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Optical protein detection based on magnetic clusters rotation

Research Paper

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Abstract

In this paper we present a simple method to quantify aggregates of 200 nm magnetic particles. This method relies on the optical and magnetic anisotropy of particle aggregates, whereas dispersed particles are optically isotropic. We orientate aggregates by applying short pulses of a magnetic field, and we measure optical density variation directly linked to this reorientation. By computing the scattering efficiency of doublets and singlets, we demonstrate the absolute quantification of a few % of doublets in a well dispersed suspension. More generally, these optical variations are related to the aggregation state of the sample. This method can be easily applied to an agglutination assay, where target proteins induce aggregation of colloidal particles. By observing only aligned clusters, we increase sensitivity and we reduce the background noise as compared to a classical agglutination assay: we obtain a detection limit on the C-reactive protein of less than 3 pM for a total assay time of 10 min.

Introduction

Immuno-detection on nanoparticles is a simple and widely used method in diagnostic tests [1]. In these assays, latex beads functionalized with antibodies are mixed with the sample. If antigens are present in the sample, they link to a first bead and by linking to a second one they form doublets or larger aggregates. As light scattering is linked to the size of aggregates, turbidimetric measurements reveal the presence of clusters and thus antigens. This method is rapid and very easy to operate, but is not sensitive enough for some analytes.

To lower the detection limit and improve the robustness of the method, various evolutions have been proposed. One approach involved the application of ultrasound to create local higher bead densities [2]. Another approach involved replacing latex particles by superparamagnetic particles. Under a 1-D magnetic field, the particles become magnetized, attract each other and assemble into chains. By bringing beads into contact, the magnetic field increases the probability of doublet formation, and so it decreases the overall test duration. Indeed, it can be shown that these latex nanoparticle based tests are diffusion limited. Microfluidic devices can also be used to locally concentrate superparamagnetic beads in order to accelerate the recognition kinetics [4] but they are less easy to implement.

In this paper, we show that by using the intrinsic optical and magnetic anisotropy of clusters, it is possible to use a magnetic field to detect scattering associated only with aggregates. Clusters are aligned using a short pulse of a low magnetic field, and this rotation induces a variation of turbidity. The amplitude of the pulses is low enough to avoid the formation of new clusters and chains. Moreover, there is no contribution from non-aggregated beads.

In the following, we first present the method and its optical basis. We will then present its application to assay the concentration of

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C-reactive protein (CRP) in solution, and compare results between the previous and the new method.

Materials and methods

Magnetic particles were purchased from Ademtech, Pessac, France. The method was first validated with *Bio-Adembeads Streptavidin* (200 nm). In order to minimize the presence of aggregates in the sample, the solution was mildly centrifuged (2000 × *g* during 10 s) prior to the test and beads were taken from the upper part of the sample. Beads were then diluted in phosphate buffered saline (PBS) + surfactant Pluronic PF127 0.3%; initial optical density indicates that the beads concentration is approximately 50 pM.

The same beads are used for the titration of biotinylated Bovine Serum Albumine (BSA-biotin). Bead concentration is 0.04% w/w—approximately 90 pM. The beads and target are diluted in PBS 0.1× + PF127 0.3%. PBS tablets, PF127 and BSA-biotin were purchased from Sigma. For the samples containing 1000 pM and 2000 pM, the optical length was 4 mm instead of 1 cm and the optical density (OD) measured was multiplied by 2.5 to get the equivalent to 1 cm.

In order to evaluate the method on a real antibody-antigen system, we have developed a C-reactive protein assay. The mean diameter of *Carboxyl-Adembeads* particles is 190 ± 60 nm, measured using dynamic light scattering (Zetasizer Nano from Malvern). Polyclonal antibodies directed again CRP are purchased from Interchim and grafted onto beads by Horiba Medical. CRP is purchased from Euromedex, concentration in stock solution is measured with a Pentra 400 (Horiba Medical).

The final cuvette volume is 150 µL: 7.5 µL of beads stored at 0.8% w/w, 7.5 µL of concentrated CRP in saponin and 135 µL of buffer containing BSA 1% w/w. The final concentration of beads is 0.04% w/w. CRP physiological concentration in plasma of healthy patients is typically less than 10 mg/L [5], corresponding to 85 nM. After a dilution 75× in saponin, CRP concentration is 1.1 nM and so the final CRP concentration in the cuvette is 56 pM. In order to produce an assay response curve and to estimate the detection limit of CRP, 11 blanks and 2 measurements at each concentration were obtained.

