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Preparation, characterization and application of urease nanoparticles for construction of an improved potentiometric urea biosensor

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ABSTRACT

The nanoparticles (NPs) aggregates of commercial urease from jack beans (*Canavalia ensiformis*) were prepared by desolvation and glutaraldehyde crosslinking and functionalized by cysteamine dihydrochloride. These enzyme nanoparticles (ENPs) were characterized by transmission electron microscopy (TEM), UV and Fourier transform infrared (FTIR) spectroscopy. The TEM images of urease NPs showed their size in the range, 18–100 nm with an average of 51.2 nm. The ENPs were more active and stable with a longer shelf life than native enzyme molecules. The ENPs were immobilized onto chitosan (CHIT) activated nitrocellulose (NC) membrane via glutaraldehyde coupling with 32.22% retention of initial activity of free urease NPs with a conjugation yield of 1.63 mg/cm^2. This NC membrane was mounted at the lower/sensitive end of the ammonium ion selective electrode (AISE) with O-ring and then electrode was connected to a digital pH meter to construct a potentiometric urea biosensor. The biosensor exhibited optimum response within 10 s at pH 5.5 and 40 °C. The biosensor was employed for measurement of potentiometric determination of urea in sera of apparently healthy and persons suffering from kidney disorders. The biosensor displayed a low detection limit of 1 μM/L with a wide working range of 2–80 μM/L (0.002–0.08 mM) and sensitivity of 23 mV/decade. The analytical recovery of added urea in serum was 106.33%. The within and between-batch coefficient of variations (CVs) of present biosensor were 0.18% and 0.32% respectively. There was a good correlation (r = 0.99) between sera urea values obtained by reference method (Enzymic colorimetric kit method) and the present biosensor. The biosensor had negligible interference from Na^+,K^+,NH^{+4} and Ca^{2+} but Mg^{2+},Cu^{2+} and ascorbic acid but had slight interference, which was overcome by specific ion selective electrode. The ENPs bound NC membrane was used maximally 8–9 times per day over a period of 180 days, when stored in 0.01 M sodium acetate buffer pH 5.5 at 4 °C.

1. Introduction

Urea (NH_2CONH_2) is a nitrogenous organic compound usually found in blood as well as other body fluids. Metabolism of proteins produces ammonia (NH_3), which in turn get converted into urea, in kidney and liver. It is commonly found in nature and its determination is one of the important aspects in medical diagnosis and farming science, environmental monitoring and food science. Urea level in urine and blood is used as a valuable marker to check kidney and liver functioning, increased catabolism of proteins, congestive heart failure, high protein diet, malnutrition, pregnancy and even shock and stress (Wei et al., 2001; Dhawan et al., 2009; Kovács et al., 2003; Bisht et al., 2005). Generally, urea level in adult human blood ranges from 2.5 to 7.5 mM (15–45 mg/dl) (Wei et al., 2001). On the other hand, very high level of urea (50–70 mM) in the blood confirms a kidney dysfunction (Wałcerz et al, 1998). The increased urea level in blood is responsible for renal failure, obstruction in urinary system, dehydration, burns, shock and bleeding in digestive track. In people suffering from chronic kidney disease (CKD) or renal failure, urea level can reach up to 10-fold, as compared to healthy persons. The annual global production of urea is likely to surpass 9.3 billion tons by 2050, the bulk of which is used as fertilizer (Tarasov et al, 2016). Urea could also be responsible for decline in soil pH (Dhawan et al., 2009). Therefore, precise measurement of urea level in blood is very crucial in various biomedical applications particularly in renal function testing, glomerular filtration rate determination and hemodialysis. Urea is chiefly accepted as the best indicator for assessing the levels of uremic toxins in humans.

A large number of conventional methods, such as colourimetry, fluorimetry, gas chromatography, enzymic colourimetry/spectrophotometry and HPLC are already available and used for urea determination at large scale (Bisht et al., 2005). Yet, these methods have one or the other disadvantages, e.g., time consuming sample pre-treatment, costly

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instrumentation, skilled operators, while some methods require long analysis time (Tiwari et al., 2009). Despite the several methods available, potentiometric and amperometric biosensing are the two methods frequently used for measuring urea concentration (Bhalinge et al., 2016). Among these two, potentiometric methods are highly selective and undoubtedly widely adopted techniques. The potentiometric biosensors have been considered as better alternative to conventional methods of urea determination, due to their ease, speed, low cost and high sensitivity. In potentiometric biosensing method, urea is hydrolyzed into ammonium (NH_4^+) and bicarbonate (HCO_3^-) ions by immobilized urease (E.C. 3.5.1.5). The concentration of urea is then measured by monitoring either the generation of ammonium ions or bicarbonate ions by their ion specific electrode (Guilbault and Tarp, 1974; Guilbault and Lubrano, 1972). The first potentiometric urease electrode was invented by Guilbault and Montalvo for determination of urea based on urease catalyzed hydrolysis (Lakard et al., 2004; Guilbault and Montalvo, 1969a, 1969b; Jiang et al., 2004). In potentiometric transducer techniques, enzymatic approaches are more selective and accurate. An enzyme-based biosensor has been developed to identify urea concentration in which enzyme layer lies directly on the surface of a sensor (Chou et al., 2008).

Amongst the different types of latest biosensors, potentiometric urea biosensors based on ion-sensitive field effect transistors (ISFET) are the best (Bergveld, 2003), because of their higher sensitivity, and fast electronic output. In urea biosensors, the urease has been immobilized onto different supports. However, direct immobilization of enzyme onto a support causes its denaturation, prompting to the loss of its activity and stability. This problem was overcome by aggregating the enzyme molecules and followed by glutaraldehyde crosslinking to form their nanoparticles (NPs), before immobilization. Due to their unique electronic, optical, mechanical, electrical, thermal and catalytic (ability to facilitate electron transfer) properties, as well as their increased surface area, the enzyme nanoparticles (ENPs) have shown great promises in improvement of enzyme electrodes (Flores et al., 2013; Kundu et al., 2013; Pundir, 2015; Pundir and Aggarwal, 2017; Narwal and Pundir, 2017). Hence the use of NPs of urease in place of native enzyme molecules could enhance the analytic performance of urea biosensor.

