This document is the accepted manuscript version of the following article:
Gerken, L. R. H., Keevend, K., Zhang, Y., Starsich, F. H. L., Eberhardt, C.,
Panzarasa, G., ... Herrmann, I. K. (2018). Lanthanide-doped hafnia nanoparticles for
multimodal theranostics: tailoring the physicochemical properties and interactions
with biological entities. ACS Applied Materials and Interfaces. <https://doi.org/10.1021/acsami.8b20334>

Lanthanide-doped Hafnia Nanoparticles for Multimodal Theranostics: Tailoring the Physicochemical Properties and Interactions with Biological Entities

Lukas R. H. Gerken¹, Kerda Keevend¹, Yucheng Zhang², Fabian H. L. Starsich³, Christian
Eberhardt⁴, Guido Panzarasa⁵, Martin T. Matter¹, Adrian Wichser^{1,6}, Andreas Boss⁴, Antonia
Neels⁷ and Inge K. Herrmann^{1,*}

¹ Particles 4D Group, Laboratory for Particles Biology Interactions, Swiss Federal Laboratories for Materials
Science and Technology (Empa), Lerchenfeldstrasse 5, CH-9014, St. Gallen, Switzerland.

² Electron Microscopy Center, Swiss Federal Laboratories for Materials Science and Technology (Empa),
Überlandstrasse 129, CH-8600, Dübendorf, Switzerland.

³ Particle Technology Laboratory, Institute of Process Engineering, Department of Mechanical and Process
Engineering, ETH Zurich, Sonneggstrasse 3, CH-8092 Zurich, Switzerland.

⁴ Department of Radiology, University Hospital Zurich, Rämistrasse 100, CH-8091 Zurich, Switzerland.

⁵ Laboratory for Biomimetic Membranes and Textiles, Swiss Federal Laboratories for Materials Science and
Technology (Empa), Lerchenfeldstrasse 5, CH-9014, St. Gallen, Switzerland.

⁶ Laboratory for Advanced Analytical Technologies, Swiss Federal Laboratories for Materials Science and
Technology (Empa), Überlandstrasse 129, CH-8600, Dübendorf, Switzerland.

⁷ Center for X-ray Analytics, Swiss Federal Laboratories for Materials Science and Technology (Empa),
Überlandstrasse 129, CH-8600, Dübendorf, Switzerland.

ABSTRACT

High-Z metal oxide nanoparticles hold promise as imaging probes and radio-enhancers. Hafnium dioxide nanoparticles have recently entered clinical evaluation. Despite promising early clinical findings, the potential of HfO₂ as a matrix for multimodal theranostics is yet to be developed. Here, we investigate the physicochemical properties and the potential of HfO₂-based nanoparticles for multimodal theranostic imaging. Undoped and lanthanide (Eu³⁺, Tb³⁺, and Gd³⁺)-doped HfO₂ nanoparticles were synthesized and functionalized with various moieties, including poly(vinylpyrrolidone) (PVP), (3-aminopropyl)triethoxysilane (APTES) and folic acid (FA). We show that different synthesis routes, including direct precipitation, microwave-assisted synthesis, and sol-gel chemistry allow preparation of hafnium dioxide particles with distinct physicochemical properties. Sol-gel based synthesis allows preparation of uniform nanoparticles with dopant incorporation efficiencies superior to the other two methods. Both luminescence and contrast properties can be tweaked by lanthanide doping. We show that MRI contrast can be unified with radio-enhancement by incorporating lanthanide dopants in the HfO₂ matrix. Importantly, ion leaching from the HfO₂ host matrix in lysosomal-like conditions was minimal. For Gd:HfO₂ nanoparticles, leaching was reduced >10× compared to Gd₂O₃ and no relevant cytotoxic effects have been observed in monocyte-derived macrophages for nanoparticle concentrations up to 250 µg/mL. Chemical surface modification allows further tailoring of the cyto- and hemocompatibility and enables functionalization with molecular targeting entities, which lead to enhanced cellular uptake. Taken together, the present study illustrates the manifold properties of HfO₂-based nanomaterials with prospective clinical utility beyond radio-enhancement.

KEYWORDS. bioimaging, functionalization, hafnium dioxide, magnetic resonance imaging, x-ray

INTRODUCTION

While treatment modalities such as surgical resection, chemotherapy and radiotherapy have improved the survival rate of patients affected by cancer, resistance acquired in the course of therapy eventually can weaken the overall therapeutic success.¹ Various approaches have been developed to enhance safety and efficacy of radiotherapy.^{1,2} Radio-enhancers hold promise to locally maximize radiation damage and increase the efficacy of cancer cell killing while limiting damage to healthy cells.³⁻⁴ Several nanoparticle-based radio-enhancers have been developed in the recent years based on high-Z materials including gold,⁵ platinum,⁶ and various metal oxides.⁷ Such nanoparticles can be designed to enter tumor cells and deposit high levels of energy upon exposure to ionizing radiation, leading to DNA damage and cell destruction.⁸ Gold⁵ and platinum⁶ have been a natural choice because of their high Z-numbers and correspondingly high mass absorption, however, their utility in terms of multimodal contrast is limited. It has also been shown that radio-sensitizing performance depends on a complex interplay of physical, chemical and biological effects rather than high mass absorption alone.⁹ The predominant physical processes for high energy deposition in nanoparticle-based radio-enhancement include element specific emission of Auger electrons for irradiation by keV beams, and Compton and electron scattering for MeV beams, which in turn depend only very little on atomic number.⁹⁻¹⁰ Interestingly, a significant additional radio-sensitization effect has also been predicted for materials with lower densities, such as Europium, Gadolinium or Ytterbium, based on reduced self-absorption of secondary electrons.⁹ To synergistically exploit high mass absorption and reduced self-absorption, hybrid materials combining high-Z materials with lighter ones are of particular interest. Metal oxides with good radio-enhancement properties and high cytocompatibility, such as bismuth oxide¹¹ and hafnium oxide¹², among others, are excellent host matrices for rare-earth element dopants giving rise to multimodal properties with promising prospective theranostic applications. However, despite encouraging advances, clinical translation of nanotechnology-based systems in general remains a slow process.¹³ Inefficient delivery and off-target effects¹⁴ of non-degradable¹⁵ radio-enhancers are major obstacles. While metals and metal oxides with high atomic numbers are ideally suited as

nanoparticulate radio-enhancers, most of these materials are chemically relatively inert and poorly degradable, with the exception of Gd_2O_3 , which releases toxic Gd^{3+} ions upon dissolution. Among the different radio-sensitizers under development for clinical use, hafnium dioxide (hafnia, HfO_2) is one of the most advanced ones.⁸ A first HfO_2 nanoparticle-based product for radio-enhancing has recently completed phase II/III studies in patients with locally advanced soft tissue sarcoma.⁸ In this case, pure (undoped) HfO_2 nanoparticles were directly injected into cancerous lesions to increase treatment efficacy without causing detectable damage to surrounding healthy tissues. Hafnium dioxide features interesting properties, including a high atomic number ($Z = 72$), high density (9.7 g/cm^3), as well as chemical and physical inertness (insulating, redox-inactive material).¹⁶ HfO_2 nanoparticles have X-ray absorption properties superior to clinically used iodine, and comparable to gold nanoparticles.¹⁷ Despite the promising findings in preclinical and clinical settings, the full potential of HfO_2 as a system for multimodal theranostics has yet to be harnessed. There is a need for nanoparticle-based radio-enhancers, which unify excellent imaging contrast properties with radio-enhancement and high biocompatibility.

Here, we seek to investigate the potential of HfO_2 -based nanoparticles for multimodal theranostic applications. We show that different synthesis routes allow preparation of HfO_2 particles with distinct physicochemical properties. Then, we illustrate how luminescence and contrast enhancement properties can be altered by lanthanide ion doping of the HfO_2 matrix, unifying radio-enhancement with multimodal imaging in a single nanoparticle formulation. We show minimal guest ion leaching from the HfO_2 matrix in lysosomal-like conditions. Finally, we report on how chemical surface modification further allows tailoring of cyto- and hemocompatibility, and bioconjugation with molecular targeting moieties.

RESULTS AND DISCUSSION

HfO_2 nanoparticles were synthesized by three different methods. Figure 1a shows transmission electron microscopy (TEM) images of HfO_2 nanoparticles prepared by direct precipitation (I),^{18,19} microwave-

assisted precipitation (II)²⁰⁻²¹ and sol-gel precipitation (III).^{17,22,23} Precipitated particles were initially amorphous as seen from the corresponding X-ray diffraction (XRD) patterns (Figure 1b). Subsequent calcination at 500°C induced a transformation into a monoclinic crystal phase. While the direct precipitation method yielded particles of irregular shape, the nanoparticles obtained by the sol-gel method were comparatively uniform and predominantly of ellipsoidal shape, similar to the particles obtained by Pinna *et al.*²⁴. XRD of the particles synthesised by the sol-gel route confirmed a monoclinic crystal phase after calcination at 600°C for 2 h. While the direct precipitation and sol-gel approaches both yielded crystalline nanoparticles with a crystalite size of 10 nm (see ESI, Table S1), the particles synthesized by the microwave-assisted approach were amorphous. Even after calcination at 500°C for 2 h, the microwave-synthesized nanoparticles remained poorly crystalline.

