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Recent Advances Incorporating Superparamagnetic Nanoparticles into Immunoassays

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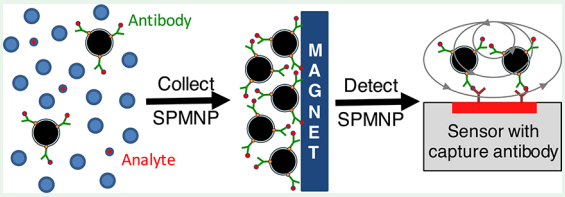
ABSTRACT: Superparamagnetic nanoparticles (SPMNPs) have attracted interest for various biomedical applications due to their unique magnetic behavior, excellent biocompatibility, easy surface modification, and low cost. Their unique magnetic properties, superparamagnetism, and magnetophoretic mobility have led to their inclusion in immunoassays to enhance biosensor sensitivity and allow for rapid detection of various analytes. In this review, we describe SPMNP characteristics valuable for incorporation into biosensors, including the use of SPMNPs to increase detection capabilities of surface plasmon resonance and giant magneto-resistive biosensors. The current status of SPMNP-based immunoassays to improve the sensitivity of rapid diagnostic tests is reviewed, and suggested strategies for the successful adoption of SPMNPs for immunoassays are presented.



KEYWORDS: *superparamagnetic nanoparticles, immunoassay, biosensor, surface plasmon resonance, giant magnetoresistive sensors, rapid diagnostic test*

INTRODUCTION

Nanotechnology is providing exciting new capabilities for research in materials science, biomedical engineering, and environmental engineering. In particular, bionanotechnology has emerged as a promising area with various commercial biomedical products already using nanoparticles.[1] The global market for nanoparticles in biotechnology, drug development, and drug delivery has been estimated to reach $79.8 billion in 2019, with a compound annual growth rate of 22%.[2] These are used for applications as varied as nanoparticle silver with antibiotic capabilities, quantum dots for noninvasive imaging, and superparamagnetic nanoparticles for enhanced oil recovery.[3,4]

Superparamagnetic nanoparticles (SPMNPs) exhibit superparamagnetic behavior (i.e., hysteresis-free reversible magnetization) and quick magnetophoretic response, which are attractive properties for biomedical applications. Indeed, a wide spectrum of applications, including drug delivery,[5−7] magnetic resonance imaging,[5,6,8,9] and hyper-thermic cancer treatments,[10−13] have been successfully developed with these particles. Localized heating using SPMNPs has also been used in nonmedical applications such as precision polymer gelation[14] (for details, see Laurent et al.[29] and Pollert and Zaveta[15]). While many different kinds of nanoparticles have been synthesized and evaluated, iron-oxide SPMNPs (synthetic γ-Fe_2O_3 or Fe_3O_4 particles with a size of ∼10 nm) are of particular interest for their excellent magnetic properties, low cost, and low toxicity.

When SPMNPs are in the presence of a controlled external magnetic field, they act as "nano-magnets" and, accordingly, acquire several useful properties: (i) they can move in a desired direction; (ii) their presence can be remotely detected; (iii) they can generate highly localized, intense heat; and (iv) they lose their magnetism instantly and completely once the external magnetic field is removed. Furthermore, the SPMNP surface can be decorated with reactive functional groups without affecting these magnetic properties. When decorated with antibodies, this allows for SPMNP-based detection of a target biological or other chemical entity. The particles can then be manipulated for sensing, filtration, fractionation, collection, and other purposes. The tremendously large surface area per mass of particle offers the potential to detect a very dilute concentration of analyte, as the complex of particles and target molecules can be magnetically concentrated 10−1000-fold for improved detection sensitivity (Figure 1).

Because of these capabilities, considerable effort has been invested in exploring the use of SPMNPs for medical, biological, and other applications.[17] Many of these require high-end sophisticated equipment and will likely be more appropriate for research laboratories and large hospitals. However, there are also opportunities to incorporate SPMNPs into rapid diagnostic test (RDT) kits. This is an attractive option, since SPMNPs have the potential to increase device

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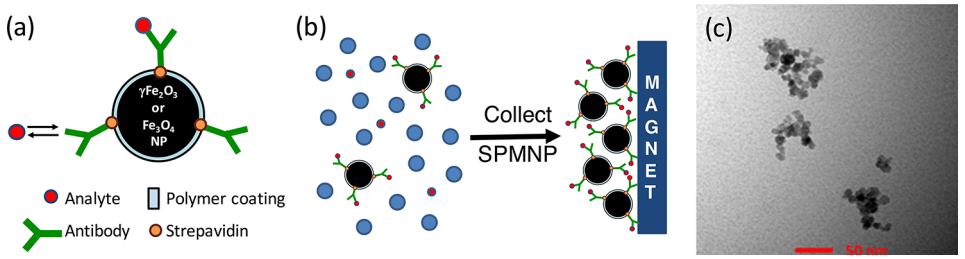

Figure 1. Modification of SPMNPs for analyte detection. (a) For incorporation into immunoassays, SPMNPs can be covalently decorated with antibodies specific for a given analyte and concentrated using an external magnet. (b) This allows for capture of the analyte in a complex mixture, even when the analyte is present at dilute concentrations. (c) Direct visualization of Fe_3O_4 SPMNPs using transmission electron microscopy. SPMNPs have spherical shape and an average diameter of ~10 nm and often form clusters or small aggregates with a size of ~50 nm. The scale bar indicates 50 nm. (c) Reproduced with permission from Ko et al. (2017).[16]

sensitivity by detecting molecules present at very dilute concentrations in biological samples such as in saliva or urine, for touch-free and rapid magnetic sensing, and for particle reuse.[16,18] Among the various SPMNPs developed, magnetite (Fe_3O_4) nanoparticles are of particular interest, because they can be produced easily and cheaply, as already demonstrated with successful applications in removing arsenic from drinking water[19] and treatment of oilfield water.[16,20] As any RDT kit, for example, for malaria detection, must be cheap, portable, and easy to use, the low-cost and large-quantity availability of magnetite nanoparticles are key advantages of SPMNP for immunoassay applications.

