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Theoretical analysis of the color tuning mechanism of oxyluciferin and 5-hydroxyoxyluciferin

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ABSTRACT

Firefly luciferase exhibits a multicolor bioluminescence, which is caused by the modulation of the emission of oxyluciferin by intermolecular interactions. One of the objectives of the present paper was analyze the possible effect of the charge density of the emitter in the color tuning. Theoretical calculations on oxyluciferin, and its respective moieties, demonstrated that no correlation between charge density and light emission can be found once solvent effects are considered. Further computational calculations demonstrate that intermolecular interactions modulate the emission by affecting the geometry of oxyluciferin, which controls the energy gap between the excited and ground state. Direct intermolecular interactions and polarity also affect the color of emission of oxyluciferin, by the same mechanism. Also, the effect of charge density modulation of Keto-(−1). A novel emitter, 5-hydroxyoxyluciferin, was considered and demonstrated to be more red-shifted that oxyluciferin.

1. Introduction

Bioluminescence is a phenomenon that occurs in living organisms in which an excited state substance is produced in an enzyme-catalyzed chemical reaction [1]. This product decays to the ground level, by emitting light. Bioluminescence is widespread in nature and has roles in sexual communication, appealing to prey and disguising. The most studied bioluminescence system is that of the North-American firefly, *Photinus pyralis* [1]. This type of insect uses an enzyme, firefly luciferase (Luc, EC 1.13.12.7), to catalyze the formation of the light emitter oxyluciferin ($OxyLH_2$) [1]. This formation occurs in a two-step reaction: in the first step, Luc catalyzes the reaction between firefly luciferin (LH_2) and adenosine-5′-triphosphate (ATP), which results in the formation of an adenylyl intermediate; in the second step, this intermediate is oxidized into $OxyLH_2$. This latter molecule is very important in this system, as besides being the light emitter, it appears to be one of the substances responsible for the flash-profile of light emitted [2–5]. The flash-profile is a term used to define the *in vitro* pattern of light emission. The *in vitro* emission of light follows, under well-defined conditions a flash pattern, with a rise in the intensity of emission that decays to low levels in a few seconds. This profile is attributed to the formation of inhibitory products, one of which is $OxyLH_2$ (competitive inhibitor of LH_2) [2–5]. In recent years, the existent bioluminescence systems have gained numerous biomedical, bioanalytical and pharmaceutical applications, among others. More specifically, it is involved in the analytical determination of adenosine-5′-triphosphate (ATP), in microbial detection, biosensing, bioimaging and is used as a gene reporter [1,6–10].

One of the more interesting and relevant characteristic of the firefly bioluminescence is its multicolor variation [1,11]. Luc is a pH-sensitive enzyme, which affects the color of the emitted light. At basic pH (pH ~7.5), $OxyLH_2$ emission has a peak at 562 nm. At acid pH (~5–6), the emission shifts to the red region of the visible spectrum (maximum at 620 nm) [11]. The understating of this feature could be of incredible importance in bioluminescence research. Lower energy emitting Luc could be used in *in vivo* medical imaging, as red light is absorbed very poorly in comparison with natural emitted light. Viviani et al. also hypothesized that the control of the multicolor bioluminescence could be used in the development of Luc as a single dual reporter gene, as a bioindicator of cellular stress and as a probe for intracellular changes of pH [12].

Numerous authors have tried to explain the color tuning mechanism. These researchers considered keto-enol tautomerism [13], rotation of the C–C bond between the benzothiazole and thiazolone rings [14], control of the resonance form of $OxyLH_2$ [15], interaction of the light emitter with a protonated basic moiety present in Luc active site [16], and the polarity and rigidity of the active site [17]. More information, regarding these and other studies, could be encountered in these four very recent reviews [11,18–20]. For the accurate study of the firefly bioluminescence is required the identification of the bioluminophore. However, the identification of Keto-(−1) as the light emitter (Fig. 1) was only achieved recently

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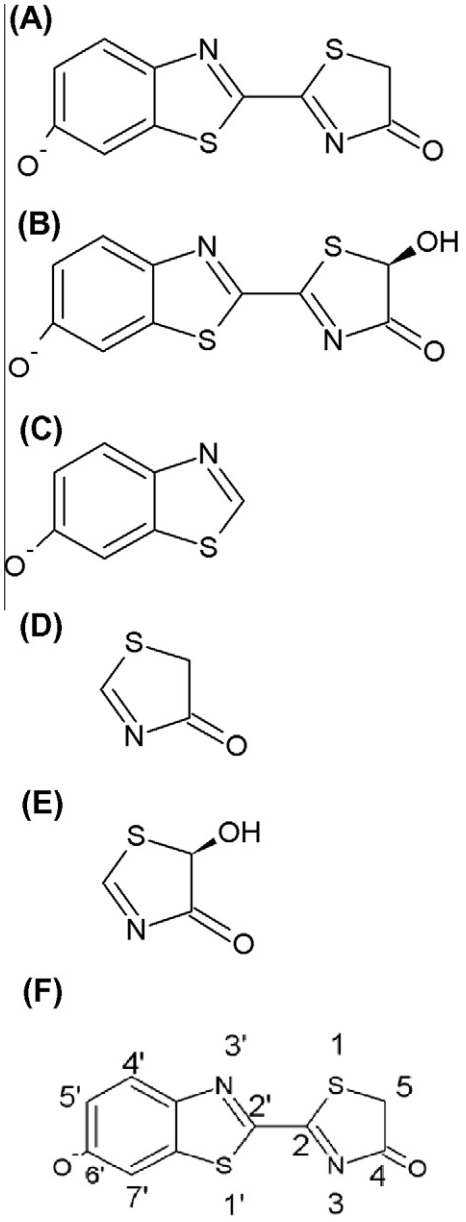

Fig. 1. Chemical structure of Keto-(−1) (A), HOxyLH2 (B), Keto-(−1) and HOxyLH2 benzothiazole moieties (C), Keto-(−1) (D) and HOxyLH2 (E) thiazolone moieties (F) indicates the numbering of the atoms of Keto-(−1).

by three different computational approaches: analysis of the dissociation and tautomeric constants of $OxyLH_2$ as a function of pH [21]; study of the direct excited-state product of firefly dioxetanone [22]; study of the possibility of keto-enol tautomerism in *Luciola cruciata* Luc (LcLuc) active site [23].

