

Video Article

Biofunctionalized Prussian Blue Nanoparticles for Multimodal Molecular Imaging Applications

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Abstract

Multimodal, molecular imaging allows the visualization of biological processes at cellular, subcellular, and molecular-level resolutions using multiple, complementary imaging techniques. These imaging agents facilitate the real-time assessment of pathways and mechanisms *in vivo*, which enhance both diagnostic and therapeutic efficacy. This article presents the protocol for the synthesis of biofunctionalized Prussian blue nanoparticles (PB NPs) - a novel class of agents for use in multimodal, molecular imaging applications. The imaging modalities incorporated in the nanoparticles, fluorescence imaging and magnetic resonance imaging (MRI), have complementary features. The PB NPs possess a core-shell design where gadolinium and manganese ions incorporated within the interstitial spaces of the PB lattice generate MRI contrast, both in T₁ and T₂-weighted sequences. The PB NPs are coated with fluorescent avidin using electrostatic self-assembly, which enables fluorescence imaging. The avidin-coated nanoparticles are modified with biotinylated ligands that confer molecular targeting capabilities to the nanoparticles. The stability and toxicity of the nanoparticles are measured, as well as their MRI relaxivities. The multimodal, molecular imaging capabilities of these biofunctionalized PB NPs are then demonstrated by using them for fluorescence imaging and molecular MRI *in vitro*.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52621/>

Introduction

Molecular imaging is the non-invasive and targeted visualization of biological processes at the cellular, subcellular, and molecular levels¹. Molecular imaging permits a specimen to remain in its native microenvironment while its endogenous pathways and mechanisms are assessed in real-time. Typically, molecular imaging involves the administration of an exogenous imaging agent in the form of a small molecule, macromolecule, or nanoparticle to visualize, target, and trace relevant physiological processes being studied². The various imaging modalities that have been explored in molecular imaging include MRI, CT, PET, SPECT, ultrasound, photoacoustics, Raman spectroscopy, bioluminescence, fluorescence, and intravital microscopy³. Multimodal imaging is the combination of two or more imaging modalities where the combination enhances the ability to visualize and characterize various biological processes and events⁴. Multimodal imaging exploits the strengths of the individual imaging techniques, while compensating for their individual limitations⁵.

This article presents the protocol for the synthesis of biofunctionalized Prussian blue nanoparticles (PB NPs) - a novel class of multimodal, molecular imaging agents. The PB NPs are utilized for fluorescence imaging and molecular MRI. PB is a pigment consisting of alternating iron (II) and iron (III) atoms in a face-centered cubic network (**Figure 1**). The PB lattice is comprised of linear cyanide ligands in a Fe^{II}-CN-Fe^{III} linkage that incorporates cations to balance charges within its three-dimensional network⁶. The ability of PB to incorporate cations into its lattice is exploited by separately loading gadolinium and manganese ions into the PB NPs for MRI contrast.

The rationale for pursuing a nanoparticle design for MRI contrast is because of the advantages this design offers relative to current MRI contrast agents. The vast majority of US FDA-approved MRI contrast agents are gadolinium chelates that are paramagnetic in nature and provide positive contrast by the spin-lattice relaxation mechanism^{6,7,8}. As compared to a single gadolinium-chelate that provides low signal intensity on its own, the incorporation of multiple gadolinium ions within the PB lattice of the nanoparticles provides enhanced signal intensity (positive contrast)^{3,9}. Further, the presence of multiple gadolinium ions within the PB lattice increases the overall spin density and the magnitude of paramagnetism of the nanoparticles, which disturbs the local magnetic field in its vicinity, thereby generating negative contrast by the spin-spin relaxation mechanism. Thus the gadolinium-containing nanoparticles function both as T₁ (positive) and T₂ (negative) contrast agents^{10,11}.

In a subset of patients with impaired renal function, the administration of gadolinium-based contrast agents has been linked to the development of nephrogenic systemic fibrosis^{8,12,13}. This observation has prompted investigations into the use of alternative paramagnetic ions as contrast

agents for MRI. Therefore, the versatile design of the nanoparticles is adapted to incorporate manganese ions within the PB lattice. Similar to gadolinium-chelates, manganese-chelates are also paramagnetic and are typically used to provide positive signal intensity in MRI^{7,14}. As with gadolinium-containing PB NPs, the manganese-containing PB NPs also function as T₁ (positive) and T₂ (negative) contrast agents.

To incorporate fluorescence imaging capabilities, the nanoparticle “cores” are coated with a “biofunctional” shell consisting of the fluorescently-labeled glycoprotein avidin (**Figure 1**). Avidin not only enables fluorescence imaging, but also serves as a docking platform for biotinylated ligands that target specific cells and tissue. The avidin–biotin bond is one of the strongest known, non-covalent bonds characterized by extremely strong binding affinity between avidin and biotin¹⁵. The attachment of biotinylated ligands to the avidin-coated PB NPs confers molecular targeting capabilities to the PB NPs.

The motivation for pursuing fluorescence and MR imaging using PB NPs is because these imaging modalities possess complementary features. Fluorescence imaging is one of the most widely used optical molecular imaging techniques, and allows for the simultaneous visualization of multiple objects at high sensitivities^{1,16,17}. Fluorescence imaging is a safe, non-invasive modality but is associated with low depths of penetration and spatial resolutions^{1,3,16}. On the other hand, MRI generates high temporal and spatial resolution non-invasively and without a need for ionizing radiation^{1,3,16}. However MRI suffers from low sensitivity. Therefore fluorescence imaging and MRI were selected as the molecular imaging techniques due to their complementary features of depth penetration, sensitivity, and spatial resolution.

This article presents the protocol for the synthesis and biofunctionalization of the PB NPs, gadolinium-containing PB NPs (GdPB), and manganese-containing PB NPs (MnPB)^{10,11}. The following methods are described: 1) measurement of size, charge, and temporal stability of the nanoparticles, 2) evaluation of cytotoxicity of the nanoparticles, 3) measurement of MRI relaxivities, and 4) utilization of the nanoparticles for fluorescence and molecular MR imaging of a population of targeted cells *in vitro*. These results demonstrate the potential of the NPs for use as multimodal, molecular imaging agents *in vivo*.

