Blu-ray optomagnetic measurement based competitive immunoassay for *Salmonella* detection

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**Abstract**

A turn-on competitive immunoassay using a low-cost Blu-ray optomagnetic setup and two differently sized magnetic particles (micron-sized particles acting as capture particles and nano-sized particles acting as detection particles) is here presented. For *Salmonella* detection, a limit of detection of $8 \times 10^3$ CFU/mL is achieved within a total assay time of 3 h. The combination of a competitive strategy and an optomagnetic setup not only enables a turn-on read-out format, but also results in a sensitivity limit about a factor of 20 times lower than of volumetric magnetic stray field detection device based immunoassays. The improvement of sensitivity is enabled by the formation of immuno-magnetic aggregates providing steric hindrance protecting the interior binding sites from interaction with the magnetic nanoparticle labels. The formation of immuno-magnetic aggregates is confirmed by fluorescence microscopy. The system exhibits no visible cross-reaction with other common pathogenic bacteria, even at concentrations as high as $10^4$ CFU/mL. Furthermore, we present results when using the setup for a qualitative and homogeneous biplex immunoassay of *Escherichia coli* and *Salmonella typhimurium*.© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

The development of sensitive assays for identification and detection of microorganisms is vital for improving diagnosis and preventing disease outbreaks. These assays also play essential roles in control programs provided by food-safety regulatory agencies and disease control and prevention agencies (Swaminathan and Feng, 1994; Hood et al., 2004; Virgin and Todd, 2011). Many of the latest generation devices, such as surface plasmon resonance (SPR) sensors (Zeng et al., 2014), nuclear magnetic resonance (NMR) devices (Harel et al., 2008), and surface enhanced Raman scattering (SERS) spectrometers (Han et al., 2009), are regarded as promising biosensor setups that have been commercialized to a large extent in order to meet the requirements of the society. Unfortunately, the relatively high cost and complexity associated with many of today’s diagnostic assay platforms limit their ability to contribute to sustainable health care systems in developing countries (Urdea et al., 2006). To address this problem, the research community has turned to nanoscale labels, e.g. colloidal gold nanoparticles (Giljohann et al., 2010), quantum dots (Medintz et al., 2005) and magnetic nanoparticles (Haun et al., 2010; Canfarotta and Piletsky, 2014) in the search for user-friendly and inexpensive diagnostic tools that can be used in remote areas where sophisticated lab facilities are not available (Kelley et al., 2014; Brandao et al., 2015).

The last decade has yielded breakthroughs within the development of nano-/microparticle based biosensors, in which the affinity capture is effectively achieved by coating dispersed nano- or microparticles with capture molecules (Gu et al., 2006; Tekin and Gijs, 2013). Due to their ability to be easily manipulated by magnetic field gradients and the negligible magnetic background of biological objects, superparamagnetic particles have been increasingly used as labels in volumetric assays (Borlido et al., 2013; Issadore et al., 2014). In biosensing platforms based on the detection of the presence or absence of magnetic particle labeled targets, the read-out is performed in various magnetic stray field detection devices, measuring changes in either static or dynamic properties of the magnetic particles upon interaction with the target, such as magnetoresistance sensors (Baselt et al., 1998; Graham et al., 2003; Dalslet et al., 2011), Hall effect devices (Mihaïlovic et al., 2005; Sandhu et al., 2007; Østerberg et al., 2014), magnetic tunnel junctions (Grancharov et al., 2005), superconducting quantum interference devices (Chemla et al., 2000; Grossman et al., 2004; Strömberg et al., 2008), or AC susceptometers (Astalan et al., 2004; Park et al., 2011; Zardán Gómez de la Torre et al., 2011). On the one hand, by employing enzymatic amplification strategies for improving the sensitivity, those magnetic biosensors are capable of detecting nucleic acid targets with a limit of detection (LOD) in the low pM range (Dalslet et al., 2011; Østerberg et al., 2014; Strömberg et al., 2008; Zardán Gómez de la
2. Experimental section

