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Triple-Quantum-Filtered ^{23}Na NMR Spectroscopy of Subcutaneously Implanted 9L Gliosarcoma in the Rat in the Presence of TmDOTP^{5-}[1]

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The utility of triple-quantum (TQ)-filtered ^{23}Na NMR spectroscopy for discriminating between intra- and extracellular Na^+ (Na_i^+ and Na_e^+, respectively) in a solid tumor *in vivo* was evaluated using TmDOTP^{5-} as a ^{23}Na shift reagent. Infusion of 80 mM TmDOTP^{5-} without added Ca^{2+} produced baseline-resolved Na_i^+ and Na_e^+ peaks in both single-quantum (SQ) and TQ-filtered ^{23}Na spectra. The Na_i^+ signal represented 22 $\pm$ 4% of the SQ spectrum, but 59 $\pm$ 10% of the TQ-filtered spectrum. Therefore, the Na_i^+ contribution in TQ-filtered spectra is much higher than in SQ spectra. Both SQ and TQ-filtered Na_i^+ signals increased by about 75% 1 h after sacrificing the animal. The TQ-filtered relaxation times did not change during this time, indicating that changes observed in TQ-filtered spectra collected with a preparation time of 3 ms represent changes in the concentration of sodium ions contributing to the TQ-filtered signal. Similar experiments were conducted without TmDOTP^{5-} to determine changes in the Na_e^+ signal in the absence of the shift reagent. The changes in total SQ and TQ-filtered signals 1 h after sacrificing the animal showed that the SQ Na_e^+ signal decreased by approximately 35%, while the TQ-filtered Na_e^+ signal did not change significantly. This demonstrates that the TQ-filtered ^{23}Na signal is relatively insensitive to changes in Na_e^+ content. To our knowledge, this work represents the first evaluation of multiple-quantum-filtered ^{23}Na spectroscopy to discriminate between intra- and extracellular Na^+ in a solid tumor *in vivo*. © 2001 Academic Press

Key Words: **^{23}Na MRS; shift reagent; tumor; multiple-quantum-filtered spectroscopy; rat.**

INTRODUCTION

Most viable cells maintain a low intracellular sodium (Na_i^+) concentration against a higher extracellular sodium (Na_e^+) concentration. This transmembrane Na^+ gradient is achieved by the action of Na^+/K^+-ATPase, which pumps three Na^+ out of the cell and two K^+ into the cell at the expense of one ATP molecule. In turn, the transmembrane Na^+ gradient is used to drive several vital physiological processes, such as maintenance of normal cell volume and transport of other ions (e.g., H^+, and Ca^{2+}) and metabolites (e.g., glucose and amino acids) into and out of the cells. The [Na_i^+] in tumors is generally elevated compared to normal tissue (*1–3*). Though the precise mechanism for increased Na_i^+ in tumors has not been elucidated, it may result from abnormalities in tumor blood flow, oxygenation, energy metabolism, and ion transport processes. Because the transmembrane Na^+ gradient plays a key role in the survival of cells in the hostile environment of the tumor, a noninvasive method for monitoring Na_i^+ in solid tumors *in vivo* will be useful in experimental and clinical cancer research.

^{23}Na nuclear magnetic resonance (NMR) provides a convenient, relatively sensitive, nondestructive method for detecting Na^+ in biological tissue and has been the focus of numerous imaging studies in animals (*4–6*) and humans (*7–9*). However, Na^+ exists in only one chemical form in tissue; consequently, its signals from intra- and extracellular compartments are coincident. One popular method for removing this degeneracy is to use a hyperfine frequency shift reagent (SR) (*10–12*). We have shown that the thulium-based SR, thulium-1,4,7,10-tetraazacyclododecane 1,4,7,10-tetrakis(methylene phosphonate) (TmDOTP^{5-}) produces baseline-resolved intra- and extracellular ^{23}Na resonances *in vivo* from a number of tissues including a subcutaneously (sc) implanted tumor with minimal physiological perturbation (*12–17*). However, an SR safe for human studies is not yet available.

An alternate potential method for discriminating between Na_i^+ and Na_e^+ is the use of multiple-quantum (MQ) filters. Because ^{23}Na has a spin quantum number I = 3/2, there are four possible spin orientations. This results in three possible single-quantum (SQ) transitions, two double-quantum (DQ) transitions, and one triple-quantum (TQ) transition. When ^{23}Na is transiently bound to macromolecules, electric field gradients created at those sites act as effective relaxation mechanisms (*18*) allowing the coherence transfer rule to be violated and MQ transitions become possible (*19, 20*). Because of the high concentration of macromolecules in the intracellular space, it was proposed that Na_i^+

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should produce a large MQ-filtered signal, while the more aqueous nature of the extracellular spaces should produce very little MQ-filtered signal (*21*). Studies on human blood samples (*22*) and various animal models (*23–26*), however, have shown a significant contribution of Na_e^+ in MQ-filtered spectra. In comparison to SQ spectra, however, MQ-filtered spectra have a much larger contribution from Na_i^+. MQ-filtration techniques have distinct advantages over SRs in that they do not require the administration of exogenous chemical agents, can be used repeatedly in serial experiments, and can be used in humans. Therefore, a ^{23}Na SR is required for absolute discrimination of Na_i^+ and Na_e^+ signals, but MQ-filtration techniques may be useful for monitoring changes in Na_i^+ content.

It has been demonstrated in the perfused rat heart and in the rat liver *in situ* that the small but measurable Na_e^+ signal that passes through a triple-quantum (TQ) filter is largely unchanged during ischemia and other physiological perturbations (*12, 24–26*). Thus, changes in total TQ-filtered ^{23}Na signal intensity in the absence of an SR may directly report changes in $[Na_i^+]$. However, changes in the TQ-filtered Na_i^+ signal of the *in situ* liver during global ischemia were shown to be different than changes in the SQ Na_i^+ signal (*12, 25*). A similar disparity between SQ and TQ-filtered signals has been seen in brain (*27*), but not in red blood cells (*28*) or heart (*26*). These findings suggest that this phenomenon may be tissue dependent. In the present study, we have investigated the use of TQ-filtered ^{23}Na MRS for monitoring changes in Na_i^+ in a subcutaneously implanted 9L glioma in rats using $TmDOTP^{5-}$ to lift the degeneracy between the Na_i^+ and Na_e^+ signals. We have compared the changes in SQ and TQ-filtered Na_i^+ and Na_e^+ signal intensities during ischemia produced by exsanguinating the animal. We believe that this study represents the first evaluation of MQ-filtered ^{23}Na spectroscopy to discriminate between intra- and extracellular Na^+ in a solid tumor *in vivo*.

