ABSTRACT: $\alpha$-Naphthyl acetate esterase ($\alpha$-NAE) and acid $\alpha$-naphthyl acetate esterase (ANAE) are two of the typical nonspecific esterases. Like other nonspecific esterases, $\alpha$-NAE and ANAE exhibit a catalytic hydrolytic function for short-chain fatty acids.\(^1\)\(^2\) That is, they can catalyze the hydrolysis reaction of naphthyl acetate derivatives to generate $\alpha$-naphthol by breaking down the acetic acid ester bond in living organisms.\(^3\) Although they belong to the nonspecific esterases, $\alpha$-NAE and ANAE exhibit unique roles in the field of cell biology and medical diagnosis due to their catalytic hydrolytic functional characteristics.\(^4\) For example, $\alpha$-NAE is a marker for leukemia diagnosis, typing and prognosis, and also for myeloid leukemia cell differentiation,\(^5\) while ANAE could be used to distinguish T lymphocytes from B lymphocytes with humoral immunity function. T lymphocytes directly attack invaders and release cytokines that can then activate other parts of the immune system, while B lymphocytes produce antibody molecules that can latch on and destroy the invading viruses or bacteria.\(^6\) But, unlike other nonspecific esterases, the catalytic hydrolytic function of $\alpha$-NAE and ANAE can only be activated by the action of two factors, that is, the enzymatic activity (biological species) and an appropriate pH (environmental conditions).\(^1\)\(^2\) $\alpha$-NAE must be at neutral pH (approximately pH 7.4), while ANAE must be at acid pH (approximately pH = 5.9–6.3). Many investigations have unequivocally demonstrated that such differences of pH conditions are key factors for activating their catalytic hydrolytic function. Importantly, such differences can affect their roles in the field of cell biology and medical diagnosis.\(^7\) More importantly, such differences in the pH of activation provide an approach for the differential detection of $\alpha$-NAE and ANAE. Thus, to that end, the rapid and highly sensitive recognition output signals that are specifically regulated by two factors (i.e., the enzymatic activity and appropriate pH conditions) become important challenges to overcome for the simultaneous and differential detection of $\alpha$-NAE and ANAE.

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In clinical diagnosis, the azo salt staining method is a gold standard for the detection of $\alpha$-NAE and ANAE for serum analysis and cell staining. Significantly, the sensitivity of the method is relatively low. Therefore, a better method for the detection of $\alpha$-NAE and ANAE is required. Toward that goal, excited-state intramolecular proton transfer (ESIPT) probes are a potential solution to that problem. Since ESIPT probes exhibit unique optical and physical properties, such as two output signals, rapid proton transfer, emission band with large stokes shift, unique four-level photochemical process, and so on, they have resulted in important fluorescence-based tools for analytical chemistry, molecular logic gates, and luminescent materials.\textsuperscript{1--10} Recently, ESIPT-based probes have been developed to monitor biomolecules or biomolecular events in a living organism, especially proteins and enzymes.\textsuperscript{11--13} Due to the extremely rapid proton transfer speed ($k_{\text{ESIPT}} > 10^{12}$ s\textsuperscript{-1}), a simple and effective 2-(2'-hydroxyphenyl) benzoxazole derivative (HBT)-based ESIPT probe was developed for the detection of ONOO$^-$ which exhibits good selectivity and a fast response time.\textsuperscript{14} Based on the transient nature of the four-level photochemical process and irreversible chemical reaction, a HBT ESIPT fluorophore, where the hydroxyl group has been protected by a tertbutyldiphenylchlorosilane, exhibited high sensitivity at the ppb level for fluoride.\textsuperscript{15} However, a HBT cyanine probe was designed to exhibit a large stokes shift 234 nm when activated at pH 5, which can effectively avoid undesirable inner-filter and/or self-reabsorption effects.\textsuperscript{16,17} These reported provide some guidance for design strategies toward ESIPT-based probes.\textsuperscript{14,18} In addition, a number of fluorescent probes combining ESIPT with AIE have been reported that overcome some of the inherent problems associated with ESIPT-based systems.\textsuperscript{19--21} Unfortunately, the application of many reported ESIPT-based probes are limited under certain circumstances (such as the detection of high-fidelity signals), since they are easily affected by environmental factors and, as such, generate off-target fluorescence changes.\textsuperscript{22} In addition, the fluorescence output signals of these probes can only be regulated by a single factor and are therefore unable to accurately monitor species that are regulated by multiple factors.\textsuperscript{23} Moreover, they are not suitable for the simultaneous and differential monitoring of multiple biological species, for example, the catalytic hydrolytic function of $\alpha$-NAE and ANAE.

