Investigating plasma volume expanders as novel macromolecular MRI-CEST contrast agents for tumor contrast-enhanced imaging

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Authors:

Lorena Consolino, PhD,[1,4] Pietro Irrera, PhD,[1,2] Feriel Romdhane, PhD,[1] Annasofia Anemone, PhD,[1] and Dario Livio Longo, PhD[3]

[1] *Centro di Imaging Molecolare, Dipartimento di Biotecnologie Molecolari e Scienze per la Salute, Università degli Studi di Torino, Torino, Italia*
[2] *Università degli Studi della Campania "Luigi Vanvitelli", Caserta, Italia*
[3] *Istituto di Biostrutture e Bioimmagini (IBB), Consiglio Nazionale delle Ricerche (CNR), Torino, Italia*

Present address:

[4] *Institute for Experimental Molecular Imaging, Department of Nanomedicines and Theranostics, RWTH Aachen University, Aachen, Germany*

Corresponding author

Dario Livio Longo

Istituto di Biostrutture e Bioimmagini (IBB), Consiglio Nazionale delle Ricerche (CNR)

Via Nizza, 52 10126 Torino

dariolivio.longo@cnr.it; dario.longo@unito.it

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ABSTRACT

Purpose: The aim of this study was to investigate two clinically approved plasma volume expanders (dextran70 and voluven) as macromolecular MRI-CEST contrast agents to assess tumor vascular properties.

Methods: CEST contrast efficiency of both molecules (6% w/v) was *in vitro* measured at various irradiation saturation powers (1–6 μT for 5 seconds) and pH values (range 5.5–7.9) and the exchange rate of hydroxyl protons was calculated. *In vivo* studies in a murine adenocarcinoma model (n = 4 mice for each contrast agent) upon intravenous injection provided CEST-derived perfusion tumor properties that were compared with those obtained with a Gadolinium-based blood-pool agent (Gd-AAZTA-Madec).

Results: *In vitro* measurements demonstrated a marked CEST contrast dependency to pH, with higher CEST contrast at lower pH values for both molecules. The measured prototropic exchange rates confirmed a base-catalyzed exchange rate that was faster for dextran70 in comparison to voluven. Both molecules showed similar CEST contrast increase (ΔST% > 3%) in the tumor tissue up to 30 min post-injection, with heterogeneous accumulation. In tumors receiving both CEST and T_{1w} agents, voxel-by-voxel analysis indicate moderate spatial correlation of perfusion properties between voluven/dextran70 and Gd-AAZTA-Madec, suggesting different distribution patterns according to their molecular size.

Conclusions: The obtained results demonstrated that both voluven and dextran70 can be exploited as MRI-CEST contrast agents for evaluating tumor enhancement properties. Their increased accumulation in tumors and prolonged contrast enhancement indicate their use as blood-pool MRI-CEST agents to interrogate tumor vascularization.

Keywords (max 6)

Magnetic Resonance Imaging - MRI; Chemical Exchange Saturation Transfer - CEST; macromolecular agent; Gadolinium; plasma volume expander

1. INTRODUCTION

Dextrans and hydroxyethyl starches (HES) are synthetic colloids of macromolecular size generally used in the clinical setting as plasma volume expanders to restore blood volume deficit after surgical hemorrhages or trauma (1-4). In addition to the post-operative practice, the capability of plasma volume expanders to remain confined in the vascular compartment has been widely exploited for several preclinical applications, mainly to improve vascular perfusion and assess the vascular integrity/permeability of angiogenic vessels (5-7). In oncology, monitoring the extravasation of dextran molecules with increasing molecular weight indicated the ideal cutoff for optimal tissue penetration and accumulation, with significant improvement for therapeutic treatments (8).

Current imaging techniques require the chemical modification (usually the conjugation with a proper imaging reporter) of these molecules, with the subsequent generation of new chemical entities that need a new Food and Drug Administration (FDA) approval for their use in patients or have been applied for visualizing not clinically approved dextrans for in vivo applications (9-11).

