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click for updatesCite this: *Chem. Sci.*, 2015, 6, 1979Chemiluminescent probes for imaging H<sub>2</sub>S in living animals†J. Cao,<sup>ab</sup> R. Lopez,<sup>c</sup> J. M. Thacker,<sup>a</sup> J. Y. Moon,<sup>a</sup> C. Jiang,<sup>d</sup> S. N. S. Morris,<sup>e</sup>  
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Hydrogen sulphide (H<sub>2</sub>S) is an endogenous mediator of human health and disease, but precise measurement in living cells and animals remains a considerable challenge. We report the total chemical synthesis and characterization of three 1,2-dioxetane chemiluminescent reaction-based H<sub>2</sub>S probes, CHS-1, CHS-2, and CHS-3. Upon treatment with H<sub>2</sub>S at physiological pH, these probes display instantaneous light emission that is sustained for over an hour with high selectivity against other reactive sulphur, oxygen, and nitrogen species. Analysis of the phenol/phenolate equilibrium and atomic charges has provided a generally applicable predictive model to design improved chemiluminescent probes. The utility of these chemiluminescent reagents was demonstrated by applying CHS-3 to detect cellularly generated H<sub>2</sub>S using a multi-well plate reader and to image H<sub>2</sub>S in living mice using CCD camera technology.

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## Introduction

Hydrogen sulphide (H<sub>2</sub>S) is increasingly recognized as an important mediator of mammalian physiology and pathology, playing roles in vasorelaxation,<sup>1</sup> angiogenesis,<sup>2</sup> redox regulation,<sup>3</sup> neuromodulation,<sup>4</sup> lifespan,<sup>5</sup> Huntington's disease,<sup>6</sup> Down syndrome,<sup>7</sup> diabetes,<sup>8</sup> and cancer.<sup>9</sup> In mammals, endogenous H<sub>2</sub>S is generated from cystathionine  $\beta$ -synthase (CBS),<sup>10</sup> cystathionine  $\gamma$ -lyase (CSE),<sup>11</sup> and 3-mercaptopyruvate sulphur transferase (3MST).<sup>12</sup> Similar to its reactive cousins, nitric oxide (NO) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>),<sup>13</sup> H<sub>2</sub>S mediates cellular function *via* direct chemical interaction with biological molecules and can have widely disparate effects that depend on concentration, tissue localization, and the molecular environment.<sup>14–16</sup> For example, H<sub>2</sub>S in colon cancer promotes tumour growth by stimulating angiogenesis and supporting cellular energetics,<sup>17</sup> whilst H<sub>2</sub>S in prostate cancer slows cell growth, disrupts androgen receptor transactivation, and reduces angiogenesis by inhibiting the function of hypoxia-inducible

factor 1.<sup>18</sup> Given the delicate site- and concentration-dependent actions of H<sub>2</sub>S, easy methods for accurate spatiotemporal detection in living cells and animals are in urgent demand and promise to significantly contribute to an increased understanding of this reactive signalling molecule.

Common methods of H<sub>2</sub>S detection, including the methylene blue assay, ion-selective electrodes, amperometric sensors, and gas chromatography, have varying strengths and weaknesses, but all generally fall short of being able to detect H<sub>2</sub>S inside of intact living organisms.<sup>19</sup> Fluorescent probes offer the ability to target specific analytes<sup>20,21</sup> and there has been an explosion of recent activity in the development of dyes responsive to H<sub>2</sub>S and its derivatives.<sup>22–24</sup> Unfortunately, imaging endogenous H<sub>2</sub>S remains rare, and often requires advanced confocal microscopy setups to allow precise same-cell tracking or two-photon excitation coupled with ratiometric imaging.<sup>25,26</sup> This instrumentation is unavailable to many researchers, reducing the potential impact of these newly developed tools.<sup>27</sup> Furthermore, there are few examples of *in vivo* imaging of H<sub>2</sub>S,<sup>28</sup> and imaging deep mammalian tissue remains a frontier field for H<sub>2</sub>S detection. At the heart of the matter is a lack of sensitivity and depth penetration due to background autofluorescence, light scattering, photoactivation/photobleaching, and probe kinetics that requires long incubation times to accumulate signal. In order to address these technological challenges and develop tools to better elucidate the precise mechanisms of H<sub>2</sub>S production and function, we have herein developed a series of selective and sensitive chemiluminescent probes for rapid imaging of H<sub>2</sub>S in living animals.

