Homogeneous immunoassay based on gold nanoparticles and visible absorption detection

UNIVERSITÀ DEGLI STUDI DI TORINO

HOMOGENEOUS IMMUNOASSAY BASED ON GOLD NANOPARTICLES AND VISIBLE ABSORPTION DETECTION

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Abstract

A sensitive homogeneous immunoassay, using human serum albumin (HSA) as a model analyte coupled with simple visible absorption detection has been developed. The new assay is based on the use of gold nanoparticles functionalized with the target protein, which compete with the analyte for the binding of a specific polyclonal antibody. The binding of antibodies to the functionalized nanoparticles determines a shift of the visible absorption maximum of the gold colloid and quantification of the analyte could be obtained as the competitive inhibition of the binding of antibodies to the nanoparticles. The proposed immunoassay has been optimized and successfully applied to measuring HSA in human urine samples, in which results agreed well with those obtained by a nephelometric reference method.

Keywords: Gold nanoparticles; homogeneous immunoassay; Human Serum Albumin in urine; visible absorption detection

Introduction

Immunochemical methods of analysis are widely employed because of their simplicity, cost-effectiveness, rapidity and limited need for skilled personnel, instrumentation and sample treatment. Fields of application of immunoassays (IAs) vary from clinical diagnostics to environmental analysis, to food safety assessment and the target analytes cover the widest range of molecular weight, from small organic molecules to bio-molecules of hundreds of kilodaltons. The large majority of the current immunoassays are performed in heterogeneous formats, mostly as the Enzyme-linked Immunosorbent Assay (ELISA) format or as the non competitive sandwich assay format (for high-molecular-mass analyte determination). These formats, and particularly the sandwich IA, allow high sensitivity, wide dynamic range and high specificity to be obtained. However, the heterogeneous IAs suffer some fundamental weaknesses since they are usually time-consuming, difficult to automatize or transfer into on-field portable devices [1-3]. These drawbacks are mainly due to the need for several subsequent separation and washing steps. On the other hand, homogeneous IAs, which are based on the measurement of some characteristics of the tracer (which is modulated by binding with the analyte) do not require separation steps. As a consequence, they are potentially swifter and easy to automatize. Nevertheless, they are usually less sensitive than heterogeneous IAs [3]. Several homogeneous IA formats have been described, based on the use of tracers which exploit various physical mechanisms which allow the free and the analyte-bound form to be distinguished: fluorescence polarization [4,5]; fluorescence resonance energy transfer [6,7], bioluminescence resonance energy transfer [1,8]; and surface plasmon resonance [9,10]; and agglutination reaction [2,3,11]. The latter mechanism was demonstrated to overcome one of the major drawbacks of homogeneous IAs, such as its limited sensitivity [3].

Very recently, the development of analytical methods, which are fast and mainly require the minimal and easier field applied instrumentation, has become a major goal [12,13].

In this paper, a sensitive homogeneous immunoassay is described, which uses functionalized gold nanoparticle as probes coupled with a simple visible absorption measurement. Gold nanoparticles show strong light absorption in the visible region which results from the coherent oscillation of the

free electrons of the nanoparticles on the particle surface called the surface plasmon resonance (SPR) and their optical properties vary with their shapes and sizes [14,15]. A modification of the metal surface, which causes a variation of the refractive index at the surface, determines a variation of the SPR spectrum [16]. The surface plasmon resonance of gold nanoparticles has a broad application and has drawn great attention in recent years [3,15,17-19]. As exhaustively described by Yu and Irudayaraj [20 and references herein], the red-shift of the resonant band, due to aggregation of gold nanoparticles, has been exploited to develop colorimetric sensors for the detection of DNA and for the monitoring of kinetics of antibody-antigen interactions. According to the same authors, the wavelength shift of the plasmon band has a great potential for biosensing, mainly because it only depends on changes in the dielectric properties in the immediate vicinity of the nanoparticles and is not determined by concentration of particles. Nevertheless, the same authors question the real possibility of exploiting the wavelength shift of the plasmon resonant band due to aggregation of spherical gold nanoparticles for detection purposes. In their opinion, aggregation results in a large wavelength shift, but contemporary in a widening of the plasmon peak which results in poor resolution.