METHODS

Tumor cell culture. 9L tumor cells were maintained in cell culture in McCoy's 5a supplemented with fetal calf serum (5–10%) and 20 mM Hepes, at pH 7.4. Cells were maintained in experimental growth by routine passage, twice weekly. New cultures were started from frozen stocks every 3 months. Tumors were started by trypsinizing cultures, stopping the trypsin with spent media, and centrifuging at 2000 *g* for 2 min at 4°C. Spent medium was decanted and the cell pellets were rinsed in phosphate-buffered saline (PBS), pH 7.4. Cells were concentrated to 10^7/ml in PBS.

Rat tumor model. All animal protocols were approved by the University Laboratory Animal Research committee of The University of Pennsylvania. Male Fisher 344 rats, weighing 125–150 g, were injected with $\sim 10^6$ 9L cells sc in the flank. When the tumor size reached ~2 cm diameter, the animals were prepared for NMR experiments. The animals were initially anesthetized by an intraperitoneal (ip) injection of a 0.2-ml mixture of ketamine (91 mg/ml) and acepromazine (0.91 mg/ml). One jugular vein and a carotid artery were cannulated through a midline neck incision. The jugular vein was used to infuse $TmDOTP^{5-}$, while the arterial catheter was connected to a pressure transducer (Ohmeda Medical Devices, Madison, WI) and used to measure pulse pressure and heart rate on a digital blood pressure monitor (Columbia Instruments, Columbus, OH). A tracheotomy was performed and the rats were mechanically ventilated at 80 breaths/min with a tidal volume of 2.5 cm^3 using a Harvard Model 680 Rodent Respirator (Harvard Apparatus, South Natick, MA). An ip catheter was used to provide additional anesthesia during the NMR experiment.

SR infusion protocol. $Na_4HTmDOTP$ (molecular weight 860) was purchased from Macrocyclics Inc. (Richardson, TX). Stock solutions of 80 mM $TmDOTP^{5-}$ were prepared as described earlier (*13*). Infusion of SR was initiated at a rate of 2 ml/h. After 10 min, the rate was gradually increased to 6 ml/h over a period of 40 min and then maintained at this level for 20 min. After achieving a chemical shift difference of 3–4 ppm between Na_e^+ and Na_i^+ resonances (typically 45–60 min after the start of SR infusion), the infusion rate was reduced to 1–2 ml/h to maintain a steady shift. No Ca^{2+} was added during infusion of the SR.

SQ and TQ-filtered ^{23}Na spectral data collection. All NMR experiments were performed on a 9.4-T, 8.9-cm vertical bore magnet interfaced to a Varian INOVA console (Varian, Inc., Palo Alto, CA). A 1.5-cm-diameter single-turn surface coil, tunable to 106 MHz, for ^{23}Na was placed over the tumor. A small glass bulb containing 5 mM $TmDOTP^{5-}$ and 60 mM NaCl was placed on top of the surface coil as an external reference. The typical nominal 90° pulse width at the reference bulb was 30 μs.

SQ ^{23}Na spectra were collected using a simple pulse–acquire sequence with a 30 μs excitation pulse and a 10 μs spectrometer dead time followed by acquisition of 1024 complex data points over a spectral width of 10,000 Hz. A predelay of 100 ms was used and 128 signal averages were collected for each SQ spectrum. TQ-filtered spectra were collected with the following pulse sequence (*12*),

$$(\theta)_\Phi\text{–}\tau/2\text{–}((\theta)_\Phi\text{–}(2\theta)_{\Phi+\pi/2}\text{–}(\theta)_\Phi)\text{–}\tau/2 - (\theta)_{\Phi+\pi/2}\text{–}\delta\text{–}(\theta)_{\pi/2}\text{–}(t_{acq})_\pm,$$

where θ is a nominal 90° pulse, τ is the MQ preparation time, δ is the MQ evolution time, and t_{acq} is the data acquisition time. The composite (θ–2θ–θ) pulse is placed at the center of the preparation time to refocus magnetic field inhomogeneities and chemical shift. TQ coherences were selected using the 48-step phase cycling scheme (*12*). The basic phase cycling scheme for selective detection of TQ coherences consisted of six steps in which the phase $\Phi = n\pi/3$, where n was cycled through 0, 1, 2, 3, 4, 5. The FIDs resulting from the pulse sequence were alternately added to and subtracted from computer memory. CYCLOPS phase cycling was included with the basic phase

cycling by incrementing all the pulse phases in steps of $\pi/2$ and appropriately routing the quadrature data in computer memory, giving a 24-step phase cycle. In addition, the phase of the composite (θ–2θ–θ) refocusing pulse was incremented by π giving a 48-step phase cycling scheme. As in the SQ experiment, the θ pulse width was 30 μs. A 10 μs dead time was followed by acquisition of 1024 complex data points over a spectral width of 10,000 Hz. Typically, a preparation delay (τ) of 3 ms and an evolution delay (δ) of 20 μs were used. The value of τ was selected by initially collecting a series of TQ-filtered spectra with various τ delays and choosing the value that gave the maximum signal intensity. The δ delay was kept as short as possible to reduce signal loss due to TQ T_2 relaxation. A predelay of 100 ms was used and 384 signal averages were collected for each TQ-filtered spectrum.

Modulation of TQ-filtered Na_e^+ *signal.* Modulation of the TQ-filtered Na_e^+ signal from the tumor was measured as a function of the evolution time to authenticate the true TQ nature of the signal. A true TQ-filtered signal is modulated according to the equation (*29*),

$$I = I_0 \cos(6\pi\nu\delta)\, e^{-\delta/T_{TQ}}, \qquad [1]$$

where I is the amplitude of the modulated signal, I_0 is the signal amplitude at $\delta = 0$, ν is the frequency offset in Hz, and T_{TQ} is the TQ transverse relaxation time. Therefore, a true TQ-filtered signal would have a maximum positive signal intensity at $\delta = 0$, a null at $\delta = (12\nu)^{-1}$, and a maximum negative intensity at $\delta = (6\nu)^{-1}$. The Na_e^+ signal from the tumor was first shifted by about 4 ppm from the Na_i^+ peak by infusing the SR. The spectrometer frequency was then set on the Na_i^+ signal, and TQ-filtered ^{23}Na spectra were collected with δ delays corresponding to the positive maximum, null, and negative maximum modulation points of the Na_e^+ signal.

