In Vivo Targeting of Underglycosylated MUC-1 Tumor Antigen Using a Multimodal Imaging Probe

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ABSTRACT

One of the most difficult challenges of oncology is to improve methods for early tumor detection, which is crucial for the success of cancer therapy and greatly improves the survival rate. Underglycosylated mucin-1 antigen (uMUC-1) is one of the early hallmarks of tumorgenesis and is overexpressed and underglycosylated on almost all human epithelial cell adenocarcinomas as well as in nonepithelial cancer cell lines, as well as in hematological malignancies such as multiple myeloma, and some B-cell non-Hodgkin lymphomas. In this study, we designed, synthesized, and tested a novel multimodal imaging probe specifically recognizing in vivo uMUC-1 antigen in an animal model of human cancer. Furthermore, in vivo magnetic resonance- and near-infrared-imaging experiments on tumor-bearing animals showed specific accumulation of the probe in uMUC-1-positive tumors and virtually no signal in control tumors. We expect that this probe has a potential to greatly aid in screening prospective patients for early cancer detection and in monitoring the efficacy of drug therapy.

INTRODUCTION

To improve noninvasive methods for early tumor detection and to follow tumor progression/regression during the course of therapy, new contrast agents specifically targeting tumors in combination with high-resolution imaging methods are required. In our search for a suitable target, we focused our attention on epithelial cell mucin, the product of the MUC-1 gene. Mucin-1 is a transmembrane molecule, expressed by most glandular epithelial cells (1, 2). Several important features make mucin-1 an attractive molecule for targeted imaging of tumors.

First, MUC-1 is overexpressed on almost all human epithelial cell adenocarcinomas, including >90% of human breast (3–6), ovarian (5–8), pancreatic (9), colorectal (10), lung (11, 12), prostate (13), colon (9), and gastric carcinomas (14). Moreover, MUC-1 expression has been demonstrated in nonepithelial cancer cell lines [astrocytoma, melanoma, and neuroblastoma (15)], as well as in hematological malignancies such as multiple myeloma and some B-cell non-Hodgkin lymphomas (16–18), in total constituting >50% of all cancers in humans (19). The cellular distribution of MUC-1 is allocated to the cytoplasm, as well as to the cell surface (4).

Second, in adenocarcinomatous tissue, as the result of the lost gland architecture, MUC-1 is ubiquitously expressed all over the cell surface (20). Because of its rod-like structure, the molecule extends >100–200 nm above the surface, which is 5–10-fold the length of most membrane molecules (21). This feature makes MUC-1 an accessible target for imaging and, possibly, therapeutic probes.

Third, whereas in normal tissues mucin-1 is heavily glycosylated (50–90% of its molecular mass is due to carbohydrates), in neoplastic tissues, MUC-1 is underglycosylated. The biochemical basis of mucin-1 underglycosylation in tumors has been well investigated. Normal mucin-1 consists of a tandemly repeated 20-amino acid sequence found in the extracellular portion of the molecule and present in 30–90 copies, constituting a variable number of tandem repeat polymorphism. In the malignant state, the oligosaccharide chains are prematurely terminated by the addition of sialic acids (20). Reduced glycosylation permits the immune system to access the peptide core of the tumor-associated underglycosylated mucin-1 antigen (uMUC-1; Ref. 22) and reveals epitopes, which in the normal cell are masked. This feature makes it possible to design probes that discriminate between normal cells and adenocarcinoma cells.

Fourth, the extracellular domain of uMUC-1, defined by the presence of the PDTDTR sequence (Fig. 1A), extends above the cell surface, thus interfering with the interaction between adhesion molecules on the tumor cell surface and their ligands on lymphocytes, aiding in the inaccessibility of tumor epitopes to immune recognition (23). Therefore, there is no tendency for tumor antigen down-regulation in response to immunotherapy, and uMUC-1 expression remains homogeneously up-regulated during the life of the tumor (24) and tumor metastases (6). These features are important in designing probes for different stages of tumor progression.

