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Nanomaterial and interface advances in immunoassay biosensors

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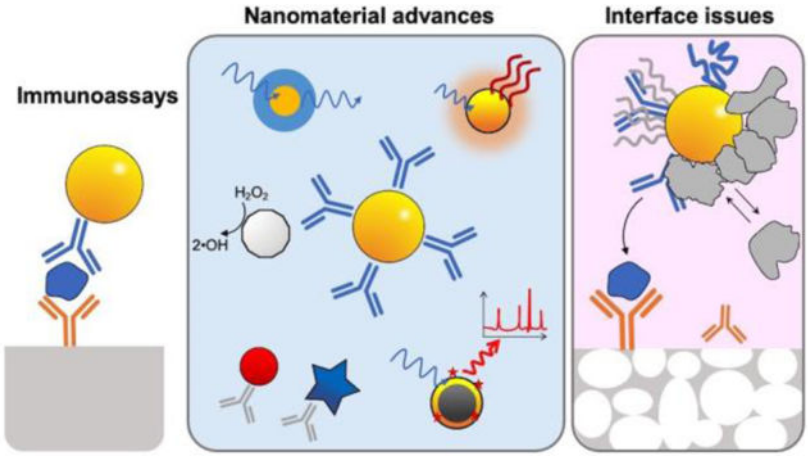
Abstract

Biosensors have been used for a remarkable array of applications, including infectious diseases, environmental monitoring, cancer diagnosis, food safety, and numerous others. In particular, the global COVID-19 pandemic has exposed a need for rapid tests, so the type of biosensor that has gained considerable interest recently are immunoassays, which are used for rapid diagnostics. The performance of paper-based lateral flow and dipstick immunoassays is influenced by the physical properties of the nanoparticles (NPs), NP-antibody conjugates, and paper substrate. Many materials innovations have enhanced diagnostics by increasing sensitivity or enabling unique readouts. However, negative side effects can arise at the interface between the biological sample and biomolecules and the NP or paper substrate, such as non-specific adsorption and protein denaturation. In this Perspective, we discuss the immunoassay components and highlight chemistry and materials innovations that can improve sensitivity. We also explore the range of bio-interface issues that can present challenges for immunoassays.

Graphical Abstract



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Keywords

Biosensors; rapid diagnostics; nano-bio interfaces; immunoassay; nanoparticles; lateral flow assay

Introduction

Biosensors of all types are incredibly useful and have been developed for a wide range of applications. These include ELISA assays for biomarkers of disease, drug tests, as well as sensors for environmental monitoring, agricultural crops and aquaculture. Biosensor development draws on multiple disciplines, incorporating aspects of analytical chemistry, physical chemistry, molecular biology, materials science, electrical engineering, signal processing, and medicine. Biosensors can take on many forms, with glucose sensors and pregnancy tests being the most common. Diagnostics are the subset of biosensors which translate the result into a decision-making tool. Diagnostics markets are growing, where the rapid diagnostics market alone is projected to be USD$72B in 2027, an increase from USD $43.2B in 2022. [1] Much of this is driven by the demand for COVID-19 rapid tests, which surged during the global pandemic. [2–5] Because of this sudden and acute need, there was a concerted push to create new rapid tests for diagnosing patients, and currently multiple manufacturing routes for diagnostics now exist.

Biosensors have benefited from materials innovations. Inorganic materials have been exploited for their magnetic, optical, catalytic, and electrochemical properties, which can be leveraged to increase sensitivity, introduce multiplexing, and many other novel capabilities. The unique properties of materials have yielded advances in other applications such as cancer imaging and therapy. However, biosensor development has shed light on the fact that interfaces between biological molecules and inorganic materials create a whole set of challenges. These interface issues arise from immobilizing biological molecules onto abiotic systems such as nanoparticles or surfaces, or incorporating inorganic nanomaterials into biological fluids and environments. Unfortunately, these nano-bio interface issues simply cannot be ignored and need to be controlled, because diagnostics must be operated in complex biological fluids such as saliva, urine, and blood.

Therefore, for constructing successful biosensors and diagnostics, there is a need to better understand and control the nano-bio interface, where nanomaterial innovations must go hand-in-hand with the interface issues that may arise. Here, we discuss the basics of biosensors and then discuss both the materials innovations that impart new capabilities to biosensors as well as the unique challenges of the nano-bio interface and discuss strategies to control it. While there are many reviews that cover nano-bio interfaces, [6, 7] what has been relatively unexplored are those with a focus on immunoassays, which has interfaces that inherently more complex. We wish to underscore the significance of this type of biosensor as they are used in COVID-19 rapid diagnostics, and so the nano-bio interfaces in these systems carry special consequences because they are used as a tool for decision-making for patient treatment or quarantining, so phenomena such as non-specific adsorption can result in false positives.

We first describe the basic components of a biosensor, then discuss different innovations in nanomaterials used in immunoassays, followed by discussion of the properties of the nano-bio interface.

Biosensor basics

Biosensors are designed to detect a sample analyte and produce a signal, and have a basic structure shown (Figure 1). The **analyte** is recognized by a **receptor**, typically a biological recognition element. The specific interaction changes a measurable quantity (color, fluorescence, pH) and the binding event is transduced by the **signal transducer** into a signal for readout, which is followed by data processing of the signal.

Analyte—The analyte is the target species for detection, and in biological and medical applications can be a protein biomarker, nucleic acid, or small molecule. For diagnostics, the choice of the target biomarker is critical as it is used as an indicator for infection. It should be present at a detectable concentration, but not have high background levels. The target levels for infected patients needs to be known so that the sensor limit of detection (LOD) can be benchmarked against it, and is a measure of whether the sensor is sufficient. Unfortunately, biomarker levels are not always known, especially if the disease is newly emerging. To complicate matters further, biomarker levels vary with the biological fluid (blood vs. urine vs. saliva) and the time course of infection. For sensors for pathogenic bacteria in water or food, the relevant factor is the amount that will make one sick, or the infectious dose (ID50), which can vary with disease. For certain strains of *E. coli* O157:H7, this number is just 100 cells, but for *V. parahaemolyticus,* this number is 3×10^6 cfu for ingestion.[8, 9]

Some viral infections have biomarkers that are relatively easy to detect and thus are good indicators of infection. For flaviviruses such as dengue and zika, the biomarker is typically nonstructural protein 1 (NS1), which is secreted into the blood stream at high concentrations. In addition, NS1 is present soon after infection during the window when a patient is exhibiting non-specific symptoms such as fever. This is before the immune response mounts and IgG and IgM are produced. Thus, detection of NS1 can be used in a diagnostic tool to determine infection. On the other hand, for SARS-CoV-2, and some other coronaviruses and other diseases, there is no secreted protein at high enough concentrations, so an antigen test must detect the proteins of the virus itself.

