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On-chip real-time monitoring of multiple displacement amplification of DNA

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ABSTRACT

The integration of systems for real-time monitoring of DNA amplification reactions is rather limited, because the heating system, the temperature control and the detection system are usually realized with separate modules. In this work, a lab-on-a-chip system for real-time monitoring of the multiple displacement amplification reaction (MDA) is presented. The amplification and detection unit consists of a system-on-glass (SoG) coupled to a microfluidic chip. The SoG includes thin film metallic resistors and amorphous silicon temperature sensors to control the temperature, as well as amorphous silicon photosensors and an interference filter for fluorescence detection on a single glass plate. The amplification reaction is carried out in the microfluidic chip made of cyclic olefin copolymer (COC). By using this setup, the multiple displacement amplification reaction can be monitored in real-time using the fluorophore $[Ru(phen)_2(dppz)]^{2+}$.

1. Introduction

The polymerase chain reaction (PCR) is widely used for DNA amplification, but requires thermal cycling. For isothermal amplification reactions such thermal cycling is not required, which makes (micro-) systems for isothermal amplification simpler and less energy-consuming, and therefore attractive for portable, battery-operated instruments. Another characteristic of isothermal reactions is that the rate of the enzyme activity is the limiting factor instead of the rate of thermal cycling, as with PCR.

MDA is an isothermal amplification technique carried out at 30 °C that makes use of strand displacement, resulting in average product lengths of more than 10 kb [1,2]. This low temperature can be used since random hexamer primers and the polymerase phi29 are used for the amplification. Dean et al. showed that with only 1–10 DNA copies 20–30 μg (in a volume of 100 μL) of DNA product can be produced. An amplification plateau is reached after 4–6 h of incubation, whereby the yield is almost independent of the initial amount of DNA (100 fg – 10 ng) [1]. It is not necessary to denature the DNA sample before the start of the MDA reaction; in fact the most efficient amplification in terms of yield is obtained without the denaturation step [1,3]. Kumar et al. used the GenomiPhi V2 kit to amplify the DNA of a single human cell. Within 4 h they acquired 4–7 μg (in 20 μL) of DNA with this kit [4]. It is, however, known that the MDA reaction can give background amplification (i.e. a false positive signal), especially after a long incubation time. Blainey et al. identified that the source of this background is from high molecular weight contaminants (contained in the polymerase mix) and is not due to the MDA reagents [5]. MDA has two main drawbacks: the amplification is non-specific due to the random primers (also synthesis of DNA contamination or primer dimers) and uneven representation of the template due to amplification bias [6,7]. Rhee et al. showed that upon limiting the reaction volume, by using microfluidic digital droplet MDA, the competition among DNA fragments for primers and polymerases reduces, resulting in a more uniform

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coverage, lower bias and non-specific amplification when compared with conventional MDA [7]. Rhee et al. created 200 pL droplets containing MDA mix, SYBR Green and DNA within a polydimethylsiloxane (PDMS) chip. Subsequently, the droplets were transferred into a Mylar/PMMA collection chip and incubated for 18 h at 30 °C in a thermocycler. A strong fluorescent signal, originating from the condensed DNA structures, could be observed with an inverted microscope [8]. Yang et al. developed a PDMS based microfluidic device for single cell whole genome amplification with the GenomiPhi V2 kit. In a volume of only 23.85 nL they obtained around 8 ng as measured with a Qubit 2.0 system. This implies a more than 1000-fold amplification of DNA using an AmpliSpeed slide cycler to apply a temperature of 34 °C for 2 h to the chip [9]. Yang et al. also used the Repli-g single cell kit for amplification in a PDMS microfluidic device, which was incubated in a water bath for 16 h at 30 °C. The latter gave a 10 000-fold amplification as was determined with a Qubit 2.0 system (on average 91.4 ng from a single cell). The NTC (i.e. chambers without cells) showed a background of 51.84 ng [10].

