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Multifunctional terpolymeric MRI contrast agent with superior signal enhancement in blood and tumor

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ABSTRACT

A new multifunctional terpolymeric system for simultaneous imaging and drug delivery has been recently developed in our laboratory. Herein we report the investigation of terpolymeric contrast agent for magnetic resonance imaging and doxorubicin (Dox) delivery. The polymer was synthesized by graft polymerization of methacrylic acid (MAA) and polysorbate 80 (PS 80) onto starch with multiple, chemically bound diethylenetriaminepenta acetic acid (DTPA) groups for gadolinium chelating. The terpolymer self-assembled to form nanoparticles upon addition of doxorubicin which binds with the PMAA chain. The physicochemical, biological and pharmacokinetic properties of the polymeric system were characterized and their contrast enhancement capability was evaluated *in vitro* and *in vivo*. The polymer was able to load gadolinium with high thermodynamic stability and exhibited low cytotoxicity. The Gd-loaded polymer (PolyGd), and Gd-Dox co-loaded nanoparticles (PolyGd-Dox) significantly enhanced MR signals, with ionic T_1 relaxivities 3–5 times higher than those from Omniscan®, a small molecule contrast agent. *In vivo* studies showed superior and prolonged contrast enhancement compared to Omniscan® at one fourth the equivalent dose, without adverse effects. The PolyGd and PolyGd-Dox accumulated in the tumor and painted the tumor boundaries clearly for at least 48 h. The PolyGd also enhanced angiogram contrast with contrast to noise ratio values of up to 55-fold and a blood half-life time of 200 min. Seven days after intravenous administration, only relatively small amounts of gadolinium could be detected in the major organs of the mice (supplementary materials). These results suggest that the new terpolymeric system is useful as a theranostic platform for contrast enhanced MR imaging of vasculature and tumor as well as Dox delivery.

1. Introduction

Magnetic resonance imaging (MRI) is a powerful diagnostic modality because it provides non-invasive 3D visualization of anatomy within an arbitrary plane with superb soft tissue contrast. MRI is also a powerful analytical modality because it enables investigation of vascular and tissue physiology and pathology using quantitative biomarkers [1,2]. Soft tissue contrast in magnetic resonance (MR) images is multi-factorial, depending on the imaging method, protocol and the relaxation time constants of tissues (*e.g.* T_1, T_2). Exogenous paramagnetic contrast agents (*e.g.*, Gd^{3+}, Fe^{3+}, and Mn^{2+} complexes) are commonly used to alter the relaxation rates of the surrounding water protons and thereby accentuate vascular and soft tissue contrast in certain applications [3].

Gadolinium (Gd^{3+}) is the primary paramagnetic molecule used for MRI due to its high relaxation efficiency and large magnetic moment [4–6]. However, gadolinium in its free form is highly toxic to biological systems, hence Gd^{3+} contrast agents are formulated as stable, water-soluble chelates to improve their clinical safety profile [7,8]. The contrast enhancing capacity, termed 'relaxivity', of a Gd^{3+} based contrast agent is related to the number of exchangeable water molecules in the inner coordination sphere of the Gd^{3+} ion [3–5]. Unfortunately, the complexation of Gd^{3+} by organic chelators reduces the number of inner sphere water molecules. Hence, one of the major challenges in the design of Gd^{3+} based MRI contrast agents is to increase their relaxivity while minimizing their toxic side effects.

Clinically used small molecule Gd^{3+} contrast agents such as diethylenetriaminepentaacetic acid gadolinium (Magnevist®) and diethylenetriamine pentaacetic acid bismethylamide gadolinium (Omniscan®) are non-toxic yet exhibit relatively low T_1 relaxivities, rapid vascular extravasation into the extra-cellular space, non-specific distribution to the whole body, and fast renal clearance leading to a short time window for contrast enhanced imaging. To overcome these limitations, macromolecular MRI contrast agents such as PEG, poly(L-lysine), poly(glutamic acid), dendrimers, dextran, and supramolecular complexes including liposomes, micelles, and other systems have been studied and demonstrated to exhibit higher T_1 relaxivities

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and longer circulation time in the blood stream [9,10] These macromolecular and supramolecular systems also enable passive targeting of tumors owing to leaky vasculature and under-development of surrounding lymph vessels known as the enhanced permeability and retention (EPR) effect [11,12].

