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Functional Biosensing Platform for Urea Detection: Copolymer of Fc-Substituted 2,5-di(thienyl)pyrrole and 3,4-ethylenedioxythiophene

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Biosensing devices for urea detection have become extensively researched as the analysis of urea levels is imperative in biological fluids indicating disorders of renal, hepatic, nervous and blood circulatory systems. The current work describes the development of two biosensing platforms for urea based on electrochemical deposition of ferrocene-substituted 2,5-di(thienyl)pyrrole (SNS-Fc) and copolymerization with 3,4-ethylenedioxythiophene (EDOT) (SNS-Fc-co-EDOT) followed by coupling with multi-wall carbon nanotubes (MWCNTs) and immobilization of Urease (Urs) through cross-linking. Optimum operational parameters (pH, applied potential) and design parameters (enzyme units, cross-linker concentration) were thoroughly investigated. The analytical comparison between P(SNS-Fc)/CNT/Urease and P(SNS-Fc-co-EDOT)/CNT/Urease showed a linear range between 0.01–0.20 and 0.01–0.15 mM, respectively with superior sensitivity (13.49 mAM^{-2} cm^{-2}) and LOD (1.9 μM) for the latter. Little to no interference was observed leading to accurate urea detection in real samples.
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Urea is one of the main final products of protein metabolism and has great significance in clinical analysis, food chemistry (determining milk adulteration) and environmental monitoring (it plays an important role in the marine nitrogen cycle).[1] The analysis of urea levels is most imperative in biological fluids since it can indicate disorders of renal, hepatic, nervous and blood circulatory systems. Various analytical methods (chromatographic, chemiluminometric, colorimetric, spectrophotometric, fluorometric) are available for this purpose and albeit precise, they are complicated and require time-consuming sample pre-treatment, expensive instrumental set-up and trained persons to operate.[2,3]

The demand of sensitive, selective, and preferably low-cost techniques for urea detection has led to the extended development of biosensors. Biosensing devices have been constructed and successfully applied for an extended array of bio-assays. They can include several bio-receptors, transducers and different types of physico-chemical interactions depending on the type of detection mechanism and chosen analyte. Urea detection biosensors are highlighted due to high specificity and ease of fabrication, allowing online detection of a broad spectrum of analytes in complex matrices (e.g. blood, urine, food, water).[4–6]

Urease (urea amidohydrolase, EC 3.5.1.5) is a highly proficient enzyme, widely distributed in nature, whose catalytic role is the hydrolysis of urea with carbonic acid and ammonia as final products. It is the biocatalyst of choice for enzymatic detection devices and its catalysis is markedly noted by the increase of pH in the reaction microenvironment.[7] As such, the foremost efforts in urease biosensors have been in the development of pH-sensitive, conductometric and potentiometric devices due to particular sensitivity to the change in pH resulted from enzymatic catalysis. Guilbault and Montalvo (1969)[8] pioneered the field by developing a potentiometric urease electrode for determination of urea through the variation in potential of a cation-selective glass electrode sensitive to ammonium ions, proportionally to urea concentration. Since then, increasing focus has been placed on the development of potentiometric urea biosensors.[9] Many different versions of this biosensor have been described since including miniaturized urea electrodes based on ISFET transducers.[4] However, the selectivity of potentiometric devices has proven troublesome, as other ionic species (e.g. potassium) can cause significant signal interference. Thus, the field has been extended towards different types of detection methods.[10]

Conductometric methods for urea detection are based on the change in the resistance of a solution during enzymatic catalysis. Screen-printed interdigitated electrode arrays incorporating sol-gel immobilized urease for detection of increased solution conductivity have been reported.[11,12] Although facility in technique, a number of influences on the conductivity of the solution may alter the reliability of this type of biosensor. Simple manometric techniques have also been applied by measurement of the gas quantity (NH_3, CO_2) produced during enzymatic catalysis. However, manometric sensors require a sealed system and large headspace gas volume relative to the sample volume, thus their application is rather complex.[13]

