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Linker-free conjugation and specific cell targeting of antibody functionalized iron-oxide nanoparticles

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Abstract

Specific targeting is a key step to realize the full potential of iron oxide nanoparticles in biomedical applications, especially tumor-associated diagnosis and therapy. Here, we developed anti-GD2 antibody conjugated iron oxide nanoparticles for highly efficient neuroblastoma cell targeting. The antibody conjugation was achieved through an easy, linker-free method based on catechol reactions. The targeting efficiency and specificity of the antibody-conjugated nanoparticles to GD2-positive neuroblastoma cells were confirmed by flow cytometry, fluorescence microscopy, Prussian blue staining and transmission electron microscopy. These detailed studies indicated that the receptor-recognition capability of the antibody was fully retained after conjugation and the conjugated nanoparticles quickly attached to GD2-positive cells within four hours. Interestingly, longer treatment (12 h) led the cell membrane-bound nanoparticles to be internalized into cytosol, either by directly penetrating the cell membrane or escaping from the endosomes. Last but importantly, the uniquely designed functional surfaces of the nanoparticles allow easy conjugation of other bioactive molecules.

Introduction

Iron oxide nanoparticles are being actively explored for drug delivery,^{1, 2} cancer therapy *via* magnetic hyperthermia,³ and diagnostic imaging.⁴ In particular, these nanoparticles have been clinically used as contrast agents for magnetic resonance imaging (MRI),⁵ or iron deficiency therapy,⁶ suggesting their great potential in nanomedicine. A significant

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challenge of using nanoparticles for *in vivo* diagnostic or therapeutic applications is the delivery efficiency to targeted locations. In fact, several Food and Drug Administration (FDA) approved MRI contrast agents were taken off the market due to lack of clinical use, owing to the fact that these nanoparticles could only passively accumulate in the liver or spleen because of the lack of targeting moieties and surface modifications protecting them from non-specific uptake.^{7, 8} To fully realize the potential of iron oxide nanoparticles in nanomedicine, a key step is to effectively attach targeting, therapeutic, or other functional molecules onto the nanoparticle surface to increase the targeting efficiency, broaden the applicability and minimize the administration dose. Therefore, it is essential to develop a highly efficient, facile, and versatile approach to attaching desired molecules onto iron oxide nanoparticle surfaces.

Among the various targeting molecules, antibody and antibody fragments are some of the most promising moieties for targeted cancer therapy, because of the high affinity and molecular specificity for an antigenic target. The GD2 disialoganglioside is an antigen expressed on neuroblastoma cancer cells, most melanomas and a large fraction of small cell lung cancers and other tumors of neuroectodermal origin.^{9, 10} These tumors, specifically in the advanced stages of disease, are difficult to treat with conventional therapies, and many patients die despite highly toxic treatment regimens.¹¹⁻¹³ Therefore, novel and targeted treatment approaches are urgently needed. Since GD2 expression on healthy tissue is restricted to the cerebellum and certain peripheral nerve tissue at very low levels,¹⁴ it has been considered a very attractive antibody target especially for neuroblastoma. Hu14.18MoAb (hu14.18-K322A) is a humanized anti-GD2 antibody currently being investigated in a phase-I immunotherapy study in neuroblastoma patients at St. Jude Children's Research Hospital, Memphis, TN.^{15, 16} Given the clinical relevance, this antibody was utilized as a model system to test the conjugation method.

Several strategies have been applied to conjugate antibodies or other molecules onto iron oxide nanoparticle surfaces.¹⁷⁻²² The most common approach is the linker chemistry, where chemical linkers cross-link nanoparticles and conjugating molecules.^{23, 24} Even though a number of chemical linkers are available, the entire chemical linker approach suffers from a number of disadvantages. First, special reaction conditions must be met depending on the chemical linker, such as acidic condition (pH 4.5-5.5) for carbodiimide (EDC) chemical linker, pH 7.2-8.0 at 4 °C for N-hydroxysuccinimide (NHS) ester cross-linker, and reducing condition for maleimide chemistry. Second, low conjugation efficiency is always a concern because of competing reactions. For example, the EDC/NHS linker directly links carboxylic and amino groups, for conjugating molecules with multiple carboxylic and amino groups (e.g., proteins). EDC/NHS chemistry causes cross conjugation, thus greatly decreasing the conjugation efficiency.^{25, 26} Finally, multiple cleaning steps are necessary to remove the excess chemical linkers and other assisting reagents.

