of New York

Hilliard Kutscher
University at Buffalo - The State University of New York

Haoyuan Huang
University at Buffalo - The State University of New York

Steven Turowski
Roswell Park Comprehensive Cancer Center

Joseph A Spemyak
Roswell Park Comprehensive Cancer Center

Anurag Singh
Roswell Park Comprehensive Cancer Center

Jonathan F Lovell
University at Buffalo - The State University of New York

Mukund Seshadri
Roswell Park Comprehensive Cancer Center

Paras Prasad (pnprasad@buffalo.edu)
University at Buffalo - The State University of New York

Research

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Abstract

**Background:** In this study, we report on the synthesis, imaging and radiosensitizing properties of ultrasmall $\beta$-NaGdF$_4$:Yb50% nanoparticles as a multifunctional theranostic platform. The synthesized nanoparticles act as potent bimodal contrast agents with superior imaging properties compared to existing agents used for magnetic resonance imaging (MRI) and computed tomography (CT). Clonogenic assays demonstrated that these nanoparticles can act as effective radiosensitizers, provided the nanoparticles are taken up intracellularly.

**Results:** Our ultrasmall $\beta$-NaGdF$_4$:Yb50% nanoparticles demonstrate improvement in T1-weighted contrast over the standard clinical MR imaging agent Gd-DTPA and similar CT signal enhancement capabilities as commercial agent iohexol. A 2 Gy dose of X-ray induced ~20% decrease in colony survival when C6 rat glial cells were incubated with non-targeted nanoparticles (NaGdF$_4$:Yb50%), whereas the same X-ray dose resulted in a ~60% decrease in colony survival with targeted nanoparticles conjugated to folic acid (NaGdF$_4$:Yb50%-FA). Intravenous administration of nanoparticles resulted in clearance through urine and feces within a short duration, based on the *ex vivo* analysis of Gd$^{3+}$ ions via ICP-MS.

**Conclusion:** These biocompatible and in vivo clearable ultra-small NaGdF$_4$:Yb50% are promising candidates for further evaluation in image-guided radiotherapy applications.

Background

Clinically relevant, multifunctional nanoparticles that combine diagnostic and therapeutic platforms are of high scientific interest, with significant societal impact. [1-3] However, these theranostic nanomaterials often result in complex and large-sized structures in order to accommodate the various components that provide multi-functionality. In addition, complicated synthesis methods are difficult to reproduce and can be impractical for large-scale processing.

In order to be clinically relevant, nanomaterials need to exhibit biocompatibility and ideally undergo rapid clearance from the body. [4] Nanoparticles are primarily taken up and eventually cleared by the reticuloendothelial system (RES) or kidneys. However, retention in the liver or spleen can take up a long time depending on their size and surface chemistry. [5, 6] These accumulations in the RES and the slow elimination, taking months or even years to clear the body, can be problematic for clinical translation. On the other hand, clearance through the kidneys occurs quickly, as the nanoparticles are filtered from the blood and excreted out. This significantly reduces the risk of potential toxicity, which makes the renal clearance pathway an attractive route of elimination. [7] However, glomerular filtration is strongly dependent on size, with a hydrodynamic diameter filtration-size threshold of $< 6$nm. [8] Thus, the development of ultrasmall, biocompatible, multifunctional nanoparticles is of high interest for potential clinical use.[8-10]
One of the promising applications of theranostic nanoparticles is their ability to enhance radiotherapeutic efficacy. Radiation therapy (RT) is an integral part of clinical management of most solid tumors, and remains one of the most cost-effective treatments for cancer patients.[11] However, not all patients respond to RT, and disease recurrence remains a significant clinical problem.[12]

The use of nanoparticles to ‘sensitize’ tumors to RT could potentially enable lowering of the total radiation dose administered to patients, without compromising efficacy. Furthermore, radiation-induced normal tissue toxicity often contributes to a poor quality of life (QOL) in patients. Optimal use of these nanoparticles in combination with RT may therefore minimize collateral radiation damage to normal tissues and potentially improve QOL.

In this regard, there is growing interest in the use of metal nanoparticles as radiosensitizers. Upon irradiation, metal nanoparticles, with their high surface area and surface chemistries, have shown intrinsic radiocatalytic activities in water producing reactive oxygen species (ROS), thus, effectively increasing the overall radical concentration. [13] Furthermore, interactions of X-ray with metals with high atomic number (high Z) are known to enhance the photoelectric and Compton effects that result in radiation dose-enhancements.[11] Most studies have focused on gold (Z=79) nanoparticles and significant evidences have been reported to demonstrate their ability to increase the therapeutic ratio of radiotherapy. [14-17] Hafnium oxide (Z=72) developed by NanoBiotix (France), has already shown success in the clinic as efficient radiation enhancers on patients requiring preoperative radiotherapy.[18]

Other metal nanoparticles that have shown great promise in augmenting radiotherapy include bismuth (Z=83) [19]; platinum (Z=78) [20, 21] and iron oxide[22, 23] having both radiosensitizing and hyperthermic [24] properties.

