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Stability Evaluation of Protein Coating for Sensing: an Application to Silicon Based Lab-on-chip Device

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Abstract: Protein coating on solid surface is an essential passivation method to achieve surface biocompatibility for sensing applications. In this paper it was investigated the stability of Bovine Serum Albumine (BSA) coating at different pH values, time and temperature conditions by monitoring the PCR performances on a silicon based lab on chip and by simple contact angle measurement of the coated surface. BSA was deposited on epoxy-silane silicon oxide surface and the coating fully characterized. By means of contact angle values, we found that BSA coating shows surface wettability properties stable under thermal and pH variation, while not treated substrates are very sensible to both pH and temperature stressing. Results here shown indicate that BSA-coated surface is stable in acid, neutral and basic conditions except for pH≥12. *Copyright © 2012 IFSA.*

Keywords: PCR, Contact angle, Silane, Surface wettability.

1. Introduction

The implementation of miniaturizated device in the molecular diagnostics field to perform a variety of chemical [1-2], enzymatic reactions [3-5] and to set up detection methods such microarrays, real-time PCR, has stimulated many research laboratories to focus on the role of surface chemistry in reactions performed inside the microfabricated devices. Many materials are proposed to achieve the appropriate biocompatibility reactions, such as (BSA) [5, 6], parylene [7-8], polyethylene glycol (PEG) [9-10] and polyvinylpyrrolidone (PVP) [10]. The biocompatibility must be guaranteed for all devices, whatever is their material composition such as silicon, plastic, silicon oxide, glass, metal and the relative surface derivatization processes. Among these, BSA is reported as the most useful, low cost, and biocompatible material used for lab-on-chip as PCR microreactors and nucleic acids microarray

devices and for this reason, it is recommended by many companies (Termo Scientifics, NUNC, Perkin Elmer and Schott) [11]. To date, in literature It is reported that enzymatic reactions strongly depend on the surface features. As example, the PCR efficiency decreases enhancing the hydrophilicity of surface and therefore it is proposed the BSA coating to minimize the absorption of PCR reagents (polymerase enzyme, DNA template, primers, salts) on PCR microreactor surface. Similar advantages in using BSA can be found in microarray devices to reduce the non-specific binding of labeled target on surface in order to increase the signal/background value. The proper degree of surface hydrophilicity determines specific wettability properties.

In this paper, with the aim to give a contribution on the establishment of the wettability stability of BSA coating, we explored the i) surface wettability of BSA coating at different pH values, time and temperature by simple static contact angle measurements and the ii) PCR performances by means of an integrated PCR/microarray Lab-on-Chip (loC) device of In-Check platform [5] to provide the chip with biocompatibility features. BSA coating was previously characterized by XPS and TEM analysis.

2. Experimental

2.1. Materials and Methods

Chemicals: Hydrogen peroxide (29 %) ammonium hydroxide (25 %) hydrochloridric acid (37 %) and methanol for wet processes were purchased by Sigma Aldrich and were used as received. Anhydrous toluene and glycidoxypropriltrimethoxysilane (GOPS) for silanization step were purchased by Sigma Aldrich and were used as received. Chemicals for passivation steps and stability testing: Sodium Dodecyl Sulfate, Sodium chloride, Sodium Citrate, Disodium Hydrogen Phosphate, Potassium Chloride, Sodium Acetate, Ammonium Acetate, Tris[hydroxymethyl]aminomethane (TRIS) and Sodium Hydroxide were purchased from Sigma Adrich and were used as received. Bovine serum albumin fraction V (BSA) has been purchased from Euroclone and was used as received.

Substrates: the substrates used to monitor the surface contact angle and the PCR performances at different stressing condition are TEOS slide and Lab-on-Chip (LoC) respectively. The TEOS slide internally manufactured consists in a silicon slide (size 2.5 × 7 cm) covered by amorphous silicon oxide film (850 nm ± 35 nm) deposited by TEOS precursor with PE-CVD technique, and properly coated by BSA. The contact angles were measured on TEOS slide before and after the chemically/physically treatment. The labonchip is a silicon based device developed and manufactured by ST-Microelectronics (Fig. 1); it integrates a PCR module to perform the DNA amplification with a microarray module for the detection of nucleic acids.

