Bioorganic & Medicinal Chemistry Letters 27 (2017) 3417–3422

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Development of an ON/OFF switchable fluorescent probe targeting His tag fused proteins in living cells

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ARTICLE INFO

Article history:
Received 24 April 2017
Revised 29 May 2017
Accepted 30 May 2017
Available online 31 May 2017

Keywords:
Cell-penetrating peptide
Maleimide
ON/OFF switch
His tag fused protein

ABSTRACT

The fluorescent labeling of target proteins is useful for analyzing their functions and localization in cells, and several fluorescent probes have been developed. However, the fusion of tags such as green fluorescent protein (GFP) to target proteins occasionally affects their functions and/or localization in living cells. Therefore, an imaging method that uses short peptide tags such as hexa-histidine (the His tag) has been attracting increasing attention. Few studies have investigated ON/OFF switchable fluorescent probes for intracellular His-tagged proteins. We herein developed a novel ON/OFF switchable probe for imaging targeted intracellular proteins fused with a CH6 tag, which is composed of one cysteine residue and six histidine residues.

The fluorescent labeling of target proteins is useful for analyzing their functions and localization in cells, and a number of fluorescent probes have been developed to date. The green fluorescent protein (GFP) is a representative fluorescent probe and the genetic fusion of GFP to target proteins is broadly used to visualize the behaviors of these proteins in cells.[1,2] However, the fusion of GFP to a target protein of interest (POI) sometimes affects its function and/or localization due to the large molecular size of GFP.[3] In order to overcome these issues, several fluorescent labeling methods using short peptide tags such as the FlAsH,[4] ReAsH[5] tags, and tetraserine motifs[6] have recently been reported. The hexa-histidine tag (His tag) is a well-known short peptide tag that interacts with metal ions such as copper (Cu), nickel (Ni), and cobalt (Co) through non-covalent bonds. Therefore, the His tag represents a promising approach to POI labeling because its molecular size has minimal effects,[7] and it is often utilized in the affinity purification of genetically modified proteins. Several Ni (II)-NTA (nickel-nitrilotriacetic acid)-based probes that target His-tagged proteins have been reported to date.[8–10] However, Ni (II)-NTA-based probes for His-tagged proteins are limited to the labeling of membrane proteins because Ni (II)-NTA itself is impermeable to the cell membrane. Recent studies reported that a Ni (II)-NTA-based fluorescent probe bearing a chloroacetamide moiety targeted the CH6 tag, which comprises one cysteine residue and the His tag, through a covalent bond between a cysteine residue on the CH6 tag and the chloroacetamide group on the fluorescent probe, thereby increasing labeling efficiency.[11,12]

The ON/OFF switching of fluorescence is also important for improving its sensitivity. An ON/Off switchable probe, which only emits fluorescence when it interacts with POI, efficiently enables observations of the behavior of POI in living cells without the washing of excess amounts of the fluorescent probe.[13–15] Therefore, ON/OFF switchable probes have been attracting increasing attention as an effective imaging tool.

We herein developed an ON/OFF switchable imaging method that targets intracellular CH6-tagged POI in living cells. We specifically designed and synthesized fluorescent probe **1** and the carrier peptide **R9-H4A2**, as shown in Fig. 1. 7-(Diethylamino) coumarin as a fluorophore was conjugated with three Ni (II)-NTA moieties to strengthen binding affinity to the His tag,[16] and with a maleimide moiety not only to form a covalent bond with the cysteine residue of the CH6 tag, but also to quench fluorescence by photoinduced electron transfer (PeT).[17] We also synthesized the carrier peptide **R9-H4A2**, consisting of the representative cell-penetrating peptide nona-arginine (R9) and **H4A2** (Sequence: HAHAHH, H: Histidine, A: Alanine) fragment. The **H4A2** moiety interacts with probe **1** through the Ni-NTA moiety and delivers hydrophilic fluorescent probe **1** into cells by the R9 fragment. Due to the substitution of two histidines with two alanines, the binding affinity of the **H4A2** moiety is weaker than that of the His tag to POI. Therefore, we expected probe **1** to dissociate from the carrier peptide and target the CH6 tag in cells.

In the fairly recent past, we have developed the small molecules that mediated degradation of His-tag fused protein using the same

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http://dx.doi.org/10.1016/j.bmcl.2017.05.087
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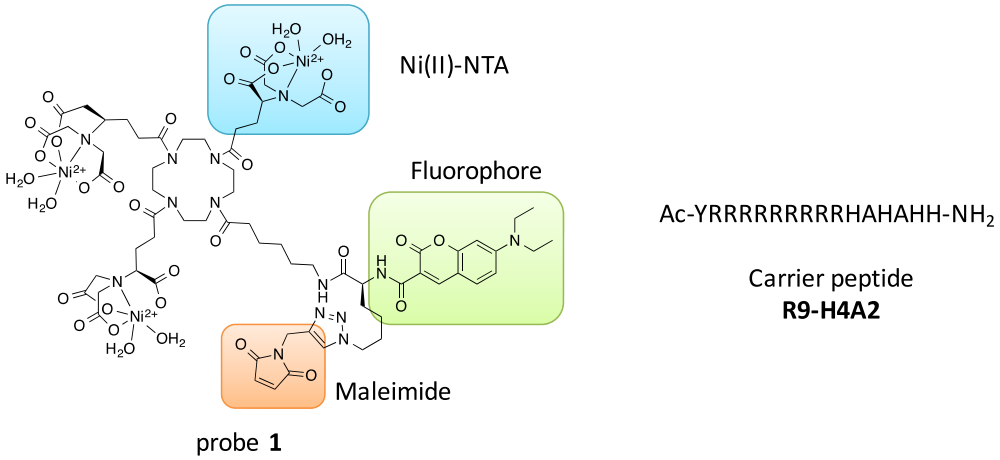

