



# Protein Encapsulation: A Nanocarrier Approach to the Fluorescence Imaging of an Enzyme-Based Biomarker

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Here, we report a new pentafluoropropanamido rhodamine fluorescent probe (ACS-HNE) that allows for the selective detection of neutrophil elastase (NE). ACS-HNE displayed high sensitivity, with a low limit of detection (<5.3 nM), and excellent selectivity toward elastase over other relevant biological analytes and enzymes. The comparatively poor solubility and cell permeability of neat ACS-HNE was improved by creating an ACS-HNE-albumin complex; this approach allowed for improvements in the *in situ* visualization of elastase activity in RAW 264.7 cells relative to ACS-HNE alone. The present study thus serves to demonstrate a simple universal strategy that may be used to overcome cell impermeability and solubility limitations, and to prepare probes suitable for the cellular imaging of enzymatic activity *in vitro*.

**Keywords:** elastase detection, BSA-based nanocarrier, nanocarrier-based enzyme detection, fluorescence imaging, cell imaging

## INTRODUCTION

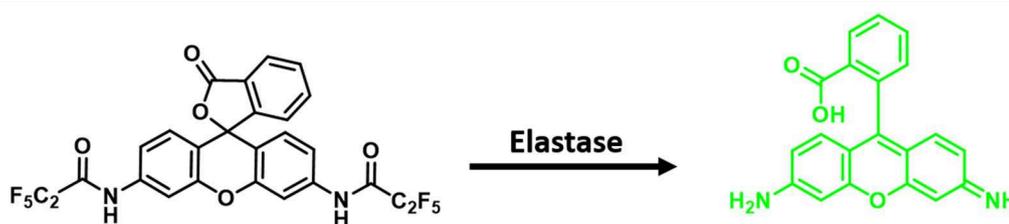
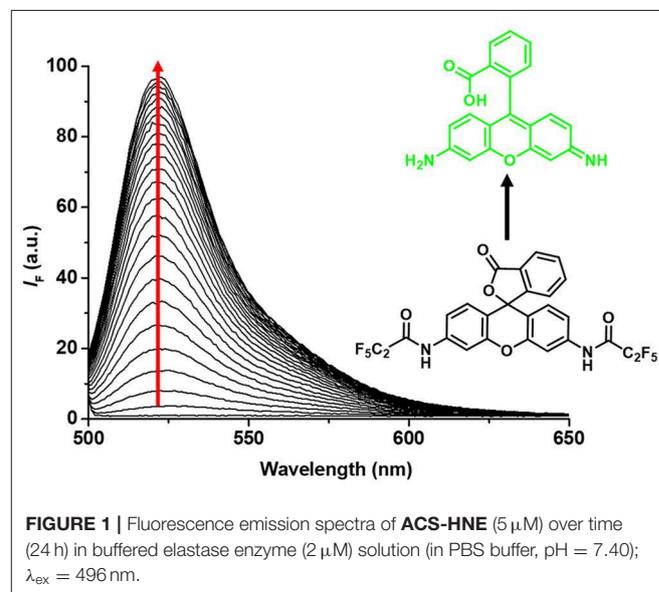
Fluorescent probes have found widespread application in imaging biomarkers relevant to physiological and pathological cellular pathways (Kobayashi et al., 2010; Chan et al., 2012; Wu et al., 2017; Erbas-Cakmak et al., 2018; Sedgwick et al., 2018d). Within this paradigm, reaction-based systems containing an integrated reactive motif offer specificity in probing biochemical processes with concomitant utility as a diagnostic tool for medical applications (Caramello et al., 1993; Burgos-Barragan et al., 2017; Xiao et al., 2018; Akashi et al., 2019). Common dye scaffolds are, however, still limited and generally rely upon flat polyaromatic frameworks. Such systems are typically restricted by their poor solubility and cell permeability. In an attempt to overcome these limitations, supramolecular nanocarrier constructs have been devised in an effort to enhance solubility, photophysical properties, and chemoselectivity (Dondon and Fery-Forgues, 2001; Sheng et al., 2014; Chen and Liu, 2016; Fu et al., 2018; Yan et al., 2018; Gao et al., 2019; Miranda-Apodaca et al., 2019).

