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## **Re-evaluation of the water exchange lifetime value across Red Blood Cells membrane**

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### **Abstract**

The water exchange lifetime ( $\tau_i$ ) through Red Blood Cells (RBCs) membranes can be measured by analyzing the water protons bi-exponential  $T_1$  and  $T_2$  curves when RBCs are suspended in a medium supplemented with paramagnetic species. Since the seminal papers published in the early '70 of the previous century, paramagnetic  $Mn^{2+}$  ions were used for doping the extracellular compartment in the RBCs suspension. The obtained  $\tau_i$  values fall in the range of 9.8-14 ms. Conversely, other physic-chemical measurements afforded longer  $\tau_i$  values.

Herein, it is shown that the replacement of  $Mn^{2+}$  with the highly stable, hydrophilic Gd(III) complexes used as paramagnetic Magnetic Resonance Imaging (MRI) contrast agents led to measure  $\tau_i$  values of  $19.1 \pm 0.65$  ms at 25°C. The observed difference is ascribed to the occurrence of enhanced permeability of RBC membrane in the presence of  $Mn^{2+}$  ions. This view finds support from the observation that an analogous behavior was shown in the presence of other divalent cations, such  $Ca^{2+}$  and  $Zn^{2+}$  ions. A possible role of scramblase has been hypothesized.

Finally,  $\tau_i$  has been measured in presence of alcohols to show that the herein proposed method can detect minor changes in RBC membranes' stiffness upon the incorporation of aliphatic alcohols.

**Keywords:** Red Blood Cells; water permeability; water exchange rate; Gd-complexes; Relaxometry

### **Abbreviations:**

RBCs: Red Blood cells

MRI: Magnetic Resonance Imaging

IR: Inversion Recovery

2SX: Two site exchange

EDTA: Ethylenediamine tetraacetic acid

PS: Phosphatidylserine

PBS: Phosphate Buffer Solution

BSA: Bovine Serum Albumin

## 1. Introduction

The measure of water exchange rate across Red Blood Cells (RBCs) membrane has received great attention since the seventies of the previous century [1]. Erythrocytes membrane permeability is considered a biomarker of different pathological states as it has been found altered in several hereditary hematological disorders, such as sickle cell anemia [2], hereditary spherocytosis, stomatocytosis, and xerocytosis [3], as well as in infectious diseases like malaria [4].

The method of choice to measure RBCs membrane permeability to water is based on the measurement of  $T_1$  or  $T_2$  of a RBCs suspension in the presence of paramagnetic Mn(II) ions in the suspending medium. In principle, the presence of Mn(II) ions can make the overall water proton relaxation curve resolvable into two components, namely, a fast component corresponding to water protons in the extracellular, Mn-doped, compartment and a slow component corresponding to the inner cell compartment whose relaxation is modulated by the rate of water exchange across the cellular membrane. The experimental work-up relies on the fitting of the experimental data to a theoretical bi-exponential curve calculated for the effect of two-site exchange.

The reported erythrocyte water exchange times ( $\tau_i$ ) ranged from 11 to 21.7 ms [5-7]. Although many causes may be invoked to account for such a large dispersion (effect of field strength, pulse technique, temperature, blood storage, etc.) it was early recognized that the concentration of  $Mn^{2+}$  strongly affect the water exchange time (the higher  $[Mn^{2+}]$ , the lower the resulting exchange time) [6]. The nature of the interactions that may occur between  $Mn^{2+}$  ions and the RBCs have not been elucidated although it was surmised that the role of manganese may be associated to the kind of aggregates (*Rouleaux* formation) which RBCs form in plasma. In spite of the lack of an improved protocol that overcomes the reported limitations there has been a general acceptance that the mean lifetime ( $\tau_i$ ) of water in fresh, normal erythrocytes fall between the extremes of 9.8 and 14 ms, at ambient temperature [6].

On this basis, we decided to re-investigate this matter in order to acquire a reliable value for this important parameter. Since Mn(II) ions, when added to the suspending medium, may have a

number of interactions with the biomolecules present in the solution as well as on the RBC surface, it was deemed useful to use paramagnetic agents characterized by having no ability of interaction with the surrounding molecules but water. For such purpose, suitable systems are represented by Gd-based contrast agents widely used in clinical settings as vascular, extracellular MRI contrast agents. Gd(III) ion has seven unpaired electrons and, in the considered MRI agents, is octacoordinated by the ligand's donor atoms leaving one coordination position to a water molecule that is in fast exchange with the bulk solvent water molecules [8]. The good ability to enhance the water proton relaxation rate is expressed by their relaxivity ( $4.4\text{-}4.7\text{ mM}^{-1}\text{s}^{-1}$  at 0.5T and 298K) *i.e.* about half of the relaxivity shown by  $\text{Mn}^{2+}$  ions ( $7.8\text{ mM}^{-1}\text{s}^{-1}$ ) [9].

