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3-(10′-Phenothiazinyl)propionic acid is a potent primary enhancer of peroxidase-induced chemiluminescence and its application in sensitive ELISA of methylglyoxal-modified low density lipoprotein

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ABSTRACT

Using a full factorial design the optimization of experimental conditions of enhanced chemiluminescence reaction (ECR) catalyzed by horseradish peroxidase (HRP) in the presence of 3-(10′-phenothiazinyl) propionic acid (PPA) as a primary enhancer was performed. The effect of concentrations of PPA, hydrogen peroxide, MORPH, luminol, and Tris on a ratio of peroxidase-catalyzed CL to background was studied. The detection limit value of HRP in ECR with PPA was 0.09 pM. Using PPA the ultra-sensitive chemiluminescent ELISA for determination of methylglyoxal-modified low density lipoprotein was developed. The detection limit value for the developed method was 0.5 ng mL^{-1}. The obtained results open up very promising perspectives for using PPA to improve the sensitivity of enzyme immunoassay kits.

1. Introduction

Enzyme immunoassay is one of the widely used methods in current analytical practice [1,2]. In this assay horseradish peroxidase (HRP) is commonly used as a label of immunoreagents. Multiple detection methods for peroxidase activity are applied, including colorimetry, fluorimetry and chemiluminescence (CL). CL is markedly more sensitive than other methods [3,4]. This method is based on the enzymatic oxidation of luminol by peroxides in the presence of peroxidase under mild alkaline conditions. Luminol oxidation leads to the formation of a 3-aminophthalate ion in an excited state, which emits light when returning to the ground state.

Since HRP is a poor catalyst in luminol oxidation, certain compounds known as primary enhancers are added to the substrate mixture to increase CL intensity [5]. The mechanism of enhanced chemiluminescence reaction (ECR), where luminol and a primary enhancer are oxidized simultaneously, was reported previously [6,7]. At the first step of ECR the enhancer, which is a more active substrate for HRP than luminol, is oxidized by hydrogen peroxide in the presence of HRP according to "ping-pong" mechanism (Eqs. (1)–(3)):

$$E+H_2O_2 \rightarrow EI+H_2O \quad (1)$$

$$EI+SH \rightarrow EII+S^{\bullet} \quad (2)$$

$$EII+SH \rightarrow E+S^{\bullet}+H_2O \quad (3)$$

where SH—enhancer, $S^{\bullet}$—radical product of one-electron oxidation of enhancer, E is the ferric enzyme (resting state), EI и EII—compound I and compound II, the oxidized intermediates of peroxidase, which are by two and one oxidation equivalents above the resting state, respectively.

Then, the formed radical product ($S^{\bullet}$), using its oxidative potential, reacts with luminol molecule (Eq. (4)). The final product of this reaction is also 3-aminophthalate (Eq. (4)).

$$S^{\bullet}+\text{luminol} \rightarrow SH+\text{3-aminophthalate}+N_2+h\nu \quad (4)$$

Abbreviations: Horseradish peroxidase, (HRP); enzyme-linked immunosorbent assay, (ELISA); chemiluminescence, (CL); 3-(10′-phenothiazinyl)propionic acid, (PPA); 3-(10′-phenothiazinyl)-propane-1-sulfonate, (SPTZ); 4-morpholinopyridine, (MORPH); enhanced chemuliminescence reaction, (ECR); methylglyoxal, (MG); low density lipoprotein, (LDL); relative luminescence units, (RLU); coefficient of variation, (CV).

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Therefore, the enhancers play a role of mediators in the peroxidase catalysis [8], and their presence in the reaction solution does not affect chemical nature of the final product, but increases CL intensity due to their higher reactivity towards Compound I and Compound II than that of luminol.

Although a number of compounds were successfully used in the enhancement of HRP-induced CL [9–11], presently the most effective enhancer is 3-(10′-phenothiazinyl)propane-1-sulfonate (SPTZ) [12,13]. Furthermore, it was shown that an introduction of some 4-dialkylaminopyridines such as 4-morpholinopyridine (MORPH), 4-dimethylaminopyridine and 4-pyrrolidinopyridine to a reaction mixture containing luminol, hydrogen peroxide and SPTZ resulted in the increase of CL intensity [14,15]. As 4-dialkylaminopyridines enhanced CL only in the presence of a primary enhancer (SPTZ) and did not act as enhancers in the absence of SPTZ, these compounds were named as secondary enhancers. The mechanism of the action of 4-dialkylaminopyridines as secondary enhancers was reported recently [15]. The use of SPTZ and MORPH allowed developing of ultra-sensitive enzyme immunoassays for the determination of human thyroglobulin, ochratoxin A and aflatoxin B1 [16–18].

