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RESEARCH PAPER

Engineering the metal sensitive sites in *Macrolampis* sp2 firefly luciferase and use as a novel bioluminescent ratiometric biosensor for heavy metals

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Abstract Most luminescent biosensors for heavy metals are fluorescent and rely on intensity measurements, whereas a few are ratiometric and rely on spectral changes. Bioluminescent biosensors for heavy metals are less common. Firefly luciferases have been coupled to responsive promoters for mercury and arsenium, and used as *light on* biosensors. Firefly luciferase bioluminescence spectrum is naturally sensitive to heavy metal cations such as zinc and mercury and to pH. Although pH sensitivity of firefly luciferases was shown to be useful for ratiometric estimation of intracellular pH, its potential use for ratiometric estimation of heavy metals was never considered. Using the yellow-emitting *Macrolampis* sp2 firefly luciferase and site-directed mutagenesis, we show that the residues H310 and E354 constitute two critical sites for metal sensitivity that can be engineered to increase sensitivity to zinc, nickel, and mercury. A linear relationship between cation concentration and the ratio of bioluminescence intensities at 550 and 610 nm allowed, for the first time, the ratiometric estimation of heavy metals concentrations down to 0.10 mM, demonstrating the potential applicability of firefly luciferases as enzymatic and intracellular ratiometric metal biosensors.

Keywords Metal binding sites · Luciferases · pH sensitivity · Reporter gene · Metal biosensor · Ratiometric curve

Abbreviations

Mac-H310A	*Macrolampis* sp2 firefly luciferase with substitution of histidine to alanine on residue 310
Mac-H310C	*Macrolampis* sp2 firefly luciferase with substitution of histidine to cysteine on residue 310
Mac-H310C/N354C	*Macrolampis* sp2 firefly luciferase with substitution of histidine to cysteine on residue 310 and asparagine to cysteine on residue 354.
Mac-N354C	*Macrolampis* sp2 firefly luciferase with substitution of asparagine to cysteine on residue 354
Mac-N354E	*Macrolampis* sp2 firefly luciferase with substitution of asparagine to glutamic acid on residue 354
Mac-N354H	*Macrolampis* sp2 firefly luciferase with substitution of asparagine to histidine on residue 354
pMac	*Macrolampis* sp2 firefly luciferase

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Introduction

Bioluminescence, the emission of visible light by living organisms [1], has been used for bioanalytical purposes for a long time. Luciferins and luciferases are used as bioanalytical reagents, and their genes are used as reporter genes for bioimaging and biosensors [2–7].

Bioluminescent biosensors rely on luciferases or photoproteins, enzymes which require oxygen to oxidize the substrate luciferin and produce light in a quantitative fashion. In the enzymatic bioluminescent biosensors, the enzyme is generally immobilized and directly or indirectly used to detect a specific analyte [8, 9], whereas in cell biosensors a luciferase reporter gene is used to transform the cell, and luciferase is quantitatively expressed in the case of *light on* biosensors, or its bioluminescence is inhibited by toxic agents that affect the metabolic state of the cell in *light off* biosensors.

Heavy metal biosensors are especially important owing to increasing concerns about environmental contamination, especially with mercury, cadmium, and lead, and also because some metals such as zinc and copper may display important physiological and pathological fluctuations inside the cells [10–12]. Most luminescent biosensors rely on fluorescence, especially fluorescence intensity. Similarly, bioluminescent biosensors rely on intensity. Bacterial luciferases have been used as *light off* biosensors, whereas firefly luciferases have been usually used in *light on* biosensors which are induced by heavy metals such as mercury and arsenite [9, 13–15]. On the other hand, luminescent biosensors that rely on ratiometric analysis of emission spectra are less common, but have the advantage of being more specific and sensitive.

The bioluminescence spectrum of firefly luciferases is well known to be naturally sensitive to heavy metals and pH, undergoing a large red shift at acidic pH, higher temperatures, and presence of heavy metals [16, 17]. This red shift is not a true bathochromic shift of the spectrum, but a change in the proportion of green and red emitter distribution which may result from induced conformational changes which affect the active site microenvironment around the oxyluciferin emitter, affecting bioluminescence colors [18–21]. However, the specific sites and mechanism responsible for such spectral sensitivity in firefly luciferases are not well understood. Although the firefly luciferase spectral sensitivity to pH and heavy metals has been usually considered a drawback for some bioanalytical applications, it could be potentially useful for ratiometric analysis. We recently showed for the first time that pH sensitivity of *Macrolampis* sp2 firefly luciferase can be harnessed to ratiometrically estimate intracellular pH in bacteria [22].

Using the Brazilian *Macrolampis* sp2 yellow-emitting firefly luciferase and site-directed mutagenesis, we show here that the residues H310 and E354 constitute two important sites for metal sensitivity. This information was used to design new luciferases with increased spectral sensitivities for specific metals, allowing us to ratiometrically estimate the concentrations of nickel, zinc, and mercury. These engineered luciferases offer potential applicability as novel kinds of bioluminescent/ratiometric intracellular metal biosensors using a single firefly luciferase gene.

Materials and methods

Escherichia coli and plasmids

The plasmid pMac (pPro, Invitrogen) harboring the luciferase cDNA from *Macrolampis* sp2 was previously constructed [23]. To construct N-terminal histidine-tagged *Macrolampis* sp2 firefly luciferase expression vector using pCold II vector (Takara, Japan), the luciferase gene was amplified using the following *primers*: Mac-NDE (GCC GCA TAT GGA AGA CGA AAA AAA) and M13-20 (GTA AAA CGA CGG CCAG) and PCR Master mix kit (Promega, USA) to amplify the DNA in a thermal cycler (one cycle at 95 °C for 5 min; 30 cycles at 95 °C for 30 s, 50 °C for 1 min, and 68 °C for 2 min; and 68 °C for 10 min for the final extension). The amplified product was purified by using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and digested with *Xho*I and *Nde*I restriction enzymes. This restriction product was purified and ligated into pCold II previously digested with the same restriction enzymes using DNA Ligation Kit (Takara, Japan) and finally used to transform *E. coli* XL1-Blue cells (Agilent Technologies, USA).

Site-directed mutagenesis

Site-directed mutagenesis was performed using a "QuikChange Site-Directed Mutagenesis" kit (Agilent Technologies, EUA). The plasmids containing the luciferase cDNA were amplified using *Pfu* turbo polymerase and two complementary primers containing the desired mutation, using a thermal cycler (one cycle at 95 °C for 2 min; 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 7 min; and 72 °C for 10 min for the final extension). After amplification, mutated plasmids containing staggered nicks were generated. The products were treated with *Dpn*I to digest nonmutated parental plasmids and used directly to transform *E. coli* XL1-Blue cells (Agilent Technologies, USA). The following primers and their respective reverse complements were used to create the following mutants: Mac-H310C (GTC TAA TTT GTG CGA AAT TGC TTC), Mac-N354C (CAT TAC ACC GTG TGG AGA TGA TAA GC) and Mac-N354H (CAT TAC ACC GCA TGG AGA TGA TAA GC). The mutant Mac-N354E was previously prepared [23], and the double-mutant Mac-H310C/N354C was prepared using the mutated cDNA Mac-H310C as base.

Luciferase expression

For expression of high levels of luciferases, *E. coli* BL21-DE3 cells (Agilent Technologies, USA) were used. Bacteria transformed with *Macrolampis* sp2 firefly luciferase and its mutants were grown in LB medium/ampicillin liquid cultures at 37 °C until $A_{600} \approx 0.40$, and then induced with 1 mM IPTG until $A_{600} \approx 1.5$ at 20 °C.