^{23}Na relaxation time measurement. A change in the fast and slow transverse relaxation times (T_{2f} and T_{2s}, respectively) of ^{23}Na could cause a significant change in the observed TQ-filtered signal intensity for a given τ value according to the relation (*12*),

$$I = A\left(e^{-\tau/T_{2s}} - e^{-\tau/T_{2f}}\right), \qquad [2]$$

where A is a constant related to the number of nuclei that produce the TQ-filtered Na^+ signal. Therefore, T_{2s} and T_{2f} of intra- and extracellular sodium in the tumor were measured before and 1 h after sacrificing the animal. TQ-filtered spectra were collected at 15 values of τ ranging from 0.38 to 96 ms, whereas δ was kept constant at 20 μs. All other spectrometer and pulse sequence parameters were set as described above. TQ-filtered ^{23}Na relaxation curves were produced by plotting signal area against preparation time. Fast and slow transverse relaxation times were determined by fitting the data points to a biexponential curve. The effect of lineshape on A was ignored because the linewidth is determined mainly by field inhomogeneity effects and by line broadening applied to the free-induction decay (FID) before Fourier transformation. To minimize the effect of time-related changes in $[Na_i^+]$ during the measurement, the data were collected in eight blocks of 48 transients for each τ value. In addition, the order of the τ values was randomized.

Experimental protocol. The magnet was shimmed on the SQ ^{23}Na FID to a linewidth of 35–50 Hz. A SQ ^{23}Na spectrum and a TQ-filtered ^{23}Na relaxation curve were collected. $TmDOTP^{5-}$ was infused to shift the Na_e^+ peak to 3–4 ppm. A SQ spectrum and another TQ-filtered relaxation curve were obtained with the stable Na_e^+ shift. Tumor ischemia was produced by terminating the animals because it is very difficult to stop blood flow to the tumor by clamping its base while the animal is in the magnet. Animals ($n = 5$) were terminated by withdrawing blood through the arterial catheter. This method of killing the animal was chosen because it provides a quick and certain death at a specified time point. One could terminate the animal by giving KCl or an overdose of anesthesia, but we have found that these interventions reduce the Na^+ shift. SQ and TQ-filtered spectra ($\tau = 3$ ms) were collected continuously for the next hour. Another TQ-filtered relaxation curve was then collected to determine T_{2f} and T_{2s} 1 h after death. To determine the changes in SQ and TQ-filtered Na_e^+ signal in the absence of SR, the experimental protocol was repeated without the infusion of $TmDOTP^{5-}$ in a second set of animals ($n = 3$). The true MQ nature of the Na_e^+ signal was tested in a third set of animals ($n = 3$). After achieving a stable Na_e^+ shift, TQ-filtered spectra were collected with different δ values corresponding to the positive maximum, null, and negative maximum modulation points.

Data analysis. SQ and TQ-filtered FIDs were Fourier-transformed after baseline correction and multiplication by a single exponential corresponding to a 10 Hz line broadening using Nuts (Acorn NMR, Livermore, CA). SQ resonance areas were determined by spectral curve fitting and TQ-filtered resonance areas were determined by integration using Nuts. Curve fitting was used for SQ spectra to minimize errors due to overlapping shoulders of the Na_e^+ and Na_i^+ peaks. On the other hand, TQ-filtered spectra did not have overlapping peaks, but have a much more complicated lineshape than SQ spectra because a TQ-filtered signal is a 1 : 1 difference of two Lorentzians of equal areas but different widths (*12*). Therefore, TQ-filtered spectra were integrated after magnitude calculation. Because only relative changes in SQ and TQ-filtered signals were measured, two different methods for measuring SQ and TQ-filtered peak areas could be used.

Values for A, T_{2s}, and T_{2f} were determined by fitting the TQ-filtered peak areas to Eq. [2]. The relaxation times of Na_e^+ in the absence of $TmDOTP^{5-}$ were calculated by subtracting the raw relaxation data points of Na_i^+ from the corresponding raw relaxation data points of total Na^+ without the SR. The subtracted curve was then fit to Eq. [2]. These calculations assume that the presence of $TmDOTP^{5-}$ in the extracellular space does not change the relaxation time of Na_i^+. The fact that we observed

no change in the Na_i^+ linewidth during infusion of increasing quantities of $TmDOTP^{5-}$ suggests that this assumption should be valid.

RESULTS

As reported previously (*12–17, 25*), infusion of 80 mM $TmDOTP^{5-}$ without added Ca^{2+} did not dramatically affect the developed pulse pressure or heart rate; a decrease in mean arterial blood pressure from 100–110 to 70–80 mm Hg was typical. Figure 1 shows SQ and TQ-filtered spectra collected before and after infusion of SR. The TQ-filtered spectra were collected at a preparation delay value that gave the maximum signal amplitude ($\tau = 3$ ms). Before SR infusion, the SQ spectrum contains two signals: the signal at 0 ppm represents the total sodium in the tumor and the signal at 8 ppm arises from the reference bulb. The TQ-filtered spectrum shows only the tumor Na^+ resonance at 0 ppm. The fact that the aqueous ^{23}Na signal from the reference bulb does not appear in the TQ-filtered spectrum indicates successful filtration of the SQ coherences.

After infusion of the SR, the SQ spectrum clearly shows three resonances: Na_i^+ at 0 ppm, Na_e^+ at 3 ppm, and the reference signal at 8 ppm. The Na_i^+ and Na_e^+ resonances are also clearly resolved in the TQ-filtered ^{23}Na spectrum after infusion of the SR. It is apparent from Fig. 1 that the TQ-filtered ^{23}Na spectrum contains a much greater Na_i^+ contribution than the SQ spectrum. Averaged over 5 animals, the SQ Na_i^+ contribution was $22 \pm 4\%$ (mean $\pm$ SEM) of the total SQ ^{23}Na signal while TQ-filtered Na_i^+ contribution was $59 \pm 10\%$ of the total TQ-filtered signal. Once discernible from the Na_e^+ resonance, the Na_i^+ resonance intensity did not change with further infusion of the SR, suggesting that $TmDOTP^{5-}$ does not alter the Na_i^+ concentration in the tumor at the doses required to resolve the two signals.

