Promega

Spectrum Compact CE System

Sanger Sequencing and Fragment/STR Analysis

- benchtop 4-capillary electrophoresis instrument for 4-, 5- and 6-dye chemistries and up to 32 samples in a single run
- compatible with a broad range of commercial chemistries for DNA sequencing, forensic STR profiling, MLPA, MSI and many more
- flexible raw data analysis
- prefilled, plug-and-play consumables for maximal flexibility and efficiency
- controlled by the integrated touchscreen or by Remote Access Software
- on-site instrument and software installation, operational and application trainings

www.promega.com/spectrum-compact-system

Spectrum
COMPACT
CE SYSTEM

Jason G. Kralj
Matthew S. Munson
David Ross

National Institute of Standards and Technology, Gaithersburg, MD, USA

Received November 19, 2013
Revised March 5, 2014
Accepted March 5, 2014

Research Article

Total protein quantitation using the bicinchoninic acid assay and gradient elution moving boundary electrophoresis

We investigated the ability of gradient elution moving boundary electrophoresis (GEMBE) with capacitively coupled contactless conductivity detection (C^4D) to assay total protein concentration using the bicinchoninic acid (BCA) reaction. We chose this format because GEMBE-C^4D behaves as a concentration dependent detection system, unlike optical methods that also rely on pathlength (due to Beer's law). This system tolerates proteins well compared with other capillary electrophoretic methods, allowing the capillary to be reused without coatings or additional hydroxide wash steps. The typical reaction protocol was modified by reducing the pH slightly from 11.25 to 9.4, which enabled elimination of tartrate from the reagents. We estimated that copper (I) could be detected at approximately 3.0 μmol/L, which agrees with similar GEMBE and CZE systems utilizing C^4D. Under conditions similar to the BCA "micro method" assay, we determined the LOD for three common proteins (insulin, BSA, and bovine gamma globulin) and found that they agree well with the existing spectroscopic detection methods. Further, we investigated how long reaction times impact the LOD and found that the conversion was proportional to log(time). This indicated that little sensitivity is gained by extending the reaction past 1 h. Hence, GEMBE provides an alternative platform for total protein assays while maintaining the excellent sensitivity of the optical-based methods.

Keywords:
Bicinchoninic acid (BCA) / Capacitively coupled contactless conductivity detection (C4D) / Gradient elution moving boundary electrophoresis (GEMBE) / Total protein assay
DOI 10.1002/elps.201400025

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Total protein assays are routinely performed using the biuret reaction, which reduces Cu(II) to Cu(I) in the presence of amines from the protein under basic conditions, and produces a color change [1]. This method was improved to enhance the sensitivity of the Cu(I) species using bicinchoninic acid, which forms a complex that absorbs strongly at 562 nm giving an intense purple color that can be measured spectroscopically, and is referred to as the bicinchoninic acid (BCA) or Smith protein assay [2]. This assay has been used for decades due to its simplicity and robustness. The assay tolerates of a range of potential interferrants commonly found in biological samples with the exception of high concentrations of glucose, some nitrogen-containing compounds, and chelating reagents [2]. A recent publication has described work to demonstrate detection of immobilized protein using a combination of electrophoresis and acid-base titration in moving reaction boundary electrophoresis (MRBE) [3–5]. Protein concentrations on the order of mg/mL were detected visually and showed good agreement with the Kjeldahl protein assay method, and MRBE was shown to work with common biological samples such as milk powder and serum.

It is increasingly common to need to analyze proteins in the range of 1–10 μg/mL. At these levels, proteins would likely be isolated products to achieve accurate quantitation, though less-refined samples may also lend themselves to this

Correspondence: Dr. Jason G. Kralj, National Institute of Standards and Technology, 100 Bureau Dr MS 8312, Gaithersburg, MD 20899 USA
E-mail: Jason.kralj@nist.gov
Fax: +1-301-330-3447

Abbreviations: BCA, bicinchoninic acid; **BGG**, bovine gamma globulin; **GEMBE**, gradient elution moving boundary electrophoresis

Colour Online: See the article online to view Figs. 2–4 in colour.