Besides the chemical linker chemistry, specific molecular recognition based on biotin-streptavidin is another common strategy.²⁷ The biotin-avidin interaction requires prior attachment of biotin molecules onto nanoparticles. The biotin-labeled nanoparticles react with any biotin-binding protein, reducing the specificity. In addition, biotin is a natural biological molecule, causing concerns about the specificity and background when

performing assays involving biotin-rich tissues and extracts (e.g., brain, liver, milk, or eggs).²⁸

Many of these conjugation approaches were using iron oxide nanoparticles produced in aqueous environment (e.g. co-precipitation method), where the quality of the nanoparticles (e.g. size, size distribution and magnetic properties) was suboptimal. High quality iron oxide NPs with respect to monodispersity, size distribution, magnetic properties and crystallinity are usually produced in organic solvents at high temperatures.²⁹⁻³³ Developing conjugation strategies for high quality nanoparticles remains a challenge and essential need with regards to their applicability in the biomedical field.

In this paper, we developed anti-GD2 antibody conjugated iron oxide nanoparticles via a facile conjugation approach based on catechol reactions. This conjugation approach was evaluated using a clinically relevant antibody as a model system. Specifically, iron oxide nanoparticles were functionalized with dopamine molecules through amino group attachment, leaving the catechol groups on the nanoparticle surfaces for further conjugation. Upon activation at basic conditions, the catechol groups can be oxidized into quinone and subsequently react with amino or thiol groups of the antibodies. After conjugation, the targeting efficiency of the antibodies on nanoparticles was evaluated on GD2-positive neuroblastoma cells. The uniquely designed functional surfaces of the nanoparticles will allow effective conjugation of biological molecules without the need for any type of chemical linkers. Eliminating the use of chemical linkers significantly simplifies the conjugation process, potentially avoids interactions with the biological activity of the targeting moiety, increases the efficiency of the conjugation and last but not least reduces the requirements of well-trained personnel. The method described herein for antibody conjugation to iron oxide nanoparticles can be readily extended to the conjugation of other biological molecules with iron oxide nanoparticles.

Experimental

Materials

All the chemical reagents were commercially purchased and used without further purification. These reagents include (FeCl₃, ACROS, 98 %), sodium oleate (TCL, 95 %), oleic acid (OA, Fisher, 95 %), trioctylphosphine oxide (TOPO, 90%), 1-octadecene (Sigma-Aldrich, 90 %), chloroform (Sigma-Aldrich, 99%), dimethyl sulfoxide (DMSO, VWR, 99 %), Bis-Tris (C₈H₁₉O₅N, Fisher, enzyme grade), sodium chloride (NaCl, ACROS, 99+%), and Hu 14.18 MoAb (in PBS, 100 mM Arginine, 0.03 % Tween-80). Trypsin-versene mixture (0.05%, Lonza), fetal bovine serum (FBS, Thermo Scientific), Iscove's Modified Dulbecco's Media (IMDM) and Eagle's minimal essential medium (EMEM) were purchased from ATCC. Poly-L-lysine (Mw: 150,000-300,000 g/mol, Sigma-Aldrich, 0.1% w/v in water), paraformaldehyde (Alfa Aesar, 97%), and Prussian blue iron stain kit (Polysciences, Inc.) (Solution A: Potassium ferrocyanide aqueous (C₆N₆FeK₄, 4%), Solution B: Hydrochloric acid (HCl, 4%), Solution C: Nuclear fast red aqueous (C₁₄H₈NNaO₇S, 1%) were also used.

Nanoparticle synthesis

Iron oxide nanoparticles were produced by heating up the iron oleate precursor (2.5 g, 2.8 mmol) in 1-octadecene (10 mL, 90%) in the presence of TOPO (90%)/OA (97%) (TOPO-0.2 g, 0.5 mmol, OA-0.22 mL, 0.7 mmol) following our previously published procedures.³⁴⁻³⁶ After two and half hour reaction at 320 °C, the nanoparticles were washed and collected for surface modification. The morphology and size of the iron oxide nanoparticles were examined under transmission electron microscopy (TEM, FEI Tecnai, F-20, and 200 kV).