Fig. 1. Lab-On-Chip.

The silicon device has four pairs of resistors for heating and sensors to monitor the temperature on hybridization area. During the PCR/hybridization reactions to avoid the liquid evaporation a set of clamps were designed for the LoC. The PCR module are composed by two silicon microreactor

(volume 12 uL) covered by amorphous silicon oxide film deposited by TEOS precursor with PE-CVD technique, and properly coated by BSA.

Equipments: Teflon tanks for wet processes were manufactured by SPM. Glove box in nitrogen atmosphere used for silanization process was purchased from BRAUM. Contact angle measurement was performed with a KSV CAM 200 equipment (water drop volume 1ul) supplied by NORDTESTS. Incubator used for BSA passivation process was purchased by RS Biotech (INOVA 44). Spectrum from X ray photoelectron Spectroscopy (XPS) was acquired under vacuum (2×10^{-9} Torr) with a PHI ESCA/SAM 5600 Multitechnique equipped with a monochromatic source of Al Kpass energy of 11.75 eV and an acceptance angle of ± 7º and several take-off angles (θtake-off = 10º, 80º). High Resolution Transmission Electron Microscopy (HRTEM) and Energy Filtered Transmission Electron Microscopy (EFTEM) were performed with JEOL 2010F instrument operating at 200 kV and equipped with Oxford electron dispersive spectrometer and Gatan image filtering apparatus. The carbon elemental mapping was obtained by using the three-window method on the k edges of the element. Samples were observed in the cross-sectional configuration after mechanical thinning and argon ion milling. To avoid an undesired intermixing between epoxy-glue, used in sandwiching samples, and surface organic layer, surface of each sample was previously covered with a thin aluminum layer (~50 nm). Aluminum layer was deposited by thermally evaporation on the substrate at room temperature. The experimental conditions were soft enough to ensure a minimal alteration of surface during deposition.

2.2. Substrate Coating Preparation

Cleaning and activation processes: TEOS slide and LoC were cleaned by standard cleaning process (H_2O:NH_4OH:H_2O_2) in a Teflon tank, rinsed with deionized water and dried by nitrogen flow; the activation step was carried out using an acid methanolic solution CH_3OH:HCl, rinsed by deionized water and dried by nitrogen flow.

Silanization process: the substrates were silanized in nitrogen atmosphere by dipping in GOPS solution. Silanized substrates were rinsed and dried in vacuum chamber Silanized substrates were stored in vacuum condition until passivation process.

BSA passivation coating: Silanized substrates were dipped in BSA aqueous solution. Substrates were rinsed in deionized water and dried by a nitrogen flow. All processes described above were performed in a clean room (class 1000).

2.3. Substrate Coating Characterization

Epoxysilane coating was confirmed by XPS analysis. XPS measurements were performed after each wet process on cleaned, silanized and BSA-coated substrates and the spectra in the region from 85 to 420 eV have been shown in Fig. 2 for comparison.

Epoxysilane coating was confirmed by C 1s signal and ratio C 1s / Si 2p values that increase from naked to silanized surface as reported in Table 1. The trace of carbon present on naked oxide surface after cleaning is attributable to the adventitious carbon, always present in XPS spectra of sample exposed to air.

As regards the BSA coating the Fig. 2 (c) shows the XPS spectra of BSA-coated substrate, in particular the increasing of C 1s at 285 eV signal value and the presence of N 1s at 400 eV signal (see Table 1) is compatible with the presence of a protein layer on epoxysilane surface.

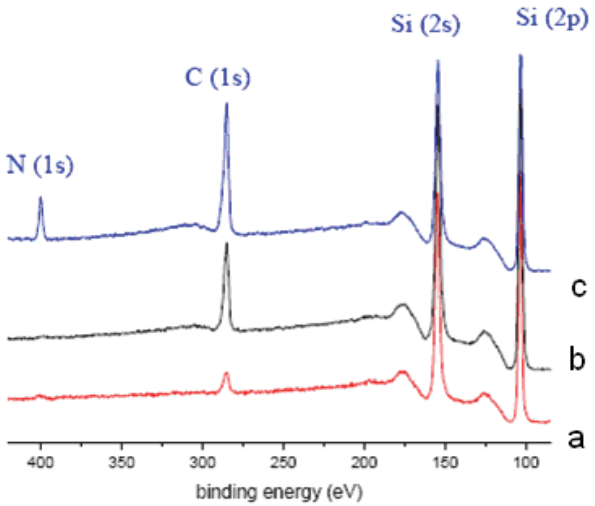

Fig. 2. XPS spectra of naked (a), silanized (b), and BSA-coated (c) substrate.