In the present work, the dependence of the visible absorption from the size and refractive index of gold nanoparticles has been exploited to develop a one-step immunoassay in homogeneous solution. The optimization of the assay parameters (ie: antibody and particle dilution, incubation time, buffers and additives) allowed us to control nanoparticle aggregation. Therefore, broadening of the resonant peak was limited and the analyte quantification could be obtained by measuring the visible absorption maxima of solutions by using a common spectrophotometer.

The approach has been applied to a model system constituted by human serum albumin (HSA) as analyte, by polyclonal antibodies as specific ligands and by HSA modified gold nanoparticles. The competitive immunoassay was optimized for different parameters, such as nanoparticle dimension, polyclonal antibody concentration, and incubation time. In addition, the influence of pH and of salt nature and concentration has been assessed. Finally, this homogeneous IA was evaluated and

validated by measuring eight human urine samples and comparing results obtained by newly developed IA with a nephelometric reference method.

Experimental section

Materials

The tetrachloroauric(III) acid, the human serum albumin (fraction V), the animal gelatine, and the anti-HSA antibody (rabbit polyclonal antiserum, affinity purified) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were obtained from VWR International (Milan, Italy). Human urine samples were kindly supplied by the analytical laboratory of the Mauriziano hospital (Turin, Italy). HSA concentration was assessed by the in-house validated automated nephelometric assay (LOD 2 mg/L).

Preparation of Colloidal Gold and coating with HSA

Two batches of gold colloids were synthesized using the sodium citrate method as previously described [21,22]. Briefly, 1 ml and 2 ml of sodium citrate (1% p/v) were added to 100ml of a 0.01% solution of tetrachloroauric acid under vigorous stirring and warming (100°C). The resulting colloidal gold solutions were characterized by their visible absorption spectra and checked by transmission electron microscope to determine the nanoparticle dimensions (as the mean value of the distribution of 300 nanoparticles).

The colloidal gold solutions were adjusted to pH 8.5 with a 50mM carbonate buffer (pH 9.6) for coating with HSA. The optimum concentration of the protein for conjugation was then determined. 200 µl of a 1% (w/v) HSA solution in borate buffer (BB, 20 mM, pH 8.0) was added to 2 mL of pH-adjusted colloidal gold solution. After 30’ incubation at room temperature, the mixture was centrifuged twice at 14000 rpm at 25°C for 20 min, and the pellet was re-suspended in borate buffer. Finally, 0.1% sodium azide was added as preservative and stored at 4°C until use. The

stability of the coated nanoparticles was assessed during a 3 month-storage at 4°C. The visible spectrum of the solutions containing the coated nanoparticles was also registered.

Homogeneous non competitive immunoassay

Coated gold nanoparticles diluted with BB to obtain a final OD of 0.2 at their λ_{max} (200 µl) were incubated overnight at room temperature with 200 µl of the anti-HSA antibody (dilution varying from 1:100 to 1:1600, v/v). Three buffers were considered to dilute the antibody: BB; BB containing variable percentages of animal gelatine (0.05-0.2%, w/v); and BB containing variable percentages of Tween 20 (BBT, 0.025-0.2% w/v). Visible spectra were recorded and the wavelength of the maximum (λ_{max}) was plotted against the log of the antibody dilutions.

Homogeneous competitive immunoassay

Antiserum was diluted to 1:300 (v/v) in BBT. A volume of 150 µl of diluted antiserum was added to 150 µL of HSA at concentrations ranging from 0 to 100 mg/L and incubated in duplicate in BBT for 30 min. Two hundred microliters of the mixture were mixed with coated gold nanoparticles diluted with BBT (OD 0.2) and incubated for 30 min. Visible spectra were recorded and the difference between λ_{max} of each standard solution and λ_{max} of the blank was plotted against the log of the HSA concentrations. Calibration curves were determined by a nonlinear regression analysis of the data using the four-parameter logistic equation [24].

