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<th>Rapid determination of vitamin B12 concentration with a chemiluminescence lab on a chip</th>
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<td><strong>Author(s)</strong></td>
<td>Lok, Khoi Seng; Siti Zubaidah Abdul Muttalib; Lee, Peter Peng Foo; Kwok, Yien Chian; Nguyen, Nam-Trung</td>
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Rapid determination of vitamin B₁₂ concentration with a chemiluminescence lab on a chip

Khoi Seng Lok, Siti Zubaidah binte Abdul Muttalib, Peter Peng Foo Lee, Yien Chian Kwok and Nam-Trung Nguyen

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Introduction

Vitamin B₁₂ (cobalamin) is an organic complex that contains a cobalt ion in its structure. This vitamin is an important coenzyme for cell development and growth, and its deficiency leads to weakness, fatigue, anemia, constipation, weight loss, pernicious anemia and nerve degeneration. Peculiarly, vitamin B₁₂ is essential for the human diet, but cannot be synthesized by the body. This vitamin has to be obtained from natural sources such as fish, dairy products, egg, meat and poultry. However, compared to other vitamins, the daily requirement of vitamin B₁₂ is relatively low. The determination of vitamin B₁₂ in human serum at picogram level becomes necessary for the detection of its deficiency. Hence, sensitive analytical detection devices are required. To counter the deficiency of vitamin B₁₂, many supplements are available in the form of tablets, capsules and injections. Correspondingly, related quality checks of these products are needed. Furthermore, vitamin B₁₂ analysis is important for the determination of its quantity and activity in food and microbial fermentation broth, as well as for the assessment of chemical properties of algal vitamin B₁₂.

Methods such as microbiological assay, radioisotopic assay, spectrophotometry, high-performance liquid chromatography, fluorometric assay, capillary electrophoresis, chemiluminescence (CL), enzyme-linked immunosorbent assay and surface plasmon resonance-based biosensor were developed for quantifying Vitamin B₁₂ for various applications. Among all these different methods, the sensitivity of the CL-based assay is the highest and can be as low as 5 pg/ml. Qin et al. first introduced this concept in 1997. The injected vitamin B₁₂ sample was acidified with diluted HCl in a mixing coil, followed by pH adjustment with Na₂CO₃. Next, the acidified vitamin B₁₂ sample was mixed with luminol and ad-hoc generated H₂O₂. Finally, CL signal was presented to the flow cell sensor. Sou and Hou reported a similar setup, but they enhanced the CL reaction with dissolved oxygen and performed pre-treatments to the samples. Akhay and Gok introduced a new external acidification method followed by the regular FIA-CL sensor. Recently, Kumar et al. reported a CL-based method using urea-H₂O₂ for determining the concentration of vitamin B₁₂ using a luminometer platform. All these works aimed to advance the chemical procedures associated with the conventional FIA-CL sensors or luminometers. Despite the numerous vitamin B₁₂ assays that are currently available, there is a need for improved assays to overcome the shortcomings of the above state-of-the-art techniques. These shortcomings include the lack of sensitivity, difficulties encountered in standardization of test procedure, and the impracticable use of high-volumes in certain situations (e.g., in hospitals). Moreover, commercial non-isotopic immunoassays for the detection of vitamin B₁₂ are not available. The recent advancement of lab-on-a-chip technology would allow the implementation of a vitamin B₁₂ assay into microfluidic system.

Lab-on-a-chip (LOC) or micro total analysis system (µTAS) aims to implement chemical analysis onto a miniature platform. Microchannel networks can be integrated into a microchip to allow mixing of reagents, reaction and detection. Specialised microchips had been made for CL detection in an array of analytes, including the determination of hydrogen peroxide in rainwater, glucose, luciferase, cobalt(II) ions, benzyl peroxide in flour etc. Microfluidic devices offer rapid analysis with high sensitivities, low costs and parallelization, which can address the shortcomings of the existing vitamin B₁₂ assays.

