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Chemical Engineering Journal 101 (2004) 333–338

Chemical Engineering Journal

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Investigations on the compatibility of chemically oxidized silicon (SiO_x)-surfaces for applications towards chip-based polymerase chain reaction

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Received 30 July 2003; accepted 28 October 2003

Abstract

Several designs of micro-fabricated PCR-chips made in silicon have been developed. Upon miniaturization the surface-to-volume ratio (SVR) increases and therefore, effects related to the non-specific adsorption of *Taq* DNA polymerase and template DNA to chip-surfaces become significant.

To repress the surface-mediated inhibition of these biological macro-molecules: (1) the PCR-compatibility of SiO_x-surfaces, (2) the combination of silanization and dynamic coating with BSA on SiO_x-surfaces on the PCR-efficiency, and (3) the stability of these surface-passivating films during PCR were investigated off-chip. (4) Finally, on-chip PCR-experiments were carried out under optimized reaction conditions.

$(CH_3)_3SiCl$, $(CH_2)_2SiCl_2$, $[(CH_3)_3Si_2]NH$ and $CH_3(CH_2)_2SiCl_3$ were used to passivate SiO_x-surfaces. The PCR was performed according to published procedures and the yield of the PCR-products was determined by gel electrophoresis analysis. To follow the degradation of the surface-passivating films contact angles were measured. It could be demonstrated that: (1) SiO_x-surfaces were an inhibitor of the PCR; (2) the PCR-efficiency of silanized SiO_x-surfaces was in the order: $CH_3(CH_2)_2SiCl_3 \approx (CH_3)_2SiCl_2 > CH_3SiCl_3 > [(CH_3)_3Si_2]NH$ and the amount of PCR-products was reduced from run to run; (3) the stability of the surface-passivating films resembled the same trend and none of these surfaces were stable for more than three consecutive PCR runs; (4) the specificity and product yield of the on-chip PCR was found to be equivalent to a conventional one, using a $(CH_3)_2SiCl_2$-modified PCR-chip with a power consumption of 2.7 W, heating (cooling) rates of up to 50 K s^{-1} (4) and reaction volumes in the range of 1–4 μl.

Based on this test configuration the silanization of SiO_x-surfaces alone will not be suited for multiple or long-term applications due to the degradation of the surface-passivating films.

Keywords: Miniaturized PCR; PCR-chip; Lab-on-a-chip; Surface-compatibility

1. Introduction

The invention of the PCR had a major impact in molecular biology [1]. The PCR is an enzyme-catalyzed, temperature-controlled reaction, which amplifies minute traces of template DNA to detectable levels, and as such the use of the PCR within the context of sample pre-treatment is obvious [2].

The mechanistic simplicity of the PCR-process—repeated cycling between three different reaction temperatures—made it an ideal candidate for miniaturization during the last decade [3]. The potential merits of miniaturized PCR-chip devices compared with conventional thermocyclers are low power consumption, fast reaction time and reduced amount of sample and reagent.

Another important issue upon miniaturization is the domination of surface-related effects, especially the non-specific adsorption of biological macro-molecules like *Taq* DNA polymerase and template DNA to glass-, polymer-, native silicon-, SiO_x- and silicon nitride-surfaces, because of an increasing SVR.

In order to perform the PCR in a micro-environment special attention must therefore be paid to the condition of the internal surfaces, which are contacted with the PCR-reaction mixture. For this purpose mere dynamic coating of the corresponding surfaces with BSA, salmon sperm,

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doi:10.1016/j.cej.2003.10.016

glycerol, Tween® 20, etc. as PCR-buffer ingredients (covalent) modification with organic mono- and/or multi-layers or a combination thereof is utilized [4]. However, although the surface-treatment is key to an effective PCR, the amplification yields of chip-based PCR were often inconsistent and not always comparable with those of a conventional PCR.

Herein: (1) the PCR-compatibility of SiO_x-surfaces, (2) the combination of silanization and dynamic coating with BSA on SiO_x-surfaces on the PCR-efficiency, and (3) the stability of these surface-passivating films during PCR were investigated off-chip. (4) Finally, on-chip PCR-experiments were carried out under optimized reaction conditions.

