FC-DIRECTED ANTIBODY CONJUGATION OF MAGNETIC NANOPARTICLES FOR ENHANCED MOLECULAR TARGETING

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In this study, we report the fabrication of engineered iron oxide magnetic nanoparticles (MNPs) functionalized with anti-human epidermal growth factor receptor type 2 (HER2) antibody to target the tumor antigen HER2. The Fc-directed conjugation of antibodies to the MNPs aids their efficient immunospecific targeting through free Fab portions. The directional specificity of conjugation was verified on a macrophage cell line. Immunofluorescence studies on macrophages treated with functionalized MNPs and free anti-HER2 antibody revealed that the antibody molecules bind to the MNPs predominantly through their Fc portion. Different cell lines with different HER2 expression levels were used to test the specificity of our functionalized nanoprobe for molecular targeting applications. The results of cell line targeting demonstrate that these engineered MNPs are able to differentiate between cell lines with different levels of HER2 expression.

Keywords: Nanoprobe; molecular imaging; cancer.

1. Introduction

Nanoparticles play an important role as imaging contrast agents in various biomedical imaging modalities including fluorescence microscopy,1–4 positron emission tomography (PET),5 single photon emission computed tomography (SPECT),5,6 ultrasound imaging,7 magnetic resonance imaging (MRI),8,9 plasmon resonance scattering,10 optical coherence tomography (OCT),11,12 and magnetomotive OCT (MM-OCT).13 With the recent developments in the synthesis and functionalization of nanoparticles, the field of nanomedicine holds a promising future with potential applications in the early diagnosis of disease,14 site-specific drug delivery15–18 and therapeutic applications utilizing hyperthermia.19,20 Various molecular imaging agents such as quantum dots,2–4 gold nanoshells,14 carbon nanotubes,19 gold nanoparticles,10 gadolinium nanoparticles,21 superparamagnetic iron oxide nanoparticles,8,9,16
promising role as molecular nanoprobes for imaging and hyperthermia applications. Iron oxide nanoparticles possess unique paramagnetic properties that result in strong T2 and T2* contrast, making them ideal for MRI studies. Their paramagnetic nature, iron oxide magnetic nanoparticles (MNP), can be modulated by an external magnetic field, creating dynamic contrast in MM-OCT.

External magnetic field, creating dynamic contrast in MM-OCT.

Among the various biomarkers, one of the most well-known targeted proteins is the human epidermal growth factor receptor 2 (HER2, also known as c-erbB-2 oncoprotein), which is a receptor protein overexpressed in about 30% of human invasive breast carcinomas. This receptor is expressed at the membrane surfaces making it easier to target by the nanoparticle conjugates.

In this manuscript we demonstrate the advantage of Fc-directed conjugation of iron oxide MNPs in active targeting applications. We describe the fabrication of engineered iron oxide MNPs coated with dextran and functionalized with anti-HER2 antibody to target HER2 antigen in human breast carcinomas. The Fc-directed specificity of conjugation is verified on a macrophage cell line. Immunostaining of macrophages treated with functionalized MNPs and free anti-HER2 antibodies showed that the antibody molecules bind to the MNPs predominantly through its Fc portion. Different cell lines with different HER2 expression levels were used to test the specificity of our functionalized nanoprobe for molecular targeting applications. These Fc-directed MNPs can act as multimodal imaging agents with in vitro and in vivo applications in magnetomotive imaging, targeted drug delivery, and therapeutic applications.

2. Materials and Methods

2.1. Preparation of targeted iron oxide MNPs

Iron oxide-dextran nanoparticles (Fe-Dex-MNPs) were synthesized by the co-precipitation of ferrous and ferric salts in the presence of the polymer dextran in alkaline medium following standard protocols. A mixed solution of ferrous and ferric ions in a molar ratio equal to 0.57 was prepared from 6.4% FeCl3·6H2O and 15.1% FeCl3·6H2O in deaerated, distilled water. An equal volume of a 20% (w/v) polymer solution in distilled water was then mixed with the iron solution and kept at a constant temperature of 60°C for 15 minutes under nitrogen purging to avoid oxidation. An approximately equal volume of 7.5% aqueous ammonia solution was added dropwise to the iron-polymer mixture to maintain the pH at 11.5 during heating at 60°C for 15 minutes. 

