Scalable DNA-Based Magnetic Nanoparticle Agglutination Assay for Bacterial Detection in Patient Samples

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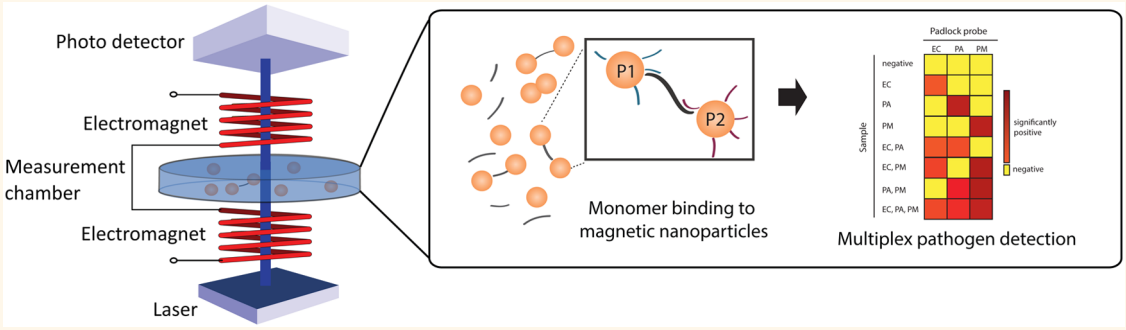
ABSTRACT



We demonstrate a nanoparticle-based assay for the detection of bacteria causing urinary tract infections in patient samples with a total assay time of 4 h. This time is significantly shorter than the current gold standard, plate culture, which can take several days depending on the pathogen. The assay is based on padlock probe recognition followed by two cycles of rolling circle amplification (RCA) to form DNA coils corresponding to the target bacterial DNA. The readout of the RCA products is based on optomagnetic measurements of the specific agglutination of DNA-bound magnetic nanoparticles (MNPs) using low-cost optoelectronic components from Blu-ray drives. We implement a detection approach, which relies on the monomerization of the RCA products, the use of the monomers to link and agglutinate two populations of MNPs functionalized with universal nontarget specific detection probes and on the introduction of a magnetic incubation scheme. This enables multiplex detection of *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* at clinically relevant concentrations, demonstrating a factor of 30 improvement in sensitivity compared to previous MNP-based detection schemes. Thanks to the universal probes, the same set of functionalized MNPs can be used to read out products from a multitude of RCA targets, making the approach truly scalable for parallel detection of multiple bacteria in a future integrated point of care molecular diagnostics system.

KEYWORDS: magnetic nanoparticles · padlock probes · rolling circle amplification · molecular diagnostics

There is a need for fast and accurate testing in microbiological diagnostics to ensure rapid, adequate and targeted treatment. The standard method for detecting bacteria in the bloodstream and other biological fluids is bacterial culture, which takes one to several days depending on the pathogen and thus can delay correct treatment.[1,2] Urinary tract infection (UTI) is one of the most frequent bacterial infections in humans with a high disease burden. Clinical guidelines for UTI suggest empirical antimicrobial therapy based on local antibiotic resistance rates until specific therapy can be directed toward the known pathogen.[3] Hence, culture based analysis of urine is required to confirm diagnosis and initiate optimal antimicrobial therapy. Novel rapid diagnostic tests for the causative pathogen would enable clinicians to cut turnaround and analysis time and quickly administer the *right* drug the *first* time.

In the past decades, several new methods have been developed for bacterial pathogen identification, primarily based on polymerase chain reaction (PCR)[4] and mass-spectrometry (MS).[5,6] Although being more rapid than plate culture,[7−10] most of these

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methods are not yet widely used in routine diagnostics either due to lack in sensitivity, high equipment or reagent costs or due to their laborious nature demanding automation. MALDI-TOF MS is implemented in routine hospital diagnostics, but has a longer turnaround time as it usually requires a single bacterial colony to achieve the required specificity.[11,12] Additionally, the miniaturization of these techniques has been proven challenging, hence limiting their potential integration in compact systems to be used at the point-of-care level, *i.e.*, directly at the patient or doctor site. Thus, a major breakthrough would be represented by a simple and multiplex molecular assay that requires minimal amplification and that can be implemented on an inexpensive cartridge and reader.

In this work, we propose a novel solution for rapid identification of bacteria in urine samples based on rolling circle amplification (RCA), magnetic nanoparticles (MNPs) and a simple reader based on low-cost optical components.

