Development of a Voltammetric Sensor for Theophylline with Sol-Gel Immobilised Molecularly Imprinted Polymer Particles

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Abstract

Application of Molecularly Imprinted Polymers (MIPs) to sensor substrates holds great promise within the field of electrochemical sensing due to their low price, tailored selectivity and facile synthesis protocols. Though MIPs can be synthesised directly onto the surface of sensors via layer or film deposition, this can be difficult due to the high number of interdependent steps involved in their synthesis. For this reason, synthesis of MIP particles is more frequently employed by synthetic and non-specialist laboratories alike. There is, however a lack of immobilisation protocols for these particles. Herein, there is presented a sol-gel based immobilisation method for MIP particles for the development of an electrochemical sensor. The macroporous precipitation-polymerised particles were imprinted with Theophylline, combined with graphite in the sol-gel and deposited on an electrode surface. The sensor was tested using differential pulse voltammetry. A limit of detection of 1µM and a relative standard deviation of 6.85% was observed for the primary analyte. The electrode was regenerated via a thermal washing process with a signal loss of 29.3% following the initial regeneration and 2.35% per subsequent regeneration.

Keywords

Molecularly Imprinted Polymers (MIP); Sol-Gel; Graphite; Theophylline; Voltammetry

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Introduction

The necessity for novel qualitative sensor systems with the capacity to detect targeted molecular compounds is ever-present. For several decades, sensors based on biological recognition, such as those utilising enzymes, antibodies, microorganisms or aptamers, have received a majority of interest within this field with their superior recognition properties [1]. Biomolecules do, however, suffer from a generally poor chemical and physical stability as well as being costly to synthesize or purchase and thus in more recent years, artificial receptors have been garnered with increasing attention [2].

Molecular imprinting embodies the creation of a tailored binding site of the selected template molecule. The binding sites that are created can be highly specific and have the ability to discriminate between structurally similar compounds as well as chiral molecules [3,4]. This field is most commonly embodied by Molecularly Imprinted Polymers (MIPs) which have received increasing interest over the last two decades [5].Though there are main two strategies for the interaction events within each binding site, covalent and non-covalent, the non-covalent is afforded greater usage due to the speed and ease of synthesis, facile post-binding regeneration and the greater level of functionality-potential at the binding site [6]. The synthesis protocol for such MIPs consists of a preassembly step where a specific template molecule is mixed with a functional monomer. This is followed by the cross-linkage of the functional monomer with a secondary co-polymer, which fixes at proper distance and geometry the binding sites and the removal of the template (Fig. 1). The popularity of MIPs comes from their low cost, ease of preparation and high stability [7].

Given their advantages molecular imprinting appears equally enticing to laboratories regardless of the level of in-house synthetic expertise. There are, however, some drawbacks and discouragements while attempting to imprint polymers. Of the two most common structural forms of MIPs, particles and deposited films, both are accompanied by disadvantages which hinder the exploitation of molecular imprinting as an analytical technique. While particles are easier to synthesize compared to films[8], the number of immobilisation methods for MIP particles available in the literature is low. Indeed, in a 2010 review of the state-of-the-art of imprinting in electrochemistry [9], of 61 papers reporting immobilisation onto sensors, only 5 were stated to have used electrode-immobilised particulate MIPs; 4 further cited articles employed columns or flow systems which must compromise between long extraction times or the use of higher volumes of polymer which increases the risk of template leaching [10].

Theophylline, the molecule chosen as molecular template for this work, is a methylxanthine alkaloid of the purine family and is present in cocoa beans, teas and a range of other beverages and plant materials along with its structural analogues caffeine and theobromine. For over 70 years it has been used to treat

maladies of the airway such as asthma and chronic obstructive pulmonary disease (COPD). Its low cost and high availability has made it one of the most widely prescribed drugs for such conditions [11]. Its simple structure and close similarities to its analogues (Figure 1) make it an attractive molecule to imprint. Its low cost and toxicity also increases the attraction of using it in prototype studies [12,13,4,14].

Conventional methods for the detection of methylxanthines are based on gas and liquid chromatography, the advantages of which are clouded by the requirement of bulky, expensive equipment and trained operators to run the analysis [15]. Electrochemical methods on the other hand, embodied by voltammetric, potentiometric, amperometric and piezoelectric devices, were until relatively recently not commonly used for their detection [16]. This is due to their extremely high oxidation potentials, observed with common electrochemical systems incorporating metallic and/or carbon-based electrodes, which make the final signal poorer, with background noise created from oxidative currents and limited reproducibility [17,13] .

Synthesis techniques of particulate MIPs consist of bulk, suspension, emulsion, two-step swelling and precipitation polymerisations [18]. The disadvantage of bulk imprinting, the most commonly used technique, lies in the high volume of material and template that is required by the synthesis. Obtained monolith particles can also have a low capacity due to binding-site heterogeneity and poor site accessibility stemming from the grinding process needed to break the polymer brick [19], thus there is a need to investigate alternative synthesis strategies.

<Figure 1>

Though MIPs have already been successfully applied to the majority of the contemporary transduction mechanisms [20], there is instead a deficit in the availability of facile immobilisation methods for particulate MIPs onto the surface of available transducers for rapid sensing of the targeted molecule.

The key difficulty in the search for an effective immobilisation method for particulate MIPs lies in the compromise between extraction or measurement time and reproducibility. When using monolithic MIP particles, a large quantity must be used in order to negate the potential heterogeneity of the measurement due to the aforementioned accessibility issues. When such particles are immobilised in lower quantities, in a membrane for example, the decreased availability of the binding sites lead to undesirably high variability between measurements [4]. Incorporation of the monoliths inside the electrode increases the robustness of the sensor and allows the use of more aggressive strategies to be used for signal amplification [13] though this method does not allow for the removal of the template upon re-binding and thus effective regeneration of the sensor is not possible.

The use of agarose gel has also been documented as a method to immobilise MIPs onto sensor-surfaces [19,21,2]. In these cases, the use of more uniform precipitated MIP microparticles allowed for a greater level of reproducibility though the location of the particles underneath a gel membrane required an extended diffusion time with respect to membrane thickness, which in turn is affected by the applied voltage potentials.

Sol-gel immobilised MIPs have been reported in which high voltage potentials (1.4-2.0V) are incorporated into the sensing protocol [22-24]; these with the use of linearly polymerised MIPs based on the 'Takagishi' method [25]. A second defect might be the low levels of crosslinking polymerisation that can also lead to polymer chain migration, breakages and flaws in the polymer network when swelling occurs; thus, a substrate must be used during polymerisation to ensure polymer integrity [26].

