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Optical and Magnetic Resonance Imaging Using Fluorous Colloidal Nanoparticles

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Abstract

Improved imaging of cancerous tissue has the potential to aid prognosis and improve patient outcome through longitudinal imaging of treatment response and disease progression. While nuclear imaging has made headway in cancer imaging, fluorinated tracers that enable magnetic resonance imaging (¹⁹F MRI) hold promise, particularly for repeated imaging sessions because nonionizing radiation is used. Fluorine MRI detects molecular signatures by imaging a fluorinated tracer and takes advantage of the spatial and anatomical resolution afforded by MRI. This manuscript describes a fluorous polymeric nanoparticle that is capable of ¹⁹F MR imaging and fluorescent tracking for in vitro and in vivo monitoring of immune cells and cancerous tissue. The fluorous particle is derived from low-molecular-weight amphiphilic copolymers that self-assemble into micelles with a hydrodynamic diameter of 260 nm. The polymer is MR-active at concentrations as low as 2.1 mM in phantom imaging studies. The fluorinated particle

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Notes

The authors declare no competing financial interest.

Supporting Information

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demonstrated rapid uptake into immune cells for potential cell-tracking or delineation of the tumor microenvironment and showed negligible toxicity. Systemic administration indicates significant uptake into two tumor types, triple-negative breast cancer and ovarian cancer, with little accumulation in off-target tissue. These results indicate a robust platform imaging agent capable of immune cell tracking and systemic disease monitoring with exceptional uptake of the nanoparticle in multiple cancer models.

Graphical abstract

INTRODUCTION

Nanomedicine has the potential to improve the delivery of therapeutics and imaging agents to diseased sites, while reducing side effects, resulting in safer and more efficacious treatments and tissue-specific imaging. In particular, molecular imaging of cancer holds great promise in revolutionizing the way physicians treat patients by enabling prognosis and disease monitoring over time. Two of the most popular cancer imaging modalities are positron emission tomography/computed tomography (PET/CT) imaging and magnetic resonance imaging (MRI), both providing clinicians with spatial representation of cancerous tissue.^{1,2} During PET imaging a radioactively labeled tracer is administered, which is selectively internalized into rapidly dividing cells and emits γ radiation that is captured as an image.³ The PET scan is then coregistered with a CT image to determine the location of cancerous tissue.⁴ PET, however, is limited by radioisotope half-life and tracer availability and is hindered by poor spatial and 3D resolution. Furthermore, the use of radioactive tracers for long-term imaging of disease progression raises concerns about overall radiation burden and long-term toxicity.⁵ By contrast, MRI holds great promise for anatomically precise imaging of tumor tissues.⁶ MRI provides high spatial resolution, deep tissue penetration, and 3D resolution.⁷ Additionally, MRI has the advantage that it does not subject patients to ionizing radiation and thus may be a better alternative for long-term disease monitoring. Traditional MRI relies on differential relaxation profiles of protons that provide contrast between pathological and physiological features.⁸ Contrast in MRI can be improved by using gadolinium chelates or iron oxide nanoparticles to further differentiate local environments.^{9–12} The heavy metal toxicity of gadolinium chelates can be problematic for patients with compromised kidney function, especially when tracking a disease over a prolonged time-course.¹³ Furthermore, gadolinium chelates show long-term deposition in brain tissue.^{14,15} While the biological effects of this are under investigation, alternative contrast agents are needed should neural toxicity become correlated to gadolinium use.

Fluorine (¹⁹F) MRI is a promising alternative imaging technique that eliminates repeated exposure to ionizing radiation and to heavy metal contrast agents while providing 3D

imaging resolution. The natural isotope of fluorine is MR-active, nearly as sensitive as hydrogen, and fluorinated compounds are generally considered to be nontoxic.¹⁶ Fluorinated MR agents are tracer technologies because the fluorine contained in the body is not visible on MRI scans.¹⁷ To use this method clinically, the fluorine image is acquired and coregistered with a ¹H image, allowing for high-contrast imaging of the tracer.^{18,19} Perfluorocarbon emulsions are commonly used as fluorinated tracers for MRI technology.²⁰ These emulsions were first investigated as blood substitutes and contrast agents via systemic targeting of diseased tissue, but long clearance times coupled to accumulation in the liver and spleen raised safety concerns, which limited clinical translation for these compounds.^{20–22} To mitigate safety concerns associated with systemic distribution of perfluorocarbon emulsions, these tracers are administered to immune cells either in situ enabling real-time cell tracking using ¹⁹F MRI.^{23–25} In addition to real-time imaging, quantitative fluorine concentration can be determined from the MR image and correlated to cell count, allowing for so-called “in vivo cytometry”.²⁶ A recent clinical trial demonstrated in vivo dendritic cell tracking of a perfluoropolyether emulsion with ¹⁹F MRI in patients with metastatic colorectal cancer.²⁷ While emulsion technology holds promise for immunotracking, the technique is limited to cellular labeling as accumulation of perfluorocarbons raises safety concerns. To extend fluorinated tracer technology toward longitudinal monitoring of diseased states, limitations in distribution must be overcome.