The molecular approach is based on binding of single-stranded oligonucleotides—so-called padlock probes—followed by highly specific enzymatic ligation to form topologically locked DNA circles that allow identification of the bacterial target DNA.[13] Ligated padlock probes can be amplified by RCA resulting in linear concatemers that collapse into μm-sized coil-like objects.[14,15] One hour of RCA amplifies the target sequence approximately 1000 times.[14] Higher amplification and thus higher sensitivity can be achieved by monomerization of RCA coils and subsequent religation and amplification *via* another round of RCA, *i.e.*, circle-to-circle amplification (C2CA).[16] The high specificity of the ligase to only seal perfectly matched sequences allows target discrimination down to single nucleotide resolution, enabling direct genotyping of mutations.[17] In addition, as each assay only requires a single padlock probe for identification with minimal interactions between probes for different sites, highly multiplexed analysis of pathogens and resistance markers can routinely be achieved.[18]

Detection of RCA products is often based on colorimetric[19,20] or fluorescence[21,22] analysis and recently other methods employing MNPs for readout of RCA coils have been established.[23,24] Colorimetric detection is simple but consists of multiple reaction steps, which is suboptimal for microfluidic integration.[25] The MNP based methods have so far suffered from limited sensitivity,[23,24,26] and together with fluorescence readout, they require expensive and sophisticated instruments, which hamper the integration of RCA in devices for point-of-care diagnostics.[27] In this work, the amplified bacterial targets—either intact RCA coils or RCA coils digested into monomers—are captured and bound by detection probes on magnetic nanoparticles in a microfluidic disk chamber. The presence of target DNA triggers MNP agglutination causing a change in the rotational dynamics of MNPs in the suspension when actuated by an external magnetic field. This change can be optically sensed by measuring the modulation of the light transmitted through the sample using simple and inexpensive optical components.

For the first time, we demonstrate sensitivity levels compatible with clinical needs. These are achieved by introducing a new scheme where monomerized RCA products induce MNP agglutination and by enhancing the specific agglutination *via* application of a strong magnetic field. The nanoparticle based readout approach is made universal and scalable by using a single set of detection probes designed to match generic sequences located on the backbone (and hence target unspecific part) of the padlock probes. As a core result, we demonstrate correct identification of three bacterial species and 100% successful detection of bacteria causing UTI in urine from patient samples in less than 4 h by combining off-chip sample preparation and amplification with a low-cost reader that automatically performs the incubation steps and the readout of up to 18 samples on a single low-cost polymer centrifugal microfluidic disk.

RESULTS AND DISCUSSION

The presented novel approach for rapid identification of bacteria is illustrated in Figure 1. Enriched bacteria from urine samples are lysed and target DNA is captured onto magnetic microbeads. After capture, padlock probes are hybridized to the bacterial DNA *via* their target specific complementary arms[13] and subsequently ligated and amplified by C2CA.[16] Intact RCA coils or RCA coils digested into monomers are next mixed with MNPs. Two populations of identical MNPs are functionalized with detection probes P1 and P2, matching separate regions on the backbone of the padlock probes. The presence of monomers amplified from the padlock probes will link the two types of MNPs and induce agglutination. The presence and amount of MNP clusters is optically determined and quantified by measuring modulation of the light transmitted through the sample upon application of an AC magnetic field. As a key feature, the two detection probes are directed against the nontarget specific sequence in the amplification products. This results in a generic detection system where a simple set of nanoparticles coated with the same probe set can be universally applied for any kind of target enabling simple multiplexed detection of different pathogens.

The picture of the setup in Figure 1 illustrates the main component of the reader, which embeds a blue laser (λ = 405 nm) from a Blu-ray optical pick-up unit, a photodiode and an electromagnet that provides a uniaxial magnetic field oscillating at a frequency *f*. The optomagnetic detection principle exploits the rotation dynamics of MNPs having a remnant magnetic moment.[28] The uniaxial AC sinusoidal magnetic field applied to the MNP suspension causes a torque on the

