Lateral flow immunoassays for myco- and phycotoxins: a review

UNIVERSITÀ DEGLI STUDI DI TORINO

Formattato: Inglese (Regno Unito)

Formattato: Italiano (Italia)

LATERAL FLOW IMMUNOASSAYS FOR MYCO- AND PHYCOTOXINS: A REVIEW

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Abstract

Natural toxin (such as myco- and phycotoxin) contamination of food involves safety and economic concerns, thus much effort is devoted to the development of screening methods which enable them to be continuously and widely monitored in food and feed. More generally speaking, rapid and non-instrumental assays for the detection of various food contaminants are generating ever increasing scientific and technological interest because they allow high-throughput, economical on-site monitoring of such contaminants. Among rapid methods for the first level screening of food contaminants, lateral flow immunoassay (LFIA) technology, also named immunochromatographic assay or immune-gold colloid immunoassay, has recently drawn scientific and industrial interest because of the attractive property of allowing very rapid, one-step, *in situ* analyses to be carried out. This review focuses on new aspects of the development and optimization of lateral flow devices for myco- and phycotoxin detection, including strategies for the management of matrix interference and, particularly, for the investigation of improvements obtained through signal enhancing strategies or through the application of non-gold nanoparticle signal reporters.

Keywords Rapid methods, Food analysis, Immunogold colloidal immunoassay.

List of abbreviations

AFB1, Aflatoxin B1; AFB2, Aflatoxin B2; AFM1, Aflatoxin M1; AFs, aflatoxins; ASP, amnesic shellfish poisoning; BSA, bovine serum albumin; DA, domoic acid; DON, deoxynivalenol; DSP, diarrhetic shellfish poisoning; FMB1, fumonisin B1; FMB2, fumonisin B2; FMs, total fumonisins; GNP, gold nanoparticle; LFD, lateral flow device; LFIA, lateral flow immunoassay; LOD, limit of detection; MC, microcystin; OA, okadaic acid; OTA, ochratoxin A; PbTx, brevitoxin; PEG, polyethylene glycol; PSP, paralytic shellfish poisoning; PVA, polyvinyl alcohol; PVP, polyvinyl pyrrolidone; STX, saxitoxin; T2, T-2 toxin; ZEA, zearalenone.

Introduction

Myco- and phycotoxins are natural metabolites produced by fungi and microalgae respectively which can affect countless varieties of food. The most common mycotoxins are produced by fungi of the genera Aspergillus (aflatoxins, ochratoxins, patulin), Fusarium (fumonisins, trichothecenes, zearalenone), and Penicillium (ochratoxins, patulin). A non-exhaustive list of commodities potentially affected by these fungi includes cereals (especially corn, wheat, rice), nuts, peanuts, spices, coffee, tea, apples, grapes, cottonseed, and soybeans. Crops can be infected pre-, during and post-harvest. Due to the relative stability of fungal toxins to thermal and chemical stresses, they are found on commodities despite the elimination of mould, after long periods of storage, and also after the transformation of raw materials; therefore the presence of mycotoxin contaminations has been ascertained in commodities such as composite feed, flour, bakery products, roasted coffee, roasted peanuts, raisins, beer, wine, and apple juice. Moreover, products of the animal metabolism of mycotoxins could retain toxicity, such as in the case of AFB1 which is metabolized into AFM1 and excreted into milk. Meat, milk and derived products can consequently also be implicated in the spreading of toxins. The chronic toxicity of mycotoxins covers a wide range of adverse effects such as carcinogenicity (AFB1, AFM1), genotoxicity (T2), mutagenicity (OTA), teratogenicity (DON), immune- suppression and/or toxicity (OTA, DON, T2), nephrotoxicity (FMs), hepatotoxicity (OTA, FMs), and endocrine disruption (ZEA). Acute toxicity has also been demonstrated for patulin (gastrointestinal lesions), DON (vomiting, feed refusal) and T2 (vomiting, diarrhoea, haemorrhages). Regulations defining maximum admissible levels for major mycotoxins in numerous commodities exist all over the world, variable in the µg-mg/kg range, except for limits imposed by the European Union for AFM1 in milk which are in the ng/kg range [1].

Phycotoxins, more properly called ‘marine and fresh water toxins’, belong to many different groups of small or medium size compounds (300-3000 Da ca.). Besides the chemical variability of classes, each class group includes several compounds characterized by similar structures, which are either produced by algae or are secondary products derived from metabolism in fish and shellfish of the primary algae toxin. The algae responsible for phycotoxin production and excretion belong to the cyanobacterium (STX, MCs, nodularins) and dinoflagellate (STX, OA, PbTx, DA) groups. The risk posed by these contaminants is increased by the fact that excreted toxins accumulate and concentrate in different aquatic organisms; including feed-filtering bivalves (mussels, clams, oysters), shellfish and herbivorous fish, and subsequently enter the food chain and cause toxicosis in consumers such as predatory fish, marine mammals, birds, and humans. Human poisoning due to ingestion of seafood contaminated by phycotoxins has frequently occurred; each group of

compounds is accountable for a distinct poisoning action. STX is one of the best known neurotoxins (in fact it is also listed as a chemical weapon) and is responsible for the Paralytic Shellfish Poisoning (PSP); adverse neurological effects are also caused by PbTxs, collectively known as the Neurotoxic Shellfish Poisoning (NSP). OA adverse effects interest the gastrointestinal tract (Diarrhetic Shellfish Poisoning, DSP) to which, in addition, neurological symptoms determine the Amnesic Shellfish Poisoning (ASP) due to the intake of DA. Given the strong acute toxicity of phycotoxins, chronic diseases have not been yet demonstrated. As is true for mycotoxins, cooking and transformations of food are inadequate to destroy phycotoxins; therefore maximum admissible levels for these contaminants in seafood and bodies of waters have been set to prevent severe risk for human health associated to consumption of contaminated seafood [2,3].

Natural toxin contaminations of food have great economic concerns besides safety issues, thus much effort is devoted to the development of rapid, cheap, and simple screening methods and, at the same time, to the optimization of accurate, sensitive, multi-residue instrumental methods. Several validated sampling and analytical methods for measuring mycotoxins are available [see, for example, ref 4-8]; the development of analytical methods for phycotoxin is a more recent goal. However, screening and instrumental methods for their assessment in water and seafood have been published and recently reviewed [9-11]. However, affordable monitoring of myco- and phycotoxins to assure food safety requires high-throughput and economical methods of detection. In addition to those priorities, little or no sample treatment, user-friendliness, employment of non-hazardous chemicals, and *in situ* applicability would be welcome attributes. With regards to mycotoxin analysis, additional requisites of low detection limits (especially for aflatoxins and ochratoxin A) and adaptability to very differing commodities are also preferable. Conversely, low detection limits are not of great concern in phycotoxin analyses, whose principal requirement is that of being class-selective instead of compound-selective, namely analytical methods capable of measuring numerous structurally differing compounds at the same time.

