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Alkaline phosphatase assay using a near-infrared fluorescent substrate merocyanine 700 phosphate

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ABSTRACT

Alkaline phosphatase (ALP) is a phosphomonoester hydrolase that is commonly used as a conjugating enzyme in biological research. A wide variety of substrates have been developed to assay its activity. In this study, we developed an ALP assay method utilizing merocyanine 700 (MC700) based substrate MC700 phosphate (MC700p). MC700 is a near-infrared fluorescent merocyanine dye, and has excitation/emission maxima at 686 nm/722 nm in ALP assay buffer. Upon hydrolysis by ALP, MC700p is converted to MC700. The fluorescence of MC700 is dependent on the pH and detergent concentration in the buffer. The fluorescence signal produced by MC700p hydrolysis is linearly related to the ALP amount and substrate concentration. A stop solution containing EDTA could be used to stop the ALP/MC700p reaction. It was also demonstrated that MC700p could substitute pNpp as the ALP substrate in a commercial 17β-Estradiol enzyme immunoassay kit.

1. Introduction

Alkaline phosphatase (ALP, EC 3.1.3.1) is a phosphomonoester hydrolase enzyme that catalyzes the cleavage of phosphate esters with the formation of the corresponding alcohol (or phenol) and a phosphate ion. It is characterized by high pH optima and broad substrate specificity [1,2]. The change of ALP activity has been used as a diagnostic marker for certain diseases, such as hepatobiliary disease, bone disorders, and malignant tumors [2,3]. In the dairy industry, ALP activity is an indicator for adequate pasteurization of milk [4]. In the biological research field, ALP is conjugated on antibodies for enzyme immunoassay (EIA) [5], and protein detection on blotting membranes [6,7]. ALP has also been utilized in DNA hybridization analysis [8,9], *in situ* hybridization [10] and fusion protein tag technology [11].

A wide variety of substrates have been developed for ALP activity detection. The most commonly used colorimetric substrate, *p*-nitrophenyl phosphate (pNpp), is colorless. Upon hydrolysis by ALP, it is cleaved into the yellow *p*-nitrophenol whose absorbance peaks at 405 nm under alkaline pH [3,12]. More sensitive methods that utilize chemiluminescent substrate [8,13,14], bioluminescent substrate [1,15], or fluorescent substrate [4,6,7,9,10] have also been developed. More recently, surface enhanced resonance Raman scattering [16] or electrochemical property [17] of ALP hydrolysis products were exploited to measure ALP activity.

We report here the development of an ALP assay method utilizing a near-infrared (NIR) fluorescent merocyanine dye based substrate. Merocyanines belong to the class of polymethine dyes, among which merocyanine 540 (MC540) was the most widely studied. In cell biology, MC540 was generally considered as a fluorescent membrane probe, although attempts have been made to use it as an indicator for proliferating cells [18,19]. In this study, a NIR fluorescent merocyanine 700 dye (MC700) and its phospharylated derivative MC700 phosphate (MC700p) were synthesized. Upon hydrolysis by ALP, MC700p is converted into MC700, which has excitation/emission maxima at 686 nm/722 nm respectively. Because MC700 fluorescence is in the NIR spectral region (700–900 nm), in which the autofluorescence background is lower compared to the visible spectrum, the fluorescence signal can be detected at a high signal to noise ratio. We investigated the factors that affect the fluorescence signal when MC700p was employed as a substrate to assay ALP activity. In addition, MC700p was tested as an ALP substrate using a 17β-Estradiol (E2) EIA kit.

2. Experimental

2.1. Chemicals

The bovine intestinal ALP was purchased from Sigma (St. Louis, MO, USA). 7-Hydroxy-9H(I,3-dichloro-9,9-dimethylacridin-

Abbreviations: ALP, alkaline phosphatase; DDAO, 7-hydroxy-9H(I,3-dichloro-9,9-dimethylacridin-2-one); DDAOp, 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate; E2, 17β-Estradiol; EIA, enzyme immunoassay; MC540, merocyanine 540; MC700, merocyanine 700; MC700p, MC700 phosphate; NIR, near-infrared; pNpp, *p*-nitrophenyl phosphate; RFU, relative fluorescence unit.

