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Compatibility of Superparamagnetic Iron Oxide Nanoparticle Labeling for ^1H MRI Cell Tracking with ^{31}P MRS for Bioenergetic Measurements

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Abstract

Labeling of cells with superparamagnetic iron oxide nanoparticles permits cell tracking by ^1H MRI while ^{31}P MRS allows non-invasive evaluation of cellular bioenergetics. We evaluated the compatibility of these two techniques by obtaining ^{31}P NMR spectra of iron-labeled and unlabeled immobilized C2C12 myoblast cells in vitro. Broadened but usable ^{31}P spectra were obtained, and peak area ratios of resonances corresponding to intracellular metabolites showed no significant differences between labeled and unlabeled cell populations. We conclude that ^{31}P NMR spectra can be obtained from cells labeled with sufficient iron to permit visualization by ^1H imaging protocols and that these spectra have sufficient quality to be used in assessing metabolic status. This result introduces the possibility of using localized ^{31}P MRS to evaluate the viability of iron-labeled therapeutic cells as well as surrounding host tissue in vivo.

Keywords

cell tracking; bioenergetics; phosphorus; P31 NMR; SPIO; MRI; cell labeling; viability

INTRODUCTION

The MRI visualization and tracking of cells by means of in vitro labeling with superparamagnetic iron oxide (SPIO) nanoparticles and microparticles has been a subject of intense interest in recent years. In particular, MRI has been used to locate labeled therapeutic cells in vivo, for instance following direct injection into myocardium (1) or during homing toward a stroke lesion following remote injection (2). Despite these advances, no non-invasive means to evaluate the bioenergetic state of these labeled cells has been reported thus far. This is of particular concern as it has become widely recognized that a large fraction of therapeutic cells die shortly after systemic administration or engraftment, despite the possible persistence of MRI contrast at the injection or homing site (3) (4) (5). Moreover, there is a need to evaluate the bioenergetic status of damaged tissue undergoing repair by therapeutic cells over time. Due to its ability to assess the concentration of high-energy phosphates, intracellular pH and metabolite reaction rates and fluxes in vivo, ^{31}P

MRS offers a highly attractive means for obtaining these physiological data, but the acquisition of usable ^{31}P NMR data is expected to be hampered by the broadening effect induced by the presence of intracellular SPIO particles in close vicinity to the metabolites of interest. Indeed, the same local B_0 field inhomogeneity that enables relatively small numbers of iron-labeled cells to be detected in a T_2^* -weighted ^1H MRI scan might render important species such as ATP and phosphocreatine (PCr) totally invisible in a ^{31}P NMR spectrum of the same cells. It is the goal of the present work to evaluate the feasibility of obtaining ^{31}P NMR spectra from a population of SPIO-labeled cells contained in a MR-compatible perfusion chamber. Care was taken to ensure, by means of histological and flow cytometric assays, that labeled and unlabeled (control) cells possessed comparable viability and function, both prior and subsequent to NMR scanning, so that differences in their ^{31}P spectra could be attributed uniquely to the presence of intracellular nanoparticles. Photomicrographs of Prussian blue-stained cells were studied to obtain a semi-quantitative evaluation of the distribution of SPIO nanoparticles among labeled cells as we anticipated that knowledge of this distribution would be needed to interpret the effects of iron labeling on their ^{31}P NMR spectra. Finally, quantitative measurements on ^{31}P spectra of labeled and unlabeled cells were compared statistically to identify differences which would impact the use of these data for the bioenergetic evaluation of SPIO-labeled cells.

METHODS

Cell Culture Techniques

C2C12 myoblasts, a murine cell line widely used in cardiac (6) and skeletal (7) muscle tissue engineering and cardiac repair studies (8), demonstrates myotube formation in vitro in response to reduced serum concentration (9). C2C12 cells were grown in DMEM media containing 25 mM D-glucose (Invitrogen, Carlsbad, CA) supplemented with 20% v/v fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah) and 1% v/v penicillin-streptomycin reagent (Invitrogen). The high serum concentration was chosen to prevent differentiation of the myoblasts into myocytes. Cells were grown in standard 162 cm² tissue culture flasks in a 5% v/v CO₂:air atmosphere at 37 °C. Cells were passaged at 70% confluence with media changes every 3-4 days.