An abundant number of investigations have focused on the potential to use uMUC-1 as a target for immunotherapy. Multiple monoclonal antibodies have been produced to recognize the immunogenic APDTRP sequence of the tandem repeat (Fig. 1A; Refs. 25–29). However, when antibodies were used as targeting molecules, the immunogenicity and long plasma half-life of these proteins were detrimental (30). Consequently, the use of small peptides instead may eliminate these shortcomings because peptide ligands are nonimmunogenic and have high affinity and selectivity for receptors. In this study, we used a synthetic peptide, designated EPPT1 (YCAREPPTTRFYAWG), derived from the CDR3 Vh region of a monoclonal antibody (ASM2) raised against human epithelial cancer cells (25, 31). Analysis of peptide structure revealed a β-strand type conformation as the active binding site (32). The EPPT1 synthetic peptide has significant affinity (Kd = 20 μM) for the uMUC-1-derived peptide PDTDTR (Fig. 1A). In a previous study, the EPPT1 peptide, labeled with (99mTc), was used to image breast carcinomas in vivo (31).

All of the features of the uMUC-1 protein listed above (overexpression throughout the cytoplasm and the cell surface, aberrant glycosylation exclusively on tumor cells, and stability of the protein core) make this molecule an ideal candidate for a potential imaging target.

It is clear that to target this tumor antigen, a combination of imaging modalities that would allow one to extract anatomical, physiological, and molecular information would be highly advantageous. For example, magnetic resonance (MR) imaging can provide high spatial resolution on the order of tens of mm/voxel both in small animals and in humans (33). Optical imaging in the near-infrared (NIR) region between 700 and 900 nm has a low absorption by intrinsic photoactive biomolecules and allows light to penetrate several centimeters into the tissue, a depth that is sufficient to image practically all small animals (34). Hence, imaging in the NIR region has minimal tissue autofluo-
rescence, which dramatically improves the target/background ratio. Therefore, by combining MR and optical imaging modalities, it is possible to obtain multiple imaging data using the advantages of both methods.

In this study, we combined two imaging modalities that use cross-linked iron oxide (CLIO) nanoparticles as an MR-imaging contrast agent (35) and Cy5.5 dye as a near-infrared fluorescence (NIRF) optical probe. Previously, CLIO and other iron oxides have been successfully attached to a variety of molecules, including proteins and small peptides (36–40). The NIRF dye Cy5.5 has been used to label peptides cleavable by proteases (41–43). Combined MR/optical probes for imaging protease activity have also been recently introduced (44, 45). Here, we synthesized and tested both in vitro and in vivo a multimodal imaging probe targeting the uMUC-1 tumor antigen. This probe consists of CLIO, modified with Cy5.5 dye, and carrying EPPT peptides attached to the dextran coat of the nanoparticle. It was shown to (a) specifically recognize tumors in vivo, (b) produce high-resolution signal on MR images, and (c) allow for real-time data acquisition by NIRF imaging.

Overall, we believe that this study is not only novel but also highly relevant to many cancer studies, their basic biology, and therapy. The knowledge gained from it would give us the ability to detect and follow the early progression of cancer, which would greatly enhance pharmacological intervention against this disease.

MATERIALS AND METHODS

Synthesis and Characterization of the CLIO-EPPT Probe

To synthesize a multi-imaging probe with a high affinity for tumor markers, we used a previously developed method of attaching peptides to CLIO nanoparticles through thioether linkage (44). Synthesis was performed in three steps. In the first step, we synthesized a modified version of the previously described EPPT1 peptide (25). The original peptide sequence (YCAREPPTRTFAYWG) was based on the CDR 3 VH and framework regions of the murine antitumor monoclonal antibody ASM2 against the underglycosylated polymorphic epithelial human mucin epitope PDTRP. This sequence was modified to introduce (a) a FITC label for subsequent fluorescence microscopy analysis and (b) an available SH-group for attachment to the nanoparticle through thioether linkage. The FITC label was introduced by adding a FITC-labeled Lys on the COOH terminus, whereas the SH-group came from an additional Cys on the NH2 terminus of the peptide. The second Cys within the sequence was protected by the addition of an acetoxymethyl group. A 6-aminohexanoic acid linker (AHA-linker) was added between the unprotected Cys and the beginning of the sequence. The final peptide, designated EPPT, had the following sequence: C-AHA-Y-C(ACM)-A-R-E-P-P-T-R-T-F-A-Y-W-G-K(FITC) (italic font indicates the original sequence). The peptide was synthesized on an automatic synthesizer (PS3; Rainin, Woburn, MA) using Fmoc chemistry with 2-(1H-benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate and N-hydroxybenzotriazole. They were cleaved from Rink amidide 4-methyldihydrolamine resin (Novabiochem, San Diego, CA) with 5 ml of trifluoroacetic acid/thioanisole/ethanedithiol/anisole.
(90/5/3/2) and purified by C18 reverse phase high-performance liquid chromatography. By matrix-assisted desorption ionization mass spectrometry, EPPT was 2591.93 calculated versus 2592.95 found (M + 1).

