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Human Protamine-1 as an MRI Reporter Gene Based on Chemical Exchange

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Abstract

Genetically engineered reporters have revolutionized the understanding of many biological processes. MRI-based reporter genes can dramatically improve our ability to monitor dynamic gene expression and allow coregistration of subcellular genetic information with high-resolution anatomical images. We have developed a biocompatible MRI reporter gene based on a human gene, the human protamine-1 (hPRM1). The arginine-rich hPRM1 (47% arginine residues) generates high MRI contrast based on the chemical exchange saturation transfer (CEST) contrast mechanism. The 51 amino acid-long hPRM1 protein was fully synthesized using microwave-assisted technology, and the CEST characteristics of this protein were compared to other CEST-based contrast agents. Both bacterial and human cells were engineered to express an optimized hPRM1 gene and showed higher CEST contrast compared to controls. Live cells expressing the hPRM1 reporter gene, and embedded in three-dimensional culture, also generated higher CEST contrast compared to wild-type live cells.

The chemistry of amino acids, which makes them differ so much from each other, provides many possibilities for engineering new proteins or assigning a new function to an existing protein. With that in mind, several proteins have been engineered to function as sensors for magnetic resonance imaging (MRI), based on their ability to enhance contrast via transverse relaxation,^{1–4} longitudinal relaxation,^{5–7} and chemical exchange saturation transfer (CEST).^{8–11} CEST is a relatively new contrast mechanism that allows versatile applications.^{12–22} Since the arginine-rich protein, protamine, purified from salmon sperm (protamine sulfate), is an exceptionally good CEST contrast agent,²³ which has been used successfully to monitor controlled drug release,²¹ we hypothesized that an arginine-rich protein would be a good candidate for a reporter gene. However, due to the fact that protamine sulfate is purified from salmon sperm, it could potentially induce an immune response when expressed in mammals. We, therefore, decided to clone the human protamine-1 (hPRM1), which shares high homology with salmon protamine, and, because it is an endogenous protein, would be better tolerated by human cells. Protamines are a family of basic proteins that predominantly exist in male germ cells, and are chemically defined by a sequence that contains at least 45% of positively charged amino acids (arginine, lysine, and histidine), with at least 30% of the amino acids as arginine residues.²⁴ Among the human protamines, hPRM1 has the highest abundance of arginine residues.²⁵ Thus, the main goal of this study was to investigate the potential and limitations of using a naturally occurring human protein as a CEST-MRI reporter gene, in the context of pure synthetic protein, cell lysates in the presence of nonspecific background (CEST contrast derived from other intracellular molecules) and in live cells. Consequently, the findings from this study may provide a better understanding of the CEST contrast generated by natural cellular proteins. Moreover, it represents a new avenue for designing optimized CEST reporter genes that may eventually be applied for *in vivo* imaging.

In this study, following the synthesis, purification, and CEST characterization of the 51 amino acid-long hPRM1 protein, we have genetically engineered both prokaryotic (*Escherichia coli*, *E. coli*) and eukaryotic mammalian (human embryonic kidney, HEK 293) cells to express the hPRM1 gene. In both cell types, high CEST contrast was associated with the guanidyl and amide exchangeable protons. We also demonstrate that the recombinant hPRM1 can generate high CEST contrast in intact live cells encapsulated in alginate capsules.

In order to evaluate the feasibility of hPRM1 as a CEST-based reporter gene, we initially compared it with protamine sulfate and poly-L-lysine, both of which are well-characterized CEST agents,^{23,26} with the latter having been used as a CEST reporter gene.⁸ Toward this end, we synthesized a 51-amino-acid-long polypeptide with the same amino acid sequence as that coded by the hPRM1 gene, using microwave-assisted technology. Successful completion of the full-length hPRM1 synthesis and its purity were confirmed with