With this research, the hydrolysis reaction of naphthyl acetate derivatives was selected as the specific recognition reaction. Considering their catalytic hydrolytic function that is affected by the two factors, pH and the enzymatic activity, $\alpha$-NAE and ANAE were selected as biomarker targets for the design of a synergistically activated ESIPT-based probe. Therefore HBT-NA was developed, where the fluorogen is 2-(benzo[d]oxazol-2-yl)phenol. In the absence of $\alpha$-NAE or ANAE, HBT-NA emitted a weak blue fluorescence signal at 392 nm. But in the presence of $\alpha$-NAE and ANAE, HBT-NA can emit differential fluorescence signals at different wavelengths to generate both ratiometric and off-on responses. We anticipated that the differential fluorescence signals of HBT-NA could be used to detect $\alpha$-NAE and ANAE sensitively and specifically in cells simultaneously. Given the molecules’ differential fluorescence signals toward $\alpha$-NAE and ANAE, we anticipated that HBT-NA could accurately type T lymphocytes and B lymphocytes and simultaneously evaluate their immunocompetence.
1H); 13C NMR (151 MHz, DMSO-δ6) δ: 169.37, 154.96, 151.61, 135.68, 132.84, 129.25, 128.31, 126.83, 126.11, 125.01, 124.88, 123.67, 122.87, 122.19, 120.26, 110.59. HRMS: m/z calcd for C19H13NO3 +H+: 304.0974, found, 304.0977.

Synthesis of 2-(Benzo[d]oxazol-2-yl)naphthalen-1-yl Acetate (Product, HBT-NA). HBT (0.36 mmol, 180 mg) and triethylamine (0.43 mmol, 43 mg) were dissolved in dichloromethane and stirred under N2 for 10 min. Then, acetyl chloride (0.42 mmol, 33 mg) was dissolved into dichloromethane and was added dropwise to the mixture at 0 °C. After addition, the mixture was stirred for 2.0 h at room temperature and monitored using TLC. When the reaction was complete, 80 mL of water was added to the mixture to quench the reaction. The mixture was separated, and the organic phase was collected. Crude HBT-NA was obtained on the removal of the solvent under reduced pressure. HBT-NA was then purified using silica gel chromatography with petroleum ether/ethanol acetate (100:1 to 30:1, v/v) as an eluent. HBT-NA (2-(benzo[d]oxazol-2-yl)naphthalen-1-yl acetate) was obtained as a white solid (236 mg). Yield: 78%. 1H NMR (600 MHz, DMSO-d6): δ: 8.38 (dd, J = 8.6, 4.4 Hz, 1H), 8.13 (dd, J = 8.1 Hz, 1H), 7.94 (dd, J = 7.9 Hz, 1H), 7.93–7.88 (m, 2H), 7.86 (dd, J = 8.7 Hz, 1H), 7.62–7.56 (m, 2H), 7.55–7.50 (m, 1H), 7.46–7.38 (m, 1H), 2.65 (s, 3H). 13C NMR (151 MHz, CDCl3) δ: 169.17, 162.85, 152.96, 145.23, 135.38, 135.27, 128.11, 127.94, 127.39, 127.36, 126.60, 126.44, 125.95, 125.50, 123.41, 122.52, 122.39, 121.39, 21.70. HRMS: m/z calcd for C19H13NO3 +H+: 304.0974, found, 304.0977.

Monitoring the Structural Changes of HBT-NA Using 1H NMR. The products formed during the reaction of HBT-NA with α-NAE/ANAE were purified as follows: (1) the mixture was separated using an ultrafiltration tube, and the filtrate with a molecular weight below 1000 was obtained; (2) the filtrate was freeze-dried; and (3) then purified using silica gel column chromatography with DCM/ethyl acetate as an eluent. Changes of HBT-NA during the reaction process were monitored using high-performance liquid-liquid high-resolution mass spectrometry. HBT-NA (5.0 mM) and the corresponding equivalent ratio of α-NAE or ANAE in PBS were used for all of the reactions.

