Apparent exchange rate for breast cancer characterization

Samo Lasic\textsuperscript{a},* Stina Oredsson\textsuperscript{b}, Savannah C. Partridge\textsuperscript{c}, Lao H. Saal\textsuperscript{d}, Daniel Topgaard\textsuperscript{e}, Markus Nilsson\textsuperscript{f} and Karin Bryskhe\textsuperscript{a}

Although diffusion MRI has shown promise for the characterization of breast cancer, it has low specificity to malignant subtypes. Higher specificity might be achieved if the effects of cell morphology and molecular exchange across cell membranes could be disentangled. The quantification of exchange might thus allow the differentiation of different types of breast cancer cells. Based on differences in diffusion rates between the intra- and extracellular compartments, filter exchange spectroscopy/imaging (FEXSY/FEXI) provides non-invasive quantification of the apparent exchange rate (AXR) of water between the two compartments. To test the feasibility of FEXSY for the differentiation of different breast cancer cells, we performed experiments on several breast epithelial cell lines in vitro. Furthermore, we performed the first in vivo FEXI measurement of water exchange in human breast. In cell suspensions, pulsed gradient spin-echo experiments with large $b$ values and variable pulse duration allow the characterization of the intracellular compartment, whereas FEXSY provides a quantification of AXR. These experiments are very sensitive to the physiological state of cells and can be used to establish reliable protocols for the culture and harvesting of cells. Our results suggest that different breast cancer subtypes can be distinguished on the basis of their AXR values in cell suspensions. Time-resolved measurements allow the monitoring of the physiological state of cells in suspensions over the time-scale of hours, and reveal an abrupt disintegration of the intracellular compartment. In vivo, exchange can be detected in a tumor, whereas, in normal tissue, the exchange rate is outside the range experimentally accessible for FEXI. At present, low signal-to-noise ratio and limited scan time allows the quantification of AXR only in a region of interest of relatively large tumors. © 2016 The Authors. NMR in Biomedicine published by John Wiley & Sons Ltd.

Additional supporting information may be found in the online version of this article at the publisher’s web site.

Keywords: pulsed gradient spin echo; diffusion; MRI; water exchange; AXR; permeability; cell; cancer

INTRODUCTION

Breast cancer is the most common cancer and leading cause of cancer-related death of women worldwide (1). The disease is heterogeneous, both biologically and clinically, and at least four well-characterized molecular subtypes of breast cancer have been repeatedly observed (2). At present, we lack diagnostic tests and biomarkers to accurately predict breast cancer outcome, and over one-quarter of women diagnosed with breast cancer will die from late recurrences. Novel non-invasive imaging methods may help us to better understand breast cancer biology and may have diagnostic, prognostic and treatment-predictive value.

Diffusion-weighted imaging (DWI) has shown promise for the detection and characterization of breast cancer. The added value of DWI for reducing false positives in breast MRI is currently being investigated in a large multicenter trial sponsored by the National Institutes of Health (3). Although breast malignancies can be differentiated from many benign lesions on the basis of the

\* Correspondence to: S. Lasic, CR Development AB, Chemical Center, Box 124, SE 22100 Lund, Sweden.
E-mail: samo@crdev.se

\textsuperscript{a} S. Lasic, K. Bryskhe
CR Development AB, Lund, Sweden

\textsuperscript{b} S. Oredsson
Department of Biology, Lund University, Lund, Sweden

\textsuperscript{c} S. C. Partridge
Department of Radiology, University of Washington, Seattle Cancer Care Alliance, Seattle, Western Australia, USA

\textsuperscript{d} L. H. Saal
Division of Oncology and Pathology, Department of Clinical Sciences, Lund University Cancer Center, Lund, Sweden

\textsuperscript{e} D. Topgaard
Center for Chemistry and Chemical Engineering, Lund University

\textsuperscript{f} M. Nilsson
Lund University Bioimaging Center, Lund University, Lund, Sweden

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Abbreviations used: ADC, apparent diffusion coefficient; ATCC, American Type Culture Collection; AXR, apparent exchange rate; DCE, diffusion contrast-enhanced; DDE, double diffusion encoding; DWI, diffusion-weighted imaging; EPI, echo planar imaging; ER, estrogen receptor; FEXSY/FEXI, filter exchange spectroscopy/imaging; GPA, Gaussian phase approximation; HER2, human epidermal growth factor receptor 2; PGSE, pulsed gradient spin-echo; PR, progesterone receptor; ROI, region of interest; SDE, single diffusion ending; SNR, signal-to-noise ratio.

NMR Biomed. 2016; 29: 631–639 © 2016 The Authors. NMR in Biomedicine published by John Wiley & Sons Ltd.
apparent diffusion coefficient (ADC), there is substantial overlap between malignant histological subtypes (4).