Theoretical calculations on Keto-(−1) complexed with small molecules revealed that intermolecular interactions have a very significant effect in the color tuning mechanism, by originating different emission wavelengths for the same luminophore [21,24–26]. The key importance of intermolecular interactions was further demonstrated by computational studies of the light emission of Keto-(−1) in both Luc and LcLuc active sites [23,27–31]. Overall, it is thought that the most important intermolecular interactions are electrostatic, hydrogen-bonding and π–π stacking. Furthermore, the color of bioluminescence is also modulated by the polarity of the microenvironment [16,17,24–27,30,32]. Therefore, the effect of active site molecules in the emission maxima of Keto-(−1) is fairly clarified. However, in order to rationally tune the color of bioluminescence we need to understand why intermolecular interactions have this effect on the excited state of the luminophore. Several groups have proposed that shifts in the emission are controlled by modulation of the charge density of the emitter, but no conclusive proof of this has been presented so far [15,23,27,28,30].

The objective of the computational study is the further clarification of the color tuning mechanism. To this end we have studied the first excited state of Keto-(−1) and of its benzothiazole and thiazolone moieties, separately (Fig. 1), both *in vacuo* and in solution (explicit and explicit/implicit water). Within this work, we intend to study the delocalization of charge within the moieties of the emitter and its effect on light emission. Also if intermolecular interactions modulate the color of emission by modifying the charge density of the emitter, it is reasonable to assume that the same effect can be achieved by altering the chemical structure of $OxyLH_2$. Therefore, to prove this assumption we have studied (*in vacuo*, and in explicit and explicit/implicit water) the photophysical properties of the first excited state of a novel emitter, 5-hydroxyOxyLH$_2$ ($HOxyLH_2$) and its separate moieties (Fig. 1). With these calculations, we could compare the properties of these molecules that caused their different wavelength maxima. However, our calculations demonstrated that charge density modulation cannot explain the color tuning mechanism. Further calculations on the ground and excited state of Keto-(−1) and $HOxyLH_2$, revealed that intermolecular interactions modulate the emission by modifying the geometry of the emitter and by direct interaction. These two factors can then red-shift the emission by decreasing the energy gap between the emitting excited state and the ground state. The polarity of the microenvironment modulates the color of emission by the same mechanism.

2. Computational methods

The initial structures of Keto-(−1) and $HOxyLH_2$ were constructed with the software Avogadro [33]. An initial guess was obtained by using Avogadro geometry optimization function, and subsequent ground state optimizations were performed by using the Density Functional Theory (DFT) functional O3LYP [34–36] and the 6-31+G(d,p) basis set, with no solvent effects. The excited state geometries were obtained by using configuration interaction with single excitations (CIS) [37] and the 6-31G(d) basis set. The first excited state refers to a fluorescence state (π–π^* excitation). We have used the CIS method for excited state geometry optimizations as other authors have stated that it can be compared with methods of higher levels of theory [28]. The separated moieties were obtained by substitution of the other moiety with a hydrogen atom, with the software Avogadro. This approach was adopted by the fact that our objective was to study the effect of charge transfer in the color tuning mechanism. So, with this approach there are not changes in geometry between a sole moiety and its corresponding moiety in the full emitter, which could influence the emission of the studied molecules. The emission energies of the emitters were computed at the time-dependent (TD) [38,39] O3LYP/6-31+G(d,p) level of theory, with no solvent effects. Despite some critics to the use of TD-DFT in the study of the multicolor bioluminescence, due to errors regarding charge transfer (CT) states [40,41] the work of another authors demonstrated that CT is small on planar $OxyLH_2$, devaluing this flaw of TD-DFT [42]. Moreover this functional provided good results for dehydroluciferin, an $OxyLH_2$ analogue [43].

To study the effect of intermolecular interactions in the emission of Keto-(−1), $HOxyLH_2$ and respective moieties, molecular mechanics methods were used. TIP3P water molecules were added

up to 12 Å by the LEAP module of the AMBER 11 suite of programs [44]. The excited state CIS/6-31G(d) geometries of the emitters were used in their parameterization with the ANTECHAMBER module of AMBER and the general AMBER force field [45]. One phase of energy minimizations (30,000 steps) was performed, by using the Not (just) Another Molecular Dynamics program (NAMD) molecular dynamic code with AMBER potential functions, parameters and file formats [46]. In this process, the Particle Mesh Ewals method was used to include the long-range interactions [47]. All the minimization steps were performed in a NVT ensemble, with a temperature of 298.15 K. In the end of the minimizations, the studied molecules and the water molecules up to 3 Å were withdrawn from the six final structures. These models were then subjected to emission energies calculation, at the TD-O3LYP/6-31+G(d) level of theory. The basis set used in these calculations was reduced, due to the high number of atoms involved. Some calculations were performed with implicit solvation, by using the conductor-like polarized continuum model (CPCM) [48]. The dielectric constant used was that of water. All DFT/TD-DFT/CIS calculations were carried out with the Gaussian 03 program package [49].