Macromolecular systems (10^4 to 10^6 Da) are advantageous over small molecules due to their increased tumor selectivity (12). Tumors present a heterogeneous and aberrant vascular network that facilitates the extravasation of macromolecular systems (based on the "enhanced permeability and retention"- EPR - effect) and determines their passive accumulation in tumors over healthy tissues. This effect has been exploited to improve the selective visualization of tumor angiogenesis by prompting the design of novel nanoprobes that exhibit increased plasma long-life retention, protection from enzymatic degradation and controlled released (13,14). In this particular framework, magnetic resonance imaging (MRI) counts a wide range of nanosized probes able to provide accurate functional properties in combination with high spatio-temporal resolution (15). An important class of perfusion Gd-based molecules are indeed the blood-pool agents that non-covalently bind to the serum albumin and are able to report antiangiogenic therapies responses, mainly in association with the Dynamic Contrast Enhanced (DCE)-MRI (16-25). Recent developments have exploited nanosized systems based on different platforms to increase further both contrast efficiency and vascular retention (26-

34). However, the selective advantage of Gd-based macromolecular contrast agents is counterbalanced by their longer elimination time from the plasma circulation and by safety issues related to the suspected Gd accumulation in normal tissue, with consequent implications for clinical approval (35,36).

In the last two decades, a new MRI approach able to detect molecules possessing exchangeable protons has been proposed, based on the chemical exchange saturation transfer (CEST) technique (37). Low-molecular weight iodinated agents, clinically approved for X-ray investigations, have been exploited as perfusion agents for MRI-CEST application. (38,39). In addition, CEST agents based on polymers or liposomes have been designed as vascular agents, exhibiting good CEST contrast, despite some of them have not been tested *in vivo* yet (40-46). Also molecules that possess exchangeable hydroxyl groups (-OH), as dextrans, can be detected by MRI-CEST without extra labelling procedures (47-53). Recently, it was demonstrated that dextran molecules with different molecular weights are able to provide a size-dependent CEST contrast (54). However, most of the reported dextrans are not FDA-approved for clinical use, limiting their potential application to the clinical settings.

The aim of this work was to investigate the capability of two clinically approved plasma volume expanders, voluven (hydroxyethyl starch, 130 kDa) and dextran70 (70 kDa) to generate MRI-CEST contrast. Therefore, CEST properties were firstly *in vitro* characterized and then *in vivo* assessed in a preclinical murine model of breast cancer. In addition, the distribution in the tumor tissue of these macromolecules has been compared to a preclinical Gd-based blood-pool contrast agent, to validate their ability for assessing tumor vasculature properties.

2. METHODS

2.1 Chemicals

Dextran70 was purchased from Sigma-Aldrich. Voluven® (Hydroxyethyl starch 130/0.4) was purchased by Fresenius Kabi Italia SpA. Gd-AAZTA-Madec was generously provided by Cage Chemicals (Novara, Italy). All other chemicals were purchased from Sigma–Aldrich.

2.2 Size distribution profiles

The mean diameter of dextran70, voluven and bovine serum albumin (BSA) was determined using a dynamic light scattering (DLS) Malvern Zetasizer 3000HS (Malvern, U.K.) at a concentration of 6% w/v.

2.3 In vitro MRI CEST acquisition

Seven phantoms containing 10 mM phosphate buffer solution of dextran70 and voluven were prepared at a concentration of 6% w/v and titrated over a range of 5.5-7.9 pH units. In vitro MRI-CEST images were acquired on a vertical 7T scanner (Bruker, Ettlingen, Germany) using a fast spin-echo sequence with centric encoding. Presaturation pulses varying in power (B1= 1, 2, 3, 4, 5 and 6.0 μT) were applied for 5 s at 37 °C. A modified RARE sequence including a magnetization transfer module was used to acquire CEST-weighted images from -10 to +10 ppm with increments of 0.1 ppm around the water resonance with following parameters: TR=10 s; TE: 3.5 ms; FOV: 30 mm; MTX: 64.

