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A novel H_2O_2-triggered anti-Fenton fluorescent pro-chelator excitable with visible light†

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Abstract

A chelator and a pro-chelator that can be activated by H_2O_2 and subsequently sequesters iron and attenuates the Fenton reaction have been developed; both molecules are fluorescent excitable by visible light, and H_2O_2-activation, as well as iron-chelation, induces remarkable changes in fluorescence.

Abnormal accumulation of redox metals (*e.g.*, iron and copper) in certain tissues in the body and the metal-promoted Fenton reactions (eqn (1)), which yield highly reactive and deleterious oxidizing species, have been implicated in the pathogenesis of Parkinson's disease (PD), Alzheimer's disease (AD), atherosclerosis, hemochromatosis, liver damage, diabetes and cancer, *etc*.[1] H_2O_2, one of the key reactants in the Fenton reactions, is also one of the major endogenous reactive oxygen species (ROS).[1] H_2O_2 is essential for cellular activities (*e.g.*, as a second messenger) but can be extremely toxic to cells at high concentrations.[1] The production of H_2O_2 is significantly increased, but its elimination mechanisms become impaired, in patients with neurodegenerative disease.[1d,e] Elevated levels of H_2O_2 and the Fenton reactions contribute to oxidative stress and neurodegeneration. Anti-Fenton agents offer a promise in treatment and prevention of these diseases. We and others have been developing Fenton antidotes *via* an H_2O_2-triggered prochelator activation and a subsequent metal caging strategy.[2,3] As the process involves consumption of H_2O_2 by the prochelator, subsequent sequestration of the metal and prevention Fenton reactions, we called it an ''anti-Fenton reaction''.[2] The advantage of this strategy is that chelation can only be triggered by toxic levels of H_2O_2 and thus will not interfere with healthy metal homeostasis. However, direct measurement of the efficacy and cellular fate of these novel compounds, currently being tested in living systems, is hindered by the lack of an easily trackable marker.

$$Fe^{II}(Cu^{I})+H_2O_2 \rightarrow Fe^{III}(Cu^{II})+HO^{-}+HO^{\bullet} \quad (1)$$

To overcome these drawbacks, we have developed our second generation prochelator, 2-boronobenzaldehyde benzoyl-hydrazone, with an anthracene fluorophore tag (**SBH-B-AN**), and the corresponding active chelator with the same tag (**SBH-AN**). Both the fluorophore-tagged prochelator and chelator give a strong fluorescent light upon excitation by UV or visible light (400 nm), thus providing an easy trace of their cellular uptake and distribution *via* a fluorescent microscope. Furthermore, the activation of the prochelator by H_2O_2 induces a new

†Electronic supplementary information (ESI) available: Details on synthesis, absorption and fluorescence spectra and the deoxyribose assay.

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fluorescence peak centred at 480 nm, while the iron chelation induces a marked quenching of the fluorescence, thus allowing the anti-Fenton process to be monitored in realtime by fluorescence techniques.

SBH-AN and prochelotor **SBH-B-AN** were synthesized by a 3-step procedure (Scheme 1 and ESI†). The iron-binding moiety salicylaldehyde benzoylhydrazone (SBH) was chosen because of its comparable iron-binding affinity to the SIH analogues,[4] a better lipophilicity (therefore potentially higher cell penetration) and a slower hydrolysis ($t_{\frac{1}{2}} = 7.9$ h) rate under physiological relevant conditions.[5]

First, we examined the photophysical properties of **SBH-AN** and the prochelotor **SBH-B-AN** at pH 7.30. **SBH-AN** displays a spectrum similar to that of a combination of the SBH moiety and the anthracene moiety (Fig. 1(A)), assignable to the π–π* transitions.[6,7] Replacing the –OH group with a boronic acid group in **SBH-B-AN** significantly changes the absorption of the SBH moiety in the UV region (270–330 nm) and the visible region (390–470 nm), while little change is observable for the anthracene moiety. Upon excitation by UV light (≤360 nm), both compounds showed a similar photoluminescence response and give a blue fluorescence, due to the anthracene fluorophore.[7] Interestingly, upon excitation at 400 nm (the phenolate absorption), different emission profiles were observed: the prochelator **SBH-B-AN** showed a single fluorescence band at 440 nm while the active chelator **SBH-AN** displayed a strong additional fluorescence band at ~480 nm (Fig. 1(B)). This characteristic allows for realtime fluorescent monitoring the response of **SBH-B-AN** to H_2O_2.

