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Improved Sensitivity and Wide Range Detection of Small Analytes Using a Two-Antigen-Combined Competitive Immunoassay

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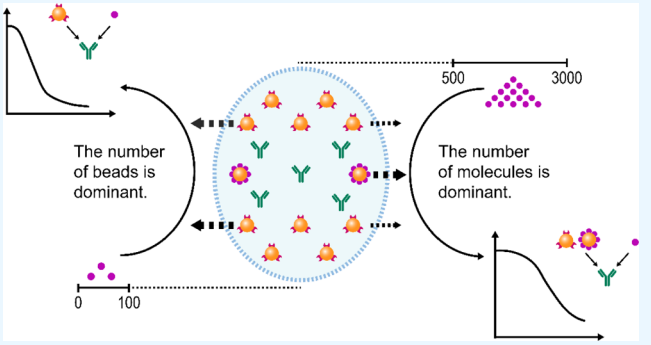
ABSTRACT: Competitive immunoassays have unique advantages in the detection of small molecules and are widely used in clinical practice. However, the concentrations of some analytes usually vary greatly among different populations, which makes it difficult to balance the sensitivity and detection range of competitive immunoassays. Studies have shown that using haptens with weaker affinity for specific antibodies as competitive antigens can help improve the sensitivity of the method. Here, we developed a competitive light initiated chemiluminescence assay based on the combination of antigens with different affinities, which has high sensitivity and wide detection range. As a proof of concept, estradiol was used as the analyte. After the mixing ratio was optimized, the two labeled haptens played different competitive roles due to the different concentrations of estradiol to be tested, which improved the sensitivity of estradiol detection, while ensuring a certain detection range. The limit of detection of this method was 5.30 pg/mL, which is lower than most current estradiol immunoassay kits. Good linearity (R^2 = 0.9902) was obtained between estradiol concentrations of 17.07−2376.22 pg/mL. This study provides a new solution for the detection of small molecule biomarkers with a large concentration span, which also has considerable potential in other immunological detection methods.



1. INTRODUCTION

Since the establishment of radioimmunoassay (RIA) in 1959, various labeled immunoassay techniques have come out one after another and have been widely used in the detection of various substances.[1] In clinical testing, there are small molecules containing only a single epitope, known as haptens, such as testosterone, progesterone, and estradiol in sex hormones, which are steroidal in nature and are mostly detected using competitive immunoassays.[2,3] A variety of small molecule detection methods based on the competition format have been developed, such as fluorescence immunoassays, chemiluminescence, and electrochemiluminescence immunoassays.[4−6] In competitive immunoassay, the labeled hapten is used as a competitive antigen and competes with the analyte to bind a limited amount of specific antibodies to form immune complexes, respectively.[7,8] After the free labeled hapten is separated, the signal value of the labeled hapten−antibody complex in the bound state is measured, and the concentration of the analyte can be converted from the dose−response curve. The curve of competitive immunoassay is an inverted "S" curve.[9] The head and tail curves tend to be flat, and the central part of the curve is nearly straight, which is the ideal detection range, that is, the linear interval, but this interval is narrow. When the detection of a clinical indicator requires both high sensitivity and a wide detection range, conventional competitive immunoassay methods often fail to meet the clinical requirements.[10−12] It has been found that the need for sensitivity can be met if labeled haptens with weaker affinity for specific antibodies are used as competitive antigens.[13,14] In the detection of some small molecule hormones, structural analogues can be used instead of small molecule hormones as labeled competitors to enhance the sensitivity of the analysis, such as estriol instead of estradiol and dihydrotestosterone instead of testosterone.[15,16] On the other hand, the use of labeled haptens with higher affinity to specific antibodies as competitive antigens allows for a wider detection range. Using the abovementioned principles, two reagents can be developed for the detection of samples containing high or low levels of the analyte, respectively. However, this approach will increase the cost of reagents and the workload of testing personnel.

The simultaneous use of two competitive antigens with different affinities based on light-initiated chemiluminescence immunoassay (LiCA) provides a new way to solve the abovementioned problems. LiCA is the latest generation of

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chemiluminescence technology.[17,18] It is based on the antigen−antibody reaction to bring sensibeads and chemibeads close to each other to achieve the delivery of high-energy reactive oxygen species and induce a photo-excited chemiluminescence process to detect the light signal in a homogeneous system.[19] The nanospheres have small diameters and good suspension in the liquid phase, making the antigen−antibody more likely to collide and interact with each other, which greatly improves the detection sensitivity and shortens the detection time. In addition, the nanospheres have a large specific surface area, and the local concentration of biomolecules can be controlled by the coating ratio of antigen or antibody molecules on the chemibeads.

Based on the diffusion characteristics of nanospheres in the liquid phase, that is, the collision probability between high-concentration nanoparticles and molecules in the liquid phase is higher than that of low-concentration particles, we propose the following assumptions. In LiCA-based competitive immunoassays, if haptens and hapten analogues with different affinities to specific antibodies are separately coated on chemibeads, the two competitive antigens can be distributed in the liquid phase with the concentration of chemibeads. After the two chemibeads are mixed, by adjusting the coating ratio of the competitive antigen on the chemibeads and the mixing ratio of the two chemibeads, the labeled hapten analogues compete with the low-level analyte to improve the detection sensitivity, while the labeled hapten competes with high-level analyte to broaden the detection range.