Figure 1. Transmission electron micrographs of hafnium dioxide nanoparticles prepared by direct precipitation (I), microwave-assisted precipitation (II) and sol-gel precipitation (III) methods (a). Corresponding X-ray diffraction patterns (b).

To investigate the potential of HfO₂ as a host matrix for rare-earth element ions, the nanoparticles were doped with different rare-earth elements, introducing luminescent Eu³⁺, Tb³⁺ ions or magnetic Gd³⁺ ions. To assess the incorporation of dopants into the crystal lattice, inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis, XRD and selected area diffraction (SAED) analysis were

performed. The XRD patterns for different undoped and doped HfO₂ nanoparticles following calcination at 600°C are shown in Figure 2. While undoped HfO₂ is present predominantly as monoclinic phase, a continuous transition into the cubic phase is detected with an increasing concentration of trivalent rare-earth element dopants. For both synthesis routes, particles with a dopant concentration of 5 mol% show co-existence of monoclinic and cubic phases for both Eu³⁺ and Tb³⁺. On the other hand, the particles doped with 10 mol% of trivalent atoms (Eu³⁺, Tb³⁺) show a cubic phase, regardless of synthesis method. However, dopants are not always reliably incorporated, as in the case of precipitated 10 mol% Eu:HfO₂ (Table S1, ESI). Instead of 10 mol% the nanoparticles only contained 5 mol% of Eu³⁺. Consequently, the XRD pattern of the precipitated Eu³⁺-doped HfO₂ showed no stabilized cubic phase. Generally, the deviation of doping concentration found experimentally compared to the theoretical concentration is considerably higher for precipitated particles (relative mean deviation 51%) than for sol-gel particles (relative mean deviation 15%), suggesting that for the incorporation of rare-earth elements the sol-gel method is preferred.

The changes in crystal structure were further investigated by selective area electron diffraction (SAED). Figure 2c shows the indexed crystal planes for sol-gel synthesized HfO₂ nanoparticles undoped and doped with 5 and 10 mol% Eu³⁺, respectively. The ring patterns indicate the presence of randomly-oriented nanocrystals. Table S2 (ESI) shows the indexed and measured interplanar *d**hkl* spacings along with values from the database.²⁵ In agreement with XRD, the diffraction pattern changes from a monoclinic polymorph (undoped HfO₂) to a cubic polymorph in the case of 10 mol% Eu: HfO₂ nanoparticles. The (11-1) and the (111) plane merge into each other indicating higher symmetry. This change in crystal structure is also apparent in the Terbium-doped samples. SAED patterns and corresponding interplanar spacings can be found in Figure S1 and Table S2 (ESI), respectively. The stabilization of the cubic phase due to incorporation of trivalent ions into the crystal lattice is in agreement with previous reports. Lauria and colleagues²⁶ found a phase change at 6.5 mol% Eu³⁺. Several studies²⁷⁻²⁸ state that the phase change due to the trivalent ion incorporation is stabilized by the generation of structural oxygen vacancies for

charge compensation. Rauwel *et al.*,²⁸ Lu *et al.*,²⁹ and Kumar *et al.*³⁰ were able to stabilize the cubic phase without using dopants by synthesizing the particles in a more reductive environment. The incorporation of Eu^{3+} and Tb^{3+} ions into the HfO_2 nanoparticles lead to luminescence in the visible region (Figure 3). The emission intensities after excitation with UV-light are related to the dopants and their energy levels. For $\text{Tb}:\text{HfO}_2$, the emission lines at 490, 544, 588 and 622 nm originate from the $^5\text{D}_4 \rightarrow ^7\text{F}_{6,5,4,3}$ transitions within the 4f electron configuration of terbium (Figure 3a).

Figure 2. X-ray diffraction patterns for undoped and rare-earth element (Tb^{3+} , Eu^{3+})-doped hafnium dioxide nanoparticles synthesized by sol-gel (a) and direct precipitation (b). Diffraction patterns for monoclinic and cubic hafnium dioxide are shown for reference. Selected area electron diffraction (SAED) patterns with d_{hkl} indexing for monoclinic and cubic phases confirm the transition from the monoclinic to cubic phase with increasing concentration of dopants (c).

The 10 mol% Tb:HfO₂ nanoparticles show emission intensities four-times stronger than the one with 5 mol% doping. The maximum excitation intensity for the Tb³⁺ emission at 544 nm is found, at 267.2(2) nm (4.640(4) eV) and 268.7(2) nm (4.614(3) eV) for 10 and 5 mol% Tb³⁺ doping, respectively. These excitation peaks can be referenced as charge transfer bands (CTB) and are attributed to an O²⁻ → Tb³⁺ electron transfer reducing Tb³⁺ to Tb²⁺, in analogy to Eu³⁺-doped materials.³¹⁻³² The more energetic but smaller excitation peaks were found at 221.1(8) nm (5.61(2) eV) for 10 mol% doping and 229.5(7) nm (5.40(2) eV) for 5 mol% doping. These values are close to the reported band gap value of 5.7 eV for HfO₂ and can be assigned to the conduction bands (CB) of the host lattice.³³ The blueshift in the CB with increased Tb³⁺ concentration can be attributed to the decreased crystal size induced by the doping (quantum confinement effect).³¹

Europium emission lines at 592, 612, 630, 654, 712 and 746 nm (Figure 3b) correspond to the ⁵D₀ → ⁷F_{1,2,3,4,5} transitions.³² In contrast to Tb:HfO₂ nanoparticles, the luminescence intensity is reduced with the higher level of doping. The ⁵D₀ → ⁷F₂-emission of the Eu:HfO₂ nanocrystals is maximally excited at 240.4(2) nm (5.157(4) eV) for the 10 mol% doping and 231.6(3) nm (5.353(7) eV) for the 5 mol% doping, respectively, corresponding to the CTB. The bands at 208.0(2) nm (5.961(6) eV) for 10 mol% and 209.5(3) nm (5.92(1) eV) for 5 mol% Eu³⁺ correspond to the CB. A similar red shift of the CTB and a blue shift of the CB was also found by Boukerika *et al.*³¹ in 1 mol% Eu³⁺ doped Y₂O₃ for low annealing temperatures, which was explained by a smaller crystallite size. Jayaraman *et al.*¹⁸ have also found a blue shift of the energy gap with decreased size of HfO₂ nanoparticles. In contrast to Tb:HfO₂, both excitation bands lie within the reported band gap values (5-6 eV).³⁴ Compared to Tb:HfO₂, the Eu:HfO₂ has a smaller d_{XRD}, explaining the higher CB excitation energy. This band arrangement excludes energy-back transfer processes³⁵ and thus the lower emission for the higher dopant concentration is most likely caused by concentration quenching. When dopant distance is decreased there is an increased probability to reach a path of non-radiative decay and the excitation energy is transferred between many ions in the time necessary for the radiative decay.³⁶ This effect was not apparent in Tb:HfO₂ nanoparticles when

comparing 5 mol% vs. 10 mol% dopant concentrations. However, another study has found an optimal Tb^{3+} concentration of 7% and significant concentration quenching for Tb^{3+} concentrations above 10%.³⁷

Figure 3. Excitation and emission spectra for hafnium dioxide nanoparticles doped with 5 and 10% terbium (a) and europium (b). Lifetime bi-exponential fits for (Tb³⁺, Eu³⁺)-doped nanocrystals (c). Photographs of the different colloids under UV-light (254 nm) (d).

Time-resolved measurements of the most intense Eu^{3+} and Tb^{3+} emissions show lifetimes in the range of milliseconds. Figure 3c shows the bi-exponential curve fits to the time resolved emission data. For 5 and 10 mol% Eu^{3+} doped nanoparticles the mean lifetimes are $\tau_{5\text{Eu}} = 0.582(56)$ ms and $\tau_{10\text{Eu}} = 0.725(62)$ ms. These are slightly shorter than the 1.2 ms lifetimes observed in Eu^{3+} doped 200 nm thin HfO_2 films prepared by atomic layer deposition³⁸ but longer than the 116 μs in Eu^{3+} -doped thin HfO_2 films prepared by sol-gel³⁹, and comparable to values found by Lauria *et al.* for $\text{Eu}:\text{HfO}_2$ nanoparticles.²⁶ For Tb^{3+} -doped nanoparticles, the lifetimes are slightly longer: $\tau_{5\text{Tb}} = 1.29(31)$ ms and $\tau_{10\text{Tb}} = 0.947(79)$ ms. These values are significantly higher than the 134 μs reported for sol-gel-prepared Tb^{3+} -doped HfO_2 films.³⁹ The

photoluminescence of Tb^{3+} and Eu^{3+} doped HfO_2 nanoparticles can easily be observed under UV light (Figure 3d).

