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Activatable Interpolymer Complex-Superparamagnetic Iron Oxide Nanoparticles as Magnetic Resonance Contrast Agents Sensitive to Oxidative Stress

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Abstract

Magnetic resonance contrast agents that can be activated in response to specific triggers hold potential as molecular biosensors that may be of great utility in non-invasive disease diagnosis. We developed an activatable agent based on superparamagnetic iron oxide nanoparticles (SPIOs) that is sensitive to oxidative stress, a factor in the pathophysiology of numerous diseases. SPIOs were coated with poly(ethylene glycol) (PEG) and complexed with poly(gallol), a synthetic tannin. Hydrogen bonding between PEG and poly(gallol) creates a complexed layer around the SPIO that decreases the interaction of solute water with the SPIO, attenuating its magnetic resonance relaxivity. The complexed interpolymer nanoparticle is in an OFF state (decreased T_2 contrast), where the contrast agent has a low T_2 relaxivity of $7 \pm 2 \text{ mM}^{-1} \text{ s}^{-1}$. In the presence of superoxides, the poly(gallol) is oxidized and the polymers decomplex, allowing solute water to again interact with the SPIO, representing an ON state (increased T_2 contrast) with a T_2 relaxivity of $70 \pm 10 \text{ mM}^{-1} \text{ s}^{-1}$. These contrast agents show promise as effective sensors for diseases characterized in part by oxidative stress such as atherosclerosis, diabetes, and cancer.

Graphical abstract

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Keywords

magnetic resonance imaging; activatable contrast; nanoparticles; oxidative stress; superparamagnetic iron oxide nanoparticles

1. Introduction

The transverse relaxation time (T_2) in magnetic resonance (MR) imaging characterizes the time required for dephasing of proton spins [1, 2]. T_2 can be decreased by exogenous contrast agents, which are under active investigation in the field of molecular imaging to target molecular markers of diseases [3, 4]. Since faster dephasing, i.e. a decrease in T_2 , results in diminished signal, these contrast agents are described as showing negative contrast. One common approach to achieving accumulation of the contrast agent at a disease site is to actively target a receptor or biomolecule [5–7], causing a correlation between local contrast and the prevalence of a disease-specific target [8]. Alternatively, recent progress in activatable MR molecular imaging has achieved a type of functional imaging in which a contrast agent exhibits greater or lesser relaxivity, that is, effect on T_1 or T_2 , in response to environmental factors such as the presence of an enzyme [9], light [10], temperature [11], or pH level [12]. In this sense, such activatable agents act as microenvironment biosensors.

A great deal of recent work has demonstrated activatable agents based on paramagnetic gadolinium [13–22], but there are few reports of such use of superparamagnetic iron oxide nanoparticles (SPIOs), which exhibit particularly high T_2 relaxivity (r_2) and biocompatibility [23–25]. Coatings greatly affect the relaxivity of the SPIOs [26–30], providing an opportunity for the tailored design of SPIO-based biosensors. Unlike gadolinium, SPIOs generally have low T_1 relaxivity but high T_2 and T_2^* relaxivity. T_2 relaxation refers to decay of transverse magnetization caused by spin-spin relaxation, whereas T_2^* relaxation refers to transverse magnetization decay caused by a combination of spin-spin relaxation and magnetic field inhomogeneity. Since the contribution of SPIOs to magnetic field inhomogeneity is long-range (μm to mm), these effects are likely much harder to silence using a nanoparticle surface coating. Therefore, our investigations primarily focused on the T_2 relaxivity of coated SPIOs.

Here we describe the development of an SPIO-based activatable MR contrast agent incorporating a polymer coating that is responsive to oxidative stress produced by local reactive oxygen species (ROS), which are known to contribute to the progression of a number of pathologies [31–33]. Oxidative stress can lead to chronic inflammation, as is seen in numerous diseases including atherosclerosis [34–36], cancer [37, 38], diabetes [34, 35,

39], and pulmonary diseases [40–42]. Thus, a contrast agent sensitive to oxidative stress may be of potential utility in the diagnosis and ongoing evaluation of a number of diseases.

We present the synthesis and characterization of SPIOs with a coating of interpolymer complexed (IPC) poly(ethylene glycol) (PEG) and poly(gallol), a synthetic dextran-based tannin-inspired polymer [43]. Hydrophilic PEG is able to colloidally stabilize nanoparticles and increase blood circulation time by reducing opsonization and immune response [44–46]. Poly(gallol) polymers are designed to mimic the natural structure of tannins, exhibiting anti-oxidant, anti-inflammatory, and anti-microbial properties [43, 47]. In this nanoparticle, interpolymer complexation relies on hydrogen bonding between PEG and poly(gallol) (Figure 1A). We demonstrate that the presence of solute water surrounding the SPIOs is reduced upon coating them with the complexed PEG and poly(gallol), greatly decreasing their T_2 relaxivity. However, when reactive oxygen species oxidize the phenolic groups on the poly(gallol), the polymer side groups lose the hydroxyl needed for complexation with PEG (Figure 1A). The disruption of this hydrogen bonding between the two polymers upon oxidation of the poly(gallol) allows for more water to reach the SPIO, and the T_2 relaxivity is regained.

