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Noninvasive and Multiplex Self-Test of Kidney Disease Biomarkers with Graphene-Based Lab-on-a-Chip (G-LOC): Toward Digital Diagnostics in the Hands of Patients

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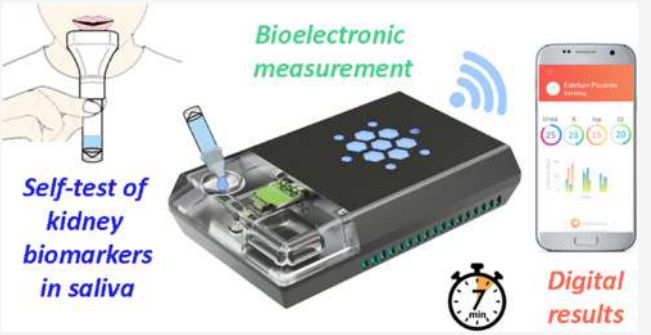
ABSTRACT: Chronic kidney disease is one of the major health issues worldwide. However, diagnosis is now highly centralized in large laboratories, resulting in low access to patient monitoring and poor personalized treatments. This work reports the development of a graphene-based lab-on-a-chip (G-LOC) for the digital testing of renal function biomarkers in serum and saliva samples. G-LOC integrates multiple bioelectronic sensors with a microfluidic system that enables multiplex self-testing of urea, potassium, sodium, and chloride. The linearity, limit of detection (LOD), accuracy, and coefficient of variability (CV) were studied. Accuracy values higher than 95.5% and CV lower than 9% were obtained for all of the biomarkers. The analytical performance was compared against three reference lab benchtop analyzers by measuring healthy- and renal-failure-level samples of serum. From receiver operating characteristic (ROC) plots, sensitivities (%) of 99.7, 97.6, 99.1, and 89.0 were obtained for urea, potassium, sodium, and chloride, respectively. Then, the test was evaluated in noninvasive saliva samples and compared against reference methods. Correlation and Bland−Altman plots showed good correlation and agreement of the G-LOC with the reference methods. It is noteworthy that the precision of G-LOC was similar to better than benchtop lab analyzers, with the advantage of being highly portable. Finally, a user testing study was conducted. The analytical performance obtained with untrained volunteers was similar to that obtained with trained chemists. Additionally, based on a user experience survey, G-LOC was found to have very simple usability and would be suitable for at-home diagnostics.



INTRODUCTION

Chronic kidney disease (CKD) has emerged as one of the most significant causes of death and suffering in the 21st century.[1] According to the World Health Organization (WHO), CKD ranked as the 10th leading cause of death globally in 2020.[2] However, predictions from the Global Burden of Disease indicate that it will increase to the fifth highest cause by 2040.[3] CKD affects more than 10% of the global population, which amounts to more than 800 million individuals. It is typically defined as a progressive condition characterized by a reduction in kidney function, with a glomerular filtration rate (GFR) of less than 60 mL/min per 1.73 m^2 or abnormalities detected through laboratory testing.[4] As CKD progresses, it ultimately leads to end-stage kidney failure. Renal replacement therapy, such as dialysis or kidney transplantation, is necessary to sustain the lives of these individuals. Due to a shortage of kidney donors and the development of comorbidities associated with age that often prevent kidney transplantation, dialysis remains the prevailing treatment option. Unfortunately, this treatment is associated with a significantly reduced quality of life, high mortality rates, and expensive medical care. In many parts of the world, annual dialysis costs range between US$35,000 and US$100,000 per patient.[5] In 2019, treatment for kidney failure consumed 6.7% of the total Medicare budget (the largest national health insurance program in the United States) to care for less than 1% of the covered Medicare population.[6] These factors contribute to kidney failure becoming one of the most pressing challenges in public health today.

Preserving kidney function can improve outcomes, slow disease progression, and can be achieved through pharmacological and nonpharmacological strategies, such as dietary and lifestyle adjustments.[1] Therefore, it is crucial to identify CKD at an early stage, monitor it regularly once diagnosed, and treat

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it with preservation practices or medical interventions. Additionally, patients on dialysis treatment or those who have had an organ transplant require frequent monitoring to evaluate the progress of the treatment. The GFR value allows assessment of the kidney function. Unfortunately, measuring GFR is time-consuming, as it requires comparative measurements of substances in both blood and urine. Consequently, kidney disease diagnosis and hemodialysis treatment are typically controlled using endogenous biomarkers such as blood creatinine, urea, and potassium, among others.[7−9] While blood is the standard sample, numerous studies have documented saliva as a noninvasive sample for monitoring kidney function by the determination of urea, potassium, sodium, and chloride levels.[10−16] Routine clinical analysis of CKD biomarkers is done in centralized laboratories or hospitals using benchtop autoanalyzers and requires the extraction of blood and sample preprocessing to measure the biomarkers in serum. Although these methodologies are widely validated, their high equipment cost, requirement of specialized technicians, and big equipment size limit their use in clinical laboratories and hospitals. To achieve a real decentralization and democratization of kidney malfunction diagnosis and monitoring, testing should be done by point-of-care (POC) tools.[17,18] The WHO and key opinion leaders in healthcare recommend that POC measurements follow the REASSURED features,[19−21] that is, real-time connectivity, easy sample collection, affordable, sensitive, selective, user-friendly, rapid, equipment-free, and deliverable to the end user. In particular, for decentralized at-home diagnostics, real-time connectivity, which means the use of smart mobile devices to power the detection, improve the test through data science tools, read results, and provide required data to decision-makers, is a paramount feature.

