Chronocoulometric determination of urea in human serum using an inkjet printed biosensor

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A biosensor for the determination of urea in human serum was fabricated using a combination of inkjet printed polyaniline nanoparticles and inkjet printed urease enzyme deposited sequentially onto screen-printed carbon paste electrodes. Chronocoulometry was used to measure the decomposition of urea via the doping of ammonium at the polyaniline-modified electrode surface at -0.3 V vs. Ag/AgCl. Ammonium could be measured in the range from 0.1 to 100 mM. Urea could be measured by the sensor in the range of 2 to 12 mM (r^2=0.98). The enzyme biosensor was correlated against a spectrophotometric assay for urea in 15 normal human serum samples which yielded a correlation coefficient of 0.85. Bland-Altman plots showed that in the range of 5.8 to 6.6 mM urea, the developed sensor had an average positive experimental bias of 0.12 mM (<2% RSD) over the reference method.

Keywords: Urea, polyaniline, nanoparticle, inkjet, chronocoulometry

1. Introduction

The detection of urea is of great interest in biomedical and clinical analysis. Indeed, an increase of urea concentration in blood and a reduced level of urine is a strong indication of renal dysfunction. The clinically relevant range of blood urea is 2.5 to 7.9 mM [1]. The determination of urea in body fluids is one of the most frequent analyses in clinical laboratories. The determination of urea is generally performed with enzyme–based biosensors. Enzymatic reactions of non-ionic substrates often produce ionic products. Therefore a variety of biosensors have been developed for the selective determination of many substances using ion-selective membranes in combination with suitable enzymes.

For the determination of urea, enzymatic biosensors are based on urease. Typical urea biosensors utilise urease aminohydrolase which catalyses the breakdown of urea into ammonium ions and bicarbonate ions according to Equation 1:

$$\text{Urea} + 2H_2O + H^+ \xrightarrow{urease} 2NH^+_4 + HCO^-_3 \quad (1)$$

In the case of conventional urea sensors, pH [2-6] and NH_4^+ [7-9] selective electrodes have been used to detect hydrogen ions and ammonium ions, respectively, that are produced by the enzymatic reaction. The major problem for pH-sensitive electrodes is that the sensor response is strongly dependent on the buffering capacity of the sample. Indeed, the change of pH which occurs during the enzyme-catalysed reaction, is compensated by the buffer used, which leads to a narrow dynamic range and a loss in sensor sensitivity [7]. Several materials have selectivity towards ammonia including certain ionophores such as nonactin [8] and conducting polymers such as polyaniline [9] and polypyrrole [10]. Amperometric [11] and potentiometric methods can be applied through the use of urease-modified pH and ion-selective electrodes for the detection of ammonium ions. In particular, polyaniline nanoparticle films have recently been shown to have excellent sensitivity to ammonium in water with a detection limit of 3.17 μM [12]. Other polyaniline-based biosensor platforms have been demonstrated to detect enzymatically produced ammonium ions according to Equation 1 [13, 14]. Only in the latter instance was the urease enzyme immobilized to

the polyaniline – this was achieved both through casting and electrochemical deposition to the electrochemically grown polymer film.

Electroactive polyaniline films have been routinely fabricated electrochemically which is not an amenable process for mass production and therefore not viable for a low cost, single-shot biosensor. More recently, there have been reports on polyaniline materials with higher processabilities, such as those synthesised chemically using improved dopant materials [15, 16], nano-dispersions [17] and wet-spun fibres [18, 19]. These can then be deposited using methods such as chemical vapour deposition (CVD), drop-coating, dip-coating, spin-coating, etc. Aqueous-based polyaniline nanoparticle dispersions have been deposited by piezoelectric-based inkjet printing [20]. This printing technique is versatile, easily controllable in terms of pattern and thickness, and is suitable for scale-up and large-scale production of sensor platforms. Thus by exploiting it to deposit these stable polyaniline nanoparticles (onto disposable carbon-paste screen-printed electrodes), it provides a powerful technique to fabricate a sensor platform capable of ammonium ion detection. Thus, a combination of inkjet printed polyaniline nanoparticles with printed enzymes would prove useful in the fabrication of low cost, point of care biosensors.

To incorporate biological functionalities onto solid materials, bioagents should first be delivered to the solid support, and followed by immobilization. A number of techniques have been used to deposit solutions of bioactive materials onto solid supports. Covalent attachment of the biomolecule to the substrate is one of the most elegant immobilization methods available, but others as adsorption, entrapment and cross-linking are often used. Some contact deposition techniques include microspotting [21], microcontact printing [22], and photolithography [23] and some noncontact deposition systems include proximal and distal electrospray deposition [24], ink-jet and biological laser printing [25]. Recently, there is a growing interest in the use of ink-jet technology for printing biomaterials [26]. Relatively small-dispensed volume (10-20 picoliter per drop), non-contact operation, speed and comparatively high spatial resolution are some advantages of this technology. Moreover, the use of an array of nozzles connected to a device-driving electronic system allows a very good control degree over the layout of the micro deposited pattern [27].

