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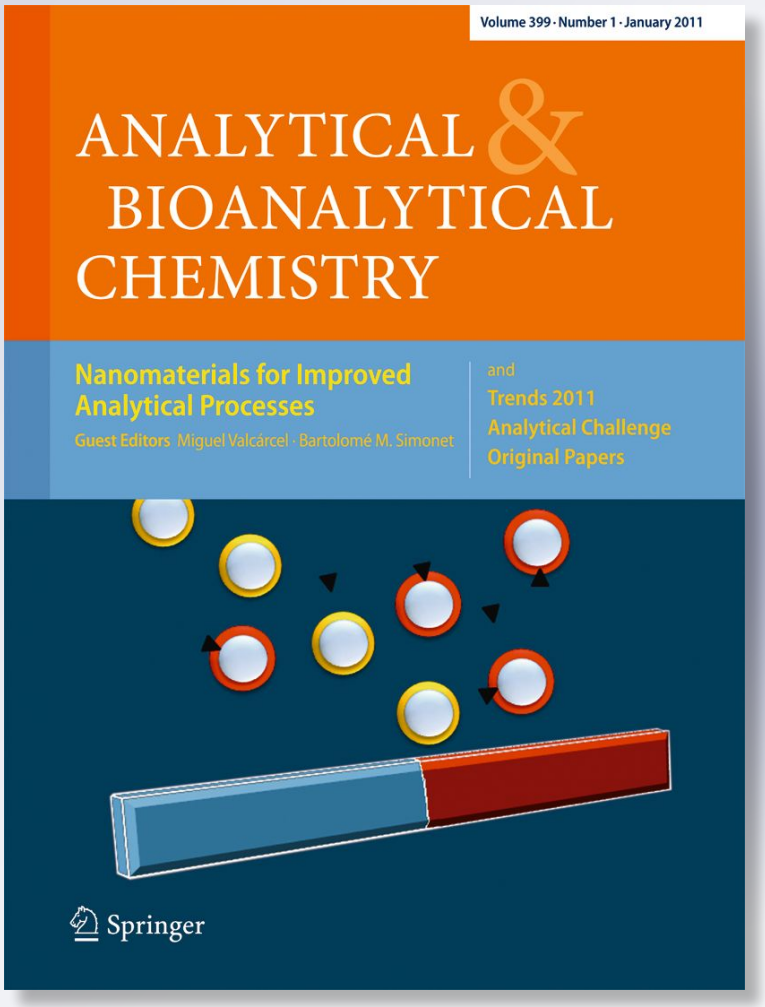

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ORIGINAL PAPER

Gold nanoparticle-based fluorescence immunoassay for malaria antigen detection

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Abstract The development of rapid detection assays for malaria diagnostics is an area of intensive research, as the traditional microscopic analysis of blood smears is cumbersome and requires skilled personnel. Here, we describe a simple and sensitive immunoassay that successfully detects malaria antigens in infected blood cultures. This homogeneous assay is based on the fluorescence quenching of cyanine 3B (Cy3B)-labeled recombinant *Plasmodium falciparum* heat shock protein 70 (*Pf*Hsp70) upon binding to gold nanoparticles (AuNPs) functionalized with an anti-Hsp70 monoclonal antibody. Upon competition with the free antigen, the Cy3B-labeled recombinant *Pf*Hsp70 is released to solution resulting in an increase of fluorescence intensity. Two types of AuNP-antibody conjugates were used as probes, one obtained by electrostatic adsorption of the antibody on AuNPs surface and the other by covalent bonding using protein cross-linking agents. In comparison with cross-linked antibodies, electrostatic adsorption of the antibodies to the AuNPs surfaces generated conjugates with increased activity and linearity of response, within a range of antigen concentration from 8.2 to 23.8 $\mu g.mL^{-1}$. The estimated LOD for the assay is 2.4 $\mu g.mL^{-1}$ and the LOQ is 7.3 $\mu g.mL^{-1}$. The fluorescence immunoassay was successfully applied to the detection of antigen in malaria-infected

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human blood cultures at a 3% parasitemia level, and is assumed to detect parasite densities as low as 1,000 parasites.μL^{-1}.

Keywords Malaria diagnosis · Gold nanoparticles · Nanodiagnosis · Heat shock protein · Fluorescence detection · Immunoassay · Plasmodium

Abbreviations

AuNP	Gold nanoparticle
AuNP-MUA	Mercaptoundecanoic acid functionalized AuNP
EDC	1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
Hsp70	Heat shock protein 70
LOD	Limit of detection
LOQ	Limit of quantitation
MUA	Mercaptoundecanoic acid
NHS	*N*-hydroxysuccinimide
*Pf*Hsp70	*Plasmodium falciparum* Heat shock protein 70
RBC	Red blood cell