Spectrophotometric measurements and calculations

Visible spectra were recorded on a Varian Cary1E spectrophotometer by setting parameters as follows: scan range 500-600nm; bandwidth 0.5 nm; scan rate 60 nm/min; scan interval 0.1 nm. Quartz cells were used, and washed with a commercial washing solution for denaturing protein between each measurement. Alternatively, plastic disposable cells can be used.

Spectra obtained as ASCII data were smoothed over using the SigmaPlot 9.0 software (Negative exponential function, sampling proportion 0.1; polynomial degree 1; intervals 300; bandwidth method: nearest neighbours). To obtain the λ_{max} value, the first derivative of the smoothed spectra was calculated and set to zero. At the same time, λ_{max} was also evaluated as the value automatically given by the spectrophotometer software.

Results and discussion

Characterization of the coated gold nanoparticles and of their binding with the antiserum

Two different batches of gold nanoparticles were prepared by reducing the tetrachloroauric acid with a varying amount of sodium citrate. Visible spectra demonstrated different absorption maxima (520 nm for the higher amount of reducing agent was added and 530 nm for the lower), which means that nanoparticles with different dimensions were obtained, as expected [25]. This finding was further confirmed by using the Trasmission Electron Microscopy (TEM), which showed that gold nanoparticles characterized by a λ_{max} of 520 nm (Au520) had a mean diameter of 18 ± 3, whereas the gold nanoparticles with the λ_{max} of 530 nm (Au530) had a mean diameter of 33 ± 4.

Both colloids were coated with the Human Serum Albumin, which allowed modifying the Vis spectra. In particular, λ_{max} was increased for both colloids by about 8-9 nanometres (Figure 1), and this absorbance shift was considered as the confirmation of the protein absorption onto the particle surface.

The binding capacity of the antiserum towards the two solutions of coated gold nanoparticles was evaluated in non competitive assays. Nanoparticles were incubated with a varying amount of antiserum. In the meantime, the addition of proteins or surfactants aimed at reducing coated particles and/or antibody absorption onto the plastic tube was evaluated. Experimental results showed that the addition of Tween 20 at concentrations from 0.05% to 0.2% (w/v) allowed the prevention of non-specific absorption and aggregation of the coated

nanoparticles, which resulted in higher absorbance and more reproducible measures (data not shown). Contrarily, the use of animal gelatine, even if effective in reducing non-specific adsorption and aggregation, interfered with the binding between the antibody and the coated particles (data not shown). Therefore, a BB containing the 0.05% (w/v) of Tween 20 (BBT) was established as the diluent for both colloidal solutions and the antiserum. Figure 2 shows the curves obtained in these conditions by plotting λ_{max} as a function of 1/dilution of the antibody for the two colloidal solutions. The first observation to be made is that the presence of the antiserum strongly affects absorption spectra of the coated colloids (see also Figure 1), in a way which is correlated to the amount of antibody present. Therefore, the binding of the antibody to the coated nanoparticles can be simply and directly evidenced by observing the shift of the absorption maxima in the Visible region.

The IC_{50} for the two curves show themselves to be very similar (antibody dilution 1:550-1:600), whereas the difference between λ_{max} of the colloidal solution in the absence of the antibody and in the presence of an excess of antibody varied considerably: 14 nm for the Au520 and 23.4 nm for the Au530 preparation (Figure 1). This large difference in the absorption maxima in the presence of the antibody was suggested to be due not only to the antibodies on the surface of the coated nanoparticles but also to the formation of large aggregates between many particles kept together by the antibody itself. In fact, being bivalent, each antibody molecule can bind two HSA molecules absorbed onto two different nanoparticles, acting as a bridge. This hypothesis was confirmed by acquiring TEM images of the coated Au530 with and without the antibody added (Figure 3). When an excess of antibody is added, very large aggregates of particles are formed, which are responsible for the large shift of the visible absorption maximum. Since the largest λ_{max} shift was supposed to be able to determine a higher sensitivity (i.e. higher slope of the curve), subsequent experiments were conducted using only the Au530 colloid.