3. Results and discussion

The emission wavelength and the oscillator strength of Keto-(−1), $HOxyLH_2$ and respective moieties are indicated in Table 1. In Table 2 are indicated the atomic Mulliken charge of the benzothiazole and the thiazolone moieties of the emitters. Analysis of Table 1 shows that *in vacuo* Keto-(−1) and $HOxyLH_2$ are green emitters. The benzothiazole moieties of Keto-(−1) and $HOxyLH_2$ are violet emitters, while theirs thiazolone moieties emit outside of the visible spectrum.

Analysis of Tables 1 and 2 indicates that charge density modulation may explain the multicolor light emission. In the case of Keto-(−1) and its benzothiazole moiety, there is effectively a decrease of the negative charge in this moiety with increasing emission wavelength (405–504 nm). There is also an increase in the negative charge of the thiazolone moiety of Keto-(−1) with increasing wavelength maxima (287–504 nm). It should be noted that the negative charge of the sole benzothiazole moiety is much higher than in Keto-(−1). The inverse is true for the sole thiazolone moiety, which negative charge is lower than in Keto-(−1). This indicate that, when in conjunction by forming Keto-(−1), there is a delocalization of the negative charge from the benzothiazole to the thiazolone moiety. This delocalization may then be responsible for the higher wavelength maximum observed for the bioluminescence emitter, $OxyLH_2$. In the case of $HOxyLH_2$, a higher negative charge is also seen in its sole benzothiazole moiety (386 nm) than in its full form (510 nm). For the thiazolone moiety, an increase in the negative charge is accompanied by an increase in the emission wavelength (245–510 nm).

Table 1
Emission wavelength (λ_{em}, in nm) and oscillator strength (f) of Keto-(−1), $HOxyLH_2$ and their respective moieties.

	TD-O3LYP/6-31+G(d,p)	
	λ_{em}	F
Keto-(−1)	504	0.5442
Keto-(−1) benzothiazole moiety	405	0.0469
Keto-(−1) thiazolone moiety	287	0.0836
$HOxyLH_2$	510	0.5452
$HOxyLH_2$ benzothiazole moiety	386	0.2290
$HOxyLH_2$ thiazolone moiety	245	0.0510

Table 2
Atomic Mulliken charges of Keto-(−1), $HOxyLH_2$ and their respective moieties.

	TD-O3LYP/6-31+G(d,p)	
	Benzothiazole moiety	Thiazolone moiety
Keto-(−1)	−0.691	−0.311
Keto-(−1) benzothiazole moiety	−1.139	
Keto-(−1) thiazolone moiety	−0.199	
$HOxyLH_2$	−0.679	−0.322
$HOxyLH_2$ benzothiazole moiety	−1.139	
$HOxyLH_2$ thiazolone moiety	−0.205	

Having studied these six emitters *in vacuo*, we have passed to the study of the effect exerted by intermolecular interactions in the emission of these molecules. This type of study can be used to try to extrapolate the possible behavior of these emitters in the active site of Luc, and to further confirm the mechanism by which the intermolecular interactions affect the emission of $OxyLH_2$ and analogs. We have chosen to perform this subsequent study in explicit and implicit water, as the choice of a more homogeneous microenvironment is expected to simplify the effect exerted by intermolecular interactions in the excited state properties of the emitters. Thereby allowing focusing in the internal modifications of Keto-(−1) and analogs, which affect the color of light emitted. Furthermore, it should be noted that recent works are highlighting the importance of studying this type of system by modeling correctly both implicit and explicit solvation, due to their color tuning capability [21,24,27,50].

In Table 3 are presented the emission wavelength and oscillator strengths of Keto-(−1), $HOxyLH_2$ and respective moieties, in explicit water and in explicit/implicit water. These models were obtained by performing molecular mechanics based calculations using NAMD, as indicated in the Computational methods section. The geometries of the emitters, which resulted from the molecular mechanics calculations, were also used in *in vacuo* calculations of the wavelength maxima. Analysis of these results further supports our working hypothesis. The emission maxima of the emitters, when solvated with explicit and/or implicit water, suffer from significant shifts when in comparison with the *in vacuo* emission energies (Table 3). Keto-(−1) is still a green emitter, but suffers from a red-shift of 9–14 nm. The emission of the benzothiazole moiety of Keto-(−1) is also affected, with a blue-shift of 18–32 nm, falling outside of the visible spectrum. For the contrary, the thiazolone moiety is red-shifted (5–58 nm). $HOxyLH_2$ suffers from a significant red-shift. When explicit and explicit/implicit solvation is considered, a red-shift of 20–30 nm is verified. Thus, the present results further state that intermolecular interactions can affect the color of the emission, which is also modulated by the polarity of the microenvironment. We also wish to note the good correlation between the emission maxima of Keto-(−1) in explicit and explicit/implicit water with known experimental results [32].

Having analyzed the emission wavelength of Keto-(−1) and analogs in solution, we have studied their atomic Mulliken charge in order to verify if the mechanism of charge density modulation still applies in these calculations. These results are presented in Table 4.

