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Research Paper / Araştırma Makalesi

A Chemiluminescence Based Optical Biosensor Coupled with Immunomagnetic Separation for the Detection of β-Glucuronidase from *Escherichia coli*

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ABSTRACT

A biosensor was developed using a magnetic focusing optical waveguide (glass tubular probe) for the detection of glucuronidase (GUS) from *Escherichia coli* based on immunomagnetic separation and chemiluminescent measurement. The magnetic focusing resulted in a lower chemiluminescent signal than that obtained without magnetic-focusing, mainly due to the diffusion of the chemiluminescent reaction products into the solution rather than concentrating around the GUS enzyme captured by the antibodies on the paramagnetic beads. A microwell format was also tested for the chemiluminescent assay. A linear relationship was observed between log-GUS concentration and log-chemiluminescent signal for the microwell and magnetic focusing formats with a limit of detection as low as 100 and 1000 pg GUS/mL, respectively. The microwell format had the potential to be improved for higher sensitivity. The procedure was simple and rapid, and the whole assay could be completed in 70 min.

Keywords: Glucuronidase, Chemiluminescence, Biosensor, Immunomagnetic separation, Magnetic focusing

Escherichia coli β-Glukuronidaz Enziminin Saptanmasında İmmunomanyetik Ayırmayla Birleştirilmiş Kemiluminesans Temelli Optik Biyosensör

ÖZ

Bu araştırmada *Escherichia coli* glukorunidaz (GUS) enziminin saptanması amacıyla immunomanyetik ayırma ve kemiluminesans yöntemlerini esas alan biyosensör geliştirilmiştir. Biyosensörde, ucunda odaklayıcı bir mıknatıs bulunan ve tüp biçiminde camdan yapılmış ışık dalga iletim kılavuzu kullanılmıştır. Kemiluminesans tepkime ürünleri paramanyetik kürecikler üzerinde antikorlar tarafından yakalanan GUS enziminin çevresine yoğunlaşmak yerine reaksiyon çözeltisine dağılmıştır. Bu nedenle manyetik odaklama yöntemiyle saptanan kemiluminesans sinyali manyetik odaklama olmadan saptanan sinyale göre daha düşük olmuştur. Ayrıca mikrokuyucuk içerisinde de kemiluminesans yöntemi uygulanmıştır. Logaritmik GUS konsantrasyonu ile logaritmik kemiluminesans ölçüm değerleri arasında doğrusal bir ilişki olduğu görülmüş, mikrokuyucuk ve manyetik odaklama yöntemlerinin duyarlılığı sırasıyla 100 ve 1000 pg GUS/mL olarak saptanmıştır. Mikrokuyucuk yönteminin daha fazla geliştirilme potansiyeli olduğu anlaşılmaktadır. Yöntem basit ve hızlı olup tüm işlemler 70 dakika sürmektedir.

Anahtar Kelimeler: Glukuronidaz, Kemiluminesans, Biyosensör, İmmünomanyetik ayırma, Manyetik odaklama

INTRODUCTION

As a member of coliform group bacteria, *Escherichia coli* (*E. coli*) is often preferred as an indicator microorganisms of fecal and possible pathogen contamination from human or animal origin to monitor the microbiological safety of foods and water supplies [1, 2]. Current detection methods for coliforms and *E. coli* takes up to 48 hours for presumptive and 96-120 hours for the complete result [2]. Methods based on the β-glucuronidase (GUS) enzyme detection of *E. coli* in food and water with the incorporation of chromogenic and fluorogenic substrates shortened the presumptive detection time to 24-48 hours [2]. Although these methods have the advantage of high accuracy, they are still complicated in operation, lack of specificity and time-consuming. Therefore, the rapid, sensitive and more practical technique for *E. coli* detection is very important for environmental monitoring and food industry [2, 3].