Triggered chemiluminescence emission can provide a highly sensitive readout of biological analytes.<sup>29</sup> Chemiluminescence

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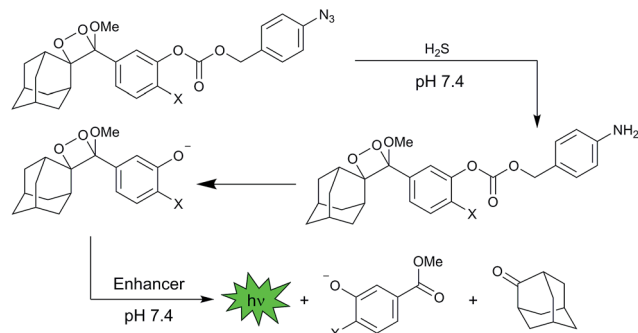
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Scheme 1 Spiroadamantane 1,2-dioxetanes for chemiluminescent  $\text{H}_2\text{S}$  detection at neutral pH.

doesn't require light excitation, thereby drastically reducing background from autofluorescence and photoactivation of azide functional groups. Whereas bioluminescence (chemiluminescence derived from living systems that express bioluminescent enzymes such as luciferase) has found wide application for preclinical analysis of biological parameters using genetically modified organisms,<sup>30</sup> small molecule chemiluminescence can be used with wild-type animals and opens up exciting opportunities for clinical imaging. Recently, there have appeared select examples of chemiluminescent agents for the detection of  $\text{H}_2\text{S}$  *in vitro*.<sup>31,32</sup> Although quite promising, the need for enzymatic additives and alkaline conditions introduces cytotoxicity and can potentially release sulphide from proteins and other base-labile sulphur pools,<sup>33,34</sup> ultimately hindering their potential for whole animal imaging.

To overcome these issues and expand the scope of chemiluminescent detection technology for small molecule biological analytes, we focused on using sterically stabilized 1,2-dioxetane systems<sup>35</sup> to develop a series of new chemiluminescent  $\text{H}_2\text{S}$  probes that display instantaneous light production under biologically compatible conditions. Sterically stabilized dioxetanes have been used for femtogram detection of enzymatic analytes<sup>36</sup> and have demonstrated potential for *in vivo* imaging.<sup>37</sup> We introduce three first generation chemiluminescent  $\text{H}_2\text{S}$  probes, **CHS-1**, **CHS-2**, and **CHS-3**, derived from sterically stabilized spiroadamantane 1,2-dioxetane scaffolds modified with self-immolative 4-azidobenzyl carbonates as the  $\text{H}_2\text{S}$  response site (Scheme 1).<sup>38</sup> This article reports their synthetic preparation, optical response and selectivity for  $\text{H}_2\text{S}$ , experimental and computational mechanistic investigations of their chemiluminescent response, detection of cellular  $\text{H}_2\text{S}$  using a multi-well plate reader, and a noteworthy demonstration of whole animal chemiluminescent imaging of  $\text{H}_2\text{S}$ .

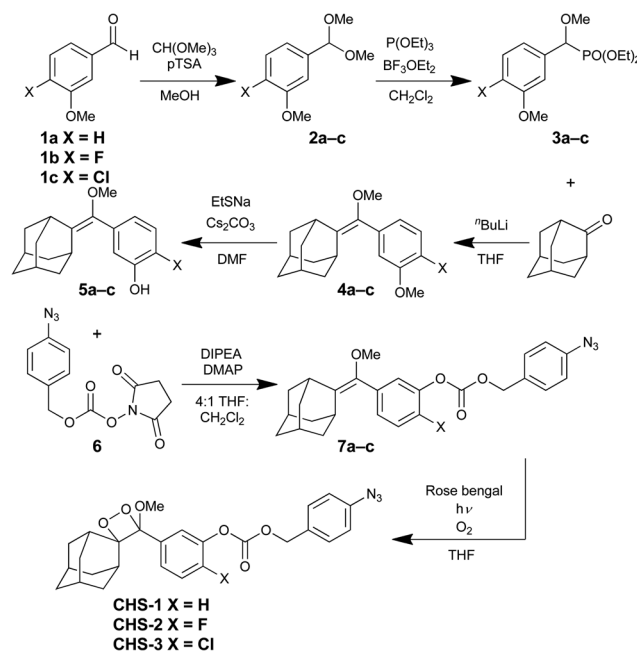
## Results and discussion

### Design and synthesis of CHS-1, CHS-2, and CHS-3

The **CHS** probe series was designed such that chemiluminescent emission would be initiated by the  $\text{H}_2\text{S}$ -mediated reduction of the azide group, followed by self-immolative carbonate cleavage yielding the free phenolate bearing the 1,2-dioxetane