A screening of some *N*-alkylphenothiazines as primary enhancers showed that phenothiazines carrying positive charged groups had no an enhancement ability, whereas phenothiazines with negative charged groups including 3-(10′-phenothiazinyl)propionic acid (PPA, Fig. 1) increased significantly an intensity of CL [19,20]. In this paper we describe the optimization of the experimental conditions of the HRP-catalyzed oxidation of luminol by hydrogen peroxide in the presence of PPA and MORPH as enhancers using a full factorial design. The obtained results demonstrated that PPA is a potent primary enhancer of peroxidase-induced chemiluminescence. The combination of PPA and MORPH was successfully applied in development of sensitive CL ELISA for the determination of methylglyoxal (MG) -modified low density lipoprotein (LDL).

2. Experimental

2.1. Reagents and materials

Horseradish peroxidase (isoenzyme *c*, RZ 3.0) was purchased from Sigma (USA) and used without further purification. Sodium 3-(10′-phenothiazinyl)propane-1-sulfonate (SPTZ) and 3-(10′-phenothiazinyl)propionic acid (PPA) and were synthesized as described in Refs. [14,21], respectively. 4-Morpholinopyridine (MORPH) was from Aldrich (USA); luminol and Tris were from Sigma (USA), H_2O_2 (30%) from ChimMed (Russia); 3,3′,5,5′-tetramethylbenzidine (TMB) from AppliChem GmbH (Germany). The concentration of HRP was measured by using $\varepsilon_{402}=102{,}000\ M^{-1}\ cm^{-1}$ [22]. The H_2O_2 concentration was determined by monitoring A_{240}, using $\varepsilon=43.6\ M^{-1}\ cm^{-1}$ [12]. The required dilutions of H_2O_2 were prepared daily. Monoclonal anti-MG-LDL antibody was a gift of Dr. T. Vlasik (Russian Cardiology Research Center, Russia).

The conjugate of streptavidin and HRP (Stv-HRP) was purchased from SDT GmbH (Germany). MG-LDL were prepared as described in Ref. [27]. The biotinylation of the monoclonal anti-LDL antibody was carried out as described previously [28].

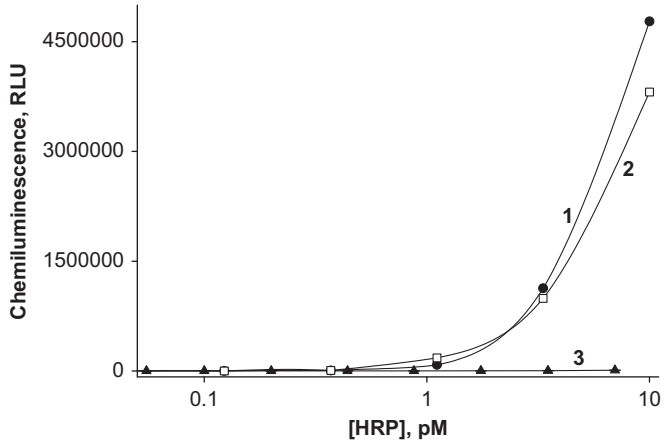

Fig. 1. Dependence of CL intensity produced upon the HRP-catalyzed oxidation of luminol in the presence of (1) PPA, (2) SPTZ and (3) *p*-iodophenol as primary enhancers on the HRP concentration. The experimental conditions: (1) 100 mM Tris, pH 8.3, containing 1.0 mM luminol, 5.2 mM PPA, 9.3 mM MORPH, and 3.0 mM H_2O_2; (2) 80 mM Tris, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM MORPH, and 1.75 mM H_2O_2 [12]; (3) 100 mM Tris, pH 8.4, containing 1.0 mM luminol, 1.0 mM 4-iodophenol, 0.5 mM H_2O_2 [13]. Each point represents the mean of duplicates. Vertical bars indicate ± S.D. about the mean.