Continued advances in imaging methods coupled with fluorescent probe technologies have made real-time monitoring of enzymatic activity a viable tool for understanding fundamental biological processes (Liu et al., 2018; Yang et al., 2019). Our own efforts have focused on the development of fluorescent-based probes for the detection of biologically relevant species that are thought to be intimately involved in a number of pathological processes, such as inflammation, neurodegenerative diseases and cancer (Sedgwick et al., 2017, 2018a,b,c,d; Wu et al., 2017, 2018; Odyniec et al., 2018; Gwynne et al., 2019). In the context of these efforts, we turned our attention toward the detection of the enzyme neutrophil elastase (NE). NE is a serine protease primarily secreted by neutrophils during an inflammatory response. NE possesses important protective functions, which include the remodeling of the extracellular matrix. It also acts as a host defense against bacterial infections. NE is found in inflamed tissues and wound exudate (Mitra et al., 2013). Elevated levels of NE have been associated with a number of inflammatory-related diseases, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), acute lung injury (ALI), and acute respiratory distress syndrome (ARDS) (Belaouaj et al., 1998; Shapiro et al., 2003; Sly et al., 2009; Korkmaz et al., 2010). In order to utilize NE as a potential therapeutic target for disease treatments, new systems elucidating its function in disease are essential (Henriksen and Sallénave, 2008; Ho et al., 2014).

Current methods for determining NE activity utilize a combination of indirect separation methods (i.e. HPLC or LC-MS) and direct electrochemical, UV-Vis spectroscopic, or fluorescence-based probes (Bieth et al., 1974; Wang et al., 2008; González-Fernández et al., 2018). These latter optical methods have proven useful for monitoring NE, but remain cost prohibitive due to the use of peptide-based substrates (Wang et al., 2008; Gehrig et al., 2012; Kasperkiewicz et al., 2014; Schulz-Fincke et al., 2018). Yang and co-workers recently reported a simple non-peptide-based strategy for the selective detection of elastase (Sun et al., 2013). Their coumarin-derived fluorescent probe was functionalised with a reactive pentafluoropropionamide unit, which served as a substrate for NE that, in turn, served to unveil the activated fluorescent dye. Unfortunately, the short excitation wavelength characteristic of many coumarin-systems limited the utility of this system as tool

for monitoring elastase activity, and apparently precluded cellular imaging experiments.

Here we report a non-peptide rhodamine fluorescent probe for the detection of elastase (ACS-HNE)—Scheme 1. This new system is based on the use of a rhodamine-based fluorophore. This was considered attractive from a design perspective since this fluorophore core is readily subject to synthetic modification. Moreover, rhodamine derivatives typically display high fluorescent quantum yields and display photophysical properties appropriate for *in vitro* study (Chan et al., 2012; Bhuniya et al., 2014). However, as true for many near-planar dye systems, probes based on rhodamine often suffer from poor solubility or a tendency to aggregate in aqueous milieus. In the present instance, we have built upon recent protein-based nanocarrier strategies (Han et al., 2020), to create an ACS-HNE/ Bovine Serum Albumin (BSA) hybrid that displays enhanced solubility relative to ACS-HNE and which acts as a fluorescent probe that allows for enzyme-based imaging in RAW 264.7 cells. Despite the recent report of an NIR probe for NE detection *in vitro* and *in vivo* (Liu et al., 2019), we believe the rhodamine scaffold of ACS-HNE offers an excellent platform for further derivatisation. In addition, this BSA-nanocarrier system represents a global strategy for



researchers to overcome the solubility issues associated with hydrophobic fluorescent imaging agents designed to detect enzyme-based biomarkers.

## RESULTS AND DISCUSSION

### Chemistry

Briefly, ACS-HNE was synthesized in one step from commercially available rhodamine 110 (RH110) by dissolving in *N,N*-dimethylformamide, deprotonating with sodium hydride at 0°C, and acylating with pentafluoropropionic anhydride. The product was isolated, after purification by column chromatography, in 43% yield—Scheme S1.

