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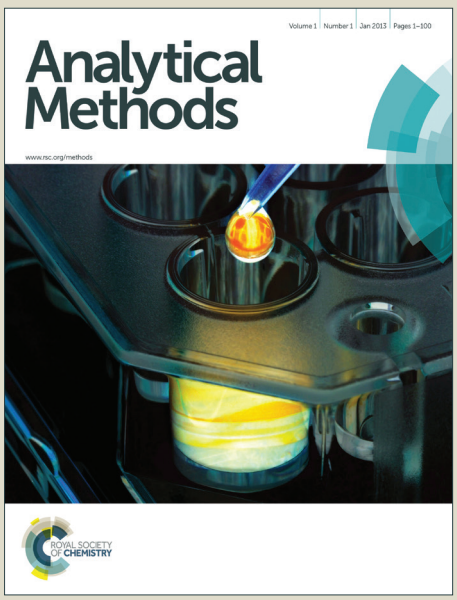

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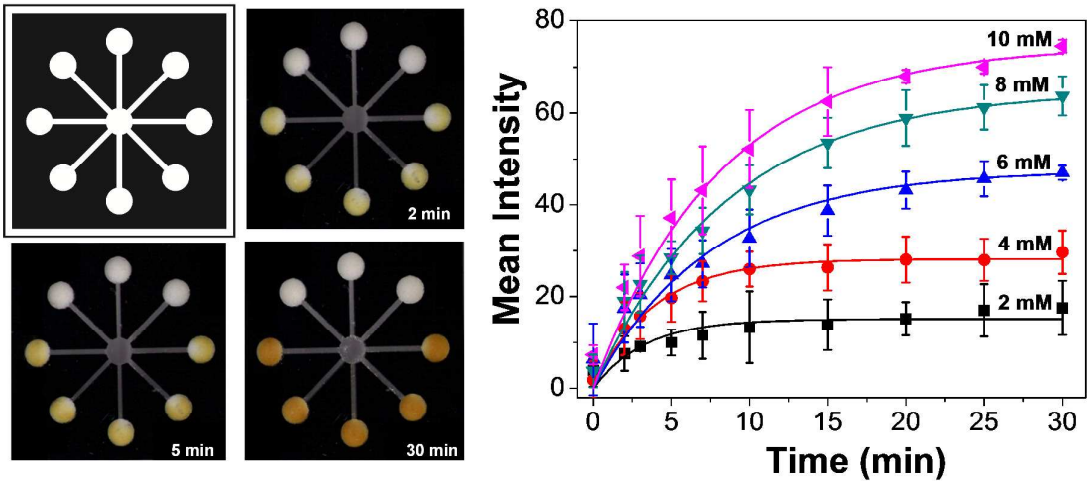
Graphical abstract

This report describes a kinetic study for the glucose oxidase reaction on microfluidic toner-based analytical devices based on digital image analysis.



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ARTICLE

Kinetic study of glucose oxidase on microfluidic toner-based analytical devices for clinical diagnostics with image-based detection

Karoliny Almeida Oliveira,[a] Paula Beatriz Medrado e Silva,[a] Fabrício Ribeiro de Souza,[a] Felipe Terra Martins[a] and Wendell Karlos Tomazelli Coltro[a,b,*]

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This report describes for the first time a kinetic study for the glucose oxidase reaction on microfluidic toner-based analytical devices (μTADs) based on colorimetric measurements performed by a scanner. μTADs were fabricated by a laser-printing process in a format defined by eight detection zones interconnected by microfluidic channels and one central zone to sample inlet (SI). Detection zones were filled with a cellulose paste and then spotted with potassium iodide and a mixture of glucose oxidase-horseradish peroxidase enzymes. Assays were performed by adding 60 μL of glucose or artificial serum sample solutions to the SI zone, which promoted the quick distribution of the sample through microfluidic channels by capillary action towards the detection zones. The hydrogen peroxide produced in the enzymatic reaction was monitored during a period between 0 and 30 minutes for five initial glucose concentrations ranging from 2 to 10 mM. The values achieved for the Michaelis-Menten constant (K_m), the turnover number (K_{cat}) and maximum reaction rate (V_{max}) were 8.9 ± 1.3 mM, 41.2 ± 14.0 s^{-1} and 19.8 ± 6.7 mM min^{-1}, respectively. The kinetic parameters found on μTADs with digital images are in agreement with the data found by other detection methods already reported in literature. The optimum reaction time achieved by the kinetic study has allowed to quantitatively determining the glucose concentration levels in serum samples with accuracy higher than 95%. Based on the proof of clinical suitability of the microfluidic platform described herein, we believe that kinetic studies of other enzymatic assays associated with clinical diagnostics can be similarly explored to provide a full knowledge of biochemical reactions in living systems.
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Introduction

