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The Benefit of High Field MRI to quantify Myeloperoxidase Activity using a Gd-DTPA Derivated Contrast Agent

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Contents

1 Abstract

Systemic inflammation can negatively influence the size and outcome of cerebral ischemia. Myeloperoxidase (MPO), a key inflammatory component, expressed by activated neutrophils and macrophages/microglia can form highly reactive species and radicalized oxygen species with hydrogen peroxide which can cause additional damage in cerebral ischemia. MPO could serve as a biomarker for inflammation and could be used for a noninvasive method to sense infarct size. A specially designed, enzyme-activatied DTPA-bisamide (Gd-DTPA-BPA) was used as a contrast agent (CA) to quantify MPO activity. This CA has the ability to polymerize in presence of peroxidase and hydrogen peroxide, resulting in a net increase in longitudinal relaxivity (r_1). This particular communication describes the attempt to use Gd-DTPA-BPA to noninvasively image MPO in 72 hours post-stroke mice at a high field strength of 12 Tesla (T) where no changes in relaxivity are expected and all changes in pharmacokinetic properties at a DCE experiment can be related to polymerization. A lower field nuclear magnetic resonance (NMR) spectroscope ($0.47\,T$) was used to characterize the behavior of Gd-DTPA-BPA in presence of peroxidase and bovine serum albumin (BSA). Dynamic Contrast Enhanced (DCE) Imaging was performed 150 minutes at an ischemic/reperfusion animal model. It could be shown that presence of peroxidase and BSA results in a dramatic increase in relaxivity at low field which is not the case at high field, no change in relaxivity was observed. With the DCE experiment no specific MPO sensitivity was detected. In order to make more specific statements on sensing MPO in inflammation regions, further experiments would be necessary. The choice of an appropriate control agent should be the main focus in future experiment to achieve significant data.

2 Introduction

Over the last years magnetic resonance imaging (MRI), a powerful and non invasive technique, has become a leading tool to obtain high resolution images of the anatomical as well as physiological (functional imaging) structure of the body.

In an increasing number of MRI scans, in both research and clinical applications, imaging results benefit from using biocompatible, paramagnetic complexes, so called contrast agents (CAs), which further enhance the image contrast by increasing the nuclear relaxation rate of water protons in the surrounding tissue. [1] [2]

Nowadays most approved CAs are based on gadolinium (Gd^{3+}), a paramagnetic and potentially toxic rare earth. This as lanthanide considered metal has seven unpaired electrons, nine binding coordination sites (eight are occupied by a ligand) and its symmetric S-state provides a favorable electronic relaxation time for the interaction with water molecules. Gd^{3+} is a highly charged and toxic heavy metal and can disrupt the criticaly Ca^{2+}-required signaling. In order to administer it as a CA, proper ligands, that actually do remain chelated in the body, have to be chosen to keep toxicity to the lowest possible level.

The fundamental reason to use CAs is based on their ability to increase the longitudinal and transverse relaxation rate of water protons, $R_1 = \frac{1}{T_1}$ and $R_2 = \frac{1}{T_2}$, respectively (where T_1 is the longitudinal relaxation time constant and T_2 is the transverse relaxation time constant), which increases local intensity in MRI images in T_1 weighted puls sequences. [3] Maximizing the relaxivity r_1 and r_2, which are defined as the increase of relaxation rates, R_1 and R_2 respectively, in presence of CA, per concentration (mM) of agent, is of special interest in order to keep the dose of the administered CA as low as

possible while achieving a maximum level of image contrast. [3]

Maximizing the relaxivity can be tackled in different ways while designing a new contrast agent as more than one parameter are capable of increasing the water proton relaxation rate: q the number of water molecules coordinated to the complex, τ_M their mean residence lifetime, τ_R the reorientationial correlation time as well as τ_{S1} and τ_{S2} the longitudinal and transverse electron spin relaxation times, respectively. The latter two are frequency dependent and so is relaxivity. At low frequencies (f$\leq 1\,kHz$) relaxivity is mostly characterized by τ_S, however at high frequencies it is controlled by τ_M and mainly by τ_R. τ_R is strictly related to size and molecular weight of the CA complex. To achieve a higher water proton relaxation rate at high field this parameter has to be longer. Increasing the number of coordinated water protons (q) would have the same effect but would most likely lead to decreasing the stability constant and thereby to a higher toxicity. Both covalent and non covalent host–guest bondings, which lead to a slower tumbling of the whole complex and furthermore to increased relaxation rates, have been reported.

