An AIE-based enzyme-activatable fluorescence indicator for Western blot assay: Quantitative expression of proteins with reproducible stable signal and wide linear range

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Abstract
Western blot is a commonly used experimental method to analyze the protein expression. However, the most commonly used chromogenic indicator based on chemiluminescence is limited by narrow linear range and unstable quantitative reproducibility, whereas the recently developed fluorescent indicator suffers from poor detection limit. Herein, we report an enzyme-activatable fluorescence indicator to quantitate proteins with reproducible stable signal and wide linear range, through introducing the hydrophilic alkaline phosphatase (ALP)-triggered phosphoric acid moiety into our established aggregation-induced emission (AIE) building block of quinoline-malononitrile (QM). In this strategy, the indicator DQM-ALP disperses well in both aqueous and lipid environments to exhibit initial “off” fluorescence, but when exposing to the ALP-coupled secondary antibody on the PVDF membrane, the specific enzymatic turnover would liberate hydrophobic AIE luminogen (AIEgen) QM-OH to emit strong luminescence, thereby achieving an ideal “off-on” state for sensitively imaging proteins with high signal-to-noise (S/N) ratio. Moreover, benefiting from the excellent signal stability of AIE fluorophore, DQM-ALP indicator exhibits superior quantitative analysis of protein expression with high reproducibility. Upon taking advantage of the AIEgens to reduce high concentration-induced luminance quenching, the linear quantification range is extremely expanded. In contrast with the traditional chemiluminescent indicator, the AIE-based enzyme-activatable indicator DQM-ALP not only greatly improves the signal stability for quantitative reproducibility, but also expands the linear quantification range, and further provides a practical alternative reagent for fluorescence Western blot assay.

KEYWORDS
aggregation-induced emission, amphiphilic fluorescence indicator, Western blot assay

1 | INTRODUCTION
Western blot is a commonly used experimental method to determine the relative protein expression in complex biological samples. It is conducted by separating proteins from cells or tissues by electrophoresis, transferring proteins to the solid phase support polyvinylidene fluoride (PVDF) membrane, and the proteins are recognized by specific primary antibody, then the signals are amplified by enzyme-coupled secondary antibody, finally the commercial chromogenic indicator Luminol is added on the membrane, followed by imaging immediately (Figure 1A). However, the most commonly used chemiluminescence-based chromogenic indicator sometimes underperform in precisely detecting the expression of proteins owing to the inherent weakness of narrow linear range and unstable quantitative reproducibility.
As the directly grafting fluorophores to the primary or secondary antibodies, the recently reported fluorescence indicator for quantitative analysis of proteins is too low in concentration to trace proteins. Moreover, the aggregation-caused quenching (ACQ) phenomenon greatly narrows the linear quantitative range.

Herein, an aggregation-induced emission (AIE)-based enzyme-activatable fluorescence indicator was designed and constructed through introducing the alkaline phosphatase’s (ALP) substrate, hydrophilic phosphate group, into our established AIE building block of quinoline-malononitrile (QM). This “amphiphilic” AIEgen DQM-ALP exhibits initial “off” fluorescence because of the superior dispersity in both aqueous and lipid environments, whereas it could be cleaved to liberate hydrophobic QM-OH to aggregate and emit strong luminescence when exposing to the ALP-coupled secondary antibody on the PVDF membrane (Figure 1C). This ALP-activatable fluorescent indicator bestows the following features: (i) Guaranteeing high signal-to-noise (S/N) ratio of amphiphilic DQM-ALP through the excellent dispersity in both hydrophobic environments; (ii) Enabling high selectivity owing to the specific recognition between ALP-coupled secondary antibody and the phosphate group; (iii) Expanding the linear quantification range with assistance of AIEgens overcoming the high concentration-induced luminescence quenching; (iv) Processing stable quantitative reproducibility as a result of the excellent photostability of AIEgens. All these advantages enable the “amphiphilic” DQM-ALP indicator quantifying proteins with reproducible stable signal and wide linear range, thereby providing a practical alternative to the commercially available chromogenic Luminol in the fluorescence-based Western blot assay.

RESULTS AND DISCUSSION

2.1 Constructing enzyme-activatable AIE fluorescence indicator for Western blot

Chemiluminescence-based indicator is commonly used in Western blot assay, where the protein sample are labeled with the sequence of primary antibody and horseradish peroxidase (HRP)-coupled secondary antibody, then the commercial Luminol and oxidizing reagent are added on the PVDF membrane, followed by imaging immediately (emission wavelength: 425 nm). However, chemiluminescence indicators not only show poor linear relationship between the expression of proteins and signal intensity, but also suffer from unstable reproducibility. As a consequence, a chromogenic indicator with strong signal stability and high sensitivity is urgently needed for quantitative analysis of the proteins in a wide linear range.

