Chapter

Antibody-Antigen Binding Events: The Effects of Antibody Orientation and Antigen Properties on the Immunoassay Sensitivity

Vanessa Susini, Chiara Sanguinetti, Silvia Ursino, Laura Caponi and Maria Franzini

Abstract

The sensitivity of an immunoassay depends on a complex combination of the physicochemical characteristics of antigens, antibodies, and reaction surfaces, which are the main elements on which the analytical principle of this technique is based. Among these characteristics is possible to include the type of surfaces, the affinity and avidity constants of antibodies, as well as antigen properties. This chapter focuses on the importance of the capturing surface in determining the analytical sensitivity of an immunoassay. It is an established knowledge that the sensitivity of immunoassays is affected by the orientation, the valence, and the spatial distribution of the capturing antibody. In addition, the size and the number of epitopes on the antigens (monovalent or multivalent) can influence the performances of these assays. In this chapter, the authors discuss how the combination of these factors reflects on the sensitivity of immunoassays.

Keywords: antibody, antigens, ELISAs, LFIAs, immunoassay, oriented-binding, affinity, avidity

1. Introduction

Enzyme immunoassays are based on the use of antibodies (Abs) to detect specific antigens (Ags). Among them, the enzyme-linked immunosorbent assay (ELISA) is the most frequently used. ELISA is a heterogeneous enzyme immunoassay technique, where one of the reaction components is nonspecifically adsorbed or covalently bound to the surface of a solid phase; the latter being usually provided by 96-well or 384-well polystyrene plates. The most common approach to using the ELISA technique is the "sandwich" type in which the antigen is bound by a so-called "capture antibody" immobilized on the solid surface. Then, an enzyme-labeled antibody (Ab*) is added to form an Ab-Ag-Ab* sandwich. The immunocomplexes are revealed

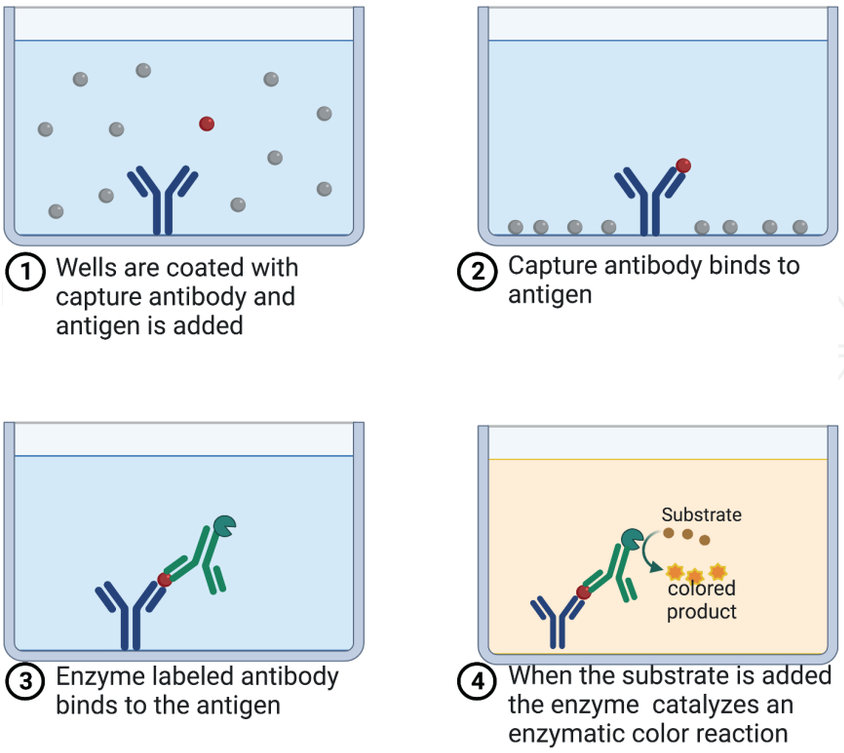

Figure 1.
Main phases of ELISA sandwich.

by adding the enzyme-substrate, which is converted to a detectable product whose amount is proportional to the quantity of Ag [1–3] (**Figure 1**).

