Fluorogenic detection of cyanide ions in pure aqueous media through intramolecular crossed-benzoin reaction: limitations unveiled and possible solutions

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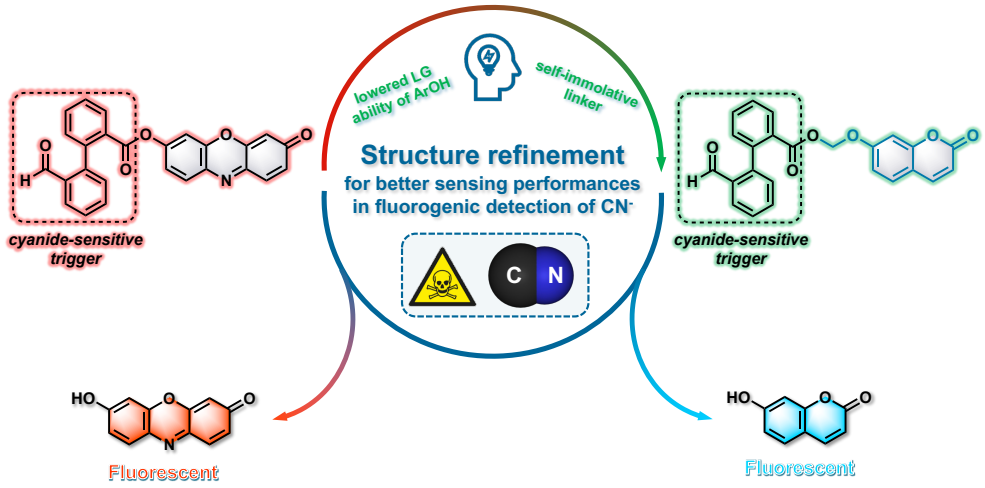

Abstract: Reaction-based fluorogenic sensing of lethal cyanide anion in aqueous matrices remains a big challenge. We have revisited the approach proposed by the Kim group (*Chem. Commun.* 2015, *51*, 7709-7712) and highlighted its limitations related to poor aqueous stability of probes and impossibility to achieve molecular amplification despite the assumed catalytic activation mechanism. Self-immolative linker strategies were considered to obtain usable cyanide-responsive chemodosimeters and statistical analyses of fluorescence data have been deepened to accurately delineate their sensing performances, especially limit of detection (LOD).

Environmental pollution by cyanides (CN^-) and related cyano compounds is clearly a topical issue of major importance. Indeed, a number of industries or industrial processes (*e.g.*, gold mining, petrochemistry, steel manufacturing, …) have widely used and continue to use cyanides, and thus they are largely responsible for persistent pollution by this lethal anion, especially in aqueous matrices.[1] This fact is exacerbated by deviant behaviours (*e.g.*, deliberate waste dumping or chemical terrorism) that constantly pose heavy threats to humans and environment. Consequently, detection and quantification of such toxic contaminant is routinely conducted using different analytical methods (*e.g.*, chromatographic, electrochemical, spectrophotometric techniques, ...)[2] in order to check that national or international legislations (*e.g.*, maximum acceptable concentration (MAC) of CN^- in drinking water is fixed at 2.7 μM by the World Health Organization (WHO)[3]) are complied with. However, the high cost of these analytical instruments, tedious sample preparation steps, requirement of trained professionals and dedicated laboratories, have led to a quest for cheaper and easily usable analytical sensing technologies.[4] Owing to its positive attributes including high sensitivity, signal output in short time scale, facile implementation within miniaturized, low-cost and portable devices, fluorescence spectroscopy quickly established itself as a valuable alternative. The development of a cyanide-responsive molecular fluorescent probe is needed to convert the molecular recognition event into a measurable signal according to intensiometric (ideally "OFF-ON") or ratiometric mode.[5] Depending on the reversibility or not of the reaction between the probe and analyte, either a fluorescent chemosensor or a fluorescent chemodosimeter is rationally designed.[6] Over the past 25 years, a myriad of cyanide-responsive fluorescent probes have been reported and the most widely used sensing mechanisms are compiled in some comprehensive reviews.[7] Due to the difficulties associated with cyanide ion selectivity in complex aqueous matrices, the reaction-based approach is preferred to minimize interactions with interfering analytes. In this context, the nucleophilicity of CN^- anion is often exploited in carbonyl or Michael-type addition reactions that lead to an hypsochromic shift of excitation/emission spectra of probe, thus producing a

