Magnetic Resonance Materials in Physics, Biology and Medicine (2022) 35:683–694
https://doi.org/10.1007/s10334-021-00983-4

RESEARCH ARTICLE

# Metabolite interactions in prostatic fluid mimics assessed by $^{1}$H NMR

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Received: 1 August 2021 / Revised: 1 December 2021 / Accepted: 3 December 2021 / Published online: 17 December 2021


## Abstract

**Introduction** Molecular interactions in prostatic fluid are of biological interest and may affect MRI and MRS of the prostate. We investigated the existence of interactions between the major components of this fluid: spermine, citrate and myoinositol, metal ions, including zinc, and proteins.

**Materials and methods** Solutions of 90 mM citrate, 18 mM spermine and 6 mM myo-inositol, mimicking expressed prostatic fluid, were investigated by 1H NMR using changes in T2 relaxation and chemical shift as markers for interactions.

**Results and discussion** Adding to this metabolite mixture the ions $Na^{+}$ , $K^{+}$, $Ca^{++}$, $Mg^{++}$ and $Zn^{++}$, decreased the T2 relaxation times of citrate and spermine protons by factors of 3 and 2, respectively, with Zn++ causing the largest effect, indicating ion–metabolite interactions. The T2 of 18 mM spermine dropped by a factor of 2 upon addition with 90 mM citrate, but no effect on T2 was seen with myo-inositol pointing to a specific citrate-spermine interaction. Moreover, the T2 of citrate in the presence of spermine decreased by adding metal ions and increasing amounts of $Zn^{++}$, indicating complexation of citrate and spermine with metal ions, particularly with Zn. The addition of bovine serum albumin (BSA), as an index protein, substantially further decreased the T2 of spermine and citrate implying the formation of a transient spermine-metal ion-citrate-BSA complex.

Finally, we found that the T2 of citrate in extracellular fluid of prostate cancer cells, as a mimic of fluid in cancerous prostates, decreased by adding fetal calf serum, indicating protein binding.

**Keywords** Prostatic fluid · Prostate cancer · Metabolites · Metal ions · Bovine serum albumin (BSA)

## Introduction

In vivo MR spectroscopy (MRS) of the prostate is used to report on metabolites dominant in the intracellular space, e.g. choline and creatine, or in the luminal space, e.g. citrate and spermine [1, 2]. Signals of these metabolites are often employed in the localization of cancer tissue in the prostate and to assess its aggressiveness [1–6]. About 70% of prostate cancers (PCa) arise in the peripheral zone (PZ) [7], in which epithelial cells reside that contribute to prostatic fluid secretions. This is of interest as the composition of this fluid changes with the prostate tissue state, as was shown by NMR for the concentrations of the main fluid metabolites: citrate, myo-inositol and spermine [8]. The authors of aforementioned paper also found a correlation between the secretion of citrate and spermine and speculated that these compounds may occur as a complex. The measurement of citrate concentrations in prostatic fluid has been shown to outperform the prostate-specific antigen (PSA) test for detecting PCa [9], especially in the differentiation from benign prostatic hyperplasia [10]. Next to that of citrate, also the prostatic fluid levels of myo-inositol and spermine are decreased in the presence of PCa [8, 11, 12].

The divalent metal ions $Ca^{++}$, $Mg^{++}$ and $Zn^{++}$, which are present in prostatic fluid [13], and pH have been shown to affect the proton NMR signals of citrate [14, 15]. In particular, the effect of zinc on the citrate chemical-shift and J-coupling may be relevant as its concentration (on average about 7–9 mM in prostatic fluid of healthy volunteers [16]) is 90–95% lower in both prostatic tissue and fluid of PCa patients [17, 18]. A colorimetry test for prostate cancer

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based on zinc levels in expressed prostatic fluid has been proposed [19]. The $^{1}$H NMR properties of spermine are influenced by proteins, divalent cations, temperature and pH [20]. Free proteins are another group of molecules occurring in prostate-associated fluids of which some major ones are: PSA, prostatic acid phosphatase, PSP-94 and serum albumin [21–23]. Proteomic studies indicate that in prostatic fluid and cells the protein content changes in composition upon malignant transformation [24–28].

These in vitro studies essentially only addressed the interactions of single compounds in isolation, while in reality prostatic fluid is composed of a mixture of compounds. Therefore, in this study, we examined how the $^{1}$H NMR signals of citrate, spermine and myo-inositol, are influenced by metal ions, in particular zinc, and proteins in solution mixtures of these compounds mimicking the composition of prostatic fluid. We added bovine serum albumine (BSA) to mimic its protein content. In addition, we studied the effect of Zn and proteins on the $^{1}$H signals of citrate in extracellular fluids of prostate cancer cells, in which citrate, spermine and proteins occur at much lower levels than in prostatic fluid of healthy volunteers, and which is more representative of the prostatic fluid composition in PCa [12].

The specific aim of this study was to identify the presence of molecular complexes in the prostatic fluid mimics, firstly employing changes in proton *T*2 relaxation of the metabolites, but also of chemical shift and J-coupling of their $^{1}$H signals. As changes in NMR parameters of metabolite signals may be associated with conditions of the prostate, in particular PCa, the interactions identified in this study may contribute to the interpretation of MRS data employed in prostate diagnostics. Alternatively, as signal shape and integral in MR spectra depend on the echo time of the pulse sequence, a change in NMR properties due to tissue conditions of the prostate may require modifications in the design and timing of pulse sequences for optimal assessments.

## Methods

### Prostate fluid mimic sample preparation

All products were obtained from Sigma-Aldrich. Stock solutions were prepared of spermine tetrahydrochloride ($C_{10}H_{26}N_4$, 4HCl), myo-inositol ($C_6H_{12}O_6$), tri-sodium citrate dihydrate ($HOC(COONa)(CH_2COONa)_2$, $2H_2O$), $ZnCl_2$, $CaCl_2$, $2H_2O$, $MgCl_2$ and KCl, dissolved in MiliQ water and stored at 4 °C. A stock solution of bovine serum albumin (BSA) was prepared in phosphate-buffered saline (PBS, ~0.8% NaCl, salt concentration 154 mM, pH of 7.4), filtered using Millex® GP 0.22 µm, with a final concentration of 100 g/L BSA as measured with a Cary300 spectrophotometer using an absorbance of 6.5 for 1% solution of protein at 280 nm.

