A review of enhancers for chemiluminescence enzyme immunoassay

Ge Chen, Maojun Jin, Pengfei Du, Chan Zhang, Xueyan Cui, Yudan Zhang, Jing Wang, Fen Jin, Yongxin She, Hua Shao, Shanshan Wang and Lufei Zheng

Key Laboratory for Agro-Products Quality and Food Safety, Chinese Academy of Agricultural Sciences, Institute of Quality Standards & Testing Technology for Agro-Products, Beijing, People’s Republic of China

ABSTRACT
There is increasing interest for chemiluminescence (CL) detection with the characteristics of simplicity, low cost and high sensitivity, especially wide application of enhancers in CL detection to increased signals, prolonged luminescence time and enhanced intensity. In this review, the applications of primary enhancers and secondary enhancers were mainly described in the horseradish peroxidase–luminol system of CL enzyme immunoassay for light delay in the course of the reaction with improved sensitivity. The present review on enhancers covers the papers since 1983. Future research needs to develop novel enhancers with less interference and better performance. With wide utilization of enhancers in the CL system, the CL immunodetection technology showed a good potential and wide development prospects in food and medicine fields.

1. Introduction

In 1977, Halman first established chemiluminescence (CL) immunoassay to detect micro-organisms by combining the CL method with immunoassay (Halman, Velan, & Sery, 1977). CL immunoassay involves highly selective immunoassay and extremely sensitive CL analysis, with the latter characterized by a wide linear range and low background noises with cheap equipment but a lack of selectivity (Lin, 2008). The CL mechanism is caused by energy release in a chemical reaction, which triggers light generation. Without external light sources, CL exhibits many advantages, such as excellent sensitivity, a low limit of detection (LOD) and a wide operating range (Duan, Cai, Luo, Chen, & Zou, 2015). This method not only avoids the pollution risk of radioimmunoassay caused by radioactive isotope employment, but also overcomes the disadvantages of complex instruments and background interference. It has drawn close attention of researchers for its high sensitivity, convenience, safety, low price and low radioactivity (Liu, Lin, & Lin, 2010). Therefore, CL has been widely used in a number of fields, including chemistry (Tian et al., 2015), pharmacy (Palmiere et al., 2015), molecular biology (Bouchez-Mahiout et al., 2015) and many others.
et al., 2010), clinical medicine (Luo, Lin, Zheng, Lin, & Chi, 2015; Thompson, Blaszykowksi, Sheikh, & Romaschin, 2015), contamination residues (Du, Zhang, Zhang, Wen, & Saren, 2014; Jin et al., 2013), environmental science (Hochel, Viochna, & Musil, 2003) and food science (Li et al., 2015; Zhang, He, Chen, & Huang, 2013). However conventional CL immunoassay is defective with weak signals, low intensity and short luminescence time. In order to improve the luminescence signals and sensitivity, an enhancer was added to the CL immunoassay, which plays a role of mediators in the peroxidase catalysis of CL reaction. The enhancers do not act as more efficient emitters but exert their action earlier in the complex reaction between peroxidase, oxidant and luminol (Thorpe, Kricka, Moseley, & Witehead, 1985). When used with an enhancer, CL immunoassay becomes advantageous with increased signals, prolonged luminescence time and enhanced intensity (Dotsikas & Loukas, 2004). Compared to conventional enzyme immunoassay, this technique is more frequently used due to cheap instruments and reagents, rapid and simple operations, and reliable test results. Enhanced chemiluminescence enzyme immunoassay (CLEIA) has the lowest LOD of 10^{-18} to 10^{-15} mol L^{-1}, which improves the sensitivity by three to five orders of magnitude when compared with traditional enzyme immunoassay (Bao et al., 2004; Yang et al., 1994).

As defined in previous studies, CL immunoassay causes luminescence materials catalyzed by horseradish peroxidase (HRP) and alkaline phosphatase (ALP) using oxidants to actively luminesce during a transition from the excited state to the ground state. Typical results show weak luminous intensity, and therefore some oxidants or compounds are required to enhance the intensity of luminescence materials to improve instrumental testing results and increase its sensitivity (Lu, 2015).

