Original Article

Paper-Based Device for First Level Screening of Nitric Oxide in Saliva of Caries Active and Caries Free Children

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Abstract

Background: For most developing nations, the development of point-of-care diagnostics is an essential program as it provides a facile and acceptable route to reach the lowest strata of their population. Saliva which is composite, offers a wide range of information on the physiological requisites, is one of the most resourceful body fluids.

Objectives: Nitric Oxide (NO) is a reliable biomarker, and the determination of NO in saliva provides a methodology to distinguish between patients with and without caries. This work is based on the standardization of fabrication of paper-based devices for the first level screening of caries-free and caries active children based on NO spotting.

Materials and Methods: Using spectrophotometer, a standard graph of concentration of nitrite vs. absorbance was plotted to understand the linear range of nitrite (indirect NO level).

Results: This work established the significance of paper-based microfluidics for the nitric oxide concentration range from 10 μM to 950 μM. First level screening of saliva samples of caries active and caries-free children for NO detection using paper-based device indicated that NO could be used as a biomarker using this device.

Conclusions: Nitric oxide as a biomarker for identifying caries in children has already been established, and this work furthers this effort in developing a paper-based microfluidic approach. Further studies are warranted to establish the use of this device for NO screening of this disease.

Key words : Paper Microfluidics; Nitric Oxide; Saliva; Caries.

Introduction

L – Arginine is an essential amino acid. The guanidine group present in N – terminal is oxidized by the presence of enzyme nitric oxide synthase, resulting in the release of endogenous nitric oxide. Nitric oxide synthase is present in two functional forms. Constitutive forms generally generate less amount of nitric oxide (NO), and they are endothelial or neuronal origin. Smooth muscle cells, endothelial cells, macrophages, neutrophils highly express nitrogen oxide synthase that increases the level of NO. NO has strong reactivity with oxides like superoxide and with oxygen. NO is water-soluble gas and increased or decreased levels in saliva or urine, or serum is an indicator of various diseases or inflammatory processes.[1,2] The human salivary proteome reveals that saliva expressed peptides and proteins that are microbial genesis, and the number extending to more than 3000.

Moreover, saliva is a beneficial diagnostic fluid as its collection is very cheap, and the collection procedure categorized as non – invasive methods.[3] Next-generation sequencing reveals that the number of microbial florae in the oral cavity can be as high as 10,000. For many diseases like heart disease, patients are susceptible to infectious diseases associated with bacteria, especially dentalcaries that are caused by invasive pathogens.[4]

Such patients are more likely to have increased risk of diseases like Dental Caries (DC), defect in enamel, etc. pH of the saliva plays vital role in the identification of dental caries and its development. DC process mainly involves the demineralization and remineralization process. DC results in demineralization of dental enamel. Bacteria produce various kinds of acid, which is a by-product of the fermentation process.[5, 6] These organic acids thus lead to demineralization in DC. Saliva can protect against DC in various ways like preventing the accumulation of plaque, minimizing enamel solubility, buffering capacity, and also a role in antimicrobial activity. The presence of NO in the saliva is due to the presence of dietary nitrates. The presence of NO can protect dental enamel from caries, and its reduction can lead to the survival of cryogenic bacteria, which in turn results in DC. Therefore, it is essential to realize that the salivary NO level is an indicator for diagnosing early childhood DC development.[7]

Earlier reports enlist a detailed review of various analytical techniques to assay NO bioactivity.[8] The paper concludes with a statement that determination of NO, which is a significant bio-marker, can be done by rapid bedside point–of–care devices by thorough validation. The use of oral fluids for disease diagnostics in recent times has emerged as very timely and reliable source of early detection of biomarkers.[9] Reports elsewhere provided a pilot study on patients affected with periodontitis and found that they correlate with salivary and serum NO markers.[10] In 2014,for the first time researchers used a paper-based device to determine nitrate level (indirect NO level) using Griess Reagent.[11] In 2018, researchers could fabricate a paper-based device for the simultaneous detection of pH and nitrate.[12]

The health of the population can be monitored by analyzing biological fluids; however, in rural regions with less industrialization, under emergency circumstances or a personalized home check, this becomes difficult. Though regular laboratory type of equipments or instruments provide measurements that are quantitative of analytes present in biological samples under emergency and stressful situations, they are not apt as the need for trained personnel or expensive tools or the need for a considerable amount of biological samples.[13] A fundamental requirement for the development of such paper-based systems lies in the generation of hydrophobic barriers that define the hydrophilic channels and test zones of the paper substrate.[14] Several fabrication methods have been reported in the literature to identify the hydrophobic barriers, wax printing being one of the most. This fabrication method enables the generation of hydrophobic barriers in paper substrates by directly printing them with wax-based ink.[15]

