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Direct Determination of Urinary Lysozyme Using Surface Plasmon Resonance Light-Scattering of Gold Nanoparticles

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ABSTRACT

The purpose of this study was to establish a simple and sensitive analytical method for lysozyme using Plasmon Resonance Light-Scattering (PRLS) technique with Gold Nanoparticles (AuNPs) as the probe. Nanomolar level of lysozyme induced AuNPs aggregation with enhanced PRLS. For 1.4 nM citrate-capped AuNPs (13 nm in diameter), the linear range of the calibration curve was 15-50 nM with a detection limit of 13.1 nM for lysozyme. Six nanomolar lysozyme can produce an observable PRLS enhancement. Most potential interfering substances present in urine had a negligible effect on the determination. The interference from human serum albumin in the urinary sample can be reduced by precipitating the albumin with ethanol at pH 4.8-4.9. The 90.1-118.2% recovery was achieved for 8 individual lysozyme-spiked urinary samples. This simple and sensitive method for lysozyme does not require sample clean-up and AuNPs modification, thus provided an alternative for urinary lysozyme determination.

1. Introduction

Tubular proteinuria detection is important in basic medical research and clinical diagnosis. The occurrence of tubular proteinuria was mainly due to reabsorption obstacle of renal tubular for low-molecular-weight proteins[1]. When renal tubular disease occurs, low-molecular-weight proteins cannot be reabsorbed by renal tubules and are excreted into the urine, thus have been considered as the marker of renal tubular disease (or injury) [2].

Lysozyme, also called muramidase, is a bacteriolytic enzyme derived primarily from leucocytes, and is an important defense molecule of the innate immune system, which is important in mediating protection against microbial invasion[3]. Like other low-molecular-weight proteins, lysozyme can be filtrated easily by glomerulus and efficiently reabsorbed by proximal tubular cells [4]. Normally, lysozyme and other low-molecular-weight proteins are virtually absent in the finally excreted urine of healthy subjects. But lysozymuria, a urinary excretion of elevated levels of lysozyme, occurs because of renal tubular injury, thus is a useful indicator of damage to the tubular cells of the kidney and a diagnostic aid in monocytic myelomonocytic leukemia[2,5–6]. Therefore, it is often necessary to determine qualitatively and quantitatively lysozyme in urine.

Traditional methods, such as turbidimetric detection[7], lysoplate assay[8] and lysorocket electrophoresis[9], are widely used for estimating lysozyme. Although these approaches have made great contributions toward the assay of lysozyme, limitations, such as lower sensitivity, interference from other proteins and sophisticated procedures, still exist. Immunoassay[10–12] and enzyme-linked immunosorbent assay (ELISA) [13–14] have very desirable selectivity and do not require intensive sample clean-up, but require expensive antibodies and are time-consuming. The assay of lysozyme in a real sample is often associated with separation techniques such as high-performance liquid chromatography[15], capillary electrophoresis[16] and ion-exchange chromatography[17]. These methods have the desired sensitivity and selectivity, but require extensive sample clean-up, relatively expensive instrumentation, and skilled personnel. Surface plasma resonance (SPR)[18–19] and quartz crystal microbalance (QCM)[20], as effective research tools for biomacromolecules, have been widely applied to the detection of lysozyme using immune-reaction or recognition with polymeric molecule layers. However, complex modification processes, expensive equipments and poor reproducibility cannot be avoided, which make it hard to be applied to routine analysis of lysozyme. Recently, a variety of aptamer-based analytical methods have been developed for lysozyme detection, which involve fluorescence[21–22], electrochemistry[23], electrochemiluminescence (ECL)[24], resonance light -scattering method [25], surface plasma resonance (SPR)[26], and quartz crystal microbalance[27]. The selectivity is

