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On-hand tool for ammonium and urea determination in saliva to monitor chronic kidney disease – Design of a couple of microfluidic paper-based devices

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ABSTRACT

In this work, two microfluidic paper-based analytical devices (μPADs) were developed for the quantification of urea and NH_x in human saliva to aid in the diagnosis/monitoring of chronic kidney disease (CKD). The NH_x determination was based on the conversion of ammonium to ammonia, followed by its diffusion through a hydrophobic membrane and then the color change of bromothymol blue (BTB) indicator. In the urea determination, prior to the ammonium conversion and BTB color change, the enzymatic conversion of urea into ammonium was produced, using urease. Several optimization studies were carried out to attain a quantification range of 0.10–5.0 mM with 0.032 mM limit of detection for the NH_x μPAD, and a determination range of 0.16–5.0 mM with 0.049 mM limit of detection for the urea μPAD. The method accuracy was assessed, and the measurements obtained with NH_x μPAD were compared with the ones obtained from an ammonia ion selective electrode; while the measurements of the urea μPAD were compared with the ones obtained from a commercially available kit. There were no statistically significant differences between methods, proving that both NH_x and urea μPAD were effective on-hand tools for CKD monitoring in saliva. To evaluate their functionality as point-of-care devices, stability studies were also performed and revealed that both NH_x and urea μPAD were stable when stored in a vacuum for 2 and 1 month, respectively. After the sample introduction, the NH_x μPAD could be scanned within the first 2 h and the urea μPAD within 1 h.

1. Introduction

The human body is an intricate and complex ‘machine’ in which every organ has a purpose. The kidneys, among other functions, are responsible for blood filtration, removing wastes and toxins, and regulating blood chemicals that are essential to life [1].

Chronic Kidney Disease (CKD) is a condition in which the kidneys malfunction and are unable to properly filtrate blood, causing the accumulation of metabolic toxins and waste, harmful to the human body [2]. Because there is no cure for CKD, early detection is key for slowing the disease progression, avoiding other complications, and maintaining the patients quality of life [1,3]. Particularly in economically fragile countries with limited access to the required healthcare and where the lack of diagnosis is a possible death sentence [3,4], even though advances in diagnosis and treatment technologies have significantly increased over the years, point-of-care affordable detection methods are still in need.

Urea is a nitrogenous product of the protein metabolism by the urea cycle, in which toxic NH_x is converted in urea [5–7]. Since its primary form of secretion is the kidney, urea is one of the most used biomarkers for kidney failure [8,9]. Furthermore, urea is present in several body fluids like blood, urine, and saliva [9]. Although the standardized sample used for urea determination is blood, it requires a painful, time-consuming, invasive collection [7]. Besides, CKD patients are more at risk of developing blood-borne diseases [2]. A viable alternative sample is saliva since it involves an easy, quick, painless, non-invasive collection. Furthermore, not only there are several studies that reported a good correlation between blood and salivary concentrations of urea, but there are also studies that reported a significant difference between the salivary urea concentration in healthy individuals (average) and in CKD patients (average) [2,7,8,10–12]. With the increase of salivary urea, urease-producing microorganisms can significantly grow, consequently

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leading to an increase of salivary ammonium. It has been previously reported that the average urea and ammonia concentrations found in CKD patients' saliva is 16.7 mM and 32.9 mM, respectively, as opposed to the urea and ammonia concentrations found in healthy individuals, 2.99 mM and 7.70 mM, respectively [12].

For ammonium and urea detection, several analytical methods have already been reported such as colorimetric, fluorometric, spectrophotometry, chromatography, among others. However, most of these conventional techniques are time consuming, require sample preparation, involve costly equipment and require specialized personnel to perform the analysis [7,8]. On the other hand, point-of-care devices have gained a great interest in the past decade, since are capable of performing the analytical determinations in less time and with a more simple, economic and user-friendly procedure [9].

Among other types of point-of-care biosensors, microfluidic paper-based analytical devices (μPADs) are a recent concept that has gained a lot of attention in the last few years. First presented by Whitesides' group in 2007, this type of device relies on the combination of a hydrophilic and a hydrophobic area [13]. The hydrophilic area is a platform for the reaction and it is usually composed of filter paper. The use of paper as a platform for analytical determinations is possible due to the microchannels in the cellulose fibers where the capillary force produced by the interaction between cohesion and adhesion forces is responsible for the flow of analytes and reagents without the need for external driving devices [13]. Furthermore, paper is a low cost alternative, available in several porosities and thicknesses, easy to store and transport, and compatible with biological samples [14]. On the other hand, the hydrophobic area is the barrier that limits the reaction and prevents contamination. The most commonly reported fabrication approach are wax printing, photolithography, inject printing, paper folding, and plasma treatment [13,15,16]. Additionally, μPADs can also be assembled in a two-dimensional or a three-dimensional structure. While the 2D-μPADs are simpler and rely on the lateral flow, the 3D-μPADs, constructed by stacking and folding, make use of both lateral and vertical flow, which gives a higher control and flow speed and allows the possibility of performing several chemical reactions in a specific order [13,16–18]. Since every technique has its own set of advantages and limitations, the fabrication and assembly method should be carefully chosen according to the device's objective [13,15].

