



Lab-on-a-disc agglutination assay for protein detection by optomagnetic readout and optical imaging using nano- and micro-sized magnetic beads

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ABSTRACT

We present a biosensing platform for the detection of proteins based on agglutination of aptamer coated magnetic nano- or microbeads. The assay, from sample to answer, is integrated on an automated, low-cost microfluidic disc platform. This ensures fast and reliable results due to a minimum of manual steps involved. The detection of the target protein was achieved in two ways: (1) optomagnetic readout using magnetic nanobeads (MNBs); (2) optical imaging using magnetic microbeads (MMBs). The optomagnetic readout of agglutination is based on optical measurement of the dynamics of MNB aggregates whereas the imaging method is based on direct visualization and quantification of the average size of MMB aggregates. By enhancing magnetic particle agglutination via application of strong magnetic field pulses, we obtained identical limits of detection of 25 pM with the same sample-to-answer time (15 min 30 s) using the two differently sized beads for the two detection methods. In both cases a sample volume of only 10 μ l is required. The demonstrated automation, low sample-to-answer time and portability of both detection instruments as well as integration of the assay on a low-cost disc are important steps for the implementation of these as portable tools in an out-of-lab setting.

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1. Introduction

Magnetic nano- and microbeads are being widely used for sensing of various biomolecules in different microfluidic systems (Antunes et al., 2015; Kwakye et al., 2006; Steigert et al., 2005; Wang et al., 2013; Zaytseva et al., 2005; Choi et al., 2002). Magnetic beads (MBs) can be utilized for detection of pathogens (Mezger et al., 2015; Beyor et al., 2008; El-Boubbou et al., 2007; Gu et al., 2006; Li et al., 2013; Wang and Gan, 2009; Zaytseva et al., 2005), small molecules (Yang et al., 2016; Wu et al., 2011; Zhang et al., 2013) and proteins (Nam et al., 2003; Tsai et al., 2007; Horng et al., 2006), for drug delivery (Chertok et al., 2008; Gonzales and Krishnan, 2005; Jain et al., 2008; Wilson et al., 2005), and even for testing certain characteristics of a drug (Quan et al., 2015). Due to the weak magnetic properties of biomolecules, a readout based on magnetic beads (MBs) is insensitive to chemical and biological parameters that may affect other readout techniques and is thus highly attractive (Yang et al., 2016; Bejhed et al., 2015; Hecht et al.,

2013). In addition, due to the simple readout, the detection devices can be produced at a low cost, facilitating their usage in resource-limited settings (Yager et al., 2008).

Assays based on agglutination of MBs, i.e., the formation of MB clusters in order to detect biomarkers, are being widely studied due to the simplicity of the readout (Donolato et al., 2015; Göransson et al., 2010; Ranzoni et al., 2012). In these assays, the beads are coated with molecules with specific affinity to bind a target molecule. The presence of the target molecule causes the MBs to bind together to form aggregates of different sizes and shapes. Based on the physical characteristics of the aggregates as well as their rotational dynamics in response to an external oscillating magnetic field, the concentration of the target molecule can be determined. Magnetic bead-based agglutination assays are promising for point-of-care (POC) diagnostics.

However, in several studies of agglutination assays using magnetic beads, the use of complex microfluidics, sophisticated readout methods, large sample volume and lack of automation have limited their real point-of-care potential. For instance, Chen et al. (2013) developed a microfluidic chip-based assay using aptamer-functionalized magnetic beads labelled with fluorescent dye Cy3 for detecting thrombin. 50 μ l of biotinylated Aptamer II

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labelled with Cy3 was incubated with 50 μ l thrombin solution for 30 min. Aptamer I functionalized with magnetic beads was then loaded into the chip through external tubing. Since the chip was placed under a permanent magnet, the aptamer-functionalized beads were aggregated. Subsequently, aptamer II labelled with Cy3 and functionalized with thrombin was loaded into the chip to react with aptamer I. Finally, any unreacted aptamer II was washed out using an external syringe pump followed by fluorescent measurement with a microarray scanner. Thus, the whole process involved large assay time, multiple manual steps and external components.

Tennico et al. (2010) performed a similar study on a further developed chip, but used quantum dot nanocrystals instead of Cy3 dye and integrated a nanopore on the chip to connect with external syringe pump for washing in an automated fashion resulting in less reagent consumption and a shorter reaction time. The steps of the assay included: loading of 3 μ l aptamer I functionalized with magnetic beads into reaction chamber followed by magnetic bead aggregation by on-chip magnets, rinsing of excess aptamers using external syringe pump, addition of 3 μ l of thrombin solution into the reaction chamber followed by 5 min incubation period, rinsing and washing with binding buffer, addition of 6 μ l aptamer II labelled with quantum dot nanocrystals into reaction chamber followed by further 5 min incubation, and finally a last washing step before measurement by a fluorescent microscope. Although the sample consumption and assay time was reduced compared to that presented in Chen et al. (2013), it still suffered from the limitations imposed by use of syringe pump as well as multiple assay steps including washing and rinsing, which makes it difficult to use it in an out-of-lab setting.

Finally, a magnetic microbead-based fluorescent-independent agglutination assay using the same aptamers as used in the above-mentioned studies for detecting thrombin in buffer solution was reported by Hecht et al. (2013). The rotational period of the formed aggregates as well as their shape, size and fractal dimension were measured to quantify the concentration of thrombin. There, the sample-to-answer time was more than 40 min and the assay required multiple manual steps.

Here, we present a conventional sandwich agglutination assay for detection of the model protein thrombin in buffer (Hecht et al., 2013), but the assay steps are integrated and automated on a low-cost microfluidic disc. As a significant achievement, thanks to the introduction of an on-disc magnetic field assisted incubation protocol (Antunes et al., 2015), we reduce the sample-to-result time from 40 min to 15 min 30 s while utilizing only 10 μ l of sample. We verify that an optimum incubation of the samples with magnetic beads under magnetic field, hereafter termed 'magnetic incubation', effectively enhances the MB agglutination (Antunes et al., 2015; Ranzoni et al., 2012; Baudry et al., 2006) and results in fast detection. By utilizing the magnetic incubation protocol, we detect different concentrations of thrombin based only on the size distribution of MB aggregates.

We validate the lab-on-a-disc agglutination assay for both micro (1 μ m) and nano (100 nm)-sized magnetic beads. We use two different detection methods for the MNB and MMB-based assays. The detection method used for MNB-based assay is the previously presented optomagnetic readout method (Mezger et al., 2015; Bejjhed et al., 2015; Donolato et al., 2015). The optomagnetic setup (Donolato et al., 2015) uses a Blu-ray optical pickup unit (OPU) as the excitation element and a photodetector as the sensing element. An AC magnetic field excitation is provided by an off-chip electromagnet. The frequency-dependent modulation of the optical transmission signal correlates with the hydrodynamic size of the MNB aggregates and enables a sensitive and quantitative detection of a target molecule based on the aggregate size (Donolato et al., 2015). The detection method used for the MMB-

based assay is an optical imaging method that detects the area of particle aggregates formed due to the presence of thrombin by fast direct imaging. We use a commercial imaging and scanning instrument 'oCelloScope' (Philips BioCell), which combines the optical sectioning principle of confocal microscopy with an automated scanning principle. A detailed description of the oCelloScope setup can be found in (Fredborg et al., 2013) and in the [Supplementary material Section S2](#). The automation, user-friendliness, fast detection and portability of both detection instruments along with the integration of the assay steps on the disc make both systems strong candidates for a simple sample-to-answer device for use in an out-of-lab setting.

2. Experimental

2.1. Materials and chemicals

The assay platform in this work is a microfluidic disc. Each disc (thickness: 2 mm) contains eight microfluidic units each with three inlet chambers, a mixing/measuring chamber and a pneumatic chamber for ensuring pneumatic mixing (Fig. 1a and b). The disc was fabricated from three layers of Polymethylmethacrylate (PMMA) bonded by pressure sensitive adhesive (PSA) in less than 20 min. The detailed fabrication procedure of the disc is presented in (Donolato et al., 2015) and in the [Supplementary material Section S1](#).