Surface modification

The iron oxide nanoparticles were functionalized with dopamine through a similar procedure used to attach charged polymers onto iron oxide nanoparticle surface.³⁶ In brief, 1 mL of iron oxide nanoparticles in chloroform (5 mg/mL) was mixed with dopamine-HCl (1.7 mg) in 49 mL of DMSO. After 48 h mixing at room temperature, the iron oxide nanoparticles were collected by centrifugation and re-dispersed in water (1 mg/mL) for further conjugation. The surface functionalization and conjugation were verified by Fourier transform infrared spectroscopy (FTIR). The hydrodynamic sizes and the surface charges of the nanoparticles were measured using a Malvern Zetasizer Nano series dynamic light scattering (DLS).

Antibody conjugation

The dopamine functionalization of iron oxide nanoparticles allows for easy attachment of proteins through thiol or amino groups upon activation.³⁷ The catechol groups on the nanoparticle surfaces can be easily oxidized into dopamine quinone at higher pH (>8.5), creating an active surface for the conjugation. The clinical-grade, humanized, monoclonal anti-GD2 Hu14.18K322A antibody (referred to as hu14.18MoAb in this manuscript) was produced at Children's GMP LLC, Memphis, TN and kindly provided by Dr. R. Barfield. Briefly, 1 mL of activated, dopamine-coated iron oxide nanoparticles (1 mg/mL) was simply mixed with 100 μ L of hu14.18MoAb (8.6 mg/mL) in Bis-Tris (10 mM)-NaCl (10 mM) buffer for four hours. After centrifugation (10 min, 15000 rpm) and cleaning twice with Bis-Tris buffer solution to remove free antibodies, the cleaned, conjugated nanoparticles were re-dispersed in Bis-Tris (20 mM)-NaCl (20 mM) solution at a concentration of 1 mg/mL for cellular study. In addition to FTIR and DLS measurements, the presence of the antibody on nanoparticle surfaces was visualized by TEM negative staining technique. The TEM grid with antibody conjugated nanoparticles were immersed into uranyl acetate solution (2 %) with the sample side facing down for 10 min, and then rinsed in distilled water and Reynolds' lead citrate for seven minutes. After washing off the excess staining solution with 0.1 M NaOH and DI water, the stained TEM grids were dried on a filter paper and examined under TEM (Hitachi 7860).

Cell culture

The GD2-positive neuroblastoma cell line (CHLA-20), was kindly provided by Dr. Wayne A. Warner (Children's Hospital Los Angeles). The GD2 negative PC-3 prostate cancer cell line was obtained from ATCC. CHLA-20 cells were cultured in IMDM supplemented with

15% (v/v) FBS while PC-3 cells were grown in EMEM supplemented with 10% (v/v) FBS. Primary cultures of skin fibroblasts, cultured in DMEM supplemented with 10% (v/v) FBS, were used as normal cell controls. All the cells were incubated at 37 °C under a humidified atmosphere with 5% CO₂. The cell morphology and growth was daily monitored. Cell passage was performed every four days by detaching cells with trypsin-versene and re-growing in 75 cm² flasks.

Cellular targeting with antibody conjugated nanoparticles

To study the antibody activity after conjugation, CHLA-20 cells were treated with antibody-conjugated and unconjugated nanoparticles under the same conditions. Briefly, 2 mL of cells (5×10^4 cells/mL) were seeded in 6-well plates and cultured overnight. Subsequently, 400 μ L of nanoparticles (1 mg/mL) in 1.6 mL medium were added into each well and incubated for four or 12 hour. Finally, the nanoparticle treated cells were detached with trypsin-versene and washed three times with 1X PBS for further analysis.