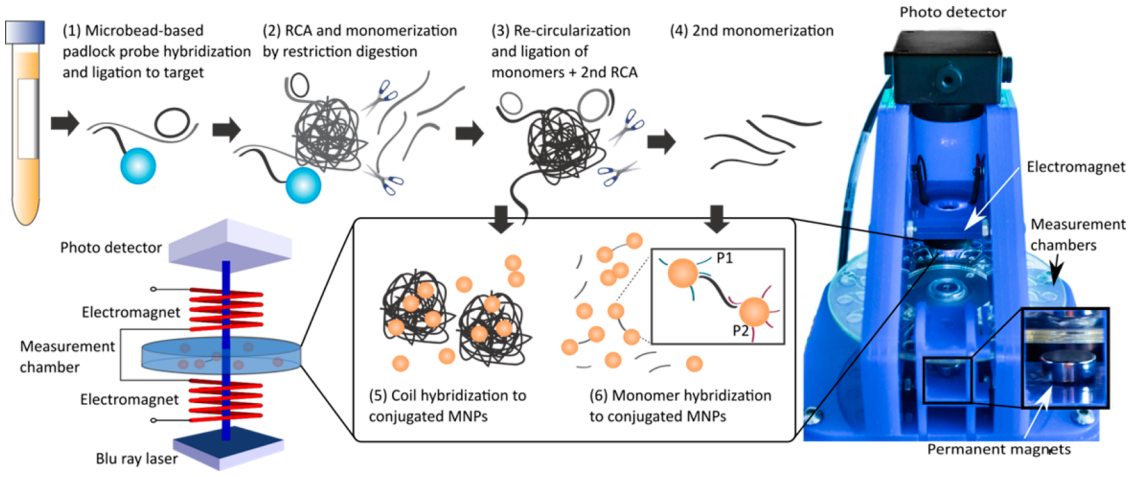

Figure 1. Schematic C2CA protocol and detection setup. (1) Capture of DNA targets from urine samples on magnetic microbeads (blue). Specific padlocks are circularized and enzymatically ligated to form a circle. (2) Amplification of circularized padlock probes by RCA and restriction digestion (illustrated by scissors) of the concatenated RCA product coil to monomers. (3) Recircularization of monomers followed by second RCA in solution. (4) Second digestion of RCA products for monomer detection. On-disk hybridization of (5) coils or (6) monomers to mixture of MNPs (orange), conjugated with probes P1 and P2. Samples are mixed in a microfluidic disk chamber by disk shaking. Magnetic incubation is performed by shaking the disk between two permanent magnets. Optomagnetic spectra are recorded using the illustrated setup by measuring the optical transmission modulation of blue laser light *vs* frequency of the applied magnetic field.

magnetic moments of the MNPs and forces them to rotate and cyclically align with the external field variation.

The optical anisotropy of the particles used in this work is linked to the remnant magnetic moment of the particles such that the field driven alignment of the individual MNP magnetic moments causes an increase in intensity of the light transmitted through the sample. However, when the field is reduced to zero, the alignment is lost due to the thermal agitation[28] and the amount of light transmitted decreases. The corresponding light modulation is monitored by measuring the phase lag φ of the second harmonic component of the photodetector signal.[29] At low frequencies the MNPs have time to align with the field, with positive and negative field values being alike. As a consequence, the observed transmitted light modulation is in-phase with the applied magnetic field ($\varphi = 0$). At higher frequencies the MNPs cannot follow the field variation and lag behind the field causing a positive phase lag. Thus, from the frequency dependence of φ it is possible to gain information on the rotational dynamics of MNPs, which can be directly related to magnetic susceptibility measurements.[28,30,31] The blue light (λ = 405 nm) was chosen as we observed the largest signal at this wavelength.

When MNPs aggregate due to the presence and binding of amplification product, their response lags behind the magnetic field at lower frequencies due to the larger hydrodynamic size. In this case a decrease in transmission with a negative phase lag is observed due to the size dependent change of the nanoparticle scattering properties upon agglutination.[32]

As a first step, we evaluate the system response when MNPs with detection probes P1 and P2 are mixed

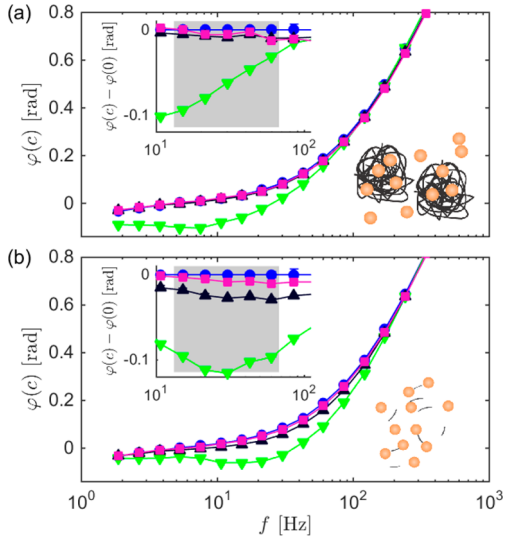

Figure 2. Optomagnetic detection of rolling circle amplification products. Magnetic phase lag $\varphi(c)$ spectra obtained for three concentrations c with $c/c_0 = 10^{-2}$ (inverted green triangles), 10^{-3} (black triangles), 10^{-4} (magenta squares) and 0 (negative control, blue circles), where c_0 is the stock solution corresponding to 1.2×10^9 CFU/mL of cultured *E. coli*. Panels (a) and (b) show results for coils and monomers obtained for optical incubation conditions (55 °C for coils and RT for monomers).

with intact RCA coils or RCA coils digested into monomers, respectively, and incubated using two different temperature conditions. Figure 2a shows $\varphi(c)$ *vs* f for the indicated relative concentrations c/c_0 of RCA products (coils) obtained by dilution, where the stock c_0 was formed from a sample with 1.2×10^9 colony forming units (CFU) of *Escherichia coli* per mL. For the highest coil concentrations, the phase has decreased at frequencies below 70 Hz, which suggests the presence

of aggregates. However, for lower coil concentrations the signal cannot be distinguished from that obtained for $c = 0$. Using monomers to link MNPs with detection probes P1 and P2 rather than binding the MNPs to coils is expected to increase sensitivity due to the higher number of available and exposed binding sites for the smaller and faster diffusing monomers. To test this hypothesis, identical amounts of the above RCA products were monomerized and serially diluted to allow for a comparison. Figure 2b shows $\varphi(c)$ *vs* f for detection of monomers formed from the indicated initial RCA product concentrations, c. For higher values of c, $\varphi(c)$ decreases for f between 10 and 100 Hz due to the formation of specific MNP aggregates. This can best be observed in the inset of Figure 2b where the data for $c = 0$ were subtracted from the sample signal. We found that $\bar{\varphi}(c)$, defined as the mean value of $\varphi(c)$ between $f = 15$ and 60 Hz, indicated in gray in the inset, correlated strongest with the target concentration.