The MBs used in this study are streptavidin coated beads with diameters of 100 nm and 1 μ m, respectively. The particular bead size was chosen as previous studies showed that the optomagnetic signal is more sensitive to formation of aggregates when the MNB size is about 100 nm (Donolato et al., 2015; Yang et al., 2016); and the 1 μ m bead size was reported as optimum for MMBs based on sedimentation rate and available binding sites (Hecht et al., 2013), making it an ideal candidate for the optical imaging study. The MNBs were purchased from Micromod (Micromod Partikeltechnologie GmbH, Rostock, Germany). The MMBs (T1 Dynabeads) as well as the human alpha thrombin (product code T6884) were purchased from Sigma-Aldrich. Three different buffer solutions were prepared for washing, aptamer binding and thrombin binding, the details of which are provided in [Supplementary material Section S1](#). Two widely used anti-thrombin aptamers functionalized with 5' biotin and consisting of 29-mer and 15-mer with 20 base poly-T tails (Table 1) were purchased from DNA technology (Denmark). We have used these two particular aptamers because of their well-proven specificity to bind with thrombin molecule (Bock et al., 1992; Tasset et al., 1997).

2.2. Magnetic bead functionalization

We prepared two sets of aptamer-functionalized MBs, one for nano-sized beads and the other for micro-sized beads. An aliquot of 10 μ l of streptavidin-coated MNBs (10 mg/ml) was washed three times with the washing buffer, resuspended in 100 μ l of aptamer-binding buffer and subsequently split into two equal aliquots (Hecht et al., 2013). 4 μ l of 50 μ M biotinylated aptamer solution (each of 29-mer or 15-mer) was added to each aliquot and incubated for 45 min at room temperature to functionalize the beads with the specific aptamers by forming the biotin-streptavidin bonds. The MNB solution was then washed three times with thrombin-binding buffer and finally resuspended at a bead concentration of 0.1 mg/ml (Antunes et al., 2015). The same protocol was followed for functionalizing the MMBs.

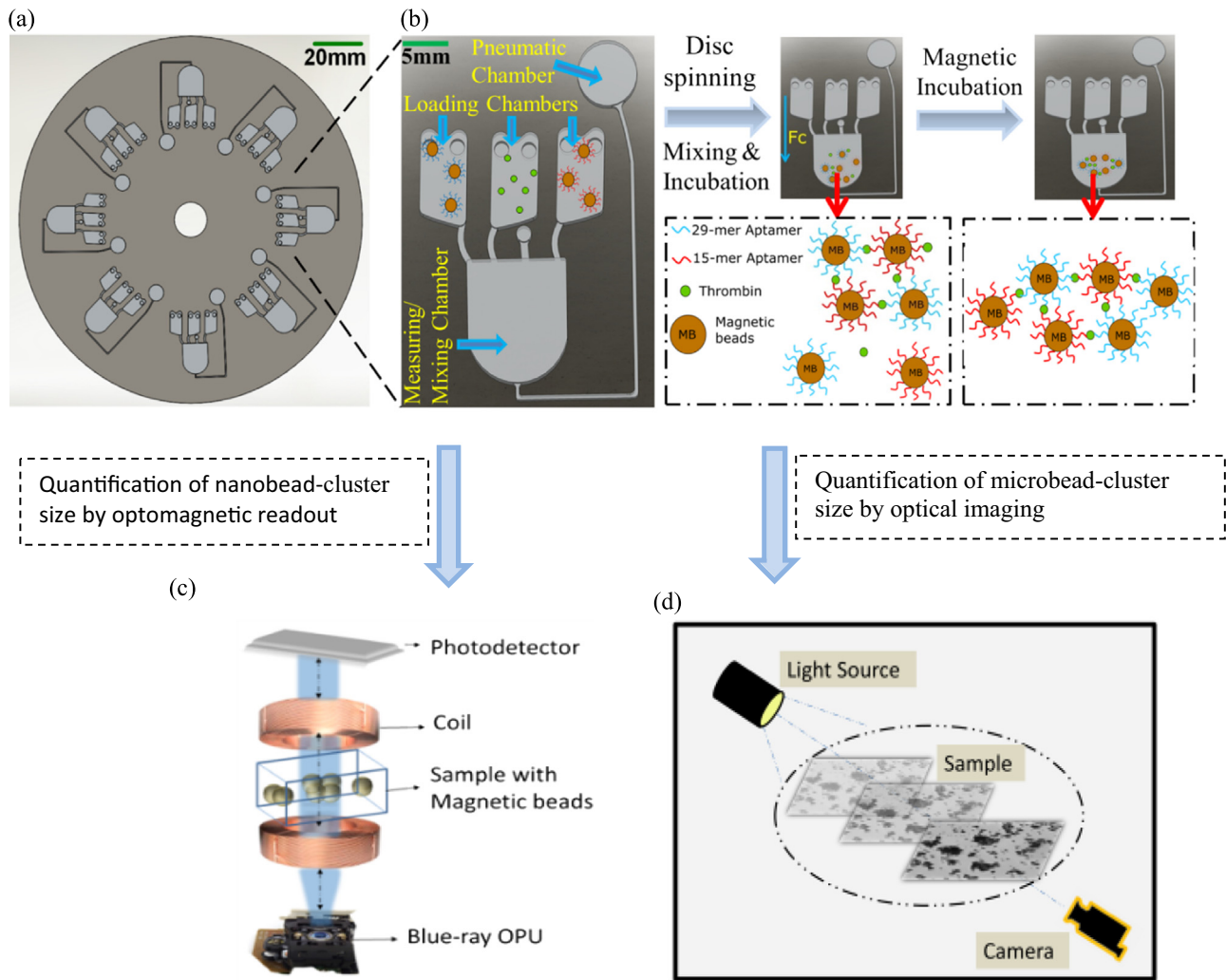


Fig. 1. (a) Schematic of the microfluidic disc with eight repeating functional units. (b) Illustration of one of the functional repeating units of the disc indicating different chambers followed by MB aggregation formation due to the presence of thrombin. (c) Schematic of the functional unit of the optomagnetic readout method for quantifying the nanobead cluster size (Supplementary material Section S2). (d) Schematic of the functional unit of optical imaging method for quantifying the microbead cluster size (Supplementary material Section S2).

Table 1
Sequence of thrombin-binding aptamers used in study.

Aptamer	Sequence
15-mer DNA aptamer	5'-Biotin-TTT TTT TTT TTT TTT TTT TT GGT TGG TGT GGT TGG-3'
29-mer DNA aptamer	5'-Biotin-TTT TTT TTT TTT TTT TTT TTT TT AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'

2.3. Experimental procedure

An aptamer-based agglutination assay was used for thrombin detection. The aptamers used in this study have the specific affinity to bind to two structurally opposite sites of the thrombin molecule. The experimental procedure consisted in mounting the disc at the specific location of the optomagnetic setup followed by injecting aptamer-functionalized magnetic beads (MBs) (10 μ l of each type) and thrombin solution (10 μ l) into the three separate loading chambers of one of the microfluidic units of the disc (Fig. 1b). No pretreatments were made in the microfluidic units of the disc.

Spinning of the disc transferred the liquids of the loading chamber to the mixing/measuring chamber (Fig. 1b). On-disc mixing of the liquids was performed by a pneumatic mixing

routine for 30 s (Noroozi et al., 2011). Subsequently, the solution was incubated on-disc at RT for 10 min in order to facilitate binding between the aptamers and the thrombin molecules. The incubation caused the formation of MB-aptamer-thrombin bonds, which resulted in aggregation of MBs as illustrated in Fig. 1b. Subsequently, 5 min of magnetic incubation was performed to further stimulate and enhance MB agglutination. The magnetic incubation consisted of repeated cycles of two steps: (1) incubation in a strong magnetic field (60 mT) between two permanent magnets to promote formation of MB clusters; (2) mixing by shaking the disc to break unspecific bindings and to facilitate re-orientation of the beads (Antunes et al., 2015). The magnetic incubation protocol was previously optimized in (Antunes et al., 2015) for similar magnetic nanobeads. With a minor modification, we have employed this magnetic incubation protocol on both the nanobeads and the microbeads. Further information on the magnetic incubation protocol is provided in the Supplementary material Section S2. Finally, after magnetic incubation, the MNB-based samples were measured by the optomagnetic readout method and the MMB-based samples were measured by the optical imaging method. For the optical imaging method, the disc was unmounted from the optomagnetic setup and transferred to the oCelloScope for scanning. The entire lab-on-a-disc agglutination assay including the time for incubation took 15 min 30 s while

requiring only 10 μl of sample volume.