In this work, two microfluidic paper-based analytical devices using a vertical flow approach were developed for the determination of NH_x and urea. In both devices, the detection relies on the diffusion of NH_3 (g) through a gas-diffusion membrane to produce a colorimetric change of a pH indicator. The use of vertical flow ensures that the sample (and/or standards) go through the paper disc with reagent and through the hydrophobic membrane, promoting the determination specificity and the device accuracy. This feature resulted in a 3D structure (lateral flow within the paper disc coupled to vertical flow through the various layers). The urea determination was possible due to the use of urease, which is a nickel-dependent metallo-enzyme, commonly used in urea determination biosensors since it selectively catalyzes the dissociation of urea in ammonia and carbon dioxide [7,8].

2. Materials and methods

2.1. Reagents and solutions

All the following solutions were prepared with analytical grade chemicals and Milli-Q water, (resistivity >18 MΩ·cm, Millipore, USA).

Standard stock solutions of 10 mM ammonium chloride (Merck) and 10 mM of urea (Sigma) were prepared monthly by dissolving approximately 26.0 mg and 31.0 mg, respectively, of previously dried solids (overnight at 100 °C) in 50 mL of water. The working standards were weekly prepared from the stock solution in a range of 0.10 –5.00 mM.

A 5 M sodium hydroxide stock solution was prepared by dissolving 20 g of the solid pellets in 100 mL of water. A fivefold dilution was prepared weekly to a final concentration of 1 M.

A 0.2 M phosphate buffer solution was prepared by dissolving 4.5 g of $K_2HPO_4\ 3H_2O$ (Merck) in 100 mL of water, and the pH was adjusted to 7. This solution was stored in the refrigerator at 2–8 °C.

The urease enzyme (from *Canavalia ensiformis* (Jack Bean), Sigma-Aldrich) solution of 125 U/mL was prepared by dissolving 32 mg of the lyophilized powder in 10 mL of phosphate buffer and was stored refrigerated at 2–8 °C.

The color reagent, 2 mM bromothymol blue indicator (BTB) solution, was prepared by dissolving 12.5 mg of BTB powder (Merck, Darmstadt, Germany) in 10 mL of ethanol (Panreac, 99.8% (v/v) Barcelona, Spain) and the pH adjusted to 6.5.

2.2. Design of the developed μPADs

The assembly of the developed μPADs consists of the alignment of 32 detection units (Fig. 1) in a distribution of 4 columns × 8 lines, inside a laminating pouch (125 μm, A6 size, Leitz). Each unit comprises 4 layers staked between the two sheets of the laminating pouch and aligned with the sample hole. The top sheet of the laminating pouch was perforated with 3 mm diameter holes (laser cutting machine, FDA, Model 3040) prior to the laminating process, for posterior sample insertion (L1 in Fig. 1).

In the μPAD for the NH_x determination, the first layer below the sample hole consists of a 9.5 mm diameter of Whatman 4 (W4) filter paper disc without any reagent or solution (E in Fig. 1). This layer was aligned on top of a 9.5 mm diameter W4 filter paper loaded with 15 μL of NaOH 1 M (OH in Fig. 1) to attain the conversion of ammonium to ammonia. Both these layers were placed over a 1.27 cm diameter hydrophobic Durapore Membrane (0.45 μm porosity, Merck) promoting that only the formed gaseous ammonia would go through (M in Fig. 1). The bottom layer (B in Fig. 1) consisted of a 9.5 mm diameter of Whatman 1 (W1) filter paper loaded with 15 μL of BTB 2 mM (pH = 6.5).

The μPAD for urea determination consisted of the same E layer (9.5 mm W4 filter paper discs, without any reagent or solution) as the first layer but with a different second layer (U in Fig. 1) consisting of a of 9.5 mm diameter W4 discs with 15 μL per disc of urease 125 U/mL. These two layers were also over the hydrophobic membrane, as the same M layer (1.27 cm diameter hydrophobic Durapore membrane with 0.45 μm porosity, Merck). The bottom layer (B in Fig. 1) was also the same as for the NH_x determination μPAD (9.5 mm diameter W1 discs loaded with 15 μL per disc of BTB 2 mM).

Before the alignment, all discs loaded with solutions/reagents were dried in an oven: the filter paper discs loaded with NaOH and BTB were dried at 50 °C for 20 min and 10 min, respectively and the filter paper discs loaded with urease were dried at 37 °C for 20 min.

After the alignment, the laminating pouch passes through the laminator to melt and seal the plastic pouch, consequently creating a physical separation between all sets of units.

2.3. Determination procedures

For the NH_x determination, 25 μL of sample/standard were loaded through the sample hole and, after this volume is completely absorbed (<1min), the holes were covered with adhesive tape to prevent contamination (of handling saliva) and possible ammonia loss by evaporation.

In contact with NaOH (OH in Fig. 1) NH_4^+ is converted to dissolved gaseous NH_3 which diffuses through the hydrophobic membrane (M in Fig. 1) to the BTB layer causing a color change from yellow to green while being converted back to ammonium. The cover of the sample holes ensures that the dissolved gaseous NH_3 goes through the hydrophobic membrane and gets converted into ammonium, thus contributing to the selectivity of the method. To capture this color change, the bottom side of the μPAD was scanned (Canon LiDE 120) 15 min after the sample/standard placement.