2. Materials and Methods

2.1. Materials

The metallic precursors iron (II) chloride (FeCl_2), iron (III) chloride (FeCl_3), N,N' -Diisopropylcarbodiimide (DIC), 4-dimethylamino-pyridine (DMAP), dimethyl sulfoxide (DMSO), dichloromethane (DCM), dimethylformamide (DMF), Pd black, sodium carbonate (Na_2CO_3), dextran, xanthine oxidase from bovine milk (XOD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3,4,5-tris(benzyloxy)benzoic acid (TBBA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Ammonium hydroxide (NH_4OH , 28–30 wt %) was purchased from BDH (Poole, Dorset, UK). Polyethylene glycol with various molecular weights was purchased from Sigma-Aldrich and Alfa Aesar (Heysham, Lancashire, UK). Phosphotungstic acid hydrate powder was purchased from Alfa Aesar (Heysham, Lancashire, UK). Sodium phosphate monobasic, sodium phosphate dibasic, and potassium hydroxide (KOH) were purchased from Amresco (Solon, OH, USA). Hypoxanthine (HX) was purchased from Research Products International (RPI) Corp. (Mt. Prospect, IL, USA). All chemicals used were of analytical grade.

2.2. Preparation of Superparamagnetic Iron Oxide Nanoparticles (SPIOs)

For the whole process (Scheme 1), N_2 purged DI water was used to minimize any unexpected oxidation. A modified coprecipitation method was used for preparation of SPIOs [24, 48–51]. First, 2 mmol of FeCl_3 and 1 mmol of FeCl_2 were dissolved in two separate beakers containing 50 mL of deionized (DI) water each. The FeCl_2 solution was added dropwise to the FeCl_3 solution with magnetic stirring. The resulting 100 mL mixture of FeCl_2 and FeCl_3 solutions was mixed well for 30 minutes under a nitrogen atmosphere with vigorous magnetic stirring. The mixture was heated to 80°C using a silicone oil bath, at which point 30 mL of 1M aqueous NH_4OH solution was added dropwise and left to react for 5 hours under nitrogen atmosphere. The solution was then cooled to room temperature and

sonicated in a bath sonicator for 1 hour. Afterwards, the solution was washed with DI water until the pH was approximately 7. The black precipitate was separated via magnetic decantation and centrifugation three times. The final product was redispersed into DI water to create a suspension containing 2 mg of SPIOs per mL.

2.3. Preparation of PEGylated SPIOs

PEGylated SPIOs were formed via adsorption of PEG on the surface of SPIOs. To vary the PEG surface coverage per particle, four separate PEG solutions were created with PEG 6 kDa, PEG 10 kDa, PEG 35 kDa, and PEG 300 kDa. PEG with lower molecular weight was expected to form a less dense polymer coating, while PEG with higher molecular weight was expected to form a high-density polymer coating on the surface of particles [52]. Each PEG solution was mixed with SPIOs at a PEG:SPIO molar ratio of approximately 18,000:1, 9,000:1, 4500:1, and 150:1 (corresponding to PEG: Fe₃O₄ molar ratios of 2:1, 1:1, 1:2, and 1:64) for 6, 10, 35, and 300 kDa PEG molecular weights, respectively. The suspension of SPIOs and PEG solution was stirred for 24 hours with a magnetic stirrer at 1000 rpm. The PEGylated SPIOs were separated with a magnet, and the supernatant was decanted. The solution was washed 3 times with DI water to remove any excess polymer.

2.4. Preparation of poly(gallol)

Poly(gallol) was prepared as described previously [43]. Briefly, TBBA molecules were conjugated to a linear dextran backbone of either 40 or 200 kDa molecular weight and were then converted to phenols via a palladium black catalyzed deprotection reaction. The conjugation of BBAs used an optimized Steglich esterification to bind the carboxyl groups on the BBAs to the free hydroxyl groups on dextran. The type of BBA used and its feed ratio determined the molecular structure of the final polymer and the density of phenolic units. In order to ensure complete deprotection, the feed ratio of free hydroxyl groups on dextran to TBBA was limited to 2:1. Higher degrees of substitution resulted in an inefficient and incomplete deprotection. Poly(gallol)s were dissolved in a 1 M sodium carbonate buffer (pH 8) to convert the phenolic units to phenoxide salts, making them water soluble while limiting self-complexation and oxidation during storage. These poly(gallate) salts were lyophilized for storage until use [43].

2.5. Preparation of complexed IPC-SPIOs

Complexed IPC-SPIOs were formed by hydrogen bonding between PEG and poly(gallol) [43]. Uncoated SPIOs and PEG 6, 10, 35, and 300 kDa-SPIOs were separately diluted to achieve a concentration of 2 mg of SPIOs per mL. The lyophilized 40 kDa and 200 kDa sodium poly(gallate)s were reconstituted in deionized water at a concentration of 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL. Each suspension of uncoated SPIOs or PEGylated SPIOs was mixed with an equal volume of sodium poly(gallate) polymer solution for one hour. Complexation was initiated upon addition of sodium phosphate buffer at a concentration of 1 M and pH 7.4 to convert the poly(gallate) salts into poly(gallol) polymers capable of hydrogen bonding with PEG. In order to have a final concentration of approximately 400 μ M of iron and 100 mM of sodium phosphate buffer, a volume ratio of 9:9:2 SPIO suspension: poly(gallate) solution: sodium phosphate buffer was used. Complexation

proceeded over several hours, as monitored by UV/Vis spectroscopy, detailed below, prior to characterization of particles.

2.6. Preparation of decomplexed IPC-SPIOs

The decomplexation reaction required an oxidative environment, which was created using xanthine oxidase and hypoxanthine [43]. XOD is an enzyme that produces superoxide (O_2^-) in the presence of its substrate, HX. When superoxide oxidizes the hydroxyl groups on the poly(gallol), the hydrogen bonding necessary for complexation is disrupted. A 30 mM solution of HX was created by dissolving HX in a 50 mM aqueous stock solution of KOH. Subsequently, the XOD (0.015 units) solution was mixed with 100 mM phosphate buffer, to provide excess oxidative species to the reaction [43]. The complexed IPC-SPIOs were added to HX and XOD for decomplexation at 37°C. In order to have a final concentration of approximately 400 μ M of iron and 100 mM of phosphate buffer, a ratio of 20 parts complexed IPC-SPIO suspension: 2 parts HX solution : 0.7 parts XOD solution was used. Serial dilutions were prepared and used fresh.

