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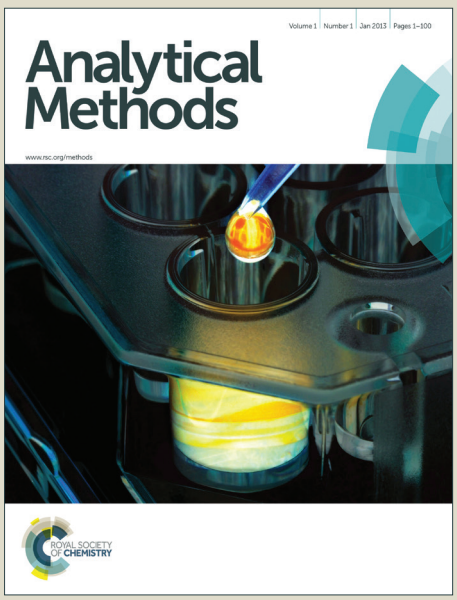

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ARTICLE

A Modified Quick PETIA of Detecting Anti-CCP Antibodies in Human Serum

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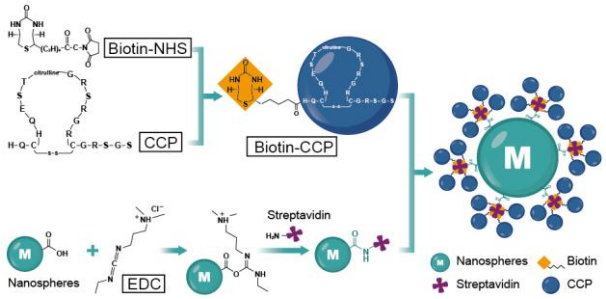

Modified Quick PETIA of Detecting Anti-CCP: Apply streptavidin-biotin pair as a connecting arm between polystyrene nanospheres and ccp.

Running tittle

Modified Quick PETIA of Detecting Anti-CCP

Disclosure

The authors have declared no conflicts of interest.

Acknowledgments

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A Modified Quick PETIA of Detecting Anti-CCP Antibodies in Human Serum

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Abstract

In this paper, a modified Particle Enhanced Turbidimetric Immunoassay (PETIA) was established to facilitate the detection of anti-cyclic citrullinated peptide (anti-CCP) antibodies in human serum. The modified method improved the detection sensitivity by applying streptavidin-biotin pair as a connecting arm to conjugate CCP antigens on the surface of polystyrene nanospheres. With the advantages of the nanoparticles and streptavidin-biotin pair, the modified PETIA exhibited a wide linear range for anti-CCP of 13-430 U/mL, with a low detection limit of 12 U/mL. Satisfactory reproducibility of the immunoassay was demonstrated. Good correlations were obtained in the analysis between the modified method and the commercial ELISA kit. The ROC curve was plotted based on the results from measuring anti-CCP antibodies in 185 clinical serum specimens. The area under the ROC curve was 0.872. With cut-off value of 27 U/mL，diagnostic sensitivity and specificity were 75.68% and 91.89% respectively. The detecting time by this new approach was only 20 minutes. In summary, this modified method can be used to detect anti-CCP antibodies in human serum practically and rapidly.

Keywords: PETIA，CCP，RA, streptavidin

Introduction

Anti-cyclic citrullinated peptide antibody (anti-CCP) is considered to be a valuable serological marker for rheumatoid arthritis (RA) diagnosis[1-3]. In 2009, anti-CCP was brought into the new Criteria for RA from the American College of Rheumatology and the European League Against Rheumatism[4]. Being present early in the disease process, anti-CCP testing is particularly useful in the diagnosis of RA, being present early in the disease process, and able to predict severe diseases and irreversible damages[3, 5]. In addition, the titers might be early predictors of the efficacy of anti-TNF therapy.

It is reported that there are several methods generally applied to detect anti-CCP including the enzyme-linked immunosorbent assay (ELISA), the colloidal gold immunochromatography assay (GICA), and recently-developed chemiluminescence analysis[6-8]. ELISA has advantages of low cost, high sensitivity and non-toxicity, etc，but disadvantages of narrow detection range , long detection time and less automation. Chemiluminescenceis method is characterized with high sensitivity, specificity, reproducibility, wide detection range, easily-operated automation and standardization, etc.. However, due to its high cost, it is not yet been widely used in clinical diagnosis. In addition, another detection method, GICA is report to be with low sensitivity and specificity, and difficult to automation and standardization, even with short detection time.