Homogeneous competitive immunoassay

Optimized conditions for carrying out a competitive immunoassay for measuring HSA were defined by a standard checkerboard assay, in which the appropriate antigen concentrations (defined as the optical density of the coated Au530) and antibody dilutions were selected. An antibody diluted at 1:600 (v/v) and the coated Au530 at 0.2 OD were selected as the most suitable on the basis of the IC_{50} value that was the lowest.

The incubation time was also optimized by comparing dose-response curves. The 30' pre-incubation between sample and antibody was not investigated, whereas the incubation of the mixture in the presence of the coated nanoparticles was varied from 5 min to 4 h. A 30' incubation was selected as a satisfactory compromise between the lowest IC_{50} and a blank λ_{max} shift around 10-15 nm.

Figure 4 shows a typical inhibition curve obtained under optimized conditions by plotting the $\Delta\lambda_{max}$ against HSA concentration, where:

$\Delta\lambda_{max}$ = $\left|\right.$ (λ_{max} observed in the absence of the analyte) – (λ_{max} observed for a certain analyte concentration) $\left|\right.$

The IC_{50} value of the assay was 0.4 mg/L (6 nM) with a detection limit, calculated as the concentration corresponding to the blank plus three standard deviations of the blank, was 0.06 mg/L (about 1 nM).

λ_{max} values were both determined as the automatic instrumental value and more accurately calculated as described in the Experimental section. No significant differences were observed upon applying the two methods, except when non-specific aggregation occurred (such as in the case of using an excess of salt, acidic pH or prolonged incubation in the absence of the analyte).

Influence of ionic strength, pH and buffer salt on the assay

Competitive immunoassays were carried out by varying the ionic strength, the pH and the buffer salt to evaluate the influence of experimental conditions on the response of the assay. Ionic strength (meaning the concentration of NaCl added to the borate buffer) was varied between 10 and

200 mM. The curve was not affected for NaCl concentration below 50 mM. On the other hand, at higher ionic strength the absorption maximum of the colloids in the absence of the analyte strongly increased and secondary maxima appeared at even higher wavelengths. In addition, in these conditions, the identification of the λ_{max} value became very uncertain. The hypothesis was made that non-specific aggregation of nanoparticles was due to the presence of salt and not to the binding of the antibody.

The buffer salt was also demonstrated to influence assay performance. Calibration curves for HSA were prepared by using: borate, phosphate, phosphate/citrate, TRIS, and HEPES as diluent for coated gold, antibody and analyte (buffer concentration = 10 mM; pH =8; 0.05% v/v Tween added). Generally, all buffer salts slightly increased λ_{max} shift when compared to borate buffer, which was initially used to optimize the assay. In particular, the phosphate/citrate buffer allows the observing of the largest λ_{max} shift. However, the same buffered solution determined the shift of the absorption maximum of the coated nanoparticles even in the absence of the antibody, which means that buffer salts caused non-specific aggregation of coated gold particles. Therefore, it was concluded that the increase of λ_{max} variation observed for the various buffer salts was associated with non-specific phenomena and borate buffer was confirmed as being the best choice, also because of the best precision of data obtained.

The influence of pH variation was also evaluated in the range between 5 and 8. As expected, acidic pH affected the assay performance, which, however, was very similar at pH 7 and 8. Since a phosphate/citrate buffer was used to carry out this investigation, it can be supposed that lowering the pH not only affected the competition reaction, but also introduced non-specific aggregation due to the presence of the citrate salt.

