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Research Article

RECENT DEVELOPMENT OF FLUOROGENIC PROBES TO DETECT HISTONE DEACETYLASE (HDAC) ENZYME ACTIVITY IN VARIOUS APPROACHES

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A B S T R A C T

Histone deacetylases (HDACs) are important enzymatic regulators of many cellular processes such as cell cycle, gene expression and tumorigenesis. HDACs enzymes are the major targets of drug development for diseases such as neurological diseases, metabolic diseases and cancer. Due to have attracting attention of HDACs, the detection of HDAC activities is key factor to medical sciences as well as basic biological research. Detection by fluorescence techniques is highly attractive due to its high sensitivity and real-time detection approach in a non-destructive way. Recently, various research have been made to develop the investigation of deacetylation of HDAC substrates based on intramolecular transesterification reaction, aggregation induced emission, intramolecular imine bond formation etc. This review article covers the design of fluorogenic probes and mechanistic behaviour for detection of HDAC activity.

INTRODUCTION

Genetic information encoded in DNA is mostly identical in every eukaryotic cell. However, cells in different tissues can have widely different gene expression patterns and thus can exhibit specialized functions. Gene expression in different types of cell needs to be maintained appropriately; inappropriate expression leads to disease. In eukaryotes, the chromatin structure i.e. the packaging of DNA with histone proteins, are thought to contribute to the control of gene expression. Histone post-translational modifications (PTMs) include methylation, acetylation, phosphorylation, ubiquitylation, and others,[1,2] and these type of modifications are believed to contribute to the control of gene expression. Therefore, an appropriate balance of stability and dynamics in histone PTMs is necessary for accurate gene expression. Epigenetic regulation of gene expression plays an crucial role in development and prevention of diseases.[3] Modification of DNA or histone proteins is controlled by the gene expression, without any change of genetic information encoded in DNA sequences. The reversible acetylation of lysine residues near the N-terminus of nucleosomal histones by histone deacetylases (HDACs) and histone acetyltransferases (HATs) controls chromatin structure and transcriptional activity.[3,4] The activities of both HATs and HDACs directly induced various biological phenomena including apoptosis,[5] angiogenesis,[6] and the pathogenesis of malignant diseases.[7]

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Histone acetylation catalysed by HATs generally leads to transcriptional activation, whereas deacetylation activity executed by HDACs results in transcriptional repression.[8]

HDACs enzymes are the major targets of drug development for diseases such as neurological diseases, metabolic diseases and cancer.[9] Due to have attracting attention of HDACs, the detection of HDAC activities is key factor to medical sciences as well as basic biological research. Although various researches have been made to develop the investigation of deacetylation of HDAC substrates, all of these methods require multiple steps for the detection of enzyme activity. Classical assays for the determination of HDAC activity were based on the incubation of the enzyme with [^{3}H]acetylated histones[10] and peptide substrates.[11] Also the radio-active assays require the separation of the product from the substrate, and this process limits the assay high throughput. A non-isotopic HDAC assay based on fluorophore-conjugated peptide was reported earlier and this method also involve two-step procedure.[12] Generally, in the first step of this assay, deacetylation of the peptides by HDAC is introduced. And in the second step, the enzyme reaction is detected via trypsin cleavage of the fluorophore from the peptides. The main limitation of this assay is that it is not able to permit continuous monitoring of the enzyme activity. However, despite this attention, few systems capable of the fluorogenic detection of HDAC activity in various approaches have been reported recently. This review covers the recent developments to date in the design of fluorogenic probes for detection of HDAC activity including our own contribution in the area. The review deals mainly with the fluorescence behaviour as sensing response and within each section, structural features,

the response observed and details of the sensing mechanism used by the probe are highlighted.

Fluorogenic Probes for HDACs activity

Kikuchi and co-workers were the first to describe a peptide based fluorogenic probe (**1**) that can detect enzymatic deacetylation upon merely mixing the probe and enzyme (HDAC), without additional treatment.[13] Successful detection of enzymatic activity is achieved by utilizing the intramolecular transesterification mechanism for the first time. The structure of the probe consists of a peptide with HDAC substrate moiety i.e. the N-terminal region of histone H3, and a coumarin unit used as the fluorophore (**Figure 1**). The peptide contains an acetyl-Lys (K4(Ac)) at the fourth position in its sequence. In order to perform the deacetylation reaction, an electrophilic carbonate ester was designed and incorporated into the 7-hydroxy position of the coumarin unit. Here, this carbonate ester moiety in the 7-hydroxy position of coumarin behaves as a fluorogenic switch. It is well established that the fluorescence quenching occurs due to acylation of 7-hydrocy position of coumarin derivatives.[14] Moreover, the electrophilic carbonate ester is reactive with the intramolecular nucleophile, the ester may transformed into free hydroxy group at the 7-position of coumarin by the deacetylated product (**2**) through nucleophilic attack of amine (**Scheme 1**). Thus, the amine generated by enzymatic deacetylation triggers spontaneous intramolecular transesterification in the probe, which turns on the masked fluorescence of coumarin. This transesterification reaction produces a fluorescent molecule (**2**) after HADC activity. Therefore, it was established that HDAC activity could be detected by the fluorescence enhancement of the probe, induced as a result of enzymatic deacetylation.

