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ORIGINAL PAPER

Chemiluminescence of horseradish peroxidase in water–ionic liquid microemulsion: an approach from catalytic point of view

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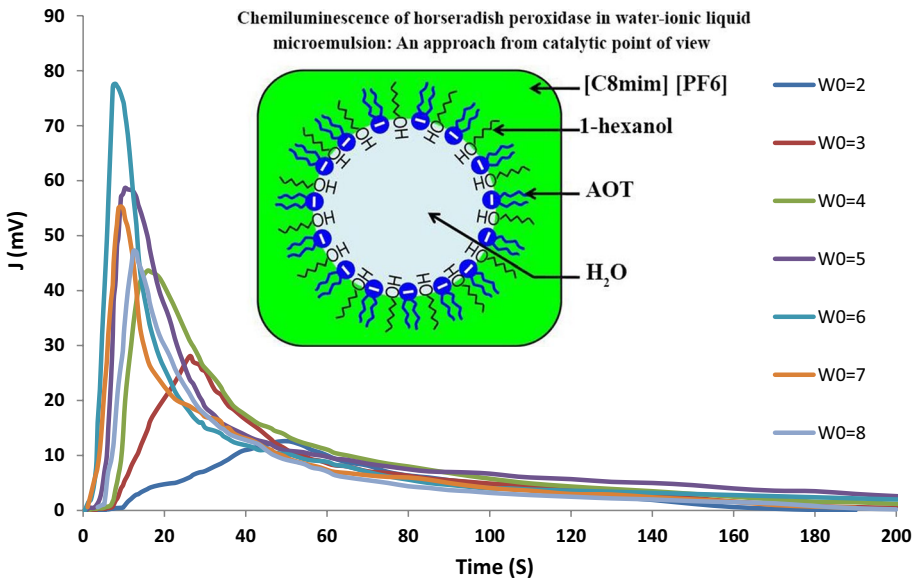
Abstract

Microemulsions of ionic liquids (ILs) are added to enhance the activity of peroxidase. From a catalytic standpoint, we provide the first research of the chemiluminescence (CL) features of luminol microencapsulated with horseradish peroxidase (HRP) in water–ionic liquid (W/IL) microemulsions. Water/AOT (sodium bis (2-ethyl-1-hexyl) sulfosuccinate)/hydrophobic IL [C8mim] [PF6] (1-octyl-3-methylimidazolium hexafluorophosphate)/1-hexanol constituted the system. Experiments demonstrated that the CL kinetic parameters, such as pseudo-first-order rise and fall rate constants for the chemiluminescence burst, CL intensity at time, maximum level intensity, total light yield, the intensity values at maximum CL, and time to reach maximum intensity, are dramatically affected as a function of W_0 (water/surfactant), co-surfactant percentage, pH, and reactant concentrations. At $W_0 = 6$, the maximum performance was achieved. The system exhibits more chemiluminescence properties than an enzymatic aqueous CL system and may be promoted without the need of an enhancer.

Graphical abstract



Keywords Water–ionic liquid microemulsion · Enzymatic chemiluminescence · Horseradish peroxidase · Kinetic parameters

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Introduction

The enzyme horseradish peroxidase (HRP, EC 1.11.1.7) is well known for its high substrate specificity and efficiency in oxidizing a wide range of organic compounds, including phenols, biphenols, anilines, benzidines, and related heteroaromatic compounds. It is also used in immunohistochemistry, Western blots, dot blots, and ELISAs (Ryan et al. 1994). The appeal of HRP primary is due to its affordability, accessibility, low side product production, and action across a wide pH and temperature range (Ryan et al. 1994; Veitch 2004; Veitch and Smith 2000). However, this item significantly reduces the potential applications of enzyme in systems with little or no water content. Micro-heterogeneous systems such as micellar systems (Motlekar and Bhagwat 2001; Gębicka and Pawlak 1997; Parida et al. 1991; Passos et al. 2008; Ghasemi et al. 2023; Li et al. 2021) and microemulsions (Li and Huang 2021; Mahiuddin et al. 2005; Krickl et al. 2018; Tzika et al. 2011; Moniruzzaman et al. 2009; Bauduin et al. 2005; Ghasemi et al. 2022) have been developed to get around this obstacle.

The definition of a microemulsion is "a thermodynamically stable isotropic transparent solution of either a hydrophilic or hydrophobic nature, together with an amphiphilic component, their difference from conventional emulsions lying not only on their significantly smaller structural size (3–100 nm) but also on their thermodynamic stability, two properties which are translated in the long-lived stabilization of mixed polar/apolar systems which is not otherwise feasible" (Cates et al. 1988; Gradzielski et al. 2021; Kale and Deore 2017; Tartaro et al. 2020). The introduction of microemulsions as potential methods to enhance the enzymology in diverse media has occurred in recent years (Tzika et al. 2011; Moniruzzaman et al. 2009; Mitsou et al. 2017; Bose et al. 2022).

Despite these advantages, the organic solvents used in enzymatic microemulsions are very flammable and potentially harmful. Due to their unique properties such as low vapor pressure, wide liquid range, good dissolution properties, and high thermal stability, ionic liquids (ILs, salts melting below 100 °C) have attracted a lot of attention in recent years as a potential replacement for or way to mitigate the effects of organic solvents (Hejazifar et al. 2020). ILs retain the catalytic activity of a wide variety of enzymes. These include lipases, alcohol dehydrogenases, proteases, oxidoreductases, and many more. Compared to enzymes in molecular organic solvents, those in ILs are more active, stable, and selective, and they can be recovered and recycled more easily (Moniruzzaman et al. 2008a; Eastoe et al. 2005; Qiu and Texter 2008; Kuchlyan and Kundu 2016; Kaur et al. 2023).