Performing a magnetic field dependent study where longitudinal relaxation rates over a wide range of magnetic field strength (corresponding to proton Lamour frequency) are recorded, leads to the so called nuclear magnetic relaxation dispersion (NMRD). NMRD profiles for Gd^{3+} complexes interacting with slowly rotating systems like proteins are highly diagnostic. They show a relaxivity peak at 20–40 MHz which dramatically drops with increasing field strength. [4, 5]

Cerebral ischemia initiates a complex cascade of biochemical and molecular changes, which involves the production of reactive oxygen species and inflammatory reactions which can contribute to stroke growth. It has been shown that stroke patients with systemic inflammation suffer a much higher damage. [6, 7] Antiinflammatory therapy has shown a decrease in infarct size and improvement in neurological sequelaes. Traditionally inflammation in stroke has been identified by histopathology as neutrophil infiltration, which highly correlates with ischemic damage. [8] MPO is the most prevalent component in azurophilic granules in neutrophils and therefore has been used as a histopathological

marker for neutrophils. [9] Also the myeloid line expresses MPO, especially monocytes and macrophages/microglia. MPO interacts with hydrogen peroxide to form highly reactive species and radicalized oxygen species, which induces apoptosis and therefore MPO is a key component of inflammation. [10] This means MPO could be used as a biomarker for noninvasive MRI methods to sense inflammation. [2]

Literature shows two different Gd-DTPA derivatives for peroxidase imaging, which polymerize in the presence of a peroxidase. The polymerization process relies on the oxidation of phenolic substrates in presence of the enzyme and hydrogen peroxide in order to form oligomers. [1] Recently one of these agents was also used for an attempt to sense the enzyme MPO. [2]

This communication describes the attempt to use Gd-DTPA-BPA (one of the previous described Gd derivatives) to noninvasively image MPO in 72 hours post-stroke mice at a high field strength of 12 Tesla (T). As reported, at particular high field strength like $12\,T$, relaxivity changes due to increasing τ_R are expected to be very limited. [4, 5] No relaxivity changes at $12\,T$ were assumed for the dynamic contrast enhanced (DCE) imaging whereby all changes in pharmacokinetic properties can be related to polymerization. A lower field nuclear magnetic resonance (NMR) spectroscope ($0.47\,T$) was used to characterize the behavior of Gd-DTPA-BPA in presence of peroxidase, like demonstrated in the literature [1] and additionally in presence of bovine serum albumin (BSA). As a consequence of expected Gd-DTPA-BPA – BSA binding, which was shown to strongly effect the pharmacokinetic properties [11], contrary to the literature [2], MultiHance® was chosen as control agent for the DCE experiments. DCE Imaging was performed longer than shown in the literature [2] due to the fact that the intensity shown did not seem to reach a maximum, the experiment was extended to 150 minutes in order to see a bigger discrepancy to the control component.

3 Materials and Methodes

3.1 Contrast Agents

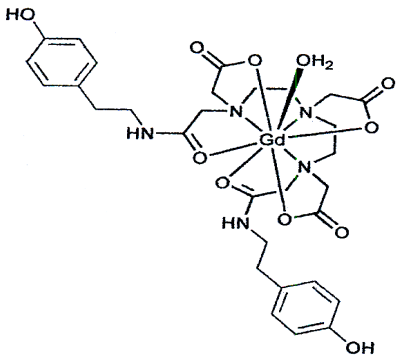

Figure 3.1: GD-DTPA-BPA

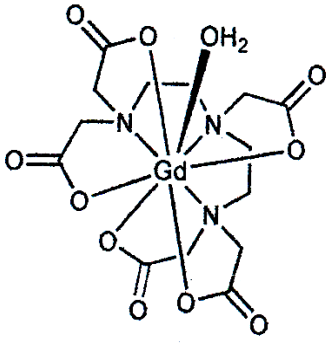

Figure 3.2: GD-DTPA

Gd-DTPA-BPA was synthesized as described [1].

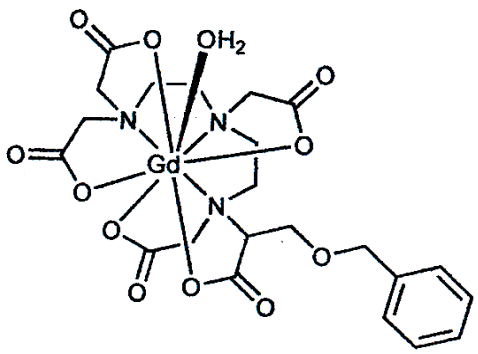

Figure 3.3: Multihance

(9R,S)-2,5,8-tris(carboxymethyl)- 12-phenyl-11-oxa-2,5,8-triazadodecane-1,9-dicarboxylic acid, gadolinium complex (MultiHance®)for clinical use ($500\,mM$), were purchased from Bracco Diagnostic Inc. (Princeton, New Jersey, USA).

