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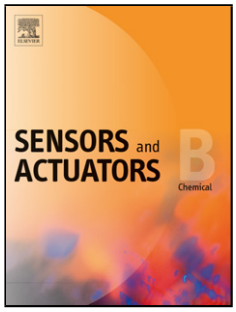

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PII: S0925-4005(12)01024-6
DOI: doi:10.1016/j.snb.2012.09.110
Reference: SNB 14633

To appear in: *Sensors and Actuators B*

Received date: 28-6-2012
Revised date: 21-9-2012
Accepted date: 30-9-2012

Please cite this article as: J. Besecker, K.A. Cornell, G. Hampikian, Dynamic passivation with BSA overcomes LTCC mediated inhibition of PCR, *Sensors and Actuators B: Chemical* (2010), doi:10.1016/j.snb.2012.09.110

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Dynamic passivation with BSA overcomes LTCC mediated inhibition of PCR

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Abstract

The increasing use of low temperature co-fired ceramic (LTCC) for the fabrication of biological microfluidic devices necessitates further research on LTCC biocompatibility. In this study we explore the inhibitory effect of DuPont's 951 LTCC on Polymerase Chain Reaction (PCR), and demonstrate a novel mechanism to increase biocompatibility between LTCC and PCR with the addition of a common passivation substance, bovine serum albumin (BSA). We show that DuPont's 951 LTCC binds negatively charged proteins including BSA and ovalbumin (OVA). This is a significant discovery as proteins (enzymes) are an essential component of most biological reactions, and a frequent addition to microfluidic devices. A proposed model for LTCC inhibition of PCR by enzyme adsorption is presented.

Keywords: LTCC, Polymerase chain reaction (PCR), Microfluidics, Bovine serum albumin (BSA), Taq Polymerase, Inhibition

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1. Introduction

During the last two decades there has been abundant research focused on the production of micro-total analysis systems (μTAS), commonly referred to as lab-on-a-chip. One of the main areas of research has been focused on the development of μTAS for genetic based testing. There are many applications for these mini genetic analyzers in clinical, forensic, and environmental testing, including bio-warfare agent detection. Some advantages to miniaturization of these systems include: faster processing time, use of less consumables, lower power consumption, and lighter weight (particularly for field applications) [1, 2].

A common component of genetic μTAS analyzers is polymerase chain reaction (PCR) [2-6], and for PCR to be practical on these micro-chips, the platform used for typical PCR reactions needs to be miniaturized (μPCR). Traditional stand-alone PCR systems are bulky, have relatively long transition times between temperatures, and require a substantial amount of energy, making them less than ideal for use in a μTAS system.

PCR was first described in 1986 by Dr. Kerry Mullis as a way to enzymatically amplify specific sections of DNA in vitro [7]. Today PCR is a well-established technique that has provided major advancements in research, diagnostic, and clinical procedures. There are relatively few components required for PCR: tris-based buffer, $MgCl_2$, free nucleotides (dNTP's), thermo-stable enzyme (i.e. Taq Polymerase), site-specific primers, and template DNA. The process works by repeatedly cycling the reaction components through three different temperatures: denaturation at 95˚, annealing at 55˚-65˚, and extension at 72˚ [8]. The temperature cycling is achieved by running the reactions through a thermocycler. After each cycle of PCR, the number of target DNA molecules is doubled. This exponential amplification allows tremendous magnification of small quantities of DNA in a short period of time.

Materials in use today for the fabrication of μPCR devices are glass, silicon, plastic, and to a lesser extent ceramic tape [1, 9-11]. The majority of work on μPCR devices is done with silicon [4, 11]. A major draw back to using silicon, glass, or plastic is the expense of manufacturing the devices.

For use on a μTAS platform μPCR devices need to be small, lightweight, durable and inexpensive [12]. A novel polymer, low temperature co-fired ceramic (LTCC), fulfills these requirements and shows great promise for the production of μPCR devices. LTCC is a ceramic material which in the green state (unfired) is composed of 45% glasses, such as PbO and SiO_2; 40% Alumina, Al_2O_3; and 15% organic binders. The material is supplied as a thin flexible "tape", which can be worked and stacked in layers to make various types of devices. Our lab is currently working on constructing a continuous flow μPCR device [13] out of low temperature ceramic tape (LTCC). It has good thermal properties, affords easy integration of electrical and mechanical components, and fabrication of the fully integrated three-dimensional devices is relatively simple and inexpensive [13-15]. Once fired, the devices are highly resistant to chemical and thermal degradation [13, 16]. Another advantage in using LTCC is that it possesses the same thermal expansion coefficient as the silver paste used for the circuits. This gives the potential for the device to be autoclaved.

Recently there have been reports of PCR devices made of LTCC [10, 17, 18]. However, it has been shown that not all LTCC is compatible with standard PCR

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reactions. When PCR is carried out in the presence of specific types of LTCC the reaction can be inhibited [14], which is also a problem found with silicon PCR devices [19-22]. This has been a major challenge in fabricating reliable μPCR devices from LTCC and silicon.