In this work, a theophylline-imprinted polymer-incorporating a voltammetric sensor, is presented. Microspherical macroporous MIP particles were immobilised using the Sol-Gel technique together with graphite as the conducting media on the surface of a carbon electrode. The theophylline-imprinted polymer was synthesised using standard protocols of precipitation polymerisation and their morphology and size distribution was confirmed via Scanning Electron Microscopy (SEM). Prepared Sol-Gel membranes were also characterised using confocal microscopy and SEM. Primary response and electrode regeneration was investigated using adsorptive stripping voltammetry (ASV) employing the differential pulse technique. Cross response to other methylxanthines, caffeine and theobromine was fully characterised. The limit of detection of the electrode was also demonstrated using chronoamperometry.

Experimental

Reagents and chemicals

50 μm particle size graphite powder was purchased from Merck (Merck, Darmstadt, Germany). Epotek H77 resin and its corresponding hardener (Epoxy Technology, Billerica, MA, USA) were also used in the electrode fabrication. All reagents were analytical reagent grade. All solvents were purchased from Scharlab, (Barcelona, Spain). Theophylline, Theobromine, Caffeine, Methacrylic Acid (MAA), Ethylene glycol dimethyl acrylate (EGDMA), Tetraethyl orthosilane (TEOS) and inhibitor removal columns were purchased from Sigma-Aldrich (St.Louis, MO). The radical initiator 2,2'-Azobis(2,4-dimethylvaleronitrile) (AIVN) was purchased from Wako Chemicals GmbH (Neuss, Germany). All other acids and Potassium Hydrogen Phthalate ($C_8H_5KO_4$) were purchased from Panreac (Barcelona, Spain).

Preparation of Theophylline-Imprinted Polymer Particles

The protocol for the precipitation polymerisation synthesis of the MIP particles was taken from the literature [12]. In brief, inhibitor in the MAA and EGDMA was removed immediately prior to use via passage through separate inhibitor removal columns; 0.255 mmol of Theophylline and 0.911 mmol of MAA were mixed with 40 ml of acetonitrile for 10 min; 3.64 mmol of EGDMA and 0.852 mmol of AIVN were then added. The solution was sonicated under vacuum and purged with nitrogen for 10 minutes, sealed and placed in a 60ºC water bath for 16 hours. A control non-imprinted polymer (NIP) was also created using an identical procedure with the omission of theophylline. The MIP particles were separated via centrifugation at 4500 RPM for 10 min. The particles were washed using 9:1 methanol: acetic acid solution for 1 hour, at which point the washing solvent was refreshed. This was repeated 5 times to ensure the complete removal of the template molecule; the particles were then rinsed again with methanol only and dried at 70ºC.

Electrode preparation and MIP-Sol-Gel Immobilisation and Regeneration

The electrodes used for experimentation were epoxy-graphite composite electrodes of normal use in the laboratory of the authors and were prepared using a previously published in-house protocol with a final geometric surface area of 0.28 mm^2 [27]. Following the curing duration, the electrodes were wet-polished with 400 grit abrasive-paper and degreased with acetone. The immobilisation of the MIP particles onto the surface the epoxy-graphite electrode was adapted from a previously published sol-gel protocol [23]. In this, 0.5 ml TEOS, 0.5 ml ethanol, 0.25 ml water and 25 µl 0.1 M HCl was combined and stirred vigorously for 35 min and rested for approximately 45 min. 200 µl of this liquid was combined with 7 mg of graphite and 40 µl of a MIP-DMF suspension consisting of 15 mg of MIP particles and 1 ml of DMF. The control was fabricated identically with the substitution of the NIP for the MIP. This mixture was shaken for 10 min at 1400 RPM. 10 µl of this solution was deposited in the centre of each electrode and evenly distributed using a home-made spin coater at 1400 RPM for 60 seconds. The electrodes were dried at atmospheric pressure at 5ºC overnight and conditioned in water for one hour before use. For regeneration experiments, the electrodes were immersed in 0.05 M HCl at 60-65ºC for 10 min and then conditioned in water for one hour before subsequent use.

Apparatus

All polymerisations were done in a water bath controlled with a Huber CC1 thermoregulation pump (Huber Kaeltemaschinenbau GmbH, Offenburg, Germany). All experiments were conducted using a commercial 52-61 platinum combined Ag/AgCl reference and counter electrode (Crison Instruments, Barcelona Spain). All voltammetric measurements were carried out using a DropSens µStat8000 multi-potentiostat/galvanostat. Chronoamperometry

measurements were executed using an Autolab PGStat 20 (Metrohm Autolab B.V, Utrecht, The Netherlands). SEM analysis was executed using a MERLIN FE-SEM (Zeiss GmbH, Jena, Germany). Confocal microscopy was done with a Leica DCM-3D system (Wetzlar, Germany).

Electrochemical measurements

Differential Pulse Voltammetry in all experiments was performed with a scan range between 1 and 1.7 V, a pulse potential of 0.01 V, a duration of 300 ms and a scan rate of 0.04 V/s. A base line measurement was taken at t=0 from which all proceeding measures were subtracted. Chronoamperometry experiments were performed at 1.18 V. All measurements were done in pH=3 C8H5KO4 buffer with the pH adjusted using 0.1 M HCl.

Results and Discussion

Particle Synthesis and Sol-Gel Immobilisation

Precipitated microspheres were used for sol-gel immobilisation due to their small size and high homogeneity. The synthesis protocol used employed a molar ratio of 1:4 MAA: EGDMA to ensure high crosslinking and optimal rigidity to maintain binding-site morphology within the polymer matrix.

The yield following template removal was 64% by weight (563 mg), 24% less than that given by Ye *et al.* [12]. This drop in yield efficiency is speculated to have originated from the lower centrifugation speeds that were used during the washing of the particles. SEM analysis confirmed the formation of highly uniform spherical particles (Fig 2a); at the higher magnification shown on the right, a roughness or 'wrinkled' surface was observed, cited as an advantageous attribute of macroporous particles which cause an increment in surface area. The pore structure is permanent does not require solvent-related swelling to provide access to the pores **[26]**. A subsequent statistical analysis (Fig 2b) found the particle size distribution to be in agreement with that published in the literature. 54% of the particle yield was made up of sizes at 970 nm while 96% of the yield was above 665 nm. In contrast to the large positive skew seen in the distribution of the MIP particle synthesis, the NIP control saw a quite narrow (σ = 33.1 nm) normal distribution of particle sizes; with 46% of the yield being sized at the mean value and 86% of the total being at or above the mean of 116 nm, shown in Fig. 2c. The greatly diminished particle size demonstrates the sensitivity of precipitation polymerisation protocols to the choice of template.