Due to this limitation, a conjugation method should be designed to target the Fc fragment (non-antigen binding fragment) of an antibody molecule through its carbonyl, hydroxyl, sulfhydryl or thiol groups.

Commonly, the contrast agent is targeted to a specific receptor or antigen by conjugating the nanoparticles and the conjugating ligand or proteins. The contrast agent is targeted to a specific receptor or antigen by using a specific antibody molecule or its Fab fragment (antigen-binding fragment). Most conjugation methods using a carbodiimide mediator target the primary amino-group present at the Fab fragment or active site of the antibody molecule. Using this conjugation method, the final product has a limited number of functional and active groups for efficient targeting.

To overcome this limitation, a conjugation method should be designed to target the Fc fragment (non-antigen binding fragment) of an antibody molecule through its carbonyl, hydroxyl, sulfhydryl or thiol groups.

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vigorous stirring. The unbound dextran was separated from MNPs by molecular sieve chromatography using a Sephadex G-300 column equilibrated with 0.01 M phosphate buffer at pH 7.4. After fractionation, the anthrone assay was used to determine the presence of any unbound dextran in the eluted fractions.

Fc-directed conjugation of the antibody molecules is made possible through reductive amination coupling between the free amino groups in the Fc-region of the antibody and reactive aldehyde groups. To create reactive aldehyde groups on the MNP surfaces, oxidation of dextran is carried out under mild conditions. A volume of 0.25 ml of 25 mmol/L NaIO₄ (final concentration 5 mmol/L) was used to oxidize 1 ml of Fe-Dex-MNPs. The reaction was kept away from light and oxygen, and was constantly stirred (150 r/min). Next, 0.2 ml of 2 mol/L ethylene glycol was added and stirred for another 30 minutes to terminate oxidation. Excess periodate was removed by dialyzing the suspension for 24 hours against 0.01 mol/L PBS at 4°C. A quantity of 10 to 25 µg/mg of rabbit polyclonal anti-HER2 antibody (c-erbB-2/neu, Thermo Fisher Scientific, Cat. #RB-103PABX) was added to the oxidized Fe-Dex-MNPs under dark conditions at 4°C for 8 h. This step was followed by reduction with 0.5 mol/L NaBH₄ for 30 minutes to stabilize the new configuration. Uncoupled antibody was separated from conjugated particles by gel filtration chromatography on a Sephacryl S 300 column. The final antibody/nanoparticle ratio (valence) was determined using a bicinchoninic acid (BCA) assay (Protein Quantitation Assay, Pierce, Rockford, IL, USA).

2.2. Particle size determination using TEM
MNPs in 20 to 40 µl aqueous (0.01 M phosphate buffer pH 7.4) suspensions were placed on glow-discharged (DPG-1 portable glow-discharge system, Denton Vacuum Inc., Moorestown N.J.) 200-mesh carbon-stabilized Formvar-coated copper TEM grids (Cat. No. 01811, Ted Pella Inc., Redding CA), and the liquid was allowed to evaporate. Grids were then imaged using a transmission electron microscope (TEM) (Philips CM200, FEI Co., Hillsboro, OR) at 120 kV. Images were collected using a TVIPS 2k x 2k Peltier-cooled CCD camera (Tietz Video and Image Processing Systems GmbH, Gauting, Germany). The scale bar was automatically added by the TEM software.

2.3. Cell culture
Human umbilical vein endothelial cells (HUVEC) (Cambrex Bio Science Walkersville, Inc.) and rat mammary adenocarcinoma tumor (MAT) cells (13762 MAT BIII, ATCC) were used because they display little to moderate amounts of HER2 expression, respectively. A human breast adenocarcinoma cell line (SKBR-3, ATCC) was used as a positive cell line for HER2 overexpression. Mouse macrophages (MØ, ATCC) were used as a source of Fc receptor-expressing cells to test the free and bound Fc portions of the antibody molecules used for conjugation. A human breast primary ductal carcinoma cell line (CRL-2314, ATCC) was used as a cell line with minimal HER2 expression.