### Spectroscopic Studies of ACS-HNE

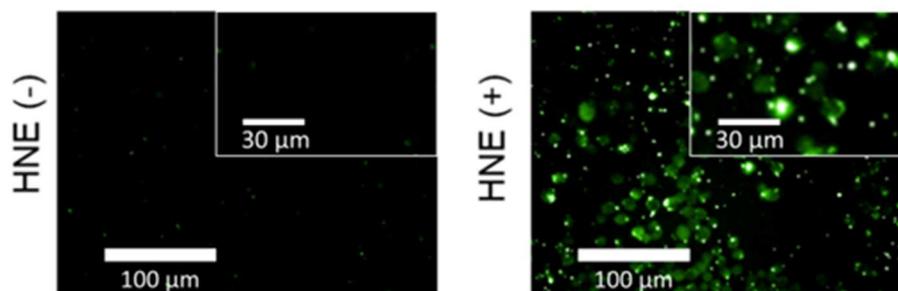
With ACS-HNE in hand, UV-Vis and fluorescence spectroscopic experiments were carried out to evaluate whether this putative probe could be used to monitor NE activity. As shown in Figures S1, S2, the addition of elastase (2 μM in PBS) led to a large increase in UV-Vis absorption at ~490 nm, as would be expected for the enzyme-based release of rhodamine 110 in accord with Scheme 1. A strong increase in the fluorescence intensity ( $I_F$ ) was also observed after the addition of elastase (2 μM in PBS)—Figure 1. Dose-response studies involving fluorescence monitoring revealed a linear increase in emission intensity with increasing enzyme concentration

(Figures S3, S4). Such behavior is fully consistent with the expected Michaelis–Menten kinetics (Nelson and Cox, 2005). Limit of detection (LoD) values for NE using ACS-HNE were calculated using the assay developed by Schönherr and co-workers (Sadat Ebrahimi et al., 2015).

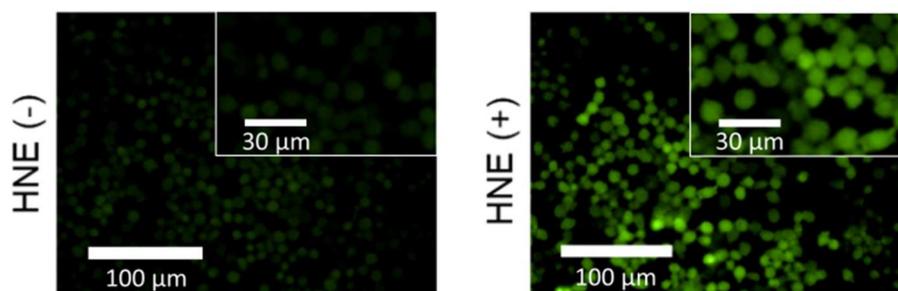
The apparent pseudo-first order rate constant for the reaction (Figure S4) and the LoD value for the detection of RH110 (Figure S5) were determined to be  $0.3 \text{ min}^{-1} \mu\text{M}^{-1}$  and 1.7 nM, respectively. At set times, ACS-HNE demonstrated high sensitivity toward elastase. For instance, LoD values of 5.3 and 2.6 nM were calculated at 60 and 120 min, respectively (Figure S6). These low LoD values were comparable to previously reported elastase detection systems (Sun et al., 2013; Ebrahimi et al., 2015; Liu et al., 2019). The selectivity of ACS-HNE was also tested by treating it with other potentially competing enzymes and biologically relevant analytes. As illustrated in Figure S7, ACS-HNE displayed excellent selectivity for elastase over a number of potential interferants, including trypsin, glutathione (GSH), and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ).

### Cellular Imaging of ACS-HNE and ACS-HNE/BSA

In light of the excellent selectivity for NE displayed by ACS-HNE, we turned our attention toward evaluating it as a potential



**FIGURE 2** | Widefield fluorescence micrographs of RAW 264.7 cells incubated with ACS-HNE (20 μM) before (–) or after (+) addition of human NE (HNE, 4 ng μL<sup>-1</sup>, 100 μL); Excitation and emission wavelengths for ACS-HNE are 460–490 nm and 500–550 nm, respectively. The insert shows magnified sections of the corresponding fluorescence micrograph.