Protocol

1. Synthesis of PB NPs, GdPB, and MnPB

Synthesis of the nanoparticles (PB NPs, GdPB, or MnPB) is achieved using a one-pot synthesis scheme by performing the steps detailed below:

1. Prepare solution 'A' containing 5 ml of 5 mM potassium hexacyanoferrate (II) in deionized (DI) water. Depending on the type of nanoparticle being synthesized — PB NPs, GdPB, or MnPB, prepare solution 'B' as follows:
 1. For PB NPs: prepare 10 ml of a solution containing 2.5 mM iron (III) chloride in DI water.
 2. For GdPB NPs: prepare 10 ml of a solution containing 2.5 mM each of gadolinium (III) nitrate and iron (III) chloride in DI water
 3. For MnPB NPs: prepare 10 ml of a solution containing 2.5 mM each of manganese (II) chloride and iron (III) chloride in DI water.
2. Add solution 'B' to a round-bottom flask, and stir the flask contents at room temperature (RT) and 1,000 rpm. Add solution 'A' drop-wise into the round-bottom flask containing solution 'B'. Control the flow rate for the addition of solution 'A' to 'B' by a peristaltic pump set to dispense approximately 10 ml/hr.
3. Continue stirring at 1,000 rpm at RT for an additional 30 min after the addition of solution 'A' to 'B' is complete. Stop stirring and collect the mixture.
4. Transfer aliquots of the mixture into microcentrifuge tubes to rinse the nanoparticles free of unreacted components. Add 5 M NaCl (0.2 ml NaCl/ml reaction mixture) to each aliquot to assist in particle collection by centrifugation.
5. Centrifuge each aliquot of nanoparticles for at least 10 min at 20,000 × g. After centrifugation, carefully remove the supernatant.
6. Resuspend each nanoparticle pellet in 1 ml DI water via sonication using a microtip (sonication pulse on/off = 1/1 sec, amplitude = 50%, duration = 5 sec, sonicator power rating = 125 W) to break up the pellet.
7. Repeat steps 1.4–1.6 at least 3× to ensure that the nanoparticles are free of components of the initial reaction and reaction byproducts. After the final centrifugation, resuspend particles in 1 ml DI water.

2. Biofunctionalization of PB NPs, GdPB, and MnPB

Biofunctionalization of the nanoparticles involves coating of the nanoparticle “cores” with avidin and adding biotinylated ligands as described below:

1. Coating of the Nanoparticles with Fluorescent Avidin
Coating of the nanoparticles with fluorescent avidin is achieved using electrostatic self-assembly where positively charged avidin (pI ~10.5) is coated on to negatively charged nanoparticles as follows¹⁸:
 1. Prepare suspensions of the PB NPs, GdPB, or MnPB in 1 ml DI water. Filter Alexa Fluor 488-labeled avidin (A488; reconstituted in DI water) through a 0.2 µm nylon microcentrifuge filter at 14,000 × g for 10 min. Add ≤0.2 mg avidin/mg nanoparticles. Add each filtered aliquot of A488 separately to the aliquots of PB NPs, GdPB, or MnPB.
 2. Contact the nanoparticles with A488 for 2–4 hr with gentle shaking or rotation at 4 °C. Protect the samples from light using aluminum foil.
NOTE: This step yields A488 coated PB NPs (PB-A488), GdPB (GdPB-A488), and MnPB (MnPB-A488).
2. Attachment of Biotinylated Antibodies
Attachment of biotinylated targeting antibodies onto the avidin-coated nanoparticles is achieved using avidin-biotin interactions as follows:
 1. Prepare suspensions of the avidin coated nanoparticles — PB-A488, GdPB-A488, and MnPB-A488 in 1 ml DI water. Filter biotinylated antibody (as supplied by the manufacturer) through a 0.2 µm nylon microcentrifuge filter at 14,000 × g for 10 min.

NOTE: Here, the present study uses biotinylated anti-neuron-glia antigen 2 (ANG2) that targets NG2 overexpressed within cells and tissues of the central nervous system and biotinylated anti-human eotaxin-3 (Eot3) that targets receptors overexpressed on eosinophils or eosinophilic cell lines.

2. Add each filtered aliquot of biotinylated antibody separately to the aliquots of the avidin-coated nanoparticles. Add ≤ 0.05 mg biotinylated antibody/mg avidin-coated nanoparticles. Contact the avidin-coated nanoparticles with the biotinylated antibodies (ANG2 or Eot3) for 2-4 hr with gentle shaking or rotation at 4 °C. Protect the samples from light using aluminum foil.

NOTE: This step yields antibody coated nanoparticles (e.g. GdPB-A488-Eot3 and MnPB-A488-ANG2).

3. Sizing, Zeta Potential, and Temporal Stability of the Nanoparticles

The size distribution, charge, and stability of the nanoparticles are measured using dynamic light scattering (DLS) methods as described below:

1. Sizing of the Nanoparticles

Sizing of the nanoparticles is achieved using dynamic light scattering as follows:

1. Add 10 μ l of the nanoparticle sample (1 mg/ml) to 990 μ l of DI water in a disposable plastic cuvette.
NOTE: This is representative value for a good DLS signal.
2. Cap the cuvette and vortex to mix well. Place the cuvette in a system used to analyze particle size in order to measure the size of the nanoparticles. Carry out particle size analysis at a measurement angle of 173°.

2. Zeta Potential of the Nanoparticles

Zeta potential of the nanoparticles is measured using phase analysis light scattering as follows:

1. Add 100 μ l of the nanoparticle sample (1 mg/ml) to 900 μ l of DI water in a disposable plastic capillary cell. This value is representative for a good zeta potential measurement.
2. Place the capillary cell in a system used to analyze zeta potential in order to measure the zeta potential of the nanoparticles using default parameters at 25 °C.

3. Temporal Stability of the Nanoparticles

Temporal stability of the nanoparticles is measured using dynamic light scattering as follows:

1. Assess the stability of nanoparticles by measuring the sizes of the nanoparticles in DI water as well as Dulbecco's Modified Eagle's Medium (DMEM) as described in section 3.1.
2. Repeat the size measurements in DI water and DMEM once/day over a period of 5 days.

4. Cytotoxicity of the Nanoparticles

Cytotoxicity of the nanoparticles is measured using an XTT cell proliferation assay as follows:

1. Seed 10,000-15,000 cells/well of each cell type studied (Neuro2a, BSG D10, EoL-1, and OE21) in a 96-well plate. Ensure that the total volume of seeded cells does not exceed 0.2 ml/well. Incubate seeded cells overnight at 37 °C and 5% CO₂.
2. Contacting nanoparticles with the cells:
 1. Incubate the cells with varying concentrations of nanoparticles (0.01 – 0.5 mg/ml). Refer to Table 1 as a representative table that describes the amounts of the nanoparticles (in 50 μ l) added to the cells after removing 50 μ l of medium/well.
 1. To account for any interfering absorbance of the nanoparticles within the assay, add a blank consisting of the appropriate concentration of nanoparticles (0-0.5 mg/ml; in equivalence to the amounts added to the cells) to medium without cells.
 2. Incubate cells with nanoparticles overnight at 37 °C and 5% CO₂. Aspirate media from each well and rinse with staining buffer comprised of 5% fetal bovine serum (FBS) in phosphate-buffered solution (PBS). Add 100 μ l of media without phenol red and incubate at 37 °C and 5% CO₂ for 16-18 hr.
3. Prepare the XTT Cell Proliferation Assay working solution by mixing the kit components (XTT Reagent and XTT Activator) as per the manufacturer's specifications. Aspirate the media from the wells and add 100 μ l of RPMI (RPMI w/o phenol red + 10% FBS + 1 $\times$ Pen-Strep) or appropriate medium for the cell type studied. Add 50 μ l of XTT working solution to each well and incubate at 37 °C and 5% CO₂ for 2-2.5 hr.
4. Measure the absorbance at 490 nm, with a reference wavelength of 630 nm to correct for fingerprints or smudges. Calculate the corrected absorbance for each well ($A_{490} - A_{630}$) and average the readings for replicates.
5. Subtract the blank readings from each well value to calculate the final corrected absorbance values. Calculate the survival percentage for each sample by normalizing the final corrected absorbance to that of untreated cells without nanoparticles. Plot survival percentage as a function of nanoparticle concentration.