Given the higher fluorescence sensitivity, we took the commercial alkaline phosphatase (ALP)-coupled secondary antibody into account, and constructed an ALP-activatable fluorescent indicator for Western blot assay through introducing the hydrophilic phosphate group into our established AIE building block of quinoline-malononitrile (QM). In this strategy, the AIE building block of QM with the superiority of weak emission in dispersed state and strong luminescence in high concentration was utilized as core structure to
overcome the concentration-induced quenching effect, for the sake of improving the linear quantitative range. Subsequently, the π-conjugated backbone was extended for longer emission wavelength, and the hydroxyl group was introduced to afford the intermediate QM-OH. Finally, the hydrophilic phosphate group was grafted on the hydroxyl group to give the desirable sensor DQM-ALP. It is anticipated that the incorporated phosphate group could play two roles, that is, well regulating the solubility or dispersion in both hydrophilic and hydrophobic systems with desirably initial “fluorescence-off” state for high S/N ratio, and behaving as the substrate to ALP for high specificity. In this regard, the indicator was virtually non-emissive in both aqueous and lipid media, but when exposed to the ALP-coupled secondary antibody on the PVDF membrane, the indicator would be activated by specific enzymatic turnover to liberate hydrophobic AIEgens QM-OH to emit strong luminance, resulted in high-sensitive quantitatively detecting proteins with “off-on” pattern in Western blot assay (Figure 1C). The DQM-ALP was synthesized (Scheme S1†), the chemical structures of DQM-ALP and intermediates were well confirmed by $^1$H and $^{13}$C NMR, and high-resolution mass spectroscopy (HRMS) in Supporting Information.

2.2 Photophysical properties of DQM-ALP and QM-OH

The photophysical properties of both DQM-ALP and QM-OH were studied in DMSO-Water mixtures with different volume fraction of water. Both DQM-ALP and QM-OH exhibited a broad absorption band ranging from 300 to 550 nm (Figure 2A,D). The absorbance wavelength of DQM-ALP slightly blue shifted and the intensity gradually decreased when the water fraction increased from 0% to 95% (Figure 2A). While the absorbance wavelength of QM-OH slightly blue shifted when the water fraction increased from 0% to 80%, then red shifted when the water fraction increased from 80% to 95% (Figure 2D).

The hydrophilic phosphate group was supposed to well regulate the specific solubility of DQM-ALP in both hydrophilic environment, thus generating nonsusceptible initial “fluorescence-off” signal. To validate the “amphiphilicity” of DQM-ALP, the fluorescence intensity was detected in a series of solution mixtures. As expected, DQM-ALP did not emit any fluorescence signal in DMSO-Water (Figure 2B,C) and THF-Water (Figures S1 and S2) and Ethanol-Water (Figures S3 and S4) with any water fractions, suggestive of the specific “amphiphilicity” of DQM-ALP with free intramolecular motions in good disperse state. Moreover, the intrinsic AIE behavior of DQM-ALP was demonstrated with increasing viscosity. The DQM-ALP emitted strong fluorescence with the increasing fraction of glycerin ($f_g$), as a result of intramolecular (RIM) mechanism, in which high viscosity restricted the free motion to release energy (Figure S5). Specifically, the fluorescence intensity of DQM-ALP at $f_g = 90\%$ could reach 33-folds than its initial intensity in 99% water (Figure S5). In brief, the ALP-activatable “amphiphilic” AIEgen DQM-ALP possessed superior solubility in both hydrophilic and lipophilic solutions, which can successfully avoid the undesirable nonspecific aggregation, thereby guaranteeing the high S/N ratio to ALP-coupled secondary antibody.
2.3 | Specifically sensing mechanism and spectral response of DQM-ALP to ALP

