

REVIEW

MRI AS A POTENTIAL TOOL FOR MONITORING OF THERAPEUTIC MONOCLONAL ANTIBODY DISTRIBUTION IN CANCER PATIENTS

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Abstract: Imaging and quantification of MAb distribution by MRI after administration *in vivo* can improve patient outcomes, although it is not routinely used in the clinic hence the motivation for the development of new MAb imaging methodologies. Improvements in imaging and quantification of antibody distributions can be made by conjugating MAbs to “hotspot” atoms such as ¹⁹F as ¹⁸F. Currently, research investigating the incorporation of ¹⁹F to antibody delivery systems has been limited to trastuzumab perfluorocarbon emulsions and this method has not been extended to other clinically approved antibodies. The methodologies of ¹⁹F MRI can improve the current limitations of antibody immunotherapy such as quantitative visualization of targeted surface antigens expressed on tumor cells.

Keywords: monoclonal antibody, fluorine, magnetic resonance imaging, cancer diagnostics

The development of magnetic resonance imaging (MRI) diagnostic constructs consisting of monoclonal antibodies (MAbs) functionalized with contrast agents continues to be an active area of research. There is currently great interest in the design and implementation of MAb-based MRI imaging agents as the selectivity of MAbs for specific receptors at nanomolar and sub-nanomolar concentration leads to high sensitivity and specificity and MAbs can be chemically modified to serve as imaging reporters without disrupting their ability to target receptors (1). The idea that antigen targets are selectively targeted by antibodies began with Ehrlich's side-chain theory published in 1897 (2). To detect the efficiency of inhibition of targeted antigens, additional modification of monoclonal antibodies is required. In total, 30 therapeutic antibodies from a group of 160 in clinical trials and 300 in preclinical trials were approved for use in the United States and Europe in 2013 (3). An additional 70 monoclonal antibodies are expected to be approved at current rates by 2020 (4). It is estimated that 0.01% of an intravenously applied dose of monoclonal antibody per gram of tissue localizes in a solid tumor after 24 h (5). The ability of monoclonal

antibodies to specifically target and bind to surface receptors has led to great advances in therapy, yet there is a need to further develop imaging and quantification through modifications.

The ability to visualize and quantify MAb distribution after administration *in vivo* is extremely desirable and is the impetus for the development of new imaging methods (6-8). Of particular interest are two of the most modified monoclonal antibodies for MRI imaging: epidermal growth factor receptor (ErbB1/EGFR) targeting Cetuximab and the ErbB2/HER2 targeting antibody Trastuzumab (9, 10). Modifications of bevacizumab which targets vascular endothelial growth factor (VEGF) have also been reported to a lesser extent (11). Monoclonal antibodies have also been modified as diagnostic reporting agents for positron emission tomography (PET), and fluorescence imaging or as dual imaging agents (1, 12). A large body of research has been performed in studying combined therapy of MAbs and cytotoxic drugs and MAb-cytotoxic drug conjugates for increasing therapeutic efficacy, yet relatively few works have reported on MAb-conjugates detectable by MRI. Here, we will focus on the advantages and disadvantages of MAbs

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conjugates that have been designed thus far for MRI. The contrast agents that have been conjugated to MABs to visualize their distribution include paramagnetic gadolinium ion (Gd^{3+}), superparamagnetic iron oxide nanoparticles (IONP), and heteronuclear fluorine-19 atoms (^{19}F) (13). The conjugation of paramagnetic contrast agents with antibodies lowers the spin-lattice (T_1) and spin-spin (T_2) relaxation times of water allowing for enhanced contrast in targeted tumor tissue. There is a pressing need for effective, non-invasive diagnostic techniques for monitoring the location and distribution of MABs for diagnostics. While contrast-enhanced MRI can provide high sensitivity for detection, ^{19}F MRI can provide even greater contrast due to a lack of tissue background. ^{19}F MRI can also characterize the oxygenation of tissue by relaxation time measurements (14). In studies of MABs distributions in tissue, the application of 1H MRI has been limited because of the large mass of water which results in very broad signals. Monoclonal antibody- ^{19}F constructs should be designed to enhance visualization of their distribution for diagnostics. A wide range of chemical shifts for the ^{19}F nucleus ensures good separation of signals in different environments (15). ^{19}F MRI offers a quantitative way of *in vitro* imaging with high ^{19}F sensitivity (0.83 relative to 1H MR), an impressively large chemical shift range (~400 ppm), and zero background signal in biological samples (16, 17). In the case of ^{19}F antibody labels, signal intensity (spin density), T_1 and T_2 of ^{19}F may be measured and complemented with anatomical water proton T_1 and T_2 measurements.

