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# Microfluidic Approach for Measurements of pH, $O_2$, and $CO_2$ in Saliva

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In this paper, we propose a microfluidic approach for measuring pH, dissolved oxygen ($O_2$), and carbon dioxide ($CO_2$) in human saliva. The proposed innovative method combines the advantages of a microfluidic approach, i.e., small amounts of samples and reagents and precise control of the experimental conditions, with rapid measurements of significant parameters of saliva. The novel design of a microfluidic chip with integrated commercially available PreSens sensors was used for examining the effect of Chlorhexidine on artificial saliva (AS), stimulated saliva (SS), and non-stimulated saliva (NSS). The measurement results showed that for persons with an initially low saliva pH, the use of Chlorhexidine increased the pH, and afterward, the pH value returned to the initial value or higher. However, measurements of volunteers with initial pH close to neutral showed that Chlorhexidine reduced the pH value, increasing the risk of erosion and demineralization. In conclusion, the proposed methodology showed potential for precise measurements of pH in saliva samples; however, further research is required to examine the influence of the sample collection method on the amounts of $O_2$ and $CO_2$ in saliva.

## 1. Introduction

Saliva is a complex biofluid that contains up to 99% water and different constituents including proteins, enzymes, and antibodies. Owing to the complexity of the saliva content, a lot of information about oral and general health, diseases, and allergies can be identified from saliva samples. Consequently, salivary diagnostics and theranostics have become an attractive field in the research and development of sensing devices for point-of-care (PoC) applications.(1)

Small amounts of samples and reagents and precise control of the measurement conditions are the main advantages of the microfluidic approach for sensing applications. The combination of the microfluidic approach and the integration of different sensors into a multifunctional lab-on-a-chip (LoC) system with the simple and non-invasive collection of saliva samples will lead to the realization of fast-sensing tools with good specificity and selectivity.(2)

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Different sensors have recently been developed for measurements of glucose[3] and nitrate and nitrite,[4] and the detection of bacterial pathogens,[5] biomarkers,[6] and diseases[7] in saliva. In addition to the detection of constituents of saliva, pH, $O_2$ content, and $CO_2$ content are important parameters that give information about the physicochemical properties of saliva and the chemical and biochemical processes that occur in the oral cavity.

A normal pH for the saliva of healthy persons is in the range of 6.2 to 7.6.[8] Owing to the buffering systems in the human body, the pH of all the fluids remains nearly constant. Therefore, even after consuming drinks and foods or applying acidic solutions, the salivary pH remains relatively stable. A recent review showed that the salivary pH may itself be used as a quick chairside diagnostic biomarker for numerous oral health conditions.[8]

Changes in the salivary pH are most evident instantly after the use of mouthwash, but the pH should return to its normal value within 30 min.[9] Dental erosion as a consequence of using mouthwashes has been discussed for decades. It was suggested that mouthwashes with a lower pH, such as Chlorhexidine, one of the most widely used oral rinse solutions, should never be applied long term.[10] It has been recently confirmed that Chlorhexidine could affect the oral microbiome and lead to increased environmental acidity.[11] In this study, the effect of Chlorhexidine on the pH, $O_2$, and $CO_2$ in saliva was examined using artificial and natural saliva.

The amounts of $CO_2$ and $O_2$ in saliva samples have rarely been analyzed in previous studies. The amount of $CO_2$ is usually estimated through the relation between the concentration of $CO_2$ in saliva and the pH value of saliva, which is based on the chemical reaction of the dissolution of $CO_2$ in water.[12] The products of the reaction are hydrogen and bicarbonate ions, which directly affect the pH value. Despite the fact that oxygen plays a significant role in chemical and biochemical processes in the oral cavity, no reliable quantitative data exist concerning its concentration in saliva,[13] although studies have shown that enzymatic reactions of glucose oxidase and catalase are responsible for the complete consumption of oxygen in saliva samples.[14]

The aim of this research is to miniaturize the system used for measurements of pH, $O_2$, and $CO_2$ in saliva and enable simultaneous measurements of all three parameters. A low-cost microfluidic chip with a novel design was produced using a hybrid fabrication technology that combines laser micromachining, xurography, and cold lamination, and enables the integration of commercially available PreSens sensors for pH, $O_2$, and $CO_2$.[15,16] The measurements were performed with artificial saliva (AS) and the natural saliva of five healthy volunteers. In addition, the effect of Chlorhexidine on the parameters pH, $O_2$ content, and $CO_2$ content was examined using non-stimulated saliva (NSS) and stimulated saliva (SS) samples.

## 2. Materials and Methods

### 2.1 Materials

The microfluidic chip used in this study for saliva analysis consisted of layers of poly(methyl methacrylate) (PMMA), a transparent and biocompatible polymer, with a thickness of 2 mm and bonding layers made of double-sided 3M 9088 tape (3M™ GPT-020F). A solution of

0.1% Chlorhexidine digluconate (Eludril Classic, Pierre Fabre Medicament) was used in an experiment with AS (Apoteka Beograd) and the natural saliva of five volunteers.

### 2.1.1 Equipment

PMMA layers were cut with a $CO_2$ laser (Gravograph LS1000XP). The 3M double-sided adhesive tape was cut to the required design with a Plotter Cutter (CE6000-60 PLUS, Graphtec America) with a 45° cutting blade (CB09U) and a cutting mat (12" Silhouette Cameo Cutting Mat). Bonding between the PMMA layers using the 3M tape was achieved through a cold lamination process with a uniaxial press (Carver 3895CEB). PreSens sensors (Precision Sensing) with precise plugs (SensorPlugs) for pH, $CO_2$, and $O_2$ were used for measurements, and a benchtop pH meter (Hanna HI5522) was used for calibration of the pH sensor.