Spermine, myo-inositol, citrate, $CaCl_2$, $MgCl_2$ and KCl had final concentrations in the NMR solution of 18, 6, 90, 18, 15, 61 mM, respectively, according to the average composition of expressed human prostatic fluid as reported in [13]. $ZnCl_2$ was used at concentrations of 4 or 9 mM and BSA at concentrations of 5–50 g/L. Samples contained 10% $D_2O$ as NMR lock signal and no additional PBS was used to avoid precipitations. The pH of the samples was set to $7.1 \pm 0.05$ using NaOH and HCl solutions and monitoring the pH until the desired pH was reached. All samples were measured after 12 h to allow the solution to equilibrate.

### Cell assay and medium preparation

The metastatic cell line LNCaP (ATCC CRL-1740), derived from a human metastasis of prostate adenocarcinoma, was a gift from Prof. Gary J. Miller from the University of Colorado Health Sciences Center, Denver, CO, USA. To produce extracellular fluid for the CPMG *T*2 measurements ~ $30 \times 10^6$ LNCaP cells were used. Six flasks of LNCaP were grown to full confluency in full medium (RPMI-1640 + 2 mM Gln + S/P + 10% fetal calf serum (fcs)), which contains 3–4 g/L BSA and zinc in trace amounts [29, 30]. and subsequently incubated in RPMI-1640 + 2 mM Gln + S/P without serum. For three flasks per cell line, the medium was also supplemented with 50 µM $Zn^{2+}$, added from a $ZnCl_2$ solution. After 48 h the cells were counted and then the media was collected and immediately placed on ice. Detached cells possibly present in the media were removed by centrifugation (3750 rpm, 2 °C, 10 min). To aliquots of all media samples 10% D2O was added and either 10% fetal calf serum, or the same volume of PBS buffer (creating samples without BSA). The pH was adjusted to pH 7.05.

### NMR Spectroscopy

NMR examinations were performed of samples in 5 mm NMR tubes on a Bruker Avance III spectrometer operating at 500 MHz and equipped with a Bruker smart probe BBO 500 MHz.

Prostatic fluid mimic and cell media samples of 600 µL were measured by 1D $^{1}$H NMR at 298 K using a simple pulse acquire sequence with a 30° excitation pulse (BW = 6 kHz, AQ = 3 s, dw = 83.20 µs, TR = 6 s, resolution = 0.33 Hz/point). Pulse calibrations were done for each sample to assure proper quantification and the gain value was fixed to assure comparison between spectra. Temperature was kept constant at 298 $^{0}$ K.

The 1D $^{1}$H NMR spectra were processed using Bruker Topspin 1.2 and ACDlabs 12.0 including removal of the $H_2O$ peak. Processing involved apodization, phasing,

baseline correction (the baseline was constructed by taking the first 16 points of the FID.), peak picking and integration of the peak-area between two chemical shift positions in the nearest noise symmetric at each side of the peak.

For the *T*2 measurements, a CPMG sequence was used starting with two water signal saturation pulses of 3 s each followed by a 90° excitation pulse. Thirteen or fourteen CPMG times were used between 1 and 2048 ms, the repetition time was 8 s and the used echo spacing was 0.5 ms for the prostate fluid mimic or 1 ms for the extracellular fluid. To obtain *T*2 values the signal intensity decays as a function of time were fitted, using a mono-exponential equation, ($S = S_0 \exp\{^{-t}/_{T2}\}$), in Matlab (Math Works).

In the case of prostate fluid mimics, the signal intensities (peak areas) were determined as described above for 1D NMR spectra. Because of the lower signal intensity of citrate in the extracellular media compared to the prostatic mimic fluid, and the presence of some other metabolites in the spectral region of citrate, we fitted the four peaks of the citrate quartet in the extracellular media separately for all separate spectra at the different echo times using *j*MRUI AMARES [31, 32]. The mean chemical shift (±0.01 ppm soft constraints) and linewidth (±0.3 Hz soft constraints) derived from the first three echo times were used as prior knowledge in AMARES and Gaussian lineshapes were assumed. *T*2 values were compared using independent two-sample *t* tests to determine if the addition of zinc and addition of fetal calf serum to the media influenced the *T*2 relaxation rates of the citrate protons.

## Results

To study metabolite interactions in prostatic fluid mimics, different solution compositions were made by mixing the metabolites citrate, spermine and myoinositol with (a) inorganic ions, commonly found in prostatic fluids, (b) zinc at different concentrations, as variations in this concentration are known to play an important role in the functioning of the prostate and development of PCa [19, 33, 34], and (c) BSA, as an index protein, to determine the effect of unspecific binding of metabolites to prostatic fluid proteins.

The $^{1}$H NMR spectra of the prostatic fluid mimic showed well-resolved signals for the selected metabolites (Fig. 1). The chemical shifts of the spermine proton signals were confirmed using 2D $^{1}$H/$^{13}$C NMR of a sample at pH 9.4 for an optimal separation of spermine signals [20, 35]. (Fig. 1,. Supplementary materials, Figs. S1-S4).

To quantify the effect of molecular interactions on the signals of different metabolites in these solutions we measured their proton *T*2, peak area, peak height, chemical shift and J-coupling. Though all these parameters can be used to identify interactions, the *T*2 relaxation rates are most valuable because they are most sensitive to binding [36].

In these investigations, we analyzed the spermine multiplet at 1.77 ppm (spm5) and the myo-inositol multiplet at 4.05 ppm (myo5), as their *T*2s appeared to be most sensitive to interactions, their resonances are well resolved and their scalar couplings are small. For *T*2 assessments of citrate protons, we selected its downfield doublet signals (cit_down).