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2-one) (DDAO) and 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate (DDAOp) were from Invitrogen (Carlsbad, CA, USA) and dissolved in DMSO as the stock solution. MC700 and MC700p were synthesized at LI-COR Biosciences (Lincoln, NE, USA) and reconstituted in DMSO as the stock solutions.

2.2. Determination of MC700 spectra

The absorbance spectrum of MC700 was measured using an Agilent 8453 Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The fluorescence spectrum was measured using a PTI QuantaMaster luminescence spectrometer (Photon Technology International, Birmingham, NJ, USA). The spectra were normalized by defining the peak value as 1.0. The molar extinction coefficient at maximum absorbance (ε) was calculated using the formula: $\varepsilon = A/cl$ (A, absorbance at the peak; c, dye concentration; l, length of light path).

2.3. Determination of pH and detergent effect on MC700 absorbance and fluorescence signal

MC700 was diluted to a final concentration of 5 μM in Tris buffers with different pH. HCl or NaOH were used to adjust the pH. The absorbance spectra were measured and the values at peak (686 nm) were recorded. The same solutions were used to measure the fluorescence signals on an Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) using the 700-nm channel (excitation/emission at 685/720 nm).

To determine the detergent effect, MC700 was diluted to 5 μM in Tris buffers (pH 9.5) containing different concentrations of Triton X-100. The fluorescence signals were measured on an Odyssey Infrared Imaging System.

2.4. ALP activity assay

Unless otherwise noted, the reaction was conducted in ALP assay buffer which is consisted of 50 mM Tris–HCl (pH 9.5), 0.4% Triton X-100, 1 mM $MgCl_2$. ALP and MC700p substrate were added and mixed before transferring to a 96-well plate (50 μl per well). The reaction mixture was incubated at room temperature for designated time periods and scanned on an Odyssey Infrared Imaging System. For those experiments requiring a stop solution, the stop solution (25 μl per well) was added. The stop solution is consisted of 50 mM Tris–HCl (pH 9.5), 0.4% Triton X-100, 5 mM EDTA.

2.5. 17β-Estradiol assay

17β-Estradiol (E2) assay was conducted using a commercial EIA kit from Assay Designs (Ann Arbor, MI, USA). The assay was carried out according to the manufacturer's instructions with modifications. Briefly, E2 samples (100 μl each) were added to the bottom of wells pre-coated with a donkey anti-sheep antibody, followed by adding 50 μl of E2-ALP conjugate, and 50 μl of sheep anti-E2 antibody. The mixture was incubated at room temperature with shaking for 2 h. After washing, 100 μl of ALP assay buffer containing 5 μM MC700p substrate was added. The ALP reaction was conducted at room temperature for 1 h and terminated by adding 50 μl of stop solution.

2.6. Statistical analysis

At least three replicates were used for each data point. When applicable, results were presented as mean ± SD.

