

Size-tuneable nanometric MRI contrast agents for the imaging of molecular weight dependent transport processes

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Abbreviated Title Page

1. Manuscript title:

Size-tuneable nanometric MRI contrast agents for the imaging of molecular weight dependent transport processes

2. Manuscript type:

Original research

3. Advance(s) in Knowledge (5 sentences):

1. Precise control of glycol chitosan (GC) polymer chemistry facilitates synthesis of Gadolinium based MRI contrast agents with identical physicochemical properties but defined molecular weight
2. GC-DTAP-Gd provides enhanced contrast over an extended duration depending on molecular weight MW ($\geq 1h$ for 40kD).
3. Kidneys show early detailed enhancement of medulla, papilla and renal pelvis suggesting renal elimination.
4. Imaging of the head with GC-DTPA-Gd allowed detailed anatomical identification of specific blood vessels in particular with the high MW agent.
5. Sequential high-resolution isotropic imaging of established A431 xenograft flank tumours with DTPA-Gd and GC-DTPA-Gd demonstrated that the initial delivery of the contrast agents was well correlated with blood supply but subsequent tissue transport was heterogeneous and primarily by diffusion, limited by molecular weight.

4. Implication(s) for Patient Care (if any)

MRI imaging of molecular weight dependent transport processes which could potentially lead to clinical biomarkers for molecular weight dependent drug transport and support selection of suitable tumour models for pre-clinical development.

5. One sentence summary statement

Precise control of glycol chitosan polymer chemistry facilitates synthesis of Gd based MRI contrast agents with defined molecular weights which allow isotropic high-resolution three-dimensional imaging of molecular weight dependent transport processes that could serve as predictive biomarkers for drug transport.

6. Abstract (250)

Purpose: To evaluate size-tuneable nanomeric glycol-chitosan-DTPA-Gd conjugates as MRI contrast agents for the imaging of molecular weight (MW) dependent transport processes.

Material & Methods: Glycol chitosans (GC) – DTPA conjugates of precisely controlled MWs were synthesised and evaluated in mice against Gd-DTPA using times series of high-resolution MRI images of trunk, head, and xenograft flank tumours. All animal studies were approved by the local ethics committee and the UK authorities.

Results: GC-DTPA modification ratio was one DTPA per 3.9 – 5.13 of GC monomers. GC-DTPA-Gd provided overall superior contrast compared to Gd-DTPA with the duration of the enhancement depending on MW (≥ 1 h for 40kD). Kidneys showed early enhancement also in the renal pelvis suggesting renal elimination. Imaging of the head with GC-DTPA-Gd allowed detailed anatomical identification of specific blood vessels in particular with the high MW agent. Sequential high-resolution isotropic imaging of established A431 xenograft flank tumours with DTPA-Gd and GC-DTPA-Gd demonstrated that the initial delivery of the contrast agents was well correlated with blood supply. Subsequent tissue transport was primarily by diffusion and was limited by molecular weight. The data also highlight the role of heterogeneity in CA distribution that was again more prominent for the high MW agent.

Conclusion: GC-DTPA-Gd with identical physical chemical properties but precisely controlled MW allow isotropic high-resolution three-dimensional imaging of molecular weight dependent transport processes which could potentially lead to clinical biomarkers for molecular weight dependent drug transport and support selection of suitable tumour models for pre-clinical development.

7. Introduction (400/394)

Around 50% of MRI studies use contrast agents (CA) such as Gadolinium (Gd) [1] which is typically administered in a chelated form to avoid the toxicity from free Gd. Most CAs in current clinical use are extracellular fluid agents that distribute readily to all tissues and are typically excreted rapidly by renal filtration due to their low molecular weight (MW)[2]. However, CAs that remain limited to the blood vessels and have an extended plasma half-life are also useful, e.g. for imaging studies of longer duration and imaging of the blood vessels in MR angiography. Such blood pool CAs would typically require molecular weights large enough to avoid extravasation and ready renal filtration, e.g. by binding of Gd-chelates to albumin (MW69kD, 7-8 nm) [3, 4]. Gadofosveset trisodium (Ablavar®, Bayer Schering Pharma AG) is the first FDA licensed blood pool CA on the market [5]. Blood pool agents provide potential advantages for the visualisation of peripheral vascular pathology, vascular aspects of CNS or kidney disease, and cancer. For example, the progression of solid tumours requires the obligatory activation of the 'angiogenic switch', i.e. the activation of pathways linked to the development of new blood vessels[6]. The developing neo-vasculature is heterogeneous with atypical morphology, and has reduced hydrodynamic and transport functionality. The discontinuous endothelial lining allows extravasation of macromolecules and nano-sized particulates[7] with a consequent increase in interstitial pressure that hampers nutrient and drug transport into the tumour parenchyma [8] and has been linked to poor prognosis and drug resistance [9]. The effects are thought to be particularly important for the transport of higher MW therapeutics such as anti-bodies, macromolecules, particulate drug carriers, and gene delivery systems [10]. Understanding the impact of MW on transport processes from blood vessels into tissues thus becomes important not only as a diagnostic feature but also as a potential predictor and biomarker for transport of such agents in specific tumours.