Alternative approaches to conventional microbiological tests based on immunoassays, molecular techniques, immunomagnetic separation and biosensors are finding a wide range of commercial applications in the area of food safety [4, 5] with a trend of miniaturization and automation of these techniques [6]. Among them, biosensors and immunosensors are receiving attention and finding increasing application due to recent developments in nanotechnology, electronic and fiber optic technology, and instrumentation to detect microorganisms and other analytes [7-9]. Biosensors can detect and/or quantify chemical or biological molecules of interest when there is an interaction between the target molecule or analyte (such as an enzyme, toxin, antibody, receptor, DNA or microbial cell), and a biological sensing element connected to a transducing system. The transducer converts the observed biological change (physical or chemical) into a measurable response, most often an electrical signal proportional to analyte concentration. The transducer can be electrochemical [10] e.g. impedimetric [11], optical, e.g. fiber optic wave guides [12] and surface plasmon resonans (SPR) [13], mass change based such as quartz crystal microbalance (QCM) [14], and thermal [15]. The number of different types of biosensors is quite large and numerous research efforts have been made during the past decades with novel futures that have been devised for the detection of various pathogenic microorganisms and microbial toxins [16]. However, relatively less research efforts have been focused on developing biosensors for the detection of *E. coli*.

In recent years, alternative approaches that are combination of immunoassay, micro or nanospheres, immunobiosensors with various instrumentation and analytical procedures have been reported for the detection of *E. coli*. A separated electrode QCM biosensor based on the impedance alteration when the bacteria changed the chemical composition of the medium was used to detect *E. coli* in pure culture with a sensitivity ranging from 10^1 to 10^6 cells/mL, in the range of 10 h to 1 h [17]. Bouvrette and Luong [18] developed a flow injection analysis (FIA) immunosensor based on the fluorescence detection of glucuronidase enzyme to detect *E. coli* in artificially contaminated food samples with a detection limit of $5x10^7$ CFU/mL. A biosensor based on an acousto-gravimetric flexural plate wave (FPW) transducer and an immunoaffinity layer on the transducer membrane for the detection of *E. coli* was studied by Pyun et al. [19]. Another type of biosensor using electronic noses based on characterizing complex vapors and aromas by an array of QCM was developed by McEntegart et al. [20] for the detection of *E. coli* when the level of cells was $5x10^8$ organisms/mL in a growth medium. A microelectromechanical system based biosensor which utilized ssDNA to capture *E. coli* RNA with amperometric methods has been described by Gau et al. [21]. Gfeller et al. [22] developed a resonance-frequency-based mass sensor using an oscillating cantilever for the detection of active *E. coli* in less than 1 h. Ertl et al. [23] used lectins on a screen-printed biosensor array to rapidly detect and distinguish *E. coli* from other bacteria based on the recognition of distinct surface lipopolysaccharide structures. Ercole et al. [24] described an antibody based potentiometric biosensor based on the detection of pH variations due to NH_3 production by an urease-*E. coli* antibody conjugate to detect *E. coli* cells, with a sensitivity of 10 cells/ml in 1.5 h, in vegetable food. An electrochemical immunoassay for rapid detection of *E. coli* in surface water by anodic stripping voltammetry based on core–shell Cu@Au nanoparticles as anti-*E. coli* antibody labels was described by Zhang et al. [25]. Dudak and Boyacı [13] immobilized biotin labelled anti *E. coli* antibody on the gold surface of SPR sensor chip and they were able to detect, with a real time detection, $2x10^4$ *E. coli* cells/mL of water at comparable sensitivity to traditional method.

Immunomagnetic cell separation (IMS) method that combines the specificity of antibodies with a rapid concentration of the target cell [26] from heterogeneous cell suspension and food matrixes using magnetic particles in micron sizes [27, 28] offer new alternatives when coupled with chemiluminescent detection [29]. Chemiluminescence is the emission of light from a chemical reaction without an external light source and can be easily detected by a portable luminometer, simpler than fluorescence for instrumentation i.e. decrease in cost of the assay. Chemiluminescent reaction is much more sensitive than colorimetric and fluorescent techniques and can be coupled to an antigen-antibody interaction [30-32]. On the other hand, the use of optical fibers for transmitting the signal, serving as a transducer, is well known for biosensor applications [12].

In our earlier study, we developed an enzyme capture immunoassay for the detection of *E. coli*, in which anti-*E. coli* GUS antibodies were covalently immobilized on magnetic beads and used for the immunocapture assay [32]. In the assay, GUS enzyme from *E. coli* culture was captured using the prepared immunomagnetic-beads and the chemiluminometric measurement of GUS using a 1,2-dioxetene derivative as substrate were achieved. We were able to detect as low as 1 cfu/mL initial *E. coli*

within 8 hours of incubation in the growth medium. The developed method was also tested in food samples [33].

