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A Microfluidic Approach for Biosensing DNA within Forensics

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Abstract: Reducing the risk of (cross-)contamination, improving the chain of custody, providing fast analysis times and options of direct analysis at crime scenes: these requirements within forensic DNA analysis can be met upon using microfluidic devices. To become generally applied in forensics, the most important requirements for microfluidic devices are: analysis time, method of DNA detection and biocompatibility of used materials. In this work an overview is provided about biosensing of DNA, by DNA profiling via standard short tandem repeat (STR) analysis or by next generation sequencing. The material of which a forensic microfluidic device is made is crucial: it should for example not inhibit DNA amplification and its thermal conductivity and optical transparency should be suitable for achieving fast analysis. The characteristics of three materials frequently used materials, i.e., glass, silicon and PDMS, are given, in addition to a promising alternative, viz. cyclic olefin copolymer (COC). New experimental findings are presented about the biocompatibility of COC and the use of COC chips for multiple displacement amplification and real-time monitoring of DNA amplification.

Keywords: biosensing; DNA analysis; forensics

1. Introduction

Sampling and securing traces at a crime scene is a crucial step in the police investigation process. Information obtained at this stage immediately gives direction to the investigation. Nowadays it takes weeks to get from collection of the evidence to a report provided by a forensic laboratory. By using microfluidic devices, also called chips, for carrying out (part of) the analysis directly at the crime scene, valuable information can be available at a much earlier stage in the investigation process. Such microfluidic devices are portable, need minimal analyte volumes and bring quick analysis times. Since sample handling in microfluidic devices is done in a sealed microfluidic environment, chips also improve the chain of custody and lower the risk of (cross-)contamination. As such, (forensic) interest in DNA analysis in microfluidic devices has arisen, with a preference for single use (disposable) chips.

Several systems have been developed that integrate the complete process from sampling to result in a single microfluidic device. The focus for these systems is on sequencing [1] or DNA amplification, often with the detection on-chip, but without the integration of sample preparation [2,3]. Commercial systems are available, although their deployment is still limited. For instance the RapidHIT produces a full short tandem repeat (STR) profile for 5–7 samples simultaneously in 90 min [4]. More recently

the ANDE Rapid DNA system has been approved by the Federal Bureau of Investigation, but also with this machine it takes about 90 min from sample to result [5].

Having a fast analysis is of utmost importance at the scene of crime, for instance in the case of the identification of human remains or in sexual assault cases [6,7]. This is one of the motivations for the interest in microfluidic devices, which will be elaborated in the following section. Subsequently, two microfluidic DNA biosensing examples will be explored, namely DNA profiling by short tandem repeat (STR) analysis and by next generation sequencing (NGS). The biochemical compatibility of the materials used for microfluidic devices is an important requirement for forensic DNA analysis. Three commonly used materials (i.e., silicon, glass and PDMS) will be compared in terms of biocompatibility, thermal conductivity and optical transparency. The latter two characteristics play a role in fast amplification protocols and optical detection. An interesting alternative, cyclic olefin copolymer (COC), will be discussed on the basis of our own experimental findings, which demonstrates that this promising material can compete with reported materials for disposable forensic microfluidic devices. Finally, results regarding the use of COC microfluidic devices for multiple displacement amplification and on-chip real-time fast analysis of DNA amplification will be shown.

2. The Need for Speed

As published before by us [2], when performed at the forensic laboratory the conventional DNA analysis process might require days, as a consequence of which the outcome may have become less relevant for the initial phase of the criminal investigation as run by the police forces [8]. Such a time span may give a perpetrator the opportunity to remove relevant traces, to disappear or even to commit another crime [9]. The first hours of investigation are not without reason called the 'golden hours'. This motivates why there is a strong need for relevant information becoming available as quickly as possible [10]. Devices that supply police investigators at the crime scene with immediate information are especially valuable, since direct analysis assists fast and effective development of the case scenario.

Over the years devices for carrying out DNA analysis became more and more important as forensic evidence. In 1991, when the polymerase chain reaction (PCR) technique to amplify DNA was introduced within forensic research in the Netherlands, the minimal amount of DNA needed for a reliable result was 1 ng (corresponding to about 170 cells). Nowadays the amount necessary to obtain a complete and accurate STR profile is 100 pg. This means that even from a contact trace a reliable profile can be obtained, and only 1-10 cells are needed to obtain a complete DNA profile by using low copy number (LCN) PCR [11,12]. The DNA success rate highly depends on the DNA concentration; basically all traces with a concentration above 100 pg/µL result in DNA profiles that can be used for DNA database storage [13].

