ESR – Note

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In Vivo EPR Extracellular pH-metry in Tumors Using a Triphosphonated Trityl Radical

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Purpose: The ability to assess the extracellular pH (pHe) is an important issue in oncology, because extracellular acidification is associated with tumor aggressiveness and resistance to cytotoxic therapies. In this study, a stable triphosphonated triarylmethyl (TPTAM) radical was qualified as a pHe electron paramagnetic resonance (EPR) molecular reporter.

Methods: Calibration of hyperfine splitting as a function of pH was performed using a 1.2-GHz EPR spectrometer. Gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) was used as an extracellular paramagnetic broadening agent to assess the localization of TPTAM when incubated with cells. In vivo EPR pH-metry was performed in MDA, SiHa, and TLT tumor models and in muscle. Bicarbonate therapy was used to modulate the tumor pHe. EPR measurements were compared with microelectrode readouts.

Results: The hyperfine splitting of TPTAM was strongly pH-dependent around the pKa of the probe (pKa = 6.99). Experiments with Gd-DTPA demonstrated that TPTAM remained in the extracellular compartment. pHe was found to be more acidic in the MDA, SiHa, and TLT tumor models compared with muscle. Treatment of animals by bicarbonate induced an increase in pHe in tumors: similar variations in pHe were found when using in vivo EPR or invasive microelectrodes measurements.

Conclusion: This study demonstrates the potential usefulness of TPTAM for monitoring pHe in tumors. **Magn Reson Med 77:2438–2443, 2017. © 2016 International Society for Magnetic Resonance in Medicine**

Key words: in vivo EPR; in vivo ESR; pH; trityls; tumors

INTRODUCTION

pH plays a critical role in various physiological processes and is closely regulated. A breakdown of normal pH homeostasis is involved in several pathologies such as inflammation, ischemia, myocardial infarction, gastric ulceration, renal failure, and chronic pulmonary diseases (1). In solid tumors, a low extracellular pH (pHe) is associated with a metastatic phenotype and poor prognosis (2). Moreover, a low pHe in tumors reduces the effectiveness of some chemotherapeutic agents (3,4). Attempts to increase the pHe of the tumor microenvironment by systemic buffers (e.g., using sodium bicarbonate) have been shown to be effective to increase the therapeutic effectiveness of doxorubicin and reduce the formation of spontaneous metastases in the metastatic breast cancer mouse model (5–8). Therefore, the noninvasive monitoring of pHe is highly relevant for characterizing the tumor microenvironment and for optimizing the therapeutic strategy.

NMR and low-field electron paramagnetic resonance (EPR) spectroscopy are the principal techniques for noninvasive assessment of pHe. The pHe measurement relies on exogenous molecular probes that are weak acids or bases with pH-sensitive spectral properties. The principle of EPR pH-metry is to assess the change in the hyperfine splitting as a function of pH. Nitroxides are the most widely used EPR pH-sensitive probes (9–14). However, the use of nitroxides *in vivo* may be impeded by their fast bioreduction into EPR-silent hydroxylamines. Trityl radicals, otherwise known as TAMs (tetrathiatriarylmethyl radicals), have a higher stability in living tissues compared with nitroxides. This explains why the synthesis of these compounds has been developed recently for several biomedical applications such as measurement of oxygenation, superoxide anion, redox status, thiols, inorganic phosphate, and pH (15–21). To get pH-sensitive probes, the TAMs have been functionalized with ionizable groups (22,23). We recently described the synthesis of triphosphonated triarylmethyl (TPTAM) radical (Fig. 1) (24). Triphosphonated TAM was preferred over the monophosphonated derivatives as a result of a higher hydrophilicity and a smaller tendency to aggregate upon acidification of the media. This probe has an excellent aqueous solubility, a high stability, and narrow line widths and presents a pH-sensitive spectrum in the physiological range (24). The aim of the present study was to assess the suitability of TPTAM to monitor the pHe in vivo. For this purpose, we first calibrated the pH dependence of TPTAM using low-frequency (1.2-GHz) EPR spectrometry. Second, we assessed the localization of the probe when incubated in the presence of cells. Third, we evaluated the ability of TPTAM to report on the pHe in vivo in tumors and in muscle. Fourth, we monitored the effect of a systemic pH buffering therapy with a bicarbonate treatment (25) and compared in vivo EPR pH-metry measurements with invasive microelectrode readouts.