Table 1. XPS Elemental analysis of cleaned, silanized and BSA-coated surfaces.

Substrate	[C]% Si2p	[C]% C1s	[C]% N1s	[C1s]/[Si2p]
Cleaned	30.48	4.03	-	0.13
Silanized	28.74	10.24	-	0.36
BSA coated	24.77	17.31	2.46	0.70

BSA passivation coating was confirmed by TEM investigations. In particular, after thermal evaporation of a protective Al layer (ca 50 nm) the interface was investigated by cross section analysis with both HRTEM and carbon mapping by EFTEM.

Fig. 3 (a) reports the morphology of the interface inspected by HRTEM. A rough surface at the edge Al/silicon oxide is evident. The energy filtered TEM investigation of this interface (Fig. 3 (b)) clearly shows a carbon layer in correspondence of the interface. The thickness of this layer is not constant and its morphology follows the silicon oxide surface roughness.

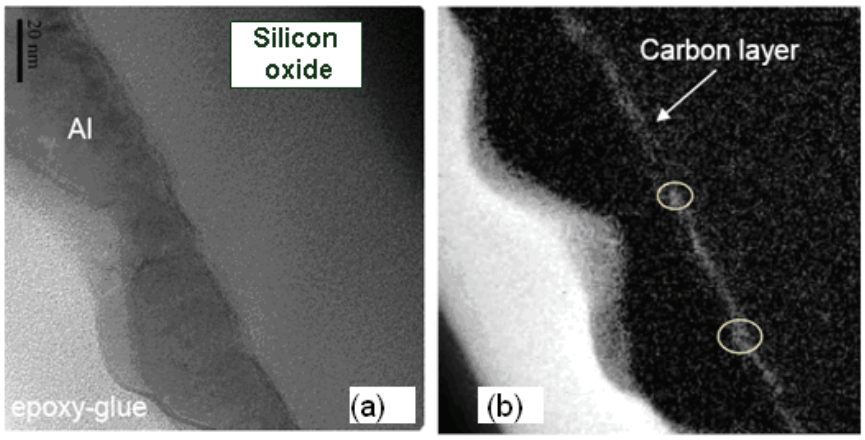

Fig. 3. Silicon oxide/Al interface: (a) cross–sectional HRTEM image; (b) EFTEM carbon map. Interfacial regions with higher carbon amount are marked with the circle.

2.4. PCR: Primer Design and Selection

The PCR performances were evaluated by complete Human beta Globine (HBB) gene consisted of 1920 bps in length, 3 small exons and 2 introns. In Table 2 the target sequence was reported together with the identification of reverse and forward regions able to amplify a 240 bp fragment. In order to prevent the formation of stable secondary structures like hairpin, self dimer and cross dimer, primers with the following criteria were designed: ΔG values had to be higher than -2 for hairpin and higher than -5 for self dimer and cross dimer. The upstream forward primer was designed as complementary to the antisense strand of the β-globin gene (position 72 through 90) while the downstream reverse primer complemented the sense strand (from position 294 to 277). Sequences of primers are respectively: 5'-CAGGGCAGTAACGGCAGA-3' (18 bases) for reverse primer and 5'- GAAGAGCCAAGGACAGGTA -3' (19 bases) for forward primer.

Table 2. Identification of regions for reverse and forward primers.