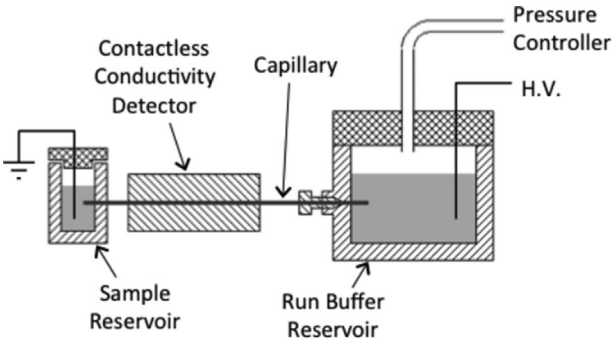

Figure 1. Schematic of GEMBE setup.

assay if potential interferrant levels are low. The BCA "micro method" would appear to suit this assay range [2]. According to Beer's law, the absorbance of the analyte is proportional to the pathlength and concentration. Conventional spectroscopy requires relatively high sample volumes of approximately 0.25 to 1.5 mL, and thus several micrograms of protein. One way to maintain the pathlength while reducing volume is to use low-volume 384-well microplates, though this would be wasteful since rarely are hundreds of assays required. If the analysis could be performed using a strictly concentration dependent detector, it would be possible to reduce the sample requirement by an order of magnitude or more compared with the conventional approach.

Electrophoresis of the Cu(I)-BCA complex (likely the formation of 2 BCA molecules complexing a single Cu(I) ion) is possible due to a net negative 2 charge. The Cu(I)-BCA complex can be separated from BCA and the BCA-Cu(II) complex (possibly either one or two BCA molecules complexing with the Cu(II)) in solution [6]. Gradient elution moving boundary electrophoresis (GEMBE) is a recently described method for electrophoretic separations that is designed for simplicity in field-portable and multiplexed applications [7].

With GEMBE, a combination of electrophoresis and bulk solution counterflow is used to provide separation. The typical hardware required for GEMBE is shown schematically in Fig. 1. GEMBE differs from conventional CZE in that there is no defined injection. Instead, the sample is placed in contact with the inlet end of the capillary (in the sample reservoir) and remains there throughout the separation. Typically, the polarity of the applied voltage is chosen so that the analytes of interest will be electrophoretically driven into the capillary from the sample reservoir toward the run buffer reservoir, and the counterflow will be directed from the run buffer reservoir toward the sample reservoir. The counterflow is usually a combination of pressure-driven flow and electroosmosis. A pressure is applied to the head space of the run buffer reservoir to control the magnitude of the counterflow so that it can be easily varied over time. At the beginning of a GEMBE separation, the applied pressure is high, resulting in a counterflow velocity greater than the electrophoretic velocity of all the analytes of interest. The pressure is then reduced gradually over the course of a separation, allowing the analytes to enter sequentially, in order from highest to lowest electrophoretic mobility. Once in the capillary, the analytes migrate in the form of a moving boundary and are detected as a step change in detector response. Theoretical and experimental work has shown that GEMBE can provide separations with resolution similar to that of CZE in a similar separation time [8, 9]. With GEMBE, however, the pressure ramp rate (or counterflow acceleration) is typically adjusted to provide the required resolution instead of the length or electroosmotic mobility of the capillary as with CZE. Consequently, the capillaries used for GEMBE are typically much shorter than those used for CZE.

With GEMBE, the counterflow is used to exclude the proteins and some other potential interferents (e.g. particulates) in the sample from entering the separation channel [10, 11]. Additionally, the separation is typically tuned to analyze only the species of interest to reduce the overall run time. Here, we focused on demonstrating the technique under the "best case" scenario to understand the performance and limitations of the method.