2.1. Reagents and bacterial strains

Ultrapure grade phosphate buffered saline (PBS, 20 ×) was purchased from AMRESCO (Solon, USA) and was diluted to 1 × before being used as the incubation/reaction/washing buffer and blank control sample. Biotinylated rabbit anti Salmonella group antibody (biotinylated polyclonal antibody, with 6–8 biotin groups per antibody according to the manufacturer) and rabbit anti E. coli (polyclonal antibody) were purchased from AbSerotec. Avidin modified 5 μM MNPs (product code 08-18-503), streptavidin modified 100 nm MNPs (product code 10-19-102) and protein A modified 250 nm MNPs (product code 09-20-252) were purchased from Micromod (Rostock, Germany). Biotin (5-fluorescein) conjugate was purchased from Sigma-Aldrich and dissolved in DMSO (purchased from Sigma). Heat-killed S. typhimurium cells (Catalog No. 50-74-01, KPL, Gaithersburg, USA) were used as the positive control sample. Enterococcus faecalis (CCUG 9997), E. coli (CCUG 17620), Staphylococcus aureus (CCUG 17621), Proteus mirabilis (CCUG 26767) and Klebsiella oxytoca (CCUG 42935) were obtained from the Department of Medical Sciences, Clinical Microbiology and Infectious Medicine, Uppsala University. All bacteria were suspended in PBS and stored at 4 °C.

2.2. Conjugation of magnetic particles and magnetic nanoparticles with antibodies

For the competitive immunoassay, polyclonal antibodies were conjugated to avidin-coated MNPs (to obtain capture particles, here denoted Ab-MPs) by mixing biotinylated rabbit anti Salmonella group antibody (4 mg/mL) and 5 μM MNPs (25 mg/mL, 3.5 × 10^8 particles/mL) in a volumetric ratio of 1:2 followed by incubation at 37 °C for 2 h to perform the avidin–biotin reaction. After washing twice using a magnetic separation stand, the Ab-MPs were resuspended at a concentration of 25 mg/mL (3.5 × 10^8 particles/mL) in PBS.

For the direct immunoassay, antibody-conjugated 100 nm MNPs were prepared by mixing biotinylated rabbit anti Salmonella group Ab (4 mg/mL) and 100 nm streptavidin-coated MNPs (10 mg/mL) in a volumetric ratio of 3:4 followed by incubation at 37 °C for 2 h. After washing two times, the Ab-MNPs were resuspended at a concentration of 10 mg/mL in PBS.

For the biplex assay, detection MNPs for E. coli were prepared by mixing anti E. coli Ab (4 mg/mL) and protein A modified 250 nm MNPs (10 mg/mL) in a volumetric ratio of 3:4 followed by incubation at 37 °C for 2 h and resuspended at a concentration of 10 mg/mL.

Ab-MPs and Ab-MNPs were stored at 4 °C for further use.

2.3. Competitive immunoassay for Salmonella

The competitive immunoassay consisted of three independent steps: capture, labeling and detection (see Scheme 1). First, the sample (blank, positive or negative) was mixed with Ab-MPs and incubated at 37 °C for 1.5 h. The mixture was shaken every 20 min to avoid sedimentation. After incubation and magnetic separation, the immuno–magnetic aggregates were washed once and resuspended (keeping the volume unchanged) in PBS. Thereafter streptavidin modified 100 nm magnetic nanoparticles, serving as detection particles, were added and the final concentration of MNPs was about 0.1 mg/mL. The MNPs were incubated with the immuno-magnetic aggregates at 37 °C for 1 h. After this step, 65 μL of the suspension was transferred into an UV-transparent disposable cuvette (REF 67.758.001, SARTSEDT, Nürnberg, Germany). In order to let the immuno-magnetic aggregates sediment

Torre et al., 2011). On the other hand, when employing a clinical affinity capture strategy, e.g. a bacterial immunoassay, the sensitivity of those devices (LOD of higher than 10^6 CFU/mL) is inferior compared to that of a miniaturized nuclear magnetic resonance (NMR) system (LOD of 10^3 CFU/mL), which benefits from a built-in signal amplification (since millions of water molecules can be affected by one individual magnetic particle) (Grossman et al., 2004; Chen et al., 2015). Furthermore, all those aforementioned biosensors require expensive instrumentation.