SEQUENCE	SIZE (n° of bases)
GGTATGGGGCCAAGAGATATATCTTAGAGGGAGGGCTGAGGGTTTGAAGT	50
forward →	
CCAACTCCTAAGCCAGTGCCAGAAGAGCCAAGGACAGGTACGGCTGTCAT	100
AA	
CACTTAGACCTCACCCTGTGGAGCCATACCCTAGGGTTGGCC TCTACT	150
CCCAGGAGCAGGGAGGGCAGGAGCCAGGGCTGGGCATAAAAGTCAGGGCA	200
GAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCA	250
ACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCG	300
← reverse	
TTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCC	350

2.5. PCR: Preparation of the PCR Reagent and the DNA Template

DNA amplifications were performed in PCR mix aliquots of 25 µl using 4 U Hotstart Taq plus DNA polymerase, 200 µM each dNTP (dATP, dCTP, dGTP and dTTP), 1 µM primer, 1 µM human DNA, 1X PCR buffer and sterile water on a Epperdorf Mastercycler Gradient (NY USA). PCR reaction began with a denaturation step at 95 °C for 5 min and followed by 25 cycles of amplification (20 s at 95 °C, 45 s at 61 °C, 72 s at 30 °C). The PCR products and their size were measured (in the range of 15 -1500 base pairs), by Agilent 2100 Bioanalyzer (Agilent Waldbronn Germany), a microfluidics-based platform for sizing and quantification of nucleic acid samples, using planar microfluidic electrophoretic lab-on-chip. Each chip was manually loaded with 12 nucleic acid samples. The movement of samples through the microchannels is controlled by a series of electrodes. The marker mixture (loaded in the first position: L) consisted of a buffer containing lower (15 bp) and upper (1500 bp) molecular size markers, used by the Bioanalyzer as internal references. The upper marker was also used as a reference for calculating the concentration of DNA fragments in each sample.

3. Discussion

BSA-coated TEOS slides and LoCs were dipped into different buffer solutions at pH 4.5, 7.0, 8.3 and 12.0, and heated at 55 and 95°C, the surface water contact angles were evaluated at different time (0, 1, 2, 4 hours) while the PCR performances were evaluated at time zero and after the stressing process (4 hours). The buffers solutions composition used for the stressing test were: acetate at pH 4.5, buffer phosphate at pH 7.0, buffer TRIS at pH 8,3 and NaOH at pH 12.0; for all solutions the ionic strength was corrected to 0,60 M with potassium chloride. Three TEOS slide were investigated for each tested point; in each slide six water contact angle values were evaluated. No relevant changes on water contact angle values have been observed when BSA-coated substrates were dipped into solution at acid pH 4,5 as reported in Fig. 4.

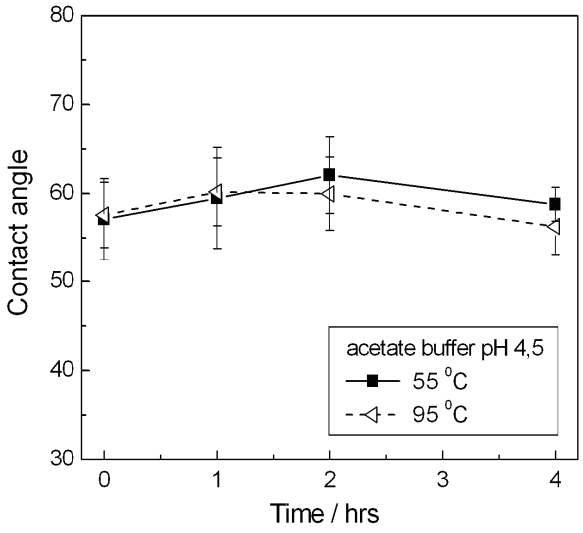

Fig. 4. BSA-coated substrate water contact angle variation at acid pH=4.5, temperatures 55 °C and 95 °C.

Experimental data from treatments with solution at neutral pH (Fig. 5) did not show any relevant water contact angle variation at both tested temperature, except for a negligible increment of hydrophilic surface after thermal treatment although the variation is within the experimental error.

BSA-coating was investigated with basic (pH 8,3) in Tris-HCl. Also in this case Fig. 6 did not show any relevant water contact angle variation at both tested temperatures.

Results showed that at pH from 4.5 to 8.3, no significant water contact angle variations have been observed for both temperatures, indicating any relevant BSA coated surface wettability modification.

Differently from the results showed above, BSA-coated substrate dipped into sodium hydroxide solution at high pH values (pH=12) showed some increasing of surface hydrophilicity, evidenced by water contact angle value decreasing (Fig. 7).

