

AD_____

Award Number: W81XWH-04-1-0341

TITLE: Magnetic Resonance Imaging of Polymeric Drug Delivery Systems in Breast Cancer Solid Tumors

PRINCIPAL INVESTIGATOR: Bahar Zarabi
Hamid Ghandehari, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland, Baltimore
Baltimore, MD 21201-1082

REPORT DATE: July 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-07-2007		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 1 Jul 2004 – 30 Jun 2007	
4. TITLE AND SUBTITLE Magnetic Resonance Imaging of Polymeric Drug Delivery Systems in Breast Cancer Solid Tumors				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0341	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Bahar Zarabi and Hamid Ghandehari, Ph.D. E-Mail: bzara001@umaryland.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Maryland, Baltimore Baltimore, MD 21201-1082				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES – Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT The overall purpose of this research is to develop a polymeric drug delivery system containing magnetic resonance contrast agents for the treatment of breast cancer. This drug-imaging agent delivery system will allow the follow up of the fate of the drug delivery system and its relation to reduced tumor mass, improved efficacy and reduced toxicity in individual patients. In three years, progress was made in the following areas: 1) Synthesis, characterization, relaxivity and stability measurements of polymer-nitroxide/dinitroxide conjugates. 2) Synthesis, characterization, relaxivity measurement, pH stability measurement, challenge study in the presence of a different chelator and cytotoxicity test of polymer- gadolinium conjugates with and without doxorubicin on cancerous and non-cancerous cell lines. In addition, a series of polymer- contrast agent conjugates targetable to macrophages were synthesized, characterized, and evaluated in vitro. A no cost extension is requested to complete the project.					
15. SUBJECT TERMS Polymers, drug delivery, breast cancer, contrast agent, MRI					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 29	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	1
Body.....	1
Key Research Accomplishments.....	5
Reportable Outcomes.....	6
Conclusions.....	7
References.....	7
Appendixes.....	8

INTRODUCTION

The **long term objective** of this research is to develop a polymeric drug delivery system containing magnetic resonance contrast agents for treatment of breast cancer. This will allow the follow up of the fate of the drug delivery system and its relation to reduced tumor mass, improved efficacy and reduced toxicity in individual patients. Two specific aims were proposed:

- 1) To synthesize a series of polymer-drug-imaging agent conjugates.
- 2) To characterize the conjugates by physicochemical methods.

In year one, progress was made to partially accomplish Aims 1 & 2, i.e., we synthesized and characterized the polymer-nitroxide and polymer-dinitroxide conjugates without the drug. In doing so Tasks 1, 2, and 3 of year one and Task 1 of year two of Statement of Work were accomplished. In addition, we synthesized and characterized polymer-linked gadolinium conjugate.

In year two, progress was made to partially accomplish Aims 1 & 2 using gadolinium as a contrast agent. In doing so Tasks 2 (synthesis), 3 (characterization), and 4 (concluding year 2 / strategizing for year 3) of year two and Task 1 of year three (relaxivity measurements) outlined in the Statement of Work were accomplished. In addition related areas for targeted delivery to macrophages were explored as outlined in the body of this report.

In year three, progress was made to accomplish Aims 1 & 2 using gadolinium as a contrast agent. In doing so Tasks 1 and 2 (relaxivity measurements and synthesis) of year three outlined in the Statement of Work were accomplished. As of June 1 until September 8th the predoctoral fellow will be on maternity leave. Upon return she will complete task 3 of year 3 which is concluding the project. A no cost extension of the remainder of the fellowship is requested to accommodate completion of the project until November 07. In addition to the above, stability and cytotoxicity of these polymers were explored as outlined in the body of this report.

BODY

A. Synthesis of the proposed comonomers and copolymers

Comonomers, (2-hydroxypropyl)methacrylamide (HPMA)¹ and methacryloylglycylglycyl- p-nitrophenyl ester (MA-GG-ONp), a reactive comonomer ², were prepared as described previously. Then, HPMA copolymeric precursors containing side chains terminated in 5, 15, and 30 mole% of p-nitrophenyl ester (ONp) were synthesized by free radical precipitation copolymerization using AIBN as the initiator. In the second step, a mononitroxide, namely 3-(aminomethyl) 2,2,5,5-tetramethyl-1-pyrrolidinoxyl, and dicyclic analogs of this compound

terminated in secondary amine (dinitroxide) were attached to the polymeric precursors by an aminolysis reaction (Appendix 1).

B. Characterization of the proposed conjugates

HPMA copolymer p-nitrophenyl ester (ONp) were characterized for the contents of ONp spectrophotometrically ($\lambda_{\text{max}}=400$) and molecular weight and molecular weight distribution by size exclusion chromatography (Superose 12 HR 10/30 column, Pharmacia, NJ). The conjugates were characterized for nitroxide and dinitroxide content using UV spectrophotometry ($\lambda_{\text{max}}=260$) (Appendix 1).

The r_1 relaxivity of HPMA copolymer- linked nitroxides and dinitroxides, mononitroxide, dinitroxide, and gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) were calculated from T_1 (relaxation time) measurements at room temperature. Solutions of each sample were diluted in deionized water at four concentrations (from 0.1 to 0.015 mM) and were imaged using 1.5 T MR system (Eclipse, Philips Medical System, Cleveland, OH and Sigma). T_1 was measured using an inversion recovery fast spin echo imaging sequence using inversion times (TI) of 50, 100, 200, 400, 700, 1400, 2000, and 2800 ms, an echo time (TE) of 12 ms, and an echo train length of 8 at a repeat time TR of 6000 ms. All images were obtained from a single axial slice with a 20×15 cm field of view (FOV), 3 mm slice thickness, 256×192 matrix and one excitation. Images were transferred to an independent workstation (SGI, O200) for the calculation of T_1 from the images obtained at various inversion times. T_1 for each solution and deionized water were calculated using MATLAB (The Mathworks, Inc., Natick, MA). The r_1 values of each solution were calculated, using a least squares fit, as the slope of $(1/T_{1, \text{solution}} - 1/T_{1, \text{water}})$ versus concentration of contrast agent (mM), where $T_{1, \text{solution}}$ is the T_1 of each dilution of the contrast agent and $T_{1, \text{water}}$ is the T_1 of water without contrast agent (Appendix 1).

For stability measurement, the reduction of HPMA copolymer-nitroxide and –dinitroxide (50 μM) conjugates in the presence of glutathione (1mM) was evaluated by Electron Paramagnetic Resonance (EPR) spectroscopy (E-109, Varian Associates, CA) at room temperature (Appendix 1).

Since the conversion rate of dinitroxide was low (approximately 50%) the relaxivity of these conjugates were not as expected. Then based on different synthetic methods we increased the conversion rate of dinitroxide to about 80% but we did not get higher relaxivity compared to nitroxide conjugates. Therefore in addition to what was proposed we synthesized and characterized a polymer-linked gadolinium conjugate.

B. Synthesis of novel conjugates

We made progress in synthesis and characterization of the copolymers N-(2-hydroxypropyl)methacrylamide (HPMA)¹, and Methacryloylglycylphenylalanylleucylglycyl-doxorubicin (MA-GFLG-dox), a reactive comonomer with a lysosomally degradable linker³ were prepared as described previously. We used biodegradable spacer (GFLG) for drug attachment for therapy purposes. Comonomer aminopropylmethacrylamide-benzyl-1,4,7,10

tetraazacyclododecane-1,4,7,10 tetraacetic acid (APMA-benzyl-DOTA) was synthesized by reacting N-(3-aminopropylmethacrylamide) (APMA) with p-isothiocyanatobenzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10 tetraacetic acid (p-SCN-Bz-DOTA) in dry dimethylsulfoxide (DMSO). The p-SCN-Bz-DOTA was reacted at 1.2 molar excess to APMA. HPMa copolymer conjugates with or without dox were synthesized by a modified two-step procedure. Briefly, in the first step the polymeric precursors containing side chains terminated in DOTA were synthesized by free radical precipitation copolymerization of the monomers of HPMa, APMA-benzyl-DOTA, and MA-GFLG-dox in predetermined molar compositions (Appendix 3, Table 1). All polymerization were carried out in acetone / DMSO using AIBN as the initiator. The ratio of monomers: initiator: solvent in the feed were kept constant at 12.5: 0.6: 86.9 (weight %), respectively. The comonomer mixture was sealed in an ampoule under nitrogen and stirred at 50 °C for 24 h. The polymers were isolated by precipitation of resulting solution into ether. In the second step, the DOTA molecules in the side chain of the polymeric precursors were chelated to gadolinium (Gd). Briefly polymer-DOTA conjugates and GdCl₃·6H₂O (1.5:1 molar equivalents relative to the theoretical DOTA content) were dissolved in deionized water. The pH of the solution was maintained at 5.0-5.5 overnight by gradual addition of 1 N NaOH solution. EDTA disodium salt dihydrate was added into the solutions to chelate the excess Gd. After stirring for 30 min, the milky solution was purified over a PD10 size exclusion column (GE Healthcare, NJ, USA), to remove the EDTA-chelated Gd and other unreacted low molecular weight monomers from the polymeric conjugates. The polymer conjugates were dissolved in deionized water, dialyzed and lyophilized.

The proposed polymers target solid tumors by a passive process of enhanced permeability and retention effect. It is of interest to develop systems that target metastases. Therefore in addition to what was proposed we also synthesized macrophage targetable polymer-linked gadolinium conjugates with implications in imaging and drug delivery to metastatic sites (Appendix 2).

C. Characterization of the novel conjugates

The characteristics of the copolymers are reported in Table 1 of Appendix 3. All polymer-contrast agent conjugates were characterized for their Gd content by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Galbraith, Knoxville, TN). Doxorubicin content was determined by UV spectrophotometry ($\lambda_{\text{max}} = 484 \text{ nm}$). The molecular weight and molecular weight distribution of the polymeric conjugates were estimated by size exclusion chromatography (SEC) on a Superose 12 HR 10/30 column (GE Healthcare, Piscataway, NJ) using a Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare) and HPMa homopolymer fractions of known molecular weight as standards.

The r_1 relaxivity of HPMa copolymer-Gd chelates were calculated from T_1 (relaxation time) measurements at room temperature as described in part B.

The feed content of comonomers was determined based on previous study in the literature ⁴. The proposed polymers target solid tumors by a passive process of enhanced permeability and retention effect. In addition to what was proposed we also characterized the macrophage

targetable polymer-linked gadolinium conjugates and evaluated the uptake of these copolymers in vitro (Appendix 2). Also, we have done the following studies (Appendix 3):

Stability studies

The stability of the HPMA- contrast agent complex was evaluated across a range of pH (Appendix 3, Table 2). Aliquots of 2mg/ml HPMA- contrast agent complex were incubated at pH 3, 5 and 7 for 1, 3 and 5 days at room temperature. At each time point samples were passed through PD10 size exclusion column (GE Healthcare, NJ, USA), to remove any decomplexed Gd and all other low molecular weight compounds from the polymeric conjugates. Then, samples were lyophilized and Gd content of polymeric conjugates was measured by ICP-OES. Results were reported as % Gd bound compared to (P-(DOTA-Gd) (Appendix 3, Table 1). The free Gd content of the polymers was determined using Arsenaso III assay⁵.