By comparing the atomic charges, we see that the blue-shifted sole benzothiazole moiety of Keto-(−1) has always a higher negative charge than the benzothiazole moiety of the red-shifted full Keto-(−1). However, the negative charge of the benzothiazole moiety of full Keto-(−1) increases with increasing wavelength maximum, when we compare *in vacuo* Keto-(−1) and the emitter in explicit water. In the comparison between explicit and explicit/implicit models, the negative charge of the benzothiazole of full Keto-(−1) decreases with increasing emission wavelength. For the contrary, in the case of the sole benzothiazole moiety, its negative charge decreases with decreasing emission wavelength. This indi-

Table 3
Emission wavelength (λ_{em}, in nm) and oscillator strength (f) of Keto-(−1), $HOxyLH_2$ and their respective moieties, at the TD-O3LYP/6-31+G(d) level of theory.

	In vacuo		Explicit water		Explicit/implicit water	
	λ_{em}	f	λ_{em}	f	λ_{em}	f
Keto-(−1)	541	0.4865	554	0.2784	563	0.5412
Keto-(−1) benzothiazole moiety	412	0.1507	394	0.1049	362	0.2230
Keto-(−1) thiazolone moiety	216	0.0995	274	0.0677	279	0.1208
$HOxyLH_2$	540	0.4566	560	0.2975	590	0.4692
$HOxyLH_2$ benzothiazole moiety	413	0.1548	391	0.1563	376	0.1934
$HOxyLH_2$ thiazolone moiety	290	0.0632	401	0.0052	325	0.0154

Table 4
Atomic Mulliken charges of Keto-(−1), $HOxyLH_2$ and their respective moieties, at the TD-O3LYP/6-31+G(d) level of theory.

	In vacuo		Explicit water		Explicit/implicit water	
Keto-(−1)	−0.790	−0.209	−1.301	−0.229	−1.275	−0.146
Keto-(−1) benzothiazole moiety	−1.191		−1.732		−1.674	
Keto-(−1) thiazolone moiety	−0.253		−0.375		−0.378	
$HOxyLH_2$	−0.620	−0.380	−0.915	−0.992	−0.814	−0.961
$HOxyLH_2$ benzothiazole moiety	−1.191		−1.827		−1.793	
$HOxyLH_2$ thiazolone moiety	−0.259		−0.482		−0.425	

cates that for the benzothiazole moiety of Keto-(−1), no visible trend can be observed regarding the control of the color of light emitted by charge density modulation. For the thiazolone moiety of Keto-(−1), the situation is similar. The negative charge of the thiazolone moiety of red-shifted full Keto-(−1) is always smaller than the negative charge of its blue-shifted sole thiazolone moiety. When we compare the emission of *in vacuo* and in explicit solvent of full Keto-(−1), it can be seen that the negative charge of the thiazolone moiety increases with increasing emission wavelength. For the contrary, for the comparison between explicit and explicit/implicit solvent, the negative charge of the thiazolone moiety decreases with increasing wavelength maximum. For the sole thiazolone moiety of Keto-(−1), the negative charge increases with increasing emission wavelength. In conclusion, the present results indicate that there is no correlation between the charge density of Keto-(−1) and its emission wavelength. Therefore, and contrary to our expectations, the modulation of charge density is not involved in the color tuning mechanism of Keto-(−1).

Analysis of Table 4 indicates that charge density modulation may also not be involved in the color tuning mechanism of $HOxyLH_2$. The negative charge of its blue-shifted sole benzothiazole moiety is always higher than the negative charge of the benzothiazole of the red-shifted full $HOxyLH_2$. For the sole benzothiazole moiety of $HOxyLH_2$, there is an increase in the negative charge with decreasing wavelength maximum, when we compare the *in vacuo* and the explicit solvation results. In the comparison between the explicit and explicit/implicit solvation results, the negative charge of the sole benzothiazole moiety decreases with decreasing emission wavelength. The behavior of the benzothiazole moiety of full $HOxyLH_2$ is identical to that of its sole benzothiazole moiety. For the thiazolone moiety of $HOxyLH_2$, its negative charge is always higher in the full form of the emitter than in the sole moiety. For both the full form and the sole thiazolone moiety of $HOxyLH_2$, its negative charge is higher when only explicit solvation is used, despite the higher emission wavelength being calculated in other solvation conditions.

The results presented so far in this paper further demonstrate that intermolecular interactions can effectively modulate the emission wavelength of $OxyLH_2$ and analogs. However intermolecular interactions do not tune the color of emission by modulating the charge density of the emitter. Thus other factors are responsible for the modulation, by intermolecular interactions, of the color of light emitted by Keto-(−1) and analogs. Comparison between the emission wavelength presented in Tables 1 and 3, demonstrates that there is a more significant shift in the emission between the *in vacuo* results of the two tables, than between the comparison between the *in vacuo* and explicit solvent results of Table 3. It should be pointed out that the gas phase emission wavelength of Keto-(−1) (504 nm) and $HOxyLH_2$ (511 nm), at the TD-O3LYP/6-31+G(d), are very similar to the ones calculated at the TD-O3LYP/6-31+G(d,p) level of theory (Table 1). This indicates that the most important effect exerted by intermolecular interactions is the modulation of the excited state geometry of the emitter, thereby modulating the energy level of the excited state. If intermolecular interactions could decrease the energy of the excited state, in the comparison with the gas phase, it could explain the red-shift of the emission. To test this hypothesis we have calculated the *in vacuo* (from the geometries represented in Fig. 1) and solvated bond lengths of Keto-(−1) and $HOxyLH_2$. The bond lengths are represented in Fig. 2. Also, as the thiazolone moieties of the emitters present some distortion when in comparison with the *in vacuo* structures, the angles of the thiazolone moieties are presented in Table 5.

