We demonstrated a unique approach that combines a layer-by-layer (LbL) self-assembly method with dendrimer chemistry to functionalize Fe$_3$O$_4$ nanoparticles (NPs) for specific targeting and imaging of cancer cells. In this approach, positively charged Fe$_3$O$_4$ NPs (8.4 nm in diameter) synthesized by controlled co-precipitation of Fe$^{II}$ and Fe$^{III}$ ions were modified with a bilayer composed of polystyrene sulfonate sodium salt and folic acid (FA)- and fluorescein isothiocyanate (FI)-functionalized poly(amidoamine) dendrimers of generation 5 (G5.NH$_2$-FI-FA) through electrostatic LbL assembly, followed by an acetylation reaction to neutralize the remaining surface amine groups of G5 dendrimers. Combined flow cytometry, confocal microscopy, transmission electron microscopy, and magnetic resonance imaging studies show that Fe$_3$O$_4$/PSS/G5.NHAc-FI-FA NPs can specifically target cancer cells overexpressing FA receptors. The present approach to functionalizing Fe$_3$O$_4$ NPs opens a new avenue to fabricating various NPs for numerous biological sensing and therapeutic applications.

1. Introduction

Magnetic nanoparticles (NPs) have many interesting biomedical applications.[1–5] They have been used as contrast agents for magnetic resonance (MR) imaging and colloidal mediators for magnetic hyperthermia of cancer.[6] One major issue related to cancer applications of NPs is the lack of specific binding of NPs to cancer cells. In order to achieve the specific targeting of cancer cells, various proteins such as transferrin,[7–10] herceptin,[11,12] and chlorotoxin[13] have been conjugated onto iron oxide NP surfaces. The applied protein ligands display inherent immunogenicity. The specific ligand-receptor or antibody-antigen interaction occurs on the cell membrane, thereby limiting the intracellular uptake of conjugated NPs.[11,12] It is believed that these disadvantages can be overcome by using a small molecular weight ligand linked to iron oxide NPs. Several groups have investigated the conjugation of folic acid (FA) onto iron oxide NPs for targeting cancer cells.[14–16] One major concern is that the conjugation methods employed have involved complicated multi-step synthesis and modification procedures. In most cases, this has made it difficult to simultaneously couple other molecules onto iron oxide NP surfaces to achieve multifunctionalities. Therefore, the fabrication of targeted iron oxide NPs that also have conjugated drug or imaging molecules remains a challenge.

One approach relevant to NP surface modifications is the electrostatic layer-by-layer self-assembly technique[17–19] that allows the creation of ultra-thin functional films on NP surfaces.[20–24] The biofunctionality of the NPs is able to be tuned by deposition of functional polyelectrolytes or biomacromolecules on the NP surfaces.[25,26] For example, Wang et al constructed CdTe quantum dot/polyelectrolyte (PE) multilayers onto polystyrene microparticles, followed by an assembly of an outermost layer of anti-immunoglobulin for subsequent immunosensing.[25] Caruso and co-workers demonstrated that the biotin-functionalized Au NP/polyelectrolyte-coated latexes terminated by FI-anti-biotin IgG can be used for homogeneous, competitive fluorescence quenching immunoassay of biotin molecules.[26] In another recent work of the same group, it is shown that multilayered sub-micrometer sized polystyrene particles modified with a humanized A33 monoclonal antibody can specifically target A33 antigen-expressing LIM1215 colorectal cancer cells.[27]