### 2.1.2 Design and fabrication of microfluidic chip

The proposed microfluidic chip is composed of three transparent layers. The top layer contains inlet/outlet holes whose diameters were adapted for pipetting the sample during the filling of the chip. In addition, three holes with a diameter of 2.1 mm were made in the top layer for installing the commercially available PreSens sensors for pH, $CO_2$, and $O_2$. The sensors are in direct contact with the sample through the holes, which enables real-time measurement of the parameters in the sample. The reservoir for the sample, made in the middle layer of the chip, has a volume of 1.8 $mm^3$. The proposed design avoids using external actuators (syringes or pressure pumps) for filling and also requires small amounts of samples. The specifically designed curved edges in the chip prevent the formation of bubbles inside the reservoir. The proposed microfluidic platform has the potential for the additional integration of different sensors and actuators to realize more complex, multifunctional platforms.

The microfluidic chip was designed using a rapid and cost-effective hybrid fabrication technology that combines laser micromachining and xurography. The aim was to use rapid and cost-effective fabrication technology and biocompatible transparent materials. Therefore, PMMA was cut with the $CO_2$ laser and the 3M double-sided adhesive tape was cut with the Plotter Cutter in the same design. The double-sided adhesive tape enables rapid and reliable cold lamination bonding between layers, forming a durable structure that did not show any leakage. The multilayer structure of the microfluidic chip is presented in Fig. 1.

## 2.2 Methods

### 2.2.1 Experimental setup

Figure 2 presents the experimental setup. The PreSens SensorPlug sensors for pH, $CO_2$, and $O_2$ were placed in the special holes at the top layer of the microfluidic chip to provide direct contact with the sample. Optical fibers were connected between the sensors and the measurement device, and the whole system was connected with PreSens Measurement Studio 2 (PMS2) software. The pH and $O_2$ sensors were calibrated in accordance with the user manuals. The $CO_2$ sensor was delivered pre-calibrated.

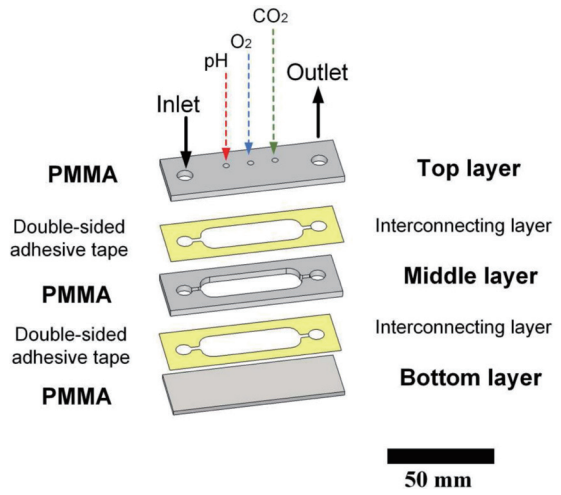


Fig. 1. (Color online) Multilayer structure of the microfluidic chip fabricated by combining laser micromachining, xurography, and cold lamination bonding. The top, middle, and bottom layers were made of PMMA, while the interconnecting layers were made of 3M double-sided adhesive tape.

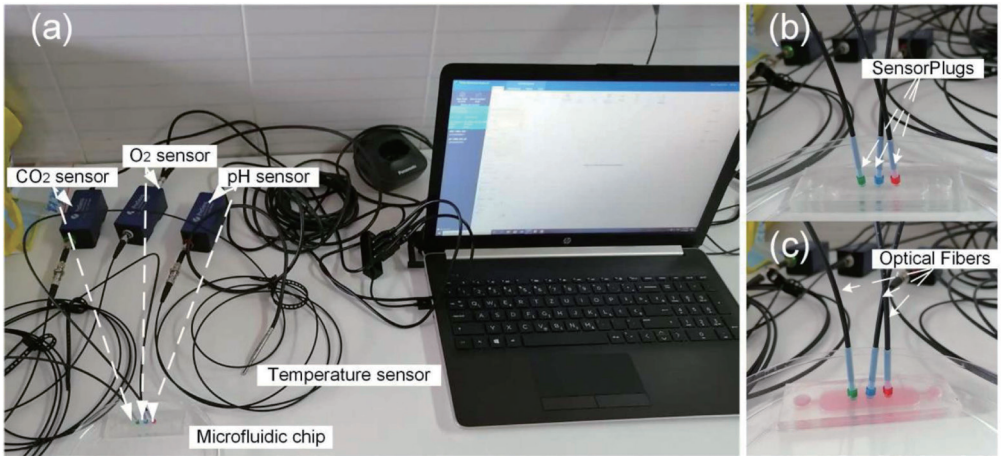


Fig. 2. (Color online) Experimental setup. (a) Microfluidic chip with saliva sample connected to PreSens sensors for measurements of pH, $O_2$, and $CO_2$; (b) microfluidic chip with saliva; (c) microfluidic chip with a sample of saliva with Chlorhexidine.

Considering that the range that the pH sensor can measure is between 5.5 and 8.5, four different solutions for pH sensor calibration were made and the pH values were measured with a benchtop pH meter. The solutions were made of phosphate-buffered saline (PBS) titrated with 0.1 M HCl to obtain solutions with different pH values. The pH values used for the calibration and the phase results are presented in Table 1.[16]

For the $O_2$ sensor calibration, air-saturated and oxygen-free water were prepared for two-point calibration. For the preparation of oxygen-free water, 1 g of sodium sulfite ($Na_2SO_3$) and 50 µl of cobalt nitrate [$Co(NO_3)_2$] standard solution [ρ(Co) = 1000 mg/l; in 0.5 mol/l nitric acid] were dissolved in 100 ml of water. Air-saturated water was made by creating air bubbles in 100 ml of water using an air pump for 20 min. The phase and temperature measurements for the two calibrating samples are presented in Table 2.[16]

Table 1
Calibration parameters for pH sensor (data from Ref. 16).

| pH | Phase (°) |
|---|---|
| 5.7 | 52.5810 |
| 6.3 | 48.0080 |
| 6.7 | 42.3190 |
| 8.46 | 21.7290 |