The light source is a very stable Coherent's ULN-Series diode laser (typical 0.06% RMS noise), emitting polarized light at 635 nm. Behind the cuvette, the beam is analyzed by a photodiode – Thorlabs, PDA36A-EC – and the voltage is measured by a multimeter Agilent 34410A. A first coil – inner diameter 10 cm – generates an axial high magnetic field B_A in the axis of the laser. Transversal field B_T, perpendicular to the laser, is generated by two smaller coils located inside the previous coil. This second field is also perpendicular to the vertical polarization of the laser. The sample is placed in a spectrometer cell; the optical path length is 10 mm. No analyzer is placed after the cuvette; i.e. dichroism is also considered. The setup is represented in Fig. 1.

The optical density is defined as $OD = \log_{10}(I_0/I)$, where I_0 is the laser intensity and I the intensity after the cuvette. After 1 min of sample stabilization, a first optical measurement is performed. Then, 5 magnetizations at 20 mT are applied – from 10 to 160 s – for a total magnetization of 5 min. Each magnetization is followed by 20 s of resting time. The field amplitude must be large enough to allow chain formation, and it can be either axial or perpendicular to the laser (it is axial in our setup). During this step, specific bonds are formed between beads. At the end, when the field is switched off, a final relaxation time of 100 s enables a fraction of non-specific doublets to dissociate. The second measurement is performed after this relaxation step. The total duration of the test is 10 min.

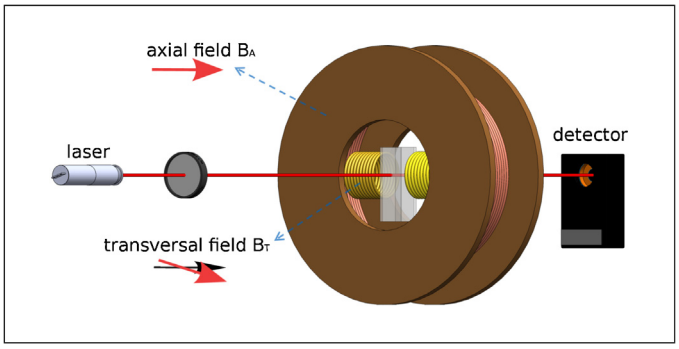

FIG. 1

Experimental setup. The two coils generate magnetic fields perpendicular to laser polarization.

Two methods are compared in this paper. First, the classical optical density method. Here, the difference between the initial and final values, noted ΔOD is measured, and the value recorded provides information on the number of clusters formed under magnetization and also on the possible drift of optical density of the system.

Second, the new method. Here, the ''Clusters Orientation Measurement'' (COM) consists of applying 250 ms of an axial magnetic field – 5 mT – and 250 ms of a transversal field – 5 mT – separated by a relaxation of 250 ms. The COM amplitude is the difference between the maximum and the minimum of optical density during the sequence of magnetic pulses—an example is shown in Fig. 2. The difference between the initial (before magnetization) and final measurement (after magnetization) is called ΔCOM. We will show that this parameter is proportional to the number of clusters created during the high magnetic field phase. We will also show that our method is of particular interest for the smallest aggregates created—e.g. the doublets.

Results and discussion

COM measurements

The sample analyzed in Fig. 2 is composed of pure *Bio-Adembeads Streptavidin* beads. The time response of the optical density during the sequence is shown on Fig. 2(b). We record an increase in the optical density during the application of the axial field and a reduction during the application of the transversal field. When the fields are switched off, OD relaxes to the initial value, indicating no aggregation due to these magnetic fields. We will show that this observation can be qualitatively explained by the reorientation of small aggregates, i.e. doublets already in the solution. In a second part, we will show that this method can also be used to assay antigen concentration as ΔCOM is proportional to the amount of binding proteins.

