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A rapid fluorescence detecting platform: applicable to sense carnitine and chloramphenicol in food samples

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Abstract

In the present work, a new Coenzyme A (CoA) fluorescence platform has been described for the sensitive determination of CoA, carnitine and chloramphenicol. Previously, our research group reported a long-wavelength latent florescent probe, termed "BCC". Here, we extend the application of BCC as an off-on fluorescence ratiometric indicator for the detection of acetyl CoA with linear range of 0.5–10 μM and limit of detection (LOD) of 0.18 μM. We established a novel platform for the determination of carnitine and chloramphenicol in presence of acetyl-CoA and their corresponding acetyl-CoA-transferring enzymes (chloramphenicol acetyltransferase (CAT) and carnitine acetyltransferase (CrAT)) respectively. The proposed method detects carnitine and chloramphenicol in the linear range of 0.5–10 μM. The LOD for the determination of carnitine and chloramphenicol were found to be 0.5 μM and 0.3 μM respectively. Moreover, BCC is provided a facile assay platform for real-time monitoring of CAT and CrAT enzymatic activity in the presence of their corresponding substrates and acetyl-CoA. Practical feasibility of the proposed method has been demonstrated in food samples such as milk, powdered milk and honey and the observed appreciable recoveries revealing the promising practicality of the proposed method.

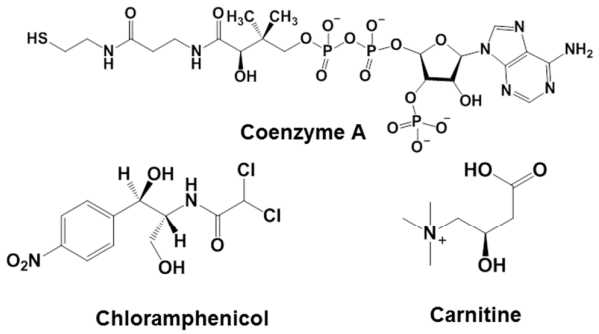
Keywords: Carnitine, Chloramphenicol, fluorescence, Acetyltransferase, Coenzyme A, food samples.

1. Introduction

Coenzyme A (CoA), a sulfhydryl thiol composed of units derived from cysteine (Cys), pantothenic acid and adenosine triphosphate (ATP) is involved in acyl-group transfers in many enzymatic reactions.[1] CoA facilitates many chemical reactions in cells including the metabolism of amino acids, carbohydrates and lipids[2] and also contributes to the bacterial detoxification.[3] Most importantly, CoA initiates the tricarboxylic acid cycle, which produces approximately 90% of the energy required for life processes. Therefore, there is a significant need for precise, sensitive, and simple methods for the accurate determination of CoA concentration[1]. The design of thiol-detecting molecular probes has attracted significant research attention because of their numerous applications in broad areas of chemistry and biology. [4-9]. However, most of the molecular probe design efforts are focused on the detection of Cys and glutathione (GSH), while less attention has been paid to the detection of CoA. [10, 11] Previously, our research group has successfully prepared and characterized a long-wavelength florescent probe, termed "BCC" for the determination of Cys and GSH.[12] The fluorogenic chemical transformation of BCC is triggered by thiols through tandem reactions: thiol induces benzoquinone reduction, followed by a quinone-methide-type rearrangement reaction

which ejects fluorogenic coumarin.[13] This reaction is spontaneous and irreversible at physiological temperature in aqueous media. In this paper, we are extending the application of BCC for the sensitive determination of CoA under physiological conditions. To the best of our knowledge, this is the first report for the detection of CoA based on long-wavelength latent fluorogenic probe. Moreover, we seek to apply BCC as a simple platform for assaying acetyl-CoA-transferring enzymatic activity.