Diffusional exchange of water between microenvironments with different apparent diffusivities, e.g., intra- and extracellular space, can be quantified by filter exchange spectroscopy (FEXSY) (5) or filter exchange imaging (FEXI) (6,7). FEXSY was initially applied to yeast cell suspensions to measure the cell membrane permeability (5). To quantify exchange in more complex systems, such as tissue or in cell suspensions with a polydispersed intracellular compartment, e.g., cells of various shapes and sizes, the concept of the apparent exchange rate (AXR) was developed (6). AXR is applicable under low signal-to-noise ratio (SNR) conditions and can be mapped in vivo in standard clinical scanners (4). AXR may provide a unique assessment of tissue organization and physiology, and therefore constitutes a valuable new biomarker for breast lesion characterization. For example, the cell membrane permeability of tumor cells may differ depending on the cancer subtype, proliferative capacity or tumor microenvironment in vivo, and may have relevance for drug delivery through tissues and into target cells. AXR could also be used to study cell morphology and membrane integrity (8).

The purpose of this study was to test the feasibility of FEXI for the differentiation of breast cancer cell lines based on AXR and for the measurement of AXR of human breast tumors in vivo. In addition to FEXSY, we performed complementary single pulsed gradient spin-echo (PGSE) experiments on all the cell lines in vitro. The use of an extended range of $b$ values in the PGSE experiment allowed us to assess the reliability of AXR and to obtain additional morphological data.

MATERIALS AND METHODS

Intracellular diffusion by PGSE

To estimate the fraction of intracellular water $f_i$ in cell suspensions with largely polydispersed cell morphology, we acquired data from a single diffusion ending (SDE) sequence, i.e., PGSE with variable pulse length. For analysis, we use a phenomenological size distribution and known expressions for restricted diffusion in the Gaussian phase approximation (GPA).

In the PGSE experiment, the sensitivity to diffusion is adjusted by the strength $G$, duration $\delta$ and the interval between the leading edges of the gradient pulses $\Delta$. The echo attenuation is proportional to the diffusion weighting factor $b = \gamma G G F \Delta t_d$, where $\gamma$ is the nuclear gyromagnetic ratio and $t_d = \Delta - \delta/3$ is the diffusion time.

In a porous medium, restrictions, e.g., cell membranes, yield an apparent diffusivity $D$ that is reduced compared with the bulk value $D_0$. In GPA and for simple geometries (planar, cylindrical and spherical), expressions for $D$ have been derived (9). For spheres with radius $R$, the apparent diffusivity is given by (10,11):

$$D_{\Delta, \delta, R} = \frac{2}{ \Delta - \delta/3 } \sum_{n=1}^{\infty} \frac{2 \alpha_m^2 D_0 \delta - 2 L(\delta) + 2L(\Delta) - L(\Delta - \delta) - L(\Delta + \delta)} { \alpha_m^2 (4n^2 - 2)(\alpha_m^2 D_0 \delta)^2 }$$

In Equation [11], $L(t) = \exp(-\alpha_m^2 D_0 t)$ and $\alpha_m$ is the $m$th root of:

$$J_{3/2}(\alpha_m R) - \alpha_m R J_{5/2}(\alpha_m R) = 0$$

where $J_n$ is the $n$th-order Bessel function of the first kind.

In general, the echo attenuation is given by a superposition of contributions from all compartments with different apparent diffusivities $D_c$:

$$S(b) = S_0 \sum_{n} f_n \exp[-D_n b]$$

where $S_0$ is the relaxation-weighted signal intensity, $b$ is the diffusion weighting factor and $f_n$ are the relative signal contributions from different compartments, where $\sum f_n = 1$. It should be noted that $f_n$ may account for different relaxation rates in different compartments. ADC is determined as the attenuation in the limit of low $b$ and is given by:

$$\text{ADC} = -\lim_{b \to 0} \frac{\partial}{\partial b} \left( \frac{S(b)}{S_0} \right) = \sum_{n} f_n D_n.$$  

A cell suspension represents a two-compartment system with an intra- and extracellular compartment. If the cells are of rather uniform size and shape, the signal attenuation measured with $b$ values up to $10^{12} \text{s/m}^2$ exhibits a bi-exponential decay (5,6,9). In tissue or in suspensions in which the cell morphology is polydispersed, this behavior is more complex, and may have relevance for drug delivery through tissues and into target cells. AXR could also be used to study cell morphology and membrane integrity (8).

AXR measurement

A double diffusion encoding (DDE) sequence (12) for AXR measurement consists of two PGSE blocks separated by a variable mixing time $t_{mv}$, as depicted in Fig. 1a. After application of the first PGSE block, according to Equation [3], the signal contributions with higher diffusivity are attenuated more than those with lower diffusivity (Fig. 1b). This results in a reduced ADC according to Equation [4]. Although the first PGSE block acts as a diffusion filter (‘filter block’), providing diffusion weighting $b_0$, the second block is used to detect the apparent diffusivity (‘detection block’) with variable diffusion weighting $b$. During $t_{mv}$, molecular exchange causes the redistribution of signal between compartments and the filtered ADC is gradually restored to its equilibrium value (Fig. 1b).
i.e. using a single PGSE experiment, yielding the attenuation as:

\[ S(b, t_m) = S_0(t_m) \exp[-\text{ADC}'(t_m) \cdot b], \]

where \( \text{ADC}'(t_m) \) denotes the filtered ADC and \( S_0(t_m) \) is the relaxation-weighted and filtered signal intensity. ADC \( (t_m) \) is given according to first-order reaction kinetics (6) by:

\[ \text{ADC}'(t_m) = \text{ADC}[1 - \sigma \exp(-\text{AXR} \cdot t_m)], \]

where ADC is the equilibrium ADC and \( \sigma \) represents the efficiency of the diffusion filter in the range 0–1. It should be noted that \( \sigma \) depends on the diffusion filter applied and on the comparison between the intracellular water fraction \( f_i \) before and after filtering (see equation [9] in ref. (6)). For \( f_i \) approaching zero, the filter efficiency becomes zero and AXR cannot be determined. Furthermore, at higher AXR, the intracellular/extracellular contrast in PGSE blocks is expected to gradually decrease leading to lower \( \sigma \). A larger \( \sigma \), as a result of increased filter strength, provides a larger dynamic range of ADC values at the expense of reduced SNR (6).