An alternative option for magnetic particle labeled target detection is to make use of optomagnetic (magneto-optic) sensor systems, which measure a modulation of an optical signal related to the Brownian relaxation dynamics of magnetic particles (Aurich et al., 2007; Chung et al., 2008; Donolato et al., 2015a). Although the optomagnetic devices have provided significant reductions in instrumentation cost, their performance regarding magnetic particle labeled target detection is similar to the magnetic stray field detection systems discussed above. Chung et al. (2008) estimated that the minimum detectable number of MNPs for their optomagnetic setup was 10^7. Aurich et al. (2007) employed a similar optomagnetic system and a homogeneous immunoassay strategy to detection human eotaxin and human IgG, and the LOD was found to be 1 nM and 70 pM, respectively. So far, optomagnetic sensors have not been reported for the detection of bacteria or cells. In addition, to the best of our knowledge, all reported volumetric magnetic stray field detection devices and optomagnetic device based immunoassays have employed direct immunoassay strategies, which means that they are negative (turn-off) assays, implying that it can be difficult to judge whether the reduction in sensor signal is due to poor assay performance or the presence of target (van Lierop et al., 2011).

Here, we present a novel Salmonella detection methodology using a low-cost Blu-ray optomagnetic setup. The utilization of a competitive turn-on immunoassay strategy associated with two kinds of magnetic particles (5 μm and 100 nm magnetic particles) gives a LOD of 8 × 10^4 CFU/mL within a total assay time of 3 h. Similar bioassay methods have been reported by Donolato et al. (2015a, 2015b) and Bejhed et al. (2015) for DNA detection, but this is the first time that the Blu-ray optomagnetic sensor is reported for a turn-on immunoassay strategy. In this work, for the competitive immunoassay scheme, bacteria are affinity captured by biotinylated-antibody coated 5 μm magnetic particles (Ab-MPs) to form immuno-magnetic aggregates. For certain bacterial concentrations, large immuno-magnetic aggregates are formed, i.e. clusters of capture MPs and bacteria. Thereafter, the aggregates are incubated with streptavidin-coated 100 nm magnetic nanoparticles (MNPs). Finally, the unreacted (non-bound) MNPs in the suspension are detected by the Blu-ray optomagnetic setup. The existence of immuno-magnetic aggregates containing clusters of Ab-MPs is confirmed using fluorescence microscopy. Furthermore, the competitive strategy is compared with a direct immunoassay scheme involving direct binding of Ab-conjugated 100 nm MNPs to bacteria, which shows that the competitive strategy is about 20 times more sensitive. Finally, we present a homogeneous and biplex detection strategy for Salmonella typhimurium and Escherichia coli. In the biplex detection, E. coli directly binds to Ab-250 nm detection MNPs, i.e. a direct detection strategy, while simultaneously S. typhimurium binds to the Ab-MPs to form immuno-magnetic aggregates (thereby preventing binding of detection streptavidin-100 nm MNPs to Ab-MPs), i.e. a competitive strategy. In addition, it is worth mentioning that the cost of this read-out system is very low in comparison to other devices, e.g. only a few percent of the cost for a miniaturized NMR device (about 50,000€).
spectra measured for a labelling time of 60 min. is closely normalized with respect to the peak value of $V_0$. was signiﬁcantly to be smaller than for a bacteria containing sample. The peak was directly employed as $V_0$ measured at 0 min for a pure MNP suspension. From top to bottom, polyclonal antibody against Salmonella are added to the sample, positive (top row) or blank (bottom row) and incubated. Labelling: Streptavidin modiﬁed 100 nm magnetic nanoparticles, serving as detection nanoparticles, are added followed by incubation. Detection: The suspension is transferred into a cuvette and the immuno-magnetic nanoparticles, serving as detection nanoparticles, are added followed by incubation. Detection: The suspension is transferred into a cuvette and the immuno-magnetic clusters are left to sediment to the bottom of the cuvette prior to performing target quantiﬁcation in a Blu-ray optomagnetic system.