**FIGURE 3** | Widefield fluorescence micrographs of RAW 264.7 cells incubated with ACS-HNE/BSA (20/100 μM) before (–) or after (+) addition of human NE (HNE, 4 ng μL<sup>-1</sup>, 100 μL); Excitation and emission wavelengths for ACS-HNE are 460–490 nm and 500–550 nm, respectively. The insert shows magnified sections of the corresponding fluorescence micrograph.

probe for cellular imaging. Predictive cytotoxicity experiments involving ACS-HNE revealed minimal cytotoxicity, which we took as a favorable augury for cellular imaging experiments (Figure S8). As shown in Figure 2, no initial fluorescence emission was observed in RAW 264.7 cells when incubated with ACS-HNE (20  $\mu\text{M}$ ). Upon exposure to exogenous elastase (4 ng  $\mu\text{L}^{-1}$ , 100  $\mu\text{L}$ ), a substantial increase in the fluorescence intensity was observed. Not surprisingly, due to the high lipophilicity of the dye scaffold, ACS-HNE displayed poor cell permeability with resultant precipitation, as seen by the fluorescent “spots” around each cell.

To overcome the issues of poor cell permeability and solubility, we applied a protein nanocarrier encapsulation strategy that involved treatment with the natural transport protein BSA. Previous studies have demonstrated BSA as an attractive candidate for the targeted intracellular delivery of therapeutics (Karami et al., 2020). Therefore, we rationalized that the use of BSA would overcome these current limitations by facilitating the effective cellular uptake of ACS-HNE. In accordance with the previously reported protocol, ACS-HNE was mixed with BSA at a molar ratio of 1:5 (ACS-HNE/BSA = 20  $\mu\text{M}$ /100  $\mu\text{M}$ ) prior to carrying out cellular imaging. The resulting ACS-HNE/BSA hybrid was subsequently added to the cells. As can be seen in Figure 3 and Figure S9, a low background fluorescence intensity was observed. The subsequent addition of exogenous elastase (154.4 nM, 100  $\mu\text{L}$ ) resulted in a 2.5-fold increase in the fluorescence intensity with little evidence of precipitation. This increase in fluorescence was attributed to the cellular uptake of exogenous elastase (Houghton et al., 2010) and reaction with ACS-HNE. This level of enhancement demonstrates the effective enzyme-based imaging of probe-albumin complexes and highlights the utility of this method as a means to increase solubility and cellular uptake for probes whose utility might otherwise be limited.

## CONCLUSIONS

In summary, we have developed a novel rhodamine-based fluorescent probe (ACS-HNE) prepared using a straightforward one-step reaction procedure. Synthetic attachment of the pendant pentafluoropropionamide functionality to a rhodamine core endows ACS-HNE with high sensitivity and selectivity toward elastase. This, in turn, permitted quantification with a low limit of detection (5.3 nM at an observation time of 60 min). The comparatively low cell permeability and poor solubility of native ACS-HNE was enhanced using a protein nanocarrier-based strategy involving formation an ACS-HNE/BSA hybrid prior to cell imaging. The combination of fluorescent probe development and nanocarrier solubilization has facilitated the development of new class of non-peptide-based fluorescent probes suitable for monitoring elastase activity *in vitro* (Bhuniya et al., 2014). We are currently extending the reaction-based fluorescence modulation and nanoparticle solubilisation approach to create new *in vitro* enzyme-specific sensor systems.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

## AUTHOR CONTRIBUTIONS

ZJ and AS carried out synthetic and spectroscopic experiments and co-wrote the manuscript with H-HH who also carried out the cellular imaging experiments. GW, LG, and JB carried out background experiments and helped with the preparation of the manuscript. X-PH and AJ are supervisors of H-HH, GW, and LG. SB, HS, JS, and TJ all conceived the idea and helped with the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2020.00389/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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