Among rapid methods for the first level screening of food contaminants lateral flow immunoassay (LFIA) technology (also named immunochromatographic assay or immune-gold colloid immunoassay, IGC) has recently generated scientific and industrial interest, because of the attractive property of allowing very rapid, one-step, *in situ* analyses to be carried out. Starting from the early 2000s, scientific papers and commercial devices (for a list of commercial LFDs for mycotoxin detection validated by USDA-GIPSA see ref [12]) aimed at measuring natural toxins in food and feed have appeared, and, in the last few years a certain amount of literature on this subject has become available, including comprehensive and critical reviews [13-14]. Although new applications appear daily in the literature, little innovation nor real breakthroughs in materials,

protocols or signalling have been described and discussed. The research is still driven by applicative concerns and by the demand for rapidly functioning devices, therefore the strategy applied is the exploitation of well-established practices and the focusing of effort in the development of good antibodies. This review will focus on highlighting new perspectives and alternative routes that could be explored in: (i) the development and optimization of lateral flow devices (LFDs), including a discussion of established protocols for preparing components of the LFD; (ii) the management of matrix interference caused by food components; and (iii) the investigation of alternative signalling through signal enhancing strategies or through the application of non-gold nanoparticle reporters.

Competitive lateral flow immunoassays for myco- and phycotoxins

As mycotoxins and phycotoxins are low-molecular-mass compounds, immunoassays in a competitive format should be conceived to measure them. The same principles and reagents as in the microwell-type immunoassays could be applied, except for the fact that separation of bound and unbound antibody sites is obtained by means of the lateral flow on a suitable support (the membrane). A liquid flow transports immunoreagents along the membrane where they encounter their counterparts and immunoreactions take place in a spatially confined zone of the membrane itself. With few exceptions, the indirect competitive format (in which the antigen is coated on the membrane and the antibody is labelled, Figure 1) is strongly preferred to the direct format (in which the antigen is labelled and the antibody is coated onto the membrane, Figure 2), though no experimental data supports the option of the first approach over the second one. On the contrary, when the two formats were compared, sometimes the direct format some other times the indirect format were preferred [15-16]. The principles of the indirect competitive immunochromatographic assay have been widely described and are schematized in Figure 1. Briefly, a labelled specific antibody is suspended in a liquid sample and flows through the membrane where it first encounters the coated antigen (test line, T-line). In the absence of the target compound in the sample (negative sample, Figure 1a), labelled antibodies bind to the coated antigen and are focused on the T-line, so that a visible (detectable) line is formed. When the target is present in the sample above the lower detectable concentration level (positive sample, Figure 1b), labelled antibody sites are saturated and could not bind to the coated antigen, resulting in a non-visible (undetectable) T-line. Usually, a second control line (C-line) follows and is constituted by secondary anti-species antibodies which capture any excess of specific antibodies. The appearance of a C-line can be regarded simply as the confirmation of the correct development of the assay (reagents and materials integrity) or else can be exploited to calculate the T/C signal ratio with the aim of normalizing strip-to-strip variations

[17] or can also be regarded as an internal standard to which the intensity of the T-line is compared to determine positivity/negativity [18-19].

Materials

In addition to the porous membrane (almost exclusively nitrocellulose) which assures lateral flow, LFDs usually include an absorbent pad positioned at the top of the membrane to increase the volume of the flowing liquid, a sample pad to assure contact between the liquid sample and the membrane, and a rigid backing. The simplest LFD is a dipstick, which is dipped directly into the sample solution. Labelled antibodies can be added to the sample as a concentrated suspension or provided in a lyophilized form to be re-suspended by the sample itself. Alternatively, the labelled antibody can be pre-adsorbed onto a releasing pad (usually a glass fibre pad, though more materials are available through commercial partners) which partially overlaps the membrane. The liquid sample flow itself causes the re-suspension of the adsorbed labelled antibodies when carrying out the assay. A further sample pad, usually made of cellulose and sometimes soaked with proteins and/or surfactants, may be added with the aim of reducing matrix interference in such a way that it overlaps the membrane or the releasing pad [16,20]. Besides the most popular dipstick format, some authors described LFDs in which the strip is inserted into a rigid plastic cassette provided with a sample well and a reading window. The main advantage of these housings is the guarantee of a reproducible compression of all components in the overlapping zones, which assures faster and more reproducible flows.

Types and quality of materials are generally regarded as well established and optimization in this field is limited to the variation of the porosity of the nitrocellulose membrane to modify the flow rate [21-23], although when various membrane suppliers were compared significantly better performances were observed when using AE99 and Prima 40 (Whatman) rather than Immunopore FP, Sartorius CN140 (Sartorius), and Hi-flow135 (Millipore) membranes [24]. Furthermore, membrane pore size is not the only nor the predominant factor affecting flow rates, especially when food samples should be analysed. Plenty of contrasting effects influence the capillary flow, including but not limited to: the viscosity of the liquid, the volume of the sample, the length and type of adsorbent and sample pads, the presence of micro-dispersed insoluble matter in the liquid, the amount and nature of organic solvents, and the addition of surfactants. A strong flow rate conditioning is due to membrane treatments carried out by some authors with the intent of saturating the nitrocellulose binding attitude towards proteins after line depositions. Membrane saturation has been demonstrated to be particularly effective in limiting matrix interference in mycotoxin determination in cereals [17,20-22 25-29], and has been reported also for improving the

determination of brevitoxins in molluscs [30]. For the purpose, the nitrocellulose membrane is soaked with buffers which contain proteins (mostly BSA [17,20-22,25-26,30], but also casein [23], ovalbumin [27] and skimmed milk) and several variant blocking agents (PVA, PVP, dextran, PEG [23-24]). Membrane saturation strongly affects the capillary flow, which becomes substantially independent of its pore size and hardly compatible with acceptable run times. The joint use of some surfactants as flow modifiers is mandatory, especially with casein, to aid liquid flow. SDS was reported as serving the purpose by Xu et al [30]. Nevertheless, Tween 20 in significant amounts (>0.1%) is frequently preferred and can be added to the membrane blocking solution directly, or to the solutions used in the subsequent washing of the membrane itself, or to the labelled antibody solution, or, lastly, to the sample. By the use of such flow modifiers run times are reduced to 10-15 min, which represents the upper limit for methods considered as a truly rapid. Remarkable exceptions to that are represented by some LFIAs for phycotoxins which require more than 30 minutes to allow the definitive judgment of results [18-19,31-32], which is a considerable and excessive time interval for a so-called “rapid analysis”.

