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RESEARCH PAPER

Microbial biosensing of ciprofloxacin residues in food by a portable lens-free CCD-based analyzer

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Abstract

We present a rapid and simple approach for sensitive detection of antibiotic residues in food samples based on luminescence induction by live bacterial sensor strains integrated into a CCD-based lens-free optical analyzer (LumiSense). Using ciprofloxacin as a model antibiotic, we demonstrate response times of between 20 and 80 min, and detection thresholds of 8 ng/mL for milk, egg white, and chicken essence, and 64 ng/mL for egg yolk. These values are below the minimal allowed values as defined by European Union regulations. Although not intended to replace traditional analytical equipment and regulation-approved methods, LumiSense and similar systems, sample preparation for which involves only simple mixing, dilution, and homogenization, may nevertheless provide a simple means for high-throughput food sample screening.

Keywords Antibiotics · Whole cell biosensor · Microbial biosensor · Milk · Eggs · Chicken essence

Introduction

As global demand for animal protein grows, so does the use of antibiotic compounds in animal husbandry. The amount of antibiotics globally consumed by livestock (estimated to be at least 63,200 tons in 2010 [1, 2]), already significantly higher than that consumed by humans, is expected to increase to more than 100,000 tons by 2030 to meet the increasing demand of the ever-growing human population [2, 3]. Part of the use of antibiotics is for disease treatment, but quantitatively more significant is their mass application for disease prevention as well as for "growth promotion." One immediate highly undesirable outcome of these practices is the global increase in the prevalence of antibiotic resistance [4]; another is the growing risk of human exposure to antibiotics in food, most significantly meat and other animal-derived products, such as milk and eggs.

As a direct consequence of this risk, regulatory authorities worldwide have limited the amount of antibiotic residues allowed in food. In the European Union, for instance, the maximal allowed concentrations of antibiotic compounds in food are strictly regulated [5]. In parallel, accurate and sensitive analytical methods for the quantification of such traces are continuously being developed; an apparent trend in recent years is the adaptation of these methods to high-throughput screening applications [6–10]. Liquid chromatography–tandem mass spectrometry (LC/MS/MS) has a quantification range of 0.5–200 ng/g, and has been applied for detection of antibiotics in milk products from the market [9]. Lower detection limits, down to 0.13–1.26 ng/mL in milk powder, were reported by Chiesa et al. [10].

In addition to chemical/physical analytical approaches, there has been a growing interest in the application for this purpose of

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biosensors [7] based on the interactions of the target antibiotics with antibodies [11], aptamers [12], enzymes [13], or molecularly imprinted polymers [14]. Another potentially useful option is biosensors based on live bacterial cells as the sensing entities, since bacteria are the most obvious "test organisms" for assaying the presence of antimicrobial compounds [15]. Indeed, genetically engineered bacterial bioreporters were developed in which genes encoding a reporter entity (often bacterial bioluminescence genes, *luxCDABE*) were fused downstream of an antibiotic-induced gene promoter [16–18]. Such bacterial sensor strains report by a dose-dependent luminescent signal on the presence of the target antibiotics. However, the concentrations at which such compounds inhibit bacterial activity are often significantly higher than the required detection thresholds for antibiotic materials in food. Consequently, several molecular approaches have been used to enhance the performance of bacterial antibiotic sensors and reduce detection thresholds to practical levels [19].

To turn a bacterial bioreporter strain into a biosensor device, it needs to be integrated into a hardware platform that will not only house the live cells but will also serve to transduce their biological signals into a quantitative response [20]. We recently described [21] a portable platform for such purposes, based on a bioluminescent 16-plex whole cell sensor array on a chip with an integrated heater and a temperature sensor. The bioluminescence induced by simulated contaminant events was detected within 15–45 min, measured as time-lapse changes in the bioluminescence by a single windowless linear charge-coupled device (CCD). This article describes the application of an improved design of this device for the rapid and sensitive detection of trace antibiotics in food products, with use of ciprofloxacin (CIP) as the model antibiotic.