Nanoparticle contrast agents have improved imaging of cancers by enabling both passive and active targeting of cancerous tissue.²⁸ Successful nanoparticles for imaging have come from a host of materials including metals,^{29–32} ceramics,^{33,34} biological particles,^{35–41} and self-assembled polymers.^{42–45} When using polymers, most often controlled radical polymerization (CRP) methods are used to synthesize nanoparticles for biomedical applications as CRP gives rise to polymers with precisely defined molecular weights.⁴⁶ CRP techniques are tolerant to diverse functional groups and allow for synthetic ease in creating block copolymers.⁴⁷ Fluorinated copolymers synthesized via CRP have been extensively studied by Whittaker et al. as tracers for ¹⁹F MRI.^{48–53} Amphiphilic hyperbranched copolymers decorated with folate targeting moieties demonstrated the ability to image melanomas using ¹⁹F MRI both in vitro and in vivo. The targeted particle showed increased accumulation in the tumor; however, off-target accumulation was prevalent, as is often seen for nanomaterials.⁵² The size and surface chemistry of these nanoparticles can dictate either their rapid renal clearance or clearance by the RES system, leading to accumulation in the liver and spleen.⁵⁴ To effectively image tumors, the particle must localize in diseased tissue and avoid long-term accumulation in off-target tissue.

We describe a multimodal imaging agent derived from amphiphilic fluororous copolymers that self-assemble into micelles. Specifically, a low-molecular-weight copolymer was synthesized to have equimolar ratios of trifluoroethyl methacrylate (TFEMA) and oligo(ethylene glycol) methyl ether methacrylate (OEGMEMA). This composition maximized fluorine content to enhance the signal for MR imaging while promoting colloidal assembly. The biocompatibility and imaging capability were evaluated using a combination of phantom imaging and tissue culture as well as mouse models of breast and ovarian cancer. Ultimately, this manuscript details a low-molecular-weight polymer colloidal nanoparticle that could find use in either cell tracking or direct in vivo imaging of tumor tissue.

METHODS

Synthesis of OEGMEMA/TFEMA Copolymer (1)

Azide initiator (A, Scheme 1) (0.104 g, 0.32 mmol) and OEGMEMA (1.212 g, 2.6 mmol) were dissolved in 500 μL of methanol in a 10 mL Schlenk flask equipped with a magnetic stir bar and degassed by N_2 bubbling for a minimum of 30 min. In a separate 10 mL Schlenk flask, Cu(II)Br_2 (0.075 g, 0.32 mmol) and bipyridine (0.99 g, 0.64 mmol) were dissolved in 1 mL of 1:1 DMF/MeOH solution and degassed for a minimum of 30 min by N_2 bubbling. TFEMA was deoxygenated in bulk (450 μL , 3.2 mmol) and was added via air-free syringe techniques to the deoxygenated initiator and OEGMEMA solution. The mixture was degassed for approximately 5 additional minutes. TFEMA is volatile and can evaporate during the deoxygenation process. Cu(I)Br (46 mg, 0.32 mmol) was added to the catalyst system as a solid under positive N_2 pressure and rapidly stirred to promote dissolution. The catalyst system was transferred to the monomer and initiator via syringe. The polymerization proceeded under positive nitrogen pressure for 36 h at room temperature and was then quenched by exposure to air. The copolymer was passed through a neutral alumina column using THF as a mobile phase to remove copper. Solvent was removed under reduced pressure, and the crude polymer was dissolved in a minimal amount of THF and precipitated in cold diethyl ether. Then, the mixture was transferred to a 50 mL falcon tube and centrifuged at 7500 rpm, 10 min, 4 $^{\circ}\text{C}$, and the diethyl ether layer was decanted. Precipitation and centrifugation were repeated three times to yield pure polymer product. Yield: 92.5%

Dynamic Light Scattering—Dynamic light scattering (DLS) measurements were conducted on **1** to determine particle size in ultrapure water. **1** was dissolved in ultrapure water to a concentration of 10 mg mL^{-1} and vortexed for 30 s to facilitate micelle formation. Serial dilutions were conducted to determine a critical micelle concentration (CMC) between 15 and 30 μM .

