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Tailored Magnetic Nanoparticles for Direct and Sensitive Detection of Biomolecules in Biological Samples

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ABSTRACT

We developed nanoparticles with tailored magnetic properties for direct and sensitive detection of biomolecules in biological samples in a single step. Thermally blocked nanoparticles obtained by thermal hydrolysis, functionalized with specific ligands, are mixed with sample solutions, and the variation of the magnetic relaxation due to surface binding is used to detect the presence of biomolecules. The binding significantly increases the hydrodynamic volume of nanoparticles, thus changing their Brownian relaxation frequency which is measured by a specifically developed AC susceptometer. The system was tested for the presence of *Brucella* antibodies, a dangerous pathogen causing brucellosis with severe effects both on humans and animals, in serum samples from infected cows and the surface of the nanoparticles was functionalized with lipopolysaccarides (LPS) from *Brucella abortus*. The hydrodynamic volume of LPS-functionalized particles increased by 25−35% as a result of the binding of the antibodies, measured by changes in the susceptibility in an alternating magnetic field. The method has shown high sensitivity, with detection limit of 0.05 μg·mL^{-1} of antibody in the biological samples without any pretreatment. This magnetic-based assay is very sensitive, cost-efficient, and versatile, giving a direct indication whether the animal is infected or not, making it suitable for point-of-care applications. The functionalization of tailored magnetic nanoparticles can be modified to suit numerous homogeneous assays for a wide range of applications.

There is an increasing need for developing label-free biosensors for selective detection of biomolecules directly in biological samples, such as serum, blood, or urine, etc., which can be more sensitive and less time- and labor-consuming.[1] Besides, for point-of-care applications, the assay should be simple, requiring no or minimum preparation. The possibility of analytes detection directly in biological samples will lead to more economic, simple to use, versatile, and flexible sensors that will have a great impact on point-of-care applications.[2]

A variety of diagnostic tests have been developed for the detection and quantification of biological molecules, e.g., antibodies, in different biological fluids. Traditional immunoassays depend on the use of radioactive, fluorescent, or enzyme-labeled antibodies as analytical tools to monitor biomolecular events.[3] The enzyme-linked immunosorbent assay (ELISA) is the most widely used for the detection of antibody−antigen interactions,[4] both in routine diagnostics and in research. These kinds of assays have a main limitation due to the need of time-consuming pretreatment required for biological samples preparation prior to analyses.[1−5]

Nanotechnology offers an attractive route for identification of biomolecules in vitro and in vivo with high sensitivity through immunoassays that require small amounts of sample and short preparation times compared to traditional diagnostic methods.[6,7] Superparamagnetic iron oxide nanoparticles are used in several biomedical applications,[8] such as MRI visualization,[9] cell separation,[8] as well as quantification of biomolecular targets in cell lysates and tissue extracts.[3,10] They have also been used to detect larger biological entities, such as bacteria, in solutions.[11,12]

In most cases, commercial beads containing magnetic multicores with different surface layers have been used,[13−15] but these detection systems have poor sensitivity and limited detection range due to a wide bead and magnetic domain size distribution. To overcome these limitations, we present a novel strategy for sensitive detection of biomolecules in biological fluids using specific antibodies in serum as a demonstrating example. The sensing principle used in this

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work is based on susceptibility measurements in an alternating (AC) magnetic field of the Brownian relaxation of functionalized magnetic nanoparticles.[14−16] AC susceptibility is an excellent detection technique, since it is simple and direct and can be carried out in a compact device equipped with a simplified readout system. Recently, the detection of specific DNA strands after rolling circle amplification with commercial magnetic beads has been carried out measuring large changes in Brownian relaxation.[16]

In the current study, the key component of the detection of biomolecules is magnetic nanoparticles with a narrow size distribution, consisting of a single magnetic core. Compared to multicore beads (containing more than one magnetic single-domain), single core magnetic particles (with only one single-domain) show superior performances when used to monitor adsorption induced changes of the Brownian relaxation measured with AC susceptometry. For a given total magnetic mass, the signal-to-noise ratio increases linearly with the volume of the nanoparticle. This is due to the fact that single core nanoparticles have a larger low field susceptibility compared to multicore nanoparticles for corresponding total size of the magnetic single domains.[17] Furthermore, the smaller nanoparticles are more sensitive to changes of its AC susceptibility than to adsorption-induced coverage changes. For example, the diameter of the single core nanoparticles that we synthesized is only 5 times larger than the *Brucella* antibodies (2−5 nm);[18] therefore the expected change in diameter due to binding is large.