The cell binding was evaluated by flow cytometry and fluorescence microscopy. Specifically, cells treated with nanoparticles for one hour were further incubated with a fluorescent goat-anti-human IgG Alexa Fluor 488 antibody. After incubation and washing, the cells were analyzed on a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec), or on a Nikon Eclipse Ti-U fluorescence microscope with Intensilight C-HGFI, equipped with a DS-QiMc digital camera and Nis-Elements D3.10 software. In flow cytometry, propidium iodide was added to samples to allow the evaluation and exclusion of dead cells from the analysis; for fluorescence microscopy, DAPI was used as nuclear counter stain.

For iron staining, nanoparticle treated cells were attached onto the X-TRA permanent positively charged glass slides (Leica Biosystems, Inc) (1×10^5 cells/slide) by cytopsin. The attached cells were fixed with cold acetone for 10 min followed by the two-step Prussian blue staining at room temperature. Briefly, pre-mixed solutions of hydrochloric acid-potassium ferrocyanide were added to the cell-mounted slide for 30 min. This step was repeated once for fully staining the antibody-conjugated nanoparticles taken up by cells. After rinsing off the excess stain solution with water, nuclear fast red solution was added to stain the nuclei and cytoplasm of cells for two minutes. Finally, the stained cells were dehydrated in a graded series of ethanol and xylene and fastened with the cover slip for staining pattern observation under inverted phase microscope.

The iron uptake by cells was quantified by ferrozine colorimetry.³⁸ Briefly, 5×10^5 cells were treated with 60 μ g/ml nanoparticles for one hour at room temperature. After several washes, the cells were then lysed with 0.5 M NaOH. The cell lysate was acidified with HCl and oxidized with 4% (w/v) KMnO₄ for two hours at 60°C. After reduction with 1M L-ascorbic acid, the ferrous ions were quantified by measuring the absorption at 570nm of their reaction product with 6.5 mM ferrozine, in the presence of 2.5 mM ammonium acetate and 6.5 mM neocuproine.

Cell targeting and competition experiments

To study the specific targeting ability of antibody-conjugated nanoparticles, CHLA-20 and PC-3 cells were treated with antibody-conjugated and unconjugated nanoparticles under the

same conditions. To further confirm that indeed the interaction of GD2 receptor and hu14.18MoAb was responsible for the cellular uptake, a competition inhibition experiment was conducted. Specifically, 2 mL of CHLA-20 cells were seeded into 6-well plates (5×10^4 cells/mL) and cultured to 80% confluency before use. Free Hu 14.18 MoAb (100 nM) was then added to each well. After four hours incubation, 400 μ L of antibody-conjugated nanoparticles (1 mg/mL) in 1.6 mL medium were added to both blocked and unblocked CHLA-20 cell cultures and incubated for four hours. For both experiments, ultrathin sections of nanoparticle-treated cells were prepared and scanned under TEM (Hitachi 7860, 120 kV). Specially, for the preparation of thin cell sections for TEM analysis, the NP-treated cells were firstly collected, and washed three times with SPB (sodium-phosphate buffer). SPB solution was simply prepared by mixing equal volume of 0.2 M Na₂HPO₄·7H₂O and 0.2 M NaH₂PO₄·H₂O. Subsequently, the collected cells were fixed with SPB-diluted glutaraldehyde solution (2.5 %) at 4 °C for 30 min. After three washes with SPB, the cells were post-fixed with SPB-diluted osmium tetroxide (2 %) for 20 min at room temperature. After removing the free fixation agents with SPB, the cell samples were dehydrated in a graded series of ethanol (25, 50, 75, 95, and 100 %) and then infiltrated within 100 % resin solution. Finally, the solidified cell-resin blocks were trimmed and sectioned. The optimal cell section thickness was selected as 90 nm for this work.

Results and discussion

Synthesis and functionalization of dopamine-coated iron oxide nanoparticles

The anti-GD2 antibody conjugated iron oxide nanoparticle first involved nanoparticle synthesis and dopamine surface functionalization steps. Iron oxide nanoparticles were synthesized using our modified heat up method,^{34-37, 39, 40} which produces nanoparticles with controlled size and narrow size distribution. After synthesis, the nanoparticles are only soluble in organic solvent, and must be transferred into aqueous solution for biological studies. We have recently developed a ligand exchange method to attach charged molecules onto iron oxide nanoparticle surfaces for water solubility.³⁶ The success of this approach is based on the design of introducing a ligand with low affinity to the iron oxide nanoparticle (e.g., TOPO) as a co-capping molecule during synthesis. The introduction of TOPO molecules is critical for the surface functionalization, where the weaker binding affinity to iron oxide surfaces of TOPO molecules⁴¹ and its bulky C₈ tails⁴² create preferred sites or “naked” spots on the nanoparticle surfaces for hydrophilic ligands to attach or bind, ensuring an effective ligand exchange process.