Transmission Electron Microscopy—Transmission electron microscopy (TEM) was performed on **1**. In brief, **1** was dissolved in ultrapure water to a concentration of 10 mg mL^{-1} and vortexed for 30 s to facilitate micelle formation. The solution was placed onto carbon-coated copper grids and negatively stained with 1% (w/v) phosphotungstic acid for 5 min prior to imaging. Samples were analyzed with a Zeiss Libra 200Fe transmission electron microscope operated at 200 kV.

Synthesis of Cy5-Labeled Polymer (2)

Copolymer **1** (0.025 g, 4.5 μmol) was dissolved in deionized water to a concentration of 10 mg mL^{-1} in a round-bottomed flask equipped with a magnetic stir bar; Cy5-alkyne (480 μL , 10 mg mL^{-1} in DMSO, 6.8 μmol) was added to the copolymer solution. The following aqueous solutions were added to the reaction mixture: A premixed solution of CuSO_4 (15 μL : 50 mM) and THPTA (5 μL : 50 mM) was followed by freshly made sodium ascorbate (20 μL , 100 mM). The reaction proceeded for 1 h, stirring at room temperature. The resulting product was purified via centrifugal filtration using a 3500 molecular weight cutoff

spin filter at 7500 rpm, 15 min until the flow-through no longer showed any Cy5 absorbance via UV–visible spectroscopy. Yield: 86.2%

¹⁹F Magnetic Resonance Imaging Spectroscopy

¹⁹F MRI spectroscopy was used to evaluate copolymer properties. The copolymer was dissolved in deionized water at concentrations from 1 to 10 mM into dram vials and placed into the 9.4T Preclinical Bruker BioSpin. The particles were evaluated using 90° nonselective radiofrequency excitation pulses with 65 μ s duration and 45 kHz acquisition bandwidth. 256 signal averages were acquired using a 400 ms repetition time. ¹⁹F spin–lattice relaxation time (T_1) was determined using standard inversion recovery sequences with nine inversion times ranging from 1 to 2560 ms for copolymer **1** at 10 mM at 25 °C. ¹⁹F spin–spin relaxation time (T_2) was measured using single echo spin echo, with a total of 12 echoes, and echo times were from 600 to 6400 μ s for copolymer **1** at 10 mM at 25 °C. Data acquired from T_1 and T_2 experiments were fit with a nonlinear least-squares curve to estimate spin–lattice and spin–spin relaxation time.

¹⁹F Magnetic Resonance Imaging Phantoms

Copolymer **1** was dissolved in deionized water and placed in dram vials with concentrations ranging from 2 to 9 mM. All polymer samples were arranged in a falcon tube, and the falcon tube was filled with water to minimize free volume. MRI images were acquired using Ultrashort TE (UTE) imaging, with imaging parameters of 8 × 8 cm FOV, 1.25 mm in plane resolution, and 1 cm slice thickness. Spatial encoding of the sample was performed using a 100-spoke radial readout trajectory with 125 μ s echo time and 60 ms repetition time. 80 averages were acquired, totaling 8 min of acquisition time.

Cell Culture—Macrophage cells (RAW 264.7 cells (ATCC)) and triple-negative breast cancer (TNBC) cells (MDA-MB-231 (a generous gift from Dr. Schiemann, Case Western Reserve University)) were maintained in Dubelco's minimal essential medium (DMEM) at 37 °C in a 5% CO₂ humidified air environment. The medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin. Hyperaggressive ovarian cancer cells (ID8-Defb29/Vegf (a generous gift from Dr. Steven Fiering, Geisel School of Medicine, Dartmouth College)) were maintained in RPMI 1640 media supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 1 × 100 U penicillin/streptomycin. Expression of luciferase was achieved by vesicular stomatitis virus-glycoprotein (VSVG) retroviral transduction of pBabe and selection with 5 μ g/mL puromycin (SCBT). In brief, 10 μ g of plasmids VSVG and pBabe-puro Luc was added to GP2-293 packaging cell line (maintained in complete DMEM) following a 30 min incubation with 60 μ L of TransIT (Mirus). (GP2-293 cells and plasmids were a generous gift from Dr. Schiemann, Case Western Reserve University.) 48 h following the addition of plasmid, media was collected, filtered to remove cellular debris, and added to ID8-Defb29/Vegf cells in the presence of Polybrene (8 μ g/mL, SCBT) in a ratio of 50:50 with modified RPMI 1640 media typically used with this cell line. ID8-Defb29/Vegf cells were treated with the retroviral particles for 24 h, allowed to recover in fresh media for 24 h, and selected for transduction with puromycin for 5 days. Expression of luciferase was confirmed by IVIS Spectrum BLI

(PerkinElmer) imaging of 5000 to 100 000 cells per well in a 96-well plate in the presence of 15 μg luciferin/well.