Recently, amperometric urea sensing has gained interest as it can provide selectivity and accuracy of analysis in a straight-forward manner. A comparison between amperometric and potentiometric transducers based on the same matrix has been reported, with better performance for the former.[1] The amperometric principle of detection can be considered somewhat laborious, as the products resulted from urea hydrolysis are not electroactive and the oxidation of ammonia/ammonium ions to nitrogen is difficult to achieve at the electrode surface.[14] Most reports employ one of two approaches: (i) the use of a second enzyme, such as glutamate dehydrogenase (GLDH), which is interconnected to the biocatalytic cycle of urease and requires NAD(P)H as cofactor, which can also play the role of a redox mediator;[15,16] (ii) the use of (nano)matrices within intrinsic conducting properties which can catalyze ammonia electrooxidation accompanied or not by redox shuttles.[17,18] Although the former approach is established in urea bio-detection, it proves to be cost-ineffective and more challenging as it requires immobilization of two different biorecognition elements. Therefore, the latter option has become increasingly studied especially given the tremendous progress that has been made in nanotechnology within the last decade. More specifically, composite nanomaterials including redox mediators, conducting polymers and/or metal nanoparticles have been utilized for a synergistic improved performance of urea biosensors.[6]

On this note, conducting polymers (CPs) have been some of the most researched materials in the fabrication of urea detection devices ever since the initial attempts, and can still be considered the "building blocks" of modern biosensors.[19,20] Several CPs-based urea biosensors have been developed by entrapment of urease within electro-synthesized matrices since they meet the requirements of biocompatibility more so than many inorganic transducers, provide fast and efficient electron transfer (allowing both electronic and ionic transport) as well as facility in deposition on the desired type of electrode.[21] Stable immobilization of

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the biorecognition element onto CP matrices (polyaniline (PANI), polypyrrole (PPy), polythiophene (PTh)) is a significant strategy for development of enduring and efficient biosensing devices. In addition, the ions released as a product of urea hydrolysis, can serve as dopants for the polymers eliciting an electrochemical response. Many studies have focused on enzyme immobilization on PPy due to its high biocompatibility and low deposition potential along with efficiency in mediating ammonia detection.[22–26] Yet, the poor morphology of the PPy film and susceptibility to oxidative damage are frequent issues. As such, research was directed towards functionalization and copolymerization of pyrrole for better performance in urea biosensing including polypyrrole–polyvinyl sulfonate,[16] poly(N-glycidylpyrrole-co-pyrrole),[27] N-3-aminopropylpyrrole-co-pyrrole,[28] poly(glutaraldehyde-co-pyrrole,[14] polypyrrole/poly(ortho-phenylenediamine)[29] etc. In comparison, polythiophene (PTh) has been less employed, mainly due to the high electrodeposition potential required albeit its superior electrochemical features. One of the few reports describes a stable matrix for urease immobilization based on a semiconductor thiophene copolymer.[30] In the last decade, the synthesis of hybrid conducting polymer matrices such as 2,5-di(thienyl)pyrroles (SNSs) has been explored and proved highly promising for applications ranging from optoelectronics, photovoltaics to, most recently, biosensors. This type of structures showed good results in glucose biosensing and are promising for the development of detection devices for other important metabolites.

Considering that most reports thus far have been focused on CPs with various modifications, employing hybrid conducting matrices containing both thiophene and pyrrole units appears as promising. Additionally, SNS polymers offer a tremendous potential for functionalization through N-substitution of the pyrrole fragment. A variety of moieties can be tethered to the hybrid monomeric molecules without adversely influencing the electrochemical properties of the final polymer. A conducting platform that contains functional groups with the ability to connect to the desired bio-element appears very promising for achieving stable immobilization and adequate orientation of biorecognition elements in biosensing. In this regard, SNS derivatives exhibit favorable features such as facility in synthesis due to lowered oxidation potential, stable electrochemical behaviour with the added benefit of tailor-made functionality.[31]

