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Nanosensors

Quantum-Dot-Based FRET Detection of Histone Acetyltransferase Activity**

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The eukaryotic genome is condensed within the nucleus as chromatin, which consists of double-stranded DNA in tight association with histone proteins. The N-terminal 'tails' of histone proteins are subject to a range of reversible covalent modifications that serve to define the transcriptional state of genes and play a key role in the recruitment of other effectors of DNA metabolism and maintenance of genome integrity.[1] The complex interplay of these modifications defines an underlying epigenetic code that determines DNA accessibility and regulation of gene expression. Dysregulation of many enzymes involved in epigenetic regulation is often accompanied by the development of numerous disease states and selective small-molecule inhibition of such enzymes presents novel routes for therapeutic intervention.[2] Histone deacetylases (HDACs), which catalyze the removal of acetyl groups from the ε-amino group of the lysine side chain, have attracted substantial attention as anticancer targets and several HDAC inhibitors have been developed that have achieved notable clinical success.[3] There is now a growing body of evidence that the antagonistic lysine acetylation reaction, catalyzed by histone acetyltransferases (HATs), and aberrant HAT activity are associated with numerous pathological conditions, including cancer, neurodegeneration, chronic inflammation, and HIV infection and, as such, HATs are considered to be a novel emerging class of drug target.[4]

The development of new pharmacological agents that inhibit HAT activity is critically dependent on the availability of simple, sensitive, quantitative, and robust functional assays. Methods of detecting HAT activity are typically based on radioisotope labeling of histone substrates using [^{3}H]/[^{14}C]-acetyl coenzyme A (CoA) or enzyme-linked immunosorbent assay (ELISA)-based detection using acetyl lysine specific antibodies. However, because of their associated disposal hazards and heterogeneous nature, these approaches are not generally considered suitable for high-throughput screening.[5]

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Several homogeneous assays have been developed that measure the generation of HAT reaction by-products (i.e., HS-CoA) by means of enzyme-coupled reactions that generate reduced cofactors and a colorimetric signal, or through the use of thiol-reactive dyes that react to produce a fluorescent adduct. These coupled assays, however, are often susceptible to fluorescence interference (that originates from the test compounds themselves, or the biological components of such assays) and furthermore can not be used to directly measure the acetylated enzyme reaction products.[5b,6] Environment-sensitive dye-labeled peptide substrates have also been developed that undergo modest shifts in fluorescence emission maximum in response to enzyme-mediated acetylation, but may not necessarily accurately mimic endogenous substrates because of the presence of bulky aromatic substituents.[7]

Herein we demonstrate a new nanosensor approach for the detection of HAT-mediated acetylation using quantum-dot (QDot)-based Förster resonance energy transfer (FRET) donors. Quantum dots are excellent reagents for FRET-based biosensing because of their broad excitation spectra, tunable peak emission wavelength, and large effective Stokes shift, and have numerous applications in bioanalytical chemistry.[8] FRET-based detection of HAT activity has several advantages over traditional assays owing to their ability to conduct homogeneous single-phase enzyme reactions and the dual-wavelength readout that provides an internal normalization mechanism. Furthermore, the use of fluorophores with red-shifted emission signals that are well separated from the donor excitation wavelength provides a convenient route to minimize background signal that originates from autofluorescence interference.[9] In contrast to other nanoparticle-enabled enzyme sensors, including protease sensors based on the cleavage of a quencher molecule, the system we report here circumvents the requirement to prefunctionalize the QDot surface and thus affords significantly enhanced assay simplicity and throughput.[10] In fact, we believe that this format offers a more generic approach to enzyme detection since enzyme–nanoparticle interactions do not need to be taken into account during assay development.

Our FRET-based HAT sensing approach is illustrated in Figure 1. A hexahistidine (His_6)-appended synthetic substrate peptide, based on the H4 N-terminal histone tail sequence, is incubated with the p300 HAT in the presence of acetyl-CoA. The peptide substrate is preferentially acetylated at the Lys_6 position.[11] The acetylated peptide can then bind to the ZnS QDot surface by His_6-mediated metal-affinity self-assembly.[12] An acceptor-dye-labeled acetyl lysine specific antibody binds to the acetylated peptide/QDot complex and results in QDot–dye energy transfer. The strong distance dependence