Graphene sensors are a highly attractive technology for REASSURED tests.[22] This is because graphene is a two-dimensional, semiconducting nanomaterial with high carrier mobility, exceptional sensitivity toward interfacial changes, and the capability for virtually instantaneous measurements using small sample volumes.[23−25] The integration of specific recognition elements (e.g., antibodies,[26] ionophores,[27] enzymes,[28,29] etc.[30]) on the surface of graphene allows for the detection of target biomolecules through electronic readings. The response of these sensors normally arises from interfacial potential changes over the graphene when the binding between the recognition element and the target occurs.[22,24] In addition, the output of these sensors is easily digitalized and can be postprocessed with data science models to enhance test performance.[27,31] For example, Khan et al. reported a paper-based electrical biosensor chip to quantify salivary cortisol at the point-of-care (POC) level. A high specificity of the sensor chip to detect cortisol was achieved by conjugating anticortisol antibody on top of gold microelectrodes with poly(styrene)-*block*-poly(acrylic acid) polymer and graphene nanoplatelets.[26] Recently, we have demonstrated the scaled manufacturing and the validation of graphene chips for the portable detection of COVID-19 antigens and biomarkers.[32−34] In these mentioned works, monoclonal antibodies were anchored on graphene chips for the detection of the SARS-CoV-2 spike antigen in human nasopharyngeal swab samples. Despite having excellent portability, this tool has the disadvantage of requiring sample placement through pipetting and, therefore, is suitable only for use by a specialized technician. Furthermore, it has been shown that integrating graphene sensors with various biotechnologies, such as aptamers and CRISPR-Cas, can lead to the creation of new diagnostic tools.[35−37] Nevertheless, these tools have the drawbacks of requiring several sample pretreatment steps using laboratory equipment and the pipetting of the sample onto the sensor surface to be carried out inside a lab. Despite the significant progress accomplished in this field over the past decade, achieving a level of usability suitable for self-tests (i.e., very easy handling and no sample pretreatment) has not been fully realized. In this regard, microfluidic lab-on-a-chip (LOC) may provide a solution by integrating multiple laboratory functions and graphene sensors inside a plug-and-sense cassette, thereby simplifying the usability.[38,39]

In this study, we developed a graphene-based lab-on-a-chip (G-LOC) technology that is user-friendly and allows untrained individuals to perform multiplexed digital self-tests for several renal biomarkers. G-LOC contains multiple bioelectronic sensors with a microfluidic system to perform highly accurate and portable diagnostics with a very simple usability. The developed test quantitatively determines urea, potassium, sodium, and chloride just from a few drops of sample. The graphene sensors were fabricated to achieve functionality using a scalable all-solution processed approach,[40] and recognition elements specific to renal biomarkers were integrated onto their surface. The G-LOC test was evaluated using both serum samples and noninvasive saliva samples. The test performance was assessed in terms of precision, accuracy, linearity, detection range, sensitivity, sensibility, and the receiver operating characteristic (ROC). A comparative study was conducted between G-LOC and reference methodologies based on laboratory benchtop analyzers. Correlation regression and Bland−Altman plots were used to compare the methods. Finally, the analytical performance and the user experience of G-LOC being used as self-test were evaluated by a group of nontrained volunteers. These results were compared to those obtained by trained chemists.

MATERIALS AND METHODS

Reagents and Equipment. Analytical grade KNO_3 (>99.0%), $NaNO_3$ (>99.0%), and urea (99.0%) were sourced from Sigma-Aldrich. Synthetic standard solutions and calibration solutions were prepared using Milli-Q water. Control-assayed serum samples Stanbio Ser-T-Fy I (normal range values) and Stanbio Ser-T-Fy II (abnormal range values) were obtained from Fisher Scientific. The control-assayed serums are certified reference materials (CRMs) that are accompanied by the quantitative concentrations of the analytes measured with FDA-approved reference methods. The saliva samples, belonging to the healthy authors of this document, were collected, processed, and stored following a rigorous protocol described below. The multiplex electrochemical graphene-based biosensors GS-X11 and the portable measurement station Zaphyrus-W20 were provided by Gisens Biotech (Berkeley).

Graphene Lab-on-a-Chip (G-LOC) Biosensor Fabrication. G-LOC is a bioelectronic cassette prepared to integrate graphene-based multiplex electrochemical biosensors GS-X11 (Gisens Biotech, Berkeley), which were manufactured as previously described.[34,40] The sensor surface contains graphene nanosheets of micrometer length-size and is modified with thin membranes containing selective ionophores (potassium ionophore I, selectophore sodium ionophore X) that respond to K^+ and Na^+, respectively. For the urea sensor,

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the graphene sensors are modified with an ammonium selective membrane (selectophore ammonium ionophore I) and a urease entrapped in a polymeric film.[40] The cassettes are also endowed with a microfluidic system for sample elution and laminar flow onto the surface sensor (placed in the sensing chamber). A USB-c port guarantees the connection between the G-LOC and the electrochemical measurement station Zaphyrus-W20. The cassette body is manufactured by a three-dimensional (3D) printer from UV resin (Clear Resin, Form Laboratories) and cleaned with isopropanol and ethanol. After the graphene biosensor was placed in the cassette, it was rinsed with deionized water, dried, and stored in the refrigerator at 5 °C until use.

Bioelectronic Measurements. Bioelectronic measurements were conducted using the portable measurement station Zaphyrus-W20 (Gisens Biotech, Berkeley). This portable device can measure various electrochemical observables (potential and current, among others). A schematic of the architecture with the main electronic components is shown in Figure S1. For the tests conducted here, the V_G potentials of each graphene sensor are measured simultaneously versus the reference electrode. G-LOC cassette is connected via a USB-c to Zaphyrus-W20, which is linked via Bluetooth to the software G-Soft v2 (Gisens Biotech, Berkeley, CA) on the user's mobile device or computer. G-Soft v2 allows real-time control of the self-test and visualization of the results through a user-friendly graphical interface. To perform the self-test, the first step is to add 6 drops (using a plastic dropper) of the calibration solution onto the inlet chamber of the microfluidic cassette. The calibration measurement takes 2.5 min. Then, the serum, saliva, or synthetic sample is mixed with a buffer solution and dropped into G-LOC. After 4 min of measurement, the quantitative results for each biomarker are calculated by G-Soft v2 and shown on the software screen.

Determination of Response Noise, Linearity, Accuracy, Precision, and LOD. The root-mean-square of the response noise (R_{RMS}) was also determined. R_{RMS} was obtained from 1 min measurements using a set of 10 sensors. First, the mean values of noise response $\langle R \rangle$ were calculated and then these values were extracted from the R (i.e., $R - \langle R \rangle$). The analytical performance of the device was determined through the calculation of analytical parameters such as linearity, accuracy, precision, limit of detection, and coefficient of variability. The obtained parameters and designed assays were determined in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI—EP09 Guideline).[41] These parameters were obtained by measuring synthetic solutions prepared with biomarker concentrations ranging from healthy to kidney failure individuals. Solutions were prepared using analytical grade reagents. Linear regression and Student's t test analysis were carried out.