The equilibrium ADC can be estimated by omitting the filter block, i.e., using a single PGSE experiment, yielding the attenuation as:

\[ S(b) = S_0 \exp[-\text{ADC} \cdot b], \]

where \( S_0 \) is the relaxation-weighted non-filtered intensity.

### In vitro experiments

**Human breast epithelial cell lines**

One non-cancerous breast epithelial cell line derived from a reduction mammoplasty, MCF-10 A, and 10 breast cancer cell lines representing three different breast cancer subtypes [luminal: MCF-7, CAMA-1 and T-47D; human epidermal growth factor receptor 2 (HER2): SK-BR-3, ZR-75-30 and HCC202; basal: HCC1937, L56Br-C1, SUM149 and MDA-MB-436] were used. Initial tests were performed with a subset of these cell lines to establish optimal experimental conditions. The cells were cultured as monolayers in the medium as described in ref. (13). All cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA), with the exception of L56Br-C1, which was established in Lund (14).

The NMR experiments required a minimum cell number of more than \( 50 \times 10^6 \) cells. Cells were seeded in hydrophobic Petri dishes [as suggested by ATCC] and harvested during the exponential growth phase by gentle scraping. For comparison, selected cell lines were also grown as monolayers in roller bottles and harvested using Accutase (Sigma, Stockholm, Sweden) or by scraping. Except where explicitly stated, all the presented results refer to cells grown in Petri dishes and harvested by scraping. Following harvesting, the cells were suspended in phosphate-buffered supplemented saline and transferred to 5-mm disposable NMR tubes 150 min before starting the measurements and kept on ice. The samples were centrifuged mildly (at 1000 \( g \) for 2 min) 1 h before measurement (fixed protocol for all samples). Further details on sample preparation are given in Supporting information.

For scanning electron microscopy, cells harvested by scraping were fixed in 4% paraformaldehyde in phosphate-buffered saline at \( 4 \) °C, and then dehydrated in a series with ethanol with increasing concentration. The cells were mounted and sputter coated with gold-palladium (15 nm) before examination in a JEOL JSM-5600 LV microscope (JEOL, Tokyo).

### NMR acquisition

**In vitro experiments** were performed at \( 37 \) °C using a, Bruker, Karlsruhe, Germany Avance II 200 spectrometer (4.7 T, Bruker DIF-25 probe, Bruker variable temperature unit with thermostat air flow, ±0.1 °C accuracy). The PGSE and FEXSY sequences were performed in an alternating fashion repeatedly up to 20 h. One PGSE experiment followed five FEXSY experiments. The sequence of these six experiments was repeated approximately every 30.5 min. Signal intensities were extracted by integration of the water peak.

Single PGSE experiments similar to those described in ref. (9) were performed using six different pulse durations \( \delta \) logarithmically spaced between 1 and 20 ms. For each \( \delta \) value, the pulse separation \( \Delta \) was adjusted to give a diffusion time \( t_d \) of 20 ms. Gradient amplitudes were adjusted for each \( \delta \) value to yield a fixed set of 22 logarithmically spaced values in the range 0.0132–9.1 ms/\( \mu \)m². A constant TE of 50 ms was used.

The experimental protocol for FEXSY was similar to that described in refs. (6,7). Experiments employed seven mixing times linearly spaced between 11 and 260 ms, gradient pulses of duration \( \delta = 10 \) ms, separated by \( \Delta = 12 \) ms, and eight \( b \) values linearly spaced between 0.00347 and 0.347 ms/\( \mu \)m². The equilibrium ADC was determined by omitting the filter PGSE in the FEXSY sequence. In filtering, \( b_s = 1.58 \) ms/\( \mu \)m² was used. The value of \( b_s \) was optimized empirically to maximize the filtering efficiency \( \sigma \) while providing sufficient SNR for the detection block. In each experiment, 128 transients were acquired during 3 min.
In vivo experiments.

Subjects

With institutional review board approval, in vivo imaging was performed in eight consenting female patients with known invasive breast cancer (invasive ductal carcinoma) who were undergoing breast MRI to evaluate the extent of disease and/or to monitor therapy. Subjects ranged in age from 42 to 63 years (median, 54.5 years). All patients had diagnostic core biopsy prior to MRI: median of 26.5 days (range, 7–53 days) after biopsy. Tumors ranged in size from 14 to 99 mm (median, 27 mm).

