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Ultrasensitive Detection of *Salmonella* and *Listeria monocytogenes* by Small-Molecule Chemiluminescence Probes

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Abstract: *Detection of Salmonella and L. monocytogenes in food samples by current diagnostic methods requires relatively long time to results (2–6 days). Furthermore, the ability to perform environmental monitoring at the factory site for these pathogens is limited due to the need for laboratory facilities. Herein, we report new chemiluminescence probes for the ultrasensitive direct detection of viable pathogenic bacteria. The probes are composed of a bright phenoxy-dioxetane luminophore masked by triggering group, which is activated by a specific bacterial enzyme, and could detect their corresponding bacteria with an LOD value of about 600-fold lower than that of fluorescent probes. Moreover, we were able to detect a minimum of 10 Salmonella cells within 6 h incubation. The assay allows for bacterial enrichment and detection in one test tube without further sample preparation. We anticipate that this design strategy will be used to prepare analogous chemiluminescence probes for other enzymes relevant to specific bacteria detection and point-of-care diagnostics.*

Amongst known human pathogenic bacteria there are several that can lead to foodborne illness, resulting from the consumption of contaminated food. Most of such illnesses are infections, caused by a variety of pathogenic bacteria, viruses and parasites.[1] Owing to the widespread occurrence and hazard of food-borne pathogenic bacteria, there is an obvious need to detect and identify the source, both as either a food contaminant or after animal/human infection.[2] Two major pathogens responsible for food contamination are *Salmonella* and *Listeria monocytogenes*.[3] The share of tests for these bacteria in the global food testing market is 40% and 31%, respectively.[4] Both bacteria are important opportunistic pathogens, which cause zoonotic diseases, for example, listeriosis and salmonellosis. The bacteria cannot be transmitted directly from human to human. Instead, fecal–oral infection takes place almost exclusively after the consumption of contaminated food. Every year, *Salmonella* is estimated to cause circa one million foodborne illnesses in the US, with 19000 hospitalizations and 380 deaths.[5] Human pathogenic *Listeria*, *L. monocytogenes*, is also widely distributed with infections resulting in a high mortality rate (greater than 20%).[6]

Detection of *Salmonella* and *L. monocytogenes* from food samples is currently performed by applying ISO-certified reference methods (ISO 6579 and ISO 11290, respectively).[7–9] These methods apply different enrichment cultures and plating on selective agar media for the detection of bacteria. Presumptive colonies have to be confirmed after the initial isolation. In case of *L. monocytogenes*, a safe negative result is available after 96 h (4 days). Positive results are available after 96–144 h (4–6 days), depending on the growth of the bacteria. In case of *Salmonella* a negative result is available after 66 h (< 3 days), while a positive result is available after 114 h (< 5 days). Rapid alternative methods such as immunoassays or DNA amplification (PCR in particular) have improved initial time to results but typically still require bacterial pre-enrichment of 16–48 h[10] while the detection time is reduced to a range from hours to few minutes.[11–13] As these molecular methods detect DNA or cell surface antigens (genotypic), a confirmatory test to show bacterial viability (phenotypic) is needed before taking action. Furthermore, a point-of-care test is difficult to achieve as transfer of samples after pathogen enrichment requires a biosafety laboratory. Outsourcing pathogen tests to external service laboratories adds transport time to the availability of results. Lateral flow assays (LFAs) are also widely used as a rapid detection method for various monitoring and diagnostic purposes, including bacterial detection.[14]

Food manufacturers today rely on random sample testing of finished products. Hence, faster methods exhibiting better sensitivity and specificity, which can be performed at the point of care, are desired in food processing (environmental monitoring). Significant effort has gone into the development of detection methods, to allow for fast, accurate, and cost-efficient detection and identification of food-borne pathogenic bacteria. Considering all requirements, specifically the need for phenotypic testing, today's most widespread meth-

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ods for pathogen detection and enumeration remain selective chromogenic and fluorogenic probes.[6,15–18]

Chemiluminogenic assays for enzymatic detection have been shown to be orders of magnitude more sensitive than equivalent fluorescence detection assays.[19–21] Recently, our group has explored new approaches for amplifying chemiluminescence light intensity[22–24] under physiological conditions.[25–34] A remarkable enhancement of light emission was obtained by simply improving the emissive nature of the excited species, formed during the chemiexcitation of Schaap's dioxetanes.[35–37] Phenoxy-dioxetane probes, bearing conjugated electron-withdrawing substituent at their *ortho* position, could release benzoate derivative, during their chemiexcitation, which was found to be highly emissive under aqueous conditions.[29] These new phenoxy-dioxetane luminophores exhibited light emission intensity of up to 3000-fold greater than that of the original Schaap's dioxetanes. As such, we sought to utilize our chemiluminescence luminophores to design probes for detection of *Salmonella* and *L. monocytogenes*. Herein, we report new efficient chemiluminescence probes for the ultrasensitive direct detection of two types of pathogenic bacteria.