Competitive challenge study

The stability of Gd-DOTA complex was evaluated in presence of a range of excess concentrations of a competitive chelator namely EDTA (Appendix 3, Table 3). Briefly polymer-DOTA conjugates and GdCl₃.6H₂O (1.5:1 molar equivalents relative to the theoretical DOTA content) were dissolved in deionized water. The pH of the solution was maintained at 5.0-5.5 overnight by gradual addition of 1 N NaOH solution. The solution was divided into four equal volumes and EDTA disodium salt dihydrate was added into the solutions at 1, 5, 25, and 125 times of Gd concentration. Polymeric solution with (1:1 EDTA:Gd) concentration was treated as a control. After stirring for 30 min, the milky solutions were purified over a PD10 size exclusion column (GE Healthcare, NJ, USA), to remove the EDTA-chelated Gd and other unreacted low molecular weight monomers from the polymeric conjugate. Then samples were lyophilized and Gd content of polymeric conjugates was measured by ICP-OES. Results were reported as % Gd bound compared to (P-(DOTA-Gd) (Appendix 3, Table 1). The free Gd content of the polymers was determined using Arsenaso III assay⁵.

Cytotoxicity studies

The toxicity of polymeric conjugates was assessed using a model breast cancer cell line (MDA-MB-435S) and a non-cancerous fibroblast cell line (NIH/3T3). Cells were seeded on 96-well culture plates at a concentration of 3000 cells/ well and allowed to attach for 24 h at 37 °C and in humidified atmosphere of 5% CO₂. Then the medium was removed and 100 µl of HPMA copolymer-(DOTA-Gd) conjugate in DMEM (10% serum) were added to obtain final concentrations of (1 to 1000 µM (Gd equivalent)). MTT assay was performed at 24, 48, and 72 h to determine time dependent effects on toxicity. The same experiment was performed with HPMA copolymer-DOTA-Dox conjugates (with and without Gd) at concentrations between 1 to 10000 nM (Dox equivalent). Cells were assayed at 560 nm on a microplate reader (SPECTRAmax plus, Molecular Devices, Sunnyvale, CA). The toxicity of the conjugates in all experiments was expressed as % of viable cells (Appendix 3, Figure 2, 3 & 4). Statistical significance of differences in toxicity between different samples was analyzed using two-tailed unpaired student t-test.

In the remaining no cost extension time I plan to finish writing new manuscripts (one original research article and one review) and disseminate the results in a conference.

KEY RESEARCH ACCOMPLISHMENTS

- 1) Synthesis and characterization of polymer-linked nitroxides: Compared to free nitroxides HPMA copolymer conjugated nitroxides demonstrated higher relaxivities than Gd-DTPA.
- 2) Stability studies of polymer-linked nitroxides: Polymeric conjugated nitroxides show higher stabilities than previously synthesized free nitroxides
- 3) Synthesis and characterization of macrophage targeted HPMA copolymer - gadolinium conjugates: All targetable HPMA-linked Gd conjugates exhibited relaxation (r_1) values up to seven times greater than a commercially available Gd-DOTA contrast agent (Dotarem®).
- 4) In vitro uptake study of targetable HPMA copolymer gadolinium conjugates: The uptake of all the targetable conjugates increased with increase in ManN concentration at all the time points. Incubation of macrophages with polymeric conjugates at 4°C resulted in significantly reduced uptake when compared to those carried out at 37°C suggesting the involvement of an energy dependent process. Polymer-chelated Gd showed higher trend of uptake but the difference between conjugate uptake with and without Gd was not significant. After mannose treatment the uptake of P₄, P₃, and P₂ was the same as P₁. These results suggest that uptake of polymeric conjugates is mediated primarily by mannose receptors. The lack of complete inhibition indicates that a secondary mechanism such as adsorptive endocytosis of the polymers may also exist Uptake studies carried out with quenching of extracellular fluorescence using trypan blue resulted in the decrease of measured uptake values by 12.5-17.9% for all the polymers. The decrease in % of uptake after incubating the cells with trypan blue suggests that not all of the polymer conjugates are internalized and that some of the conjugates are associated with the surface of macrophages.
- 5) Synthesis and characterization of polymer- gadolinium- doxorubicin and polymer-gadolinium conjugates: Both HPMA-linked Gd conjugates (with and without doxorubicin) exhibited relaxation (r_1) values higher than a commercially available Gd-DOTA contrast agent (Dotarem®). Polymeric conjugate with Dox exhibited 1.6 times higher relaxivity than polymeric conjugate without Dox.
- 6) Competitive challenge study of HPMA copolymer gadolinium conjugate:Gd labeling in the presence of increasing concentrations of a competitive chelator EDTA, resulted in lower Gd content of polymeric conjugates. The amount of Gd decomplexed over 30 min increased linearly with respect to the concentration of added EDTA compared to control. Arsenazo III assay showed less than 2% free Gd in each sample suggesting more than 98% of Gd was chelated in each sample.

- 7) Stability study of HPMA copolymer gadolinium conjugate: Stability studies were performed in physiological and acidic pH conditions. Results showed that at pH 7 and 5 less than 5% of Gd was decomplexed in 5 days suggesting the high kinetic stability of the Gd-DOTA complex. At these pH values decomplexation was not observed beyond 3 days. Under highly acidic condition (pH=3), 85.8% of Gd remained chelated even after 5 days. Arsenazo III assay showed less than 2% free Gd in each sample suggesting more than 98% of Gd was chelated in each sample.
- 8) In vitro cytotoxicity study of HPMA copolymer gadolinium conjugates with and without doxorubicin: The results of time and concentration dependent cytotoxicity of HPMA-Gd conjugate (without Dox) on MDA-MB-435 is presented as percentage of viable cells following treatment with the polymer conjugate and was compared to Magnevist (a commercially available Gd chelate contrast agent) at incremental polymer concentrations (1, 10, 100 and 1000 μ M Gd equivalent). At concentrations between 1 and 100 μ M, polymer-Gd conjugate showed significantly lower toxicity compared to Magnevist (Gd-DTPA) after 72 h ($p < 0.019$). There is no significant difference between these compounds after 24 and 48 h at concentrations between 1 and 100 μ M equivalent of Gd. At 1000 μ M Gd equivalent concentration polymer-Gd conjugate showed significantly higher toxicity than Magnevist after 48 and 72 h ($p < 0.025$). There is no significant difference between these compounds after 24 h at this concentration. The same experiment was performed on NIH/3T3 cell line. Polymer-Gd conjugate showed higher trend of % of viable cells on healthy fibroblast cell line but at each concentration, there is no significant difference between the cytotoxicity of the same conjugates. Also, Toxicity of HPMA-DOTA-Gd conjugates with and without Dox on MDA-MB-435 cell line was compared to each other after 24, 48 and 72 h (Figure 4). No significant difference in toxicity of polymer-drug conjugates with and without Gd was observed, suggesting Gd does not interfere with the effect of Dox. Toxicity of Polymeric Dox conjugate is significantly less than free Dox suggesting a slower endocytic mechanism of uptake for the conjugates compare to rapid diffusion of free Dox.

REPORTABLE OUTCOMES

- 1) Poster presentation in Era of Hope (2005) (Appendix 1).
- 2) Departmental seminar (2005).
- 3) Presentation of this research at the University of Maryland Graduate Research Conference in Baltimore (local meeting) (2006).
- 4) Presentation of this research at the University of Maryland Pharmacy Research Day in Baltimore (local meeting) (2006).

5) Presentation of this research at the Annual American Association of Pharmaceutical Scientist Biotechnology Meeting in Boston (National) (2006).

6) Publish of an original research article to *Molecular Pharmaceutics* 2006 Sep-Oct; 3(5):550-7 (Appendix 2).

During the no cost extension period the following will be accomplished.

1) Presentation of this research at the Controlled Release Society Meeting in Long Beach, California (2007).

2) Preparation of an original research article and a review article (2007).

CONCLUSIONS

In summary progress was made in the synthesis, characterization and *in vitro* evaluation of polymer-linked contrast agent conjugates with and without doxorubicin. In addition a series of polymeric conjugates targetable to macrophages were synthesized and characterized for which a manuscript was published. Two additional manuscripts and a presentation are under way which will be completed during the no cost extension period.

REFERENCES

1. Strohalm, J.; Kopecek, J. *Angew. Makromol. Chem.* **1978**, 70, 109.
2. Rejmanova P.; Labsky J.; Kopecek J. *Makromol. Chem.* **1977**, 178, 2159.
3. Ulbrich, K.; Subr, V.; Strohalm, J.; Plocova, D.; Jelinkova, M.; Rihova, B. *J. Controlled Rel.* **2000**, 64, 63.
4. Wang, D.; Miller, S. C.; Sima, M.; Parker, D.; Buswell, H.; Goodrich, C. H.; Kopeckova, P.; Kopecek, J. *Pharm. Res.* **2004**, 21, 1741.
5. Gouin, S; Winnik, F. M. *Bioconjugate Chem.* **2001**, 12, 372.

APPENDIXES

Appendix 1: Bahar Zarabi, Jiachen Zhuo, John Weaver, Gerald Rosen, Rao Gullapalli, and Hamid Ghandehari. Synthesis and characterization of a novel macromolecular magnetic resonance imaging contrast agent. Poster Presentation at the 4th Era of Hope Meeting, Philadelphia, PA, June 8-11, 2005.

Appendix 2: Bahar Zarabi, Anjan Nan, Jiachen Zhuo, Rao Gullapalli, and Hamid Ghandehari, Macrophage Targeted N-(2-hydroxypropyl)methacrylamide (HPMA) Conjugates for Magnetic Resonance Imaging, *Molecular Pharmaceutics*, 3(5), **2006**, 550-557.

Appendix 3: Chemical structure and characteristics of polymer-linked gadolinium conjugates with and without drug.

Appendix 1: Bahar Zarabi, Jiachen Zhuo, John Weaver, Gerald Rosen, Rao Gullapalli, and Hamid Ghandehari. Synthesis and characterization of a novel macromolecular magnetic resonance imaging contrast agent. Poster Presentation at the 4th Era of Hope Meeting, Philadelphia, PA, June 8-11, 2005.

OBJECTIVE

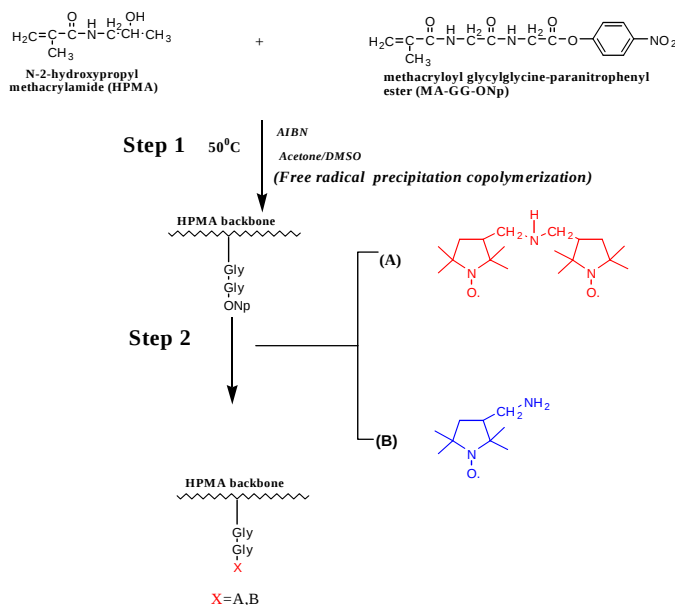
To synthesize and characterize novel N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-nitroxide conjugates with improved relaxivity for Magnetic Resonance Imaging (MRI) of breast cancer solid tumors.

