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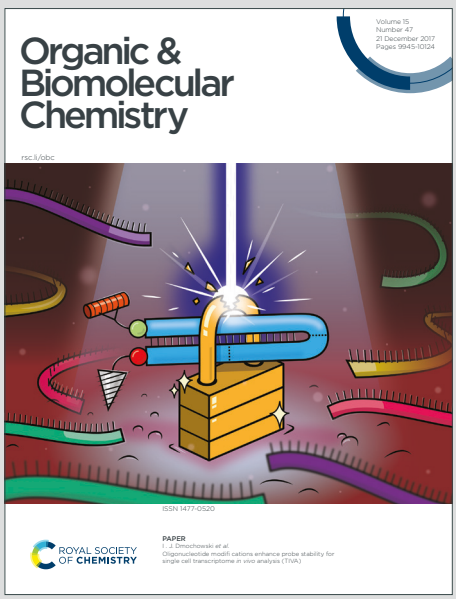

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COMMUNICATION

Aldol sensors-inspired fluorescent probes for measuring protein citrullination

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Protein citrullination is one important posttranslational modification on arginine residue. However, high quality fluorescent probes for measuring citrullination level and capturing citrullinated proteins are quite limited. Inspired by the similarity between acid-promoted citrulline-labeling reaction and Aldol reaction, here we present "turn-on" and "turn-off" fluorescent probes for measuring citrulline levels based on the scaffold of Aldol sensors. Further application of the modified probe showed great potential to simultaneously monitor and capture citrullinated peptides.

Over the past few decades, it is increasingly recognized that posttranslational modifications (PTMs) of protein are widespread and they are crucial for cell state determination.[1] Among the amino acid residues subjected to PTMs, arginine is one of the major players. With a unique pendant guanidinium group enriched in hydrogen-bonding capability, arginine residue is currently known to subject to four major PTMs: methylation, citrullination, ADP-ribosylation, and phosphorylation.[2] Protein citrullination, which is catalyzed by five peptidyl arginine deiminase (PADs), converts positively charged guanidino-containing arginine residue to neutral ureido-containing citrulline residue (Scheme 1A, first step).[3] Recent studies suggest that dysregulation of protein citrullination is highly involved in tumorigenesis, and also in inflammatory diseases especially rheumatoid arthritis (RA).[4] Therefore, citrullination level measurement and citrullinated protein capture are of great value for elucidating the biological function of protein citrullination.

The conversion of arginine residue to citrulline residue results in minimal disturbance on molecular weight and molecular size. However, dramatic change in hydrophobicity, electrostatic interaction and chemical reactivity are noticed in this process. Based on these differences, several strategies for measuring protein citrullination level were developed[5] including color development reagent (COLDER) assay, anti-citrulline antibody, ammonia release assay, fluorescent substrate-based assay, fluorescence polarization, supramolecular host sensor,[6] reactive fluorescent turn-on probes. Among these methods, acid-promoted condensation reaction between ureido group and 1,2-dicarbonyl group (represented by phenylglyoxal derivatives and their hydrates) constitutes the basis for chemically characterizing protein citrullination level and capturing citrullinated proteins (Scheme 1A, second step). This reaction, which is generally believed to go through an "addition-cyclization" process, has long been adopted to screen PAD inhibitors with COLDER assay still being widely used nowadays.[7] Meanwhile, phenylglyoxal-functionalized beads and phenylglyoxal-biotin conjugate (**Biotin-PEG-GBA**) were synthesized to selectively capture and enrich citrullinated peptides (Scheme 1B).[8] And based on this, Thompson et al. developed a rhodamine–phenylglyoxal (**Rh–PG**) conjugate. After reacting this probe with cell lysates or purified protein under acidic condition, the citrullinated proteins can be visualized by gel separation and fluorescent imaging, resulting in comparable outcomes as antibody-based method.[9] Recently, Nakagawa group developed a series of fluorescent turn-on probes built on glyoxal-fluorescein hybrids. These probes are represented by compound **4MEBz-FluME** in Scheme 1B. The fluorescence of these probes can be turned off via an intramolecular donor-excited photoinduced electron transfer (d-PeT) mechanism. Upon reacting with citrulline, the electron-deficiency of glyoxal group was diminished and the fluorescence was resumed.[10]