Introduction

Malaria is caused by protozoan parasites of the genus *Plasmodium* (*P.*), with one species, *Plasmodium falciparum*, being responsible for the vast majority of the mortality and morbidity associated with this disease. Malaria infection is initiated when an infected *Anopheles* mosquito injects *Plasmodium* sporozoites into the bloodstream of a mammalian host during a blood meal. These parasites then travel to the liver where they undergo a unidirectional and clinically silent multiplication phase that culminates in the release of thousands of newly formed merozoites into the blood, leading to the appearance of disease symptoms [1]. Despite its huge burden, with 40% of the world's population at risk of infection, the clinical diagnosis of malaria is often not straightforward partly because its symptoms (high fevers, shaking chills, headache, and flulike illness) resemble those of other febrile illnesses. Although light microscopy analysis is still considered the standard method for parasite-based malarial diagnosis, it is labor-intensive, requires technical expertise, and has a long turn-around time [2, 3]. Therefore, there is an urgent need to develop rapid, sensitive, and cost-effective tests for both high- and low-resource settings [4]. Simpler and easier to interpret methods requiring minimal infrastructure and expertise and with shorter turn-around time have been developed including immunochromatographic tests or rapid diagnostic tests (RDTs). Commercially available RDTs allow the detection of different malarial antigens including parasite histidine rich protein, lactate dehydrogenase, and aldolase [2]. However, the sensitivity of such tests may be dramatically affected by field conditions such as heat and humidity [5]. These tests may also generate false positives due to cross-reactivity with the rheumatoid factor that is present in high titer in blood of patients suffering from diseases such as rheumatoid arthritis [6]. A more sensitive method for the detection of malarial infection is the detection of parasitic nucleic acid via PCR. Although this method is sensitive and can be used to differentiate between malarial species, the capital and operating costs are high and advanced infrastructure and trained personnel are crucial to prevent contamination [2].

Gold nanoparticles (AuNPs) are ideal candidates for biosensing assays due to their unique characteristics, namely high solubility in water, and easily tailored synthesis for suitable morphology, size dispersion and surface functionalities [7]. Recently, fluorescence based approaches have been explored in conjugation to AuNP towards the establishment of immunoassays [8–11]. AuNPs can cause fluorescence enhancement or fluorescence quenching of molecules in their vicinity, depending on the distance between the nanoparticle and the fluorophore [12]. It is also known that AuNPs are able to quench fluorescence with larger efficiencies than organic quenchers, and are more stable than the latter [13]. As more basic studies unveil the complicated mechanisms underlying these processes, these effects have been used in immunosensors in a variety of formats, including a typical sandwich assay [11], and involving the utilization of magnetic beads [8]. The Feldmann group presented a competitive immunoassay for digoxigenin, using the BSA-coupled drug, with a LOD in the nanomolar range [9] and a cardiac troponin T non-competitive sandwich immunoassay that reached a LOD in the picomolar range [10], the lowest value reported so far for this type of antigen-detection assay.

Here, we present an immunoassay that relies on the use of mercaptoundecanoic acid (MUA)-capped AuNPs (AuNP-MUA) conjugated with 2E6, a mouse IgG monoclonal antibody that specifically recognizes *P. falciparum* heat shock protein 70 (*Pf*Hsp70) [14]. Heat shock proteins are immunodominant antigens recognized by the host immune system in various infectious diseases [15]. Antibodies against *Pf*Hsp70, could be detected in the sera of people exposed to *P. falciparum* using ELISA [16], and the antigen was previously detected in the pellet of saponin-treated *P. falciparum*-infected RBCs using Western Blot analysis [17]. A rapid agglutination-based assay has been recently described utilizing polystyrene particles functionalized with either malaria antigen or antibodies (monoclonal against *Pf*Hsp70 or polyclonal against *P. falciparum*), with sensitivities ranging from around 84–90%, for the detection of either antigen or antibody in clinical samples [18] .

Here, we use a monoclonal antibody for recombinant *Pf*Hsp70 antigen quantitative detection using a gold-nanoparticle-based fluorescent immunoassay. This assay could also detect the *Pf*Hsp70 antigen in a *P. falciparum*-infected human blood culture, but not in the blood of a healthy donor, suggesting that this antibody–antigen set can be used in the development of a diagnostic test for malaria in clinical samples.

Materials and methods

Chemicals and reagents

Protein concentration was determined by the bicinchoninic acid method [19]. UltraPure agarose (Invitrogen) was used in the agarose gels. Nitrocellulose membranes were from Schleicher and Schull (BioScience). Horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG were from GE Healthcare. SDS-PAGE reagents were from BioRad. All other chemicals and reagents were from Sigma Aldrich and were of the highest purity available.

Synthesis and functionalization of AuNPs

Gold nanoparticles with an average diameter of 15–20 nm were synthesized by the citrate method originally described by Turkevich, with minor alterations [20, 21]. The concentration of AuNPs in the final colloidal solution was determined by UV/Vis spectrophotometry as previously described [22]. To allow uniformity of negative charge at the surface of the AuNPs and easy functionalization with the antibody by chemical cross-linking (see below), the citrate capping of the synthesized AuNPs was replaced by MUA. The AuNPs solution was incubated overnight at 4 °C with MUA at a 1:120 molar ratio, and the functionalized AuNP-MUA were utilized without further treatment.

