Rapid Assay of Trace Ceruloplasmin Using an Immunonanogold Resonance Scattering Spectral Probe

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Nanogold particles of size 15 nm were used to label goat anti-human ceruloplasmin (GCP) to obtain an immunonanogold probe (AuGCP) for ceruloplasmin (CP). In a pH 7.8 citric acid- Na_2HPO_4 buffer solution and in the presence of polyethylene glycol (PEG), an immunoreaction between AuGCP and CP took place, and the released nanogold particles aggregated to bigger clusters, which caused the resonance scattering (RS) intensity at 552 nm ($I_{552\text{nm}}$) to be enhanced greatly. The enhanced intensity ΔI was proportional to the CP concentration (C_{CP}) in the range from 0.0030 to 1.26 $\mu\text{g/mL}$, with a regress equation of $\Delta I = 101.0C_{\text{CP}} + 1.8$, a correlation coefficient of 0.9970, and a detection limit of 1.1 ng/mL CP. This simple and sensitive RS immunoassay was applied to the determination of CP in human plasma, with satisfactory results.

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Introduction

Ceruloplasmin (CP), a copperish α_2 -glycoprotein, which is mainly synthesized by the liver,¹ existing in the plasma of all vertebrate species, exhibited oxidase activity toward some aromatic amines and phenols. Due to its higher oxidizing activity, CP is also called ferroxidase. Recent studies showed that CP was helpful to Fe metabolism because of its catalytic oxidation of Fe(II) to Fe(III).² Nevertheless, although the precise physiological functions of CP are unknown, it has antioxidant activity by promoting Fe mobilization³ and preventing the metal-catalysis of free radicals and the formation of lipid peroxide in tissue. Therefore, CP played an important role in maintaining body homeostasis and guaranteeing its normal function.⁴ In fact, CP is an acute-phase protein with its serum concentration that increases during pregnancy, infection, tissue injury, and certain malignant disorders.² CP is considered as a useful laboratory test for Wilson's disease and Menkes' syndrome. Its serum concentration is decreased in up to 95% of symptomatic patients.^{5,6} In addition, CP may be decreased in other disease states, like malnutrition, severe liver diseases and nephrotic syndrome.⁵ Furthermore, the content of CP may also be low in patients with hereditary hypoceruloplasminemia. Thus, the measurement of CP is of great significance in the diagnosis of some diseases. At present, several methods have been employed for the determination of CP in human serum, including enzymatic assay and immunoassay. Based on the enzymatic oxidase activity of CP on the substrate of *p*-phenylenediamine (PPD), a PPD colorimetry was established.⁷ However, the assay is non-specific, and some anions inhibited oxidative activity. Schosinsky *et al.* have improved the method with *N,N'*-dimethylphenylenediamine (DPPD)³ as a substrate,

because the product is more stable.^{8,9} Immunoreaction has been widely used in biochemical analysis, since it has high specificity. To enhance the sensitivity, radioactive, fluorescence and enzyme-labeled assays were developed.¹⁰⁻¹³ Several immunoassays, including spectrophotometry, immunoturbidimetric assay, rocket immunoelectrophoresis, radial immunodiffusion, kinetic turbidimetry and bi-antibody radioimmune methods have been reported for CP.^{6,14-19} Among these methods, the immunoturbidimetric assay is simple and rapid, but requires large amounts of precious antisera. Radial immunodiffusion is simple and more sensitive, but it was time-consuming and a harmful reagent was used. Rocket immunoelectrophoresis has a high specificity, and uses smaller amounts of antisera (5 - 10 μL), but the requirements of experiment are very strict.

Nanogold particles have become the fourth immune label because of their specific physical and chemical characteristics, high electron density and good biocompatibility. At present, the detection techniques for the nanogold-labeled immunoassay mainly include spectrophotometry,²⁰ chemiluminescence,^{21,22} mass spectrometry,^{23,24} resonance scattering spectrometry^{25,26} and electrochemical methods.^{27,28} A resonance scattering (RS) technique was sensitive, rapid and simple, and has been applied to the analysis of trace inorganic and organic compounds.²⁹⁻³³ Recently, coupling immunoreaction and the RS effect of nanogold, an immunonanogold RS spectral assay, has been developed, with higher sensitivity and selectivity.^{34,35} However, there has been no report about the immunonanogold RS assay for CP. In the present work, a highly sensitive and selective RS method for CP was established.