The development of multifunctional nanoparticles has attracted tremendous interests with the potential to revolutionize the diagnosis and treatment of cancer. These nanoparticle systems, also referred to as nanotheranostics, have been envisioned as novel contrast agents for non-invasive molecular imaging and targeted drug delivery [13,14]. Specific examples of nanotheranostic systems, such as polymer micelles, polymer nanoparticles, liposomes, and solid lipid nanoparticles have been reviewed by others [15,16]. Recently, we have designed a new nano-theranostic platform consisting of a graft terpolymer of starch with poly(methacrylic acid) (PMAA), and polysorbate 80 (PS 80) and demonstrated their usefulness for pH-dependent release of doxorubicin and overcoming multidrug resistance in breast cancer cells [17,18]. Starch is a polysaccharide with a good biocompatibility and biodegradability profile. Starch based derivatives such as carboxymethyl starch and acetyl starch are already used in humans as plasma expanders [19,20]. They are generally well tolerated because of their lower immunologic potential as compared to dextran. Unlike albumin, starch contains no to a little amount of peptide components that may be immunologically active and induce antibody production [21]. The abundant hydroxyl groups on the backbone of starch also offer multiple reaction sites for graft polymerization and conjugating other functional groups. Grafting PMAA renders more functionality to the starch. The PMAA components can load Dox effectively and covalently link with a fluorescence dye, producing a nano-theranostic system for simultaneous *in vivo* fluorescence imaging and inhibition of tumor growth as shown by our previous studies [18,22]. The Dox-loaded terpolymer system showed high tumor accumulation and significantly greater antitumor efficacy than free Dox [22].

Given these useful properties of this multifunctional terpolymer system and potentially higher contrast enhancement of hydrogel polymers [23], herein we covalently linked diethylenetriamine pentaacetic acid (DTPA) to the starch component of the terpolymer for chelating Gd^{3+} and investigated its potential as a carrier system for simultaneous delivery of Dox and Gd^{3+}. The properties of the terpolymeric system as an MRI contrast agent, such as the stability, T_1 relaxivity, and cytotoxicity have been studied *in vitro*. Upon loading of Dox, the Gd^{3+} loaded terpolymer (PolyGd) self-assembles into stable nanoparticles (PolyGd-Dox). The pharmacokinetics and MR contrast enhancement properties of the PolyGd and PolyGd-Dox have been evaluated *in vivo* using healthy and tumor-bearing mice.

2. Materials and methods

2.1. Chemicals

Soluble corn starch (MW = 11,000 g/mol), methacrylic acid (MAA), sodium thiosulfate (STS), potassium persulfate (KPS), polysorbate 80 (PS 80), sodium dodecyl sulfate (SDS), gadolinium chloride hexahydrate (Gd), diethylenetriaminepenta acetic acid (DTPA), and all other chemicals unless otherwise mentioned were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Acetic anhydride, pyridine, and sodium hydroxide (NaOH) were purchased from Fisher Scientific Canada (Ottawa, ON, Canada). DTPA-bis-anhydride was synthesized in house according to the method described by Andersen et al. [24]. Doxorubicin hydrochloride (Dox) was purchased from Polymed Therapeutics (Houston, TX, USA). All cell culture plasticware was purchased from Sarstedt (Montreal, QC, Canada). Cell culture medium, α-modified minimal essential medium, and phosphate buffered saline (PBS) were obtained from the Ontario Cancer Institute (Toronto, ON, Canada). Fetal bovine serum (FBS) and trypsin were purchased from Invitrogen, Inc. (Burlington, ON, Canada).