2. Experimental

2.1. Materials

All chemicals (supplier) had reagent grade or better and were used without further purification. If not stated otherwise all chemicals were purchased from Merck. For aqueous solutions de-ionized water was purified by ultra-filtration (Millipore). Substrate surfaces were dried using a stream of compressed air or nitrogen. For silanization reactions toluene was distilled over sodium. Following silanes were used {formula}: chlorotrimethylsilane $\{(CH_3)_3SiCl\}$ (Sigma), dichlorodimethylsilane $\{(CH_3)_2SiCl_2\}$ (Pharmacia Biotech), hexamethyldisilazane $\{[(CH_3)_3Si_2]NH\}$ (Roth), and trichloropropylsilane $\{CH_3(CH_2)_2SiCl_3\}$ (Aldrich).

2.2. Equipment

For comparison off-chip PCR-reactions were carried out using a Mastercycler® gradient-thermocycler (Eppendorf) equipped with 200 μl polypropylene tubes (Roth). Detailed specifications of the PCR-chips used for the following experiments are given elsewhere [5]. For conventional PCR-reactions (testing the long-term stability of the surface coatings) reaction volumes of 25 μl (200) were used. Electrophoresis was performed in an EasyCast™-electrophoresis system (Biometra). The corresponding product bands were recorded using an E.A.S.Y.-gel documentation system (Herolab). Contact angle measurements were carried out with an OCA 20-contact angle meter (dataphysics).

3. Methods

3.1. Surface modification

First, a native 2 in. silicon⟨1 0 0⟩-wafer (VEB Spurenmetalle Freiberg) was cut into pieces of 0.5 mm × 0.5 mm, chemically activated in a 3:1 (v:v) mixture of sulfuric acid and 30% hydrogen peroxide for 1 h, vigorously washed with copious amounts of water and blown dry with nitrogen. The substrates were then immersed into a 10 mM solution of $(CH_3)_3SiCl$, $[(CH_3)_3Si_2]NH$, or $CH_3(CH_2)_2SiCl_3$ in toluene at room temperature for 3 h, rinsed successively with toluene, ethanol, acetone and water, blown dry with nitrogen, and finally dried at 100 °C for 1 h. The silanization with $(CH_3)_2SiCl_2$ followed the manufacturer's guideline. Substrates prepared according to this procedure showed the following contact angles with water: $(CH_3)_3SiCl$ 80°, $(CH_3)_2SiCl_2$ 98°, $[(CH_3)_3Si_2]NH$ 48°, and $CH_3(CH_2)_2SiCl_3$ 87°. Furthermore, to simulate a high SVR, silicon powder was modified the same way as outlined above. Both the PCR-compatibility of different surface coatings and their long-term stability were first investigated off-chip in the conventional thermocycler, thereby soaking one piece of silanized silicon or 5, 10, 15 and 20 mg of silanized silicon powder in the PCR-reaction mixture and PCR-buffer, respectively. Between consecutive experiments the silanized substrates were rinsed with water and blown dry with nitrogen. Immediately thereafter, the corresponding contact angles were determined.

3.2. PCR-protocol

For all PCR-reactions a *Taq* PCR Core Kit (Qiagen) was used. The template was a mutant of a gene encoding for glutaraldehyde-3-phosphate-dehydrogenase (GAPDH) and the length of the amplicon was 379 bp. The primer sequences (MWG Biotech) used were: primer GAPDH A1 (5′-CCAGTGAGCTTCCCGTTCAGC-3′) and primer GAPDH SO (5′-CCCATCACCATCTTCCAGGAGC-3′) [6]. The PCR-reaction mixture consisted of: 27 μl of water, 10 μl of Q-solution, 5 μl of 5× Qiagen PCR-buffer, 5 μl of 1.5 μM BSA, 1 μl of 10 mM dNTPs, 0.5 μl of 100 μM of GAPDH A1 and GAPDH SO, 0.5 μl of <0.5 μg/μl template DNA and 0.5 μl of 5 U/μl *Taq* DNA polymerase resulting in a total volume of 50 μl. For on-chip PCR-reactions 1–4 μl of this PCR-reaction mixture was used. Both the on- and off-chip PCR-reactions were run in parallel using aliquots of the same reaction mixture under identical reaction conditions. The reaction mixture was initially heated to 94 °C for 180 s and then amplified for 25 cycles: 94 °C for 20 s, 50 °C for 20 s and 68 °C for 40 s. A final extension was added at 68 °C for 180 s. After completion of the reaction the PCR-products were analyzed by gel electrophoresis. For slab gel electrophoresis a 3% agarose gel was prepared in 1× TAE buffer, pH 8.0, which was pre-stained with 1.25 μM ethidium bromide (Sigma) [7]. ØX174 RT DNA *Hinc* II (Advanced Biotechnologies) was used as size marker. The electrophoresis was performed at 110 V for 30 min.

