Supporting Information.

Biophysical characterization of human protamine-1 as a responsive CEST MR contrast agent

Nikita Oskolkov[1,2], Amnon Bar-Shir[1,3], Kannie W.Y. Chan[1,2], Xiaolei Song[1,2]

Peter C.M. van Zijl[1,2], Jeff W.M. Bulte[1-3], Assaf A. Gilad[1-3], and Michael T. McMahon[1,2,*]*

[1]Russell H. Morgan Department of Radiology and Radiological Science, The Johns Hopkins University School of Medicine, Baltimore, MD, United States,

[2]F.M. Kirby Research Center for Functional Brain Imaging, Kennedy Krieger Institute, Baltimore, MD, United States,

[3]Cellular Imaging Section and Vascular Biology Program, Institute for Cell Engineering, Baltimore, MD, United States

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MATERIALS AND METHODS

Peptide synthesis. The synthesis of the three protamine peptides (**Table 1**) was performed on a microwave-assisted peptide synthesizer Liberty1 (CEM Corporation, Matthews, NC, USA) using N-fluorenylmethyloxycarbonyl (Fmoc) chemistry with O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as activator.[1] For synthesis of phosphorylated peptides Fmoc-*O*-benzylphospho-L-serine monomer was used in order to get phosphorylated peptides without post-synthetic peptide modifications. The arginine residues were attached using double couplings with standard coupling conditions. The loading of Rink-amide MBHA resin was decreased down to 0.1 mmol/g in order to increase the efficiency of each coupling by reduction of steric effect. The time of cleavage from the resin was increase up to 6 hours due to the high content of 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl-protected (Pbf) arginine residues in the peptide sequences. As a result the yield of the synthesis was more than 90% calculated by resin growth after the synthesis and the crude purity was about ~50% according to preparative run HPLC profile (data not shown). After resin cleavage using a standard protocol[2] the crude peptide was purified using HPLC on a C12 reverse-phase column and the molecular weight of hPRM1 was confirmed by mass-spectrometry. In addition to mass-spectrometry analysis the acid-urea electrophoresis was performed as described to confirm MW of prepared peptides.[3] The purity of synthetic protamine and its analogs were analyzed by analytical HPLC and were more than 90% pure (Figure S1).

The molecular weight of highly cationic peptides was also confirmed using acid-urea gel electrophoresis, which is based on differences in size and effective charge. This analysis was performed in comparison with Poly-L-Lysine (~280 kDa) and naturally occurring salmon protamine sulfate (~4.2 kDa), which have different sizes and charges. The band

of synthetic h-PRM1 was between low MW protamine sulfate and high MW poly-L-Lysine (lane 3, Figure S2), which demonstrate the presence of our synthetic hPRM1 in the desired molecular weight range. The presence of disulfide bridges was verified by Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), which is widely used to detect free thiol groups in solution by measuring the absorbance of visible light at 412 nm. [4]

Solution preparation. All peptide solutions were prepared at different concentrations (1-5 mg/ml) using phosphate-buffered solution (PBS) or ddH_2O and pH was adjusted to physiological pH prior analysis. The solubility of peptides was sufficentlly good to produce 5 mg/ml solutions in order to obtain proper CEST contrast. Nucleotides were dissolved at 0.1-0.2 M concentrations in PBS and pH was adjusted as above.

Circular Dichroism (CD). CD spectra were collected at 25 °C on a Jasco J-715 spectropolarimeter. Ellipticity-dependent wavelength spectra of PS and peptide solutions were obtained in a 1 mm quartz cell. Mean residue ellipticity [θ] was calculated using ridge regression (CONTIN)[5] and self-consistent methods (SELCON3)[6] on DichroWeb software.[7, 8]

Dynamic Light Scattering (DLS). Hydrodynamic mean diameter of the particles was determined by dynamic light scattering studies using a Zetasizer Nano-S apparatus (Malvern Instruments, United Kingdom). Peptide solutions were prepared in PBS and water. All results were based on three or four measurements from two independent samples. All data were converted to ''relative by intensity'' plots from where the mean hydrodynamic diameter was derived.