Kidney Biomarker Detection in Serum Samples and Method Comparison. With the aim of conducting a benchmark analysis of G-LOC in real complex samples, its analytical performance was compared to certified reference material (CRM) samples. Human serum at two different concentrations, Stanbio Ser-T-Fy I and Stanbio Ser-T-Fy II, corresponding to a healthy range and kidney failure range, were used. These CRM serums are accompanied by the concentration values of each biomarker determined using three standard clinical quantitative techniques. Thus, a benchmark was conducted between the values obtained by G-LOC technology and the values reported on the data sheet of the CRM serums. The study was performed following precautions and recommendations according to the Biosafety level II as established in Biosafety in Microbiological and Biomedical Laboratories by the CDC. More than 100 G-LOC tests were measured. For the statistical analysis of the results, receiver operating characteristic (ROC) curves were constructed and a t test was applied for mean comparison (with a significance level of 0.0001) between both serum levels. The ROC curve is a graphical representation of sensitivity (or the true positive rate) versus the false positive rate (100—specificity) for a binary classification system as the discrimination threshold is varied.

Kidney Biomarker Detection in Saliva and Method Comparison. The performance of the G-LOC was assessed using human saliva samples. The proposed assay aimed to compare the results obtained with G-LOC with a reference method. The reference method for potassium and sodium consisted of ion-selective electrodes, composed of glass capillaries modified with selective membranes prepared as previously reported[42] and a reference Ag/AgCl electrode from BASi (Indiana). The reference method for urea was a commercial kit for clinical use (Uremia from the Wiener Lab) based on an indirect spectroscopic determination. It measures the absorbance of an obtained colorimetric compound at 540 nm. Absorbance was measured using an HP 8453E UV−vis spectrophotometer in a quartz cuvette. Increasing concentrations of renal biomarkers were prepared by spiking the saliva samples with high-concentration standard solutions. Concentration ranges from healthy individuals to those with renal failure were covered. A total of 16 increasing concentrations were measured for the entire assay. Saliva samples used were provided by the authors of this study, and the sample collection was carried out according to the following protocol, which is based on previous works:[10,12,43] first, the volunteer/individual must refrain from consuming food or drinks and avoid brushing their teeth for at least 2 h before sample collection. To begin, the individual must rinse his/her mouth three times with tap water, place the water in his/her mouth, and then discard it. Next, any remaining saliva in the mouth should be swallowed, followed by a 1 min waiting period. After this period, the sample should be collected by spitting into a sample collector over the following 5 min. If necessary, the sample was stored at −20 °C until processing (see additional considerations in the Supporting Information (SI)).

Statistical Analysis of Results. For data analysis, correlation graphs of analyte concentrations measured by G-LOC and the reference method were constructed as functions of the analytical concentrations. The linearity of the response of both methods was assessed (at a significance level of 0.05), and the slope and R^2 values of the correlation graph were obtained. Additionally, Bland−Altman plots were constructed where the difference between the values obtained by G-LOC and the reference method was plotted against the average of the concentrations measured at each point. Favorable results are indicated by data points close to zero and an equilibrium distribution around zero for the entire concentration interval tested.

Self-Testing: User Testing and User Experience. Functionality self-test and user experience studies were conducted on the G-LOC for renal biomarkers. This study spanned a 3 day period and involved the participation of 13 nonexpert male and female volunteers aged between 20 and 70

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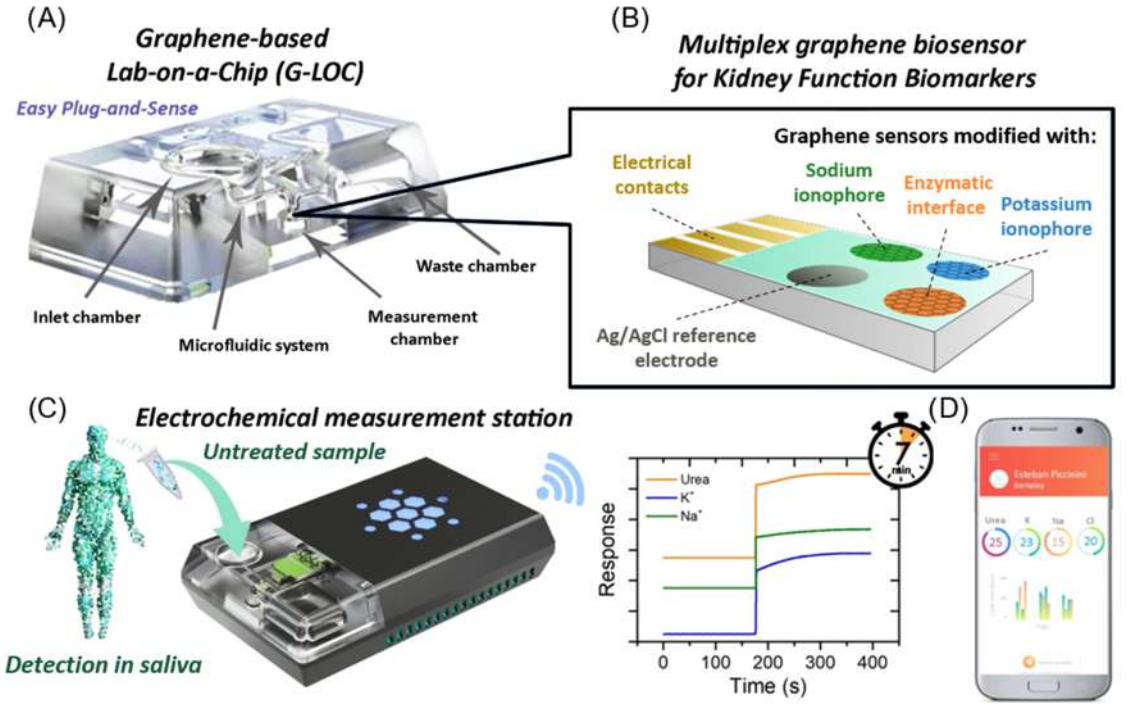

Figure 1. (A) Microfluidic system description of the graphene-based lab-on-a-chip (G-LOC). (B) Schematic representation of the multiplex biosensor for kidney function biomarkers. (C) Bioelectronics assay using saliva or serum. G-LOC is connected to the electrochemical measurement station by an easy plug-and-sense manner. Simultaneous response was measured for each biomarker as a function of time during the test running. (D) Test results are displayed in software for mobile devices.

years. They were provided with a one-page instruction manual and tasked with determining the concentration of analytes present in a synthetic standard sample that simulates saliva. The assay was conducted three times by each volunteer without receiving any assistance or additional information beyond what was provided in the instruction manual. To assess the precision and accuracy obtained by untrained individuals, the results were compared with those obtained by trained technicians. For quantitative analysis, the average value, standard deviation (SD), and coefficient of variability (CV) were calculated for each biomarker and compared. Regarding the user experience, volunteers were requested to complete a survey that encompassed various practical aspects related to the correct operation of the self-test as well as opinions and general preferences regarding its use compared to other alternatives currently available.