3. Results and discussion

3.1. Time-lapse analysis without magnetic incubation

We first performed a time-lapse analysis without magnetic incubation for a specific concentration of thrombin (2 nM) to investigate the kinetics of the MB aggregation. For the time-lapse analysis by optical imaging of MMBs, the sample-filled measurement chamber was kept under the oCelloScope and scanned every 15 min using a routine-setup of the control software until the MMB aggregation saturated. Similarly, for the optomagnetic readout method using MNBs, time-lapse measurements were performed until signal saturation was observed in the optomagnetic spectra.

Fig. 2a shows the in-phase part of the normalized optomagnetic spectra (V_2/V_0 vs. f) after incubation at the indicated times. Here, V_2 is the in-phase component of the 2nd harmonic of the transmitted light intensity with respect to the magnetic field excitation and V_0 is the average value of the transmitted light intensity. The signal increase at low frequencies that peaks near $f=7.5$ Hz indicates the formation and growth of MNB aggregates over time due to the presence of thrombin. Fig. 2b shows the V_2/V_0 -values at $f=7.5$ Hz vs. time. The results indicate that the signal without magnetic incubation reached saturation after approximately 50 min.

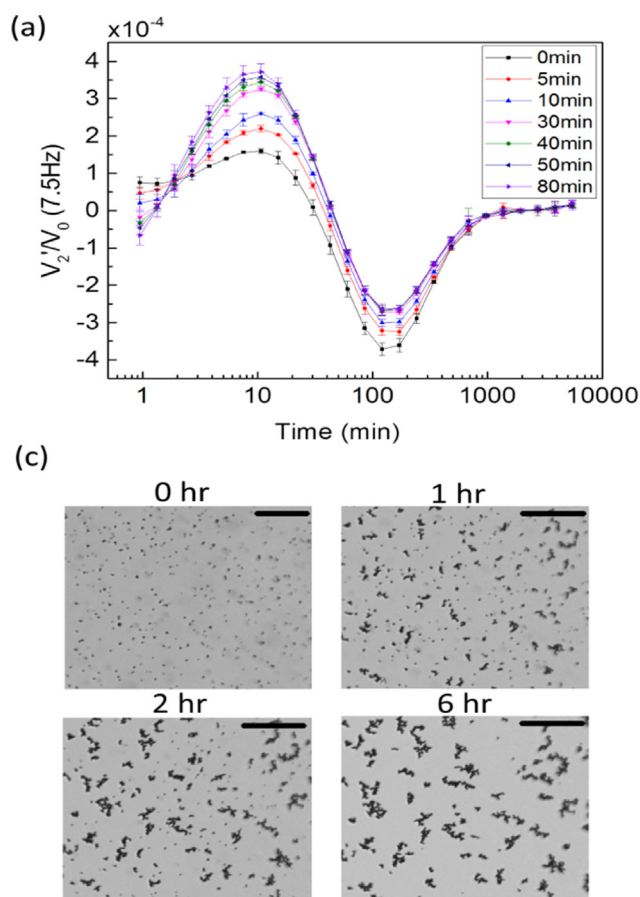


Fig. 2c and d show optical images and the corresponding mean area of MMB aggregates vs. incubation time, respectively. For the investigated concentration of thrombin (2 nM), the aggregate area saturated after approximately 6 h.

The time-lapse analysis using the two methods confirmed that the MNBs aggregated much faster than the MMBs due to their shorter diffusion time (Gundersen and Palmer, 2007). However, the times needed for both assays were still prohibitively long from a POC application perspective. In this regard, effective manipulation of the aggregate formation by magnetic field can speed up the aggregation resulting in faster detection as well as a lower limit of detection (Antunes et al., 2015; Ranzoni et al., 2012; Baudry et al., 2006). Thus, we designed our incubation strategy for further experiments as 10 min incubation at RT followed by 5 min of magnetic incubation cycles.

3.2. Optomagnetic study with magnetic incubation

For a quantitative analysis of MNB-based assay involving magnetic incubation, we performed an optomagnetic study of six samples with thrombin concentrations ranging from $c=0$ (blank) to $c=2$ nM (2000 pM). Fig. 3a shows the in-phase part of the normalized optomagnetic spectra (V_2/V_0 vs. f) measured after magnetic incubation for samples with the indicated thrombin concentrations. From Fig. 3a it is observed that due to the formation of MNB aggregates, higher thrombin concentrations caused an increase of the peak value of the signal as well as a shift of the peak to lower frequencies.

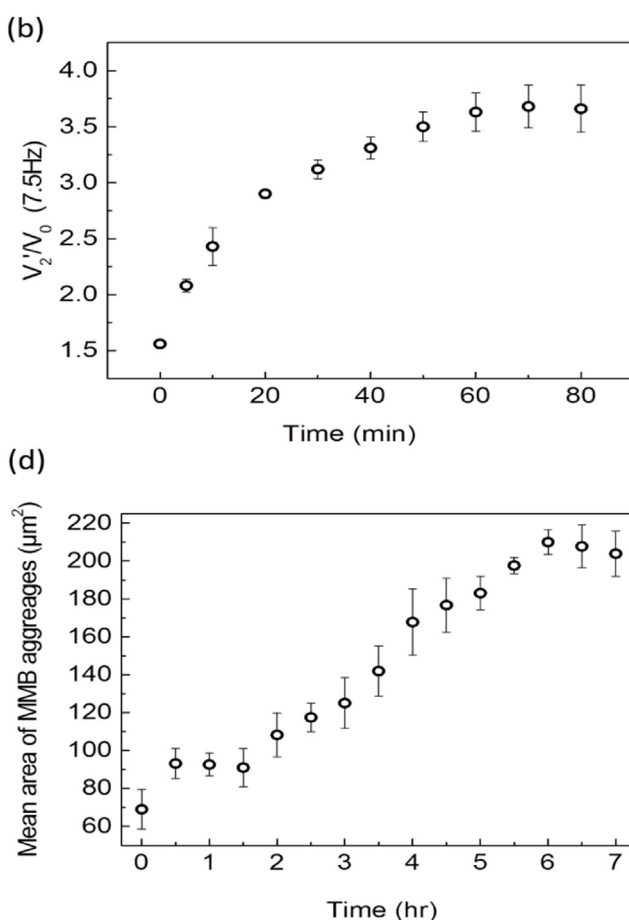


Fig. 2. Results vs. time without magnetic incubation for incubation of aptamer functionalized MBs with 2 nM of thrombin. (a) Optomagnetic spectra (V_2/V_0 vs. f) at different time points. Occurrence of a signal at low frequencies indicates formation and growth of MNB aggregates. (b) Low-frequency signal V_2/V_0 (7.5Hz) vs. incubation time. (c) oCelloScope images of aggregation state obtained after the indicated incubation times (all images are shown in [Supplementary material Section S4](#)). (d) Mean area of MMB aggregates vs. incubation time. Error bars indicate standard deviation obtained from triplicate measurements. Scale bars indicate 60 μm .