Furthermore, incorporation of a mediator unit has been another relevant approach reported. From natural dyes (e.g. hematein[32]) and other redox-sensitive probes[33] (e.g. polytoluidine blue[34]) to (poly)vinylferrocene[35] and nickel hexacyanoferrate,[36] the electron transfer efficiency in urea biosensing has been enhanced by incorporation of a mediator unit. Ferrocene is one of the most efficient redox molecules due to its low oxidation potential, lack of pH susceptibility, stability in its redox states and fast electron transfer ability.[37] Previous works employed polymers of Fc-functionalized pyrrole[38,39] and thiophene[40,41] and an amino-substituted poly SNS coated with Fc for urea detection was recently reported.[42] Given the usual hindrances in classical mediated biosensors such as diffusion or leaching of mediator from the electrode (which leadsto deficient electron transport) and considering the tremendous potential for functionalization of SNS molecules, N-substitution with a Fc graft appears as a promising approach. As a result, a conducting material with intrinsic redox activity is rendered leading to the development of a so-called "reagentless" system.[43,44] The electrochemical properties of SNS structures can be further enhanced through copolymerization with other conducting materials, such as 3,4-ethylenedioxythiphene (EDOT); this approach has proved successful in electrochromic studies[45] by improving the electrochemical properties of the polymers (due to increased conjugation length and decreased band gap).[46,47] Albeit in incipient stages of research for biosensing devices, there is potential for enhanced electron kinetics leading to efficient transduction mechanism and, thus, fast and accurate analyte detection.[31] Conducting co-polymers of functional SNSs (e.g. 2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)amido ferrocenyl dithiophosphonate) with EDOT have been applied for glucose sensing with good results.[48] In the case of amperometric detection of urea, since the products from enzymatic catalysis are not easily oxidizable at the electrode surface, an improvement in electrochemical characteristics of the biosensor platform should provide enhanced analytical performance.

Therefore, the current study proposes not only the polymerization of a Fc-substituted SNS but also its copolymerization and application in biosensing of urea. The synthesis of the monomers and subsequent (co)polymerization of poly (SNS-Fc) and poly (SNS-Fc-EDOT) films has been previously reported in detail.[49] Glucose biosensors based on these two conducting matrices have been recently reported by our group showing adequate performance,[43] thus this study attempts to provide an analysis of their performance in urea detection, which is herein reported for the first time.

Given the outstanding merits of carbon nanoelements in biosensing (excellent electrical conductivity and efficiency in electron transfer, thermal and mechanical stability) and successful impact thus far in urea detection,[50–52] multi-walled carbon nanotubes (MWCNTs) were further included in the design of the two urea biosensors. The biosensing platforms based on the coupling of Fc-functionalized hybrid (co)polymers with MWCNTs have been evaluated by comparison of analytical characteristics, interference effects and accuracy in urea determination in real samples.

Experimental

Materials.—Urease (EC 3.5.1.5. from Jack beans) was purchased from Alfa Aesar. $LiClO_4$, $NaClO_4$, Urea, Multi-walled carbon nanotubes (MWCNT) (O.D. xl 6–9 nm × 5 μm, >95% (carbon)), sodium dodecyl sulfate (SDS), ethanol and acetonitrile were purchased from Sigma. All other chemicals were of analytical grade and purchased either from Merck or from Sigma. The urine sample used for real sample analysis was kindly gifted by local laboratory.

Instrumentation.—All amperometric measurements were performed with the potentiostat GAMRY Ref. 600 (GAMRY Instruments Inc., Pennsylvania, USA) in a three-electrode cell configuration consisting of a platinum foil electrode (0.5 cm^2) as the working electrode. A platinum wire was used as the counter electrode and Ag/AgCl (3 M KCl saturated with AgCl as an internal solution, BASI) was used as the reference electrode.