While still in the early stages, several recent studies have reported SPMNP-based immunoassays for disease detection in developing countries. For example, Castilho et al.[21] reported an electrochemical magneto immunoassay to detect the malarial *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2) with higher sensitivity as compared to conventional methods. Nash et al.[22] used a mixture of polymer-coated SPMNPs and gold nanoparticles (AuNPs) to concentrate captured antigens 50-fold before use in commercial lateral flow assays to detect two malarial antigens, namely, pan-aldose and *Pf*HRP2.

This review focuses first on the characteristics of SPMNPs that are relevant for biosensor applications and describes biosensors currently in development for SPMNP-based immunoassays. The detection sensitivity of reported SPMNP-based immunoassays is compared with conventional methods including enzyme-linked immune-sorbent assay (ELISA) and commercial RDT kits for various analytes, with a special emphasis on malaria antigens. Finally, we describe strategies for improving the sensitivity and practical usability of SPMNP-based immunoassays.

■ ATTRACTIVE CHARACTERISTICS OF MAGNETIC NANOPARTICLES FOR IMMUNOASSAY APPLICATIONS

There is an extensive literature covering SPMNP fundamentals, their behavior, and applications.[23,24] Therefore, in this review, we describe only those unique SPMNP properties relevant for immunoassay development.

Magnetic Behavior. The most useful SPMNP feature in this respect is their magnetic ability, which can be described quantitatively and predictably with physical analyses. When SPMNPs are subjected to an external magnetic field (H), they magnetize and generate an induced field (M) (see, e.g., Guimaraes[23]):

$$\vec{\mathbf{M}} = \chi \vec{\mathbf{H}}$$

where

$$\chi = \frac{\pi}{18}\phi\mu_0\frac{M_d{}^2 d^3}{kT}$$

is the magnetic susceptibility of the SPMNP dispersion, ϕ is the volume fraction of the particles; μ_o is magnetic permittivity in free space (or in vacuum); M_d is the bulk magnetization of the material; d is the diameter of the particle; k is the Boltzmann constant, and T is the absolute temperature.

The implication of this magnetic susceptibility is twofold. First, nanoparticles dispersed in a biological medium can collect upon application of a magnetic field, allowing quantification and analyses of the nanoparticles. Second, as the magnetization is proportional to the SPMNP volume fraction, shown in the equation above, the nanoparticle concentration (and accordingly the concentration of attached analytes) can be measured. The SPMNPs can be ushered in a desired direction or to a desired location by application of a magnetic field gradient, because they experience the following force:[25]

$$\vec{\mathbf{F}}_\mathbf{m} = \mu_0 V_p M_p \cdot \nabla H$$

where V_P is the SPMNP volume; M_P is their magnetization; and ∇H is the gradient of the external magnetic field. Therefore, by collecting the SPMNPs to an analysis location, the concentration of SPMNPs and associated analytes can be increased many-fold.

Synthesis. Various types of magnetic nanoparticles (MNPs) have been synthesized, including those based on iron oxides (e.g., γ-Fe_2O_3 and Fe_3O_4), pure metals such as Fe, Co, and Ni, spinel-type ferromagnets (e.g., $MgFe_2O_4$ and $CoFe_2O_4$), alloys (e.g., $CoPt_3$ and FePt), and multifunctional composites (e.g., Fe_3O_4–Ag, Fe_3O_4–Au, and FePt-Ag).[26,27] These can be synthesized by wet chemical methods[28] (e.g., coprecipitation, high-temperature reactions, reactions in steric environments, sol–gel reactions, decomposition of organometallic precursors, polyol methods, etc.), physical methods (e.g., gas-phase deposition and electron-beam lithography), and microbial methods.[26] The most popular synthesis method is coprecipitation, because this technique can achieve a narrow size distribution (10–50 nm) of nanoparticles.[28,29] Iron oxide nanoparticles (e.g., magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) with superparamagnetism are most widely used in biomedical applications due to their low toxicity, excellent biocompatibility, facile surface modification, and low cost.

Superparamagnetism. Because of their small size and high surface area per volume, SPMNPs have different properties compared with those of bulk materials, including

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magnetic behavior and lower melting and boiling points.[30] Namely, in the absence of an external magnetic field, the spins of SPMNPs can orient randomly resulting in zero magnetic moment. However, in the presence of a magnetic field, a high magnetic response is induced. Superparamagnetism appears in ferromagnetic nanoparticles with sizes ranging from a few nanometers to several tenths of nanometers.[31] This characteristic is highly dependent on the size, material, temperature, and surface modification of the nanoparticle.[32] This hysteresis-free, reversible magnetization property allows for a rapid "on−off" switching capability that can be controlled by the presence of an external magnet.[30] In the presence of a magnetic field, the SPMNPs will aggregate, but when the field is removed, the magnetization rapidly drops to zero, allowing the particles to redisperse. In the case of an immunoassay, this allows for reuse of the sensor and potentially even the same SPMNPs for subsequent detection events.