We investigated the effect of temperature on the DNA hybridization during the incubation. Samples were either mixed and incubated at 55 °C (0.5 h) and subsequently kept at room temperature (2h) or they were incubated continuously at room temperature (2.5 h) (Supporting Information, Figure S1a). The results showed that the binding of MNPs to RCA products (coils) was optimal at 55 °C (Figure 2a), whereas the binding of MNPs to monomers was more efficient at room temperature (Figure 2b). We attribute this effect to the increased accessibility of binding sites in the coils at elevated temperatures due to the reduced likelihood of forming secondary structures. However, the hybridization off-rate likely had a larger impact for the smaller monomers as compared to the coils at 55 °C, which may explain their higher binding efficiency at room temperature. Using the best incubation method, the limit of detection (LOD) for coils corresponded to about $c/c_0 = 1.4 \times 10^{-3}$, whereas for monomers it corresponded to $c/c_0 = 2 \times 10^{-4}$ (Figure S1a). Therefore, using monomers instead of RCA coils as templates for clustering of MNPs, lowered the LOD by a factor of 7 compared to previous work.[28]

To further improve the hybridization kinetics of the detection of monomers, we investigated the effect of applying a strong magnetic field during incubation. Two detection chambers were measured simultaneously where one chamber was exposed to the magnets while the other was not (Figure 3a). The magnetic incubation consisted of 20 cycles of shaking the sample in the permanent magnetic field for 4 s followed by shaking outside the magnetic field for 8 s. This speeds up the hybridization kinetics and removed nonspecific aggregation. The data suggests that the magnetic incubation step increases the local concentration of MNPs and as a consequence it not only accelerates up the kinetics for the formation of specific MNP clusters, but also shifts the equilibrium of the reaction. A significant

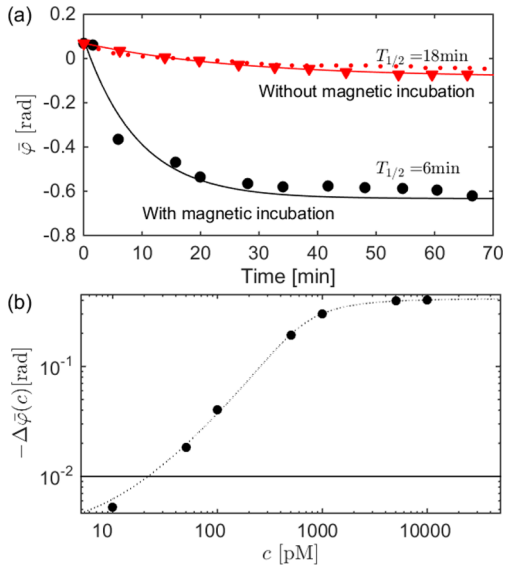

Figure 3. Hybridization kinetics without and with magnetic incubation. (a) $\bar{\varphi}$ *vs* time for DNA monomers formed from genomic *E. coli* DNA from bacterial culture ($c/c_0 = 10^{-2}$). The average phase signal is measured at RT without magnetic incubation (red dots (no shaking), red triangles (with shaking)) and with repeated cycles of magnetic incubation (20 cycles) followed by measurements (black circles), respectively. The time axis for the data generated without shaking (red dots) has been divided by a factor of 5 to allow the slower kinetics to fit in the graph. The half-lifes ($T_{1/2}$) of exponential fits to the data are indicated in the figure. (b) Values of $-\Delta\bar{\varphi}(c)$ *vs* concentration c of the synthetic DNA target monomer, where $\Delta\bar{\varphi}(c)$ is the average phase change observed upon 7 min of magnetic incubation (35 cycles). The solid line indicates the LOD calculated as the mean plus three times the standard deviation of the $c = 0$ sample (measured in triplet).

change in signal between the two cases can already be observed after the first step of magnetic incubation.

Magnetic incubation of only 80 s (without shaking of the disk) improved the LOD of the monomerized RCA products by a factor of 3 and 160 s of magnetic incubation improved the LOD further by a factor of about two (*cf.* Figure S1). Using 80 s of magnetic incubation in the detection, we estimate an LOD of $c/c_0 = 8 \times 10^{-5}$ corresponding to 1×10^5 CFU/mL. As the $c = 0$ signal is not affected by magnetic incubation (figure S2), measuring the sample directly after mixing as well as after magnetic incubation allows to use the sample itself as an internal negative reference. We therefore define $\Delta\bar{\varphi}(c)$ as the *change* in phase averaged in the region $f = 15$ Hz to $f = 60$ Hz between the reference measurement performed right after mixing to that performed after magnetic incubation. Note that this change is negative when $c > 0$ (*cf.* Figure S1).