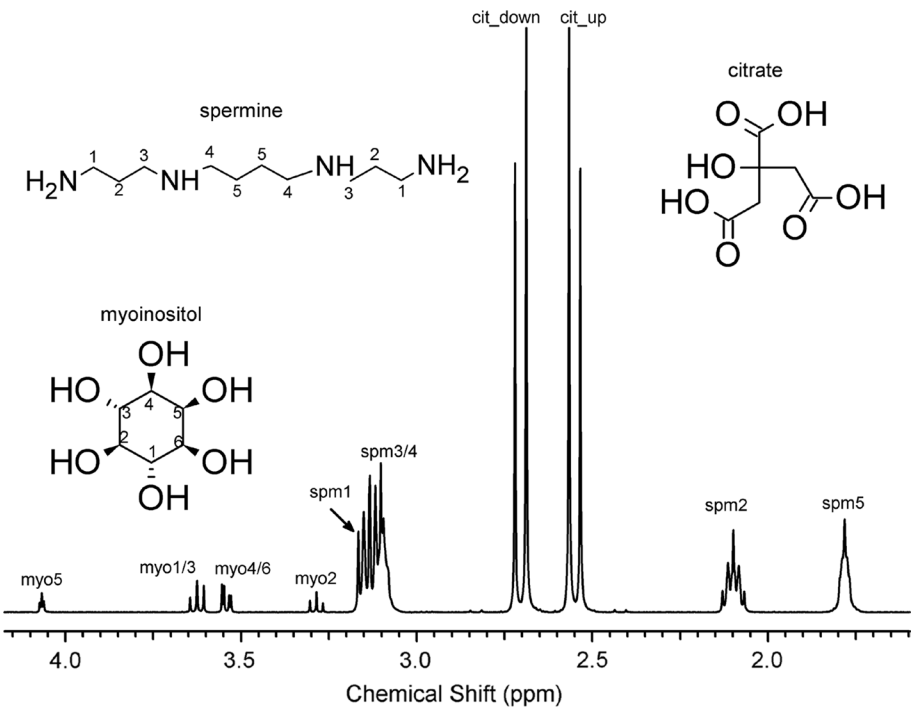


**Fig. 1** 1D $^{1}$H NMR spectrum obtained at 500 MHz of the prostatic fluid mimic at 298 K. No BSA or Zn was added. The labeling of the protons on the molecules correspond to the numbers at the end of the signal abbreviations—cit: citrate; myo: myoinositol; spm: spermine. Citrate doublets are labeled cit_down and cit_up to represent the doublets resonating downfield and upfield, respectively

Although the decay of both doublets could be fitted to a mono-exponential equation under all experimental conditions (supplemental material), the upfield signals of citrate at BSA concentrations ≥ 15 g/L showed a different intensity or phase at long echo times (Fig. 2). All the selected signals showed a clear mono-exponential decay as a function of the CPMG echo time and no indications for additional relaxation effects were observed (see supplementary info Fig. S7). Under all conditions, the *T*2 value of the upfield and downfield signal of citrate were similar (i.e. ≤ 5% difference).

## T2 relaxation assessments: interactions of metabolites with cations and BSA

First, we investigated interactions of the individual metabolites citrate, myo-inositol and spermine, at constant concentration in separate solutions, with metallic ions (a mixture of $Ca^{++}$, $Mg^{++}$, $K^{+}$ and $Na^{+}$), and with Zn (Fig. 3a). The *T*2 of citrate protons decreases as ions are added and decreases further at higher zinc concentrations. The *T*2 of spermine protons stays relatively constant at ~ 0.66 s when ions are supplied to the solution, but drops by a factor of 2 after 9 mM of zinc is added. Remarkably, the *T*2 of myo-inositol protons increased with the addition of ions, but decreased as the concentration of zinc increases. In agreement with this observation, the myo-inositol signal in the absence of metal ions was found to be broad, a phenomenon that we will explain in the discussion section.

Adding BSA to a concentration of 15 g/L, which is the average total protein content reported for prostatic fluid of non-cancerous prostates [37], to the metabolite solutions without ions, substantially decreased the *T*2 values of citrate and spermine protons. The *T*2 of myo-inositol protons was not much different (Table 1). Adding ions and Zn to these solutions had little effect on *T*2, but for citrate protons *T*2 dropped from 0.18 to 0.11 s (Fig. 3c).

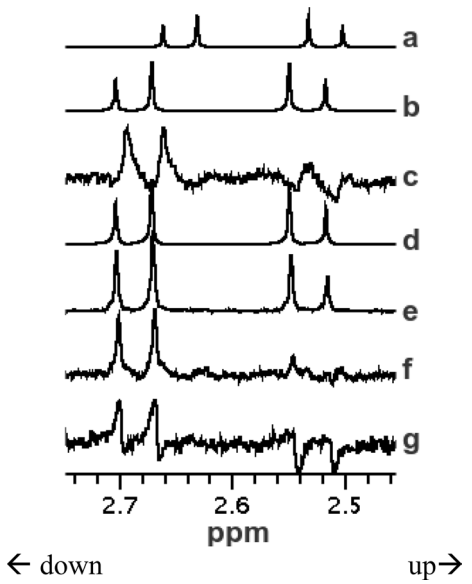


**Fig. 2** Part of the $^{1}$H NMR spectra of prostate fluid mimic showing only the citrate signals. The spectra were obtained with a CPMG sequence with an echo time of 0.63 s (echo spacing 0.5 ms). The spectra are from samples with the following composition (**a**) mixture of metabolites citrate (90 mM), myo-inositol (11 mM) and spermine (18 mM) (met_mix); **b** mix_met and all metallic ions with Zn at 9 mM; **c** met_mix and all metallic ions with Zn at 4 mM and 50 g/L BSA; **d** met_mix and all metallic ions with Zn at 9 mM and 5 g/L BSA (fourfold zoomed in in vertical direction); **e** met_mix and all metallic ions with Zn at 9 mM and 15 g/L BSA (twofold zoomed in in vertical direction); **f** met_mix and all metallic ions with Zn at 9 mM and 30 g/L BSA; **g** met_mix and all metallic ions with Zn at 9 mM and 50 g/L BSA (twofold zoomed in vertical direction). Metallic ions are: $Ca^{++}$, $Mg^{++}$, $K^{+}$, $Na^{+}$ and $Zn^{++}$. Myo5 was used as chemical shift reference at 4.05 ppm. A representative spectrum of citrate in extracellular fluid of LNCaP cells is shown in figure S5

## T2 relaxation assessments: interactions between metabolites with and without cations and BSA

To assess possible interactions among metabolites we mixed spermine, myo-inositol and citrate in solution (Met_mix). With respect to the proton *T*2 of the individual components this did not affect the *T*2 of citrate protons, but that of spermine decreased (from 0.66 to 0.37 s) and that of myo-inositol increased (Fig. 3b). Upon addition of ions to this mixture, the *T*2 of citrate protons decreased and then upon adding 9 mM Zn the *T*2 of the protons of all three compounds decreased, to similar values as for myo-inositol and citrate individually and for spermine somewhat lower than for this component individually (Fig. 3b; Table 1).

Then we investigated which bi-molecular interactions were responsible for these effects. In a combination of only citrate and spermine without ions the *T*2 of spermine protons decreased to the same extent as adding all three metabolites together (from 0.66 to 0.37 s), but this decrease is not observed in the combination of spermine with myo-inositol, without citrate (Table 1), indicating that citrate interacts with spermine. The *T*2 of citrate is, however, not affected by this interaction. An interaction with the citrate counterion sodium is unlikely as adding other cations had no effect on the proton *T*2 of an individual solution of spermine (Fig. 3a). Myo-inositol addition did not affect the *T*2 of spermine and citrate protons. However, the myo-inositol *T*2 value was slightly lower in the presence of citrate than spermine (0.83 vs. 1.08 s) (Table 1).