5. MRI Relaxivities of the PB NPs, GdPB, and MnPB

MRI relaxivity is measured using T₁- and T₂-weighted sequences by preparing an MRI "phantom" using a 96-well plate containing nanoparticles as described below:

1. Phantom Preparation

1. Prepare a 96-well plate with each well containing 100 μ l nanoparticles (PB NPs, GdPB, or MnPB) at the appropriate concentration. Beginning with a concentration of 0.4 mM for each type of nanoparticle, serially dilute the nanoparticles 2 $\times$ using DI water until a concentration of 2.4×10^{-5} M is reached (this requires 14 dilutions and 15 wells).

2. Add 100 μ l of molten 1% agarose solution in DI water into each well and mix well. Allow the gel to solidify at 4 °C for 12 hr.
NOTE: This yields a phantom containing serial dilutions of the nanoparticles in solidified agarose.
3. Place the phantom in a horizontal 3 T clinical magnet under a solid block of 2% agar (150 cm³). Secure the phantom and block of agar within the center of an 8-channel HD brain coil. Measure relaxation times using 0.5 mm thick coronal slices at the mid-height of the 96-well plate¹¹.
2. T₁- and T₂-Weighted Sequences
Use the following representative settings for acquiring the sequences in the clinical magnet.
NOTE: These values are optimized for the nanoparticles used in the present study:
 1. Acquire T₁-weighted (T1W) MR images using the following fluid attenuated inversion recovery (FLAIR) sequence: Echo train (ET) = 8, Repetition time (TR) = 2,300 msec, Echo time (TE) = 24.4 msec, Matrix size = 512 x 224, Field of view (FOV) = 16 x 16 cm².
 2. Acquire T₂-weighted (T2W) MR images using the following fast relaxation fast spin echo (FRFSE) sequence: ET = 21; TR = 3,500 msec; TE = 104 msec; Matrix size = 512 x 224; FOV = 16 x 16 cm².
 3. Acquire T1W and T2W MR images at 127 MHz (3 T) at the following inversion times: 50, 177, 432, 942, 1,961, and 4,000 msec.
3. Measuring the MRI Relaxivities
 1. After acquiring T1W and T2W images using the sequences described in section 5.2, measure the signal intensity for each well using ImageJ, henceforth designated as the region of interest (ROI) by selecting each ROI using the oval crop button located on the main toolbar, then selecting Analyze > Measure.
NOTE: A pop-up should display the mean signal intensity of each ROI under the column labeled "Mean".
 2. For each ROI, plot the measured signal intensity against its specific inversion time. If needed, invert points (readings) that generate an initial "bubble" or outliers at the beginning of the plot (lower inversion times).
NOTE: These readings are generated at low inversion times because MRI measures the magnitude or absolute value of images rather than their actual (negative) value, which needs to be accounted/corrected for¹⁹.
 3. Prepare an exponential fit for each plot of signal intensity for the ROIs (SI) vs. inversion times (T_i) as described in section 5.2. Plot T_i on the x-axis, SI on the y-axis; α and Δ are constants determined by regression, and T₁ or T₂ is the variable to be solved:

$$SI(t) = \alpha e^{\frac{-T_i}{t}} + \Delta$$

where $t = T_1, T_2$.

4. Solve for T₁ or T₂ using the above equation and plot the inverse of the calculated values (1/T₁ and 1/T₂) against the concentrations of the nanoparticles used in each ROI.
5. Calculate the slope of the linear graph which yields the relaxivities r₁ and r₂.
NOTE: The following section of the protocol describes the application of the nanoparticles for fluorescent labeling and generating MRI contrast in targeted cells.

6. Fluorescent Labeling of Targeted Cells Using the Nanoparticles — Confocal Microscopy

NOTE: The nanoparticles (PB NPs, GdPB, and MnPB) can be used to fluorescently label a population of targeted cells (monitored by confocal microscopy) as follows:

1. Synthesis of the Fluorescent Nanoparticles
 1. Synthesize GdPB-A488, GdPB-A488-Eot3, MnPB-A488, MnPB-A488-ANG2 for fluorescent labeling of a population of targeted cells by following the steps detailed in sections 1 and 2.
2. Preparation of Cells for Fluorescence Targeting
 1. Coat no. 1.5 micro cover glasses by dipping them in a solution of 0.002% poly(L-lysine) hydrobromide for 90 min. Remove the cover glasses from the solution and allow them to dry for 24 hr.
 2. Seed the cells (e.g. EoL-1, BSG D10, SUDIPG1) on the coated cover glasses placed in a 6-well plate and incubate at 37 °C and 5% CO₂ for at least 16 hr. Rinse the cells with a 1× PBS solution and (optional) fix with 10% formaldehyde in neutral buffer solution for 15 min. Stain cells with 5 μ M red-orange cell-permeant dye in PBS at 37 °C for 30 min. Subsequently, rinse cells with PBS.
3. >Fluorescent Labeling of Targeted Cells
 1. Add 1% bovine serum albumin (BSA; in DI water) to the cells to minimize non-specific binding on the cells, and incubate at 37 °C for 1 hr.
 2. Incubate cells with the nanoparticles in 1% BSA for 1 hr. For EoL-1: incubate with 0.2 mg/ml GdPB-A488 or GdPB-A488-Eot3; For BSG D10 and SUDIPG1: incubate with 0.2 mg/ml MnPB-A488 or MnPB-A488-ANG2. Rinse the cells with PBS 3× to remove unbound nanoparticles.
 3. Carefully invert the cover glass (containing cells and nanoparticles) on a microscope slide, ensuring that there are no trapped air bubbles. Seal the edge between the cover glass and microscope slide by carefully applying clear nail polish. Image the cells using a confocal laser scanning microscope.

7. Fluorescent Labeling of Targeted Cells Using the Nanoparticles — Flow Cytometry

The nanoparticles (PB NPs, GdPB, and MnPB) can be used to fluorescently label a population of targeted cells (monitored by flow cytometry) as follows:

1. Prepare suspensions of the cells being targeted in PBS. For pure culture studies, the suspensions consist of a single cell type (e.g. BSG D10); resuspend a cell pellet of BSG D10 cells in PBS at 100,000 cells/ml (10 ml total). For mixed culture studies, the suspensions consist of at least two cell types (e.g. EoL-1 and OE21); similar to BSG D10, resuspend cell pellets of EoL-1 and OE21 in PBS at 100,000 cells/ml each (10 ml total).
 1. Prepare varying ratios of EoL-1:OE21 1:0 (2 ml EoL-1), 3:1 (1.5 ml EoL-1, 0.5 ml OE21), 1:1 (1 ml each of EoL-1 and OE21), 1:3 (0.5 ml EoL-1, 1.5 ml OE21), 0:1 (2 ml OE21).
2. Block the cells (pure and mixed suspensions) being targeted by the nanoparticles with 2 ml of 5% BSA to minimize non-specific binding.
3. Add 1 ml (1 mg/ml) nanoparticles to the cells and incubate for 1 hr. For BSG D10, incubate cells with MnPB-A488, MnPB-A488-AbC (control antibody), or MnPB-A488-ANG2. For mixtures of EoL-1 and OE21, incubate the mixtures with a fixed amount of GdPB-A488-Eot3.
4. Rinse the cells free of unbound nanoparticles by spinning down the samples at $1,000 \times g$ for 5 min at least 3 $\times$ to remove unbound particles. Resuspend the cells in 1 ml PBS and fix with 10% formaldehyde in PBS. Stain cells by incubating with 10 μ g/ml 7-Aminoactinomycin D in PBS on ice for 30 min. Rinse with PBS, then analyze 10,000 gated cells from each sample using a flow cytometer.