Chloramphenicol (**scheme 1**) is a potent broad-spectrum antibacterial agent that has been widely used since the 1950s to treat food-producing animals.[14, 15] It is relatively inexpensive, highly effective and has good pharmacokinetic properties. As a result, it is extensively used for controlling mammalian, poultry, aquatic and bee diseases around the world.[16, 17] However, it is associated with serious adverse effects such as rapid, serious toxic effects, especially bone marrow depression. The adverse effects is particularly severe when it assumes the form of dose-independent and fatal aplastic anemia.[18] The European Union (EU) has prohibited the use of chloramphenicol for veterinary use in 1994 and no maximum residue limit has been established for this antibiotic. Therefore, monitoring chloramphenicol residue in food is a critical food safety issue.[19] Chloramphenicol acetyltransferase (CAT) is the most commonly encountered effectors of chloramphenicol in eubacteria. Hence, closely monitoring the amount of chloramphenicol administered to livestock and tracking CAT activities has been important for the food safety goals.[16]



Scheme 1: The chemical structures of carnitine and chloramphenicol

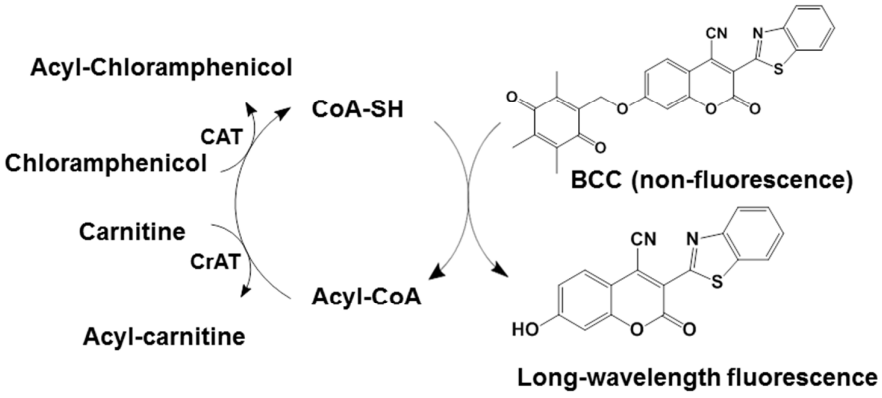
L-Carnitine (**scheme 1**) is an endogenous molecule involved in fatty acid metabolism which is biosynthesized within the human body using amino acids, L-lysine and L-methionine as substrates.[20] L-carnitine is facilitating the transport of fatty acid chains into the mitochondrial matrix through the activity of carnitine acyltransferase (CrAT) and allowing the cells to break down fat to obtain energy from stored fat reserves. Carnitine is also found in many foods including fish, poultry, dairy products, seeds, nuts and honey.[20] Disturbances in the carnitine homeostasis have serious troubles to the human health. Moreover, recent studies are started to shed light on the beneficial effects of carnitine

in various clinical therapies. Therefore, determination of carnitine is extremely important for clinical diagnosis and for the maintenance of carnitine homeostasis. [21] CrAT, a membrane-bound mitochondrial enzyme, is part of the carnitine system that maintains acetyl-CoA/CoA homeostasis. CrAT is also involved in detoxification of a certain pool of metabolized branched-chain fatty acyl esters generated by amino acid catabolism.[22]

Analytical methods such as high-performance liquid chromatography (HPLC) with UV or fluorescence detection,[23, 24] capillary electrophoresis[25, 26], gas chromatography–mass spectrometry (GC-MS) [27] and flow injection analysis[28] were developed for the determination of carnitine and mostly these are associated with pre-derivatization steps. Similarly, several analytical methods such as HPLC[29], GC-MS[30], liquid chromatography–electrospray negative ionization tandem mass spectrometry[31], colorimetric methods [32]. However, there are very few reports available in the literature based on latent fluorogenic probe for the determination of chloramphenicol[33] and carnitine[34]. In the present work, for the first time we are describing a latent fluorogenic probe for the determination of chloramphenicol and carnitine.