The present report describes the preparation & characterization of nanoparticles of urease and their immobilization onto nitrocellulose (NC) membrane to construct an improved potentiometric urea biosensor using an ammonia ions selective electrode(AISE).

2. Experimental

2.1. Major reagents and materials used

Urease from Jack beans (Canavalia ensiformis) and glutaraldehyde (25%), urea, chitosan(CHIT), ethanol, and methanol from SISCO Research Lab, Mumbai, Nessler Reagent from Hi-Media Lab. Pvt. Ltd. Mumbai, NC membrane from Whatman, GmbH-Dassel, Germany through Genetrix, New Delhi and cysteamine dihydrochloride from Fluka, Mumbai were used. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) was used in all experiments. Reference filling solution (30 ml supplied with electrode) /0.1 M NaCl, standard Ionic Strength Adjuster (ISA) solution for preparing standards and samples were used.

2.2. Major instruments used

Ammonium ion (NH_4^+) selective electrode (AISE) from Van London-phoenix Co., USA through Spectrum Hisar as transducer and digital Ion/pH meter (model no. 111from EI, India) were used for the construction of potentiometric urea biosensor. UV–vis–NIR spectrophotometer (Lambda 750, Perkin Elmer, USA) for UV–visible spectra, FTIR spectrophotometer (Perkin Elmer, USA) for Fourier transform infrared (FTIR) spectra and Spectronic20 (Thermo, USA) for measuring absorbance. The transmission electron microscope (TEM) (H-7500, Hitachi, Japan) for taking TEM image of urease NPs and scanning electron microscope (SEM, JSM-6100 Jeol, Japan) for SEM images of electrode were used at Advanced Instrumentation Research Facility (AIRF), Punjab University Chandigarh.

2.3. Assay of urease

Assay of urease was based on the measurement of ammonia produced from hydrolysis of urea by urease and carried out in a 15 ml test tube containing a reaction mixture of 0.9 ml of 0.05 M Tri-acetate buffer (pH 7.3), 0.1 ml of 0.1 M urea. The reaction was started by adding 0.1 ml of dissolved urease (1 mg/ml of 0.1 M sodium phosphate buffer, pH 6.0). The control received 0.1 ml heat denatured enzyme in place of native enzyme. After incubating the reaction mixture at 37 °C in a waterbath shaker for 10 min, the reaction was stopped by adding 1.0 ml 10% trichloroacetic acid (TCA) and followed by adding 1.0 ml of Nessler's reagent with swirlings. The absorbance of yellow color produced in the reaction, was measured at 405 nm against blank. In case of blank, the urease solution was replaced by 0.1 M sodium phosphate buffer (PB), pH6.0 (Panpae et al., 2006). The amount of NH_4^+ generated during the assay, was intraploted from standard curve between NH_4Cl concentrations vs. A_{405}. One Unit of enzyme is defined as the amount of enzyme required to liberate 1 µmol ammonia from hydrolysis of urea in 1 min under the standard assay/ test conditions. The native/free urease had an activity of 6.8 µmoles of NH_4^+/min/ml.

$$H_2N - CO - NH_2 + 2H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + CO_3^{2-}$$

2.4. Preparation of urease nanoparticles (urease NPs)

Urease NPs were prepared by desolvation method using ethanol as desolvating agent. Firstly 6 ml of absolute ethanol was added to 3 ml of enzyme solution (1 mg/ml) at a dropping rate of 0.1–0.2 ml/min. under constant stirring at 500 rpm. This resulted into the aggregation of enzyme molecules into enzyme nanoparticles (ENPs). Now, 1.8 ml of 2.5% glutaraldehyde was added to urease NPs suspension under continuous stirring at 500 rpm at 4 °C for 24 h, ensuring the cross-linking of urease NPs and thus forming ENPs. Amino groups (-NH_2) were introduced on urease-NPs aggregates by the addition of 0.12 g of solidcysteamine dihydrochloride under constant stirring for 5–6 h. Finally, urease NPs were separated from enzyme solution by centrifugation at 1200 rpm at 4 °C for 10 min, followed by their dispersion into 2.0 ml 0.1 M sodium phosphate buffer (PB) (pH 6.0) and then sonication for 5 min (Kundu et al., 2013). The suspension of urease NPs was stored at 4 °C, when not in use.

2.5. Characterization of urease NPs

Urease NPs were characterized by taking their images in transmission electron microscope, recording their UV–visible spectra in a UV spectrophotometer and FTIR spectra in FTIR spectrometer.

2.6. Immobilization of urease NPs onto chemically modified NC membrane

The urease NP aggregates were immobilized covalently onto NC membrane. Firstly NC membrane (1×1.5 cm^2) was washed with DW to remove any debris and then air-dried for 30 min. The membrane was treated with 0.2% chitosan (in 2% acetic acid) for 24 h at room temperature to introduce –NH_2 groups on its surface. Unbound -NH_2 groups were removed by washing with 10% methanol followed by air drying for 30 min. The membrane was activated by immersing it

in 2.5% glutaraldehyde (in 0.1 M PB, pH 7.3) for 2 h at room temperature (Kundu et al., 2013). The membrane was washed thoroughly with 0.1 M PB, pH 7.3. The suspension of urease-NPs aggregates (0.5 ml) was spread over activated NC membrane, uniformly and kept it overnight at 4 °C for immobilization. The urease NPs aggregates bound NC membrane was washed with 0.1 M PB, pH 7.3 and the urease NPs aggregates discard was tested for enzyme activity and protein. Urease NPs aggregates bound NC membrane was also tested for enzyme activity as described above. The resulting urease NPs aggregates bound NC membrane was used as working membrane and stored in 0.1 M sodium acetate buffer, pH 5.5 at 4 °C, when not in use. The SEM images of bare NC membrane and urease NPs aggregates bound NC membrane were taken in a scanning electron microscope.