Human urine samples

In order to verify the possibility of applying the proposed homogenous competitive assay for the determination of HSA in real matrices, two samples of human urine were analyzed. The two

samples which were kindly supplied by the Analytical Laboratory of the Mauriziano Hospital (Turin) were known to be a blank (HSA < 2 mg/L) and a high-positive sample (HSA = 246 mg/L). They were simply diluted with the BBT and analyzed by the developed assay. Table 1 shows that already a 1:10 dilution of the human urine with the BBT allowed us to clearly distinguish between negative and positive samples. HSA showed itself to be detectable also in the blank sample, which could be compatible with the lower LOD of the developed method or, most probably may indicate the presence of some matrix effects. The high-positive sample, on the other hand, was greatly underestimated. Nevertheless, the 1:50 dilution of samples allowed us to offset matrix interference. This finding was further confirmed by carrying out inhibition curves with standards prepared in a buffered solution and in the blank sample diluted 1:50: the two curves completely matched (data not shown). In conclusion, urine samples could be directly measured by the developed assay after a 1:50 dilution, thus the LOD of the method became 3 mg/L in human urine, which is comparable to the LOD of the methods usually employed to measure HSA in urine: In addition, it allows us to discriminate between healthy subjects and patients affected by microalbuminuria [25]. Indeed, the cut-off level for the microalbuminuria is set at 20 mg/L, which corresponds to the IC_{50} of the inhibition curve, thus to the region of maximum sensitivity.

A cross-validation of the homogeneous assay with a nephelometric reference assay was carried out by measuring HSA concentration in eight human urine samples, representing very different contamination levels (0-250 mg/L). Agreeing results were obtained with the two methods (Figure 5): the linear regression analysis yielded a good correlation between the methods (y=0.96x-1.8, r^2 = 0.997, n=8). These results demonstrated the validity of the developed assay in the entire range of albumin concentration investigated, which comprehends the possibility to detect both microalbuminuria and albuminuria.

The precision of the method was evaluated at a preliminary level by analyzing replicates of two urine samples, at HSA concentrations corresponding to the lower and the upper limit of the curve (Table 2). The values of RSD% were calculated at each nominal concentration and were

considered acceptable according to FDA requirements for the validation of bioanalytical methods [26].

These results proved that the developed homogeneous immunoassay can be applied for the measurement of the target protein in real samples at levels of clinical relevance, with accuracy comparable with that obtained through the reference method.

In conclusion, the newly developed homogeneous IA allows the rapid and accurate determination of the target analyte after a simple dilution of samples and using a common spectrophotometer as the unique instrumentation required. Therefore, it can be easily automated and potentially transferred in a portable format.

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Figure 1: Visible spectra recorded for solutions which contained: Au530 (bold line); Au530 coated with HSA (dashed line); Au530 coated with HSA and incubated with the polyclonal antibody (Ab dilution 1:600) for 30 min. Scan interval was set at 1 nm.

Figure 2: Noncompetitive binding of antiserum against HSA towards the two coated gold colloids (○ Au520 and △ Au530).

Figure 3: TEM images of the coated Au530 in the absence (a) and in the presence of the antibody (b)

Figure 4: A typical inhibition curve obtained under optimized conditions for the developed homogeneous immunoassay.

Figure 5: Correlation of results obtained by both developed homogeneous immunoassay and reference nephelometric assay for the human albumin detection on human urine samples. The linear regression analysis yielded a good correlation between methods (y=0.96x-1.8, r^2 = 0.997)

Tables

Table 1. Matrix interference on the developed homogeneous assay as a function of the human urine sample dilution.

Sample dilution	Estimated conc of HSA ± SD (mg/L)	
	blank sample (HSA < 2 mg/L)	high-positive sample (HSA = 246 mg/L)
1:10	0.9 ± 0.2	131 ± 17
1:50	< LOD	261 ± 39
1:200	< LOD	252 ± 38

Table 2

HSA concentration according to the reference method (mg/L)	measured HSA concentration ± SD (mg/L)	RSD% [a]
6	6.9 ± 1.5	21.7
246	261 ± 39	14.9