A magnetic focusing immunosensor has been developed by Pivarnik et al. [34] for the detection of *Salmonella* serotype (ser.) Typhimurium based on the concentration of a complex of immunomagnetic beads-Salmonella-fluorescent label in front of a magnetic probe that is attached to excitation and detection fibers. The probe was then modified into a tubular optical waveguide [35, 36].

To date, an enzyme capture immunoassay coupled with IMS using chemiluminescent reaction in a biosensor system has not been previously reported for the detection of *E. coli* GUS enzyme. This study extends our former work on the enzyme capture immunoassay and sensitive chemiluminometric detection of *E. coli* to a new bioosensor system using a magnetic focusing probe. Therefore, the objectives of the study were to design an optical biosensor using a magnetic focusing optical waveguide probe coupled with a chemiluminescent enzme capture immunoassay, and also to develop a multiwell format for the detection of GUS from *E. coli*.

MATERIALS and METHODS

Biosensor Design

The new magnetic focusing biosensor was constructed to accommodate chemiluminescent immunocapture assay as shown in Figure 1. The biosensor assembly consisted of (1) a magnetic focusing optical waveguide as a probe in front of a cuvette assembly and (2) a flexible fiber-optic light guide (Edmund Industrial Optics, Barrington, NJ, USA), 6.4 mm in diameter and 630 mm in length, connected to a luminometer (St. John Associates, Beltsville, MD, USA). The focusing probe was modified from a patented tubular optical waveguide [36] that had been developed from the magnetic focusing fiber optic probe used by Pivarnik et al. [34]. In the present study, the probe length and diameter differed from the previous design [35, 36] and was attached to a luminometer instead of being connected to an excitation light source. Also, waveguide did not have the sidearm optical fiber connected to the excitation source. The waveguide probe was made at the Physics Department at the University of Rhode Island by glass-blowing a borosilicate glass tube and tapering it gradually at the front end. A tapered magnet rated at 4600 Gauss, Neodymium 27 cylindrical plug magnet (Herbach and Rademan Company, NJ, USA) was fitted inside the tubular waveguide. It was possible to slide the magnet inside the waveguide with the movement of an external metal object. The front end of the waveguide served as the optical probe while the rear end served as the connector to the signal transmission fiber [35]. The probe assembly had a diameter of 7.2 mm at the largest section and a total length of 60 mm with a 1 mm wall thickness. The diameters of the tapered front and rear end of the probe were 2.5 mm and 6.1 mm, respectively. The flat front end of the probe was aligned in front of a plastic cuvette in vertically adjustable cuvette holder (Oriel Instruments, Stratford, MA) with a three screw adjustable ring mount measuring 53 mm in diameter. The cuvette holder was installed on a platform that could be moved on an x-axis metric stage to position and provide full contact between the cuvette wall and the flat cut front end of the magnetic probe. The rear end of the waveguide was matched to the tip of a flexible fiber-optic bundle using an index-matching (optical couplant) gel (Fiber Instrument Sales Inc, Oriskany, NY), which was secured by a ferrule and a fiber bundle connector assembly (Oriel). The fiber-optic bundle was attached to the luminometer that was connected to a MacLab/4 interface (ADInstruments, Castle Hill, Australia).

Preparation of Anti-Glucuronidase (anti-GUS) Immunomagnetic Beads

Anti-GUS antibodies were covalently immobilized on magnetic beads following the method given by Tağı et al. [32]. Carboxylated magnetic beads (Dynabeads M-270 , 2.8 μm diameter, $2x10^9$ beads/mL , 30 mg/mL) (Dynabeads M-270, Thermofisher Scientific., Grand Island, NY, USA) were washed with cold deionized water three times using a magnetic particle concentrator (Dynabeads MPC®-6; Thermofisher Scientific, Grand Island, NY, USA). The beads were activated by resuspending in 1 mL of 0.005 M 1-cyclo-hexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluensulfonate (CMC) (Sigma, St. Louis, USA) solution and incubated for 10 min at 4°C with a slow tilt rotation at 20 rpm using a Dynal sample mixer (Dynabeads®, Thermofisher Scientific). After incubation, and removing the supernatant with magnetic particle concentrator, 0.6 mL of 0.005 M CMC and 0.4 mL of 0.3 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 4.8, was added and incubated for 30 min at 4°C. To coat the magnetic particles, with GUS antibodies, the bead suspension was washed with cold 0.1 M MES, then 0.35 mL of 0.1 M MES containing 0.3 mg anti-β-glucuronidase rabbit polyclonal antibody (GUS-IgG) (Molecular Probes, Eugene OR, USA) was added to the beads (3 μg ligand for $1x10^7$ beads), and the antibody-immunomagnetic bead mixture was incubated for 2 h at 4°C with the sample mixer. The antibody-conjugated magnetic beads were then mixed with IgG free bovine serum albumin (BSA, Sigma) for blocking and incubated for another 4 h in the same condition as above and were washed with PBS (pH 7.4) containing 0.1% (v/v) Tween 20 and triton X-100 (PBS-TX). Beads were stored in PBS containing 0.5% (w/v) BSA and 0.02% (w/v) sodium azide at 4°C.