## 2. Material and Methods

### 2.1 Chemicals

ProHance<sup>®</sup> was kindly provided by Bracco Imaging S.p.A (Colleretto Giacosa, Torino, Italy). Dotarem<sup>®</sup>, Magnevist<sup>®</sup> and Gadovist<sup>®</sup> were purchased from Guerbet, Schering and Bayer, respectively. Ficoll Hystopaque<sup>1039</sup>, heparin and all other chemicals were purchased from Sigma-Aldrich Co. LLC.

### 2.2 Isolation of Red Blood Cells

Red Blood Cells were separated from healthy human donors' blood (*provided by A.O.U. Città della Salute e della Scienza, Torino*) by using the Ficoll Hystopaque<sup>1039</sup> methodology. The cells' suspension diluted 1:1 with PBS was centrifuged for 30 minutes without brake at 1500 rpm and 25°C. Red Blood Cells were then separated from the other components, washed three times with PBS supplemented with heparin and centrifuged at 2300 rpm for 10 min, 4°C. The amount of Hb was evaluated by measuring the Absorbance in the SORET band region (413 nm) by using a 6715 UV-Vis Spectrophotometer Jenway (Bibby Scientific Limited, Beacon Road, Stone, Staffordshire, ST15 OSA, UK).

### 2.3 <sup>1</sup>H-relaxometric measurements

To 200  $\mu\text{L}$  of the RBCs suspension (hematocrit 50%), 100  $\mu\text{L}$  of the Gd-complexes solutions were added to reach the final Gd-concentrations of 10,11,12,13,14,15,16,17,18,19 and 20 mM (mmoles of Gd per L of extracellular volume).

Then 100  $\mu\text{L}$  of the suspensions of RBCs and Gd-complexes were transferred to 5 mm NMR tubes and  $R_1$  ( $1/T_1$ ) was measured at 0.5T and 25°C on a Stellar Spinmaster relaxometer (Stelar, Mede,

Pavia) by means of the inversion recovery (IR) pulse sequence (64 increasing delay times). Each time evolution of the magnetization in the IR experiment was analyzed on the basis of the two site exchange (2SX) model (See Appendix section).

#### ***2.4 Evaluation of RBCs water exchange rate in presence of metal ions***

In order to evaluate the change of water exchange rate through RBC membranes in presence of metal ions, RBC samples were incubated at 25°C in presence of 10mM MgCl<sub>2</sub>, CaCl<sub>2</sub>, ZnCl<sub>2</sub>, SrCl<sub>2</sub> and ProHance® 15mM. The relaxometric measurements have been carried out as described above immediately after the addition of the metal and for the following 24h.

Moreover, to assess whether the effect of the added metal ions was reversible or not, EDTA 10mM was added to the RBCs/ProHance® suspension and R<sub>1</sub> measured immediately after addition of EDTA.

#### ***2.5 Evaluation of Phosphatidylserine exposure on outer Red Blood cell membrane***

The exposure of Phosphatidylserine (PS) on the outer cell membrane was evaluated by using the commercial Muse™ Annexin V & Dead Cell Assay that utilizes Annexin V to detect PS on the external membrane of cells.

Briefly, RBCs were incubated in presence of 10mM of MgCl<sub>2</sub>, CaCl<sub>2</sub>, MnCl<sub>2</sub>, ZnCl<sub>2</sub> or SrCl<sub>2</sub> for 15 min, then washed and suspended in fresh PBS with 1% BSA at the concentration of 5x10<sup>6</sup> RBCs/mL. As control, untreated RBCs were used. Then, 100µL of RBCs suspension were added with 100 µL of Muse™ Annexin V Reagent. The samples were mixed thoroughly by pipetting up and down and then stained for 20 minutes at room temperature in the dark. After the treatment each sample was loaded onto Muse™ Cell Analyzer (Millipore, Merck KGaA, Darmstadt, Germany) and the amount of RBCs positive for PS was counted.

The relative exposure of PS was evaluated for samples incubated in the presence of divalent metal ions in respect to untreated RBCs. Each experiment was repeated in triplicate.

#### ***2.6 Evaluation of RBCs water exchange rate upon treatment with aliphatic alcohols***

RBCs were incubated at 25°C in presence of Methanol, Ethanol, and 1-Propanol at a concentration of 0.5M and ProHance® in the range 10-20mM. Then relaxometric measurements have been carried out as reported in section 2.3 immediately after the addition of the aliphatic alcohols and for the following 3h.