A material that inhibits PCR (or other types of enzymatic reactions) may do so in two possible ways: either an inhibitory substance is leaching from the solid surface into the reaction, or essential PCR reagents are being adsorbed to the surface of the material. It has been shown that the enzyme Taq polymerase adsorbs to bare silicon, which is the main cause of PCR inhibition by silicon [19-23]. Studies have also shown that serum proteins bind to certain ceramic substances such as synthetic hydroxyapatite, and Al_2O_3. The main serum proteins found to bind these ceramic substances are albumin, and alpha-1-anti-trypsin [24]. The degree of protein binding to a ceramic is dependent on multiple factors such as surface charge (zeta-potential), and hydrophobicity. Micro-pores on the surface of a ceramic have also been shown to increase the level of protein adsorption to calcium phosphate based ceramics [25]. Since proteins have a tendency to unfold when they bind to the surface of a material, the conformational changes result in loss of protein activity [26]. Therefore, it is important to consider the binding effects of vessel materials that are in contact with the protein components of any reaction.

There are two common passivation procedures used to overcome PCR inhibition in device materials: static and dynamic. Static passivation involves pre-treating the surface of the material with a substance that is compatible with a PCR reaction. Dynamic passivation involves including passivation reagents in the PCR reaction mixture to make the material compatible with the reaction. Common passivation substances in use today are polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), and bovine serum albumin (BSA), which are assumed to preferentially bind to the surface of the material, leaving essential PCR components free in the reaction mixture [1, 4, 19]. BSA is a PCR additive that is used when inhibitors may be present in the reaction [27-29] and is commonly used passivation reagent in μPCR devices [2, 4, 11, 30].

To date it has been shown that specific types of LTCC inhibit PCR reactions, although the mechanism of inhibition has not been described. The aim of this study is to characterize LTCC inhibition of PCR, and to demonstrate a reliable way to overcome this inhibition through dynamic passivation with BSA.

2. Materials and Methods

2.1 LTCC

All LTCC used in this experiment was DuPont's 951 LTCC Green Tape (Research Triangle Park, NC). The composition of this tape is a trade secret, however the disclosed components are: 45% glasses, such as PbO and SiO_2; 40% Alumina, Al_2O_3; and 15% organic binders (MSDS). Prior to being added to PCR reactions, fired LTCC was washed two times in nanopure water to remove any soluble surface impurities. Single layer LTCC 951 Green Tape was machine cut then fired at 350˚C for one hour, then the temperature was ramped up 5˚C per minute to 850˚C and the LTCC is fired overnight at this final temperature.

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2.2 PCR in the presence of LTCC

To establish the effect DuPont's 951 LTCC on PCR, reactions were carried out in 0.2ml thin walled PCR tubes (Fisher) with increasing surface area of LTCC. Thermocycling was achieved using an MJ Research Mini-Cycler (Bio-Rad). PCR reactions had a final volume of 25 μl and contained 1 unit of DNA Polymerase, 100mM Tris-HCl (pH 9.0 at 25ºC), 1.5mM $MgCl_2$, 50mM KCL, 0.2mM dNTP's, 100nM each forward and reverse primer, and template DNA. Positive and negative control reactions were run to ensure proper PCR conditions.

Once it was determined that DuPont's 951 LTCC inhibits PCR, subsequent PCR reactions contained inhibitory amounts of LTCC with dimensions of 3x3x0.263mm (approximate surface area= 21.156mm^2). Control PCR reactions were also run containing 3x3x0.263mm pieces of plastic that were cut from 0.2ml thin walled PCR tubes (Fisher) to ensure that volume exclusion in the reaction was not the cause of inhibition. To determine if Taq polymerase was a limiting reagent in PCR reactions containing LTCC, PCR reactions were performed as above with increasing amounts of Taq polymerase (Fisher Scientific). Dynamic passivation reactions were performed to establish if the common passivation substance BSA could help overcome inhibition of PCR by LTCC. PCR reactions had a final volume of 25 μl and contained 1 unit of DNA Polymerase (Fisher Scientific), 100mM Tris-HCl (pH 9.0 at 25ºC), 0.2mM dNTP's, varying concentrations of $MgCl_2$, 50mM KCL, 0.4 mg/ml BSA (Fisher Scientific) 100nM each forward and reverse primer, and template DNA. Positive and negative controls were run to ensure that PCR reactions were working properly, and that no spurious amplification was taking place. PCR reactions were visualized using 2.0% ethidium bromide stained agarose gel.

2.3 Fluorimetry

Fragments (3x3x0.263mm) of LTCC and plastic volumetric controls (cut from PCR tubes) were soaked for two hours in 25μl of 1.5 mg/ml fluorescein isothiocyanate (FITC) labeled bovine serum albumin (BSA) or water (substrate fluoresce controls). Labeled BSA contained 5.5 fluorescent units per BSA (FBSA). After soaking, the LTCC was rinsed three times in 100μl of 75mM Tris-HCl pH 8.6 (Sigma) to remove unbound proteins. The LTCC was then washed in 80ul of 100mM glycine (Sigma) pH 2.6 to remove any proteins that had bound to the surface of the LTCC. Each glycine wash was aspirated and pooled into groups of three, each group was added to a single well of a black 96 well plate for a total volume of 240 μl glycine per well. Each glycine well was neutralized with 60 μl of 1 M Tris pH 8.0, to bring the total well volume to 300μl. Wells containing known amounts of FBSA ranging from 0.0mg to 0.8mg were analyzed to generate a standard curve, which was used to estimate the amount of protein bound to the surface of the LTCC. Standards contained the same ratio of Tris/glycine (1:5) as the samples measured above. The amount of fluorescence detected in the glycine washes was used to calculate the level of protein bound to the surface of the LTCC. Fluorescence was detected using Cytoflour 2300 (Millipore). The experiment was performed in triplicate to establish a mean and standard deviation of the amount of FBSA bound to

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LTCC.