INTRODUCTION

Early detection of tumors and characterization of their response to therapy are fundamental challenges in the quest to improve cancer survival rates (1). Dynamic magnetic resonance imaging is often used for diagnosis and prognosis of solid tumors. The most commonly used magnetic resonance contrast agents (CAs) are gadolinium chelates. However, these agents are not tissue selective and generally have a short residence time in tumor. Recent investigations have demonstrated that macromolecular CAs have longer lifetime in the blood pool and higher accumulation in solid tumors in comparison to low molecular weight CAs. N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers are one class of polymeric carriers that show promise for targeted delivery of drugs and imaging agents. These polymers are non-immunogenic and can be tailored to the characteristics of the specific target (2). We previously investigated the potential of HPMA copolymer-mononitroxide conjugates as MRI contrast agent (3). Challenges for the use of nitroxide based contrast agents in MRI are their low magnetic relaxivities and high bioreduction rate. Compounds that contain multiple nitroxides have been shown to have higher relaxivities and stabilities (4). The objective of this research was to attach dinitroxides to HPMA copolymers and evaluate their relaxivity and bioreduction.

EXPERIMENTAL METHODS

SYNTHESIS OF POLYMER-IMAGING AGENT CONJUGATES (SCHEME 1)



Synthesis of HPMA copolymer - contrast agent conjugates: First, HPMA copolymeric precursors containing side chains terminated in 5, 15, and 30 mole% of p-nitrophenyl ester (ONp) were synthesized by free radical precipitation copolymerization using AIBN as the initiator (Scheme 1). In the second step, a mononitroxide, namely 3- (aminomethyl) 2,2,5,5-tetramethyl-1-pyrrolidinoxyl, and dicyclic analogs of this compound terminated in secondary amine (dinitroxide) were attached to the polymeric precursors by an aminolysis reaction.

Characterization of conjugates: HPMA copolymer p-nitrophenyl ester (ONp) were characterized for the contents of ONp spectrophotometrically ($\lambda_{\text{max}}=400$) and molecular weight and molecular weight distribution by size exclusion chromatography (Superose 12 HR 10/30 column, Pharmacia, NJ) (Table1). The conjugates were characterized for nitroxide and dinitroxide content using UV spectrophotometry ($\lambda_{\text{max}}=260$).

PHYSICOCHEMICAL CHARACTERIZATION (Table 1)

sample	HPMA (mole%)	MA-G-G-ONP (mole%)	ONP content (mmole/g polymer)	Mw (g/mole)	n ^a
P1	70	30	1.41±.037	22000	1.5
P2	85	15	0.696±.012	41000	1.8
P3	95	5	0.273±.005	37000	1.3

^a Polydispersity

Relaxivity measurements: The T1 relaxivity (r1) of HPMA copolymer-linked nitroxides and dinitroxides were calculated at room temperature and 1.5 tesla (T). The relaxivity of mononitroxide, dinitroxide, and gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) were also measured as controls (Table 2).

Stability test: The reduction of HPMA copolymer-nitroxide and –dinitroxide (50 μM) conjugates in the presence of glutathione (1mM) was evaluated by Electron Paramagnetic Resonance (EPR) spectroscopy (E-109, Varian Associates, CA) at room temperature (Figure1).

RESULTS AND DISCUSSIONS

Relaxivity of conjugated nitroxides and dinitroxides when attached to polymers increased compared to free nitroxide and dinitroxide (Table 2). These macromolecules are more efficient with respect to their relaxivity as a consequence of their increased rotational correlation time resulting from their high molecular weight and higher nitroxide content. The relaxivity of polymeric conjugates of nitroxides and dinitroxides exceeded that of standard CA Gd-DTPA. Results suggest that the relaxivity of HPMA copolymer-dinitroxide conjugates with 50 mole% conversion for P2 and P3 is less than and for P1 equal to r1 relaxivity of HPMA copolymer-mononitroxide conjugates with 100 mole% conversion. The reasons for this anomalous behavior are subject of further investigation. In vitro stability test in the presence of glutathione showed that the polymeric conjugates and selected structures of free dinitroxide and mononitroxide are relatively stable (Figure 1).

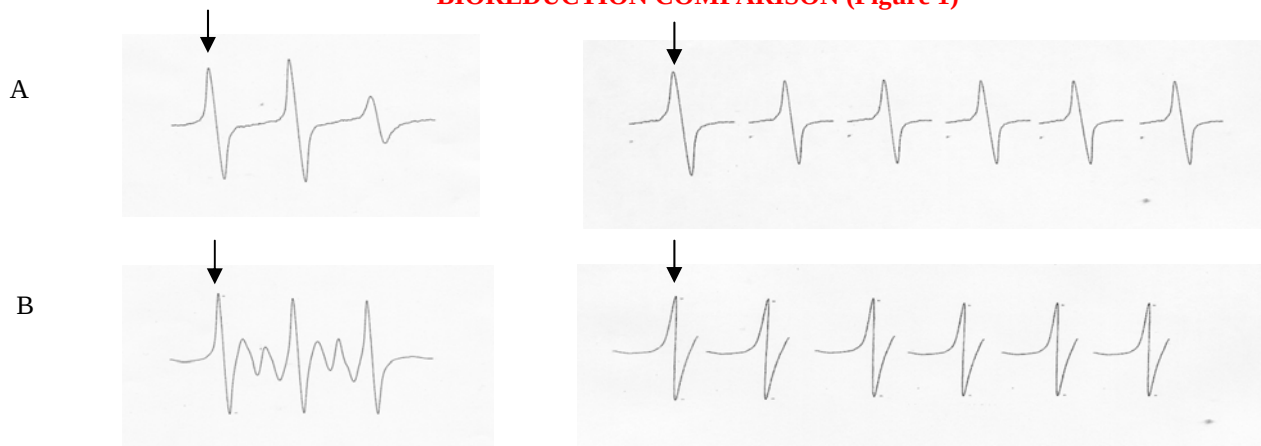
CHARACTERIZATION OF POLYMER-LINKED NITROXIDES (Table 2)

SampleNo.	Sample Description	Nitroxide Content (mmole/gpolymer)		Relaxivity ^b (mM ⁻¹ .s ⁻¹)
		Nitroxide	Dinitroxide (2°) ^a	
1	P1-Nox	1.43±0.004	-	10.38
2	P1-Dinox (2°)	-	0.764±0.054	10.58
3	P2-Nox	0.704±0.019	-	11.83
4	P2-Dinox (2°)	-	0.375±0.021	8.61
5	P3-Nox	0.278±0.005	-	4.24
6	P3-Dinox (2°)	-	0.136±0.005	2.86
7	Nitroxide	-	-	0.26
8	Dinitroxide (2°)	-	-	0.52
9	Gd-DTPA	-	-	5.2

^a Secondary amine dinitroxide

^b Data for relaxivity are the average of duplicate experiments

BIOREDUCTION COMPARISON (Figure 1)



A) 1: Sample 8 EPR; 2: Sample 8 EPR at different time points in the presence of glutathione.

B) 1: Sample 6 EPR; 2: Sample 6 EPR at different time points in the presence of glutathione.

CONCLUSIONS

- ❑ Compared to free nitroxides HPMA copolymer conjugated nitroxides demonstrated higher relaxivities than Gd-DTPA.
- ❑ Selected free and polymeric conjugated nitroxides show higher stabilities than previously synthesized free nitroxides (5).
- ❑ These studies demonstrate the potential of HPMA copolymer-nitroxide conjugates for imaging of breast cancer tumors.

REFERENCES

- ❑ 1. R. Weissleder et al., Nat Biotechnol.17 (1999) 375
- ❑ 2. J. Kopecek et al., Eur J Pharma Biopharm.50 (2000) 61
- ❑ 3.Y. Huang et al., Macrom Biosci. 3 (2003) 647
- ❑ 4.C. Winalski et al., Mag Reson Med. 48 (2002) 965
- ❑ 5.G. Rosen et al., Macromolecules. 36 (2003) 1021

ACKNOWLEDGEMENTS

This work was supported by predoctoral DOD fellowship W81XWH0410341, and NIH-1R21CA095005-01.

Appendix 2: Bahar Zarabi, Anjan Nan, Jiachen Zhuo, Rao Gullapalli, and Hamid Ghandehari, Macrophage Targeted N-(2-hydroxypropyl)methacrylamide (HPMA) Conjugates for Magnetic Resonance Imaging, *Molecular Pharmaceutics*, 3(5), **2006**, 550-557.

Macrophage Targeted N-(2-Hydroxypropyl)methacrylamide Conjugates for Magnetic Resonance Imaging

Bahar Zarabi,^{†,‡,§} Anjan Nan,^{†,‡} Jiachen Zhuo,^{||} Rao Gullapalli,^{‡,||} and
Hamidreza Ghandehari^{*,†,‡}

Department of Pharmaceutical Sciences, Center for Nanomedicine and Cellular Delivery,
and Department of Radiology, University of Maryland, Baltimore, Maryland 21201

Received June 30, 2006

Abstract: This study describes the synthesis, characterization and in vitro evaluation of targetable N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer–gadolinium (Gd) chelates for enhanced magnetic resonance imaging (MRI) of macrophages. Copolymers of HPMA, methacryloylglycylglycyl-mannosamine (MA-GG-ManN), aminopropylmethacrylamide-benzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (APMA-DOTA), and 5-(3-(methacryloylaminopropyl)thioureidyl) fluorescein (MA-AP-FITC) were synthesized and characterized. Gd was chelated to the polymeric precursors. The conjugates were characterized for gadolinium content by inductively coupled plasma optical emission spectrometry (ICP-OES) and T_1 relaxivity (r_1) at room temperature and 1.5 T. The effect of ManN content on mannose receptor mediated uptake of THP-1 human macrophages was evaluated as a function of time and temperature. The polymer conjugates showed relaxivities in the range of 21.8–24.9 s⁻¹ mM⁻¹ Gd. Relaxivities of the conjugates per mM Gd were up to 7 times higher than that of a commercially available MR contrast agent Gd-DOTA. Significantly ($p < 0.042$) higher uptake was observed for targeted conjugates compared to nontargeted conjugates. The uptake of polymeric conjugates was time and concentration dependent and appears to be mannose receptor mediated. The increased relaxivity coupled with the ability to target these carriers to cells containing ManN receptors shows promise for the application of these agents in clinical MR imaging of macrophage mediated malignancies.

Keywords: HPMA copolymers; targeted delivery; contrast agent; magnetic resonance imaging; relaxivity

Introduction

Activated macrophages play an important role in many pathophysiological processes such as inflammatory diseases,

autoimmune diseases, cancer, atherosclerosis, neurological disorders, organ rejection, and bacterial soft-tissue infections. Early detection and noninvasive monitoring of these conditions are critical for successful intervention. The role of magnetic resonance imaging (MRI) in the detection of macrophage activity is rapidly evolving.¹ Compared to conventional imaging methods such as ultrasound, scintigraphy, computed tomography, and radiography, MRI provides a high spatial resolution in detection. Due to this

* Corresponding author. Mailing address: Center for Nanomedicine & Cellular Delivery, Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 Penn Street, Baltimore, MD 21201. Tel: (410) 706-8650. Fax: (410) 706-5017. E-mail: hghandeh@rx.umaryland.edu.

[†] Department of Pharmaceutical Sciences.

[‡] Center for Nanomedicine and Cellular Delivery.

[§] E-mail: bzara001@umaryland.edu.