MRI. MR data of synthetic peptides at different pH values, concentrations and complexed with nucleotides or heparin solutions were acquired on an 11.7 T Bruker Avance system

(Bruker Biosciences, Billerica, MA, USA) using a 20 mm birdcage transmit/receive coil and saturation pulses with B_1= 1.2 μT, 2.4 μT, 3.6 μT, 4.7 μT, 5.9 μT, 7.2 μT and 10.8 μT, T_{sat} = 4 s followed by a RARE readout (RARE=8, TR/TE=6000ms/19.09ms). The CEST contrast was calculated as MTR_{asym} function:

$$MTR_{asym} = \frac{S_w(-\Delta w) - S_w(+\Delta w)}{S_{0w}}$$

Signal intensity data (S_w) were processed using Matlab and Prism 6 software.

Statistical analysis. Values in all experiments are represented as mean ± SEM of three independent experiments done in duplicate.

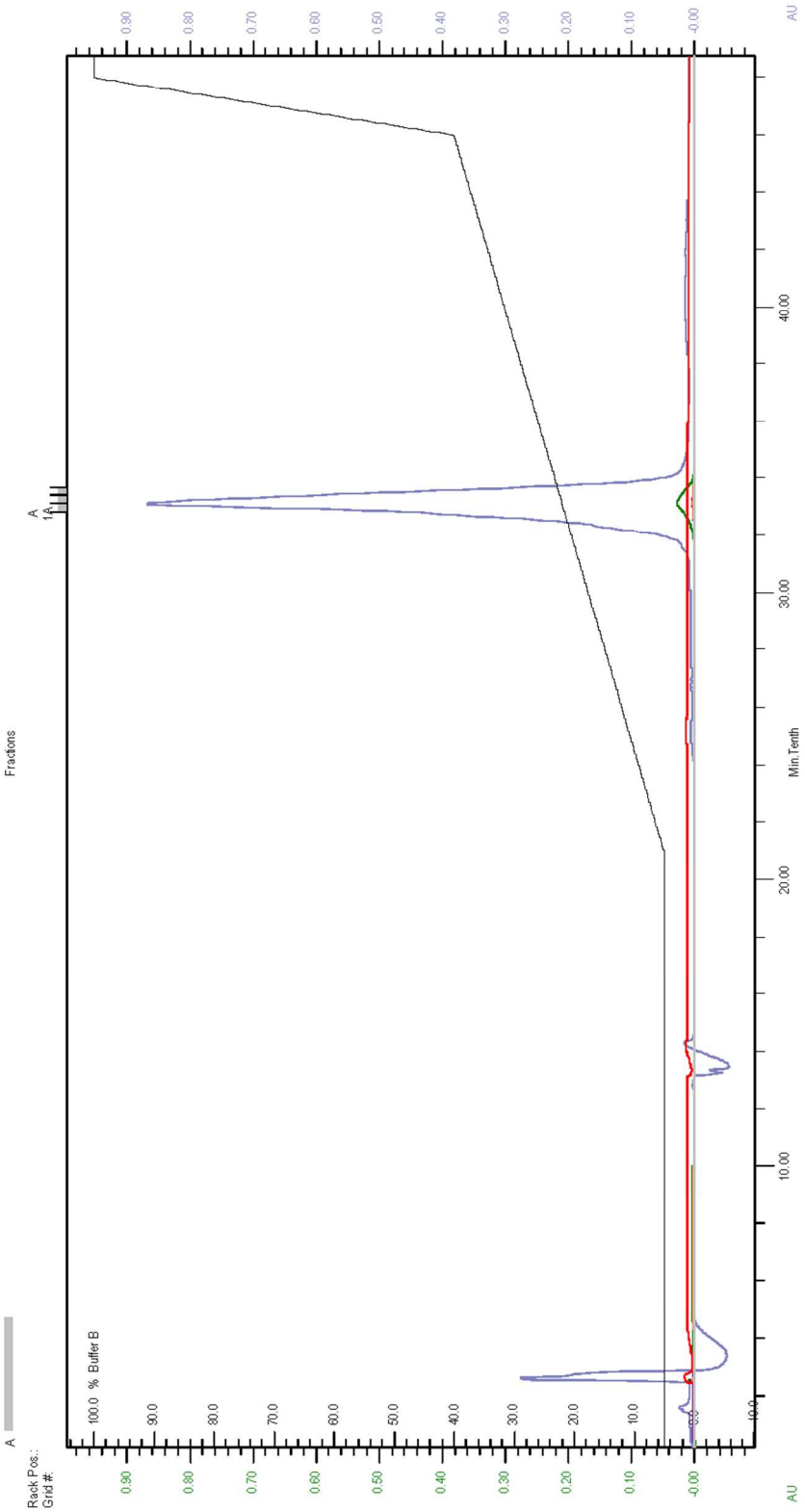

Figure S1. Analytical HPLC profile of purified peptide product of hPRM1.

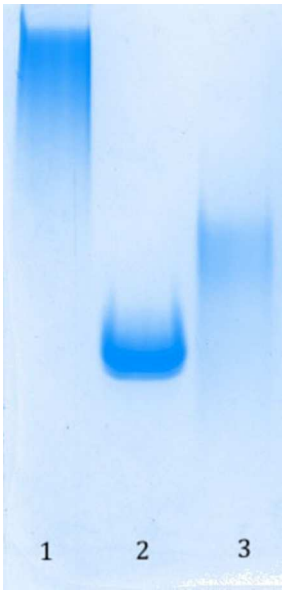

Figure S2. Acid-Urea gel electrophoresis of Poly-L-Lysine (1), Salmon Protamine Sulfate (2) and synthetic h-PRM1 (3)