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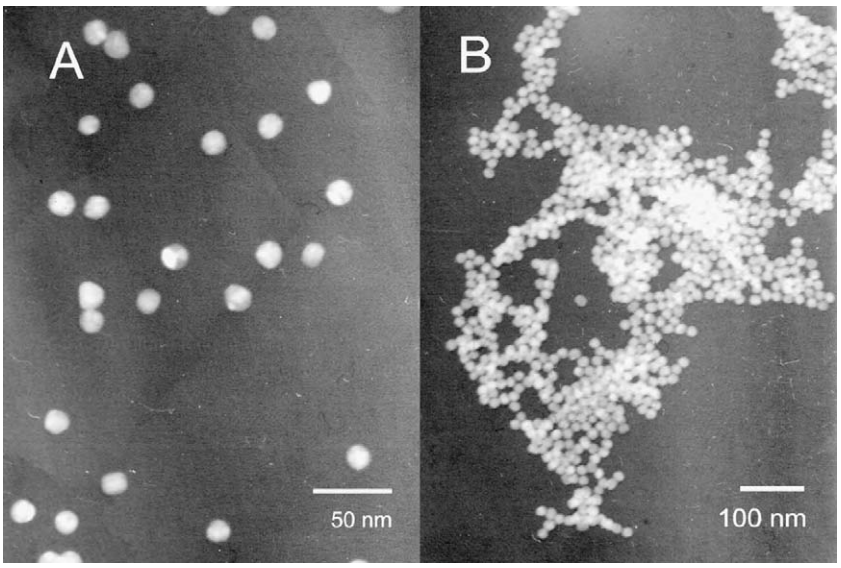

Fig. 1. TEM Images demonstrating the lysozyme-induced aggregation of AuNPs. (A) AuNPs; (B) aggregation of AuNPs in the presence of 40 nM lysozyme.

achieved by the specific binding between lysozyme and its aptamer. However, most aptamer-based sensors involves multiple incubation steps, preparation of labeled aptamer, attachment of aptamers to the sensor surface, thus the assay procedure becomes complicated and its potential as a simple method for routine analysis is compromised. Therefore, it is most desirable to develop a simple, fast, low cost method for many situations, especially the clinical diagnostic laboratories for urinary samples.

Citrate-capped AuNPs have negative charges on the surface and easy to aggregate in the presence of target molecules with positive charges. This results in a color change and provides an simple colorimetric method useful for many applications[28]. The enhancement of light-scattering associated with the plasmon absorption band can also be used for development of ultra-sensitive method with a simple steady-state fluorimeter[29–30]. By synchronously scanning the excitation and emission monochromators at $\Delta\lambda$=0 nm, AuNPs has a light-scattering peak at a wavelength close to the plasmon absorption band. The scattering intensity can be enhanced when AuNPs aggregates are produced with the addition of analytes or the increase of ionic strength.

Lysozyme is different from other proteins because it has a high isoelectric point at about 11 which makes it cationic at pH about 7, while most other proteins with pI less than 6 remain negatively charged. We found that lysozyme at nanomolar level can induce aggregation of AuNPs with an enhanced PRLS at 550-560 nm, a wavelength very close to the plasmon absorption band of AuNPs. At the plasmon resonance wavelength region, most naturally occurring species do not absorb light thus interference from coexisting species can be avoided. The interference from human serum albumin (HSA), the major interfering protein in urinary sample, can be reduced by precipitation with alcohol at a pH close to the pI of the albumin. Based on the above observations, we aim at developing a simple and sensitive method with a common fluorimeter for the determination of lyozyme using PRLS of AuNPs, in hopes that this method does not involve modification of AuNPs and complicate sample pretreatment.

2. Experimental

2.1. Reagents

Chloroauric acid ($HAuCl_4{\cdot}4H_2O$) (AR) was purchased from Shenyang Research Institute of Nonferrous Metals (Shenyang, China), trisodium citrate (AR) from Beijing Chemical Plant (Beijing, China) and Lysozyme (AR) was obtained from Sigma-Aldrich. A 1.0 μM lysozyme stock solution was prepared by dissolving weighed amount of lysozyme in water. Working solutions of the desired concentration were prepared by subsequently dilution of the stock solution. All other chemicals, unless mentioned otherwise, were of analytical grade (AR). Doubly distilled water was used throughout the study.