The remarkable advances in the research field related to the micro total analysis systems (μTAS) have led to the development of a wide range of applications including diagnostic assays, biosensing, and drug discovery[1,2]. The impressive growing seen in literature is associated with the advantages provided by the miniaturization like low sample consumption, short analysis time, minimal waste generation and others[1-3].

Concerning the clinical interests, some diagnostic assays are carried out with specific enzymes that catalyze biochemical reactions in living systems[4,5]. The profile of many enzymatic assays is often described by the Michaelis-Menten model allowing to determine the most important reaction kinetic parameters[4]. The enzyme assay kinetics is conventionally studied by UV-visible spectrophotometry, which requires higher amount of reagents and samples when compared to chip-based assays[6,7]. Furthermore, the analysis of enzymatic kinetics using conventional techniques is time-consuming once it requires a large number of individual experiments[8].

Due to the attractive features offered by μTAS, different authors have reported kinetic studies in microfluidic devices fabricated on glass[6,9,10] or polymer[5,11-13] platforms using fluorescence[6,12], bioluminescence[11] or amperometric[5,9,10,13] detectors. Microfluidic toner-based analytical devices (μTADs) have emerged as one of the cheapest and simplest platforms for bioassays on point-of-care testing (PoC)[13]. This substrate is composed of a toner layer laser printed on a polyester surface. The printed layout can be laminated with single or multiple films to create microfluidic structures in a matter of minutes. μTADs were proposed around ten years ago[14] and their use in clinical applications have been recently demonstrated. Some examples involve the use of electrophoresis microchips and printed microzones for the detection of C-reactive protein[15] and dengue virus biomarkers[16], respectively. In addition, μTADs have been also explored to demonstrate the simultaneous detection of glucose, cholesterol and protein in human serum samples[17]. In this platform, the sample is distributed towards the detection zones by capillary action through laser-printed microfluidic channels previously defined by three polyester films and two toner layers. The multilayer microfluidic device

ensures the spontaneous transport of the sample under a flow rate estimated in ca. 6.0 mm/s. The amount of sample delivered to the detection zones by capillary action has been enough to promote a uniform color development at the whole zone[17].

The use of μTADs for clinical studies has been recently reported based on digital images captured with cell-phone camera[16] or desktop scanner[17]. The major advantages of using digital-imaging methods are regarded to their simplicity, portability and, mainly, global affordability. In addition, the use of image-based detection in association with disposable microfluidic platforms has received considerable attention due to the capability of performing clinical assays in the PoC without benchtop equipment[17-28]. However, it is important to note that the colorimetric detection of enzymatic assays is time-dependent, i.e., the color intensity changes according to the reaction time. For this reason, the analytical reliability of colorimetric measurements for clinical purposes requires a systematic study to understand the enzymatic kinetics yielding precise and accurate diagnostics in biological fluids.

In this context, this paper describes for the first time a kinetic study for the GOx reaction on μTADs based on colorimetric measurements in order to demonstrate the clinical feasibility of the proposed approach for the determination of glucose concentration levels in serum samples. The colorimetric detection of glucose involves its conversion to gluconic acid and hydrogen peroxide catalysed by the glucose oxidase (GOx) enzyme. This reaction occurs in the presence of horseradish peroxidase (HRP) which catalyses the reduction of the hydrogen peroxide and the oxidation of iodide to molecular iodine. The enzymatic reactions promote changes in the color from colorless to brown[19,29]. As proof-of-concept, the kinetic parameters (Michaelis-Menten and catalytic constants and maximum reaction rate) for the GOx reaction were determined and compared to the values found with other detection methods reported in literature. This enzyme assay has been chosen as model due to its clinical feasibility regarded to the quantification of glucose in biological samples, being therefore useful in diagnostics of diabetic patients[10].