In this work, we report on the fabrication of a biosensor using a combination of inkjet printed materials. The derived biosensor was applied to the determination of urea in serum using chronocoulometric analysis.

2. Experimental

2.1. Materials

Aniline (242284) was distilled before use and stored under liquid nitrogen. Ammonium persulfate (215589) and sodium dodecyl sulfate (L4509) were purchased from Aldrich and used as received. Dodecylbenzenesulfonic acid (DBSA-D0989) was purchased from Tokyo Kasei Kogyo Co. Ltd. A Dialysis membrane (D9402), 12 kDa molecular weight cut-off, was purchased from Sigma and soaked in Milli-Q water before use. Carbon paste ink (C10903D14) was purchased from Gwent Electronic Materials, UK. PET (175 µm) was purchased from Gwent Electronics, UK. Urease (U4002) from *Canavalia ensiformis* (Jack bean) type IX (50 kU - 100 kU fraction) with a specific activity of 70400 U/g purchased from Aldrich. Disodium hydrogen phosphate was purchased from Riedel-de Haën (30472), potassium dihydrogen phosphate, 99% (221309) and Triton X-100 (93426) were purchased from Aldrich. Urea assay Kit (ab83362) purchased from Abcam plc UK. All solutions were prepared with Milli-Q deionised water with a resistivity greater than 18 MΩ.

2.2. Buffers

0.1 M phosphate buffer was made by dissolving 4.68 g KH_2PO_4 (0.03442 mol) and 11.67 g $Na_2HPO_4{\cdot}2H_2O$ (0.06558 mol) in 1 l Milli-Q water. The pH was then adjusted using NaOH to bring the pH to 7.12.

2.3. Instrumentation

Inkjet printing was carried out using a Dimatix 2831 printer (Fuji Dimatix). All electrochemical protocols were performed on a CH601C Electrochemical analyser with CHI601 software, using chronocoulometry. An in-house fabricated batch cell of 2 ml maximum and 200 µl minimum volume was used for all electrochemical measurements, which had an integrated Ag/AgCl wire reference electrode and platinum wire auxiliary electrode. A Tecan i-control microplate reader with Nunclon 96 flat bottom polystyrol plate was used for measuring absorbance (A_{570nm}).

2.4. Fabrication of Inkjet Printed Urea Biosensors

Carbon paste screen-printed electrodes were fabricated in-house using a DEK 248 screen-printer according to Grennan et al., [28]. Briefly, electrodes were screen-

printed onto pre-shrunk PET substrate. A layer of silver was deposited using the required patterned screen. For the carbon-paste working electrodes, a layer of carbon paste ink, followed by an insulation layer to eliminate cross-talk and to define the working electrode area (7.07 mm^2) was deposited on top of the silver as the working electrode.

Polyaniline nanoparticles (NanoPANI) were synthesised according to Morrin et al., [20] using dodecylbenzenesulphonic acid as both micelle stabiliser and dopant. After purification of the nanoparticles (by centrifugation and dialysis), the dispersion was filtered through a 0.45 µm and thereafter 0.2 µm filter to remove large particle aggregates. The filtered ink was then poured into a Dimatix cartridge (DMC-11610) and was inkjet printed to the working electrode area of the carbon-paste screen-printed electrodes using an acceleration voltage of 16 V and a pitch spacing of 20 µm. The circular print pattern was designed by computer software and had an area of 7.07 mm^2. Typically, 20 electrodes were fabricated during a print run, where electrodes were printed with a single layer of nanoPANI. The nanoPANI-modified electrodes were stored in sealed vials before use. Ink cartridges were cleaned with both deionized water and buffer before filling with the enzyme ink to avoid clogging of the nozzles.

Urease (50 mg) containing glycerol (0.1% v/v) and Triton X-100 (0.01% v/v) was mixed with 1 ml of phosphate buffer pH 7.12 (0.1 M) and inkjet printed onto the nanoPANI-modified electrodes using an acceleration voltage of 16 V, pitch spacing of 20 µm and a firing frequency of 5 KHz. The modified electrodes were dried at 4^oC. The amount of enzyme ink sample deposited was estimated gravimetrically by firing all 16 printer nozzles for a given time at 5 KHz into a tared weighing boat.