A series of experiments were carried out to gain insight into the activation of DQM-ALP with hydrophilic alkaline phosphatase. The reaction mechanism between DQM-ALP and ALP was revealed by the high-performance liquid chromatography (HPLC), electrospray ionization (ESI)-MS spectra and fluorescence spectrum. First, the retention time of pure DQM-ALP was 4.4 min in HPLC spectrum, but after incubating the DQM-ALP with the ALP for 20 min, a new peak appeared at 7.9 min, which was in accordance with the retention time of pure QM-OH (Figure 3A). Moreover, the peaks of QM-OH were also found at m/z 338.1307 in the ESI-MS spectra after incubating DQM-ALP with ALP for 20 min (Figure 3B). In addition, DQM-ALP emitted no fluorescence in aqueous solution, but exhibited strong signal after incubating with ALP for 20 min (Figure 3C). This new spectra was in accordance of the QM-OH (Figure 2F), but it could be blocked by ALP inhibitor, sodium orthovanadate, thus demonstrative of the specific cleavage property of ALP to phosphate group (Figure 3C). All these observations confirmed that the DQM-ALP with specific substrate of ALP, phosphate group, could be hydrolyzed by breaking the O-P bond upon enzyme-reactive reaction, so as to generate QM-OH and emit strong fluorescent signal for detection.

The selectivity of DQM-ALP for ALP was then evaluated using a series of relevant components in Western blot assay. As shown in Figure 3D, DQM-ALP displayed no fluorescence response toward other interfering species, indicative of a specific ALP-activatable indicator of DQM-ALP for Western blot assay. Then, the optimal action time was studied by incubating DQM-ALP (10 μM) with ALP (150 U/L) for 30 min at room temperature, and both the absorption (Figure S7) and fluorescence spectra (Figure 3E) were measured every 2 min. The absorption intensity decreased with increased incubation time in aqueous solution (Tris/DMSO = 95: 5, v/v, 50 mM, pH 7.4), indicative of gradual consumption of DQM-ALP and the generation of QM-OH (Figure S7). In the fluorescence spectra (Figure 3E), the fluorescence intensity at 560 nm gradually increased with time till the growth curve tended to be flat at 20 min, clearly indicative of the optimal action time of 20 min (Figure 3F).

To further estimate the quantitative analysis capability for ALP, DQM-ALP (10 μM) was incubated with different concentrations of ALP (0, 60, 80, 100, 120, 150 U/L) for 10 min. As expected, the fluorescence intensity increased (Figure 3G) on an ALP concentration dependent manner (Figure 3H) along with the absorbance of DQM-ALP decreased (Figure S7). A good liner relationship was obtained in the ALP concentration range of 60–150 U/L with R² of 0.991 (Figure 3H). Moreover, the excellent liner relationship between DQM-ALP and ALP was also observed from 0 to 150 U/L with R² of 0.995 at 37 °C (The optimum temperature of enzymatic reaction; Figure S8). These results revealed that DQM-ALP indicator possessed the quantitative analysis capability for ALP with a broad linear range.

2.4 | Excellent signal stability of fluorescence indicator

The traditional Western blot assay is based on the principle of chemiluminescence, which exhibits strong signal in the initial stage but decreases rapidly because the luminescent substrate is gradually consumed with time (Figure 1B) [43, 44]. Therefore, the chemiluminescence indicators underperform in quantitative reproducible testing in Western blot assay [45, 46]. In this work, the AIE-fluorophore was supposed to promote the signal stability with the excellent photostability of AIEgen. Therefore, DQM-ALP was incubated with ALP to evaluate the signal stability with the chemical chemiluminescence indicator as control. As shown in Figure 4A, strong luminescence was emitted immediately after mixing the commercial luminescent substance and the HRP (1 U/mL) into a 3 mL cuvette, then the luminescence signal was rapidly decreased upon a time-dependent manner. According to the statistics result, the luminescence intensity rarely changed in the first min, then rapidly decayed to 30% after 3 min, and finally disappeared completely after 5 min (Figure 4B). The sharp intensity decline curve indicated the fast consumption of chemiluminescence indicator (Figure 4B). In the contrast, strong fluorescence signal was observed after incubating the DQM-ALP with ALP in a 3 mL cuvette, and the signal intensity rarely changed in the following 20 min (Figure 4C; Figure S9). The extremely slow fluorescence intensity decline curve was observed for DQM-ALP sensor, that is, the fluorescence remained 98% of initial intensity after 3 min, 90% after 5 min, and even 72% after 20 min exposing to the UV lamp irradiation, suggestive of slow consumption of the fluorescence of AIEgen indicator (Figure 4D).

