



Novel carbon nanotube-based potentiometric sensor for ascorbic acid detection. Unveiling evidence on surface interactions

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ABSTRACT

Ascorbic Acid (AA) has been the center of controversial dialogues and studies when it comes to cancer research, though recently it has gained interest as an anti-tumor agent. Here, we present an electroanalytical concept to determine AA based on a new fundamental finding: the reversible interaction of AA with carbon nanotubes (CNTs) under zero-current measurements (potentiometry). This interaction is systemically studied under several experimental conditions, aiming to provide specificity with the combination of a thin film of CNTs with a nanometer-sized plasticized polymeric membrane (ca. 300 nm in thickness). The resulting sensor shows a consistent sensitivity to AA of -33.53 ± 2.57 mV/decade ($n = 17$), presenting a linear range of response that includes from normal physiological to pharmacological AA concentrations (10–200 μ M and 0.2–1 mM, respectively). Importantly, interferences such as uric acid (UA), sodium ion and lactate have a limited influence on the potentiometric response, in contrast to previously published sensors. In addition to the experimental evidence, computational simulations on the interactions of AA and UA with a graphene-based model were performed to provide insights in describing the very distinct experimental responses that were observed. Thus, the formulated hypothesis is supported by both experimental data and simulations, which has not been reported before, to the best of our knowledge. Furthermore, we demonstrate the suitability of the developed sensor for real sample analysis (undiluted human serum, saliva and urine). The significance of the developed sensor is three-fold: 1) analytical performance addressing several applications, 2) enhanced selectivity, specially towards UA, 3) simplicity of the concept in terms of materials and preparation that makes it compatible with micro- and nano-electrodes for further analytical applications never explored until now (e.g., intracellular measurements, nano-electrochemistry, etc.)

1. Introduction

In today's world, where the average lifespan of humans is higher than ever and as individuals are exposed to many harmful materials throughout their lives, the risk of cancer occurring is increasing. [1] The mutation from a healthy cell into a cancer bypasses several critical safety checks, and this results in multiple changes in both the microenvironment, as well as the cell itself. [2–4] Considering the inhibition of this development path, a compound which usage has been controversial in the treatment of cancer has been Ascorbic Acid (AA), also known as Vitamin C. In 1976, a pioneer clinical study was conducted by Cameron and Pauling, where a comparison between 100 cancer patients treated

with AA and 1000 cancer patients who did not receive any additional AA to what they assimilated by diet was performed. [5] The trial revealed very promising results, though there was no placebo group to compare the results with. In contrast, a follow up study by the Mayo Clinic, considering indeed such a placebo control group, showed no significant difference between AA-treated patients and non-treated ones. [6] However, it has to be pointed out that, in the study by Cameron and Pauling, they administered intra-venous injections of AA, reaching higher concentrations of AA in the blood than the oral supplements of AA proposed by the Mayo Clinic. This was a kind of starting point for the controversy of the use of AA in cancer research, which has been going on to this day.

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In vitro trials on cancer cells have exhibited interesting results, especially when the concentration of AA reaches the mM range, which is not attainable with oral doses only. [7,8] A strategy that would help to understand levels and method of administration necessary for AA action to be significant at the cellular level is to measure AA concentrations locally around the cells at the same time as inside the cells. Nevertheless, this is not an easy task considering the requirements and the available portfolio of analytical techniques: as such, addressing AA intracellular measurements is an analytical challenge. To observe the effect of a high AA concentration on a single cell, a few criteria must be met: the spatial and temporal resolution must be good enough to allow for dynamic measurements of a single or few cells. Utilizing methods such as chromatography needs the intracellular constituents in a rather high volume, and no temporal data will be obtained unless several successive experiments are accomplished. [9] More suitable alternatives are electrochemical sensors, which has already demonstrated enabling single-cell and close-to-cell measurements for other analytes rather than AA, [10, 11] but also, fluorescent trackers such as quantum dots. [12] However, possibilities for intracellular measurements lack.

AA has been characterized and measured by means of different voltammetry and amperometry methods. AA presents an oxidation potential of approx. 0.4 V vs. a saturated Ag/AgCl reference electrode. This potential also happens to be the oxidation potential of other compounds present in biological fluids, such as the neurotransmitter dopamine (DA) and a product of our metabolism, Uric Acid (UA), making these compounds hard to distinguish from each other. Strategies of discriminating between AA, DA, and UA have been investigated, such as measuring the O₂ consumption by Ascorbate Oxidase (AO) at a lower potential, avoiding oxidation of other similar compounds. [13] Also, AO has been used to construct amperometric biosensors meant for food, and measuring the resulting current at higher voltages as the enzyme oxidizes AA to dehydroascorbic acid. [14] Possible interferences commonly appearing in food products were found to have little effect on the sensor.

Several papers investigated the differentiation of AA, UA, and DA via different modifications of the electrode surface to obtain a simultaneous measurement of these three compounds. [15–21] Among the choices of materials, carbon nanotubes (CNTs) and polymers are especially interesting due to their good electrical properties as well as their capabilities to be functionalized to fit a specific purpose. These electrodes succeed in the compound discrimination to a certain degree; however, applying a potential with the subsequent current passing through the electrodes in order to oxidize AA in complex samples can easily result in surface inactivation. [22] Many other molecules and proteins may interact, bind, polymerize, or otherwise foul the surface, lowering the signal related to the analyte and rendering the sensor inert over time, which in turn reduces the real-world applicability. [23] In an attempt to reduce this inactivation issue, autooxidation of AA was proposed by having a second electrode with a higher overpotential than that needed to oxidize AA as the reference, resulting in AA being oxidized at the working electrode without any external current. [24,25] In all these cases, it is difficult to translate the reported approaches into a nanoelectrode capable of providing measurements at the single cell level.

While being an option to determine AA, potentiometric sensors reported for AA have been only few, mainly relying on indirect measurements. For example, taking advantage of the ability of AA to convert iodate into iodide, which would then be measured by an iodide ion-selective electrode. [26,27] Nevertheless, this method is limited by the fact that in a complex matrix (i.e., real samples), other antioxidant compounds might have the same effect in iodate, such as citric or oxalic acid, [28] and is as such not specific to AA. Reports about non-enzymatic potentiometric AA sensors are based on: i) metal ions such as copper to catalyze AA oxidation, [29] ii) imprinted polymers for capturing the AA, [30] or iii) membranes traditionally containing an ionophore [31] or being produced by electrochemical methods in turn. [32] Such sensors did demonstrate adequate selectivity for AA in contrast to the iodate sensors. Notably, in the case of the membrane containing an AA

ionophore, the interactions resulted in a super-Nernstian response with underlying mechanisms which are poorly understood. [31]

In this paper, we develop and demonstrate for the first time a potentiometric sensor based on a thin-layer (nanometer-sized in thickness) membrane–CNTs tandem for AA detection. In addition, computational simulations for insights into the mechanism are performed. The experimental evidence accompanied by calculations lead to the hypothesis that unmodified CNTs, with few inherent surface groups, have a specific affinity to AA over other possible compounds (e.g., UA), a finding which, to the best of our knowledge, has not been reported before. Utilizing the combined chemical, physical, and electrical properties of CNTs being confined in a plasticized polymeric matrix of ca. 230 nm of thickness, a new sensor capable of measuring AA in undiluted serum samples is here presented. Importantly, the conceived approach is easy to be further translated to the nanopipette format to address the desired cell-based measurements but also fundamental studies at the nanoelectrochemistry domain. Accordingly, the significance of the developed sensor is three-fold: 1) analytical performance addressing several applications, 2) enhanced selectivity, specially towards UA, 3) simplicity of the concept in terms of materials and preparation that makes it compatible with micro- and nano-electrodes for further analytical applications never explored until now (e.g., intracellular measurements, nanoelectrochemistry, etc.)