Both gadolinium-based (Z=64) and ytterbium-based (Z=70) nanoparticles have garnered attention as theranostic platforms. Gd-based nanoparticles have been shown to enhance magnetic resonance (MR) imaging contrast and have been found to induce X-ray dose enhancement, making them ideal candidates for combined imaging and therapy, and are currently in Phase I clinical trial for the treatment of multiple brain metastases.[25-27] Yb-based nanoparticles have been developed as bimodal probes for X-Ray computed tomography (CT) and near infrared-to-near infrared fluorescence imaging. [28] The high X-ray attenuation of Yb enabled its use as a theranostic agent, with both tumor imaging and radiosensitization functions. [29]

In this study, we present nanoparticles containing Gd and Yb as candidates for combined imaging and therapy in a single ultra-small nanoplatform for cancer therapy. The combination of Gd and Yb allows for the nanocrystal serving as a bimodal imaging probe for MR and CT examinations. MR imaging is best suited for soft tissue imaging, while X-ray CT is ideal for hard tissues or bone. In addition, reducing the size of the nanoparticles to sub-5nm increases the surface Gd\(^{3+}\) accessible to H\(_2\)O which leads to higher T1 relaxivities in comparison to larger nanoparticles,[30] while allowing complete elimination from the body within days (i.e., 4 days) through hepatic and renal clearance, as revealed from our ICP-MS analysis of Gd\(^{3+}\). The first reported use of combined Gd and Yb as an MR/CT probe was in the form of
NaGdF$_4$:Yb20% doped with 2% Erbium(Er) for additional optical imaging capability. [31] In addition, the CT signal was enhanced by increasing the amount of Yb from 20% to 80% (i.e., NaYbF$_4$:Gd20%). [32] However, for obtaining the preferred thermodynamically stable hexagonal phase, both these preparations resulted in large-sized nanoparticles (i.e. > 20 nm). In addition, prior to the synthesis of the nanocrystals, an initial step to prepare the lanthanide precursors is needed. Here, we present a more practical and user-friendly approach introducing a single-step method that allows a precise control of size, uniformity and crystal phase. Because MRI is the more sensitive modality, it is ideal to have a higher ratio of Yb to Gd which would result in much higher X-ray attenuation. On the other hand, increasing the concentration of Yb generally produces larger nanoparticles. [31-33] Thus, to guarantee that the ultra-small size is maintained in the hexagonal phase, our nanoparticles were designed with an equimolar amount of Gd$^{3+}$ and Yb$^{3+}$ ions.

To ensure effective radiosensitization, these nanoparticles were modified for targeted delivery by conjugating folic acid to their surface, ensuring optimal cellular uptake by cancer cells, which ultimately significantly decreased the number of surviving colonies following a clinically relevant X-ray exposure. Ultra-small size nanoparticles are also ideal for radiotherapy due to low self-absorption of electrons resulting in higher Auger electron yield. [34] These emitted secondary electrons have very low energy and thus short-range to produce localized cellular damage.

We evaluated the efficacy of this nanoplatform in an in vitro clonogenic assay using C6 rat glioblastoma cells. Glioblastoma multiforme (GBM) is a grade IV tumor and represents about 15% of all primary brain tumors. It is the most aggressive and infiltrative form of gliomas, quickly spreading in all parts of the brain. [35] The average survival for GBM is 12–15 months using the current standard of care treatment, and the determination of treatment response and clinical decision-making are based on the accuracy of radiographic assessment. [36] A major factor that contributes to poor prognosis in GBM patients is the limited response to treatment caused by the inability of most chemotherapeutic agents to cross the blood-brain barrier (BBB). We demonstrate here that the ultrasmall size of the nanoparticles conjugated with folic acid can take advantage of the folate receptor expressed at the BBB [37, 38] to facilitate the transport of the nanoparticles across BBB. Advanced MRI and CT imaging techniques such as dynamic contrast-enhanced (DCE) imaging, which monitors the temporal changes in contrast enhancement in blood vessels and tissues to provide a time-concentration curve, are promising non-invasive methods with moderate to high accuracy in stratifying tumors and discriminating recurrent lesions and treatment-related changes. [39-42] Given the marked signal enhancement on both MR and CT imaging, our nanoparticles could enable accurate diagnosis of disease progression of GBM.

With its facile synthesis, highly uniform size distribution, ultrasmall size and easily tailored surface, these novel nanoparticles present a promising, translatable theranostic platform with high tumor uptake, favorable biodistribution and route of elimination.

Results
Formation of Ultrasmall $\beta$-NaGdF$_4$:Yb50%

Uniform sub-5 nm NaGdF$_4$:Yb50% nanoparticles in a thermodynamically stable, hexagonal phase ($\beta$-phase) were successfully synthesized. Analysis of more than 100 nanoparticles from TEM images reveals a normal size distribution with an average diameter of 3.44 nm ± 0.72 nm (Figure 1a-c). The formation of ultrasmall $\beta$-NaGdF$_4$:Yb50% nanoparticles is confirmed by its very broad X-ray diffraction patterns, which conform to the standard XRD peaks of the hexagonal $\beta$-phase NaGdF$_4$ (JCPDS 27-0699) (Figure 1d). Elemental analysis of Gd and Yb content shows the respective actual molar percentages to be 52.18% and 47.82%, a clear indication that the desired stoichiometric amount of Yb$^{3+}$ ions was successfully doped into the NaGdF$_4$ nanoparticle.