## 2.7 Statistical Analysis

Data are represented as mean $\pm$ SD. Statistical significance of results was evaluated by using a unpaired two-tails Student's t-test. Results were considered significant at 99% (\*\*) if  $P < 0.01$  and at 99.9% (\*\*\*) if  $P < 0.001$ .

## 3. Results and Discussion

By applying the same two-site exchange equations used in Mn(II) studies, the water exchange lifetime ( $\tau_i$ ) has been determined from the  $T_1$ -data measured, at 0.5T, from RBCs suspensions added of the paramagnetic Gd-complexes at 298K (hematocrit = 33%). The  $T_1$  data were collected over the 10-20 mM concentration range for four commercial Gd(III)-containing agents, namely ProHance<sup>®</sup> (Bracco Imaging), Dotarem<sup>®</sup> (Guerbet), Magnevist<sup>®</sup> (Schering), Gadovist<sup>®</sup> (Bayer HealthCare Pharmaceuticals) (Figure 1A). The time evolution of the magnetization in the Inversion Recovery (IR) experiment was analyzed on the basis of the two site exchange (2SX) model (see Appendix section) for each experimental data point.

Figure 1B reports the obtained  $\tau_i$  values as a function of the applied concentration of the paramagnetic complexes. The mean  $\tau_i$  values obtained by using the four Gd-complexes are not significantly different one from each other (Figure 1C) and, interestingly, the mean value (19.1 $\pm$ 0.65 ms) resulted to be markedly longer than the previously reported ones obtained using Mn<sup>2+</sup> ions as doping agent (ca. 10 ms). Actually the longer  $\tau_i$  is well consistent with the exchange lifetime values obtained with other methodologies, i.e. the scintillation counting of the self-exchange of radioactive <sup>3</sup>H<sub>2</sub>O ( $\tau_i$  = 18.3 ms) [10], or the spectrophotometric analysis of H<sub>2</sub>O/D<sub>2</sub>O suspended RBCs labelled with a fluorescent probe which emission is related to the H<sub>2</sub>O:D<sub>2</sub>O ratio ( $\tau_i$  = 23.5 ms) [11], or the measure of <sup>2</sup>H<sub>2</sub>O NMR resonance quadrupolar splitting ( $\tau_i$  = 20 ms) [12].

We hypothesized that the different values obtained by applying the methods based on the use of MnCl<sub>2</sub> (literature) or of Gd-containing agents (this work) may be related to the interactions Mn<sup>2+</sup> ions have with RBCs that, in turn, lead to enhanced membrane permeability. In order to get more insight into the understanding of an active role of metal ions in RBCs membrane permeability, the relaxation times of the RBCs/ProHance<sup>®</sup> (15 $\pm$ 1 mM) suspensions were measured in the presence of Ca<sup>2+</sup> and Zn<sup>2+</sup> ions, at 10 mM concentration (Figure 2A) as a function of time. The reported ordinate values of Fig. 2A were obtained by dividing the observed blood relaxation rate constants (subtracted of the neat unlabeled blood contribution) by the mmol(Prohance)<sup>®</sup>/L(extracellular

volume), i.e.  $\Delta R_1/[Gd]_{ex}$ . This representation of the data allows to get rid of inaccuracies in the individual sample's preparation. The relaxation rate constant of ProHance<sup>®</sup> in the RBCs suspension, before the addition of  $Ca^{2+}$  and  $Zn^{2+}$ , is lower than that measured in PBS ( $4.8 \text{ mM}^{-1}\text{s}^{-1}$ ) due to the “quenching” effect brought about by a limited exchange rate through RBCs membranes. Interestingly the presence of these divalent ions caused a marked  $R_1$  increase that was not instantaneous with the addition of  $CaCl_2$  and  $ZnCl_2$ , but required few hours to reach the maximum value. After this time,  $R_1$  remained constant over a period of 24h even upon addition of an equivalent of EDTA to sequester the free divalent metal ions. The effect of the addition of  $Mn^{2+}$  ions on the relaxivity was not directly investigated due to the paramagnetism of manganese ion which would interfere with the observed relaxation rate constant. Moreover, additional experiments showed that no effect on the relaxivity of ProHance<sup>®</sup> occurs in the presence of 10 mM  $CaCl_2$  and  $ZnCl_2$  in PBS in the absence of RBCs (data not shown). Fitting the single IR data points of figure 2A to the 2SX model equations allowed to determine the membrane exchange life times ( $\tau_i$ ) at each investigated time point. These values have been reported in Fig. 2B as a function of time. The  $R_1$  increase yields a corresponding decrease of  $\tau_i$  that, after 4 h from the administration of the salts, reaches the plateau value of 10 ms, i.e. in the range of the values previously reported for the Mn-based experiments (Figure 2B). This behavior was not observed upon the addition of  $MgCl_2$  or  $SrCl_2$  (Figure 2A and 2B). The time constants for the  $M^{2+}$  induced  $\tau_i$  decrease, extracted from fitting of data in Fig. 2B, were 4 min, 5min, 11h and 25 h upon the addition of  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Mg^{2+}$  and  $Sr^{2+}$ , respectively. Thus, it was supposed that the overall similarity of  $Ca^{2+}$ ,  $Zn^{2+}$  and  $Mn^{2+}$  may be considered responsible for the observed similar effects on the RBC's membrane permeability [13,14].