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ratiometric fluorogenic response.[8] However, a majority of these fluorescent chemodosimeters are used in presence of a large amount of organic co-solvent (often DMSO) due to lack of solubility, high aggregation propensity leading to fluorescence quenching (ACQ) or limited nucleophilic reactivity of CN^- anion. In order to overcome these limitations, and to obtain a high-performance fluorogenic "OFF-ON" probe, a cutting-edge reaction-based approach was proposed by the Kim group in 2015.[9] A strategy of protection-deprotection of the resorufin phenol moiety was established using a cyanide-assisted intramolecular crossed-benzoin reaction. **IND-1** was studied in PBS (pH 7.4, containing 1% DMSO, v/v) and a limit of detection (LOD) of 4 nM was claimed. Sensing ability in complex biological systems was also evaluated in cyanide-doped living cells (HeLa and A549 cell lines). As part of a research program devoted to the development of a novel strategy for quick on-site detection of cyanide ions through a simple "mix and read" approach using a portable fluorometer, we have reconsidered the chemistry, aqueous stability and sensing performances of fluorescent chemodosimeter **IND-1**. Thanks to the implementation of a robust analytical methodology based on fluorescence assays and RP-HPLC-MS analyses, we have highlighted some major limitations associated with the fluorogenic intramolecular crossed-benzoin reaction, not disclosed in the Communication published by the Kim group. The most critical is the marked propensity of **IND-1** to undergo undesired hydrolytic activation at physiological pH and in the absence of CN^- ions, thus negatively impacting its sensing performances (*vide infra*). In order to overcome these unexpected difficulties, and to obtain truly usable cyanide-responsive fluorescent probes based on this water-compatible cascade process, we have considered the use of other phenol-based fluorophores (displaying lower leaving group ability)[10] and assessed the potential of the first self-immolative fluorogenic systems responsive to this analyte in aqueous matrices (Fig. 1).[11] Highlights of this study and recommendations for in-depth validation and evaluation of sensing performances (especially LOD) of fluorescent cyanide chemodosimeters intended to be used in pure aqueous media, are reported herein.

First, the cyanide biphenyl-type trigger directly installed onto the optically tuneable phenol group of resorufin and umbelliferone through an ester linkage, was readily prepared through a Suzuki-Miyaura cross-coupling reaction followed by ester saponification, according to protocols reported by Penhoat *et al.*[12] (for synthetic scheme S1 and experimental details, see Supporting Information). Blue-cyan emissive umbelliferone was selected as an alternative to resorufin due to a higher pKa value for its phenol moiety (7.8 *vs.* 5.9)[10,13] making this fluorophore a worse leaving group, particularly well-suited for conducting a comparative study on the stability of the ester linkage of both probes **IND-1** and **1**, in physiological pH buffer conditions (for all experimental details related to optimized synthesis, purification and characterization of probes **IND-1** and **1**, see Supporting Information).

With the two probes in hand, we first examined their fluorescence response to cyanide analyte through *in vitro* time-course assays, conducted in PBS (10 mM phosphate + 2.7 mM KCl + 137 mM NaCl, pH 7.5) at 25 °C, and under the experimental conditions used by the Kim group[9], to allow fair and accurate comparisons of our respective results. Briefly, 10 μM solutions of probes **IND-1** and **1**, were incubated in PBS alone or in PBS with different amounts of KCN (1-20 equiv.). Firstly, for **IND-1,** we observed a spontaneous hydrolysis of this probe upon its incubation in PBS alone as supported by the gradual increase of fluorescence emission at 595 nm (assigned to the emissive phenolate form of the released resorufin), and a quite similar fluorescence kinetics curve was obtained after addition of 10 equiv. of KCN (Fig. 2A).

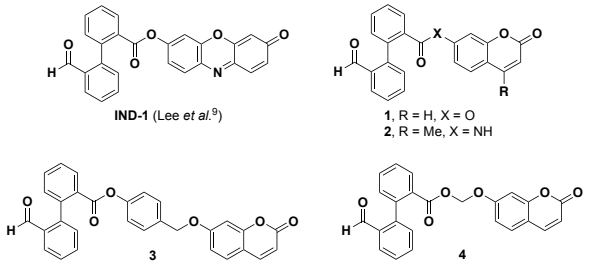

Fig. 1 Structures of cyanide-responsive fluorogenic probes studied in this work.