to the bottom of the cuvette, thus not contributing to the measured optomagnetic response, the cuvette was left on the bench for 20 min prior to optomagnetic read-out.

2.4. Optomagnetic measurement setup

A detailed description is provided in Supplementary Material, section S1. The output from one measurement (used for target quantiﬁcation) is the total intensity of transmitted laser light, $V_0$, the in-phase, $V_2$, and the out-of-phase, $V_2$, components of the complex second harmonic signal of the transmitted light. In the competitive immunoassay approach, $V_2$ was normalized with respect to the simultaneously measured value of $V_0$ to compensate for the variations in laser light intensity, particle concentration and cuvette reﬂection/absorption. In the direct immunoassay and homogeneous biplex immunoassay, $V_2$ was directly employed as the output signal for target quantiﬁcation since $V_0$ was signiﬁcantly inﬂuenced by the concentration of bacteria in the cuvette. Measurements of $V_2$ and $V_0$ at room temperature were performed in the frequency range 10–10,000 Hz, using an AC magnetic excitation ﬁeld amplitude of 2.6 mT.

Scheme 1. Schematic illustration of the Blu-ray optomagnetic measurement based competitive immunoassay for S. typhimurium detection. The assay consists of three independent steps (from left to right in the illustration): capture, labelling and detection. Capture: 5 μm-sized magnetic particles equipped with biotinylated polyclonal antibody against Salmonella are added to the sample, positive (top row) or blank (bottom row) and incubated. Labelling: Streptavidin modiﬁed 100 nm magnetic nanoparticles, serving as detection nanoparticles, are added followed by incubation. Detection: The suspension is transferred into a cuvette and the immuno-magnetic clusters are left to sediment to the bottom of the cuvette prior to performing target quantiﬁcation in a Blu-ray optomagnetic system.

2.5. Fluorescence microscopy study of the immuno-magnetic aggregations

A detailed description is provided in Supplementary Material, section S2. Immuno-magnetic aggregates were incubated ﬁrst with MNPs and thereafter with biotin (5-ﬂuorescein) conjugates, and studied in a ﬂuorescence microscope (Axioplan II, Zeiss).

2.6. Homogeneous biplex immunoassay for S. typhimurium and E. coli

A detailed description is provided in Supplementary Material, section S3. Two different kinds of antibody conjugated magnetic particles were added to the sample and incubated. After incubation, the immuno-magnetic aggregates were reacted with MNPs and detected by the optomagnetic read-out.

3. Results and discussion

3.1. Establishment of the competitive immunoassay

Scheme 1 shows the principle behind the competitive immunoassay. In summary, if S. typhimurium cells and/or fragments are present, a part of the streptavidin MNPs will attach to the Ab-MPs by the streptavidin-biotin reaction, causing the peak value of $V_2/V_0$ to be smaller than for a bacteria containing sample. The peak amplitude can therefore be used for qualitative detection of S. typhimurium in the sample.

