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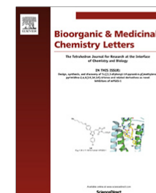
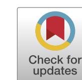


Development of a fluorescent probe for detection of citrulline based on photo-induced electron transfer



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ABSTRACT

Peptidyl arginine deiminases (PADs) catalyze the post-translational deimination of peptidyl arginine residues to form citrulline residues. Aberrant citrullination of histones by one of the PAD isozymes, PAD4, is associated with various diseases, including rheumatoid arthritis, so high-throughput screening systems are needed to identify PAD4 inhibitors as chemical tools to investigate the role of PAD4, and as candidate therapeutic agents. Here, we utilized the addition-cyclization reaction between phenylglyoxal and citrulline under acidic conditions to design turn-on fluorescent probes for citrulline based on the donor-excited photoinduced electron transfer (d-PeT) mechanism. Among several derivatives of phenylglyoxal bearing a fluorescent moiety, we found that **FGME** enabled detection of citrulline without a neutralization process, and we used it to establish a simple methodology for turn-on fluorescence detection of citrulline.

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Peptidyl arginine deiminases (PADs)[1] catalyze post-translational deimination of peptidyl arginine residues to form pepidyl citrulline residues.[2,3] Under physiological conditions, this citrullination reaction converts the guanidino group carrying a positive charge to a neutral urea group, which alters the hydrophobicity and electrostatic interactions of the protein, leading to alterations of molecular folding and function. Aberrant levels of PAD activity are associated with various diseases, including rheumatoid arthritis (RA),[1,4] multiple sclerosis (MS),[5] Alzheimer's disease (AD),[6] and several cancers.[7,8]

In humans, five highly homologous PADs, i.e., PAD1-4 and PAD6, are encoded, and PAD activity is known to be increased in a Ca^{2+} concentration-dependent manner.[9–11] Among these PADs, PAD4 is overexpressed in tumors of various organs and is considered to be involved in tumorigenesis.[7,8] In addition, PAD4 contains a nuclear localization signal (NLS), and appears to play an important role in nuclear functions, such as epigenetic control.[12,13] PAD4 induces citrullination of a number of nuclear proteins, including histones. A simple and sensitive detection method for PAD activity is needed to investigate these phenomena and to assist in drug discovery.

So far, there have been several reports on the detection of PAD4 activity, including an ELISA method,[14] a method based on detection of ammonia gas,[15] and an enzymatic reaction method using a fluorescent probe.[16,17] However, those methods all have various drawbacks in terms of cost, reproducibility, or false positive results, so Thompson et al have developed a HTS system based on a fluorescence polarization.[18] Although their strategy shows great utility and accomplished HTS successfully, as they mentioned, their assay has limitations such as a bias toward irreversible inhibitors.

In this study we report a new turn-on fluorescent probe for detecting PAD4 citrullination activity. We focused on the addition-cyclization reaction of phenylglyoxal (**PG**) and citrulline under acidic conditions[19] and hypothesized that this reaction could be utilized for fluorescence off/on switching based on donor-excited photoinduced electron transfer (d-PeT).[20–22] Based on this idea, we synthesized d-PeT-based citrulline probes containing a glyoxal moiety and a fluorescein moiety, and succeeded in establishing a simple methodology for detecting PAD4 citrullination activity.

Strategy and molecular design for PAD activity detection

Recently, Fleckenstein and co-workers reported a method for detecting citrullinated proteins by modification with **PG** under acidic conditions using mass spectrometry (Scheme 1).[17] Adapting this specific addition-cyclization reaction between **PG** and citrulline for fluorescence detection, we designed and synthesized a fluorescent probe **Flu-Glyoxal** (**FG**) consisting of a glyoxal moiety and a fluorescein fluorophore (Fig. 1). We expected that the fluorescence of **FG** would be quenched via a donor-excited photoinduced electron transfer (d-PeT)-type mechanism due to the electron-deficient glyoxal moiety, but that **FG** would be converted to a strongly

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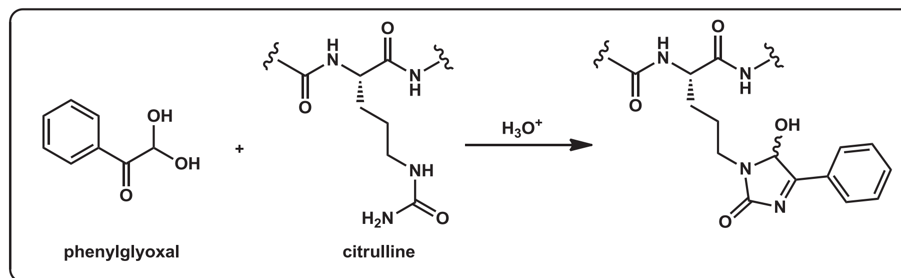


Scheme 1. The specific cyclization reaction between phenylglyoxal and citrulline.[17]

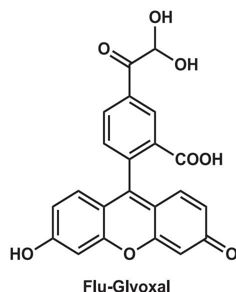


Fig. 1. The structure of **Flu-glyoxal** (**FG**).

fluorescent FG-citrulline cycloadduct (**FG-Cit**) when the addition-cyclization reaction took place (Scheme 2). By coupling this reaction with enzymatic citrulline formation, we considered that PAD activity could be measured quantitatively from the fluorescence readout.

