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Laura Anfossi[1] & Fabio Di Nardo[1] & Alida Russo[2] & Simone Cavalera[1] & Cristina Giovannoli[1] & Giulia Spano[1] & Sabine Baumgartner[3] & Kathrin Lauter[3] & Claudio Baggiani[1]

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Abstract

In this study, we report the simultaneous use of gold and silver nanoparticles to set a multicolor multiplex lateral flow immunoassay (xLFIA). Silver nanoparticles (AgNPs), spherical in shape and characterized by a brilliant yellow color, were obtained by a new viable one-step synthetic protocol. AgNPs were stable over time and acceptably robust to conditions used for fabricating LFIA devices. These AgNPs were employed as a colorimetric probe in combination with two different kinds of gold nanoparticles (AuNPs) to set a visual xLFIA for detecting allergens. Surface plasmon resonance peaks of probes (AgNPs, spherical and desert rose-like AuNPs) were centered at 420, 525, and 620 nm, respectively. Therefore, the xLFIA output was easily interpreted through a Byellow magenta cyan (YMC)^ color code. The prospect of the YMC xLFIA was demonstrated by simultaneously detecting three major allergens in bakery products. Antibodies directed towards casein, ovalbumin, and hazelnut allergenic proteins were individually adsorbed onto metal nanoparticles to produce three differently colored specific probes. These were inserted in a LFIA comprising three lines, each responsive for one allergen. The trichromatic xLFIAwas able to detect allergenic proteins at levels as low as 0.1 mg/l and enabled the easy identification of the allergens in commercial biscuits based on the color of the probes.

Keywords Immunochromatographic strip test . Multiplex detection . Ovalbumin . Casein . Hazelnut . Colorimetry

Introduction

Simultaneous on-site measurement of different substances from a single sample, called multiplexed point-of-need testing, is an emerging issue to achieve efficient and highthroughput detection. The lateral flow assay (LFA) has wellestablished and prevailing advantages over alternative pointof-need analytical platforms; however, one of the incoming challenges for the LFA technology is exactly multiplexing [1]. In order to answer to the pressing demand for multiplexing LFA, several approaches have been exploited. In one sense, antibodies or other recognition materials that are class-specific to contaminants have been developed to produce classselective methods [2]. However, multiplexed LFA (xLFA) is realized primarily through the following approaches: (i) combining several strips (each targeting an individual analyte) in a single cassette, (ii) spatially separating the detection sites on one strip, and (iii) using signal reporters that provide distinguishable signals [3]. The first approach has major limitations, such as being expensive (the material expenditure for the fabrication of the device increases proportionally with the number of analytes being detected) and requiring large sample volumes. Therefore, the second strategy has been exploited more frequently [4–6]. In this case, the LFA strip comprises several successive zones each containing a different capturing reagent with specificity towards a different target. Usually, all capturing reagents bind to probes fabricated by using the same signal reporter. The signal generated is localized in different zones of the strip that, therefore, respond to the presence of different target compounds. This approach decreases expenditure on assay materials and sample volumes required.