Tentatively, one may suggest that a decrease of  $\tau_i$  could be related to Plasma Membrane Phospholipid Scramblase activity, a  $Ca^{2+}$  depending enzyme for which an analogous effect from  $Mn^{2+}$  and  $Zn^{2+}$  ions was already reported [15]. Scramblase activates the flip-flop exchange of phospholipids in the cellular membrane and one may assume that such process induces an increase of their permeability [16]. In order to evaluate if divalent metal ions can trigger the membrane phospholipids flip-flop exchange, RBCs incubated in presence of  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$  and  $Sr^{2+}$  have been tested for the exposure of Phosphatidylserine (PS) on the outer cell membrane. The PS is known to be present in the inner layer of plasmatic membrane but not in the extracellular layer [17,18]. By using an annexin V-based colorimetric assay, the relative exposure of PS was evaluated for samples incubated in presence of divalent metal ions in respect to untreated RBCs (Figure 3). This value is almost nil in untreated RBCs and in cells treated with  $Sr^{2+}$  and  $Mg^{2+}$  while it increases

significantly upon the treatment with  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$ . The order of PS exposure ( $\text{Ca}^{2+} > \text{Mn}^{2+} > \text{Zn}^{2+} > \text{Mg}^{2+} > \text{Sr}^{2+}$ ) is the same reported for divalent metal ions Scramblase affinity [15] and reflects the one herein found for the effect of membrane's water exchange rate on the relaxation rate constant of ProHance<sup>®</sup>/RBCs suspensions.

The availability of a robust method to measure water exchange across the plasmalemma membrane of RBCs may open new directions in the use of this parameter as biomarker of diseased states.

We tested the method in a typical experiment of modification of RBCs membrane fluidity upon the addition of aliphatic alcohols. It is well established [19-22] that aliphatic alcohols can be incorporated in the cells' membrane yielding an increase of the stability of the phospholipid bilayer which is expected to lead to a concomitant decrease of the water exchange rate between the intracellular and extracellular compartments. In Figure 4 the calculated  $\tau_i$  values for RBC/ProHance<sup>®</sup> in the presence of alcohols such as Methanol, Ethanol, and 1-Propanol are reported. Whereas the addition of Methanol does not cause any significant change in  $\tau_i$ , Ethanol and 1-Propanol do it. This is consistent with the view that the degree of stiffness induced by the aliphatic alcohols depends on the length of the aliphatic chain (i.e.  $\tau_i$  is longer for 1-Propanol containing membranes in respect to the Ethanol and Methanol containing ones) [23].

In summary, the herein reported results show that the relaxometric method based on the use of highly stable Gd-containing agents is very robust and easy to implement. Moreover, the comparison with previously reported results calls for caution when referring to  $\tau_i$  values which have been determined by using  $\text{Mn}^{2+}$  ions for decreasing the relaxation times of the extracellular compartment. The observation that  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$  ions cause a marked enhancement of RBCs membrane permeability supports the view that  $\text{Mn}^{2+}$  could be responsible for modifications of the membrane that yielded shorter  $\tau_i$  values. The use of Gd(III)-based paramagnetic complexes is a good solution to remove this drawback.

#### 4. Appendix

The time evolution of the magnetization in the IR experiments was analyzed on the basis of the two site exchange (2SX) model [24-26].