2.5. Chronocoulometry

The chronocoulometric response of the nanoPANI sensor towards ammonium was performed in the three-electrode batch cell, as described above, using the inkjet printed nanoPANI electrode as the working electrode. Initially, 900 µl of phosphate buffer (0.1 M, pH 7.12) was added to the cell and held at -0.5 V for 360 s before being stepped to the equilibrium potential (0.07 V), followed by addition of 100 µl ammonium chloride standards for 50 s. Finally, the potential was stepped to -0.3 V

and the cathodic charge past (ΔQ) was monitored for 50 s. All measurements were performed at 25±1 ◦C.

For the measurement of urea, electrodes modified with both nanoPANI and urease enzyme were used. Following application of -0.5 V for 360 s to 900 µl of buffer, 100 µl of urea solution were added to the cell and allowed to pre-incubate at 0.07 V vs. Ag/AgCl. Ammonium was measured chronocoulometrically by stepping the potential to -0.3 V vs. Ag/AgCl and monitoring the cathodic charge. All measurements were performed in triplicate.

2.6. Determination of serum urea with the inkjet printed Urease/NanoPANI biosensor

Blood samples (5 ml) were taken from 15 healthy, locally recruited volunteers following ethical approval and kept at room temperature for 1 h to clot. The samples were centrifuged at 5000 rpm for 5 min, and the serum was collected and stored at 4^{o}C until use. Urea content was determined in these serum samples using the inkjet printed urease/ NanoPANI biosensor according to the method above, except that the urea standard was replaced by serum, as well as by the standard spectrophotometric enzymatic kit method which was carried out according to the manufacturer's specification.

3. Results and Discussion

Point of care diagnostic sensors need to possess several characteristics such as low sample volume, rapid assay time and ease of use. In addition to this, the fabrication and production methodology must be such that they can be manufactured rapidly in large numbers to allow scale up and reduce individual device costs. In this regard, the development of printed biosensor electrode strips has been performed for some 20 years now, particularly in the area of glucose sensing where screen printing has been a key fabrication technology. However, other print production methodologies are finding application in sensor fabrication, including inkjet printing as it is a low volume, patternable, non-contact process with low volume and low ink viscosity requirements.

3.1. Chronocoulometric measurement of ammonium at the nanoPANI electrode

Several electrochemical techniques are suitable for the measurement of ammonium and ammonia in polyaniline. These include impedimetric/conductimetric techniques and amperometric techniques. However, impedimetry/conductimetry, while good for gas phase measurements [29], are not particularly suited to solution phase measurements. Amperometry has been shown to be a useful technique for monitoring ammonia as ammonium in solution. Chronocoulometry is a related technique in which the integral of current is measured over time [30]. In this way, the cumulative response of a process over some given time interval can be measured, rather than its rate. In the context of ammonium measurement at polyaniline electrodes, it has been shown that the ammonium dopes the polymer which becomes oxidized. The film is restored to its reduced state resulting in a cathodic current at a suitably applied potential [12, 13].

To exploit this method a film of inkjet printed polyaniline nanoparticles (nanoPANI) was fully reduced at -0.5 V vs. Ag/AgCl. The potential was then stepped to the equilibrium potential for the ammonia-modified film (approx. 0.07 V) where the ammonium was allowed to equilibrate with the polymer film. Lastly, the potential was stepped to -0.3 V to drive the charge equilibration of the polymer in a manner proportional to the ammonium concentration. The results of this can be seen in Fig. 1

which shows the coulometric responses of the polymer-modified electrodes as they were stepped from 0.07 V to -0.3 V, whereupon they produced a cathodic charge transfer composed of a double layer charging response (Q_{dl}), as evidenced by the control and an additional charge dependent on the reduction of ammonium adsorbed on the film (Q_{ads}) and some fraction of the ammonium chloride reduced following its diffusion from solution (Q_{diff}):

$$Q_{total} = Q_{dl} + Q_{ads} + Q_{diff} \quad (2)$$

A single layer of the printed nanoPANI was found to be capable of measuring differences in ammonium chloride concentration from 0.1 to 100 mM in 40 s ($\mathbf{y}$= 3.55 x 10^{-4} Log$\mathbf{x}$+ 2.89 x 10^{-5}, r^2=0.98) which is within the range of molar equivalents of urea in human blood (2.5 – 7.9 mM), assuming full conversion of urea to ammonia. Several factors potentially dictate the response characteristics of the film. One factor is the adsorption capacity and proton exchange capacity of the film which will limit the total charge capacity of the film. This capacity can be tuned by controlling the film layer thickness. However, for the purposes of this assay, a single print of the nanoPANI was shown to be adequate. Inter-electrode variability was assessed for five electrodes at 1 mM ammonium chloride yielding a CV of 7.3%.