Electrochemical characterization and preparation of enzyme electrodes.—The synthesis of SNS-Fc and subsequent (co)-polymerization with EDOT were previously described.[43,49] Shortly, SNS-Fc (2 mg) was dissolved in 5 ml of ACN and, for copolymerization, 5 μl of EDOT was also introduced into the electrolysis cell containing $LiClO_4$. The film was prepared dynamically scanning the potential between 0.0 V and 1.0 V at a scan rate of 100 mV s^{-1}.

After polymerization, MWCNTs (1.0 mg) were dissolved in 1.0 ml 98% ethanol and drop-coated onto the polymer covered electrode followed by drying at ambient temperature. For the enzyme immobilization, 2 mg Urease (~30.0U) and 1.0% glutaraldehyde (12.5 μl) in phosphate buffer solution (PBS 0.1 M, pH 7.0) were spread over the surface of MWCNT-modified polymer coated electrode and allowed to dry at ambient conditions for 1 h.

Principle of measurements.—All experiments were carried out at ambient conditions in a standard electrochemical cell containing 10 ml PBS with controlled stirring. After each run, the electrode was washed with distilled water and the buffer was refreshed. After signal equilibration in buffer was acquired (at applied potential of −0.2 V (vs. Ag/AgCl)), the substrate was progressively added to the medium and the current response was recorded.

Results and Discussion

The (co)polymerization and electrochemical behaviour of the two hybrid polymeric platforms was previously disclosed[43] showing a charge-transfer controlled reaction in correlation with the active centers

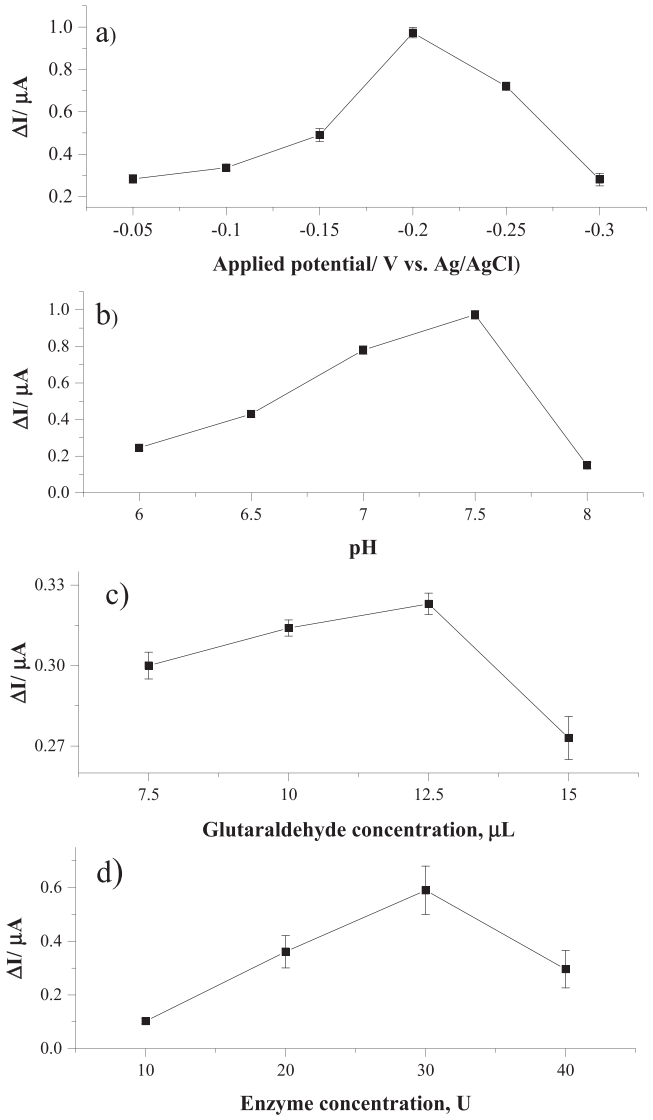

Figure 1. Optimum biosensor design parameters for P(SNS-Fc)/CNT/Urease (red) and P (SNS-Fc–co-EDOT)/CNT/Urease (black); (a) applied potential; (b) pH; (c) cross-linker concentration; (d) enzyme concentration; room temperature, additions of 0.25 mM urea.

on the electrode surface. The comparison between the homopolymer poly (SNS-Fc) and the copolymer poly (SNS-Fc-co-EDOT) proved increased electrochemical activity for the latter. This will be herein tested in performance towards urea detection.