■ RESULTS AND DISCUSSION

The developed graphene-based lab-on-a-chip (G-LOC) is a multiplexed and simple-handling test that uses graphene sensors integrated in a microfluidic cassette (see Figure 1A). The microfluidic system had a sample inlet, an air bubble trap, a measurement chamber, and a waste chamber. The microfluidic system was designed in such a way that when a few drops of the sample are placed in the sample inlet, the liquid flows through the system until it reaches the measurement chamber. Any excess sample ends up in the waste chamber. This system has very simple usability, is suitable for nontrained people, and is specifically designed for self-test uses. The measurement chamber of the cassette contains a chip with three graphene-based sensors (see Figure 1B) that were manufactured in fabrication facilities using a previously reported scalable method.[32,34] Using this approach, each graphene-based sensor has thin films containing the corresponding recognition element (urease, K^+ ionophore, Na^+ ionophore) to detect quantitatively the specific renal function biomarkers (urea, potassium, and sodium). The measurement chamber assures a laminar flow of the sample onto the graphene sensor, thus improving its signal amplitude and reducing the test duration. Moreover, chloride concentration was estimated using a predictive model that uses sodium and potassium concentrations in combination with the sample type (serum, saliva) as input. The model was trained using public clinical databases and is a tool integrated into the software G-Soft v2. The technological convergence presented in G-LOC gathers a very simple usability, such as the easiest lateral-flow self-tests, together with the ultrahigh sensitivity and ease of digitalization of graphene chips.

To perform the renal function biomarker quantitative assay, the G-LOC cassette is connected to a portable electrochemical measurement station (Zaphyrus-W20, Figure 1C) in a plug-and-sense manner. The multiplex assay consists, first, of the addition of 6 drops of calibration solution, followed by 6 drops of the collected sample as explained in the Materials and Methods section. The portable measurement station features intelligent software that can detect whether the cassette and the sample are properly placed. If necessary, it alerts the user to reconnect the cassette or rerun the sample placement. In this way, the portable measurement station ensured success in handling the test method. Additionally, Zaphyrus-W20 does the electronic readout and sends the results in digital format to a mobile App or computer software (Figure 1D). The mobile App processes the data, displays the results, and stores the historical levels of each biomarker.

The root-mean-square of the response noise (R_{RMS}) is an important parameter to evaluate the inherent noise of the

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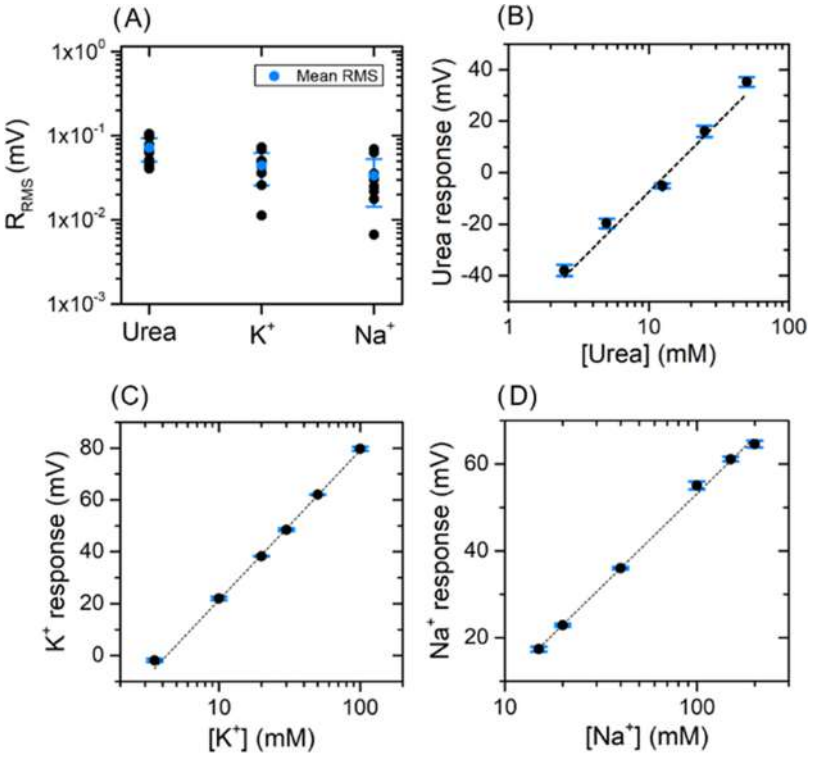

Figure 2. (A) Root-mean-square response (R_{RMS}) values (black) and mean value (blue) of a set of urea, potassium, and sodium bioelectronic measurements acquired with the G-LOC. Bioelectronic response as a function of the logarithm of the concentration of urea (B), potassium (C), and sodium (D). The dashed lines represent the linear fit of the two data sets. Error bars correspond to the standard deviations for three measurements of the same concentration.

electronic test (Figure 2A). R_{RMS} average values of 7.1×10^{-2}, 4.4×10^{-2}, and 3.34×10^{-2} mV were obtained for urea, potassium, and sodium, respectively. Figure 2B–D shows the response of the urea, potassium, and sodium tests as a function of concentration measured using 3 replicates per concentration of standard samples. In the presence of the analytes, the interfacial potential of each graphene sensor increases linearly over a wide range with the logarithm of the biomarker concentration.

The fundamentals for each sensing mechanism were described in previous works.[32,34,44] For the case of the urea sensor, the response is associated with the hydrolysis of urea, catalyzed by the enzyme urease, whose reaction products yield a potential shift at the graphene surface.[34,44] On the other hand, potassium and sodium cations interact with ionophores contained in ion-selective membranes (ISMs) on the graphene sensors and generate a potential difference between the conducting/membrane interface and the sample solution.[34,45,46] This architecture possesses the capability for electronic and instantaneous transduction of concentration changes (of the selective ions) in potential changes. The observed potential for each graphene sensor (V_G) is determined by the following simplified equation (see the Supporting Information for more details)[27,34,47]

$$V_G = V_* + 2.3\frac{RT}{zF}\log(C_E)$$

where V_* is a constant term, C_E is the concentration of the selective ion in the solution, R is the molar gas constant, T is the temperature, F is the Faraday constant, and z is the charge number of the ion.

