Superparamagnetic Nanosensors

Magnetic Sensors for Protease Assays

*Ming Zhao, Lee Josephson, Yi Tang, and Ralph Weissleder**

Sensitive protease assays play an important role in elucidating protease function and in developing effective and selective anti-protease therapeutics. Although assays based on radioisotopes excel in sensitivity, the use of ionizing radiation often impedes a more general adaptation. Fluorogenic substrates with an internally quenched fluorophore are designed to produce fluorescent emission upon cleavage in assays based on fluorescence resonance energy transfer (FRET).[1,2] While the widespread application of such assays underscores their usefulness and practicality, issues intrinsic or secondary to the quenching mechanism must be resolved for each substrate.[1,3]

Here we describe a protease assay system that uses superparamagnetic nanoparticles as magnetic relaxation switches (MRS)[4] and determines protease activity from the spin–spin relaxation time (T_2) of water. The MRS approach differs from the method based on paramagnetic agents, in which enzyme activity is measured through changes in the spin–lattice relaxation time (T_1) of water.[5,6] The current assay employs peptide substrates that consist of a central sequence for protease recognition and cleavage, flanked by a biotinylated residue on each terminal. The bi-biotinylated peptide (BBP) substrates interact with avidin-magnetic nanoparticles (CLIO-A) and induce a clustered state with high T_2 relaxivity (Figure 1). When present, proteases cleave the

[*] Prof. R. Weissleder, M. Zhao, L. Josephson, Y. Tang
Center For Molecular Imaging Research
Massachusetts General Hospital, Harvard Medical School
Building 149, 13th Street, Room 5403, Charlestown, MA 02129 (USA)
Fax: (+1) 617-726-5709
E-mail: weissleder@helix.mgh.harvard.edu

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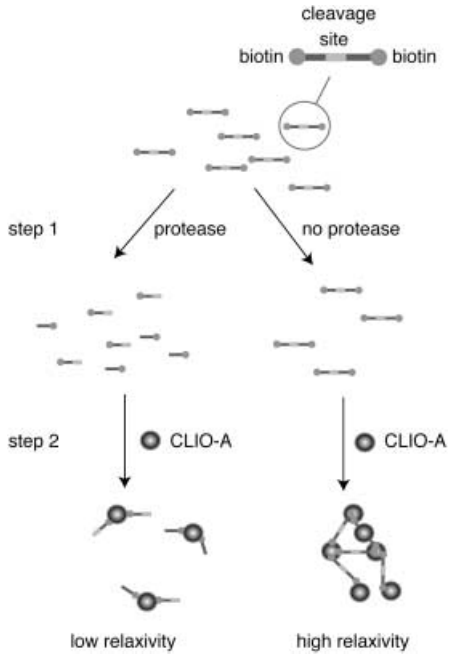

Figure 1. Schematic view of the BBP-MRS assay. The BBP substrate contains a central sequence with a cleavage site and flanking terminal biotins (insert). BBP induces cluster formation among CLIO-A nanoparticles, which results in high T_2 relaxivity (right). A protease converts BBP into mono-biotinylated fragments, abolishing the cluster-inducing activity; this is accompanied by low T_2 relaxivity (left).

substrate sequence between the two biotins (Btns), which results in mono-biotinylated fragments that interact with CLIO-A nanoparticles without inducing aggregation. Such a substrate design obviates the necessity of fluorescent quenching, and the magnetic resonance measurements can be conducted with optically opaque media such as tissue culture medium and tissue extracts.