NaREF$_4$ (RE = rare earth) nanoparticles are known to exist in two phases, the metastable cubic $\alpha$-phase and the thermodynamically stable hexagonal $\beta$-phase.\[^{43}\] This difference in stability has been exploited in the focusing of particle size distribution, wherein the more soluble $\alpha$-phase nanoparticles serve as sacrificial precursors to form the thermodynamically preferred $\beta$-phase with narrow distribution.\[^{44-46}\] This method typically results in larger nanoparticles, although Haase et al has successfully synthesized 5.6 nm $\beta$-NaYF$_4$:Yb 20%, Er 2% nanoparticles by heating 10 nm sacrificial $\alpha$-NaYF$_4$:Yb 20%, Er 2%.\[^{46}\] However a more practical and user-friendly approach is to have a single-step method that will allow precise control of size, uniformity and crystal phase.

Surface modification of the nanoparticle surface was achieved through ligand exchange by allowing the nanoparticles in chloroform solution, and the L-cysteine and DTPA anhydride in basic water (pH 9) to mix for 24 h. The hydrodynamic diameter measured by dynamic light scattering (DLS) showed an increase in the hydration shell from 4.1 nm (in hexane) to 5.1 nm (in H$_2$O) (Figure S1) after surface modification. TEM images did not show clustering or aggregation of the nanoparticles suspended in H$_2$O (Figure S2). This successful coating of the ligands on the nanoparticle surface also provided additional functional groups (i.e., amine and carboxylate) to allow bioconjugation of targeting ligands.

Gd$^{3+}$ Leaching, Cytotoxicity and Biodistribution

The stability of NaGdF$_4$ was evaluated by measuring the Gd$^{3+}$ ion leakage from the crystal matrix. An analysis of Gd$^{3+}$ leaching shows less than 0.1% Gd$^{3+}$ ions were present when dialyzed against H$_2$O. Solutions of DMEM with 10% FBS, and DMEM with 10% FBS supplemented with 10 mM phosphate, incubated at 37°C, were utilized to mimic physiological conditions and to assess the effect of elevated phosphate levels on the stability of the nanoparticles. After 3 days of dialysis, ~2% of the Gd$^{3+}$ was observed in dialysate; this rose to ~3% at higher phosphate concentrations (Figure 3a).

The effect of the nanoparticles on cell viability was studied by monitoring the mitochondrial metabolic activity through the standard MTS assay. C6 cells remained 100% viable after 12 and 24h incubation at up to 1 mg/mL (Figure 3b). More importantly, cells remained 100% viable even with increased incubation
time (48h), at 125 μg/mL. It is generally accepted that nanoparticle toxicity is concentration- and time-
dependent.[47, 48] Similarly, further increase in the concentration of the nanoparticles to 1 mg/mL at
prolonged exposure time (i.e., 48 h) resulted in increased cytotoxicity (50% of cell viability).

Passive biodistribution and clearance studies revealed that less than 0.5% of the nanoparticles remained
in the organs after 4 days, as detected by ICP-MS (Figure 3c). After 4 h, 33% of the nanoparticles were
eliminated through the urine and 21% through the feces (Figure 3d). The remaining nanoparticles were
eliminated mostly through the feces over a period of 4 days (Figure 3d).

**Nanoparticles for MRI and CT Imaging**

The potential of these nanoparticles as a bimodal imaging probe for both MR and CT imaging was
evaluated by measuring their T1 relaxivity ($r_1$) and the Hounsfield unit (HU) values, respectively. The
relaxivity of the nanoparticles was compared to Gd-DTPA (Magnevist®) at 25°C and 37°C by measuring
T1 rates of a series of solutions containing increasing Gd$^{3+}$ molar concentrations (as determined by ICP-
OES). There is a linear relationship between the Gd$^{3+}$ concentration and the longitudinal relaxation rate
(1/T1), and $r_1$ values are determined from the slope of the resulting linear plots (Figure S3). A pseudo-
colorized, T1-weighted spin echo image (TE/TR = 8.5/500ms) for saline, 200 μM Gd-DTPA and
nanoparticles (200 μM [Gd]) demonstrates the improvement in T1-weighted contrast of the nanoparticles
over the standard clinical MR imaging agent Gd-DTPA (Figure 4b).

The Hounsfield unit (HU) value, determined from the slope of the linear plot of HU as a function of the
concentration, can indicate if the nanoparticles can serve as a CT contrast agent. There is a linear
correlation between the increasing contrast agent concentration and the CT signal intensity for both the
commercial agent iohexol and the nanoparticle solution (Figures 5a and 5c). The nanoparticles and
iohexol show almost identical line slopes (Figures 5b and 5d) indicating similar signal enhancement
capabilities. Setting the HU value of water as zero, the calculated slope for the HU value for the ultrasmall
NaGdF$_4$:Yb50% is approximately 26 HU while that of the iohexol is about 23 HU.