Cell Labeling

A sterile suspension of SPIO nanoparticles (Feridex IV, Advanced Magnetix, Cambridge, MA) was diluted 1:14 in serum-free OptiMEM I media (Invitrogen) to give an iron concentration of 0.8 mg/ml. An equal volume of Lipofectamine 2000 transfection agent (1 mg/ml; Invitrogen) diluted 1:50 in OptiMEM I was mixed with the diluted SPIO suspension and incubated for 15 minutes at room temperature to coat the SPIO particles with cationic lipid. Media was removed from a tissue culture flask containing approximately 3×10^4 cells/cm² and replaced with 20 ml of OptiMEM I. 1 ml of the SPIO-Lipofectamine mixture was then added drop-wise to the media to give final concentrations of 20 µg Fe/ml and 0.5 µg/ml Lipofectamine 2000. The flask was then incubated for 12 hours at 37 °C in a 5% CO₂ atmosphere to permit cellular uptake of the SPIO particles. After labeling, cells were released with 0.25% trypsin solution (Invitrogen), pelleted and washed three times with sterile Dulbecco's phosphate buffered saline (DPBS; Invitrogen) to remove SPIO particles not internalized by cells.

Cell Viability Assays

Aliquots of labeled and unlabeled (control) cells, which were exposed to neither SPIO particles nor Lipofectamine, were stained with Trypan blue (Sigma, St. Louis, MO), transferred to a hemacytometer and examined under a microscope. Total cell number and percent viability were calculated from counts of stained and unstained cells. Separate

aliquots, containing 3×10^5 to 5×10^5 cells each, were washed twice in DPBS, resuspended in 100 μ l annexin V binding buffer (BD Pharmingen, San Diego, CA) and incubated with 5 μ l FITC-conjugated annexin V reagent (BD Pharmingen) with or without 10 μ l of 50 μ g/ml propidium iodide solution (PI, BD Pharmingen) for 15 minutes in the dark. These cells were then analyzed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA), yielding percentages of apoptotic (annexin V positive, PI negative), necrotic and viable cells. Trypan blue and flow cytometric assays for cell viability were performed on labeled and unlabeled cells both before and after MRI and NMR scanning in perfused gel beads, as described below.

Quantitative Iron Uptake Assay

A relaxometric assay was employed to measure SPIO uptake (10). Aliquots of ca. 2×10^5 labeled cells were counted then digested in 1 ml of 6M HCl for 1 hr at room temperature and pipetted into individual wells of a home-built 96-well assay plate fabricated from a solid block of polyetherimide (ULTEM) plastic. Eight standard samples with iron concentrations ranging from 0 to 10 μ g/ml were prepared by diluting 1000 ppm Fe atomic absorption standard solution (Sigma-Aldrich) and were pipetted into adjacent wells of the plate. T_2 -weighted images of the plate were obtained with a 7 Tesla 30 cm bore Bruker Biospec MRI scanner (Bruker Biospin MRI GmbH, Ettlingen, Germany) using an 80-echo CPMG sequence with TR/TE = 2 s / 20 ms, FOV = 5×5 cm, MTX = 256×256 , slice thickness = 2 mm and NEX = 2. A three-parameter fit was performed on average pixel intensities of ROI's defined within each well to determine T_2 values. A standard curve was constructed from a fit of the $R_2 = 1/T_2$ versus [Fe] data to the function $R_2 = R_{20} + C [\text{Fe}]$ ($R^2 \geq 0.98$). Iron concentration within each cell digest sample was then calculated from its R_2 value and expressed as picograms of iron per cell (pg Fe/cell). Averages were obtained from three separately-cultured cell populations labeled under identical conditions.

Characterization of SPIO Uptake Distribution

To visualize SPIO uptake via Prussian blue staining, additional aliquots of labeled and unlabeled cells were fixed in 10% formalin, exposed to a freshly-prepared solution of 0.12 M $\text{K}_4\text{Fe}(\text{CN})_6$ in 1M HCl (Sigma-Aldrich) for 20 minutes and examined under a microscope. Clusters of SPIO particles were visible as dark blue-black features. To obtain an estimate of the distribution of SPIO particles among labeled cells, photographs of fields of approximately 100 Prussian blue-stained cells were analyzed. Regions of interest were manually drawn around each cell and an intensity threshold was established empirically to identify SPIO clusters by their low intensity. The number of these dark pixels within each region of interest was then calculated by MetaMorph (Molecular Devices, Sunnyvale, CA). Assays for iron uptake and distribution were performed on aliquots of SPIO-labeled cells prior to NMR and MRI scanning.

