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Design and synthesis of small molecule-conjugated photoaffinity nanoprobes for a streamlined analysis of binding proteins

Kaori Sakurai*, Amane Kato, Keisuke Adachi

Tokyo University of Agriculture and Technology, 2-28-16, Naka-cho, Koganei-shi, Tokyo, Japan

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ABSTRACT

We designed and synthesized a set of photoaffinity nanoprobes, which multivalently display a small molecule ligand and a photoreactive group on gold nanoparticles. Due to the typically hydrophobic nature of these two functional groups, a hydrophilic spacer was additionally introduced to co-functionalize the nanoprobes to maintain their dispersibility in aqueous buffer solutions. Photoaffinity labeling studies using the nanoprobes composed of different ratios of three functional groups showed that including high density of the spacer group attenuates crosslinking efficiency. Comparative analysis of the reactivity among three major photoreactive groups suggested that unlike in the context of conventional photoaffinity probes, arylazide group enables the most selective crosslinking of a model small molecule binding protein.

Photoaffinity probes provide powerful chemical tools for target identification and target engagement studies of bioactive small molecules.[1] Development of appropriate probes is critical for successful photoaffinity labeling, which can fulfill the requirements of maintaining bioactivity of the parent molecule and providing optimal positioning of a photoreactive group. However, it is typically not a trivial task.[2] Covalent capture of small molecule binding proteins by photoaffinity labeling is often low yielding. New photoreactive groups have been reported but certain technical difficulties remain with them in terms of synthetic accessibility and chemical stability.[3] When clickable tags are used as a reporter group,[4] identification of probe-labeled proteins is a multistep and laborious process, requiring conjugation to an affinity tag or affinity resins such as azide-PEG-biotin then subsequent affinity purification. Gold nanoparticles (AuNPs) offer a suitable scaffold to rapidly assemble multiple functionalities to generate various designs of photoaffinity probes.[5] We recently reported AuNP-based photoaffinity probes, which implement both affinity enhancement and highly efficient purification of specific binding proteins for rapid exploration of carbohydrate-binding proteins in one-pot.[6] We are interested in expanding the scope of AuNP-based photoaffinity probes so that they can be applied in the target identification of various small molecule drugs and natural products other than carbohydrate ligands. There have been several studies, where AuNPs were successfully functionalized with small molecule ligands for applications in aqueous buffer solutions such as for biosensing.[7] However, it has also been known that the utility of AuNP-based tools is limited by their tendency to flocculate and to form irreversible aggregates thereby lacking long-term colloidal stability.[8] In this study, we designed and synthesized new photoaffinity nanoprobes based on AuNPs to multivalently display a small molecule ligand for streamlined analysis of the binding proteins (Fig. 1). We found that including a hydrophilic spacer group as a part of the nanoprobe design is critical in presenting dense arrays of hydrophobic small molecule ligands and photoreactive groups on the AuNP surface while maintaining them water dispersible. The new photoaffinity nanoprobes allowed facile capture and detection of small molecule binding proteins. The ease of modular functionalization on AuNPs is a particular advantage in the straightforward optimization of the probe design, which is often a laborious and time-consuming step in the photoaffinity labeling studies.

To explore the utility of photoaffinity nanoprobes, we designed probes displaying an hCAII inhibitor sulfamoylbenzoic acid derivative (SBz) as a model system and one of the three major photoreactive groups, diazirine (DA), arylazide (AA), or benzophenone (BP) (Scheme 1a and b).[2,9] Based on our previous work,[6] lipoic acid was employed as a AuNP functionalization tail moiety to form stable bivalent S-Au bonds. To provide hydrophilic probe surfaces, a polyethylene glycol (PEG) linker was used to unite the lipoic acid moiety and a head group presenting a small-molecule protein binding ligand or a photoreactive group (Scheme 1a). AuNPs with an approximately 13 nm diameter were prepared according to a previously reported method.[10] To investigate if AuNP-based probes can be used to detect small molecule-protein interactions by photoaffinity labeling, a photoaffinity

* Corresponding author.
E-mail address: sakuraik@cc.tuat.ac.jp (K. Sakurai).