Analysis of both Fig. 2 and Table 5, it can be seen that intermolecular interactions effectively induce changes in the geometry of the emitters. In solution, the thiazolone moiety of both Keto-(−1) and $HOxyLH_2$ suffers from clear distortion. Moreover, despite not being drastic, it can be seen that the interaction with water molecules caused changes in the bond lengths of the emitters. In Keto-(−1), this change is more clearly seen in the benzothiazole moiety. For the contrary, in the case of $HOxyLH_2$, the thiazolone moiety appears to be the one more affected by intermolecular interactions. Thus, this indicates that intermolecular interactions modulate slightly the excited state geometry of the emitter, which is in line with the clear but not drastic shift in the emission wavelength. To analyze this new hypothesis, we have calculated the stability of Keto-(−1) and $HOxyLH_2$ different geometries. With this objective, we have calculated the energies of those geometries both at the excited and ground state, which are presented in Table 6. For a better comparison, the energies of the solvated geometries of the emitters were calculated without any explicit or implicit solvation. By analysis of this Table, it can be seen that gas phase and solvated geometries, of both molecules, have different energies in both the ground and excited state. In the case of Keto-(−1), the solvated geometry is 0.038 hartrees less stable (in the ground state) than the gas phase geometry. In the excited state, the solvated geometry

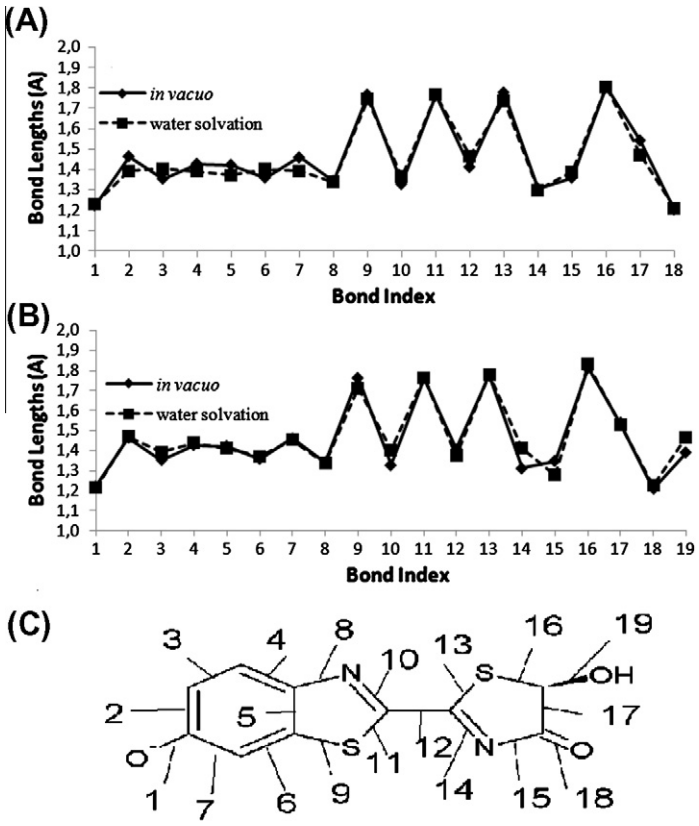

Fig. 2. Bond lengths of the excited state of Keto-(−1) (A), and HOxyLH2 (B). (C) identify the bond numbers used in the above graphics.

Table 5
Angles (in degrees) of the thiazolone moiety of excited state Keto-(−1) and $HOxyLH_2$, both in the gas phase and in water.

Angles	Keto-(−1)		$HOxyLH_2$	
	Gas phase	Water	Gas phase	Water
$C_{2'}-C_2-S_1$	119.07	122.89	119.43	124.26
$C_{2'}-C_2-N_3$	123.58	121.21	123.03	121.41
$C_2-S_1-C_5$	88.87	87.58	88.62	90.76
$N_3-C_4-C_5$	113.66	109.51	114.66	120.58
C_5-C_4-O	120.07	123.16	117.69	124.57
C_4-C_5-OH			110.56	117.83

Table 6
Energies (in hartrees) of the ground and excited state Keto-(−1) and $HOxyLH_2$, at the TD-O3LYP/6-31+G(d) and O3LYP/6-31+G(d) level of theory.

	Keto-(−1)		$HOxyLH_2$	
	Gas phase geometry	Solvated geometry	Gas phase geometry	Solvated geometry
Ground state	−1440.278	−1440.240	−1515.459	−1515.406
Excited state	−1440.188	−1440.155	−1515.370	−1515.322

is 0.033 hartrees less stable than the gas phase geometry. In the case of $HOxyLH_2$, the solvated geometry is 0.053 hartrees less stable (in the ground state) than the gas phase geometry. In the excited state, the solvated geometry is 0.048 hartrees less stable than the gas phase geometry. Thus, these results indicate that modulation of the geometry can affect the energy of both ground and excited states. Moreover, the different destabilization of the ground and excited state energies of Keto-(−1), by solvent-dependent geometry modification, caused a 0.006 hartrees decrease in the energy gap between the two states. This decrease corresponds to a 37 nm red-shift, which is the shift verified between the emission of the gas phase (504, Table 1) and the solvated geometry (541, Table 3) of Keto-(−1). The destabilization of the ground and excited states of $HOxyLH_2$ caused a 0.005 hartrees decrease in the energy gap between the two states, which corresponds to a 30 nm red-shift. Indeed, this was the red-shift verified between the emission of the gas phase (510, Table 1) and the solvated geometry (540, Table 3) of $HOxyLH_2$. Therefore, with these results we can state that changes in the geometry of the emitter affect the energy of both the ground and excited states, but with different extents. These differences in the stabilization/destabilization of both states will modify the energy gap between the ground and excited state, thereby originating shifts in the emission.