Determination of the Number of Magnetic Beads

In order to verify the number of magnetic beads either before antibody immobilization or in the assay buffer, a Petroff-Hausser blood counting chamber (Hausser Scientific Co., Horsham, PA, USA) was used. The bead suspension in PBS-TX was transferred to fill the counting chamber and covered with a cover glass, and counted on a systematically selected 10 fields in each counting field using a light microscope (total magnification, x450-675).

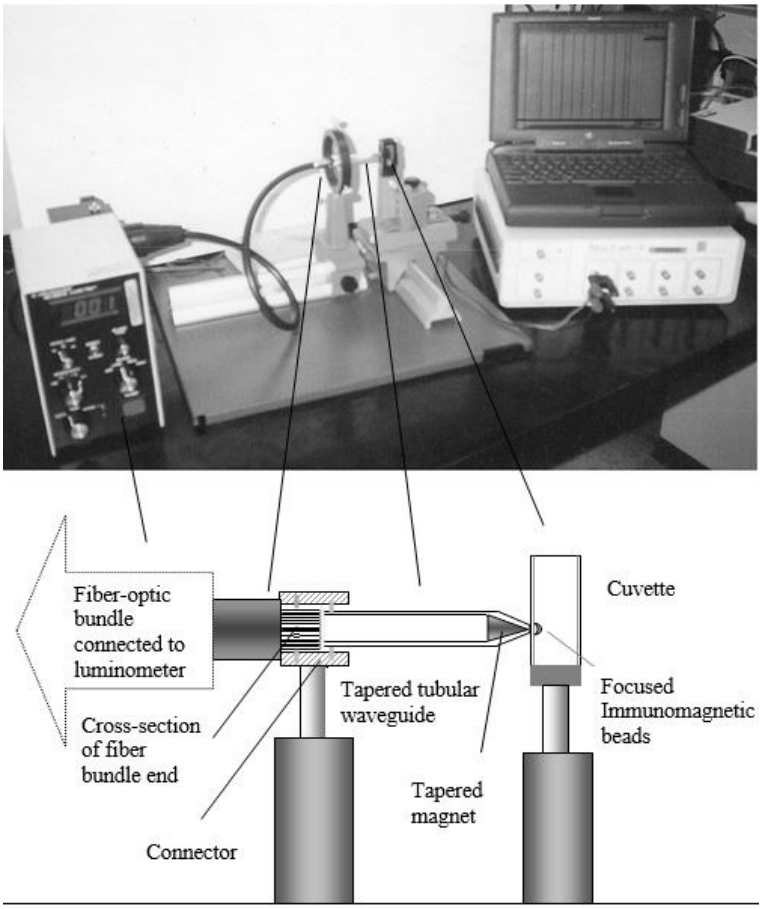

Figure 1. Tubular tapered waveguide positioned in front of a chemiluminescent reaction cuvette. The waveguide was connected to the fiber bundle transmitting the light to the luminometer

Determination of the Protein Concentration

GUS enzyme protein was determined based on the method of Bradford using the Bio-Rad Protein assay kit (Bio-Rad Labs., Hercules, CA) in a Perkin Elmer Lambda 4B spectrophotometer (Waltham, MA, USA). Bovine serum albumin (Sigma) was used as the standard protein.