<Figure 2>

Optimisation and Determination Sol-Gel Conditions

In order to obtain a sensing surface usable for voltammetry, the obtained MIP microspheres were immobilised within a Sol-Gel matrix. This was done by modifying a procedure determined by Patel *et al.* [23,28,22]. Optimal results were seen when the electrodes were chilled following the deposition and distribution (spin coating) and allowed to dry over night at ambient pressure. Though signal intensity was seen to be directly proportional to the final gel thickness, an optimised deposited volume of 10 µl on the graphite-epoxy composite electrode was chosen as larger deposition volumes were seen to increase the instance of crack formation on the gel surface; a finding consistent with the literature [29]. Though it is possible to negate the instance of crack altogether as well as achieve extremely high film uniformity via multiple depositions [30], the signal augmentation that was seen with the single deposition was not replicated when multiple layers were built up on the electrode surface. The use of high-potential voltammetric cycling or sustained high voltage pre-treatments were seen to be detrimental to the structural integrity of the sol-gel and thus obliged the subtraction of interfering background currents prior to each measurement. Performance of measurements in strong acids in order to increase the signal to noise ratio, as used for the detection of similar compounds [13], also was not feasible as the acid caused the gel deterioration due to continued hydrolysis of the membrane.

In the first instance, a standard phosphate salt buffer was chosen as a detection medium. Though many groups report the use of neutral to slightly basic pH buffers when performing MIP-template binding experiments, acidic pH buffers are also widely used for signal augmentation [31,32] and indeed, a measuring pH of 3 was chosen in keeping with the recommendations of Spataru *et al*. [17] whereby pH=3 and below was said to be the most suitable for methylxanthine analysis.

Upon deposition, the Sol-Gel mixture was evenly distributed using a home-made spin coater. When dried, the thickness of the membrane was measured using a 3D confocal microscope (Fig 3a). It was observed that though the greatest thickness remained at the deposition site, the overall distribution of membrane thickness was relatively uniform varying from approximately 200 µm at the extreme peripheries of the electrode to 300 µm at the original deposition point. This variation was seen to arise from the decrease in solution thickness, as well as graphite particle quantity, which occurred when the graphite particle size was increased while the mass proportion remained constant thus decreasing the in-situ availability of the graphite causing a radial gradient to be observed. Increasing the mass proportion of the graphite to compensate for this reduction, though aiding in the homogeneous distribution of the gel upon deposition provoked an increased occurrence of crack formation in the Sol-Gel surface upon drying which obliged a reduction in deposition volume and decreased signal strength.

<Figure 3>

Due to their lower density relative to the graphite-Sol-Gel suspension, it was seen that the MIP particles preferentially occupied the surface of the Sol-Gel electrode (Fig. 3b & c). This separation was seen to occur during the spin coating procedure in which mechanical spreading of the deposition also caused the repositioning of the suspension's components. This orientation is advantageous as it reduces the incidence of non-specific interactions between the analyte solution and the graphite particles and thus maximises the specific binding events between the MIP particles and the target analyte. The high availability of the MIP particles at the surface of the sensor also reduces the immersion time in the analyte required before measurement can occur.

Electrochemical Characterisation

The devised theophylline sensor made from the immobilisation of MIP microspheres in a Sol-Gel matrix was subsequently used in adsorptive stripping voltammetric determination of the alkaloid. An accumulation time of 5 min was chosen for measurements, as a minimal signal increase occurred subsequent to this which was also accompanied by an increased inter-electrode response variation (Fig. 4).

<Figure 4>

When different methylxanthines were assayed with the prepared electrode, oxidation peaks of 1.18, 1.32 and 1.35 V were observed for Theophylline, Theobromine and Caffeine, respectively (Fig. 5). The peak cross-responses of Theobromine and Caffeine at pH = 3 were consistent with that set forward by Spataru et al. [17]. The peaks observed for the secondary analytes were broader than that of theophylline most probably due to the lower incidence of binding events occurring at the surface of the MIPs.

<Figure 5>

Signal current intensities were extracted from the profiles by subtracting the background signal and averages were made (n=5). Relative standard deviation (RSD) was calculated as 6.85% for the primary target and an intensity of 258.1% (relative to control NIP-Sol-Gel electrode) (Figure 6). Lesser differentiation was seen between the caffeine and theobromine with intensities being only 125.6% and 119.7% relative to their controls. The minor intensity decrease seen in the

case of theobromine relative to caffeine is speculated to stem from solubility related molecular aggregation occurring within the solution [33].

<Figure 6>

Regeneration of the MIP-Sol-Gel immobilised electrode was attempted. The temperatures, 60-65ºC, duration, 10 min, and acidic strength 0.05 M HCl, were optimised. Similar to that found during the optimisation of the deposition of the gel, strong acid was detrimental to the integrity of the sol-gel structure. Similarly, extended periods at high temperatures were seen to cause a similar effect; thus the parameters were determined. It was also seen that use of the electrode immediately following regeneration caused erratic results; this is believed to be due to the electrode body acting as a thermal battery, thus a relaxation period was required to return the electrodes to a base level.

As was observed by Alizadeh et al. [13], measures subsequent to the initial binding event decreased in intensity. Such was the behaviour seen in the MIP-Sol-Gel electrode whereby all measures following the first were approximately 70.7% of the initial measurement (Figure 7). It was seen, however, that the linear pattern seen from the first regeneration cycle one was consistent for over 40 regeneration cycles (not shown) with an average signal loss of 2.35% per cycle. The initial 29.3% decrease, followed by a greatly reduced loss of signal is due to the occupation of the deeply-seated, high affinity binding sites within the MIP particle structure which cannot be easily cleaned following the initial binding event. The subsequent preferential recognition of the analyte exhibited by the MIP-Sol-Gel electrode can be attributed to binding sites located on the ‘wrinkled’ surface of the particles which are not present on the surface of the NIP particles

<Figure 7>

Lower limit of response of the Sol-gel immobilised MIP was seen to be 1 µM, consistent with the LOD observed when MIP particles synthesised using an identical protocol were immobilised via agarose gel [19], shown in Figure 8. A lower limit of detection (LOD) was not possible to confirm due to the decreasing signal-to-noise ratio caused by the aforementioned interference originating from the problem of overlapping oxidative currents coming from the electrode inherent to the detection of methylxanthines.

<Figure 8>

Conclusion

A MIP-incorporating voltammetric sensor, responsive to the alkaloid theophylline has been presented. A modified Sol-gel protocol was exploited to immobilise MIP microspherical particles onto epoxy-graphite electrodes which were synthesised in-house. Particle morphology, size distribution and Sol-Gel deposition uniformity were confirmed using SEM, statistical and confocal microscopy analysis, respectively. Aggressive pre-treatments were negated with the subtraction of a base line from each measure to remove noise coming from the overlapping oxidation currents. Primary response, cross response to other alkaloids, caffeine and theobromine, and electrode regeneration was investigated using adsorptive stripping voltammetry.