Surface Modification for Stability in Aqueous Solution. A small particle size (5−25 nm) with a narrow size distribution is necessary to yield not only superparamagnetic character but also uniform physical and chemical properties.[33] However, because of the high surface area-to-volume ratios and the hydrophobic nature of bare magnetic nanoparticles, these readily aggregate, causing several problems. Large nanoparticle clusters experience strong magnetic dipole−dipole attractions between clusters, leading to ferromagnetic behavior, in which clusters retain magnetic properties even after the magnetic field is removed.[34] Plasma proteins also readily coat the individual or clustered SPMNPs, which can interfere with analyte detection.

Surface modifications can help to passivate the surface and maintain SPMNP dispersion stability. Coating the nanoparticles with a shell of polymeric materials (e.g., poly(vinylpyrrolidone) [PVP], poly(lactic-glycolic acid) [PLGA], poly(ethylene glycol) [PEG], and poly(vinyl acetate) [PVA]), ionic and nonionic amphiphiles (e.g., oleic acid and stearic acid), inorganic materials (e.g., silica and gold), and carbon-based materials (e.g., carbon precursor such as glucose)[35] are all strategies used to stabilize SPMNPs. The coating provides repulsion (of either electrostatic or steric/entropic origin) between the nanoparticles to overcome the attractive van der Waals forces, thereby preventing aggregation.[36] Indeed, Torrisi et al.[37] demonstrated that PEG-coated SPMNPs are stable in cell culture media for over one month. The uses of polymer-coated SPMNPs in biomedical applications and their morphology, advantages, and magnetization were recently reviewed in Wu et al.[36]

Surface Functionalization for Analyte Detection. Surface functionalization of SPMNPs for effective immobilization of protein is critical for developing immunoassays. There have been various attempts to functionalize nanoparticles using covalent bonding, noncovalent bonding (e.g., physical adsorption), and affinity bonding (e.g., streptavidin−biotin interaction).[68,69] For most biological assays, the SPMNPs require a specific binding functionality to recognize the desired analyte, in addition to the reduced aggregation and nonspecific protein adsorption conferred by a polymeric coating. A convenient and modular approach is to first functionalize the particles with streptavidin, which can then capture biotinylated antibodies. Streptavidin is a homotetrameric protein from the actinobacterium *Streptomycetes* that can bind up to four molecules of biotin with extremely high affinity (equilibrium dissociation constant K_d of $\sim 1 \times 10^{-14}$ M). Because of biotin's small size (244 Da), it can be conjugated to larger proteins such as antibodies with minimal effect on their properties. The streptavidin−biotin system has been successfully used for immunoassays as well as solid-phase assays using magnetic and polystyrene nanoparticles.[61,62,64,70]

For nanoparticle decoration, a simple approach is to use a polymer coating with functional groups that can covalently bond to streptavidin. For example, the carboxylic acid groups present on polymers such as carboxyl-PEG can react with the terminal amino groups of surface-exposed lysine residues in strepavidin. The subsequent capture of biotinylated ligands is quite efficient, due to the high affinity of the interaction. Eberbeck et al.[71] reported that ∼85% of SPMNPs decorated with streptavidin bound to biotinylated beads, while only 20% of SPMNPS decorated with antibiotin-antibody bound to biotinylated beads. Other approaches to immobilize streptavidin include the addition of a six-residue peptide linker, including a single unpaired cysteine, to facilitate thiol-based conjugation methods and act as a tether to separate streptavidin from the solid surface.[72] Ylikotila et al.[73] introduced active thiol groups through primary amines in the streptavidin, providing higher biotin binding efficiency of streptavidin conjugated flat solid surface.

Magnetophoresis. Magnetophoresis refers to the controlled motion of SPMNPs in a viscous medium induced by the application of an external magnetic field.[38] This useful characteristic can be used to isolate, wash, and concentrate SPMNPs and any attached material. Purification and enrichment of dilute SPMNP samples in a biological medium (e.g., blood, saliva, etc.) can be performed by three simple steps. First, an external magnetic field is applied to collect SPMNP and associated material, the supernatant is removed, and the SPMNPs are washed if necessary before resuspension in a smaller volume of buffer. The concentrated sample can be obtained by small-scale magnetophoresis, negating the need for electricity or specialized machinery such as centrifugation. This is the key advantage of SPMNPs for biomedical applications, especially RDT and other diagnostics, because the SPMNPs conjugated with the analyte can be purified and concentrated before detection, which improves the signal-to-noise ratio and detection sensitivity.[22,39] Indeed, SPMNPs have been successfully used to separate and purify specific cells,[40] antigens,[22] and DNA strands.[41,42] Previous studies[43−45] have demonstrated that the average magnetophoretic velocities of microbeads conjugated with analyte were proportional to the analyte concentration.