Preparation and characterization of AuNP-antibody conjugates

The anti-*Pf*Hsp70 monoclonal antibody (2E6) was obtained from a hybridoma culture and purified using Protein G sepharose affinity chromatography (see Electronic supplementary material). The formation of AuNP-antibody conjugates between the AuNP-MUA and the 2E6 antibody (AuNP-MUA-2E6) was performed in the presence and in the absence of EDC/NHS cross-linkers. In the absence of the EDC/NHS cross-linkers, the 2E6 antibody binds to AuNP-MUA mostly through electrostatic interactions. However, in the presence of the EDC/NHS cross-linkers, a covalent amide bond is formed between the carboxylic terminal groups of MUA and amine groups present in the amino acid side-chains of the antibody [23]. These concentrations were optimized for all antibody to AuNP-MUA molar ratios, based on the presence of non-aggregated conjugates in agarose gels (data not shown). Stock solutions of EDC and NHS, 280 and 120 mM respectively, were prepared by dissolving the respective salts in milli-Q water and were added to the antibody solutions (final volume of 2 mL), to a final NHS and EDC to MUA molar ratios of 10 and 23.3, respectively. The 2E6 antibody in 5 mM phosphate buffer, pH 7.0, was added to AuNP-MUA at molar ratios of antibody to AuNP-MUA ranging from 5 to 400. Conjugates were incubated overnight at 4 °C, or for 2 h at room temperature for EDC/NHS conjugates. After conjugation, non-bound antibody was removed by centrifugation. All conjugates were prepared in glass containers instead of plastic vials to minimize adsorption to the walls.

Agarose gel electrophoresis

Formation of the AuNP-antibody conjugates was confirmed by separation on agarose gel electrophoresis (0.5% agarose, 0.125×TAE buffer, pH 8.4, at 10 V/cm for 30 min), using the Mini-Protean system (BioRad). After incubation, the conjugates were pelleted by centrifugation, resuspended in 27 μL of the supernatant and mixed with 3 μL of glycerol (87%) before loading. Electrophoretic mobility (μ) of the AuNP-antibody conjugates in agarose gels was calculated as previously described [24, 25].

Zeta potential measurements

Zeta potential measurements were performed using a previously reported method [26] in order to determine the binding constant for the conjugation of 2E6 to AuNP-MUA. A Zetasizer Nano-ZS (Malvern Instruments) instrument was used with a 4 mW He–Ne laser (633 nm) and fixed 17° scattering angle. One mililiter of sample was loaded into the disposable clear zeta cell (Malvern, UK). The experiments were initiated 15 min after the sample had reached thermal equilibrium. Each sample was measured four times and each measurement consisted of 100 acquisitions.

Zeta potential values were calculated by the Malvern software package using Henry's equation that relates electrophoretic mobility to zeta potential (Eq. 1):

$$\mu = \frac{2\varepsilon\zeta}{3\eta} f(\kappa R) \quad (1)$$

where μ is the electrophoretic mobility, ε and η are the dielectric constant and viscosity of the solvent, respectively, and ζ is the zeta potential. Henry's function, $f(\kappa R)$, was approximated by the Smoluchowski approximation, $f(\kappa R)=1.5$, because the zeta potential measurements were performed in aqueous solution.

Data obtained for different [2E6]/[AuNP-MUA] ratios was fitted to a Langmuir adsorption isotherm (Eq. 2) using Origin 8.0 Pro software.

Western blot analysis of *Pf*Hsp70 antigen in blood samples

Sample preparation

P. falciparum-infected red blood cell (RBC) cultures were prepared by separating human RBCs by Ficoll gradient from freshly collected buffy coats discarded from blood donations of healthy adults. A *P. falciparum* 3D7 culture was then started by adding parasitized RBC to freshly prepared RBC to a hematocrit of 3% and grown in complete medium. Venous blood collected from a healthy individual in a tube with heparin to prevent blood clotting was used as control. RBC numbers in infected and non-infected blood samples were normalized to 4.56×10^9 cells.

RBCs were separated from whole blood samples, treated with saponin and the pellets used to perform Western blot analysis. Briefly, whole blood was centrifuged at 800×g for 5 min at 4 °C, plasma was removed and RBCs were washed in PBS followed by lysis in saponin (0.15% in PBS) [17]. The resulting pellets were washed and boiled for 5 min in Laemmli buffer. Protein extracts (30 μl) were separated under denaturing conditions on a 12.5% polyacrylamide gel.

Western blotting and immunoblotting

For Western blotting, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes. Membranes were blocked in 5% *w/v* of non-fat dry milk in PBS containing 0.05% Tween 20. After blocking, membranes were incubated with monoclonal 2E6 antibody against *Pf*Hsp70 or anti-actin primary antibody, used as a loading control. Primary antibody binding was detected by using horseradish peroxidase (HRP)-labeled anti-mouse or anti-rabbit IgG respectively. Antibodies were diluted in 5% *w/v* of non-fat dry milk in 0.05% PBST and nitrocellulose membranes overlaid with antibodies for 1 h at room temperature. The blots were developed by addition of SuperSignal West Pico Chemiluminescent Substrate (Thermo-Scientific). Alternatively, after blocking, membranes were incubated with AuNP-MUA-2E6 conjugates, prepared in the absence of EDC/NHS cross-linkers (molar ratio of 2E6 antibody to AuNP of 25:1), for 1 h at room temperature.

Production and purification of recombinant *Pf*Hsp70 antigen

Recombinant *Pf*Hsp70 antigen was obtained from an overproducing *E. coli* strain, previously transformed with the pQE30/PfHsp70 plasmid [27]. The His-tagged *Pf*Hsp70 was purified by Ni-NTA affinity chromatography (Qiagen, Germany). For a complete description of the experimental procedures, see the Electronic supplementary material.