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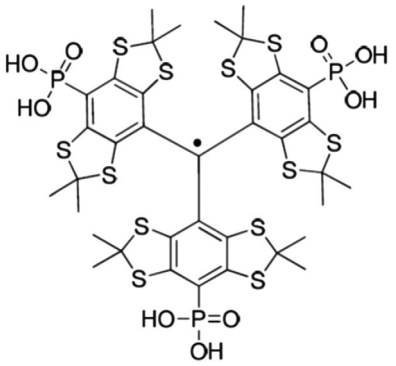

FIG. 1. Chemical structure of TPTAM.

METHODS

Synthesis

The triphosphonated tetrathiatriarylmethyl radical (TPTAM) (Fig. 1) was synthesized as described previously (24).

Cell Culture Conditions

The leukemic cell line K562 was purchased from European Collection of Cell Cultures and maintained in RPMI 1640 medium (Gibco) supplemented with fetal calf serum (10%), penicillin-streptomycin (1%), and gentamicin (50 μg/mL) at 37 °C in humidified 5% CO_2. SiHa human cervix carcinoma cells and MDA-mb-231 human breast carcinoma cells were obtained from American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium containing D-glucose (4500 mg/L), L-glutamine (4 mM), heat-inactivated fetal bovine serum (10%), and penicillin-streptomycin (1%) at 37 °C in humidified 5% CO_2.

L-Band EPR Studies

Measurements (calibration and in vivo experiments) were performed using an L-band EPR spectrometer (Magnettech, Berlin, Germany) operating at 1.2 GHz and equipped with an external loop-gap resonator. The typical instrument parameters were as follows: B_0 field = 42 mT; modulation amplitude = 0.05 mT; modulation frequency = 100 kHz; sweep width = 5 mT; sweep time = 30 s; and number of scans = 5.

pH Calibration at 1.2 GHz

A solution of 100 μM of TPTAM in 2 mM phosphate-buffered saline (150 mM NaCl) was titrated by addition of small volumes of HCl or NaOH to achieve the required pH value. The pH was measured using a WTW inoLab pH-meter (WTW, Weilheim, Germany) equipped with a pH electrode (WTW, Sentix81). The electrode was calibrated using standard buffer solutions of pH 4.0 and 7.0 at 37 °C. Temperature was maintained at 37 °C using a water bath attached to a thermostat. The hyperfine splitting constant was measured as the distance between the two high-field components of the quartet. To obtain the pKa value of the probe, the experimental dependence of hyperfine splitting on pH was fitted using the standard Henderson-Hasselbalch titration equation (GraphPad Prism 5.0).

Localization of the Probe in the Presence of Cells

TPTAM (100 μM) was incubated in the presence of leukemic cells K562 (10^7 cells/mL) with (or without) the cell membrane-impermeant broadening agent gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA, 20 mM; Sigma-Aldrich, St. Louis, Missouri, USA). Gd-DTPA dramatically decreases the relaxation times of other paramagnetic agents and abolishes any EPR signal contribution coming from the extracellular compartment (26). EPR spectra were recorded at 37 °C using a Bruker EMX EPR spectrometer (Bruker, Rheinstetten, Germany) operating at 9.8 GHz. A total of 400 μL of the cellular suspension were transferred into a quartz flat cell (ER 160 FC-Q, Bruker, Germany). The acquisition settings were as follows: B_0 field = 350.1 mT; sweep width = 2 mT; modulation frequency = 10 kHz; modulation amplitude = 0.002 mT; time constant = 20.48 ms; conversion time = 20.48 ms; sweep time = 41.94 s; number of points = 2048; and number of scans = 3.