Luciferase extraction and purification

Bacteria expressing luciferases were harvested by centrifugation at 2500×*g* for 15 min at 4 °C. The resulting pellet was resuspended in extraction buffer (25 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 % Triton X-100, 10 % glycerol, 2 mM DTT, and protease inhibitor cocktail for crude extracts; and 50 mM sodium phosphate buffer pH 7.0, 300 mM NaCl, 10 mM imidazole, 1 mM DTT, and protease inhibitor cocktail for purification), ultrasonicated (Misonix, USA) five times, and finally centrifuged at 15,000×*g* for 15 min at 4 °C. The N-terminal histidine-tagged *Macrolampis* sp2 firefly luciferase expressed from pCold II vector was further purified by agarose-nickel affinity chromatography followed by dialysis. Samples were analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

We compared the effect of cation concentration on the bioluminescence spectra of *Macrolampis* sp2 firefly luciferase in crude extracts and in purified samples. Ni^{2+} spectra did not show any difference between the crude extract and purified enzymes. However, Hg^{2+} and Zn^{2+} displayed a larger red shift on the spectra of the purified enzyme in relation to crude extracts at concentrations of 0.5 mM and 4.0 mM, respectively (from 585 nm to 602 nm for Hg^{2+} and from 580 nm to 584 nm for Zn^{2+}), as the expected result of non-specific competitive binding of these metals by other proteins in the crude extracts (data not shown).

Luminometric assays of in vivo and in vitro bioluminescence

Bioluminescence activities of liquid cultures of *E. coli* expressing beetle luciferases and luciferase in vitro assays were measured using an AB2200 luminometer (ATTO, Japan). For in vivo bioluminescence of cell suspensions, 10 μL of 10 mM D-luciferin in 50 mM sodium citrate buffer pH 5.0 was mixed with 90 μL of culture in the luminometer tube, and intensity was measured in counts per second (cps) for 10 s. For in vitro assays, 80 μL of 0.10 M Tris-HCl buffer pH 8.0 was mixed with 5 μL of 10 mM D-luciferin, 10 μL of luciferase, and 5 μL of a solution containing 40 mM ATP and 80 mM $MgSO_4$. The reported values are the result of at least three independent assays, each one measured in triplicate.

Bioluminescence spectra

Bioluminescence spectra were recorded using an ATTO high sensitivity spectroluminometer with cooled CCD camera (LumiF SpectroCapture AB-1850, ATTO, Japan). For this assay, 10 μL of luciferase was mixed with 90 μL of assay solution (80 μL of 0.10 M Tris-HCl buffer pH 8.0, 5 μL of 10 mM D-luciferin, and 5 μL of a solution containing 40 mM ATP and 80 mM $MgSO_4$) in a transparent microtube. The emission slit was kept at 1 mm, but the integration time and sensitivity were varied according to the luminescence intensity of the sample from 10 s to 5 min. The reported spectra are the result of at least three independent assays.

Effect of divalent metals on bioluminescence spectra

The effect of divalent metals like Zn^{2+}, Ni^{2+}, Hg^{2+}, and Ag^{+} on luciferases activities was measured in the luminometer and their effect on the bioluminescence spectra was measured in the spectroluminometer by adding 0.5–40 μL of the respective salts ($ZnSO_4$, $NiSO_4$, and $AgNO_3$ at 20 mM and $HgCl_2$ at 0.1, 1, and 10 mM) to the assay solution.

To determine the dose/effect of metals on bioluminescence spectra, 10 μL of luciferase was mixed with 50–80 μL of 0.10 M Tris-HCl buffer pH 8.0, 5 μL of 10 mM D-luciferin, 5 μL of a solution containing 40 mM ATP and 80 mM $MgSO_4$ and each salt solution described earlier. For the metal salts $ZnSO_4$, $NiSO_4$, and $AgNO_3$ at 20 mM, the volumes used were 0.5, 2.5, 5, 10, 20, and 40 μL (final concentration of 0.1, 0.5, 1, 2, 4, and 8 mM, respectively); and for $HgCl_2$ at 0.1 mM and 1 mM, the volumes used were 1 and 5 μL (final concentration of 1 μM, 5 μM; 10 μM and 50 μM, respectively); and for $HgCl_2$ at 10 mM, the volumes used were 1, 5, 10, and 20 μL (final concentration of 0.1, 0.5, 1, and 2 mM, respectively). The reported spectra are the result of at least three independent assays.

Ratiometric analysis of bioluminescence spectra

The bioluminescence intensities in the green (I_{green}) and red (I_{red}) were used to calculate the ratio of intensities of bioluminescence spectra (R) at $\lambda_{green} = 563$ nm for green and $\lambda_{red} = 616$ nm for red. Then we plotted the ratio $R = I_{red}/I_{green}$ versus metal concentration. To estimate the spectral sensitivity (S) of the luciferases to the metal concentration, we calculated the ratio between the spectral shift in reciprocal of wavelength (cm^{-1}) and the concentration of the metal.

Results and discussion

Identification of metal sensitive sites in firefly luciferases

The main relevant substitution important for bioluminescence spectra and pH sensitivity that distinguishes *Macrolampis* sp2 firefly luciferase from the closer *Photinus pyralis* luciferase (91 % identity) and the green-emitting *Cratomorphus distinctus* luciferase (83 % identity) is the substitution of the well-conserved E354 by asparagine (E354N) [23]. We previously showed that this natural substitution, E354N, in *Macrolampis* sp2 firefly luciferase is responsible for its characteristic broader and red-shifted bioluminescence spectrum [23]: the mutation N354E narrowed down the spectrum, which became very similar to that of *P. pyralis* that naturally displays glutamate (E354) at this position. Similarly, the reverse mutation E354N in the green-emitting luciferase of *C. distinctus* had the opposite effect, resulting in a broader and red-shifted spectrum. Because the residue E354 may form a salt bridge with H310, we also investigated mutations of the residue H310, showing that the mutation H310A had no effect on the bioluminescence spectrum of *Macrolampis* sp2 firefly luciferase at pH 8.0 [23]. Altogether these results indicate that the residues H310 and E354 are important for pH-sensitivity mediation and bioluminescence colors in firefly luciferases [23]. Therefore, we decided to investigate by site-directed mutagenesis whether the residues H310 and E354 could be also involved in metal sensitivity.

We first compared the effect of Zn^{2+}, Ni^{2+}, and Hg^{2+} on the bioluminescence spectra of the wild-type luciferase and the mutant Mac-N354E (Fig. 1; and Fig. S1 in the Electronic Supplementary Material, ESM). The mutant Mac-N354E displayed a larger red shift in the presence of Zn^{2+}, similar to that observed for the wild-type *C. distinctus* and *P. pyralis* luciferases that naturally display the substitution N354E (Fig. 1). These results indicate that the residue E354 is critical for metal sensitivity in firefly luciferases.

Then, we checked the possible involvement of H310 in metal spectral sensitivity. However, in contrast to the wild-type luciferase, the metal cations did not cause any effect in the bioluminescence spectrum of H310A, with the exception of Hg^{2+} at higher concentration than 0.5 mM, and Ni^{2+} above 2 mM (ESM Figs. S2a and S2b, respectively). These results are consistent with an auxiliary role of H310 in binding metals such as Zn^{2+}.