The superior sensitivity of our method has been tested by detecting *Brucella* antibodies directly in serum samples from animals and monoclonal antibodies in buffer solutions. *Brucella* bacteria are the agents causing brucellosis, a worldwide zoonosis with severe effects on both human and animal health. A large family of related bacteria has been identified, infecting both humans and domestic animals.[19] The most widespread form is caused by *B. abortus* and, more recently, *B. melitensis* which affects mainly cattle.[20] Therefore we have chosen to detect Brucella antibodies to validate the assay and the detection system.

Despite the large number of reports on the synthesis of superparamagnetic nanoparticles with different methods,[21] including coprecipitation, hydrothermal synthesis, microemulsion, and thermal decomposition in organic solvents, very few methods allow the preparation of thermally blocked nanoparticles with mean size just above the critical minimal limit necessary for magnetic thermal blockage.[22] In this study, we used a modified synthesis procedure with controlled hydrolysis of chelate metal alkoxide complexes at high temperature in solutions of a chelating alcohol. Typically, ferrous and ferric chloride solution in *N*-methyl diethanolamine (NMDEA) was added to a solution of NMDEA containing sodium hydroxide. The reaction mixture was mechanically stirred at room temperature for 4 h, after which the temperature was raised slowly to 210 °C and maintained for 5 h. The suspension was then slowly cooled to room temperature and the solid product formed was isolated by centrifugation, washed four times with a mixture of ethanol and ethyl acetate and finally redispersed in water.

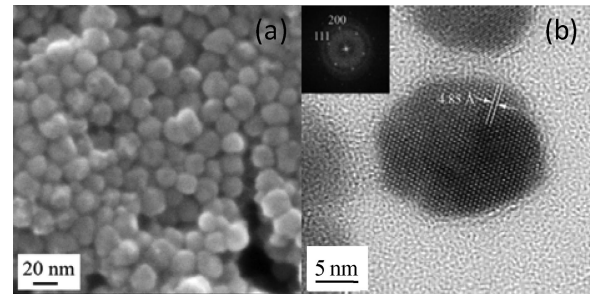

Figure 1. (a) SEM secondary electron image of pristine iron oxide nanoparticles. Specimens were prepared by applying a few drops of the nanoparticle suspension on a standard aluminum stub. (SEM image recorded at 10 kV with a LEO 55 Ultra equipped with a field emission gun.) (b) High-resolution TEM image showing a single crystal nanoparticles imaged along the zone axis [0$\bar{1}$1]. Inset shows the electron diffraction pattern confirming the magnetite structure.

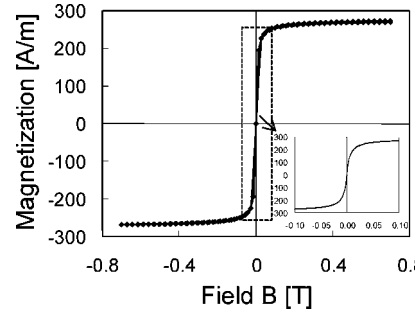

Figure 2. Magnetization vs applied field curve of the magnetic particle system at room temperature in two field ranges, ±0.7 T and ±0.1 T (the inset figure) measured using VSM.

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to confirm particle size, morphology, and crystal structure. Figure 1a shows an overview of the iron oxide nanoparticles. The average diameter of the nanoparticles was 19.5 nm (standard deviation 4 nm) as measured from 300 particles, and this size is just above the critical superparamagnetic limit of 12−15 nm.[21] High-resolution transmission electron microscopy (HRTEM) showed that most of the particles are single crystals (Figure 1b). The magnetite structure (Fe_3O_4, ICDD No. 88-0866) was confirmed by selected area electron diffraction and energy dispersive X-ray (EDX) analysis. In addition, X-ray powder diffraction analysis also confirmed that the nanoparticles have the magnetite crystal structure.

