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Gd-labeled glycol chitosan as a pH-responsive magnetic resonance imaging agent for detecting acidic tumor microenvironments

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Abstract

Neoplastic lesions can create a hostile tumor microenvironment with low extracellular pH. It is commonly believed that these conditions can contribute to tumor progression and resistance to therapy. We report the development and characterization of a pH-responsive magnetic resonance imaging contrast agent, for imaging the acidic tumor microenvironment. The preparation included conjugation of 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid 1-(2,5-dioxo-1-pyrrolidinyl) ester (DOTA-NHS) to the surface of a water soluble glycol chitosan (GC) polymer, which contains pH titrable primary amines, followed by gadolinium complexation (GC-NH₂-GdDOTA). GC-NH₂-GdDOTA had a chelate to polymer ratio of approximately 1:24 and a molar relaxivity of 9.1 mM⁻¹s⁻¹. GC-NH₂-GdDOTA demonstrated pH-dependent cellular association *in vitro* compared to the control. It also generated a 2.4-fold enhancement in signal in tumor bearing mice 2 h post-injection. These findings suggest that glycol chitosan coupled with contrast agents can provide important diagnostic information about the tumor microenvironment.

INTRODUCTION

It is generally accepted that early detection of breast cancer can greatly improve the chances of patient survival. This is supported by a recent study that showed mammography screening led to a 28–65% reduction in the rate of breast cancer mortality in the U.S. between 1975 and 2000.¹ Despite the proven effectiveness of mammography, it has been estimated that this method of screening has a sensitivity range of only 75–95% with a specificity of 90–95%.^{1, 2} Gadolinium (Gd)-enhanced magnetic resonance (MR) imaging of the breast can provide improved sensitivity, ranging from 88–95%; however, the specificity of Gd-enhanced MR imaging is actually quite poor, only 30–80%.³ The limited specificity of MR imaging is generally attributed to the fact that many benign lesions as well as presumably normal tissue may enhance after the administration of Gd chelates.⁴ This can result in an increase in the amount of material excised, additional excisions, or both.^{5, 6}

Numerous studies have shown that the extracellular pH (pHe) of human and animal tumors can reach values approaching 6.0,^{7, 8} and that a sub-physiologic pHe can stimulate *in vitro* invasion and *in vivo* metastases.^{9, 10} Therefore, it has been hypothesized that detection of tumor acidosis may provide a mechanism for improved cancer detection. Accordingly,

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Supporting Information Available: NMR spectra and HPLC chromatograms of native GC and GC-NH₂-DOTA. This information is available free of charge via Internet at <http://pubs.acs.org>

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various techniques and imaging strategies have been developed for measuring pH *in vivo*, most of which are based on magnetic resonance spectroscopy (MRS). Reported MRS techniques include ^{31}P imaging of 3-aminopropylphosphate (3-APP), ^1H imaging of imidazole-based compounds such as IEPA, and most recently, ^{13}C imaging of hyperpolarized bicarbonate ($\text{H}_{13}\text{CO}_3^-$) and $^{13}\text{CO}_2$.^{11–16} A variety of paramagnetic chemical exchange saturation transfer (PARACEST) MRI contrast agents have also been used to measure *in vivo* tumor pH.^{7, 17, 18} While each of these techniques has shown promise, advancements must still be made to improve the spatiotemporal resolution and sensitivity on 1.5 T scanners before these techniques can be adopted for routine clinical use.

Recently, we developed a pH-responsive MR imaging agent consisting of glycol chitosan-coated superparamagnetic iron oxide (SPIO) that specifically accumulated at sites of low pH.¹⁹ Tumor accumulation was driven by the pH-titratable amino groups on glycol chitosan which have an aggregate pKa of ~6.5. At sub-physiologic pH, the pH-responsive SPIO possessed a net-positive surface charge that led to strong electrostatic interactions with the surrounding environment. However, under normal physiological conditions the SPIO had a net neutral charge and showed lower tumor accumulation. Although, this contrast agent demonstrated an improved ability to accumulate within the acidic tumor microenvironment, SPIO-based imaging requires a long delay between contrast agent administration and imaging, typically 24 h, has a long residence time, and is typically used to generate negative T_2 contrast. In part, because of these characteristics, SPIO-based agents have not yet been widely adopted for routine use in the clinic.