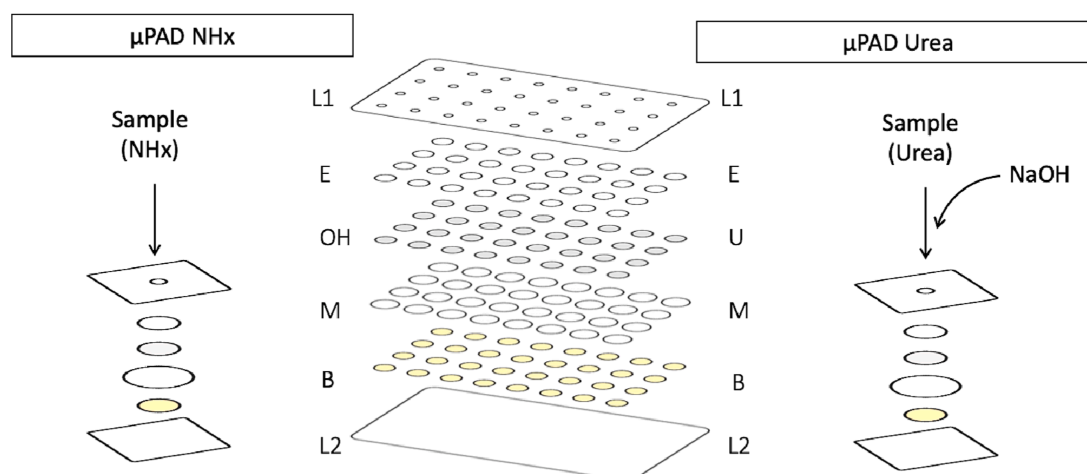

Fig. 1. Schematic representation of the assembly of the developed μPADs for NH_x (left) and urea (right) determination; L1, top layer of the laminating pouch; L2, bottom layer of the laminating pouch; E, empty layer; OH, NaOH layer (15 μL per disc); U, urease layer (15 μL per disc); M, hydrophobic membrane layer; B, BTB layer (15 μL per disc).

In the urea determination, 20 μL of sample/standard were inserted into the sample hole and a waiting period of 15 min was accounted to enable the enzymatic conversion of urea into ammonium to occur (Enzymatic Reaction Time, ERT). Then, 10 μL of NaOH 1 M was added to the μPAD also through the sample hole to promote the conversion of the formed ammonium to ammonia, so it can diffuse through the hydrophobic membrane to the BTB layer. Like the ammonium determination, the sample holes were covered with adhesive tape, and the bottom side of the device was scanned, 20 min after the NaOH placement (Color Reaction Time, CRT).

The scanned images were processed through ImageJ (National Institutes of Health, USA) by converting them into RGB plots and using the red filter to measure the color intensity. The use of the red filter can be justified by the complementary colors wheel since red is the complementary color of green obtained from the pH reaction.

For each unit, an option was made to do the measurements using a circular selection of 200×200 pixels, since it allowed a better adjustment to the BTB disc area. The intensity values were then converted into absorbance values using the formula: $A = \log_{10}(I_B/I_S)$, where I_B is the intensity of the blank signal, obtained when loading with deionized water, and I_S is the intensity of the standards/sample signal. For each reading of blank or standard/sample, 6 measurements were obtained, and outliers were removed when necessary. The remaining replicates were used in the average calculations.

The concentration obtained from the urea determination μPAD corresponds to $[NH_x + Urea]$, therefore the concentration of urea ([Urea]) was calculated by subtracting the concentration of NH_x ($[NH_x]$) from the concentration obtained from the urea μPAD ($[NH_x + Urea]$).

2.4. Saliva samples

The saliva samples used in this work were collected as "blind samples" from volunteers with their informed consent, and different colleting processes were used. Samples were analyzed after collection (when possible) and stored in the freezer when not in use.

2.4.1. Saliva collection using gauze

Saliva samples were collected by placing a 5×5 cm sterile gauze (Wells) in the mouth for approximately 2 min. The gauze was then placed in a 5 mL sterile syringe and squeezed to remove the saliva from the gauze to a 5 mL plastic tube.

2.4.2. Saliva collection by swish

Saliva samples were collected by swishing 5 or 10 mL of deionized water for about 1 min and retrieving the swished water into a plastic tube.

2.4.3. Saliva collection by spiting – CKD patients

Saliva samples collected from patients with CKD undergoing peritoneal dialysis (PD) at least 1 h after eating or performing oral hygiene procedure, by spitting into a sterile tube for 5 min were supplied as blind samples. These samples were stored in the freezer and, due to the low quantity of sample supplied and the expected high content of the analytes, all samples were diluted 10 times with synthetic saliva [19] before analysis.

2.5. Comparison methods – Validation process

To assess the accuracy of the measurements provided by the NH_x determination μPAD, 15 saliva samples were analyzed both with the developed device and with an ammonia selective electrode (OrionTM, ThermoFisher Scientific, USA). The potentiometric determination was performed by mixing 10 mL of water with 1 mL of standard/sample and adding 250 μL of NaOH 5 M immediately before the measurement, as recommended by the manufacturer.

The accuracy of the urea determination μPAD was evaluated by comparing the measurements of #11 saliva samples obtained from the developed device, and the measurements of the samples obtained from a commercially available urea/ammonia determination kit (K-URAMR 04/20; Megazyme).

3. Results and discussion

3.1. Ammonium/ammonia μPAD

The μPAD assembly was based upon the work described by Thepchuay et al [20] which consisted of three-layer units including a hydrophobic membrane as the middle layer. The diameter of the paper discs (9.5 mm) and of a hydrophobic membrane (12.7 mm) were adopted from that work. Consequently, the volumes of the color reagent, in the bottom layer, and of hydroxide, in the top layer, were also set from the published work as 15 μL (reported as the maximum volume for the 9.5 mm paper disc).