Table 2
Calibration parameters for $O_2$ sensor. $T_0$ and $T_{100}$ are the temperatures for oxygen-free and air-saturated water, respectively.

| | |
|---|---|
| Phase of oxygen-free water | 54.88° ($T_0$ = 27.33 °C) |
| Phase of air-saturated water | 21.98° ($T_{100}$ = 17.3 °C) |

Five healthy volunteers (two males and three females) participated in this research. The ages of volunteers 1 to 5 were 23, 28, 39, 45, and 49, respectively. Prior to collecting the samples, the volunteers did not brush their teeth or consume food and drink for at least 1 h. Samples were collected as follows: NSS was collected by volunteers using the "spitting method".[17] After that, SS was collected by spitting into glasses after chewing sterile paraffin wax for a few minutes.[18] Thereafter, the same collection methods were used 30 min after rinsing the mouth with Chlorhexidine solution (0.1% Chlorhexidine) for 1 min. For measurement purposes, SS and NSS were mixed with Chlorhexidine for 1 min in a 1:1 ratio. Measurements were performed with PreSens sensors in the following order: NSS, NSS mixed with Chlorhexidine, SS, SS mixed with Chlorhexidine, NSS after 30 min, and SS after 30 min. Saliva samples were injected into the microfluidic chip manually using a syringe.

### 2.2.2 Statistics

The data are presented in the form of the mean value and standard deviation. To evaluate how far data were from normality, the Shapiro–Wilk test was used. For inference, owing to the deviation of the distribution from normality, the significance of the difference between the examined research groups was tested by the Kruskal–Wallis test. The Statistical Package for Social Sciences (SPSS 20.0) was used for all statistical calculations together with Jamovi software (version 0.9.2.8).

## 3. Results

### 3.1 Chlorhexidine and AS

The initial experiments were performed with AS and AS mixed with Chlorhexidine. Firstly, pH, $O_2$, and $CO_2$ were measured separately for samples of AS and Chlorhexidine, and afterward, the AS and Chlorhexidine were mixed in a ratio of 1:1. The mean value and standard deviation of the results are presented in Fig. 3.

It can be seen that the measured pH value of AS was 7.078(9), which corresponds to a neutral environment, and after mixing with Chlorhexidine of pH 6.141(22) in a ratio of 1:1, the pH shifted to an acidic value of 6.72(3). The mixing process corresponds to mixing mouthwash with saliva inside the mouth. According to Ref. 12, $CO_2$ is dissolved in the saliva or in a bicarbonate form. The chemical reactions occurring in saliva,[12]

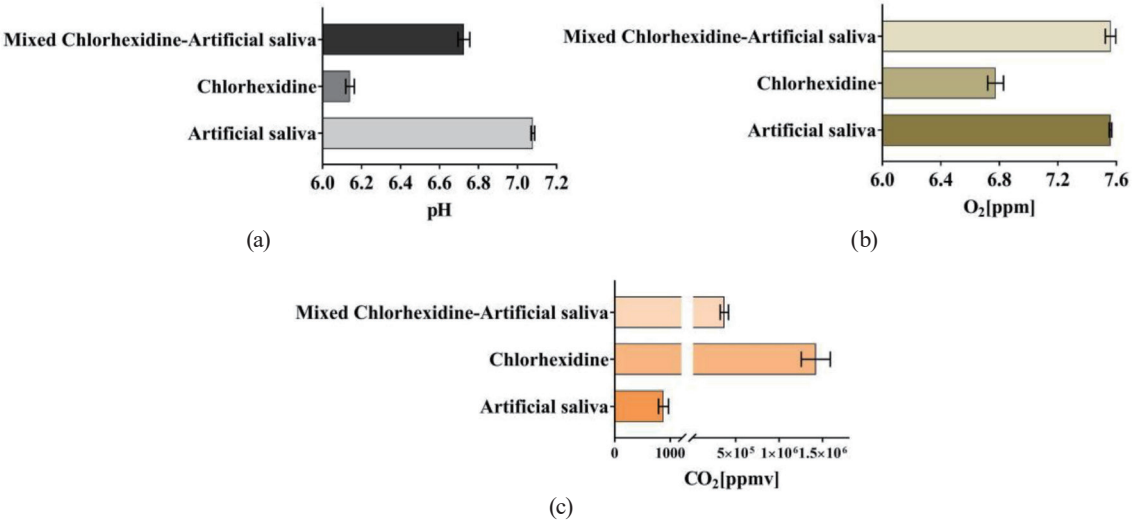


Fig. 3. (Color online) Measurement results of AS, Chlorhexidine, and AS mixed with Chlorhexidine. The results are presented mean values with standard deviation. (a) pH results; (b) $O_2$ results; (c) $CO_2$ results.

$$CO_2 \text{ dissolved} + H_2O \leftrightarrows H^+ + HCO_3^-, \quad (1)$$

indicate the relation between the $CO_2$ concentration and pH value. It is expected that for higher pH values, the concentration of $CO_2$ will be lower due to $CO_2$ dissolving in water and vice versa. Figure 3 presents mean values and standard deviations for measurements of pH, $O_2$, and $CO_2$ for AS, Chlorhexidine, and mixed AS and Chlorhexidine. In Figs. 3(a) and 3(c), the previous conclusion corresponds to the measured results for AS: the mean concentration of dissolved $CO_2$ for AS is $8.8(9) \times 10^2$ ppmv and pH = 7.078,[9] and the mean concentration of $CO_2$ for AS mixed with Chlorhexidine is $3.7(5) \times 10^5$ ppmv and pH = 6.72(3). The mean concentration of the measured dissolved oxygen in AS is 7.560(8) ppm, and it can be seen from Fig. 3(b) that in the case of AS, Chlorhexidine does not affect the amount of oxygen: the concentration of oxygen for AS mixed with Chlorhexidine is 7.56(3) ppm.