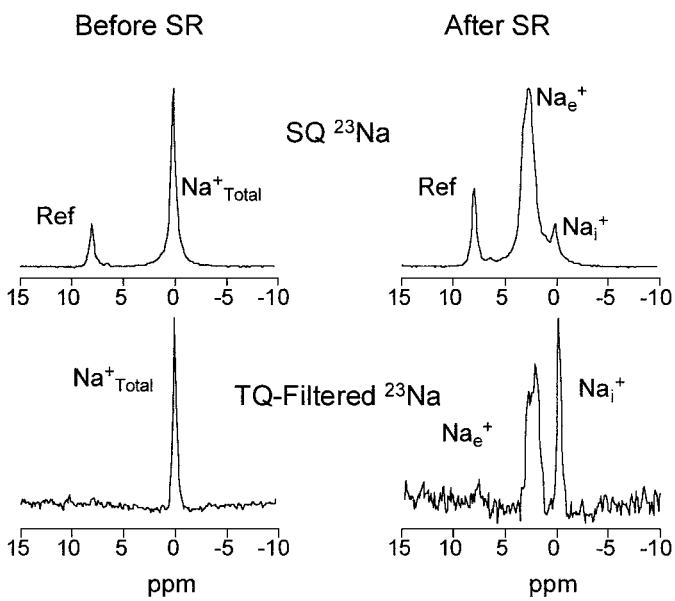

FIG. 1. SQ (top) and TQ-filtered (bottom) ^{23}Na spectra before (left) and after (right) SR infusion. Two signals are present in the SQ spectrum before infusion of SR: total Na^+ in the tumor at 0.0 ppm and the signal from the reference bulb at 8 ppm. Only the tumor signal is present in the TQ-filtered spectrum before the SR. The lack of signal from the aqueous solution in the reference bulb demonstrates successful filtration of SQ coherences in the TQ-filtered spectrum. After infusion of the SR, three signals are seen in the SQ spectrum: tumor Na_i^+ at 0.0 ppm, tumor Na_e^+ at 3 ppm, and the reference signal at 8 ppm. After infusion, the two signals corresponding to Na_i^+ and Na_e^+ can also be seen in the TQ-filtered spectrum. It is clear from the spectra collected after SR infusion that the TQ-filtered spectrum contains a much higher Na_i^+ component than the SQ spectrum.

The ^{23}Na signal from the reference bulb containing a known amount of Na^+ can be used to convert the SQ ^{23}Na signal intensities into intra- and extracellular Na^+ concentrations, as described previously (*16*). While these measurements were not performed in the present research, we have conducted similar experiments showing that SQ ^{23}Na NMR gives identical values compared to ICP spectroscopy (*17*). The [Na_i^+] in 9L gliosarcoma was 19 mM. This is similar to many normal tissues such as liver and kidney. However, [Na_i^+] in the brain is only 5–12 mM. Thus, the glioma has approximately twice Na_i^+ concentration compared to the normal brain.

Quantitation of TQ-filtered ^{23}Na signal intensity using similar procedures is not straightforward because of the use of a surface coil. The TQ-filtered signal intensity (peak height) from Na_i^+ was approximately 7–10% of the SQ signal intensity in the 9L glioma. In a homogeneous RF field, the TQ-filtered signal intensity is expected to be 25–40% of the SQ signal intensity for a value of τ which gives maximum TQ-filtered signal intensity (*12*). The TQ-filtered signal intensity was lower in our experiments because we used a surface coil. A surface coil produces a nonuniform magnetic field, causing variations in the flip angle at different regions of the tumor. Single-quantum and MQ-filtered ^{23}Na signals have different flip angle dependences (*30*), and therefore, the two signals cannot be compared without correcting for these effects.

To prove the true MQ nature of the Na_e^+ signal, the amplitude of the shifted peak was modulated by varying the δ delay. Figure 2 shows TQ-filtered ^{23}Na spectra obtained from the *in vivo* tumor with the Na_e^+ resonance shifted by 3.9 ppm. The δ delay was set to 20 μs (left), 202 μs (middle), and 404 μs (right). As in Fig. 1, the preparation delay was set to 3 ms. With the shortest δ delay ($\delta = 20$ μs), Na_e^+ appears at a positive maximum. With $\delta = 202$ μs, the shifted peak is approximately nulled. The slight Na_e^+ signal intensity at this δ may arise from unstable shift, biological motion, or inhomogeneous distribution of SR in the extracellular spaces. The Na_e^+ signal reaches a negative maximum with $\delta = 404$ μs. Because the Na_e^+ resonance shows the expected modulation as a function of δ delay, we conclude that the Na_e^+ signal is of true TQ-filtered origin. Similar results were obtained after the animal was sacrificed (data not shown).

Figure 3 shows the relative changes in SQ (solid rectangles) and TQ-filtered (open circles) Na_i^+ signals before and up to

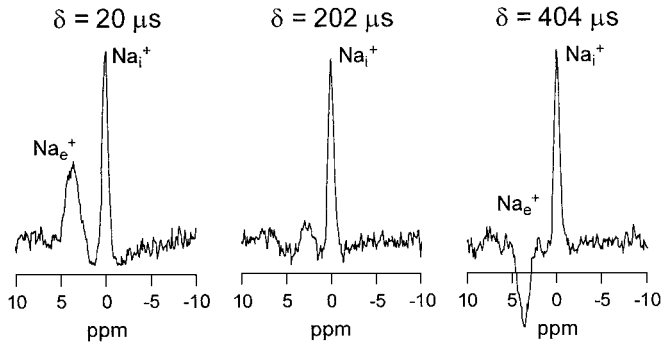

FIG. 2. TQ-filtered ^{23}Na spectra obtained with $\delta = 20\,\mu$s (left), 202 μs (middle), and 404 μs (right). Since the Na_e^+ signal is shifted 3.9 ppm relative to Na_i^+, it is modulated according to Eq. [1]. With Na_i^+ set on resonance, the Na_e^+ signal appears as a positive maximum (left), null (center), and a negative maximum (right). Therefore, we conclude that the Na_e^+ signal is of true MQ origin because it displays the proper modulation characteristics.