2.7. Characterization

2.7.1. Transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDS)—Transmission electron microscopy (TEM, JEOL JEM 2100F, Japan) was performed at a voltage of 200 kV to assess the size and morphology of the synthesized particles. Several drops of the diluted suspension were placed on the surface of an ultrathin carbon film on lacey carbon support film grid (400 mesh, Ted Pella Inc., Redding, CA, USA). For negative staining, a few drops of 2% phosphotungstic acid aqueous solution were placed on top of the sample TEM grids. The sample TEM grids were dried in a vacuum desiccator overnight. During TEM imaging, energy dispersive X-ray spectroscopy (EDS) was used for a quantitative elemental analysis of uncoated SPIOs (Fe_3O_4). ImageJ (NIH, Bethesda, MD, USA) was used to measure the particle size of uncoated SPIOs and PEG-SPIOs [53].

2.7.2. Scanning electron microscopy (SEM)—Uncoated SPIOs and PEG-SPIOs were characterized by field-emission scanning electron microscopy (FE-SEM, Supra 55 VP, Carl Zeiss, Oberkochen, Germany) with an accelerating voltage of 2 kV. Each dried sample was placed on a metallic pin stub with adhesive and coated with carbon (Cressington 208C High Vacuum Turbo Carbon Coater, Ted Pella Inc.).

2.7.3. Dynamic light scattering (DLS) and zeta-potential—The hydrodynamic size (diameter), size distribution, polydispersity index (PDI), and zeta-potential of each sample were determined by dynamic light scattering (Zetasizer, Malvern NanoZS, Worcestershire, UK) at room temperature. For DLS, all samples were suspended in water at a SPIO concentration of 0.05 mg/mL, and placed in a folded capillary cuvette cell.

2.7.4. Nanoparticle tracking analysis (NTA)—The hydrodynamic size (diameter) and concentration of each sample were analyzed by NTA (Malvern NanoSight NS300, Worcestershire, UK) using a 532 nm continuous wave (CW) green laser. All samples were introduced into the sample chamber through the inlet tubing by disposable syringe. All

measurements were performed at room temperature. The scientific CMOS (SCMOS) camera captured movement of the nanoparticles' Brownian motion. Then, NTA software (NTA 3.2 Dev Build 3.2.16) was used to identify, track, and size particles individually within each frame.

2.7.5. Fourier transform infrared spectroscopy (FT-IR)—Fourier transform infrared spectroscopy (FT-IR Nicolet 8700 Spectrometer) was used to examine the characteristic bands of PEG 300 kDa, 200 kDa dextran backbone poly(gallol), complexed IPC-SPIOs, and decomplexed IPC-SPIOs. PEG signal was outside the detectable range with the Nicolet Spectrometer, so the PEG spectrum was instead collected using attenuated total reflectance (ATR), which resulted in narrower peaks due to the differences in data collection modes. Samples were deposited onto polished 13mm × 2mm KBr discs (International Crystal Labs, NJ, USA), and spectra were acquired in the range of approximately 2000 - 650 cm⁻¹.

2.7.6 Ultraviolet-visible spectroscopy—Ultraviolet-visible spectroscopy (Synergy H1 Microplate reader, BioTek, VT, USA) was used to examine the turbidity of various formulations with 40 and 200 kDa dextran backbone poly(gallol) during complexation and in response to superoxide by measuring the absorbance of each sample at 550 nm and 37°C. For this study, three different concentrations (1, 0.5, 0.25 mg/mL) of poly(gallol) were used and the control lacked phosphate buffer. Relative absorbance was calculated as the change in absorbance divided by the absorbance at zero time.

2.7.7. Measurement of magnetic resonance relaxivity—MR relaxivity of suspensions of nanocomplexes was measured. To determine T₂, T₂*, and T₁ relaxivities, samples of particles were suspended at various concentrations in phosphate buffer in a custom built, susceptibility-matched ULTEM 96-well plate [54] and scanned on a 3 T MR scanner using a knee coil (Philips 3T Achieva MRI, Best, Netherlands). In preliminary studies, complexed IPC-SPIOs made with 40 kDa dextran backbone poly(gallol) and PEG 6, 10, 35, or 300 kDa were scanned. In studies with 200 kDa dextran backbone poly(gallol), un-complexed, complexed, and decomplexed PEG 300 kDa-SPIOs were prepared and scanned. Uncoated SPIOs, uncoated SPIOs exposed to poly(gallol), and water were scanned as controls. A volume of 700 µL of each sample was placed into a well of the custom plate with approximate concentrations of 50, 100, 200, and 400 µM of iron. MR images were acquired for a single, 10 mm thick slice with coronal orientation (i.e. in a plane parallel to the surface of the well plate) and with a field of view of 75 × 75 mm. T₂-weighted images of particle suspensions were acquired using a multi-spin echo sequence with echo time (TE) = 7.5 ms, matrix size (MTX) = 256 × 256 pixels, echo train length (ETL) = 32, repetition time (TR) = 680 ms and number of averages (NSA) = 2, resulting in a total scan time of 5.8 minutes. T₂*-weighted images of particle suspensions were acquired using a multi-gradient echo sequence with 25 degree flip angle, TE = 3.9 ms, ETL = 30, MTX = 244 × 195 pixels, TR = 500 ms and NSA = 2, resulting in a total scan time of 3.3 minutes. T₁-weighted images of particle suspensions were acquired using a Look-Locker gradient recalled echo (GRE) sequence with 14 degree flip angle, TE = 4.5 ms, MTX = 192 × 176 pixels, EPI readout (ETL = 3), TR = 4 s, 55 equally-spaced inversion times (TI) = 50, 100, ..., 2750 ms and NSA = 1, resulting in a total scan time of 3.9 minutes. For studies done with 200 kDa

dextran backbone poly(gallol), iron concentrations were measured after MR scanning by inductively coupled plasma optical emission spectrometry (ICP-OES).