CL-based assays, utilizing luminol, lophile or gallic acid can be used for the detection of cobalt ions. Cobalt(II) ion serves as an effective catalyst in the oxidation of luminol, in the presence of hydrogen peroxide in alkaline medium condition. This reaction emits blue light at a wavelength of 425 nm. But, the cobalt in vitamin B₁₂ complex itself does not catalyse the CL reaction. The solution of vitamin B₁₂ has to be acidified to set the cobalt ions free for catalysis and detect presented ions. The amount of cobalt(II) ions detected correlates directly to the concentration of the vitamin. Therefore, the acidification process is crucial for the determination of the vitamin presented. A variety of methods have been implemented. Kumar et al.
acidified the vitamin B₁₂ solution with 9 M of HNO₃. Excess HNO₃ was then removed with 5.5 M of HCl, followed by dilution without heating to dryness. The sample was mixed directly with diluted HCl in an acidification channel, followed by its immediate detection using a luminol-peroxide CL assay in the microchannel network. In the second practice, the vitamin B₁₂ samples were externally acidified in a digester. The acidified extracts were then quantitatively analysed using the CL assay in a microfluidic system. A milder acidification procedure was also introduced and tested. Real samples using vitamin B₁₂ tablets and hen eggs were analysed. The LOC device shows good correlations between the emitted CL intensities and vitamin B₁₂ concentrations. The device reported here is small in size, light in weight, portable and has an analysis performance comparable to current FIA/CL detection instruments. These features allow the use of the our system in the field and at remote locations.

**Experimental**

**Conceptual design of the microfluidic system**

Figure 1(a) shows the schematic diagram of the detection concept. The continuous-flow system consists of programmable infusion pumps, a microchip, and a detector. The infusion pumps introduce the reagents continuously. The microchip houses the microchannel network. The detector records a steady CL signal emitted from the reaction. The conventional luminol-peroxide CL chemistry was adopted. The system was designed to operate in two modes, I and II. In mode I, vitamin B₁₂ sample was directly acidified in the microfluidic device. Vitamin B₁₂ sample, hydrochloric acid (HCl), sodium hydroxide (NaOH), luminol and hydrogen peroxide (H₂O₂) were introduced into the system through inlets A, B, C, D and E respectively, Fig. 1(a). Vitamin B₁₂ sample was mixed with diluted HCl to liberate the cobalt(II) ions in the acidification channel. Next, NaOH was introduced to neutralize the acidified sample. This mixture was then mixed with luminol and H₂O₂ in the reaction channel. The emitted CL signal from the mixture was detected in the detection channel. In mode II, the acidification of the vitamin B₁₂ sample was carried out outside the microfluidic system. The inlets A and B of the microchip were blocked. The treated vitamin B₁₂ sample, luminol and hydrogen peroxide (H₂O₂) were introduced into the system through inlets C, D and E respectively, Fig. 1(c). The vitamin B₁₂ sample was mixed with luminol and H₂O₂ in the reaction channel.

A multi-syringe (KDS220, KD Scientific, MA, USA) and a double-syringe infusion pump (KDS200, KD Scientific, MA, USA) was used to deliver the reagents such as HCl, NaOH, luminol and H₂O₂. Another single-syringe infusion pump (KDS100, KD Scientific, MA, USA) was used to deliver the test sample, in this case, the acidified vitamin B₁₂ solution, deionised water or hydrochloric acid (HCl). The reagents were stored in disposable 5-ml Terumo syringes to reduce cross-contaminations. Although, the different inlet streams may be adjusted to run at different flow rates, the same flow rate was used in our work to simplify the operation of the system. The flow rate of each syringe was set at 0.5 ml/min, giving a total flow rate of 2.5 ml/min and 1.5 ml/min in the mode I and II, respectively.

In this paper, we first explored the use of a continuous-flow microfluidic device for the CL detection of cobalt(II) ions in vitamin B₁₂. A LOC device containing two passive micromixers and a double spiral microchannel network was designed and fabricated. The micromixer resolved the mixing issue of the laminar flow of a fluid in microchannels, allowing rapid reagents and sample mixing and faster detection. The double spiral microchannel network allows a better presentation of CL signals to a photon detector as compared to a single spiral in the conventional flow cell. This device was specially designed to embrace two current laboratory practices. In the first practice, the vitamin B₁₂ sample was mixed directly with diluted HCl in an acidification channel, followed by its immediate detection using a luminol-peroxide CL assay in the microchannel network.
Bone structure in each turn, providing six periods of mixing formed by a clockwise spiral and an anti-clockwise spiral which turning width of the channel is 2.8 mm. The double spiral was a height of 0.9 mm and a width of 1 mm. The spiral channels overlay each other perfectly. The spiral channels are connected to a via hole in the centre of the spirals. The spiral channels have a height of 0.2 mm and a width of 0.5 mm. This micromixer adopts the working principle of chaotic advection similar to our previously published work.\(^{24}\) The micromixer has two periods of herring-bone structure and 0.4 mm deep. The herring-bone structure has a height of 0.2 mm and a width of 0.5 mm. This micromixer adopts the working principle of chaotic advection similar to our previously published work.\(^{24}\)

Figure 2(a) depicts the microchannel network of the microchip. This network was fabricated into three layers. The first layer works as the cover for the microchannels in the second layer. The microchannel network in the second layer consists of a passive micromixer and a clock-wise spiral microchannel for detection. The third layer contains another passive micromixer together with a matching anti-clock-wise spiral microchannel. The passive serpentine micromixer is 62 mm long, 1.8 mm wide and 3 mm in thickness.