Optimization of biosensor design parameters.—To obtain the best biosensor performance, main design parameters were optimized via variation of each specific one while maintaining the others fixed. Since this is the first time an amperometric urea biosensor based on these conducting platforms is reported, optimization of working conditions was firstly required. The optimum operational pH is particularly significant in maintaining the enzyme activity, whose catalysis is responsible for analyte determination. The pH found optimum for amperometric measurement was 7.5 (Fig. 1b), close to the pH of human blood,[26] making it adequate for clinical analysis. The change in conducting matrix did not affect the optimum pH, which is in proximity to the optimum pH of free urease (7.0–7.5),[53] showing minimal disruption of the enzyme activity upon immobilization. The applied potential was optimized at −0.2 V vs Ag/AgCl (Fig. 1a), similar to several reports based on CP matrices employed for urea determination.[14,26]

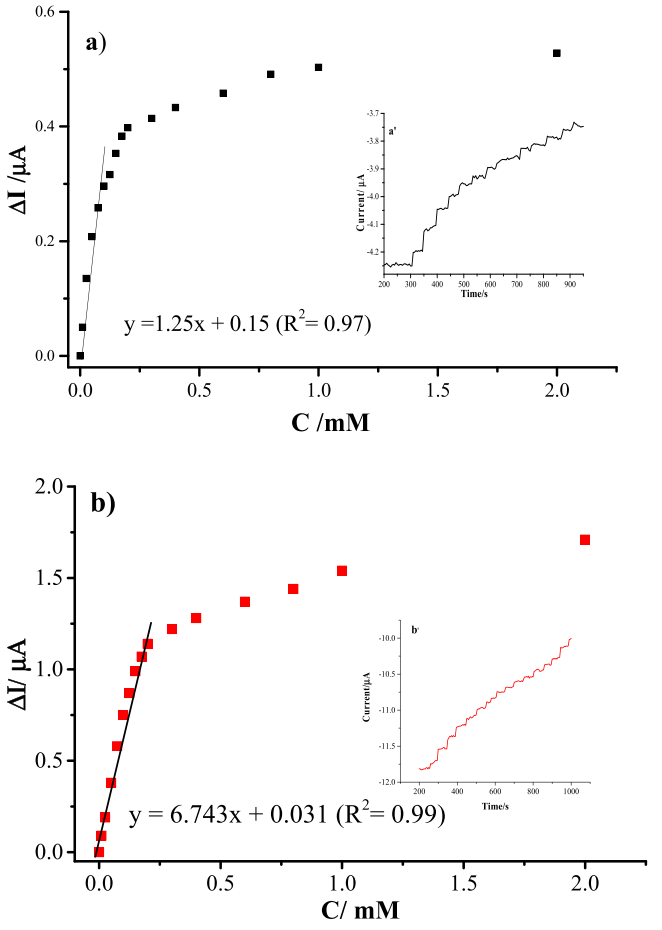

Figure 2. Biosensing performance of P(SNS-Fc)/CNT/Urease (black) and P (SNS-Fc-co-EDOT)/CNT/Urease (red): (a), (b) hyperbolic calibration curves (a′), (b′) insets represent samples of amperometric response.

The concentration of immobilized enzyme and cross-linker were further investigated as they represent two of the most crucial parameters. An increasing amount of Urease from 10 to 40U was immobilized on the conducting platforms and the best amperometric signal was observed at 30U (Fig. 1d). Likewise, an increasing volume of glutaraldehyde (1%) was used for cross-linking and optimized at 12.5 μl (Fig. 1c).