The light-emission mechanism of our phenoxy-dioxetane chemiluminescence probes is initiated by the removal of a triggering responsive group, which serves as the substrate of a specific bacterial enzyme (Figure 1 A). Once the trigger is cleaved, spontaneous 1,6-elimination of a spacer occurs to yield a phenolate intermediate. This phenolate decomposes through a chemiexcitation process, to emit intense green light. Probe **CLSP** is composed of phenoxy-dioxetane masked with a C8-ester group, which is a known substrate of a *Salmonella* esterase.[16] Similarly, probe **CLLP** is composed of phenoxy-dioxetane masked with a myo-inositol 1-phosphate group; a known substrate of the virulence factor phosphatidylinositol-specific phospholipase C (PI-PLC), which is produced only by the pathogenic *L. monocytogenes* (Figure 1 B).[38]

The synthesis of the *Salmonella* probe, **CLSP**, was performed as described in the Figure 2. In brief, an esterification reaction between caprylic acid and 4-hydroxybenzyl alcohol afforded compound **1a**, which was then converted to its iodine derivative **1b**. The latter was reacted with phenol **1c** to produce ether **1d**. Oxidation of the enol-ether function of **1d** with singlet oxygen afforded the corresponding dioxetane **CLSP**.

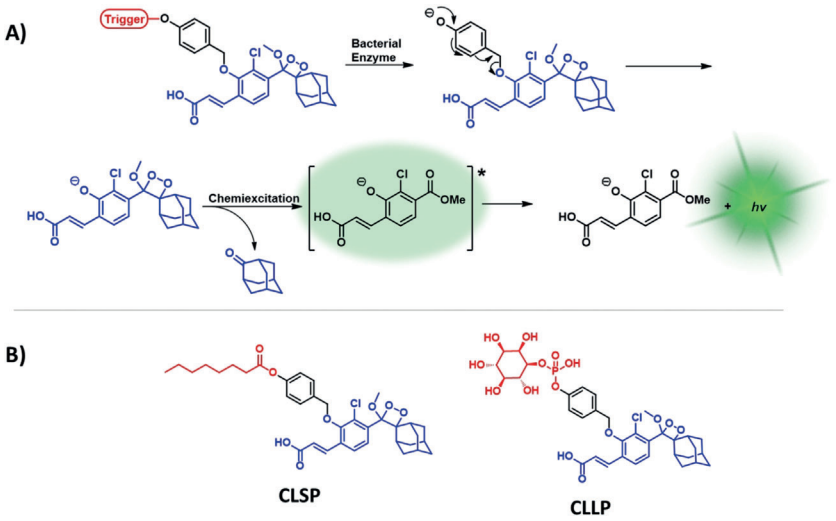

Figure 1. A) General structure and chemiexcitation pathway of probes designed for the detection of bacterial enzymes. B) Molecular structures of chemiluminescence probes **CLSP** and **CLLP** for detection of *Salmonella* and *Listeria monocytogenes*, respectively.

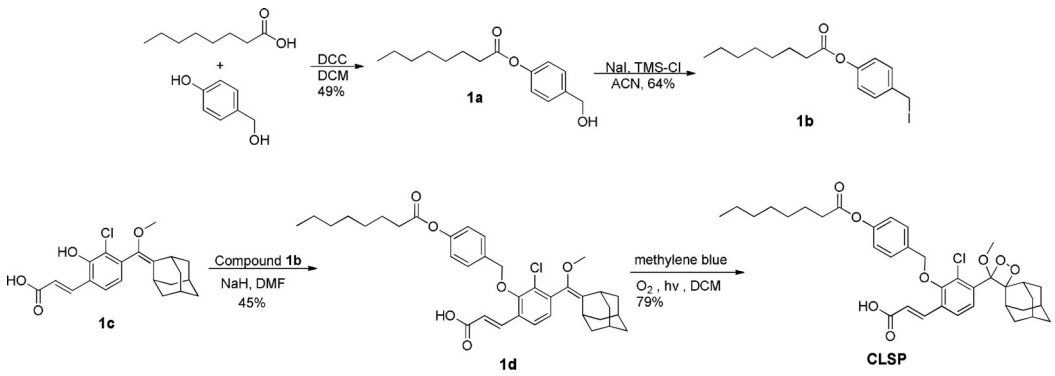

Figure 2. General synthetic scheme for **CLSP**.

