Appl Biochem Biotechnol
DOI 10.1007/s12010-014-0985-0

Flow Injection Analysis Biosensor for Urea Analysis in Urine Using Enzyme Thermistor

Geetesh K. Mishra · Atul Sharma · Kanchanmala Deshpande · Sunil Bhand

Received: 14 January 2014 / Accepted: 19 May 2014

Abstract There is a need for analytical methods capable of monitoring urea levels in urine for patients under clinical monitoring to appraise renal function. Herein, we present a practical method to quantify levels of urea in human urine samples using flow injection analysis-enzyme thermistor (FIA-ET) biosensor. The biosensor comprises a covalently immobilized enzyme urease (Jack bean) on aminated silica support, which selectively hydrolyzes the urea present in the sample. Under optimized conditions, the developed biosensor showed a linear response in the range of 10–1,000 mM, R^2=0.99, and response time of 90 s in 100 mM phosphate buffer (PB) (flow rate of 0.5 mL/min, sample volume of 0.1 mL, and pH 7.2). The urea-spiked human urine samples showed minimal matrix interference in the range of 10–1,000 mM. Recoveries were obtained (92.26–99.80 %) in the spiked urine samples. The reliability and reproducibility of the developed biosensor were found satisfactory with percent relative standard deviation (% RSD)=0.741. The developed biosensor showed excellent operational stability up to 30 weeks with 20 % loss in original response when used continuously at room temperature. These results indicate that the developed biosensor could be very effective to detect low and high levels of urea in urine samples.

Keywords Urea · Urine · Enzyme thermistor · Immobilized urease · Aminated silica gel

G. K. Mishra · A. Sharma · K. Deshpande · S. Bhand (✉)
Biosensor Laboratory, Department of Chemistry, Birla Institute of Technology and Science, Pilani, KK Birla Goa Campus, Goa 403726, India
e-mail: sunil17_bhand@yahoo.com

G. K. Mishra
e-mail: geeteshmishra26@gmail.com

A. Sharma
e-mail: atulsharma407g@rediffmail.com

K. Deshpande
e-mail: kanchi_43@yahoo.com

Present Address:
K. Deshpande
Department of Chemistry, Goa University, Goa 403206, India

Published online: 07 June 2014

Introduction

Determination of urea has numerous applications in a wide range of fields such as clinical diagnostics, environmental monitoring, and food science. However, the most important application of urea determination lies in clinical analysis to appraise renal functions. Being a waste product of the kidney, urea is an important biomarker that is routinely monitored to determine the functional ability of the renal system [1, 2]. The normal range of urea in urine and blood is between 8 and 20 mg/dL [3]. Elevated urea levels in serum and urine are indicative of renal dysfunction, urinary tract obstruction, dehydration, diabetes, shock, burns, and gastrointestinal bleeding. However, reduced urea level may cause hepatic failure, nephritic syndrome, and cachexia [4].

In recent years, several analytical methods such as reverse phase ultra-fast liquid chromatography [5], colorimetric method based on the reaction of urea and diacetyl-monoxime derivatives [6], infrared spectrometry [7], and high performance liquid chromatography [8] have been utilized for urea determination. Furthermore, biosensor techniques such as potentiometry, conductimetry, amperometry, and coulometry have also been extensively studied for the detection of urea by urease-catalyzed hydrolysis products [9–12].

The growing demand for clinical diagnostics in relation to kidney diseases has stipulated the development of new methods for the rapid and accurate measurement of urea in samples like urine and serum. Thermal biosensors have been considered to be a practical alternative due to their robustness, simplicity, low cost, and extended stability. Another advantage of thermal biosensors is the universal detection principle that has been combined with the specificity of biological reactions. A number of enzymatic reactions for the analysis of urea, glucose, lactate, cholesterol, heavy metals, and fructose have been studied by thermal biosensors among which urea detection was studied extensively in serum and milk [13–18].

Recently, we have demonstrated a flow injection analysis-enzyme thermistor (FIA-ET) biosensor for urea adulteration in milk [19], which was for the first time studied by this instrument comprising urease (Jack bean, EC 3.5.1.5) immobilized on controlled pore glass (CPG). The objective of the present work is to demonstrate the application of the enzyme thermistor for the analysis of urea in urine samples. A low-cost amine-functionalized silica gel microparticle matrix was used as column material for the immobilization of urease. Herein, we present a highly stable, sensitive, and economical FIA-ET biosensor for the analysis of urea in urine samples. The presented biosensor can facilitate the regular and continuous monitoring of abnormal urea concentrations to keep track of the functional ability of the renal system. Another important feature of the presented biosensor is the lower detection limit (10 mM) and an excellent dynamic range of detection—10-1,000 mM urea with a sample throughput of more than 20 within an hour.

Experimental

Reagents and Materials

Enzyme urease (Jack beans lyophilized 5 U mg^{-1}, EC 3.5.1.5), urea, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate monohydrate, glutaraldehyde solution

25 %, and tri-ethanol amine were obtained from Merck, Germany. Functionalized silica gel (amino-3, ca. 1.4 mmol, particle size 40–83 μm) was procured from Across Organics, NJ, USA. All other chemicals used were of GR grade.