2.4 Fluorescent Microscopy

Fragments (3x3x0.263mm) of LTCC were soaked for two hours in a 1.5 mg/ml solution of FITC labeled ovalbumin (FOVA). FOVA contained 4.0 FITC units per ovalbumin molecule. Other fragments were soaked in a solution containing 1.5mg/ml FOVA along with un-labeled BSA (7.5mg/ml) to demonstrate competitive binding. Control fragments of LTCC without FOVA incubation were also imaged. LTCC was removed from the FOVA and FOVA+BSA solutions and rinsed three times in 1ml of 75mM Tris pH 8.6 to remove any unbound proteins from the surface. Some of the LTCC fragments were also rinsed in 100mM glycine to remove bound proteins, demonstrating that the glycine wash was sufficient to remove any bound proteins. The fragments of LTCC were then viewed under an Olympus BX60 fluorescent microscope. Fluorescence indicates that labeled proteins are bound to the surface of the LTCC. An untreated fragment of LTCC was also viewed under the microscope to ensure that LTCC does emit background fluorescence under the conditions used.

2.3 Densitometry/Statistics

Multiplex PCR reactions were performed to gather quantitative data on the inhibitory effect of LTCC on PCR, and the recovery effects of BSA on this inhibition. Multiplex PCR reactions were carried out using 2X ABgene® Master Mix. Reactions had a final volume of 25ml and contained 0.625 units of Thermoprime Plus DNA Polymerase, 75mM Tris-HCl (pH 8.8 at 25ºC), 20mM $(NH_4)_2SO_4$, 3.0mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2mM each dNTP, 100nM each forward and reverse primer, and 1µl of each target DNA. Three different treatments (multiplex + BSA, multiplex + LTCC, and multiplex + BSA and LTCC) were run along with a control (multiplex) to observe the effect of BSA, LTCC, and BSA with LTCC on the multiplex PCR reaction. Multiplex reactions containing BSA and or LTCC had a final concentration of 0.4 mg/ml BSA, and a 21.156 mm^2 piece of LTCC. Multiplex reactions were visualized using 12% polyacrylamide gel electrophoresis and post stained with ethidium bromide. Gels were imaged with a Bio-Rad Gel Doc XR molecular imager using a UV filter. Densitometry was performed using Bio-Rad 1-D Analysis Software to quantify total ng of DNA produced for all PCR products in the multiplex reactions. Statistical analysis was performed on the densitometry results from four different multiplex reactions of each treatment and control in SAS® using analysis of variance (ANOVA). A p value of 0.05 was used for significance.

3. Results and Discussion

3.1 LTCC Inhibition of PCR

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Previously it has been demonstrated that compatibility issues exists between PCR reactions and specific types LTCC material. We investigated the correlation between the surface area of exposed DuPont's 951 LTCC and PCR inhibition, by agarose gel electrophoresis after PCR. PCR reactions were performed with increasing amounts of exposed DuPont's 951 LTCC surface area (Fig. 1). PCR products were visualized using agarose gel electrophoresis. The intensity of the bands in the gel is a relative measure of the efficiency of the PCR reaction. Brighter bands indicate more amplified product. As the amount of LTCC was increased in the reactions, a decrease in PCR product was observed. When a fragment of LTCC with surface area of 21.156mm^2 is added to a 25ul PCR reaction, a noticeable decrease in PCR product is observed (Fig. 1). Greater amounts of LTCC had increasing inhibitory effects. This decrease in product demonstrates that the presence of LTCC has an inhibitory effect on PCR reactions. These results indicate that PCR can proceed in the presence of DuPont's 951 LTCC however, it has an inhibitory effect. As more LTCC is added the reaction is progressively inhibited until no detectable amplification can be viewed via ethidium bromide stained agarose gel electrophoresis. This is contrary to recent studies where it is reported that DuPont's 951 LTCC has no inhibitory effect on PCR [14, 31]. This may be due to differences in surface area that was in contact with the PCR reaction, or to variations in the material from different lots. We suggest that further research be performed on the biocompatibility of DuPont's 951 LTCC from various lots. Other materials, such as silicon, which are being used for the construction of μPCR devices, also show partial inhibition of PCR reactions through the adsorption of the enzyme Taq polymerase [19-22].

3.2 LTCC and Proteins in PCR Reaction Mix

3.2.1Taq Polymerase is limiting in the presence of LTCC

Increasing Taq polymerase levels in PCR reactions has been shown to reduce inhibition by silicon [19]. We investigated this approach in experiments with increasing concentrations of the enzyme Taq polymerase in PCR reactions. As the concentration of Taq was increased in reactions containing a 21.156mm^2 piece DuPont's 951 LTCC, the efficiency of the reaction also increased (Fig. 2). When five units of Taq were used in a PCR reaction containing a piece of LTCC, the PCR product is comparable to the control reaction containing only one unit of Taq. This result suggested that some interaction between the LTCC and Taq polymerase occurred to reduce enzyme activity. At lower concentrations much of the polymerase may be adsorbed to the surface of the LTCC, which has been shown to be the cause of inhibition in reactions in the presence of silicon [19-22]. However, when the concentration of polymerase is increased, more of the enzyme remains in solution, resulting in the concomitant increase in PCR product. Our results confirm that there is a correlation between the concentration of Taq and PCR efficiency in reactions that contain DuPont's 951 LTCC, and that Taq polymerase is a limiting factor in a PCR reaction containing LTCC.