We hypothesised that polymeric MRI CAs of variable MW but identical physicochemical properties could be a useful tool for the study of such molecular weight dependent transvascular transport processes and chose to test this by creating CAs based on glycol chitosan (GC), a versatile biocompatible carbohydrate polymer widely used in the development of nanomedicines and nano-diagnostics.

8. Materials and Methods (800/788)

8.1. Synthesis of GC-DTPA

All reagents were purchased from Sigma-Aldrich, UK. Glycol chitosan (GC) of varying molecular weight was obtained by acid degradation [11]. Briefly, GC with a degree of polymerisation of 2,500 was degraded (2 h, 48 h) and recovered by filtration, dialysis, and freeze-drying. The depolymerised GC was then reacted with *p*-SCN-Bn-DTPA (2-(4-isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid, Macrocyclics, USA) in alkaline conditions (24h) and the product obtained after dialysis and freeze-drying. The polymer MW was determined by gel permeation chromatography and multi-angle laser light scattering (GPC/MALLS; Dawn EOS, Wyatt Technology) [11]. ¹H-NMR

and ^1H - ^1H COSY (Bruker AMX 400 MHz spectrometer) were performed on GC, *p*-SCN-Bn-DTPA, and GC-DTPA conjugates using deuterated water (D_2O). Elemental analysis was carried out on a Thermo Finnigan EA 1112 instrument (Thermo Scientific).

8.2. Gadolinium Loading

GC-DTPA conjugate and gadolinium (0.5 mmol) were dissolved in imidazole buffer (pH=6.8), mixed and stirred over night to form a precipitate (GC-DTPA-Gd) which was re-dissolved at pH7 to precipitate free Gd. After Gd removal by filtration (0.45 μm) and dialysis the final product was obtained by freeze-drying. The Gd loading of the GC-DTPA polymer was confirmed using isothermal titration calorimetry (ITC), the arsenazo assay, and inductively coupled plasma-atomic emission (ICP-AE): For the ITC (Microcal VP-ITC, MicroCal Inc., USA) Gd chloride hexahydrate (6.5mM), DTPA and GC-DTPA (0.2mM DTPA equivalent) were dissolved (0.1M HCl/NaOH buffer, pH = 2) and de-gassed (ThermoVac, MicroCal Inc., USA). Then aliquots of Gd (20–25 \times 5 μL , Δt 6 mins) were injected into the ITC cell filled with either DTPA or GC-DTPA ($n=3$). Free Gd in solutions of DTPA or GC-DTPA was quantified using the absorbance of a Gd-arsenazo complex (653 nm) [12]. The Gd content in a range of samples of GC-DTPA-GD was measured using ICP-AE (138 UL Trace, Jobin Yvon, UK; Vista Pro, Varian Inc.) and Gd standards (0-40 mg L $^{-1}$) at 310 nm.

8.2.1. Animal Preparation and Maintenance

All studies adhered to current national and local regulations and guidelines [13]. Female nude mice (CD1-nu, ~20 g) were anaesthetized (2–3% isoflurane in 70/30 N_2/O_2) via a facemask. Temperature (water jacket), respiration and ECG were monitored using a BIOPAC system (Goleta, CA, U.S.A.). Xenograft tumours, established by subcutaneous injection of 1×10^6 A431 epidermoid carcinoma cells (ATCC CRL-1555), were used when vascularized ($\phi > 5$ mm 10+ days).