Recent advances in dendrimer chemistry show that poly(amidoamine) (PAMAM) dendrimers can be covalently linked with defined numbers of targeting ligands, imaging dyes, and drugs, thus providing a platform for the specific targeting, imaging, and treatment of cancer.[28–32] We here attempt to combine the unique features of dendrimer chemistry with the versatile, LbL self-assembly technique that provides a facile
approach to fabricating multifunctional iron oxide NPs for targeting and imaging of cancer cells. To accomplish this, we assembled iron oxide NPs with a polyelectrolyte (polystyrene sulfonate sodium salt, PSS) and a generation 5 PAMAM dendrimer prefunctionalized with FA and fluorescein isothiocyanate (FI) moieties (G5.NH2-FI-FA) on the surface of iron oxide NPs using the LbL self-assembly technique. The PSS/G5.NH2-FI-FA bilayer-coated iron oxide NPs were then subjected to an acetylation reaction to neutralize the remaining terminal amine groups of the dendrimers (Fig. 1). The formed FI- and FA-functionalized iron oxide NPs display very high specific binding affinity to cancer cells overexpressing FA receptors (FAR), as demonstrated by flow cytometry, confocal microscopy, transmission electron microscopy (TEM), and MR imaging. To our knowledge, this is the first example of the fabrication of multifunctional iron oxide NPs by combining the LbL self-assembly technique with dendrimer chemistry.

2. Results and Discussion

2.1. Characterization of Functionalized Iron Oxide Nanoparticles

The magnetic iron oxide (Fe3O4) NPs were synthesized by controlled co-precipitation of Fe II and Fe III ions according to the literature.[33] The synthesized Fe3O4 NPs (8.4±1.4 nm in diameter as verified by TEM) are positively charged (zeta potential = +42.02 mV), which allows the subsequent self-assembly of a negatively charged PSS polyelectrolyte followed by a positively charged G5.NH2-FI-FA or G5.NH2-FI dendrimer. The Fe3O4/PSS-G5.NH2-FI-FA or Fe3O4/PSS-G5.NH2-FI NPs formed were subjected to an acetylation reaction to neutralize the remaining terminal amine groups of the dendrimers (Fig. 1). Zeta potential measurements were used to monitor each step of the coating and functionalization of Fe3O4 NPs (Table 1). The alternating charge reversal of Fe3O4 NPs after coating with PSS and G5.NH2-FI or G5.NH2-FI-FA dendrimers indicates the successful electrostatic assembly. After the acetylation reaction, the zeta potentials of both Fe3O4/PSS/G5.NHAc-FI and Fe3O4/PSS/G5.NHAc-FI-FA significantly decreased due to the conversion of the dendrimer surface amine groups to acetamide groups. It is interesting to note that the zeta potentials of neither Fe3O4/PSS/G5.NHAc-FI nor Fe3O4/PSS/G5.NHAc-FI-FA NPs are close to zero. This implies that some of the dendrimer terminal amines that interact with PSS polymer chains due to electrostatic interaction cannot be acetylated. We believe that the few remaining positive charges of Fe3O4 NPs would not cause significant non-specific binding with tumor cells, because the outermost surface amines of dendrimer layer are acetylated. The functionalized Fe3O4 NPs with the bilayer coating and acetylation reaction are colloidal stable in aqueous solution as well as in cell culture medium for at least 6 months at concentrations up to 10 mg mL–1. The selection of the bilayer coating in this study is to demonstrate a concept that the LbL assembly combined with dendrimer chemistry can be used to functionalize preformed NPs. We also think that several layers’ coating with the outermost dendrimer layer may be applicable for improving the mechanical stability of the particles for in vivo studies. However, more layers’ coating may increase the risk of weakening the colloidal stability of the particles. It is worthwhile to note that the approach combining the LbL assembly method with dendrimer chemistry is essential to make the iron oxide NPs functional biologically. It is known that carboxyl-terminated PAMAM dendrimers can be self-assembled onto iron oxide nanoparticle surfaces.[34,35] Our unpublished data show that iron oxide NPs directly modified with carboxyl acid-terminated dendrimers functionalized with FA moieties through electrostatic interaction do not specifically bind KB cells overexpressing FA receptors, presumably due to the carboxyl groups on the dendrimer surface. Fabrication of iron oxide NPs with neutral or close to neutral surface charge is essential to facilitate the specific binding through receptor-mediated endocytosis.