In the second step, we labeled aminated CLIO (CLIO-NH₂) with Cy5.5 dye. CLIO-NH₂ was prepared as described previously (44, 46). It consists of a core of superparamagnetic iron oxide and a cross-linked coating of dextran with amino groups. To attach the Cy5.5 monofunctional dye to nanoparticles, 1 ml of aminated CLIO ([Fe] = 11.45 mg/ml), with the pH adjusted to 9.6 with 0.5 M sodium bicarbonate, was added to 1 mg of Cy5.5 dye (catalogue no. Q15408; Amersham-Pharmacia) and incubated on a rotator overnight at room temperature. After incubation, the mixture was purified from nonreacted dye on a G-25 Sephadex column equilibrated with 20 mM sodium citrate buffer with 0.15 M NaCl (pH 8.0). In the third step of the synthesis, we attached EPPT peptides to CLIO. To synthesize the thioether-bonded conjugate CLIO-EPPT, from benign breast fibrocystic disease and a primary embryonic kidney 293-negative U87 glioblastoma line and normal lines (MCF10-A cells derived (colon); and ChaGo-K-1 (lung). Human control cell lines included a uMUC-75-1 (breast); BT-20 (breast); HT-29 (colon); CAPAN-2 (pancreas); LS174T (pancreas); ChaGo-K-1 (lung); HT-29) and a variety of human uMUC-1-positive tumor cell lines of different organs: ZR-75-1 (breast); BT-20 (breast); HT-29 (colon); CAPAN-2 (pancreas); LS174T (colon); and ChaGo-K-1 (lung). Human control cell lines included a uMUC-1-negative tumor or normal cells.

Specificity of the CLIO-EPPT Probe

Quantitative Cell Binding Assay. To image tumors in vivo, tumor cells have to be efficiently labeled with a superparamagnetic compound. To calculate the amount of iron associated with a fixed number of cells, we used a variety of human uMUC-1-positive tumor cell lines derived from different organs: ZR-75-1 (breast); BT-20 (breast); HT-29 (colon); CAPAN-2 (pancreas); LS174T (colon); and ChaGo-K-1 (lung). Human control cell lines included a uMUC-1-negative tumor or normal cells. For cell binding analysis and biodistribution studies, the probe Cy5.5-CLIO-EPPT-FITC, designated CLIO-EPPT, was radioiodinated using the Iodogen method (Pierce, Rockford, IL). The synthesis and schematic representation of CLIO-EPPT are shown in Fig. 1B.

The number of peptides/CLIO particle was determined spectrophotometrically as the number of FITC groups attached to a single CLIO particle measured at 494 nm. The peptide to particle ratio was obtained from concentrations of peptide and iron assuming 2064 iron atoms/particle (47). Iron concentration was determined spectrophotometrically (38). For the Cy5.5 dye, the number of dyes/particle was obtained from absorption at 678 nm and an extinction coefficient of 250,000 M⁻¹cm⁻¹ (Amersham-Pharmacia, product information) and assuming 2064 iron atoms/particle. The R1 and R2 relaxivities (change in relaxation rate/mM) were measured at 37°C using a 0.47T Bruker NMR-120 Minispec nuclear magnetic resonance spectrometer operating at 20MHz. Nanoparticle size was determined by light scattering (submicron particle size analyzer Coulter N-4; Coulter, Hialeah, FL).

The absorption spectrum of the probe was obtained using a Hitachi 3500 spectrophotometer.