2. Experimental

2.1. Reagents and materials

L-Ascorbic Acid, NaCl, KCl, Na₂HPO₄•2H₂O, NaHCO₃, NaOH, KH₂PO₄, MgCl₂, Sodium L-Lactate, NH₄Cl, KSCN, THF, and Tris (hydroxymethyl)aminomethane were purchased from VWR (vwr.com). Sodium-Citrate monobasic, Urea, Na₂SO₄, Creatinine, Tridodecylmethylammonium chloride (TDMACl), Sodium tetrakis[3,5-bis(trifluoromethyl) phenyl]borate (NaTFPB), Tetradodecylammonium tetrakis(4-chlorophenyl)borate (ETH 500), Poly(vinyl chloride), Bis(2-ethylhexyl) sebacate (DOS), and sulfuric acid were obtained from Merck (sigmaaldrich.com). Dopamine hydrochloride, 99 %, Glucose, Uric Acid, and HCl were purchased from Thermo Scientific (vwr.com). All these reagents were of analytical grade or higher. Bovine Serum Albumin was of molecular biology grade and purchased from VWR (vwr.com). All solutions were prepared with ultrapure water (Milli-Q, 18.2 MΩ cm, merckmillipore.com).

Multi-walled Carbon Nanotubes (outer diameter 30–50 nm, length 50 μm, purity > 95 wt%, ash > 1.5 wt%, specific surface area > 60 m²/g) was purchased from HeJi, and used as received (unmodified), and with two different modifications: carboxylated (c-CNTs) or functionalized with dodecyl groups NH-C18 (d-CNTs). Real samples were obtained from the IMIB biobank (Instituto Murciano de Investigación Biosanitaria). Tested samples were urine, serum, and saliva, and the experiments were approved by and conducted in accordance with the Ethical Committee of Universidad Católica San Antonio de Murcia (CE062308), received and kept at –80°C until used. Samples used for fluorescence were first deproteinized with a 10 kDa cutoff centrifuge filter as described by the ascorbic assay kit manual.

2.2. Electrochemical measurements

All the electrochemical measurements were conducted in 10 mM PBS consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ dihydrate, and 1.8 mM KH₂PO₄ (ionic strength of 0.169 mol/L) and with a pH 7.4 unless otherwise indicated. Voltammetry was performed with a PGSTAT101, using the NOVA 2.1.6 software from Metrohm with a single junction Ag/AgCl 3 M KCl reference electrode from Metrohm (6.0733.100), and a platinum rod counter electrode also from Metrohm. Potentiometric experiments were performed with a Lawson 16 interface potentiometer, using the corresponding EMF16 software and a Metrohm

double junction reference electrode (6.0726.100) (3 M KCl inner filling and 1 M LiAcO outer filling solutions). Potentiometric experiments were run while stirring of the solution at 350 rpm using a magnetic stirrer (IKA Color Squid S000, Germany). The working electrode in the experiments was of three types: 1) raw glassy carbon electrode (GCE), 2) GCE modified with CNTs, c-CNTs or d-CNTs, with or without a plasticized polymeric membrane, or 3) unmodified platinum disk electrode (Pt). GCEs (6.09395.014) and Pt electrodes (6.1204.310), both with a diameter of 3 mm, were purchased from Metrohm.

2.3. Preparation of the CNT- and CNT/membrane-based electrodes

For both amperometric and potentiometric measurements the CNT modified GCEs, from now on mentioned as CNT, c-CNT, or d-CNT electrodes, were prepared as following. A mask was 3D-printed from CPE-plus filament with an UltiMaker S5, with dimensions of 5.3 mm for the inner hole diameter facilitating CNT deposition, 12.2 mm as the outer diameter and 1 mm for the wall thickness. A silicone ring was allocated between the mask and GCE as a seal and to allow the later disassembling. Subsequently, 10 μL of a 1 mg/mL CNT/THF solution were deposited on the GCE surface through the mask and left to dry in air at room temperature. The process was repeated 10 times, for a total deposited volume of 100 μL . When a membrane is required on top of the CNT film, a volume of 30 μL of the corresponding membrane cocktail was drop casted on the CNT surface while the electrode was rotated at 1500 rpm for 1 min using a rotating disk electrode (Autolab RDE, Metrohm Autolab B.V., Utrecht, The Netherlands), following an adaptation of the protocol reported elsewhere for the preparation of thin-layer membranes of ca. 230 nm of thickness. [33,34] The membrane cocktail compositions herein investigated are presented in Table S1.

2.4. Standard addition procedure coupled with in-sample calibration of electrodes

A protocol for standard additions in real samples of unknown concentrations was adopted due to three reasons: i) to minimize sample exposure time to air and light, both of which degrade AA at room temperature, ii) to induce the conditioning of the membrane with AA before the electrode is exposed to the sample, and iii) standardizing the initial potential step of AA for a posterior standard addition program that is more reproducible and applicable to any sample. Accordingly, the established procedure was as follows. First, a stable potential readout from the GCE/CNT/membrane electrodes in 10 μM AA in PBS is obtained. Thereafter, the sample is added to the solution, with a 1:2 sample:PBS ratio, and the signal is registered until a stable potential is attained. Then, the standard addition program is performed. Finally, high AA concentrations are added for individual calibrations of the electrodes to account for possible matrix effects as well as for small individual differences in the sensitivity of the electrodes. The resulting measurements were fitted with MatLab software using a custom script, taking all dilutions and additions into account, from which the starting concentrations in the samples were calculated.

2.5. Accuracy study using a fluorescence assay kit

Fluorescence-based Ascorbic Acid Assay Kit was purchased from Sigma-Aldrich (MAK074) and used following the manufacturer indications. Briefly, these steps were accomplished: 1) Filling of the wells on an opaque fluorescence 96-well plate to a total of 120 μL in each, consisting of the provided AA buffer and sample or AA standard for calibration. 2) Adding 30 μL of the catalyst to each well, as well as 50 μL mastermix, consisting of a 46:2:2 ratio of AA buffer, probe, and enzyme mix respectively, all of which is provided by the kit. 3) This mixture is left in the dark to react for 5 min and measured as single point data with an excitation and emission wavelength of 535 nm and 587 nm, respectively. [35] Fluorescence data was gathered with a Fluoromax Plus

Spectro Fluorometer from Horiba, with an attached MicroMax 384microwell-plate reader.

2.6. Simulations

DFT calculations and Molecular Dynamics (MD) Simulations are described in detail in the [supporting information](#).