![Figure 2 Lab on a chip for determination of vitamin B\(_{12}\) concentration: (a) microchannel network (A to E are inlets; W is outlet to waste; arrows indicate the fluid flow); (b) Fabricated microchip. The chip measures 45 mm × 30 mm × 3 mm.](image)

and reflected the CL light back to the detector. A plano-convex lens (30 mm in diameter, 30 mm focal length, VIS0 coating, Edmund optics, Singapore) focuses the scattered CL light into the 8-mm aperture of the photon detector. The detector is based on a photomultiplier tube (PMT) sensor, made especially for low-level light detection. The sensor is sensitive for wavelengths between 300 nm and 650 nm, highly responsive to typical 420 nm light at 270,000 per s.pW. Integration time can be set between 10 to 10,000 ms.

Figure 2(b) shows the microfabrication process of the fabricated microchip. The PMMA parts were subsequently aligned and secured with masking tapes. The assembled parts were subsequently sandwiched between two clean polished silicon wafer and thermally bonded at 165°C and low pressure.\(^{25}\) Metal tubes were attached to the device by epoxy adhesive to work as inlets and outlets. The final dimensions of the fabricated microchip were 45 mm in length, 30 mm in wide and 3 mm in thickness.

### Data analysis

The minimum sample volumes required for the analysis in modes I and II were 30 µl and 50 µl, respectively. The corresponding minimum analytical times were 3.6 s and 6 s for modes I and II, respectively. In order to achieve steady CL signals longer analysis duration was used in our experiments. The CL signals were captured by the photon detector at a 50-ms time intervals. This time interval was chosen to detect the signal produced by the investigated concentrations of the analyte without saturation. A customized program was written to log the captured data points in a computer. A sample size of at least 1000 data points was collected for each run, and each experiment was repeated at least five times. The data were subsequently imported and analyzed using Microsoft Excel. The photon counts were averaged and standard deviations were calculated. All the experiments and readings were conducted and recorded in a dark room to reduce background noise.

Background signals were recorded by running the CL assay in the system with deionized water. A solution of 1.0 M HCl was used to flush out the residual cobalt(II) ions in the microchip between each experimental run.

### Chemical reagents

All the reagents were of analytical grade, and prepared fresh before each experiment. Ultrapure water (18.2 MΩ cm) was obtained from a Milli-Q purification system (Millipore, Japan). Luminol and pure crystalline vitamin B\(_{12}\) were purchased from Sigma-Aldrich (USA). 30% v/v hydrogen peroxide (H\(_2\)O\(_2\)) and bicarbonate buffer (pH 10) were obtained from Scharlau (Spain). A stock solution of luminol (10 mM) was prepared by dissolving the luminol in 1.5 ml of 0.1 M sodium hydroxide made up to 10 ml in bicarbonate buffer. Further dilutions of this stock solution were prepared using bicarbonate buffer to obtain concentrations of 1 µM to 1 mM luminol solutions for the analysis. An amount of 1 mg of vitamin B\(_{12}\) was dissolved in 1 ml of bicarbonate buffer in running deionised water for a minute and wiped cleanly. A high pressure air gun was used to remove any remaining dust particles. The PMMA parts were subsequently aligned and assembled. The parts were then secured with masking tapes. The assembled parts were subsequently sandwiched between two clean polished silicon wafer and thermally bonded at 165°C and low pressure.\(^{25}\) Metal tubes were attached to the device by epoxy adhesive to work as inlets and outlets. The final dimensions of the fabricated microchip were 45 mm in length, 30 mm in wide and 3 mm in thickness.

### Micro fabrication

The LOC device and the holder were designed using CorelDraw (Corel Co., Canada), a vector graphics software. A commercial CO\(_2\) laser system (Universal M-300, Universal Laser Systems Inc., Arizona, USA) was used to transfer the designs to a transparent Polymethyl methacrylate (PMMA) substrate. For the lens holder, a 2-mm thick PMMA sheet was used. The machined parts were assembled and covered with black paper. PMMA sheet of 1-mm thickness was used for the LOC device. After machining, the parts were washed in 99% denatured ethanol for 30 seconds, followed by rising in running deionised water for a minute and wiped cleanly. A high pressure air gun was used to remove any remaining dust particles. The PMMA parts were subsequently aligned and assembled. The parts were then secured with masking tapes. The assembled parts were subsequently sandwiched between two clean polished silicon wafer and thermally bonded at 165°C and low pressure.\(^{25}\) Metal tubes were attached to the device by epoxy adhesive to work as inlets and outlets. The final dimensions of the fabricated microchip were 45 mm in length, 30 mm in wide and 3 mm in thickness.
buffer to give a stock solution of vitamin B₁₂ at 1 mg/ml. This stock solution was either acidified or used directly. Further dilutions of this stock solution were prepared in bicarbonate buffer to obtain concentrations of 1 pg/ml to 1 µg/ml of vitamin B₁₂ after the acidification.