### 3.2 Volunteers

The effect of Chlorhexidine during and after usage was examined for samples from five healthy volunteers using NSS and SS. The results of pH, $O_2$, and $CO_2$ are presented in Figs. 4 and 5. Figures 4 and 5 present the same results from a different point of view. Concretely, from Fig. 4 it is easier to follow each volunteer before, with, and after Chlorhexidine while from Fig. 5 it is easier to compare the results from non-stimulated and stimulated saliva. Both approaches are equally important for the potential applications with a big number of volunteers. The initial pH of volunteer 1 was 6.764(4), which was the highest pH among the volunteers, indicating a healthy near-neutral environment. The other volunteers had low initial values of pH of less than 6, which can be an indication of oral and other health problems. Consequently, volunteer 1 can be considered as an example of a healthy person, allowing us to examine how Chlorhexidine

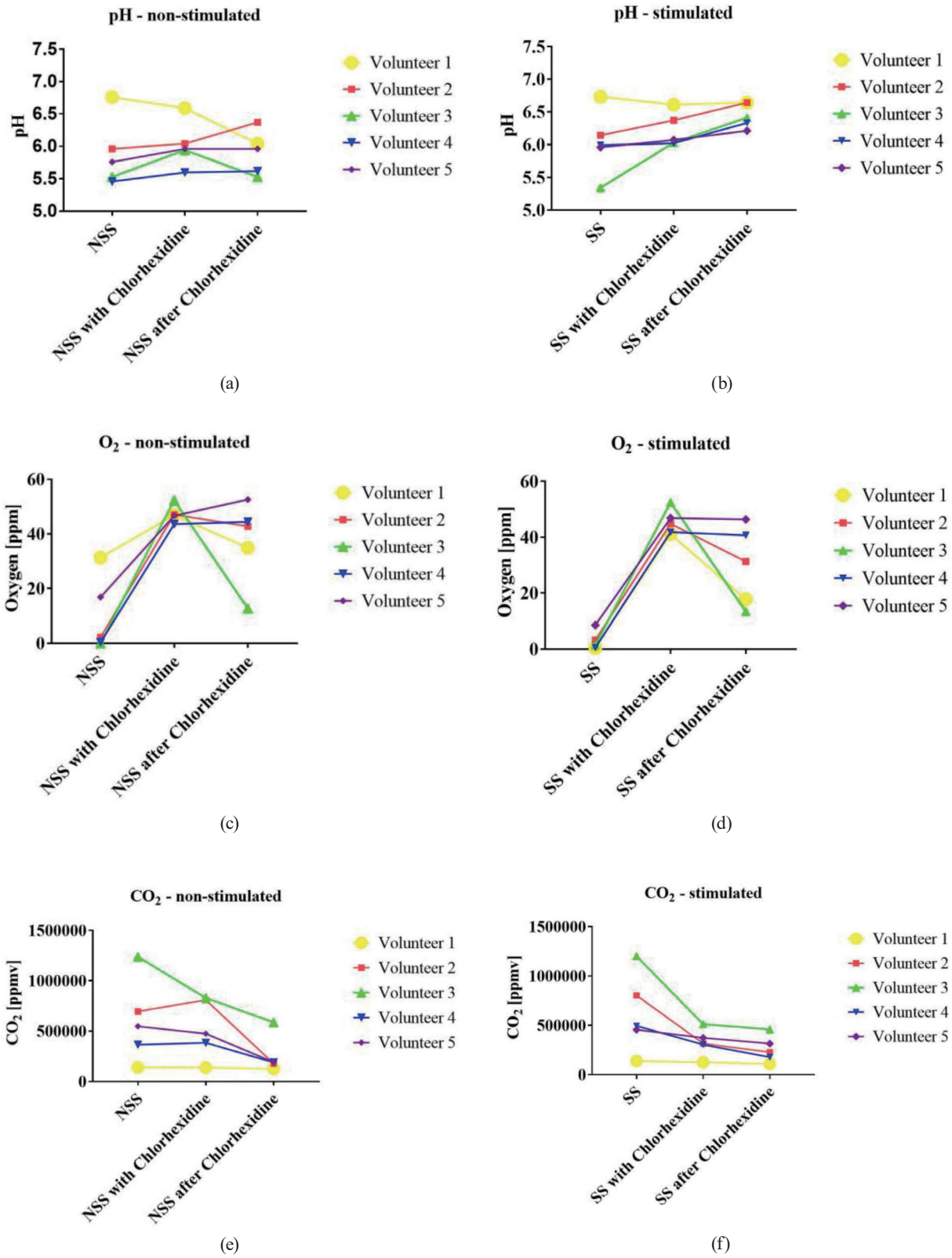


Fig. 4. (Color online) Measured pH, $O_2$, and $CO_2$ parameters for NSS and SS with and after using Chlorhexidine. (a) pH of NSS; (b) pH of SS; (c) $O_2$ of NSS; (d) $O_2$ of SS; (e) $CO_2$ of NSS; (f) $CO_2$ of SS.