The self-assembled bilayers of PSS/G5.NHAc-FI and PSS/G5.NHAc-FI-FA on Fe3O4 NPs were also characterized by TEM imaging. The TEM image of PSS/G5.NHAc-FI-FA-coated Fe3O4 NPs (Fig. 2a) shows that, after the bilayer self-assembly and chemical functionalization, the particles display similar morphology to the ones before self-assembly (Fig. S4, Supporting Information). The aggregated nanoparticles shown in the TEM image

Table 1. Zeta potential values of Fe3O4 NPs after each step modification.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Zeta potential [mV]</th>
</tr>
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<tbody>
<tr>
<td>Fe3O4</td>
<td>+42.02</td>
</tr>
<tr>
<td>Fe3O4/PSS</td>
<td>–45.04</td>
</tr>
<tr>
<td>Fe3O4/PSS/G5.NH2-FI</td>
<td>+52.81</td>
</tr>
<tr>
<td>Fe3O4/PSS/G5.NH2-FI-FA</td>
<td>+43.08</td>
</tr>
<tr>
<td>Fe3O4/PSS/G5.NHAc-FI</td>
<td>+31.48</td>
</tr>
<tr>
<td>Fe3O4/PSS/G5.NHAc-FI-FA</td>
<td>+23.86</td>
</tr>
</tbody>
</table>

Figure 1. Schematic representation of the fabrication of targeted iron oxide NPs.
PSS/G5.NHAc-FI or Fe3O4/PSS/G5.NHAc-FI-FA NPs display solution. As mentioned above, the functionalized Fe3O4 NPs are stable in both aqueous solution and cell culture medium. A negatively stained (phosphotungstic acid) TEM image (Fig. 2b) clearly shows that all Fe3O4 NPs are surrounded with the bright rings of the polymer bilayers of PSS/G5.NHAc-FI-FA, further confirming the successful self-assembly process. PSS/G5.NHAc-FI-coated Fe3O4 NPs display similar polymer ring structures to those of PSS/G5.NHAc-FI-FA-coated Fe3O4 NPs as observed from the negatively stained TEM images (data not shown).

2.2. Cytotoxicity Assay

The cytotoxicity of the functionalized Fe3O4 NPs was evaluated by fluorescein diacetate (FDA) and propidium iodide (PI) staining and by observing cell morphology changes after incubation with the Fe3O4 NPs for 96 h. Cell viability data (Fig. S5, Supporting Information) show that the KB cells (a human epithelial carcinoma cell line) treated by functionalized Fe3O4 NPs with or without FA conjugation display similar percentage of FDA positive cells to the KB cells treated by unmodified Fe3O4 NPs at an Fe concentration of 0–150 µg mL⁻¹. Phase contrast microscopy images show that even at a Fe concentration of up to 150 µg mL⁻¹, KB cells treated with either Fe3O4/PSS/G5.NHAc-FI-FA or Fe3O4/PSS/G5.NHAc-FI-FA NPs display the same morphology as those treated with PBS buffer (Fig. S6, Supporting Information), indicating that the PSS/dendrimer bilayer functionalized Fe3O4 NPs are biocompatible at an Fe concentration of up to 150 µg mL⁻¹.