As a consequence of these findings, the optimal method for measuring a single monomer sample was defined as follows. The sample was mixed with the MNB suspension and loaded directly onto the disk. Then, the reference spectrum of the sample was measured, followed by 35 cycles (7 min) of magnetic incubation and measurement of the final spectrum of the sample. This approach, which was used in all

TABLE 1. Oligonucleotide Sequences

name	sequence
E. coli padlock probe	5′-TTAATACCTT TGCTCATTGA CAGAGTGTAT GCAGCTCCTC AGTATAGTCG ATAGTCACGG CTACTTTTGG AAGGGAGTAA AG-3′
P. mirabilis padlock probe	5′-GACCTTGCAC TATCGGATGA GAGTGTATGC AGCTCCTCAG TATAGTCGAT AGTCACGGCT ACTTTTGGGG CTCTTCG-3′
P. aeruginosa padlock probe	5′-AGGGAGAAAG TGAGAGTGTA TGCAGCTCCT CAGTATAGTC GATAGTCACG GCTACTTTTC CGCATACGTC CTG-3′
E. coli capture oligonucleotide	5′-CTCTCTCTCT CTCTCTCTCT CTCTCTGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAA-3′
P. mirabilis capture oligonucleotide	5′-CTCTCTCTCT CTCTCTCTCT CTCTCTGGAT TAGCTAGTAG GTGGGGTAAA GGCTCACCTA GGCGAC-3′
P. aeruginosa capture oligonucleotide	5′-CTCTCTCTCT CTCTCTCTCT CTCTCTCGGA CCTCACGCTA TCAGATGAGC CTAGGTCGGA TTAGCTA-3′
Primer	5′-TACTGAGGAG CTGCATACAC-3′
Restriction oligonucleotide	5′-GTGTATGCAG CTCCTCAGTA-3′
P. aeruginosa synthetic target	5′- AGGCCTTTAC CCCACCAACT AGCTAATCCG ACCTAGGCTC ATCTGATAGCG TGAGGTCCGA AGATCCCCCA CTTTCTCCCT CAGGACGTAT GCGGTA-3′
Detection probe P1	5′-Biotin-CTTCT+CC+TC+AG+TATAGTC-3′[a]
Detection probe P2	5′-Biotin-CTTAG+AG+TG+TA+TGCAG-3′
Detection probe P3	5′-Cy3-AGTAGCCGTG TTCUUUU-3′[b]
Detection probe P4	5′-Cy3-ACTATCGACT TTCUUUU-3′[b]

[a] + before a base indicates LNA residues. [b] The four last bases are 2′-O-Methyl RNA bases.

studies below, is advantageous compared to detection of intact RCA coils as it both simplifies the assay and reduces the time, since no preincubation at elevated temperatures is needed.

We characterized the optimized method with a synthetic *Pseudomonas aeruginosa* monomer target (Table 1) in order to establish an exact value for the LOD of DNA monomers. Figure 3b shows the obtained sigmoidal dose response of $-\Delta\bar{\varphi}(c)$ that gives an LOD of 50 pM and a dynamic range of 2 orders of magnitude.

As many infections, including urinary tract infections, can be caused by more than one pathogen, it is desirable to simultaneously detect multiple pathogens with high specificity. For this reason we investigated the parallel detection of three common urinary pathogens. *E. coli*, *Proteus mirabilis* and *P. aeruginosa* were cultured separately and diluted to approximately 10^7 CFU/mL. The specificity of padlock probes was first shown by targeting cultures containing a single bacterial strain (Figure 4). Next, to mimic mixed infections, all three or only two bacterial strains were mixed in all possible combinations and then separately probed for and measured using the same set of detection probes. No unspecific signal from any of the tested padlock probes was detected. Furthermore, we were able to correctly identify mixed infections with our assay setup (Figure 4, Table S1).

To demonstrate the capability of the presented technology for use in clinical diagnostics, we analyzed 28 urine samples that were sent to the hospital laboratory for further microbiological analysis. For this proof of concept study we choose to only detect *E. coli* as it is by far the most common pathogen causing UTI. According to the clinical laboratory results, eight samples were *E. coli* positive, including two samples that were coinfected by another bacterial species. Seven samples contained bacteria not targeted in this study and 13 samples showed no growth when cultured on agar plates. With our assay, we accurately detected seven out of eight *E. coli* positive samples (Figure 5 and Table S2).

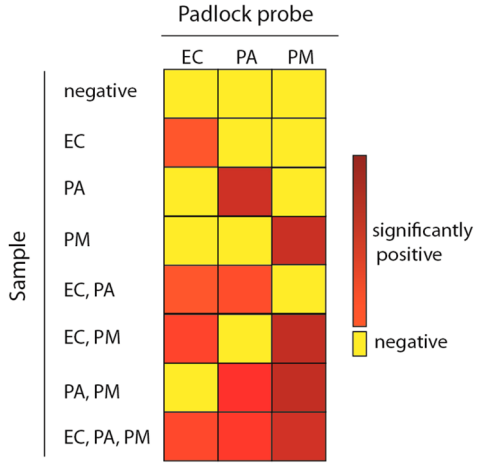

Figure 4. Multiplex pathogen detection. Heat map showing specifically acquired signal for each padlock probe and its corresponding template from bacterial culture. Signals above the LOD are indicated as positive with a significant shift (>3 s.d. of negative reference sample).