Even with these advances, there is still a great shortage of chemical probes suitable for performing multiple functions such as detecting and capturing protein citrullination to meet the diverse biochemical study needs, especially probes with low fluorescence background. In view of this, herein we explored a novel type of bifunctional glyoxal-based citrulline-reactive fluorescent probes. This is inspired by the previously reported Aldol sensors which could be fluorescently switched on/off to monitor the activity of Aldol enzymes (Scheme 2A).[11] These sensors are a set of aromatic aldehydes with or without conjugated triple or double bond. Depending on the existence of the conjugation pendant, the aldehyde/aldol pair can be either fluorescently turned on or turned off. When there is no π-conjugated bond (exemplified by compound **A** in Scheme 2A),

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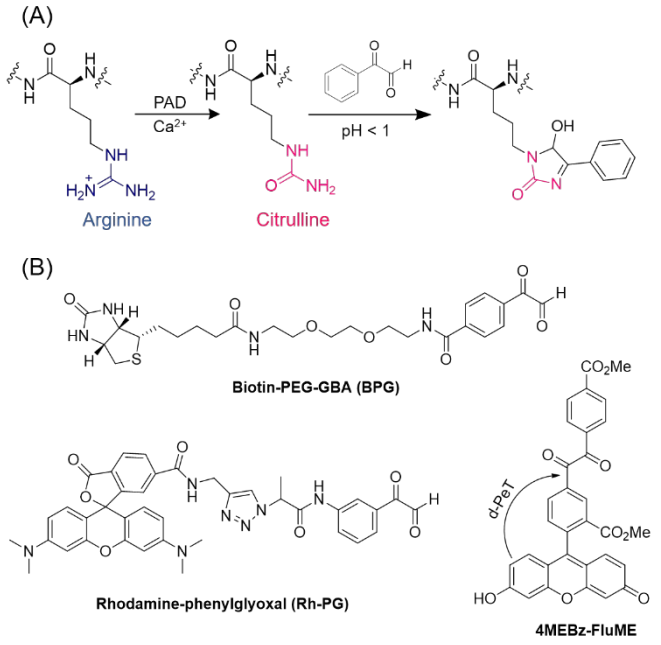
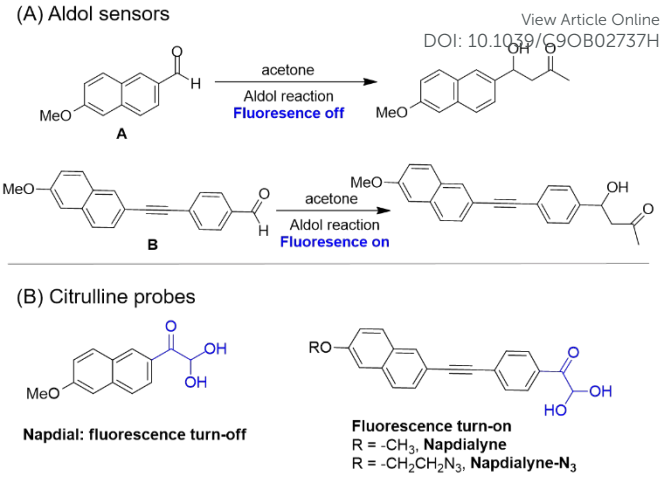
the fluorescence is turned off after Aldol reaction because the aromatic aldehyde system was disrupted. In contrast, when there is conjugated bond (exemplified by compound **B** in Scheme 2A), the electron-deficient formyl group quenches the fluorescence of aromatic system resulting in low fluorescence background, and the fluorescence of probe can be recovered when the formyl group was disrupted after Aldol reaction.