2.7. Construction of urease NPs/NC membrane/AIS electrode

An ion-selective electrode (ISE) also known as ammonium ion specific electrode, acts as a transducer/sensor that converts the activity of a specific ions dissolved in a urea solution into an electrical potential, which can be measured by a digital ion/voltmeter. The lower sensing part of the electrode is usually made of NH_4^+ specific membrane along with a reference electrode. Firstly, the small black cap from the bottom of NH_4^+ selective electrode (ISE) was removed and then rubber insert covering the filling hole of reference chamber of electrode was also removed. Now, the electrode was filled with the reference filling solution (0.1 M NaCl) provided with the ISE. The ISE was shaken gently downward to remove the air bubbles, which might be trapped over the NC membrane. The ISE was immersed into deionized water for 30 min before its first use.

2.8. Construction and response measurement of potentiometric urea biosensor

Two standards of ammonium chloride (NH_4Cl) of 1 ppm and 1000 ppm were prepared by diluting 1000 ppm standard solution, whose concentration was near the expected sample concentration. The urease-NPs bound NC membrane was mounted over the lower/sensitive end of the ISE with the help of O-ring and the electrode was connected to a digital ion/pH meter. Then it was immersed into a mixture of 100 ml l ppm standard NH_4Cl and 10 ml of ionic strength adjuster (ISA) (0.25 M magnesium acetate + 0.5 M acetic acid in continuously stirred 500 ml DW) in a 200 ml beaker and the solution was stirred thoroughly. After 1 min, the value in the memory was fixed according to the meter manufacturer's calibration instructions, which was 117 mV. Then the AISE/electrode tip was rinsed with DW and wiped with the tissue paper. The electrode was placed into a beaker containing 1000 ppm standard NH_4Cl solution and10 ml of ISA in a 200 ml beaker and the solution was stirred thoroughly. When a stable reading was displayed, the value in mV value was recorded, which was 138 mV. Now the electrode was washed with DW, dried with tissue paper and immersed into 100 ml sodium phosphate buffer (0.1 M, pH 7.0) containing 0.1 ml of 0.1 M urea. After 1 min, the reading in mV was recorded directly from the meter display, which was 115 mV. The electrode was re-calibrated every 1–2 h by simply repeating the above steps.

2.9. Optimization of improved potentiometric urea biosensor

To optimize the present urea biosensor, the effects of pH, incubation temperature, time, and substrate (urea) concentration on biosensor response were studied. To determine the optimal pH, the pH of reaction buffer was varied between 5.0 and 8.0 at an interval of 0.5 M sodium citrate buffer in pH range 4–5.5 and 0.05 M sodium phosphate buffer at pH 6–7.5. Similarly to study the optimal temperature, the reaction mixture was incubated at different temperatures range (25 – 55 °C) at an interval of 5°C in a temperature controlled chamber.

2.10. Application of improved urea biosensor

The sera samples of apparently healthy and diseased persons suffering from kidney disorders were collected from hospital of local Pt. BDS Postgraduate Institute of Medical Sciences and their urea content was determined by the present biosensor in the same manner as described for its response measurement under its optimum conditions, except that urea was replaced by serum (0.1 ml), which was added into 10 ml reaction buffer (0.1 M sodium acetate buffer, pH 5.5). Urea concentration was intrapolated from standard curve between urea concentration and biosensor response in volts, prepared under optimum conditions.

2.11. Evaluation of urea biosensor

The evaluation of the urea biosensor was done by calculating its analytical performance in terms of % analytical recovery, limit of detection (LOD), precision and correlation.

2.12. Storage stability of urease enzyme immobilized NC membrane

The storage stability of urease NPs-NC membrane was studied for 3 months by measuring its response weekly. The membrane was stored in 0.1 M sodium acetate buffer, pH 5.5 at 4 °C, when not in use after washing in DW.

3. Results and discussion

3.1. Preparation of urease NPs and their aggregates

Urease NPs aggregates were prepared by desolvation method using ethanol. The urease-NPs aggregates were formed by dropwise addition of ethanol to the native urease solution, while maintaining the temp at 4 °C. Urease NPs were formed due to decrease of hydration layer around the native urease molecules. The urease molecules interacted with each other by forces like Vander Waals, hydrophobic and electrostatic interactions, resulting into formation of urease-NP aggregates. These enzyme aggregates were cross-linked by reaction of $-NH_2$ groups, which were introduced by cysteamine dihydrochloride on the enzyme surface with a bi-functional cross-linking agent such as glutaraldehyde (Fig. 1), rendering the aggregates permanently insoluble, while maintaining their pre-organized structures and hence their activity. There was a significant increase in the activity of urease NPs aggregates as compared to the native urease molecules, because its nanoparticle aggregates can orient themselves in such a way that their active site become more accessible to the substrate, without undergoing any structural change. This activation might be due to conformational changes, induced in the enzyme molecules during aggregation.

3.2. Characterization of urease NPs

The morphology and the size of urease NPs aggregates, as examined by TEM, is given in Fig. 2A. The diameter of urease NPs was in the range, 20–100 nm with an average of 53 nm, while the same for native monomeric urease was 13 nm by TEM (Turbett et al., 1992). This indicated that 4–7 molecules of native urease were aggregated to form one NP of urease in spherical shape. These urease NPs aggregates were further characterized by UV–visible absorption spectroscopy. UV–visible absorption spectra of native urease showed a peak at 280 nm, which was due to absorption by peptide bond and aromatic amino acids. The spectra of urease NPs showed a major shift of peak of peptide bond up to 10 from 3.8 of native urease molecules. Moreover, the peak of aromatic bond of urease shifted from 230 to 280 nm after NPs synthesis (urease NPs). There was an increase in this absorbance peak, in case of urease NPs aggregates, which revealed that the urease molecules were concentrated without any change in the primary