Separation of the Cu(I)-BCA is advantageous because it may be assayed in the separation capillary by capactively coupled contactless conductivity detection (C^4D). This detection scheme is effectively pathlength independent, unlike spectroscopy. Hence, small amounts of protein can be assayed in small volumes to achieve the same information. C^4D works as its name implies [12–14]. The benefits of this approach are that (i) the electrodes can be kept separate from the surrounding fluid to avoid fouling; (ii) modularity of the detector simplifies construction and capillary replacement; and (iii) detection limits are in the high nmol/L to low μmol/L range.

In this work, we examined the capability of GEMBE to separate and detect Cu(I)-BCA from the background of reagents, and to modify the BCA reaction around GEMBE for low-protein assays in a way similar to the "micro" BCA assay [2]. We found that reducing the pH allowed us to eliminate one of the reagents from the original BCA assay (tartrate) and improve the separation of the Cu(I) complex while having a minimal affect on the reaction. Calibration experiments demonstrated that we could achieve a 3.0 μmol/L LOD of the Cu(I)-BCA complex using GEMBE with C^4D. This LOD was comparable to that found with the "micro method" BCA assay with absorbance measured at 562 nm in a 96-well plate reader format [2]. Using GEMBE with the modified BCA assay, we assayed three common proteins (insulin, BGG, BSA) and found that 3 μg/mL protein was routinely detected, with the LOD dependent on the protein and ranging from 0.4 to 2.0 μg/mL. Further analysis of the reaction kinetics revealed that Cu(I)-BCA complex formation was proportional to the log(time), and hence there are severe time requirements for increasing conversion past what is achieved in 20 min to 1 h.

2 Materials and methods

2.1 Chemicals and Buffers

The run buffer used for GEMBE separations was 25 mmol/L carbonate pH 9.4 (BupH carbonate-bicarbonate buffer pack,

Pierce, IL) in water obtained through reverse osmosis and filtration to 18.2 MΩ-cm purity. BCA (sodium salt, hydrated) was also obtained from Pierce. Copper (II) sulfate pentahydrate, copper (I) chloride, 5 mol/L sodium chloride solution, and insulin (human, recombinant expressed in yeast) were obtained from Sigma-Aldrich. BGG and BSA standards (2 mg/mL) were obtained from Thermo Scientific in 1 mL ampules.

A CuCl standard solution was prepared by adding powdered CuCl to 25 mmol/L BCA and 25 mmol/L carbonate buffer to bring the concentration of CuCl to 5 mmol/L. Once the CuCl dissolved, it was diluted to 0.2 mmol/L CuCl, 1 mmol/L BCA using 25 mmol/L carbonate buffer. This was stored at room temperature and used for approximately 1 month. We observed no significant change in the solution over that time. Calibration solutions were prepared using 1 mmol/L BCA, x mmol/L CuCl, and (200-x) mmol/L $CuSO_4{\cdot}5\ H_2O$; where x is a value between 0 and 200. This was to simulate conversion of the Cu(II) to Cu(I) and keep the total copper concentration constant.

A 10× reaction buffer was prepared at 10 mmol/L BCA, 2 mmol/L $CuSO_4{\cdot}5\ H_2O$, 25 mmol/L carbonate pH 9.4. This was stored at room temperature and used within 2 weeks. Note that tartrate was omitted from the reaction buffer, as it is no longer needed at this pH to stabilize the Cu(II). Insulin was received as 25 mg powder and dissolved using 25 mmol/L carbonate buffer to a concentration of 10 mg/mL. BGG and BSA were diluted from their ampules with 150 mmol/L sodium chloride solution to maintain salt levels in the original samples. Typically, fourfold dilution series of the protein were prepared from 200 μg/mL to 0.195 μg/mL and run immediately. Typical reactions contained 50 μL of 10× reaction buffer, 473.5 μL of carbonate buffer, and 12.5 μL of protein sample. The reaction volume was 500 μL in a 1.5 mL microcentrifuge tube (Eppendorf), unless otherwise noted.