Flow Cytometry—RAW 264.7 cell monolayers were washed three times with PBS and detached using enzyme-free Hanks' -based cell dissociation buffer (Fisher), pelleted, and resuspended in fresh media. Cells were then seeded in triplicate in 96-well v-bottom plates (2.5×10^5 cells/well, 200 μL /well) and incubated with 2 or 20 μm of Cy5 polymer nanoparticle **2** for 30 min and 3 h at 37 °C in humidified air (5% CO_2). Cells were then washed twice with FACS buffer (1 mM EDTA, 25 mM HEPES, 1% (v/v) FBS in PBS, pH 7.0) and fixed with 2% (v/v) paraformaldehyde for 10 min at room temperature. Cells were washed twice, resuspended in 200 μL of FACS buffer, and stored at 4 °C until analysis. Cells were analyzed using a BD LSR II flow cytometer with a 633 nm laser, and a minimum of 10 000 events per sample were collected. Data were analyzed using FloJo 8.6.3 software.

Cytotoxicity Assays—RAW 264.7 cells were seeded (5×10^3 cells in 200 μL of complete DMEM) in a sterile, tissue-culture-treated, 96-well clear bottom plate overnight at 37 °C in humidified air (5% CO_2). After 24 h, the cells were washed and media was replaced, and **1** dissolved in complete growth media was added to the cells at concentrations ranging from 0.1 to 10 mg mL^{-1} ($n = 6$). After 24 h, the wells were washed twice with PBS and replaced with fresh media. MTT solution was added (100 μL /well, 85:15 (v/v) complete DMEM/MTT solution, 5 mg mL^{-1} in PBS) and incubated in humidified air (5% CO_2) at 37 °C for 4 h. Media was aspirated and DMSO was added (100 μL /well) to dissolve formazan crystals. The plate was placed on an orbital shaker for 30 min in the dark at 100 rpm. The dissolved formazan was analyzed at 570 nm using a Biotek Synergy HT microplate reader. Cell viability was determined by normalizing treated cells as a percentage relative to the untreated cells. Values are presented as averages $\pm$ standard deviation. Statistical analysis was carried out using GraphPad Prism software.

Confocal Microscopy—Localization of the particle in the cell was evaluated by confocal microscopy. RAW 264.7 cells were seeded (1.25×10^5 in 1.5 mL of complete DMEM) on coverslips in an untreated six-well plate for 24 h at 37 °C in humidified air (5% CO_2). Cells were rinsed with PBS before the addition of **2** to a final concentration of 1 nM ($\sim 10^6$ polymers per cell) and incubated for 3 h. Cells were washed three times with PBS, then replaced with fresh media and incubated overnight at 37 °C in humidified air (5% CO_2) to allow particle uptake. Cells were washed with PBS then fixed using DPBS with 4% (v/v) paraformaldehyde and 0.3% (v/v) glutaraldehyde for 5 min at room temperature. After washing three times with DPBS, cell membranes were stained using 1 μL of wheat germ agglutinin (Invitrogen) in DPBS with 5% (v/v) goat serum for 45 min. Cells were washed three times with DPBS, and coverslips were mounted using Fluoroshield with DAPI and sealed using clear nail polish. Images were acquired on a Leica TCS SPE Confocal microscope equipped with 40 $\times$ and 63 $\times$ oil immersion objectives.

TNBC Targeting—All animal studies were carried out using IACUC-approved protocols. Female athymic NCR nu/nu mice were injected with 2 million MDA-MB-231 cells suspended in 100 μL of 50% media, 50% matrigel subcutaneously in the right flank. Mice

were maintained on an alfalfa free diet (Teklad) to reduce autofluorescence. Once established, tumors were monitored daily and biodistribution was performed when tumors were $\sim 800 \text{ mm}^3$. The Cy5 polymer nanoparticle **2** was administered intravenously ($100 \mu\text{L}$ at 10 mg mL^{-1} in sterile PBS; this corresponds to a 5 g/kg polymer/body weight dose). Phosphate-buffered saline was administered for control animals. 24 h following injections, mice were sacrificed and imaged using the Maestro fluorescence imager. Exposure time for Cy5 was 30 s. Following whole animal imaging, organs and tumor tissue were excised, imaged, and analyzed separately via Maestro.

OVCA Targeting—Animal studies were carried out using IACUC-approved protocols. Female C57BL6 mice were injected intraperitoneally with luciferin-positive ID8-Defb29/Vegf-A. Tumors were monitored weekly using IVIS imager, and tumors were allowed to grow for 4 weeks. The Cy5 polymer nanoparticle **2** was administered in the intraperitoneal (IP) space (a $100 \mu\text{L}$ injection at a concentration of $10 \text{ mg particle/mL}$ in sterile PBS corresponding to a 5 g/kg particle/body weight dose), and phosphate-buffered saline was administered as a control for the experiment, $n = 3$. Following 24 h of incubation the mice were injected with luciferin (15 mg mL^{-1} , $150 \mu\text{L}$ intraperitoneally), then humanely euthanized and visualized via IVIS Spectrum from PerkinElmer for luminescence and fluorescence imaging. Exposure time for luciferin imaging was 60 s and that for Cy5 imaging was 30 s. Following whole animal imaging, organs and tumor tissue were excised and imaged separately to better determine organ distribution.