The incorporation of ^{19}F nuclei with monoclonal antibodies is an underutilized approach for tumor tissue imaging. To date, the bulk of research investigating ^{19}F -monoclonal antibody constructs has been performed with Trastuzumab conjugated to, and delivered by, perfluorocarbon emulsions (18-19). This research has clearly shown that analysis of ^{19}F labeled Trastuzumab delivery systems affords quantification and imaging of targeted HER2 receptors. Other advantages of the study of ^{19}F -MABs conjugates include the potential for modification for either enhanced lipophilicity or water solubility and cellular uptake. We will argue that ^{19}F conjugation should be investigated in a wider range of monoclonal antibodies for MRI cancer imaging diagnostics. Recently reported ^{19}F MRI nanoparticle probes that consist of a perfluorocarbon core encapsulated with silica might be potentially exploited to serve as MABs imaging conjugates (20-22).

Molecules or nanoparticles that contain ^{19}F are particularly well-suited for conjugation to MABs for

analysis by ^{19}F MRI due to 100% ^{19}F abundance. The high ^{19}F gyromagnetic ratio (40.05 MHz/T) which is about 6% lower than that for protons allows for the use of the same hardware instrumentation used for 1H MRI adjusted for ^{19}F (15).

Methods of imaging of monoclonal antibodies by 1H MRI

Imaging of MABs conjugates that are constructed with paramagnetic contrast agents by MRI detects distribution based on differences in T_1 and T_2 of water protons in tissue. Paramagnetic ions including Gd^{3+} , manganese (Mn^{2+}) and copper ion (Cu^{2+}) result in a shortening of water proton T_1 resulting in a bright tissue contrast image. Dark tissue contrast has been achieved by the conjugation of MABs to superparamagnetic iron oxide which lowers T_2 (23). Monoclonal antibody-contrast agent conjugates that influence the T_1 and T_2 of water have been designed using a variety of methods that have overwhelmingly utilized paramagnetic Gd^{3+} or superparamagnetic iron oxide. Gadolinium-based MABs conjugates including attachment of chelates such as Gd-diethylenetriaminepentaacetic acid (Gd-DTPA) (24) and isothiocyanobenzyl-ethylenediaminetetraacetic acid (Gd-EDTA) (25), Gd^{3+} binding proteins (26), Gd^{3+} incorporated polymeric nanoparticles (27), Gd^{3+} Mesoporous silica nanoparticles (28, 29), and cationic Gd^{3+} -liposomes (30). Spin-spin relaxation contrast agents conjugated to MABs include superparamagnetic iron oxide nanoparticles (31), and magnetic albumin nanospheres (32). Often, nanoparticles can also be designed to include a fluorescent dye in addition to an MRI contrast agent (32) or a chemotherapeutic drug such as gemcitabine (33). Superparamagnetic iron oxide nanoparticles have been employed in conjunction with Trastuzumab to image HER2 receptor overexpression in breast cancer that was readily tracked by MRI (34). Streptavidin conjugated superparamagnetic iron oxide nanoparticles were directed HER2 bound trastuzumab and this resulted in strong T_2 contrast. Moreover, the level of HER2 expression was in proportion to T_2 contrast and 10^6 HER2 receptors per cell were estimated. Adolphi et al. conjugated trastuzumab with SHP-20 carboxyl iron oxide nanoparticles via carbodiimide coupling (35). Spin-spin relaxation imaging by MRI and magnetic relaxometry were both performed before and after the binding of this trastuzumab construct to HER2 receptors in nude mice. Interestingly, the authors state that magnetic relaxometry was better suited for receptor quantification than T_2 MRI contrast in part due to the difficulty of imparting an echo time (TE) of $\ll 5$ ms in a clinical scanner.

The potential for imaging monoclonal antibodies by heteronuclear MRI

As previously mentioned, the design of MAb conjugates for distribution imaging by ^1H MRI typically contain contrast agents such as Gd^{3+} , superparamagnetic iron oxide, and their variations that shorten the T_1 and T_2 of water protons in tissue. These MAb conjugates thus far have led to great advances in the ability to visualize the distribution of antibodies in tissue. However, a commonly held view by us and others is that the visualization and quantification of antibody distributions could be improved by implementing so-called heteronuclear "hotspot" atoms that are commonly used in NMR such as ^{13}C , ^{31}P and ^{19}F (13). Heteronuclear MRI avoids the large intrinsic background imaging of water protons, which are essential for anatomical detail but can interfere with distinguishing healthy and cancerous regions of tissue. Very few studies thus far have conjugated heteronuclear atoms to antibodies to provide a second imaging mode, and, of the available heteroatoms, only ^{19}F concentrations (10^{-3} $\mu\text{mol/g}$ wet tissue) are below MR detection limit (15).