Adding 15 g/L BSA to the mixture of metabolites in the absence of ions, increased the *T*2 of myo-inositol somewhat (Fig. 3d). The *T*2 of spermine in the Met_mix solution with 15 g/L BSA was slightly lower (~ 0.16 s) than when not mixed with other metabolites (~ 0.26 s).

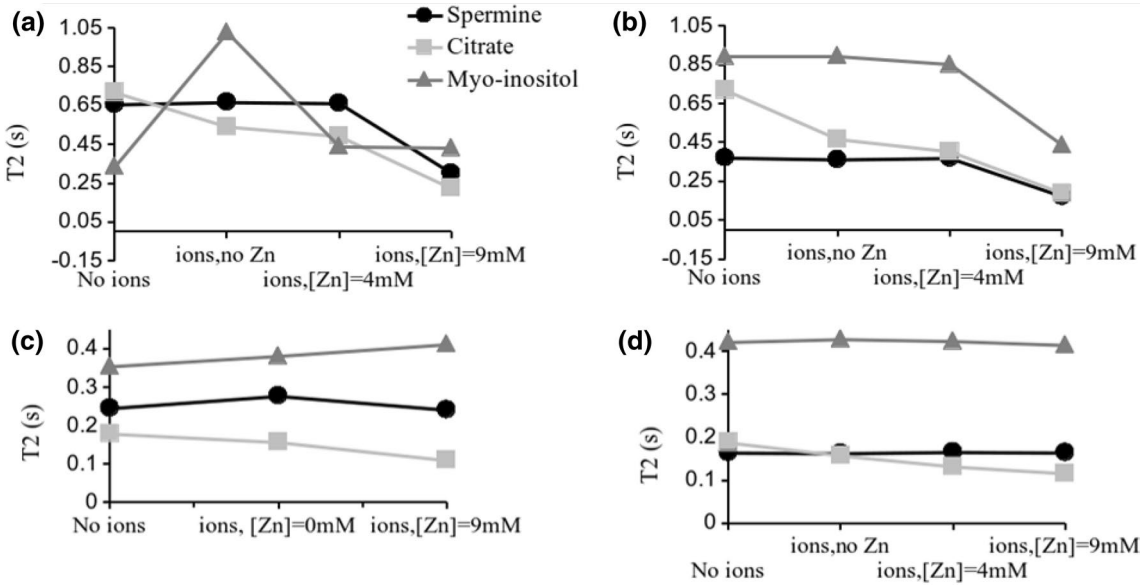


**Fig. 3** *T*2 relaxation times of protons of citrate (cit_up), myo-inositol (myo5) and spermine (spm5). These proton relaxation times were measured individually (**a**, **c**) or mixed (**b**, **d**). **c**, **d** present *T*2 values, obtained in the presence of 15 g/L BSA. The x-axis represents the presence of metal ions (calcium, magnesium, potassium, sodium) and zinc. The zinc concentration was varied. Note that all metal cations were dissolved with $Cl^-$ as counterion

Elevating the BSA concentration from 5 to 50 g/L decreased the *T*2s of myo-inositol and spermine only slightly with little further effect of Zn (Fig. 4a, c). In contrast we observed a strong decrease of the *T*2 of citrate (Fig. 4b). Its *T*2 drops by nearly a factor of 2 when the BSA concentration was elevated from 5 to 50 g/L, which further decreased by a similar factor upon adding 9 mM Zn (Fig. 4b; Table 1). These *T*2 changes concern the protons of the downfield doublet of citrate. Similar changes were found for the upfield doublet, but for all BSA solutions the *T*2 of these protons was 5% lower than of the downfield protons. Most remarkably, for the protons of the upfield doublet a unique signal modulation is observed with increasing BSA levels, but only at long CPMG echo times above 500 ms (Fig. 2).

## T2 relaxation of citrate protons in extracellular fluid

Finally, we studied the effect of adding Zn and proteins on the *T*2 of citrate in extracellular fluid of prostate cancer cells grown in RPMI-1640 medium. We calculated that the citrate concentration in this fluid was about 0.65 ± 0.11 mM ($N = 3$), which is representative of prostatic fluid in very high-risk prostate cancer [12, 38]. In the absence of additions, the average *T*2 of citrate protons in extracellular fluid was found to be 420 ± 17 ms for cit_down and 454 ± 25 ms for cit_up, which is comparable to that of citrate in the prostatic mimic fluid with ions and 4 mM Zn. The addition of 50 µM Zn to this fluid had no effect on the *T*2 of citrate (cit_down 421 ± 25 ms and cit_up 464 ± 28 ms) (Fig. 5). However, adding 10% fetal calf serum decreased the average *T*2 significantly to about 200 ± 10 ms/227 ± 12 ms (with Zn/without Zn) for cit_down, or 262 ± 13 ms/274 ± 14 ms (with Zn/without Zn) for cit_up (Fig. 5).

## Peak height and integral

To evaluate changes in peak height and integral we normalized them relative to the metabolite peaks from the mixture without metal ions and BSA. The citrate peak height was the most affected by additions to the fluid mimic, it lowered to ~ 62% in the presence of metal ions. A further decrease down to 30% was observed when mixed with both metal ions and 50 g/L BSA (Table 2). Spermine and myo-inositol showed a decrease in peak height to 79% and 54% respectively, in the presence of metal ions with 4 mM zinc and 50 g/L BSA. As normalized signal integrals of the metabolites did not change for different sample compositions (Supplement, Table S1) it follows that the peak height changes arise from changes in peak width and thus in *T*2* relaxation times.

## Chemical shift and J-coupling

The presence of BSA in the mixtures precluded the use of TSP as an internal reference, as it binds to BSA [39]. As myo-inositol showed the most stable *T*2 relaxation time in Met_mix solutions for all preparations, it apparently interacts least with other components. Thus, we used the