BIOSENSORS BASED ON MAGNETIC NANOPARTICLES

Because of their high biocompatibility and potential for sensitive detection of analytes with a high signal-to-noise ratio and a short analysis time,[26] SPMNPs are finding applications in biosensors using various detection methods.[46] The SPMNPs can be used to facilitate sample concentration, amplify the signal, or directly contribute to the detected signal.

Reported devices include voltammetric, electro-chemiluminescent, superconducting quantum interference device (SQUID) sensors, nuclear magnetic resonance (NMR), and surface plasmon resonance (SPR). In addition, there are spintronic sensors such as giant magneto-resistance (GMR) and tunneling magnetoresistance (TMR), which measure magnetoresistance changes caused by binding target analytes to the SPMNPs. Voltammetric sensors measure electrochemical

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Table 1. SPR and GMR Sensors Based on Magnetic Nanoparticles

sensor	SPMNP composition	NP size	analyte	detection limit	detection range	ref
SPR	streptavidin conjugated Fe_2O_3 magnetic nanoparticles	50 nm	brain natriuretic peptide	ND	0.025−1 ng/mL	82
	streptavidin conjugated superparamagnetic nanoparticles	50 nm	Staphylococcal enterotoxin B	ND	0.10−10 ng/mL	83
	tosyl-activated superparamagnetic nanoparticles	1 μm	prostate specific antigen	10 fg/mL	1 fg/mL−100 ng/mL	52
	magnetic nanoparticles with iron oxide core	220 ± 63 nm	beta human chorionic gonadotropin	0.45 pM	ND	84
	carboxyl group modified Fe_3O_4 magnetic nanoparticles	10.5 nm	thrombin	0.017 nM	0.27−27 nM	85
	core/shell Fe_3O_4/SiO_2 nanoparticles	16 nm	rabbit IgG	ND	1.25−20 μg/mL	86
	core/shell $Fe_3O_4/Ag/SiO_2$ nanoparticles	19 nm	rabbit IgG	ND	0.30−20 μg/mL	86
	core/shell Fe_3O_4/Au nanoparticles modified with 3-mercaptopropionic acid (MPA)	8−30 nm	human IgM	ND	0.30−20 μg/mL	87
	Iron oxide carboxyl-modified magnetic nanoparticles	200 nm	Ochratoxin A	0.94 ng/mL	1−50 ng/mL	88
	core/shell Fe_3O_4/Au nanoparticles	25−30 nm	A-fetoprotein	0.65 ng/mL	1.0−200.0 ng/mL	54
	Fe_3O_4−Au nanorod (50 × 15 with 0.05 mmol/L $AgNO_3$, 65 × 30 with 0.1 mmol/L $AgNO_3$)		goat IgM	ND	0.15−40.00 μg/mL	55
	Fe_3O_4/Ag/Au nanocomposite	35 nm	Dog IgG	0.15 μg/mL	0.15−40.00 μg/mL	56
	carboxyl group modified Fe_3O_4 magnetic nanoparticles	6.53 ± 0.22 nm	*Salmonella enteritidis*	14 cfu/mL	14−1.4 × 10^9 cfu/mL	89
GMR	streptavidin conjugated superparamagnetic nanoparticles	300 nm	parathyroid hormone	10 pM	ND	90
	cubic FeCo nanoparticles	12.8 ± 1.58 nm	Interleukin-6	ND	125 fM−41.5 pM	91
	streptavidin conjugated Fe_2O_3 nanoparticles	50 nm	Aflatoxin B1	50 pg/mL	0.050−50 ng/mL	62
	streptavidin conjugated microbeads	ND	allergen Ara h 1	7. 0 ng/mL	7.0−>2000 ng/mL	61
	streptavidin conjugated microbeads	ND	allergen Ara h 2	0.2 ng/mL	0.2−>250 ng/mL	61
	streptavidin conjugated microbeads	ND	allergen gliadin	1.5 ng/mL	1.5−4000 ng/mL	61
	streptavidin conjugated cubic FeCo nanoparticles	12.8 ± 1.58 nm	endoglin	83 fM	ND	70
	streptavidin conjugated superparamagnetic nanoparticles	50 nm	Staphylococcal enterotoxin A	0.1 ng/mL	ND	64
	streptavidin conjugated superparamagnetic nanoparticles	50 nm	toxic shock syndrome toxin	0.3 ng/mL	ND	64
	streptavidin conjugated magnetic nanoparticles	ND	Flt3 ligand	ND	0.020−3 μg/mL	59
	streptavidin conjugated magnetic nanoparticles	ND	serum amyloid A1	ND	3−50 ng/mL	59
	streptavidin conjugated magnetic nanoparticles	ND	influenza A virus	1.5 × 10^2 TCID[a] 50/mL	1.5 × 10^2−1.0 × 10^5 TCID 50/mL	66

[a]TCID, tissue culture infective dose.

signal changes (e.g., voltage or current), while electrochemiluminescent sensors detect photons released from chemical reactions. For example, Li et al. (2013)[47] developed an electro-chemiluminescent sensor to detect *Bacillus thuringiensis Cry1Ac* using a gold-coated iron oxide nanoparticle. In this study, a primary antibody was complexed with the nanoparticles and immobilized on a glass carbon electrode using an external magnet. Next, antigen was captured and detected with a second antibody followed by a glucose oxidase conjugated antibody and signal produced by the reaction of luminol and hydrogen peroxide in the presence of the enzyme.