[a] n=6

Figure 1

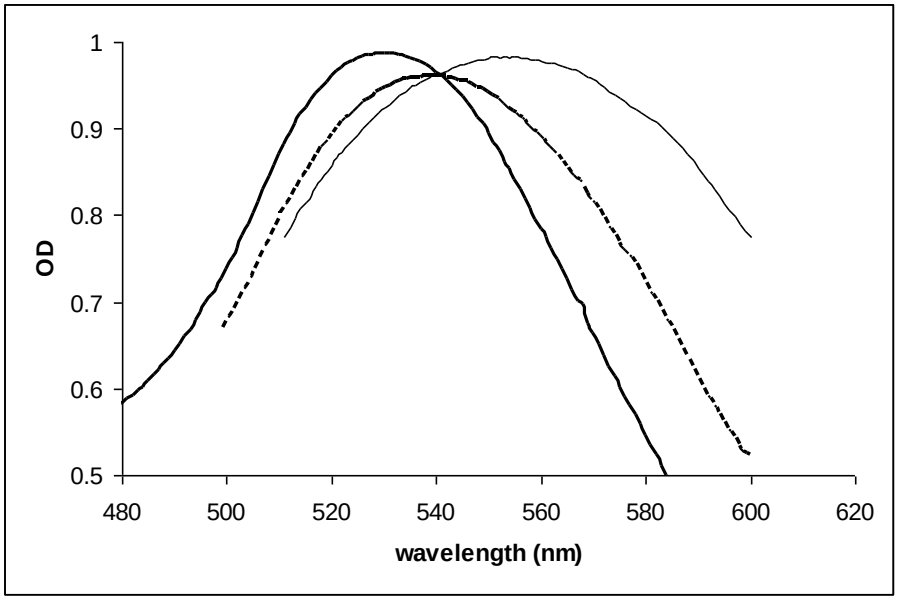


Figure 2

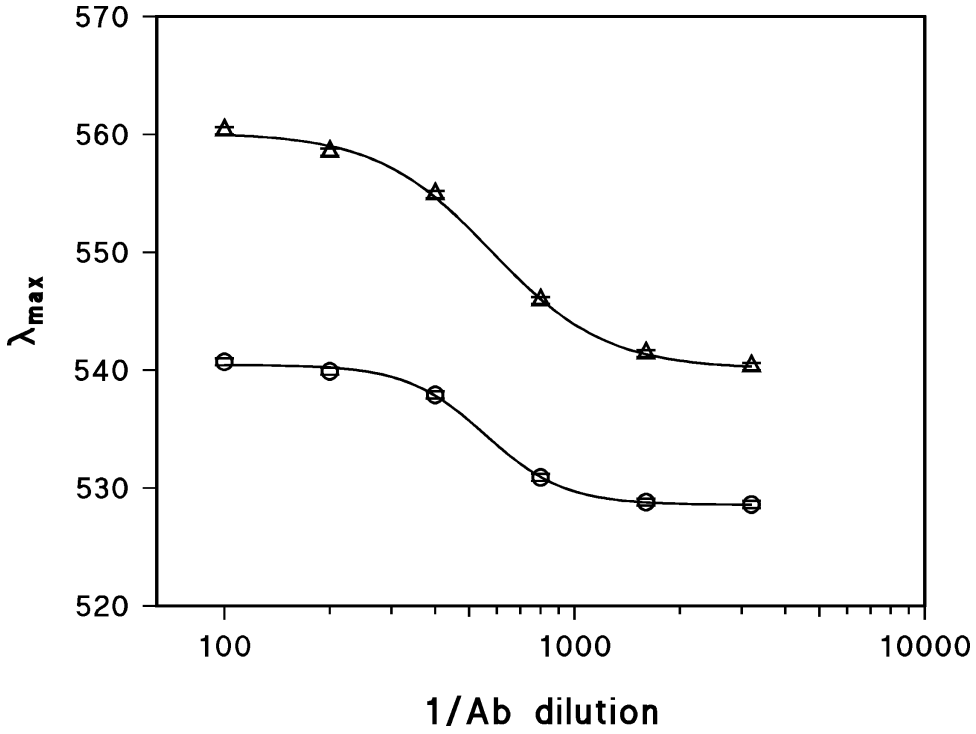
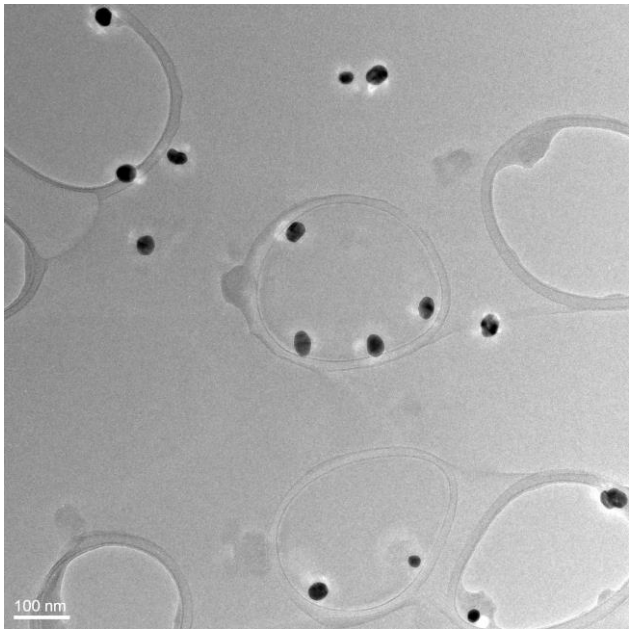