Here, we report the development of a pH-responsive, gadolinium (Gd)-based MR contrast agent that was designed to allow for rapid imaging (i.e. within several hours) and to generate positive T_1 -contrast. Similar to the pH-responsive SPIO, these agents take advantage of the unique pKa value of glycol chitosan, which promotes electrostatic interactions with surrounding cells and extracellular structures strictly within acidic tumor microenvironments but otherwise remains relatively inert (Figure 1).²⁰ The pH-responsive Gd agents were prepared through the reaction of glycol chitosan (10–30 kDa) with 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid 1-(2,5-dioxo-1-pyrrolidinyl) ester (DOTA-NHS) followed by Gd loading (GC-NH₂-GdDOTA). To create a control agent, the remaining amine groups on the glycol chitosan were blocked with glycidol to eliminate pH responsiveness (GC-OH-GdDOTA).

RESULT AND DISCUSSION

Synthesis of GC-NH₂-GdDOTA and GC-OH-GdDOTA

This report describes an efficient synthesis of a glycol chitosan-based T_1 MRI contrast agent. The compound (GC-NH₂-GdDOTA) was prepared by conjugating DOTA-NHS ester (50% by weight) to glycol chitosan (10–30 kDa; Figure 1) followed by gadolinium complexation. This allowed the retention of some functional free amines that are pH-responsive. The control (GC-OH-GdDOTA) was prepared by blocking the remaining free amines with glycidol to eliminate pH sensitivity.

Purified GC Characterization

^1H NMR characterization of GC and GC-NH₂-DOTA is shown in figure S1. The peak at 1.8 ppm arises from the methyl of the acetyl group while the peaks from 2.6 to 3.1 ppm arise from protons at positions 2, 7 and 8. Previous reports showed the presence of the acetyl group to a certain extent in commercial chitosans due to incomplete alkaline deacetylation during the process of preparation.^{21, 22} The peaks from 3.3 to 4.2 ppm are attributed to protons at positions 3 to 6, and the peak at 4.4 ppm arises from protons at position 1. The

chemical shifts of protons at positions 1 and 2 are subject to change depending on the pH.²² Conjugation between DOTA-NHS ester and GC is evidenced by the down field shift of methyl signal at 1.8 ppm to 2.1 ppm while the chemical shifts of the ligand overlap with those of GC between 3.3 to 4.2 ppm. Such overlapping has also been observed when DOTA is conjugated to a dendrimer.²³

Characterization of the GC-NH₂-GdDOTA and GC-OH-GdDOTA

The GC-NH₂-GdDOTA conjugate had a relaxivity (r_1) value of 9.1 mM⁻¹ s⁻¹ per gadolinium ion (Figure 2), which is significantly greater than that of Gd-DOTA (r_1 = 3.9 mM⁻¹ s⁻¹). It is well documented that the increase in relaxivity with size can primarily be attributed to an increase in the rotational correlation time (τ_c) of the chelated Gd; however, changes in the inner-sphere water exchange rate and the internal motion will also influence the measured relaxivities.^{24–27} GC-NH₂-GdDOTA (~22,000 Da) produced a lower relaxivity than polyamidoamine dendrimer (G3)-[Gd-C-DOTA]⁻¹ (r_1 = 10.9 mM⁻¹ s⁻¹), which has a lower molecular weight (14,000 Da)²⁸, indicating that size is not the only factor influencing relaxivity. Notably, the control agent, GC-OH-GdDOTA, has a similar relaxivity (9.5 mM⁻¹ s⁻¹) as GC-NH₂-GdDOTA.