Materials and methods

The LumiSense system

The CCD-based platform used for monitoring the luminescent responses of the bioreporter bacteria to the presence of CIP, the model antibiotic used in this study, was a significantly improved version of the system (LumiSense) described by Tsai et al. [21]. Briefly, LumiSense is a portable system with integrated microfluidic sample chips, a bacterial incubation chip (BacChip), and a linear CCD as the photodetection component. Some major components of the LumiSense system are illustrated in Fig. 1. The lid, which includes layers for sample introduction and storage, covers the BacChip and forms a watertight seal to allow direct contact between the liquid samples and the immobilized bacterial reporters. The BacChip is an aluminum chip that holds 16 wells (each 1 mm × 2.5 mm) to accommodate polymer-immobilized bacteria (4.5 μL per well), and its temperature is maintained at 37 °C by an integrated heater and a temperature sensor. The sample chamber layer separates the wells into four groups, four wells in each. A small protrusion structure allows the BacChip to fit into a slot in the linear CCD chip in very close proximity to the CCD sensing elements, to achieve lensless imaging of the bacterial luminescence. The top-down arrangement of sample, bacterial well, and CCD ensures that luminescence is detected with no interference by food sample turbidity. The detailed fabrication procedures for the BacChip and other component layers were described previously [21–23]. The improvements incorporated into the new design include the following:

1. A grounded metal box cover was added, shielding the BacChip and the CCD. This allowed the blocking of all optical interference and reduced the background static electrical discharge, a frequent phenomenon in the original version, to a unobservable level.
2. An additional lightproof cover was included, encompassing the entire LumiSense system, reducing external light leakage to the CCD to a minimum.

As a consequence of these two modifications, the background signal measured by the linear CCD was drastically reduced: the average luminescence of 16 sample wells was reduced from approximately 803,000 ± 152,000 units to approximately 39,900 ± 13,600 units. Additionally, the temporal variation of the background signal was reduced by approximately 80% (from approximately 6020 units to approximately 1280 units; see Table S1). Another modification included an enhancement of the BacChip surface hydrophilicity by oxygen plasma treatment (described in detail later). This has completely abolished the trapping of air bubbles in the wells during the loading of the bacterial reporters, and had a major effect on the BacChip performance: the chip loading time was shortened from approximately 10 min to less than 1 min per chip, and the variability in luminescence between wells was dramatically reduced. As shown in Fig. S1, the standard deviation changed from approximately 30% (977,000 ± 290,800) to approximately 2.4% (2,080,000 ± 50,800).

Bacterial reporter strain, growth conditions, and testing setup

The antibiotic-responsive microbial reporter strain used in this study was an *Escherichia coli* RFM strain [24] harboring a fusion of the *E. coli recA* gene promoter to the *Photorhabdus luminescens luxCDABE* bioluminescence gene cassette [25]. This reporter strain was previously shown [18] to respond to the presence of specific antibiotic compounds by a dose-dependent bioluminescent signal. The bacteria were grown overnight in lysogeny broth (LB; Difco catalog no. 244620) in the presence of ampicillin (100 mg/L) at 37 °C, diluted 12-fold in the same medium without the antibiotic, and regrown to an optical density at 620 nm of 0.2 (Metertech photometer, model 6+). A culture aliquot (6 mL) was centrifuged (16,000*g*, Eppendorf 5415 R), and the pellet was