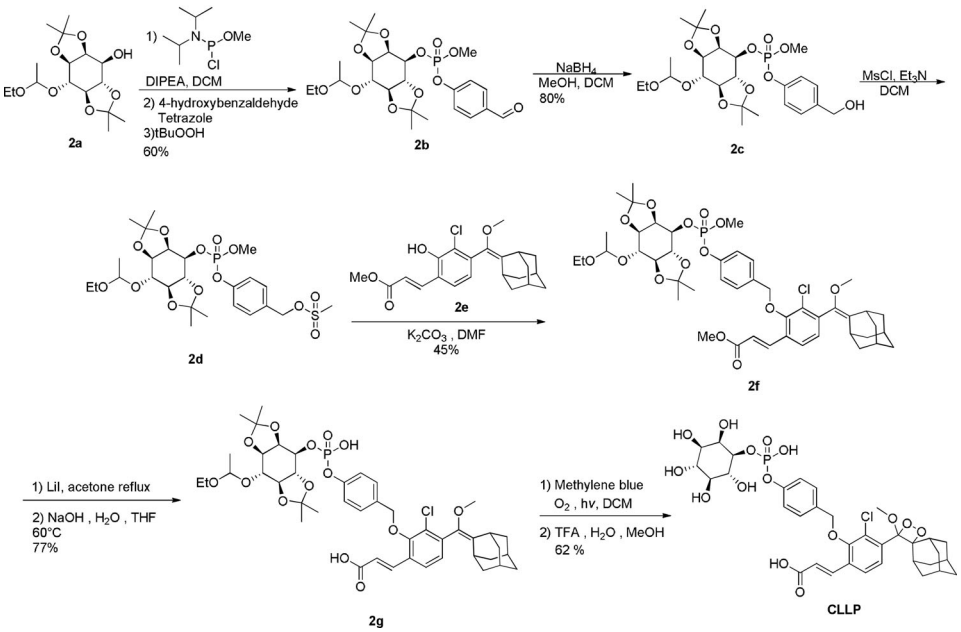

Figure 3. General synthetic scheme for **CLLP**.

The synthesis of the *Listeria* probe, **CLLP**, was performed as described in the Figure 3. A reaction between penta-protected inositol derivative **2a** and 4-hydroxybenzaldehyde under the indicated conditions afforded phosphate **2b**. The aldehyde functionality of **2b** was reduced to its benzylalcohol derivative **2c**, which was further reacted with mesyl-chloride to give compound **2d**. The latter was reacted through an S_N2 reaction with phenol **2e** to afford ether **2f**. Compound **2f** was treated with lithium iodide to selectively remove the methyl phosphonate protecting group. Additional alkaline hydrolysis, to remove the methyl from the acrylate substituent, yielded compound **2g**. Oxidation of the enol-ether function of **2g** with singlet oxygen afforded the corresponding dioxetane **CLLP**.

The chemiluminescence emission profile of **CLSP** over time was measured in physiological buffer, in the presence and in the absence of porcine liver esterase (PLE). The total light emission signal observed upon activation with PLE was about 300-fold higher than that obtained in the absence of the enzyme (Figure 4). This result is remarkable, especially given the fact that **CLSP** is composed of an activated phenolic ester, which suffers of a relatively high rate of background hydrolysis. Similarly, the chemiluminescence emission profile of **CLLP** was measured in the presence and in the absence of PI-PLC. The total light emission signal upon activation by PI-PLC was almost 700-fold greater than the background signal. In addition, both **CLSP** and **CLLP** show a logarithmic correlation between probe concentration and enzyme concentration, enabling quantitative enzyme detection (see Supporting Information, Figures S1 and S2).