To evaluate the kinetics of the competitive immunoassay, Ab-MPs of $2 \times 10^6$ particles/ml were incubated with a positive control sample (S. typhimurium of $10^7$ CFU/ml) and a blank control sample (PBS) at 37 °C for 1.5 h (as a capture step). After washing, streptavidin modiﬁed MNPs were added to the suspensions to a ﬁnal concentration of 0.1 mg/ml (as a labelling step). Thereafter $V_2$ and $V_0$ were recorded as a function of labelling time (cf. Fig. 1A). As shown in Fig. 1B, the frequency dependence of $V_2/V_0$ is closely related to the Brownian relaxation dynamics of the MNPs and

![Fig. 1](image-url). Panel A: Peak value of $V_2/V_0$ vs. labelling time normalized with respect to the peak value of $V_2/V_0$ measured at 0 min for a pure MNP suspension. From top to bottom, the curves represent pure MNP sample, [MPs] sample, [Ab-MPs + positive control + MNPs] sample and [Ab-MPs + blank control + MNPs] sample, respectively. Panel B: $V_2/V_0$ spectra measured for a labelling time of 60 min.
shows a peak at approximately 200 Hz. The normalized peak value of the pure MNP suspension $(V_2/V_0)_{\text{max, MNP, 0 min}}$ was chosen as a reference value for normalizing the peak values of the other samples. From the results shown in Fig. 1A, it can be seen that $(V_2/V_0)_{\text{max}}$ decreases with increasing labelling time. For the pure MNP sample, the decrease is due to the fact that a small amount of MNPs adheres to the inner wall of the EP tube, thus not contributing to the modulation of the transmitted light. The peak value of the [MPs (no antibody) + MNPs] sample is slightly lower than that of the pure MNP sample. We attribute such features to inter-assay variations and/or to magnetic interaction fields due to MPs sediment, which attract MNPs to the bottom of the cuvette and prevent them from contributing to the measured optomagnetic response. For the [Ab-MPs + positive control + MNPs] and [Ab-MPs + blank control + MNPs] samples, the peak decrease is mainly related to the specific (biotin–streptavidin) and nonspecific (bacterial surface-streptavidin) bindings of MNPs to immunomagnetic aggregates. To maintain a balance between the total labelling time and signal-to-noise ratio, 60 min was chosen as the reaction time of the labelling step.

3.2. Qualitative detection of S. typhimurium

For the competitive immunoassay strategy (Scheme 1), it can be concluded that the amount of Ab-MPs used to capture the bacteria is strongly correlated to the sensitivity and specificity of the system. On the one hand, the smaller amount of Ab-MPs used, the fewer bacteria are needed to block the biotinylated antibodies from interaction with the subsequently added streptavidin MNPs, and thus the lower the LOD will be. On the other hand, the smaller amounts of Ab-MPs used, the fewer MNPs can bind to MPs, and thus the lower the signal-to-noise ratio will be. To optimize the amount of Ab-MPs, a series of Ab-MP concentrations (from $2 \times 10^3$ to $2 \times 10^6$ particles/mL) was chosen for reacting with 0.1 mg/mL streptavidin MNPs, and a cut-off value was calculated as the average of $(V_2/V_0)_{\text{max}}$ for a triplicate measurement using a blank control sample (PBS) minus three times the standard deviation of the triplicate (Fig. 2A). Although the dose-response curve indicates that the smallest concentration below the cut-off value (red dashed line) was $3 \times 10^4$ particles/mL, the signal-to-noise ratio for this amount of Ab-MPs was too low to be used for further

![Fig. 2](image-url). Panel A: Peak value of $V_2/V_0$ vs. Ab-MP concentration. Panels B, C and D show peak value of $V_2/V_0$ vs. S. typhimurium concentration when using an Ab-MP concentration of $1.25 \times 10^5, 5 \times 10^5, 2 \times 10^6$ particles/mL, respectively. Note that the peak values in panels B–D have been normalized with respect to the peak values of the blank controls. Error bars indicate one standard deviation based on three independent measurements. Dashed lines indicate the cut-off value calculated from blank control samples.
experiments. The coefficients of variation for each point of the curve shown in Fig. 2A were less than 4.8%.