In order to check for the possible interference of His-tag on the bioluminescence spectra of recombinant luciferases in the presence of nickel, we compared the effect of this metal on the bioluminescence spectra of different constructs of *Macrolampis* sp2 firefly luciferase with (pCold-Mac, pPro-Mac) and without histidine-tags (pBl-Mac). However, no differences could be detected in the peak and shape of the bioluminescence spectra of His-tagged and non-His-tagged luciferases (results not shown).

Design of new metal-sensitive sites in *Macrolampis* sp2 firefly luciferase

Considering that the residues H310 and E354 in firefly luciferases are involved in metal spectral sensitivity, we decided to investigate whether site-directed mutagenesis could be used to engineer more specific and sensitive metal binding sites, by substituting the natural residues by other residues with known metal-chelating properties such as histidines and cysteines. Thus, by using *Macrolampis* sp2 firefly luciferase, we prepared the single mutants Mac-N354H, Mac-N354C, and Mac-H310C, and double mutant Mac-H310C/N354C, and analyzed the effect of Zn^{2+}, Ni^{2+}, and Hg^{2+} on their bioluminescence spectra. These mutations caused only minor effects on the bioluminescence spectra at pH 8.0 (Table 1).

The mutants displayed increased magnitude of the spectral shifts with metals in the following order: (Mac-N354H) $Hg^{2+} > Ni^{2+} > Zn^{2+}$; (Mac-H310C) $Hg^{2+} > Ag^{+} > Zn^{2+} > Ni^{2+}$; (Mac-N354C) $Hg^{2+} > Zn^{2+} > Ag^{+}$; and (Mac-H310C/N354C) $Zn^{2+} > Hg^{2+}$ (Figs. 2, 3, 4, and ESM Fig. S3). Although these results are not simple to rationalize, they in general indicate that the presence of cysteine at position 354 increase the sensitivity to mercury and zinc, which is consistent with the well-known affinity of sulfhydryls for Hg^{2+} ions. Furthermore, because Hg^{2+} and Zn^{2+} display larger van der Waals radia, they are likely to be better accommodated in the space left by the substitution of the larger side chains of H310 and N354 by the smaller side chains of cysteines.

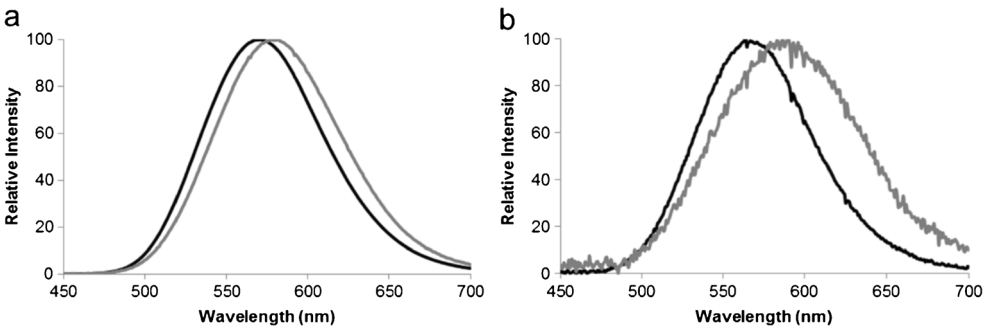

Fig. 1 Red-shift effect of 2 mM $ZnSO_4$ (*gray lines*) on the bioluminescence spectra of *Macrolampis* sp2 (**a**) firefly luciferase and Mac-N354E (**b**) (*black lines*)

Table 1 Spectral sensitivity and effect of metal concentration on the luminescence activity of *Macrolampis* sp2 firefly luciferase and its mutants

Luciferase	λ_{max} (nm)	$ZnSO_4$		$HgCl_2$		$NiSO_4$
		Relative activity[a] (%)	Spectral sensitivity[b] (cm^{-1} mM^{-1})	Relative activity[a] (%)	Spectral sensitivity[b] (cm^{-1} mM^{-1})	Relative activity[a] (%)
Wild type	570	77	454	18	830	90
Mac-N354H	568	20	1080	7	1740	23
Mac-H310C	573	20	1080	11	–	85
Mac-N354C	564	8	10,750	6	7780	72
Mac-H310C/N354C	571	15	1820	4	–	–

The complete table is available in the ESM (Table S1)

[a] Relative activity was measured in presence of metal at 1 mM

[b] Spectral sensitivity was measured as the slope of the effect of metal concentration on the spectral shift from Fig. 5