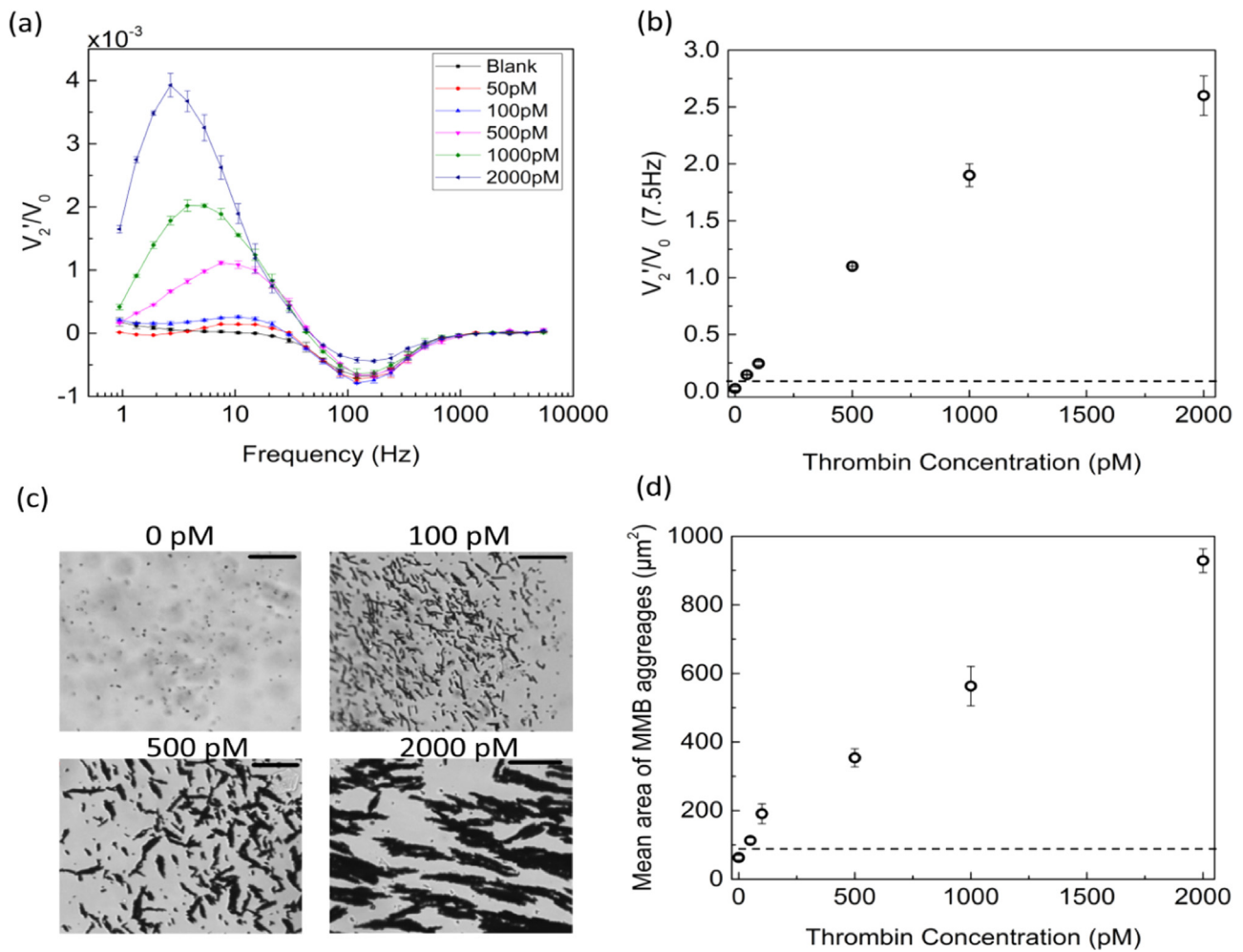


Fig. 3. Results vs. indicated concentrations c of thrombin buffer when magnetic incubation is performed. (a) In-phase optomagnetic spectra of MNBs. (b) Corresponding values of V_2/V_0 (7.5 Hz) vs. c . (c) oCelloScope images of four different MMB samples (full set of images is given in [Supplementary material Section S4](#)). (d) Corresponding mean area of MMB aggregates vs. c . The black dotted lines in (b) and (d) represent the mean blank signal plus the 3 times of its standard deviation, which is used for calculating the LODs. Error bars are standard deviations obtained from triplicate measurements. Scale bars indicate 60 μm .

A dose-response curve (Fig. 3b) was obtained by measuring the V_2/V_0 value at $f=7.5$ Hz corresponding to the position of the peak in the spectra for lower thrombin concentrations. From the dose-response curve, we found a limit of detection (LOD) of 25 pM. This value was obtained from the signal level corresponding to the mean value of the blank signal plus three times its standard deviation.

Compared to the time-lapse analysis without magnetic incubation, the magnetic incubation reduced the required time to form effective clusters by 35 min. In addition, comparing Figs. 2a and 3a, it is observed that the magnetic incubation shifted the frequency of the peak for $c=2$ nM from $f=10$ Hz to 2.66 Hz. This indicates that the magnetic incubation resulted in the formation of larger clusters than without magnetic incubation. Furthermore, comparing Figs. 2b and 3b, the amplitude of the signal at 7.5 Hz increased a factor of about 7 from 3.5×10^{-4} to 2.6×10^{-3} . Thus, the magnetic incubation also provided a significant signal enhancement.

3.3. Optical imaging study

For a quantitative analysis of the MMB-based assay, we performed optical imaging study for the same concentrations as studied above after performing the magnetic incubation procedure. Fig. 3c shows the images of the MMB clusters scanned by the

oCelloScope for the four indicated values of c . The images clearly illustrate that higher values of c resulted in formation of larger clusters. The blank sample showed very little or no aggregation of the MMBs, whereas the sample with $c=2000$ pM showed very large MMB aggregates.

Using the optical sectioning principle, the scanning created multiple stacks of images and from the image stack, the instrument software created tracks of each scanned aggregate, which facilitated calculation of the average projected area of each aggregate. This was averaged over all MMB aggregates to obtain the mean aggregate area of the population of MMB aggregates (detailed information in [Supplementary material Section S4](#)). Fig. 3d shows this mean area of MMB aggregates vs. c . From this dose-response curve, we found an LOD of 25 pM, which is the same value as obtained using the optomagnetic readout.

We have maintained a level of statistical analysis of our experimental results similar to other comparable studies (Yang et al., 2016; Donolato et al., 2015; Hecht et al., 2013). In order to ensure repeatability, all experiments were performed in triplicate. Standard statistical techniques, which take into account the standard deviation (S.D.), e.g., $3 \times \text{S.D.}$ criterion, were employed to establish the limit of detection of the assay (25 pM). The error bars in Figs. 2 and 3, representing the standard deviation, are very small compared to the measured signal. This shows the repeatability of the proposed biosensing system.

The images of MMB aggregates for different thrombin concentration in Fig. 3c obtained with magnetic incubation differ significantly from those in Fig. 2c obtained without magnetic incubation. With magnetic incubation, the MMBs formed a tightly packed elongated structure due to the applied magnetic force and torque, whereas without magnetic incubation, the MMBs formed branched clusters. Moreover, comparing Figs. 2d and 3d, it is observed that the magnetic incubation protocol reduced the time to form effective aggregates of the MMBs from about 6 h to 15 min while the average area of the aggregates increased by a factor of 4.4 from 209.9 μm^2 to 928.9 μm^2 . This also indicates that the magnetic incubation protocol with cycles of magnetic field application and mixing by shaking enabled strong interaction both between MMBs and between the MMBs and the target molecules. Furthermore, as no magnetic field was applied during the shaking step, this step promoted disruption of unspecific bindings between MMBs.

4. Conclusion

We have presented a centrifugal microfluidics platform for aptamer-based protein detection in solution by use of magnetic nano- and microbeads. The biosensor can be applied to agglutination assays based on magnetic nanobeads or magnetic microbeads to detect other clinically significant target molecules that have affinity towards certain aptamers. The results presented here illustrate the potential of a compact lab-on-a-disc agglutination assay facilitating automation, low sample volume (10 μl) and a short sample-to-answer time. The integration of the assay in a microfluidic disc enabled the automation of sample handling, mixing and magnetic incubation, which play a major role in increasing reliability and repeatability of the assay as well as for the user-friendliness of the technique. The optomagnetic readout using the Blu-ray OPU and the photodetector produced results on magnetic nanobeads that correlated well with those obtained on magnetic microbeads using direct real-time imaging in the oCelloScope. Thus, these two methods support each other for the usage of small and large particles for a particular assay and further validation of assay. Further, the magnetic incubation protocol significantly shortened the aggregate formation time and enhanced the size of the aggregates such that these were detectable by the two readout techniques for low concentrations of thrombin. In addition, although MMBs require much more time than MNBs to form aggregates by diffusion, the magnetic incubation facilitated detectable aggregation for both bead sizes with the same LODs and the same total assay time. In future study, we will extend our current work to more complex sample matrices, such as blood plasma, where the plasma samples can be obtained via centrifugal separation from a whole blood sample by a simple extension of the presented disc system with no need for user intervention or advanced sample preparation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in

the online version at <http://dx.doi.org/10.1016/j.bios.2016.05.023>.