ELISAs were first described in 1971 by Engvall and Perlmann as a rapid and sensitive method for detection and quantification of an antigen [4]. Today, this method is widely used in many different contexts, such as in clinical chemistry, environmental analysis, or quality control laboratories.

The lateral flow immunoassay (LFIA) or immunochromatographic strip is another widely used immunoassay format. Its success is mainly due to fast result (> 30 min), low development costs, and ease of production [5]. LFIAs contain all the required reagents within the strip itself that is usually supplied as a plastic cassette. The sample is loaded into a well that is in proximity to the conjugate pad containing the detection Abs that are conjugated with latex or gold microparticles. The Ags bind to the detection Abs and the so-formed immunocomplexes flow by capillary forces along a solid phase constituted by paper or nitrocellulose. The immunocomplexes are bound by the capturing Abs, which are coated on a specific region of the solid phase that is called "test line." The presence of the looked-for Ag is highlighted by the appearance of a colored line [3] (**Figure 2**). For a detailed description of LFIAs see Ref. [6]

LFIAs are used in medicine for the qualitative and quantitative detection of specific Ags, but they are also employed in veterinary medicine, food and environmental science, and even by police forces and regulatory authorities for the rapid detection of drugs [5].

The wide use of both ELISAs and LFIAs justifies the many ongoing studies aimed at improving their analytical performances.

Although the affinity of the antibody for its antigen may be considered the main factor determining the sensitivity of immunoassays, the question is far more complex. The overall design of ELISAs and LFIAs relies on a stepwise addition of "layers,"