4. Results and discussion

4.1. PCR-compatibility of silicon

Due to its superior thermal properties and outstanding position in micro-machining technologies silicon, besides

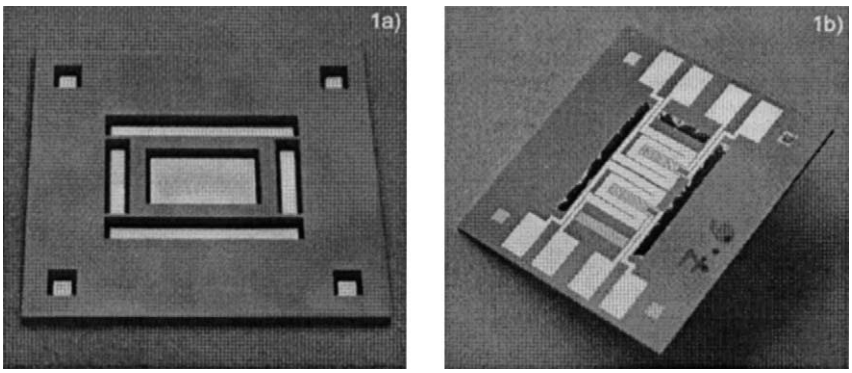

Fig. 1. (a) and (b) Front- and back-side view of a stationary-working, chamber-type chip-thermocycler. The reaction chamber is thermally isolated from the surrounding by incorporation of thermal gaps. The thermal management of the chip is accomplished by platinum-thin film transducers, whereby the periphery is contacted via aluminum-pads.

glass, is the material of choice for the construction of chip-based thermocyclers.

Upon miniaturization, the SVR increases: dependent from the reaction volume, 200 μl polypropylene PCR-tubes (PCR-chips used in this study) have a SVR in the range of 1–2 mm^{-1} (5–10) (Fig. 1a and b). Therefore, the non-specific adsorption and thus the potential inactivation of components of the PCR-reaction mixture, especially *Taq* DNA polymerase and DNA template, becomes a critical issue. Initial tests using SiO_x-piece as component of the PCR-reaction mixture revealed that there was some degree of inhibition of the PCR (Fig. 2a). This effect became more pronounced, if SiO_x-powder with its inherent large surface area was used instead: the higher the amount of SiO_x-powder added, the lower was the yield of the PCR-products (Fig. 2b).

This inhibition effect of SiO_x-surfaces was consistent with previous findings [8]. However, the addition of BSA in the PCR-reaction buffer was essential to get reliable results on bare silicon, whereby its concentration is dependent on the history of the silicon used, i.e., pre-treatment, source, impurities, etc. (data not shown).

4.2. Effect of silanization

Apart from higher concentrations of *Taq* DNA polymerase or template DNA in the PCR-reaction buffer, mere dynamic coating of the SiO_x-surface with PCR-buffer ingredients like BSA, salmon sperm, glycerol, etc., covalent modification with organic mono- or multi-layers was exploited to render the SiO_x-surfaces more PCR-compatible [4].