Using Rayleigh's equation, which correlates the intensity of the scattered light – measured by dynamic light scattering - to molecular weight, it was determined that there were approximately 24 DOTA per glycol chitosan agent (Table 1). This estimate is based on the change in molecular weight upon derivatization of GC with DOTA.

Similar values were obtained when size-exclusion HPLC (SE-HPLC) was used to estimate the molecular weights of GC and GC-NH₂-DOTA. The SE-HPLC chromatograms of the pre- and post conjugated polymers are provided in Figure S2. The elution curves appear broad, but this is consistent with what has previously been reported in the literature.^{22, 29, 30} Since GC-OH-GdDOTA was synthesized by direct chemical blockade of the pH-sensitive amino groups with glycidol, the level of DOTA labeling was identical to GC-NH₂-DOTA. The hydrodynamic size of the GC-OH-GdDOTA (8.7 nm) was also found to be nearly identical to GC-NH₂-GdDOTA (8.5 nm), based on dynamic light scattering measurements (Figure 3).

Consistent with the presence of free and titratable amines along the glycol chitosan chain even after Gd-loading, GC-NH₂-GdDOTA exhibited a zeta potential that varied as a function of pH, similar to unmodified glycol chitosan. At physiologic pH, the net zeta potential of GC-NH₂-GdDOTA was near neutral (+1.9 mV), but increased as the pH was reduced (Figure 4A). At pH 6.0 the zeta potential of GC-NH₂-GdDOTA was +8.4 mV. In contrast, GC-OH-GdDOTA exhibited a near-neutral zeta potential regardless of pH. The physical-chemical properties of GC-NH₂-GdDOTA and GC-OH-GdDOTA are summarized in Table 2.

In Vitro Cellular Association Studies

When T6–17 tumor cells were incubated with GC-NH₂-GdDOTA in media that had its pH adjusted between 6.0 and 7.4, it was found that the cells exhibited pronounced pH dependence in their T_1 relaxation times. The cells that were incubated with GC-NH₂-GdDOTA in pH 6.0 media exhibited the highest extent of labeling, i.e. shortest T_1 relaxation times (Figure 4B), compared with unlabeled control cells. A decreasing extent of cell labeling was observed as the pH was increased, with little cell labeling observed at pH 7.4. Cells incubated with GC-OH-GdDOTA did not show any pH-dependence in T_1 relaxation time, with all samples exhibiting similar T_1 measurements as unlabeled cells. These results indicate that the pH-responsive behavior of GC-NH₂-GdDOTA allows for an

increase in the extent of cellular association within acidic environments, likely through electrostatic interactions.

Contrast-Enhanced *In Vivo* MR Imaging

To assess the ability of GC-NH₂-GdDOTA to provide tumor specific MR contrast, T6–17 flank tumors were grown in nude mice to a diameter of approximately 8 mm, and then either GC-NH₂-GdDOTA or GC-OH-GdDOTA was administered intravenously at a dose of 0.03 mmol Gd/kg. This is less than 1/3 of the clinically recommended dose of 0.1 mmol Gd/kg for Gd-based agents. A significant contrast enhancement in *T*₁-weighted images was clearly visible for as long as 4 h following injection of GC-NH₂-GdDOTA, with the greatest contrast observed at 2 h post-injection (Figure 5). Little to no contrast enhancement was observed 24 h post-injections, which is suggestive of rapid clearance. It is expected that the ability to image within 2 h or less from the time of injection is a significant advantage over SPIO-based agents, which typically require imaging 24 h post injection. A short delay between imaging and injection is expected to improve patient compliance, since the entire procedure can be carried out in a single outpatient visit.

It should be noted that MR imaging did reveal a slight global enhancement in background signal over the first 4 h post injection, suggestive of some degree of non-specific interactions between the contrast agent and normal tissue. This is somewhat consistent with the low levels of association that we observed between GC-NH₂-GdDOTA and cells in culture at pH 7.4 (Figure 4B). However, this did not seem to interfere with the preferential accumulation of GC-NH₂-GdDOTA within the tumor, relative to muscle, and the enhancement was only transient. It is hypothesized that these non-specific interactions could be reduced by adjusting the net charge of GC-NH₂-GdDOTA closer to neutral, at pH 7.4. This can potentially be achieved by incorporating a small number of carboxyl groups into the glycol chitosan backbone or by using 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (p-SCN-Bn-DOTA) as the chelate. When complexed with Gd, p-SCN-Bn-DOTA possesses a net charge of -1.