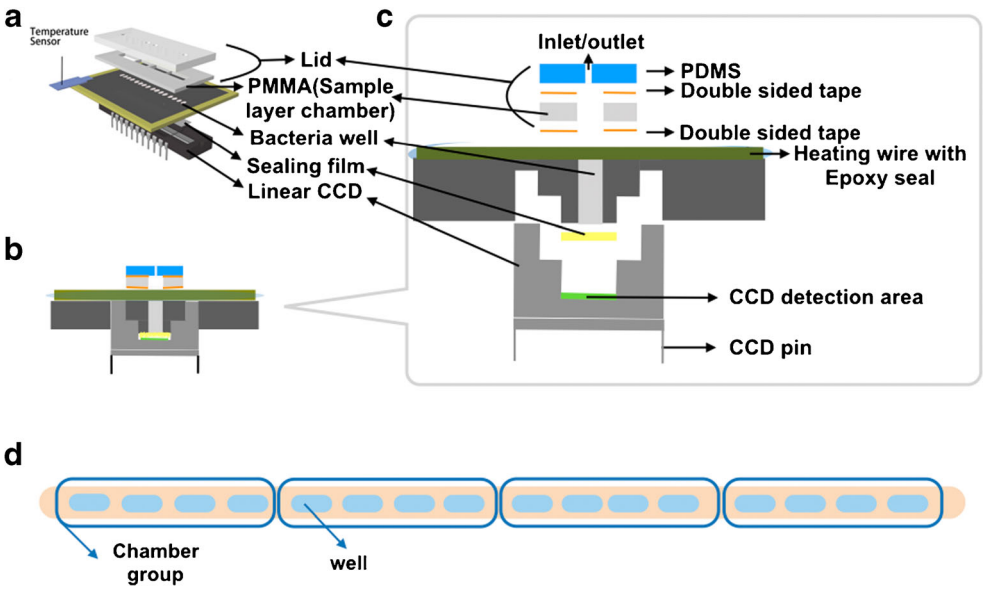

Fig. 1 The integrated microfluidic chip, the bacterial incubation chip (BacChip), and a linear charge-coupled device (CCD) photodetector (a). The green frame surrounding the BacChip denotes the integrated heater. Cross-sectional side view (b) and the assembly of the BacChip with other components (c). Top view showing the arrangement of sample wells and chamber groups on the BacChip (d). PDMS polydimethylsiloxane, PMMA poly(methyl methacrylate)

resuspended in 150 μL LB containing 0.4% (v/v) alginic acid. Then, 4.2 μL (approximately 1.3×10^7 cells) of the bacterial suspension was pipetted into each well of the BacChip, which had earlier undergone an oxygen plasma cleaner treatment (Nordson March AP-600) for 1 min at 85 W. This increased the chip surface hydrophilicity and prevented air bubble formation. Increased hydrophilicity was confirmed by examination of the water contact angle, which was reduced from 85° on the native aluminum surface to 15° on the plasma-treated surface (Fig. S2). The contact angle was measured by a contact angle goniometer with use of the image of a sessile drop at the points of intersection (three-phase contact points). To solidify the bacterial solution, 0.5 μL of 2.5% $CaCl_2$ (dissolved in LB) was then added to each well, following which the BacChip was sealed with the lid and stored at 4 °C for 30 min to ensure solidification of the bacterial suspension. As the sensing element of the microbial bioreporter used in this study, the *recA* gene promoter, may potentially also be induced by unknown nonantibiotic components in the complex food samples tested, the baseline control for all samples included the nonspiked food sample at the required dilution.

CIP and food sample preparation

CIP was obtained from Sigma-Aldrich (catalog no. 17850). A stock solution (10 mg/L) was prepared in deionized water; light exposure was avoided to prevent decomposition. This solution was then diluted by different food samples to the desired CIP concentration (1–1024 μg/L).

CIP-spiked chicken essence (CE) samples for LC/MS/MS were prepared by dilution of the CIP stock with the original CE. The samples were then sent to a certified service laboratory (Intertek Testing Services Taiwan) and analyzed as per MOHW Food No. 1021950329 Announcement (9/6/2013) - Method of Test for Veterinary Drug Residues in Foods - Method for Multiresidue Analysis (2) [26] by LC/MS/MS analysis.

Milk (Costco Kirkland Signature whole milk), chicken eggs, and CE (BRAND'S®) were purchased from a local supermarket. Milk was spiked with CIP without prior dilution or homogenization. The egg white (EW) and egg yolk (EY) were separated by decantation and separate sonication for homogenization (QSonic Q700, amplitude 35) for 3 min. EW was homogenized and spiked with CIP without prior dilution. EY was diluted in LB to the desired concentration before homogenization, and CE was similarly diluted but without homogenization. Fresh samples were prepared before each experiment. The CE acquired was confirmed to be free of CIP by LC/MS/MS by the certified service laboratory mentioned earlier.

Data acquisition and analysis

Luminescence of the bacteria in the BacChip was detected by the CCD and recorded by PC-based software (LumiLogger) as previously described [21]. After mounting of the loaded BacChip, the integrated heater was turned on, reaching a temperature of 37 °C in about 5 min. Following the introduction of liquid samples at time 0, time-lapse measurement of luminescence intensity (LI) was recorded in each well at 3-min intervals for 120 min. For each time point, LI was integrated for 6.5 s; the latter value, currently a consequence of the hardware configuration, could be extended in future studies to lower the detection limit.