**Nanoparticles as a Radiosensitizer**

A clonogenic assay was used to investigate the potential of the nanoparticles as radiosensitizers in a rat
C6 glioma cell line. To ensure that cell death was not due to any inherent toxicity of the nanoparticles, the
concentration was kept at 100 μg/mL, which still maintained more than 90% cell viability even after 48h
incubation (Figure 3b). Colony formation of the cells without nanoparticles and without X-ray radiation
treatment served as control. C6 cells incubated with nanoparticles but not subjected to X-ray radiation did
not reduce surviving colonies, confirming that the nanoparticle concentration was not cytotoxic (Figure
6). Irradiation alone of the cells with a 2 Gy dose did not result in any significant cell reproductive death.
Cells treated with non-targeted NaGdF$_4$:Yb50% nanoparticles showed a 16% decrease in surviving
colonies, in comparison to cells treated only with X-ray radiation. In comparison, targeted NaGdF$_4$:Yb50%-
FA nanoparticles demonstrated superior efficacy with only 40% surviving colonies when treated with 2 Gy radiation.

**Nanoparticles cross the Blood-Brain-Barrier**

To further test the potential application of these ultrasmall nanoparticles to treat brain tumors, the ability to cross the blood-brain barrier (BBB) was explored utilizing a previously reported cell-based two-chamber *in vitro* transwell model of the BBB.[49, 50] Both non-targeted and FA-targeted nanoparticles demonstrate the ability to cross the BBB (Figure 7). After 3 h, only ~5% of the non-targeted NaGdF$_4$:Yb$_{50\%}$ nanoparticles crossed the BBB, whereas ~17% of the targeted NaGdF$_4$:Yb$_{50\%}$-FA crossed. The rate of cell uptake was very gradual for the non-targeted NaGdF$_4$:Yb$_{50\%}$ nanoparticles, and only ~14% were able to cross in 24 h. The targeted NaGdF$_4$:Yb$_{50\%}$-FA nanoparticles saturated uptake at 24 h, and ~34% of the nanoparticles were able to cross BBB at 24 h. Both the non-targeted and targeted nanoparticle uptake had little further uptake between 24 and 72 h.

**Discussion**

To produce uniform ultra-small size nanoparticles utilizing a single-step method, it is critical that sufficient nucleation occurs to ensure uniformity, and the reaction temperature (e.g. 270°C) is reduced to decrease the particle size.[30, 51, 52] It is well established that the hexagonal β-phase NaGdF$_4$ nanoparticles readily form at reaction temperatures below 300°C.[30, 51] This is due to the large radius of the light lanthanide Gd$_{3+}$ ion that is more polarizable and susceptible to the electron cloud distortion required for the cubic-to-hexagonal phase transformation.[33, 53, 54] However, incorporation of the smaller Yb$_{3+}$ ions into the NaGdF$_4$ nanoparticles resulted in an increased free-energy barrier with regards to the formation of the hexagonal phase nanoparticles. Thus, significant doping of the Yb$_{3+}$ ion into the host lattice favors the formation of the cubic phase nanoparticles, which are easily produced due to the high surface energy of the ultra-small nanoparticles. This is in agreement with the results of our synthesis of pure NaGdF$_4$, pure NaYbF$_4$, and NaGdF$_4$:Yb$_{50\%}$ nanoparticles (Figure S4). Allowing nucleation at room temperature for 30 minutes and subsequently growing the nanoparticles at 260°C for 10 minutes yielded hexagonal NaGdF$_4$, while both pure NaYbF$_4$ and NaGdF$_4$:Yb$_{50\%}$ resulted in cubic phase nanoparticles as evidenced by their respective XRD patterns (Figure S1). One way to achieve hexagonal β-NaGdF$_4$:Yb$_{50\%}$ is to increase the temperature to 300°C, but this also leads to formation of larger nanoparticles (~12 nm).[33] Hence, in order to form hexagonal NaGdF$_4$:Yb$_{50\%}$, nucleation and growth are allowed to take place for 24 h to facilitate the formation of thermodynamically stable, hexagonal nanocrystals, while still maintaining the nanoparticle growth reaction temperature at 260°C for 10 minutes to tune the size of the nanoparticles. Pure NaYbF$_4$ was also synthesized with 24 h nucleation to check if β-NaYbF$_4$ can form under such conditions. The XRD pattern (Figure S5) revealed a pure cubic α-phase, indicating that the reaction conditions were not sufficient to transform to hexagonal NaYbF$_4$. Cubic nanoparticle formation
was expected since the formulation did not contain Gd$^{3+}$ ions, which have been established to lower the energy barrier for phase transformation of NaYbF$_4$. [33]

To render the $\beta$-NaGdF$_4$:Yb50% nanoparticles useful for biological applications, it is necessary to modify the hydrophobic oleic-capped surface with a biocompatible, hydrophilic ligand. The proximity of water protons to the surface of the nanoparticles is critical in achieving high T1 relaxivity, which can be controlled through a surface coating strategy.[55] Phase transfer via ligand exchange was then performed to ensure efficient surface hydration. Removal of oleic acid avoids the formation of long hydrophobic chains that could render the Gd on the surface of the nanoparticles inaccessible to water.[56] In this case, cysteine-DTPA replaced oleic acid on the surface of the nanoparticles to form a stable monodisperse aqueous suspension. The small increase in the hydrodynamic diameter post-surface modification, indicates the formation of a compact hydrophilic surface.

