Electronic Supplementary Material

Multicolor immunochromatographic strip test based on gold nanoparticles for the determination of aflatoxin B1 and fumonisins

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Characterization of spherical GNP

Red spherical GNPs were obtained through the largely employed protocol that involves the reduction of boiling HAuCl4 by means of sodium citrate [1-2].

GNPs were characterized by a nearly spherical shape (Fig S1a) and their size was calculated from TEM micrographs. Using 180 nanoparticles, a mean diameter of 25 ± nm was obtained, with an acceptable distribution of dimension around it (Fig. S1b)

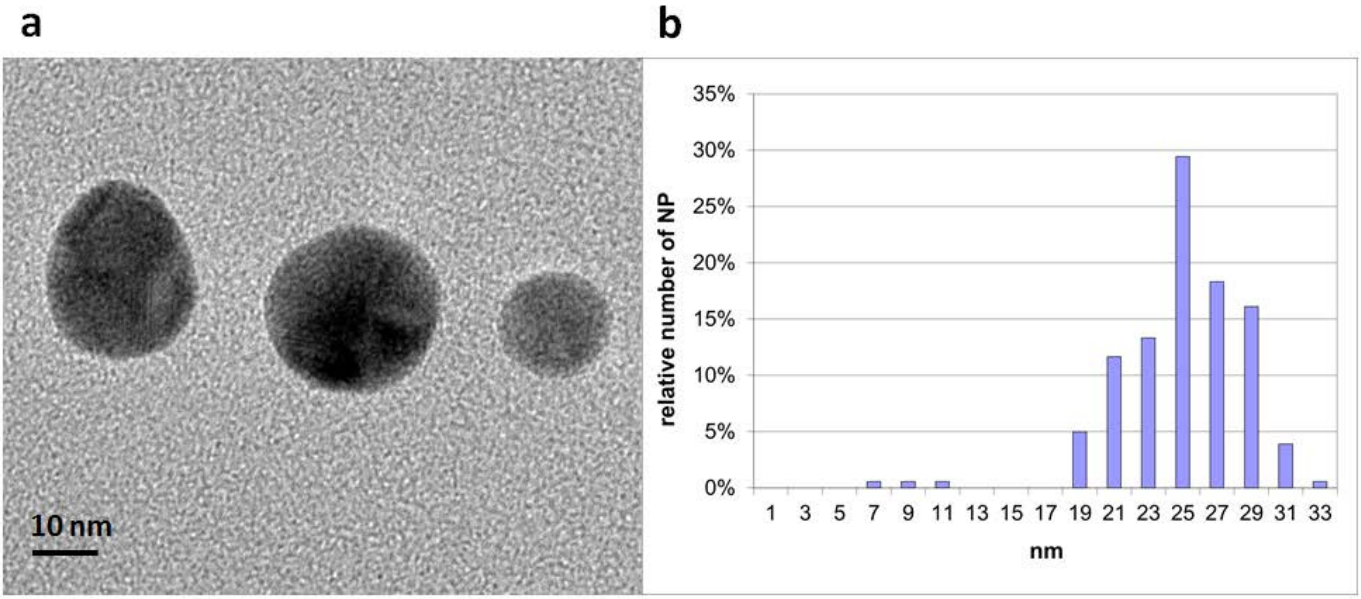

Fig. S1. (a) TEM micrograph of the red (spherical) GNPs (250000 x magnification). (b) Distribution of diameter of spherical GNPs (180 NPs)