We chose dopamine as the surface functionalization molecule because of the easy oxidation property of the catechol group. The reaction was performed in DMSO, a non-aqueous solution, which limits the formation of poly-dopamine like in many other aqueous reactions.⁴³⁻⁴⁷ The use of dopamine molecules as functional surface coatings is key to the subsequent conjugation, where the amino group of the dopamine molecule attaches to the iron oxide nanoparticle surfaces, leaving the catechol group out. The catechol groups on the nanoparticle surfaces can be easily oxidized into dopamine quinone at higher pH (>8.5), creating an active surface for further conjugation. The activated dopamine groups allow for the direct conjugation of biological molecules containing amine and/or thiol groups through

Michael addition and/or Schiff base formation.^{48, 49} The amino group attachment is different from iron oxide nanoparticles synthesized *via* the co-precipitation method at high pH, where the nanoparticles have surface bounded with hydroxyl groups. These surface hydroxyl groups interact with the catechol groups of dopamine molecules and leave the amino groups out.^{50, 51} The availability of the catechol groups on the nanoparticle surfaces is critically important to the direct conjugation of biological molecules. Importantly, this conjugation can be generalized for attaching other biological molecules to the iron oxide nanoparticle surfaces.

Fig. 1a shows the TEM image of the well-dispersed dopamine-coated, 12 nm iron oxide nanoparticles in water. The high resolution TEM image indicated that the nanoparticles were highly crystalline (Fig. 1b). The sharper lattice fringes and the clean boundary also suggested the absence of thick poly-dopamine layer, because the formation of thick poly-dopamine layer has been observed on several nanoparticle systems.⁴⁴⁻⁴⁷ This observation also supports our hypothesis that the amino group of the dopamine molecule was attached onto iron oxide nanoparticle surface, which limited the formation of thick poly-dopamine layer. However, it is possible to form some patches of thin poly-dopamine due to the presence of excess free dopamine after water dispersion, as shown in selected HRTEM images (Fig. S1).

The amino group attachment was consistent with our previous studies showing that the amino group has a preferable binding to iron oxide nanoparticles over other functional groups.^{36, 37, 41} The hydrodynamic size of the dopamine-coated nanoparticles increased to 24 nm (Fig. 1c), compared to the 16 nm in organic solvent (Fig. S2). The presence of the catechol groups on the dopamine-coated nanoparticle surface was also supported by the negative zeta-potential (−44 mV, Fig. 1d). If catechol functional groups are attached to the nanoparticles, the amino groups on the nanoparticle surfaces would lead to a positive zeta-potential. The catechol groups on the nanoparticle surfaces can be easily oxidized into dopamine-quinone at higher pH (>8.5), creating an active surface for further effective conjugation of antibody.

The IR spectra of the dopamine-functionalized iron oxide nanoparticles before and after activation were shown in Fig. 2. Compared with the IR spectrum before activation, a peak at 1647 cm^{−1} appeared, which is the characteristic of -C=O band in quinone structure.⁵² Accordingly, the characteristic band of -C-O at 1282 cm^{−1} disappeared after activation. In addition, the typical phenol alcohol band at 1065 cm^{−1} disappeared, and a strong -CH=CH- ring breathing mode at 956 cm^{−1} showed up. All these IR absorption changes indicated the dopamine oxidation process on the nanoparticle surfaces. The activated dopamine groups allowed for the direct conjugation of biological molecules through Michael addition and/or Schiff base formation.^{48, 49} Because of the stability issue with the Schiff base,⁵³ the Michael addition reaction commonly happens.⁵⁴

Synthesis and functionalization of dopamine-coated iron oxide nanoparticles

Fig. 3a shows the negative stained TEM image of the hu14.18MoAb conjugated iron oxide nanoparticles, where the lighter shells around the nanoparticles were from the antibodies. Depending on the orientation of the antibody, the shell region can be larger or small. The

tiny black spots around the nanoparticles were from the staining solution, where possible undissolved uranyl acetate stain or lead carbonate precipitation from lead citrate stain absorbed CO₂ from air.