Because of its low background light, straightforward equipment, quicker reaction rate, and greater dynamic range, chemiluminescence (CL, i.e., the creation of light from a chemical reaction) methods have been extensively employed in clinical chemistry, biochemistry, and environmental chemistry (Garcia-Campana and Baeyens 2001; Lin and Yamada 2003; Yu and Zhao 2021; Biparva et al. 2014, 2016; Kazemi et al. 2012). The most effective chemiluminescent process that an enzyme can catalyze is the oxidation of luminol by hydrogen peroxide when HRP is present and mildly alkaline conditions are present (Misra and Squatrito 1982). When a luminol is oxidized, a 3-aminophthalate ion is created, which, when it returns to its ground state, emits light. The maximal emission wavelength, 425 nm, is employed in immunoassays, metabolic pathway monitoring, inorganic and organic component detection, enzymatic reaction evaluation, and blood detection at crime scenes (Misra and Squatrito 1982; Zhang et al. 2018; Nakamura and Nakamura 1998).

Surprisingly, only one publication has been reported on the peroxidase–luminol CL system in microemulsions (Puchkaev and Metelitsa 1993), and to the best of our knowledge, very few studies have been published on the use of microemulsions as media and for CL reactions (Thompson and McBee 1988; Cohen and Magdassi 1996; Kamyshny and Magdassi 1998; Ishimaru et al. 1998; Huang and Hohn 2010; Murillo Pulgarin et al. 2010). In this study, we used chemiluminescence for the first time to examine how HRP behaved when it was incubated with IL microemulsions. Then, emission was examined from a catalytic perspective. The implications of numerous physicochemical microemulsion factors that have an impact on the CL system are thoroughly examined.

Experimental

Materials

The surfactant AOT, sodium bis(2-ethyl-1-hexyl)sulfosuccinate (> 99%), horseradish peroxidase (HRP, EC 1.11.1.7), and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were bought from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and applied without further purification. Hydrogen peroxide (H_2O_2) (35% W/W), [C8mim] [PF6] (1-octyl-3-methylimidazolium hexafluorophosphate), mannitol, desferrioxamine, α-tocopherol, NaN_3, thiourea, and cysteamine were from Fluka (Basel, Switzerland). 1-Hexanol was provided from Romil (Romil, England). Tris-phosphate buffer was utilized via the work.

Apparatus

A Berthold tube luminometer was used to conduct the CL kinetic experiments (Berthold Detection System, Sirius L, Pforzheim, Germany). The kinetic chemiluminescence parameters were determined using the nonlinear least-squares curve fitting tool KINFIT. The appropriate intensity vs. time charts were used to analyze the experimental results, including the time to attain maximum intensity and maximum intensity. Studies on steady-state chemiluminescence were conducted using a Cary-Eclipse fluorescence spectrophotometer (Agilent, Australia). The spectroscopic research was done using T92 + (PG Instruments, UK). The Multimeter 8603 was used to test pH (AZ instrument, Taiwan). At a constant temperature of 27 °C, all tests were conducted. Dynamic light scattering (DLS) studies were done on a DLS Analyzer Nanotrac Wave II (Microtrac, USA).

Preparation of IL microemulsions

Dissolving HRP in hydrophobic ionic solutions is notoriously difficult. Possible solutions to this problem include using an appropriate surfactant to stabilize the water domains in an IL continuous phase (also known as W/IL microemulsions). The great stability and activity of the enzyme molecules in the micro-heterogeneous medium is a result of their isolation from the organic solvent by a layer of water and surfactant molecules (Moniruzzaman et al. 2009). The necessary quantity of AOT was dissolved in IL that included 10% (V/V) 1-hexanol. Next, a little bit of buffer solution was added to create a microemulsion. To assure the clarity and stability of the stock solution, it was mixed in a vortex mixer for one full minute. Microemulsions with the necessary water concentration were prepared by adding the appropriate volume of buffer solution (Moniruzzaman et al. 2008b), and the molar ratio of water-to-AOT (W_0) was determined by subtracting the quantity of water soluble in pure IL without surfactant from the total water concentration.

Preparation of reaction solutions

The HRP stock solutions were produced using the buffer solution. The peroxidase concentration was determined using spectrophotometric measurement, with a molar absorption coefficient of 9.1×10^4 M^{-1} cm^{-1} at 403 nm (Moniruzzaman et al. 2009). To prepare the H_2O_2 solutions for the experiment, the concentrated solution was diluted with buffer. For the luminol solutions, 1-mM Tris-buffer solution was used (pH 9.5). The following is an example of a typical experiment for enzymatic CL reactions using the AOT/water/IL/1-hexanol systems. It took 80 µL of luminol solution and measured amounts of buffer solution added to 1 mL of stock solution to get the desired W_0. The solution was vortexed for 20 s to achieve macroscopic uniformity. After adding 40 µL of HRP, we gently mixed and kept the contents at 27 °C until they were ready to be blended with the reaction mixture at room temperature. To initiate the CL reaction, 50 µL of H_2O_2 were injected. The enzyme and substrate concentrations were stated as the total concentration, rather than the volume fraction of water droplets in the W/IL microemulsions or the partitioning coefficient of substrates in the bulk IL and microaggregate.