The experiments described herein were conducted in quadruplicate, with use of at least three separate BacChip units, on separate days; the luminescence values presented are averages of the data collected in all experiments. The time at which the signal intensity rose above the baseline luminescence (on-time) was determined as follows. The baseline LI and the

standard deviation of each chamber group were determined by the LI of the first five data points (3, 6, 9, 12, and 15 min) in the time-lapse recording. When the LI of a group was higher than the baseline LI by a difference higher than three times the standard deviation, the signal in the group was treated as significant, and the time of this data point was marked as the on-time. LI at the last time point (120 min) is denoted as LI-120.

Results and discussion

Detection of CIP in milk: reaction kinetics

We first examined bacterial light emission stimulated by CIP in whole milk. Luminescence was observed starting from around 40 min after sample introduction, as shown in Fig. 2a (64–1024 ng/mL) and Fig. 2b (0-8 ng/mL). The on-time for the 8 ng/mL sample was 42 min, whereas that for the 4 ng/mL sample was calculated as insignificant, even though an increase in LI was apparent (Fig. 2b).

Even at the highest CIP level tested (1024 ng/mL), LI displayed a monotonous increase during the 120-min observation time, indicating that all concentrations tested were in the subinhibitory range. The dynamic range derived from our current data is from about 4 ng/mL to at least 1024 ng/mL. Higher concentrations were not tested, and the upper limit of the dynamic range was not sought, since these values would be considerably higher than the expected range in food samples. Our lowest detectable CIP level in milk, 8 ng/mL, is close to but lower than the maximum allowed residue limit, 10 ng/mL, set by the Taiwan Food and Drug Administration [26], which employs LC/MS/MS to analyze CIP and other animal drugs; it is also lower than the level set by European Commission Regulation (EU) No 37/2010 (100 ng/g) [5]. Thus, our bacterial bioreporter response range, in combination with the LumiSense hardware system, appears to be functional at the required CIP concentration range in food samples.

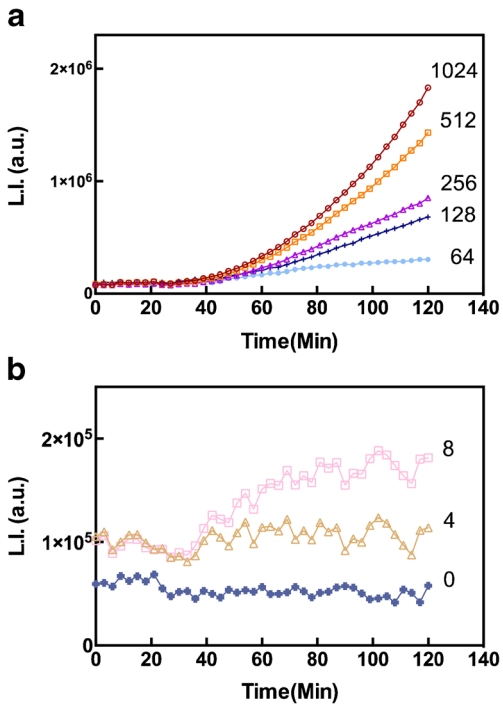

Fig. 2 Luminescence intensity (L.I.) development versus time from undiluted milk spiked with ciprofloxacin at 64–1024 ng/mL (**a**) and 0–8 ng/mL (**b**). The numbers beside the lines denote the corresponding concentrations in nanograms per milliliter. a.u. arbitrary unit