Experimental

Reagents and apparatus

Goat anti-human ceruloplasmin (GCP) and ceruloplasmin (CP) were purchased from Beijia Biological Reagents Ltd. Co.,

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Shanghai, China. A 10.8 $\mu\text{g/mL}$ CP working solution was obtained by diluting the stock solution (360 mg/mL) with water. A working GCP solution was prepared as follows: we put 1.0 mL of the GCP serum into a 25-mL volumetric flask, and diluted to the marker with water. Then, 1.0% HAuCl_4 (National Pharmaceutical Group Chemical Reagents Co., China) and 1.0% trisodium citrate (Shanghai Chemical Reagent Plant, Shanghai, China) were used to prepare nanogold. A 0.10 mol/L Na_2HPO_4 solution and 0.10 mol/L citric acid were used to prepare different pH phosphate buffer solutions according to a certain volume ratio. PEG-6000, PEG-4000, PEG-10000, PEG-20000 and KCl were also used. All reagents were of analytical grade and the water was doubly distilled.

A Model Cary Eclipse fluorescence spectrophotometer (Varian Company, Palo Alto, CA) was used to record the RS intensity, with excited and emission slits of 5 nm, and PMT a voltage of 400 V. A Model H-600 transmission electron microscope (TEM) (Electronic Stock Ltd. Co., Japan), a Model 79-1 magnetic heat agitator (Zhongda Instrumental Plant, Jiangsu, China), and a Model SK8200LH ultrasonic reactor (Kedao Ultrasonic Instrument Ltd. Co., Shanghai, China) were used.

Preparation of the colloidal gold

Nanogold particles with sizes of 10, 15 and 30 nm were prepared by the addition of 6.0, 3.0 and 2.0 mL 1% trisodium citrate, respectively, according to a procedure described in a reference.³⁵ All glassware used in the following procedures were thoroughly cleaned with freshly prepared $\text{HNO}_3\text{-HCl}$ ($\text{HNO}_3\text{:HCl} = 1\text{:3}$, by volume) and washed with double-distilled water. In brief, 100 mL of a 0.01% HAuCl_4 solution was heated to boiling. After 2 min, a certain amount of a 1% sodium citrate solution was added quickly with vigorous stirring. The color immediately changed from pale yellow to purple, then to dark red, and to bright red at the end. After about 7 min, the heating was stopped. The solution was stirred continuously until being cooled to room temperature. Finally, the cooled solution was diluted with water to 100 mL and stored in a refrigerator at 4°C.

Preparation of the immunonanogold probe for GCP

Effect of the pH on the labeling. Because the combination of nanogold with GCP is done by physical force, a successful combination depends on the pH. The RS technique was used to test the influence of different pH conditions on nanogold labeling. We adjusted the pH from 4.0 to 10.0 in 1.0 mL of a 57.96 $\mu\text{g/mL}$ nanogold solution. When the pH was <8.5 , the addition of a GCP working solution did not coat the nanogold particle and the RS intensity at 552 nm ($I_{552\text{nm}}$) was greatly enhanced since nanogold aggregated when 100 μL of 10% KCl was added. When pH was >8.5 , the $I_{552\text{nm}}$ was least and stable. It could be interpreted that the coating of nanogold particles by GCP prevented the aggregation of nanogold particles by the KCl. A pH of 8.5 was considered to be suitable for labeling GCP.

Selection of GCP amounts. The effect of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55 μL GCP working solutions on the $I_{552\text{nm}}$ was considered. $I_{552\text{nm}}$ was stronger in tubes with 0 – 25 μL GCP working solutions than those with 30 – 55 μL GCP working solutions, which remained stable. In the experiment, a 30.0 μL GCP working solution was chosen for labeling 1.0 mL of nanogold in sizes of 10 and 15 nm; a 45.0- μL GCP working solution was needed when labeling 1.0 mL of nanogold for a size of 30 nm.