The fabrication of earliest microfluidic paper-based analytical devices is using photoresist to define flow boundaries; however, the cost of photoresist and the potential for background reactivity makes this fabrication method less than desirable. The limitations of photoresist methods led to the development of low-cost fabrication methods that made use of wax and similar materials instead of photoresist. Creating barriers made from wax provides a low cost, easily accessible fabrication technology using relatively inert materials to contain the fluid flow. Wax in particular, can be applied to the paper using a variety of techniques and readily melts through the paper substrate with heat to create a three-dimensional barrier.[166]

Based on the identified opportunities in the literature, this study aims to develop first-level screening, a paper-based diagnostic device for the detection of nitric oxide in caries active and caries-free children.

Materials and Methods

1. Chemicals/Materials used

Sodium Nitrate, 1-(1-Napthylethylenediamine), Sulphanilamide, Phosphoric Acid, Whatman No1 Filter Paper, Wax, glass slide.

2. Strip Fabrication

The glass slide (template) was made ready with the button cell that acted as a mask. Whatman no.1 filter paper cut was in such a way that it was made to fit the size of the glass slide, aligned and compressed. Then it was immersed into the heating mantle containing the hot wax and was taken out (this whole process carried out within 2-5 seconds). Further, it was air-dried for 2-5 minutes at room temperature. The mask in the template created a fluid cavity in the strip of 7 mm, and the length of the glass slide was about 7.8 cm. The gap between each mask for the cavity was aligned such that the distance between them was about 0.5 cm. Such alignment created a paper strip containing five fluidic chambers. Fluidic chambers surrounded by the hydrophobic wall and the reaction zone had a capacity of 5 µL. Five fluidic chambers can be used for detection by this fabricated paper strip. These strips were used in the following process.[17]

3. Preparation of Griess reagent for estimation of NO by UV-Vis Spectroscopy and by paper-based detection

For the estimation of NO[18], Griess reagent for reaction and sodium nitrate as the standard used. Forthe preparation of 1mM of sodium nitrate, 3.45 g of sodium nitrite dissolved in 50 mL of distilled water, which makes 1 M. From this 10 µL solution was taken and added in 9.990 mL of milli-Q-water

that makes the 1 mM sodium nitrate solution. Griess reagent was prepared by adding 500 µL of 0.1% N-(1-Naphthyl) ethylenediamineto 500 µL sulphanilamide and dispersed together. 0.1 % N-(1-Naphthyl) ethylenediamine was dissolved in distilled water and mixed well further stored at 4°C. 8.61 g of sulphanilamide and 2.5 mL of phosphoric acid dissolved in 50 mL of distilled water, these two used in the preparation of Griess reagent. Griess reagent is prepared freshly for each usage.

4. Saliva Sample collection

Institutional Human Ethics Committee of Chettinad Academy of Research & Education (CARE) approved the study (Proposal No: 156/IHEC/12-16). Children between the age group of 6-12 years of Caries active and Healthy participants were selected. The informed consent form was obtained from the children's parents. The volunteers were chosen from the Chettinad Dental Hospital and Research Institute, Kelambakkam. The exclusion criteria include children with fever, immune-compromised patients, any other dental disease like periodontitis and participants with any other infectious diseases. The children with caries identified with the help of a dental physician and the preliminary details recorded and samples were collected accordingly.

Sample Group: On total 4 number of samples were used and divided into 2 control and 2 active samples respectively

Sample Collection: The participants were first instructed to rinse their mouth with water and the secreted saliva collected in the centrifuge tube, the participants were asked not to cough up mucus, and the collected samples stored at -80°C. The stored samples were centrifuged at 4500 rpm for 15 min, the pellet was discarded, and the supernatant was stored for further study.

5. Estimation of NO by UV-V is Spectroscopy

A standard plot was drawn using 20 different concentrations of sodium nitrite (1.5 µM to 31 µM). Different concentrations of sodium nitrite was taken in separate tubes and freshly prepared Griess reagent was added. The solution was incubated for 10 mins and reading was recorded using a spectrophotometer. The standard plot was drawn and accordingly, the concentration of NO in saliva was determined using the standard graph.