RESULTS AND DISCUSSION

Polymer Synthesis and Characterization

Fluorous copolymers were designed to meet several goals for applications in fluorine MR and optical imaging as well as tumor targeting. First, a high fluorine content was incorporated that also generated a single NMR signal. We postulated that this would drive nanoscale colloidal assembly through phase separation while enhancing MR sensitivity. The colloidal particles were anticipated to be within the size regime to harness the enhanced permeability and retention (EPR) effect and passively accumulate in cancerous tissue. Furthermore, individual polymers were targeted to be of low molecular weight. The small size of individual polymers would allow for renal clearance once diluted below the CMC in vivo. Finally, telechelic polymers were implemented that could undergo postpolymerization reactions to introduce alternative imaging modalities.

A random copolymer of TFEMA and OEGMEMA was synthesized via atom transfer radical polymerization (ATRP) using an azide-terminated initiator (Scheme 1). The azido end group was installed to facilitate the copper-catalyzed azide–alkyne cycloaddition (CuAAC) for postpolymerization modification. Polymerizations were carried out with equimolar ratios of TFEMA to OEGMEMA in the reaction mixture with a target molecular weight of 5 kDa. Following polymerization, monomer conversion was monitored via ^1H NMR of the crude polymer product and indicated near-complete conversion of the monomers, with 94 and 97% conversion of OEGMEMA and TFEMA, respectively. Monomer composition in the feedstock closely correlated to monomer distribution in the polymer, as confirmed by ^1H

NMR. Methylene protons adjacent to the ester on the OEGMEMA appear at 4.06 ppm, whereas the protons adjacent to those on the CF₃ group of TFEMA appear at 4.36 ppm in the ¹H NMR (Figure 1A) and indicate a 1:1 composition of the copolymer. Literature data indicate that this is likely a statistically random copolymer and the composition of the polymer is expected to show no sequence bias toward gradient or block copolymers.^{55,56} The 1:1 ratio of monomers was the highest fluorine content that allowed for the polymer to be dispersible in aqueous systems; higher molar fractions of TFEMA resulted in phase separation. The chemical shift for the polymer by ¹⁹F NMR appears as a single resonance at -76.5 ppm, with noticeable peak broadening in the polymer spectra when compared with the TFEMA monomer (Figure 1B). In water, the copolymer self-assembles into micelles where fluorinated components sequester into a hydrophobic core and OEGMEMA units interface with water to minimize overall surface energy. This self-assembly is evident in the ¹⁹F NMR of the copolymer dissolved in D₂O, confirmed by a significant peak broadening when compared with CDCl₃, a good solvent for the polymer (Figure S1).⁵⁷ Polymer molecular weight was characterized by both gel permeation chromatography (GPC) and NMR. GPC indicated a number-average molecular weight (M_n) of 10.5 kDa and a dispersity index (M_w/M_n) of 1.14 (Figure S2) when calibrated against polystyrene (PS) standards. This larger than expected mass from GPC is attributed to comparing versus a molecular weight standard curve generated from polystyrene standards or potentially to self-assembly. This comparison can overestimate the molecular mass of unknowns due to differences in architecture and structure between the standard and the sample of interest.⁵⁸ Because GPC was unreliable, molecular weight was further confirmed by end-group analysis. Number-average molecular mass (M_n) was determined by integrating the resonance peak from the geminal dimethyl group derived from the initiator versus TFEMA and OEGMEMA in the backbone. This corresponded to a M_n of ~5500 g/mol or approximately nine repeat units of each monomer. The polymer end group was further modified with ethynylpyrene using optimized ligand-accelerated CuAAC conditions in organic solvent to confirm molecular weight and end-group modification yield. This reaction proceeds with high efficiency and allowed for comparison between the pyrene protons and protons from each respective monomer unit in the polymer by NMR. The M_n of the polymer was confirmed to be ~5.5 kDa by NMR and indicated near quantitative conversion of the azide end group using ligand-accelerated CuAAC conditions (Figure S3).