Affinity agent—The receptor in a biosensor is responsible for binding to the target. Depending on the target, this affinity reagent can be antibodies, proteins, peptides, or complementary nucleic acids (Figure 1). Typical affinities for the biomarker can vary, where antibody affinity is usually in the nanomolar (nM) range, while peptide affinities are in the 100s of nM range. [10] In some cases, an enzyme can be used as the recognition agent, where it uses the analyte as a substrate to convert into a product, and the presence of the product is read out. This is the mechanism of glucose biosensors that utilize the enzyme glucose oxidase to convert glucose into a product. In addition, sensors must be designed to be specific, where the binding agent is designed to bind exclusively to the target. Predominantly, antibodies are used as biorecognition reagents due to their specific binding

and high affinities for targets, but antibodies can exhibit cross reactivity. Some infectious diseases are closely related, where targets for the nonstructural protein 1 (NS1) for dengue and zika can be as high as 70–80%.[11] Thus, one has to take into account for species that may be present for which the antibodies could be cross-reactive, and also to verify that the sensor does not pick these up. However, sensors can also be designed to bind selectively, where the targets are exposed to an array of affinity agents, and the binding pattern is used for target identification. [12]

The choice of the biorecognition affinity agent depends on the epitope on the target. Sandwich immunoassays require two binding events, so the target must be able to bind simultaneously to two antibodies (antibody "pairing"). Thus, the epitopes must not interfere with the other's ability to be recognized by its respective antibody. Polyclonal antibodies can bind to multiple epitopes, whereas monoclonals bind to only a single one. Monoclonals can be more desirable due to the higher specificity of the reaction, but because polyclonals can bind to multiple epitopes, they can have a higher probability of binding the target.

The availability of the affinity reagents must also be considered, where high cost and limited access can hinder the utility of a sensor or diagnostic. [13] Antibodies are highly attractive as affinity agents in sensors, but they must be raised against a specific against the target, a process that is expensive and time consuming, requiring up to 24 months.[14] One of the major limiting steps in antibody production is the cell line creation. [15] While commercial sources are widely available, cost can be a major barrier. Antibodies also are highly sensitive to temperature and environmental conditions, so stability can be an issue. In contrast, DNA and peptides are convenient as affinity agents as custom species can be synthesized commercially. In particular, the custom synthesis of nucleic acids is now inexpensive, facilitating the development of biosensors using DNA as capture, detection, or amplification agents.

Signal transducer—Once the binding event occurs, a signal must be transduced, where a measurable readout changes upon binding of the analyte to the receptor. The most common signal in immunoassays are visual readouts, which makes them amenable for low-cost diagnostics. Often this is achieved by gold NPs, which have a strong optical absorption and large molar extinction coefficient due to their surface plasmon resonance (SPR). Readout can be visualized by eye, or by mobile phone cameras. Other signals for readouts include fluorescence, Raman spectroscopy, magnetic, electrochemical readouts, piezoelectric, and mechanical where binding results in deflection of microcantilevers. [16]

The most relevant performance metric of a biosensor is its limit of detection (LOD), which is the lowest readable signal that is statistically significant above the background, which is defined as 3X the standard deviation of the signal when the analyte concentration is zero. Quantification of the limit of detection (LOD) of the assay is achieved done by measuring the signal as a function of analyte concentration, fitting it to a curve, and then extrapolating the concentration for which the signal is above background. For diagnostics, their usefulness is based on their ability to detect or exclude infection in a patient. The sensitivity and specificity are the relevant performance factors that quantify its accuracy, defined as:

$$Sensitivity = \frac{TP}{(TP + FN)} \quad (1)$$

$$Specificity = \frac{TN}{(TN + FP)} \quad (2)$$

where TP = True positives, TN = true negative, FN = False negative, and FP = false positive. [17] To measure sensitivity and specificity, the assay is tested on a pool of patient samples which have been measured against a gold standard, such as PCR.

Examples of Immunoassay innovations

Commercially available rapid diagnostics are predominantly paper immunoassays. This is because they are easy to use, where minimal sample preparation steps are required, and are typically made of low-cost materials, which enables manufacturing at scale, and thus are widely deployable. For immunoassays, the recognition element are antibodies specific for the biomarker, which can be an antigen, an antibody produced in an immune response, or even a small molecule. The signal transducer for rapid diagnostics are gold NPs that produce a visual color that can be seen by eye.

Immunoassays are inherently a transient binding event,[18] where the dynamics require the binding events to happen in a particular window. Thus, immunoassay performance is limited by the diffusion of the analyte and the NP-antibody conjugate. In addition, the binding reaction occurs as the sample flows through the strip, so it is under fluid flow and not under equilibrium conditions.[19]

One of the major drawbacks of traditional LFAs are their low sensitivity and specificity, because the visual readout requires a high enough concentrations of the target to generate a visual signal.[20] Readout can be negatively impacted by lighting conditions, which can result in incorrect test interpretation. To improve sensitivity and extend assay working ranges, a growing diversity of labels have been explored that exploit the unique properties of NPs, where different NP readouts can yield different limits of detection (Figure 2). In this section, we highlight some of the ways in which NP properties have been exploited in immunoassays (Figure 3a).