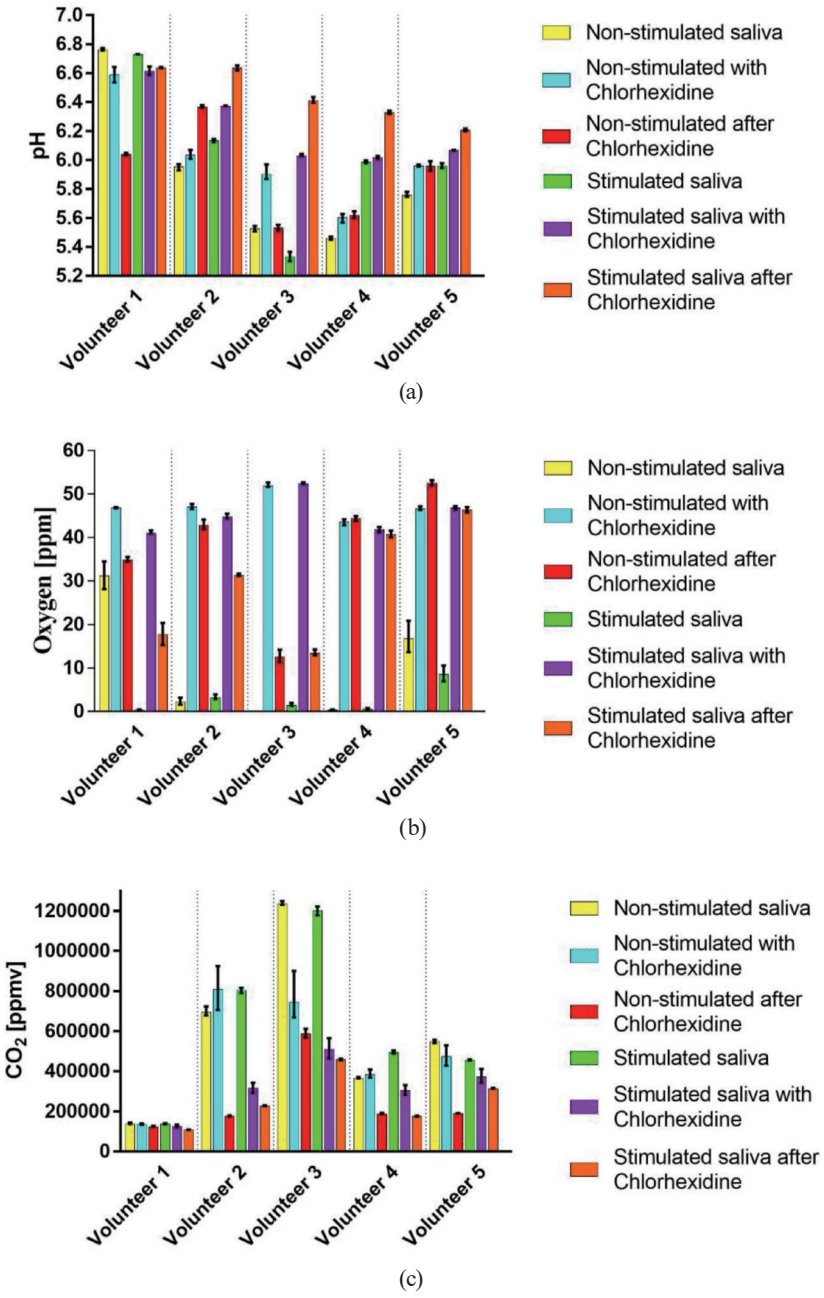


Fig. 5. (Color online) Comparation of pH, $O_2$, and $CO_2$ for volunteers with and after using Chlorhexidine for NSS and SS. (a) pH; (b) $O_2$; (c) $CO_2$.

influences the three parameters. Figures 4(a) and 4(b) show that the pH value of human saliva mixed with Chlorhexidine decreased only for volunteer 1, whose initial pH value was greater than that of Chlorhexidine, whereas the pH value increased for the other four volunteers. Measurements were also performed 30 min after using the Chlorhexidine mouthwash, and it can be seen that the pH value of NSS was restored to the initial value for volunteer 3, it increased for volunteers 2, 4, and 5, and decreased by 10% for volunteer 1. In the case of SS, all volunteers

except for volunteer 1 showed an increase in the pH value after using the mouthwash because of the higher pH value of the mouthwash, whereas the pH of volunteer 1 showed little variation. In conclusion, the effect of mouthwash is beneficial for persons with a low saliva pH, whereas for persons whose saliva pH is nearly neutral, the pH value is imbalanced after using a mouthwash, increasing the risk of demineralization and erosion of the enamel.

The results for $O_2$ and $CO_2$ have to be treated with caution due to the complexity of the sample collection. Firstly, the samples were collected in a glass, transferred to a syringe, and then injected into the chip. On the other hand, the samples were measured immediately after collection, so the time allowed for gas transition was minimized. The initial results for NSS and SS of the volunteers showed a small concentration of oxygen in the samples and a larger concentration of oxygen for the samples mixed with Chlorhexidine. By comparing the concentrations of oxygen in AS from Fig. 3(b) and in human saliva from Figs. 4(c) and 4(d), it can be seen that Chlorhexidine did not change the concentration of oxygen in the AS, in contrast to the increase in the concentration of oxygen observed for NSS and SS. From Figs. 4(e) and 4(f), it can be seen that the concentration of $CO_2$ for volunteer 1 was very low, corresponding to a near-neutral environment according to Eq. (1). Other volunteers had lower pH values and more $CO_2$ in their saliva.

We estimated the influence of Chlorhexidine by examining the pH values of NSS and SS with Chlorhexidine (ch) and after using Chlorhexidine (a_ch) in Fig. 6, where the values above the graph represent the initial pH value of the saliva for each volunteer.

Figure 6(a) shows the pH for NSS, while Fig. 6(b) shows the pH results for SS with paraffin wax. SS is more alkaline than NSS. Consequently, the pH of SS before using Chlorhexidine was higher than that of NSS before using Chlorhexidine for volunteers 2, 4, and 5. Volunteer 1 had almost the same pH value for NSS and SS, and volunteer 3 had a lower pH value for SS before using Chlorhexidine than that of NSS before using Chlorhexidine.