Experimental Section

Materials, Chemicals and Samples

Cellulose powder, GOx (138 U/mg), D-glucose, HRP (73 U/mg), potassium iodide, sodium monohydrogen phosphate, and sodium dihydrogen phosphate were acquired from Sigma Aldrich Co. (Saint Louis, MO, USA). Polyester sheets (A4 size, model CG 3300) and toner cartridge (model CB435A) were purchased from 3M (São Paulo, SP, Brazil) and Hewlett-Packard (Palo Alto, CA, USA), respectively. The artificial serum samples (levels I and II) were obtained from Doles Reagentes (Goiânia, GO, Brazil). All chemicals were used as received without further purification.

Fabrication of the toner-based microfluidic devices

μTADs were fabricated in multilayers according to a procedure recently reported[17] and depicted in Figure 1. Briefly, the device layout and its mirror image were drawn in Corel Draw software version 13 (Corel Corporation, Ottawa, Canada) and printed on a polyester film (see steps I and II in Fig. 2a) using a laser printer model P1102w with 1200-dpi resolution (Hewlett-Packard LaserJet, Palo Alto, CA, USA).

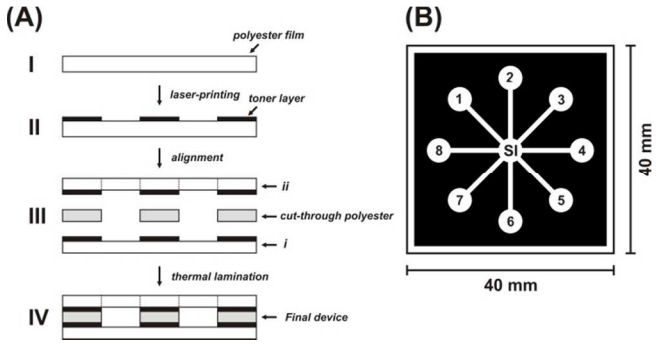

Figure 1. Representation of (A) the fabrication scheme of μTADs and (B) the layout design used to investigate the enzymatic reaction kinetics. In (A), the labels *i* and *ii* mean the printed and its perforated mirror layout, respectively. In (B), the zones labelled from 1 to 3 and from 4 to 8 indicate the detection zones for control and glucose assays, respectively. Also in (A), the label SI means the sample inlet zone.

A cut-through polyester film previously prepared in a local cutting service with a laser engraver (Ordones Laser Ltda, Goiânia, GO, Brazil) was inserted between both printed images to enhance the channel depth. Before laminating, all zones in the upper piece of polyester film were perforated with a paper punch to allow access to the microfluidic channels. The three polyester pieces were then aligned (step III in Fig. 1a) and thermally laminated at 150°C under a rate of 50 cm/min. The fabrication time to obtain the final device (step IV in Fig. 1a) is lower than 10 min. The layout of the proposed μTADs (40 mm × 40 mm) consisted of eight test zones (labelled from 1-8) interconnected by microfluidic channels and one central zone to sample inlet (SI) (see Fig. 1b). All channels were 10-mm long, 1-mm wide, and *ca.* 110-μm deep.

Colorimetric detection

The images were captured with the scanner mode of a DeskJet multifunction printer (Hewlett-Packard, model F4280) using a 600-dpi resolution. In order to investigate the enzyme reaction kinetics, all images were scanned from 0 to 30 min after adding glucose in SI zone. The recorded images were converted to a 8-bit grayscale in CorelPhoto-Paint software and the arithmetic mean of pixel intensity within each test zone was used to quantify the colorimetric measurement.

Glucose assay

Prior to performing colorimetric assays, all detection zones were filled with a paste made of cellulose powder and water (1:4 m/m). Aliquots of 10 μL of paste were manually added to each test zone with a micropipette and allowed to dry at room temperature during 30 min. Afterwards, zones were prepared according to a procedure described elsewhere[29]. Briefly, an 8 μL-aliquot of a potassium iodide solution (0.6 M) was added on all detection zones and dried at room temperature. Then, five zones (labelled from 4 to 8) were spotted with 8 μL of a solution containing GOx-HRP (5:1) prepared in 100 mM phosphate buffer (pH 6) and allowed to dry at room temperature during 20 min. The other three zones (labeled from 1 to 3) previously spotted with potassium iodide solution were used as control zones. Assays were performed by adding 60 μL of standard or artificial sample solutions to the SI zone, which

promoted the quick distribution of the sample through microfluidic channels by capillary action towards the detection zones.