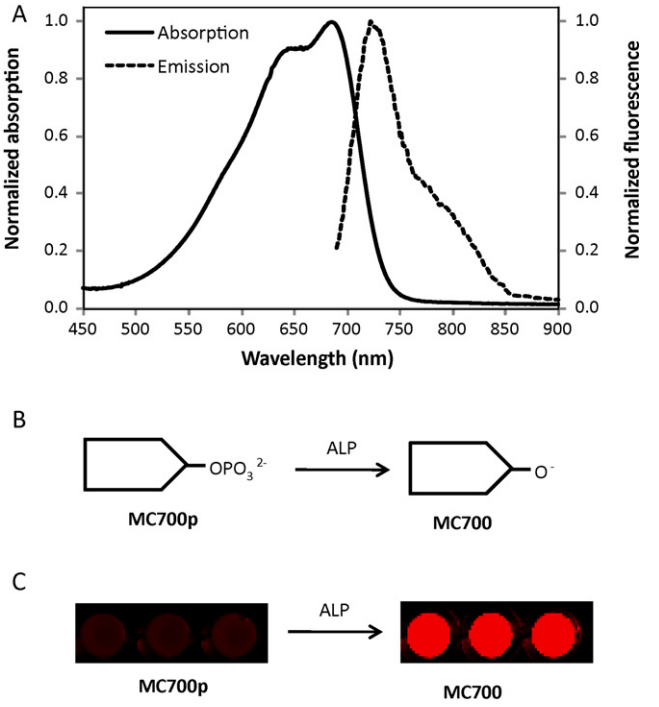

Fig. 1. Merocyanine 700 (MC700) and merocyanine 700 phosphate (MC700p). (A) Absorption and emission spectra of MC700 in ALP assay buffer. The absorption and emission maxima were 686 nm and 722 nm, respectively. To measure the emission spectrum, MC700 solution was excited at 686 nm. (B) and (C) ALP catalyzed hydrolysis converted MC700p to MC700, which could be detected on an Odyssey Infrared Imaging System at 700-nm channel.

3. Results

3.1. Characterization of MC700 and MC700p

In ALP assay buffer, MC700 has absorption and emission peaks at 686 nm and 722 nm, respectively (Fig. 1A), which are substantially red-shifted from those of MC700p (absorption/emission: 442 nm/638 nm). This big red shift allows the detection of MC700 fluorescence signal in the presence of MC700p. When scanned on an Odyssey Infrared Imaging System at 700-nm channel, the fluorescence signal of MC700p was barely detectable. Reaction in the presence of ALP produced a strong fluorescence signal, presumably from the hydrolysis product MC700 (Fig. 1B and C).

3.2. The MC700 signal is dependent on pH and detergent concentration

Because the ALP enzymatic activity is reflected by the signal intensity of MC700, which is produce by MC700p hydrolysis, it is of interest to investigate the factors that affect the MC700 signal intensity. First, we examined the effect of buffer pH. The absorbance and fluorescence signal of MC700 in solutions with different pH values were determined. With the increase of pH, the absorbance peak was shifted from 477 nm (pH 6.0) to 668 nm (pH 9.0) (Fig. 2A). It is noteworthy that the shape and peak value of MC700 spectrum in Tris buffer (pH 9.5) was different from those of MC700 in ALP assay buffer (Supplementary Fig. S1). The 18-nm red shift (from 668 nm to 686 nm) of MC700 absorbance peak in ALP assay buffer might be caused by Triton X-100, which has also been shown to induce a 10-nm red shift in the MC540 spectrum [18]. The peak values of MC700 absorbance (A668) at different pH were summarized in Fig. 2B. A668 value increased with the increase of pH, and reached the maximum at pH 9.0–9.5. The molar extinction coeffi-