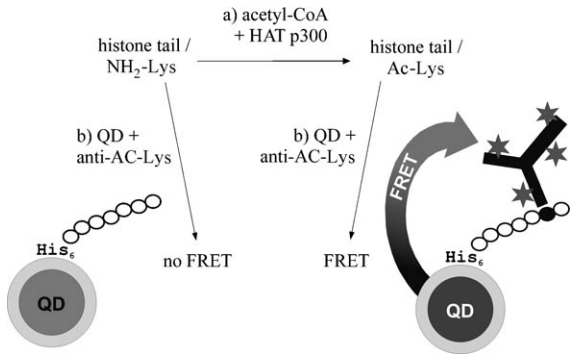

Figure 1. QDot FRET-based detection of p300 HAT activity. a) Lysine-containing histone tail substrate peptide was incubated with p300 HAT and acetyl-coA at 30 °C. b) Aliquots of peptide solution at varying degrees of acetylation (i.e., different timepoints) were added to solutions of CdSe/ZnS core/shell quantum dots and dye-labeled anti-acetyl lysine. The resulting bioconjugates formed by His-tag/metal-affinity coordination (multiple peptides per QD; one peptide shown for clarity), and antibody–antigen biorecognition exhibited characteristic emission spectra associated with a FRET process; the magnitude of the FRET process correlated with the extent of acetylation by p300 HAT.

of the FRET process prevents unbound-dye-labeled antibodies from contributing to the signal and enables a convenient, simple homogeneous detection scheme.

Aliquots of the p300 HAT reaction mixture were periodically transferred to an aqueous buffer detection solution containing mercaptopropionic acid capped QDots (emission maximum 605 nm) and Alexa Fluor 647 labeled anti-acetyl lysine antibody. This FRET pair affords a Förster radius of 72 Å[13], well-separated donor and acceptor emission peaks, and minimal direct excitation of the acceptor at the QDot excitation wavelength.[14] Steady-state photoluminescence spectra revealed a rapid increase in dye-specific fluorescence and corresponding decrease in QDot-specific emission as a function of time (Figure 2), consistent with an enzyme-dependent FRET process. However, sensitized acceptor

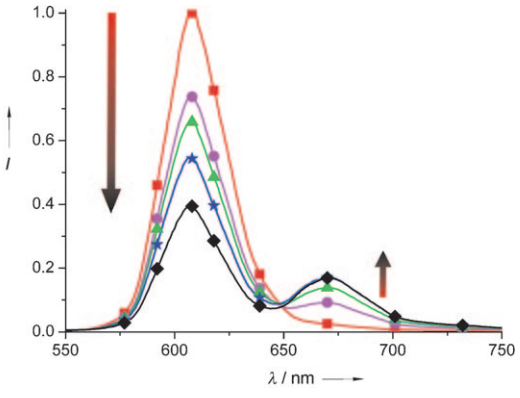

Figure 2. Photoluminescence spectra taken at 15 min intervals after addition of 320 nM p300 HAT. ■ 0 min, ● 15 min, ▲ 30 min, ★ 45 min, ◆ 60 min. The magnitude of QDot–dye energy transfer was monitored by calculating the ratio of the emission peaks at 670 and 605 nm. The red–black arrows indicate decreased QDot emission coupled with increased dye emission, which correspond to a greater degree of acetylation by p300 HAT.

emission was not observed in control experiments in which either p300 HAT or acetyl-CoA were omitted from the reaction mixture, thus confirming the specificity of the nanosensor.

The kinetics of immunocomplex formation were monitored by following the ratio of the dye- and QDot-specific emissions (670 and 605 nm) following addition of an enzymatically acetylated peptide to the QDot/anti-acetyl lysine solution. The 670/605 nm signal increased rapidly, on the order of seconds, in the case of peptide that had been incubated with both p300 HAT and acetyl-CoA, and reached saturation after approximately 30 minutes. Again, this spectral response was not observed in control experiments that excluded either p300 HAT or acetyl-CoA from the reaction medium, and is consistent with the hypothesis that the observed increase in dye emission and decrease in QDot emission was due to FRET driven by the activity of p300 HAT. We did not see any evidence for direct antibody binding to the QDot surface, thus suggesting that the inclusion of bovine serum albumin (BSA) in the medium was sufficient to prevent nonspecific binding.

As proof-of-concept, we further investigated the ability of our HAT nanosensor to quantitatively assess inhibitor potency by exposing p300 HAT to serial dilutions of the known selective small-molecule inhibitor and antiproliferative agent, anacardic acid.[15] p300 HAT was preincubated with solutions of anacardic acid in dimethylsulfoxide (DMSO) prior to initiating the reaction by the addition of acetyl-CoA. The reaction products were subsequently detected by QDot–immuno FRET, as described, and the ratio of emission intensities at 670 and 605 nm was plotted as a function of inhibitor concentration (Figure 3).

