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COMMUNICATION

Protein assemblies by site-specific avidin–biotin interactions†

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Exploiting self-assembly systems with biological building blocks is of significant interest in the fabrication of advanced biomaterials. We assessed the potential use of site-specific ligand labeling of protein building blocks in designing functional protein self-assemblies by combining site-specifically biotinylated bacterial alkaline phosphatase (as a bidentate or tetradentate ligand unit) and streptavidin (as a tetrameric receptor).

In nature, the self-assembly of proteins plays an essential role in the formation of higher order structures, such as virus capsids, microtubes and amyloids. In the fields of biotechnology and nanotechnology, self-assembly processes found in living systems have been exploited to design and fabricate advanced materials.[1–3] Although the use of proteins as building blocks has been challenging, the design of novel self-assembly strategies using these macromolecules has been realized.[4–10] For example, Yeates and co-workers reported a general strategy for designing protein self-assemblies with AB-type fusion proteins, in which proteins A and B form self-assembling oligomers,[11] whereas Cornelissen *et al.* demonstrated the formation of self-assembly architectures with protein–synthetic polymer hybrid amphiphiles.[12] As shown in the latter case, the assembly of not only simple (structural) proteins but functional building blocks such as enzymes and antibodies is of particular importance, because protein supramolecular complexes (PSCs) are shaped with functional attributes.[13–14] Examples include enzyme/protein immobilization with a self-assembling unit[15] and synthetic protein scaffolds for generating efficient cascade reactions by localizing the concentration of enzymes.[16] Consequently, the rational design of functional PSCs has potential value in many fields.

Although excellent examples of designed protein assemblies were demonstrated,[17] forming PSCs without impairing function is generally difficult because protein function relies solely on the tertiary structure. The first issue in the design of PSCs is the selection of a suitable driving force to facilitate the assembly process. Controlling noncovalent (and intrinsically nonspecific) interactions between protein units such as hydrogen bonding, van der Waals interactions and π–π stacking represents a challenge in the design of PSCs. Conversely, specific interactions between certain ligands and receptors have been used as important tools in the *de novo* design of PSCs due to the applicability and the ease of adding multiplicity. In this research, a strong and specific molecular interaction between streptavidin (SA), a receptor protein, and biotin, a small molecular ligand, was selected as a driving force for protein assemblies. The avidin–biotin interaction is recognized as a facile molecular tool in facilitating the assembly or immobilization of proteins.[18] SA forms a tetramer in solution and possesses one biotin binding site per monomeric unit. Biotinylated proteins will thus undergo spontaneous formation of PSCs by simply mixing with SA.

In terms of protein labeling with ligands, chemical modification generally provides the simplest approach. Chemically reactive amino acids on the surface of proteins can be modified with a ligand; however, such modifications often impair the function of the protein because controlling the sites and the degree of labeling are difficult. This leads to (random) chemical modification unsuitable for controllable protein self-assembly. Maintaining solubility and avoiding unfolding (and steric hindrance) caused by molecular proximity represents other crucial issues that need to be considered in the design of PSCs. For these reasons we investigated the potential of enzymatic peptide tag-specific modification by a protein cross-linking enzyme, microbial transglutaminase (MTG), in the preparation of PSCs. MTG catalyzes the acyl-transfer between the side chains of glutamine and lysine or amino groups, and is a valuable tool for post-translational protein labeling. Ligand molecules are easily cross-linked to MTG-recognizable Gln- or Lys-containing peptide tags genetically incorporated into the N- and/or C-termini of target proteins. Additionally, a highly soluble, flexible linker can be introduced, which leads to an increase in the solubility and reduces protein density and unfolding.[19]

In this communication, we report on the effects of the number and the location of a ligand on a protein unit on the formation of PSCs based on the SA–biotin interaction (Fig. 1). Bacterial alkaline phosphatase (AP), a symmetric protein dimer, was selected as a model. The MTG-reactive Gln-containing peptide tag (MLAQGS, abbreviated as Q-tag) was genetically introduced at the N- and/or C-terminus of AP (Table 1).[20] The purity of the recombinant APs were verified by SDS-PAGE analysis (Fig. S1, ESI†).