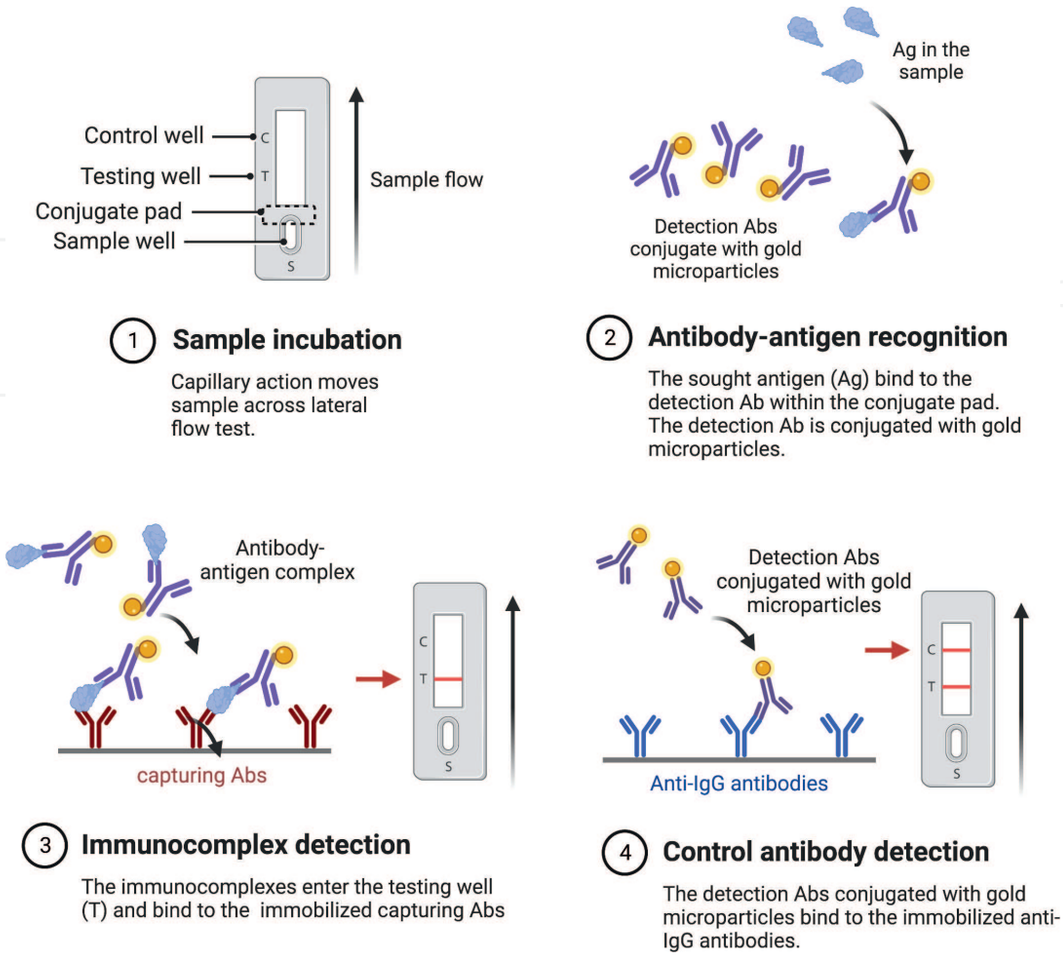

Figure 2.
Representation of the working principle of LFIA.

where each one affects the performance of the following one. Indeed, the type of surfaces, the distribution of Abs, and the physicochemical properties of Ags contribute to determining the analytical performances of immunoassays.

The aim of this chapter is to describe the importance of these features in the development of ELISAs and LFIAs.

2. Antibodies adsorption

ELISA is particularly useful when testing for low levels of analytes in biological samples since the antigen is concentrated by the antibody, which coats the solid surface. Since 90s, researchers have studied how antibodies interact with different plastic surfaces to obtain an efficient binding of the antigen. Stevens and coworkers investigated the adsorption of antibodies on several types of polystyrene microtiter plates with different protein binding capacities [7]. The authors observed two possible patterns of binding. The first followed the Langmuir model, according to which Abs were adsorbed as a monolayer without lateral interactions among them. The second pattern, instead, was characterized by the presence of Ab-Ab interactions. Interestingly, Abs coated according to the second pattern were more susceptible to desorption in the subsequent steps of the ELISA. This could be due to a supersaturating concentration

of coated antibodies resulting in the formation of protein multilayers, which are less stable than protein adsorbed directly on hydrophobic surfaces of polystyrene [8, 9]. Therefore, it is recommended the choice of surface materials that allow the formation of Ab monolayers.

Several researchers studied the Abs adsorption on planar surfaces with the aim to describe their distribution and orientation in monolayers. When adsorbed on polystyrene surfaces, Abs can assume four different orientations: end-on (Fab-up or Fab-down), side-on, or flat-on (**Figure 3**).

Buijs and coworkers [10] established the mass of Ab monolayers composed of only one of the possible orientations. The flat-on orientation allowed the binding of 200 ng/cm^2 Abs and the end-on orientation of 370 ng/cm^2 or 550 ng/cm^2, depending on the distance between fragment antigen-binding regions (Fabs). Quartz crystal microbalance measurements showed that monolayers obtained on planar polystyrene by random adsorption contained 468 ng/cm^2 Abs. This value corresponded to a mixture of antibodies orientations, in fact, Abs are initially adsorbed in a flat-on orientation, then the residual binding sites are filled up by Abs in an end-on orientation [11–14].

Several studies showed that the formation of an Ab monolayer depends on the concentration of the solution used to coat polystyrene surfaces. The use of coating solutions containing Abs up to 20 μg/ml was associated with a progressive increase of both adsorbed Abs molecules and antigen-binding capacity. Solutions exceeding the 20 μg/ml threshold favored the deposition of Abs in multilayers that negatively affected the antigen-binding capacity of the surface since the access of antigens to the binding sites is limited by steric hindrance [7, 12, 13, 15–18].