Animals and Tumor Models

Experiments were performed on three different tumor models. The tumors were inoculated intramuscularly by injection of a suspension (100 μL) of cells in the rear leg of mice. TLT (hepatocarcinoma; 10^6 cells from ascite dispersed in saline solution 0.9 %) were injected in 4- to 6-week-old NMRI male mice. MDA-mb-231 cells (mammary adenocarcinoma, 10^7 cells from in vitro culture, suspended in Hank's balanced salt solution [HBSS]) were injected in 4- to 6-week-old nude NMRI female mice. SiHa cells (cervical carcinoma, 10^7 cells from in vitro culture, suspended in HBSS) were injected in 4- to 6-week-old nude Balb-c male mice. The mice were supplied by Janvier Labs (Mayenne, France). The pHe measurements were performed when the tumor size reached 8 ± 0.5 mm. All animal experiments were performed in compliance with national and university animal care regulations.

Treatment

The effect of bicarbonate therapy was assessed in mice bearing the TLT tumor model. The treatment ($NaHCO_3$ 200 mM in drinking water) was initiated concomitantly with the tumor inoculation to neutralize the acidic character of tumors with a physiological systemic buffer (5).

In Vivo EPR Experiments

Animals were anesthetized by isoflurane inhalation (3% for induction and 1% for maintenance, mixed with air). A circulating warm water (37 °C) blanket was used to keep the mice at normothermia. A solution of the TPTAM dissolved in distilled water (5 mM) was titrated to get a pH of 7.3–7.4 without addition of any buffer. A total of 20 μL of this solution was injected in the tissues along a single track (5 mm distance), allowing the distribution of the probe within the tissue of interest. Due to the presence of the bicarbonate buffer (>20 mM) in the extracellular space, the effect of TPTAM on the pHe can

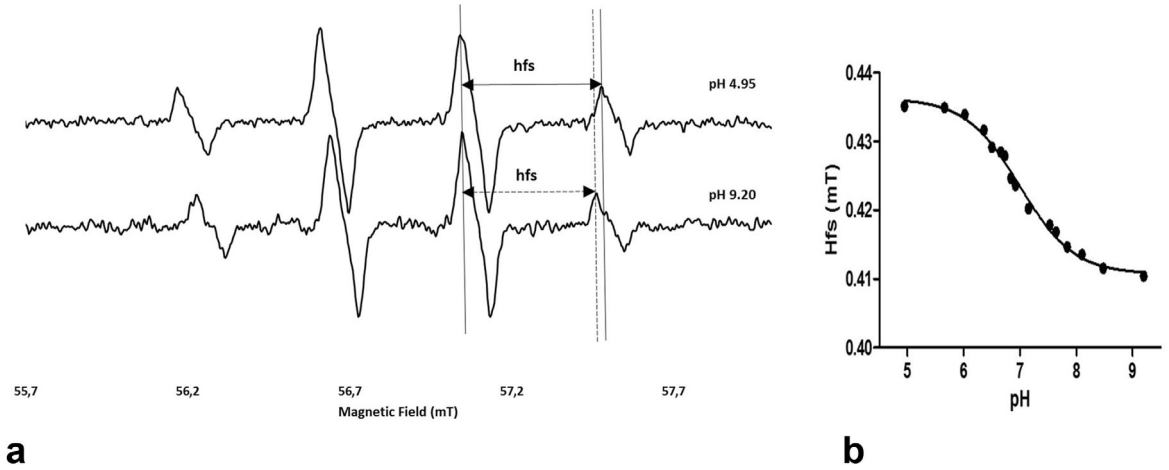

FIG. 2. Sensitivity of the EPR spectrum to changes in pH. (**a**) EPR spectra recorded at pH = 4.95 and pH = 9.20. Note the change in hyperfine splitting (hfs) constant. (**b**) Titration of the hyperfine splitting as a function of pH (37 °C, spectra recorded at 1.2 GHz). The titration curve revealed a pKa of 6.99 ± 0.05.

