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# Click functionalized biocompatible gadolinium oxide core-shell nanocarriers for imaging of breast cancer cells†

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Site-specific delivery using functionalized nanocarriers is in high demand in imaging applications of modern clinical research. To improve the imaging capabilities of conventionally used contrast agents and expand the targeting accuracy, functional gadolinium oxide based nanocarriers originated from homogeneous core shells structures ( $Gd_2O_3@SiO_2@Fe_3O_4$ ) were developed using a multilayer formation approach. The synthesis and chemical configuration for the covalent binding of macrocyclic chelating agents and estrogen targeting molecules on these nanocarriers were designed by a two-step chemical synthesis method. Initially,  $SiO_2@Fe_3O_4$  structures were prepared and encapsulated with a homogenous thin  $Gd_2O_3$  overlayer. The exterior surface of the as-prepared carriers offered chemical binding with a breast cancer specific estrogen molecule, covalently grafted through a Click-Chemistry protocol. In the next step, to enhance the diagnostic imaging capabilities of these carriers, thiocyanate-linked chelator molecule, DOTA, was attached to the surface of estrogen bound  $Gd_2O_3@SiO_2@Fe_3O_4$  using basic reaction conditions. The active amino groups before and after conjugation of estrogen molecules on the surface were quantified using a fluorescamine based approach. Due to the covalent binding of the macrocyclic chelator to the  $Gd_2O_3@SiO_2@Fe_3O_4$  surface, core shell carriers showed potential radiolabeling efficiency using positron emitter radionuclide, gallium-68 ( $^{68}Ga$ ). Intracellular uptake of estrogen-conjugated carriers was evaluated with MCF7 breast cancer cell lines using confocal laser scanning microscopy and fluorescent flow cytometry. In addition, *in vitro* cytotoxicity studies of functional nanocarriers as compared to bare nanoparticles showed reduced toxicity to HEK-293 cells demonstrating the role of surface attached molecules in preventing direct exposure of the  $Gd_2O_3$  surface to the cells. The as-developed gadolinium based nanocarriers presented excellent capabilities as biocompatible target-specific imaging probes which indicates great potential in the field of dual-mode contrast agents.

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## Introduction

Experimental pathways unifying imaging and therapeutic entities in a single carrier are desired for developing new therapeutics probes capable of diagnosing the disease and delivering the therapeutic in a simultaneous action.<sup>1</sup> Additionally, by integrating multiple imaging components within one

nanoparticulate platform, hybrid materials may become robust contrast agents (CA) for dual mode imaging to enhance the diagnosis accuracy.<sup>2</sup> In this context, nanoparticles (NPs) with surface-grafted functional ligands that can selectively link to an imaging unit as well as a drug molecule are of increasing interest.<sup>3</sup>

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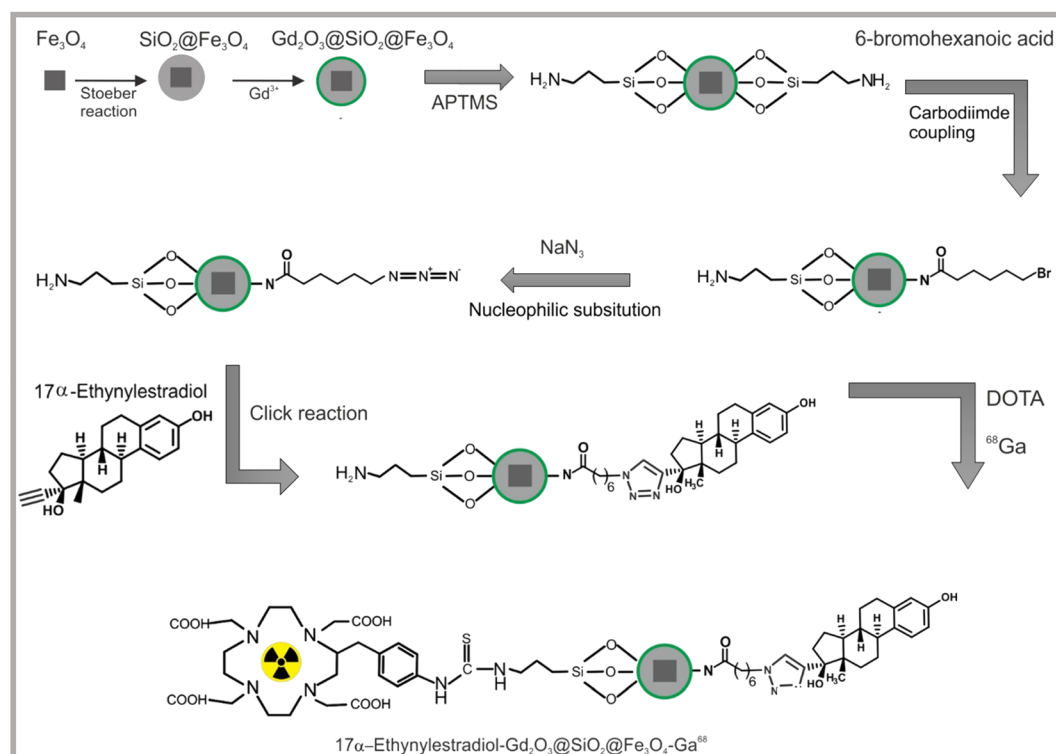


The next-generation positive ( $T_1$ ) MRI contrast agents' gadolinium oxide ( $Gd_2O_3$ ) NPs are growing as an excellent alternative to the current clinically available  $Gd^{3+}$ -chelates based CAs on account of their high spin paramagnetism, high stability in physiological milieu, lower toxicity, and better bioavailability achievable through surface conjugation of targeting units.<sup>4–6</sup> One of the major obstacles in using Gd-based materials for *in vivo* experiments is the known toxic effect of the  $Gd^{3+}$  ion.<sup>7–9</sup> Although gadolinium ions can be toxic, however, the  $Gd_2O_3$  nanoparticles can be designed as benign materials and currently being investigated as promising materials for various MRI applications. For instance, BSA and lactobionic acid coated gadolinium-based NPs are known as non-toxic to the different cell lines at the higher concentration and for a longer period of time.<sup>10,11</sup> In another study,  $Gd_2O_3$  particles showed excellent (100%) cell viability after their injection into mice up to 4 weeks.<sup>9</sup>

Tumor imaging *via* MRI can be upgraded by active and passive targeting and literature shows several reports on the use of gadolinium as a contrast agent in different biological assays. For example, gadolinium oxide particles covered with polymers such as polyacrylic acid or conjugating sericin enhanced magnetic resonance imaging with higher  $T_1$  relativity at tumor site in human brain glioma cells.<sup>12,13</sup> Also doping the gadolinium oxide particles with mesoporous silica enhanced  $T_1$  contrast of gadolinium due to silica geometrical confinement.<sup>14–16</sup> Recent studies have revealed that the conjugation of  $Gd^{3+}$  complexes with iron oxide NPs, and the

combination of  $Gd_2O_3$  NPs with other magnetic materials, such as  $Fe_3O_4$  NPs into single composite particles leads to the enhancement of  $T_1$  and  $T_2$  contrast in MRI. In case of a thin gadolinium shell, nanocomposites provided higher relativity than a thick shell during MRI examinations.<sup>18</sup> *In vivo* and *in vitro* studies during different applications, the gadolinium labeled iron oxide nanoparticles showed brighter-effects in  $T_1$ -weighted MRI and iron nanoparticles were used as spin-spin relaxation ( $T_2$ ).<sup>19</sup> To significantly enhance the  $T_1$  relaxation time without decreasing the  $T_2$  relaxation, iron-doped gadolinium nanoparticles were further studied by combining with hydrogenated silica to form a core-shell nanostructure with *in vivo* imaging with a significant improvement in  $T_1/T_2$  dual mode.<sup>20</sup> These results evidently showed that gadolinium labeled iron-silica nanoparticles have potential as a contrast agent in magnetic resonance imaging. However, the optimal design of efficient and nontoxic  $Gd_2O_3$  nanomaterials remains to date a stimulating approach for the development of biocompatible materials for cancer-specific imaging applications.

The surface chemistry of NPs is an important factor for their biomedical application, as colloidal NPs come into contact with the biological environment through their surfaces.<sup>21,22</sup> In general, one of the most critical aspects in the development of optimal nanoparticles is their colloidal stability and uncoated particles tend to aggregate in their dispersion in cell culture media exhibiting a significant toxic effect that limits their performance. Therefore, modifying the surface of NPs with organic molecules or hydrophilic polymers is crucial to improve



**Scheme 1** Schematic illustration of the design and functionalization of  $Gd_2O_3@SiO_2@Fe_3O_4$  ( $Fe_3O_4$ : cubes) nanocarriers with silane linker, estrogen and chelator molecules.

their stability and inherent water dispersibility.<sup>23–28</sup> Our study demonstrate biocompatible  $\text{Gd}_2\text{O}_3$  based core shell nanocarriers to boost cancer cells intracellular uptake *via* ligand–receptor binding interactions. In addition, study also showed that amino silane rooted  $\text{Gd}_2\text{O}_3$  carriers prevented the leaching of gadolinium ions which is of tremendous value to generate gadolinium based biocompatible nanomaterials for health-care application. The remaining amine residues were quantified on the surface of  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  nanocarriers and facilitated the binding of DOTA molecules to enable imaging of the cancer cells (Scheme 1).

## Results and discussion

### Design and synthesis of multilayered core-shell

Homogenous structures of  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  nanocarriers were chemically prepared based on multilayer assisted core shell approach. The formation of gadolinium oxide layer on core shell nanomaterials consists of a magnetite core, a middle  $\text{SiO}_2$  shell as a separating layer and outer a thin  $\text{Gd}_2\text{O}_3$  layer.<sup>28–30</sup> The  $\text{SiO}_2$  shell shields the magnetic dipole interaction of  $\text{Fe}_3\text{O}_4$  NPs to reduce the quenching effect on T1 contrast material, and consequently prevents the perturbation of the relaxation properties of T1 contrast material.<sup>31,32</sup>

The pathway of  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  synthesis first includes, solvothermal method and the use of inorganic iron precursor in the presence of DMF at high temperature for the formation of nanocubes. Following, these cubes were coated with a homogeneous silica shell using controlled reaction condition *via* a Stöber reaction. The silica shell on iron oxide cubes was chemically covered with a thin layer of gadolinium oxide. The carriers were extended for click conjugation strategies: APTMs was covalently attached on the surface of nanocarriers and quantified for amino active surface. Amino-terminated  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers were linked with  $-\text{Br}$  and  $-\text{N}_3$  molecules *via* nucleophilic substitution reaction. The estrogen molecules with active alkyne groups were interacted with azide modified  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  *via* Click conjugation approach. The chelator molecules were covalently binded on estrogen-capped carriers *via* a chemical reaction using  $-\text{SCN}$ -linked DOTA compound under basic conditions.

Following, in a multistep click chemistry approach, the surface of  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  nanocarriers was evaluated for the quantification of active amino groups.