1 h after sacrificing the animal. The SQ and TQ-filtered Na_i^+ resonance areas were normalized to their respective value before sacrificing the animal. One hour after death, both SQ and TQ-filtered Na_i^+ signals reached a steady value approximately 75% higher than their values before death. Thus, we can see that relative changes in SQ and TQ-filtered Na_i^+ signals after death were similar.

Since the TQ-filtered spectra obtained after death are collected at a single τ delay, changes in transverse relaxation times could cause significant errors in estimating TQ-filtered longitudinal magnetization. TQ-filtered ^{23}Na relaxation data were collected before and 1 h after sacrificing the animal to determine if T_{2s} and T_{2f} relaxation times had changed. Figure 4 shows TQ-filtered

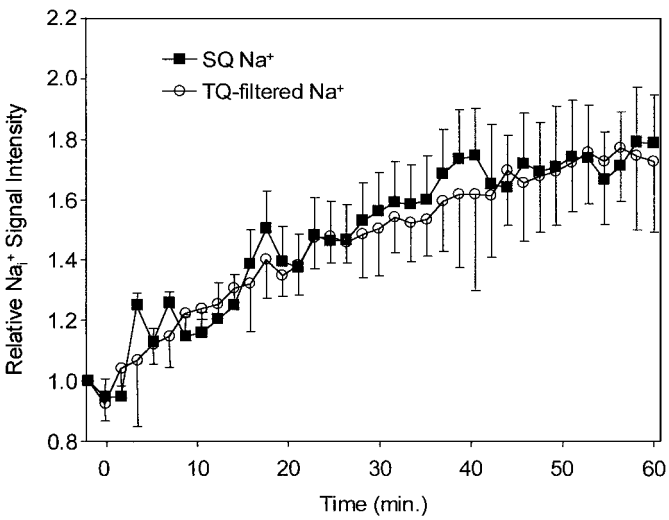

FIG. 3. Relative changes in SQ (solid rectangles) and TQ-filtered (open circles) Na_i^+ resonance areas before and up to 1 h after death. TQ-filtered spectra were collected with $\tau = 3$ ms. The animal was sacrificed at Time 0. One hour after death, SQ and TQ-filtered Na_i^+ signals increased by approximately 75% compared to their values before death.

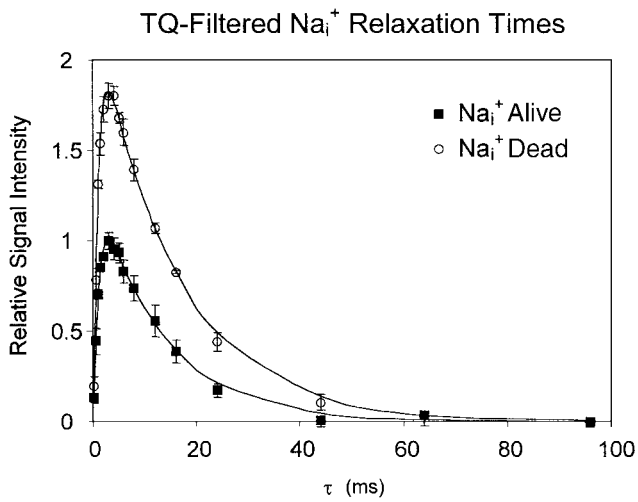

FIG. 4. TQ-filtered Na_i^+ relaxation curves obtained before (solid squares) and 1 h after (open circles) sacrificing the animal. Before sacrificing the animal, $T_{2s} = 13.1 \pm 1.2$ ms and $T_{2f} = 1.00 \pm 0.13$ ms. The value of τ_{opt} before death was 2.78 ms. After death, $T_{2s} = 15.5 \pm 0.6$ ms and $T_{2f} = 0.87 \pm 0.05$ ms, corresponding to a τ_{opt} of 2.65 ms. The fast and slow relaxation times were not significantly different before and 1 h after sacrificing the animal ($P > 0.1$). The solid lines represent least-squares fits to the data points.

Na_i^+ signal intensity plotted against τ delay before (solid rectangles) and after (open circles) sacrificing the animal. As indicated in Fig. 3, the Na_i^+ signal intensity increased by about 75% after sacrificing the animal. The transverse relaxation times for Na_i^+, however, did not significantly change after death. As shown in Table 1, T_{2s} and T_{2f} for TQ-filtered Na_i^+ signal were 13.1 $\pm$ 1.2 and 1.00 $\pm$ 0.13 ms, respectively, before terminating the animals, and 15.5 $\pm$ 0.6 and 0.87 $\pm$ 0.05 after terminating the animals ($P > 0.1$). The optimum τ delay to achieve maximum TQ-filtered Na_i^+ signal intensity before and after death are 2.78 and 2.65 ms, respectively. Both of these values are very close to the τ delay of 3 ms that we used to collect the TQ-filtered spectra after sacrificing the animal. The slight variations in T_{2s} and T_{2f} before and after death can produce only 6 % variations in TQ-filtered signal intensity at 3 ms τ delay.

To determine the effects of sacrificing the animal on the SQ and TQ-filtered Na_e^+ signals, we repeated the experiments without infusing the SR. The presence of $TmDOTP^{5-}$ in the extracellular space greatly alters the relaxation characteristics of the Na_e^+ signal. As shown in Table 1, the T_{2s} and T_{2f} of TQ-filtered Na_e^+ in the presence of SR were 13.0 $\pm$ 1.5 and 0.97 $\pm$ 0.19 ms, respectively. These relaxation times in the absence of SR were 38.7 $\pm$ 5.4 and 1.18 $\pm$ 0.17 ms, respectively (see below). Because TQ-filtered signal strongly depends on ^{23}Na relaxation characteristics, the presence of SR can alter the TQ-filtered signal drastically.

