Chemiluminescence: From mechanism to applications in biological imaging and therapy

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INTRODUCTION

Optical imaging is a noninvasive imaging approach with high sensitivity and spatiotemporal resolution.[1] However, photoluminescence imaging suffers from severe light scattering, tissue absorption and autofluorescence, leading to a low SBR and sensitivity. Chemiluminescence (CL) is the emission of light as a result of chemical reaction, which is in stark contrast to the photoluminescence that the emissive excited state is formed through external light irradiation. This makes CL show great advantages over the photoluminescence in imaging, achieving deeper tissue penetration, higher SBR and sensitivity, as the external excitation source is not required.[2] For instance, increased tissue penetration depth has been reported (2.5 cm for CL and 1 cm for fluorescence imaging of NIR-I dye).[2] Inspired by the above-mentioned merits, the application of CL in bioimaging is booming in the recent few years.

Photodynamic therapy (PDT) has attracted great attention as a novel strategy for the treatment of cancer.[3] Generally, three elements, that is, triplet photosensitizer, oxygen and light, are required for PDT. The triplet photosensitizer can be promoted to its triplet state upon light irradiation, which reacts with molecular oxygen to produce reactive oxygen species (ROS) to kill tumor cells. Spatiotemporal selectivity can be achieved as the PDT process is controlled by the implement of light, that is, the generation of toxic ROS can be initiated upon the light irradiation, and terminated once cessation of the light irradiation. However, due to the poor light penetration ability of visible light (<2 mm) and NIR light (~1 cm),[2] the clinical PDT is still limited to the treatment of superficial diseases such as skin cancers. To break the...
light-related restrictions regarding to the size and localization of tumor, considerable efforts have been invested, such as the development of two-photon absorption photosensitizers, and the use of X-ray or ultrasound instead of light to initiate the therapy.[4–6] CL initiated PDT is such an external excitation source-free strategy, hence it can be applied to treat deeply seated large tumors.[7–14] However, as the CL is uncontrollable once it is generated, CL initiated PDT is still limited to conception.

Concerning the increased interest and investigation on CL, there have been a few reviews that nicely summarized the development of CL and its biological applications.[15–20] Instead, this review focuses on the theoretical aspects and molecular design strategies for better biological application efficacy. Specifically, some theoretical issues of CL mechanism, CL quantum yield and chemiexcited state will be introduced. Then the application of CL in bioimaging is highlighted. In particular, strategies to modulate the brightness and the wavelength of CL, two of the most crucial parameters in bioimaging, are summarized. Finally, the conception of CL initiated PDT is introduced.

**GENERALITIES**

**Mechanisms**

The mechanisms of chemiluminescence are diverse and controversial. It is widely acknowledged that electron transfer is involved before the emitting of chemiluminescence.[21,22] Instead, this review focuses on the reverse of photoexcitation-induced electron transfer process (Scheme 1), and light is the product of the former while the initiator of the latter.

$$A + B \rightarrow A^+ + B^- \quad (\text{Scheme 1})$$

$$A^+ + B^- \rightarrow C^* + D \rightarrow C + D + \text{hv} \quad (\text{Scheme 2})$$

The photoexcitation-induced electron transfer process is presented in Scheme 1. When a fluorophore A is hit by light, the molecule is promoted to its electronically excited state (A*), followed by the thermodynamically allowed electron transfer process (the energy level of electron transfer excited state is lower than the singlet excited state). In this process, light is the initiator and a portion of light energy is converted into chemical energy and stored in the products.

In the case that the energy level of electron transfer state is higher than the excited state, the photo-induced electron transfer process is not accessible. However, if the radical cation and radical anion (A*+ + B*) can be produced by other strategies such as chemical reactions, the charge recombination process can populate the excited state (C*). If the excited state C* is an emissive state, light can be observed, which is the so-called chemiluminescence, that is, A*+ + B → C* + D → C + D + hv. The chemiluminescence process converted chemical energy into light energy, which resembles the reverse process of photochemical electron transfer. The electron transfer induced the CL, and light is the product.

The general CL process includes three crucial steps: (1) the formation of high-energy intermediate such as 1,2-dioxetane, 1,2-dioxetanone, and 1,2-dioxetanediene,[15] which can release sufficient energy to produce a photon of visible light (energy in the range of 40–70 kcal/mol); (2) the decomposition of the high-energy intermediate and chemiexcitation to the excited state; (3) emission from the excited state.

The precise mechanism is unclear and diverse for many chemiluminescent reactions. Some reported mechanisms are even controversial.[23] Herein we do not intend to introduce all the diverse mechanisms, while we focus on the following two representative mechanisms: (1) chemical initiated intramolecular electron exchange, leading to the decomposition of high-energy intermediate and formation of the emissive excited state, exemplified with the phenolate-substituted dioxetanes; (2) chemical initiated intermolecular electron exchange, exemplified with the peroxyoxalate in the presence of activator, which forms the singlet excited state of activator.[15]

**Intermolecular chemically initiated electron exchange luminescence (CIEEL) exemplified with peroxyoxalate**

The intermolecular CIEEL mechanism was first proposed by Schuster and coworkers.[21,22] They reported that the nonemissive (or weakly emissive) high-energy intermediates such as diphenoyl peroxide and dimethyldioxetanone can induce CL, upon addition of aromatic hydrocarbons (activator) with low oxidation potential and high fluorescence quantum yield. The CL intensity (of the activator) is highly dependent on the concentration and the oxidation potential of the activator, hence intermolecular electron transfer process is proposed to be involved.

