Gold-Labeled Nanoparticle-Based Immunoresonance Scattering Spectral Assay for Trace Apolipoprotein AI and Apolipoprotein B

Zhiliang Jiang,1,2* Shuangjiao Sun,1 Aihui Liang,2 Wenxin Huang,1 and Aimiao Qin2

Background: Apolipoprotein AI (ApoAI) and ApoB are risk indicators of cardiovascular disease. We describe the use of immunoresonance scattering to measure the ApoAI and ApoB in serum.

Methods: We used a trisodium citrate method to prepare 9.0-nm gold nanoparticles labeled with goat anti-human ApoAI and ApoB antibodies. The immune reaction between gold-labeled antibodies and antigens took place in Na2HPO4-NaH2PO4 buffer solution (pH 6.4 for ApoAI and pH 6.0 for ApoB) in the presence of 75 g/L polyethylene glycol (PEG). We used a transmission electron microscope to observe the shape of the gold nanoparticles. Results were compared with those obtained by immunoturbidimetric methods. Twenty-five human serum samples were assayed by the immunoresonance scattering assay preset with the data indicated and by an immunoturbidimetric assay.

Results: The presence of PEG greatly enhanced the intensity of resonance-scattering peaks at 560 nm. The intensity (ΔI) was proportional to concentration at 0.00833–0.3333 mg/L ApoAI and 0.00197–0.1972 mg/L ApoB. The detection limits were 2.04 and 0.96 μg/L for ApoAI and ApoB, respectively. The results for human serum samples were in agreement with those obtained with an immunoturbidimetric method. Linear regression analysis revealed a correlation coefficient, slope, and intercept of 0.915, 0.966, and 68.53 mg/L, respectively, for ApoAI and 0.919, 0.996, and 15.46 mg/L for ApoB.

Conclusion: This method showed high sensitivity and good selectivity for quantitative determination of ApoAI and ApoB in human serum, with satisfactory results.

Apolipoproteins are the protein component of lipoproteins. Apolipoprotein AI (ApoAI) is the major protein in HDL (1), and apolipoprotein B (ApoB) is the major protein in LDL. ApoB maintains the structure of proteins, transports lipid, and regulates lipid metabolism (2). ApoAI and ApoB are risk predictors of cardiovascular disease, as are total cholesterol, HDL-cholesterol, and LDL-cholesterol (3, 4). Increased ApoAI is associated with decreased risk of coronary heart disease, whereas increased ApoB is associated with increased risk (5–7). ApoAI and ApoB are better predictors of cardiovascular disease risk than the corresponding lipoprotein cholesterol (3, 4, 8). Methods for measuring ApoAI and ApoB include radial immunodiffusion (9), enzyme immunoassays (10), radioactivity immunoanalysis (11), ELISA (12), capillary electrophoresis (13), immunonephelometric assays (14), and immunoturbidimetric assays (14). Among these methods, radial immunodiffusion is simple, but it can be time-consuming and unreliable. Compared with other methods, RIA and ELISA are more sensitive and use smaller amounts of antisera, but some of the reagents used are harmful. Immunoturbidimetric assays are simple and rapid, but they require large amounts of antisera and cannot provide measurements at nanogram concentrations. Gold immunolabeling has been applied in medicine and food safety because it is a rapid and simple technique that does not use harmful reagents (15). Resonance scattering (RS) spectral analysis can rapidly analyze nanogram concentrations of proteins (16–20). Recent studies have shown that liquid-phase gold nanoparticles have a strong resonance-scattering effect (21). We combined an immunoreaction and the resonance-scattering effects of gold nanoparticles to establish a nano-gold-labeled immunoresonance scattering spectral method to quantita-
tively and qualitatively determine ApoAI and ApoB in human serum.

**Materials and Methods**

**REAGENTS AND APPARATUS**

We obtained H\textsubscript{2}AuCl\textsubscript{4} from the National Pharmaceutical Group Chemical Reagents Company, China. Goat antihuman ApoAI and ApoB antisera, ApoAI, and ApoB were purchased from Shengfeng Biological Reagents Limited Company. We used 0.20 mol/L stock solutions of Na\textsubscript{2}HPO\textsubscript{4} and NaH\textsubscript{2}PO\textsubscript{4} to prepare phosphate buffer (PB) solution.