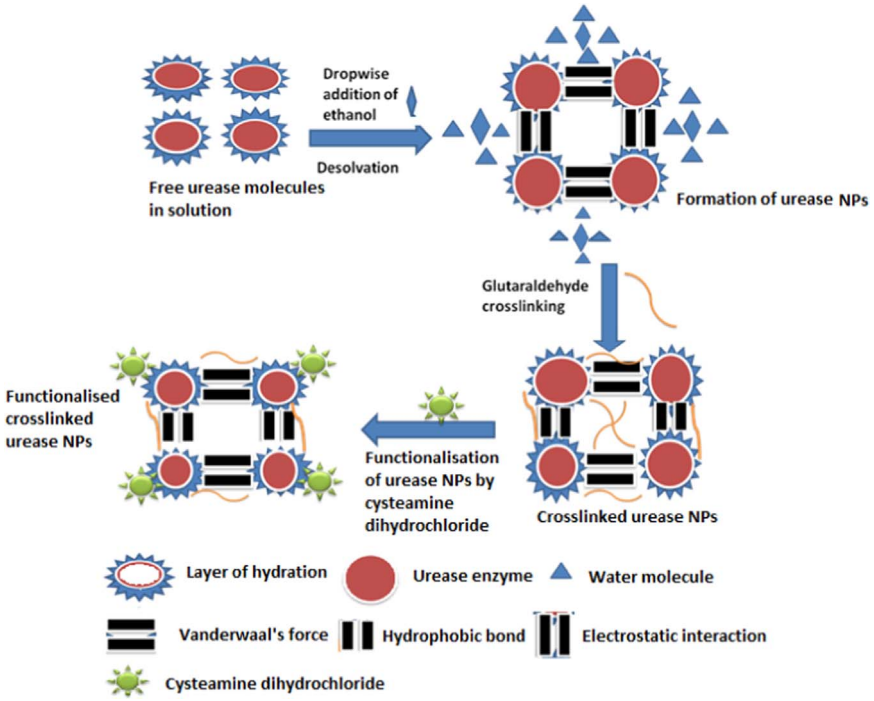

Fig. 1. Schematic representation of Cross-linked urease NPs aggregates formation by desolvation process.

structure, confirming the formation of urease NPs aggregates (Fig. 2B). A comparison of UV spectra of native urease and urease NPs showed a gradual increase in absorbance of the urease NPs in contrast to native urease.

3.3. Immobilization of urease-NP aggregates onto NC membrane

The urease-NPs were immobilized covalently onto CHIT activated NC membrane through glutaraldehyde coupling. Firstly, the NC membrane was activated by introducing free amino groups on the membrane by chitosan. These free-NH_2 groups on NC membrane reacted with the –CHO groups of bifunctional glutaraldehyde. Now the other free –CHO groups of glutaraldehyde reacted with $-NH_2$ groups on the surface of urease NPs aggregates, forming the Schiff base between activated NC membrane and urease NPs aggregates confirming the immobilization of urease NPs aggregates onto the NC membrane (Fig. 3A).

3.4. Characterization of urease NPs bound NC membrane

3.4.1. By SEM image

The SEM images of bare NC membrane showed a hollow beaded morphology, while urease NPs aggregates bound NC membrane showed clusters of beads of urease NPs in the form of beaded structures throughout the surface of the NC membrane (Fig. 3B), which confirms the immobilization of urease-NPs aggregates onto NC membrane. The immobilized enzyme retained 86.72% retention of its initial activity of native enzyme and a conjugation yield of 1.63 mg/cm^2, showing increase in the activity of enzyme after covalent immobilization of urease NPs onto NC membrane through glutaraldehyde coupling between NH_2 groups of cysteamine-dihydrochloride functionalized urease NPs and CHIT decorated NC membrane.

3.4.2. By FTIR spectra

In Fig. 3C, curve 1 from 4000 to 500 cm^{-1} wavenumbers shows FTIR spectra for native urease, while curve 2 shows the spectra for urease NPs aggregates. The FTIR spectra of urease and urease-NPs showed the stretching vibration bands at transmittance (3433.1, 3433.2 cm^{-1}) with –NH and –OH stretching due to cysteamine dihydrochloride, (2067.51, 2076.62 cm^{-1}) with N-H and C=N stretching, (1637.12, 1637.23 cm^{-1}) with C=C stretching due to presence of glutaraldehyde, (1164.55, 1167.65 cm^{-1}) with C-N stretching, (1078.53, 1079.61 cm^{-1}) with C-O stretching, (936.58, 937.67 cm^{-1}) with =C-H bending, (670.34, 685.48 cm^{-1}) with C-H and C=C bending respectively. An extra peak at 989.67 cm^{-1} appeared in urease NPs, which may be due to C-O stretching.

3.5. Construction of potentiometric urea biosensor

A potentiometric urea biosensor was constructed by mounting urease NPs aggregates bound NC membrane over the lower/sensitive end of the ammonium ion selective electrode(AISE) with the help of O-ring and connecting it to a pH meter (Fig. 4). The potentiometric response was based on AISE,which offers advantages such as simple procedure, relatively fast response time, non-destructive analysis, wide linear range with moderate selectivity and being extensively applied for the determination of many ions. A potential is generated, due to NH_4^+ concentration in the reaction buffer as function of the time, when the NC membrane bound urease hydrolyse urea into NH_4^+ and HCO_3^-. The potentiometric measurements were carried out by NH_4^+ selective electrode.

3.6. Response measurement of urea biosensor

The response time of biosensor was studied between 10 s and 120 s at an interval of 10 s. When urea was added into the reaction buffer (0.1 M sodium acetate buffer, pH 5.5), the biosensor responded rapidly to the substrate and achieved 98% of steady current within 20–42 s (Fig. 5A). This response time was comparable to that of earlier reported potentiometric urea biosensors based on PANI/cellulose (24 s), urease/MWCNTs/SiO2/ITO (24 s), MWCNTs/silica composite material (24 s), glutaraldehyde & polyethylene (30 s), glutaraldehyde solution with glass sealed metal microelectrode (30 s), poly film (50 s), PVF matrix (60 s), urease-immobilized polypyrrole (60 s), urease-immobilized polypyrrole (60 s), CNT and polyion complex film (60–90 s), PVA with styryl pyridinium groups and ITO glass plate (60–120 s), PVC-COOH polymer (60–120 s), urease/BSA-PPy/ITO (70–90 s), gelatin beads (120 s), poly(N-vinylcarbazole/stearic acid) LB film on ammonium electrode