However, the chemiexcitation quantum yields (ΦCE) of diphenyl peroxide and dimethyl-1,2-dioxetanone, two model high-energy intermediate in Schuster’s intermolecular CIEEL mechanism, were found to be severely overestimated: Catalani and Wilson reported that the chemiexcitation quantum yield of diphenyl peroxide is only $2 \times 10^{-5}$ einstein/mol,[25] which is 4 orders of magnitude lower than the 0.1 einstein/mol reported by Schuster.[26,27] Later, Baader showed that the chemiexcitation quantum yield of dimethyl-1,2-dioxetanone system (0.001 einstein/mol) is also significantly lower than the reported value (0.1 einstein/mol).[28] Hence the intermolecular CIEEL mechanism for these high-energy intermediate systems are challenged.[18]

In stark contrast to the extremely low chemiexcitation efficiency, the peroxyoxalate reaction shows dramatically high chemiexcitation quantum yield (ΦCE ∼ 60%). Peroxyoxalate reaction is first reported by Chandross,[29] which is one of the brightest CL reactions and widely used in "glow stick."[30] Till now, peroxyoxalate reaction has been the only reaction that clearly shows intermolecular CIEEL mechanism. Among the peroxyoxalate reactions, the most typical one is the bis(2,4,6-trichlorophenyl) oxalate (TCPO), hydrogen peroxide and activator system. Baader et al. investigated the TCPO CL system in detail by using different activators such as rubrene (RUB), perylene (PER), 9,10-diphenylanthracene...
As shown in Figure 1, despite the result of DCNA, the chemiexcitation quantum yield is found to be positively related to the free energy change of back electron transfer (ACT\textsuperscript{\textbullet} + CO\textsuperscript{\textbullet} \rightarrow ACT\textsuperscript{\textast} + CO\textsubscript{2}), which verifies the intermolecular chemically initiated electron exchange mechanism of the TCPO system.

Hence the intermolecular CIEEL of the TCPO system can be depicted as follows: The first step is the generation of the high-energy intermediate (1,2-dioxetanedione), followed by the charge transfer between the activator and the 1,2-dioxetanedione. Then intermolecular electron transfer from the activator to the 1,2-dioxetanedione occurred irreversibly, due to the elongation and cleavage of the O–O bond. Subsequently, the C–C bond is cleaved, which leads to the formation of a new radical ion pair within the solvent cage. Back electron transfer populates the chemiexcited singlet state of activator, which eventually emits the CL.\textsuperscript{[23]} Concerning such a high chemiexcitation quantum yield, the intermolecular electron transfer and back electron transfer must be efficient. Hence it is proposed that both the intermolecular electron transfer and back electron transfer occurred within the solvent cage.

Intramolecular CIEEL exemplified with phenoxy substituted dioxetanes

The representative example of intramolecular CIEEL mechanism is the CL of dioxetanes.\textsuperscript{[31]} Among the dioxetanes, Schaap’s dioxetane, being triggerable and thermal stable at room temperature,\textsuperscript{[32]} is one of the most attractive skeletons. As shown in Figure 2, the Schaap’s dioxetane consists four parts\textsuperscript{[33,34]}: (1) the 1,2-dioxetane group is the energy source. This is totally different from other multicomponent CL systems such as peroxyxylate or luminol system, where the energy source is not directly presented in the molecular skeleton but generated as an intermediate; (2) the adamantylidene moiety is employed as the stabilizer, which ensures the thermal stability at room temperature; (3) the aromatic ring is the fluorophore that eventually emits CL; and (4) the phenol substituted group (Figure 2) is the triggerable group, which can be removed on demand to form a phenolate ion at physiological pH condition.\textsuperscript{[33,34]}

The exact intramolecular CIEEL mechanism of Schaap’s dioxetane is as follows. The first step is the removal of triggerable group, which initiates the intramolecular electron transfer from the phenolate ion to the dioxetane group, leading to the breaking of the O–O bond, that is, the decomposition of dioxetane. As the phenolate ion can be more easily oxidized...
than the parent phenol, the decomposition rate constant of the dioxetane bearing phenolate ion can be up to six order of magnitude than that of its phenol form.\[33\] Hence the removal of triggerable group, that is, the deprotection of the phenolate ion, initiates the intramolecular CIEEL. As the broken of O–O bond can form either compound 2a or 2b (Figure 2), there are two possible pathways to induce CL: For path A, the intermediate 2a decomposed to 3a and adamantanone; then an intramolecular back electron transfer results to the excited state of 4 (4*), which emits CL during its decay to ground state 4. While for path B, compound 2b decomposed to the solvent-caged radical-ion pair 3b, followed by an intermolecular back electron transfer and formation of excited state of 4 (4*). Again, the decay of 4* to its ground state 4 leads to CL.

The exact pathway is still elusive and both pathways, that is, intramolecular and intermolecular back electron transfer, are plausible.\[35–37\]

### Energy considerations of chemiexcitation

1,2-Dioxetanes, 1,2-dioxetanones, and 1,2-dioxetanediones are typical high-energy intermediates. Is their energy sufficient to produce an excited state? To address such a concern, herein, a well-studied tetramethyldioxetane (TMDO) is described (Figure 3A). The reaction enthalpy ($\Delta H_0$) and the activation enthalpy ($\Delta H^\ddagger$) are experimentally determined to be –61 and 25 kcal/mol, respectively. Hence, TMDO has a total energy of 86 kcal/mol, which is higher than the energy of the singlet and triplet excited states of acetone (84 and 78 kcal/mol, respectively). This energy diagram shows that the high-energy TMDO is sufficient for the chemiexcitation of acetone.\[18\] Hence the high-energy character of the intermediate is crucial for chemiexcitation.