In the study of enhanced CL reaction (Easton, Simmonds, Rakishev, Egorov, & Candeias, 1996; Lind, Merenyi, & Eriksen, 1983; Vlasenko et al., 1989), a simultaneous oxidation mechanism was postulated for luminol and an enhancer, complying with multiple enzyme-triggered oxidative reactions of the enhancer happening at the first stage according to the “ping-pong” mechanism.

\[ E + H_2O_2 \Rightarrow EI, \]
\[ EI + SH \Rightarrow EII + S^*, \]
\[ EII + SH \Rightarrow E + S^*. \]

where SH is the substrate enhancer, S* is the radical oxidation product of the substrate enhancer, and E, EI and EII are different types of peroxidase (Sakharov & Vdovenko, 2013).

The common enhancers introduced of this review were phenolic and its derivatives, N-phenoxazine derivatives, D-luciferin, metallic ion and other enhancers. Based on previous studies, the phenolic and its derivatives enhancer (especially 4-phenolic containing halogen of the phenolic) activities are relatively high efficient than other enhancers because of the compounds containing phenoxide group to catalyze the CL reagents oxidation reaction. 4-phenolic containing halogen of the phenolic and its derivatives is the potent enhancer in future CLEIA. In this review, the CL system with the enhancer applications of HRP–luminol and ALP–AMPPD was mainly described in food and medicine fields.
2. Mechanism of the enhanced HRP–luminol CL system

HRP–luminol CL reaction was performed as follows: luminol catalyzed to the ground state by HRP and an enhancer in alkaline and oxidant condition was accompanied by light quantum emission. The mechanism of HRP–luminol-based CL reaction is shown in Figure 1.

Sakharov and Vdovenko (2013) have proposed hypothesis of the mechanism of co-enhancer. In the mechanism of co-enhancer based on the mechanism of HRP–luminol-based CL reaction, the secondary enhancer accelerates peroxidase-catalyzed primary enhancer by hydrogen peroxide to produce cation radical for increasing CL intensity.

Luminol is a low-cost substrate, and HRP is the most extensively used enzyme conveniently and cheaply acquired from enzyme immunoassay. This assay has relatively low luminous efficiency as a disadvantage, which requires special chemical materials for luminous intensity enhancement. Enhancers can strengthen luminescence signals, delay the luminescence reaction and therefore improve the detection sensitivity, stability and simplicity of CLEIA (Yu, Sheng, Zhao, & Fan, 2016).

2.1. Applications of the enhanced HRP–luminol system

2.1.1. Primary enhancers and their applications

Many enhancers have been applied in CLEIA in researches. Original enhancers include the smoked stone (Johnson, 1989), surface-active groups and aromatic substituent groups. However, their efficiency is unsatisfactory, and the luminescence time is not extended as expected. In recent years, a number of high-efficiency enhancers have been proposed in many fields.

Whitehead, Thorpe, Carter, Groucutt, and Kricka (1983) have added D-luciferin (4,5-dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazole-carboxylic acid) to HRP-catalyzed luminescence of a cyclic hydrazide for enhancing HRP-catalyzed light emission. The enhanced assays have been applied to the immunoassay of serum α-fetoprotein, thyroxine, digoxin, hepatitis B surface antigen, immunoglobulin E and rubella virus antibody (Whitehead et al., 1983).

![Figure 1](image-url)  
*HRP: horseradish peroxidase; SH: substrate enhancer

**Figure 1.** Mechanism of enhanced CL reaction-catalyzed HRP.
There is an increasing interest in phenolic derivatives as the enhancers of CL reaction. Thorpe et al. (1985) studied the phenols as the enhancers of the CL HRP–luminol–H₂O₂ reaction. The emission was enhanced up to 2500-fold with adding p-iodophenol or p-phenylphenol as compared with the unenhanced reaction. The phenols were applied to CL immunoassay for human choriogonadotropin digoxin and factor VIII-related antigen in their study.

Zhao (2001) has proposed p-phenolic derivatives as CLEIA enhancers, including 4-tert-butylphenol and 1-methyl-4-hydroxybenzene. The strongest peak of 4-tert-butylphenol occurred at about 30–60 s, and that of the other was displayed at about 30–75 s. The luminescence intensity was relatively stable until 3 min.