Conventional PETIA develops recently and becomes more stable and accurate based on homogeneous immunity turbidimetric detection of body fluid proteins[9, 10]. The method is applied to detect the target antigen through conjugation with specific monoclonal antibody which is coated on polystyrene nanospheres surface to capture corresponding antigen. Due to the connection of antigen with antibody, the absorbance of the reaction solution is changed in the short duration time, and the change of absorbance is correlated with the change of concentration of the test antigen[11]. This detection mode has been used in some macromolecular protein antigens, such as C-reactive protein, rheumatoid factor etc[12, 13]. PETIA is characterized as the method to be simple, rapid, accurate, low cost, and easily automated and standardized[14-16]. But, the relatively low sensitivity compared with Chemiluminescenceis method existed in PETIA. Polystyrene nanospheres used in the conventional PETIA have been successfully employed in many areas of research, including cell separation, biomolecule detection, DNA extraction and various immunoassay methodologies[17-20]. Polystyrene nanospheres with bioactive molecules such as antibodies are very useful tools for immunoassays such as PETIA. In conventional PETIA, the polystyrene nanospheres are applied as the detection vectors. The polystyrene nanospheres with antibodies could be almost prepared through chemical coupling with different crosslinking arms. In general, Bovine serum albumin (BSA) or other unrelated proteins are used as the crosslinking arm to couple the antibodies with polystyrene nanospheres.

Unlike the conventional PETIA, the modified PETIA in this study is established for the measurement of anti-CCP. We applied streptavidin-biotin pair as a connecting arm to conjugate CCP on the surface of polystyrene nanospheres to facilitate the detection of anti-CCP antibodies in human serum. Comparing with the ELISA for anti-CCP, the modified method possesses apparent advantages of short analysis time and simple operation. Hence, this method has significant promise and could be further developed for practical clinical detection of anti-CCP.

Experimental section

Reagents and apparatus

NHS(N-Hydroxysuccinimide), EDC(1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride), Tween-20, PEG6000(polyethylene glycol 6000), MES(2-(N-morpholino)ethane sulfonic acid), DMSO(dimethyl sulfoxide), SA(streptavidin), NHS-Biotin (Biotinyl-N-Hydroxysuccinimide Ester) were purchased from Sigma. Carboxylated polystyrene nanospheres was purchased from Bangs Laboratories Co. Analytical grade water was prepared with 18.2MΩcm, by Millipore MilliQ system.

Enzyme-linked immunosorbent assay (ELISA) kit and anti-CCP electrochemiluminescence kit were purchased from AESKU (Germany) and ROCHE (Switzerland) respectively. PD MiniTrap G-10 desalting columns were purchased from GE (USA). SpectraMax M5 multifunctional microplate reader was purchased from MD (USA).

Preparation of Anti-CCP Polypeptide Antigen

Anti-CCP polypeptide was sequenced as HQCHQESTCitrullineGRSRGRCGRSGS, synthesized by China Peptide Co. according to the protocol of solid-phase synthesis of peptides by Daveyand[21] and modified methods by Hammer & Albericio[22]. The purity of synthetic polypeptide was greater than 95% with separation and purification by high performance liquid chromatography.

Fixing Process of Anti-CCP Polypeptide Antigen to polystyrene nanospheres

As shown in Figure 1, the crosslinking process between synthesized CCP and polystyrene nanospheres were divided into three steps.

First, synthesized CCP was biotinylated in the amino-terminal, followed by isolation and purification of the biotinylated CCP. The procedures were divided by several steps, CCP was dissolved in carbonate buffer (0.1 M pH 9.5) with concentration of 20 mg/mL. The activated carboxyl NHS-Biotin was also dissolved in DMSO with concentration of 22 mg/mL. Solution of CCP and solution of NHS-Biotin were mixed in a volume ratio of 10:1, and incubated at room temperature for 4 h. After completion of the reaction, biotinylated CCP was dialyzed through the dialysis tubing and purified at 2-8 °C.

Secondly, streptavidin-labeled nanoparticles were prepared by using carbodiimide reaction. 25 μL fresh EDC

solution (10 mg/mL) was mixed with 40μL NHS solution (10 mg/mL) and 10mg carboxylated polystyrene nanospheres, to activate carboxyl group on the surface of nanospheres. Nanosphere suspension was washed in 1mL reaction buffer (0.1 M MES, pH 6.0) for three times after reaction at room temperature for 20 min, then 100 μg streptavidin-biotin was added to activate nanospheres with incubation for 2.5 h at room temperature. After the streptavidin-labeled nanospheres were washed for 3 times by washing buffer, the nanospheres were incubated with 3 mL blocking buffer (0.5 M BSA-containing phosphate buffer, pH 7.4) at room temperature for 1 h. With further washing of streptavidin-labeled nanospheres, the nanospheres were resuspended in PBS buffer and stored at 4°C. Finally, biotinylated CCP was specifically bound with nanospheres labeled with streptavidin. 1mL10% labeled streptavidin nanospheres were washed with PBS buffer (0.1M, pH7.4), mixed with 1.92mg biotinylated CCP, and incubated at room temperature for 30min. The reaction product was separated, purified by centrifugation and resuspended in storage buffer (containing 0.05% BSA and 0.1M glycine buffer of 0.05% NaN3, pH 8.4).