To demonstrate the principle of the BBP-MRS assay, cluster formation in CLIO-A was induced by using various amounts of the generic substrate Btn-$(G)_4$RRRR$(G)_3$K(Btn). The transformation of the nanoparticle conjugates from a monodispersed to higher order clustered state was accompanied by a linear increase in the T_2 relaxivity R_2 (which is defined as the slope of the plot of the relaxation rate ($1/t$, s^{-1}) against nanosensor concentration (Fe, mM); Figure 2a). Subsequently, when we synthesized a more specific substrate for trypsin, Btn-GPARLAIK(Btn),[7,8] and used quantitative HPLC as a reference for the BBP-MRS assay, the changes in T_2 were correlated to the extent of substrate hydrolysis. The enzyme kinetics constants k_{cat} and K_m were determined to be 71.6 s^{-1} and 80.4 μM, respectively, which are in the same range as previously published values for both native peptide and FRET substrates for trypsin.[7,8] According to the HPLC profile and MS data, the site of hydrolysis is between positions P_1 and P_1', also consistent with the literature.[8,9] These observations strongly indicate that the BBP substrates retain native activities to proteolytic recognition and hydrolysis, while carrying molecular ligands designed for magnetic resonance readout. To demonstrate the feasibility of using the BBP-MRS assay in a high-throughput fashion, we imaged a 96-well microtiter plate (200 μL per well) with a 1.5 T clinical MR imaging system as described previously (Figure 2b).[10] With this methodology we have shown that stacked microtiter plates with up to 1536 wells per plate and 10 μL per well can be imaged simultaneously, providing data on thousands of samples in tens of minutes.[10]

Next, we investigated whether this technique could be used to quantitate a clinically relevant protease such as renin and how sensitive the magnetic assay would be. Renin is a protease secreted by juxtaglomerular cells in the kidney and plays an important role in blood pressure regulation.[11] Figure 3 summarizes the direct comparison of the assays against purified enzyme with FRET[12] and BBP substrates. The detection limit of the current method was calculated to be 69 ng mL^{-1} h^{-1} (equivalent to 31 nM h^{-1} of BBP hydrolyzed), which is in a range similar to that of published values obtained with FRET substrates for renin assay.[12] In comparison, FRET assays have the advantage of being continuous measurements. However, while the inner filter effect often limits the useful substrate concentration of FRET assays to micromolar range,[3] a given BBP substrate may be used at much higher concentrations. The ability to use a wide range of substrate concentrations facilitates measurements for the maximal rate of hydrolysis in enzyme kinetics studies. Another demonstration of the indifference of MRS to interferences was performed with renin in the presence of

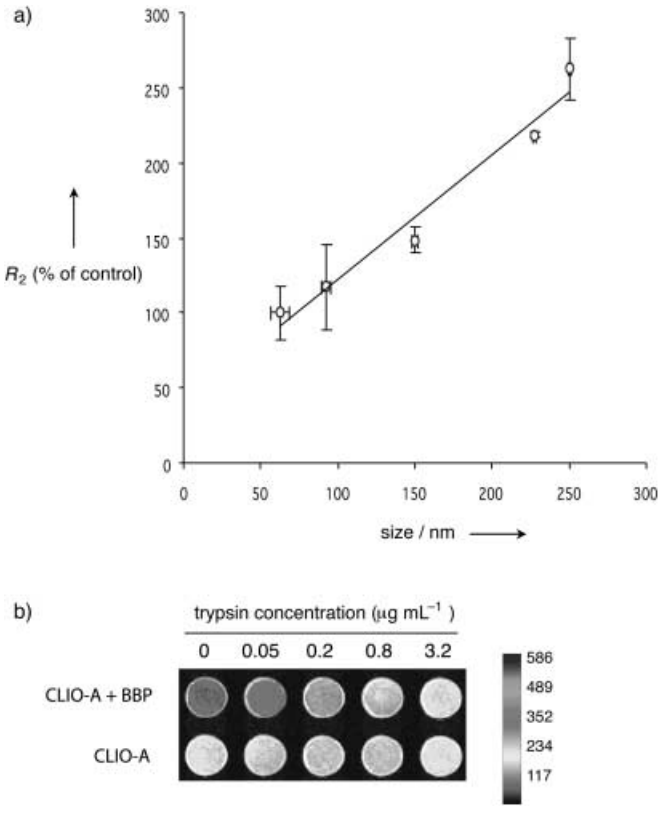

Figure 2. a) Plot of the increase in the average cluster size and the increase in relaxivity R_2 among CLIO-A nanoparticles, in the presence of BBP. b) T_2 mapping of 96-well plate samples measuring trypsin activity. The upper row contains CLIO-A and trypsin from 0 to 3.2 μg mL^{-1}. In the lower row are reference wells with CLIO-A only. T_2 values are coded according to the bar on the right.