2.2. Instrumentation

Resonance light-scattering spectra were recorded by synchronous scans with $\Delta\lambda$=0 nm on Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Japan). The plasmon absorption spectra were obtained with a Hitachi U-3010 spectrophotometer (Hitachi, Japan). Transmission electron microscopy (TEM) was performed with JEOL-200CX (JEOL, Japan). A 5 μL of gold nanoparticle sample was dropped on the carbon-coated copper grids (obtained from PELCO, USA), air-dried, and the samples were examined by TEM.

2.3. Preparation of colloidal gold nanoparticles (AuNPs)

AuNPs were synthesized by citrate reduction of $HAuCl_4$[31]. All glassware was cleaned with freshly prepared aqua regia (3 parts HCl, 1 part HNO_3) and rinsed thoroughly with water. Ninety seven milliliters of 0.01% $HAuCl_4$ aqueous solution was brought to a reflux with stirring. Then 3 mL of 38.8 mM trisodium citrate solution was added. The resulting dark red solution was kept refluxing for 15 min, cooled to room temperature, and subsequently filtered through 0.22 μm membrane. The particle size as determined by transmission electron microscopy (TEM) was about 13 nm (Fig. 1). The characteristic plasmon absorbance band centered at about 519 nm (Fig. 2). The molar concentration of AuNPs was about 3.5 nM[32].

2.4. General procedure

Fifty microlitres of the lysozyme standard solution at the desired concentration was mixed with 500 μL AuNPs by vortexing. After 3 min at room temperature, the mixture was used for plasmon absorption and light-scattering measurements.

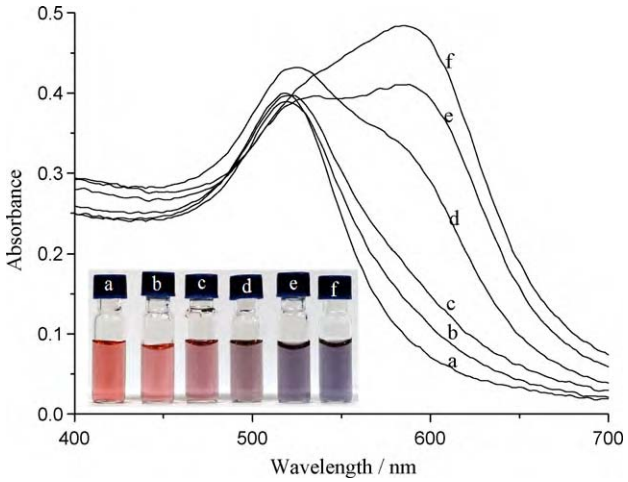

Fig. 2. The plasmon absorption spectra of AuNPs upon interacting with lysozyme, associated with a red-to-blue color change. AuNPs, 1.4 nM; lysozyme from a to f, 0, 15, 20, 30, 40, 50 nM, respectively.

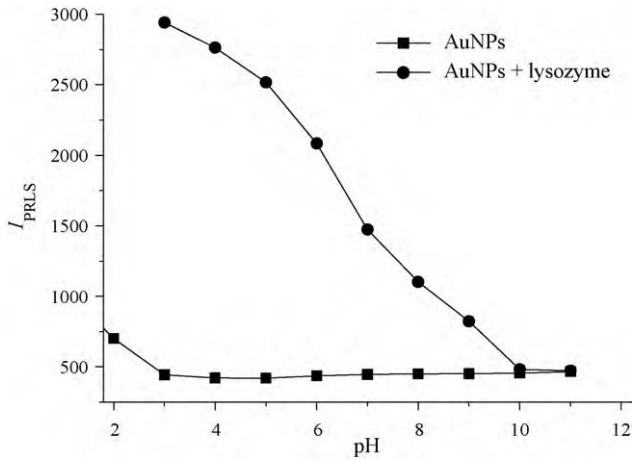

Fig. 4. The pH dependence of the PRLS intensity of AuNPs and AuNPs-lysozyme system. AuNPs, 1.4 nM; lysozyme, 30 nM.