Based on the abovementioned assumptions, we established a LiCA-based quantitative detection method for estradiol, which can improve the sensitivity of estradiol detection while ensuring a certain detection range. 17β-Estradiol is the most biologically active estrogen, which can promote the development of the reproductive system and maintain the secondary sexual characteristics of women.[20] The determination of estradiol can be used to clinically elucidate the diseases of the hypothalamic−pituitary−gonadal axis and assist in the diagnosis of precocious puberty in children.[21,22] It can also be used for *in vitro* fertilization (IVF) to monitor fertility treatments and to determine the timing of ovulation.[23] The distribution of estradiol concentrations in prepubertal children, adolescents, men, premenopausal and postmenopausal women, and pregnant women spanned several orders of magnitude (40−2000 pg/mL).[24−26] Therefore, it is necessary to develop a quantitative detection method for estradiol with high sensitivity and wide detection range. In this study, estriol and estradiol (see Figure 1 for chemical structure) linked to bovine serum albumin (BSA) were separately coated on chemibeads as competitive antigens and constituted a mixed reagent. The coating ratio and mixing ratio of the two chemibeads were adjusted, so that the two competitive antigens can selectively act according to the concentration of estradiol to be detected, which improves the sensitivity of LiCA to detect estradiol and at the same time ensures an appropriate detection range.

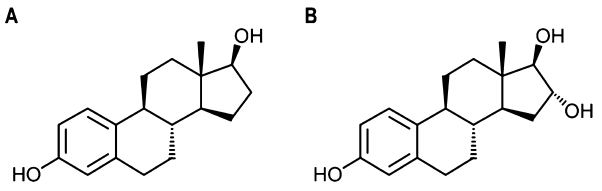

Figure 1. Chemical structures of (A) estradiol and (B) estriol.

2. EXPERIMENTAL SECTION

2.1. Sera Samples. Serum from patients with estradiol in Tianjin Hexi Obstetrics and Gynecology Hospital from January 2022 to May 2022 was collected and stored at −80 °C until use. The study was approved by the Ethics Committee of Tianjin Medical University (TMUHMEC2017008) and conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all human subjects.

2.2. Reagents and Equipment. Estradiol-3-BSA (E2-BSA) and estriol-BSA (E3-BSA) were purchased from EastCoast Bio (Maryland Heights, USA). Anti-estradiol antibody was purchased from Abcam (Cambridge, UK). Estradiol International Reference Material (BCR 576,577,578) was purchased from LGC standards (Teddington, UK). Estradiol quality controls were purchased from Bio-Rad (California, USA). Chemibeads and streptavidin-coated sensibeads were obtained from Beyond Biotech (Shanghai, China). All the tests were performed using LICA-HT analyzer (Chemclin, Beijing, China).

2.3. Preparation of Estriol and Estradiol-Conjugated Chemibeads. Coupling of E2-BSA and E3-BSA to chemibeads was performed according to the procedure in previous studies.[18] The general steps are as follows: first, 2 mg of chemibeads was washed with distilled water and resuspended in 0.05 M carbonate buffer (pH 9.6). A certain volume of E2-BSA or E3-BSA antigen solution was added, and the mixture was shaken at 37 °C overnight. Then, 40 μL of $NaBH_4$ (8 mg/mL) solution and 10 μL of glycine (75 mg/mL) were added sequentially. After washing twice with distilled water, the microsphere pellet was resuspended in 200 μL of preservation solution (25 mM HEPES + 1% BSA + 0.1% ProClin-300) to a final concentration of 10 mg/mL and stored at 4 °C in the dark. Antigen-coupled chemibeads with different coating ratios can be obtained by controlling the amount of antigen added. E2-BSA-coupled chemibeads were defined as CB-E2 in this study, and E3-BSA-coupled chemibeads were defined as CB-E3.

2.4. Competitive Determination of Estradiol Using CB-E3 or CB-E2 Alone. First, after diluting the biotinylated anti-E2 antibody to a certain concentration, it was pre-incubated with a series of concentration estradiol solutions at 37 °C for 15 min. Then, diluted CB-E2 (or CB-E3) was added and incubated again at 37 °C for 15 min. Finally, 175 μL of streptavidin-coated sensibeads was added in the dark and incubated at 37 °C for 15 min, and the signal value was obtained. The logarithm of the estradiol concentration was taken as the abscissa and B/B_0 (percentage of calibrator or sample signal to zero signal ratio) as the ordinate to draw the competition curve. The differences in sensitivity and linear range of the method were observed when E3-BSA and E2-BSA were used as competitive antigens, respectively.