Fig. 1.

3.2. Optimisation of the inkjet printed nanoPANI/Urease enzyme biosensor

For full printed fabrication of the sensor, deposition of urease using ink jet printing was chosen. In producing a formulation suitable for inkjet printing, it should be noted that the conventional additives used to optimize ink rheological parameters may produce inactivation or denaturation of enzyme. A suitable bioink formulation must maintain the activity of the enzyme while at the same time produce stable and repeatable drops for piezoelectric jetting. In order to jet the enzyme ink, the viscosity and surface tension of the ink had to be adjusted to optimum values (30 $mN.m^{-1}$ and 5 cps) as suggested by [27]. To adjust the enzyme ink surface tension, the non-ionic

surfactant Triton X-100, was used in preference to anionic and cationic surfactants due to their reduced impact on enzyme activity [31].

An additional problem that needs to be addressed for reliable jetting is the 'first drop problem' [32]. This problem is caused by evaporation of solvent at the nozzles during idle periods. The evaporation results in local changes in the ink composition and reheological properties, which lead to potential clogging of the nozzles. To reduce the evaporation and to enhance the ink performance, 0.1% glycerol was added to the formulation as the humectant [27]. It was observed that it did not affect the printing and the first drop problem was avoided.

Urease enzyme solutions made up to 25, 50 and 100 mg/ml were assessed for their deposition via inkjet printing. 100 mg/ml was found to occasionally block the print head and so 50 mg/ml was chosen as an upper concentration for bio-ink formulation. The enzyme was typically deposited in four deposition and drying cycles. The surface coverage of the urease ink used for printing was 0.652 $\mu l/cm^2$. Given that the area of the circular printed electrode was 0.0707 cm^2, the volume of urease used was 0.46 μl per layer or 1.85 μl for four layers with a CV of 8.0% (n= 3). This equated to a mass of enzyme of 92.5 μg per electrode.

Chronocoulometric detection of urea was performed in a similar manner to that of ammonium except that the inkjet printed nanoPANI/Urease biosensor was pre-incubated with 5 mM urea at the equilibrium potential for a period of time before stepping to the reduction potential of -0.3 V vs. Ag/AgCl. The effect of pre-incubation time on the coulometric response at 50 s is shown in Fig. 2. It was shown that the coulometric response increased with increasing incubation time and that after approx. 150 s, the response was beginning to plateau. In this instance, the printed enzyme may be either non-covalently deposited on the polymer surface and/or free to dissolve in solution, bringing about near full conversion of the urea to ammonium and bicarbonate. As a result, all further measurements were performed with pre-incubation at the equilibrium potential for 150 s.

Fig. 2.

Based on the optimized fabrication and assay conditions, the biosensor was used to measure a series of urea concentrations from 0 to 12 mM (Fig. 3). This gave a linear response in the region of 2 to 12 mM with a slope of 6.7 μC/mM and an r^2 of 0.98 (n=3). This is within the appropriate range for clinical measurements of urea in human blood.

Relatively little is yet known about the impact of piezoelectric inkjet printing on enzyme activity and stability. Earlier works involving incorporation of enzymes into thick film pastes did lead to significant decreases in enzyme activity and reduced stability [33]. This may be due to the more complex ink formulation requirements to achieve the necessary screen printing rheological and processing parameters. Piezoelectric inkjet printing has been shown to lead to reductions in enzyme activity [34]. It has been suggested that this is related to the print processing parameters, particularly the acceleration voltage to eject the droplet. Cook et al. used a Microfab system which required ejection voltages of 40 to 80 V. Our work has shown that optimum ejection and activity is seen at much lower voltages (16 V) with the Dimatix system. Other work by us (unpublished data) has also shown that there is negligible loss in activity following ejection and following deposition (approx. 2%) using this instrument and these parameters. In terms of enzyme stability, any effect will thus be brought about by its deposition onto the polyaniline nanoparticle film [35]. Polyaniline has been shown to be a good surface for the immobilization of urease, showing no increased reduction in enzymatic activity as a consequence of immobilization. Nevertheless, further study is required to demonstrate the long term stability of these devices.

Fig. 3.