Static and dynamic magnetic measurements were carried out on solutions of nanoparticles suspended in MilliQ purified water. Results from static measurements performed using a commercial vibrating sample magnetometer (VSM) at room temperature, presented in Figure 2, show a sharp decrease of the magnetization at low fields and magnetization saturates at moderate fields (about 0.4 T).

The low field magnetic susceptibility (the initial susceptibility) of nanoparticles suspension is high compared to commercial multi core particles. This, together with the fact that the magnetization of the nanoparticle system saturates at moderate fields, is characteristic of the single core nature

of the particles and the size of the magnetic core (high particle magnetic moment).

When the nanoparticles are suspended in liquid and the internal Neél relaxation in the single-domain is longer than the relaxation due to motion of the whole particle, the particles relax mainly through the Brownian relaxation process. For such particles the hydrodynamic size can be determined by magnetic measurements.[23] The Brownian relaxation time, τ_B can be described by

$$\tau_B = \frac{\pi D_H^3 \eta}{2kT} \quad (1)$$

where D_H is the hydrodynamic diameter of the particle, η the viscosity of the liquid, k the Boltzmann constant, and T the temperature. The experimental susceptibility data were fitted to a Debye model including the particle size distribution[24] according to

$$\chi = \int \frac{\chi_0}{1 + j\omega\tau_{eff}(D_H)} f(D_H)\, dD_H \quad (2)$$

where χ is the complex magnetic susceptibility, $f(D_H)$ the hydrodynamic size distribution of the particles, ω the angular frequency ($2\pi f$, where f is the excitation frequency), τ_{eff} the effective relaxation time of the magnetic particles (that includes both the Néel and Brownian relaxation process), and χ_0 is the DC susceptibility. AC susceptometry can be used to measure the magnetic relaxation of pristine nanoparticles in suspension.

AC susceptometry has been performed using a detection system designed in-house (described elsewhere[16]) that allows measurements of dynamic magnetic properties at much higher frequencies than commercially available systems. The setup is simple since it is based on an exciting coil (in which the sample is inserted) and a measuring one, it operates at room temperature and it requires only a few seconds to acquire the data. It was necessary to develop a system working over a wide frequency range because the small size of the nanoparticles used in this study give Brownian relaxation frequencies around several tens of kilohertz. The measurement principle is based on the frequency dependence of the real and imaginary parts of the magnetic susceptibility, which is sensitive to the increase in the hydrodynamic volume of the magnetic nanoparticles before and after binding biomolecules. From the measurements and the fitting procedure, it is possible to calculate the hydrodynamic size distribution of the nanoparticle system. The experimental data and the results of the fitting are shown in Figure 3.

Figure 3 shows the real (curve a) and imaginary (curve b) parts of the frequency-dependent complex magnetization for the native iron oxide nanoparticles. The imaginary part of the complex susceptibility has a maximum at about 50 kHz for the native nanoparticles suspended in MilliQ purified water, due to the Brownian relaxation. From the result of the fitting, we can determine a median diameter of the particles of about 20 nm with a size distribution width of 8 nm, which is in a good agreement with the results from TEM data. Photon correlation spectroscopy (PCS) data also confirm the size of the pristine nanoparticles. The magnetic, hydrodynamic, and crystalline sizes are quite similar due to

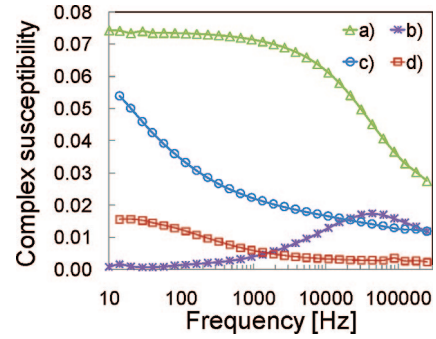

Figure 3. AC susceptibility measurements in the range 1−250 kHz for pristine iron oxide nanoparticles and lipopolysaccharide (LPS) functionalized nanoparticles. Curves a and b give the real and imaginary parts of the magnetic susceptibility for pristine iron oxide nanoparticles, respectively. Curves c and d give the real and imaginary parts of the magnetic susceptibility data obtained for LPS-bound particles, respectively. A very large frequency shift is observed for LPS particles indicating a large increase in hydrodynamic volume due to LPS particles binding. The solid lines are obtained by fitting the data points to eq 2.