3.2. Design of the μPAD – Sample volume

The work described by Thepchuay et al [20] reported the use of 12 μL of sample/standard volume, as the maximum amount to be absorbed so

aiming to increase the sample volume it was necessary to add a filter paper layer to the device. Therefore, a μPAD with four layers was prepared by adding an empty Whatman 1 filter paper disc (9.5 mm diameter) on top of the three layers described by Thepchuay et al [20]. In the newly designed μPAD, with a four-layer structure, sample volumes of 15, 20, 25, and 30 μL (ESM-Fig. 1) were tested. It was also possible to observe that there were no significant differences in sensitivity for the sample volumes of 15, 20 and 25 μL (overlapping of the 5% relative deviation intervals). However, when a 30 μL sample volume was placed in the device, a sensitivity decrease (relative deviation of the slope >20%) was observed, probably because the volume was not completely absorbed. Therefore, to obtain the highest possible sensitivity, the sample volume chosen to continue the optimization studies was set to 25 μL.

3.3. Paper selection

The porosity of the filter paper can affect the reactions on the microfluidic device so within the Whatman qualitative paper, different pore sizes were tested: Whatman Grade 1 (W1) with 11 μm pore size, Whatman Grade 4 (W4) with 20–25 μm pore size and Whatman Grade 5 (W5) with 2.5 μm pore size. The most widely used (and cheaper) filter paper is W1 so it is the base choice. Considering that the top layer of the designed μPAD was an empty layer, the first studies were made for the second layer (loaded with NaOH) keeping W1 in the empty top layer.

The studies were performed by establishing calibration curves (#3 standards) and comparing the calibration curve slope; no standards were removed as outliers (Fig. 2).

When using either W1 and W4 in the second layer there were no significant differences in the sensitivity (overlapping of the relative error intervals) but W4 presented a higher correlation factor (Fig. 2A); so, the W4 was chosen as a compromise solution between sensitivity and correlation factor.

Then, the first layer filter paper was also tested using the same filter papers W1, W4, and W5 (Fig. 2B). Again, the paper that presented the best compromise solution with high sensitivity and high correlation factor was the W4 filter paper. In conclusion, the paper chosen for the first two layers of the μPAD was W4. This choice intended to ensure that they were not the limiting factor for the gas diffusion rate.

3.4. Hydrophobic membrane

The hydrophobic membrane layer, the third layer of the device, is an essential layer for the device selectivity in the NH_4^+ determination since it ensures that only the gaseous NH_3 diffuses to the detection layer (bottom layer). Two hydrophobic membranes were tested, Mitex (5 μm porosity) and Durapore (0.45 μm porosity), preparing two μPAD with each membrane to obtain calibration curves. The results (ESM-Fig. 2) showed that the sensitivity values were not statistically different (relative deviation of the slope <10%), and so the Durapore membrane was chosen as a more economical choice.

3.5. BTB and NaOH concentration

The influence of the BTB concentration in the absorbance signal was evaluated and BTB concentrations of 1, 2, and 3 mM were prepared and used on the designed μPAD (Fig. 3A). Then, the absorbance signal of a 1 mM ammonium standard was calculated for each BTB concentration, and it was possible to observe an over 2.5-fold increase when using 2 mM BTB when compared with 1 mM BTB. However, between using 2 mM and 3 mM BTB, there was no significant difference in the absorbance signal (<10% relative deviation) so 2 mM BTB was the BTB concentration chosen.

The influence of the NaOH concentration in the calibration curve slope (device sensitivity) was also studied as an essential step for ammonium conversion to ammonia to ensure diffusion through the hydrophobic membrane. Concentrations of 0.1, 0.5, 1, and 1.5 M NaOH were tested (Fig. 3B), and although there was a significant increase in sensitivity when the concentration increased from 0.1 to 1 M, no significant difference was found between 1 and 1.5 M, so the concentration of 1 M was chosen.

3.6. Influence of adhesive tape – Biological samples handling

Because the developed device is intended for application to saliva samples, the covering of the sample hole after sample loading is a safety and security procedure for the operator.

As the μPAD for NH_x determination involves the production and diffusion of the formed gaseous ammonia, it was especially important to evaluate the influence of applying adhesive tape over the sample holes. Two μPADs were prepared and ammonium standards were loaded; then, in one μPAD, the sample holes were covered with adhesive tape immediately after the standard insertion, and in the other μPAD the sample holes were not covered and no significant differences between the two calibration curves were observed. Still, based on the application to biological samples, the covering with adhesive tape was employed.