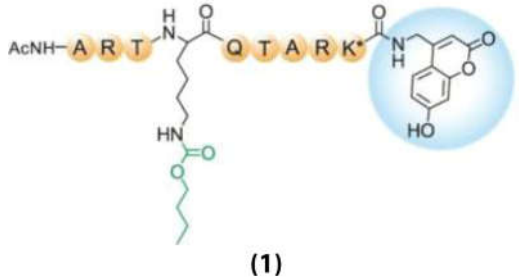
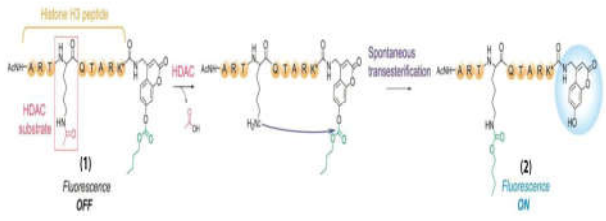
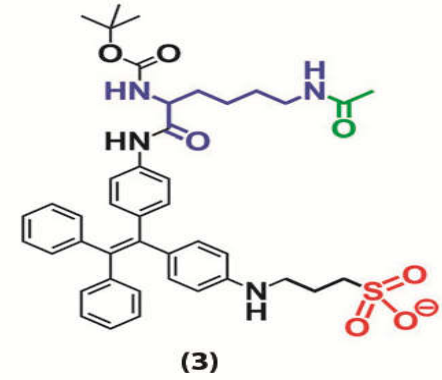


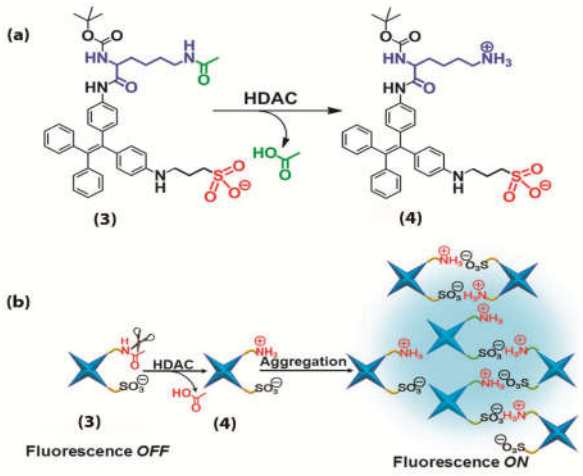
Figure 1 Structure of the peptide based probe 1.



Scheme 1 Mechanism of detection of HDAC activity by the probe **1** using a one-step procedure. Reprinted with permission from ref. 13. Copyright 2012 ACS.