Having determined the effect exerted by the modulation of the geometry of the emitter in the emission, we have tried to understand the effect exerted by direct intermolecular interactions between the emitter and other molecules, and by polarity of the microenvironment. The presented study indicated that these factors can red- or -blue-shift the emission by modulating the energy gap between the ground and excited state, of the studied emitter. To test this hypothesis, we have calculated the energy, of both the excited and ground state, of Keto-(−1) and $HOxyLH_2$ in explicit and explicit/implicit water. The same geometries were also used in *in vacuo* calculations. The results, calculated at the TD-O3LYP/6-31+G(d) level of theory, are presented in Table 7.

These results further demonstrate the effect exerted by direct intermolecular interactions and polarity. For Keto-(−1), we can see that direct intermolecular interactions diminishes the energy gap between the ground and excited, explaining the 13 nm red-shift (541–554 nm, Table 3). When an implicit solvation is introduced in the calculations, a further decrease in the energy gap be-

Table 7
Energies (in hartrees) of the ground and excited state Keto-(−1) and $HOxyLH_2$, at the TD-O3LYP/6-31+G(d) and O3LYP/6-31+G(d) level of theory.

	Keto-(−1)		$HOxyLH_2$	
	Ground state	Excited state	Ground state	Excited state
In vacuo	−1440.240	−1440.155	−1515.406	−1515.322
Explicit water	−2357.026	−2356.944	−2814.185	−2814.104
Explicit/implicit water	−2357.193	−2357.113	−2814.390	−2814.313

tween the ground and excited state is observed. This decrease is also in line with the 22 nm red-shift (541–563 nm, Table 3). Intermolecular interactions and polarity modulate the emission of $HOxyLH_2$ in a similar way.

In conclusion, our results indicate that the emission maxima of $OxyLH_2$ and analogues are modulated by three different factors: direct intermolecular interactions; modulation of their geometry, also controlled by intermolecular interactions; polarity of the microenvironment. Furthermore, all of these factors appear to control the color of emission by affecting both the energy of the ground and of the excited state. This allows us to define a mechanism for the different wavelength maxima verified for the various firefly luciferases (538–623 nm) [12]. All of these enzymes present, with various extents, active sites with different amino-acids constitutions. This will lead to different types and/or strength of intermolecular interactions that the active site molecules can perform with the universal firefly bioluminophore (Keto-(−1)). These differences will then affect the emission by modulating differently the geometry of Keto-(−1), and by direct intermolecular interaction (also depending of the constitution of the active site). Also, the different constitution of the active site will affect its polarity, leading to more tuning of the color of bioluminescence. Therefore, if the differences in types/strength of intermolecular interactions and polarity are small between the active sites of different luciferases, the differences between the emission maxima will also be small. This can be seen in the small differences between the green maxima presented by various luciferases (538–570 nm) [12]. If the differences between the active sites are more significant, the difference between the wavelength maxima will also be more relevant. This can be seen in the difference between the emission of LcLuc at basic (561 nm) and at acid pH (609 nm), which is thought to be caused by different conformations of the active site [17,27].

4. Conclusion

In this paper we have studied the role of intermolecular interactions and polarity in the light emission of Keto-(−1) and a novel emitter, $HOxyLH_2$. This study was performed calculating the first excited state of the emitters, and their separate moieties, at the CIS and TD-DFT level of theory. Further calculations were performed in explicit and explicit/implicit water, by means of molecular mechanics energy minimizations and TD-DFT calculations.

The results obtained in this paper allow us to define the color tuning mechanism of Keto-(−1), both in solution and in Luc active site (by extrapolation). Our results demonstrate that intermolecular interactions can affect the geometry of the emitter, thus modulating the energy difference between the emitting excited and ground, which can originate different emission wavelengths. Besides affecting the geometry of the emitter, direct intermolecular interactions between Keto-(−1) and other molecules can further affect the energy difference between the ground and excited state. The polarity of the microenvironment can also modulate the emission, by modulating the energy gap between the emitting excited and ground states. Therefore different solvents and different active site conformations/constitutions, originate different intermolecular interactions networks between the emitter and other neighboring molecules, leading to different energy gaps and subsequently different emission wavelengths. This mechanism can then explain the different emission wavelength of Keto-(−1) in different solvents, in different Luc species and at different pH (due to its effect in pH-sensitive Luc).

A novel emitter was also considered, by hydroxylation of the C_5 carbon of Keto-(−1). $HOxyLH_2$ could be of use in *in vivo* Luc-based applications, due to its higher emission wavelength.