8. Generating MRI Contrast on Targeted Cells Using the Nanoparticles

The nanoparticles (PB NPs, GdPB, and MnPB) can be used to generate MRI contrast (in both T_1 - and T_2 -weighted sequences) in a population of targeted cells as follows:

1. Phantom Preparation
 1. Grow cells (e.g. BSG D10) in T75 flasks until ~80% confluence. Use appropriate growth medium and conditions. For BSG D10, grow the cells in DMEM + 10% FBS + 1 $\times$ Pen-Strep at 37 $^{\circ}$ C and 5% CO_2 .
 2. Rinse cells free of medium with 5 ml PBS and block the cells with 5 ml 1% BSA in PBS for 1 hr to minimize non-specific binding. Add 5 ml (0.5 mg/ml) nanoparticles to the cells. For BSG D10, incubate the cells with MnPB-A488, MnPB-A488-AbC (control antibody), and MnPB-A488-ANG2 for 1 hr.
 3. Rinse cells 3 $\times$ with 5 ml PBS to remove unbound nanoparticles. Trypsinize the cells by incubating the cells with 2 ml Trypsin EDTA 0.25% solution 1 $\times$ for 5 min at 37 $^{\circ}$ C and 5% CO_2 to detach them from the flask for phantom preparation. Add 8 ml of DMEM to quench the trypsinization of cells.
 4. Collect the cells by centrifuging them at $1,000 \times g$ for 5 min; aspirate out the supernatant. Resuspend the cells in 1 ml PBS and add 1 ml 10% formaldehyde in PBS to fix the cells. Add 100 μ l of each sample to a separate well of a 96-well plate.
 5. Add 100 μ l of molten 1% agarose solution in DI water into each well and mix well by pipetting up and down. Allow the gel to solidify at 4 $^{\circ}$ C for 12 hr.
NOTE: This yields a phantom containing cells with attached nanoparticles in solidified agarose.
 6. Place the phantom in a horizontal 3 T clinical magnet next to a solid block of 2% agar (150 cm^3). Secure the phantom and block of agar within the center of an 8-channel HD brain coil. Measure relaxation times using 0.5-mm thick coronal slices at mid-height of the 96-well plate¹¹.
2. T_1 - and T_2 -Weighted Sequences

Use the following settings for acquiring the sequences in the clinical MRI magnet:

 1. Acquire T1W MR images using the following spin echo sequence: Echo train (ET) = 1, Repetition time (TR) = 650 msec, Echo time (TE) = 11 msec, Matrix size = 320 x 256, Field of view (FOV) = 10 x 10 cm^2 .
 2. Acquire T2W MR images using the following FRFSE sequence: ET = 28; TR = 3,000 msec; TE = 101 msec; Matrix size = 384 x 288; FOV = 10 x 10 cm^2 .
3. Post-acquisition Processing
 1. For easier reading, convert the original gray scale images into a color scale image using ImageJ: Select Image > Type > 8-Bit, to convert the image to gray scale.
 2. Calculate the normalized intensity for each sample after subtracting the signal contribution from the agarose solution.

Representative Results

Using the one-pot synthesis scheme, nanoparticles of PB NPs (mean diameter 78.8 nm, polydispersity index (PDI) = 0.230; calculated by the dynamic light scattering instrument), GdPB (mean diameter 164.2 nm, PDI = 0.102), or MnPB (mean diameter 122.4 nm, PDI = 0.124) that are monodisperse (as measured by DLS) can be consistently synthesized (**Figure 2A**). The measured zeta potentials of the synthesized nanoparticles are less than -30 mV (**Figure 2B**), indicating moderate stability of the particles based on their surface charges. The synthesized nanoparticles exhibit adequate temporal stability over a period of 5 days as indicated by their consistent sizes (hydrodynamic diameters; **Figure 2C**).

When co-incubated with cells, the nanoparticles (PB NPs, GdPB, and MnPB) exhibit negligible cytotoxicity to the cells below certain threshold concentrations (**Figure 3**). Cytotoxicity studies of PB NPs on Neuro2a cells indicate negligible cytotoxicity when co-incubated with Neuro2a at concentrations lower than 0.67×10^{-6} mg/cell (**Figure 3A**). Cytotoxicity studies conducted by co-incubation of GdPB with EoL-1 and OE21 cells indicate negligible cytotoxicity of GdPB on both cell types at concentrations lower than 0.25×10^{-6} mg/cell (**Figure 3B**). Similar, cytotoxicity studies indicate negligible cytotoxicity of MnPB when co-incubated with BSG D10 at concentrations lower than 0.25×10^{-6} mg/cell (**Figure 3C**). The additional cytotoxicity of MnPB and GdPB can be attributed to the presence of additional ions (Mn^{2+} for MnPB and Gd^{3+} for GdPB) within the nanoparticle core.

MRI relaxivity studies were conducted using phantoms comprised of varying concentrations of PB NPs, GdPB, and MnPB. The studies indicate the utility of the nanoparticles as MRI contrast agents in both T1W and T2W sequences (**Figure 4**). This is demonstrated by the increased

hyperintensities (positive contrast) in T_1 -weighted sequences and increased hypointensities (negative contrast) in T_2 -weighted sequences with increasing concentrations of both GdPB (**Figure 4A**) and MnPB (**Figure 4B**). Based on the relaxivity measurements, MnPB is a moderate T_1 agent and a strong T_2 agent while GdPB is a strong T_1 agent and a moderate T_2 agent (**Figure 4C**). The measured relaxivities of GdPB and MnPB compare favorably with those of clinically approved contrast agents^{10, 11}.

The biofunctionalized PB NPs are able to fluorescently label a population of targeted cells *in vitro* (**Figure 5**). When biofunctionalized with both fluorescent avidin (A488) and biotinylated anti-human eotaxin-3 antibody (Eot3), GdPB can fluorescently target a population of EoL-1 cells (**Figure 5B**). Control GdPB nanoparticles without Eot3 exhibit negligible binding (**Figure 5A**). Similarly, when GdPB is biofunctionalized with both fluorescent avidin (A488) and biotinylated anti-neuronal glial antigen-2 (ANG2), the nanoparticles can fluorescently target populations of BSG D10 and SUDIPG1 neurospheres (**Figures 5D and F**), while control nanoparticles without the targeting antibody are unable to fluorescently label the cells (**Figures 5C and E**). Thus, efficient targeting and fluorescent labeling require the presence of both fluorescent avidin and the biotinylated targeting ligand.