Collection of Human Serum Sample

185 serum samples were collected from Nanfang Hospital inpatients, including 111 RA patients, 49 patients with other rheumatic diseases (17 patients with osteoarthritis, 11 patients with ankylosing spondylitis, 10 patients with systemic lupus erythematosus, 9 patients with connective tissue disease, 1 patient with Sjogren's syndrome, 1 patients with reactive arthritis) and 25 healthy control serum specimens, all RA patients were enrolled by 2010 ACR / EULAR revised diagnostic criteria and exclusion criteria. Age and sex distribution between the groups was not statistically significant. All serum samples were collected and stored at -20℃.Collection of these clinical samples was approved by the Ethical Committee of Science and Technology Department of Southern Medical University.

Establishment of modified PETIA System

Modified PETIA system was comprised of reagent R1 of glycine buffer containing 100 mM glycine, 300 mM NaCl, 0.1% Tween-20, 0.05% NaN3 (pH8.4) and reagent R2 containing 0.5% CCP polypeptide antigen-labeled polystyrene nanospheres, 100 mmol/L glycine, 0.05% BSA, 0.05% NaN3 (pH8.4). Modified PETIA reaction process was shown in Figure 2. 6μL serum was placed in wells, adding 200μL R1 reagent, which were incubated for 5 min at 37°C. Then 50μL R2 reagent was added, and the absorbance at 562nm was recorded. The absorbance at 562nm was recorded again after incubation for 15 min at 37°C, and the change of the absorbance (ΔA) was calculated. The concentration of anti-CCP in test serum that correlated with ΔA was calculated according to the calibration curve of anti-CCP. The procedure was shown in Figure 2.

Statistical Analysis

Mean and standard deviation of absorbance changes were calculated by SPSS13.0. The scatter plots were obtained by correlation of change of absorbance value (y) with anti-CCP concentrations (x) and linear equation $y = A + B \times x$ was calculated by SPSS13.0. In addition, correlation and linear regression between the values from commercial ELISA kit and modified PETIA were analyzed via Pearson mathematical method. $P < 0.05$ (analyzed by paired t-test analysis) was considered to be statistically significant.

Results and Discussions

Optimization of the preparation of polystyrene nanospheres coupled with synthesized **CCP**

Conventional PETIA adopted the pattern of detection antibodies or antigen protein through the particles coupled with antibodies. Under this pattern, the antibodies could be easily coupled with the microspheres by physical adsorption or chemical binding method. In the present study, a modified PETIA was taken to detect anti-CCP in human serum. So the preparation of polystyrene nanospheres coupled with CCP was the critical step. In our previous experiment, the BSA was applied as the crosslinking arm between CCP and the nanospheres to ensure the CCP adequate to capture the anti-CCP in serum. The result was unsatisfactory because of the composition complexity of BSA (the results were not shown). The Streptavidin-biotin was provided with the advantage of specific and efficient binding mode between streptavidin and biotin. Thus, we improved the procedure. Streptavidin-biotin as a cross-linking arm to link synthesized CCP to the surface of nanoparticles was applied and optimized in the present study, and the CCP-labeled nanospheres had been successfully produced.

The crosslink of SA with carboxylated polystyrene nanospheres is dependent on the factors such as the amount of EDC, incubation time, PH value etc. Since high concentration EDC might cause irreversible aggravation of carboxylated microspheres, we needed to ensure the SA labeling amount and reduce the EDC amount as much as possible. As shown in Figure 3A, the difference of the SA label amount was not significant when the concentration of EDC was higher than 1.2 g/L. Thus, 1.2g/L was the optimum concentration of EDC. Maximum SA labeling amount was present in the buffer with pH 7.0 and 7.4, though there was no statistically difference between buffers of pH 6.5 and 7.8. Therefore, pH 7.4 was chosen as the optimum PH value for the reaction buffer (Figure 3C). SA crosslinking amount was increased with extended incubation time once amount of EDC and the reaction pH were unchanged, as shown in Figure 3B, the steady state of SA crosslinking had been reached after 2.5h incubation. Then, the correlation between SA conjugate amount with adding amount of SA was investigated with the fixed condition of EDC concentration (1.2 g/L), reaction buffer with pH 7.4, and reaction time of 2.5 h, as shown in Figure 3D. The results showed that SA conjugation amount was gradually increased with the addition of increasing amounts of SA, and the highest SA binding was obtained when adding amount of SA reached to 2mg. SA

crosslinked polystyrene nanospheres were subsequently bound with biotinlyated-CCP, and synthesized CCP was stably and specifically conjugated to the surface of the polystyrene nanospheres to achieve the fixation of synthesized CCP on the nanospheres.