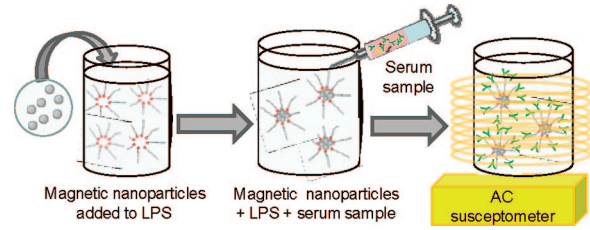

Figure 4. Schematic illustration of the detection scheme. First thermally blocked nanoparticles with tailored properties are synthesized, then specific molecules for the analyte to be detected are bound to the surface of the pristine nanoparticles. After this, the functionalized nanoparticles are mixed to a biological sample to analyze and finally the magnetic relaxation of the mixture is measured. A frequency shift is observed for LPS functionalized nanoparticles compared to pristine ones due to an increase in hydrodynamic volume. A further increase in volume occurs when the analyte to detect binds to the functionalized nanoparticles.

the fact that the prepared nanoparticles are highly crystalline and have a hydrophobic surface that reduces the absorption of water molecules.

The detection scheme of the assay, depicted in Figure 4, is based on a few steps: synthesis of thermally blocked nanoparticles with tailored properties, binding of specific molecules for the analyte to be detected to the surface of the pristine nanoparticles, mixing the functionalized nanoparticles to a biological sample to analyze, measuring the magnetic relaxation of the mixture. As a result of the binding of the analyte molecules to the functionalized nanoparticles, the hydrodynamic size will increase, which can be determined by an AC susceptometer.

To demonstrate the validity of the approach, we studied the specific detection of *Brucella* antibodies by functionalizing the nanoparticles with lipopolysaccarides (LPS) as corresponding antigen. Using other types of LPS or antigens for the functionalization step, it will be possible to detect different antibodies and other biomolecules. The antigen in the form of lyophilized *Brucella abortus* lipopolysaccharide (LPS) (obtained from Department for Environment, Food

and Rural Affairs (DERFA), United Kingdom) was directly immobilized on pristine nanoparticles. The antigen was attached to the particles by adsorption through van der Waals interactions, as the surface of the particles is hydrophobic and thus there is no need for chemical coupling of *Brucella* antigen. This conjugation strategy has the advantage over the use of chemical binding of the antibodies to the surface of the nanoparticles, because it does not affect the bioactivity of the antibodies and also requires fewer functionalization steps resulting in minimal loss of nanoparticles and antigens during processing. Iron oxide nanoparticles have been exposed to high antigen concentration (7 mg/mL), far above the critical micelle concentration (cmc) for the antigen. Since LPSs are amphiphilic molecules, they form aggregates of different shapes depending on concentration, temperature, and ionic strength, etc. The exact nature of the attachment of LPS to magnetic particles is not well understood. It is plausible that LPS monomers attach to the surface of magnetic particles resulting in the formation of aggregates. The obtained suspension is stable in serum indicating that LPS has a stabilizing effect, blocking nonspecific binding via its polysaccharide chains. This is consistent with the known ability of LPS to block nonspecific adsorption of proteins.[20]

The procedures of the test are simple and straightforward: A small sample of suspension containing nanoparticles (500 μL, [Fe] = 4.1 mg/mL) is mixed with 100 μL of LPS solution (7 mg/mL LPS in water) and sonicated by pulsed ultrasound cycles for 2 $\times$ 20 min. The LPS-functionalized nanoparticles form a suspension which is stable for several days without noticeable aggregation. A small sample of the LPS-functionalized nanoparticles is added to the biological solution (serum with or without the antibodies, i.e., positive or negative serum, respectively). AC susceptibility of the mixture is measured.