3.7. Urea μPAD

In the μPAD for urea determination, the color reaction was the same

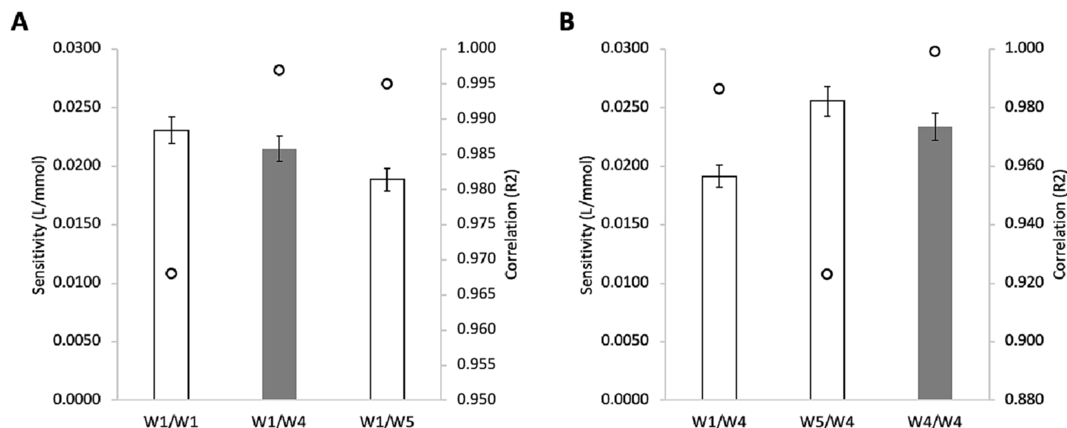

Fig. 2. Study of the influence of different filter paper porosities (W1: 11 μm; W4: 20 – 25 μm; W5: 2.5 μm) on the calibration curve slope (bars) and correlation factor (bullets) of the NH_x μPAD; **A**) study of the second layer loaded with NaOH; **B**) study of the top (empty) layer; the dark grey bar represents the chosen combination, and the error bars correspond to the 10% relative error.

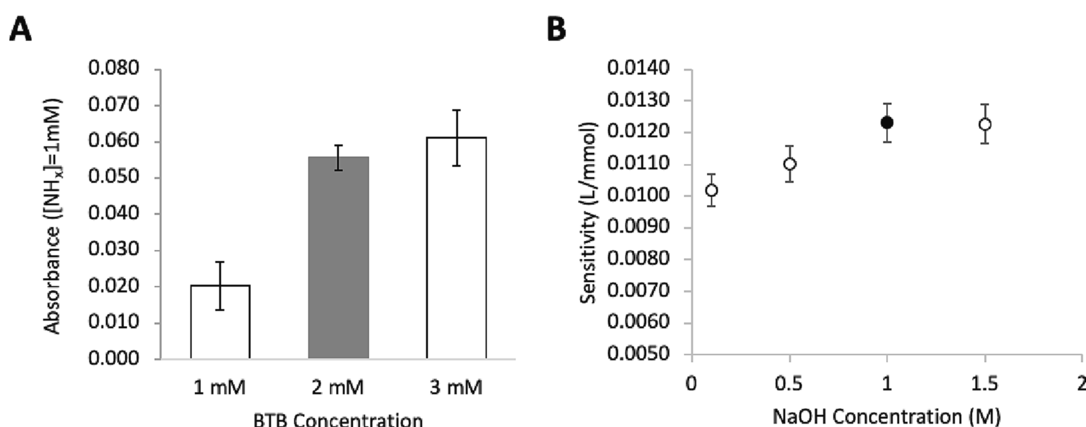

Fig. 3. Study of the influence of the reagents: **A)** BTB concentration influence in the absorbance signal obtained when using a 1 mM NH_x standard; the error bars correspond to the standard deviation of the respective absorbance measurement; the grey bar corresponds to the concentration chosen; **B)** NaOH concentration influence in the sensitivity of the calibration curve; the error bars correspond to a 5% deviation; the point in black corresponds to the chosen concentration.

as in the NH_x μPAD, based upon the color change of BTB in the bottom layer after the ammonium diffusion through the hydrophobic membrane. This meant that both the membrane layer and the BTB layer were kept from the optimization studies of the NH_x μPAD (section 3.1.).

3.8. Enzymatic reaction

To achieve the urea determination, the enzymatic reaction that converts urea to ammonia using urease enzyme was chosen and then the formed ammonia would be determined following the process of the NH_x μPAD. In this context, the urease enzyme had to be incorporated into the μPAD assembly and several possible units alignment were tested (Fig. 4).

First, the NaOH in the second layer was replaced by urease enzyme (Fig. 4A) and this layout was tested with urease concentrations ranging from 0.1 to 100 U/mL.

However, when standards of urea were placed on the device, no color was observed and consequently no signal was obtained from the scans. The same results were obtained when the urease was placed on the first layer (Fig. 4B). To evaluate if the problem was due to the enzymatic reaction, standards of NH_3 were placed on these two layouts (Fig. 4A and B) and it was expected that the NH_3 would reach the bottom layer and induce a change in pH which consequently would lead to a change in color. However, that wasn't observed, which led us to conclude that the buffer used in the urease preparation was interfering with the NH_3/NH_4^+ equilibrium.

To fix this issue, a μPAD was prepared with urease on the first layer and NaOH on the second (Fig. 4C). This would allow the urea conversion to NH_3/NH_4^+ and, with the NaOH in the second layer, the NH_3/NH_4^+ equilibrium would favor the presence of NH_3. Nevertheless, this layout also didn't produce color when standards of urea were inserted. Color was only observed when standards of NH_3 or NH_4^+ were placed on the device, which indicated that the NaOH was allowing the NH_3 to reach the BTB layer but was interfering in the urease conversion efficiency.

The strategy found for the removal of this interference was to prepare a μPAD with just urease on the second layer (Fig. 4D), to inject the standard/sample in the device, and allow the enzymatic reaction to occur for about 20 min and, only after, to insert the NaOH to convert the NH_4^+ formed to NH_3. This approach allowed the urea to be converted to NH_4^+ by the enzyme without any interference, after which the addition of the NaOH to the device led to the conversion of NH_4^+ to NH_3, causing the gradual increase of color of the BTB.