Good photostability is another key role for quantitative reproducible test in fluorescence Western blot assay. Herein, the photostability of both DQM-ALP sensor and the enzyme-cleaved product QM-OH was conducted with the lasting irradiation (Hamamatsu, LC8 Lightningcure, 200 W) using the commercial indocyanine green (ICG) as the control. The absorption intensity of ICG rapidly decreased to the minimum level after 2 min, suggesting that ICG was subject to
irreversible photobleaching (Figure 4E). In contrast, 95% initial intensity of DQM-ALP remained after exposing to the high-density light for 2 min, while 85% initial intensity of QM-OH remained after 2 min (Figure 4F). The absorbance half-time of QM-OH (9 min) was 27-fold longer than that of ICG, suggestive of the long-term imaging ability for Western blot assay. These data provide a direct evidence that amphiphilic AIEgen DQM-ALP indicator is much more suitable for stable quantitative reproducible expression of protein than commercial luminescent substance in Western blot assay.

2.5 Stable quantitative reproducibility of AIE-based DQM-ALP indicator

Encouraged by the above results that the enzyme-activatable DQM-ALP indicator could selectively detect ALP with excellent stability, the indicator was supposed to precisely reflect the protein amount through detecting the ALP-coupled secondary antibodies. In this work, the intracellular proteins were extracted from different cells, human breast cancer cell line (MCF-7), human ovarian carcinoma cell (SKOV-3), human lung adenocarcinoma cell (H-1975), human breast cancer cells (MBA-MD-231) and human gastric adenocarcinoma cells (SGC-7901), quantified by BCA Protein Assay Kit, then added into the gel for series Western blot assay steps including electrophoresis procedure, membrane transferring, blocking, primary antibody incubation (β-Catenin, Catalase, Ras, and GAPDH) and secondary antibody incubation (HRP-coupled or ALP-coupled) (Figure 5A,B). As expected, all proteins bands were clearly displayed after incubating with both commercial Luminol and AIE-active fluorescence indicator DQM-ALP, and the intensity of protein was comparable in these two methods (Figure 5A,B). These results demonstrated that the AIE-active DQM-ALP is an effective
The short emission time of commercial chemiluminescence indicator required fast images collection, caused inconsistent data, and increased the workload of researchers.\cite{45-46} Inspired with the slow consumption and strong photostability of AIEgens, the “amphiphilic” AIE-active DQM-ALP was supposed to long-time imaging proteins in Western blot assay. Take this into consideration, Western blot assay was carried out and output the signal by both chemiluminescence and fluorescence, then the luminescence images were captured at different time points (Figure 5C; Figure S10). In the initial stage of imaging, the chemiluminescence reagent was brighter than fluorophore with higher $S/N$ ratio. However, with the extension of time, in chemiluminescence imaging group, all protein bands became much shallower and decreased rapidly to an invisible level after 30 min, so as to generate smaller $S/N$ ratio. On the contrary, the gray intensity slowly decreased with time in fluorescence imaging group, and all those bands were still clearly observed after 180 min, suggestive of stable quantitative reproducibility with higher $S/N$ ratio (Figure 5C).

The statistical data of both chemiluminescence and fluorescence gray images further verified the long-time imaging property of amphiphilic AIE-active DQM-ALP for Western blot assay. For chemiluminescence staining, all these bands’ gray intensity rapidly decreased to undetectable after 30 min. In particular, Ras intensity remained only 22% of initial intensity after 15 min. Even for $\alpha$-tubulin, which band intensity decreased slowest, just remained 40% of initial intensity after 15 min (Figure 5D). While for DQM-ALP staining, over 50% of the gray intensity was remained for all proteins bands after 30 min, and over 40% was exhibited after 3 h. Specifically, almost no intensity reduction happened for Catalase in the first 30 min, and over 80% of initial intensity was maintained after 3 h (Figure 5D). These results demonstrated that the enzyme-activatable fluorescence indicator DQM-ALP can not only be used for qualitative detection, but also process strong signal stability for reproducible test in Western blot assay.
2.6 | Wide linear quantitative range in fluorescence Western blot assay

High linear correlation between the protein amount and the response signal is the prerequisite for quantitative analysis of protein expression in Western blot assay. However, the commonly used chemiluminescence indicator always exhibited narrow linear range because of the high concentration-induced luminescence quenching or overexposure (Figure S11-S14). [8–11] Inspired by the previous experimental results, the fluorescence intensity of the DQM-ALP was linearly correlated well with the concentration of ALP, so that the good linear correlation relationship was expected between the signal intensity and the ALP-coupled secondary antibody amount. Herein, the protein samples (290, 230, 170, 140, 120, 60 μg) were added into the gel for electrophoresing, following by membrane transferring, blocking, incubating with primary antibody, ALP-coupled secondary antibody, and DQM-ALP indicator for imaging.