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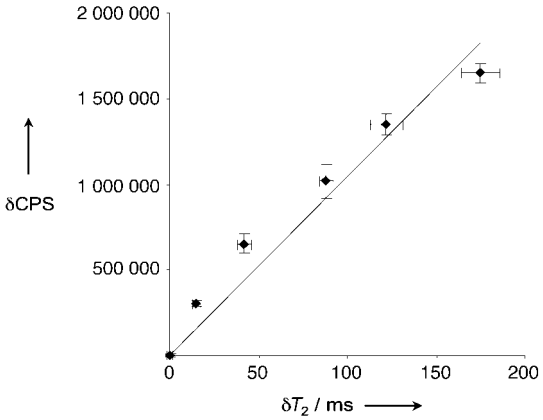

Figure 3. Direct comparison between FRET (*y* axis) and magnetic (*x* axis) assays. CPS = counts per second.

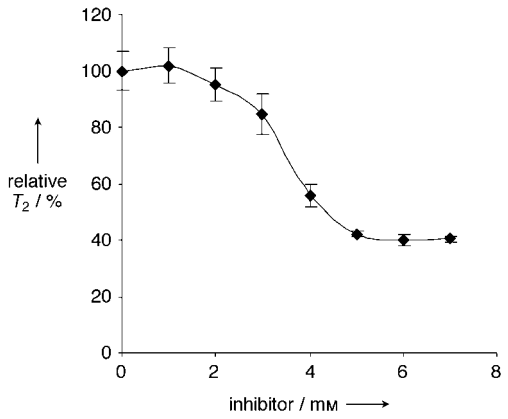

Figure 4. MMP-2 activity from the cell culture media of HT1080 fibrosarcoma cells was shown by the BBP-MRS assay to be inhibited by the presence of EDTA in a dose-dependent manner.

red blood cells. A solution of 2% whole blood in buffer reduced the fluorescence of the FRET substrate to near background levels, which makes it imposssible to perform FRET measurements. In contrast, the MRS assay was unaffected.

Proteases play key roles in diverse diseases.[13–17] In subsequent studies we wished to explore whether the BBP-MRS assay could be used to measure inhibition of matrix metalloproteinase 2 (MMP-2), a protease linked to metastasis and tumor angiogenesis.[13] The substrate Btn-GGPLGVRGK(Btn) was incubated with purified, active MMP-2, which led to hydrolysis at the expected cleavage site,[18] as confirmed by HPLC and mass spectrometry. Subsequently, we measured the concentration of MMP-2 in cell-culture supernatant from HT1080 fibrosarcoma cells without any purification.[19] Apart from MMP-2, the collected media also contained a host of biomolecules that contribute to a broad range of background absorbance at wavelengths less than 600 nm, which can be problematic to fluorescent measurements. However, with the MRS-BBP method, MMP-2 activity could be determined in unpurified samples (19 ng mL^{-1} from 24 h growth media), whereas fluorescence measurements under similar conditions required the assay to be run in buffer. Since MMP-2 activity is zinc dependent,[20] EDTA (ethylenediaminetetraacetate) as a model inhibitor was able to inhibit MMP-2 activity in a dose-dependent fashion (Figure 4). Similar results were also observed with other more specific MMP inhibitors (data not shown).

The MRS protease assay we describe utilizes bi-biotinylated peptides readily made by solid-phase peptide synthesis. Yet the assay format could, in theory, be extended to other hydrolytic enzymes (nucleases, polysaccharidases, lipases). Since the protease recognizes the bi-biotinylated peptide in solution, immobilization of the peptide is not required. Unlike fluorescence-based protease assays, the MRS method can accommodate a range of assay conditions, including high turbidity and extreme pH and temperatures.[21] Owing to the penetrating nature of the radio-frequency radiation it employs, the method is also free from light interferences. Finally, in terms of instrumentation, T_2 measurements can be conducted with individual samples in single tubes by using MR relaxometers or with microtiter plates in a high-throughput format.