The results from Figs. 6(a) and 6(b) showed that the pH value of human saliva mixed with Chlorhexidine decreased only for volunteer 1, whose saliva had a pH value greater than that of

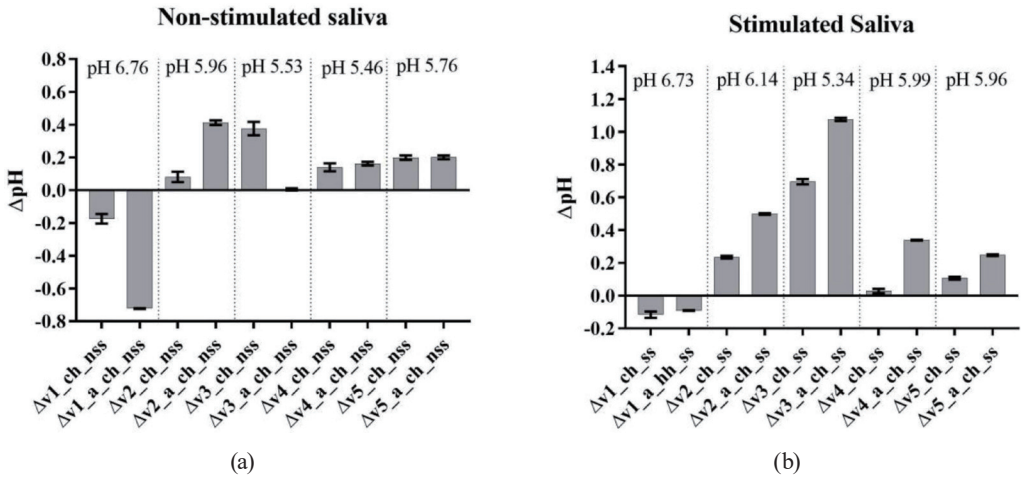


Fig. 6. Difference between pH value of natural saliva and (a) NSS with Chlorhexidine (ch_nss) and NSS after using Chlorhexidine (ch_a_nss); (b) SS with Chlorhexidine (ch_ss) and SS after using Chlorhexidine (a_ch_ss).

Chlorhexidine, whereas for the other four volunteers, the pH value increased after mixing with Chlorhexidine. Thirty minutes after using the mouthwash, the pH value of NSS was restored to the initial value for volunteer 3, increased for volunteers 2, 4, and 5, and decreased by 10% for volunteer 1. In the case of SS, the pH values of volunteers 2–5 increased after using the mouthwash, whereas that of volunteer 1 showed little variation.

### 3.3 Statistics

The mean values of the parameters pH, $O_2$, and $CO_2$ for all NSS and SS samples, all samples mixed with Chlorhexidine, and all samples measured 30 min after using Chlorhexidine are presented in Table 3. The results that are outside the recommended range of values for PreSens sensors are labeled in the table. In addition, Kruskal–Wallis analysis was performed to determine whether there was a statistically significant difference between measurements to determine the reliability of the measured results. The analysis showed that $p < 0.001$ for all measurements, which shows that the differences between the mean of the measured results are statistically significant.

## 4. Discussion

Recently proposed microfluidic platforms combine various biocompatible materials and fabrication technologies.[19,20] Commonly used materials for the fabrication of microfluidic chips are polymers such as poly(dimethylsiloxane) (PDMS), PMMA, polyvinyl chloride (PVC),

Table 3
Mean values of measurements for non-stimulated human saliva (NSS), stimulated human saliva (SS), NSS with Chlorhexidine (NSS_Ch), SS with Chlorhexidine (SS_Ch), NSS after using Chlorhexidine (NSS_a_Ch), and SS after using Chlorhexidine (SS_a_Ch).

| Volunteer | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| NSS pH | 6.76 | 5.96 | 5.53 | 5.46* | 5.76 |
| NSS Ch pH | 6.59 | 6.04 | 5.94 | 5.60 | 5.96 |
| NSS a Ch pH | 6.04 | 6.37 | 5.53 | 5.62 | 5.96 |
| SS pH | 6.73 | 6.14 | 5.34* | 5.99 | 5.96 |
| SS Ch pH | 6.61 | 6.37 | 6.03 | 6.02 | 6.07 |
| SS a Ch pH | 6.64 | 6.64 | 6.41 | 6.33 | 6.21 |
| NSS $O_2$ | 31.31 | 2.23 | 0.08 | 0.36 | 16.84 |
| NSS Ch $O_2$ | 46.86 | 47.09 | 52.32 | 43.59 | 46.69 |
| NSS a Ch $O_2$ | 34.93 | 42.80 | 12.66 | 44.44 | 52.59 |
| SS $O_2$ | 0.43 | 3.30 | 1.49 | 0.59 | 8.57 |
| SS Ch $O_2$ | 41.10 | 44.81 | 52.46 | 41.83 | 46.89 |
| SS a Ch $O_2$ | 17.79 | 31.34 | 13.45 | 40.73 | 46.38 |
| NSS $CO_2$ | 140045.35 | 695440.39 | 1238049.14 | 365804.47 | 548102.12 |
| NSS Ch $CO_2$ | 137628.46 | 808949.62 | 831078.05 | 384436.26 | 473825.02 |
| NSS a Ch $CO_2$ | 124728.64 | 177765.83 | 587093.33 | 189011.29 | 191430.86 |
| SS $CO_2$ | 138933.11 | 803707.66 | 1202196.36 | 493938.21 | 455156.38 |
| SS Ch $CO_2$ | 127170.66 | 315363.05 | 512157.38 | 304383.58 | 373142.45 |
| SS a Ch $CO_2$ | 109622.67 | 227674.60 | 458639.60 | 177346.92 | 313819.54 |

*Result outside the recommended range of values.

different glasses, ceramic materials such as low-temperature co-fired ceramics (LTCCs), and papers in combination, which are subjected to different bonding techniques. The aim of the above-mentioned technologies is to find an optimal, cost-effective, and rapid fabrication solution that uses biocompatible and transparent materials, and enables the integration of different sensing components into multifunctional, portable systems. Our miniaturized multifunctional system was fabricated by combining laser cutting of PMMA and cutting 3M double-sided tapes using xurography. The proposed technologies enable the precise and rapid fabrication of microfluidic chips, and the chamber design enables the integration of different sensors for simultaneous measurements in the sample. The commercially available PreSens sensors have shown high sensitivity and the potential for simple integration in microfluidic systems. The proposed setup for measuring pH, $O_2$, and $CO_2$ in saliva has good potential for establishing a new methodology for measuring the important parameters in saliva.