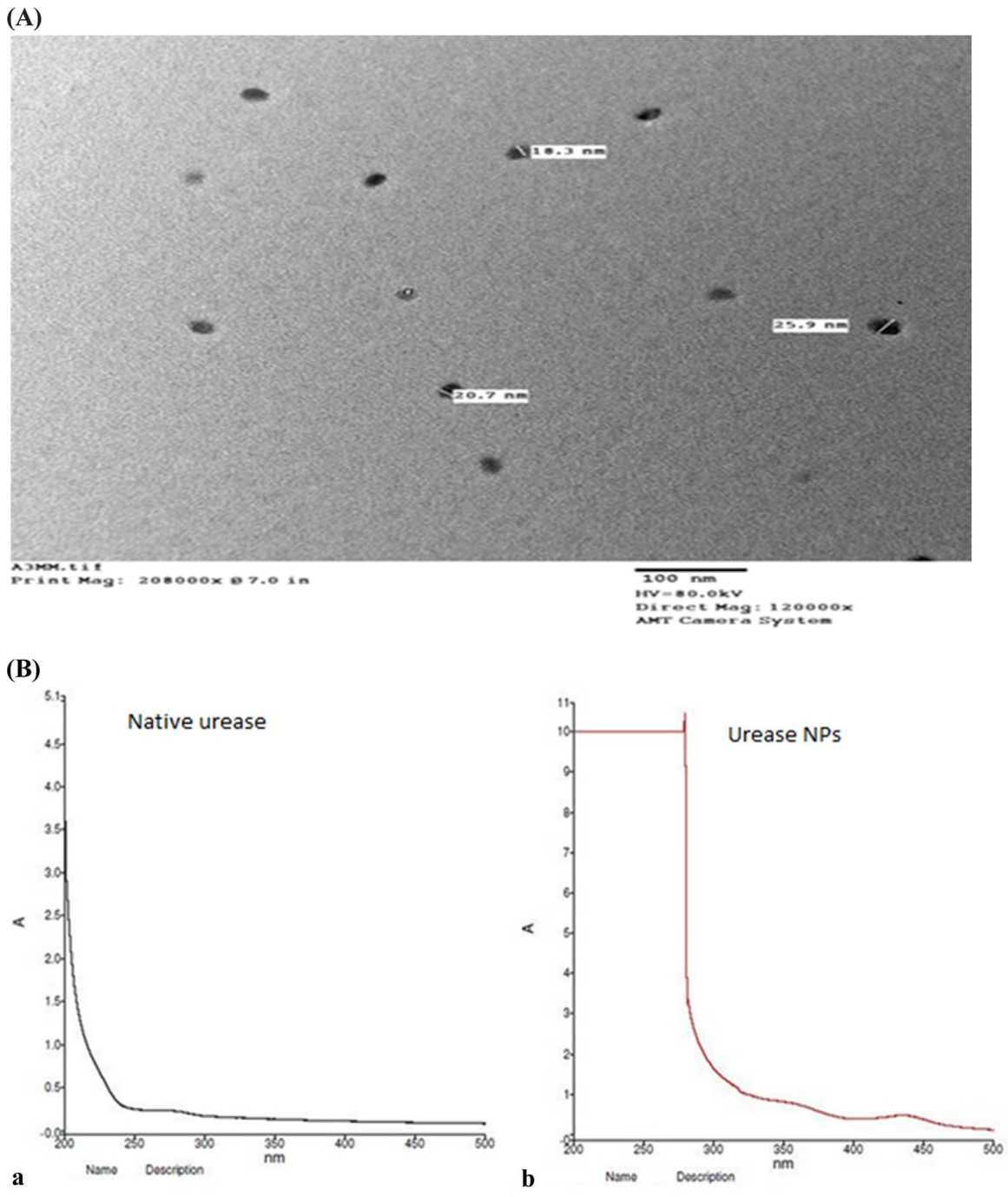

Fig. 2. (A) TEM images of urease NPs. (B) UV spectra of native urease and urease NPs.

(120 s), Whatman paper (120 s), PVA-PAA composite matrix(120 s), polyacetate membrane (180 s), chitosan membrane (4.66 min), chitosan membrane (5 min), ammonium ion sensitive electrode (5 min), liquid photopolymerizable N-pyrrolidone (5–10 min), N-pyrrolidone (5–10 min), thiophene copolymer (6 min), silica gel NPs (9 min), SOI, silicon nanochannel fabrication (10 min), Sol-gel(10–15 min), nylon net (15 min), nylon net ammonium ion-selective electrode(15 min) and biocomposite nonwoven nanofiber (20 min)(Table S1).

3.7. Optimization of urea biosensor

The immobilized urease NP aggregates based biosensor exhibited its optimum response (Fig. 5B) at pH 5.5, which is lower than free urease (pH at 7.0). This decrease in optimum pH could be resulted from the loss of $-NH_2$ groups on the surface of the enzyme. The optimum incubation temperature was in the range, 35–45 °C with maximum at 40 °C (Fig. 5C), which is higher than of native urease molecules (25 °C) enzyme. This increase in optimum temperature of urease could be due to enhanced thermo-stability of ENPs aggregates, which arise due to aggregation of enzyme molecules and their crosslinking. There was a hyperbolic relationship between biosensor response and urea concentration in the range 1–350 μM. The response was steady/constant state after 80 μM. (Fig. 5D). The working range of the present urea biosensor was from 2 to 80 μM (0.002–0.08 mM),which is lower than potentiometric urea biosensors based on urease/BSA/PPy/ITO (0.000066–0.75 mM) (Ahuja

et al., 2008), PVAc-PE (urease) GC/Urease/PPD (10 μM–1 mM) (Chirizzi and Malitesta, 2011), chitosan–poly(vinyl alcohol) hydrogel (0.002 μM–2 mM) (Zeng et al., 2015), AISE (0.013–10 mM) (Chou et al., 2008), PANI/cellulose (0.04 ~ 36 mM) (Ahuja et al., 2008), SMn2/TiO2 sensing memb.(0.008–3 mM) (Chen et al., 2009), polyurethane-acrylate-photocurable-polymeric membrane(0.24–216 mM) (Puig Lleixa et al., 1999), PVA/SbQ photopolymer(0.5–40 mM) (Velychko et al., 2016), urease-immobilized polypyrrole (0.5–10 mM) (Prissanaroon-Ouajai et al., 2015), polyelectrolyte cojugated electrode (0–50 mM) (Chaudhari et al., 2017), chitosan-iron Oxide magnetic NPs (0.1 – 80 mM) (Ali et al., 2013), PAN membrane(1–100 mM) (Ramesh et al., 2015), thiophene copolymer (0.99–4.97 mM) (Lai et al., 2017), PVA/PAA composite matrix (1–1000 mM) (Jha et al., 2008), and Urease/MWCNT/SiO_2/ ITO (0.0218–1070 mM) (Ahuja et al., 2011) but higher than based on gold nanoparticles (0.0001–0.002 μM) (Yang et al., 2005) and nanostructured polyaniline/nafion (0.00001–0.0005 mM) (Do et al., 2011) (Table S1). The linear range of present biosensor is also better than the reference methods i.e. colorimetric method (0.22–4.04 mM) and enzymic colorimetric method (0.25–44 mM) (Urea Kit by Abbot Clinical Chemistry, USA).