Labeling of the *Pf*Hsp70 antigen with Cy3B

For the fluorescence competitive immunoassay, the *Pf*Hsp70 antigen was labeled with Cy3B (Cy3B-*N*-hidroxisuccinimidine monoester, GE Healthcare). *Pf*Hsp70 in PBS, pH 7.4, was incubated with Cy3B at a molar ratio of 1:3, for 2.5 h at room temperature. Excess free dye was removed by ultrafiltration using PBS as washing buffer until a clear eluate was obtained. The Cy3B labeled *Pf*Hsp70 solution was concentrated and its absorbance spectrum was compared with that of free Cy3B (Fig. S1, Electronic Supplementary Material). A red shift in the peak maximum occurred from 558 nm to 566 nm upon labeling, proving effective labeling. The solution of labeled antigen was stored at −20 °C until use.

Competitive immunoassay experiments

Optical density spectra were measured with a Varian Cary 50 UV/Vis spectrophotometer. Fluorescence measurements were on a Varian Cary Eclipse spectrophotometer controlled by the Cary Eclipse software (Version 1.1, Varian), using a 70 μl fluorescence quartz microcell (Hellma, Germany). Emission spectra were recorded in the 500–700 nm range, with 480 nm excitation. AuNP-MUA-2E6 conjugates used for the immunoassay contained a 25:1, 2E6 antibody to AuNP-MUA molar ratio (prepared in the presence and absence of EDC/NHS cross-linkers). BSA blocking was performed to avoid unspecific antigen binding to the AuNP-MUA-2E6 conjugates. In a typical experiment, 2 mL of BSA (3 μM in 5 mM phosphate buffer, pH 7.0) were added to 2 mL of 2 nM AuNP-MUA-2E6 conjugates at a 500 BSA molar excess. Incubation was performed for 30 min at RT. This solution was diluted to 30 mL with 5 mM phosphate buffer, pH 7.0, and excess BSA was removed by centrifugation, followed by pellet resuspension in the same buffer for a final blocked AuNP-MUA-2E6 concentration of 0.85 nM. In a typical fluorimetric reaction assay, the solution mixture contained the *Pf*Hsp70 antigen (or PBS for blank measurements), BSA-blocked AuNP-MUA-2E6 conjugates, and labeled *Pf*Hsp70 antigen at a 100-fold molar excess in relation to AuNP-MUA-2E6. Fluorescence measurements were performed after 3 h of incubation at room temperature.

Results and discussion

Several complementary approaches were followed for the establishment of an AuNP-based immunoassay for malaria antigen detection. These included the optimized formation of the AuNP-antibody conjugates; the proof-of-concept for

the utilization of the particular malaria antigen/monoclonal antibody pair; the application of the assay for detecting recombinant antigen; and, antigen detection in infected human blood culture samples.

Fluorescence immunoassay concept

The proposed homogenous immunoassay for the detection of *Pf*Hsp70 is based on competition between the antigen and its Cy3B-labeled counterpart for the active sites of 2E6 antibodies conjugated to AuNP-MUA (Fig. 1). The spectral overlap between the optical density spectrum of AuNP-MUA-2E6 conjugates and the photoluminescence spectrum of Cy3B-labeled antigen (Fig. 1a) allows for effective quenching of the Cy3B fluorescence emission, by the AuNPs present in the AuNP-MUA-2E6 conjugates, upon binding of the labeled antigen to the specific antibody binding site. Cy3B was chosen as the fluorescence marker because of this extensive spectral overlap between its photoluminescence spectrum (emission maximum at 580 nm) and the broad AuNP plasmon resonance band, centered at 520 nm. Also, Cy3B is only weakly influenced by tryptophan quenching, as all cyanine dyes, but presents increased stability and quantum efficiency compared to other such dyes [28]. Figure 1b shows photoluminescence spectra that correspond to the assay principle: when the Cy3B-labeled antigen binds to the AuNP-MUA-2E6 conjugates, the photoluminescence intensity of Cy3B is low due to quenching by the AuNPs (dashed trace). When the non-labeled antigen (analyte) competes for binding to the AuNP-MUA-2E6 conjugates, an increased amount of Cy3B-labeled antigen stays free in solution and consequently an increase in the photoluminescence intensity is observed (solid trace). This increase in fluorescence intensity can be as high as 100% as presented in Fig. 1b. This system was successfully used for quantitative detection of recombinant *Pf*Hsp70 in a proof-of-concept for the assay, and for detection of native *Pf*Hsp70 antigen in malaria-infected blood samples.