Next, we sought to evaluate the ability of the **CLSP** and **CLLP** probes to detect *Salmonella* and *L. monocytogenes*, respectively. In order to identify one type of bacteria among others, it is particularly important to find out whether the chemiluminescent signal of the probe can be selectively

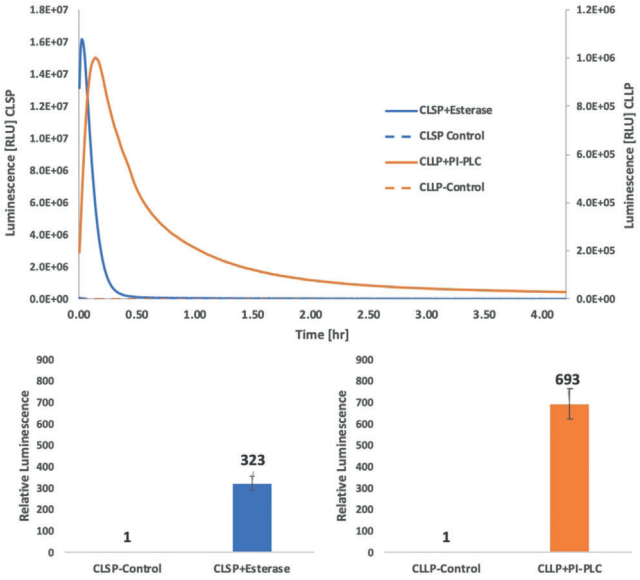

Figure 4. Chemiluminescence kinetic profiles of **CLSP** or **CLLP** [10 μM] in PBS (pH 7.4, 10% DMSO) in the presence or absence (control) of porcine liver esterase [1 U mL^{-1}] or PI-PLC [0.5 U mL^{-1}], respectively, at room temperature (top). Relative total light units (RLU) emitted, normalized to that of the control (bottom).

produced by its corresponding specific bacterial strain. Therefore, probes **CLSP** or **CLLP** were added to cultures of several appropriate strains of bacteria, preincubated at 37 °C for 6 h. For the *Salmonella* detection, **CLSP** was tested with the following bacterial strains: *Escherichia coli* ATCC 25922 (*E. coli*), *Citrobacter freundii* ATCC 8090, *Salmonella* Typhimurium (D) ATCC 14028 (*S.* Typhimurium), *Salmonella* Enteritidis (D) ATCC 13076 (*S.* Enteritidis 1) and *Salmonella* Enteritidis RKI 05/07992 (*S.* Enteritidis. 2). Both *E. coli* and *C. freundii* served as negative controls, as they do not possess

a C8-esterase. Moreover, these bacteria may be present in stool samples tested for *Salmonella*, as they occur in the intestinal tracts of animals and humans.[39,40] Additionally, some serotypes of *E. coli* can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls owing to food contamination, similarly to *Salmonella*.[41]

For *L. monocytogenes* detection, **CLLP** was added to cultures of the following bacterial strains: *E. coli* ATCC 25922, *Listeria innocua* (6a) ATCC 33090, *L. monocytogenes* (4b) ATCC 19115 (*L. monocytogenes* 1) and *Listeria monocytogenes* ATCC 7644 (*L. monocytogenes* 2). Both *E. coli* and *L. innocua* served as negative controls, as they do not possess a PI-PLC enzyme.[38] Moreover, they may be present in food samples tested for *L. monocytogenes*, as *L. innocua* occurs in food sources, and some serotypes of *E. coli* may as well. Additionally, as mentioned above, *E. coli* may also be present in stool samples.[40–42]

Both probes were also added to sterile saline, and the luminescence amounted was used to normalize the measured signal obtained for each bacterial strain. The signal intensities, obtained by each sample with the appropriate probe, are shown in Figure 4. Probe **CLSP** was able to differentiate between the *E. coli* and *C. freundii* controls and the *Salmonella* strains, with a signal enhancement of up to 693-fold for the *Salmonella* strains compared to the sterile control. Furthermore, the obtained light emission intensity, allowed **CLSP** to visually distinguish between *Salmonella* (*S.* Enteritidis RKI 05/07992; *S.* Enteritidis 2) and *E. coli* (*E. coli* ATCC 25922), using a high-resolution digital camera (Figure 5B.