By the use of rapid identification technologies, like real-time analysis of DNA, investigators might identify a leading scenario early in the investigation process. This information can give direction to the further search for traces. Verification of a scenario can take place already at the crime scene and the scenario can be further reconstructed and adapted during the investigation process. De Gruijter et al. showed that receiving information concerning identification already at the crime scene helps to construct a more accurate scenario [14].

3. Biosensing of DNA

In this section various options regarding biosensing of DNA using the microfluidic approach. To generate DNA profiles STR analysis is the method of use will be described. Recently, with the introduction of sequencing techniques, also NGS methods are upcoming for forensic DNA profiling.

3.1. STR Analysis

STR analysis is the method within forensic science to generate DNA profiles. After amplification of the DNA, by PCR, the separation and detection of the amplified DNA must be carried out, which is usually accomplished by capillary electrophoresis (CE) [15].

Chips for PCR can be divided in two categories: well-based and continuous-flow chips [16]. In the first case, a well-based system (Figure 1A), the complete microdevice is sequentially cooled and heated. Drawback of a well-based system is the long time per cycle, caused by the relatively large total thermal mass of the system [16,17]. Continuous-flow chips can be further split up in three categories: fixed-loop, closed-loop and oscillatory chips. A fixed-loop microdevice (Figure 1B) contains zones with different temperatures through which the sample is moved. The numbers of meanders in the microdevice defines the number of thermal cycles. The time of each step is typically controlled by the length of the meander in a specific temperature zone in combination with the flow rate. In a closed-loop chip (Figure 1D) the circuit is fixed through which the sample is moved, while the number of thermal cycles can vary. Additionally, in an oscillatory microdevice the number of cycles can be varied (Figure 1C). The different zones of the microdevice are held at different temperatures and the sample is shunted back and forth between these different zones [16].

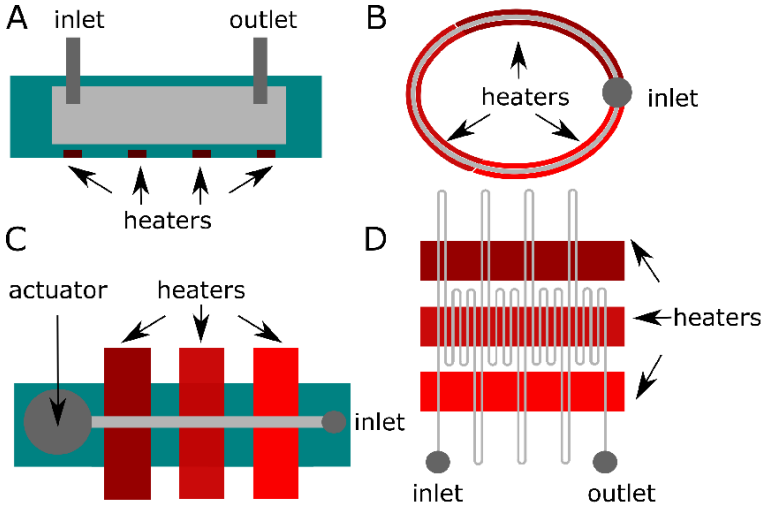

Figure 1. Examples of polymerase chain reaction (PCR) chip designs: (**A**) well-based (cross-section), (**B**) fixed-loop (top view), (**C**) oscillatory (top view) and (**D**) closed-loop systems (top view). The microfluidic amplification chamber (well-based) or channel (continous-flow) is given in grey. The temperature zones (continuous-flow) are given in the various shades of red.

In order to obtain a STR profile, in forensics there is significant interest in chips for CE to accomplish this [18]. By using such devices the required reagent and sample volumes are decreased, as well as the total analysis time [15]. Typical duration times of the separation are about 5–15 min [19–21].

3.2. Next Generation Sequencing

DNA sequencing is an essential technique within biotechnology, virology and medical diagnostics. In addition, for forensics these techniques have gained attention. In cases of a limited amount of DNA or highly degraded DNA the current NGS systems can help out.