The potential toxicity of the non-targeted nanoparticles was investigated to assess their practical usability in a biological environment. One major challenge in the development of a Gd-based contrast agent is the inherent toxicity of the Gd$^{3+}$ ion when dissociated from its chelate in vivo.[57] In the nanocrystal form (i.e., NaGdF$_4$), the hexagonal phase provides a stable matrix that eliminates transmetallation with endogenous metal ions (i.e., Cu$^{2+}$, Zn$^{2+}$, Fe$^{2+}$/Fe$^{3+}$) [58-61] and hinders any leaching of toxic, free Gd$^{3+}$ ions. [62, 63] The very low concentration of Gd$^{3+}$ when dialyzed against H$_2$O demonstrates the high stability of the nanoparticles against dissolution attributed to their thermodynamically stable hexagonal phase. [64] However, the presence of elevated phosphate levels resulted in a significant increase in leakage, although still a low percentage of Gd$^{3+}$, indicating the stability of the nanoparticles in a physiological environment.

It has been demonstrated that the capping ligand has stabilizing effects and can sequester the free Gd$^{3+}$ ions through chelation.[52, 65, 66] To further investigate and minimize the Gd$^{3+}$ leakage, two strategies could be pursued to improve the design of the surface ligand in relation to Gd$^{3+}$ release. First, the amount of DTPA conjugated to cysteine could be optimized. Second, DTPA can be replaced with other polyaminocarboxylate ligands such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and derivatives, which are known to form lanthanide complexes with high kinetic stability.[67, 68]

No intrinsic cytotoxicity from the nanoparticles was observed at a concentration as high as 125 µg/mL, even at prolonged exposure time (i.e., 48 h). In vivo clearance study show that the nanoparticles are cleared from the body within days (i.e., 4 days). Furthermore, the fact that the nanoparticles can be cleared through hepatobiliary excretion, indicates a decrease in kidney load compared to commercially available Gd$^{3+}$ chelates for MRI (i.e., Gd-DTPA) [69] which are primarily cleared renally. This can potentially avoid contrast-induced nephropathy, a form of acute renal failure caused by exposure to the contrast media, and may lower the risk for developing nephrogenic systemic fibrosis, triggered in patients with advanced kidney disease.[70]
After establishing the biocompatibility of the nanoparticles, their ability to be used for dual MR/CT imaging was verified. *In vitro* experiments revealed a substantially higher T1-relaxivity of the nanoparticles compared to a commercial Gd\(^{3+}\) chelate at both room temperature and at physiological temperature (37°C) (Figure 4a), which may be attributed to the slower tumbling rate of the nanoparticle than the chelate.\(^{[71]}\) The higher T1 relaxivity values exhibited by the ultra-small NaGdF\(_4\):Yb50\% nanoparticles compared to clinically-utilized Gd-DTPA, and their low \(r_2/r_1\) ratio value falling below 2 (1.47 at T = 25°C and 1.31 T = 37°C calculated from Figure S3) demonstrate their potential to serve as an effective T1 MR imaging contrast agent.\(^{[72]}\) In addition, the high atomic number of Yb induced enhanced CT signal comparable with iohexol. These results confirm the promise of these nanoparticles in MR/CT multimodal imaging.

The radiosensitization effect of the ultrasmall NaGdF\(_4\):Yb50\% nanoparticles was then assessed in rat C6 glioma cell line. The survival and the reproductive integrity of the irradiated cells with and without nanoparticle treatment were evaluated through colony formation. One strategy to target the delivery of nanoparticles is to exploit the overexpressed folate receptor, found in many cancer cell lines. C6 cells internalize folic acid-conjugated particles through caveolae-mediated endocytosis.\(^{[73]}\) Taking advantage of the highly expressed folate-receptors on C6 glioma cells, nanoparticles with conjugated folic acid (NaGdF\(_4\):Yb50\%-FA) were prepared to improve cellular uptake.

Clonogenic assessment showed increased colony formations with the non-irradiated cells incubated with non-targeted and targeted ultrasmall NaGdF\(_4\):Yb50\% nanoparticles (Figure 6) in comparison to the untreated control cells. When cells are exposed to environmental stress, such as the presence of nanoparticles, autophagy can be induced as an adaptive response, upregulating expressions of genes and proteins that induce cytoprotection and promote cell survival.\(^{[74-76]}\) Irradiation of cells without nanoparticles did not result in any significant effect on the colony area at 2 Gy dose, suggesting the low intrinsic radiosensitivity of C6 cells.\(^{[77]}\) Nevertheless, they seemed to form more smaller colonies indicating some effect on their reproductive capacity. On the other hand, both the non-targeted and targeted nanoparticles have clearly shown radiosensitization. These results are in agreement with the recent study investigating the cytotoxicity and radiosensitization of several rare-earth oxide nanoparticles (i.e. Ce, Nd, Gd, La), wherein Gd\(_2\)O\(_3\) nanoparticles have shown significant radiosensitization and have generated additional ROS in U-87 MG cell line upon irradiation, without intrinsic toxicity.\(^{[78]}\) As evidenced by a significant difference in the surviving colonies between the non-targeted (NaGdF\(_4\):Yb50\%) and the targeted (NaGdF\(_4\):Yb50\%-FA) nanoparticles at the same concentration, it is imperative that the nanoparticles be associated with the cells to induce effective damage. A new study has shown near complete destruction of tumor spheroids of human ovarian cancer (OVCAR8) when incubated with gadolinium loaded mesoporous silica nanoparticles (Gd-MSN) prior to exposure to monochromatic 50.25 KeV X-rays.\(^{[79]}\) It is worth noting that the Gd-MSNs accumulated in the lysosomes located close to the cell nucleus. This highlights the importance not only of the energy compatibility made possible by using tunable monochromatic beam radiation, but also by the proximity of the radiosensitizers to the nucleus in order to destroy the DNA of the tumor cells. This is due to the low energy and consequent short-range...
characteristics of the Auger electrons from the Gd$^{3+}$ and Yb$^{3+}$ ions in the nanoparticles provide for the possibility of a highly targeted radiation therapy.