Similarly, probe **CLLP** was able to differentiate between the *E. coli* and *L. innocua* controls and the *L. monocytogenes* strains, with a signal-to-noise ratio of up to 10000-fold higher for the *L. monocytogenes* strains than that of the sterile control. Remarkably, the signal intensity produced by the pathogenic *L. monocytogenes* strains was about three orders of magnitude greater than that of the non-pathogenic *L. innocua.* This enables us to differentiate between the pathogenic and non-pathogenic *Listeria* within a short time after addition of **CLLP** (Figure 5D).

With chemiluminescence probes **CLSP** and **CLLP** in hand, we next sought to compare their ability to detect *Salmonella* and *L. monocytogenes* with that of currently existing fluorescence methods. 4-Methylumbelliferyl caprylate (**MUCAP**) and 4-methylumbelliferyl myo-inositol 1-phosphate (**MUMIP**) are two commercially available fluorogenic probes for the detection of *Salmonella* and *L. monocytogenes*, respectively.[16,42] **MUCAP** and **MUMIP** are mostly used in chromogenic nutrient agar plates for confirmatory testing.[18,43] The mode of action of these probes is based on removal of a triggering enzyme-labile group by the *Salmo-*

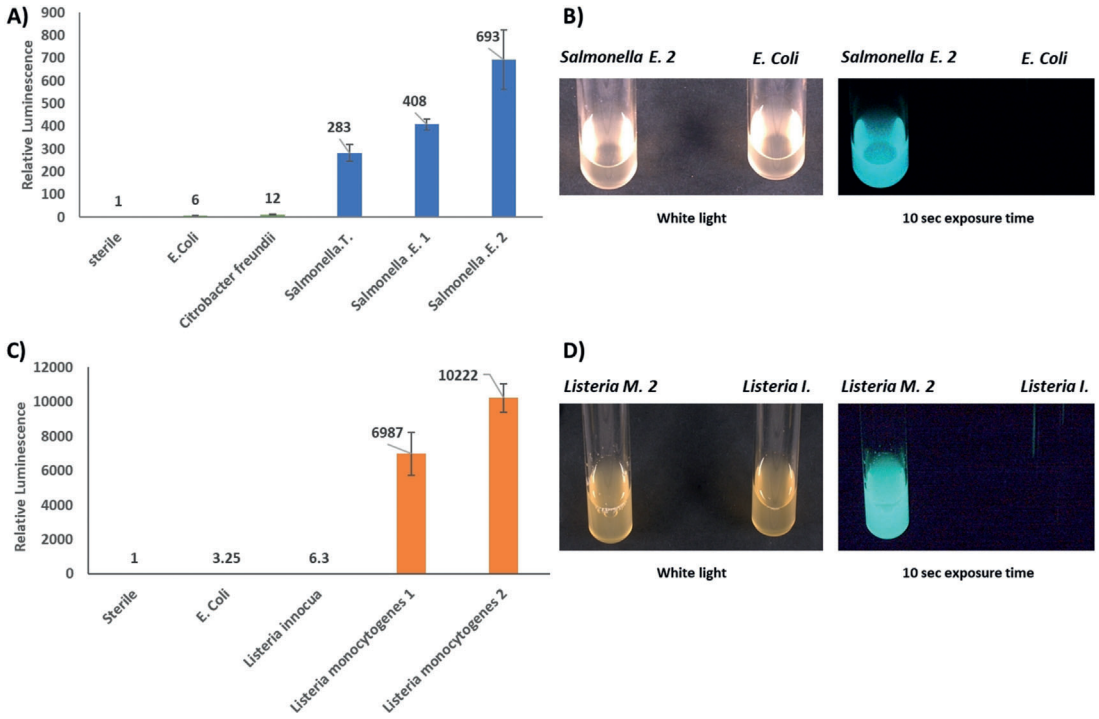

Figure 5. A) Relative Luminescence (RLU) emitted from cultures of different bacterial strains (inoculated with 1×10^5 CFU mL^{-1}, incubation time 6 h) after the addition of **CLSP** [10 μM] (normalized to that of **CLSP**, which was added to sterile growth medium), 27–30 min after addition of the probe. B) Images of *Salmonella* (*S.* Enteritidis RKI 05/07992; *S.* Enteritidis 2) and *E. coli* (*E. coli* ATCC 25922) tube cultures (incubated overnight, ca. 2×10^9 CFU mL^{-1}) after the addition of **CLSP** [20 μM] taken with a high-resolution, high quantum efficiency digital camera, with white light illumination turned on (left) and off (right). C) RLU emitted from cultures of different bacterial strains (inoculated with 1×10^5 CFU mL^{-1}, incubation time 6 h) after the addition of **CLLP** [10 μM] (normalized to that of **CLLP**, which was added to sterile growth medium), 8 min after addition of the probe D) Images of *L. monocytogenes* ATCC 7644 (*L. monocytogenes* 2) and *E. coli* (*E. coli* ATCC 25922) tube cultures (incubated overnight, ca. 2×10^9 CFU mL^{-1}) after the addition of **CLLP** [67 μM] with white light illumination turned on (left) and off (right).

