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Application of a novel co-enzyme reactor in chemiluminescence flow-through biosensor for determination of lactose

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ABSTRACT

A novel enzyme reactor with co-immobilization of β-galactosidase and glucose oxidase in calcium alginate fiber (CAF) and amine modified nanosized mesoporous silica (AMNMS) was prepared which incorporate the adsorption and catalysis of AMNMS with the cage effect of the polymer to increase catalytic activity and stability of immobilized enzyme. The enzyme reactor was applied to prepare a chemiluminescence (CL) flow-through biosensor for determination of lactose combined with a novel luminol-diperiodatonickelate (DPN) CL system we reported. It shows that the CL flow-through biosensor possesses long lifetime, high stability, high catalytic activity and sensitivity. The relative CL intensity was linear with the lactose concentration in the range of 8×10^{-8}–4×10^{-6} g mL^{-1} with the detection limit of 2.7×10^{-8} g mL^{-1} (3σ). It has been successfully applied to the determination of lactose in milk.

1. Introduction

Free enzyme is usually not stable and cannot be easily reused, so enzyme immobilization techniques which have attracted much attention in these years can strengthen the needs for enzyme stability and reuse. Immobilized enzyme holds good stability against heat, organic solvents, temperature and pH which can improve the accuracy and reproducibility of the analytical method. So immobilized enzymes are widely used in biocatalysis, bioprocessing and biospecific detection. They are used in batchwise experiments or packed into columns and used in a flow system as immobilized enzyme reactors [1]. Immobilized enzyme reactors used in flow system holds easy automation and control of a continuous process with respect to a batch process which has been used in on-line analytical system. Enzymes may be immobilized by a variety of methods, which can be broadly classified as physical and chemical [2]. But all the methods present advantages and drawbacks. Adsorption is simple, cheap and effective but frequently reversible, covalent attachment and crosslinking are effective and durable, but expensive and easily worsening the enzyme performance, and in membrane reactor-confinement, entrapment and microencapsulation diffusional problems are inherent. Therefore immobilized enzymes often fail to retain their native stability and activity at a certain extent.

Mesoporous silica (MS) was widely used in enzyme immobilization due to tunable and uniform pore system, functionalizable surfaces, and restricted nanospaces. The surface functional groups play important roles in enzyme immobilization by changing the surface charge of MS for controlling electrostatic interaction with adsorbed enzyme. One can modify MS surface to bind positively charged enzyme or negatively charged enzyme [3]. Recently, many research groups have immobilized enzyme on MS which showed great improvement on enzyme stability, catalytic activity, products specificity, and resistance to extreme environmental conditions [4–6].

Alginate is one of the most promising natural polymers used in immobilization enzyme [7–12] for applications in a variety of fields because enzymes can retain their activity in alginate hydrogels thanks to its swollen and non-toxic environment. The size of the beads is an important factor for applications of calcium alginate, since it has been reported that smaller beads are more biocompatible than larger beads [13] and lower shear forces due to reduced size may increase their long-time stability [14]. In this work, CAF about 60 μm in diameter was prepared to immobilize enzyme. But it is concerned that enzyme leakage is always a big disadvantage while using calcium alginate gel matrix for enzyme immobilization [15–17]. Therefore, MS can be combined with CAF to improve the efficiently encapsulate enzyme to prepare enzyme reactor. The

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novel enzyme reactor increases the catalytic activity and stability of immobilized enzyme by incorporating the adsorption of enzyme on MS in the cage effect of the polymer.