Experimental Section

BBP synthesis: Fmoc amino acids were obtained from Calbiochem, NovaBiochem (San Diego, CA). BBPs were synthesized by using an automated peptide synthesizer (PS3, Rainin, Woburn, MA), and biotinylated at the N-terminal NH_2 and C-terminal lysine by reaction with NHS-biotin (Pierce) after removal of the Fmoc and 2-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) protecting groups. A second method for making BBPs employed Fmoc-Lys(Btn)-OH in automated peptide synthesis. BBPs were cleaved from the resin and purified by HPLC, and their molecular weights were determined by mass spectrometry. The following BBPs were synthesized: BTN-$(G)_4$RRRR$(G)_3$K(BTN) (M_w calcd: 1622.9, found: 1622.9); trypsin substrate BTN-GPARLAIK(BTN) (M_w calcd: 1276.6, found: 1276.6); MMP-2 substrate BTN-GGPLGVRGK(BTN) (M_w calcd: 1291.4, found: 1291.4), and renin substrate RK(Btn)IHPFHL-VIHTK(Btn)R (M_w calcd: 2233.6, found: 2233.6).

CLIO-A synthesis: Aminated CLIO (CLIO-NH_2) was prepared as described elsewhere.[22] *N*-Succinimidyl *S*-acetylthioacetate (SATA), *N*-succinimidyl 6-[3′-(2-pyridyldithio)propionamido]hexanoate (Lc-SPDP), and neutravidin were obtained from Pierce. Neutravidin is a modified form of avidin with a near-neutral isoelectric point and is free of carbohydrate units to minimize nonspecific binding. To neutravidin (10 mg mL^{-1}, 0.1 mL) was added SATA (11 mg mL^{-1}, 10 μL), and the mixture was allowed to stand for 2 h at room temperature before it was dialyzed and deacetylated with hydroxylamine HCl (Sigma). Thiolated neutravidin was incubated with Lc-SPDP activated CLIO-NH_2. The average number of neutravidin per CLIO particle was determined as described,[23] and CLIO-A was purified by using a magnetic separation column. The hydrodynamic size was determined by using a Zetasizer 1000 SHA (Malvern Instruments).

Protease assays: A solution of the BBP substrate was subjected to protease digestion. Aliquots were removed at defined time intervals and added to CLIO-A, typically at 15 μg Fe per mL, in the presence of protease inhibitors to prevent neutravidin degradation. After 1 h at room temperature, T_2 was measured on a 0.47 T benchtop spectrometer (Bruker). When appropriate, reverse-phase (C18) HPLC was employed as a reference method (a gradient from 10 to 80% solvent A (0.1% trifluoroacetic acid in H_2O as solvent A; 10% solvent A and 90% acetonitrile as solvent B)). The percentage of hydrolysis was calculated from the peak areas and correlated to T_2. K_m was estimated by using the Lineweaver–Burk plot; k_{cat} was deduced from the

equation $k_{cat}/K_m = K_{obs}/Et$, given that substrate concentration was substantially lower than the estimated K_m value.

Comparison with FRET: BBP and FRET (Molecular Probes) substrates were incubated in the same cuvettes (0.5 mL, 1 cm) at 2 μM, with final concentrations of renin (CalBiochem) of 0.78, 1.56, and 3.13 mU. After 8 h, emission at 490 nm (excitation 340 nm) was measured with a Fluorolog spectrofluorometer (Jobin Yuon Inc.). Immediately following fluorescent measurements, T_2 values were obtained. In addition, both assays were repeated in the presence of 2% mouse whole blood.

MMP-2 assay: A solution of MMP-2 BBP substrate (10 $\mu g\,mL^{-1}$) was incubated with various amounts of active recombinant MMP-2 (CalBiochem) at 37°C (50 mM HEPES, 10 mM of $CaCl_2$ and 0.005% Brij (Sigma), pH 7.4). T_2 measurements were performed as described above. HT1080 fibrosarcoma cells were cultured in MEM (Gibco) to confluence, with 10% fetal calf serum. The cells were washed twice, and cultured in serum-free MEM for 24 h. The media were collected and centrifuged, and MMP-2 BBP substrate was added to give a final concentration of 10 $\mu g\,mL^{-1}$. EDTA (0–5 mM) was included as an MMP-2 inhibitor in some of the samples. After incubation for 12 h at 37°C, magnetic resonance measurements were conducted as described above.