electrospray ionization mass spectrometry (ESI-MS), HPLC, and acetic acid-urea gel electrophoresis (Figure 1a). To compare the CEST contrast generated by these three proteins, 5 mg mL⁻¹ of each peptide/protein was dissolved in PBS (approximately 35 mM of amide protons per sample). As can be concluded from both the MTR_{asym} plots and maps (Figure 1b,c), the total CEST contrast from both protamines was considerably higher than that of poly-L-lysine. This is because poly-L-lysine has a peak only at the amide proton resonance frequency (3.6 ppm offset with one amide per residue), while both protamine sulfate and hPRM-1 displayed two distinct peaks, specifically, for the amide protons of the protein backbone (at 3.6 ppm) and the guanidyl protons of the arginine side chain (at the 1.5 ppm offset from the water protons with four of these protons per arginine). It is important to note that the amine group on the lysine side chain does not generate high CEST contrast due to the fast exchange rate (k_{ex}) of these protons compared to their chemical shift from water ($\Delta\omega$).²⁷ These exchangeable protons do not fulfill the slow-to-intermediate exchange regime ($\Delta\omega > k_{ex}$) required for CEST contrast on the 500 MHz scanner that was used in this study. This observation is in agreement with previous reports showing a lack of CEST contrast from the amine-exchangeable protons of lysine-rich peptides and proteins.^{20,23,8} To further evaluate the feasibility of hPRM1 as a CEST reporter at lower magnetic field strengths relevant to clinical imaging, we performed a CEST-MRI experiment at 3 T (128 MHz) using a clinical scanner. As shown in Figure 1d, CEST contrast could be obtained from both the guanidyl protons at $\Delta\omega = 1.5$ ppm and the amide protons at $\Delta\omega = 3.6$ ppm, similar to the results obtained at 11.7 T (compare Figure 1b). For clinical MRI scanners, the specific absorption rate (SAR) and hardware limitations imposed by the FDA for human use currently prevent optimal CEST MRI measurements for small volume samples and small animals. Aside from the field strength, it is therefore not surprising that CEST contrast is lower compared to that obtained at 11.7 T. Nevertheless, these results indicate that hPRM1 is clearly detectable at lower clinical field strength.

To investigate the feasibility of hPRM1 as a genetically encoded reporter, we initially expressed the protein in a prokaryotic expression system, *E. coli*, a widely used platform for the development of reporter genes.²⁸ However, a major challenge in expressing an arginine-rich protein is that four of the six arginine codons (i.e., AGA, AGG, CGG, and CGA) are rarely translated in *E. coli* (termed rare codons; Figure 2b) and could cause translational errors and lower levels of protein expression.²⁹ We, therefore, designed a synthetic gene encoding hPRM1, optimized for *E. coli*. In this synthetic gene, all the original arginine rare-codons were replaced with the arginine codons CGU and CGC that are used more frequently by *E. coli*, and the gene was cloned under the T7 promoter. The hPRM1 gene was fused to the myc-tag and the six-histidine tag at the C-terminal of the protein for detection with immunohistochemistry. Western blot analysis using an anti-His antibody (Figure 2c) demonstrated that recombinant hPRM1 was, indeed, expressed in *E. coli* after codon optimization, compared to the human gene.

To evaluate the CEST capabilities of the recombinant hPRM1, *E. coli* cells expressing the hPRM1 were lysed, and the total protein content of the cells was dialyzed against PBS (pH = 7.4). As control, a recombinant *E. coli* cytosine deaminase (CD) was used since it is a bacterial protein that does not require gene optimization. For both samples, the total protein concentration, which was determined with a bicinchoninic acid (BCA) assay, was adjusted to the same amount for all samples. As can be seen in Figure 2, the solutions that contained hPRM1 generated higher MTR_{asym} values compared to CD. Similar to the synthetic hPRM1, the highest MTR_{asym} value was obtained at the 1.5 ppm offset from the water frequency, where significant differences were obtained between hPRM1 and CD (Figure 2e, $n = 4$, $p < 0.001$). At the 3.6 ppm frequency offset, the differences between hPRM1 and CD lysates were also significant ($p < 0.01$). It is important to note that, since a cell extract was

used, the CEST contrast obtained for the CD containing lysate is from the total cellular proteins and not specifically from the CD.

To examine the feasibility of detecting hPRM1 in mammalian cells using CEST, we constructed a lentivirus that encodes the hPRM1 under the cytomegalovirus (CMV) promoter, and transduced human embryonic kidney (HEK293) cells (Figure 3a). As can be seen in the MTR_{asym} plots in Figure 3b, significantly higher CEST contrast (MTR_{asym} values) were obtained for the 293^{hPRM1} lysate, compared to the lysate from wild-type cells (293^{wt}). The MTR_{asym} maps (Figure 3c), obtained following saturation at the guanidyl (1.5 ppm) and amide (3.6 ppm) resonance frequencies, visualize the higher CEST contrast for the 293^{hPRM1} compared to nonexpressing cells.