2.4 Animal studies

Male 8 to 10 weeks old BALB/C mice (Charles River Laboratories Italia S.r.l., Calco, Italy) were used. Mice were maintained at the animal facility and treated in accordance with the university's ethical committee and European guidelines (Directive 2010/63). Murine breast cancer HER2+ TS/A

cell line were used for *in vivo* experiment. This cell line derives from a spontaneous mammary adenocarcinoma in a BALB/c female mouse (36). Cells were grown in RPMI medium containing 10 % (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. RPMI, FBS and Trypsin were purchased from Lonza (Lonza Sales AG, Verviers, Belgium). The penicillin–streptomycin mixture was purchased from Sigma Chemical Co., St. Louis, MO, USA. Cells were incubated in 75-cm^2 flasks in a humidified 5% CO2 incubator at 37 °C. At confluence, TS/A cells were detached by adding 1 mL of Trypsin-EDTA solution [0.25 % (w/v) Trypsin- 0.53 mM EDTA]. 2.5 x 10^5 TS/A cells resuspended in PBS were subcutaneously inoculated into both flanks of BALB/c mice. For the MRI-CEST experiments (n=4, 8 tumors in total), mice intravenously received 250 µL of voluven or dextran70 through a catheter inserted in the lateral vein of mice tail. For the MRI-CEST study combined with T_{1w} Gd-enhanced experiments (n=4, 8 tumors in total), mice received voluven or dextran70, followed 20 minutes later by a 0.05 mmol Gd/ kg body weight injection of Gd-AAZTA-Madec, slowly injected through the same catheter without removing the animal from the MRI scanner.

2.5 *In vivo* MR imaging

MR images were acquired on a Bruker Avance 7T MRI scanner (Bruker BioSpin MRI). BALB/c mice bearing mammary adenocarcinoma tumors (inoculated subcutaneously in both flanks with murine TS/A HER2+ cancer cells) were anaesthetized with a mixture of tiletamine/zolazepam (Zoletil 100; Vibac) 20 mg/kg and xylazine (Rompun; Bayer) 5 mg/kg and their breath rate was monitored during the acquisition by a respiratory air pillow (SA Instruments,). After the scout images acquisition, T_{2w} anatomical image in the central part of the tumor was set up as a reference image using a RARE sequence (TR = 4000 ms; TE = 35.5 ms; number of slices = 1; slice thickness = 1.5 mm; FOV = 30 mm; MTX = 256 x 256; NEX = 2; acquisition time = 2 m 8 s). MRI-CEST experiments (n=4 mice, 8 tumors in total) were performed before and after voluven or dextran70 injection (dose: 0.6 g/kg b.w.) by acquiring Z-spectra in the frequency offset range ±10 ppm with a centric encoded single-shot RARE sequence (TR = 6000 ms; TE = 4 ms; number of slices = 1; slice thickness = 1.5 mm;

FOV = 30 mm; MTX = 96 x 96; NEX = 1; acquisition time = 8 m 12 s) preceded by a 1.5 μT CW block presaturation pulse for 5s. For the MRI-CEST acquisition combined with T_{1w} Gd-enhanced experiments, T_1-weighted images before and after Gd-AAZTA Madec injection (dose 0.05 mmol Gd/ kg b.w.) were acquired 20 minutes after the last CEST Z-spectrum acquisition by maintaining the same geometry, orientation and spatial resolution of the MRI-CEST images. An axial 2D fast low angle shot (FLASH) gradient echo sequence with the following parameters was used: TR = 70 ms; TE = 1.5 ms; flip angle = 45°; number of slices = 1; FOV = 30 mm; MTX = 96 x 96; NEX = 6; acquisition time = 42 s.

2.6 Data analysis

Home-made scripts implemented in MATLAB R2015 (The Mathworks, Inc., Natick, MA, USA) were used for analyzing all MRI-CEST and T_{1w} images.