Acidification of vitamin B₁₂

In mode I, using the same working principle in Qin et al.’s work, 0.2 M HCl was used to acidify vitamin B₁₂ directly in the microchip. In mode II, a milder acidification method was devised from a combination of the work done by Kumar et al. and Akbay and Gok. 0.4 ml of the stock solution (1mg/ml) was added to 4 ml of 5M nitric acid (HNO₃) and heated to dryness on a heating magnetic stirrer inside a fume hood. Subsequently, 4 ml of 3M HCl was added to remove any traces of HNO₃ in the solution, giving a concentration of 100 µg/ml of acidified vitamin B₁₂. The solution was then heated at approximately 95°C for 4 minutes and cooled. The actual concentration of the acidified stock vitamin B₁₂ was verified by UV-visible spectrophotometry. The residue was subsequently diluted to concentrations of 1 pg/ml to 1 µg/ml in bicarbonate buffer.

Preparations of vitamin B₁₂ supplements and egg yolk

The preparation process of the samples were adopted from Hou and Song. Vitamin B₁₂ tablets were obtained from a local nutraceutical retailer, GNC. The tablets were weighed and grounded to a fine powder. An amount of 500µg of vitamin B₁₂ powder was accurately weighed and dissolved into 100 ml deionised (DI) water in a clean, sterilised conical flask. An amount of 0.5 ml of this prepared vitamin B₁₂ solution was then added to 5.0 ml of HCl and further diluted with 25 ml DI-water in a 25-ml flask. Eggs were purchased from a local supermarket. They were pre-treated according to the literature. Approximately 5.0 g of boiled egg yolk was weighed, grounded and acidified with 50 ml of 0.5 M HCl acid in a digester. The egg yolk was then digested ultrasonically until a homogeneous mixture was obtained. The mixture was centrifuged and the supernatant was obtained and filtered. The filtrates were then further analysed. All processed samples were analyzed by our CL lab-on-a-chip system and UV-visible spectrophotometry. With the determination of vitamin B₁₂ in UV-visible spectrophotometry, a calibration curve was obtained by serial dilutions of the vitamin B₁₂ solution at a wavelength of 361 nm. The concentrations of vitamin B₁₂ in the samples were determined by comparing the recorded value to this calibration curve.

Results and discussion

Fabricated microchip

As mentioned above, the LOC devices were fabricated in polymethyl methacrylate (PMMA) substrate, using laser ablation and thermal bonding, Fig. 2(b). PMMA is a well suited material for this application as compared to other polymeric substrates. The inert and hydrophobic surface of PMMA leads to minimum interaction with the reagents. Therefore, this material provides an excellent resistance against alkaline and acidic chemicals. This property is especially important for a vitamin B₁₂ assay as the samples are treated in an acidic medium, followed by the detection in an alkaline medium. A 3-mm thick PMMA sheet has a transmission of visible light up to 92% leading to a good sensitivity for CL measurement.

Optimization of reaction conditions

Since the microchip was designed to operate in two different modes, the device has to be optimized separately. In mode I, the system has five inlets (Fig. 2(a)) which determine the flow rates of the reagents and need to be optimized accordingly. The flow rates of the reagents affect the final pH level of the mixture. In mode II, the system has three inlets. The concentration of H₂O₂ and luminol are the main factors that affect the CL reaction.

Effect of flow rate in mode I. First, the optimum flow rate of the microfluidic system was determined. Reagents of 0.2 M HCl, 1 µg/ml vitamin B₁₂, 0.2 M NaOH, 1 mM Luminol and 3% v/v H₂O₂ were introduced into the microchip at flow rates ranging from 0.5 to 3.5 ml/min. The CL intensity recorded against the different flow rates are shown in Fig. 3(a). As expected, CL intensity increased with increasing flow rates from 0.5 to 2.5 ml/min. However, if the flow rate increases beyond 2.5 ml/min, the CL intensity decreases drastically from 3.2×10⁴ to 3.7×10³. At a high flow rate, the residence time in the micromixer becomes shorter (1.037 seconds for a flow rate of 2.5 ml/min), and the acidified samples may not mix well with NaOH before entering the detection channel. The subsequent luminol CL reaction was carried out in an unfavourable acidic condition, resulting in a poor CL signal. Since the flow rate of 2.5 ml/min gave the highest CL signal, this flow rate was used in our subsequent experiments.