Figure 3

(a)



(b)

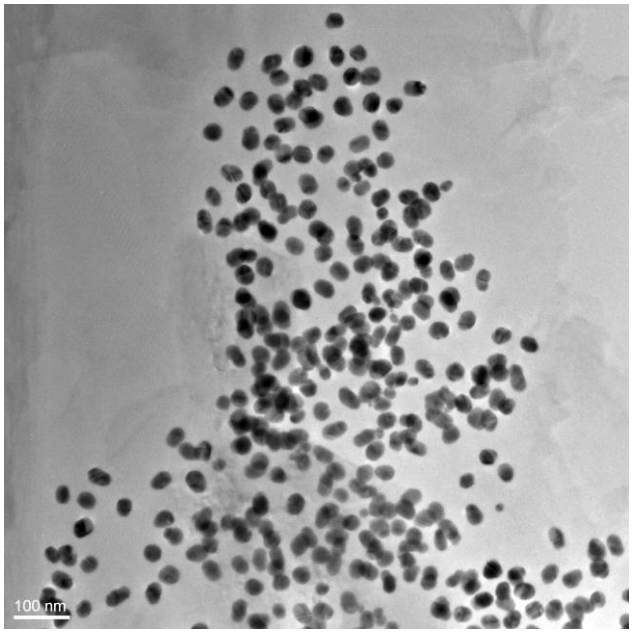


Figure 4

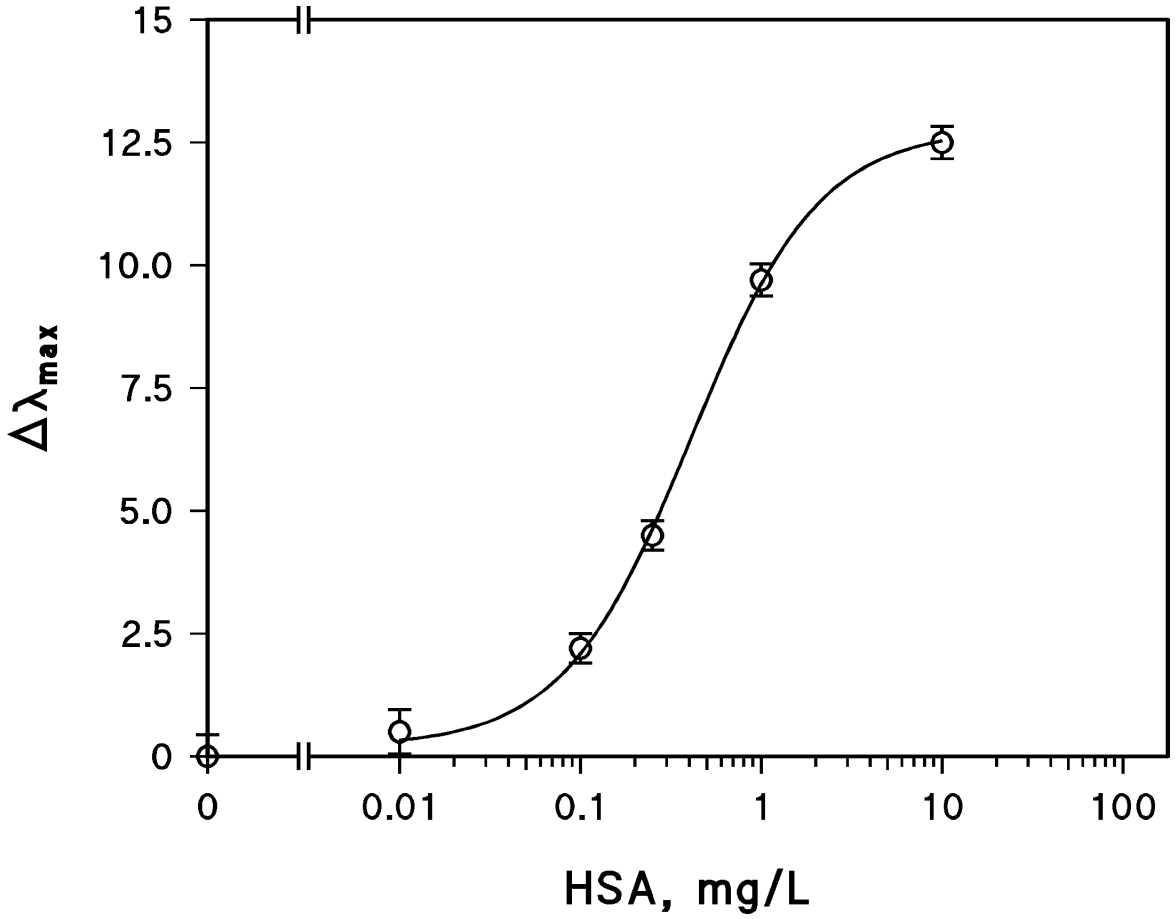