**Table 1** *T*2 values of protons of spermine (spm5), citrate (cit_down) and myo-inositol (myo5) in different sample compositions

| | Metal ions | BSA (g/L) | Metabolite *T*2 (s) | | |
|---|---|---|---|---|---|
| | | | Spm5 | Cit_down | Myo5 |
| Spermine, citrate and myo-inositol (not mixed) | No ions | 0 | 0.657 | 0.719 | 0.338 |
| | ions,no Zn | | 0.669 | 0.543 | 1.029 |
| | ions,[Zn] = 4 mM | | 0.663 | 0.493 | 0.440 |
| | ions,[Zn] = 9 mM | | 0.303 | 0.224 | 0.434 |
| | No ions | 15 | 0.246 | 0.179 | 0.355 |
| | ions,no Zn | | 0.278 | 0.157 | 0.382 |
| | ions,[Zn] = 9 mM | | 0.241 | 0.110 | 0.412 |
| Spermine + citrate | No ions | 0 | 0.372 | 0.720 | |
| Spermine + m-inositol | | | 0.661 | | 1.079 |
| Citrate + m-inositol | | | | 0.823 | 0.828 |
| Spermine, citrate and myo-inositol (mixed) | No ions | 0 | 0.372 | 0.721 | 0.894 |
| | ions,no Zn | | 0.363 | 0.468 | 0.897 |
| | ions,[Zn] = 4 mM | | 0.370 | 0.404 | 0.854 |
| | ions,[Zn] = 9 mM | | 0.173 | 0.190 | 0.437 |
| | No ions | 15 | 0.165 | 0.188 | 0.422 |
| | ions,no Zn | 5 | 0.167 | 0.187 | 0.434 |
| | | 15 | 0.163 | 0.158 | 0.429 |
| | | 30 | 0.156 | 0.134 | 0.440 |
| | | 50 | 0.148 | 0.114 | 0.417 |
| | ions,[Zn] = 4 mM | 5 | 0.172 | 0.166 | 0.417 |
| | | 15 | 0.166 | 0.132 | 0.424 |
| | | 30 | 0.160 | 0.104 | 0.437 |
| | | 50 | 0.152 | 0.082 | 0.423 |
| | ions,[Zn] = 9 mM | 5 | 0.166 | 0.156 | 0.434 |
| | | 15 | 0.163 | 0.121 | 0.415 |
| | | 30 | 0.158 | 0.093 | 0.417 |
| | | 50 | 0.153 | 0.069 | 0.416 |

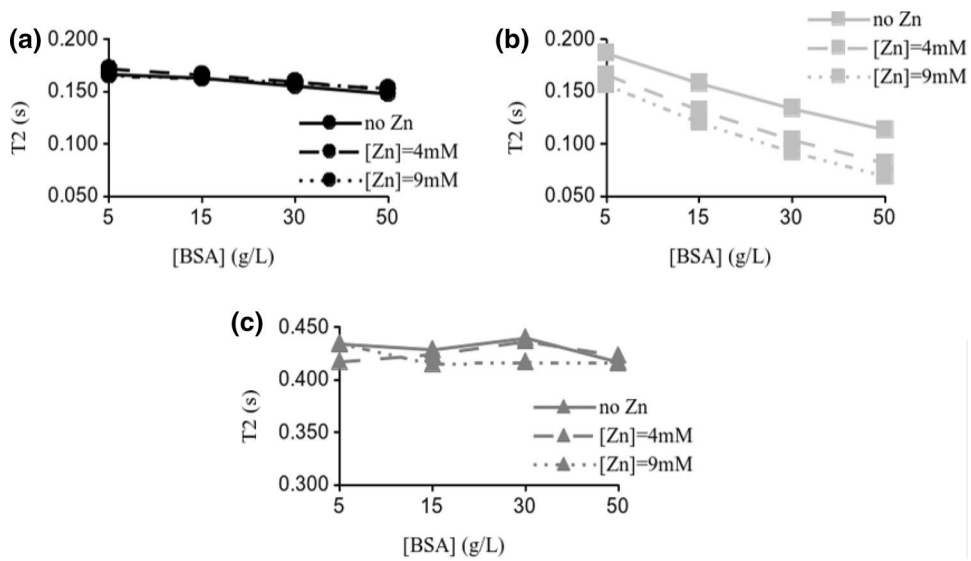


**Fig. 4** Combined effect of zinc and BSA concentration on the *T*2 of **a** spermine, **b** citrate, **c** myo-inositol in mixture. All mixtures contained spermine tetrahydrochloride, myo-inositol, tri-sodium citrate, calcium di-chloride di-hydrated, magnesium di-chloride and potassium chloride at concentrations of 18, 11, 90, 18, 15, 61 mM, respectively. Zinc and BSA concentration are given in the figure's legend and x-axis, respectively

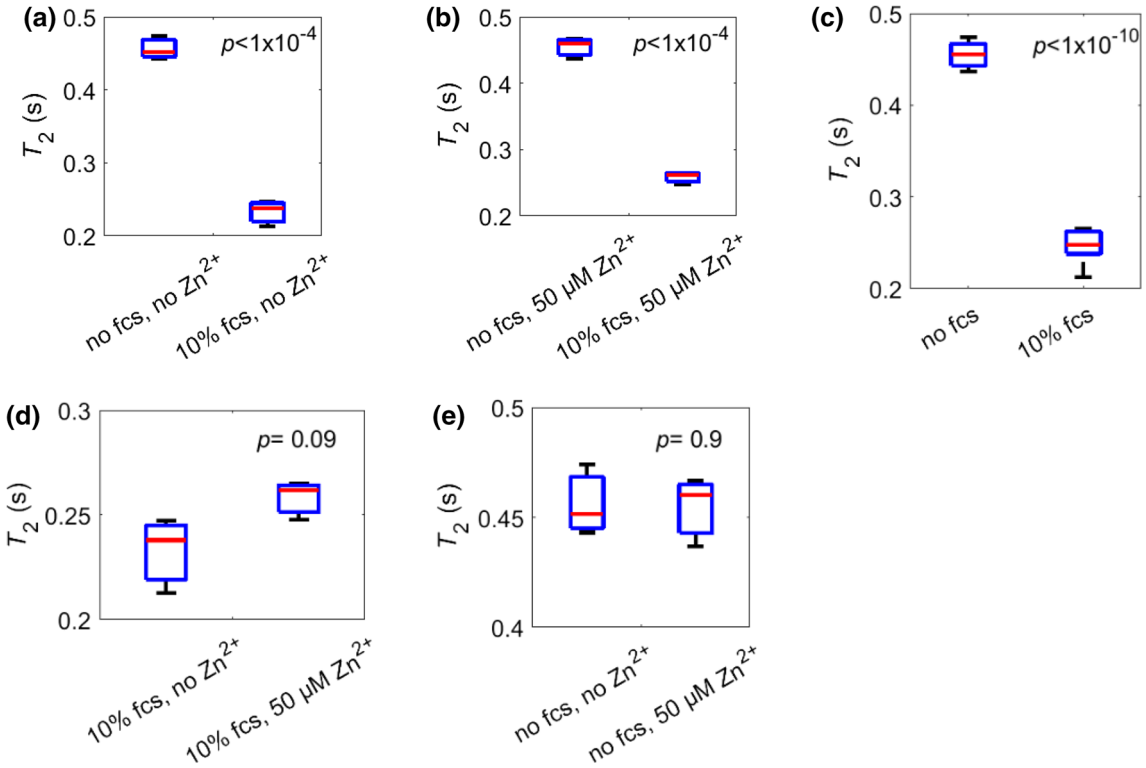


**Fig. 5** Boxplots of *T*2 values found for citrate for the different combinations of supplements added to the extracellular fluid of LNCaP cells. When no zinc or fcs is added to the extracellular fluid, the *T*2 is significantly higher compared to when 10% fcs is added (**a**). This effect is still seen in the presence of 50 μM zinc (**b**) and when these measurements are pooled (**c**). No differences were found between samples with or without 50 μM zinc(II) supplementation, whether there is 10% fcs present (**d**) or not (e)