The analytical performance of the test was evaluated by measuring standard samples. A linear function of the potential with the logarithm of the concentration was observed for the three sensors. The slope, coefficient of determination (R^2), linear range, LOD, accuracy, and coefficient of variability are summarized in Table 1. The kidney biomarker test displayed

Table 1. Analytical Performance of G-LOC

	urea	K^+	Na^+
linear range (mEq/L)	1–50	1–100	2–200
slope (mV/decade)	54.3 ± 2.0	48.6 ± 2.3	52.1 ± 5.8
R^2	0.963	0.996	0.996
LOD (mEq/L)	0.2	0.5	2.5
accuracy (%)[a]	96.5	97.5	95.5
CV (%)[a]	9.0	7.2	5.7

[a]For concentrations of urea = 2.5 mmol/L, potassium = 20 mEq/L, and sodium = 15 mEq/L.

good linearity (R^2 values of 0.963, 0.996, and 0.996 for urea, potassium, and sodium, respectively) in a linear range of 2 orders of magnitude that includes the clinical range of concentration of both samples of interest, blood and saliva. For concentrations typically presented in saliva, urea = 2.5 mmol/L, potassium = 20 mEq/L, and sodium = 15 mEq/L, the G-LOC test showed an accuracy of 96.5, 97.5, and 95.5%, respectively. The K^+ and Na^+ slopes are in the range of those previously reported for all-solid-state ISM developed for the detection in biological samples.[27,47,48] The difference in the K^+ slope with respect to its ideal value (59 mV/decade) is due to the use of a buffer solution that is necessary to measure both serum and saliva samples (see Figure S2 and the SI for more

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details). The selectivity coefficients of the ISM sensors were estimated by a separate solution method (see the SI for more details). The obtained values (Table S1) are similar to those previously reported by other authors using membranes prepared with the same ionophores. Moreover, the water-layer test was performed,[49] demonstrating that the potential measurements show negligible drift due to the water-layer effect (see Figure S3 in the SI).

Renal dysfunction normally leads to out-of-range urea and electrolyte levels in blood.[50−52] Furthermore, clinicians make diagnostic decisions using the levels of these biomarkers for renal patients with different disease stages and treatments. For example, urea is used as a serum marker of uremic solute retention and elimination. For hemodialysis and peritoneal dialysis patients, the degree of urea clearance correlates with clinical outcome, and it is used to model hemodialysis adequacy over time.[53] The blood urea is measured before and after dialysis to estimate the K_t/V_{urea} and thereby assess whether the dialysis dose was sufficient or if a dialysis time correction needs to be applied. Although urea levels exhibit a nonlinear and inverse relationship with GFR, it can be used to estimate GFR if extrarenal factors that influence its endogenous production (high protein intake, critical, gastrointestinal hemorrhage, or drug therapy) are taken into account.[51] In addition, it has been demonstrated that remote monitoring helps improve the personalization of automated peritoneal dialysis prescriptions, enables early intervention, and reduces the frequency of in-person visits for emergency problems.[54]

Electrolyte panel, including potassium, sodium, and chloride, is frequently used to screen for an electrolyte imbalance to both diagnosis and management of renal, endocrine, acid−base, water balance, and many other conditions.[52] High K^+ levels (hyperkalemia) in CKD patients occur due to a high dietary K intake relative to reduced renal function, metabolic acidosis causing extracellular K^+ shift, and the use of renin−angiotensin−aldosterone system blockers inhibiting renal K^+ excretion. Hyperkalemia can lead to adverse cardiac effects including arrhythmias, heart block, fibrillation, and death. Severe hyperkalemia is common in end-stage renal disease patients (ESRF), affecting ∼13% of hemodialysis patients,[7] and is being one of the main decompensation reasons. Thus, controlling serum potassium is an important practice in patients with maintenance dialysis patients.

Urea, potassium, sodium, and chloride levels in blood normally are in the range of 1.8−7.1 mM (5−20 mg/dL), 3.5−5, 135−145, and 96−106 mEq/L for healthy people.[55] On the other hand, acute kidney failure patients (or end-stage CKD) can present blood levels in the range of 15−33 mmol/L urea (41-96 mg/dL), 5.0−6.2 mmol/L potassium, and disturbed sodium and chloride concentrations.[11,15,56,57]

G-LOC performance with serum samples was evaluated using certified reference material (CRM) of two different levels, healthy and renal failure levels. First, the G-LOC test-to-test dispersion was evaluated by measuring more than 100 tests (Figure 3A−D). As proved with a statistical t test analysis, the method can differentiate healthy and unhealthy samples with a significance level (p) of 0.0001. Then, the G-LOC results were compared versus three lab benchtop analyzer reference methods including SIRRUS, an FDA-approved analyzer. Average values and standard deviation of G-LOC are compared to the benchtop analyzers in Table 2. In terms of the standard deviation (SD), G-LOC performed similarly and

Table 2. Result Comparison between G-LOC and Reference Methods for Healthy- (H) and Renal-Failure (RF)-Level Samples of Serum[a]

		H−S		RF-S	
biomarker	method	mean	SD	mean	SD
urea mmol/L	G-LOC	5.2	0.7	13.8	3.3
	SIRRUS	4.3	0.7	14.6	2.8
	liqui-UV	6.8	2.1	23.6	7.1
	altair	6.4	1.4	18.9	3.9
K^+ mEq/L	G-LOC	3.7	0.3	6.8	0.6
	SIRRUS	3.8	0.8	6.1	0.8
	turbidim.	3.8	1.1	6.1	1.8
	altair	3.7	0.8	6.1	0.8
Na^+ mEq/L	G-LOC	132	13	172	10
	SIRRUS	139	6	171	6
	U.A.[b]	114	34	154	46
	altair	132	6	171	6
Cl^- mEq/L	G-LOC	89	8	112	9
	SIRRUS	91	8	117	9
	M.T.[b]	115	23	134	27
	altair	88	6	113	9

[a]The reference method values were extracted from their datasheets. [b]U.A. for uranyl acetate method, and M.T. for mercuric thiocyanate method.

in some cases better than the benchtop reference methodologies. Considering the average value of the three reference methods, G-LOC accuracy was calculated. Values of 89.1, 98.3, 97.4, and 97.8% were obtained for urea, potassium, sodium, and chloride, respectively.