Yang et al. utilized 4-(4′-iodo) phenylphenol (IPP) as a novel signal enhancer to combine with double-codified gold nanoparticles (DC-AuNPs) modified with HRP-conjugated anti-alpha-fetoprotein (AFP) to detect AFP. The results demonstrated that the enhanced CL method combined with AuNPs used as an anti-AFP–HRP carrier achieved outstanding sensitivity and provided a linear range of AFP from 0.008 to 0.3 ng mL⁻¹ with an excessively low LOD of 5 pg mL⁻¹, much lower than the LOD of classical enzyme-linked immunosorbent assay (ELISA). The new system could be easily converted into many immunodetection methods or DNA analysis (Yang, Guo, Bi, & Zhang, 2009). 4-(1, 2, 4-triazole-1-yl) phenol (TRP) as a fresh enhancer in the luminol–H₂O₂–HRP system was investigated in Yang, Guo, and Mei (2009) to detect H₂O₂ with immobilized HRP using magnetic beads as a solid carrier. The results showed that the wide linear range for H₂O₂ was 2.0×10⁻⁶ g mL⁻¹ to 1.0×10⁻³ g mL⁻¹ and the detection limit of H₂O₂ was 2.0×10⁻⁶ g mL⁻¹, and that it could be developed into a sensor to detect H₂O₂ in rainwater. Compared to the conventional enhancer 4-iodophenol (PIP) in immunoassay, the luminescence intensity of the technique was better and the luminescence time was longer with the addition of TRP. 4-Iodophenol (PIP) effectively functioned as an enhancer in HRP-catalyzed luminol CL in liposomes. They applied 4-iodophenol as an enhancer in direct detection of HRP encapsulated in liposomes by using luminol CL. The result revealed that the CL intensity observed in liposomes was a factor of 150 greater than a lipid-free bulk solution. Compared with a lipid-free bulk solution, the detection limit in direct detection of HRP encapsulated in liposomes was more sensitive (Kamidate, Maruya, Tani, & Ishida, 2009).

Yan-li et al. (2012) studied the effects of luminol signal enhancers on the CL intensity and kinetics in three p-phenol derivatives, which were 4-(1-imidazolyl)-phenol (4-IMP), 4-hydroxybi-phenyl (4-BIP) and 4-hydroxy-4′-iodobiphenyl (HIOP). The employment of a p-phenol derivative as an enhancer in a luminol–H₂O₂–HRP system can result in dramatic changes of CL intensity and kinetics, as well as the characteristics of an assay (detection limit and concentration range). Compared with 4-IMP and HIOP, 4-BIP had superiority in the stability of CL. The enhancer that produces the most intense signal also produces the most sensitive assay. Therefore, 4-BIP was a more useful enhancer of the luminol–H₂O₂–HRP CL system in future research.

Kim, Kim, Rho, and Lee (2014) explored the role of p-iodophenol in enzyme assay and developed enhanced 1, 1′-oxalylidimidazole chemiluminescence enzyme immunoassay (ODI-CLEIA) to continuously quantify the trace levels of triple tumor markers. The results demonstrated that the CL signal intensity with the addition of p-iodophenol was about ninefold higher than that without p-iodophenol. The enhanced ODI-CLEIA
could consecutively and quickly quantify triple markers using the same incubation time, proving to be more sensitive than conventional ELISA, which was capable of quantifying them separately and slowly with different incubation time. In addition, the accuracy, precision and recovery of the enhanced ODI-CLEIA with the existence of \( p \)-iodophenol were satisfactory under a statistical error range. In the research of ELISA by enhanced CL detection for standardization of estrogenic miroestrol (ME) in Pueraria candollei Graham ex Benth, Yusakul et al. (2015) developed enhanced CL enzyme-linked immunosorbent assay (ECL-ELISA) in the presence of 4-(1-imidazolyl) phenol as an enhancer. Compared with the LOD (0.73 ng mL\(^{-1}\)) of the colorimetric ELISA, the IC\(_{50}\) value of ECL-ELISA was 3.47 ng mL\(^{-1}\), which improved the colorimetric ELISA (IC\(_{50} = 147.09\) ng mL\(^{-1}\)). The IC\(_{50}\) value was decreased from 147.09 ng mL\(^{-1}\) in colorimetric ELISA to 3.47 ng mL\(^{-1}\) in ECL-ELISA. The ECL-ELISA system showed linearity within a concentration range of 0.31–10.00 ng mL\(^{-1}\), for which the RSDs were both less than 10% for intra- and inter-plate determination. The ECL-ELISA was reliable for the determination of ME, being proved by a high recovery percentage (101.22–103.06%). The result revealed that the 4-(1-imidazolyl) phenol application in CL-ELISA enhanced the sensitivity and accuracy of ECL-ELISA to determine the trace level of ME in the biological fluids of experimental animals and humans for pharmacokinetic investigations.