**Table 2** Intensities normalized relative to metabolites peaks of mixture of spermine, citrate and myo-inositol without metal ions and BSA

| | BSA (g/L) | Signals intensities | | |
|---|---|---|---|---|
| | | Spermine | Citrate | Myo-inositol |
| No ions | 0 | 1.00 | 1.00 | 1.00 |
| Ions, no Zn | 0 | 0.94 | 0.63 | 0.87 |
| Ions, [Zn] = 4 mM | 0 | 0.94 | 0.63 | 0.87 |
| Ions, [Zn] = 9 mM | 0 | 1.05 | 0.62 | 0.87 |
| No ions | 15 | 0.98 | 0.66 | 0.87 |
| | 5 | 1.05 | 0.60 | 0.87 |
| Ions, no Zn | 15 | 0.94 | 0.57 | 0.87 |
| | 30 | 0.94 | 0.48 | 0.87 |
| | 50 | 0.87 | 0.45 | 0.87 |
| | 5 | 0.94 | 0.56 | 0.87 |
| Ions, [Zn] = 4 mM | 15 | 1.05 | 0.52 | 0.87 |
| | 30 | 0.94 | 0.46 | 0.87 |
| | 50 | 0.79 | 0.30 | 0.54 |
| | 5 | 1.05 | 0.58 | 0.87 |
| Ions, [Zn] = 9 mM | 15 | 1.05 | 0.51 | 0.87 |
| | 30 | 1.05 | 0.41 | 0.87 |
| | 50 | 1.02 | 0.34 | 0.87 |

chemical shift of the well-resolved signal Myo5, as an internal reference (Fig. 1). In this analysis, we compared the chemical shifts of the metabolite peaks from those in the mixtures containing no metal ions and BSA.

Adding BSA had no or a minimal effect on the chemical shifts of the resonances of the metabolites. E.g. in the presence of 15 g/L BSA, without metal ions, only a 1 Hz shift was observed for Spm1 and Cit_down, compared to the mixture free of both metal ions and BSA. Adding metal ions caused obvious changes in the chemical shifts of both citrate and spermine proton resonances, but very little or none in the shifts of myo-inositol resonances (Table 3). The largest shifts were observed for Spm5 (−6 Hz) and Cit_down (12 Hz). Adding 9 mM zinc further changed the chemical shift of the proton signals of these compounds. The largest relative chemical shift differences were observed for spermine and citrate in solutions with ions, 9 mM Zn and BSA, with the biggest shift for Cit_down (20 Hz). Overall, the least to most affected metabolite signals were Spm1, Spm3/4, Spm2 < Cit_up < Spm5 < Cit_down upon adding metal ions and zinc (Table 3).

To evaluate the potential effects of protein and cation binding on coupling between the citrate protons, their J-couplings were calculated (Table 4). Upon increasing metal ions

**Table 3** Chemical shift change in Hz compared to peaks from the sample without metal ions and absence of BSA. All samples contain metabolite mixtures of citrate, myo-inositol and spermine. The signal myo5 (Fig. 1) was used as an internal reference. The changes in chemical shift were rounded to integer values for readability. Chemical shift change in samples with only myo-inositol can be found in Table S2

| | | Signals Chemical shift wrt Myo5 [Hz] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | BSA (g/L) | Spm5 | Spm2 | Cit_up | Cit_down | Spm3/4 | Spm1 | Myo2 | Myo4/6 | Myo1/3 | |
| No ions | 0 | 1.79 | 2.1 | 2.54 | 2.64 | 3.09 | 3.15 | 3.28 | 3.54 | 3.62 | ppm |
| Ions, no Zn | 0 | − 5 | − 2 | 1 | 12 | 3 | 2 | 1 | 1 | 0 | Hz |
| Ions, [Zn] = 4 mM | 0 | − 5 | − 2 | 1 | 12 | 3 | 2 | 1 | 1 | 0 | |
| Ions, [Zn] = 9 mM | 0 | − 6 | − 3 | 6 | 17 | 2 | 2 | 1 | 1 | 0 | |
| No ions | 15 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | |
| Ions, no Zn | 5 | − 5 | − 2 | 1 | 12 | 3 | 2 | 1 | 1 | 0 | |
| | 15 | − 5 | − 2 | 1 | 12 | 3 | 2 | 1 | 1 | − 1 | |
| | 30 | − 5 | − 2 | 0 | 12 | 3 | 2 | 1 | 1 | − 1 | |
| | 50 | − 4 | − 2 | 0 | 12 | 2 | 3 | 1 | 1 | − 1 | |
| Ions, [Zn] = 4 mM | 5 | − 6 | − 3 | 5 | 17 | 3 | 3 | 1 | 1 | 0 | |
| | 15 | − 6 | − 3 | 5 | 16 | 2 | 3 | 1 | 1 | − 1 | |
| | 30 | − 5 | − 3 | 4 | 16 | 2 | 3 | 1 | 1 | − 1 | |
| | 50 | − 5 | − 2 | 3 | 15 | 2 | 3 | 1 | 1 | − 1 | |
| Ions, [Zn] = 9 mM | 5 | − 7 | − 3 | 8 | 20 | 3 | 3 | 1 | 1 | − 1 | |
| | 15 | − 6 | − 3 | 8 | 20 | 3 | 3 | 1 | 1 | − 1 | |
| | 30 | − 6 | − 2 | 7 | 19 | **3** | 3 | 1 | 1 | − 1 | |
| | 50 | − 5 | − 2 | 5 | 18 | 3 | **3** | 1 | 1 | − 1 | |

and Zn to 9 mM the J-coupling increased by about 1 Hz (15.1–16.1 Hz). Adding BSA at any condition did not further affect the J-coupling. In extracellular fluid, the addition of fcs significantly changed the J-coupling in the upfield citrate doublet (by 1 Hz) and the chemical shift difference between the two methylenes (by about 6 Hz) (Table 4).