The adsorption of molecules to polystyrene surfaces can be mediated by intermolecular attraction forces (i.e., van der Waals forces), hydrophilic bonds (e.g., hydrogen bonds), or hydrophobic interactions. The type of bond predominantly used by proteins depends on the prevalence of hydrophobic or hydrophilic amino acid residues [19]. Based on this, several types of adsorbent polystyrene surfaces have been developed to promote the adsorption of predominantly hydrophobic or hydrophilic proteins. Regarding Abs, their adsorption is maximized on microtiter plates made from polystyrene modified by adding hydrophilic groups. These types of plates favor also more orderly adsorption of Abs through the interaction between the carbohydrate moieties in their Fc fragments and the hydrophilic groups of the polystyrene surfaces [19].

Physical adsorption of Abs was mainly studied on polystyrene surfaces, but the same principles can be extended to other kinds of solid surfaces such as paper and cellulose membranes. In fact, physical adsorption of Abs may occur on these kinds

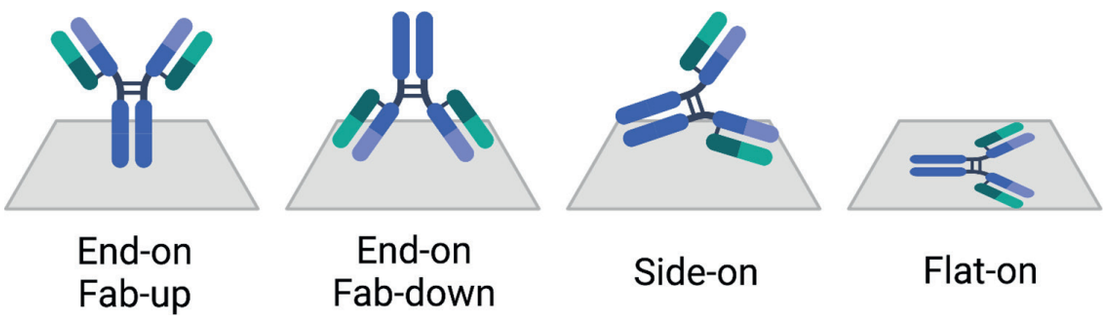

Figure 3.
Different antibodies orientations on polystyrene surface.

of surfaces by van der Waals forces, hydrogen bonds, and hydrophobic interactions. But the leading forces involved in Abs binding on cellulose membrane are the electrostatic interaction between cationic groups of proteins and anionic groups of cellulose itself [20]. Despite the hydrophilic nature of cellulose, also in these cases a partial denaturation and random orientation of Abs have been observed [21, 22]. Currently, researchers are investigating strategies to increase the sensitivity of paper or cellulose-based immunoassays by functionalizing them with suitable chemical compounds to obtain a more homogeneous capturing surface without affecting the folding of Abs. These approaches will be discussed in the next session.

3. Antibodies orientation

As described above, Abs are adsorbed on polystyrene in different orientations that can affect the biological function of Abs. Three out of the four possible orientations (i.e., Fab-down, side-on, or a flat-on) cause the reduction or the loss of antigen-binding capacity [12]. In fact, only 5–10% of the antigen-binding sites are effectively available when Abs are randomly adsorbed on the polystyrene surface [23]. Moreover, the adsorption on polystyrene can induce a partial denaturation of Abs [11, 23–26]. Overall, the spontaneous adsorption of Abs on polystyrene is a widely applied technique due to its ease of use, but it reduces the total antigen-binding capacity of capturing surfaces, and it is associated with a poor signal-to-noise ratio. It is now well established that surfaces coated with Abs uniformly bound in the Fab-up orientation lead to a significant improvement of ELISAs sensitivity. For this reason, several researchers have studied strategies to orient Abs on planar surfaces while preserving their original folding (**Figure 4**).

The first method described to orient Abs is the covalent coupling of their amine or carboxylic groups on chemically activated surfaces [27–31]. This method allowed an improvement in ELISAs sensitivity, but not a uniform distribution of Abs since amino and carboxylic groups are throughout the Ab structure [32]. For this reason, the covalent coupling is not a site-controlled orientation method.