8.3. MR Imaging

All MR imaging was performed on a Bruker Biospin Advance using a 7T horizontal 30cm bore magnet. Trunk imaging was carried out with a 35mm RF resonator coil (gradient maximum 200mT m $^{-1}$), other images used a Bruker micro-imaging gradient insert (BG-6, 60mm ID, 100A amplifier; gradient maximum 1000 mTm $^{-1}$, rise time of 50 μs) or a dedicated optimized tumour solenoid RF micro-coil, built into the animal cradle [14].

The trunk with a focus on the kidneys was imaged using a four slice multi slice multi echo (MSME) 2D sequence with in-plane isotropic voxels 150 \times 150 μm^2 , slice thickness of 2 mm, in-plane FoV 60 \times 34 mm. The sequence ($T_E=10.5$ ms, $T_R=75$ ms, a flip angle of 90 $^\circ$ with a 180 $^\circ$ re-focusing pulse and four averages) required a total scan time of 1 minute 7 seconds.

The brain was imaged using a 3D FLASH sequence with isotropic voxels 160 \times 160 \times 160 μm^3 and a field of view 20 \times 16 \times 16 mm 3 . This sequence used an echo time of 3.7 ms, a repetition time of 20 ms, a flip angle of 30 $^\circ$, and one average. The total scan time was 3 minutes 20 seconds.

A 3D FLASH sequence was also used to image tumours using isotropic $100\ \mu\text{m}^3$ voxels in a field of view $80\ \text{mm}^3$ with an echo time of 3.5 ms, a repetition time of 25.0 ms, a flip angle of 30° , and either two or four averages with total scan times of 5 min 20 sec and 10 min 40 sec, respectively. Tumour angiography studies used a multi-slice 2D time of flight (TOF) gradient echo sequence sliced perpendicular to the axis of the coil or sagittal in anatomical terms with isotropic $100\ \mu\text{m}^3$ voxels in-plane resolution and 60 sequential slices of $100\ \mu\text{m}$ thickness. The echo time used was 5 ms and the recovery time was 40 ms with 8 averages.

Raw data was exported as free induction decay (FID) file and then processed using Interactive Data Language (IDL, RSI, Boulder, Colorado, U.S.A.) routines.

9. Results (1000/1062)

9.1. GC-DTPA Synthesis

The acid degradation of glycol chitosan reduces the polymer MW in a nonlinear manner following a first order reaction with a first order rate constant of -1.346 ($M_n = 94448 e^{-1.346t} + 7041$, $r^2 = 0.99901$) [15]. The molecular weight of the resultant polymer can be selected based on the choice of reaction conditions, in particular reaction time and temperature [15]. The reaction produces polymers of low polydispersity ($M_w/M_n \sim 1.07$ - 1.3) with a yield inversely proportional to the degradation time (49-75%, low MW polymer is lost in dialysis) and a weight average MW of 37.8 ± 1.4 kDa (GC_{40}), 16.6 ± 1.6 kDa (GC_{16}) and 12.3 ± 1.9 kDa (GC_{12}), respectively.

The yield of the GC-DTPA conjugation step was 61–86% with the freeze-dried product obtained as an off-white/yellow cotton like product. The NMR analysis confirms the product and very low N-acetylation levels (< 0.05 mol% at 2.0 ppm; Figure 1). Signals from the polymer and DTPA overlap but the benzene group at $\delta 7.3$ – 7.5 indicates the presence of the benzyl isothiocyanate moiety of *p*-SCN-Bn-DTPA. Furthermore, a new peak for the GC anomeric proton that is normally masked becomes detectable at 4.4 ppm, presumably as a result of conformational changes.

The conjugation level of GC-DTPA was measured using elemental analysis, an arsenazo III assay, and ITC. Based on the elemental nitrogen and sulphur the number of DTPA moles per 100 moles of the monomer GC was calculated as 5.15% (GC_{40} -DTPA), 3.96% (GC_{16} -DTPA-1), and 4.89% (GC_{12} -DTPA-2), respectively (Tables

Table 1). Gd^{3+} ions bind to arsenazo III to form the colorimetrically detectable complex ($\log K = 15.85$ [12]) only once DTPA has been saturated with Gd to form the DTPA-Gd complex ($\log K = 22.46$ [16]). The colorimetric Gd arsenazo III complex thus allows measurement of free and GC-DTPA chelated Gd from which the level of conjugation was determined to be 4.83% (GC_{40} -DTPA), 3.94% (GC_{16} -DTPA-1), and 5.37% (GC_{12} -DTPA-2), respectively (Tables Table 1).