As previously observed, with a few noticeable exceptions which will be discussed below, colloidal gold is the signal reporter of choice to label antibodies. Gold nanoparticles (GNPs) of about 40 nm mean diameter provide good properties in terms of handling during conjugation to antibodies, stability and, above all, line detectability. In addition, the availability of commercial GNP suspensions aside, the preparation of GNPs of predictable dimensions via the Frens and Turkevich [33] methods is relatively economical, easy and rapid. The conjugation of GNPs to antibodies follows established protocols as well, including: (i) the determination of the saturation quantity of antibodies, according to Horisberg and Rosset [34]; (ii) the incubation of antibodies and GNPs in mild alkaline conditions for a time variable from a few tens of minutes to some hours; (iii) the overcoating of the potentially free GNP surface with an excess of BSA; and (iv) the washings of unbound antibodies by repeated centrifugations and re-suspensions of the pellet. Finally, GNP-labelled antibodies (GNP-Ab) are typically re-suspended in buffered solutions supplemented with high concentrations of proteins and sucrose for long-term storage. Occasionally, one of the listed steps is subject to verification and optimization, as, for example in the works of Xiulan et al [25] and of Tippkotter et al [35], where the optimum pH for the GNP-Ab conjugation was observed to depend on the antibody’s pI. Tippkotter et al also studied the course of the gold-antibody conjugation reaction finding that 30-40’ incubation at room temperature is sufficient to completely saturate the GNP surface, when working with an excess of antibodies and at pH > pI.

Development and optimization

The development of an LFD implies the identification of balanced conditions between the amounts of following reagents: the coated antigen of the T-line, the antibody conjugate to GNPs, and, less importantly, the secondary antibodies of the C-line. Checkerboard titrations are conducted to achieve the best sensitivity and good detectability of signals, similarly to the schematic of microwell-based immunoassays. Guidelines for selecting best conditions, which would specially apply to visual devices, were recently highlighted in a critical review of Krska and Molinelli and can be summarised as: the presence of an intense and reproducible C-line, the complete disappearance of the T-line at and above the designated cut-off level, and the absence of background colouring of the membrane [13]. The recent tendency to provide a semi-quantitative evaluation of results by means of some instrumental reader permits these criteria to be less stringent, by background subtraction and normalization of colour intensity. Moreover, in place of designating the cut-off level with the naked eye the definition of detection limit similar to microwell-based immunoassays is feasible. The major advantage is the improved detectability, mostly when the slope of the inhibition curve is limited. A reduction of the uncertainty in the attribution of samples near to the cut-off level and of the number of false positives / false negatives could hopefully be also attained. The requirement of a reproducible C-line has been addressed by some authors by introducing a second couple of affinity reagents, completely independent from the antibody-antigen interaction which takes place in the test zone. Kim et al, for example, coated streptavidin to form the C-line and mixed a labelled biotin to the immunoreagents in such a way that the intensity of the C-line was completely unaffected by the immunoreaction and therefore by the presence and amount of the target compound in the sample [16].

Surprisingly, some factors which are recognized as crucial for determining sensitivity in competitive microwell based immunoassays are rarely evaluated when developing LFIAs; such as, for example, the nature of the coated antigen (number of antigen moieties per molecule of carrier protein and use of heterologous antigen). In this regard, Xu et al compared performances of three protein conjugates of DON as the coated antigen of the T-line and demonstrated that the use of a cationised BSA as the carrier protein improves sensitivity, probably because of the different reactivity towards the derivatised DON used to synthesize the antigen [24]. Liu et al showed better sensitivity for a LFD in which an ovalbumin instead of a polylysine conjugate of OTA was used to form the T-line [36]. A slight sensitivity improvement in the detection of microcystins and nodularins was also reported by Kreich et al when a heterologous conjugate rather than the immunogen itself was used to form the T-line [37]. Contrarily, Kolosova et al observed better performances when the T-line was constituted by a homologous rather than a heterologous conjugate when compared to the immunogen [29].

Likewise, studies aimed at demonstrating the convenience of the practice of saturating GNPs with the specific antibody rather than working in a limited specific antibody concentration are still lacking. The work of Laycock et al, although unfortunately not very detailed, suggests that decreasing the amount of antibodies in the GNP-antibody preparation would strongly positively influence the sensitivity of the resulting LFDs [18].

Papers dealing with additional critical points in the optimization of LFDs (which have been recently underlined in some critical reviews [14,38-39]) have also been published such as: (i) addressing the defect of quality control and enforcing correct attribution of positivity/negativity, (ii) evaluating cross-reactivities to other toxins, (iii) extending applicability by counteracting matrix interference. The latter being a major concern is discussed separately. With validation and quality control purposes, the accuracy of commercial LFDs for detecting STX and DA, meaning the ability of correctly individuating positive and negative samples, was investigated together with the degree of agreement of results provided by several operators [40-41]. As expected, the number of incorrect attributions was dependent on the level of sample contamination. In particular, no false negatives (n = 77) were observed when STX contamination was higher than twice the cut-off level, while 3% of false negatives (n = 135) was recorded when STX concentrations lie between the cut-off and twice the cut-off level. A value as high as 18% (n = 335) of false positive was also assessed. Interestingly, while the judgment of the sole colour intensity of the T-line by the naked eye showed some discrepancy, a strong agreement between the 8 participants in the trials was obtained in the interpretation of results, thanks to the use of the C-line intensity as a colour reference. Results on the DA system reflect the same qualitative behaviour. The definition of a cut-off level is intrinsically a source of uncertainty, as first pointed out by Kolosova et al who proposed the definition of an indicator range of analyte concentrations within which the colour of the T-line gradually faded rather than a cut-off level [23]. Most frequently the positivity is assigned to samples that produce a complete disappearance of the T-line at the expense of detectability. The reduction of uncertainty and misinterpretation of signals in qualitative LFDs has been pursued by some authors by the provision of some kind of colour reference. Tsao et al proposed the use of a control strip to judge the result in the measurement of DA in mussel extract by a qualitative dipstick [42]. Laycock et al assumed the colour of the C-line as the reference intensity for a negative sample and defined the T-line colour as being half the C-line colour or weaker as the indication of positivity [31]. The same approach was used by Komano et al in the evaluation of a commercial LFD for measuring PSP toxins [19]. The availability of portable readers, which allow an objective measurement and a numerical comparison of line colours has to be regarded in this context not only as a step towards

(semi-)quantitative measurements, but primarily as the achievement of a simpler positive/negative discrimination and a higher sensitivity [20,35,43].

Cross-reactivity towards other mycotoxins compared to the target compound has also started to be evaluated. Wang et al showed no interference of DON, ZEA and FMB1 in the determination of OTA [28]; Shim et al reported no interference of OTA, ZEA, citrinine, patulin, and T2 in the determination of aflatoxins [44]; and Molinelli et al assessed a negligible cross-reactivity of ZEA and several trichothecenes in the measurement of FMs [22]. Moreover, reported developments of multi-residue LFDs also contribute to regarding the interference due to other toxins as a presumably false problem. Not the same applies to cross-reactivity towards toxin derivatives which result from the reaction of the target toxin with some matrix components (proteins, starch,…). The recognition of such derivatives by antibodies directed towards the toxin has been demonstrated to allow the detection of hidden mycotoxins in an ELISA for measuring Fms and has been suggested as a fascinating explanation for the general over-estimation of immunoassay techniques towards chromatographic methods in the quantification of mycotoxins [45]. The LFIA capability of measuring masked mycotoxins would deserve more investigations and could also account for deviations when LFD quantitation is compared to HPLC results.