The antibody conjugation shifted the zeta-potential of the nanoparticles from -44 eV to -34 eV (Fig. 3b). Even though the zeta-potential increased about 10 mV, the suspension of the antibody conjugated nanoparticle solution was still stable. After antibody conjugation, the hydrodynamic sizes of the nanoparticles increased about 20 nm, another indication of antibody attachment (Fig. 3c). The FTIR spectrum of the antibody conjugated nanoparticles (Fig. 3d) showed clear amide I (1633 cm^{-1}) and amide II (1520 cm^{-1}) bands, also suggesting the attachment of antibodies. After conjugation, the amine or thiol groups normally attached to the fourth position adjacent to a hydroxyl group through Michael addition and the quinone shifted back to hydroxyl groups. This process was supported by the IR spectrum of antibody-conjugated nanoparticles, where hydroxyl and its C-O bands at 1065 and 1005 cm^{-1} were clearly seen, compared with the strong $-\text{CH}=\text{CH}-$ ring breathing peak at 956 cm^{-1} (Fig. 2). In fact, the IR bands in the range of $900\text{--}1100\text{ cm}^{-1}$ of the antibody conjugated nanoparticles was very similar to the dopamine coated nanoparticles before oxidation.

Cell targeting evaluation of antibody-conjugated iron oxide nanoparticles

To evaluate the targeting capability of the hu14.18MoAb after attaching on the iron oxide nanoparticles, cell targeting experiments were performed on GD2-positive cell lines (CHLA-20) and GD2 negative control cell lines (PC-3) or normal fibroblasts. CHLA-20 neuroblastoma cells have a high level of expression of GD2 antigen on the cell surface while PC-3 cells and normal fibroblasts do not express the GD2 receptor, serving as suitable negative controls [55]. The localization of the nanoparticles on CHLA-20 cell surface was visualized by fluorescence microscopy using green-fluorescent Alexa 488-labeled anti-human IgG antibody. The lack of green fluorescence after the treatment of cells with unconjugated nanoparticles and anti-human IgG antibody (Fig. 4a) indicated the absence of a nonspecific reaction of the detection system used. The sharp green shell around the cell surface (Fig. 4b) suggested a remarkably high affinity and magnitude of binding of the antibody-conjugated nanoparticles to GD2-positive cells.

In contrast, the antibody-conjugated nanoparticles did not bind to GD2-negative cells (such as normal fibroblasts, Fig. 4c, 4d), indicating their capability to GD2 receptor recognition with high specificity. The binding of antibody-conjugated nanoparticles to GD2-positive cells (CHLA-20) was quantified by flow cytometry, yielding an up to 250-fold increase of the mean fluorescence intensity above the autofluorescence of the cells (Fig. 4e), whereas the binding to GD2-negative cells (such as normal fibroblasts and PC-3) did not increase the cell fluorescence more than 1.2 fold (data not shown), strongly supporting the binding specificity of antibody-conjugated nanoparticles. The antibody binding increased with increasing nanoparticle concentrations and reached saturation at a Fe concentration of $\sim 60\text{ }\mu\text{g/mL}$ (corresponding with $2\text{ }\mu\text{g}$ antibody/million cells) (Fig. 4f). A comparison of the binding of the antibody-conjugated nanoparticles with the original hu14.18MoAb by flow cytometry demonstrated only very minor differences in binding, especially after reaching

saturation (Fig. 4f), indicating that the conjugation process did not substantially alter the antibody binding properties.