(Scheme 1). The negatively charged phenolate will emit light spontaneously upon decomposition *via* an intramolecular chemically initiated electron exchange luminescence (CIEEL) mechanism.<sup>39–41</sup> This newly produced light can be observed directly or by transfer of its energy to acceptor molecules like quantum dots, rhodamine, or fluorescein.<sup>42</sup> In our studies, we employ a commercially available Emerald II Enhancer solution that consists of a cationic polymer and a dye with similar photophysical properties to fluorescein. The polymer reduces water-induced quenching by providing a hydrophobic environment for the chemiluminescent reaction and the dye effectively red shifts the luminescence emission making it more amenable for biological imaging applications.

By adapting a literature procedure,<sup>43</sup> we optimized an efficient and modular synthetic route to access the probes **CHS-1**, **CHS-2**, and **CHS-3** (Scheme 2). First, unsubstituted, fluorinated, and chlorinated 3-methoxybenzaldehyde derivatives **1a–c** were reacted with trimethyl orthoformate in the presence of *p*-toluenesulfonic acid to give acetals **2a–c**. These acetals were subjected to triethyl phosphite and boron trifluoride diethyl etherate at 0 °C, which afforded the diethyl methoxy (3-methoxyphenyl) methyl phosphonates **3a–c**. Next, enol ethers **4a–c** were obtained through Horner–Wadsworth–Emmons reaction by treating phosphonates **3a–c** with  $^t\text{BuLi}$  and 2-adamantanone.<sup>44</sup> Nucleophilic demethylation using sodium ethanethiolate provided the phenols **5a–c**. The activated ester **6**, prepared according to analogous literature procedures, was coupled to the phenols to provide **7a–c**, azide-bearing precursors to the final 1,2-dioxetanes.<sup>45,46</sup> Finally, a [2 + 2] cycloaddition with singlet oxygen was accomplished by bubbling oxygen through a solution of **7a–c** and the sensitizer Rose bengal with visible light irradiation, delivering the chemiluminescent probes **CHS-1**, **CHS-2**, and **CHS-3**.



Scheme 2 Syntheses of **CHS-1**, **CHS-2**, and **CHS-3**.



## Response and selectivity

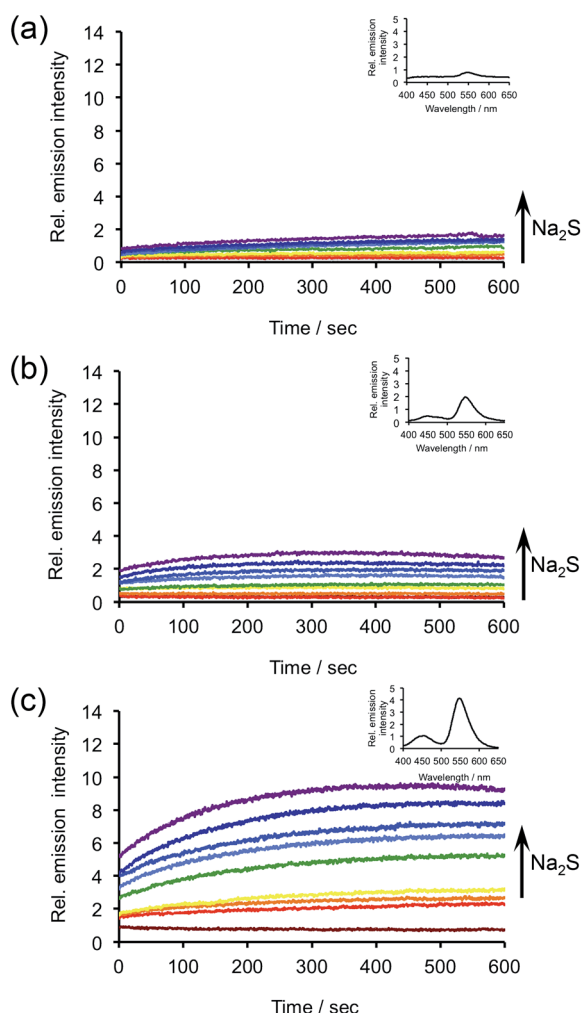
With the first three **CHS** probes in hand, we proceeded to measure their luminescent responses to  $\text{H}_2\text{S}$  using an F-7000 Hitachi spectrophotometer. At pH 7.4, treatment of the **CHS** probes with  $\text{H}_2\text{S}$  resulted in instantaneous luminescent emission that increased over a course of 10 minutes in a dose-dependent manner (Fig. 1). Addition of the fluorescein-based Emerald II Enhancer provided a red-shifted peak centred at 545 nm compared to the 470 nm emission of the phenolate (Fig. 1, insets). Under these physiologically relevant conditions, we observed an increase in the integrated luminescent response to  $\text{H}_2\text{S}$  from **CHS-1** to **CHS-2** to **CHS-3**, giving 5-fold, 4-fold, and 12-fold turn-on responses respectively. It should be noted that no background corrections were performed and the values in Fig. 1 are direct instrumental values to provide an accurate comparison between probes. At pH 10, **CHS-1** provided the highest chemiluminescence intensity and response, giving a 7-