A limit of detection consistent with the state-of-the-art was demonstrated using chronoamperometry. To the best of the authors' knowledge, this is the first report of an acrylate-based MIP particle being used for quantitative electrochemical measurements using Sol-Gel immobilisation methods. The use of such provides an immobilisation technique that greatly reduces the conditioning time required for each measurement relative to other similar immobilisation techniques in the literature and also provides a reduced RSD% in comparison to other MIP particle immobilisation methods which use monolith MIPs. The presented procedure might be extended to many equivalent compounds with the condition that they should be electroactive and can be used as a facile tool for rapid prototyping of synthesised MIP particles.

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Figure Captions

Fig. 1 Molecular structure of Caffeine, Theophylline and Theobromine (a); Schematic of theophylline-imprinted binding site consisting of pre-assembly of the functional monomer and the template, polymerization of the crosslinking polymer and the subsequent extraction of the template (b).

Fig. 2 (a) SEM of uniform spherical theophylline-Imprinted Polymer particles. (b) Statistical analysis of MIP particle size distribution with a mean of 819 nm and standard deviation of 153 nm. (c) Statistical analysis of NIP particle size distribution with a mean of 116 nm and standard deviation of 33 nm.

Fig. 3 3-dimensional surface profile and film-thickness of Sol-gel on electrode surface obtained by confocal microscopy examination (a) SEM images of a deposited Sol-Gel membranes containing graphite only (b) and the Sol-Gel immobilised MIP microspheres (c).

Fig. 4 Dynamic response of MIP-Sol-Gel electrode (●) and NIP-Sol-Gel control electrode (◌) toward the primary imprinted methylxanthine (theophylline) in a pH =3 phosphate buffer solution at a concentration of 0.27 mmol/l.

Fig. 5 Differential pulse voltammetry response intensities of the MIP-Sol-Gel electrode (Solid line) and NIP-Sol-Gel electrode (broken line) for caffeine, theophylline and theobromine at a concentration of 0.27 mmol/l and t = 5 min.

Fig. 6 Intensities of response profiles of the MIP-Sol-Gel electrode (solid fill) and NIP-Sol-Gel electrode (white fill) caffeine, theophylline and theobromine at analyte concentrations of 0.27 mmol/l.

Fig. 7 Regeneration of MIP-Sol-Gel electrode (●) and NIP-Sol-Gel control electrode (◌) using thermal-acidic treatment calculated from n = 5 replications.

Fig. 8 Obtained calibration with the MIP-Sol-Gel electrode (applied potential: 1.18V; R=0.9917; y = 53.436x + 0.0053).

Figure 1

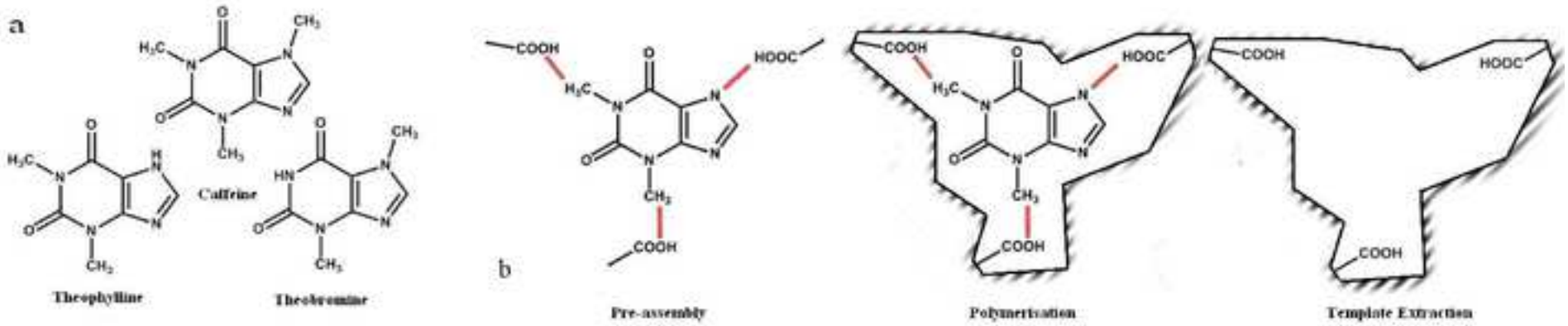
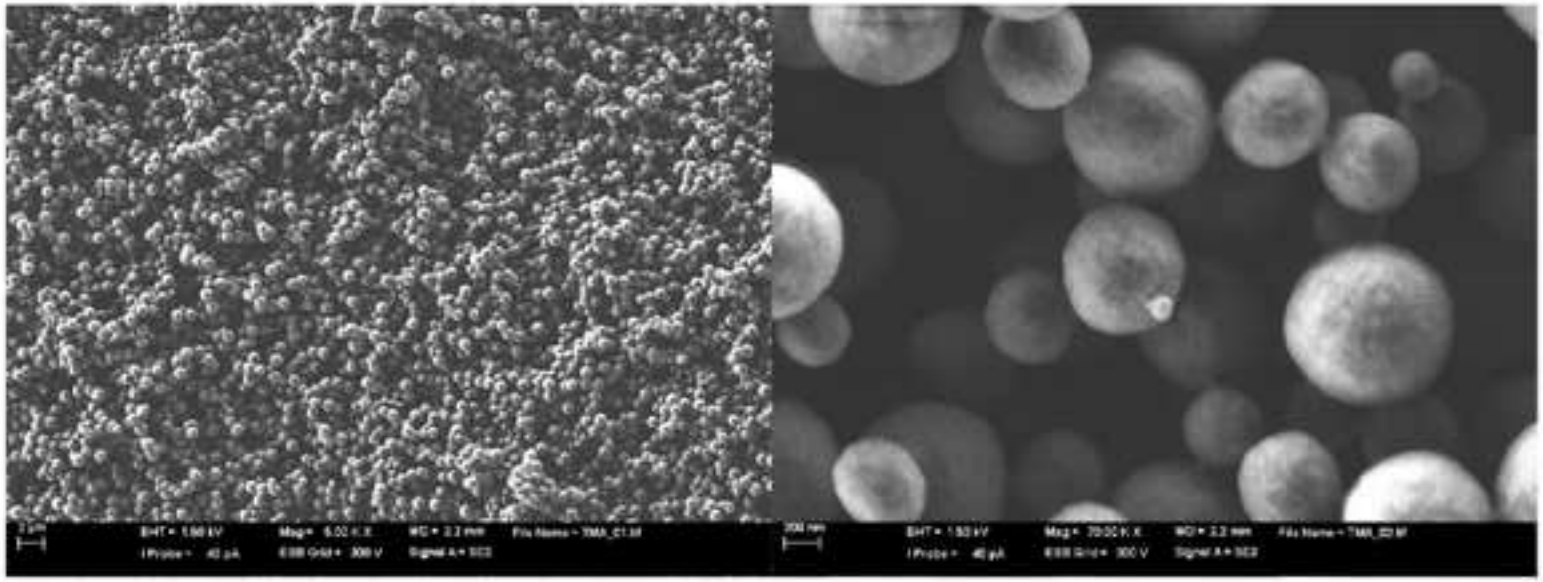
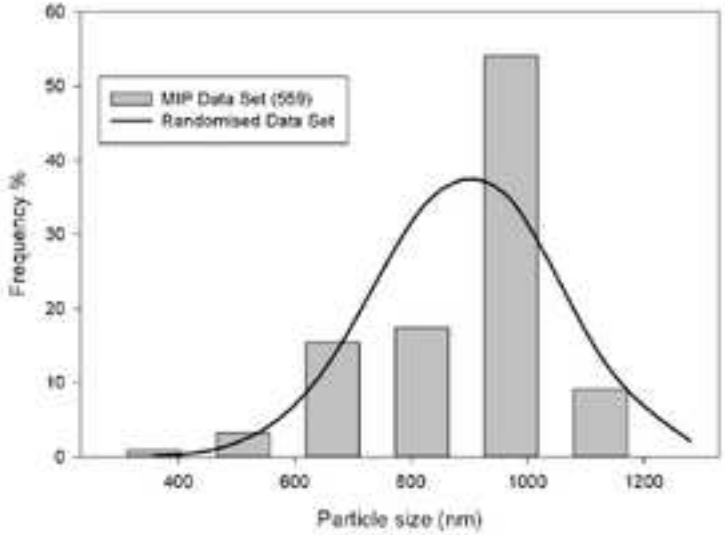