2.2. Chemiluminescent determination of peroxidase activity

HRP activity was assayed as follows: 200 μL of 20–80 mM Tris buffer, pH 8.3 containing hydrogen peroxide, luminol, PPA and MORPH were placed in wells of black polystyrene plates (MaxiSorp, NUNC, Denmark) for chemiluminescent enzyme immunoassay. Then the enzymatic reaction was initiated by adding 50 μL of peroxidase solution in Tris buffer at relevant pH and concentration. CL was measured at room temperature on a reader Zenyth 1100 and 3100 (Anthos, Austria). The CL signal in the absence of the enzyme was taken as background value. The light intensity was expressed in relative luminescence units (RLU).

2.3. Experimental design

Full (2^5) factorial design was used to optimize the experimental conditions of performance of ECR catalyzed by HRP. The effect of five factors such as concentrations of luminol, hydrogen peroxide, PPA, MORPH, and Tris was evaluated. The used concentrations of variables are presented in Table 1. The efficiency of ECR performance was evaluated as a ratio of peroxidase-catalyzed CL to background. The obtained results were analyzed using Statgraphics XV.I.

2.4. Determination of MG-LDL by ELISA

The determination of MG-LDL was carried out using 96-wells black (the assay with CL detection (CL-ELISA)) and transparent (the assay with colorimetric detection) polystyrene plates (High Binding, Corning, USA). The plates were coated by adding into each well 100 μL of anti-MG-LDL-mAb (5 μg mL^{-1}) dissolved in 50 mM carbonate buffer, pH 9.5 and incubated at 4 °C overnight. The plate was then washed using PBS with 0.05% Tween 20 (PBST) four times. Subsequently, 100 μL of MG-LDL (0.14–100.0 ng mL^{-1})

Table 1
The variable values used in the optimization of experimental conditions of ECR catalyzed by HRP.

Variables (mM)	Levels		
	Low (−1)	Centered (0)	High (+1)
X_1—[PPA]	0.01	4.0	8.0
X_2—[H_2O_2]	0.1	1.6	3.2
X_3—[MORPH]	0.01	7.5	15
X_4—[luminol]	0.001	0.6	1.2
X_5—[Tris]	20	60	100

soluble in PBST with 1% BSA were added to wells. The plates were incubated for 1 h at 37 °C and then were washed as described above. Then 100 μL of biotinylated anti-MG-LDL-mAb (dilution 1:50) dissolved in PBST with 1% BSA was added to each well. The plates were incubated for 1 h at 37 °C and then washed with PBST four times. One hundred microliters of the conjugate of streptavidin and HRP (Stv-HRP, dilution 1:400 in C-ELISA and 1:50 in colorimetric ELISA) dissolved in PBST with 1% BSA were added to each well. The plates were incubated for 30 min at 37 °C and then washed with PBST four times. Finally, 100 μL of freshly prepared substrate solution (100 mM Tris, pH 8.3, containing 1 mM luminol, 5.2 mM PPA, 9.3 mM MORPH, and 3 mM H_2O_2) in CL-ELISA or TMB solution (100 mM citrate buffer, pH 5.0, containing 1.45 mM TMB and 7 mM H_2O_2) in colorimetric ELISA were added to each well and stirred. Intensities obtained were monitored at room temperature on a reader Zenyth 1100 and 3100 (Anthos, Austria).

3. Results and discussion

3.1. Optimization of the conditions of enhanced chemiluminescence reaction with PPA

To optimize the experimental conditions of performance of HRP-catalyzed ECR with PPA as the primary enhancer we applied the full (2^5) factorial design. In our work the concentrations of luminol, hydrogen peroxide, PPA, MORPH, and Tris in the reaction solution were selected as independent. MORPH was introduced to the reaction solution as a secondary enhancer. The used values of variables are presented in Table 1. Preliminary the values of these variables were determined using "one-variable-a time" method. A ratio of CL intensity formed upon the enzymatic reaction to background (S/N) was used to estimate the efficiency of ECR performance.