Finally, to demonstrate the ability to image hPRM1 expression in live cells with CEST, we used a three-dimensional culture of alginate-encapsulated 293^{wt} and 293^{hPRM1} cells (Figure 4a). As expected, encapsulated live cells expressing the hPRM1 generated a higher CEST contrast compared to nonexpressing wild-type cells (Figure 4b). This observation was found for saturation at both the 1.5 ppm or 3.6 ppm offsets from the water resonance. However, as compare to cell lysates, in the live encapsulated cells, only at the frequency offset of the guanidyl protons, that is, $\Delta\omega = 1.5$ ppm, a significant different ($p < 0.05$) was obtained. It should be noted that the contrast *in vivo* might be different. On the one hand, the cell density is 2 orders of magnitude higher *in vivo* in a normal tissue (1×10^9 cells/mL) than in the phantom setup of the microcapsules, in which approximately 2×10^7 cells/mL is suspended in alginate. Therefore, it is anticipated that *in vivo* the difference in contrast between expressing and nonexpressing cells will be higher. On the other hand, it might be the case that not all the cells *in vivo* will express high levels of the transgene; this may compromise CEST contrast. In addition, other factors *in vivo*, such as magnetization transfer and background signal from nonhomogenous tissue can compromise the accuracy of measuring the CEST contrast.

Note that a small CEST effect is observed along the edges of the tubes (see also Figures 1c and 3c). This effect is due to the presence of glass–fluid and air–glass interfaces, substances with a different magnetic susceptibility. This results in water line broadening due to an apparent decrease in T_2^* of the sample causing broad shifts in the water resonance frequency offsets. This artifact is commonly seen *in vitro* for phantom studies and does not affect the statistical differences between the studied groups.²⁰ One suggested approach to circumvent this phenomenon may be to surround the phantoms with other media that lack protons, such as Fluorinert FC-43.³⁰

In a previous study, we have demonstrated that an artificial CEST based reporter gene that encodes to a lysine-rich protein (LRP) generates a detectable CEST contrast both *in vitro* and *in vivo*, in a preclinical animal model of brain tumor.⁸ There are several advantages and disadvantages for using the hPRM1 versus the LRP as a reporter gene. In contrast to the hPRM1, which is a human gene and therefore was well adopted for expression in human cells throughout the evolution, the LRP is a synthetic gene that has multiple DNA repeats (lysine is encoded only by AAA and AAG). In this sense, it might be recognize by the cells as a foreign DNA. The DNA repeat may result instability of the DNA and loss of parts of the gene via recombination. One alternative is to optimize the LRP by redesigning of the gene. Another alternative is to use a human gene (such as hPRM1) as a backbone for designing a new reporter. It is likely that the hPRM1 far from an optimized reporter, and further improvements are warranted. For example, removal or replacement of putative phosphorylation sites might affect cellular localization,^{31,32} since the addition of a phosphate group might affect the protein's charge, as well as its secondary and tertiary structure. It has been demonstrated previously that protein phosphorylation can reduce the

CEST contrast derived from arginine-rich domains of proteins.¹⁰ One alternative that should be considered to improve the performance of hPRM1 as a CEST reporter gene is to use directed evolution. This methodology has been used to improve the MRI contrast from metalloproteins.⁷ Therefore, alternating the protein sequence may result in higher CEST contrast as was shown for different polypeptides.^{20,23}

In summary, we have developed a novel MRI reporter gene based on a human gene, the human protamine-1. Following its full-length synthesis and characterization, we demonstrated its successful expression and detection with CEST-MRI in prokaryotic and eukaryotic cells, as well as in three-dimensional cell cultures. The results presented in the current study are promising and yet future *in vivo* studies are warranted.

METHODS

Poly-L-lysine (MW 15–30 kDa, P-7890) and protamine sulfate (P-3369) were purchased from Sigma-Aldrich (St. Louis, MO).

hPRM1 Synthesis

The synthesis of the 51 amino acid long hPRM1 protein was performed on a microwave-assisted peptide synthesizer Liberty1 (CEM, USA) using N-fluorenylmethyloxycarbonyl (Fmoc) chemistry with O-Benzotriazole-N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU) as an activator. The crude peptide was purified using HPLC on a C12 reverse-phase column, and pure synthetic hPRM1 was confirmed by acetic acid-urea gel electrophoresis (Figure 1a).