fold increase in photon emission in the first 10 minutes after adding 200  $\mu\text{M}$   $\text{H}_2\text{S}$  (Fig. S1†). Under these alkaline conditions, the  $\text{H}_2\text{S}$ -triggered chemiluminescent emissions of the fluorinated **CHS-2** and chlorinated **CHS-3** were lower than **CHS-1**. The background signal also increases with **CHS-2** and **CHS-3**, reducing the relative increases over control to 2-fold and 3-fold respectively. A key advantage of these probes is that they display an immediate concentration-dependent emission of light that persists for over an hour (Fig. S2†), an ideal property for the high-throughput detection and imaging of  $\text{H}_2\text{S}$  generated in biological environments.

We next tested the selectivity of **CHS-1**, **CHS-2**, and **CHS-3** for  $\text{H}_2\text{S}$  against other biologically relevant reactive sulphur, oxygen, and nitrogen (RSO<sub>N</sub>) species. The response of **CHS-1** to 200  $\mu\text{M}$   $\text{Na}_2\text{S}$  in 20 mM HEPES buffered to pH 7.4 was tested against other RSO<sub>N</sub> species by adding 5 mM reduced glutathione (GSH), 1 mM L-cysteine and homocysteine, and 200  $\mu\text{M}$  of S-nitrosoglutathione, sulfite ( $\text{SO}_3^{2-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hypochlorite ( $\text{OCl}^-$ ), *tert*-butyl hydroperoxide (*t*-BuOOH), nitroxyl ( $\text{HNO}$ ), nitric oxide ( $\text{NO}$ ), and nitrite ( $\text{NO}_2^-$ ). None of the species tested displayed significant increases in luminescence intensity over the blank control (Fig. 2a). Additionally, the response to  $\text{Na}_2\text{S}$  was minimally perturbed by the presence of physiological levels of GSH, L-cysteine, and homocysteine. We observed similar results when **CHS-2** and **CHS-3** were evaluated for their selectivity in 20 mM HEPES at pH 7.4 (Fig. 2b and c). These response and selectivity data demonstrate that **CHS-1**, **CHS-2**, and **CHS-3** are able to detect  $\text{H}_2\text{S}$  at physiologically relevant pH with minimal interference from competing analytes.

## Mechanistic studies of chemiluminescent $\text{H}_2\text{S}$ detection

We next sought to understand the factors that lead to increased light production and optimized response for 1,2-dioxetane chemiluminescent probes. First, we investigated the role of the phenol/phenolate equilibrium. Since the measurement of the  $\text{pK}_\text{a}$  of the phenolate dioxetane products of the **CHS** probes is complicated by their rapid chemiluminescent decomposition, we used the reported experimental values of phenol, 2-fluorophenol, and 2-chlorophenol as an approximation.<sup>47</sup> We plotted the integrated chemiluminescent emission of **CHS-1**, **CHS-2**, and **CHS-3** in response to 200  $\mu\text{M}$   $\text{Na}_2\text{S}$  at pH 7.4 and pH 10 (Fig. 3a and b) against the experimental  $\text{pK}_\text{a}$  values for phenol, 2-fluorophenol, and 2-chlorophenol (Fig. 3c and d). The  $\text{H}_2\text{S}$ -stimulated emission intensity at pH 7.4 displays an increasing trend with increased phenol acidity, indicating that ionization of the phenol is critical to attaining good luminescent response under these conditions (Fig. 3c). On the other hand, there is no clear correlation with phenol  $\text{pK}_\text{a}$  and the chemiluminescent response at pH 10 (Fig. 3d). We further investigated the nature of the chemiluminescent emission by performing quantum chemistry calculations on the phenolate structures released from **CHS-1**, **CHS-2**, and **CHS-3** after reaction with  $\text{Na}_2\text{S}$  (Tables S1–S3†). Geometries were optimized using density functional theory (DFT) at the B3LYP/6-311+G(d,p) level of theory, and the charge on each atom was