For this purpose SiO_x-surfaces were modified with silanes of the general formula R_nSiCl_{4-n}, whereby the silanes differed with respect to their number of chlorine-substituents. Experiments performed with silanized SiO_x-pieces as component of the PCR-reaction mixture showed a marked difference: the amount of PCR-products decreased with decreasing number of chlorine-substituents (Fig. 3). This trend was also observed in the case of silanized SiO_x-powder (data not shown). But, in all cases

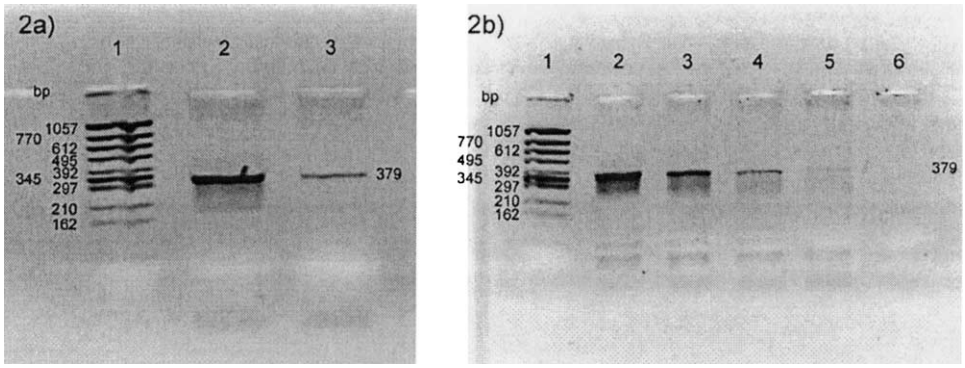

Fig. 2. (a) Gel electrophoresis analysis of PCR-products: inhibition effect caused by the addition of a 0.5 mm × 0.5 mm SiO_x-piece. Lane 1, marker; lane 2, reference control without SiO_x-piece; lane 3, addition of one SiO_x-piece. (b) Gel electrophoresis analysis of PCR-products: inhibition effect caused by the addition of SiO_x-powder. Lane 1, marker; lane 2, reference control without SiO_x-powder; lanes 3, 4, 5 and 6, addition of 5, 10, 15 and 20 mg of SiO_x-powder, respectively.

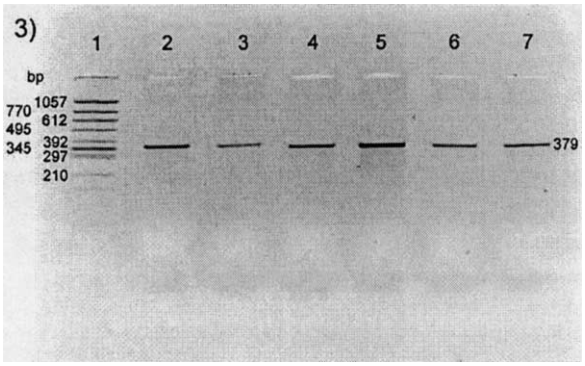

Fig. 3. Gel electrophoresis analysis of PCR-products: dependence on the PCR-efficiency by silanization of SiO_x-surfaces. Lane 1, marker; lane 2, reference control without SiO_x-piece; lane 3, SiO_x-piece without modification; lane 4, 5, 6 and 7, SiO_x-piece modified with $[(CH_3)_3Si_2]NH$, $(CH_3)_2SiCl_2$, $(CH_3)_3SiCl$ and $CH_3(CH_2)_2SiCl_3$, respectively.

the amount of PCR-products was reduced from run to run without regeneration of the surface-passivating films. Normally, silanization of SiO_x-surfaces was effective for a maximum of three consecutive PCR runs. These findings correlated with tests regarding the stability of the surface-passivating films, which was also in the order: $CH_3(CH_2)_2SiCl_3 \approx (CH_3)_2SiCl_2 > CH_3SiCl_3 > [(CH_3)_3Si_2]NH$. A speculation for this trend will be given in the next session.

4.3. Stability of the surface-passivating films

Modification of a hydrophilic SiO_x-surface with short-chain, aliphatic silanes generates a hydrophobic surface, whereby a covalent silyl-ether bond is formed. It is also known that the silyl-ether bond is base-labile [9], i.e., it is cleaved at elevated pH values, which will then again result in a hydrophilic surface. Therefore, contact angle measurements provide a good means to gain information about the current state of the surface during the course of the PCR.

