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ARTICLE TYPE

A solid-phase affinity labeling method for target-selective isolation and modification of proteins

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Solid-phase affinity labeling of a target protein, peanut agglutinin (PNA), with the specifically designed chemical tool 1 selectively and effectively furnished the labeled PNA. Furthermore, this method was applicable to native human carbonic anhydrase II in red blood cell lysate using the chemical tool 2 without the need for tedious manipulations.

The development of novel methods to selectively isolate or modify a specific target protein by incorporation of functional groups, such as biotin, fluorescent probes, or affinity tags, has attracted much attention in the fields of chemistry, biology, and medicine. Efficient chemistry-based approaches, such as affinity labeling methods, for the specific modification of target proteins without the need for genetic manipulation and loss of the original biological activity have recently been developed.¹ However, tedious manipulations are still needed for solution-phase chemical approaches to the purification and isolation of labeled proteins: 1) separation of labeled from unlabeled protein can be problematic due to the small difference in molecular weight between them and 2) separation of ligand molecule used for affinity labeling from the target protein can be difficult due to the high affinity between them. This report describes a solid-phase affinity labeling method utilizing a biocompatible polymer for the target-selective isolation and modification of proteins without the need for tedious manipulations.

To develop the solid-phase affinity labeling method, solid-supported chemical tools containing three functionalities in the molecular structure were designed, as shown in Fig. 1a. One is a ligand moiety that binds to a target protein selectively; the second is an alkylating group, 2-bromoacetamide, that can form a covalent bond with the target protein; and the third is a ligand exchange site, a hydrazone group, that can remove the labeled target protein from the solid support and introduce a reactive handle, such as an azide moiety that is useful for Staudinger ligation² and copper-catalyzed Huisgen [3+2] cycloaddition³ reactions under physiological conditions. PEGA resin was chosen as the solid support.⁴ PEGA resin is porous and hydrophilic,

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suitable for both chemical and enzymatic reactions⁵ and for screening biomolecules such as antibodies, lectins, and proteins.⁶ The strategy is illustrated in Fig. 1b. Recognition of the target protein by the ligand molecule in the solid-supported chemical tool triggered alkylation of the nucleophilic amino acid residue on the target protein with the 2-bromoacetamide group of the solid-supported chemical tool. Subsequent washing of the resulting resin to remove the unlabeled target protein and other proteins provided the resin-bound target protein 3. The hydrazone-oxime ligand exchange reaction^{1h,1k,7} of 3 with an *O*-alkyloxyamine, followed by washing of the resulting resin, provided the ligand-bound PEGA resin 4 and the pure labeled target protein 5. In addition, when an azide-functionalized *O*-alkyloxyamine was used for the ligand exchange reaction, the labeled target protein 5 with an azide group (R = N₃) was obtained and derivatized by subsequent copper-catalyzed Huisgen reaction with an acetylene-appended functional molecule to furnish the modified target protein 6. No complicated manipulations were required.