MRI acquisition

Imaging was performed on a Philips, Best, The Netherlands 3-T Achieva TX scanner with a 16-channel breast coil. The parameters of the FEXI protocol in vivo were as follows: $\delta = 10$ ms; $\Delta = 21$ ms; $t_m = 12$ ms ($\times2$); $250$ ms ($\times2$); $b_1 = 0.3$ ms/μm$^2$; $b = 0.044$ ms/μm$^2$ ($\times3$); $0.51$ ms/μm$^2$ ($\times6$). Here, ‘$\times2$’ means that the acquisition was repeated twice with the same parameter, and correspondingly for ‘$\times3$’ and ‘$\times6$’. The values of $t_m$ and $b_1$ were selected with an optimization minimizing noise propagation, assuming ADC values of $1.3–1.9$ μm$^2$/ms (8,15). FEXI was performed with diffusion encoding applied along three orthogonal directions. Seven 5-mm-thick slices were acquired using echo planar imaging (EPI) with $3 \times 3$-mm$^2$ spatial resolution, TR = 3 s and a total scan time of 6 min and 6 s. Because standard fat suppression did not perform well, we isolated the water and fat signals by chemical shift separation.

Image analysis

A region of interest (ROI) was placed in the tumor region on FEXI images by identifying the lesion location and extent on contrast-enhanced MRI images acquired during the same breast MRI examination. Care was taken to avoid areas of necrosis or biopsy-related artifacts within the tumor. For AXR quantification purposes, the signal was averaged over all directions and over all voxels within the ROI. Data could be analyzed only if the following criteria were fulfilled: (i) for application of the AXR model, the filter efficiency needed to be sufficient to detect a significant change in diffusivity during the applied mixing times, resulting in AXR values in the range $1–10$ s$^{-1}$; (ii) chemical shift separation was sufficient for patients with a small- or medium-sized breast; (iii) for sufficient chemical shift separation, the lesion needed to be far away from the chest wall; (iv) for sufficient SNR, the lesion needed to be at least 1 cm wide.

Parameter estimation

All parameters were obtained by non-linear least-squares fitting implemented in Matlab. Errors corresponding to the standard deviation were estimated by Monte Carlo analysis (16) using 1000 fitting iterations. Rician noise, estimated from the fitted residuals, was added to the original data for each fitting iteration. The mean and standard deviation of the fitting parameters were calculated from the resulting parameter distributions.

The data measured by the single PGSE experiment were fitted by Equation [5] using the restricted diffusion model in GPA [Equations [1] and [2]] for the intracellular compartment and the log-normal size distribution [Equation [6]]. The following parameters were fitted: the diffusion coefficients for the intra- and extracellular compartments, $D_0$ and $D_\infty$, respectively, the intracellular fraction $f_c$, mean size $\mu$ and dispersion coefficient $s$. The size distribution $P(R)$ was generated using 100 linearly spaced bins between 0.01 and 100 μm. The signal attenuation data measured using the FEXSY/FEXI protocol were fitted by Equations [7] and [9] using AXR, ADC, $\sigma$, $S$, and a set of $S_i$ as adjustable parameters.

RESULTS AND DISCUSSION

Single PGSE and FEXI on cell suspensions

Single PGSE provided a complementary measure of cell integrity in terms of the amount of intracellular water, and thus served to assess the reliability of the AXR measure. In addition, single PGSE provided an estimate of cell size. The results of single PGSE experiments are presented first, followed by the results of FEXSY and the analysis of AXR. Lastly, time-resolved parameters from both experiments are presented.

Probing of the intracellular compartment by single PGSE

In all cases, a polydispersed intracellular compartment was inferred from the multi-exponential signal attenuation in the PGSE experiments. The SNRs were in the range from approximately 620 to 20 (from lowest to highest $b$ values). An example dataset for MCF-7 cells is shown in Fig. 2. For the MCF-7 cells, the analysis yielded $\mu = 13.2 \pm 0.5$ μm, $s = 0.57 \pm 0.03$, $f_c = 0.52 \pm 0.02$, $D_0 = 1.53 \pm 0.03$ μm$^2$/ms and $D_\infty = 2.79 \pm 0.05$ μm$^2$/ms. The distribution mean size (Fig. 2b) corresponded to the mean cell size visible on the scanning electron micrograph (Fig. 2c). An accurate estimation of the size distribution based on micrographs would be challenging as the cells were scraped and thus not spherical. Cells look clumpy and can appear very large because of cytoplasmic extrusions that do not contribute to the intracellular volume. The log-normal size distributions have often been used within the diffusion NMR community (17–23) and in cell biology (24). This simple parametric distribution fitted well to all of our datasets. It should be noted that the size distribution estimated here could be biased if the different cell sizes have different relaxation times. Although the log-normal distribution of spherical restrictions is a rather crude approximation for the cell morphology, the analysis of the PGSE data allowed a robust estimation of the intracellular fraction $f_c$ which was the primary aim of this analysis. The value of $f_c$ observed in MCF-7 cells represents a typical value for all cell suspensions measured in this study. It should be noted that this value depends on cell packing, which was affected by our centrifugation protocol. Calculation of the probability distribution of diffusion coefficients $P(D)$, based on the estimated $P(R)$, confirmed that an adequate diffusive contrast between the intra- and extracellular compartments existed, providing the necessary precondition for AXR quantification (Fig. 2d).