3. Results and discussion

3.1. Voltammetric characterization of the CNT-based GCEs

Cyclic voltammetry (CV) experiments utilizing GCEs unmodified or modified with CNTs were performed in AA and UA solutions, as well as a mixture of these. The concentration of 1 mM was selected because this level considers both pharmacological and physiological values of interest of AA, and it is easily observed in a CV. For UA, 35 μM was chosen based on its solubility in aqueous solutions. The results are presented in Fig. 1. It was evident that the CNTs facilitated the oxidation of AA with respect to UA: two peaks at ca. -20 and 350 mV were observed in Fig. 1b versus a single peak at ca. 500 mV in Fig. 1a. Moreover, the cathodic wave was visible only in the presence of CNTs. In the case of the bare GCE, the found electrochemical behavior was similar as that previously reported: the typical non-reversible feature involving a two-electron transfer reaction, without being able to distinguish between AA and UA oxidation, which appears in the potential window from 0.3 V to 0.7 V with an anodic peak at ~ 0.5 V. From this experiment, the preference of CNTs to catalyze AA oxidation respect to UA was evidenced, certainly due to any kind of AA-CNTs interaction, and inspired us to continue investigating on it bearing in mind its electroanalytical exploitation.

Figure S1 in the [Supporting Information](#) displays both the CVs and background corrected data for the same sample solutions but using CNTs, carboxylated CNTs (c-CNTs) and functionalized CNTs (d-CNTs) deposited on the GCE surface. This experiment was designed aiming to understand whether any possible interaction of the AA with the CNTs is mainly due to the C structure. Two distinct voltammetric peaks were observed for AA and UA at ca. -0.02 V and 0.35 V on both CNTs and c-CNTs electrodes, respectively, finding an analogous behavior to that reported elsewhere. [36,37] With d-CNTs, the AA peak presented a shift, appearing at ca. 100 mV compared to the previous position of -20 mV. Effectively, the discrimination between AA and UA is worse on d-CNTs compared to CNTs and c-CNTs. Notably, owing to the high capacitive contribution from the CNTs to the overall electrochemical signal, background corrected CVs are more appealing to be analyzed to guarantee appropriate conclusions. In any case, successive scans led to a gradual decrease of the peak current, as AA was consumed at the electrode surface due to the partial irreversibility of its oxidation process, with a decrease from 26.5 μA in the first scan to 14.5 μA in the 9th scan (considering CNTs as an example, Figure S2 in the [Supporting Information](#)). From the 6th scan, a steady state current was displayed, because of the establishment of the diffusion layer of AA near the electrodes surface, with the potential window from -0.2 V to 0.8 V and scan rate of 100 mV/s.

3.2. Investigation of the potentiometric response of CNT-based GCEs

The pKa of AA is ~ 4.17 , meaning that a physiological pH, it will be mainly in the form of the monovalent ascorbate anion (AA^-). Accordingly, a negative potentiometric response is expected at increasing AA concentrations considering zero current conditions. Moreover, if the response is purely generated by the AA^- following a traditional potentiometric response, a slope close to -59 mV/dec should be manifested. Fig. 2a depicts the potentiometric dynamic response of GCEs modified with CNTs, c-CNTs and d-CNTs towards increasing AA concentrations in the range from 3.16 μM to 10 mM, including the AA levels expected in human blood ($10 - 200$ μM) [38]. A potential decrease was observed in

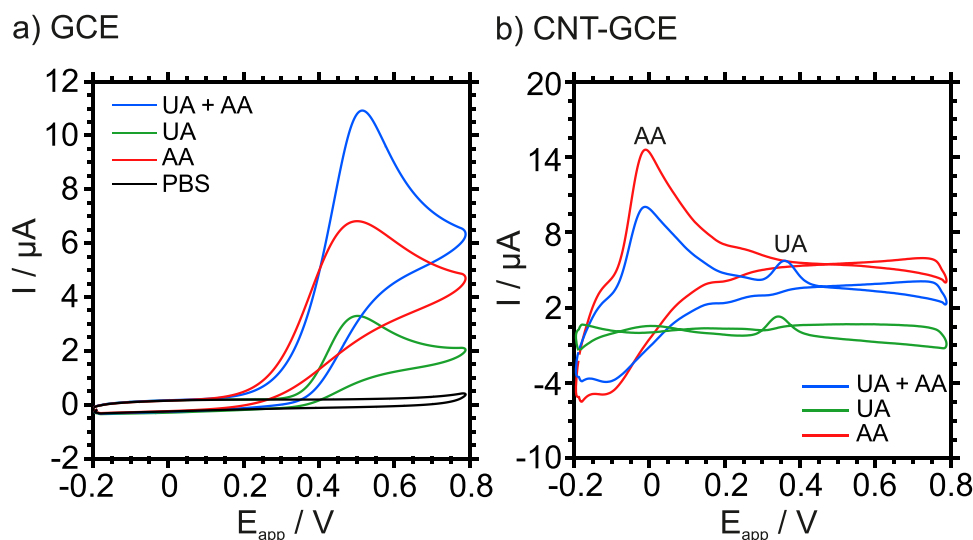


Fig. 1. (a) CVs provided by the bare GCE in PBS (black), 1 mM AA (red), 35 μ M UA (green), and 1 mM AA + 35 μ M UA (blue) solutions. (b) Background corrected CVs provided by the CNT-electrodes in 1 mM AA (red), 35 μ M UA (green), and 1 mM AA + 35 μ M UA (blue) solutions. Scan rate of 100 mV/s.

the three cases, with similar response times (t_{95} , defined as the time needed to achieve a response of 95 % of the total signal): 238–16 s for CNTs, 292–21 s for c-CNTs and 260–62 s for d-CNTs in the entire concentration range of the experiment.

When inspecting the corresponding calibration graphs prepared by plotting the logarithmic AA concentration versus the steady-state potential, it was observed that the linear range of response (LRR) started between 10 and 31 μ M and extends to at least 10 mM. Higher concentrations were not investigated, considering the levels expected in real samples/cases. The limit of detection (LOD) was not fundamentally explored, as it would be much lower than the concentrations of interest in view of the clear signal even at the first tested concentration of 3.16 μ M. The slopes found for all three types of CNTs were similar, around -23 mV/dec (Table 1). This is lower than the expected Nernstian response of -59 mV/dec for a single charged anion, suggesting that there are other mechanisms responsible for the potentiometric response rather than the traditional ones, which will be explored in this work. Moreover, the fact that the sensitivity of the electrode was kept unaffected by surface modification of the CNTs pointed out an interaction between the CNT backbone and AA.

One can realize that this aspect is surprisingly overlooked in many reports, despite the number of publications investigating voltammetric peak separation between AA and other compounds based on CNTs as well as potentiometric sensors.^{30,31,39,40} For example, a near-Nernstian response of -57 mV/dec with a LRR from 10 μ M to 2 mM (at pH 5.5) was revealed when using a molecularly imprinted polymer approach to enhance AA selectivity.^[30] In contrast, with a graphite electrode modified with a traditional polymeric membrane containing an AA ionophore, a pH dependent, super-Nernstian response in the range from -73 to -100 mV/dec (pH from 5 to 9) was found within a linear range of 5 μ M to 5 mM.^[31] The authors mentioned uncertainties in the working mechanism underlying the electrode, but lacking a formal study of the interactions of AA with the electrode surface. As the nature of the CNT demonstrated a non-significant influence on the potentiometric response, unmodified CNTs were selected for further experiments, therefore resulting in the simplest system.