Stabilization of DR-GNP by shielding their surface with antibodies

Otherwise to what happens for red spherical GNPs, an insufficient amount of antibodies (Ab) adsorbed on DR-GNPs did not produce a change in the SPR but (e.g. in the color of the sol) but caused the blue color of the sol turning to colorless with a gradual decrease of the OD (Fig. S2a). Figure S2b shows UV-vis spectra obtained upon mixing the DR-GNPs with increasing amount of Ab (from 0 to 10 µg added to 0.5 ml of DR-GNPs with initial optical density 0.5). The color tone varied upon the addition of largely insufficient amounts of the Ab (<4 µg) while starting from the addition of 4 µg, the λmax remained unchanged (630 nm). However, a perceivable change in the intensity of the coloring was observed, which was confirmed by increasing OD values measured for Ab up to 8 µg. No further variations were observed for the addition of larger amounts of the Ab. TEM images helped to confirmed that the colorless sol obtained for insufficient amount of antibodies adsorbed corresponded to aggregated DR-GNPs (inset figure S2b). Hence, the observed decrease in OD can be explained by the simultaneous increase of aggregates that are colorless when the GNP covering with Ab is insufficient at preventing aggregation. When the OD remains almost unchanged, the absence of aggregate fractions is quite certain and, therefore, the corresponding amount of Ab is the minimum needed for DR-GNPs stabilization. DR-GNP titer should be defined accordingly, as the minimum amount of Ab that does not cause OD variation rather than color tone variation.

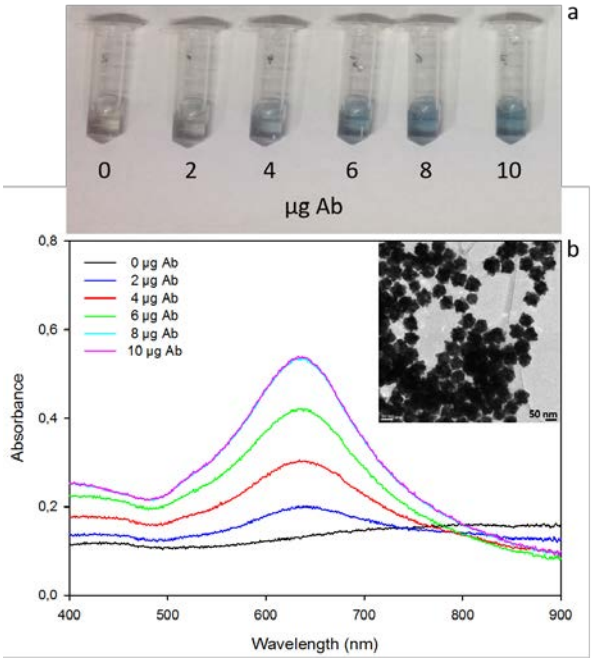

Fig. S2. (a) Visual result of the DR-GNPs titration (referred to 0.5 mL of DR-GNPs). (b) UV-vis spectra of DR-GNPs upon addition of 0 (black), 2 (blue), 4 (red), 6 (green), 8 (cyan) and 10 (pink) µg of anti-AFB1 Ab. Inset: TEM micrograph of the colorless sol obtained upon addition of 0 µg of anti-AFB1 Ab (25000 x magnification)

Optimal pH and concentrations for the conjugation of DR-GNPs to antibodies

The optimal pH for the conjugation of DR-GNPs to Ab was defined as the one providing the higher sensitivity. Therefore, the DR-GNP solution was divided into four aliquots and each was adjusted to a different pH (5, 6, 7, and 8) by adding carbonate buffer (sodium carbonate-sodium bicarbonate 50 mM pH 9.6). The pH-adjusted sol were conjugated to the same amount of Ab directed towards AFB1 (10 µg Ab per 1 mL DR-GNP) and tested by using three levels of AFB1 (0-1-10 ng mL^{-1}) diluted in phosphate buffer with 1% BSA and 0.1% Tween 20 added. Signals of Test (T) and Control (C) lines were measured to calculate the T/C ratio. The T/C ratio for each AFB1 concentration (B) was then normalized by dividing it by the T/C ratio measured for the blank (B_0). The optimal pH, which maximized the inhibition of binding of DR-GNP-Ab to the Test line (lower B/B_0 values), resulted to be 5 (fig.S3). The sensitivity was comparable at basic and neutral pH, while increased in acidic conditions.

