

# A Novel Fluorescent Probe for Detecting Hydrogen Sulfide in Osteoblasts During Lipopolysaccharide-Mediated Inflammation Under Periodontitis

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## Research Article

**Keywords:** Periodontitis, hydrogen sulfide, fluorescence probe, osteogenesis, LPS

**Posted Date:** August 6th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-776276/v1>

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1 *A novel fluorescent probe for detecting hydrogen sulfide in osteoblasts during*  
2 *lipopolysaccharide-mediated inflammation under periodontitis*

3 **Title page**

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12 **Author contributions**

13 Xiaoya Lu, Yi Chen and Yue Wu performed the bioimaging experiments together.

14 Hanchuang Zhu synthesized the H<sub>2</sub>S fluorescence probe.

15 Shengyun Huang, Baocun Zhu and Dongsheng Zhang conceived the idea and directed  
16 the work.

17 All authors contributed to data analysis, manuscript writing and participated in  
18 research discussions.

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22

23 **Abstract**

24 *Periodontitis, one of the most common chronic inflammatory diseases, affects the*  
25 *quality of life. Osteogenesis makes an important role of the disease. There is a*  
26 *connection between hydrogen sulfide (H<sub>2</sub>S) and periodontitis, but according to the*  
27 *study has been published, the precise role of H<sub>2</sub>S in inflammation remains in doubt.*  
28 *The main reason of the lack of research is that H<sub>2</sub>S is an endogenous gasotransmitter,*  
29 *difficult to discern through testing. So, we synthesis a novel fluorescence probe which*  
30 *can detective H<sub>2</sub>S in vitro. By using the novel H<sub>2</sub>S fluorescence probe, we found that*  
31 *H<sub>2</sub>S changes in osteoblasts mainly by cystathionine- $\gamma$ -lyase, and H<sub>2</sub>S increases under*  
32 *LPS stimulation. H<sub>2</sub>S could be a potential marker for diagnosis of inflammatory*  
33 *diseases of bone, and might help deeper studies of the changes of H<sub>2</sub>S level and promote*  
34 *the progression on the researches about pathogenesis of periodontitis.*

35

36 **Keywords**

37 *Periodontitis, hydrogen sulfide, fluorescence probe, osteogenesis, LPS*

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45 **Introduction**

46 *Periodontitis as one of the most common chronic inflammatory diseases, afflicting man.*

47 *It can leading cause of bone resorption, even worse tooth loss. Under normal*

48 *physiologic conditions, the balance of osteoclasts and osteoblasts is tightly related to*

49 *avoid the loss of bone. The breakdown of the balance will cause diseases. Avoiding*

50 *alveolar bone destruction is an important problem to control the periodontitis.*

51 *However, the detailed mechanism of periodontitis is still largely unknown.*

52 *Lipopolysaccharide (LPS), a major toxic factor of gram-negative bacteria, plays a*

53 *main role in periodontitis. It can cause periodontitis by modulating the activity of the*

54 *host defenses [1], inducing a hypoxic phase [2] etc., and it eventually stimulates bone*

55 *resorption [3]. LPS may leads to inflammatory response in osteoclasts and osteoblast,*

56 *which may results in a disorder in the balance of osteoclasts and osteoblasts even cell*

57 *death, leading to accelerating bone loss [4]. For experimental researches, LPS*

58 *stimulated the rat gingival sulcus every day in order to obtain an experimental*

59 *periodontitis model by immunizing it with the antigen [5]. LPS treated cells are in a*

60 *similar situation as well. Halitosis is one of the clinical features of periodontitis, and*

61 *Hydrogen sulfide (H<sub>2</sub>S) is the main unbearable stinky smell of periodontitis and may*

62 *play a significant role in its development.*

63 *Biothiols are indispensable in human physiology, which are in a vital branch of reactive*

64 *sulfur species (RSS) family. H<sub>2</sub>S is an endogenous gasotransmitter, which is*

65 *well-known for its stinky smell like rotten eggs. H<sub>2</sub>S is produced by the*