The main aim of the presence work is to develop a new fluorescence platform based on BCC for the sensitive determination of CoA, carnitine and chloramphenicol. The preparation of BCC and assay procedures involve very simple protocols and reproducible. The proposed latent fluorogenic probe based approach sensitively detects chloramphenicol and carnitine present in real samples milk, powdered milk and honey validating practical feasibility of the proposed method. In addition, BCC is a convenient latent fluorogenic probe for real-time spectrophotometric monitoring of CrAT and CAT activity. The assay platform provides a facile method to monitor enzyme activities without involving tedious protocols and hazardous radioactive materials. Especially, BCC is proved as beneficial fluorogenic indicator for monitoring stably transfected CAT reporter genes and identifying CAT-targeted antibacterial agents in future high-throughput screens.

The schematic representation of the work has been outlined as scheme 2. BCC is co-incubated with the acetyl-CoA-transferring enzymes (CAT and CrAT) and their corresponding substrates, chloramphenicol and carnitine respectively. Both CAT and CrAT catalyze the reversible transfer of acetyl group from acetyl-CoA to chloramphenicol and carnitine respectively to yield the corresponding acyl-products, concomitantly revealing the sulfuryl moieties of CoA [35, 22]. The sulfuryl moiety of CoA reacts with BCC and undergoes cascade reactions which unmask the cloaked fluorogenic coumarin (**Scheme 2**).



Scheme 2. A fluorescence detecting platform based on BCC and acetyl-CoA-transferring enzymes for the determination of chloramphenicol and carnitine.

2. Experimental

2.1. Materials and Methods

CoA, acetyl-CoA, CAT (E. C. 2.3.1.28; from *Escherichia coli*), and CrAT (E.C. 1.2.1.5; from pigeon muscle) were purchased from Sigma-Aldrich Co. (St. Louis, MO). For the practicality experiments, organic wild flower honey was purchased from a local supermarket, while milk and powdered milk were acquired from Hidaka Hokkaido milk products. All other chemicals were purchased from Acros Organics, Sigma-Aldrich, Showa Chemical Industry Co. or TCI America, and used without further purification. BCC was prepared according to our previously reported procedure.[12] Fluorescence measurements were carried out in 50 mM phosphate buffer (pH 7.8) solution using fluorescence-grade quartz cuvettes and a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer.

2.2. Preparation of stock solutions

The stock solution of BCC was prepared by dissolving 1 mg BCC in 41.4 mL of dimethyl sulfoxide (DMSO) and diluting in phosphate buffer (50 mM $H_2PO_4^-/HPO_4^{2-}$ and 2 mM EDTA adjusted to pH 7.8 with 1 M NaOH); the DMSO concentration never exceeded 10% (v/v) in any experiment. Acetyl-CoA solutions were prepared by dissolving 100 mg acetyl-CoA in 12.08 mL of distilled water. CoA (1 mM), carnitine (10 mM) and chloramphenicol (10 mM) standards are prepared in phosphate buffer.

2.3. Sample preparation

Powdered milk (1 g) was weighed into a conical flask and dissolved by adding 10 mL of water. 1 mL of this solution was transferred to a microcentrifuge tube, to which 50 µL of 0.25 M perchloric acid was added. The solution was mixed and centrifuged at 3000 × g for 10 min. The upper layer was removed and filtered through 0.22 µm pore microfilters into another microcentrifuge tube, after which the pH of the solution was adjusted to pH 7 with 1 M NaOH. Samples containing chloramphenicol were prepared in the same manner by spiking 1 mL powdered milk solutions with the addition of chloramphenicol before adding perchloric acid.

2.4. Assay procedure for the real sample application

Assays were conducted in phosphate buffer containing milk, powdered milk and phosphate buffer containing honey. The basic reaction mixture contains BCC (50 µM), acetyl-CoA (10 mM), enzyme (CrAT or CAT) and substrate (carnitine or chloramphenicol, 0-10 µM) in phosphate buffer. The milk and powdered milk-containing reactions consists of 100 µL of the basic reaction mixture plus 100 µL of milk or powdered milk solution. The honey containing reaction consisted of the basic reaction mixture with the addition of 0.1 g honey. For all reactions, samples were mixed by vortexing, incubated at 37°C for 1 h and analyzed by fluorescence spectroscopy.