A concentration series of *S. typhimurium* was measured using the competitive immunoassay. The immunoassay was performed using Ab-MP concentrations of $1.25 \times 10^5$, $5 \times 10^5$ and $2 \times 10^6$ particles/mL, and the results of those measurements are shown in Fig. 2B–D, respectively. Normalized peak values of the blank controls ($((V_j/V_0)_{\text{max, Blank Control}}$) were used not only to calculate the cut-off values (average signal for a triplicate measurement plus three times the standard deviation of the triplicate) for each experiment, but also to normalize the signals to compensate for the inter-assay variations. As mentioned earlier, the LOD for *S. typhimurium* improves upon decreasing the amount of Ab-MPs, and the best LOD $8 \times 10^4$ CFU/mL was achieved for an Ab-MP concentration of $1.25 \times 10^3$ particles/mL (Fig. 2B). Interestingly, the smallest measured *S. typhimurium* concentration above the cut-off value corresponded well with the concentration of Ab-MPs used, implying that large immuno-magnetic aggregates were efficiently formed when having a MP/bacteria ratio of 1:1. The coefficients of variation for each point of the curves shown in Fig. 2B–D were less than 3.7%, 5.4% and 6.7% respectively.

The shape of the dose-response curves in Fig. 2B–D is governed mainly by two mechanisms: (1) formation of immuno-magnetic aggregates containing clusters of Ab-MPs, providing steric hindrance preventing streptavidin-MNPs from binding to biotinylated antibodies inside clusters; and (2) saturation of Ab-MPs by bacteria, which prevents direct binding of streptavidin-MNPs to Ab-MPs. Immuno-magnetic aggregates containing clusters of Ab-MPs form below a certain bacterial concentration limit, which depends on the concentration of MNPs (around 1:1 ratio of MP/bacteria). The Ab-MP cluster size increases with increasing bacterial concentration up to this limit, which results in an increasing dose-response curve. Above this limit the aggregates containing several Ab-MPs no longer form, and the immuno-magnetic aggregates mainly contain single Ab-MP. At even higher bacterial concentrations, more and more bacteria bind to the surfaces of Ab-MPs, yielding a dose-response increasing with bacterial concentration and eventually to a plateau in the curve when the Ab-MPs become saturated with bacteria. We thus infer that the sudden decrease of signal between the peak and the plateau represents a situation where aggregates containing clusters of Ab-MPs cannot form, but instead the immuno-magnetic aggregates appear as single Ab-MPs with only a few bound bacteria each, thus having free binding sites for MNPs.

In another test described in Supplementary Material, section S4, the Ab-MPs/bacteria incubation time was shortened to 0.5 h to investigate its influence on the sensitivity of the assay. In the measurement employing $5 \times 10^5$ particles/mL Ab-MPs, the sensitivity was not significantly affected by the smaller incubation time compared with the dose-response curve in Fig. 2C. To balance the sensitivity and stability, subsequent competitive immunoassay tests were performed using an Ab-MPs concentration of $5 \times 10^5$ particles/mL.

### 3.3. Fluorescence microscopy study of Ab-MP/bacteria binding

The fluorescence photomicrographs (Fig. 3) confirm the existence of immuno-magnetic aggregates containing clusters of Ab-MPs and also show that the formation of such immuno-magnetic aggregates mainly exists below a certain bacterial concentration limit. It should be mentioned that the small biotin (5-fluorescein) molecule can easily diffuse into the interior of the immuno-magnetic aggregations and bind to both free avidin groups on the MNPs and to free streptavidin groups on the MNPs. This enables easy and straightforward visualization of free Ab-MPs and immuno-magnetic aggregations in a fluorescence microscope.