3.9. Urease and NaOH concentration

Several urease concentrations, in the range of 50 – 150 U/mL, were tested and the results showed that the sensitivity of the reaction (calibration curve slope) increased with the increase of urease concentration (Fig. 5A). The increase was significant up to 125 U/mL but smaller when using 150 U/mL (relative deviation of the slope <10 %) so 125 U/mL of urease was chosen to continue the studies.

The NaOH was now added after the sample/standard and to ensure the complete absorption into the μPAD the volume used was set to 10 μL.

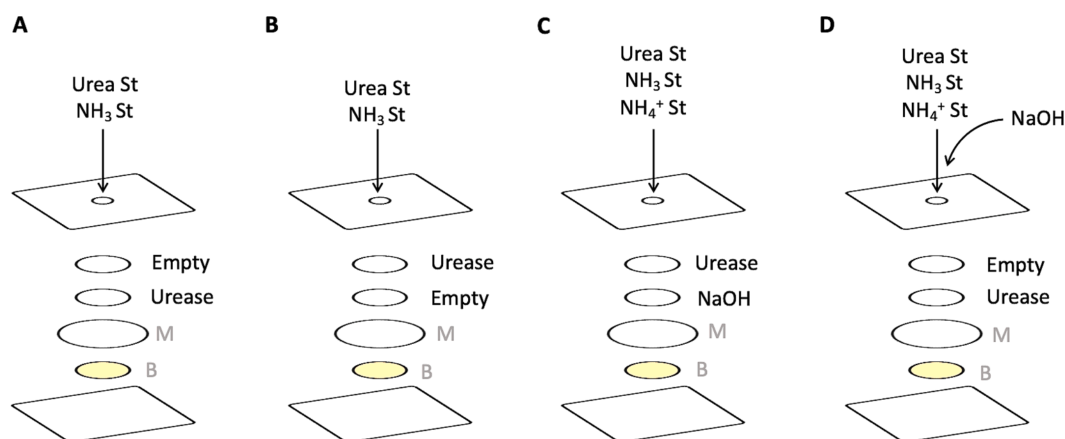

Fig. 4. Schematic representation of four different unit assemblies for the urea μPAD tested with indication of the different standard solutions tested; St, standards; M, hydrophobic membrane layer; B, BTB color reagent layer.

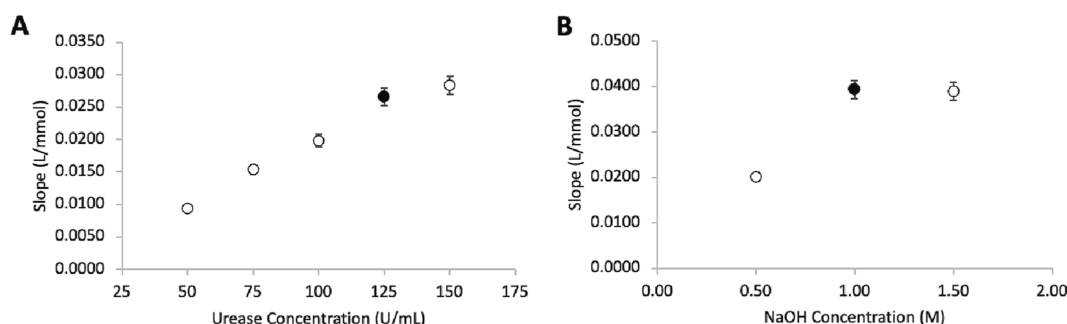

Fig. 5. Study of the influence of the Urease (A) and NaOH (B) concentrations in the μPADs calibration curve slope; the points in black represent the chosen values.

The concentration previously chosen of 1 M was revisited hydroxide solutions of 0.5, 1, and 2 M of were tested. Still 1 M proved to be a better compromise choice of highest sensitivity and reagent consumption (Fig. 5B).

3.10. Time for the enzymatic and colour reaction

In the urea determination, both the enzymatic reaction time (ERT) and color reaction time (CRT) influence was assessed in the quantification sensitivity. Considering that the addition of hydroxide (loading NaOH) inactivated the enzyme and promoted the conversion of the formed ammonium to ammonia, the ERT corresponds to the time interval between the sample and the hydroxide loading. After the NaOH loading, when the ammonia is being formed, the time interval up to the device scanning corresponds to the CRT. For the urea quantification, a combined study of different ERT and CRT was carried out and calibration curves with different time intervals between sample and NaOH loading (ERT assessing) and between NaOH loading and device scanning (CRT assessing) were established. The calibration curves were compared to determine the best combination of ERT and CRT (Table 1). The established calibration curves, within the urea concentration range of 0.1 to 5 mM, presented two distinct slopes indicating two linear ranges: 0.1 – 1 mM and 1 – 5 mM.

In both defined concentration ranges, the sensitivity (calibration curve slope) increased with the increase of ERT from 10 to 20 min in all tested CRTs and in most cases, it also increases from 20 to 30 min, but not for all the tested CRT.

Additionally, for a set time interval ERT, the sensitivity also increased with the increase of the CRT. However, because the purpose of the device is to provide a fast measurement, a maximum combined time (ERT + CRT) of 35 min was established, as a compromise solution between sensitivity and analysis time, so the combinations with analysis time (grey shadowed cells in Table 1) were discarded. The chosen combination was an ERT of 15 min and a CRT of 20 min since it presented a higher sensitivity in both concentration ranges (numbers in bold on Table 1).