2.5. Competitive Determination of Estradiol Using a Mixture of CB-E3 and CB-E2. First, CB-E2 (Ag/bead = 1:250) and CB-E3 (Ag/bead = 1:1000) with the same concentration but different coating ratios were prepared, and CB-E2 was diluted at a ratio of 1:5000. At the same time, CB-E3 was diluted at the ratio of 1:5000, 1:1000, and 1:250, respectively. The diluted CB-E2 and CB-E3 were used as competitive antigens to detect a series of estradiol solutions

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Table 1. Different Competitive Antigen Detection Conditions

condition	1	2	3	4	5	6	7
competitive antigen no. 1	CB-E2				CB-E2	CB-E2	CB-E2
coating ratio no. 1	1:5000				1:5000	1:5000	1:5000
competitive antigen no. 2		CB-E3	CB-E3	CB-E3	CB-E3	CB-E3	CB-E3
coating ratio no. 2		1:5000	1: 1000	1: 250	1:5000	1:1000	1:250

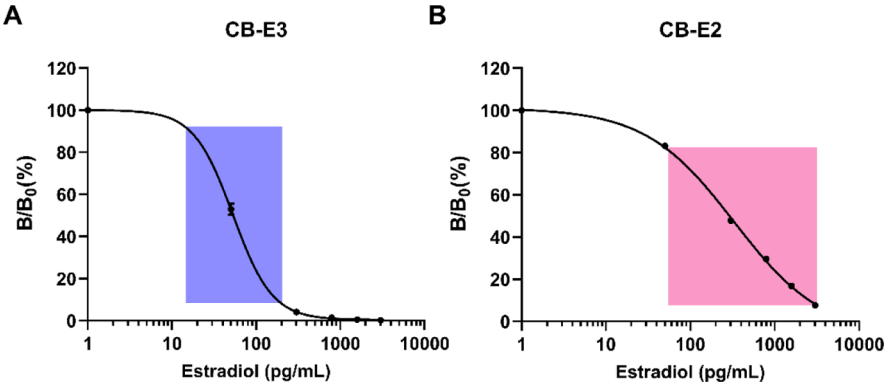

Figure 2. Dose−response curves for the detection of estradiol using different competitive antigens. (A) CB-E3 was used as competitive antigen; (B) CB-E2 was used as competitive antigen. The colored rectangles indicate the linear range.

(conditions 1−4). Then, the diluted CB-E2 was mixed with 3 different dilution ratios of CB-E3, so that the concentration ratios of CB-E3 and CB-E2 were 1:1, 5:1, and 20:1 respectively (conditions 5−7). A series of concentrations of estradiol solutions were detected under these three conditions. The ratios of the signals produced when CB-E3 and CB-E2 acted alone versus in combination were calculated (Table 1).

2.6. Optimization of the LiCA-E2 Method Combining Two Competitive Antigens. First, the coating ratio of CB-E3 and CB-E2 was optimized. Since CB-E3 was used for sensitivity enhancement, estradiol solutions at concentrations of 0, 5, 10, 15, 20, 25, 30, 40, and 50 pg/mL were prepared, and then, different coating ratios of CB-E3 were used for the detection of these solutions. Since CB-E2 was used to broaden the upper limit of detection (LoD), estradiol solutions were prepared at concentrations of 100, 200, 500, 1000, 1500, 2000, 2500, 3000, and 3500 pg/mL. Then, different coating ratios of CB-E2 were used to detect these solutions.

Subsequently, the mixing ratio of CB-E3 and CB-E2 was optimized. The CB-E2 with the optimal coating ratio was diluted to a certain concentration, and then, CB-E3 with different concentrations of the optimal coating ratio was added to it. Under the condition of CB-E2 acting alone and under the condition of mixing with CB-E3, a series of concentrations of estradiol solutions were detected respectively. Changes in low-end sensitivity and high-end discrimination under different conditions were compared. In addition, the concentrations of biotinylated anti-E2 antibody, reaction buffer, and E2 release reagent were also optimized.

2.7. Performance Assessment of LiCA-E2 Assay. According to the CLSI-EP05-A3 guideline document,[27] three levels of E2 quality controls were used for the precision assessment of this method. Each sample was tested five times per day for 5 consecutive days. Mean, standard deviation and coefficient of variation (CV) were calculated.

The ability of LiCA to detect estradiol was assessed according to the CLSI-EP17-A2 guideline document.[28] First, five blank samples without estradiol were selected to confirm the limit of blank (LoB). One of the samples was tested every day, and the test was repeated 12 times. Then, five low-level samples with estradiol concentrations between LoB and 4LoB were selected to obtain the LoD, one of the samples was tested each day, and each sample was tested in 12 replicates. To obtain the limit of quantification (LoQ), a low-value sample with an estradiol concentration of 59.89 pg/mL was serially diluted to five concentrations. Each sample was tested 20 times. The estradiol concentration when the CV is less than 20% was defined as the LoQ.

According to the CLSI-EP06 guideline document,[29] a low-value serum and high-value serum were mixed in different proportions to prepare 11 concentration samples to evaluate linearity. The 11 samples were detected by the LiCA-E2 method and the method using CB-E3 as a competitor, respectively, and the linear ranges of the two methods were compared.