References

- Antunes, P., Watterson, D., Parmvi, M., Burger, R., Boisen, A., Young, P., Cooper, M.A., Hansen, M.F., Ranzoni, A., Donolato, M., 2015. Quantification of NS1 dengue biomarker in serum via optomagnetic nanocluster detection. *Sci. Rep.* 5, 16145. <http://dx.doi.org/10.1038/srep16145>.
- Baudry, J., Rouzeau, C., Goubault, C., Robic, C., Cohen-Tannoudji, L., Koenig, a, Bertrand, E., Bibette, J., 2006. Acceleration of the recognition rate between grafted ligands and receptors with magnetic forces. *Proc. Natl. Acad. Sci. USA* 103, 16076–16078. <http://dx.doi.org/10.1073/pnas.0607991103>.
- Bejhed, R.S., de la Torre, T.Z.G., Donolato, M., Hansen, M.F., Svedlindh, P., Strömberg, M., 2015. Turn-on optomagnetic bacterial DNA sequence detection using volume-amplified magnetic nanobeads. *Biosens. Bioelectron.* 66, 405–411. <http://dx.doi.org/10.1016/j.bios.2014.11.048>.
- Beyor, N., Seo, T.S., Liu, P., Mathies, R., 2008. Immunomagnetic bead-based cell concentration microdevice for dilute pathogen detection. *Biomed. Microdevices* 10, 909–917. <http://dx.doi.org/10.1007/s10544-008-9206-3>.
- Bock, L.C., Griffin, L.C., Latham, J.A., Vermaas, E.H., Toole, J.J., 1992. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* 355 (6360), 564–566. <http://dx.doi.org/10.1038/355564a0>.
- Chen, F.Y., Wang, Z., Li, P., Lian, H.Z., Chen, H.Y., 2013. Aptamer-based thrombin assay on microfluidic platform. *Electrophoresis* 34, 3260–3266. <http://dx.doi.org/10.1002/elps.201300338>.
- Chertok, B., Moffat, B., David, A.E., Yu, F., Bergemann, C., Ross, B.D., Yang, V.C., 2008. Iron oxide nanoparticles as a drug delivery vehicle for MRI monitored magnetic targeting of brain tumors. *Biomaterials* 29, 487–496. <http://dx.doi.org/10.1016/j.biomaterials.2007.08.050>.
- Choi, J.-W., Oh, K.W., Thomas, J.H., Heineman, W.R., Halsall, H.B., Nevin, J.H., Helmicki, A.J., Henderson, H.T., Ahn, C.H., 2002. An integrated microfluidic biochemical detection system for protein analysis with magnetic bead-based sampling capabilities. *Lab Chip* 2, 27. <http://dx.doi.org/10.1039/b107540n>.
- Donolato, M., Antunes, P., de la Torre, T.Z.G., Hwu, E.-T., Chen, C.-H., Burger, R., Rizzi, G., Bosco, F.G., Strömberg, M., Boisen, A., Hansen, M.F., 2015. Quantification of rolling circle amplified DNA using magnetic nanobeads and a Blu-ray optical pick-up unit. *Biosens. Bioelectron.* 67, 649–655. <http://dx.doi.org/10.1016/j.bios.2014.09.097>.
- El-Boubbou, K., Gruden, C., Huang, X., 2007. Magnetic glyco-nanoparticles: a unique tool for rapid pathogen detection, decontamination, and strain differentiation. *J. Am. Chem. Soc.* 129, 13392–13393. <http://dx.doi.org/10.1021/ja076086e>.
- Fredborg, M., Andersen, K.R., Jorgensen, E., Droce, a, Olesen, T., Jensen, B.B., Rosenvinge, F.S., Sondergaard, T.E., 2013. Real-time optical antimicrobial susceptibility testing. *J. Clin. Microbiol.* 51, 2047–2053. <http://dx.doi.org/10.1128/JCM.00440-13>.
- Gonzales, M., Krishnan, K.M., 2005. Synthesis of magnetoliposomes with mono-disperse iron oxide nanocrystal cores for hyperthermia. *J. Magn. Magn. Mater.* 293, 265–270. <http://dx.doi.org/10.1016/j.jmmm.2005.02.020>.
- Göransson, J., Zardán Gómez De La Torre, T., Strömberg, M., Russell, C., Svedlindh, P., Strömberg, M., Nilsson, M., 2010. Sensitive detection of bacterial DNA by magnetic nanoparticles. *Anal. Chem.* 82, 9138–9140.
- Gu, H., Xu, K., Xu, C., Xu, B., 2006. Biofunctional magnetic nanoparticles for protein separation and pathogen detection. *Chem. Commun.*, 941–949. <http://dx.doi.org/10.1039/b514130c>.
- Gundersen, S.L., Palmer, A.F., 2007. Conjugation of methoxypolyethylene glycol to the surface of bovine red blood cells. *Biotechnol. Bioeng.* 96, 1199–1210. <http://dx.doi.org/10.1002/bit>.
- Hecht, A., Commiskey, P., Shah, N., Kopelman, R., 2013. Bead assembly magnetorotation as a signal transduction method for protein detection. *Biosens. Bioelectron.* 48, 26–32. <http://dx.doi.org/10.1016/j.bios.2013.03.073>.
- Hong, H.E., Yang, S.Y., Hong, C.Y., Liu, C.M., Tsai, P.S., Yang, H.C., Wu, C.C., 2006. Biofunctionalized magnetic nanoparticles for high-sensitivity immunomagnetic detection of human C-reactive protein. *Appl. Phys. Lett.* 88. <http://dx.doi.org/10.1063/1.2207990>.
- Jain, T.K., Richey, J., Strand, M., Leslie-Pelecky, D.L., Flask, Ca, Labhasetwar, V., 2008. Magnetic nanoparticles with dual functional properties: drug delivery and magnetic resonance imaging. *Biomaterials* 29, 4012–4021. <http://dx.doi.org/10.1016/j.biomaterials.2008.07.004>.
- Kwakye, S., Goral, V.N., Baeumner, A.J., 2006. Electrochemical microfluidic biosensor for nucleic acid detection with integrated minipotentiostat. *Biosens. Bioelectron.* 21, 2217–2223. <http://dx.doi.org/10.1016/j.bios.2005.11.017>.
- Li, S., Liu, H., Deng, Y., Lin, L., He, N., 2013. Development of a magnetic nanoparticles microarray for simultaneous and simple detection of foodborne pathogens. *J. Biomed. Nanotechnol.* 9, 1254–1260.
- Mezger, A., Fock, J., Antunes, P., Østerberg, F.W., Boisen, A., Nilsson, M., Hansen, M.F., Ahlford, M.D., 2015. Scalable DNA-based magnetic nanoparticle agglutination assay for bacterial detection in patient samples. *ACS Nano* 9, 7374–7382.
- Nam, J.-M., Thaxton, C.S., Mirkin, Ca, 2003. Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. *Science* 301, 1884–1886. <http://dx.doi.org/10.1126/science.1088755>.
- Norozi, Z., Kido, H., Peytavi, R., Nakajima-Sasaki, R., Jasinskas, A., Micic, M., Felgner, P.L., Madou, M.J., 2011. A multiplexed immunoassay system based upon reciprocating centrifugal microfluidics. *Rev. Sci. Instrum.* 82, 064303. <http://dx.doi.org/10.1063/1.3587555>.