Results and discussion

Characteristics of CL system

Examining the kinetic parameters is essential to ascertain a more in-depth understanding of the chemiluminescence process. As a result, we employed a simple model pooled at the intermediate level (Dye and Nicely 1971; Hadd et al. 2000):

$$R \xrightarrow{Kr} X \xrightarrow{Kf} P \tag{1}$$

Both phases of the process are irreversible first-order reactions, as shown by the pools of reactants, intermediates, and products denoted by R, X, and P, respectively. The integrated rate equation of CL intensity vs. time is provided by: Where CL signal is proportional to the concentration of intermediate X.

$$I_{(t)} = \frac{\mathrm{Mk}r}{kf - kr}\left(e^{-kr\,t} - e^{-kf\,t}\right) \tag{2}$$

As a result, $I(t)$ represents the CL intensity at time t, M is the theoretical maximum intensity that would result from a complete conversion of the reactants into a CL-generating material, and K_r and K_f are the first-order rate constants for the rise (faster step) and fall (slower step) of the CL burst, respectively (Dye and Nicely 1971). This model's additional benefits include the ability to estimate the intensity at maximum level (J) and the parameters M, K_r, and K_f. The time of maximum intensity (T_{max}) and the total yield(Y) presented as follows:

$$T_{\mathrm{Max}} = \frac{\mathrm{Ln}\left(\frac{k_f}{k_r}\right)}{kf - kr} \tag{3}$$

$$J = M\left(\frac{kf}{kr}\right)\left(e^{-kr\,t} - e^{-kf\,t}\right) \tag{4}$$

$$Y = \int_0^{\infty} I_{(t)}\,\mathrm{d}t = \frac{M}{kf} \tag{5}$$

Using the related CL intensity–time plots as a guide, the computerized nonlinear least-squares curve fitting tool KINFIT was utilized to assess the M, K_r, and K_f values (Dye and Nicely 1971). Using the discovered K_r, K_f, and M values, the remaining parameters J, T_{max}, and Y were then deduced from Eqs. (3)–(5). The resulting intensity vs. time charts were then used to calculate the experimental time to obtain maximum intensity and maximum intensity.

While maintaining constant the concentrations of the other factors, the effect of H_2O_2 concentration on the kinetic CL parameters was examined. Table 1 and Fig. 1 show the findings. It is clear that when the concentration of H_2O_2 is raised from 0.5 to 1.68 mM, both rate constants, CL intensity, and quantum yield clearly rise to their maximum value, while the duration to attain maximum CL decreases (Figs. 1a–d). The pseudo-first-order rise (K_r) and fall rate constant (K_f) linearly increases with the regression equations $K_r = 0.0917\ [H_2O_2] + 0.4598$ with a relatively large intercept of about 0.4598 s^{-1} and equation $K_f = 0.002\ [H_2O_2] + 0.0058$, respectively, which is representative of a first-order reaction that is zero-order at the hydrogen peroxide concentration used (Figs. 1e-f). All parameters decreased when concentration was raised to 1.68 mM, with the exception of duration to maximum CL. This finding may be explained by a number of factors: The polar substrate H_2O_2 is distributed efficiently in the IL phase, in contrast with how poorly it is soluble in a polar organic solvent. Here, the aqueous pseudo-phase's concentration is decreased by H_2O_2 partitioning in the IL continuous phase, and the enzyme can only access the H_2O_2 that has been solubilized there (Azevedo et al. 2001). It is important to note that peroxide serves as an enzyme inhibitor at greater doses (Metelitza et al. 1992). Additionally, a greater peroxide concentration hinders the process by lowering superoxide anion concentrations, which accelerates the destruction of luminol (Hoshino and Hinze 1987).

The following phase was looking at how the W_0 value (water content:concentration ratio of water to surfactant) affected the CL of the W/IL microemulsion. Water-in-IL microemulsions, like many W/O microemulsions, exhibit a spherical droplet form for which the droplet radius is directly proportional to W_0 value. As a result, the W_0 value may be changed to alter the microenvironment surrounding the enzyme. The formation of microemulsions in ionic liquids occurred between a W_0 value of less than nine (Moniruzzaman et al. 2009; Metelitza et al. 1992), it should be highlighted. Above this water content, the system splits into two stages. Up until $W_0 = 6$, the system was approaching a critical value for the generation of IL microemulsions and had reached an equilibrium value, as shown in Fig. 2 and Table 2, and this was assessed. The intensity vs. time curve in different W_0s is shown in Fig. 2a. After mixing, the peak intensity rises quickly and reaches its peak in a few seconds. The light intensity gradually decreases from its peak over significantly longer times. The time to attain maximum CL was lowered by nearly six times, the CL intensity increased by about six times, and the rise in K_r was around 8.25 times (see Figs. 2b, c and Table 2). This finding may be explained by the enzyme's proximity to the very minimum quantity of water required for maximum catalytic activity. For the enzyme to function well in microemulsions, it has to be suitably hydrated. At the low W_0 value, a significant portion of water is firmly bonded to the AOT head groups (Azevedo et al. 2001). Because less water is available to hydrate the enzyme, the HRP reaction of luminol is better catalyzed and may experience structural changes with an increase in water concentration. Due to dilution of reactants at higher W_0 (increased micelles and/or water pool), CL parameters reduced (Hoshino and Hinze 1987). In order to evaluation of size distribution of microemulsion droplet at optimum condition, DLS technique was performed. The size of (hydrodynamic diameter) distributions of the microemulsions obtained, which is one of the most commonly used methods to estimate the size distribution of the aggregates in microemulsions (Hyde 2001). As illustrated, the droplet is obviously spherical with average size of 29.21 nm.