### Chemiexcited state and photoexcited state

A common strategy to study the chemiluminescence property is to measure the fluorescence emission spectra of the product, which assume that the emissive state of the decomposed peroxide (chemiexcited state that emits CL) is exactly the same as the excited state of the product (photoexcited state that emits fluorescence). However, the chemiluminescence emission and fluorescence emission spectra do not have to be identical. By adopting different starting geometries for computation, Rocas-Sanjuán et al. shows that the chemiexcited state and the photoexcited state can be totally different, either in electronic structures or molecular geometries (as shown in Figure 3B).\[38\] The chemiexcited state has a charge transfer character, while the photoexcited state is a delocalized excited state. Apparently, they are also different in energy. As there is an energy barrier between the two states, the decomposition of the peroxide leads to the formation of the chemiexcited state, which emits CL instead of transforming to the photoexcited state. This indicates that the CL emission of the peroxide and the fluorescence emission of the product can be different,\[38,39\] which is experimentally confirmed later.\[40\]

The distinguish of the chemiexcited state and the photoexcited state is very important. For instance, it is helpful to understand the pronouncedly different chemiexcitation quantum yield of 3-(2’-spirodadamantyl)-4-methoxy-4-(3’’-hydroxyphenyl)-1,2-dioxetane (m-AMPD) and its para isomer p-AMPD. A computation study conducted by Liu et al.\[41\] shows that the m-AMPD is not stable and transforms to the photoexcited state rapidly, which is responsible for the eventual light emission. While in stark contrast, the chemiexcited state of p-AMPD is rather stable, as there is a large energy barrier from the chemiexcited state to the photoexcited state. According to the computed oscillator strengths, the chemiexcited state is less emissive as compared with the photoexcited state, which perfectly explained the significantly lower CL quantum yield of p-AMPD.

### The quantum yield of chemiluminescence

The quantum yield of chemiluminescence is a vital parameter to evaluate the chemiluminescence efficiency of a variety of CL systems. According to definition, the CL quantum yield is the mols of photons emitted divided by the mols of limiting reagent (Equation 1). To convert the CL emission intensity (generally in arbitrary units, e.g., counts/s) to the mols of photons emitted (in einstein), a modified luminol standard is commonly used to calibrate the unit (Equation 2).\[16,42\] However, as the PMT detector has different sensitivity in different wavelength, another calibration must be performed, that is, $f_{\text{PMT}}$ (Equation 3), especially for those CL systems whose CL emission wavelength is significantly different from that of the luminol. Usually, this calibration should be done with a calibration file measured with a standard light source, and in this way all the wavelengths are calibrated. In this situation, the calibration factor can be ignored, that is, $f_{\text{PMT}} = 1$. However, if the whole emission spectrum is not calibrated, this calibration factor $f_{\text{PMT}}$ can be approximately estimated by the maximum emission wavelength of the sample and luminol CL.
which should be available from the manufacturer.\cite{23,28}

\[
\phi_{CL} = \frac{\text{mols of photons emitted}}{\text{mols of Limiting reagent}},
\]

(1)

\[
\phi_{CL} = \phi_{\text{lum}} \times \frac{Q \times n_{\text{lum}}}{n \times Q_{\text{lum}}},
\]

(2)

\[
\phi_{CL} = \phi_{\text{lum}} \times \frac{Q \times n_{\text{lum}}}{n \times Q_{\text{lum}}} \times f_{\text{PMT}},
\]

(3)

\[
\phi_{CL} = \phi_{\text{CE}} \times \phi_{F},
\]

(4)

where $\phi_{CL}$ is the chemiluminescence quantum yield of the sample; $\phi_{\text{lum}}$ is the chemiluminescence quantum yield of the luminol system; the $Q$ is the total photon numbers (with different wavelength/energy) emitted over the entire time course; the $n$ is the mols of the limiting reagent; the $f_{\text{PMT}}$ is the calibration factor of PMT detector; the subscript lum stands for luminol; $\phi_{\text{CE}}$ is the chemiexcitation quantum yield and $\phi_{F}$ is the fluorescence quantum yield of the emitter.

It should be noted that one should pay attention to the ordinate of their CL emission spectra, as the CL intensity and the CL photon numbers (in counts) are totally different. The CL intensity is not only related to the number of photons, but also depends on the frequency of the photon. A conversion from the CL intensity to the photon numbers is further required, with the consideration of photon frequency (or wavelength). Hence a fluorescence spectrometer with Time-Correlated Single Photon Counting (TCSPC) detector that can directly provide the information on photon numbers is more convenient for the calculation of CL quantum yield.

The unit of the CL quantum yield is einsteins/mol. For instance, the CL of luminol system is $1.29 \times 10^{-2}$ einsteins/mol\cite{16,42a} which means 1 mol luminol can produce $1.29 \times 10^{-2}$ mol photons. The theoretical maximum CL quantum yield is 1 einsteins/mol, which means at most 1 mol
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As an external excitation-free technique, CL is expected to have high SBR and sensitivity, and has been widely applied in bioimaging.\(^{[42b]}\) For instance, luminol CL system and peroxoyxalate CL system have been utilized to the detection of ROS (such as H\(_2\)O\(_2\)) and specific enzymes.\(^{[43,44]}\) Dioxetanes with a trigger group (Figure 2) are more versatile and can detect a variety of substrates such as reactive oxygen species (ROS), enzymes, reactive carbonyl species, etc.\(^{[17,45–51]}\) In addition, the enol-ether precursor of dioxetane can be utilized as singlet oxygen sensor for the real-time monitoring of singlet oxygen production under physiological conditions.\(^{[52a,52b]}\) Herein, this review do not intend to list those probes one by one, as they have been nicely introduced by many reviews.\(^{[19]}\) Instead, we would like to summarize the strategies on the modulation of the brightness and the wavelength of CL, which are two of the most crucial parameters in bioimaging, as brighter CL (a higher CL quantum yield) and NIR CL emission are beneficial to achieve higher SBR and deeper tissue penetration in bioimaging.