*T*₁-weighted images of mice injected with GC-OH-GdDOTA did not reveal any discernable enhancement within the tumor at any of the time points studied. These findings support the tumor specificity of GC-NH₂-GdDOTA. Further, they provide initial evidence that the contrast enhancement observed with GC-NH₂-GdDOTA was not the result of enhanced permeability and retention (EPR) alone, since the two agents have similar physical properties. To further establish that the tumor-specific accumulation of GC-NH₂-GdDOTA was pH-mediated, an additional control study was performed whereby GC-NH₂-GdDOTA was administered to tumor bearing mice that received sodium bicarbonate in their drinking water. It has been previously established that sodium bicarbonate can neutralize the pH in tumors.^{31, 32} Consistent with pH-dependent accumulation, no significant contrast enhancement was observed in the tumors of mice receiving sodium bicarbonate. These studies provide strong evidence that retention of GC-NH₂-GdDOTA within the tumors of mice not receiving sodium bicarbonate, between 1 to 4 h post-injection, is dictated by pH.

The relative signal intensity (rSI) on MR images was determined by dividing the signal intensity of operator-defined regions of interest in the tumor by that of paraspinal muscle on a slice by slice basis. This was done to account for variations in absolute signal resulting from RF coil positioning. The signal change was then determined by calculating the relative signal enhancement (rSE), which is defined as the quotient of the rSI in the pre- and post-contrast MR images. It was found that GC-NH₂-GdDOTA yielded a rSE as high as 2.4 at 2 h post-injection (Figure 6), while GC-OH-GdDOTA led to a rSE near unity at all time points, which represents little to no contrast enhancement. Similarly, mice that had sodium

bicarbonate in their drinking water also had an rSE near unity, following the administration of GC-NH₂-GdDOTA.

CONCLUSION

In summary, we have described and successfully validated a complementary approach to MRS imaging for the identification of sites with sub-physiologic pH. Our results provide strong evidence that glycol chitosan coupled with contrast agents will exhibit longer retention at sites of low extracellular pH and may thus provide important diagnostic information about the tumor microenvironment. A benefit of using Gd-based contrast agents for imaging acidic lesions is that they can be detected on clinical scanners with high spatiotemporal resolution compared with MR spectroscopy and PARACEST agents. The pH-responsive Gd-complex will likely not provide a quantitative measure of pH, but if a pH map is desirable, then MRS imaging can subsequently be performed over lesions that have first been identified with the Gd-complexes. We believe that this approach can significantly reduce the acquisition time required for MR spectroscopy since fewer (more predictive) sites can be selected for MRS imaging. We plan to further validate this contrast agent with the long-term goal of using it for clinical cancer imaging.

It should be noted that various Gd-labeled macromolecular contrast agents have been evaluated in the past (e.g. Albumin-Gd-DTPA,³³ Dextran-Gd-DTPA,³⁴ Polylysine-Gd-DTPA,³⁵ and MPEG-polylysine-Gd-DTPA³⁶) with marginal success.³⁷ However, an important distinction between these agents and GC-NH₂-GdDOTA, is that these prior agents were designed to serve as blood pool agents. Therefore, they possessed high molecular weights (>30 kDa, and typically >100 kDa) to encourage long circulation times. Macromolecules of this size typically have a high degree of polydispersity and poor excretion profiles. The GC-NH₂-GdDOTA developed here was specifically designed with a low molecular weight (10–30 kDa), to allow for rapid clearance and efficient excretion. Extensive studies to evaluate the excretion profile and long-term retention of GC-NH₂-GdDOTA are ongoing.