MDA can be used as technique to amplify the DNA prior to further (forensic) analysis, since the DNA yield from forensic samples is often limited. Sufficient DNA for sequencing from even a single cell can be achieved [6]. Despite the possible non-specific amplification and amplification bias of the MDA reaction, Ballantyne et al. showed that it is possible to use MDA to amplify genomic DNA from small amounts of template, 5 pg to 1 ng, for downstream short tandem repeat (STR) multiplex genotyping [11]. The same group also investigated two different methods for WGA: the GenomiPhi and the GenomePlex kits. The two kits were tested on STR genotyping of low copy number (LCN) and degraded DNA samples. They showed that by using WGA the quality and quantity of DNA can be increased and that it has the potential to improve STR typing from difficult samples in forensic casework. Both kits could amplify the LCN DNA, but only GenomiPhi showed an increase in profiling success. The profiling success from digested DNA was improved by both kits [12].

These examples exemplify that MDA can be performed successfully on-chip. However, the heating system, the temperature control and the detection occurred with separate modules, which limits the portability of the system. Combining sensors and/or actuators for two different physical parameters into one system (e.g. light and temperature sensors [13] or a heater and temperature sensors [14]) increases the integration level. Integration of different functionalities into one lab-on-a-chip (LoC) system leads to a compact transportable system, which would be useful for various applications in the field, such as direct analysis at crime scenes. MDA is chosen, since it can be used to amplify DNA for further (forensic) DNA analysis. A LoC with integrated functionalities can also be used to monitor other amplification reactions, catalytic tests, chemical tests or immunological tests. COC is a copolymer based on linear and cyclic olefins, which is widely used for food packaging and medical/diagnostic disposables. COC is also an upcoming material for microfluidic devices [15–17]. Serra et al. used COC, in combination with PCR sealing tape to close the chip, for the amplification of template DNA by PCR. The product was analyzed on gel and showed the expected amplicon [18]. Gulliksen et al. used a COC chip with an aluminum block as heater to carry out real-time nucleic acid sequence-based amplification (NASBA). With this chip they could successfully detect cancer markers with detection limits comparable to conventional laboratory systems [19].

To monitor the MDA reaction real-time a `light switch' complex is used. $[Ru(phen)_2(dppz)]^{2+}$ is a fluorophore that shows no photoluminescence in aqueous solution, but displays intense photoluminescence in the presence of DNA [20,21]. This fluorophore has been applied for the detection of DNA [20,22,23], however, never for real-time monitoring of amplification procedures performed in LoC devices.

In this work, a LOC system suitable for on-chip real-time monitoring of MDA with the fluorescent dye $[Ru(phen)_2(dppz)]^{2+}$ is presented. In this system a microfluidic chip made of cyclic olefin copolymer (COC) is coupled to a system-on-glass (SoG). In the microfluidic chip the MDA reaction is executed with DNA samples of different concentrations and origin, whereas all the sensors (temperature and photosensors) and actuators (heaters) and an interference filter needed to control and monitor the reaction in real-time are integrated into the system-on-glass.

2. Materials and methods

2.1. Materials

MDA was performed using the illustra GenomiPhi V2 DNA Amplification Kit purchased from Fisher Scientific. Calf thymus DNA was obtained from Sigma-Aldrich. The fluorophore $[Ru(phen)_2(dppz)]^{2+}$ was synthesized following the procedure described elsewhere [20].

2.2. COC chip fabrication

SolidWorks was used to design the chip. The microfluidic channels of the COC chips were milled in a 85 mm x 54 mm x 2 mm COC plate (DENZ Bio-Medical grade 6013) using a Sherline 5410 Deluxe Mill with a 1.0 mm diameter mill. The in- and outlet of the COC chips were drilled and had a diameter of 1.5 mm in order to be compatible with standard pipette tips. Therefore, the chip could be easily filled by using a pipette (standard tip). Each COC chip has a meandering channel with a width and a depth of both 1 mm and a total volume of approximately 55 µL (Fig. 1). Close to the reaction channel a reference channel was present, in which a thermocouple could be inserted.