be neglected. The tissue of interest was positioned in the L-band loop resonator. The spectra were recorded immediately after injection and positioning.

pH Micro-electrode Measurements

We compared the pHe measurements obtained using in vivo EPR with readouts obtained by pH microelectrodes in the TLT tumor model (separate cohorts). Measurements were achieved using a pH-sensitive glass electrode mounted into the protected-tip of a 21-gauge needle (MI-408; Microelectrodes, Bedford, New Hampshire, USA). An Ag/AgCl electrode with a flexible barrel was used as a reference electrode (MI-402; Microelectrodes). pH was determined using a WTW inoLab pH-meter. The electrodes were calibrated before insertion using two-point calibration in standard buffers of pH 4.0 and 7.0 at 37 °C. The calibration was verified by inserting the electrodes into a pH 7.4 buffer at 37 °C. The tissue was exposed through a narrow incision. Using a stereotaxic micromanipulator, the electrode was advanced a few millimeters into the tissue through the incision and fixed in place. The reference electrode was placed in the adjacent subcutaneous tissue.

Statistical Analysis

Results are presented as the mean ± SE. Comparisons were made using a Student *t* test or one-way analysis of variance along with post hoc Bonferonni's multiple comparison tests. $P < 0.05$, $P < 0.01$, or $P < 0.001$ were considered statistically significant.

RESULTS

The EPR spectrum of TPTAM is split into a quartet because of the presence of three phosphorus atoms with a nuclear spin of ½. As shown in Figure 2a, the hyperfine splitting, measured as the distance between the two high-field components, is strongly dependent on the pH of the solution. The titration of the TPTAM at 1.2 GHz (the frequency used for the in vivo experiments) shows a strong dependence of hyperfine splitting around the pKa value (Fig. 2b). From the analysis of this titration curve, the pKa value was estimated to be 6.99 ± 0.05.

To assess the localization of the TPTAM when incubated in the presence of cells, we used Gd-DTPA as an EPR broadening agent. Gd-DTPA was selected because it remains exclusively in the extracellular compartment and is nontoxic for cells (26). By subtracting the EPR spectrum recorded with Gd-DTPA to the spectrum recorded without Gd-DTPA, the contribution of the intracellular probe can be calculated. As shown in Figure 3, the EPR signal of the TPTAM was completely abolished in the presence of Gd-DTPA. This means that TPTAM remains exclusively in the extracellular compartment with no residual signal coming from the intracellular compartment. As a result, any pH value recorded with TPTAM in a biological sample may be considered as a measurement of the pHe.

Figure 4 illustrates the capacity of the probe to report pHe in living organisms. The tumor pHe was significantly lower in the three tumor models tested compared with the pHe measured in the muscular tissue. Average pHe values were 6.83 ± 0.02, 6.63 ± 0.02, and 6.85 ± 0.04 in MDA-mb-231 (n = 5), SiHa (n = 3), and TLT tumor models (n = 11), respectively. These results are consistent with the acidic character of the tumor

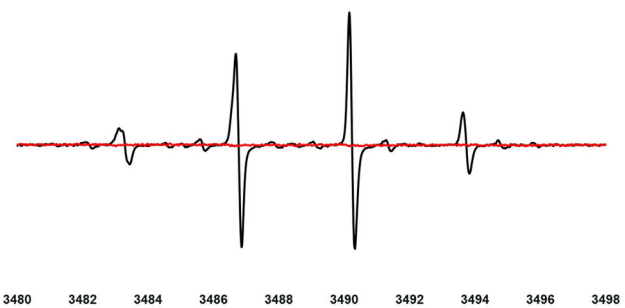

FIG. 3. Localization of TPTAM in the presence of cells. The EPR spectra were recorded in the presence of tumor cells K562 without (black line) or with (red line) Gd-DTPA 20 mM. Note that the EPR signal is completely abolished in the presence of the extracellular broadening agent.