However, the number of capturing reagents that can be arranged in a single strip is limited. In addition, interpreting the qualitative results of a multi-zone lateral flow assay is not as simple as that of a conventional single-parameter assay [7]. This difficulty can be overcome by using differently colored probes. For the purpose, the unique luminescence property of nanomaterials like quantum dots is especially advantageous. Quantum dots are able to emit light at different wavelengths while excited by a single source. Thus, they are preferred signal reporters for the implementing of LFA multiplexing, in which each target compound is revealed by the light emitted at a different wavelength [7–10]. However, fluorescence nanoparticles require an external device for promoting excitation. Colored nanoparticles that enable equipment-free detection are strongly preferable for point-ofneed testing. Equipment-free multicolor xLFAs have been reported, based on the use of colored latex beads [2] and noble metal nanoparticles [11–13]. Noble metal nanoparticles are characterized by strong surface plasmon resonance (SPR) bands, which frequency depends on the size and shape of the nanoparticle, besides the nature of the metal and the composition of the surrounding medium. Therefore, Lee et al. used two kinds of gold nanoparticles that differed in shape and consequently had different SPR bands, for establishing twocolor LFIAs for discriminating the serological response to acute febrile illness [11]. A single antigen was spotted onto the membrane that was able to bind indifferently to immunoglobulins G and M, while probes were specific for just one class of immunoglobulins. In such a way, the coloring of the spot furnished double information: the appearance of whatever color meant illness, while the specific color enabled the identification of the type of the immune response. Similarly, our group exploited two gold nanoparticle probes to establish an xLFA for the simultaneous detection of two food contaminants [12]. In that work, two lines of capturing reagents were placed onto the membrane, each one responsive for a single contaminant. The use of differently colored probes allowed for the easier interpretation of the assay output. Yen et al. used three differently colored silver nanoparticle labels in an xLFIA for the simultaneous diagnosis of three infectious diseases [13]. Authors prepared orange, pink, and green AgNPs and exploited the probes both in the single line strategy (which, however, needed processing of the images to extract information on individual tests) and in separate lines.

Silver nanoparticles (AgNPs) candidate as preferential labels for colorimetric xLFA, as they can be obtained in very different shapes and color [14], thus allowing for expanding ideally the number of different probes available. However, this kind of nanoparticles has been rarely used as the probe in LFAs and has major shortcomings, such as the limited stability over time [14, 15] and the difficulty of producing monodisperse nanoparticles [16, 17].

In this study, we report the simultaneous use of gold and silver nanoparticles to set a qualitative multicolor xLFIA. Two kinds of AuNPs that differed in shape (spherical and desert rose–like AuNPs) were prepared. Their SPR bands were at 525 nm and 620 nm, respectively, which correspond to the red—magenta (M) and the blue—cyan (C) colors. AgNPs with a SPR band at ca 420 nm were also obtained through a new viable one-step seed-mediated approach. The color of the silver-based probe was yellow (Y). Therefore, the output of the xLFIA employing AgNPs and spherical and desert rose

AuNPs can be interpreted basing on an BYMC^ color code. The innovative combination of probes facilitates the visual readout also because probes were based on primary colors. To illustrate the advantages of the combined use of the three metal nanoparticles for enabling simple and equipment-free multiplexing LFA, the simultaneous detection of three allergenic proteins was used as a model system. Food allergy is a public health concern and an important food safety issue, especially in industrialized countries. The presence of undeclared allergenic ingredients or the presence of traces of allergens due to contamination during food processing poses a great health risk to susceptible individuals. Clinical symptoms of food allergies can range from minor digestive disorders and skin irritations to severe, potentially life-threatening symptoms. The only way to manage food allergies is allergen avoidance. Therefore, EU regulation enforces manufacturers declaring the presence of allergenic ingredients in food products [18].

The risks associated with the presence of hidden allergens in food have increased the need for rapid, sensitive, and reliable methods for detecting and identifying allergenic ingredients in food products. Conventionally, allergenic ingredients are identified by DNA- and protein-based methods, the first encompassing PCR and the second enzyme-linked immunosorbent assay (ELISA) methods. However, both techniques usually are performed in the laboratory and require considerable time and reagent consumption and expensive detection instruments [19, 20]. Several point-of-need testing devices for detecting allergens have made available on the market [19] and have been reported in the literature [21–27]. However, most devices are intended for the identification of a single allergen while multiplexing is realized through complicated and expensive instrumental techniques [28, 29]. In this work, we present an xLFIA enabling the simultaneous detection and identification of three largely employed and risky allergenic ingredients [30], based on the combined use of three metal nanoparticles as colored probes. At the best of our knowledge, this is the first LFIA that employs gold and silver nanoparticles simultaneously. Their combination to set a multi- chromatic xLFIA enables expanding the multiplexing capability of the technique, while retaining most advantages of traditional visual LFIA, such as equipment-free detection (by using colored probes), simplicity of result decoding also by untrained end-users (one color/ one target), cost-effectiveness (basing on well-established protocols and cheap materials), and sensitivity.

