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A diazirine-based photoaffinity probe for facile and efficient aptamer–protein covalent conjugation†

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Huimin Zhang, Yanling Song, Yuan Zou, Yun Ge, Yuan An, Yanli Ma, Zhi Zhu* and Chaoyong James Yang*

A photo-reactive functional labelling reagent, diazirine phosphoramidite, was designed and synthesized for easy and flexible site-specific labelling of oligonucleotides with the diazirine moiety. The new reagent allows facile photo-crosslinking of oligonucleotide with its interacting partner for a variety of applications, including tertiary structure determination, molecular interaction study and biomarker discovery.

The identification of biomarkers associated with disease states is extremely important for disease diagnosis and treatment. The combination of chromatographic techniques with mass spectrometry (MS), which enables researchers to analyse the entire cell proteome and identify cell-specific proteins, has become one of the most widely used methods for biomarker discovery.[1] Although this method has enabled the discovery of several important proteins, it is time-consuming, labor-intensive and inefficient. Thus it is highly desirable to develop rapid, facile, and efficient methods for biomarker discovery.

Recently, an aptamer-based "fishing" method was developed for identification of diseased cell related biomarkers.[2] Aptamers are single-stranded oligonucleotides generated by a well-established method named SELEX (Systematic Evolution of Ligands by EXponential enrichment).[3] When using whole disease cell as the target, a panel of aptamers can be generated by cell-SELEX without the knowledge of cell membrane characteristics.[4] Then, specifically bound molecules on the cell surface can potentially be identified as biomarkers using the aptamer-based "fishing" method. Although it is a straightforward and promising method for biomarker discovery, one major challenge is the dissociation of the aptamer–target complex during the identification process due to the unstable non-covalent interactions between the aptamer and the target. If this non-covalent interaction can be transformed into a covalent bond, the stable aptamer–target complex can be obtained with higher purity by harsh washing, greatly facilitating the process of biomarker discovery on cell membranes.

Photoaffinity probes are promising ligands for the formation of the covalent bonding with good spatial and temporal resolution. Currently, they have been widely used to covalently link protein with small molecule or another protein to study their interactions.[5] The probes contain at least two parts: an affinity unit and a photo-reactive moiety.[6] Azide, benzophenone and diazirine are common photo-reactive groups that can generate highly reactive species such as nitrene, reactive triplet carbonyl state and carbene under UV irradiation.[7] Among them, azide is most widely used because it is easy to synthesize.[8] However, it generates nitrene that could lead to the undesired side product of ketimines.[9] Moreover, azide requires the irradiation with destructive short-wavelength UV (<300 nm). Benzophenone can be photo-activated by long-wavelength UV (~350 nm) but needs a long photo-irradiation time for the reaction.[10]

Diazirine is one of the most effective photo-reactive groups because it can generate a highly active carbene intermediate which can even be inserted into the inactive C–H bond.[7,11] Meanwhile, the photoreaction of diazirine uses long-wavelength UV and short irradiation time, which causes less damage to biomolecules and minimizes non-specific labelling. Therefore, diazirine has been widely used for a variety of photo-labelling, such as the covalent linking of a ligand to its target for the investigation of ligand–receptor interactions,[12] identification of enzyme inhibitor binding sites,[13] and discovery of the key amino acid residues in protein–protein interactions.[14] Various diazirine analogs of nucleic acids have also been developed for DNA modification, such as 2,3-dideoxyuridylate with a diazirine on the C5-position.[15] A simpler and more popular method for DNA labelling is based on the coupling of an amine-modified oligonucleotide with the succinimide ester of diazirine. Unfortunately, the low reaction efficiency and modification site restriction greatly impede further application of these modified oligonucleotides.