Cell Proliferation Assay

In order to examine whether SPIO labeling had an effect on the rate of cell division, aliquots containing 5000 labeled or unlabeled cells were plated on standard tissue culture flasks. Flasks were incubated under standard tissue culture conditions using the media described above. Cells were passaged at 80% confluency. After 3 or 5 days incubation, cells were released by trypsinization and counted using a hemacytometer. This cell proliferation assay was performed for cells prior to MRI and NMR scanning.

Cell Differentiation Assay

To determine whether SPIO labeling adversely affected the potential of C2C12 myoblasts to differentiate into viable myotubes, approximately 10^5 labeled or unlabeled C2C12 cells were

seeded in individual wells of 24-well tissue culture plates pre-coated with Matrigel (Sigma) and incubated in media containing 5% FBS in place of the 20% concentration used to maintain the cell line in the myoblast phenotype. After four days of incubation in media with reduced serum concentration, cells were observed by light microscopy at 50X magnification. Myotube formation was quantified using the method of Bernardini et al (11). The C2C12 cells used for the differentiation assay were not used in NMR or MRI experiments.

Immobilization and perfusion of labeled cells

In preparation for MRI and NMR scanning, cells (ca. 2.0×10^7 per experiment) were suspended in a sterile 1.2% solution of sodium alginate (Sigma) in 0.15 M NaCl. This suspension was added dropwise to 100 mM CaCl_2 to form beads of 2.5-3 mm diameter containing ca. 4×10^5 cells per bead. Beads were washed three times in DPBS, suspended in DMEM media containing 20% fetal bovine serum and packed into a home-built MR-compatible perfusion system consisting of a 10 mm screw-top NMR tube fitted with porous Teflon plates (Small Parts, Inc.) threaded onto a central PEEK media inlet tube. A 1 mm diameter glass capillary containing 0.4 M methylenediphosphonic acid (MDP; Sigma) was inserted into the central tube as a ^{31}P NMR chemical shift standard. Media was circulated through this system via a peristaltic pump using a loop of gas-permeable silicone tubing enclosed in a 95% air: 5% CO_2 v/v atmosphere (12). The temperatures of the media reservoir and NMR tube were maintained at $37.0 \pm 0.1^\circ\text{C}$ via a water bath and the NMR probe heater system, respectively.

Magnetic resonance imaging and spectroscopy of iron-labeled cells

^1H MRI and ^{31}P MRS data were acquired for the perfused cell beads using a 9.4T/105 mm vertical magnet (MagneX Scientific, Ltd., Oxford, UK) interfaced to a Bruker DMX400 spectrometer and equipped with a three-axis 1000 mT/m shielded gradient set and 15 mm ^1H birdcage resonator or 10 mm $^1\text{H}/\text{X}$ broadband probe (Bruker BioSpin NMR, Rheinstetten, Germany). Prior to the acquisition of each image or spectrum, unlocalized manual shimming was performed on the ^1H signal from H_2O . ^1H gradient echo images were acquired with a single, 0.5 mm thick sagittal slice, 3.0×1.2 cm FOV ($V \times H$), 256×256 matrix size, $\text{TR} = 3$ s, $\text{TE} = 1.5$ ms and $\text{NEX} = 1$. Spin echo images were acquired with identical parameters except for an echo time of 7.2 ms. MRI signal acquisition was triggered by a piezoelectric sensor (PowerLab, AD Instruments, Castle Hill, Australia) attached to the silicone perfusion tubing in order to eliminate artifacts associated with flowing media. Nonlocalized ^{31}P NMR spectra were acquired without ^1H decoupling using a one-pulse sequence with $\text{TR} = 2$ s, $10 \mu\text{s}$ 90° rectangular pulses, 4096 complex points sampled at a rate of 10 kHz and 27000 averages, saved to disk as 60 blocks of 450 scans each. After acquisition of ^{31}P NMR spectra, cells were released from gel beads by exposure to 25 mM EDTA for 10 min. After washing with DPBS, the recovered cells were examined using the Trypan blue and flow cytometry assays described above.