On the other hand, cross-reactivity studies in the development of LFIAs for phycotoxins deserve a separate discussion. Assays in this field should be oriented to the determination of a class of related compounds rather than of a specific target, as the relevant information is the potential toxicity of the sample and this is associated to numerous compounds, though being strongly variable for individual compounds. For instance Laycock et al highlighted that a commercial LFD developed for measuring STX showed a certain broad selectivity, which, however, enabled the detection of some mildly toxic STX parent compounds, while highly toxic members of the PSP's family were poorly detected [18]. This aspect is partially counterbalanced by the fact that tolerable limits of phycotoxins in waters and food rarely represent a constraint compared to typical sensitivities of LFIAs and enable the detection of either low cross-reacting compounds. Excessive sensitivity for low toxic parent compounds was accounted for the high false positive rates observed in some LFDs which measure phycotoxins [40].

In addition to the aforementioned concerns, two other aspects should draw more attention to the development of lateral flow-based immunoassays, such as the stability (of separate components and of the complete ready-to-use device) and the ruggedness of the methods of analysis. Ruggedness of the LFDs and of the analytical methods should be primarily evaluated since they are claimed to serve as point-of-use tests (without environmental constraints like, for example, temperature and humidity) and are intended to be used by non-trained personnel [46]. The effect of environmental

variations (in particular as regards the ambient temperature) was studied during the optimization of LFDs for FMs, AFs and OTA and reproducible results were attained for temperatures varying from 22 to 30°C [17,26-27]. As part of ruggedness evaluation, some authors highlighted a great inconsistency of results for small variations in the sample matrix, even simply in terms of grain size for cereal samples [22] or changing from drinking water to river water [16]. When evaluated, the stability achieved turned out to be limited. Storage at pH 9 of GNP-antibody conjugates has been monitored by Tipptokker et al at 4 and -18°C (with glycerol added) and stability was maintained for 120 days [35]. Nevertheless, dipsticks for MC developed by the same authors were showed as being stable at room temperature for no more than 20 days. Similar short stability at room temperature was verified by Shim et al for dipstick-format strips aimed at measuring OTA and ZEA in corn [44]. Wang et al also stated one-month stability (at 4°C) of their devices for measuring OTA by using an aptamer-quantum dot approach [47]. Only Molinelli et al reported longer storage stability (4 months at room temperature for their LFD for T2 toxin [21] and one year at 4°C for the components of the device aimed at detecting FMs [22]). In these cases, however, the signal reporter (GNP-antibody) was stored apart from the strip and as a solution rather than in lyophilized form or adsorbed onto a releasing pad.

Application of LFDs in food analysis

The major concern in the development of LFDs for phycotoxins and primarily for mycotoxins are the unpredictable effects due to food components co-extracted from the sample beyond the target and which affect not only the antigen-antibody interaction on which the immunoassay is based, but also the mechanics of the device itself. From this general observation the difficulty arises in defining appropriate standards for calibration and the fact that individual foods require distinct devices to be developed for them (which means not only that a different calibration of the same device is required, but even that several devices, each characterized by its own materials and/or treatments, ought to be devoted to various food materials). In addition to that, some authors experienced the apparently inexplicable failure of recovery experiments conducted on fortified materials and the incongruity of results attained for artificially and naturally contaminated samples, which makes the definition of calibrators disappointingly arduous. Therefore, the group of Molinelli pointed out the necessity of matrix-matched calibrations [21-22], as also experienced in our group's works [17,26-27] and recommended the use of naturally contaminated samples blended in varying proportions with blank samples as calibrators to overcome both matrix interference and non-matching between fortified and naturally contaminated materials. Similar dependence of results on the sample characteristics has also been underlined in the application of LFDs for MCs in water

[16,35] and for STXs in shellfish and phytoplankton, therefore matrix-matched calibrators in the form of fortified samples were exploited in these works as well [18].

Nevertheless, it should be noted that the majority of authors reported calibration of newly developed LFDs by the means of standard toxins diluted in buffers (to which methanol is often added in variable proportions). The observation is valid for both myco- and phycotoxin determination and, with the above discussed exceptions, interference from matrix components is generally regarded as insignificant, given a limited dilution (1:2 – 1: 10) of sample extracts. Partly, the same LFD materials help to abate interferences (by filtering particulate matter and adsorbing various co-extract components); secondly, counteracting strategies can be implemented by pre-soaking pads with suitable matrix modifiers (buffer salts, surfactants, proteins, …). The ultimate goal is the minimizing and simplifying of sample processing to render LFDs suitable for an effective on site usage. Thus, typically liquid samples are directly analysed (or simply diluted before analysis), whereas solid samples are extracted with aqueous methanol. Several papers agree that methanol in proportions below 30-35% does not affect assay performances; therefore, established protocols for extracting target toxins from food, which typically involve the use of methanol/water mixtures could be safely employed. An adequate dilution of methanolic extracts suffices to attain conditions suited for directly submitting diluted extracts for the analysis (see for example ref 42 and 29 for phyco- and mycotoxins respectively).

Mycotoxins

Rapid and affordable analytical methods to monitor major mycotoxins in food and feed at virtually every stage of the production chain, preferably right at the place of production or processing are strongly pursued. The non-uniform distribution of such contaminants in commodities would further prompt the increase of the number of controlled samples, given acceptable costs and time investment. Therefore, lateral flow technology has promptly been exploited to develop disposable devices for the qualitative assessment of mycotoxin presence in varying commodities. Indeed, industrial rather than scientific research on this topic came first and remains the major propellant of new and forthcoming developments.

An extensive list of papers regarding devices aimed at measuring mycotoxins in food and feed is given in Table 1 (validated LFIA methods) and Table 2. The prominence of AFs in mycotoxins controls is also testified by the prevalence of LFDs developed to detect these contaminants [20,25,44,48-50]. Major aflatoxins were detected in grain and feed, typically after methanol/water extraction followed by dilution to reduce the proportion of the organic solvent. Visual devices have been developed which enables aflatoxin detection at levels complying with the legislation in force.

An aqueous extracting medium associated to a LFD with instrumental detection was also proven to allow total aflatoxin quantification in maize samples [26].

Very recently, Wang et al first described a LFD for the detection of AFM1 [51]. The cut-off level (0.5-1 µg/l) is just above the eligible value required by the US regulation [52] and far beyond the more severe limits imposed by the European Union for this contaminant [1]. However, it is a thoroughly sensitive and rapid assay, provided that the whole analytical procedure can be completed in 10 minutes, as no sample treatment is required. The validation of a commercial device aimed at quantitatively measuring AFM1 in milk was also described [53]. As the result of an interlaboratory trial, which involved 21 participants, the ROSA Charm Aflatoxin M1™ was verified at four levels above and two below the declared LOD (0.4 µg/l). Less than 5% of false negative (n=83) and no false positive below 300 ng/l were found. For contaminations between 350 and 450 ng/l false positivity increased from 21 to 93%. Some LFDs based on non-GNP reporters have also been proposed by using aflatoxins as system models (*vide infra*) [54-56].

Besides aflatoxins, rapid detection of OTA has attracted great attention due to the toxicity and the widespread presence of such contaminant in differing kinds of food [57]. The applicability of LFDs exploiting GNP reporters was demonstrated in various cereals [27-28, 58-59], beverages [28], and in coffee [28, 60]. Moreover, instrumental recording allowed high sensitivity to be achieved using the device developed by Urusov et al [43] and in the quantitative assay developed by our group (LOD 1.5 µg/kg in maize and wheat) [27].