As a control experiment, the AA calibration was additionally performed at the same experimental conditions as with the CNT-based electrodes but using a 3-mm Pt disc electrode. As observed in Fig. 2c, the Pt electrode presented a response time ranging from 125 s to 12 s, slope of -54.8 mV/dec and a LRR from 3.16 μ M to 10 mM (see Table 1). Three main differences with the CNT-based electrode were manifested: 1) the response time was almost twice as fast; 2) the LRR was wider,

especially considering the lowest AA concentrations; 3) a close to Nernstian response, as expected by a single negatively charged molecule as the AA at the experimental pH. Consequently, the CNT-AA output signal provided by the CNT-based electrode must be influenced by something extra than the expected traditional potentiometry readout, otherwise the signal would be like that provided by the Pt electrode.

To verify that the potentiometric response was not generated by a change in the redox potential of the sample solution at increasing AA concentrations, varying ratios of the redox couple Fe(III)/Fe(II) at a set concentration of 1 mM in 0.1 M KNO_3 background solution were investigated with the CNT-GCE and the Pt electrode. The results are shown in Fig. 2d. The calibrations of both electrodes were practically indistinguishable from each other and as such, the differences found between the CNT-based and the Pt electrodes for the AA calibration must be caused by some interaction between the AA and CNT backbone, and not by just a change of ionic species in the solution. Notably, this is likely the reason why CNTs have been used to discriminate between AA and other compounds using voltammetry.^[17,20,37,39,40]

Considering the pK_a of 4.17 for the AA, the AA/AA^- molar ratio will increase while decreasing the pH. Thus, if the CNT-GCE responds only to the anionic form of the AA according to a traditional potentiometric behavior, a lower potentiometric response would be expected with a higher AA/AA^- molar ratio. To this end, experiments at different pHs (7.4, 4.0 and 0.2) adjusted by the background solution were additionally accomplished. The results are presented in Figs. 2e and 2f. A slope of ca. -24 mV/dec was obtained irrespective of the sample pH, indicating that the potential change is not affected by the charge of the AA. A feature that was found to vary with the pH was the response time (t_{95}), being faster at physiological pH. As detailed in Table S2 in the Supporting Information, t_{95} values calculated at pH of 0.2 and 4.0 were higher than the equivalent ones in PBS. Variations connected to the first concentration jump (i.e., the very first stabilization time for the electrode when facing AA in the sample) may be related to different kinetics for the AA-CNT interaction in connection to the pH (and therefore AA/AA^- ratio) change: ca. 1573 ± 92 s, 988 ± 89 s and 238 ± 47 s ($n = 3$) for pH values of 0.2, 4.0 and 7.4. Nonetheless, the LRR was nearly the same for the three tested pHs, with 31.6 μ M to > 10 mM for pH 4 and 7.4, and reaching 10 μ M to > 1 mM in the case of pH 0.2.

Regarding the reversibility of the potentiometric response, and so the CNT-AA interaction, the CNT-GCE was immersed multiple times ($n = 20$) in solutions switching between 0.1 mM and 1 mM AA concentration. The results (Fig. 2g) revealed a rather reversible response, with the [average $\pm$ standard deviation] for the potential jump observed

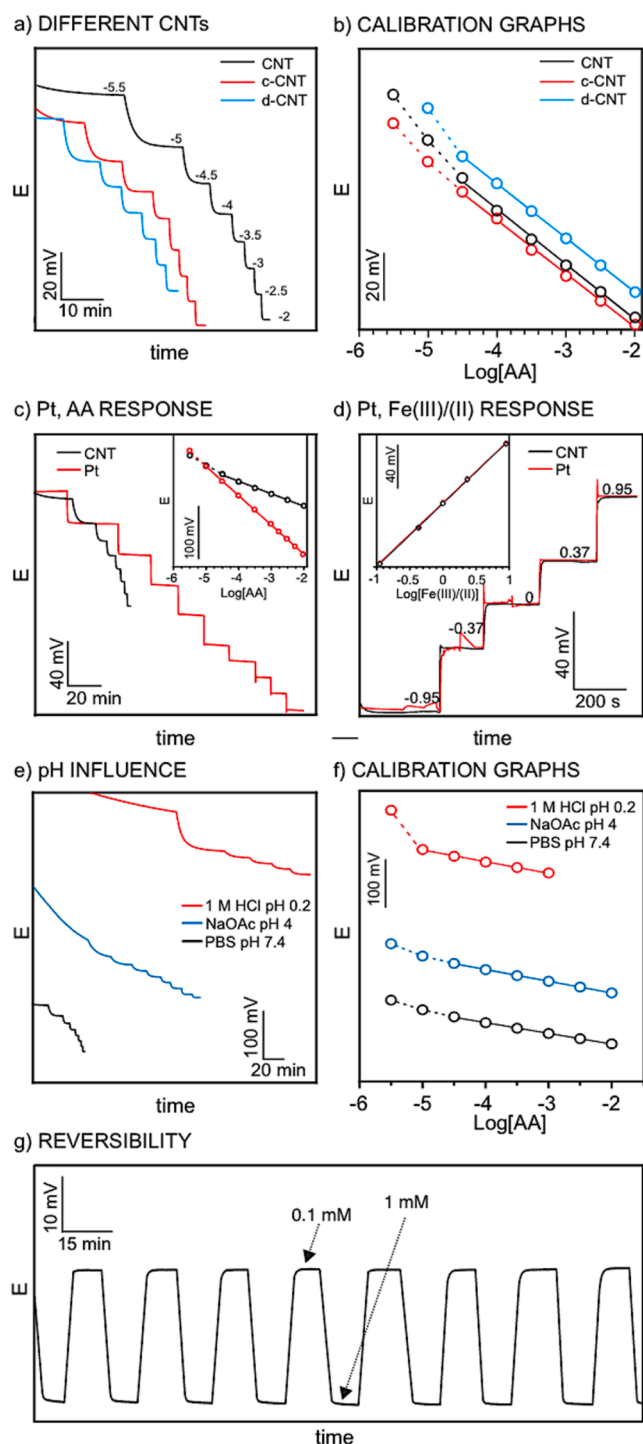


Fig. 2. (a) Potentiometric responses of electrodes based on CNTs, c-CNTs, and d-CNTs to increasing concentrations of AA. Numbers correspond to the total log [AA] concentration in 10 mM PBS solution. (b) Corresponding calibration graphs. (c) Potentiometric response of CNT and Pt electrodes using the same conditions as (a). Inset shows the corresponding calibration graphs. Slopes of -22.66 ± 0.69 mV/dec ($n = 3$) and -54.84 ± 2.19 mV/dec ($n = 1$) for CNTs and Pt. (d) Potentiometric responses of the electrode based on CNTs and a Pt electrode obtained by changing the $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio with a constant concentration of 1 mM. Inset shows the corresponding calibration graphs, with slopes of 57.65 ± 0.034 mV/dec ($n = 3$) and 57.20 mV/dec ($n = 1$) for CNTs and Pt. (e) Potentiometric responses of the electrode based on CNTs in solutions with fixed pHs of 7.4, 4, and 0.2. (f) Corresponding calibration graphs. (g) Potentiometric response of an electrode based on CNTs changing between AA concentrations of 0.1–1 mM in 10 mM PBS.