Figure 3E−H shows the receiver operating characteristic (ROC) curve for serum measurements. Red dots in ROC plots indicate the biomarker threshold concentrations for healthy individuals as previously reported in clinical studies. The sensitivity and sensibility in such concentrations are 99.7 and 100% for urea (7.1 mmol/L), 97.6 and 99.2% for potassium (5.5 mM), 99.1 and 80.2% for sodium (150 mM), and 89.0 and 93.9% for chloride (106 mM), respectively. Moreover, the interference of a wide variety of biomolecules that are present in blood was evaluated (Table S2 in the SI). The performance of the G-LOC test was not reduced in the presence of the studied interferents at clinical concentrations.

It is well reported that urea, potassium, sodium, and chloride levels in saliva are good biomarkers of CKD. Figure 4B synthesizes the biomarker concentration for healthy and severe-to-acute renal failure groups (data were collected from previously reported works; see Table S3 in the SI).[11−16] For healthy people, average levels of urea, potassium, sodium, and chloride are typically in the range of 7.5−11.3 mmol/L, 22.2−25.1, 8.0−9.6, and 16−24.8 mEq/L, respectively. On the other hand, severe-to-acute kidney failure patients present average levels in the range of 26.3−29.4 mmol/L, 34.0−37.3, 15.5−16.5, and 24.7−34.1 mEq/L, respectively. For all of these biomarkers, statistically significant differences were found between healthy and severe-to-acute renal failure groups.[12−15] Despite the demographic differences among the study groups of the research works, the average concentrations and differences between healthy and renal groups show good concordance. The small variations in average levels or SD values may be related to the fact that in each work, a different sampling protocol was used. Moreover, it was demonstrated that biomarkers in saliva can be used to differentiate disease

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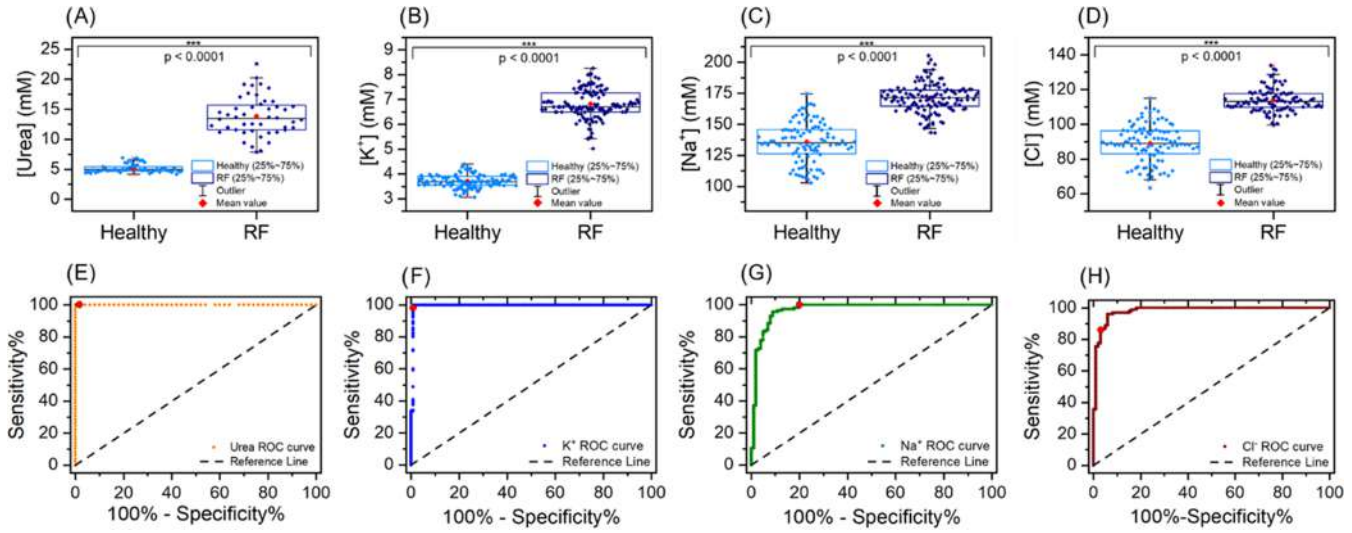

Figure 3. Box charts of the concentration of urea (A), potassium (B), sodium (C), and chloride (D) measured in serum samples with analyte levels of healthy individuals (light blue) and renal failure (RF, blue) individuals. For all biomarkers, the sample populations are significantly different at the 0.0001 significance level. Receiver operating characteristic (ROC) plots for the G-LOC determination of urea (E), potassium (F), sodium (G), and chloride (H). Sensitivity and specificity conditions at the clinical cutoff values are shown with red dots.