ITC was used to measure heat enthalpy of the GC-DTPA/ Gd^{3+} interactions for GC_{16} -DTPA and to determine the conjugation level by comparison with free DTPA and Gd (Figure 2). At the acidic low pH used in this experiment ($\text{pH}=2$), the carboxylic groups of DTPA are protonated (un-ionised) and interactions with Gd^{3+} occur at a slower rate. Initial Gd injections produce large peaks resulting from the binding of Gd^{3+} to DTPA. As the concentration of free DTPA reduces the heat reduces to the heat of dilution represented by the small peaks at the end of the curve. The

stoichiometry of binding is 0.4993:1 (GC₁₆-DTPA:Gd) corresponding to a GC:DTPA molar ratio of 3.79 ± 0.05 .

The yield for the off-white to yellow freeze-dried product was 217 mg (GC₄₀-DTPA) and 120 mg (GC₁₆-DTPA), respectively. The average ratio (ICP-AES) of Gd per mg of GC-DTPA-Gd was 0.079 ± 0.003 mg for GC₄₀-DTPA-Gd, 0.100 ± 0.005 mg for GC₁₆-DTPA-Gd, and 0.073 ± 0.005 mg for GC₁₂-DTPA-Gd.

9.2. Magnetic Resonance Imaging Results

The initial biodistribution was imaged at 1 min and 29min post the intravenous administration of GC₁₆-DTPA-Gd ($0.1 \text{ mmole Gd kg}^{-1}$). The MR imaging of the trunk showed very clearly highlighted kidneys when compared to the pre-administration image (Figure 3). Immediately after injection detailed imaging shows GC₄₀-DTPA-Gd and GC₁₂-DTPA-Gd in the kidneys with rapid passage to renal pelvis and then ureter. Although the level of contrast decreased over time, the kidneys remained highlighted. Magnevist, when administered at the same concentration of Gd, yielded only low level of dynamic contrast enhancement which was somewhat more pronounced after 30 minutes (Figure 3). Renal enhancement has been suggested as being indicative of contrast agent removal from the blood and elimination by the kidneys [17]. The higher molecular weight GC₄₀-DTPA-Gd continued to be present in the kidney for a longer period of time ($\geq 30 \text{ min}$) suggesting a longer plasma residence time consistent with a larger polymer MW. The data suggest these CAs are filtered by the kidneys and eliminated in the urine.

The imaging properties of Magnevist, GC₄₀-DTPA-Gd, and GC₁₂-DTPA-Gd were compared further imaging the cranium (Figure 4); the data is visualised using maximum intensity projections (MIPs) in three directions before and after (3 min 20 sec) administration. Magnevist produces a diffuse enhancement because of leakage of contrast material into the brain parenchyma and surrounding tissues. Conversely, GC₄₀-DTPA-Gd remains confined to the blood pool giving a much clearer enhancement of the brain vasculature while GC₁₂-DTPA-Gd shows an intermediate effect.

This blood pool selectivity dramatically changes the structures and detail that can be visualised from the volume data as shown by enhancement volume projections (Figure 4). These visualise regions with a level of enhancement greater than 20% based on the differential between pre-injection and post-injection contrast ($t=3 \text{ min}20 \text{ sec}$).

The CAs show a distinct distribution with signal time profiles dependent on anatomical location. Regions of interest (ROI) representing brain parenchyma, blood vessels, and surrounding tissue were identified and signal intensities compared over time (Figure 5). All CAs show a sharp initial enhancement in the major blood vessel. While both Magnevist and GC₁₂-DTPA-Gd show a brief peak the higher MW GC₄₀-DTPA-Gd shows a sustained enhancement suggesting a prolonged plasma half-life compared to the lower MW agents. However, the signal decay at one hour suggests signal intensities would reach pre-contrast levels within two hours. Both polymeric CAs GC₄₀-DTPA-Gd and GC₁₂-DTPA-Gd do not enhance contrast in the brain parenchyma or the surrounding connective tissue. Conversely the connective tissue region shows the greatest enhancement and longest time to peak when imaged with Magnevist, suggestive of a diffusion dependent accumulation. The vascular contrast enhancement using the GC₄₀-DTPA-Gd is sufficient to allow anatomical identification of specific larger blood vessels using regional maximum intensity projections.