Orientation of doublets: Magnetic anisotropy

The magnetic beads used here are superparamagnetic. When an external magnetic field is applied, beads in solution rapidly acquire a magnetic moment parallel to the external field, and proportional to its amplitude. In the second step, due to anisotropy of the

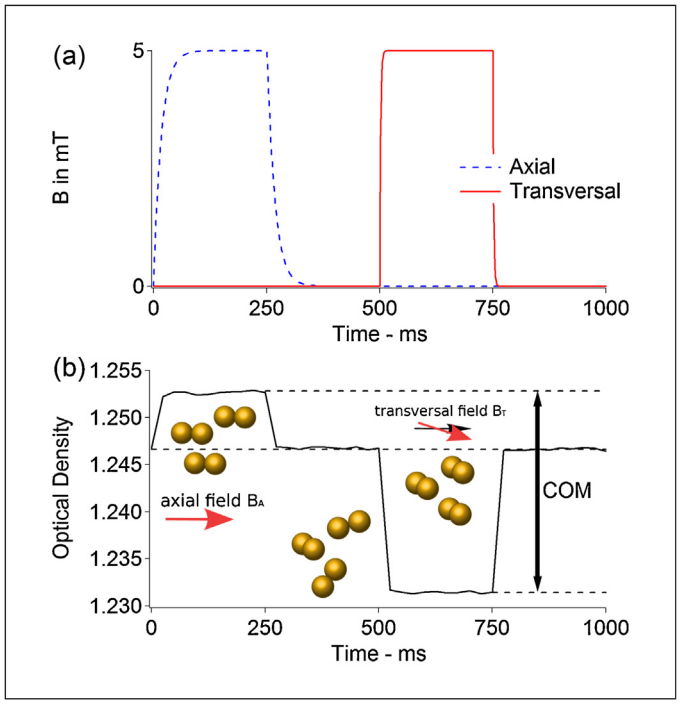

FIG. 2

COM principle. (a) Sequence of applied magnetic pulses of axial and transversal field for the orientation measurement. (b) Associated optical density variations. On this example COM amplitude is 21 mOD.

dipole–dipole interactions, the beads self-assemble into chains. At the same time, if a cluster of beads is already formed in solution, it will align in the direction of an applied magnetic field: each bead bears a magnetic moment always parallel to the field, so the anisotropic cluster will rotate to align the dipoles. We will show in this part that it is possible to adjust the experimental parameters in order to observe cluster rotation without the formation of chains.

Fig. 3 shows the time evolution of the optical density after the application of a magnetic field. For $B < 6$ mT, the optical density quickly reaches a plateau, whereas it continuously increases with time if $B > 6$ mT. This threshold corresponds to the chaining process, which it occurs when the interaction energy between beads is higher than the thermal energy, k_BT [6]:

$$U_m = \frac{\pi R^3 \chi B^2}{18\mu_0} \geq k_B T \quad (1)$$

where μ_0 the magnetic permeability, R the radius of the bead, k_B Boltzman's constant and T the temperature (the magnetic susceptibility of a particle is close to 1 [7]). Estimation with our conditions gives $B \approx 5$ mT, which is in good agreement with the experimental data.

Below this chaining field, the plateau value increases with the field amplitude: clusters are more aligned with the direction of the field at higher amplitude, i.e. the thermal torque acting on the clusters cannot be neglected. Experimentally, we have chosen $B = 5$ mT for the COM detection, which provides a trade-off between a good alignment of the doublets and no chain formation. The pulse of magnetic field must be long enough to enable clusters to rotate. For 5 mT, 250 ms is sufficient to reach the steady state.

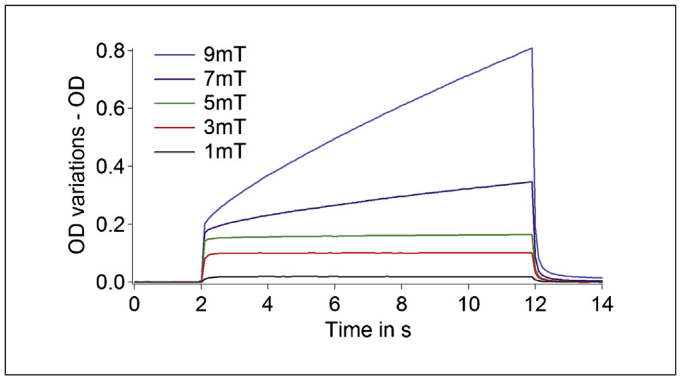

FIG. 3

Optical density variations of non grafted beads (0.04% w/w) under different values of axial magnetic field. Field is applied during 10 s.

Doublet quantification: Optical anisotropy

It can be shown that an elongated object will scatter light differently depending on its orientation [8], this is the essence of our method. In the case of doublets, there is no analytical solution to the problem, and numerical simulation has to be used. We will first recall how to compute the turbidity variation in the classical method [3] and then we will explain the COM computation.