Materials and methods

Immunoreagents, chemicals, and materials

Milk casein (CAS), bovine serum albumin (BSA), egg chicken albumin (OVA), antiOVA antibody (rabbit polyclonal immunoglobulin obtained by ammonium sulfate precipitation), antirabbit immunoglobulins, and antimouse immunoglobulins goat polyclonal antibodies were obtained from Sigma–Aldrich (St. Louis,MO, USA). A pair of monoclonal antibodies directed towards hazelnut proteins (antiHNP) was kindly provided by prof. Sabine Baumgartner (Boku University, Austria). The antiCAS antibody (rabbit polyclonal immunoglobulin obtained by ammonium sulfate precipitation) was kindly supplied by EuroClone Spa (Milano, Italy). Other chemicals were purchased from VWR International (Milan, Italy).

Millipore High Flow (HF) 180, absorbent cellulose pad,

and glass fiber conjugate pad were obtained from Merck Millipore (Billerica, MA, USA). Whatman Standard 14 pad was obtained from Whatman International Ltd. (Maidstone, England). The LFIA cassettes were furnished by Kinbio (China).

Metal nanoparticles

Magenta gold nanoparticles with a SPR band at about 525 nm (M-AuNPs) were obtained from tetrachloauric acid by the citrate reduction method, as described in [12]. A typical protocol involves adding 1 mL of 1% sodium citrate to 100 mL of boiling 1% tetrachloauric acid under vigorous stirring. After the color turned to ruby red (indicating successful formation of AuNPs), the solution was cooled down to room temperature and stored at 4 °C for subsequent conjugation to biomolecules.
Cyan gold nanoparticles (C-AuNPs) were obtained by a seeding growth approach [12]. Briefly, AuNP seeds (mean diameter ca 10 nm) were prepared as described above for MAuNPs except for the volume of sodium citrate that was 2 ml.
0.1 ml of AuNP seeds (optical density 1) were added to 10 ml of a mixture that contained tetrachloauric acid (20 nmol) and sodium citrate (7.5 nmol) and stirred for 2 min in order to homogenize. Then, 1.5 ml of hydroquinone (30 mM) was quickly added to the solution. The solution was kept under stirring for further 20 min. The obtained colloid exhibits a blue color, which corresponded to a SPR band centered at ca 620.
Silver nanoparticles with a SPR band at ca 420 nm (YAgNPs) were synthesized based on a one-step seed-mediated growth method inspired by the protocol described in [31]. A small amount of $NaBH_4$ was used as the reducing agent to form seeds while excess of hydroquinonewas used to promote the growth of seeds. In details, 0.1 ml of $NaBH_4$ (0.2mM) was quickly added to 20 mL of pre-mixed silver nitrate (0.2 mM) and sodium citrate (0.2 mM) under vigorous stirring. After waiting 3 min for allowing seeds formation (the solution turned light yellow), 0.4 ml of hydroquinone (0.05mM, freshly prepared in MilliQ water) was added quickly and kept for further 10 min under stirring. The mixture turned red-orange. It was aged at room temperature without stirring for further 2 h during which the colloid turned brilliant yellow.
Metal NPs were characterized by recording their UV-vis spectra on a Varian Cary 1E spectrophotometer (Agilent Technologies, USA) and by transmission electron microscopy using a Jeol 3010-UHR (Jeol Ltd., Japan) high-resolution transmission electron microscope (HR-TEM) equipped with a LaB6 filament operating at 300 kVand with an Oxford Inca Energy TEM 300 X-ray EDS analyzer. For TEM imaging, a drop of the metal NP aqueous suspension was put on a copper grid covered with a lacey carbon film for the analysis.