6. Detection of NO by paper-based method in saliva

The fabricated strip was used to understand the change in the intensity of color by varying the concentration of NO. Accordingly, 2 µL of 20 different concentrations ranging from 10 to 950 µM was added to the cavity of paper strip and compared with the chamber that did not contain sodium nitrite. Different concentrations of sodium nitrate was added in the paper strip and allowed to dry at room temperature for 2 mins. 2 µL of Griess reagent was loaded into the fluid cavity. Visible color development was noticed in most of the concentrations, at lower concentration the color change visibility was less when compared to that at higher concentration. For determination of NO in saliva, samples control, 2 µL of control/caries active samples were loaded, and 2 µL of Griess reagent was added to the cavity of the paper strip. The strip was dried at room temperature for 2 mins. The color change indicated the level of NO in caries active and caries free saliva sample. The strips were photographed for comparison.

Results

1. Fabrication of Paper-Based Microfluidic Device

Paper-based fluidic device was fabricated using wax templates and standardized. The steps involved in the fabrication are depicted in Fig. 1. Each strip fabricated contained five chambers for analyte detection.

2. Standard plot-concentration of sodium nitrate versus absorbance

Increasing concentrations of sodium nitrite (indirect NO level 1.5 – 31 µM) was allowed to react with Griess reagent, and the absorbance spectra was recorded. It is the preliminary test for the detection of nitrite ions present in the solution. The test is based upon the formation of chromophore when Griess reagent reacts with the solution containing nitrite. In this reaction, the acidified nitrite undergoes diazotization with sulphanilamide. It leads to the production of diazonium salt, which undergoes a coupling reaction with the naphthylethylenediamine and forms a pink color diazo compound that gives characteristic peak in the absorption spectra. The λmax was found to be at 540 nm. Standard graph was plotted and this plot can be used to determine the unknown concentration of nitric oxide in saliva (Fig. 2 A and Fig 2 B)

3. Paper-based device spotted with increasing concentration of sodium nitrite

Fig.3 provides information about the images of a paper-based device with an increasing concentrationsof nitric oxide. The concentrations used in this study is in the range of 10 µM to 950 µM. Note that 1 indicates no nitrite added to strip and 2 to 20 indicates increasing concentration of nitrite from 10, 50, 100, 150 to 950 µM.