The capability of the biofunctionalized nanoparticles to fluorescently label a population of cells, as quantified by flow cytometry, confirms the need for both fluorescent avidin and biotinylated-targeting ligand for effective fluorescent labeling (**Figure 6**). BSG D10 cells contacted with MnPB containing both A488 and ANG2 (MnPB-A488-ANG2) exhibit increased fluorescence (**Figure 6A**) and percentage of fluorescently-labeled cells (**Figure 6B**) when compared to control fluorescent nanoparticles with a control antibody (MnPB-A488-AbC) and without an antibody (MnPB-A488). The biofunctionalized nanoparticles are able to fluorescently target a specific sub-population of cells within a cell mixture (**Figure 6C**). This is demonstrated as fixed amounts of EoL-1-targeting, fluorescent GdPB (GdPB-A488-Eot3) are contacted with targeted cells (EoL-1) and control cells (OE21). Fluorescence increases as proportions of EoL-1 are increased (increased Alexa Fluor 488 signal intensity; **Figure 6C**) within the mixture. This indicates the specificity of the nanoparticles for this sub-population of cells within the cell mixture.

The biofunctionalized PB NPs increase MRI contrast in a population of targeted cells (**Figure 7**). When BSG D10 cells are contacted with equivalent concentrations of experimental (MnPB-A488-ANG2) and control (MnPB-A488-AbC and MnPB-A488) nanoparticles, phantoms exhibit increased hyperintensity for cells contacted with MnPB-A488-ANG2 compared to controls in T1W sequences, and increased hypointensity for cells contacted with MnPB-A488-ANG2 compared to controls in T2W sequences (**Figure 7A**). Image analysis of the ROIs within the phantom confirm this trend where cells contacted with experimental particles exhibit significantly increased intensity when compared to controls in T1W sequences and significantly decreased intensity relative to controls in T2W sequences (**Figure 7B**).

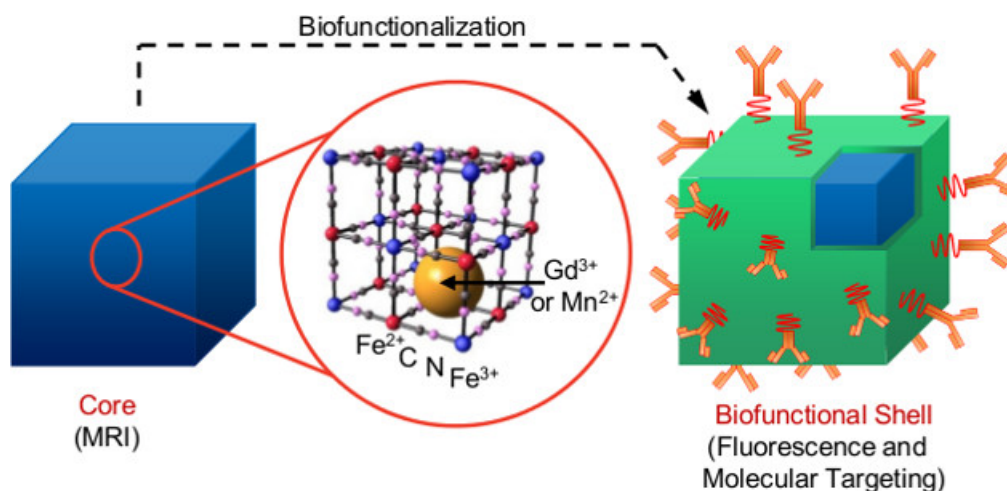


Figure 1: The core-shell design of the PB NPs. The core consists of a PB lattice, which is comprised of linear cyanide ligands in a $\text{Fe}^{\text{II}}-\text{CN}-\text{Fe}^{\text{III}}$ linkage. These linkages enable PB NPs to incorporate cations within its three-dimensional network as a means of balancing charges⁵. This cation-binding ability of Prussian blue is utilized to load gadolinium and manganese ions within the lattice, which provides MRI contrast. The core is coated with a biofunctional shell comprised of fluorescent avidin to enable fluorescence imaging and biotinylated ligands to enable molecular targeting.

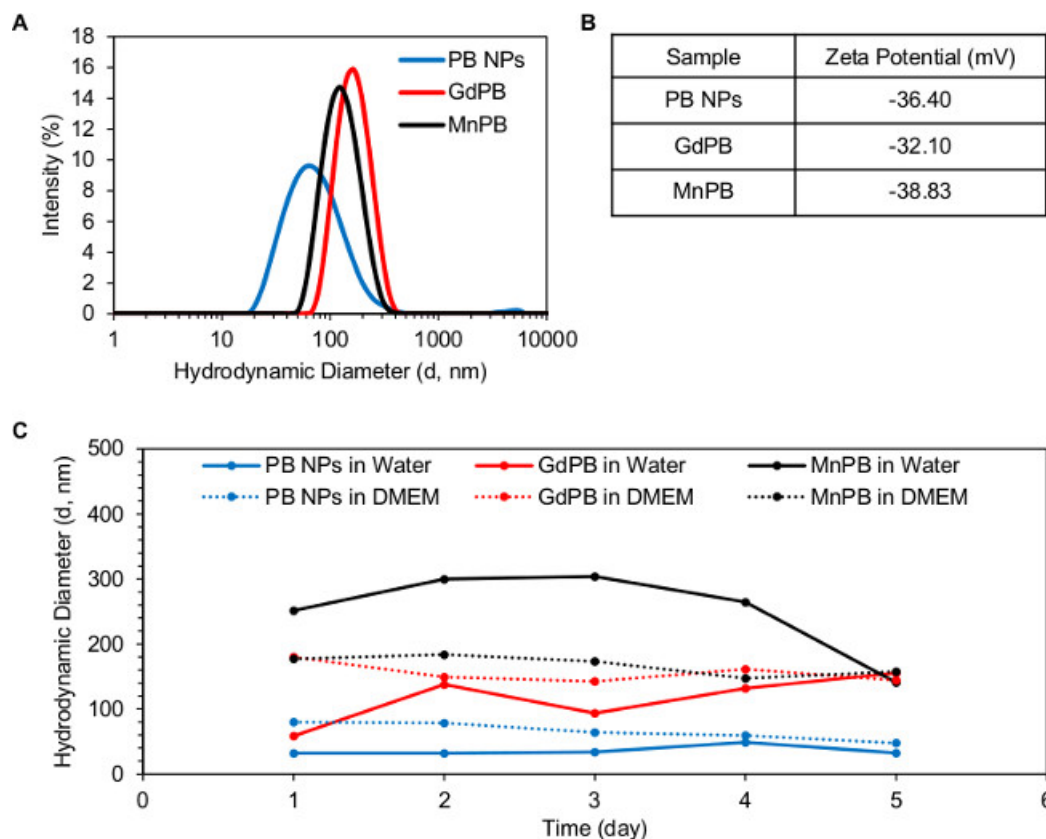


Figure 2: Size, charge, and stability of the PB NPs. (A) Size distributions of PB (blue), GdPB (red) and MnPB (black) nanoparticles measured by DLS. (B) Zeta potentials of PB NPs, GdPB, and MnPB. (C) Temporal stability of PB (blue), GdPB (red) and MnPB (black) nanoparticles in water (solid) and DMEM (dotted) for five days after their synthesis, measured by DLS.