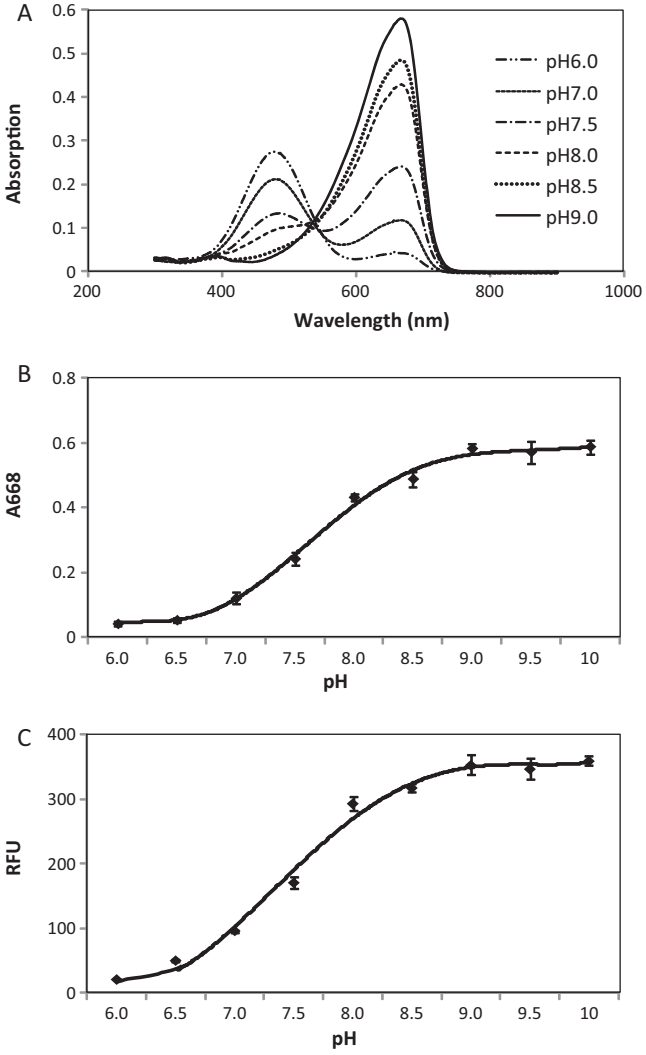

Fig. 2. Effect of pH on MC700 absorbance and MC700 fluorescence signal. (A) Absorption spectra for MC700 in Tris buffer with different pH. MC700 was diluted in 50 mM Tris buffer to a final concentration of 5 μM for spectra measurement. (B) Quantification of MC700 absorbance at 668 nm (A668). (C) Fluorescence signals of the same MC700 solutions as in (B). RFU, relative fluorescence unit.

cient of MC700 in Tris buffer (pH 9.5) is about 96,000 M^{-1} cm^{-1}. The same solution was used to measure the fluorescence signal. As expected, the fluorescence signal showed a similar trend as A668 (Fig. 2C), suggesting that the fluorescence signal is proportional to the absorbance at 668 nm.

As the detergent could enhance the fluorescence signal of some fluorescent dyes, such as DDAO [20], we studied whether the detergent had a similar effect on MC700 fluorescence signal. At concentrations below 0.4%, Triton X-100 enhanced MC700 fluorescence signal in a dose-dependent manner (Fig. 3A). However, little increase could be achieved when Triton X-100 concentration exceeded 0.4%.

The detergent effect on the fluorescence signal from MC700p hydrolysis in the presence of ALP was also investigated. As the detergent could affect ALP enzymatic reaction, as well as the fluorescence signal of the product MC700, the curve was slightly different from that of MC700 alone. Triton X-100 had a positive effect on MC700p hydrolysis signal, which was similar to the MC700 curve. However, the signal intensity reached the highest level at a lower concentration of Triton X-100 (0.3%) (Fig. 3B).