Preparation of NP-Ab probes

The pH of the AuNP solutions was adjusted to 8 (M-AuNPs) and 6 (C-AuNPs) by means of sodium carbonate-sodium bicarbonate buffer (50 mM, pH 9.6) prior to conjugation with antibodies. Y-AgNPs showed a pH value of 5.4 and were used without adjusting pH.
Labelling of antibodies with metal NPs was achieved through passive adsorption of proteins onto NP surface. Gold NPs were conjugated to antibodies as reported in [12]. Labelling of antibodies with Y-AgNPs followed the same approach, with minor modification. Briefly, the minimum amount of antibodies required for inhibiting AgNP aggregation (upon addition of 1% NaCl) was established according to

Horisberg and Rosset [32]. This quantity of antibody was added to the Y-AgNP solution (supplemented with 10% v/v of borate buffer 20 mM, pH 8.0) and incubated for 30 min at 37 °C. Then, 0.1% (w/v) of BSA was added to saturate YAgNPs unbound to antibodies (10 min at 37 °C) and the YAgNP-Ab probe was recovered by centrifugation (14,000 rpm for 10 min).

Fabrication of the xLFIA device

The configuration of the color-encoded multiplex strip is shown in Fig. 1a. Strips were prepared from nitrocellulose membranes (Hi-flow plus 180) employing an XYZ3050 platform (Biodot, Irvine, CA, USA) for coating capturing reagents to form three test and one control lines. The antiCAS (0.4 mg ml^{-1}), antiOVA (0.5 mg ml^{-1}), and antiHNP (1.0 mg ml^{-1}) antibodies formed the T_{CAS}, T_{OVA}, and T_{HNP} test lines, respectively. A mixture of antirabbit (0.25 mg ml^{-1}) and antimouse (0.4 mg ml^{-1}) immunoglobulin antibodies was used to form the control line. All reagents were lined at 1 μL cm^{-1}, keeping a distance of 3 mm between the lines. As far as the HNP system, one available monoclonal antibody was lined onto the membrane, while the second was labelled with the M-AuNPs. For CAS and OVA systems, the polyclonal antibody was used both as the capturing antibody (to form the test line) and as the detection antibody (to prepare probes). The three antibodies, each labelled by a different metal NPs were mixed at their optimal optical density (M-AuNP OD 2; CAuNP OD 2, Y-AgNP OD 3) and applied to the conjugate pad by dipping the pad in themix and drying at roomtemperature for at least 2 h. The conjugate pad was previously treated with borate buffer (20mMpH 8.0) supplemented with 1% w/v BSA, 2% w/v sucrose, 0.25% v/v Tween 20, and 0.02% w/v sodium azide, and dried at 60 °C for 60 min. For cross-reactivity evaluation, each NP-Ab probe was applied separately to the conjugate pad. Membranes were dried at 37 °C for 60 min under vacuum and then laminated with sample, conjugate, and absorbent pads. Assembled membranes were cut into strips (4-mm width) by means of a CM4000 guillotine (Biodot, Irvine, CA), inserted in a plastic cassette (Kinbio Tech, Shanghai, China), and stored in plastic bags containing silica at room temperature until use.

Samples and sample preparation

Hazelnut flower and biscuits were purchased from local producers and from market brands. About 100 g of biscuits was ground with a cooking blender and 1 g of powder or hazelnut flower was extracted with 10 ml of 2× Tris buffer (Tris 25 mM, pH 7.0, 250 mM NaCl) [33]. After homogenization by manual shaking, the suspension was shaken for 30 min and centrifuged for 5 min at 5000 rpm. The upper fat layer was discarded and the limpid supernatant was diluted 1:10 with Tris buffer and used for the multicolor xLFIA analysis.

The multicolor xLFIA

Sample extracts or protein standards diluted in Tris buffer (25 mM, pH 7.0, 250 mM NaCl) were pipetted in the sample well of the plastic cassette in order to start the capillary migration process. After 10 min, the results were qualitatively estimated by the naked eye. Images of the cassette were also recorded by a portable scanner (OpticSlim 550 scanner, Plustek Technology GmbH, Norderstedt, Germany) and the area of the colored lines was quantified by means of the QuantiScan 3.0 software (Biosoft, Cambridge, UK).

