8. เอกสารอ้างอิง

Alexeev, V. L., Sharma, A. C., Goponenko, A. V., Das, S., Lednev, I. K., Wilcox, C. S., Finegold, D. N. and Asher, S. A., *Analytical Chemistry*. 75 (2003) 2316-2323.

Annaka, M. and Tanaka, T., *Nature* 355 (1992) 430-432.

AOAC International, Method Validation Programs (OMA/PVM Department), including Appendix D: Guidelines for collaborative study procedures to validate characteristics of a method of analysis, 2000. http://www.aoac.org/vmeth/devmethno.htm.

Blake, D. A. and McLean, N. V. 1989. A Colorimetric Assay for the Measurement of D-Glucose Consumption by Cultured Cells.

Choodum, A. Thavarungkul, P., Kanatharana, P., Smith, N.W., *Chromatographia.* 69 (2009)[a] 481-488.

Choodum, A. Thavarungkul, P., Kanatharana, P., Smith, N.W., *Analytical Sciences* 25 (2009)[b] 517-522.

Dean, K.E.S., Horgan, A.M., Marshall, A.J., Kaliban, S., Pritchard, J., *Chemical Communications.* (2006) 3507-3509.

Gabai, R., Sallacan, N., Chegel, V., Bourenko, T., Katz, E. and Willner, I., Jounal of Physical Chemistry Part B 105 (2001) 8196-8202.

Guide EURACHEM, The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics, 1998. http://www.eurachem.org.

Hoare, T., Pelton, R., *Macromolecules.* 40 (2007) 670-678.

Ivanov, A. E., Galaev, I. Y. and Mattiasson, B., *Macromolecular Bioscience.* 5 (2005) 795-800.

Ivanov, A.E., Thammakhet, C., Kuzimenkova, M.V., Thavarungkul, P., Kanatharana, P., Mikhalovska, L.I., Mikhalovsky, S.V., Galaev, I.Y., Mattiasson, B., *Journal of Molecular Recognition.* 21 (2008) 89-95.

Kataoka, K., Miyazaki, H., Bunya, M., Okano, T. and Sakurai, Y., *Journal of American Chemical Society.* 120 (1998) 12694-12695.

Kuzimenkova, M.V., Ivanov, A.E., Thammakhet, C., Mikhalovska, L.I., Galaev, I.Y., Thavarungkul, P., Kanatharana, P. Mattiasson, B. *Polymer.* 49 (2008) 1444-1454.

Lee, M.C., Kabilan, S., Hussain, A., Yang, X., Blyth, J., Lowe, C.R., *Anaytical Chemistry.* 76 (2004) 748-5755.

Lorand, J.P., Edwards, J.O., *Journal of Organic Chemistry.* 24 (1959) 769-774.

Matsumoto, A., Kurata, T., Shiino, D. and Kataoka, K., *Macromolecules.* 37 (2004)[a] 1502-1510.

Matsumoto, A., Yoshida, R. and Kataoka, K., *Biomacromolecules.* 5 (2004)[b] 1038-1045.

Miller, G.L., Analytical Chemistry. 31 (1959) 426-428.

Park, S., Boo, H., Chung, T. D., *Analytica Chimica Acta.* 556 (2006) 46-57.

Position Statement-Urine Glucose Monitoring, International Diabetes Federation. retrieved on July 4, 2010. http://www.idf.org/Position_statementsurine_monitoring.

R Development Core Team, R: A language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2006.

Ren, L., Liu, Z., Dong, M., Ye, M., Zou, H., *Journal of Chromatography A.* 1216 (2009) 4768-4774.

Springsteen, G. and Wang, B. A., *Tetrahedron* 58 (2002) 5291-5300.

Suzuki, A. and Tanaka, T., *Nature* 346 (1990) 345-347.

Taverniers, I., Loose, M.D., Bockstaele, E.V. *TrAC, Trends in Analytical Chemistry.* 23 (2004) 535-552.

Urine test AUTION™ Sticks. Retrieved on September 10, 2010. http://www.arkray.co.jp/english/products/urinalysis.html.

Wang, W., Gao, X., Wang, B., *Current Organic Chemistry.* 6 (2002) 1285-1317.

WHO. (2011). "Diabetes" Retrieved Feb 8, 2011, from http://www.who.int/mediacentre/factsheets/ fs312/en/index.html

Wu, B., Zhang, G., Shuang, S., Choi, M. M. F., *Talanta.* 64 (2004) 546-553.

ภาคผนวก

View Letter

Close

Date: Apr 04, 2011
To: "Chongdee Thammakhet" tchongdee@gmail.com
From: "Analytica Chimica Acta, Editorial" aca@elsevier.com
Subject: ACA-11-137R1: Final Decision

Ms. No.: ACA-11-137R1

Dear Dr. Thammakhet,

On behalf of the editor handling your manuscript, I am pleased to inform you about the acceptance of the manuscript entitled:

"Development of an On-column Affinity Smart Polymer Gel Glucose Sensor"
by Panote Thavarungkul, D.Phil; Proespichaya Kanatharana, Ph.D.

The publisher will send a transfer of copyright form and galley proofs to you in due course. For questions with regard to proofs, publication date or reprints of your article, please contact our Author Support Department (fax: +353 61-709100; e-mail: authorsupport@elsevier.com).

Yours sincerely,

Tanya Devanny
Journal Manager
Analytica Chimica Acta
E-mail: aca@elsevier.com

Close

Development of an On-column Affinity Smart Polymer Gel Glucose Sensor

Chongdee Thammakhet[a,b,c,*], Panote Thavarungkul[a,b,d], Proespichaya Kanatharana[a,b,c]

[a]*Trace Analysis and Biosensor Research Center, Prince of Songkla University, Hat Yai 90112, Thailand*

[b]*Center for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai 90112, Thailand*

[c]*Department of Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai 90112, Thailand*

[d]*Department of Physics, Faculty of Science, Prince of Songkla University, Hat Yai 90112, Thailand*

* Corresponding author: Department of Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand. Tel.: +66 7428 8429; Fax: +66 7455 8841

E-mail address: chongdee.t@psu.ac.th (C. Thammakhet)

Abstract

An on-column affinity smart polymer gel glucose sensor was developed as a non-enzymatic glucose sensor. A copolymer of 3-acrylamidophenylboronic acid and acrylamide, the so called "smart polymer", was synthesized *in situ* in a 5 cm long capillary tube with a detection window to provide the on-column detection. The optical density of this semitransparent affinity smart polymer gel, coated inside the tube, decreased with increasing glucose concentration and was detected using a UV-Vis detector at 500 nm. The capillary tube was incorporated into a flow injection system. Under optimum conditions, a linear dynamic range of 0.5-16.0 mM with a limit of detection of 0.5 mM (S/N $\geq$ 3) was obtained. A single coated affinity smart polymer gel had good stability for up to 250 consecutive injections with relative standard deviation of less than 5%. The analysis time for each injection was 6 minutes. Ten glucose samples prepared in distilled water were analyzed by the developed method and the results compared well with those obtained from the conventional dinitrosalicylic acid (DNS) method (P>0.05). Real urine samples with known glucose levels were analyzed and the developed sensor provided comparable results to those

from the normal strip test technique. Acceptable percentage recoveries, ranging from 88±2% to 103±4% from the spiked urine sample, were obtained.

Keywords: non-enzymatic glucose sensor, on-column detection, phenylboronic acid, affinity smart polymer gel

1. Introduction

The determination of glucose is very important in various processes, for example, the monitoring of glucose level in either blood or urine for the treatment of diabetes or the monitoring of cell growth in biotechnology and food industries where glucose is the primary carbon source in most fermentation processes [1, 2]. The most commonly used method is by an enzyme based sensor [3] because of its high selectivity to glucose. However, one of the major problems is the low stability of the test enzymes. Their activities can be easily affected by temperature, pH, humidity and toxic chemicals [1, 4-6]. Moreover, immobilization of an enzyme on the electrode surface is needed and this may also decrease enzyme activity [6]. In addition, the amount of dissolved oxygen in the sample can affect the accuracy of the results and needs to be controlled [1, 4, 7]. For these reasons, a non-enzymatic glucose sensor has been considered to overcome the previously mentioned drawbacks [6, 8]. One interesting approach is the use of a smart polymeric material containing a glucose selective ligand, such as phenylboronic acid [9, 10] because the smart polymer can reversibly change its physico-chemical property according to the change in glucose concentration [11-14].