Table 1
Influence of both the enzymatic reaction time (ERT) and color reaction time (CRT) on the calibration curve slope (sensitivity).

Urea dynamic range	ERT	CRT		
		10 min	*15* min	*20* min
0.1 – 1 mM	*10* min	0.0517	0.0711	0.0856
	20 min	0.0530	**0.0740**	0.0969
	30 min	0.0568	0.0772	0.0943
1 – 5 mM	*10* min	0.0150	0.0219	0.0196
	20 min	0.0225	**0.0226**	0.0260
	30 min	0.0194	0.0260	0.0212

3.11. Features

The main characteristics of both the developed μPADs such as dynamic range, average calibration curve, limit of detection (LOD) and quantification (LOQ), and relative standard deviation (RSD) are summarized in Table 2.

In both determinations, the quantification range was divided in two linear dynamic ranges because of the difference in calibration curve slopes (ESM Fig. 3).

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the concentration corresponding to three and ten times the standard deviation of the intercept ($n = 4$), respectively, according to IUPAC recommendations [21]. A difference between the LOD of urea determination and the LOD of NH_x determination can be explained by the addition of the enzymatic reaction and the extra steps in the quantification process.

The repeatability of the developed μPADs was evaluated calculating the relative standard deviation (RSD) of a sample measurement, obtained dividing the standard deviation of the sample measurements ($n = 4$) by the average of those values.

3.12. Devices stability

Because μPADs are devices that typically are intended for on-hand, point-of-care application, it was vital to assess their stability both in

Table 2
Features of the developed μPADs for the determination of NH_x and Urea; S, calibration curve slope; b, calibration curve intercept; SD, standard deviation; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

Analyte	Dynamic Range (mM)	Calibration Curves [a] A = S (±SD) × [Analyte] + b (±SD)	% RSD (mM)	LOD, [b] mM	LOQ, [b] mM
NH_x	0.105 – 1.00	A = 0.0869 (±0.0076) × $[NH_x]$ + 0.003 (±0.004) R^2 = 0.992 (±0.004)	5.5 (0.866)	0.032	0.105
	1.00 – 5.00	A = 0.0184 (±0.0014) × $[NH_x]$ + 0.064 (±0.015) R^2 = 0.992 (±0.009)	4.7 (3.93)		
Urea	0.163 – 1.00	A = 0.0823 (±0.0011) × [Urea] + 0.003 (±0.004) R^2 = 0.991 (±0.004)	3.2 (0.928)	0.049	0.163
	1.00 – 5.00	A = 0.0165 (±0.0017) × [Urea] + 0.073 (±0.001) R^2 = 0.992 (±0.002)	3.8 (4.32)		

[a] n = 3.
[b] n = 4.

storage before use and after the colored product formed, after the sample analysis.

To assess the storage stability, μPADs were prepared and placed in clear plastic bags (Lacor, 69053, Bergara, Spain), shielded from light, under two different atmospheric conditions, air, and vacuum, for different periods of time. The vacuum atmosphere was achieved using a vacuum packaging machine (Henkovac—MINI/120-ST ECO,'s-Hertogenbosch, The Netherlands). After each tested period, standards were loaded in the stored μPAD and the calibration curve obtained was compared with one obtained from freshly prepared μPADs, calculating the relative deviation between the slopes (RD <10%, no significant differences).

The μPAD developed for NHx determination showed to be stable for at least 2 months when stored in a vacuum (RD −5%). On the other hand, the urea determination μPAD was stable for only 1 month in a vacuum atmosphere (RD 4%). Because enzymes are sensitive to temperature, additional studies were performed to evaluate the effect of the storage temperature on the device sensitivity. μPADs were prepared and stored in a vacuum atmosphere at room temperature (21 °C), 4 °C, and −20 °C. In all the storage conditions used, the devices were stable for 1 month. When storing for 2 months, a decrease in sensitivity was observed for all tested temperatures, −34% for 4 °C and −20 °C storage, and −90% in relative deviation of the slope for room temperature storage.

To study the stability of the colored product, μPADs for each determination were prepared to obtain a calibration curve and then scanned several times, up to 3 h. It was possible to conclude that the color formed in the NH_x and urea μPADs was stable for 2 and 1 h, respectively.

3.13. Application to saliva samples

The accuracy of the developed μPAD for NHx determination was evaluated by comparing the results obtained from #15 saliva samples analyzed with the developed device and with an ammonia selective electrode (ASE), as a reference method and a linear correlation between the two set of results was established (ESM Fig. 4).

The obtained equation $[NH_x]_{\mu PAD} = 0.974(\pm 0.078) \times [NH_x]_{ASE} + 0.065 (\pm 0.195)$, where the values in brackets corresponds to 95% confidence interval, proved that there was no statistically differences between the two sets of results because the slope and the intercept were not statistically different from 1 and 0, respectively.

Since in the urea determination, urea is converted into NH_4^+, and NH_3 is the analyte that is detected, the μPAD for urea determination is in fact the sum of NH_x and urea in the sample. Therefore, 11 saliva samples were analyzed with both developed devices, and the results were compared with the results obtained with a commercially available ammonia/urea determination kit (Table 3). The concentration of urea was obtained by calculation ($[Urea]_{calc}$), the concentration of NH_x ($[NH_x]_{\mu PAD}$) was subtracted to the measurement obtained from the urea μPAD ($[NH_x + Urea]_{\mu PAD}$), and the value compared with the kit measurement ($[Urea]_{kit}$).