Diethylenetriaminepentaacetic acid gadolinium(III) dihydrogen salt hydrate (Gd-DTPA) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) in his pure form (97%) and dissolved in buffer ($120\,mM$ NaCl, $20\,mM$ Hepes and de-ionized water, pH$\sim$8). For all experiments the same buffer was used.

3.2 Stock Solutions

Freshly prepared solutions were used in all experiments to avoid oxidation.

Horseradish peroxidase (HRP) that is widely used in immunochemistry was used to proof the principles. The essentially salt-free and lyophilized powder, was purchased from Sigma-Aldrich with an activity of $1000\,Units/mg$ (designation of the manufacturer). It was dissolved in buffer ($4\,mg/ml$) and stored refrigerated.

Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich as essentially fatty acid free and lyophilized powder ($\geq$96%) and was dissolved in buffer ($1.5\,mM$) every day right before measurements.

3.3 Sample Preparation

Horseradish peroxidase time course. Samples were prepared as close to the measurement as possible. Samples were prepared with CA and BSA at a concentration of $0.5\,mM$ and $0.6\,mM$, respectively, and an excess of 3% H_2O_2. HRP in different amounts, at a range of $0.004\,U - 4\,U$, was added.

BSA Binding. Samples were prepared with a CA concentration of $0.05\,mM$, BSA was successively added up to concentrations of $0.62\,mM$. In order to not dilute the sample as BSA was added, the titrant was also prepared with a concentration of $0.05\,mM$ CA.

Gd-DTPA-BPA MRI Imaging. For this experiment samples were prepared in the same manner as for the time course experiment but with a CA concentration of only $0.1\,mM$ and HRP was added immediately. Assays for BSA measurement were prepared with a CA concentration of $0.1\,mM$ and varying amounts of BSA ranging from $0.1\,mM$ to $0.7\,mM$.

3.4 Relaxometry at 0.47T

Samples were prepared like described under 3.3 and preheated to 37°C about 20min prior to the measurement. All T_1 measurements were accomplished on a $20\,MHz$ Brucker "The minixpec mq 20 NMR" (Bruker, Karlsruhe, Germany) using an inversion recovery sequence with TR $\geq 5 * T_1$ and 15 TI points automatically spaced up to TR. Every data point was obtained by using 4 averages, T_1 was fitted automatically by the Brucker software the minispec (V 2.58). Temperature was controlled to 37° by using a Julabo F 25 water circulation heating unit.

3.5 Relaxometry at 11.75T

Samples were prepared like described under 3.3 and preheated to 37°C about 20min prior to the measurement. Samples were kept in flexible Falcon wellplates 96-well, cut to 4 x 4 wells, covered with a sticky lid to fix them. Relaxation times at high field were

obtained at an $11.74\,T$, $31\,cm$ inner diameter (ID) horizontal clear bore magnet (Magnex Scientific/Bruker Instrument, Bruker, Karlsruhe, Germany) with a $9\,cm$ ID gradient coil set insert using a $72\,mm$ ID Brucker Quad Transmit Coil, utilizing an inversion recovery sequence with TR= $5000\,ms$, TI=50, 75, 100, 150, 200, 250, 300, 400, 500, 600, 700, 1000, 2500, 3000, $5000\,ms$ for the HRP experiment and TR= $7000\,ms$, TI=50, 75, 100, 150, 200, 300, 400, 500, 600, 700, 1000, 200, 3000, 5000, $7000\,ms$ for the BSA experiment. Samples were temperature controlled at 37°C during the whole imaging process with a hot air stream using a small animal heating system (Small Animal Instruments, Inc, Stony Brook, New York). To obtain the mean signal intensity a fixed region of interest (ROI) was drawn over each well. The data was fitted to the equation:

$$I_x = I_0 * |1 - 2 * e^{\frac{-TI}{T_1}}| \tag{3.1}$$

with T_1 and I_0 being fitted and I_x as the Intensity gained at a certain time point TI. All image processing was performed with Jim V. 5.0 (Xinapse Systems Ltd, U. K.).