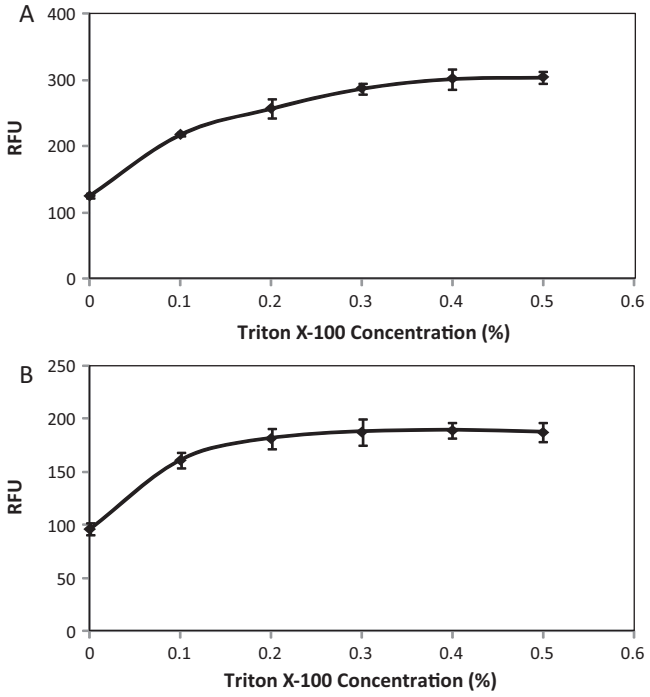

Fig. 3. Effect of detergent on MC700 fluorescence signal and ALP catalyzed MC700p hydrolysis. (A) Fluorescence signal of MC700 in the presence of different concentrations of Triton X-100. MC700 was diluted to a final concentration of 5 μM in 50 mM Tris buffer (pH 9.5). Triton X-100 was added to different concentrations. (B) MC700p was diluted to a final concentration of 5 μM in the same buffers as in (A). After adding ALP (0.01 U per 50 μl), the reaction solutions were incubated for 1 h at room temperature before measuring the fluorescence signals.

3.3. The stability of MC700 and MC700p

The stability of the MC700 signal was determined by incubating 5 μM MC700 in ALP assay buffer at room temperature for various time periods. The fluorescence signal was measured after the incubation. As a comparison, the fluorescence signal of DDAO was also determined under the same conditions. Consistent with our previous report [20], the DDAO signal is stable. The DDAO signal intensity barely changed during the 6 h of incubation. On the contrary, the signal intensity of MC700 decreased slightly during the incubation (Fig. 4A). The remaining signal intensities were about 95% and 82% of the initial intensity after 1 h and 6 h of incubation, respectively.

The signal intensities increased over time for both MC700p and DDAOp (Fig. 4B), indicating that both chemicals were dephosphorylated by non-enzymatic hydrolysis, resulting in the production of the corresponding fluorescent products. However, the magnitude of the signal increase of MC700p was less than that of DDAOp. After 1 h, the signal intensities were about 1.04 and 1.36 folds of the initial intensities for MC700p and DDAOp, respectively. After 6 h, the signal intensities increased to 1.85 and 3.53 folds of the initial intensities (Fig. 4B).

3.4. The fluorescence signal produced by MC700p hydrolysis is dependent on the ALP enzyme amount and MC700p concentration

The effect of ALP amount on the fluorescence signal from MC700p hydrolysis was studied by incubating different amounts of ALP with 5 μM MC700p. The signal from the reaction was measured after 30 min and 60 min, respectively. A linear relationship was observed between the MC700p hydrolysis signal and the ALP amount in the range tested (Fig. 5A). The R^2 values were 0.9975 and 0.9948 for 30 min and 60 min, respectively. Similarly, the effect of MC700p concentration was investigated by incubating a fixed