The apparent Km for urease NPs bound to NC membrane was calculated from Lineweaver- Burk plot (Fig. 5E) at different urea concentrations between 0.0032–0.0046 μmol and found to be 4.932448 μmol/L. The smaller Km values of membrane bound urease NPs indicates that the diffusion of substrate and product into and out of the membrane was slightly easier in the present NC membrane rather than in the earlier matrices (Table S1).

3.8. Evaluation of urea biosensor

3.8.1. LOD

The detection limit of the present biosensor was 1 μmol/L, which is lower than previously reported potentiometric urea biosensors based on PVAc-PE (urease) (12 μM), AISE (26 μM), PVA-PAA composite matrix (1000 μM), polyacetate membrane (5000 μM), PANI/cellulose (1000 μM), nanobiocomposite of chitosan-iron oxide magnetic NPs(3000 μM) but higher than those based on gold nanoparticles (0.1 μM), nano-structured polyaniline//Nafion membrane (0.05 μM) and CHIT/PVA hydrogel (0.001 μM) (Table S1). The LOD of present biosensor is also lower than the reference methods i.e. colorimetric method (0.22 mM) and enzymic colorimetric method (0.25 mM) (Urea Kit by Abbot Clinical Chemistry, USA).

3.8.2. Sensitivity

The sensitivity of present improved urea biosensor 23 mV/decade, which is lower than potentiometric urea biosensors based on PVAc-PE (urease) GC/Ur/PPD (28 mV) (Chirizzi and Malitesta, 2011), polymer (aniline-co-o-phenylene diamine) polymeric matrix (31.12 mV) (Ma et al., 2016), PPy/$NaHCO_3$ (32 mV) (Komaba et al., 1997), polyacetate-membrane(42 mV) (Medeiros et al., 2011), urease-immobilized onto polypyrrole (43.4 mV) (Prissanaroon-Ouajai et al., 2015), PVA PE (urease) (52 mV) (Eggenstein et al., 1999), nanobiocomposite of CHIT-iron oxide magnetic NPs (57 mV) (Ali et al., 2013), AISE based solid state biosensor (57.59 mV) (Chou et al., 2008) polyurethane-acrylate-photocurable-polymeric-membrane(58 mV) (Puig-Lleixà et al., 1999), C60-urease (59.67 mV) (Saeedfar et al., 2013), carbon

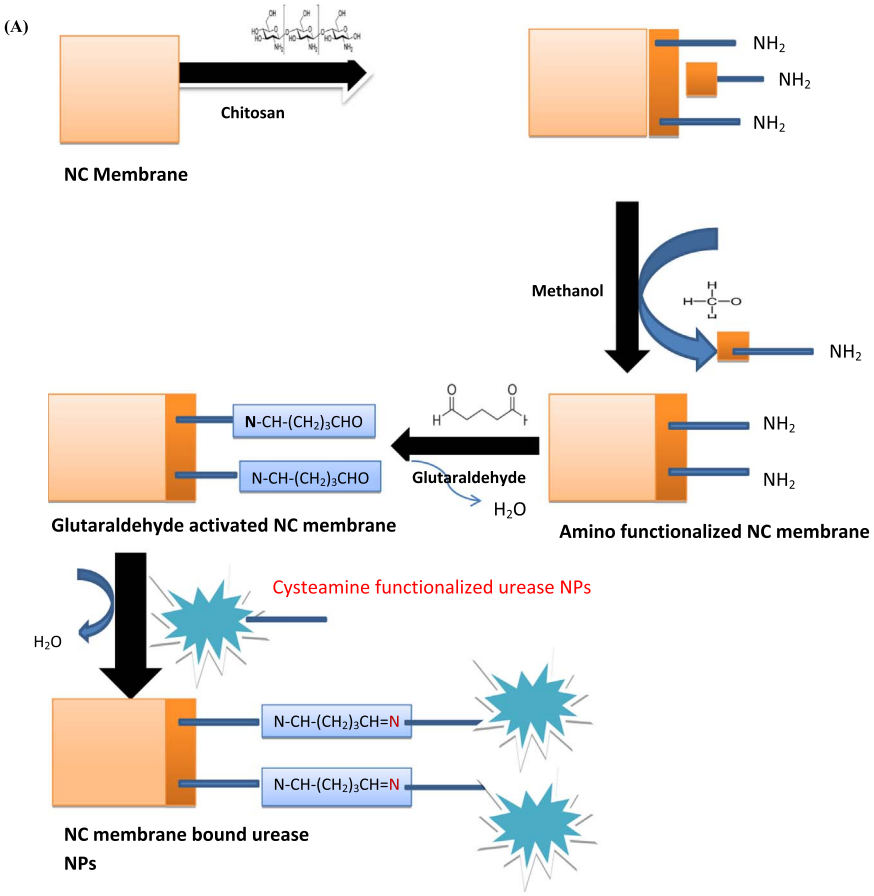

Fig. 3. (A) Schematic representation of steps/chemical reactions involved in the fabrication of urease NPs/NC membrane. (B) SEM of bare NC membrane and NC membrane bound to urease NPs. (C) FTIR spectra of native urease and urease of nanoparticles(a).