Scheme 1. (A) PAD-catalyzed protein citrullination and chemoselective reaction between citrulline and phenylglyoxal under acidic condition; (B) Previously reported phenylglyoxal analogues for protein citrullination measurement.

In this work, we reasoned that the reaction between glyoxal group and citrulline residue could result in diminishing of the electro-deficiency on glyoxal group, and this is similar to the outcome of Aldol reaction. Based on this, we firstly built two probes (namely Napdial and Napdialyne) consisting of a glyoxal group and a fluorescence turn on/off group (Scheme 2B). Further, we modified the methoxyl group on Napdialyne to introduce a N_3 handle (namely Napdial-N_3) for a second function such as capturing citrullinated peptide. Since these probes are predominantly existed in glyoxal hydrate form, the hydrate structure rather than aldehyde structure was adopted in this report.



Scheme 2. Aldol sensors-inspired fluorescent probes for measuring and capturing protein citrullination.

The syntheses of compounds Napdial and Napdialyne are outlined in Figure 1. Briefly, Napdial can be prepared by a one-step SeO_2-oxidation starting from acetonaphthone analogue. For probe Napdialyne, the key intermediate **3** can be accessed by a Pd-catalyzed Sonogashira coupling reaction between terminal alkyne compound **2** and 2-bromo-6-methoxynaphthalene.[11c] However, subsequent oxidation of compound **3** using classic SeO_2-oxidation led to no reaction, probably due to the electron-withdrawing effect of alkynyl substitute. Instead, a combination of I_2/DMSO yielded the desired glyoxal product with satisfactory yield.[12] For compound Napdial, ^{1}H NMR and ^{13}C NMR spectra showed that it exists as ca. 1:1 ratio of hydrate state and glyoxal state. For compound Napdialyne, NMR spectra suggested that it is a hydrate. The reason of incomplete hydration of Napdial is not clear at this moment. For both compounds, mass spectrum displayed glyoxal forms rather than hydrate form, probably due to water loss during ion fragmentation.

With Napdial and Napdialyne in hand, we next tested their reactivity with free L-citrulline. As expected, both probes reacted smoothly with L-citrulline under acidic condition (TFA/H_2O 1:1), and the adducts (Napdial-Cit and Napdialyne-Cit) can be successfully separated as pure compounds and their structures were confirmed.

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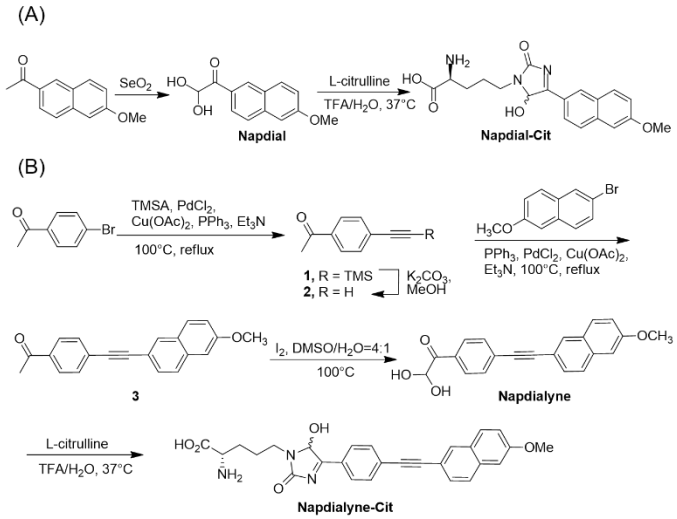

Figure 1 Synthetic routine to probes Napdial and Napdialyne, and their reactions with L-citrulline.