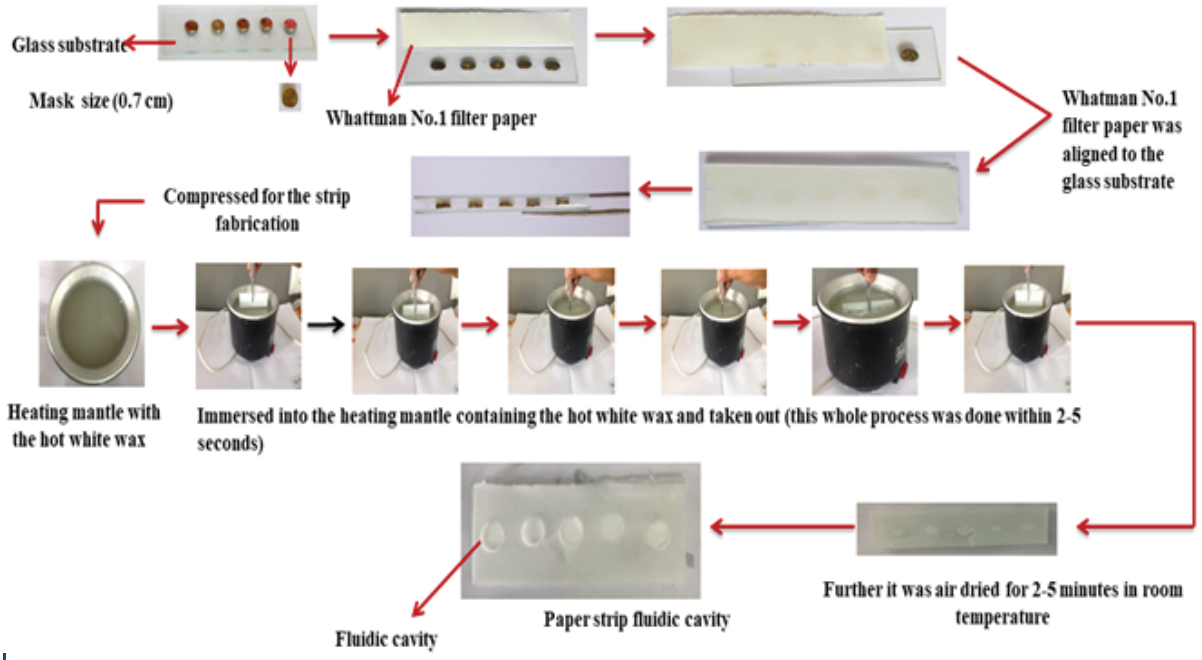

Figure 1: 1Steps involved in the fabrication of paper-based device

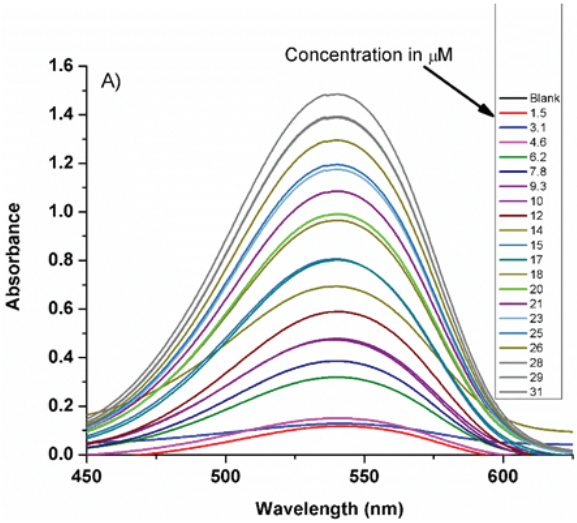

Figure 2a: Absorption spectra of increasing concentration of nitrite

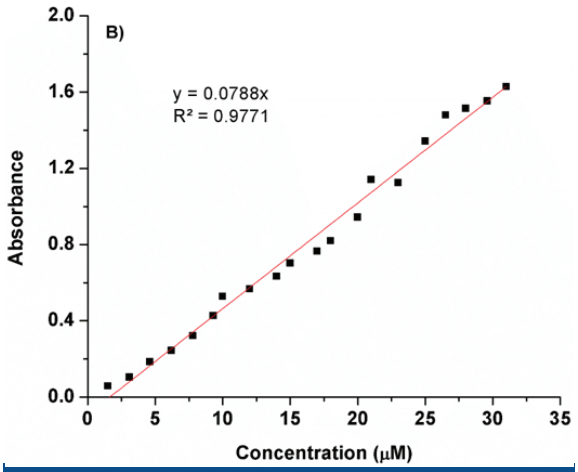

Figure 2b: Nitrite Calibration curves for the determination of NO using the method of Griess reaction

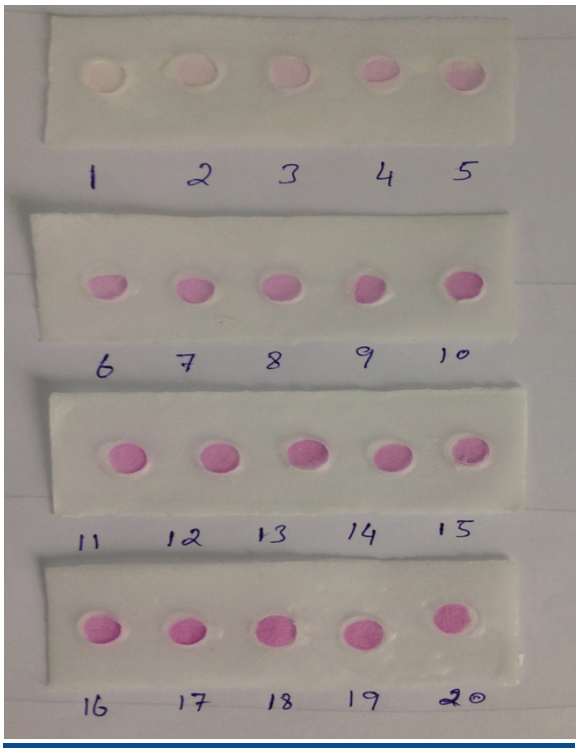

Figure 3: Paper-based device spotted with increasing concentrations of sodium nitrite.

4. Paper-based device for detection of NO in saliva

Fig.4 depicts the screening of NO level in caries-free (C1 and C2) and caries active (A1 and A2) participants. Initial results suggested that caries-free indicated spotting of NO as seen by light pink color, while caries active did not show any change in color of cavity when the sample was placed.