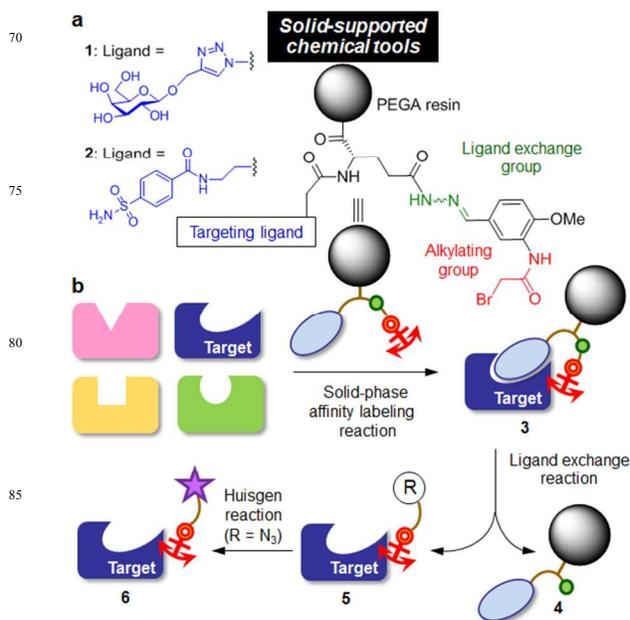
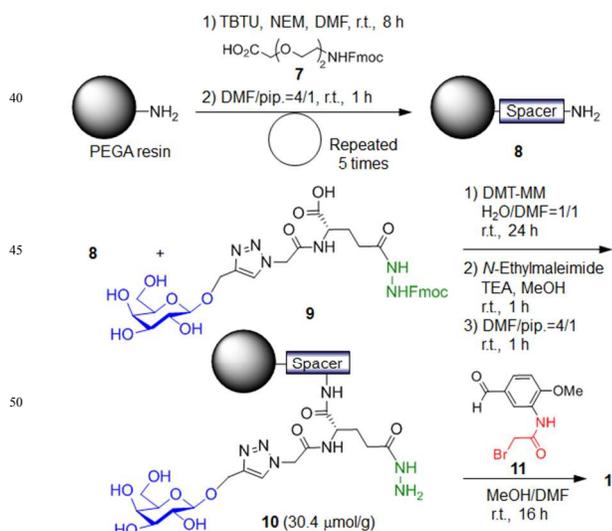


Fig. 1 (a) Molecular design and structure of solid-supported chemical tools 1 and 2, and (b) representation of the solid-phase affinity labeling method.

To investigate our hypothesis, we first selected β -D-galactoside and peanut agglutinin (PNA) as a ligand molecule and a target protein, respectively, and designed the solid-supported chemical tool **1**. It is known that the interactions between monosaccharides and lectins are generally weak, and the association constant for binding of methyl β -D-galactoside to PNA is $1.18 \times 10^3 \text{ M}^{-1}$.⁸ Therefore, we envisaged that if target-selective isolation and modification of PNA with **1** was feasible, applications to other target receptor proteins by changing the ligand moiety would be possible. The synthesis of designed solid-supported chemical tool **1** is shown in Scheme 1. Starting with amino-functionalized PEGA₈₀₀ resin (50 $\mu\text{mol/g}$, swollen form in MeOH), spacer unit **7**⁹ was elongated on the resin by an amide coupling reaction using TBTU/NEM, followed by deprotection of the Fmoc group with 20% piperidine in DMF. This reaction sequence was repeated five times to obtain the five spacer units of the elongated amino resin **8**. Complete conversion at each coupling step was confirmed through a negative Kaiser test.¹⁰ Then, amidation of **8** with **9** (see ESI Scheme S1†) was performed using DMT-MM¹¹ in DMF/H₂O for 24 h, and the remaining free amino groups were capped with *N*-ethylmaleimide. Completion of the capping reaction was confirmed through a negative Kaiser test. Removal of the Fmoc group with 20% piperidine in DMF provided **10**. Measurement of the UV absorbance of the piperidine-dibenzofulvene adduct (301 nm) formed during the Fmoc deprotection reaction indicated that the loading value of **10** was 30.4 $\mu\text{mol/g}$. Treatment of **10** with aldehyde **11** (see ESI Scheme S2†) furnished **1**.

With the solid-supported chemical tool **1** successfully created, the solid-phase affinity labeling reaction of PNA was examined in HEPES buffer (50 mM, pH 8.0) at room temperature (Fig. 2a). Note that 4.0 equiv. of PNA (200 μM) to the galactose ligand on **1** were used in this reaction, because PNA exists as a tetramer at pH 8,¹² and only one-fourth of the PNA was expected to be labeled on the solid surface. After incubation of **1** with PNA for 24 h, the reaction mixture was filtered, and the resulting resin



Scheme 1 Synthetic scheme of **1**. TBTU = *N,N,N',N'*-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate; NEM = *N*-ethylmorpholine; DMF = *N,N*-dimethylformamide; pip. = piperidine; DMT-MM = 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride; TEA = triethylamine.