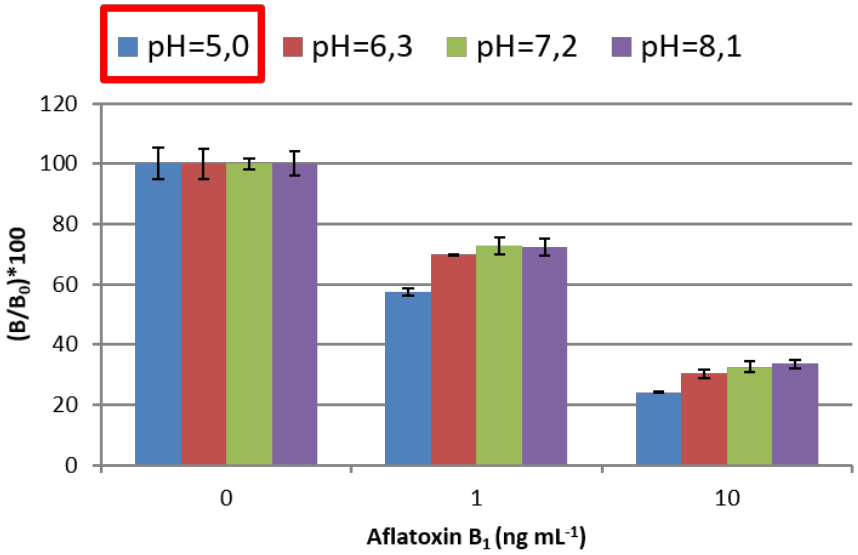

Fig. S3. Inhibition of DR-GNP-Ab binding to the Test line at four pH values. The lower the B/B_0 value, the most efficient the inhibition.

The same experiment was also conducted by varying the concentrations of DR-GNP-Ab (expressed as OD of the DR-GNP conjugates). As shown in fig. S4, a limited increase of sensitivity could be obtained by lowering the OD from 2 to 0.5. Nevertheless, this result was achieved at the expenses of the absolute signal intensity. Thus, we decided to use concentrated DR-GNP-Ab conjugates (OD= 2) for easier visualization

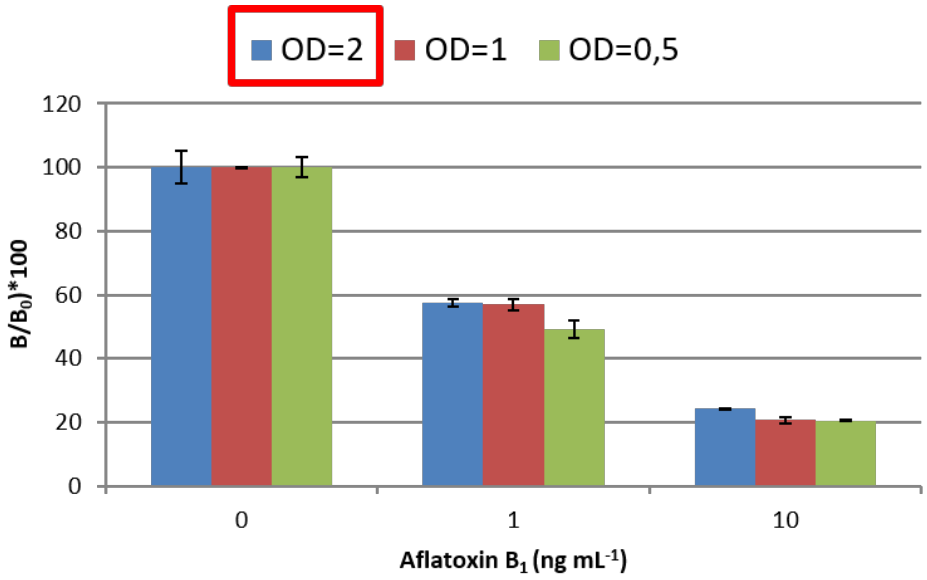

Fig. S4. Inhibition of DR-GNP-Ab binding to the Test line as a function of the concentration of the conjugates. The lower the B/B_0 value, the most efficient the inhibition.

Comparison of the sensitivity obtained by using DR- and s-GNPs conjugated to antibodies at varying pH and tested at different ODs

The evaluations of optimum pH and ODs were performed also for s-GNPs in order to compare the two GNP preparations.