The eighth sample did not have a CFU count exceeding the threshold for UTI (10^5 CFU/mL).[33] No false-positive signals were observed in samples containing other bacterial species than *E. coli*, including Gram-negative mixed flora. Thus, the assay delivered the required sensitivity with 100% specificity strongly correlating with the result obtained by standard plate culture from the clinical laboratory.

Even though current results are limited to urinary tract samples, which have a high positive CFU threshold (usually 10^5 CFU/mL), this method is fully compatible with a large variety of pathogens, as the assay dynamic range can be tuned by increasing the RCA time or by introducing an additional round of RCA. Both these approaches will increase the number of monomers by a factor of 1000 per 60 min amplification cycle. As a main advantage compared to PCR based methods only a quick washing step is needed to purify the sample prior to amplification, which makes this

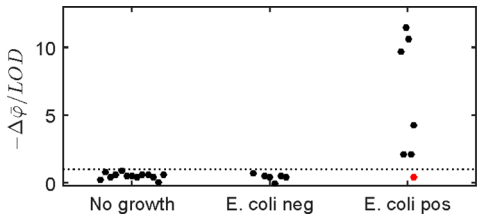

Figure 5. Patient samples analysis. A set of 28 patient samples was analyzed in a blinded fashion. The horizontal axis shows the sample classification obtained from the routine clinical laboratory test and the vertical axis shows the optomagnetic signal normalized with the LOD. The horizontal line indicates the LOD. The data point in red did not yield a positive result by our assay as it had a CFU count below the diagnostic threshold for UTI.

assay potentially suitable for direct analysis of several different specimen types, such as blood, sputum and swabs from mucosal surfaces. This also applies to MALDI-TOF, which, however, to gain optimal specificity and to deduce polymicrobial samples, requires bacterial colonies as starting material thus significantly increasing the total analysis time. Our overall assay strategy can be used to identify antibiotic resistances, which, for instance is a major limitation of MALDI-TOF as associated proteins are expressed at a low level or are similar to other bacterial proteins.

The presented assay features several characteristics beneficial for a future integration of the sample processing with the optomagnetic readout to form a complete analysis system: (i) The use of an isothermal amplification method eliminates the need for fast temperature changes and elevated temperatures and thus, demands on electronics as well as materials are much lower. (ii) The nature of the protocol is highly repetitive, which reduces the number of different actuators required. (iii) Magnetic microparticles are used as solid phase support during the amplification, which increases reaction kinetics as a higher concentration of padlock probes can be used and it allows for washing or replacement of liquids with no need for additional chambers. (iv) Multiplexed analysis can be achieved by parallelizing the readout of multiple pathogens in any microfluidic support using MNPs coated with the same set of detection probes and target specific padlock probes in separate chambers. This capability paves the way to a large number of applications, *e.g.*, within detection of infectious diseases and of cancer biomarkers.

Recently Chung *et al.* have demonstrated the use of a magneto-DNA nanoparticle system for rapid pathogen detection, where MNP agglutination is sensed using a highly sensitive nuclear magnetic resonance based readout.[7] A smart and sensitive detection of oligonucleotides has also been demonstrated with the so-called "pinwheel effect", based on magnetic microbead agglutination.[34] In addition to the different agglutination tests mentioned above, we demonstrate that by using MNPs in combination with magnetic incubation, molecular assay time can be dramatically reduced and high sensitivity can be obtained by simply measuring optically the phase lag spectrum of the MNP response.

CONCLUSION

In summary, we presented a molecular detection strategy using padlock probe recognition and on-bead isothermal RCA combined with the on-disk automated optomagnetic nanoparticle readout. The employed molecular approach does not require rapid changes of temperature or extensive DNA preparation, and padlock probes offer high specificity and multiplexing capability. Amplification products are detected using an optomagnetic readout, which provides high sensitivity and multiplexing capability using low-cost optical components.

In the present study we demonstrated the use of MNPs coated with universal readout probes matching generic sequences on the backbone of the padlock in an agglutination assay format. Moreover, we presented a new scheme for the detection of RCA monomers as alternative to RCA coils and demonstrated an increase in the sensitivity of the nanoparticle based readout by a factor of about 10. The use of a magnetic incubation step further increased the sensitivity by a factor of 3, such that the present work represents an improvement by a factor of 30 compared to other MNP based readout schemes. In the presented detection scheme, the sample itself prior to magnetic incubation is used as a reference for the measurement after magnetic incubation. This is possible because of the slow reaction kinetics without magnetic incubation and the short measurement time.

We employed the above assay format to successfully identify *E. coli* in clinical samples with no false negatives and a total assay time, including sample preparation, amplification and detection below 4 h. This implies that using the presented technology the correct diagnosis can be achieved with a significant gain in time as compared to standard microbiology methods. In addition, the use of low-cost optical components and a plastic microfluidic disk for automated multichamber sample readout proves the scalability and commercial potential of the presented methodology.