3.6 Animal Model preparation

Strokes were introduced by Amy Bruestle (PhD candidate at the Stenzel-Poore Lab) using a ischemic/reperfusion Model. Mice were subjected to 45 min middle cerebral artery occlusion (MCAO) using the monofilament suture method as previously reported in the literature [12]. Body weights were monitored prior to and following MCAO. Mice were anesthetized by isoflurane inhalation (1.75%/l O_2). Blocking the middle cerebral artery was performed by a silicone-coated 8-0 monofilament nylon surgical suture that was threaded through the external carotid to the internal carotid and finally blocked the bifurcation into the MCA and anterior cerebral artery. The filament was maintained intraluminally for 45 min and then removed, thereby restoring blood flow. Cerebral blood flow (CBF) was monitored throughout the surgery by laser Doppler flowmetry (Periflow 5000; Perimed, Sweden).

3.7 Contrast Agent for Dynamic Contrast Enhanced MRI

GD-DTPA-BPA was dissolved in a solution of heparin (1.5%)(heparin sodium injection 1000USP Units/ml, APP Pharmaceuticals, Schaumburg, Illinois, USA), to avoid blood clotting in the tail vein injection, and saline to a concentration of $100\,mM$ and neutralized with NaOH.

MultiHance® ($500\,mM$) was diluted in the heparin-saline solution to a concentration of $100\,mM$.

3.8 Dynamic Contrast Enhanced MRI

Mice were prepared as shown in 3.6 and imaged 72 hours post-stroke. Gd-DTPA-BPA and the control (MultiHance®) were administered in two different mice. The mice were benumbed with ketamin and kept anesthetized during the whole measurement with isoflurane. A bolus of CA ($0.3\,mM/kg$), prepared as shown in 3.7, was administered per tail vain injection ($1\,ml/min$) using an injection pump (Harvard Apparatus, PHD 2000, Massachusetts, USA). DCE was realized at an $11.74\,T$, $31\,cm$ inner diameter (ID) horizontal clear bore magnet (Magnex Scientific/Bruker Instrument, Bruker, Karlsruhe, Germany) with a $9\,cm$ ID gradient coil set insert using a brucker mouse coil, utilizing a flash sequence with TE=1,636 ms, TR=238 ms, FOV=25 mm x 25 mm, matrix=256 x 256, 20 slices with a thickness of 0,5 mm, interslice thickness=0,5 mm and using a number of averages of one. Scan time for one repetition of 1m 15ms resulting in a total scan time for 15 repetitions of 2h 32m 19s 200ms.

4 Results

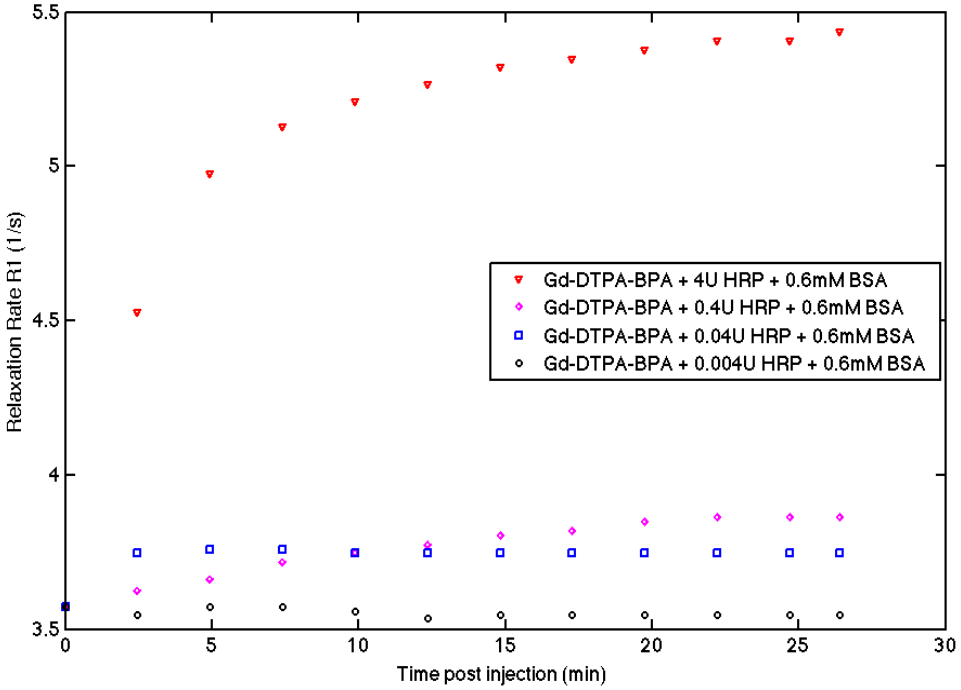

Figure 4.1: $0.5\,mM$ Gd-DTPA-BPA in presence of $0.6\,mM$ BSA, hydrogen peroxide and varying concentrations of HRP, temperature = 37°C, established at $20\,MHz$