After milling the chips were thoroughly cleaned with MilliQ water (i.e. demineralized and deionized water) and subsequently placed in ethanol in a sonicator (15 min) and finally blown dry with nitrogen gas. To regain optical transparency after milling, the chips were exposed to cyclohexane vapor at 60 °C for 1 min to decrease the surface roughness as suggested by Ogilvie et al. [24]. The channel was closed by

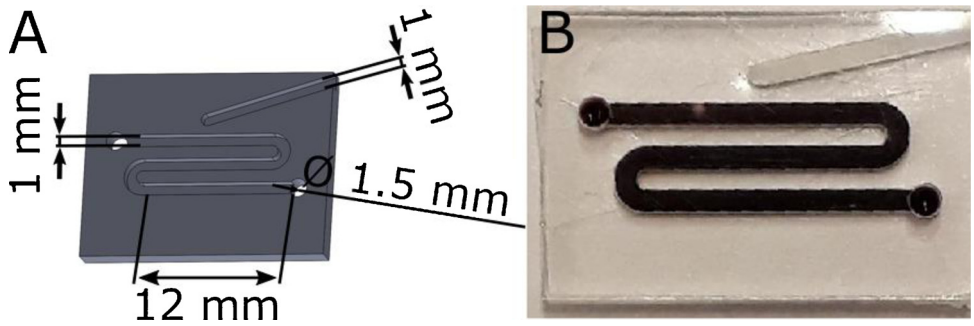

Fig. 1. The design of the COC chip for real-time monitoring of the MDA reaction with A) a schematic representation of the COC chip with the used dimensions and B) a photo of the COC chip filled with dark blue food dye to visualize the channel geometry.

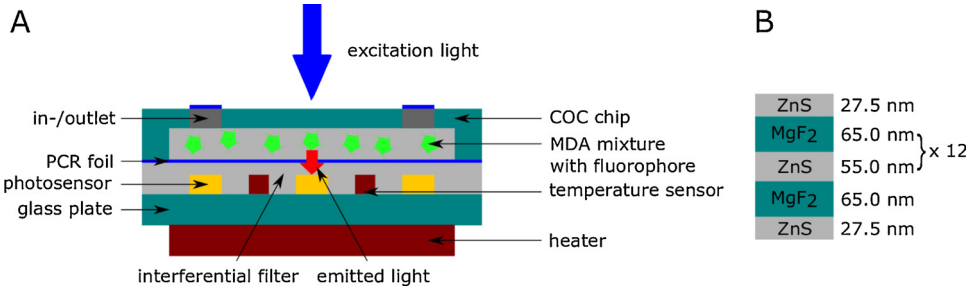

Fig. 2. A schematic overview of A) the cross-section of the system-on-glass and B) the details of the configuration of the interference filter.

laminating the chip with a piece of PCR foil (Microseal `B' Adhesive Seals, Bio-Rad). After filling the chip with the pre-mixed MDA mixture, the in- and outlet were also sealed with a small piece of PCR foil, creating a well based system. The use of sealing tape or adhesive foil has proven to be robust and suitable for biological applications [18].

2.3. SoG fabrication

The SoG integrates the following functions on a single glass plate: i) a metallic film electro resistive heater [14,22,25–27], ii) p-i-n doped hydrogenated amorphous silicon (a-Si:H) temperature sensors [13,14,22,25], iii) p-i-n doped a-Si:H photosensors [25,28] and iv) a long-pass interference filter [23,29].

A schematic cross section of the SoG is given in Fig. 2. The heater was fabricated on the bottom side of the glass plate, while the other components (i.e. temperature and light sensors, interference filter) were fabricated on the top side. As shown in Figs. 2 and 3 the COC chip was directly placed on top of the SoG.