These disappointing results differ completely from those reported by the Kim group and question the trustworthiness of their positive findings about sensing performances of **IND-1**. Furthermore, at 10 μM, we noted partial precipitation of **IND-1** in this buffer (see Fig. S12 in Supporting Information) and a self-quenching of resorufin at concentrations higher than 5 μM was highlighted through a concentration-dependent fluorescence study (see Fig. S10 in Supporting Information). These further observations enable us to affirm that the reported LOD value of 4 nM does not reflect the real sensing performances of this fluorescent chemodosimeter. The aqueous instability was also largely favoured when a lower but more relevant concentration of probe (1.0 μM) was used (see Fig. S11 in Supporting Information). Even more surprisingly, for the coumarin-based probe **1**, we also noted a non-negligible hydrolysis upon its incubation in PBS alone, thus suggesting that the ester linkage of cyanide-responsive trigger, is fragile whatever the phenol-based fluorophore used (Fig. 2B). However, its fluorogenic response at 450 nm towards cyanide ions (10 equiv. KCN) is a bit better than that obtained with **IND-1**. According to normalized data (Fig. 2C), the two probes showed a modest maximum of fluorescence intensity (F.I.) ratio.

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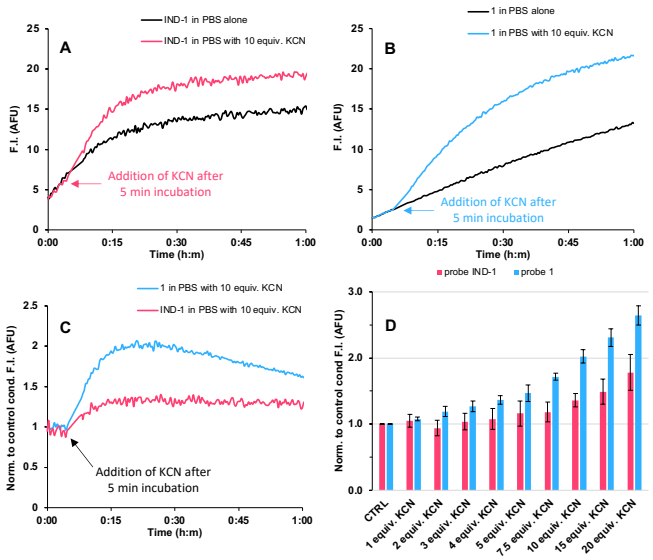

Fig. 2 (A-B) Time-dependent changes in the fluorescence intensity of fluorogenic probes **IND-1** and **1** in the presence of KCN, in PBS (pH 7.5) at 25 °C. (C) Normalised mean values of F.I. to control condition (PBS alone). (D) Bar chart of the normalised mean values of fluorescence intensity to control condition after 15 min of incubation with KCN for **IND-1** and **1**. All experiment were independently repeated thrice. Error bars represent standard deviation. See Supporting Information for parameters used for fluorescence kinetics.

A second feature of the Kim's reaction-based cyanide sensing strategy related to the catalytic mechanism assumed for the activation of **IND-1** raised issues. Indeed, in view of the concomitant release of fluorescent resorufin, 9,10-phenanthrenequinone and the cyanide anion itself (Fig. 3), the recycling of this latter analyte should be effective to produce formal molecular target amplification through its further reaction with multiple reporter molecules **IND-1**. Curiously, this valuable asset was not mentioned and studied whereas it would be particularly useful for achieving trace-level detection.[14] Furthermore, for practical sensing applications of such probe, a dramatic different methodology than the one used by the Kim group for LOD determination, should be implemented to perform quantification of analyte, through prior generation of a calibration curve using known concentrations of cyanide ion and for a given set time point (*vide infra*). In order to gain insights into the activation mechanism of probes **IND-1** and **1**, reaction mixtures corresponding to their incubation in PBS alone or in the presence of 10 equiv. KCN, were subjected to RP-HPLC-ESI-MS analyses (both full scan and single ion monitoring (SIM) modes); this latter one being considered to enhance detection sensitivity of poorly ionizable compounds such as 9,10-phenanthrenequinone (see Figs. S16-S17 in Supporting Information). After 4 h of incubation whatever the probe studied and the conditions used (*i.e.*, absence or presence of CN^- analyte), we observed on all elution profiles a peak assigned to the phenol-based fluorophore (resorufin, t_R = 2.9 min, *m/z* = 214.1 $[M + H]^+$, calcd for $C_{12}H_8NO_3^+$ 214.0; umbelliferone, t_R = 2.5 min, *m/z* = 163.1 $[M + H]^+$, calcd for $C_9H_7O_3^+$ 163.0). We also noticed the formation of 2'-formyl[1,1'-biphenyl]-2-carboxylic acid (t_R = 3.4 min, *m/z* = 225.1 $[M - H]^-$, calcd for $C_{14}H_9O_3^-$ 225.2) thus proving that undesirable hydrolysis of ester linkage of **IND-1** and **1** readily occurs in PBS. For the assays conducted with cyanide analyte (10 equiv.), two further peaks were observed at t_R = 3.7 min and t_R = 3.9 min, identified as 9,10-phenanthrenequinone (MS(ESI+): *m/z* = 209.1 $[M + H]^+$, calcd for $C_{14}H_9O_2^+$ 209.1) and cyanohydrin intermediate **5** (MS(ESI+): *m/z* = 236.2 $[M + H]^+$, calcd for $C_{15}H_{10}NO_2^+$ 236.1) respectively.