After obtaining the MR images, regions of interest (ROI) were drawn for each of the 96 wells, and the average pixel intensity in each ROI as a function of echo time (TE) or inversion time (TI) was measured using ImageJ software (NIH, Bethesda, MD, USA) [53]. These data, omitting the first echo time, were fit to the following T_2 , T_2^* , and T_1 equations using MATLAB® (The MathWorks Inc., Natick, MA, USA):

$$\text{For } T_2, M(TE) = A + M_0 \times e^{-(TE/T_2)},$$

$$\text{For } T_2^*, M(TE) = A + M_0 \times e^{-(TE/T_2^*)},$$

$$\text{For } T_1, \text{abs}[M(TI)] = \text{abs}[A + M_0 \times (1 - 2B \times e^{-(TI/T_1^*)})]$$

Here, A is a variable offset that partially accounts for the presence of Rician noise in the magnitude MR images while M_0 is the T_1 -weighted proton density, B accounts for incomplete inversion and can be initially set to “1” and T_1^* is an apparent T_1 related to the true T_1 by the equation $1/T_1 = 1/T_1^* + \ln(\cos(\alpha))/\tau$. Here, α is the Look-Locker flip angle (14 degrees) and τ is the Look-Locker inter-pulse delay (50 ms) [55].

2.7.8. Inductively coupled plasma optical emission spectrometry (ICP-OES) assay for iron content—After MR scans, particle suspensions were diluted 1:1000 in 3% HNO_3 , and the iron content was determined using ICP-OES (Varian Vista-MPX CCD Simultaneous ICP-OES, Varian Inc., Melbourne, Australia). Relaxivity was calculated as the slope of the linear regression of $R_2 = 1/T_2$, $R_2^* = 1/T_2^*$, or $R_1 = 1/T_1$ versus iron concentration, and the standard error of the regression was reported.

2.7.9. Statistics—Mean size data were compared using a one-way ANOVA and post-hoc Student’s t-test with Tukey corrections to determine the p-values. A one-way ANOVA and post-hoc Student’s t-test with Tukey corrections were used to compare mean relativities of different sample types. All statistical analysis was done in Microsoft Excel® (Redmond, WA, USA) or MATLAB® (The MathWorks Inc., Natick, MA, USA).

3. Results and discussion

3.1. Preparation and characterization of nanoparticles

As described previously [43], the synthesis of poly(gallol) consisted of conjugating benzyloxy benzoic acids as side groups to a linear dextran backbone of either 40 or 200 kDa molecular weight and converting the benzoic acids to phenols via a palladium black catalyzed deprotection reaction. Prior to lyophilization, the poly(gallol) was converted to sodium poly(gallate) by dissolution in a 1 M sodium carbonate buffer (pH 8) to change the

phenolic units to phenoxide salts, which are water soluble and more easily storable (Figure 1B).

As illustrated in Scheme 1, the SPIOs were created by coprecipitation of FeCl_2 , FeCl_3 , and NH_4OH in DI water (step 1) [24, 48–51]. Despite many advantages of the aqueous coprecipitation method for SPIOs synthesis such as ease of implementation, fast reaction, less use of hazardous materials, and high yield, resultant particles are typically quite polydisperse. SPIOs were PEGylated by adsorption of PEG of various molecular weights to the SPIO surface, and the poly(gallate) polymer solutions at various concentrations were mixed with the particles. Complexation was initiated by adding pH 7.4 phosphate buffer to protonate the poly(gallate) polymers back to poly(gallol)s (Figure 1B), allowing for hydrogen bonds to form between the poly(gallol) and PEG at the surface of the SPIOs to form a thick coating [43, 49]. The response of IPC-SPIOs to oxidation was observed in the presence of superoxide (O_2^-) created by the reaction of XOD and HX. Complexed and decomplexed particles were analyzed extensively, as detailed below.

Uncoated SPIOs, PEG 300 kDa-SPIOs, and PEG 300 kDa-SPIOs negatively stained with 2% phosphotungstic acid aqueous solution were imaged using TEM; representative images are shown in Figure 2A–C. SPIOs were spherical in shape, although particles clumped as an artifact of drying (Figure 2A–C), with the PEG 300 kDa-SPIOs exhibiting a gray halo around the darker SPIO particles, attributable to the polymer coating (Figure 2B). The negative staining technique was used to study the morphological shape and thickness of the dry-state of the polymer coating (Figure 2C). From the TEM images, the mean and standard deviation of particle size for uncoated SPIOs and the thickness of the PEG coating were measured to be 11 ± 4 nm ($n=101$) and 2.9 ± 1 nm ($n=49$), respectively. The EDS spectrum of the uncoated SPIOs (Figure 2D) showed peaks for iron and oxygen as expected, while the grid and Si (Li) detector caused peaks for carbon, copper, and silicon. These EDS data did not show any additional elemental peaks from impurities.