**Pretreatment of goat anti-human apolipoprotein antisera.** We used the improved method (22) to prepare colloidal gold by adding H\textsubscript{2}AuCl\textsubscript{4} solution to a boiled solution of trisodium citrate while stirring, not following the common, reversed procedure. With this method we obtained gold colloid with a consistent particle size of 8–10 nm.

**Identification of colloidal gold.** We used a transmission electron microscope to determine the size and uniformity of the gold particles. Examination of the gold nanoparticles (see Fig. 1 in the online Data Supplement) showed that the gold nanoparticles were not clearly observable because they were coated tightly by colloidal gold. We eliminated redundant electrolytes from the apolipoprotein antisera before labeling by dialysis for 30 h in doubly distilled water.

**Adjustment of the colloidal gold pH.** Because the combination of colloidal gold with ApoAI and ApoB antisera is done by physical force, successful combination depends on the pH. In this experiment, we used an RS method to test the influence of different pH conditions on colloidal gold labeling. We adjusted the pH in 1.0-mL aliquots of 58 mg/L colloidal gold solution by adding 0.20 mol/L K\textsubscript{2}CO\textsubscript{3} and 0.10 mol/L HCl to each tube and then adding 25 µg of ApoAI antiserum or 25 µg of ApoB antiserum, respectively. After 5 min, we added 0.10 mL of 100 g/L KCl solution; 2 h later, we diluted the solution with doubly distilled water to 3.0 mL. We then used immuno-resonance scattering to determine the scattering intensity at 560 nm (23, 24). At a pH < 7.0, in the case of ApoAI, the addition of the antiserum did not stabilize the gold nanoparticles (see Table 1 in the online Data Supplement). When the pH was > 7.0, the intensity decreased and the system stabilized because coating of the gold particles by the ApoAI antiserum prevented aggregation of the colloidal gold by the KCl solution. In addition, pH 7.5 optimized labeling of ApoB antiserum with colloidal gold (see Table 1 in the online Data Supplement). Before adjusting pH, we used a 1 g/L PEG-20000 to stabilize the colloidal gold (25).

**Selection of the ratio between colloidal gold and the antibody.** We added 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 55 µg of ApoAI and ApoB antisera to 1.0 mL of a 58 mg/L colloidal gold solution. The pH of the colloidal gold solution had been adjusted to 7.0–7.5. After 5 min, we added 0.10 mL of 100 g/L KCl solution and mixed well. After 2 h, we measured the resonance-scattering intensities in each tube. Resonance-scattering intensities were stronger in the tubes with 0–30 µg of antiserum than in the tubes with 35–55 µg of antiserum, which remained stable (see Table 2 in the online Data Supplement). Thus, 35 µg was the minimum amount of antiserum that stabilized 1.0 mL of colloidal gold solution.

**Preparation of gold-labeled goat anti-human ApoAI and ApoB antisera.** We adjusted 100 mL of a colloidal gold solution to pH 7.0–7.5. During magnetic stirring, we added 3.5 mg of ApoAI antiserum to 100 mL of colloidal gold, maintaining the dropping time for 5 min, and 1.75 mL of 30 g/L PEG-20000 as stabilizer, to a final concentration of ~0.5 g/L. The mixture was stirred for 15 min and kept at 4 °C. The labeling procedures for ApoB antisera were the same as for ApoAI. The gold-labeled antisera were not purified by centrifugation, and the results were consistent with those for purified material. Micrographs of gold-labeled ApoAI antisera (see Fig. 2 in the online Data Supplement) showed that the gold nanoparticles were not clearly observable because they were coated tightly by antisera.