The results of testing three different buffer concentrations (at 20, 30 and 50 mM) to ensure sufficient buffer capacity are shown in Fig. 3. The highest intensity and fastest increasing rate constant were reported at 50 mM, suggesting enhanced reactivity of superoxide anion. The influence of the pH of the aqueous pseudo-phase was studied in the CL microemulsions system. Since measuring the pH of a swimming pool's

Table 1 CL kinetic parameters as function of H_2O_2 concentration. Experimental conditions are the same as described in Fig. 1

Parameter	$[H_2O_2]$ mM	K_r (s^{-1})	K_f (s^{-1})	M (mV)	J (mV)	J_{exp} (mV)	I (mV)	T_{exp} (s)	T (s)	Y
H_2O_2	0.5	0.504 ± 0.018	0.0068 ± 0.0006	39.44 ± 0.74	42.51	45.55	37.23	11.52	8.71	5800
	1.0	0.558 ± 0.009	0.0081 ± 0.0002	45.89 ± 0.30	49.23	50.25	43.05	8.34	7.75	5780
	1.4	0.598 ± 0.03	0.0088 ± 0.0001	56.38 ± 0.78	60.080	58.82	52.55	8.00	7.17	6334
	1.68	0.608 ± 0.026	0.0091 ± 0.0009	65.88 ± 0.91	70.11	71.94	62.24	7.68	7.00	7239
	2.0	0.59 ± 0.036	0.008 ± 0.001	58.06 ± 0.46	62.08	60.71	55.12	7.84	7.21	6673
	2.3	0.573 ± 0.038	0.0081 ± 0.0007	49.27 ± 0.38	53.36	51.14	46.19	8.10	7.62	6082

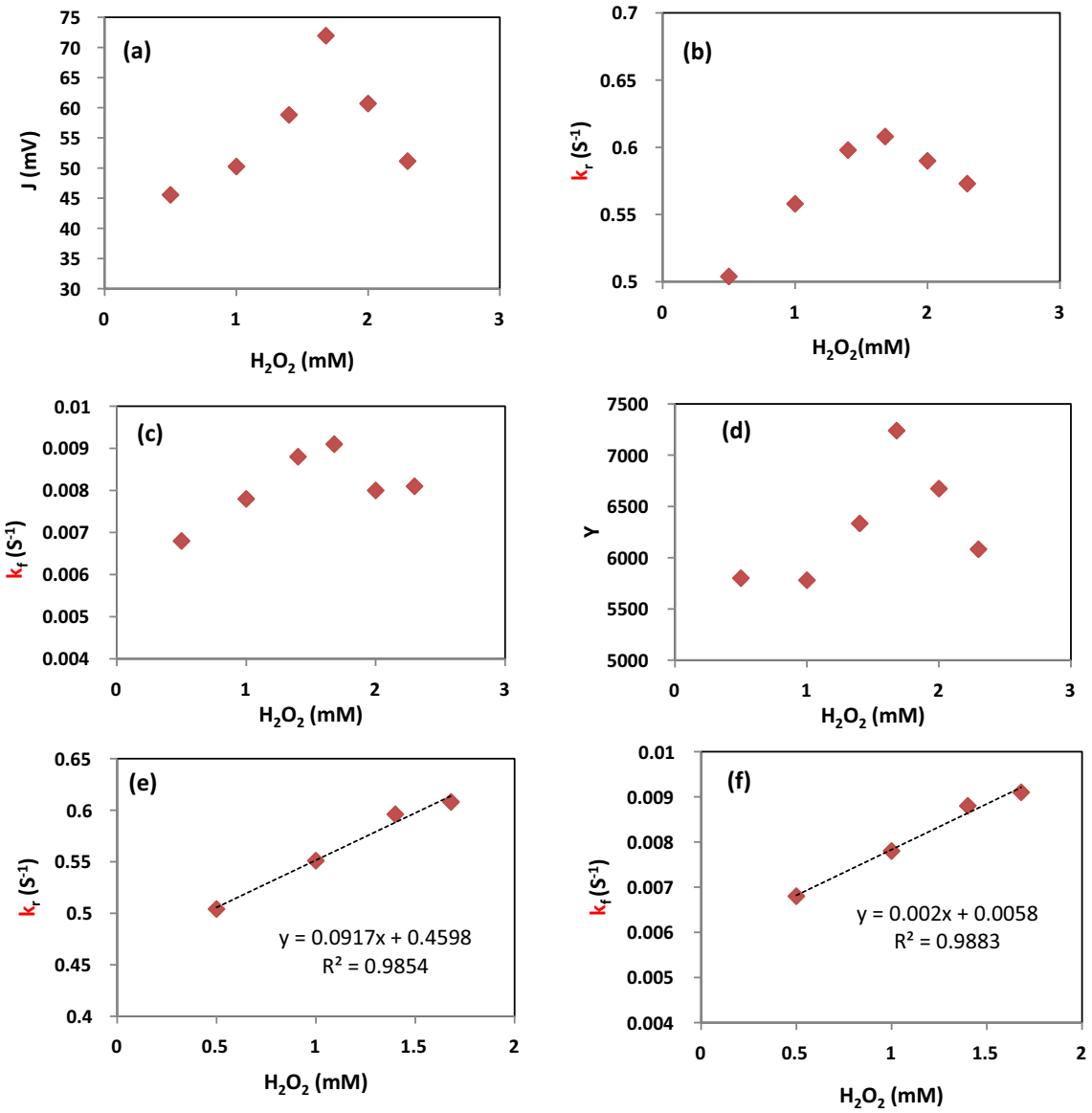

Fig. 1 Effect of concentration of H_2O_2 on **a** *J*, **b** K_r, **c** K_f, **d** *Y*, **e** regression equation of k_r, and **f** regression equation of K_f. Conditions were: [Luminol] = 2 mM, [Buffer] = 50 mM, pH = 9.5, [HRP] = 0.16 µM,[AOT] = 200 mM, W_0 = 6, and 1-Hexanol = 8%

water is problematic, the values provided below refer to the buffer's pH just before it is dissolved in IL microemulsions. We examined HRP-catalyzed oxidation of luminol in W/IL microemulsions as a function of pH at 50-mM buffer concentration, holding enzyme, and substrate concentrations constant. The usual pH profile of W/IL microemulsions is shown in Fig. 4 and tabulated below. At pHs 7.25 and 8.60, HRP CL oxidation of luminol was shown to be fastest both when no enhancer was present and when para-iodophenol was used. Previously, the CL process was reported by Metelitza et al. 1992, in a water–oil microemulsion at pH 9.5. Our results also showed that the optimal pH for HRP catalytic activity in the W/IL system was 9.5 (Table 3).