a
Caffeine
Theophylline
Theobromine
b
COOH
HOOC
Pre-assembly
Polymerization
Template Extraction

Figure2

a



b


MIP Data Set (559)
Randomised Data Set
Frequency %
Particle size (nm)
0
10
20
30
40
50
60
400
600
800
1000
1200

c

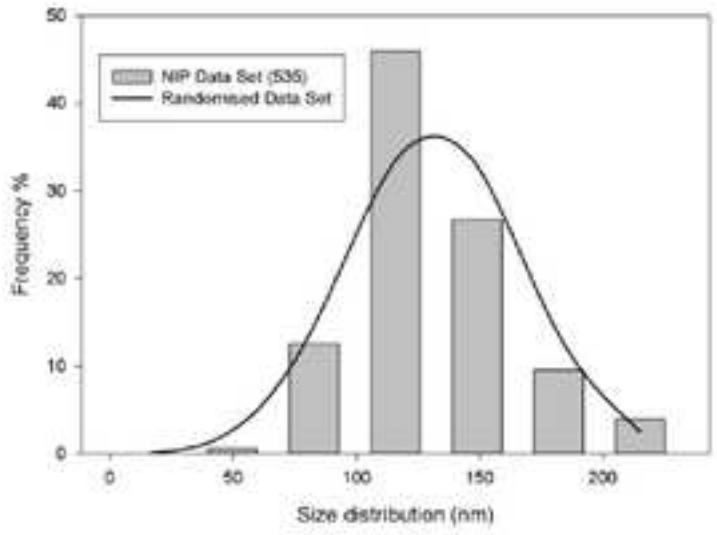

NIP Data Set (535)
Randomised Data Set
Frequency %
Size distribution (nm)
0
10
20
30
40
50
0
50
100
150
200