3. Result and discussion

3.1. Spectral Observations

Lysozyme caused AuNPs to aggregate as verified by TEM (Fig. 1). Dramatic changes in surface plasmon absorbance of AuNPs were observed shortly after mixing AuNPs with lysozyme solutions (Fig. 2). The color change with AuNPs aggregation can be easily visualized as showed in insert of Fig. 2. The citrate-capped AuNPs with about 13 nm diameter have a intense plasmon absorption at 519 nm. The addition of lysozyme produces a distinctive red-to-blue color change, associated with a red shift in the absorption band from 519 to 534 nm and a later appearance of new absorption peak near 600 nm. Change in plasmon absorption of AuNPs indicates that the microenvironment around the surface of AuNPs changed with the aggregation as the inter-particle distance decreased. The degree of aggregation was dependent on the concentration of lysozyme.

Along with the plasmon absorption change upon addition of lysozyme to AuNPs, the PRLS intensity of AuNPs was enhanced with sensitivity higher than the plasmon absorption. The PRLS intensity increased linearly with the addition of lysozyme in the range of 15-50 nM (Fig. 3 and inset). AuNPs had a weak PRLS at about 550 nm. With the addition of lysozyme, PRLS intensity of AuNPs was greatly enhanced associated with a small red shift of the maximum scattered peak. Shoulder PRLS peaks at about 610-630 nm were also observed. At these wavelengths, the PRLS intensities were proportional to the concentration of lysozyme and all can be applied to determination of lysozyme. PRLS at 550 nm was chosen for measurement because the slope sensitivity of the calibration curve was the highest.

3.2. Optimization of General Procedure

pH is a very important factor because pH influences the surface charge of AuNPs and the net charge of lysozyme. The PRLS intensity of citrate-capped AuNPs was stable at pH from 4 to 12 (Fig. 4). At pH < 4, the dissociation of citric acid is inhibited and the adsorption of citrate towards AuNPs was reduced. As a result, the negative charges on the AuNPs surface were reduced, and aggregation of AuNPs occured, thus intensity of PRLS increased. For lysozyme-AuNPs system, the PRLS intensity was high at pH 4 but decreased as pH increased. As pH was brought to a value greater than the isoelectric point of lysozyme (pI = 11), lysozyme became negatively charged, and there was no enhacement of PRLS observed. The above observation indicates that the electrostatic interaction is the major driving force for interaction between lysozyme and AuNPs. Sensitivity was high at acidic pH, however, trace amounts of other proteins might interfere with the detection because proteins such

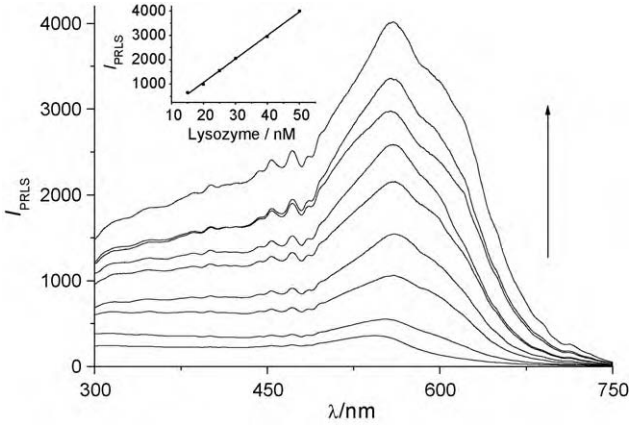

Fig. 3. Change of PRLS spectra of 1.4 nM AuNPs upon interaction with lysozyme. Lysozyme from the bottom to the top, 0, 15, 20, 25, 30, 35, 40, 45 and 50 nM, respectively.