In order to correlate the surface wettability stability with PCR performances, BSA-coated LoCs were chemically and thermally stressed and PCR performances evaluated. The BSA-coated LoCs stressed at pH 4.5, 7.0, and 8.3 did not show appreciable PCR performance variaration, while at pH 12 for both temperatute 55 and 95 °C, the PCR efficiency decrease are reported in Fig. 8.

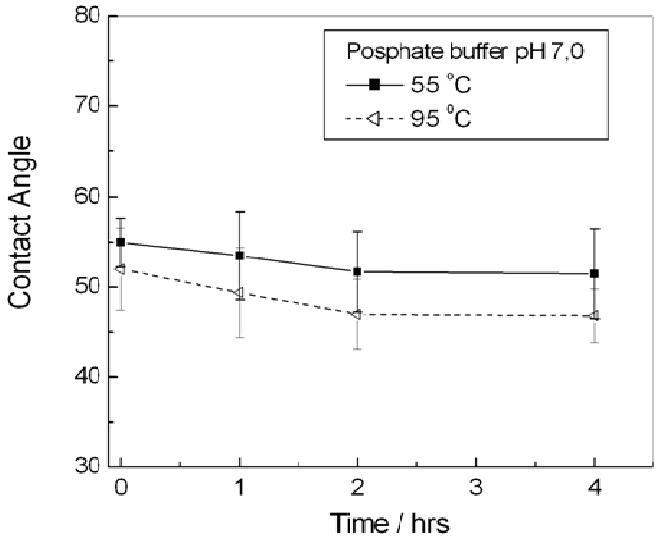

Fig. 5. Contact angle variation of BSA-coated substrate at neutral pH at 55 and 95 °C.

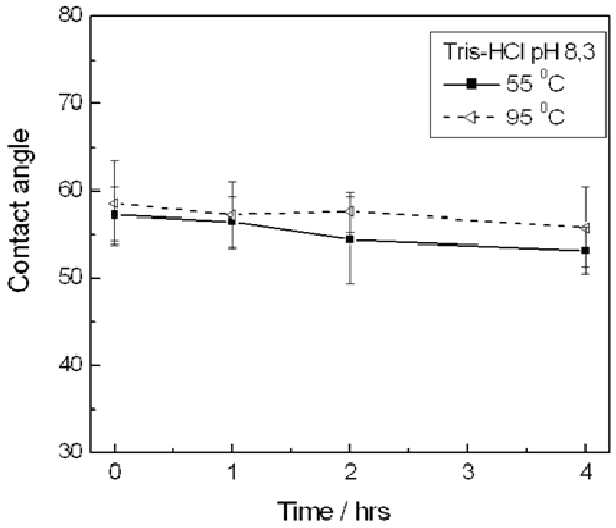

Fig. 6. BSA-coated substrate water contact angle variation at basic pH=8.3 to 55° C and 95° C.

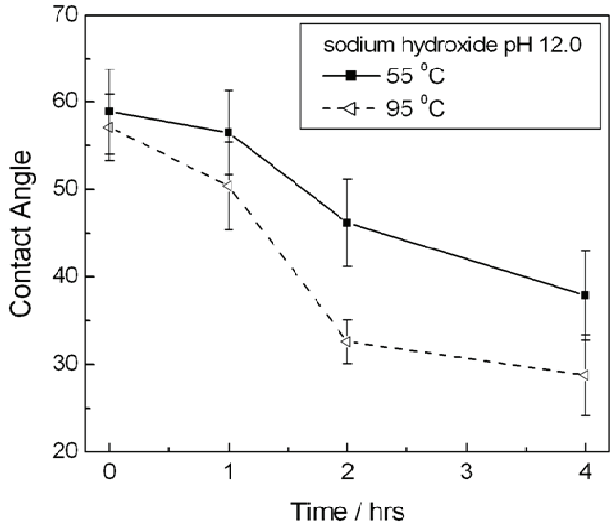

Fig. 7. Water contact angle variation of BSA-coated substrate at basic pH 12 to 55 and 95 °C.