Preparation of the 15 nm immunonanogold. We adjusted 100 mL of a nanogold solution to pH 8.5 under magnetic stirring by adding 3.2 mL of 0.1 mol/L K_2CO_3 solutions. We then added

Fig. 1 TEM of AuGCP and AuGCP-CP (magnified 60000 times). A, AuGCP; B, AuGCP-CP system.

3.0 mL of a GCP working solution slowly to the above colloidal gold solution, while controlling the dropping time to within 5 min. After stirring for about 6 min, we added 1.80 mL of 3% PEG-20000 as a stabilizer, to a final concentration of 0.05%. The mixture was stirred for 15 min and stored in a refrigerator at 4°C.

Procedure

A 0.30 mL pH 7.8 citric acid- Na_2HPO_4 buffer solution, 0.60 mL of a 53.58 $\mu\text{g/mL}$ AuGCP (calculated as Au) solution, a certain quantity of the CP working solution and 0.50 mL of 20% PEG-6000 were added to 5 mL graduated tubes successively. Then, the mixed solution was diluted to 3.0 mL with water and mixed well. Subsequently, the tubes were deposited in an ultrasonic reactor under ultrasonic irradiation (59 kHz) for 20 min of incubation at room temperature. The RS spectra were recorded by means of synchronous scanning; the excited wavelength, λ_{ex} , the emission wavelength, λ_{em} , ($\lambda_{\text{ex}} - \lambda_{\text{em}} = \Delta\lambda = 0$) and the RS intensity at 552 nm ($I_{552\text{nm}}$) were recorded. The I_b values for the blank solutions without CP were also measured. The values $\Delta I = I_{552\text{nm}} - I_b$ were calculated.

Results and Discussion

It is known that the antibody was globulin, and most of the antigens also were protein. They were water-soluble macromolecular proteins, since they contained many amidogen and carboxyl residues in charges. Because the stereo structure anastomoses and the charge were opposite between GCP and CP, they could attract and combine each other specifically. A nanogold particle has a high electron density, and easily combines with biological macromolecules, such as protein, DNA, RNA and glycoprotein. The nanogold conjugated with GCP to form nanogold-labeled GCP (AuGCP), which was proved by transmission electron microscopy (TEM), as shown in Fig. 1A. The AuGCP can combine with CP to form nanogold-labeled immunocomplexes in the presence of PEG by the van der Waals' force, the hydrophobic force, the Coulomb attracting force and the hydrogen bond binding force. After the immunoreaction, the nanogold particles would be released and aggregated to form bigger clusters, as shown in Fig. 1B. The aggregated nanogold particles resulted in an enhancement of $I_{552\text{nm}}$. In a certain CP concentration range, with a raised concentration of CP, more nanogold particles would be released, the stronger would be the aggregation degree of nanogold particles.²⁵ The $I_{552\text{nm}}$ become enhanced linearly with the CP concentration. Thus, an immunonanogold RS assay for CP was proposed.

Fig. 2 RS spectra of the AuGCP-CP-PEG6000 system. a – d: pH, 7.8; AuGCP, 10.72 $\mu\text{g/mL}$; PEG-6000, 33.33 mg/mL . CP: a, 0; b, 0.18; c, 0.72; d, 1.26 $\mu\text{g/mL}$.

Fig. 3 Effect of the AuGCP concentration. pH, 7.8; CP, 0.36 $\mu\text{g/mL}$; PEG6000, 33.33 mg/mL .

Resonance scattering spectra

GCP, CP and their immuno-complex have a weak RS signal, because both GCP and CP are water-soluble macromolecular proteins. Nanogold particles have a weak RS at 522 nm. AuGCP also has weak RS peaks at that wavelength. After the immune reactions between AuGCP and CP took place under a suitable condition, the immuno-complex formed, and the coated nanogold particles were released from the AuGCP. The released gold nanoparticles aggregated in the presence of PEG, which led to a great enhancement of the intensity at 522 nm (Fig. 2). A wavelength of 552 nm was chosen for use.