2.3. Flow Cytometry

The FA and the dye FI modified onto the G5 dendrimer surface were used as a targeting ligand and an imaging molecule, respectively. This affords the functionalized Fe3O4 NPs with both targeting and imaging functionalities. Folic acid receptor (FAR) is well known to be overexpressed in several human carcinomas including breast, ovary, endometrium, kidney, lung, head and neck, brain, and myeloid cancers.[36–38] In this study, KB cells expressing both high- and low-levels of FAR (denoted as KB-HFAR and KB-LFAR, respectively) were selected for the intracellular uptake of functionalized Fe3O4 NPs. Figure 3 illustrates the binding of PSS/G5.NHAc-FI-FA- and PSS/G5.NHAc-FI-coated Fe3O4 NPs (Fe concentration = 4.5 µg mL⁻¹) in KB-HFAR and KB-LFAR cells investigated by flow cytometry. It is clear that after binding of PSS/G5.NHAc-FI-FA-coated Fe3O4 NPs with KB-HFAR cells, the fluorescence signal significantly increases (Fig. 3a). In contrast, PSS/G5.NHAc-FI-modified Fe3O4 NPs without FA conjugation display a similar fluorescence signal to the PBS control, suggesting no measurable binding to KB-HFAR cells. For KB-LFAR cells, neither Fe3O4/PSS/G5.NHAc-FI nor Fe3O4/PSS/G5.NHAc-FI-FA NPs display significant binding (Fig. 3b). This indicates that the binding of the FA-modified Fe3O4 NPs to KB cells is mediated by the FAR. We also investigated the dose-dependent cellular uptake of the functionalized Fe3O4 NPs (Fig. 3c and d). At an Fe concentration above 2.3 µg mL⁻¹, KB-HFAR cells exposed to Fe3O4/PSS/G5.NHAc-FI-FA NPs show remarkably higher fluorescence signals than those treated with Fe3O4/PSS/G5.NHAc-FI NPs without FA (Fig. 3c). This again implies that the high affinity of FAR mediates specific uptake of the NPs.

Both Fe3O4/PSS/G5.NHAc-FI and Fe3O4/PSS/G5.NHAc-FI-FA NPs display much less uptake in KB-LFAR cells than in KB-HFAR cells, even at an Fe concentration of up to 90 µg mL⁻¹ (Fig. 3d). However, in the studied concentration range, Fe3O4 NPs with FA conjugation exhibit more uptake in KB-LFAR cells than those without FA modification, which is quite different than that of single FA-modified G5 dendrimers.[30] It implies that the FA-modified Fe3O4 NPs display higher binding sensitivity than that of FA-modified G5 dendrimers. The higher binding capacity of FA-modified Fe3O4 NPs may stem from the polyvalency effect due to multiple FA ligands presented onto each Fe3O4 NP surface.[39,40] The number of FA ligands (n) per Fe3O4 NP can be calculated according to the following equation:

\[ n = n_1 \times \frac{4\pi r_1^2}{\pi r_2^2} \]  

where \( r_1 \) and \( r_2 \) are the radius of PSS-modified Fe3O4 NPs and G5.NHAc-FI-FA dendrimers, respectively, and \( n_1 \) is the number of FA moieties per G5 dendrimer. Note that the calculation is based on the following assumptions: (1) a densely packed monolayer of G5.NHAc-FI-FA dendrimer is presented onto the Fe3O4 NP surfaces; (2) each dendrimer molecule shows a pancake shape when deposited onto the Fe3O4 NP surfaces[41,42] and the diameter of the pancake shape does not change significantly, compared with that of dendrimers in solution; (3) the PSS polymer layer thickness is 2 nm[43] and (4) there are half the number of FA (2.4) moieties presented in each dendrimer molecule available for binding (based on the geometry of dendrimer shape and stochastic distribution of FA moieties onto each G5.NHAc-FI-FA dendrimer). The number...
of FA moieties per Fe₃O₄ NPs was calculated to be ∼35.6, using the average diameter of PSS-coated Fe₃O₄ NPs (10.4 nm) and G5 dendrimers (5.4 nm). The larger number of FA moieties per Fe₃O₄ NP compared with single FA-modified dendrimer (4.8 FA per dendrimer) facilitates the polyvalency effect, thereby significantly increasing the binding affinity of Fe₃O₄ NPs on each KB cell. It should be noted that in the in vitro binding experiment, the use of KB-LFAR cells conveys similar information to the free-FA blocking experiment performed with dendrimer nanodevices in our group.