The SQUID immunoassays rely on an immobilized antibody that captures antigen that is in turn detected by a second biotinylated antibody, and finally, avidin-coated SPMNPs. The signal results from the slowly decaying magnetic flux of immobilized SPMNPs when the magnetic field is turned off. Free SPMNPs cannot be detected due to the random orientation of the dipole moments resulting from Brownian motion.[48,49] Antibody-bound SPMNPs cannot rotate, and thus relaxation occurs slowly after magnetization by this Neel mechanism, producing a decaying magnetic signal. NMR measures the ^{1}H proton signal. Because more surrounding water protons can be affected by MNPs than other materials,[50] MNP-based nuclear magnetic resonance has higher detection sensitivity than regular nuclear magnetic resonance. This modification has the potential to improve magnetic resonance imaging.[51]

The SPR and GMR sensors, which work by measuring optical and magnetic field changes, respectively, are being combined with SPMNP-based immunoassays. Therefore, we briefly describe the SPR and GMR phenomena and discuss the use of SPMNPs to improve SPR and GMR sensor sensitivity in recent studies (summarized in Table 1).

Surface Plasmon Resonance (SPR) Sensors. SPR is an optical method that measures refractive index changes caused by an increase of mass on the metallic sensor surface and corresponding change in refractive index. In this case, the mass increases when analytes are captured by immobilized antibodies. SPR response units are related to the amount of bound analyte per test area (one response unit ≈ 1 pg/mm^2).[52] SPR has the advantages of rapid and label-free detection, real-time analysis, and small sample volume requirements. Thus, SPR biosensors have been widely used for qualitative and quantitative measurements of biomolecular interactions.[53] However, since the SPR signal is related to the mass of protein bound to the sensor surface, it has poor sensitivity for small molecular weight molecules or low concentrations due to the

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small refractive index changes created by binding of these analytes on the SPR surfaces.[54]

SPMNPs have been used to amplify the signal in SPR biosensors simply due to their large mass, concentrate antigens, and higher refractive index, and, when the SPMNP includes silver or gold, they produce an SPR signal by their ability to propagate surface plasmons. For instance, antibody immobilized on the sensor surface can be used to capture an analyte, which is then bound by a second antibody decorating an SPMNP, which greatly increases the refractive index relative to the analyte alone (Figure 2).[55−58] Wang et al.[56] was able to

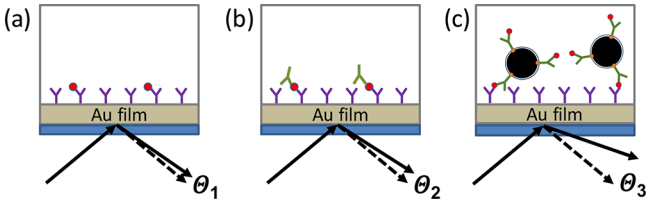

Figure 2. SPMNPs to enhance SPR sensitivity. (a) SPR devices typically function by immobilizing a capture antibody on a supported gold film. Polarized light is refracted by the gold film at an angle Θ_1 greater than would be expected due to the mass of bound antigen affecting the surface plasmon resonance. If the antigen has a small mass, the angle is small and can be difficult to detect. (b) Detection sensitivity can be increased by detecting bound antigen with a second detection antibody, thereby increasing the amount of mass bound and the reflection angle Θ_2 per bound antigen. (c) Sensitivity can be further increased by using a detection antibody bound to an SPMNP with a large total mass, resulting in a much larger angle Θ_3 per antigen molecule bound, and thereby lowering the concentration of antigen that can be reliably detected.

detect canine immunoglobulins at a concentration range of 1.25−20.00 μg/mL using a standard SPR detection strategy, but the detection range improved to 0.15−40.00 μg/mL when SPMNPs/Ag composite was included. Additionally, biological analytes associated with SPMNPs can be concentrated and washed using an external magnet before application to the SPR (Figure 2). This purification strategy efficiently removes nontarget analytes present in biological samples, thereby reducing background signal. Finally, using magnetic pillars, SPMNPs can be attached and immobilized on the SPR surface to enhance the biosensor sensitivity.[55,56]

Giant Magnetoresistive (GMR) Sensors. Magnetoresistance is the dependence of a material's electrical resistance to an externally applied magnetic field. GMR is a quantum mechanical effect that causes a significant change in electrical resistance of a thin-film layered structure induced by changing the external magnetic field.[59]

The GMR sensor developed by Osterfeid et al.[60] utilized antibody-conjugated magnetic nanoparticles to measure antigen concentrations (Figure 3). They used spin-valve GMR sensors consisting of the top "free" ferromagnetic layer and the bottom "fixed" ferromagnetic layer.[61] A capture antibody was immobilized on the sensor surface and used to capture antigen from a complex sample. This was in turn recognized by a second biotinylated detection antibody, which binds a separate site on the antigen. Finally, streptavidin-coated SPMNPs bind the biotinylated antibody to generate a change in the magnetization of the top free layer. The electrical resistance of the sensor changes in real-time, resulting in the detected signal.