66 *sulfur-containing materials cysteine, homocysteine or*

67 3-mercaptopyruvate.  $H_2S$  is transformed by cystathionine- $\beta$ -synthase (CBS),  
68 cystathionine- $\gamma$ -lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST)[6].  
69 Most researchers previously believe that  $H_2S$  can promote the pathogenesis of  
70 periodontitis, and hugely harm to their periodontal tissue [7]. But recently, there is  
71 evidence shows that  $H_2S$  might be useful in cell protection. For exogenous  $H_2S$ , it can  
72 promote LPS-induced apoptosis of osteoblast cells, which might represent a new  
73 direction in the treatment of osteomyelitis [8]. When oxidative damage occurs,  $H_2S$  can  
74 increase cell viability and reduce cell apoptosis.  $H_2S$  might have a advantageous  
75 effect, because according to the research, NaHS treatment can produce  
76 anti-inflammatory effects via NO and TNF- $\alpha$  [9]. Besides,  $H_2S$  can protect cell injury  
77 by regulating oxidative stress, mitochondrial function, and inflammation. It also have  
78 the ability to potentially prevent bone loss in periodontitis [10]. So, there is a  
79 connection between  $H_2S$  and periodontitis, but until now, the precise role of  $H_2S$  in  
80 inflammation remains unknown.

81 Most of the studies focus on the effect of the  $H_2S$ , not many about  $H_2S$  changes under  
82 stimulation. Researchers often use Western blot, immunohistochemical staining, and  
83 some other methods to detect the  $H_2S$  changes indirectly. Recently, there are some  
84 direct techniques to detect  $H_2S$ , such as chromatography, electrochemistry and  
85 colorimetry [11]. But a technique that can detect  $H_2S$  directly in living cells is still  
86 needed.  $H_2S$ -fluorescence probes, which is high-speed developing, is considered as one  
87 of the most helpful instrument areas in the field of  $H_2S$  biology [12]. We previously  
88 designed a  $H_2S$  probe, which consists of a 4-chloro-1,8-naphthalimide as fluorophore

89 and introduces hydrophobic chains (dodecylamine) and hydrogen sulfide recognition  
90 groups (azide groups) (Figure 1 a). The introduction of the electron-withdrawing azide  
91 group changes the push-pull system and quenches the fluorescence. It is noteworthy  
92 that the reaction is easy to carry out and the yield is high. When the probe reacts with  
93 hydrogen sulfide, the azide group is reduced to an amino group. Because the amino  
94 group acts as an electron-donating group, the effect of intramolecular charge transfer  
95 is enhanced, and the fluorescence is recovered. The probe is able to directly measure  
96 the real time H<sub>2</sub>S level in living cells. Overall, because of high resolution and sensitivity  
97 of the H<sub>2</sub>S probe make it a helpful tool. There are some studies showing that H<sub>2</sub>S  
98 fluorescence probe can detect endogenous H<sub>2</sub>S in real-time and in situ. However, most  
99 of them use tumor cells instead of somatic cells. If the probe could be used in  
100 somatic cells, it can broaden the diagnose and treatment applications of H<sub>2</sub>S. By using  
101 a novel H<sub>2</sub>S fluorescence probe, we found that H<sub>2</sub>S changes in osteoblast mainly by  
102 CSE, and H<sub>2</sub>S increases under LPS stimulation.

103

## 104 **Materials and methods**

### 105 1. Regents

106 The hydrogen sulfide fluorescent probe was provided by Professor Baocun Zhu (School  
107 of Resources and Environment, University of Jinan, Jinan, China). The mother  
108 solution of the probe was prepared with DMSO (Sigma-aldrich, USA) and  
109 dichloromethane. The probe concentration was 1 mM. The test concentration was  
110 10 μM and the experiment was carried out at room temperature (25°C).