Colorimetric—NPs possess a surface plasmon resonance (SPR) which gives rise to an intense optical absorption, resulting in their strong color that can be seen by eye. Typically, visual readout of LFAs using the colorimetric properties of Au NPs achieves LODs in the ng/mL range. However, other strongly absorbing species as substitutes for Au NPs can improve sensitivity. Gold nanoshell particles (AuNSs), which are 150 nm gold coated silica particles, absorb light more strongly than smaller spherical Au NPs due to their larger size and large scattering cross sections. [21] Substituting AuNSs for the probe could improve detection of serum thyroid stimulating hormone (TSH) with a 26X decrease in LOD.[22]

Carbon nanotubes also possess strong optical features and have a black color due to their broad absorbance, and were similarly used as a label to detect rabbit IgG down to 1.3 pg/mL, an LOD 1000X lower compared to assays with Au NPs.[16]

The plasmonic properties of Au NPs have been investigated in the literature for decades, where the factors influencing their absorption spectra are now well understood.[23, 24] Classical Mie scattering theory and plasmon hybridization[25] has been used successfully to describe the strength of the SPR and how it is influenced by particle size and shape with high precision. This has enabled the tuning of the optical properties gold NPs by changing their size or shape. Au NP synthesis approaches can now achieve finely-tuned sizes and complex shapes, such as gold nanostars, nanorods, cubes, shells, and many others, which has facilitated changing NP dimensions to improve the intensity of the test line.[26, 27] In particular, it has enabled multiplexed readouts by making it multicolor (Figure 3b).[28] Di Nardo et al. [29] used red and blue gold NPs for the detection of aflatoxin B1 and type-B fumonisins which are important in food safety. Yen et al. created a multiplexed test for dengue, yellow fever, and Ebola viruses using silver nanoprisms of different colors.[30]

The aggregation of gold NPs can result in a color change due to the shift in SPR peak wavelength when in proximity of another NP.[31, 32] This has been exploited in many different formats for detection of nucleic acid acids, ions, and small molecules in solution or blood,[33] and more recently has been combined with LFAs to improve the colorimetric detection of adenoside and other biomarkers.[32, 34, 35]

Fluorescence—Another route to increase immunoassay performance is to use fluorescent reporters, as fluorescence measurements can reach sensitivities higher than colorimetric readouts. Typically fluorescent NPs or quantum dots (QDs) are substituted for Au NPs. QDs are attractive as they are robust to photobleaching compared to fluorescent dyes, and due to their size-dependent photoluminescence, enabling tunable colors by the changing QD core size (Figure 3c). For example, Li et al. used QDs in LFAs to improve the detection of nitrated ceruloplasmin, a biomarker for cardiovascular disease and lung cancer and could ultimately reach an LOD of 8 ng/mL.[36] Taranova et al. [37] used multiple colors for multiplexed assay of multiple antibiotics in milk (ofloxacin, chloramphenicol, and streptomycin), where presence of each antibiotic was correlated with a specific emission line, demonstrating the multiplexed capabilities of QDs, or as “barcodes” for multiplexed detection of hepatitis B, hepatitis C, and HIV.[38]

Combining fluorophores with gold NPs can enhance the fluorescent readout. Zhang et al. used this approach for multiplexed detection of three organophosphate pesticides (triazophos, parathion, and chlorpyrifos) in agricultural products such as rice, wheat, and fruits and vegetables. [39]

Fluorescent upconverting NPs (UCNPs) possess advantages over traditional dyes due to their higher photostability and strong fluorescence intensities. [40, 41] The upconversion allows for excitation in the NIR and emission in the visible, and this in combination with their sharp and narrow emission bands facilitates better separation of signal from background and thus lower background noise. The typical composition are rare earth doped NPs such as

Yb^{3+}/Er^{3+} doped $NaYF_4$ and numerous others. Liu et al. used UCNPs as labels in an IgG immunoassay and could achieve an LOD 0.1 ng/mL.[42]

Fluorescence lifetime measurements can be used to yield higher sensitivities. Lanthanide ions such as europium and terbium have advantageous intrinsic fluorescent properties such as long-lived fluorescence, narrow linewidths, and large Stokes shifts. Their long fluorescence lifetimes have been leveraged for time-resolved measurements, where the short lived autofluorescent background can be eliminated, resulting in high signal to noise ratios. There are examples in the literature using europium-doped particles of polystyrene on the label antibody in LFAs, measured by commercial LFA readers which have a fluorescent lifetime measurement option. Juntunen et al. compared a fluorescence lifetime LFA for prostate specific antigen (PSA) with a visual readout. They measured an LOD of 0.08 ng/mL, with up to a 3-fold increase in sensitivity relative to gold NPs. [43, 44] Liu et al. achieved an LOD of 5 pg/mL of HIV-1 p24 antigen, which is valuable for HIV detection as p24 levels in patients are low. [45]

SERS—Another technique that can increase immunoassay sensitivity is surface enhanced Raman spectroscopy (SERS), which has been successful in detecting analytes at trace amounts, down to attomoles. The Raman signal of a small molecule reporter is enhanced several orders of magnitude by its conjugation to a gold or silver NP. For detection of biological species, the nanotag format is often used, where a NP-reporter is linked to an antibody specific for the biomarker of interest, and this antibody is responsible for recognition of the target. Presence of the biomarker is read out by the Raman signal of the reporter at the test line. Compared to a visual read out, SERS detection in immunoassays can have a sensitivity higher by several orders of magnitude.[46] SERS nanotags have been used for detection of staphylococcal enterotoxin B, HIV DNA, and many other targets (Figure 3d).[47–50] Using AuAg nanoshells [51] instead of a spherical gold NPs can increase the enhancement due to the properties of Ag. signal for the interferon biomarker myxovirus resistance protein A (MxA) which can be used to distinguish viral vs. bacterial infections, and is present in at levels too low for detection by traditional LFAs. [52] In the case of bacterial pathogens, the increase in sensitivity can remove the need for culturing to grow bacteria to a sufficient level for detection, which is a time-consuming step and must be done in a lab.[53]

SERS readouts become much more powerful with multiplexing, which can be achieved if multiple reporter molecules with distinct spectra are used in the nanotags.[54] This has been demonstrated for the zika and dengue biomarker nonstructural protein 1 (NS1), where one nanotag was specific to dengue, and had one Raman reporter 1,2-bis(4-pyridyl)ethylene (BPE) and the other was specific to zika, and had a different reporter, 4-mercaptobenzoic acid (MBA) that had a distinct spectra from BPS.[49] Other routes to increase SERS multiplexing utilize combinations of reporter in to create bar codes that can achieve 40–50 unique labels.[55, 56] Recent advances in SERS instrumentation have led to portable and even handheld spectrometers, which further popularized SERS as an analytical tool. [57]