The SoG fabrication (details in the supplementary material) started with the deposition of the heater material, followed by the deposition of the doped a-Si:H layers and ended with the deposition of the interference filter. The temperature sensors and photosensors were manufactured simultaneously using a plasma enhanced chemical vapor deposition (PECVD) layered deposition process. The long-pass interference filter is composed of alternating layers of zinc sulphide (ZnS) and magnesium fluoride (MgF_2) (Fig. 2B) that were fabricated by electron beam physical vapor deposition (EBPVD) directly over the a-Si:H junctions, at a deposition pressure of 10^{-4} mbar and with a growth rate of 1.5 nm/min. The filter was designed with XOP freeware software and the IMD extension to block the excitation peak at 450 nm. The spectral responsivity of the filter was calculated as the ratio between the photocurrent and the radiation power impinging on the junction, whereby the sensors were biased in short-circuit conditions.

2.4. Dye characterization

In order to characterize the absorption, excitation and emission spectra of $[Ru(phen)_2(dppz)]^{2+}$, a Tecan M200 PRO multimode reader (operated by Tecan I-control software) in combination with a Corning 96 flat bottom black polystyrene microplate was used.

2.5. Off-chip MDA procedure

Isothermal amplification of the DNA was carried out in a total volume of 10 µL per experiment with a conventional thermocycler (Applied Biosystems 2720). MDA mix was made with reaction buffer, sample buffer, enzyme mix, DNA and dye solution in a ratio of 9:5:1:1:4, respectively. In order to monitor the reaction in real-time, the amount of sample buffer from the original protocol was lowered and exchanged by the $[Ru(phen)_2(dppz)]^{2+}$ dye. It has been reported that the amount of sample buffer can be lowered without affecting the amplification yield or kinetics of the reaction [4]. The thermal protocol was set to 45 cycles of 2 min at 30 °C. At the end the sample was heated to 65 °C for 10 min to inactivate the polymerase. All experiments were carried out in at least duplicate.

A stock concentration of 1 mg/µL calf thymus DNA was made (verified with a NanoDrop 1000, Thermo Fischer) and a 10-fold serial dilution series of 0.001–10 ng/µL were prepared. As no template control (NTC) MilliQ water was added to the mixture instead of DNA. End concentrations of 1.33 µM, 2.13 µM and 2.66 µM $Ru(phen)_2(dppz)]^{2+}$ were used to determine the optimal dye concentration. The thermal protocol was set to a total run time of 2 h at 30 °C. After the run time the samples were heated to 65 °C for 10 min to inactivate the polymerase.

After amplification, the samples were analyzed by gel electrophoresis by using 1% agarose gel stained with ethidium bromide (EtBr) and 5 µL of amplified sample in combination with ladders of 1 kb and 100 bp. The gel was run at 100 V for 15 min. Subsequently, the agarose gels were analyzed with a GelDoc Bio-Rad gel documentation system.

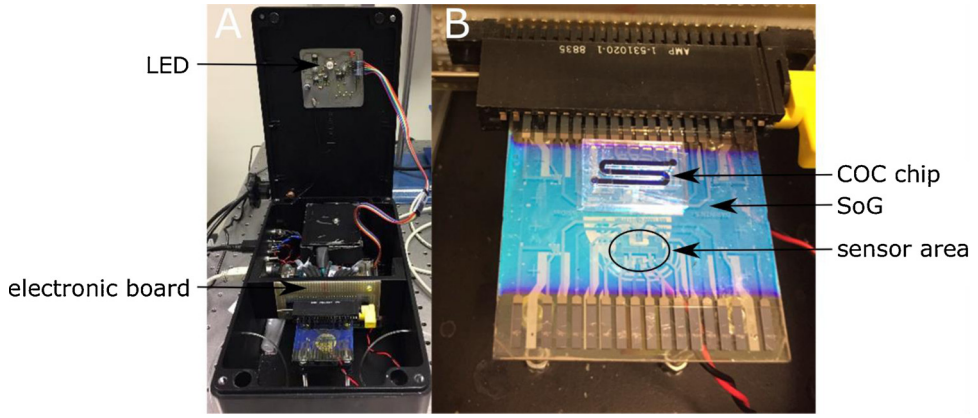

Fig. 3. Image of the lab-on-a-chip system for real-time monitoring of the multiple displacement reaction. The cyclic olefin copolymer chip was filled with blue food dye to visualize the channel geometry.