Gd-DTPA-BPA in the presence of $0.6\,mM$ BSA has shown a 52% increase in R_1 when treated with the enzyme HRP ($4\,U$) (Fig. 4.1). Decreased specific protein activity of $0.4\,U$, $0.04\,U$ has proven an increase in R_1 of 8% and 5%, respectively. Treating the CA with $0.004\,U$ HRP has not shown any increase of the relaxation rate. In presence of a specific protein activity of $4\,U$ HRP, more than 80% of the maximum R_1 value was reached within 5 minutes.

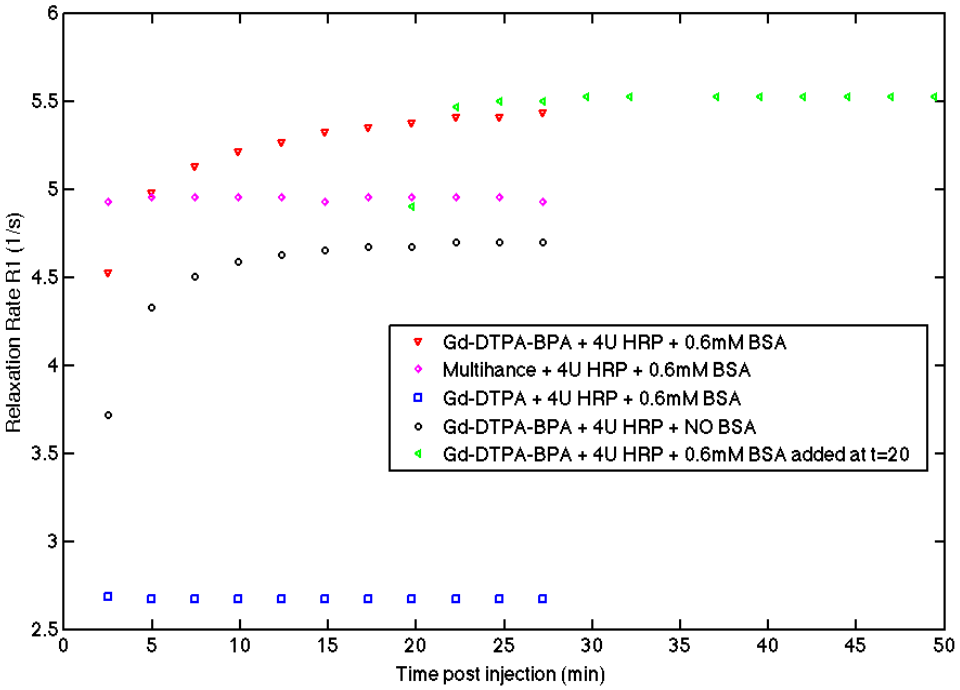

Figure 4.2: Gd-DTPA, MultiHance® and Gd-DTPA-BPA at a concentration of $0.5\,mM$ in the presence of $0.6\,mM$ BSA, hydrogen peroxide and $4\,U$ of HRP. Also presented Gd-DTPA-BPA treated with $4\,U$ of HRP and hydrogen peroxide, NO BSA added to this sample, temperature = 37°C, established at $20\,MHz$

Compared to Gd-DTPA, which does not show any change in relaxation rate in presence of $0.6\,mM$ BSA and treated with the enzyme HRP ($4\,U$), Gd-DTPA-BPA shows a 103% increase of R_1 25 minutes post treatment with the same proteins (Fig. 4.2). Multihance does not show any change in R_1 either when treated with this two proteins. Compared to Multihance, Gd-DTPA-BPA has shown a 10% higher R_1-value at a given time point of 25 minutes. In absence of BSA, Gd-DTPA-BPA showed a change in relaxation rate of more then 69%, relatively to pretreatment with HRP, 25 minutes after treatment with $4\,U$ of enzyme. Compared to Gd-DTPA, this depicts a change in R_1 of more than 75%. Adding BSA to an already reacted solution of Gd-DTPA-BPA and HRP has shown a further increase in R_1 of about 12% relatively to its start point. R_1 enhancement of

Gd-DTPA-BPA treated with $4\,U$ HRP in presence and absence of BSA was proven to be 52% and 69%, respectively, 25 minutes post treatment (Fig. 4.2), which describes a 17% increased R_1 value when BSA is present.