nella C8 esterase or by the PI-PLC enzyme to release a fluorescent coumarin derivative (4-methylumbelliferone). This fluorophore produces a measurable fluorescence signal ($\lambda_{max} = 460$ nm) with an excitation at wavelength of 360 nm.

Figure 6 shows the signal-to-noise ratios obtained by each chemiluminescence probe (**CLSP** and **CLLP**) and by their fluorogenic commercial analogues (**MUCAP** and **MUMIP**)

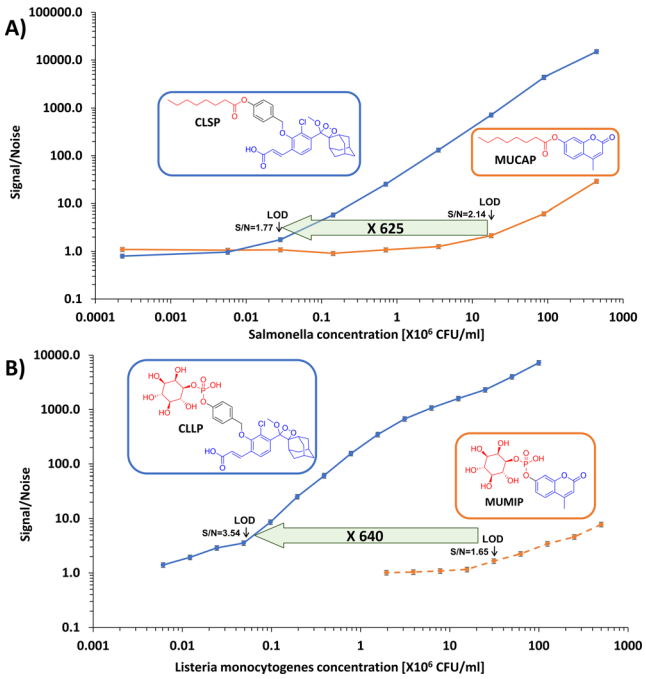

Figure 6. A) Signal/noise ratio values for **CLSP** [10 μM] and **MUCAP** [100 μM] plotted against different *Salmonella* concentrations (*S.* Enteritidis RKI 05/07992; *S.* Enteritidis 2) using logarithmic scales. B) Signal/noise ratio values for **CLLP** [10 μM] and **MUMIP** [100 μM] plotted against different *L. monocytogenes* concentrations (*L. monocytogenes* (4b) ATCC 19115) using logarithmic scales.

for different bacteria concentrations (for *Salmonella* and *L. monocytogenes*, respectively). Remarkably **CLSP**, exhibited a limit of detection (LOD) value of 28.8×10^3 CFU mL^{-1}, while MUCAP detected *Salmonella* with an LOD value of $17\,800 \times 10^3$ CFU mL^{-1}. Similarly, **CLLP** exhibited an LOD value of 48.8×10^3 CFU mL^{-1}, while **MUMIP** detected *L. monoctogenes* with an LOD value of $31\,250 \times 10^3$ CFU mL^{-1}. The enhanced sensitivity observed by **CLSP** and **CLLP** (625 and 640-fold, respectively) clearly demonstrate the advantage of our chemiluminescence substrates versus currently existing fluorescence substrates for bacterial detection assays.

The performed chemiluminescence assays showed a positive logarithmic correlation between the signal-to-noise ratio and bacteria concentration, even at a concentration range as low as the limit of detection, making this assay suitable for quantitative as well as qualitative assays (see Figures S3 and S4). In addition, chemiluminescence assays require significantly less substrate than fluorescence assays (the chemiluminescence data obtained in Figure 6 were obtained with probe concentrations 1/10 of that required for the fluorescence commercial analogues).

