

Protein assemblies by site-specific avidin-biotin interactions

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Experimental

Materials and Instruments

Biotin-AC5-OSu was purchased from Dojindo Laboratories (Kumamoto, Japan). Biotin-PEO4-Amine (Molecular Biosciences, Inc.) was purchased from Takara Bio Inc. (Otsu, Japan). Streptavidin was purchased from Promega KK (Tokyo, Japan). All other reagents were purchased and used as received. Prepacked columns including Ni-NTA columns (His-Trap HP column 5 mL), an anion-exchange column (HiTrap DEAE FF 5 mL), a size-exclusion chromatography column (Superdex 200 10/ 300 GL) and full-range rainbow molecular weight markers were purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). All chromatography experiments were conducted on a BioLogic DuoFlow chromatography system (Bio-Rad Laboratories, Inc. Hercules, CA).

Preparation of specific peptide-fused recombinant APs

NQ-AP was prepared as NQ6-AP as previously described. Specific peptide tags, MLAQGS (abbreviated as Q-tag) was genetically attached to the C-terminus of NQ-AP. The resultant C-terminal Q-tagged AP was designated as NQ-AP-CQ (Table 1). The plasmid vector pET22b(+) was employed for the final vector construction. All the recombinant APs were expressed in *E. coli* strain BL21(DE3) (EMD Biosciences, San Diego, CA, USA) and were purified with a hexahistidine tag (His-tag) attached to the C-terminus of a recombinant AP using a Ni-NTA column (Table 1). Fractions containing APs were collected and desalted into 20 mM Tris-HCl (pH 8.0) on PD 10 Sepharose columns. The AP solutions were applied to DEAE columns and the fractions that exhibited AP enzymatic activity were collected and then concentrated to a volume of 0.9 mL using an ultrafiltration membrane (MWCO 30 kDa). The AP solutions were next purified using a SEC column equilibrated with the SEC mobile phase (50 mM Tris- HCl, 150 mM NaCl, pH 7.5). All of the AP solutions were applied to the SEC column using a 1 mL sample loop and size exclusion chromatography was carried out at flow rate of 0.5 mL/min. Relative peak areas were quantitated by integrating the absorbance at 280 nm. Finally fractions containing APs were collected. To identify purifications, the samples of NQ-AP, NQ-AP-CQ and SA (0.5 mg/mL) were mixed with the standard sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (12 vol% 2-mercaptoethanol, 4 wt% SDS, 20 vol% glycerol in 100 mM Tris-HCl, pH 6.8). After the electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB) R-250 (Quick-CBB, Wako Laboratory Chemicals, Osaka, Japan).

The full sequence of the wild-type AP is as follows:

TPEMPVLENRAAQGDITAPGGARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNY
AEGAGGFFKGIDALPLTGQYTHYALNKKTKGKPDYVTDSAASATAWSTGVKTYNGALGVDIHE
KDHPTILEMAKAAGLATGNVSTAELQDATPAALVAHVTSRKCYPGSAATSEKCPGNALEKGGK
GSITEQLLNARADVTLGGGAKTFAETATAGEWQGKTLREQAQARGYQLVSDAASLNSVTEAN
QQKPLLGLFADGNMPVRWLGPKATYHGNIDKPAVTCCTPNPQRNDSVPTLAQMTDKAIELLSK
NEKGFLLQVEGASIDKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTADHAHAS
QIVAPDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQRLRIAAYGPHAANVVGLTDQT
DLFYTMKAALGLK

Chemical biotinylation

NHS-PEO4-biotin (10, 100 and 1000 μ M) was added to an aqueous solution of a NQ-AP sample (10 μ M in 150 mM boric acid, pH 9) at 4 °C. After 3 h, chemically biotinylated AP was prepared by passing through a Ni-NTA column.

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Enzymatic biotinylation (NQ-AP)

Biotin-PEO4-Amine (200 μ M) and MTG (1U) were added to an aqueous solution of NQ-AP (10 μ M in 25 mM Tris-HCl, pH 7.4) at 4 °C. After 3 h, enzymatic site-specific biotinylated AP (Nb-AP) was obtained by passing through a Ni-NTA column.

Enzymatic biotinylation (NQ-AP-CQ)

Biotin-PEO4-Amine (400 μ M) and MTG (1U) were added to an aqueous solution of NQ-AP-CQ (10 μ M in 25 mM Tris-HCl, pH 7.4) at 4 °C. After 3 h, enzymatic site-specific biotinylated AP (Nb-AP-Cb) was obtained by passing through a Ni-NTA column.