We analyzed how the amount of 1-hexanol present in W/IL microemulsions affected the CL system within (see Table 4 and Fig. 5). Generally speaking, 1-alcohols slow down enzyme reactions. For preparing W/IL microemulsions using AOT as the surfactant, 10% (V/V) 1-hexanol in IL is required (Moniruzzaman et al. 2009; Roy et al. 2006). Figure 5 and Table 4 show the result of progressively substituting 1-hexanol for IL in W/IL microemulsions at a constant surfactant concentration and W_0 value. The intensity and rate constants of CL were found to be drastically diminished by increasing 1-hexanol concentration. Increasing 1-hexanol concentration is likely due to the following factors that contribute to low enzymatic activity.

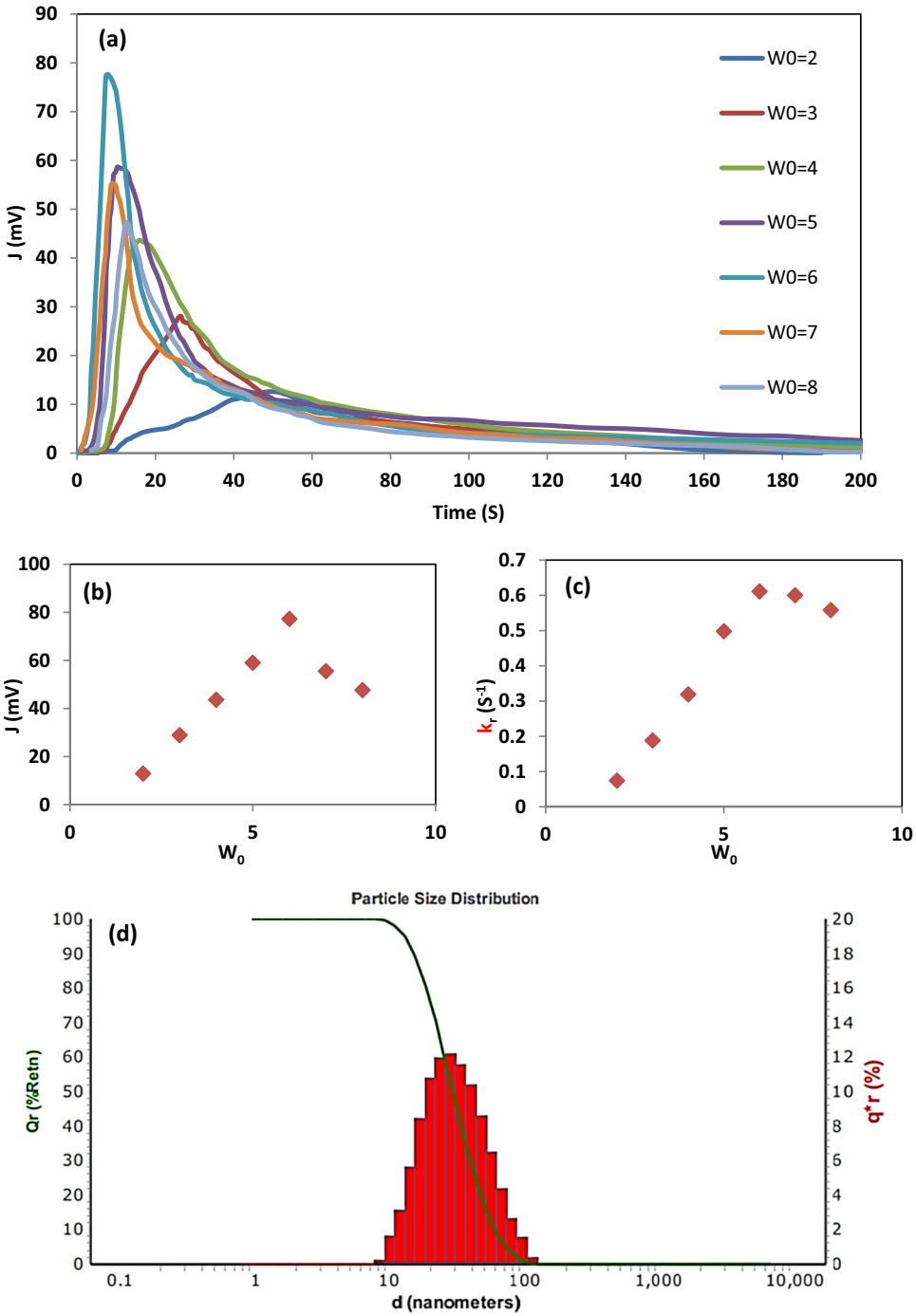

Fig. 2 **a** Intensity vs. time profiles as function of W_0; **b** J as function of W_0; **c** K_r as function of W_0; and **d** size distribution of microemulsion at $W_0 = 6$. Conditions were: [Luminol] = 2 mM, [Buffer] = 50 mM, pH = 9.5, [HRP] = 0.16 µM, [AOT] = 200 mM, $[H_2O_2]$ = 1.68 mM, and 1-Hexanol = 8%

To begin, it stands to reason that the interfacial 1-hexanol concentration would increase proportionally to the total solution concentration. A higher concentration of 1-hexanol is clearly observed in the vicinity of the HRP active site. HRP is inactive because 1-alcohols can denature the enzyme structure. Second, it's possible that the decreased catalytic activity of HRP was caused by the new microemulsion's altered structure and rising 1-hexanol content. However, regardless of the 1-hexanol concentration used to produce the systems, all of the microemulsions were found to be stable and transparent.