Micelle formation and other self-assembly processes are common for fluorinated polymers and have been previously observed.^{59,56,55} The self-assembly of the polymer in water was probed using DLS. The copolymer was dispersed in ultrapure water and vigorously vortexed to form a stable colloidal dispersion. The hydrodynamic diameter of the particles was 260 nm, and colloidal assembly was confirmed from concentrations ranging from 15 μ M to 10 mM (Figure S4). At concentrations below 15 μ M, no reliable signal from the DLS was seen, which likely indicates the CMC. Particles remained stable for up to 4 months and likely longer because no changes in size distribution occurred over the period studied. TEM was used to further verify self-assembly (Figure 2B). TEM micrographs indicate a smaller particle diameter than that given by DLS, as would be expected upon drying of the amphiphilic particles. The particles in solution are fully solvated, and thus DLS is a more

appropriate estimate than TEM micrographs. While not precise in determining dimensions, TEM confirms colloidal assembly of particles.

Phantom MRI Studies

Polymer nanoparticle **1** was evaluated for its ability to serve as a fluorine MR contrast agent. Experiments were conducted to benchmark lower limits of detection and to confirm that signal intensity correlates linearly with concentration. The linear relationship between fluorine concentration and signal intensity has previously been exploited in fluorinated tracer technology to determine apparent fluorine concentration in cells and at sites of accumulation.²⁷ **1** was dispersed in water at concentrations ranging from 1 to 8 mM (5.5 to 44 mg mL⁻¹) and placed in the isocenter of a 9.4T Bruker (Bruker Biospin, Billerica, MA) preclinical MR scanner. ¹⁹F nonlocalized FIDs were acquired using 2048 points at 45 kHz bandwidth, with 90° excitation, 400 ms TR, and 256 signal averages. ¹⁹F spectra (Figure 3A) were obtained after 100 Hz line broadening and Fourier transform. Spectra of **1** showed singlet lineshapes with fluorine concentration-dependent MRS signal intensity. This singlet line shape improves MRI properties when compared with fluorocarbon emulsions, as it will maximize signal-to-noise (SNR) from the fluorine nuclei and mitigate artifacts. Artifacts can lead to “ghost” signals in the resulting image, giving rise to ambiguity in tracer location.^{16,60} Enhanced SNR at low concentrations predicts clinical utility for potential imaging agents because low concentrations can be unambiguously imaged. The peak to background SNR was measured to be 25–410 depending on concentration (Figure 3B). At the lowest concentration (1 mM), the SNR for **1** is 40, an order of magnitude improvement over the current state of the art in polymer-fluorinated nanoparticle tracers. (SNRs were reported between 2 and 6 at 10 mM.)⁶¹ This signal intensity indicates that colloidal nanoparticle **1** shows comparable imaging feasibility when compared with other fluorinated polymeric systems.

¹⁹F Spin–lattice (T_1) and spin–spin (T_2) relaxation times were measured for the copolymer **1** in ultrapure water at 25 °C (Figure S6). T_1 was determined to be 380 ms, which is relatively short and consistent with other fluorinated polymeric particles that report T_1 times between 300 and 600 ms.⁵⁹ Short spin–lattice relaxation times (T_1) are promising in imaging, as they provide quick recovery and give rise to shorter data acquisition times.^{18,62} Spin–spin (T_2) relaxation time was measured as 2.0 ms, which is also relatively short. This short T_2 relaxation time is likely the result of confinement of fluorinated pendants in the micelle core, which can lead to a reduction in signal compared with fully solvated fluorinated pendants.^{48,51} The T_1 and T_2 relaxation times of copolymer **1** are comparable to polymeric fluorine agents.

Because of the promising SNR found in nonlocalized ¹⁹F MR experiments, ¹⁹F imaging was performed to assess imaging feasibility. Copolymer-filled vials of varying concentration were arranged at the isocenter of MR scanner. Proton imaging was used to perform field corrections and assess geometry (Figure S7). Resulting ¹⁹F ultrashort echo time (UTE) images showed minimal imaging artifacts, with a strong signal intensity at 8.8 mM concentration and weaker but visible signals at 3.0 and 2.1 mM.

Cell Viability and Uptake in Macrophages

Macrophages are found at sites of inflammation and measurement of macrophage burden can enable prognosis; for example, in the setting of atherosclerosis, high macrophage burden may be an indicator of a vulnerable plaque. Furthermore, macrophages can be targets for therapeutic intervention; for instance, the reprogramming macrophages from M2 to M1 phenotype in cancer may restore the cancer immunity cycle.^{63,64} Besides in vivo targeting applications, current fluorine MRI largely focuses on imaging immune cells by ex vivo cell labeling, followed by reinjection of the cells back into the host.⁶⁵ To demonstrate potential utility for cell-tracking applications, cellular uptake and biocompatibility of the fluororous colloidal contrast agents were evaluated in macrophages. Cytotoxicity of the polymers with RAW 264.7 murine macrophages was evaluated using an MTT assay at concentrations up to 10 mg mL⁻¹ following 24 h of incubation with the polymer. Cells remained completely viable at the highest concentrations, demonstrating biocompatibility (Figure 4A).