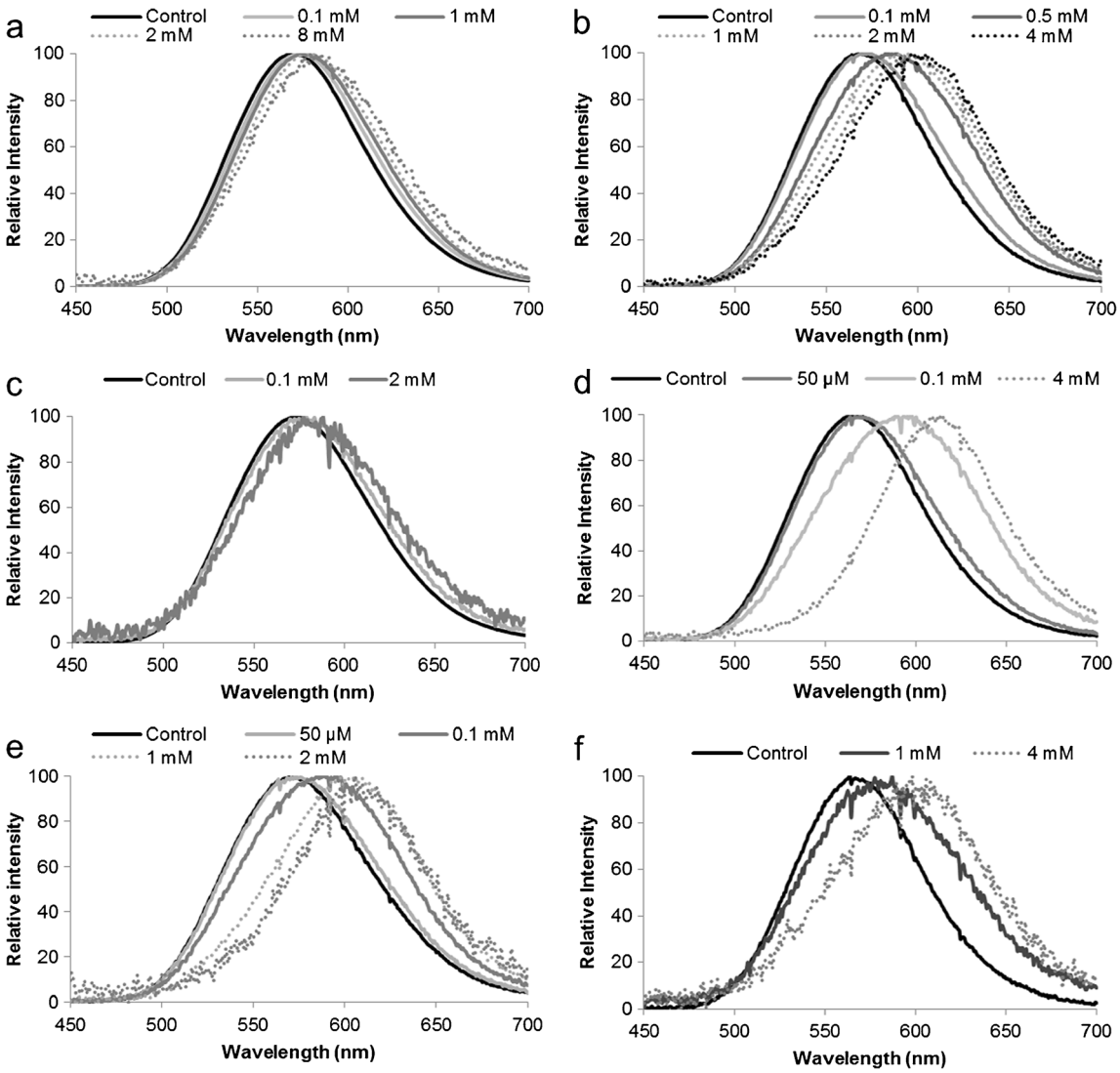

Fig. 2 Red-shift effect of different concentrations of $ZnSO_4$ on the bioluminescence spectra of *Macrolampis* sp2 firefly luciferase and its mutants: wild type (**a**), Mac-N354H (**b**), Mac-H310C (**c**), Mac-N354C (**d**), Mac-H310C/N315C (**e**), and Mac-N354E (**f**)

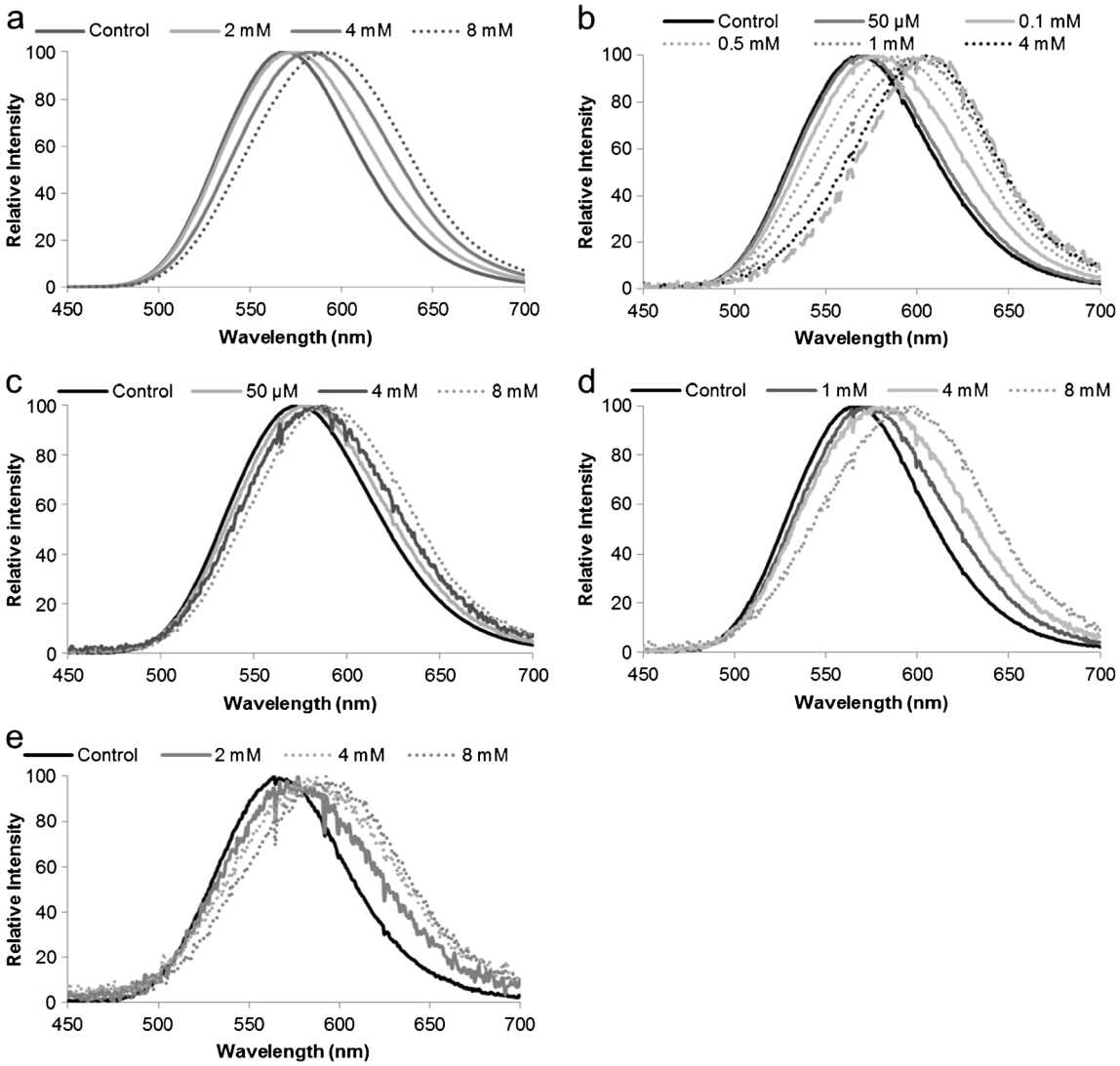

Fig. 3 Red-shift effect of different concentrations of $NiSO_4$ on the bioluminescence spectra of *Macrolampis* sp2 firefly luciferase and its mutants: wild type (**a**), Mac-N354H (**b**), Mac-H310C (**c**), Mac-N354C (**d**), and Mac-N354E (**e**)

Comparing with the wild-type *Macrolampis* sp2 firefly luciferase, the mutants Mac-N354H, Mac-N354C, Mac-N354E, and Mac-H310C/N354C displayed a larger red shift with Zn^{2+} (Fig. 2b, d, f, and e, respectively), whereas the mutation Mac-H310C had no effect (Fig. 2c). The results of Fig. 2b and d clearly show that the mutation of N354 by His or Cys considerably increased the magnitude of the red shift in the presence of Zn^{2+}, supporting the importance of the presence of a chelating group such as sulfhydryl or imidazole at this position. Finally the bioluminescence spectrum of the double mutant Mac-H310C/N354C (Fig. 2e) was considerably affected by Zn^{2+}.

In the case of Ni^{2+}, the mutant Mac-H310C resulted in a small red shift on the spectra (Fig. 3c), whereas the wild-type luciferase, Mac-N354H, Mac-N354C, and Mac-N354E displayed larger red shifts (Fig. 3a, b, d, and e, respectively). Furthermore, the replacement of His by Cys at position 310 reduced the sensitivity to Ni^{2+}. These results are fully consistent with the higher affinity of histidines for Ni^{2+}, and their importance at the positions 310 and 354 for nickel binding in firefly luciferases.

Regarding Hg^{2+}, all luciferases (wild type and mutants) showed large spectral shifts (Fig. 4). The wild-type luciferase showed an increased sensitivity for this cation when compared to Zn^{2+} and Ni^{2+}. The mutant Mac-N354C displayed the largest effect (Fig. 4d). Among the mutants, however, substitution of H310C and the double mutant Mac-H310C/N354C decreased the magnitude of the red shift (Fig. 4c and e, respectively), whereas the mutants Mac-N354H and Mac-N354C increased the sensitivity to this metal (Fig. 4b and d, respectively). These results indicate the major importance of position 354 for binding mercury.