Investigating the effects of altering HRP concentration on the CL parameters produced the results shown in Table 5 and Fig. 6. Under the experimental circumstances, there is obviously a good association between solution HRP catalytic activity and a linear relationship for enzyme concentrations

Table 2 Kinetic parameters as a function of W_0. Experimental conditions are the same as described in Fig. 2

Parameter		K_r (s^{-1})	K_f (s^{-1})	M (mV)	J (mV)	J_{exp} (mV)	I (mV)	T_{exp} (s)	T	Y
W_0	2	0.074 ± 0.004	0.0040 ± 0.0006	10.31 ± 0.14	12.26	12.9	8.89	46.3	40.26	2577
	3	0.188 ± 0.007	0.0069 ± 0.0010	27.78 ± 0.19	31.02	28.88	25.01	27.15	18.18	4026
	4	0.319 ± 0.010	0.0060 ± 0.0004	40.15 ± 0.44	42.98	43.62	37.94	15.8	12.55	6691.6
	5	0.498 ± 0.008	0.0080 ± 0.0007	52.9 ± 0.28	56.77	58.98	50.01	10.68	8.22	6612
	6	0.611 ± 0.009	0.0090 ± 0.0003	65.4 ± 0.7	69.11	77.24	61.48	7.7	7.01	7266
	7	0.600 ± 0.018	0.0090 ± 0.0008	51.08 ± 0.36	54.03	55.5	48.11	9.3	7.14	5675
	8	0.558 ± 0.013	0.0070 ± 0.0004	46.45 ± 0.92	50.15	47.66	43.26	12.4	8.02	6635

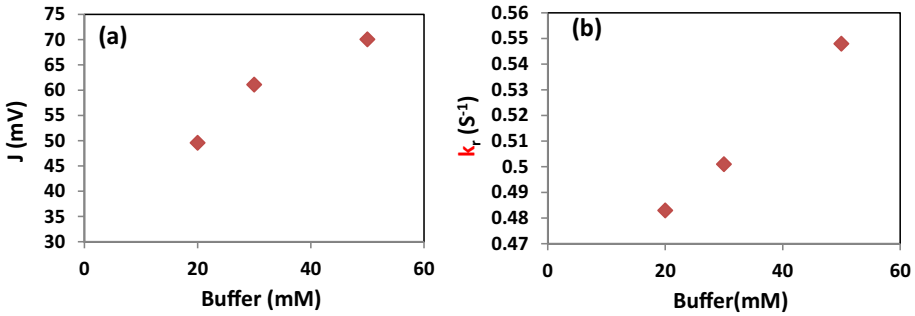

Fig. 3 Effect of buffer concentration on **a** maximum intensity and **b** rate constant. Conditions were: [Luminol] = 2 mM, pH = 9.5, [HRP] = 0.16 µM, [AOT] = 200 mM, [H_2O_2] = 1.68 mM, W_0 = 6, and 1-Hexanol = 8%

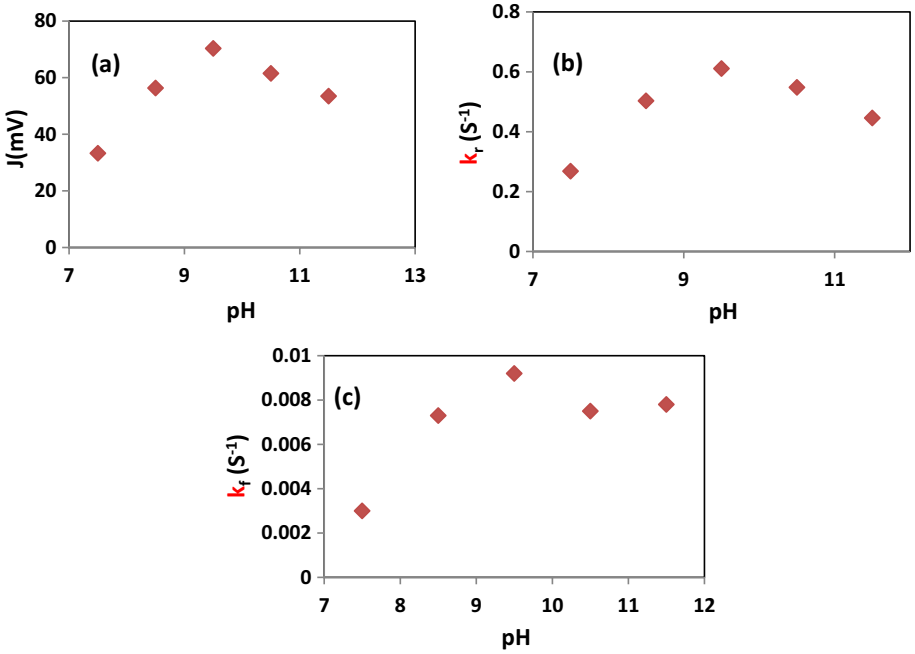

Fig. 4 Dependence of CL parameters on pH **a** maximum intensity, **b** rise rate constant and **c** fall rate constant. Condition were: [Luminol] = 2 mM, [Buffer] = 50 mM, [HRP] = 0.16 µM, [AOT] = 200 mM, [H_2O_2] = 1.68 mM, W_0 = 6, and 1-Hexanol = 8%

between 0.008 m and 0.16 m. The nonlinear correlation and waning intensity at increasing enzyme concentrations indicate additional reaction limiting condition (Bhandari et al. 2010).