The power of sequencing is that standard STR-profiles can be generated, but also DNA repeats can be sequenced to look for polymorphisms. To acquire information on ancestry, paternity or phenotype additional single-nucleotide polymorphisms (SNPs) can be analyzed with NGS techniques. However, the NGS workflow takes much longer than the conventional STR-profiling workflow (typically days versus hours).

There are several NGS machines on the market, such as the systems from Life Technologies, Illumina and PacBio. For forensic applications basically only the MiSeq/HiSeq (Illumina) and the Ion Torrent are used, with Nanopore as an upcoming sequencing system for forensics. A more elaborate review on NGS for forensics is given elsewhere [22].

3.2.1. MiSeq/HiSeq

Solexa, later on acquired by Illumina, made a sequencing system based on sequencing-by-synthesis in combination with reversible dye terminators and a planar solid glass support. The adapters are placed at the ends of the DNA sequence. [23–26]. Sequences complementary to the adapters 'A' and 'B' are present on the complete inside of these flow cell lanes [27]. Once the other end of the target DNA hybridizes to the complementary sequence present on the support, a bridge structure arises [24]. Each one of those bridge amplified clusters contains a unique DNA template, which is subsequently primed and sequenced [26]. There are different fluorescent labels present on each of the ddNTPs and a removable blocking group. The DNA sequence can be determined by completing the template one base at a time and recording of the fluorescent signal with a CCD camera. The HiSeq series, and later the MiSeq series, succeeded the Solexa Genome Analyzer [28].

3.2.2. Ion Torrent

The Ion Personal Genome Machine (PGM) was introduced by Ion Torrent at the end of 2010 [28,29]. This sequencing-by-synthesis method starts with emulsion PCR on beads. When a new nucleotide binds, upon pyrophosphate cleavage, a proton releases which is detected by monitoring the potential [28]. A well plate ensures that the release of these protons can be localized and retained. It is possible to sequence homopolymeric regions of the template DNA, since the signal is proportional to the amount of protons released. Data collection is done with a complementary metal-oxide semiconductor (CMOS) sensor array chip located at the bottom of the well plate. These chips can measure millions to billions of simultaneous sequencing reactions [30]. By measuring the potential, the system is faster, cheaper and accomplished with smaller instruments in comparison to systems that are based on fluorescence read-out [28].

3.2.3. Nanopore

A single DNA molecule can be read by the use of nanopores. This can be accomplished without the need for amplification or expensive fluorescent labels [31]. The detection principle of nanopore based systems depends on the ion current, which is generated when a charged molecule, such as a DNA molecule, passes through a nanoscale pore in a membrane resulting in a change of the ionic current. The four different bases produce four distinct current levels, which can be used for sequencing the DNA strand [31,32].

3.3. Conclusion

Flow cells, which are part of most NGS machines, have nanopatterned features in the microfluidic channels. These nanosized wells make it possible to obtain a high data density by patterned clustering of DNA fragments [33]. Microfluidic devices or flow cells can be used for STR analysis and for NGS, respectively. With NGS techniques more genetic information can be obtained besides the standard DNA profile, but the complete analysis process takes longer than standard STR analysis.

4. Biocompatibility of Materials for Forensic Microfluidic Devices

The material choice for (forensic) microfluidic chips is very critical. Each material has its own characteristics in the areas of, for instance, transparency, chemical resistance, thermal conductivity and certainly also biochemical compatibility, as shown in Table 1. Although more chip materials are available, such as poly(methyl methacrylate) (PMMA), polystyrene (PS), polyethylene terephthalate (PET) and polytetrafluoroethylene (PTFE). In this article only glass, silicon and the plastics PDMS and COC are considered, since these materials are mostly reported as chip materials for forensic microfluidic devices.

Table 1. Characteristics of different chip materials.