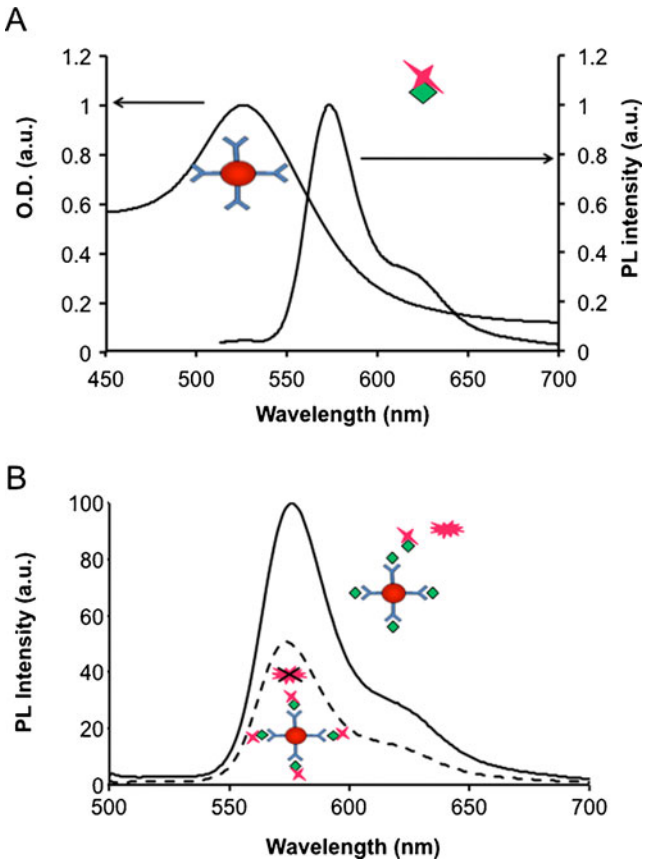

Fig. 1 Principle of the fluorescence competitive immunoassay for the detection of *Pf*Hsp70. **A** Spectral overlap between the optical density spectrum of AuNP-MUA-2E6 conjugates ("OD" *yy* axis) and the photoluminescence spectrum of Cy3B-labeled *Pf*Hsp70 ("PL intensity" *yy* axis) allows effective quenching of the Cy3B fluorescence emission. **B** The photoluminescence intensity of Cy3B in the presence of AuNP-MUA-2E6 conjugates is low due to quenching by the AuNPs (*dashed trace*). When the *Pf*Hsp70 antigen (analyte) binds to the AuNP-MUA-2E6 conjugates, an increased amount of Cy3B-labeled *Pf*Hsp70 stays free in solution and consequently an increase in the photoluminescence intensity is observed (*solid trace*). *Red circles* are AuNPs, *blue "Y"* are antibodies, *green diamonds* represent the antigen, and *pink stars* represent the Cy3B label

AuNP-antibody conjugates

Although many studies on the characterization of AuNP-protein conjugates rely solely on the analysis of their migration pattern on agarose gel electrophoresis, this pattern is known to be influenced by several factors such as charge, volume and mass of the conjugates. On the other hand, zeta potential measurements provide reliable information on nanoparticle superficial charge but can be negatively influenced by the soft protein shell present on the conjugates and, as all light dispersion techniques, are prone to errors related to the presence of small amounts of large size impurities. It is therefore important to provide a complementary approach using these two techniques in which agarose gels provide information on relative volumes and masses of the AuNP-antibody conjugates, and zeta potential measures superficial charges and, indirectly, gives an indication of the degree of antibody coverage of the AuNP surfaces.

AuNP-MUA-2E6 conjugates were formed either by direct adsorption of the antibody to the MUA-functionalized AuNPs (physisorption), or by cross-linking both components using EDC/NHS chemistry (chemisorption). The MUA functionalization present in the AuNPs is intended to promote these controlled conjugation processes, avoiding unspecific binding through the antibodies exposed –SH groups. Agarose gel electrophoresis and zeta potential

analyses were used to evaluate the robustness and binding properties of the AuNP-antibody conjugates. Figure 2a shows agarose gel electrophoresis of increasing molar ratios of antibody to AuNP-MUA both in the presence (left panel) and absence (right panel) of EDC/NHS cross-linking agents. Noticeable differences can be observed in the electrophoretic mobility of the samples for both conjugation procedures (insets in Fig. 2a). Whereas in the presence of the EDC/NHS cross-linking agents the electrophoretic mobility is the same for all antibody to AuNP-MUA molar ratios tested (ca. −0.6 μm cm V^{-1} s^{-1}), in the case of the non-chemical conjugation approach the electrophoretic mobility of the samples increases up to ca. −0.4 μm cm V^{-1} s^{-1}