The MOE Key Laboratory of Spectrochemical Analysis & Instrumentation, Key Laboratory for Chemical Biology of Fujian Province, State Key Laboratory of Physical Chemistry of Solid Surfaces, Department of Chemical Biology, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, P. R. China. E-mail: cyyang@xmu.edu.cn, zhuzhi@xmu.edu.cn

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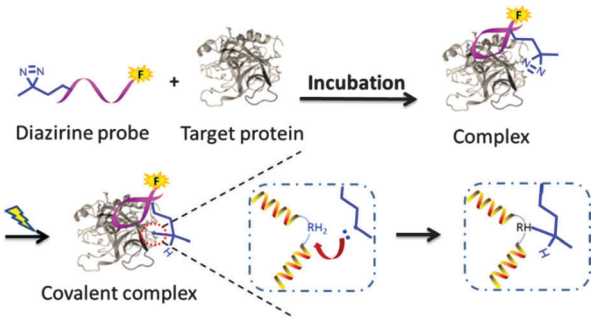

Scheme 1 Photo-initiated efficient covalent coupling of diazirine modified aptamer probe with its target protein for biomarker discovery.

Therefore, it is of great importance to develop diazirine probes for simple, facile and efficient labelling on DNA ligands, such as aptamers, for DNA–protein interaction study and aptamer-based biomarker discovery.

Herein we developed a new diazirine probe for simple, facile and efficient labelling of DNA ligands. Our diazirine phosphoramidite (diazidite) can be chemically synthesized by a simple procedure and used for easy and flexible site-specific labelling of a DNA sequence with an automated DNA synthesizer. Using the resulting diazirine-labelled aptamer to interact with the target, a covalent bond can be formed between the aptamer and the target by 365 nm irradiation. By fishing out such covalent complexes and analysing them with the help of MS, the possible biomarkers on the disease cell surface can then be discovered. According to the principle described in Scheme 1, diazirine is inserted into a certain position of the aptamer, and the high affinity and selectivity of the aptamer enable specific capture of target protein. As a proof-of-concept, we adopted two known aptamer targets, streptavidin (SA) and thrombin (TMB) to verify the feasibility of photo-crosslinking capability of diazirine-labelled aptamers with target proteins. Additionally, we compared the photo-crosslinking efficiency of our probe with that of the widely used I-dU probe.

The synthesis of diazidite is shown in Fig. 1. The diazirine–COOH (**2**) was synthesized from 4-oxopentanoic acid (**1**) by reacting with NH_2OSO_3H in liquid ammonia and oxidized by iodine.[16] The diazirine–COOH was reacted with 6-amino-2-hydroxymethylhexan-1-ol to obtain two hydroxyl groups.

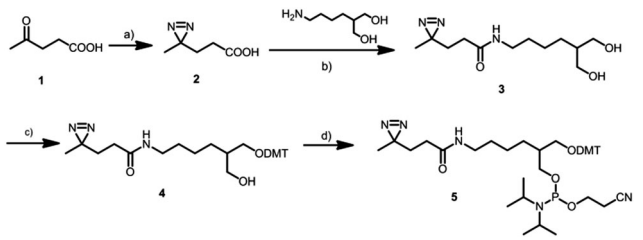

Fig. 1 Synthesis of diazirine phosphoramidite: (a) **1** NH_3 (liquid), NH_2OSO_3H, MeOH; **2** I_2, Et_3N, MeOH, 0 °C; (b) DCC, HOBt, DMF, room temperature, 24 h; (c) DMT-Cl, pyridine, CH_2Cl_2, DMAP, ice bath, and then room temperature about 24 h; (d) P-(N-iPr_2)$_2$(OCH_2CH_2CN), DIPEA, CH_2Cl_2, 0 °C, about 4 h.

With 4-4′-dimethoxytriphenylmethyl chloride (DMT-Cl), one of the hydroxyl groups was then protected by DMT, while the other hydroxyl was coupled with 2-cyanoethyl diisopropylchlorophosphoramidite to generate the final product (**5**). The DMT-protected diazidite is highly compatible with the general DNA synthesis protocol. Moreover, ten carbons have been introduced between the diazirine group and the backbone of DNA to enable appropriate flexibility for diazirine to react with targets.