111 *DL-propargylglycine (PAG) (cystathionine  $\gamma$ -lyase inhibitor, Sigma-Aldrich),*  
112 *Cysteine (Cys), NaHS, lipopolysaccharide (LPS) (Sigma-aldrich, USA), cell counting*  
113 *kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan).*

#### 114 2. *MC3T3-E1 cell culture*

115 *The murine calvaria-derived MC3T3-E1 osteoblast-like cell line (Procell CL-0378,*  
116 *subclone 14) was provided by Procell Life Science and Technology CO.,Ltd. Cells were*  
117 *seeded at  $5 \times 10^4$  cells/ml into 25 cm<sup>2</sup> flasks and maintained in  $\alpha$ -MEM, supplemented*  
118 *with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were*  
119 *maintained in an incubator containing a 5% carbon dioxide/air environment at 37°C.*

#### 120 3. *Toxicity Analysis:*

121 *The influence of the H<sub>2</sub>S probe on MC3T3-E1 cell was examined by CCK-8. Briefly,*  
122 *MC3T3-E1 cells, seeded at a density of  $5 \times 10^4$  cells/ml on a 96-well plate, were*  
123 *maintained at 37°C in a 5% CO<sub>2</sub>, 95% air incubator for 24 h. Then the cells were*  
124 *incubated with different concentrations (0, 5, 10, 20, 25, 37.5, 50, 75 and 100  $\mu$  M) of*  
125 *probe suspended in culture medium for 24 h. Same as the probe group, the other plate*  
126 *of cells incubated with same concentrations (0, 5, 10, 20, 25, 37.5, 50, 75 and 100  $\mu$  M)*  
127 *of solvent. Subsequently, CCK-8 solution was added into each well for 2 h, and*  
128 *measured the absorbance at 450 nm was measured.*

129 4. *Application of H<sub>2</sub>S probe to access exogenous H<sub>2</sub>S levels: The cells were pre-treated*  
130 *with NaHS (50, 100, 150, 500  $\mu$  M) for 30 minutes, then, treated with the H<sub>2</sub>S*  
131 *probe (10  $\mu$  M) for 30 minutes. Fluorescence and bright field images were collected after*  
132 *PBS washing for three times. Green fluorescence was observed under the confocal*

133 microscope at excitation wavelengths of 405 nm. In order to control exposure, Smart  
134 Gain was kept at the same voltage in every photographs.

135 5. Application of H<sub>2</sub>S probe to access endogenous H<sub>2</sub>S levels:: In the periodontium of  
136 mammalian host, H<sub>2</sub>S is produced using Cys mainly by CSE and CBS. The cells were  
137 pre-treated with Cys (100 μ M, 200 μ M) for 30 minutes, then, treated with the H<sub>2</sub>S  
138 probe (10 μ M) for 30min. Fluorescence and bright field images were collected after  
139 PBS washing for three times. Green fluorescence was observed under the confocal  
140 microscope at excitation wavelengths of 405 nm. In order to control exposure, Smart  
141 Gain was kept at the same voltage in every photographs.

142 PAG is an irreversible inhibitor of CSE. It can blocking-up the produce of endogenous  
143 H<sub>2</sub>S in MC3T3-E1. Therefore, we pre-treated cells with 50 μ M PAG, 30min, then  
144 cells were treated with or without Cys for 30 min. Last, fluorescence was examined as  
145 before, Smart Gain was kept at the same voltage in every photographs.

146 6. Addition of lipopolysaccharide (LPS) for inducing inflammation and assessment  
147 with H<sub>2</sub>S probe: The cells were incubated with 1, 2 μ g/ml LPS for one day.  
148 Subsequently, the culture dish was washed with PBS for three times and incubated  
149 with 10 μ M probe for 30 min. Then, the cells were washed with PBS, then the  
150 fluorescence imaging was examined by confocal microscope, Smart Gain was kept at  
151 the same voltage in every photographs.