Chemiluminescent Immunocapture Immunoassay Procedure

Three assay formats were studied: a; measurement in luminometer (luminometer format), b; magnetic focusing, b1; without magnetic focusing, and c; in microwells of microtiter plate (microwell format). The chemiluminescent immunocapture immunoassay procedure was adapted from the method described by Tağı et al. [32]. Glucuronidase (GUS) enzyme from *E. coli* (Sigma) was commercially obtained. A ratio of 5 µL of magnetic beads (equal to ~$5x10^6$) to 1 mL of GUS enzyme aliquot in 50 mM PBS, pH 7.4, was used for the assay. The reaction mixture in a 2 mL volume screw cap sterile microcentrifuge tube (Denville Scientific Inc., Holliston, MA, USA) was mixed by rotating and tumbling, using a Dynabeads® sample mixer (Thermofisher Scientific., Grand Island, NY, USA) at a rotation speed of 20 rpm to allow GUS binding to the IgG immobilized on the beads at 37°C for 30 minutes. Following the incubation, the magnetic beads-enzyme complex was collected on the inner side of the tube for 1.5 min using the magnetic particle concentrator and the reaction fluid was removed by aspiration. Then the beads were resuspended in 1.5 mL PBS containing 0.05% Tween 20 (v/v) and vortexed briefly at moderate speed. The washing process was repeated three times. After the final wash, the beads-enzyme complex was resuspended in 50 µL of 50 mM PBS-ET and was used for each developed format as follows.

Luminometer Format (a)

The magnetic beads-enzyme complex in PBS-ET was transferred into a 9 x 42 mm size luminometer tube (St. John Associates) that contained 180 µL of adamantyl 1,2-dioxetane arylglucuronide substrate Glucuron™, (Tropix, Applied Biosystems, Foster City, CA, USA) at a final concentration of 120 µM. The assay mix was incubated at 37°C for 30 min. Following the incubation and brief mixing, 300 µL of light emission accelerator solution Emerald™ (Applied Biosystems) was injected manually into the reaction mixture to trigger the light in the luminometer tube that was placed in the luminometer chamber. Chemiluminescence intensity

was recorded as peak value after 5 seconds delay by a MacLab/4 interface (ADInstruments, Castle Hill, Australia). Enzyme activity was expressed as net millivolt (mV) light intensity peak subtracting the background (blank) intensity.

Magnetic-Focusing Format (b)

The magnetic beads-enzyme complex in PBS-ET was transferred into a 12 x 50 mm semi-micro plastic (polystyrene) cuvette (St. John Associates, Beltsville, MD). The cuvette was preincubated at 37°C for 3 min, and then 180 µL of Glucuron™, was added in the assay solution that was incubated at 37°C for 30 min in a water bath. Following the incubation and brief mixing, the cuvette was placed in front of the magnetic probe attached to a Lumi-Tec Luminometer as decribed below. After magnetic focusing for a total of 3 min, which is sufficient to collect all beads, 300 µL of light emission accelerator solution (Emerald™, Tropix, Applied Biosystems, MA, USA) was injected manually to the reaction mixture, and then chemiluminescence intensity was measured by the luminometer and recorded as peak by a MacLab/4 interface (ADI Instruments). Enzyme activity was expressed as net milivolt (mV) light intensity peak, subtracting the background (blank) intensity.

Format without Magnetic Focusing (b1)

In order to compare the efficiency of magnetic-focusing to the measurement of chemiluminescence of immunomagnetic beads-GUS in suspension, chemiluminescence measurement was repeated for the same concentration of GUS without magnetic-focusing under the same measurement conditions as above. This was done by sliding the tapered magnet from the front end of the tubular optical waveguide backward inside the tubular wave guide by applying an external metal rod. In this case, chemiluminescent reaction was carried out as above after the cuvette was placed in front of the tubular waveguide without magnetic-focusing.

Microwell Format (c)

Application of the chemiluminescent assay in microwells of microtiter plate was done as follows. After immunocapture of GUS in an enzyme solution and following washing steps, as described above, 5 µL of immunomagnetic particles were resuspended in 25 µL of PBS-ET and added into microwells of a 96-well polystyrene flat bottom microtiter plate (Nunc®, Sigma) containing 90 µL of glucuron (assay volume was reduced almost by half to fit the working volume of microwell of the plate). Following incubation at 37°C for 30 minutes, 180 µL of a light emission accelerator solution was injected into the reaction mixture in each well, and chemiluminescent intensity was measured by a flexible fiber-optic light guide (Edmund Industrial optics, NJ), 6.4 mm in diameter and 630 mm in length, adapted to the luminometer. The light guide was positioned with a supporting fitting over the well to collect the light upon the chemiluminescent reaction as shown in Figure 2.