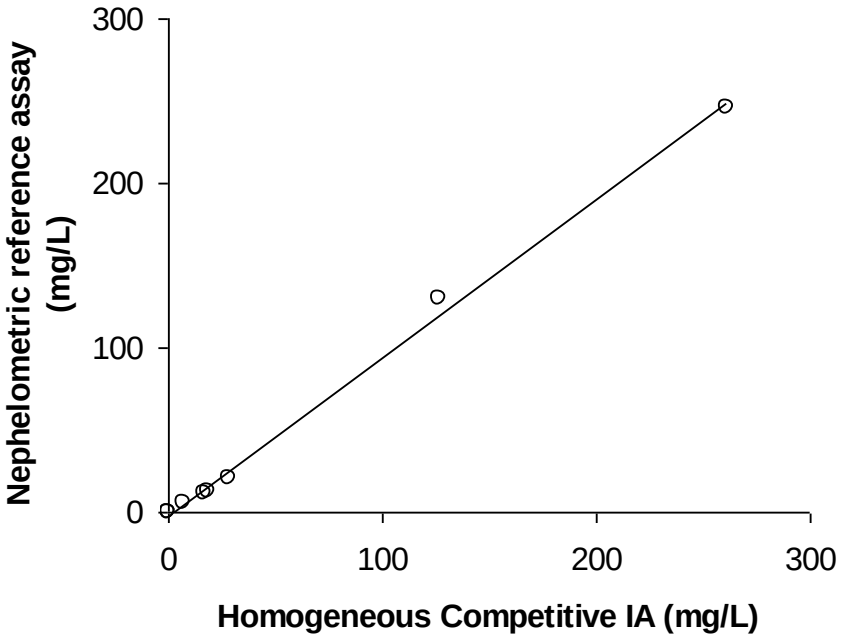


Figure 5


Nephelometric reference assay (mg/L)
300
200
100
0
0
100
200
300
Homogeneous Competitive IA (mg/L)