Figure3

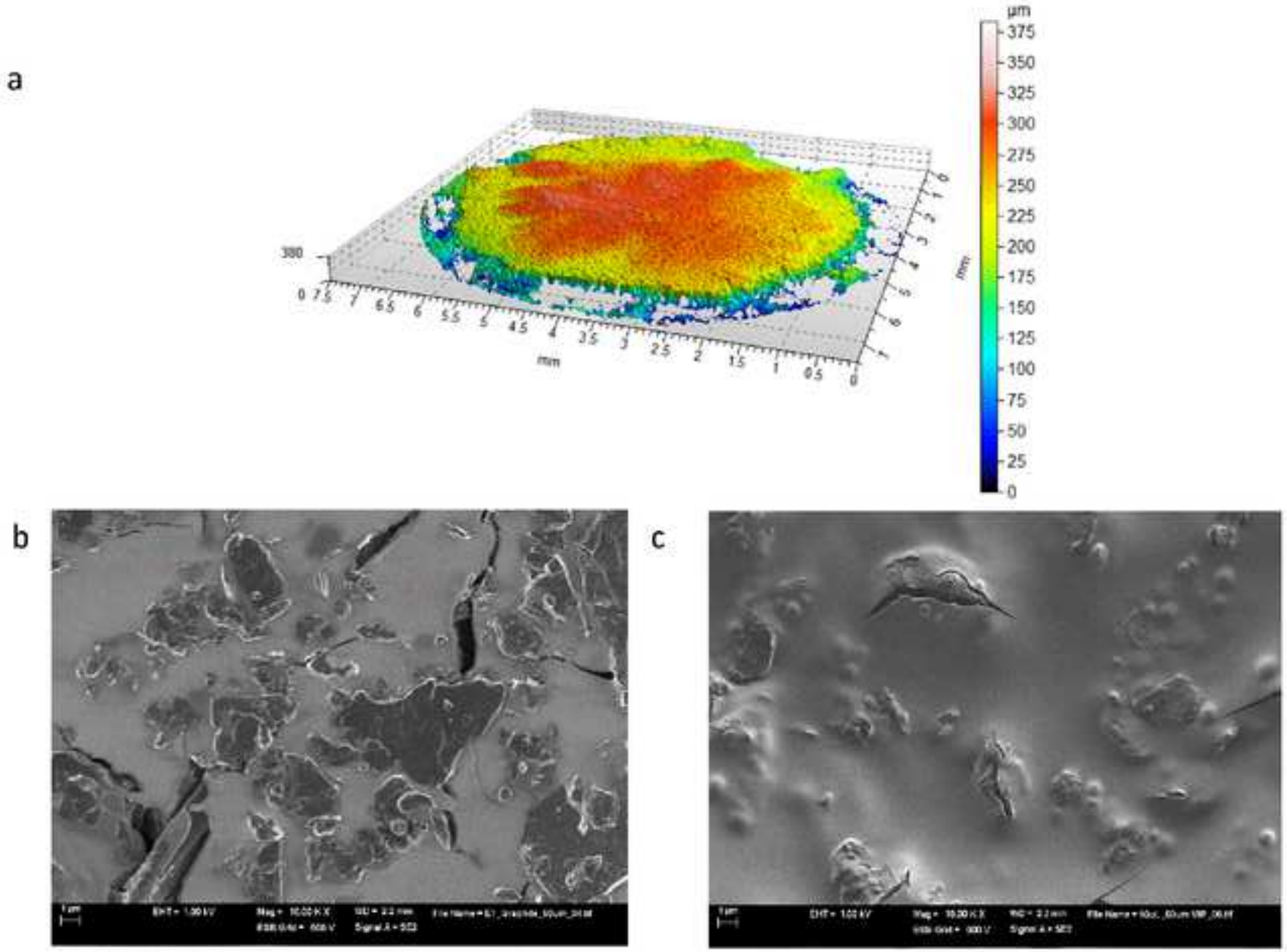

a
μm
375
350
325
300
275
250
225
200
175
150
125
100
75
50
25
0
mm
b
c

Figure4

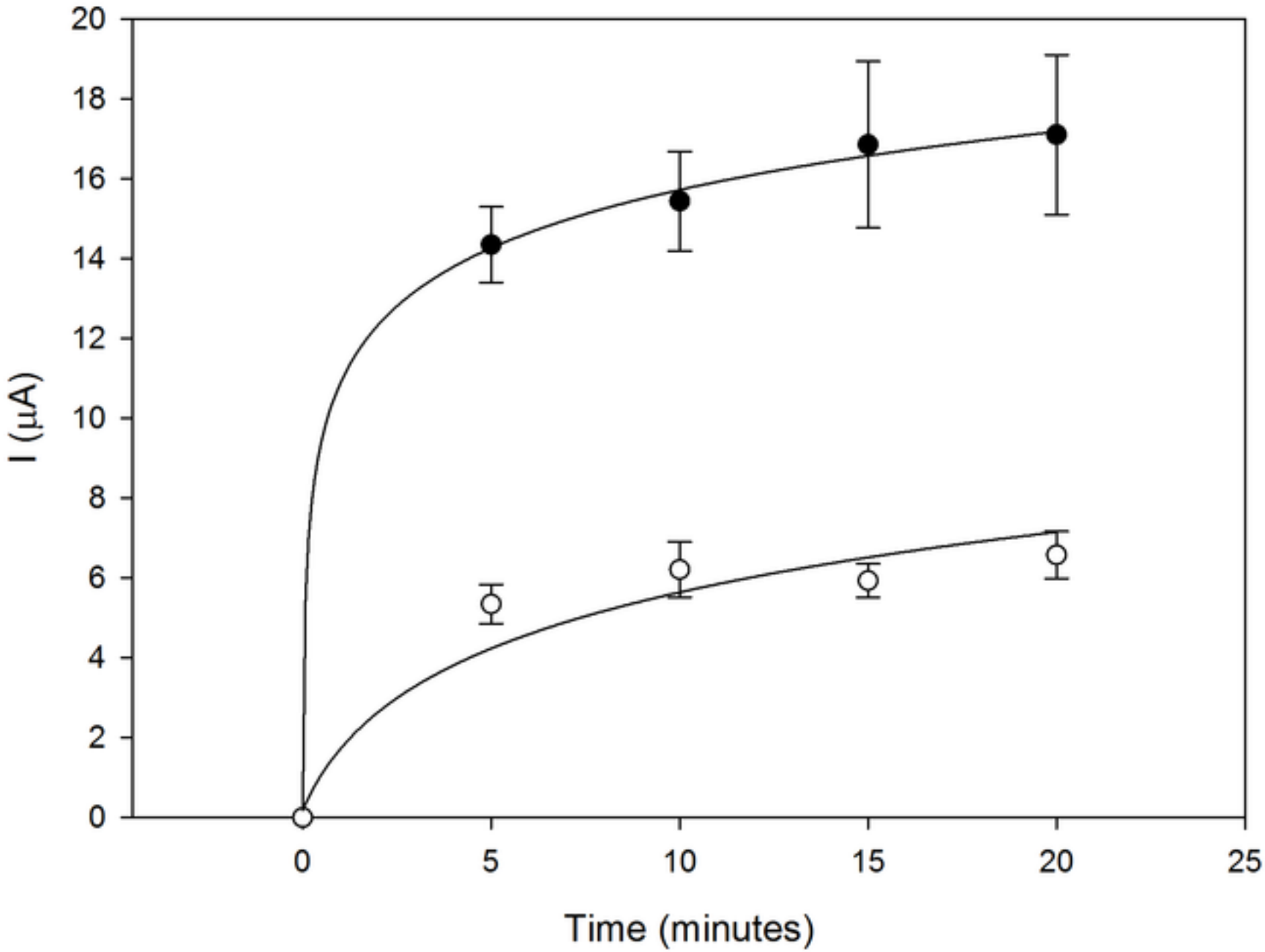