Solution Preparation

Phosphate buffer (PB) 100 mM, pH 7.2, was prepared by mixing 100 mM of sodium dihydrogen phosphate monohydrate and 100 mM of disodium hydrogen phosphate monohydrate in double distilled water. PB was degassed before the analysis. A stock solution of urea (1,000 mM) was prepared by dissolving an appropriate amount of urea in 100 mL of PB (100 mM, pH 7.2). The working solution of urea was freshly prepared prior to use.

Enzyme Column Preparation

Enzyme immobilization on amine-functionalized silica gel was achieved by glutaraldehyde cross-linking as reported earlier with slight modifications as presented in Fig. 1a. In brief, urease was covalently immobilized on amine-functionalized silica gel according to the following procedure: 250 mg aminated silica gel was activated with 2.5 % glutaraldehyde in 100 mM PB, pH 7.2. The reaction was allowed to take place for at least 1 h inside the desiccators, under reduced pressure. The activation of the aminated silica gel support was confirmed by the presence of a brick red color (formation of Schiff base). Activated silica gel was successively washed with double distilled water and PB. Urease 200 mg (1,000 IU) dissolved in 1 mL PB was added to the wet activated silica gel. The coupling reaction was allowed to proceed for 30 min at room temperature and overnight at 4 °C. The enzyme preparation was washed with PB. Tri-ethanolamine (200 mM) was added to terminate all the unreacted groups and then washed with 100 mM PB, pH 7.2. The immobilized urease was finally packed into a Delrin column by a slurry packing method.

Instrumentation

The schematic setup for the FIA-ET biosensor for urea analysis is presented in Fig. 1b. The experimental setup consists of a peristaltic pump (Gilson Minipuls Evolution II, France), a six-port injection valve (Rheodyne, Cotati, USA), sample loop (0.1 mL), enzyme thermistor, Wheatstone bridge equipped with a chopper-stabilized amplifier, and Pico logger data recorder (Picotech, UK). PTFE tubings (0.8 mm i.d.) were used for the connection. PB (100 mM, pH 7.2) was used as the carrier buffer.

Procedure

For urea analysis, calibration standards (10–1,000 mM) were prepared by dilution of urea stock solution (1,000 mM) in 100 mM PB, pH 7.2. The immobilized urease column was mounted inside the ET and was allowed to equilibrate with the surrounding. Degassed carrier buffer was passed at an optimum flow rate of 0.5 mL min^{-1} at which a stable baseline was achieved. Samples were injected and data collected using a data acquisition system connected to the Wheatstone bridge. All measurements were carried out by