2.4. Confocal Microscopy Observations

The self-assembly of G5.NHAc-FI-FA dendrimers onto Fe₃O₄ NPs also affords the utilization of confocal microscopic imaging of the intracellular uptake of Fe₃O₄ NPs. It is clear that after treatment with Fe₃O₄/PSS/G5.NHAc-FI-FA NPs for 2 h, the green FI fluorescence signals appear in the cytosol of KB-HFAR cells (Fig. 4c). In contrast, KB-HFAR cells treated with Fe₃O₄/PSS/G5.NHAc-FI without FA conjugation do not show a FI fluorescence signal, similar to the KB-HFAR cells treated with PBS buffer (Fig. 4a and b). The confocal imaging data suggest that the intracellular uptake of Fe₃O₄/PSS/G5.NHAc-FI-FA NPs into KB-HFAR cells is through the FAR-mediated endocytosis.

2.5. Transmission Electron Microscopy (TEM) Imaging

The specific intracellular uptake of FA-modified Fe₃O₄ NPs was further verified by TEM. The TEM imaging technique allows for clear identification of the Fe₃O₄ NPs in different cellular entities. TEM images of KB-HFAR cells treated with Fe₃O₄/PSS/G5.NHAc-FI-FA NPs for 2 h show that the NPs distributed predominantly into the vacuoles of the cells (Fig. 5a and b), whereas the lyosomes and the nucleus do not show any uptake of Fe₃O₄/PSS/G5.NHAc-FI-FA NPs. In contrast, we did not observe any significant uptake of Fe₃O₄/PSS/G5.NHAc-FI NPs without FA modification (Fig. 5c). There was only minimal uptake of Fe₃O₄/PSS/G5.NHAc-FI NPs randomly distributed in the vacuoles of some cells (Fig. 5d, Supporting Information), which was undetectable using confocal microscopy. This minimal uptake might be related to diffusion-
driven non-specific binding since control cells without treatment of Fe3O4 NPs did not show any internalized NPs. The TEM studies underline the high specificity of FA-modified Fe3O4 NPs for targeting KB-HFAR cells, in agreement with the confocal imaging data.

2.6. Magnetic Resonance (MR) Imaging

MR imaging is often used for the diagnosis and staging of cancer. Iron oxide NPs affect the MR signal by dephasing transverse magnetization and hence reducing the value of T2. A targeted iron oxide NP would have a major benefit in cancer imaging by specifically detecting tumors that over-express the FAR. To study the effect of Fe3O4/PSS/G5.NHAc-FI-FA NPs on cancer cells, we measured the T2 of KB-HFAR cells exposed to differing concentrations of Fe3O4/PSS/G5.NHAc-FI-FA NPs. The T2 values of KB-HFAR cell pellets treated with Fe3O4/PSS/G5.NHAc-FI-FA NPs dramatically decreased as a function of Fe concentration (Table 2). In contrast, the decreasing trend of T2 values as a function of Fe concentration for the same KB cells treated with Fe3O4/PSS/G5.NHAc-FI NPs is significantly less than Fe3O4/PSS/G5.NHAc-FI-FA NPs. In the T2-weighted spin-echo MR images (the color change from red to purple indicates the gradual decrease of MR signal intensity, which is similar to those reported based on the intensity of black color\([11,12]\)) obtained using an Fe concentration of 11.3 \( \mu \)g/mL, Fe3O4/PSS/G5.NHAc-FI-FA NPs reduces the signal intensity to 50% of the initial value (PBS control) whereas Fe3O4/PSS/G5.NHAc-FI-FA NPs dramatically decreased as a function of Fe concentration (Table 2). In contrast, the decreasing trend of T2 values as a function of Fe concentration for the same KB cells treated with Fe3O4/PSS/G5.NHAc-FI-FA NPs.

Table 2. MR signals of KB-HFAR cells treated with functionalized Fe3O4 NPs.