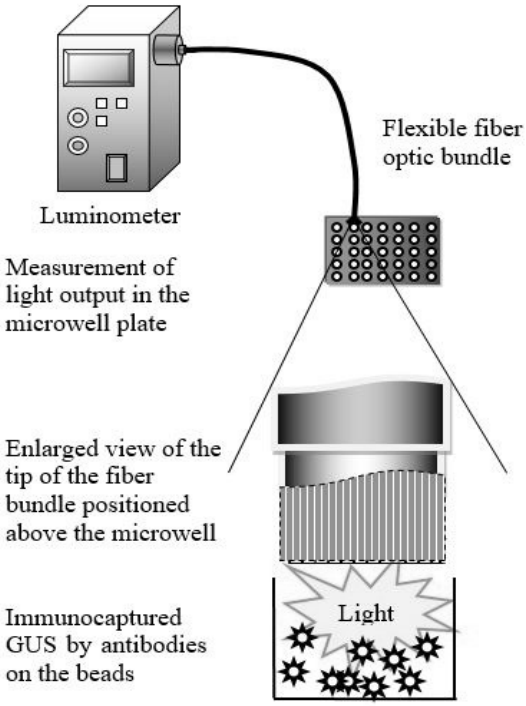

Figure 2. Schematic representation of chemiluminescent GUS detection in microwell format

Dose Response Curve

The calibration curve was prepared by increasing concentrations of GUS using GUS stock solution (1 mg/mL) to give final protein levels between 1 and $1x10^6$ pg/mL prepared in 50 mM sodium phosphate buffer, pH 7.0, containing 5 mM EDTA. In order to test the detection limit for the each immunocapture assay format, except the format without magnetic focusing (b1), "chemiluminescent immunocapture immunoassay procedure" given above was followed.

Statistical Analysis

Data were reported as the means of 3-5 replicates with the standard deviation (SD) for each mean. The relationship between chemiluminescence and GUS enzyme concentration was established by a linear correlation plot (Microsoft Corporation. 2013. Microsoft Excel User's Guide).

RESULTS and DISCUSSION

The chemiluminescent assay was done with magnetic focusing, without magnetic focusing and microwell formats and compared to the luminometer format using a serially diluted GUS enzyme solution under the same assay conditions given in the material methods section. The limit of detection for GUS enzyme was determined to be three times the standard deviation of the noise (blank signal) for each assay format as commonly practiced. GUS activity that was measured by chemiluminescent assay was plotted against increasing concentrations of GUS to obtain dose-response curve. A log-log plote showed a linear relationship between chemiluminescence intensity and amount of GUS (Fig. 3). The data and the plots represented a typical chemiluminescent immunoassay characteristic for the substrate, adamantly glucuronide [31]. The results were comparable to reported studies [37, 38]. The slopes for the luminometer and microwell formats were higher than that for the magnetic focusing format. The detectable lowest concentration of GUS was 1 and $1x10^3$ pg/mL for the luminometer and magnetic focusing formats, respectively while it was $1x10^2$ pg/mL for the microwell format. The detection limits for the tree formats were lower than a recently reported limit of detection as $1x10^4$ pg/mL for GUS measured with an amperometric detection method [39]. Although the correlation coefficient values were high for each assay format, the estimated value of either GUS or chemiluminescent signal were found to be below or above the experimental values. For example, when the GUS concentration was $1x10^2$ pg and $1x10^5$ pg/mL for the luminometer format, the corresponding calculated values from the chemiluminescent assay were 81 and 80500 respectively, whereas the predicted value from the dose response plot were 150 and 49000. A similar concern was expressed in a study by Ye et al. [37] where they established calibration curve between chemiluminescence signal and *E. coli* O157:H7 cells based on horseradish peroxidase (HRP) labeled detection by a fiber optic biosensor. Therefore, in the present study, GUS enzyme detection was compared experimentally for each format by using fixed amount of GUS enzyme for a better comparison as shown in Table 1 and 2.