Another approach consisted of the immobilization of Abs based on Ab-biotin/streptavidin-surface complexes that increased the availability of antigen-binding sites up to 70% when Ab was randomly biotinylated [23, 33, 34]. Peluso and coworkers compared the effect of the random or site-direct biotinylation of Abs and Fab fragments on their binding capacity [35]. In this work, Abs were specifically biotinylated on the oligosaccharide moiety of the Fc fragment, while Fab were biotinylated on the reduced thiols of the hinge region. Results showed that site-direct biotinylation of Abs and Fab' provided 10- and 5-fold higher signals, respectively, in comparison with the randomly biotinylated counterpart. As a result of these observations, several site-direct methods to orient Abs have been developed.

A common specific method to orient Abs takes advantage of intermediate proteins that bind the Fc fragment of Abs [36]. Protein A and protein G are cell wall proteins expressed by *Staphylococcus aureus* and *Streptococcus* species, respectively. Both proteins A and G specifically bind amino acid residues at the interface between the constant domains C_{H2} and C_{H3} of mammalian IgGs [37].

Protein A binds all human immunoglobulin G (IgG) subclasses except for IgG3. It can also bind human IgA, IgM, IgE, and mouse IgG2a, IgG2b, and IgG3 [38]. Protein G recognizes Fc domains of all human IgG subclasses, as well as rabbit, mouse, and goat IgGs [39].

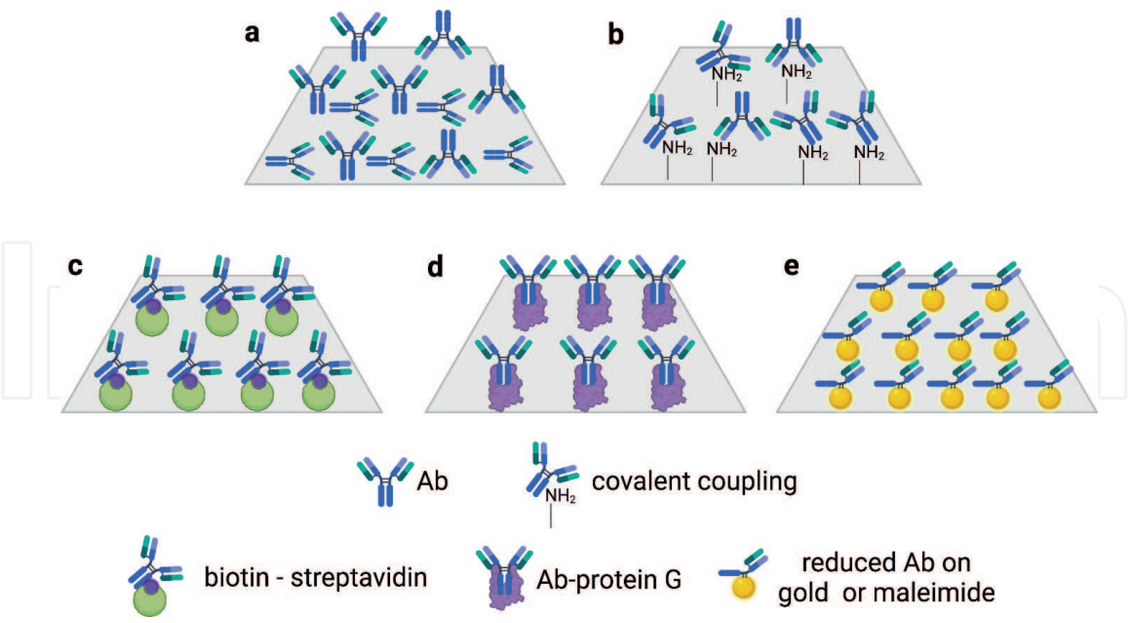

Figure 4.
Antibodies orientation strategies on polystyrene surface. a) Random orientation by adsorption. b) Random orientation by covalent coupling. c) Site-direct orientation by biotinylated antibody and streptavidin-coated surface. d) Site-direct orientation by protein G-coated surface. e) Site-direct orientation by reduced ab on gold or maleimide surface.