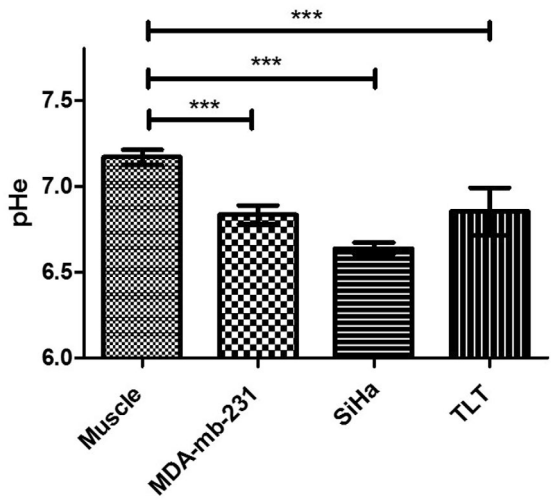

FIG. 4. pHe values recorded in vivo (1.2 GHz) in muscle and in the MDA-mb-231, SiHa, and TLT tumor models. Note that pHe is more acidic in tumors than in muscle. $^{***}P < 0.001$.

microenvironment (2). By comparison, pHe was 7.17 ± 0.02 in the muscle ($n = 6$).

We also tested the ability of TPTAM to assess changes in pHe induced by bicarbonate therapy. Low-frequency EPR experiments showed that the treatment with bicarbonate increased significantly the pHe of 6.85 ± 0.04 ($n = 11$) to 7.04 ± 0.02 ($n = 8$) in the TLT tumor model (Fig. 5a). The pH was also measured using pH microelectrode in the same tumor model: pH values recorded were 6.84 ± 0.01 and 7.07 ± 0.01 in the control and the bicarbonate treated group, respectively (Fig. 5b). The pH values obtained are not significantly different between both techniques (unpaired *t* test with Welch's correction).

DISCUSSION

The ability to measure pHe noninvasively remains an important goal when considering the crucial role played by the acid–base imbalance in tumor aggressiveness and mechanisms of drug resistance. Several methods have been developed to match this biomedical need, such as fluorescence imaging, MR spectroscopy, and chemical exchange saturation transfer. EPR pH-metry may also offer an opportunity to measure this parameter. It should be noted that considerable progress has been made recently in low-field EPR instrumentation (250 MHz to 1.2 GHz), and EPR measurements in living animals can provide significant insights to physiology, pathophysiology, and pharmacology (27). Moreover, recent developments have also demonstrated the possible application of EPR in the clinic (28). Our present study was performed to qualify TPTAM as sensor of pHe using in vivo EPR. In our previous paper, in which we described the synthesis of TPTAM (24), we also reported the sensitivity of the EPR spectrum to the pH. The pKa values determined by fitting the experimental titration curve were $pKa1 = 1.3$ and $pKa2 = 7.1$. These experiments were performed at 25 °C using an X-Band (9 GHz). Here, we focus on the calibration in the conditions used for in vivo studies, namely a calibration at 37 °C and using a L-Band (1.2 GHz) EPR spectrometer. The pKa of interest was equal to 6.99 ± 0.05. This value is optimal for measurements in biological media, especially in the tumor microenvironment, as we may expect values varying between 6.5 and 7.5 according to the literature (29,30). Importantly, we found that TPTAM is exclusively distributed in the extracellular space (Fig. 3). This observation can be explained by the charged nature of the phosphonic acid moieties (at physiological pH), preventing them from crossing the cell membrane. This feature is crucial because tumor microenvironment is characterized by a reversed pHi-pHe gradient with a pHi (7.2–7.7) that is higher than the pHe (6.5–7.1). (30,31). We then used TPTAM for the first time in vivo with the aim to measure the pHe in tumors. For this proof-of-concept study, we used intratumoral administration instead of intravenous administration to reduce the amount of trityl to be injected. Interestingly, a recent EPR imaging study of