Figure5

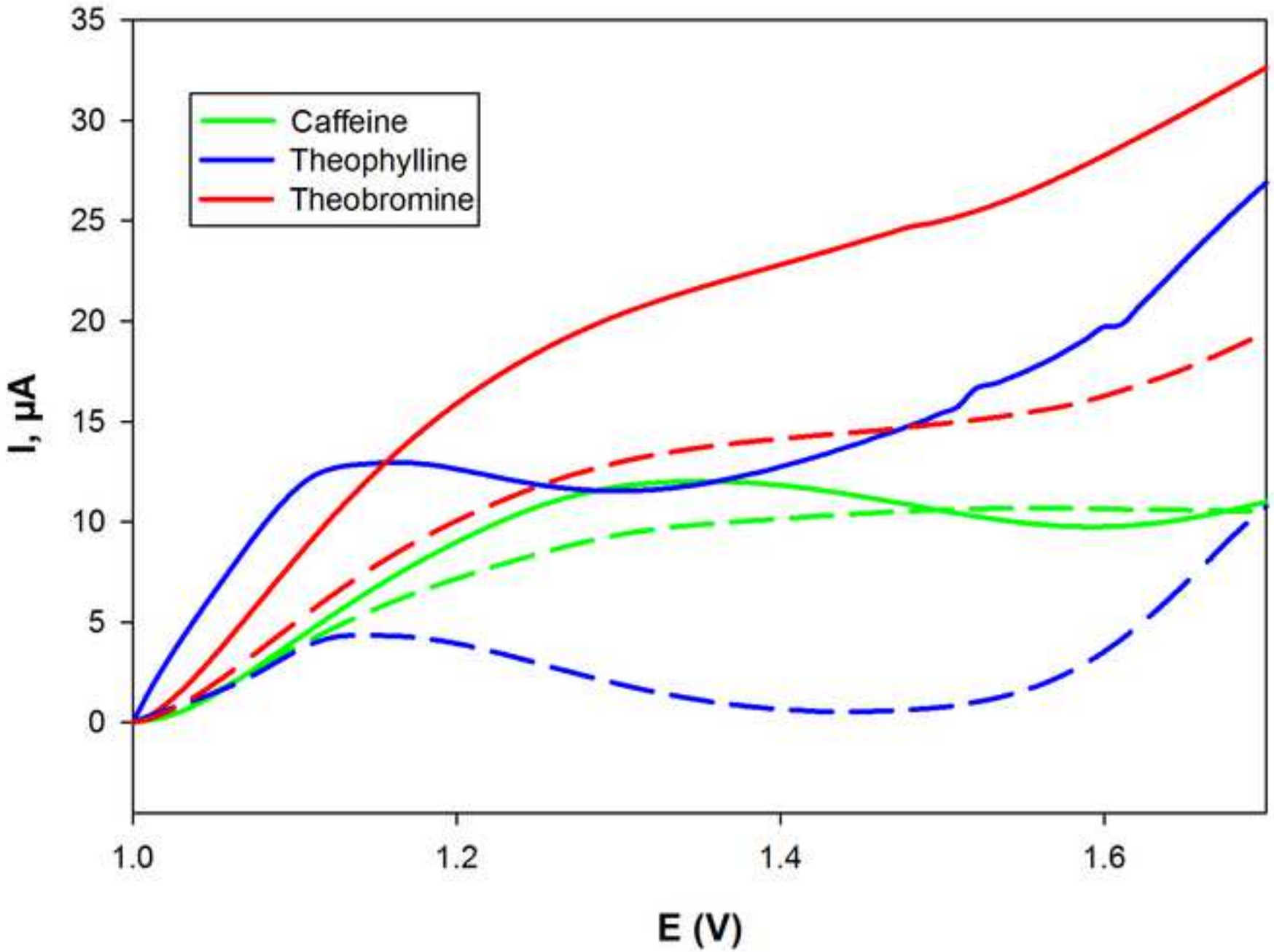

Caffeine
Theophylline
Theobromine
I, µA
E (V)
35
30
25
20
15
10
5
0
1.0
1.2
1.4
1.6