Among the phenol enhancers, the enhancing ability of common phenol enhancers with halogen elements at the para-position of phenol (such as IPP and BIP) was better than the enhancer with other groups at the para-position attributed to the different 4-substituent group. That because of the \( p \)-substituent is considered to have two functions. One is increasing the electron density on phenolic hydroxyl oxygen by its electron donating nature. The other function is to localize the radical electron on the \( p \)-position by the inductive effect of the para-position. Halogen elements as electron donors group at the para-position of phenol have a great influence on the O-H key dissociation of the phenol group, further influencing the stabilization of the phenoxy radical (Yang et al., 2015).

The N-alkylated phenothiazine also could be used as an enhancer in luminol–oxidant–HRP system. N-alkylated phenothiazine is a highly advantageous enhancer and increases light output (up to 10-fold) observed in their presence can be translated into a corresponding improvement in sensitivity of chemiluminescent assays. The intensity of the light emission can be further increased by adding redox mediators such as phenothiazines and lowering the LOD for HRP from 50 to 8 amol (Marzocchi et al., 2008).

N-alkyl phenothiazines with different ionic groups were studied as the enhancers of CL catalyzed by soybean peroxidase in Vdovenko, Vorobiev, and Sakharov (2013). The CL intensity was improved with the addition of the co-enhancer SPTZ and MORP. Under the enhanced CLEIA system, the analytical parameters decreased the lower detection limit (LDL) of SbP to 0.03 pM. The enhancer provided new opportunities for the increase in the sensitivity of analyte determination by CLEIA. Sakharov et al. (2013) reported that 3-(10’-phenothiazinyl) propionic acid (PPA) was a strong primary enhancer for peroxidase-induced CL, which was applied to sensitive chemiluminescent ELISA the for determination of methylglyoxal-modified low-density lipoprotein. Using PPA as an HRP–luminol–\( \text{H}_2\text{O}_2 \) enhancer, the developed method achieved a low LOD value of 0.5 ng mL\(^{-1}\) and opened up very promising perspectives to improve the sensitivity of enzyme immunoassay kits.
3-(10′-phenothiazinyl)-propane-1-sulfonate was used in the HRP–luminol–H₂O₂ system of CLEIA. In this study, the signal intensity of CL was improved and CL kinetics extended the emission time to 30 min. The stability and the repeatability were better than the unenhanced the CL immunoassay. In addition, 3-(10′-phenothiazinyl)-propane-1-sulfonate used as an enhancer was soluble in water and convenient to apply in immunoassay. The result demonstrated that the 3-(10′-phenothiazinyl)-propane-1-sulfonate as an enhancer has a wide application prospect in CLEIA and Western blot (Qiu et al., 2011).

Ichibangase et al. evaluated lophine derivatives as L-012 (luminol analog)-dependent CL enhancers for measuring HRP and H₂O₂ in their study. In their previous research, four lophine-based CL enhancers of the HRP-catalyzed CL oxidation of luminol were developed, namely 2-(4-hydroxyphenyl)-4,5-diphenylimidazole (HDI), 2-(4-hydroxyphenyl)-4,5-di(2-pyridyl)imidazole (HPI), 4-(4,5-diphenyl-1H-imidazol-2-yl)-phenylboronic acid (DPA) and 4-[4,5-di(2-pyridyl)-1H-imidazol-2-yl]-phenylboronic acid (DPPA). In the study, they discovered the strongest enhancement with HPI resulted from a lower blank emission compared with that of 4-iodophenol. HPI was therefore the best L-012-dependent CL enhancer, and each optimized condition using HPI as the L-012-CL enhancer could be successfully applied (Ichibangase, Ohba, Kishikawa, Nakashima, & Kuroda, 2014).