Protein Sequence Alignment

The sequences of protamine sulfate and hPRM1 were aligned using the “Pairwise Sequence Alignment” tool (the public databases of EMBL-EBI, <http://www.ebi.ac.uk>), which is used to identify regions of similarity that may indicate functional, structural, and/or evolutionary relationships between the two protein sequences.

Cloning

The gene encoding to hPRM1 (NM_002761) was obtained from Origene (Rockville, MD). A synthetic gene encoding *E. coli*-optimized hPRM1 was obtained from Blue Heron Biotechnology (Bothell, WA). Both hPRM1 genes, as well as the Cytosine Deaminase (CD) gene were subcloned into the pEXP5-CT expression vector (Invitrogen, Carlsbad, CA), and expressed in-frame with a 6-histidine tag under a T7 promoter. The mammalian-optimized gene was cloned into a pLenti-6- V5/DEST vector (Invitrogen) under a cytomegalo-virus (CMV) promoter and a fused V5-tag.

Expression in *E. coli*

BL21 (DE3) (Invitrogen) cells were transformed with both *E. coli*- and mammalian-optimized pEXP-5-CT-hPRM1 and pEXP-5-CT-CD. After induction in Magic Media (Invitrogen) at 30 °C for 18 h, the total cellular protein was extracted and dialyzed against 10 mM PBS, pH = 7.4. The total protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL). The expression was validated by Western blot or dot blot using an anti-six histidine (anti-His) antibody (Invitrogen).

Expression in Mammalian Cells

Viruses (pLenti-hPRM1) were propagated in Human Embryonic Kidney 293 cells (HEK-293FT). Expressing cells were selected using blasticidin antibiotic for 14 days; 1×10^7 cells were lysed using Mammalian Protein Extraction Reagent (MPER, Thermo Fisher Scientific), and anti-V5 antibody (Invitrogen) was used for Western blot analyses. For CEST-MRI, cell lysates were dialyzed against PBS and total protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific).

Cell Encapsulation

Human embryonic kidney (HEK293) cells expressing hPRM1 (293^{hPRM1}) and wild type nonexpressing control cells (293^{wt}) were encapsulated in alginate microcapsules, as previously described.³³ Briefly, a mixture of alginate (NovaMatrix, Sandvika, Norway) and either 293^{hPRM1} or 293^{wt} cells (1×10^7 cells/mL) were suspended at 100 μ L/min into a Petri dish containing 20 mM BaCl₂ in 10 mM HEPES using a syringe pump. Beads containing cells surrounded by the first layer of alginate were collected. The resultant microcapsules containing cells (~300 cells/microcapsule) were rinsed with saline solution, followed by cross-linking with 0.1% poly-L-lysine for 5 min and applying a second layer of alginate. The microcapsules were then washed and incubated with the medium.

CEST-MRI

All CEST-MRI experiments were performed on a vertical bore 11.7 T Bruker Avance system. (i) *CEST of protein solutions*: Pure proteins or total proteins from cell lysates were either dissolved or dialyzed against PBS, and CEST experiments were performed as previously described²⁰ using 30 μ L of protein solution, placed in 1 mm glass capillaries. A modified RARE (TR/TE = 6000/9.4 ms) sequence, including a magnetization transfer module with $B_1 = 4.7 \mu$ T and saturation time (t_{sat}) = 4000 ms, was used to acquire CEST-weighted images from -5 to +5 ppm, in increments of 0.2 ppm around the water resonance, which was assigned as 0 ppm. The absolute water resonant frequency shift was measured using a modified WASSR method, with the same parameters as for CEST imaging, except for a TR = 1.5 s and a saturation pulse with $B_1 = 0.5 \mu$ T and $t_{\text{sat}} = 250$ ms and a Z-spectral acquisition from -1 to +1 ppm with 0.1 ppm steps, which was used for B_0 correction for each voxel using MatLab. The MTR asymmetry as a percentage of available water signal ($\text{MTR}_{\text{asym}} = 100 \times [S_{-\Delta\omega} - S_{+\Delta\omega}]/S_0$) was computed at different offsets, $\Delta\omega$. Slice thickness = 1 mm, FOV = 11×11 mm², matrix size = 64×32 , and resolution = 0.17×0.34 mm² were used for each CEST/WASSR experiment. (ii) *CEST of encapsulated living cells*: 5-mm NMR tubes were filled with microcapsules containing either 293^{hPRM1} or 293^{wt} cells. The same parameters were used as for the protein solutions, except that a magnetization transfer module with $B_1 = 3.6 \mu$ T and $t_{\text{sat}} = 3000$ ms was used to acquire CEST-weighted images from -5 to +5 ppm, in increments of 0.25 ppm. (iii) *CEST of pure protein solutions at 3 T*: Solutions of pure synthetic human protamine-1 and protamine sulfate (5 mg mL⁻¹ in PBS, pH = 7.4) in 5 mm NMR tubes were placed in a 3 T Philips Achieva system (Philips Healthcare, Best, The Netherlands) using a body coil for transmitting and a 32-channel head coil for reception. CEST data were using a Spin Echo sequence (TR/TE = 12000/7.1 ms), including a magnetization transfer module with $B_1 = 2.0 \mu$ T and saturation time (t_{sat}) = 4000 ms. CEST-weighted images were acquired from -12 to +12 ppm and in increments of 0.3 ppm around the water resonance, which was assigned as 0 ppm. Other parameters were slice thickness = 5 mm, FOV = 200×176 mm², and an in-plane resolution of 1.04×1.05 mm².