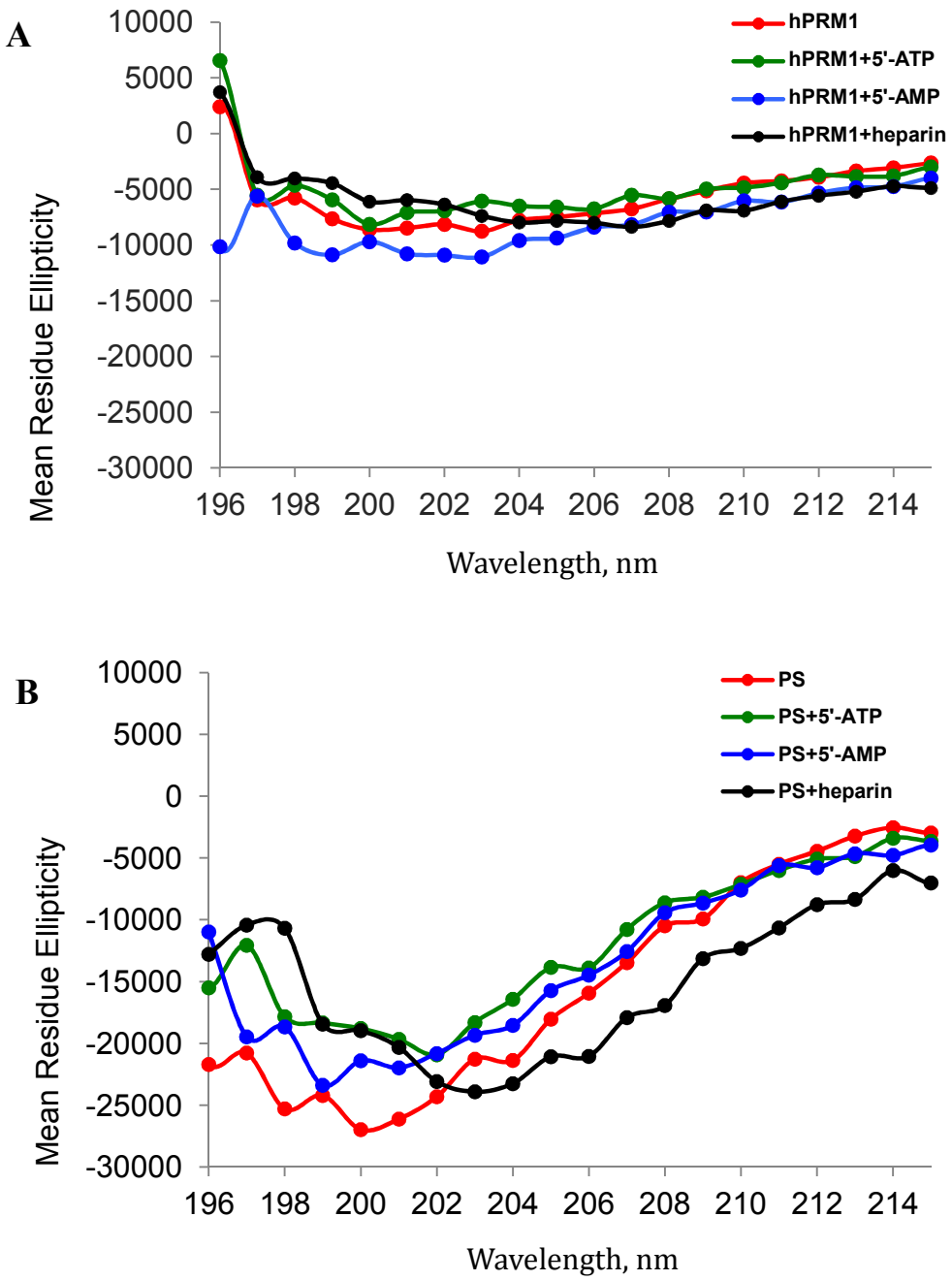

Figure S3. UV-CD spectra of synthetic hPRM1 (A) and Protamine sulfate (B) alone and complexed with 5'-ATP, 5'-AMP represented as Mean Residue Ellipticity MRE [θ], which is measured in the range of 196-215 nm and reported as degrees cm^2 $dmol^{-1}$ $residue^{-1}$ .The concentration of protamines is 0.1 mg/ml and the final concentration of heparin is 0.06 mg/ml and nucleotides are 200 μM.