**Fig. 1** Time scans of the chemiluminescent emission at 545 nm from (a) 40  $\mu\text{M}$  **CHS-1**, (b) 40  $\mu\text{M}$  **CHS-2**, or (c) 40  $\mu\text{M}$  **CHS-3** and 0, 5, 10, 20, 40, 80, 100, 150, 200  $\mu\text{M}$   $\text{Na}_2\text{S}$  in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. Insets are chemiluminescence spectra of (a) 40  $\mu\text{M}$  **CHS-1**, (b) 40  $\mu\text{M}$  **CHS-2**, and (c) 40  $\mu\text{M}$  **CHS-3** and 200  $\mu\text{M}$   $\text{Na}_2\text{S}$ , acquired immediately after adding probes.



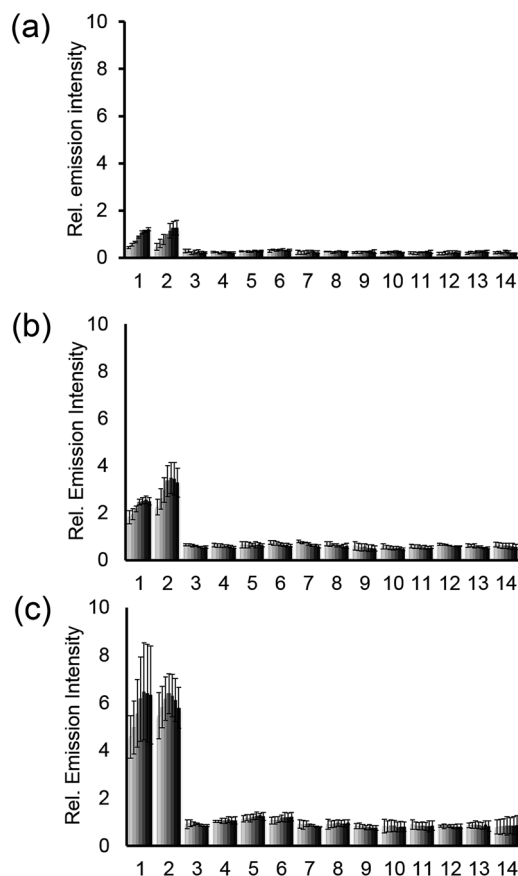


Fig. 2 Chemiluminescent responses of (a) 40  $\mu$ M CHS-1, (b) 40  $\mu$ M CHS-2, or (c) 40  $\mu$ M CHS-3 to biologically relevant RSON species in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. Bars represent chemiluminescent emission at 545 nm and 30, 60, 120, 240, 360, 480 and 600 s after addition of RSON species. Data shown are for 5 mM glutathione, 1 mM cysteine and homocysteine, and 200  $\mu$ M for other RSON species. Legend: (1)  $\text{Na}_2\text{S}$ ; (2)  $\text{Na}_2\text{S}$ , glutathione, L-cysteine, and homocysteine; (3) glutathione; (4) S-nitrosoglutathione; (5) L-cysteine; (6) homocysteine; (7)  $\text{HNO}$ ; (8)  $\text{NO}$ ; (9)  $\text{NaNO}_2$ ; (10)  $\text{Na}_2\text{SO}_3$ ; (11)  $\text{H}_2\text{O}_2$ ; (12)  $\text{NaClO}$ ; (13)  $^t\text{BuOOH}$ ; (14) blank. Error bars are  $\pm$  S.D.