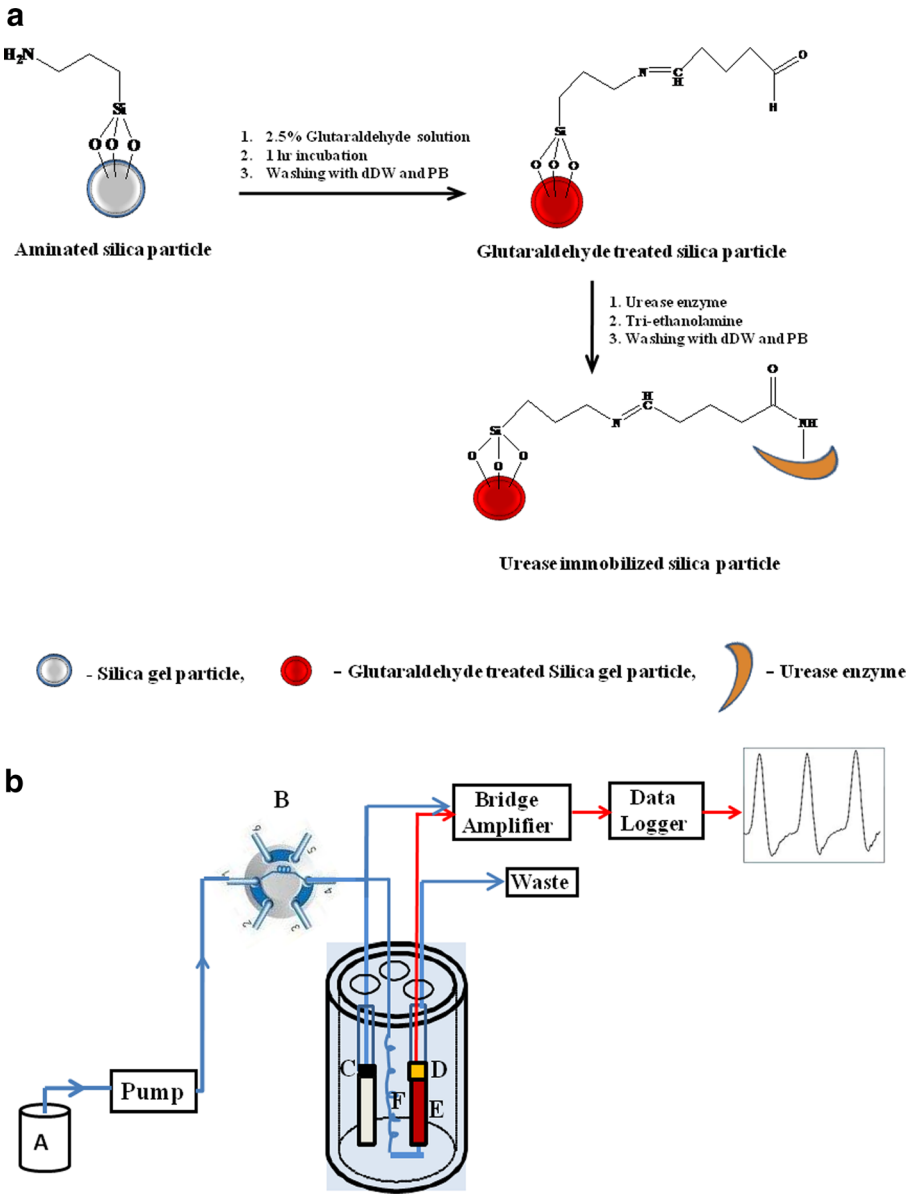

Fig. 1 **a** Schematic representation of immobilization of enzyme urease to aminated silica matrix. **b** Schematic representation of the experimental setup of the FIA-ET biosensor for urea analysis in urine (*A* buffer reservoir, *B* injection valve, *C* reference probe, *D* thermistor, *E* immobilized urease column, *F* heat exchanger)

injection of 0.1 mL sample at a flow rate of 0.5 mL min^{-1}. After each sample analysis, the sample loop was thoroughly rinsed with PB. The urine samples were spiked with a known amount of urea and, after matrix matching with appropriate dilutions, were injected into the FIA-ET system.

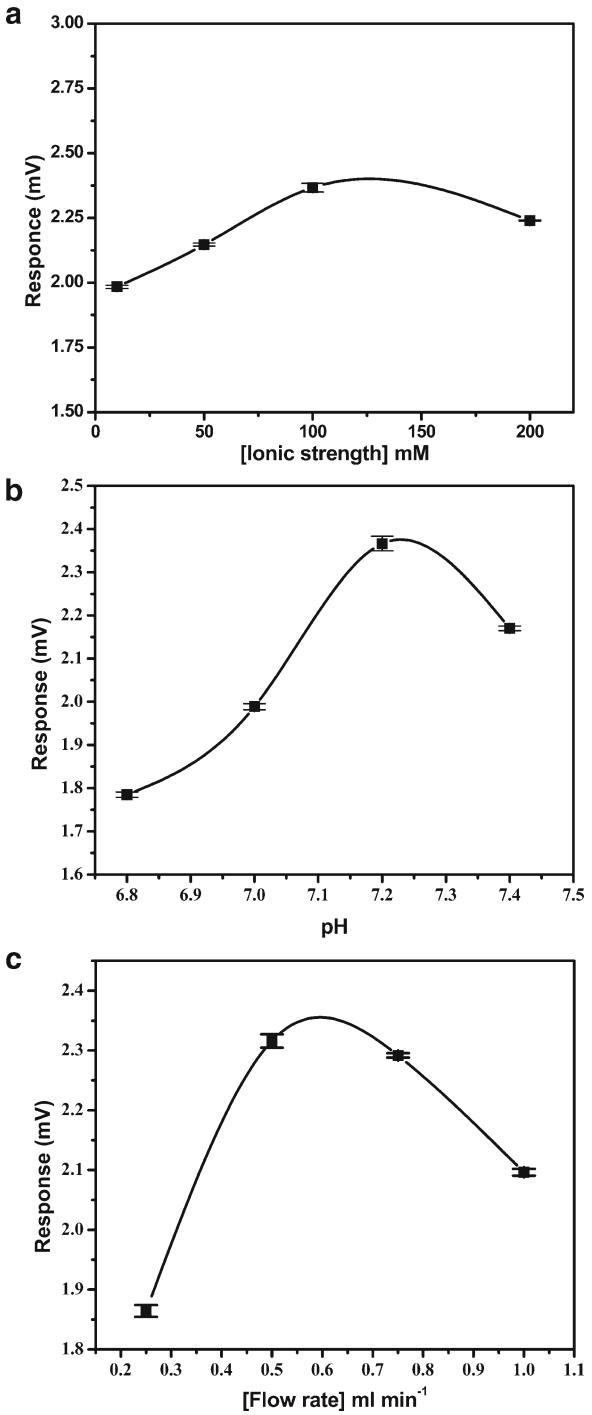

Fig. 2 **a** Effect of ionic strength of PB on the response of the FIA-ET biosensor to 0.1 mL injection of 200 mM urea. **b** Effect of pH of PB on the response of the FIA-ET biosensor to 0.1 mL injection of 200 mM urea. **c** Effect of flow rate of PB on the response of the FIA-ET biosensor to 0.1 mL injection of 200 mM urea

Result and Discussion

Optimization of the FIA-ET Urea Biosensor

A series of experiments were performed to determine the influence of ionic strength and pH of PB, the flow rate and sample volume on the activity of enzyme urease, and the sensitivity of the biosensor. Measurements for optimization of the flow system were carried out with varying ionic strength (10, 50, 100, and 200 mM) and pH (6.8–7.4) for PB. Flow rates of 0.25, 0.5, 0.75, and 1.0 mL min^{-1} and sample volumes of 0.05 and 0.1 mL were investigated against 200 mM urea. As presented in Fig. 2a–c, the results indicate that the performance of the FIA-ET was highly reproducible under optimized conditions of PB (ionic strength of 100 mM, pH 7.2, flow rate of 0.5 mL min^{-1}, and sample volume of 0.1 mL).