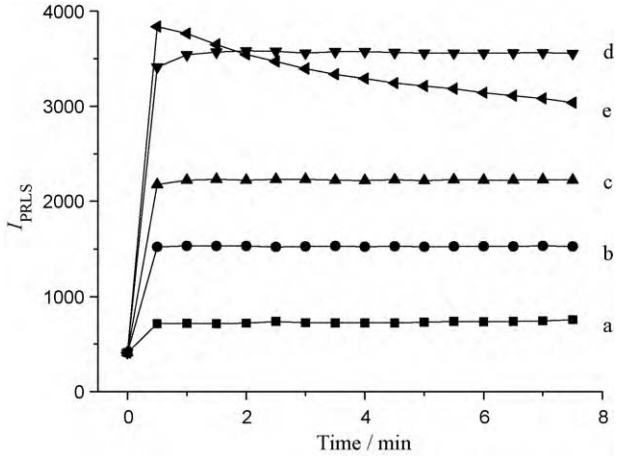

Fig. 5. The time dependence of PRLS intensity. AuNPs, 1.4 nM; lysozyme from a to e, 20, 30, 40, 50, and 60 nM, respectively.

Table 1
Linear regression results of lysozyme calibration curves at different AuNPs concentrations.

AuNPs (nM)	Linear Regression Equation (c, nmol/L)	Regression Coefficient	Linear Range (nmol/L)	Detection Limit (nmol/L)
2.4	I = 115.2 c - 1567.6	0.994	20 - 60	17.4
1.4	I = 96.6 c - 866.9	0.998	15 - 50	13.1
0.88	I = 98.7 c - 413.2	0.998	10 - 40	7.9

as albumin (pI = 4.9) and γ-globulin (pI = 5.5) have a positive net charge at low pH.The AuNPs synthesis medium with a pH of 6.5 was used without introducing other buffers for the citrate medium possessed reasonably good buffer capacity in the range of pH 2-7 ($pK_{a(1\text{-}3)}$ are 3.0, 4.4 and 6.1 at I = 0.1 mol·L^{-1}), and the experimental procedure was simplified.

The rate for the PRLS intensity to reach the maximum was lysozyme concentration dependent (Fig. 5). Generally, after thoroughly mixing the intensity of the test solution reached the maximum in 1 min. Lysozyme-AuNPs system showed a good stability at tested concentration of lysozyme. With lysozyme at 60 nM, a concentration beyond the upper limit of linear range (Fig. 5 e), the PRLS intensity of solution decreased gradually after reaching maximum value. The possible reason is that sedimentation occurs after the aggregation reached the limit within which the colloidal solution stays well suspended. In this study, the PRLS intensity was measured after 3 min.

The calibration curves for lysozyme were obtained with different concentration of AuNPs. The linear range was expanded with comparable linear regression slopes. The detection limit increased as AuNPs with higher concentration was used (Table 1). In this study, we selected 1.4 nM AuNPs for all tests.