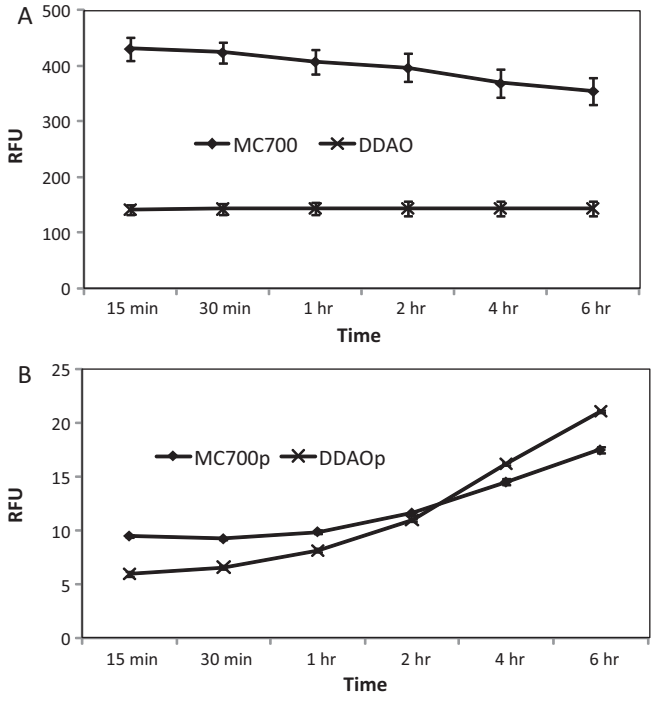

Fig. 4. Stability comparison between MC700 and DDAO, and between MC700p and DDAOp. (A) Time course of fluorescence signals from 5 μM MC700 or 5 μM DDAO in ALP assay buffer. (B) Time course of fluorescence signals produced by 5 μM MC700p or 5 μM DDAOp in ALP assay buffer.

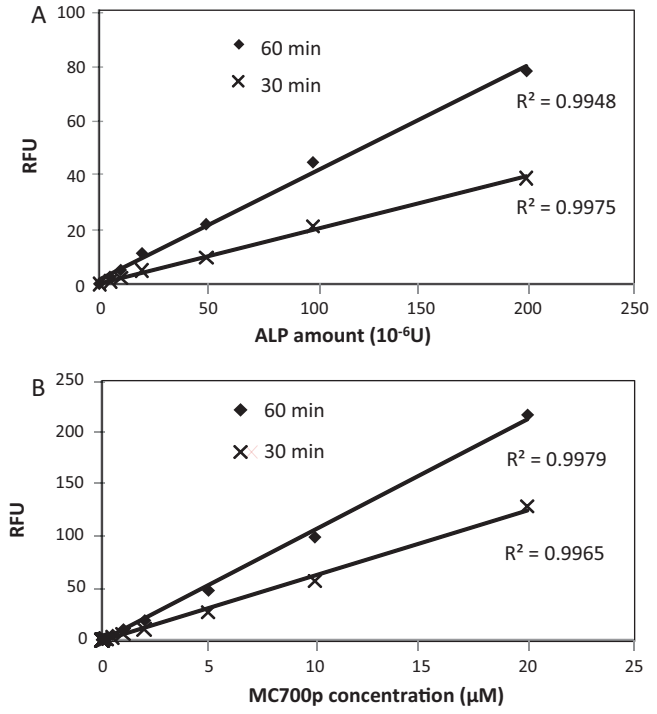

Fig. 5. The fluorescence signal produced by MC700p hydrolysis is linearly related to the ALP amount and substrate concentration. (A) The fluorescence signal produced by 5 μM MC700p after incubation with different amounts of ALP. Signal intensities were determined after 30 min and 60 min of reaction. (B) The fluorescence signal produced by different concentrations of MC700p in the presence of ALP (10^{-4} U per 50 μl). Signal intensities were determined after 30 min and 60 min of reaction.

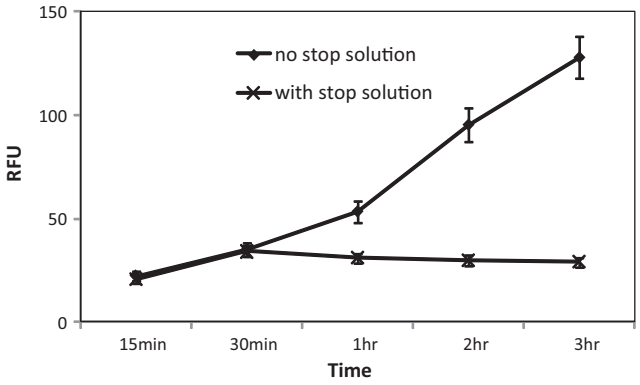

Fig. 6. Termination of the MC700p hydrolysis reaction by the stop solution. MC700p was diluted to a final concentration of 5 μM in ALP assay buffer before adding ALP (10^{-4} U per 50 μl). Stop solution was added after 30 min of reaction.

amount of ALP (10^{-4} U per 50 μl) with different concentrations of MC700p. Again, the relationship between the signal intensity and the MC700p concentration was linear, with R^2 values at 0.9965 and 0.9979 for 30 min and 60 min, respectively (Fig. 5B).