In detail, primarily, hydrophilic (PVP-capped  $\text{Fe}_3\text{O}_4$ , quasi-cubic) and hydrophobic (oleic acid-capped  $\text{Fe}_3\text{O}_4$ , spherical) iron oxide particles, which differ substantially in size, shape, and surface texture were prepared and coated with a homogeneous silica shell to ensure stability and active linker conjugation.<sup>33</sup> Meanwhile, in case of the oleic acid-capped iron oxide particles, the oleic acid molecules were chemically adsorbed at NPs surfaces through the co-ordination between the metal atom and the  $-\text{COO}$  groups.<sup>34</sup> As a result of this chemical interaction, the hydrophobic part of oleic acid heading outwards leads to the nonpolar nature of the oleic layer around NPs surface. Therefore, to confine and control the  $\text{SiO}_2$  coating and to stimulate the diffusion of the  $\text{Fe}_3\text{O}_4$  NPs into the aqueous phase, non-ionic surfactant and micelles were employed. Herein, we applied an alternative strategy to use Stöber method for silica coated spherical iron oxide NPs instead of micro-emulsion approach. The treatment of nanocarriers with HCl (pH range: 3–4) resulted in the protonation of the oleate ligand and consequently the release of oleic acid from the NPs surface.<sup>35,36</sup> Therefore, the NPs retained their hydrophilic properties due to the presence of the  $-\text{OH}$  groups on the surface. The formation of  $\text{Gd}_2\text{O}_3$  shell onto the surface of  $\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers was attained through homogeneous precipitation of  $\text{Gd}^{3+}$  ion by the decomposition of urea at an elevated temperature.<sup>37</sup> Controlled-precipitation reaction (pH > 6) offered the formation of  $\text{Gd}(\text{OH})\text{CO}_3$  which coagulated on the negatively charged silanol groups at NP-surface. It in turn inhibits the dissolution of  $\text{SiO}_2$  layer and prevents possible agglomeration. Thermal treatment of  $\text{Gd}(\text{OH})\text{CO}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  NPs at 800 °C led to conversion of  $\text{Gd}(\text{OH})\text{CO}_3$  to  $\text{Gd}_2\text{O}_3$  layer offering monodispersed  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  structures. The crystallinity of all samples was investigated by powder X-ray diffraction measurements (Fig. 1). For both, nanocubes and nanospheres, all diffraction peaks are consistent with the standard pattern of pure  $\text{Fe}_3\text{O}_4$  which crystallized in an inverse spinel cubic space group. The broadening of the peaks in the case of nanospheres indicates the small size of the NPs, which is confirmed by TEM analysis. XRD pattern of  $\text{SiO}_2@\text{Fe}_3\text{O}_4$  NPs

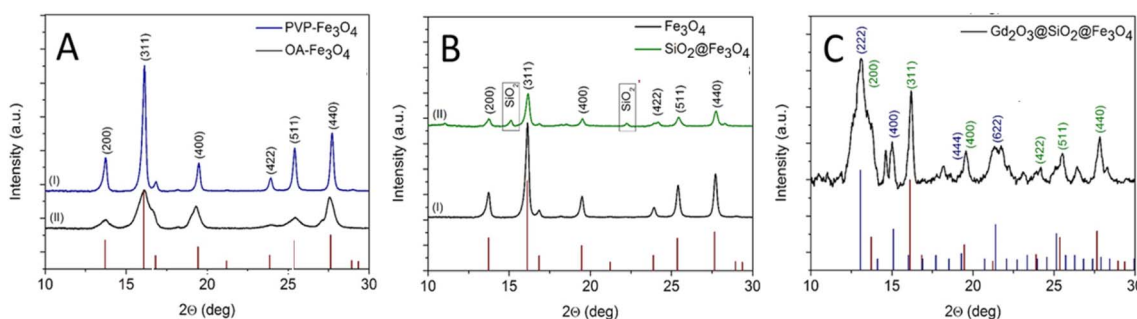


Fig. 1 X-ray diffraction patterns of: (A) iron oxide cubes and spheres, (I) PVP-capped  $\text{Fe}_3\text{O}_4$  nanocubes, and (II) OA-capped  $\text{Fe}_3\text{O}_4$  nanospheres, (B)  $\text{Fe}_3\text{O}_4$  nanocubes (I) before and (II) after coating with  $\text{SiO}_2$  layer, and (C) the as-prepared core-shell  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  ( $\text{Fe}_3\text{O}_4$  cubes as core) after calcination at 800 °C for 2 h.



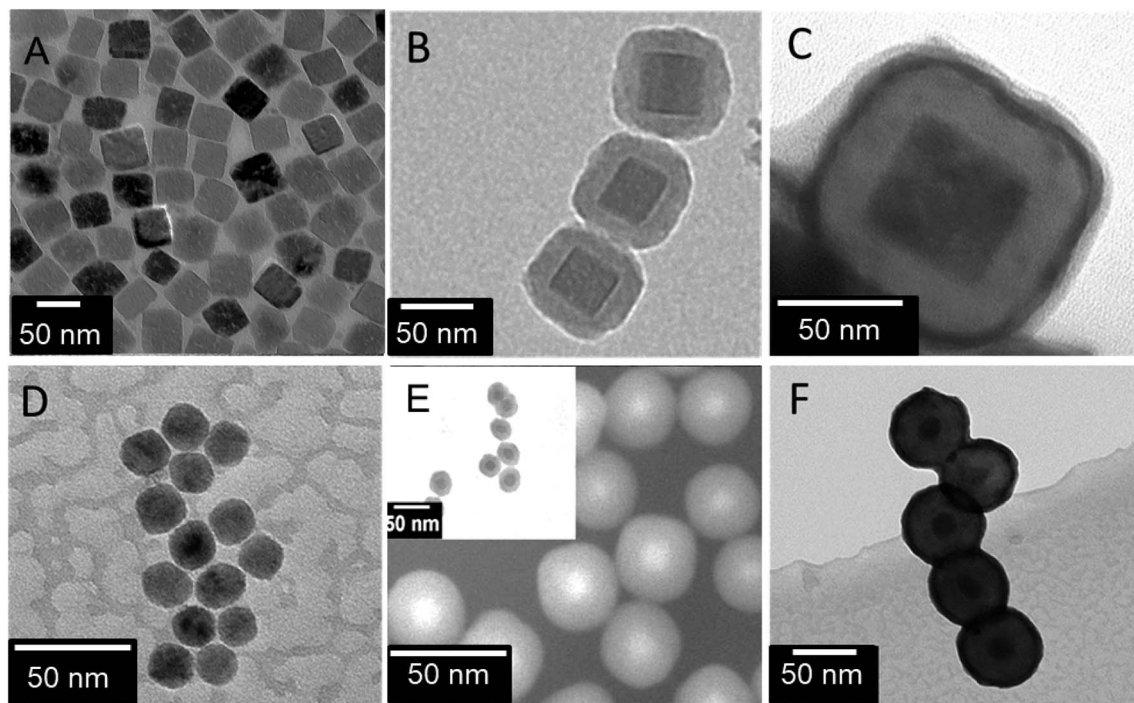


Fig. 2 Microscopic images: (A) PVP-capped- $\text{Fe}_3\text{O}_4$  nanocubes, (B)  $\text{SiO}_2@\text{Fe}_3\text{O}_4$ (cubes), (C)  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ (cubes), (D) OA- $\text{Fe}_3\text{O}_4$  nanospheres, (E)  $\text{SiO}_2@\text{Fe}_3\text{O}_4$ (spheres), and (F)  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ (spheres).

exhibited diffraction peaks similar to that of  $\text{Fe}_3\text{O}_4$  NPs. All peaks indexed to the cubic inverse spinel structure of  $\text{Fe}_3\text{O}_4$  confirmed that core maintained its crystallinity after  $\text{SiO}_2$  coating. Two additional peaks with low intensity were observed, probably due to the poorly crystalline state of  $\text{SiO}_2$  shell.<sup>38,39</sup> The diffraction pattern of  $\text{Gd}(\text{OH})\text{CO}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  showed key characteristic peaks of  $\text{Fe}_3\text{O}_4$ , (ICDD: C72-2303). The weak intensity of the peaks can be assigned to the formation of the additional layers on the surface of  $\text{Fe}_3\text{O}_4$  core. No other peaks were observed indicating the amorphous nature of the  $\text{Gd}(\text{OH})\text{CO}_3$ .<sup>40</sup> After annealing at 800 °C (2 h), additional peaks appeared which can be directly marked as a cubic crystalline phase of  $\text{Gd}_2\text{O}_3$  according to ICDD: 00-012-0797. The low intensity of the signals can be attributed to the thin thickness of  $\text{Gd}_2\text{O}_3$  layer.

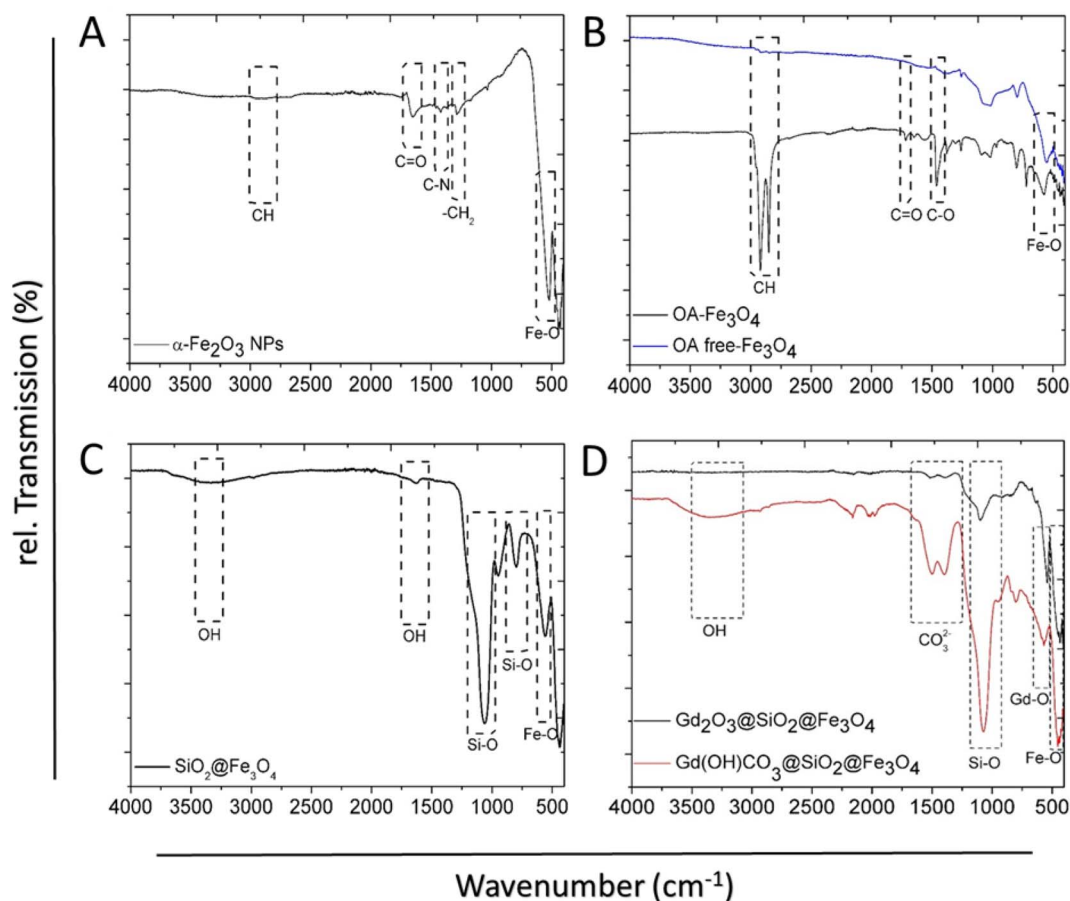
The transmission electron microscopy (TEM) analysis of iron oxide revealed monodispersed nanocubes with uniform structure and an average diameter of  $40 \pm 4$  nm (Fig. 2A). The controlled-quasi cubic geometry of the resulted nanocubes is dedicated to PVP which aids NPs to select their positions during the course of orientation attachment, and thus, formation of the regular geometry.<sup>41</sup> The TEM analysis of other iron oxide particles showed the presence of nanospheres in the sample with an average size of  $13 \pm 2$  nm (Fig. 2D). These uncapped spherical  $\text{Fe}_3\text{O}_4$  NPs did not show any agglomeration trends after the detachment of oleic acid which can be due to the presence of characteristic surface charge (Fig. S2†). It has been shown in the literature that the uncovered  $\text{Fe}_3\text{O}_4$  NPs are agglomerated compared to the OA-covered  $\text{Fe}_3\text{O}_4$  NPs.<sup>42</sup> Fig. 2B shows that  $\text{SiO}_2@\text{Fe}_3\text{O}_4$ (cubes) acquired a typical core shell