3.2.2 BSA alleviates the need for excess Taq polymerase during PCR in the presence of LTCC

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Once it was established that LTCC inhibited PCR reactions, and this could be corrected through increasing the concentration of Taq polymerase in the reaction, we hypothesized the mechanism of inhibition was protein adsorption. We predicted that BSA (which has a similar molecular weight and isoelectric point to Taq polymerase) added to our PCR reactions would preferentially/competitively passivate the LTCC, resulting in more Taq polymerase molecules available in the solution to catalyze the reaction. BSA is a common additive that is used when inhibitors may be present in the PCR reaction [27-29], and is often used in passivation of μPCR devices [2, 4, 11, 30]. Figure 3 shows long exposures of agarose gels demonstrating the results of PCR reactions carried out with (+) and without (-) 21mm^2 LTCC. Numbers above wells indicate final concentration of $MgCl_2$ used in the reaction. In the figure, the panel on the left shows reactions that did not contain BSA. The panel on the right shows reactions that contained a final BSA concentration of 0.4mg/ml. All reactions contained 1 unit Taq polymerase. In the reactions that contained LTCC and BSA, PCR inhibition is greatly reduced. We believe the increased amplification is due to BSA preferentially binding to the surface of the LTCC.

In order to quantify the inhibitory effects of LTCC on PCR multiplex PCR, and the recovery effect of BSA on this inhibition, reactions were carried out in the presence of BSA and or LTCC. Three different treatments (multiplex + BSA, multiplex + LTCC, and multiplex + BSA and LTCC) were run along with a control (multiplex) to observe the effect of BSA, LTCC, and BSA with LTCC. To gather quantitative data on the inhibitory effect of LTCC, densitometry was performed on 4 different multiplex reactions of each treatment and the control (Fig. 4). The addition of 0.4 mg/ml BSA to the multiplex alone (control) did not have any significant effect on the outcome of the reaction ($p < .2286$). When a 21-mm^2 fragment of LTCC was added to the multiplex, there was a significant decrease in the amount of PCR product ($p < .0001$). Successful multiplex reactions were performed in the presence of LTCC with the addition of 0.4 mg/ml BSA. BSA (0.4mg/ml) was shown to significantly ($p < .0002$) enhance multiplex PCR product formation in the presence of LTCC.

3.2.3 BSA binds to LTCC

In order to investigate if proteins like BSA and Taq polymerase may be adsorbing to LTCC, and to show that BSA preferentially binds to LTCC, we performed a fluorescent binding assay using FITC-labeled BSA. Pieces of LTCC (21mm^2), or plastic controls, were soaked in FITC labeled BSA, rinsed in 75mM Tris-HCl and then washed with glycine buffer (pH 2.5) to remove any bound protein. The glycine washes from three separate pieces of LTCC (or plastic) were aspirated and pooled into a single well of a 96 well plate. The amount of labeled protein liberated from the surface of the LTCC and plastic samples was determined using fluorimetry (Fig. 5). Elevated fluorescence was present in the LTCC glycine washes indicating that proteins had bound to the ceramic (left bar). Reduced levels of fluorescence were present in the plastic (control) glycine washes (right bar). No fluorescence was eluted from untreated LTCC (center bar), indicating that the fluorescence measured in the experiment was due to bound FBSA and

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not the elution of some uncharacterized fluorescent species from the LTCC polymer. It is clear from the results that there was a significant amount of protein bound to the LTCC.

Using a standard curve of FBSA fluorescence (0.0 μg to 0.8 μg), the BSA bound per 21 mm^2 fragment of LTCC (Fig. 5) was calculated to be approximately 43 ng (647.27 femto moles), or $1.843x10^7$ BSA molecules bound per μm^2 of LTCC. This indicates that there are ample binding sites for proteins on the surface of the LTCC. The PCR reactions used to study inhibition of PCR by LTCC contained 62.5 ng of Taq polymerase. With a 21 mm^2 piece of LTCC able to bind 43 ng of BSA, it is possible to estimate that 68.8% of the Taq polymerase in the reaction could be bound to the LTCC. Removing this much Taq from a reaction would result in a considerable decrease of PCR product, and is a likely cause of PCR inhibition by LTCC.

3.2.4 BSA competes with other protein molecules (ovalbumin) for LTCC binding

Once it had been shown that FITC-labeled BSA bound to LTCC, a study was performed to confirm these LTCC-protein binding results and to determine whether this binding was competitive with other similar protein molecules. For these studies, LTCC was first soaked in a solution of FOVA, rinsed in Tris buffer (pH 8.6) to remove unbound proteins, and the ceramic chip viewed using fluorescence microscopy (Fig. 6). Panel A shows a fluorescence micrograph of a piece of LTCC that had been immersed in FOVA for one hour, and exposed for 600 milliseconds. The green fluorescence in the image shows where FOVA is bound to the LTCC. Areas of more intense fluorescence indicate a higher density of protein bound. The micrograph in Panel B shows a piece of LTCC that was soaked in a mixture of FOVA and non-labeled BSA (exposure time of 600milliseconds). Addition of non-labeled BSA (panel B) reduced fluorescence significantly (compared to panel A), demonstrating that BSA competes for binding with FOVA, and showing that LTCC specifically binds protein (labeled or not). Panel C is a longer exposure of the image in panel B, demonstrating that a reduced amount of FOVA is still bound to the LTCC. In conclusion, figure 6 demonstrates the ability of LTCC to competitively bind two negatively charged proteins: bovine serum albumin and ovalbumin.