Kinetics monitoring

Enzyme assays were carried out to determine the GOx activity on the proposed microfluidic device. The hydrogen peroxide produced in the enzymatic reaction was monitored over time by a scanner for five initial glucose concentrations (*[Glu]*) ranging from 2 to 10 mM. Scanned images were analyzed using Corel PhotoPaint® software in order to extract the mean color intensity on each detection zone. Experimental data were fitted according to a first-order exponential growth model using the Microcal™ Origin™ 8.0 software for determination of Michaelis-Menten constant.

Biological samples

The optimum reaction time has been fixed to determine the glucose concentration in biological samples. Two artificial human serum samples (levels I and II) were used to evaluate the analytical feasibility of μTADs. Prior to assays, artificial serum samples were diluted in water and analysed as described earlier. The accuracy of the analytical determination was evaluated based on recovery experiments, in which samples were spiked with four concentration levels of glucose (from 2 to 5 mM).

Results and discussion

Colorimetric measurements on μTADs

The colorimetric measurements for the GOx reaction have demonstrated a strong dependence on the reaction time. As it can be seen in the optical micrographs presented in Fig. 2, the color intensity for the same assay (glucose concentration fixed at 10 mM) performed on μTADs gradually increases from 2 to 30 minutes (see zones labelled by 4-8). The color inside control zones labelled from 1 to 3 did not exhibit any change due to the absence of enzymes and color indicator.

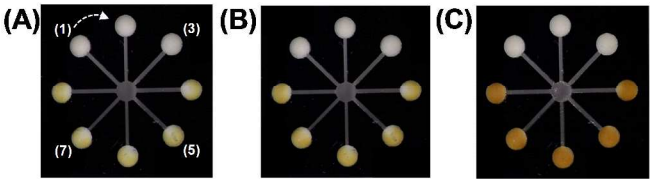

Figure 2. Images captured for glucose colorimetric assay after (A) 2, (B) 5 and (C) 30 minutes. Zones labelled as 1-3 and 4-8 were used for control and glucose assays, respectively.

Monitoring of the Michaelis-Menten kinetics

Before discussing the kinetics for the GOx reaction, it is important to highlight that the colorimetric detection of glucose occurs in the presence of both GOx and HRP enzymes. In addition, it is well-known that the enzymatic activity for the immobilized GOx on microfluidic reactor is lower than in free solution. For this reason, the conjugation of both enzymes in a cascade scheme has been essential for sensing applications[30,31]. As mentioned earlier, HRP is involved in the second chemical reaction responsible for the development of color inside detection zones. When compared to the GOx reaction, the HRP reaction kinetics is much faster, where the catalytic efficiency is higher in magnitude (more than 100×)[30,31]. Based on this statement, we assumed that the GOx kinetics is no significantly affected by the HRP reaction rate, *i.e.*, the global reaction rate is mainly driven by the GOx kinetics.

Due to the time-dependence presented in Figure 2, the analytical response of the glucose assay was monitored during a period between 0 and 30 minutes for glucose concentration levels ranging from 2 to 10 mM. The results are shown in Figure 3, in which each point mean the average value of the color intensity recorded in five detection zones at the same device. The experimental data depicted in Figure 3 were fitted by an exponential growth equation (eqn (1)):

$$I = I_{max}\left(1 - e^{-bt}\right) \qquad \textbf{(Equation 1)}$$

where I is the color intensity of the zone at a time point, I_{max} is the maximum color intensity captured over time, b refers to the rate constant for each condition and t means the reaction time. This exponential model has well-fitted all data measured over time. For all curves, the coefficients of determination (R^2) ranged from 0.962 to 0.985.

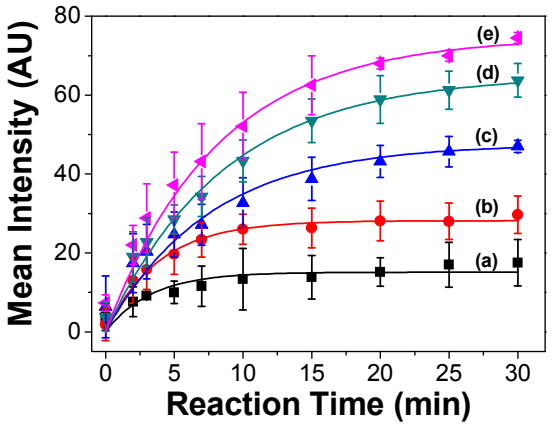

Figure 3. Monitoring of the mean color intensity over time for the glucose enzymatic assay keeping the glucose concentration at (a) 2, (b) 4, (c) 6, (d) 8 and (e) 10 mM. Experimental data were fitted using Eq. 1. The mean intensity for the control zones was subtracted from the values recorded in the glucose detection zones.