As shown in Figure 6A, the fluorescence signal intensity was well dependent upon the decreasing of protein concentration for all tested protein types in fluorescence based Western blot assay. Specifically, all bands were completely presented with the loading amount of 290 μg protein, then the bands’ intensity gradually weakened when the loading amount reduced to 120 μg protein, the intensity finally decreased to the minimum until the loading amount reduced to 60 μg protein, and the Ras was almost invisible to the naked eye with the smallest loading amount (Figure 6A and Figure S15). It is worth noting that due to the good stability of the fluorescent Western blot, the protein band still maintained strong fluorescence signal after 60 min (Figure 6B and Figure S16), 2 (Figure S17), and 24 h (Figure S18), and these bands’ intensity always exhibited protein concentration dependent manner.

The statistical data of all bands’ gray was analyzed to show the linear correlation between the protein amount and the response signal. As shown in Figure 6, all tested protein types presented excellent linear relationship between proteins amounts and gray intensities. The $R^2$ is 0.962 for β-Catenin (Figure 6C), 0.928 for Catalase (Figure 6D), 0.909 for Caspase 3 (Figure 6E), and 0.944 for Ras (Figure 6F). All these $R^2$ values were bigger than 0.90, which illustrated the practicability of DQM-ALP for quantitative detection of protein. Owing to the time-independent property of fluorescence DQM-ALP sensor, the excellent linear relationship was maintained after 60 min with the $R^2$ of 0.971 for β-Catenin (Figure 6G), 0.964 for Catalase (Figure 6H), 0.928 for Caspase 3 (Figure 6I), and 0.908 for Ras (Figure 6J). Because the fluorescence imaging was less affected by time, the linear relationship between these high expressed proteins and response signal could be maintained for up to 24 hours (Figure S18). These results confirmed that the enzyme-activatable fluorescence indicator DQM-ALP could reproducibly...
FIGURE 6  DQM-ALP staining proteins with different loading amount. Western blot protein membranes with various protein amounts were stained with the fluorescence DQM-ALP for 20 min and stored for (A) 0 min and (B) 60 min. Corresponding linear plot of relative gray values of (C) β-catenin, (D) catalase, (E) caspase 3, (F) Ras after storing the protein membrane for 0 min, and (G) β-catenin, (H) catalase, (I) caspase 3, (J) Ras after storing the protein membrane for 60 min. Exposure time: 30 s.

3 | CONCLUSION

Proteins play an important role in the catalysis, transportation and immunity of organism, and the abnormal expression is closely related to disease occurrence. However, the commercial chemiluminescence indicator for Western blot is not ideal for quantitatively analyzing the protein amount because of the narrow linear range and unstable quantitative reproducibility. An AIE-based amphiphilic ALP-activatable indicator DQM-ALP was developed by introducing hydrophilic phosphate group to our established AIE building block of QM with excellent photostability. The synergistic effect of both hydrophilic group phosphate and hydrophobic unit QM was supposed to generate amphiphilic AIE property of DQM-ALP, thus generating non-susceptible initial “fluorescence-off” signal, while the enzyme-product QM-OH exhibited typical AIE property in aqueous environment to emit strong fluorescence, which guaranteed the high S/N ratio to ALP-coupled secondary antibody.

In the Western blot experiment, owing to the inherent advantages of fluorophores and the strong photostability of AIEgens, the signal of QM-OH lasted more than 20 min, which was much longer than that of commercial luminescent, suggestive of the stable imaging characteristics. In the Western blot assay, the amphiphilic AIEgen DQM-ALP can successfully detect various proteins in many kinds of cells, and further spread in long-time quantitative imaging proteins with good correlation (higher than 0.90) in a wide linear range. The staining time can even be extended to 24 h due to the excellent photostability of QM-OH. Overall, aided by the advantages of AIE phenomenon, we successfully develop an amphiphilic ALP-cleavable indicator DQM-ALP, and further demonstrate the applicability of quantifying proteins with reproducible stable signal and wide linear range specific quantitative in Western blot assay. This work not only provides an ideal tool for stable accurate detection of ALP, but also offers a practical alternative to the commercially available chromogenic Luminol in the fluorescence-based Western blot assay.

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CONFLICTS OF INTEREST
There are no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT
The data used to support the findings of this study are included within the article and the supporting information.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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