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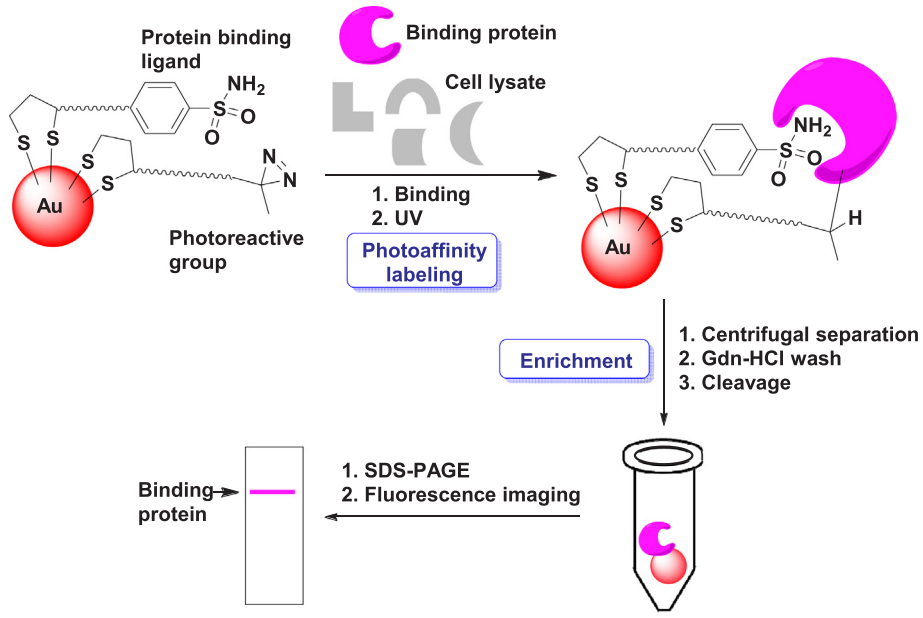

Fig. 1. Photoaffinity labeling scheme to capture and enrich binding proteins using small molecule-conjugated photoaffinity nanoprobes.

nanoprobe **4** was prepared with a mixed presentation of the hCAII ligand (**1**, SBz) and diazirine group (**2a**, DA) at a ratio of 2:1 (Scheme 1b). As a reference, nanoprobe **5** was also prepared, which only presents the hCAII ligand (**1**). Initial attempts to functionalize AuNPs by the ligand exchange method in aqueous buffer[6,11] proved problematic because of severe flocculation resulting in precipitation. As it was likely due to poor miscibility between the hydrophobic ligands and anionic AuNPs, citrate-coated AuNPs were transferred from the citrate solution to DMF by centrifugation to remove the aqueous supernatant solution and the ligand exchange reaction was performed in DMF. The PEG-lipoic acid based building block presenting SBz (**1**) and a photoreactive group **2a** mixed at a desired ratio were assembled on AuNPs by treating with citrate-stabilized AuNPs to give nanoprobe **4**–**5**. It was found that the functionalized AuNPs tend to stick to microcentrifuge tubes when they are transferred to an aqueous buffer solution. We thought this was due to the hydrophobic surface formed by densely functionalized hydrophobic ligands on AuNPs. We therefore decided to incorporate a hydrophilic spacer bearing a hydroxyl-terminated PEG linker (**3**) into the new design of nanoprobes to confer colloidal stability (Scheme 1b).[12] Control nanoprobes presenting the hCAII ligand (SBz, **1**) and a spacer group **3** were initially prepared with two different molar ratios (**6**, SBz: spacer = 1:1 or **7**, SBz: spacer = 1:2) to evaluate the effect of spacer density to their dispersibility in water. Both nanoprobes were found to be water dispersible as judged by UV–VIS analysis and no precipitates were observed. We then prepared photoaffinity nanoprobes with different composition of SBz, a photoreactive group and a spacer (**8**–**11**, Scheme 2). Similar to probe **6**–**7**, nanoprobes **8**–**11** showed no apparent precipitation or color changes, showing the desired water dispersibility conferred by the hydrophilic PEG spacer.