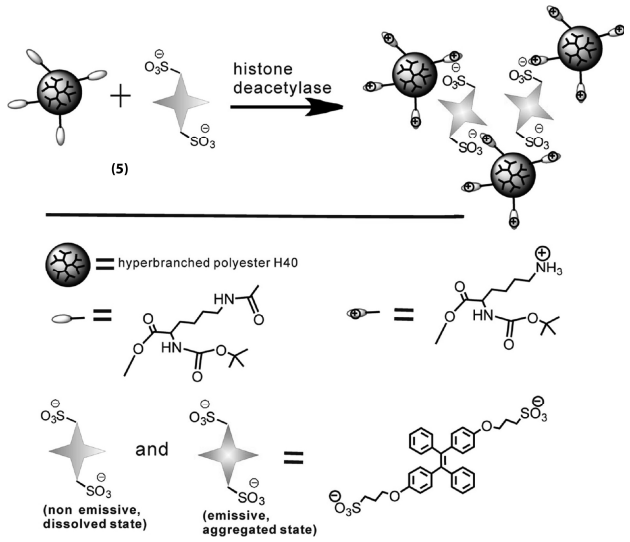
After this contribution, Kikuchi and co-workers again reported a different type of fluorescent probe (**3**) direct one-step detection of HDAC activity based on aggregation induced emission (AIE) mechanism.[15] According to the probe design strategy, a tetraphenylethylene (TPE) molecule was employed as fluorophore based on aggregation-induced emission (AIE) as a switching mechanism. N-□-t-Butoxycarbonyl-N-□-acetyl-L-lysine was attached to one of the amino groups of **3** and 1,3-propanesultone was reacted with the other amino group to form the probe structure (**Figure 2**). A recent breakthrough in the synthesis of fluorescent organic dyes, which induce AIE phenomena, has attracted special attention.[16] TPE derivatives are AIE-active and weakly fluorescent in solution and these dyes become highly fluorescent upon aggregation.[17] Due to the fast rotation of the phenyl rings and partial twisting of the CQC bond the fluorescence is quenched in dilute solution.[18] On the other hand, aggregation of TPE molecules restrict the rotation of the phenyl groups resulting in fluorescence enhancement. This unique luminescence behavior of TPE has been harnessed for the development of biological sensors,[19] solid-state lighting materials,[20] and luminescent polymers.[21] Here, the sulphonate unit was chosen to increase the water solubility as well as to serve as a negative charged terminal. In this acetylated state, the probe shows weak fluorescence due to the lack of aggregation of the probe. The deacetylation of the probe with HDAC generates compound **4**, which possesses primary aliphatic □-amine of lysine (**Scheme 2**). Since the pKa of this amine is close to 10.5, the amine was protonated under physiological conditions (pH 8.0). As a result, the newly generated cationic unit would trigger the aggregation of the probe (**Scheme 2b**) owing to the probable electrostatic interaction among the negatively charged sulphonate unit and the cationic N-□-ammonium ion. This phenomenon restricts the free rotation of the phenyl groups and thus, compound **4** becomes highly fluorescent.





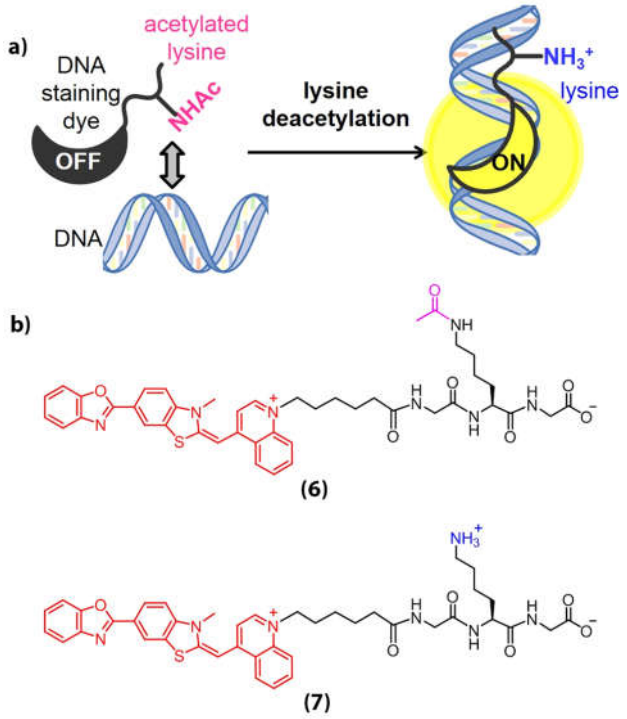
Scheme 2 (a) Proposed enzymatic reaction of **3** with HDAC. (b) Schematic representation of the AIE enhancement upon the reaction of **3** with enzyme.

Inspired by the revolutionary work of Kikuchi, Zeng and Wu group reported a HDAC detection method based on AIE platform.[22] Zeng and Wu co-workers prepared a hyperbranched polyester-based one-step fluorescent assay for HDAC. The hyperbranched polyester was employed as the detection platform in this assay, due to its water-solubility, high molecular weight nature, globular architecture and high number of chain end functionalities.[23-25] This assay system (**5**) consists of two components: the first one is the hyperbranched polyester coupled with acetylated lysine groups (electroneutral) and the second is the negatively charged TPE derivative containing two sulfonic acid groups (TPE-$2SO_3^-$). In the absence of HDAC, the two-component assay system is nonfluorescent. Although, when the HDAC is added in this mixture, the acetyl moieties are removed from hyperbranched polymer derivative. Thus it generates the polymer with a large number of ε-amine groups which become protonated under physiological conditions. Consequently, the electroneutral polymer globules become positively charged and interaction occurs between the globules and the negatively charged TPE-$2SO_3^-$ molecules; leads to the formation of nanoaggregate. The schematic illustration for the fluorescent turn-on detection is shown in **Scheme 3**.