Selection of the pH, type, and volume of buffer solution

We tested the influence of pH 6.6 – 8.0 on the ΔI . When the pH was 7.8, the system had the maximum ΔI . A pH of 7.8 was chosen for use. We also tested the effect of citric acid- Na_2HPO_4 and Na_2HPO_4 - NaH_2PO_4 , Na_2HPO_4 - KH_2PO_4 with a pH of 7.8 on ΔI . The results demonstrated that 0.30 mL of a pH 7.8 citric acid- Na_2HPO_4 buffer, gave the biggest ΔI , and was thus chosen for use.

Effect of the AuGCP concentration

The influence of the AuGCP concentration on ΔI was studied. Figure 3 shows that in a certain range of concentrations, ΔI increased with an increase of the AuGCP concentration. ΔI attained the maximum when the AuGCP concentration was 10.72 $\mu\text{g/mL}$. Thus, 10.72 $\mu\text{g/mL}$ AuGCP was chosen.

Effect of the PEG concentration

PEG is a polymer of ethylene with no charge. A suitable

Fig. 4 Effect of different types of PEG and concentration on ΔI . Conditions: pH, 7.8; AuGCP, 10.72 $\mu\text{g/mL}$; CP, 0.36 $\mu\text{g/mL}$. PEG: a, 6000; b, 4000; c, 10000; d, 20000.

Fig. 5 Effect of the incubation time on ΔI . Conditions: pH, 7.8; AuGCP, 10.72 $\mu\text{g/mL}$; CP, 0.36 $\mu\text{g/mL}$; PEG, 6000. a, 20°C, ultrasonic irradiation; b, 37°C; c, 20°C.

concentration of PEG would lead to aggregation of the nanogold particles to enhance the detection signals. We considered the influence of PEG-4000, PEG-6000, PEG-10000 and PEG-20000 on ΔI (see Fig. 4). To a certain degree, the four PEG would enhance the ΔI , maximum values of ΔI for the system occurring at a PEG-6000 concentration of 33.33 mg/mL . Thus, 33.33 mg/mL PEG-6000 was selected for use.

Effect of the incubation time and the temperature

The influence of the incubation time from 0 to 60 min on ΔI was investigated the results are shown in Fig. 5. Under ultrasonic irradiation at room temperature, the reaction was relatively quick, and the incubation time was evidently reduced. It was completed within 20 min, and the ΔI value was comparatively stable. In 37°C water, the reaction was relatively slow, and the reaction completed within 40 min. However, at 20°C room temperature, the reaction was more slow, and the reaction still continued even after 60 min. Thus, an incubation time of 20 min under ultrasonic irradiation was selected for use.

Linear relationship

Under the optimal condition, different C_{CP} values were tested, and working curves were drawn according the relationship between C_{CP} and their corresponding ΔI . The data were linearly correlated according to the equation $\Delta I = 101.0C_{\text{CP}} + 1.8$, a correlation coefficient of $r = 0.9970$ in the 0.0030 – 1.26 $\mu\text{g/mL}$ concentration range and a 1.1 ng/mL detection limit (DL). The

Table 1 Analytical features for the four PEG systems

PEG	Regression equation	Linear range/ $\mu\text{g mL}^{-1}$	Correlation coefficient	Detection limit/ ng mL^{-1}
PEG-4000	$\Delta I = 62.1C_{\text{CP}} + 2.4$	0.0045 – 0.72	0.9938	1.4
PEG-6000	$\Delta I = 101.0C_{\text{CP}} + 1.8$	0.0030 – 1.26	0.9970	1.1
PEG-10000	$\Delta I = 73.8C_{\text{CP}} + 2.1$	0.0054 – 1.10	0.9949	1.3
PEG-20000	$\Delta I = 76.2C_{\text{CP}} + 1.0$	0.0090 – 1.10	0.9947	1.5

