Plasmonic sensing through bioconjugation of Ag nanoparticles: Towards the development of immunoassays for ultralow quantification of antigens in colloidal dispersions

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Abstract

The combination of the optical properties of silver nanoparticles with some of the tool-kits of the widespread used ELISA method for antigen quantification immunooassays, give rise to an enzyme free, low cost, fast and more sensitive optical method denoted as Intensity Depletion Immuno-linked Assay (IDILA), that can be performed directly in colloidal dispersions without any immobilization of the capture antibody, antigen, secondary and primary antibodies on a substrate. The capabilities of the method for quantifying antigens at ultralow concentrations in colloidal dispersions as well as its performance are demonstrated for specific antigens of importance in medicine and in food science. Copyright © 2018 VBRI Press.

Keywords: Plasmonic nanoparticles, antigen quantification, bioconjugation, immunoassay.

Introduction

One of the most popular tools in biochemistry for antigen detection is the Enzyme-Linked Immuno-sorbent Assay (ELISA). It is used, for instance in the clinical analysis for the prognosis and diagnostics of several diseases [1-3], as well as in other areas such as the control of the quality of food[5-7], bio-technology[8,9], environmental monitoring of pollutants[10,11], defense and biotechnology.[12-14]

In the standard ELISA (see Fig.1), the antigen to be detected in a fluid phase is immobilized on a substrate, to a specific capture antibody, which is itself subsequently detected by a secondary, enzyme-coupled antibody that upon reacting with a substrate, yields a visible color change or fluorescence, indicating the presence of the antigen.[15]

Although this method has demonstrated to be robust and of easy implementation in laboratories, it has some shortcomings such as the several steps involved and the occasionally nonspecific binding of the antigen to the substrate that could lead to false positive results.

One of the most significant improvements of the ELISA assay performed in recent years is the so-called plasmonic ELISA (pELISA).[16] The basis of this method relies on the extraordinary signal amplification
generated by the enzyme using the unique optical properties of noble metal nanoparticles (NPs). These properties arise from the Localized Surface Plasmon Resonance (LSPR), a phenomenon generated by the collective excitation of the conduction electrons of noble metals induced upon illumination with light.[17] The frequency of the LSPR is highly dependent on NP size, shape, and composition as well as on the external dielectric environment and clusterization degree.[17-19] The absorption as well as the scattering cross section of Ag or Au NPs are highly enhanced at the LSPR frequency. The extinction cross section is the contribution from both scattering plus absorption cross sections. For relatively small NPs (around 15-20 nm diameter) absorption dominates the extinction spectra, while for NPs of large size (roughly above 100 nm) scattering is the main contribution.[17] For NPs of intermediate size both scattering and absorption processes are important. It should be noted that extinction spectra are the far field optical property measured in a conventional UV-Vis spectrophotometer. Using a dark field illumination and collecting the light using a high NA objective, the light scattered by individual nanoparticles can be detected as bright color points by the eye.[20] Furthermore, by collecting the scattering signal of a single NP and due to the extreme sensibility of the LSPR wavelength to the external dielectric environment, the binding of specific analytes to the NP surface can be monitored following the spectral shift of the LSPR. In this way, extremely sensitive LSPR sensors have been developed.[21-25]

Many different mechanisms for amplification of the signal in pELISA have been developed in recent years. All of them are based on inducing a change of color using the plasmonic properties of noble metal NPs. The product generated by the enzyme can produce, for instance, the agglomeration of a colloidal dispersion of Au/Ag NPs, the formation and growth of new NPs or the etching of noble metal NPs.[26-32] These different types of modulating the state of the plasmonic nanostructures, yields as a result a pronounced color change in colloidal solution as a function of the analyte concentration. In this way, the pELISA methods already mentioned are able to detect very low concentrations of antigens, offering relatively low cost, reliable and quick semi-quantitative determination. In spite of these significant improvements of the ELISA method by introducing plasmonic properties as signal traducers, its format is still the same, since in any case it involves detecting the antigen by its immobilization on a solid substrate (microplates) using the specific antibody, and the need to label it with a primary or secondary antibody with an enzyme. Therefore, the several time-consuming steps of incubation, washing and immobilization cannot be avoided together with the need to use an enzyme labelled antibody.

In order to surmount these shortcomings, we have developed a new method denoted as IDILA (Intensity Depletion Immunolinked Assay) that combines the plasmonic properties of silver nanoparticles with the biorecognition properties of the molecules used in the ELISA assay (biotinylated specific antibody and antigen) but with the important advantage that it can be performed totally in a colloidal dispersion, it does not require using an enzyme, and can be performed in almost one step.[33] This means that does not require high amounts of reagents to be immobilized, implying short experiment lengths and extremely low amounts of reagents, making it feasible for a fraction of the common price per experiment of ELISA (See table S1 in the Supporting Information).