To confirm the co-localization of the nanoparticles with antibody, Prussian blue iron staining was performed on CHLA-20 cells treated with conjugated and unconjugated nanoparticles. CHLA-20 cells treated with unconjugated, dopamine-coated nanoparticles only showed occasional big blue spots from nanoparticle aggregates (Fig. 5a). In contrast, the cells treated with antibody conjugated nanoparticles showed clear blue shells around the cells, confirming the presence of the iron oxide nanoparticles around the cell membranes (Fig. 5b). In conjunction with the fluorescent microscopy image, this observation demonstrated the co-localization of nanoparticles and antibodies. The cellular surface iron content was assessed by ferrozine colorimetry and increased significantly in cells treated with antibody conjugated iron oxide nanoparticles (Fig. 5c). The flow cytometry evaluation of the biocompatibility of the iron oxide nanoparticles by propidium iodide exclusion, following cell treatment with medium alone (no NP), unconjugated (NP) or antibody-conjugated nanoparticles (antibody-NP) suggested that the conjugation did not alter the cell viability significantly when co-cultured with either normal fibroblasts or tumor cells. (Fig. 5d).

Cellular uptake of antibody-conjugated nanoparticles

The detailed cellular uptake of the antibody-conjugated nanoparticles was further studied using TEM. Fig. 6 shows the time-dependent cellular uptake and distribution of nanoparticles on GD2 positive CHLA-20 cells and GD2-negative PC-3 cells. The GD2 binding of the antibody-conjugated nanoparticles was compared with the unconjugated, dopamine-coated nanoparticles. After four hours incubation, the cellular uptake and localization of unconjugated nanoparticles on CHLA-20 and PC-3 cells showed little difference (Fig. 6a-6d). The unconjugated nanoparticles were primarily taken up through endocytosis and ended up inside endosomal- or lysosomal-like organelles (red circles). The corresponding detailed information was shown in Fig. 6b, 6d. In contrast, after four hours incubation with antibody-conjugated nanoparticles, many nanoparticles were attached onto CHLA-20 plasma membranes (Fig. 6e), and the nanoparticle morphology could be clearly observed at higher magnification (Fig. 6f). No cell membrane anchored nanoparticles were observed on PC-3 cells, which lack GD2 cell receptors (Fig. 6g). Instead, antibody-conjugated nanoparticles were localized inside endosomal- or lysosomal-like organelles, indicated by the visible membrane edge in Fig. 6h. This study demonstrated the specific GD2 recognition and binding of the antibody-conjugated nanoparticles. Besides the high selective targeting capacity, the internalized conjugated nanoparticles were well dispersed inside the cells without morphology change and free of aggregation.

At 12 hours, nanoparticles on CHLA-20 cell membranes were internalized, leading to much cleaner cell surfaces. Some internalized nanoparticles were found inside endosomal- or lysosomal-like organelles but some nanoparticles were localized in the cytosol because the nanoparticles might enter cells by penetrating the plasma membrane or endosomal or lysosomal-like organelles membranes were ruptured (blue-dashed circles in Fig. 6i and the enlarged image of Fig. 6j). At 12 hours, not much difference was observed on TEM images

of the PC-3 cells treated antibody-conjugated nanoparticles, compared with that from the four hour treatment (Fig. 6k). The internalized, conjugated nanoparticles were still located in endosomal- or lysosomal-like organelles with the clearly observed membrane (Fig. 6l). This result suggested that the hu14.18MoAb-GD2 interaction was responsible for the specific uptake of antibody-conjugated nanoparticles by GD2-positive cells.

To further confirm the role of the hu14.18MoAb in the recognition of nanoparticles by CHLA-20 cells, an antibody competition experiment was performed (Fig. 7). The CHLA-20 cells were first treated with free hu14.18MoAb (100 nM) for four hours, followed by the addition of antibody-conjugated nanoparticles for another four hours. The incubation of CHLA-20 cells with free hu14.18MoAb was designed to block all the GD2 receptors on the cell surfaces. Such a blockade would subsequently limit the GD2 recognition and binding of the antibody-conjugated nanoparticles. Then, the cellular uptake was compared with unblocked CHLA-20 cells treated with antibody-conjugated nanoparticles. Fig. 7a shows the TEM images of the unblocked CHLA-20 cells treated with antibody-conjugated nanoparticles. Without the blockage of GD2 receptors by free antibody, the nanoparticles were primarily localized on the surface of the CHLA-20 cells (Fig. 7b), similar to Fig. 6c. After blockage of the GD2 receptors by free antibodies, the nanoparticle surface recognition and binding were barely seen and the internalization was significantly reduced (Figure 7c, 7d). These results demonstrated that the enhanced cell binding in GD2-positive neuroblastoma cells was a direct result of GD2-hu14.18MoAb interactions.