Figure 5 shows the relative changes in total tissue SQ and TQ-filtered ^{23}Na signal intensity before and up to 1 h after sacrificing the animal. The total tissue SQ signal intensity decreased by approximately 10% after exsanguination. At the same

TABLE 1
Experimental TQ-Filtered ^{23}Na Transverse Relaxation Times (in ms) in the sc Implanted 9L Glioma

	Before terminating the animal		After terminating the animal		Percentage change in TQ-filtered ^{23}Na signal intensity at $\tau = 3$ ms
	T_{2s}	T_{2f}	T_{2s}	T_{2f}	
Total tissue Na^+ in the absence of SR	32.9 ± 1.2	0.83 ± 0.06	21.6 ± 0.9	0.87 ± 0.02	5.4%
Na_i^+ in the presence of SR	13.1 ± 1.2	1.00 ± 0.13	15.5 ± 0.6	0.87 ± 0.05	−6.2%
Na_e^+ in the presence of SR	13.0 ± 1.5	0.97 ± 0.19	12.1 ± 1.2	0.74 ± 0.18	−1.9%
Na_e^+ in the absence of SR	38.7 ± 5.4	1.18 ± 0.17	28.5 ± 11.4	0.94 ± 0.38	−1.4%

Note. The relaxation times of Na_e^+ in the absence of $TmDOTP^{5-}$ were calculated by subtracting the raw relaxation data points of Na_i^+ from the corresponding raw relaxation data points of total Na^+ without the SR. The last column lists the percentage change in the observed TQ-filtered signal intensity at $\tau = 3$ ms because of the change in ^{23}Na relaxation time on terminating the animal.

time the TQ-filtered signal intensity increased by approximately 42%. The decrease in SQ ^{23}Na signal intensity was because of a decrease in Na_e^+ from blood loss. The TQ-filtered signal intensity increased largely because of an increase in Na_i^+ concentration. These changes in SQ and TQ-filtered ^{23}Na signal intensity clearly demonstrate that simple SQ ^{23}Na NMR spectroscopy and imaging may not be able to detect tissue damage in certain situations and TQ-filtered ^{23}Na NMR techniques are sensitive to changes in Na_i^+.

The T_{2s} and T_{2f} of total tissue TQ-filtered Na^+ signal in the absence of SR were 32.9 ± 1.2 and 0.83 ± 0.06 ms, respectively. One hour after death, T_{2s} decreased to 21.6 ± 0.9 ms, but T_{2f} (0.87 ± 0.02 ms) did not change significantly. The decrease in the T_{2s} was largely because of an increase in Na_i^+. Figure 6 shows the TQ-filtered relaxation curves of the Na_e^+ signal calculated by subtracting the Na_i^+ relaxation data points from the total Na^+ relaxation data points. As with the Na_i^+ signal shown in Fig. 4, there is no significant difference in relaxation times before and 1 h after sacrificing the animal (before death; $T_{2s} = 38.7 \pm 5.4$ ms, $T_{2f} = 1.18 \pm 0.17$ ms; after death; $T_{2s} = 28.5 \pm 11.4$ ms, $T_{2f} = 0.94 \pm 0.38$ ms, $P > 0.1$). The standard errors in these relaxation times are much greater than the Na_i^+ relaxation times,

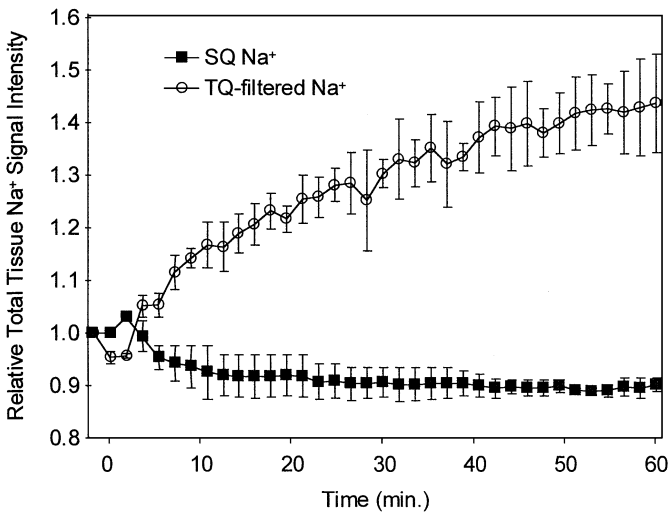

FIG. 5. Relative changes in total tissue SQ (solid rectangles) and TQ-filtered (open circles) ^{23}Na resonance areas before and up to 1 h after death. TQ-filtered spectra were collected with $\tau = 3$ ms. The animal was sacrificed at Time 0. One hour after death, total tumor SQ signal decreased by 10% compared to before death while the total TQ-filtered ^{23}Na signal increased by approximately 45%.

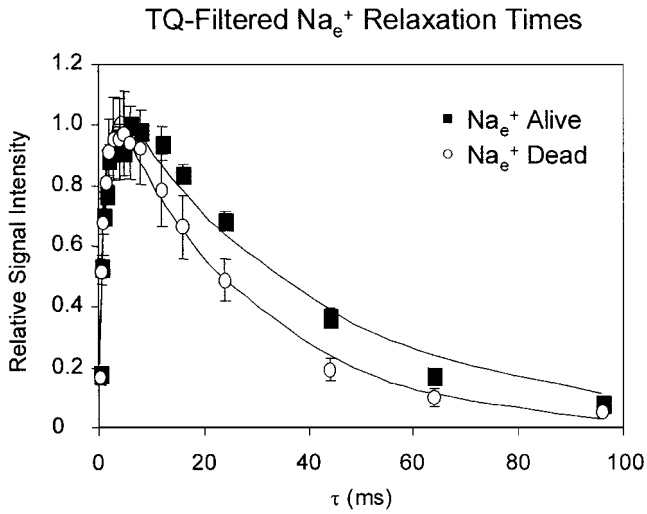

FIG. 6. TQ-filtered Na_e^+ relaxation curves obtained before (solid squares) and 1 h after (open circles) sacrificing the animal. Before sacrificing the animal, $T_{2s} = 38.7 \pm 5.4$ ms and $T_{2f} = 1.18 \pm 0.17$ ms, corresponding to a τ_{opt} of 4.25 ms. After death, $T_{2s} = 28.5 \pm 11.4$ ms and $T_{2f} = 0.94 \pm 0.38$ ms, corresponding to a τ_{opt} of 3.32 ms. As was seen for Na_i^+, the fast and slow relaxation times were not significantly different before and 1 h after sacrificing the animal ($P > 0.1$). The large errors seen in these relaxation time measurements are due to animal to animal variability. The solid lines represent least-squares fits to the data points.

because the calculation of Na_e^+ relaxation time requires measurements from two groups of animals (the Na_i^+ measurements from animals with SR are subtracted from the total Na^+ measurements from animals without SR to obtain the Na_e^+ signal). The animal to animal variability causes these measurements to have very large variations. The Na_e^+ relaxation times correspond to τ_{opt} values of 4.25 ms before death and 3.32 ms after death. A change in T_{2s} and T_{2f} from 38.7 and 1.18 ms to 28.5 and 0.94 ms, respectively, can produce only a 1–2% increase in TQ-filtered signal intensity at 3 ms τ delay. Thus, it is safe to assume that the changes in Na_e^+ transverse relaxation times after sacrificing the animal did not alter the observed TQ-filtered ^{23}Na signal intensity.