In order to further explore the multimodal properties and particularly the possibility of developing HfO_2 nanoparticle-based agents for MRI-guided radiotherapy, Gd:HfO_2 nanoparticles (10 mol% doping) and pure gadolinium oxide (Gd_2O_3) nanoparticles were synthesized via the same sol-gel method in addition to the above particles. Gadolinium is a magnetic resonance imaging (MRI) active trivalent ion. Gd_2O_3 nanoparticles have been suggested for dual imaging⁴⁰ and radio-enhancement.⁹ However, toxicity of Gd_2O_3 , and released Gd^{3+} in particular, remain a major obstacle. Therefore, Gd:HfO_2 nanoparticles were synthesized and performance was compared to pure Gd_2O_3 . Both Gd:HfO_2 and pure Gd_2O_3 nanoparticles show a cubic structure and a crystallite size of 6(2) nm as revealed by XRD measurements (Figure 4a, Table S1, ESI). The size and crystallinity of the nanoparticles was confirmed by TEM and SAED images (Figure S2, ESI). Similar to the trivalent Tb^{3+} and Eu^{3+} doping, 10 mol% Gd^{3+} -ion dopant concentration stabilizes the cubic phase of HfO_2 and reduces the crystallite size. The 10 mol% dopant concentration was confirmed by ICP-MS (Table S1, ESI).

The MRI contrast properties were determined for undoped HfO_2 , Gd:HfO_2 and Tb:HfO_2 nanoparticles, and compared to pure Gd_2O_3 (Figure 4b,c). Undoped HfO_2 nanoparticles showed neither T_1 nor T_2 contrast. There was also no T_1 contrast seen for Gd:HfO_2 and Tb:HfO_2 nanoparticles. On the other hand, T_2 contrast properties of HfO_2 could be significantly improved by incorporating either Gd^{3+} or Tb^{3+} ions thus potentially enabling image-guided procedures (see also ESI, Table S5 for a comparison to r_2 values reported for comparable systems). Tb:HfO_2 nanoparticles are of particular interest because of their superior cytocompatibility compared to Gd_2O_3 (*vide infra*).

Figure 4. XRD pattern of HfO₂, gadolinium oxide and Gd:HfO₂ nanoparticles including reference patterns for cubic and monoclinic HfO₂ (a). Magnetic resonance imaging (MRI) T₁ (b) and T₂ (c) properties of un-doped, 10% Gd:HfO₂, and 10% Tb:HfO₂ nanoparticles. Pure gadolinium oxide is shown as a reference. Ion leaching from the HfO₂ matrix compared to pure gadolinium oxide in lysosomal buffer (d).

For prospective applications in theranostics, radio-enhancement properties of the doped HfO₂-based nanoparticles are particularly interesting especially for image-guided therapy applications. We therefore

assessed the nanoparticle radio-enhancement factors in a modified colonogenic assay using CaCo-2 cells. At 2 Gy, HfO₂ nanoparticles showed a nanoparticle enhancement ratio (NER2) of 0.75 ± 0.14 . Dopants in this case had only minor additional effects on the NER (*e.g.*, NER2 of 0.75 ± 0.08 (Tb:HfO₂)). These values are comparable to values found for other nanoparticle-based radio-enhancers in similar settings.⁴¹ Taken together, these results illustrate that HfO₂ nanoparticles can be transformed into multimodal theranostic agents for combined CT, MRI imaging and radio-enhancement by quantitative lanthanide-doping.

In clinical practice, leaching of rare-earth ions, and particularly gadolinium ions, has become a major concern. This is particularly important in the context of nephrogenic systemic fibrosis (NSF), which is believed to be a consequence of Gd³⁺ ion leaching and accumulation following exposure to gadolinium-based contrast agents.⁴² Thus, we explored whether HfO₂ can be employed as a stable host lattice to limit Gd³⁺ or Tb³⁺ leaching. To determine ion leaching from the matrix in relevant conditions, 10 mol% Gd:HfO₂ and 10 mol% Tb:HfO₂ nanoparticles were dispersed at 1 mg/mL concentration in citrate buffer at lysosomal pH (pH 4.5). Subsequent ICP-OES measurements of the supernatants reveal that after 1 day of incubation at 37 °C, 4 to 5 µg/mL of Tb³⁺ and Gd³⁺ ions leached into the medium, corresponding to 5 to 7 % of the total lanthanide dopant content (Figure 4d). This relates to the 10⁻⁵ – fold amount per kg body weight of the lethal dose (LD50) of free gadolinium ions, which is 100-200 mg kg⁻¹.⁴³ Only a marginal increase in Gd³⁺ ion concentration was found at day 11 for the Gd:HfO₂ nanoparticles. Hafnium ions leached out only to an amount of 0.1% of the total hafnium concentration after 24 hours and to 0.3% at day 11. Compared to the pure Gd₂O₃ phase, which dissolved almost completely (94%) already after 1 hour, HfO₂ is much more stable and Gd³⁺ ions stay incorporated to a high extent (leaching only up to 8.6% of total Gd³⁺-ion content after 11 days). These results indicate that HfO₂ does serve as a chemically stable matrix for the incorporation of toxic guest ions, such as Gd³⁺, thus exhibiting potential for the development of safer multimodal radio-enhancers, as compared to G₂O₃.

Apart from imaging performance and minimal guest ion leaching, high cyto- and hemocompatibility in absence of radiation remain key elements for clinical applications. Therefore, primary and secondary surface functionalization of HfO₂-based nanoparticles has been investigated. Polyvinylpyrrolidone (PVP) was chosen as an effective dispersant with well-documented biocompatibility.⁴⁴ (3-Aminopropyl)-triethoxysilane (APTES) was used to provide reactive amine groups on the nanoparticle surface for further functionalization. Fourier transform-infrared spectroscopy confirms the successful functionalization of the nanoparticle surface by showing the presence of characteristic functional groups, such as the carbonyl stretching at 1650 cm⁻¹ in case of PVP-functionalized particles (Figure 5a). For APTES-functionalized nanoparticles, the silane and amine stretchings around 980, 1600 and 3310 cm⁻¹ and the CH₂ bands around 2920 cm⁻¹ can be identified (Figure 5b). Surface functionalization with polyvinylpyrrolidone (PVP) or (3-aminopropyl)triethoxysilane increased dispersability and decreased the hydrodynamic size (z-average (DLS) HfO₂ (unfunctionalized) = 635 nm, PVP@HfO₂ = 553 nm and APTES@HfO₂ = 559 nm) (Table S3, ESI). While DLS is the most widely used method to assess hydrodynamic particle sizes, it has previously been shown that it overestimates the fraction of larger particles in polydisperse samples.⁴⁵ Nanoparticle tracking analysis (Nanosight) showed a main particle population of 40-400 nm with a mode of ~160 nm. NTA measurements showed no indication of nanoparticle agglomerates larger than 400 nm, neither in ddH₂O nor cell culture medium (see ESI, Figure S3). The influence of functionalization on surface charge has been investigated (Table S4, ESI). Zeta-potential measurements show a surface charge of -45.6 ± 2.6 mV for unfunctionalized HfO₂ nanoparticles. Gd₂O₃ nanoparticles have a surface charge of -22.4 ± 1.2 mV at neutral pH. APTES and PVP functionalized nanoparticles show surface charges of -21.6 ± 0.9 and -7.3 ± 0.6 mV, respectively. PVP- and APTES- functionalization led to a pronounced change in surface charge comparable to previous studies on bare and amino-functionalized SiO₂ nanoparticles.⁴⁶

In order to explore further bio-functionalization, the primary amine groups from APTES were conjugated to folic acid (FA). FA can be used to selectively target FA-receptor overexpressing tumor cells.⁴⁷

Following conjugation to folic acid moieties, a dot blot analysis was performed on the FA-APTES@HfO₂ particles using an FA-specific antibody conjugated to an Alexa 488 dye. Fluorescence intensity measurements in comparison to APTES-functionalized HfO₂ nanoparticles confirmed the presence of folic acid on the surface of FA-conjugated particles (Figure 5c). The corresponding collected intensity ratios of FA-APTES@HfO₂ (Figure 5c, top) versus APTES@HfO₂ (Figure 5c, bottom) were 1.11, 1.18 and 1.34 for 5, 10 and 15 μ L particle suspension (1 mg/mL), respectively. The dot blot assay confirms successful secondary functionalization and demonstrates that the folic acid is available to a folic acid specific antibody for binding.

Figure 5. Fourier-transform infrared spectra of unfunctionalized, PVP-functionalized (a) and APTES-functionalized (b) HfO₂ nanoparticles. A PVP polymer spectrum is shown for reference (b). Dot blot assay on FA-functionalized HfO₂ nanoparticles using an FA-antibody and an increasing nanoparticle dose (c). APTES functionalized nanoparticles with no folic acid are shown as reference. The

corresponding relative fluorescence intensity is shown below. Association and uptake of functionalized of PVP and FA-functionalized HfO_2 nanoparticles measured by flow cytometry (d). Transmission electron micrographs of Caco-2 following HfO_2 nanoparticles exposure (e). The Feret diameter of intracellular agglomerates was determined and compared to the Feret diameter found for NBTXR3 undoped hafnia nanoparticles ($N > 100$) (f). The distance from the nuclear membrane was measured and illustrates accumulation of HfO_2 nanoparticles in the perinuclear region ($N > 100$) (g). The cumulative distribution shows that $\sim 50\%$ of the nanoparticles are found within a distance of $\leq 1 \mu\text{m}$ from the nuclear membrane (h).