3.3. Correlation of the nanoPANI/Urease biosensor with spectrophotometric enzyme kit in normal human serum samples

The inkjet printed nanoPANI/Urease biosensor was correlated against a commercially available colourimetric kit assay for measuring urea in human serum. Fig. 4 shows the

results of 15 normal human samples performed with both the biosensor (Test method) and the spectrophotometric assay (Reference method). The assays had a correlation coefficient of 0.85. Most of the serum samples had a urea concentration that clustered around 5.8 to 6.8 mM according to the spectrophotometric assay. A single sample lay outside this range, being 3.1 mM. All these values were in line with the expected assay range for normal serum urea concentrations [1]. The least squares regression gave a slope of 0.84 and an intercept of 0.89 which suggests that, at the low end of the assay range, the biosensor test method overestimates the urea concentration compared with the reference method, but that, at higher concentrations, the biosensor was underestimating. This can be seen more clearly in the Bland-Altman plot in Fig. 5. For the lowest urea concentration, the difference between biosensor overestimated the value by some 0.8 mM as compared to the average of the two tests, which is an approximate 25% divergence. However, for all other samples, the biosensor underestimated by only 0.12±0.08 mM compared with the average of the two test results. This represents a deviation of less than 2% in the 5.8 to 6.8 mM range. The within (intra-day) and between batch (inter-day) CVs for urea determination in serum by the present method were found to be <5% and <7%, respectively (n=6).

Fig. 4.

Fig. 5.

Many examples of urease biosensors exist in the literature, particularly electrochemical and optical devices. In addition, several have used conducting polymer materials as the selective agent, most notably, polyaniline. Luo and Do [13] used electropolymerised films of PANI doped with Nafion®. In a similar manner to that shown here, they showed the onset of reduction at approx. -0.17 V vs. Ag/AgCl. Although they established a linear range of urea in the clinically relevant range of 6-60 mg/dL (1 – 10 mM), it is well known that the reproducible, large scale production of electropolymerised PANI films is a significant barrier to widespread application. Other groups continue to use membrane layers to achieve selectivity. For example, Trivedi et al., [36] recently used a double membrane layer to produce an ammonium ion sensitive potentiometric sensor for urea. However, such systems still suffer from pH dependence. More recently, Malinoski et al., [37] used aqueous polyaniline nanoparticle dispersions, again as the basis of a potentiometric urea biosensor which demonstrated a non-linear potentiometric response from 1 to 6 mM. None of these works demonstrated the application of the assay device in human blood or serum, or correlated against available tests.

The work presented here is a combination of the use of conducting polymer nanoparticles in combination with inkjet printing of these, along with the enzyme, urease, to solve the problems associated with reproducible mass production of conducting polymer-based biosensors. In addition, the sensor was shown to be applicable over the relevant clinical range of 2.5 to 7.9 mM urea in real human plasma samples, with excellent correlation with established tests.

4. Conclusions

A biosensor using inkjet printed polyaniline nanoparticles and urease enzyme was constructed. The device was shown to be sensitive to ammonium in solution in the range of 0.1 to 100 mM using chronocoulometry. The inkjet printed biosensor was also shown to have a linear response to urea in the range of 2 to 12 mM (r^2=0.98), and

when compared to a colorimetric enzyme kit for urea determination in human serum samples was found to have a correlation coefficient of 0.85.

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Figure legends

Fig 1. Chronocoulometric response of the nanoPANI electrode to ammonium as ammonium chloride. Electrodes were poised at the equilibrium potential of 0.07 V vs. Ag/AgCl for 50 s upon the addition of the ammonium chloride and then stepped to -0.3 V vs. Ag/AgCl for 50 s over which time, the coulometric responses were monitored. Cathodic currents are shown as positive. Ammonium chloride concentration increases in the direction of the arrow from 0, 0.1, 0.5, 1, 5, 10, 50 and 100 mM.

Fig. 2. The effect of time on the coulometric response from the nanoPANI/Urease biosensor in the presence of 5 mM urea. Chronoculometric response taken at 50 s following step potential from 0.07 V to -0.3 V vs. Ag/AgCl. All measurements were performed at 25±1^{o}C (n=3).

Fig. 3. Calibration of the nanoPANI/Urease sensor after addition of urea, pre-incubation at 0.07 V for 150 s and stepped to -0.3 V vs. Ag/AgCl, followed by measurement of cathodic charge passed after 50 s (n=3). From 2 to 12 mM, slope = 6.7 µC/mM, intercept = 60.1 µC, r^2=0.98.

Fig. 4. Correlation of the nanoPANI/Urease biosensor with a spectrophotometric enzyme assay kit for the determination of urea in 15 human serum samples. Intercept = 0.89, slope = 0.84 and r^2 = 0.85. Inset shows the cluster of 14 samples from 5.8 to 6.8 mM.

Fig. 5. Bland-Altman plot comparing the nanoPANI/Urease biosensor (Test) with the spectrophotometric assay (Reference).