Possible mechanism

Luminol (5-amino-2, 3 dihydrophthalazine-1, 4-dione) emits light by a chemiluminescent process that involves the splitting of an oxygen–oxygen bond (Bhandari et al. 2010). The light produced by this reaction occurs at 425 nm. As

Table 3 Effect of pH on CL parameters. Experimental conditions are the same as described in Fig. 4

Parameter		K_r (s^{-1})	K_f (s^{-1})	M (mV)	J (mV)	J_{exp} (mV)	I (mV)	T_{exp} (s)	T	Y
pH	7.5	0.268 ± 0.027	0.0030 ± 0.0004	36.3 ± 0.3	38.44	33.28	34.68	29	17.12	12,100
	8.5	0.503 ± 0.019	0.0073 ± 0.0002	50.8 ± 0.4	54.58	56.36	47.96	13.4	8.27	6972
	9.5	0.611 ± 0.014	0.0092 ± 0.0008	63.4 ± 0.3	68.21	70.34	59.22	8.6	7.12	6980
	10.0	0.548 ± 0.036	0.0075 ± 0.0010	57.00 ± 0.67	60.58	63.51	53.14	10.3	8.01	7588
	11.0	0.446 ± 0.042	0.0078 ± 0.0008	50.5 ± 0.8	53.71	53.47	47.65	11.5	9.78	64,668

Table 4 Effect of 1-hexanol content on the CL system encapsulated in water-in-IL microemulsion. Experimental conditions are the same as described in Fig. 5

Parameter	%	K_r (s^{-1})	K_f (s^{-1})	M (mV)	J (mV)	J_{exp} (mV)	I (mV)	T_{exp} (s)	T	Y
1-Hexanol	8	0.602 ± 0.024	0.0083 ± 0.0004	59.88 ± 0.66	63.98	68.05	57.34	8.13	7.33	7214
	16	0.507 ± 0.051	0.0077 ± 0.0007	43.74 ± 0.64	46.03	45.94	41.26	8.84	8.35	5680
	24	0.390 ± 0.019	0.0066 ± 0.0006	31.98 ± 0.59	33.67	31.4	30.08	13.5	10.63	4845
	32	0.211 ± 0.041	0.0057 ± 0.0011	20.14 ± 0.7	22.72	19.73	18.26	23	17.22	3533
	40	0.144 ± 0.033	0.0053 ± 0.0009	14.26 ± 0.62	16.23	12.38	12.33	30.2	24	2690

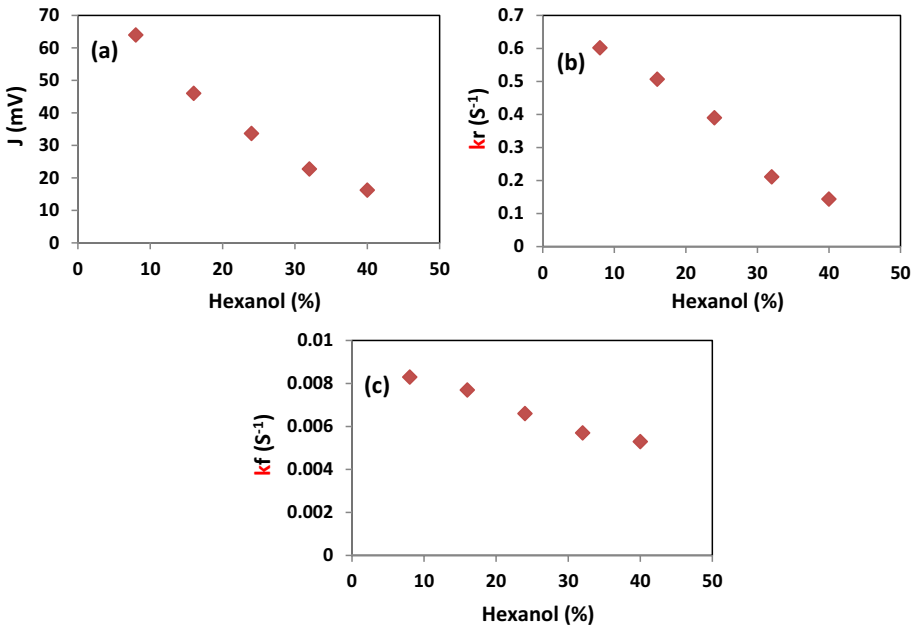

Fig. 5 Effect of co-surfactant content (%) on CL parameters **a** J, **b** K_r, and **c** K_f. Conditions were: [Luminol] = 2 mM, [Buffer] = 50 mM, pH = 9.5, [HRP] = 0.16 µM, [AOT] = 200 mM, $[H_2O_2]$ = 1.68 mM, and W_0 = 6

Table 5 CL parameters as a function of enzyme concentration. Experimental conditions are the same as described in Fig. 6

CL parameter	µM	K_r (s^{-1})	K_f (s^{-1})	M (mV)	J (mV)	J_{exp} (mV)	I (mV)	T_{exp} (s)	T	Y
HRP	0.008	0.531 ± 0.014	0.0069 ± 0.0004	33.20 ± 0.28	35.23	33.28	31.49	8.11	8.14	4811
	0.03	0.546 ± 0.019	0.0074 ± 0.0009	40.23 ± 0.36	42.11	41.05	38.02	8.15	8.02	5436
	0.07	0.563 ± 0.025	0.0073 ± 0.001	47.26 ± 0.92	50.81	48.89	45.13	8.05	7.96	6473
	0.11	0.586 ± 0.039	0.0081 ± 0.0003	59.64 ± 0.6	62.98	64.12	55.37	7.94	7.39	7362
	0.16	0.602 ± 0.057	0.0088 ± 0.0005	69.49 ± 0.44	74.17	74.89	65.02	7.83	7.08	7897