A number of phenylboronic acid based smart polymeric glucose sensors have been studied in the past few years. Kabilan and co-workers constructed a holographic sensor that showed a wavelength shift after binding with glucose [11]. This sensor worked well at pH 9.0. In the same year, the same group improved their holographic sensor to be able to operate

at under pH 7.4 by changing the monomer from 4-vinylphenylboronic acid to 3-aminophenylboronic acid [12]. This supported a former finding that incorporation of an amino group into a phenylboronate polymer protected the borate-diol complex from hydrolysis under physiological conditions of pH [2, 15]. In our earlier work, we also presented an optical polymeric gel glucose sensor by detecting the optical density change (at 500 nm) of the copolymer of 3-acrylamido phenylboronic acid and acrylamide coated on a glass slide in the presence of glucose. However, the response time of the sensor, 40-60 minutes, was too slow for practical use [13, 14].

In this article, we have proposed a capillary microfluidic flow injection system to overcome such a limitation. The concept is based on the advantages of the microliter flow detection system employed in both micro-high performance liquid chromatography (μ-HPLC) and capillary electrochromatography (CEC) because such a system uses a small column, a minimal amount of mobile phase, a small sample volume and on-column detection. This was applied in combination with the convenient detection of the change in optical density of the affinity smart polymer gel in the presence of glucose. This was done by incorporating a small capillary, with an inside coating of the smart polymeric gel network, into a flow injection system. Glucose was then passed through the polymeric network of the smart polymer gel coated inside the capillary instead of only on the polymer surface as in the earlier work [13, 14]. It was expected that the analysis time would be improved and the shape of the signal would be easier to handle. To obtain the highest sensitivity of the sensor, the affecting parameters were first optimized and the analytical performances of the sensor were then evaluated. The developed sensor was then applied for the analysis of glucose in real samples, *i.e.*, glucose in urine samples.

77 **2. Experimental**

78 ***2.1 Materials***

79 3-Aminophenylboronic acid monohydrate (98% purity), *D*-galactose, *D*-mannose,
80 sucrose and lactose were from Aldrich (St. Louis, MO, USA). Acrylamide (≥99% purity),
81 *N,N,N',N'*-tetramethylethylenediamine (TEMED) (99% purity), ammonium persulphate (≥ 98%
82 purity) were the products of Sigma Chemical Co. (St. Louis, MO, USA). Acryloyl chloride (≥
83 96% purity) and *D*-(+)-glucose anhydrous were obtained from Fluka (Stienheim, France).
84 Sodium hydroxide and hydrochloric acid were from LAB-SCAN Analytical Science,
85 (Labscan Asia Co., Ltd., Bangkok, Thailand). Sodium dihydrogen orthophosphate and
86 disodium hydrogen orthophosphate were products of APS Finechem (NSW, Australia). *N*-
87 acryloyl-*m*-aminophenylboronic acid (NAAPBA) was prepared as described elsewhere [16].
88 Fused silica capillary tubings with various internal diameters (I.D.) were from Polymicro
89 Technology (Arizona, USA)

90

91 ***2.2 Instrumentation***

92 The flow injection system (Fig. 1a) consisted of a pump (400 Solvent Delivery
93 System, Applied Biosystem, USA) to drive the carrier buffer, an injection valve (6 port valve,
94 Valco Instruments Co. Inc., USA) where the sample and regeneration solutions were
95 introduced and a UV-Vis detector (757 Absorbance Detector, Applied Biosystem, USA) for
96 on-column detection. The detection window in the middle of the coated capillary was
97 positioned at the level of the detection cell (Fig. 1b). The on-column detection was based on
98 the optical density change caused by the swelling and shrinking of the gel, observed at 500
99 nm [13-14].

100

101

102 ***2.3 Procedures***

103 ***2.3.1 In situ synthesis of the on-column affinity smart polymer gel***

104 To obtain the affinity smart polymer gel of a 3-acrylamidophenylboronic acid
105 acrylamide copolymer (NAAPBA-co-AA), 9.6 mg of NAAPBA and 30.6 mg of acrylamide
106 were dissolved in 0.5 mL of ultrapure water at 60°C. The mixture was gently agitated by
107 manual shaking for dissolution. Two microliters of TEMED was added and gently agitated by
108 manual shaking for 1 minute for the solution to be completely mixed. The solution was then
109 degassed by purging with nitrogen. This solution was the "monomer mixture". The "initiator
110 solution" was prepared by dissolving 19.9 mg of ammonium persulphate in 0.5 mL of
111 ultrapure water.

112 A fused silica capillary tube, 5 cm long with an I.D. of 0.32 mm, was first
113 used to optimize the affecting parameters. A detection window was made in the middle of the
114 tube by heating the capillary with a custom-made heating coil. In this process, an
115 approximately 2 mm length of the polyimide layer was removed from the tubes outer surface
116 leaving a clear portion of the capillary for the on-column UV detection window.

117 Twenty microliters of the monomer mixture was mixed with 10 μL of the
118 initiator solution. One end of the capillary tube, with its detection window, was immediately
119 dipped into the solution and the solution was drawn into the tube by capillary action. This
120 amount of solution could prepare 3 tubes. After a few minutes the semitransparent affinity
121 smart polymer gel was formed inside the capillary without further treatment, ready to be set
122 into the flow injection system. Phosphate buffer which was used as the carrier buffer in the
123 flow injection system was run through the three serially connected coated tubes for 30
124 minutes to stabilize the coated gel and to flush any remaining excess monomer mixture out of
125 the tubes. The tubes can be stored at room temperature before use.

126

127 ***2.3.2 Detection principle of the sensor***

128 In principle, the functional groups of the phenylboronic acid affinity smart
129 polymer gel exist in equilibrium between the neutral trigonal planar and tetrahedral anionic
130 forms. Glucose favors binding to the charged form. Therefore, when the concentration of
131 glucose increases, the equilibrium shifts increasingly towards the direction of the charged
132 borate. The concentration difference between ions in the gels and those in the surrounding
133 solution induces the Donnan potential and results in the swelling of the affinity smart
134 polymer gel [14]. The level of swelling is reflected by the decrease in the measured optical
135 density. Since the binding of boronic acid with glucose is by a reversible covalent bond,
136 when glucose is released from the affinity smart polymer gel, the gel shrinks and the optical
137 density returns to the original signal prior to the binding.

138 With this principle, the carrier buffer was initially passed through the affinity
139 smart polymer gel to obtain the absorbance baseline (Fig. 2 inset). When the standard
140 solution of glucose was injected, the binding of glucose and phenylboronic acid in the affinity
141 smart polymer gel caused the gel to swell. At this moment the observed optical density
142 decreased. The chart recorder (BBC Goerz Metrawatt, Australia) was connected in such a
143 way that the measured signal (mV) will rise when the optical density decreases, (signal "*a*" in
144 Fig. 2 inset). Since the binding between glucose and phenylboronic acid is favored by a basic
145 solution, an acidic solution (phosphate buffer with an acidic pH) was employed to speed up
146 the release of glucose from the gel and to allow the gel to be reused. This regeneration
147 solution was introduced into the system *via* the injection valve; the dissociation between
148 glucose and phenylboronic acid caused the gel to shrink. This could be observed by the rapid
149 decrease of the signal (signal "*b*" in Fig. 2 inset). After the flow of the carrier buffer the pH
150 was quickly adjusted and the optical density returned to the original baseline within a few
151 seconds (signal "*c*" in Fig. 2 inset) and the next analysis can be immediately performed.

152 To obtain the best performance of the sensor, affecting parameters such as the
153 concentration and pH of the carrier buffer and regeneration solutions, carrier flow rate and
154 sample volume were optimized (Table 1) by varying a single parameter and keeping others
155 constant. The highest signal with the shortest analysis time was used as the criteria to select
156 the optimum conditions. Under optimum conditions, performances of the sensor, for
157 example, linearity, limit of detection, accuracy, and precision were evaluated.
158

159 ***2.3.3 Methods comparison***

160 In order to compare the performance of the on-column affinity smart polymer
161 gel sensor with the conventional method, 10 glucose samples, prepared in distilled water so
162 that any matrix effect was minimal were first tested. Each sample was analyzed with the
163 developed sensor and the conventional dinitrosalicylic acid (DNS) method [17] based on the
164 oxidation of the aldehyde functional group present in glucose and the reduction of 3,5-
165 dinitrosalicylic acid in an alkaline solution. The absorbance of the red-brown color compound
166 of 3-amino, 5-nitrosalicylic acid was measured at 575 nm with a spectrophotometer. The
167 results analyzed by both methods were statistically tested with the Wilcoxon Signed Rank
168 technique.
169

170 ***2.3.4 Real sample analysis***

171 Three urine samples from Songklanagarind Hospital, Hat Yai, Thailand were
172 analyzed. The samples were first passed through a filter paper (Whatman® grade 1, pore size
173 11 μm, Whatman International Ltd., Maidstone, England) to remove any particulates. Urine
174 samples spiked with a series of standard solutions of glucose were prepared to produce a
175 matrix matched calibration curve to investigate the matrix effect [18]. A standard curve was
176 also prepared using standard glucose solution. The slopes of the two curves (standard and

177 matrix matched curves) were compared. If there is a significant difference between the
178 slopes, the matrix matched calibration curve will be used for the analysis of glucose in real
179 samples. The obtained concentrations of glucose in the urine samples as analyzed by the
180 affinity smart polymer gel sensor were then compared with the values obtained from the
181 hospital, analyzed using the strip test (ARKRAY AUTION^{TM} Sticks, ARKRAY, Inc. Kyoto,
182 Japan), and the recoveries were calculated followed the EURACHEM guideline [18].
183

184 **3. Results and discussion**

185 ***3.1 Affinity smart polymer gel morphology***

186 The morphology of the affinity smart polymer gel as observed by scanning electron
187 microscopy (SEM) (JSM 5200 microscope, JEOL, USA) showed a porous three dimensional
188 network (Fig. 1c). This connected network of pores and channels allows fluid to flow through
189 the gel inside the capillary tube. This porous network also provides a high surface area for the
190 binding between glucose and phenylboronic acid.
191

192 ***3.2 Optimization of the on-column affinity smart polymer gel sensor***

193 *3.2.1 Flow injection system*

194 To obtain the best performance, parameters affecting the binding between the gel and
195 glucose in the flow injection system were optimized as follows.