Photothermal—Gold NPs possess unique photothermal properties, [60] where laser excitation at their SPR can result in their rapid heating. Thus, laser excitation of the LFA

can be used to increase their contrast in thermal imaging by a thermal camera or NIR detector. This approach, thermal contrast amplification (TCA) was used to increase detection sensitivity of cryptococcal antigen for AIDS and also SARS-CoV-2, up to a 50-fold improvement over a visual readout. [61, 62] In addition, readers can be made to be portable and low-cost, demonstrated on immunoassay strips for human chorionic gonadotropin (hCG). [63]

Electrochemical—NPs have also been used to enhance readouts beyond visual and optical modalities, such as in electrochemical detection. Electrochemical sensing has been made more accessible with the availability of low-cost disposable electrodes. [64] Using the NP in the NP-antibody conjugate signal, electrochemical detection of α-Enolase (ENO1), a biomarker for lung cancer, was possible by square wave voltammetry (Figure 3e).[58]

Magnetic NPs—Using superparamagnetic NPs for the label allows for magnetic readout of LFAs. This was demonstrated on detection of hCG using magnetite NPs on the antibody and readout of the test strips with a magnetic assay reader (MAR), resulting in a higher sensitivity compared to traditional LFAs.[65] Novel combinations of gold and magnetic NPs with the thermoresponsive polymer poly(N-isopropylacrylamide) (pNIPAm) enabled stimuli-responsive magnetic concentration for detection of malarial biomarkers pan-aldolase and *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2).[66] Other materials innovations with magnetic NPs include coating superparamagnetic Fe_3O_4 with a carbon coating in a “nanoflower” form to detect extracellular vesicles in an immunoassay, exploiting sample concentration using a magnet. [67]

Post-processing catalytic activity—NPs can act as enzyme mimics, or “nanozymes,” where they are used in place of a peroxidase (*e.g.* horseradish peroxidase) to catalyze a reaction. [68] Au@Pt[69] used the Pt shell to oxidize a substrate 3,3’,5,5’-tetramethylbenzidine, TMB, and has the benefit of amplifying the signal. Porous Pt core-shell NPs can catalytically enhance the signal for detection of p24 for HIV, which relies on a chromogenic substrate, 4-chloro-1-naphthol/3,3′-diaminobenzidine, tetrahydrochloride, which produces an insoluble black product in the presence of hydrogen peroxide, and thus resulting in a darker band at the test line. [70]

In addition to changing the NP material, signal can be increased by post-binding enhancement techniques, such as silver staining of the immobilized gold NPs.[71] Isotachaphoresis, an electrokinetic preconcentration and separation technique, has been used to improve transport of target analytes to the LFA capture line, increasing sensitivity as high as two orders of magnitude [72]. Magnetic focusing of magnetic NPs can also concentrate the particles to increase test line signal. [73]

Going beyond the materials alone—One can expand immunoassay capabilities by thinking beyond the probe material properties. While cross-reactivity is normally considered to be a drawback, it can be actually leveraged to detect emerging infectious diseases. By exploiting cross-reactivity of antibodies, one can use immunoassay as a selective array instead of a specific sensor. In combination with multicolor NPs [30, 74] and machine learning (ML), the assay can be trained to detect targets that the antibodies were not raised against. This was demonstrated for detecting yellow fever virus (YFV) biomarkers using dengue

(DENV) and zika (ZIKV) antibodies, and could be used to “hack” existing dengue rapid diagnostics to detect YFV (Figure 3f). [59]

Interface issues for biosensors

A major challenge for biosensors is the interface, where phenomena such as surface fouling and non-specific adsorption can be detrimental for signal. Biotic-abiotic interfaces encounter undesirable side effects at length scales spanning from the macroscopic to the nanoscale. The nano-bio interface has been particularly problematic, as nanomedicine applications have suffered from complications due to non-specific adsorption and protein corona formation. However, these interface issues are more challenging in diagnostics and carry more serious consequences.[19] Non-specific adsorption to NPs in diagnostics it has serious consequences as it can yield false positives, which impacts patient treatment and quarantining. In sandwich immunoassays there are two biotic-abiotic interfaces: 1) between the antibody and the NP label, and 2) between the antibody and the paper substrate. Additionally, there is a protein corona that can form around the NP-antibody conjugate, and finally the binding event occurs under fluid flow.[75–77] Drying of the NP-antibody conjugates in the conjugate pad can also cause precipitation and thus contribute to loss of signal.

Because COVID-19 rapid tests are nearly ubiquitous, it is easy to be lulled into the thinking that all interface issues have been solved. However, they are still very problematic, and unfortunately severely limit diagnostic capabilities. For example, rapid point of care COVID-19 antigen tests cannot be used at the early infection stage when viral levels are low, so often users are advised to take the test twice, with a day or two apart for a reliable answer. This can be attributed to not enough signal to noise, which is hampered by NP-antibody precipitation, protein corona formation, and issues with the sample matrices. To further complicate the matter, variability arises from patient to patient, and tests from different commercial suppliers can yield different results depending on what protein is used as the target.[78] Evidently, the limited performance of rapid antigen tests have restricted their use to certain situations. Thus, it is important to understand the bio-nano interface issues in immunoassays.

Issues faced by the nano-bio interface in immunoassays can be separated into the categories of a) the conjugate, b) the sample matrix and protein corona effects, c) the substrate, and d) Ab interference in the pair (Figure 4).