The results of CL determinations at different experiment conditions are presented in Table 2. The analysis of the obtained results was performed using Statgraphics XV.I that allowed a determination of the dependence of a ratio of CL intensity formed upon the enzymatic reaction to background (Y) upon the concentrations of PPA (X_1), hydrogen peroxide (X_2), MORPH (X_3), luminol (X_4), and Tris (X_5) (Eq. (5)).

$$Y = 63,855 - 169X_1 + 7576X_2 + 9282X_3 + 10,248X_4 + 2158X_5 - 46,686X_1^2 + 3982X_1X_2 + 683X_1X_3 + 687X_1X_4 - 2171X_1X_5 - 1789X_2^2 + 5354X_2X_3 + 5003X_2X_4 + 2372X_2X_5 - 25,458X_3^2 + 7031X_3X_4 - 1770X_3X_5 - 7569X_4^2 + 1677X_4X_5 + 26,062X_5^2 \quad (5)$$

The ANOVA (analysis of variance) showed that at a significance level ≥95% (with a confidence interval p-value ≤0.05), this model (although with a lack-of-fit) is a reasonably good representation of the data including significant contribution of linear ([luminol] and [MORPH]) and square ([PPA] × [PPA]) effects. This fact is also confirmed by a reasonable coefficient of the determination (R^2 = 71.3%). Eq. (5) allowed a calculation of a combination of concentrations of the reacting substances to obtain the maximum value of S/N ratio. The calculated conditions were the following: 100 mM Tris, pH 8.3, containing 1.0 mM luminol, 5.2 mM PPA, 9.3 mM MORPH, and 3.0 mM H_2O_2.

3.2. Analytical parameters of chemiluminescent assay of HRP

Under the optimized conditions the dependence of CL intensity on the HRP concentration was determined (Fig. 1). In the case of PPA the value of detection limit of HRP was 0.09 pM. This value was equal to the detection limit obtained at the use of SPTZ and significantly lower than that at the use of p-iodophenol (1.1 pM [13]), which widely uses in CL immunoassay [29–31]. It should be noted that the sensitivity of HRP determination with PPA defined as the calibration curve slope in the linear range was higher than that with SPTZ (Fig. 1). The obtained results demonstrated that PPA is the potent primary enhancer of HRP-induced CL. This fact opens up very promising perspectives for using PPA in development of sensitive CL-ELISA kits.

3.3. Chemiluminescent ELISA of methylglyoxal-modified low density lipoprotein

To demonstrate it, we used PPA in a CL-ELISA for determination of MG-LDL. Recent studies have shown that human LDL modified by MG accounts for 5% of LDL in healthy people and increases by up to fourfold in patients with diabetes [23,24]. Since the modification of LDL with MG may be connected with the increased risk of atherosclerosis and diabetes, it is necessary to have some

Table 2
Full factorial design used in the optimization of experimental conditions of ECR catalyzed by HRP.

№	Variable level					Ratio of HRP-induced CL to background
	X_1	X_2	X_3	X_4	X_5	
Main block						
1	−1	−1	−1	−1	−1	476
2	+1	−1	−1	−1	−1	3
3	−1	+1	−1	−1	−1	150
4	+1	+1	−1	−1	−1	3
5	−1	−1	+1	−1	−1	5,603
6	+1	−1	+1	−1	−1	7
7	−1	+1	+1	−1	−1	1,084
8	+1	+1	+1	−1	−1	174
9	−1	−1	−1	+1	−1	6,319
10	+1	−1	−1	+1	−1	15
11	−1	+1	−1	+1	−1	783
12	+1	+1	−1	+1	−1	23
13	−1	−1	+1	+1	−1	26,510
14	+1	−1	+1	+1	−1	22
15	−1	+1	+1	+1	−1	71
16	+1	+1	+1	+1	−1	71,474
17	−1	−1	−1	−1	+1	800
18	+1	−1	−1	−1	+1	3
19	−1	+1	−1	−1	+1	100
20	+1	+1	−1	−1	+1	25
21	−1	−1	+1	−1	+1	4,803
22	+1	−1	+1	−1	+1	7
23	−1	+1	+1	−1	+1	2,542
24	+1	+1	+1	−1	+1	327
25	−1	−1	−1	+1	+1	6,408
26	+1	−1	−1	+1	+1	15
27	−1	+1	−1	+1	+1	23
28	+1	+1	−1	+1	+1	26
29	−1	−1	+1	+1	+1	16,894
30	+1	−1	+1	+1	+1	17
31	−1	+1	+1	+1	+1	72,108
32	+1	+1	+1	+1	+1	64,507
Additional block						
33	−1	0	0	0	0	10,003
34	+1	0	0	0	0	12.295
35	0	−1	0	0	0	14
36	0	+1	0	0	0	112,078
37	0	0	−1	0	0	71
38	0	0	+1	0	0	64,682
39	0	0	0	−1	0	610
40	0	0	0	+1	0	99,922
41	0	0	0	0	−1	75,159
42	0	0	0	0	+1	92,634
43	0	0	0	0	0	87,937