Table 1. Physical properties of the CLIO-EPPT probe

<table>
<thead>
<tr>
<th>Compound</th>
<th>Size (nm)</th>
<th>R1 (s⁻¹ mM⁻¹)</th>
<th>R2 (s⁻¹ mM⁻¹)</th>
<th>Peptides/particle</th>
<th>Cy5.5/particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLIO-NH₂</td>
<td>29.5</td>
<td>22.54</td>
<td>43.69</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>CLIO-EPPT</td>
<td>35.8</td>
<td>26.43</td>
<td>53.44</td>
<td>14</td>
<td>5</td>
</tr>
</tbody>
</table>

For biodistribution, female nulnu mice (Massachusetts General Hospital Radiation Oncology breeding facilities) were injected bilaterally with uMUC-1-negative (U87) and uMUC-1-positive tumors of different tissue origins (ChaGo-K-1, LS174T, HT-29, and U87) cell lines was visualized by fluorescence microscopy. Briefly, cells were grown overnight on coverslips, fixed in 4% paraformaldehyde, incubated with CLIO-EPPT, and washed. Cells were initially identified under a bright-field microscope and then subjected to correlative dual-channel fluorescence microscopy in the green (for FITC detection) and NIR (for Cy5.5 detection) channels, using an inverted fluorescence microscope (Zeiss Axiosvert 100TV, Zeiss, Wetzlar, Germany). Images were collected using a cooled charge-coupled device (Photometrics, Tucson, AZ) with appropriate excitation and emission filters (Omega Optical, Brattleboro, VT).

In Vivo MR Imaging

For MR imaging, mice were injected bilaterally with uMUC-1-negative (U87) and uMUC-1-positive (LS174T, ChaGo-K-1, HT-29, and CAPAN-2) tumors (n = 3 each tumor). After tumors reached ~0.5 cm in diameter, mice were injected i.v. with the 125I-labeled CLIO-EPPT probe (10 mg Fe/kg). Twenty four h later, animals were sacrificed. Tumors, fluids, and organs were removed and weighed. Organ-associated radioactivity was then counted in a gamma counter. Biodistribution results were expressed as the percentage of the injected dose/g of tissue (% of injected dose/g).

For image analysis, mice were injected with uMUC-1-positive (LS174T, ChaGo-K-1, HT-29, and CAPAN-2) tumors (n = 3 each tumor). After tumors reached ~0.5 cm in diameter, mice were injected i.v. with the 125I-labeled CLIO-EPPT probe (10 mg Fe/kg). MR imaging was performed before and 24 h after injections on animals anesthetized i.p. with a ketamine/xylazine mixture (80 mg/kg/12 mg/kg; Parke-Davis, Morris Plains, NJ/Miles Inc., Shawnee Mission, KS). MR imaging was performed using a 9.4T Bruker horizontal bore scanner (Billerica, MA) equipped with ParaVision 3.0 software. The imaging protocol consisted of coronal and transverse T2-weighted spin echo (SE) pulse sequences. To produce T2 maps, the following imaging parameters were used: SE TR/TE = 3000/8, 16, 24, 32, 40, 48, 56, 64; FoV = 40 × 40 mm; matrix size 128 × 128; slice thickness = 0.5 mm (total five slices); resolution 312 × 312 μm; and an imaging time of 12 min and 48 s.
In Vivo NIRF Imaging

NIRF optical imaging was performed immediately after the MR-imaging sessions. For NIRF imaging, animals were treated as specified above and placed into a whole-mouse imaging system, a modification of the commercially available chemiluminescence system (Kodak), equipped with a band-pass filter at 630 nm and a long-pass filter at 700 nm (Omega Optical, Brattleboro, VT) as described previously (41). Image reconstruction was performed using CMIR Image v0.1/H9252 software (Dr. Edward Graves, Stanford University, CA).

All experiments, including animals, were performed in compliance with institutional guidelines and according to protocol no. 2003N000056 approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital.

Fluorescence Microscopy of Tumors ex Vivo

Localization of CLIO-EPPT in tumors after the MR- and NIRF-imaging sessions was performed on 7-μm frozen tumor sections. Sections were fixed in acetone, washed, and analyzed by multichannel fluorescence microscopy in the green (for FITC detection) and NIR (for Cy5.5 detection) channels.

Statistical Analysis

All data were represented as means ± SD. Statistical analysis was performed using a two-tailed Student t test.