Matrix Matching

Urine samples from different individuals were collected and the matrix effect in the analysis of urea in the urine sample was investigated. Various measurements of ionic strength and pH of urine samples were performed for appropriate dilutions of urine with PB. It was observed that 1:4 dilution of the urine sample with PB was the most responsive for urea analysis. To obtain the calibration curve for urea in spiked urine samples, a simple dilution strategy was developed to avoid the effect of ionic strength and pH on the urease column. Urine analyses were carried out within the same day after sample preparation.

Surface Characterization of the Silica Matrix

The surface morphology of the aminated silica matrix was characterized using a JEOL (JSM 6560 LV) Scanning Electron Microscope (SEM) operated at×400 magnification and an accelerating voltage of 8 kV as presented in Fig. 3a(A, B). Figure 3a(A) shows the bare surface of the silica particle before the attachment of the enzyme molecule. Figure 3a(B) shows the enzyme molecule attached to the silica matrix. From both figures and enlargements, it is clearly distinct that the enzyme molecules are coupled to the silica matrix. Furthermore, Fourier transform infrared (FT-IR) spectroscopy was also applied to measure the surface properties of the aminated silica matrix coupled to the enzyme. The surface was characterized before and after immobilizing the enzyme to the silica particles. Spectra were recorded using IR Affinity 1 (SHIMADZU, Japan) with attenuated total reflectance (ATR) attachment Specac Diamond ATR AQUA using 40 scans at 4 cm^{-1} resolution collected under vacuum condition. The ATR FT-IR spectra are shown in Fig. 3b. The insets in Fig. 3b(1a, 2a, 1b, 3a) show the results for amine-functionalized silica surface. In Fig. 3a(1a), the presence of two peaks at 2,963 and 2,858 cm^{-1} represents the *as*CH_2 and *s*CH_2 vibration. In Fig. 3b(1b), four different peaks at 1,562, 1,506, 1,488, and 1,471 cm^{-1} are due to the asymmetric and symmetric bending of primary amine. The presence of one peak at 669 cm^{-1} with two associated peaks at 686 and 648 cm^{-1} confirms the presence of –Si–C– bond in amine-functionalized silica (not shown in inset). In Fig. 3b(2a), the formation of Schiff base after treatment with glutaraldehyde is confirmed by the appearance of two different peaks at 1,702 for –C=N– of Schiff base and 1,651 cm^{-1} –CO of free aldehyde group. In Fig. 3b(3a), the presence of peak at 1,641 cm^{-1} is attributed to –C=O of peptide bond between immobilized urease enzyme and glutaraldehyde-activated silica particle. In Fig. 3b(3a), the presence of peaks at 1,528 and 1,477 cm^{-1} with two associated peaks at 1,459 and 1,443 cm^{-1} is due to asymmetric and symmetric bending vibration of –NH– of secondary amide. The peaks that appear at 1,234 and 1,076 cm^{-1} are

due to –C–N– and –Si–O–Si bond. A broad band from 3,257 cm^{-1} may be attributed to –NH– stretching vibration.

Calibration of Urea Biosensor in PB

The packed column with immobilized urease enzyme was installed inside the thermistor and allowed to equilibrate with the carrier buffer. The filtered and degassed PB was pumped manifold via FIA until a constant baseline was achieved. Mock urea standard solutions in the range of 10–1,000 mM (1 mM urea corresponds to 60.06 ppm) were prepared in PB and injected into the flow stream. Response signals were recorded using a data logger acquisition system connected to the Wheatstone bridge and plotted as current (mV) vs. urea (mM). All samples were measured in triplicates. The Michaelis constant, K_M, for urea was calculated using Lineweaver–Burk plot and found to be 448 mM. A good linear response was obtained