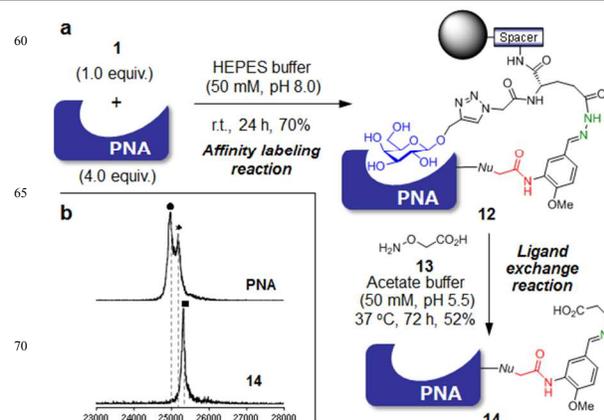


Fig. 2 a) Solid-phase affinity labeling reaction of PNA using **1**, and ligand exchange reaction of **12** with **13**. b) MALDI-TOF MS analysis of PNA (●) (M_w 24991) and the labeled PNA **14** (■) (M_w 25317) (The asterisk (*) is the peak of PNA + matrix).

was washed twice with the reaction buffer to remove unreacted PNA. Conversion of the labeling reaction was calculated based on the recovered PNA concentration estimated by the Bradford method.¹³ Results showed that the labeling reaction of PNA with **1** proceeded effectively to give resin-bound PNA **12** in 70% yield. This indicates that PNA effectively bound to the galactose ligand and reacted with the 2-bromoacetamide moiety on the solid support. Next, the hydrazone-oxime ligand exchange reaction was examined using aminoxyacetic acid (**13**) in acetate buffer (50 mM, pH 5.5) at 37 °C to cleave labeled PNA from the solid support. The ligand exchange reaction also proceeded to give **14** in 52% yield. In addition, MALDI-TOF MS data indicated that a peak corresponding to the labeled PNA **14** clearly appeared as a single peak after the cleavage reaction (Fig. 2b).

Next, to confirm protein selectivity, the affinity labeling reaction was conducted in the presence of an equimolar mixture (25 μM , 4.0 equiv. to **1**) of four proteins, bovine serum albumin (BSA), fetuin, PNA, and ribonuclease A (RNase A), using **1**, and ligand exchange with **13** (Fig. 3a). The progress of each reaction step was monitored by SDS-PAGE (Fig. 3b). Results showed that only the SDS-PAGE band corresponding to the target protein PNA was reduced after affinity labeling compared to that before

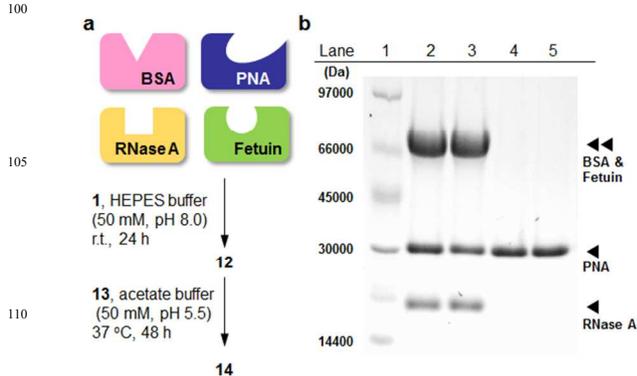


Fig. 3 (a) Reaction scheme for solid-phase affinity labeling reaction of PNA using **1** in the protein mixtures (BSA, fetuin, PNA, and RNase A), and ligand exchange reaction with **13**. (b) SDS-PAGE analysis of the affinity labeling reaction and ligand exchange reaction by SYPRO Ruby staining. Lane 1, size marker; lane 2, before affinity labeling; lane 3, after affinity labeling; lane 4, authentic standard of PNA; lane 5, after ligand exchange.

the labeling reaction (Fig. 3b, lanes 2 and 3). Furthermore, after ligand exchange, labeled PNA **14** was effectively and selectively isolated from the other three proteins, which do not possess affinity toward the galactose ligand in **1** (Fig. 3b, lane 5). These results clearly indicate that target-selective isolation of protein was feasible,¹⁴ even in protein mixtures, using this solid-phase affinity labeling method under mild aqueous conditions without the need for complicated manipulations.