Interestingly, in an earlier study, Schaap's adamantylidene-dioxetane was applied to prepare a chemiluminescent probe for PI-PLC enzyme using the same triggering substrate demonstrated in this work.[44] However, this dioxetane suffers from an extremely weak light emission efficiency under aqueous conditions and is completely unusable without additives at physiological pH values. Therefore, a long incubation time (up to 5 days) and autoradiography film had to be used in order to detect a measurable signal. The **CLLP** probe described in this study produces a light emission intensity more than three orders of magnitude higher than the classic Schaap's adamantylidene-dioxetane under physiological conditions without any additives.

To further establish that our probes can be used for environmental sampling, we tested whether **CLSP** is able to detect dry stressed *Salmonella* recovered from stainless steel plates. According to certification guidelines for food diagnostics, *Salmonella* presence/absence needs to be detected from stainless steel surfaces partially at low concentration (25–75 %) and fully at elevated concentrations (100 %) in defined time to results.[45] We plated low or high concentrations of *Salmonella* (200–600 CFU or $1.0–2.0 \times 10^4$ CFU respectively) and let them dry for 2.5 h. Afterwards, we recovered the *Salmonella* bacteria using a standard swab and transferred them into an enrichment medium. After 16 h of incubation **CLSP** was added and the luminescence signal was measured (Figure 7 A).

Light emission from either a low or high amount of *Salmonella* showed a typical chemiluminescence kinetic profile with an increase in signal, which decays over time (see Supporting Information, Figure S5). At high concentrations (Figure 7 B) 100 % of the swabs gave a high signal-to-background ratio (close to 5000:1) and all samples could be differentiated from the control. For low concentrations (Figure 7 B) 88 % of the swab samples produced a high signal-to-background ratio (close to 2000:1). For 12 % of the swabs, no signal above background could be detected. We attribute this fact to the typically low recovery rates of swabs, which we measured to be between 1–2 % (data not shown). This makes it likely that swabbing a low number of bacteria from a stainless-steel plate will result in some samples not containing any *Salmonella* for enrichment and later for enzyme activity detection.[46] In order to be useful in the food industry, several strains of *Salmonella* need to be detected, therefore we show that **CLSP** can detect a variety of strains of *Salmonella* (see Supporting Information, Figure S6). The obtained results fill some key certification requirements as stated by the Association of Official Agricultural Chemists (AOAC).

Currently many bacterial detection methods are designed for a qualitative endpoint determination. This has the disadvantage that potentially highly contaminated samples can only be detected after the same time as minimal contaminated samples. A chemiluminescence dynamic monitoring method has the potential to monitor samples right from the start, thereby detecting highly contaminated samples earlier. To this end, we inoculated *Salmonella* cells in different