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References

[1] S.M. Marques, J.C.G. Esteves da Silva, Firefly bioluminescence: a mechanistic approach of luciferase catalyzed reactions, IUBMB Life 61 (2009) 6–17.
[2] J.C.G. Esteves da Silva, J.M.C.S. Magalhães, R. Fontes, Identification of enzyme produced firefly oxyluciferin by reverse phase HPLC, Tetrahedron Lett. 42 (2001) 8173–8176.
[3] C. Ribeiro, J.C.G. Esteves da Silva, Kinetics of inhibition of firefly luciferase and oxyluciferin and dehydroluciferyl-adenylate, Photochem. Photobiol. Sci. (2008) 1085–1090.
[4] J.M. Leitão, J.C.G. Esteves da Silva, Firefly luciferase inhibition, J. Photochem. Photobiol. 101 (2010) 1–8.
[5] L. Pinto da Silva, J.C.G. Esteves da Silva, Kinetics of inhibition of firefly luciferase by dehydroluciferyl-coenzyme A, dehydroluciferin and l-luciferin, Photochem. Photobiol. Sci. 10 (2011) 1039–1045.
[6] A. Roda, P. Pasini, M. Mirasoli, E. Michelini, M. Guardigli, Biotechnological applications of bioluminescence and chemiluminescence, Trends. Biotechnol. 22 (2004) 295–303.
[7] K.E. Luker, G.D. Luker, Applications of bioluminescence imaging to antiviral research and therapy: multiple luciferases enzymes and quantitation, Antiviral Res. 78 (2008) 179–187.
[8] F. Fan, K.V. Wood, Bioluminescent assays for high-throughput screening, Assay Drug Dev. Technol. 5 (2007) 127–136.
[9] B.R. Branchini, T.R. Southworth, N.F. Khattak, E. Michelini, A. Roda, Red- and green-emitting firefly luciferase mutants for bioluminescent reporter applications, Anal. Biochem. 345 (2005) 140–148.
[10] T.C. Doyle, S.M. Burns, C.H. Contag, In vivo bioluminescence imaging for integrated studies of infection, Cell Microbiol. 6 (2004) 303–317.
[11] L. Pinto da Silva, J.C.G. Esteves da Silva, Computational studies of the luciferase light-emitting product: oxyluciferin, J. Chem. Theory Comput. 7 (2011) 809–817.
[12] V.R. Viviani, F.G.C. Arnoldi, A.J.S. Neto, T.L. Oehlmeyer, E.J.H. Bechara, Y. Ohmiya, The structural origin and biological function of pH-sensitivity in firefly luciferases, Photochem. Photobiol. Sci. 7 (2008) 159–169.
[13] E.H. White, E. Rapaport, H.H. Seliger, T.A. Hopkins, The chemi- and bioluminescence of firefly luciferin: An efficient chemical production of electronically excited states, Bioorg. Chem. 1 (1971) 92–122.
[14] F. McCapra, D.J. Gilfoyle, D.W. Young, N.J. Church, P. Spencer, Bioluminescence and Chemiluminescence. Fundamental and Applied Aspects, Wiley, New York, 1971.
[15] B.R. Branchini, T.L. Southworth, M.H. Murtiashow, R.A. Magyar, S.A. Gonzalez, M.C. Ruggiero, J.G. Stroh, An alternative mechanism of bioluminescence color determination in firefly luciferase, Biochemistry 43 (2004) 7255–7262.
[16] T. Hirano, Y. Hasumi, K. Ohtsuka, S. Maki, H. Niwa, M. Yamaji, D. Hashizume, Spectroscopic studies of the light-color modulation mechanism of firefly (beetle) bioluminescence, J. Am. Chem. Soc. 131 (2009) 2385–2396.
[17] T. Nakatsu, Y. Ichiyama, J. Hiratake, A. Saldanha, N. Kobashi, K. Sakata, H. Kato, Structural basis for the spectral difference in luciferase bioluminescence, Nature 440 (2006) 372–376.
[18] S. Hosseinkhani, Molecular enigma of multicolor bioluminescence of firefly luciferase, Cell Mol. Life Sci. 68 (2011) 1167–1182.
[19] I. Navizet, Y.J. Liu, N. Ferré, D. Roca-Sanjuán, R. Lindh, The chemistry of bioluminescence. an analysis of chemical functionalities, Chem. Phys. Chem. 12 (2011) 3064–3076.
[20] J.Y. Hasegawa, K.J. Fujimoto, H. Nakatsuji, Color tuning in photofunctional proteins, Chem. Phys. Chem. 12 (2011) 3106–3115.

