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Application of arsenazo III in the preparation and characterization of an albumin-linked, gadolinium-based macromolecular magnetic resonance contrast agent

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Abstract

A macromolecular magnetic resonance contrast agent (MMCA) was prepared by linking bovine serum albumin (BSA) to gadolinium (Gd) via a chelating agent, diethylenetriaminepentaacetic acid (DTPA). Colorimetric testing with 2,7-bis(*o*-arsenophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid (arsenazo III) was performed to check for the appearance of free gadolinium during preparation and to quantify the Gd content in the final product. The complex was purified by dialysis, concentrated by lyophilyzation and characterized by magnetic resonance (MR) proton relaxation times. The resultant product had a molecular weight of about 90 kDa, Gd:BSA ratio of 14:1, and T_1 and T_2 relaxation times of 128.3 and 48.9 ms, respectively, at a field strength of 7 Tesla (T) and at 20% concentration. Contrast enhancement of Gadomer-17 (a dendritic MMCA) and Gd-linked to BSA (Gd–BSA) was sequentially evaluated in a rat brain gliosarcoma model ($n = 5$) by MR imaging (MRI). Following intravenous injection, the blood concentration of Gadomer-17 fell rapidly, whereas that of Gd–BSA was almost constant for the duration of imaging. The areas of enhancement of both MMCAs were comparable. The spatial distribution of Gd–BSA showed good agreement with Evans blue-tagged albumin. Treatment with dexamethasone decreased Gd–BSA enhancement in the tumor. These results suggest that the arsenazo III method is applicable in preparing Gd–BSA to image brain tumors and their response to treatment. This simple method may also be useful for preparing other gadolinium-linked MMCAs.

Keywords: Brain tumor; Gadomer-17; Gd–DTPA; MRI

1. Introduction

Many disease states of the brain are characterized by leaky vasculature. One noninvasive way to visualize diseased vasculature is by magnetic resonance imaging (MRI) of contrast agents that remain intravascular in normal brain capillaries, but leak into the extravascular space if the vasculature is damaged. The most commonly used MR contrast agents are based on the paramagnetic lanthanide, gadolinium (Gd). Since unbound gadolinium is toxic, it is usually linked to chelating agents such as diethylenetriaminepentaacetic acid (DTPA). The resultant contrast agent, Gd–DTPA, has a molecular weight of ∼550 Da, is well tolerated clinically (Niendorf et al., 1985; Weinmann et al., 1984) and has been employed to detect blood–brain barrier (BBB) opening by MRI. The small molecular weight and size (∼14 Å) of this contrast agent help in detecting increases in vascular permeability in some cerebral pathologies, e.g., stroke (Knight et al., 2005; Merten et al., 1999). Under conditions of sustained high permeability such as in tumor vasculature, however, Gd–DTPA tends to underestimate tumor size, possibly because of its rapid diffusion from the site of leakage and likely back-flux into the leaky vasculature (Adam et al., 1996; Su et al., 1999). In addition, it has been shown that Gd–DTPA failed to reliably differentiate

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between pre- and post-drug treatment effects on tumor vasculature (Turetschek et al., 2003).

The preceding suggests that it would be important to develop macromolecular contrast agents (MMCA) to assess the pathophysiology of cerebral tumors and evaluate their response to therapy (Adam et al., 1996; Pathak et al., 2005; Su et al., 1999). If the MMCA is formed with a bioactive moiety, then various molecular processes linked to it may also be imaged, tracked, and quantitated by MRI (Hawkins et al., 1990; Kiessling et al., 2002; Kornguth et al., 1987). In addition, such MMCAs may possess plasma half-lives different than that of Gd–DTPA, and this difference may be advantageous in determining the ongoing pathology of a particular cerebral lesion.