To explore whether the folic acid functionalization also results in increased cellular uptake, we measured particle uptake by flow cytometry. While PVP nanoparticles show little association with cells and only limited uptake within the first 2 hours, the distinct increase in side scatter indicates that FA-functionalized HfO_2 nanoparticles are readily associated to the cellular membrane and taken up by cells (Figure 5d). Cellular uptake was confirmed by transmission electron microscopy (Figure 5e). Intracellular agglomerate sizes (mean Feret diameter of 410 nm [CI 330, 489]) were comparable to previous studies⁴⁸ on undoped hafnia nanoparticles (NBTXR3) in various cell types and tumor tissues (mean Feret diameter of 280 nm [CI 230, 322] assessed by analyzing > 100 events in published TEM images of PANC-1 and 42-MG-BA cells) (see Figure 5f). Additionally, assessment of the distance to the nuclear membrane relative to the outer cell membrane indicates preferential accumulation of the hafnia nanoparticles in the perinuclear regions of the cells (Figure 5g). Around 50% of the particles were found within $\leq 1 \mu\text{m}$ distance from the nuclear membrane and $>75\%$ of the particles were localized within $\leq 2 \mu\text{m}$ distance (Figure 5h). This is in line with the observed radio-enhancement effects and with literature studies reporting radio-enhancement effects for nanoparticles accumulating in perinuclear regions.⁴⁹

Next, cyto- and hemocompatibility was assessed for the different particles and surface functionalizations (Figure 6). Commercial fumed silica nanoparticles (Aerosil 200) were included as particle based positive control with known cytolytic and procoagulant activity. Generally, HfO_2 exhibited a consistently low toxicity, in agreement with previous reports.⁵⁰⁻⁵¹ Lactate dehydrogenase (LDH) release and MTS activity were measured for different concentrations of HfO_2 -based nanoparticles on monocyte-derived macrophages (MDM).⁵² Exposure for 24 hours showed minimal toxicity (Figure 6a,b). Only pure Gd_2O_3

and APTES functionalized HfO_2 nanoparticles (APTES@HfO_2) showed relevant membrane damage and reduced metabolic activity at concentrations $> 100 \mu\text{g per mL}$, similar to damage observed for fumed silica (A200). Neither undoped nor Tb:HfO_2 , Eu:HfO_2 or Gd:HfO_2 nanoparticles showed apparent cytotoxicity, which is in good agreement with the minimal ion leaching observed for lanthanide-doped HfO_2 compared to pure Gd_2O_3 (Figure 4d). In terms of hemocompatibility, hemolysis and coagulation are among the most critical parameters (along with the activation of the complement). No effect on hemolysis, *i.e.* red blood cell damage, was observed for HfO_2 -based nanoparticles (Figure 6c).

Figure 6. Cytotoxicity of different HfO_2 -based nanoparticles assessed by lactate dehydrogenase (LDH) assay (a) and MTS assay (b) in monocyte derived macrophages (MDM) following 24 hours exposure. * indicates $p < 0.05$ compared to negative control. Blood compatibility of selected nanoparticles (concentration of $100 \mu\text{g/mL}$) assessed in a hemolysis (c) and a plasma clotting assay (d). PBS served as negative control (NC), Triton X-100 (0.1%) as positive control (PC). Aerosil200 (A200, fumed SiO_2) served as particle-based positive control.

In the plasma coagulation assay, unfunctionalized HfO_2 particles led to a pronounced plasma coagulation activation compared to positive control (Figure 6d). Similar to commercially available fumed silica

(Aerosil A200), with a well-documented procoagulant activity,⁵³ complete fibrin polymerization was reached within the first 15 min for HfO₂-based nanoparticles. These findings are in contrast to a previous study¹² showing no activation of the blood coagulation cascade in a thrombin activated assay.⁵⁴ However, the activation by exogenous thrombin might have masked the procoagulant effects of the nanoparticles in this case.⁵⁵ We investigated whether the activation of the plasma coagulation cascade could be attenuated by surface modification. Indeed, PVP surface modification markedly reduced plasma coagulation compared to bare nanoparticles attenuating the thrombogenicity, which is in line with the well-documented hemocompatibility of PVP.⁵⁶ These results illustrate that surface modification is pivotal for a safe and efficient use of HfO₂-based multimodal imaging probes.

CONCLUSION

This work demonstrates the manifold prospects of hafnium dioxide as a host matrix for multimodal theranostic imaging. We show that the sol-gel method enables superior control over particle size distribution and dopant ion incorporation efficiency compared to the other methods investigated. Luminescence, MR, and CT properties of HfO₂ nanoparticles can be tuned by introducing lanthanide guest ions. The host matrix stability of HfO₂ efficiently prevents ion leaching, which is also confirmed by the high cytocompatibility of doped HfO₂ nanoparticles, compared to *e.g.*, pure Gd₂O₃. Apart from stable guest ion incorporation into the HfO₂ matrix, appropriate surface modification is pivotal to reduce potentially adverse procoagulant effects of HfO₂ and to introduce molecular targeting entities. While more detailed investigations on compatibility and therapeutic efficacy are needed, the present study illustrates diverse properties of HfO₂-based nanomaterials beyond radio-enhancing and the potential of HfO₂ as a new generation multimodal theranostic agent for image-guided radiotherapy.

EXPERIMENTAL SECTION

Materials. Hafnium(IV) chloride (99.9% trace metal basis), terbium(III) nitrate hexahydrate (99.999% trace metals basis), europium(III) chloride hexahydrate (99.99% trace metals basis) and gadolinium nitrate hexahydrate (99.99% trace metals basis), (3-Aminopropyl)triethoxysilane (APTES, 99%) and polyvinylpyrrolidone (PVP360, avg. M_w 360 kDa) were purchased from Sigma-Aldrich.

Direct precipitation method. For the synthesis of HfO_2 nanoparticles via a direct precipitation route a previously described method was used.^{18,19} An aqueous solution of 0.4M NaOH was added drop-wise to an equivalent volumetric amount of 0.1M HfCl_4 and the solution was stirred magnetically with a Teflon coated bar over night. Afterwards, the dispersion was centrifuged ($3000 \times g$) and precipitates were washed three times with ddH_2O . After discarding the supernatant, the resulting white product was dried in an oven for three hours in air before it was calcined for 2 hours at 500°C . After cooling to room temperature, the sample was mortared to yield a white powder. To prepare doped samples, the appropriate molar amount of terbium(III) nitrate hexahydrate or europium(III) chloride hexahydrate was dissolved in the aqueous HfCl_4 solution before adding the aqueous NaOH solution.

Microwave-assisted precipitation method. For the microwave-assisted synthesis of HfO_2 nanoparticles, a procedure similar to the precipitation route was followed. However, after the mixing of the precursors, the reaction mixture was put into a glass microwave tube and microwaved for three hours at 120°C with a maximum power of 1200 W. After cooling to room temperature, the precipitate was washed ($3\times$) and centrifuged at $3000 \times g$ for 20 min. Then the precipitate was dried at 80°C for 24 hours.

Sol-gel method. The sol-gel method was performed according to previous reports.^{17,22,23} In short, 0.05 mol of citric acid was added to 24 mL of ddH_2O to yield 31 mL of 2 M aqueous citric acid solution. To this, 2 g (0.00625 mol) of HfCl_4 (or $\text{Gd}(\text{NO}_3)_3 \times 6\text{H}_2\text{O}$ for gadolinium oxide) was added and stirred over night. On the next day, 0.2 mol of ethylene glycol (1:4 ratio of citric acid:ethylene glycol) was added to the precursor while stirring in an oil bath at 90°C . The high temperature was used to support

polyesterification and was kept for three hours. To remove the water, the resulting viscous but transparent product was dried at 130°C over night. After the drying step, the resulting dried gel was calcined in a preheated oven at 600°C for 2 hours yielding a white powder. To obtain doped samples, the appropriate molar amount of doping precursor was added to the citric acid solution during synthesis.

X-Ray powder diffraction (XRD). The analysis of the crystal structure was performed using a Stoe Imaging Plate Diffractometer System IPDS II (STOE & Cie GmbH, Darmstadt, Germany, 2015). Diffraction patterns were collected using MoK α radiation ($\lambda = 0.71073 \text{ \AA}$, beam diameter 0.5 mm). Two-dimensional diffraction images (10 min exposure time) were obtained at an image plate distance of 200 mm with a continued sample rotation; the intensity integration was done over the entire image (360°). The resolution was $D_{min} = 24.00 \text{ \AA}$ and $D_{max} = 1.04 \text{ \AA}$. Crystallite sizes were calculated by applying the Scherrer equation to the (1-11) peak at $2\theta = 12.9^\circ$ for monoclinic and to the (111) peak at $2\theta = 13.8^\circ$ for cubic phases.