In addition to being used as a contrast agent for detecting lesions with sub-physiologic pH, it is expected that GC-NH₂-GdDOTA may also be used for MRI-guided biopsies. Currently, difficulties can arise during these procedures, due to decreasing target visibility during intervention as small Gd chelates are “washed out” of the tumor, i.e. “vanishing target” phenomenon. In a recent study, the vanishing target phenomenon occurred in 63% of the patients, which necessitated an additional contrast-enhanced dynamic series to verify the position of the core-needle with respect to the lesion.³⁸ Loss of contrast can also lead to poor sampling of the lesion. It has been reported, that missed rates of needle localized breast biopsies can be as high as 17.9%.³⁹

Since GC-NH₂-GdDOTA exhibited longer retention times within tumors, compared with control agents, it could provide a longer time window for biopsies and thus improve the quality of MR-guided tissue sampling.

EXPERIMENTAL SECTION

Materials

Glycol chitosan was purchased from Wako Chemicals (Richmond, VA). 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid 1-(2,5-dioxo-1-pyrrolidiny) ester (DOTA-NHS) was prepared as described in the literature.⁴⁰ All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Surface Conjugation of Glycol Chitosan

High molecular weight glycol chitosan was degraded as previously described,¹⁹ with slight modifications. Briefly, 8 g of ≈ 400 kDa glycol chitosan was dissolved in 160 mL of 6 M HCl and heated to 80°C for 3 h. The material was cooled on ice and immediately neutralized with the solid sodium carbonate to terminate degradation. Excess base was removed by centrifugation, while diafiltration membranes (100, 50, 30 and 10 kDa MWCO) were used sequentially to desalt and fractionate the material. The Fraction collected between 10 – 30 kDa was washed with water for three cycles using a 10 MWCO membrane filter, vacuum dried and used without any further purification.

Gd-labeled glycol chitosan (GC-NH₂-GdDOTA) was prepared as follows: 1.0 g of glycol chitosan (10 – 30 kDa fraction) was dissolved in 30 mL of conjugation buffer (0.48 M NaHCO₃, 0.02 M Na₂CO₃, 1.5 M NaCl, and 0.005 M EDTA), and 0.5 g (1.0 mmol) of DOTA-NHS was added in portions while the pH was maintained at 8.5 with 1M NaOH. The mixture was stirred at 45°C for 24 h. The solution was cooled to room temperature and centrifuged to remove large particulates. The buffer was exchanged to 1× acetate buffer (pH 6.0) using centrifugation (10 kDa MWCO; Millipore, Billerica, MA) to achieve the final volume of 30 mL. 0.5 g (1.1 mmol) of Gd(NO₃)₃·6H₂O was added and the solution was heated to 100°C for 24 h. The solution was cooled to room temperature, centrifuged and filtered through a 0.22 μ m membrane filter. The buffer was then exchanged to 1X PBS using centrifugation (10 kDa MWCO) to achieve the final volume of 2 mL. The flow-through was collected to ensure that no unchelated gadolinium ions were present in the sample. Gadolinium ion concentration of both the concentrated retentate and flow-through was determined by ICP-OES analysis using a Genesis ICP-OES (Spectro Analytical Instruments GMBH; Kleve, Germany) at the Department of Earth and Environmental Sciences, University of Pennsylvania, PA, USA. No gadolinium ions were detected in the flow through (i.e. below the detection limit), confirming >95% purity of the final compound.

Preparation of GC-OH-GdDOTA

To a mixture of 1.5 mL of GC-NH₂-GdDOTA in 1X PBS, 3 mL of glycidol was added and the suspension was incubated at 30° C for 24 h. The reaction mixture was cooled to room temperature and centrifuged up to five cycles using a 10 kDa MWCO membrane and 1X PBS to remove excess glycidol. The volume was reduced from 10mL to 1.5 mL for each centrifugation cycle resulting in removal of >99.99% of the glycidol. The final volume was retained at 1.5 mL and the concentration of Gd³⁺ ion was determined by ICP-OES.