CL method has been used widely in many fields due to low detection limits, wide linear dynamic range and rapid response with simple instrumentation. Nowadays, many enzyme-based CL biosensors have been reported [18–23]. In this work, it is explored that the feasibility of using AMNMS to increase the activity and stability of encapsulated enzyme in CAF. A CL flow-through biosensor was made for determination of lactose by combining a bi-enzyme reactor of co-immobilizing β-galactosidase (p*I* = 4.61) and glucose oxidase (p*I* = 4.0) in CAF–AMNMS with a new luminol-DPN CL system we reported [24]. In the CL system, DPN plays two important roles of catalyzer and oxidant [25], and catalysis is primary. The measurement principle was based on the following reactions:

$$\text{lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \beta\text{-D-galactose} + \beta\text{-D-glucose}$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-glucono-1, 5-lactone} + H_2O_2$$

$$\text{luminol} + H_2O_2 + OH^- \xrightarrow{\text{DPN}} \text{3-aminophthalate} + h\nu(425\,\text{nm})$$

The current novel CL flow-through biosensor with CAF–AMNMS as enzyme immobilization platform offers several advantages: (I) enzymes retain their activity in CAF–AMNMS thanks to its swollen and non-toxic environment; (II) smaller diameter CAF offers better transportation [26], dispersion, mechanical strength, easier implantation, good stability, and potential access to new implantation sites [27]; (III) combination the adsorption of enzyme on AMNMS with the cage effect of the polymer can increase the capability of enzyme immobilization; (IV) the huge surface area of AMNMS improves enzyme stability and catalytic activity; (V) the proposed biosensor possesses excellent sensitivity to determine lactose combined with novel luminol-DPN system. Therefore, a novel CL flow-through biosensor with high sensitivity, long lifetime, and high catalytic activity was developed and successfully applied to the determination of lactose in milk.

2. Experimental

2.1. Chemical and reagents

Luminol stock solution (1.0×10^{-2} mol L^{-1}) was prepared by dissolving 1.772 g luminol (>95%, Shaanxi Normal University, China) in 1 L 0.1 mol L^{-1} carbonate buffer and left to stand for approximately 24 h before use and stored in the dark. β-Galactosidase and glucose oxidase were obtained from Sigma (USA). Tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB) and calcium chloride anhydrous were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Potassium persulfate from Shanghai Aijian Chemical Reagent Company, and sodium alginate, lactose, potassium hydroxide, sodium nitrate, sodium periodate and nickel sulfate from Shanghai Chemical Reagent Company. All reagents were of analytical grade and deionized and double-distilled water was used throughout.

2.2. Apparatus

The flow system employed in this work is shown in Fig. 1. Two peristaltic pumps (Remex Analytical Instrument Co. Ltd., Xi'an, China) were used to deliver all flow streams. Polytetrafluoroethylene (PTFE) flow tubes (0.8 mm i.d.) were used to connect all the components in the flow system. Injection was done using an eight-way injection valve equipped with a sample loop (90 μL). CAF or CAF–AMNMS with immobilized enzymes was packed into a glass tube (length: 55 mm, i.d.: 3 mm). The flow cell was made by coiling 20 cm of colorless glass tube (2 mm i.d.) into a spiral disk shape and was located directly facing the window of the photomultiplier tube (PMT). The CL signal was monitored using an IFFM-A multifunction chemiluminescence analyzer (Remex Analytical Instrument Co. Ltd., Xi'an, China). The UV absorbance was detected with Hitachi U-3900 spectrophotometer (Hitachi Ltd., Japan).

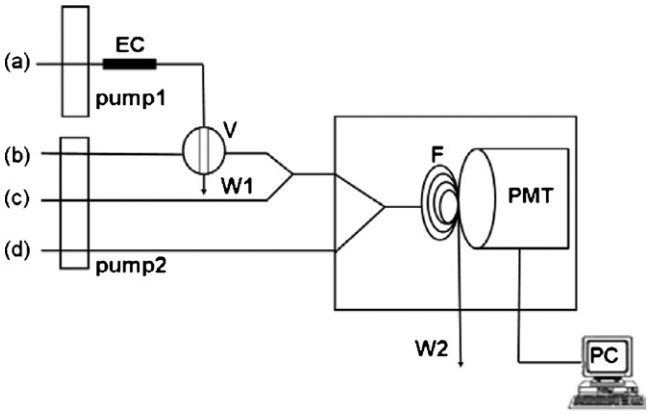

Fig. 1. Schematic diagram of the CL flow-through biosensor for the determination of lactose (a) lactose standard solution or samples; (b) distilled water; (c) luminol solution; (d) DPN solution; EC: enzymes reactor; V: injection valve; F: spiral glass cell; PMT: photomultiplier tube; pump1, pump2: peristaltic pump; W1, W2: waste.