Effect of HCl concentration in mode I. The concentration of HCl played an important role in mode I. To investigate the effect of HCl concentration, solutions of 0 to 0.5 M HCl, 1 µg/ml vitamin B₁₂, 0.2 M NaOH, 1 mM Luminol and 3% v/v H₂O₂ were introduced into the system. Figure 3(b) shows the effect of HCl on the CL intensity. CL intensity increased from 0 M to 0.2 M HCl, because the amount of liberated cobalt ions increases with more a higher acid concentration. However, the CL intensity decreased rapidly from 0.2 M to 0.5 M HCl, because the excess acid alters the pH condition unfavourable for the subsequent luminol-CL reaction.

Effect of NaOH concentration in mode I. The concentration of HCl was fixed at 0.2 M to study the effect of NaOH concentration on this system. NaOH can neutralise the acidified sample. Furthermore, it can be used to adjust the pH level of the reaction. Solutions of 0.2 M HCl, 1 µg/ml vitamin B₁₂, 0 to 1 M of NaOH, 1mM Luminol and 3% v/v H₂O₂ were introduced into the system. The effect of NaOH on CL intensity is shown in Fig. 3(c). Figure 3(c) clearly shows that 0.2 M of NaOH gave the highest CL signal. At low concentrations of less than 0.2 M NaOH, the amount of NaOH introduced may not be sufficient to neutralize the acidified sample completely. At higher concentrations of more than 0.2 M NaOH, the amount of NaOH makes the pH level too alkaline, which is unfavourable for CL reaction.
Further investigation was done on how the addition of acid might have changed the pH level in a carbonate buffer. Experiments were carried out using test tubes and a pH meter. The solution of 0.1 M HCl was added to carbonate buffer in different portions. Table 1 shows how the pH of the solution that may be affected by different amounts of HCl. The result explains why the CL reaction was suppressed when NaOH failed to mix well and to neutralize the acidified sample at high flow rates, because NaOH cannot neutralize the acidified sample completely, Fig. 3(a). A high concentration of HCl also suppresses the CL reaction, because NaOH cannot neutralize the acidified sample completely, Fig. 3(b).

Table 1 pH values after adding 0.1 M HCl to carbonate buffer

<table>
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<tr>
<th>HCl/NaOH ratio</th>
<th>pH</th>
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<tr>
<td>1:1</td>
<td>1.85</td>
</tr>
<tr>
<td>1:2</td>
<td>6.02</td>
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<tr>
<td>1:3</td>
<td>6.81</td>
</tr>
<tr>
<td>1:4</td>
<td>7.33</td>
</tr>
<tr>
<td>0:1</td>
<td>9.94</td>
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</table>

The molar ratio between HCl and NaOH can significantly affect the CL intensity, Fig. 3(d). If the ratio of HCl to NaOH increases from 0 to 1, the CL intensity also increases. This phenomenon can be explained by the fact that the cobalt(II) ions were better liberated with a stronger acid. When the equal amount of HCl and NaOH was used, the CL intensity was the highest, because a mole of HCl is required to neutralize a mole of NaOH to give a neutral salt at pH 7. Hence, the amount of NaOH added to HCl must be in equal or more, to achieve a pH level of 7 or higher. Surprisingly, additional amounts of NaOH did not seem to aid the reaction, because a high pH level inhibits the CL reaction.

Effect of flow rate in mode II. The flow rate in mode II was determined separately because in this mode three reagents were used instead of five. Various flow rates from 0.5 to 3.5 ml/min were used to introduce 0.1 µg/ml vitamin B12, 1 mM luminol and 3% H2O2 into the system. The recorded CL intensity increased with an increasing flow rate used, giving a similar result as our previous finding. This is simply because more reagents were pumped into the system per unit time leading to a brighter CL intensity.

Effect of concentration of (a) H2O2 and (b) luminol on CL intensity in mode II.

Fig. 4 Effect of concentration of (a) H2O2 and (b) luminol on CL intensity emitted from the reaction. Since the CL intensity peaked at 3% v/v H2O2, this concentration was used in subsequent experiments.

Effect of luminol in mode II. Finally, the concentration of luminol in mode II was optimized. Various concentrations of luminol from 1 µM to 1 mM, together with 3% v/v H2O2, and 0.1 µg/ml vitamin B12 were introduced into the microfluidic system. Figure 4(b) shows the CL intensity emitted versus the concentration of luminol. The CL intensity increased with increasing concentrations of luminol, reaching a maximum at 5 µM, followed by a decline as the concentration of luminol increased further. Since the CL intensity reached a maximum at 5 µM of luminol, this concentration was used in the later experiments.