Synthesis, photochemical characteristics, and reaction of FG

FG and **FG-Cit** were synthesized as shown in Scheme 3. Briefly, after iodination of **5-aminofluorescein** at the amino group, the two phenolic hydroxyl groups were protected with acetyl groups.[23] Then, in order to obtain the precursor for the oxidation reaction, an acyl group was introduced into the benzene ring with acetic anhydride and palladium catalyst, and the two acetyl ester groups were deprotected under basic conditions. This precursor was led to **FG** by oxidation reaction with selenium dioxide. Finally, the resulting **FG** was reacted with commercial L-citrulline under acidic conditions to obtain the cyclized product **FG-Cit** as the TFA salt. Compounds were purified by HPLC, and the structures were confirmed by HRMS.

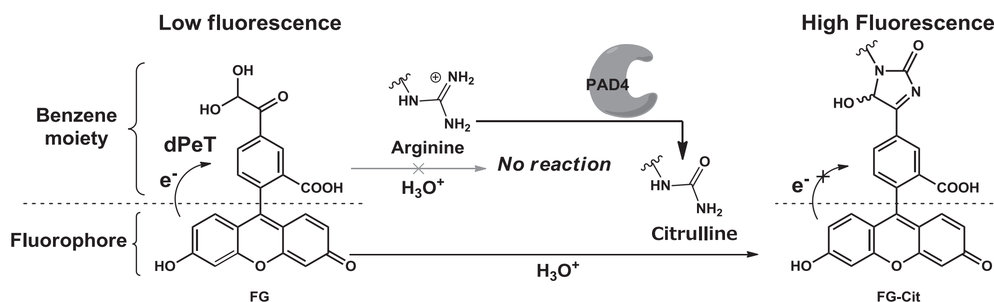
We measured the absorption and fluorescence spectra of **FG** and **FG-Cit**, and calculated their fluorescence quantum yields by using fluorescein as a reference compound (Fig. 2, Table 1). The results showed that the fluorescence of **FG** was efficiently quenched, whereas the fluorescence intensity of **FG-Cit** was close to that of fluorescein. This result indicates that **FG** is potentially available to measure citrulline formation in terms of fluorescence intensity change after the reaction of **FG** with citrulline to form the **FG-Cit** adduct.

Next, we examined the fluorescence response in the reaction of **FG** with citrulline. A solution of citrulline (10 mM) was incubated with **FG** (100 μM) under an acidic condition (pH 1) for various times, and the reaction mixtures were analyzed by HPLC (Figs. 3A, B and S1). From the results (Fig. 3A), citrulline decreased and the fluorescence product linearly increased up to at least 120 min. Therefore, we set the reaction time of **FG** with citrulline as 120 min. Under this condition, the fluorescence was also linearly correlated with the concentration of citrulline at a fixed concentration of **FG** (Fig. 3B). This result indicated that the concentration change of citrulline in the test solution could be evaluated by measuring the fluorescence of the reaction product.

Detection of enzymatic citrulline formation with FG

We then examined the fluorescence response of **FG** to the citrulline derivative formed by the enzymatic reaction of PAD4 with an arginine derivative (Fig. 4). **BAEE**, which is hydrolyzed to **BCEE** by PAD4,[24] was incubated with PAD4 under physiological conditions. After the enzymatic reaction, the reaction mixture was acidified with TFA, and further incubated with **FG**. We optimized the reaction time for the enzymatic reaction at fixed concentrations of **BAEE** and PAD4 (10 μM and 200 nM respectively). HPLC analysis of **BAEE** and **BCEE** confirmed that the PAD4 reaction should be performed in neutral buffer solution, and the decrease of **BAEE** and the formation of **BCEE** were linearly dependent on the incubation time with PAD4 up to 30 min (Figs. 5 and S2). Therefore, we set the enzymatic reaction time at 30 min.

Next, we examined whether PAD4 activity was detectable under the optimized condition; i.e., the mixture after enzymatic reaction was directly acidified and incubated with **FG** (Fig. 6). The fluorescence intensity was significantly increased in the presence of PAD4, but not in its absence (**BAEE** alone). In addition, a



Scheme 2. Schematic illustration of our strategy for detection of citrulline by **FG**.

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