**Modulation of the brightness of CL**

The brightness of CL is similar to that of the faint starlight at night, which is close to the absolute threshold of human visual function.\(^{[52c]}\) Most CL is weak (Table 1), which limited the bioimaging SBR and sensitivity. Brighter CL is highly desired and elegant efforts have been made. For instance, horseradish peroxidase (HRP) and transition metal cations\(^{[53,54]}\) are proved to be efficient enhancers to the luminol CL. Bovine serum albumin is a qualified enhancer as it can increase the CL by approximately one order of magnitude by providing a hydrophobic microenvironment.\(^{[55]}\) Molecular structure derivatization is found to significantly affect the chemiexcitation quantum yield and hence induce a pronounced variation of CL quantum yield.\(^{[56–58]}\) This review summarizes these strategies and introduces their application in bioimaging. Generally speaking, most of these strategies are based on either increase of the chemiexcitation quantum yield or increase of the fluorescence quantum yield (Equation 4). Other strategies such as domino effect and autoinduction are also introduced.

**Table 1** A summary of the photophysic parameters of the chemiluminescent compounds introduced in this paper

| Probe          | \(\lambda_{\text{em}}\) (nm) | \(\Phi_{\text{CL}}\) | Reference            |
|----------------|-----------------------------|----------------------|----------------------|
| 6,8-Dialkyl luminol | 461                        | 2.37 \times 10^{-1}  | Chem. Eur. J., 2015, 21, 9975 |
| Luminol       | 425                        | 1.2 \times 10^{-2}   | Photochem. Photobiol., 2007, 83, 1205 |
| Luminol       | 425                        | 1.29 \times 10^{-2}  | ACS Nano, 2016, 10, 6400 |
| Shaap’s dioxetane (skeleton A) | 470                        | 3.2 \times 10^{-5}   | ACS Cent. Sci., 2017, 3, 349 |
| Shaap’s dioxetane (skeleton B) | 525                        | 9.8 \times 10^{-2}   | ACS Cent. Sci., 2017, 3, 349 |
| Shaap’s dioxetane (skeleton C) | 690                        | 1.12 \times 10^{-2}  | J. Am. Chem. Soc., 2017, 139, 13243 |
| TCPO + semiconducting polymer | 436–700                    | 8.18 \times 10^{-5} to 2.3 \times 10^{-2} | ACS Nano, 2016, 10, 6400 |
| Probe 1       | 470                        | 3.3 \times 10^{-5}   | J. Am. Chem. Soc., 2016, 138, 13438 |
| Probe 2       | 535                        | 3.8 \times 10^{-3}   | J. Am. Chem. Soc., 2016, 138, 13438 |
| Probe 3       | 714                        | 9 \times 10^{-4}     | J. Am. Chem. Soc., 2016, 138, 13438 |
| TPE-CLA       | 500                        | \sim b)               | Anal. Chem., 2017, 89, 7210 |
| DPD-O         | 660                        | 8.2 \times 10^{-3}   | J. Am. Chem. Soc., 2017, 139, 13243 |
| DPD-S         | 760                        | 2.3 \times 10^{-3}   | Angew. Chem., Int. Ed., 2020, 59, 1 |
| DPD-Se        | 780                        | 1.2 \times 10^{-3}   | Angew. Chem., Int. Ed., 2020, 59, 1 |

\(^{a}\)In einstein/mol.
\(^{b}\)Not reported.

photons can be produced by 1 mol substrates. Moreover, once the CL quantum yield and the fluorescence quantum yield of the emitter are known, the chemiexcitation quantum yield (\(\Phi_{\text{CE}}\)) can be readily calculated according to the Equation 4.
unsubstituted luminol and dialkylated luminol shows a significant different energy of conical intersection state. Due to the steric gearing in dialkylated luminol, the weak O=O bond is prolonged and the aromatic ring is more twisted. This leads to a higher energy of CL and hence a more efficient population of excited state of the product C. The “steric gearing” perfectly explained the higher CL quantum yield of dialkylated luminol. Although the author pointed out that this steric hindrance effect on the CL intensity should be general, more structures are expected to verify this postulation.

On the other hand, the molecular skeleton can affect the fluorescence quantum yield, which consequently affect the CL quantum yield. A typical example is Doron Shabat’s dioxetane (skeleton B) in Figure 4b, which is redesigned based on the Schaap’s dioxetane (skeleton A) in Figure 4b. By introducing an electron withdrawing group (methyl acrylate or acrylonitrile group) to the ortho position of the phenolic oxygen, a significantly increased fluorescence quantum yield in aqueous solution is observed, leading to a three orders of magnitude enhancement on CL quantum yield (ΦCL = 9.8% for skeleton B and ΦCL = 0.0032% for skeleton A). In stark contrast, the para substitution does not induce pronounced enhancement to the fluorescence quantum yield. Hence the structure–efficiency relationship and the mechanism of the significantly improved fluorescence quantum yield of this new skeleton B are still not clear. A better understanding on this issue should be crucial for the future molecular design. Inspired by the pronounced improvement on the CL brightness (Figure 4b), the phenols were protected by a triggerable group that is activatable by β-galactosidase and used for cell imaging. At an exposure time of 40 s, high-quality CL image of the HEK293-LacZ cells (with β-galactosidase overexpressed) can be obtained. Whereas no signal can be observed for HEK293-WT cells (with low level expression of β-galactosidase). Previously, limited to the weak CL emission, the CL of Schaap’s adamantylidene 1,2-dioxetane has rarely been applied to cell imaging with a direct CL mode. The discovery of the new highly emissive skeleton B opens up a new avenue for bioimaging.