Nuclear Magnetic Resonance Spectroscopy

¹H NMR spectra were acquired with a Bruker Avance-360 spectrometer equipped with a 5 mm manual switching QNP probe with gradient and variable temperature capability. Samples were prepared at 100 mg/mL in deuterium oxide. The pH of the samples was ~ 7.5 . Spectral data were collected in Bruker's Topspin software.

Characterization of the GC-NH₂-GdDOTA and GC-OH-GdDOTA

Samples were diluted to a final concentration of 100 μ g Gd/mL in pH 7.4 phosphate buffered saline to determine the hydrodynamic diameter by dynamic light scattering (DLS). Measurements were acquired with a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) using the non-invasive backscatter (NIBS) mode. The longitudinal (R_1) relaxivity of the two formulations were calculated by plotting the reciprocal of the T1 relaxation time versus the gadolinium concentration using a Bruker mq60 tabletop MR relaxometer operating at 1.41 T. For zeta-potential pH titrations, 10 mM HEPES buffered water was prepared with pH values ranging from 6.0 to 7.4 in 0.2 unit increments. Each

glycol chitosan sample was diluted to a final concentration of 100 µg Gd/mL in the buffer at each pH and the mean nanoparticle zeta potential was measured using a Zetasizer Nano-ZS.

The number of DOTA per GC was determined by measuring the molecular weight of GC before and after derivatization with DOTA. By calculating the difference in molecular weight and dividing by the molecular weight of DOTA (387 Da) it was possible to estimate the average number of DOTA per GC molecule. The molecular weight was determined using two different methods. In the first method molecular weight was determined by applying the Rayleigh equation:

$$\frac{KC}{R_\theta} = \left(\frac{1}{M} + 2A_2C \right)$$

where C is the sample concentration, R_θ is the Rayleigh ratio (i.e. the ratio of scattered light to incident light of the sample), M is the molecular weight of the sample, A_2 is the 2nd Virial Coefficient, and K is the optical constant:

$$K = \frac{2\pi^2}{\lambda_0^4 N_A} \left(n_0 \frac{dn}{dc} \right)^2$$

where N_A is Avogadro's constant, λ_0 is the laser wavelength, n_0 is the solvent refractive index, and dn/dc is the differential refractive index increment. The differential refractive index increment (dn/dc) of chitosan was obtained by measuring the samples at different concentrations on a Reichert AR2000 digital refractometer.

The intensity of scattered light $\left(\frac{KC}{R_\theta} \right)$ was measured on a Zetasizer Nano ZS at various concentrations (C). This was compared with the scattering produced from a standard (i.e.

Toluene). The molecular weight was then determined by creating a Debye plot $\left(\frac{KC}{R_\theta} \text{ vs. } C \right)$,

which has a y-intercept of $\left(\frac{1}{M} \right)$. All calculations were performed using an application that is available within the Zetasizer Nano ZS software package.

The second method that was utilized to determine molecular weight was size-exclusion HPLC (SE-HPLC). SE-HPLC was performed using a Varian Prostar (Groton, CT) equipped with model 210 solvent delivery module and a model 340 UV detector (λ 254 nm). Size-exclusion chromatography was performed on a Protein Pak 300SW 10 µm, 7.8 mm × 300 mm (Waters, Milford, MA), using phosphate buffered saline (1X PBS) solution as the eluent at 1.0 mL/min. Biorad gel filtration standard, which consists of five different known protein samples, was used for SE-HPLC to determine the molecular weight of GC and GC-DOTA. Briefly, the standard was run and the retention times were plotted against their molecular weights. The pre- and post-conjugated samples were run and their respective retention time were inserted to the standard plot to obtain the estimated molecular weights. Again, the difference between the two values was used to estimate the number of conjugated DOTA.

Cell Culture

T6–17 mouse fibroblasts (a derivative of the NIH/3T3 line) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin at 37°C and 5% CO₂.