The change of AC susceptibility of the nanoparticles after LPS functionalization (Figure 3) shows that LPS-functionalized nanoparticles exhibit a frequency maximum at about 20 Hz (Figure 3, curves c and d), which is considerably lower than the maximum observed for pristine nanoparticles without the LPS (about 50 kHz). This dramatic shift in frequency for the maximum in the imaginary part of the susceptibility indicates that the hydrodynamic diameters of the LPS-functionalized nanoparticles increases more than 10 times as compared with that of the pristine nanoparticles (from 20 to $\sim$260 nm). The hydrodynamic size of the nanoparticles determined by AC susceptometry is in agreement with the measurements done using photon correlation spectroscopy. The measurements also indicate that the particle size distribution is monomodal and there is no significant amount of pristine nanoparticles in the suspension.

The assay for the detection of *Brucella abortus* antibodies uses LPS-functionalized nanoparticles that were first diluted to a concentration of 2 mg/mL with respect to Fe. The serum samples were diluted 6 times with MilliQ water before the measurements. Aliquots of 30 μL of nanoparticle suspension were mixed either with 5 μL of (i) positive serum (containing anti-*Brucella* antibodies) from infected cows, (ii) negative serum (containing no anti-*Brucella* antibodies) from noninfected animal, or a mixture containing (iii) 5%, (iv) 10%, and (v) 50% positive serum.

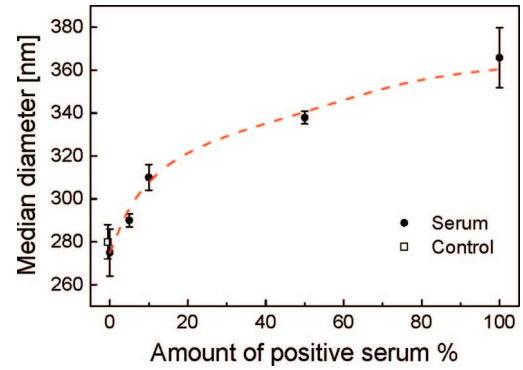

Figure 5. Hydrodynamic median diameter of LPS functionalized nanoparticles derived from magnetic susceptibility measurements vs amount of positive serum. Iron oxide nanoparticles, after LPS treatment, bind anti-*Brucella* antibodies contained in positive serum, resulting in an increase of their hydrodynamic volume. Repeated measurements on each samples showed virtually no spread. Error bars represent the standard deviation. The dashed line only represents the trend.

As control sample (noted as control in Figure 5) MilliQ purified water containing NaCl (with a salt concentration corresponding to the one in serum samples) was added to a sample with magnetic nanoparticles. This reference was chosen because magnetic nanoparticles tend to agglomerate slowly (on the order of days) in solutions with high ionic strength. The samples were incubated for 1 h at room temperature on a shaking board after which the magnetic susceptibility measurements were performed. LPS-functionalized nanoparticles did not show aggregation when mixed with positive serum or with the *Brucella* antibodies in a buffer solution. However, their hydrodynamic size changes as a result of binding of the antibodies to LPS.

The detection method is based on magnetic response of the LPS-functionalized nanoparticles, and it results in an increase of their size which is significantly different for particles not bound to anti-*Brucella* antibodies (ABA). The magnetic susceptibility results are summarized in Figure 5.

From the AC susceptibility data, no increase in median particle diameter could be observed upon mixing LPS-functionalized nanoparticles suspension with negative serum. When LPS-functionalized nanoparticles are added to positive serum, a large increase in hydrodynamic diameter is obtained. When LPS-functionalized nanoparticles are exposed to solutions with increasing positive serum concentrations (5%, 10%, and 50%), the hydrodynamic sizes increase accordingly.