In this study, Ab-MPs with a concentration of $5 \times 10^5$ particles/mL were reacted with samples containing different concentrations of bacteria. Because of steric hindrance, biotinylated antibodies cannot block all the biotin binding sites on the surface of avidin-MPs, implying that the bacteria–Ab-MPs conjugates can still be labeled by the biotin (5-fluorescein) conjugate.
When exposed to a high concentration of *S. typhimurium* (i.e., 10^7 CFU/mL), Ab-MPs were covered by excessive bacterial fragments and could, thus, not form aggregates containing clusters of Ab-MPs (Fig. 3A). This in turn resulted in that only a few MNPs (with bound fluorescent labels) were bound to the Ab-MPs, in accordance with the observed plateau at the position of 10^7 CFU/mL in Fig. 2C. As the concentration ratio of bacteria/Ab-MPs was reduced, non-saturated immuno-magnetic aggregates containing single Ab-MPs coexisting with a few aggregates containing small clusters of Ab-MPs were observed (Fig. 3B), but these immuno-magnetic aggregates cannot sterically block the binding sites for MNPs, in accordance with the valley at the position of 2 x 10^6 CFU/mL in Fig. 2C. Immuno-magnetic aggregates containing large clusters of Ab-MPs (Fig. 3C) create steric hindrance around the interior binding sites which reduces the amount of bound streptavidin MNPs, thus giving rise to a peak around 10^6 CFU/mL *S. typhimurium* in Fig. 2C. In Fig. 3D, Ab-MPs were still free after being incubated with the blank control sample (PBS), and the fluorescence intensity was slightly higher than for the MNPs in the other images. This observation can be explained by the fact that in this case a large number of MNPs (with fluorescent labels on surface) were attached to the Ab-MPs.

3.4. Comparison of competitive and direct immunoassay strategies

By using the same reagents and read-out device, a direct immunoassay strategy for *S. typhimurium* detection was evaluated and compared with the competitive assay approach (details are given in Supplementary Material, section S5). The LOD for the direct immunoassay was found to be approximately 2 x 10^4 CFU/mL (cf. Fig. S4), which corroborates previous results (5.6 x 10^5 CFU/mL) reported by Grossman et al. (2004). A dynamic range of at least two orders of magnitude is obtained for the direct immunoassay strategy. A competitive immunoassay is generally less sensitive than the corresponding direct assay, but in this study it was proven to be more sensitive. This could be explained by the fact that the amount of biotin binding sites on the surface of the Ab-MPs is much larger and easier to control than the amount of antigen sites on the surface of typical bacteria. In addition, the formation of immuno-magnetic aggregates containing clusters of Ab-MPs strongly reduces the number of available binding sites, thus further enhancing the sensitivity. In the direct immunoassay strategy, immuno-magnetic aggregates could also be formed by Ab-MNPs and bacteria. (Wang et al., 2015) However, cross-linking between Ab-MNPs and bacteria means that the bacteria are not saturated with Ab-MNPs (bacteria are sharing the labels), and hence, the sensitivity is limited. If we regard the 5 pm Ab-MPs as “ideal bacteria” with target molecules covering their entire surface, the LOD of a direct immunoassay for this “bacteria” would be 3 x 10^5 particles/mL, which can be appreciated by inspection of Fig. 2A. Since it is nearly impossible to achieve such an ideal situation in real clinical tests, the competitive assay strategy performs better than the direct immunoassay strategy. This can be explained by the fact that the strength of antibody–antigen interaction (employed in the direct immunoassay) is variable and not as strong as the biotin–streptavidin pair used in this competitive immunoassay.