The specificity of LiCA for the detection of estradiol was assessed according to the CLSI-EP07 guideline document.[30] The interference of common chemicals in serum with this method was first assessed. Estradiol samples with low, middle, and high levels were selected in duplicate, with common interfering substances added to one as the interference sample, and the other with the same volume of diluent as the basic sample. The concentration of estradiol in the interference sample and the basic sample was simultaneously detected by LiCA method, and the interference rate was calculated. In addition, the cross-reactivity of substances with similar structures to estradiol in the detection of estradiol by LiCA was evaluated. Estrone, estriol, testosterone, and 17-hydroxyprogesterone were added to blank samples without estradiol, and the estradiol concentration in these samples was measured by the LiCA-E2 method. The cross-reactivity rate was then calculated.

According to the CLSI-EP09 guideline document,[31] 110 serum samples with estradiol concentrations ranging from 8.79 to 2322.04 pg/mL were detected by Cobas[32] and LiCA methods, respectively, and the consistency of the detection results of the two methods was compared.

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2.8. Statistical Analysis. Data processing was performed using SPSS 12.0 (IBM, New York, USA). Method comparison was performed using MedCalc (MedCalc Software Ltd., Ostend, Belgium).

3. RESULTS AND DISCUSSION

3.1. Comparison of the Competitive Ability of CB-E3 and CB-E2. Based on the LiCA method, the competitive ability of CB-E3 and CB-E2 as competitive antigens was verified. As shown in Figure 2A, CB-E3 as a competitive antigen was beneficial to improve the analytical sensitivity, but the upper LoD was low. As shown in Figure 2B, the sensitivity of the method was lower when CB-E2 was used as a competitive antigen, but the upper LoD was higher. If the two antigens were used in combination, the sensitivity may be improved and the upper LoD may be broadened at the same time.

3.2. Determination of Estradiol Using a Mixture of CB-E3 and CB-E2. Figure 3 shows the schematic diagram of

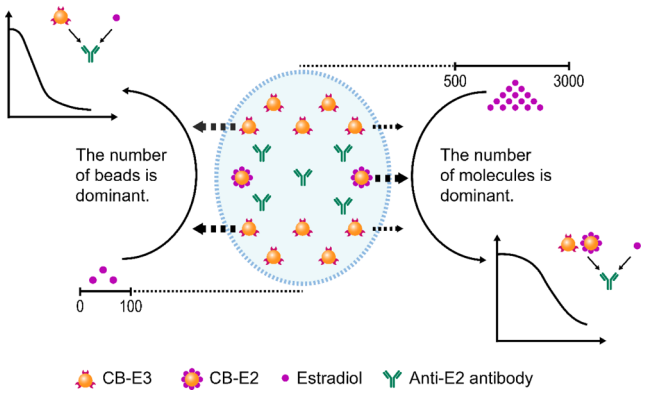

Figure 3. Schematic diagram of mixing two immobilized competitive antigens with different affinities to detect estradiol.

the combined use of immobilized competitive antigens with different affinities to detect estradiol. E3-BSA was coated on the chemibead surface with a low coating ratio (CB-E3), and E2-BSA was coated on the chemibead surface with a high coating ratio (CB-*n**E2). The two chemibeads were then mixed, and the concentration of CB-E3 was higher than that of CB-n*E2 (n*CB-E3 > CB-n*E2). Then, CB-E3 and CB-E2 were used as competitive antigens to detect estradiol.

When the concentration of the antigen to be detected is low, the chance of immobilized antigen binding to the antibody is highly dependent on the number of beads. Therefore, when low levels of estradiol samples are detected, n*CB-E3 takes advantage of the number of beads, diffuses to the end of estradiol and anti-E2 antibody, and competes with estradiol for binding to the anti-E2 antibody. Since the affinity of E3-BSA to the antibody is weaker than that of estradiol, estradiol is preferentially bound to the antibody, which can improve the sensitivity of the estradiol assay. When the concentration of estradiol in the sample is high, estradiol takes advantage of its molecular number to rapidly disperse and distribute around the antibody and the two labeled antigens. In this case, the chance of antibody−antigen binding is no longer affected by the concentration of beads. Despite the small concentration of CB-n*E2, the high amount of E2-BSA coated on the chemibeads plays a major competitive role, competing with estradiol for binding to the antibodies, thereby broadening the upper LoD.

The hypothesis that the immobilized antigens played different competitive roles depending on the concentration of estradiol was verified in the condition of combined use of CB-E3 and CB-E2. Figures 4 and S1 show the signal generated when CB-E3 and CB-E2 were used alone to detect different concentrations of estradiol as a percentage of the signal generated when they were used together. Our previous experiments confirmed that the sum of the signals generated when the two antigens were used alone was equivalent to the signals generated when the two antigens were used together. Therefore, in Figures 4 and S1, the proportion of signals produced by CB-E2 and CB-E3 corresponds to the respective competitive roles of the two antigens in the detection of estradiol under the combined conditions.