To rule out effects correlated with the non-specific adsorption of ingredients of the PCR-reaction buffer, especially BSA, *Taq* DNA polymerase and template DNA, which also renders the silanized surface hydrophilic, experiments with silanized SiO_x-pieces were conducted with PCR-buffer and water. By increasing cycling times the contact angles of the surface-passivating films decreased with different rates in PCR-buffer, whereas in the water they remained unchanged (Fig. 4a and b).

Taking into account a pH value of up to 9.3 and an overpressure of up to 1 bar during denaturation [10], a decreasing contact angle with water means a forthcoming degradation of the silanized surface by hydrolysis of the silyl-ether bond. Thereby, the enhanced stability of $(CH_3)_2SiCl_2$- and $CH_3(CH_2)_2SiCl_3$-modified silicon-surfaces towards wet-chemical etching was attributed to the formation of

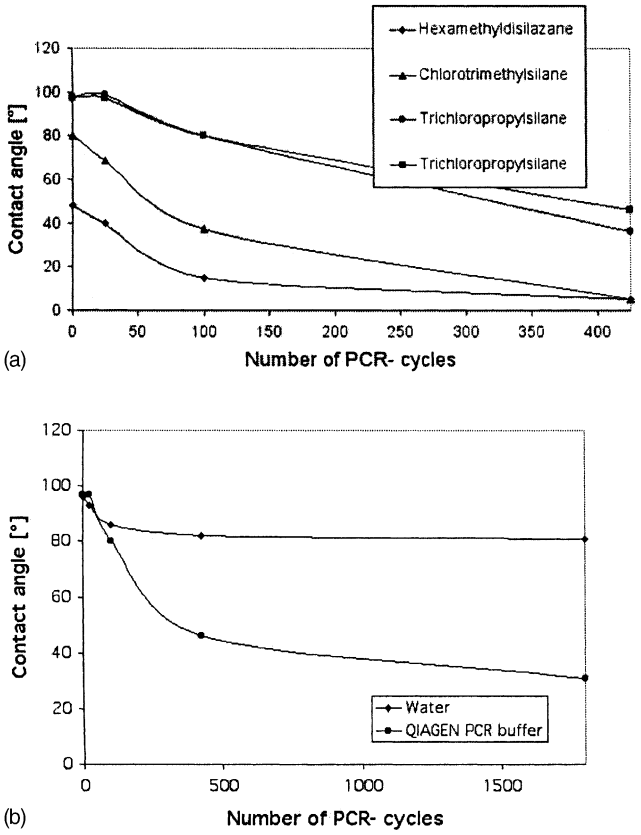

Fig. 4. (a) Contact angle of water at SiO_x-pieces modified with different silanes in dependence on the cycling time. (b) Contact angle of water at SiO_x-pieces modified with $(CH_3)_2SiCl_2$ in water and PCR-buffer in dependence on the cycling time.

multi-layers by cross polymerization of the additional chlorine-substituents.

4.4. On-chip PCR

In contrast to conventional PCR miniaturized PCR benefits from lower power consumption, a reduced amount of sample and reagents and shorter reaction times. Typically, the PCR-chip (conventional thermocycler) used herein had a power consumption of 2.7 W (1500), the PCR-reaction volume was 1–4 μl (25) and the total reaction time was 44 min (73), thereby achieving experimental heating and cooling rates of up to 50 (3) and $4\,K\,s^{-1}$ (1), respectively (Figs. 1 and 5a).

On-chip experiments using the optimized reaction conditions obtained so far demonstrated that the specificity and product yield was equivalent to those run in conventional polypropylene tubes (Fig. 5b). Similar to the results outlined above appropriate surface passivation and PCR mixture composition turned out to be the most critical factors for a successful on-chip PCR. However, to get reproducible results with this set-up the chip-surface had to be regenerated after every PCR run.