2.6. On-chip MDA procedure

2.6.1. Calf thymus DNA amplification

The SoG was connected to a custom-made electronic control and readout board in order to control the temperature of the heaters and to acquire the photocurrent [30]. These components were placed inside a metallic box (26 cm x 16 cm x 9 cm) to prevent electromagnetic interference. Home-made software was used to monitor the photocurrent in time, with a time interval of 200 ms. The excitation source was a PG2C1-450 light emitting diode (LED) (450 nm, full width at half maximum (FWHM) 35 nm) obtained from Roithner Lasertechnik GmbH. A commercial band-pass filter (450 nm, FWHM 25 nm) purchased from Corion Corporation was placed between the LED and the COC chip.

On-chip MDA was carried out by pipetting 55 µL of the pre-mixed MDA mixture, in the same ratio as for the off-chip experiments, into the COC chip. Also NTC runs were carried out. After sealing the in- and outlet (Microseal `B' Adhesive Seals, Bio-Rad), the chip was manually aligned with the SoG in such a way that the sensors were located underneath the microfluidic channel.

Of each mixture a drop of the on-chip amplified sample was spotted on a glass petri dish and analyzed with a microscope using an exposure time of 250 ms. The fluorescence images were taken with an inverted phase contrast fluorescence microscope (Leica DMIL) with a 10 X objective (100 X total magnification), equipped with a 100 W mercury vapor lamp and a TRITC filter set. The microscope was coupled to a high resolution charge coupled device (CCD) photocamera (Jenoptik and Zeiss AxioCam ICc3) operated by Zen 2011 software.

2.6.2. cDNA amplification

To further evidence the functionality of the device for on-chip real-time amplification monitoring, cDNA was amplified. This cDNA was obtained by extracting RNA (TRIzol RNA Isolation Reagents from Thermo Fisher) from K562 bone marrow cells from Homo Sapiens following the protocol given by the manufacturer (ATCC® CCL-243™). The concentration of the extracted RNA was 5 ng/µL, as was verified by the NanoDrop.

2.7. DNA quantification

The off-chip and on-chip samples with a dye concentration of 1.33 µM and the various initial DNA concentrations (0.01–10 ng/µL) were quantified with a Qubit fluorometric quantification system (Thermo Fisher) in combination with the dsDNA HS kit (Thermo Fisher), following the protocol of the manufacturer.

3. Results and discussion

3.1. SoG characterization

The SoG reported in this work is based on a previously reported article [25] with an additional interference filter [23,29]. The filter is designed in such a way that the wavelengths centered around the excitation peak of 450 nm are removed, while the emitted radiation is transmitted without attenuation. The filter transmittance is around 90% for wavelengths above 550 nm. Due to these characteristics the excitation wavelength is prohibited from reaching the photosensors, while the fluorescent radiation is transmitted to these sensors. Details of the filter characteristics are given in the supplementary material, as well as more details about the characterization of the SoG. Details of the characterization of the heater and sensors are published previously [13,14,25] and summarized in the supplementary material.

3.2. Dye characterization

The absorption spectra of the free $[Ru(phen)_2(dppz)]^{2+}$ as well as of the $[Ru(phen)_2(dppz)]^{2+}$/DNA complex showed a maximum at 440 nm, using 6.65 µM of $[Ru(phen)_2(dppz)]^{2+}$ and 10 µg of DNA. Setting the emission wavelength at 610 nm, the excitation maximum of the complex occurred at 400 nm, while the free dye did not show an excitation peak. Both the free dye and the complex have an emission peak at 510 nm upon excitation at 440 nm. Upon binding of the $[Ru(phen)_2(dppz)]^{2+}$ to the DNA, an additional peak was observed at 620 nm. These results were in accordance with those previously reported [20,21]. $[Ru(phen)_2(dppz)]^{2+}$ was selected as dye since it has a large Stokes shift, which enhances the filter performance.