Table 1

Calibration parameters of the different electrode configurations towards increasing AA concentrations.

Electrode	Slope (mV/dec)	Intercept (mV)	t_{95} in the LRR (s)
CNT ($n = 12$)	-22.66 ± 0.69	-177.10 ± 6.09	237.7 ± 46.7
CNT-M1 ($n = 17$)	-33.53 ± 2.57	-174.22 ± 6.55	1524.8 ± 234.9
CNT-M2 ($n = 3$)	-33.25 ± 0.42	-184.45 ± 1.05	1199.4 ± 63.7
CNT-M3 ($n = 3$)	-33.59 ± 6.22	-182.32 ± 4.98	3502.5 ± 941.4
CNT-M4 ($n = 3$)	-31.51 ± 1.91	-172.58 ± 1.93	1136.0 ± 54.5
Pt disc ($n = 6$)	-54.84 ± 2.19	-93.61 ± 27.68	125.3 ± 33.6

when changing the concentrations was 21.67 ± 0.27 mV, and a 1.2 % of RSD when comparing two subsequent measurements of the same concentration over the 20 cycles. Moreover, t_{95} was 33.7 ± 1.4 s for increasing concentration changes, and 79.6 ± 0.95 s for decreasing concentration variations. In addition, it can be seen in Table 1 that the CNTs have a good reproducibility, both in the slope and the intercept, with less than 1 mV and 6 mV deviations, respectively.

Considering selectivity, as above described (Fig. 1), UA is a common interferent of AA measurements when using voltametric methods. A preliminary test with UA in PBS on the CNT-electrode was performed to realize if this is also the case in potentiometric measurements. Figure S3 in the Supporting Information shows the potentiometric response at increasing UA concentrations in the range from 25 μM to 316 μM . It presented a positive response of ca. 9.6 mV/dec, being unaffected by the pH. This behavior was unexpected, i.e., in principle, a negative response should appear similar as for AA. Therefore, both AA and UA were considered in the simulations herein performed (see below). In any case, the positive outcome was that the UA exhibited a reduced potentiometric response in comparison to that of AA. Notably, other interferences were considered only once the membrane was implemented in the electrode (see below).

3.3. Investigation of the potentiometric response of membrane-CNT-GCE systems

The potentiometric response of membrane-CNT electrodes towards AA was evaluated to understand the possibility of membrane utility in enhancing the overall performance, including selectivity. The initial hypothesis was that the CNTs will present a double role: as the ion-to-electron transducer but also a kind of AA ionophore/receptor when wrapped within the membrane phase. A membrane with a thickness in the hundreds of nanometers was used aiming to minimize the diffusion time of AA from the sample towards the CNT layer as much as possible, in contrast to the traditional hundreds of micrometers used in potentiometric sensors. [41] Notably, this sort of membranes can be used from a couple of days to a week taking special care of the rinsing process in order to preserve the initial response. The first membrane tested was based on a simple PVC/DOS membrane, labelled as M1 (Table S1 in the Supporting Information). The potentiometric response at increasing AA concentrations together with that observed in the case of the CNT-GCE are displayed in Fig. 3a. Fig. 3b shows the corresponding calibration graphs.

The first effect that could be realized was an increase by approx. 10 mV/dec in the sensitivity with the membrane deposited on top of the CNTs: -23 versus -33 mV/dec for bare CNT and CNT-M1 respectively. This is likely due to the rough nature of the CNTs, where the membrane might be unevenly distributed across the surface, with nanopores and holes throughout it. This would in turn allow for different diffusion and interaction, changing the whole surface similar as a thin film system. [42] Secondly, the shift of the LRR, which was found to range from 31.6 μM to 10 μM . Thirdly, there was an increase in the t_{95} for the entire AA concentration range that was tested, as summarized in Table S3 in the Supporting Information. Indeed, the t_{95} was strongly influenced by the concentration of AA present in the sample, similarly to the CNT-GCEs, ranging from 1500 s to 200 s.

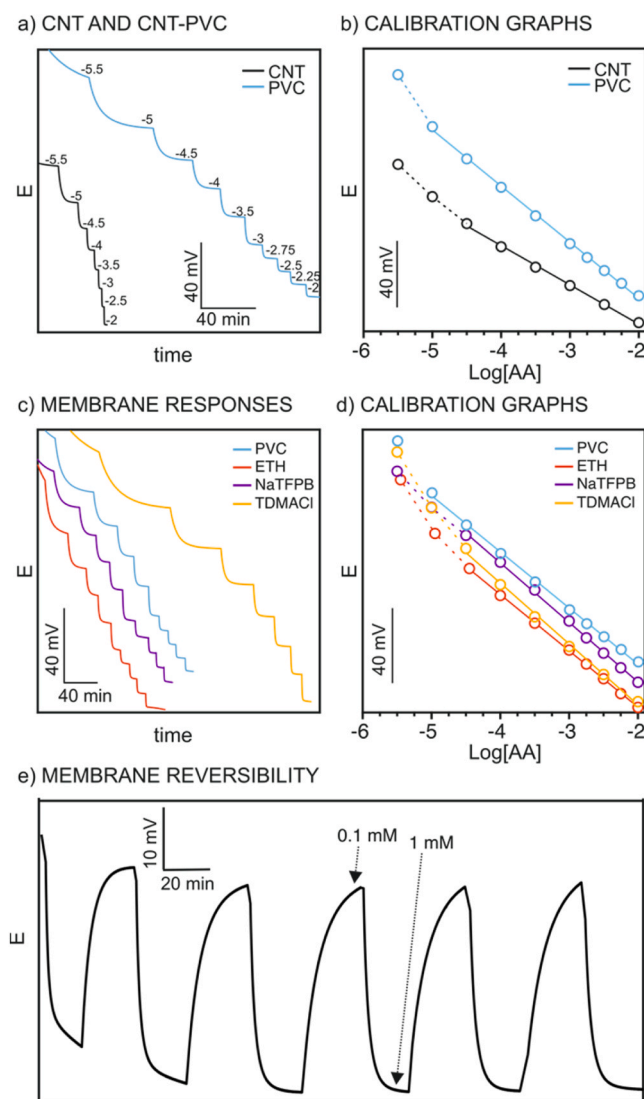


Fig. 3. (a) Potentiometric responses provided by the CNT- (black) and CNT-M1 (blue) electrodes. Numbers correspond to the total log[AA] concentration in 10 mM PBS solution. (b) Corresponding calibration graph. (c) Potentiometric response of AA observed in CNT-electrodes comprising the M1–M4 using the same conditions as in (a). (d) Corresponding calibration graphs. (e) Potentiometric response of the CNT-M1 electrode changing between 0.1 and 1 mM of AA in PBS.