3.5. Stop solution

Many applications require the enzymatic reaction be stopped after a certain period. Several recipes are being used to stop the ALP reaction. These recipes normally contain Na_2CO_3, NaOH or EDTA. However, high concentrations of Na_2CO_3 or NaOH, which are needed to stop the reaction, decreased the MC700 signal significantly (data not shown). Therefore, a solution containing 5 mM EDTA was used to stop the ALP/MC700p reaction in this study. The ALP enzymatic reaction was terminated immediately after the addition of the stop solution, as indicated by the flat curve (Fig. 6). Actually, the signal intensity decreased slightly, possibly due to the signal reduction of the hydrolysis product MC700 in ALP assay buffer, a phenomenon that was observed in Fig. 4A. When no stop solution was used, the signal intensity kept increasing owing to the continuous accumulation of MC700 (Fig. 6).

3.6. E2 assay using MC700p as ALP substrate

ALP conjugates are widely used in EIA assays. In these assays, pNpp is often employed as the substrate to measure the ALP activity, which reflects the amount of ALP conjugated molecules. In this study, we tested the MC700p substrate using an E2 EIA kit. In this kit, ALP-conjugated E2 competes the anti-E2 antibody with free E2 in the sample. As a result, the signal from the ALP activity is reversely related to the E2 content in the sample. The signal intensity of blank (B0) containing no E2 was arbitrarily set as 100%. Signal intensities of the samples (B) were less than that of B0. Thus the values of B/B0 were less than 100%. As shown in Fig. 7, the B/B0 values of MC700p were very close to the B/B0 values of pNpp, suggesting that MC700p could substitute pNpp in this application. DDAOp was also used for comparison. The B/B0 values of DDAOp showed a similar trend as compared to those of MC700p in the E2 concentration range used in this study (Fig. 7).

4. Discussion

Fluorescence technology is widely used in biological research. Most fluorophores currently in use are in the visible spectrum range. Although these visible fluorophores with various colors are routinely used in the research field, they do have limitations, such as high autofluorescence, limited tissue penetration and high sig-

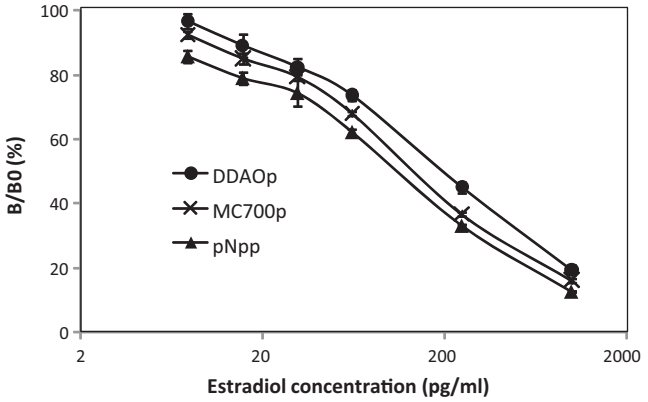

Fig. 7. Application of MC700p as ALP substrate in E2 EIA assay. An E2 EIA kit containing the ALP-conjugated E2 was used. MC700p substrate was compared with the conventional colorimetric pNpp substrate.

nal to noise ratio [21,22]. These problems could be solved partly by exploiting fluorophores in the NIR spectrum range because NIR light has the advantage of deeper penetration capability and reduced background from cells, cell debris, buffer components and plastic materials [23].