196 *3.2.1.1 Concentration and pH of regeneration solution*

197 Since glucose prefers to bind with borate in the charged form rather than with
198 its neutral form, in the acidic pH range a reversible reaction will occur and glucose will be
199 rapidly released from the binding. To obtain the quickest dissociation of the binding between
200 glucose and phenylboronic acid, the regeneration solution was optimized. The concentrations
201 of phosphate buffer used for the regeneration were investigated at 15, 20, 25 and 30 mM,

each with pH values of 5.00, 5.50, 6.00, 6.50 and 7.00. The initial conditions were: carrier buffer 10 mM phosphate buffer at pH 7.30, sample volume 200 μL and sample flow rate 0.05 mL min^{-1}.

Neither the concentration nor the pH had a significant effect on the peak height but had some effect on the analysis time. When the pH was decreased from 7.00 to 5.00, the dissociation time (time from point "1" to "2" of signal *b* in Fig. 2 inset) between glucose and phenylboronic acid decreased from a few minutes to a few seconds while the return to the baseline time increased. In the case of the concentration, the higher the concentration the shorter was the time to return to baseline. For example, at 15 mM pH 5.50, the return to baseline time (time from point "2" to "3" of signal *c* in Fig. 2 inset) is 10 minutes, whereas it only took 5 minutes for the 20 mM to 30 mM buffer. Therefore, a 20 mM regeneration phosphate buffer at pH 5.50 was used for further optimization since it provided a relatively fast dissociation time (15 seconds) and a short time to return to the baseline (5 minutes).

3.2.1.2 Concentration and pH of carrier buffer

The concentrations of carrier phosphate buffer were investigated at 10, 15, 20 and 25 mM each with pH ranges from 7.00 to 8.00, at 0.25 intervals of pH. The optical density change increased with buffer concentration from 10 mM to 15 mM for all studied pHs then leveled off for higher concentrations, whereas the optical density change increased with pH for all concentrations (Fig. 3). This is because in the basic range phenylboronic acid is mostly in the charged form favoring glucose binding. For all concentrations, when the pH was increased from 7.50 to 7.75, the optical density increased by 3.8±0.3 times while the increase of pH from 7.75 to 8.00 increased the optical density change by only 1.3±0.1 times. Therefore, 15 mM phosphate buffer pH 7.75 was chosen for further experiments.

227 *3.2.1.3 Sample volume and carrier flow rate*

228 Sample volumes of 100, 200, 300 and 400 μL and a carrier buffer flow rate of
229 0.02 to 0.10 mL min^{-1} (0.02 mL min^{-1} interval) were then simultaneously optimized. For all
230 sample volumes, the smaller the flow rate the higher and broader was the peak. The analysis
231 time was also longer. For example, the signal from a 100 μL volume at 0.02 mL min^{-1} was
232 15.5±0.2 mV with an analysis time of 11 minutes whereas for 0.10 mL min^{-1} the signal was
233 only 6.5±0.3 mV with a 2 minute analysis time. This is because at a slower flow rate, glucose
234 has a longer time to be retained and to interact with the phenylboronic acid. For a larger
235 sample volume, the signal was higher with a longer analysis time. For example, at a 0.10 mL
236 min^{-1} flow rate , when the sample volume was increased from 100 μL to 400 μL, the signal
237 increased from 6.5±0.3 mV (2 minutes) to 14.9±0.7 mV (5 minutes), respectively. Creating
238 the right balance between a high signal and a short analysis time, a sample volume of 300 μL
239 and a flow rate of 0.08 mL min^{-1} were selected for further use.

240 All optimized parameters and their optimum values are summarized in Table
241 1. With these conditions the analysis of glucose can be complete within 6 minutes and a
242 signal of 15.6±0.3 mV from a glucose concentration of 10.0 mM was obtained.

243

244 *3.2.2 Internal diameter of the on-column affinity smart polymer gel sensor*

245 The effect of the I.D. of the capillary tube (0.25 mm, 0.32 mm and 0.53 mm) on the
246 sensitivity (slope of the calibration curve) of the on-column affinity smart polymer gel
247 glucose sensor is shown in Fig. 4. The wider capillary tube provided a higher sensitivity. This
248 was because the larger tube can accommodate more affinity smart polymer gel that can
249 interact with glucose and result in a higher optical density change. In addition, the wider tube
250 also provided a longer path length for the light resulting in an increase in the sensitivity
251 according to Beer's law.

252 However, when the glucose standard solution higher than 8.0 mM was injected into
253 the 0.53 mm capillary tube, the signal leveled off from the linear range at a rather low
254 glucose concentration (10.0 mM) (Fig. 4). During this time, some gel was observed to be
255 emerging from the tube at the outlet of the flow injection system. This might be because the
256 affinity smart polymer was only maintained inside the tube by physical adsorption without
257 any cross-linking agent, and the polymeric network of the gel formed inside the capillary tube
258 is physical cross-linked during polymerization [13, 19]. Therefore, with a wider tube, it is
259 likely that the gel in the middle could be removed from the capillary tube during the swelling
260 especially at higher concentration of glucose when there is more swelling. After some of the
261 coated gel was removed, the optical density change did not increase with glucose
262 concentration resulting in a narrower linearity and higher RSDs. Even though the 0.53 mm
263 I.D. provided the highest sensitivity, in order to maintain the repeatability of the signal at the
264 higher concentration, the 0.32 mm I.D. capillary was selected for further investigations.
265
266 ***3.3 Analytical performance and method validation***
267 *3.3.1 Linearity and limit of detection*
268 Using the optimum conditions (Table 1), the linearity of the on-column affinity smart
269 polymer gel sensor was evaluated by injecting glucose standard solutions from 0.5 mM to 18
270 mM (Fig. 2). The optical density changes were plotted against the concentrations of glucose
271 (data not shown). The linear dynamic range was found to be between 0.5 and 16.0 mM
272 [$y(mV) = (7.1\pm0.2)\ x(mM) - (4\pm2)$, $R^2 = 0.994$] with a limit of detection of 0.5 mM ($S/N \geq 3$).
273 These detection range and detection limit are comparable to some recent glucose sensors
274 based on phenylboronic acid [20-22]. However, the method proposed in this work is easy; the
275 detection of glucose is simple and possible to be carried out automatically.
276

277 *3.3.2 Intra- and inter-day precisions*

278 The sensor precision was evaluated within a single day (intraday precision) and on
279 three consecutive days (interday precision). On each day, 15 repetitive injections of an 8.0
280 mM standard glucose solution (midpoint of the linear dynamic range) were performed with
281 the same coated gel. For the intra-day precision, the relative standard deviations were 3.7%,
282 1.5% and 2.5% for the first, second and third days, respectively. The inter-day precision was
283 then calculated based on the same data as the intra-day precision and the relative standard
284 deviation of 2.8% was obtained [23]. Both sets of these values indicated that the on-column
285 affinity smart polymer gel sensor provided acceptable precision as recommended by AOAC
286 (RSD of 3.7% at the concentration of 8.0 mM glucose) [24].