	Glass Borofloat	**Silicon With Layer of Native Oxide**	**PDMS Sylgard 184**	**COC Topas 6013**
Wettability Contact angle (H_2O)	Hydrophilic ≈ 35° [34]	Hydrophilic ≈ 57° [35]	Hydrophobic ≈ 105° [36]	Hydrophobic ≈ 88° [37–39]
Inhibitory (to amplification) Applied coating (to avoid inhibition)	Yes PEG 8000, PVP, EPDMA [47,48]	Yes SurfaSil, C_3H_9SiCl, $C_3H_7Cl_3Si$ [49,50]	Yes Parylene, BSA [40–44]	Yes BSA, PEG [45,46]
Transparancy Light transmission (350–750 nm)	Transparant ≈ 91% [51]	Opaque [17] 0%	Transparant ≈ 90% [52]	Transparant ≈ 91% [39,53]
Thermal conductivity W/m · K	Medium 1.2 (90 °C) [51,54]	High 156 (300 K) [55]	Low 0.27 (unknown K) [54,56]	Low 0.12–0.15 (20 °C) [53]
Chemical resistance Affected by	High HF, NaOH [51]	High KOH [57]	Low Chloroform, benzene, ethanol, acetone [58,59]	High Non-polar organic solvents [39,53]

By using microfluidic devices for the amplification reaction, instead of polypropylene tubes or well plates in combination with a conventional thermocycler, an enormous gain in time is achieved. Forensic chips are for single-use only, i.e., disposable, and therefore these chips can be fabricated of a cheap base-material, provided that it meets all requirements. However, material selection is not always simple and easy, because the material itself can disadvantageously influence (some of) the steps in the process of DNA analysis, such as PCR and detection.

Biochemical compatibility of materials for forensic chips is of great importance, since an interaction of the used chemicals with the walls of a microfluidic channel can result in slowing down of the reaction or even total inhibition. The high surface-to-volume ratio of microfluidic devices is a disadvantage in this case. However, such unwanted interaction between the chip material and used chemicals can be avoided by either passivation of the walls of the reaction chamber prior to use, called passive coating, or by so-called dynamic coating by adding components to the used chemicals, e.g., in the amplification mixture [60].

Besides biochemical compatibility, also the wettability of the chip material is of great importance. Microfluidic devices for droplet PCR are usually made of PDMS [61–66], since the interior of a PDMS channels is hydrophobic and aqueous droplets-in-oil are easier generated in channels of which the surface has this wetting state (compared to a hydrophilic state of the surface of a microfluidic channel). In case of water-in-oil droplets, the template DNA that is present in the aqueous phase cannot adsorb to the interior of the fluidic channel because of the hydrophobic property of the oil [67]. On the other hand, the PCR mixture, as well as other reaction mixtures used for DNA analysis (e.g., lysing agents), are aqueous solutions. By using hydrophilic channel walls it is easier to introduce these kind of solutions into the chip without bubble formation [17].

Since the fabrication of microfluidic channels in glass and silicon often requires cleanroom facilities, due to which the production costs for such chips are relatively high, chips made of these materials are not very suitable as disposables (i.e., for single use only) [17]. Plastic devices can be made using e.g., hot embossing, micromilling, casting or injection molding, for which a cleanroom environment is not necessary, which makes such devices cheaper. Due to their relatively low price plastic chips are (most) appropriate for single-use (i.e., disposable), which in particular circumvents cross-contamination issues [68,69].

Whereas plastic chips are attractive because of their bio(chemical)compatibility, another advantage of this material is its low thermal conductivity [70]. Materials with a low thermal conductivity are desirable for continuous-flow devices for PCR (which requires different temperature zones in the chip), for which reason plastic as well as glass are attractive [68]. As such, the majority of chips for analysis of biological fluids is fabricated from PDMS or PMMA [64,71–76], although glass and silicon are also occasionally utilized [77–79].

The next subsections contain detailed information about various aspects, such as transparency, thermal conductivity and bio(chemical)compatibility of glass, silicon, COC and PMDS as chip material for forensic microfluidic devices.

4.1. Glass

Due to its transparency in the visible range, which gives opportunities for optical detection, glass is widely used a material for (forensic) DNA analysis on-chip. PCR and CE can be easily integrated on-chip due to the beneficial electro-osmotic-flow/electrical characteristics of glass [17,80,81].