Protein G/A were first used in affinity chromatography to purify Abs [40], then their use was extended to immunoassays applications. The first described application was a biosensor for human IgGs detection based on protein A and quartz crystal microbalance [41]. This approach was then applied by Prusak-Sochaczewski and Luong, that described the use of protein A for Ab immobilization in immunoassays [42]. Since then, a number of immunoassays that use protein A/G to orient Abs on the surface were developed; for extensive review elsewhere [43, 44]. It could be argued that also the orientation of protein A/G might affect the overall binding capacity of capturing surface. Orientation of protein A/G affects the Ab density, but experimental results showed that the maximum increase in antigen-binding capacity was obtained with non-oriented protein G/A-Ab complexes compared to randomly adsorbed Ab. Protein A/G orientation did not provide a further significant increase [43].

Another interesting method of site-directed Ab immobilization involves disulfide bridges located in the hinge region of Abs. These disulfide bridges can be specifically reduced by mild reducing agents, such as tris(2-carboxyethyl)phosphine hydrochloride (TCEP), dithiothreitol (DTT), or 2-mercaptoethanolamine (2-MEA). The so-obtained monovalent Abs can self-assemble and spontaneously orient on conveniently functionalized surfaces [45–49]. The functionalization of surfaces includes the layering of gold [50], maleimides [51], and pyridyl disulfides [52]. ELISAs, performed with oriented monovalent antibodies, showed improved antigen-binding capacity compared to the same assay performed with randomly adsorbed Abs [53].

The contribution of reduction and orientation on the antigen-binding capacity was investigated separately by coating monovalent and whole Abs on polystyrene or maleimide functionalized microplates. Results highlighted that the chemical reduction by itself increased the antigen-binding capacity of Abs, probably as a consequence of folding rearrangements that influence the affinity for the antigen-binding site. Orientation was shown to improve both the sensitivity and the reproducibility of the results likely providing a more homogeneous capturing surface [54].

Some of the approaches previously described to orient Abs on ELISA surfaces have now been applied also to LFIAs to improve their sensitivity. Capturing Abs on the test line (**Figure 2**) can be oriented by using fusion proteins that have a binding site both for cellulose and Fc fragments of Abs. Yang and coworkers [21] developed a fusion protein composed of a cellulose-binding module and the Fc-binding domain of protein A. Authors found that the orientation of capturing Abs on the test line, obtained by the developed fusion protein, increases the sensitivity of the LFIAs when compared to randomly adsorbed Abs [21]. Another interesting approach involves the orientation of the detection Abs instead of the capturing ones. It was shown that also the orientation of Abs on the detection beads increased the sensitivity of the assay and this increase was obtained even if the amount of immobilized Abs was lower in comparison with a random absorption on the beads [55, 56].

To conclude, orientation of Abs increases the overall antigen-binding capacity of ELISA and LFIA surfaces and this contributes to enhance the sensitivity of these kinds of assays. This is especially true for small antigens that have a Stoke radius smaller than those of Abs since, in this case, the simultaneous binding of two antigens for Ab molecule is possible [35].

The contribution of analyte and antibody properties to the sensitivity of oriented ELISAs is discussed in the next section.

4. Effects of antibody and antigen properties on the sensitivity of immunoassays

In the development of immunoassays, one of the key steps is the selection of the Ab to be immobilized on the surface. In this regard, the two factors certainly taken into consideration are the specificity toward the Ag of interest and the affinity constant of the Ab for the selected epitope on the Ag. Anyway, the orientation of the Ab, its avidity, and the physicochemical characteristics of the antigens (size, mono-multivalency) should be also considered.