2.2 Apparatus/operation

Samples were incubated at 60°C in a water bath (Neslab RTE-210) with foam floats. After reaction, the tubes were placed on ice or in a refrigerator (4°C) for a few minutes up to overnight until being run on GEMBE. We measured step heights from sample replicates analyzed on different days (separated by approximately 18 h) and found no change.

The details of the GEMBE apparatus (Fig. 1) are described elsewhere. [10] A 5 cm fused silica capillary (360 μm od, 15 μm id) was threaded through a TraceDec C^4D detector (Innovative Sensor Technologies). The pressure controller was a Mensor 600 Series. For these assays, 2 mL of carbonate run buffer were used and changed at least once per day. The sample reservoir was filled with 0.2 mL of sample each run. After each run, the sample cup was rinsed once with 0.2 mL of the next sample. Alternatively, we also found no difference when rinsing with 0.2 mL of carbonate run buffer.

For each GEMBE separation, the applied voltage was set at +3000 V (600 V/cm field strength). The pressure was initially set at +1000 Pa and held constant for approximately 20 s, then set at approximately −8700 Pa for 15 s, followed by a ramp rate of −12.5 Pa/s. The pressure ramp lasted approximately 300 s, after which the pressure was reset to +1000 Pa. The run control and C^4D data-logging was performed using a custom-written Labview (National Instruments, TX) program. Total GEMBE analysis time was approximately 6 min for each sample, with steps resolved for the Cu(I)-BCA complex and the mixture of BCA and [BCA-Cu(II)] complex.

2.3 Data analysis

Electropherograms were fit to a sum of two error functions and a quadratic baseline. A 90 s time window centered approximately at the time between the two steps was used for all data fits. The sum of square errors was minimized for the data range using initial estimates for step heights, widths, positions, and baseline.

$$s = A_1\mathrm{erf}\left(\frac{t-x_1}{w_1}\right) + A_2\mathrm{erf}\left(\frac{t-x_2}{w_2}\right) + a\,(t-t_0)^2 + b\,(t-t_0) + c \quad (1)$$

Where s is the signal, t is the time, A are half the step heights, subscript 1 is Cu(I)-BCA and 2 is a mixture of BCA and [BCA-Cu(II)], x are the inflection points (step locations), and w the characteristic step widths; t_0, a, b, and c are baseline fit parameters. Some day-to-day variability of the detector response was observed, but the variability was consistent for all steps. Thus, the relative magnitude of the Cu(I)-BCA step was defined as:

$$\phi \equiv \frac{A_1}{A_1 + A_2} \quad (2)$$

and used as the response variable to generate the calibration curve and to estimate Cu(II) reduction from total protein. Values for ϕ ranged between 0.01 and 0.7 in most cases.

The LOD was estimated by determining the concentration necessary to produce a signal (relative magnitude) that differed by 3× the SD from the signal obtained with blank (zero concentration) samples.

3 Results and discussion

3.1 Reaction and GEMBE separation optimization

Smith et al. noted that the optimum detection conditions were pH 11.25 for the spectroscopic BCA assay, with significant reductions in signal for deviations of ±0.75 pH units [2]. For the GEMBE-BCA assay, we found a different set of conditions to be optimal. At pH 11, the reaction for reducing Cu(II) to Cu(I) proceeded well as indicated by the generation of intense purple color of the sample, but the Cu(I)-BCA complex was unresolvable from the other reagents using GEMBE. Cyclodextran species to improve the separation were found to react with the copper, and thus were not used; though others have employed them for similar separations [6]. At pH 9.4, the reaction could still proceed at a rate similar to

pH 11, and electrophoretic separation of the Cu(I)-BCA complex was possible. In addition, we found that at the lower pH, tartrate was no longer needed to stabilize the Cu(II), so it was eliminated from the reaction mixture. We did not examine smaller pH changes, as we had achieved resolution of the desired compounds while maintaining reaction rate and eliminating a reagent.