Uncoated SPIOs and PEG 300 kDa-SPIOs were also imaged by SEM (Figure 2E–F), which showed particles with a relatively spherical morphology and an average diameter slightly increasing with the addition of the polymer coatings from around 19 ± 3 nm ($n = 50$) for uncoated SPIOs to around 22 ± 3 nm ($n = 50$) for PEG 300 kDa-SPIOs. The complexed and decomplexed IPC-SPIOs were not suitable for imaging by TEM or SEM due to degradation of the polymer coatings under the electron beam.

As summarized in Table 1, nanoparticle hydrodynamic size was measured by DLS and NTA, and zeta potential was measured using electrophoretic DLS. NTA and DLS techniques measured the hydrodynamic radius in aqueous solutions, which was larger than the dry size measured by SEM and TEM due to solvated polymers and bound water. The mean hydrodynamic diameter of particles increased with the addition of PEG and poly(gallol) to the complex. Additionally, particle size increased during decomplexation, likely demonstrating swelling behavior. The zeta potential of uncoated SPIOs in DI water was -34 mV, similar to published values [56–58]. PEG-SPIOs had a slightly increased zeta potential of -27 mV, compared to uncoated SPIOs, indicating charge shielding due to particle surface coating with PEG [59, 60]. The zeta potential also increased towards neutral after

interpolymer complexation with poly(gallol) and again after decomplexation of the IPC-SPIOs. The increase in size of the SPIO polymer coatings moves the slipping plane farther from the SPIO surface, which may explain why the zeta potential shifts towards neutral [61, 62].

FT-IR spectroscopy was performed to demonstrate the successful attachment of PEG and complexation of poly(gallol) on the particles. Figure 3 shows FT-IR spectra of PEG 300 kDa (A), 200 kDa dextran backbone poly(gallol) (B), and complexed (C) and decomplexed (D) IPC-SPIOs (synthesized with PEG 300 kDa and 200 kDa dextran backbone poly(gallol)). The PEG spectrum showed peaks for CH₂ bending at 1466 cm⁻¹ and 1341 cm⁻¹, C-O-C, OH, and C-O-H stretching at 1279 cm⁻¹, C-O stretching at 1241 cm⁻¹, OH and C-O-H stretching at 1098 cm⁻¹, and C-H bending at 960 cm⁻¹ and 841 cm⁻¹. Poly(gallol) exhibited absorbance at 1590-1700 cm⁻¹, due to C=O stretching in the ester bonds, 1415-1570 cm⁻¹ due to the C=C stretching in the aromatic rings, 1003-1390 cm⁻¹ due to the combination of C-O stretching and in-plane aromatic C-H bending, and 750-900 cm⁻¹ due to the out-of-plane aromatic C-H bending. Figure 3 shows that the peaks in the range of 1590-1700 cm⁻¹ characteristic of poly(gallol) (B) and peaks in the range of 840-960 cm⁻¹ characteristic of PEG (A) were present in the spectrum of complexed IPC-SPIOs (C). This along with the previously published data [43] implies that the two polymers, PEG and poly(gallol), have entangled and created a complexed network. The FT-IR spectrum of decomplexed IPC-SPIOs shows significant overlap between characteristic peaks of PEG and poly(gallol), indicating that both polymers are still present in the swollen particles. Residual xanthine oxidase, hypoxanthine, and ammonium sulfate with peaks at 1632 cm⁻¹, 1680 cm⁻¹, and 1420-1450 cm⁻¹ and 1100 cm⁻¹, respectively, may contribute to peak broadening in Figure 3D (decomplexed IPC-SPIOs).

Based on previous work with IPC particles without SPIOs [43], we initially hypothesized that during decomplexation, the oxidized poly(gallol) would fully detach from the particles due to a disruption of hydrogen bonding between PEG and poly(gallol). However, the particle size, FT-IR spectra, and zeta potential of IPC-SPIOs indicated that decomplexation-dependent swelling of the particles likely occurred as some, but not all, hydrogen bonding was lost between poly(gallol) and PEG. Since the design of the nanocomplexes for MR imaging depends on access of water to the central SPIO, swelling of the particles during decomplexation of IPC-SPIOs was expected to demonstrate a substantial change in relaxivity, as was confirmed in our MR studies below.

3.2. MRI-Based T₂-weighted deactivation of complexed IPC-SPIOs

A preliminary investigation of the MR properties of complexed IPC-SPIOs using 40 kDa dextran backbone poly(gallol), a SPIO core of 11 ± 4 nm and varying molecular weights of PEG was conducted in a 3T scanner by suspending particles in water in a custom well plate. Complexed IPC-SPIOs were synthesized by separately combining 1 mg/mL 40 kDa dextran backbone poly(gallol) with serial dilutions of uncoated SPIOs, PEG 6 kDa-SPIOs, PEG 10 kDa-SPIOs, PEG 35 kDa-SPIOs, or PEG 300 kDa-SPIOs. For these samples, measurement of iron content was not performed, so the absolute relaxivities are not reported here. The MR signal arising from complexed PEG 6 kDa-SPIOs, PEG 10 kDa-SPIOs, and PEG 35 kDa-

SPIOs samples were all concentration dependent, based on the serial dilutions made. However, the MR signal from the nanocomplexes that made use of 300 kDa molecular weight PEG did not significantly vary with concentration, indicating that these complexes had much lower relaxivity than the other complexes. This lower relaxivity in the complexed, OFF-state is a desirable property for our contrast agents. Uncoated SPIOs mixed with poly(gallol) had the highest signal, likely indicating that the large reduction in relaxivity observed when PEG-SPIOs are mixed with poly(gallol) is due to interpolymer complexation, not merely coating the particle surface with poly(gallol). Low and intermediate molecular weight PEG was not studied further due to its intermediate effect on relaxivity when complexed in the IPC-SPIOs; further studies were conducted with PEG 300 kDa.