In several reported studies, folate-conjugated drug delivery systems have shown significant nuclear uptake.[80-82] Folic-acid modified silica nanoparticles (FAMSNs) with 100 nm diameter have been observed to accumulate in both the nuclei and the cytoplasm, while unmodified MSNs were found only in the cytoplasm, which confirmed the role of folic acid receptors in the nuclear uptake.[81] Presence of folic acid receptor α (FRα) in the nuclear membrane has been reported. [83, 84] It has also been demonstrated that in the presence of folic acid, FRα translocates to the nucleus. [83, 85] This mechanism of folic acid is highly compatible in the targeted delivery of radiosensitizers. Combined with the additional multi-modal imaging capabilities of the nanoparticles, localization in the tumor can be ensured prior to irradiation, therefore the damage to the surrounding normal cells is minimized if not completely prevented. Furthermore, in vitro transmigration assay confirms that both non-targeted and targeted nanoparticle were able to cross the BBB, with the folic acid-modified nanoparticles being 2.4-fold higher. These results further confirm the effectiveness of using folic acid as a target molecule to facilitate transport through BBB.

This study has several limitations. Firstly, although the equimolar ratio of Gd and Yb have shown to achieve the desired properties of CT and MR contrast enhancement and radiosensitization, an optimal ratio between Gd and Yb can only be determined by preparing these hexagonal ultrasmall nanoparticles with different Gd and Yb ratios. Secondly, in vivo MR and CT imaging still need to be performed in order to evaluate the efficacy of these nanoparticles as dual contrast agents. Thirdly, the in vivo biodistribution and clearance studies were not performed in GBM-bearing mice to evaluate the percentage and half-life of the nanoparticles that cross the BBB of a diseased animal model. As folate receptors are overexpressed in GBM, it is possible that a higher nanoparticle concentration will be internalized by the brain tumors, which could affect the biodistribution in the brain. A survival study of post-irradiated mice with and without these radiosensitizers have yet to be done in order to assess their safety and efficacy in vivo.

Conclusions

A novel, ultrasmall sub-5 nm NaGdF$_4$:Yb50% formulation designed to combine imaging and therapy was successfully synthesized and surface modified to render biocompatibility and enhanced cellular uptake. Co-doping of Gd and Yb in equimolar amount allowed the formation of the hexagonal phase of the nanoparticle as well as imparting the nanoparticle with multifunctionality to be used as a bimodal probe for both MR and CT imaging with excellent T1 contrast for MRI and Hounsfield unit (HU) for CT imaging. Bioconjugation of folic acid to the surface of these nanoparticles facilitated BBB crossing and increased cellular uptake to enable efficient radiosensitization effects from the emitted low energy Auger electrons in brain cancer cells. In vitro radiosensitization experiments in rat C6 glioma cells showed the FA-targeted nanoparticles as very promising radiosensitizers. Hence, these ultrasmall nanoparticles should be further developed to serve as a promising theranostic platform for image-guided radiotherapy.
Materials And Methods

Materials

Gadolinium chloride hexahydrate (99.999%), ytterbium chloride hexahydrate (99.9%), ammonium fluoride (99.99), sodium hydroxide (97%), oleic acid (90%), 1-octadecene (90%), oleylamine (70%), L-cysteine (97%), diethylenetriaminepentaacetic dianhydride (98%), and H2O2 (30%) were purchased from Sigma-Aldrich. Methanol (ACS reagent grade, ≥ 99.8%), hexane (ACS reagent grade, ≥ 98.5%), and chloroform (ACS reagent grade, ≥ 99.8%) were purchased from Fisher Scientific. Gadolinium and ytterbium standards for ICP are from Inorganic Ventures and high purity nitric acid for quantitative trace metal analysis at the ppb level is from BDH Aristar® Plus. All materials were used as received.

Synthesis

Synthesis of Ultrasmall α-NaGdF4:Yb50%. Ultrasmall nanoparticles were synthesized by modification of a previously reported procedure.[30, 86] To a 100mL three-neck flask containing 0.5 mmol of GdCl3 × 6H2O and 0.5 mmol of YbCl3 × 6H2O were added 9 mL of oleic acid and 15 mL octadecene. The mixture was heated to 160°C and maintained for 1h under argon gas with constant stirring and then cooled to room temperature. A solution of methanol (10 mL) containing 4 mmol NH4F and 2.5 mmol NaOH was added and the mixture was stirred for 30 minutes. The temperature is then increased to 100°C and maintained for 30 minutes to remove methanol. The solution was then heated at 260°C for 10min before cooling to room temperature. The nanoparticles were collected by adding an excess amount of ethanol and centrifuged at 7000 rcf for 5 min. The precipitate was washed with ethanol and finally dispersed in 10 mL hexane for further uses.