Feng et al. applied phenolphthalin in the HRP–luminol–H₂O₂ system of CLEIA in detection, improving the intensity and extending the luminescence time to 30 min and acquired a low LOD (1.25 pg mL⁻¹). Phenolphthalin as an enhancer has advantages, such as low-cost and convenience, over the other enhancers. The CL immunoassays were more stable and precise with the addition of phenolphthalin as the enhancer for the actual sample analysis (Feng, Cai, Li, Li, & Cao, 2014).

Other than organic phenols and their derivatives as single enhancers, metallic ions could be used as enhancers in CLEIA research. For example, Coteur and Dubois (2004) have discovered zinc enhancement on the signal of luminol-enhanced CL in the research of metal interactions with peroxidase-mediated luminol-enhanced CL. Zinc stabilized the luminol radical, increasing the half-life of this radical in the medium. Pal and Bhand (2015) utilized zinc oxide nanoparticle to enhance CL signals of luminol and LOD for the determination of carcinoma embryonic antigen (CEA) in human serum. The CL signal of luminol tripled over the conventional ELISA and the limit of quantitation became as low as 1 pg mL⁻¹. The single enhancer application is listed in Table 1.

2.1.2. Applications of secondary enhancers

A number of single enhancers were successfully used in the enhancement of peroxidase-induced CL, and the co-enhancer of the luminol–H₂O₂–HRP CL system currently showed high sensitivity and good linearity. An increasing number of enhancers have been applied in CLEIA research.

Tao et al. (2014) used an enhancer in the development of highly sensitive CLEIA as the substrate in their study. They designed catalysis based on enhanced chemiluminescence reaction (ECR) by horseradish peroxidase (HRP-C) in the presence of the enhancers 3-(10′-phenothiazinyl)-propane-1-sulfonate (SPTZ) and 4-morpholinopyridine (MORP). The signal was enhanced significantly and the light emission time was extended. The SPTZ and MORP had synergistic effects in the HRP–H₂O₂–luminol system of CLEIA. In their study, the MORP is a secondary enhancer to assist SPTZ to promote light
emission. The LOD of this new chemiluminescent cocktail is 0.33 pg/well for HRP, lower than that of the commercial super signal substrate. The results demonstrated that the chemiluminescent cocktail could significantly boost the light output of HRP-catalyzed ECR and therefore improved the sensitivity in the presence of the co-enhancer of SPTZ and MROH.

SPTZ and MORP were used as enhancers in Vdovenko, Demiyanova, Chemleva, and Sakharov (2012) to improve the sensitivity of CLEIA. The sensitivity of HRP-C determination was improved by 2355-fold without a change in the LOD and the enhancers were applied to phenol determination using CL produced upon HRP-catalyzed oxidation of luminol. The phenol assay with SPTZ and MORPH not only had much higher sensitivity and an LDL value than the assay without enhancers, but also provided the concentration of HRP 40 times less than the assay without them. These results indicated that the synergistic effect of SPTZ and MORPH was widely applied in assays (Vdovenko, Papper, Marks, & Sakharov, 2014). A direct competitive CL-ELISA was proposed for detecting aflatoxin M1 (AFM1). In this research, a mixture of SPTZ and MORPH was used as the co-enhancer to intensify peroxidase-induced CL for AFM1 detection. The low LOD value and dynamic operating range of CL-ELISA for AFM1 were 0.001 ng/mL and 0.002–0.0075 ng mL\(^{-1}\), respectively, and the measurements of AFM1 at the concentrations below the maximum acceptable limit were allowed. The recoveries of intra- and inter-assay were 81.5–117.6% and 86–110.6%, respectively. The result demonstrated that ultra-sensitive CL-ELISA was developed in the presence of SPTZ and MORPH (Vdovenko, Papper, Marks, & Sakharov, 2014). Luo et al. (2002) studied the synergistic strategy of sodium tetraphenyl borate (NaTPB) and para-phenylphenol (PPP) on CL. In their study, the enhanced efficiency was the strongest when the concentrations of NaTPB and PPP were 6 \times 10^{-4} \text{ mol L}^{-1} and 4 \times 10^{-4} \text{ mol L}^{-1}, respectively. When flow injection chemiluminescence (FI–CL) was used for the determination of the CLEIA system, the LOD could reach 8.46 \times 10^{-12} \text{ g mL}^{-1}. The sensitivity and repeatability of this method were better than traditional methods. This research showed that NaTPB and PPP on CL had a synergistic effect.