Current methods and future directions of imaging monoclonal antibodies by ^{19}F MRI

The primary applications of ^{19}F MRI in cancer research are the imaging and tracking of ^{19}F labeled anticancer agents *in vivo*, *in vitro* and *ex vivo* as they offer a controlled and systematic way to investigate cellular and molecular properties associated with the disease. Additional applications of ^{19}F MR are in tissue characterization, quantification of drugs, and cellular tracking, tissue pH measurements and detection of ^{19}F labeled cells. ^{19}F MR provides only localized detection and visualization of ^{19}F labeled cells and ^1H MR image overlays are used in conjunction to give anatomical detail. Other applications include tissue perfusion, tissue oximetry and visualization of labeled cells.

Imaging and quantification of ^{19}F conjugated MAb distributions by ^{19}F MRI have many advantages and is an underutilized methodology with the potential for advancing diagnostic imaging. Potential improvements in MAb immunotherapy and diagnostics can be sought by incorporation ^{19}F nuclei into antibody delivery systems involving new synthetic strategies for tracking and visualization of ^{19}F -MAb conjugates (36-38). Targeted delivery ^{19}F -MAbs could also potentially serve to alleviate side effects that are observed commonly with chemotherapy strategies for cancer treatment. There may be other undiscovered advantages of developing ^{19}F

conjugated MAbs. Potential conjugation of MAbs with compounds containing ^{19}F may lead to delivery systems with other desirable properties in addition to enabling unambiguous imaging and antibody quantification by MRI such as increased lipophilicity, biocompatibility and persistence within the tissue (35-38). The carbon-fluorine bond has an average bond strength of 485 kJ/mol (43) and is the strongest covalent bond found in organic molecules rendering ^{19}F conjugates inert.

Imaging of ^{19}F -MAb conjugate delivery systems must be conducted at 1.5 or 3.0 Tesla to be applicable clinically (15). It has been estimated that *in vivo* ^{19}F MRI requires concentrations of ^{19}F labeled compounds to be ≥ 200 nmol/g tissue for detection at clinical field strengths of 1.0–2.0 Tesla, ≥ 50 nmol/g at 7.0 Tesla, ≥ 5 -10 nmol/g at 11.7 Tesla (44). All these attributes suggest that imaging and quantification of MAb distribution can be enhanced by the development of ^{19}F -conjugated MAbs.

Quantification of ^{19}F labeled MAbs *in vitro*

Quantification of cells *in vitro* performed with the use of MRI is non-invasive and non-destructive to cell physiology and morphology. However, this technique requires a high concentration of cells (on the order of 10^6). There is a diagnostic need to quantify MAbs in cells and quantify targeted sites in cancer tissue. The goal of targeted anticancer MAb therapy is to localize the MAbs and monitor binding. The main disadvantage of conventional chemotherapy is nonspecific drug delivery and toxicity. In oncology, the major drugs used in targeted anticancer therapy are monoclonal antibodies. Therefore, molecular imaging promises to play a decisive role in the noninvasive detection of cancer since molecular targets of interest are well known (39-42). Due to the absence of natural fluorine in the cells, there is no background noise making this method very sensitive. Therefore, quantification by the use of fluorine labeled MAbs is very efficient.

The quantifications of fluorine labeled MAbs by ^{19}F signal intensity (SI) in tissue or cells are performed in 5 steps:

1. A calibration curve is prepared with no less than 12 different concentrations of the selected fluorine compound. The calibration curve is a function of ^{19}F SI vs. ^{19}F concentration;
2. The MAbs is labeled with a fluorine marker with a known concentration of ^{19}F ;
3. The cells are treated with the fluorine labeled MAbs;
4. The ^{19}F SI of the MAb-conjugate is measured in the treated cells;

5. The use of a calibration curve is necessary to estimate MABs uptake in tissue.

Quantification of the number of dead cells after treatment of cells with fluorine labeled MABs in tissue are also performed in 4 steps:

1. A calibration curve is prepared with 12 different cell concentrations and per one selected concentration of fluorine labeled MABs. The calibration curve is a function of ^{19}F SI vs. the number of cells that are labeled with MABs;
2. A biological sample with an unknown number of cells is treated with the same concentration of MABs-conjugate;
3. The ^{19}F SI of the biological sample is measured;
4. The number of treated cells is estimated based on ^{19}F SI.