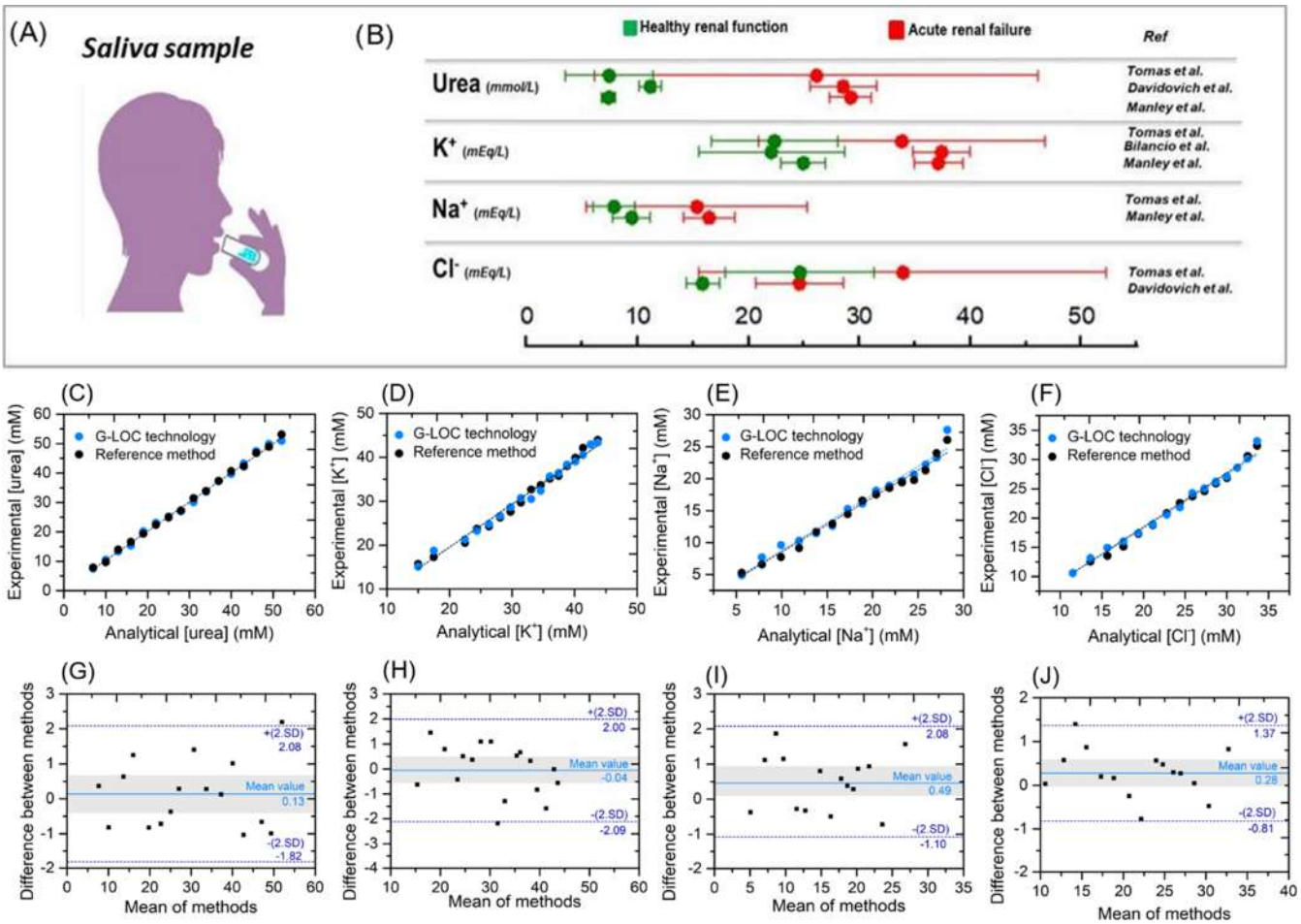

Figure 4. (A) Illustration of noninvasive saliva collection. (B) Summary of the renal biomarker mean values in saliva for healthy individuals (green) and acute renal failure patients (red). Error bars correspond to SD. Data was extracted from reported clinical studies. Correlation plots of urea (C), potassium (D), sodium (E), and chloride (F) concentrations measured in salivary samples by G-LOC (light blue) and a reference method (black) in contrast to their analytical values. The dashed lines represent the linear fit of the data set for each method. Bland−Altman plots (in mM units) of urea (G), potassium (H), sodium (I), and chloride (J) determination between the G-LOC technology and the reference method. The gray shadow area corresponds to the 95% confidence interval of the mean value.

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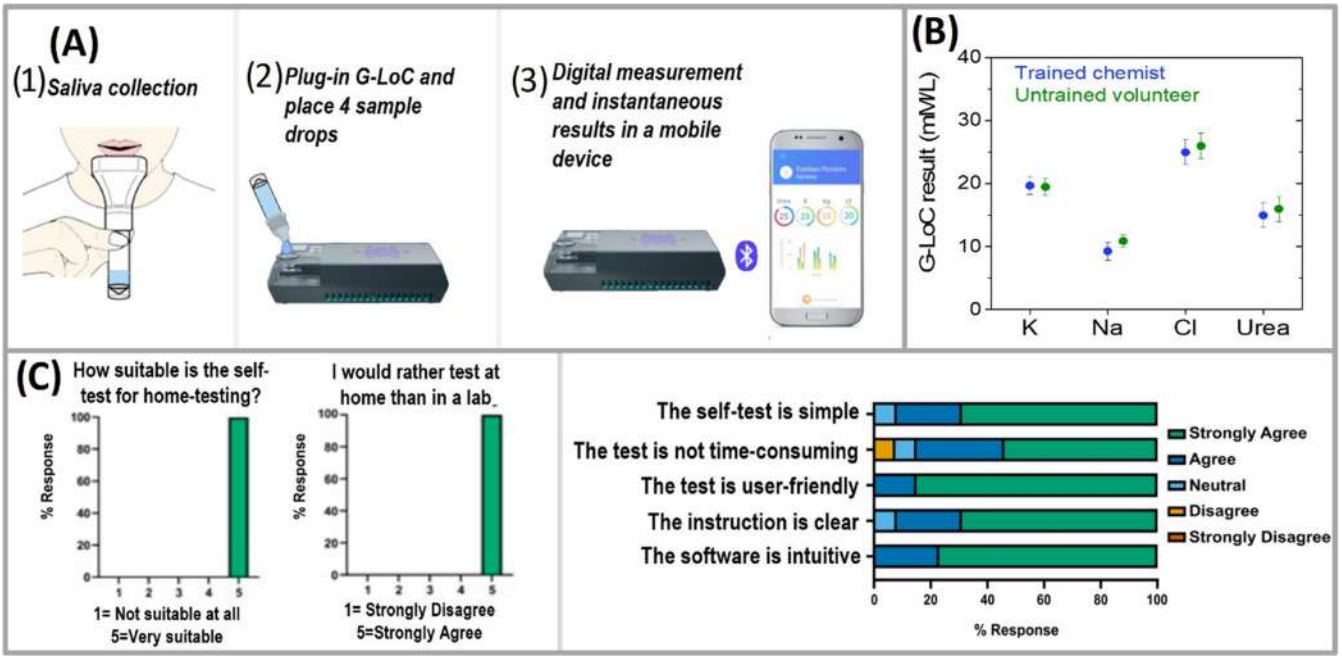

Figure 5. (A) Schematic of the G-LOC self-test protocol for the determination of kidney biomarkers in saliva. (B) User testing quantitative results obtained from the G-LOC test performed by trained chemists (blue) and untrained volunteers (green). For all biomarkers, t-Student test showed nonsignificant differences between both groups at a significance level of 0.001. (C) Survey results of the user experience for the G-LOC test.

stages of CKD.[12] In addition, urea in saliva can be used to assess the effectiveness of the dialysis session.[16] As the biomarker levels in saliva considerably change regarding the renal condition, and in addition, saliva is noninvasive and easily collected, this sample offers great advantages compared to blood to unlock the simple self-test (or at-home test) of kidney function.