3.14. Sampling procedure

There are several methods of saliva collection already reported, so to develop a simple, user-friendly, point-of-care method of CKD biomarkers determination, it was also important to test different sampling processes.

The three sampling procedures tested consisted of collection using a piece of gauze [14]; collection by swishing with Milli-Q water [22]; and collection by spitting [12]. The quantification of urea and ammonium was effectively attained independently of the collection procedure used, so it is possible to conclude that the developed μPADs work with all three tested sampling methodologies. This can be an important feature due to some practical issues observed.

The use of gauze to collect saliva showed to be a simple and efficient procedure since it allowed the collection of higher amounts of saliva (1 to 3 mL) without altering the sample matrix. However, some children and elderly individuals did find the gauze collection procedure somewhat difficult and uncomfortable.

The saliva collection procedure used with CKD patients was by spitting and the samples were diluted with synthetic saliva (10-fold dilution) to maintain uniformity in the sample matrix The saliva collection proved to be ineffective since in some cases <0.1 mL was obtained (in 5 min given to the patients). The use of synthetic saliva in the dilutions would be a good solution since it would minimize the changes in the sample matrix or viscosity.

The collection by swishing avoids some of the limitations of the other procedures since it was very simple and assessable to all age groups and would facilitate sampling from CKD patients with an incorporated dilution. However, that is its main drawback, the inability to control the dilution factor.

Even though the tested saliva sampling collection each has advantages and limitations, they all can be used with to the developed devices.

4. Conclusions

In this work, the determination of urea and NH_x in human saliva samples was accomplished in 35 and 15 min, respectively, with two newly developed microfluidic paper-based analytical devices (μPADs), which could be used as a tool in the diagnosis and monitoring of CKD. The NH_x determination μPAD was capable of quantifying ammonia in a range of 0.105–5.0 mM and provided limits of detection and quantification of 0.032 mM and 0.105 mM, respectively. The urea determination μPAD was capable of quantifying urea in a range of 0.163–5.0 mM and provided limits of detection and quantification of 0.049 mM and 0.163

Table 3
Analysis of saliva samples with the developed μPADs and comparison of the results obtained with the measurements obtained with the commercially available kit; RD, relative deviation.

Saliva sample ID	Collection Method	NH_x determination			Urea determination				
		$[NH_x]_{\mu PAD}$ (mM)	$[NH_x]_{kit}$ (mM)	RD (%)	$[NH_x + Urea]_{\mu PAD}$ (mM)	$[NH_x]_{\mu PAD}$ (mM)	$[Urea]_{calc.}$ (mM)	$[Urea]_{kit}$ (mM)	RD (%)
S1	Gauze	1.42±0.08	1.53	−7.2%	2.47±0.12	1.42±0.08	1.04	1.16	−9.7%
S2	Gauze	1.18±0.13	1.29	−9.1%	2.73±0.20	1.18±0.13	1.56	1.50	3.6%
S3	Gauze	1.74±0.12	1.74	−0.2%	3.09±0.27	1.74±0.12	1.36	1.39	−2.4%
S4	Gauze	0.934±0.057	0.995	−6.2%	2.77±0.26	0.934±0.057	1.83	1.87	−2.0%
S5	Gauze	3.18±0.10	3.01	5.8%	3.97±0.33	3.18±0.10	0.788	0.780	1.0%
S6	Swishing	0.692±0.011	0.744	−7.0%	0.992±0.075	0.692±0.011	0.301	0.297	1.3%
S7	Swishing	1.11±0.18	1.22	−9.1%	1.29±0.09	1.11±0.18	0.181	<0.212	–
S8	Swishing	0.113±0.022	<0.384	–	0.301±0.012	0.113±0.022	0.188	<0.212	–
S9	Swishing	0.709±0.019	0.661	7.3%	0.873±0.047	0.709±0.019	0.164	<0.212	–
S10	Spiting	0.365±0.016	<0.384	–	2.47±0.02	0.365±0.016	2.11	2.02	4.5%
S11	Spiting	0.193±0.007	<0.384	–	1.99±0.27	0.193±0.007	1.79	1.68	6.2%

mM, respectively.

The use of a gas-diffusion hydrophobic membrane and of the urease enzyme makes the developed μPADs very selective and efficient devices for determinations in human saliva samples since it eliminates possible sample matrix interferences without requiring any pre-treatment.

Moreover, the developed devices were successfully applied in human saliva samples of both healthy individuals and CKD patients.

Because the main application proposed for these devices was to facilitate health diagnosis in a very simple, on-hand, point-of-care manner, the stability of the developed devices was tested. The NH_x μPADs were stable for at least 2 months when stored in a vacuum, and the color formed when placing the sample was stable for scanning for up to 2 h. The urea μPADs, on the other hand, could remain in vacuum storage for just 1 month and, after placement of the sample, should be scanned within 1 h.

Ammonia and urea are well-known biomarkers for several human health conditions [12], and there are some reports of paper-based devices developed for their determination. However, as far as we know, the μPAD developed in this work can be applied directly to saliva samples without any sample pretreatment and presents lower LOD and LOQ than the few articles reported [8,23,24].

In addition to the advantages already mentioned, the developed devices are also disposable by incineration, which besides being environmentally friendly, is also important when handling biological samples. Furthermore, the use of several replicates and the possibility of removing outliers nearly eliminates the main downside of these types of manually assembled devices, which is the possible shifting of the discs during assembly and lamination, which can lead to uneven distribution and decreased reproducibility.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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