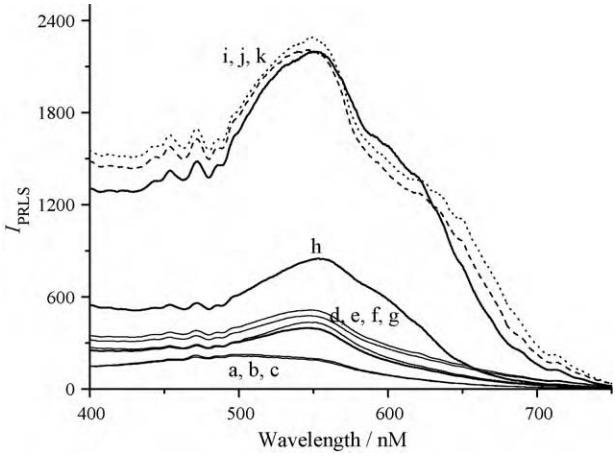

Fig. 6. PRLS spectra of lysozyme (a), HSA (b), ethanol (c), AuNPs (d), AuNPs with ethanol (e), AuNPs with HSA (f), AuNPs with HSA and ethanol (g), AuNPs with lysozyme and HSA (h), AuNPs with lysozyme (i, solid line in bold), AuNPs with lysozyme, HSA and ethanol (j, dashed line), AuNPs with lysozyme and ethanol (k, dotted line). AuNPs, 1.4 nM; lysozyme, 30 nM; HSA, 20 nM; ethanol, 60% (v/v).

3.3. Selectivity and Sensitivity

The interferences from some metal ions, inorganic anions, carbonhydrates, proteins, amino acids, and other entities that may be present in urine specimens were evaluated (Table 2). The concentrations of foreign substances selected were used at their expected upper limit in human urine. Most foreign substances tested had negligible effects on the PRLS intensity of test system, supporting the application of the proposed method for urine analysis.

HSA is one of the major proteins existing in the urinary specimens, it seriously interferes with determination because albumin binds lysozyme[33–34]. HSA (pI = 4.8-4.9) with negative charges at the test pH could reduce the amount of positive charge of lysozyme via electrostatic interaction with lysozyme, thus reduced the aggregation of AuNPs. Therefore, Efforts must be taken to reduce the interferences from HSA.

Table 2
Effect of potentially interfering substances on PRLS of AuNPs-lysozyme system.

Substances Added	Concentration/mM	I_{PRLS}, changed/%
KCl	1.0×10^2	-4.5 ± 1.4
NaCl	2.5×10^2	5.5 ± 0.4
Mg^{2+}	8.0	-4.9 ± 2.6
Zn^{2+}	2.0×10^{-2}	2.0 ± 1.6
Ca^{2+}	8.0	-0.9 ± 2.1
NH_4^+; SO_4^{2-}	80	-1.2 ± 6.8
$C_2O_4^{2-}$	0.40	0.4 ± 3.7
PO_4^{3-}	20	-0.2 ± 4.0
Urea	2%	-2.1 ± 3.1
Creatinine	20	-1.9 ± 2.2
Citric Acid	2.0	-3.5 ± 4.3
Glucose	5.0	-4.3 ± 2.3
cAMP	1.0×10^{-2}	-2.5 ± 1.7
Chondroitin Sulfate	40 mg L^{-1}	2.7 ± 3.3
Cysteine	0.40	9.1 ± 4.7
Glutamine	0.80	-0.6 ± 1.4
Phenylalanine	0.80	1.5 ± 5.5
Tyrosine	0.80	6.8 ± 5.3
γ-Globulin	50 mg L^{-1}	4.6 ± 1.1

Note: All the inorganic salts were added as chloride; cAMP was Cyclic Adenosine Monophosphate; concentration of lysozyme was 30 nM.

The experiment was carried out according to the procedure as follows: The specimen containing HSA and lysozyme was adjusted to pH 4.8-4.9, ethanol was added to make a final solution of 60%, and the resulted solution was incubated in a water bath for 1 h at 4 °C. After filtered through the 0.22 μm membrane, the solution was ready to be tested. Ethanol can reduce the solubility of albumin[35–36] and denature albumin to some extent, thus moderates the interaction between albumin and lysozyme. The assay was performed at pH 4.8-4.9, about the pI of albumin, at which the solubility of albumin is the minimum.

The experimental results showed that the above approach was feasible. First, the analyte (i.e. lysozyme) and reagents, such as HSA and ethanol, showed negligible light-scattering (Fig. 6, curves a, b, and c, respectively) themselves; their effects (curves e, f, and g, respectively) on the PRLS of AuNPs (curve d) were also minimal. Second, the addition of ethanol minimized the effect of HSA on the determination. The enhancement of PRLS of AuNPs by lysozyme (curve i, solid line in bold) was greatly compromised in the presence of HSA (curve h). Ethanol was able to effectively eliminate the interference from HSA (curve j, dashed line) while showing a minimal impact on the PRLS of lysozyme-AuNPs (curve k, dotted line). The amount of ethanol was also investigated (Fig. 7), and the satisfactory results could be achieved with 60% (v/v) ehtanol in the final solution.