Experiments were carried out by exploiting DR- and s-GNP conjugated at pH 5-6-7-8 and tested by varying their amount (ODs). Fig S5 shows the comparison of the inhibition of DR- and s-GNP binding to the Test line obtained by increasing the amount of AFB1 for conjugates prepared at pH 5. In summary, DR- and s-GNPs showed very similar behaviors. Some advantages due to the use of DR-GNPs were observed only for diluted GNP conjugates, where the sensitivity slightly increased. Similar trends were observed when conjugation was carried out at pH 6-7-8 (data not shown).

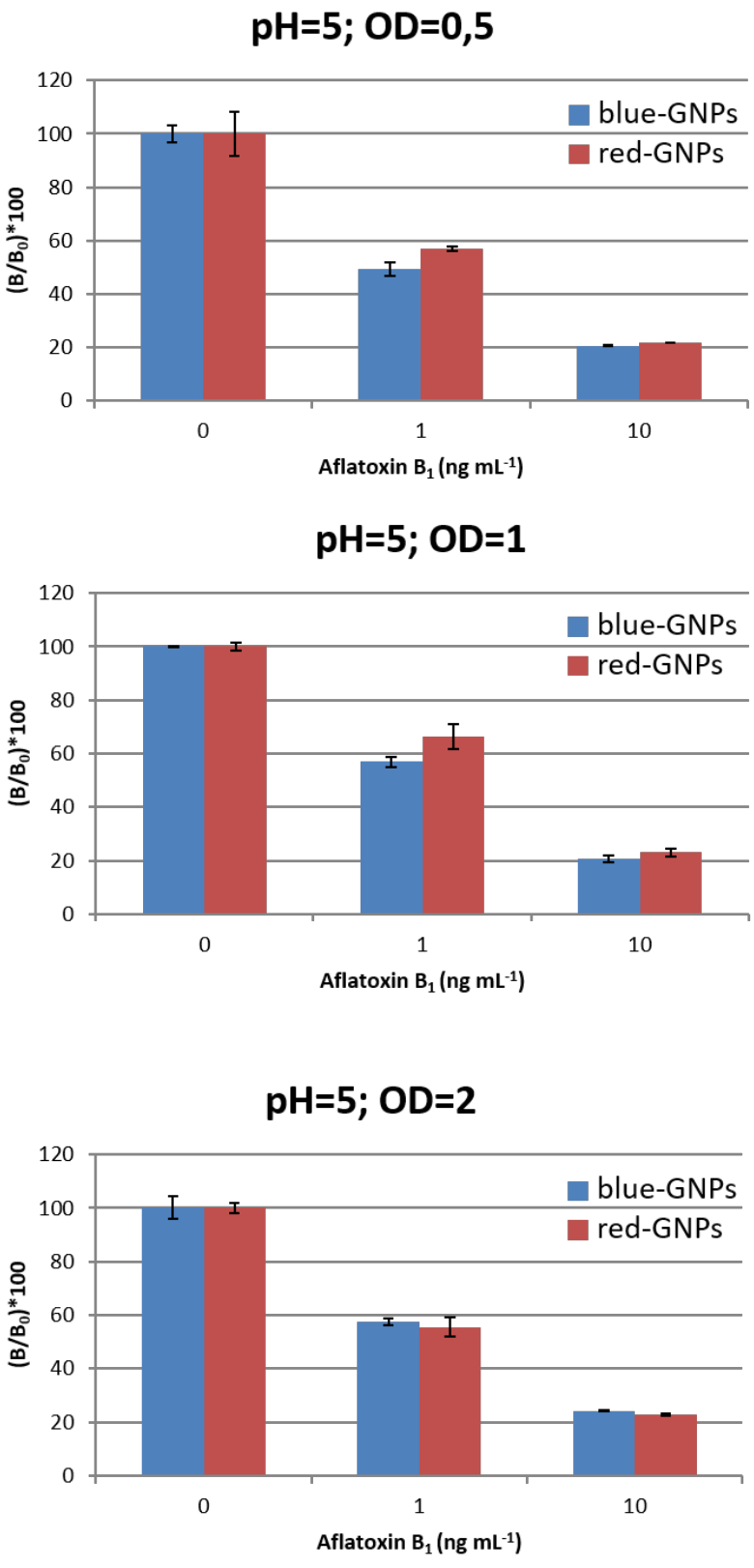

Fig. S5. Comparison of the inhibition of binding to Test line for DR- and s-GNPs conjugates prepared at pH 5 and tested at different OD values.