One doublet scatters more light than two isolated singlets, the ratio between these two light scattered intensities for randomly oriented doublets is called α. This value can be estimated by using a T-matrix method and a freely available software [9]. Parameters needed are particle optical index – $1.78 + 0.02i$ [7] – and particle radius—100 nm. With these conditions, it can be checked numerically that a doublet will absorb almost the same amount of light as two separate beads, so we will considerer only the contribution of scattering in the following calculation. With Q_{scat} defined as scattering efficiency of a singlet, h the optical path length and R the geometric radius of particle; optical density for a solution containing initially N_{sing} singlets and N_{doub} doublets per unit volume is:

$$OD_{initial} = h\pi R^2 Q_{scat} \frac{N_{sing} + \alpha . N_{doub}}{\ln(10)} \quad (2)$$

During the test ΔN_{doub} are formed; leading to a final optical density:

$$OD_{final} = h\pi R^2 Q_{scat} \frac{(N_{sing} - 2\Delta N_{doub}) + \alpha(N_{doub} + \Delta N_{doub})}{\ln(10)} \quad (3)$$

Variation of optical density $OD_{final} - OD_{initial}$ during the test is then:

$$\Delta OD = h\pi R^2 Q_{scat} \frac{(\alpha - 2)\Delta N_{doub}}{\ln(10)} \quad (4)$$

With our conditions, we obtain numerically $Q_{scat} = 0.23$ and $\alpha = 2.45$; so 1 pM of antigen which creates 1 pM of doublets should increase optical density of 8.5 mOD.

Concerning the COM; it uses the property that doublets scatter light differently, if they are parallel to laser beam – aligned with B_A – or if they are transversal to it—aligned with B_T. In the first case the ratio between scattered light by one doublet and two separate singlets is named β_A; the ratio is β_T in the second case. Numerical simulations give $\beta_A = 2.73$ and $\beta_T = 1.73$, compared to the average

value for randomly oriented doublets $\alpha = 2.45$ obtained previously. With an axial field, a doublet will scatter more light than without field, whereas with a transverse field, it should scatter less light. This coincides with the experimental data obtained for a well dispersed sample, where we observe a small number of small aggregates (doublets), as shown on Fig. 2. According to these ratio values—α, β_A, β_T, optical variation during the application of a transversal field must be larger than during the application of an axial field: $(\alpha - \beta_T) = 2.6(\beta_A - \alpha)$. This ratio is also obtained in Fig. 2, where variations during transversal and axial field are 15 and 5.8 mOD respectively. The measured ratio is 2.6, which supports our assumption that aggregates are mainly doublets in this example.

The amplitude of a COM is the optical density difference when clusters are aligned with these two fields:

$$\mathrm{COM} = h\pi a^2 Q_{\mathrm{scat}} \frac{(\beta_A - \beta_T) N_{\mathrm{doub}}}{\ln(10)} \quad (5)$$

In our condition, 1 pM of analyte should result in a signal of 19 mOD, which more than doubles the ΔOD signal since pulses of magnetic field align the doublets and maximize the amount of scattering. Furthermore, this signal is specifically associated with the formation of doublets, as such, it should be less prone to noise and drift associated with non-reactive beads in solution. Even in a well dispersed colloidal sample, there is always a small number of doublets or even larger clusters, as demonstrated by Fig. 2 and the experimentally measured COM signal of 21 mOD. If we assume again that these are doublets, the COM measurement reveals that around 1 pM of doublets are present and 4% of the beads are aggregated. This value is small enough to justify our assumption that the only aggregates present in solution are doublets.

Clusters orientation assay

In a bioassay, we are less interested by the initial aggregation state of the solution, so we will measure the variation between final and initial measurement:

$$\Delta\mathrm{COM} = h\pi a^2 Q_{\mathrm{scat}} \frac{(\beta_A - \beta_T) \Delta N_{\mathrm{doub}}}{\ln(10)} \quad (6)$$

ΔCOM measurements for different concentrations of target proteins are shown in Fig. 4(a), along with the classical turbidity measurements. At the beginning of the curve we observe a linear increase of the signal as a function of target concentration. This corresponds to the formation of small aggregates – e.g. doublets – due to the presence of BSA-biotin. Signal variations, higher than ΔOD, make our method particularly relevant in this range. The initial slopes are 70% of our numerical estimation for both methods, indicating that not all the BSA-biotins are forming doublets. These differences could also be due to small errors on the particles optical index and size determination, leading to an overestimation of the numerical slope. It may also come from thermal motion: doublets are not perfectly aligned with the field, reducing in effect the value $(\beta_A - \beta_T)$. Indeed, higher fields lead to chaining as explained before.