It is noteworthy that the level of creatinine in saliva has been shown to exhibit a lower to similar significance in differentiating stages of chronic kidney disease compared to that of urea and potassium.[12,58] In addition, creatinine has the disadvantage of being present in a low concentration in saliva, within the range of tens of μM, almost 10−100 times lower than in blood,[15] making it challenging to develop quantitative tests.

G-LOC was tested in saliva samples with increasing concentrations of the biomarkers. Samples were prepared by the addition of the biomarker reagents to a saliva sample of a healthy volunteer of known initial concentrations. Figure 4C−F (light blue dots) shows the concentration determined by G-LOC as a function of the analytical concentration. Moreover, the G-LOC performance was compared against a reference method using liquid junction ion-selective electrodes (Figure 4C−F, black dots). Slope (and R^2 values) was obtained from G-LOC, $m_{GLOC}(R^2)$, and a reference method, $m_{ref}(R^2)$. For urea, m_{GLOC} = 0.992 (R^2 = 0.996) and m_{ref} = 0.997 (R^2 = 0.998). For K^+, m_{GLOC} = 0.974 (R^2 = 0.999) and m_{ref} = 0.978 (R^2 = 0.999). For Na^+, m_{GLOC} = 0.877 (R^2 = 0.998) and m_{ref} = 0.854 (R^2 = 0.999). For Cl^-, m_{GLOC} = 0.924 (R^2 = 0.999) and m_{ref} = 0.915 (R^2 = 0.999). Slopes and goodness of the linear regression for G-LOC are similar to those obtained by the reference methods. Moreover, the direct correlation of the concentrations obtained by G-LOC and the reference method (see Figure S4 in the SI) evidence a very good method concordance.

Bland−Altman plots were employed to determine the agreement and bias between G-LOC and the reference method (Figure 4G−J). In this type of plot, a low bias is reflected with values close to the zero-reference line. Moreover, limits of agreement lines (2SD) represent how variable these results are from the difference. Thus, the closer these lines are to the mean, the higher the precision. The obtained bias values were 0.13 mM (95% CI [−0.4−0.65 mM]) for urea, −0.04 mM (95% CI [−0.57−0.48 mM]) for potassium, 0.49 mM (95% CI [0.05−0.93 mM]) for sodium, and 0.24 mM (95% CI [−0.02−0.58 mM]) for chloride. With the performance of G-LOC, it is evident that it is possible to quantify biomarkers in saliva (Figure 5A) with accuracy and precision that would allow for the differentiation between healthy people and those with acute renal failure. Moreover, since urea and potassium are considerably different between CKD stages,[12,59] G-LOC would also be able to differentiate CKD stages using saliva samples.

Regarding the long-term stability of the test, K^+ and Na^+ sensors retained more than 95% of their initial slope value for 12 months, if stored, as detailed in the Materials and Methods section. On the other hand, due to urease loss of activity, urea sensors retained 95% of the initial performance for 60 days. The long-term stability of urea sensors agrees with other approaches previously reported.[60,61] The multiplex sensors are meant to be disposable; nevertheless, we proved that they can be reused up to 20 times, losing less than 3% of their slope.

Finally, a user testing study was conducted to assess the performance and user experience of G-LOC as a self-test. Untrained volunteers with diverse backgrounds and ages participated in the study, performing the G-LOC test three times each in a home environment, solely relying on the user manual as their guide. The tests were conducted by using sample standards, enabling a quantitative comparison between the results of untrained volunteers and those of trained chemists (the authors of this study). Remarkably, the average

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values and standard deviations obtained from both groups are very similar (Figure 5B). Moreover, t-Student statistical tests showed a nonsignificant difference between results obtained by untrained volunteers and trained chemists. Moreover, a survey of the user experience was completed by volunteers (Figure 5C). All of the volunteers (100%) indicated that "G-LOC self-test is very suitable for at-home use" and "they would rather monitor their health using the self-test instead attending a clinical laboratory". Moreover, 100% of the volunteers strongly agree or agree that the test is user-friendly and the software is intuitive. Therefore, the results obtained from the quantitative analysis and survey strongly indicate that G-LOC would be suitable for at-home self-testing.

CONCLUSIONS

Our study introduces the groundbreaking graphene-based lab-on-a-chip (G-LOC) technology to multiplex and digitally self-test several renal biomarkers. Through the combination of multiple bioelectronic sensors within a microfluidic system, G-LOC ensures high accuracy and very simple usability that is suitable for patient self-testing. The multiplex test was evaluated in serum and noninvasive saliva. With just a few drops of sample, G-LOC quantitatively measured urea, potassium, sodium, and chloride, offering portable monitoring of renal health. The evaluation of the analytical performance using both serum and saliva samples proved to give very good results across various parameters including precision, accuracy, linearity, detection range, sensitivity, and sensibility. Comparative studies against reference methodologies based on laboratory benchtop analyzers were carried out and assessed through correlation regression and Bland–Altman plots. Remarkably, G-LOC showed analytical performance similar to that of the lab benchtop analyzers. In addition, the G-LOC analytical performance and user experience were evaluated by untrained volunteers evidencing exceptionally simple usability with as good accuracy and precision as those obtained by trained chemists. These results strongly indicate that G-LOC would be suitable for at-home self-testing of kidney function. This tool could be very useful for (i) early-stage CKD screening; (ii) monitoring patients diagnosed with CKD to personalize their pharmacological treatment; (iii) evaluating the success of treatment for patients on at-home peritoneal dialysis; and (iv) monitoring whether organ rejection occurs for recently transplanted patients. Therefore, G-LOC may represent a paradigm shift in renal biomarker diagnostics, offering a portable, accurate, and user-friendly solution with the potential to revolutionize healthcare decentralization and patient outcomes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.3c05148.

> Architecture of the electrochemical measurement station; sensing mechanism; potassium sensor slope; selectivity coefficients; water-layer test results; interference evaluation; biomarker levels in saliva according to the literature; additional considerations for saliva sampling and detection; and method concordance plot for saliva samples (PDF)

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Notes

The authors declare the following competing financial interest(s): E.P., W.A.M and O.A. are scientific advisors of GISENS BIOTECH through a contract between UNLP, CONICET and GISENS BIOTECH. T.C. and J.M.P. are recently or presently employed by GISENS BIOTECH. The other authors declare no competing interests.

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