MR imaging: Samples of 200 μL were loaded into a 96-well plate and imaged on a 1.5 T superconducting magnet (Signa 5.0; GE Medical Systems, Milwaukee, WI) using a 5-inch surface coil and a T_2-weighted spin echo sequence: 3000/85 (TR/TE), 1 mm slice thickness, 6 × 6 cm field of view, 256 × 256 imaging matrix. To obtain a T_2 map, echo time (TE) values between 20 to 400 ms were used; the T_2 maps were calculated by using Matlab (Matlab 6.0; The MathWorks, Natick, MA).

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[1] E. D. Matayoshi, G. T. Wang, G. A. Krafft, J. Erickson, *Science* **1990**, *247*, 954.
[2] G. B. Irvine, M. Ennis, C. H. Williams, *Anal. Biochem.* **1990**, *85*, 304.
[3] B. P. Holskin, M. Bukhtiyarova, B. M. Dunn, P. Baur, J. de Chastonay, M. W. Pennington, *Anal. Biochem.* **1995**, *226*, 148.
[4] L. Josephson, J. M. Perez, R. Weissleder, *Angew. Chem.* **2001**, *113*, 3304; *Angew. Chem. Int. Ed.* **2001**, *40*, 3204.
[5] A. Y. Louie, M. M. Hüber, E. T. Ahrens, U. Rothbächer, R. Moats, R. E. Jacobs, S. E. Fraser, T. J. Meade, *Nat. Biotechnol.* **2000**, *18*, 321.
[6] A. Bogdanov, L. Matuszewski, C. Bremer, A. Petrovsky, R. Weissleder, *Mol. Imaging* **2002**, *1*, 16.
[7] V. Schellenberger, C. W. Turck, L. Hedstrom, W. J. Rutter, *Biochemistry* **1993**, *32*, 4349.
[8] S. Grahn, D. Ullmann, H. D. Jakubke, *Anal. Biochem.* **1998**, *265*, 225.
[9] I. Schechter, A. Berger, *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
[10] D. Hogemann, V. Ntziachristos, L. Josephson, R. Weissleder, *Bioconjugate Chem.* **2002**, *13*, 116.
[11] L. M. Resnick, J. H. Laragh, *Am. J. Cardiol.* **1985**, *56*, 68.
[12] G. T. Wang, C. C. Chung, T. F. Holzman, G. A. Krafft, *Anal. Biochem.* **1993**, *210*, 351.
[13] A. John, B. Tuszynski, *Pathol. Oncol. Res.* **2001**, *7*, 14.
[14] Z. S. Galis, J. J. Khatri, *Circ. Res.* **2002**, *90*, 251.
[15] R. A. Nixon, *Ann. N. Y. Acad. Sci.* **2000**, *924*, 117.
[16] J. A. Tavel, *Expert Opin. Invest. Drugs* **2000**, *9*, 917.
[17] G. Giannelli, S. Antonaci, *Histol. Histopathol.* **2002**, *17*, 339.
[18] E. I. Chen, S. J. Kridel, E. W. Howard, W. Li, A. Godzik, J. W. Smith, *J. Biol. Chem.* **2002**, *277*, 4485.
[19] H. J. Cha, S. K. Bae, H. Y. Lee, O. H. Lee, H. Sato, M. Seiki, B. C. Park, K. W. Kim, *Cancer Res.* **1996**, *56*, 2281.
[20] B. Isaksen, M. K. Fagerhol, *Mol. Pathol.* **2001**, *54*, 289.
[21] J. Perez, L. Josephson, T. O'Loughlin, D. Hoegeman, R. Weissleder, *Nat. Biotechnol.* **2002**, *20*, 816
[22] L. Josephson, C. H. Tung, A. Moore, R. Weissleder, *Bioconjugate Chem.* **1999**, *10*, 186.
[23] M. Zhao, M. F. Kircher, L. Josephson, R. Weissleder, *Bioconjugate Chem.* **2002**, *13*, 840.

  0044-8249/03/11512-1416 $ 20.00+.50/0