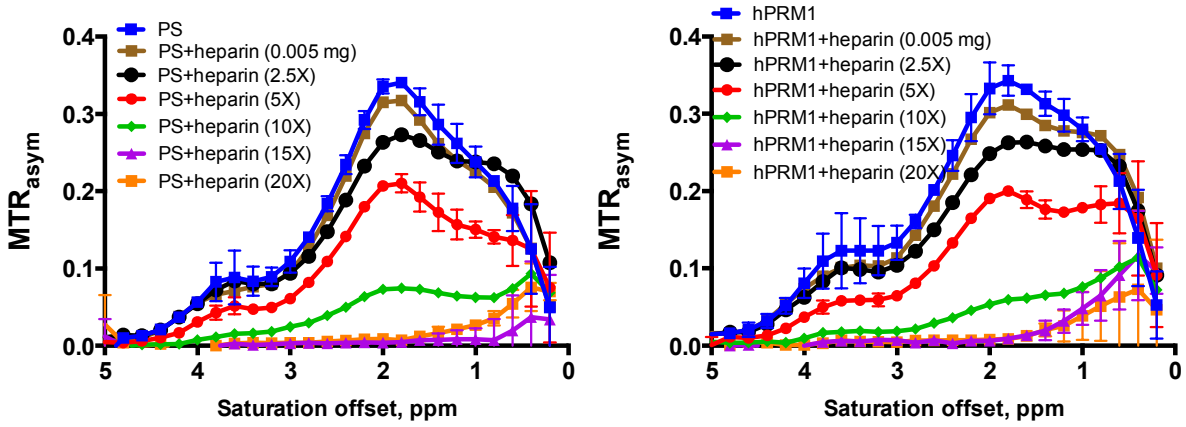

Figure S4. MTR_{asym} of hPRM1 and PS solution (5 mg/ml) condensed using various amounts of heparin with the measurements performed using a saturation pulse with B_1 = 3.6 μT and T_{sat} = 4 s. Each value represents the mean at least three independent experiments (mean±SEM, n=3).

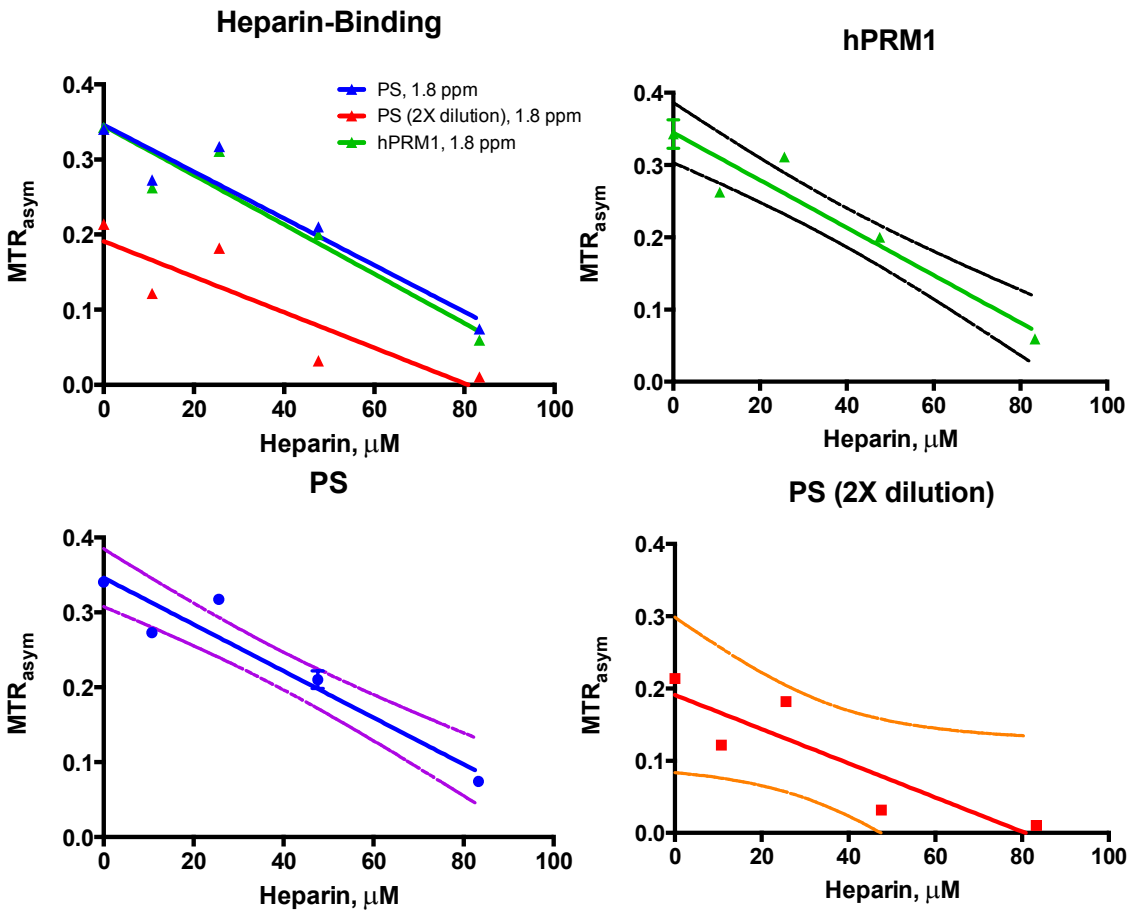

Figure S5. Scatchard plots of PS and hPRM1 complexed with different amounts of heparin. The dashed lines above and below the best-fit solid line define the 95% confidence interval.