Having prepared both probes and their citrulline adducts, a proof-of-concept study was conducted by comparing their fluorescence spectra (Fig. 2). As expected, probe Napdial showed a fluorescence upon UV irradiation at 320 nm with a maximum emission wavelength at 470 nm, while its purified adduct Napdial-Cit showed no fluorescence in this range (Fig. 2A). On the contrary, probe Napdialyne displayed no fluorescence, while its purified adduct Napdialyne-Cit displayed a strong fluorescence with a maximum emission wavelength at 375 nm (Fig. 2B). The "turn-on" and "turn-off" fluorescence properties of these two citrulline adducts are very similar to the products of Aldol sensors, confirming our hypothesis that the glyoxal moiety is critical for modulating the fluorescence of citrulline probe just as that of formyl group for Aldol sensors. Meanwhile, the ratio of Napdialyne-Cit/Napdialyne fluorescence intensities was 340-fold at maximum emission wavelength. This extra low fluorescent background indicated that the fluorescence of probe Napdialyne can be sufficiently quenched intramolecularly, which is especially useful for measuring low concentration of citrullinated product when high concentration of probe is present. The quantum yields of Napdialyne and Napdialyne-Cit were listed in Table S1.

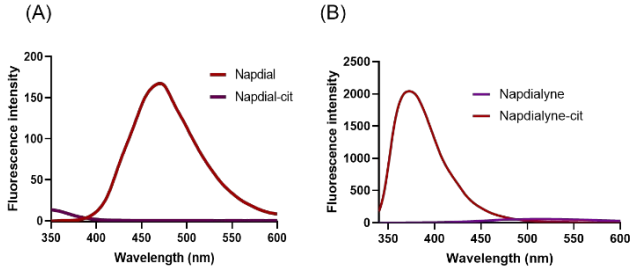

Figure 2 Fluorescence emission spectra comparison of Napdial, Napdialyne and their citrulline adducts at 10 μM concentrations, λ_{ex} = 320 nm. For Napdial and Napdial-Cit in panel A, solvent: CH_3OH; For Napdialyne and Napdialyne-Cit in panel B, solvent: DMSO.

Next, the fluorescence response of probes to citrulline concentration was assessed. Briefly, probe Napdial (100 μM) or Napdialyne (50 μM) was incubated with different concentrations of L-citrulline in the presence of 20% trichloroacetic acid, then the reaction mixture was diluted ten times before fluorescence measurement. The whole procedure is relatively simple, and extra separation or neutralization step was not needed. For probe Napdial, it exhibited a linear decrease in the range of 0-600 μM L-citrulline reaction concentration at emission wavelength of 450 nm (Fig. 3B-C), which is consistent with its mechanism as a "turn-off" probe (Fig. 3A). For "turn-on" probe Napdialyne, a linear increase was observed at L-citrulline reaction concentration from 0 to 250 μM (Fig. 4B-C), suggesting that this probe is more sensitive to detect low concentration of citrulline. Further, the reaction condition and dilution solvents were investigated (Fig. 4C and Fig. S1), and results showed that fluorescence intensity was briefly linearly increased over time at 50 °C reaction temperature and the optimal dilution solvent was DMSO.

Meanwhile, we also measured the selectivity profile of Napdialyne. As shown in Fig. S2, Napdialyne displayed low fluorescence intensity towards arginine, lysine or buffers (HEPES, Tris), which is consistent with the previous report that the glyoxal compounds selectively react with citrulline residue under acidic condition.