MRS data processing and statistics

^{31}P data were zero-filled from 4K to 8K complex points and exponentially multiplied with a line-broadening factor of 12 Hz prior to Fourier transformation. Following spline baseline correction and chemical shift calibration with $\text{MDP} = 0$ ppm, Lorentzian deconvolution was performed to obtain widths and areas for each of the major spectral peaks. Peak assignments were made by referring to published spectra of isolated cardiac myocytes (13-15). Calculation of pH from the chemical shift of inorganic phosphate (Pi) was performed based on the calibration of Gadian (16). Blocks of scans were selected for addition and final processing based on stability of the Pi frequency to within 5 Hz and stability of the NMR-determined pH to within 0.1 pH units over the course of the data collection. The number of usable blocks meeting these criteria resulted in summed FID's containing from 12150 to

20700 (mean $\pm$ std. dev.: 17775 ± 3620) averages for unlabeled cells and from 9450 to 25650 (mean $\pm$ std. dev.: 15621 ± 6515) averages for labeled cells. Incorporation of all usable scans in the analysis permitted working with spectra of maximal SNR. Quantitative data are presented as mean $\pm$ standard deviation. Comparisons between unlabeled and labeled preparations and between pre-NMR and post-NMR measurements were made using a two-tailed unpaired Student's t-test assuming unequal variances or a two-tailed paired t-test, respectively. A p value of 0.05 or smaller was taken to indicate a statistically-significant difference.

RESULTS

SPIO Uptake by C2C12 Myoblasts

NMR relaxometry measurements of iron concentration in acid digests of SPIO-labeled C2C12 cells indicated an average uptake of 0.81 ± 0.08 pg Fe/cell ($n=3$). Microscopic observation of Prussian blue stained cells revealed extensive, non-uniform uptake of SPIO particles by the C2C12 myoblasts (Fig. 1). Quantitative analysis of this photomicrograph indicated a broad, asymmetric distribution of SPIO particles per cell, with all cells labeled to some extent.

Cell Viability

Both Trypan blue ($n=6$) and flow cytometric (FACS) assays ($n=4$) indicated slightly lower viability for SPIO-labeled C2C12 cells when compared to unlabeled cells. Results prior to NMR analysis were as follows. Trypan blue positive for dead cells: unlabeled, $3.8 \pm 0.2\%$, labeled, $5.2 \pm 0.1\%$, $p < 0.05$. Necrotic cells by FACS: unlabeled, $5.0 \pm 0.9\%$; labeled, $5.6 \pm 0.4\%$, NS; apoptotic cells by FACS: unlabeled, $8.8 \pm 0.2\%$; labeled, $11.6 \pm 0.6\%$; $p < 0.05$. No significant differences were found in viability indices between cells examined before versus after immobilization in alginate beads, perfusion during MRI and NMR scanning and release from the beads with EDTA treatment.

Proliferation and Differentiation of Labeled Myoblasts

After 3 days of culture, SPIO-labeled C2C12 cells increased in number by a factor of 4.9 ± 0.1 while unlabeled cells exhibited a 4.5 ± 0.1 fold increase ($n=3$ each, $p = 0.007$). However, after 5 days of culture, no significant difference was observed in proliferation factor between labeled and unlabeled myoblasts (labeled: 22.2 ± 1.5 , unlabeled: 21.4 ± 0.7 ; $n=3$ each, $p = 0.477$). In addition, SPIO-labeled and unlabeled C2C12 cells showed similar myotubule formation following culture in media with 5% FBS. Specifically, the differentiation assay detected 83 ± 3 myotube intersection points per mm^2 for unlabeled C2C12 cells versus 82 ± 4 intersections per mm^2 for SPIO-labeled cells ($n=3$ each, $p = 0.67$).

^1H MRI and ^{31}P MRS of labeled cells

Proton NMR linewidths after shimming were generally greater for SPIO-labeled preparations than for unlabeled preparations (70.7 ± 48.0 Hz vs. 32.2 ± 19.3 Hz, respectively, $n=6$ each), however, this difference was not statistically significant. Gradient echo (Fig. 2) and spin echo images (data not shown) showed excellent contrast for beads containing SPIO-labeled cells despite the short echo times used. Fig. 3 shows representative ^{31}P spectra of labeled (right) and unlabeled (left) C2C12 cells. All major peaks were visible and quantifiable in spite of labeling-induced broadening.