As is evident from Fig. 2a, the diffusive contrast between the two compartments depends on the pulse duration $\delta$. The multi-exponential decays observed in the single PGSE experiment (Fig. 2), caused by a polydispersed intracellular compartment, suggested that the analysis of exchange in terms of a single exchange rate or intracellular lifetime (5) was not feasible. Instead, the AXR analysis (6) needed to be used to gain information about an average or ‘apparent’ exchange rate. Analysis of the PGSE experiments presented here [see Equations [1]–[6]] assumed that the exchange was negligible during the PGSE sequence. This assumption was supported by FEXSY experiments in all cell suspensions, indicating that exchange took place at a relatively longer time-scale. Exchange during a single
PGSE would cause a negative bias in the estimated intracellular fraction and a positive bias in estimated cell sizes.

**Determination of AXR by FEXSY**

An example of FEXSY data and the AXR analysis for MCF-7 cells is shown in Fig. 3. Diffusion filtering resulted in a reduced ADC' at short $t_m$, which gradually approached the equilibrium value at prolonged $t_m$. The SNRs were in the range from approximately 670 to 410 (for non-filtered data from lowest to highest $b$ values).

The optimal fit of Equations [7]–[9] to the data, shown in Fig. 3a, yielded AXR = 5.0 ± 0.7 s$^{-1}$, ADC = 1.482 ± 0.001 μm$^2$/ms and $\sigma = 0.36 ± 0.01$. Examples of ADC'($t_m$) for all cell lines are included in the Supporting information. Keeping the timing parameters of the PGSE blocks constant decouples the effects of morphology and exchange in FEXSY (6). However, fast exchange diminishes the diffusion contrast between the intracellular and extracellular compartments, leading to a reduced precision of AXR estimations.

It has been shown that the AXR analysis can be successfully applied to a minimal dataset consisting of only two $t_m$ values (8,15). This is important in our in vivo experiments, where a set of nine diffusion-encoded images at two short $t_m$ values (with filter active and non-active) and two long $t_m$ values (both with filter active) was collected. To verify the feasibility of AXR analysis when only two mixing times are used, we performed a similar analysis on a reduced dataset. Retaining only the data at the shortest and two longest mixing times (four $t_m$ values in total), which is equivalent to the in vivo protocol, yields AXR = 5.0 ± 1.3 s$^{-1}$, which
is the same result as obtained from the entire dataset, but with decreased precision.

**Time-resolved single PGSE and FEXSY**

The results of repeated experiments performed with MCF-7 cells are shown in Fig. 4. Cells were grown in Petri dishes or roller bottles and harvested by scraping (Fig. 4a) or grown in roller bottles and harvested using Accutase (Fig. 4b). Each experiment was repeated twice. The intracellular fraction $f_i$ and the ADC parameters inform us how the cell membrane integrity changes over time and allows the inference of the lifetime of the cells (stability of the samples). Initially, a gradual increase in $f_i$ and decrease in ADC could be observed in all cases, which was a consequence of cell sedimentation. After several hours, an abrupt drop in $f_i$ and increase in ADC indicates the breakdown of the intracellular compartment. It should be noted that, by ‘breakdown’, we refer to the loss of the ability of the membrane to separate the intra- and extracellular water populations, i.e. to limit water exchange between the two compartments. This breakdown time limit, chosen when a systematic decrease in $f_i$ and increase in ADC started, was used to identify the time interval during which AXR could not be reliably determined (gray markers in Fig. 4).

$f_i$ and ADC depend largely on cell packing. However, AXR reflects the physiological condition of the cell membranes. In multi-compartment systems, AXR is difficult to relate to the true microscopic exchange rates (25). Assuming constant membrane permeability, AXR is expected to increase/decrease when the fractional population of intracellular water increases/decreases. This is a consequence of the equilibrium exchange between the compartments. Such a mechanism might be responsible for the initial slight increase and subsequent decrease in AXR values observed in Fig. 4.

It is notable that cell membrane breakdown (start of the gray line) occurred earlier in MCF-7 cells harvested by Accutase (Fig. 4b, at around 9h) than in cells harvested by scraping (Fig. 4a, between 12 and 15h). In addition, scrapings of cells seeded in hydrophobic Petri dishes resulted in a longer cell survival relative to scrapings of cells in roller bottles (Fig. 4a). Fairly constant and reproducible AXR values were observed within the first 6h of measurement for all duplicate samples shown in Fig. 4. No significant differences in AXR were observed between cells harvested from scrapings grown on Petri dishes or in roller bottles. However, approximately double AXR values were observed for cells harvested by Accutase (Fig. 4b) compared with scrapings (Fig. 4a). At high AXR (Fig. 4b), the values were less stable as a consequence of the limited range of AXR values accessible in FEXSY (6). From these results, we conclude that Accutase has a disruptive effect on the cell membrane. This is probably related to the effects of hyaluronidase, present in Accutase, on the glycocalyx, a polysaccharide-containing coating that surrounds cells and functions as a hydrated gel, and thus may affect water transport (26–28). However, from the observation that Accutase treatment resulted in a larger AXR than did scraping treatment, we chose to grow cells in hydrophobic Petri dishes and to harvest them by scraping in further experiments across different cell lines.