First, the effect of biotinylation on enzymatic activities of AP was analyzed using *p*-nitrophenylphosphate as a substrate

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Table 1 Recombinant APs prepared in this study

Enzyme	Amino acid sequence of the N- and C-terminal regions of the recombinant APs[a]		
wild-type AP	TPEMP	- - - - -	ALGLK
NQ-AP	MLAQGSTPEMP	- - - - -	ALGLKLEHHHHHH
NQ-AP-CQ	MLAQGSTPEMP	- - - - -	ALGLKLEHHHHHHMLAQGS

[a] Full sequence of wild-type AP is shown in the ESI.

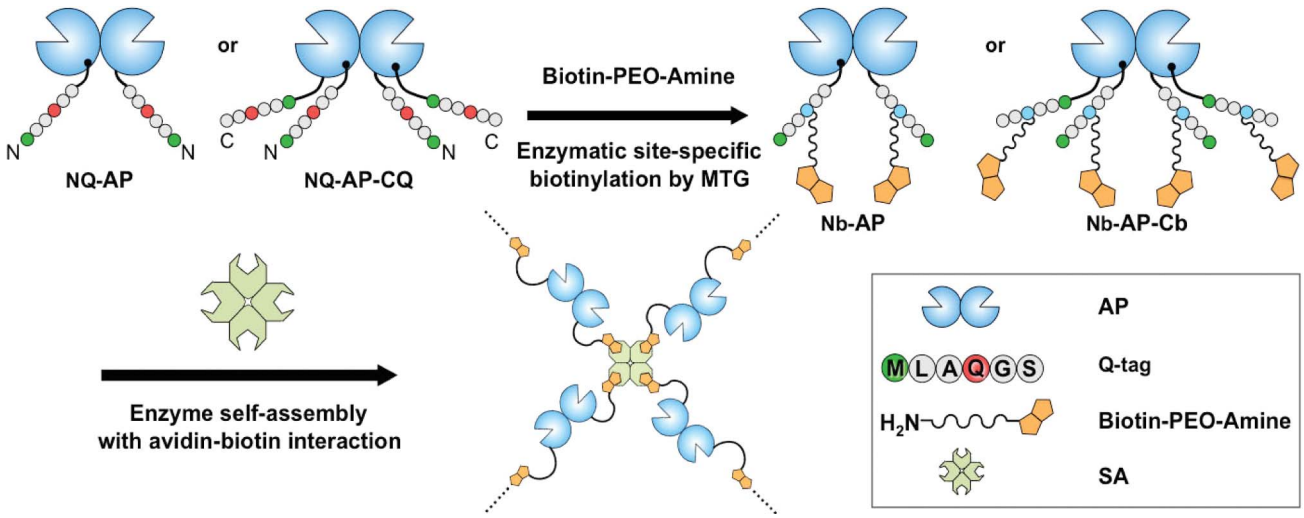

Fig. 1 Scheme for the protein supramolecular complex formation between AP and SA using site-specific avidin–biotin interactions.

and following the increase in the absorbance at 410 nm due to *p*-nitrophenol production. The degree of biotinylation was determined by calculating the molar ratio of biotin incorporated into a protein using the 4′-hydroxyazobenzene-2-carboxylic acid-avidin method.[21] When APs were biotinylated with different concentrations of a chemical modifier (biotin-AC5-OSu, Dojindo Laboratories, Kumamoto, Japan) with 1, 10 and 100 equivalents of AP, biotinylated APs (abbreviated as b-APs) showed 92, 84, 65% of the initial activity, respectively, suggesting up to 35% of the original activity was lost. The degrees of biotinylation were 0.82, 3.45, 7.65 per monomeric unit of AP, indicating that the gradual inactivation of b-APs was caused by the higher degree of biotinylation. By contrast, when site-specific biotinylation was conducted with MTG and 10 (for NQ-AP) or 20 (for NQ-AP-CQ) equivalents of a biotin derivative with a primary amine ((+)-biotinyl-3,6,9,trioxaundecanediamine, biotin-PEO-Amine, Molecular Biosciences, Inc.), the reduction in the catalytic activities of both b-APs (Nb-AP and Nb-AP-Cb) was within 1.2%. The degree of Nb-AP and Nb-AP-Cb was 0.92 ± 0.07 and 1.85 ± 0.11, respectively, thus quantitative protein labeling was achieved.