**IMMUNORESONANCE SCATTERING ASSAY PROCEDURE**

PB solution (0.50 mL of pH 6.4 solution for ApoAI or 0.30 mL of pH 6.2 solution for ApoB), gold-labeled antisera (1.2 mL of 58 mg/L gold-labeled ApoAI antisera or 1.2 mL of 58 mg/L gold-labeled ApoB antisera), a certain quantity of ApoAI (or ApoB), and 0.45 mL of 500 g/L PEG-6000 were successively added to 10-mL graduated tubes. The mixed solutions were diluted to 3.0 mL with distilled water.
water, mixed well, and then deposited in an ultrasonic reactor (59 KHz) for 15 min at 37 °C. Suitable volumes of the prepared solutions were transferred to a quartz cell. The low sensitive file setting and a longitudinal coordinate scale of 6 were chosen, and the synchronous scattering spectrum of the system was recorded by means of synchronous scanning of excitation wavelength ($\lambda_{ex}$) and emission wavelength ($\lambda_{em}$; $\Delta \lambda = 0$) on the Model RF-540 spectrofluorophotometer. The RS intensity (IRS) at 560 nm was then recorded. The IRS values of blank solutions with no ApoAI or ApoB ($[IRS]_b$) were also measured. The values for $\Delta I = IRS - [IRS]_b$ were calculated.

Results and Discussion

The gold nanoparticles were found opposite to the globular heads at the N-terminal ends of the heavy chains of the antibodies (26). In the absence of PEG, gold-labeled ApoAI and ApoB antisera can combine with ApoAI and ApoB, respectively, to form gold-labeled immunocomplexes (ICs). The resulting RS intensity is very weak, however. Results indicate that the gold particle is not released from the IC and that the gold nanoparticles do not aggregate to form larger particles. In the presence of PEG, the labeled gold nanoparticles were liberated from the antisera and aggregated to form large particles (Fig. 1), with the immune reaction continuing. Because of the correlation of RS intensity with the size of the gold particles (22, 23), the intensity was greatly enhanced. The process is shown in Fig. 2. The RS intensity increased linearly with ApoAI and ApoB concentration.

RS spectrum

ApoAI and ApoB are water-soluble macromolecular hydrophilic colloidal proteins with weak synchronous scattering. There are 2 resonance-scattering peaks, at 340 and 400 nm, and a synchronous scattering peak at 470 nm, where the apparatus has the strongest emission (27). After idiosyncratic reactions take place between the apolipopro-
teins and their respective antisera, the IC forms and aggregates to a certain degree because of its hydrophobic properties, enhancing the RS intensities. The strongest RS peaks of the IC are at 340 and 520 nm. Colloidal gold exhibits 3 RS peaks, at 320, 390, and 560 nm, and an apparatus self-producing synchronous scattering peak at 470 nm (24). Among those peaks, the peak intensity at 560 nm is the strongest (24). The small size of the gold nanoparticles used to label the antisera led to weak RS intensities for ApoAI (see Fig. 3) and ApoB (see Fig. 3 in the online Data Supplement). Immune reactions between the apolipoproteins and their respective antisera led to IC formation, release of colloidal gold from the antisera, and aggregation through the action of PEG. Intensities increased linearly with ApoAI and ApoB concentrations.

**SELECTION OF THE pH, TYPE, AND VOLUME OF BUFFER SOLUTION**

We tested the influence of PB solution (pH 5.8–8.0) and Tris-HCl (pH 7.0–8.5) buffer solution on the ΔIRS. PB solution had a positive effect on the system and was chosen for use. The maximum ΔIRS occurred in PB solution at pH 6.4 for ApoAI and ApoB solution at pH 6.0 for ApoB. As a result of tests of buffer solution concentration, we chose 0.030 mol/L PB solution for ApoAI and 0.020 mol/L PB solution for ApoB.

**EFFECT OF GOLD-Labeled ApoAI AND ApoB ANTISERUM CONCENTRATIONS**

We investigated the influence on ΔIRS of different concentrations of gold-labeled antisera. In a certain range of concentrations, with the increase in gold-labeled antisera, the value of ΔIRS increased. The maximum ΔIRS values for both assays were attained at a gold-labeled antisera concentration of 23.2 mg/L.

**EFFECT OF PEG CONCENTRATION**

PEG can bring about the aggregation of the IC, causing the release of gold particles. This aggregation is reversible, and the deposited proteins have no effect on bioactivity (28). Our results demonstrated that ΔIRS values increased greatly with increased PEG concentrations (see Figs. 4 and 5 in the online Data Supplement) owing to gold nanoparticle aggregation. Maximum ΔIRS values for the ApoAI and ApoB systems occurred at a PEG-6000 concentration of 75 g/L.