Optimization of the modified PETIA

In this study, we introduced a modified PETIA based on the nanospheres coupled with CCP by the crosslinking arm of streptavidin-biotin. The antigen of CCP was immobilized on the surface of nanospheres, and a one-step reaction was carried out. The analytical performance was significantly influenced by several reaction parameters, including PEG6000 concentration, nanoparticle concentration and the serum incubation volume.

As the catalyst to improve the crosslink of antigen with antibody, PEG6000 concentration (2.4-4.4%) was crucial to determine the efficiency of conjugation of antigen and antibody. Therefore, the optimum PEG6000 concentration was characterized from concentration of 2.4% to concentration of 4.4% (Figure 4A). The absorbance which showed the crosslink of antigen and antibody was upregulated with the increase of PEG6000 concentration. However, the upregulation of ΔA was obviously reduced when the concentration of PEG6000 was more than 3.6%. The increasing of PEG6000 concentration could accelerate the antigen-antibody reaction, reduce equilibrium time. The function of PEG6000 was to release electronic layer and hydration shell around the antigen-antibody conjugate, and improve the formation of antigen-antibody complexes. But high PEG6000 concentration could lead to non-specific conjugation. Therefore, 3.6% of PEG6000 concentration was chosen as the optimum PEG6000 concentration.

Nanoparticles were solid phase carrier to capture antibody and enhance antibody conjugation. Therefore, the nanoparticle concentration was optimized to improve sensitivity of the assay without cost increase as much as possible. The calibrated serum of 200 U/mL anti-CCP was chosen for the optimization of nanoparticle concentrations from 0.3-0.6% in the present study. The results revealed that the absorbance increased with the increase of nanoparticles concentration (Figure 4B), but the increase slowed down with CCP labeled nanoparticles concentration more than 0.5%. Meanwhile, the concentration of CCP-labeled nanospheres affected the length of the detection time. High concentrations of CCP-labeled nanospheres could cause the extension time of reaction equilibrium, thereby cause the increasing of detection time of anti-CCP. Therefore, 0.5% of CCP-labeled nanospheres was chosen to be optimum nanosphere concentration.

The ratio of concentrations of anti-CCP in serum sample and CCP-labeled nanospheres was pivotal factor for absorbance measurement. In the present study, the human serum sample with 200 U/mL anti-CCP had been chosen to investigate the optimum serum incubation volume. As shown in Figure 4C, the results revealed that the change

of absorbance was not statistically different when the serum volume was lower than 5 μL. However, the absorbance variation was reduced with serum volume to be higher than 6 μL. Therefore, it could be assumed that 5-6 μL of serum was the optimum incubation volume for the detection. In practical work, increase of the test samples volume could reduce the detection error, and the reproducibility of results will be better. Thus, 6 μL of serum was considered as the optimum incubation volume.

The detection system of the modified PETIA for detection of anti-CCP in human serum was optimized and established with 3.6% of PEG6000 concentration, 0.5% of CCP polypeptide antigen labeled nanospheres, and 6 μL of serum.

Linear range of the particle-enhanced turbidimetric immunoassay

The series of concentrations of anti-CCP (7, 13, 26, 54, 108, 215, 430, 860U/mL) were continuously tested three times for each concentration under the optimum conditions, and the relation between the means and ΔA was calculated to generate the calibration curve. The results were fit by SPSS13.0 to a regression curve. The average deviation from linearity (ADL) of the best-fit regression curve was calculated, and was compared with percent bound (PctBnd, which lower than 5% considered to be linear). The results showed that linear range of this modified PETIA was from 13 to 430 U/mL. Blank sample repeatedly tested for 20 times, mean and standard deviation was calculated. The resulting mean plus 3 SD was defined as the limit of detection (LOB), which is 12 U/mL. Comparing with commercial ELISA with linear range of 0-300 U/mL, the modified method showed wider detection range in the high level concentration of anti-CCP.