We selected 60°C as the incubation temperature because it generated no significant signal in the blank samples over 1 h and strong Cu(I) conversion when relatively small amounts of protein were present. This compares well with previous work [2]. We found that high temperatures (e.g. 90°C) led to significant Cu(I) formation in blank (protein-free) samples. While test (positive) samples generated high amounts of detectable Cu(I)-BCA complexes, the background subtraction can introduce significant variability. Blank samples run at 60°C produced values of ϕ from 0.01 at 1 h, and up to 0.03 over 24 h, demonstrating the specificity of the reaction under these conditions.

3.2 Instrument response calibration to Cu(I)

Before examining the effect of protein concentration, we investigated how Cu(I), the reporter molecule, affected the C^4D signal. Instrument response curves for various concentrations of Cu(I) were generated between 0 and 200 μmol/L to estimate the LOD and working range. In every case, the total Cu concentration was held constant at 200 μmol/L. In Fig. 2A, a series of signal versus time electropherograms (shown offset for visual clarity) were plotted using different CuCl concentrations. Assays of each CuCl concentration were repeated three times. We observed that the first species of interest enters the detector around 260 to 280 s (indicated as 1), with the step size affected by the CuCl concentration. Hence, it was identified as BCA-Cu(I) complex. The second step (2) appeared at approximately 300 s, and is the sum of free BCA and BCA-Cu(II) complex. The sum of the two steps is nearly constant, as we kept $[BCA]_0$ and total copper concentration constant. In Fig. 2B, ϕ versus CuCl concentration is slightly nonlinear as the ratio of excess BCA to Cu(I) decreases. As such, a second-order polynomial was used to fit the instrument response. CuCl LOD was found to be 3.0 μmol/L, and is comparable to the LOD obtained for other inorganic and organic ions with both GEMBE [10] and CZE [12, 15] with C^4D detection.

We did not investigate the effect of additional species, such as other transition metals, divalent IIA metals, or anions, that could coelute with the Cu(I)-BCA complex and/or the (BCA-Cu(II) complex and BCA) mixture. However, we do not anticipate that these would be a major concern, because their concentrations would likely be low under typical assay conditions (especially if assaying a purified protein product) where the protein sample constitutes approximately 2.5% of the reaction mixture. Clearly, suitable blanks and calibration samples would need to be run to determine the LOD of a specific type of protein sample and its matrix.