To demonstrate the applicability of this method, human carbonic anhydrase II (hCAII) and benzenesulfonamide were chosen as a combination of the target protein and its specific ligand,^{1h,11,15} respectively, and the solid-supported chemical tool **2** was designed. After chemical synthesis of **2** (see ESI Schemes S3 and S4†), solid-phase affinity labeling of purified hCAII (5 μ M) using **2** in HEPES buffer (50 mM, pH 8.0) for 24 h at room temperature, and ligand exchange in acetate buffer (50 mM, pH 5.5) for 24 h at room temperature with **13**, were conducted. Results showed that both affinity labeling and ligand exchange proceeded effectively to give resin-bound hCAII **15** and labeled hCAII **17** in 69% and 68% yields, respectively (see ESI Scheme S5†). The labeling site of **17** was determined by the peptide mapping experiment using Lysyl endopeptidase.^{1d} Results clarified that the labeling site of **17** was His-3 (see ESI Fig. S1†). Next, the affinity labeling of native hCAII in cell lysate of human red blood cells (hRBCs)¹¹ using **2**, and ligand exchange were examined under the same reaction conditions (Fig. 4a). The progress of each reaction step was monitored by SDS-PAGE (Fig. 4b). Results showed that only the SDS-PAGE band corresponding to the target protein hCAII was clearly reduced after affinity labeling compared to before the labeling reaction (Fig. 4b, lanes 2 and 3). Furthermore, after ligand exchange with **13**, labeled hCAII **17** was effectively and selectively isolated from the other proteins (Fig. 4b, lane 4).

Finally, to demonstrate the target selective isolation and modification of native hCAII in cell lysate, affinity labeling using **2**, ligand exchange with **16**, and subsequent Huisgen reaction using acetylene-appended fluorescein **19**¹⁶ were examined. The reaction progress was analyzed by MALDI-TOF MS and SDS-PAGE. Results showed that both affinity labeling in hRBC lysate and ligand exchange with **16** proceeded effectively and selectively to provide azide-labeled hCAII **18**¹⁷ (Fig. 4b, lane 5). In addition, Huisgen reaction of **18** with **19** in the presence of $[\text{Cu}(\text{MeCN})_4]\text{PF}_6$ and triazole ligand **20**¹⁸ in 25 mM HEPES buffer (pH 8.0) for 2 h at room temperature afforded fluorescein-labeled hCAII **21**. The MALDI-TOF MS data indicated that a new peak corresponding to **21** appeared after the Huisgen reaction (see ESI Fig. S3†). Furthermore, the SDS-PAGE band corresponding to **21** was clearly detected as a single band by not only CBB staining but also in-gel fluorescence image (Fig. 4c). These results indicate that this solid-phase affinity labeling method was applicable for target-selective isolation and modification of the native protein even in human cell lysate under mild aqueous conditions.

In conclusion, a novel solid-phase affinity labeling method was developed for target-selective isolation and modification of proteins under mild aqueous conditions without the need for tedious manipulations, which are typically needed in conventional methods. In addition, this method is quite novel