Antibody conjugation to NPs—Conjugation of the label antibody to the NP that gives rise to a signal must be done in such a way that it does not interfere with the antibody binding, or result in an inappropriate orientation. If the conjugation chemistry is not site-specific (*i.e.*, targets any amine), it can potentially link to the binding site and negatively impact target binding. There are studies quantifying the effect of the conjugation on the functionality of the antibody and its important consequence on impacting binding to the target.[79]

Many click chemistry approaches are available for antibody conjugation, which often use bifunctional linkers that link on one end to functional groups on the antibody (R-NH_2, R-COO) and the other end to the NP. [80] Thiols are used predominantly for gold

NPs due to the Au-S interaction, and ligands with dithiols can improve binding.[81] For silica coated particles, silane chemistry is used for bioconjugation. Nanoparticles can also be functionalized with surface ligands with functional groups that can be covalently linked to. Popular routes include N-hydroxy succinimide (NHS) ester, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and maleimide conjugation chemistries, which link to primary amines, carboxylic acid groups, or azides, so nanoparticle ligands with these terminal groups are utilized.

However, these chemistries target functional groups on the antibody that are not site-specific, and thus can label anywhere on the antibody, including the variable region. Thus, there is the probability that conjugation could interfere with binding to the target. To address this, Kumar et al. developed a directed conjugation route[82] that targets the glycosylations on the Fc portion of the antibody, leaving the antigen-binding portion, or Fab region, unhindered. The linker is heterobifunctional and has an amide-bonded adipic hydrazide on one end, and a dithiol tether on the other end that attaches to the gold NPs.

On the other hand, others have determined that simply adsorbing the antibody on the NP surface is sufficient for functionality.[83, 84] which is a much simpler route than click chemistry. Lowering the pH (7.5 vs. 8.0 or 8.5) can optimize antibody orientation with respect to binding the target, as the surface charge distribution on the antibody becomes more uniformly negative at higher pH. This was measured by NP tracking analysis, which quantified the protein layer thickness on the particle, yielding information on the antibody orientation.

Effect of protein coronas—Protein coronas are the clouds of proteins that form nearly instantaneously around NPs when they are introduced into biological fluids that have proteins present at very high concentrations. Because immunoassays are often run directly in blood or saliva, protein coronas are definitely present in immunoassays. There is evidence in the literature of the protein corona impacting immunoassays that use fluorescence readouts.[85] Characterization of protein coronas by SDS-PAGE and LC-MS/MS has shown that only 20–30% adsorbed proteins on a NP surface are able to bind to a target.[86]

Some approaches have utilized surface passivation chemistries to mitigate the protein corona and non-specific adsorption to NPs, which is usually performed after conjugation as a backfill step. Because polyethylene glycol (PEG) is relatively inert, PEGylation is the most common route, as it is used heavily with NPs, substrates, and flat surfaces. This involves backfilling with a thiolated PEG, PEG-SH, which binds to the Au NP surface by the thiol, allowing the PEG to block adsorption. [87, 88] For NPs made of metal oxides (Fe_3O_4, $MnFe_2O_4$, $CoFe_2O_4$, etc.) COO^- groups can coordinate to the NP surface.[89, 90] For silica coated particles, silanes are used as linking groups. However, protein corona formation and non-specific adsorption to PEGylated NPs can still occur.[91] Additional strategies include the use of polymer stabilizers.[92] Zwitterion ligands can completely prevent protein corona formation by preventing ion pairing between proteins and the surface charges.[93, 94] Moyano et al. studied a series of zwitterion terminal groups on gold NPs and found that they can completely prevent formation of a hard corona, improving NP stability and blood circulation time.[95]

Finally, it should be noted that protein coronas can actually be helpful in dipstick sandwich immunoassays.[96] Strips run in buffer (*e.g.* PBS) can exhibit non-specific adsorption and result in false positives. When the media is human serum (HS), a corona can form around the NP due to all of the proteins present in HS at high concentrations. The corona actually reduces non-specific adsorption to both the NP-antibody conjugate and the paper substrate, thus reducing the probability of a false positive signal.

Ultimately, protein coronas and sample matrix effects cannot be ignored in lateral flow and dipstick immunoassays.[97] Sample matrices are highly variable, varying from patient to patient, and properties are different by how and when the sample is collected among many other factors. Finally, the properties of biological fluids used in diagnostics are very different, where urine vs. blood vs. serum vs. saliva have different protein concentrations, viscosities, and working volumes, so the diagnostic must be designed for a particular fluid.

Substrate issues—Lateral flow assays use substrates of paper, which is an extremely versatile material. Paper possesses advantages due to its porosity, low-cost, ease of manufacturability at scale, and ability to perform separations and filtrations.[98, 99] The breadth of its unique properties have accelerated diagnostic test development. Immunoassays primarily use nitrocellulose, which can be deposited in a multitude of ways, such as inkjet printing, spotting, spray drying, and screen printing. Because paper provides a good scattering background, it facilitates visual readout, and also can be used for fluorescence and electrochemistry.[100] On the other hand, paper can be a challenging substrate, as it is porous, so it can sequester the target, and non-specific adsorption to the paper or precipitation during fluid flow can cause test line smearing. Routes to block paper with the protein bovine serum albumin (BSA) have been the most common approach to reduce this phenomenon. Alternative passivation molecules have been explored such as poly(oligoethylene glycol methacrylate) [101] and the amphiphilic sugar n-dodecyl β-D-maltoside.[102] Scherag et al. [103] argue that the analyte can still exchange with blocking agents *via* the Vroman effect or adsorb to polymer coatings, so they develop photo-cross-linkable copolymers of polystyrene and polydimethylacrylamide that are better at reducing non-specific adsorption. The hydrogels are much more repellent to analyte adsorption tissue transglutaminase (tTG), a marker for celiac disease.

Conclusions

While nanomaterials offer multiple attractive ways for enhancing diagnostics and have been the source of numerous innovations, many challenges remain for their true implementation. The significant lag in their widespread use in commercial assays [104] can be attributed to the tradeoff between sufficient novelty for scientific publication vs. bridging the manufacturing gaps to make a diagnostic truly deployable. In general, the drive to innovate has led to a domination of publications on technical advancements, with papers that discuss manufacturing or clinical evaluations of tests a rarity. For nanotechnology-enabled infectious disease diagnostics, the system must be robust and reproducible, which is challenging as the nanomaterials must be stable with a respect to a range of environmental conditions. This is often achieved by optimizing surface chemistry and running conditions, which can be more of an art than a science. However, this may be an opportunity to use ML to arrive at desired

conditions more efficiently, as ML has been demonstrated for small molecule synthesis, materials design, NP synthesis, and many other chemical systems. [105, 106]

There are numerous challenges associated with making a diagnostic operational with patient samples under real field conditions, which are very different from controlled scientific lab environments.[107] Infrastructure and instrumentation accessibility place many constraints on integrated hardware, such as the lack of ground electricity or the existence of large temperature gradients and high humidity in the local climate. Thus, devices must be rugged enough to withstand these field conditions.