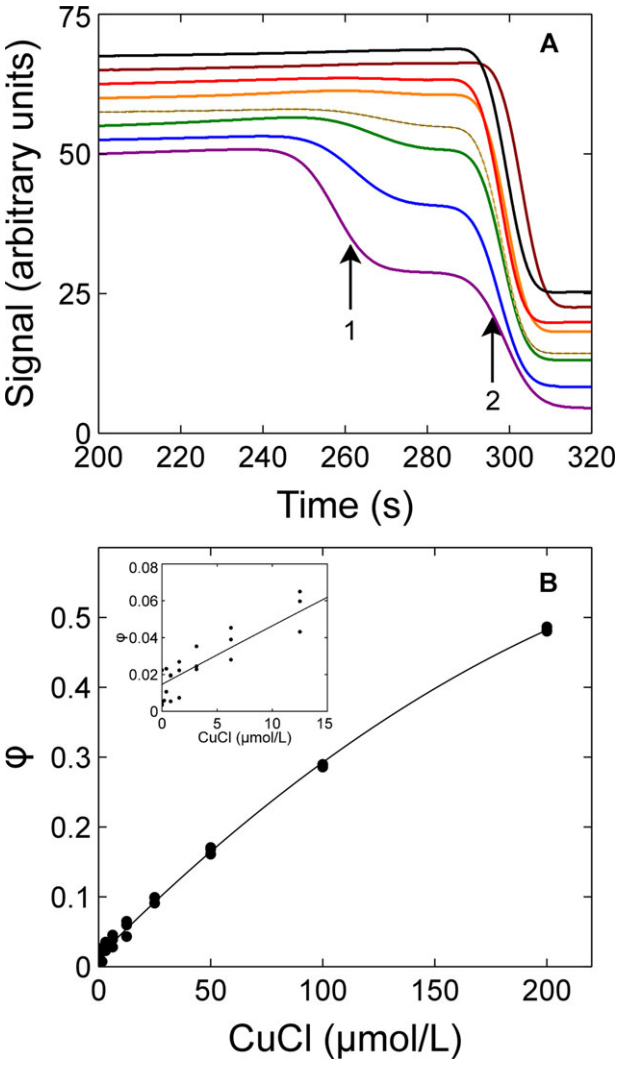

Figure 2. (A) GEMBE electropherograms for dilutions of CuCl used for calibration. The first step at approximately 270 s is from the Cu(I)-BCA complex, and step 2 at approximately 300 s is the remaining BCA and BCA-Cu(II) complex. From top to bottom, the traces represent CuCl concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50, 100, and 200 μmol/L. In all samples, total copper concentration was kept constant at 200 μmol/L. (B) The relative step size (ϕ) is determined by data fitting and used to generate calibration data. The inset shows the individual assay data from low CuCl concentration samples.

3.3 Protein analyses

The three test proteins were insulin, BSA, and BGG. Insulin is a relatively short peptide with a high concentration of known reactive residues (Cys, Trp, Tyr), and has been demonstrated to rapidly reduce Cu(II) [2, 16]. BSA and BGG are more indicative of larger proteins and are routinely used as standards for assay calibration. Figure 3 shows the level of $Cu(I)BCA_2$ generated versus the input protein concentration. There were clear differences between the reaction rates involving different proteins, which is consistent with

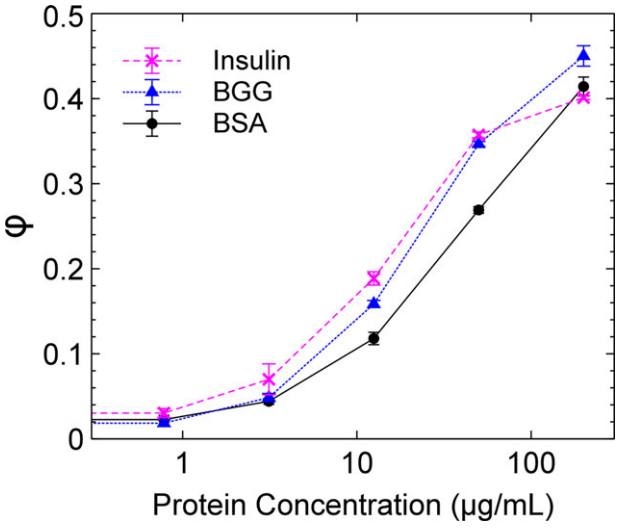

Figure 3. Relative step size versus protein concentration. Different proteins were subjected to the modified-BCA reaction and GEMBE analysis. Dilution of each protein shows the reaction is dependent on protein amount and composition, as previously observed. The error bars are SDs from 3–5 replicates.