**EFFECT OF ULTRASONIC IRRADIATION INCUBATION TIME**

We tested the influence of ultrasonic irradiation incubation time from 0 to 40 min on the IRS values of the ApoAI and ApoB systems. Under the conditions of ultrasonic irradiation at 37 °C, when the ApoAI (or ApoB) concentration was higher, the incubation was quicker, and incubation was slower when the ApoAI (or ApoB) concentration was lower. When the incubation lasted 15 min, all reactions ran to completion and the experimental results were stable. We therefore chose ultrasonic irradiation at 37 °C for 15 min for the 2 systems. The results of the experiment indicate that the ΔI was slower when PEG-6000 was added after the incubation of gold-labeled antibody and antigen than when PEG-6000 was added immediately before the incubation.

**LINEAR RANGE**

Using solutions with different concentrations, we tested the relationship between ApoAI and ApoB concentrations (x) and their corresponding intensities (ΔI; y). The linear range for ApoAI was 0.00833–0.3333 mg/L, the linear regression equation was ΔI = 180.5x + 5.96, the correlation coefficient was 0.9978, and the detection limit was 2.04 µg/L; for ApoB, the linear range was 0.00197–0.1972 mg/L, the regression equation was ΔI = 579.6x – 0.96, the correlation coefficient was 0.9988, and the detection limit was 0.96 µg/L. The IRS values were 77.4 for ApoAI at 0.3333 mg/L and 123.1 for ApoB at 0.1972 mg/L.

**EFFECTS OF INTERFERING SUBSTANCES**

We tested the effects of potentially interfering substances on the measurement of ApoAI and ApoB. When ApoAI and ApoB concentrations were 60.0 mg/L with relative

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### Table 1. Selectivity of the assay (0.06 mg/L ApoB).

<table>
<thead>
<tr>
<th>Coexisting substance</th>
<th>Tolerance, mg/L</th>
<th>Relative error, %</th>
<th>Coexisting substance</th>
<th>Tolerance, mg/L</th>
<th>Relative error, %</th>
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<tbody>
<tr>
<td>BSA</td>
<td>17</td>
<td>−1.0</td>
<td>Ascorbic acid</td>
<td>117</td>
<td>+1.6</td>
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<tr>
<td>Urea</td>
<td>200</td>
<td>−4.5</td>
<td>Aspartic acid</td>
<td>20</td>
<td>+3.5</td>
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<tr>
<td>Glycine</td>
<td>167</td>
<td>−1.6</td>
<td>Tryptophan</td>
<td>7</td>
<td>−2.4</td>
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<tr>
<td>Cane sugar</td>
<td>167</td>
<td>−3.5</td>
<td>l-Tyrosine</td>
<td>33</td>
<td>−4.4</td>
</tr>
<tr>
<td>Methionine</td>
<td>7</td>
<td>−4.1</td>
<td>Folic acid</td>
<td>3</td>
<td>−2.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>7</td>
<td>−6.8</td>
<td>Vitamin B₁</td>
<td>2</td>
<td>−4.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>33</td>
<td>−4.3</td>
<td>Sodium oxalate</td>
<td>167</td>
<td>−5.7</td>
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<tr>
<td>L-Arginine</td>
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<td>−3.9</td>
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<td>1667</td>
<td>−4.8</td>
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<tr>
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<td>−5.8</td>
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<tr>
<td>EDTA</td>
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<td>K⁺ Cl⁻</td>
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<td>Human IgM</td>
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<tr>
<td>Human IgA</td>
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<td>+5.5</td>
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</tbody>
</table>

*a* BSA, bovine serum albumin; HSA, human serum albumin.
error of ± 8%, the examined substances did not significantly interfere with the analysis (Table 1).

**Comparison Study**

We analyzed 25 serum samples obtained from apparently healthy men at No. 5 Hospital of Guilin City (Figs. 4 and 5; also see Tables 3 and 4 in the online Data Supplement). Their mean (SD) totals by this assay were 1263 (107.6) mg/L for ApoAI and 853.2 (81.9) mg/L for ApoB. The samples were also assayed by immunoturbidimetry (14).

The linear regression analysis revealed a correlation coefficient, slope, and intercept of 0.915, 0.966, and 68.53 mg/L for ApoAI, and 0.919, 0.996, and 15.46 mg/L for ApoB, respectively. The results obtained with both methods were consistent with published reference intervals for serum (29).

In conclusion, using this novel approach we were able to determine the concentrations of ApoAI and ApoB.

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**References**