Because the relaxation times of TQ-filtered Na_i^+ and Na_e^+ signals (in the absence of SR) do not change significantly on sacrificing the animal, the changes in the Na_e^+ signal in the absence of the SR was calculated by subtracting the Na_i^+ signal intensity in the presence of the SR from total signal intensity without the SR. The total tissue SQ and TQ-filtered signal intensities were normalized to 1 for both groups of animals (one group without SR and the other group with SR). The relative Na_i^+ contribution was calculated from the animals that received the SR and was subtracted from the total Na^+ signal intensity measured from animals without the SR. These calculations yielded the relative contribution of Na_e^+ to the SQ and TQ-filtered signals relative to the total tissue SQ and TQ-filtered Na^+ signal before terminating the animal. The results of these calculations are shown in Fig. 7. Again, the error bars in the data are relatively large because data from two different groups of animals (one terminated in the presence of SR and the other in the absence of SR) were used in the calculations. The errors were calculated by taking the root-mean-square of the errors in both the groups. The relative contribution of SQ Na_e^+ signal to the total tissue decreased from ~80 to ~55% 1 h after death, while the relative contribution of TQ-filtered Na_e^+ signal remained approximately 40%. These data suggest that although TQ-filtered ^{23}Na signal contains a significant contribution from Na_e^+, this signal is insensitive to possible changes from Na_e^+ and may be considered as a constant background signal. Thus, TQ-filtered ^{23}Na NMR can be used for monitoring changes in Na_i^+ in the tumor model.

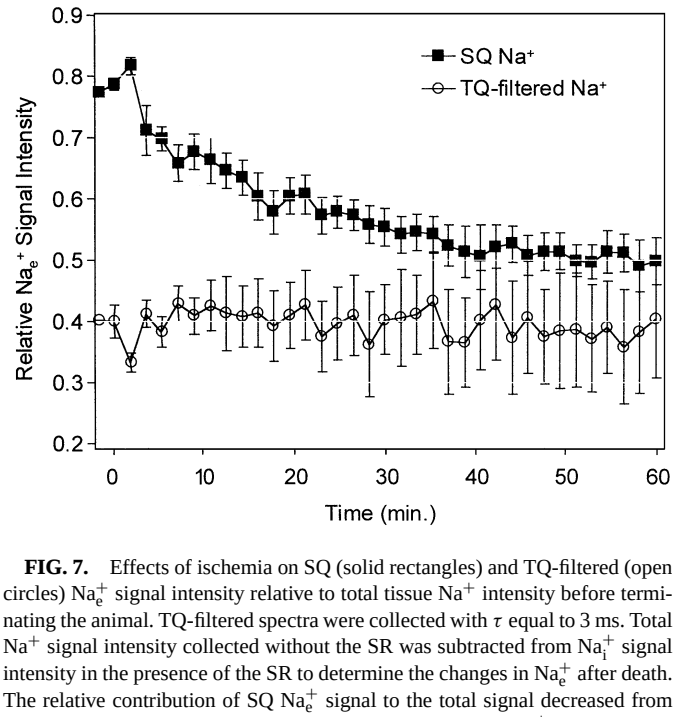

FIG. 7. Effects of ischemia on SQ (solid rectangles) and TQ-filtered (open circles) Na_e^+ signal intensity relative to total tissue Na^+ intensity before terminating the animal. TQ-filtered spectra were collected with τ equal to 3 ms. Total Na^+ signal intensity collected without the SR was subtracted from Na_i^+ signal intensity in the presence of the SR to determine the changes in Na_e^+ after death. The relative contribution of SQ Na_e^+ signal to the total signal decreased from ~80 to 55%, while the relative contribution of TQ-filtered Na_e^+ signal did not change significantly.

DISCUSSION

We have evaluated, for the first time, the utility of TQ-filtered ^{23}Na spectroscopy for discriminating between Na_i^+ and Na_e^+ in a solid tumor *in vivo* using $TmDOTP^{5-}$. We have demonstrated that the Na_i^+ signal represents about 22% of the SQ spectrum, but about 60% of the TQ-filtered spectrum. Therefore, the Na_i^+ contribution in TQ-filtered spectra is much higher than in SQ spectra. The observed 40% contribution from Na_e^+ in TQ-filtered spectra from the 9L glioma is similar to the contributions reported from the *in vivo* rat liver (*25*) and the perfused rat heart (*24*).

We also observed that both SQ and TQ-filtered Na_i^+ signals increase by about 75% 1 h after sacrificing the animal. The TQ-filtered relaxation times do not change during this time, indicating that changes observed by TQ-filtered spectra with δ equal to 3 ms represent changes in TQ-filtered magnetization. We conducted similar experiments without the SR to determine changes in the Na_e^+ signal. By subtracting the SQ and TQ-filtered Na_i^+ signals from the total SQ and TQ-filtered signals, we see that the SQ Na_e^+ signal decreases 35%, while the TQ-filtered Na_e^+ signal does not change significantly. This demonstrates that the TQ-filtered ^{23}Na signal is relatively insensitive to changes in Na_e^+ content. Similar results have also been demonstrated in the rat liver (*25*). The TQ-filtered Na_e^+ relaxation times are also statistically identical before and 1 h after sacrificing the animal.

Both the observations that Na_i^+ contributes a larger percentage to the TQ-filtered spectrum than the SQ spectrum and that Na_e^+ decreases with time in the SQ spectrum but not in the TQ-filtered spectrum suggest that there are at least two different pools of Na_e^+; one "free" pool that does not pass through a MQ filter, and another "bound" pool that interacts with macromolecules and can pass through a MQ filter. Srinivasan *et al.* (*31*) have recently shown that addition of Li^+ quenches both DQ- and TQ-filtered ^{23}Na resonance from unsealed and cytoskeleton-depleted human red blood cells because of competitive binding of Li^+ at the Na^+ binding sites on the plasma membrane. This strongly suggests that the TQ-filtered Na_e^+ signal originates from interaction of Na_e^+ with extracellular membrane proteins. Because changes in Na_e^+ signal intensity in SQ ^{23}Na spectra result from a change

in extracellular volume and not Na_e^+ concentration, the pool of sodium that interacts with the extracellular membrane proteins may not change with changes in Na_e^+ contents. This may explain why the TQ-filtered Na_e^+ signal is relatively constant despite a decrease in the SQ Na_e^+ signal.