Formation of PSCs was first examined by the addition of SA to the solution of chemically labeled b-APs at different molar ratios of the monomeric units of SA (mSA) and b-AP (mAP). The b-AP with the lowest degree of biotinylation forms negligible amounts of PSCs (Fig. S2-A, ESI†). In contrast, the other b-APs with higher degrees of biotinylation form aggregates and finally precipitated as the molar ratio of the mSA to the mAP in solution increased (Fig. S2-B and C, ESI†). This is presumably due to the introduction of higher degrees of random modification.

The site-specifically monobiotinylated bidentate AP (Nb-AP) showed a different behavior. When the samples were prepared under conditions where the molar ratios of mSA to mAP were 1/4, 1/2 and 1, PSCs formation was observed (Fig. 2A). Analysis with size-exclusion chromatography showed that the fraction of bAP gradually reduced as the SA molar ratio increased, and a new intense peak at the elution volume of 12 mL was observed. The apparent molecular weight of the PSCs at this elution volume, which was calculated by a standard curve (Fig. S3, ESI†), was *ca.* 268 kDa, which corresponds closely to the theoretical molecular weight of a $(Nb\text{-}AP)_2(SA)$ complex (249.6 kDa; a schematic model is shown in the inset 2 of Fig. 2C). We anticipated that this is caused by intramolecular avidin–biotin interactions prior to the intermolecular interaction upon the binding of one biotin moiety of Nb-AP to SA because the two N-terminals of an AP dimer would be facing the same direction (Fig. S4 and S5, ESI†). Gaussian peak deconvolution of the SEC traces of PSCs (Fig. S6, ESI†) confirmed that a plausible $(Nb\text{-}AP)_2(SA)$ complex is predominantly formed under all the conditions (Table 2). New

Table 2 Fractional area percentage of the PSCs with Nb-AP calculated by Gaussian peak deconvolution of the SEC traces of PSCs. The ratio of biotin binding sites of SA to biotin groups of AP is 1/4 (b), 1/2 (c), and 1 (d). The fraction numbers are depicted in Fig. 2

	1	2	3	AP	SA
(b)	—[a]	39.1	—[a]	60.9	—[a]
(c)	10.2	59.1	—[a]	30.5	—[a]
(d)	23.0	55.4	9.8	8.7	3.1

[a] The assignment of the corresponding peak failed to fit the experimental data in the Gaussian peak deconvolution.