Chemiluminescence resonance energy transfer (CRET)

CRET is an important strategy to amplify the CL emission. For instance, recall from “Mechanisms” section that the high-energy intermediate 1,2-dioxetanedione is nonemissive, the addition of highly emissive aromatic hydrocarbons (activator) significantly enhances the CL of peroxynitrate. The excited nonemissive species are converted into the highly emissive excited states through the electron transfer and back electron transfer process, making the CL possible and observable. This might be regarded as a kind of intermolecular CRET though the electron exchange. Based on this strategy, Pu et al. screened a variety of semiconducting polymers (PFO, PFVA, PFPV, PFPT, and PFODBT) as the activator, and found a good correlation of CL quantum yield with the energy interval of the LUMO of TCPO and HOMO of semiconducting polymer (Figure 5b). By varying the activators, the CL quantum yields varies from 2.18 × 10⁻⁵ einsteins/mol to 2.3 × 10⁻² einsteins/mol. After an optimization of the CL quantum yield, a NIR dye (NIR775) was doped, and ultrasensitive detection of H₂O₂ in peritonitis models in vivo was achieved (Figure 5c).

Intramolecular CRET is a more common and efficient strategy to amplify the CL emission. In this strategy, a highly emissive fluorophore (energy acceptor) is attached to the weakly emissive CL skeleton (energy donor). Similar to the Förster Resonance Energy Transfer (FRET), CRET is a nonradiative energy transfer process between energy donor and energy acceptor. The energy of the CL donor in its excited state can be transferred to the acceptor in its ground state, resulting to the fluorescence emission of the energy acceptor. As shown in Figure 6, it is possible to efficiently populate the excited state of energy acceptor if the CRET is efficient and competitive to the nonradiative decay of the excited state of the energy donor.

To ensure efficient CRET, the selection of the fluorophore (energy acceptor) and the distance between the energy donor/acceptor are crucial. First, the two components should have good spectral overlap, that is, the emission spectrum of the CL skeleton and the absorption spectrum of the selected fluorophore should be overlapped. Second, as the energy transfer efficiency is highly dependent on the distance between the energy donor/acceptor, a short distance will ensure a highly efficient CRET. The intramolecular CRET (two components attached in one molecule) usually have a much higher efficiency than the two components mechanically mixed in the solvents.

Hence, intramolecular CRET strategy can be utilized to enhance CL emission.

Doran Shabat tethered highly emissive dyes such as fluorescein and quinone-cyanine to the dioxetane. Different from the parent dioxetane (Probe 1) that shows a typical weak CL emission of dioxetane at 470 nm (ΦCL = 0.033%), the fluorescein tethered dioxetane (Probe 2) and the quinone-cyanine tethered dioxetane (Probe 3) show dramatically enhanced CL at 535 nm (ΦCL = 0.38%) and 714 nm (ΦCL = 0.09%), corresponding to the emission of fluorescein and quinone-cyanine, respectively. This
result indicates that the CRET from the dioxetane to the dyes occurred. Inspired by the significantly enhanced CL quantum yield, the Probe 2 and Probe 3 were tested for the detection of β-galactosidase (Figure 6D–G). Upon addition of β-galactosidase in aqueous solution, Probe 2 shows brightest CL emission, followed by the Probe 3 and Probe 1 (Figure 6D). This is in well accordance with the CL quantum yields. However, in whole-body imaging in vivo, the Probe 3 shows highest CL signal due to its NIR character (Figure 6F), which highlights the benefit of long wavelength CL in bioimaging. The strategies to modulate the wavelength (color) of CL will be introduced in “Modulation of the wavelength of CL” section in detail. Anyway, the intramolecular CRET strategy has been proved to be effective on the improvement of the CL quantum yield.

Aggregation-induced emission (AIE)

As the bioimaging has to be conducted in physiological conditions, the fluorescence quantum yield of the emitter in aqueous solution has to be considered. Unfortunately, most of the organic compounds suffer from greatly reduced fluorescence emission due to aggregation in aqueous solution or the interaction of the excited fluorophores with surrounding water molecules. Same problem exists for CL emitters. Aggregation-induced emission (AIE) compounds are reported to show enhanced emission upon aggregation in aqueous solution, which solved the fluorescence quenching problem to some extent. Recently, this strategy has been utilized to enhance the CL emission in aqueous solution. Wang et al. reported a probe (TPE-CLA) based on an AIE platform for the detection of superoxide anion in dual mode (CL mode and fluorescence mode). As shown in Figure 7, imidazopyrazinone (CLA) is selected as reactive motif for the detection of superoxide anion in CL mode, and the tetraphenylethene (TPE) is selected as the AIE motif to enhance the fluorescence/CL emission. Upon addition of superoxide anion, the CL emission centered at 500 nm is turned on. As the maximum of CL emission of CLA is at 380 nm, the CL emission of the TPE-CLA is attributed to the TPE moiety, which is in consistent with the fluorescence emission spectrum of TPE. The probe was applied to image native superoxide anion in Raw264.7 cells and stimulated superoxide anion in inflamed mice, as well as endogenous superoxide anion induced by acetaminophen in HL-7702 cells. Compared with the CL of commercial CLA, the TPE decorated CLA (TPE-CLA) exhibits significantly higher CL intensity, which indicates the feasibility of this strategy to utilize the AIE moiety to enhance the CL emission in aqueous solution.