In a two compartment system, the observed water proton relaxation rate constant is determined by the relaxivity of each compartment and the water exchange rate between the compartments. The intracellular ( $R_1^{\text{in}}$ ) and extracellular ( $R_1^{\text{ex}}$ ) relaxation rate constants can be written as:



$$R_1^{in} = \frac{1}{2}(R_{1ex}^0 + r_{1ex} \times [Gd]_{ex} + R_{1in}^0 + k_{in} + k_{ex}) + \frac{1}{2}\{[(R_{1ex}^0 + r_{1ex} \times [Gd]_{ex}) - R_{1in}^0 + k_{ex} - k_{in}]^2 + 4k_{in}k_{ex}\}^{1/2} \quad (A.1)$$

$$R_1^{ex} = \frac{1}{2}(R_{1ex}^0 + r_{1ex} \times [Gd]_{ex} + R_{1in}^0 + k_{in} + k_{ex}) - \frac{1}{2}\{[(R_{1ex}^0 + r_{1ex} \times [Gd]_{ex}) - R_{1in}^0 + k_{ex} - k_{in}]^2 + 4k_{in}k_{ex}\}^{1/2} \quad (A.2)$$

Where  $R_{1ex}^0$  and  $R_{1in}^0$  are the intrinsic relaxation rate constants of extra-cellular and intra-cellular compartments in the absence of the Gd-complex and in the absence of exchange,  $r_{1ex}$  and  $[Gd]_{ex}$  are, respectively, the millimolar relaxivity and the concentration of the Gd-complex in the extracellular compartment, and  $k_{ex}$  ( $1/\tau_{ex}$ ) and  $k_{in}$  ( $1/\tau_i$ ) are the water exchange rates constants from intra- to extra- and from extra- to intra-cellular compartments, respectively.

The time evolution of  $M_z$  in the IR experiment is given by:

$$M_{z(t)} = M_0 \left\{ 1 - 2 \left[ (1 - a_{in})e^{(-tR_1^{ex})} + a_{in}e^{(-tR_1^{in})} \right] \right\} \quad (A.3)$$

Where  $M_0$  is the equilibrium magnetization and  $a_{in}$  is given by:

$$a_{in} = \frac{1}{2} - \frac{1}{2} \left( \frac{(R_{1ex}^0 + r_{1ex} \times [Gd]_{ex}) - R_{1in}^0}{\{[(R_{1ex}^0 + r_{1ex} \times [Gd]_{ex}) - R_{1in}^0 + k_{ex} - k_{in}]^2 + 4k_{in}k_{ex}\}^{1/2}} (2v_{in} - 1) + k_{in} + k_{ex} \right) \quad (A.4)$$

The equilibrium mass action relationships (A.5) and (A.6) have to be taken into account.

$$v_{in} \times k_{in} = v_{ex} \times k_{ex} \quad (A.5)$$

$$v_{in} + v_{ex} = 1 \quad (A.6)$$

Where  $v_{in}$  and  $v_{ex}$  are the intra-cellular and extracellular volume fractions.

The time evolution of  $M_z$  in the IR experiment (two explicative cases are reported in Figure 5) of each RBCs/Gd-complex suspension was fitted to extract  $k_{in}$  ( $1/\tau_i$ ) by using equations A.1-A.6 and introducing the extracellular and intracellular intrinsic relaxation rate constants ( $R_{1ex}^0 = 0.5 \text{ s}^{-1}$  and  $R_{1in}^0 = 2.0 \text{ s}^{-1}$ ), the total Gd concentration, the intracellular volume fraction ( $v_{in} = 0.26$ ) and the relaxivity of the Gd-complexes ( $r_{1ex} = 4.7 \text{ mM}^{-1}\text{s}^{-1}$  for Dotarem<sup>®</sup>, Magnevist<sup>®</sup> and Gadovist<sup>®</sup> and  $4.8 \text{ mM}^{-1}\text{s}^{-1}$  for ProHance<sup>®</sup>) as fixed parameters.

The intracellular volume fraction ( $v_{in}$ ) has been calculated by multiplying the number of RBCs contained in 200  $\mu\text{l}$  of RBCs suspension (ca.  $1 \times 10^9$ ) for the volume of each RBC ( $9.8 \times 10^{-14} \text{ l}$ ) referred to the total suspension volume; the value has been corrected to account for the fact that

only 70% of the intracellular volume is accessible to water molecules, as the remaining 30% is occupied by cells membranes [27].

The relaxation rate constant of intra-cellular compartment ( $R_{1in}^0$ ) has been determined by measuring the longitudinal relaxation rate of water protons of a RBCs suspension added of the same volume of PBS as that used in the experiments with the Gd-complexes. In the absence of any paramagnetic complex, the fast exchange limit (FXL) condition occurs and the observed relaxation rate constant is given by:

$$R_1 = R_{1ex}^0 v_{ex} + R_{1in}^0 v_{in} \quad (\text{A.7})$$

Fixing  $R_{1ex}^0$  to  $0.5 \text{ s}^{-1}$  and knowing  $v_{in}$  and  $v_{ex}$ ,  $R_{1in}^0$  has been calculated to be  $2.0 \text{ s}^{-1}$ . This value is consistent with the expected value for intracellular Hemoglobin concentrations of 5-6 mM.