Precision of the modified PETIA

Precision of the modified PETIA was evaluated by calculating standard deviation and coefficient of variation of results for intra-assay, between-run, within-day and total-assay according to EP5-A Guidance by CLSI[23]. Briefly, we analyzed two separate run with two test samples of high and low concentration of anti-CCP daily, the detection time between two separate run were greater than 2hrs. The experiment lasted for 20 days. The standard deviation and coefficient of variation (CV) of the testing results in 20 days were calculated according to equations in EP5-A Guidance. The CVs at the concentrations of 20U/mL and 200 U/mL were 3.657% and 2.956% for intra-assay, 6.684% and 4.705% for inter-assay, 5.335% and5.425% for daily assay, and the total variation coefficients were 10.135% and 8.343%. The results are within the criteria recommended by CLSI guidance. Therefore, the precision of the modified PETIA established in this study was within an acceptable range.

Comparison of the modified PETIA to the commercial ELISA kit

ELISA method is the most well-applied detection method to measure anti-CCP in serum. Thus, the performance of

the modified PETIA was evaluated by comparing with a commercial ELISA kit. 40 fresh serum samples were collected and randomly divided into five groups. Each serum was randomly numbered. Serum samples in each group were detected by modified PETIA and ELISA in accordance with the numbering sequence, and were measured again in the same day with reverse order for the consecutive five days. The correlation equation y = 0.9784x +5.8937 was derived by linear regression analysis (Figure 5), in this equation, *x* represented the calculated concentration of anti-CCP from ELISA; *y* represented the calculated concentration of anti-CCP from modified PETIA. Data showed good correlation between modified PETIA and ELISA with the correlation coefficient of 0.981. The detection time of modified PETIA was only 20min, which was much less than detection time (approximate 3h) of commercial ELISA kit.

Clinical Application

Anti-CCP antibodies of serum from 111 RA patients, 49 patients with other rheumatic diseases and 25 healthy control subjects had been detected by using the modified PETIA, and ROC (Receiver Operating Characteristic) curve of data from modified PETIA were generated (Figure 6). The area under the ROC curve was 0.872 (95% confidence interval to be 0.820-0.924). 27 U/mL was the optimum cut-off threshold of the modified PETIA according to the ROC analysis. The diagnostic sensitivity and specificity of modified PETIA for RA patients were 75.68% and 91.89%, and positive predictive value was 93.33% with 27 U/mL of anti-CCP concentration cut-off threshold in serum (Table 1). The reports showed that the sensitivity and specificity of ELISA for RA patients were 41% -87.6% and 88.9%-98%[24-26], while the sensitivity and specificity of chemiluminescence were 67.7%-77.4% and 91.5%-98%[28].'In our research, we found that the sensitivity of ELISA and chemiluminescence were 77.7% and 80.0% respectively, while the specificity was 76.5% and 97.06% respectively (data did not show). Therefore, diagnostic efficiency of chemiluminescence is considered to be the best for CCP-antibody detecting. The diagnostic efficiency of our modified PETIA is between ELISA and chemiluminescence.'.Therefore, the modified PETIA could be applied as an effective method for clinical diagnosis of RA patients.

Conclusion

In conclusion, a modified PETIA to detect anti-CCP in serum was established. It was characterized that applying streptavidin-biotin pair as a cross-linking arm to conjugate synthesized CCP on the surface of polystyrene nanospheres to facilitate the detection of anti-CCP in human serum. The modified PETIA possessed several advantages over previous methods, including short analytical time, broad dynamic assay ranges, and cost-effectiveness. Additionally, the modified method showed good properties for detecting of anti-CCP in human serum with acceptable reproducibility and clinical diagnostic efficacy. Therefore, the modified PETIA could be

developed into a commercial kit of anti-CCP detection for clinical use. Moreover, the PETIA which was modified by the combination of streptavidin-biotin pair and polystyrene nanospheres may provide an interesting tool for the determination of other specific antibodies with small molecules in clinical laboratories.

Acknowledgments

This work was supported by the Guangdong Province production science research fund (grant number 2010B090400542, 2012B091100461) and the Bureau of Science and Technology of the Guangzhou Municipality (grant number 2012J4300074). We would like to thank all the related committees and departments.

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Figure legends/Table Captions

Figure. 1 The procedure of the crosslinking between CCP and polystyrene nanospheres

Figure. 2 Schematic illustration of the particle-enhanced turbidimetric immunoassay.

Figure. 3 Optimization of the conditions for the labeling between streptavidin and nanoparticles

Figure. 4 Optimization of the PETIA assay.

Figure. 5 Plot of the results obtained using the modified PETIA method versus ELISA. commercial kits for anti-CCP detection in 40 serum samples with a correlation coefficient of 0.981.

Figure. 6 ROC analysis of anti-CCP antibodies detected by the proposed PETIA assay.