The behavior seen in Figure 3 may be explained by the Michaelis-Menten kinetic model[4,32] as expressed according to eqn (2):

$$V_o = \frac{V_{max}[Glu]}{K_m + [Glu]} \qquad \textbf{(Equation 2)}$$

where V_0 means the initial reaction velocity, V_{max} is the maximum velocity, *[Glu]* refers to the glucose concentration and, K_m, is the Michaelis-Menten constant. From the data presented in Figure 3, it can be inferred that the greater the glucose concentration, the higher the initial reaction velocity. Based on the enzymatic assay, the substrate is consumed over time to convert glucose in GA and hydrogen peroxide. Consequently, the mean color intensity reaches a constant plateau indicating the saturation of the analytical signal which may be associated with the total consumption of glucose. This behavior has been similar to the glucose concentration levels from 2 to 10 mM. The maximum enzyme velocity is regarded

to the turnover number, K_{cat}, which represents the maximum number of molecules converted to product per second in each active site of the enzyme. The K_{cat} can be estimated by the ratio between the V_{max} and the enzyme concentration[4,33].

The K_m and K_{cat} values were determined for the GOx reaction through the Michaelis-Menten model. Initially, the V_0 values were calculated using the first derivative of the exponential curves presented in Figure 3. Then, the reciprocal V_0 values were plotted against the respective reciprocal glucose concentrations generating the Lineweaver-Burk plot (Figure 4), which represents a linearized version of the Michaelis-Menten equation. The linear model equation[4] is shown inset Figure 4.

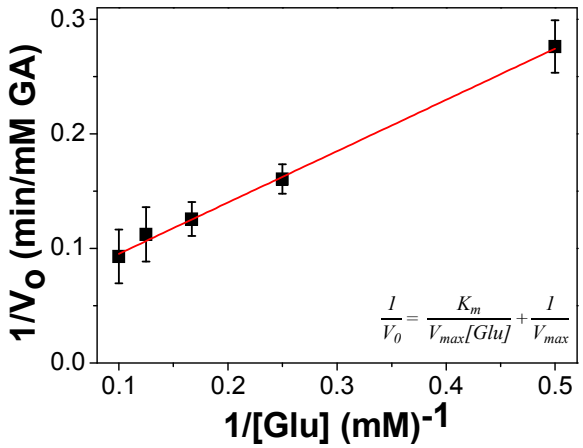

Figure 4. Lineweaver-Burk plot for the GOx reaction.

According to the linear form of the Michaelis-Menten equation, the angular and linear coefficients of the curve shown in Figure 4 are represented by K_m/V_{max}, and $1/V_{max}$, respectively. Consequently, the extrapolated *1/[Glu]* intercept is $-1/K_m$. When setting V_0 to $V_{max}/2$, it can be inferred that *[Glu]* is equal to K_m, namely, the *[Glu]* leading to the half of maximum reaction rate that is the main affinity indicator of an enzyme for its substrate on specific conditions. For the GOx reaction, the K_m and V_{max} values found with colorimetric detection on μTADs were 8.9 ± 1.3 mM and 19.8 ± 6.7 mM min^{-1}, respectively.

Concerning the K_m, the value achieved is lower than that of the enzyme in solution (25—27 mM)[9,34]. This reflects a higher affinity of GOx for glucose when supported on microfluidic strutuctures[5,9,10,13,33]. Different authors have reported studies focused on the determination of kinetic parameters regarded to GOx by using mainly electrochemical methods. Liu and co-workers[13] integrated an enzyme microreactor into a printed polymer microchip and based on this arrangement, they achieved a K_m value of 2.6 mM. He *et al.*[8] found a K_m value of 7.2 mM based on using of an integrated microfluidic electrochemical cell. Wang and co-workers[10] reported a K_m value of 6.0 mM determined with microchip electrophoresis coupled with amperometric detection. More recently, Wang *et al.*[5] showed that the K_m depends on the channel depth. The authors demonstrated that the K_m rises from 4.02 to 10.06 mM when the channel depth reduces from 140 to 55 nm. Likewise the K_m, the V_{max} achieved with colorimetric detection is also in agreement with the results found in literature[9,33].