287

288 *3.3.3 Operational stability of the smart polymer gel sensor*

289 The operational stability was studied for the number of injections that a single coated
290 affinity smart polymer gel could be used. The standard solution of glucose (8.0 mM, the
291 middle point of the linearity) was consecutively injected into the flow system. Peak heights
292 obtained from each injection were converted into a percentage response based on the 100%
293 response of the first injection. The plot between percentage responses against the number of
294 injection (Fig. 5) showed that for the affinity smart polymer gel the average response for 250
295 injections was 98±5%. With this injection numbers, the responses are within ±10% (t_{L10}) of
296 the signal from the first injection [23]. The reduction of the signal was only 0.036% for each
297 injection showing a very good repeatability with a relative standard deviation of 5%. This
298 250-times-injection was therefore considered to allow for enough use for each sensor and to
299 finish at least one set of experiments. In addition, the proposed sensor does not need to be
300 stored at 4°C as is always the case for an enzyme based sensor. As reported in the previous

301 work, a freshly prepared affinity smart polymer gel can be used every day for at least one
302 month without changing its sensitivity [14].
303
304 *3.3.4 Tube-to-tube coating reproducibility*
305 The tube-to-tube coating reproducibility of the affinity smart polymer gel was studied.
306 Six capillary tubes were coated with the same monomer mixture and initiator solution and
307 tested in the flow injection system using 1.0-16.0 mM standard solutions of glucose. The
308 sensitivity (slope of the calibration curve) obtained from each tube was compared. The results
309 in Fig. 6 showed that the sensitivities of each gel are significantly different. This might be
310 because the coating of the gel inside the capillary tube merely relies on the physical
311 adsorption on the capillary wall surface. When the capillary tube was set into the flow
312 injection system and the carrier buffer was initially passed through the gel, some parts of the
313 gel that were not directly attached to the surface might be lost with the buffer resulting in a
314 different amount of gel inside the tube leading to the different sensitivities. However, with the
315 operational stability of 250 times (reported in section 3.3.3) the different sensitivity from the
316 different coating tube will not cause any problem since at least one set of experiment can be
317 finished with the use of a single coated affinity smart polymer gel. In the case where
318 fabrication reproducibility is required, chemically attachment of the polymer gel to the
319 surface might be employed. For example the capillary surface could be silanised and then
320 cross-linked with the polymer gel. Additional cross-linking agent could also be added to
321 provide the chemical cross-linked within the polymer gel [13]. With this procedure, the
322 coating reproducibility might be improved.
323
324
325

326 *3.3.5 Selectivity of affinity smart polymer gel*

327 It is well known that phenylboronic acid is a ligand that can form a reversible
328 covalent bond to a variety of *cis*-diol containing compounds [12, 25-26]. Therefore, any
329 sensor system utilizing boronic acid ligands could be susceptible to interference from
330 competing *cis*-diol containing compounds in the sensing environment. The degree of cross-
331 reactivity of the on-column affinity smart polymer gel to other saccharides was then studied.
332 Mannose and galactose were two monosaccharides selected for this test since they have
333 similar structures to glucose. Sucrose and lactose were the two disaccharides that were also
334 used in this investigation.

335 A calibration curve was prepared for each sugar in the concentration range of 1.0 to
336 16.0 mM. Only the response to glucose provided the linear relationship from 1.0 mM to 16.0
337 mM whereas the linear dynamic ranges obtained from mannose, galactose, sucrose and
338 lactose were 4.0-16.0, 1.0-8.0, 2.0-8.0 mM and 4.0-16.0 mM, respectively. The sensitivities
339 of the on-column affinity smart polymer gel are shown in Fig. 7.

340 As can be seen the affinity smart polymer gel sensor was not selective for glucose,
341 and this agreed well with previous work [14, 26]. This sensor responded to mannose and
342 galactose with almost similar sensitivity whereas the sensitivities to sucrose and lactose were
343 very poor (almost 9 times lower). This is probably because of the steric effect caused by the
344 presence of a glycosidic bond and the fact that certain diol groups were no longer available
345 for binding [12]. Therefore, the benefit of this newly developed sensor is to be used as a
346 glucose, mannose or galactose sensor where it is known *prior* to the test that either of these
347 sugars are the only monosaccharide present , or it could be used as a monosaccharide
348 screening sensor. For example, it can be used for on-line monitoring of glucose in a
349 bioprocess or fermentation process. To improve the selectivity future work might include the

investigation of the different structure of phenylboronic acid receptor. The change of the positions and orientations of boronic acid may favor one saccharide over the other [27].

3.3.6 Comparison with dinitrosalicylic acid (DNS) method

In order to compare the performance of the on-column affinity smart polymer gel sensor with the conventional method, 10 glucose samples in the range of 5.0-50.0 mM, prepared in distilled water where the matrix effect is minimal were tested. These glucose concentrations are within the determination range of the strip test technique (2.8- 55.0 mM) [28]. Each sample was analyzed with the smart polymer gel sensor and the conventional DNS method [17]. Each sample was diluted with the different dilution factor to obtain the concentration in the linear range and analyzed by both methods. The obtained signals were converted to glucose concentrations by the linear equation of the calibration curve of each technique and multiplied by the dilution factor. The comparison of glucose concentration from both methods was shown in Fig. 8. Using the Wilcoxon Signed Rank test, it was found that there is no significant difference between the two methods at the confidence level of 95% ($P>0.05$).

3.3.7 Real sample analysis

To confirm the applicability of the on-column affinity smart polymer gel sensor for the analysis of glucose in real samples, the developed method was used to detect urine glucose. Although, the monitoring of glucose in urine cannot be used to substitute for blood glucose, it can still provide useful information when blood glucose monitoring is not available [29-30]. Three urine samples obtained from the Songklanagarind Hospital, Hat Yai, Thailand were analyzed.

374 The samples were filtered and first tested to see if there was any effect due to the
375 matrix. Filtered samples were spiked with three different concentrations of glucose to
376 produce the matrix matched calibration curve (4 calibration points including unspiked urine
377 samples with 3 replications for each concentration). The slopes of these matrix matched
378 calibration curves were compared with that of the standard curve. The significant difference
379 was determined using two ways ANOVA by R software [31]. The result showed that the
380 slopes of the matrix matched and standard curves were significantly different ($P>0.05$), and
381 clearly indicated that the matrix had some influence on the analysis. Therefore, the matrix
382 matched calibration curve was used to determine the concentration of glucose in urine
383 samples.

384 The signals from three un-spiked urine samples were 9.7, 7.2 and 9.0 mV and the
385 corresponding linear equations were $y = (5.3\pm0.2)x + (9.6\pm0.8)$, $y = (2.8\pm0.2)x + (7\pm1)$ and
386 $y = (3.1\pm0.1)x + (8.9\pm0.2)$, respectively. The concentrations of glucose in the samples were
387 calculated from the extrapolation of the y-intercept of the matrix matched calibration curve
388 obtained from each sample, and they were found to be 1.8±0.2 mM, 2.5±0.4 mM and 2.8±0.1
389 mM for samples no. 1, 2 and 3, respectively (Table 2). The results of these three samples
390 provided by the hospital using the strip test were labeled at "trace level" (Table 2) meaning
391 that the concentrations of glucose in urine samples were less than 50 mg dL^{-1} (2.8 mM)
392 which is the limit of detection of this strip test [28]. In other words, the lowest concentration
393 of glucose that can be reported by the strip test is 2.8 mM. Whereas the proposed sensor, that
394 provided a 5.6 times lower detection limit, could report the lowest concentration of glucose at
395 0.5 mM.

396 Normally the concentration of glucose in urine is between 0.0-0.8 mM and when
397 glucose blood level exceeds the renal threshold of 10 - 12 mM, it is excreted in the urine.
398 This may indicate the presence of renal glycosuria or diabetes mellitus [32]. Therefore, if the

399 method used to determine the concentration of glucose can provide more quantitative results,
400 it will provide further useful diagnostic information. From the results, the proposed sensor
401 can indicate very low quantitative concentrations that are normally identified only as "trace
402 levels" in the normal strip test.

403 To further validate the system, the recoveries were tested and calculated according to
404 the guideline from EURACHEM [18]. The concentration found in spiked urine sample was
405 subtracted with that of un-spiked sample and it was then divided with the fortified
406 concentration. The recovery was obtained by multiplying this value by a hundred. The
407 recoveries were obtained in the range of 88±2 to 103±4 (Table 2).

408

409 **4. Conclusions**

410 A simple on-column detection, non-enzymatic glucose sensor, based on the use of an
411 affinity smart polymer gel containing a chemoselective ligand, phenylboronic acid, was
412 obtained. This affinity smart polymer gel sensor provided good operational stability; a single
413 sensor can be reused up to 250 times with only a 0.036% reduction of the signal for each
414 injection and a short analysis time of 6 minutes per analysis. The limit of detection of the
415 sensor (0.5 mM) is suitable for analysis of glucose in several types of samples, such as urine,
416 human serum or cell growth media. Especially for glucose monitoring in urine, the developed
417 sensor provide more than a factor of 5 lower detection limit compared with that of the strip
418 test. Even though this affinity smart polymer gel does not display specificity to a particular
419 monosaccharide, in the case where it is known a priori that any of these would be the
420 dominant sugar, this sensor would be suitable. For example the use of this sensor to
421 determine glucose in the urine and blood as well as in some bioprocess where glucose is used
422 as the primary carbon source in cell growth media.