structure with dark ( $\text{Fe}_3\text{O}_4$  core) and light contrast ( $\text{SiO}_2$  shell thickness of  $20 \pm 2$  nm). Similar trends of homogeneous shell development ( $30 \pm 10$  nm) were observed for  $\text{SiO}_2@\text{Fe}_3\text{O}_4$ (spheres) structures originated from spherical iron oxide (Fig. 2E). After,  $\text{Gd}_2\text{O}_3$  coating, both,  $\text{SiO}_2@\text{Fe}_3\text{O}_4$ (cubes) and  $\text{SiO}_2@\text{Fe}_3\text{O}_4$ (spheres) exhibited smooth and thin  $\text{Gd}_2\text{O}_3$  layer formation ( $6 \pm 2$  nm and  $15 \pm 5$  nm respectively) with uniform solid core-shell structure. The images showed that the final morphology of the materials  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ (cubes) or  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ (spheres) obtained is influenced by the selection of the core material (Fig. 2C and F). More thickness of the  $\text{Gd}_2\text{O}_3$  layer on  $\text{SiO}_2@\text{Fe}_3\text{O}_4$ (spheres) as compared to  $\text{SiO}_2@\text{Fe}_3\text{O}_4$ (cubes) may be due to the concentration of  $\text{Gd}^{3+}$  salt precursor during synthesis. Selected area electron diffraction (SAED) pattern showed polycrystalline nature of the  $\text{Gd}_2\text{O}_3$ -shell (Fig. S-3C†). The composition of the NP was further confirmed by Energy-Dispersive X-ray analysis (EDX) which displayed surface elements distribution of iron (Fe), silicon (Si), gadolinium (Gd) and oxygen (O) elements in the core shell NPs (Fig. S-3D†).<sup>43</sup>

FT-IR spectra demonstrated distinct peaks of  $\alpha\text{-Fe}_2\text{O}_3$  NPs, whereas the vibration bands at 2946, 1650, 1420, and  $1276\text{ cm}^{-1}$  are attributed to the CH, C=O, C-N, and  $-\text{CH}_2$ , respectively, from PVP at NPs surface Fig. 3A. The strong peak at  $523\text{ cm}^{-1}$  is ascribed to the Fe-O vibration of  $\text{Fe}_2\text{O}_3$ . The successful release of oleic acid from the surface of the NPs was confirmed by FT-IR. The spectrum of OA- $\text{Fe}_3\text{O}_4$  NPs showed the characteristic peaks which indicate the presence of the OA-capping ligands at the NPs surface (Fig. 3B).<sup>44</sup> The peaks at  $2915\text{ cm}^{-1}$  and  $2845\text{ cm}^{-1}$  can be related to the asymmetric and symmetric







**Fig. 3** FT-IR spectra of: (A) PVP-capped  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>, (B) OA-capped Fe<sub>3</sub>O<sub>4</sub> (black) and uncapped Fe<sub>3</sub>O<sub>4</sub> (blue), (C) SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub>, and (D) amorphous Gd(OH)CO<sub>3</sub> shell of the precursor, and successful conversion to crystalline Gd<sub>2</sub>O<sub>3</sub> layer after calcination.

stretching of  $-\text{CH}_2$  groups. These peaks are absent in the OA-free NPs (Fig. 3B upper). Additionally, in contrast to the OA-NPs the peaks at  $1711\text{ cm}^{-1}$  and  $1457\text{ cm}^{-1}$  originate from the asymmetric and symmetric  $-\text{COO}$  stretching are not observed in the OA free NPs. The band observed around  $570\text{ cm}^{-1}$  in both spectra is assigned to the Fe-O vibration. Surface group analysis of SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> provided further confirmation for the formation of SiO<sub>2</sub> shell around Fe<sub>3</sub>O<sub>4</sub> NPs (Fig. 3C). The broad band at  $3346\text{ cm}^{-1}$  corresponds to the  $-\text{OH}$  group from silanol group at NPs surface as well as the water molecules, which can be absorbed on the surface. This band is correlated with another band at  $1617\text{ cm}^{-1}$  which is attributed to deformation vibration of the water molecules. The sharp band at  $1060\text{ cm}^{-1}$  can be assigned to Si-O-Si asymmetric stretching vibration and the lowest intense band at  $798\text{ cm}^{-1}$  is ascribed to Si-O-Si symmetric stretching vibration. Peak at  $556\text{ cm}^{-1}$  is related to the Fe-O vibration. For Gd(OH)CO<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> (Fig. 3D), a strong absorption band at  $1520\text{ cm}^{-1}$  arises from the vibration of  $\text{CO}_3^{2-}$  group of amorphous Gd(OH)CO<sub>3</sub> shell. In addition, the broad band at  $3330\text{ cm}^{-1}$  can be assigned to the vibrations of  $-\text{OH}$  groups. A strong band at  $1093\text{ cm}^{-1}$  is assigned to the Si-O-Si asymmetric stretching vibration, whereas the other

band at  $798\text{ cm}^{-1}$  is attributed to the Si-O-Si symmetric stretching vibration.

From the spectra of Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub>, the intensity of characteristic bands of Gd(OH)CO<sub>3</sub> amorphous shell remarkably decreased after calcination (Fig. 3D). The two intense absorption bands located at  $544$  and  $445\text{ cm}^{-1}$  are accounting for Gd-O stretching vibration of the Gd<sub>2</sub>O<sub>3</sub> layer and Fe-O vibration of Fe<sub>3</sub>O<sub>4</sub> core, respectively. The FT-IR studies provided further evidence that the amorphous Gd(OH)CO<sub>3</sub> shell can be efficiently converted to Gd<sub>2</sub>O<sub>3</sub> during the calcination process, which is consistent with the results obtained from XRD and EDX.

The magnetic measurements of the SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> and Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> carriers showed an effect on the magnetic behavior of the SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> NPs after the coating of Gd<sub>2</sub>O<sub>3</sub> layer to the composite material (Fig. 4). There is a distinct separation of field-cooled (FC) and zero field-cooled (ZFC) curves, as expected for the superparamagnetic behavior of the Fe<sub>3</sub>O<sub>4</sub> cores (Fig. 4B). The diversion of FC and ZFC curves starts already at  $270\text{ K}$ , and agree with the high temperature maximum of both curves. Although the signal is relatively weak, the behavior is mimicking that of pure Fe<sub>3</sub>O<sub>4</sub> NPs,<sup>26</sup> however, there is an additional "tail" at lower temperatures in both FC and ZFC



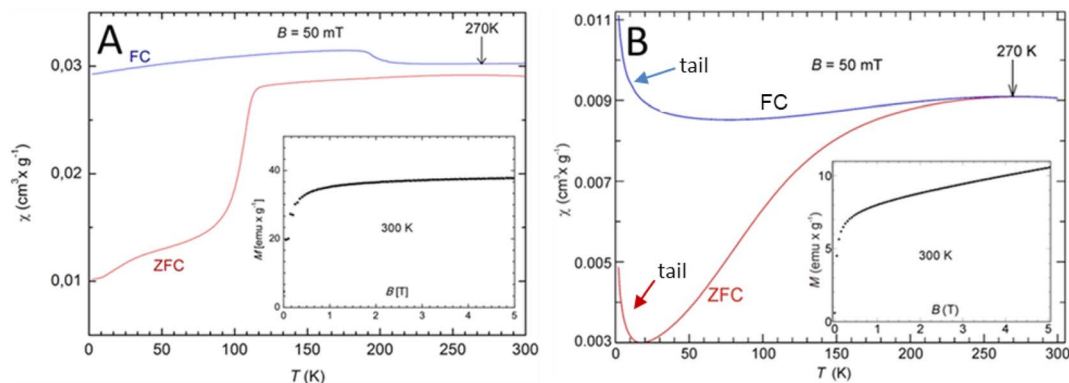


Fig. 4 Temperature dependent magnetic susceptibility ( $\chi$ ) of: (A)  $\text{SiO}_2@Fe_3O_4$  and (B)  $\text{Gd}_2O_3@SiO_2@Fe_3O_4$  and its field dependent magnetization ( $M$ ) at 300 K (inset). All data are normalized to the sample mass in g. FC (field cooled) and zero field-cooled (ZFC) data are presented and they superimpose only above the marked 270 K.

curves, indicating the presence of the paramagnetic Gd-moments. Moreover, there is no clear blocking temperature, as was observed at about 120 K for the composite  $\text{SiO}_2@Fe_3O_4$  material without the  $\text{Gd}_2O_3$  shell (Fig. 4A).<sup>33</sup> The room-temperature magnetization (inset Fig. 4B) can be explained by a superparamagnetic part, which is saturated already at low fields, and a paramagnetic part, causing a linear increase with field on top of the saturation part. The absolute signal is also significantly small per gram, here  $\sim 7$  as compared to about  $\sim 35$   $\text{emu g}^{-1}$  for  $\text{SiO}_2@Fe_3O_4$ , which is due to the relatively high density and, thus, the absolute weight of  $\text{Gd}_2O_3$  relative to  $\text{SiO}_2@Fe_3O_4$ . The addition of  $\text{Gd}_2O_3$  to the  $\text{SiO}_2@Fe_3O_4$  composite adds a paramagnetic component across the whole measured temperature range and seems to prevent a clear magnetic blocking to occur between the  $Fe_3O_4$  cores. Hence, the paramagnetic  $\text{Gd}_2O_3$  shell seems to screen the core ferromagnetic moment of  $Fe_3O_4$ . This perhaps allows the magnetic NPs to act more like individual entities.