^{||} Department of Radiology.

(1) Weinmann, H. J.; Ebert, W.; Misselwitz, B.; Schmitt-Willich, H. Tissue-Specific MR Contrast Agents. *Eur. J. Radiol.* **2003**, *46*, 33–44.

advantage there is an increasing demand for development of sensitive and well-tolerated MRI agents that can be rapidly translated from small animal models into patients with diseases that involve mediation of activated macrophages. Findings of several studies have shown the feasibility and clinical potential of macrophage-specific MR imaging following intravenous administration of iron oxide particles.^{2,3} Due to the negative contrast, however, differentiation between signal loss caused by iron and native low signal in tissue may be problematic. It is therefore preferable to achieve positive contrast using agents such as gadolinium (Gd).

Gd-based macromolecular contrast agents provide positive contrast and have several advantages over conventional small molecular weight agents. First, the attachment of multiple contrast agents to a macromolecular carrier such as a polymer increases the circulation half-life of the contrast agent, which can lead to increased local concentration at certain anatomical (e.g., liver) or pathological (e.g., solid tumor) sites. Second, due to decrease in molecular motion of the macromolecule, the relaxivity of the contrast agent increases. Third, because of prolonged intravascular retention time of macromolecular contrast agents, imaging of multiple body regions without repeated dosing of contrast agent is possible.⁴ Finally, by passive or active targeting of the macromolecular carrier, it is possible to target the contrast agent to specific cells further enhancing contrast.⁵

Mannose receptors are c-type lectin containing multiple carbohydrate-recognition domains.⁶ They are expressed primarily on macrophages and dendritic cells as well as some endothelial cells.^{6,7} Due to the enhanced expression of mannose receptors in activated macrophages and the ability of these receptors to recycle, ligand uptake by such cells is essentially continuous, allowing accumulation of large quantities of ligands intracellularly.^{7,8} These properties make

mannose receptor an attractive target for delivery of diagnostic or therapeutic agents.

N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers are nontoxic water-soluble synthetic polymeric carriers that have been extensively evaluated for safety and efficacy and are currently in clinical trials for targeted cancer chemotherapy.⁹ Previously HPMA copolymers containing pendant saccharide moieties were evaluated for their bioadhesive properties¹⁰ and for targeted delivery of antileishmanial compounds to liver macrophages.¹¹ The potential of these copolymers for passive delivery of MR contrast agents has also recently been reported.^{12,13} Active (receptor-mediated) targeting of HPMA-based MR contrast agents to macrophages, however, remains unexplored. Here we report the synthesis, physicochemical characterization, and in vitro cellular uptake of HPMA copolymer–Gd chelates containing mannosamine in the side chains for active targeting to macrophages.

Experimental Section

Chemicals and Reagents. *N,N'*-Azobisisobutyronitrile (AIBN) and gadolinium(III) chloride hexahydrate (GdCl₃·6H₂O) were obtained from Aldrich (Milwaukee, WI). *N*-(3-Aminopropyl)methacrylamide (APMA) was obtained from Polysciences, Inc. (Warrington, PA). *p*-Isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (*p*-SCN-Bz-DOTA) was obtained from Macrocyclics (Dallas, TX). *N,N,N',N'*-Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA disodium salt dihydrate) was obtained from USB Corporation (Cleveland, OH). Trypan blue stains 0.4% and 2-mercaptoethanol were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from QBI (Gaithersburg, MD). Phorbol myristate 13-acetate (PMA) was obtained from Promega (Madison, WI). All other

- (2) Thorek, D. L.; Chen, A. K.; Czupryna, J.; Tsourkas, A. Superparamagnetic Iron Oxide Nanoparticle Probes for Molecular Imaging. *Ann. Biomed. Eng.* **2006**, *34*, 23–38. Review.
- (3) Corot, C.; Perty, K. G.; Trivedi, R.; Saleh, A.; Jonkmanns, C.; Le Bas, J. F.; Blezer, E.; Rausch, M.; Brochet, B.; Foster-Gareau, P.; Baleriaux, D.; Gaillard, S.; Douset, V. Macrophage Imaging in Central Nervous System and in Carotid Atherosclerotic Plaque using Ultrasmall Superparamagnetic Iron Oxide in Magnetic Resonance Imaging. *Invest. Radiol.* **2004**, *39*, 619–25. Review.
- (4) Bogdanov, A. A., Jr.; Weissleder, R.; Frank, H. W.; Bogdanova, A. V.; Nossif, N.; Schaffer, B. K.; Tsai, E. A New Macromolecule as a Contrast Agent for MR Angiography: Presentation, Properties, and Animal Studies. *Radiology* **1993**, *187*, 701–706.
- (5) Orang-Khadivi, K.; Pierceet, B. L.; Ollom, C. M.; Floyd, L. J.; Siegel, R. L.; Williams, R. F. New Magnetic Resonance Imaging for the Detection of Breast Cancer. *Breast Cancer Res. Treat.* **1994**, *32*, 119–135.
- (6) Taylor, M. E. Evaluation of a Family of Receptors Containing Multiple C-Type Carbohydrate-Recognition Domains. *Glycobiology* **1997**, *7*, v–viii.
- (7) Stahl, P. D.; Ezekowitz, R. A. B. The Mannose Receptor is a Pattern Recognition Receptor Involved in Host Defense. *Curr. Opin. Immunol.* **1998**, *10*, 50–55.

- (8) Sallusto, F.; Cella, M.; Danieli, C.; Lanzavecchia, A. Dendritic Cells Use Macropinocytosis and the Mannose Receptor to Concentrate Macromolecules in the Major Histocompatibility Complex Class II Compartment: Downregulation by Cytokines and Bacterial Products. *J. Exp. Med.* **1995**, *182*, 389–400.
- (9) Kopecek, J.; Kopeckova, P.; Minko, T.; Lu, Z. HPMA Copolymer-Anticancer Drug Conjugates: Design, Activity, and Mechanism of Action. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 61–81.
- (10) Rath, R. C.; Kopeckova, P.; Rihova, B.; Kopecek, J. *N*-(2-Hydroxypropyl)methacrylamide Copolymers Containing Pendant Saccharide Moieties: Synthesis and Bioadhesive Properties. *J. Polym. Sci., Part A: Polym. Chem.* **1991**, *29*, 1895–1902.
- (11) Nan, A.; Croft, S. L.; Yardley, V.; Ghandehari, H. Targetable Water-Soluble Polymer-Drug Conjugates for the Treatment of Visceral Leishmaniasis. *J. Controlled Release* **2003**, *94*, 115–127.
- (12) Huang, Y.; Nan, A.; Rosen, G. M.; Winalski, C. A.; Schneider, E.; Tsai, P.; Ghandehari, H. *N*-(2-Hydroxypropyl)methacrylamide (HPMA) Copolymer-Linked Nitroxide: Potential Magnetic Resonance Contrast Agents. *Macromol. Biosci.* **2003**, *3*, 647–652.
- (13) Wang, D.; Miller, S. C.; Sima, M.; Parker, D.; Buswell, H.; Goodrich, C. H.; Kopeckova, P.; Kopecek, J. The Arthrotropism of Macromolecules in Adjuvant-Induced Arthritis Rat Model: a Preliminary Study. *Pharm. Res.* **2004**, *21*, 1741–1749.

Table 1. Physicochemical Characteristics of Targetable HPMA Copolymer–Contrast Agent Conjugates

sample ^a	feed comonomer composition (mol %)				polymer characteristics ^d (mmol/g polymer)				<i>M_w</i> ^b	<i>n</i> ^c	relaxivity (s ⁻¹ mM ⁻¹ Gd)
	HPMA	ManN	DOTA	FITC	DOTA content ^d	ManN content ^d	FITC content ^d	Gd content ^d			
P ₀	88	0	10	2	0.452 ± 0.01	0	0.053 ± 0.02	0.43	52 000	1.6	21.8
P ₁	86	2	10	2	0.332 ± 0.01	0.097 ± 0.03	0.083 ± 0.02	0.25	63 000	1.6	21.5
P ₂	84	4	10	2	0.325 ± 0.02	0.199 ± 0.04	0.095 ± 0.02	0.21	59 000	1.8	24.4
P ₃	80	8	10	2	0.313 ± 0.01	0.272 ± 0.05	0.083 ± 0.02	0.21	58 000	1.7	24.4
P ₄	72	16	10	2	0.290 ± 0.02	0.498 ± 0.04	0.072 ± 0.02	0.15	58 000	1.8	24.9
Gd-DOTA											3.4

^a For structures of polymer–contrast agent conjugates see Figure 1. ^b Weight average molecular weight of polymer precursor. ^c Polydispersity index. ^d Polymer contrast agent conjugate.

chemicals were obtained from Sigma (St. Louis, MO) and were of reagent grade.

Cell Culture. Human monocyte cell line THP-1 (ATCC TIB 202; ATCC, Manassas, VA) was cultured in modified RPMI 1640 (ATCC) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 0.05 mM 2-mercaptoethanol. Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂. Phorbol myristate 13-acetate (PMA) 160 nM was applied to monocyte cultures. After incubation with PMA for 48 h, monocytes were differentiated to macrophages. Macrophages were washed with modified RPMI medium containing 10% fetal bovine serum to eliminate the effect of PMA.

Synthesis and Characterization of Polymer–Contrast Agent Conjugates. Monomer Synthesis. *N*-(2-Hydroxypropyl)methacrylamide (HPMA),¹⁴ 5-[3-(methacryloylamino-propyl)thioureidyl] fluorescein (MA-AP-FITC),¹⁵ and methacryloylglycylglycylmannosamine (MA-GG-ManN)¹⁰ were prepared as described previously. Comonomer aminopropyl-methacrylamide-benzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (APMA-benzyl-DOTA) was synthesized by reacting *N*-(3-aminopropyl)methacrylamide (APMA) with *p*-isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (*p*-SCN-Bz-DOTA) in dry dimethyl sulfoxide (DMSO). The *p*-SCN-Bz-DOTA was reacted at 1.2 molar excess to APMA.

Polymer Synthesis. HPMA copolymer conjugates with or without ManN were synthesized by a modified two-step procedure. Briefly, in the first step the polymeric precursors containing side chains terminated in DOTA were synthesized by free radical precipitation copolymerization of the monomers of HPMA, APMA-benzyl-DOTA, MA-AP-FITC, and MA-GG-ManN in predetermined molar compositions (Table 1). All polymerizations were carried out in acetone/DMSO using AIBN as the initiator. The ratio of monomers:initiator:solvent in the feed was kept constant at 12.5:0.6:86.9 (wt

%), respectively. The comonomer mixture was sealed in an ampule under nitrogen and stirred at 50 °C for 24 h. The polymers were isolated by precipitation of the resulting solution into ether. The contents of side chains terminating in DOTA were determined by UV spectrophotometry (λ_{max} = 274 nm). In the second step, the DOTA molecules in the side chain of the polymeric precursors were chelated to gadolinium (Gd) as described elsewhere.¹³ Briefly polymer–DOTA conjugates and GdCl₃·6H₂O (1.5:1 molar equiv relative to the DOTA content) were dissolved in deionized water. The pH of the solution was maintained at 5–5.5 overnight by gradual addition of 1 N NaOH solution. EDTA disodium salt dihydrate was added into the solutions to chelate the excess Gd. After stirring for 30 min, the milky solution was purified over a PD10 size exclusion column (GE Healthcare, NJ), to remove the EDTA-chelated Gd and other unreacted low molecular weight monomers from the polymeric conjugates. The polymer conjugates were dissolved in deionized water, dialyzed, and lyophilized. The chemical structure of the macromolecular contrast agent is shown in Figure 1.