- doi.org/10.1063/1.3597578.
- Quan, X., Uddin, R., Heiskanen, A., Parmvi, M., Nilson, K., Donolato, M., Hansen, M.F., Rena, G., Boisen, A., 2015. The copper binding properties of metformin – QCM-D, XPS and nanobead agglomeration. *Chem. Commun.* 12, 1–4. <http://dx.doi.org/10.1039/C5CC04321B>.
- Ranzoni, A., Sabatte, G., van Ijzendoorn, L.J., Prins, M.W.J., 2012. One-step homogeneous magnetic nanoparticle immunoassay for biomarker detection directly in blood plasma. *ACS Nano* 6, 3134–3141.
- Steigert, J., Grumann, M., Brenner, T., Mittenbuhler, K., Nann, T., Ruhe, J., Moser, I., Haerberle, S., Riegger, L., Riegler, J., 2005. Integrated sample preparation, reaction, and detection on a high-frequency centrifugal microfluidic platform. *J. Assoc. Lab. Autom.* 10, 331–341. <http://dx.doi.org/10.1016/j.jala.2005.07.002>.
- Tasset, D.M., Kubik, M.F., Steiner, W., 1997. Oligonucleotide inhibitors of human thrombin that bind distinct epitopes. *J. Mol. Biol.* 272, 688–698. <http://dx.doi.org/10.1006/jmbi.1997.1275>.
- Tennico, Y.H., Hutanu, D., Koesdjojo, M.T., Bartel, C.M., Remcho, V.T., 2010. On-chip aptamer-based sandwich assay for thrombin detection employing magnetic beads and quantum dots. *Anal. Chem.* 82, 5591–5597. <http://dx.doi.org/10.1021/ac101269u>.
- Tsai, H.Y., Hsu, C.F., Chiu, I.W., Bor Fuh, C., 2007. Detection of C-reactive protein based on immunoassay using antibody-conjugated magnetic nanoparticles. *Anal. Chem.* 79, 8416–8419. <http://dx.doi.org/10.1021/ac071262n>.
- Wang, L., Gan, X.-X., 2009. Biomolecule-functionalized magnetic nanoparticles for flow-through quartz crystal microbalance immunoassay of aflatoxin B1. *Bio-process Biosyst. Eng.* 32, 109–116. <http://dx.doi.org/10.1007/s00449-008-0228-2>.
- Wang, Z., Fan, Y., Chen, J., Guo, Y., Wu, W., He, Y., Xu, L., F.F., 2013. A microfluidic chip-based fluorescent biosensor for the sensitive and specific detection of label-free single-base mismatch via magnetic beads-based “sandwich” hybridization strategy. *Electrophoresis* 34, 2177–2185.
- Wilson, K.S., Goff, J.D., Riffle, J.S., Harris, La, St Pierre, T.G., 2005. Polydimethylsiloxane-magnetite nanoparticle complexes and dispersions in polysiloxane carrier fluids. *Polym. Adv. Technol.* 16, 200–211. <http://dx.doi.org/10.1002/pat.572>.
- Wu, S.J., Duan, N., Wang, Z.P., Wang, H.X., 2011. Aptamer-functionalized magnetic nanoparticle-based bioassay for the detection of ochratoxin a using upconversion nanoparticles as labels. *Analyst* 136, 2306–2314. <http://dx.doi.org/10.1039/C0an00735h>.
- Yager, P., Domingo, G.J., Gerdes, J., 2008. Point-of-care diagnostics for global health. *Annu. Rev. Biomed. Eng.* 10, 107–144. <http://dx.doi.org/10.1146/annurev.bioeng.10.061807.160524>.
- Yang, J., Donolato, M., Pinto, A., Bosco, F.G., Hwu, E.-T., Chen, C.-H., Alström, T.S., Lee, G.-H., Schäfer, T., Vavassori, P., Boisen, A., Lin, Q., Hansen, M.F., 2016. Blu-ray based optomagnetic aptasensor for detection of small molecules. *Biosens. Bioelectron.* 75, 396–403. <http://dx.doi.org/10.1016/j.bios.2015.08.062>.
- Zaytseva, N.V., Goral, V.N., Montagna, Ra, Baeumner, A.J., 2005. Development of a microfluidic biosensor module for pathogen detection. *Lab Chip* 5, 805–811. <http://dx.doi.org/10.1039/b503856a>.
- Zhang, Y., Pilapong, C., Guo, Y., Ling, Z., Cespedes, O., Quirke, P., Zhou, D., 2013. Sensitive, simultaneous quantitation of two unlabeled DNA targets using a magnetic nanoparticle–enzyme sandwich assay. *Anal. Chem.* 85, 9238–9244.

Electronic Supplementary Material

Lab-on-a-disc agglutination assay for protein detection by optomagnetic readout and optical imaging using nano- and micro-sized magnetic beads

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S1. MATERIALS AND CHEMICALS

The microfluidic disc used in this study was designed using Solidworks 2014 and fabricated in-house. Each disc consisted of three layers of PMMA (thickness: 600 μm each) with two layers of PSA (thickness: 140 μm each) in order to attach the PMMA layers (Fig. S1a). The top PMMA layer contained sample loading and venting holes and middle PMMA layer contained the channels and chambers. All the microfluidic features on the PMMA layers were cut using a CO₂-laser (Epilog Mini 18, 30W, Epilog, USA). The dimensions of the channels and chambers are illustrated in Fig. S1b. The depth of the chambers and channels were 880 μm and 140 μm , respectively.

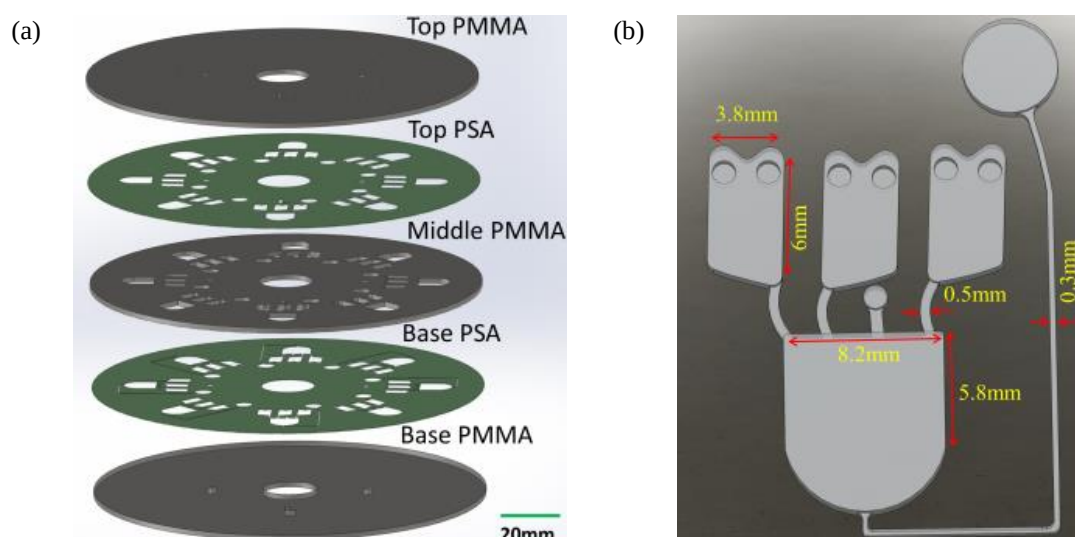


Fig. S1: (a) Schematic of five PMMA and PSA layers for forming the microfluidic disc with an overall thickness of 2.1 mm (b) The dimensions of the channels and chambers of one of the repeating units of the disc.

The buffers used in this study were same as those in (Hecht *et al.*, 2013; Centi *et al.*, 2007; Huang *et al.*, 2004).

Table 1: Buffers used in the study

Buffer name	Composition of buffer
Washing buffer	1 M NaCl, 1 mM EDTA and 20 mM Tris (pH 7.5)
Aptamer-binding buffer	1 M NaCl, 1 mM EDTA and 5 mM Tris (pH 7.5)
Thrombin-binding buffer	140 mM NaCl, 1 mM MgCl ₂ , 10 mM KCl, 50 mM Tris, 0.1% BSA, Tween-20, (pH 7.4)

S2. EXPERIMENTAL PROCEDURES

The disc was first mounted on the optomagnetic setup which consisted of a Blu-ray optical pick-up unit (OPU, $\lambda = 405$ nm), a stepper motor, two electric coils and a photodetector (Fig. S2). All on-disc sample handling including the magnetic incubation was performed by a routine-control of the stepper motor using the in-house Labview program. After sample loading, mixing and initial incubation, 5 min of magnetic incubation was performed.