**Basis of the IDILA methodology**

The IDILA method is based on the inhibition of the formation of nanoparticle dimers produced by linking two Ag NPs by a specific antibody in the presence of different concentration of the corresponding antigen to be detected. The formation of Ag dimers at a certain time, in the absence of antigen, produces a depletion of the extinction intensity (the degree of depletion depends on the fraction of dimers formed and therefore of the antibody concentration). In the presence of both the antigen and the antibody, the specific interaction between the antibody and the antigen, causes an inhibition of dimer formation. As a consequence, the fraction of dimers formed decreases as the antigen concentration increases (the comparison of the extinction intensities in the absence and in the presence of antigen is performed at the same reaction time, normally around 30 minutes, after which no change of the extinction intensity is observed).

The Ag NPs are functionalized, before adding the antigen, with biotin and streptavidin mixed in concentration ratio 1:1:1 between Ag NPs, biotin and streptavidin, in such a way to have on the average only one (or only a few) biotin-streptavidin complex per NP.

The first issue to be addressed is why the formation of silver NPs dimers causes a decrease of the extinction intensity. The answer to this issue is quite simple. Comparing the calculated extinction cross-section of 60 nm diameter Ag monomer and the corresponding dimer with a gap of around 20 nm (corresponding to the size of the molecules sandwiching the monomers, *i.e.* biotin-STV-antibody-STV-biotin, see figure 4) shows that although the average extinction cross section of a Ag dimer ( \( \sigma_{\text{Ext}} = \frac{1}{3} \sigma_x + \frac{1}{3} \sigma_y + \frac{1}{3} \sigma_z \) ) is larger by a factor of 1.5 with respect to the monomer, the quantity to be compared is the sum of the extinction cross-sections of two monomer with respect to one dimer, which is 0.75 times smaller. In addition, as the plasmon coupling in the Ag NP dimers is small due to the relatively large interparticle gap, the extinction spectra of the dimer have its peak intensity at almost the same wavelength. Considering these features, and the fact that as dimers are formed, the total number of particles (dimers plus monomers) decreases, it follows that the
extinction intensity of the colloidal dispersion should also decrease.

For the dimerization process:

\[ 2M \rightarrow D \]

where M stands for Ag monomers and D for Ag NPs dimers, the total concentration of particles at any time \( t \) ([\( NPs \)]\(_t\)) as a function of the initial concentration of NPs ([\( NPs \)]\(_0\)), and the fraction of dimers \( f_d = \frac{[D]_t}{[NPs]_t} \) is given by:

\[ [NPs]_t = [NPs]_0 (\frac{1 - f_d}{1 + f_d}) + [NPs]_0 (\frac{f_d}{1 + f_d}) \]

where the first term corresponds to the concentrations of monomers and the second term to the concentration of dimers. The extinction intensity at any time \( E_t \), is obtained multiplying each term by the corresponding extinction cross-section.

\[ E_t = [NPs]_0 (\frac{1 - f_d}{1 + f_d}) \sigma_m + [NPs]_0 (\frac{f_d}{1 + f_d}) \sigma_d \]

being \( \sigma_m \) and \( \sigma_d \) the extinction cross-section of the monomer and dimer respectively. Note that initially \( f_d = 0 \), and \( E_t = [NPs]_0 \sigma_m \) as expected while as \( f_d \rightarrow 1 \), \( E_t = \frac{1}{2}[NPs]_0 \sigma_d \), thus if \( \sigma_d = 1.5 \sigma_m \), then for a total conversion of monomer to dimers, the value of the extinction should be \( E_t = 0.75[NPs]_0 \sigma_m \), which corresponds to 25% of depletion with respect to the initial value. The addition of antigen produces a smaller fraction of dimers and as a consequence a less degree of depletion.

**Dependence of the extinction depletion on the IgG antibody concentration**

Now we will illustrate the application of this principle to the quantification of antigens.

**Fig. 3** illustrate the extinction depletion as a function of time after adding the specific capture biotinylated IgG antibodies for the detection of antigens of importance in clinical (i.e., interleukin 10 – IL-10) and food chemistry (i.e., gliadin) diagnosis.

It can be appreciated that the extinction intensity decreases with the addition of the different biotinylated IgG reaching a maximum depletion for a concentration around 1 ng/mL and then start to decrease again, until reaching almost the initial value. The initial intensity depletion implies that the formation of dimers is favored as IgG increases, the lower value corresponds to the antibody concentration where the maximum dimer fraction is produced. After this concentration value, the intensity starts to increase again, because the concentration of IgG is high enough that many of the available streptavidin sites at the surface of the Ag NPs are occupied by IgG and therefore are not able to form dimers.
The formation of dimers of Ag NPs structures is evidenced from the TEM images obtained after adding to the NPs dispersion a specific quantity of antibody at a given reaction time as shown in Fig. 4. Note also that the interparticle separation is around 20 nm in agreement with the molecular geometry.