METHODS

Bacteria Culture and Lysis. One mL of Luria Broth (LB) was inoculated with *E. coli*, *P. aeruginosa* and *P. mirabilis*,[22] respectively, and incubated overnight at 37 °C. Bacteria were diluted to the desired concentration and 100 μL of culture were distributed to the reaction vessels. The bacteria were lysed by adding 10 μL of 5 M NaOH and heated at 95 °C for 10 min. To adjust pH 10 μL of 5 M HCl and 10 μL of 1.5 M Tris-HCl were

added. Bacterial amount was quantified by plating 100 μL of an appropriate dilution onto Luria Agar. Plates were incubated overnight at 37 °C before colonies were counted manually.

Patient Samples. Urine samples, sent to the Uppsala University Hospital for analysis, were used in this study. The study on the clinical samples was approved by the ethics committee of the Faculty of Medicine, Uppsala University (01−367), and conducted according to the Declaration of Helsinki. All samples were handled anonymously according to Uppsala University Hospital rules. Ten μL of urine were added to 90 μL of LB and incubated at 37 °C for 2 h to allow growth of bacteria. All samples were lysed as described above and pH was adjusted.

Padlock Probe Hybridization, Ligation and Amplification. Padlock probes were designed for *E. coli*, *P. mirabilis* and *P. aeruginosa* and ordered from Integrated DNA Technologies (USA) along with all needed oligonucleotides for C2CA (Table 1). Prior to use, padlock probes were 5′ phosphorylated by incubating 1 μM of padlock probes in a mixture consisting of 1× PNK buffer A (50 mM Tris-HCl [pH 7.6 at 25 °C], 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, Thermo Scientific, USA), 1 mM ATP (DNA-Gdansk, Poland) and 10 U T4 Polynucleotide Kinase (Thermo Scientific) for 30 min at 37 °C. The kinase was inactivated at 75 °C for 10 min. To capture target sequences onto magnetic particles, samples were denatured at 98 °C for 5 min, followed by snap-cooling on ice. 4× B&Wtw buffer (40 mM Tris-HCl (pH 7.5), 20 mM EDTA, 0.4% Tween-20 and 4 M NaCl) was added to a final concentration of 1× together with 4 nM biotinylated capture oligo and incubated at 55 °C for 10 min. Ten μL of Dynabeads MyOne Streptavidin T1 (Invitrogen, USA) microbeads, washed three times in 1× B&Wtw buffer prior to that, were added to the sample mixture and incubated under rotation at room temperature for 10 min. To wash away LB and urine, respectively, samples were washed once in 100 μL of 1× Ampligase reaction buffer (20 mM Tris-HCl [pH 8.3], 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, and 0.01% Triton X-100, Epicenter, USA). The washing buffer was discarded and microbeads were resuspended in 1× Ampligase reaction buffer, 0.1 μM padlock probe, 0.2 μg/μL BSA and 5 U Ampligase (Epicenter). The solution was incubated at 55 °C for 10 min to allow hybridization and ligation of padlock probes to their complementary target sequences. To wash away excess padlock probes, samples were washed once in 1× Wtw buffer (10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% Tween-20 and 0.1 M NaCl). To initiate RCA, microbeads were resuspended in 1× phi29 DNA polymerase reaction buffer (33 mM Tris-acetate [pH 7.9 at 37 °C], 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20, 1 mM DTT, Thermo Scientific) supplemented with 0.2 μg/μL BSA, 125 μM dNTPs, 25 nM primer and 100 mU/μL phi29 DNA polymerase (Thermo Scientific). RCA was performed for 60 min (20 min for urinary samples) at 37 °C and the polymerase was inactivated at 65 °C for 1 min. In order to allow for further amplification, RCA products were enzymatically monomerized by adding a mixture of 1× phi29 DNA polymerase reaction buffer, 0.2 μg/μL BSA, 120 nM restriction oligo and 40 mU/μL AluI (New England Biosciences, USA) and incubated at 37 °C for 1 min. The restriction enzyme was inactivated at 65 °C for 1 min. The microbeads were removed after monomerization by transferring the supernatant to a new tube for further amplification. The washing steps were magnetically assisted using a magnetic plate holder (DynaMag-96 Side Magnet, Invitrogen). The monomers were religated, templated by undigested restriction oligo and further amplified by RCA. For this, 1× phi29 DNA polymerase reaction buffer supplemented with 0.2 μg/μL BSA, 100 μM dNTPs, 0.67 mM ATP, 14 mU/μL T4 DNA ligase (Thermo Scientific), and 60 mU/μL phi29 DNA polymerase was added to the reaction. The second RCA was incubated at 37 °C for 60 min (20 min for urinary samples) and phi29 DNA polymerase was inactivated at 65 °C for 1 min. To monomerize RCA products five μL of 1× phi29 DNA polymerase reaction buffer, 0.2 μg/μL BSA, 400 nM primer, and 270 mU/μL AluI were added and incubated at 37 °C for 10 min, followed by an incubation at 65 °C for 1 min. The above procedure resulted in samples with a volume of 55 μL.