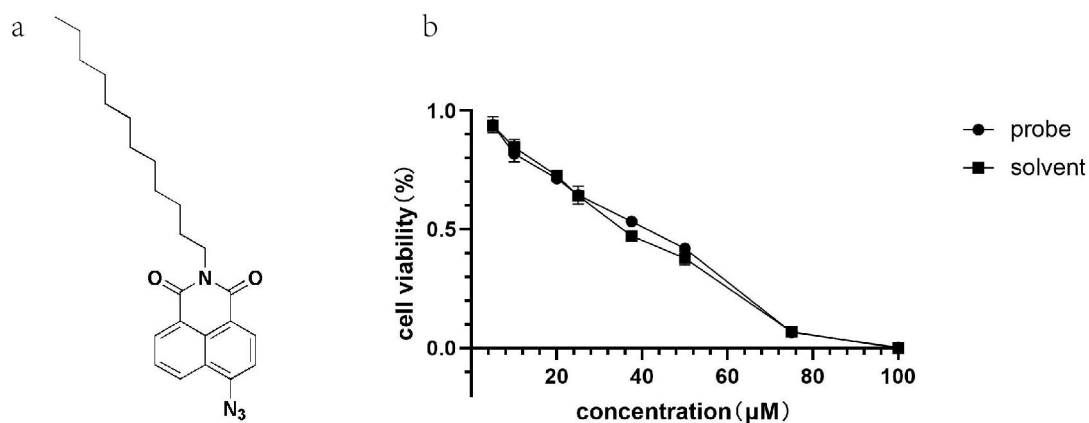
## 152 **Results**

153 Toxicity analysis: The cell's viable and healthy during the detection is a key concern.

154 Figure 1 b showed that cell viability was almost not affected by the probe at 10 μ M.



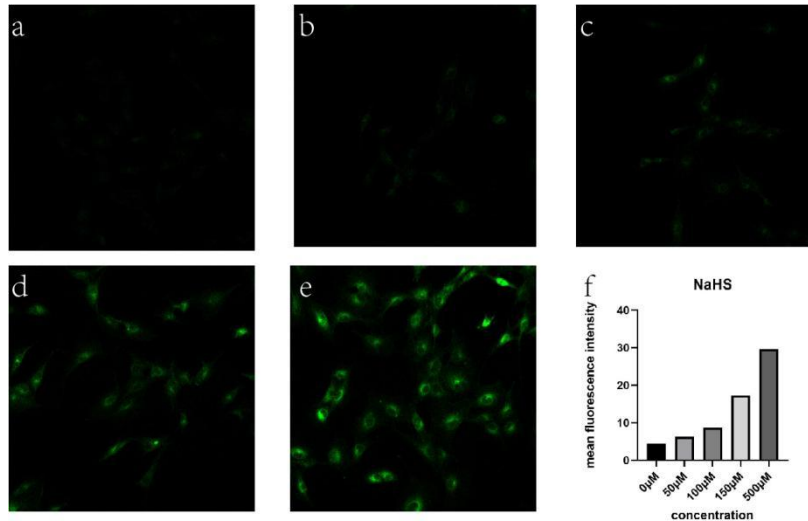
155 Toxicity is mainly introduced by solvent, DMSO and dichloromethane. The result  
156 verify that the H<sub>2</sub>S probe is harmless to the cell. Thus, the H<sub>2</sub>S probe can be used in  
157 living cells for fluorescence imaging analysis.



158  
159 Figure 1 a. Structure of H<sub>2</sub>S probe. b. toxicity Analysis.

160

161 Cell fluorescence imaging of different concentrations exogenous H<sub>2</sub>S: As shown by  
162 figure 2, with the different concentrations (0, 50, 100, 150, 500 μ M) of NaHS, a gradual  
163 increase of intensive green fluorescence was observed using 405nm as an excitation  
164 wavelength. Consistent with previous studies, the amount of H<sub>2</sub>S is one third of  
165 exogenous of NaHS. Thus, the probe was estimated detection of accuracy to 10 μ M.  
166 Fluorescent intensity is stable during the progress of takeing pictures under the  
167 confocal laser scanning microscopy. That indicated that our probe is sensitive to H<sub>2</sub>S,  
168 and it also prove that H<sub>2</sub>S probe was cell membrane permeable and can used in the  
169 normal cells for detecting intracellular H<sub>2</sub>S.