For glass surfaces it is reported that silanization of the surface is required to minimise surface adsorption of Taq polymerase. Giordano et al. investigated the use of dynamic coating (polyethylene glycol (PEG) 8000, polyvinylpyrrolidone (PVP) and hydroxyethylcellulose (HEC)) versus passive (or static) coating (epoxy (poly)dimethylacrylamide (EPDMA)) of the reaction chamber of a PCR glass chip. Covalent silanization results in 50–120% of product relative to the amount obtained with conventional PCR in polypropylene tubes. The use of HEC ends in total inhibition of the reaction, whereas the use of PEG 8000, PVP and EPDMA results in 13%, 78% and 72% of relative product amount,

respectively [48]. Other coatings that can be used to passivate the glass surface are SigmaCote [82] or dichlorodimethylsilane [83].

4.2. Silicon

Similar to glass, also silicon is frequently used as chip material, because of its high thermal conductivity. Due to the latter silicon is attractive for the fast heating and cooling as required for (well-based) PCR cycling [17]. Since silicon is a semiconductor, disadvantage of this material is that it cannot resist the high potentials needed for CE. Moreover, silicon is not transparent for the wavelengths of UV-Vis light, for which reason UV-Vis cannot be used for detection [70,84]. Based on a paper of Cho et al. [70], it is concluded that the majority of well-based PCR chips is made from silicon.

In fact, bare silicon shows inhibitory effects on the PCR reaction, which cannot be circumvented with the deposition of a silicon nitride layer. A silanizing agent followed by a polymer coating (e.g., polyglycine or polymaleimide) prevents inhibition, whereby SurfaSil shows better results than SigmaCote. An oxidised silicon surface can also prevent inhibition of the PCR reaction [49]. Felbel et al. investigated chlorotrimethylsilane, dichlorodimethylsilane, hexamethyldisilazane and trichloropropylsilane as silanization agents, which all prevent inhibition of the PCR reaction [50].

4.3. PDMS

PDMS is also reported as chip material for the DNA amplification reaction [4,85,86]. PDMS is stable over a temperature range of −50–200 °C [87]. Moreover, PDMS is biochemically compatible, easy to shape (by molding) and optically transparent [59,88]. PDMS is very suitable for cell culturing, since the permeability for gases, like O_2 and CO_2, is higher than for any other polymer [39]. However, PDMS suffers from severe bubble formation during the thermal cycling protocol, due to evaporation of the PCR mixture (through the PDMS) at temperatures above 90 °C [40,89]. PDMS is not very compatible with common solvents, such as chloroform, benzene, acetone and ethanol, since PDMS swells in these chemicals [58,59]. Furthermore, it is reported that surface treatments on PDMS are often unstable over time [39].

Although PDMS is used as coating for glass PCR chips [90,91], an important drawback of the use of uncoated PDMS is that, because of its permeability, it might result in inhibition of the amplification reaction due to absorption or adsorption of components in the amplification mixture (in particular the enzyme). In fact, this drawback can be bypassed by coating the PDMS with bovine serum albumin (BSA) [42–44]. To coat PDMS chips, Shin et al. and Prakash et al. used a parylene (dichlorodiparaxylylene) surface treatment [40,41]. PVP is also applied by Kim et al. to coat a PDMS/glass PCR chip [92].

4.4. COC

COC, nowadays frequently applied in disposables for medical diagnostics as well as for food packaging, is a promising and interesting material for chips [39,53,93]. COC is a copolymer based on linear and cyclic olefins. Since some manufacturers make COC from more than one type of monomer the 'copolymer' part is included in the name [39].

By means of a variety of techniques microfluidic networks can be realized in COC, such as injection molding, micromilling and hot embossing. The COC grade determines the exact heat deflection temperature, which lays in the range 70–170 °C [53,94]. Post-processing, two pieces of COC can be joint using thermal bonding, solvent bonding or gluing [39,93]. COC has a high rigidity and is optically transparent in the UV-range. Moreover, its water absorptivity is low (<0.01%) and it is electrically non-conductive. COC withstands a variety of chemicals (e.g., HCl, H_2SO_4, HNO_3, NaOH, EtOH and $(CH_3)_2CO$), and the material is only affected by some non-polar solvents (e.g., C_6H_{14} and C_7H_8) [39].