For efficient cell tracking, the fluorinated agent must accumulate with high efficiency in cells prior to injection back into the patient. Cellular uptake was investigated, again with murine macrophages, by both confocal microscopy and flow cytometry. To enable visualization, an alkyne-functionalized near-infrared fluorescent dye (Cy5) was covalently coupled to the azido terminus of the polymer using the ligand-accelerated CuAAC reaction (Scheme 2). Following purification, UV–visible spectroscopy confirmed attachment of the alkyne dye to the azide termini of the copolymer (Figure S8). DLS measured a hydrodynamic diameter of 180 nm for Cy5 polymer **2** dispersed in phosphate-buffered saline, a similar size regime to that of the unlabeled polymer (Figure S9). Cy5 allowed the polymer to be tracked by microscopy, flow cytometry, and in vivo optical imaging (as described in the Tumor Targeting section).

Confocal microscopy was used to visualize cell uptake of the dye labeled polymer nanoparticle. After a 3 h incubation with Cy5 polymer nanoparticle **2**, cells were fixed and stained with DAPI for the nuclei (blue), wheat germ agglutinin for the membrane (green), and the Cy5 polymer was directly visualized (red). Data indicate intracellular localization of the nanoparticles (Figure 4B). Flow cytometry was used to further probe macrophage uptake of **2**. Cells were incubated with **2** for either 30 min or 3 h at concentrations of 2 and 20 μ M. Internalization was time-dependent with increasing accumulation correlating to increased incubation time and concentration. Minimal internalization was observed at 30 min (Figure S10); however, after 3 h all cells showed significant uptake and fluorescence intensity was concentration-dependent (Figure 4C). The fluorescence signal from polymer uptake at 20 μ M was nearly an order of magnitude greater than that at 2 μ M (2800 ± 250 compared with $25\,000 \pm 4500$ (Figure 4D).

Tumor Targeting

The colloidal particles were of the appropriate size regime to harness the EPR effect, also known as passive tumor targeting. The EPR effect is a result of the unique tumor microenvironment derived from uncontrolled outgrowth, often leading to gaps in tumor vasculature on the order of hundreds of nanometers.⁶⁶ To take advantage of the EPR effect, nanoparticles need to be properly sized so as to permeate into tumor tissue from circulation

but avoid renal (>10 nm) and hepatic clearance (<500 nm).⁶⁷ The in vivo properties were assessed using two established mouse models, a TNBC xenograft model and an orthotopic syngeneic ovarian cancer (OVCA) model.⁶⁸ Both TNBC and OVCA can be highly recurrent, and drug resistance is often induced, making it imperative that these models can be monitored over long time courses.⁶⁹

Triple-Negative Breast Cancer Model—For biodistribution and tumor homing studies, the Cy5 polymer nanoparticles, **2**, were administered intravenously into female athymic mice bearing MDA-MB-231 xenografts. Targeting of therapeutics and contrast agents through molecular signatures has proven challenging for TNBC; the disease is highly heterogeneous, and these tumors do not express common biomarkers, such as HER2.⁷⁰ However, some TNBC tumors are highly vascularized, allowing passive targeting mediated through the pathophysiological features of the EPR effect.⁷¹ Cy5 polymer nanoparticle **2** was administered via tail vein injection when tumors reached 800 mm³; 24 h post-treatment, mice were sacrificed and imaged using fluorescence imaging. Fluorescent images of the excised organs show well-dispersed fluorescence throughout the tumor and minimal fluorescent signal in the liver, lungs, kidneys, and spleen (Figure 5). Imaging analysis by region of interest (ROI) revealed a 4-fold increase in fluorescence intensity in the tumor compared with the liver and kidneys (Figure S12).

Orthotopic Syngeneic Ovarian Cancer Model—Ovarian cancers are plagued by late-stage diagnosis, while inefficient chemotherapy treatments lead to platinum-resistant strains and cancer recurrence following treatment.⁶⁹ Robust, longitudinal imaging of ovarian cancer could provide a significant improvement in patient morbidity and mortality. To evaluate nanoparticle administration in ovarian cancer, an OVCA model was used. C57BL6 mice were injected intraperitoneally with luciferase-positive ID8-Defb29/Vegf-A cells, a model for aggressive metastatic disease.⁷² The luciferase-positive cells allowed for tumor tracking through luminescence imaging (Figure 6A). Mice bearing ovarian cancer were administered polymer nanoparticle **2**, injected into the IP space. After 24 h, organs were excised and visualized using an IVIS in vivo imaging system for both luminescence and fluorescence for polymer accumulation. Consistent with data obtained from the TNBC model, imaging indicates that the polymer nanoparticle accumulates primarily in tumor tissue, with negligible accumulation in the liver and spleen. A small amount of the polymer was deposited in the lungs. Future studies are required to dissect the deposition in the lungs and its cause; it is possible that following IP administration larger copolymer aggregates are formed that are subsequently filtered by the lung.⁷³ Evaluation of fluorescence via radiant efficiency of the Cy5 signal in the organs allows for quantitative analysis of particle fate. A predominate amount of the fluorescent signal is observed in the ovarian tumors over all other tissues (~3-fold higher).