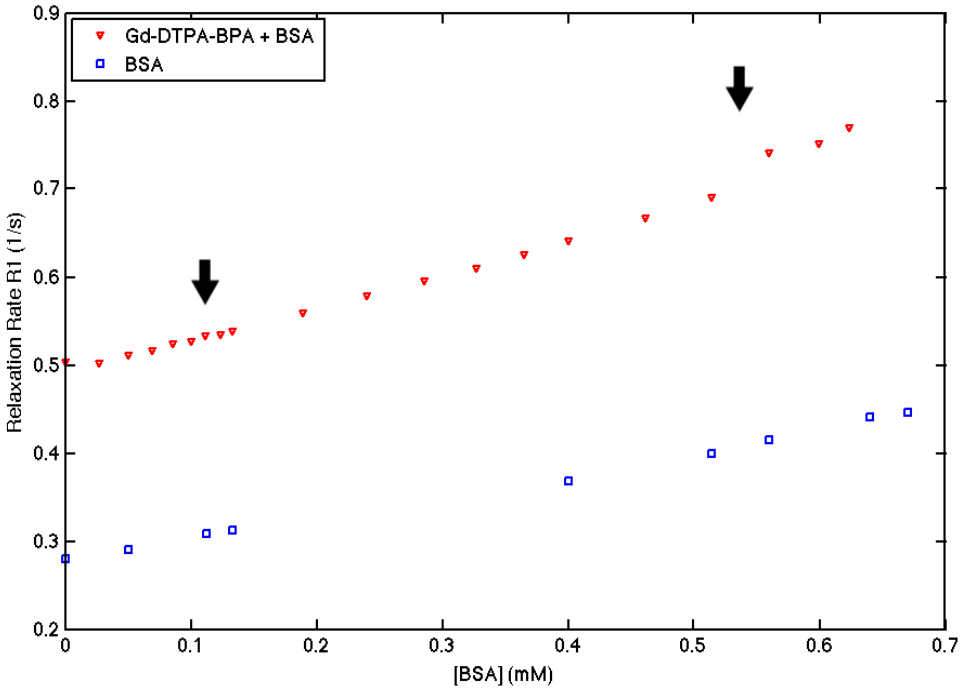

Figure 4.3: Gd-DTPA-BPA at a concentration of $0.05\,mM$ with varying concentrations of BSA in a range of $0\,mM$ – $0.6235\,mM$ also varying BSA concentrations without presence of CA, temperature = 37°C, established at $20\,MHz$

Gd-DTPA-BPA has shown a R_1 dependence on BSA concentration which can be seen in Fig 4.3. In presence of $0.6\,mM$ which depicts a 1:12 CA-BSA ratio the slope of the curve was shown as 42%. BSA without presence of CA has also shown a R_1 dependence, its slope was observed to be 25%. Two potential binding events were observed, the first at $0.1\,mM$ and the second one at $0.55\,mM$ – $0.62\,mM$ (arrows).

At 12T Gd-DTPA-BPA has not shown a clear trend of change in R_1 when treated with different concentrations of BSA (Fig. 4.4). The mean R_1 value and standard deviation were observed to be at $0.7861\,\frac{1}{s}$ and $0.0385\,\frac{1}{s}$, respectively.