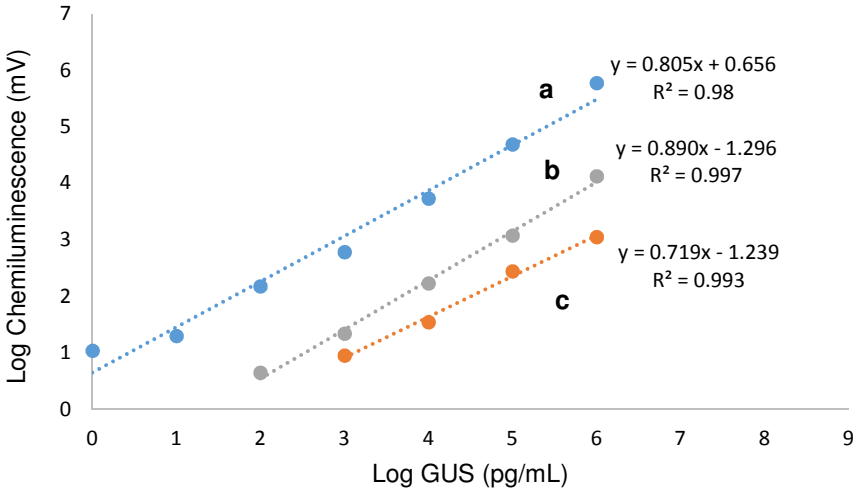

Figure 3. Comparison of log-log dose-response curves for GUS by chemiluminescent assay. a: Luminometer format, b: Microwell format, c: Magnetic focusing format

The results of chemiluminescent GUS detection with magnetic focusing and without magnetic focusing, which were done simultaneously in comparison to detection in luminometer format, were given in Table 1. Magnetic focusing did not increase the chemiluminescent signal when compared to the signal measured with the luminometer format. In contrast, a greater chemiluminescent signal was obtained with no magnetic focusing in comparison to magnetic focusing format. Chemiluminescent signal with magnetic focusing, decreased by almost half (59%) compared to without magnetic focusing, showed that focusing beads in front of the light guide actually decreased the chemiluminescent light intensity probably by blocking

the signal in the solution. In comparison to the detection of GUS in the luminometer, 90 and 220x less signal was collected when the cuvette was placed in front of a tubular waveguide with no magnetic focusing and with magnetic focusing, respectively (Table 1). In the present study, as appose to our expectation, no signal increase was obtained. However, a 20-fold increase in signal was reported by Pivarnik et al. [34] when they focused immunomagnetic bead-Salmonella-fluorescent label complex, as compared to homogenous suspension. This partially may be due to the nature of the chemiluminescent assay. Chemiluminescent reaction itself provides light without being excited by an external light source as in the fluorescence, and emission of light occurs by the free molecules in the reaction solution [30].

Table 1. Chemiluminescent GUS activity when measured in front of a tubular waveguide compared to a luminometer format ($x \pm s_x$; n=3)

Chemiluminescent GUS activity (mv)		
Detection in luminometer	Detection in front of magnetic focusing probe	
	Without magnetic focusing	Magnetic focusing
41400±8600	460±95	188.75±41

Following the incubation of bead-Ab-GUS complex with substrate and after light emission accelerator solution was injected, beads were collected in front of magnetic focusing probe and the reaction mixture aliquot was separated, then chemiluminescence was measured in these fractions for comparison of GUS activity. Chemiluminescent signal was 270 (±60) and 190 (±45) mV for the collected beads and separated free solution respectively. The results confirmed that some of the chemiluminescent enzyme reaction product diffused into the solution rather than concentrating around the GUS enzyme captured by the anti-GUS antibodies on the beads. Furthermore, the amount of immunomagnetic beads were increased from 5 µL to 10 µL to increase the captured GUS and the chemiluminescent signal (Fig. 4). However, chemiluminescent signal did not increased with the increasing amount of beads as shown in a previous study by Tağı et al. [32]. In contrast, increasing the amount of beads led to a proportional decrease in chemiluminescent signal measured, which indicated that the light signal was physically prevented from reaching to the front end of focusing waveguide resulting in a less chemiluminescent signal.

As a result, magnetic focusing under these conditions was not useful for a chemiluminescent assay which was based on the measurement of product by a captured enzyme. This approach could be more useful when used with a secondary fluorescent-labeled antibody followed by detection of the emission from the label [34, 40].