Plastic chips, either PMMA or COC, can be coated with a PEG solution [45]. A chip made from cyclic olefin polymer (COP) with a glass transition temperature of 138 °C is coated with BSA prior

to the PCR by Koh et al. [46]. Although BSA is widely used as surface treatment to prevent PCR inhibition, it might influence fluorescence detection, due to the possible interaction between BSA and the fluorescent dye or probe [17]. Panaro et al. tested the influence of dynamic coating on inhibition by adding 0.75% w/v PEG 8000 to the PCR mixture. For a large amount of plastics the addition of PEG 8000 improves the concentration of PCR product [47].

4.5. Conclusion

Silicon is a suitable material for well-based PCR systems, due to its good thermal conductivity. Glass or polymers, on the other hand, have a low thermal conductivity, making these chip materials suitable for continuous-flow PCR systems. Polymers, like COC, are also attractive for microfluidic devices for forensic DNA analysis due to their bio(chemical)compatibility, transparency and the the relatively wide variety of available fabrication techniques, which makes it possible to fabricate polymer-based chips at relatively low costs, making them suitable as disposables.

5. COC Chips for Multiple Displacement Amplification (MDA): Biocompatibility and Real-Time Analysis

As discussed in the previous section, COC is an attractive material for forensic micofluidic devices, since it has excellent bio(chemical)compatibility and chips of this material can be made at relatively low costs. This motivates our experimental investigation of COC for multiple displacement amplification (MDA) in microfluidic devices. It is noted that parts of the presented work are reported elsewhere as well by us [95–97].

Uniform amplification of small amounts of DNA is important for single-cell genomics, sequencing and forensic science. Due to amplification bias, the isothermal amplification method MDA is known for uneven representation of the template. Besides a reduction in the amount of reagents and sample, lowering the volume of the amplification reaction contributes to a more uniform amplification.

The compatibility of COC with the MDA amplification reaction is verified by performing an on-chip reaction. Two different designs of COC chips are investigated and chips with and without BSA treatment are used to investigate the need for a coating. In SolidWorks both designs are made. Design I contains a serpentine-shaped channel (width as well as depth 1.0 mm) with a volume of ca. 55 µL (Figure 2A), whereas design II comprises a straight channel (depth 0.5 mm, width 1.0 mm) with an internal volume of ca. 5 µL (Figure 2B). In the vicinity of the reaction channel a channel is present for insertion of a thermocouple that is used for temperature measurement and control. Both microfluidic layouts are milled in 2 mm thick (design I) or 1 mm thick (design II) COC plates (DENZ Bio-Medical grade 6013) using a Sherline 5410 Deluxe Mill with a 1.0 mm diameter mill, followed by drilling of fluidic accesses with a 1.5 mm diameter drill into which standard pipette tips can be mounted.

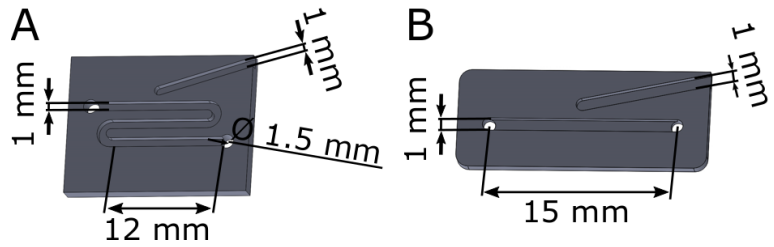

Figure 2. The schematic representation of the chips with the channel dimensions of (**A**) design I and (**B**) design II.

Post to milling and drilling, the COC chips are extensively rinsed with MilliQ water and ultrasonication (15 min) in ethanol, followed by nitrogen gas blow-drying. In order to recover the optical transparency, the surface roughness of the milled structures is lowered by following the procedure of Ogilvie et al. [98]: the chips are exposed (1 min) to cyclohexane vapor (60 °C).

Subsequently, the channel of each COC-chip is sealed by applying a piece of PCR foil (Microseal 'B' Adhesive Seals, Bio-Rad), which is a robust seal that is appropriate for biological applications [99].