Intracellular and extracellular Pi could not be resolved in these spectra, although the intracellular contribution to the Pi resonance is expected to be small given the large volume of phosphate-containing media. The pH, as calculated from the chemical shifts of Pi and γ -ATP, was 7.30 ± 0.08 ($n=6$) and 7.33 ± 0.33 ($n=7$), $p = 0.811$, for unlabeled and labeled

cells, respectively. Phosphomonoester and phosphodiester resonances were visible downfield and upfield of the Pi resonance, respectively.

No significant differences were observed in the mean chemical shifts of Pi, PCr, β -ATP, and γ -ATP relative to MDP between labeled and unlabeled samples; the change in the chemical shift of α -ATP, while reaching statistical significance, was small compared to its linewidth.

Significantly greater linewidths were observed from labeled as compared to unlabeled samples for all observed resonances except MDP and PCr (Table 1). As seen, the degree of broadening differed substantially among the metabolites. Peak area ratios with Pi as the normalizing denominator, as well as the PCr/ β -ATP ratio, were also calculated as indicators of metabolic status. In spite of the broadening of resonances, none of these ratios were statistically different between the labeled and unlabeled cells (sample number ranging from n=2 to n=7).

DISCUSSION

In the present study, C2C12 mouse myoblasts were intracellularly labeled with Feridex SPIO nanoparticles using Lipofectamine 2000 as a transfection agent via an adaptation of a protocol for labeling primary porcine myoblasts (17). Like the authors of the original protocol, we found significant uptake of SPIO particles with minimal effects on cell viability and proliferation. Using Feridex with protamine sulfate as a transfection agent, it has been previously demonstrated that C2C12 cells retained their ability to differentiate into multinucleated myotubes under low serum conditions (18). Similar results have been reported for labeling C2C12 cells with ferumoxide:poly-L-lysine complexes and MD-100 magnetodendrimers in the absence of a transfection agent (19) (20). These findings, and in particular the negligible effects on viability and proliferative capacity, are in agreement with our observations.

Our results indicate that immobilizing cells in alginate beads, perfusing them during NMR scanning and releasing them via EDTA treatment did not significantly affect cell viability. The low concentration of dead or dying cells, measured both before and after NMR scanning, indicates that the large observed inorganic phosphate peak cannot be attributed to a large fraction of metabolically-compromised cells, but rather is attributable to the use of phosphate-containing media to perfuse the cells. While precluding identification of a spectral peak uniquely reflecting intracellular Pi, this ensured the bioenergetic stability of the cells during the long period of signal averaging needed to acquire high-SNR ^{31}P NMR spectra.

The average amount of iron taken up by the C2C12 cells was somewhat smaller than that reported for ferumoxide labeling of muscle-derived mc13 stem cells (19) or rabbit skeletal myoblasts (21). However, the degree of labeling achieved was sufficient to produce marked contrast even on very mildly T_2 or T_2^* -weighted images (Fig. 2). This point is central to our investigation, in that the ability to quantify ^{31}P spectra of iron labeled cells is of interest only in the setting of sufficient labeling to produce MRI contrast.

The linewidth of the MDP resonance, used as an indicator of the quality of shimming, ranged between 51 and 79 Hz in the co-added spectra. This variation is much smaller than linewidth alterations observed for intracellular metabolites due to iron labeling; this lack of broadening is as expected, since the MDP external standard was not in close proximity to SPIO particles. Conversely, the absence of a significantly broader linewidth for PCr in the ^{31}P spectra of SPIO-labeled cells reflects the fact that only those spectra with PCr peaks sufficiently narrow to be unambiguously detectable were quantified. The increase in linewidth of the largely extracellular Pi peak is consistent with the fact that the field

distortions produced by SPIO particles extend well beyond the cell membrane, as reflected in the imaging literature (22,23).