2.5. Kinetic studies

Mixtures containing BCC (5 µM), acetyl-CoA (10 mM), enzyme (CrAT or CAT) and substrates (0-600 µM of carnitine or 0-150 µM of chloramphenicol) in phosphate buffer were analyzed by fluorescence spectroscopy after incubating for 15 min at 25°C.

3. Results and Discussion

3.1. Determination of CoA

The emission spectrum of BCC alone (5 µM BCC in 10% [v/v] DMSO in phosphate buffer, pH 7.8) is revealed a slight fluorescence (**Fig. 1**). The introduction of CoA resulted in a concentration-dependent increase in fluorescence characteristic of coumarin after 1 h (**Fig. 1**). The maximum fluorescence response was obtained at the incubation time of 1 h and therefore we have used 1 h for all the further experiments (**Fig. S1**). A characterization of the pH dependence of the fluorescence responses of BCC to added CoA revealed an optimal signal-to-noise ratio at pH 7.8 (Supplementary Information **Fig. S2**). A plot between $(I_f – I_i)/I_i$ (here, I_i= intensity in the absence of CoA, I_f= intensity in the presence of particular concentration of CoA) vs. [CoA] is exhibited a linear relationship at CoA concentrations between 0.5 and 10 µM (**Fig. 1**, inset), with a limit of detection

(LOD) of 0.18 μM. This LOD is 10-fold lower than that of current commercially available CoA fluorescence detection kits and known procedures.[36] These results suggest that BCC is a stable molecule with an intense fluorescence that can be unmasked by CoA in the nanomolar range.

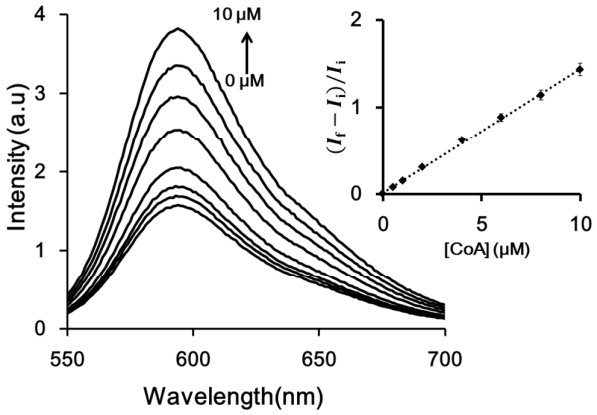


Fig. 1. Fluorescence spectra changes (λ_{ex} = 500 nm, λ_{em} = 595 nm) of BCC (5 μM) with CoA (0–10 μM) inset: "$(I_f–I_i)/I_i$ vs. [CoA] in 10% DMSO PBS pH 7.8 (v/v) with 1h of incubation. We Here, I_i= intensity in the absence of CoA, I_f= intensity in the presence of particular concentration of CoA.

3.2. Determination of chloramphenicol and carnitine

To our knowledge, only two assay platforms have been reported to date for determining chloramphenicol and carnitine concentrations based on coupling fluorogenic thiol-detection agents with corresponding acetyl-transferring enzymes.[35] We therefore assessed the utility of BCC as a new fluorimetric indicator in an acetyl-transferring enzyme assay for the determination of chloramphenicol and carnitine (**Scheme 2**), establishing an assay platform consisting of BCC, acetyl-CoA, acetyl-transferring enzyme (CAT or CrAT) and corresponding substrates (chloramphenicol or carnitine). In this platform, CAT or CrAT catalyzes the acetylation of its corresponding substrate using acetyl-CoA with the concomitant liberation of CoA-SH, which induces BCC to undergo rearrangement reactions and eject the fluorogenic coumarin. The emission spectra of BCC co-incubated with acetyl-CoA transferring enzymes and acetyl-CoA (300 μM) in the absence of substrates were included as controls. The introduction of chloramphenicol or carnitine to the solution induced a concentration-dependent increase in fluorescence characteristic of coumarin after incubating at 37°C for 1 h (**Fig. 2 and Fig. 3**). In order to reduce reagent consumption and maintain maximal assay sensitivity, we determined the optimal assay concentrations for acetyl-CoA. As summarized in the supplementary materials, the optimal signal-to-noise ratio for this reaction was observed with 0.3 mM acetyl CoA for both enzymes (**Fig. S3**) and therefore we used this concentration for further analysis. A plot of fluorescence intensity