Effect of vitamin B12 concentrations in mode I and II. To investigate if vitamin B12 can be determined using the microfluidic system by chemiluminescence, a range of concentrations of vitamin B12 samples from 1 pg/ml to 0.1 mg/ml was introduced into the system. In mode I, the vitamin B12 samples were directly acidified in the microchip. The samples, 0.2 M HCl, 0.2 M NaOH, 1 mM luminol and 3% v/v H2O2 were introduced into the microfluidic system at a flow rate of 2.5 ml/min. The HCl solution acidifies the vitamin B12 samples, liberating the cobalt(II) ions in situ. The liberated ions will in turn...
Table 2 Vitamin B12 presents in samples

<table>
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<tr>
<th>Samples</th>
<th>Label</th>
<th>Literature</th>
<th>Proposed CL method</th>
<th>UV-visible spectrophotometry</th>
</tr>
</thead>
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<tr>
<td>Vitamin B12 tablets (n = 5)</td>
<td>4.60×10⁻⁸ M</td>
<td>—</td>
<td>6.30×10⁻⁸ M g⁻¹</td>
<td>9.70×10⁻⁷ M g⁻¹</td>
</tr>
<tr>
<td>Hen egg yolks (n = 3)</td>
<td>—</td>
<td>3 – 10×10⁻⁸ g⁻¹</td>
<td>9.90×3.3×10⁻⁸ g⁻¹</td>
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</table>

Fig. 5 Effect of vitamin B₁₂ concentration on CL intensity

catalyse the CL reaction of luminol and peroxide in carbonate buffer (pH 10). NaOH was added to adjust the pH condition so that the CL reaction might be carried out in an alkaline medium. The emitted CL signal was captured by the photon detector and analysed. In mode II, the vitamin B₁₂ solutions were first acidified to release the initially bound cobalt(II) ions. The acidified samples, 3% v/v hydrogen peroxide and 5 μM luminol were delivered into the microfluidic system at a flow rate of 1.5 ml/min. The CL reaction was conducted in pH 10 carbonate buffer. Figure 5 shows the effect of vitamin B₁₂ concentrations on CL intensity for both modes.

For mode I and II, CL intensity increased with increasing amounts of vitamin B₁₂. For mode I, a linear range between 1 ng/ml and 10 μg/ml was observed from the curve (Fig. 5) with a relationship of \( y = 6.56 \times 10^3 \cdot \lg(x) + 1.23 \times 10^4 \). The relative standard deviation (RSD) was between 1.23 % and 2.31% (n = 5). Similarly, for mode II, a linear range was observed between 0.1 ng/ml and 10 μg/ml from the curve (Fig. 5). The first order correlation equation obtained was \( y = 1.27 \times 10^4 \cdot \lg(x) + 2.23 \times 10^4 \), \( R^2 = 0.994 \). The RSD was between 0.898% and 2.32% (n = 6). Comparing the gradients of the two lines obtained from the two modes, the gradient of mode II is 1.93 times more than that of mode I. Therefore, mode II is more sensitive than mode I. That means acidifying the vitamin B₁₂ externally actually produces better results. However, mode I offers a more rapid and direct analysis, when the aqueous samples of vitamin B₁₂ are used.

The background signal was measured using deionised water in replacement of the vitamin B₁₂ sample. The background signal measured was 4,402±488 and 4,426±471 counts for mode I and II respectively. The similar values indicate that the contributions of the background noise to the data are constant. The limit of detection (lod) was determined by the concentration of analyte required to give a signal equal to the background (blank) plus three times the standard deviation of the blank. For a concentration of 1 pg/ml of vitamin B₁₂, the detected signal was 8,370±150. Using first order fitting, the lod was determined for a signal of 5,866 to be 0.368 pg/ml for mode I. Similarly, for mode II, the lod was estimated to be 0.576 pg/ml. If 1 M HCl was used instead of water, the detected signal was 2,100±164 for mode I and 2,750±104 for mode II, respectively. Normally, the detection limit is lower and close to the lower value of the dynamic linear ranges, Table 3. However, this is not the case for our experiments.

The photon detector used in our experiments was not able to pick up slight variations in the signal at sub-picogram level, because of the set integration over time. The integral time may then be extended to capture more photons, with better isolation from the stray background light. However, high concentrations of analytes may also overexpose the sensor. A dynamic routine can be written to automatically adjust the integration time with the detected signal. A better photon multiple tube (PMT) with a typical sensitivity of a PMT is 4–5×10⁻⁷ per s/PW, which is 1.6 times more than the current detector. However, a more sensitive PMT will increase the cost of the device.