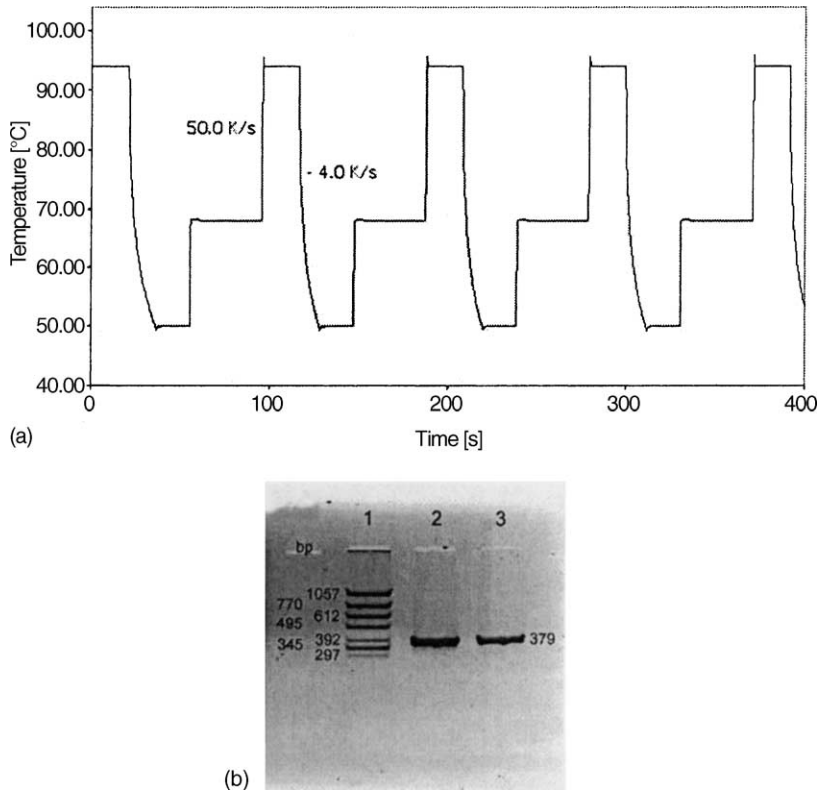

Fig. 5. (a) Heating and cooling rates of the chip-thermocycler used in a typical PCR-experiment. (b) Gel electrophoresis analysis of PCR-products: comparison of the specificity and product yield of the PCR in the conventional and chip-based thermocycler. Lane 1, marker; lane 2, conventional thermocycler; lane 3, PCR-chip.

5. Conclusions

(1) SiO_x-surfaces as component of the PCR-reaction mixture caused a distinct inhibition effect on the yield of the PCR, which could be quantified using different amounts of SiO_x-powder.
(2) Alkyl-terminated SiO_x-surfaces as component of the PCR-reaction mixture showed two tendencies: firstly, the amount of PCR-product decreased in the order of $CH_3(CH_2)_2SiCl_3 \cong (CH_3)_2SiCl_2 > (CH_3)_3SiCl > [(CH_3)_3Si_2]NH$ and secondly, in all cases the amount of PCR-product was reduced from run to run. The silanization of the SiO_x-surfaces was effective for a maximum of three consecutive PCR runs only.
(3) These findings resembled tests regarding the stability of the surface-passivating films, which was in the same order. Base- and pressure-mediated hydrolysis of the silyl-ether bond seemed to be responsible for the increasing degradation of the silanized SiO_x-surfaces.
(4) Experiments using a $(CH_3)_2SiCl_2$-modified PCR-chip in combination with dynamic coating with BSA demonstrated that the specificity and product yield of the PCR was equivalent compared to a conventional one. The reaction volume for this chip-based approach was 1–4 μl, whereby heating (cooling) rates of up to $50\,K\,s^{-1}$ (4) and a power consumption of 2.7 W were realized. However, with this set-up the chip-surface had to be regenerated after every PCR run to get reproducible results.

Acknowledgements

This work is part of a joint project with Advalytix AG and Biofrontera Pharmaceuticals AG and has been supported by the German Ministry of Education and Research under contract number 16SV1388. The authors thank their colleagues at the IPHT for technical support and helpful discussions, in particular H. Fischer, A. Ihring, M. Kielpinski, H. Porwol, M. Sossna, R. Stöpel and M. Urban.

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