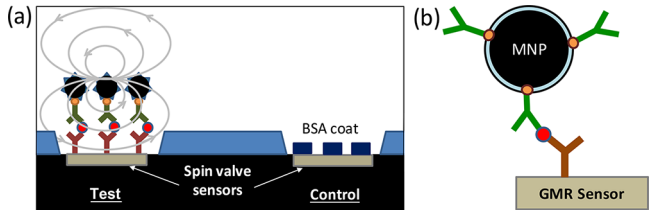

Figure 3. Spin-valve GMR sensor to detect analyte. (a) A test sensor is first functionalized with a capture antibody specific for the target analyte. This is used to capture analyte and create an immune sandwich culminating in SPMNPs. The magnetic signal from the immobilized SPMNPs enables quantification of the analyte concentration using a standard curve. A separate control sensor is coupled with bovine serum albumin (BSA) to detect background signal resulting from nonspecific binding. (b) The molecular assembly used for detection includes the capture antibody covalently coupled to the sensor surface that binds the target analyte. This is then detected by a second antibody binding a different epitope on the analyte. Since the detection antibody is biotinylated, it can in turn be bound by a streptavidin-conjugated SPMNP. This sensor was reported in Osterfeld et al.[60]

Recent studies have used GMR sensors to detect various analytes such as food allergens, potential cancer markers,[60] and mycotoxins[62] with fast detection and high sensitivity. For example, Osterfeld et al.[60] detected multiple potential cancer markers (e.g., interleukin-1-alpha, interleukin-10, etc.) at subpicomolar concentrations with a more than four-log range in sensitivity. In addition, Mak et al.[62] detected multiple mycotoxins (e.g., aflatoxin B1, zearalenone, and HT-2) in real-time using a streptavidin-linked SPMNP for detection and achieved a detection limit of 50 pg/mL. The MNPs used in GMR sensors are superparamagnetic or ferromagnetic with a high magnetic moment and large susceptibility, which is essential for magnetization in a low magnetic field. In addition, the SPMNPs must have a homogeneous size distribution and high stability in physiological solutions to ensure a stable relationship between number of analyte-complexed SPMNPs and the resulting magnetic signal.[26,63]

CURRENT DEVELOPMENT OF SPMNP-BASED IMMUNOASSAYS

Immunoassays that incorporate SPMNPs are under development for monitoring of various infectious diseases, including malaria and staphylococcal enterotoxins. Here, we describe these assays and compare their detection sensitivity to conventional assays including commercial RDT kits and ELISA.

Nash et al.[22] developed a system with polymer-coated SPMNPs and gold nanoparticles to concentrate malaria antigens before application to commercial lateral flow assays. The SPMNPs and AuNPs were decorated with the thermally responsive polymer poly(*N*-isopropylacrylamide) (pNIPAm). The coated AuNPs were next conjugated to streptavidin and coated with a biotinylated antibody recognizing a malaria antigen (either aldolase, which is common to all malarial parasites, or *Pf*HRP2). The particles were then incubated with human plasma samples spiked with malaria antigens and combined with the SPMNPs. Heating for 15 min at 40 °C resulted in polymer phase transition and coaggregation of the AuNPs and SPMNPs, which were then concentrated using an external magnet for an additional 15 min at 40 °C, resulting in a 50-fold reduction in volume (Figure 4). The concentrated AuNPs-antigen-SPMNP mixture was directly applied to existing

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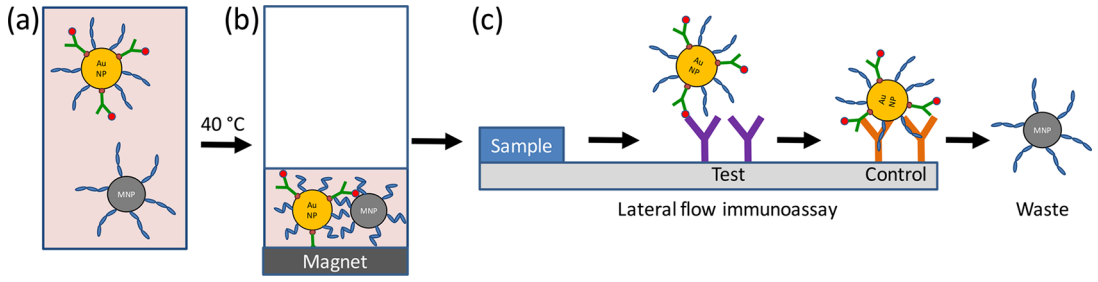

Figure 4. Sample concentration using mixed a AuNP/SPMNP system for lateral flow immunoassays. (a) Polymer and antibody-coated AuNPs were combined with polymer-coated SPMNPs and human plasma spiked with malaria antigens. (b) Heating to 40 °C induced a polymer phase transition and coaggregation of the AuNPs and SPMNPs, which were collected using an external magnet. The supernatant was removed, and the particles were redispersed in a smaller volume of buffer. (c) The concentrated particles were directly applied to a standard lateral flow immunoassay and migrated down the paper strip by capillary action. The gold nanoparticles accumulated at the test and control lines, allowing for visual inspection of the results, while the SPMNPs were transported into the waste. Figure inspired by Nash et al.[22]

lateral flow immunoassays with control and test lines. This enrichment method improved detection sensitivity 4.4-fold over a conventional commercial assay, as measured by visual inspection and integrated pixel intensity.