Transmission electron microscopy (TEM). Nanoparticle morphology was assessed using a JEOL 2200FS TEM operated at 200 kV for diffraction contrast imaging and selected area electron diffraction (SAED). Indexing of diffraction rings was done manually by measuring the interplanar spacings and comparing them to the database.²⁵

Photoluminescence. Photoluminescence spectra were measured at room temperature using a Varian Cary Eclipse Fluorescence spectrophotometer. The excitation light source was a xenon lamp with built-in monochromator grating. Steady state emission and excitation spectra were taken under phosphorescence mode, with a delay time of 0.1 ms, a gate time of 5 ms and a total decay time of 10 ms. For time resolved photoluminescence, the decay signal of 544 nm under 267 nm excitation for terbium-doped samples, and the 612 nm emission under 230 nm excitation for europium-doped samples were studied. The concentration of the measured sample colloid was 1.1 mg/mL. The sample powder was dispersed in ddH₂O, sonicated with a micro-tip sonicator for 4 min at 30% amplitude and 40% duty cycle.

Elemental analysis. Elemental analysis was performed on an inductively coupled plasma atomic emission spectrometer (ICP-OES, Perkin Elmer, Optima 3000). Total digestion of HfO₂ nanoparticles was done at 180 °C using 1 mL HF, 1mL HNO₃ and 1 mL H₂O.

Magnetic Resonance Imaging. Nanoparticles were embedded in 5% Agar for MRI measurements. For agar embedding, 0.225 g of agar (Sigma-Aldrich) was mixed with 15 mL of water and heated for 20 min at 100 °C. This agar mixture was mixed with an equivalent volumetric amount of aqueous nanoparticle suspension to yield the required final nanoparticle concentrations. All measurements were performed on a 3 Tesla whole-body scanner (Magnetom Skyra, Siemens Healthcare, Erlangen, Germany) at room temperature. The signal was received using a 64-channel head coil, while the built-in body transmit coil was used for spin excitation. For quantification of the longitudinal relaxation times (T₁ times), a two-dimensional turbo spin echo sequence with inversion recovery preparation was acquired with increasing inversion times (TR= 10000 ms, TE = 13 ms, BW = 130 Hz/px, turbo factor = 5, voxel size = 0.7x0.7x4.0 mm³, TI = 25-50-100-200-400-700-1200-2000-3500-8000 ms, acquisition time 33:40 min:sec). Transverse relaxation times (T₂ times) were determined from the signal acquired using a two dimensional spin echo sequence for increasing times to echo (TR= 5000 ms, BW = 310 Hz/px, voxel size = 0.9x0.9x4.0 mm³, TE =7-12-20-30-45-65-100-150-250-400 ms, acquisition time 50:20 min:sec). MR-images were processed offline for pixel-wise quantification of the relaxation rates R₁ and R₂ using routines written in Matlab (MATLAB Release 2012, The MathWorks, Inc., Natick, Massachusetts, United States). For all curve fitting procedures, non-linear least square fits to the signal intensities were performed based on the Levenberg-Marquardt-Algorithm (Matlab “lsqcurvefit”).

The longitudinal relaxation rate R₁ was estimated by fitting of the inversion recovery MR-signal $S(TI)$ to the function:

$$S(TI) = S(0)(1 - 2 * e^{-TI \cdot R_1}) + N, \quad (1)$$

where S(0) represents the equilibrium magnetization and N accounts for the image noise.

The relaxation rate R_2 was calculated by fitting the signal intensities for increasing TE to the equation:

$$S(TE_i) = S(0) \cdot e^{-TE_i R_2} + N, \quad (2)$$

where $S(0)$ is the signal at TE = 0 ms.

The molar relaxivities r_1 and r_2 were estimated from the relaxation rates $R_{1,2}(c)$ measured at different particle concentrations c according to

$$R_1(c) = R_{1,0} + r_1 * c \quad (3)$$

and

$$R_2(c) = R_{2,0} + r_2 * c \quad (4)$$

by linear regression.

Ion leaching. For ion leaching experiments, 1 mg/mL of particles (HfO_2 ; HfO_2 :10%Tb or HfO_2 :10%Gd) were dispersed in K-citrate buffer and prepared by sonication (2 min; 70% amplitude; 3:1 duty cycle; micro tip). The 100mM K-citrate buffer was prepared using citric acid and potassium citrate and pH was adjusted to 4.5 (lysosomal pH). The nanoparticle-suspensions were aliquoted into volumes of 1.5 mL and put on a shaker at 37°C. Three aliquots of each condition were collected for elemental analysis at the respective time intervals. The samples were centrifuged at 20000 $\times g$ for 20 min. 0.8 mL of supernatant was collected and stored in a freezer at -20°C prior to elemental analysis. For elemental analysis, supernatants were thawed and diluted 10-fold in HNO_3 . Samples were analyzed by ICP-MS (Element 2, Thermo Fischer). Only the ion leaching from Gd_2O_3 samples in citrate buffer was analyzed by ICP-OES (Optima 3000, Perkin Elmer).

Surface functionalization. Surface functionalization of the nanoparticles with PVP was performed by adding 1 wt% PVP to a dispersion containing 60-80 mM as synthesized HfO_2 powder in ddH₂O. The

dispersion was stirred overnight and washed, thereafter, 3 times with ddH₂O. Surface functionalization with APTES was performed by adding 4 mL of 28% ammonium hydroxide to 0.5 g of HfO₂ nanoparticles suspended in 16 mL ddH₂O. Ethanol was added to reach a total volume of 150 mL. The mixture was kept at 30°C. Then, 25 µL of APTES was added and the mixture was allowed to react overnight. Thereafter, unreacted APTES was removed by washing with ethanol or ddH₂O and centrifuging at 3000 ×g for 30 min for at least 3 times. For folic acid coupling, APTES functionalized nanoparticles were reacted with a folic acid NHS ester conjugate (100 fold excess) in bicarbonate buffer (pH 8) for 3 hrs.

Dynamic light scattering (DLS) and zeta potential. Measurements to assess hydrodynamic size and surface charge were performed on a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, United Kingdom) with a 90deg scattering angle. The colloid concentration (particles in ddH₂O) used was ~ 1 mg/mL corresponding to an attenuator position around 8 and the derived count rate was between 4000 and 5000 kcps. A refractive index 2.1 for HfO₂ was used. The viscosity of water was used for the colloid. The particles were sonicated using a Branson Digital Sonifier Type 450 (Branson Ultrasonics, Danbury, USA) with a 3/4" High Gain Horn for 10 min, using 70% amplitude and a duty cycle of 3:1. The colloid was cooled in an ice bath during sonication. DLS was measured within 10-20 min. For zeta potential measurements, the particle concentration was adjusted to 1 mM and 10 vol% of PBS were added. The used attenuator was 6 (7 in only one sample: HfO₂:10%Eu). The conductivity was in all cases ~ 2 mS/cm, except for the PVP functionalized sample, where it was ~ 1 mS/cm.

Nanoparticle tracking analysis, NTA. In addition to the DLS, Nanoparticle Tracking Analysis (NTA) was used to assess particle sizes (NanoSight NS500, Malvern Instruments Ltd., Worcestershire, United Kingdom). For comparison with DLS, a 1 mg/mL NP solution was sonicated for 10 min in a bath before measurement and 1 part was diluted with either 2 parts of ddH₂O or 2 parts of filtered (800 µm) phenolred free medium (MEM). Analysis of 1498 frames was done with a camera level of 6-7.

Attenuated Total Reflectance (ATR). ATR was measured using a Varian 640-IR Spectrometer to assess vibrational surface states. Samples were mortared prior to measuring.

Dot blot. Dot-Blot Immunoassay was performed at room temperature like in ⁵⁷. FA-functionalized and APTES-functionalized (without FA) nanoparticles were deposited at the same concentration (1mg/mL) on an absorbent filter paper (Bio Dot SF Filter Paper, Bio Rad), which was cut in round circles fitting in the wells of a 48-well-plate. The nanoparticle suspension was loaded on the membrane by adding drops of 5 μ L. After drying, the membranes were blocked by soaking in 0.6 mL 5% (w/v) BSA (bovine serum albumin) in TBST (20mM Tris-HCL, 150mM NaCl, 0.05% (v/v) Tween20, pH 7.6) for 1 h on a shaker. After discarding the blocking solution, 0.12 mL of monoclonal antifolic acid primary antibody diluted 1:500 in 5% BSA in TBST was added to the membranes for 1 h. The membranes were washed 3 times for 5 min in TBST. Then goat anti-mouse IgG secondary antibody (Alexa Fluor 488 or 633) diluted 1:200 in TBST was added to the membrane for 1 h in the dark. Afterwards, the membrane was washed again with TBST three times. The well-plate with the membranes was protected from light until fluorescence was measured using a microplate reader or it was imaged using a TECAN LS Reloaded microarray reader.

