

## Synthesis of human protamine-1 (hPRM1), a novel CEST contrast agent

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**Target audience:** Researchers interested in molecular imaging, MRI reporter genes, and diamagnetic CEST contrast agents.

**Purpose:** Protamines form a class of low-molecular-weight, arginine-rich, nuclear proteins that allow for dense packing of DNA in germ cells to protect chromosomal DNA. In addition salmon protamine sulfate (PS) is currently in use as an antidote for heparin poisoning, reversing the anticoagulation effects [1]. Protamine is also in use for more than 75 years in the Protamine Zinc Insulin (PZI) formulation of insulin to slow down release and increase action time [2] and has been proposed as a reporter gene for imaging gene expression [3]. The source of protamine in these applications is mainly natural, purified from fish, or recombinant, which can result in some difficulties during the purification process. A synthetic analog of protamine could be used as an alternative to natural proteins. Protamines are known to produce much higher chemical exchange saturation transfer (CEST) contrast than other natural proteins or synthetic peptides due to the large percentage of arginine in their sequence [4]. So a synthetic hPRM1 may also be used as a biologically compatible CEST contrast agent.

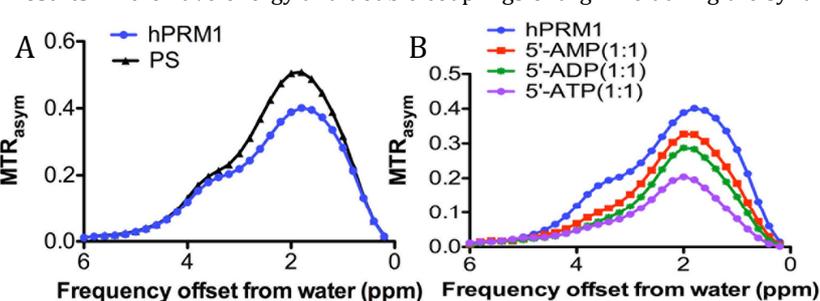
Here we report the synthesis and purification of the 51 amino acid long human Protamine-1 (hPRM1) protein (**Table 1**) using solid phase peptide synthesis (SPPS) with high yield. This compound, which is comparable with the shorter, naturally occurring salmon protamine, was tested for its ability to interact with DNA and nucleotides and for its CEST contrast.

**Methods:** The peptide synthesis was performed on a microwave-assisted peptide synthesizer Liberty1 (CEM, USA) using N-fluorenylmethoxycarbonyl (Fmoc) chemistry with O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as activator. The crude peptide was purified using HPLC on a C12 reverse-phase column and the molecular weight of hPRM1 was confirmed by mass-spectrometry and urea electrophoresis. The purity of synthetic protamine was analyzed by analytical HPLC (>90% pure). CEST weighted images from -5 to 5ppm (step=0.2ppm) of synthesized and naturally occurring protamine solutions in PBS (pH=7.2-7.4) with or without nucleotides were acquired on an 11.7T scanner using a saturation pulse with B1=4.7μT, T<sub>sat</sub>=4s at 310K followed by a RARE readout (TR/TE=6000/9.4ms). Data processing was performed using custom-written scripts in Matlab. Z-spectra were calculated from the mean of ROI for each sample after B0 correction.  $MTR_{asym} = (S-\Delta\omega - S+\Delta\omega) / S-\Delta\omega$  was computed at different offsets Δω (i.e. +1.5 ppm and +3.6 ppm).

**Table 1.** The list of compounds used in this study.

Name	Sequence	MW, Da
Human protamine-1 (hPRM1)	MARYRCCRSQSRSRYRQRQSRRRRRRSCQTRRRAMRCCRPRYPRCRRH-NH <sub>2</sub>	6822
Salmon Protamine sulfate (PS)	PRRRSSSRPVRRRRRPVSRRRRRRGGRRR-OH	4250

**Results:** Microwave energy and double couplings of arginine during the synthesis of human Protamine-1 significantly increased the



**Fig.1** Comparison of CEST contrast of PS and hPRM1 (**A**) and  $MTR_{asym}$  of hPRM1 with different nucleotides (**B**)

final product yield of hPRM1 and also reduced the time of the synthesis. As a result the yield before resin cleavage was approximately 90%, based on the peptidyl-resin growth after synthesis and approximately 60% after cleavage from the resin. The fraction isolated during the purification does generate good CEST contrast compared to PS. The maximum contrast occurred at a chemical shift 1.8 ppm, which is associated with the exchangeable protons in guanidine group of arginines. Synthetic human protamine-1 (hPRM1) produces ~40%  $MTR_{asym}$  (**Fig.1A**) at 5 mg/ml (0.733 mM). MR data of synthetic hPRM1 complexed with nucleotides showed that there is a drop in CEST contrast due to

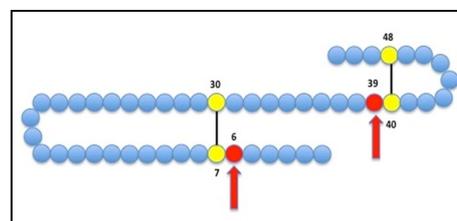
strong interaction of hPRM1 with nucleotides (**Fig 1B**), which again demonstrate that synthetic analog is comparable with naturally occurring proteins. In order to compare the molecular weight of synthetic protein with salmon protamine sulfate the urea-acid gel electrophoresis was used, which was based on the level of positively charged residues in protein structure.

**Discussion:** The conformation of protamine alone or complexed with different nucleotides is still under investigation. The structure is determined by cysteine-rich domains. The model proposed by Vilfan [5] for bull protamine showed that 4 cysteines form intramolecular disulfide bonds and the other cysteines participate in intermolecular bonding. Based on this, we have proposed the model shown in **Fig. 2** for the hPRM1 conformation. CEST contrast of hPRM1 is comparable to PS, with the small difference explainable by the arginine content of hPRM1 (~40%) being lower than in protamine sulfate (~55%). These structural and imaging features of hPRM1 could make synthetic protamine a very promising biomaterial for future *in vivo* studies.

**Conclusion:** This study shows that hPRM1 can be synthesized with a strong yield using a microwave synthesizer. This protein generates good CEST contrast and can interact with nucleotides similar to the naturally occurring salmon protamine sulfate.

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**References:** 1. Sie, P., et al., Thromb Res, 1989. 54(1): p. 63-74. 2. Owens, D.R., Diabetes Technol Ther, 2011. 13 Suppl 1: p. S5-14. 3. Bar-Shir, A., et al., Genetic Engineering of Human Protamine-1 for use as MRI Reporter Gene Based on Proton Exchange. in Proc. Intl. Soc. Mag. Reson. Med. 2011. 4. McMahon, M.T., et al., Magn Reson Med, 2008. 60(4): p. 803-12. 5. Vilfan, I.D., et al., J Biol Chem, 2004. 279(19): p. 20088-95.



**Fig.2** A proposed model of protamine with cysteines involved in intramolecular disulfide bonding (yellow) and intermolecular bonding