2.3. Preparation of AMNMS

2.3.1. Preparation of nanosized MS

A typical synthesis gel was prepared by adding 5.4 mL TEOS to an aqueous solution containing 1.1 g CTAB and 0.31 g NaOH and 270 mL deionized water. After stirring for about 2 h at room temperature, the resulting product was filtered, washed three times with distilled water, dried at ambient temperature, followed by calcination in air at 550 °C for 5 h to obtain nanosized MS [28].

2.3.2. Pore size expansion

0.56 g nanosized MS was added into 2 mL mixture solution including distilled water, NaCl, LiCl, and KNO_3 at a proportion of 70:20: 5:5 (wt) then heated at 300 °C for 2 h to obtain remodeled MS [29]. The remodeled MS was washed with distilled water and dried at ambient temperature.

2.3.3. Preparation of AMNMS

AMNMS was obtained when 0.5 g of nanosized MS or remodeled nanosized MS was refluxed in 30 mL dry toluene solution containing 0.5 mL 3-aminopropyltrimethoxysilane (97%, Aldrich) at 100 °C for 12 h [30]. The obtained AMNMS was washed with ethanol, distilled water and dried at ambient temperature.

2.4. Enzymes immobilization in AMNMS

2 mL mixing enzymes solution (0.01 M pH 7.4 phosphate buffer, 0.3 mg β-galactosidase, 2.0 mg glucose oxidase) was added to 20 mg AMNMS or remodeled AMNMS in a 5 mL graduated centrifuge tube. The mixture was stirred for 4 h at room temperature. The AMNMS or remodeled AMNMS immobilized enzymes was separated from solution by centrifugation and washed with distilled water.

2.5. Immobilization of enzymes in CAF

0.5 mL enzymes solution mixed with 5 mL 5.0% aqueous sodium alginate solution and stirred for 5 min. The mixed solution obtained

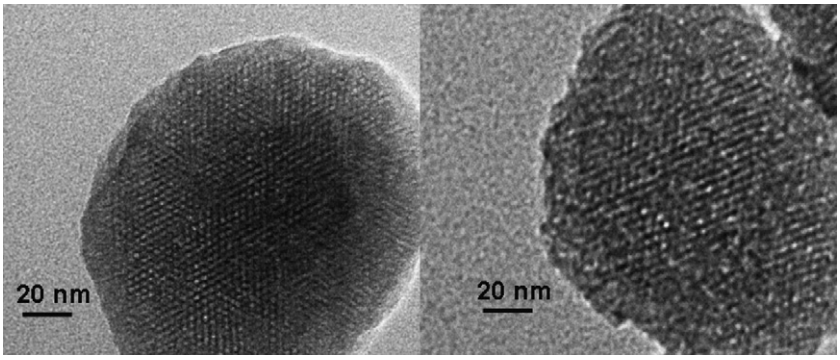

Fig. 2. TEM image of AMNMS (left) and remodeled AMNMS (right).

was placed in a syringe and injected into 4% calcium chloride solution with needle (0.45 mm i.d.) inserting in calcium chloride solution, forming CAF and thus entrapping enzymes. The fibers were allowed to harden for 2 h at 4 °C. The obtained fibers were washed and stretched in distilled water, then dried. The dry fibers were cut into 8 cm long pieces, and packed into a glass column filled with a small amount of glass wool at both ends to prevent the fibers from being washed away by the solution. The enzyme reactor was store at 4 °C until further use. The reactor was denoted as sensor A.

2.6. Immobilization of enzymes in CAF–AMNMS

The obtained AMNMS or remodeled AMNMS with immobilization enzymes which was well dispersed in 0.5 mL of distilled water by ultrasonic prior to use. Then the mixture solution was mixed with 5 mL 5.0% aqueous sodium alginate solution and the rest of procedure was as the same as Section 2.5. The reactor with AMNMS or remodeled AMNMS was denoted respectively as sensor B or sensor C.