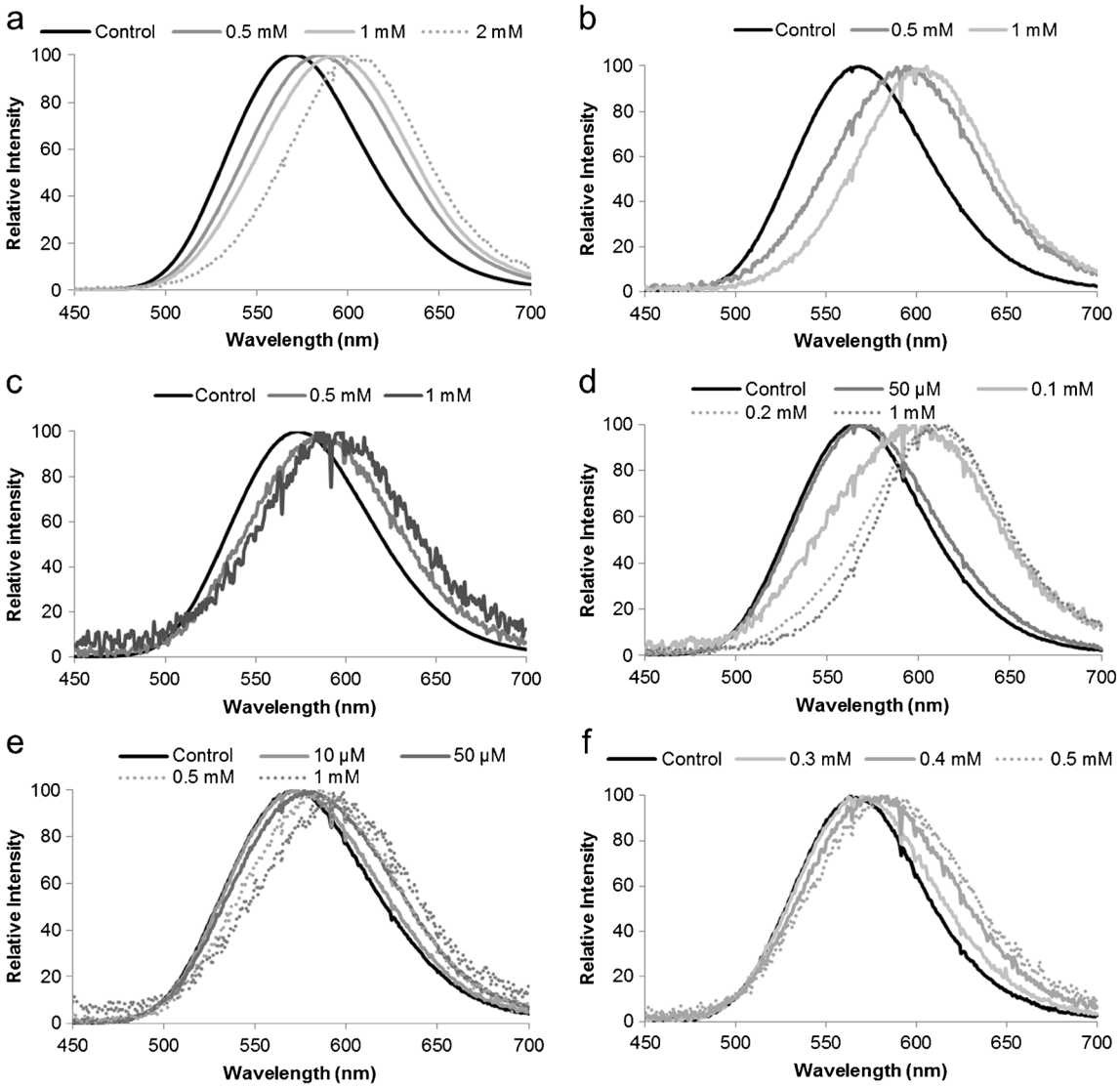

Fig. 4 Red-shift effect of different concentrations of $HgCl_2$ on the bioluminescence spectra of *Macrolampis* sp2 firefly luciferase and its mutants: wild type (**a**), Mac-N354H (**b**), Mac-H310C (**c**), Mac-N354C (**d**), Mac-H310C/N354C (**e**), and Mac-N354E (**f**)

When we compared the substitutions N354H and N354C with the natural substitution N354E (glutamate is conserved in firefly luciferases with the exception of *Macrolampis* sp2), the mutant Mac-N354E showed a smaller red shift for Hg^{2+} and Zn^{2+} comparing with Mac-N354C, but similar to Mac-N354H, which is consistent with the high affinity of imidazole and sulfhydryl groups for these cations.

Altogether these results confirm the importance of negatively charged glutamate at position 354, and other chelating residues such as cysteines and histidines at position 310 and 354 for sensitivity to divalent metals, especially histidines. Among all these mutants, the most sensitive to these metals are those at position 354 which include a cysteine (for Hg^{2+} and Zn^{2+}), a histidine (for Ni^{2+} and Zn^{2+}), or a glutamate (for Zn^{2+}); Mac-N354C is the most sensitive for Hg^{2+}, causing a larger red shift at lower concentrations.

Spectral sensitivity to the metal

The spectral sensitivity of the bioluminescence to metals can be regarded as a function of the magnitude of the shift in relation to the concentration of the metal: the larger the red shift and the smaller the metal concentration, the larger the spectral sensitivity to the metal. Therefore we used as a sensitivity parameter (S) the ratio of the spectral shift in reciprocal of wavelength ΔF (cm^{-1}) and the concentration of the metal (mM). Figure 5 and Table 1 summarize the sensitivity of luciferase mutants to different metals. Among all mutants, N354C displayed the highest sensitivity to both Zn^{2+} and Hg^{2+} (Figs. 2d and 4d), with the lowest detection limits (<100 µM), whereas the mutant Mac-N354H was the most sensitive for Ni^{2+} (Fig. 3b).

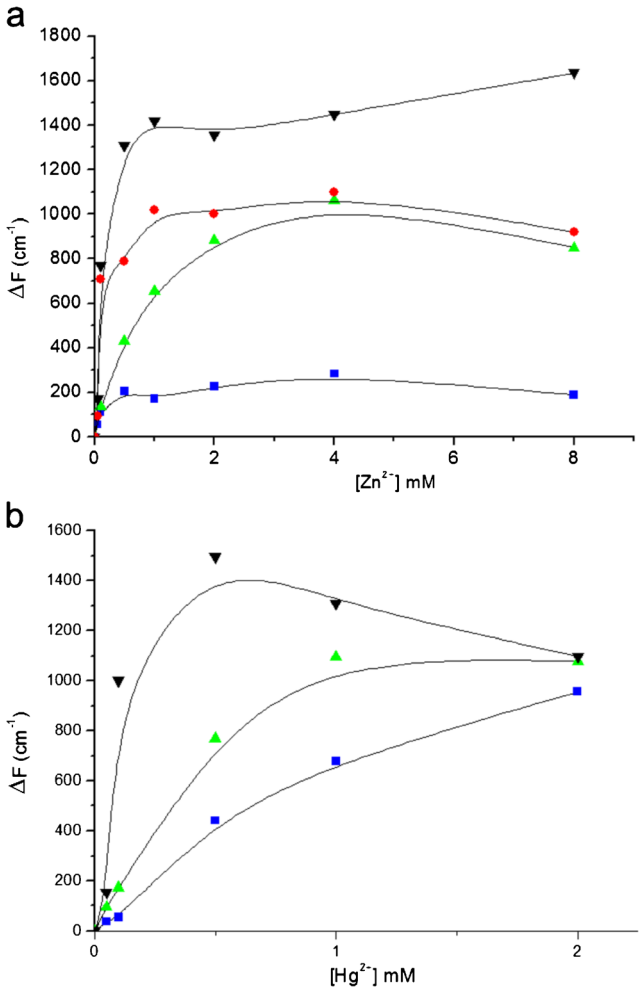

Fig. 5 Effect of metal concentration (mM) on the spectral shift in reciprocal of wavelength ΔF (cm^{-1}); *Macrolampis* sp2 (*blue*), Mac-N354H (*green*), Mac-H310C/N354C (*red*), and Mac-N354C (*black*)