This indicates that only antibodies specific to the LPS *Brucella* antigen bind to the nanoparticles giving us the possibility to distinguish between infected and noninfected animals. Negative serum does not contain any *Brucella* antibodies and indeed does not result in any change of nanoparticle size. However, there might be a slight difference in total protein and antibody concentration because the serum samples are obtained from different animals.

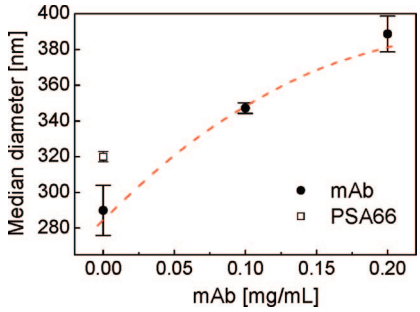

Figure 6. Hydrodynamic median diameter of LPS-functionalized nanoparticles derived from magnetic susceptibility measurements. Iron oxide nanoparticles, after LPS treatment, bind anti-*Brucella* monoclonal antibodies Bx88 but do not bind nonspecific PSA66 antibodies. Repeated measurements on each sample showed virtually no spread. Error bars represent the standard deviation. The dashed line only represents the trend.

In order to confirm the specificity of LPS-functionalized nanoparticles, further experiments were performed with monoclonal antibodies specific to *Brucella* (Bx88, obtained from HyTest LtD, Finland) in buffer solution as well as with a control of nonspecific antibodies (PSA66, prostate-specific antigen IgG antibodies, obtained from CanAg Diagnostics, now Fujirebio Diagnostics, Sweden). The exposure of LPS-functionalized nanoparticles to the anti-*Brucella* antibody Bx88 produces a significant shift in the maximum of the imaginary magnetic susceptibility. In comparison, the nonspecific PSA66 antibodies used as a negative control did not give any significant shift (see Figure 6). This is reflected in a large increase in hydrodynamic size of the LPS-functionalized nanoparticles exposed to Bx88 antibodies as compared with the exposure of LPS-functionalized nanoparticles to PSA66 antibodies, as in Figure 6.

The data in Figure 6 confirm the high specificity of the LPS-functionalized nanoparticles that are capable to sense specifically anti-*Brucella* specific monoclonal antibody and not other kind of antibodies, i.e., PSA66.

In order to investigate the sensitivity of the nanoparticle-based sensor, detection experiments were performed by diluting the original positive serum solution 400 and 4000 times with MilliQ purified water. The data did not show any tend in the case of 4000 times dilution. Therefore we can conclude that our nanoparticle-based biosensor system is capable of detecting anti-*Brucella* specific antibodies in serum with concentrations close to 0.05 μg/mL of *Brucella* antibodies. The upper limit of detection (dynamic range) depends on the total number of particles in the sample. If we consider the lowest particle concentration that we can detect (1 mg Fe/mL), we estimate the upper limit of detection in 0.2 mg/mL. Our detection system is therefore capable of distinguishing between infected and noninfected animals using small serum samples even with low antibodies concentration. Traditional assays, such as ELISA, can have a better sensitivity, but they require laborious sample treatments, such as separation, preparation, and purification, while our system is capable of detecting biomolecules directly in serum or other biological fluids even at low concentrations without any pretreatments.

In conclusion, we have reported on the fabrication tailored thermally blocked iron oxide nanoparticles and demonstrated their use for highly sensitive detection of *Brucella* antibodies directly in biological fluids, such as serum, through magnetic susceptibility measurements. This approach, apart from being sensitive and fast, gives a direct answer if the animal is infected or not and requires no or minimum sample preparation. The use of induction techniques as detection system allows the construction of an automated, compact, simple-to-use, and fast instrument at low cost suitable for use at point-of-care by nonspecialists. The assay is also very versatile, and by choosing different antigen molecules for the functionalization, it is possible to detect different analytes directly in biological fluids to build a modular system for multianalyte detection.

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Supporting Information Available: Figures showing TEM study of pristine iron oxide nanoparticles, PCS studies of iron oxide nanoparticles before and after LPS functionalization, and study on the dynamic range of the nanoparticle-based sensor. This material is available free of charge via the Internet at http://pubs.acs.org.

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