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## Figure Legends

**Figure 1:** A) Structures of the four Gd-based MRI contrast agents; B) Membrane exchange life times ( $\tau_i$ ) of RBCs calculated by the addition of four Gd-based MRI CAs as a function of their concentration in the external environment of RBCs; C) mean  $\tau_i \pm \text{SD}$  values determined with the four CAs.

**Figure 2:** A) Time evolution of the change in  $\Delta R_1/[\text{Gd}]_{ex}$  in a suspension of RBCs containing ProHance® ( $15 \pm 1 \text{ mM}$ ) upon the addition of  $\text{CaCl}_2$ ,  $\text{ZnCl}_2$ ,  $\text{SrCl}_2$  and  $\text{MgCl}_2$  ( $10 \text{ mM}$ ); B) Time dependence of the membrane exchange life times ( $\tau_i$ ) determined by fitting the single IR data points of figure 2A to the 2SX model equations upon addition of  $\text{CaCl}_2$ ,  $\text{ZnCl}_2$ ,  $\text{SrCl}_2$  and  $\text{MgCl}_2$ .

**Figure 3:** Relative exposure of PS for samples incubated in presence of divalent metal ions in respect to untreated RBCs evaluated with the Muse™ Annexin V & Dead Cell Assay. Each experiment was repeated in triplicate.

**Figure 4:** Alcohols (Methanol, Ethanol and 1-Propanol) effect on the mean  $\tau_i \pm \text{SD}$  values determined by adding ProHance® to RBCs.

**Figure 5:** Explicative Time evolutions of the Magnetization in the Inversion recovery measure of the observed relaxation rate constants of a suspension of RBCs added with ProHance® 10 mM (●) and ProHance® 10 mM in the presence of 1-Propanol 0.5M (○). Each data set was fitted with equations A1-A6 of the appendix ( $R^2=0.9999$  (●) and  $R^2=0.9996$  (○)).

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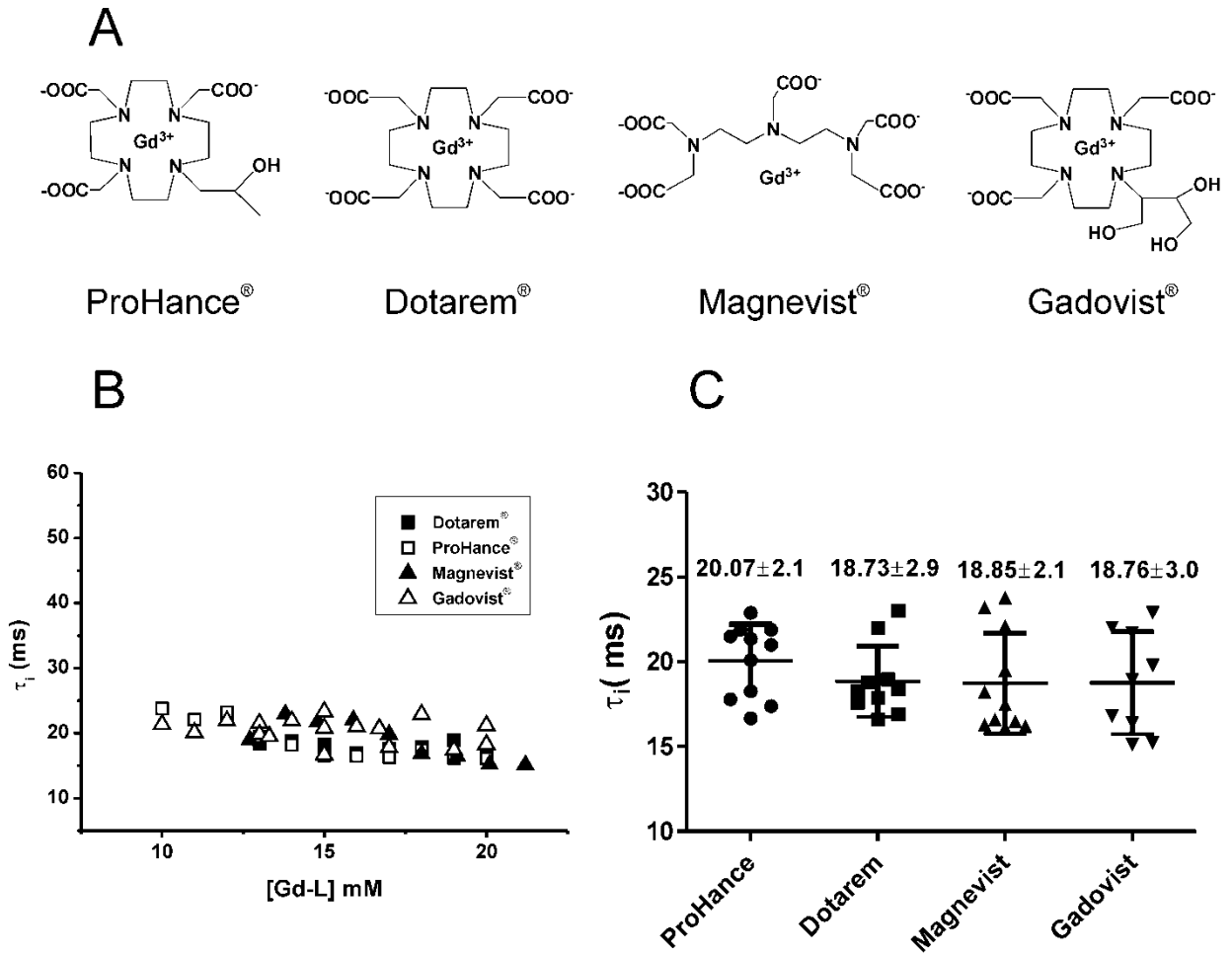