<table>
<thead>
<tr>
<th>Fe Concentration [( \mu g/mL )]</th>
<th>T1 [s]</th>
<th>T2 [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe3O4-FI[a]</td>
<td>2.10 ± 0.07</td>
<td>2.01 ± 0.07</td>
</tr>
<tr>
<td>Fe3O4/FI-FA[b]</td>
<td>2.24 ± 0.07</td>
<td>2.26 ± 0.03</td>
</tr>
<tr>
<td>Fe3O4-FI[a]</td>
<td>2.24 ± 0.07</td>
<td>2.26 ± 0.03</td>
</tr>
<tr>
<td>Fe3O4/FI-FA[b]</td>
<td>2.24 ± 0.24</td>
<td>2.26 ± 0.03</td>
</tr>
</tbody>
</table>

[a] Denotes Fe3O4/PSS/G5.NHAc-FI NPs.
[b] Denotes Fe3O4/PSS/G5.NHAc-FI-FA NPs.
[c] PBS buffer.

The dose-dependent quantitative MR signal intensity shown in Figure 6b shows a significant MR signal intensity decrease with the increase of Fe concentration for KB-HFAR cells incubated with Fe3O4/PSS/G5.NHAc-FI-FA NPs. The non-specific uptake of Fe3O4/PSS/G5.NHAc-FI-FA NPs is significantly less than Fe3O4/PSS/G5.NHAc-FI NPs. In the T2-weighted spin-echo MR images (the color change from red to purple indicates the gradual decrease of MR signal intensity, which is similar to those reported based on the intensity of black color\([11,12]\)) obtained using an Fe concentration of 11.3 \( \mu \)g/mL, Fe3O4/PSS/G5.NHAc-FI-FA NPs reduces the signal intensity to 50% of the initial value (PBS control) whereas Fe3O4/PSS/G5.NHAc-FI-FA NPs reduces the signal to about 89% of the initial value (Fig. 6a). This suggested that Fe3O4/PSS/G5.NHAc-FI-FA NPs can specifically hamper the MR signal through FAR-mediated binding and endocytosis. At higher Fe concentrations (e.g., 45 \( \mu \)g/mL), non-specific binding of Fe3O4/PSS/G5.NHAc-FI NPs without FA conjugation occurred with KB-HFAR cells. The dose-dependent quantitative MR signal intensity shown in Figure 6b shows a significant MR signal intensity decrease with the increase of Fe concentration for KB-HFAR cells incubated with Fe3O4/PSS/G5.NHAc-FI-FA NPs. The non-specific uptake of Fe3O4/PSS/G5.NHAc-FI-FA NPs at higher Fe concentrations can also be inferred from...
3. Conclusions

In summary, flow cytometry, confocal microscopy, TEM, and MR imaging studies all demonstrated that Fe₃O₄/PSS/G₅.NHAc-FI-FA NPs specifically bind to KB-HFAR cells. In contrast, Fe₃O₄/PSS/G₅.NHAc-FI NPs that lack surface FA moieties did not display binding to the KB cells, suggesting that the binding was mediated by the FAR. The approach to functionalizing Fe₃O₄ NPs using LbL self-assembly and dendrimer chemistry may be applicable for various NPs and targeting ligands (e.g., sugars, peptides, hormones and the like), thereby providing a general strategy to fabricating various NPs for a range of biological sensing and therapeutics applications. For in vivo applications, the stability of the fabricated Fe₃O₄ NPs may need to be further improved by increasing the polymer layer thickness, which can be achieved by increasing the number of polymer layers via the LbL self-assembly technique. In addition, the mechanical stability of the polymer coating may also need to be improved through shell cross-linking. These experiments and improvements are currently being developed in our lab, and should provide a truly unique approach to functionalized NPs.