2.7. Procedures

During the enzymatic reaction, a lactose molecule creates equivalent H_2O_2 by reacting with β-galactosidase and glucose oxidase. The determination of H_2O_2 was based on the CL reaction of luminol-DPN in alkaline medium. The CL flow-through biosensor used for the determination of lactose is shown in Fig. 1. When the analyte solution passed through the immobilized bi-enzymes reactor, lactose was transformed to H_2O_2 by enzymes. Then 90 μL H_2O_2 produced was injected into the carrier stream to react with luminol and DPN to produce CL. The CL signal was detected by IFFS-A multifunction chemiluminescence analyzer. The concentration of sample was quantified by the relative peak height of the CL intensity.

3. Results and discussion

3.1. Characterization of AMNMS

TEM images of AMNMS and remodeled AMNMS were taken using a JEM-2100 transmission electron microscopy (JOEC, Japan) (Fig. 2). It can be clearly observed that remodeled AMNMS possesses bigger mesoporous cavities which were more suitable for enzyme immobilization [29]. The ability of adsorption was confirmed by the UV–vis absorption spectra (Fig. 4).

3.2. Characterization of CAF

Alginate beads have been extensively used in entrapped enzyme due to their excellent biocompatibility, non-toxicity, and potential bioactivity. In this work, CAF was prepared to immobilize enzyme. Immobilization of enzymes or microorganisms in CAF has two major advantages over immobilization in alginate beads: diffusion in alginate fibre with smaller diameter is better than that in alginate beads [31] and alginate fibers retain more enzymes compared to alginate beads [32]. Morphology of CAF was recorded using environmental scanning electron microscopy (SEM, Quanta 200, FEI Company, Japan) (Fig. 3). It was concluded that the diameter of fiber was about 60 μm. Though enzymes retain their activity in alginate hydrogels thanks to its swollen and non-toxic environment, leakage is a big problem due to the fact that even large enzymes with molecular weights of 300 kDa can leak out from CA [33–35]. In this work, the leakage of enzymes was more remarkably improved by immobilized enzymes in CAF–AMNMS than CAF. Two biosensors were prepared by AMNMS and remodeled AMNMS combined with CAF to immobilize enzymes and studied the effect on CL response respectively.

3.3. The ability of enzymes immobilized in AMNMS

The ability of enzymes in AMNMS or remodeled AMNMS was measured by adding 20 mg AMNMS to the mixture of two enzymes solution and stirred for 4 h at room temperature. The supernatant was separated from the solid material by centrifugation. The ability of immobilized enzyme in AMNMS was calculated from the different absorbance of enzymes solution and supernatant after

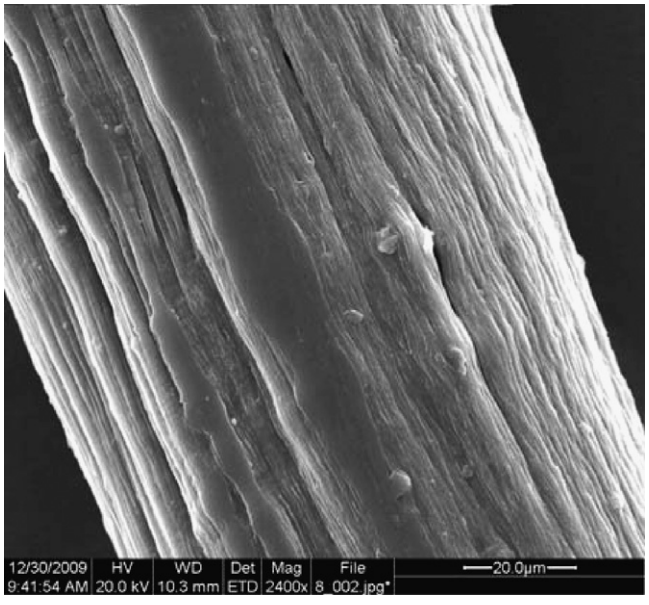

Fig. 3. The SEM image of calcium alginate fiber.