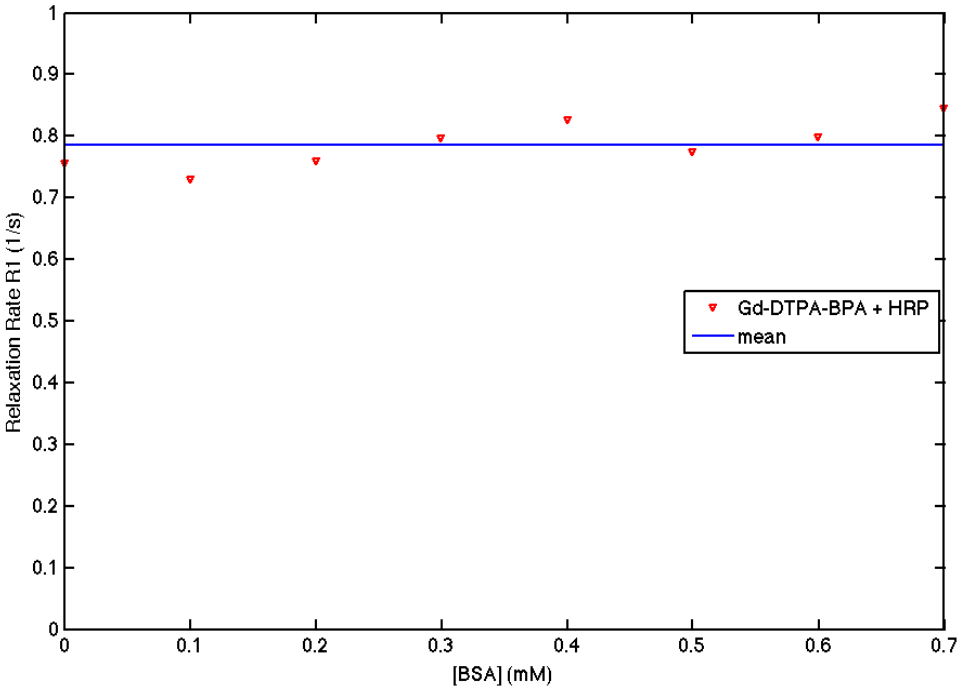

Figure 4.4: Gd-DTPA-BPA at a concentration of $0.1\,mM$ with varying concentrations of BSA in a range of $0\,mM$–$0.7\,mM$, temperature = 37°C, established at $500\,MHz$

Gd-DTPA-BPA has shown a maximum of intensity after approx. 100 minutes post injection and only a slightly decreased intensity level at later time points (Fig. 4.5). A maximum of the control component was observed at approx. 60 minutes post injection.

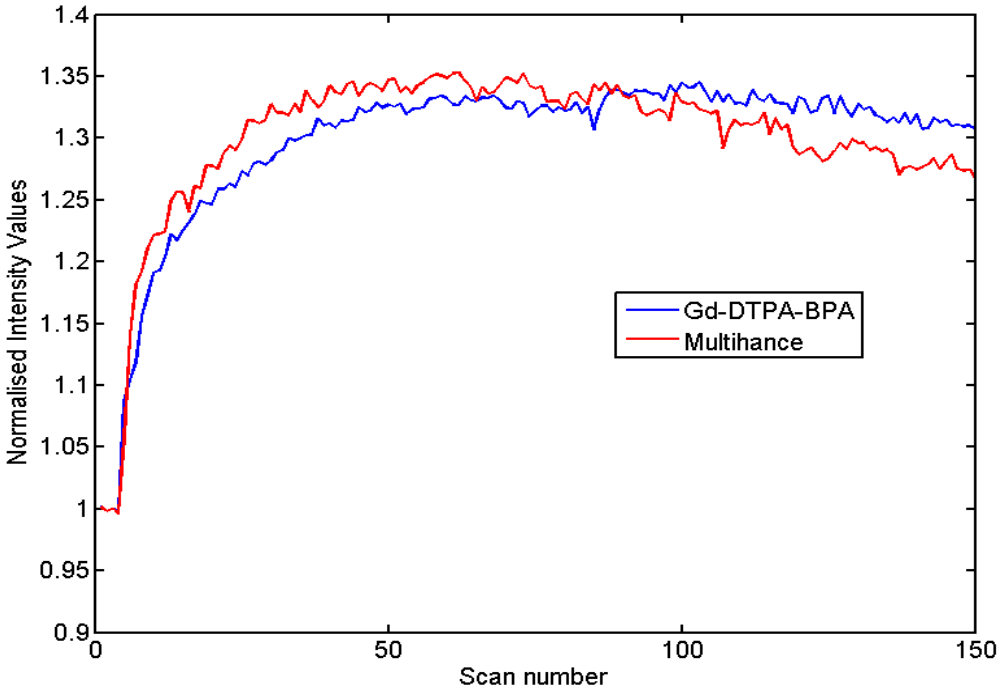

Figure 4.5: Normalized intensity values of the 72 hours post-stroke DCE experiment (scan number is approx. minutes), temperature = 37°C, established at 500 MHz

5 Discussion

In this present study a Gd-DTPA derivative (GD-DTPA-BPA) has shown an increase in R_1 of more than 50% when treated with the enzyme HRP ($4\,U$) and with BSA ($0.64\,U$) (Fig. 4.1) which coincides with the literature. [1] Detectable changes in R_1 could be seen down to a specific enzyme activity of $0.4\,U$ (5%). At a specific enzyme activity of $0.04\,U$ an increase in R_1 was observed, but the fact that R_1 reached a constant level right after injection leads to the conclusion that this is not an effect of polymerization but an effect of adding some impurities. A further decrease of enzyme activity did not show any significant change in R_1 which was expected and also shown in the literature. [1] Nevertheless this fact could play an unknown and very important role in the detection of MPO in early stages of inflammation where the enzyme presence would be fairly small and thus undetectable. The differences in R_1 could be explained by different levels of polymerization due to different specific enzyme concentrations. [1] Higher polymerization levels will enlarge the size and molecular weight of the CA complex, both which are strictly tied to enhancement of τ_R. Furthermore this polymerization reaction seems to be fairly fast (Fig. 4.1) which could play a major role in *in vivo* measurements to immediately initiate the polymerization process in presence of the enzyme and thus limit the risk of getting washed out before reacting with the enzyme.