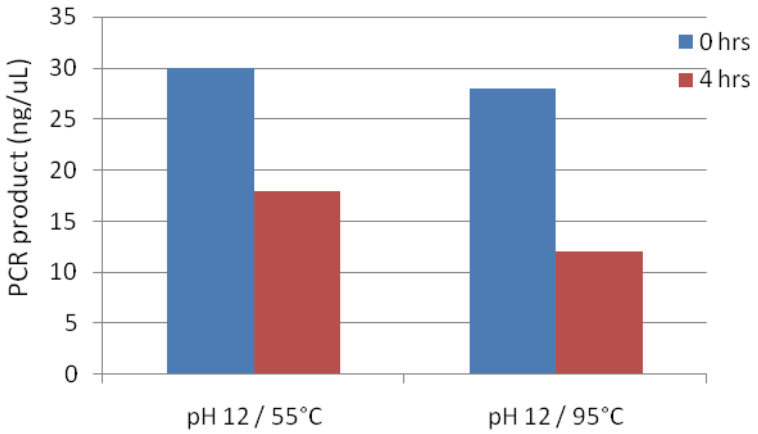

Fig. 8. PCR product yield BSA-coated LoCs at pH 12 to 55 and 95 °C.

The surface wettability of BSA-coated and uncoated (epoxy-silane derivate) substrates were investigated at neutral and basic pH, at temperature 95 °C and at different times, for comparison; for all solutions the ionic strength was corrected to 0,60 M with potassium chloride.

Fig. 9 reports the BSA-coated and uncoated water contact angle variations on substrates treated at neutral pH (phosphate buffer), for respectively 30, 60, 90 and 120 min at 95°C. It is shown that after half hour the water contact angle value of uncoated substrate decreases, to indicate the epoxysilane coating modification; on the contrary, the BSA coated water contact angle values slightly changed, indicating a good thermal and pH surface wettability stability in the time frame of investigation.

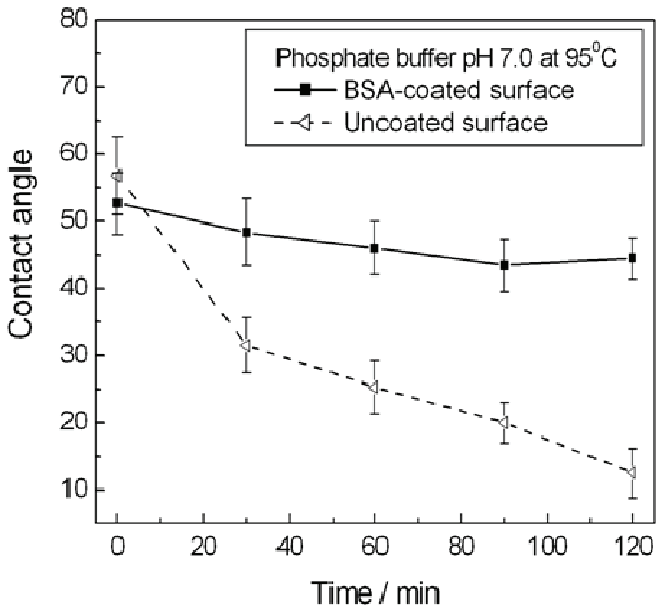

Fig. 9. Contact angle decreasing of BSA-coated and uncoated substrate at neutral pH at different time.

Fig. 10 reports the BSA-coated and uncoated water contact angle variations on substrates treated at basic pH (TRIS buffer), for respectively 30, 60, 90 and 120 min at 95 °C. Also in this case after 30 min. the water contact angle value of uncoated substrate decreases, once again indicating the epoxysilane coating modification; while, similarly at neutral pH, the BSA coated water contact angle values did not change.

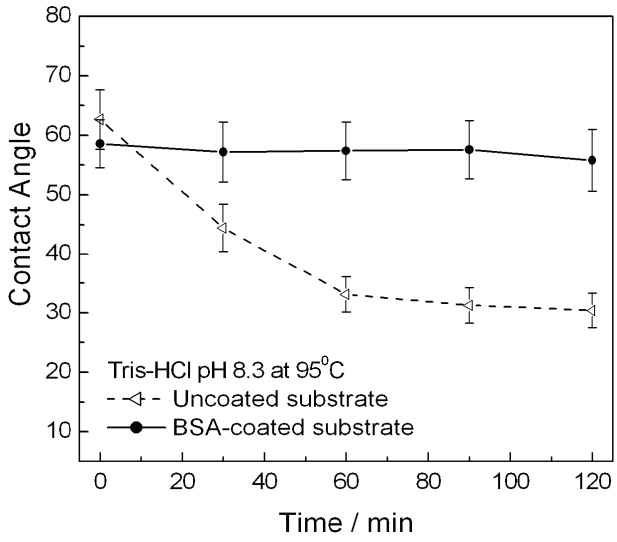

Fig. 10. Contact angle decreasing of BSA-coated and uncoated substrate at basic pH (8,3) to different time.