In addition to K_m and V_{max} values, the K_{cat} was also estimated using eqn (3) taking into account that all GOx added on the detection zone was kept immobilized on the cellulose paste. In this case, the enzyme concentration, *[E]*, was 8.0 μM. In this way, the K_{cat} was estimated to be 41.2 ± 14.0 s^{-1} and represent the maximum number of molecules converted to product for second in each active site. This value is slightly different from those reported by other authors[5,9,33]. He *et al.* achieved a K_{cat} value of 13.4 s^{-1} for immobilized GOx on polymer monoliths inside microfluidic electrochemical system[9]. Wang *et al.* showed that the K_{cat} depends on the channel dimensions[5]. The authors found that this constant increases from 134.7 to 150.0 s^{-1} when the channel width reduces from 140 to 55 nm. The discrepancy between the value found in the proposed device and the results reported in literature may be associated with surface chemistry involved in the immobilization process as well as with detection system.

Assays with biological samples

Based on the kinetic study, the reaction time of 15 min has been chosen as optimum to proceed with the analytical determination of glucose in two artificial human serum samples. The colorimetric response has exhibited a linear behaviour for a glucose concentration range between 0 and 10 mM (Figure 5). The analytical sensitivity and the limit of detection (LD) achieved were 6.38 (AU/mM) and 1.2 mM, respectively. The LD value found was calculated based on the color intensity resulting from the ratio between three times the standard deviation for the control zone and the angular coefficient of the analytical curve.

The LD found in this current report is slightly lower than that reported by de Souza and co-workers[17] (1.7 mM) and Lim *et al.*[35] (2.0 mM) and higher than those found by Martinez *et al.*[29] (0.5 mM), Lankelma *et al.*[36] (0.2 mM) and Chen *et al.*[37] (0.2 mM). The normal levels of glucose in serum and urine samples include the ranges from 3.8 to 5.5 mM and from 0.1 to 0.8 mM, respectively[17,19,29]. When the glucose concentration is higher than 7.0 mM (serum) and 1.4 mM (urine), the diagnostic can represent an indicative of some diseases as diabetes, for example[19]. As documented by the World Health Organization (WHO), long-time diabetes causes damage to eyes, kidneys, and nerves. Furthermore, it increases the risk of heart disease, stroke, and insufficiency in blood flow to legs[38]. As depicted in Figure 5, it can be inferred that the linear range and the sensitivity found for colorimetric detection on μTADs are useful for clinical applications.

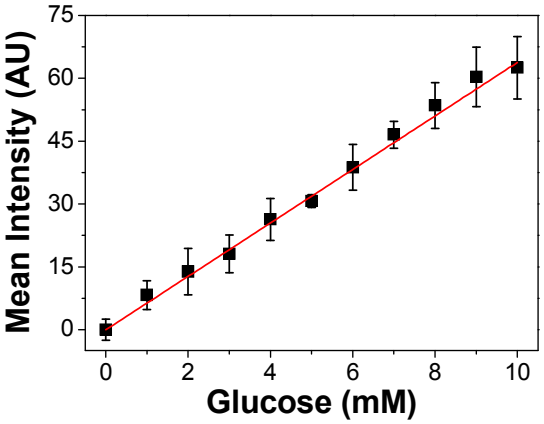

Figure 5. Analytical curve for the glucose colorimetric assay. The mean color intensity for the control zones was subtracted from the values recorded in the glucose detection zones.

In order to demonstrate the clinical feasibility, the glucose concentration has been determined in two artificial human serum samples (levels I and II). The serum samples were diluted in water and directly analysed on μTADs. The glucose concentrations achieved in both samples are depicted in Table 1. In comparison with the certified values by the supplier, the concentrations found on serum samples presented an error of 7.5 (level I) and 2.1% (level II) for the samples analysed. The accuracy of the assays performed on μTADs was investigated by spiking the biological samples with four glucose concentration levels (2—5 mM). As it can be visualized in Table 1, the recovery ranged from 95.5 to 99.6%. The glucose concentrations in serum samples were calculated taking into account the average value of color intensity recorded on five detection zones. The analytical response on μTADs was monitored during four consecutive weeks using three devices by week. The analysis of the results achieved from five independent measurements per concentration of glucose showed that the color intensity on each device was not statistically different from each other at the confidence level of 0.05 (data not shown).