At higher concentrations we have clusters bigger than doublets, so the previous computation could not be directly applied; quantification now relies on a calibration step, as for all immunoassays. It may also be possible to extend the previous optical model if an absolute quantification proves to be necessary for a particular application. If the target concentration is increased further, up to 1 nM, the COM signal decreases, so the accessible dynamical

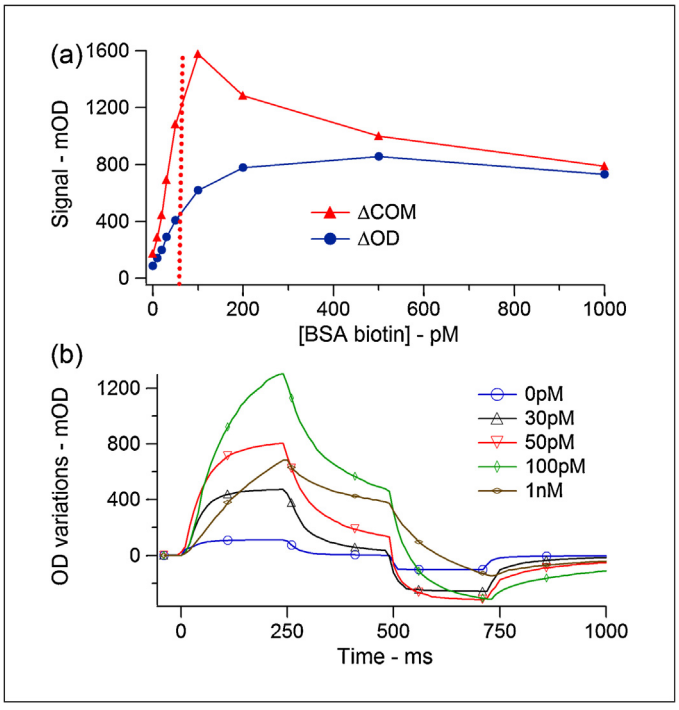

FIG. 4

(a) ΔCOM and ΔOD for different BSA-biotin concentrations. The signal is linear until [BSA-biotin] ~ 80 pM (dashed line). (b) Time traces of the optical density for different BSA-biotin concentrations.

range is typically between 1 and 100 pM of BSA. In order to understand why the signal is decreasing, we may observe the sample response to a sequence of magnetic pulses as for Fig. 2. Fig. 4(b) shows such variations at the end of the test. For 100 pM of BSA and more, the aggregates are now too big to rotate completely during the 250 ms of magnetic field application. Therefore this problem could be remedied by using a longer time of magnetization, for example 5 s. If, as in our actual set-up, heating of the transverse coil with time is an issue, it may be also possible to systematically extrapolate the stationary COM signal from the time traces for large aggregates. It is also interesting to note from Fig. 4(b) that as the target concentration is increases, most of the COM signal is associated with the axial field application, as such a system with only one axial magnetic field, as for a classical magnetic agglutination assay, may be enough for most of the applications.

CRP testing

We will now present the detection of CRP in a buffer with 1% w/w of BSA. The experimental results – on Fig. 5 – show that 1 pM of agent increases the signal by 2.7 mOD/pM for ΔOD and by 3.9 mOD/pM for the ΔCOM. Differences between experimental and theoretical values (respectively, 8.5 and 19 mOD/pM) could be explained by the fact that each protein does not induce the formation of one doublet. We can suppose that approximately 25% of the antigens create a link between particles, as both the ΔOD and the ΔCOM signals are around 4 times smaller than their theoretical values. We now calculate the limit of detection, defined as the concentration read from the curve at a response level equal to the mean negative control level plus three times the

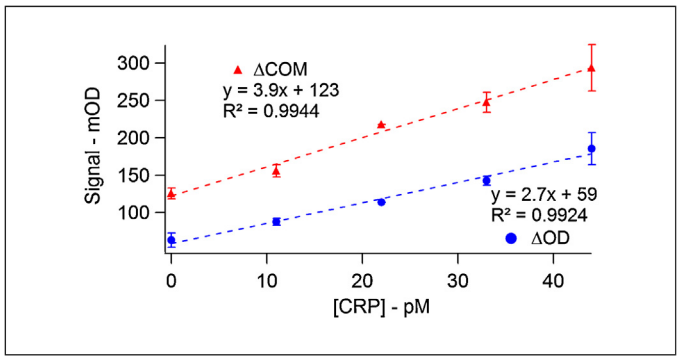

FIG. 5

ΔCOM and ΔOD variations for different CRP in buffer with BSA. The slope is 3.9 mOD/pM for the ΔCOM measure, and 2.7 mOD/pM for the classical OD method.