calculated using an electrostatic potential (ESP) model at the M06/6-311+G(d,p) level of theory. All calculations were carried out with the integral equation formalism polarizable continuum model (IEF-PCM) in water as solvent using the Gaussian 09 program package. While we found no correlation between the charge on the phenolic oxygen (O8) and the chemiluminescent response at pH 7.4 (Fig. 3e), an excellent correlation ( $R^2 > 0.99$ ) was observed with the response at pH 10 (Fig. 3f). ESP charge calculations performed with B3LYP and  $\omega$ B97XD functionals also provided good correlations ( $R^2 > 0.97$ ) of the O8 charge and the chemiluminescent response at pH 10 (Fig. S3†). These data indicate that at lower pH, the chemiluminescent emission is governed by the equilibrium between the protonated phenol and unprotonated phenolate oxygen, and are in agreement with previous observations.<sup>48</sup> CHS-3 is the most readily ionized and therefore shows the highest chemiluminescent emission. On the other hand, at pH 10 the phenolate species dominates for all three deprotected

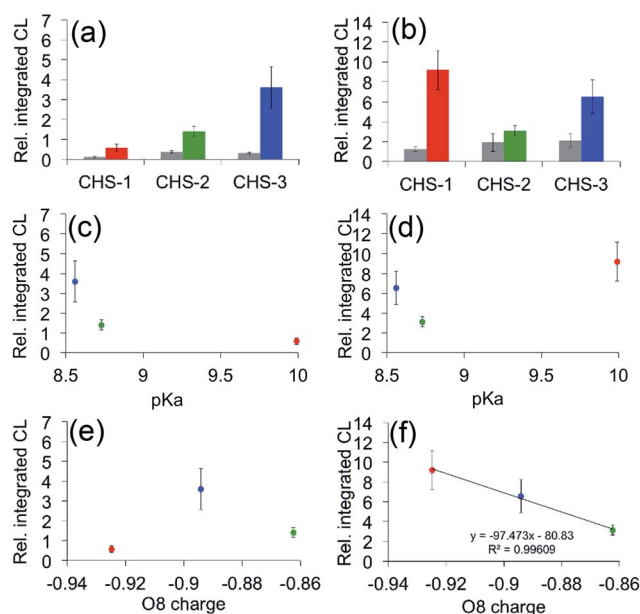


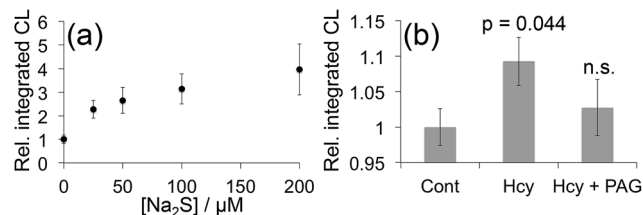
Fig. 3 Analysis of chemiluminescent responses. (a and b) Bar graphs for the integrated chemiluminescent emission over 10 min at (a) pH 7.4 or (b) pH 10 of CHS-1, CHS-2, and CHS-3 to 0  $\mu$ M  $\text{Na}_2\text{S}$  (grey bars) and 200  $\mu$ M  $\text{Na}_2\text{S}$  (colored bars). (c and d) Plot of the integrated chemiluminescent emission over 10 min at (c) pH 7.4 or (d) pH 10 of 200  $\mu$ M  $\text{Na}_2\text{S}$  and 40  $\mu$ M CHS-1 (red), CHS-2 (green) and CHS-3 (blue) versus experimental  $pK_a$  values of model compounds phenol, 2-fluorophenol, and 2-chlorophenol. (e and f) Plot of the integrated chemiluminescent emission over 10 min at (e) pH 7.4 or (f) pH 10 of 200  $\mu$ M  $\text{Na}_2\text{S}$  and 40  $\mu$ M CHS-1 (red), CHS-2 (green) and CHS-3 (blue) versus the calculated atomic charge on the phenolate oxygen (O8). All luminescent measurements were acquired in 20 mM HEPES buffer (pH 7.4) or 100 mM glycine buffer (pH 10) containing 20% Emerald II Enhancer. The reported values are averages of the integrated emission intensities over 10 min ( $n = 4-7$ ). Error bars represent  $\pm$  S.D. The ESP atomic charges were calculated at the M06/6-311+G(d,p) level of theory using geometries optimized at the B3LYP/6-311+G(d,p) level of theory. Calculations were carried out with the IEF-PCM water solvation model using the Gaussian 09 program package.

dioxetanes and the efficiency of chemiluminescent emission is predicted by the more negative O8 charge density, probably due to an increased propensity towards initiation of the first intramolecular electron transfer step of CIEEL.<sup>49,50</sup> These data reveal trends that, when taken together, provide a powerful predictive model for the design of improved chemiluminescent reagents.