The importance of Ag properties on the effect of uniform orientation of Abs has been investigated by a few authors. Trilling and coworkers [57] studied how the dimension of Ags affects the sensitivity of ELISAs based on oriented Abs. Considering the Stoke radius of Ags, the authors showed that the greatest advantage in using oriented ELISAs was observed with small Ags, since they could interact with all the accessible antigen-binding sites. Whereas oriented ELISAs for large Ags did not allow for a significant increase in sensitivity in comparison with the non-oriented counterpart. This is probably due to the steric hindrance of Ags that limits access to neighboring antigen-binding sites. These results find confirmation in publications by other researchers [35, 54].

Studies aimed to investigate oriented ELISAs analytical performances in the presence of different concentrations of Ags revealed the importance of considering the affinity of Ab in the setup of ELISAs. The affinity of an Ab for its Ag can be described by the dissociation constant which is a measure of the strength of the interaction between an epitope and a single antigen-binding site. Collected data suggested that affinity is a major determinant of the analytical sensitivity of all types of ELISAs [57], especially in the oriented ELISAs in which the maximum advantage was observed for Ag concentration below the dissociation constant [35, 58].

Avidity is a measure of the overall strength of binding of Ab-Ag complexes, and it depends on the affinity and valency of both Abs and Ags. The role of avidity is

especially evident with multimeric Ags, which can engage multiple antigen-binding sites by the repeated epitopes exposed on their surface. The multiple binding leads to the stabilization of the Ab-Ag complexes, suggesting that avidity is the leading force involved in the binding of multimeric Ags. This occurs in both oriented and non-oriented ELISAs [59, 60]. A clue to the role of affinity and avidity on the analytical sensitivity of ELISAs was obtained by comparing a non-oriented and an oriented ELISA for ferritin, a multimeric protein. The same capturing Ab was used for the two assays: in the non-oriented assay polystyrene was coated with whole Ab, while in the oriented assay reduced monovalent Ab was used. As mentioned above, chemical reduction improves the affinity of the antigen-binding site [54]. No differences were observed between the two types of ELISAs for ferritin. These data suggested that the avidity is predominant in affinity when considering multimeric Ags [58].

In light of the described studies, it is possible to conclude that Ag properties, such as dimension and valency, have an important role in the setup of ELISAs, and for extension also in the setup of LFIAs.

5. Conclusions

The formation of the Ab-Ag immunocomplex is one of the factors determining the sensitivity of ELISAs and LFIAs. Its formation is dependent both on the orientation and density of Abs coated on the surface and Ags properties. In ELISAs, the selected polystyrene and the concentration of the Abs coating solution should ensure the formation of a monolayer of Abs in which each Ab molecule interacts directly with the surface. This is the condition that ensures the maximum stability of the binding between Abs and polystyrene but not the maximum accessibility of antigen binding sites. The direct interaction with the surface (polystyrene, paper, or cellulose membrane) can cause a partial denaturation of Abs and the random orientation can mask an unpredictable amount of antigen binding sites. Indeed, it was estimated that only 5–10% of the antigen-binding sites remain effectively available.

To overcome these limits, several strategies have been developed to preserve Abs folding and to orient them with their antigen-binding sites facing outwards the surface. These strategies showed to be efficient in enhancing the sensitivity of ELISAs and LFIAs, besides, studies to develop them stand out the importance of Ags characteristics in determining the formation of Ab-Ag complexes. It can be easily understood that the quantification of small Ags takes advantage of the orientation of Abs as small Ags can effectively use the greater number of accessible antigen-binding sites provided by orientation. It is not so clear the role of the Abs coated surface on the binding of multivalent Ags that can engage multiple antigen-binding sites. Thus, the main property of Abs contributing to the formation of Ab-Ag complexes is avidity, which is often overlooked.

In conclusion, a broader perspective that goes beyond the selection of Abs just on the basis of affinity should be introduced in ELISAs and LFIAs development. Indeed, the best solution to favor the antigen-binding event should be tailored to both the Abs and Ags properties.

Conflict of interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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