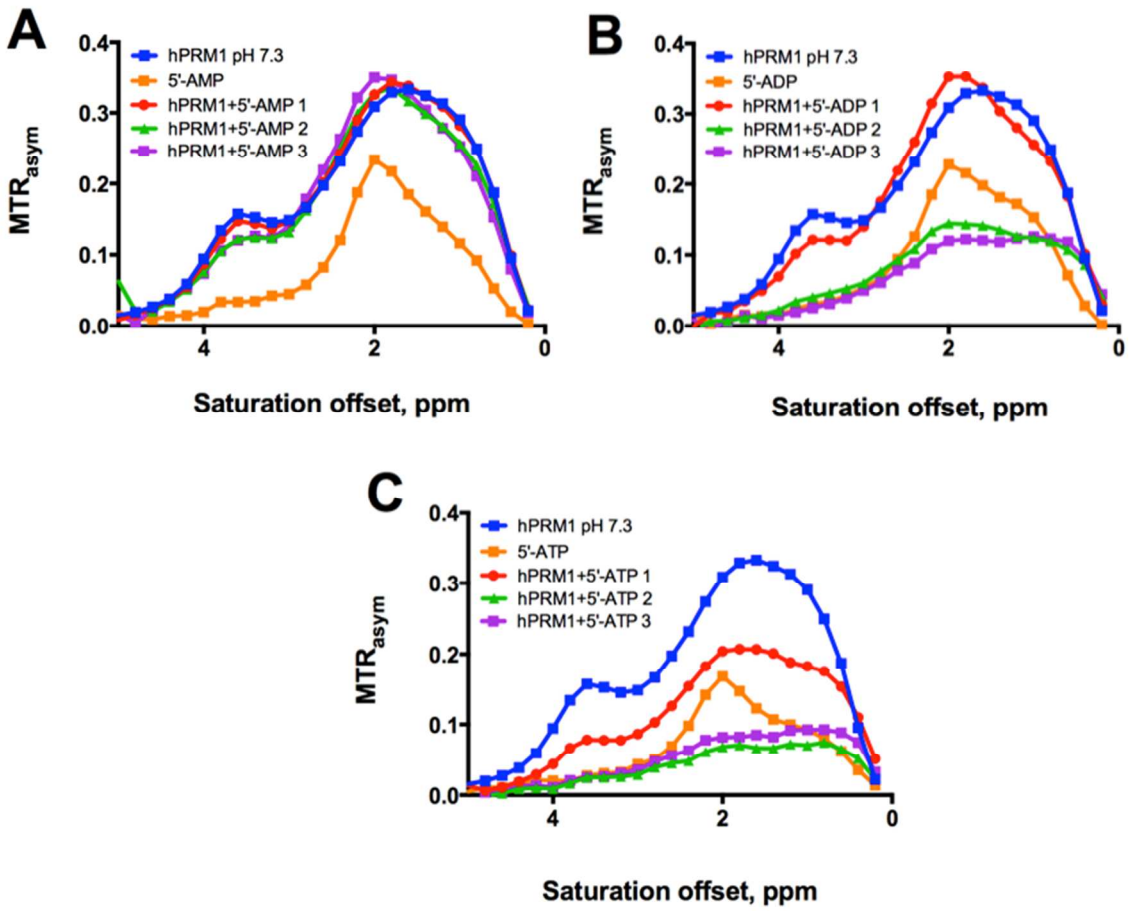

Figure S6. MTR_{asym} of hPRM1 (5 mg/ml) condensed with various concentrations of nucleotides: 5'-AMP (A); 5'-ADP (B); 5'-ATP (C) starting at the volume ratio (hPRM1: nucleoside) of 2:1 (1), 1:1 (2) and 1:2 (3) using a saturation pulse with $B_1 = 3.6$ μT, $T_{sat} =$ 4 s. The concentrations of nucleosides are 0.1 M for 5'-AMP and 0.2M for 5'-ADP and 5'-ATP.