Cell lines and culture conditions. The Caco-2 cell line (ACC169) was acquired from 'Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures' (Braunschweig, Germany). Undifferentiated cells were grown in Minimum Essential Medium Eagle (MEM, Sigma-Aldrich, M2279) with 10% Fetal Calve Serum (FCS, Sigma-Aldrich, F9665), 1% L-Glutamine (Sigma Aldrich, G7513), 1% Penicillin-Streptomycin-Neomycin (PSN, Sigma-Aldrich, P4083) and 1% Non-Essential Amino Acids (Sigma-Aldrich, M7145) in addition. They were maintained in an incubator at 37 °C under 5% CO₂ humidified atmosphere. Routinely sub-culturing was done twice a week at 70-80% confluence by treatment with 0.5% Trypsin-EDTA (Sigma-Aldrich, T3924).

The THP-1 cell line (TIB-202) was acquired from American Type Culture Collection. THP-1 cells were grown in suspension and routinely subcultured once a week in RPMI-1640 culture medium (Sigma-Aldrich, RO883) with 10% FCS, 1% L-Glutamine and 1% PSN in addition.

Cellular Uptake. For cellular uptake experiments, 4×10^5 Caco-2 cells were seeded in a T25 flask with supplemented medium. After 24 h, cells were washed with PBS and the medium was replaced by 3 mL of folic acid (FA) free medium. All conditions were incubated under standard culturing conditions for 1 h. Nanoparticles were suspended in milliQ water and tip sonicated shortly before the addition to cells. Then, 100 μ L of either PVP@HfO₂ or FA-APTES@HfO₂ functionalized particles at a concentration of 1 mg/mL, or milliQ water (control) was added. After another 1 h of incubation under standard culturing conditions, cells were washed with PBS and collected using Accutase under standard procedures. Cells were suspended in 2 mL PBS, strained using a 100 μ m cell strainer and put on ice. PI staining (5 μ L per 500 mL sample) was added before analyzing the samples by flow cytometry. For analysis, cells were gated using quadrant gates excluding debris. The gated cells were further single-cell discriminated using a FS-H vs. FS-A plot. The gated single cells were then separated into Live and Dead cells using a histogram plot for the PI fluorescence and only live cells were used for further analysis. Negative control cells (without PI staining) showed no fluorescence. The gated numbers of live single cells were between 5 and 10×10^3 cells.

EM sample preparation, imaging and analysis. For imaging particles in cells with electron microscopy, 0.2 Mio Caco-2 cells were seeded in a T75 flask and grown over 2 days. On the third day, the cell medium was replaced by new medium in which an aqueous HfO₂ NP dispersion was added such that the final concentration was 0.2 mg/mL. The amount of ddH₂O was 10%. Cells with NPs were incubated for 24 hours. After washing with prewarmed PBS and trypsinizing, cells were incubated for 2.5 h at room temperature in 4% methanol-free paraformaldehyde (PFA). The cell pellets were washed 3 times with ddH₂O, twice with cacodylate buffer (0.1 M) and then stained with 2% osmium tetroxide and 1.5% potassium ferricyanide for 1 h. Afterwards, they were gradually dehydrated using an ethanol gradient

(50%, 60%, 70%, 80%, 90%, 100%, 100%, 100%) and embedded in epoxy resin (EPON 812), first together with ethanol (50%) for 10 h and then pure for three days in the fridge. Resin blocks were then cured in the oven at 60°C, trimmed with a razor blade, sectioned in 100-nm slices using an ultramicrotome and imaged in a FEI Helios scanning transmission electron microscope. Analysis of EM images was done using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA). The region of interests were cropped and the NP agglomerate Feret diameters (the longest distance between any two points along the selection boundary) and outlines were determined (see ESI, Figure S4). The shortest distances from the NP agglomerate outline to the nuclear membrane or outer cell membrane was measured manually.

Cell irradiation experiments. For cell irradiation experiments, 200'000 Caco-2 cells were seeded in 12-well-plates in 0.9 mL supplemented MEM 48 h prior to irradiation. After 24 h incubation in cell culture medium, 0.1 mL of 5 mg/mL particle suspensions (HfO_2 and HfO_2 :10%Tb) in ddH₂O was added, so that the final NP concentration was 0.5 mg/mL per 4 cm² growth area. For cells without nanoparticles, 0.1 mL of ddH₂O was added. Cells were irradiated with 150 keV photons (source) reaching 1 Gy/min. An aluminium foil (1.2 cm thickness) was placed above the well-plate, to filter out low energy photons and a dosimeter was placed next to the plate. For each condition, three replicates were done. Positive controls included cells with 0.1% Triton X or 100 µM CdSO₄. Cells not exposed to radiation were used as negative controls. After irradiation, for all conditions the same volume of cells dispersed in media, corresponding to roughly 5'000 cells in the untreated population, was reseeded in 96-wells. MTS was then measured according to the manufacturer's protocol 5 days after the irradiation exposure and nanoparticle enhancement ratios were calculated by dividing the viability value for nanoparticle-containing cells by nanoparticle-free cells.

LDH assay. A membrane damage assay were performed based on protocols from the manufacturer for the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corporation, USA). Briefly, 50'000 THP-1 cells were seeded into 48-well-plates with 225 µL RPMI-1640 culture medium supplemented with 100

1
2
3
4
5
6 nM phorbol-12-myristate 13-acetate (PMA, Sigma) and incubated for 72 hours under standard culture
7
8 conditions to obtain monocyte-derived macrophages (MDM).⁵⁸ Prior to exposure experiments, medium
9
10 was exchanged to PMA-free culture medium. To assess membrane damage potential of the nanoparticles,
11
12 25 μ L of according aqueous particle concentrations (0.25, 1, 2.5 mg/mL) were added to each well after 2
13
14 min tip sonication, so that the maximum amount of water was 10%. Triton X-100 (Sigma-Aldrich, X-
15
16 100) 1% was used as positive control. Cells were then incubated for 24 hours. Thereafter, 50 μ L of the
17
18 supernatants were transferred into a new 96-well-plate. Then, 50 μ L CytoTox 96 Reagent was added and
19
20 the plate was incubated at room temperature in the dark for 30 min. Eventually, 50 μ L of stop-solution
21
22 (Promega, G183A) was added and the absorbance was measured at 490 nm with a plate reader (Mithras2
23
24 LB 943). The data was analyzed by subtracting the background (medium and LDH reagent) from all
25
26 absorbance values. Values are expressed relative to the positive control.
27
28

29
30 **MTS Assay.** The MTS assay was performed according to the manufacturer's protocol. After the
31
32 supernatant was removed for LDH measurements, medium with MTS substrate was added to the cells and
33
34 incubated for 3 hours. Afterwards, optical absorbance was measured at 490 nm using the plate reader
35
36 (Mithras2 LB 943).
37

38
39 **Hemocompatibility.** After obtaining written informed consent (ethical approval, EKSG
40
41 PB_2016_00816), whole blood from healthy volunteers was collected in tubes containing 0.109 M
42
43 sodium citrate. Hemolysis and coagulation assays were performed as described previously.⁵⁹ Red blood
44
45 cells were resuspended in PBS, washed three times and the hemoglobin concentration was adjusted to 30
46
47 mg/mL, while the plasma was used for coagulation studies. Positive and negative control were 1% Triton
48
49 X solution and PBS, respectively. For the hemolysis measurement, 180 μ L red blood cells and 20 μ L of
50
51 nanoparticle suspension (undoped, doped and functionalized nanoparticles) were mixed to obtain a final
52
53 nanoparticle concentration of 100 μ g/mL and incubated for 3 hours at 37 °C. After centrifugation at 6000
54
55 $\times$ g for 5 min, 100 μ L of supernatant was transferred to a 96-well-plate and absorption at 570 nm
56
57 (hemoglobin) was measured with a Mithras2 LB 943 multimode microplate reader. For coagulation
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

experiments, 20 μL of the same nanoparticle suspensions were added to tubes containing 180 μL blood plasma, incubated at 37 $^{\circ}\text{C}$ for 30 min and centrifuged at 6000 $\times g$ for 5 min. Supernatants (100 μL) were transferred to 96-well-plate, recalcified with 65 μL of 50 mM CaCl_2 solution and absorbance was measured at 405 nm in 50 s intervals during 1 h with the plate reader (Mithras2 LB 943) to assess fibrin polymerization. Aerosil 200 was used as nanoparticle-based positive control.

ACKNOWLEDGEMENT

We acknowledge financial support by the Olga Mayenfisch Foundation, Zurich, Switzerland and the SNSF. We thank Anja Huch for technical assistance with the ultramicrotome.

ASSOCIATED CONTENT

Supporting Information. Tables: XRD, elemental analysis, lattice indexing, DLS and Zeta-potential, r_2 relaxivities. Figures: TEM and SAED images, DLS and NTA data, Image analysis data (file type: PDF).

AUTHOR INFORMATION

Corresponding Author

*Email: inge.herrmann@empa.ch

Notes

There are no conflicts of interests to declare.