Cross-interference among the assays for the three allergens was studied at different levels: (i) the mutual interactions of probes comprising different metal NPs and different antibodies were checked by recording TEM images of the mixed YAgNP-antiOVA, M-AuNP-antiHNP, and C-AuNP-antiCAS; (ii) the cross-reactivity was verified by applying each single probe separately to the multiplex LFIA and observing the specific binding to the corresponding test line (besides to the control line); and (iii) the reciprocal independency of assays was evaluated by applying a mixture of the three allergens in the multiplex LFIA comprising three test lines and three mixed probes. For these experiments, standard solutions of CAS (0.1 mg/l) and OVA (0.1 mg/l) diluted in Tris buffer and the raw hazelnut extract diluted 1/100 in Tris buffer were used. For the estimation of the visual limit of detection (vLOD), standard solutions of OVA and CAS at 1 g/l were prepared by dissolving the lyophilized protein in phosphate buffer Hazelnut proteins were obtained as a crude extract from a hazelnut flower sample bought on a local market. Standards of proteins and hazelnut crude extract were serially diluted with Tris buffer (base 10) and tested in three replicates by the YMC xLFIA. The color produced at each test line was quantified by means of the software Quantiscan (Biosoft, UK) from images of the strips obtained by a scanner.

Results and discussion

Metal NP probes

Gold nanoparticles with SPR bands at ca 525 and 620 nm, resulting in magenta and cyan colors were obtained as previously described [12]. In particular, magenta gold nanoparticles (M-AuNP) with spherical shape and mean diameter at ca 30 nm were obtained by the reduction of tetrachloroauric acid mediated by citrate [34] (see Electronic Supplementary Material (ESM) Fig. S1). Cyan gold nanoparticles (CAuNPs) were prepared according to a seed-mediated growth approach [12] that furnished desert rose–like NPs, with an estimated hydrodynamic diameter of ca 75 nm (ESMFig. S1). Yellow silver nanoparticles (Y-AgNPs) showing a SPR band at ca 420 nm were obtained by a one-step seed-mediated approach that modified the synthetic route firstly proposed by Homan et al. [31]. Differently from most protocols, seeds were directly generated during the synthesis. A limited amount of a strong reducing agent ($NaBH_4$) was added to produce nuclei, followed by the addition of an excess of a secondary reducing agent (hydroquinone). This last is known to require metallic seeds for promoting reduction of metal ions. Seeds act as a catalyst for the reduction of further metal ions, which enlarge the original nuclei. The optimal protocol for obtaining spherical, homogeneous, and bright yellow AgNPs was established as follows: the molar rate between $NaBH_4$ and $AgNO_3$ and between hydroquinone and AgNO3 were 1:200 and 5:1, respectively. The overall volume was 20 ml ($AgNO_3$ concentration 0.2 mM). AgNPs were prepared at room temperature under vigorous stirring and aged without stirring for further 2 h. Y-AgNPs produced as described were characterized by UV-vis spectrometry (ESM Fig. S2a) and TEM imaging (ESM Fig. S2b). A strong SPR band at 420 nm was obtained, as desired and Y-AgNPs shown to be almost spherical in shape, with an estimated diameter of ca 10–15 nm. Stability of Y-AgNPs over time was confirmed up to 7 days by UV-vis spectrometry (ESM Fig. S3).