Second, we examined the spectroscopic changes when prochelotor **SBH-B-AN** reacted with H_2O_2. Upon adding H_2O_2 to **SBH-B-AN** in DMF (*N*,*N*-dimethylformide)–KPB (potassium phosphate buffer) (20 mM, pH 7.3, v/v, 1 : 1), no change occurred to the fluorescence peak at 440 nm, while a new peak emerged at 480 nm and grew over time (Fig. 2(A)). The final fluorescence spectrum of the mixture almost matches that of **SBH-AN**, implying a conversion of **SBH-B-AN** to **SBH-AN**. A 5.5-fold increase in intensity at 520 nm was observed.

To further characterize the chemistry of the conversion, the reaction was followed by ^{1}H NMR in dimethylsulfoxide (DMSO). After incubating **SBH-B-AN** with H_2O_2, ^{1}H NMR peaks (Fig. S1†) for **SBH-B-AN** (δ11.05(s), –NH–; δ8.76(s), –CH═N–; δ8.50(s), –B(OH)$_2$) gradually decreased in intensity, while the peaks corresponding to **SBH-AN** (δ12.08(s) –NH–; 11.30(s), –OH–; 8.64(s), –CH═N–) appeared simultaneously and increased in intensity with time. The peaks (δ7.44–7.37(m), 7.30(t) and 6.95–6.90(m), salicyl-aldehyde) also underwent similar conversions. Meanwhile, the peak for boric acid, the deprotection product of boronic acid by H_2O_2, appeared at δ6.55(s). No ^{1}H NMR change was observed for the anthracene moiety due to its distance from the reaction site. In ~4 h, **SBH-B-AN** had been cleanly converted to **SBH-AN** with no intermediate formed, as indicated by the ^{1}H NMR spectra. The conversion from **SBH-B-AN** to **SBH-AN** was also confirmed by UV-vis difference spectra in DMF–KPB (20 mM, pH 7.3, v/v, 1 : 1) (Fig. S2†). With a ten-fold excess of H_2O_2, the conversion reaction is pseudo-first order with an apparent rate constant (K_{obs}) of 4.37×10^{-4} s^{-1}.

Third, we investigated whether **SBH-AN** and **SBH-B-AN** bind iron under physiological relevant conditions and whether the chelation can be detected by fluorescent techniques. Since metal binding to SBH induces characteristic changes to the absorption bands,[6,8] UV-vis titration was performed first to probe the binding between **SBH-AN** and Fe^{III} in potassium phosphate buffer (KPB), pH 7.30. Although the changes in spectra were largely obstructed by the strong absorption from the anthracene moiety in the UV region, evident spectroscopic changes due to iron-binding were observed (Fig. S3†): a decrease in intensity around 330 nm with simultaneous increases in intensity around 370 nm and in the visible region (400–500 nm). These changes match those reported for Fe–SBH complexation,[8] suggesting the

formation of specific **SBH-AN**–Fe complexes. However, when a similar titration was performed with the pro-chelator, no change in absorption was observed in the UV-vis region (Fig. S4†), indicating no interaction between the pro-chelator with Fe^{III} under the conditions applied. However, upon the addition of H_2O_2 to the mixture, characteristic absorption bands for Fe-binding emerged and increased in intensity (Fig. S5†), suggesting the formation of **SBH-AN**–Fe complexes. The spectroscopic changes indicate that H_2O_2 "activates" **SBH-B-AN** to **SBH-AN**, which subsequently chelates iron (Fig. S6†). Interestingly, iron-binding also induces marked fluorescent changes. Titration of the chelator **SBH-AN** with Fe^{III} induced a quenching of fluorescence for both the 440 nm and 480 nm bands (Fig. 2(B)), presumably due to metal-coordination-induced molecular collisional quenching. In contrast, similar titration experiments with the prochelator **SBH-B-AN** induced little fluorescent change (Fig. S7†).

Finally, we tested the anti-Fenton activities of **SBH-AN** and **SBH-B-AN** using a 2-deoxyribose degradation assay.[2] As shown in Fig. 3, both **SBH-B-AN** and **SBH-AN** at a 2 : 1 chelator :Fe ratio significantly inhibited hydroxyl radical-induced 2-deoxyribose degradation, indicating that **SBH-B-AN** and **SBH-AN** efficiently attenuate the Fenton reaction under physiologically relevant conditions.