Synthesis of Ultrasmall β-NaGdF4:Yb50%. Ultrasmall nanoparticles were synthesized following the procedure described for α-NaGdF4:Yb50%, except the solution was stirred for 24 h after the addition of methanol solution (10 mL) containing NH4F (4 mmol) and NaOH (2.5 mmol).

Synthesis of Ultrasmall β-NaGdF4. Ultrasmall nanoparticles were synthesized following the procedure described for α-NaGdF4:Yb50%, except 1.0 mmol of GdCl3 × 6H2O was used.

Synthesis of Ultrasmall α-NaYbF4. Following the procedure described for both α-NaGdF4:Yb50% and β-NaGdF4:Yb50%, except using 1.0 mmol of YbCl3 × 6H2O, resulted in cubic ultrasmall nanoparticles only.

Ligand Exchange Surface Modification. L-Cysteine (60 mg) and diethylenetriaminepentaacetic (DTPA) dianhydride (20 mg) were dissolved in 30 mL H2O at pH 9 in a 100 mL round bottom flask. To this aqueous solution was added 10 mL chloroform solution containing 10 mg of the oleic-capped ultrasmall nanoparticles. The biphasic mixture was stirred vigorously overnight at room temperature to facilitate the transfer of the nanoparticle to the water phase. Excess ligand was removed by twice centrifugation using
Vivaspin-20 centrifugal filters (10kDa MWCO) at 3000 rcf for 15 minutes and the collected nanoparticles were redispersed in water and filtered through a 0.2 μm syringe filter.

**Folic-acid Functionalized Ultrasmall Nanoparticles (FA-NaGdF₄:Yb50%).** Five hundred microliters of folic acid dissolved in DMSO (25 mg/mL) in the presence of triethylamine (6.25 μL) was incubated with 6.5 mg of NHS and 6.25 mg of DCC in the dark overnight and then passed through a 0.2 μm filter. The resulting NHS-activated folic acid was then covalently linked to the amino surface of the nanoparticles provided by cysteine ligand by incubating overnight. The resulting NaGdF₄:Yb50%-FA was centrifuged at 16000 rcf for 15 minutes, washed twice and stored in 1 mL H₂O for future use.

**Characterization**

The size and the morphology of the resulting nanoparticles were characterized by transmission electron microscopy (TEM) using a JEM-2010 microscope at an acceleration voltage of 200 kV. The hydrodynamic size was determined using Malvern Zetasizer NanoZS90. Powder X-ray diffraction (XRD) patterns were recorded by a RigakuUltima IV diffractometer, using Cu Kα radiation ($\lambda = 0.15418$ nm). The 2θ angle of the XRD spectra was recorded at a scanning rate of 1°/min. Inductively coupled plasma-optical emission spectrometer (ICP-OES) analysis was performed using a Thermo Scientific iCAP 6000 instrument. CT tests were performed on microCTInveon model scanner (Siemens Medical Solutions USA, Inc.). T1 and T2 rates of the nanoparticles were measured on a 4.7T preclinical MR scanner using increasing concentrations at both 25°C and 37°C with an inversion-recovery, balanced steady-state free precession (IR-bSSFP) sequence, and a multiecho CPMG scan, respectively, as described elsewhere. [87] T1 and T2 relaxivities (mM⁻¹·s⁻¹) of the nanoparticles were compared to the commercially-available Gd-DTPA contrast agent, Magnevist®.

**Elemental analysis using ICP-OES**

Acid digestion was performed by dissolving 0.15 mg of the nanoparticles in 0.5 mL concentrated high purity HNO₃ acid overnight and diluting with a 2% HNO₃ solution to a total volume of 15 mL. The single element standards were prepared with the same acid solution.

**Gd³⁺ ion leaching**

The nanoparticles (5 mL, 1 mM Gd) were loaded into a dialysis tubing (Spectrum, 3.5 kD cut-off) and incubated in H₂O, or DMEM with 10% fetal bovine serum (FBS), or DMEM with 10% FBS supplemented with 10mM phosphate, at 37°C under sink conditions, with rocking for 3 days. The amount of released Gd³⁺ ions in each solution was measured using ICP-OES.

**Biodistribution and Clearance**

Animal experiments were performed in compliance with guidelines set by the University at Buffalo Institutional Animal Care and Use Committee. Female CD-1 mice were injected intravenously via tail vein
with the nanoparticles in 5% dextrose in water at a dose of 2 mg/kg and housed in metabolic cages for 4 days with free access to water and a standard laboratory diet. Urine and feces were collected separately every 4 h and the mice were sacrificed after 96 h through cervical dislocation. Feces and organs including liver, spleen, kidney, brain, heart and lungs were harvested, frozen and weighed prior to digestion. The urine, feces, and isolated organs were individually placed in a screw cap polypropylene sample tube and to each were added 3 mL of concentrated nitric acid and 2 mL peroxide (30% by weight) and pre-digested for 24 h. The tubes were then placed in a sonicated water bath for a total of 8 h until the samples were completely dissolved. After digestion, each sample was diluted to 100 mL with a 2% solution of nitric acid. The samples were then passed through a 0.2 μm filter and the Gd content was quantified with inductively coupled plasma mass spectrometry (ICP-MS) utilizing a Thermo Scientific XSERIES 2 ICPMS Single Quadrupole Mass Spectrometer.