4. Conclusion

Our results clearly demonstrate that DuPont's 951 LTCC had an inhibitory effect on PCR. We have determined a reliable way to overcome LTCC inhibition of PCR by the addition of BSA as a passivating agent in the reaction. We have demonstrated that two net negatively charged proteins (BSA and OVA) bind to LTCC in a competitive manner. Loss of PCR activity appears to be overcome by BSA molecules preferentially adsorbing to the LTCC which increases the Taq polymerase available to perform the enzymatic reaction. While the mechanism of protein binding to LTCC is not fully resolved, it is clear that interactions exist between proteins and the surface of the LTCC. From these result we propose that LTCC inhibits PCR reactions by adsorbing Taq polymerase, and that dynamic passivation with BSA is sufficient to rescue reaction efficiency.

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Research supported by the Environmental Protection Agency, Boise State University Sensor Program (CN X97031101-0).

References

[1] L.J. Kricka, P. Wilding, Microchip PCR, Anal. Bioanal. Chem. 377 (2003) 820-825.
[2] C. Zhang, D. Xing, Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends, Nucleic Acids Res. 35 (2007) 4223-4237.
[3] Y.H.Chang, G.B.Lee, F.C. Huang, Y.Y. Chen, J.L. Lin, Integrated polymerase chain reaction chips utilizing digital microfluidics, Biomed. Microdevices 8 (2006) 215-225.
[4] Zhang C, Xu J, Ma W, Zheng W, PCR microfluidic devices for DNA amplification, Biotechnol. Adv. 24 (2006) 243-284.
[5] Y. Li, C. Zhang, D. Xing, Integrated microfluidic reverse transcription-polymerase chain reaction for rapid detection of food- or waterborne pathogenic rotavirus, Anal. Biochem. 415 (2011) 87-96.
[6] S. Park, Y. Zhang, S. Lin, T.H. Wang, S. Yang, Advances in microfluidic PCR for point-of-care infectious disease diagnostics, Biotechnol. Adv. 29 (2011) 830-839.
[7] K. Mullis, F. Faloona, S. Scharf, R. Saiki, G. Horn, H. Elrlich, Specific Enzymatic Amplification of DNA in vitro: The Polymerase Chain Reaction, Cold Spring Harb. Symp. Quant. Biol. 51 (1986) 263-273.
[8] R.K. Saiki, S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, N. Arnheim, Enzymatic Amplification of Î²-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia, Science. 230 (1985) 1350-1354.
[9] P. Neuzil, J. Pipper, T.M. Hsieh, Disposable Real-Time MicroPCR Device: Lab-On-a-Chip at a Low Cost, Mol. BioSyst. 2 (2006) 292-298.
[10] P. Bembnowicz, P. Herbut, M. Malodobra, A. Karpiewska, L. Golonka, A. Jonkisz, T. Dobosz, The low temperature co-fired ceramics (LTCC) chip for polymerase chain reaction (PCR) application, Optica Applicata. 41 (2011) 471-480.
[11] R. Kodzius, K. Xiao, J. Wu, X. Yi, X. Gong, I.G. Foulds, W. Wen, Inhibitory effect of common microfluidic materials on PCR outcome, Sens. Actuators B 161 (2012) 349-358.
[12] D.M. Bravata, V. Sundaram, K.M. McDonald, K.L. Smith, H. Szeto, M.D. Schleinitz, D.K.Owens, Evaluating Detection and Diagnostic Decision Support Systems for Bioterrorism Response, Emerg. Infect. Dis. 10 (2004) 100-108.
[13] K. Moeller, J. Besecker, G. Hampikian, A. Moll, D. Plumlee, J. Youngsman, J.M. Hampikian, A Prototype Continuous Flow Polymerase Chain Reaction LTCC Device, Mat. Sci. Forum 539-543 (2007) 523-528.
[14] P. Bembnowicz, M. Malodobra, W. Kubicki, P. Szczepanska, A. Gorecka-Drzazga, J. Dziuban, A. Jonkisz, A. Karpiewska, T. Dobosz, L. Golonka, Preliminary studies on LTCC based PCR microreactor, Sens. Actuators B 150 (2010) 715-721.

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DOI: 10.1016/j.snb.2012.09.110