The efficiency of the surface-functionalized nanocarriers in biological systems is influenced by the physical and chemical nature of the surface-attached groups.<sup>26,46,47</sup> Since both carriers contain a thin gadolinium oxide layer, only the multilayer  $\text{Gd}_2O_3@SiO_2@Fe_3O_4$  with cube-based nanocarriers were extended for chemical conjugation strategies to prove the conjugation concept. The  $\text{Gd}_2O_3@SiO_2@Fe_3O_4$  were modified with APTMS molecules to offer free amino ( $-NH_2$ ) groups. Attachment of APTMS not only provided free amino groups for further ligand conjugation, nevertheless also offered better safety to  $\text{Gd}_2O_3@SiO_2@Fe_3O_4$  nanocarriers evident from toxicity assay. It reveals the coverage of the  $\text{Gd}_2O_3$  surface with silane linkers, could prevent its direct exposure to the cells. FT-IR analysis demonstrated the binding of APTMS groups on  $\text{Gd}_2O_3@SiO_2@Fe_3O_4$  surface evident in the increased intensity of Si-O-Si band at  $1093\text{ cm}^{-1}$ , when compared to bare particles (Fig. 5A). The peaks at  $1687$  and  $1380\text{ cm}^{-1}$  belonged to the  $-NH$  bending and C-N stretching vibrations, respectively, whereas the minor peak at  $2933\text{ cm}^{-1}$  is attributed C-H vibrations. Surface modification of the as-developed carriers with estrogen molecule ( $17\alpha$ -ethynylestradiol) was carried out *via* click

chemistry approach.<sup>48</sup> As a first step of click-functionalization sequences, 6-bromohexanoic acid molecules were covalently binded to the surface of particles by DCC/NHS carbodiimide chemistry at  $60^\circ\text{C}$ . The carboxylic groups were activated by the addition of coupling reagent dicyclohexylcarbodiimide (DCC) to create amine-reactive *O*-acylisourea ester. Following, the activated carboxylic acid were readily reacted with amino groups at the NPs surface to afford bromo groups on nanoparticles ( $-Br@NPs$ ), Fig. 5B. After bromohexanoic acid attachment on particles, the FTIR spectrum showed a strong band due to C=O vibration at  $1717\text{ cm}^{-1}$ , and the stretching vibrations of the C-H at  $2965\text{--}2850\text{ cm}^{-1}$  indicating the successful on surface attachment of the bromo-acid (Fig. 5B). Subsequently, the bromide-terminated NPs were substituted with azide groups ( $-N_3$ ) in a nucleophilic substitution  $SN_2$  reaction with sodium azide. The absorption band at  $2099\text{ cm}^{-1}$  corresponds to the  $-N_3$  stretching vibrational mode verified the conjugation of 6-azido-hexanoate on the surface of particles (Fig. 5C).<sup>49,50</sup> The absorption band at  $2990\text{ cm}^{-1}$  owing to the C-H vibration mode showed that the bromine precursor maintained its properties on the surface without any degradation. Successful immobilization of estrogen molecule onto the  $N_3@NPs$  surface was accomplished by an azide-alkyne cycloaddition reaction.<sup>51</sup> In Fig. 5D the characteristic peak of the azide vibration is completely vanished, while new absorption bands at  $1624$  and  $1440\text{ cm}^{-1}$  owing to triazole ring appeared, demonstrating the completion of click reaction on NPs surface.<sup>52</sup> All FT-IR spectra for the modified core shell NPs demonstrated the vibration bands of the Si-O, Gd-O, and Fe-O suggesting the unchanged configuration and structure of the core shell NPs.

Moreover, the XRD pattern of estrogen- $\text{Gd}_2O_3@SiO_2@Fe_3O_4$  carriers that the crystallinity of the material did not change during click modification (Fig. S5†).

To evaluate the average hydrodynamic radius of bare and surface functionalized nanocarriers, dynamic light scattering measurements were conducted at pH 7.0 in water. APTMS- $\text{Gd}_2O_3@SiO_2@Fe_3O_4$  showed an average hydrodynamic diameter of  $259 \pm 11$  which is larger than the diameter shown by bare  $\text{Gd}_2O_3@SiO_2@Fe_3O_4$  indicating a change on the surface of



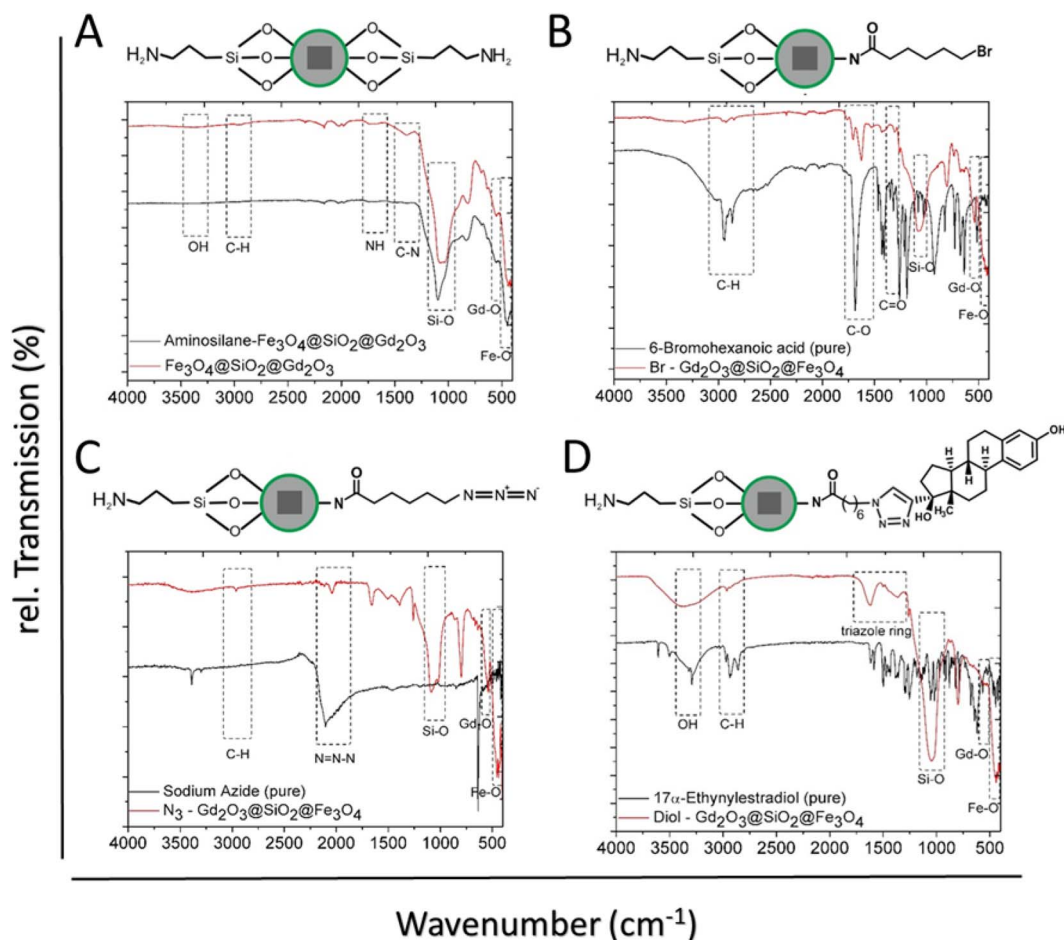


Fig. 5 The FTIR-spectra of: (A) APTMS-capped  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  NPs, (B) attachment of the bromooctanoic acid to the surface, (C) attachment of azide groups, and (D) click chemistry between 17 $\alpha$ -ethynylestradiol and the azide-terminated  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  NPs.

particles after APTMS conjugation. Nanocarriers showed a further increase in hydrodynamic diameter of particles due to the binding of additional molecules including bromo and estrogen onto the surface (Table S1†). To evaluate the average surface charge of the bare and surface functionalized nanocarriers, zeta potential measurements were conducted at pH 7.0 in water. Bare  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  exhibited a negative value of  $-10.1$  mV due to the presence of hydroxyl groups on bare surface. After amino silane coupling, these values moved to  $+11.6$  mV, ascribed to the positive charge from protonated amine groups in aqueous solution. After bromo and estrogen conjugation to these carriers, the surface charge showed a huge shift to  $-13.0$  mV and  $-15.8$  mV respectively due to the consumption of amino groups during carbodiimide coupling reaction between  $-\text{NH}_2$  capped nanocarriers and bromo hexanoic acid (bromo: more electron density).

The thermal degradation behaviour of the bare, intermediate steps and estrogen-functionalized  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers were observed using thermogravimetric (TGA) measurements in the temperature range of  $30$ – $900$   $^{\circ}\text{C}$  at a heating rate of  $10$   $^{\circ}\text{C}$   $\text{min}^{-1}$  under the flow of nitrogen. TGA analysis showed that bare  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  particles are stable up to  $300$   $^{\circ}\text{C}$ . At this decomposition temperature, the total mass loss is 6%

which could be attributed to evaporation of the residual adsorbed solvent and water molecules on the  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  particles. APTMS-capped  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers showed a mass loss of in two steps. After solvent evaporation at  $250$   $^{\circ}\text{C}$ , the results show approximately 5% mass loss followed by second mass loss of 24% (with a total of loss of 31%), occurred in the temperature window from  $250$  to  $900$   $^{\circ}\text{C}$  which is much higher than that of bare  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  particles. This increase in mass loss can be attributed to the thermal decomposition of the surface-grafted aminosilane (APTMS) molecules (Fig. 6B). After surface modification with 6-bromohexanoic acid, the Br-conjugated  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  particles showed a total mass loss of 28%. At initial decomposition temperature of  $300$   $^{\circ}\text{C}$ , a mass loss of 6% occurred due to the removal of adsorbed solvent molecules. The other mass loss of 22% from  $300$  to  $900$   $^{\circ}\text{C}$  displayed the decomposition of bromo group related content *i.e.*, alkyl chain and amide bond (Fig. 6C). Thermal degradation of  $\text{N}_3$ -conjugated  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  particles demonstrated a total mass loss of 18% (Fig. 6D) for the removal of azide group related content. The deviation in results as compared to Br-conjugated  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  particles can be attributed to the different amount of NPs that present in the measured sample as well as the size distribution of NPs.