In summary, we have developed a novel fluorescent pro-chelator, **SBH-B-AN**, and an active chelator **SBH-AN**, both excitable by non-damaging visible light. **SBH-B-AN** can sense H_2O_2 and be converted to **SBH-AN**, which subsequently sequesters iron and attenuates the Fenton reaction. The H_2O_2-activation and the iron-chelation processes produce specific fluorescent changes. These novel properties may be utilized in the realtime monitoring of cellular oxidative stress status (H_2O_2 levels) and in tracking their anti-Fenton efficacy and metabolism in living systems by fluorescent techniques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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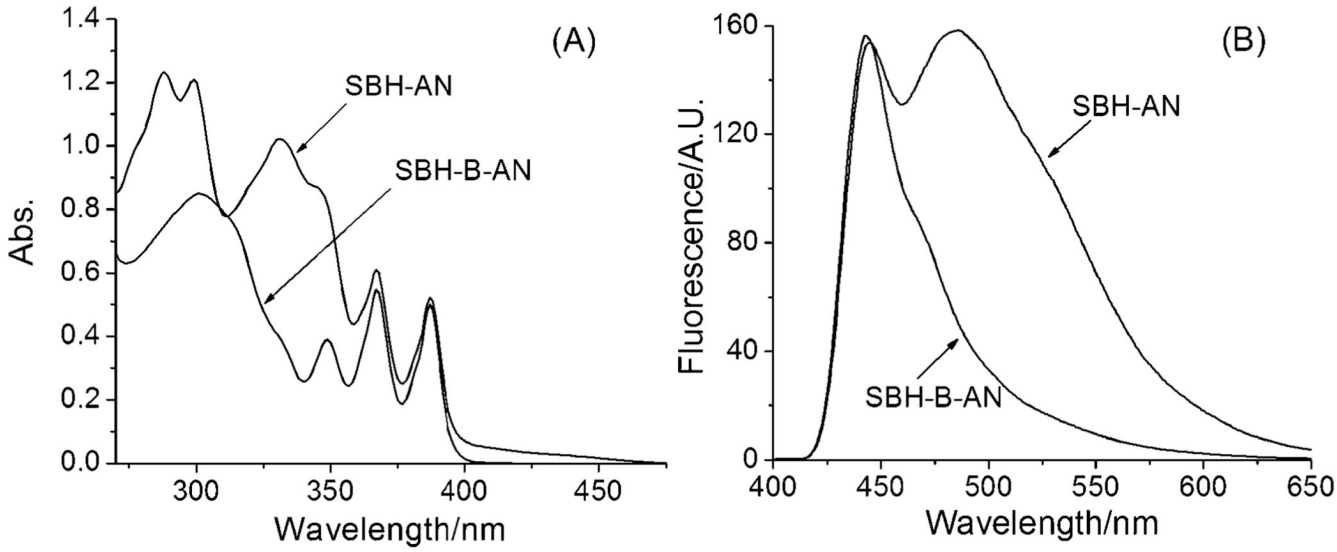


Fig. 1.

(A) UV-vis spectra and (B) fluorescence spectra (λ_{ex} = 400 nm, with a 415 nm cutoff filter) of 10 μM **SBH-B-AN** and **SBH-AN** in DMF/KPB (1 : 1), pH 7.30, at 298 K.