In vitro Z-spectra were fitted by simultaneous multiple Z-spectra Bloch-McConnell fit as previously reported (55,56). A two-pools exchange models was exploited, with one pool (pool A) that describes the proton pool of bulk water molecules (with chemical shift set at 0 ppm) and only one pool (pool B) describing the hydroxyl protons, with chemical shift δ = 1.0 ppm (57). We only considered the direct proton exchange rate (k_b) between hydroxyl groups and water pool. The fraction of the mobile protons (f_b) relative to exchangeable water protons was fixed and directly given by the concentration fraction of the two molecules; (f_b = number of protons per glucose moiety x [Glc] / (2[H_2O]) = 3 x 0.332 M / 111 M), was set to 0.0089 and 0.0087 for voluven and dextran70 protons, respectively. According to experimental T_1, the water pool relaxation value provided to the fit was R_{1A} = 0.34-0.38 Hz for both voluven and dextran70. In addition, the pH-dependence of the calculated exchange rate can be described by the following equation, including only the base- and water-catalyzed exchange:

$$k_b = \{k_1 * k_w/[H_3O^+] + k_2\}(1 - f_b) \qquad \text{eq. (1)}$$

from which the base (k_1) and water (k_2) exchange catalyzed terms were calculated (58).

For *in vivo* images, both CEST and T_{1w} images were analyzed on a voxel-by-voxel basis. The Z-spectra were B_0-shift corrected after interpolation by smoothing splines, noisy Z-spectra removed when $R2 < 0.999$ and saturation transfer efficiency (ST%) was measured by punctual analysis (59). Difference CEST contrast maps (ΔST%) and signal intensities enhancement (SIenh%) were calculated between pre- and post-injection images of voluven/dextran70 and Gd-AAZTA-Madec, respectively. Post- to pre-injection ST% contrast subtraction was performed to reduce confounding effect related to endogenous CEST contributions. Time-averaged tumor to muscle (T/M) ratio for ΔST% contrast was calculated for the four time points. In addition, the extravasation fraction estimates were calculated for each agent as the percentage of pixels showing a ΔST% or a SIenh% above the threshold of 2% and 15% for CEST and T_{1w} images, respectively (49,60,61). Enhancement and extravasation values have been calculated after 8 minutes post-injection for voluven, dextran70 and for Gd-AAZTA Madec.

For voxelwise spatial correlation, the two-dimensional correlation coefficient was calculated in the same tumor region in both enhanced and extravasation parametric maps obtained from CEST and Gd-based images. Spatial similarity maps have been color-coded as blue pixels where both contrast agents have been detected, whereas red and green colors have been assigned to pixels with only CEST and Gd-based contrast, respectively.

2.7 Statistical analysis

All results were expressed as mean ± standard deviation (SD). The statistical significance of the differences between the means of contrast enhancement and extravasation values was calculated using a Student's t-test. P values less than 0.05 were considered statistically significant. The calculations were performed with GraphPad Prism (GraphPad Software, La Jolla, CA).

3. RESULTS

3.1 Characterization of *in vitro* CEST contrast

Figure 1 shows the chemical structure of voluven and dextran70 that possess several hydroxyl groups with mobile exchangeable protons (Figure 1A). These macromolecules have a molecular weight of 130 kDa and 70 kDa, respectively. To further evaluate their distribution *in vivo* in comparison to an albumin-binding blood pool agent (Gd-AAZTA Madec), the hydrodynamic diameters of voluven, dextran70 and of BSA were calculated by DLS measurements (Figure 1B). The investigated molecules show similar size distribution profiles, with a diameter of 11 ± 2 nm (voluven) and 14 ± 3 nm (dextran70). Although BSA (60 kDa) and dextran70 have similar molecular weight, the hydrodynamic diameter of BSA (6 ± 2 nm) is more than two-fold smaller in comparison to dextran70. Their capability to generate CEST contrast can be observed by plotting the normalized water signal intensity as a function of the irradiated chemical shift, or Z-spectra (Figure 2A). The CEST contrast peaks at approximately 1 ppm (0.9 and 1.1 ppm for voluven and dextran70, respectively) that corresponds to the expected resonance frequency of the hydroxyl protons embedded in glucose polymers (Figure 2B). The measured ST% contrast is affected by multiple factors, including concentration, magnetic field strength, saturation power/duration and the exchange rate of the mobile protons (37). Considering that the exchange rate of mobile protons is strongly affected by the pH, the dependency of CEST contrast on pH was measured for both molecules in phantoms titrated at different pH (5.5-7.9). Figure 2C shows that both voluven and dextran70 exhibit a pH-dependent CEST contrast. In particular, the hydroxyl protons of both molecules shows a slight increase in CEST contrast (ST%) from pH 5.5 to pH 6.3 and a drop in ST% starting at pH 6.3-6.7 in a pH-dependent manner when a saturation pulse of 2μT is applied. We observed statistically higher ST% contrast for dextran70 at more acidic pH, conversely, voluven showed higher ST at more neutral pH values (Figure 2C).