REFERENCES

- (1) Barker, H. E.; Paget, J. T. E.; Khan, A. A.; Harrington, K. J. The Tumour Microenvironment after Radiotherapy: Mechanisms of Resistance and Recurrence. *Nature Reviews Cancer* **2015**, *15*, 409.
- (2) Wardman, P. Chemical Radiosensitizers for Use in Radiotherapy. *Clinical Oncology* **2007**, *19* (6), 397-417.
- (3) Harrington, K. J.; Billingham, L. J.; Brunner, T. B.; Burnet, N. G.; Chan, C. S.; Hoskin, P.; Mackay, R. I.; Maughan, T. S.; Macdougall, J.; McKenna, W. G.; Nutting, C. M.; Oliver, A.; Plummer, R.; Stratford, I. J.; Illidge, T. Guidelines for Preclinical and Early Phase Clinical Assessment of Novel Radiosensitisers. *British Journal Of Cancer* **2011**, *105*, 628.
- (4) Su, X.-Y.; Liu, P.-D.; Wu, H.; Gu, N. Enhancement of Radiosensitization by Metal-Based Nanoparticles in Cancer Radiation Therapy. *Cancer Biology & Medicine* **2014**, *11* (2), 86-91.
- (5) Hainfeld, J. F.; Dilmanian, F. A.; Slatkin, D. N.; Smilowitz, H. M. Radiotherapy Enhancement with Gold Nanoparticles. *Journal of Pharmacy and Pharmacology* **2008**, *60* (8), 977-985.
- (6) Erika, P.; Samuel, L.; Hynd, R.; Noriko, U.; Katsumi, K.; Yoshiya, F.; Claude Le, S.; Sandrine, L. Platinum Nanoparticles: A Promising Material for Future Cancer Therapy? *Nanotechnology* **2010**, *21* (8), 085103.
- (7) Zdenka, K.; Sandrine, L. Nanoparticle Radio-Enhancement: Principles, Progress and Application to Cancer Treatment. *Physics in Medicine & Biology* **2018**, *63* (2), 02TR01.
- (8) Bonvalot, S.; Le Pechoux, C.; De Baere, T.; Kantor, G.; Buy, X.; Stoeckle, E.; Terrier, P.; Sargos, P.; Coindre, J. M.; Lassau, N.; Ait Sarkouh, R.; Dimitriu, M.; Borghi, E.; Levy, L.; Deutsch, E.; Soria, J.-C. First-in-Human Study Testing a New Radioenhancer Using Nanoparticles (NBTXR3) Activated by Radiation Therapy in Patients with Locally Advanced Soft Tissue Sarcomas. *Clinical Cancer Research* **2017**, *23* (4), 908-917.
- (9) McMahon, S. J.; Paganetti, H.; Prise, K. M. Optimising Element Choice for Nanoparticle Radiosensitisers. *Nanoscale* **2016**, *8* (1), 581-589.
- (10) Sancey, L.; Lux, F.; Kotb, S.; Roux, S.; Dufort, S.; Bianchi, A.; Crémillieux, Y.; Fries, P.; Coll, J. L.; Rodriguez-Lafrasse, C.; Janier, M.; Dutreix, M.; Barberi-Heyob, M.; Boschetti, F.; Denat, F.; Louis, C.; Porcel, E.; Lacombe, S.; Le Duc, G.; Deutsch, E.; Perfettini, J. L.; Detappe, A.; Verry, C.; Berbeco, R.; Butterworth, K. T.; McMahon, S. J.; Prise, K. M.; Perriat, P.; Tillement, O. The Use of Theranostic Gadolinium-Based Nanoprobes to Improve Radiotherapy Efficacy. *The British Journal of Radiology* **2014**, *87* (1041), 20140134.
- (11) Stewart, C.; Konstantinov, K.; McKinnon, S.; Guatelli, S.; Lerch, M.; Rosenfeld, A.; Tehei, M.; Corde, S. First Proof of Bismuth Oxide Nanoparticles as Efficient Radiosensitisers on Highly Radioresistant Cancer Cells. *Physica Medica* **2016**, *32* (11), 1444-1452.
- (12) Furasova, A. D.; Fakhardo, A. F.; Milichko, V. A.; Tervoort, E.; Niederberger, M.; Vinogradov, V. V. Synthesis of a Rare-Earth Doped Hafnia Hydrosol: Towards Injectable Luminescent Nanocolloids. *Colloids and Surfaces B: Biointerfaces* **2017**, *154*, 21-26.
- (13) Rösslein, M.; Liptrott, N. J.; Owen, A.; Boisseau, P.; Wick, P.; Herrmann, I. K. Sound Understanding of Environmental, Health and Safety, Clinical, and Market Aspects Is Imperative to Clinical Translation of Nanomedicines. *Nanotoxicology* **2017**, *11* (2), 147-149.

- (14) Deng, J.; Xu, S.; Hu, W.; Xun, X.; Zheng, L.; Su, M. Tumor Targeted, Stealthy and Degradable Bismuth Nanoparticles for Enhanced X-Ray Radiation Therapy of Breast Cancer. *Biomaterials* **2018**, *154*, 24-33.
- (15) Heath, J. R. Nanotechnologies for Biomedical Science and Translational Medicine. *Proceedings of the National Academy of Sciences* **2015**, *112* (47), 14436-14443.
- (16) Liu, C.; Hajagos, T. J.; Kishpaugh, D.; Jin, Y.; Hu, W.; Chen, Q.; Pei, Q. Facile Single-Precursor Synthesis and Surface Modification of Hafnium Oxide Nanoparticles for Nanocomposite Γ -Ray Scintillators. *Advanced Functional Materials* **2015**, *25* (29), 4607-4616.
- (17) McGinnity, T. L.; Dominguez, O.; Curtis, T. E.; Nallathamby, P. D.; Hoffman, A. J.; Roeder, R. K. Hafnia (HfO₂) Nanoparticles as an X-Ray Contrast Agent and Mid-Infrared Biosensor. *Nanoscale* **2016**, *8* (28), 13627-13637.
- (18) Jayaraman, V.; Bhavesh, G.; Chinnathambi, S.; Ganesan, S.; Aruna, P. Synthesis and Characterization of Hafnium Oxide Nanoparticles for Bio-Safety. *Materials Express* **2014**, *4* (5), 375-383.
- (19) Ramadoss, A.; Krishnamoorthy, K.; Kim, S. J. Novel Synthesis of Hafnium Oxide Nanoparticles by Precipitation Method and Its Characterization. *Materials Research Bulletin* **2012**, *47* (9), 2680-2684.
- (20) Eliziário, S.; Cavalcante, L.; Sczancoski, J.; Pizani, P.; Varela, J.; Espinosa, J.; Longo, E. Morphology and Photoluminescence of HfO₂ obtained by Microwave-Hydrothermal. *Nanoscale Research Letters* **2009**, *4* (11), 1371.
- (21) De Roo, J.; De Keukeleere, K.; Feys, J.; Lommens, P.; Hens, Z.; Van Driessche, I. Fast, Microwave-Assisted Synthesis of Monodisperse HfO₂ Nanoparticles. *Journal of Nanoparticle Research* **2013**, *15* (7), 1778.
- (22) Galvez-Barboza, S.; Gonzalez, L. A.; Puente-Urbina, B. A.; Saucedo-Salazar, E. M.; Garcia-Cerda, L. A. Preparation and Characterization of Ce-Doped HfO₂ Nanoparticles. *Journal of Alloys and Compounds* **2015**, *643*, S62-S66.
- (23) Ramos-Gonzalez, R.; Garcia-Cerda, L. A.; Alshareef, H. N.; Gnade, B. E.; Quevedo-Lopez, M. A. Study of Hafnium (IV) Oxide Nanoparticles Synthesized by Polymerized Complex and Polymer Precursor Derived Sol-Gel Methods. *Advanced Electron Microscopy and Nanomaterials* **2010**, *644*, 75-78.
- (24) Pinna, N.; Garnweitner, G.; Antonietti, M.; Niederberger, M. Non-Aqueous Synthesis of High-Purity Metal Oxide Nanopowders Using an Ether Elimination Process. *Advanced Materials* **2004**, *16* (23-24), 2196-2200.
- (25) Xu, Y. B.; Yamazaki, M.; Villars, P. Inorganic Materials Database for Exploring the Nature of Material. *Japanese Journal of Applied Physics* **2011**, *50* (11).
- (26) Lauria, A.; Villa, I.; Fasoli, M.; Niederberger, M.; Vedda, A. Multifunctional Role of Rare Earth Doping in Optical Materials: Nonaqueous Sol-Gel Synthesis of Stabilized Cubic HfO₂ Luminescent Nanoparticles. *Acs Nano* **2013**, *7* (8), 7041-7052.
- (27) Jiang, C. L.; Wang, F.; Wu, N. Q.; Liu, X. G. Up- and Down-Conversion Cubic Zirconia and Hafnia Nanobelts. *Advanced Materials* **2008**, *20* (24), 4826-4829.
- (28) Rauwel, P.; Rauwel, E.; Persson, C.; Sunding, M. F.; Galeckas, A. One Step Synthesis of Pure Cubic and Monoclinic HfO₂ Nanoparticles: Correlating the Structure to the Electronic Properties of the Two Polymorphs. *Journal of Applied Physics* **2012**, *112* (10), 104107.