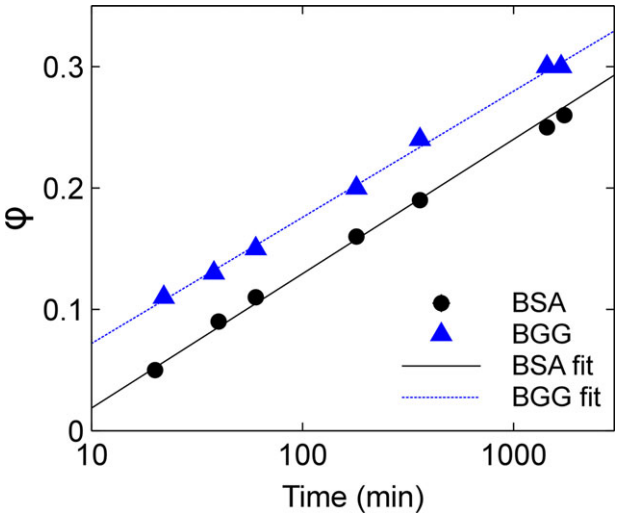

Figure 4. Kinetic response of 10 μg/mL protein subjected to the modified BCA reaction and GEMBE analysis. To determine whether increasing reaction time could improve the LOD, we assayed BSA and BGG over approximately 29 h. Conversion was observed to be proportional with log(time). Since Cu(II) is still available, this would seem to indicate that active amine groups of the protein are reacting/decaying, and that the different AAs (and/or combinations) have significantly different kinetic parameters resulting in slower Cu(I)-complex generation.

previous findings [1, 2, 16]. We found that 3–100 μg/mL of protein was routinely detectable using these conditions and represents a typical working range for protein samples. The LOD for the BSA, BGG, and insulin were approximately 2.0, 1.4, and 0.4 μg/mL, respectively. Most spectroscopic methods appear to have a similar LOD, with the same or slightly smaller working range [1]. We have included a comparison of BSA and BGG data from GEMBE-C^{4}D with that obtained using optical absorbance in the Supporting Information. Concordance between the methods was good.

Particulates and/or turbid solutions, though rare in most protein assay situations, could interfere with conventional spectroscopy by light scattering. GEMBE could be employed to directly assay such samples, without the need for any additional cleanup [10]. This analysis is also amenable to multiplexed detection, currently capable of handling tens of samples at a time [17]. While the samples in this study focused on relatively "clean" samples and low throughput to determine assay performance limitations, one should be able to extend BCA/GEMBE assays to complex, less refined biosamples including those with, for example, particulates and liposomes, all without significantly compromising sensitivity or throughput.

3.4 Extended BCA reaction studies

We explored the possibility of improving LOD by increasing the reaction time. This assay does not normally reach an endpoint [1], thus increasing conversion of the reaction could improve the LOD. We took aliquots of a reaction mixture and incubated them at 60°C for times ranging from 20 min to 29 h using BGG and BSA samples, with the assay results in Fig. 4.

Values of ϕ appeared to follow a logarithmic dependence with time. Each curve was fit empirically using:

$$\phi = m \cdot \ln(t) + b \quad (3)$$

where b and m are the fit parameters, and t is time. From Fig. 4, we see clear differences in values of b. The values for m were not statistically significantly different for BGG and BSA (average ± 95% CI: 0.045 ± 0.03 and 0.047 ± 0.02, respectively). This might be explained by a reaction that was limited by Cu(I) dissociation from the variety of peptide complexes, after an initial phase where reactive sites are rapidly consumed and Cu(I) release was fast [18]. However, it is not clear that b and m have significant fundamental value, since there is no established kinetic model for this particular reaction scheme.

At 24 h, ϕ approached 0.3 for 10 μg/mL BGG, versus 0.15 at 1 h. It is clear that little is gained by increasing the reaction time to increase conversion, as only slightly improved sensitivity is possible unless further refinements to the reaction chemistry are undertaken.

4 Concluding remarks

Total protein assays using a modified BCA reaction can be readily monitored using GEMBE with C^{4}D. Cu(I)-complex detection limits were in the μmol/L range, which were generated from 3 to 100 μg/mL levels of protein in approximately 1 h at 60°C. The original reaction was simplified by eliminating tartrate and reducing the pH to 9.4 from 11.25,

which stabilized the Cu(II) reagent, and did not significantly affect the reaction rate. This reduction in pH enabled electrophoretic separation of the Cu(I) complex. The LODs using GEMBE with C^4D for BSA, BGG, and insulin were 2.0, 1.4, and 0.4 μg/mL, respectively. These values were approximately the same as BCA "micro method" assay using spectroscopy [1, 2]. The reaction conversion appears to increase with log(t), resulting in slow reaction progress past approximately 1 h. Hence, reaction times much longer than 1 h are not a viable option for improving the LOD.