2.2. Cell line and maintenance

The murine breast carcinoma cell line EMT6/WT was initially provided by Dr. Ian Tannock (Ontario Cancer Institute, Toronto, ON, Canada) and is now maintained in our laboratory. Monolayers of cells were cultured on 75 cm^2 polystyrene tissue culture at 37 °C in 5% CO2/95% air humidified incubator. Cancer cells were maintained in α-minimal essential medium, supplemented with 10% FBS. Cells grown to confluence were trypsinized with 0.05% trypsin–EDTA, diluted (1/10) in a fresh growth medium and reseeded.

2.3. Preparation of the Gd^{3+} loaded PMAA-g-St-DTPA polymer (PolyGd)

The PolyGd was synthesized by first conjugating DTPA to the starch followed by grafting PMAA and PS 80 onto starch using a novel dispersion polymerization method described in detail previously [17]. Subsequently, Gd^{3+} was chelated to the DTPA component to form PolyGd (Fig. 1).

2.3.1. Synthesis of St-DTPA

Starch (3 g) was dissolved in 50 ml of dry dimethyl sulphoxide (DMSO) followed by addition of 1.5 g of DTPA-bis-anhydride. The solution was stirred at room temperature for 24 h, dialysed against DMSO for 48 h, and subsequently against water for another 48 h. The product (St-DTPA) was then dried in an oven at 50 °C overnight. The DTPA conjugation was confirmed by ^{1}H NMR (supplementary materials, Fig. S1).

2.3.2. Synthesis of PMAA-PS 80-g-St-DTPA polymer

The PMAA-g-St-DTPA polymer was synthesized using a modification of a one-pot dispersion polymerization method developed in our laboratory [17]. Briefly, 1.55 g of St-DTPA was dissolved in 150 ml of distilled water by heating at 70 °C for 30 min. The solution was purged with N_2 for 30 min to remove any dissolved oxygen. Subsequently, 0.25 g of SDS, 1.5 g of PS 80, 0.18 g of KPS and 0.25 g of STS were added to the St-DTPA solution while being stirred. After 10 min, the reaction was started by addition of 2 g of nitrogen purged MAA. Opalescence appeared after 5 min and the reaction was continued for 8 h at 70 °C to ensure complete grafting. The product was dialyzed (molecular weight cut-off 25,000 g/mol) extensively against warm water for 3 days, against methanol for another 2 days and against water again for another 2 days. The purified polymer was then dried at 50 °C for 24 h, and stored in a desiccator for future use.

2.3.3. Loading of Gd^{3+} onto PMAA-PS 80-g-St-DTPA polymer

The PMAA-PS 80-g-St-DTPA polymer (0.5 g) was dispersed in 10 ml of distilled deionized water (DDIW). The pH was adjusted to 6.5 using 0.1 N NaOH. Ten milliliters of aqueous solution of gadolinium chloride hexahydrate (10 mg/ml) was then added drop-wise while stirring, and the pH of the reaction was kept at 6.5 with the 0.1 N NaOH throughout the experiment. After stirring for 1 h, the product was dialysed exhaustively against 0.9% NaCl until no free Gd^{+3} was detected in the wash medium using the xylenol orange test [25]. The product (called PolyGd) was then neutralized, freeze-dried and stored for future use. The Gd^{3+} content in the product was measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Optima 7300, PerkinElmer, Shelton, CT, USA). The weight averaged molecular weight of PolyGd was measured using a static light scattering method described elsewhere [26].

2.4. Determination of the Gd^{3+} binding constant

A complexometric competition binding assay was performed to measure the binding constant of Gd^{3+} to PMAA-PS 80-g-St-DTPA polymer according to a method described in detail previously [27]. Xylenol orange was used as the competitive colorimetric indicator,