Determination of vitamin B₁₂ in nutritional supplements and egg yolk

Since mode II gave better detection capabilities, the proposed method was used to further determine the concentrations of vitamin B₁₂ present in real samples of vitamin B₁₂ supplemental tablets and in hen egg yolks. The preparation of these samples was discussed earlier in experimental section. UV-VIS spectrophotometry was used as a reference method for comparison.

Table 2 shows the vitamin B₁₂ present in samples of vitamin B₁₂ supplemental tablets and in hen egg yolks, obtained using the proposed CL and the reference methods. In both supplemental tablets and egg yolks, the results obtained from the proposed CL methods deviated slightly from the value indicated on the nutritional label, reference value obtained from the literature and data obtained from the UV-VIS spectrophotometry. These differences were found to be statistically insignificant (P > 0.05) when the two sample groups were made to undergo two-tailed unpaired T-tests. The CL microfluidic system showed promising analytical results for the determination of vitamin B₁₂ concentrations in both supplemental tablets and egg yolks.

Further experiments can be conducted to study the feasibility of this method in the quantification of vitamin B₁₂ in human serum and other naturally occurring food sources. This method may then be applied to the healthcare industry or in the quality control of food products.

Acidification of vitamin B₁₂

The acidification of vitamin B₁₂ is crucial in order to liberate the cobalt(II) ions from its organic complex. The freed cobalt(II) ions can then take part in the CL-based assay. Prior to this, pre-treatment of samples was required to
extract the vitamin B12 from binding proteins through heating, enzymatic digestion or ethanol precipitation from solid animal products, food samples, human plasma, bile, and feces. In these treatments, the pH level was adjusted to an acidic condition of 4.8, thus allowing the release of the cobalt(II) ions in directly. In this case, module II of the microfluidic system can be useful for such samples.

The effectiveness of the liberation of cobalt(II) ions is highly dependent on the concentrations of HNO3 and HCl. The method used in mode II was devised from a combination of Kumar et al. and Akbay and Gok. The key difference of our current method compared to the previous works are the smaller amount of corrosive acids and thus safer in handling.

### Limits and merits of our system

The main objective of this study was to develop a LOC system for the determination of vitamin B12 concentration. A common well-known reaction, luminol-peroxide CL, was adopted in our study. It would have been too ambitious and redundant to explore new chemical procedures and developing the LOC system simultaneously. We were confident that the working chemical procedures reported by other14, 16, 19, 20 to be able to function in our system and perhaps give even better results than the current procedure. Sample determination in pharmaceuticals, human serum, egg yolk and for other applications is of present interest. Our present work is different from the previously published works. The main concept of Qin et al.19 aimed at a reusable system. The experiments were done in a connected anion-exchange and glass flow cell columns. The column with immobilized luminol on resins could be used over 500 times with ad-hoc generated peroxide. There is only one mode that is using HCl to mix with vitamin B12 injection samples.

In our current work, we pioneered the initiatives to fabricate the traditional CL assays into a continuous-flow LOC system for vitamin B12 determination. Moreover, we also introduced a new concept of designing a dual-mode chip for two laboratory practices. Most chip designs are now specialised only for a specific assay, which in turn limits the usage of the chip. Compared to our previous work,59 the length of each micromixer was shortened from 202 mm to 62 mm. This design reduced the size of the microchip without hindering its performance.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>CL Method</th>
<th>Ranges (ng/ml)</th>
<th>LOD (ng/ml)</th>
<th>Min. analytical time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qin et al.22</td>
<td>1997</td>
<td>Flow sensors</td>
<td>4.00×10^-10 - 1.00×10^-7</td>
<td>0.00×10^-7</td>
<td>100</td>
</tr>
<tr>
<td>Song and Hou20</td>
<td>2003</td>
<td>Flow injection</td>
<td>2.00×10^-6 - 1.20×10^-7</td>
<td>1.00×10^-7</td>
<td>10</td>
</tr>
<tr>
<td>Akbay and Gok16</td>
<td>2008</td>
<td>Flow injection</td>
<td>8.69×10^-10 - 8.69×10^-7</td>
<td>8.69×10^-7</td>
<td>10</td>
</tr>
<tr>
<td>Kumar et al.18</td>
<td>2009</td>
<td>Luminometer</td>
<td>1.00×10^-4 - 1.00×10^-7</td>
<td>1.00×10^-7</td>
<td>60</td>
</tr>
<tr>
<td>Our current method</td>
<td>2011</td>
<td>LOC-Mode I, LOC-Mode II</td>
<td>1.00×10^-10 - 1.00×10^-7</td>
<td>1.00×10^-10</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Although inference study of the CL assay was not done in our work, we could easily postulate the results based on the current literatures.22, 23, 59 We would expect equal amount of Co²⁺, Cu²⁺, and Fe³⁺ to interfere heavily with the determination of vitamin B12. These cations are well-known metallic catalysts for the luminol CL reaction. The tolerable concentration ratios with respect to 10 ng/ml vitamin B12 for interference at the 5% level would be more than 1000 for ions such as Al³⁺, Ag⁺, Ba²⁺, Ca²⁺, Cd²⁺, Cr³⁺, Fe³⁺, Hg²⁺, Mg²⁺, Mn²⁺, Ni²⁺, Pb²⁺, Sn²⁺, Sr²⁺ and Zn²⁺. These unbounded ions will mildly interfere with the assays. However, they are not typically found in human serum. High concentrations of Co²⁺, Cu²⁺, and Fe³⁺ will result in a signal peak with a shorter height and a broader base, and vice versa. Hence, there will be a variation in the results if the same analysis is performed in different laboratories or by different personnel. This problem will not occur with a continuous-flow microfluidic system. The signal is generated continuously, and the signal strength will be constant if the flow rate remains constant. Therefore, the measurement procedure can be standardized.