Repeated experiments indicated differences in the time course of AXR values for different cell lines (Fig. 5). The abrupt drop in $f_i$, exemplified by MCF-7 cells in Fig. 4, was observed for all cell lines. In Fig. 5, AXR values are shown within a limited time range, with the ‘cut-off’ time (corresponding to the disintegration of the intracellular compartment) depending on the cell line. For most of the cell lines, the AXR values decreased over time, with the exceptions of MCF-7 and HCC202, were increasing AXR values were observed. However, in the 1–1.8h interval, AXR values were constant within experimental precision for all samples. For this time interval, AXR values ranged from 3.3 s$^{-1}$ for T-47D cells to 12 s$^{-1}$ for MDA-MB-436 cells. For normal cells, MCF-10 A, AXR was approximately 5.6 s$^{-1}$. Representative examples of ADC($t_m$) are included in Supporting information. The generation of rigorous statistical information

**Figure 4.** Monitoring MCF-7 human breast cancer cells over an extended period of time. Shown are the results for the intracellular fraction $f_i$, apparent diffusion coefficient (ADC) and apparent exchange rate (AXR) from repeated measurements on duplicate samples with MCF-7 cells grown in roller bottles (RB) and on Petri dishes (PD) harvested by scraping (S) or by Accutase (A) treatment. The results for cells harvested by scraping are shown in (a), whereas the results for cells harvested by Accutase are shown in (b). The results shown in gray were obtained in a time interval in which the diffusion contrast between intra- and extracellular compartments could not be reliably detected. The beginning of this interval was chosen when a systematic increase in ADC started, also reflected by a systematic decrease in $f_i$ values. The initial increase in $f_i$ and decrease in ADC can be attributed to the sedimentation of cells.
needed to classify different cell lines based on AXR values and the time course of other observable parameters was beyond the scope of this study.

Presently, we have no clear explanation for the differences in results for the breast cancer cell lines and MCF-10 A cells; however, it is interesting to note that, in this proof-of-concept series, the majority of the more aggressive breast cancer subtypes (three of the four basal subtypes and two of the three HER2 subtype breast cancer cell lines) had AXR values higher than the median and average AXR, and also higher than that of the normal epithelium-derived cell line MCF-10 A. Further studies are required to explain the above observations.

FEXI for in vivo breast tumor characterization

The analysis criteria stated in the ‘Image analysis’ section were fulfilled for four of eight cases. For three of the four analyzable cases, SNRs were relatively low and AXRs were high with large uncertainties, i.e. 4.6 ± 1.0, 5.1 ± 2.3 and 8.1 ± 3.0 s⁻¹. Here, we show the remaining case, in which conditions for AXR analysis were good and provided the highest precision in our study. In Fig. 6a, an enhancing invasive ductal carcinoma [Nottingham Grade I, estrogen receptor (ER)-positive/progesterone receptor (PR)-positive/HER2-negative] of the right breast from a 51-year-old woman is visible in the dynamic contrast-enhanced (DCE) image (top), and the tumor ROI is outlined in the $b = 0$ s/mm² FEXI image (bottom). The size of the tumor was estimated to $14 \times 17 \times 15$ mm³ on DCE MRI. The tumor tissue micrograph is available in Supporting information. The SNR was too low for reliable voxel-based analysis, but averaging the signal across the tumor ROI allows an ROI-averaged AXR value to be obtained. For the ROI-averaged data, the SNRs were in the range from approximately 100 to 30 (non-filtered, from lowest to highest $b$ values). The effects of exchange are clearly visible on the ADC($t_m$) plot in Fig. 6b. Application of Equations [7]–[9] to analyze

Figure 5. Apparent exchange rate (AXR) in different human breast epithelial cell lines monitored over an extended period of time. Continuous application of filter exchange spectroscopy (FEXSY) allows the AXR value to be monitored in cell suspensions of 11 different human breast epithelial cell lines (see labels on the right), including one from normal ducts (MCF-10 A) and several from different cancer subtypes. All cells were grown on Petri dishes and harvested by scraping. The AXR values are shown for the time interval during which the intracellular compartment could be reliably detected, characterized by large $f_i$ values (see the example in Fig. 4). The color coding corresponds to an ascending order of AXR values from blue to red.

Figure 6. Apparent exchange rate (AXR) in breast tumor in vivo. (a) Tumor is clearly visible in the dynamic contrast-enhanced (DCE) image (top) and the region of interest (ROI) for filter exchange imaging (FEXI) analysis is outlined in the $b = 0$ FEXI image (see green line, bottom). (b) AXR analysis of the average signal across the tumor ROI. The plot shows the filtered apparent diffusion coefficient (ADC) as a function of mixing time, ADC($t_m$). Data at $t_m < 0$ correspond to the equilibrium ADC measured without the filter pulsed gradient spin-echo (PGSE) block at $t_m = 12$ ms. The solid line was calculated with Equation [8] and corresponds to the optimal fitting parameters: AXR = $2.8 \pm 0.5$ s⁻¹, ADC = $1.343 \pm 0.008$ $\mu$m²/ms and $\sigma = 0.18 \pm 0.01$. 
the average ROI signal yielded AXR = 2.8 ± 0.5 s⁻¹, ADC = 1.343 ± 0.008 μm²/ms and α = 0.18 ± 0.01. The fitted ADC (fADC) is shown as a solid line in Fig. 6b. On pathology, the tumor was determined to be a low-grade invasive ductal carcinoma with less aggressive features and luminal A molecular subtype (Nottingham grade I, ER-positive/PR-positive/HER2-negative). In normal tissue, the AXR was outside the experimental range (5–8), suggesting AXR values either below 1 s⁻¹ or above 20 s⁻¹. It should be noted that possible effects of perfusion were not accounted for in our analysis. Such effects could introduce a compartment that is in slow exchange, and could thus cause a negative bias in AXR.