A fluorescent dye was used as the label in the work of Wang et al [47], who also replaced the use of a specific antibody with that of an aptamer capable of selective recognition properties towards the target toxin. Feasibility of the developed test to assess OTA contamination in red wines was established. An interesting approach has been proposed by Lai et al who profited from the fact that a peptide mimicking OTA had been previously described [61]. LF strips prepared by spraying the mimotope peptide or an OTA-BSA conjugate onto the membrane showed similar performances in terms of detection limit (10 ng/ml with visual evaluation), reproducibility and degree of agreement to a classic microwell-based immunoassay [59]. As mimotope peptides exist for other mycotoxins [61 and reference herein] this approach could be extended with the advantage of avoiding handling of large amounts of toxic compounds typically involved in the synthesis of toxin conjugates. OTA has also been determined in association with other mycotoxins (AFB1 [62] and ZEA [63]) in multi-analyte devices.

LFDs for the detection of FMs were among the first to be described [64] and various concerns in their development were accurately discussed by Molinelli et al [22]. Recent papers have reported an improved sensitivity by exploiting LFDs with both visual [65] and instrumental [17] detection.

Some examples of lateral flow immunoassays for the detection of most relevant tricothecenes (DON in ref [23 and 24]; T-2 in ref [21]) and one applying to ZEA detection [66] can also be found in the literature.

Phycotoxins

Lateral flow immunoassays for principal phycotoxins started to be published from 2003 when Kim et al first reported a quantitative assay for measuring MC in water exploiting a fluorescent reporter [16]. Following works by Pyo et al [67] and Kreich et al [37] also investigated the use of fluorescent labels, sulforodamine B encapsulated in liposome and quantum dots respectively, in the development of LFDs for MCs. On the other hand, Tipptokker et al exploited a more classical GNP reporter for the same purpose. In this work, authors widely studied the interaction between antibodies and gold nanoparticles, in terms of dependence on time, antibody concentration and pH for achieving optimal GNP stabilization [35]. The optimized LFD had a visual cut-off of 5 µg/l in water, while measurement of line intensities by means of a CCD camera allowed 5-fold improvement in sensitivity. Responses of the LFD differed depending on which matrix was tested (buffered solution, drinking water, salt water), though surprisingly the cut-off level remained the same. Moreover, the interpretation of results could be rendered independent of matrix interference by using the C- line signal as a reference, because the matrix equally affected both lines. Usability of the LFD to measure MCs in mussel extracts, after a simple filtration of sample extracts, was also demonstrated.

Only one LFD for the detection of brevitoxin has been described so far [30]. This is a qualitative assay, which employed GNPs as the signal reporter, and was applied to toxin detection in molluscs. The visual cut-off was set at 10 ng/ml in buffered solutions and at 20 ng/ml in food extracts. Extraction of PbTx was obtained as follows: molluscs were whisked within a DMSO/water mixture, which was then centrifuged and filtered prior to being put into contact with the strips. The chromatographic run takes 10'. Authors also evaluated strip stability (6 weeks at 4°C) and cross-reactivity towards other marine toxins. The system exhibited good recognition properties towards most NSP and no interference from phycotoxins belonging to different classes (MCs, DSP, and ASP). Moreover, LFD performances agreed well with results obtained through a parallel ELISA method.

Commercial systems for measuring PSP, DSP and ASP have been available since the early 2000s and had been the objects of several evaluation studies [19,31-32,40-41]. Accuracy of the devices through comparison to instrumental methods of analysis and agreement degree between results provided by different operators were assessed and allowed authors to confirm the reliability of the

assays. In addition, comparisons to other screening methods were performed, such as in the work of Laycock et al [18] where the predictability of the toxicity of the LFD was compared to that of the most frequently used screening method, which is a bioassay. In fact, a measure of toxin concentrations can be regarded as less informative than the immediate measure of toxicity, though the two are related when mixtures of differing toxins are considered. Conversely, the same is not true when a single toxin is determined, because it could not be representative of the overall toxicity of the sample, therefore a broad selectivity is indispensable for LFDs developed in this field. Authors screened more than 3000 samples from different countries during a 5 year survey and highlighted a close agreement between the two methods, thus demonstrating the value of the LFD as a screening tool for extensive monitoring of PSP.

In addition to validation and comparative studies, the production of a monoclonal antibody for DA and its exploitation in the development of a qualitative lateral flow immunoassay for detecting ASP toxins in mussels was described by Tsao et al [42]. The optimized dipstick was prepared by pipetting an OVA conjugate of the target toxin as a T-spot and using the selected mAb (GNP-labelled) as the reporter. After a 10-minute development, the strip was visually evaluated by the naked eye, providing an indicator range between 1-5 ng/ml, which is well below the statutory maximum admissible level for DA in the tissue of mussels. The assay was applied to assess the presence of DA in mussel samples, which were undergone an extraction with aqueous methanol followed by a centrifugation and the dilution of extracts with a phosphate buffer to eliminate the interference from the organic solvent in the assay.

STX, besides being a marine contaminant concerning food safety, is also listed as a chemical weapon, therefore LFDs aimed at its detection could also be used as disposable tools for field checks against terrorism (such as in airports, frontier checks, etc.). For this purpose, Komano et al [19] investigated the influence of several potential interfering agents in the assay for determining STX via a commercial LFD, such as: white flour, alkali, acids, oxidants, and reducing reagents. The assay proved to be sufficiently robust, although some of tested agents invalidated the test (hydrochloric acid, formaldehyde, sodium hydroxide, wheat flour), and hypochlorite produced false negatives.

Available literature concerning LFIA of phycotoxins is summarised in Table 3.

Advances towards multi-residue analyses and high sensitivity

Multi-analyte LFDs

One of the benefits of lateral flow technology is represented by the easy implementation of multi-residue analyses. In theory, it suffices to add one or more T-lines to an existing LFD and to mix the

partner labelled antibodies to increment the number of analytes to be determined. This is true in so far as cross-reactivity between target compounds to be simultaneously determined is negligible and samples could be treated in the same manner, as attested in the papers of Shim and co-workers [63,66]. In their work, authors established optimized conditions for the simultaneous detection of OTA and ZEA and OTA and AFB1 in corn by means of two LFDs, which completely resembled individual assays and performed likewise. The comparison of the multi-analyte and single target devices developed by Kolosova's group and separately published [23,29] also confirms that the simultaneous detection of two analytes could be obtained by transferring optimal conditions for each separate LFIA into a single device. As a part of a project funded by the European Union, a LF device has been developed and fully validated which allows the simultaneous detection of up to six Fusarium toxins (DON, ZEA, T-2/HT-2, and Fms) in cereals [68]. Also in this case, authors developed antibodies directed towards each separate toxin and merely mixed them after GNP-labelling. The strips are prepared by dispensing four test lines, each made by individual toxin conjugate, and by a unique control line. Results are instrumentally evaluated and allow discrimination between positive and negative samples according to European legislation in force.