4. Experimental

Materials: Ethylenediamine core amine-terminated PAMAM dendrimers of generation 5 (G₅.NH₂) with a polydispersity index less than 1.08 were purchased from Dendritech (Midland, MI). FA, FI, Ferric chloride hexahydrate (FeCl₃ · 6H₂O > 99 %), ferrous chloride tetrahydrate (FeCl₂ · 4H₂O > 99 %) and hydrochloric acid. All other chemicals and solvents were purchased from Aldrich (St. Louis, MO) and used as received. KB cells were from American Type Tissue Collection (ATCC, Rockville, Maryland). Penicillin, streptomycin, fetal bovine serum (FBS) were purchased from Sigma (St. Louis, MO). Trypsin-EDTA, Dulbecco’s PBS, and RPMI 1640 medium (with or without FA) was obtained from Gibco-BRL (Gaithersburg, MD). Water used in all experiments was purified using Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with resistivity higher than 18 MΩ cm. Cellulose dialysis membranes (MWCO = 10000) were purchased from Fisher.

Synthesis of Iron Oxide NPs: The Fe₃O₄ NPs were synthesized by controlled co-precipitation of Fe²⁺ and Fe³⁺ ions according to a method described elsewhere [33]. Briefly, 25 mL of 1 M FeCl₂ · 4H₂O, 0.5 M FeCl₃ · 6H₂O and 0.4 M HCl mixture solution was prepared in water under vigorous stirring. The co-precipitation of Fe₃O₄ NPs was carried out in a three-neck round-bottom flask. The above mixture solution was added to 250 mL of 0.5 M NaOH, which was preheated to 80 °C before the co-precipitation reaction. The reaction was protected under N₂ atmosphere and was vigorously stirred. Black powder was collected by sedimentation with the help of an external magnetic field and washed several times with water until stable ferrofluid was obtained. Finally, the particles were dispersed in water.

Synthesis of FI- and FA-Functionalized Dendrimers: Amine-terminated G₅ dendrimer (G₅.NH₂) was conjugated with FA or both FA and FI moieties, according to previously published reports [29, 46, 47]. Briefly, G₅.NH₂-FI (30 mg, 0.0010501 mmol) in DMSO (12 mL) was dissolved in an ambient DMSO (24 mL). To the above solution was added dropwise a solution of FI (4.4 mg, 0.00063275 mmol) in DMSO (24 mL) under vigorous stirring at room temperature. The reaction was stopped after 24 h. The mixture was dialyzed against PBS buffer (3 times 4 l) and water (3 times 4 l) for 3 days through a 10 000 MWCO membrane. Lyophilization gave G₅.NH₂-FI as an orange solid (60.6 mg, yield 94.0 %).

For the synthesis of G₅.NH₂-FI-FA, FA (3.7 mg, 0.0084004 mmol) and EDC (9.3 mg, 0.020101 mmol) were dissolved in DMSO (3 mL) and the mixture was stirred at room temperature for 3 h to activate the γ-carboxylic acid of FA. The resulting solution was added dropwise to a solution of G₅.NH₂-FI (30 mg, 0.0010501 mmol) in DMSO (12 mL) under vigorous stirring at room temperature. After 3 days, the reaction mixture was dialyzed through a 10 000 MWCO membrane against PBS buffer (3 times 4 l) and water (3 times 4 l) for 3 days, followed by lyophilization to give G₅.NH₂-FI-FA (31.2 mg, yield 96.4 %). The G₅.NH₂-FI and G₅.NH₂-FI-FA conjugates were characterized by ¹H NMR, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, and UV-vis spectrometry (Supporting Information, Figs. S1–3) [29, 46]. The numbers of FA and FI moieties conjugated onto each G₅ dendrimer were estimated by comparing the differences between the integration values of ¹H NMR signals associated with dendrimers and the FA and FI moieties. The average numbers of FI and FA moieties conjugated onto each G₅ dendrimer were estimated to be 4.5 and 4.8, respectively. The molecular weights of G₅.NH₂-FI and G₅.NH₂-FI-FA conjugates were determined to be 29 564 and 33 484, respectively.