To further understand the complexation and decomplexation of these nanoparticles, the UV/Vis absorbance of various formulations was monitored over time at 550 nm for changes in turbidity, which is indicative of nanocomplex size. SPIOs coated with PEG 300 kDa were complexed with poly(gallol) synthesized with either a 40 or 200 kDa dextran backbone at three separate concentrations. Absorbance was measured for 6 hours during complexation (Figure 4). Turbidity increased before leveling off or slightly dropping, likely indicating the completion of complexation. The negative control that lacked phosphate buffer showed insignificant change in turbidity (Figure 4). Following 6 hours of monitoring complexation, IPC-SPIOs were exposed to the superoxide product of HX and XOD to initiate decomplexation. During decomplexation, when poly(gallol)s are oxidized and lose hydrogen bonding with PEG, the absorbance at 550 nm increases, which likely indicated swelling. Controls lacking superoxide showed minimal change in turbidity, confirming minimal if any spontaneous decomplexation within this time period. Furthermore, these absorbance data indicated that the 200 kDa dextran backbone poly(gallol) IPC-SPIOs complexed readily and were much more responsive in swelling in the presence of superoxides compared to 40 kDa dextran backbone poly(gallol) IPC-SPIOs. It is hypothesized that the higher molecular weight dextran backbone promoted more hydrogen bonding with the high molecular weight PEG, which could then be disrupted due to oxidation of the poly(gallol). Subsequent studies utilized 200 kDa dextran backbone poly(gallol) as this formed IPC-SPIOs that had greater swelling during decomplexation and were expected to result in more pronounced changes in relaxivity in response to oxidation.

3.3. MRI-Based T₂-weighted activation of complexed IPC-SPIOs

Finally, the MR relaxivity of IPC-SPIO particles containing 200 kDa dextran backbone poly(gallol) complexed with 300 kDa PEG was measured using a 3T scanner, and inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to measure iron content. Decomplexed IPC-SPIOs were formed by placing complexed IPC-SPIOs in an oxidative environment created by XOD and HX. MR relaxivities of uncoated SPIOs, PEG-SPIOs, complexed IPC-SPIOs, and decomplexed IPC-SPIOs were measured (Figure 5) using a spin echo sequence with varying TE, with images of each complex at the highest iron concentration tested shown in Figure 5B. Negative contrast agents result in rapid loss of image intensity with increasing TE as a consequence of shortened T₂. Uncoated SPIOs (1) had the highest T₂ relaxivity of $470 \pm 30 \text{ mM}^{-1} \cdot \text{s}^{-1}$ while PEG-SPIOs (2) had a relaxivity of

350 $\pm$ 30 mM⁻¹·s⁻¹. Despite low PDI values measured from DLS, the high r_2 value of uncoated SPIOs is likely due to polydispersity of the sample. Additionally, the effect of poly(gallol) concentration on T₂ relaxivity was measured by varying the concentrations of poly(gallol) (1, 0.5, 0.25, 0.125, and 0.0625 mg/mL) complexed with PEG 300 kDa-SPIOs (Table 2). A poly(gallol) concentration of 0.25 mg/mL yielded the greatest difference between complexed and decomplexed relaxivities, with a complexed relaxivity of 7 $\pm$ 2 mM⁻¹·s⁻¹ and decomplexed relaxivity of 70 $\pm$ 10 mM⁻¹·s⁻¹. Spin echo images of the complexed IPC-SPIOs (lane 3 in Fig. 8B) decreased in intensity only at long TE, indicating that the polymer coating greatly attenuated the relaxivity of the SPIOs. Finally, images of the decomplexed particles (lane 4) show a substantial increase in T₂ relaxivity. This recovery of relaxivity is dependent upon the concentration of poly(gallol), as shown in Table 2, with the ratio of decomplexed to complexed relaxivity being 2.5 $\pm$ 0.2^a, 5.7 $\pm$ 0.6^b, 10.3 $\pm$ 0.4^c, 10.6 $\pm$ 1.3^c, and 7.2 $\pm$ 1.7^{b,c} with poly(gallol) concentrations of 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL, respectively (mean $\pm$ standard error, results that do not share letter superscripts are significantly different ($p < 0.05$)). These data indicate that IPC-SPIOs can be formulated to increase relaxivity up to 10-fold upon exposure to physiologically relevant ROS such as superoxides.