It has been reported that ^{19}F labeled trastuzumab directed to HER2 can be imaged and quantified by ^{19}F MRI. Unmodified trastuzumab has been reported to inhibit breast cancer carcinoma by 30% – 50% at doses of 10 $\mu\text{g}/\text{mL}$ – 1000 $\mu\text{g}/\text{mL}$ over 72 h (41). Incorporating ^{19}F into antibody delivery systems affords quantification of targeted HER2 receptors, enhanced water solubility and uptake (18). The delivery of trastuzumab to HER2 overexpressing MCF-7 cells *ex vivo* has been accomplished using ^{19}F -containing perfluorocarbon (PFC) emulsions at 9.4 Tesla. Perfluorocarbons are hydrocarbon analogs where ^1H is substituted for ^{19}F that are non-toxic and biologically stable. These ^{19}F emulsion antibody delivery systems were composed of trastuzumab conjugated to emulsions containing a liquid PFC core containing with or without an encapsulating Lipoplex lipid monolayer. The PFCs evaluated as Trastuzumab delivery emulsions were perfluoro crown ether (PFCE) and perfluorooctyl bromide (PFOB). The delivery systems were not only visible by ^{19}F MRI, but showed increased anticancer activity compared to Trastuzumab alone. Percent viabilities of $54 \pm 2\%$, $50 \pm 3\%$ and $45 \pm 1\%$ were reported in treated MCF-7 cells for Trastuzumab, Trastuzumab-PFOB, and Trastuzumab-PFOB-Lipoplex respectively after 72 hours of treatment (14). A similar trend was found for Trastuzumab PFCE emulsions where percent viabilities of $54 \pm 2\%$, $49 \pm 3\%$, and $43 \pm 5\%$ for trastuzumab, trastuzumab-PFCE, and trastuzumab-PFCE-Lipoplex respectively were reported in treated MCF-7 cells after 72 h of treatment (18). The EC_{50} values for these ^{19}F -containing trastuzumab delivery systems decreased compared to the unmodified antibody. The reported percent decreases in EC_{50} were as follows: trastuzumab-PFOB (7%), trastuzumab-PFOB-Lipoplex (27%), trastuzumab-PFCE (3%),

and trastuzumab/PFCE/lipoplex (35.5%) (18). Clearly, fluorinated emulsion conjugates enhanced the anticancer activity of trastuzumab *ex vivo*. In addition to the enhancement of activity, the number of cells that had internalized trastuzumab was estimated by ^{19}F MR signal intensity.

An additional feature of PFC labeled MABs constructs of note is the potential to monitor differences in the T_1 of ^{19}F as a function of tissue oxygenation. Since oxygen is paramagnetic, it can act as a contrast agent itself, lowering or increasing the T_1 of ^{19}F depending on local concentration (45). The T_1 of ^{19}F is lowered in the presence of oxygen, thus it is inversely proportional to the concentration of dissolved molecular oxygen, and tumor tissue hypoxia can be inferred by T_1 measurements (14, 18). PFC emulsions do have the drawback of ^{19}F peak splitting which tends to lessen signal intensity (46). Tracking and imaging of MABs conjugated with ^{19}F substituents by ^{19}F MRI has been demonstrated and has great future potential.

MRI imaging agents containing ^{19}F have been used clinically for reasons of cell quantification by direct detection discussed earlier (47). The delivery of *ex vivo* ^{19}F labeled dendritic cells to patients with colorectal adenocarcinoma was imaged by 3T MRI; the labeled dendritic cells appeared as “hotspots” at the site of injection (48). Stromal vascular fraction (SVF) cells were labeled with CS-1000 (PFC nanoemulsion) prior to transplantation in breast cancer patients with radiation-induced fibrosis and monitored by ^{19}F MRI at 3T (49). The authors claim that CS-1000 may discriminate between live and dead cells as ^{19}F is only retained in live stem cells, unlike superparamagnetic iron oxide.

A potential MABs- ^{19}F conjugate must contain a high number of equivalent ^{19}F atoms for imaging and quantification. Two ^{19}F agents that have been reported recently that could potentially enhance MABs imaging and quantification that have not been utilized as MABs conjugates to date. The first candidate is a recently reported PFC encapsulated with silica called Fluorine accumulated silica nanoparticle for MRI contrast enhancement (FLAME). We suggest that the design of new classes of MABs- ^{19}F conjugates could benefit from recent developments that have been reported in the synthesis of these easily functionalized silica encapsulated perfluorocarbons. Matsushita et al. developed a specific ^{19}F MRI nanoparticle probe consisting of a core of liquid PFCE surrounded by a modifiable silica shell (20). The ^{19}F nanoparticle probes were constructed to overcome two main problems that are encountered in the design of ^{19}F conjugates. The first problem