### Using CHS-3 to detect cellular $\text{H}_2\text{S}$

Our results show that CHS-3 provides the most robust light emission at physiological pH when compared to CHS-1 and CHS-2. We therefore examined the ability of CHS-3 to detect cellular  $\text{H}_2\text{S}$  production in human lung adenocarcinoma epithelial cells (A549). These cells express the enzyme cystathionine  $\gamma$ -lyase (CSE),<sup>51</sup> which can utilize homocysteine (Hcy) as a substrate for  $\text{H}_2\text{S}$  production. We first demonstrated the ability of CHS-3 to detect exogenous  $\text{Na}_2\text{S}$  in a multi-well plate reader format (Fig. 4a), and determined an estimated detection limit (blank control + 3 S.D.) of 5.4  $\mu$ M. We then incubated A549 cells with Hcy, a substrate for the enzyme CSE. This resulted in





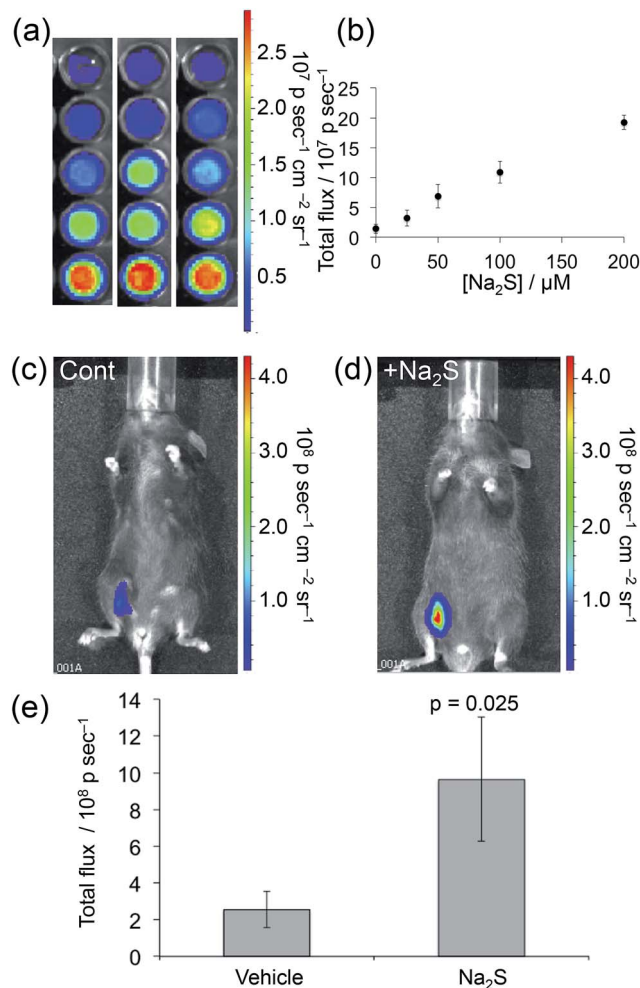
**Fig. 4** Detection of cellular  $H_2S$  using a multi-well plate reader. (a) Luminescent responses of 40  $\mu M$  **CHS-3** and 0, 25, 50, 100, and 200  $\mu M$   $Na_2S$  in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. (b) A549 cells were treated with a vehicle control (Cont), 200  $\mu M$  Hcy (Hcy), or 200  $\mu M$  Hcy after being pre-treated with 200  $\mu M$  PAG (Hcy + PAG) for 20 min. 20 min after incubating with Hcy or vehicle, the cells were washed and treated with 40  $\mu M$  **CHS-3** and 125  $\mu L$  Emerald II Enhancer. The reported values represent the average luminescent intensity of replicate experiments ( $n = 12$ ,  $p = 0.044$ ). Error bars represent  $\pm$  S.E.M.

$\sim 10\%$  increase ( $n = 12$ ,  $p = 0.044$ ) in luminescent emission from **CHS-3** compared with vehicle treated cells (Fig. 4b). Pre-incubation with the CSE inhibitor  $D,L$ -propargylglycine (PAG) before adding Hcy attenuates signal observed from **CHS-3**. Although the observed increase is small, it is statistically significant and the efficacy of **CHS-3** for endpoint detection of  $H_2S$  generated by whole cells at physiological pH is an important advance that sets the stage for imaging  $H_2S$  in living animals.