3.2 Determination of hydroxyl exchange rate

To calculate the exchange rate (k_b) for the hydroxyls protons, multi-B_1-Z-spectra for each pH value were simultaneously fitted by the numerical Bloch-McConnell equations. Figure 3A shows representative fitted Z-spectra at pH 7.4 for the two macromolecules. The exchange rates have been calculated as a function of pH in the range of 6.0-7.4 units (Figure 3B, Table 1). Voluven and dextran70 showed similar k_b at low pH, whereas at higher pH values the exchange rates appear consistently different, with dextran70 showing a two-fold faster exchange rate in comparison to voluven (k_b = 5000 ±180 Hz and 2300 ±50 Hz for dextran70 and voluven, respectively, at pH 7.4). From these curves, the calculated catalyzed exchange terms were $6.24 \cdot 10^9$ dm^3 mol^{-1} s^{-1} (k_1) and 908 s^{-1} (k_2) for voluven and $1.69 \cdot 10^{10}$ dm^3 mol^{-1} s^{-1} (k_1) and 891 s^{-1} (k_2) for dextran70, respectively. The faster exchange rates when moving from acidic to neutral pH values reduce the labelling efficiency at a specified saturation power, hence resulting in a lower CEST contrast at higher pH values.

3.3 *In vivo* CEST contrast enhancement and comparison with Gd-based blood pool agent in tumors

To further evaluate their CEST properties, voluven and dextran70 were injected in BALB/c mice bearing breast cancer tumors and CEST contrast was evaluated along time. Figure 4 shows a marked increase of the CEST signal inside the tumor regions following voluven or dextran70 injection. Additional regions, such as bowel and kidneys, reported also an increase in contrast along time, because of the presence of CEST artifacts related to movement of the intestinal tracts or following the accumulation in the kidneys of these molecules by renal filtration. To better characterize the increase of the CEST contrast, we calculated the difference in CEST contrast between pre- and post-injection CEST images. Figures 5A and B report that voluven and dextran70, respectively, were able to provide prolonged CEST contrast into the tumor tissue up to 32 minutes post injection, although a statistical significant difference between tumor and control muscle regions was observed only at 8 minutes post injection. The average increase in contrast from baseline values (ΔST%) for both the

molecules was ca. 3%, whereas values obtained in control muscle tissue where below or equal to 2%. Time-averaged tumor-muscle (T/M) ratios of voluven and dextran70 ΔST% were 1.2 ±0.2 and 1.3 ±0.3 for dextran70 and voluven, respectively (Figure 5C).

Representative ΔST% maps of the tumor and control muscle regions overimposed on T_{2w} images are shown in Figure 5D. Color-coded pixels reported heterogeneous distribution of the contrast in the tumor region, with some areas showing poor contrast enhancement and other showing similar CEST contrast along time.

Moreover, the capability of these macromolecules to accumulate into the tumor tissue and to generate contrast enhancement was compared to that provided by Gd-AAZTA-Madec, a Gd-based blood pool agent with comparable size. Gd-AAZTA-Madec was injected 20 minutes after voluven or dextran70 by sequential injection through the same catheter. This approach ensured the maintenance of the same anatomical position to compare CEST and Gd-derived estimates on a voxel-by-voxel basis. As previously observed, similar contrast was provided by both voluven and dextran70 in MRI-CEST images of TS/A tumors (Figure 6A). The mean contrast enhancement values measured in tumor ROIs were ΔST% equal to 4.5 ± 0.3 and 4.8 ± 0.4 for voluven and dextran70, respectively. Gd-AAZTA-Madec provided a similar T_{1w} contrast enhancement in the tumors of the two groups, with a signal intensity enhancement (ΔSI%) of 31± 2 for voluven and 26 ± 2 for dextran70 (Figure 6C). Figures 6B and 6D report the mean extravasation fractions of the investigated molecules. These results indicated that a similar percentage of pixels showed CEST and T_{1w} contrast enhancement, resulting in similar mean extravasation fraction for all these molecules (45 ± 3, 39 ± 3 for voluven and dextran70, respectively; 56 ± 5 and 44 ± 4 for Gd-AAZTA-Madec injected in the same mice after voluven and dextran70, respectively).