Prior to performing the MDA reaction, some chips of design I are coated with BSA (1% (10 µg/µL)). The MDA reactions (GenomiPhi V2 kit from GE Healthcare) are carried out in the COC chips that are incubated in a water bath of 32 °C for 2h. The manufacturer of the MDA kit states that this kit is optimized for whole genome amplification from at least 10 ng [100]. Next to that an input concentration of 10 ng/µL is used, since off-chip results showed an enormous increase in the DNA concentration within 2 h [97]. It is noted that in case of design I 10 µL of the MDA mix (reaction buffer, sample buffer, enzyme mix, DNA and MilliQ in a ratio of 9:5:1:1:4, respectively) is injected in each chip, whereas the injected volume is 5 µL in case of design II. Post to loading of the indicated volume of MDA mixture, the fluidic accesses of the chips are closed with PCR foil. In addition, 10 µL MDA mixture is pipetted into a 500 µL Eppendorf polypropylene (PP) vial as a control. The concentration of double-stranded DNA (dsDNA) is determined with the Qubit™ dsDNA HS Assay Kit and the fluorescence is measured using the Qubit™ 3.0 fluorometer (both from Thermo Fisher Scientific). All samples are measured in triplicate.

Table 2 summarizes the outcome of the DNA quantification of the experiments with chip design I. As can be seen, the concentration of DNA in uncoated chips is well above 200 ng/µL (after a 2 h run), which is in agreement with the control vials, runs in a thermocycler (see [95,97] for more details) and literature [100–102]. Remarkable is that use of a BSA coating in the COC microfluidic channels clearly negatively affects the MDA reaction, in fact it results in inhibition, whereas such coating is known as method to prevent non-specific binding to COC surfaces [46,103–105].

Table 2. The concentration of double-stranded DNA (dsDNA) obtained after 2 h of amplification in the Eppendorf vials and chips of design I with and without BSA coating.

	Run 1 (ng/µL)	Run 2 (ng/µL)	Run 3 (ng/µL)	Run 4 (ng/µL)	Average [a] (ng/µL)
Eppendorf vial	204 ± 7	218 ± 8	265 ± 13	200 ± 16	222 ± 30
Chip without BSA coating	118 ± 6	209 ± 6	295 ± 13	233 ± 20	214 ± 73
Chip with BSA coating	79 ± 3	[b]	86 ± 2	62 ± 3	76 ± 12

[a] The standard deviation was taken from the average values of the four runs. [b] The seal of the chip was not secured properly and came off, hence no quantification measurement could be carried out.

The adsorption of polymerase on the channel walls can be prevented by the addition of BSA to the amplification mix or by coating the channel walls with BSA [106–108]. Erill et al. suggest to use a concentration of 2.5 µg/µL (0.25%) [107]. Taylor et al. state that high BSA concentrations (>0.15%) result in a lower yield compared to absence of BSA in the amplification mixture and that increasing the BSA concentration will finally result in total inhibition of the PCR reaction. The optimum BSA concentration for their silicon chip is found to be to be 0.05% resulting in a 2-fold increase in yield [108]. Kodzius et al. conclude that 2 µg/µL (0.2%) of BSA does not have any negative influence on the PCR reaction [106]. Kreader et al. recommend to use 0.2–0.4 µg/µL (0.02–0.04%) to relieve the inhibition of humic acids in PCR [109]. Qin et al. studied the optimal pH and BSA concentration to coat their Norland Optical Adhesive (NOA) chip. They report that the absorption capacity of the BSA to the NOA surface does not increase any further after 1 h. An acidic environment (pH 4) of the BSA solution shows a higher PCR efficiency than neutral pH. A BSA concentration of 5 µg/µL (0.5%) gives the best results. Using a lower concentration might not be sufficient, while a higher concentration can lead to PCR inhibition [110]. According to Zhang et al. [17], another reason for obtaining a lower DNA concentration upon using a BSA coating could be that salts present in the amplification mixtures (e.g., KCl, $MgCl_2$, $MgSO_4$ or NaOH) negatively affect the coating.

By using X-ray photo electron spectroscopy and contact angle measurements Sweryda-Krawiec et al. determined the mode of surface passivation by BSA. On a hydrophilic substrate BSA surface passivation shows two modes: it covers the surface and it can interact with

an initially deposited layer for further adsorption. Surface passivation of a hydrophobic surface is reached in one step. The measured contact angle is the same after completion of the adsorption reaction [111]. Additionally, the BSA surface coverage is at maximum 53% for a complete monolayer on hydrophobic surfaces, while on hydrophilic surfaces an almost full monolayer can be present [112]. COC is hydrophobic, probably resulting in an incomplete monolayer of BSA.