[15] L. Golonka, P. Bembnowicz, D. Jurkow, K. Malecha, H. Roguszczak, R. Tadaszak, Low temperature co-fired ceramics (LTCC) Microsystems, Optica Applicata 41 (2011) 383-388.
[16] G.A. GroB, T. Thelemann, S. Schneider, D. Boskovic, J.M. Kohler, Fabrication and fluidic characterization of static micromixers made of low temperature cofired ceramic (LTCC), Chem. Eng. Sci. 63 (2008) 2773-2784.
[17] D.J. Sadler, C. Rajnish, P. Robert, C. Chou, F. Zenhausern, Thermal Management of BioMEMS: Temperature Control for Ceramic-Based PCR and DNA Detection Devices, IEEE Transactions on components and packaging technologies 26 (2003) 309-316.
[18] M. Malodobra, P. Bembnowicz, A. Jonkisz, A. Karpiewska, P. Sniadek, A. Gorecka-Drzazga, L. Golonka, T. Dobosz, Multiplex miniSTR PCR in miniaturized system based on LTCC technology – first report, Recent Researches in Geography, Geology, Energy, Environment and Biomedicine (2011) 31-34.
[19] I. Erill, S. Campoy, N. Erill, J. Barbé, J. Aguiló, Biochemical analysis and optimization of inhibition and adsorption phenomena in glass-silicon PCR-chips, Sens. Actuators B 96 (2003) 685.
[20] W. Wang, H.B. Wang, Z.X. Li, Z.Y. Guo, Silicon inhibition effects on the polymerase chain reaction: a real-time detection approach, J. Biomed. Mater. Res. A 77 (2006) 28-34.
[21] T.B. Christensen, C.M. Pedersen, K.G. Gröndahl, T.G. Jensen, A. Sekulovic, D.D. Bang, A. Wolff, PCR biocompatibility of lab-on-a-chip and MEMS materials, J. Micromech. Microeng. 17 (2007) 1527-1532.
[22] C. Potrich, L. Lunelli, S. Forti, D. Vozzi, L. Pasquardini, L. Vanzetti, C. Panciatichi, M. Anderle, C. Pederzolli, Effect of materials for micro-electro-mechanical systems on PCR yield, Eru. Biophys. J. 39 (2010) 979-986.
[23] M.A. Shoffner, J. Cheng, G.E. Hvichia, L.J. Kricka, P. Wilding, Chip PCR. I. Surface Passivation of Microfabricated Silicon-Glass Chips for PCR, Nucl. Acids Res. 24 (1996) 375-379.
[24] D.H. Sun, M.C.D. Trindade, Y. Nakashima, W.J. Maloney, S.B. Goodman, D.J. Schurman L.R. Smith, Human Serum Opsonization of Orthopedic Biomaterial Particles: Protein-binding and Monocyte/Macrophage Activation in vitro, J. Biomed. Mater. Res. 65A (2003) 290-298.
[25] M. Rouahi, E. Champion, O. Gallet, A. Jada, K. Anselme, Physico-chemical characteristics and protein adsorption potential of hydroxyapatite particles: Influence on in vitro biocompatibility of ceramics after sintering, Colloids Surf B: Biointerfaces 47 (2006) 10.
[26] Å. Rosengren, E. Pavlovic, S. Oscarsson, A. Krajewski, A. Ravaglioli, A. Piancastelli, Plasma protein adsorption pattern on characterized ceramic biomaterials, Biomaterials 23 (2002) 1237.
[27] C.A. Kreader, Relief of Amplification Inhibition in PCR with Bovine Serum Albumin or T4 Gene 32 Protein, Appl. Environ. Microbiol. 62 (1996) 1102-1106.
[28] T.A. Giambernardi, U. Rodeck, R.J. Klebe, Bovine Serum Albumin Reverses Inhibition of RT-PCR by Melanin, Biotechniques 25 (1998) 564-566.

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DOI: 10.1016/j.snb.2012.09.110

[29] P. Rådström, R. Knutsson, P. Wolffs, M. Lövenklev, C. Löfström, Pre-PCR Processing Strategies to Generate PCR-Compatible Samples, Mol. Biotechnol. 26 (2004) 133-146.
[30] T.B. Taylor, E.S. Winn-Deen, E. Picozza, T.M. Woudenberg, M. Albin, Optimization of the performance of the polymerase chain reaction in silicon-based microstructures, Nucleic Acids Res. 25 (1997) 3164-3168.
[31] H. Bartsch de Torres, C. Rensch, M. Fischer, A. Schober, M. Hoffmann, J. Müller, Thick film flow sensor for biological microsystems, Sens. Actuators A 160 (2010) 109-115.

Biographies

Jason Besecker is research associate at Boise State University in the laboratory of Dr. Greg Hampikian. Jason received his BS (2005), and MS (2010) degrees in biology from Boise State University, Boise, ID, United States. He received his doctor of optometry (OD) degree from Indiana University School of Optometry in 2012. His research interests include biocompatibility of microfluidic devices, clinical/field applications of microfluidic detection systems.

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Kenneth A. Cornell is an Associate Professor in the Department of Chemistry & Biochemistry at Boise State University. He earned a Ph.D. in Biochemistry (1997) from Oregon Health & Sciences University, and was the recipient of an NIH pre-doctoral fellowship in Hematology/Oncology. Following post-doctoral work at the Portland VA Medical Center, he worked in the medical devices industry to develop antimicrobial coatings for wound care products and implants. Research in his lab involves the examination of new antibiotics and mucosal vaccines, and the development of microfluidic medical diagnostic devices for point of care applications.

Figure(s)

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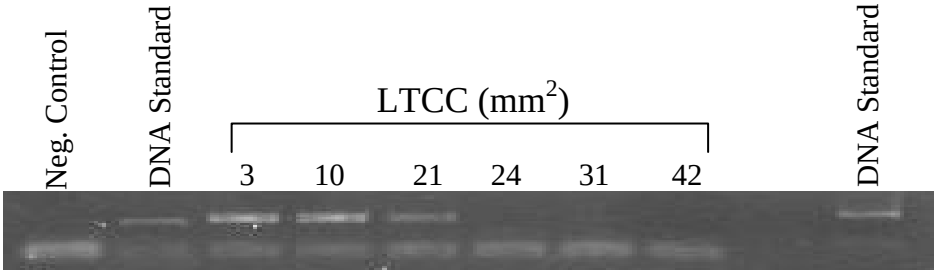

Figure 1. DuPont 951 LTCC inhibits PCR reactions. Reactions were performed with increasing surface area of LTCC (3-42 mm^2). Noticeable reduction in PCR product is seen with the addition of 21 mm^2 LTCC to the reaction. Above 21mm^2 LTCC, no visible PCR product could be detected using 2.0% agarose gel electrophoresis.