Table 1 Comparison of the glucose concentrations determined in two artificial human serum samples and presentation of the recovery values achieved.

Serum Samples	Certified values/mM	Found values/mM	Recovery (%)
Level I	4.0 ± 0.7	4.3 ± 0.5	96.0 – 99.6
Level II	14.0 ± 2.7	14.3 ± 1.2	95.5 – 96.5

Conclusions

In summary, this report has described for the first time the use of μTADs with colorimetric detection to determine the kinetic constants for enzymatic reactions. The values achieved for K_m (8.9 ± 1.3 mM), K_{cat} (41.2 ± 14.0 s^{-1}) and V_{max} (19.8 ± 6.7 mM min^{-1}) are in agreement with the data reported in literature using different microfluidic platforms[13,39] as well as other detection systems (e.g., electrochemical systems)[5,9,10]. In addition, the kinetic monitoring has allowed reaching a relatively short optimum reaction time of 15 min, which was then chosen to quantitatively determine the glucose concentration in two artificial human serum samples with accuracy higher than 95%, being therefore suitable to clinical purposes. As a consequence of this kinetic investigation, we believe that other enzyme assays involved in clinical diagnostics as well as another studies associated with physiology and pathology can be also investigated on μTADs with colorimetric detection.

μTADs offer some attractive features including extremely low-cost ($0.10 per device), short processing time (less than 10 min), biocompatibility, affordability and capability of mass production. Furthermore, they are lightweight, disposable and present good optical transparency thus enabling to be coupled with optical detectors. Likewise paper-based devices[29], the proposed μTADs have exhibited great potential to perform clinical assays based on use of digital imaging[16,17]. Due to the noticeable advantages, μTADs are suitable to be implemented in places with low resources-settings or in applications where the sample-in-answer-out capability is required.

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Notes

[a]*Instituto de Química, Universidade Federal de Goiás, Campus Samambaia, 74001-970, Goiânia, GO, Brazil. Fax/Tel: 55 62 3521 1167, *E-mail: wendell@ufg.br*
[b]*Instituto Nacional de Ciência e Tecnologia de Bioanalítica, 13083-970, Campinas, SP, Brazil.*

References

1. M. L. Kovarik, D. M. Ornoff, A. T. Melvin, N. C. Dobes, Y. L. Wang, A. J. Dickinson, P. C. Gach, P. K. Shah and N. L. Allbritton, *Anal. Chem.*, 2013, **85**, 451-472.
2. M. L. Kovarik, P. C. Gach, D. M. Ornoff, Y. L. Wang, J. Balowski, L. Farrag and N. L. Allbritton, *Anal. Chem.*, 2012, **84**, 516-540.
3. A. Arora, G. Simone, G. B. Salieb-Beugelaar, J. T. Kim and A. Manz, *Anal. Chem.*, 2010, **82**, 4830-4847.
4. S. L. Sjostrom, H. N. Joensson and H. A. Svahn, *Lab Chip*, 2013, **13**, 1754-1761.
5. C. Wang, Z. H. Sheng, J. Ouyang, J. J. Xu, H. Y. Chen and X. H. Xia, *Chemphyschem*, 2012, **13**, 762-768.
6. A. G. Hadd, D. E. Raymond, J. W. Halliwell, S. C. Jacobson and J. M. Ramsey, *Anal. Chem.*, 1997, **69**, 3407-3412.
7. X. D. Cao, Y. Li, Z. Q. Zhang, J. C. Yu, J. Qian and S. Q. Liu, *Analyst*, 2012, **137**, 5785-5791.
8. S. Matosevic, N. Szita and F. Baganz, *J. Chem. Technol. Biot.*, 2011, **86**, 325-334.
9. P. He, G. Greenway and S. J. Haswell, *Microfluid. Nanofluid.*, 2010, **8**, 565-573.
10. J. Wang, M. P. Chatrathi, B. M. Tian and R. Polsky, *Anal. Chem.*, 2000, **72**, 2514-2518.
11. W. D. Ristenpart, J. D. Wan and H. A. Stone, *Anal. Chem.*, 2008, **80**, 3270-3276.
12. M. Srisa-Art, E. C. Dyson, A. J. Demello and J. B. Edel, *Anal. Chem.*, 2008, **80**, 7063-7067.
13. A. L. Liu, T. Zhou, F. Y. He, J. J. Xu, Y. Lu, H. Y. Chen and X. H. Xia, *Lab Chip*, 2006, **6**, 811-818.
14. C. L. do Lago, H. D. T. da Silva, C. A. Neves, J. G. A. Brito-Neto and J. A. F. da Silva, *Anal. Chem.*, 2003, **75**, 3853-3858.
15. A. R. Kim, J. Y. Kim, K. Choi and D. S. Chung, *Talanta*, 2013, **109**, 20-25.
16. K. A. Oliveira, C. R. de Oliveira, L. A. da Silveira and W. K. T. Coltro, *Analyst*, 2013, **138**, 1114-1121.
17. F. R. de Souza, G. L. Alves and W. K. T. Coltro, *Anal. Chem.*, 2012, **84**, 9002-9007.
18. K. M. Schilling, D. Jauregui and A. W. Martinez, *Lab Chip*, 2013, **13**, 628-631.