versus concentration of chloramphenicol (**inset to Fig. 2)** and carnitine (**inset to Fig. 3**) revealed a linear relationship with linear range of 0.5–10 μM for both the analytes. The LOD for the determination of chloramphenicol and carnitine were calculated as 0.5 μM and 0.3 μM, respectively. Thus, the latent fluorophore BCC is a sensitive ratiometric fluorescence indicator for the detection of chloramphenicol and carnitine.

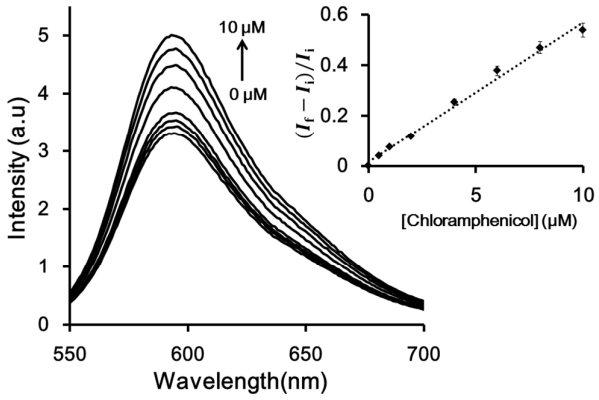


Fig. 2. Fluorescence spectra changes (λ_{ex} = 500 nm, λ_{em} = 595 nm) of BCC (5 μM) with chloramphenicol (0–10 μM) inset: (I_f–I_i/I_i) vs. [chloramphenicol] in 10% DMSO PBS (pH 7.8) (v/v), 300 μM of acetyl CoA and 1 unit of CAT with 1h of incubation. Here, I_i= intensity in the absence of chloramphenicol, I_f= intensity in the presence of particular concentration of chloramphenicol.

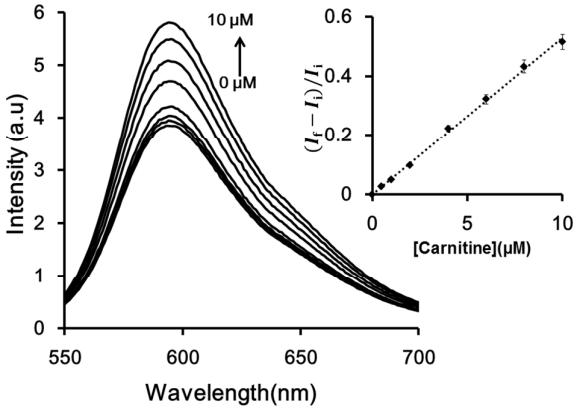


Fig. 3. Fluorescence spectra changes (λ_{ex} = 500 nm, λ_{em} = 595 nm) of BCC (5 μM) with carnitine (0–10 μM). Inset: (I_f–I_i/I_i) vs. [carnitine] in 10% DMSO PBS (pH 7.8) (v/v), 300 μM of Acetyl CoA and 0.1 unit of CrAT with 1h of incubation. Here, I_i= intensity in the absence of carnitine, I_f= intensity in the presence of particular concentration of carnitine.