The as-synthesized diazidite was applied to label the streptavidin aptamer[17] as an example to demonstrate the feasibility for covalent binding with streptavidin (SA) by UV irradiation. Since the highly active carbene intermediate generated by diazirine can react only with nearby molecules, the suitable diazirine labelling sites on the aptamer needed to be identified for highly efficient photo-crosslinking with the target, while maintaining the binding affinity. According to the secondary structure of the SA–aptamer, three sites were chosen. The resulting diazirine modified SA–aptamers (Table S1, ESI†) were synthesized and labelled with FITC for easy characterization. SA-beads were used as targets so that the formation of covalent bond could be directly detected by flow cytometry. The modified SA–aptamers were incubated with SA-beads in the dark at 37 °C for 30 min. After washing away the excess or nonspecific sequences, the resulting complexes were irradiated with 365 nm UV light on ice. Afterwards, the complex was subjected to 10 M urea washing. Since urea can destroy the non-covalent interaction of the SA–aptamer with SA, the non-crosslinked SA–aptamers could be washed away from the SA-beads, leaving the beads nonfluorescent, while the covalently bound aptamers stayed with SA, yielding fluorescent beads. Flow cytometry was used to detect the fluorescence intensity of SA-beads after different treatments.

First, the effect of the modification site on aptamer binding affinity was investigated. As shown in Fig. S1 (ESI†), compared with the unmodified SA–aptamer, different insertion locations of diazirine have distinct influence on aptamer binding. This suggested that the inserted diazidite may disturb the secondary structure of aptamer and affect its binding affinity. Then, the crosslinking capability of each modified aptamer was evaluated (Fig. S2, ESI†). Among them, SA-6-drz has shown the best crosslinking efficiency. As shown in Fig. 2A, after treatment with 10 M urea, the fluorescence intensity of SA-beads with unmodified SA–aptamer was greatly decreased due to the destruction of noncovalent interactions by urea. However, the fluorescent intensity of SA-beads with SA-6-drz did not exhibit a significant change after UV irradiation and washing with 10 M urea, suggesting that the covalent bond was successfully formed between the aptamer and the protein. To verify that the covalent bond formation was indeed the result of UV irradiation, the SA-6-drz sample was treated with different irradiation times. In Fig. 2B, as the irradiation time increased, the fluorescence intensity of SA-beads treated with urea also increased, indicating that the quantity of covalent complexes increased with longer irradiation. These results demonstrated that diazirine labelled aptamer could still bind the protein and formed the covalent bond by photo-irradiation.

Previously, I-dU was used for photoaffinity labelling of aptamers for biomarker discovery.[18] The photo-crosslinking

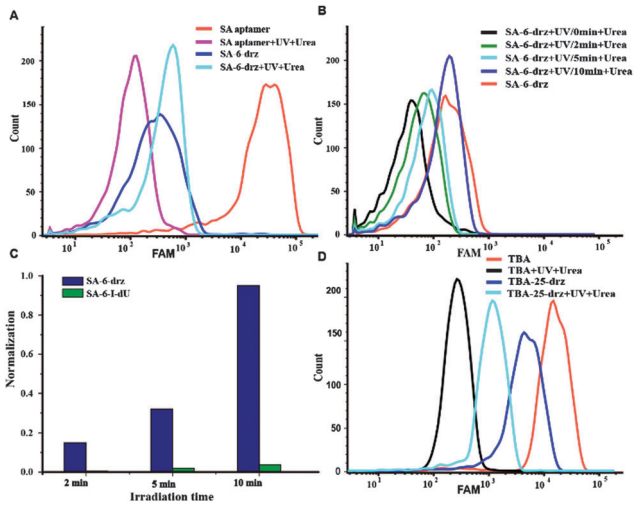

Fig. 2 (A) Binding of aptamer sequences SA and SA-6-drz to SA beads before and after UV/urea treatment. (B) Effect of UV irradiation time on the crosslinking efficiency of modified aptamer sequence SA-6-drz to SA beads. (C) Comparison of the cross-linking efficiency between diazirine and I-dU modified probes under their respective optimized conditions. (D) Binding of aptamer sequences TBA and TBA-25-drz to thrombin beads before and after UV/urea treatment.