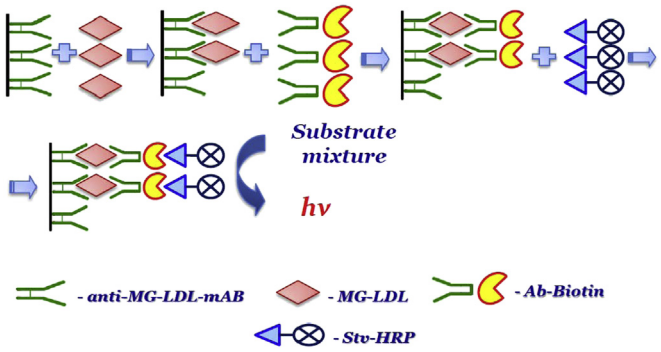

Fig. 2. Scheme of sandwich ELISA for determination of methylglyoxal-modified low density lipoprotein used in this work.

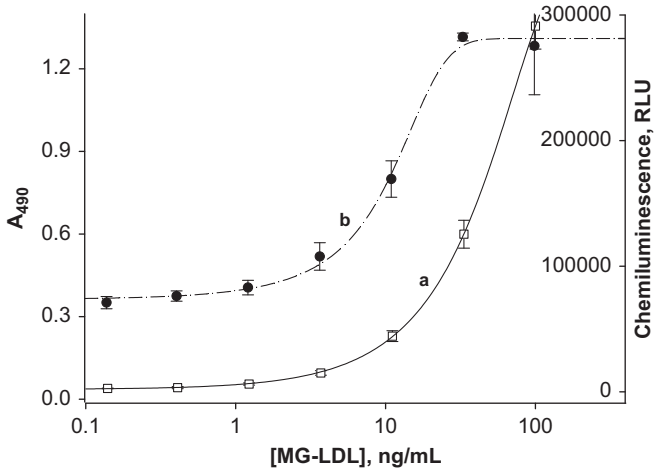

Fig. 3. Calibration curves for determination of MG-LDL by ELISA using (a) chemiluminescent and (b) colorimetric methods to estimate peroxidase activity. Each point represents the mean of duplicates. Vertical bars indicate ±S.D. about the mean.

methods which could control the MG-LDL level in human serum. This stimulated us to develop the sensitive immunoassay for determination of MG-LDL.

In the present work we used a sandwich format of ELISA (Fig. 2). To increase of the sensitivity a streptavidin-biotin pair was used in the assay construction [25,26]. The obtained calibration curves for determination of MG-LDL were presented in Fig. 3. The curve *a* was obtained at the use of CL-ELISA with PPA as the primary enhancer. The detection limit value for the developed method was 0.5 ng mL^{-1}. However, if under the same experimental conditions, when for the HRP determination the colorimetric method with TMB was used instead the chemiluminescent one, the sensitivity was extremely low (data not shown). To improve the sensitivity we increased the concentration of Stv-HRP conjugate (dilution 1:50 instead 1:400). At the increased concentration conjugate the detection limit value for the colorimetric ELISA of MG-LDL was 1.6 ng mL^{-1} (Fig. 3, curve *b*). Thus, the replacement of the colorimetric method of HRP activity measurement with the chemiluminescent one using PPA allowed improving the sensitivity of the assay of MG-LDL and simultaneously 8-fold diminishing the consumption of Stv-HRP conjugate.

4. Conclusions

In enhanced CL reaction a lot of substances (luminol, hydrogen peroxide, PPA, MORPH, HRP, Tris) take place. To optimize experimental conditions of this reaction we used a full factorial design, which previously was successfully used by us to optimize the conditions of ECR with SPTZ [12]. Under the favorable conditions and using PPA the value of detection limit of HRP was 0.09 pM. This value was equal to the detection limit obtained at the use of SPTZ and significantly lower than that at the use of *p*-iodophenol. This opens up very promising perspectives for using PPA to develop enzyme immunoassay kits with improved sensitivity. To prove it we developed the CL-ELISA with PPA use to determine MG-LDL, which was more sensitive than the ELISA with colorimetric detection.

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