To prove that the observed fluorescence of BCC is originates from CoA, two separate experiments were carried out in 10% DMSO PBS (pH 7.8) (v/v) containing (1) 100 μM of acetyl CoA and (2) 10 μM of acetyl CoA (**Fig. S4**). Although the concentration of Acetyl CoA is ten times higher

than that of CoA, Acetyl CoA does not able to reveal the fluorescence of BCC which indicating that acetyl CoA is incapable to trigger the signal revealing mechanism of BCC. However, CoA able to reveal the fluorescence of BCC even in the presence of 10 μM concentration which is evident from the observation of highly enhanced fluorescence signal compared with background signal. Therefore, the observed fluorescence of BBC should be originated from CoA rather than Acyl-CoA.

In our previous report, we have used BCC for the detection of cysteine, homocysteine and glutathione.[12] However, these thiols did not affect the carnitine and chloramphenicol assay in the sense that here the assay involves different assay pathways consisting of selective acetyl-CoA transferring enzymes (CAT or CrAT) with the presence of acetyl-CoA (scheme 2). Only carnitine and chloramphenicol are capable to follow the schematic procedure explained in scheme 2 in the presence of their respective acetyl-CoA transferring enzymes. Moreover, we have carried out control experiments in the absence of analytes (carnitine and chloramphenicol) and subtract the background response, by this way minimal interference (if any present in biological and food samples) also avoided. It is worth to mention that the assay procedure of our BCC platform is similar to the commercial carnitine and chloramphenicol fluorometric assay kits. The real sample analyses (explained in the 3.4) carried out in milk, powdered milk and honey samples presented acceptable recoveries with less than 2% error which are clearly indicating other analytes which are coexisted in the real samples did not affect the determination of carnitine and chloramphenicol.

Moreover, in our previous report, we have inferred that BCC has great selectivity that the amino acids (Gly, Ala, Ser, Thr, Val, Leu, Ile, Met, Pro, Phe, Tyr, Try, Glu, Gln, His, Lys, Arg, Asn and Asp) and biological reductants such as ascorbic acid, dopamine, histamine, uric acid and NADH were unable to trigger the ejection of fluorogenic coumarin from BCC[12]. Real sample studies has shown acceptable recovery results which clearly revealed that the proposed fluorescence detecting platform is highly selective and sensitive for the determination of carnitine and chloramphenicol.

3.3. Apparent Kinetic parameters

BCC is also a convenient fluorogenic substance for indirect spectrophotometric monitoring of various kinetic parameters of both enzymes. The solution containing BCC and acetyl-CoA with either carnitine or chloramphenicol and the corresponding enzymes become fluorescent within 15 min (**Fig. S5, Fig. S6**). In contrast, incubation of BCC, acetyl-CoA and enzymes alone resulted in no increase in fluorescence; thus, BCC is not a substrate of either CrAT or CAT (**Fig. 4**). The apparent kinetic parameters of the acetyl-transferring reactions for carnitine and chloramphenicol with the corresponding enzymes using BCC as a spectrophotometric reporter were also determined.

A double-reciprocal plot of fluorescence signal appearance rate versus different concentrations of carnitine and chloramphenicol is shown in **Figure 4**. Michaelis–Menten equation has been used to calculate K_m and V_{max} values. The apparent K_m values for the acetyl-transferring enzymatic reaction for carnitine and chloramphenicol with their corresponding enzymes were 191.7 ± 9.1 and 42.7 ± 3.9 μM, respectively, and the corresponding V_{max} values were 18.6 ± 2.8 and 1.65 ± 0.2 μmol min^{-1} mg of $enzyme^{-1}$. The K_m values determined using BCC as the spectrophotometric reporter are comparable to those previously reported for carnitine (244 μM) and chloramphenicol (33 μM).[36, 37] However, the apparent V_{max} values for the acetyl-transferring reaction for carnitine and chloramphenicol with the corresponding enzymes obtained using BCC are much smaller than previously reported values of 98 and 2.71 μmol min^{-1} mg of $protein^{-1}$, respectively.[38] These literature values of V_{max} were determined by either direct spectrophotometric monitoring of CoA formation or direct autoradiographic detection of acetyl-^{14}C-chloramphenicol formation. The apparent V_{max} determined using BCC is an indirect measurement of the rate of CoA formation. After the acetyl group is transferred to the substrate, the liberated CoA reacts with BCC to induce the release of fluorogenic coumarin; thus, the apparent V_{max} determined here incorporates the rates of both the acetyl-transferring reaction and the CoA- induced release of the fluorogenic coumarin. Our findings suggest that the CoA-induced coumarin-release step prolongs the apparent rate of the overall reaction, resulting in a reduced V_{max} value. Thus, the CoA-induced release of fluorogenic coumarin could be the rate-limiting step in the overall reaction. Collectively, these results indicate that BCC is a convenient latent fluorogenic probe for real-time spectrophotometric monitoring of CrAT and CAT activity.