Orlov et al.[64,65] developed separate magnetic immunoassays to detect staphylococcal enterotoxins in contaminated foods and water and the cancer marker prostate specific antigen. In the first case, they created an immune sandwich with a capture antibody on three-dimensional fiber filters, followed by toxin and detection antibody coupled to a magnetic nanoparticle.[64] This was able to detect concentrations as low as 4 pg/mL from a 30 mL sample. In the second case, they combined a traditional lateral immunoassay with capture antibodies linked to 200 nm magnetic nanoparticles and thicker nitrocellulose (260 μm) to allow access to a larger sample volume and magnetic particle quantification.[65] This resulted in a detection limit of 25 pg/mL in serum.

Finally, Ng et al.[61] demonstrated detection of several food allergens using GMR sensor arrays. With a molecular assembly comprised of food allergens, then biotinylated antibodies followed by streptavidin-conjugated SPMNPs, the resistance of GMR sensors correlated with allergen concentrations. The authors showed that limit of detection (LOD) of GMR sensor assays were approximately an order of magnitude higher than for a conventional ELISA, ranging from 0.2 to 7.0 ng/mL (Table 2). Furthermore, multiple food allergens (peanut Arah1, peanut Arah2, and wheat gliadin) were detected with little to no cross-reactivity.

Table 2. Comparison of SPMNPs Immunoassays and Conventional ELISA Limit of Detection

analyte	ELISA LOD	sensor type	SPMNPs immunoassay LOD	ref
peanut Ara h 1	31.5 ng/mL	GMR	7.0 ng/mL	61
peanut Ara h 2	2 ng/mL	GMR	0.2 ng/mL	61
wheat gliadin	40 ng/mL	GMR	1.5 ng/mL	61
Staphylococcal enterotoxins (SEs)	0.1−0.5 ng/mL	magnetic particle quantification (MPQ) method	0.3 ng/mL (express MIA)	64
			10 pg/mL (HV MIA)	
prostate specific antigen (PSA)	~100 pg/mL	magnetic particle quantification (MPQ) method	25 pg/mL	65

Nash et al.[22] and Orlov et al.[65] developed modified lateral flow immunoassays, which used color changes and a magnetic particle quantification reader for quantitative detection of the results, respectively. The diagnostic methods developed in these two studies would be appropriate for qualitative biomarker detection in the field. The GMR sensors reported by Nash et al.[22] can incorporate multiple sensor arrays in one GMR chip, which allows for simultaneous detection of several biomarkers from one sample. It was reported that GMR bioassays can be portable, sensitive immunoassays for on-site application and thus have potential applications for developing world diagnostics to support sero-epidemiology studies.[66,67]

STRATEGIES TO IMPROVE SPMNP-BASED IMMUNOASSAYS

Increase Magnetic Nanoparticle Stability. Even with the polymer coatings described above, SPMNPs remain subject to aggregation in complex cell culture media and sera, which have a higher ionic strength (~150 mM) than buffers often used for nonbiological applications.[74] To further stabilize the SPMNPs, copolymers have employed to coat the particles. These copolymers are typically random combinations of two monomers: one provides an "anchor" functionality, while the other confers chemical functionality. The anchor monomer adsorbs to or forms covalent bonds with the SPMNP surface, allowing the "functional" monomers to extend from the SPMNP surface, preventing SPMNPs aggregation via electrostatic stabilization or steric repulsion. Additionally, the functional monomer often provides chemical reactivity to connect to streptavidin or antibodies.

In one example, Jon et al.[75] synthesized a random copolymer composed of (trimethoxysilyl) propyl methacrylate (the anchor) and PEG methacrylate (the functional), denoted as poly(TMSMA-r-PEGMA). The trimethoxysilyl group is surface-reactive, forming covalent bonds with the solid oxide (SiO_2) surface, effectively anchoring the copolymer to the surface. The hydrophilic PEG components extend away from the surface, into the aqueous solvent, acting as an aggregation and protein-resistant shell via steric exclusion.[76] The authors reported that the TMSMA-r-PEGMA copolymer significantly reduced nonspecific protein adsorption by insulin, lysozyme, and fibrinogen on Si/SiO_2 wafers. Lee et al.[74] also successfully synthesized poly(TMSMA-r-PEGMA)-coated magnetic nanoparticles and reported that these particles resisted aggregation during one month of storage in phosphate-buffered saline.

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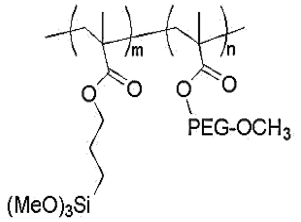
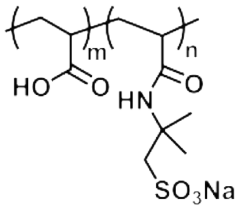
Table 3. Copolymer-Coated Magnetic Nanoparticles

Co-polymer	Co-polymer structure	DLS size of co-polymer coated SPMNPs	Resistance to aggregation	Ref.
TMSMA-r-PEGMA		16.0 ± 2.2 nm 12.3 ± 1.2 nm (size depends on surface modification methods)	SPMNPs were stable in PBS buffer solution and a broad range of pH (from 1 to 10), and did not form any aggregations over 1 month.	74
AMPS-*co*-AA		156 ± 5 nm (in API brine)	SPMNPs were stable in DI water and API brine for several months due to the electrosteric stabilization (zeta potential was lower than -40 mV)	77