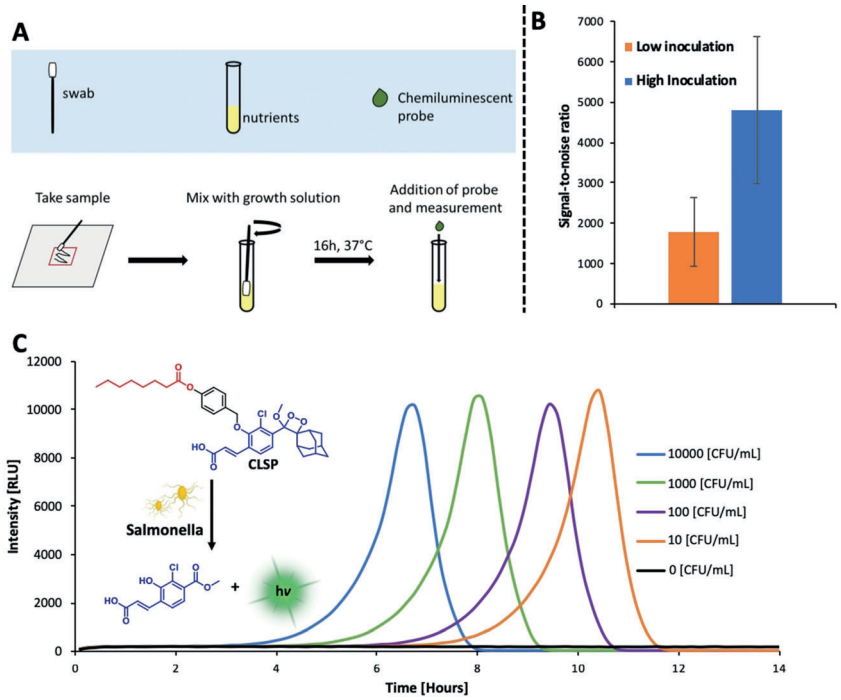

Figure 7. A) Either a low amount of *Salmonella* (200–600 CFU) or high amount ($1.0–2.0\times10^4$ CFU) was plated on a stainless-steel plate. After 2.5 h of drying bacteria were partially recovered by swabs and grown in enrichment medium for 16 h. Afterwards, **CLSP** was added [10 μM] and the luminescence signal monitored. B) Luminescence signal detected from a *Salmonella* strain recovered after dry stress on a stainless steel plate. The bars illustrate the signal/noise ratio after 10 min of incubation. C) Dynamic monitoring of luminescence signal with different starting concentrations of *Salmonella*. *Salmonella* were inoculated in different log concentrations (10–10000 CFU mL^{-1}) and placed together with **CLSP** (10 μM). Luminescence signal was constantly monitored over 14 h.

concentrations with **CLSP** and monitored how the luminescence signal developed during bacterial growth (Figure 7C). As expected, **CLSP** is stable in an enrichment medium and yields light signals curves depending on the starting number of bacteria and time of growth. Higher contamination levels could be detected in around half the time compared to low contamination levels. Such experiments may offer the ability not only to detect specific bacteria but also to give information about the initial level of contamination; which can be valuable to determine if acceptable levels of environmental bacteria have been breached in a high-risk environment.

In summary, we have developed two new chemiluminescence probes, **CLSP** and **CLLP**, for the direct detection of two of the most widely distributed and deadliest food-borne pathogenic bacteria, *Salmonella* and *Listeria monocytogenes*. The probes were composed of a phenoxy-dioxetane luminophore masked by a triggering group, which is designed for activation by a specific bacterial enzyme. Remarkably, **CLSP** and **CLLP** were able to distinguish *Salmonella* or *L. monocytogenes* strains from other bacteria that may occur in samples tested for food-borne pathogens. Furthermore, **CLLP** was able to differentiate between two strains of *Listeria*, the pathogenic *L. monocytogenes* and non-pathogenic *L. innocua*. Similarly, **CLSP** could visually differentiate between *Salmonella* and *E. coli* strains. The two chemiluminescence probes could detect their corresponding bacteria with an LOD value of about 600-fold more sensitive than that of fluorescent probes. Moreover, the *Salmonella* probe was able to detect a minimum of 10 pathogenic bacterial cells after only 6 h of incubation (Tables S2 and S3). As such, the current report exemplifies the most sensitive luminogenic bacterial enzymatic assays known to date. We anticipate that the design strategy presented in this study will be broadly used to prepare analogous chemiluminescence probes for other enzymes relevant to selective bacteria detection. Such chemiluminescence probes could serve as a supportive technological platform, which is complementary with current state-of-the-art molecular biology methods.

Acknowledgements

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Conflict of interest

NEMIS Technologies AG is a Swiss startup developing diagnostic detection kits for food safety applications. Biosynth AG is a chemical synthesis company based in Switzerland producing diagnostic molecules for detection applications.

Keywords: bacterial probes · chemiluminescence · dioxetanes · Listeria · Salmonella

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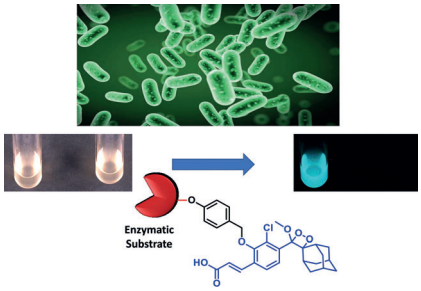
Research Articles

Analytical Methods

M. Roth-Konforti, O. Green, M. Hupfeld, L. Fieseler, N. Heinrich, J. Ihssen, R. Vorberg, L. Wick, U. Spitz,* D. Shabat* _____ ■■■■–■■■■

Ultrasensitive Detection of *Salmonella* and *Listeria monocytogenes* by Small-Molecule Chemiluminescence Probes



Salmon and Lister would be proud: The development of new chemiluminescence probes for the direct detection of two of the most widely distributed and deadliest food-borne pathogenic bacteria, *Salmonella* and *Listeria monocytogenes*, is described. The two probes could detect their corresponding bacteria with a limit of detection about 600-fold lower than that of fluorescent probes.