### Table 1. The application of single enhancer in HRP CL system.

| Enhancer | Target analytes | Luminescence signal | Time | LOD | Ref |
|----------|-----------------|---------------------|------|-----|-----|
| 4-tert-butylphenol and p-cresol | — | Enhanced 20 times | Prolonged | — | Zhao (2001) |
| 4-(4-iodo) phenylphenol | α-fetoprotein | Enhanced | Prolonged | 5 pg/mL | Yang, Guo, Bi, et al. (2009) |
| 4-(1,2,4-triazole-1-yl) phenol | H\(_2\O\) | Enhanced | Prolonged | 2.0 \times 10^{-6} M | Yang, Guo, Bi, et al. (2009) |
| 3-(10'-Phenothiazinyl) propionic acid | Methylglyoxal-modified low density lipoprotein | Enhanced | Prolonged | 0.5 ng/mL | Sakharov et al. (2013) |
| 3-phenothiazinepropyl sulfate | p-iodophenol | Enhanced | Prolonged 30 min | — | Qiu et al. (2011) |
| 4-(1-imidazolyl) phenol | Tumor markers(AF, CEA, PSA) | Enhanced 9 times | Prolonged | — | Kim et al. (2014) |
| zinc | Miroestrol | Enhanced | Prolonged | 0.73 ng/mL | Yusakul et al. (2015) |
| Zinc oxide nanoparticle | The vitro contaminants | Enhanced | Prolonged | 20 ng/mL | Coteur and Dubois (2004) |

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In the study of bromophenol red and bovine serum albumin (BSA) as the luminol signal co-enhancers in chemiluminescent detection of sequence-specific DNA, the CL signal was increased up to $6 \times 10^4$ when bromophenol red and BSA were placed into the luminol–$\text{H}_2\text{O}_2$–HRP system as the co-enhancers. The results indicated that BSA in the bromophenol red-enhanced CL system showed 36 times stronger CL signals than that without BSA addition. In this study, BSA played a role in the bromophenol red-enhanced CL system. The enhanced effect of bromophenol red was compared with that of PIP, which is a commonly used enhancer in the luminol–$\text{H}_2\text{O}_2$–HRP system. Therefore, sequence-specific DNA based on a magnetic separation process was detected by the novel luminol–$\text{H}_2\text{O}_2$–HRP–bromophenol red–BSA CL system. The LOD of the target DNA was 0.4 fmol, which was sensitively detected using the proposed CL system without any amplification process. The novel luminol–$\text{H}_2\text{O}_2$–HRP–bromophenol red–BSA CL system might be a promising perspective for the sensitivity improvement in CL detection of sequence-specific DNA using bromophenol red and BSA (Yu et al., 2016). The co-enhancer applications are listed in Table 2.

### Table 2. The application of co-enhancer in HRP CL system.

| Enhancer | Target analytes | Luminescence signal | Time | LOD | Ref |
|----------|-----------------|---------------------|------|-----|-----|
| 3-(10′-phenothiazinyl)-propane-1-sulfonate and 4-morpholinopyridine | Chloramphenicol | Enhanced | Prolonged | 1.6 ng/L | Tao et al. (2014) |
| 3-(10′-phenothiazinyl)-propane-1-sulfonate and 4-morpholinopyridine | Aflatoxin M1 | Enhanced | Prolonged | 0.001 ng/mL | Vdovenko, Lu, et al. (2014) |
| 3-(10′-phenothiazinyl)-propane-1-sulfonate and 4-morpholinopyridine | Phenol | Enhanced | Prolonged | — | Vdovenko, Papper, et al. (2014) |
| 3-(10′-phenothiazinyl)-propane-1-sulfonate and 4-morpholinopyridine | — | Enhanced | Prolonged | 0.25 pM | Vdovenko et al. (2012) |
| sodium tetrphenyl borate (NaTPB) and para-phenylphenol (PPP) | — | Enhanced | Prolonged | 8.46$\times 10^{-12}$ g/mL | Luo et al. (2002) |
| bromophenol red and BSA | Sequence-specific DNA | Enhanced | Prolonged | 0.4 fmol | Yu et al. (2016) |