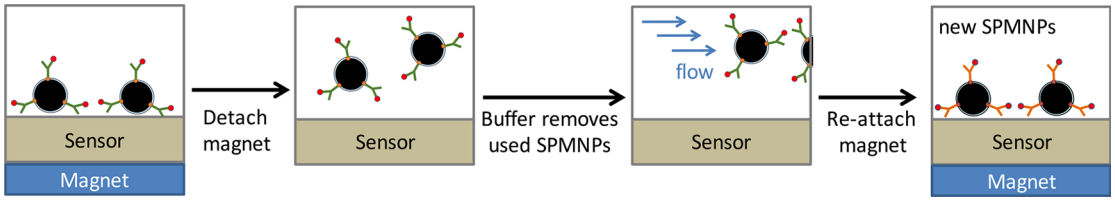


Figure 5. Reusable SPMNP immunoassays. After analyte capture by both the antibody-coated SPMNPs, antigen-bound SPMNPs are concentrated at the sensor surface by a magnet for signal detection. These are then released by detaching the external magnet and removed by flowing buffer solution. New SPMNPs are easily attached on the sensor surface by attaching the external magnet and ready for a new assay.

These promising results have prompted exploration with other copolymers to stabilize and functionalize SPMNPs (Table 3). Ureña-Benavides et al.[77] used poly(2-acrylamido-2-methyl-1-propanesulfonic acid-*co*-acrylic acid), denoted as poly(AMPS-*co*-AA), to stabilize magnetic iron oxide nanoparticles under harsh conditions such as high salinity and high temperatures observed in enhanced oil recovery operations. In this copolymer, poly(AMPS-*co*-AA), the AA groups have anchor function, which form strong complexes with SPMNPs, and the AMPS groups support electrosteric stabilization. The authors reported that poly(AMPS-*co*-AA)-coated SPMNPs were stable against aggregation at 90 °C in standard American Petroleum Institute (API) brine (8 wt % NaCl and 2 wt % $CaCl_2$) for 24 h. The hydrodynamic diameter of poly(AMPS-*co*-AA)-coated SPMNPs was measured by dynamic light scattering after 24 h at 90 °C in standard API brine and found to be 183 ± 58, which is slightly higher than 136 ± 25 measured in API brine at room temperature. Thus, surface modification of SPMNPs with copolymeric materials is likely necessary immunoassay applications, which require a similar high stability in biological media.

In addition to increased stability, polymeric coatings can provide other functions to improve immunoassay performance. Thermally responsive polymers, such as pNIPAm, have been conjugated to SPMNPs to purify and to concentrate AuNP-immune complexes, as described above for detection of malaria antigens.[22] Similarly, gold-magnetite composite nanoparticles coated with poly(acrylic acid) were conjugated with *Treponema pallidum* (Tp) antigens and used to detect the presence of anti-Tp antibodies by lateral flow immunoassay. This resulted in a detection limitation as low as 1 national clinical unit/mL.[78] Finally, SPMNPs and the anticancer drug doxorubicin were coencapsulated with a biocompatible amphiphilic block copolymer and conjugated with an antibody recognizing the breast cancer antigen HER2. These were then able to detect breast cancer cells by magnetic resonance imaging and demonstrated high sensitivity in mice,[79] suggesting the approach may be useful for immunoassays as well.

Reusable Immunoassays. Immunosensors whose surfaces can be regenerated and reused without loss of sensitivity are of great interest for reducing assay cost and waste and may be especially attractive for developing world applications. Thus, far, immunoassays have been regenerated by detaching bound antigen−antibody complexes with treatment such as low pH, which disrupts the complex but also often leads to loss or inactivation the immobilized antibody.[80] In one example, Kandimalla et al.[81] compared different antigen-dissociating agents observing that Gly-HCl (pH 2.3) buffer with 1% dimethyl sulfoxide (DMSO) was most effective, removing 97% of the bound material.

In contrast, SPMNP immunoassays can be easily regenerated by replacing the used nanoparticles with fresh ones. In essence, SPMNPs are first immobilized on the sensor surface using an external magnet. Thus, contaminated SPMNPs are released by detaching the external magnet and removed by flowing buffer solution. The introduction of new SPMNPs and their immobilization with the magnet results in a fresh surface for

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a new assay. Previous studies successfully regenerated SPMNPs immunoassays by these simple steps[54,55,78,80] (Figure 5).

CONCLUSION AND OUTLOOK

Current RDT assays are inexpensive, simple to use with high sensitivity, and provide rapid results. However, the desire to achieve ever-lower detection limits and analyze samples with very low analyte concentrations (namely, urine and saliva) demands analytical techniques with far greater sensitivity. The inclusion of superparamagnetic SPMNPs in immunoassays is a promising strategy to increase assay sensitivity by facilitating sample concentration, amplify or directly produce signal, depending on the specific sensor used. In this review, we introduced strategies by which SPMNPs can be generated, incorporated into immunoassays, and discuss recent efforts to develop SPMNP-based immunoassays. On the basis of these initial reports, assays using SPMNPs are likely to become increasingly common tool for diagnostics.

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Notes

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ABBREVIATIONS

AuNP, gold nanoparticle
ELISA, enzyme-linked immune-sorbent assay
*Pf*HRP2, *Plasmodium falciparum* histidine-rich protein 2
GMR, giant magneto-resistive
LOD, limit of detection
MNP, magnetic nanoparticle
RDT, rapid diagnostic test
SPMNP, superparamagnetic nanoparticle
SPR, surface plasma resonance
SQUID, superconducting quantum interference device

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