![Fig 4.](image)

**Fig 4.** (a) Scheme showing the dimensions of the molecular sandwich between two Ag NPs. (b) Representative TEM image of the dimers formed after 27 min of the addition of IgG-gliadin.

The regime for the applicability of the IDILA technique is the one that favor the dimer formation. In this regime, the presence of the antigen of interest according to the nature of the IgG used, will inhibit, according to its concentration the dimer formation.

**On the general capabilities of the IDILA method for quantification of any type of antigen**

One important question which arises is if once the intensity depletion in the presence of the biotinylated antibody, the capability biotin-STV functionalized Ag NPs is a general feature, independent of the antigen nature. In order to answer this issue, we have performed experiments using antigens of different composition and size. Fig. 5 depicts the significant change on the degree of depletion of the extinction intensity upon the addition of pico-molar concentrations of antigen to a colloidal dispersion of streptavidin-biotin functionalized Ag NPs at the same time (27 minutes) of reaction time. A comparison of the degree of depletion of the extinction spectra intensity with and without the antigen, show that the degree of depletion decreases as the antigen concentration increases, till reaching an asymptotic value where the formation of dimers is completely disfavored.

![Fig 5.](image)

**Fig 5.** (a) Fractional extinction depletion (normalized to the initial extinction) in the presence of 1 ng/mL of IgG antibody as a function of the antigen concentration for a) IL-10 antigen (b) Gliadin antigen.

The reason of the behavior mentioned above can be explained in a simple way. At low antigen concentrations, a small quantity of Ag monomers can form dimers as a small fraction of monomers are bounded to the antibody and the remaining nanoparticles are functionalized with the antigen. This is translated in an increase of the intensity of the extinction as the antigen concentration increase (less formation of dimers). At high antigen concentrations dimer formation is completely inhibited because all antibodies are functionalized with antigens and therefore any is able to react with another nanoparticle to form a dimer or the biotinylated antibody hides their biotin groups (for instance by a conformational change when it is functionalized with the antigen), leading in any case to the same result. The underlying microscopic changes in antibody conformation or the specific changes induced by the molecular binding of the antibody to the antigen is a research topic under development.

The analytical performance of the IDILA methodology for the quantification of the two antigens discussed in the present article are shown in Table 1. The comparison of the detection sensitivity and limit of detection (LOD) between IDILA and ELISA revealed that the sensitivity is improved by IDILA by 1000-10000 folds over the traditional colorimetric assay.

**Table 1.** Comparison of the analytical parameters between IDILA and ELISA.

| Antigen     | IDILA      | ELISA      |
|-------------|------------|------------|
| IL-10       | (0.06±0.02) pg/mL | (1.5±0.1) x10^3 pg/mL |
| Gliadin     | (5 ± 1) pg/mL     | (5±0.1) x10^3 pg/mL* |
| LOD         | 0.8 pg/mL        | 0.52 pg/mL     | 1.82 ng/mL |

*data of commercially available ultra-sensitive ELISA kits from ThermoFisher.
Conclusions

In this perspective article, we have outlined the main principles underlying a new form of immunoassay that combines the unique optical properties of Ag plasmonic NPs, the strong streptavidin–biotin interactions and affinity between the antigen and its specific biotinylated antibody. This new method, denoted as intensity depletion immunoassay (IDILA), is based on the depletion of the intensity of extinction caused by the formation of silver nanoparticle dimers between biot-STV functionalized Ag NPs and the antibody. This depletion occurs because the extinction cross-section of two Ag NPs monomers is significantly greater than the corresponding Ag NP dimer. It was demonstrated that it is not expected to work with Au NPs since for this metal this condition is not fulfilled. The presence of different antigen concentrations, inhibits the process of dimer formation and therefore the extinction depletion varies with an upper bound equal to zero (when the antigen concentration is so high that dimers are not formed) and a lower bound that depends on the fraction of dimers formed (in the absence of the antigen).

This method proved to be robust, fast (< 2 hours), cheap, sensitive, accurate and potentially adaptable to any antigen (here we showed its performance for detection and quantification of IL-10 and Gliadin antigens, important for clinical and food chemistry diagnosis, respectively).

There are still many avenues that still need to be addressed in order to further prove/improve the capabilities of the IDILA method such as the role played by the initial concentration of the functionalized Ag NPs, the influence of the Ag NP size and shape, the dependence of its sensitivity upon the concentration of the antibody, for just mentioning the main issues under current study in our laboratories.

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Author’s contributions

Authors have no competing financial interests.

Supporting information

Materials and methods for the theoretical simulations, extinction depletion studies, TEM images, and fractional extinction depletion in the presence of a specific antigen. Table of comparative costs between IDILA and ELISA. Supporting information are available from VBRI Press.

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