The concentration of $[Ru(phen)_2(dppz)]^{2+}$ for real-time monitoring of MDA was optimized by performing an off-chip experiment with a thermocycler. The amplified samples were analyzed by gel electrophoresis (Figure SM3 of the supplementary material). The analysis showed that the concentration of $[Ru(phen)_2(dppz)]^{2+}$ has an influence on the yield of amplification. Amplification was observed for samples amplified using 1.33 µM $[Ru(phen)_2(dppz)]^{2+}$ in combination with an input of 10 ng/µL, 1 ng/µL and 0.1 ng/µL of calf thymus DNA. Higher concentrations, as well as lower concentrations of $[Ru(phen)_2(dppz)]^{2+}$ displayed some or complete inhibition. Therefore 1.33 µM $[Ru(phen)_2(dppz)]^{2+}$ has been used for the on-chip experiments.

3.3. On-chip real-time monitoring

3.3.1. Calf thymus DNA amplification

Real-time monitoring of the MDA reaction was carried out for a total run time of 1 h, as can be seen in Fig. 4A. Each curve represents the

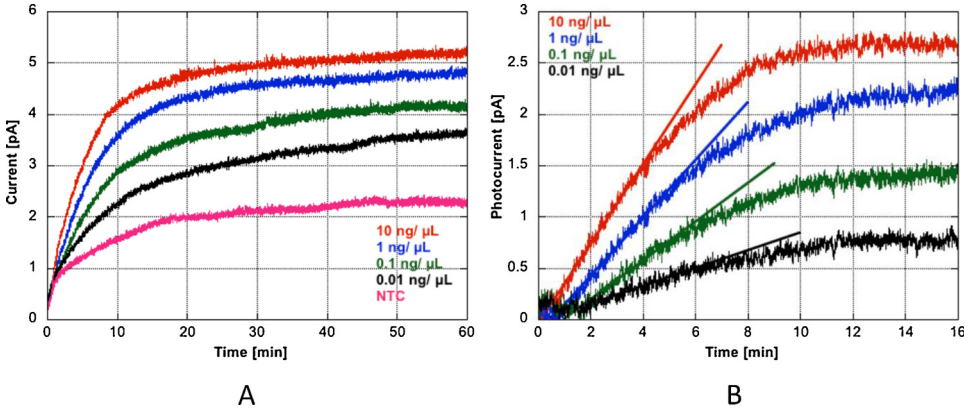

Fig. 4. On-chip real-time monitoring of the MDA reaction (amplification curves) showing the increase of the photocurrent as function of time for A) 0.01–10 ng/µL of input DNA and the NTC and B) a zoom-in after NTC subtraction. Each curve is an average of three measurements with a standard deviation below 10%.