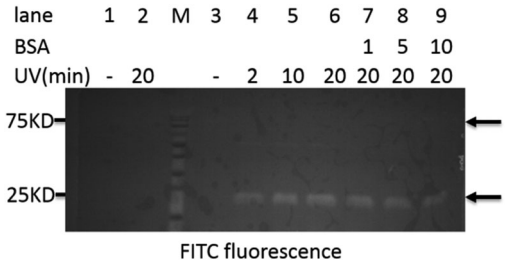

Fig. 3 SDS-PAGE analysis of photoaffinity labelling of SA with diazirine-labelled-aptamer in the presence of a high background protein (BSA). M: marker.

efficiency of the diazidite was then compared with the commonly used I-dU modification. To ensure fair comparison, the probe SA-6-I-dU was synthesized using the same process as that of SA-6-drz. SA-beads were incubated with 200 nM of either SA-6-I-dU or SA-6-drz for 30 min at 37 °C, and then irradiated with 308 or 365 nm UV light at the same power on ice, respectively. As shown in Fig. 2C, with all three irradiation time durations (2, 5 and 10 min), the SA-6-drz treated sample displayed a significantly higher crosslinking efficiency than that of SA-6-I-dU, demonstrating the excellent labelling efficiency of the diazirine over the I-dU based photoaffinity probe.

In order to validate whether SA-6-drz can photo-crosslink with SA in the high background of irrelevant proteins, the reaction of SA-6-drz and SA was performed in the presence of ten-fold concentrations of bovine serum albumin (BSA), and the result was analysed by SDS-PAGE. Because noncovalent interactions are disrupted in SDS-PAGE, only covalent bonding as the result of photo-crosslinking will result in the formation of the protein–aptamer complex. The SDS-PAGE results were obtained by recording the fluorescence image of FITC-labelled SA-6-drz. As shown in Fig. 3, after UV irradiation, a band corresponding to streptavidin–aptamer complexes (25 kD) was observed (lanes 4–6) and it increased with irradiation time, but there was no BSA–DNA complex band (~75 kD) even with 1-, 5- or 10-fold excess BSA (lanes 7–9). These results revealed that diazirine modified aptamer had high selectivity for photo-crosslinking with the target protein, suggesting the potential of the diazidite for use in biomarker discovery with aptamers generated from cell-SELEX.

To demonstrate the generality of the diazidite for aptamer labelling to crosslink protein, thrombin (TMB) and its 29-mer aptamer[19] were chosen as another example. The secondary structure of the G-rich thrombin aptamer is different from the hairpin-loop structure of the SA–aptamer. To find the most suitable photo-crosslinking site, we inserted diazirine into four different sites, and used the same method to test whether the probe could covalently link to the target protein. As shown in Fig. 2D, although there was a slight decrease in the binding capacity of TBA-25-drz, it can still be covalently linked to target after UV irradiation. And the other modified aptamers cannot form covalent binding with the protein beads due to the loss of binding affinity (Fig. S3, ESI†).

In conclusion, we have designed and synthesized a photo-crosslinking reagent, diazidite, which can be used for site-specific labelling of aptamer sequences directly by the general DNA synthesis protocol. The synthesis of diazidite is simple and straightforward. Diazirine has the advantages of high crosslinking efficiency, small size and excellent chemically stability, which are highly desirable for use in aptamer labelling. We demonstrated that by choosing the right modification position, the diazirine modified streptavidin and thrombin aptamers have high efficiency and specificity for photo-crosslinking with their corresponding target proteins. The diazidite probe is thus useful for biomarker discovery by providing a novel and efficient strategy for covalent labelling of biomarkers with aptamers generated from cell-SELEX. Moreover, because it can be inserted into any site of a DNA/RNA sequence, the diazidite is potentially useful for the determination of DNA/RNA secondary structures and identification of nucleic acid–protein interaction binding sites.

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