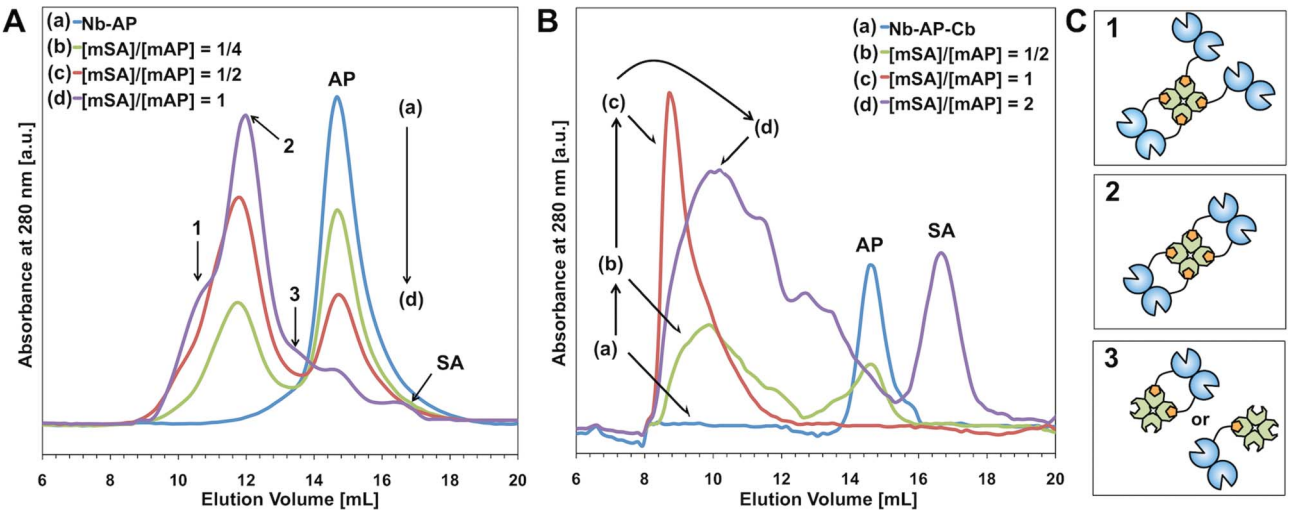

Fig. 2 Formation of PSCs with Nb-AP (A) and Nb-AP-Cb (B) at different molar ratios of monomeric SA and AP characterised by size-exclusion chromatography. Blue curves: biotinylated APs; green, red, and purple curves: the ratio of biotin binding-sites of SA to biotin groups of AP is 1/4, 1/2, and 1. (C) Possible structures of PSCs that correspond to the numbered fraction in A.

shoulder peaks in the SEC trace (1 and 3 in Fig. 2A) are likely to be byproducts generated with the residual monodentate AP (the inset 1 and 3 of Fig. 2C).

In the case of Nb-AP-Cb with tetradentate ligands, the molar ratio of mSA to mAP was adjusted to 1/2, 1 and 2. An equimolar amount of SA (tetramer) and b-AP (dimer) was present in the final ratio. The formation of PSCs ~750 kDa in size (at a peak elution volume of 9.9 mL) was evident when [mSA]/[mAP] = 1/2 ((b) in Fig. 2B). When equimolar amounts of mSA and mAP were used, (*i.e.*, [total biotin binding-site]/[total biotin moiety] = 1/2), large PSCs (> about 1.3 MDa) were excluded in the void volume ((c) in Fig. 2B), implying that all the SA and b-AP molecules participated in the formation of PSCs (Fig. S6-D, ESI†). Further addition of SA resulted in a shift to smaller PSCs with a residual SA fraction ((d) in Fig. 2B). The change in the chromatograms indicates the existence of different types of PSCs at each step. Note that at [mSA]/[mAP] = 1, relatively large PSCs formed even in the presence of excess free biotin ligands. At [mSA]/[mAP] = 2, a number of new peaks were observed (Table S2 and Fig. S6-E, ESI†), implying the presence of complex equilibria between the protein components. The residual SA fraction suggests that some biotin groups of PSCs were inaccessible and uncomplexed with SA. Additionally, comparing the results of Nb-AP-Cb with those of Nb-AP, increasing the interaction points per monomeric protein unit promoted intermolecular interactions, making it possible to form high-molecular-weight PSCs.

Under the condition that almost all SA and b-AP were involved in the formation of PSCs ([mSA]/[mAP] = 1 for both Nb-AP and Nb-AP-Cb), the resulting complexes were further characterized. Dynamic light scattering showed the formation of PSCs with an average diameter of 16.0 and 31.9 nm for Nb-AP and Nb-AP-Cb, respectively (Fig. 3 and Table S3, ESI†). This is in good agreement with the chromatographic analysis showing the enlargement of PSCs by increasing the ligand-receptor interaction points. Although the spatial location of the ligand introduction sites on symmetric protein scaffolds should be considered for further growth of PSCs, enzymatic site-specific and stoichiometric ligand labeling will aid in the design and fabrication of functional PSCs.

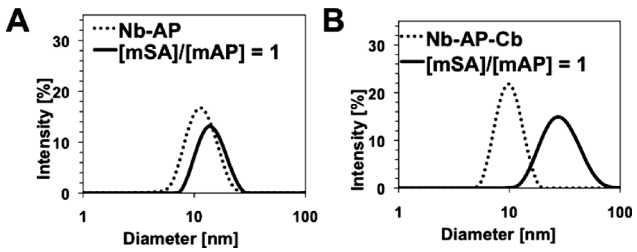

Fig. 3 Dynamic light scattering data of Nb-AP (A) and Nb-AP-Cb (B). Black dotted curves: enzymatic biotinylated AP; black curves: PSCs prepared at an equimolar amount of the monomeric protein unit ([mSA]/[mAP] = 1).

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