Furthermore, sample preparation remains a major barrier to test robustness, precision, and deployment. Multiple preparation steps are often required before a sample goes into the test. While lab on a chip systems seek to go from a raw sample to answer, there are only a few examples of low-cost, fully-integrated systems.[108] Most rapid diagnostics largely rely on antibodies and proteins that bind to antibodies, and production of new antibodies is costly and requires time and significant infrastructure. [109]

Outbreaks of new and re-emerging diseases are occurring more rapidly[110] and on much larger scales, as we have witnessed with the COVID-19 global pandemic. Immunoassays have risen in importance due to these growing threats from infectious diseases. Advantages of nanomaterials are that their properties are complementary to the biology, where they can be used to improve diagnostic performance or enable novel readouts beyond the traditional visual signal. However, the disadvantages of nanomaterials is that their integration into diagnostics that are used on biological samples can be thwarted by interface issues, and thus these surface effects must be accounted for. The ideal approach is to account for both aspects in immunoassay design, where potential interface issues are addressed in nanomaterial choice.

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BIOGRAPHIES

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ABBREVIATIONS

BSA	Bovine serum albumin
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
Fab	Fragment antigen-binding
Fc	Fragment crystallizable region
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LFA	lateral flow assay
LOD	limit of detection
ML	machine learning
NHS	N-hydroxy succinimide
NP	nanoparticle
PBS	Phosphate Buffered Saline
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SERS	surface enhanced Raman spectroscopy
SPR	surface plasmon resonance
tTG	tissue transglutaminase

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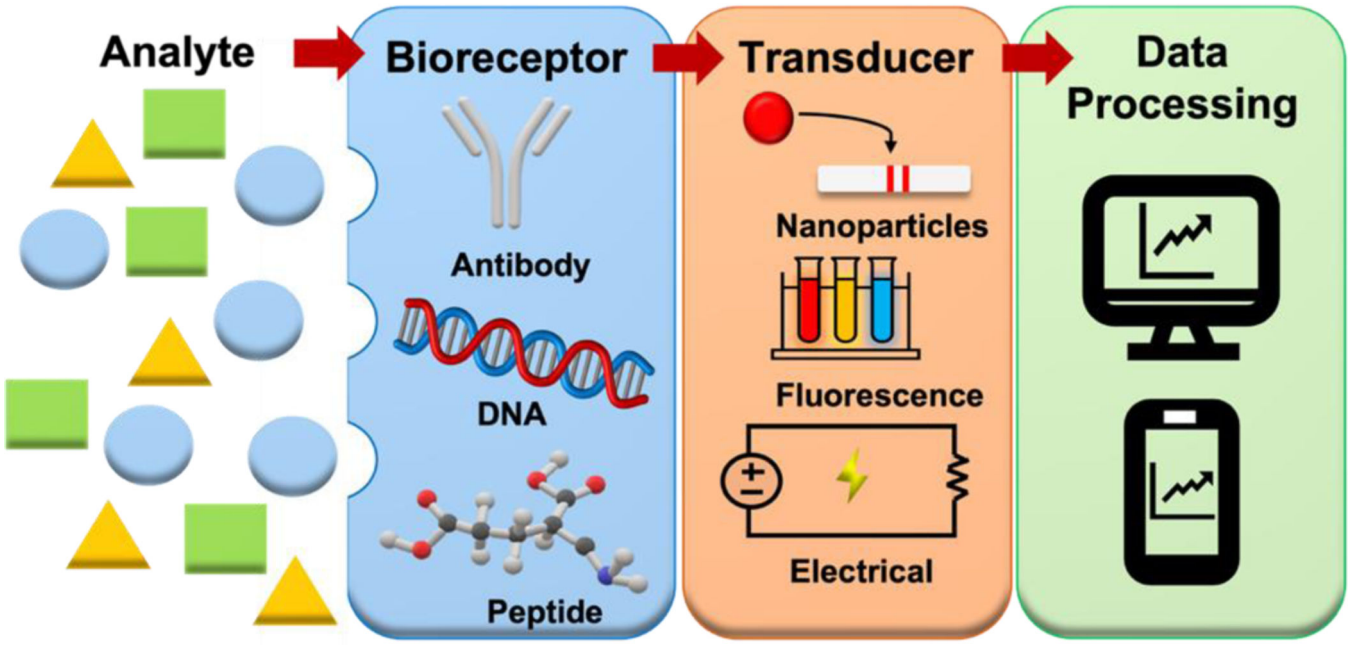

Figure 1.

Basic biosensor components: the analyte, bioreceptor, and transducer. The signal is analyzed by data processing. The bioreceptor can be a range of different bioaffinity agents, including antibodies, DNA, and peptides.