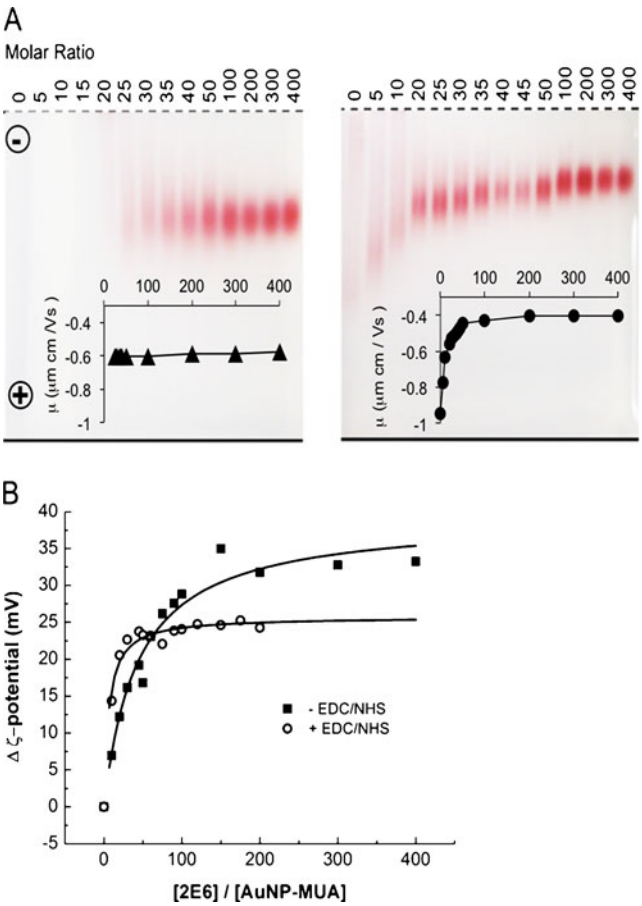

Fig. 2 Formation of the AuNP-MUA-2E6 conjugates at different antibody to AuNP-MUA molar ratios. **A** Agarose gel electrophoresis of conjugates formed in the presence (*left panel*) or in the absence (*right panel*) of EDC/NHS cross-linkers (*left panel*). The 2E6 to AuNP-MUA molar ratios are indicated in the top of each well, and the "+" and "−"signs indicate electrodes polarity. *Insets* electrophoretic mobility (μ) of the conjugates vs. 2E6 to AuNP-MUA molar ratios, calculated from observed band migrations. **B** Variation of the value of the zeta potential for AuNP-MUA-2E6 conjugates in relation to AuNP-MUA alone (Δζ-potential), as a function of 2E6 to AuNP-MUA molar ratio. *Black squares* and *empty circles* denote conjugates prepared in the absence or presence of EDC/NHS cross-linkers, respectively. *Solid lines* depict fittings to a Langmuir-type adsorption isotherm (Eq. 2)

at a AuNP-MUA molar ratio of ca. 100, stabilizing at that value for higher molar ratios. These results suggest that EDC/NHS cross-linking forms more compact AuNP-antibody conjugates with lower amounts of conjugated antibodies than their non-cross-linked counterparts.

Figure 2b presents the variation of the zeta potential, in relation to AuNP-MUA alone, for AuNP-MUA-2E6 conjugates formed with different 2E6 to AuNP-MUA molar ratios, both in the presence and in the absence of EDC/NHS cross-linking agents. The binding constant for both types of conjugates could be determined by fitting a Langmuir-type curve to the experimental data [26] (Eq. 2).

$$\Delta\zeta = \frac{\Delta\zeta_{\max} K_L R}{1 + K_L R} \tag{2}$$

in which Δζ is the variation in the zeta potential measured for each 2E6 to AuNP-MUA molar ratio in relation to the zeta potential value for AuNP-MUA alone; $\Delta\zeta_{max}$ is the maximum value for Δζ-potential as [2E6]/[AuNP-MUA] increases and K_L is a binding constant corresponding to the value of the inverse of the concentrations ratio, for one-half of $\Delta\zeta_{max}$. The binding constant for the formation of the AuNP-MUA-2E6 conjugates was determined to be K_L=0.161 in the presence of the EDC/NHS cross-linking agents, and K_L=0.023 for the conjugates formed by simple incubation. Also, a larger variation on the zeta potential value can be observed for the conjugates formed by simple incubation (ca. 35 mV) in comparison with ca. 25 mV for the conjugates formed in the presence of EDC/NHS cross-linking agents. This observation indicates adsorption of a ca. 30% larger amount of antibody to the AuNP-antibody conjugates formed by electrostatic and van der Waals forces, in relation to the ones where the antibody and the AuNP-MUA are covalently bound.

Taken together, the agarose gel electrophoresis and zeta potential results indicate that chemical cross-linking with EDC/NHS affords AuNP-MUA-2E6 conjugates with an antibody corona containing a smaller number of antibodies, in comparison with conjugates formed in the absence of those cross-linking chemicals. The activity of both types of conjugates on the immunoassay is evaluated in "Competitive immunoassay experiments" section.

Hsp70 antigen/2E6 monoclonal antibody pair for *Plasmodium* detection

Western blot analyses were performed to investigate whether *Pf*Hsp70 can be recognized by the monoclonal antibody 2E6, and used as a diagnostic target for malaria in blood samples. Western blot analysis of whole blood samples revealed several bands, possibly resulting from cross-reactivity of the antibody with blood antigens, with an additional band in the *P. falciparum*-infected blood,

corresponding to *Pf*Hsp70 (data not shown). However, analysis of the pellet fraction of saponin-treated isolated RBCs showed a single band in the infected samples, which was absent from the non-infected saponin-treated isolated RBC samples (Fig. S2, Electronic Supplementary Material). An intense red ~70 kDa band was observed in saponin pellets of the infected, but not of the non-infected RBC, in blots overlaid with AuNP-MUA-2E6 conjugates (Figure S2A, Electronic Supplementary Material). A band of the same size was identified by immunoblotting using 2E6 as primary antibody and a HRP-labeled secondary antibody (Figure S2B, Electronic Supplementary Material). These data show that the 2E6 monoclonal antibody can be used to specifically detect *P. falciparum* Hsp70 protein in infected blood samples. It was also possible to directly identify that antigen using AuNP-MUA-2E6 conjugates, avoiding the need for secondary antibody detection. Purified *Pf*Hsp70 antigen was used as positive control in these assays confirming the detection of this protein in the infected blood samples. Additional, less intense, bands identified following immunoblotting of the purified *Pf*Hsp70 protein with the 2E6 antibody (right lane in Fig. S2B, Electronic Supplementary Material) can be attributed to degradation of the purified *Pf*Hsp70 protein. Nevertheless, results clearly show that *Pf*Hsp70 can be specifically detected by the AuNP-MUA-2E6 conjugates in saponin extracts of infected RBC samples, supporting the utilization of *Pf*Hsp70 as an antigen-target in a AuNP-based immunoassay for malaria diagnosis.