Significant uptake in tumors is observed for both the TNBC and OVCA models with little accumulation in off-target tissue. The EPR effect is likely responsible for the tumor homing as both cancers have been reported as extensively vascularized.^{66,74} Importantly, polymer accumulation in off-target tissues is minimal. We speculate that once in circulation, polymer micelles are diluted below the CMC and can be cleared renally once dissociated into

unimers. Taking these results together, the fluororous copolymer provides a robust platform for imaging multiple cancer models with predominant accumulation in tumors through passive mechanisms. The increased partitioning toward the tumor versus nontarget organs using **2** is interesting and may allow for theranostic intervention. We envision the use of fluororous nanoparticles first to screen whether a patient presents a tumor enabled EPR targeting; this could be monitored through MRI. If the patient tests positive, then a drug-loaded formulation of the same chemistry could be used to treat the disease. The integrated imaging approach would enable the measurement of treatment success and disease regression.

CONCLUSIONS

The utility of a water-dispersible fluorinated colloidal platform was described that exhibits exceptional partitioning into tumor tissue with the potential for multimodal imaging. Macrophages showed uptake of the particles when exposed in vitro and cell toxicity was not observed, attesting to the biocompatible design of the formulation. The composition of the polymer allowed for both ^{19}F MR imaging as well as optical fluorescence imaging of cells and multiple tumor models. Administration of the particle into both TNBC and OVCA mouse models showed the versatility of fluorinated assemblies to passively target tumor tissue through both intravenous and IP delivery routes. In summary, this work describes the use of a simple copolymer formulation that shows significant uptake into tumors with little off-target accumulation. Future work will focus on enhancing ^{19}F MRI properties for longitudinal monitoring of disease states and theranostic design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fluorous copolymer characterization. (A) Labeled ^1H NMR spectrum of (1) copolymer in CDCl_3 at 600 MHz. (B) ^{19}F NMR spectra of the copolymer **1** in CDCl_3 at 600 MHz.

Figure 2. Nanoparticle characterization. (A) Dynamic light scattering (DLS) of copolymer **1** in water (10 mg mL^{-1} , 1 mM). (B) Transmission electron micrograph (TEM) of **1** in water stained with 1% (v/v) phosphotungstic acid. Scale bar is 100 nm.

Figure 3.

Magnetic resonance characterization. (A) Magnetic resonance spectra (MRS) of copolymer **1** at concentrations from 1 to 8 mM. (B) Plot of signal peak area versus concentration. Line fit is linear, $R^2 = 0.99$. (C) Phantom ^{19}F imaging experiments of copolymer **1**.

Figure 4.

Cellular viability and uptake assays. (A) MTT assay of copolymer **1** with RAW 264.7 cells. Viability is expressed as a percentage of untreated cells. (B) Confocal microscopy images of RAW 264.7 cells after 24 h of incubation with Cy5 polymer nanoparticle **2**. Nuclei stained using DAPI (blue), membrane stained using wheat germ agglutinin (green), and the Cy5 polymer is imaged in red. Scale bar = 10 μ m. (C) Representative histograms displaying flow cytometry data at 3 h of incubation time with particle at 2 and 20 μ M. (D) Bar graph of fluorescence intensity; 2-way ANOVA statistics were performed on flow cytometry data to determine statistical significance.

Figure 5.
Representative Maestro imaging of excised organs from TNBC model 24 h postinjection.
(A) Control mouse injected with PBS and (B) Cy5 polymer nanoparticle.

Figure 6.

Ovarian cancer model. (A) Representative luminescence images emitted from tumors imaged using IVIS. Imaging occurred 24 h after administration of **2**. (B) Representative fluorescence images visualized with IVIS. Imaging occurred 24 h after administration of **2**. (C) Quantitative ROI analysis of excised organs indicating total radiant efficiency per unit area. *** $p < 0.001$.

Scheme 1.
Synthetic Scheme for Fluorinated Copolymers

Scheme 2.

Cy5 Dye Labeling of Polymer 1 via Ligand-Accelerated CuAAC Reaction