Based on the differential contrast of the high and low MW polymers these CAs were applied to the investigation of molecular weight dependent transport processes in solid tumours. Transport limitations of individual tumours were visualised by sequential imaging of low and high MW CAs administered to the same animal and same tumour, separated by a washout period (Figure 6). For all agents initial delivery of both agents to the tumour was correlated with blood supply. As would be expected the lower molecular weight material exhibited greater diffusion and consequently covered a larger area of the tumour. A volume projection highlights the overall disparity in tumour coverage of the two agents. The heterogeneity of distribution relates to identifiable anatomical structural detail but is also linked to the difference in CA molecular weight.

10. Discussion (800)

Molecular weight is one of the key factors determining biological behaviour of molecules in terms of biodistribution, and vascular and tissue transport. Here we explore the use of biocompatible polymers with defined and tailored molecular weights as the basis of a family of MRI CAs to explore these processes by MRI. Such CAs, having the same physical chemistry but different molecular weights could be adapted to serve preferentially as blood pool CAs or to study macromolecular transport processes.

The potential for polymers based CAs with enhanced blood residence time has previously been explored. When poly-L-lysine-GTPA of increasing MW were explored as potential blood pool agents it has previously been shown that plasma half-life is increased and renal clearance reduced with increasing MW [18]. However, agents such as carboxy methyl dextran-GTPA have limited utility in exploring MW dependent processes because of their broad MW distribution (10-163 kD)[19]. On the other hand, CAs with a defined molecular structure, such as dendrimers, possess monodisperse MWs but do not allow continuous tuning of polymer molecular weight[20].

MW is not only a key determinant of a CAs potential utility as a blood pool agent but can also be used to assess pathological transport processes. Once tumours are able to induce the growth of new vasculature (angiogenic switch) they may proliferate and metastasise. The developing 'leaky' vasculature allows extravasation of macromolecules that leads to an increase in interstitial pressure leading to diffusion limited transport into the tumour parenchyma which affects low MW drugs [21] but even more higher MW therapeutics such as anti-bodies, macromolecules, particulate drug carriers, gene delivery systems [10], and which has been linked to poor prognosis and drug resistance [9].

MRI has been applied in to understand and quantify such transport processes, in particular by applying DCE-MRI and associated mathematical derivation of parameters such as transfer coefficient, vascular volume fraction and measures of permeability and perfusion, typically applying models based on the unidirectional transfer of contrast material between compartments such as the Tofts two compartment model [22] and the three-compartment model [23].

The current study analyses the relationships between blood supply and contrast agent delivery directly and in high resolution. The data emphasises the important role of underlying anatomical structures and local tissue heterogeneity in determining the detailed spatiotemporal distribution of molecules of different molecular weight.

The concept of using MRI contrast agents as surrogates for drugs to investigate tumour delivery and transport issues itself is not new. Both low molecular weight contrast materials [24] and macromolecular contrast materials [25] have been used as to predict the delivery and transport of drugs of a similar molecular weight. In both cases the delivery of the drug confirmed by histology proved to be well correlated with the prediction by MRI. Furthermore studies have been performed in which contrast agents with different molecular weights have been serially administered to the same tumour in order to optimise modelling procedures [26, 27]. The studies reported here would appear to be the first time that these two concepts (the use of contrast agents as drug surrogates and the serial administration of contrast agents with different molecular weights) have been brought together.