Highly labeled cells may give rise to metabolite peaks too broad to be detectable, in which case the integrated ^{31}P NMR spectral intensity from the ensemble of labeled cells will be less than that arising from an equal number of unlabeled cells. We estimated the extent of this effect by comparing the ratio of metabolite peak areas to the area of the total Pi peak in labeled and unlabeled preparations. As noted, the small size of the PCr and ATP/ADP peaks and the small number of non-viable cells indicate that the Pi peak is dominated by extracellular Pi. It is reasonable to assume that all extracellular Pi is visible, given its modest increase in linewidth upon labeling. Thus, the observation that ATP/total Pi ratios were not significantly different between labeled and unlabeled cells suggests that no more than a small fraction of cells possess unobservable metabolites. Further, the lack of a significant effect of SPIO labeling on the chemical shifts of γ -ATP and total Pi suggests that ^{31}P NMR could be used to measure intracellular pH in SPIO-labeled cells maintained in low-phosphate media. The lack of pronounced changes in metabolite peak positions with SPIO labeling confirms that these peaks can be assigned based on chemical shifts observed for unlabeled samples. Similarly, the consistency of peak area ratios from cells with and without labeling indicates that the bioenergetic status of SPIO-labeled cells can be evaluated in the same way as for unlabeled cells.

Light microscopy of Prussian blue stained myoblasts (Fig. 1) indicated that all cells were labeled to some extent. This confirms that the spectra we observed for SPIO-labeled populations did not arise just from a small sub-population of unlabeled cells. Since SPIO nanoparticles within a cell may induce a B_0 field distortion well beyond the dimensions of the cell (22,23) and cells were dispersed in alginate gel at a high density in our experiments, it is likely that the line broadening of intracellular metabolite peaks was attributable to the presence of both intracellular SPIO particles and particles within nearby cells.

Heterogeneity of iron uptake among SPIO-labeled cells, as evidenced by photomicrographs of Prussian blue stained cells, is a ubiquitous phenomenon (1,18,24-27). This may have minimal impact on the ability to track relatively large clusters of labeled cells in vivo. However, the line shapes and widths of metabolite peaks in ^{31}P NMR spectra of labeled cells would show a dependence on the SPIO distribution, with heavily-labeled cells yielding broad components superimposed on narrower lines arising from lightly-labeled cells. A simulation of the resulting spectrum (28) would require knowledge of the SPIO distribution, available from magnetophoresis studies (29) or by measuring the volumes of hypointense features in a 3D gradient echo image of a dilute suspension of individual labeled cells (30,31). Alternatively, one could simplify the interpretation of ^{31}P NMR spectra of SPIO-labeled cells by preparing a homogeneously-labeled population, either by modifications to the labeling procedure (32) or through magnetic sorting (33).

Metabolite peaks in our spectra of concentrated suspensions of SPIO-labeled myoblasts were not greatly broader than in localized ^{31}P spectra of in vivo mouse myocardium acquired at the same field strength (34). This suggests that ^{31}P resolution and signal-to-noise ratios should not be dramatically worse in localized in vivo spectra of tissues containing SPIO-labeled cells as compared to tissues devoid of labeled cells.

The application of localized ^{31}P MRS to the study of bioenergetics in labeled therapeutic cells and surrounding tissue in vivo will require overcoming several technical challenges. The large voxel sizes required for ^{31}P MRS will inevitably include both labeled and unlabeled cells. However, with appropriate choice of SPIO labeling concentration, it may be possible to separate these components due to differences in linewidth, either by filtering

during acquisition or by post-processing. In addition, even when these components cannot be separated, ^{31}P MRS may still be used to characterize the bioenergetic response of surrounding tissue to cell therapy. Moreover, it is evident that voxels placed adjacent to but outside the region of ^1H MRI hypointensity should yield high-quality ^{31}P spectra. This has been illustrated in previous work (35) in which well-resolved ^{31}P spectra of a hepatic tumor were obtained with suppression of the signal from surrounding liver by pre-administration of a large intravenous dose of SPIO nanoparticles. It was also found that at SPIO doses sufficiently low to minimally broaden ^{31}P spectral lines from normal liver, good ^1H MRI contrast could be obtained between liver and adjacent tumor. Thus, as we have demonstrated here as well, there exists an SPIO concentration at which labeled cells are conspicuous on ^1H MR images yet ^{31}P MR spectra are not excessively perturbed.

CONCLUSIONS

Usable ^{31}P NMR spectra can be obtained from immobilized, perfused cells despite the presence of intracellular SPIO particles at a concentration sufficient for visualization by ^1H MRI. These spectra can be qualitatively interpreted in the same way as those of unlabeled cells, potentially permitting MRI-based cell tracking techniques to be paired with a non-invasive assessment of bioenergetic status in both therapeutic cells and host tissue.