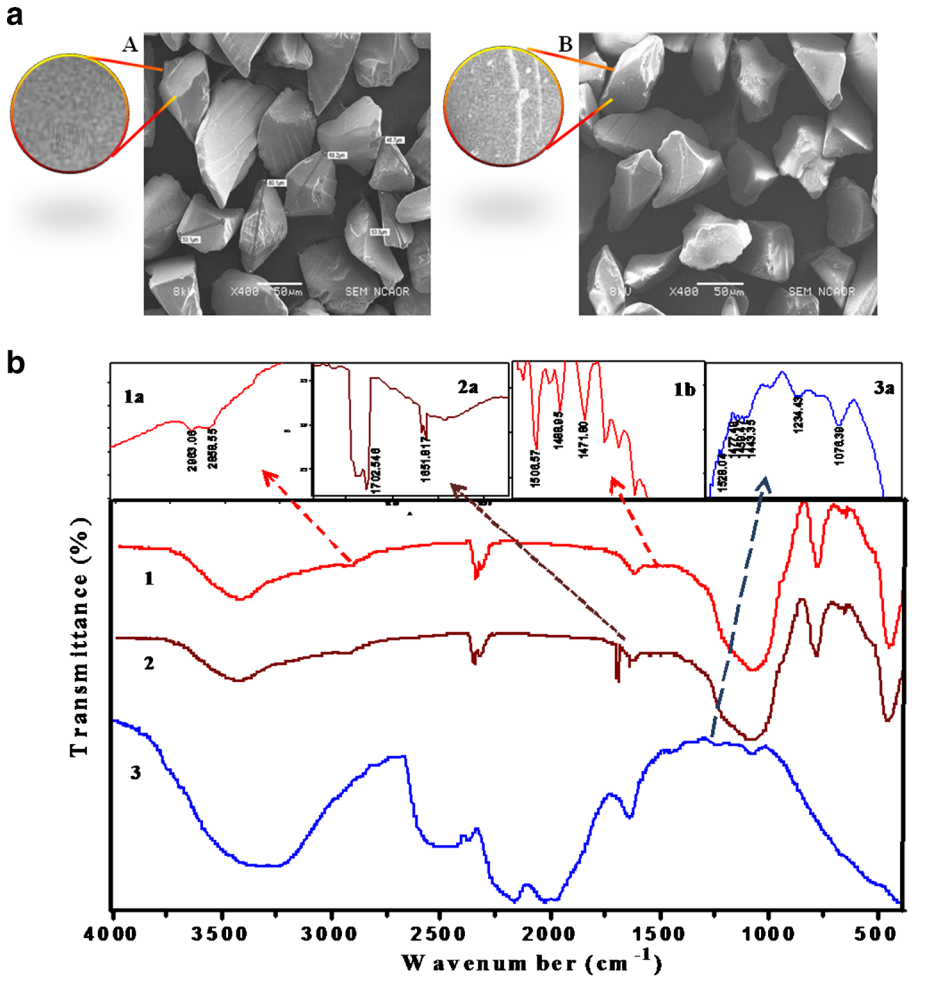

Fig. 3 **a** SEM micrograph of the bare and enzyme-coupled silica matrix at magnification of×400. **b** FT-IR characterization of *1* aminated silica matrix, *2* glutaraldehyde-activated silica matrix, and *3* immobilized urease on silica matrix. *Inset*: *1a*, *1b*, *2a*, and *3a* represent the corresponding FT-IR characteristic peaks