AgNPs as probes for LFIA

AuNPs are widely used as probes for developing colorimetric

LFIA. As regards AgNPs, few papers reported their employment in LFIA-based systems [13, 35]. Therefore, a preliminary investigation on their suitability as a LFIA probe was conducted, and optimal protocols for their conjugation to antibodies were established. At this purpose, the following parameters were studied: pH of the sol, amount of the antibody, separation of AgNP-Ab conjugate from unconjugated antibody by centrifugation. The pH of the Y-AgNPs was 5.4 ± 0.1. Attempts made to adjust the pH to neutral (7.0) and basic (8.0) for allowing better interaction with polyclonal antibodies [36] determined flocculation; therefore, functionalization of Y-AgNPs with antibodies was conducted without pH correction. The optimal antibody amount for stabilizing Y-AgNP suspension was established by serially diluting the antibody and adding 10 μl of this solution to 1 ml of the Y-AgNP preparation. Upon addition of concentrated NaCl (10% w/v, 0.1 ml), uncapped Y-AgNPs aggregated, while those shielded by a layer of antibodies showed a persistent yellow color. Similarly to AuNPs, the optimal amount of antibody for conjugation to AgNP was defined as the one allowing the sol to retain the initial brilliant yellow color. However, differently to AuNPs, not only the lack of sufficient antibody but also the excess of antibodies destabilized Y-AgNPs (ESM Fig. S4). Hence, the identification of the correct amount of antibody to be conjugated to AgNPs is more critical. Finally, the protocol for adsorbing antibodies to YAgNPs was adapted from that used for AuNPs. Modifications were needed to obtain stable suspensions. In particular, repeated centrifugations (to remove the unbound antibody) should be avoided to prevent nanoparticle aggregation and low yield of conjugation. The SPR band of Y-AgNPs conjugated to Ab showed a red-shift of ca 10 nm compared to the bare YAgNPs (ESM Fig. S2a) that was considered as a confirmation of successful labelling of antibodies with AgNPs.

The BYMC^ xLFIA

The multicolor LFIA is based on the two-site immunometric format, in which three specific antibodies (one for each allergen) are coated separately onto the membrane to form three test lines. Detection antibodies specific for the three allergens are individually labelled with the metal NP probe (Fig. 1a). The formation of the Bsandwich complex^ (i.e., coated antibody/ allergen/labelled antibody) is revealed by the accumulation of the metal NPs at the test line, which results in the appearance of a visible line. The color of the line is determined by the metal NP; therefore, the use of differently colored metal NPs allows for easily identifying a particular allergen besides detecting its presence (Fig. 1b). In the example of application developed, the presence of CAS, OVA, and HNP in the sample was revealed by the formation of a cyan, yellow, and magenta test line, respectively. The control line, which served to confirm assay validity, was able to capture all labelled antibodies, so the line appeared brown-colored as resulting from mixing the colors of the three metal NP probes (Fig. 1c). Preliminary, non-specific interactions among the different kinds of probes was excluded by TEM imaging; examining images of the mixture containing conjugates between antibodies and M-AuNPs, C-AuNPs, and Y-AgNPs (ESM Fig. S5), no aggregation among NPs of the same or of different shape was seen. The cross-reaction of probeswith capturing reagents coated onto the membrane was also evaluated by applying separately metal NP-antibody conjugates to the multiplex LFIA device, which included the three test lines. The mutual interference resulted negligible, as also confirmed by applying the mix including the three probes to the multiplex LFIA (Fig. 2) and analyzing

the three proteins singularly. Furthermore, the result obtained by analyzing a mix including the three proteins was comparable to those obtained when each allergen was analyzed separately. In conclusion, apparently no mutual interactions among probes and between assays were observed, which is the condition for developing amultiplex assay. Each analytical system was individually optimized by a checkerboard titration approach to define the amount of capture antibody to be lined onto the membrane and the amount of the probe. The so-defined optimal conditions were employed without further optimization in the xLFIA.