To further investigate their distribution pattern into the tumor tissue, a voxelwise comparison was performed. Representative parametric maps of the investigated molecules overimposed on T_{2w} anatomical images are shown in Figure 7, as CEST (Figure 7A) and T_{1w}-contrast enhancement maps

(Figure 7B), showing only pixels with ΔST% or ΔSI% values greater than zero, to better highlight the detection of the contrast agents inside the tumor. Voluven showed a significant higher spatial correlation for the enhancement maps with Gd-AAZTA-Madec in comparison to dextran70 (0.52 ± 0.03 and 0.40 ± 0.03, for voluven and dextran70, respectively, Figure 8A). A similar trend was reported for the spatial correlation of the extravasation maps, where voluven showed higher correlation with Gd-AAZTA-Madec in comparison to dextran70 (spatial correlation of 0.47 ± 0.05 and 0.39 ± 0.03 for voluven and dextran70, respectively, Figure 8B). Representative similarity maps with color-coded pixels (Figure 7C) showed that CEST molecules differently distributed in tumor tissue in comparison to Gd-AAZTA-Madec.

4. DISCUSSION

Macromolecular systems able to accurately map tumor vascularity and permeability properties are currently missing in the MRI-CEST setting, although the recent exploitation of low-molecular weight iodinated contrast media as innovative perfusion agents for MRI-CEST visualization (38). Here we propose two macromolecules, voluven and dextran70, with high translational potential due to their clinical approval as plasma volume expanders. Our results demonstrated that dextran70 and voluven can generate moderate and prolonged MRI-CEST contrast *in vivo* upon the extravasation and retention in the extravascular-extracellular space. Furthermore, despite the amount of extravasation of these molecules was comparable to that of a Gd-based blood-pool agent, they showed a slightly different spatial distribution within the tumor region, suggesting their potential for visualizing tumor regions with different vascularization and permeability levels.

In the conventional MRI context, dextran-based nanoparticles conjugated to Gd-complexes have already shown promising results for the *in vivo* monitoring of vascular properties, however a clear limitation in clinical translation relies in their increased retention times of Gadolinium within the body (9,62). Conversely, MRI-CEST technique can provide an indirect visualization of these

molecules without the need of additional imaging moiety that may strongly affect their pharmacokinetic and clearance properties. Recently, Li et al. demonstrated that dextran molecules can be visualized by MRI without the need for the labelling with a dedicated imaging reporter (54). We further extended this concept, by investigating FDA-approved plasma volume expanders as novel macromolecular CEST agents for characterizing tumor vascular properties.

Both molecules showed *in vitro* a pH-dependent CEST contrast due to a base-catalyzed prototropic exchange rate in the investigated pH range. Interestingly, a slightly higher CEST contrast efficiency was reported for voluven at more neutral pH values, whereas dextran70 showed higher CEST contrast at more acidic pH values. A plausible explanation might be related to the different structures: linear for dextran70 and more ramified for voluven, that may affect the exchange rates of the hydroxylic protons differently responding to pH changes. In fact, the investigated voluven shows a molar substitution degree of 0.4 that results in a high degree of branching in comparison to dextran70, likely resulting in a reduced solvent exposure for those protons in the inner region of the molecule due to a more compact conformation of voluven in respect to dextran70 (63).