Optimization of sample dilution

As described above, satisfactory luminescence induced by the CIP-spiked milk was obtained with the use of an undiluted sample. In contrast, for two of the other samples tested, EY and CE, stronger responses of the reporter bacteria were obtained in diluted samples. Figure 3 presents, as an example, the effect exerted by CE concentration on both LI and the on-time, obtained with two different diluents, LB and water. In both media, the luminescent response to a single CIP concentration (128 ng/mL) decreased with increasing CE concentration, indicating an inhibitory effect on the response. This effect could be due to either absorption of the antibiotic molecules to an undefined component of the CE, which would reduce their availability to the bacteria, or a deleterious effect of high CE concentrations on bacterial activity. It can also be observed in Fig. 3 that sample dilution in LB yielded better results compared with the use of water as a diluent; the signal intensity was higher and the on-time (the time point at which the luminescent response becomes statistically significant) was shorter. Clearly, nutritional components supplied by LB are essential for optimal bacterial responses to the presence of CIP. On the basis of these results, in all subsequent experiments CE and EY samples were diluted to one eighth of the original concentration in LB before the tests. A corollary of the data in Fig. 3 is that when no increase in luminescence is observed, the lack of response may be interpreted as either that

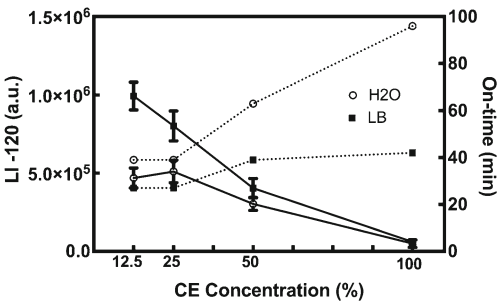

Fig. 3 Luminescence intensity at 120 min (LI-120; solid lines) and on-time (dashed lines) for different chicken essence (CE) dilutions in either water (circles) or lysogeny broth (LB; squares). The ciprofloxacin concentration in all samples was 128 ng/mL. Luminescence intensity is depicted in the instrument's arbitrary units (a.u.)

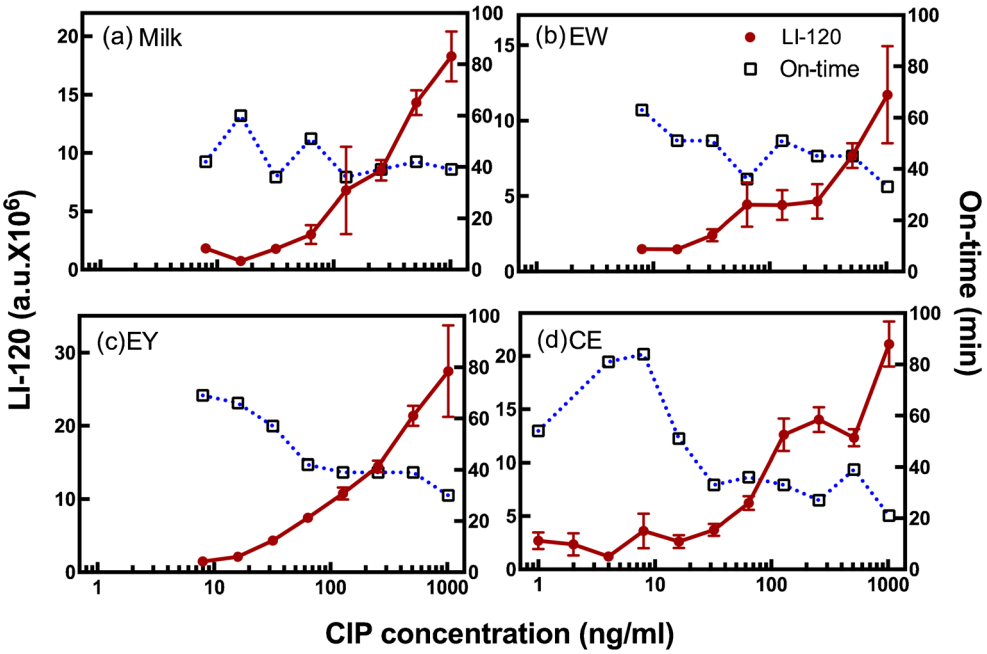

Fig. 4 Dependency of luminescence intensity (circles) and on-time (squares) on ciprofloxacin (CIP) concentration in milk (a), egg white (EW; b), egg yolk (EY; c), and chicken essence (CE; d). a.u. arbitrary unit, LI-120 luminescence intensity at 120 min

the sample is antibiotic-free or that the reporter bacteria are negatively affected by the sample's toxicity. The two options may be distinguished by a dilution of the sample, as demonstrated in Fig. 3: if a diluted sample displays an enhanced response, this is clearly a case of a positive antibiotic-induced response masked by an interfering sample composition. The effect of the high sample concentration appears not to be unique to CIP detection; it has also been observed with nalidixic acid, a known model inducer of the *recA::lux* bioreporter, as the target molecule (data not shown).