Flow Cytometry for Typing of Lymphocytes and Analysis of Immunocompetence. Pure and highly immunoreactive T lymphocytes, B lymphocytes, and nontyped lymphocytes were used in this work, which were obtained from mice. The pure and highly immunoreactive T lymphocytes and B lymphocytes were used as control groups and were incubated with HBT-NA (2.0 μmol) for 30 min, where the excitation wavelength for the blue channel was 352 nm, the detection wavelength for the blue channel was 390–440 nm, the excitation wavelength for the green channel was 413 nm, and detection wavelength for the green channel was 500–560 nm. The threshold value was set by the fluorescence intensity of the control group (pure and highly immunoreactive T lymphocyte group and the pure and highly immunoreactive B lymphocyte group). The immunocompetence was analyzed using the activity of α-NAE and ANAE.

RESULTS AND DISCUSSION

Molecular Design for the Differential Detection of α-NAE and ANAE. We designed a dual-factor synergistically regulated ESIP-based probe (HBT-NA) for the simultaneous and differential monitoring of changes in the catalytic hydrolytic function of α-NAE and ANAE using the high-fidelity output signal. The selectivity, sensitivity, and dual-factor synergistic activation of the probe for the catalytic hydrolytic function of α-NAE and ANAE are key points for the molecular design. First, 2-(benzo[d]oxazol-2-yl)phenol (HBT, Scheme 1) was selected as the platform due to the efficient four-level photochemical capability, which is advantageous to improve the sensitivity and response speed. More importantly, the molecular design
platform is more susceptible to proton transfer under neutral conditions, which is key to realizing the differential monitoring of changes in the catalytic hydrolytic function of $\alpha$-NAE and ANAE, as part of the two factors required to activate the recognition output signals, i.e., appropriate pH conditions. To achieve high selectivity for nonspecific esterases, the naphthalen-1-yl acetate (NA, Scheme 1) was added to the probe as the specific activation group. This is the second of the two factors required to activate the recognition output signals, i.e., enzymatic activity. Based on this design strategy, i.e., differential regulation of the ESIPT process using two-factors, we anticipated that HBT-NA could simultaneously monitor in real time the catalytic hydrolytic function of $\alpha$-NAE and ANAE using differential output signals. The high-fidelity differential output signals could then be used to precisely type T lymphocytes and B lymphocytes among nontyped lymphocytes and simultaneously evaluate their immunocompetence. The molecular structure and properties of HBT-NA and intermediate products are given in Scheme 1 (See Supporting Information for characterization data).

**Spectral Changes of HBT-NA Toward $\alpha$-NAE and ANAE.** The spectral response including the absorption spectra and emission spectra of HBT-NA for $\alpha$-NAE and ANAE were investigated using buffers with different pH values. The absorption spectra (Figure S1a) and the optical data (Table S1) indicated that HBT-NA ($5.0 \mu M$, $\varepsilon = 10655 M^{-1} \text{cm}^{-1}$) exhibited an absorption peak at 320 nm at pH 7.4. With increasing $\alpha$-NAE, the absorption peak does not change, but its intensity increases slightly in PBS buffer (pH = 7.4, Figure S1a), while there is a significant fluorescence enhancement in the emission spectra for $\alpha$-NAE (Figure 1a). In the absence of $\alpha$-NAE, HBT-NA was weakly fluorescent ($F_{392 \text{nm}}$/$\Phi_{\text{HBT-NA}}$ = 0.13, $\lambda_{\text{em-max}}$ = 392 nm, Table S1) in PBS buffer solutions (pH = 7.4, Figure 1a). When HBT-NA reacted with $\alpha$-NAE, the fluorescence intensity was significantly enhanced with increasing $\alpha$-NAE (0–25 U) at 392 nm at neutral pH (PBS buffer solutions, pH = 7.4) over a very short time (approximately 60 s, Figure S1b). The fluorescence quantum yield increases to 0.27 when the activity of $\alpha$-NAE is increased to 25 U ($\Phi_{\text{HBT-NA}}$ $\approx 0.21$) and then plateaus. Furthermore, the fluorescence enhancement of HBT-NA for $\alpha$-NAE ($F_{392 \text{nm}}$/$F_{392 \text{nm}}$) exhibited a good linear relationship ($F_{392 \text{nm}}$/$F_{392 \text{nm}}$ = 0.042 $C_{\text{HBT-NA}} + 1.1$, $R^2 = 0.99$) with the activity of $\alpha$-NAE (0–25 U, Figure 1b), producing a $v_{\text{max}}$ of 3.750 $\mu$mol/L S (Figure S1c). However, for ANAE at neutral pH, no absorption or emission spectral changes of HBT-NA were observed (Figure S1d) even after 2 h. This is because ANAE was not active at neutral pH.