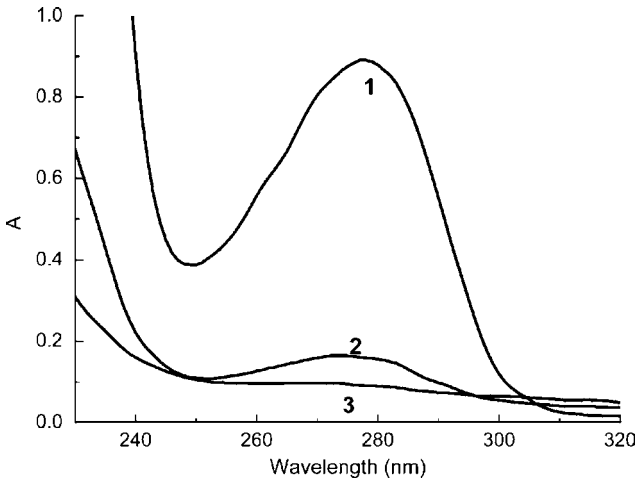

Fig. 4. UV–vis spectra of enzymes solution (pH 7.4). (1) The mixture of two enzymes solution; (2) after adsorption to AMNMS; (3) after adsorption to remodeled AMNMS.

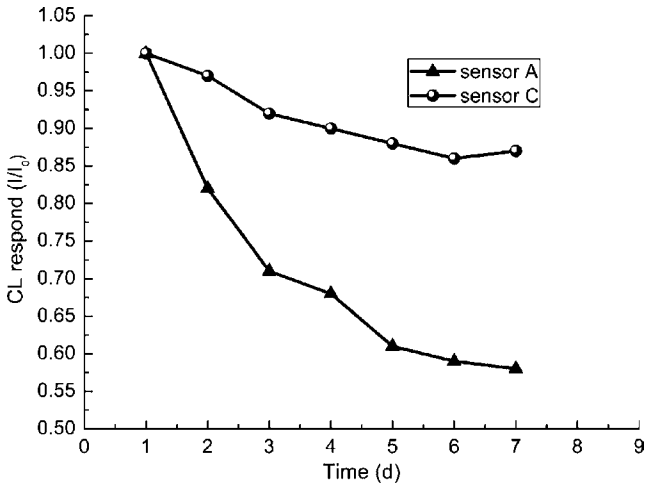

Fig. 5. The variety of CL responds versus to initial value.

addition of the support (Fig. 4). It is showed that remodeled AMNMS shows larger adsorption capacity than AMNMS. So it is concluded that remodeled AMNMS possesses more proper pore for enzymes immobilization when a mixture of three salts was used to enlarge the pore size of AMNMS.

3.4. Optimization of experiment procedure

To achieve the highest sensitivity, the optimal conditions for the flow-through CL biosensor was investigated by the relative CL intensity (lactose: 1.0×10^{-6} g mL^{-1}) at different concentration level of luminol, DPN and potassium hydroxide in DPN and flow rate of pump. The optimal conditions for the flow-through CL biosensor are shown in Table 1.

3.5. Lifetime of the biosensor

The lifetime of biosensor depended mainly on the enzymatic stability and activity. The storage stability of the sensors at 4 °C was examined by periodically checking their CL response (Fig. 5). It was found that during the first six days CL intensity of sensor C dropped to approximately 87% of its initial value and that after the first week the response of the biosensor for the same lactose solution nearly kept constant over the two months storage period. But the value of sensor A was dropped to 58% after the first six days then to 30% after two months. Therefore, incorporating remodeled AMNMS into CAF would be an effective approach to efficiently encapsulate enzymes, which could combine the adsorption of enzyme on AMNMS with the cage effect of the polymer and potentially increase the activity and stability of the immobilized enzyme.

3.6. Stability of the biosensor

The operational stability of the sensor C was studied by recording more than 100 successive assays of 1.0×10^{-6} g mL^{-1} lactose with the relative standard deviation of 6.1%. The result of part of reproducibility experiment is shown in Fig. 6. And the CL intensity of the biosensor for the same lactose solution almost did not clearly decrease. Obviously, high stability and activity of immobilization enzyme on AMNMS-CAF were obtained.