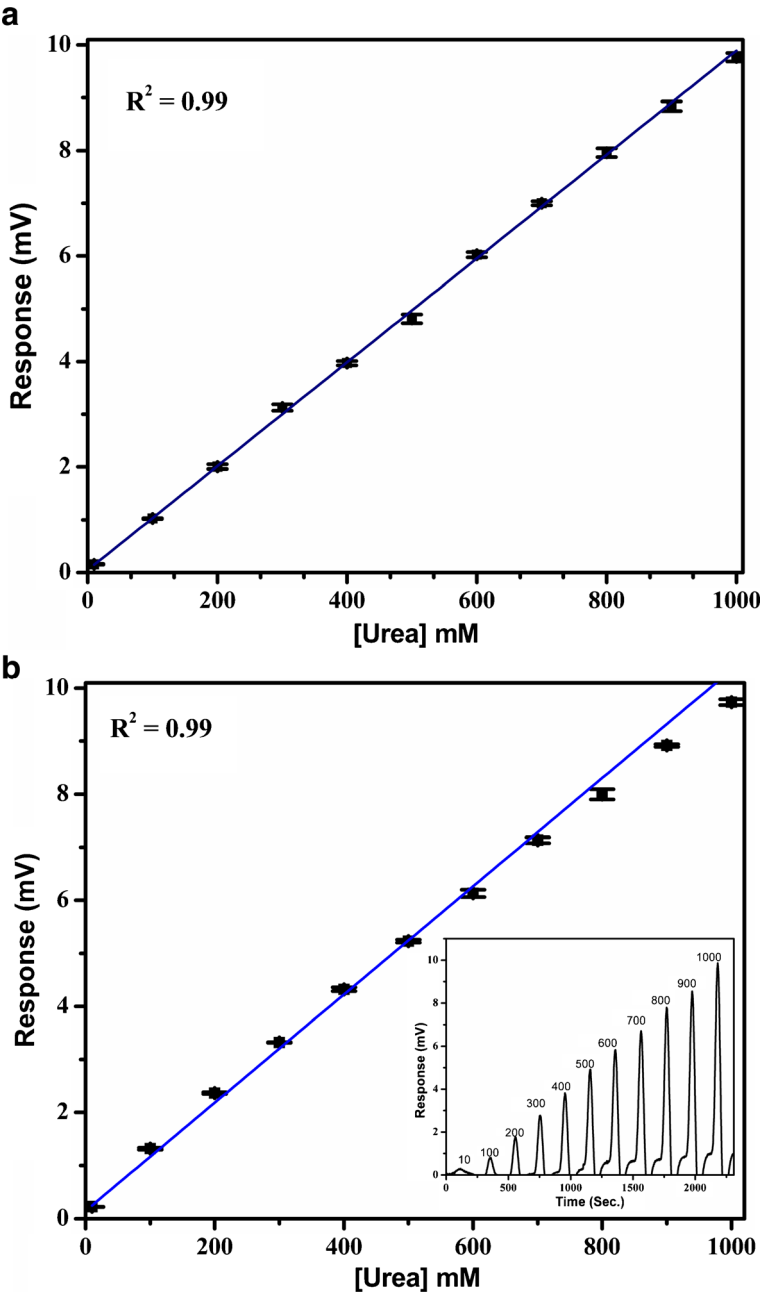

Fig. 4 **a** Calibration plot obtained for urea using the FIA-ET biosensor in 100 mM PB, pH 7.2 for 0.1 mL of sample injection, flow rate of 0.5 mL min^{-1} at 30 °C, R^2=0.99, % RSD=0.02. **b** Calibration plot obtained for urea-spiked urine samples using the FIA-ET biosensor in 100 mM PB, pH 7.2 for 0.1 mL of sample injection, flow rate of 0.5 mL min^{-1} at 30 °C, R^2=0.99, % RSD=0.741. *Inset*: real-time response obtained from the FIA-ET urea biosensor for urea concentrations 10–1,000 mM

Table 1 Comparison of the FIA-ET biosensor with other reported biosensor methods for the analysis of urea

S. no	Matrix	Transducer	Dynamic range	References
1	Urine	Thermal biosensor	10–1,000 mM	Present work
2	Urine, blood, water	Capacitance biosensor	0.1 pM–10 mM	[1]
3	Urine	Potentiometric bioelectronic tongue	1–1,000 mM	[2]
4	Blood	Potentiometric	1–1,000 mM	[20]
5	Blood, serum	Potentiometric	0.05–0.072 mM	[21]
6	Urine, blood	Colorimetric	0.17–0.44 M	[22]
7	Urine, blood	Amperometric	10–250 μM	[23]
8	Urine	Enzyme-based biosensor	0–714.3 mM	[24]
9	Urine, serum	Enzyme-based biosensor	0–100 mM	[25]
10	Urine, serum	Amperometric biosensor	0.1–35 mM	[26]

for urea hydrolysis in the range of 10–1,000 mM, which is presented in Fig. 4a. Linear fit of data shows a good correlation between urea and immobilized urease (R^2=0.99, percent relative standard deviation (% RSD)=0.02).

Calibration of Urea Biosensor in Urine

Urine samples spiked with urea standard solutions (10–1,000 mM) were injected into the flow stream and the response signal was recorded. The FIA-ET biosensor showed an excellent dynamic range for urea present in spiked urine in the range of 10–1,000 mM with good linearity and the minimum detection limit was 10 mM. The results are presented as Fig. 4b (R^2=0.99, % RSD=0.960). Various biosensors reported in the literature for the analysis of urea are listed in Table 1. Most of the reported biosensors used urease as biorecognition element and have good detection range. However, the presented FIA-ET urea biosensor is capable of detecting urea in a urine sample in a broad dynamic range of

Table 2 Determination of recovery from urine samples spiked with known amounts of urea using the FIA-ET urea biosensor

Urine samples	Urea added (mM) (n=3)	Urea found (mM) (n=3)	% Recovery	Mean ± SD	% RSD
Sample 1	0.5328	0.5219	97.97	0.97±0.016	1.64
Sample 2	1.0237	0.9445	92.26	0.94±0.012	1.28
Sample 3	2.0086	1.9767	98.41	1.97±0.071	3.60
Sample 4	3.1277	2.9511	94.36	2.95±0.016	0.54
Sample 5	3.9692	3.9613	99.80	3.96±0.016	0.40
Sample 6	4.8124	4.7315	98.32	4.73±0.022	0.46
Sample 7	6.0250	5.7695	95.76	5.77±0.034	0.59
Sample 8	6.9983	6.7612	96.61	6.76±0.021	0.31
Sample 9	7.9598	7.6810	96.50	7.68±0.106	1.38
Sample 10	8.8393	8.5633	96.88	8.56±0.123	0.14
Sample 11	9.7647	9.3846	96.11	9.38±0.022	0.23