However, ANAE in acid solutions (PBS buffer, pH = 6.0) can cause significant changes of HBT-NA within 2 min in the absorption and emission spectra. With increasing ANAE to 25 U, the absorption peak was red-shifted from 320 nm to 370 nm and 400 nm in PBS buffer solution (pH = 6.0, Figure S1e), While the fluorescence spectra exhibited significant changes toward ANAE (Figure 1c). In the absence of ANAE, HBT-NA emitted a weak fluorescence ($\Phi_{\text{HBT-NA}} = 0.11, \lambda_{\text{em-max}} = 392 \text{nm}$) in PBS buffer solution (pH = 6.0). However, when HBT-NA reacts with ANAE, a strong fluorescence at 505 and 353 nm (pH = 6.0) was observed (Figure 1c), and the intensity increased with increasing ANAE (0–50 U). A good linear relationship ($F_{505 \text{nm}}/F_{392 \text{nm}} = 0.83$ $C_{\text{ANAE}} + 1.95$, $R^2 = 0.99$, Figure 1d) was obtained between ANAE (0–25 U) and the fluorescence intensity ratio at 505 and 392 nm ($F_{505 \text{nm}}/F_{392 \text{nm}}$). Significantly, these differential spectral changes of HBT-NA toward $\alpha$-NAE and ANAE are complete within 50 s and then plateau (Figure S1f), which is extremely conducive for the real-time differential monitoring of ANAE. The $v_{\text{max}}$ with ANAE was determined to be 6.124 $\mu$mol/L S (Figure S1g).

Subsequently, the selectivity of HBT-NA for $\alpha$-NAE and ANAE was evaluated. As shown in Figure 2a (pH = 7.4) and Figure 2b (pH = 6.0), there were no changes observed for 11 kinds of lipases (cholinesterase, alkaline phosphatase, nuclease, phospholipase, sulfatase, sphingomyelinase, hepatic lipase, endothelial lipase, lipoprotein lipase, lysosomal acid lipase, acid cholesteryl ester hydrolase) at different pH values, and similar results were obtained using 13 kinds of ions and 13 kinds of bioactive small molecules (Figure S2). These results indicated that the monitoring ability of HBT-NA for $\alpha$-NAE and ANAE was highly specific.

**Mechanism of Spectral Changes of HBT-NA with $\alpha$-NAE and ANAE.** To explain the spectral changes (Figure 3a) of HBT-NA for $\alpha$-NAE and ANAE, HPLC (Figure 3b) and Gaussian 16 (Figure 3c) were used to analyze the recognition process. The Gaussian 16 (Figure 3c) results indicated that HBT-NA exhibits maximum absorption and emission peaks at 307 and 393 nm, respectively, which were very close to the experimental results (Figures 1a and S1a). In pH = 7.4 PBS buffer, the chromatographic peak of HBT-NA (Mr = 303.0888) appeared at 7.75 min. When HBT-NA reacted with $\alpha$-NAE, a new chromatographic peak at 9.05 min in pH = 7.4 PBS buffer was observed, which can be assigned to HBT (Mr = 261.0779) which is the enol (E) form (Figure 3a). The energy gap ($\Delta E$)
between the HOMO and LUMO of HBT is in line with that of HBT-NA (Figure 3c). But, the electron density of the oxygen of reacts with at pH = 7.4. (c) The orbital energy of HOMO and LUMO of the theoretical calculation and the spectral data in Figure 1. The wavelength of the enol (E) form of intensity at 392 nm is enhanced, which belongs to the emission between the HOMO and LUMO of Figure 3.