Table 1
The optimal condition for the flow-through CL biosensor.

Effect parameters	Range of study	Optimal condition
Luminol concentration	6.0×10^{-8}–1.0×10^{-6} mol L^{-1}	2.0×10^{-7} mol L^{-1}
DPN concentration	1.0×10^{-5}–8.0×10^{-4} mol L^{-1}	8.0×10^{-5} mol L^{-1}
KOH concentration	5.0×10^{-3}–0.4 mol L^{-1}	8.0×10^{-2} mol L^{-1}
Pump 1 flow rate	0.5–3.0 mL min^{-1}	0.4 mL min^{-1}
Pump 2 flow rate	0.5–3.0 mL min^{-1}	3.0 mL min^{-1}

3.7. Interference studies

The effects of foreign substances in milk were tested by analyzing a standard solution of lactose (4.0×10^{-7} g mL^{-1}) to which increase amounts of interfering substances was added. The tolerable concentration ratios were taken with respect to 4.0×10^{-7} g mL^{-1} lactose for relative error less than 5%. The results are listed in Table 2. The interference of glucose can be cancelled by a single glucose biosensor when samples including glucose.

3.8. Performance of biosensor for lactose measurements

Under the selected conditions given above, the CL responses about sensor A, sensor B and sensor C were compared (Fig. 7). It is indicated that the responses of sensors B and C in which the AMNMS is employed, are more sensitive than sensor A. The higher response value of sensors B and C attributes to the huge surface area provided by AMNMS to improve enzyme stability and catalytic activity. AMNMS can adsorb the enzymes intensively and prevent enzyme leaking from platform because modified MS surface with amine groups can bind negatively charged enzymes by electro-

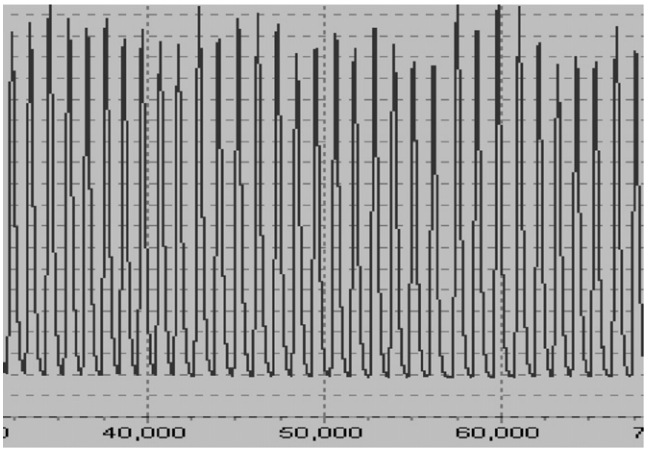

Fig. 6. The reproducibility of the CL flow-through biosensor for the determination of 1.0×10^{-6} g mL^{-1} lactose.

Table 2
The effect of interference of substances on the determination of 4.0×10^{-7} g mL^{-1} lactose under the optimum conditions.

Interfering substances	Tolerable concentration ratio
Na^+, Ca^{2+}, NO_3^-, NH_4^+, and SO_4^{2-}	1000
Lysine, creatinine, glutamic acid, leucine, serine, and asparagine	500
Lactic acid and ascorbic acid	50

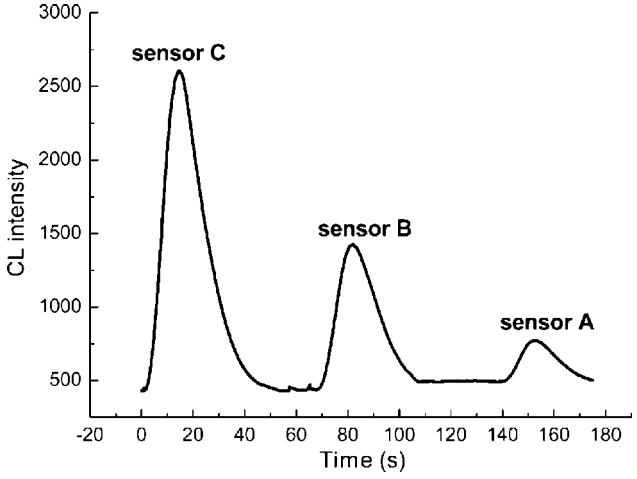

Fig. 7. The CL response of three sensors (lactose concentration: 1.0×10^{-6} g mL^{-1}).