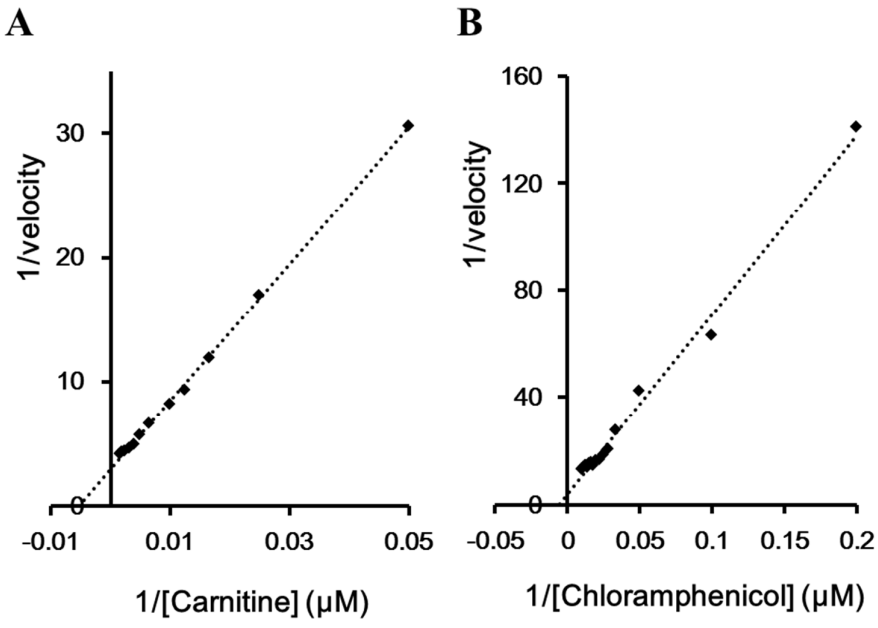


Fig. 4. Double-reciprocal (Lineweaver–Burk) plots of velocity (μM/s) versus substrate

concentration (μM). (A) carnitine (0-600 μM); (B) chloramphenicol (0-100 μM).

Fluorescent or absorbance probes are often used in experimental protocols to report on the activity of enzyme-catalyzed reactions. Such assays are important for monitoring and quantifying promoter strength in genomic studies of gene expression. CAT is commonly used as a reporter gene in such genomic research.[39, 40] A current, commonly used protocol for assaying CAT reporter plasmids relies on monitoring the acetylation of [^{14}C] chloramphenicol by acetyl-CoA.[22] The procedure involves tedious extraction and separation steps that pose difficulties for many labs that lack the proper equipment and requires the handling of radioactive materials, which creates disposal issues. Furthermore, bacteria overexpress CAT as a means for overcoming the toxicity of chloramphenicol, making CAT a target for the design of inhibitors to enhance the efficacy of chloramphenicol.[16] The fluorescence signal exhibited by BCC is specific for CAT in the presence of chloramphenicol and acetyl-CoA, and the assay platform itself provides a facile method for monitoring enzyme activities without requiring tedious processes or hazardous radioactive materials. Thus, BCC would be a useful fluorogenic indicator for monitoring stably transfected CAT reporter genes and identifying CAT-targeted antibacterial agents in future high-throughput screens.