Analytical characterization of the urea biosensors.—The performance of the two biosensing platforms P(SNS-Fc)/CNT/Urease and P(SNS-Fc–co-EDOT)/CNT/Urease was further evaluated by comparison under previously optimized conditions. The amperometric response (Figs. 2a, 2b) of the copolymeric biosensing platform was over five times higher than that of the homopolymer-based one within a similar linear range up to 0.15 mM for the former and up to 0.20 mM for the latter. The calibration curves derived from the chronoamperometric measurements (Insets 2a', 2b') proved a sensitivity of 13.5 μA mM^{-2} cm^{-2} for P(SNS-Fc–co-EDOT)/CNT/Urease in comparison with 2.5 μA mM^{-2} cm^{-2} for P(SNS-Fc)/CNT/Urease. A similar difference was observed in comparison of LOD values from 52.8 μM for the homopolymer biosensor to a minimal value of 1.9 μM for the copolymer one.

The analytical data reported herein is comparable (or superior) to reported analogues (Table I). It is reasonable that the analytical characteristics of the P(SNS-Fc) platform are in accordance to reported work involving redox moieties or linked to conductive matrices such as Poly(vinylferrocene), Poly (N-glycidylpyrrole-co-pyrrole) or Poly (toluidine blue). Yet, the copolymer platform proved up to ten times higher sensitivity and very good value for

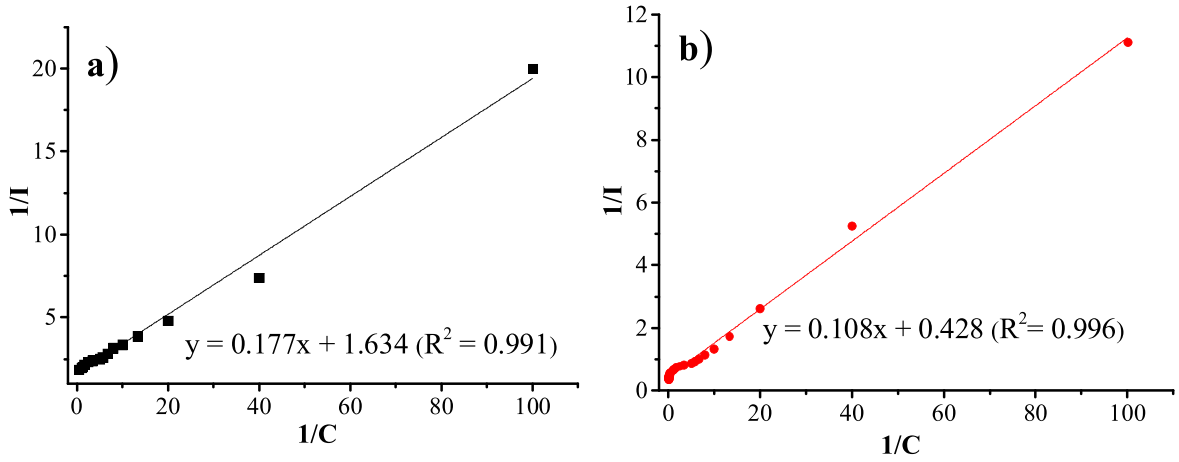

Figure 3. Lineweaver-Burk plots of the developed biosensors: (a) P(SNS-Fc)/CNT/Urs (black) and (b) P(SNS-Fc-co-EDOT)/CNT/Urs (red).

Table I. Analytical characteristics of reported analogues.