RESULTS

Physicochemical Properties and in Vitro Specificity of the Novel CLIO-EPPT Probe. CLIO-EPPT constitutes a triple labeled nanoparticle, consisting of FITC on the uMUC-1-specific EPPT peptide (for fluorescence microscopy), superparamagnetic iron oxide (a MRI label), and Cy5.5 attached to the CLIO (for NIRF imaging). Furthermore, an available Tyr within the EPPT peptide was iodinated for quantitative cell binding studies and biodistribution. The physical properties of the CLIO-EPPT probe are summarized in Table 1. The synthesized probe had approximately the same size and R1 and R2 relaxivities as the parental compound CLIO-NH2. The absorption spectrum of CLIO-EPPT shown on Fig. 1c demonstrates three peaks associated with the absorption of Cy5.5 dye at 675 nm, FITC at 494 nm, and iron oxide absorption in the UV part of the spectrum. These peaks coincided with the corresponding peaks of the mixture of free compounds at the same concentration; therefore, we concluded that the obtained nanoparticles consisted of Cy5.5-labeled CLIO, conjugated to FITC-labeled EPPT peptides.

To define the specificity of CLIO-EPPT for uMUC-1, we performed a quantitative cell binding assay in which we quantified the amount of iron associated with adenocarcinoma and control cell lines (Fig. 2). The U87 and 293 cell lines shown to express no MUC-1, and the MCF10-A normal breast cell line expressing normally glycosylated MUC-1 (see Supplementary Fig. 1)3 displayed a significantly lower uptake of CLIO-EPPT compared with the adenocarcinoma cell lines (P < 0.05).

To further characterize the in vitro specificity of CLIO-EPPT for adenocarcinomas, we performed flow cytometric analysis of probe binding to selected adenocarcinoma and control lines (Fig. 3A). We evaluated staining in both the FL1 (derived from the FITC label) and the FL4 (derived from the Cy5.5 label) channels. As expected, the U87 control line showed no binding. In contrast, the adenocarcinoma cell lines bound the probe and displayed diverse stain-

3 Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).
ing intensities, consistent with variable glycosylation. Surprisingly, the relative binding of CLIO-EPPT by the adenocarcinoma cell lines did not directly parallel differential epitope exposure as determined by fluorescence-activated cell sorting with a uMUC-1-specific monoclonal antibody (Supplementary Fig. 2 online), possibly as a result of differences in the precise core peptide epitopes targeted by the probe and the monoclonal antibody. Additional experiments determined differential availability of uMUC-1 epitopes by immunostaining with an antibody specific to the APDTR core peptide epitope on the uMUC-1 protein and showed positive staining for adenocarcinoma cell lines and negative staining for control uMUC-1-negative lines (Supplementary Fig. 3 online). Fluorescence microscopy experiments in which adenocarcinoma and control cells were incubated with the probe confirmed the fluorescence-activated cell sorting data (Fig. 3B). Except for the U87 glioblastoma line, all of the adenocarcinoma cell lines stained strongly in both channels and showed colocalization of the two signals. In contrast, the 293 primary embryonic kidney cell line, shown to express no MUC-1, and the normal breast MCF10-A cell line, displaying normally glycosylated MUC-1, produced no staining in either the FITC or the Cy5.5 channel (data not shown).

**In Vivo Imaging of a Mouse Model of Human Cancer Using the Multimodal CLIO-EPPT Probe.** *In vivo* MR imaging was performed on animals bearing bilateral uMUC-1-positive and uMUC-1-negative tumors before and 24 h after the injection of the probe. Representative images of T2 maps are shown in Fig. 4. Additional T2-weighted images are presented in Supplementary Fig. 4 online. After administration of the probe, no significant change in signal intensity was observed in uMUC-1-negative tumors, indicating that there was little or no accumulation of the probe. In contrast, a significant signal reduction was observed in some regions of uMUC-1-positive tumors (a 52% decrease for LS174T tumors (shown), a 53% decrease for CAPAN-2 and a 43% decrease for ChaGo-K-1 and HT-29 tumors versus a 13–18% decrease in control U87 tumors).