Unlike Gd-DTPA-BPA, other CAs like MultiHance® and Gd-DTPA have shown no change in T_1 at all in presence of the enzyme HRP (Fig. 4.2), which was expected due to missing polymerization abilities. As anticipated, the presence of BSA seems to have an effect on the Gd-DTPA-BPA–HRP reaction. In absence of BSA the relative increase of R_1 was seen to be higher than in presence of BSA (Fig. 4.2). This observation could be

due to BSA occupation of important binding sites which are used for polymerization and thus stopping this process if a CA molecule, bound to BSA, is attached to an existing polymer. Despite this effect, the total R_1 value in presence of BSA is higher than without BSA. This effect could be explained in terms of BSA binding to free, not polymerized Gd-DTPA which would lead to a further increase ina R_1. Moreover it does not seem to make any difference which of the proteins is present earlier, the resultant final R_1 values (25 min post injection) differ approx. 1.5% (Fig. 4.2). The small increase in R_1 by adding BSA to an already reacted solution of Gd-DTPA-BPA and HRP leads to the conclusion that the main part of R_1 enhancement seems to be due to polymerization of Gd-DTPA-BPA in presence of HRP.

Gd-DTPA-BPA, when treated with different concentrations of BSA, has shown a different slope of R_1 change compared to the R_1 dependence of BSA in absence of CA (Fig. 4.3). Gd-DTPA-BPA has also shown two potential binding events at $0.1\,mM$ and $0.55\,mM$ (arrows). These effects are strongly indicative of weak binding to BSA but would have to be confirmed by a different experiments such as displacement of fluorescent probes as shown in the literature. [13]

At a high field of $12\,T$ no change in R_1 was seen when Gd-DTPA-BPA was treated with HRP over a range of $4\,U$ – $0.004\,U$ specific enzyme activity, which coincides with the literature. [4, 5] This further clarifies the hypothesis of limited relaxation rates at very high field strength regardless of the CA. Small changes in R_1 in the BSA and HRP experiments are the result of known field inhomogeneity throughout the coil and temperature variation. This inhomogeneity makes a proper and accurate T_1 measurement throughout a well plate almost impossible. To avoid this problem in the future, measurements should be realized on a $500\,MHz$ NMR spectrometer, which would also provide a better temperature control.

Gd-DTPA-BPA has shown a later intensity maximum, post injection, than the control in DCE imaging and also seemed to dim slower, which could probably be explained by polymerization in presence of MPO in the stroke region but it could be due to differences in stroke properties (infarct size, level of blood-brain barrier (BBB) disruption)

in different mice, or due to differences in BSA binding as well. Both CAs were still detectable even 24 hours post injection (data not shown), which was especially surprising for the control. No specific MPO sensitivity could be shown in this experiment. In order to make more specific statements on sensing MPO in inflammation regions with Gd-DTPA-BPA, further experiments would be necessary. The choice of an appropriate control is affecting the resulting data in a big way, and therefore of prime importance. As consequence of shown Gd-DTPA-BPA binding to BSA, Gd-DTPA does not seem to be an appropriate control for this approach. Due to its structure the specific control (not charged) (Fig. 5.1) may be the best approach, although the unknown charge of Gd-DTPA-BPA may play an important role in pharmacokinetic properties and thus a comparison to this control is probably also inappropriate. To confirm MPO sensing ability, imaging MPO knockout mice would probably give evidence, although it is not known how MPO knockout affects the stroke region, the inflammation and further BBB disruption, which is required for the CA to be able to get into the inflamed brain region.

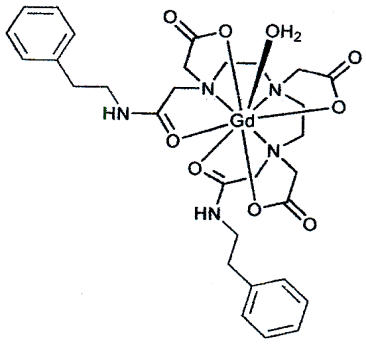

Figure 5.1: Control synthesize by Woods M.

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