Figure 1

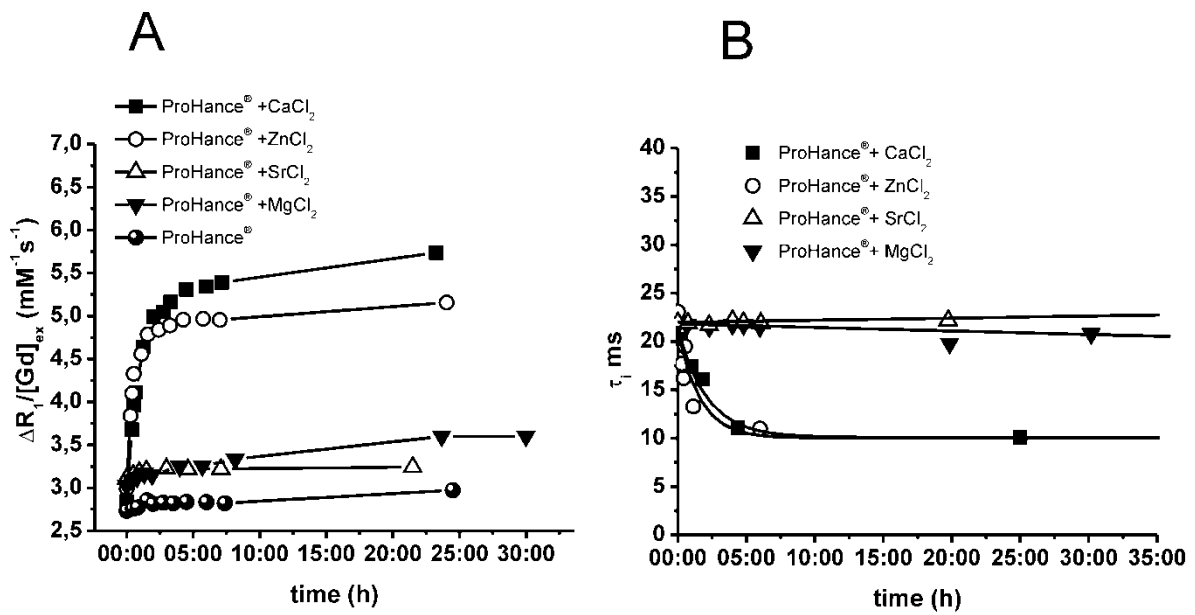


Figure 2

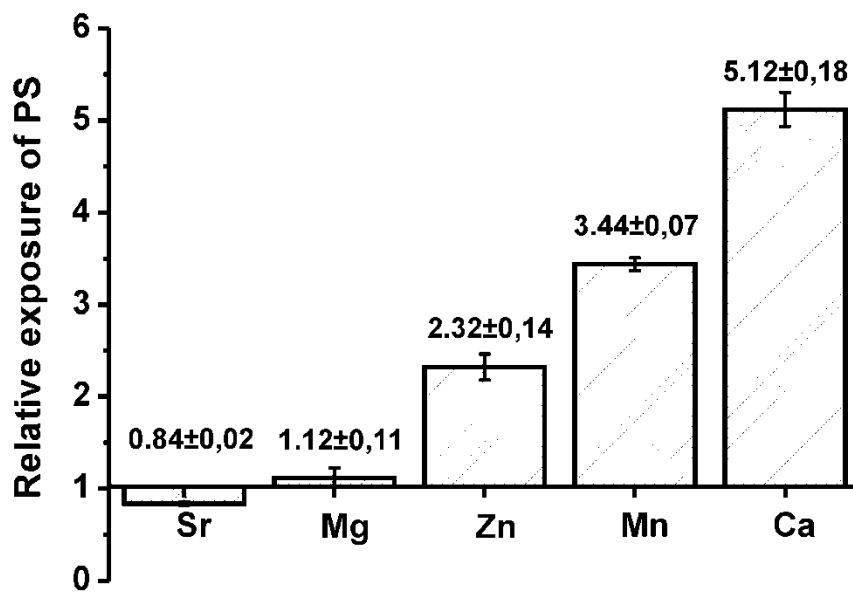