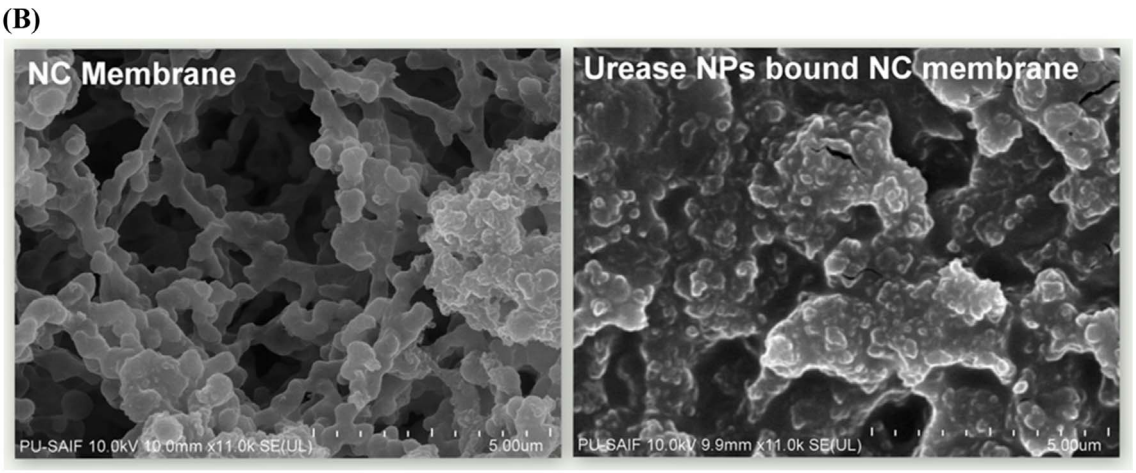

Fig. 3. (*continued*)

nanotubes and polyion complex film (59.1 mV) (Nguyen and Yoon, 2015), modified MWCNTs on SPEs (59.7 mV) (Saeedfar et al., 2017), PPy (63 mV) (Jin and Paek, 2003), thiophene copolymer(100–300 mV) (Lai et al., 2017) & SOI silicon nanochannel based biosensor (600–700 mV) (Chen et al., 2008) and higher than PANI/Cellulose (17.3 mV) (Ahuja et al., 2008) (Table S1).

3.8.3. Analytical recovery

The average analytic recoveries of added urea into blood serum at levels of 6.0 mM, 8.0 mM and 12.0 mM were 107.1%, 111.5% and 101.15%, demonstrating the high reliability of the present biosensor (Table S2).

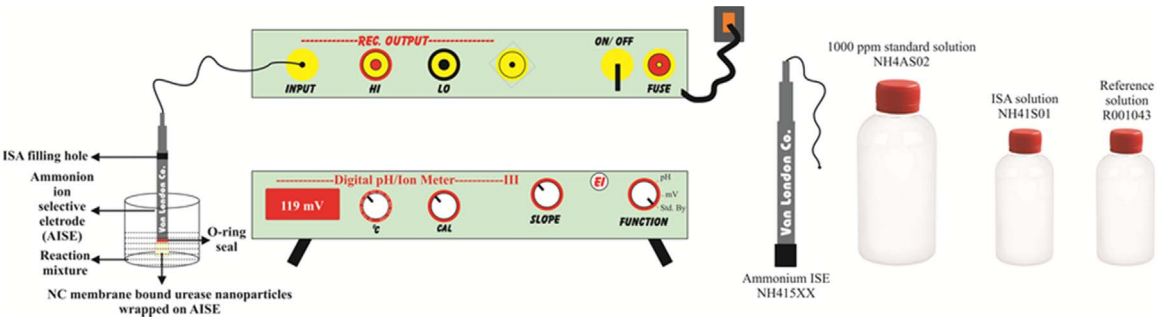

Fig. 4. A potentiometric urea biosensor based on urease NPs aggregates immobilized on NC membrane over the lower sensitive end of AISE.