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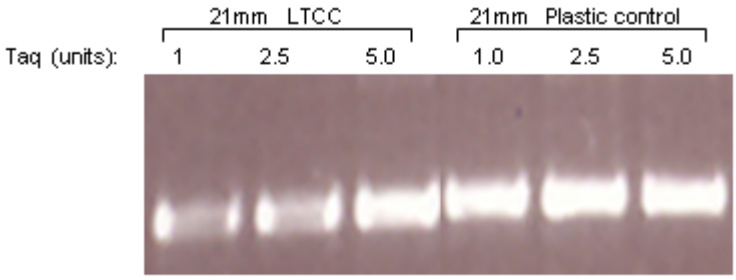

Figure 2. Increasing Taq polymerase concentration overcomes LTCC inhibition. Increasing amounts of Taq polymerase (1-5 units) were added to PCR reactions in the presence of LTCC or a plastic control. An increase in PCR product was observed with an increase in the concentration of Taq polymerase in the LTCC reactions, suggesting that increasing the enzyme concentration overcomes inhibition by LTCC. The plastic control had no effect on the enzyme reaction.

Figure(s)

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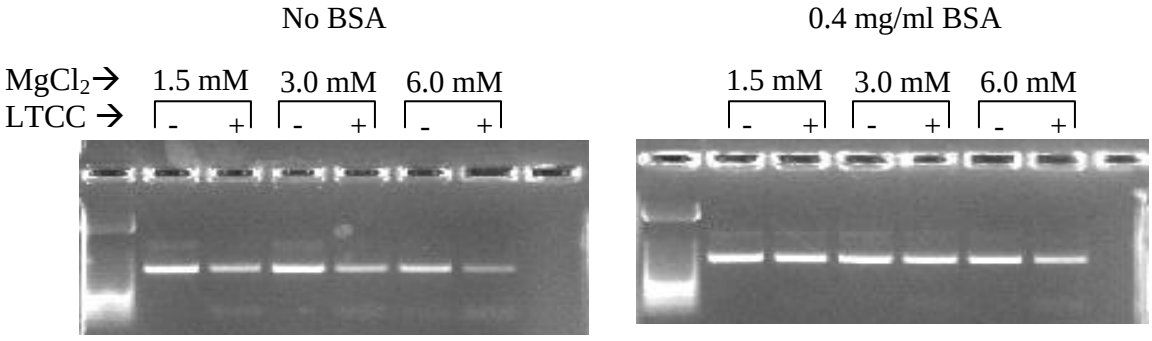

Figure 3. BSA alleviates DuPont 951 LTCC inhibition of PCR. Ethidium bromide stained 2% agarose gels show the results of PCR reactions carried out with (+) and without (-) 21mm^2 LTCC. Numbers above wells indicate final concentration of $MgCl_2$ used in the reaction. In the figure on the left, the reactions did not contain BSA. The figure on the right shows reactions that contained a final BSA concentration of 0.4mg/ml. In the reactions that contained LTCC and BSA, PCR inhibition by LTCC is visibly reduced.

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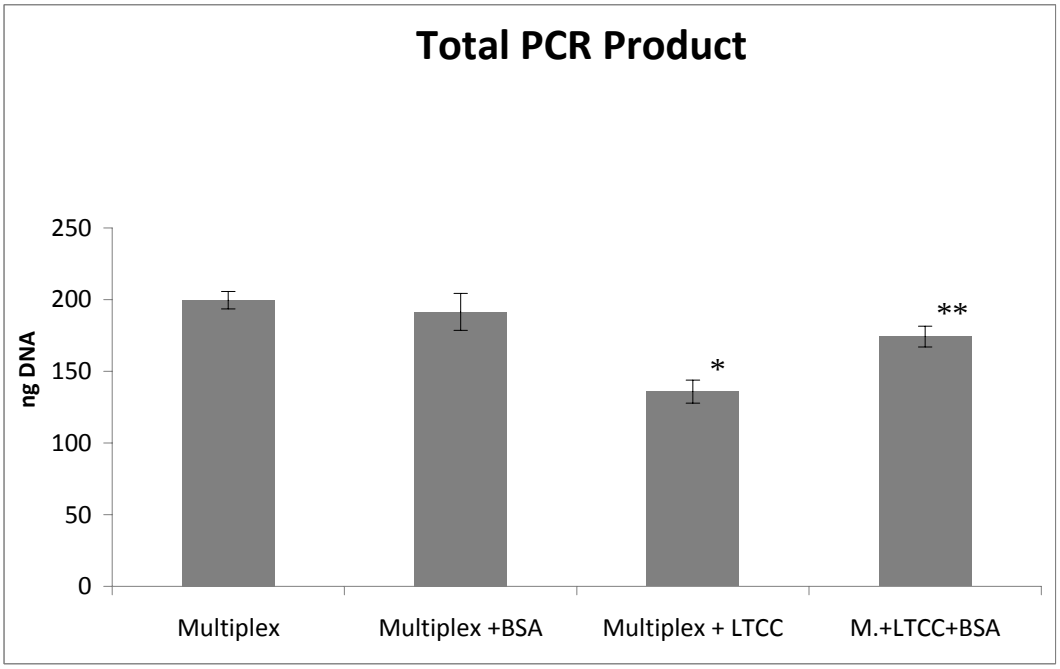

Figure 4. BSA alleviates LTCC inhibition of PCR. Multiplex PCR reactions were performed in the absence (columns 1 and 2 on left) and presence of LTCC (columns 3 and 4 on right). LTCC significantly (*, $p < .0001$) decreased the total amount of PCR product obtained by the multiplex PCR (column 3 on right). BSA (0.4mg/ml) significantly (**, $p < .0002$) enhanced multiplex PCR product in the presence of LTCC.