Although in literature it is reported that silane coating is sensitive to basic pH because of to the hydrolytic effect on disiloxy bonds (R-Si-O-Si) of the Silane-surface arrangement, the data above reported clearly show that the epoxysilane coating is also sensible to the buffer composition. In fact at basic pH with TRIS buffer, the sample surface does not achieve high hydrophilicity as expected if compared with the sample surface treated at neutral pH in phosphate buffer. This could be due to a different chemical composition of the two buffers. In particular, the amino groups present in the molecule of TRIS buffer, at basic pH, could be covalent linked to the epoxysilane ring, probably creating a coating with a specific surface wettability. This new coating creation is further suggested by the asymptotic behavior of water contact angle value versus the reaction time, showed in Fig. 10. Oppositely, in the phosphate buffer, at neutral pH, the main reaction mechanism seems to be addressable to the hydrolytic effect on disiloxy bonds, confirmed by the different kinetics reported in Fig. 9, and by the low contact angle value typical of naked surface.

In order to correlate the surface wettability after stressing with the PCR performances BSA-coated and uncoated LoCs were chemically and thermally stressed at pH 7 and 95 °C for 2 hours and the PCR performances were measured before and after the stressing process. In Fig. 11 are reported the PCR yield product (ng/uL) performed on stressed LoCs.

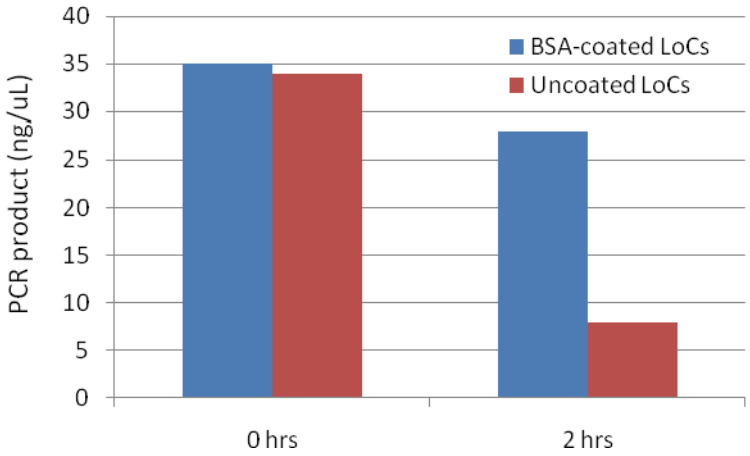

Fig. 11. PCR product yield for BSA-coated and uncoated LoCs before and after stressed process at pH 7, to 95 °C.

4. Conclusions

Data here reported indicate that BSA treatment improves thermal and pH stability of surface wettability, while uncoated substrate wettability is very sensible to both pH and temperature stressing. It is reported that disiloxy bonds (R-Si-O-Si), are cleaved at elevated pH values. Moreover at elevate pH value (pH=12) the contact angle measured in BSA-coated and uncoated substrate decreases with different weight, confirming the protective rule of BSA coating. Additionally, it was observed that the epoxysilane coating is also sensible to the buffer composition. Indeed, in amino based buffer (TRIS), differently from buffer phosphate yielding low contact angle values, at basic pH the sample surface does not achieve high hydrophilicity, as expected probably because a protective layer has been formed, thanks to TRIS amino termination, whose presence is reasonably confirmed by the asymptotic behavior of water contact angle and its value higher than buffer phosphate treated surface.

Therefore contact angle measurement provides a good means to gain information about the state of the surface wettability. Indeed it is usually observed that PCR efficiency depends on surface wettability.

Thus a reliable and stable surface wettability is crucial for PCR reproducibility performed into PCR microreactor. In this view BSA coating is a good method to perform a PCR-friendly surface finishing.

Further investigations will be addressed to study the BSA coating stability at different ionic strength values and humidity levels of finished surfaces storage.

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