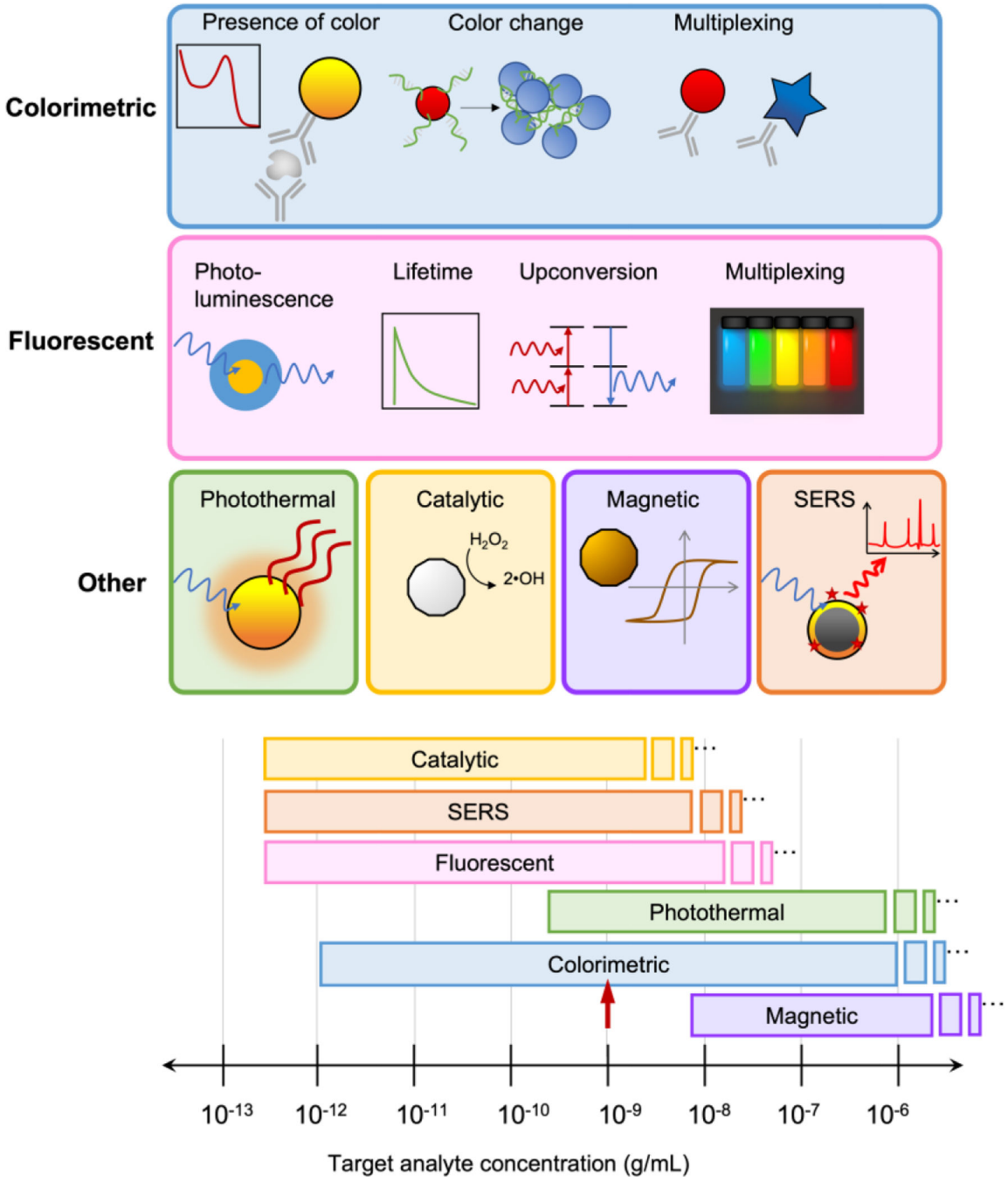

Figure 2.

Different readouts from NP labels and typical working ranges of sensitivity, for colorimetric, fluorescence, and others. The LOD of a traditional visually read out immunoassay is ñg/mL (red arrow).

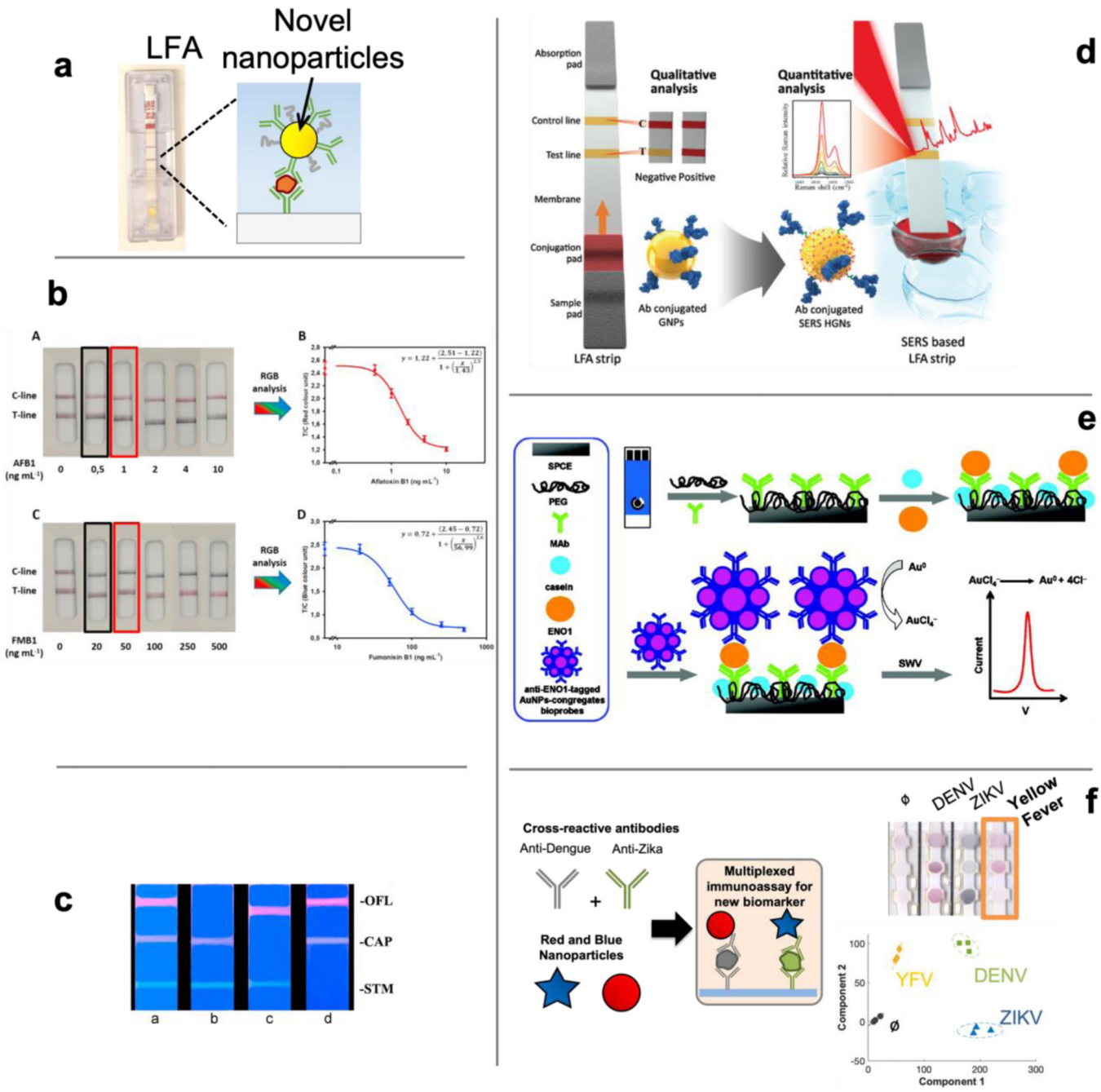

Figure 3.