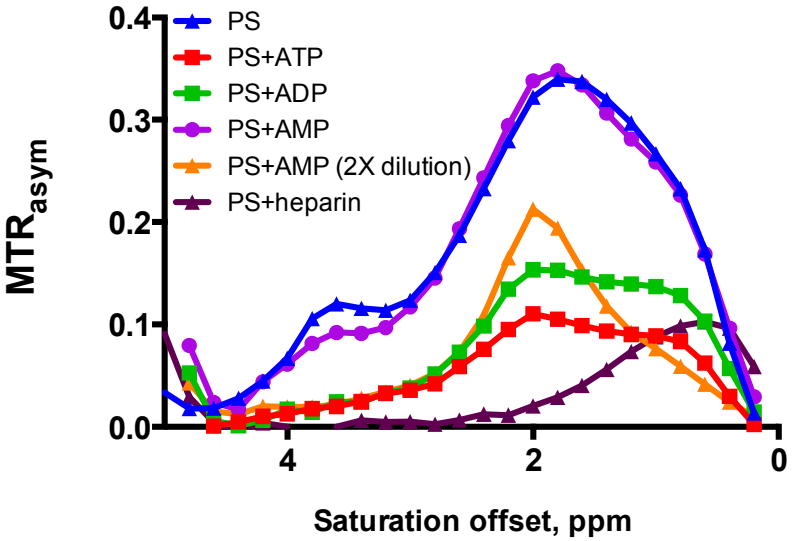

Figure S7. MTR_{asym} of protamine sulfate complexed with nucleotides at PS: nucleoside volume ratio of 2:1 and 0.075 mg of heparin (PS concentration 0.733 mM). Conditions: B_1 = 3.6 μT, T_{sat} = 4 s. The concentrations of nucleosides are 0.1 M for 5'-AMP and 0.2 M for 5'-ADP and 5'-ATP.

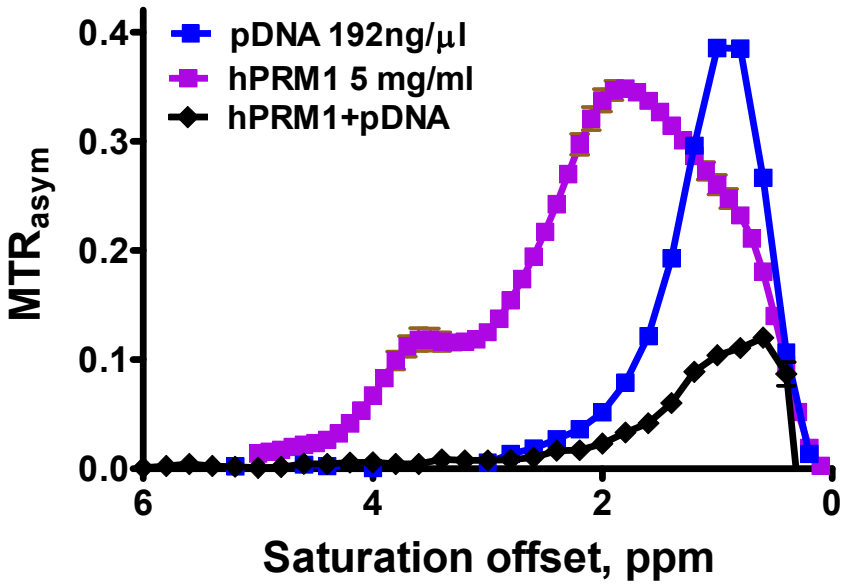

Figure S8. MTR_{asym} of hPRM1 complexed with pDNA at a hPRM1: pDNA volume ratio of 1:1. Conditions: hPRM1 - 5 mg/ml, B_1 = 3.6 µT, T_{sat} = 4 s. The concentration of pDNA is 192 ng/µl.