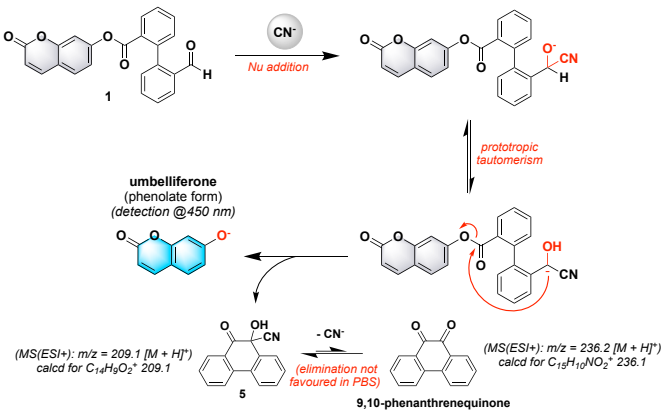

Fig. 3 Revised detection mechanism of cyanide ions based on fluorogenic intramolecular cross-benzoin reaction. Please note: for self-immolative probes **3** and **4**, an elimination step (release of PHBA or formaldehyde respectively) complete this domino process.

The formation of **5** as a stable product in PBS was not considered by the Kim group, and two distinct reaction pathways may be suggested to explain it: (1) nucleophilic addition of CN^- onto activated carbonyl of newly formed 9,10-phenanthrenequinone, and (2) premature ending of cyanide-mediated intramolecular crossed-benzoin reaction. We ruled out the first hypothesis by conducting further assay involving incubation of commercial 9,10-phenanthrenequinone with 10 equiv. of cyanide ion in PBS (containing 1% DMSO) and the non-formation of **5** was unambiguously confirmed by RP-HPLC-ESI-MS analyses (see Fig. S18 in Supporting Information). Consequently, we assumed that the last elimination step leading to the release of CN^- and formation of 9,10-phenanthrenequinone, is not really favoured in PBS, and the activation mechanism of probe can be revised as shown in Fig. 3, with **5** as major end product. Our study clearly demonstrates the non-catalytic feature of this intramolecular crossed-benzoin reaction explaining among other why the fluorogenic response is crescent for increasing amounts of analyte.

To solve inherent problem associated with the poor aqueous stability of **IND-1** and **1**, we first thought applying the probe design principle to an aniline-based fluorophore (7-amino-4-methylcoumarin) thus leading to **2** which has a more stable amide linkage between trigger and reporter units. Unfortunately, this chemodosimeter was found to be unreactive towards cyanide ion as supported by the lack of a fluorogenic response (see Fig. S7 in Supporting Information). A valuable alternative to reach hydrolytic stability while keeping reactivity towards analyte, is often based on the use of a self-immolative linker.[11] In this context, we first considered the installation of well-known and reliable *para*-hydroxybenzyl moiety using a multi-step synthetic route whose key step is the selective phenol esterification of *para*-hydroxybenzyl alcohol