## Discussion

The $^{1}$H NMR spectra of prostatic fluid mimics are similar to those of prostate secretions previously measured by Lynch et al. [8, 40]. These authors speculated that an interaction exists between spermine and citrate in prostatic fluid. The results of our studies, specifically of the *T*2 relaxation times investigation, indeed demonstrate the existence of such an interaction. In addition, the changes in *T*2 relaxation suggest that the citrate-spermine complex binds to metal ions, in particular zinc [41], and interacts with proteins (BSA). Changes in other NMR parameters, such as in chemical shift and peak height of citrate resonances confirm it interacts with metal ions, Zn and BSA, while the observed changes in J-coupling are indicative of metal ion and Zn interactions.

The *T*2 values of spermine protons dropped by a factor of 2 when mixed with 90 mM citrate, indicating binding between spermine and citrate. However, the presence of spermine in our experiments did not change the *T*2 of citrate protons, an observation that is explained below. In addition, the lower citrate proton *T*2 relaxation value after adding bivalent metallic ions indicates that citrate interacts with these ions. Further, citrate *T*2 values are lower in the presence of spermine and increasing zinc concentration. Overall, our results strongly suggest the formation of a transient spermine-metal-ion-citrate complex. These observations confirm previous studies showing that polyamines, such as spermine, stabilize metal-citrate complexes by secondary sphere interactions [42, 43]. In prostate biofluids, either intra- or extracellularly, Zn is considered to mainly occur in a complex with citrate [44]. It is thought that when zinc is bound in this zinc-citrate complex, it is involved in transport and in reactions or interactions, whereas free zinc ions are not.

To explain why, in the absence of ions and of Zn, spermine proton *T*2 decreases but not that of citrate, as other experiments indicate that they form a complex, we refer to the difference in fraction bound and free spermine and citrate. As the peak integrals of the proton signals of the metabolites are not affected by any of the solution conditions and no additional relaxation effects were observed in the signal *T*2 decay curves we assume that metabolite interactions occur in the fast exchange regime [36].

$$T2_{\mathrm{obs}} = T2_{\mathrm{b}} \times F_{\mathrm{b}} + T2_{f} \times F_{\mathrm{f}} \quad (1)$$

**Table 4** Difference in chemical shift between the centers of the citrate methylene doublets ($\Delta\delta$) and their respective J-coupling (Hz) for both prostate mimic with and without BSA and ions (Zn), and for extracellular fluid of LNCaP cells with or without fcs and Zn supplementation

| | BSA (g/L) | Δδ between methylenes | J-coupling [Hz] | |
|---|---|---|---|---|
| | | | Cit_down | Cit_up |
| No ions | 0 | 64.5 | 15.1 | 15.1 |
| Ions, no Zn | 0 | 75.7 | 15.8 | 15.7 |
| Ions, [Zn] = 4 mM | 0 | 76.3 | 15.9 | 15.9 |
| Ions, [Zn] = 9 mM | 0 | 77.0 | 16.1 | 16.0 |
| No ions | 15 | 65.2 | 15.1 | 15.1 |
| | 5 | 76.0 | 15.8 | 15.8 |
| Ions, no Zn | 15 | 76.2 | 15.8 | 15.8 |
| | 30 | 76.7 | 15.8 | 15.8 |
| | 50 | 77.0 | 15.8 | 15.8 |
| | 5 | 76.6 | 15.9 | 15.9 |
| Ions, [Zn] = 4 mM | 15 | 76.7 | 15.9 | 15.9 |
| | 30 | 77.0 | 15.9 | 15.9 |
| | 50 | 77.5 | 15.9 | 15.9 |
| | 5 | 77.1 | 16.1 | 16.0 |
| Ions, [Zn] = 9 mM | 15 | 77.4 | 16.0 | 16.0 |
| | 30 | 77.6 | 16.0 | 16.0 |
| | 50 | 78.0 | 16.0 | 16.0 |
| | fcs | Δδ between methylenes | Cit_down | Cit_up |
| [Zn] = 50 µM | 0 | 73.06 ± 0.39 | 15.81 ± 0.02 | 15.80 ± 0.02 |
| No Zn | 0 | 73.23 ± 0.44 | 15.86 ± 0.02 | 15.80 ± 0.03 |
| [Zn] = 50 µM | 10% | 76.34 ± 0.18 | 15.81 ± 0.03 | 15.93 ± 0.02 |
| No Zn | 10% | 76.40 ± 0.04 | 15.83 ± 0.00 | 15.92 ± 0.02 |

Here, the observed $T2$ ($T2_{obs}$) depends on the fraction bound ($F_b$, spermine-citrate complex) and free ($F_f$, the individual metabolites); $T2_b$ is the $T2$ in the bound state, i.e. in/of the complex, and $T2_f$ is the $T2$ in the free state. For spermine, with its concentration being five times lower than citrate, the fraction $F_b$ will be larger for spermine than for citrate upon binding, assuming that citrate and spermine interact in a close to 1:1 ratio (Eq. 1). This explains the observed decrease in $T2$ of spermine and unaffected $T2$ of citrate, although we cannot exclude the contribution of a smaller $T2_b$ of bound spermine protons versus that of bound citrate. In the presence of ions and Zn we also observe a moderate decrease in $T2$ of citrate upon addition of spermine which might indicate a larger fraction bound citrate ($F_b$, spermine-metal ion-citrate complex) and/or a change in its $T2_b$ in this complex compared to the metal ion free complex.

To investigate the effect of BSA on the proton $T2$ of the individual metabolites, with and without metallic ions, we studied mixtures containing 15 g/L BSA, which is the average protein content in prostatic fluid of non-cancerous prostates [37]. The strong decrease in $T2$ of spermine and in particular citrate protons indicate a substantial interaction of these metabolites with BSA. A previous study indicated that the NMR signals of citrate bound to BSA may broaden beyond detection [45], meaning a very short $T2_b$. However, because the BSA concentration in our study was two or three orders of magnitude less than the metabolite concentrations (75–750 nM BSA vs. 90 mM citrate in the prostate mimic, and 45–60 nM BSA vs. 0.7 mM citrate for the extracellular fluid) the fraction of bound citrate will be very small and binding to BSA is not expected to affect the integral of the citrate signal, as was indeed observed. Because we did not observe additional relaxation effects in the signal decay curves we assume that also this interaction is in the fast exchange regime. As there may be more than one binding site or mode on BSA the measured $T2$ may represent an average value of different interactions.

In the mixture of the three metabolites the $T2$ value of citrate dropped by a factor of 2 either by increasing BSA concentration from 5 to 50 g/L or by increasing zinc from 0 to 9 mM. Thus citrate, apart from the spermine-metal ion-citrate complex, has free citrate, $F_f$, available to form further complexes with zinc and to interact with BSA. Parallelly, since increased zinc concentration did not lower spermine $T2$, it appears that any metal ion present was adequate to form the spermine-metal ion-citrate complex [14]. Finally,

it is possible that the addition of zinc dichloride contributes to higher levels of chlorine ions that bind to spermine and that this contributes to the *T*2 decrease.