Membranes M2–M4 (Table S1 in the Supporting Information) containing cation exchanger (NaTFPB), anion exchanger (TDMACl) or a lipophilic salt (ETH500) were also tested. The corresponding time traces and calibrations are provided in Fig. 3c and Fig. 3d, while the calibration parameters are shown in Table 1. When comparing the results of the CNT-electrode vs. M1–M4, the addition of a membrane irrespective of the composition was found to increase the sensitivity by ca. 10 mV/dec, but the addition of either an ion-exchanger or the lipophilic salt did not significantly change either the sensitivity or the intercept. However, TDMACl increased the t_{95} especially at lower concentrations (3503 s vs. 1525 s when stepping from -5.5 to -5 compared to the M1 membrane). This is likely explained by the additional pairing of AA^- with the TDMA^+ counterpart in addition to the CNT interaction, which does not occur in the rest of the membranes.

According to the results, membrane M1 was selected for further studies. Reversibility of this electrode was evaluated following the same experimental protocol as for the CNT-GCE. As observed in Fig. 3e, the results presented a potential step of -31.38 ± 2.97 , with 9.5 % RSD

($n = 3$). However, if the first cycle is excluded, evidently the electrode was not fully conditioned yet, the reversibility was refined: slope of -30.18 ± 0.52 mV, intercept at -165.61 ± 9.33 mV and a 1.5 % RSD. These values for the membrane-based electrode rather agree with the results found for the CNT-GCE.

3.4. Selectivity study of CNT- and CNT-M1-based electrodes

A series of experiments were conducted to assess certain interferences, acknowledging those species mainly present in biological fluids that are prone to provide a potentiometric response. [38,43,44] Notably, as the PBS used for the experiments conducted until this moment of our research contains significant amounts of potassium, sodium, and chloride ions, a medium based on TRIS buffer at pH of 7.4 was used to test these ions as interferences. Figure S4 in the Supporting Information depicts the dynamic potentiometric responses of the selected substances, while the total potential jumps at the tested concentration ranges are presented in Table 2. For potentiometric ISEs, the apparent selectivity coefficient is typically used to describe the electrode performance. However, this is namely when investigating the interferences with the same charge sign. [45] Because we have a collection of differently charged ions, we decided to refer to the total potential jump to evaluate any possible effect.

In the case of UA, lactate, glucose, creatinine, urea, citrate, MgCl_2 and CaCl_2 , the response towards 10 μM AA in PBS was first recorded and then, when this was constant, increasing concentrations of the corresponding interference were gradually added (Figures S4a–S4j). Only for UA, CaCl_2 and MgCl_2 some positive responses were observed, but far from the Nernstian behavior and always being lower for the electrode containing the membrane (total potential changes for M1 of 10.46 ± 0.15 mV, 17.51 ± 2.08 mV, and 15.14 ± 0.85 mV respectively, Table 2). For UA, as in the case of the CNT-GCE, a positive potentiometric response was found with M1, though in contrast to the response for AA, the sensitivity did not change when the membrane is added. These findings are further explored in our simulation studies (see below), to understand the differences between AA and UA.

When investigating NaCl and KCl in TRIS buffer, the same protocol as for the previous compounds was followed (Figures S4f and S4h). Significantly long measurements were needed due to stabilization issues. Also, drifting signals were observed for the CNT-GCE, with the obtained results being difficult to be analyzed. Nevertheless, for the M1-based electrode, the total potential jumps pointed out negligible interferences from the sodium and potassium ions (10.19 ± 1.70 mV and 1.66 ± 10.49 mV respectively for a concentration change higher than 3 orders of magnitude, Table 2).

The study of the pH interference was conducted in PBS, increasing the pH with NaOH additions and decreasing it with HCl additions while measuring the resulting pH with a commercial pH electrode. The dynamic potentiometric traces are presented in Figure S5 in the Supporting Information. For CNTs there was a clear potential shift with the addition of NaOH or HCl, with a slope of 36.13 ± 3.36 mV/pH between pH 6.6 and 8.3. With the CNT-M1 the potential shift was less obvious, with a

Table 2

Total potential change of interferences in the presence of 10 μM AA.

Compound ($n = 3$)	CNT (mV)	CNT-M1 (mV)	logC range
Uric Acid	9.94 ± 0.79	10.46 ± 0.15	from -4.6 to -3.5
Glucose	1.04 ± 0.08	-0.37 ± 0.73	from -5 to -2
Na-lactate	1.08 ± 0.13	0.65 ± 0.34	from -5 to -2.3
CaCl_2	33.11 ± 0.63	17.51 ± 2.08	from -6 to -2.3
Creatinine	2.42 ± 0.31	2.53 ± 0.65	from -5 to -2.3
NaCl	12.93 ± 0.91	10.19 ± 1.70	from -4.8 to -1
MgCl_2	17.32 ± 0.92	15.14 ± 0.85	from -6 to -2.4
KCl	6.92 ± 0.46	1.66 ± 10.49	from -5 to -1.6
Urea	0.47 ± 0.27	0.11 ± 0.06	from -6 to -2.3
Citrate	0.30 ± 0.33	-0.16 ± 0.22	from -5.5 to -2.5

slope of 9.26 ± 2.30 mV/pH in the same range of pH. This change in sensitivity could be explained by the much lower ability of the involved ions to interact with the CNTs. Overall, the addition of the membrane lowers the interferences from other ions to a suitable degree.

Additionally, AA calibrations were performed in artificial biofluids, namely artificial saliva (ASa), urine (AU) and serum (ASe), to understand if the response provided by CNT-M1 electrodes suffers from any matrix effects in view of the just described responses. The results are provided in Fig. 4 and Table 3. The AA response in artificial biofluids did not significantly change in terms of sensitivity compared to PBS, with the LRR displayed in ASa being slightly wider. While the developed electrode revealed potential applicability in real biological samples at this stage, the provided response was found to be significantly slow in certain media, especially at the lowest assayed concentrations (e.g., from -6 to -4.5 in $\log[\text{AA}]$ units in saliva).

3.5. Investigation of the mechanism

The AA-CNT interaction was evaluated in terms of energy and compared to UA-CNT. A simulation was conducted with focus on investigating the binding energies (ΔG_{bind}) of AA and UA on graphene in aqueous and CH_2Cl_2 (DCM) environments, mimicking both the CNT-sample (CNT-aqueous) and CNT-membrane (CNT-organic) phases. To accurately describe the non-covalent interactions between graphene and the acids, the epsilon value of the graphene carbon atoms was increased, in accordance with prior studies of catalysts adhered to graphene, [46] as shown in Table S4. Notably, graphene was selected to simplify the simulation process and because the needed parameters and conditions are better established than for the CNTs. Also, because of the empirical evidence of AA interaction with the carbon bone of the various CNTs, graphene was considered a suitable general model. It was assumed that the size of the AA molecule versus the CNTs is very small, thus making graphene a reasonable model for the large nanotube. The ΔG_{bind} values are derived from the potential of mean force (PMF) obtained through a series of umbrella sampling simulations. The lowest pKa values of AA and UA are 4.17 and 5.6 ($\text{AAH} \rightleftharpoons \text{AA}^- + \text{H}^+$, $\text{UAH} \rightleftharpoons \text{UA}^- + \text{H}^+$), and as such, the ΔG_{bind} of their first dissociation products (AA^- and UA^-) were investigated as well, because these would be the main form at physiological pH.