Serum albumin is the most abundant protein in plasma, is relatively large (68 kDa), binds many endogeneous materials and drugs, and delivers them to the capillaries as it circulates (Adzamli et al., 2003; Su et al., 1999). Albumin-linked MR contrast agents have been suggested to be particularly useful in estimating blood-volume and perfusion dependent contrast enhancement of brain tissue (Schmiedl et al., 1986). Albumin also has a long biological half-life and is preferentially accumulated in some carcinomas and facilitates prolonged MR imaging (Kiessling et al., 2002). In this report we describe a simple method to prepare a large MMCA by covalently linking bovine serum albumin (BSA) to Gd–DTPA. A novel, arsenazo III-based reaction was employed to detect unbound gadolinium in the reaction mixture (Fritz et al., 1958) and to quantitate the gadolinium content of the final product (Cassidy et al., 1986; Fritz et al., 1958; Hvattum et al., 1995). The contrast enhancement and blood-to-brain influx of this preparation, viz., Gd–BSA, and of a large, dendritic Gd-linked contrast agent, Gadomer-17 (kind gift of Schering AG, Berlin, Germany), were evaluated in a rat 9L gliosarcoma tumor model. At the end of the MRI data collection, the distribution of Evans blue (a vital dye that labels plasma albumin by instantly and completely binding to it upon intravenous injection) over a 25 min period was determined in fixed tissue samples and these images were compared to those of Gd–BSA. Gadomer-17 has a molecular weight of 17 kDa, but an effective size of about 34 kDa with its hydration rings (Misselwitz et al., 2001). It has been used in many experimental studies on tumor permeability and drug response (Su, 1999; Su et al., 1999; Turetschek et al., 2004) and is the nearest established experimental MMCA in molecular weight to Gd–BSA. Therefore, we hypothesized and tested that both Gadomer-17 and Gd–BSA would give comparable areas of enhancement in the 9L gliosarcoma tumor and that Gd–BSA distribution will be similar to that of Evans blue.

2. Materials and methods

2.1. Preparation of Gd–BSA

All reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA) and used as received. The coupling of DTPA and gadolinium to BSA was performed by modifying published procedures (Hnatowich et al., 1982; Ogan et al., 1987; Schmiedl et al., 1986; Su et al., 1995). To 5 g of BSA in 250 ml of citrate buffer at pH 6.5, 10.0 g of DTPA–dianhydride was slowly added with constant stirring. The pH was maintained between 5.5 and 6.5 by adding 3N NaOH as needed. After stirring for 1 h at room temperature, 12 g of gadolinium chloride hexahydrate ($GdCl_3 \cdot 6H_2O$) dissolved in 10.0 ml of distilled water were carefully added in batches of 200 μl with constant stirring to the reaction mixture. Each successive addition of gadolinium persisted a little longer in the reaction mixture indicating progressively slower incorporation into the complex. After every addition, time was given for complete dissolution of $GdCl_3$. During the addition of $GdCl_3$ the pH of the reaction mixture was again maintained between 5.5 and 6.5 with 3N NaOH. A 10 μmol solution of arsenazo III in acetate buffer at pH 5.0 was prepared following published reports (Fritz et al., 1958). A series of 20 μl drops of arsenazo III were arranged in a 96-well microplate. After each addition and complete dissolution of $GdCl_3$, a 5 μl droplet from the reaction mixture was added to one drop of arsenazo III in a well and mixed completely. The arsenazo III solution that was initially bright pink in color would turn light-to-dark purple with the addition of chelated gadolinium. When arsenazo III began turning green, which indicates the presence of unbound gadolinium, further addition of $GdCl_3$ was stopped.

The product was stirred in a cold-room overnight and then dialyzed (10,000 Da cut-off dialysis membrane) for 3 days with three changes of 0.1 M citrate buffer per day and for three more days with three changes of distilled water per day. The solution was subsequently lyophilyzed to dryness. The dried product was reconstituted in 10 ml of de-ionized water and tested for protein content with a kit (Pierce, Rockford, IL, USA).

The gadolinium content in the Gd–BSA preparation was analyzed by arsenazo III reagent (pH 1.0) prepared by dissolving 12.3 mg arsenazo III in 100 ml of 0.1 M HNO_3 containing 10 mmol urea and 0.1% Triton X100 as a stabilizer. The product was read at a wavelength of 630 nm against known gadolinium standards of 1 mmol $GdCl_3 \cdot 6H_2O$ diluted from 100 to 10% (Cassidy et al., 1986; Fritz et al., 1958; Hvattum et al., 1995). The molecular weight was determined by sodium dodecylsulphate (SDS) electrophoresis. The Gd–BSA prepared thusly was evaluated for its relaxation properties and compared against Gadomer-17 for in vivo MR contrast enhancement with a 9L gliosarcoma brain tumor model.

2.2. Implantation of tumor cells

Five adult male Fischer rats (Charles River Breeding Laboratories, Wilmington, MA, USA) weighing between 200 and 240 g were used in the study. All animal use protocols were performed with approval from the institutional animal care and use committee, in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal facility. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg; Abbott Laboratories, North Chicago, IL, USA) and positioned in a stereotaxic head frame (David Kopf Instruments, Tujunga, CA, USA). Aseptic surgical techniques were used to open the scalp in the midline. A 1.0 mm burr-hole was made in the skull 2.5 mm anterior to bregma and