Self-immolative domino

Dendritic amplification is a concept that an initial stimulus triggers the disassembly of the dendrimer and release of large amount of dendrimer fragments. Suppose the number of the released dendrimer fragments is $N$, the signal can be amplified by $N$ folds. Akkaya et al. reported the self-immolative dendrons for CL signal amplification. However, multistep synthesis of dendrimers and large steric hindrance limit the number of tail groups. To address these challenges, self-immolative polymeric molecules are designed for CL amplification. As shown in Figure 8A, the polymer consists a fluoride-responsive substrate (silyl-phenolic ether) as head-trigger and ca. 20 repeat units of Schaap’s adamantylidene-dioxetane for CL emission. The monomer, dimer, and trimer are also synthesized and studied for comparison. The $^1$H-NMR spectra of the polymer upon addition of tetra-n-butylammonium fluoride (TABF) were recorded, unveiling the mechanism of the amplified CL. As the first step, the removal of the substrate by TABF triggered the quinone-methide elimination and lead to the disassembly of...
the polymer. Then the resulted monomeric units react with nucleophile (such as water molecule in the environment), followed by the decomposition through CIEEL and generation of CL emission. The polymer shows a significantly amplified CL output as compared with monomer, dimer, and trimer.

As shown in Figure 8C, different concentrations of the polymer (1 eq.) and monomer (20 eq.)/dimer (10 eq.)/trimer (6.5 eq.) are adopted to ensure the same amount of dioxetane units. Subsequently, same amount of TABF (1 eq.) is added to the polymer, trimer, dimer, and monomer, respectively. The CL intensity of each pair is recorded. As compared with the monomer, the polymer, trimer, and dimer show an CL intensity enhancement of 20 folds, 3 folds, and 2 folds, respectively. These results indicate that the amplification magnitude is highly related to the length of the chain (the number of the CL units). This self-immolative
domino-like polymer is proved to show pronounced CL amplification effect. The generality of this strategy was also demonstrated with other substrates (such as allyl-ether substrate activatable by Pd(0) and phenyl-boronic ester substrate activatable by hydrogen peroxide).

Autoinduction (positive feedback loop)

Autoinduction is used to amplify the fluorescence emission signal. Recently, this strategy is utilized to achieve CL amplification. As shown in Figure 8D, the DIO-F consists of three components: the fluoride-responsive substrate (OTBS) as head-trigger, Schaap’s adamantylidene-dioxetane for CL emission, and the internal fluoride ion source, which is responsible for the CL amplification. Upon recognition of the external fluoride ion, the probe undergoes the quinone-methide elimination and subsequently CL emission. This is accompanied with fresh fluoride ion (from the molecule) autoinduction, which initiates the CL of another molecule and lead to CL amplification. The triplet peaks at −202 and −200 ppm in 19F-NMR, which are characteristic peaks for benzylic fluoride, gradually disappeared after the addition of TBAF. This result further supports the elimination and release of fluoride ion. This self-supplementary and autoinduction dramatically lowered the limit of detection (LOD) to fluoride ions. The integrations of the CL of DIO-F incubated with different concentrations of TBAF (1 eq., 0.01 eq.) are found to be similar. Moreover, with same amount of fluoride ions added (0.001 eq.), the CL of DIO-F is 219-fold stronger than that of the reference CL probe without autoinduction effect involved. These results indicate that the autoinduction strategy successfully achieved the CL amplification. However, due to the high sensitivity, the background signal is also strong.

Modulation of the wavelength of CL

Compared with the light in short wavelength, NIR light has deeper tissue penetration ability due to the reduced light-tissue interactions. However, typical CL systems show CL emission in blue region. For instance, the CL emissions of luminol system, Schaap’s adamantylidene 1,2-dioxetane system, and imidazopyrazinone (CLA) system are centered at 425, 470, and 380 nm, respectively. Elegant efforts have been made to redshift the CL emission. Herein, this review summarizes those strategies and introduces their application in bioimaging.

Molecular skeleton

The most direct way to redshift the CL emission is through the structure modification of the CL skeleton. Once the π-conjugation of the CL skeleton is extended, the CL emission will be redshifted accordingly. For instance, the engineering of the wavelength of luminol CL has been achieved by replacing the benzene with a naphthalene core, and the standard blue (420 nm) CL emission is shifted to green (varied from 490 to 590 nm, depending on the substituted position of the amine group). Conjugation of the electron-withdrawing group (EWG) such as acrylic acid at the ortho position of the phenol not only leads to a redshifted emission in green region (~540 nm), but also an augmented CL intensity. Conjugation of the electron-donating group (EDG) dicyanomethylchromone at the para position of the phenol induces a further redshift to NIR region.
FIGURE 8 (A) Molecular structure and CL amplification mechanism and (B) total emitted photons of the monomer, dimer, trimer, and polymer, respectively. (C) Chemiluminescence signal obtained from incubation of tetra-n-butylammonium fluoride (TBAF) (50 μM) with polymer (50 μM), trimer (330 μM), dimer (500 μM), and monomer (1000 μM) in DMSO/H2O (99:1) at room temperature. (D) Autoinductive disassembly and chemiluminescence emission of DIO-F triggered by fluoride. Reproduced with permission: copyright 2017, American Chemical Society[74] (~690 nm).[82] The advantage of this redshifted CL emission is demonstrated by Pu et al.[83] The authors designed two renal-clearable near-infrared CL reporters (NCR1 and NCR2), responsive to O2•− and ONOO−, respectively, for the early detection of drug-induced acute kidney injury (AKI), which is at least 24 h earlier than histological analysis. In comparison with the green CL reporters (GCR1 and GCR2), the NIR CL sensors (NCR1 and NCR2) achieved deeper tissue-penetration and higher SBR (Figure 9B). On the basis of NIR phenol substituted dioxetane DPD-O reported by Shabat’s group, recently, Pu et al. reported a record long near-infrared chemiluminescent probe, by replacing the O atom with S or Se atom (DPD-S and DPD-Se, Figure 9F). The wavelength of CL is further redshifted to 780 nm (Figure 9G).[84]

However, although engineering the CL skeleton structure is the most direct way to redshift the CL, one must be careful as this engineering may have adverse impact on other parameters (such as chemiexcitation quantum yields).[32] Moreover, the derivatization of the CL skeleton may complicate the structures and require multisteps of synthesis.