Electrode coating	Linearity (mM)	Sensitivity (μA mM^{-2} cm^{-2})	K_m (mM)	LOD (μM)	References
Poly(N-glycidylpyrrole-co-pyrrole)	0.1−0.7	4.5	2.21	20	27
Poly (VinylFerrocene)	0.001−0.25	—	25.4	1	35
Nylon net	0.01−0.3	—	—	10	10
Poly toluidine blue	up to 0.8	0.98		20	34
Polyaniline-Nafion	0.001−1.0	—	—	0.5	55
n-eicosane-graphite	0.01−0.25	1.95		3	32
Fc-PAMAM/MWCNT	0.2–1.8	1.085	—	50	56
P(SNS-Fc)/MWCNT	0.01–0.20	2.5	0.1	52.8	This work
P(SNS-Fc-co-EDOT)/MWCNT	0.01–0.15	13.5	0.252	1.9	

LOD. Such a difference in analytical figures is most assuredly given by the superior electrochemical features of the conducting matrix. The copolymer platform possesses increased conjugation length and decreased band gap, thus superior electrochemical properties. It may be proof that such properties of the copolymer matrix are optimum for oxidation of the products derived from the enzymatic hydrolysis of urea. Certainly, the effect of MWCNTs incorporated within the matrix must be taken into account as well. Considering the low concentration range with minimal LOD, the P(SNS-Fc-co-EDOT) biosensor appears promising for analysis of trace amounts of urea as required in environmental monitoring.[54] Further investigations were solely focused on this biosensing platform as it has most potential for practical determination of urea.

Lineweaver-Burk plots derived from calibration curves (Fig. 3) reveal much lower values of the apparent Michaelis-Menten constant (K_m) for both biosensors (0.1 and 0.252 mM) than those reported for poly (vinylferrocene) or poly (N-glycidylpyrrole-co-pyrrole) biosensing platforms (Table I) albeit the similarity in the type of biosensor platform. It is presumably the result of incorporation of MWCNTs, that facilitate efficient contact between biorecognition element and transducer, thus the affinity of the enzyme towards analyte appears enhanced.

Stability of the biosensor.—The loss in detection ability during consecutive measurements or over time is one of the most serious limitations in the practical application of biosensors.[57] The reusability of P(SNS-Fc–co-EDOT)/CNT/Urease biosensor was studied by performing over 20 consecutive measurements with a standard deviation of 0.095 and coefficient of variation (CV) of 2.67% (n = 8). Around 90% of initial activity was maintained during first

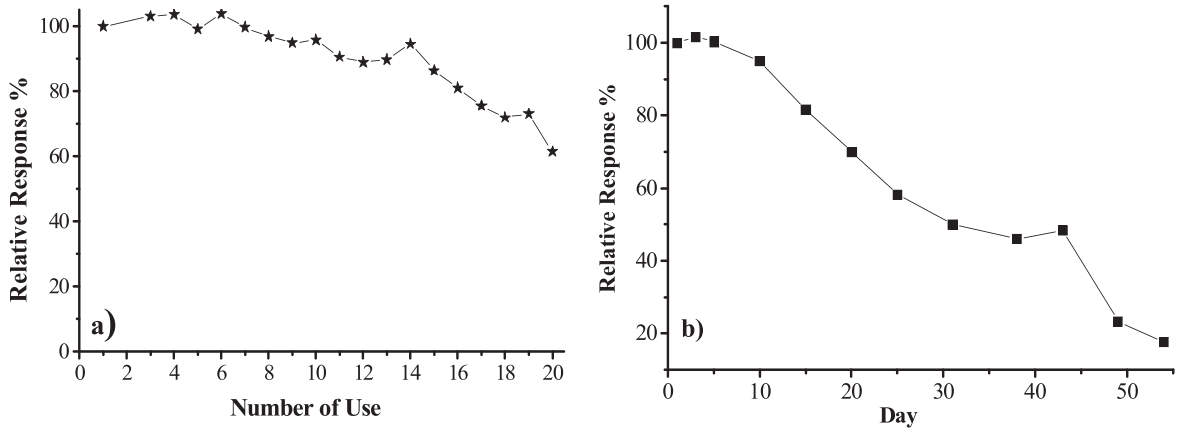

Figure 4. (a) Operational stability and (b) Shelf-life of the P(SNS-Fc-co-EDOT)/CNT/Urease biosensor.