standard deviation estimated from the negative control. We obtain detection limits of 7.4 pM for ΔOD and 3.0 pM for ΔCOM. We gain at two levels: first, as expected, the new method is less noisy ($\sigma_{\Delta OD}$ = 6.8 mOD vs $\sigma_{\Delta COM}$ = 3.9 mOD, 11 blanks), and second the slope of the dose–response curve is higher ($slope_{\Delta OD}$ = 2.7 mOD/pM vs $slope_{\Delta COM}$ = 3.9 mOD/pM). Our new method allows us to divide detection limit by two; with only minor modifications to our experimental setup.

Moreover, this direct optical quantification provides interesting information in order to optimize an immunoassay. For a given assay format, beads surface coating, antibodies choice and their grafting procedure are known to affect drastically the overall test performance, but there are no straightforward rules to optimize a test. Intermediate characterization, such as antibodies density, their orientation etc are very interesting but are tedious to measure. Here, the dose–response slope can be directly compared to its optimal value. For example, the BSA-biotin assay is near its optimum, however it should be possible to improve by a factor 4 the reactivity of the CRP system. In the case of future development of this assay, we should try other antibodies, and/or optimize the grafting procedure. The first COM measurement provides information on the aggregation state of the initial sample, before agglutination due to proteins, which could be used to check the quality of the reactive beads.

Comparison with other methods

It has to be noticed that magneto rotation has already been used for diagnostic applications, albeit using different implementations. For example, the rotational diffusion of nanoparticles [10] or microparticles [11] may be affected by antigens capture, thus allowing its detection. Another idea was to use chain rotation as micromixer to enhance the capture of proteins from solution: Park et al. [12] measured subnanomolar concentration in 30 s. The most integrated and sensitive platform to date has been developed by Ranzoni et al. [13]. In these experiments, the optical and magnetic anisotropy of the clusters are also used to measure only the signal associated to clusters, and not the strong background by the non-aggregated beads. By using a rotating magnetic field, they were able to measure oscillation of the scattered light at that frequency and could obtain a limit of detection of 5 pM in human plasma in a total assay time of 3 min. From a physical point of view, a frequency response as in Ranzoni et al. or an impulse response like in our case should lead to similar results, but the setups have different practical implementation that may slightly affect their performance. The differences may be more related to the antigen and antibody nature, and particularly the antibodies grafting procedure. If our design does not improve the assay performance, our work focuses on the comprehension of the physical processes in magnetic agglutination assays. Moreover, our system, in its simplest version could be implemented without modification to any magnetic agglutination assay, since only a time variation of a magnetic field with a fixed direction is necessary for the method.

Conclusions

Fields can be employed to accelerate the formation of aggregates in agglutination immunoassay. In this paper, we show that the field could also be used to improve the quantification step. Particles aggregates are anisotropic light scatters, but they are randomly oriented owing to Brownian motion. Here, we use a pulse of magnetic field to align them to maximize the amount of light scattering. Moreover, as single particles are optically isotropic, only the signal associated to aggregates is measured, removing a large background signal.

A precise optical characterization of our system allows us to use the optimum optical and magnetic geometries, and to obtain an absolute quantification of the number of doublets presented in solution in the limit of very small aggregates. We applied our detection to detect CRP in a buffer spiked with BSA, showing a twofold increase of the detection limit as compare to a classical magnetic immunoassay.

Acknowledgments

The authors have made the following declarations about their contributions: Conceived and designed the experiments: E Compain, E Brient-Litzler, J Bibette, J Baudry. Performed the experiments: D Ramiandrisoa, A Daynes. Analyzed the data: D Ramiandrisoa, E Brient-Litzler, E Compain, J Bibette, J Baudry. Contributed reagents/materials/analysis tools: D Ramiandrisoa, A Daynes, E Brient-Litzler, E Compain, J Baudry. Wrote the paper: D Ramiandrisoa, J Baudry. Thanks to D.W. Mackowski for making its T matrix code freely available, and J. Walters for his careful reading of the manuscript. We thank the Direction Générale de l'Armement (DGA) from the French Ministry of Defense for their constant interest and support to Bertin Technologies.

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