In vivo results showed that both voluven and dextran70 molecules exhibit similar properties in tumors in terms of CEST contrast enhancement (3-4%) and extravasation fraction (~40%), despite owing different molecular weights. These findings are in contrast with those reported by Li et al., who showed that the uptake of dextrans is size-dependent and that the CEST contrast generated in tumors decrease for molecules with bigger size (54). Considering that the molecular weight of voluven (130 kDa) is almost two-times bigger than that of dextran70 (70 kDa), one may expect a size-dependent extravasation according to the EPR effect (13). However, previous studies showed that HES with middle molecular weight and low substitution degree remains in blood as long as large-sized HES, reporting that their chemical structure and composition strongly drive their permeability properties and contribute to their intravascular retention rate (2). These observations are in accordance with our DLS measurements that show comparable dimensions for both voluven and dextran70 (11 and 14 nm, respectively), indicating that they can possess similar pharmacokinetic properties despite differences

in molecular weight. Moreover, due to their macromolecular size, both voluven and dextran70 exhibit two- to three-fold reduced extravasation fraction in comparison to Iopamidol, a small molecular weight contrast agent (39).

Quantitative analysis in tumor and control muscle regions of ΔST% CEST contrast provided an average tumor/muscle ratio for the two investigated agents of ca. 1.2-1.3, a value comparable with that observed by several Gd-based nanosized systems in murine tumor models (64-66). We could consider that the longer circulating time of voluven and dextran70 in the vascular compartment, as for other nanosystems, might contribute to an increased contrast in well perfused and vascularized regions, such as the muscles. This effect might explain the reduced CEST contrast ratio between tumor and control muscle regions at longer time points, hence reducing the calculated T/M ratio (67).

A large interest has been devoted by the MRI community to develop Gd-based agents able to bind to serum albumin. Firstly, they present enhanced contrast efficiency at low magnetic field compared to small molecular weight one. Secondly, they are exploited for a better characterization of tumor vessel permeability (23,68). To extend the concept to voluven and dextran70, we aimed to compare on a voxel-by-voxel basis their perfusion properties upon a sequential injection in the same mouse of a preclinical blood-pool agent, Gd-AAZTA-Madec (17). Although similar mean extravasation fractions values might suggest comparable permeability to the leaky tumor vessels, the three investigated molecules showed different spatial distribution within the tumor extravascular space. The irregular and hyperpermeable nature of tumor microvasculature might strongly determine the distribution pattern of macromolecules, in terms of penetration and tumor uptake (69). In addition, several studies demonstrated that the nanoparticle distribution strongly depends also on multiple factors such as shape, chemical composition and surface charge (70,71). These findings are consistent with our *in vitro* measurements showing different size and distribution profile for molecules with similar molecular weight, as for BSA and dextran70. These properties suggest that different size/shape may be relevant for the distribution pattern and extravasation in tissues. Moreover,

different rates of enzymatic degradation for voluven and dextran that occurs via α-amylase and dextranase, respectively, may also affect their extravasation and accumulation (72,73).

Additionally, the use of voluven and dextran70 as perfusion MRI-CEST agents could arise interesting perspectives in the monitoring of tumor angiogenesis. Recently, Chen et al. investigated the capability of dextrans to report vessels permeability changes in tumors upon the administration of a disrupting vascular agent (74). However, the high molecular weight (150 kDa) of the exploited dextran molecule did not provide any CEST contrast before the administration of the vascular disrupting agent, hence limiting its applicability in contrast to the presented molecules.

This study presents some limitations. Although both voluven and dextran70 can provide moderate CEST contrast in tumor tissue (3-5%), Gd-based agents are still superior in their contrast enhancement capabilities (26-31%). However, similar information in terms of extravasation and permeability can be achieved when exploiting molecules with comparable size.