The HUVEC cell line was grown using the EGM-2 BulletKit (CC-3162, Cambrex Bio Science Walkersville, Inc.). The MAT and SKBR-3 cell lines were grown in modified McCoy’s 5a medium (ATCC), and macrophages and CRL-2314 cells were grown in DMEM and RPMI 1640 (complete growth medium with 10% fetal bovine serum, ATCC) respectively. All cell lines were cultured at 37°C in a humidified CO₂ incubator (5% CO₂ and 95% air). A 1% mixture of an antibiotic-antimycotic agent (Penicillin G, Streptomycin sulfate, and Amphotericin B, Sigma, A5955) was also added to all cell culture media, except the EGM-2 BulletKit.

2.4. Specific antibodies
Rabbit polyclonal anti-HER2 antibody (c-erbB-2/HER-2/neu Ab-1 (21n) (Thermo Scientific, Cat. #RB-103PABX) was used at a concentration of 1 mg/ml to construct the targeted MNPs. The c-erbB-2 antibody has a known reactivity to HER2 for rat tumor models. For immunostaining after in vitro targeting, FITC-conjugated donkey anti-rabbit IgG (H+L) was used as a secondary antibody (Jackson Immunoresearch Laboratories, Inc.).

2.5. Cell line targeting
Approximately 10⁶ cells from each cell line were grown on sterile, round microscope cover slips (Fisher brand) in sterile Petri dishes with cell culture media. After 24 hours, the cover slips were...
washed three times in sterile $1 \times \text{PBS}$. Cells were fixed with ice-cold acetone for 15 minutes at $4^\circ\text{C}$ and kept at $-20^\circ\text{C}$ until use. Cell monolayers were washed three times using $1 \times \text{PBS}$, blocked with 10% normal donkey serum (in $1 \times \text{PBS} + 1\% \text{BSA}$) for 30 minutes at room temperature inside a humidity chamber, and washed three times for two minutes in washing buffer ($1 \times \text{PBS} + 0.1\% \text{Tween 20}$). Cover slips were then incubated with either targeting solution [anti-$\text{HER2}$ antibody ($100\mu\text{l}$ of $100 \times$ diluted), Ab-$\text{Dex-MNPs}$ ($100\mu\text{l}$ of $1 \text{mg/ml solution}$), Dex-$\text{MNPs}$ ($100\mu\text{l}$ of $1 \text{mg/ml solution}$)] or just PBS buffer ($100\mu\text{l}$), for 90 minutes at room temperature inside a humidity chamber. After incubation cover slips were washed three times for two minutes with washing buffer, and then incubated with secondary antibody (1:200 dilution) for 60 minutes inside the humidity chamber. After washing cover slips another three times for two minutes with washing buffer, they were mounted onto a microscope slide using a hard set mounting medium (H-1400, Vectashield, Vector Laboratories, Inc.). The samples were viewed with fluorescence microscopy (Axiovert 200, Carl Zeiss).

2.6. **Quantification of fluorescence signal**

All fluorescence images were acquired under the same microscope illumination, camera gain, and exposure settings. Cell line monolayers were confirmed in the field-of-view under brightfield illumination before fluorescence imaging. Analysis was performed in MatLab™ on the raw 8-bit, 1,600 $\times$ 1,200 pixel images to quantify the detected fluorescence signals. Due to cell monolayers not being uniformly distributed over the cover slips, ten representative regions of interest (ROIs) measuring 50 $\times$ 50 pixels within each image were chosen manually for the analysis. A histogram for each ROI was calculated, and the fluorescence intensity was determined as the gray level corresponding to the histogram peak. The mean fluorescence intensity over the ten ROIs was calculated for each image and divided by 256 ($2^8$ intensity values) to provide the relative fluorescence intensity (RFI). The RFIs were further normalized to the RFI signal from free anti-$\text{HER2}$ antibody, which represented the maximum binding. These normalized RFIs (NRFIs) provided quantification of the efficiency of treatment with Ab-$\text{Dex-MNPs}$ or Dex-$\text{MNPs}$.