Furthermore, it is desirable that the luminescence activity of the luciferase is not severely affected by the concentration of the metal. Among the tested cations, Zn^{2+} caused the largest red shift (26 nm for Mac-N354C and 34 nm for Mac-H310C/N354C) with the lowest impact on the bioluminescence activity at the concentration of 0.1 mM (ca. 10 % for wild type and 40 % for Mac-N354H), whereas Ni^{2+} displayed the lowest impact on bioluminescence activity (ca. 20 % for wild type and 30 % for Mac-H310C) at high concentrations (2 to 4 mM), and Hg^{2+} was the metal with highest impact on the activity (ca. 70 % for Mac H310C and Mac-H310C/N354C, 85 % for Mac-N354H, and 90 % for Mac-N354C) at low concentration (0.5 mM).

Ratiometric measurement of [Me^{2+}]

Therefore we analyzed the relationship between cation concentration and the ratio of intensities of bioluminescence spectra ($R = I_{red}/I_{green}$) in the red and green region for *Macrolampis* sp2 luciferase ($\lambda_{red} = 616$ nm; $\lambda_{green} = 563$ nm) and their mutants in order to find out whether a linear relationship could be used to estimate the concentration of certain metals in living cells.

As expected, a linear relationship between the ratio of intensities (R) and the concentration of Zn^{2+}, Ni^{2+}, Hg^{2+}, and Ag^{+} was found using different luciferases (Figs. 6, 7, 8, and ESM Figs. S4, S5, and S6).

In the more sensitive mutants, the curve of R versus the metal concentration is more steep, and extended to lower concentrations giving the detection limit of the ratiometric analysis (minimal concentration that results in a measurable spectral shift). Among the luciferase mutants, Mac-N354C, besides being the more sensitive mutant, also displayed the lowest detection limit for Zn^{2+} and Hg^{2+} (<0.1 mM), whereas the mutant Mac-N354H displayed the lowest detection limit for Ni^{2+} (<0.1 mM).

Comparison of firefly luciferases as ratiometric biosensors with other metal biosensors

Luminescent biosensors for metals can be divided into fluorescent and chemiluminescent or bioluminescent. Most of the currently used luminescent biosensors are fluorescent. The fluorescent biosensors can be further divided into those based on intensity, in which the intensity at a single wavelength increases or decreases in response to metal concentration, and ratiometric biosensors, in which there are spectral changes that can be quantified by the ratio of intensities at different wavelengths.

Most of the currently used fluorescent biosensors are based on fluorescence intensity which is linearly responsive to increasing concentrations of metal. Mercury sensors are among the most important because of the toxicity of this metal (for reviews, see [24, 25]), whereas zinc sensors that use fluorescent proteins to estimate intracellular concentrations are also important to investigate cell homeostasis (for a review, see [26]). These kind of fluorescent sensors have been used to detect Zn^{2+} in water samples and living cells in the range from 0.5 to 10 μM for Zn^{2+} [27–30] and Hg^{2+} in the range from 2 to 20 μM [31–38], displaying specificity for these metals and being used for cellular detection.

On the other hand, ratiometric fluorescent biosensors are less common, but they have advantages such as specificity and selectivity for the metals. Whereas the fluorescence intensity is sensitive to the actual concentration of the fluorophore, and self-absorption variations, the ratio of intensities at different wavelengths is insensitive to fluorophore concentration, reflecting only the actual concentration of the analyte (the metal). A ratiometric fluorescent sensor for detection of Cu^{2+} was developed, in which increasing concentrations of Cu^{2+} decrease the ratio of fluorescence intensity at 470 nm and 355 nm (I_{470}/I_{355}) [39]. Other researchers developed a