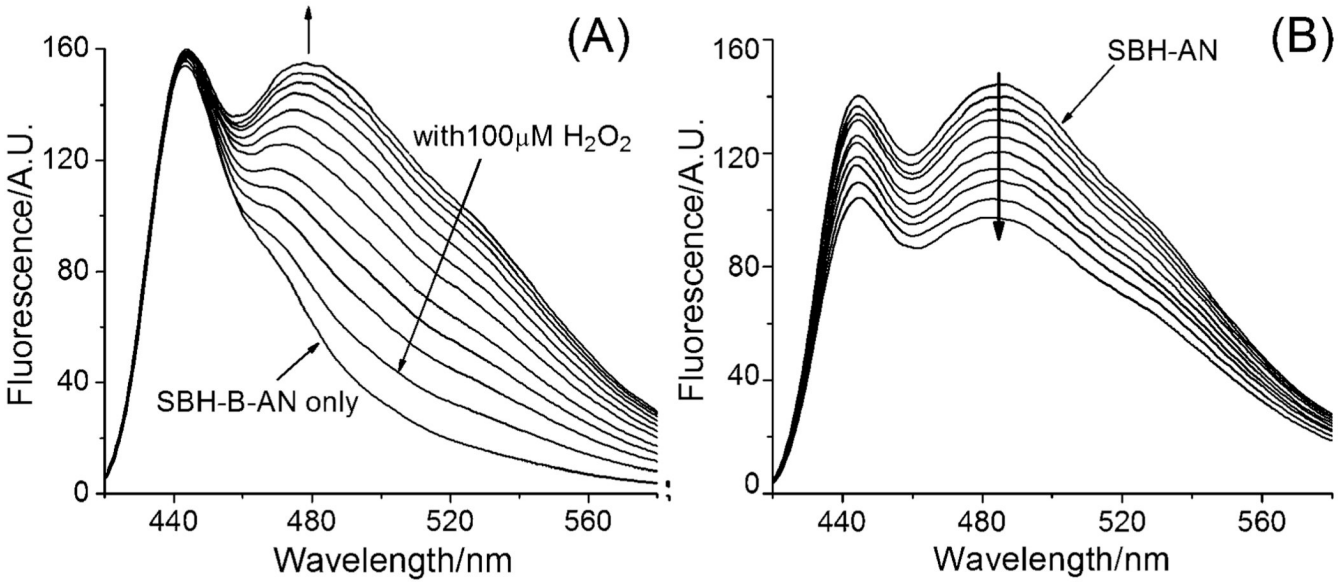


Fig. 2.

(A) Fluorescence spectra (λ_{ex} = 400 nm) of the time course of the reactions of 10 μM **SBH-B-AN** with 100 μM H_2O_2 (0 to 120 min); (B) fluorescence (λ_{ex} = 400 nm) titration of 10 μM **SBH-AN** with Fe^{III} in 1 μM increments in DMF–KPB (1 : 1), pH 7.30, at 298 K.