170

171 *Figure 2 Cell fluorescence imaging of different concentrations exogenous H<sub>2</sub>S. a-e.*

172 *Fluorescence imaging of cells incubated with different concentration of NaHS (0, 50,*

173 *100, 150, 500 μ M) f. Fluorescence intensity analysis.*

174

175 *Cell fluorescence imaging of endogenous H<sub>2</sub>S: According to the previous research, for*

176 *osteoblasts, CSE-H<sub>2</sub>S might be the major path for the H<sub>2</sub>S produced [13]. As shown by*

177 *Figure 3, the incubation of cells with 100 μ M Cys produced intensive green*

178 *fluorescence, but the fluorescence decreased when cells were incubated with 200 μ M*

179 *Cys. That means that low dose of Cys could increase H<sub>2</sub>S production, but high dose of*

180 *Cys inhibited H<sub>2</sub>S production. In order to verify whether the CSE-H<sub>2</sub>S pathway is the*

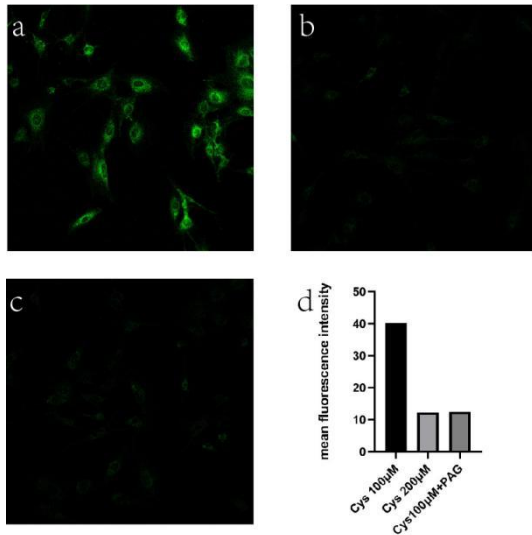
181 *main pathway to produce the H<sub>2</sub>S, we used PAG as the irreversible inhibitor to CSE.*

182 *Figure 3 showed that the intensity of fluorescence was decreased, which means the H<sub>2</sub>S*

183 *was decreased, because of the pretreatment of the inhibitor, and the intensity of PAG*

184 *group was as weak as the control group, indicating that the production of endogenous*

185 *H<sub>2</sub>S was significantly inhibited with CSE inhibitor.*



186

187 *Figure3 Cell fluorescence imaging of endogenous H<sub>2</sub>S. a. Cys 100 μ M, b. Cys 200 μ M,*

188 *c. Cys 100 μ M + PAG 50 μ M, d. Fluorescence intensity analysis.*

189

190 *Cell fluorescence imaging of LPS induced endogenous H<sub>2</sub>S: when cells were treated*

191 *with LPS (2 μ g/ml) to produce inflammation, as shown by Figure 4, intensive green*

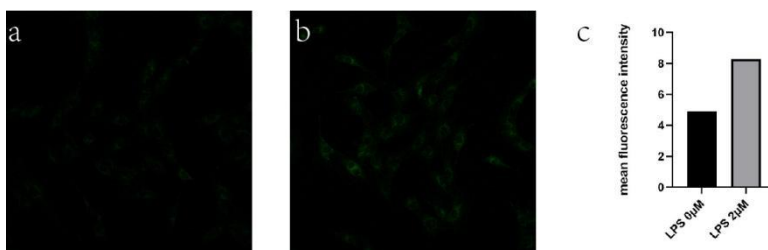
192 *fluorescence were produced compared to the control group. This indicates that when*

193 *inflammation occurs, a lot of H<sub>2</sub>S was produced. In other words, the increase of H<sub>2</sub>S*

194 *level can serve as an indicator for cells that are under the inflammation state. The*

195 *production of endogenous H<sub>2</sub>S induced by lipopolysaccharide-mediated inflammation*

196 *was successfully monitored with this H<sub>2</sub>S probe.*



197

198 *Figure4 Cell fluorescence imaging of LPS induced endogenous H<sub>2</sub>S. a. LPS 0 μ M, b.*