**Cytotoxicity Assay**

Cell viability was assessed by the PromegaCellTiter 96® AQuieux One Solution Cell Proliferation (MTS) Assay. C6 cells were seeded into a 96-well flat-bottom microplate (c.a. 10000 cells/well) at 37°C and 5% CO₂ and allowed to attach to the bottom of the microplate overnight. The cells were then treated with different concentrations of NaGdF₄·Yb50% nanoparticles for 12, 24, and 48 h. After the treatment, the cellular medium was changed to remove the nanoparticles and cell debris, and the AQuieux One Solution reagent (20 µl/well) was added to the cells and incubated for 4 h. Finally, the absorbance was measured at 490 nm using a microplate reader (Opsys MR microplate reader) to determine the percentage of viable cells in the culture relative to the control wells without nanoparticle treatment.

**Clonogenic Assay**

Clonogenic assay was performed by growing C6 cells in 6-well plates to 90% confluence and were treated with 100 µg/mL concentration of the nanoparticles overnight. Afterwards, cells were irradiated with a 2 Gy X-ray dose using the Faxitron® RX-650 X-ray Irradiator at a dose rate of 0.5 Gy/min delivered using 130 kV energy. Plates were then incubated for 4 h at 37 °C in 5% CO₂, and the cells were subsequently harvested and counted. To assess colony formation, cells were then re-plated at 1000 cells/well in 6-well plates and allowed to form colonies consisting of 50 cells. Colonies were then gently washed with Hank’s Balanced Salt Solution (Gibco® HBSS) and fixed with ice-cold methanol for 10 minutes, rinsed once again with HBSS and stained with a 0.5% crystal violet solution for another 10 minutes. Plates were then rinsed with H₂O to remove excess stain and were left to dry at room temperature. Images of the plates were then acquired and saved in the tagged image file format (Tiff). The colony area for each plate was then measured using the Colony Area plugin[88] in ImageJ. Surviving colonies were normalized against control wells without nanoparticle treatment.

**In vitro BBB Transmigration Assay**
We made and validated a cell-based in vitro transwell model of the BBB in our laboratory and used it to examine BBB properties like quantitative permeability and transendothelial migration of nanoparticles. Our 2D in vitro BBB model consists of a two-chamber transwell system in a 12 well culture plate with the upper (luminal) compartment separated from the lower (abluminal) by a semipermeable membrane (polyethylene terephthalate, PET) insert on which the Human brain microvascular endothelial cells (BMVECs) were grown to confluence on the upper side, while a confluent layer of normal human astrocytes (NHAs) was grown on the underside. After tight BBB formation was confirmed by the transendothelial electrical resistance (TEER) measurement, the dispersed nanoparticles (100 µg/mL media) were added to the upper chamber (luminal) and incubated at 37°C in 5% CO₂. Media from the lower chamber (abluminal) were collected at 1, 5, 24, 48 and 72 h incubation times and the Gd content was measured using ICP-OES. Percent transmigration was calculated relative to the initial Gd concentration of the media with 100 µg/mL nanoparticles. The TEER was measured again after their crossing of the BBB to make sure that the transmigration was not due to the compromise of BBB.

List Of Abbreviations

MRI: magnetic resonance imaging
CT: computed tomography
RT: radiotherapy
GBM: glioblastoma
BBB: blood-brain barrier
FBS: fetal bovine serum
DMEM: Dulbecco's modified Eagle medium
DTPA: diethylenetriaminepentaacetic
FA: folic acid
MTS: 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium

Declarations

Ethics Approval and Consent to Participate

Animal experiments were performed in compliance with guidelines set by the University at Buffalo Institutional Animal Care and Use Committee.

Consent for Publication
Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its additional information on file).

Competing interests

The authors declare that they have no competing interests

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Authors’ contributions

All authors listed have made substantial, direct, and intellectual contributions to the work discussed in this publication. JAD, TYO, SM, GC, and PNP designed the study. JAD, SM, AS, HLH, HH, STG, and JAS performed the experiments and analyzed the data. SM, JFL, and AKS provided resources to ensure completion of the project. MS and PNP provided the funding. PNP supervised the study. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

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Appendix A. Supporting Information

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**Figures**

![Figure 3](image_url)

Biocompatibility of H2O-dispersed β-NaGdF4:Yb50% (a) ICP-OES analysis of the Gd3+ ions leaching from the nanoparticles after 3 days of dialysis. (b) Cell viability when incubated with the nanoparticles evaluated by MTS assay. (c) Biodistribution at 4 h post-injection via tail vein measured by ICP-MS. (d) Cumulative renal and fecal clearance of the nanoparticles monitored by Gd3+ determination via ICP-MS.
Figure 6

Effect of the nanoparticle treatment on the colony formation of C6 cells following 2 Gy X-ray irradiation. The cells were incubated with the nanoparticles overnight prior to the irradiation. The surviving fraction for each treatment was tested using two-way ANOVA with Tukey’s multiple comparisons test. (*P<0.05; *** P<0.001)