3. **The principle and application of the enhanced ALP–AMPPD CL system**

The ALP–AMPPD CL reaction worked under the principle that 3-(2′-spiro adamantane)-4-methoxy-4-(3′-phosphoryloxy)phenyl-1,2-dioxetane catalyzed to the ground state by ALP and the enhancer in alkaline conditions was accompanied by light quantum emission. The principle of this CLEIA is illustrated in Figure 2.

ALP marked in CLEIA was widely employed in enzyme-linked immunoassay and nucleic acid hybridization analysis. ALP and AMPPD implemented the most important and selective CLEIA. Yang, Wang, Jiang, Bi, and Ma (1995) have studied 5-(N-tetradecanoyl)-amino-fluorescein and hexadecyltrimethylammonium bromide in their study. The results demonstrated that 5-(N-tetradecanoyl)-amino-fluorescein and hexadecyltrimethylammonium bromide could improve the luminescence intensity by about 400 times as a novel complex enhancer.
4. Applications of other enhancers

Besides the HRP–luminol–H₂O₂ and ALP–AMPPD systems, there are other systems in CLEIA applications. For example, some enhancers are catalyzed in soybean peroxidase. In Vdovenko, Ciana, & Sakharov (2009), 3-(10′-Phenothiazinyl) propane-1-sulfonate (SPTZ) was shown to be a potent enhancer of soybean peroxidase (SbP)-induced CL. The SbP–SPTZ system showed better sensitivity and an LDL with respect to the HRP–4-iodophenol system traditionally used in chemiluminescent enzyme-linked immunosorbent assay (ELISA). In another study of Vdovenko et al. (2010), the SbP/SPTZ/MORPH and HRP/PIP system was developed to detect serum thyroglobulin (Tg) in human serum. According to the comparison of these two systems, the LOD for CL-ELISA with SbP/SPTZ/MORPH was 10 times lower than that for the immunoassay with HRP/PIP. The SbP-based CL-ELISA showed a good correlation between these two immunoassays (\(y = 1.15x - 0.14; R = 0.99\)). The obtained results indicated good perspectives for the use of the SbP/SPTZ/MORPH system in the development of ultrasensitive immunoassays.

5. Conclusions and future perspectives

With the applications of enhancers in CLEIA, the sensitivity and accuracy of this method were enhanced. The enhancers had many forms, including the single enhancer, co-enhancer, and even a sensitizing solution and were widely employed in CLEIA. Unlike expensive AMPPD and acridinium ester compounds, HRP–luminol was widely employed in food processing and medical fields because of convenient luminol oxidation at a low cost. However, the enhancers had some disadvantages in practical applications. (1) The stability of the enhancers is not excellent. (2) The luminescence time attenuates extremely fast. (3) The background interference is strong. Therefore developing novel enhancers with less interference and better performance will be aimed in future research.

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**Notes on Contributor**

**Ge Chen** is responsible for designing the structure of the paper and writing paper.

**Maojun Jin** is responsible for designing the structure of the paper and writing paper.

**Pengfei Du** is responsible for searching the references of CL principle.

**Chan Zhang** is responsible for searching the references of CL principle.

**Xueyan Cui** is responsible for searching the references of CL principle.

**Yudan Zhang** is responsible for searching the references of CL principle.

**Jing Wang** is responsible for searching the references of the HRP-Luminol system application.

**Fen Jin** is responsible for searching the references of the HRP-Luminol system application.

**Yongxin She** is responsible for searching the references of the HRP-Luminol system application.

**Shangshang Wang** is responsible for searching the references of the ALP system application.

**Lufei Zheng** is responsible for searching the references of the ALP system application.

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