423

Acknowledgements

This work was supported by the Office of the Higher Education Commission and the Thailand Research Fund (TRF) granting no. MRG 5180106. Partial support from the Trace Analysis and Biosensor Research Center (TAB-RC), the Center for Innovation in Chemistry (PERCH-CIC) and the Department of Chemistry, Faculty of Science, and the Songklanakarind Hospital, Prince of Songkla University. Associate Professor Dr. Alexander E. Ivanov and Professor Dr. Bo Mattiasson are gratefully thanked for providing the opportunity for C. Thammakhet to work with this smart polymer during a research visit at the Department of Biotechnology, Lund University, Lund, Sweden. The authors would also like to thank Dr. Brian Hodgson for his help in a proof reading of the manuscript.

References

[1] X. Kang, Z. Mai, X. Zou, P. Cai, J. Mo, Analytical Biochemistry. 363 (2007) 143-150.

[2] S. Chaterji, I.K. Kwon, K. Park, Progress in Polymer Science. 32 (2007) 1083-1122.

[3] B. Peng, Y. Qin, Analytical Chemistry. 80 (2008) 6137-6141.

[4] S. Park, H. Boo, T.D. Chung, Analytica Chimica Acta. 556 (2006) 46-57.

[5] R. Wilson, A.P.F. Turner, Biosensors and Bioelectronics. 7 (1992) 165-185.

[6] J. Zhao, L. Wu, J. Zhi, The Analyst. 134 (2009) 794-799.

[7] E. Miller, M. Przybyt, Fresenius' Journal of Analytical Chemistry. 363 (1999) 103-107.

[8] S. Takahashi, J.-i. Anzai, Langmuir. 21 (2005) 5102-5107.

[9] G. Zenkl, I. Klimant, Microchimica Acta. 166 (2009) 123-131.

[10] K. Kataoka, H. Miyazaki, M. Bunya, T. Okano, Y. Sakurai, Journal of the American Chemical Society. 120 (1998) 12694-12695.

449 [11] S. Kabilan, J. Blyth, M.C. Lee, A.J. Marshall, A. Hussain, X.P. Yang, C.R. Lowe,
450 Journal of Molecular Recognition. 17 (2004) 162-166.
451 [12] M.C. Lee, S. Kabilan, A. Hussain, X. Yang, J. Blyth, C. R. Lowe, Anaytical
452 Chemistry. 76 (2004) 5748-5755.
453 [13] M.V. Kuzimenkova, A.E. Ivanov, C. Thammakhet, L.I. Mikhalovska, I.Y. Galaev, P.
454 Thavarungkul, P. Kanatharana, B. Mattiasson, Polymer. 49 (2008) 1444-1454.
455 [14] A.E. Ivanov, C. Thammakhet, M.V. Kuzimenkova, P. Thavarungkul, P. Kanatharana,
456 L.I. Mikhalovska, S.V. Mikhalovsky, I.Y. Galaev, B. Mattiasson, Journal of
457 Molecular Recognition. 21 (2008) 89-95.
458 [15] I. Hisamitsu, K. Kataoka, T. Okano, Y. Sakurai, Pharmaceutical Research, 14 (1997)
459 289-293.
460 [16] A.E. Ivanov, H. Larsson, I.Y. Galaev, B. Mattiasson, Polymer, 45 (2004) 2495-2505.
461 [17] G.L. Miller, Analytical Chemistry. 31 (1959) 426-428.
462 [18] Guide EURACHEM, The Fitness for Purpose of Analytical Methods: A Laboratory
463 Guide to Method Validation and Related Topics, 1998. http://www.eurachem.org.
464 [19] T. Hoare, R. Pelton, Macromolecules. 40 (2007) 670-678.
465 [20] W. Wu, T. Zhou, M. Aiello, S. Zhou, Biosensors and Bioelectronics. 25 (2010) 2603-
466 2610.
467 [21] G. Ye, X. Wang, Zhou, Biosensors and Bioelectronics. 26 (2010) 772-777.
468 [22] A. Tiwari, D. Terada, C. Yoshikawa, H. Kobayashi, Talanta. 82 (2010) 1725-1732.
469 [23] I. Taverniers, M. De Loose, E. Van Bockstaele, TrAC, Trends Anal. Chem. 23 (2004)
470 535-552.
471 [24] AOAC International, Method Validation Programs (OMA/PVM Department),
472 including Appendix D: Guidelines for collaborative study procedures to validate
473 characteristics of a method of analysis, 2000. http://www.aoac.org/vmeth/
474 devmethno.htm.

475 [25] W. Wang, X. Gao, B.Wang, Current Organic Chemistry. 6 (2002) 1285-1317.

476 [26] J.P. Lorand, J.O. Edwards, Journal of Organic Chemistry. 24 (1959) 769-774.

477 [27] K.E.S. Dean, A.M. Horgan, A.J. Marshall, S. Kaliban, J. Pritchard, Chemical
478 Communications. (2006) 3507-3509.

479 [28] Urine test AUTION^{TM} Sticks. retrieved on September 10, 2010. http://www.
480 arkray.co.jp/english/products/urinalysis.html.

481 [29] Position Statement - Urine Glucose Monitoring, International Diabetes Federation.
482 retrieved on July 4, 2010. http://www.idf.org/Position_statementsurine_monitoring.

483 [30] M. Miyashita, N. Ito, S. Ikeda, T. Murayama, K. Oguma, J. Kimura, Biosensors and
484 Bioelectronics. 24 (2009) 1336-1340.

485 [31] R Development Core Team, R: A language and Environment for Statistical
486 Computing, R Foundation for Statistical Computing, Vienna, Austria, 2006.

487 [32] Glucose in urine, Home test kits by Sanitoets, retrieved on July 4, 2010. http://www.
488 anytestkits.com/utk-glucose-in-urine.htm.

489

490

491

492

493

494

495

496

497

498

499

500 **Figure captions**

501

502 **Fig. 1** Schematic diagram showing the on-column affinity smart polymer gel glucose sensor
503 flow injection system (a), pictures of the detection cell and the coated smart polymer gel
504 capillary with the detection window in the middle (b) and a scanning electron microscopy
505 image at 1500× magnification of the gel (c).

506

507 **Fig. 2** Characteristics of the sensor signals at various concentrations of glucose. Inset; the
508 optical density change after (a) 6.0 mM glucose was injected, (b) regeneration solution was
509 injected to the flow injection system, (c) pH adjusted to its original value. Analytical
510 conditions: sample flow rate 0.08 mL min^{-1}, sample volume 300 μL, carrier buffer 15 mM
511 phosphate buffer pH 7.75, regeneration solution 20 mM phosphate buffer pH 5.50, detection
512 wavelength 500 nm. The time taken between points 1 and 2 is the dissociation time and
513 between point 2 and 3 is the return to baseline time.