As shown in Figure 4A,B, when the concentration of estradiol was low, the more competitive effect of E3-BSA was produced as the concentration of CB-E3 in the mixture gradually increased. On the contrary, as shown in Figure 4C,D, when the concentration of estradiol was high, with the gradual increase of the concentration of CB-E3 in the mixture, the competitive effect of E3-BSA increased, but E2-BSA still played the main competitive role. Figure S1 also shows that when detecting low concentrations of E2, increasing the concentration of CB-E3 made it possible to take advantage of the number of chemibeads and preferentially compete with estradiol for binding to specific antibodies, which was beneficial to improve the sensitivity of the method. However, it was worth noting that the ratio of CB-E3 was not as large as possible, and as shown in Figure S1C, excess CB-E3 also affected the competitive effect of CB-E2 in detecting high levels of estradiol. Therefore, the mixing ratio of CB-E3 and CB-E2 should also be appropriate.

3.3. Optimization of LiCA-E2 Assay. According to the hypothesis, E3-BSA was coupled to the chemibeads in a lower ratio and E2-BSA was coupled to the chemibeads in a higher ratio. As shown in Figure 5A, when the mass ratio of E3-BSA to chemibeads is 1:500 and 1:1000, the low-level estradiol samples have better discrimination. Among them, 1:1000 was selected as the best coating ratio of E3-BSA, which requires less E3-BSA. As shown in Figure 5B, when the mass ratio of E2-BSA to chemibeads was 1:62.5, the high-level estradiol samples have better discrimination, which was the optimal coating ratio of E2-BSA.

Then, the mixing ratio of the two was optimized. As shown in Figure 6A, when CB-E2 was used alone at a concentration of 1.0 μg/mL, the high-level samples were well discriminated, but the sensitivity was insufficient to distinguish the samples between 0 and 50 pg/mL. As shown in Figure 6B−D, with the increasing concentration of CB-E3 mixed in, the discrimination of low-level samples gradually increased. When the CB-E3 concentration was 5.0 μg/mL, samples from 20 to 50 pg/mL could be distinguished. When CB-E3 concentrations were 10.0 and 20.0 μg/mL, samples from 10 to 50 pg/mL could be distinguished. Considering the discrimination of high-level samples, the CB-E2 concentration of 1.0 μg/mL and the CB-E3 concentration of 10.0 μg/mL were used as the optimal mixing conditions.

In the competitive immunoassay, the concentration of the specific antibody is also an important factor affecting the detection sensitivity, and the number of molecules of the antibody needs to be reduced to improve the competitive

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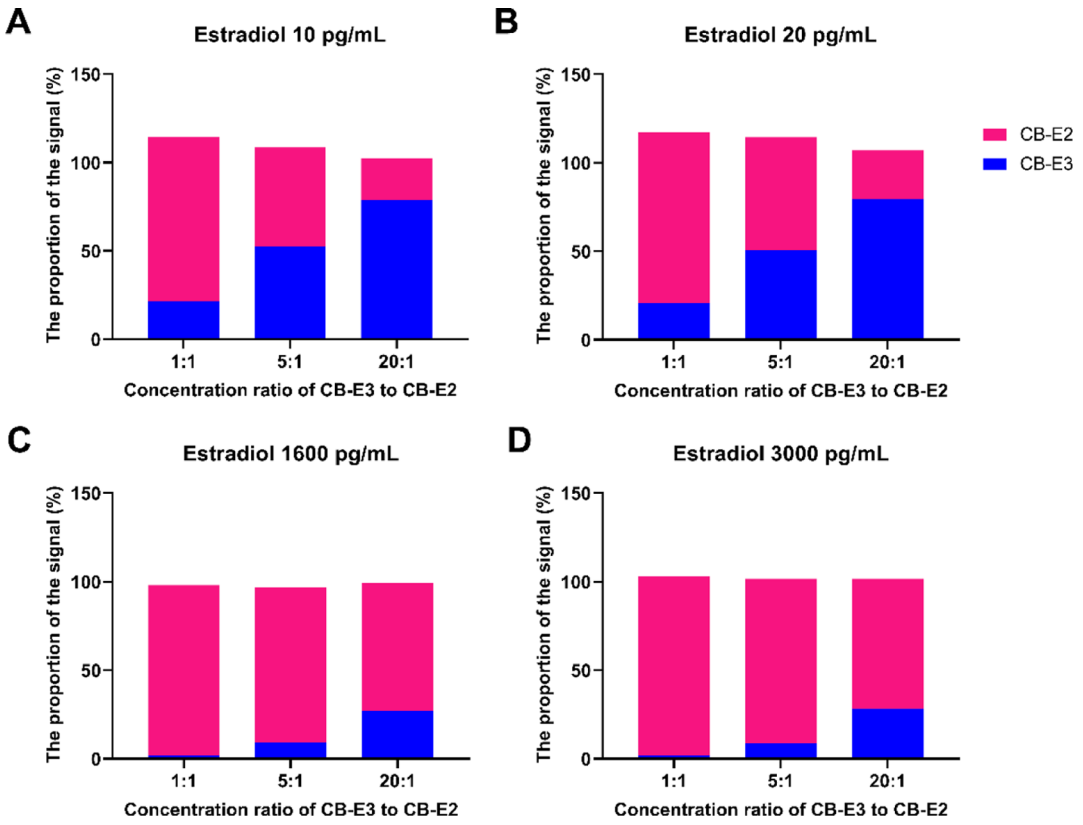

Figure 4. Percentage of signals generated by each of the two antigens in different mixing ratios to the total signal. (A,B) Low levels of estradiol at 10 and 20 pg/mL; (C,D) high levels of estradiol at 1600 and 3000 pg/mL.