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(PHBA) with an acyl-Bunte salt intermediate (see Scheme S2 in Supporting Information). This led to the self-immolative coumarin-based probe **3**. A second traceless linker belonging to the class of acetals, namely methylene aryloxy ester, was also regarded and the corresponding coumarin-based probe **4** was successfully synthesised (see Scheme S3 in Supporting Information). We next assessed the fluorogenic behaviour of these two probes under the same conditions than those previously used (*vide supra*). The stabilization effect of PHBA-based linker is too effective and a very weak increase of fluorescence emission at 450 nm, corresponding to the release of umbelliferone was observed over a prolonged period of time (Fig. 4A and Fig. S8 in Supporting Information). Therefore, no relevant conclusion can be drawn on a possible selective activation of this probe by cyanide against its non-specific hydrolysis. Conversely, the presence of acetal-based linker within the structure of **4** slowed the undesired hydrolysis process without compromising cyanide-triggered umbelliferone release. This is clearly reflected by the enhancement of the normalized F.I. (Fig. 4B) with regards to the results obtained for the linker-free probe **1.** The positive impact of the ester bond stabilization on the fluorogenic behaviour was also noted at lower cyanide doses. Indeed, after 15 min of incubation with an equimolar amount of KCN (Fig. 4D), the normalized F.I. response of **4** is multiplied by a factor of 1.3 which is close to the value 1.4 obtained for activation of **IND-1** with 10 equiv. of KCN. Furthermore, as supported by statistics and data analysis (*i.e.*, standard deviation calculation), assays conducted with **4** led to a significant improvement in the accuracy of results produced whatever the conditions tested. Finally, as already observed for **IND-1** and **1** (Fig. 2C), the maximum of F.I. ratio was obtained after 15 min of cyanide incubation (Fig. 4C). Interestingly, the plotting of normalized F.I. (at this time) as a function of KCN concentration, pointed out a good correlation (r^2 >0.995) not observed with **IND-1** and **1** (see Figs. S13-S15 in Supporting Information). A detectable concentration range of 4-8.5 μM was obtained whereas poor results were calculated for linker-free probes (LOD range = 23-49 μM, r^2 = 0.956 for **IND-1**, and 12-25 μM, r^2 = 0.988 for **1**); these results illustrate a reliable indication of LOD reachable with the Kim's chemodosimeter approach.

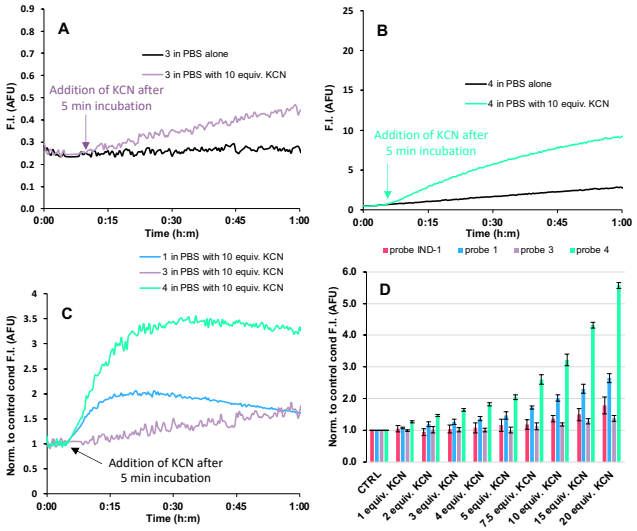

Fig. 4 (A-B) Time-dependent changes in the fluorescence intensity of self-immolative fluorogenic probes 3 and 4 in the presence of KCN, in PBS (pH 7.5) at 25 °C. (C) Normalised mean values of F.I. to control condition (PBS alone). (D) Bar chart of the normalised mean values of fluorescence intensity to control condition after 15 min of incubation with KCN for IND-1, 1, 3 and 4. All experiments were independently repeated thrice. Error bars represent standard deviation values. See Supporting Information for parameters used for fluorescence kinetics.

In conclusion, we have synthesised and re-assessed the fluorogenic properties of cyanide-responsive reaction-based probe **IND-1**. Two major weaknesses considerably impacting its sensing performances in aqueous matrices, namely poor hydrolytic stability and formation of cyanohydrin **5**, have been highlighted. In order to address the issue of non-specific hydrolysis, Kim's triggering unit was attached to the phenol moiety of umbelliferone directly or through a self-immolative spacer, leading to three novel cyanide-responsive chemodosimeters. The very comprehensive analytical methodology used to validate them associated with a rigorous statistical analysis of fluorescence data enables us to identify acetal-based fluorescent probe **4** as the best candidate for potential practical applications, even if its fluorogenic "OFF-ON" response towards cyanide ions still remains modest (LOD: 4-8.5 μM). To overcome this limitation, the implementation of the Kim's triggering moiety to zero-background "covalent-assembly" fluorescent probes whose activation leads to *in situ* formation of bright pyronin fluorophores[15], is currently under investigation by our research team and will be reported in due course.

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Data availability

The data supporting this article have been included as part of the SI file. All raw data have been deposited in dat@UBFC repository (DOI: https://doi.org/10.25666/dataubfc-2024-07-17).

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