As mentioned previously, the linear range of the calibration curve was dependent on the concentration of AuNPs. For 1.4 nM AuNPs in the final solution, the linear range was from 15 to 50 nM with the detection limit of 13.1 nM (3S/N). In fact, 6 nM lysozyme could cause an observable increase of PRLS intensity when we gradually reduced lysozyme concentration in the final test solution.

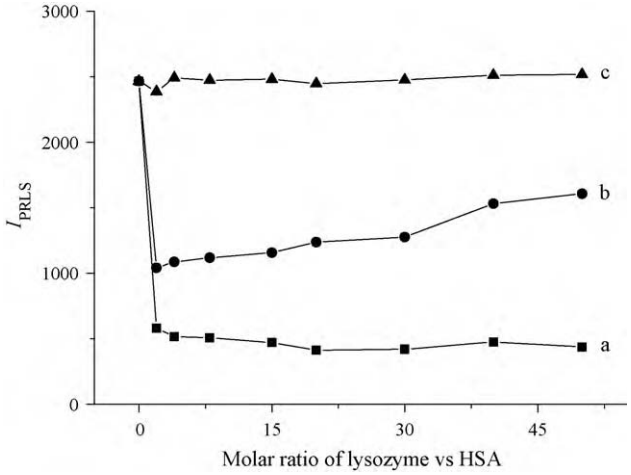

Fig. 7. Effects of ethanol on PRLS intensity of AuNPs at different molar ratio of lysozyme to HSA. Curves a to c, no ethanol, 30% (v/v) and 60% (v/v) ethanol in the final test solutions, respectively.

Table 3
Recovery of lysozyme from urinary samples (n=3).

Sample No.	Recovery/%	RSD/%
1	111.4	1.9
2	118.2	1.1
3	105.7	2.3
4	115.7	1.8
5	90.1	2.2
6	112.0	2.8
7	110.0	1.2
8	117.8	1.1

3.4. Determination Lysozyme in Urinary Specimens

We prepared lysozyme urinary sample by simply spiking lysozyme standard solution with normal human urine to mimic a patient's urine specimen. The spiked urine was adjusted to pH 4.8-4.9 and the resulted solution was mixed with ethanol by vortexing such that the concentration of ethanol in the solution was 60% (v/v). After filtered through a 0.22 μm membrane, 50 μL of so prepared urinary sample was mixed with 200 μL AuNPs, and H_2O was added to make a final test solution of 500 μL. The reagent blank, including all the ingredients added other than the lysozyme standard solution, was prepared in the same way as described above.

The urinary specimens from eight healthy individuals were obtained and each was spiked with lysozyme to make a final concentration of 37.5 nM. Recovery of lysozyme from all urinary samples was 90.1-118.2%, which demonstrated that this proposed method can be used for determination of lysozyme in urinary samples (Table 3).

4. Conclusion

The plasmon resonance light-scattering (PRLS) technique combined with AuNPs probes has been shown to be a reasonable approach for the determination of nanomolar level of lysozyme in human urine. Most potential coexisting substances in the urine specimens did not significantly interfere with the determination. The interference from human serum abumin can be reduced by precipitation with ethanol at the pH close to the pI value of albumin during sample preparation. The spiked recovery of urinary samples from 8 individuals was in the range of 90.1-118.2%. This proposed method is a simple, sensitive and rapid approach for determining lysozyme in urine that does not involve modification of AuNPs and complicated pre-treatment of urinary samples. Therefore, it can be a practical method for quantitation or detection of urinary lysozyme.

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