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Monitoring the Intracellular Activity of α-NAE and ANAE. Encouraged by the excellent differential fluorescence signals of HBT-NA in aqueous media activated by the two factors, we evaluated the system in live cells. First, HBT-NA exhibited extremely low cell toxicity toward cancer cells (Hep G2 cells), normal cells (7702 cells), and hemocytes (Figure 4a).

Furthermore, prior to the enzymatic activity analysis in living cells, the biocompatibility of HBT-NA (Figures S3–S6), including photostability, biological pH stability, and water solubility, were evaluated. HBT-NA exhibits low biotoxicity and excellent biocompatibility, making it convenient for monitoring intracellular enzyme activity. HBT-NA emits a very weak fluorescent signal in the blue channel (410–450 nm) and green channel (490–570 nm), which is almost negligible when the activities of α-NAE and ANAE are inhibited (Figure 4b). Even in an acidic environment, i.e., lysosome, negligible cellular fluorescence was observed (Figure 4b). That is to say, the environmental pH factor cannot activate differential fluorescence signals in the absence of enzyme activation. A bright fluorescence signal at 500–580 nm (green channel) was observed due to ANAE enzyme activity in the live cells (Figure 4c), whereas a bright fluorescence signal in the green channel was observed in the lysosome (Pearson coefficient = 97%) due to an acidic environment, where ANAE can exhibit enzymatic activity (Figure 4d–4f). This is mainly due to the departure of the ester and proton transfer by the hydrolytic activity of ANAE under acid conditions. More importantly, the fluorescence intensity (Fgreen channel) of the green channel gradually increases with increasing ANAE activity (Figure 4g). However, when there was only α-NAE in the live cells, only one bright fluorescence signal at 410–450 nm (blue channel, Figure 4h) was observed, and the fluorescence was only observed in the cytoplasm (Pearson coefficient = 92%) due to an acidic environment. This is because α-NAE only exhibits high enzyme activity under neutral conditions. That is, the hydrolytic activity of α-NAE can only function under these conditions. More importantly, the fluorescence intensity (Fblue channel) of the blue channel gradually increased with the increasing enzymatic activity of α-NAE (Figure 4g). These results indicate that HBT-NA can monitor the enzyme activity of α-NAE and ANAE in living cells using differential fluorescence signals.

Typing lymphocytes and Evaluating Immunocompetence. The enzyme activities of α-NAE and ANAE in lymphocytes during immune response have become one of the breakthroughs in the study of immune diseases. The level of enzyme activity can type the kind of lymphocyte (i.e., T lymphocyte or B lymphocyte) and simultaneously reflect their immune activity. Thus, the precise typing of lymphocytes and the screening of immune cells using small changes of these two enzyme activities would be beneficial to help evaluate the immune function of living organisms. In this work, lymphocytes including T lymphocytes and B lymphocytes from different samples and viral hepatitis were stained using HBT-NA (5.0 μmol) and analyzed using flow cytometry (Figure 5).

HBT-NA (5.0 μmol) exhibited fluorescence signals with different intensities in the blue channel (392 nm) and green channel (505 nm) during the different stages of viral hepatitis. This was mainly attributed to the different activities of α-NAE and ANAE during these stages. As the activities of α-NAE and ANAE increase, the fluorescence signal intensities in the blue channel and green channel exceed 10^4 au. Therefore, this fluorescence intensity was used as a threshold to divide the quadrants. The pure and highly immunoreactive T lymphocytes and B lymphocytes isolated from mouse blood were used to help illustrate the thresholds (Figure 5a,5b). Figure 5a,5b, and 5e indicated that cells are mainly distributed in the upper left and lower right quadrants. The data in the two quadrants