19. W. Dungchai, O. Chailapakul and C. S. Henry, *Anal. Chim. Acta*, 2010, **674**, 227-233.
20. S. A. Klasner, A. K. Price, K. W. Hoeman, R. S. Wilson, K. J. Bell and C. T. Culbertson, *Anal. Bioanal. Chem.*, 2010, **397**, 1821-1829.
21. A. W. Martinez, S. T. Phillips, G. M. Whitesides and E. Carrilho, *Anal. Chem.*, 2010, **82**, 3-10.
22. N. R. Pollock, J. P. Rolland, S. Kumar, P. D. Beattie, S. Jain, F. Noubary, V. L. Wong, R. A. Pohlmann, U. S. Ryan and G. M. Whitesides, *Sci. Transl. Med.*, 2012, **4,** 1-10.
23. A. Maattanen, D. Fors, S. Wang, D. Valtakari, P. Ihalainen and J. Peltonen, *Sensor Actuat. B-Chem*, 2011, **160**, 1404-1412.
24. L. Shen, J. A. Hagen and I. Papautsky, *Lab Chip*, 2012, **12**, 4240-4243.
25. M. Santhiago, E. W. Nery, G. P. Santos and L. T. Kubota, *Bioanalysis*, 2014, **6**, 89-106.
26. E. W. Nery and L. T. Kubota, *Anal. Bioanal. Chem.*, 2013, **405**, 7573-7595.
27. A. K. Yetisen, M. S. Akram and C. R. Lowe, *Lab Chip*, 2013, **13**, 2210-2251.
28. C. Parolo and A. Merkoci, *Chem. Soc. Rev.*, 2013, **42**, 450-457.
29. A. W. Martinez, S. T. Phillips, E. Carrilho, S. W. Thomas, H. Sindi and G. M. Whitesides, *Anal. Chem.*, 2008, **80**, 3699-3707.
30. P. Ciaurriz, E. Bravo and K. Hamad-Schifferli, *J. Colloid Interf. Sci.*, 2014, **414**, 73-81.
31. D. Mackey, A. J. Killard, A. Ambrosi and M. R. Smyth, *Sensor Actuat. B-Chem*, 2007, **122**, 395-402.
32. H. Jo, N. Meinhardt, Y. B. Wu, S. Kulkarni, X. Z. Hu, K. E. Low, P. L. Davies, W. F. DeGrado and D. C. Greenbaum, *J. Am. Chem. Soc.*, 2012, **134**, 17704-17713.
33. C. Wang, D. K. Ye, Y. Y. Wang, T. Lu and X. H. Xia, *Lab Chip*, 2013, **13**, 1546-1553.
34. M. J. Rogers and K. G. Brandt, *Biochemistry-Us*, 1971, **10**, 4624-4630.
35. K. R. Lim, J. M. Park, H. N. Choi and W. Y. Lee, *Microchem. J.*, 2013, **106**, 154-159.
36. J. Lankelma, Z. H. Nie, E. Carrilho and G. M. Whitesides, *Anal. Chem.*, 2012, **84**, 4147-4152.
37. X. Chen, J. Chen, F. B. Wang, X. Xiang, M. Luo, X. H. Ji and Z. K. He, *Biosens. Bioelectron.*, 2012, **35**, 363-368.
38. World Health Organization (WHO), http://www.who.int/, Accessed January 2014.
39. C. Wang, S. J. Li, Z. Q. Wu, J. J. Xu, H. Y. Chen and X. H. Xia, *Lab Chip*, 2010, **10**, 639-646.