The functionalization of AuNPs **4**–**11** was characterized by UV–VIS, SDS-PAGE and MALDI-TOF MS analysis (Figs. S1 and S2).[6,13] A slight red-shift to 527 nm were observed for the absorbance maxima in the UV–VIS spectra of nanoprobes, indicating an increase in the particle sizes by functionalization.[13] SDS-PAGE analysis showed that nanoprobes were on average uniformly functionalized as compared to unfunctionalized AuNPs, which do not electrophorese readily under the present experimental condition (Fig. S1). MALDI-TOF MS analysis demonstrated the desired ligands were attached on the AuNP scaffold.

We next evaluated the photocrosslinking efficiency toward hCAII by different design of nanoprobes **4**–**5**, **8**–**9**. Based on our previously established one-pot method for photoaffinity labeling reactions and enrichment (Fig. 1),[6] photoaffinity nanoprobes were incubated with proteins to bind at 4 °C for 1 h then were subjected to photoirradiation condition (λ = 365 nm) at 0 °C for varied duration of time. After the photoaffinity labeling step, the unreacted proteins were removed by centrifugation and by repeated washing with 3 M guanidinium hydrochloride (Gdn-HCl) buffer, which is a strong protein denaturing agent. Only the photocrosslinked proteins having covalent bonds could be enriched on AuNPs, which were then cleaved by 2-mercaptoethanol at the S-Au bond to be eluted from AuNPs. The eluted proteins were analyzed by SDS-PAGE and fluorescence imaging. Among the three diazirine nanoprobes **4**, **8**–**9**, nanoprobe **8** with a 1:1:1 ratio of SBz, DA and a spacer group displayed the highest crosslinking efficiency (Fig. 2a, lane 5, Fig. S3, lane 7). These data showed that the spacer density affected the outcomes of photoaffinity labeling. While a judicious density of the spacer group is necessary to provide water dispersive AuNPs, sufficiently high local concentration of SBz and the photoreactive group on AuNPs is apparently important to promote an efficient photocrosslinking reaction.

To compare the photocrosslinking efficiency and selectivity of different photoreactive groups (DA, AA, BP), we next employed probes **8**, **10, 11** for photoaffinity labeling reactions with hCAII in HeLa cell lysate. The ligand-dependent reactivity of nanoprobes was assessed by performing a photoaffinity labeling reaction in the presence of excess concentration of sulfamoylbenzoic acid as a competitor (10 μM), which is a known hCAII inhibitor. All three nanoprobes crosslinked hCAII with similar efficiency (Fig. 2b, lane 3, 6, 9), although significant levels of nonspecific bands were observed under all conditions with diazirine probe **8** showing the highest level of background reactivity (Fig. S4, lane 9). The results from the competitive photoaffinity labeling conditions showed different outcomes for the three photoreactive groups in terms of selectivity of the photoaffinity labeling reaction. As verified by the absence of the hCAII band under the competitive photoaffinity labeling condition in the presence of a competitor, arylazide (**10**) and benzophenone nanoprobes (**11**) enabled ligand-dependent crosslinking (Fig. 2b, lane 6, 7 and 3, 4). On the other hand, the hCAII bands were detected by the diazirine nanoprobe **8** under both reaction conditions with or without a competitor. It suggested that photocrosslinking of hCAII by diazirine nanoprobe **8** was not solely dependent on ligand binding and therefore nonselective (Fig. 2b, lane 9, and 10). These results are in contrast to our previous findings that when the reactivity of the three major photoreactive groups (DA, AA, BP) were compared in the context of conventional photoaffinity probes, diazirine group was