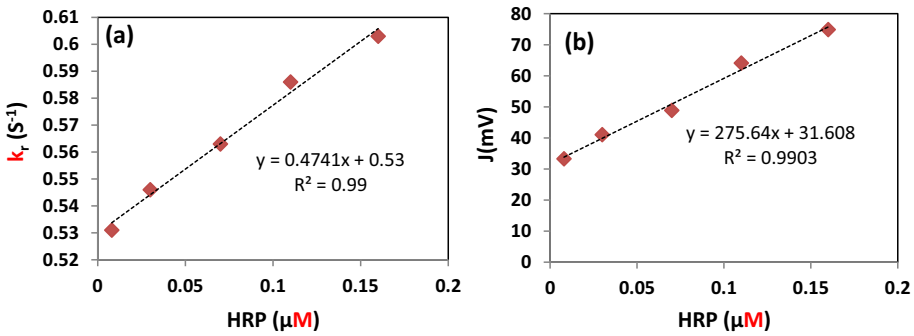

Fig. 6 Effect of enzyme concentration on intensity **a** and **b** rise rate constant. Conditions were: [Luminol] = 2 mM, [Buffer] = 50 mM, pH = 9.5, [AOT] = 200 mM, $[H_2O_2]$ = 1.68 mM, W_0 = 6, and 1-Hexanol = 8%

part of the HRP–luminol–H_2O_2 test, HRP reacts with H_2O_2 by oxygen transfer. Equations (6) and (7) show how HRP is transformed into HRP-I and HRP-II in the presence of luminol LH^- and peroxide. The luminol radical, $L^{\bullet -}$, is formed when one of these intermediates removes an electron from luminol (see Eq. 9). After entering a complicated chemical route, luminol radicals ultimately provide diazaquinone L, luminol endoperoxide LO_2^{-2}, excited 3-aminophthalate ion AP^{-2}, and nitrogen gas Eqs. (10–12). 3-aminophthalate dianion (AP^{-2}) is formed when AP^{-2} is decomposed into it and light (Eq. (14)) (Roswell and White 1978; Rauhut et al. 1966; Seitz 1978).

$$HRP + H_2O_2 \rightarrow HRP - I + H_2O \quad (6)$$

$$HRP - I + LH^- \rightarrow HRP - II + H_2O \quad (7)$$

$$HRP - II + LH^- \rightarrow HRP + L^{\cdot -} \quad (8)$$

$$LH^- \rightarrow H^+ + L^{\cdot -} \quad (9)$$

$$LH^- + LH^- \rightarrow L + LH_2 \quad (10)$$

$$2\,L^{\cdot -} \rightarrow L + LH^- \quad (11)$$

$$L + H_2O_2 \rightarrow LO_2^{2-} \quad (12)$$

$$LO_2^{2-} \rightarrow (AP^{2-}) * + N_2 \quad (13)$$

$$\left(AP^{2-}\right)^* \rightarrow AP^{2-} + hv(425\ nm) \quad (14)$$

Some antioxidants were utilized as scavengers to research the potential mechanism of CL in W/IL microemulsion: Superoxide radicals are dealt with by superoxide dismutase (SOD), hydroxyl radicals are dealt with by mannitol and desferrioxamine, singlet oxygen is dealt with by α-tocopherol and NaN_3, and hypochlorite is dealt with by thiourea and cysteamine (Nakamura and Nakamura 1998; Oosthuizen and Greyling 1999). The results of the experiments were comparable to those of aqueous mediums.

Various amounts of water have a drastic effect on CL parameters. By increasing water content up to $W_0 = 6$, the CL parameters were increased. At upper value, these parameters were noticeably decrease. This phenomena can be attributed that the stability of the dianion be affected by water content that led to change the monoanion–dianion equilibrium of Eqs. (11) and (12) (decrease dianion concentration) (Hoshino and Hinze 1987; Gorsuch and Hercules 1972; Fujiwara and Kumamaru 1994). It is important to note that the rate constant, maximum intensity, and quantum yield are all greatly improved by IL microemulsion.

Conclusion

Hence, for the first time, we surveyed the biocatalyzed chemiluminescence reaction of luminol in water–ionic liquid microemulsion. Some general conclusions are provided from these results:

- In contrast with aqueous medium, the chemiluminescence reaction of luminol catalyzed by HRP may be carried out in water-in-ionic liquid microemulsion with enhanced rate constant, intensity, and quantum yield.
- Based on kinetic studies and SOD testing, the chemiluminescence process is comparable to that seen in aqueous media.
- HRP-catalyzed chemiluminescence reaction in W/IL can be enhanced without any enhancer.
- High background emission, auto-oxidation of luminol, spontaneous decomposition of peroxide, or interferences from potentially reductive species present in solution are some issues that are frequently encountered under the strongly basic conditions necessary for the CL production process in aqueous solution and can be effectively reduced by using W/IL microemulsion.
- The employment of ILs in microemulsion may boost the sensitivity of the CL system in analytical and biologi-

cal applications due to the ascend in chemiluminescence yield.

Authors' contributions SMA worked in: methodology, investigation, formal analysis, writing—original draft preparation, and confirming of the final version. MS helped in: conceptualization, supervision, resources, reviewing, writing—original draft preparation, and confirming of final version.

Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of interest The authors declare no competing financial interest.

Ethics approval This research is not involving human or animal subject. The Razi University committee has confirmed that no ethical approved is required.

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