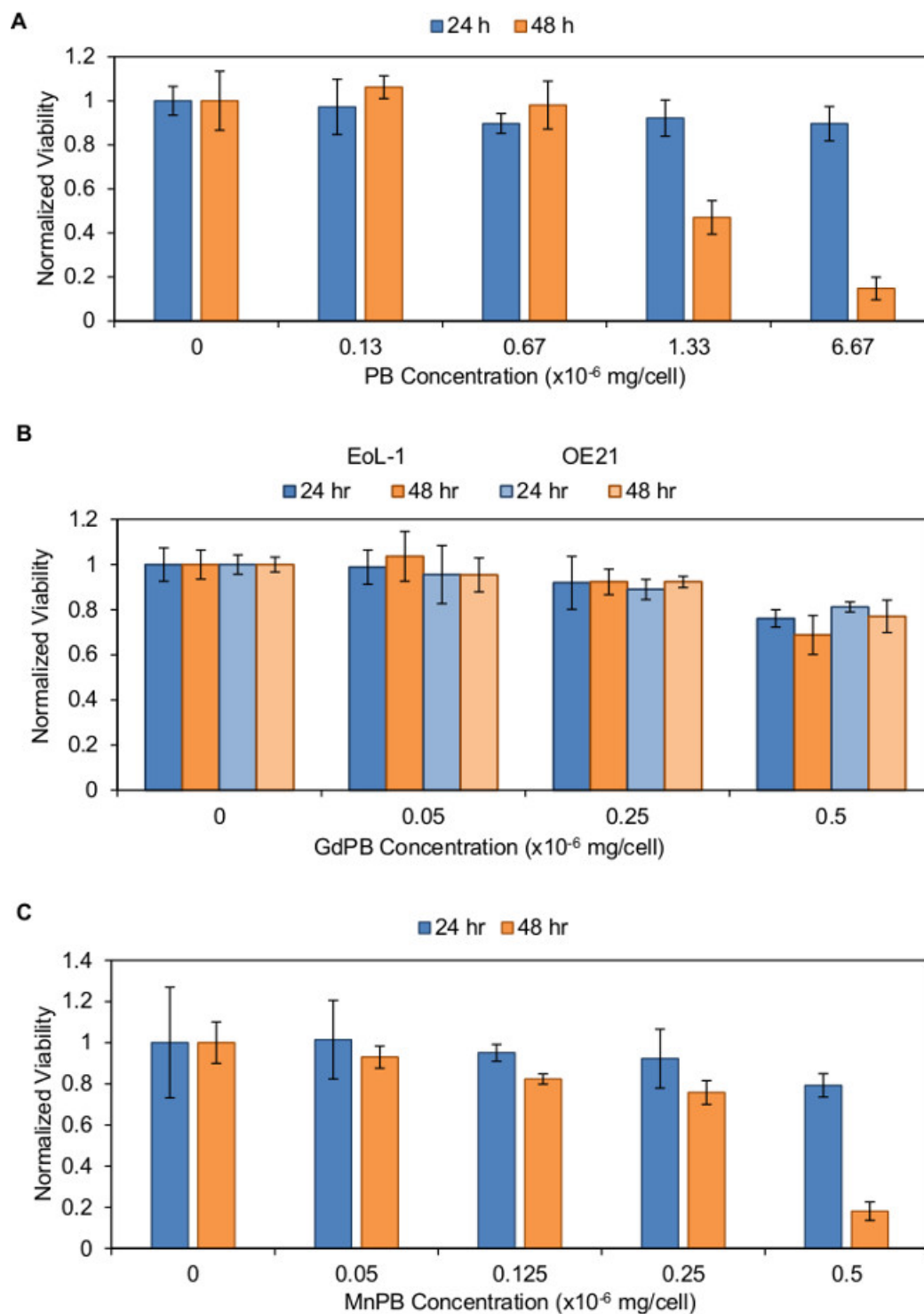
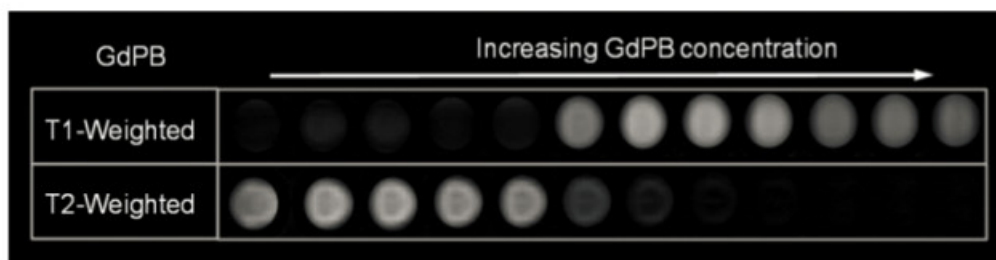
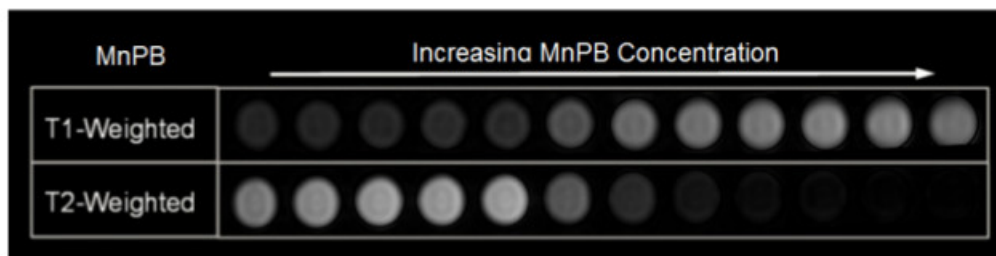


Figure 3: Cytotoxicity of the PB NPs. Cytotoxicity studies using various concentrations of (A) PB NPs added to a fixed number of Neuro2a cells (B) GdPB added to a fixed amount of EoL-1 and OE-21 cells, respectively and (C) MnPB added to a fixed number of BSG D10 cells. Cell survival rate was calculated at 24 and 48 hr. Reproduced with permission from Ref 11, copyright 2014 American Chemical Society; Ref 10 with permission from Dove Press Ltd; and Ref 21 with permission from The Royal Society of Chemistry.

A



B



C

Contrast Agent	Relaxivity ($\text{mM}^{-1} \text{s}^{-1}$)	
	r_1	r_2
PB NPs	7.9	14.4
GdPB	38.5	44.7
MnPB	15.8	143.0

Figure 4: MR images and relaxivities of GdPB and MnPB at 3 T. Hyperintensity in T_1 -weighted sequences and hypointensity in T_2 -weighted sequences of (A) GdPB and (B) MnPB as a function of concentration of the nanoparticles. (C) Tabulation of the relaxivities of PB NPs, GdPB, and MnPB measured at 3 T. Reproduced with permission from Ref 11, copyright 2014 American Chemical Society.