The visual limit of detection (vLOD) was estimated for each allergen by serially diluting the standard protein (CAS and OVA, 1 g/l) or the crude protein extract obtained from hazelnut powder (HNP) in Tris buffer and observing line intensities. A typical hook effect was visible for all three proteins at low dilution factors, not due to the probe used. Therefore, the upper limit of detection was estimated at 1 mg/l for OVA and CAS and 1/100 for hazelnut extract [37]. For all analytes, a fairly perceivable color appeared for allergen concentrations above 0.01 mg/l (HNP extract diluted 1/100,000); however, 0.1 mg/l (HNP diluted 1/10,000) was considered as a reliable estimation of the vLOD (Fig. 3). Till now, no maximum tolerable levels of allergens in food have been defined, since the threat due to consumption of food containing allergens strongly depends on the individual response of sensitized subjects. A cautious lower bound has been indicated as hundreds of micrograms. Considering this value, the reported sensitivity is sufficient for ensuring safety of food at clinically relevant levels [38]. Furthermore, the sensitivity achieved by the YMC xLFIA was comparable or even better than that reported by other LFIA for detecting single allergens by LFIA devices [21–27]. For these assays, the vLOD ranged between 1 and 10 mg/l, except for the work of Ji et al. that was able to detect as low as 0.01 mg/l Ara h1 (the major peanut allergen) [25]. It can be noticed that the amount of Y-AgNPs used was higher than those of gold-based nanoparticles (the optical densities of Y-AgNP/M-AuNP/C-AuNP in the mix were 3:2:2). The reason for the requirement of a higher amount of the AgNP probe can be ascribed to the affinity of antibodies. However, we also compared the Y-AgNP labels to MAuNPs in the same analytical system (data not shown). We verified that the different extinction coefficient and the yellow color of AgNPs, which is hardly perceived on the white membrane background, most likely justify the need of increasing the optical density of the silver probe compared to the two gold-based probes.

Detection of food allergens in bakery products by the YMC xLFIA

The Breal world^ applicability of the YMC xLFIAwas checked by assessing the presence of allergenic proteins in four biscuit samples of various compositions (Fig. 4). Samples were all industrial products from different brands and ingredients were deduced from manufacturer's label (Table 1). Compositions were very variable and comprised up to 20 different ingredients. Sample #1 was chosen because it contained high amounts of hazelnuts (12%). Samples #2 and #3 were made of several ingredients; however, they included just one allergenic ingredient within those detected by the YMC xLFIA. Sample #4 had the most complex composition and included all the allergens investigated in this work. In addition, it contained chocolate and

cocoa powders that are known to pose severe interference to immunological assays [39].

Each sample was extracted in duplicate and extracts were assayed in triplicate. The qualitative results were evaluated visually by three operators. No discordant evaluations were obtained between operators and within replicates for all samples. Based on the color code, OVA (yellow line) was clearly detected in samples #1, #2, and #4, as expected. However, a weak signal was also observed for sample #3, which label did not list eggs as an ingredient. This finding can be explained considering that the list of ingredient furnished by sellers can be unauthentic. However, a non-specific binding of the YAgNP-Ab probe cannot be excluded, due to matrix composition. According to the list of ingredients, HNP (magenta line) were detected in samples #1 and #4. Also in this case, a weak red line was observed for sample #3. Finally, CAS was detected in samples #3 and #4, which perfectly agrees with the presence of milk listed as an ingredient for these two kinds of biscuits and not for biscuits #1 and #2.

Conclusions

Silver nanoparticles, spherical in shape and characterized by a brilliant yellow color, were obtained by a new one-step assay protocol derived from a seed-mediated approach [31]. The YAgNPs were stable over time and acceptably robust to conditions used for passively adsorb antibodies and for fabricating LFIA devices. Therefore, these Y-AgNPs were conjugated to antiOVA antibodies and were employed in combination with two different kinds of gold nanoparticles as the probe for establishing a multiplex colorimetric LFIA for measuring allergens.

The multicolor strategy enabled for the facile interpretation of assay results. Indeed, the presence of a particular allergen was easily recognized by visual observation, based on the color code used: cyan/casein, yellow/ovalbumin, magenta/hazelnut proteins.

The use of differently colored probes that can be easily combined and fit with usual LFIA materials and (bio)reagents is a step towards enabling 2-D multiplexing LFAs (e.g., using multiple probes providing distinguishable signals in one detection site combined with several spatially separate detection sites) that largely expands the detection capability of the technique.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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