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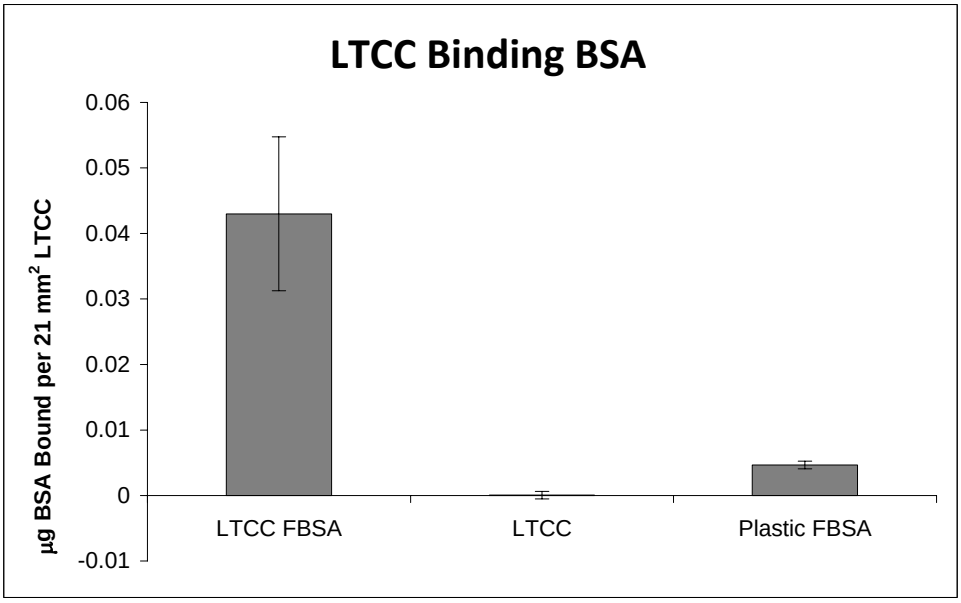

Figure 5. BSA binding to LTCC. Pieces of LTCC (21mm^2) or plastic controls were soaked in FITC labeled BSA 1.5 mg/ml for two hours. Pieces were then rinsed in Tris buffer and bound protein eluted with 100mM glycine (pH 2.5). The glycine elutions from three separate pieces of LTCC (or plastic) were combined into a single well of a 96 well plate, and neutralized with a small volume of 1M Tris buffer pH 8.0. A fluorimeter was used to measure the amount of labeled protein liberated from the surface of the LTCC and plastic samples. The appearance of fluorescence in the LTCC glycine elutions indicated that proteins had bound to the ceramic (left bar). Very little fluorescence was present in the plastic (control) glycine elutions (right bar). Pieces of LTCC that were not soaked in fluorescently labeled BSA were treated the same as above to ensure that there was no fluorescent component that could be washed from the surface of untreated ceramic (center bar).

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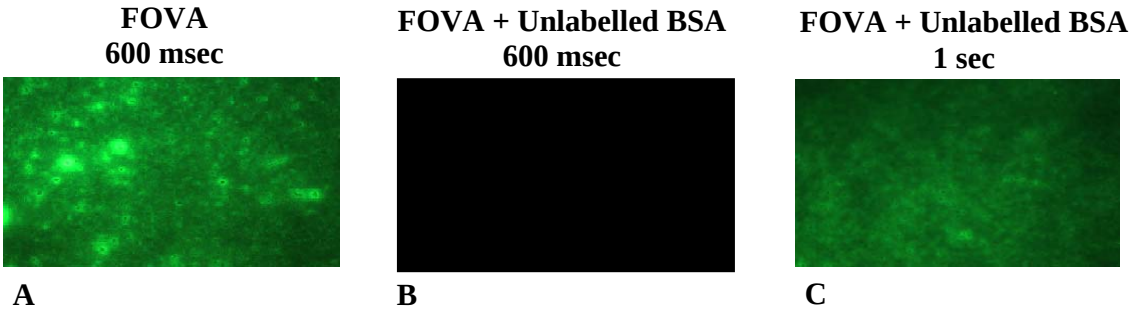

Figure 6. Fluorescence Microscopy of FOVA binding to LTCC. Exposure times (600msec-1sec) are indicated. (Panel A) LTCC was soaked in a solution of FOVA, rinsed to remove unbound protein, and then viewed using fluorescence microscopy. (Panel B) Addition of non-labeled BSA reduced fluorescence, demonstrating that LTCC specifically binds protein, and not the fluorescent tag. (Panel C) Longer image exposure (1 sec) of unlabeled BSA plus FOVA, shows that some labeled proteins are still bound in the competition experiment.