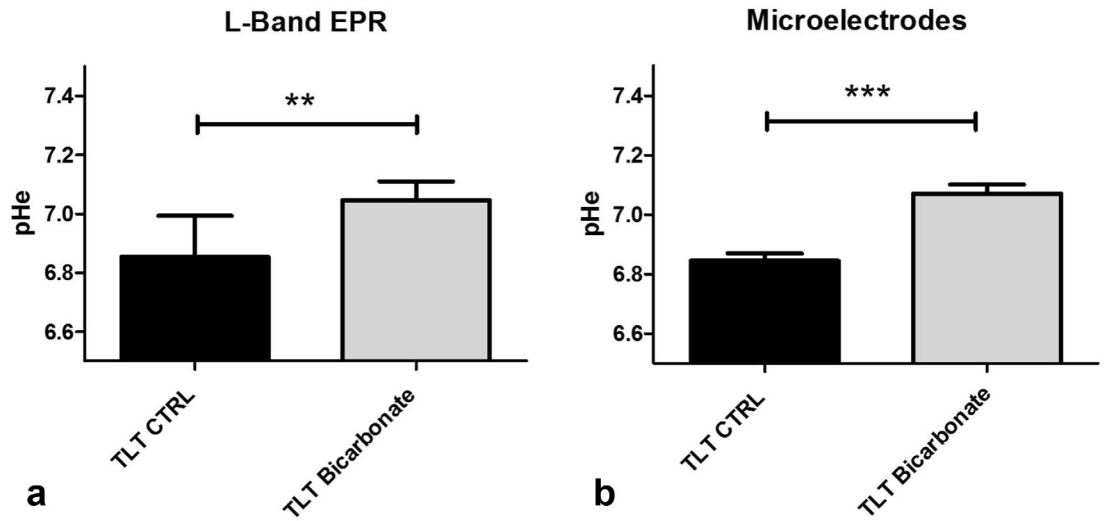

FIG. 5. Effect of bicarbonate treatment on pHe in TLT tumors. (**a**) Data from in vivo EPR. (**b**) Data from invasive pH microelectrodes. Note the consistence of the data between both readouts. $^{**}P < 0.01$. $^{***}P < 0.001$.

Epel showed similar patterns of oxygen distribution using another trityl administered by both methods (32). We did not observe any sign of toxicity using TPTAM. We found that the pHe was significantly lower in the three tumor models tested compared with the normal muscle (Fig. 4). Because one potential application of EPR pH-metry is its use as a marker of response to treatments, we also investigated its usefulness to monitor changes in pHe after use of a systemic buffer. TPTAM was sensitive enough to monitor subtle differences in pHe between the control group and bicarbonate-treated group (Fig. 5). Even more importantly, the data obtained using TPTAM in vivo were closely related to those obtained using invasive pH microelectrodes, as no significant difference was observed between both readouts. Overall, all the data reported for the present study support the potential application of TPTAM for monitoring tumor pHe in cancer research. One potential limitation of TPTAM is its rather rapid clearance after administration: the half-life was evaluated to be 10 min in tumors (data not shown). Nonetheless, this half-life is longer than that of other pHe-sensitive nitroxides such as an oxylimidazolidine coupled to glutathion (tumor half-life estimated to 3 min) (33). This longer half-life allows the possibility of pHe measurement during at least 20 min after administration of TPTAM. Further research will be needed to identify the parameters responsible for the clearance. For other trityl radicals, the washout through the blood circulation has been attributed to kidney filtration (34). However, metabolism may also play a role in the decay of the EPR signal (35).

In conclusion, this study demonstrates the potential usefulness of TPTAM to monitor pHe in tumors.

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