CRET

CRET strategy has been widely adopted to modulate the wavelength of CL of luminol system,[87,88] peroxyoxalate system,[44] and 1,2-dioxetane system.[61]

For instance, as shown in Figure 1, the excited state of high-energy intermediate 1,2-dioxetanedione can be converted to the excited state of the activator through a CRET-like process. The wavelength of the CL emission of the peroxyoxalate system can be readily tuned by varying the activator. However, the two-component nature (peroxyxalate and activator) limits the use of peroxyoxalate CL. As the intermolecular CRET is less efficient than the
FIGURE 9  (A) Molecular skeleton of Schaap's adamantylidene 1,2-dioxetane which emits blue, green or red CL. (B) Chemiluminescence imaging of GCR1 (green CL dioxetane) and NCR1 (NIR CL dioxetane) in PBS after the addition of KO2 through different thickness of chicken breast tissues. (C) Signal-to-background ratios (SBRs) for green and NIR chemiluminescence as a function of tissue depth. (D) In vivo chemiluminescence images of GCR1 and NCR1 (upper panel), and GCR2 and NCR2 (lower panel) in the presence of O2− and ONOO−, respectively. (E) SBRs for green and NIR chemiluminescence images in panel (D). (F) Molecular structure and frontier molecular orbits of DPD-O, DPD-S, and DPD-Se. (G) Normalized chemiluminescence spectra of NCPx (x = O, S or Se) in the presence of ONOO−, which forms DPD-O, DPD-S, and DPD-Se respectively, in PBS. Reproduced with permission: copyright 2020 Wiley[83] and copyright 2020 Wiley[84].

intramolecular CRET, high concentration of the fluorophore or methodology of confining the two components in vicinity is required. To address this problem, “2-in-1” peroxyoxalate chemiluminescence molecules (PO–Tz) are designed.[89] As shown in Figure 10A, the oxalate moiety is embedded into the fluorescent pyrazoline through the nitrile-imine-mediated tetrazole-ene cycloaddition (NITEC) reaction, hence the peroxyoxalate CL probes (PO–Tz) are emissive and the wavelength of the CL emission can be modulated by varying the structure of the tetrazole (Tz). Upon the addition of H2O2, the probe PO–Tz shows bright CL emission observable by the naked eye, even in the absence of base (catalyst). This probe represents a good alternative to the conventional multicomponent peroxyoxalate CL systems. Finally, the probe was constructed onto the surface of polymeric microspheres, enabling solid phase CL read-out upon recognition of H2O2.

As the CRET has been introduced thoroughly in the previous section, this section will not make more examples. The readers can refer to literatures[44,61,87,88,91] and the previous “Chemiluminescence resonance energy transfer (CRET)” section for more details.

Conformational variation

It is not surprising that one molecule can emit in different colors, which may due to structural or conformational variation. A typical example is luciferin, which emits light of both green and red light in the presence of Beetle luciferase.[18] This phenomenon can be elucidated by the conformational variation to a twisted intramolecular charge transfer (TICT) excited state.[92] Another possibility is that the keto-enol tautomerization induces such a pronounced variation of color.[93]

CL wavelength modulation of a dioxetane bearing a 3-(anthracen-9-yl)-5-hydroxyphenyl moiety by controlling the
molecular conformation was reported. The experimental results show that the wavelength of the CL emission is highly dependent on the base added. For compound Dioxetane-AN, the addition of tetrabutylammonium tert-butoxide (TBA" ⋅ tBuO−) as the base lead to a crimson light with the emission centered at 709 nm. However, a complex of crown ether with tBuOK as the base will lead to a blue shifted CL emission. The magnitude of this blue shift is highly dependent on the steric hinderance of the crown ether of the base. For example, the base with bulky crown ether ligand shows significant larger shift (Δλ > 100 nm) than other bases. Moreover, only the dioxetanes with bulky substitution (Dioxetane-AN) show such a spectral emission shift while the less bulky one (Dioxetane-H) does not show significant change. These results indicate that the color change of the CL is related to the steric effect. The author proposed that the bulky substituents lead to a smaller twisted angle between anthracene and phenolic rings (Figure 10C). The calculation results in Figure 10D show that a decrease of the twisted angle from 70° to 30° will lead to an increase of energy gap (ΔE = ELUMO − EHOMO); hence, a blue shift of the CL emission was observed.