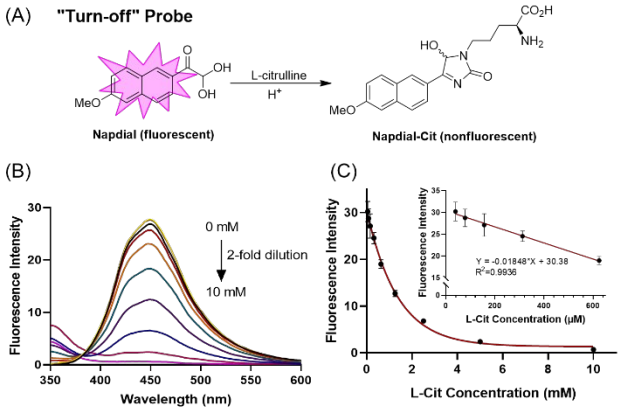

Figure 3 (A) Schematic illustration of the reaction between probe Napdial and L-citrulline; (B-C) Fluorescence spectra (B) and dose-dependent relationship of fluorescence intensity (C) on L-citrulline concentration. Reaction condition: Napdial (100 μM), L-citrulline (10 mM to 0 mM, 2-fold dilution), 20% trichloroacetic acid in ddH_2O, 50 °C, 3 h. The reaction mixture was diluted 10-times with MeOH before measurement. λ_{ex} = 324 nm, λ_{em} = 450 nm, n = 2. L-Cit: L-citrulline.

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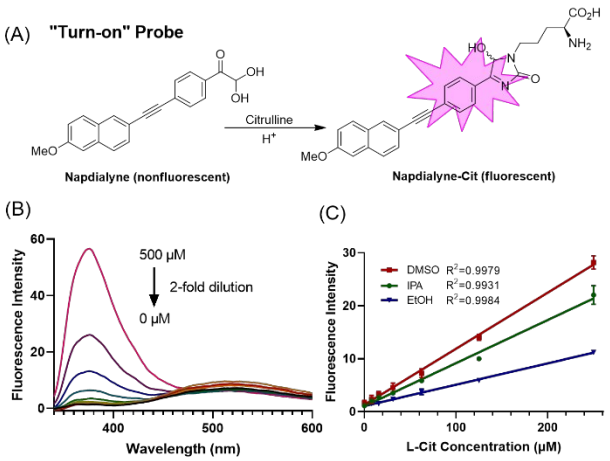

Figure 4 (A) Schematic illustration of the reaction between probe Napdialyne and L-citrulline; (B) Fluorescence spectra of Napdialyne with different concentrations of L-citrulline, λ_{ex} = 320 nm. Reaction condition: Napdialyne (50 μM), L-citrulline (0.5 mM to 0 mM, 2-fold dilution), 20% trichloroacetic acid in ddH_2O, 50 °C, 3 h. The reaction mixture was diluted 10-times with DMSO before measurement. λ_{ex} = 320 nm; (C) Dose-dependent relationship of fluorescence intensity of Napdialyne upon incubation with different concentrations of L-citrulline. The reaction conditions were the same as in panel B, except that the reaction mixtures were diluted 10-times with different solvents before measurement. For DMSO, λ_{ex} = 320 nm, λ_{em} = 376 nm; For IPA, λ_{ex} = 317 nm, λ_{em} = 360 nm; For ethanol, λ_{ex} = 315 nm, λ_{em} = 362 nm. L-Cit: L-citrulline; IPA: isopropanol. n = 2.

Encouraged by these results, we further developed a bifunctional probe for fluorescently measuring and capturing citrullinated peptides. We hypothesized that this could be achieved by replacing the methoxyl group in probe Napdialyne with a N_3-containing ethoxyl group. And the resulted probe Napdialyne-N_3 (Fig. 5A) could contain two parts: one azide part to facilitate biotin attachment and further capturing by streptavidin beads, and one fluorescent part for fluorescently tracing of citrulline conjugation. The synthetic routine to probe Napdialyne-N_3 was shown in Fig. S3, and the general procedure was similar to the synthesis of probe Napdialyne, except that Cu-free Sonogashira and Cu-free Click reactions were applied to avoid intramolecular reaction since it contains both azide group and alkynyl group.

It is known that the arginine 3 on histone 4 can be citrullinated by peptidyl arginine deiminase 4 (PAD4), so we next used citrullinated histone 4 peptide (Ac-H_4(1-13)Cit_3-NH_2, Ac-SG**Cit**GKGGKGLGKG-NH_2) as model peptide with non-citrullinated H4 peptide (Ac-H_4(1-13)-NH_2, Ac-SGRGKGGKGLGKG-NH_2) as negative control. We tested whether Napdialyne-N_3 could be used to quantify free citrulline and peptidyl citrulline. As shown in Fig. S4 and S5, a good linear relationship was obtained between fluorescence intensity and citrulline or peptidyl citrulline concentrations, with slightly decreased fluorescence intensity as compared with that of Napdialyne.