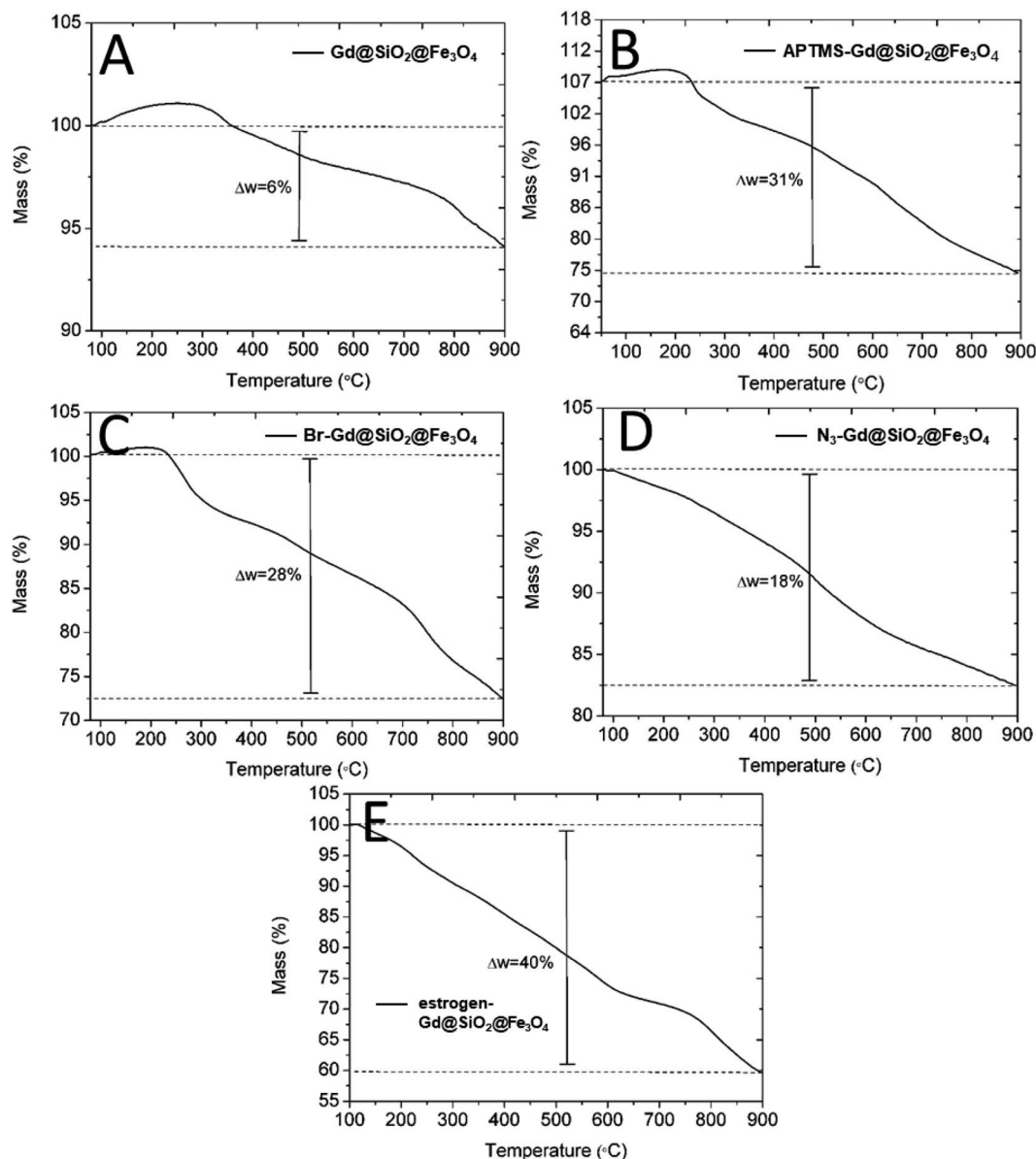


Fig. 6 Thermogravimetric analysis of the different synthetic steps of nanocarrier's functionalization: (A) bare  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ , (B) APTMS/ $\text{NH}_2\text{-Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ , (C)  $\text{Br-Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ , (D)  $\text{N}_3\text{-Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ , and (E) estrogen- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ . The initial increase of mass in the range from 0–300 °C in (A), (B) and (C) can be due to the minor fluctuation of the weighing balance of the machine due to the background noise.

After grafting 17 $\alpha$ -Ethinylestradiol on  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  surface, the larger mass loss of 40% in three steps was the consequence of the thermal decomposition of all molecules including 17 $\alpha$ -ethinylestradiol.

#### Quantification of active primary amines on the surface of nanocarriers

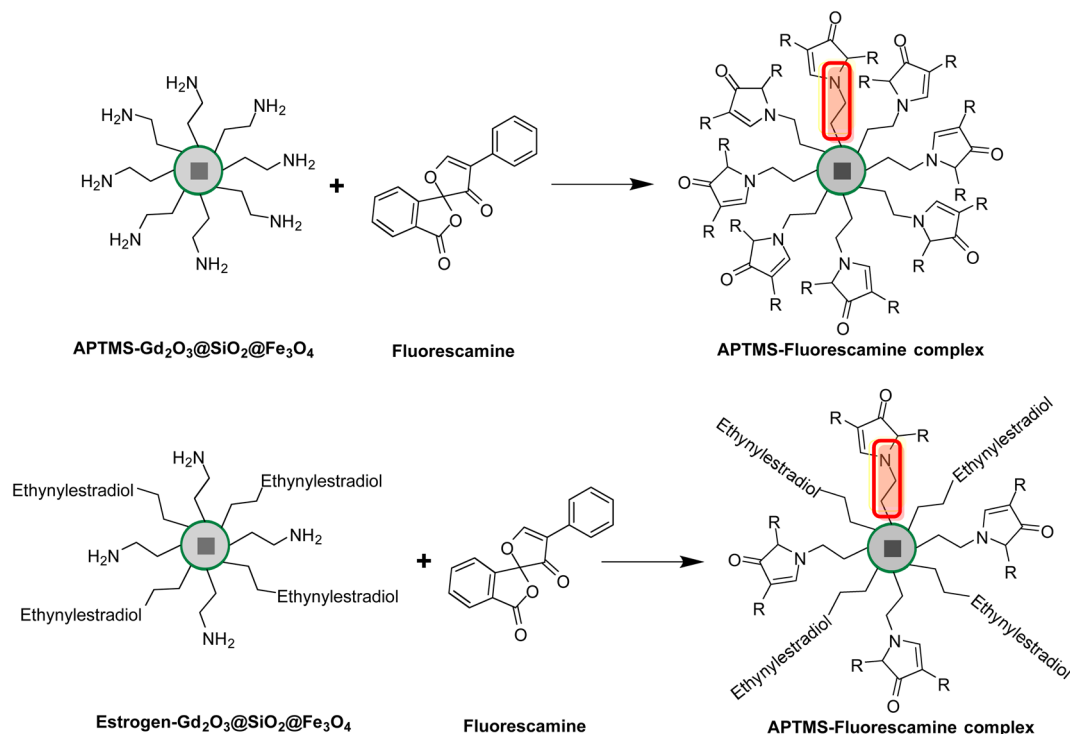
The fluorescamine molecule based approach was chosen to quantify the active amino group on the surface of functional nanocarriers.<sup>45a</sup> Precisely, fluorescamine is not UV-visible active on its own but when reacts with primary amine groups it forms a product which gives an absorbance at a wavelength of 384 nm under UV-visible radiation.<sup>45b</sup> Moreover, the unreacted

fluorescamine hydrolyzes in the presence of water and produces non UV-visible active molecules and this feature makes this approach more accurate and reliable, Scheme 2.

To calibrate this approach, the experiments were conducted with pure APTMS at its various concentrations using a constant amount of fluorescamine (stock solution: 1 mg fluorescamine/1 ml acetonitrile). The pure APTMS samples were measured by applying UV-vis spectroscopy in which the absorption of spectra generated at given wavelengths can be linked directly to the concentration of the sample, Table 1.

Based on the values, amino capped- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  and estrogen-coated  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  nanocarriers were evaluated for the quantification of active surface amino ligands.





**Scheme 2** The fluorescamine molecule based approach for the quantification of active amino groups on the surface of nanocarriers before and after the conjugation of estrogen molecules.

**Table 1** Absorbance values of different concentration of APTMS with 50  $\mu\text{l}$  of fluorescamine for calibration curve

APTMS ( $\mu\text{l}$ )	APTMS concentration ( $\mu\text{g}$ )	No of moles APTMS	Molar concentration	Absorbance (a.u.)
5.84	60	$3.3 \times 10^{-7}$	$3.28 \times 10^{-7}$	0.50971
25.33	260	$1.4 \times 10^{-6}$	$1.36 \times 10^{-6}$	1.32234
35.08	360	$2.0 \times 10^{-6}$	$1.9 \times 10^{-6}$	1.62892
44.83	460	$2.5 \times 10^{-6}$	$2.39 \times 10^{-6}$	2.20854
54.57	560	$3.1 \times 10^{-6}$	$2.93 \times 10^{-6}$	2.75921

Active amino groups were quantified with reference to the calibration curve using different concentration of APTMS with 50  $\mu\text{l}$  of fluorescamine from the stock solution. Nanocarriers functionalized with APTMS molecules showed a peak at  $\lambda_{\text{max}}$  390 nm with absorbance of 1.36 (Fig. 7C), whereas, the same amount of estrogen-capped carriers showed a peak at 390 nm with absorbance of 0.597. Table 2 (Experimental section) provides details of the quantification readout in which it can be observed that the decrease in the absorbance indicated the presence of less active amino group on the surface of estrogen capped  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  nanocarriers compared to the APTMS functionalized nanocarrier.

Since the well-known toxic effect of  $\text{Gd}^{3+}$ -chelate compounds and  $\text{Gd}_2\text{O}_3$ -NP are inhibiting their application *in vitro* and *in vivo*, the biocompatibility of the bare  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  and APTMS-capped  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  was verified using as model cell-line HEK 293 (Fig. 8). Hemmer *et al.* showed that by appropriate surface functionalization, the interaction of ion leaching can be reduced which offers reduced toxicity and

enhanced biocompatibility.<sup>36</sup> For as-synthesized bare particles showed some toxic effect to the cells (cell viability: 70%) after 48 h with 50  $\mu\text{g ml}^{-1}$  NPs concentration (Fig. 8A). However, APTMS modification on these carriers offered notable cell viability. The molecules attachment on the surface of  $\text{Gd}_2\text{O}_3$  shell, forming a protective layer on the overall core shell structure which renders its direct exposure to the cells (Fig. 8B). A minor decrease in viability of cells (78%) was observed when cells were exposed to nanocarriers with a maximum concentration of 100  $\mu\text{g ml}^{-1}$ . The results are comparable to the applied concentration in reported MRI studies, declaring that the APTMS-coating on gadolinium based carrier's offers biocompatibility and decreased toxicity for cells.

#### Radiolabeling assessment of DOTA-conjugated estrogen- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ nanocarriers

To assess the imaging capabilities of as-synthesized core shell nanocarriers, we have developed an efficient radiolabeling procedure using  $^{68}\text{Ga}$  which can be followed through radiation