Figure 3. Structural changes of HBT-NA (a) and the HPLC results of HBT-NA under different conditions (b). Black line: pure HBT-H; green line: pure HBT-NA; purple line: pure HBT; red line: HBT-NA reacts with ANAE at pH = 6.0; blue line: HBT-NA reacts with α-NAE at pH = 7.4. (c) The orbital energy of HOMO and LUMO of HBT-NA, HBT-H, and HBT using Gaussian 16.
Figure 4. continued
Figure 4. (a) Cell toxicity of HBT-NA (5.0 and 10.0 μM) for Hep G2 cells, 7702 cells, and hemocytes. (b) Control group: cell imaging of HBT-NA (5.0 μM) with no α-NAE and ANAE activity. The activities of α-NAE and ANAE were inhibited by NaF (1.0 mM). (c) ANAE group: cell imaging of HBT-NA (5.0 μM) under the activity of ANAE. (d) Colocalization experiments for HBT-NA (5.0 μM) with LysoTracker Red (1.0 μM) under the activity of ANAE. (e) Fluorescence intensity profile of the yellow line in HBT-NA channel and Lyso channel. (f) Intensity correlation plot of HBT-NA and LysoTracker Red in the same pixel between the HBT-NA channel and Lyso channel. (g) Fluorescence intensity of the blue channel and green channel in the control group, ANAE group, and α-NAE group. (h) α-NAE group: cell imaging of HBT-NA (5.0 μM) under the activity of α-NAE. (i) Colocalization experiments for HBT-NA (5.0 μM) and 5(6)-CFDA (1.0 μM) under the activity of α-NAE. (j) Fluorescence intensity profile of the
respectively are 85.4 and 86.3% for Figure 5a and 5b. For viral hepatitis samples obtained from mice Figure 5c−5e indicated that more T lymphocytes and B lymphocytes with high immune activity, respectively, enter the upper left and lower right quadrants as viral hepatitis progresses. The cell number in the upper left and lower right quadrants, respectively are 0.692 and 0.168% for the primary stage of viral hepatitis (Figure 5c, 5e), while the number of cells in the upper left and lower right quadrants, respectively are 73.5 and 21.3% for advanced-stage viral hepatitis (Figure 5d, 5e). The above results are consistent with the results of the clinical standard staining methods (see Table S2), which indicated that the immunocompetence of lymphocytes gradually increased from the primary stage to the advanced stage during the progression of viral hepatitis. In addition, this method was much easier and more convenient than the standard clinical method. Thus, HBT-NA can be used as a potential tool for the typing of lymphocytes and the analysis of immunocompetence.

**CONCLUSIONS**

The simultaneous and sensitive detection of nonspecific esterases, i.e., α-NAE and ANAE using a differential fluorescence...
signal by means of a dual-factor synergistically activated ESIP
t-based probe (HBT-NA), has been achieved. The three key
points in molecular design are selectivity, sensitivity, and dual-
factor synergistic activation. With the molecular design, we set
(1) catalytic hydrolytic function of α-NAE and ANAE and the
appropriate pH conditions as the target for the molecular design;
(2) 2-(benzo[d]oxazol-2-yl)phenol was the core of the
fluorescent probe due to rapid response and different proton
transfer under the effect of different pH; and (3) naphthalen-1-yl
acetate was used as the specific reactive group for activation by
the esterases. Based on this design strategy, HBT-NA emitted an
absorption peak at 320 nm and weak fluorescence at 392 nm at
pH 7.4. Significantly, HBT-NA generated different responses for
α-NAE and ANAE under different environmental conditions.
When HBT-NA reacted with α-NAE at pH = 7.4, the
fluorescence intensity enhanced at 392 nm within approximately
60 s. However, when HBT-NA reacted with ANAE, ratio
tic signals in the absorption and emission spectra were observed at
pH = 6.0 within 2.0 min. Such differential fluorescence signals
were used to detect the activity of α-NAE and ANAE in
solutions and live cells. Importantly, based on the differential
fluorescence signals, a highly sensitive method was developed to
distinguish type T lymphocytes and B lymphocytes among
nontyped lymphocytes using the enzyme activities of α-NAE
and ANAE. More importantly, this method can be used in real
time to evaluate the immune function of living organisms using
flow cytometry in a rapid, sensitive, and quantitative fashion.
Hence, HBT-NA could have potential applications in the
ultrasensitive detection of the enzyme activity of α-NAE and
ANAE suitable for real-time and precise typing of lymphocytes
and monitoring of immunocompetence.

■ ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge at
https://pubs.acs.org/doi/10.1021/acs.analchem.1c02945.

Procedures section, the synthesis of HBT-NA, the basic
optical data and the absorption spectra of HBT-NA, the
biocompatibility of HBT-NA, the clinical standard
staining methods for the immunocompetence of
lymphocyte, references, and attached spectra (PDF)

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The manuscript was written through contributions of all
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Notes
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