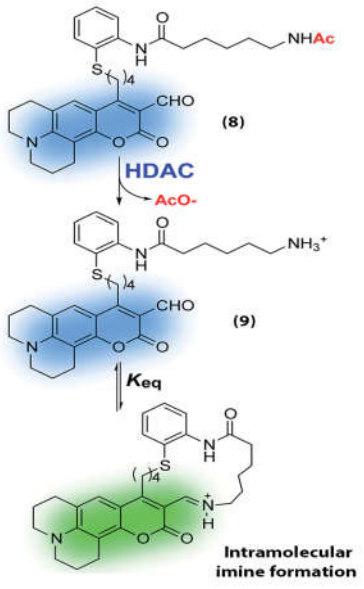
Scheme 3 Schematic illustration for the assay system **5**, and its fluorescent response to histone deacetylase. Reprinted with permission from ref. 22. Copyright 2013 ACS.

Again Kikuchi and co-workers developed a simple, rapid, and continuous method for the detection of HDAC activity.[26] They designed and synthesized a fluorogenic probe, **6**, which is consist with DNA staining dye-peptide conjugate having an acetylated lysine moiety. The acetylated probe displays very low fluorescence even in the presence of DNA owing to have its weak interaction with DNA. Upon the addition of HDAC, the probe **6** has been deacetylated by enzyme to form compound **7**, its charge state becomes more positive and it strongly interacts with DNA, resulting in an enhancement of the fluorescence signal (**Scheme 4**). This method directly monitors the enzymatic activity that enables the kinetic analysis of the enzymatic reaction. Further, this method is a rapid and simple procedure that can also be applied for the evaluation of the potencies of HDAC inhibitors. Also their system may be used for the imaging of HDAC activity in living cells, where the deacetylated shows fluorescence upon binding to intracellular DNA.



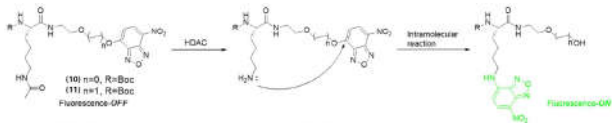
Scheme 4 Fluorogenic detection of HDAC activity using **6**. a) Schematic illustration of HDAC detection method that uses a fluorogenic probe **6** based on a DNA staining dye, b) Chemical structures of the probe **6** and deacetylated product **7**. Reprinted with permission from ref. 26. Copyright 2014 ACS.

Recently, Buccella and co-workers reported a small-molecule probe for single-step, continuous detection of deacetylase activity based on an acetyl-lysine mimic functionalized with an amine-reactive fluorophore.[27] They design a small-molecule probe (**8**) that consisting of an acetyl-lysine functionalized with an amine-reactive coumarin fuorophore. After the treatment of HDAC, the formed deacetylated product(**9**) undergo rapid intramolecular imine formation (**Scheme 5**). This reaction shows a bathochromic shift in the absorption spectrum and changes in fluorescence emission intensity that allow detection of HDAC activity of purified enzymes or in cell lysates.



Scheme 5 Deacetylation reaction by HDAC using probe **8** for single-step spectrophotometric and fluorescence based detection based on intramolecular imine formation. Reprinted with permission from ref. 27. Copyright 2015 RSC.

Very recently, Sun and co-workers designed and developed fluorogenic probes **10** and **11**, that shows one-step turn-on fluorescence for monitoring HDAC activity in a continuous manner.[28] Here nitrobenzoxadiazole (NBD) is as fluorophore moiety because of its unique physical, chemical and fluorescence properties: 1) *O*-NBD and *N*-NBD have distinctly different fluorescence properties; 2) *O*-NBD can be attacked by free amine to form *N*-NBD. As shown in **Scheme 6**, the C-terminus of N-acetyl lysine is functionalized with an *O*-NBD fluorophore such that the O-linkage of the NBD group displays very low fluorescence. Upon deacetylation with HDAC the free amine group in the lysine residue is released. The free amine undergoes spontaneous intramolecular exchange with *O*-NBD, leading to turn-on of fluorescence.



Scheme 6 Mechanistic path for HADC detection using probes **10** and **11**. Reprinted with permission from ref. 13. Copyright 2016 ACS.

CONCLUSION

The approaches discussed above in this review provide examples of the probe designing and detection mechanism of recently reported fluorogenic probes for HDAC activity. Some of the reported probes were based on AIE method using TPE-based fluorophore. Other developed probes were designed using several types of intramolecular reactions e.g. transesterification, imine bond formation etc. within the molecule after the enzymatic reaction. Despite of recent progress in the fluorescent HDAC probes, there is still a high demand to improve the practically available probes for utilization in biological samples.

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