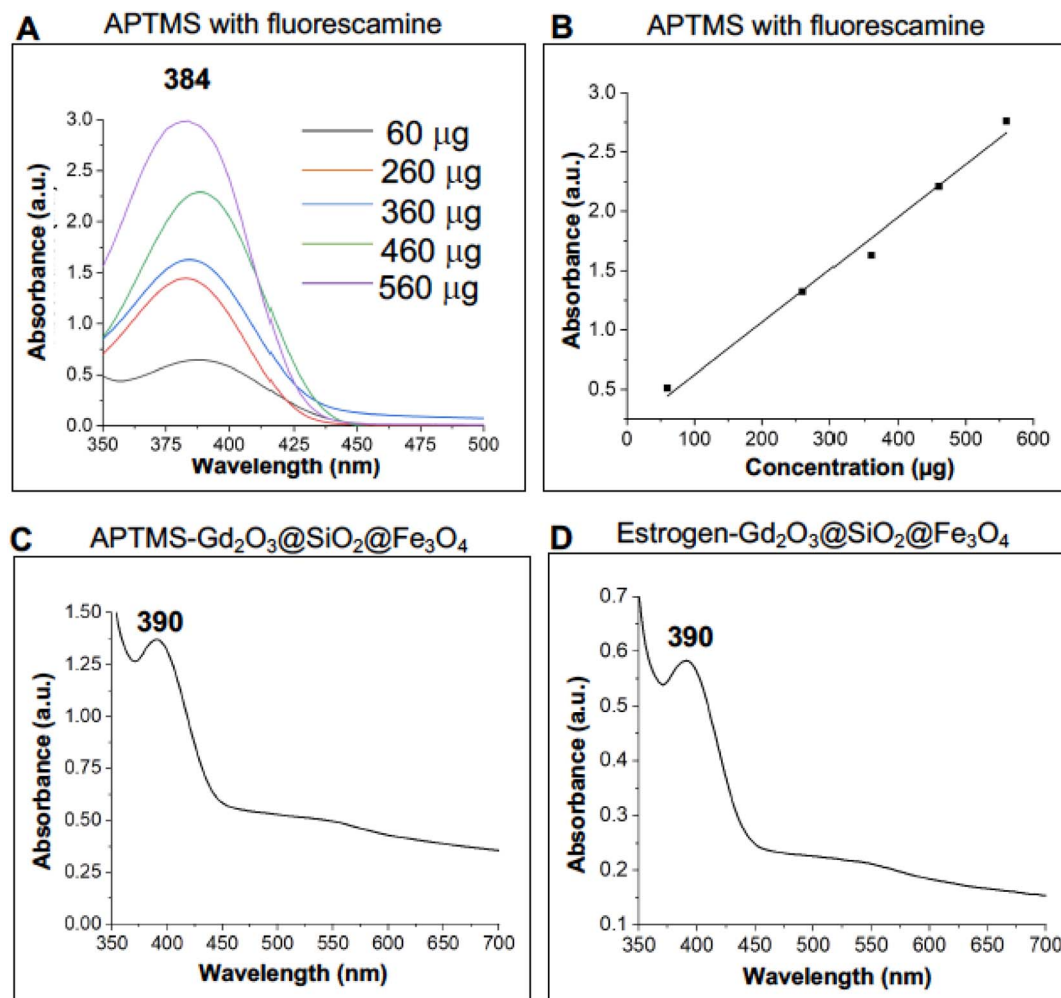


Fig. 7 Quantification of active amino groups on the surface of gadolinium based functionalized nanocarriers: (A and B) UV-visible spectra and relevant calibration curve for the quantification of pure APTMS using fluorescamine molecules. (C and D) Determination of amino groups on APTMS- $\text{Gd}_2\text{O}_3@SiO_2@Fe_3O_4$  and estrogen- $\text{Gd}_2\text{O}_3@SiO_2@Fe_3O_4$  nanocarriers.

detection as a result of radioactive decay.<sup>53</sup> To determine radioactive labeling yields, we used instant radio thin layer chromatography (ITLC) following APTMS- $\text{Gd}_2\text{O}_3@SiO_2@Fe_3O_4$  and estrogen- $\text{Gd}_2\text{O}_3@SiO_2@Fe_3O_4$  nanocarriers. The available amino groups at the carrier's surface provided the binding of *p*-SCN-Bn-DOTA through isothiocyanate linkage. Subsequently, radiolabeling experiments were conducted using  $^{68}\text{Ga}$  considering its incorporation to the vicinity of DOTA chelator. As the

half life time of  $^{68}\text{Ga}$  radionuclide is 68 min, all experiments were performed for a maximum incorporation time of 60 min. Fig. 9 shows the TLC graph for the radiolabeling efficacy of  $^{68}\text{Ga}$ -DOTA conjugated carriers. In both samples, the radiolabeling profile exhibited two different regions, the high intensity peak which correspond to the  $^{68}\text{Ga}$ -DOTA nanocarriers, and the low intense peak for the unbound  $^{68}\text{Ga}$  radionuclide. Radiochemical Yield (RCY) was estimated to be 80% for APTMS-

Table 2 Determination of active amino groups on the surface of nanocarriers

Nanocarriers	Absorbance (a.u.)	Concentration <sup>a</sup> (µg) of active amino group	No. of moles <sup>b</sup> of active amino group	Molar concentration <sup>c</sup>	No. of molecule <sup>d</sup> of active amino group
APTMS@ $SiO_2@Fe_3O_4$ (2 mg)	1.36	266	$1.48 \times 10^{-6}$	$1.409 \times 10^{-6}$	$8.9 \times 10^{17}$
Estrogen-capped $Gd_2O_3@SiO_2@Fe_3O_4$ (2 mg)	0.597	94	$5.2 \times 10^{-7}$	$4.95 \times 10^{-7}$	$3 \times 10^{17}$

<sup>a</sup> Concentration of active amino groups on the surface of nanocarriers was calculated using slope formula. <sup>b</sup> No of moles = concentration (in grams)/molar mass of APTMS. <sup>c</sup> Molar concentration = no of moles/1.050 ml. <sup>d</sup> No of molecules was calculated by multiplying the no of moles with avogadro's number.



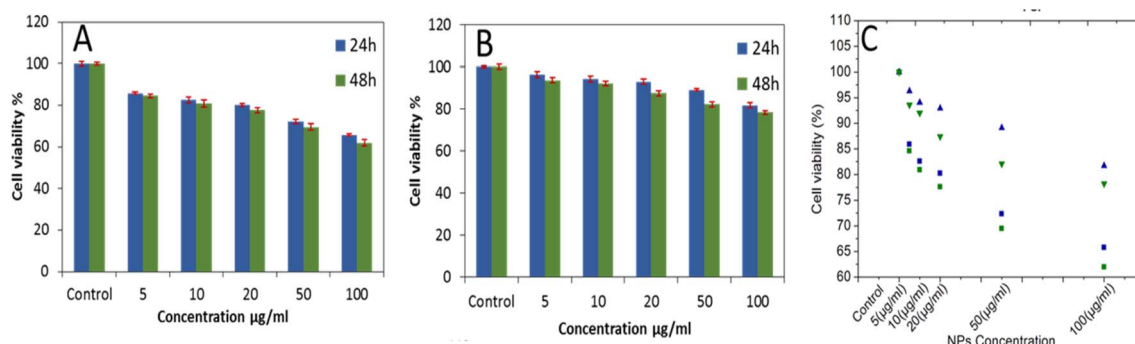


Fig. 8 *In vitro* cell viability data of cultured HEK 293 cells after incubation with: (A) bare  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ , and (B) APTMS-capped  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ , using standard MTT colorimetric assay (C) comparison of the cell viability assay: the square symbols indicate cell viabilities measured for bare  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  and the triangle symbols for APTMS-capped  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ , viabilities after 24 and 48 h of incubation are shown in blue and green, respectively.

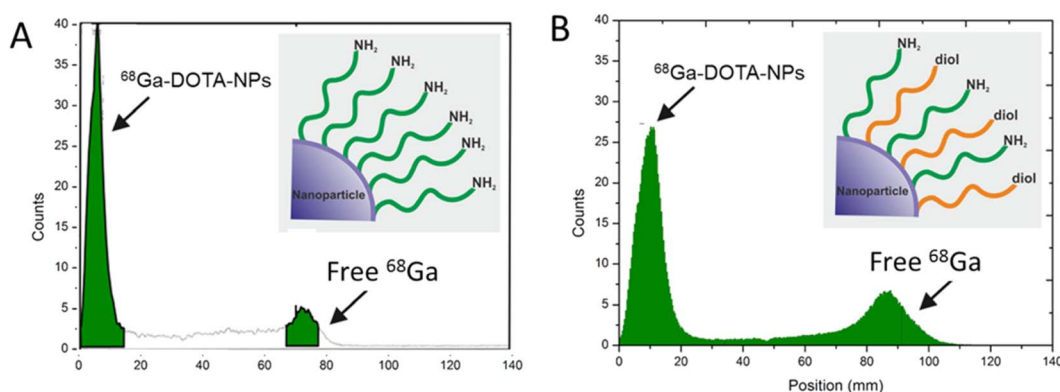


Fig. 9 TLC chromatogram of: (A) APTMS- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  after radiolabeling with  $^{68}\text{Ga}$  and (B) APTMS-estrogen  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers after radiolabeling with  $^{68}\text{Ga}$ . The radiolabeling was performed under slight acidic conditions incubating particles for 15 min at  $95^\circ\text{C}$ . The radiochemical yield was determined by thin layer chromatography (ITLC). No peak was observed for free chelator molecules indicating the covalent binding of the DOTA molecule to the estrogen-capped APTMS- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  nanocarriers.<sup>54</sup>

$\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  (Figure 9A) and 70% for estrogen- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  nanocarriers (Fig. 9B). The expected variance of radiochemical yield for both nanocarriers can be explained by the insufficient remaining  $-\text{NH}_2$  groups on the surface of estrogen- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers.

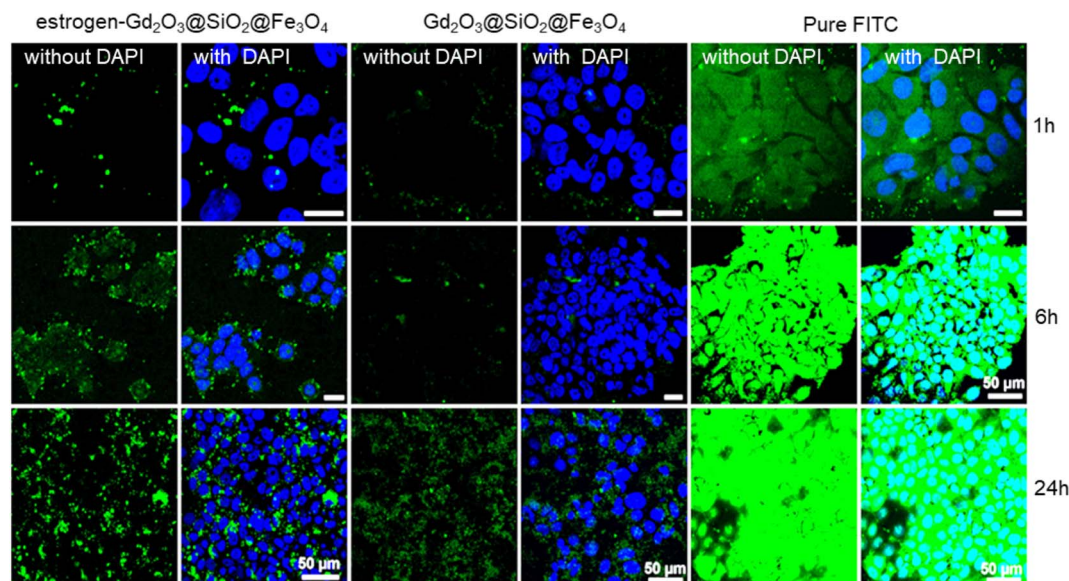
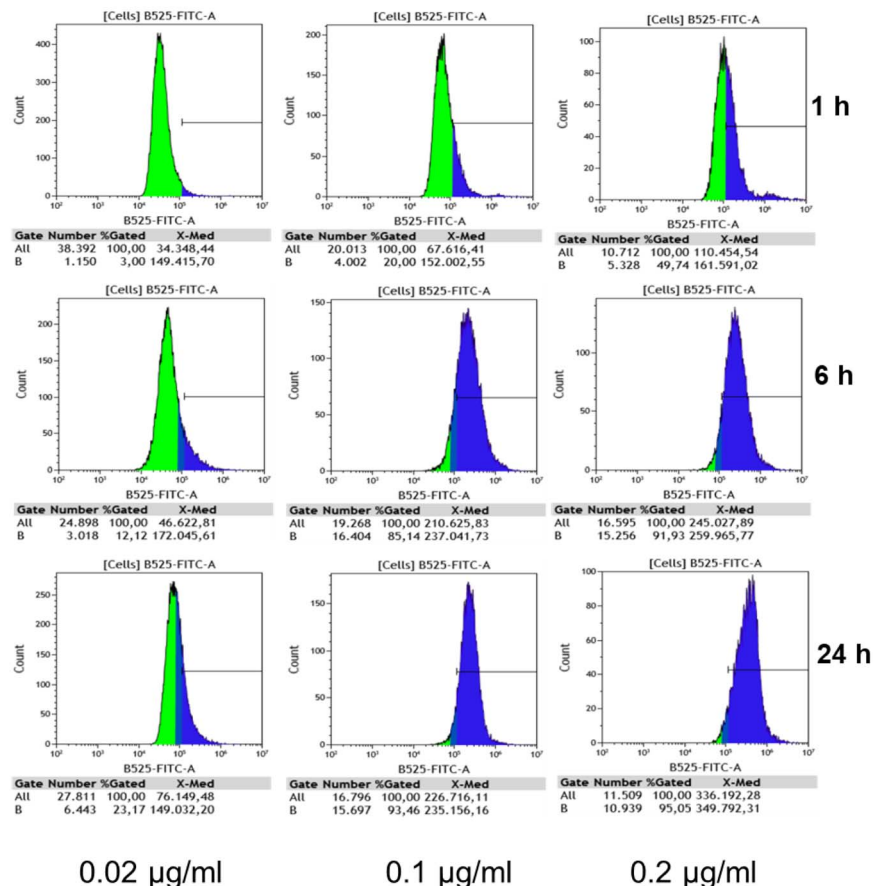
These amino groups are required for DOTA binding at the particle surface. Due to their consumption during carbodiimide crosslinking, the estrogen- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers are expected to have fewer active amino groups for chelator coupling as compared to APTMS- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  particles. Therefore, less number of DOTA ligands might be available for  $^{68}\text{Ga}$  incorporation, resulting in a lower radiolabeling yield. In general, DOTA ligands are specific and efficient chelating agents for  $\text{Gd}^{3+}$  ions. It has been reported that the DOTA ligand has a high affinity to coordinate with  $\text{Gd}^{3+}$ , while NODAGA (2-[1,4,7-triazacyclononan-1-yl-4,7-bis('Bu-ester)]-1,5-pentanedioic acid) ligands have a high affinity for  $^{68}\text{Ga}$ . Moreover, gallium complex must be stable enough to avoid *trans*-chelation. Thus, a possible competition among  $\text{Gd}^{3+}$  and  $\text{Ga}^{3+}$  ions for coordinating the DOTA ligands cannot be ruled out in the solutions, which can consequently reduce the RCY. For the labeling experiment, we used a specific activity of 12.8