Competitive immunoassay experiments

According to the detection principle of the proposed assay, fluorescence intensity increases in the presence of the analyte (*Pf*Hsp70 antigen) relative to a measurement without antigen (Fig. 1). The fluorescence intensity enhancement with increasing analyte concentration can therefore be expressed as a relative fluorescence increase (Eq. 3):

$$\mathrm{Fl} = \left(1 - \frac{\mathrm{PL(control)}}{\mathrm{PL(analyte)}}\right) \times 100 \qquad (3)$$

where PL(analyte) and PL(control) are the peak values of the photoluminescence intensities at 574 nm (the Cy3B emission maximum) of the samples containing *Pf*Hsp70 and the blank without *Pf*Hsp70, respectively.

Linear correlations between the concentration of recombinant *Pf*Hsp70 and the relative fluorescence increase for assays conducted with AuNP-MUA-2E6 conjugates formed both in the presence and in the absence of EDC/NHS cross-linking agents were observed (Fig. 3).

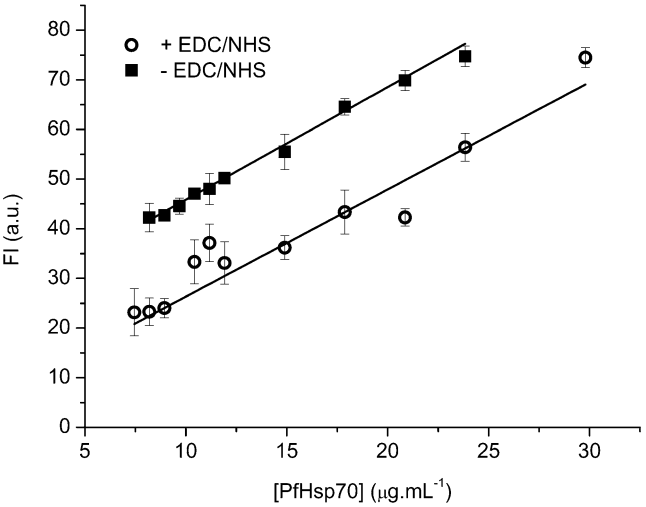

Fig. 3 Relative fluorescence increase as a function of *Pf*Hsp70 concentration. The assay employed AuNP-MUA-2E6 conjugates obtained in the absence (*black squares*, − *EDC/NHS*) and in the presence (*empty circles*, + *EDC/NHS*) of EDC/NHS cross-linking agents. *Solid lines* represent linear regression fits to the experimental data. *Error bars* represent the standard deviations from the average result of three independent measurements

As concluded from agarose gels electrophoresis and zeta potential measurements, AuNP-MUA-2E6 conjugates prepared in the presence of EDC/NHS cross-linking agents present an antibody corona containing a smaller number of antibodies per AuNP in the active conjugate than AuNP-MUA-2E6 conjugates prepared by electrostatic adsorption. However, in the fluorescent immunoassay, a greater dispersion of experimental fluorescence intensity values was observed in the case of the cross-linked conjugates as evidenced by the *R*-squared value for the linear regression of the experimental points (Fig. 3 0.917 in the case of the cross-linked conjugates, and 0.991 in the case of the non-crossed-linked conjugates). Also, the standard deviation (SD) of the assay as determined by replicate measurements of blank points for AuNP-antibody conjugates formed with and without cross-linking agents, revealed a value in the case of AuNP-MUA-2E6 conjugates formed in the presence of cross-linking agents (SD=9.94) that is almost sevenfold larger than the value for AuNP-MUA-2E6 conjugates formed by simple incubation (SD=1.52). Taken together, the data obtained for the two types of AuNP-antibody conjugates led us to conclude that the utilization of the cross-linking agents EDC/NHS in the formation of AuNP-antibody is unnecessary, and simple incubation of the nanoparticles with the antibody generates conjugates that allow better sensitivity in the proposed immunoassay for *Pf*Hsp70 antigen detection.

In the case where AuNP-MUA-2E6 conjugates were formed by simple incubation, a linear response could be obtained in the 8.2 to 23.8 μg mL^{-1} antigen concentration

range (black squares in Fig. 3). For this assay, limits of detection (LOD=3.3×SD/*m*, where SD is the standard deviation of the *yy* intercept and *m* is the slope, for the calibration curve) and limits of quantitation (LOQ=10×SD/*m*) [29] were calculated at 2.4 and 7.3 μg mL^{-1}, respectively. The intra-assay coefficient of variation (intra-assay CV (%)=(mean of the SD of ten duplicated samples/grand mean of the duplicates)×100) was further calculated to be 4.7%, while the inter-assay coefficient of variation (inter-assay CV (%)=SD of the mean of ten duplicates measured in different days/grand mean of the duplicates)×100 was estimated at 6.3%. These parameters indicate that the reproducibility of the proposed assay is within the acceptable range for an immunoassay [30].