3.4. Real sample analysis

We have determined the intrinsic amounts of carnitine and spiked chloramphenicol in food samples such as milk, powdered milk and honey using our proposed method with either CrAT (0.1 unit) or CAT (1 unit) coupled with the novel fluorimetric indicator, BCC (5 μM), and acetyl-CoA (300 μM); the results are summarized in **Table 1**. Determination of carnitine in milk by our platform was validated using an established procedure that utilizes a different fluorimetric indicator, 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) (656 μM), together with CrAT (0.1 unit) and acetyl-CoA (300 μM).[41, 42] The amounts of carnitine determined by our fluorimetric indicator in milk or milk powder are in close agreement with the results obtained with ABD-F, and are also in the range of other previously reported values.[43] Our method for determining carnitine is as sensitive as ABD-F based methods, and provides the advantage of requiring 10-fold lower amounts of fluorimetric indicator. We attempted to reduce the amount of ABD-F used in the assay, but were unable to obtain reliable measurements within 1 h. Moreover, our platform was able to successfully determine various amounts of chloramphenicol spiked directly in milk, milk powder, and honey. Unfortunately, the currently allowed residual chloramphenicol level in food is beyond our platform LOD. We are currently working to improve our detection limit by re-designing our fluorimetric indicators. Thus, BCC is an extremely sensitive latent fluorimetric indicator, and the assay system developed by our group is easy to use and does not rely on complicated procedures.

Table 1. Determination of Carnitine and Chloramphenicol present in various food samples by BCC assay platform.

Samples	Free carnitine			Chloramphenicol		
	BCC (mg/100g)	ABD-F (mg/100g)	Publish ranged (mg/100g)	Spiked (mg/100g)	Measured (mg/100g)	Recovery (%)
Milk	1.59±0.02	1.60±0.01	1.34 - 2.02	0.47	0.47±0.01	99.80%
				0.62	0.63±0.01	100.32%
				1.87	1.87±0.01	99.56%
Milk powder	13.24±0.38	13.11±0.15	11.64 - 16.89	0.46	0.45±0.01	99.39%
				0.61	0.60±0.01	99.47%
				1.82	1.85±0.02	101.62%
Honey	N.D.	N.D.	N.D.	0.48	0.48±0.01	99.28%
				0.65	0.65±0.00	101.16%
				1.94	1.93±0.01	99.46%

4. Conclusion

In summary, we have successfully implemented the first long-wavelength, latent fluorogenic substrate, BCC, as an off-on fluorimetric indicator for the determination of CoA, carnitine and chloramphenicol. The fluorescence signal generated by this assay is specific. An assay platform consisting of BCC, acetyl-CoA, and CAT or CrAT in the presence of their corresponding substrates provides a simple method for real-time monitoring of CAT and CrAT enzymatic activities. In addition, in an assay configuration that includes acetyl-CoA and CrAT but no substrate, BCC is a sensitive fluorimetric indicator for quantitatively measuring intrinsic carnitine in the nanomolar range. Moreover, real sample studies carried out in food samples revealed the promising practical feasibility of the proposed fluorimetric sensor. This BCC and acetyl-CoA transferase/acetyl-CoA assay platform is expected to be applicable for measuring a broad range of important physiological

analytes in clinical diagnostic applications.

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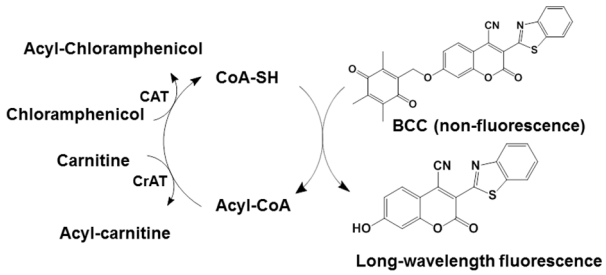

A new long-wavelength latent florescent probe, termed "BCC" for sensitive determination of Coenzyme A, carnitine and chloramphenicol.