Table. 1 Diagnostic efficiency of anti-CCP antibodies determined by PETIA method with 27UmL-1 as the cut-off value

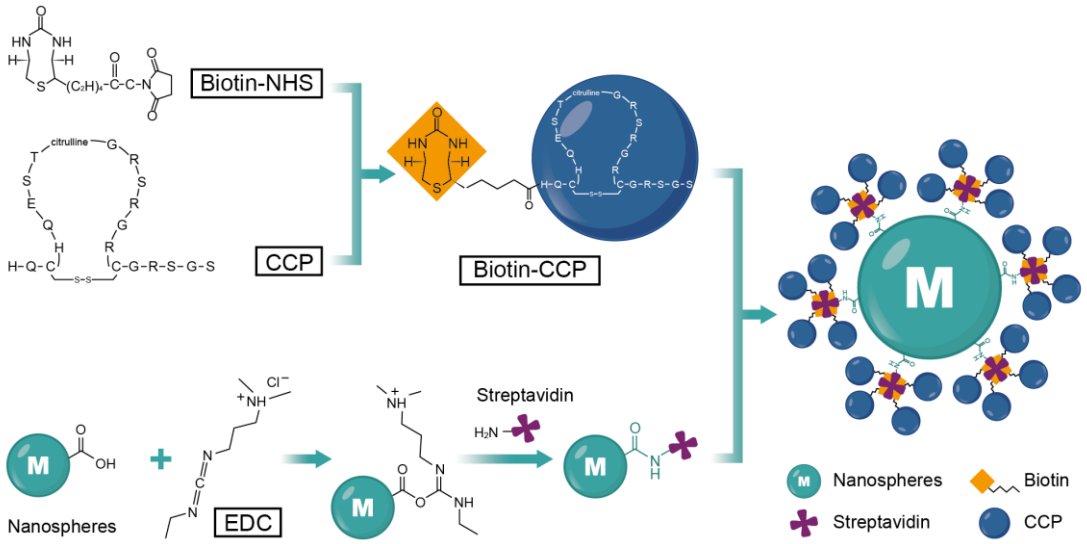


Figure.1 The procedure of the crosslinking between CCP and polystyrene nanospheres The crosslinking process between CCP and nanospheres were divided into three steps. First, CCP polypeptide antigen was biotinylated in the amino-terminal, followed by isolation and purification of the biotinylated CCP antigen. Secondly, streptavidin-labeled nanoparticles were prepared by using carbodiimide reaction between the amino of streptavidin and the carboxylated polystyrene nanospheres. Finally, biotinylated CCP peptide antigen was specifically bound with nanospheres labeled with streptavidin.

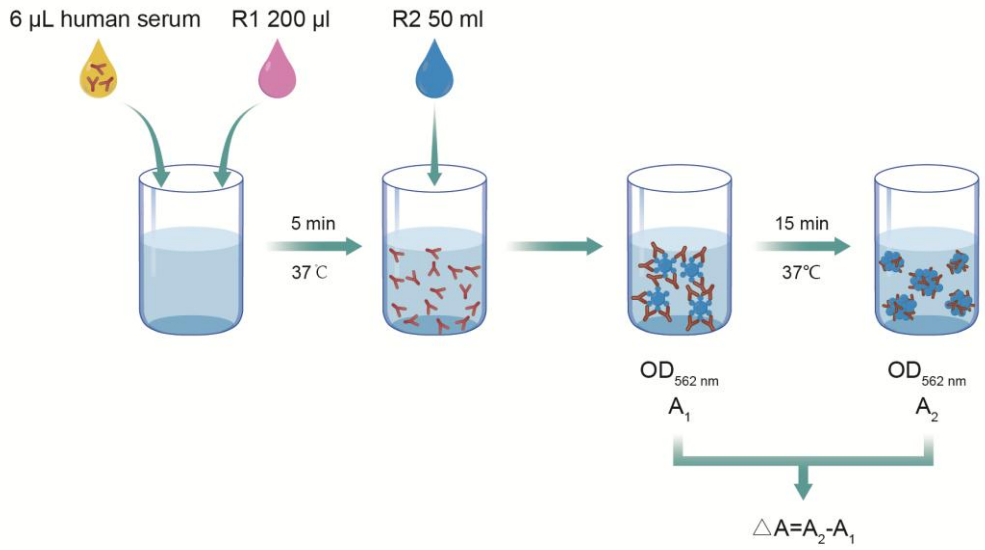


Figure. 2 Schematic illustration of the particle-enhanced turbidimetric immunoassay. 6μL serum was placed in wells, adding 200μL R1 reagent, which were incubated for 5 min at 37 ℃. Then 50μL R2 reagent was added, and the absorbance at 562nm was recorded. The absorbance at 562nm was recorded again after incubation for 15 min at 37°C, and the change of the absorbance (ΔA) was calculated. The concentration of anti-CCP in test serum that correlated with ΔA was calculated according to the calibration curve of anti-CCP.