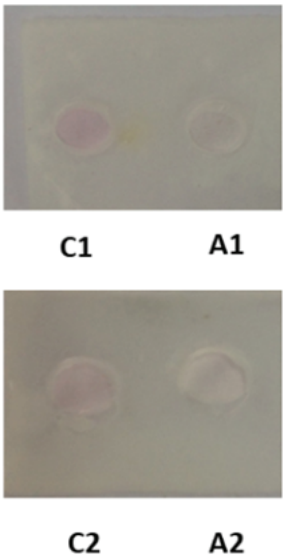

Figure 4: Caries free (C1 and C2) and Caries active (A1 and A2) spotting by paper-based device

Discussion

When compared to conventional microfluidic devices, paper-based devices offer lower fabrication costs and do not require extensive microfabrication facilities, making them practical for point of care diagnostics in limited-resources environments. For an ideal analytical test, the World Health Organization has laid seven guidelines. Specifically, tests must be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users. When compared to these benchmarks, the traditional analytical device is not adequate as a perfect analytical tool because it can be seen that they are neither equipment-free nor affordable.[19] Fig.1 describes the step-wise method by which paper-based fluidic device that can be used for analytes detection. The fluid cavity that is formed is 7 mm in diameter,which is used for analyte detection.

NO can be indirectly measured by understanding the steady decay of nitrate and nitrite with the help of a spectrophotometer. The measurement is by the reduction of nitrate to nitrite, which is then resolved by Griess reaction. This reaction involves two steps, first a diazotization reaction wherein the nitric oxide derived dinitrogen trioxide, a nitrosating agent is generated. Next is by the acid-catalyzed development of nitrous acid from nitrite. Griess reagent that contains sulphanilamide reacts with this to produce a diazonium ion. It is here that N-(1-napthyl)-ethylenediamine of Griess reagent couples with the ion to form a colored azo product that can absorb light strongly at 540 nm.[20] In the present study, a standard graph was plotted with increasing concentration of sodium nitrite. To understand the linearity effect, increasing concentrations of nitrite with change in absorbance was measured at 540 nm and it is inferred that Lambert – Beer's law is obeyed in this range. Calibration curves were constructed for the nitric oxide concentration ranging from 1.5 µM to 31 µM. Sample concentrations can be determined using linear curve fit (Fig. 2A and 2B).

There is constant supply of nitrates that is concentrated by the metabolic products in the environment of the oral cavity of human beings. It has been postulated earlier that the metabolic products of nitric oxide and nitric oxide is deleterious to humans. However in recent times, research has shown enough evidence on the favourable properties of NO including antimicrobial activity, which is very significant to the oral cavity. Enzyme nitrate reductase present in the oral cavity catalyzes the reduction of nitrate to nitrites. The acidification happens when in contact with the micro flora of plaque like Lactobacillus, Streptococcus mutants and Actinomyces. It is this acidification that results in the production of nitrogen oxides and nitrous acid, which is a complex mixture. As nitrous oxide is unstable, it rapidly decomposes to form nitric oxide.[21] Earlier studies have shown that salivary nitric oxide was higher in the caries-free group as compared to the caries-active group which suggests that increased production of nitric oxide in the oral cavity may contribute to lower caries incidence in children. In the current study, for the 4 participants included spotting on paper strip indicated a reduced level of NO (as there did not indicate a color change) in active group as compared to control group (Fig.3).

Current research has indicated a significant increase in the interest in the development of microfluidic paper-based devices that are created by patterning hydrophobic materials in the hydrophilic paper. When integrated with conventional devices microfluidic offers less cost, portable, straightforward, and it seems promising.[22] The current method is based on a single-step way that uses Whatman filter paper and wax templates designed with a pattern for the fabrication of a paper-based strip. The design developed uses effortless skills and minimal equipments that are easier for fabrication. Calibration standards were prepared using standard sodium nitrite and covered a 10 – 950 µM range of nitrite, including a blank solution (Fig 3.). When coupled with measurable means by which intensity of the color can be measured with a portable device, the process can become a standard gold technique for NO detection. Griess reagent also provided a suitable acidic environment for the reaction that played a role in the development of color.

Conclusions

Literature is upbeat on the possibilities of microfluidics as point-of-care diagnostic devices. The facile and cost effectivity of paper-based microfluidics has furthered this effort. This work looks at the development of paper-based microfluidics for distinguishing between participants with and without caries. The salient outputs from this work are optimizing the procedure for paper-based microfluidics in the estimation of NO. The methodology developed indicates a visual difference in NO content of caries active and caries-free participants. Further studies are warranted to establish the use of paper strip in differentiating caries active and caries-free detection quantitatively. In essence, this work presents a new opportunity to use a paper-based microfluidics approach to distinguish between caries active and caries-free children by the visual difference of nitric oxide level in saliva.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

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