Definition of the visual LOD (vLOD) for AFB1 and FMB1 detection in maize extract

The vLODs in maize extract were obtained by analyzing samples fortified with the two mycotoxins separately at four levels: 0-0.2-0.4-1 ng mL^{-1} for AFB1 (fig. S6) and 0-50-100-200 ng mL^{-1} for FMB1 (fig. S7). Strips were visually observed by three subjects. All of them reported a T-line signal significantly weaker than that of the T-line of a negative sample when AFB1 level was at 0.4 ng mL^{-1} and the FMB1 level was at 200 ng mL^{-1}, which corresponded to 2 µg kg^{-1} and 1000 µg kg^{-1} in the maize sample, respectively.

Fig. S6. Determination of the vLOD in maize extract for AFB1. The strip corresponding to the vLOD is highlighted in blue

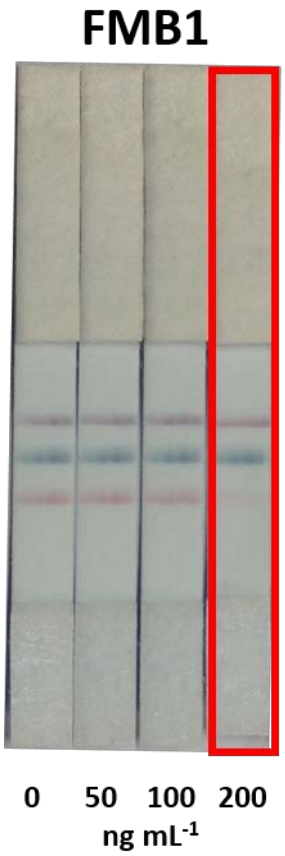

Fig. S7. Determination of the vLOD in maize extract for FMB1. The strip corresponding to the vLOD is highlighted in red

Determination of aflatoxin B1 and Fumonisins in naturally contaminated maize

Eighteen naturally contaminated maize flour samples were tested with the multicolor ICST. Each sample was extracted and checked in triplicate and the visual observation of results was done by three operators.

The judgment of results was based on color intensities of Test lines compared to those of a blank sample used as the reference. Samples were considered as positives for a particular mycotoxin if the corresponding coloring of the Test line was weaker than that of the reference, according to the cut-off values established as above described.

The results obtained through the multicolor ICST were compared with the total fumonisins concentration (FMs) intended as the sum of fumonisin B1 and fumonisin B2 and obtained by a reference LC-MS/MS method [3] and with the AFB1 content assessed by a commercial ELISA kit. The polyclonal antibodies directed towards FMB1 used to set the multicolor ICST had showed cross-reactivity for fumonisin B2 of 97 % [3]. Therefore, the assay would provide information about total fumonisins content, as requested by the European Union [4].

Table S1. Results for the analysis of naturally contaminated maize flour samples. Qualitative evaluation was performed by 3 operators in three replicates that were always in agreement between them.

Fumonisins (B1+B2)		**Aflatoxin B1**	
LC-MS/MS (µg kg^{-1}) [a]	multicolor-ICST [b]	ELISA (µg kg^{-1})	multicolor-ICST [b]
< LOD	– – –	< LOD	– – –
< LOD	– – –	< LOD	– – –
< LOD	– – –	< LOD	– – –
< LOD	– – –	< LOD	– – –
< LOD	– – –	< LOD	– – –
< LOD	– – –	< LOD	– – –
295	– – –	< LOD	– – –
980	– + +	< LOD	– – –
2430	+ + +	< LOD	– – –
3550	+ + +	< LOD	– – –
4290	+ + +	< LOD	– – –
4480	+ + +	< LOD	– – –
< LOD	– – –	5.1	+ + +
< LOD	– – –	10	+ + +
< LOD	– – –	11.4	+ + +
< LOD	– – –	14	+ + +
< LOD	– – –	16	+ + +
< LOD	– – –	16,8	+ + +

[a] Data from [3].