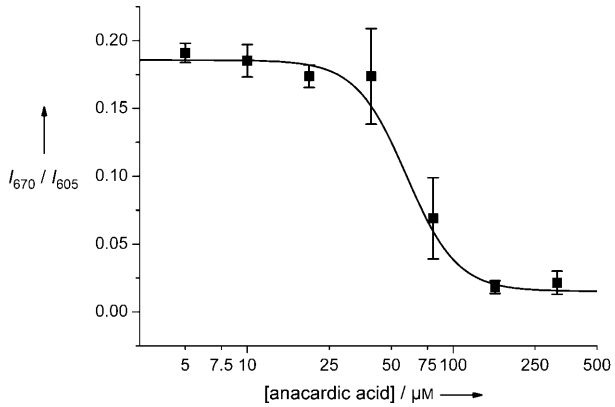

Figure 3. Dose response of p300 HAT (250 nM) to various concentrations of anacardic acid. Each data point is the average of two independent measurements.

The dose-response curve for anacardic acid was sigmoidal and provided an approximate IC_{50} value of (59 ± 12) μM; this value is within a factor of seven of the reported IC_{50} value of 8.5 μM, which was determined by isotope labeling (Figure 3).[16] This level of correspondence is easily sufficient to allow for a comparative screen of acetyltransferase inhibitors. Anacardic acid is thought to inhibit binding of acetyl-CoA to the active site of HATs;[17] hence the data

measured at high anacardic acid concentrations indicate that this assay is truly enzyme-activity-dependent rather than simply a measure of enzyme concentration.

We ascertained the limit of detection of our system using extended HAT reaction conditions (6 h reaction times) prior to the detection of acetylated products, as described above, by measuring the emission intensity ratios at 670 and 605 nm (Figure 4). We detected sub-nanomolar concentrations of p300 HAT; this sensitivity is comparable to that of radioisotope-based detection methods.[5a]

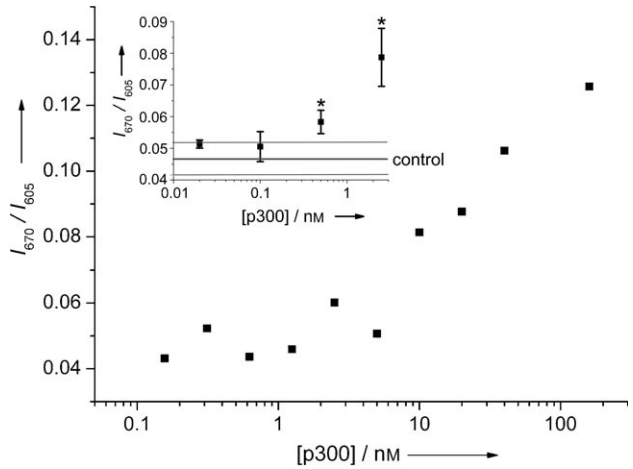

Figure 4. Ratios of the emission intensity at 670/605 nm for varying enzyme concentrations, showing an elevated response for [p300] > 10 nM. Inset: Ratios after 6 h incubation time. Asterisks indicate significant signal above control (95% confidence level, $n = 3$). The detection limit was 0.5 nM.

Whilst a range of optically active, luminescent, and magnetic nanoparticle materials has been used to devise numerous sophisticated enzyme-sensing systems, these approaches are often limited by the necessity to perform sophisticated nanoparticle–biomolecule derivitization procedures that contribute to complexity, cost, and limit assay throughput. Our sensing approach affords greater simplicity as it can be conducted without prior nanoparticle conjugation steps or additional purification.[18] Furthermore, the use of freely diffusing peptide substrates in this case, as opposed to those that have been subjected to surface immobilization, provides additional benefits, as they should provide a more accurate mimic of endogenous physiological substrates. The two-step nature of this assay means that kinetic analysis of p300 HAT is more cumbersome to perform. However, this difficulty did not prevent the use of end-point data in an inhibitor-screening format. Indeed, bound substrates often present a complicated and ill-characterized biological interface. For instance, enzyme interactions with nanoparticle-bound substrates can be strongly influenced by nanoparticle size, curvature, ligand packing density, and surface charge; all of which may have a significant effect on steric accessibility and enzyme kinetics.[19] In addition, we were unable to observe any enzyme-dependent FRET behavior in experiments that employed peptides preassembled upon the QDot surface (not shown), possibly because the sterically constrained nature of the substrate occludes access of target lysine residues to the p300 HAT active site.