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Abbreviations

SPIO	superparamagnetic iron oxide
PCr	phosphocreatine
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
FITC	fluorescein isothiocyanate
PI	propidium iodide
Pi	inorganic phosphate
CPMG	Carr-Purcell-Meiboom-Gill
FOV	field of view
MTX	matrix size
NEX	number of signal averages
ROI	region of interest
FBS	fetal bovine serum
PEEK	polyetheretherketone
MDP	methylenediphosphonic acid
EDTA	ethylenediaminetetraacetic acid
FID	free induction decay
SNR	signal-to-noise ratio

FACS	flow-activated cell sorting
PME	phosphomonoesters
PDE	phosphodiesterases
NTP	nucleotide triphosphates
NDP	nucleotide diphosphates
NAD/H	nicotinamide adenine dinucleotide (/reduced form)
NADP/H	nicotinamide adenine dinucleotide phosphate (/reduced form)
DPDE	diphosphodiesterases

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Fig. 1. Photomicrograph (300X) of Prussian blue-stained SPIO-labeled C2C12 cells indicating iron oxide nanoparticle uptake. Note that all cells in the field contain nanoparticles. Although individual SPIO particles are too small to observe, the load per cell has been estimated and reported as the number of pixels below an empirically-determined intensity threshold; the inset histogram shows the distribution within 110 labeled cells.

Fig. 2. ^1H MRI of C2C12 mouse myoblasts in perfused alginate beads. Gradient echo images ($\text{TE} = 1.5 \text{ ms}$, $\text{TR} = 3 \text{ s}$) of unlabeled cells (left) and labeled cells (right). Synchronizing MRI acquisition to media flow gave good bead contrast without blurring. Note that labeling was sufficient to give good MRI contrast for labeled C2C12 cells.

Fig. 3. ^{31}P NMR spectra of C2C12 mouse myoblasts in perfused alginate beads. Left: 2.0×10^7 unlabeled cells; 14400 averages; Right: 2.0×10^7 iron-labeled cells; 22050 averages. Spectra were acquired without ^1H decoupling using a one-pulse sequence with 90° flip angle and $\text{TR} = 2$ s and were processed with 12 Hz exponential apodization. Chemical shifts are referenced to an external MDP standard. Spectra are scaled uniformly to permit direct comparison of peak intensities. Blue curves indicate Lorentzian fits; residuals are shown in red. Magnified views of the spectral regions upfield from the Pi peak are shown as insets. As expected, metabolite peaks are broader for iron-labeled cells, although they are clearly visible and quantifiable. Metabolite chemical shifts (sample number ranging from $n=2$ to $n=7$) were unchanged by labeling, with the exception of a small but statistically significant ($p = 0.02$) change in the shift of $\alpha\text{-ATP}$ from -28.37 ± 0.03 to -28.60 ± 0.20 ppm; this change was small compared to the linewidth of the peak. The peak area ratios PCr/Pi , ATP/Pi and $\text{PCr}/\beta\text{-ATP}$ exhibited no significant differences between labeled and unlabeled cells (sample number ranging from $n=2$ to $n=7$). PME = phosphomonoesters; Pi = inorganic phosphate; PDE = phosphodiester; PCr = phosphocreatine; NTP = nucleotide triphosphates; NDP = nucleotide diphosphates; NAD/H = nicotinamide adenine dinucleotide (/reduced form); NADP/H = nicotinamide adenine dinucleotide phosphate (/reduced form); DPDE = diphosphodiester.

Table 1

³¹P linewidths in spectra of isolated, perfused C2C12 mouse myoblasts. All values were calculated as full width at half height and are shown in Hz. n indicates the number of independent experiments for each metabolite and labeling group.

	Unlabeled Cells	SPIO-labeled Cells	p (unlabeled vs labeled)
MDP	76.6 ± 11.8 (n=3)	69.4 ± 12.8 (n=7)	0.437
Total Pi	32.4 ± 10.4 (n=6)	69.1 ± 34.4 (n=7)	0.030*
PCr	34.9 ± 15.8 (n=5)	38.6 ± 8.9 (n=2)	0.716
γ-ATP	60.2 ± 19.2 (n=6)	181.3 ± 98.0 (n=7)	0.017*
α-ATP	59.0 ± 19.4 (n=6)	227.6 ± 123.8 (n=7)	0.011*
β-ATP	84.5 ± 10.6 (n=6)	169.3 ± 55.0 (n=6)	0.012*