It is interesting that a relatively low AXR observed in the examined patient presented here is consistent with the lower AXR values observed for the luminal subtype cancer cell lines. Although this study does not provide a solid basis for tumor grading, it suggests that lower AXR values might be associated with less invasive cancer types.

CONCLUSIONS

From PGSE experiments with large b values, it is evident that the intracellular compartment is polydispersed in breast cell suspensions. High f values, corresponding to large diffusion contrast between the intra- and extracellular compartments, provide the essential condition for the successful quantification of exchange. Consequently, we found that FEXSY provided a robust quantification of AXR in all cell suspensions tested. AXR values ranged from 3.3 to 12 s⁻¹, with the observation that lower AXR values tended to be associated with less invasive cancer subtypes, suggesting that different cancer subtypes may be distinguishable on the basis of their AXR values. The time-resolved experiments showed that the intracellular compartment desintegrates rather abruptly in all cell lines, indicating different lifetimes for different cell lines. The results suggest that FEXSY could serve as a useful method for the characterization of cell suspensions.

In breast tissue in vivo, AXR could be determined for the breast tumor ROI, whereas, in normal tissue, AXR was outside the FEXI experimental range. High ADC values observed in breast (4,29) suggest a rather fast exchange, probably with AXR > 20 s⁻¹. The relatively low AXR value observed in tumor (low-grade invasive ductal carcinoma, luminal A molecular subtype) was consistent with the low AXR values observed for the luminal subtype cancer cell lines. At present, the in vivo protocol yields too low an SNR to achieve AXR maps with a resolution comparable with ADC mapping, and is therefore limited to tumors larger than approximately 1 cm³. The achievement of optimal fat suppression and optimal diffusion filtering, as a result of a wide range of ADC values (4), represents a challenge for the application of FEXI in breast, and requires further protocol optimization. Our results encourage further investigations of AXR as a potential diagnostic biomarker. We anticipate that future hardware development, in terms of stronger gradients and better coil design, yielding higher SNR, will allow reliable breast AXR mapping in vivo.

ACKNOWLEDGEMENTS

This work was financially supported by the Swedish Research Council (grant numbers 2009–6794, 2011–4334, 2011-3021), the Swedish Cancer Society, Governmental Funding of Clinical Research within National Health Service, Lund University Medical Faculty, Crawford Foundation, Gunnar Nilsson Cancer Foundation, the Mrs. Berta Kamprad Foundation, National Institutes of Health/National Cancer Institute (NIH/NCI) (grant number R01 CA151326), Fred Hutchinson Cancer Research Center Support Grant Pilot Award and CR Development AB, Lund, Sweden. The authors would like to acknowledge Anthony George (Division of Oncology and Pathology, Lund University, Lund, Sweden) for technical assistance, and Ewa Dahlberg and Rita Wallén (Department of Biology, Lund University, Lund, Sweden) for help with cell culturing and scanning electron microscopy, respectively. Also acknowledged is Björn Lampinen (Clinical Sciences Lund, Medical Radiation Physics, Lund University, Lund, Sweden) for his work on FEXI protocol optimization.