MBq per microgram (32 MBq per 2.5 microgram), which agrees very well with the values reported for the specific activity of PET probe.

#### Cellular uptake of estrogen- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$ nanocarriers

Cell uptake of  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  and estrogen- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  nanocarriers was investigated with confocal microscopy and flow cytometry measurements using MCF7 breast cancer cell-line (*in vitro*). Before their testing with cancer cells, the carriers were coupled with FITC to track their localization. Confocal imaging was performed in a time dependent manner for 1, 6 and 24 h, Fig. 10. In case of nanocarriers without ligand, it can be observed that even after 24 h only a low amount of  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers are taken up by BC cells and typically located in the intercellular area (Fig. 10 upper image). In case of estrogen- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers, time dependent higher amount of particles is observed. After 24 h the nanocarriers accumulated in the intracellular spaces and are located next to the cell nucleus in the cytoplasm. Flow cytometry measurements were performed with  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  and estrogen- $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  using MCF-7 breast cancer cell



Cell uptake of estrogen-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub>

**Fig. 10** Cell uptake measured by confocal microscopy (upper image) followed by flow cytometry (lower image) using Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> and estrogen-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub>: estrogen-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> exhibited intracellular localization. Confocal laser scanning microscopy showed that nanocarriers has been internalized in cancer cells. MCF-7 cells were incubated with the equivalent concentration of nanocarriers for different time frames, 1 h, 6 h and 24 h. Incubation with estrogen-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub>, bare Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> and pure FITC were evaluated. Cells were incubated (30 min) with 4',6-diamidino-2'-phenylindole (DAPI) to give visibility to the nuclei (blue) and FITC is indicated by green fluorescence. Scale bar in each image is 50 μm. The nanocarriers were incubated with MCF7 cells lines. The cell uptake is investigated under the influence of different incubation time and nanocarriers concentration.





line. Here,  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  and cancer cells without particles were considered as control samples, (Fig. S7†). To investigate concentration and time dependent cell uptake, cancer cells were incubated with 3 different concentrations of NPs ( $0.1 \mu\text{g ml}^{-1}$ ,  $0.2 \mu\text{g ml}^{-1}$  and  $0.02 \mu\text{g ml}^{-1}$ ) following the investigation of cellular uptake with 3 time periods of 1, 6 and 24 hours. Based on the negatively charged cell membrane and positively charged APTMs-capped nanocarriers, the particles stack randomly with cancer cells by electrostatic interaction leading to a cellular uptake of over 99% for all tested time periods (1 h, 6 h, and 24 h) as well as all tested concentrations. Only in case of an incubation time for 1 h and 6 h at a concentration of  $0.02 \mu\text{g ml}^{-1}$ , the NPs exhibited an uptake between 74% and 85% respectively, Fig. S7.† The estrogen  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  showed reduced cellular uptake for all tested time periods, however, in a concentration dependent manner (Fig. 10 lower image) (possibly due to its negative surface charge). For a concentration of  $0.02 \mu\text{g ml}^{-1}$  during 1 h, nearly no nanoparticles were taken up or stack on cancer cell membrane, illustrating the influence of surface charge of nanoparticles. When estrogen  $\text{Gd}_2\text{O}_3@\text{SiO}_2@\text{Fe}_3\text{O}_4$  carriers were incubated with an increased concentration of  $0.1 \mu\text{g ml}^{-1}$  and  $0.2 \mu\text{g ml}^{-1}$ , the probes showed increasing trends for cell uptake within the first hour of incubation. Over time, the cellular uptake increased due to the affinity of  $17\alpha$ -ethynylestradiol molecules to estrogen receptors indicating receptor mediated cell uptake. The slow cell uptake over a longer period of time, which can be observed in naked nanocarriers and control samples and must be taken into account, is also striking. However, an optimal ratio between randomly (non-specific) and specific cellular uptake was achieved at a concentration of  $0.1 \mu\text{g ml}^{-1}$ . Under these conditions, 20% of all cells took up at least one NP within the first hour. After 6 hours, cellular uptake increased to 85% and after 24 hours to 93%. Thus, estrogen-conjugated carriers showed slower but specific cellular uptake within 24 hours compared to carriers without a targeting molecule. Remarkably, any cancer cell that took up at least one NP resulted in a positive signal.

## Conclusion

Layer by layer chemical method is a versatile approach to develop new core-shell design and structure of nanocarriers such as  $\text{Gd}_2\text{O}_3$  based  $\text{SiO}_2@\text{Fe}_3\text{O}_4$ . The as-developed carriers offer covalent attachment of more than one ligands quantitatively, on their surface, capable of individually addressing their targets in physiological environment on a single carrier system. In addition, binding the surface  $\text{Gd}_2\text{O}_3$  thin layer with silane precursors enables biocompatibility and offer less toxicity to cells. Surface charge and active ligands are decisive factors a significant increase in cellular uptake of particles, suggesting receptor-mediated endocytosis in MCF-7 cancer cell lines. The appropriate radiolabeling yield with  $^{68}\text{Ga}$  demonstrate its potential as diagnostic nanocarriers for breast cancer research. The developed nanocarriers could be promising dual-contrast agent for T1 & T2 MRI and PET imaging expending different radionuclides.

## Experimental section

### Materials

Gadolinium(III) nitrate hexahydrate ( $\text{Gd}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ ), iron(III) nitrate nonahydrate ( $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ ), ammonium hydroxide solution (28–30%), tetraethylorthosilicate (TEOS), 6-bromohexanoic acid, sodium azide ( $\text{NaN}_3$ ),  $17\alpha$ -ethynylestradiol 98%, dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorescamine, borate buffer ( $0.1 \text{ mol L}^{-1}$ ), acetonitrile were all supplied by Sigma-Aldrich company. *N,N*-Dimethyl formamide (DMF) was obtained from Fischer Scientific and poly(*N*-vinyl-2-pyrrolidone)  $M_w = 55,000$  (PVP) from Acros Organics. S-2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (*p*-SCN-Bn-DOTA) from macrocyclics. Phosphate-buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco. The HEK 293 cells were obtained from Leibniz Institute DSMZ-German Collection of Microorganism and Cell Culture, ACC 305. The MCF-7 is human breast cancer cell line and obtained originally by pleural effusion from a female patient with metastatic disease. All reagents and solvents were used as received without further purification.

### Methods

**Synthesis of  $\text{Fe}_3\text{O}_4$  nanocubes.** Quasi-cubes  $\alpha\text{-Fe}_2\text{O}_3$  NPs were synthesized following the procedure reported by Zheng *et al.*<sup>34</sup> Briefly, 289 mg (0.571 mmol)  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  were dissolved in 23 ml DMF followed by the addition of 628 mg (0.011 mmol) PVP (55 000). The mixture was transferred into a 50 ml Teflon-lined stainless autoclave and heated at  $180^\circ\text{C}$  for 30 h. The obtained NPs were separated from solution by centrifugation and washed several times with water and ethanol. Afterwards NPs were dried in a vacuum oven at  $60^\circ\text{C}$  for 12 h. The hematite NPs were reduced to  $\text{Fe}_3\text{O}_4$  in an oven using forming gas (5%  $\text{H}_2$ /95% Ar) at  $350^\circ\text{C}$  for 4 h with a heating rate of  $10^\circ\text{C min}^{-1}$ .<sup>33</sup>

**Synthesis of iron-oleate precursor.** Iron-oleate compound, 10.8 g of iron chloride (40 mmol) and 36.5 g of sodium oleate (120 mmol) was dissolved in a mixture of 60 ml distilled water, 80 ml ethanol, and 140 ml hexane. The resulting solution was heated to  $70^\circ\text{C}$  and kept for 4 h in same temperature. Afterwards, the upper organic layer containing iron-oleate complex was washed several times with 30 ml distilled water. After washing, the residual hexane was evaporated off yielding iron-oleate complex in a waxy solid form.<sup>55</sup>

**Synthesis of  $\text{Fe}_3\text{O}_4$  nanospheres.** 18 g (20 mmol) of the synthesized iron-oleate complex and 2.8 g of oleic acid (10 mmol) were dissolved in 100 g of 1-octadecene at ambient temperature. The reaction mixture was heated to  $320^\circ\text{C}$  with a heating rate of  $3.3^\circ\text{C min}^{-1}$ , and kept at the same temperature for another 30 min. The reaction mixture was cooled down to ambient temperature followed by the addition of 100 ml of ethanol to precipitate the  $\text{Fe}_3\text{O}_4$  particles. The obtained NPs were separated by centrifugation, washed with acetone and ethanol (8000 rpm for 15 mins, 6x) and dried overnight (16 h) at ambient temperature.<sup>55</sup>