3. Results and Discussion

3.1. **Size determination of targeted magnetic nanoparticles by TEM**

TEM analysis revealed that the size of the Dex-$\text{MNP}$ and Ab-Dex-$\text{MNP}$ particles was approximately 20 to 30 nm (Fig. 1). The MNPs appear mostly monodispersed, with little to no aggregate formation. Particles in the range of 30 to 100 nm can usually avoid rapid leakage from the blood capillaries, whereas particles larger than 100 nm move more slowly and are more susceptible to clearance by interstitial macrophages. Larger particles ($>200$ nm) are more efficient at activating the complement system and they are cleared faster from the circulation by Kupffer cells.\(^{41}\) Hence, based on the size and coating characteristics of our MNPs, a longer circulation life span for in vivo studies is expected.

![Fig. 1. TEM images of Ab-Dex-MNPs (left) and Dex-MNPs (right).](image-url)
3.2. Fc-directed method of conjugation

A conjugation method to engineer efficient antibody-targeted nanoparticles should maximize the free and specific targeting active sites. There are many different sites on an antibody molecule, such as hydroxyl, carboxyl, amino, and sulfidryl or thiol groups, that can be used for this purpose. Among them, the conjugation methods which target primary amino groups by using the reagent carbodiimide hydrochloride (EDC) [N-ethyl-N-(3-dimethylaminopropyl)] are the least effective. In each monomeric antibody molecule such as IgG, only two primary and an abundant number of secondary amino groups are present. Hence, binding the primary amino groups to a nanoparticle will significantly reduce the antibody activity and the targeting sensitivity. To overcome this problem, we used an Fc-directed conjugation, to exclude the active sites from being involved in conjugation, hence keeping them intact for active and efficient targeting.

3.3. Characteristics and advantages of our engineered MNPs

A macrophage cell line, which is known to have multiple Fc receptors on the cell surface, was used to demonstrate that the antibody molecules were mostly bound through the Fc portion on the antigen and not through the active sites (Fig. 2).

Different cell lines were used to test the activity, specificity, and sensitivity of our engineered targeted MNPs using a solid phase indirect immunofluorescence assay. The results show that binding of the Ab-coated MNPs obtained by the Fc-directed conjugation method is significantly less (Fig. 3, middle) than binding observed with free antibody molecules to the surface of macrophages (MØ). Diagram (A) illustrates that free, unbound anti-HER2 antibody can easily bind to the Fc receptors present on the surface of macrophages, while the binding of the Fc-directed MNPs, conjugated with the same antibody, will be greatly reduced or even completely blocked. The diagonal black line in the figure schematically represents the blockage of binding of targeted MNPs to the surface of macrophages through the Fc receptors.

Fig. 3. Macrophages immunostained with free anti-HER2 antibody (left), Ab-Dex-MNPs (middle), and Dex-MNPs (right). The fluorescence signal is significantly reduced in the middle image compared to the left image, which indicates reduction of MNP binding due to the Fc-directed conjugation of the antibodies to the MNPs. The right image confirms the absence of non-specific binding of MNPs to the macrophages.
3.4. Cell line targeting

To demonstrate that our engineered MNPs are capable of actively targeting HER2 receptors, immunostaining of different cell lines with different HER2 expression levels was performed. The results confirm that the engineered targeted MNPs are capable of actively targeting HER2 expression (Fig. 4) and can also differentiate between cell lines with different HER2 expression levels (Figs. 5 and 6).