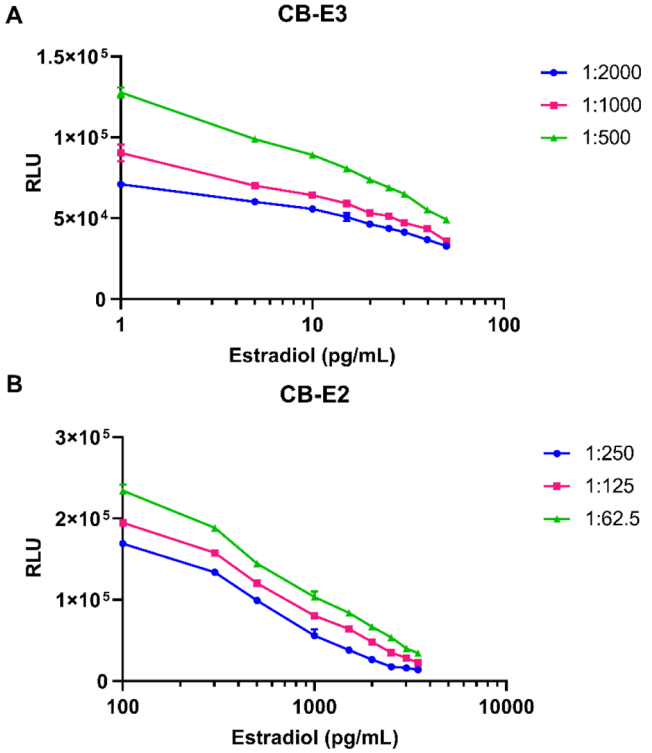

Figure 5. Dose−response curves for the detection of estradiol using competitive antigens with different coating ratios. (A) CB-E3 was used as competitive antigen; (B) CB-E2 was used as competitive antigen. RLU: relative light units.

effect. When the concentration of biotinylated anti-E2 antibody was 0.03 μg/mL, the difference in the signal produced among low, middle, and high levels of serum was the largest, which was the optimal antibody concentration (Figure S2). The reaction buffer was also optimized. Among the four buffers, 2-(*N*-morpholino)ethanesulfonic acid (MES), Tris-HCl, phosphate buffer saline, and citrate, the fitting curve R^2 value was the largest under the condition of 0.05 M MES buffer, and the fitting effect was the best (Figure S3). Since more than 95% of estradiol in serum is bound to sex hormone-binding globulin, it needs to be released from the protein by appropriate treatment for quantitative detection.[33,34] Studies have shown that proper acid treatment promotes the release of estradiol. As shown in Figure S4, when the pH of the release reagent was 6.5, the slope of the fitting curve was the largest. In addition, after estradiol was released, the mesterolone in the release reagent can bind to sex hormone-binding globulin, preventing estradiol from binding to sex hormone-binding protein again. As shown in Figure S5A, mesterolone had no significant effect on low-level serum signal. At the mesterolone concentration of 50 ng/mL, the signal values were the lowest in the intermediate- and high-level serums (Figure S5B,C). Therefore, 50 ng/mL is the optimal concentration of mesterolone.

3.4. Calibration. Estradiol solutions were diluted with charcoal-stripped human serum (Equitech Enterprises, Inc., USA) to prepare a series of estradiol calibrators at 0, 50, 300, 800, 1600, and 3000 pg/mL. All calibrators were traceable to the International Reference for Estradiol (BCR 576,577,578). The calibration curve was established with the logarithm of the estradiol concentration as the abscissa and the B/B_0 value as the ordinate. The concentration of estradiol in serum was