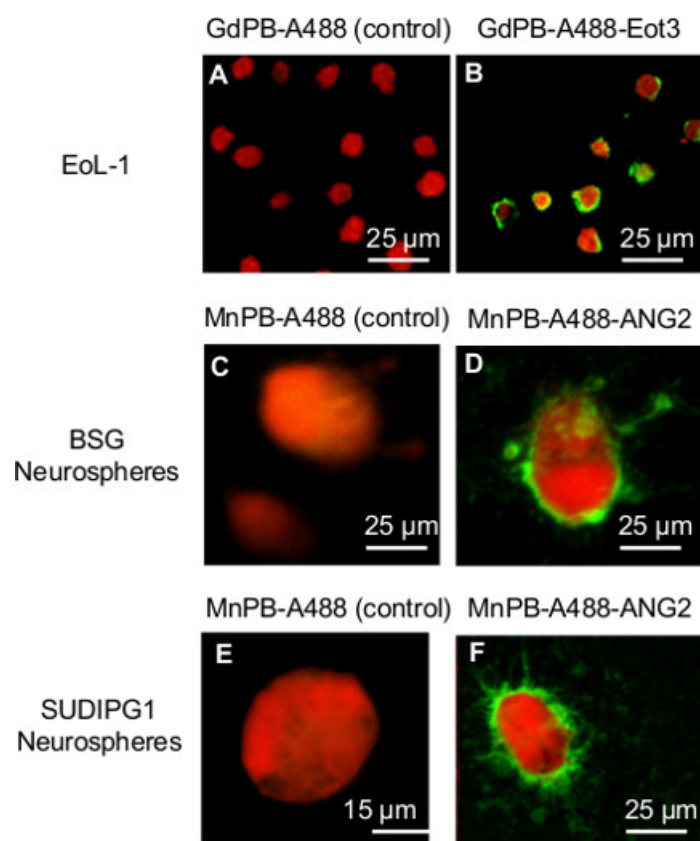


Figure 5: Fluorescent labeling of targeted cells using the biofunctionalized PB NPs, as measured by laser scanning confocal microscopy. Images of EoL-1 cells treated with (A) control (GdPB-A488) and (B) experimental (GdPB-A488-Eot3) nanoparticles. Images of BSG neurospheres treated with (C) control (MnPB-A488) and (D) experimental (MnPB-A488-ANG2) nanoparticles. Images of SUDIPG1 neurospheres treated with (E) control (MnPB-A488) and (F) experimental (MnPB-A488-ANG2) nanoparticles. Reproduced with permission from Ref 11, copyright 2014 American Chemical Society. [Please click here to view a larger version of this figure.](#)

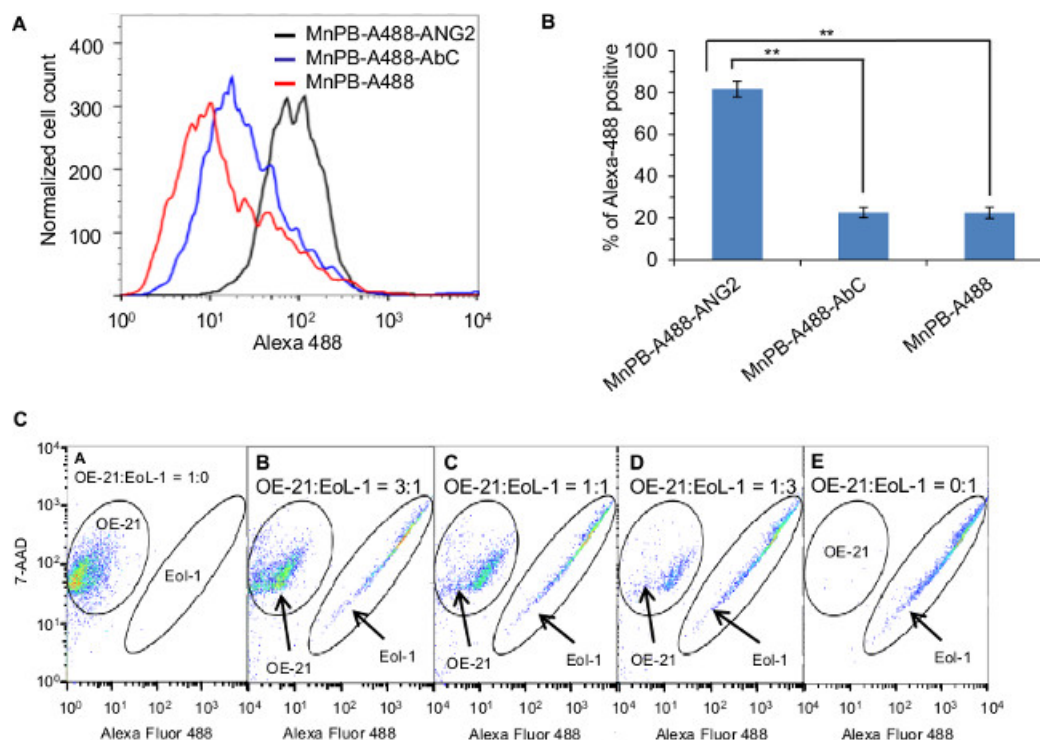


Figure 6: Fluorescent labeling of targeted cells using the biofunctionalized PB NPs, as measured by flow cytometry. (A) Flow cytometry histograms of BSG D10 cells treated with experimental (MnPB-A488-ANG2) and control (MnPB-A488-AbC and MnPB-A488) nanoparticles. (B) Percentage of BSG D10 cells from panel (A) that are fluorescent (% of Alexa-Fluor positive) upon treatment with experimental (MnPB-A488-ANG2) and control (MnPB-A488-AbC and MnPB-A488) nanoparticles, ** $p < 0.05$. (C) Flow cytometry scatter plots of a cell mixture containing varying proportions of EoL-1 (targeted cells) and OE21 (control cells) targeted by the nanoparticles (GdPB-A488-Eot3). Reproduced with permission from Ref 11, copyright 2014 American Chemical Society and from Ref 10 with permission from Dove Press Ltd. [Please click here to view a larger version of this figure.](#)