GEMBE offers an alternative platform for total protein assays, equaling the LOD performance of conventional spectroscopy while potentially reducing the amount of sample required by an order of magnitude. The specific advantages of using GEMBE in a modified BCA assay are that it is (i) pathlength independent, (ii) amenable to working with submicrogram quantities of protein, (iii) tolerates proteins without fouling the capillary, and (iv) capable of being multiplexed. Standard methods for spectroscopic detection of the Cu(I)-complex that use cuvettes or well-plates require pathlengths of several milimeters, which typically requires sample volumes of more than 250 μL. While there are low-volume-high-pathlength technologies available, they are not routinely used for this application. GEMBE detection is pathlength-independent, and volumes could potentially be reduced to 20 μL or less by using a smaller sample reservoir. As such, reducing the sample volume would require approximately 60 ng of protein, or approximately an order of magnitude reduction over conventional spectroscopy. Since protein samples are rarely available in large amounts, this would reduce the quantity of sample spent for quantification purposes.

The authors would like to acknowledge Jonathan Shackman for helpful feedback and excellent suggestions for preparing this manuscript. NIST disclaimer: Any mention of commercial products is for information only; it does not imply recommendation or endorsement by NIST. J.G.K. and D.R. are inventors on patents (describing GEMBE) assigned to the United States of America as represented by the Secretary of Commerce, The National Institute of Standards and Technology.

The authors have declared no conflict of interest.

5 References

[1] Krohn, R. I., Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.] 2011, Appendix 3, 3H.

[2] Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C., *Anal. Biochem.* 1985, *150*, 76–85.

[3] Wang, H.-Y., Guo, C.-Y., Guo, C.-G., Fan, L.-Y., Zhang, L., Cao, C.-X., *Anal. Chim. Acta* 2013, *774*, 92–99.

[4] Guo, C.-Y., Wang, H.-Y., Liu, X.-P., Fan, L.-Y., Zhang, L., Cao, C.-X., *Electrophoresis* 2013, *34*, 1343–1351.

[5] Wang, H.-Y., Li, S., Tang, Y.-Y., Dong, J.-Y., Fan, L.-Y., Cao, C.-X., *Analyst* 2013, *138*, 3544.

[6] Meng, Q., Guo, L., Shen, R., Xie, J., *Chin. J. Chromatog.* 2010, *28*, 682–687.

[7] Shackman, J. G., Munson, M. S., Ross, D., *Anal. Chem.* 2007, *79*, 565–571.

[8] Ross, D., *Electrophoresis* 2010, *31*, 3650–3657.

[9] Ross, D., *Electrophoresis* 2010, *31*, 3658–3664.

[10] Strychalski, E. A., Henry, A. C., Ross, D., *Anal. Chem.* 2009, *81*, 10201–10207.

[11] Strychalski, E. A., Henry, A. C., Ross, D., *Anal. Chem.* 2011, *83*, 6316–6322.

[12] Kuban, P., Hauser, P. C., *Electrophoresis* 2009, *30*, 176–188.

[13] Kuban, P., Hauser, P. C., *Electrophoresis* 2011, *32*, 30–42.

[14] Kuban, P., Hauser, P. C., *Electrophoresis* 2013, *34*, 55–69.

[15] Epple, R., Blanes, L., Beavis, A., Roux, C., Doble, P., *Electrophoresis* 2010, *31*, 2608–2613.

[16] Wiechelman, K. J., Braun, R. D., Fitzpatrick, J. D., *Anal. Biochem.* 1988, *175*, 231–237.

[17] Ross, D., Kralj, J. G., *Anal. Chem.* 2008, *80*, 9467–9474.

[18] Huang, T., Long, M., Huo, B., *Open Biomed. Eng. J.* 2010, *4*, 271–278.

