Introduction

Immunochemical assays founded on chemical interactions between biomolecules and metal nanoparticles (MNPs) have attracted attention of researchers due to their medical and environmental sensing applications allied to the singular optical, electronic and catalytic properties of MNPs.\(^1,2\) One major merit of using antibody–antigen interactions is based on their biospecific recognition, which provides high selectivity.\(^3\) On the other hand, silver and gold nanoparticles have a remarkably high attenuation coefficient and strongly distance-dependent optical properties.\(^4\)

The localized surface plasmon resonance shift is an optical phenomenon, characterized by the attenuance of light by MNPs (typically 1 – 100 nm) in suspension, due to the interaction of the electromagnetic radiation with the electrons in the conduction band of the metal. This phenomenon is dependent on several factors such as size, shape and distance between particles.\(^5,6\) A change in colloid color, which is accompanied by a change in the absorbance spectrum, occurs when the distance between the nanoparticles in the colloidal suspension decreases with concomitant plasmon coupling of neighboring nanoparticles.\(^7,8\)

The aggregation in a MNP-modified colloidal system due the interaction of antibodies immobilized on MNP surfaces with their antigens can be used in an immunoassay development. This aggregation depends mostly on size, charge and concentration of the antigen as well as on the size, morphology and type of the nanoparticles.\(^9\) It is known that the size of analyte biomolecule limits the application of the assay as it prevents the approximation of MNPs and thus minimizes the change in the color of the suspension.\(^1\) Drawbacks also include the time required for aggregation completion and subtle color change.\(^10\) In order to increase the sensitivity of this kind of assay, usually more sophisticated techniques are employed, such as hyper-Rayleigh scattering\(^11\) and photothermal deflection spectroscopy.\(^12\) The addition of a saline solution is also used to induce aggregation of the colloidal systems in immunoassays.\(^13\)

In this case, a stabilization effect could be observed upon antigen-antibody interaction,\(^14,15\) resulting from the variation of surface potential (zeta potential).\(^15\)

Recently, several colorimetric assays based on MNPs have been developed for the detection of small analytes,\(^16–18\) polymers,\(^19\) biomolecules,\(^20–22\) and microbes.\(^23–25\) Gold nanoparticles (AuNPs) are used in the majority of colorimetric methods because their simple preparation, high chemical stability and well known conjugation protocols.\(^26–28\) However, silver nanoparticles (AgNPs) have higher molar attenuation coefficient, lower cost and lower tendency for unspecific aggregation than AuNPs.\(^29–31\)

hIgG is a class of immunoglobulin predominant in human serum that play an important role in humoral immunity in various types of cells.\(^32\) The concentration of hIgG can be significantly altered in pathological conditions such as infections and immunodeficiencies.\(^33,34\) Radioimmunoassay, enzyme-linked immunosorbent assay (ELISA) and nephelometry are some methods used for the determination of antibodies, including hIgG.\(^35–37\) In general, these tests show high selectivity and sensitivity; however, there are some disadvantages related to the adequacy, analysis time and automation complexity.\(^38,39\)

In this current work, an effortless and time-saving immunoassay for the determination of hIgG using AgNPs is reported. The assay is based on the inhibition of the aggregation of AgNPs functionalized with anti-hIgG on the surface due to the presence of the analyte (hIgG). The analyte is detected and quantified by monitoring the color change of the colloidal suspension using a conventional UV-Vis spectrophotometer.
Experimental

Reagents and chemicals
Goat anti-Human IgG (anti-hIgG) and human serum albumin were purchased from Jackson ImmoResearch and BioRad, respectively. Mercaptosuccinic acid (MSA) and sodium citrate tribasic dehydrated were purchased from Synth. IgG from human serum and other analytical grade chemicals were purchased from Sigma-Aldrich. All reagents were used as received without further purification and the aqueous solutions were prepared with Milli-Q water (18.2 MΩ).

Procedure for preparation AgNP-antibody conjugate
A stable colloidal suspension of AgNPs (yellow staining) was prepared by reduction of AgNO₃ with NaBH₄. AgNPs with average diameter smaller than 10 nm were prepared by the dissolution of 200 μL AgNO₃ 100 mmol L⁻¹ and 200 μL sodium citrate 100 mmol L⁻¹ into 80 mL of water under vigorous stirring, followed by addition of 4.8 mL NaBH₄ 25 mmol L⁻¹ aqueous solution at once. Silver colloid solutions were stored at 4°C until use.