stated by the authors is that to obtain a strong ^{19}F signal by MRI, a large number of fluorine atoms are needed in the conjugate probe which, in turn, has the effect of decreasing water solubility (20). Secondly, as the molecular weight of the conjugate probe increases, the fluorine atoms themselves tend to become less mobile which decreases ^{19}F signal intensity by shortening T_2 (20). FLAME displays a strong single ^{19}F peak at -16.4 ppm and PEG surface modification provided persistence and *in vivo* imaging in tumor-bearing mice. FLAME was also modified by reaction with 3-aminopropyltriethoxysilane-Cy5 dye molecules for use as a dual ^{19}F MRI and near-infrared fluorescence reporter (22). To date, there are no reports as yet of the use of PFC bound silica nanoparticles as MAb conjugates. The second potential MAb conjugates are ^{19}F dendrimers. Recently, G5-PAMAM dendrimers fluorinated with between 49 and 68 ^{19}F atoms have been reported as highly efficient and non-toxic gene vectors (50). As of today, the use of fluorinated dendrimers in MAb delivery, imaging and quantification have not been explored.

Future perspective

In vitro studies

For an increase in the efficacy of Trastuzumab, it was advantageous to couple trastuzumab to perfluorooctyl bromide (PFOB) and perfluoro-15-crown-5 ether (PFCE) to form biocompatible water-oil emulsions for breast cancer cell treatment. The coupling of Trastuzumab with both above mentioned PFCs allows tracking, identification and quantification using ^{19}F MR imaging and spectroscopy. This methodology can be extended to develop new MAb imaging systems with other clinically approved antibodies. By monitoring ^{19}F signal intensity, the number of treated cells can be estimated where an increase in fluorine signal intensity corresponds to an increase in cell death. Future use of three-dimensional cancer cell cultures will provide tissue density that “mimics” *in vivo* conditions and allow for further MRI studies that can eventually be introduced clinically.

Imaging and quantification of MAb that are conjugated with a ^{19}F bearing particle such as FLAME or fluorinated dendrimers will lead to advances in MAb “hotspot” imaging and therapy.

In vivo studies

In vivo MRI techniques for quantitative measurements such as diffusion-weighted imaging (DWI), dynamic contrast-enhanced (DCE), dynamic susceptibility contrast (DSC), and diffusion-weighted imaging (DWI), chemical exchange satu-

ration transfer (CEST), and polarized MRI are still under development in clinical trials (51). MRI has been shown to be useful for monitoring and quantification MAb *in vitro* up to this point in time. We expect that in the near future, challenges will be overcome and MAb imaging and quantification *in vivo* will be established.

Executive summary

The ability to visualize and quantify MAb distribution after administration *in vivo* is extremely desirable and is the impetus for the development of new imaging methodologies.

The use of ^{19}F nuclei conjugated to monoclonal antibodies is an underutilized approach for tumor tissue imaging. Currently, the research investigating ^{19}F -monoclonal antibody constructs has been limited to Trastuzumab delivery with perfluorocarbon emulsions.

The visualization and quantification of antibody distributions could be improved by implementing so-called heteronuclear “hotspot” atoms that are commonly used in NMR, especially ^{19}F . ^{19}F MRI avoids the large intrinsic background imaging of water protons, which are essential for anatomical detail but can interfere with distinguishing healthy and cancerous regions of tissue.

The synthesis of MRI detectable MAb imaging agents that are constructed by covalent attachment of chemical moieties containing ^{19}F are needed. The selectivity of MAb for specific receptors at nanomolar and sub-nanomolar concentrations can be retained without disrupting their ability to target receptors.

The use of ^{19}F MRI will impact the field of immunotherapy by providing a critical improvement to our ability to visualize MAb uptake by cancer tissue and to quantify the response of tissue to treatment. The methodologies of MRI can improve the current limitations of antibody immunotherapy such as quantitative visualization of targeted surface antigens expressed on tumor cells.

Fluorinated trastuzumab in oil-water emulsions already has demonstrated a significant contribution to the medical field. Monitoring MAb delivery is a critical step in the application of new immunotherapies to treat cancer. The merging of immunotherapy and tumor imaging will result in improvements in MAb efficacy.

MAb- ^{19}F conjugates must contain a high number of equivalent ^{19}F atoms for imaging and quantification. Two potential conjugates that have not yet been explored as MAb imaging agents are FLAME nanoparticles and PAMAM fluorinated dendrimers.

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Conflict of interests

The authors declare no conflict of interest.

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