To support our hypothesis that the accumulation of the CLIO-EPPT probe was sufficient to produce a NIRF signal in uMUC-1-positive tumors, we subjected the same animals to optical imaging immediately after the MR-imaging session. A high-intensity NIRF signal was obtained from the uMUC-1-positive tumors (HT-29, LS174T, ChaGo-K-1, and CAPAN-2) after injection of the CLIO-EPPT probe, whereas no significant signal was observed from the control U87 tumors (Fig. 5A, LS174T versus U87). Fig. 5B shows images of HT-29- and U87-excised tumors and a piece of muscle tissue. As expected, neither the U87 tumor, nor muscle tissue produced any significant signal. However, in agreement with the *in vivo* images, uMUC-1-positive tumors produced a strong NIRF signal. These data showed that the signal obtained on NIRF images was because of the specific accumulation of the CLIO-EPPT probe in uMUC-1-positive tumors. Additional MR and NIRF images on yet another tumor line are presented in Supplementary Fig. 5 online (HT-29 versus U87). The data from the imaging studies correlated with the *in vivo* biodistribution data where on average, uMUC-1-positive tumors accumulated 3.4 times more CLIO-EPPT probe than uMUC-1-negative tumors (1.78 ± 0.2% injected dose/g; *P* < 0.0001). The rest of the organs showed a biodistribution pattern typical for parental cross-linked iron oxides (46).

Correlative dual channel fluorescence microscopy of excised tumors showed colocalization of signal in the FITC channel with fluorescence in the Cy5.5 channel, indicating accumulation of the probe in uMUC-1-positive tumors after i.v. injection (Fig. 5C). Sections from U87 tumors did not show any signal in either channel (data not shown). Microscopic data corroborated our conclusions from *in vivo* imaging that CLIO-EPPT specifically accumulated in uMUC-1-positive tumors and did not significantly accumulate in control tumors.
DISCUSSION

One of the great challenges of oncology is to improve methods for early tumor detection, which is crucial for the success of cancer therapy. We believe that the target chosen for this study is highly relevant because in 1999 alone (latest data available), cancers that express the uMUC-1 protein accounted for 72% of new cases and 66% of deaths (19). The combination of two imaging modalities instead of one provides the advantages of both. MR imaging can offer high spatial resolution and the capacity to simultaneously obtain physiological and anatomical information. Optical imaging, carried out at different resolutions and depth penetrations, allows for rapid screening, which is highly desirable in clinical use. Fluorescence-mediated tomography has recently been shown to three-dimensionally localize and quantify fluorescent probes in deep tissues with high sensitivity (48). Thus, the combination of NIRF- and MR-imaging modalities would provide comprehensive information on tumor localization, environment, and status.

In this study, we designed and tested a novel imaging probe that uses both modalities and specifically targets neoplastic tissue. The synthesized probe demonstrated specificity toward a variety of uMUC-1-positive human adenocarcinomas in vitro. Furthermore, in vivo MR and NIRF imaging experiments on tumor-bearing animals showed specific accumulation of the probe in uMUC-1-positive tumors and virtually no signal in control tumors. These results were corroborated by fluorescence microscopy of excised tumors. The uMUC-1-specific multimodal-imaging probe introduced here can significantly advance our current ability to detect primary tumors in various organs as well as tumor metastases because the targeted antigen is also ubiquitously expressed on metastasizing cancer cells (6). Although not tested in the present study, other uMUC-1-positive nonepithelial cancer cell lines [astrocytoma, melanoma, and neuroblastoma (15)], as well as hematological malignancies such as multiple myeloma and some B-cell non-Hodgkin's lymphomas can be potentially detected using the described probe. Therefore, the insight gained from this study would give us the ability to detect and follow the progression of cancer, including its advanced metastatic state. It is important to emphasize that the suggested approach can be applied to early detection of precancerous lesions because aberrant expression and underglycosylation of the uMUC-1 antigen has been found, for example, in patients with ductal hyperplasia and ductal carcinoma in situ that are at high risk to develop subsequent invasive breast carcinoma (52).

Overall, this multimodal imaging approach, in conjunction with the chosen target, would allow for (1) early cancer detection as well as staging and imaging of the recurrence of tumors and (2) monitoring of therapeutic efficacy. Thus, we believe that the results of this study could lead to the development of a versatile, clinically relevant imaging probe, particularly in view of the fact that related iron oxides have successfully been applied to clinical use (53).
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