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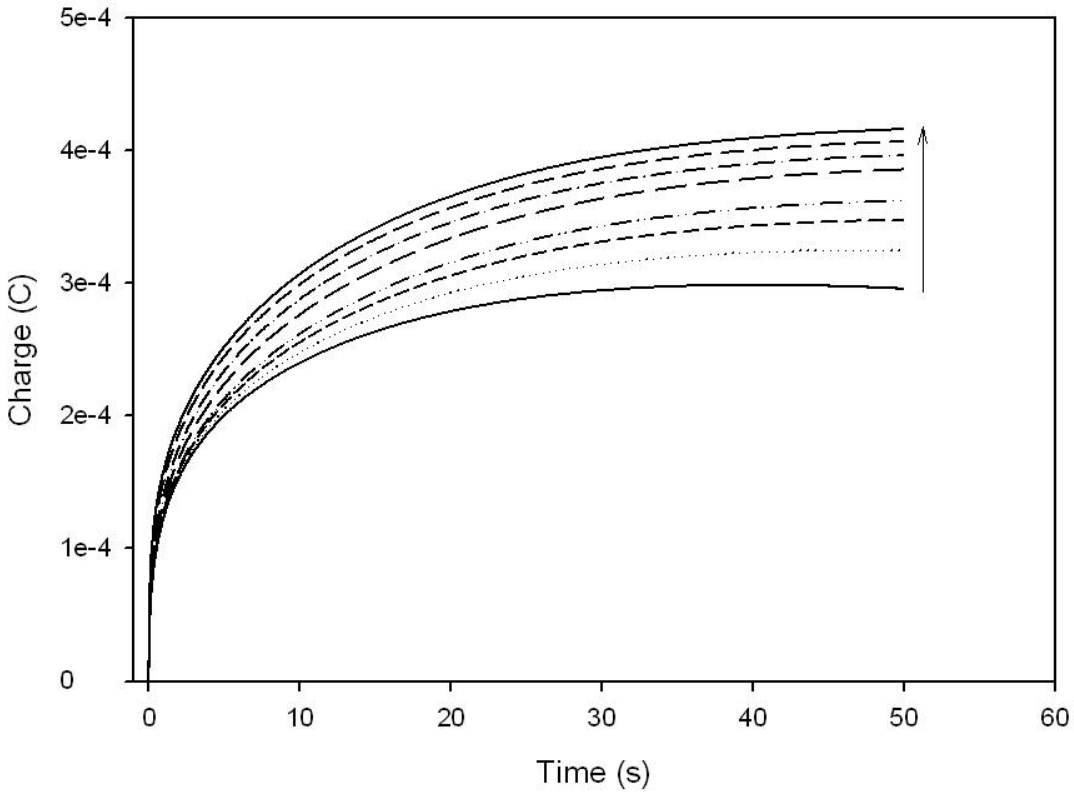

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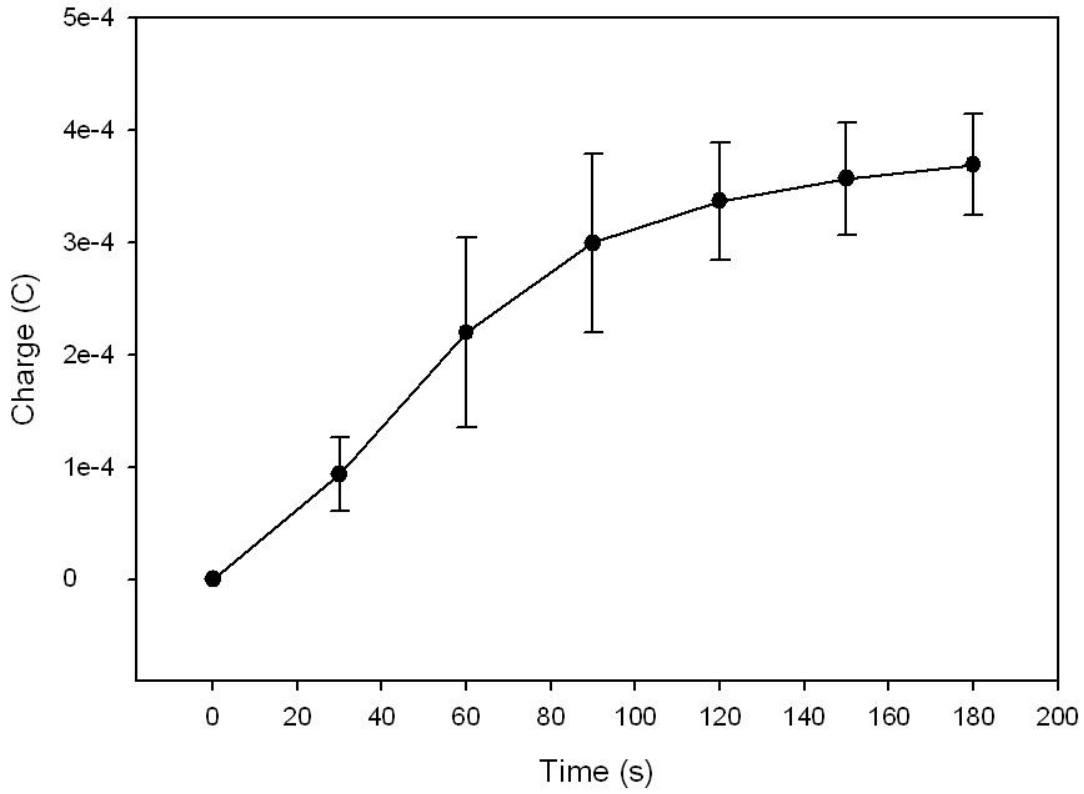
481 Fig. 2.