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Figure 1.

CEST properties of pure samples in PBS solution. (a) Acetic acid-urea polyacrylamide gel electrophoresis of positively charged proteins, poly-L-lysine, salmon protamine, and human protamine-1, stained with Coomassie blue staining. The CEST MRI characteristics of 5 mg mL⁻¹ protein is depicted as (b) MTR_{asymp} plots and (c) MTR_{asymp} maps (calculated at the 1.5 ppm and 3.6 ppm offsets from water). CEST data were acquired at 11.7 T, 37 °C, pH = 7.4, $B_1 = 4.7 \mu\text{T}$, and $t_{\text{sat}} = 4000$ ms. (d) MTR_{asymp} plots of salmon protamine and human protamine-1 acquired at 3 T, 25 °C, pH = 7.4, $B_1 = 2.0 \mu\text{T}$, and $t_{\text{sat}} = 4000$ ms.

Figure 2.

Optimization of hPRM1. (a) Amino acid sequence alignment of protamine from salmon and humans. [*] indicates a conserved residue, [:] indicates groups with a strong similarity in their properties, and [.] indicates groups of weakly similar properties. (b) Partial, arginine-rich hPRM1 sequence of human- and *E. coli*-optimized genes. (c) Western blot of recombinant hPRM1 protein expressed in *E. coli*, using the human- or the *E. coli*-optimized DNA. (d) MTR_{asym} plots and Western blot analysis (inset) of *E. coli* lysates from cells expressing either hPRM1 (red) or CD (blue). (e) The comparison of mean MTR_{asym} values of hPRM1 (red bars) and CD (blue bars) at the 1.5 and 3.6 ppm frequency offsets. The mean MTR_{asym} (+SD) were calculated from four independent measurements for each cell type ($n = 4$). CEST data were acquired at 11.7T, 37 °C, pH = 7.4, $B_1 = 4.7 \mu T$, and $t_{sat} = 4000$ ms.

Figure 3. CEST MRI of HEK293 cell extracts. (a) Western blot, using an anti-V5 antibody, showing hPRM1 expression. (b) MTR_{asym} plots ($\pm$ SD; $n = 3$). (c) Representative MTR_{asym} maps obtained at the 1.5 and 3.6 ppm frequency offsets. (d) Mean MTR_{asym} ($\pm$ SD) at the 1.5 ppm and 3.6 ppm frequency offsets. CEST data were acquired at 11.7T, 37 °C, pH = 7.4, $B_1 = 4.7$ μ T, and $t_{\text{sat}} = 4000$ ms.

Figure 4.

MR imaging of hPRM1 reporter gene expression in live cells in a three-dimensional cell culture system. (a) Bright-field microscopic images of encapsulated 293HEK cells. (b) T₂-weighted images and overlaid MTR_{asymp} maps of encapsulated cells obtained at the 1.5 and 3.6 ppm frequency offsets. (c) p-values as a function of the frequency offset from water (Dw), as calculated using a Student's *t* test (two-tailed distribution, paired test). Red line represents p-value of 0.05 (significance level). (d) Mean MTR_{asymp} ($\pm$ SD) at 1.5 ppm calculated from three samples containing encapsulated cells (293^{hPRM1} and 293^{wt}). CEST data were acquired at 11.7 T, 37 °C, pH = 7.4, B_1 = 3.6 μ T, and t_{sat} = 3000 ms.