[21] L. Pinto da Silva, J.C.G. Esteves da Silva, Computational investigation of the effect of pH on the color of firefly bioluminescence by DFT, Chem. Phys. Chem. 12 (2011) 951–960.
[22] S.F. Chen, Y.J. Liu, I. Navizet, N. Ferré, W.H. Fang, R. Lindh, Systematic theoretical investigation on the light emitter of firefly, J. Chem. Theory Comput. 7 (2011) 798–803.
[23] C.I. Song, Y.M. Rhee, Dynamics on the electronically excited state surface of the bioluminescent firefly luciferase-oxyluciferin system, J. Am. Chem. Soc. 133 (2011) 12040–12049.
[24] L. Pinto da Silva, J.C.G. Esteves da Silva, Theoretical modulation of the color of light emitted by firefly oxyluciferin, J. Comput. Chem. 32 (2011) 2654–2663.
[25] C.G. Min, A.M. Ren, J.F. Guo, Z.W. Li, L.Y. Zou, J.D. Goddard, J.K. Feng, A time-dependent density functional theory investigation on the origin of red chemiluminescence, Chem. Phys. Chem. 18 (2010) 251–259.
[26] Y.J. Liu, L. De Vico, R. Lindh, Ab initio investigation on the chemical origin of the firefly bioluminescence, J. Photochem. Photobiol. A 194 (2008) 261–267.
[27] L. Pinto da Silva, J.C.G. Esteves da Silva, Study of the effects exerted by intermolecular interactions on the firefly multicolor bioluminescence, Chem. Phys. Chem. 12 (2011) 3002–3008.
[28] N. Nakatani, J.Y. Hasegawa, H. Nakatsuji, Red light in chemiluminescence and yellow-green light in bioluminescence. Color-tuning mechanism of firefly; photinus pyralis: studied by the symmetry-adapted cluster-configuration interaction method, J. Am. Chem. Soc. 129 (2007) 8756–8765.
[29] C.G. Min, A.M. Ren, J.F. Guo, L.Y. Zou, J.D. Goddard, C.C. Sun, Theoretical investigation on the origin of yellow-green firefly bioluminescence by time-dependent density functional theory, Chem. Phys. Chem. 11 (2010) 2199–2204.
[30] I. Navizet, Y.J. Liu, N. Ferré, H.Y. Xiao, W.H. Fang, R. Lindh, Color-tuning mechanism of firefly investigated by multi-configurational perturbation method, J. Am. Chem. Soc. 132 (2010) 706–712.
[31] B.F. Milne, M.A. Marques, F. Nogueira, Fragment molecular orbital investigation of the role of AMP protonation in firefly luciferase pH-sensitivity, Phys. Chem. Chem. Phys. 12 (2010) 14285–14293.
[32] P. Naumov, Y. Ozawa, K. Ohkubo, S. Fukuzumi, Structure and spectroscopy of oxyluciferin, the light emitter of the firefly bioluminescence, J. Am. Chem. Soc. 131 (2009) 11590–11605.
[33] Avogadro: An open-source molecular builder and visualization tool. Version 1.0.0. <http://avogadro.openmolecules.net/> (accessed 10.02.11).
[34] C.T. Lee, W.T. Yang, R.G. Parr, Development of the colle-salvetti correlation-energy formula into a functional of the electron-density, Phys. Rev. B: Condens. Matter Mater. Phys. 37 (1998) 785–789.
[35] N.C. Handy, A.J. Cohen, Left-right correlation energy, Mol. Phys. 99 (2001) 403–412.
[36] W.N. Hoe, A.J. Cohen, N.C. Handy, Assessment of a new local exchange functional OPTZ, Chem. Phys. Lett. 341 (2001) 319–328.
[37] J.B. Foresman, M. Head-Gordon, J.A. Pople, M.J. Frisch, Towards a systematic molecular-orbital theory for excited-states, J. Phys. Chem. 96 (1992) 135–149.
[38] E.K.U. Gross, W. Kohn, Time-dependent density-functional theory, Adv. Quantum Chem. 21 (1990) 255–291.
[39] M.E. Casida, Recent Advances in Density Functional Methods, World Scientific, Singapore, 1995.
[40] T. Yang, J.D. Goddard, Predictions of the geometries and fluorescence emission energies of oxyluciferins, J. Phys. Chem. A 111 (2007) 4489–4497.
[41] K. Fujimoto, S. Hayashi, J.Y. Hasegawa, H. Nakatsuji, Theoretical studies on the color-tuning mechanism in retinal proteins, J. Chem. Theory Comput. 3 (2007) 605–618.
[42] Z.W. Li, A.M. Ren, J.F. Guo, T. Yang, J.D. Goddard, J.K. Feng, Color-tuning mechanism in firefly luminescence: theoretical studies on fluorescence of oxyluciferin in aqueous solution using time dependent density functional theory, J. Phys. Chem. A (2008) 9796–9800.
[43] L. Pinto da Silva, J.C.G. Esteves da Silva, Analysis of the performance of DFT functionals in the study of light emission by oxyluciferin analogues, Int. J. Quantum Chem. (2012), doi:10.1002/qua.24014.
[44] D.A. Case, T.E. Cheatham, T. Darden, H. Gohlke, R. Luo, K.M. Merz, A. Onufriev, C. Simmelring, B. Wang, R. Wodds, The amber biomolecular simulation programs, J. Comput. Chem. 26 (2005) 1668–1688.
[45] J.M. Wang, R.M. Wolf, J.W. Caldwell, P.A. Kollman, D.A. Case, Development and testing of a general amber force field, J. Comput. Chem. 25 (2004) 1157–1174.
[46] J.C. Philips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R.D. Skell, L. Kale, K. Schulten, Scalable molecular dynamics with NAMD, J. Comput. Chem. 26 (2005) 1781–1802.
[47] U. Essmann, L. Perera, M.L. Berkowitz, T. Darden, H. Lee, L.G. Pedersen, A smooth particle Mesh Ewald method, J. Chem. Phys. 103 (1995) 8577–8593.
[48] V. Barone, M. Cossi, Quantum calculation of molecular energies and energy gradients in solution by a conductor solvent model, J. Phys. Chem. A 102 (1998) 1995–2001.
[49] Gaussian 03 (Revision C.02), M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, J.A. Montgomery Jr., T. Vreven, K.N. Kudin, J.C. Burant, J.M. Millam, S.S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G.A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J.E. Knox, H.P. Hratchian, J.B. Cross, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Stratmann, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, P.Y. Ayala, K. Morokuma, G.A. Voth, P. Salvador, J.J. Dannenberg, V.G. Zakrzewski, S. Dapprich, A.D. Daniels, M.C. Strain, O. Farkas, D.K. Malick, A.D. Rabuck, K. Raghavachari, J.B. Foresman, J.V. Ortiz, Q. Cui, A.G. Baboul, S. Clifford, J. Cioslowski, B.B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R.L. Martin, D.J. Fox, T. Keith, M.A. Al-Laham, C.Y. Peng, A. Nanayakkara, M. Challacombe, P.M.W. Gill, B. Johnson, W. Chen, M.W. Wong, C. Gonzalez, J.A. Pople, Gaussian, Inc., Wallingford, CT, 2004.
[50] M. Anselmi, S. Marocchi, M. Aschi, A. Amadei, Theoretical modeling of the spectroscopic absorption properties of luciferin and oxyluciferin: a critical comparison with recent exerimental studies, Chemical Physics (2011), doi:10.1016/j.chemphys.2011.11.021.