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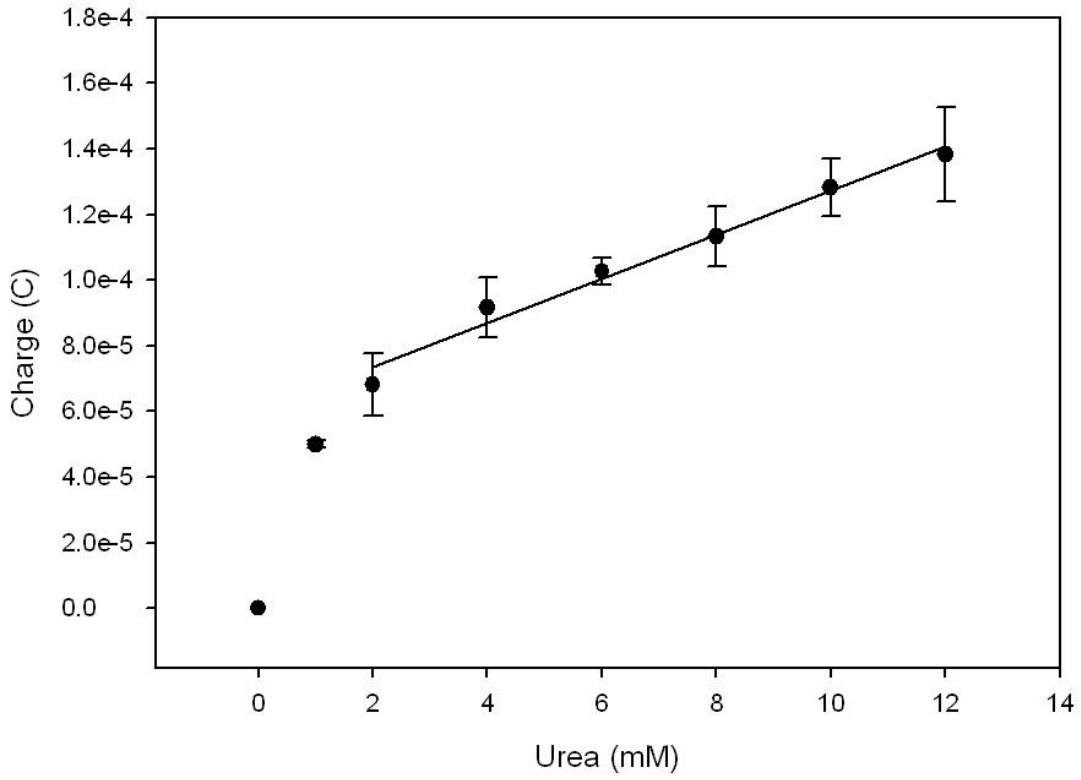