***In Vitro* Cellular Association Studies**

For *in vitro* pH studies, cell culture medium was supplemented with 25 mM HEPES buffer and prepared with pH values ranging from 6.0 to 7.4 in 0.20 unit increments. Freshly trypsinized T6–17 cells (5×10^6) were incubated with either GC-NH₂-GdDOTA or GC-OH-GdDOTA at a concentration of 80 μ g Gd/mL for 1 h at 37°C in a total volume of 1.0 mL buffered medium. Following incubation, unassociated contrast agents were removed by triplicate low-speed centrifugal washes with contrast agent-free medium of matching pH. The cell samples were then resuspended in 0.3 mL of PBS at pH 7.4, and the T_1 relaxation times of the suspensions were measured on the tabletop relaxometer.

Contrast-Enhanced *In Vivo* MR Imaging

Approximately 6 week old female nu/nu nude mice (n = 9; Charles River Laboratory, Charles River, MS) were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. Mice were anesthetized via isoflurane, and T6–17 cells were injected subcutaneously into the back right flank (2×10^6 cells in 0.2 mL of PBS). When tumors reached a size of approximately 8 mm, precontrast images were acquired using a 4.7 T small animal horizontal bore Varian INOVA system. T_1 -weighted images were acquired in the axial plane using parameters as follows: repetition time (TR) = 392 ms, echo time (TE) = 5.12 ms, FOV = 80×40 mm, flip angle = 30°, slice thickness = 0.5 mm, number of requisition = 6, matrix = 256×128 pixels. Immediately following the precontrast image acquisition, the contrast agents were administered *via* tail vein injection at a gadolinium concentration of 0.030 mmol/kg (GC-NH₂-GdDOTA n = 3; GC-OH-GdDOTA n = 3). Postcontrast images were acquired at 1 h, 2 h, 4 h, and 24 h under the same imaging parameters used for precontrast images. One group of mice (n=3) was exposed to drinking water containing 80 mM NaHCO₃ (pH 8.2) for 10 days prior to administration of GC-NH₂-GdDOTA and imaging to increase the pH in the tumor.

Image Analysis

For quantitative analysis of MR images, the relative signal intensity (rSI) of the tumor was calculated by dividing the MR signal intensities within operator-defined regions of interest by that of adjacent paraspinal muscle. The relative signal enhancement (rSE) was then calculated as the quotient of the postcontrast rSI and precontrast rSI. A *t* test (two-tailed, unequal variance) was used to compare the rSE at different time points postinjection. A *p* < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations Used

3-APP	3-aminopropylphosphate
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid 1-(2,5-dioxo-1-pyrrolidinyl)
EPR	enhanced permeability and retention

GC	glycol chitosan
Gd	gadolinium
MR	magnetic resonance
MRS	magnetic resonance spectroscopy
MWCO	molecular weight cut-off
NIBS	non-invasive back-scatter
PARACEST	paramagnetic chemical exchange saturation transfer
pHe	extracellular pH
rSE	relative signal enhancement
rSI	relative signal intensity
SE-HPLC	size exclusion high performance liquid chromatography
SPIO	superparamagnetic iron oxide

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Figure 1.

Schematic of Gd-labeled glycol chitosan (GC-NH₂-GdDOTA) and the mechanism of pH-mediated enhanced delivery within acidic microenvironments. (A) GC-NH₂-GdDOTA consists of native glycol chitosan sparsely labeled with GdDOTA. (B) A control agent, GC-OH-GdDOTA, which is unresponsive to changes in pH, was prepared via alkylation of the free amines on glycol chitosan using glycidol. (C) GC-NH₂-GdDOTA exhibits near neutral surface charges at physiologic pH due to deprotonation of the titratable amines on the glycol chitosan. The presence of a neutral surface charge minimizes nanoparticle association with blood components and normal tissue. Within acidic microenvironments, the amino groups of GC-NH₂-GdDOTA titrate to yield a positive charge leading to electrostatic interactions with negatively charged cell membranes and extracellular matrix components and resulting in enhanced retention in these areas. (D) GC-OH-GdDOTA exhibits a near neutral surface charge regardless of pH and thus does not exhibit significant electrostatic interactions with tissue at physiologic or sub-physiologic pH.