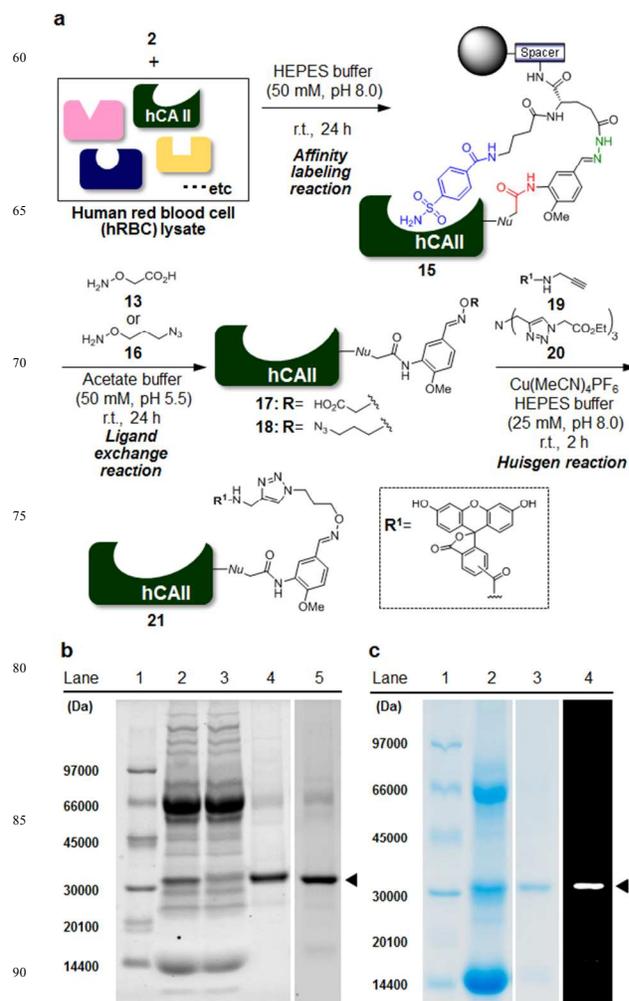


Fig. 4 a) Reaction scheme for solid-phase affinity labeling reaction of native hCAII in human red blood cell lysate using **2**, and ligand exchange reaction with **13** or **16**. b) SDS-PAGE analysis by SYPRO Ruby staining. Lane 1, size marker; lane 2, before affinity labeling; lane 3, after affinity labeling; lane 4, after ligand exchange with **13**; lane 5, after ligand exchange with **16**. c) SDS-PAGE analysis by CBB staining (lanes 1-3) and in-gel fluorescence image (lane 4). Lane 1, size marker; lane 2, before affinity labeling; lane 3, after ligand exchange with **16**; lane 4, after Huisgen reaction with **19**.

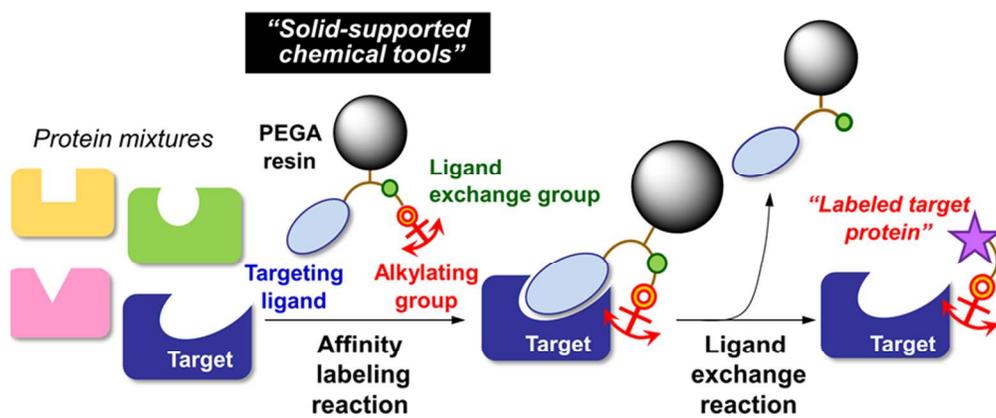
from the methodological point of view. The designed solid-supported chemical tools can be easily and effectively synthesized on biocompatible PEGA resin and then directly used for protein labeling experiments. Moreover, it was revealed that this method could be applicable for the target selective isolation and modification of proteins by changing the ligand moiety. Furthermore, it was successfully demonstrated that target-selective isolation and modification of the native protein, hCAII, was feasible, even from human cell lysate under mild aqueous conditions without need for complicated manipulations. We expect this method to be generally applicable not only for target-selective isolation and modification of unknown target receptor proteins of biologically active small molecules but also for creating multi-functional artificial proteins. These investigations are ongoing.

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