In this research 10 µg/µL (1%) is used, which is higher than recommended concentrations [106,108,109], which probably causes (partial) inhibition of the MDA reaction. Additionally, the chip is coated with BSA (passive coating/static passivation) instead of adding the BSA solution to the amplification mixture (active coating/dynamic passivation), as a consequence of which the BSA can compete with the polymerase for adsorption, leading to a lower amplification yield.

The amplification yield of chip design I is compared with that of design II (Table 3), without the use of any coating. The on-chip amplification with design I shows similar results as shown in Table 2 and the control run in the Eppendorf vial (215 ng/µL). However, the chips with the smaller internal volume, design II, shows a lower amplification yield in comparison with design I.

Table 3. The concentration of dsDNA obtained after 2 h of amplification with chips of both designs, without BSA coating.

	Run 1 (ng/µL)	Run 2 (ng/µL)	Run 3 (ng/µL)	Run 4 (ng/µL)	Average [a] (ng/µL)
Chip design I	201 ± 4	262 ± 5	231 ± 35	215 ± 6	228 ± 26
Chip design II	207 ± 4	181 ± 0	185 ± 5	141 ± 4	179 ± 28

[a] The standard deviation was taken from the average values of the four runs.

Although lowering the amplification volume gives better amplification in terms of coverage, bias and specificity [113–117], the yield is not necessarily higher as shown in these experiments. The interaction of the polymerase with the channel walls increases upon a higher surface-to-volume ratio [106]. Chip design I and chip design II have a 6:1 and 4:1 surface-to-volume ratio, respectively. This 1.5 times larger surface-to-volume ratio of design II is assumed to be the reason for the lower amplification yield of the chips with design II.

In conclusion, for COC chips with a sufficiently low surface-to-volume ratio (4:1 or lower) there is no need for (BSA) coating. In case BSA coating is required to make the chip material bio(chemical)compatible the concentration of BSA has to be dosed to an optimum value.

In fact, the presented COC chips can be used for fast analysis. More specific, design I can be used for real-time monitoring of DNA amplification with the MDA reaction down to an input concentration of 0.01 ng/µL. In COC chips of design I, the MDA reaction is performed with different DNA concentrations. All required measurement and control functionality, i.e., temperature sensors and photosensors based on amorphous silicon, thin film metallic heaters as well as an interference filter, for monitoring the reaction in real-time (based on measuring the fluorescence intensity) are realized onto a so-called system-on-glass. The COC chip is positioned on top of this system-on-glass, making the setup a compact transportable system, with the potential to be used at a crime scene. The experimental data indicate that upon using on-chip real-time detection the presence of DNA in a sample can be observed within approximately 10 minutes [96], i.e., one only has to wait until the signal exceeds the threshold [118].

6. Conclusions

Microfluidic devices, by having a closed microfluidic network, improve the chain of custody, reduce the risk of (cross-)contamination and offer fast analysis times, making them very suitable to be used directly at the crime scene. By choosing the correct chip material such microfluidic devices offer the required optical transparency, biocompatibility and thermal conductivity. COC is a bio(chemical)compatible material for which no coating of the microfluidic channel walls is required (provided that fluidic network has a sufficiently low surface-to-volume ratio) and in which microfluidic

channels can be made at relatively low costs, making them very suitable for single use only for forensic applications like DNA analysis.

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Abbreviations

The following abbreviations are used in this manuscript:

BSA	bovine serum albumin
CCD	charge coupled device
CE	capillary electrophoresis
CMOS	complementary metal-oxide semiconductor
COC	cyclic olefin copolymer
COP	cyclic olefin polymer
dsDNA	double-stranded DNA
DNA	deoxyribonucleic acid
EPDMA	epoxy (poly)dimethylacrylamide
HEC	hydroxyethylcellulose
LCN	low copy number
MDA	multiple displacement amplification
NFI	Netherlands Forensic Institute
NGS	next generation sequencing
NOA	Norland Optical Adhesive
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane
PEG	polyethylene glycol
PET	polyethylene terephthalate
PMMA	poly(methyl methacrylate)
PP	polypropylene
PTFE	polytetrafluoroethylene
PS	polystyrene
PVP	polyvinylpyrrolidone
SNP	single-nucleotide polymorphism
STR	short tandem repeat

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