In conclusion, we showed that voluven and dextran70 can be successfully visualized by MRI-CEST both in vitro and in vivo. In tumor tissue, both molecules exhibit good MRI-CEST contrast in a preclinical model of breast cancer, showing different distribution pattern based on their physical/chemical properties. Therefore, voluven and dextran70 can be considered as alternative perfusion molecules with comparable size of standard macromolecular Gd-based agents to assess tumor vascularization. In addition, the exploitation of clinical-approved plasma volume expanders as MRI contrast agents might have a relevant translational potential in the clinical setting.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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TABLES

Table 1. Measured hydroxyl exchange rates				
	Voluven		Dextran70	
pH	k_b (Hz)	Error (Hz)	k_b (Hz)	Error (Hz)
6.0	730	20	780	20
6.3	970	20	1150	20
6.7	1480	30	1890	30
7.0	1800	40	2800	70
7.4	2300	50	5000	180

Table 1. Exchange rate k_b (Hz) and corresponding error (Hz) at each investigated pH value calculated for voluven and dextran70 6% w/v solutions from the simultaneous fitting of Z-spectra at multiple B_1 values.

FIGURE LEGENDS

Figure 1. A) Chemical structure and molecular weight of the investigated molecules voluven and dextran70. B) Size distribution profiles of bovine serum albumin (BSA, green), dextran70 (blue) and voluven (red) measured by dynamic light scattering.

Figure 2. Representative CEST Z-spectra (A) reporting Normalized Intensity Values (NIV) at pH 7, calculated ST% curves at pH 7 (B) and CEST contrast dependence with pH measured in the range of 5.5–7.9 pH units (C) in phantoms containing 6% w/v of voluven and dextran70 solutions (saturation pulse power of 2 μT applied for 5 s at 37 °C).

Figure 3. Representative results of (A) Multi-B_1 fitting of Z-spectra for dextran70 (left) and voluven (right) at pH 7.4 (7T, 37°C) and (B) calculated proton exchange rates for the hydroxylic protons at several pH values for dextran70 (left) and voluven (right); solid lines show the best fits using eq. 1.

Figure 4. Representative tumor bearing mouse injected with voluven (top) or dextran70 (bottom) and, from left to right, T_{2w} images with tumor (green) and muscle (red) ROIs and CEST contrast (ST%) maps for the whole images before and 8, 16, 24 and 32 minutes after voluven or dextran70 injection.

Figure 5. A-B) Bar graphs indicate ΔST% contrast (ST% post injection – ST% pre injection) calculated in TS/A tumor (n=4 mice) and control muscle regions at different time points (8, 16, 24 and 32 min post injection) for voluven (A) and dextran (B) by applying B_1 = 1.5 μT for 5 s on a 7T MRI scanner. C) Time-averaged tumor/muscle (T/M) ratio of ΔST% contrast calculated for both voluven and dextran70. D) Representative T_{2w} images and ΔST% parametric maps overimposed on T_{2w} anatomical images for voluven (top) and dextran70 (bottom) at different time points (8, 16, 24 and 32 min post injection) and corresponding tumor (green) and muscle (red) ROIs.

Figure 6. Descriptive statistics of the contrast enhancement (A) and extravasation fraction (B) upon the sequential i.v. injection of one of the two investigated agents with the MRI-CEST approach (voluven: grey bars; dextran70: white bars) and of the contrast enhancement (C) and extravasation fraction (D) after Gd-AAZTA-Madec injection with the MRI-T_{1w} approach measured in the same ROIs encompassing the tumor regions (n=4, 8 tumors in total for each molecule).

Figure 7. Representative (A) MRI-CEST contrast enhanced maps upon i.v. injection of voluven (left panel) and dextran70 (right panel) as ΔST% maps (calculated as ST% post injection – ST% pre injection) followed by i.v. injection of Gd-AAZTA-Madec (B) as ΔSI% maps (calculated as (SI post injection – SI pre injection) / SI pre injection) overimposed on T_{2w} anatomical images in TS/A breast tumors. Corresponding similarity maps (C) shows pixels where both voluven or dextran70 and Gd-AAZTA-Madec molecules have been detected (blue pixels), whereas red pixels (CEST agents) and green pixels (Gd-based agent) indicate the presence of only one contrast agent.

Figure 8. Box-plots for spatial voxelwise correlation comparing parametric maps derived using CEST agents (voluven: grey; dextran70: white) with those from Gd-AAZTA-Madec for contrast enhancement estimates (A) and extravasation fraction (B). The central mark is the median, the edges of the boxes are the 25th and 75th percentiles and the range of the whiskers includes 5 to 95 percentiles of the data.