Figure6

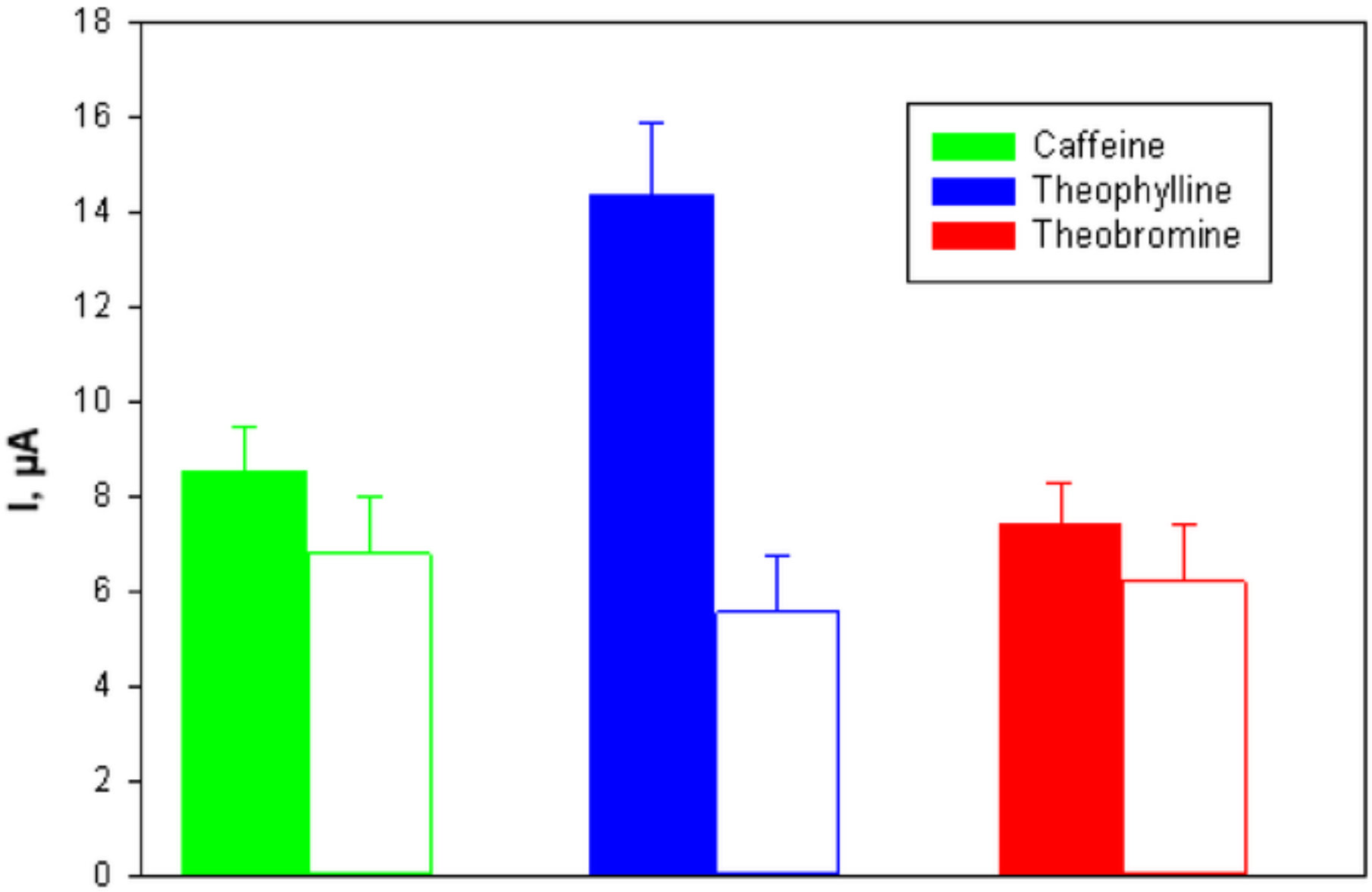


Figure7

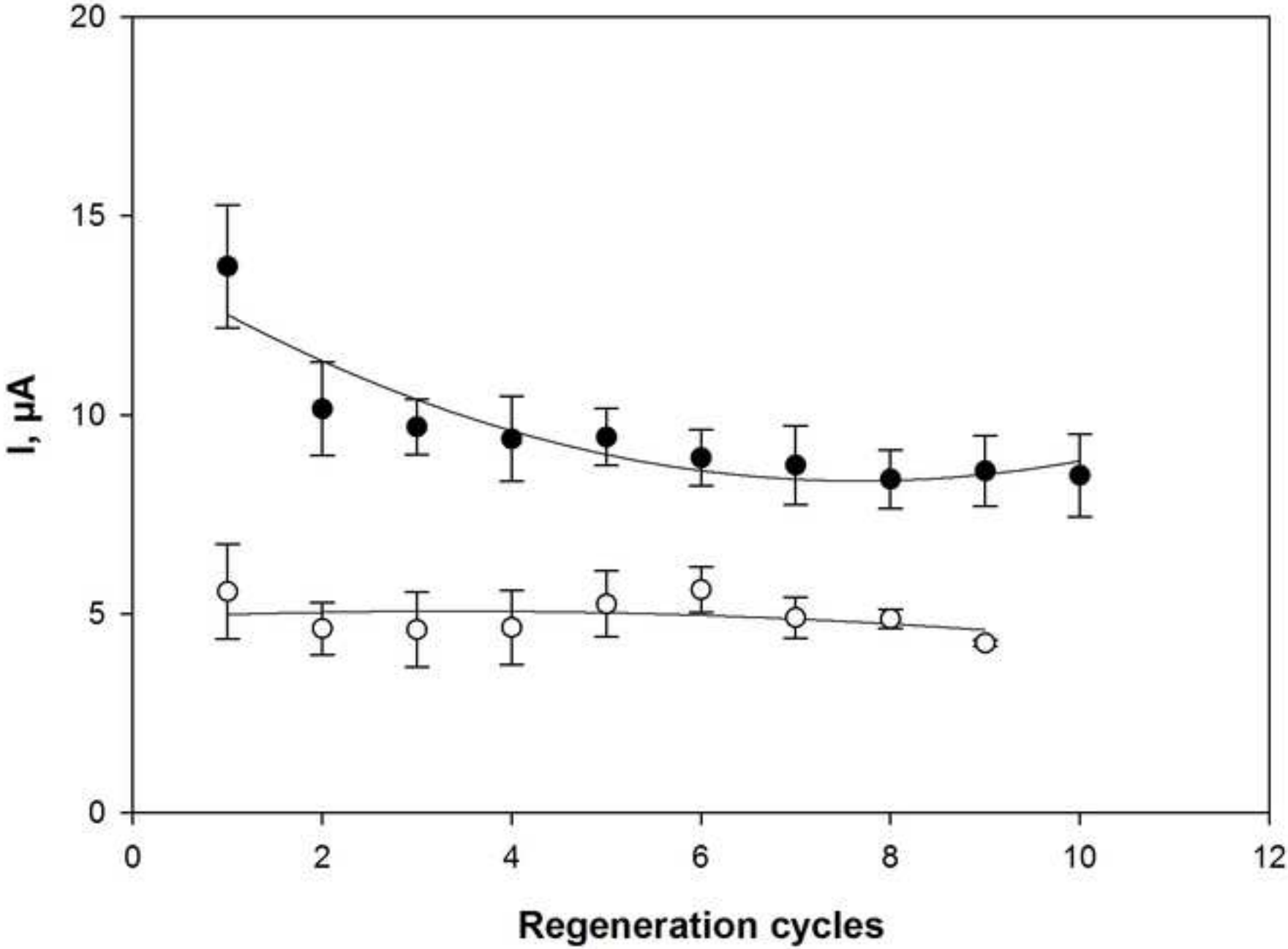
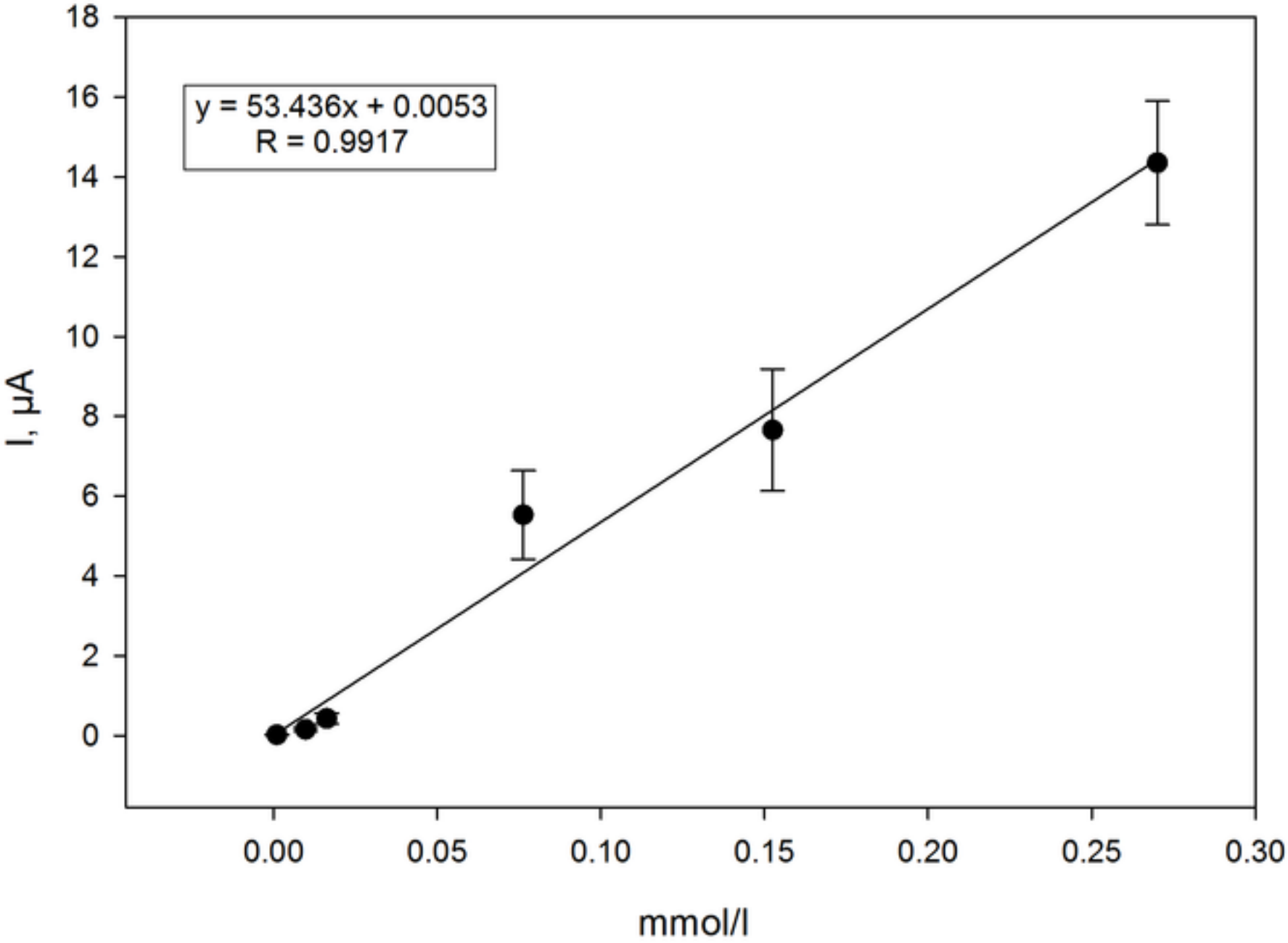


Figure 8


y = 53.436x + 0.0053
R = 0.9917
I, µA
mmol/l
18
16
14
12
10
8
6
4
2
0
0.00
0.05
0.10
0.15
0.20
0.25
0.30