One of the most important aims in the early phases of any clinical trial is to obtain all the necessary information regarding the clinical applicability of new compounds without interfering with current clinical protocols. The recruitment of healthy volunteers stands for the ideal pattern for conducting these early phases of clinical trials, because it offers the possibility of safely investigating and documenting the tolerability of the treatment or diagnostic procedure without interference from associated pathologies. As a result, this approach has promoted a new procedure for first-in-man trials, defined as "phase I" of the pre-clinical trial, which is performed very early on a limited number of healthy volunteers who are exposed to newly established methodologies.[21]

Salivary pH value contains information about the environment in the mouth.[8] In addition, salivary pH has distinct daily, monthly, and annual rhythms.[22] Changes in salivary pH were evident instantly after the use of mouthwash, but pH was restored within 30 min.[9] The time required for normal oral pH levels to be restored depends on the salivary flow. It has been reported that this is an age-related characteristic that is similar for NSS and SS.[23] In addition, it has also been observed that SS is more resistant than NSS to changes in pH when exposed to acidic substances and that SS sampling is a good method to determine buffering capacity during oral health assessment.[24] The results of the present investigation can only partially confirm these results since the SS appeared to be more capable of resisting changes in pH only in volunteer 1, the volunteer with the highest pH values for both SS and NSS, while the SS and NSS of the other four participants showed a significant drop in pH.

Chlorhexidine is one of the most widely used mouthwashes in dentistry, which acts bacteriostatically at low concentrations and has a bactericidal effect at high concentrations. It also has a fungicidal effect. As a mouthwash, it has numerous uses, including preventative plaque control, preoperative and postoperative disinfection of the oral cavity, and the treatment of gingivitis and periodontal disease.[25] On the other hand, there are also concerns about its effectiveness in specific oral health conditions owing to its low viscosity, acidity, and lack of remineralizing potential.[26] The reported pH values of Chlorhexidine solutions vary from 5.50 to 6.88,[25] consistent with the present investigation.

Data regarding the oxygen content in saliva are rather limited. Cohen *et al.* reported surprisingly low concentrations of oxygen, often less than 0.08 ppm.[13] The samples analyzed in

the present investigation may be to some extent different from the saliva present in the oral cavity due to the collection procedure and technique, the transfer to the chip, and the analysis, factors that may have significantly affected the $O_2$ content. We presume that $O_2$ is quickly dissolved in saliva and is probably consumed in numerous enzymatic reactions. The time elapsed between saliva collection and analysis could also have significantly affected the obtained results. That is probably the reason why the absolute data regarding $O_2$ content in saliva are contradictory. The majority of literature sources refer to $O_2$ concentrations in the range between 0.18 and 0.25% but do not give precise information on the method of determination, and saliva is frequently described by indefinite expressions such as "an aerated electrolyte solution".[13]

It has been reported that the concentration of dissolved $CO_2$ can be less than 1 mM in NSS[27] and reach 60 mM in highly SS.[28] The results from our study cannot confirm these findings, since these extreme variations were not observed among our SS and NSS samples, although significant variations were recorded among the five volunteers and also after the use of Chlorhexidine.

No regularity was observed for the pH, $O_2$ content, and $CO_2$ content for the five volunteers. However, in the case of an initially low saliva pH in patients, the use of Chlorhexidine increases the pH value of saliva, and in the case of an initially near-neutral pH, the pH value is reduced, which increases the risk of erosion and demineralization.

## 5. Conclusion

In this paper, we proposed a microfluidic approach for measurements of three important parameters of saliva (pH, $O_2$ content, and $CO_2$ content) in a novel microfluidic chip. The proposed microfluidic chip with a novel chamber design enables the integration of different sensors for simultaneous measurements of the different parameters of saliva, and in this study, we proposed the integration of commercially available PreSens sensors.

A rapid and simple hybrid manufacturing process that uses the laser micromachining of transparent and biocompatible PMMA, and 3M double-sided adhesive tapes cut using xurography, enabled the fabrication of the microfluidic chip in a few minutes. The proposed technology did not show any leakage or problems with bubbles. In addition, the proposed design uses small amounts of samples and reagents for experiments; the volume of saliva samples collected by the spitting method from volunteers in this study was 1.8 $mm^3$.

The proposed method of measurements has strong potential for rapid and reliable measurements of pH in saliva. However, further research is required to estimate the influence of the method of collecting saliva and the influence of inlet/outlet holes on the concentrations of $O_2$ and $CO_2$.

The results of measurements for five volunteers showed that in the case of an initially low saliva pH, the use of Chlorhexidine increased the pH, and after 30 min, the pH value was the same as the initial value or higher, which is beneficial in terms of salivary pH. On the other hand, for the volunteer whose initial pH was close to neutral, the use of Chlorhexidine reduced the pH value, increasing the risk of erosion and demineralization.

Finally, the concentrations of $O_2$ and $CO_2$ in human saliva did not show any specific trend, but the low values of standard deviations showed that during the time of measurements, the gas concentration did not change in the MF chip. Therefore, the proposed system behaves like a closed system and enables reliable measurements of gas concentration. In addition, some further research has to be done with different methods of sample collection.