Physicochemical Characterization. All polymer–contrast agent conjugates were characterized for their Gd content by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Galbraith, Knoxville, TN). The targeting moiety (ManN) content was determined by the Morgan–Elson method and UV spectroscopic measurement as described earlier.¹⁶ Briefly the ManN covalently attached to the polymer side chains was hydrolyzed under acidic conditions followed by complexation of the hydrolyzed sugar with *p*-dimethylaminobenzaldehyde to yield a colored complex, which was determined spectrophotometrically at 585 nm. DOTA and FITC contents of the final conjugates were determined by UV spectrophotometry at 274 and 492 nm, respectively. The molecular weight and molecular weight distribution of the polymeric conjugates were estimated by size exclusion chromatography (SEC) on a Superose 12 HR 10/30 column (GE Healthcare, Piscataway, NJ) using a fast protein liquid chromatography (FPLC) system (GE Health-

(14) Strohm, J.; Kopecek, J. Poly *N*-(2-Hydroxypropyl)Methacrylamide. 4. Heterogeneous Polymerization. *Angew. Makromol. Chem.* **1978**, 70, 109–118.

(15) Omelyanenko, V.; Kopeckova, P.; Gentry, C.; Kopecek, J. Targetable HPMA Copolymer-Adriamycin Conjugates. Recognition, Internalization, and Subcellular Fate. *J. Controlled Release* **1998**, 53, 25–37.

(16) Van De Loo, H. M. An Improved Method for the Quantitative Determination of Hexosamines According to Elson and Morgan. *Anal. Biochem.* **1976**, 76, 556–60.

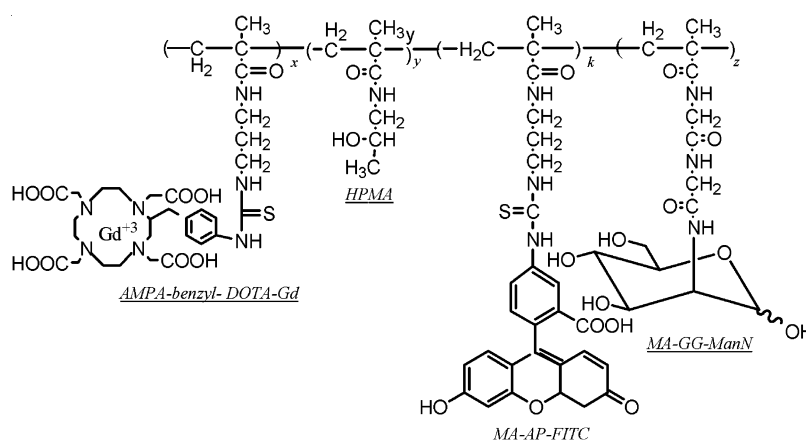


Figure 1. General structure of HPMA copolymer–DOTA (Gd)–ManN–FITC conjugates (HPMA, *N*-(2-hydroxypropyl)methacrylamide; MA–AP–FITC, 5-[3-(methacryloylamino)propyl]thioureidyl fluorescein; MA–GG–ManN, methacryloylglycylglycylmannosamine; APMA–benzyl–DOTA, aminopropylmethacrylamide–benzyl–1,4,7,10-tetraazacyclododecane–1,4,7,10-tetraacetic acid; Gd, gadolinium).

care) and HPMA homopolymer fractions of known molecular weight as standards.

Relaxivity Measurements. The r_1 relaxivities of HPMA copolymer–Gd chelates were calculated from T_1 (relaxation time) measurements at room temperature. Solutions of each sample were diluted in deionized water at four concentrations (from 0.1 to 0.015 mM) and were imaged using 1.5 T MRsystem (Eclipse, Philips Medical System, Cleveland, OH, and Sigma). T_1 was measured using an inversion recovery fast spin–echo imaging sequence using inversion times (TI) of 50, 100, 200, 400, 700, 1400, 2000, and 2800 ms, an echo time (TE) of 12 ms, and an echo train length of 8 at a repeat time TR of 6000 ms. All images were obtained from a single axial slice with a 20×15 cm field of view (FOV), 3 mm slice thickness, 256×192 matrix, and one excitation. Images were transferred to an independent workstation (SGI, O200) for the calculation of T_1 from the images obtained at various inversion times. T_1 values for each solution and deionized water were calculated using MATLAB (The Mathworks, Inc., Natick, MA). The r_1 values of each solution were calculated, using a least-squares fit, as the slope of $(1/T_{1,\text{solution}} - 1/T_{1,\text{water}})$ versus concentration of contrast agent (mM), where $T_{1,\text{solution}}$ is the T_1 of each dilution of the contrast agent and $T_{1,\text{water}}$ is the T_1 of water without contrast agent.

In Vitro Evaluation of Polymer–Contrast Agent Conjugates. Macrophage Uptake Studies. FITC (fluorescein-5-isothiocyanate) was used as a fluorescent probe to measure biorecognition and uptake of polymeric conjugates in model THP-1 human monocytes. Before analysis cells were seeded on 96-well culture plates at a concentration of 1×10^5 cells/well and treated with 160 nM PMA in modified RPMI 1640 (10% fetal bovine serum) for 48 h at 37 °C and in a humidified atmosphere of 5% CO₂. Upon treatment with PMA, THP-1 cells adhered to the dish, as the first indication of differentiation to macrophages.^{17,18} Before the uptake study, cells were washed with modified RPMI 1640 (10% fetal bovine serum) to stop the effect of PMA. One hundred microliter volumes of HPMA copolymer–DOTA conjugates (with and without Gd) in modified RPMI 1640 (10% FBS)

were added to obtain final concentrations of 2, 4, and 8 μM (ManN equivalent), respectively, for each sample. Experiments were performed at 3, 6, and 24 h to determine time dependent effects on uptake. At each time point the overlay was removed and cells washed 2 times with PBS. One hundred microliters of modified RPMI 1640 (without FBS) was subsequently added to each well, and the total fluorescence associated with the cells was determined directly on a SPECTRAMax Gemini XS fluorescent plate reader (Molecular Devices, Sunnyvale, CA) (Ex/Em 492/520). Experiments were performed at 37 °C and 4 °C to determine temperature dependent uptake. Polymers with and without Gd were compared to determine its effect on uptake.

To explore the possibility of active mannose receptor mediated uptake, additional experiments were performed with macrophages preincubated with 100 mM ManN solution for 3 h. The uptake of the conjugates in all experiments was expressed as % of fluorescence in the feed after correcting for background. Statistical significance of differences in uptake between different samples was analyzed using Student's *t* test.

Quenching of Extracellular Fluorescence. The concentration of trypan blue required to completely quench extracellular fluorescence was first determined by exposure of 100 μL /well of sample P₁ (Table 1) (2, 4, 8 μM equivalent of FITC) to 100 μL of serial dilution of the dye (62.5–4000 $\mu\text{g/mL}$) in a 96-well plate. The fluorescence intensity was measured directly in the wells using a fluorescent plate reader (Ex/Em 492/520). Wells containing only sample P₁ (Table 1) (2, 4, 8 μM equivalent of FITC) were used as controls to indicate complete quenching.

- (17) Perieto, J.; Eklund, A.; Patarroyo, M. Regulated Expression of Integrins and other Adhesion Molecules during Differentiation of Monocytes into Macrophages. *Cell. Immunol.* **1994**, *156*, 191–211.
- (18) Schwende, H.; Fitzke, E.; Ambs, P.; Dieter, P. Differences in the State of Differentiation of THP-1 Cells Induced by Phorbol Ester and 1, 25-Dihydroxyvitamin D3. *J. Leukocyte Biol.* **1996**, *59*, 555–561.

In subsequent experiments after incubation of macrophages with the polymer conjugates for 3 h, extracellular fluorescence was quenched by adding 100 μ L of trypan blue (4000 μ g/mL). The dye was removed after 1 min, and cells were washed two times with PBS. One hundred microliters of modified RPMI 1640 (without fetal bovine) was subsequently added to each well, and the intensity of intracellular fluorescence was measured directly in the wells.

Results

Synthesis and Characterization of HPMA Copolymer–Contrast Agent Conjugates. A series of HPMA-DOTA-(Gd)-FITC conjugates were synthesized with incremental variation in targeting moiety (ManN) content (Figure 1, Table 1). As control a conjugate without targeting moiety (sample P₀, Table 1) was synthesized. The incorporations of the chelating (APMA-DOTA), reporter (MA-AP-FITC), and targeting (MA-GG-ManN) comonomers were between 71% and 92%, 66% and 96%, and 78% and 98%, respectively, of the feed comonomer content. Subsequent chelation of the DOTA side chains of the conjugates with Gd resulted in Gd incorporation efficiency of 52–75% of the DOTA molecules per polymer backbone. All HPMA-linked Gd conjugates exhibited relaxation (r_1) values up to 7 times greater than a commercially available Gd-DOTA contrast agent (Dotarem)¹⁹ (Table 1). The estimated weight average molecular weight of the polymers was between 52 000 and 63 000 with polydispersity index ranging from 1.6 to 1.8 (Table 1), which was typical of similar polymeric conjugates reported in the literature.¹¹

Time and Concentration Dependent Uptake. The time and concentration dependent uptake of polymeric conjugates by macrophages was evaluated (Figure 2). The fluorescence values (expressed as % of feed content) corresponding to the uptake of conjugates were compared at three different concentrations of 2, 4, and 8 μ M (equivalent of ManN) (Figure 2, panels a, b, c). The uptake of all the targetable conjugates increased with increase in ManN concentration at all the time points. After 24 h the uptake of 4 mol % or higher ManN containing polymers was significantly ($p < 0.040$) higher than after 3 and 6 h. This was observed at both 4 and 8 μ M concentrations of ManN. However, at 2 μ M concentration, the uptake after 24 h was only significantly different ($p < 0.024$) from uptake after 3 and 6 h for sample P₄ (Table 1). There was no significant difference in uptake between 3 and 6 h at any concentration.