Fig. 5a and Fig. 5b show the PMF required to remove AA^- , AAH, UA^- and UAH a specific distance from the model surface of graphene. If PMF = 0, that is the distance with the lowest energy state, or the most stable configuration, of the molecule on the surface. In aqueous solution, the

Table 3

Calibration parameters using the CNT-M1 electrode in artificial biofluids.

Media	Slope (mV/dec)	Intercept (mV)	LRR	t_{95} in the LRR (s)
Saliva	-34.26 ± 2.73	-206.76 ± 1.99	from -5.5	1888.9 ± 370.1
Urine	-35.20 ± 1.35	-171.82 ± 6.61	from -4.5	618.3 ± 91.4
Serum	-32.55 ± 0.89	-207.51 ± 6.20	from -5	745.8 ± 82.8

values calculated for ΔG_{bind} (Table S5 in the Supporting Information) were 7.58, 8.00, 13.87, and 14.66 Kcal/mol for AA^- , AAH, UA^- , and UAH respectively. This ΔG_{bind} reflects the energy needed to completely detach these species from the most stable configuration on the graphene surface, bringing them to the bulk of the (aqueous or organic) solution. It was observed that the difference in ΔG_{bind} between the protonated and deprotonated species are not significant for both AA and UA, with less than 1 kcal/mol of difference. This is likely pointing out that the interaction between the acids and graphene is not electrostatic in nature and hence, the charge does not play a critical role in the binding energy. This conforms well to the experimental data, as the pH, and thus the AA/ AA^- ratio, does not change the sensitivity of the CNT-GCE towards AA.

When comparing the simulations of the PMF values vs. distance from the surface for AA^- and AAH in water, it is evident that AA^- starts attaching to the surface at around 0.95 nm (i.e., the point where the PMF starts to decrease), whereas AAH is at around 0.75 nm. Accordingly, the detachment starts for AAH before that for AA^- , which could be the reason for the t_{95} increasing when lowering the pH found in our potentiometric experiments. Then, in DCM, calculations provide an indication of the change in binding affinity between polar water and a non-polar medium, giving a rough estimation on the changes in ΔG_{bind} when the membrane is present. For both AA^- and UA^- , the energy needed to remove the molecule completely from the surface is reduced by half compared to water. This may be related to the increase found for the slope in the potentiometric experiments.

Regarding UA, a difference of approx. 0.1 nm for the distance in which UA^- and UAH are attracted to the surface was observed, coinciding with a slower t_{95} when decreasing the pH, similar as the effect described for AA^- and AAH. Then, we decided to explore more experimental evidence to determine the cause of the positive potentiometric response of UA. Thus, motivated by theoretical insights from the literature that simulated AA and UA on models of pristine and hydroxylated graphene, [47] the response of UA on CNTs and d-CNTs was investigated. Figure S6 in the Supporting Information depicts the potential response of UA on CNTs and d-CNTs. The electrode based on d-CNTs presented a lower positive signal than the CNTs (2.82 ± 1.62 mV/dec

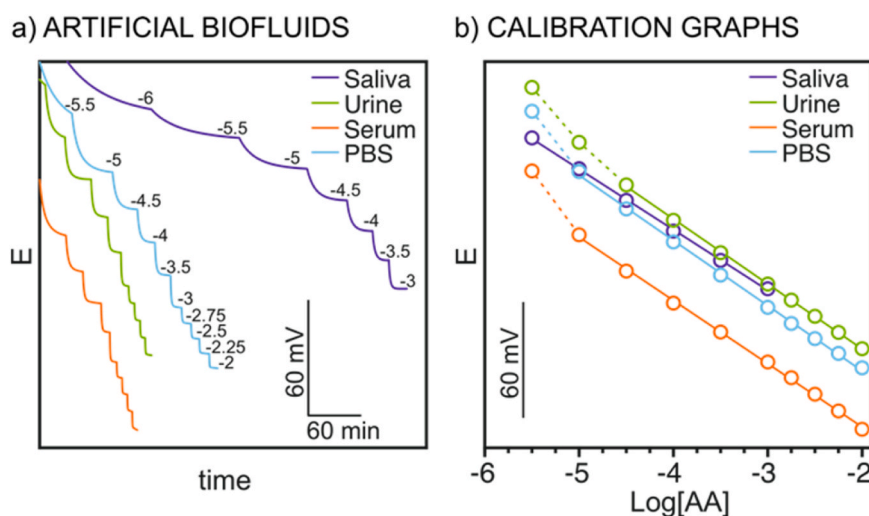


Fig. 4. (a) Potentiometric responses of AA on CNT-M1 in PBS and artificial biofluids. Numbers correspond to the total $\log[\text{AA}]$ concentration, ranging from 3.16 μM up to 10.00 mM. (b) Corresponding calibrations of the different artificial biofluids.

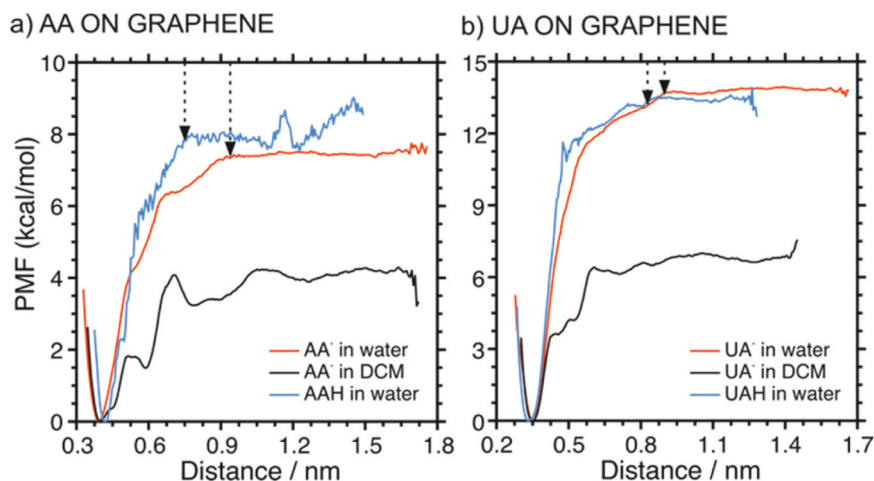


Fig. 5. Potential of mean force of (a) AA⁻ and AAH and (b) UA⁻ and UAH in water and DCM depending on the distance from the graphene surface. Dotted arrows represent the distance where the surface of the graphene will start attracting the analyte.

and 9.58 ± 0.39 mV/dec respectively). The charge difference of the molecule after binding to pristine graphene should be negative for both AA and UA, while binding to hydroxylated graphene would give a negative change in charge of the AA molecule, but a positive change in charge of the UA molecule, a similar pattern to what we see on the CNTs.