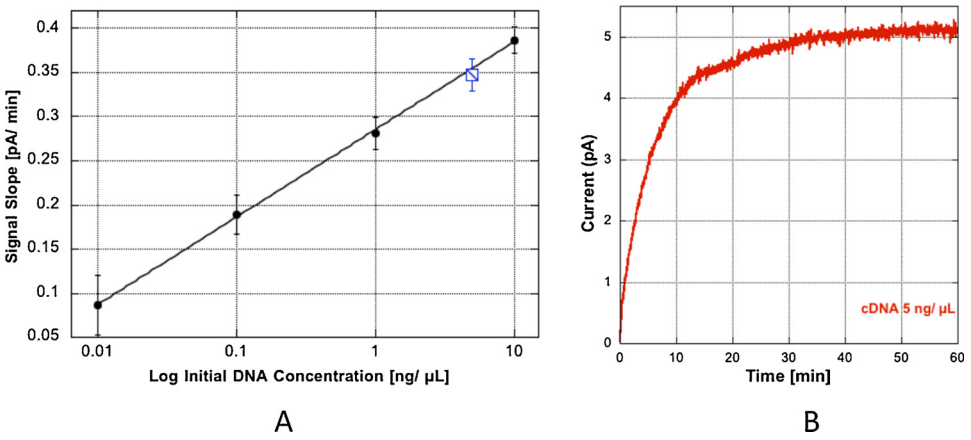

Fig. 5. A) DNA calibration curve: the slopes of the linear trend lines as a function of the initial DNA concentration (the error bars are an average of three measurements reported as standard deviation, error was below 10%) and B) on-chip real-time monitoring of the MDA reaction (amplification curve) for cDNA with an initial concentration of 5 ng/ µL (the curve is an average of three measurements with a standard deviation below 10%).

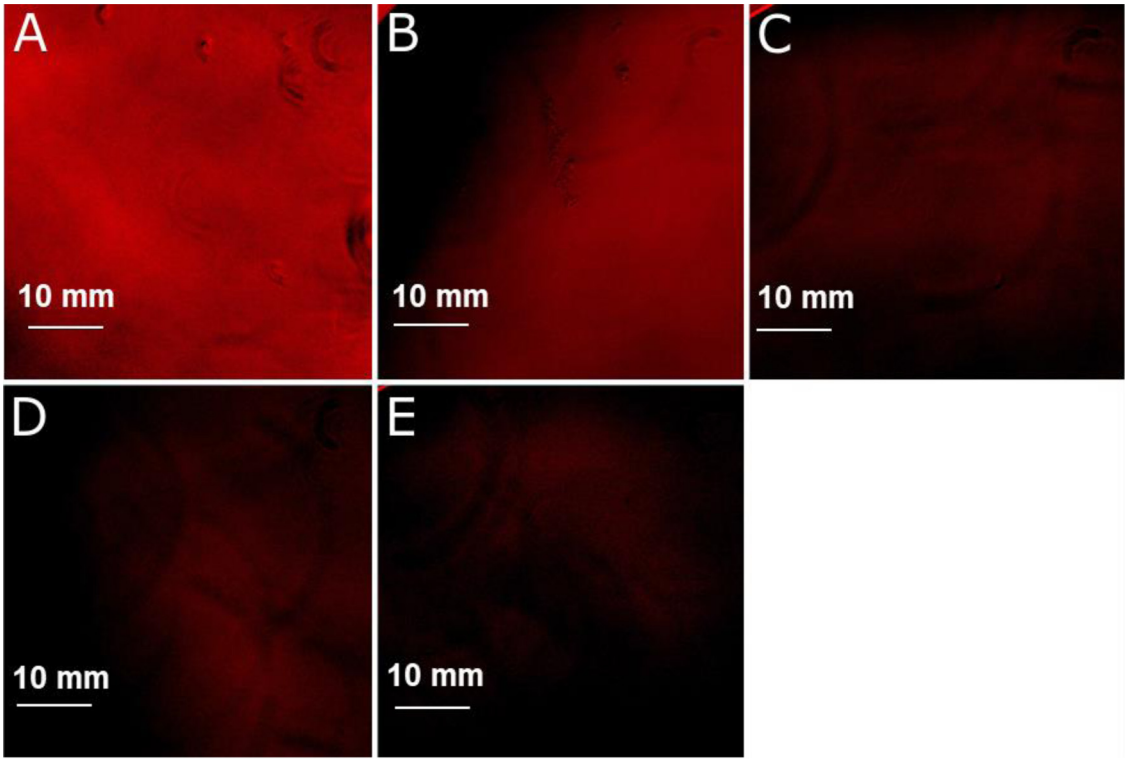

Fig. 6. Fluorescent images of samples with an initial DNA concentration of A) 10 ng/µL, B) 1 ng/µL, C) 0.1 ng/µL, D) 0.01 ng/µL and E) the NTC.

Table 1
DNA concentrations measured after the on- and off-chip experiments with a run time of 1 h.