a) Nanotechnological innovations in infectious disease diagnostics often use a novel NP engineered to have unique properties in a rapid LFA test. B) multiplexed detection of different food toxins can be achieved using red and blue gold NPs [29], c) Quantum dots can be used for a multiplexed fluorescent readout of the presence of different antibiotics in milk [37], d) SERS gold NP-reporter “nanotags” can allow sensitive SERS readout [47], e) electrochemical detection can be enhanced by gold NPs Reprinted with permission from Ho, J.-a. A.; Chang, H.-C.; Shih, N.-Y.; Wu, L.-C.; Chang, Y.-F.; Chen, C.-C.; Chou, C. Diagnostic Detection of Human Lung Cancer-Associated Antigen Using a Gold

Nanoparticle-Based Electrochemical Immunosensor. *Analytical Chemistry* 2010, 82 (14), 5944–5950. Copyright 2010 American Chemical Society. [58] f) multicolored gold NPs can be used to repurpose dengue and zika antibodies to detect yellow fever without the need for yellow fever antibodies. Adapted with permission from Rodríguez-Quijada, C.; Gomez-Marquez, J.; Hamad-Schifferli, K. Repurposing old antibodies for new diseases by exploiting cross reactivity and multicolored nanoparticles. *ACS Nano* 2020, 14 (6), 6626–6635. Copyright 2020 American Chemical Society. [59]

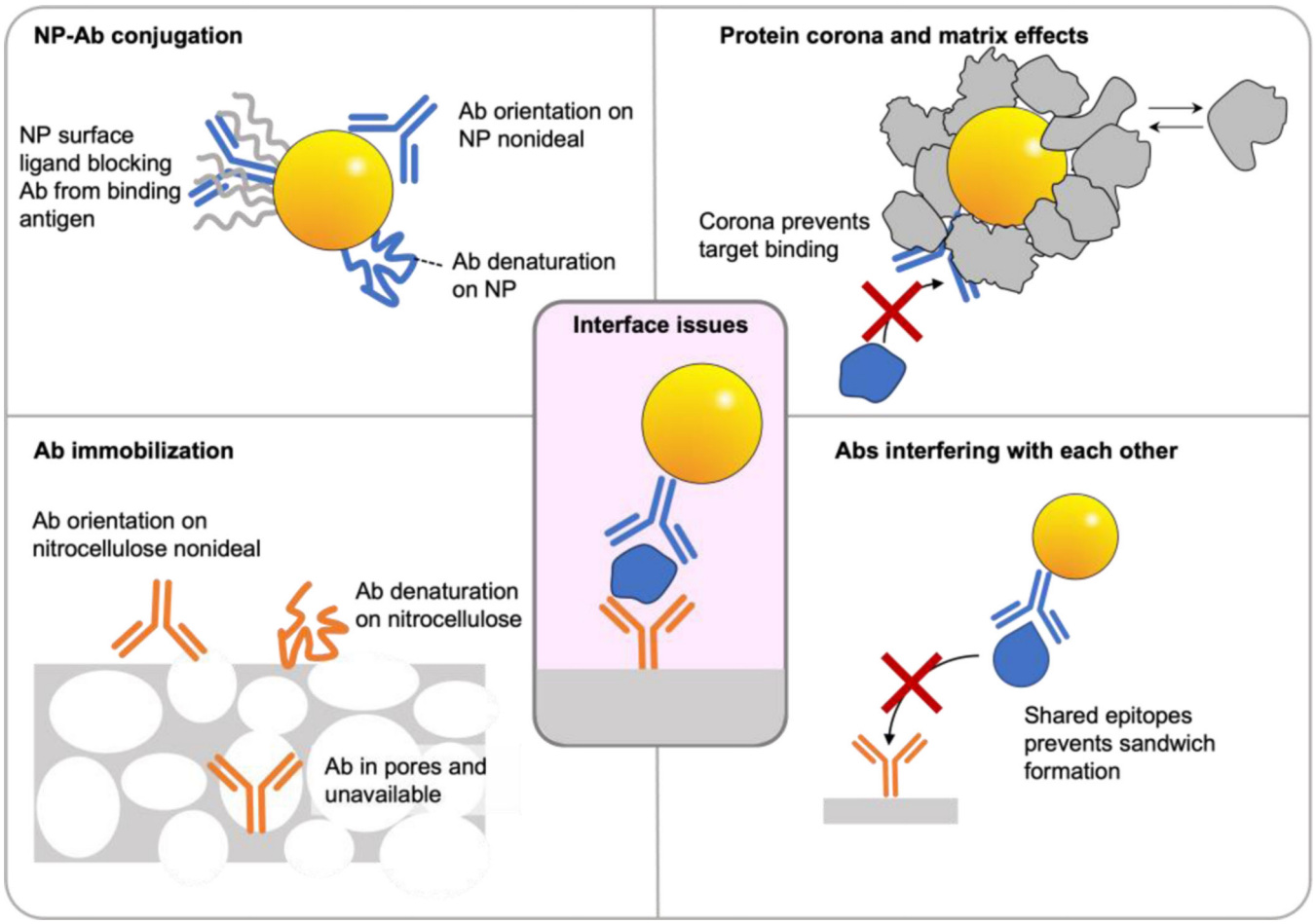

Figure 4.
Interface effects that can occur in immunoassays for the conjugate, the sample matrix,