Then, a simple three-step assay was designed for fluorescent measurement and capture of citrullinated peptides (Fig. 5A): firstly, the azide terminal of probe Napdialyne-N_3 was conjugated with DBCO-PEG_4-Biotin via strain-promoted azide-alkyne cycloaddition (SPAAC); then, the glyoxal end of the resulted conjugate was reacted with citrulline or peptidyl citrulline under acidic condition leading to fluorescent product; finally, this fluorescent product which contains a biotin tag, can be captured by streptavidin agarose beads. The details of this assay can be found in Figure. S6. We measured fluorescence intensity of the beads and supernatant after beads incubation. As shown in Figures 4B-C, the citrulline-free groups (L-arginine, peptide Ac-H_4(1-13)-NH_2) showed neglectable fluorescence in both beads and supernatant groups, which is consistent with prior finding that probe Napdialyne-N_3 selectively detect citrulline residue. In contrast, both citrulline and peptidyl citrulline groups showed strong fluorescence, and fluorescence enrichment can be observed in beads group as compared with supernatant group. This suggests that probe Napdialyne-N_3 could selectively react with citrulline or peptidyl citrulline to give fluorescent adducts which can be further captured via biotin-streptavidin interaction, and this process can be easily monitored by fluorescence readout.

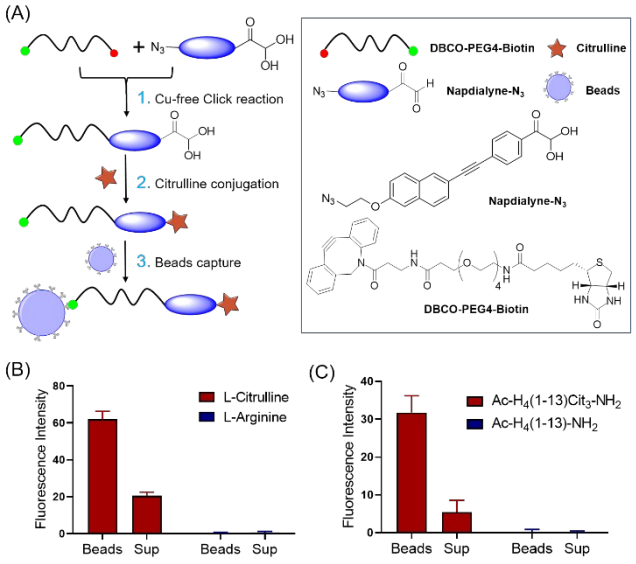

Figure 5 (A) Napdialyne-N_3-based fluorescent capture of citrulline and peptidyl citrulline; (B) Fluorescent readout of beads and supernatant in L-citrulline and L-arginine groups; (C) Fluorescent readout of beads and supernatant in peptide Ac-H_4(1-13)Cit_3-NH_2 and Ac-H_4(1-13)-NH_2 groups. Sup: supernatant. λ_{ex} = 317 nm, λ_{em} = 360 nm. n = 3.

In conclusion, inspired by the fluorescent Aldol sensors, here we provide a novel approach to address the challenge of protein citrullination analysis through "turn-on" and "turn-off" probes. Benefited from their low fluorescent background, these probes could offer sensitive and convenient readouts for citrulline level measurement. Further structure modification leads to a N_3-appendant bifunctional citrulline probe. And based on this probe, a simple three-step assay was developed to allow convenient monitoring the capturing of citrullinated peptide. It is expected that these probes could be valuable tools for studying protein citrullination at biochemical level.

Conflicts of interest

There are no conflicts to declare.

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