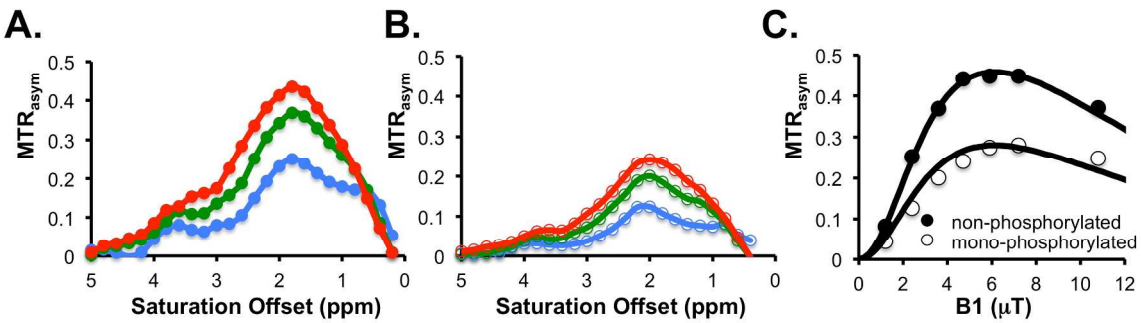

Figure S9. MTR_{asym} vs saturation offset for 0.7 mM non-phosphorylated hPRM1 (A) and mono-phosphorylated hPRM1(B). The saturation field strengths are 2.4 μT (blue), 3.6 μT (green) and 4.7 μT (red); (C) MTR_{asym} at 1.8ppm as a function of saturation field strength.

	PBS, pH 7.4	+ 5'-AMP		+5'-ATP		+ heparin	
	Size (nm)	Size (nm)	K_{eq}, $x10^{-3}$ M	Size (nm)	K_{eq} $x10^{-3}$ M	Size (nm)	K_{eq} $x10^{-6}$ M
hPRM1	**3.95**±1.1 (0.242)	**312**±25 (0.05)	108±7	**425**±32 (0.105)	35.8±6.9	**106**±19 (0.435)	304±32
PS	**2.65**±0.33 (0.265)	**2.75**±0.14 (0.175)	147±15	**926**±54 (0.305)	88.9±5	**1544**±15 (0.837)	321±33

Table S1. Average diameter (size, nm±SD) of PS and hPRM1 nanoparticles alone or complexed with AMP, 5'-ATP and heparin determined by DLS measurements at 25°C and binding constants (Keq) of protamines with 5'-AMP, 5'-ATP and heparin. Concentration of peptide solutions is 0.5 mg/ml.

	Result	**Helix (%)**	**Strand (%)**	**Turn (%)**	**Disordered (%)**
PS 0.1 mg/ml	1	7	40	12	40
	2	6	43	12	39
PS (0.1)+ 5'-ATP (1/100)	1	9	37	12	42
	2	7	39	12	43
hPRM1 0.1 mg/ml	1	0	48	12	40
	2	0	61	25	14
hPRM1 0.1 + 5'-ATP(1/100)	1	100	0	0	0
	2	100	0	0	0
hPRM1 5 mg/ml	1	15	6	19	60
	2	13	5	24	57
hPRM1 5 mg/ml + heparin	1	12	21	16	50
	2	8	24	16	53
hPRM1 5 mg/ml + DNA	1	16	6	19	60
	2	14	5	23	58

1: Closest matching solution; 2: Average of all matching solutions.

Table S2. Calculated secondary structure fractions of PS and hPRM1 alone and complexed with 5'-ATP, heparin or DNA using CONTIN algorithm (Provencher & Glockner Method).

References

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