Effect of Targeting Moiety. Conjugates with 4 mol % or higher of ManN (P₂–P₄) resulted in significantly ($p < 0.017$) higher uptake than 2 mol % ManN containing conjugate (P₁) at the same equivalent concentrations of targeting moiety and at all time points studied, namely, 3, 6, and 24 h. At 8 and 4 μ M (equivalent of ManN) concentrations, 16 mol % ManN containing conjugate showed significantly higher uptake ($p < 0.041$) than 8 mol

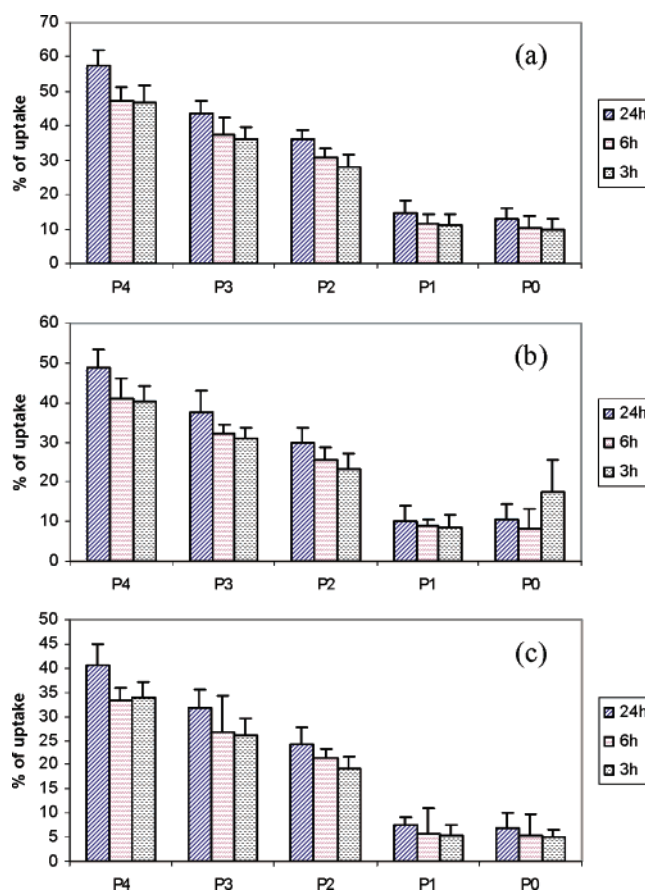


Figure 2. Time and concentration dependent uptake of targetable HPMA copolymer–contrast agent conjugates at 37 °C at (a) 8 μ M; (b) 4 μ M, and (c) 2 μ M (equivalent concentration of ManN). Uptake is expressed as mean of three samples $\pm$ standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

% after 6 and 24 h. The uptake of polymeric conjugates with 4 mol % or more ManN content was also higher than control nontargeted conjugate (P₀). Polymer with 2 mol % ManN did not show significant uptake compared to the control without targeting moiety (P₀, Figure 2). Incubation of macrophages with polymeric conjugates at 4 °C (Figure 3) resulted in significantly reduced uptake when compared to those carried out at 37 °C suggesting the involvement of an energy dependent process.

Effect of Gd on Uptake. Uptake of polymeric conjugates with and without Gd was compared to each other after 3 h (Figure 4). Polymer-chelated Gd showed a higher trend of uptake, but the difference between conjugate uptake with and without Gd was not significant.

Evidence of Mannose Receptor Mediated Uptake. The uptake of polymeric conjugates with 4, 8, and 16 mol % of targeting moiety at 2, 4, and 8 μ M after 3 h was inhibited by 65–85% upon preincubation with free ManN (Figure 5). However, the uptake of P₁ before and after treatment did not change significantly. Figure 6 showed that after mannose treatment the uptake of P₄, P₃, and P₂ was the same as that of P₁. These results suggest that uptake of polymeric

(19) GE Healthcare Website: <http://www.amershamhealth.com> (visited June 2006).

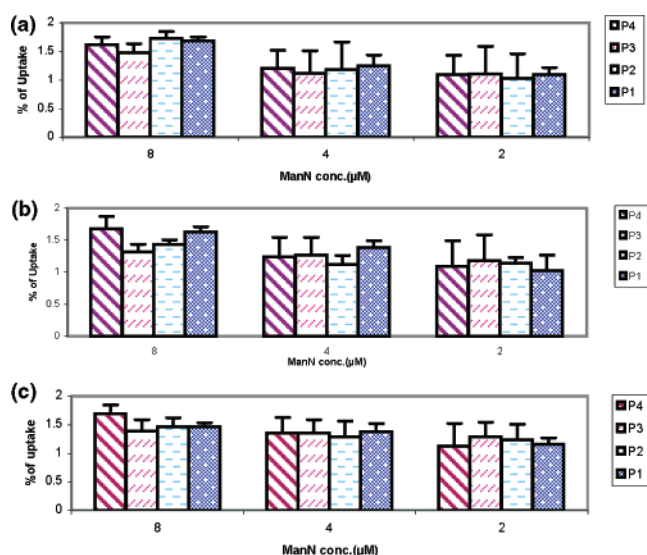


Figure 3. Time dependent uptake of targetable HPMA copolymer–contrast agent conjugates at 4 °C after: (a) 3 h; (b) 6 h; and (c) 24 h. Uptake is expressed as mean of three samples $\pm$ standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

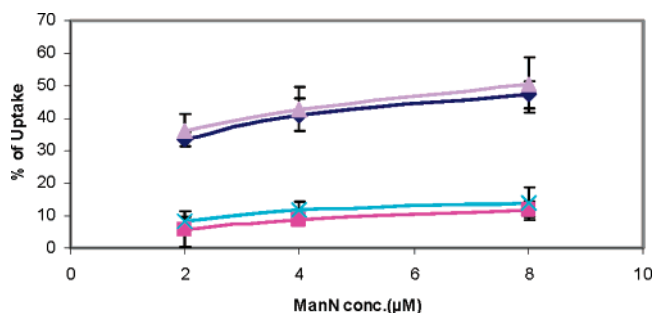


Figure 4. Effect of Gd on uptake of HPMA copolymer–contrast agent conjugates. P₄ (◆); P₁ (■); P₄–Gd (▲); P₁–Gd (×). Uptake is expressed as mean of three samples $\pm$ standard error. For structures and characteristics of samples see Figure 1 and Table 1.

conjugates is mediated primarily by mannose receptors. The lack of complete inhibition indicates that a secondary mechanism such as adsorptive endocytosis of the polymers may also exist.

Extracellular Fluorescence Quenching. In this study, we used the trypan blue dye technique to quench the extracellular fluorescence.^{20,21} Complete quenching of FITC fluorescence was obtained by 4000 $\mu\text{g/mL}$ of trypan blue (data has not been shown). This concentration was subsequently used in

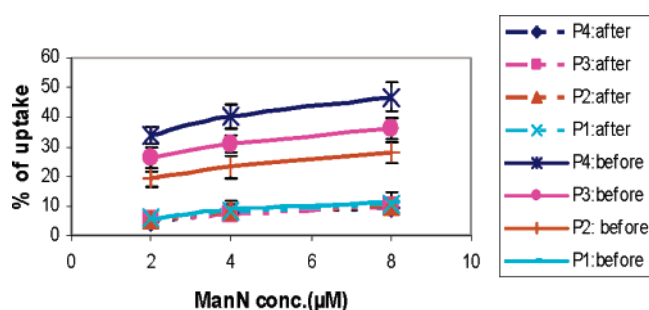


Figure 5. Effect of preincubation with free ManN on the uptake of HPMA copolymer–contrast agent conjugates by macrophages. Dashed lines show % of uptake of polymers in the presence of ManN. Full lines show % of uptake of polymers in the absence of ManN. Uptake is expressed as mean of three samples $\pm$ standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

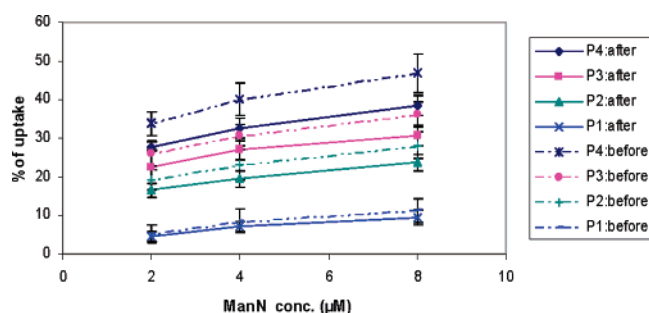


Figure 6. Effect of extracellular fluorescence quenching on the uptake of HPMA copolymer–contrast agent conjugates by macrophages. Dashed lines show % of uptake of polymers before quenching. Full lines show % of uptake of polymers after quenching. Uptake is expressed as mean of three samples $\pm$ standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

uptake measurements. Uptake studies carried out with quenching of extracellular fluorescence using trypan blue resulted in the decrease of measured uptake values by 12.5–17.9% for all the polymers (Figure 6). The decrease in % of uptake after incubating the cells with trypan blue suggests that not all of the polymer conjugates are internalized and that some of the conjugates are associated with the surface of macrophages.

Discussion

Macrophages are a major component of the mononuclear phagocyte system that play a critical role in the initiation, maintenance, and resolution of inflammation. Activated macrophages secrete multiple potent mediators of inflammation and tissue destruction, including proinflammatory cytokines (e.g., IL-1, IL-6, TNF- α), chemokines, prostaglandins, metalloproteinases, and reactive oxygen species.^{22,23} Further, activated macrophages are known to participate in

(20) Hed, J.; Hallden, G.; Johansson, S. G. O.; Larsson, P. The Use of Fluorescence Quenching in Flow Cytometry to Measure The Attachment and Ingestion Phases in Phagocytosis in Peripheral Blood without Prior Cell Separation. *J. Immunol. Methods* **1987**, *101*, 119–125.

(21) Sahlin, S.; Hed, J.; Runquist, I. Differentiation Between Attached and Ingested Immune Complexes by a Fluorescence Quenching Cytofluorometric Assay. *J. Immunol. Methods* **1983**, *60*, 115–124.

(22) Kinne, R. W.; Brauer, R.; Stuhlmüller, B.; Palombo-Kinne, E.; Burmester, G. R.; et al. Macrophages in Rheumatoid Arthritis. *Arthritis Res.* **2000**, *2*, 189–202.

antigen presentation, and thereby they are thought to contribute to the activation and proliferation of antigen specific T-cells and their consequent destructive activities.^{22,24,25} Because macrophages produce a wide range of biologically active molecules participating in both beneficial and detrimental outcomes in inflammation, therapeutic interventions targeting macrophages and their products may open new avenues for controlling inflammatory diseases. However, understanding the underlying mechanisms of macrophage action is still in question. Current strategies mostly involve invasive procedures such as blood sampling, biopsy, etc. Noninvasive external imaging techniques such as MRI offer methods for the measurement of cell behavior and biochemical events in situ and can be valuable tools for early detection and diagnosis of the diseases where macrophages are involved. The use of MR contrast agents such as Gd is limited by the sheer amount required to obtain a good signal for detection. An approach that would selectively localize a high concentration of contrast agents in the activated macrophages without compromising their essential functions can enhance contrast signal-to-background ratio significantly. In addition such an approach could also be considered as a surrogate for a similar delivery of a therapeutic payload, e.g., anticancer, antiinflammatory, or antiarthritic drugs or tumor vaccine to induce an immune response.²⁴ The macrophage mannose receptor, exclusively expressed on activated macrophages, can be used to target and localize large amounts of contrast agents for diagnostic purposes.

In this study, we evaluated macrophage targetable macromolecular contrast agents consisting of gadolinium (Gd) chelated to the backbone of water-soluble HPMA copolymers. The HPMA copolymer backbone contains a multivalency of mannosamine molecules as targeting ligands specific to the macrophage mannose receptors. The overall hypothesis behind this study was that by active targeting of Gd-polymer conjugates to the macrophages it is possible to significantly increase accumulation of contrast agent, resulting in a higher macrophage to background ratio of accumulation. HPMA copolymers are advantageous as macromolecular carriers because of the ability to tailor-make the polymer backbone and control the content of side chains by facile chemical manipulations. As a first step toward development of HPMA copolymer-ManN conjugates for targeted delivery of Gd to macrophages, we synthesized and characterized a series of these conjugates with incremental variation in their targeting moiety content. Previous work in our laboratory¹¹ has shown that HPMA conjugated to ManN can be used to actively target the macrophage mannose receptors of the liver for treatment of infectious diseases. Drawing from those

studies we designed a series of Gd containing HPMA conjugates with a range of targeting moiety content (0–16 mol %) to study the effect of ManN content on the extent of biorecognition and uptake by macrophages. This would help in identification of a lead conjugate with optimum ManN content for highest macrophage specific targeting.