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## References

1 A. Ilea, V. Andrei, C. N. Feurdean, A. M. Băbțan, N. B. Petrescu, R. S. Câmpian, A. B. Boșca, B. Ciui, M. Tertiș, R. Săndulescu, and C. Cristea: Biosens. **9** (2019) 1. https://doi.org/10.3390/bios9010027
2 C. M. Pandey, S. Augustine, S. Kumar, S. Kumar, S. Nara, S. Srivastava, and B. D. Malhotra: Biotechnol. J. **13** (2018) 1. https://doi.org/10.1002/biot.201700047
3 L. F. De Castro, S. V. De Freitas, L. C. Duarte, J. A. C. De Souza, T. R. L. C. Paixão, and W. K. T. Cotro: Anal. Bioanal. Chem. **411** (2019) 4919. https://doi.org/10.1007/s00216-019-01788-0
4 F. T. S. M. Ferreira, R. B. R Mesquita, and A. O. S. S Rangel: Talanta **219** (2020) 1. https://doi.org/10.1016/j.talanta.2020.121183
5 Z. Chen, M. G. Mauk, J. Wang, W. R. Abrams, P. L. Corstjens, R. S. Niedbala, D. Malamud, and H. H. Bau: Ann. N. Y. Acad. Sci. **1098** (2007) 429. https://doi.org/10.1196/annals.1384.024
6 B. Johannsen, L. Müller, D. Baumgartner, L. Karkossa, S. M. Früh, N. Bostanci, M. Karpíšek, R. Zengerle, N. Paust, and K. Mitsakakis: Micromach. **10** (2019) 1. https://doi.org/10.3390/mi10120833
7 J. Noiphung, M. P. Nguyen, C. Punyadeera, Y. Wan, W. Laiwattanapaisal, and C. S. Henry: Theranostics **8** (2018) 3797. https://doi.org/10.7150/thno.24941
8 S. Baliga, S. Muglikar, and R. Kale: J. Indian Soc Periodontol. **17** (2013) 461. https://doi.org/10.4103/0972-124X.118317
9 E. Tolentino, L. E. Chinellato, and O. Tarzia: J. Appl. Oral Sci. **19** (2011) 90. https://doi.org/10.1590/s1678-77572011000200002
10 H. Pontefract, J. Hughes, K. Kemp, R. Yates, R. G. Newcombe, and M. Addy: J. Clin. Periodontol. **28** (2001) 319. https://doi.org/10.1034/j.1600-051x.2001.028004319.x
11 R. Bescos, A. Ashworth, C. Cutler, Z. L. Brookes, L. Belfield, A. Rodiles, P. Casas-Agustench, G. Farnham, L. Liddle, M. Burleigh, D. White, C. Easton, and M. Hickson: Sci. Rep. **10** (2020) 1. https://doi.org/10.1038/s41598-020-61912-4
12 P. Grøn and A. C. Messer: Arch. Oral Biol. **10** (1965) 757. https://doi.org/10.1016/0003-9969(65)90129-9
13 F. Cohen, G. Burdairon, F. Rouelle, and M. Chemlam: Electroanalysis **1** (1989) 523. https://doi.org/10.1002/elan.1140010608
14 M. R. Korayem, M. Traudt, and I. Kleinberg: Arch. Oral Biol. **35** (1990) 759. https://doi.org/10.1016/0003-9969(90)90100-o
15 PreSens, "Optical Sensors for Industries and Research" 2020. [Online]. Available: https://www.presens.de/#c123 (accessed 26 November 2020).
16 I. Podunavac, S. Hinić, S. Kojić, N. Jelenčiakova, V. Radonić, B. Petrović, and G. M. Stojanović: Appl. Sci. **11** (2021) 1. https://doi.org/10.3390/app11052049
17 J. D. Rudney, R. K. Staikov, and J. D. Johnson: Arch. Oral Biol. **54** (2009) 91. https://doi.org/10.1016/j.archoralbio.2008.08.007
18 T. C. Hart, P. M. Corby, M. Hauskrecht, O. Hee Ryu, R. Pelikan, M. Valko, M. B. Oliveir, G. T. Hoehn, and W. A. Bretz: Int. J. Dent. **196721** (2011) 1. https://doi.org/10.1155/2011/196721
19 B. K. Gale, A. R. Jafek, C. J. Lambert, B. L. Goenner, H. Moghimifam, U. C. Nze, and S. K. Kamarapu: Inventions **3** (2018) 1. https://doi.org/10.3390/inventions3030060

20 S. Kojic, S. Birgermajer, V. Radonic, I. Podunavac, J. Jevremov, B. Petrovic, E. Markovic, and G. Stojanovic: Microfluid. Nanofluid. **24** (2020) 1. https://doi.org/10.1007/s10404-020-02372-0
21 G. Pasqualetti, G. Gori, C. Blandizzi, and M. Del Tacca: Eur. J. Clin. Pharmacol. **66** (2010) 647. https://doi.org/10.1007/s00228-010-0827-0
22 A. Pachori, H. Kambalimath, S. Maran, B. Niranjan, G. Bhambhani, and G. Malhotra: Int. J. Clin. Pediatr. Dent. **11** (2018) 177. https://doi.org/10.5005/jp-journals-10005-1507
23 P. Anderson, M. P. Hector, and M. A. Rampersad: Int. J. Clin. Pediatr. Dent. **11** (2001) 266. https://doi.org/10.1046/j.1365-263x.2001.00293.x
24 M. Moritsuka, Y. Kitasako, M. F. Burrow, M. Ikeda, and J. Tagami: Aust. Dent. J. **51** (2006) 170. https://doi.org/10.1111/j.1834-7819.2006.tb00422.x
25 E. Varoni, M. Tarce, G. Lodi, and A. Carrassi: Minerva Stomatologica **61** (2012) 1. PMID: 22976567
26 M. Chevalier, C. Sakarovitch, I. Precheur, J. Lamure, and V. Pouyssegur-Rougier: Acta Odontol. Scand. **73** (2015) 267. https://doi.org/10.3109/00016357.2014.923108
27 I. L. Shannon, W. A. Gibson, and H. H. Chauncey: J. Dent. Res. **42** (1963) 179. https://doi.org/10.1177/00220345630420011501
28 J. H. Thaysen, N.A. Thorn, and I. L. Schwartz: Am. J. Physiol. **178** (1954) 155. https://doi.org/10.1152/ajplegacy.1954.178.1.155