514

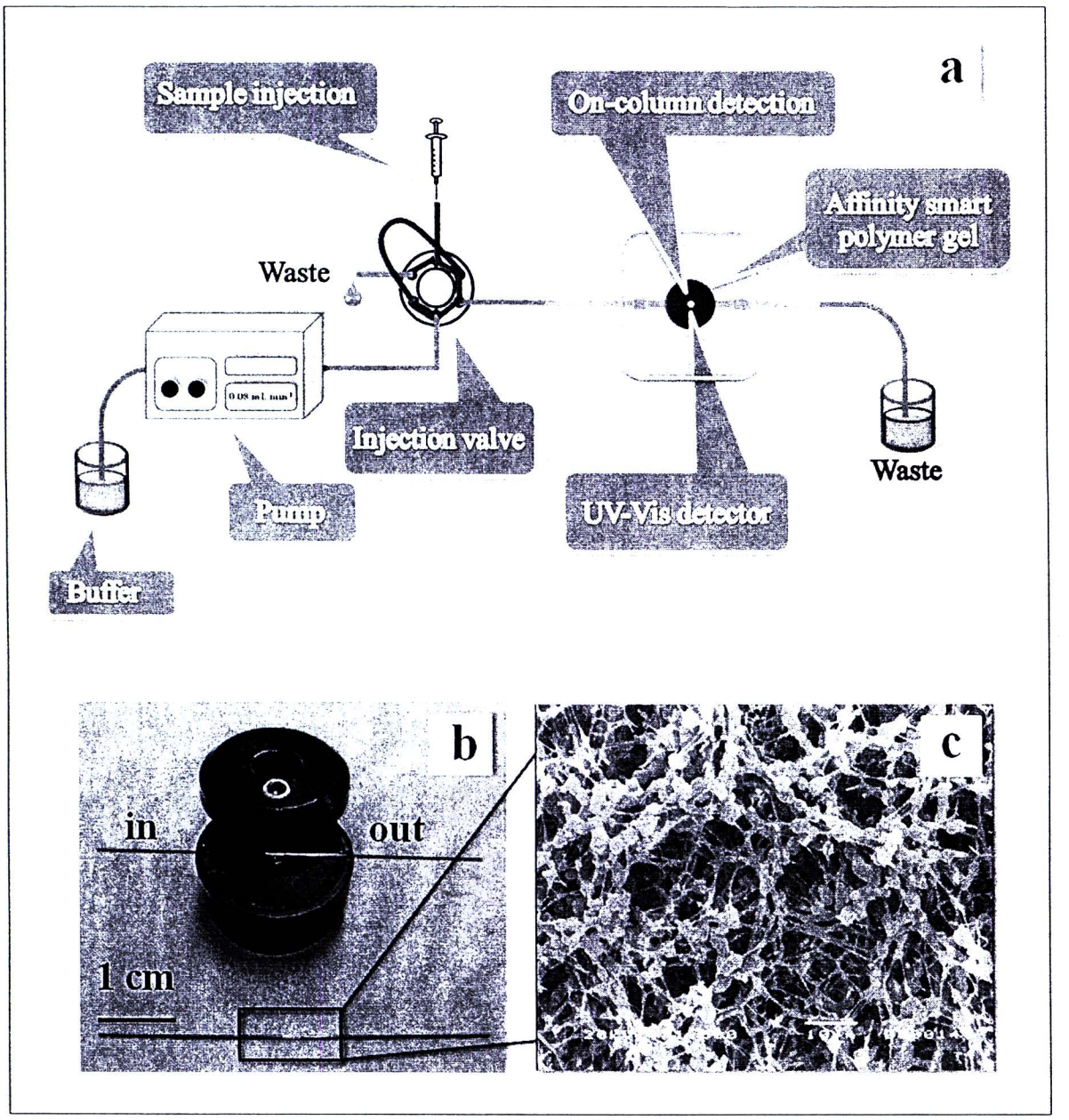
515 **Fig. 3** The effect of pH and concentration of carrier phosphate buffer on the signal of the on-
516 column affinity smart polymer gel glucose sensor. Analytical conditions: sample flow rate
517 0.05 mL min^{-1}, sample volume 200 μL, regeneration solution 20 mM phosphate buffer pH
518 5.50, glucose concentration 8.0 mM, detection wavelength 500 nm.

519

520 **Fig. 4** The effect of the internal diameter (I.D.) of the capillary tube on the sensitivity of the
521 on-column glucose sensor. Three replications were done for each point of the calibration
522 curves. Analytical conditions: sample flow rate 0.08 mL min^{-1}, sample volume 300 μL,
523 carrier buffer 15 mM phosphate buffer pH 7.75, regeneration solution 20 mM phosphate
524 buffer pH 5.50, detection wavelength 500 nm.

525 **Fig. 5** The operational stability of the affinity smart polymer gel. Analytical conditions:
526 sample flow rate 0.08 mL min^{-1}, sample volume 300 μL, carrier buffer 15 mM phosphate
527 buffer pH 7.75, regeneration solution 20 mM phosphate buffer pH 5.50, glucose
528 concentration 8.0 mM, detection wavelength 500 nm.
529
530 **Fig. 6** The sensitivities of six different coated affinity smart polymer gel sensors prepared
531 from the same coating solution. Three replications were done for each point of the calibration
532 curves. Analytical conditions: sample flow rate 0.08 mL min^{-1}, sample volume 300 μL,
533 carrier buffer 15 mM phosphate buffer pH 7.75, regeneration solution 20 mM phosphate
534 buffer pH 5.50, detection wavelength 500 nm.
535
536 **Fig. 7** The sensitivities of the on-column affinity smart polymer gel for various saccharides.
537 Three replications were done for each point of the calibration curves of each saccharide.
538 Analytical conditions: sample flow rate 0.08 mL min^{-1}, sample volume 300 μL, carrier buffer
539 15 mM phosphate buffer pH 7.75, regeneration solution 20 mM phosphate buffer pH 5.50,
540 detection wavelength 500 nm.
541
542 **Fig. 8** Comparison of glucose concentrations in samples obtained from the affinity smart
543 polymer gel sensor and DNS method (mean±SD, n=3)
544
545
546
547
548
549