Detection of *Pf*Hsp70 antigen in *P. falciparum*-infected human blood

The fluorescence immunoassay was applied for the detection of free *Pf*Hsp70 antigen in saponin-treated isolated RBCs from the blood of a non-infected, human donor and from *P. falciparum*-infected human blood cultures at a 3% level of parasitemia. Assuming a single parasite infection per RBC, parasite density in the sample corresponds to ca. 1.7×10^5 parasites μL^{-1}. Saponin extracts from RBCs of infected blood were tested in the immunoassay at several dilutions. Experimental conditions were as used for the purified recombinant *Pf*Hsp70, and the AuNP-MUA-2E6 was prepared by simple incubation of the components in the absence of cross-linking agents. The increase in fluorescence observed for different dilutions of the saponin extracts from RBCs, varied linearly with the dilution factor, for dilutions up to 160-fold. Figure 4 represents the increase in fluorescence observed for those samples, in function of the number of parasites present in each sample. The background signal that could be detected in the sample obtained from saponin-treated isolated RBCs from non-infected blood was ca. 30 in the fluorescence increase scale, a value equivalent to that obtained for a 160-fold diluted infected sample (FI=30.4). Using the next dilution available (80-fold, corresponding to a parasite density of ca. 2,000 parasites μL^{-1}), and the corresponding value of 56.9 in the fluorescence increase scale and introducing that fluorescence value in the calibration curve presented in Fig. 3 (corresponding to AuNP-antibody conjugates without EDC/NHS), a value of 14.9 μg mL^{-1} of *Pf*Hsp70 in the saponin extract of the infected blood culture was obtained.

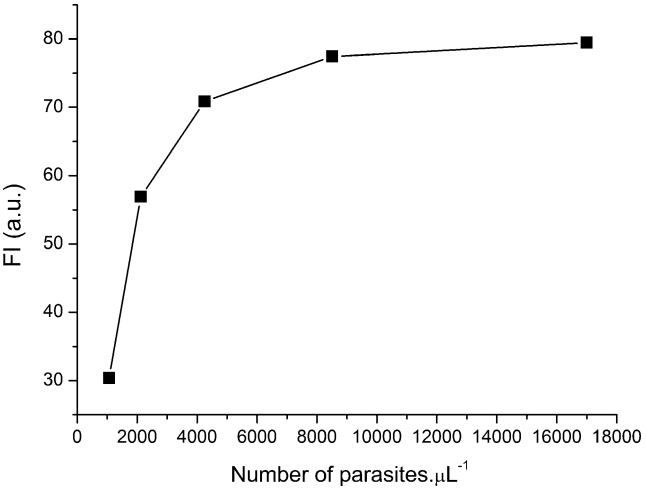

Fig. 4 Detection of *Pf*Hsp70 in a *P. falciparum*-infected blood culture. Sequential dilutions of a RBC saponin extract from a human blood culture presenting a 3% parasitemia level (corresponding to ca. 1.7×10^5 parasites μL^{-1}), were assayed. Each point averages three consecutive measurements

Conclusions

Here, we describe a simple and sensitive immunoassay for malaria diagnosis in blood samples. The assay in a homogeneous format is based on the fluorescence increase of a fluorescently labeled Hsp70 antigen from *P. falciparum* upon competition with its native counterpart, for binding to an AuNP-conjugated anti-Hsp70 monoclonal antibody. The proposed fluorescence competitive immunoassay allows the utilization of only one antibody in the assay format, making it easier to implement than non-competitive fluorescence assays based on antibody pairs [10]. Results from Western blots of saponin extracts of malaria-infected blood samples clearly showed that *Pf*Hsp70 can be specifically detected by the AuNP-MUA-2E6 conjugates, validating the proposed utilization of *Pf*Hsp70 as an antigen-target in a AuNP-based immunoassay for malaria diagnosis. Careful analysis of the formation of the AuNP-antibody conjugates indicated that although EDC/NHS cross-linking forms conjugates with an antibody corona containing a smaller number of antibodies, than their non-cross-linked counterparts, the latter have a better performance on the proposed immunoassay. The reasons for this difference might be related to loss of antibody activity imparted by the EDC/NHS cross-linking strategy. Another explanation for the observed activity difference between both types of conjugates, might be related to different orientations of the antibodies on the AuNP surface, leading to a more effective fluorescence quenching for the AuNP-MUA-2E6 conjugates prepared by electrostatic adsorption.

The proof-of-concept for the proposed fluorescence immunoassay was then established utilizing AuNP-MUA-2E6 conjugates formed by electrostatic conjugation, for the detection of recombinant *Pf*Hsp70 antigen. Under these conditions, a linear response could be obtained in the 8.2 to 23.8 μg mL^{-1} antigen concentration range, with a LOD of

2.4 μg mL^{-1} and a LOQ of 7.3 μg mL^{-1}. Finally, assay results for a malaria-infected blood culture sample indicate that the proposed immunoassay can be used to diagnose malaria infection for blood parasite densities between 1,000 and 2,000 parasites μL^{-1}. These values are considered by the World Health Organization to be below the mean parasite density found in many populations with endemic malaria, allowing the reliable identification of clinical malaria [31]. Further work is being developed to increase the sensitivity of the assay and its adaptation to direct detection in clinical samples.

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