3.5. Specificity evaluation of competitive and direct immunoassays

The specificity (selectivity) of the competitive and direct immunoassay approaches was investigated by considering five types of other common pathogenic bacteria (see Section 2.1) as negative controls (at 10^3 CFU/mL and 10^5 CFU/mL, respectively). Results presented in Fig. 4 show that the normalized peak values, (V(2)/V(0))max/(V(2)/V(0))max, Blank Control, of the negative control samples are close to 1, meaning that the response from the negative controls are similar to the blank control. Similar results were obtained for the direct immunoassay strategy (see Fig. S5), indicating that no significant amounts of bacteria other than *S. typhimurium* were captured by the Ab-MPs (in competitive immunoassay) or Ab-MNPs (in direct immunoassay). Therefore, the current approaches were able to differentiate *S. typhimurium* from other clinical common pathogenic bacteria and are therefore suitable for the specific detection of *S. typhimurium*.

3.6. Homogeneous biplex immunoassay for *S. typhimurium* and *E. coli*

This optomagnetic setup can be used for multiplex detection, since MNPs with different hydrodynamic sizes have V(2) peak/valley values at different frequencies. The V(2) valley (negative peak) of 250 nm MNPs is located at 18 Hz, while the V(2) peak of 100 nm MNPs is located at 184 Hz. Direct and competitive immunoassays were employed for *E. coli* and *S. typhimurium* using 250 nm and 100 nm MNPs as detection labels, respectively. As shown in Fig. 5A, each spectrum has its own signature in terms of valley/peak values and frequency positions. For the spectra of the *E. coli* containing samples, i.e., the yellow triangles and blue diamonds, the absolute values of the valley at 14–18 Hz are smaller than for the blank sample (red squares), since a fraction of the Ab–250 nm MNPs are bound to the *E. coli*. When measuring the spectra of *S. typhimurium* containing samples, the black circles and blue diamonds, the *Salmonella* fragments covered the Ab-MPs and left more streptavidin-100 nm MNPs free in the suspension, resulting in higher peak values at 184 Hz. In view of the homogeneous biplex immunoassay results (Fig. S5B), we conclude that the setup can be applied for qualitative multiplex immunoassays.

4. Conclusion

In summary, we have presented a turn-on competitive immunoassay using a low-cost Blu-ray optomagnetic setup for read-out. This qualitative detection strategy utilizes two differently
sized magnetic particles and gives a LOD of $8 \times 10^4$ CFU/mL $S. \text{typhimurium}$ within a total assay time of 3 h. The improvement of the sensitivity was achieved by the formation of immuno-magnetic aggregates containing clusters of Ab-MPs providing steric hindrance protecting the interior binding sites from interaction with the magnetic nanoparticle labels. The existence of immuno-magnetic aggregates containing clusters of Ab-MPs was confirmed by fluorescence microscopy. This presented method is fully compatible with a large variety of pathogens and can be used for the detection of urinary tract samples, which have a high positive CFU threshold (usually $10^9$ CFU/mL) [Mezger et al., 2015]. Moreover, our method can be easily combined with the use of a MP pre-treatment procedure to enrich the bacteria before the test, thereby further improving the LOD. The immunoassay exhibits no visible cross-reaction with other clinically common pathogenic bacteria, even at a concentration of $10^7$ CFU/mL. If a positive answer is obtained from the qualitative competitive assay, we can further quantify the bacterial concentration by employing a direct immunoassay approach. Furthermore, we have demonstrated a qualitative and homogeneous biplex immunoassay of $E. \text{coli}$ and $S. \text{typhimurium}$. In consideration of the advantages shown in this study, as well as the low-cost of the setup, the system has a great potential to be used for cost-efficient and user-friendly identification and detection of microorganisms relating to e.g. food safety.

Acknowledgments

This work was financially supported by Swedish Research Council Formas (Project no. 221-2012-444). Dr. Eva Tano at the Department of Medical Sciences, Clinical Microbiology and Infectious Medicine, Uppsala University, and Ma Jing at the Department of Immunology, Genetics and Pathology (IGP), Uppsala University are gratefully acknowledged for providing bacterial samples. Dr. Camilla Russell at IGP, Uppsala University, is gratefully acknowledged for helpful assistance when running the fluorescence microscope.

Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.08.070.