Goryacheva et al. already indicated LFIAs as a privileged way to achieve multi-detection by means of immunochemical methods in 2007 [69]. Nevertheless and despite multi-detection is encouraged by criteria of economy and increased rapidity and, furthermore, would serve to individuate the co-occurrence of mycotoxins in food, which is regarded as a major goal [63], research in this area has been thus far greatly disregarded (as already pointed out in 2009 by Ngom et al [14]). Just as before, over the last two years, very little effort has been made in this direction.

Signal enhancement and non-GNP reporters

Besides the standard approach of using GNPs as signal reporters in lateral flow assays several researches have been addressed to the investigation of potential benefits derived from using different labels, such as: fluorescent dyes, liposomes encapsulating visible or fluorescent dyes, quantum dots, magnetic nanoparticles and silver-gold nanoparticles.

Kim et al [16] used a fluorescent reporter which was exploited to label both the antibody and the antigen and consequently two different assay formats (direct and indirect competitive immunoassay) were developed and compared. The approach in which the antigen was immobilized and antibodies were labelled (indirect competitive format) proved to be more sensitive and more highly reproducible in this case, hence it was applied for measuring MC in tap water and river water. Calibration was obtained by serial dilution of the target in PBS and by plotting the signal of the T-line divided by the signal of the C-line (fluorescence measurement mediated for the area of

the line) towards MC concentration. To maintain the C-line as constant as possible, streptavidin was sprayed on the membrane and some fluorescent-labelled biotin was added to the fluorescent-labelled antibody. No matrix interference was observed when measuring MC in tap water, provided that the membrane had been saturated with a solution containing BSA, Tween 20 and PVA and that the sample pad had been soaked with PBS with BSA and Tween 20 added. Authors particularly highlighted the role of the surfactant, which strongly reduced non-specific binding of the fluorescent label to the membrane.

Fluorescent labelling has been exploited by the group of Pyo to prepare an LFD for measuring MC as well; the assay exhibited a LOD of 200 pg/ml and was able to detect all major MCs [15,67,70]. The fluorescent LFD showed lower detection limits than a parallel device in which GNPs were used as signal reporters. However, the observed sensitivity increment could also had accounted for the fact that fluorescent LFD was provided with instrumental detection while results from GNP-based LFD were visually evaluated, which generally reduces detectability, as discussed above. Conversely, the fluorescent LFD is limited by the need for an instrumental reading.

High sensitivity was also attested when liposomes encapsulating sulforodhamine B were used as reporters in the determination of microcystins and nodularins [37]. In this system, the analytical signal was considered as the rate between the T- and C-line average fluorescence instrumentally determined. To improve sensitivity, after strip development (15’), the dipsticks were dried in an air oven for 5’ to destroy liposomes and allow dye release which reduced self-quenching. By this stratagem, a 10-fold increase in sensitivity compared to an LFD with GNP reporters was obtained. Nevertheless, also in this circumstance, the GNP system was visually evaluated, thus sensitivity was surely underestimated. The use of dye-encapsulating liposomes was first proposed by Ho and Wauchope [54], who described a device in which liposomes were tagged and covalently linked to AFB1; an anti-AFB1 antibody was deposited onto the membrane to form the test zone, while the control zone was missing. Negative samples determined the focusing of liposomes in the test zone, so that colour could be revealed and related to the amount of AFB1 in the sample. The absolute limit of detection of such a device was 18 ng of AFB1 and the test could be completed in a total of 12 minutes, including sample preparation.

The fluorescence of quantum dots (QDs) was exploited by Wang et al [47] to prepare QD-labelled aptamers used as the reporters in the development of an innovative LFD for measuring OTA. The T-line was made up of a DNA probe which is able to hybridize with the aptamer, while the C-line was composed of a DNA probe binding the 18-polyA tag on the 5’- aptamer end. When OTA was present in the sample, it inhibited the binding of the labelled-aptamer to the T-line so that the

interpretation of results was as per usual. The device proved to be suitable for measuring OTA in red wines at the ng/ml levels both by visual or instrumental readings.

The increment of the specific activity of the reporter (i.e. the intensity of colour/fluorescence per molecule of labelled antibody) should enable the reduction of the number of antibodies themselves which should also mean a consequent increase of assay sensitivity. A sharp signal enhancement can be obtained when mixing the use of gold nanoparticles with silver as first highlighted in the work of Liu et al [71] for a nanoparticle-based immunoassay with ICP detection. In 2010, Liao and Li first described [56] a visual device which exploited the same principle. They prepared nanoparticles with a silver core and a gold shell which were used as the reporters in the construction of a LFD for AFB1. The toxin was determined in cereal and nut samples and performances were compared to those of a GNP-based LFIA and to those obtained through a classic microwell-based immunoassay. The authors demonstrated that the newly developed LFD was comparable to the GNP-LFD in terms of stability of components and reproducibility of signals. On the other hand, it allowed a great enhancement in sensitivity so that values as low as 0.1 ng/ml AFB1 could be measured. More recently, Wei et al [72] further confirmed the potential of the combined use of silver and gold in nanoparticles. Their application regarded a proteic target (abrin-a), therefore the scheme of the assay was quite different. Nevertheless, the rise of sensitivity obtained is impressive and could be of major interest in the development of LFIA for low-molecular-mass toxins as well, particularly when very low detection limits should be achieved, such as in the case of aflatoxin M1.

With the expectation of increasing the useful signal, magnetic nanogold microspheres with a Fe_2O_3 core and a gold shell have also been proposed [55]. The magnetic core of particles allowed authors to simplify separation steps during the labelling of antibodies and their micro- dimensions to enhance colour. A three-fold increase in sensitivity was stated for the visual detection of AFB2 compared to the use of simple gold colloid nanoparticles. However, authors did not discuss the possible adverse effects on capillary flow due to so increased particle dimensions (micro- rather than nano-metric), which could affect reproducibility and rapidity or would imply the requirement of specifically designed materials.

A lateral flow dipstick, in which the signal is not generated by coloured particle focalization, has recently been described, in which an enzymatic label has been employed [73]. In the proposed assay, unlabelled anti-FM antibodies were mixed with HRP-labelled secondary antibodies and the sample. Upon completion of the migration onto the membrane where the usual assay scheme took place, a chemiluminescent substrate was added, and the luminescence developed at the T and C-lines was recorded by means of a portable CCD camera. Quantitative measurement of FMs in maize

was obtained with a 5-fold improvement in terms of LOD in comparison to the corresponding GNP-based LFIA developed with the same immunoreagents.