After calibrating the Relative Fluorescence Intensity (RFI) to 100% obtained by treating SKBR-3 cells with commercial anti-HER2 antibody, as shown in Fig. 6, the normalized RFI (NRFI) for SKBR-3 cells treated with Ab-coated MNPs would be greater than 300. The signal from SKBR-3 cells treated with commercial anti-HER2 antibody represents the ideal binding conditions. The higher RFI obtained by Ab-coated MNPs can be attributed to multiple bound antibody molecules on each MNP, which may amplify the fluorescence intensity obtained by the indirect immunofluorescence assay. We estimated this amplification factor (AF \approx 3) by dividing the NRFI of SKBR-3 cells treated with commercial anti-HER2 antibody by the NRFI obtained from SKBR-3 cells treated with commercial anti-HER2 antibody. The AF suggests that in the conjugation process, on average, binding of three antibody molecules per MNP occurs.

If we calibrate the fluorescence signal obtained from the macrophages treated with commercial
Fig. 5. Different cell lines immunostained using free anti-HER2 (top row) and Ab-Dex-MNPs (bottom row). The cell lines are indicated above each column. The middle row shows the brightfield images of the different cell lines.

Fig. 6. Relative fluorescence intensity from different cell lines obtained using free anti-HER2 antibody and targeted Ab-Dex-MNPs. Symbol Ø in the case of CRL-2314 cell line with minimal HER2 expression, indicates that there was no fluorescence signal after exposure to free anti-HER2 antibody. Macrophages (MØ) were used to detect the fraction of Fab-conjugated MNPs. For each cell line, the difference in RFI between Ab/HER2 and Ab-MNP binding is statistically significant ($p < 0.05$).

*Value of normalized relative fluorescence intensity (NRFI) divided by amplification factor (AF).

anti-HER2 antibody to 100%, then the NRFI for macrophages treated with Ab-MNPs is calculated to be 16%, after dividing by the AF. This means that if we assume that 100% of the commercial anti-HER2 antibody have the Fc portion available to be bound to macrophage Fc receptors, only 16% of targeted MNPs may have any available terminal Fc sites for this kind of binding. Considering the fact that normal cells such as macrophages may express low levels of the HER2 marker, we can conclude that even more than 84% of the antibody molecules were attached to the MNPs through their Fc portions.

In 2007, Grützner, et al., conjugated magnetic nanoparticles using different methods and
approaches. In their well-designed two-step immunosassay, they calculated the highest percentage for a maleimide-based method to be ~80% and for a carbodiimide-based method to be ~30%. In their calculations, however, they did not consider the fact that the secondary antibody is not only recognized by the Fc portion of an antibody molecule attached to the MNP through its Fab portion, but also can fully recognize the multiple antigenic determinants present on the Fc portion of an antibody molecule, even if attached through the Fc portion. Therefore, the total measurements could potentially be at least half of the reported values. Using the carbodiimide-based method of conjugation as an example, which usually targets the primary amino group of the antibody molecule present on the Fab portion, the correct calculated percentage would likely be less than 10%. This means that at least 90% of the antibody molecules will likely be attached to the MNP through their Fab portion or their active sites. Therefore, by following the Fc-directed conjugation described in this paper, over 80% of the active sites are likely to remain free for specific and sensitive targeting.

4. Conclusion

In this study, we have engineered superparamagnetic dextran-coated iron oxide nanoparticles to be used as specific anti-HER2 antibody-targeted MNPs for active molecular targeting. In our selected way of conjugation, the Fc portion of the antibody molecule binds to the surface of the magnetic nanoparticle and the Fab portion (active site) of the antibody molecule remains intact and available for specific targeting, as was demonstrated on different cell lines with different levels of HER2 expression.

Based on the size, negative charge, hydrophilic surface, dispersibility, and Fc-directed-conjugation, this molecular probe is a promising candidate for in vivo applications due to its longer predicted circulation lifetime and reduced clearance rate by macrophages. The probe can potentially be used to enhance contrast in imaging modalities such as MRI and magnetomotive optical coherence tomography. Finally, this molecular probe has the potential for targeted in vivo therapy using magnetically induced drug release or for site-specific hyperthermia treatments in cancer therapy.

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