static interaction. The CL response of sensor C is higher than sensor B because remodeled AMNMS by pore size expanding can adsorb more enzymes. So the sensor C holds the highest sensitivity. The relative CL intensity of sensor C was linearly proportional to the lactose concentration in the range of 8.0×10^{-8} g mL^{-1}–4.0×10^{-6} g mL^{-1} and the detection limit was 2.7×10^{-8} g mL^{-1} (3σ). The regression equation was $\Delta I = 23.7C - 248.3$ (ΔI being the relative height of CL intensity and C being the lactose concentration (10^{-8} g mL^{-1})) with $R^2 = 0.9953$ (Fig. 8). Figures of merit of comparable methods for determination of lactose were shown in Table 3.

3.9. Sample analysis

To demonstrate the application of the biosensor based on glucose oxidase and β-reactor for lactose analysis. Aliquots of commercial milks were distributed in centrifuge tubes and centrifuged at 10,000 rpm for 10 min. Both tube pellet (insoluble proteins) and supernatant (milk fats) were eliminated. The clarified milk was diluted with distilled water to yield testing sample solution. The response of the sample solution was measured and compared with that of a set of lactose standard solutions. The results are shown in Table 4 which matched with lane-eynon method.

Table 3
Figures of merit of comparable methods for determination of lactose.

Method	Linear range	LOD	Reference
ELSD	0.1–12.0 g L^{-1}	0.03 g L^{-1}	[36]
Conductometric biosensor	60–800 μM	–	[37]
CL biosensor	20–200 mM	–	[38]
Amperometric biosensor	1×10^{-4}–3.5×10^{-3} M	–	[39]
Amperometric biosensor	1–5 mM	0.5 mM	[40]
	2–10 mM	1.0 mM	
	1–5 mM	0.5 mM	
The CL biosensor	8×10^{-8}–4×10^{-6} g mL^{-1}	2.7×10^{-8} g mL^{-1}	The work

Table 4
Analysis results of lactose in milk.

Samples	Found (mg mL^{-1}) ± RSD (%, $n = 3$)	Lane-eynon method (mg mL^{-1})
No. 1	37.5 ± 0.9	36.2
No. 2	38.0 ± 3.3	37.4
No. 3	33.5 ± 4.6	34.5
No. 4	40.3 ± 0.2	40.8
No. 5	31.4 ± 1.4	31.0

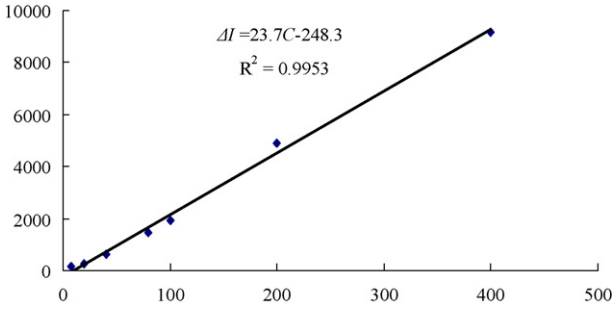

Fig. 8. The linear chart of relative CL intensity and lactose concentration.

4. Conclusion

In this paper, a novel co-enzyme reactor by using CAF–AMNMS as platform was prepared which was combined with luminol-DPN system to design a flow-through CL biosensor for the determination of lactose. It is important that CAF–AMNMS could greatly improve leakage and activity of the immobilized enzyme and stability of the biosensor. So the CL flow-through biosensor holds excellent stability with a long shelf life which possessed high sensitivity.

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