[b] (–) negative, sample T-line intensity ≥ T-line intensity of non-contaminated sample; (+) positive, sample T line intensity < T-line intensity of non-contaminated sample.

Experimental

Immunoreagents, chemicals and materials

Gold (III) chloride trihydrate (ACS reagent), Aflatoxin B1 (AFB1) and Fumonisin B1 (FMB1) Oekanal standard solutions, bovine serum albumin (BSA), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydro-chloride (EDC) and hydroquinone were obtained from Sigma–Aldrich (St. Louis, MO, USA https://www.sigmaaldrich.com). FMB1 and AFB1 powder were purchased from Fermentek (Jerusalem, Israel, http://www.fermentek.com/). Tween 20 and other chemicals were purchased from VWR International (Milan, Italy https://it.vwr.com/store/).

The anti-FMB1 antibody (rabbit polyclonal antiserum raised against FMB1, relative cross-reactivity towards fumonisin B2, FMB2, 97%) and the anti-AFB1 antibody (rabbit polyclonal antiserum rose against AFB1, relative cross-reactivity towards AFB2 11%, AFG1 32%, and AFG2 6%) were kindly supplied by EuroClone Spa (Milano, Italy, http://www.euroclonegroup.it/). The γ-globulin fraction was isolated by ammonium sulphate precipitation and used without any additional treatments. The goat anti-rabbit immunoglobulin antibody was purchased from AbCam (Cambridge, UK).

Millipore High Flow (HF) 180, absorbent cellulose pad and glass fiber conjugate pad were obtained from Merck Millipore (Billerica, MA, USA, http://www.merckmillipore.com/IT/it).

Preparation of the FMB1-BSA conjugate

The FMB1–BSA conjugate was synthesized as previously described [5]. Briefly, 6.5 mg of FMB1 dissolved in 3 mL of MES-NaCl buffer (MES 0.1 M, NaCl 0.9 M at pH 4.7) were mixed with 3 mg of BSA dissolved in 0.6 mL of water. 0.7 mL of a freshly prepared water solution of EDC (10 mg mL^{-1}) was added drop wise to the mixture and reacted for 2h at room temperature. By-products were removed by gel-filtration on a Sephadex G-25 cartridge (GE Healthcare Bio-Science, Sweden, http://www3.gehealthcare.com/en/global_gateway), by using phosphate buffer (sodium hydrogen phosphate-sodium dihydrogen phosphate 20 mM, pH 7.4) with 0.1 M NaCl added as the eluent. The concentration of the FMB1-BSA conjugate was determined by UV absorption at 280 nm.

Preparation of the AFB1-BSA conjugate

The AFB1-BSA conjugate was synthesized as previously reported [5], employing the N-hydroxysuccinimide ester method. Briefly, 5.7 mg of the AFB1-oxime hapten were activated with equimolar amounts of N-hydroxysuccinimide and N,N'-diisopropylcarbodiimide (1:1:1) in anhydrous N,N-dimethylformamide for 2 hours (4°C) and then reacted overnight at room

temperature with 5 mg of BSA dissolved in sodium bicarbonate 0.15 M at pH 8.3 and the pure conjugate was obtained from gel-filtration, as described above. AFB1-BSA concentration was determined through the Brilliant Blue Coomassie method. Conjugates were supplemented with 0.1% sodium azide and stored refrigerated.

Preparation of s-GNPs and GNPs seeds

The most common approach to synthesize s-GNPs in aqueous solutions through the tetrachloroauric acid reduction by means of sodium citrate was reported for the first time by Hauser and Lynn [6] and subsequently improved by Turkevich [1] and Frens [2]. S-GNPs with a SPR band at 525 nm and mean diameter of 30 nm were prepared as previously described [32]. Briefly, tetrachloroauric acid was dissolved in 100 mL of deionized water (0.01%, w/v) and the obtained solution was brought to boil. Then, 1 mL of 1% w/v sodium citrate aqueous solution was added to the boiling solution while stirring vigorously. The color of the solution changed gradually from light yellow to wine red thus confirming the successful reduction.