The coupling of polyclonal anti-hIgG to AgNPs was accomplished using mercaptosuccinic acid (MSA). Antibody-conjugated silver nanoparticles (AgNP-anti-hIgG) were prepared by mixing 490 μL AgNP colloidal suspension with 10 μL MSA stock solution (20 mmol L⁻¹). The mixture (500 μL) was kept under constant stirring for 150 min at 25°C. Thereafter, 10 μL anti-hlgG 15 μg mL⁻¹ and 490 μL phosphate buffer (10 mmol L⁻¹, pH 7.5) were added to the AgNP suspension. The mixture was incubated at 4°C for 18 h and the final colloidal suspension (1 mL), containing AgNP-anti-hIgG, was then used in the aggregation immunoassay.

Apparatus
AgNP size and morphology were investigated by transmission electron microscopy (MET, JEOL JEM-2100). The UV-Vis attenuation spectra were recorded using a Hitachi U-2010 spectrophotometer.

Colorimetric detection of hIgG
Ten microliters hlgG (25 – 200 ng mL⁻¹) and 10 μL of NaClO₄ (2.5 mol L⁻¹) were sequentially added to microtubes containing 1 mL of the AgNP-anti-hlgG suspension. The mixtures were incubated at 15°C for 10 min, followed by the acquisition of the attenuation spectra. The addition of NaClO₄ induces the aggregation of AgNP-anti-hlgG. The degree of aggregation of AgNP-anti-hlgG can be obtained as a ratio of dispersed nanoparticles (attenuance at 400 nm, A₄₀₀) over the attenuation intensity of the aggregated nanoparticles (attenuance at 530 nm, A₅₃₀), A₄₀₀/A₅₃₀. A linear correlation between A₄₀₀/A₅₃₀ and hlgG concentration was obtained. Moreover, bovine serum albumin (BSA; 1000 ng mL⁻¹) and rabbit IgG (rIgG, 100 ng mL⁻¹) were added in the absence and presence of hIgG to evaluate the selectivity of the immunoassay.

Results and Discussion
The AgNP formation was followed by its absorption at 390 nm, which is characteristic of spherical AgNP-observed by the transmission electron microscopy (TEM) technique (Fig. 1). As can be seen from this micrograph, the silver nanoparticles are relatively monodisperse in size and shape. The average diameter found for the AgNP was 5 nm. The micrograph also shows some crystal planes, which have an inter-planar distance of 2.37 Å corresponding to the (111) crystal plane.

The designed procedure of this colorimetric method for hIgG detection is depicted in Fig. 2. The color of the colloidal system formed by AgNP-anti-hlgG, originally yellow, turns red upon addition of NaClO₄ solution. However, in the presence of hIgG the color turns dark yellow or orange, depending on the concentration of this analyte, as a result of binding with antibodies.

This kind of assay can be developed using polyclonal or monoclonal antibodies. Polyclonal antibodies are usually cheaper and easier to obtain. Moreover, polyclonal antibodies are more stable over a broad salt concentration and pH. The coupling of anti-hlgG to AgNPs was accomplished using mercaptosuccinic acid (MSA). The sulphhydryl group of MSA binds covalently to the metal, whereas its carboxylic group interacts electrostatically with the positively charged moieties such as ammonium present in the antibody. It must be emphasized that, AgNP-anti-hlgG antibodies are produced in situ and were not isolated from the initial components. Therefore, some residual amount of MSA remains unbound in solution. However, this conjugation method proved to be effective when used in the development of immunoassays employing metal nanoparticles, and no aggregation was observed before addition of NaClO₄. Additionally, the effect of anti-hlgG concentration was investigated in the range of 150 and 750 ng mL⁻¹. The best performance was observed using 150 ng mL⁻¹ anti-hlgG. At higher anti-hlgG concentrations, the assay performance was affect by unconjugated anti-hlgG in solution.