Our data indicate that SPIOs experienced increased shielding from water when surrounded by the complexed poly(gallol)-PEG IPC, which limited the interaction of solute water with the SPIO surface. Previous work by Bao et al. described the dependence of T₂ relaxivity on the thickness of the SPIO surface coating [26, 28]. They found that a coating creates two distinct layers around the SPIO surface: 1) an exclusion radius immediately surrounding the SPIO that water cannot penetrate, and 2) a slow compartment radius in which water diffusion is slowed but water is not excluded. Their Monte Carlo simulations modeled the movement of water protons in a field with SPIOs and showed that increasing the exclusion radius dramatically decreases the effective T₂ relaxivity of the particle. However, increasing the thickness of the slow compartment radius slightly increases the T₂ relaxivity because slower water diffusion allows for increased residence time of water molecules within the inhomogeneous magnetic field produced by the iron oxide core. This slow compartment effect is outweighed by the negative effect of the exclusion radius on T₂ relaxivity, and T₂ relaxivity decreases to near zero when the exclusion radius of the coating reaches 50 nm (for a 6.6 nm diameter SPIO core), regardless of the slow compartment radius thickness. The data presented in this paper confirm experimentally that the formation of a ~70 nm interpolymer coating created by the 0.25 mg/mL 200 kDa dextran backbone poly(gallol) and PEG 300 kDa around the 11 nm SPIO core reduced the particle's T₂ relaxivity compared to the uncoated SPIO by approximately 99%. In essence, particle complexation turns OFF the T₂ contrast-causing ability of the particles. The nature of the hydrogen bond- based complexation between the poly(gallol) and PEG excludes water from the complex. Due to the pre- mixing of PEG-SPIOs and sodium poly(gallate) prior to the addition of phosphate buffer to convert the poly(gallate)s to poly(gallol)s, which initiates the complexation, we hypothesize that PEG and poly(gallol) are intertwined as a coating on the SPIO surface. PEG-poly(gallol) hydrogen bonding is disrupted upon oxidation of the poly(gallol) side groups, leading to particle swelling that concomitantly decreases the exclusion radius of the decomplexed IPC-SPIOs and increases the T₂ relaxivity. Despite the fact that the

hydrodynamic radius of the particles grows with swelling upon oxidation, the exclusion radius—the distance that water cannot penetrate—decreases. We speculate that SPIO contrast is activatable upon exposure to physiologically relevant ROS as particles decomplex and the exclusion radius of the IPC-SPIOs decrease.

As expected, the OFF/ON activation of this system was not exhibited in T_2^* relaxation. Since the contribution of SPIOs to magnetic field inhomogeneity in T_2^* contrast is long-range (μm to mm), these effects are likely much harder to silence using a nanoparticle surface coating. T_2^* relaxivities of IPC-coated particles were significantly smaller compared to PEG-coated particles, but there was no statistically significant difference in T_2^* relaxivity between complexed and decomplexed IPC-SPIOs at any of the tested poly(gallol) concentrations. Finally, T_1 relaxivity was impacted by the IPC coating in a similar manner to T_2 with complexed IPC-SPIOs exhibiting a statistically significant lower T_1 relaxivity than PEG-SPIOs at all poly(gallol) concentrations. Also, the complexed particles had a lower relaxivity than decomplexed particles at all poly(gallol) concentrations except 1 mg/mL. However, the relative change in T_1 relaxivity upon decomplexation was much smaller than that seen for T_2 relaxivity, which is attributable to the known dominant effect of SPIO particles on dephasing through establishment of microinhomogeneities in the local magnetic field.

4. CONCLUSIONS

This study shows the successful creation of an activatable MR contrast agent based on superparamagnetic iron oxide nanoparticles. Superparamagnetic nanoparticles were synthesized using the coprecipitation method and were coated with PEG via adsorption. PEGylated SPIOs were complexed with poly(gallol) to produce complexed IPC-SPIOs. Finally, complexed IPC-SPIOs were decomplexed in an oxidative environment. The complexed IPC-SPIOs had a low T_2 relaxivity, indicating that the polymer coating shielded the SPIO core from interacting with surrounding protons; this represents the OFF stage of the activatable agent. The decomplexed IPC-SPIOs showed a partial but substantial recovery of T_2 relaxivity and represent the ON stage of the agent. DLS, zeta potential, and UV/Vis spectroscopy results support evidence of a swelling upon decomplexation. This activatable characteristic of the particles carries the potential to permit measurement of local oxidative stress in a variety of disease states.

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Highlights

- An activatable T_2 magnetic resonance contrast agent has been created.
- Superparamagnetic iron oxide nanoparticles are encapsulated in complexed polymers.
- The complexed polymers swell when oxidized, activating the contrast agent.
- The activation is confirmed with measurements of MR relaxivity and size changes.

Figure 1.

(A) Poly(gallol) and poly(ethylene glycol) interact via hydrogen bonding when complexed. In the presence of ROS such as superoxides, the poly(gallol) is oxidized and the two polymers can no longer maintain this interaction, leading to decomplexation. (B) During polymer preparation, poly(gallol) is converted to sodium poly(gallate) to improve solubility and storage. During IPC-SPIO synthesis, poly(gallate) salts are returned to poly(gallol)s by adding phosphate buffer to allow for complexation between the polymer and PEG, as illustrated in (A).

Figure 2.

TEM images (scale bar of 20 nm) of uncoated SPIOs (A), PEG 300 kDa-SPIOs (B), and PEG 300 kDa-SPIOs negatively stained with 2% phosphotungstic acid (C), and EDS spectrum of uncoated-SPIOs (Fe_3O_4) (D). SEM images of uncoated SPIOs (E) and PEG 300 kDa-SPIOs (F), scale bar is 100 nm. Diameters were quantified (Table 1).

Figure 3.

Fourier transform infrared spectra of PEG 300 kDa, 200 kDa dextran backbone poly(gallol), complexed IPC-SPIOs and decomplexed IPC-SPIOs synthesized with PEG 300 kDa and 200 kDa dextran backbone poly(gallol).

Figure 4.

Relative change in UV/Vis absorbance at 550 nm over time compared to zero time, indicative of increased turbidity upon formation of IPC-SPIOs containing PEG 300 kDa and three different concentrations of 40 or 200 kDa dextran backbone poly(gallol) (A, C) and decomplexation in the presence of superoxides (B,D). For complexation, the controls contained all substituents but lack phosphate buffer. For decomplexation, the controls contained complexed particles but no superoxides. Data presented are mean $\pm$ standard deviation, $n = 3$.

Figure 5.