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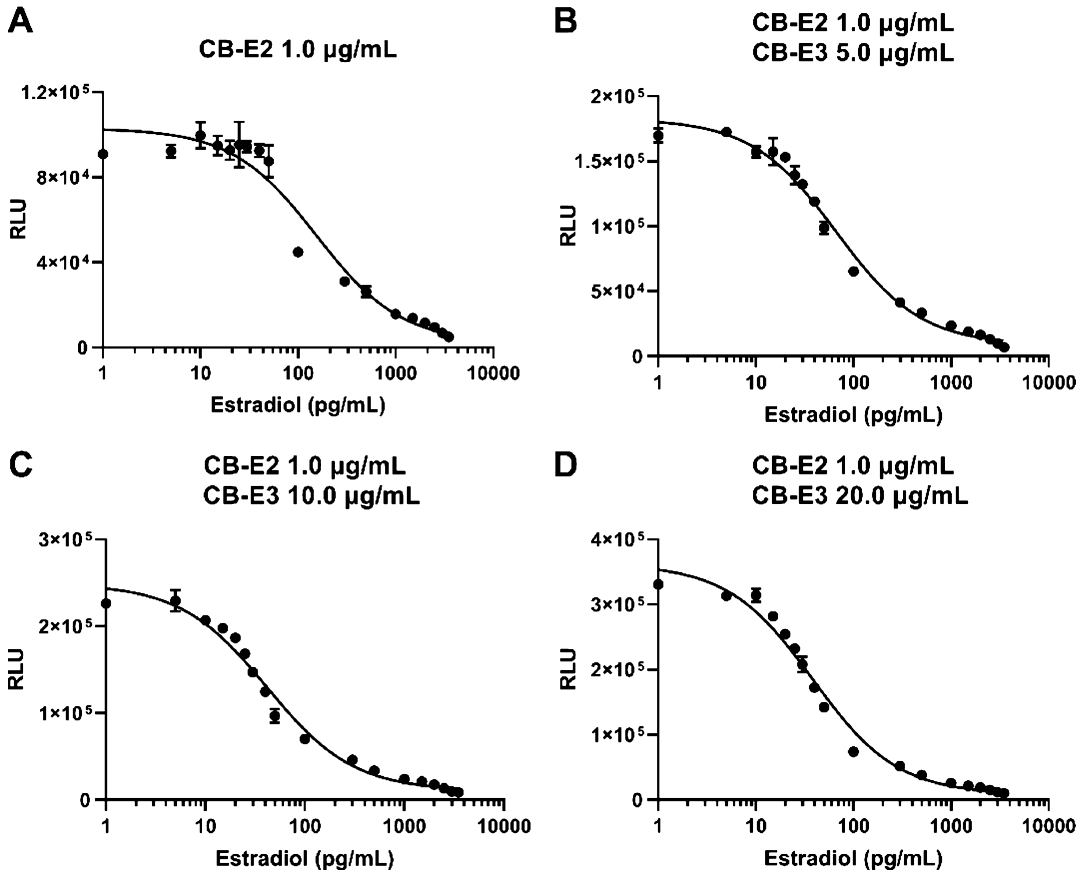

Figure 6. Optimization of the mixed concentration of two competitive antigen-coated chemibeads. (A) CB-E2 1.0 μg/mL; (B) CB-E2 1.0 μg/mL, CB-E3 5.0 μg/mL; (C) CB-E2 1.0 μg/mL, CB-E3 10.0 μg/mL; and (D) CB-E2 1.0 μg/mL, CB-E3 20.0 μg/mL. Each sample was performed in duplicate.

inversely proportional to the intensity of the signal produced. As shown in Figure 7, the best fitting curve was a four-parameter fitting curve, and the equation was

$$y = (A - D)/\left[1 + \left(\frac{x}{C}\right)^{B}\right] + D$$

$$(A = 99.98,\ B = 3.43,\ C = 1.93,\ D = -10.00,\ R^2 = 0.9998)$$

3.5. Performance Assessment of LiCA-E2 Assay. The precision of the established LiCA-E2 method was first evaluated using three levels of quality controls. As shown in Table S1, the CV of repeatability is between 6.08 and 9.72%,

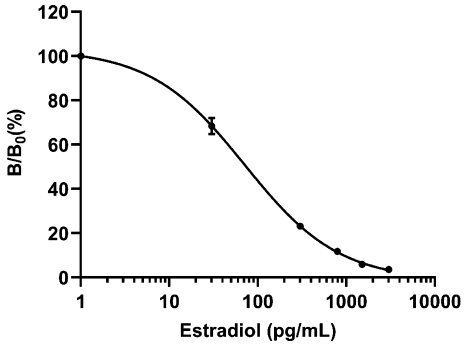

Figure 7. Calibration curve of the LiCA method to detect estradiol.

and the CV of intermediate precision is between 7.32 and 12.52%, implying acceptable reproducibility of LiCA-E2 method. Then, the detection ability of the method was evaluated. After statistics, a total of 60 results from 5 blank samples showed non-normal distribution ($p < 0.01$), and the LoB calculated by non-parametric statistical method was 1.86 pg/mL. A total of 60 results from 5 low-value samples showed homogeneity of variance, and the LoD calculated by parametric statistics was 5.30 pg/mL. When the estradiol concentration was 20.90 pg/mL, the corresponding CV was 19.82% (Table S2), which was less than 20%. Therefore, the LoQ of the LiCA-E2 method was 20.90 pg/mL. Compared with using CB-E2 alone as a competitive antigen, the sensitivity was greatly improved. As shown in Figure 8, the LiCA-E2 method had good linearity ($R^2 = 0.9902$) between the estradiol concentration of 17.07 and 2376.22 pg/mL. The linear range was broadened compared to when only CB-E3 was used as the competitive antigen (14.60−1365.14 pg/mL, $R^2 = 0.9920$). As shown in Table 2, the LiCA-E2 assay has a lower LoD and a broadened detection range compared to the luminescent oxygen channeling immunoassay (LOCI)[35] and Architect-E2 assay.[36] Compared to the Cobas-E2 assay,[32] our method has comparable sensitivity, but the upper LoD is slightly lacking. In competitive immunoassays, the characteristics of the antibody have a large impact on the detection range. Both Cobas and LiCA-E2 assay use a detection mode in which the labeled antigen competes with the antigen to be tested to bind biotinylated antibodies. The upper LoD of our method is lower than that of Cobas probably due to the different antibodies