Upon sacrificing the animal, we observed that SQ and TQ-filtered Na_i^+ signals increased in an identical fashion. Likewise, the increase in TQ-filtered Na_i^+ in the perfused heart (*32, 33*) is very similar to the increases seen in SQ Na_i^+ signal (*34*). In contrast, the TQ-filtered Na_i^+ signal in the liver (*25*) and the DQ-filtered Na_i^+ signal in the brain (*27*) increase much more than the respective SQ Na_i^+ signals. The discrepancies seen between SQ and TQ-filtered Na_i^+ signals in the brain and liver have been attributed to changes in macromolecular binding upon ischemia causing more Na^+ to undergo MQ transitions (*25*). There could either be an increase in the amount of electrostatic binding sites available to Na_i^+ or a change in the percentage of Na_i^+ contributing to the TQ-filtered signal due to the large amount of Na_e^+ entering the cell. The first hypothesis may cause a change in TQ-filtered relaxation times because the dominant factor effecting T_{2f} is macromolecular binding (*35*). In fact, the value of T_{2f} in the liver doubled after sacrificing the animal (*25*), suggesting that macromolecular binding contributes to the discrepancy between SQ and TQ-filtered Na_i^+ signals. Of course, it is also possible that both of the mechanisms contribute to the higher increase in TQ-filtered Na_i^+ compared to SQ Na_i^+. Since we observed equal changes in SQ and TQ-filtered Na_i^+ signals and the T_{2f} relaxation times do not change with sacrificing the animal, we conclude that the changes in TQ-filtered Na_i^+ signal intensity from the tumor are entirely due to Na^+ entering the cells.

In the present study a surface coil was used to obtain SQ and TQ-filtered ^{23}Na spectra from the sc implanted 9L glioma. The use of a surface coil presents three problems. First, it is very difficult to compare SQ and TQ-filtered signal intensities because the two signals have different flip-angle dependences (*30*). Second, Brown and Wimperis (*36*) have shown that if the flip angle of the refocusing pulse used in the spin-echo and multiple-quantum filtration experiments deviates from 180° then the measured transverse relaxations show a strong dependence on flip angle. They proposed the use of a MQ filter pulse sequence without the refocusing pulse to reduce this problem. However, the effects of main magnetic field inhomogeneity are not refocused with their pulse sequence. In our experiments, we used a composite refocusing pulse to avoid problems arising from both the main magnetic field inhomogeneity and the RF field inhomogeneity. We have checked the effects of RF field inhomogeneity on the relaxation time measurements with our MQ filter sequence by measuring the relaxation times of a gel phantom using a surface coil and a volume coil. Both coils gave identical values for T_{2s} and T_{2f} for the gel. Another potential problem because of the inhomogeneous RF field from a surface coil is that translational diffusion of sodium in and out of the region affected by the coil can alter the effectiveness of an MQ filter. We investigated the effect of flow on MQ-filtered ^{23}Na signal intensity and relaxation time using a phantom consisting of two cylindrical compartments. Normal saline containing 10 mM $TmDOTP^{5-}$ was circulated through the outer compartment of the phantom to mimic blood flow and the inner compartment was filled with normal saline in 10% agarose gel to induce the biexponential relaxation characteristic of Na_i^+. The flow of saline was changed to various flow rates while collecting TQ-filtered ^{23}Na spectra. We did not observe any MQ-filtered signal from the outer compartment and the signal intensity from the inner compartment did not change at any flow rate. The relaxation time values with and without flow were also identical. These observations suggest that flow does not affect the efficiency of the MQ filter in discriminating between mono- and biexponentially relaxing sodium or the measurement of relaxation times with a surface coil.

Monitoring and imaging of changes in Na_i^+ in tumors by MQ filter techniques have numerous potential applications. The Na_i^+ concentration in tumors is generally elevated compared to normal tissue (*1–3*). We have previously shown that the Na_i^+ concentration in the 9L gliosarcoma is ~19 mM (*17*). This is approximately twice compared to the Na_i^+ concentration in the normal brain. Measurement of Na_i^+ content by invasive means in a variety of cancer types has demonstrated a positive correlation between Na_i^+ content and mitotic activity (*1–3*). As mitotic activity constitutes an important prognostic factor used in the design of a therapeutic plan, assessing Na_i^+ content by MQ-filtered ^{23}Na MR spectroscopy and imaging in tumors could prove to be a valuable tool for the clinical evaluation of tumors. MQ-filtered ^{23}Na MR may also be useful in detecting early response to therapy because tumor physiology and metabolism are expected to change during the course of therapy. It would be important to be able to assess the ongoing results of therapy during the treatment course because therapy parameters or therapy type could be modified on the basis of such data. We have recently shown that chemotherapy of sc implanted 9L glioma with 1,3-bis(2-chloroethyl)-1-nitrosourea produced a 60% decrease in TQ-filtered ^{23}Na signal intensity compared to untreated tumors (*37*). Thus, MQ-filtered ^{23}Na MRS may prove useful for monitoring response to therapy. There is great interest in enhancing tumor response to nonsurgical therapy by various physiological and metabolic manipulations such as altering tumor blood flow, oxygenation, or energy metabolism. Because these manipulations are also expected to alter Na_i^+ levels in tumor and the transmembrane Na^+ gradient plays a key role in the survival of cells in the hostile environment of the tumor, MQ-filtered ^{23}Na MR techniques may also prove useful in monitoring tumor sensitization to therapy.

In conclusion, the results of these experiments show: (1) the TQ-filtered ^{23}Na signal has a much higher contribution from Na_i^+ than the SQ signal, (2) the TQ-filtered and SQ Na_i^+ signals increase in an identical fashion after sacrificing the animal, and (3) the TQ-filtered Na_e^+ signal remains relatively constant despite large changes in Na_e^+ content. These findings suggest

that changes in total TQ-filtered ^{23}Na signal intensity (without an SR) in the 9L tumor model may accurately reflect changes in Na_i^+. These techniques may prove to be useful for diagnosis and treatment of cancer.

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