550 **Fig. 1**



551

552

553

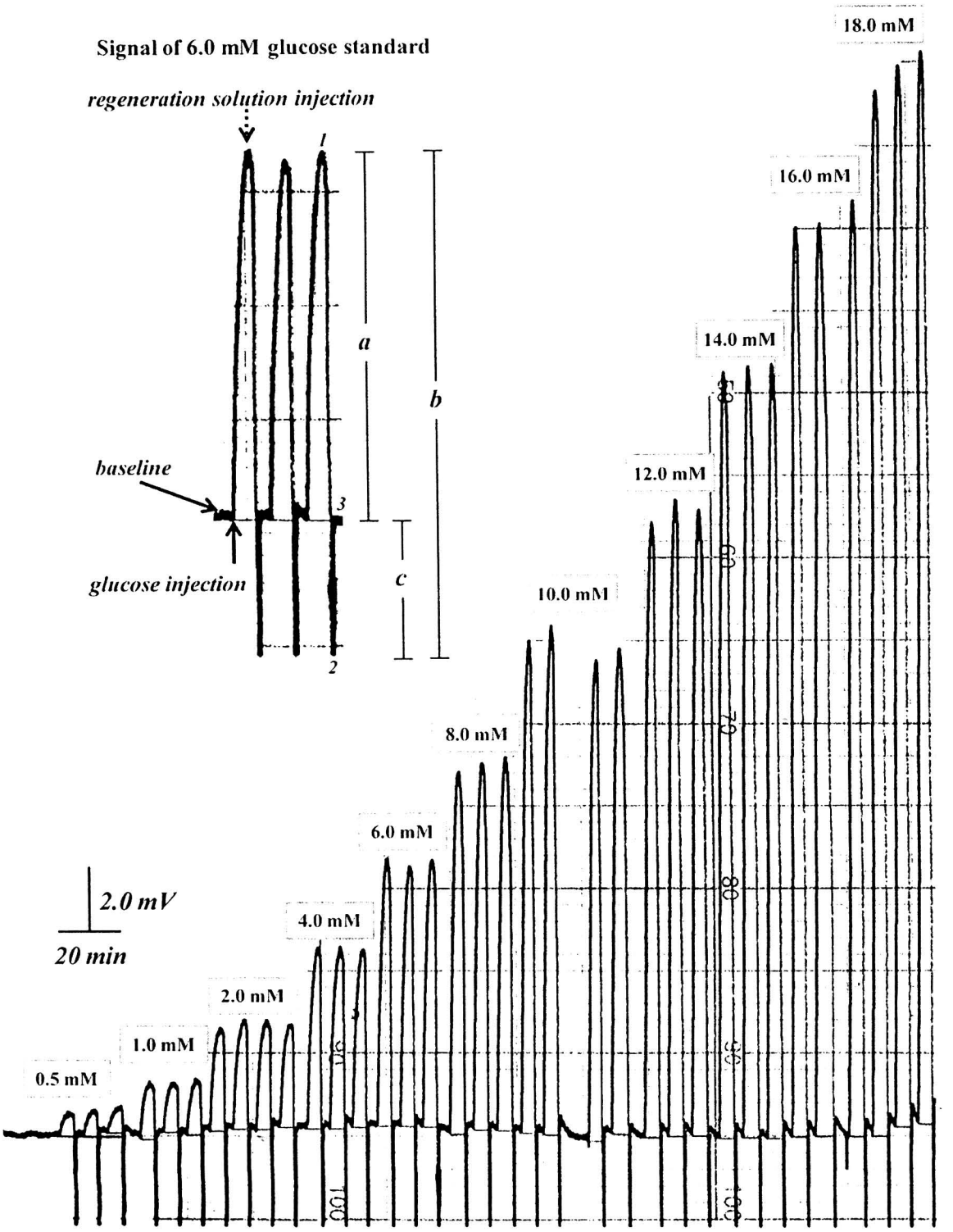
554

555

556

557

558 **Fig. 2**

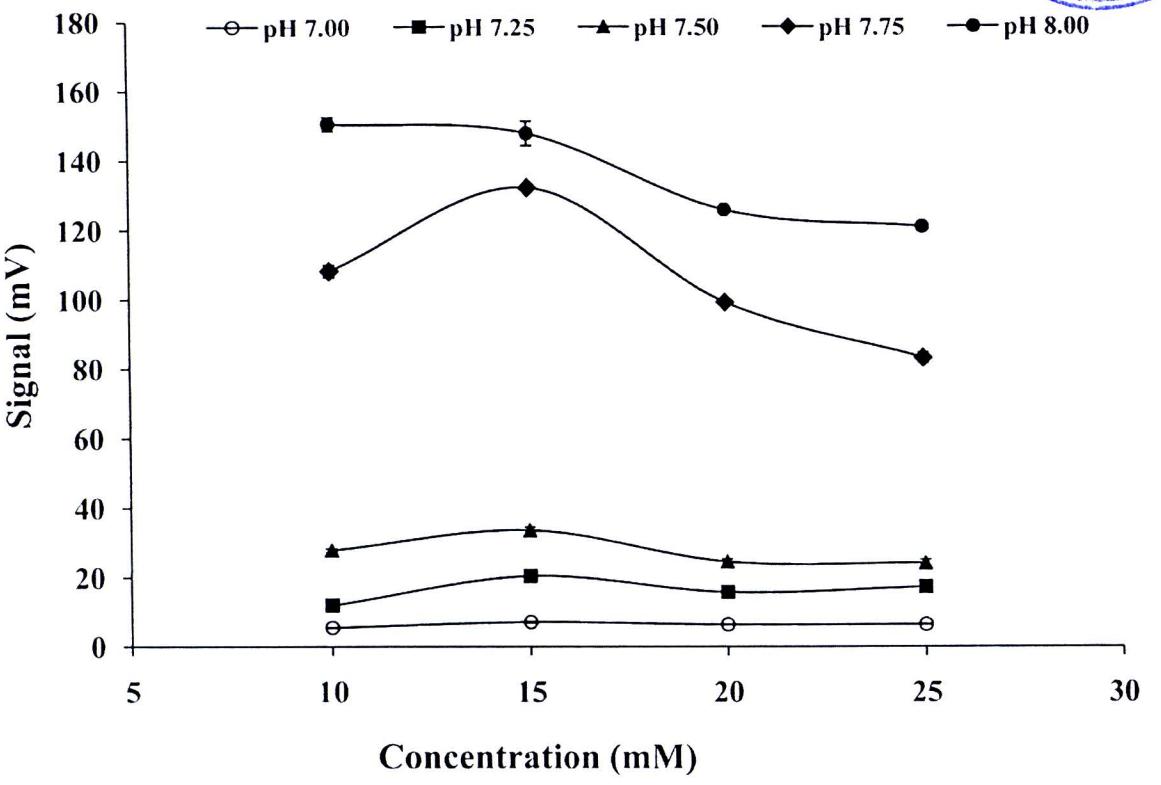


559

560

561

562 **Fig. 3**



563

564

565

566

567

568

569

570

571

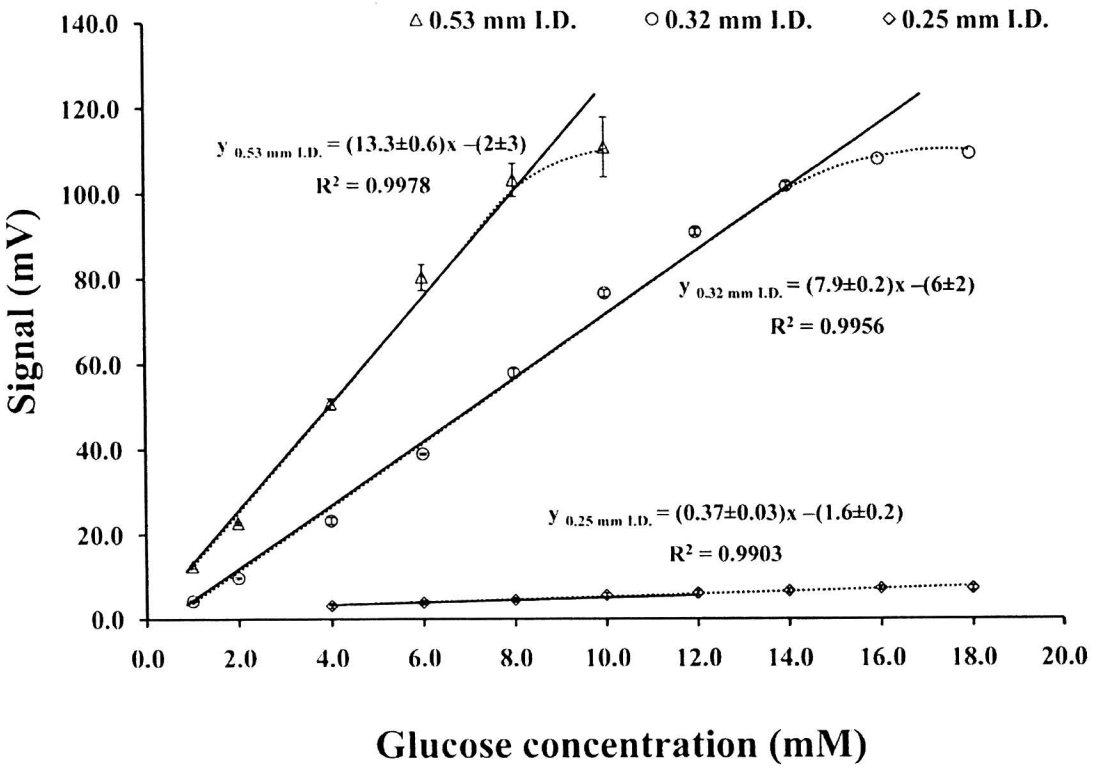
572

573

574 Thammakhet *et al.*

575

576 **Fig. 4**



577

578

579

580

581

582

583

584

585

586

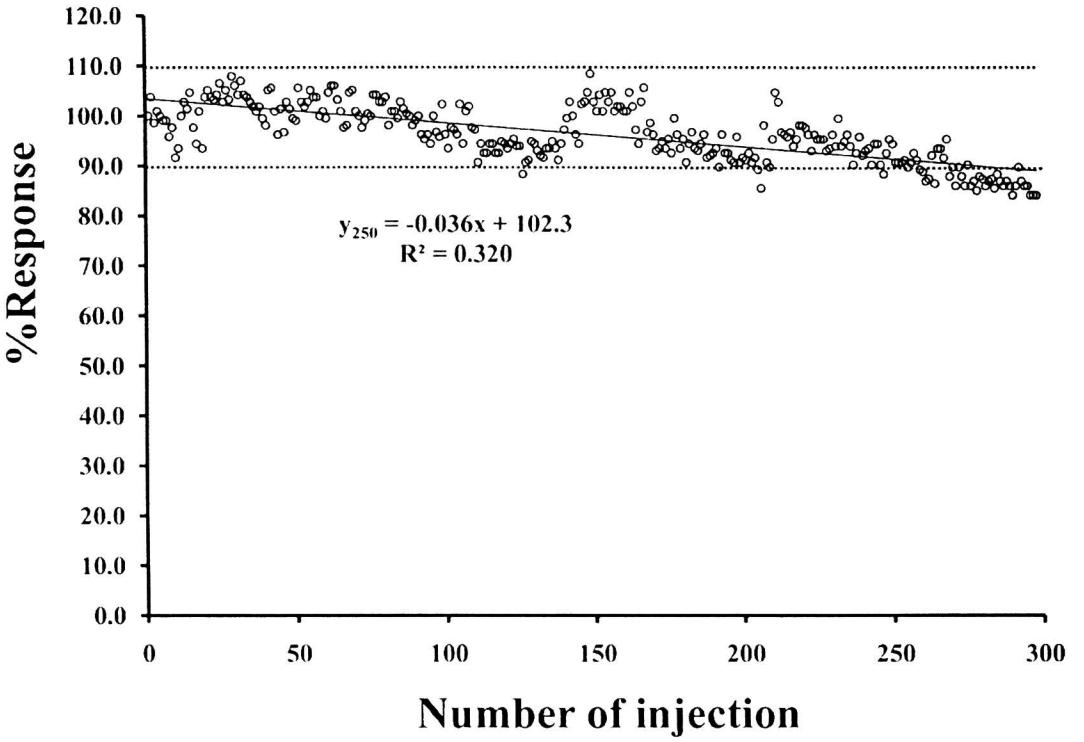
587

588

589

590 **Fig. 5**

591



592

593

594

595

596

597

598

599

600

601

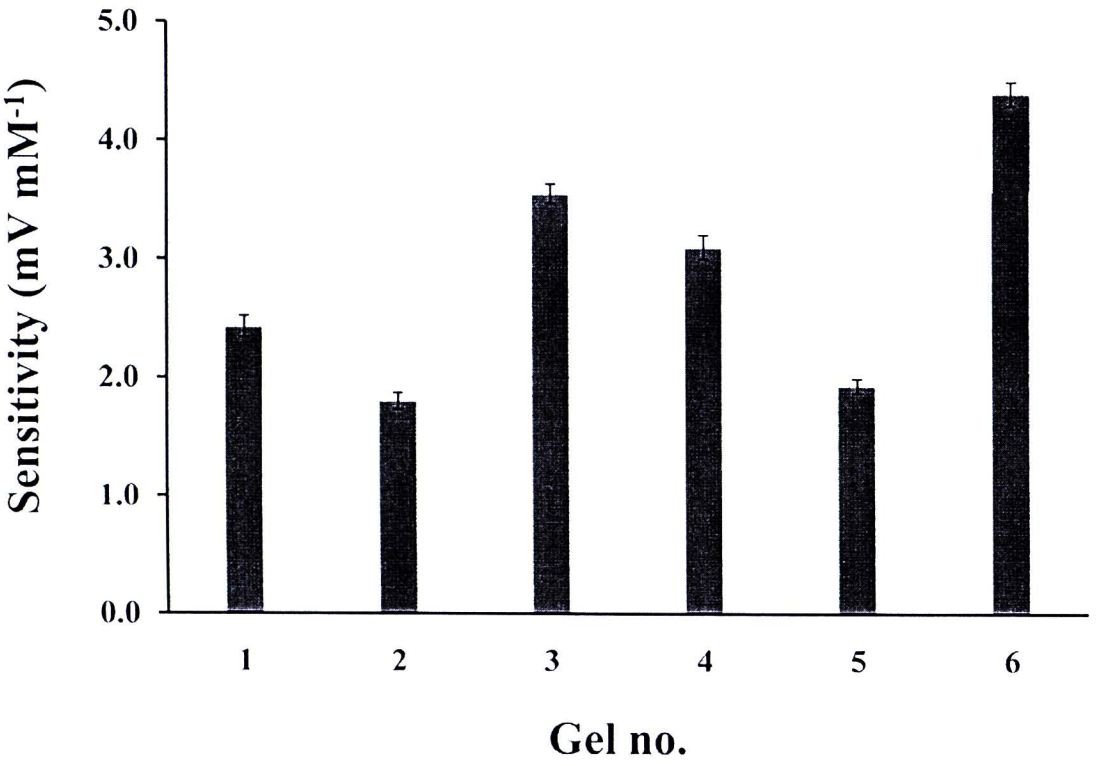
602

603

604

605 **Fig. 6**

606



607

608

609

610

611

612

613

614

615

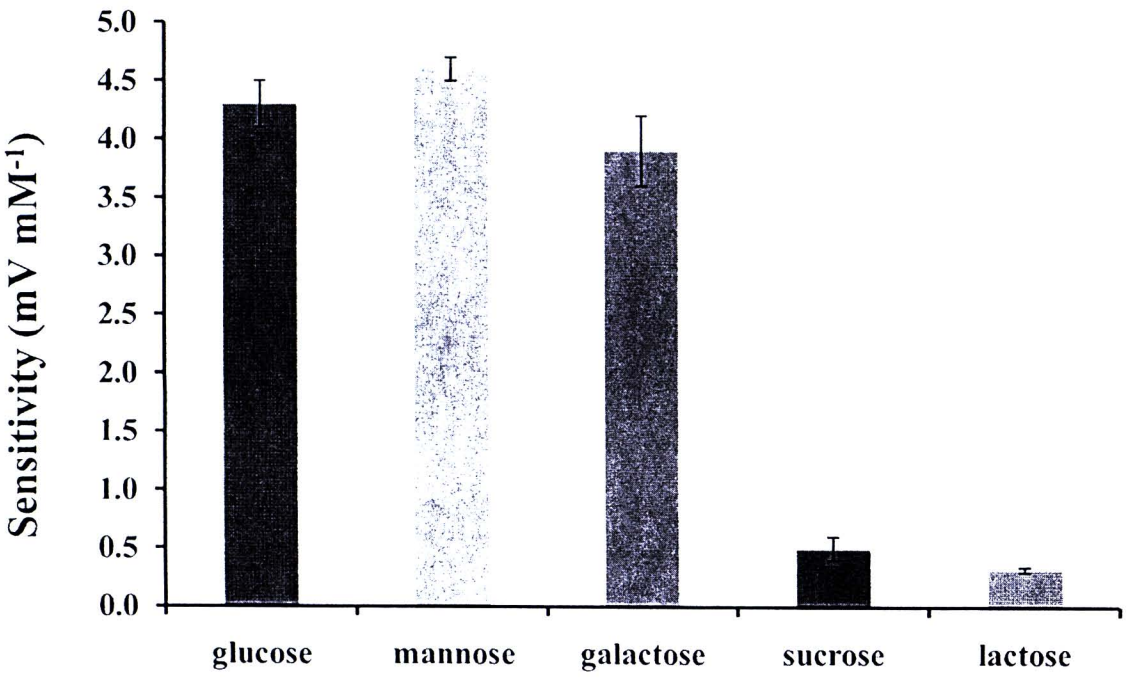
616

617

618

619 **Fig. 7**

620



621

622

623

624

625

626

627

628

629

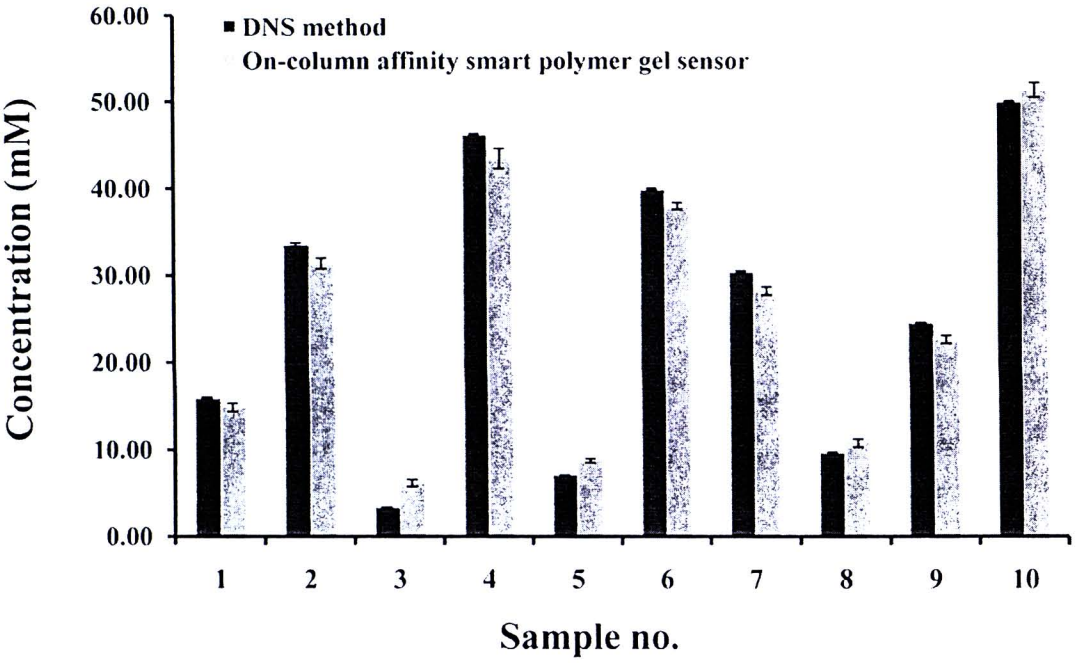
630

631

632

633

634 **Fig. 8**



635

636

637

638

639

640

641

642

643

644

645

646

647

648

649

650 **Table 1** Optimization of parameters for measuring glucose the on-column affinity smart
651 polymer gel flow injection system

Parameter	Studied range	Optimum value
Phosphate as carrier buffer		
concentration (mM)	10-25	15
pH	7.00-8.00	7.75
Phosphate as regeneration buffer		
concentration (mM)	15-30	20
pH	5.00-7.00	5.50
Sample volume (μL)	100-400	300
Flow rate (mL min^{-1})	0.02-0.10	0.08

652
653
654
655
656
657
658
659
660
661
662
663
664

665 **Table 2** The results from the analysis of three urine samples compared with that from the
666 strip test provided by the hospital

Sample no.	Concentration of glucose (mM)		%Recovery at spiked concentration of glucose[a] (mM)		
	Affinity smart polymer gel sensor[a]	Strip test	2.0	4.0	8.0
1	1.8±0.2	trace level[b]	103±4	99±1	100±6
2	2.5±0.4	trace level[b]	98±9	95±10	88±2
3	2.8±0.1	trace level[b]	91±5	96±2	99±6

667 [a] n =3

668 [b] Less than 50 mg dL^{-1} or 2.8 mM of glucose

669

670

671

672

673

674

675

676

677

678

679

680 Thammakhet *et al.*

681