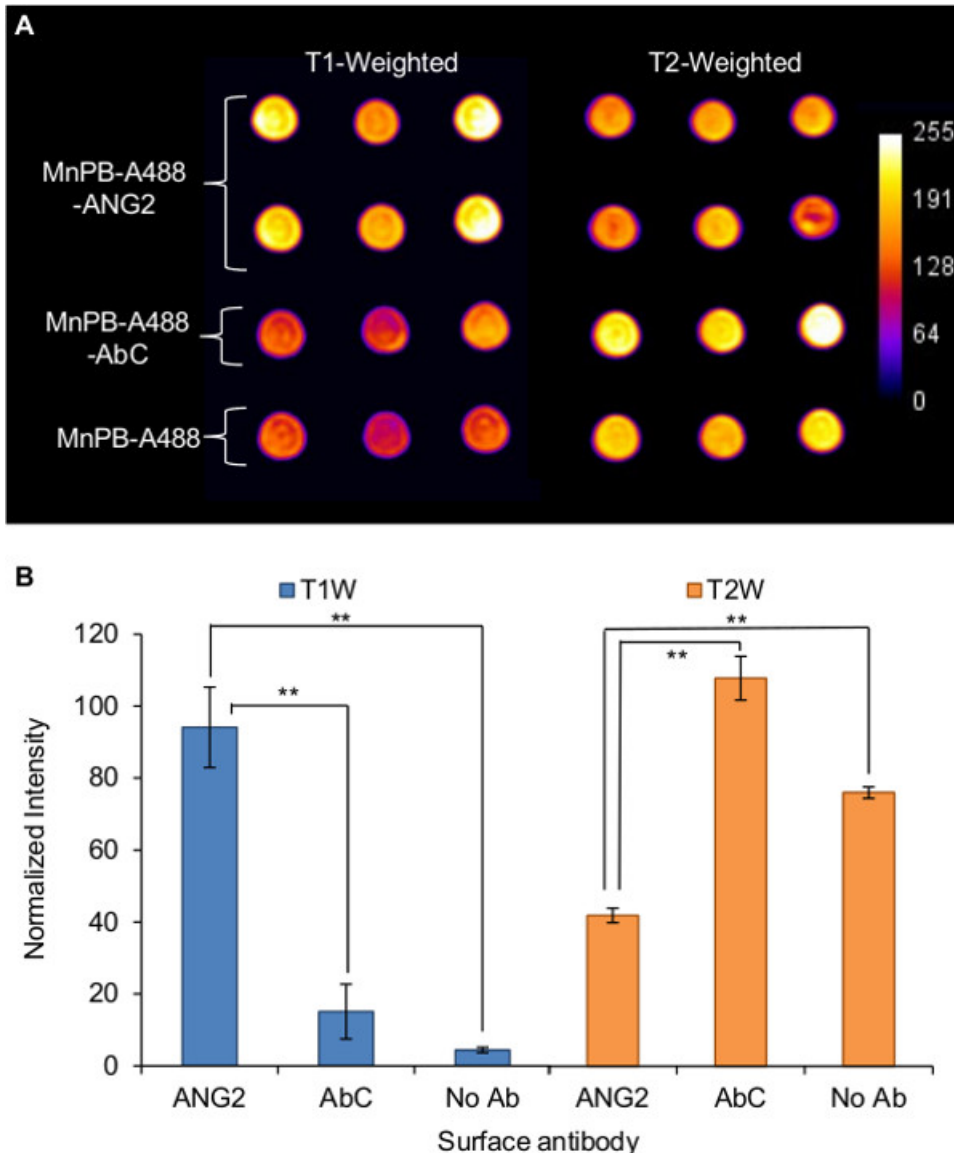


Figure 7: Increasing MRI contrast in targeted cells using the biofunctionalized PB NPs. (A) T₁-weighted and T₂-weighted contrast enhancement in phantoms comprised of a fixed number of BSG D10 cells treated with experimental (MnPB-A488-ANG2) and control (MnPB-A488-AbC and MnPB-A488) nanoparticles. (B) Normalized signal intensity for BSG D10 cells treated with experimental (MnPB-A488-ANG2) and control (MnPB-A488-AbC and MnPB-A488) nanoparticles, ** $p < 0.05$. Reproduced from Ref 10 with permission from Dove Press Ltd.

Discussion

This article has presented the methods for the synthesis of a novel class of multimodal, molecular imaging agents based on biofunctionalized Prussian blue nanoparticles. The molecular imaging modalities incorporated into the nanoparticles are fluorescence imaging and molecular MRI, due to their complementary features. The biofunctionalized Prussian blue nanoparticles have a core-shell design. The key steps in the synthesis of these nanoparticles are the: 1) one-pot synthesis which yields the cores that are comprised of Prussian blue nanoparticles (PB NPs), gadolinium-containing Prussian blue nanoparticles (GdPB), or manganese-containing Prussian blue nanoparticles (MnPB), 2) biofunctionalization of the nanoparticles using fluorescent avidin by electrostatic self-assembly, and 3) attachment of the biotinylated ligands (antibodies) to the nanoparticles using robust avidin-biotin interactions. Both fluorescent avidin and the biotinylated ligands constitute the biofunctional shell of the nanoparticles.

The one-pot synthesis yields PB NPs, GdPB, or MnPB that function both as T₁ and T₂ contrast agents for MRI. The amounts of the paramagnetic ions (gadolinium or manganese) loaded into the nanoparticle core can be altered (increased or decreased) by varying the amounts of the paramagnetic ion-containing salts (gadolinium (III) nitrate and manganese (II) chloride) in the one-pot synthesis (Step 1.2). This results in altered (increased or decreased) MRI signal intensities. However, these modifications to the synthesis scheme may result in unstable, aggregated nanoparticles. To mitigate concerns associated with aggregation, the one-pot synthesis may be modified to incorporate size-controlling capping agents such as citrate during synthesis²⁰.

Biofunctionalization of the nanoparticle cores is achieved by electrostatic self-assembly with fluorescent avidin, which enables fluorescence imaging. Electrostatic self-assembly requires opposite charges on the surface of the nanoparticles and the coating polymer (in this article, fluorescent avidin). To alter the surface functionality of the nanoparticles, avidin may be replaced by positively-charged polymers (e.g. polylysine or an amine-group containing polyethylene glycol) during the synthesis. However the relative proportions of the nanoparticles and coating polymer will have to be optimized to maintain nanoparticle size and stability and to prevent aggregation.

Addition of the biotinylated antibodies onto the avidin coated nanoparticles confers molecular targeting capabilities to the PB-based nanoparticles. This step is based on the robust interaction between avidin and biotin (equilibrium dissociation constant, $K_d \sim 10^{-15}$). As with previous steps, the relative proportions of the avidin-coated nanoparticles and biotinylated ligands need to be optimized so as to prevent the biotinylated ligand from simultaneously binding two avidin coated nanoparticles resulting in nanoparticle aggregation. For molecular targeting, the antibodies may be replaced by other targeting ligands such as antibody fragments (Fab), single-chain variable fragment (scFv), peptides, or aptamers.

The key advantages of this method for synthesizing biofunctionalized nanoparticles as multimodal, molecular imaging agents are the: 1) facile one-pot (1-step) synthesis of the nanoparticle cores, and 2) sequential contacting steps (electrostatic self-assembly and avidin-biotin interactions) for coating the nanoparticle core with a biofunctional shell. Other advantages of the nanoparticles include the fact that Prussian blue (sold as Radiogardase) is already FDA-approved for human use and that the nanoparticles that results from the one-pot synthesis scheme can be used as an MRI contrast agent both in T_1 (positive) and T_2 (negative)-weighted sequences, which is not easily achieved using other contrast agents or nanoparticle platforms without complicated synthesis schemes or specialized chemicals for synthesis of the contrast agents. A limitation of the technique is that the synthesis can lead to polydisperse nanoparticles with aggregates if the relative proportions of the reactants in both nanoparticle core synthesis and biofunctional shell coating steps are not rigorously optimized for the reactants used in those particular steps. For example, the relative proportions for coating the nanoparticles cores with avidin cannot be extended to coating the nanoparticles with polylysine without prior optimization studies.

After mastering the technique for synthesizing biofunctionalized Prussian blue nanoparticles described here, this versatile design can be modified for molecular imaging studies *in vivo*. This will require PEGylation of the nanoparticles for lower immunogenicity and longer circulation times *in vivo*. Similar to the design described here, the PB NPs can be biofunctionalized with antibodies prior to *in vivo* administration. Other studies include the use of the biofunctionalized PB NPs for theranostic (simultaneous therapy + diagnostic) applications *in vivo*. Studies investigating the use of Prussian blue nanoparticles for photothermal therapy (based on their absorbance characteristics at near infrared wavelengths) are currently underway²¹.

Disclosures

The authors have nothing to disclose.

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