Our results demonstrated successful synthesis and characterization of HPMA based macrophage targetable macromolecular contrast agents. The molecular size of the conjugates ranged between 50 and 60 kDa, which is large enough to be retained in the macrophages once internalized.²⁶ Observed relaxivities for HPMA copolymer contrast agent conjugates (Table 1) were improved over commercially available contrast agents Gd-DOTA. Conjugation of Gd-DOTA to larger macromolecules is known to increase relaxivity by reducing rotational correlation time.²⁷ This has been observed for many Gd-based complexes^{28–32} and is similarly observed for HPMA-based contrast agents. Importantly the advantage of HPMA conjugates over Gd-DOTA will be the larger molecular size which may result in longer retention time in the macrophages. Consequently it may be possible to obtain enhanced long-term imaging data due to sustained incremental accumulation of the macromolecular agent compared to Gd-DOTA.

THP-1 cells are well-known for their phagocytic properties³³ and expression of mannose receptors.³⁴ As a result this cell line was chosen as a model for our studies. A fluorimetric

- (23) Kmiec, Z. Cooperation of Liver Cells in Health and Disease. *Adv. Anat., Embryol., Cell Biol.* **2001**, *161*, iii–xiii, 1–151.
- (24) Bresnihan, B. Pathogenesis of Joint Damage in Rheumatoid Arthritis. *J. Rheumatol.* **1999**, *26*, 717–719.
- (25) Burmester, G. R.; Stuhlmüller, B.; Keyszer, G.; Kinne, R. W. Mononuclear Phagocytes and Rheumatoid Synovitis. Master-Mind or Workhorse in Arthritis? *Arthritis Rheum.* **1997**, *40*, 5–18.

- (26) Kobayashi, H.; Kawamoto, S.; Bernardo, M.; Brechbiel, M. W.; Knopp, M. V.; Choyke, P. L. Delivery of Gadolinium-Labeled Nanoparticles to the Sentinel Lymph Node: Comparison of the Sentinel Node Visualization and Estimations of Intra-Nodal Gadolinium Concentration by the Magnetic Resonance Imaging. *J. Controlled Release* **2006**, *111*, 343–351.
- (27) Meyer, D.; Schaefer, M.; Bonnemain, B. Gd-DOTA, a Potential MRI Contrast Agent. Current Status of Physicochemical Knowledge. *Invest. Radiol.* **1988**, *23* (Suppl. 1), S232–S235.
- (28) Caravan, P.; Greenfield, M.; Li, Z.; Sherry, A. The Gd³⁺ Complex of a Fatty Acid Analogue of DOTP Binds to Multiple Albumin Sites with Variable Water Relaxivities. *Inorg. Chem.* **2001**, *40*, 6580–6587.
- (29) Caravan, P.; Ellison, J.; McMurry, T.; Lauffer, R. Gadolinium-(III) Chelates as MRI Contrast Agents: Structure, Dynamics, and Applications. *Chem. Rev.* **1999**, *99*, 2293–2352.
- (30) Nivorozhkin, A.; Kolodziej, A.; Caravan, P.; Greenfield, M.; Lauffer, R.; McMurry, T. Enzyme Activated Gd³⁺ Magnetic Resonance Imaging Contrast Agents with a Prominent Receptor-Induced Magnetization Enhancement. *Angew. Chem., Int. Ed.* **2001**, *40*, 2903–2906.
- (31) Kiessling, F.; Heilmann, M.; Lammers, T.; Ulbrich, K.; Subr, V.; et al. Synthesis and Characterization of HE-24.8: A Polymeric Contrast Agent for Magnetic Resonance Angiography. *Bioconjugate Chem.* **2006**, *17*, 42–51.
- (32) Mohs, A. M.; Zong, Y.; Guo, J.; Parker, D. L.; Lu, Z.-R. PEG-g-poly(GdDTPA-co-L-cystine): Effect of PEG Chain Length on in Vivo Contrast Enhancement in MRI. *Biomacromolecules* **2005**, *6*, 2305–2311.
- (33) Tsuchiya, S.; Yamabe, M.; Yamaguchi, Y.; Kobayashi, Y.; Konno, T.; Tada, K. Establishment and Characterization of a Human Acute Monocytic Leukemia Cell Line (THP-1). *Int. J. Cancer* **1980**, *26*, 171–176.

phagocytosis assay was adopted using FITC fluorescence to compare the uptake of the various mannose containing polymeric conjugates.

The uptake data of ManN containing HPMA copolymer–DOTA conjugates suggest the involvement of mannose receptors in recognition of the conjugates by macrophages. With an increase in the ManN content of the conjugates there was a significant ($p < 0.042$) increase in their uptake, which suggests that the uptake mechanism may be an active receptor mediated process in the recognition and internalization of these polymeric conjugates. It is well established that the affinity of receptor ligand binding in active targeting is often enhanced by the multivalency of the ligands.³⁵ A significant increase in uptake from 2 to 4 mol % or higher ManN containing polymer is indicative of this multivalent effect. The uptake of 2 mol % ManN conjugate was comparable to that of the conjugate without any targeting moiety (Figure 2). These results suggest that the number of targeting moieties per polymeric backbone can influence the uptake through ManN receptors. However, further mechanistic studies need to be done to evaluate the role and quantify the ManN concentration required for maximum uptake.

An active process such as receptor mediated binding and internalization typically demonstrates saturability. In the presence of free ManN the uptake of all targetable polymeric conjugates decreased by 65–85% (Figure 5). These results suggest the competitive inhibition effect by ManN and therefore strongly support the conclusion that uptake of polymeric conjugates is mediated primarily by mannose receptors.

The limited uptake by the nontargetable HPMA conjugate (sample P₀, Figure 2) suggests the possible involvement of a passive endocytosis mechanism as well. The involvement of endocytosis for polymeric conjugates is further suggested by time dependent studies (Figure 2). The uptake after 24 h for 4 mol % or higher ManN containing polymers was

significantly ($p < 0.040$) higher than after 3 and 6 h at 8 and 4 μ M (equivalent of ManN). No significant difference between 3 and 6 h at any concentrations was observed possibly since the passive uptake of macromolecules is usually a slower kinetic process. Finally significantly reduced uptake of polymer conjugates at 4 °C compared to 37 °C suggests that an energy dependent uptake mechanism such as endocytosis may be involved. These observations are in agreement with previous results on similar polymeric carriers for the delivery of antileishmanial agents.¹¹

The current studies demonstrate the potential of HPMA copolymer–ManN–Gd conjugates as macromolecular contrast agents for enhanced MR imaging in conditions where activated macrophages are involved. The linear flexible and hydrated chains of HPMA copolymers can provide higher molar relaxivities. Covalent attachment of targeting moieties with control over content can allow optimization of macrophage localization. Control over molecular weight and charge can allow control over pharmacokinetics and biodistribution.^{36,37} Finally such conjugates can be used for simultaneous delivery of drugs and imaging agents to allow optimization of therapy to target sites.

Conclusions

HPMA copolymer–Gd conjugates containing ManN were synthesized and characterized. In vitro studies demonstrated active mannose receptor mediated uptake of the conjugates by macrophages as well as by passive endocytosis. The multivalency of ManN units on the polymer backbone resulted in significantly higher uptake than nontargetable conjugates. The conjugates showed relaxivity values ranging from 6.3- to 7.3-fold higher than Gd. These results demonstrate the potential of macrophage targeted HPMA copolymers for delivery of MR contrast agents.

Acknowledgment. This study received financial support from the NIH (R01 EB0207171) and a predoctoral DOD fellowship (W81XWH0410341).

MP060072I

- (34) Diaz-Sivestre, H.; Espinosa-Cueto, P.; Sanchez-Gonzalez, A.; Esparza-Ceron, M. A.; Pereira-Suarez, A. L.; Bernal-Fernandez, G.; Espitia, C.; Mancilla, R. The 19-kDa Antigen of Mycobacterium Tuberculosis is a Major Adhesion that Binds the Mannose Receptor of THP-1 Monocytic Cells and Promotes Phagocytosis of Mycobacteria. *Microb. Pathog.* **2005**, 39, 97–107.
- (35) Roseman, D. S.; Baenziger, J. U. The Mannose/N-Acetylgalactosamine-4-SO₄ Receptor Displays Greater Specificity for Multivalent than Monovalent Ligands. *J. Biol. Chem.* **2001**, 276, 17052–17057.

- (36) Mitra, A.; Nan, A.; Ghandehari, H.; McNeill, E.; Mulholland, J.; Line, B. R. Technetium-99m-Labeled N-(2-Hydroxypropyl)-methacrylamide Copolymers: Synthesis, Characterization and In Vivo Biodistribution. *Pharm. Res.* **2004**, 21, 1153–1159.
- (37) Kissel, M.; Peschke, P.; Subr, V.; Ulbrich, K.; Schuhmacher, J.; Debus, J.; Friedrich, E. Synthetic Macromolecular Drug Carriers: Biodistribution of Poly [(N-2-Hydroxypropyl)Methacrylamide] Copolymers and their Accumulation in Solid Rat Tumors. *PDA J. Pharm. Sci. Technol.* **2001**, 55, 191–201.

Appendix 3: Chemical structure and characteristics of polymer-linked gadolinium conjugates with and without drug.

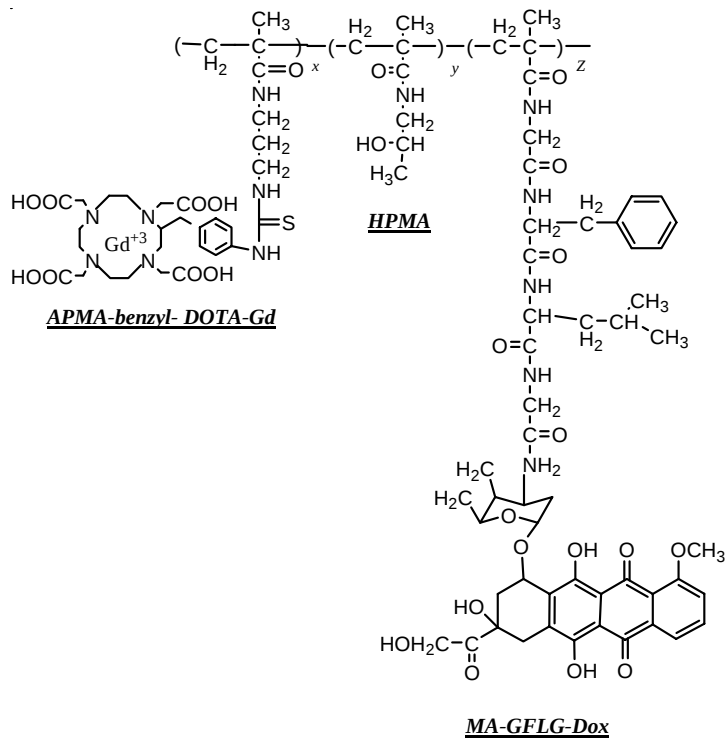


Figure 1. General structure of HPMA-copolymer-DOTA(Gd)-Dox conjugates.

Table 1. Physicochemical characteristics of HPMA copolymer - contrast agent conjugates.