Conclusions

In this paper we describe the development of a novel and facile conjugation platform for iron oxide nanoparticles. The method circumvents the use of chemical linkers, thereby eliminating the disadvantages inherent to this type of conjugation chemistry, such as non-physiological reaction conditions and competition of reactive groups leading to low conjugation efficiency. To show proof of principle with a therapeutically relevant, biologically active targeting moiety, we used a humanized antibody for conjugation to the nanoparticles. Our method utilizes a simple, one-step approach, which is both time-saving as well as economical and could facilitate large-scale production of diagnostically and therapeutically valuable iron oxide nanoparticles. The antibody-conjugated nanoparticles fully retained the antibody binding capacity and presented a high targeting selectivity on GD2-positive cells (CHLA-20), as compared to GD2-negative cells (PC-3, and normal fibroblasts). The targeting efficiency was verified with several complementary techniques, including flow cytometry, fluorescence microscopy, Prussian blue staining and transmission electron microscopy. Importantly, we observed the membrane-anchored, conjugated nanoparticles via GD2 receptors were capable of transporting into cytosols, providing a promising platform to load the cancer-curing drug on and perform targeted therapy in future applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Dopamine functionalized iron oxide nanoparticles: (a) TEM image, (b) high resolution TEM image, (c) DLS plot, and (d) zeta-potential plot.

Fig. 2.
FTIR spectra of dopamine – coated iron oxide nanoparticles before and after oxidation.

Fig. 3. Antibody conjugated iron oxide nanoparticles: (a) TEM image, (b) Zeta-potential plot, (c) DLS plot, and (d) FTIR spectrum.

Fig. 4.

Binding evaluation of hu14.18MoAb-conjugated iron oxide nanoparticles to GD2-positive cells. Fluorescence microscopy (400X) of GD2-positive cells (CHLA-20 neuroblastoma) or GD2-negative cells (normal fibroblasts) treated with unconjugated (a, c) or antibody-conjugated (b, d) nanoparticles and Alexa 488-anti-human IgG antibody. (e) Flow cytometry of cell auto-fluorescence (clear histogram) and antibody-conjugated nanoparticles bound to cells (tinted histogram). (f) Flow cytometry binding curves of hu14.18MoAb (antibody) and hu14.18MoAb-conjugated nanoparticles (antibody-NP) to CHLA-20 cells. Averages $\pm$ SD of three experiments are presented. NP, nanoparticles. DAPI, nuclear counterstain. MFI, mean fluorescence intensity. RU, relative units.

Fig. 5. Perls staining using Prussian blue reaction detecting iron for (a) unconjugated and (b) antibody-conjugated iron oxide nanoparticles. (c) Quantification by ferrozine reaction of the iron content of CHLA-20 cells following treatment with medium alone (no NP), unconjugated (NP) or antibody-conjugated nanoparticles (antibody-NP). (d) Flow cytometry evaluation of the biocompatibility of the dopamine-coated iron oxide nanoparticles by propidium iodide exclusion, following cell treatment with medium alone (no NP), unconjugated (NP) or antibody-conjugated nanoparticles (antibody-NP). Averages $\pm$ SD of three independent experiments are shown.

Fig. 6. Comparison of nanoparticle recognition and internalization on CHLA-20 and PC-3 cells: (a and c) cells treated with dopamine-coated nanoparticles for four hours, (e and g) cells treated with antibody-conjugated NPs for four hour, and (i and k) cells treated with antibody-conjugated NPs for 12 hour, (b, d, f, h, j, l) higher magnification of the areas that red/blue-dashed circle indicates.

Fig. 7. The binding competition experiments on CHLA-20 cells: (a) GD2 unblocked CHLA-20 cells treated with antibody-conjugated NPs for four hours, (c) GD2 blocked CHLA-20 cells treated with antibody-conjugated nanoparticles for four hours. (b and d) Higher magnification of the areas that red-dashed circle indicates.