Table II. Recovery rates for urea detection in real samples.

Human urine sample	Spectroscopy (UV)[a)]	P(SNS-Fc–co-EDOT) /CNT/Urs	P(SNS-Fc)/CNT/Urs
Urea content	8.997 ± 0.09	9.362 ± 0.38	11.334 ± 0.32
Recovery (%)		104.0	125.98

a) Medical lab analysis.

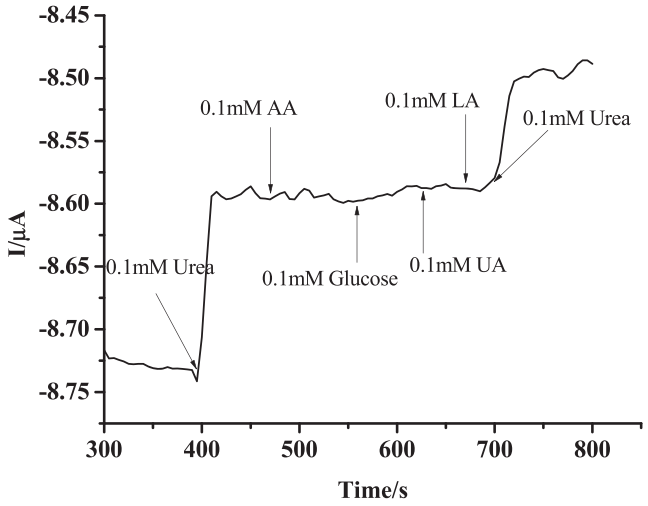

Figure 5. Interference effect on the P(SNS-Fc–co-EDOT)/CNT/Urs biosensor (−0.2 V, pH = 7.5, 0.1 M PBS, 25 °C); AA-ascorbic acid; UA-uric acid.LA-lactic acid.

15 measurements with eventual decrease down to 61%. For shelf-life investigations, the biosensor was tested every week for over 50 d maintaining 95% of its initial performance the first ten days (CV = 2.95%) followed by loss in activity reaching 50% by the 30th day (Fig. 4). Similar instances of decreased stability after 10–15 d are reported in literature for CP/MWCNTs urea biosensing platforms.[50]

Interference effects and real sample study.—The interference effects of some compounds that may be present in the real samples (glucose, uric acid, ascorbic acid, and lactic acid) on the two biosensors were further studied. Neither glucose or lactic acid interfered with the analysis, yet ascorbic and uric acid showed 8.1% and 16.2% interference, respectively for the P(SNS-Fc)/CNT/Urease platform. Much lower interference of 1.4% and 6.4% for ascorbic and uric acid, respectively was recorded for the P(SNS-Fc-co-EDOT)/CNT/Urease biosensor (Fig. 5).

As such, a 25.98% error was recorded for urea determination in human urine by the P(SNS-Fc)/CNT/Urease sensor and only 4% error was found when the urea detection was performed with the P (SNS-Fc-co-EDOT)/CNT/Urease biosensor (Table II). Thus, the copolymer platform appears quite promising for the development of urea biosensors with potential for practical applications in clinical analysis.

Conclusions

This study proposes urea detection by immobilization of Urease on hybrid functional CP (2,5-di(thienyl)pyrroles (SNSs)) and MWCNTs. For enhancement of electrochemical characteristics of the biosensor matrix, the copolymerization of the Fc-functional SNS with EDOT was performed leading to the development of two biosensors: P(SNS-Fc)/CNT/Urs and P(SNS-Fc-co-EDOT)/CNT/Urs.

The sensors were employed in urea determination and analyzed by comparison regarding analytical characteristics, interference effects and accuracy in real sample study. The copolymer platform showed superior performance with high sensitivity within a low concentration range, minimal LOD and reduced interference effect. As such, accurate determination of urea in human urine samples was achieved proving practical applicability of the proposed concept.

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