- (29) Lu, C.-H.; Raitano, J. M.; Khalid, S.; Zhang, L.; Chan, S.-W. Cubic Phase Stabilization in Nanoparticles of Hafnia-Zirconia Oxides: Particle-Size and Annealing Environment Effects. *Journal of Applied Physics* **2008**, *103* (12), 124303.
- (30) Kumar, N.; George, B. P. A.; Abrahamse, H.; Parashar, V.; Ray, S. S.; Ngila, J. C. A Novel Approach to Low-Temperature Synthesis of Cubic HfO₂ Nanostructures and Their Cytotoxicity. *Scientific Reports* **2017**, *7* (1), 9351.
- (31) Boukerika, A.; Guerbous, L. Annealing Effects on Structural and Luminescence Properties of Red Eu³⁺-Doped Y₂O₃ Nanophosphors Prepared by Sol–Gel Method. *Journal of Luminescence* **2014**, *145* (Supplement C), 148-153.
- (32) Binnemans, K. Interpretation of Europium(III) Spectra. *Coordination Chemistry Reviews* **2015**, *295* (Supplement C), 1-45.
- (33) Wilk, G. D.; Wallace, R. M.; Anthony, J. M. High-κ Gate Dielectrics: Current Status and Materials Properties Considerations. *Journal of Applied Physics* **2001**, *89* (10), 5243-5275.
- (34) Bersch, E.; Rangan, S.; Bartynski, R. A.; Garfunkel, E.; Vescovo, E. Band Offsets of Ultrathin High-κ Oxide Films with Si. *Physical Review B* **2008**, *78* (8), 085114.
- (35) Wan, N.; Xu, J.; Lin, T.; Zhang, X.; Xu, L. Energy Transfer and Enhanced Luminescence in Metal Oxide Nanoparticle and Rare Earth Codoped Silica. *Applied Physics Letters* **2008**, *92* (20), 201109.
- (36) Benz, F.; Strunk, H. P. Rare Earth Luminescence: A Way to Overcome Concentration Quenching. *AIP Advances* **2012**, *2* (4), 042115.
- (37) Montes, E.; Cerón, P.; Rivera Montalvo, T.; Guzmán, J.; García-Hipólito, M.; Soto-Guzmán, A. B.; García-Salcedo, R.; Falcony, C. Thermoluminescent Characterization of HfO₂:Tb³⁺ Synthesized by Hydrothermal Route. *Applied Radiation and Isotopes* **2014**, *83*, 196-199.
- (38) Lange, S.; Kiisk, V.; Aarik, J.; Kirm, M.; Sildos, I. Luminescence of ZrO₂ and HfO₂ Thin Films Implanted with Eu and Er Ions. *physica status solidi (c)* **2007**, *4* (3), 938-941.
- (39) Lange, S.; Kiisk, V.; Reedo, V.; Kirm, M.; Aarik, J.; Sildos, I. Luminescence of RE-Ions in HfO₂ Thin Films and Some Possible Applications. *Optical Materials* **2006**, *28* (11), 1238-1242.
- (40) Ahmad, M. W.; Xu, W.; Kim, S. J.; Baek, J. S.; Chang, Y.; Bae, J. E.; Chae, K. S.; Park, J. A.; Kim, T. J.; Lee, G. H. Potential Dual Imaging Nanoparticle: Gd₂O₃ Nanoparticle. *Scientific Reports* **2015**, *5*, 8549.
- (41) Retif, P.; Pinel, S.; Toussaint, M.; Frochot, C.; Chouikrat, R.; Bastogne, T.; Barberi-Heyob, M. Nanoparticles for Radiation Therapy Enhancement: The Key Parameters. *Theranostics* **2015**, *5* (9), 1030-1044.
- (42) Morcos, S. K. Nephrogenic Systemic Fibrosis Following the Administration of Extracellular Gadolinium Based Contrast Agents: Is the Stability of the Contrast Agent Molecule an Important Factor in the Pathogenesis of This Condition? *The British Journal of Radiology* **2007**, *80* (950), 73-76.
- (43) Zhao, Y.; Shen, Y., *Biomedical Nanomaterials*. Wiley: 2016; Vol. 12.
- (44) Koczur, K. M.; Mourdikoudis, S.; Polavarapu, L.; Skrabalak, S. E. Polyvinylpyrrolidone (PVP) in Nanoparticle Synthesis. *Dalton Transactions* **2015**, *44* (41), 17883-17905.

- (45) Filipe, V.; Hawe, A.; Jiskoot, W. Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by Nanosight for the Measurement of Nanoparticles and Protein Aggregates. *Pharmaceutical Research* **2010**, *27* (5), 796-810.
- (46) Samuel, J.; Raccurt, O.; Poncelet, O.; Auger, A.; Ling, W.-L.; Cherns, P.; Grunwald, D.; Tillement, O. Surface Characterizations of Fluorescent-Functionalized Silica Nanoparticles: From the Macroscale to the Nanoscale. *Journal of Nanoparticle Research* **2010**, *12* (6), 2255-2265.
- (47) Zwicke, G. L.; Mansoori, G. A.; Jeffery, C. J. Utilizing the Folate Receptor for Active Targeting of Cancer Nanotherapeutics. *Nano Reviews* **2012**, *3*, 10.3402/nano.v3i0.18496.
- (48) Marill, J.; Anesary, N. M.; Zhang, P.; Vivet, S.; Borghi, E.; Levy, L.; Pottier, A. Hafnium Oxide Nanoparticles: Toward an in Vitro Predictive Biological Effect? *Radiation oncology (London, England)* **2014**, *9*, 150-150.
- (49) McQuaid, H. N.; Muir, M. F.; Taggart, L. E.; McMahon, S. J.; Coulter, J. A.; Hyland, W. B.; Jain, S.; Butterworth, K. T.; Schettino, G.; Prise, K. M.; Hirst, D. G.; Botchway, S. W.; Currell, F. J. Imaging and Radiation Effects of Gold Nanoparticles in Tumour Cells. *Scientific Reports* **2016**, *6*, 19442.
- (50) Field, J. A.; Luna-Velasco, A.; Boitano, S. A.; Shadman, F.; Ratner, B. D.; Barnes, C.; Sierra-Alvarez, R. Cytotoxicity and Physicochemical Properties of Hafnium Oxide Nanoparticles. *Chemosphere* **2011**, *84* (10), 1401-1407.
- (51) Villa, I.; Villa, C.; Monguzzi, A.; Babin, V.; Tervoort, E.; Nikl, M.; Niederberger, M.; Torrente, Y.; Vedda, A.; Lauria, A. Demonstration of Cellular Imaging by Using Luminescent and Anti-Cytotoxic Europium-Doped Hafnia Nanocrystals. *Nanoscale* **2018**, *10* (17), 7933-7940.
- (52) Daigneault, M.; Preston, J. A.; Marriott, H. M.; Whyte, M. K. B.; Dockrell, D. H. The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages. *PLoS One* **2010**, *5* (1), e8668.
- (53) Keevend, K.; Panzarasa, G.; Starsich, F.; Zeltner, M.; Spyrogianni, A.; Tsolaki, E.; Fortunato, G.; Pratsinis, S.; Bertazzo, S.; Herrmann, I. K. Facile Meltpegylation of Flame-Made Luminescent Tb³⁺-Doped Yttrium Oxide Particles: Hemocompatibility, Cellular Uptake and Comparison to Silica. *Chemical Communications* **2018**.
- (54) Fröhlich, E. Action of Nanoparticles on Platelet Activation and Plasmatic Coagulation. *Current Medicinal Chemistry* **2016**, *23* (5), 408-430.
- (55) Ilinskaya, A. N.; Dobrovolskaia, M. A. Nanoparticles and the Blood Coagulation System. Part II: Safety Concerns. *Nanomedicine (London, England)* **2013**, *8* (6), 969-981.
- (56) Liu, X.; Xu, Y.; Wu, Z.; Chen, H. Poly(N-Vinylpyrrolidone)-Modified Surfaces for Biomedical Applications. *Macromolecular Bioscience* **2013**, *13* (2), 147-154.
- (57) Almeida, C. S.; Herrmann, I. K.; Howes, P. D.; Stevens, M. M. Tailoring Cellular Uptake of Conjugated Polymer Nanoparticles Using Modular Amphiphilic Peptide Capping Ligands. *Chemistry of Materials* **2015**, *27* (19), 6879-6889.
- (58) Richter, E.; Ventz, K.; Harms, M.; Mostertz, J.; Hochgräfe, F. Induction of Macrophage Function in Human THP-1 Cells Is Associated with Rewiring of MAPK Signaling and Activation of MAP3K7 (TAK1) Protein Kinase. *Frontiers in Cell and Developmental Biology* **2016**, *4*, 21.
- (59) Matter, M. T.; Starsich, F.; Galli, M.; Hilber, M.; Schlegel, A. A.; Bertazzo, S.; Pratsinis, S. E.; Herrmann, I. K. Developing a Tissue Glue by Engineering the Adhesive and Hemostatic Properties of Metal Oxide Nanoparticles. *Nanoscale* **2017**, *9* (24), 8418-8426.

TOC: TABLE OF CONTENTS GRAPHIC

TOC: TABLE OF CONTENTS GRAPHIC

304x190mm (150 x 150 DPI)