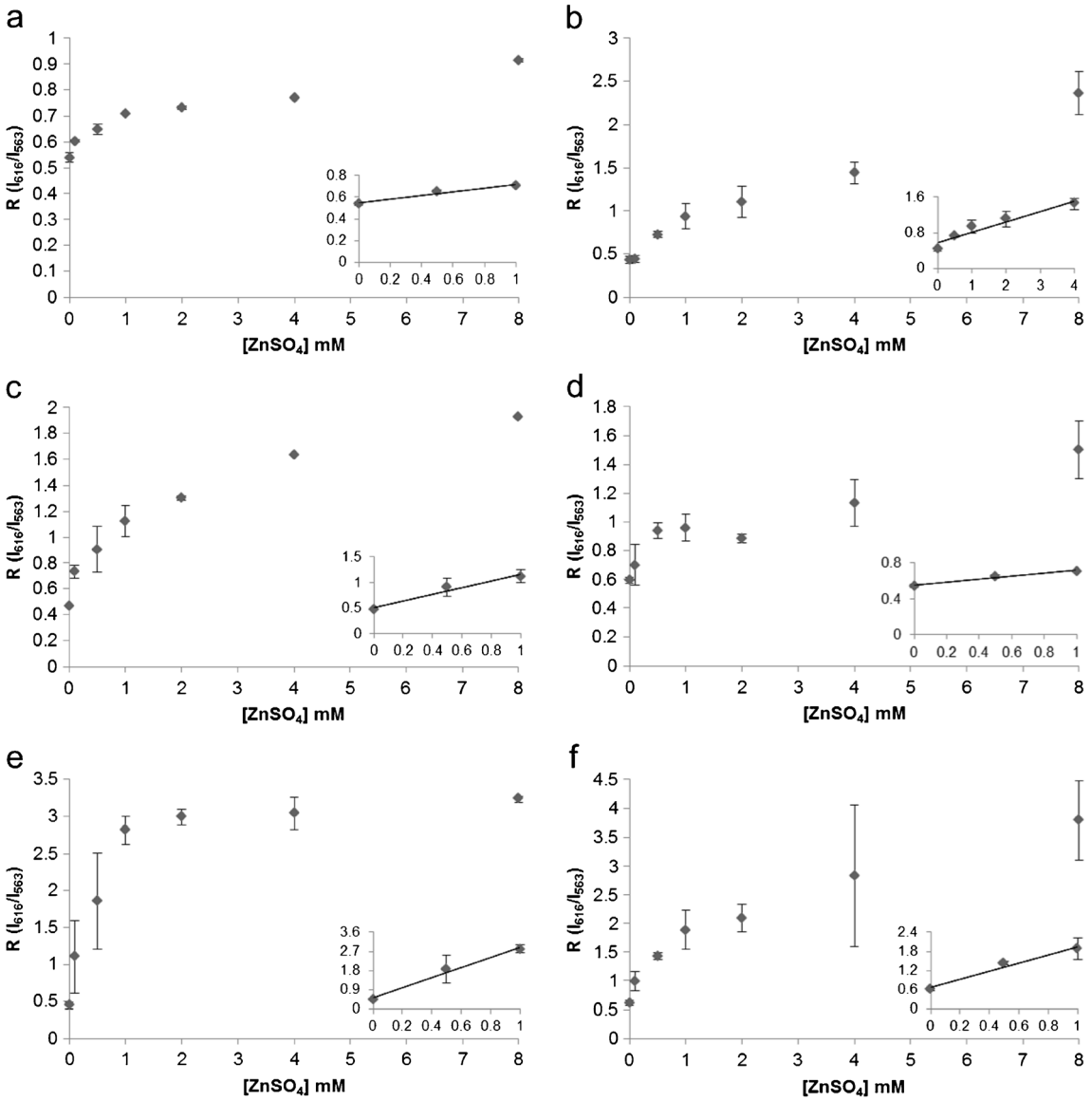

Fig. 6 Effect of $ZnSO_4$ concentration on the ratio of bioluminescence intensities at λ_{green} and λ_{red} ($R = I_{red}/I_{green}$) using the engineered *Macrolampis* sp2 firefly luciferase: wild type (**a**), Mac-N354E (**b**), Mac-N354H (**c**), Mac-H310C (**d**), Mac-N354C (**e**), and Mac-H310C/N354C (**f**). *Inset* linear range of the curve

fluorescent ratiometric sensor for Zn^{2+} based on bicarboxamidoquinoline with high selectivity, using the ratio of intensities at the wavelengths of 410 nm and 500 nm (I_{500}/I_{410}) [40]. A group of ratiometric fluorescent sensors to detect different kinds of metals consists of small fluorescent molecules with spectroscopic responses to metals such as Zn^{2+}, Hg^{2+}, and Pb^{2+}, using the intramolecular charge transfer (ICT) mechanism in which the intramolecular conjugation of the metal results in a change of the fluorescence intensity and shift of the emission wavelength. This fluorescent sensor displays different responses to different metal ions, and one fluorescence signature is assigned to each metal [41]. Despite being very sensitive, most fluorescent biosensors are not specific to one metal.

Similarly to fluorescent biosensors, bioluminescent biosensors are usually based on luminescence intensity measurements, being divided into *light off* and *light on* biosensors. Non-specific *light off* biosensors based on bacterial bioluminescence have long been used to detect heavy metals, whereas *light on* bioluminescent biosensors based on *lux-CDABE* and firefly luciferase were developed to detect the bioavailable Hg^{2+} based on the increase of the bioluminescence intensity [42, 43].

However, the use of ratiometric bioluminescent biosensors, with the exception of bioluminescence resonance energy transfer (BRET) systems which employ a bioluminescent donor protein (luciferase or photoprotein) and a fluorescent acceptor like GFP, was not reported until very recently. Recently,

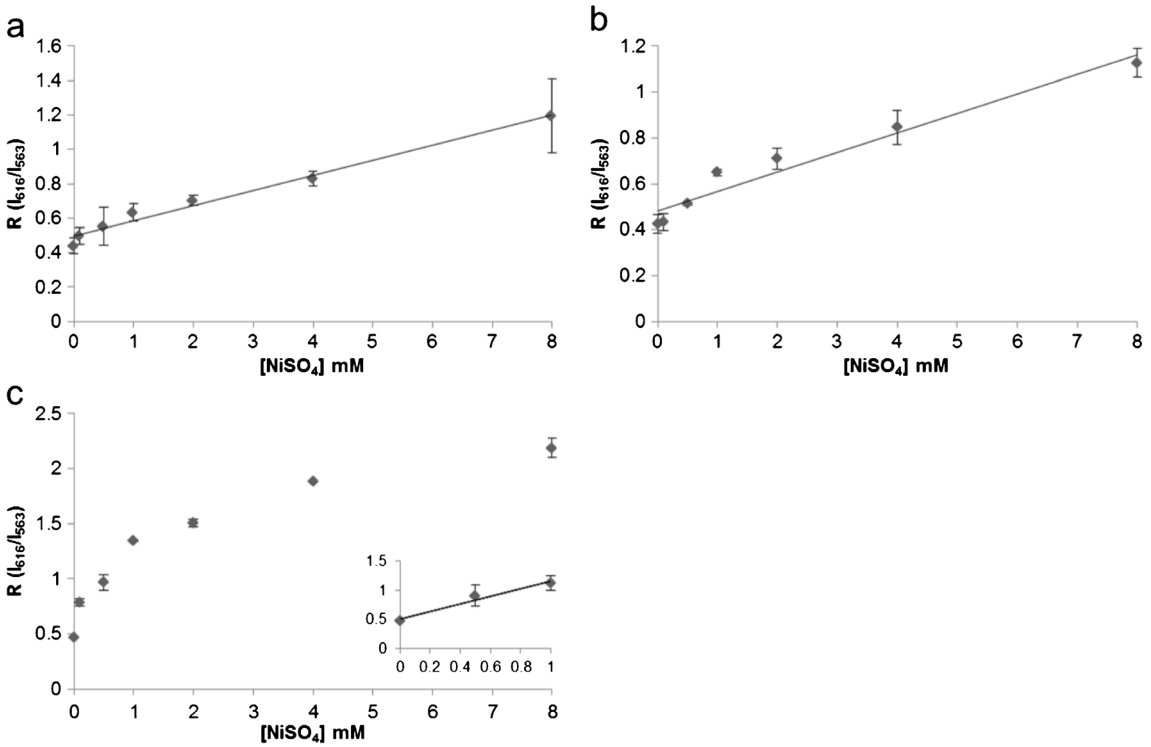

Fig. 7 Effect of $NiSO_4$ concentration on the ratio of bioluminescence intensities at λ_{green} and λ_{red} ($R = I_{red}/I_{green}$) using the engineered *Macrolampis* sp2 firefly luciferase: Mac-N354C (**a**), Mac-N354E (**b**), and Mac-N354H (**c**). *Inset* in **c**: linear range of the curve

a bioluminescent indicator of pH in living cells based on a complementation of fragments of firefly luciferase and a photoreactive protein to visualize pH change was developed [44]. We recently reported the first intracellular pH-ratiometric biosensor based on the spectral pH-sensitivity using a single firefly luciferase [22]. Therefore, on the basis of the same principle of spectral sensitivity of firefly luciferases to pH, we provide here the second case of a ratiometric bioluminescent biosensor using a single firefly luciferase, and the first case of ratiometric bioluminescent biosensor specifically aimed to detect heavy metals.

Unlike fluorescent and BRET-based ratiometric biosensors, this luciferase bioluminescent ratiometric biosensor has the advantage of using a single luciferase gene, being more reliable, and avoiding problems associated with the complexity of two protein interactions found in BRET systems; moreover, it avoids the need for light irradiation, eliminating problems such as autofluorescence, self-absorption, or phototoxicity that characterize fluorescent sensors, generating its own light in a very specific manner. Also, the ratiometric bioluminescent biosensor eliminates problems associated with changes in luciferase expression and its inactivation, which are usually drawbacks in luminescent biosensors based on intensity. Furthermore, as a result of the instability of firefly luciferases, it has the potential advantage to be used in real-time imaging.

However, the sensitivity of this ratiometric approach is still lower than usual fluorescent biosensors. The most sensitive mutant of this novel firefly luciferase-based biosensor was Mac-N354C, which has a detection limit slightly below 0.1 mM for Zn^{2+} and Hg^{2+}.