The magnetic incubation protocol consisted of constant incubation step under the magnet followed by a shaking step. In the incubation step, the measurement chamber was placed between two permanent magnets (60 mT field) for 5 s. After 5 s of incubation, the disc was shaken clockwise and counterclockwise (speed: 1000 rpm) another 5 s. Such cycles of incubation and shaking were performed for 5 min.

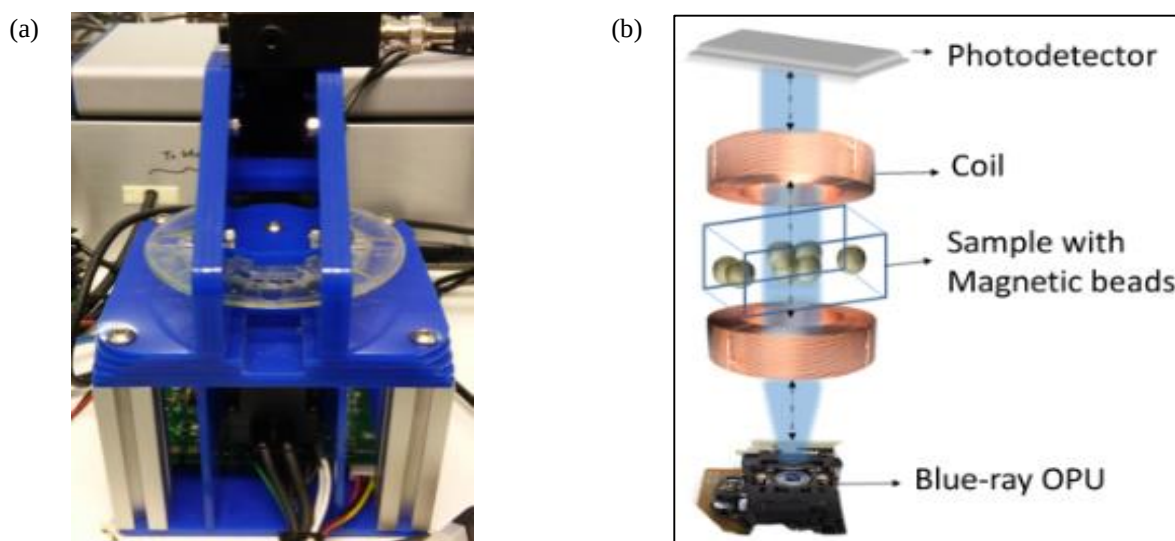


Fig. S2: (a) Experimental setup of optomagnetic readout. (b) Schematic illustration of the main functional elements of optomagnetic readout with Blu-ray OPU and the measurement geometry.

After magnetic incubation, the measurement chamber of the disc was aligned with the Blu-ray laser for measurement. The laser beam passed the chamber filled with sample through the electromagnet coils to the photodetector. The two electromagnetic coils created a uniaxial sinusoidally varying magnetic field, which caused different rotational dynamics for different hydrodynamic sizes of the MNB aggregates. The rotation of MNBs and MNB aggregates resulted in a modulation of the intensity of the laser light measured by the photodetector. The frequency dependence of this light modulation depends on the rotation dynamics defined by the hydrodynamic size of the MNB aggregates as well as their scattering properties. The 2nd harmonic normalized in-phase component of the photodetector signal, V_2'/V_0 , measured at different frequencies provides information on the aggregation states of the MNBs. Spectra of this photodetector signal were measured at 26 logarithmically equidistant points between 0.94 Hz and 5461 Hz. The time required to obtain a spectrum was approximately 1 min 30s. Further details information on the optomagnetic readout method are given in (Donolato *et al.*, 2015).

For the MMB-based samples, all assay steps including the magnetic incubation were performed using the stepper motor and the in-house Labview program of the optomagnetic setup. For scanning and quantifying the sample by optical imaging method, the disc was unmounted from the optomagnetic setup and placed on a home-built disc-holder customized to fit the oCelloScope measurement area. The purpose of the disc-holder was to position the disc within the focal plane of the camera as well as to ensure position repeatability for all experiments. The oCelloScope is an automated and portable imaging instrument with an optical resolution of 1.3 μ m. Its main functional unit consists of an illumination unit (light source), a digital camera unit and a lens system for sophisticated imaging of a particular sample (Fig. S3). The measurement chamber of the disc was placed between the light source and the camera. The instrument software 'Uniexplorer' was used to operate the instrument and run the image scanning.

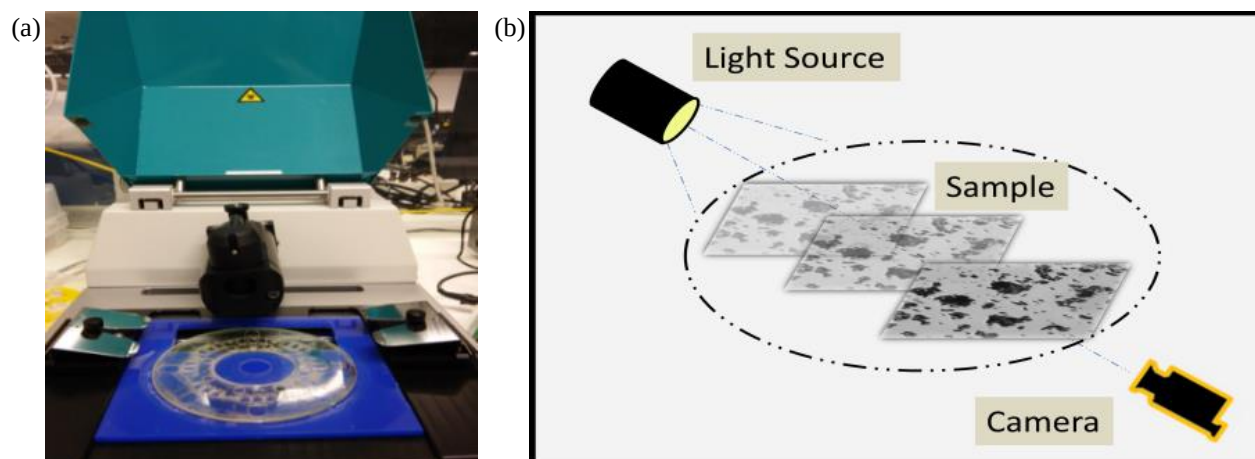


Fig.S3: (a) Experimental setup of oCelloScope with the microfluidic disc placed between the light source and camera. (b) Functional unit of oCelloScope consisting of a light source and a camera with tilted focal plane. The tilted focal plane facilitates to create a stack of images and subsequently, object stack for each scanned object by optical sectioning principle.

During scanning of a selected scan area, the light beam passed through the sample and was captured by the camera. Uniquely for the instrument, aggregates were scanned along the x-, y- and z-directions in the particular sample. This was achieved using an optical sectioning principal by tilting the focal plane 6.25 degree for recording series of images along the scan direction (vertical axis) forming an image stack. Based on the image stack, an object stack was formed for each of the objects of interest, in our case MMB aggregates. For further analysis of the object stack, physical parameters of interest were defined in the instrument software. In our case, the physical property of interest was the average area of each of the scanned objects (MMB aggregates). It is worth-mentioning that a particular region of a sample to be scanned as well as a size-range of the objects to be characterized could be pre-selected in the software. For our study, both the size-range (0 to 6000 μm^2) and the selected scan-region were kept constant for all of the measurements to ensure repeatability and reliability. The average size of the MMB aggregates was calculated by the image processing algorithm of the instrumental software. The average area mentioned in this study is the mean area, i.e. the average size of all scanned aggregates within the selected size range. The time required for scanning the sample and calculating the area of the aggregates was approximately 1 min. Further information on the oCelloScope principle is given in (Fredborg *et al.*, 2013).