The effect of ions and BSA on the *T*2 of protons in spermine, alone in solution, has been studied previously [20]. The *T*2 value of the spermine protons resonating at 1.8 ppm, measured at the same field strength, temperature and ion concentrations, except for KCl, as in our study, was longer than in our study, i.e. 0.32 versus 0.16 s [20]. Most likely this is due to the much higher percentage $D_2O$ used, as reported in [20], which lengthens *T*2 values. For normal prostatic fluid a *T*2 value of only 0.056 s was reported [20] which may indicate further molecular interactions with spermine. In agreement with this study, we did not observe an effect of ions on the *T*2 of spermine, except at the highest Zn concentration (9 mM) when the *T*2 dropped substantially in our study, which may be due to different measurement conditions (e.g. KCl presence and much lower $D_2O$ content in our fluid compositions). Also, in agreement with the study by Spencer et al. [20], we noted a large drop in *T*2 upon the addition of 15 g/L BSA.

An intriguing phenomenon is the short myo-inositol *T*2, of ~ 0.34 s, when dissolved in MiliQ water, but, mixed with metal ions (no zinc) its *T*2 became longer, i.e. ~ 1 s. This indicates that myo-inositol is free to tumble in the presence of metal ions. This can be understood from the findings of Zhao et al., who reported an increasing partial molar volume of myo-inositol in solutions of alkali metal salt [46]. This is due to interactions between myo-inositol and metal ions [47]. The *T*2 values of myo-inositol protons were shown to be around 0.35 s, using HR-MAS $^1$H-NMR measurements of tissue samples [48], which is within the range of the values we report here.

We also analyzed the effect of cations and BSA on the peak height and integral of metabolite proton signals. Of the three metabolites, the signal of citrate was most affected through the different sample compositions. Since peak height is related to *T*2 (half-width ≈ 1/*T*2), these data confirm that interactions affect citrate signals more than those of the other metabolites in the mimic. Peak integrals, which represent the total concentration of free metabolites, remained constant throughout all different sample compositions indicating fast-exchange regime interactions.

In the presence of metal ions and high BSA levels (≥ 15 g/L) we observed an asymmetry in the citrate quartet represented by different phases and intensities of the Cit_up signal at longer echo times. Although we have no proper explanation for this phenomenon it suggests that the two signal groups of citrate reflect a type of binding that may be related to the citrate orientation in the binding site of BSA. Asymmetric NMR behavior of citrate has been observed for chemical shifts and J-couplings in the presence of metal ions [14], and albumin [49].

On addition of metal ions to solutions with spermine and citrate their proton signals shifted up-field and down-field, respectively, with respect to myo-inositol. Increasing BSA concentration caused citrate signals in the prostate fluid mimic to shift further down-field. However, the two citrate doublet signals chemical shift changes are not equivalent: Cit_down shifted by 20 Hz and Cit_up by 8 Hz, increasing their shift difference by 12 Hz. On the other hand, the addition of fetal calf serum to the extracellular fluid of LNCaP cells increased the chemical shift separation with only 6 Hz. Note that BSA and citrate concentrations are much lower in the extracellular fluid of LNCaP cells compared to the concentrations in the prostate fluid mimic. Nonetheless, this parallels the citrate T2 data and indicates a preferential orientation of citrate for binding to BSA. How the citrate molecules are exactly oriented towards the protein is difficult to understand because the $CH_2$ groups cannot be distinguished from the simple NMR 1/2D $^1$H and $^{13}$C experiments employed here. The J-coupling of citrate methylene protons increases by 1 Hz with increasing metal ion concentration, and thus represents another NMR parameter to assess ion interaction [14].

In an extracellular fluid of prostate cancer cells growing in RPMI medium the concentration of citrate secreted by these cells (0.5–0.7 mM) is much lower than in normal prostate extracellular fluid (about 90 mM) [50] and therefore has some similarity with the extracellular fluid of high-risk prostate cancer tissue. In the extracellular fluid of PCa tissue, also Zn occurs at much lower levels than in normal extracellular fluid. No significant effect of the addition of 50 µM zinc(II) on the *T*2 relaxation of protons in citrate, secreted by LNCaP into the medium is seen. The molar ratio between Zn and citrate is very similar to that in the prostate fluid mimic (prostate mimic 90 mM/9 mM = 10; citrate secreted by LNCaP in medium 0.5 mM/0.050 mM = 10).

In the presence of 10% fetal calf serum the *T*2 of citrate decreases to 0.26 s compared to samples without fetal calf serum. As this amount of calf serum represents about 3–4 g/L protein [29, 51] this indicates that protein binding is also a major factor determining citrate *T*2 in prostatic fluids in cancer tissue. Lower *T*2s can be expected upon binding to higher levels of proteins, e.g. at 5 g/L we would expect a *T*2 of 1–0.15 s. In expressed prostatic fluids of prostates with tumor tissue the levels of proteins appear to be lower than in these fluids in the normal prostate [26].

The *T*2 value of 0.12 s obtained for citrate in the prostate fluid mimic, i.e. with metal ions, 9 mM zinc, and 15 g/L BSA, is slightly lower compared to the *T*2 values reported for citrate protons in the peripheral zone of the human prostate at 1.5–3 T: i.e. from 0.17 to 0.22 s [2, 52, 53]. Although *T*2 is relatively insensitive to field strength, differences are expected due to experimental variations such as in protein

content and temperature between our samples and the in vivo studies.

## Conclusion

Proton *T*2 relaxation times were found to be the best NMR parameter to identify interactions in a prostatic fluid mimic involving the three major fluid metabolites, citrate, spermine and myo-inositol. Decreases in *T*2 values reported the formation of a transient spermine-metal ion-citrate complex and its binding to proteins (BSA or fcs). Decreases in citrate signal peak heights confirm these interactions. The two methylene groups of citrate showed asymmetric NMR behavior at long echo times upon interaction of this compound with ions and BSA. Finally, the NMR observations indicate that the interactions are in the fast-exchange regime. Although these interactions were identified at higher field strength than clinically used and at room temperature, they may be a factor affecting the in vivo assessment of prostate pathologies by $^{1}$H MRSI using citrate and spermine signals.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10334-021-00983-4.

**Acknowledgements** This research was partly supported by GO-EFRO project "Ultrasense NMR" and CMI project "PET/MR". We thank Prof. Sybren Wijmenga for help in starting up this project, Kees Jansen for assistance with cell culture and Sjaak van Asten and Frank Nelissen for technical assistance.

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

**Ethical approval** No animals or human subjects were included in this study and no ethical approval is required.

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