The positive signal for UA could therefore be associated to -OH imperfections in the CNTs, which would be less pronounced after functionalizing the CNTs with a C18 chain, removing the -COOH functionalities from the c-CNTs, resulting in the lesser response of the d-CNTs. This also explains why there is not much difference between the CNTs, c-CNTs, and d-CNTs for AA, as both the body and the imperfections of the CNT would give a negative contribution of the molecule charge after binding to the surface. However, for UA, there is a mix of positive and negative contributions from the body and the imperfections, generating different directions in the potentiometric response of AA and UA on CNTs. This situation maybe responsible for a lower potentiometric response than the Nernstian one expected for a single charged anion such as AA at our experimental conditions, since it surely affects the potential interface at the CNTs-sample or CNTs-membrane interface. Truly, this effect is less significant in the case of the CNT-membrane system. Moreover, lower ΔG_{bind} values were revealed for DCM than water. The used CNT materials have indeed intrinsic imperfections, as revealed by the Raman spectra provided by the supplier: The D- and G- band ratio is similar to those for the MWCNT-COOH material used in the literature, which means the presence of surface groups. [48] Also, there should be no metallic center left in the nanotubes to catalyze AA oxidation, as there are no peaks present in the range 100–1800 cm^{-1} besides the CNT G and D band. [49]

Finally, inspecting the MD trajectories for AA and UA, some hints can be obtained about the nature of its interaction with the graphene. As observed in Figure S7, UA generally maintains a planar conformation on the graphene surface, indicating that its adsorption is mainly stabilized by π - π interactions. For AA, the five-membered ring lies nearly parallel to the graphene surface, with C-H bonds pointing towards it, suggesting that the adsorption is dominated by both the noncovalent interaction of the five-membered ring with graphene and C-H... π interactions.

3.6. Quantification of AA in real samples

The standard addition method was used to determine the AA content in unfiltered serum, urine and saliva samples by means of the CNT-M1 electrode. In essence, the corresponding sample was added to a PBS background including 10 μM of AA and then the standard addition method was performed (20, 40, 70, 100, and 150 μM of AA). Finally, an AA calibration covering the described LRR was carried out to ensure that

the electrode response was correct (1500, 3160, and 5620 μM of AA). All the samples were additionally analyzed with the commercially available fluorescence kit for comparative purposes (see the [Supporting Information](#) for more details). The potentiometric response observed along the defined protocol applied to the serum sample together with the corresponding plot of the standard addition part are presented in Fig. 6 as an example of the obtained results.

The sensitivities of the electrodes in the real samples were slightly altered compared to PBS and artificial fluids, with the slopes being -26.74 ± 0.42 , -31.94 ± 0.40 , and -37.57 ± 1.06 mV/dec for serum, saliva, and urine respectively. Compared to the artificial biofluids, both serum and saliva showed a lower sensitivity, with saliva closer to PBS and ASa than serum. In contrast, real urine, showed a higher sensitivity for AA than in PBS and AU, hinting at other interactions that have not been anticipated. Overall, the protocol established for real samples measurements easily accounts for all these variations but also, any change in the electrode response caused by its continued usage. It is also worth noticing that the t_{95} at the LRR for artificial biofluids were 745.9 s, 3568.7 s, and 618.3 s for ASe, ASa, and AU respectively, but

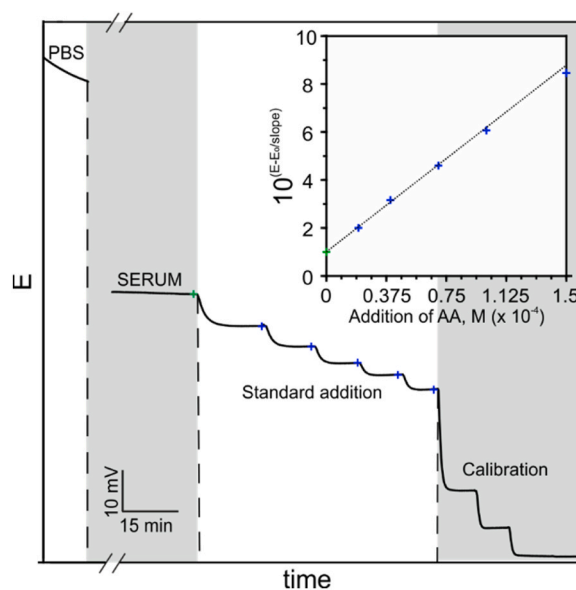


Fig. 6. Potentiometric response obtained along the experimental program developed to analyzed AA in of unfiltered serum. Inset shows the plot of the potentials selected to apply the standard addition method.

once the real samples were added, a long conditioning step appeared (approx. 5000 s for serum and 2000 s for saliva and urine). Fortunately, no fouling effect on the electrode response was detected in the experimental time frame (considering both sample measurements and overall electrode usage).

The comparison of the analytical results with those provided by a commercial fluorescence kit are provided in Table 4 for all three samples used as received. A good agreement for serum (<7 % of difference) was found. However, for saliva and urine, the discrepancies were more significant, with the value provided by the kit for saliva being outside the detection range. Notably, the kit is specially indicated for serum analysis and thus, it might not be suitable to properly validate the measurements provided by the developed electrode in other samples than serum. To investigate this, the saliva sample was spiked with a known amount of AA (30 μ M) after being filtering and just before been measured. Such a sample was also analyzed. The fluorescence showed a concentration of only $3.96 \pm 1.48 \mu$ M, while the electrodes provided a concentration of $30.4 \pm 4.8 \mu$ M. This latter corresponded to a recovery of 101.3 %, indicating that the electrodes is a more reliable method for measuring AA in saliva than the fluorescence reference kit. This conclusion was extrapolated to the case of urine.

4. Conclusions

In this work, it has been demonstrated that CNT-based electrodes modified with a very thin membrane can selectively detect AA in biological samples in a reliable way. From the analytical point of view, the linear range found for the sensor is rather wide (from 10 μ M to above 10 mM), including the expected levels in biosamples, both of physiological and pharmacological interest. It has been discovered that the sensitivity of the potentiometry readout is not affected by the sample pH, making it a tangible candidate for different sample types. While the sensor did not exhibit Nernstian behavior, the proposed mechanism behind the potentiometric response is not based on the charge of the molecule, but rather on surface interactions between AA and the CNT backbone and defects, which changes the charge distribution on the surface and inherently show a discrimination between AA and UA. This mechanism is based on both simulations of AA and UA near the surface of pristine graphene, combined with simulations of the electron-distribution change of AA and UA on graphene with defects from other works, and corresponds well to the experimental data shown here. All the described features make the CNT-membrane potentiometric concept herein developed unique for any real application. Moreover, the implicit simplicity of materials and fabrication process provides a tangible horizon for the concept to be applied to micro- and nano-analytical devices that may find suitability for fundamental nanoelectrochemistry insights as well as intracellular measurements desired in clinical research related to cancer and others. These are aspects that will be considered in our future research steps.

CRediT authorship contribution statement

Ahlquist Mårten S. G.: Writing – original draft, Software, Investigation, Formal analysis. **Gastón A. Crespo:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Cuartero Maria:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Ke Ye:** Writing – original draft, Software, Investigation, Formal analysis. **Christian Meinert Putnaergle-Bache:** Writing – original draft, Visualization, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

Table 4

Determination of AA in real samples, n = 3 (electrodes), n = 5 (kit).

Sample	Sensor (μ M)	Kit (μ M)
Serum	35.1 ± 4.3	32.69 ± 3.33
Saliva	17.6 ± 6.7	–
Saliva (30 μ M)	30.4 ± 4.8	3.96 ± 1.48
Urine	67.1 ± 30.0	17.47 ± 3.45

the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2025.138548.

Data availability

Data will be made available on request.

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