S3. Occupation of binding sites on beads

In this section, we estimate the maximum number of aptamers bound onto the surface of the magnetic beads and thus, compare the aptamer binding, i.e., thrombin binding capability of the MNBs and MMBs.

Table S1: Comparison of aptamer binding capability of MNBs and MMBs

Beads	No. of streptavidin binding sites	No. of beads/mg	Max. No. of aptamers/ bead	Max. No. of bound aptamers/ml of beads
MNB	300 pmol/mg beads (Micromod 2015)	6×10^{11} (Micromod 2015)	300	1.8×10^{15}
MMB	400 pmol/mg beads (Invitrogen 2011)	1×10^9 (Invitrogen 2011)	2.4×10^5	2.4×10^{15}

For a single experiment, we used 20 μl of aptamer-beads (10 μl of each 29-mer and 15-mer) and 10 μl of sample (thrombin solution). Thus, based on the values of Table S1, the maximum number of aptamers bound to the surface of the beads per 20 μl of solution are 3.6×10^{13} and 4.8×10^{13} for MNBs and MMBs, respectively. For the thrombin concentration of 25 pM corresponding to the LOD, the number of molecules in 10 μl sample is 1.2×10^{10} . Hence, as one thrombin molecule can bind to at most two aptamers (one 29-mer and one 15-mer aptamer), from the above-mentioned values it can be stated that although the number of aptamers on the surface of each MNB is much smaller than that on each MMB, all of the thrombin molecules were occupied in both cases.

S4. OPTICAL IMAGING RESULTS

Fig. S4 illustrates the full-set of images of the time-lapse analysis of MMB-based thrombin sample performed using the oCelloScope for 7 hours.

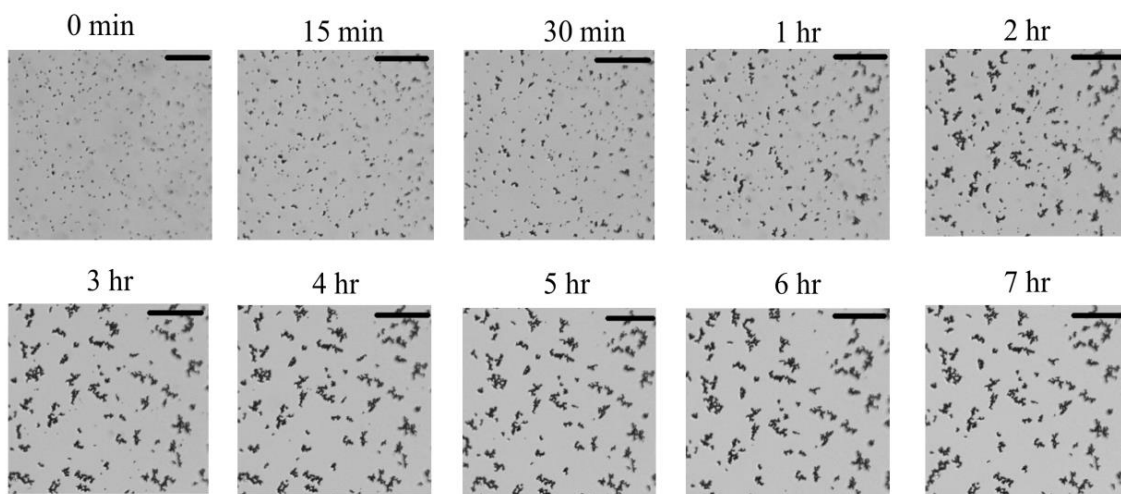


Fig. S4: oCelloscope images of MMB aggregates due to the presence of 2 nM thrombin after the indicated incubation times without application of magnetic field. Scale bars indicate 60 μm .

Fig. S5 illustrates the scanned images with oCelloScope of all the different concentrations of thrombin sample

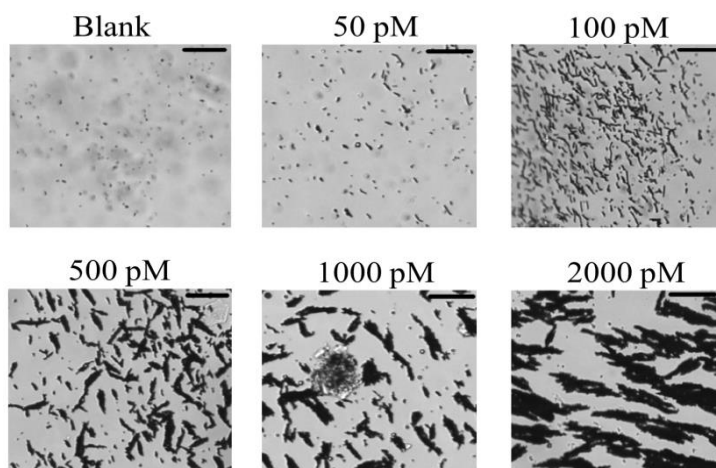


Fig. S5: oCelloscope images of MMB aggregates formed in the indicated concentrations of thrombin in buffer after magnetic incubation. Scale bars indicate 60 μm .

S5. DISCUSSION

The focus of our current study was on the biosensing platform, its automation and portability along with utilization of magnetic incubation for obtaining low and similar LOD for micro- and nanobead based assay within a short period of time. We have chosen thrombin as a model protein due to its wide popularity as an analyte in proof-of-principle studies. Thus, obtaining a very low signal-to-noise ratio was not our current focus for this study. On the contrary, the commercial assay kits are specifically designed for the detection at very low sensitivity, which is suitable to be used in clinics and hospital. Thus, the comparison between our assay and commercially available assays would be an unbalanced comparison. Nevertheless, we are discussing some aspects of the commercial assays to demonstrate the prospect of our current assay for clinical use.

The current commercial assays for thrombin detection are ELISA-based assays performed in 96-well plates. One of such commercially available kits is Thrombin (Factor II) Human ELISA Kit (ab108909) developed by Abcam plc. It is designed for quantitative detection of thrombin in cell supernatant whereas in our current work, we have performed the detection in buffer. Furthermore, the assay kit involves multiple manual steps, whereas our assay is performed in an automated fashion. Moreover, the Abcam assay kit's detection method is colorimetric and our detection method is based on the size and concentration of magnetic bead clusters.

Another example of a commercial kit is Thrombin (Human) Elisa Kit (Catalog# KA0511) developed by AbNova. Its detection method is colorimetric as well and the required sample volume is 50 μ l obtaining a LOD of 0.16 ng/ml (4 pM), whereas our required sample volume is only 10 μ l. Hence, our assay procedure and detection mechanism differ significantly from the commercial ones in terms of sample volume and automation. In future, we will optimize the assay platform for real patient sample and also focus more on obtaining lower sensitivity.

References

- Centi, S, Tombelli S, Minunni M, M.M., 2007. Aptamer-based detection of plasma proteins by an electrochemical assay coupled to magnetic beads. *Analytical Chemistry* 15, 1466–73.
- Donolato, M., Antunes, P., de la Torre, T.Z.G., Hwu, E.-T., Chen, C.-H., Burger, R., Rizzi, G., Bosco, F.G., Strømme, M., Boisen, A., Hansen, M.F., 2015. Quantification of rolling circle amplified DNA using magnetic nanobeads and a Blu-ray optical pick-up unit. *Biosensors and Bioelectronics* 67, 649–655. doi:10.1016/j.bios.2014.09.097
- Fredborg, M., Andersen, K.R., Jorgensen, E., Droce, a, Olesen, T., Jensen, B.B., Rosenvinge, F.S., Sondergaard, T.E., 2013. Real-time optical antimicrobial susceptibility testing. *J Clin Microbiol* 51, 2047–2053. doi:10.1128/JCM.00440-13
- Hecht, A., Commiskey, P., Shah, N., Kopelman, R., 2013. Bead assembly magnetorotation as a signal transduction method for protein detection. *Biosensors and Bioelectronics* 48, 26–32. doi:10.1016/j.bios.2013.03.073
- Huang, C., Cao, Z., Chang, H., Tan, W., 2004. Protein - Protein Interaction Studies Based on Molecular Aptamers by Affinity Capillary Electrophoresis 76, 6973–6981.