Here we synthesise and characterise glycol chitosan (GC-DTPA-Gd³⁺) as a biocompatible macromolecular contrast agent for which the molecular weight can be tuned precisely for various imaging purposes. We demonstrate its utility as a blood pool agent by detailed imaging of mouse brain vascular architecture, show that filtration via the kidney provides early and sustained contrast enhancement in the kidney, and provide evidence that GC-DTPA-Gd of high and low MW can be used to characterise molecular weight dependent transport in murine xenograft tumours with a high spatial resolution. The imaging also showed that the diffusion distances for higher molecular weight materials were lower than for low molecular weight materials. Furthermore, there is an increasing appreciation of the role of spatiotemporal heterogeneity of tumour transport. Data from doxorubicin treated breast cancers demonstrate this heterogeneity can persist for many hours [21] but work in experimental tumours suggests that vascular supply can also change rapidly over time [28]. We believe that isotropic high-resolution three-dimensional imaging of molecular weight dependent transport process in tumours can help to better understand the role of heterogeneity for the transport of therapies in the tumour. Studies of this type using a series of well-calibrated CAs that differ only by MW could potentially provide a surrogate biomarker for the accessibility of tumours to drug treatments (based on their molecular weight) would be expected to offer good coverage of a specific tumour. In drug development terms this may also help in the selection of a suitable tumour models to test new high MW treatments.

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12. Tables

Table 1. Characteristics of GC-DTPA conjugates and level of conjugation, represented by number of GC monomers per DTPA pendant group, measured using elemental analysis, arsenazo III assay, and ITC.

Conjugate	Time (hours)	MW kDa	GC:DTPA (mol:mol)	Elemental analysis	Conjugation level %		Average
					Arsenazo III assay	ITC	
GC ₄₀ -DTPA	2	37.8 ± 1.4	01:02	5.15	4.83±0.14	n/a	4.99
GC ₁₆ -DTPA	48	16.6 ± 1.6	01:01	3.96	3.94 ± 0.19	3.79 ± 0.05%	3.9
GC ₁₂ -DTPA	48	12.3 ± 1.9	01:02	4.89	5.37±0.06	n/a	5.13

13. Figure legends

Figure 1. Structure and annotated ¹H-NMR spectra of GC-DTPA in D₂O indicating the presence of the benzylisothiocyanate moiety of p-SCN-Bn-DTPA at δ 7.3–7.5.

Figure 2. Determination of the conjugation level of GC-DTPA/Gd³⁺ based on the measurement of heat enthalpy. Injection of Gd³⁺ (6.5 mM) into a buffered aqueous solution of GC16-DTPA (0.2 mM based on p-SCN-Bn-DTPA, 25 °C and pH = 2) yields a titration curve showing heat flow against amount of injected Gd³⁺ (top) and of enthalpy versus molar ratio (bottom).

Figure 3. Time course of trunk MR of mice imaged over 28 minutes and injected with Magnevist (top panel) and GC40-DTPA-Gd at 0.1 mmole Gd/kg-I (bottom panel). The polymer CAs shows an enhanced level of contrast over a significantly prolonged duration compared to Magnevist as well as early enhancement of the kidney parenchyma and renal pelvis.

Figure 4. MR imaging of the head imaging after administration of (a) Magnevist, (b) GC12-DTPA-Gd, and (c) GC40-DTPA-Gd. Left panel: Maximum intensity projections of the cranium in the coronal, sagittal, and transverse planes prior and 3 mins 20 sec after administration of CAs. Middle panel: Volume projections showing areas of greater than 20% enhancement after x minutes demonstrate the different levels of vascular retention of the agents. Right panel: Maximum intensity projections of murine brain imaged using GC40-DTPA-Gd allow visualisation of cranial vasculature in sufficient to allow anatomical identification of blood vessels (1-Superior sagittal sinus; 2-Lateral superior cerebellar artery; 3-Common carotid artery; 4-Posterior cerebral artery).

Figure 5. MR images and time dependent contrast intensity profiles of the mouse cranium imaged using (a) Magnevist, (b) GC12-DTPA-Gd, and (c) GC40-DTPA-Gd. A number of specific

anatomical regions of interest (ROI) were identified (square= brain parenchyma, rhombus = brain blood supply, triangle&cross= tissue surrounding the brain). The contrast intensity in those structures was then extracted from the 4D data stack and plotted over time yielding intensity-time curves.

Figure 6. Comparison of molecular weight dependent contrast enhancement in a tumour. A431 epidermoid carcinoma flank tumour in a mouse are imaged over a period of 20 min after intravenous administration of first Magnevist followed by GC40-DTPA-Gd (top panel). Changes in the distribution between low (Magnevist) and high MW CA (GC40-DTPA-Gd) between 0 to 10 minutes and 10 to 20 minutes are visualised, respectively (bottom panel, left). High and low MW agent distribution 20 minutes after injection in the same tumour is visualised as a volume projection of CA distribution (bottom right panel).