In summary, we have demonstrated that QDots can serve as efficacious reagents for the detection of HAT activity in a simple, convenient homogeneous FRET assay. We anticipate that this approach should be sufficiently versatile to provide a general platform to study the activity of other histone-modifying enzymes by simply changing the sequence of the substrate peptide and complementary modification-specific antibody. These assays may be used in drug-discovery applications to identify novel small-molecule regulators of epigenetic enzymes.

Experimental Section

Reagent preparation: The substrate peptide (H_2N-RGKGGKGLGKGGAKAHHHHHH-$CONH_2$) was prepared by standard 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase synthesis. Organic CdSe/ZnS core/shell QDots (605 nm emission maximum) were obtained from Invitrogen and rendered soluble in aqueous buffer by base-promoted ligand exchange of the native hydrophobic surfactant coating with mercaptopropionic acid (MPA). The MPA-capped QDots were separated from excess thiol by centrifugal ultrafiltration (Millipore, molecular weight cut-off 10 kDa) and stored in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; 50 mM, pH 8.0), bovine serum albumin (0.1% (w/v); assay buffer) at 4°C before use. Monoclonal anti-acetyl lysine (clone 7F8, Abcam) was labeled with the succinimidyl ester derivative of Alexa Fluor 647 (Invitrogen) according to the manufacturer's instructions (final fluorophore/protein ratio 3.3:1, determined by absorbance).

Enzyme assays: Recombinant GST-purified p300 HAT domain (Millipore) at different final concentrations was incubated with H4 substrate peptide (20 μM) and acetyl-CoA (200 μM) in assay buffer in a total volume of 10 μL at 30°C. A 1.5 μL aliquot of the reaction mixture was added to of MPA–QDots (50 nM, 12 μL) and antibody/Alexa Fluor 647 conjugate (0.5 μM) in assay buffer to reach a peptide/QDot ratio of 50:1 (to provide a maximum signal, as determined by peptide ratio titration) and the mixture was allowed to equilibrate for 30 min. Photoluminescence emission spectra were then recorded in 384-well black microplates (Corning) on a SpectraMax M^5 plate reader (Molecular Devices; 400 nm excitation, 5 nm excitation/emission slit width).

Inhibitor studies: Stock solutions and serial dilutions of anacardic acid (Enzo Life Sciences) were prepared in anhydrous DMSO and stored at −20°C before use. p300 HAT (2 μL, 100 nM), peptide (5 μL, 40 μM), and inhibitor dilution (1 μL in DMSO) were pre-equilibrated for 10 min at room temperature, prior to initiating the reaction with the addition of acetyl-CoA (1 μL, 2 mM) and incubation for 1 h at 30°C. The acetylated reaction products were then detected as described above.

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[1] M. Biel, V. Wascholowski, A. Giannis, *Angew. Chem.* **2005**, *117*, 3248–3280; *Angew. Chem. Int. Ed.* **2005**, *44*, 3186–3216.
[2] a) A. P. Feinberg, *Nature* **2007**, *447*, 433–440; b) C. Ptak, A. Petronis, *Annu. Rev. Pharmacol. Toxicol.* **2008**, *48*, 257–276.