Relaxation rate (R_2) plotted against the measured concentration of iron for SPIO particles coated with PEG 300 kDa and complexed with 0.25 mg/mL of 200 kDa dextran backbone poly(gallol), showing low relaxivity when complexed and recovering relaxivity when decomplexed (A) and T₂-weighted MR images at the highest tested iron concentration of uncoated SPIOs (1), PEG-SPIOs (PEG 300 kDa) (2), complexed IPC-SPIOs (PEG 300 kDa) (3), and decomplexed IPC-SPIOs (PEG 300 kDa) (4) showing increasing echo time (TE 7.5 ms) from left to right (B).

Scheme 1.

Schematic representing formulation of uncoated-SPIOs (step 1), PEGylated SPIOs (step 2), complexed IPC-SPIOs (step 3), and decomplexed IPC-SPIOs (step 4).

Table 1

Hydrodynamic size, polydispersity index (PDI), and zeta potential (ζ P) of uncoated-SPIOs, PEG-SPIOs, complexed IPC-SPIOs, and decomplexed IPC-SPIOs with PEG 300 kDa and 0.25 mg/mL 200 kDa dextran backbone poly(galloyl) measured in triplicate using a Malvern Zetasizer ZS. Sizes also were measured by TEM, SEM, and NTA. Values are shown as mean $\pm$ standard deviation of triplicate tests. The sizes and zeta potentials of the four types of nanoparticles were significantly different ($p < 0.05$).

Nanocomplexes	TEM Size (nm)	SEM Size (nm)	NTA Size (nm)	DLS Size (nm)	DLS PDI	DLS ζ P (mV)
<i>Uncoated SPIOs</i>	11 $\pm$ 4	19 $\pm$ 3	81 $\pm$ 21	56 $\pm$ 1	0.12 $\pm$ 0.01	-34 $\pm$ 1
<i>PEG-SPIOs</i>	3 $\pm$ 1 (coating)	22 $\pm$ 3	106 $\pm$ 28	68 $\pm$ 2	0.13 $\pm$ 0.02	-27.7 $\pm$ 0.4
<i>Complexed IPC-SPIOs</i>	-	-	205 $\pm$ 50	150 $\pm$ 3	0.15 $\pm$ 0.02	-10.4 $\pm$ 0.4
<i>Decomplexed IPC-SPIOs</i>	-	-	240 $\pm$ 60	258 $\pm$ 1	0.14 $\pm$ 0.01	-6.7 $\pm$ 0.5

Table 2

T_2 , T_2^* , and T_1 relaxivities of uncoated SPIOs ($n = 4$), PEG-SPIOs ($n = 4$), complexed IPC-SPIOs, and decomplexed IPC-SPIOs with PEG 300 kDa and 200 kDa dextran backbone poly(gallol) concentrations of 1 mg/mL ($n = 12$), 0.5 mg/mL ($n = 4$), 0.25 mg/mL ($n = 4$), 0.125 mg/mL ($n = 4$), and 0.0625 mg/mL ($n = 4$). Relaxivity expressed as linear regression best fit $\pm$ standard error of the regression.

Formulation	Poly(gallol) concentration	$r_2(\text{mM}^{-1}\cdot\text{s}^{-1})$	$r_2^*(\text{mM}^{-1}\cdot\text{s}^{-1})$	$r_1(\text{mM}^{-1}\cdot\text{s}^{-1})$
Uncoated SPIOs	--	470 ± 30	710 ± 240	4.9 ± 0.5
PEG-SPIOs	--	350 ± 30	590 ± 60	4.3 ± 0.3
Complexed IPC-SPIOs	1 mg/mL	$24 \pm 4^*$	$190 \pm 50^*$	$0.91 \pm 0.04^*$
Decomplexed IPC-SPIOs	1 mg/mL	$60 \pm 4^{*\dagger}$	$290 \pm 50^{\S}$	$0.84 \pm 0.02^*$
Complexed IPC-SPIOs	0.5 mg/mL	$14 \pm 7^*$	$200 \pm 60^*$	$0.85 \pm 0.05^*$
Decomplexed IPC-SPIOs	0.5 mg/mL	$80 \pm 20^{*++}$	$300 \pm 80^{\S}$	$1.23 \pm 0.02^{*\dagger}$
Complexed IPC-SPIOs	0.25 mg/mL	$7 \pm 2^*$	$230 \pm 50^*$	$0.70 \pm 0.04^*$
Decomplexed IPC-SPIOs	0.25 mg/mL	$70 \pm 10^{*\dagger}$	$240 \pm 50^*$	$1.14 \pm 0.07^{*\dagger}$
Complexed IPC-SPIOs	0.125 mg/mL	$6 \pm 8^*$	$210 \pm 50^*$	$0.65 \pm 0.05^*$
Decomplexed IPC-SPIOs	0.125 mg/mL	$66 \pm 8^{*\dagger}$	$230 \pm 50^*$	$1.13 \pm 0.03^{*\dagger}$
Complexed IPC-SPIOs	0.0625 mg/mL	$7 \pm 12^*$	$280 \pm 40^{\S}$	$0.4 \pm 0.2^*$
Decomplexed IPC-SPIOs	0.0625 mg/mL	$51 \pm 12^{*++}$	$210 \pm 50^*$	$1.13 \pm 0.03^{*++}$

If denoted, within categories of T_2 , T_2^* , and T_1 relaxivities, values are significantly different (§ $p < 0.05$, * $p < 0.005$) than the PEG-SPIO relaxivity; relaxivity for decomplexed particles is significantly different ($^{++}$ $p < 0.05$, † $p < 0.005$) than complexed particles at the matched poly(gallol) concentration.