GNPs seeds with a SPR band at 518 nm and a mean diameter of 11 nm were synthesized according to the aforementioned approach, using a double amount of the reducing agent. The final color of the solution changed up to orange-red thus indicated the obtainment of smaller s-GNPs.

The sol was adjusted to the desired pH with carbonate buffer.

GNPs characterization

UV-vis measurements were carried out using a Varian Cary1E spectrophotometer (Agilent technologies, Santa Clara, CA, http://www.agilent.com/en-us/), employing a 1 cm path length quartz cuvette.

Electron micrographs were obtained using a Jeol 3010-UHR (Jeol Ltd, Japan, http://www.jeol.co.jp/en/) high resolution transmission electron microscope (HRTEM) equipped with a LaB_6 filament operating at 300 kV and with an Oxford Inca Energy TEM 300 X-ray EDS analyzer. Samples for HRTEM were prepared putting a drop of the sol on a copper grid covered with a lacey carbon film.

DLS measurements were carried out using a Delsa Nano™ C Analyzer (Beckman Coulter, Milano, Italy, https://www.beckmancoulter.com) equipped with a 638 nm diode laser and a temperature control. The viscosity of the sample was assumed to be the viscosity of the dispersant (water). Measurements were performed at 25°C using a sample volume of 2 mL. The sample was measured in quintuple and the mode of the distribution was reported.

Titration of GNP for conjugation with antibodies

The optimum concentration of the polyclonal antibodies for conjugation to s-GNPs was determined according to Horisberger [7]. Briefly, increasing amounts of a 0.1 mg mL^{-1} Ab solution (0–50 µL, phosphate buffer 20 mM pH 7.4) were added to 0.5 mL of s-GNPs. After 30 min of incubation at 37 °C, 100 µL of a concentrated NaCl solution (10% w/v) were added and the color of the obtained mixture was observed after 10 min. High salt concentrations induce GNPs flocculation when an insufficient amount of antibodies has been adsorbed on the surface of the GNPs themselves. The aggregation can be easily detected because the red color of the sol turns to purple-blue. The amount of antibodies needed to stabilize GNPs was visually established as the minimum quantity that not causes the shift of the GNPs color.

The optimum concentration of the polyclonal antibodies for conjugation to DR-GNPs was determined as described above, but using a fixed amount (10 µL) of increasing concentration of the Ab diluted in phosphate buffer (0-0.1-0.2-0.3-0.4-0.5-0.6-0-7-0.8-0.9-1 mg ml^{-1}). The DR-GNPs flocculation was evaluated both visually and spectrophotometrically by recording Vis spectra in the range 400-900 nm.

Preparation of test strips

Strips for multiplex analysis were prepared from nitrocellulose membranes (Hi-flow plus 180) employing an XYZ3050 platform (Biodot, Irvine, CA, USA, https://www.biodot.com/), equipped with three BioJet Quanti™ 3000 Line Dispenser for non-contact dispensing. In particular, from bottom to top of the strip, the FMB1-BSA conjugate (0.3 mg mL^{-1}), the AFB1-BSA conjugate (0.2 mg mL^{-1}) and the goat anti-rabbit immunoglobulin antibody (1 mg mL^{-1}) diluted in buffer A were dispensed to form the T1-, T2- and the C- lines, respectively. Reagents were deposited at a flow rate of 1 µL cm^{-1}, keeping a distance of 3 mm between the lines. GNPs-labeled antibodies at different optical density (OD) were dispensed onto the conjugate pad at a flow rate of 8 µL cm^{-1} and dried at room temperature for at least 2 hours. The conjugate pad was previously treated with buffer G and dried at 60°C for 60 minutes. Membranes were dried at 37°C for 60 minutes under vacuum and then assembled with conjugate and absorbent pads, with 1-2 mm of overlap between one and the other. In the adopted strip configuration, we did not use any additional sample pads. Assembled membranes were cut into strips (5 mm width) by means of a CM4000 guillotine (Biodot, Irvine, CA, https://www.biodot.com/) and stored in plastic bags containing silica at room temperature until use.

Strips for s-GNPs and DR-GNPs comparison were prepared as previously mentioned, dispensing only the T2-line (AFB1-BSA 0.2 mg mL^{-1}) and the C-line.

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