The raw data generated in FIA consists of a series of signal peaks plotted against time. Generally, the base peak or the integral of the peak can be used for measurements. The shape of the peak is affected by the injection rate (manual and automatic). For example, a slow and large sample injection will result in a signal peak with a shorter height and a broader base, and vice versa. Hence, there will be a variation in the results if the same analysis is performed in different laboratories or by different personnel. This problem will not occur with a continuous-flow microfluidic system. The signal is generated continuously, and the signal strength will be constant if the flow rate remains constant. Therefore, the measurement procedure can be standardized.
The determination of vitamin B₁₂ concentration using a CL system is usually performed using a large luminometer or a flow-injection analyzer. Compared to these bulky and heavy instruments, our microfluidic system is small in size and portable. In a micro-flow injection system, samples are injected into a continuous flowing stream of reagents. Thus, a lot of reagents is required. However, the samples and reagents in our system were introduced together simultaneously, and the total amount of reagents used was less than a milliliter. Using a more sensitive PMT sensor would allow further reduction of the device size and sample volume. Although, infusion pumps were used in our experiments, reagents and samples may be infused manually into the device. Alternatively, light and portable infusion pumps or integrated micropumps can be designed and used instead. Analysts are able to bring the device out to the field where samples are obtained for on-the-spot analysis. Being small in size and portable, several of such microfluidic devices may be placed on a single laboratory bench. Hence, parallelization can be achieved in this manner. A continuous-flow syringe infusion pump may be used to continuously feed the device with samples and reagents. More importantly, the low-cost and disposable PMMA microchips can be replaced regularly.

Our reported system can be further modified to provide better functions. A flow injection system can be incorporated into the current system and turned this microfluidic system into an integrated FLA-LOC system to provide automation and quick analysis. An oxidising electrode may be fabricated into the microchip to provide the necessary peroxides for the CL reaction. This method can help to generate ad-hoc \( \text{H}_2\text{O}_2 \) from \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) and in turn reduces the associated issue with the preparation of \( \text{H}_2\text{O}_2 \). In addition, our system can also be used for other CL assays. For instances, suspended cultured cells can be mixed with a cell lysis buffer, and adenosine triphosphate (ATP) can be detected using luciferase assay in the mixer-detection microchannel network, using mode I. Cells extract after tissue homogenisation can be directly determined with luciferase-ATP assay in mode II. Hence, our microchip designs are not limited by the current CL assay.

Conclusions

The proficiency of a microfluidic CL system for the detection of vitamin B₁₂ was successfully demonstrated. The system was able to detect small amounts of vitamin B₁₂ concentration at picogram level. The LOC device can also determine quantitatively the concentration of vitamin B₁₂ at nanogram and microgram levels. A less corrosive method was also introduced for the acidification of vitamin B12. Determination of vitamin B₁₂ in supplementary tablets and hen egg yolk was successfully demonstrated with this system. The obtained results were in good agreement with the data obtained by UV-visible spectrophotometry. Compared to previously published works and other instrumental systems, our lab-on-a-chip system shows a great potential in providing a low-cost, portable and yet sensitive analysis.

Notes and References

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57 1. S. S. Kumar, R. S. Chouhan and M. S. Thakur, Analytical Biochemistry, 2010, 398, 139-149.
70 14. S. S. Kumar, R. S. Chouhan and M. S. Thakur, Analytical Biochemistry, 2009, 388, 312-316.