In fluorescence imaging, the fluorophores are often attached to the macromolecules such as nucleic acid, antibodies, peptides or other proteins of interest. The visualizability of this method is largely dependent on levels of the binding targets of the macromolecules. Alternatively, an enzyme can be attached to the molecule, and a fluorescence-based substrate can be used to assay the enzymatic activity. The most commonly used enzymes include horseradish peroxidase (HRP), β-galactosidase and ALP. In this case, the signal is amplified owing to the continuous accumulation of the fluorescent product from the enzymatic reaction. We reported previously a β-galactosidase activity assay method using a far-red-shifted fluorescent substrate 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside (DDAOG) [20]. The enzymatic cleavage product DDAO has excitation and emission maxima of 646 nm and 659 nm, respectively, and can be detected on the Odyssey Infrared Imaging System using the 700-nm channel.

Merocyanine dye MC540 has been used as a fluorescent membrane probe to stain the membranes of a wide variety of electrically excitable cells [19]. By employing the acidic tumor environment, it has also been demonstrated that nucleolar localization of MC540 and an elevated fluorescence signal could be used as indicators for proliferating cells [18]. Interestingly, it was noted that the detergent Triton X-100 could increase the fluorescence signal of MC540 [18]. We discovered that Triton X-100 had a similar enhancing effect on MC700 fluorescence signal. The enhancing effect of detergent on fluorescence signal has also been noticed for DDAO [20]. One possible explanation for this phenomenon is that the detergent disaggregates the dye molecules, which is beneficial to the fluorescence. The red shift induced by Triton X-100 may also contribute to the higher signal intensity because the shift is toward the optimal detection wavelength of the Odyssey Infrared Imaging System. Importantly, ALP is tolerant to Triton X-100. No significant decrease of ALP-catalyzed MC700p cleavage signal was observed in the presence of Triton X-100 at a concentration as high as 0.5%. This is in contrast to β-galactosidase, which loses activity when Triton X-100 concentration is higher than 0.3% [20]. The tolerance of ALP to Triton X-100 facilitated the inclusion of this detergent in the ALP assay buffer, which enhanced MC700 fluorescence signal and the sensitivity of the assay method based on MC700.

Another important factor that impacts MC700 fluorescence is pH. An alkaline condition is beneficial to the MC700 fluorescence signal. However, increasing pH beyond 9.5 did not further increase the fluorescence signal. Interestingly, the optimal pH for MC700 fluorescence matches perfectly with the requirement for an alkaline condition for an optimal ALP enzymatic activity.

Unlike the chemiluminescence and bioluminescence technologies, which require measuring the signal immediately after reaction [14,15], the fluorescence technology has the advantage of a wide signal detection time window [20]. However, for some unknown reason, the signal intensity of MC700 decreased overtime in ALP assay buffer. Although the signal reduction was mild (95% remaining after 1 h), it would be beneficial to measure the signal within a short period after the completion of reaction. On the other hand, the stability of the substrate MC700p is better compared to DDAOp, which is desirable for a lower background.

Estrogens, especially E2, play a crucial role in various physiological and pathological events, such as sexual development, reproductive cycle, fertility, maintenance of pregnancy, osteoporosis, cardiovascular diseases and breast cancer [24,25]. The level of E2 is normally measured using radio-immunoassay (RIA) or EIA kits. The colorimetric substrate pNpp is often used as the ALP substrate in EIA kits. Our results demonstrated that MC700p could substitute pNpp as the ALP substrate in these assays. To achieve optimal performance, further study is needed to optimize the protocol, such as MC700p concentration and ALP/MC700p reaction time. In addition to E2 EIA, MC700p could also be applied in other EIA kits that use ALP as the conjugating enzyme. In addition, fluorescence imaging has the advantage of multiple channels, which can be employed to image two or more targets simultaneously [26]. It would be attractive to combine MC700p with fluorescence-based substrates for other enzymes (such as HRP) to make multiplex EIA kits to assay multiple analytes simultaneously.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.02.035.

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