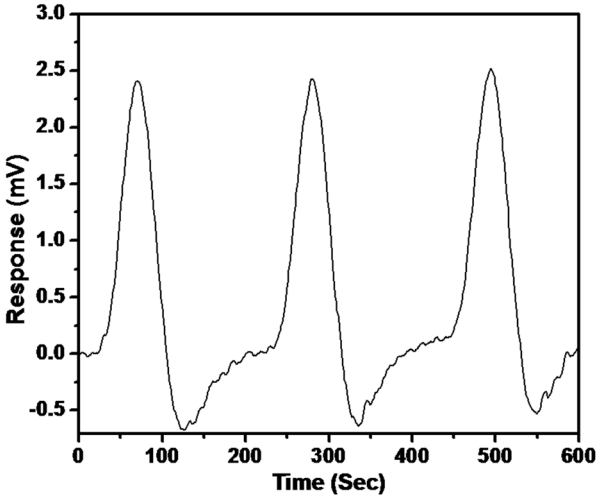

Fig. 5 Real-time triplicate sensor signal recorded from the FIA-ET urea biosensor for 0.1 mL sample of 200 mM urea, flow rate of 0.5 mL min^{-1} at 30 °C

10–1,000 mM that covers the requirement of clinical industries to determine the functional ability of the renal system.

Recovery Studies from Spiked Urine Samples

A known amount of urea standard was spiked to a diluted urine sample and analyzed using the FIA-ET biosensor. Recoveries were calculated from the obtained response signal. Table 2 shows the recovery results obtained from urine urea. Excellent recoveries were obtained in the range of 92.26–99.80 % in the spiked urine samples. This ensures the reproducibility and sustainability of the developed biosensor.

Reliability and Reproducibility of the Urea Biosensor

In order to determine the reliability and reproducibility of the developed urea biosensor, several measurements were performed in triplicate. Figure 5 shows the real-time triplicate

Table 3 Intraday and interday reliability of the FIA-ET urea biosensor

Intraday reliability				Interday reliability			
Urea concentration (mM)	Intraday mean (mV) (n=3)	Mean ± SD	% RSD	Day	Interday mean (mV) (n=3)	Mean ± SD	% RSD
100	1.3145	1.31±0.017	1.29	Day 1	2.3721	2.37±0.021	0.89
200	2.3664	2.36±0.016	0.67	Day 2	2.3605	2.36±0.019	0.80
500	5.2284	5.22±0.026	0.49	Day 3	2.3479	2.34±0.015	0.65
				Day 4	2.3518	2.35±0.002	0.10
				Day 5	2.3305	2.33±0.002	0.08

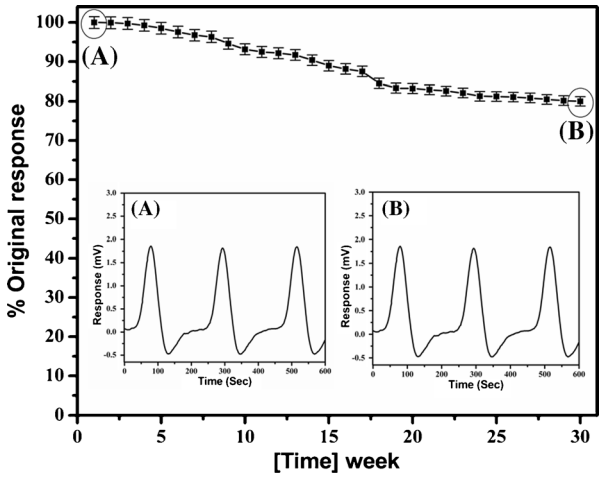

Fig. 6 Operational stability of the sensor signal, response to 0.1 mL, 200 mM urea over the period of 30 weeks at room temperature. *Inset*: real-time triplicate sensor response obtained from the FIA-ET urea biosensor against 200 mM urea of the initial week and the 30th week of operation

sensor signal recorded from the FIA-ET urea biosensor on the urea-spiked urine sample. The obtained signal confirms the reproducibility of the sensor response. Table 3 presents the intraday and interday reliability of the developed biosensor.

Stability of the Sensor Signal

Stability of the sensor signal was investigated by making repetitive measurements over a period of 30 weeks. The sensor showed good operational stability when continuously monitored at room temperature. In an earlier reported work, CPG beads (125–140 μm particle diameter and 50 nm pore size) were used as a column matrix. The column was stable up to 180 days at room temperature with about 70 % original response. In the present work, amine-functionalized silica gel microparticle (particle size 40–83 μm) was used as the column matrix to reduce the cost of the column and cost of per sample analysis. To check the stability of the sensor signal, about 20 measurements were made every week against 200 mM urea. The stability of the sensor signal is presented in Fig. 6. The sensor showed excellent stability over the initial 5 weeks with no appreciable loss of enzyme activity. After 15 weeks of continuous use, enzyme activity was found about 90 % of the original response. However, after a period of 20 weeks of operation, about 14 % decay in response was recorded. Similarly, the response was monitored within 25 and 30 weeks and signals recorded were 85 and 80 % of the original response, respectively. The presented urea biosensor is cost effective and shows good stability as reported earlier for the FIA-ET biosensor.