CHEMILUMINESCENCE INITIATED PDT

Conventional PDT requires three key elements: triplet photosensitizer (PS), oxygen, and an external light source. Upon shedding light on the tumor, the triplet photosensitizer is promoted to its triplet excited state and reacts with molecular oxygen to produce singlet oxygen (1O2), which finally kills the tumor cells (Figure 11). However, due to the limited penetration ability of red light or NIR light, till now the PDT is still limited to the treatment of superficial disease such as skin cancer. External light source-free strategy such as CL initiated PDT is of great interest and has the potential for the treatment of deep tumors. However, relative report is rare. Herein, this review will make a precise summary and critical comments on this issue.
Luminol CL system has been widely utilized as the light source of PDT.\textsuperscript{[10,11,94,95]} For instance, Wang et al. reported a luminol-cationic oligo(p-phenylene vinylene) (OPV) system (Figure 12A). The CL of luminol in the presence of hydrogen peroxide and HRP ranges from 375 to 550 nm, which is overlapped with the absorption of OPV (in the range of 350–550 nm). Moreover, the electronic interaction between the luminol dianion (the oxidation product of luminol in CL reaction) and the OPV cation may shorten the distance between the two components, which is beneficial for an efficient CRET. Hence the photosensitizer OPV can be successfully excited by the CL of luminol. Moderate (30\%) inhibition of tumor was observed for the treatment group (luminol CL system with photosensitizer OPV) as compared with the reference group (luminol CL system without photosensitizer OPV). However, the luminol CL system is intrinsically toxic (Figure 12A), which is actually not the ideal CL system of biological application.\textsuperscript{[11]}

Intermolecular CRET is less efficient as it is difficult to ensure all the components (photosensitizer and the chemiluminescence components luminol, H$_2$O$_2$, HRP) present in the same place, hence high concentration of photosensitizer is usually required.\textsuperscript{[94,95]} To solve this problem, Akkaya et al. designed the erythrosine-luminol conjugate, which combine the chemiluminescence part and photosensitizer into one molecule (Figure 12B).\textsuperscript{[12]} The embryo of this molecule is the fluorescein-luminol conjugate reported by Burgess, for which efficient through bond and through space CRET was observed.\textsuperscript{[87]} Due to the presence of iodine atoms, erythrosine is proved to be an efficient triplet photosensitizer. Upon addition of CuSO$_4$ and H$_2$O$_2$, CL of luminol is initiated and the singlet excited state of erythrosine is generated through intramolecular CRET. Subsequently, triplet excited state of erythrosine is populated, yielding the singlet oxygen in a quantum yield of 4.2\%. This moderate singlet oxygen quantum yield is reasonable as the efficiency of this multistep process depends on both the CRET efficiency and intersystem crossing (ISC) efficiency. As high concentration of H$_2$O$_2$ presents in tumor cells, the erythrosine-luminol conjugate has the potential to be applied to external light source-free PDT of deep tumor.

Previously reported CL initiated PDT reagents involve two parts, that is, CL moiety and triplet photosensitizer moiety. Multisteps are involved and intermolecular (or intramolecular) CRET efficiency greatly affects the PDT efficacy. da Silva et al. reported a molecule (Br–Cla), where CL moiety can also act as triplet photosensitizer (Figure 13).\textsuperscript{[9]} This is achieved by attaching bromine atoms to the coelenterazine, which emits CL in the presence of superoxide anion. Upon the recognition of superoxide anion, the triplet excited state of Br–Cla is directly chemiexcited and populated. The CL excited PDT efficacy was examined in tumor cells and normal cells in vitro. Selective PDT to tumor cells is achieved with a lethal concentration (IC$_{50}$) of 0.1 $\mu$M, while no toxicity is observed for normal cells. However, the PDT efficacy in vivo is not reported. More importantly, although the CL is initiated selectively in tumor cells while not in normal cells,
there is still a great concern that the uncontrollable and long duration characters of CL might cause high phototoxicity to the normal tissues/organs during the subsequent circulation in the body.

SUMMARY AND OUTLOOK

CL is an interesting phenomenon that has attracted tremendous investigation. This review introduces the mechanism of CL and its application in bioimaging and therapy (to be specifically, herein is PDT). Concerning to the external excitation-free character, higher SBR and sensitivity are expected. Moreover, this excitation source-free strategy may break through the limitation of PDT being just for superficial tumors.

However, the development of CL is still in its infancy, despite of the long history since its discovery. First, the mechanism of CL has not been well understood. Although the CIEEL is generally accepted as the mechanism of CL, there is argument on whether fully electron transfer or partial charge transfer occurred. Moreover, due to the instability of the intermediates of the CL reaction, the characterization of those intermediates is difficult. Hence even the mechanism of some typical CL systems is not fully unveiled.

Second, concerning the bioimaging, a versatile CL skeleton with high CL quantum yield under physiological condition is crucial. However, the general CL system such as peroxoxalate system is limited to the detection of reactive oxygen species and specific enzymes. 1,2-Dioxetane is more versatile but has weak CL emission ($\Phi_{CL} < 10\%$). Although elegant works have been done to improve the CL quantum yield as summarized in this review, the brightness of current CL systems is still unsatisfying. It is clear that the CL quantum yield is determined by the chemiexcitation quantum yield and the fluorescence quantum yield. However, the relationship between the chemiexcitation (or fluorescence) quantum yield and the molecular structure is unclear. Hence, much room is left for physical chemists and theoretical experts. A better understanding on these issues is crucial for the improvement of the current CL systems and the development of new CL systems.

Last but not the least, although CL initiated PDT is interesting as it is external excitation-free, there is still a great concern to its toxicity to normal tissues during the subsequent circulation in the body. Moreover, this CL initiated PDT loses the advantage of photoselectivity of PDT, as conventional PDT can be controlled by light irradiation while the CL-induced PDT is uncontrollable as soon as it is initiated. In addition, it is still an open question that whether it is feasible to use CL to initiate the sensitization, as the CL is actually very weak. Hence, these problems limited the potential of this approach at conception.

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ETHICS STATEMENT
This review article does not involve any human investigation and animal experiment.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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