Sample	Feed comonomer composition (mol%)			Polymer characteristics (mmol/g polymer)		Mw (g/mol)	n ^a	Relaxivity (s ⁻¹ mM ⁻¹ Gd)
	HPMA	APMA-DOTA	MA-GFLG-Dox	Dox content ^b	Gd content ^b			
P-(DOTA-Gd)	90	10	0	-	0.41±0.014	35000	1.6	19.6
P-(DOTA-Gd)-Dox	85	10	5	0.26±0.05	0.19±0.021	34000	1.4	32.5

^a Polydispersity index.

^b Polymer contrast agent-drug conjugate.

Table 2. pH stability results of HPMA copolymer-(DOTA-Gd) conjugate.

Days	pH=3	pH=5	pH=7
	% Gd bound ^a		
1	95.9	97.5	99.1
3	89.9	95.9	98.9
5	85.8	95.9	98.5

^a The data represent the means of duplicate points.

Table 3. Competitive challenge results of HPMA copolymer-(DOTA-Gd) conjugate.

No.	EDTA:Gd	Stability (% Gd bound) ^a
1	1:1	100
2	5:1	88.2
3	25:1	84.2
4	125:1	57.9

^a The data represent the means of duplicate points.

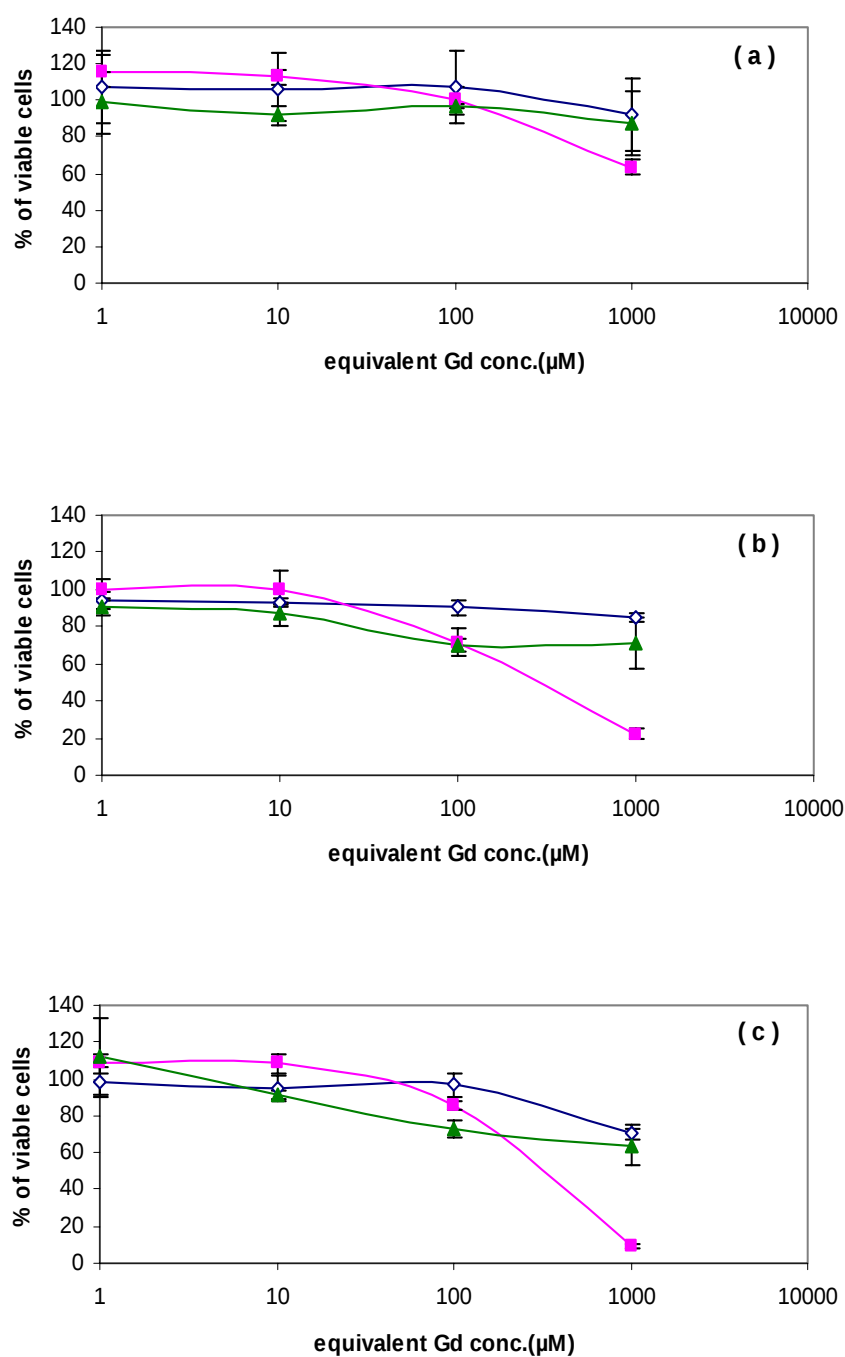


Figure 2. Cytotoxicity of HPMA copolymer-Gd conjugate on MDA-MB-435 at 37°C after: a) 24h; b) 48h; and c) 72h. GdCl₃ ($\diamond$); P-Gd ($\blacksquare$); Magnevist ($\blacktriangle$) using MTT assay. Data represent the means of triplicate points $\pm$ standard error.

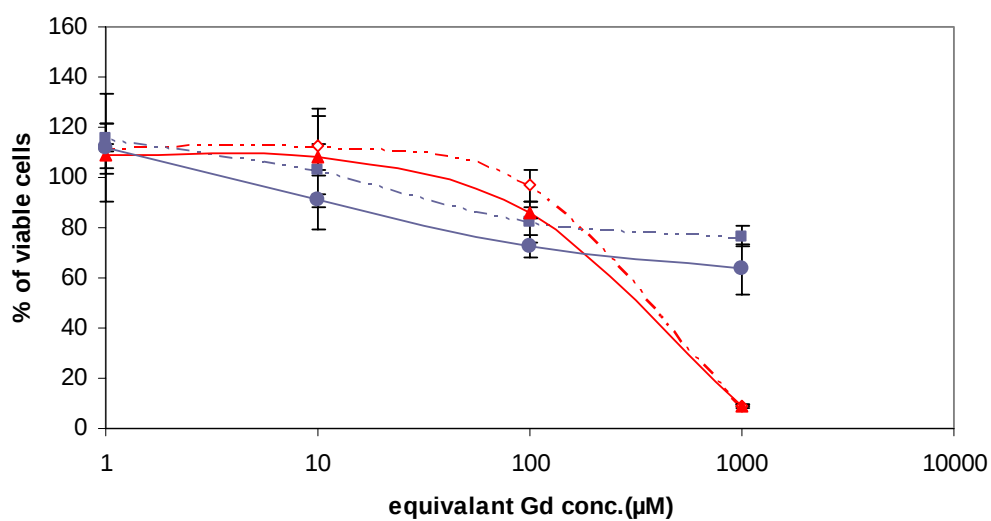


Figure 3. Comparison of cytotoxicity of varying concentrations of HPMA copolymer - Gd chelate after 72 h using MTT assay on MDA-MB-435 and NIH/3T3 cell lines .P-Gd [NIH/3T3] ($\diamond$); Magnevist [NIH/3T3] ($\blacksquare$);P-Gd [MDA-MB-435] ($\blacktriangle$); Magnevist [MDA-MB-435] ($\bullet$);Data represent the means of triplicate points $\pm$ standard error.

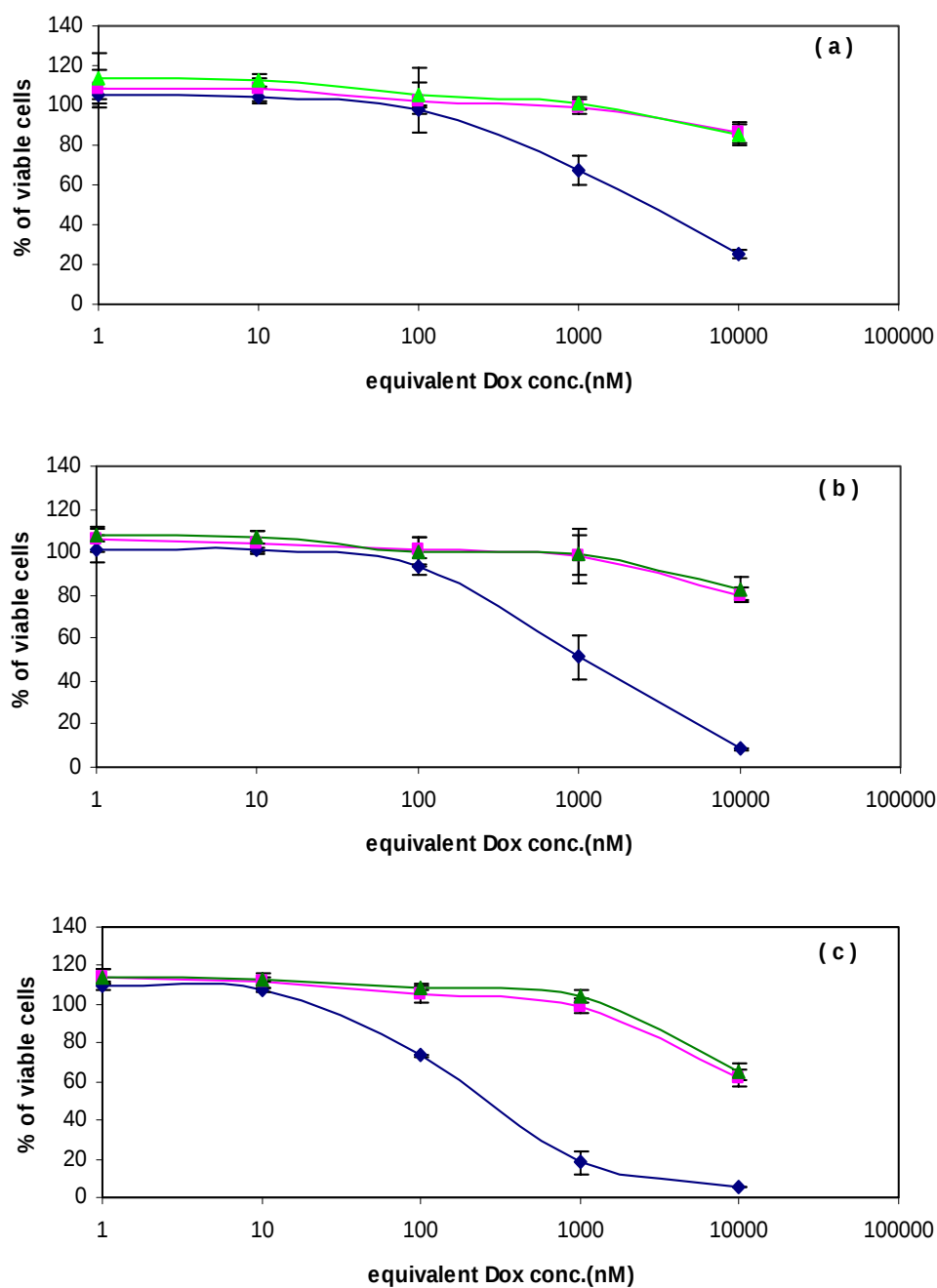


Figure 4. Effect of Gd on cytotoxicity of HPMA copolymer-Dox conjugates at 37°C after: a) 24h; b) 48h; and c) 72h. Dox (♦); P-Dox-DOTA (■); P-Dox-DOTA-Gd (▲) using MTT assay. Data represent the means of triplicate points $\pm$ standard error.