Since the immunoassay involves various ionized species, the pH effect was evaluated using phosphate buffers at pH 6.5, 7.0 and 7.5. No significant changes in the aggregation were observed at pH 7.0 for increasing concentrations of hlgG. Although hlgG has higher stability at pH 7.0, there is a tendency for aggregation due to the proximity to the isoelectric point. This pattern was also reported in other systems in which the pH was near the isoelectric point of the protein attached to the metal NP. Moreover, the charge distribution of the biomolecules involved is a factor of great influence in the stabilization process. The anomalous stability of latex colloidal particles conjugated to immunoglobulins with increasing ionic strength is attributed to hydration forces. The approximation of two charged surfaces, due to the partial dehydration of ions
adsorbed and/or presence of charged groups, results in an increase of the system energy, because of the repulsive forces.\(^{45,47}\) Usually this effect is more pronounced in hydrophilic surfaces,\(^{45}\) thus repulsive electrostatic forces were minimized at pH 7.0. On the other hand, at pH 6.5 and 7.5, a dependence on the antigen concentration and AgNP-anti-hIgG aggregation was observed. In addition, pH 7.5 provided higher assay sensitivity.

### Analytical performance of immunoassay

The response of the proposed immunoassay for quantitative detection of hIgG antigen was investigated. The test consisted of increasing the hIgG concentration (25 to 200 ng mL\(^{-1}\)), at 24 mmol L\(^{-1}\) NaClO\(_4\) and 150 ng mL\(^{-1}\) AgNP-anti-hIgG, prepared by adding 30 µmol L\(^{-1}\) MSA to a suspension of AgNP. Each UV-Vis spectrum was obtained after 10 min–time required for the aggregation to occur (Fig. 3). The response was evaluated from attenuance spectra using the ratio at 400 and 530 nm (\(A_{400}/A_{530}\)). The value \(A_{400}/A_{530}\) represents the relative amount of dispersed and aggregated AgNP-anti-hIgG, respectively.

The sensitivity of the methodology was evaluated using the ratio \(A_{400}/A_{530}\) as a function of hIgG concentration. A linear correlation was observed in the range of 25 to 200 ng mL\(^{-1}\) hIgG (\(A_{400}/A_{530} = (0.0084 \pm 0.0011) [\text{IgG}] + (1.07 \pm 0.03)\), \(r = 0.9942, p < 0.0001\), and the limit of detection (LOD) was estimated to be 11 ng mL\(^{-1}\) hIgG. The LOD was calculated considering 3 times the standard deviation of the blank signal/sensitivity (3\(\sigma/\text{slope}\)). The linear range and sensitivity of the proposed methodology is similar to some reported ELISA methods\(^{37}\) and sensitive electrochemical immunosensors.\(^{48}\) Using AuNP, and the hyper-Rayleigh scattering technique, a change of the analytical signal (aggregation) by using 10 µg mL\(^{-1}\) of hIgG was observed.\(^{11}\) Another method was developed for determining hIgG based on aggregation of AuNPs and dynamic scattering detection with a reported detection limit of 10 ng mL\(^{-1}\) hIgG.\(^{14}\) Based on these previous results, the method herein proposed is less expensive as it just requires a simple spectrophotometer.

The immunoassay selectivity was evaluated by conducting the experiment in the presence of BSA and rIgG as contaminants. The variation of attenuance signal, \(\Delta(A_{400}/A_{530})\), in the presence of hIgG (100 ng mL\(^{-1}\)), rIgG (100 ng mL\(^{-1}\)), BSA (1 µg mL\(^{-1}\)) and BSA (1 µg mL\(^{-1}\)) + hIgG (100 ng mL\(^{-1}\)). Experiments were performed in triplicate.
hand, when the assay is conducted with 100 ng mL⁻¹ hlgG in the presence of 1 μg mL⁻¹ BSA, an average signal 26% lower than hlgG alone was obtained. It must be emphasized that the concentration of BSA used was 10 times higher than hlgG. These results indicate that the method is not affected by cross-reactivity and is weakly influenced by nonspecific aggregation.

Conclusions

In conclusion, this study demonstrates that it is possible to determine hlgG concentrations higher than 25 ng mL⁻¹ by using a colorimetric immunoassay based on the aggregation inhibition of silver nanoparticles functionalized with hlgG antibody on the surface (AgNP-anti-hlgG). The method described is simple, does not require the use of sophisticated instruments and is highly promising as an alternative approach for the determination of clinical-relevant antigens.

Acknowledgements

The authors gratefully acknowledge the FAPESP and CNPq for financial support.

References

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