[3] S. Grant, C. Easley, P. Kirkpatrick, *Nat. Rev. Drug Discovery* **2007**, *6*, 21 – 22.
[4] a) L. Stimson, M. G. Rowlands, Y. M. Newbatt, N. F. Smith, F. I. Raynaud, P. Rogers, V. Bavetsias, S. Gorsuch, M. Jarman, A. Bannister, T. Kouzarides, E. McDonald, P. Workman, G. W. Aherne, *Mol. Cancer Ther.* **2005**, *4*, 1521 – 1532; b) S. R. Bhaumik, E. Smith, A. Shilatifard, *Nat. Struct. Mol. Biol.* **2007**, *14*, 1008 – 1016; c) S. C. Hodawadekar, R. Marmorstein, *Oncogene* **2007**, *26*, 5528 – 5540; d) F. J. Dekker, H. J. Haisma, *Drug Discovery Today* **2009**, *14*, 942 – 948.
[5] a) A. Poveda, R. Sendra, *Anal. Biochem.* **2008**, *383*, 296 – 300; b) G. W. Aherne, M. G. Rowlands, L. Stimson, P. Workman, *Methods* **2002**, *26*, 245 – 253.
[6] a) J. Y. Zhao, Z. Yan, Z. L. Liu, N. Wang, *Prog. Biochem. Biophys.* **2008**, *35*, 1084 – 1089; b) Y. Kim, K. G. Tanner, J. M. Denu, *Anal. Biochem.* **2000**, *280*, 308 – 314.
[7] J. Wu, Y. G. Zheng, *Anal. Biochem.* **2008**, *380*, 106 – 110.
[8] a) I. L. Medintz, H. Mattoussi, *Phys. Chem. Chem. Phys.* **2009**, *11*, 17 – 45; b) K. Boeneman, B. C. Mei, A. M. Dennis, G. Bao, J. R. Deschamps, H. Mattoussi, I. L. Medintz, *J. Am. Chem. Soc.* **2009**, *131*, 3828 – 3829; c) M. Suzuki, Y. Husimi, H. Komatsu, K. Suzuki, K. T. Douglas, *J. Am. Chem. Soc.* **2008**, *130*, 5720 – 5725; d) Z. Chen, G. Li, L. Zhang, J. Jiang, Z. Li, Z. Peng, L. Deng, *Anal. Bioanal. Chem.* **2008**, *392*, 1185 – 1188; e) C. Xu, B. Xing, J. Rao, *Biochem. Biophys. Res. Commun.* **2006**, *344*, 931 – 935.
[9] K. M. Kleman-Leyer, T. A. Klink, A. L. Kopp, T. A. Westermeyer, M. D. Koeff, B. R. Larson, T. J. Worzella, C. A. Pinchard, S. A. T. van de Kar, G. J. R. Zaman, J. J. Hornberg, R. G. Lowery, *Assay Drug Dev. Technol.* **2009**, *7*, 56 – 67.
[10] a) J. E. Ghadiali, B. E. Cohen, M. M. Stevens, *ACS Nano* **2010**, *4*, 4915 – 4919; b) S. Gupta, H. Andresen, J. E. Ghadiali, M. M. Stevens, *Small* **2010**, *6*, 1509 – 1513; c) J. E. Ghadiali, M. M. Stevens, *Adv. Mater.* **2008**, *20*, 4359 – 4363; d) R. Freeman, T. Finder, R. Gill, I. Willner, *Nano Lett.* **2010**, *10*, 2192 – 2196; e) D. Aili, M. Mager, D. Roche, M. M. Stevens, *Nano Lett.*, DOI: 10.1021/nl1024062; f) A. Laromaine, L. L. Koh, M. Murugesan, R. V. Ulijn, M. M. Stevens, *J. Am. Chem. Soc.* **2007**, *129*, 4156 – 4157.
[11] P. R. Thompson, H. Kurooka, Y. Nakatani, P. A. Cole, *J. Biol. Chem.* **2001**, *276*, 33721 – 33729.
[12] A. M. Dennis, D. C. Sotto, B. C. Mei, I. L. Medintz, H. Mattoussi, G. Bao, *Bioconjugate Chem.* **2010**, *21*, 1160 – 1170.
[13] T. T. Nikiforov, J. M. Beechem, *Anal. Biochem.* **2006**, *357*, 68 – 76.
[14] C. y. Zhang, L. Johnson, *Angew. Chem.* **2007**, *119*, 3552 – 3555; *Angew. Chem. Int. Ed.* **2007**, *46*, 3482 – 3485.
[15] J. Souto, M. Conte, R. Álvarez, A. Nebbioso, V. Carafa, L. Altucci, A. de Lera, *ChemMedChem* **2008**, *3*, 1435 – 1442.
[16] K. Balasubramanyam, V. Swaminathan, A. Ranganathan, T. K. Kundu, *J. Biol. Chem.* **2003**, *278*, 19134 – 19140.
[17] Y. L. Sun, X. F. Jiang, S. J. Chen, B. D. Price, *FEBS Lett.* **2006**, *580*, 4353 – 4356.
[18] a) G. von Maltzahn, D. H. Min, Y. Zhang, J. H. Park, T. Harris, M. Sailor, S. Bhatia, *Adv. Mater.* **2007**, *19*, 3579 – 3583; b) R. Gill, I. Willner, I. Shweky, U. Banin, *J. Phys. Chem. B* **2005**, *109*, 23715 – 23719; c) I. Willner, R. Baron, B. Willner, *Biosens. Bioelectron.* **2007**, *22*, 1841 – 1852.
[19] C.-C. You, S. S. Agasti, M. De, M. J. Knapp, V. M. Rotello, *J. Am. Chem. Soc.* **2006**, *128*, 14612 – 14618.