REFERENCES

1 Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int. J. Cancer 2015; 136: E359–E366.
2 Patani N, Martin L-A, Dowsett M. Biomarkers for the clinical management of breast cancer: international perspective. Int. J. Cancer 2013; 133(1): 1–13.
3 A multi-center study evaluating the utility of diffusion weighted imaging for detection and diagnosis of breast cancer. Available at: https://clinicaltrials.gov/ct2/show/NCT02022579?term=6702&rank=2 [accessed Feb 15 2016].
4 Partridge SC, Demartini WB, Kurland BF, Eby PR, White SW, Lehman CD. Differential diagnosis of mammographically and clinically occult breast lesions on diffusion-weighted MRI. J. Magn. Reson. Imaging 2010; 31(3): 562–570.
5 Åslund I, Nowacka A, Nilsson M, Topgaard D. Filter-exchange PGSE NMR determination of cell membrane permeability. J. Magn. Reson. 2009; 200(2): 291–295.
6 Lasić S, Nilsson M, Lätt J, Ståhlberg F, Topgaard D. Apparent exchange rate mapping with diffusion MRI. Magn. Reson. Med. 2011; 66(2): 356–365.
7 Nilsson M, Lätt J, van Westen D, Brockstedt S, Lasić S, Ståhlberg F, Topgaard D. Noninvasive mapping of water diffusional exchange in the human brain using filter-exchange imaging. Magn. Reson. Med. 2013; 69(6): 1572–1580.
8 Lampinen B, Szczepankiewicz F, van Westen D, Englund E, Sundgren P, Lätt J, Ståhlberg F, Nilsson M. Optimal experimental design for filter exchange imaging: apparent exchange rate measurements in the healthy brain and in intracranial tumors. Accepted in Magn. Reson. Med.
9 Åslund I, Topgaard D. Determination of the self-diffusion coefficient of intracellular water using PGSE NMR with variable gradient pulse length. J. Magn. Reson. 2009; 201(2): 250–254.
10 Neuman CH. Spin echo of spins diffusing in a bounded medium. J. Chem. Phys. 1974; 60(11): 4508–4511.
11 Murday JS, Cotts RM. Self-diffusion coefficient of liquid lithium. J. Chem. Phys. 1968; 48(11): 4938.
12 Shemesh N, Jespersen S, Alexander DC, Cohen Y, Drobnjak I, Lyby TB, Finsterbusch J, Koch MA, Kuder T, Lain F, Lawrenz M, Lundell H, Mitra PP, Nilsson M, Ozarslan E, Topgaard D, Westin C-F. Conventions and nomenclature for double diffusion encoding NMR and MRI. Magn. Reson. Med. 2016; 75(1): 82–87.
13 Neve RM, Chinn K, Fridlyand J, Yeh J, Baehner FL, Ferv T, Clark L, Bayani N, Coppe J-P, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo W-L, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Eisen S, Gazdar A, Gray JW. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 2006; 10(6): 515–527.
14 Johannsson OT, Staff S, Vallon-Christersson J, Kyöla S, Gudjonsson T, Rennstam K, Hedenfalk IA, Adeyinka A, Kjellén E, Wennerberg J, Baldevtorp B, Petersen OW, Olsson H, Oredsson S, Isola J, Borg A. Characterization of a novel breast carcinoma xenograft and cell line derived from a BRCA1 germ-line mutation carrier. Lab. Invest. 2003; 83(3): 387–396.
15 Alexander DC. A general framework for experiment design in diffusion MRI and its application in measuring direct tissue-microstructure features. Magn. Reson. Med. 2008; 60(2): 439–448.
16 Alper JS, Gelb RI. Standard errors and confidence intervals in nonlinear regression: comparison of Monte Carlo and parametric statistics. J. Phys. Chem. 1990; 94(2): 4747–4751.
17 Packer K, Rees C. Pulsed NMR studies of restricted diffusion. I. Droplet size distributions in emulsions. J. Colloid Interface Sci. 1972; 40(2): 206–218.
18 Fleischer G The effect of polydispersity on measuring polymer self-diffusion with the n.m.r pulsed field gradient technique. Polymer (Guildf). 1985; 26(11): 1677–1682.
19 Callaghan PT, Pinder DN. Influence of polydispersity on polymer self-diffusion measurements by pulsed field gradient nuclear magnetic resonance. Macromolecules 1985; 18(3): 373–379.
20 Lasič S, Åslund I, Topgaard D. Spectral characterization of diffusion with chemical shift resolution: highly concentrated water-in-oil emulsion. J. Magn. Reson. 2009; 199(2): 166–172.
21 Shemesh N, Ozarslan E, Bassar PJ, Cohen Y. Detecting diffusivity-diffraction patterns in size distribution phantoms using double-pulsed field gradient NMR: theory and experiments. J. Chem. Phys. 2010; 132(3): 12.
22 Ozarslan E, Shemesh N, Koay CG, Cohen Y, Bassar PJ. NMR characterization of general compartment size distributions. New J. Phys. 2011; 13: 17.
23 Grinberg F, Ciobanu L, Farrher E, Shah NJ. Diffusion kurtosis imaging and log-normal distribution function imaging enhance the visualisation of lesions in animal stroke models. NMR Biomed. 2012; 25(11): 1295–1304.
24 Celli JP, Rizvi I, Evans CL, Abu-Yousif AO, Hasan T. Quantitative imaging reveals heterogeneous growth dynamics and treatment-dependent residual tumor distributions in a three-dimensional ovarian cancer model. J. Biomed. Opt. 2014; 15(5): 10.
25 Zimmerman JR, Brittin WE. Nuclear magnetic resonance studies in multiple phase systems: lifetime of a water molecule in an adsorbing phase on silica gel. J. Phys. Chem. 1967; 61(4): 1328–1333.
26 Mitchell MJ, King MR. Physical biology in cancer. 3. The role of cell glycocalyx in vascular transport of circulating tumor cells. Am. J. Physiol. Cell Physiol. 2014; 306(2): C89–C97.
27 Rehm M, Zahler S, Lötsch M, Welsch U, Conzen P, Jacob M, Becker BF. Endothelial glycocalyx as an additional barrier determining extravasation of 6% hydroxyethyl starch or 5% albumin solutions in the coronary vascular bed. Anesthesiology 2004; 100(5): 1211–1223.
28 Rilla K, Tihonen R, Kultti A, Tammi M, Tammi R. Pericellular hyaluronan coat visualized in live cells with a fluorescent probe is scaffolded by plasma membrane protrusions. J. Histochem. Cytochem. 2008; 56(10): 901–910.
29 Partridge SC, Ziadloo A, Murthy R, White SW, Peacock S, Eby PR, DeMartini WB, Lehman CD. Diffusion tensor MRI: preliminary anisotropy measures and mapping of breast tumors. J. Magn. Reson. Imaging 2010; 31(2): 339–347.

SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article at the publisher’s website.