**Synthesis of SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> nanocarriers.** 40 mg of Fe<sub>3</sub>O<sub>4</sub> NPs (cubes or spheres) were dispersed in 60 ml water by sonication. 0.4 ml silicic acid solution [SiO<sub>x</sub>(OH)<sub>4-2x</sub>]<sub>n</sub> was prepared in prior by hydrolysis of 0.65 ml tetraethylorthosilicate (TEOS) in 5 ml distilled water at pH 3–4 and added to the NPs dispersion. The dispersion was stirred for 30 min, then 0.4 ml NH<sub>4</sub>OH (28%) was added and after aging the reaction for 3 h, the particles were collected by centrifugation and redispersed in 40 ml ethanol. Afterwards, 0.08 ml of TEOS was added to the dispersion, followed by 0.65 ml of NH<sub>4</sub>OH (28%). The reaction was stirred for 3 h at ambient temperature. Further 0.08 ml TEOS were added and the reaction mixture was kept for 12 h. The resultant SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> core shell NPs were separated by centrifugation and washed with ethanol (8000 rpm for 10 mins, 3x) and dried for 16 h at ambient temperature.<sup>33</sup>

**Synthesis of Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> nanocarriers.** 1.7 ml of 0.5 M Gd(NO<sub>3</sub>)<sub>3</sub> aqueous solution was added into a 50 ml round-bottom flask and dissolved in 10 ml of water. A 0.5067 g portion of urea was then added to the solution with stirring for 5 min to form homogeneous clear solution. Solution of well dispersed SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> (0.08345 g 10 ml<sup>-1</sup>, either with cubes as core or spheres as core were added into the above solution and sonicated for 15 min). Afterwards, the flask containing the mixture was placed in an oil bath and heated at 90 °C for 6 h under vigorous stirring. The particles were separated by centrifugation and washed three times with ethanol and dried at 60 °C overnight. The particles were thermally treated at 800 °C for 2 h with a heating rate of 10 °C min<sup>-1</sup> under an air atmosphere to afford Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> both cubes and spheres based core shell structure.<sup>18</sup>

**Synthesis of NH<sub>2</sub>-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> nanocarriers.** 50 ml ethanol was purged with an excess for nitrogen flow following by the addition of 30 mg Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> nanocarriers. These carriers were sonicated for 5 min following dropwise addition of 0.5 ml APTMS under continuous supply of nitrogen. Later, the reaction was refluxed at 75 °C for 6 h.<sup>56</sup> The modified particles were collected *via* centrifugation at 6000 rpm and consequently washed with ethanol (3x) to remove any unbound aminosilane. The particles were dried at 60 °C for 16 h at ambient temperature and tested for further characterization.

**Synthesis of Br-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> nanocarriers.** 20 mg of APTMS-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> NPs were dispersed in excess of toluene under nitrogen environment and dispersion was sonicated for 10 min. 14 mg of 6-bromohexanoic acid was first reacted with DCC (8 mg) followed by addition to nanoparticles dispersion under vigorous stirring. The dispersion was stirred for 20 h at 60 °C. The nanoparticles were separated by centrifugation and purified using ethanol with sonication followed by centrifugation at 4000 rpm for 10 min. The final construct was dried overnight (16 h) at ambient temperature.<sup>24,48</sup>

**Synthesis of azide-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> nanocarriers.** 15 mg of Br-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> were dispersed in 15 ml of DMF. The nanoparticles dispersion was treated with 13 mg of sodium azide and stirred for 48 h at 60 °C. The product was isolated by centrifugation and purified with ethanol at 6000 rpm for 10

minutes (3x). The retrieved particles were dried overnight (16 h) at ambient temperature.<sup>24,48</sup>

**Synthesis of estrogen-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> nanocarriers.** Click reaction was conducted in a with azide-Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> NPs in mixture of ethanol and water. 50 mM 17 $\alpha$ -ethynylestradiol was added to sodium citrate and CuSO<sub>4</sub>·5H<sub>2</sub>O. This solution was slowly transferred to the particles dispersion and kept under stirring (500 rpm) for 48 h at 60 °C. The estrogen-modified particles were separated by centrifugation, and washed three times with water and ethanol and dried overnight (16 h) at ambient temperature.<sup>24,48</sup>

### Quantification of active primary amines on the surface of nanocarriers

Stock solution of fluorescamine was prepared in 1 ml of acetonitrile by adding 1 mg of fluorescamine. Stock solution of APTMS was prepared by adding 500  $\mu$ l of APTMS to 50 ml of borate buffer. Different concentration of APTMS stock solution in 1 ml of borate buffer (0.1 mol L<sup>-1</sup>) were reacted with a 50  $\mu$ l of fluorescamine stock solution (Table 1) and the absorbance of fluorescamine-amine complex was calculated with UV-visible spectrophotometry. Calibration curve at the wavelength of  $\lambda_{\text{max}}$  384 nm was established using the absorbance of different concentration of APTMS with fluorescamine and the whole experiment was done in triplets (Fig. 7B). To calculate the active amino groups 2 mg of each APTMS and estrogen capped Gd<sub>2</sub>O<sub>3</sub>@SiO<sub>2</sub>@Fe<sub>3</sub>O<sub>4</sub> nanocarriers were dispersed in 1 ml of borate buffer and reacted with 50  $\mu$ l of fluorescamine stock solution.<sup>45a,b</sup> The pure APTMS samples were measured by applying UV-vis spectroscopy in which the absorption of spectra generated at given wavelengths can be linked directly to the concentration of the sample, Table 2.

The UV-visible spectra of both nanocarriers was analyzed and the concentration of active amino group on the surface of nanocarriers were calculated considering slope and intercept values obtained by calibration curve [Fig. 7B and eqn (1)]

$$y = a + bx \quad (1)$$

where  $y$  = absorbance,  $a$  = intercept,  $b$  = slope and  $x$  = concentration.  $y = a + bx$  where  $a = 0.17843$  and  $b = 0.00443$ , considering calibrating in Fig. 7B.

### Radiolabeling efficacy studies

**Synthesis of DOTA-conjugated NPs and <sup>68</sup>Ga labeling.** 1 mg of nanocarriers were suspended in 1.0 ml of 0.2 M Na<sub>2</sub>CO<sub>3</sub> buffer. A stock solution of 1 mg of *p*-SCN-Bz-DOTA was dissolved in 2.5 ml 0.2 M Na<sub>2</sub>CO<sub>3</sub> buffer solution under alkaline conditions. The reaction was incubated at ambient temperature for 18 h. After the incubation time the reaction was centrifuged for 10 min at 6000 rpm. The supernatant was removed and the nanocarriers were purified ultrapure water at 4000 rpm (3x). Later, nanocarriers were re-suspended in 950  $\mu$ l of ultrapure water for labeling experiments with gallium-68. For the labeling experiments, 50  $\mu$ l of the suspended NPs were mixed with 150  $\mu$ l



of 2 M sodium acetate buffer in a 1.5 ml reaction vial. 200  $\mu\text{l}$  of  $^{68}\text{Ga}$  ( $\sim 32$  MBq) was added to the reaction vial resulting in a solution with acidic pH. The labeling mixture was incubated at 95  $^{\circ}\text{C}$  for 20 min. The radiochemical yield was determined by instant thin layer chromatography (ITLC).<sup>57</sup>

**Cell toxicity assay.** The cytotoxicity of the  $\text{Gd}_2\text{O}_3$  nanocrystals was evaluated by means of MTT assay, using HEK 293 cells. HEK 293 cells (150  $\mu\text{l}$  of  $7.5 \times 10^4$  cells per ml) were cultured in a 96-well plate at 37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for 24 h. The nanocrystals (1 mg NPs in 1 ml sterilized water) were incubated in the medium with different concentration, (5  $\mu\text{g}$ , 10  $\mu\text{g}$ , 20  $\mu\text{g}$ , 50  $\mu\text{g}$ , 100  $\mu\text{g}$ ) over a period of 24 and 48 h at 37  $^{\circ}\text{C}$ . HEK 293 cells treated only with culture media fixed as positive control. To each well, MTT (5 mg  $\text{ml}^{-1}$ ) was added for 2 h incubation at 37  $^{\circ}\text{C}$ . Afterwards, the supernatant was removed, and 150  $\mu\text{l}$  DMSO per well were added to resolve the formazan-crystals. The absorbance was measured at 490 nm with reference wavelength of 630 nm using Elisa reader multiplate (ELX 800, BioTek Instruments, USA).<sup>58</sup>

**FACS measurements.** MCF7 breast cancer cells were cultured in 10 ml cell medium at 37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for 48 h. The cell medium was removed and the cells were washed carefully with 8 ml DPBS buffer. 4 ml trypsin was added and the cells were incubated for 5 min breaking the bonds between cells and vessel bottom. Afterwards, 5 ml cell medium including PBS was added stopping the reaction of trypsin. The cells were centrifuge at 1200 rpm for 5 min and the supernatant was discarded. The pellet was redispersed in 5 ml cell medium and transferred into a 96 well plate containing of 500 000 cells per well. Different concentrations (0.02  $\mu\text{g ml}^{-1}$ , 0.1  $\mu\text{g ml}^{-1}$ , 0.2  $\mu\text{g ml}^{-1}$ ) of nanoparticle dispersions were added to each well (except the negative control) and the well plate were incubated for 1 hour, 6 hours and 24 hours. Afterwards the cell medium was removed and the cells were washed carefully with 200  $\mu\text{l}$  DPBS buffer. 100  $\mu\text{l}$  trypsin was added and the 96 well plate was incubated for 3 min. To stop the reaction of trypsin, 150  $\mu\text{l}$  cell medium including PBS was added and the cells were transferred into a second 96 well plate. The dispersion was centrifuged 3 times at 1200 rpm for 5 min and washed with 200  $\mu\text{l}$  cell wash during each step. After the last washing step, the cells were redispersed in 100  $\mu\text{l}$  cell wash and measured (CytoFLEX LX, Beckman Coulter, Germany).<sup>24</sup>

**Instrumentation.** The X-ray diffraction (XRD) analysis of nanopowders was carried out on a STOE-STADI MP diffractometer equipped using graphite-monochromated Mo K $\alpha$  radiation (0.71073  $\text{\AA}$ ) source and operating in transmission mode. Fourier transform infrared (FTIR) spectra were recorded with PerkinElmer FTIR spectrophotometer 400 in the range 400–4000  $\text{cm}^{-1}$ . Scanning electron micrographs were performed on a Nova Nano SEM 430 Company FEI (max. acceleration voltage 30 kV). Electron-X-ray spectroscopy was performed on an Apollo X EDAX (working distance 5 mm; entry angle 35 $^{\circ}$ ). The morphologies and composition of the samples were observed by Transmission Electron Microscope (TEM) using Zeiss LEO 912 instrument equipped with LaB $_6$ -cathode operated at 120 kV high voltage. The dynamic light scattering experiments (DLS) were measured with a Malvern instruments Zetasizer Nano SZ. Phases of thin films were determined Magnetic measurements were performed with a superconducting quantum interference device (SQUID)

magnetometer (Quantum Design, MPMS XL-7). Thermogravimetric analysis (TGA) characterization was performed on Netzsch STA 449C Jupiter. Samples were characterized in  $\text{Al}_2\text{O}_3$  chamber in atmosphere of dry nitrogen or synthetic air (80%  $\text{N}_2$  + 20%  $\text{O}_2$ ), flow: 70  $\text{cm}^3 \text{min}^{-1}$ , heating ramp 10  $^{\circ}\text{C min}^{-1}$  to 900  $^{\circ}\text{C}$ .

## Conflicts of interest

The authors declare no competing financial conflict of interest.

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