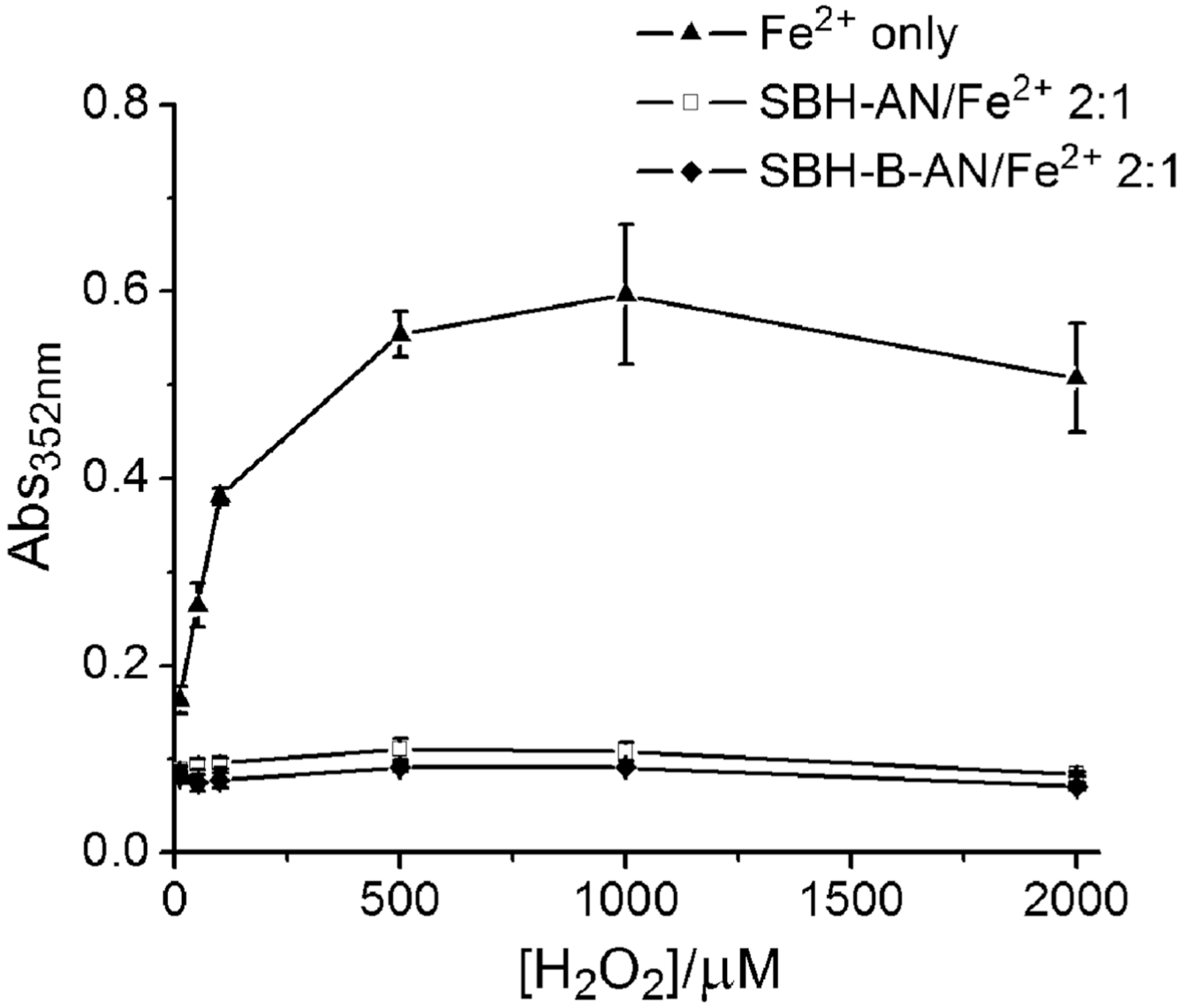
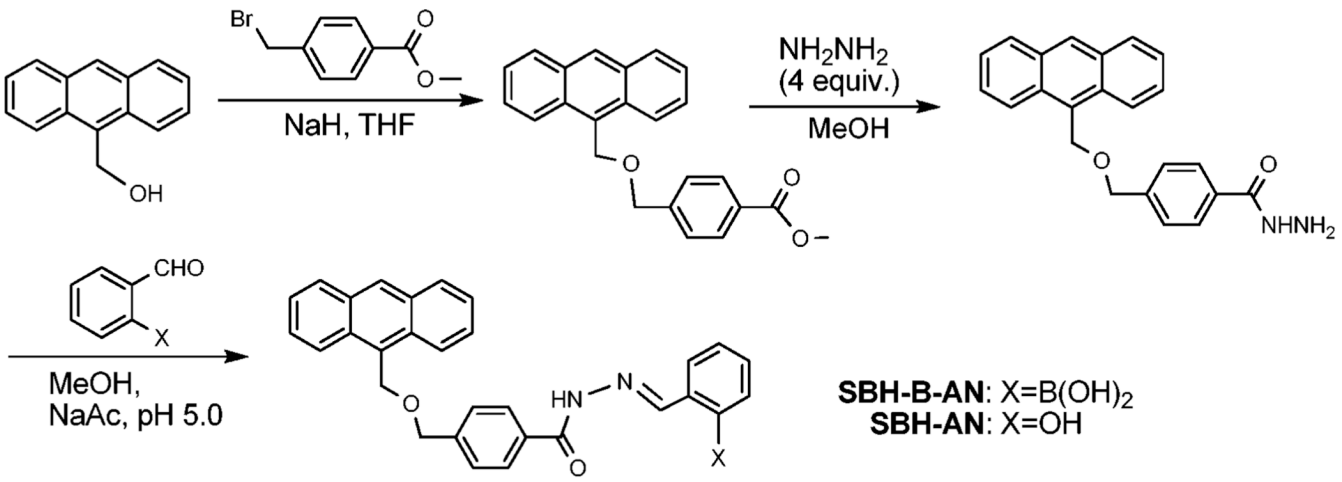


Fig. 3.

Effect of **SBH-AN** and **SBH-B-AN** on the oxidative degradation of 2-deoxyribose promoted by Fe^{II} (20 μM) in the presence of H_2O_2 and hydroascorbate (5 mM) in 20 mM KPB, pH 7.20.



Scheme 1.
Synthesis and abbreviations of the compounds.