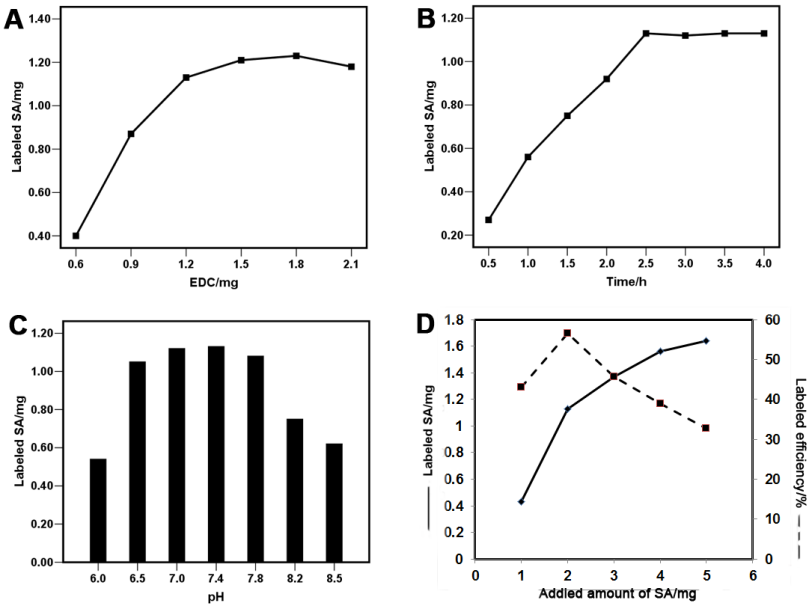


Figure. 3 Optimization of the conditions for the labeling between streptavidin and nanoparticles.(A) Effect of EDC amount to the SA labeling.(B) Effect of incubation time to the amount to the SA labeling.(C) Effect of PH value to the amount to the SA labeling.(D) Effect of SA amount to the amount to the SA labeling(solid line standfor labeled SA, dashed line standfor labeled efficiency).

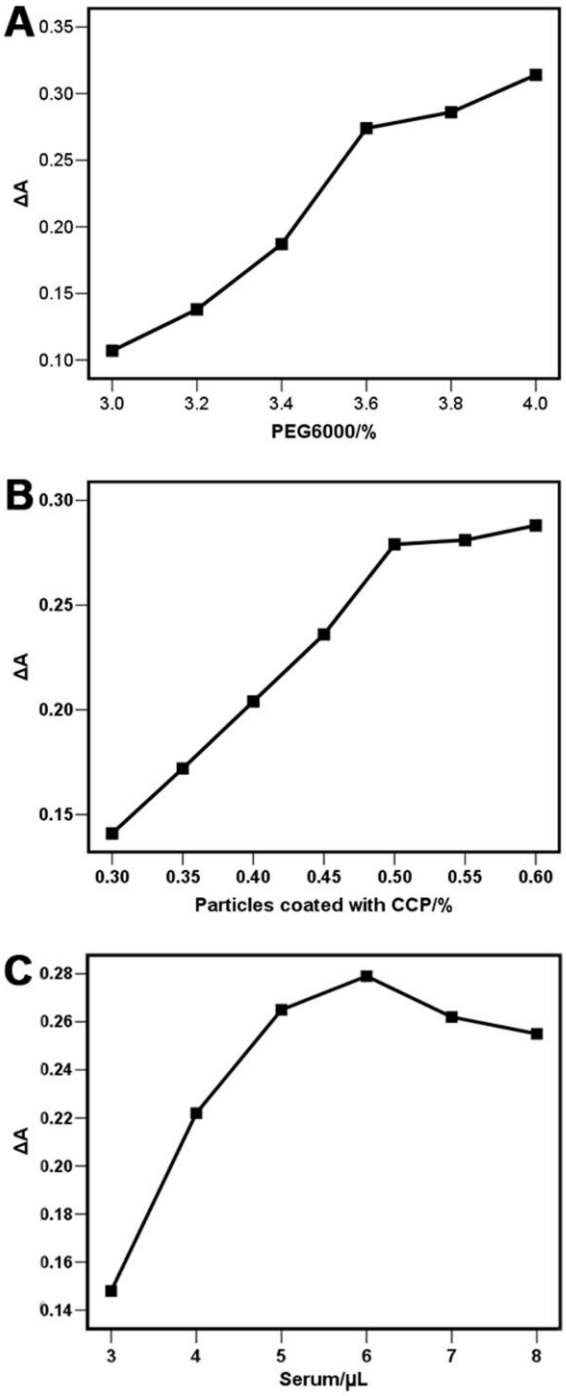


Figure. 4 Optimization of the modified PETIA assay.(A) Optimization of the concentration of PEG6000 (varied from 2.4% to 4.4%).(B) Optimization of the concentration of the particles coated with CCP (varied from 0.3% to 0.6%).(C) Optimization of the serum amount (varied from 3μL to8μL).