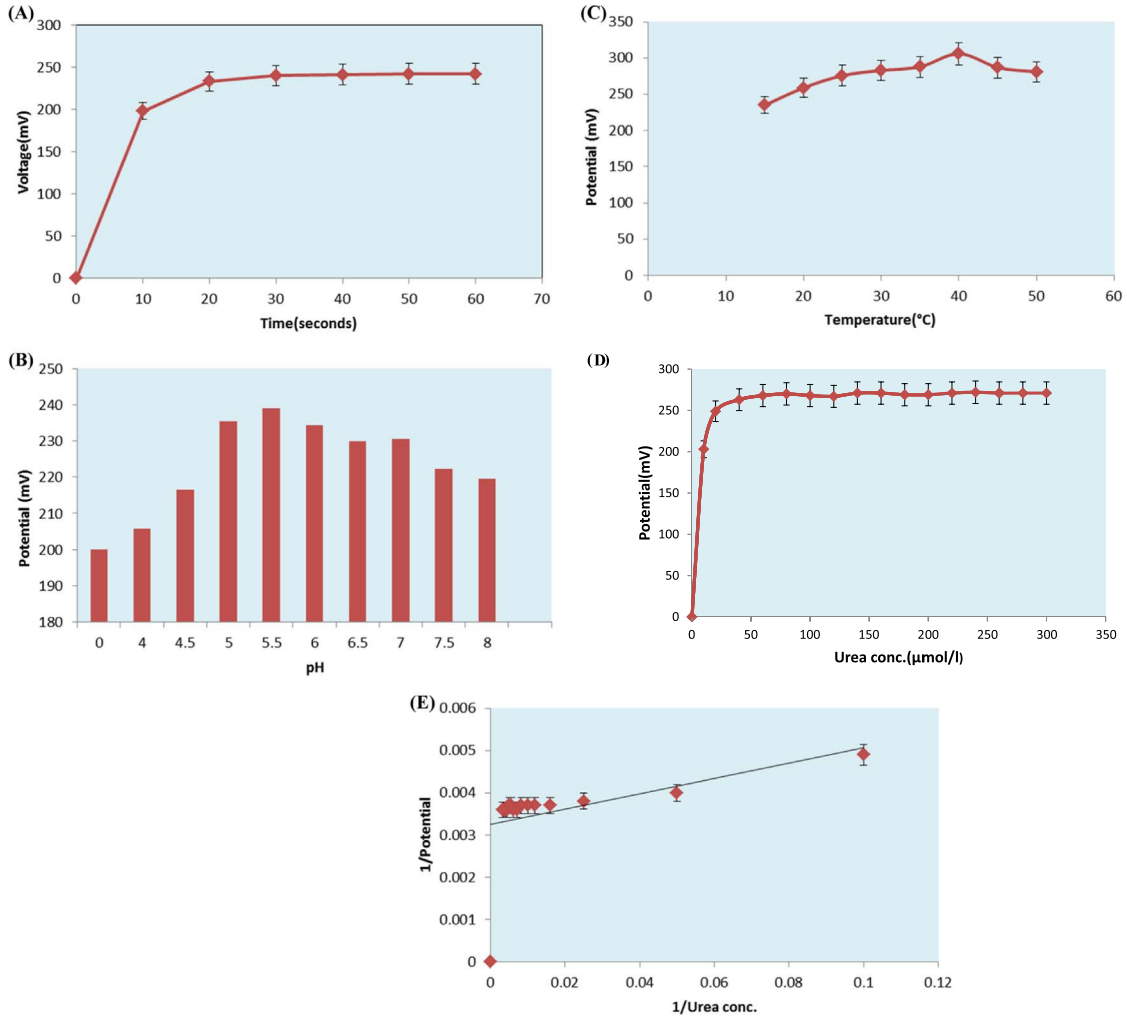

Fig. 5. (A) Influence of incubation time on the potential response of urea biosensor based on urease NPs/NC membrane. (B) Effect of pH on urea biosensor based on urease NPs/NC membrane response. (C) Effect of incubation temperature on urea biosensor based on urease NPs/NC membrane. (D) Effect of urea concentration on urea biosensor based on urease NPs/NC membrane. (E) LB plot for effect of urea concentration on urea biosensor response based on (Urease NPs/NC membrane) response (D).

3.8.4. Precision

Within and between sample coefficient of variations (CVs) for the determination of urea in blood sera on the same day and after 1 week of storage at −20 °C were 0.18% and 0.32%, respectively (Table S3). These results of precisions indicates the good reproducibility and consistency of the present biosensor method.

3.8.5. Correlation

To determine the accuracy of the present biosensor, urea values (mM) in 20 blood sera samples were determined by the present biosensor method and compared with those obtained by the reference method (Enzymic colorimetric kit method) used in hospitals. The values obtained by both the methods were correlated using regression equation. The regression plot (Fig. 6A) between the two methods was drawn and the correlation coefficient (CV) was calculated showing a good correlation (R2 = 0.9991) between the urea values measured by the reference method and present biosensor. Compared to reference methods, the present biosensing method for determination of urea in serum is more simple, sensitive, specific and rapid.

3.8.6. Storage stability of NC/Urease NPs/NH_4^+ electrode

The improved urea biosensor response measurement was 96% reproducible till 8–9 reuses, after that the membrane got saturated, washed in DW and reused after 4–5 h. The ENPs bound NC membrane was used over a period of 180 days, when stored in 0.01 M sodium acetate buffer pH 5.5 at 4 °C (Fig. 6B). The AISE electrode lasted for 6 months, when stored in DW at room temperature. Hence preparation of cross-linked urease NP aggregates increased the shelf life of immobilized urease.

3.9. Interference study

Some common cations and related metabolites were chosen to study their effect on the response of present biosensor at their physiological concentration. Urea at 1.0 mM final concentration in reaction buffer was used in all these interference studies. The biosensor had negligible interference from Na^+,K^+,NH^{+4} and Ca^{2+} but Mg^{2+},Cu^{2+} and ascorbic acid had slight interference, which was overcome by specific ion selective electrode.

3.10. Potentiometric detection of urea in serum

The level of urea in sera was, as measured by present biosensor was in the range 32.10–80 mg/dl in apparently healthy persons and 598 –884 mg/dl in kidney patients (Table S4), which are significantly higher than those in apparently healthy persons. These values are comparable to those reported for earlier urea biosensors.

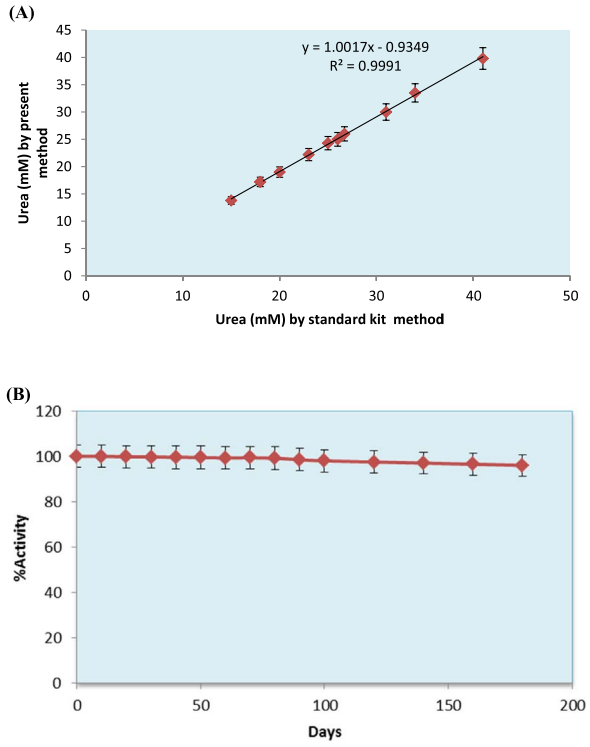

Fig. 6. (A) Correlation between sera urea values measured by standard enzymic kit method(x axis) and the present biosensor based on urease NPs/NC membrane(y axis). (B)Storage stability of urease NPs/NC membrane at 4 °C.

4. Conclusion

The NPs aggregates of urease were prepared, characterized, immobilized covalently onto NC membrane and employed for construction of a potentiometric urea biosensor using ammonium ion selective electrode. The biosensor showed improved analytical performance in terms of sensitivity (23 mV/decade), high selectivity, broader working range (2–80 μM), low LOD (1.0 μM), good reproducibility and high storage stability (6 months). It is concluded that the use of ENPs in construction of enzyme sensors not only provides their improved analytical performance but also simplify their construction.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.09.005.

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