Figure 2. Relaxivity (r_1) of (A) GC-NH₂-GdDOTA and (B) GC-OH-GdDOTA. The relaxation rates (R_1) were measured at various Gd concentrations using an MR relaxometer operating at 1.41 T (60 MHz). Linear curve fits indicated relaxivities of 9.1 and 9.5 mM⁻¹ s⁻¹ for GC-NH₂-GdDOTA and GC-OH-GdDOTA, respectively. All measurements were acquired in triplicate.

Figure 3. Dynamic light scattering (DLS) measurements of (A) GC-NH₂-GdDOTA and (B) GC-OH-GdDOTA. DLS measurements of hydrodynamic diameter were acquired using a non-invasive back-scatter (NIBS) mode. GC-NH₂-GdDOTA and GC-OH-GdDOTA were found to have average hydrodynamic diameters of 8.5 and 8.7 nm, respectively.

Figure 4.

Effect of pH on zeta potential and *in vitro* association of GC-NH₂-GdDOTA with T6-17 cells. (A) The zeta potential of GC-NH₂-GdDOTA and GC-OH-GdDOTA were recorded as a function of pH. All samples were measured in triplicate. (B) GC-NH₂-GdDOTA and GC-OH-GdDOTA (80 µg Gd/mL) were incubated with 5×10^6 T6-17 cells in culture medium buffered with 25 mM HEPES at various pH. After removal of unassociated Gd-complexes, T₁ relaxation times were measured using an MR relaxometer operating at 1.41 T (60 MHz). T₁ relaxation times were also acquired for unlabeled cells. All experiments were performed in triplicate.

Figure 5.

In vivo MR images of nu/nu mice with T6-17 flank tumors. T_1 weighted MR images in the axial plane were obtained prior to injection (precontrast) and 1 h, 2 h, 4 h and 24 h after injection (postcontrast) of 30 $\mu\text{mol/kg}$ of GC-NH₂-GdDOTA (top, n=3) or GC-NH₂-GdDOTA (middle, n=3). In a separate control study, 30 $\mu\text{mol/kg}$ of GC-NH₂-GdDOTA (n=3) was administered to tumor-bearing mice that received NaHCO₃ in their drinking water (bottom). Tumor location is indicated by white arrows.

Figure 6. Quantitative analysis of enhancement in MR tumor contrast following the administration of GC-NH₂-GdDOTA or GC-NH₂-GdDOTA. The relative signal intensity (rSI) was obtained by normalizing the signal intensity of each tumor to adjacent paraspinal muscle. The relative signal enhancement (rSE) was then calculated as the quotient of the post-contrast rSI to pre-contrast rSI. Errors are reported as standard deviations. Asterisk indicates statistical significance ($p < 0.05$).

Table 1

Estimate of DOTA loading on glycol chitosan

Method	GC (kDa)	GC-DOTA (kDa)	DOTA (kDa)	#DOTA/#amine
SE-HPLC	22.0±3.0	31.5±3.5	9.5±0.5	24±2/107
viscosity/DLS	21.7±1.1	31.0±2.2	9.3±1.1	24±3/106

Table 2

Physical and Magnetic Properties

Particle	Zeta (ζ , mV)		Size ^a (nm)	relaxivity (r_1 , mM ⁻¹ s ⁻¹)
	pH 6.0	pH 7.4		
GC-NH ₂ -GdDOTA	+8.4±1.4	+1.9±1.3	8.5±1.7	9.1±0.6
GC-OH-GdDOTA	+1.9±1.2	+1.6±1.4	8.7±1.6	9.5±0.5
Glycol Chitosan	+9.3±1.5	+2.1±1.3	8.0±1.4	----

^aThe hydrodynamic diameter of the particles at pH 7.4.