Comparatively, the fluorescent ratiometric sensor for Zn^{2+} based on bicarboxamidoquinoline described by Tian et al. [40] has a lower detection limit of 0.05 mM, whereas the label-free fluorescent DNA-based sensor for Hg^{2+} can detect concentrations lower than 0.01 mM [41]. Fluorescent biosensors are also in general more specific to different heavy metals. Although the lower sensitivity and specificity of our luciferase-based biosensor limit its potential applications in the current state of the art, it could be useful to quickly detect the presence of heavy metals in a sample using a single luciferase, based on ratiometric measurements, avoiding the need to use several fluorescent biosensors to measure different metals.

Because the estimated intracellular concentration of zinc in human cells is around 200–300 μM [45], it is in principle possible to use the mutants like Mac-N354C or H310C/N354C, which have a detection limit below 100 μM, to detect large magnitude concentration fluctuations inside these cells. However, because the free Zn^{2+} in the cytosol of several other cells is estimated to be around 0.1–1 nM [26], our biosensing approach needs further improvement of the enzyme to reach such a detection limit.

Concentrations of 100 μM of $HgCl_2$ in mammalian cells are toxic and affect the cell division, whereas lower concentrations above 10 μM $HgCl_2$ were found to cause threefold

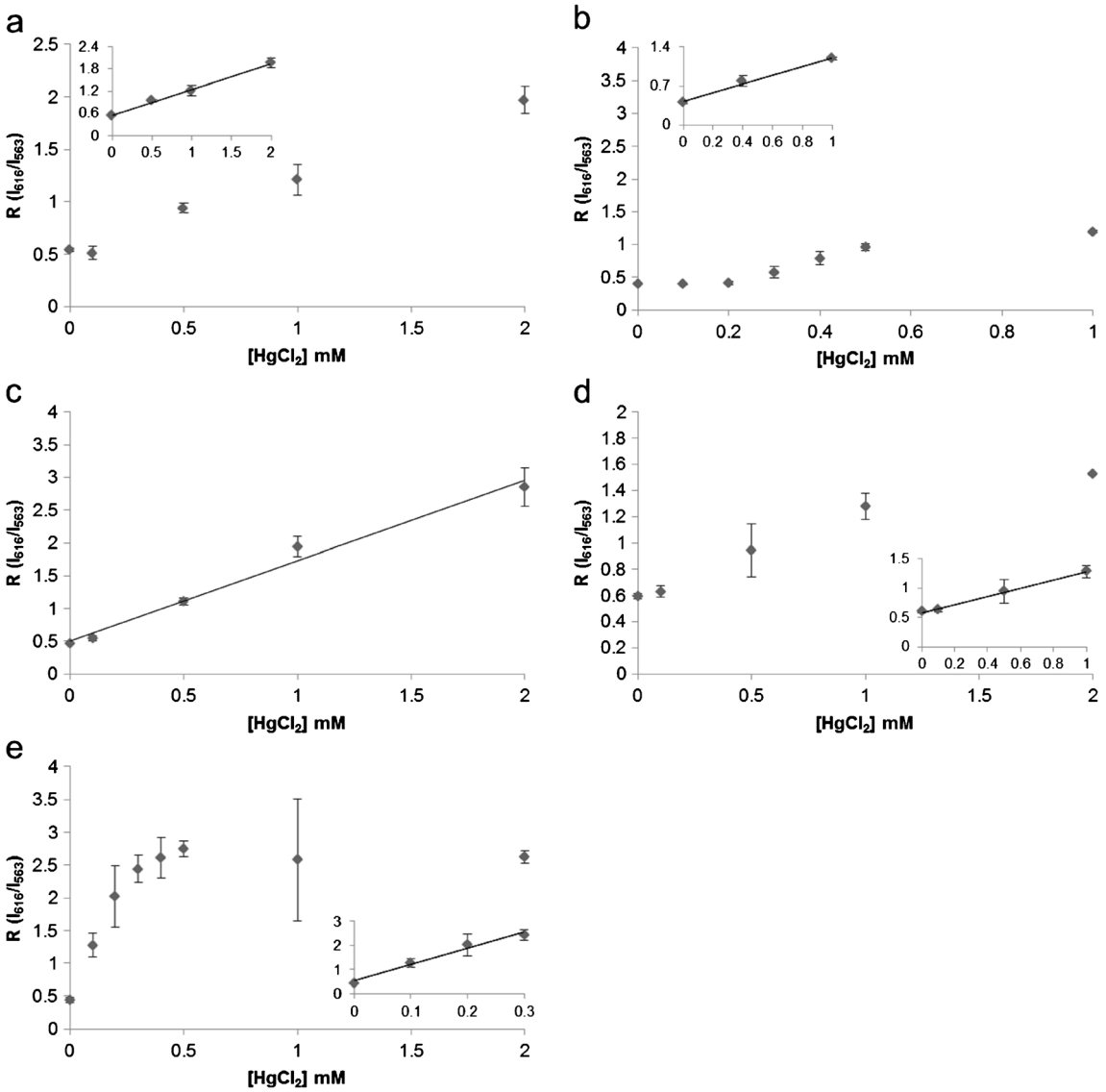

Fig. 8 Effect of $HgCl_2$ concentration on the ratio of bioluminescence intensities at λ_{green} and λ_{red} ($R = I_{red}/I_{green}$) using the engineered *Macrolampis* sp2 firefly luciferase: wild type (**a**), Mac-N354E (**b**), Mac-N354H (**c**), Mac-H310C (**d**), and Mac-N354C (**e**). *Inset* in **a**, **b**, **d**, and **e**: linear range of the curve

more abnormalities/aberrations like polycentrism of the chromosome and chromatid breakage [46]. However, to estimate the bioavailability of this metal inside cells is not easy. Among our firefly luciferase mutants, Mac-N354C was the most sensitive to Hg^{2+}, displaying a detection limit just below 100 μM.

Therefore, although in the current state this firefly luciferase-based ratiometric approach to estimate heavy metals has the drawbacks of lower sensitivity and specificity, it offers potential interest for intracellular biosensing. The main analytical advantage is that it uses a single firefly luciferase gene, avoiding problems such as autofluorescence, self-absorption, and photoxicity associated with fluorescent sensors, or complex protein interactions associated with BRET systems. Furthermore, this single luciferase-based biosensor offers the potential advantage of reporting in two dimensions in real-time analysis: (1) the intensity dimension that could be used to report location, gene expression, or ATP content; and (2) the spectral dimension (ratiometric analysis) reporting metal fluctuations inside cells. Further investigations are necessary to analyze the specificity of this luciferase-based biosensor for other heavy metals such as lead and cadmium, and to improve its sensitivity, specificity, and therefore the feasibility of application as ratiometric biosensor of metals inside cells.

Conclusion

Here we showed that the residues H310 and E354 constitute two critical metal-sensitive sites responsible for bioluminescence spectral sensitivity of firefly luciferases. By engineering these sites using site-directed mutagenesis, we increased the

sensitivity of the bioluminescence spectra to metals such as Zn^{2+}, Ni^{2+}, and Hg^{2+}. The linear ratiometric response of these engineered luciferases at two different wavelengths (green and red) allowed us to determine the concentration of metals, demonstrating the potential applicability of firefly luciferases and their genes as intracellular bioluminescent biosensors for heavy metal contamination and intoxication.

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Compliance with ethical standards

Conflict of interest A patent (Brazilian patent PI0604475-1 A2, 2006) using *Macrolampis* sp2 firefly luciferase spectral sensitivity to detect metal has been applied for. No other conflict of interest is found.

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