### In vivo imaging of $H_2S$ using **CHS-3**

We next investigated the ability of **CHS-3** to image  $H_2S$  at physiological pH using an IVIS Spectrum. An opaque 96-well plate was loaded with 0, 25, 50, 100, and 200  $\mu M$   $Na_2S$  in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. **CHS-3** was added at 40  $\mu M$  and imaged to reveal a clear increase in luminescence intensity with increasing  $H_2S$  concentrations (Fig. 5a). Averages of repeated experiments provide a good linear response in the range of 0–200  $\mu M$  when imaged 30 s after exposure to  $H_2S$  (Fig. 5b). We next applied a mouse carcass model to determine if **CHS-3** would have sufficient light output to be observable through mammalian tissue. The carcasses of sacrificed SCID/BALB-C mice were injected with **CHS-3** and either a vehicle control (Fig. S4a and b†) or 0.4  $\mu mol$   $H_2S$  (Fig. S4c and d†) into the peritoneal cavity. An increase in luminescence could be clearly observed in the peritoneum of the carcass injected with  $H_2S$  versus the carcass injected with the vehicle control.

Confident that light emission from **CHS-3** was capable of significant tissue penetration, we finally sought to establish the ability of **CHS-3** to image  $H_2S$  in living animals. C6 brown mice were administered i.p. injections on one side of their peritoneal cavity. The skin was raised during injections to avoid puncturing internal organs. Images were acquired 30 s after injecting 0.08  $\mu mol$  **CHS-3** and either 0.4  $\mu mol$   $Na_2S$  or a vehicle control ( $H_2O$ ) in 100  $\mu L$  HEPES buffered at pH 7.4 containing 20% Emerald II Enhancer. The final concentration of  $Na_2S$  in the injection was 4 mM. While vehicle control experiments produced modest signal (Fig. 5c), the mice that received  $Na_2S$  treatments displayed



**Fig. 5** Imaging  $H_2S$  using **CHS-3**. (a) Images 30 s after adding 40  $\mu M$  **CHS-3** to 0, 25, 50, 100, and 200  $\mu M$   $Na_2S$  in 20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer ( $n = 3$ ). (b) Plot of total photon flux versus  $H_2S$  concentration for the experiments described in (a). (c and d) Images of living C6 brown mice 30 s after administering i.p. injections of 0.08  $\mu mol$  **CHS-3** and (c) vehicle control or (d) 0.4  $\mu mol$   $Na_2S$  in 100  $\mu L$  20 mM HEPES buffer (pH 7.4) containing 20% Emerald II Enhancer. (e) Quantification of the total photon flux from three replicates of the experiments described in (c) and (d). Statistical analyses were performed with a two-tailed Student's  $t$ -test ( $n = 3$ ,  $p = 0.025$ ). Error bars are  $\pm$  S.D.

robust emission of light that was easily detected through their tissue (Fig. 5d). Quantification of the total photon flux from replicate experiments (Fig. S5†) revealed  $\sim 4$ -fold increase ( $n = 3$ ,  $p = 0.025$ ) in the luminescence response in the  $Na_2S$  treated mice versus vehicle controls (Fig. 5e). The agents were well tolerated and the mice showed no immediate outward signs of malaise. Taken together, these data provide a key milestone towards the development of a new class of *in vivo* imaging tools for investigating biological hydrogen sulphide.

## Conclusions

We have designed and synthesized three 1,2-dioxetane chemi-luminescent reaction-based  $H_2S$  probes, **CHS-1**, **CHS-2**, and



**CHS-3** that display immediate light emission upon reacting with H<sub>2</sub>S at physiological pH, a significant advance for H<sub>2</sub>S detection technology. These reagents provide a sensitive and selective detection platform for H<sub>2</sub>S using spectrophotometers, multi-well plate readers, and IVIS Spectrum instruments. We have provided a computational and experimentally supported mechanistic framework that serves as a predictive model for designing sterically hindered 1,2-dioxetanes with improved light emission based on the pK<sub>a</sub> of the phenol released after reacting with H<sub>2</sub>S and the relative atomic charge densities of these structures. Finally, we demonstrated that **CHS-3** was not only capable of detecting cellular H<sub>2</sub>S produced by A549 cells treated with homocysteine at neutral pH, but also has the rare ability to image H<sub>2</sub>S in living animals. While **CHS-3** provides an exciting proof of principle for *in vivo* chemiluminescence imaging, we are currently engaged in improving the sensitivity and biocompatibility of chemiluminescent 1,2-dioxetane reagents for diverse applications, and anticipate that these new optimized systems will provide powerful whole animal imaging tools across a range of biological analytes and parameters.

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