Optomagnetic Setup. Figure 1b shows a schematic of the readout system described previously by Donolato *et al.*[28] In brief, a photodetector (ThorLabs PDA36A) collected light from a laser (Sanyo Blu-ray optical pick-up, λ = 405 nm) after the light passed through the measurement chamber on a microfluidic disk. Two electromagnets positioned along the optical axis produced a sinusoidally varying magnetic field (RMS 2.4 mT) resulting in a modulation of the light transmission through the MNP suspension. The current for the electromagnets was controlled and the signal from the photodetector was collected using a data acquisition card (National Instruments 6251). The frequency *f* of the applied magnetic field was swept from 2.66 to 965.25 Hz in 17 logarithmically equidistant steps and the real (V_2') and imaginary (V_2'') components of the second harmonic photodetector signal corresponding to the signal from a lock-in amplifier were calculated using FFT. A frequency sweep was recorded in 80 s. The phase lag of the magnetic response was calculated using $\varphi = \text{atan2}(-V_2', -V_2'')/2$.[28,29] For the magnetic incubation steps an external permanent Nd−Fe−B magnet (NdFeB, φ = 10 mm, thickness = 1.5 mm) was positioned at the radial position opposite to the two electromagnets (see Figure 1). The magnetic field, measured at the center of the pool, was 250 mT.

Optomagnetic Readout Protocol. Fifteen μL of 10 mg/mL BNF-Starch-Streptavidin MNPs with a diameter of 100 nm (#10-19-102, Micromod Partikeltechnologie GmbH, Germany) was washed once with 1× Wtw buffer and incubated with biotinylated detection probes P1 or P2 for 30 min. The functionalized MNPs were washed three times in 1× Wtw and resuspended in 150 μL of B&Wtw buffer. Equal volumes of MNPs functionalized with detection probes P1 and P2 were mixed and diluted with B&Wtw to 0.1 g/L. Sixteen μL of the MNP mixture were mixed with 16 μL of the total sample volume of 55 μL (diluted in B&Wtw). The samples were loaded onto a microfluidic disk by spinning the disk at 40 rps for 5 s and measured.

We obtained the data in Figure 2. and in Figure S1 using the readout strategy described below and schematically illustrated in Figure S3a. The samples were either incubated at 55 °C or at room temperature for 30 min followed by 2 h at RT. All samples were loaded onto the disk simultaneously. The first nine (out of 18) samples on a disk were measured without the permanent magnets for magnetic incubation mounted in the setup. Then the magnets were mounted and the last nine samples were measured without exposure to the magnetic field from the permanent magnets. As the permanent magnets were mounted opposite to the readout position, the first nine samples were exposed to magnetic incubation (for 80 s) while the last nine samples were measured. Nonspecific aggregates spontaneously dissolved during the time lag (∼10 min) between the magnetic incubation and the measurement. This magnetic incubation was repeated once to obtain a total magnetic incubation time of 160 s.

All remaining data were obtained using the readout strategy described below and schematically illustrated in Figure S3b. The sample was mixed and loaded onto the microfluidic disk without a preincubation step. Subsequently, the sample was measured and magnetic incubation performed. In this case, to improve the reaction kinetics, the disk was continuously moved 10° back- and forward at 30 Hz for 4 s in the magnetic field to increase mixing of the suspension. To remove nonspecific aggregates formed under the magnetic incubation 8 s of shaking was thereafter performed outside the magnetic field. Thus, one cycle of magnetic incubation followed by mixing took 12 s. This cycle was repeated as needed to obtain the total time of magnetic incubation. The data *vs* time of magnetic incubation (Figure 3a) were obtained as repeated measurements separated by 20 magnetic incubation cycles. All remaining data were obtained using a magnetic incubation time of 7 min (35 magnetic incubation cycles).

Microfluidic Disk Fabrication. Microfluidic disks were fabricated as described previously.[28] Briefly, three 600 μm thick Poly(methyl methacrylate) (PMMA, Axxicon, Netherlands) DVD disks were joined together with pressure sensitive adhesive (PSA, 90106, Adhesive Research, Ireland). A CO_2 laser (Mini 18, 30W, Epilog, USA) was used to cut inlets in the top disk, measuring chambers and reservoirs (φ = 5 mm) in the middle disk and alignment holes in all three disks. Fluid channels were cut in two

PSA sheets using a blade cutter machine (Silhouette, USA). Finally, the PSA was laminated on the bottom and top disks, which were then aligned and bonded to the central disk containing the reservoirs. This rapid prototyping process allowed for the complete fabrication of a disk with 18 microfluidic chambers in less than 20 min.

Fluorescence Detection in Microfluidic Setup. As a control, RCA products were also detected using a commercially available fluorescence detector (Aquila400, Q-linea, Sweden). Prior to detection, RCA products were fluorescently labeled by adding 10 nM detection probe P3, 10 nM detection probe P4, 40 mM EDTA, 40 mM tris-HCl (pH 8), 0.2% Tween20, and 2 M NaCl. To allow hybridization of detection oligonucleotides, samples were incubated 2 min at 70 °C followed by 15 min at 55 °C and next counted in a flow-channel in the detector.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Tables with results for multiplex pathogen detection and patient samples. Figure with dose−response curves of optomagnetic response for incubation at RT and 55 °C without magnetic incubation and for two magnetic incubation times. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.5b02379.

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