Conclusion

The FIA-ET biosensor for the analysis of urea in human urine samples is developed and demonstrated. A low-cost urease-immobilized aminated silica gel column matrix has been tested successfully, without compromising operational stability. This significantly reduces the cost of analysis per sample. A broad dynamic range has been obtained for urine urea analysis

(10–1,000 mM). The system has been automated for real-time analysis with a response time of 90 s. Results indicate that the developed biosensor is highly stable, simple, and economical. The method was optimized and had been successfully applied to determine urea levels in urine samples. The biosensor has been found to be stable at room temperature up to 30 weeks with 20 % loss of original response. The presented biosensor can facilitate the regular and continuous monitoring of abnormal urea concentrations to keep track of the functional ability of the renal system.

Acknowledgments This work is funded by the National Agriculture Innovation Project (NAIP) C4/C30032, ICAR, and The World Bank. We offer our sincere thanks to Prof. Bengt Danielsson, Acromed AB, Lund, Sweden for scientific interaction and providing the enzyme thermistor at BITS, Pilani-KK Birla Goa Campus. We also offer our sincere thanks to Dr. Rahul Mohan, NCAOR Goa for recording the SEM micrographs.

References

1. Alizadeh, T., & Akbari, A. (2013). *Biosensors and Bioelectronics, 43*, 321–327.
2. Gutiérrez, M., Alegret, S., & Valle, M. D. (2007). *Biosensors and Bioelectronics, 22*, 2171–2178.
3. Ali, A., AlSalhi, M. S., Atif, M., Ansari, A. A., Israr, M. Q., Sadaf, J. R., et al. (2013). *Journal of Physics: Conference Series, 414*, 120–124.
4. Sumana, G., Das, M., Srivastava, S., & Malhotra, B. D. (2010). *Thin Solid Films, 519*, 1187–1191.
5. Czauderna, M., & Kowalczyk, J. (2012). *Czech Journal Animal Science, 57*(1), 19–27.
6. Goeyens, L., Kindermans, N., Yusuf, M. A., & Elskens, M. (1998). *Estuarine, Coastal and Shelf Science, 47*, 415–418.
7. Jensen, P. S., Bak, J., Ladefoged, S., & Andersson, S. (2004). *Spectrochimica Acta Part A, 60*, 899–905.
8. Clark, S., Francis, P. S., Conlan, X. A., & Barnett, N. W. (2007). *Journal of Chromatography A, 161*(1–2), 207–213.
9. Koncki, R., Radomska, A., & Glab, S. (2000). *Analytica Chimica Acta, 418*, 213–224.
10. Jdanova, S., Poyard, S., Soldatkin, A. P., Renault, N. J., & Martelet, C. (1996). *Analytica Chimica Acta, 321*, 35–40.
11. Pizzariello, A., Stredansky, M., Stredanska, S., & Stainslav, M. (2001). *Talanta, 54*, 763–772.
12. Adams, R. E., & Carr, P. W. (1978). *Analytical Chemistry, 50*, 944.
13. Danielsson, B., Mattiasson, B., & Mosbach, K. (1976). *Pure and Applied Chemistry, 51*, 1443–1457.
14. Bjarnason, B., Johansson, P., & Johansson, G. (1998). *Analytica Chimica Acta, 372*, 341–348.
15. Chen, Y., Andersson, A., Mecklenburg, M., Xie, B., & Zhou, Y. (2011). *Biosensors and Bioelectronics, 29*, 115–118.
16. Raghvan, V., Ramanathan, K., Sundaram, P. V., & Danielsson, B. (1999). *Clinica Chimica Acta, 289*, 145–158.
17. Pirvutoiu, S., Dey, E., Bhand, S., Ciucu, A., Magearu, B., & Danielsson, B. (2002). *Roumanian Biotechnology Letters, 7*, 975–986.
18. Bhand, S. G., Soundararajan, S., Wärnmark, I. S., Milea, J. S., Dey, E. S., Yakovleva, M., et al. (2010). *Analytica Chimica Acta, 668*, 13–18.
19. Mishra, G. K., Mishra, R. K., & Bhand, S. (2010). *Biosensors and Bioelectronics, 26*, 1560–1564.
20. Jha, S. K., Topkar, A., & D'Souza, S. F. (2008). *Journal of Biochemical and Biophysical Methods, 70*, 1145–1150.
21. Eggenstein, C., Borchardt, M., Diekmann, C., Grundig, B., Dumschat, C., Cammann, K., et al. (1999). *Biosensors & Bioelectronics, 14*, 33–41.
22. Verma, N., Minhas, R. K., & Kumar, S. (2012). *Advances in Applied Science Research, 3*(1), 135–141.
23. Pizzariello, A., Stredansky, M., Stredanska, S., & Miertus, S. (2001). *Talanta, 54*, 763–772.
24. Morishita, Y., Tsuji, K., Nakane, K., Soya, Y., Fukatsu, T., Yoneda, K., et al. (1997). *Clinical Chemistry, 43*(10), 1932–1936.
25. Orsonneau, J. L., Massoubre, C., Cabanes, M., & Lustenberger, P. (1992). *Clinical Chemistry, 38*(5), 619–623.
26. Tiwari, A., Aryal, S., Pilla, S., & Gong, S. (2009). *Talanta, 78*, 1401–1407.