Table 2 Analytical features for three immunonanogold AuGCP probes

Nanogold size/nm	Regression equation	Linear range/ $\mu\text{g mL}^{-1}$	Correlation coefficient	Detection limit/ ng mL^{-1}
10	$\Delta I = 73.2C_{\text{CP}} + 3.4$	0.0038 – 1.08	0.9913	1.6
15	$\Delta I = 101.0C_{\text{CP}} + 1.8$	0.0030 – 1.26	0.9970	1.1
30	$\Delta I = 144.7C_{\text{CP}} + 3.1$	0.0046 – 0.54	0.9953	3.1

definition of DL was recommended by IUPAC in 1975, DL is expressed as $DL = KS_b/m$. Where m and S_b represent the slope of the standard curve ($m = 101.0$) and the standard deviation of the blank value ($S_b = 0.0389$, $n = 20$), respectively. Based on the suggestion of IUPAC, $K = 3$ was used to calculate DL. In addition, we tested the relationship between C_{CP} and their corresponding intensities, ΔI , using 40 mg/mL PEG-4000, 20 mg/mL PEG-10000, 20 mg/mL PEG-20000 were used as sensitizing agents, respectively. As shown in Table 1, the highest sensitivity and the widest linear range occurred in the presence of 33.33 mg/mL PEG-6000. Moreover, we also tested the relationship between C_{CP} and their corresponding intensities, ΔI , for 10 and 30 nm immunonanogold AuGCP probes, respectively. As shown in Table 2, when a 15-nm immunonanogold AuGCP probe was chosen for the determination of CP, the detection limit was lower, the linear range was wider, and the preparation of 15 nm nanogold was easy.

Effect of foreign substances

According to the procedure, the influence of foreign substances on the determination of 0.36 $\mu\text{g/mL}$ CP was tested. When the relative error was within $\pm 8\%$, the tolerated amount for foreign substances was as listed in Table 3. The result showed that the foreign substances did not significantly interfere with the CP determination, which indicated that this method had good selectivity.

Analysis of samples

Ten sera samples were collected from the No. 5 Hospital of Guilin and diluted 40-fold with water. A 100- μL portion of the diluted samples was taken to determine the CP content according to the procedure. The samples were simultaneously determined by immunoturbidimetry. Using both assay results, a linear regress analysis was made, as shown in Fig. 6, with a slope value of 0.962, a relative coefficient of 0.9964 and an intercept value of 20.5 $\mu\text{g/mL}$. The results obtained with both assays were relatively good, and were consistent with published reference intervals for serum.⁵

Conclusion

Nanogold particles of size 15 nm were prepared simply, and were easy to be labeled. In this paper, 15 nm gold nanoparticles

Table 3 Effect of coexisting substances (0.36 $\mu\text{g/mL}$ CP)

Coexistent substance	Allowable conc./ $\mu\text{g mL}^{-1}$	Relative error, %
L-Lysine	40	+5.9
Urea	200	-5.7
BSA	5	+7.7
Histidine	20	+6.6
L-Cystine	5	-2.3
Glycine	250	+5.9
L-Tyrosine	39	+7.3
DL-Tryptophan	27	-6.6
VB ₆	10	-7.8
VB ₁	20	-4.3
DL-Aspartic acid	50	-5.3
Nicotinic acid	10	-6.7
IgG	4	+4.9
Vc	150	-5.1
HSA	7	-7.3
Glucose	33	-6.7
Sucrose	33	+4.1
Creatine	5	+7.3
Co ²⁺ , SO ₄ ²⁻	6	-6.9
Zn ²⁺ , SO ₄ ²⁻	11	-5.5
Ni ²⁺ , Cl ⁻	7	-7.5
Cu ²⁺ , SO ₄ ²⁻	10	+5.9

were used to label GCP to obtain an immunonanogold RS probe for CP. Based on the immunoreaction between the probe and CP, and the RS effect of nanogold, a simple and sensitive immunonanogold RS assay has been proposed for the determination of CP with high sensitivity and good selectivity. This assay has been applied to detect CP in real samples, with satisfactory results.

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Fig. 6 Linear regress analysis.

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