Fig. 1. Chemical structure of fluorescent probe **1** and the sequence of the carrier peptide **R9-H4A2**.

strategy.[18] In this report, MV-1, which induces the ubiquitination of target proteins, was conjugated with three Ni (II)-NTA moieties. The hybrid compound was internalized by the supports of carrier peptide and formed the covalent bond with cysteine residue of CH6 tagged target proteins, subsequently the selective degradation of target proteins occurred. These results indicated that the probe **1** is capable of selectively reacting with CH6 tagged proteins and visualize the target proteins in living cells.

The mechanism proposed for the ON/OFF switchable imaging method of CH6-tagged POI is shown in Fig. 2. Fluorescent probe **1** and the carrier peptide **R9-H4A2** initially form a complex through interactions between the Ni (II)-NTA and **H4A2** moieties. The complex then penetrates the cell membrane through the effects of R9. Fluorescent probe **1** dissociates from the complex and interacts with CH6-tagged POI. The cysteine residue of the CH6 tag is physically close to the maleimide moiety of **1** and forms a covalent bond. The fluorescence of probe **1** is recovered by the loss of PeT effects and enables the visualization of CH6-tagged POI (Fig. 2).

The synthetic route of fluorescent probe **1** was shown in Scheme 1. H-Glu(OBzl)-O*t*Bu was treated with *tert*-butyl 2-bromoacetate to form a NTA moiety and the benzyl group was removed using Pd/C under a H_2 atmosphere to give compound **3**. Compound **4** was synthesized by the condensation of 3 eq. compound **3** and 1,4,7,10-tetraazacyclododecane. The resulting amino group of **4** was condensed with 6-(carbobenzoxyamino)hexanoic acid [Cbz-Acp(6)-OH]] and the subsequent deprotection of the Cbz group to give compound **6**. Fmoc-azidolysine was coupled with **6** and the Fmoc group was subsequently removed by diethylamine to afford compound **8**. Compound **8** was coupled with 7-(diethylamino)coumarin, the maleimide moiety was attached by 1,3-dipolar cycloaddition, and all *tert*-butyl ester groups were then

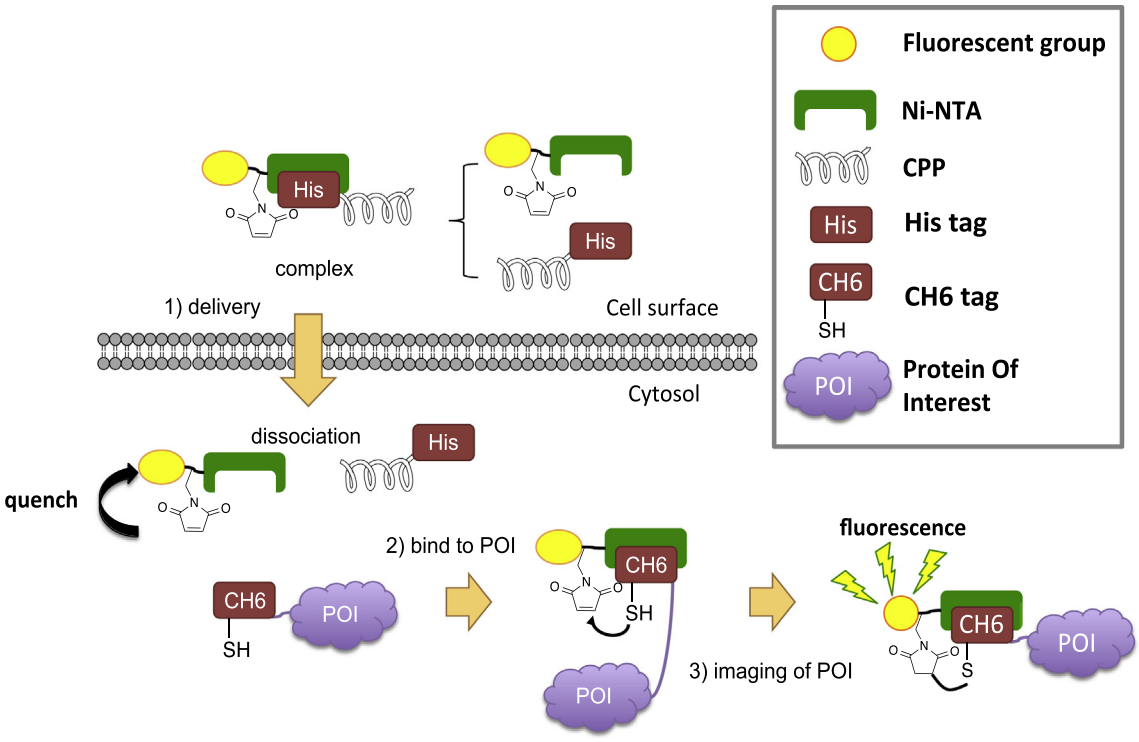

Fig. 2. Proposed mechanism for the ON/OFF switchable imaging method.