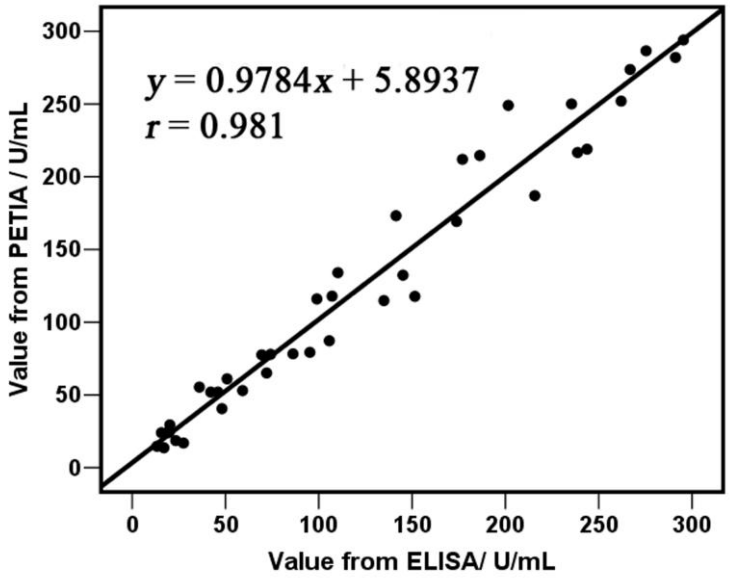


Figure. 5 Plot of the results obtained using the modified PETIA versus ELISA. commercial kits for anti-CCP detection in 40 serum samples with a correlation coefficient of 0.981.

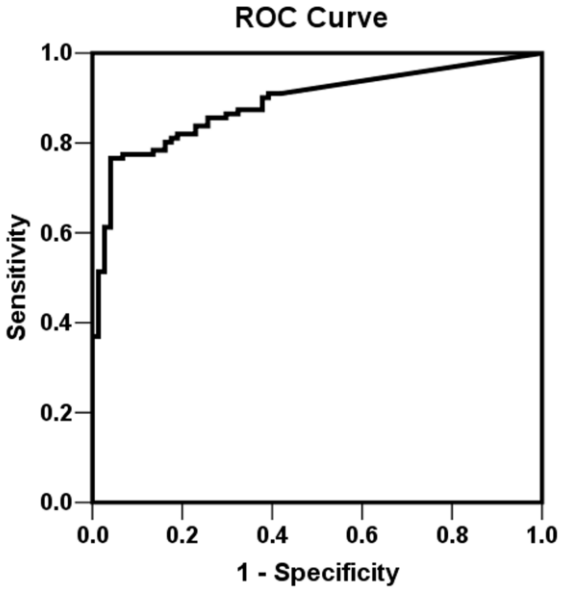


Figure. 6 ROC analysis of anti-CCP antibodies detected by the modified PETIA assay. The area under the ROC curve was 0.872 (95% confidence interval to be 0.820-0.924). 27 U/mL was the optimum cut-off threshold of the modified PETIA according to the ROC analysis.

Table.1 Diagnostic efficiency of anti-CCP antibodies determined by modified PETIA with 27UmL^{-1} as the cut-off value

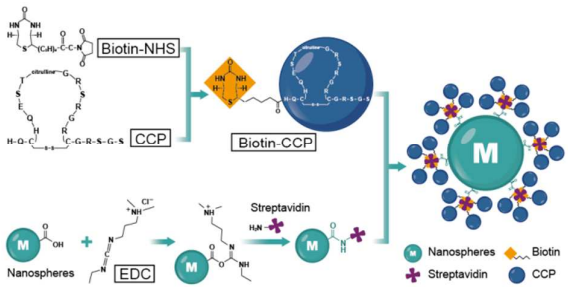
Anti-CCP antibodies	The 2010ACR/EULAR diagnostic criteria of RA		
	RA	Non-RA	Total
Positive	84	6	90
Negative	27	68	95
Total	111	74	185

Sensitivity=84/111=75.68%

Specificity=68/74=91.89%

Positive Predictive Value=84/90=93.33%

Graphical Abstract



Modified Quick PETIA of Detecting Anti-CCP: Apply streptavidin-biotin pair as a connecting arm between polystyrene nanospheres and ccp.