Figure 3

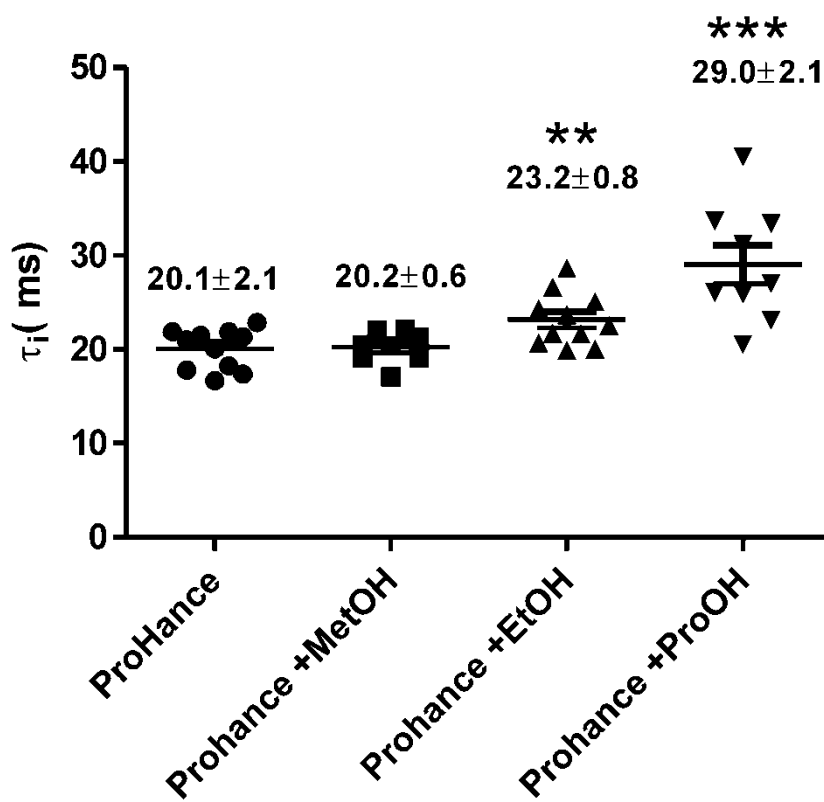


Figure 4

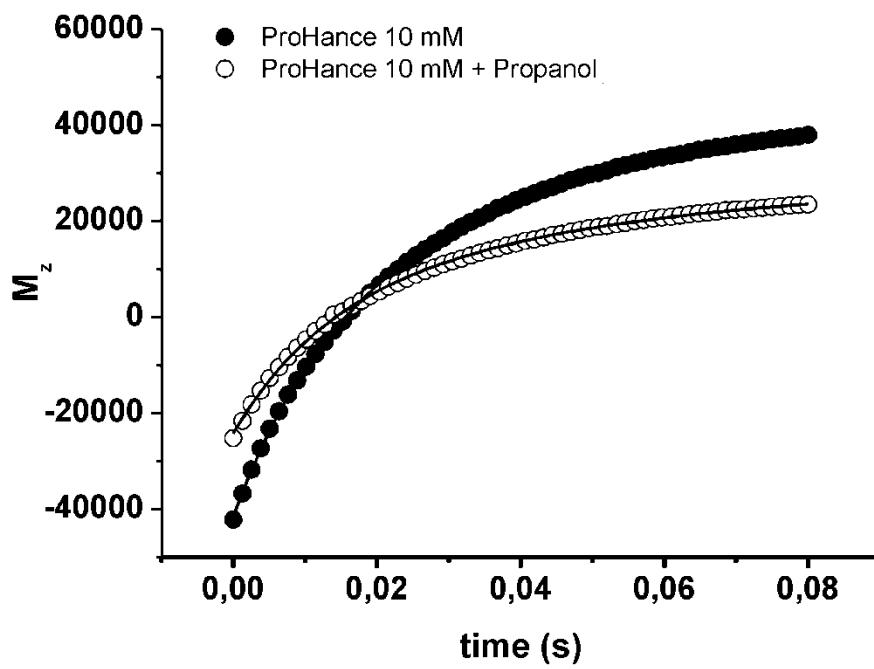


Figure 5