CIP detection in milk, EW, EY, and CE

LI in response to the presence of different CIP concentrations is shown in Fig. 4, along with the on-time measured for the same samples. For all sample types tested, LI-120 increased with increasing CIP concentration. This increase was close to linear at levels lower than 100 ng/mL, tending to flatten out at higher concentrations. This may indicate that maximal gene induction intensity has been reached, and/or that at the high levels of antibiotics, their antibacterial activity begins to cancel out the effects of gene induction.

The shortest on-time for all sample types appears to be between 20 and 40 min, with the values increasing at lower CIP concentrations up to a maximum at 60–80 min. Thus, for samples with CIP concentrations lower than 100 ng/mL, an analysis result may be obtained in less than 80 min, with shorter response times for higher concentrations.

From the data shown in Fig. 4, viscosity did not exert an effect on the luminescence response, since CIP detection in the most viscous sample (EY) was similar to that in the least viscous one (CE). Similarly, there was no interference by the opaqueness of the EY and milk samples, compared with the transparent EW and CE.

As a comparison of the LumiSense system with the standard analytical method for animal drugs, LC/MS/MS, we have listed in Table 1 the analysis results from both methods. The LC/MS/MS-determined CIP concentrations of all samples were lower than the calculated values based on the actual amounts added. This might be caused by loss during the sample manipulations involved in food sample preparation for LC/MS/MS analysis. Despite this loss, positive results were obtained when the CIP concentration was higher than the detection limit (10 ng/mL) of the service laboratory. The qualitative results showed that LumiSense could detect CIP at 1 ng/mL. The considerably simpler LumiSense system demonstrated a CIP detection capability that was superior than LC/MS/MS analysis of the same samples. Remember that the CE used for LumiSense detection was diluted to one eighth of the original concentration. With the dilution factor corrected, the lowest detected concentration corresponded to 8 ng/mL (1 ng/mL × 8) in the original CE.

Table 1 Detection of spiked ciprofloxacin (CIP) in chicken essence by liquid chromatography–tandem mass spectrometry (LC/MS/MS) and LumiSense

Final CIP concentration in sample (ng/mL)	LC/MS/MS detection (ng/mL)	LumiSense detection and on-time (min)
1	Not detected	Positive (21)
16	10	Positive (24)
128	70	Positive (39)

Conclusions

The device and method described in this communication present a simple approach for the detection of antibiotic residues in food samples based on luminescence induction by live bacterial sensor strains integrated into a CCD-based lens-free optical analyzer. The detection thresholds achieved are all within the regulatory requirements in the European Union [5] as well as those in Taiwan [26]. The lowest detected concentrations (after correction for sample dilution) were 8 ng/mL for milk, EW, and CE, and 64 ng/mL for EY. Although these values are significantly higher than those achievable by state-of-the-art analytical equipment such as LC/MS/MS systems, characterized by detection limits as low as approximately 0.1 ng/mL [10], the performance of the method presented here is not only adequate for ensuring regulatory compliance but is also advantageous by being simple and of low cost. Whereas sample preparation procedures for liquid chromatography include several elaborate and time-consuming steps such as deproteinization, defatting, and extraction [10], and require a multitude of reagents and solvents, the procedure presented here involves only simple mixing, dilution, and homogenization. The portable LumiSense system is smaller, cheaper, much simpler to maintain and operate, and provides a very effective alternative for quick and simple detection of antibiotic compounds, as presented here with CIP as an example. Although not intended to replace traditional analytical equipment and regulation-approved methods, LumiSense and similar systems may nevertheless provide a simple and effective means for high-throughput sample screening. Furthermore, by incorporation into the BacChip an array of bacterial sensor strains, "tailored" to detect specific food contaminants (e.g., antibiotics), compound classes (such as heavy metals or chlorinated pesticides), or global effects such as toxicity or genotoxicity, future generations of the device may be able to provide comprehensive screening capabilities for diverse and application-specific targets of choice.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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