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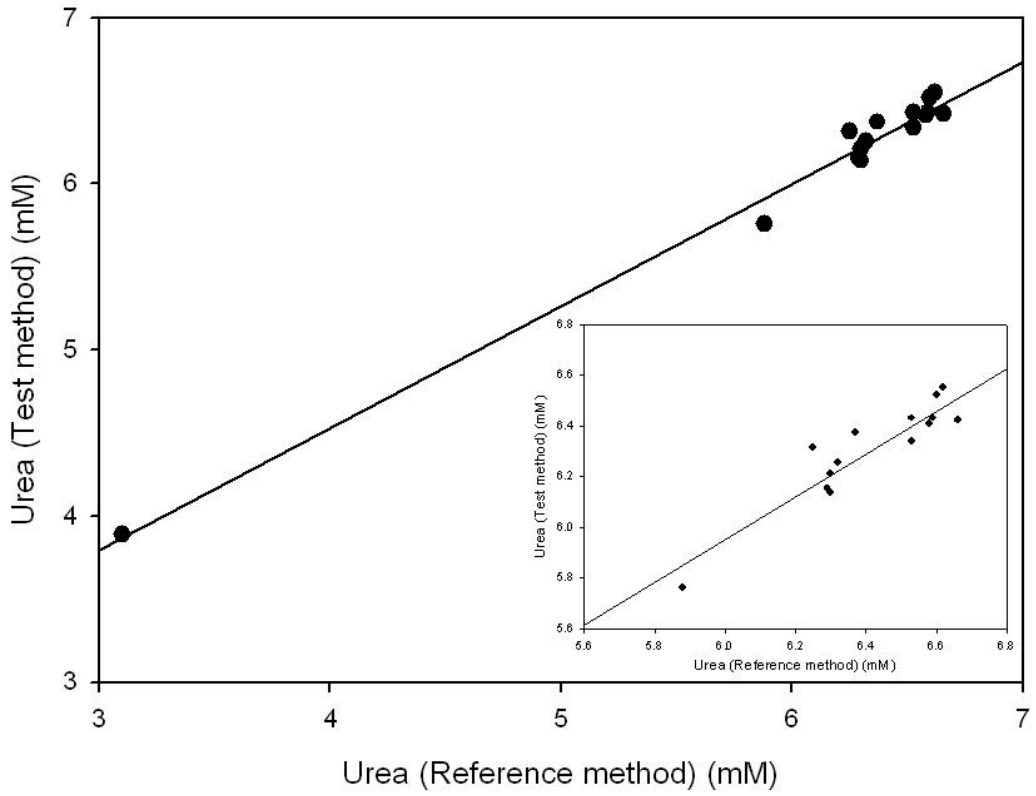
518 Fig. 4.



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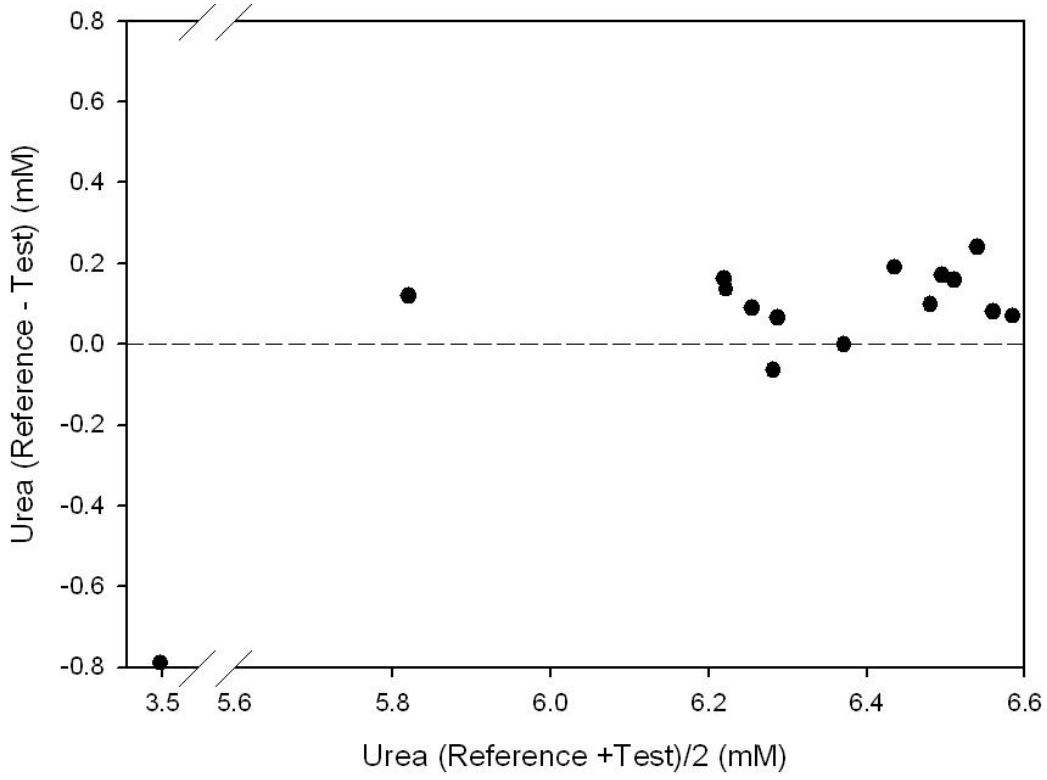
536 Fig. 5.


0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Urea (Reference - Test) (mM)
3.5
5.6
5.8
6.0
6.2
6.4
6.6
Urea (Reference +Test)/2 (mM)

537