199 *LPS 2 μ M, c. Fluorescence intensity analysis.*

200

201 **Discussion**

202 *The main aim of the experiment is to solve the problem of detection of the*  
203 *inflammation of osteoblast, furthermore, we found that H<sub>2</sub>S produced by osteoblast is*  
204 *mainly via CSE-H<sub>2</sub>S pathway. In our study, we proved that our probe can be used in*  
205 *the normal cell to detect the H<sub>2</sub>S changes, which is rarely studied. There are already a*  
206 *lot of fluorescent probes that have been devised to detect intracellular H<sub>2</sub>S levels,*  
207 *however, to our knowledge, most of these probes were successfully applied to show*  
208 *alteration of H<sub>2</sub>S levels of tumor cells or living animals [14; 15; 16]. But in our study,*  
209 *we used a novel fluorescent probe to detect alteration of H<sub>2</sub>S levels in living osteoblast*  
210 *cells with exogenous or endogenous H<sub>2</sub>S for the first time. The H<sub>2</sub>S probe possesses*  
211 *high sensitivity, selectivity, and an ultrafast response to H<sub>2</sub>S, rendering it suitable for*  
212 *detection of H<sub>2</sub>S concentration in living cells. In order to determine whether the cell*  
213 *could translate Cys to H<sub>2</sub>S, and whether the probe could visualize endogenous H<sub>2</sub>S, we*  
214 *treated the osteoblast cells with irreversible inhibitor, PAG. The result proved that*  
215 *H<sub>2</sub>S is produced mainly by CSE-H<sub>2</sub>S pathway, which had not been proved in a visual*  
216 *way before. Other researchers have proved that (CSE) majorly contributed to*  
217 *endogenous H<sub>2</sub>S production in the primary osteoblast by overexpression and*  
218 *knockdown CSE [13]. This is consistent with our results.*

219 *For the inflammation of bone, there are two proved sources of H<sub>2</sub>S: bacteria and*  
220 *macrophage. When inflammation occurs, some bacteria produced and released H<sub>2</sub>S,*  
221 *including various common gram-negative pathogens in osteomyelitis such as*

222 *Escherichia coli*, *Enterococcus faecalis*, *Enterobacter cloacae*, and *Klebsiella*  
223 *Pneumoniae*. For macrophage, research shows that the level of  $H_2S$  was improved and  
224 the expression of CSE mRNA increased because of the stimulate of LPS[17]. Our study  
225 shows that osteoblasts is the third source of  $H_2S$ . Different sources of  $H_2S$  might have  
226 interaction effect, for example,  $H_2S$  production by osteoblast might modulate  
227 macrophage polarization and contribute to bone reparation. Keeping physiological  
228 level of endogenous  $H_2S$  in PDLSCs/periodontal tissue is beneficial to maintain the  
229 homeostasis of periodontal tissue [18]. A appropriated level of  $H_2S$  may play an vital  
230 role in maintaining the homeostasis of the bone marrow system. A previous study have  
231 clarify that BMSCs can produce  $H_2S$ , regulate osteogenic differentiation and cell  
232 self-renewal, and that the lack of  $H_2S$  could lead to defects in their differentiation [19].  
233 Exogenous  $H_2S$  could protect cell injury by regulating oxidative stress, mitochondrial  
234 function, and inflammation. While when inflammation occurs,  $H_2S$  from bacteria  
235 disturbs the endogenous  $H_2S$  of osteoblast cells, leads to a negative effect. In  
236 periodontitis studies, drugs that can release  $H_2S$  have been used for the treatment,  
237 such as ATB-352, a kind of ketoprofen that can releasing  $H_2S$ . The main aim is to  
238 minimize the presence of side-effect at the gastrointestinal tract. Meanwhile they  
239 found that the reduction of the inflammation even had a beneficial effect on bone  
240 resorption or tissue damage. ATB-346, releasing  $H_2S$  like ATB-352, is beneficial for  
241 improving bone quality too [20]. Since  $H_2S$  also can promote the development of  
242 periodontitis, there are still many questions about the biological mechanisms of  $H_2S$ . It  
243 is well-know that there are many kinds of cell playing important roles in periodontitis,

244 such as periodontal ligament stem cells, osteoclasts, and immune cells. Independent  
245 detection of H<sub>2</sub>S changes in living cell might facilitate the study of the role of H<sub>2</sub>S in  
246 diseases.