DNA concentration (ng/ µL)	Off-chip amplification (ng/µL)	On-chip amplification (ng/µL)
NTC	Too low	0.9
0.01	Too low	7.8
0.1	Too low	28.1
1	38.3	85.5
10	315.0	268.0

photocurrent signal acquired during the amplification reaction for various initial DNA concentrations (0.01–10 ng/µL). For all the initial DNA concentrations an increase of the photocurrent was measured and within 60 min the plateau phase was reached and the photocurrent signal measured after this run time depends on the initial DNA concentration. A rapid increase in the first 10 min was observed, which is due to the increasing fluorescent signal derived from the DNA

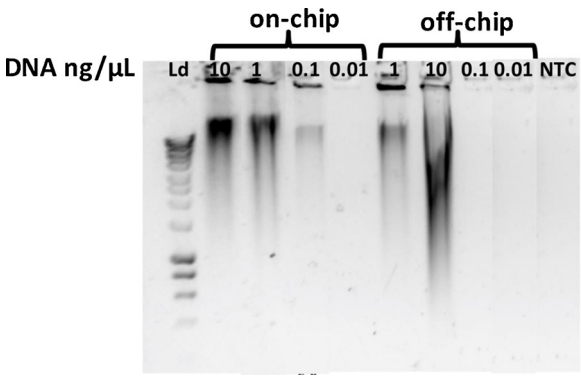

Fig. 7. Gel-electrophoresis of MDA amplification products performed using off-chip and on-chip procedures (Ld = ladder).

amplification and due to an increase of the chip temperature from room temperature to the MDA working temperature. In order to remove the signal increase originating from the heating of the chip, the signal of the NTC has been subtracted from the other curves, as shown in Fig. 4B.

Subtracting the NTC from the amplification curves gives the photocurrent increase solely due to DNA amplification. Fig. 4B shows that at the beginning of the amplification there is a linear increase of the photocurrent. A linear fit can be made, as shown in Fig. 4B. The slopes of these trend lines (for a time length of 4 min) as a function of the initial DNA concentration can be used as a calibration curve, which is presented in Fig. 5A.

Subsequently, the samples were analyzed by fluorescence microscopy (Fig. 6). The analysis displays that the intensity of the fluorescent signal increases for higher initial concentrations of dsDNA, which is in agreement with the data obtained with the LoC system.

3.3.2. cDNA amplification

In order to verify the applicability of the obtained calibration curve, also a sample with cDNA with an initial concentration of 5 ng/μL was amplified on-chip. The obtained amplification curve is plotted in Fig. 5B. The slope of the linear fitting line is determined and plotted in Fig. 5A as blue open square. This result confirms the reliability of the system for on-chip real-time monitoring and validity of the calibration curve.

3.4. DNA quantification

To compare the on- and off-chip results the DNA quantity was measured after 1 h of amplification. The data, given in Table 1, showed that the on-chip DNA amplification resulted in higher end concentrations for most of the initial concentrations of DNA upon comparison with the off-chip experiments. Presumably amplification in a chip is more efficient and/or faster than the off-chip reaction.

In order to study the differences in amplification yield between the on- and off-chip amplification experiments, a gel with both samples was run under identical experimental conditions and analyzed. Whereas for off-chip experiments the sample with an initial concentration of 0.1 ng/μL did not show amplification, the on-chip experiment did show a successful MDA reaction (Fig. 7). For an initial concentration of 1 and 10 ng/μL both on- and off-chip experiments showed a long smear in the gel, as is known for successful amplification with a high yield [1]. This data shows that the results obtained with gel electrophoresis are in agreement with data obtained with the Qubit.

4. Conclusions

With the integrated heater, sensors and interference filter on one glass plate in combination with a COC chip, the MDA reaction of dsDNA as well as cDNA could be monitored real-time by the use of the fluorophore $[Ru(phen)_2(dppz)]^{2+}$. The functionality of this LoC system, i.e. a SoG in combination with a microfluidic chip, was confirmed by fluorescent microscopy and gel electrophoresis. Qubit results showed that the on-chip amplification reactions resulted in higher DNA amplification yields in comparison with the off-chip reactions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2019.04.144.

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