Fabrication of Multi-functional Dendrimer-Functionalized Iron Oxide NPs: The LbL assembly of oppositely charged PSS and dendrimers was performed according to the literature [20, 23, 29, 48, 49]. Briefly, a solution of Fe₃O₄ NPs (5 mg in 0.5 mL water) was added with 1 mL of a PSS solution (2 mg mL⁻¹, containing 0.5 M NaCl) with occasional shaking. After adsorption of PSS for 20 min, the suspension was centrifuged at 8000 rpm for 10 min. The supernatant was then carefully removed, and the coated Fe₃O₄ NPs were washed by three alternate cycles of centrifuging and resuspending the particles in pure water. Then 1 mL of
the polymerization of Fe₃O₄ NPs were characterized by a Philips CM-100 TEM (Tilburg, UK) equipped with a standard 633 nm laser. The size and morphology were measured using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a Hamamatsu Digital Camera ORCA-HR operated at a voltage of 60 kV. Images were recorded using a Micromex ToSpec-2E spectrometer (Beverly, MA) according to a procedure described in our previous report [51]. The iron concentration of Fe₃O₄ NPs before and after surface modification was determined by atomic absorption spectrometry (AA903, ARL). A defined volume of the NPs were digested in 1.0M nitric acid before measurements. The surface potential of functionalized Fe₃O₄ NPs was measured by a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633 nm laser. The size and morphology of Fe₃O₄ NPs were characterized by a Philips CM-100 TEM equipped with a Hamamatsu digital camera controlled by AMT (advance microscopy technology) software. The KB-HFAR cells were incubated with Fe₃O₄/PSS/G5.NHAc-FI or Fe₃O₄/PSS/G5.NHAc-FI-FA NPs for 2 h at 37 °C. The medium was then removed and the cells were washed with Sorenson buffer and fixed at room temperature for 1 h using 2.5 % of glutaraldehyde in Sorenson buffer. The cells were rinsed 3 times with Sorenson buffer and fixed at room temperature for 1 h using 2.5 % of glutaraldehyde in Sorenson buffer. The cells were then washed with PBS buffer and incubated with Fe₃O₄/PSS/G5.NHAc-FI or Fe₃O₄/PSS/G5.NHAc-FI-FA NPs for 24 h at 37 °C. The nuclei were counterstained with 1 µg mL⁻¹ of Hoechst33342, using a standard procedure. Samples were scanned on an Olympus IX-71 inverted microscope, using a 60× water immersion objective and magnified with Fluovue software. Transmission Electron Microscopy (TEM): The uptake of functionalized Fe₃O₄ NPs was further examined by a Phillips CM-100 TEM microscope operating at a voltage of 60 kV. Images were recorded using a Hamamatsu digital camera controlled by AMT (advance microscopy technology) software. The KB-HFAR cells were incubated with Fe₃O₄/PSS/G5.NHAc-FI or Fe₃O₄/PSS/G5.NHAc-FI-FA NPs for 2 h at 37 °C. The medium was then removed and the cells were washed with Sorenson buffer and fixed at room temperature for 1 h using 2.5 % of glutaraldehyde in Sorenson buffer. The cells were rinsed 3 times with Sorenson buffer and fixed at room temperature for 1 h using 2.5 % of glutaraldehyde in Sorenson buffer. The cells were then washed with PBS buffer and incubated with Fe₃O₄/PSS/G5.NHAc-FI or Fe₃O₄/PSS/G5.NHAc-FI-FA NPs for 24 h at 37 °C. The nuclei were counterstained with 1 µg mL⁻¹ of Hoechst33342, using a standard procedure. Samples were scanned on an Olympus IX-71 inverted microscope, using a 60× water immersion objective and magnified with Fluovue software.