Conclusions and outlook

Despite LFIAs still being regarded in some ways as an emerging and incoming technology for mycotoxin detection [38-39], there are several examples of fully developed devices described in the literature and also available as commercial kits. Applicability of these systems in various food and feed matrices has also been demonstrated through comparison to reference analytical techniques, such as liquid chromatography, or to well-accepted screening methods, such as, for example, ELISAs. Annual updates of state-of-the-art techniques underline the growing interest in the field and the increasing relevance of this technology over more established screening techniques [6-7,57,74]. Notwithstanding the research is conditioned by the attainment of an effectively functioning device, often at the expense of true innovation, except in a few rare cases. Among conceivable routes of development some could be regarded as more attainable, such as those concerning: (i) detectability improvement, namely strategies for attaining lower detection limits (such as modifying the format of the assay, tailoring the mycotoxin conjugate used as the competitor in the T-line, varying the probe selected for antibody labelling); (ii) ruggedness, which means the reproducibility and stability of strips, the independence from prevalent ambient conditions to ensure an effective usability, and the congruence of interpretation of visual observation; (iii) adaptation of extraction protocols (which are expected to influence the mismatching experienced between fortified and naturally contaminated samples and to contribute to determining selectivity towards matrix components) with the further aim of limiting the use of hazardous chemicals to render the assays effectively applicable outside the laboratory.

Phycotoxin relevance in food safety assessment is gaining recognition and new analytical devices for the monitoring of such hazardous metabolites are appearing, though bioassays are traditionally and prevalently employed for the purpose [9,75]. Limitations to the development of LFIA, as for other immunoassays for this class of compounds, lie in the unavailability or expense of the pure toxins in such quantities as to allow the preparation of conjugates and obtain antibodies. Furthermore, phycotoxin monitoring implies the detection of groups of compounds which is a major drawback for the overly selective immunochemical techniques. Nevertheless, potential benefits of disposable point-of-use tests and of rapid and economical screening tools would promote research in this field.

Furthermore, recent breakthroughs of research in the immunoassay field could also incite to more innovation in the conception of LFIAs for myco- and phycotoxins, such as replacing antigens by

means of mimotope peptides [76], with undoubted advantages in economic and safety terms, and improving/tailoring antibody performances by exploiting phage display technology [77-78]. Particularly suggestive could be strategies aimed at the identification of synthetic selective recognition systems which would allow overcoming the need of producing specific antibodies towards toxins. Aptamers with selective binding properties towards OTA have been demonstrated to replace antibodies in the development of an effective LFD [45] and this approach could be expected to extend to other toxins. Molecularly imprinted polymers have also been described as synthetic selective ligands for myco- and phycotoxins [79-80], however their application in immunochemical methods of analysis is still challenging.

Acknowledgements

This study was supported by the Italian Ministry for Universities (PRIN, Prot. n.2007AWK85F_002)

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Figure captions

Figure 1. Schematic of the indirect format of a competitive lateral flow immunoassay. The test zone is formed by adsorbing a conjugate of the target compound (toxin). The control zone is formed by anti-species antibodies (white), reporters are specific (anti-toxin antibodies, black) and non-specific antibodies (grey) labelled with GNP.
Focalization of GNP-labelled antibodies and colour appearance on both the test and control lines occurs in the absence of the target compound (A), whereas only the control line appears when the target compound is present because saturation of specific antibodies prevents their binding in test zone (B).

Figure 2. Schematic of the direct format of a competitive lateral flow immunoassay. The test zone is formed by adsorbing the antibody specific (black) towards the target compound (toxin). The control zone is formed by a second ligand such as an antibody directed towards a non-target antigen (white) or streptavidin. Part of the GNPs are functionalized with a conjugate of the target compound, the other with the partner of the second ligand (e.g.: a non-target antigen or biotin). The biotin-streptavidin pair assures colouring of the control zone regardless of what happens in the Test zone. In the absence of the target compound, GNPs functionalized with the target conjugate are captured and colour appears in test zone (A). The target compound, if present, competes with functionalized GNPs for binding in the test zone and thus inhibits colouring of the line (B).

Table 1. Overview of the literature concerning validated lateral flow immunoassays for measuring mycotoxins

Target	Detection	Signal reporter	Cut-off / LOD	Commodities	Ref
AFB_1	visual	GNP	5 µg/kg	pig feed	48
AFs	instrumental	GNP	1 µg/kg	maize	26
AFM_1	instrumental	GNP	0.4 µg/l	milk	53
AFM_1	visual	GNP	0.5-1 µg/l	milk	51
AFB_1, OTA	visual	GNP	AFB_1: 10 µg/kg, OTA: 50µg/kg	feed	62
DON	visual	GNP	250 µg/kg	wheat, pig feed	23
DON, ZEA	visual	GNP	DON: 1500 µg/kg, ZEA: 100 µg/kg	wheat	29
FMBs	instrumental	GNP	200 µg/kg	maize	22
FMB_1	instrumental	GNP	120 µg/kg	maize	17
FMB_1	instrumental	peroxidase	25 µg/kg	maize	73
OTA	instrumental	GNP	1.5 µg/kg	maize, wheat	27
OTA, ZEA	visual	GNP	OTA: 5 µg/kg, ZEA: 10 µg/kg	corn	63
T-2	visual	GNP	100 µg/kg	wheat, oat	21

Table 2. Overview of the literature concerning LFIA developed for measuring mycotoxins. Cut-off and/or limit of detection are provided as evaluated in buffered solutions. Applicability in food samples, if preliminary evaluated, is highlighted.

Target	Detection	Signal reporter	Cut-off / LOD	Commodities	Ref
AFB_1	instrumental	dye-encapsulating liposomes	18 ng	-	54
AFB_1	visual	GNP	2.5 µg/l	-	25
AFB_1	visual / instrumental	GNP	2.5 µg/l / 0.1 µg/l	rice, corn, wheat flour	20
AFB_1	visual	GNP	0.5 µg/l	grain, feed	44
AFB_1	visual	GNP	0.5 µg/l	rice, barley, feed	50
AFB_2	visual	magnetic nanogold microspheres	0.9 µg/kg	peanuts, hazelnuts, pistachio, almonds	55
AFB_1	visual	silver-gold nanoparticles	0.1 µg/l	cereals, nuts	56
AFB_1	visual	GNP	1 µg/l (fortified sample extract)	peanuts, oil, feedstuffs	49
DON	visual	GNP	50 µg/l	wheat, maize	24
FMB_1	visual	GNP	1 µg/l	corn, barley, peanuts, oats, rice, sorghum	64
FMB_1	visual	GNP	1-5 µg/l	maize	65
OTA	visual	GNP	500 µg/l	-	58
OTA	visual	GNP	1 µg/l	cereals, raisins, beer, coffee	28
OTA	visual	GNP	5-10 µg/l	coffee	60
OTA	visual	GNP	10 µg/l	cereals, soybean	59
OTA	visual / instrumental	Fluorescent QDs	5 µg/l / 2 µg/l	red wines	47
OTA	visual / instrumental	GNP	50 µg/l / 5 µg/l	maize, barley	43
ZEA	visual	GNP	2.5 µg/l	corn	63

Table 3. Overview of the literature concerning lateral flow immunoassays developed for measuring phycotoxins