247 It was found previously that CBS and CSE were both increased in human gingival  
248 tissue during periodontitis through the technology of PCR and Western blot. However,  
249 H<sub>2</sub>S level or H<sub>2</sub>S synthesis in gingivitis and periodontitis was detected not increase  
250 after tissue homogenate [21]. This can be problematic for many reasons, such as the  
251 synthesis capacity decreased or consume increased of H<sub>2</sub>S in inflammation. But as a  
252 gasotransmitter, half of H<sub>2</sub>S can escape from medium in five minutes in tissue culture  
253 wells, which makes it hard to detect [22]. Under physiological conditions, H<sub>2</sub>S presents  
254 in three chemical ionization forms, about 18.5% H<sub>2</sub>S, 81.5% HS<sup>-</sup> and minute quantities  
255 of S<sup>2-</sup> [23]. Different detection methods might lead to different results. H<sub>2</sub>S is more  
256 permeable in plasma membranes, the solubility of H<sub>2</sub>S in lipophilic solvents is  
257 quintuple greater than in water [24], thus, fluorescence probe in theory could detect  
258 H<sub>2</sub>S more precisely. Our H<sub>2</sub>S probe might help deeper studies of the changes of H<sub>2</sub>S  
259 level and promote the progression on the researches about pathogenesis of  
260 periodontitis.

261 Fluorescence techniques is gaining widespread attention as sensors offering excellent  
262 sensitivity, good selectivity, and rapid response to changes. First of all, our probe has  
263 been shown to be sensitive for endogenous H<sub>2</sub>S detection and real-time monitoring of  
264 the changes in H<sub>2</sub>S in living cells, and it reacts quickly under physiological conditions.

265 There are some things that can be improved, for example, a more precise target of

266 probes to certain subcellular organelles, certain cells, tissues, or organs, which may be  
267 achieved by using near-infrared emit to get a greater tissue penetration and minimize  
268 the interference from background auto-fluorescence [25]. For clinical use, H<sub>2</sub>S has a  
269 potential to be used as an appropriate biomarker for the related investigations of  
270 inflammation response. However, it still requires further development.

271

## 272 **Conclusion**

273 In conclusion, it is the first experiment using H<sub>2</sub>S probe to detect H<sub>2</sub>S changes under  
274 stimulation in osteoblast in real time. We used a new hypotoxic H<sub>2</sub>S probe for  
275 exogenous and endogenous H<sub>2</sub>S detection in living osteoblast cells. Moreover, the  
276 results indicate that in osteoblast cells, H<sub>2</sub>S is produced mainly by CSE-H<sub>2</sub>S pathway  
277 directly, it also shows that under inflammation stimulation, endogenous H<sub>2</sub>S  
278 production will increase. The results suggest that H<sub>2</sub>S could be a potential marker for  
279 diagnosis of inflammatory diseases of bone, and it might help further studies for  
280 understanding the synthesis and change of H<sub>2</sub>S level in pathogenesis of periodontal  
281 disease.

282

## 283 **Funding**

284 We gratefully acknowledge financial support from Department of human resources and  
285 social security of Shandong Province (postdoctoral innovated project, 202002050),  
286 China Postdoctoral Science Foundation Grant (2019M652380), Natural Science  
287 Foundation of Shandong Province(ZR2017BH069) Shandong Provincial Medicine and

288 *Health Science and Technology Development Program(2017WS097), the National*  
289 *Natural Science Foundation of China (21777053) and A Project of Shandong Province*  
290 *Higher Educational Youth Innovation Science and Technology Program*  
291 *(2019KJD005).*

292 **Competing interests**

293 *The authors declare that they have no competing interests.*

294

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