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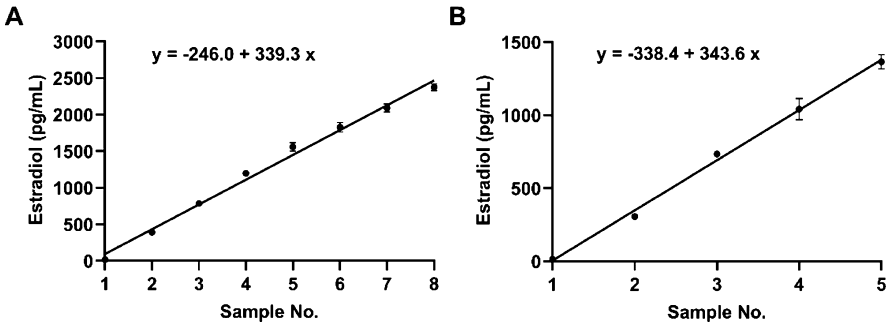

Figure 8. Linear fitting results. (A) CB-E2 and CB-E3 mixed. (B) CB-E3 used alone. The abscissa represented a series of samples with equal concentration intervals. Each sample was performed in triplicate.

Table 2. Comparison between LiCA and Other Methods to Detect Estradiol

methods	LoD (pg/mL)	detection range (pg/mL)
LOCI	11.00	11−1500
Architect	10.00	10−1000
Cobas	5.00	5.00−3000
LiCA	5.30	5.30−2376.22

used. In the follow-up work, we will optimize the antibody to obtain a wider detection range.

As shown in Table S3, when there were 200 mg/dL of hemoglobin, 20 mg/dL of bilirubin, 300 mg/dL of triglyceride, and 20 ng/mL of biotin in serum, the interference rate was less than 15%, which had no significant effect on the test results. In addition, as shown in Table S4, adding a certain concentration of estrone, estriol, testosterone, and progesterone, there was no obvious cross-reaction with estradiol. The above showed that the specificity of the LiCA-E2 method for the identification of potential interfering substances was satisfactory.

The method comparison between the established LiCA-E2 method and Cobas was evaluated using Passing−Bablok regression. As shown in Figure 9, the regression equation was $y = -2.5964 + 0.9531x$ ($r = 0.9640$). The results showed that the LiCA-E2 method had a good correlation with the Cobas method in detecting estradiol.

4. CONCLUSIONS

In this study, two haptens with different affinities to anti-E2 antibodies, E2-BSA and E3-BSA, were coated on chemibeads in different ratios. By optimizing the mixing ratio of the two kinds of chemibeads, the antigens on the chemibeads can have different competitive effects depending on the concentration of estradiol, which successfully improves the sensitivity of LiCA to detect estradiol and at the same time ensures an appropriate detection range. The optimal mixing concentration of the two chemibeads was 1.0 μg/mL for CB-E2 and 10.0 μg/mL for CB-E3. The method has good reproducibility and provides a new solution for the development of detection methods for other small molecules with different detection requirements. A limitation of this study is that when CB-E2 is used alone, the upper LoD is not sufficient to cover the IVF population. According to RIA theory, the properties of the antibody affect the detection range. If referring to the method of combining antigens, the combination of two antibodies may have a positive effect on broadening the detection range. In the future, we will continue to screen for antibodies that can generate a wider detection range to cover more people.

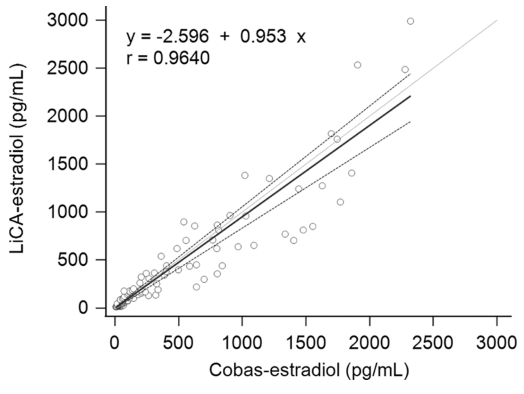

Figure 9. Passing−Bablok regression curve between LiCA and Cobas.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c06126.

Mixed two antigens produce different competitive effects; optimization of biotinylated antibody concentration, reaction buffers, E2 release reagents, and mesterolone concentration; performance assessment of LiCA-E2 assay (PDF)

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Notes

The authors declare no competing financial interest.

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