Target	**detection**	**signal reporter**	**cut-off / LOD**	**commodities**	**ref**
DA	visual	GNP	1-5 µg/l (buffer)	mussels	42
DA	visual	GNP	20,000 µg/kg (sample)	shellfish	41
MCs	instrumental	GNP	0.05 µg/l (buffer)	tap water, river water	16
MCs	visual / instrumental	GNP	5 µg/l / 1 µg/l (water sample)	drinking water, salt water, mussels	35
MCs	instrumental	Fluorescent liposomes	0.06 µg/l (buffer)	water bloom	37
MCs	instrumental	Fluorescent dye	0.05 µg/l (buffer)	water (from cell culture)	15
MCs	instrumental	Fluorescent dye	0.2 µg/l (buffer)	water (from cell culture)	70
MCs	instrumental	Fluorescent dye	0.2 µg/l (buffer)	-	67
OA	visual	GNP	80,000 µg/kg (sample)	mussels	31
OA	visual	GNP	160,000 µg/kg (sample)	bivalve molluscs	32
PbTxs	visual	GNP	10 µg/l (buffer)	molluscs	30
STXs	visual	GNP	400 µg/kg (sample)	shellfish, phytoplankton	18
STXs	visual / instrumental	GNP	12 µg/l (buffer)	-	19
STXs	visual	GNP	400 µg/kg (sample)	bivalve molluscs	40

Competitive lateral flow immunoassay for myco- or phycotoxin: the *Test* zone is formed by adsorbing a conjugate of the target compound (*toxin*); *Control* zone is formed by anti-species antibodies (*white*), reporters are specific (anti-toxin antibodies, *black*) and non-specific (*grey*) antibodies labelled with gold nanoparticles (*GNP*). Focalization of GNP-labelled antibodies determines a visible/detectable colour appearance on both the Test and Control lines, which can be related to analyte amount in a liquid sample.

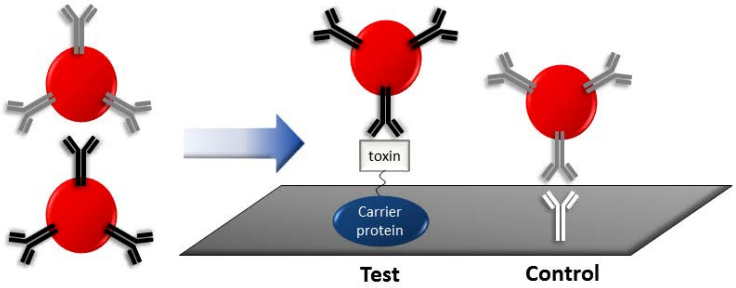


Figure 1.

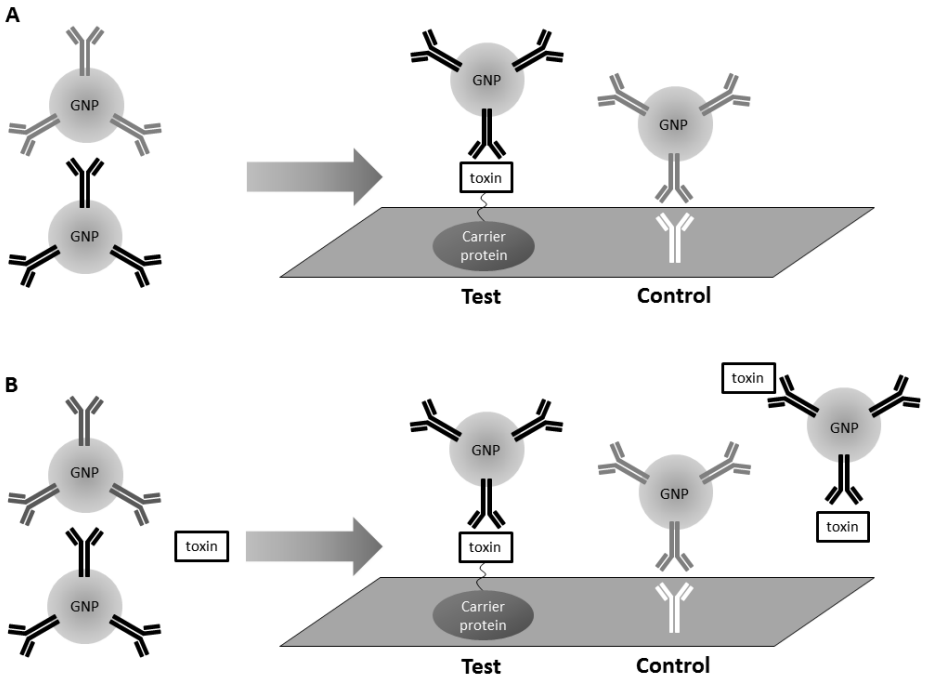
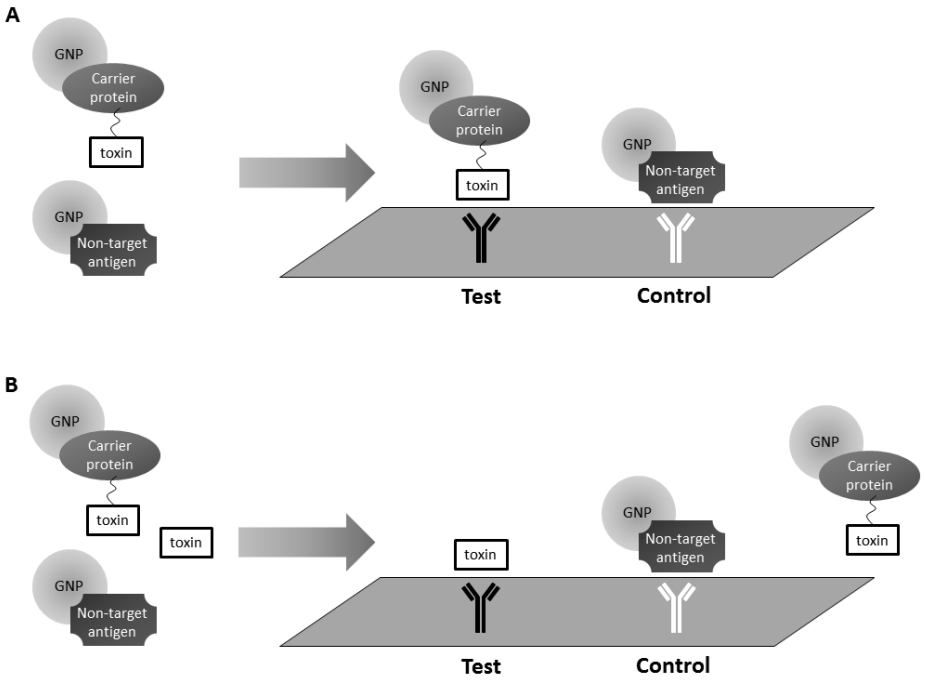

A
GNP
GNP
GNP
toxin
Carrier protein
Test
GNP
Control
B
GNP
GNP
toxin
GNP
toxin
GNP
toxin
toxin
Carrier protein
Test
Control

Figure 2


A
GNP
Carrier protein
toxin
GNP
Non-target antigen
GNP
Carrier protein
toxin
GNP
Non-target antigen
Test
Control
B
GNP
Carrier protein
toxin
toxin
GNP
Non-target antigen
toxin
GNP
Non-target antigen
GNP
Carrier protein
toxin
Test
Control