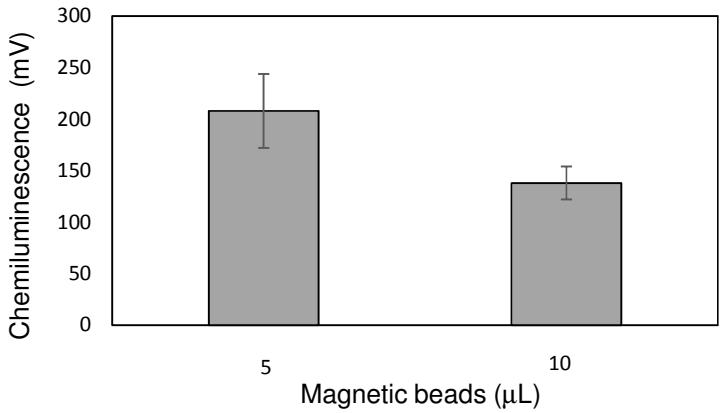

Figure 4. Effect of the amount of magnetic beads on the chemiluminescent signal when magnetic focusing format was used

A microwell format was tested for the chemiluminescent assay. In this format, a flexible fiber bundle effectively permitted the transfer of the light signal to the luminometer after the light was triggered from the enzyme-magnetic beads reaction mixture in a microwell. The presence of GUS enzyme at 100 pg/mL as minimum concentration was detected by this format. There was a 52x decrease in chemiluminescent signal compared to detection by the luminometer format.

The main reason for the decrease in signal, to a large extent, was probably the decrease in the volume of light emission accelerator almost by half to fit the total assay volume to the standard shallow micro well size. Also delay due to the manual measurement of the light with the fiber bundle might cause signal decrease. Standard multi-well microtitre plates come in a range of sizes, with shallow well plates having well volumes on the order of 200 to 300 µL and deep well plates typically having well

volumes of 1.2 -2.0 mL. Use of commercially available deep well plate system [41] with elevated well walls with higher volume capacity may provide a higher loading capacity thus increase in chemiluminescence signal.

Table 2. Chemiluminescent GUS activity when measured in a microwell format, compared to a luminometer format ($x \pm s_x$; n=3)

Chemiluminescent GUS activity (mv)	
Detection in luminometer	Detection in microwell
37700±7300	720±200

The presence of GUS enzyme in aliquots could be detected in a total of 70 min including IMS capture of GUS (30 min), washing steps (5 min), enzyme–substrate reaction and measurement (35 min). This was relatively a shorter assay time due to direct immunocapture of enzyme and detection of enzyme activity directly without the need for a secondary labeled antibody, which was comparable with that of other reported immunoassay based methods. For example, a chemiluminescence biosensor that was based on the antibody– coated bead – bacteria –peroxidase labeled antibody sandwich complexes were used for the detection of *E. coli* O157:H7 and the assay took 1.5 hours [37]. Amperometric detection method for *E. coli* beta-galactosidase by bead-based immunoassay required less than 60 min [42]. Another study using immunomagnetic separation and sandwich assay with secondary antibodies labelled with quantum dot using fluorescence detection needed 90 minutes for *E. coli* detection in water [13]. Zhang et al. [25] could detect *E. coli* in 2 h using an electrochemical immunoassay by anodic stripping voltammetry (ASV) based on core–shell Cu@Au nanoparticles (NPs) as anti-*E. coli* antibody labels.

Delay in GUS measurement was due to use of manual injection followed by manually positioning the front end of the fiber bundle over the well to collect the light upon chemiluminescent reaction. Another contributing factor was the signal loss in the fiber as reported by the manufacturer [43]. A 12% light loss in the 63 cm flexible fiber bundle was calculated based on the manufacturer data. Therefore, use of fiber with different properties such as larger diameter and shorter length can be considered to reduce light loss and this way some signal increase could be achieved. On the other hand, the system is amenable to automation and lends itself to redesign and portability due to the use of a flexible fiber-optic bundle and the multiwell format. In addition, the decrease in sensitivity due to the delay caused by manual injection could be compensated by combining automated injection system [44] with positioning of the fiber bundle end above or inside the well.

CONCLUSIONS

In contrast to our expectation, magnetic focusing system did not improve chemiluminescent signal for the measurement of *E. coli* GUS, and was not more sensitive than our original immunomagnetic enzyme capture immunoassay. In that sense, magnetic focusing system can be considered unsuitable. In spite of that, magnetic focusing system itself seems to working and worth improving with different approach or format which could eliminate masking effect of clustered immunomagnetic beads in front of the capture probe. The response of microwell format was better than the immunomagnetic focusing system. The microwell format, on the other hand, had the potential to be improved. Consequently, the developed microwell format could be used for the measurement or detection of GUS from live *E. coli*, which could eventually be used for the detection of active *E. coli* cell in culture or in foods after testing with food system. However, the system should be tested with *E. coli* culture to establish detection time and detection limit.

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