Calculation of biotinylation degree

2-Hydroxyazobenzen-4'-carboxylic acid (HABA) reagent (14.0 mg HABA in 500 μ L DMSO) was prepared and 200 μ L of this reagent was added to 10 mg avidin, and the volume made up to 20 mL (HABA-Avidin reagent). 900 μ L of the Avidin-HABA Reagent was pipetted into a 1 mL cuvette and the OD at 500 nm measured (Abs_A). Next, 100 μ L of the biotinylated sample was mixed. After 30 min, the OD at 500 nm was measured (Abs_B). The Abs_A and Abs_B values were used in combination with the below equation (Eq. 1) to compare the value and concentration of biotinylated samples; the biotinylation degree was calculated.

$$[\text{Biotin}] (\text{mol/l}) = 10^{-3} \times (0.9 \times Abs_A - Abs_B) / 34 \quad (\text{Eq. 1})$$

Measurement of enzymatic activity

The enzymatic activities of the APs were analyzed by dephosphorylation of *p*-nitrophenylphosphate (*p*-NPP). 10 μ L of the AP samples (2 μ M) were pipetted into 990 μ L of aqueous solution (1 mM *p*-NPP in 1 M Tris-HCl, pH 8.0) in a 1 mL quartz cell for UV-vis spectroscopic measurements. In 1 M Tris-HCl buffer (pH 8.0), hydrolysis of the substrate (1 mM) was followed by an increase in the absorbance at 410 nm (derived from *p*-nitrophenol) using a UV-vis spectrogram leader. The OD 410 nm was recorded for 3 min at 25 °C and the values were used to calculate the mOD_{410}/min and the relative activity. Enzymatic activity before biotinylation was assumed to be 100%.

Self-assembly of biotinylated APs

An aqueous SA solution (150 μ M in TBS buffer; 137 mM NaCl, 2.68 mM KCl, 25 mM *tris*(hydroxymethyl)aminomethane, pH 7.4, filtered through a 0.02 μ m filter) was added gradually to 500 μ L of biotinylated APs in TBS buffer (2 μ M) under vigorous stirring. The final volume of the SA solution added was 13.3 μ L. All the samples were analyzed after incubation at 25 °C for 30 min.

Dynamic light scattering (DLS) analysis

Chemically biotinylated AP (2 μ M) was mixed with 0.25, 0.5, 1 equiv. of SA and prepared to a final volume of 500 μ L of TBS buffer.

Nb-AP (2 μ M) or Nb-AP-Cb (2 μ M) was mixed with 1 equiv. of SA (based on the monomeric unit of each protein) and the solution was made up to a final volume of 500 μ L using TBS buffer. In the assembled samples, the ratios of biotin binding-sites of SA to biotin groups of AP were 1 (for Nb-AP) and 1/2 (for Nb-AP-Cb). After incubation for 30 min, the particle sizes of assembled APs were evaluated with a Zetasizer Nano ZS (Malvern, Worcestershire, UK) using the incident He-Ne laser (633nm). The cuvette holder was controlled at 25 °C. The detector position was set at 173°. The autocorrelation function of the scattered light intensity as a function of time was measured several

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times and their averaged data were analyzed by the cumulant method (ISO 13321). Assuming a homogeneous spherical particle, the hydrodynamic particle diameter (d_H) was calculated by the Stokes-Einstein equation (Eq. 2), where η is solvent viscosity, D is the diffusion coefficient, k_B is Boltzmann's constant and T is the absolute temperature.

$$d_H = (k_B T) / (3\pi\eta D) \quad (\text{Eq. 2})$$

Size exclusion chromatography (SEC)

The assembled samples (Nb-AP and Nb-AP-Cb) were diluted up to 1 mL with TBS buffer. All of the samples were applied to the SEC column using a 1 mL sample loop and size exclusion chromatography was carried out at flow rate of 0.5 mL/min.

The Gaussian peak deconvolution of the SEC traces

Gaussian peak deconvolution of the SEC trace of PSCs was performed on a personal computer using a software of IGOR Pro 6.0 (Oregon, USA). The peak areas of each fraction of PSCs were integrated to calculate the fractional percentage of each PSCs.

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Table S1. Data of the SEC markers. (Components, molecular weights, elution volumes and K_{av} , the value calculated from Eq. 3.)

	Components	MW [Da]	V_E [mL]	K_{av}
1	Ovalbumin	43,000	15.66	0.449050495
2	Conalbumin	75,000	14.73	0.388118812
3	Aldolase	158,000	13.32	0.295049505
4	Ferritin	440,000	10.97	0.139933993
V_0	BlueDextran	200,0000	8.85	-

$$K_{av} = (V_E - V_0)/(V_C - V_0) \quad \dots\dots(\text{Eq. 3})$$

V_C : Column volume, 24 [mL].

Table S2 Fractional area percentages of the PSCs with Nb-AP-Cb, which were calculated from Gaussian peak deconvolution of the SEC trace of PSCs (Fig. 2B). The ratio of biotin binding-sites of SA to biotin groups of AP is 1/2(c), and 1(d). The numbers of PSCs were presented in Fig. S6.

Nb-AP-Cb	4	5	6	7	8	9	AP	SA
(c) ^a	-	-	-	-	-	-	-	-
(d)	8.1	19.0	17.6	21.0	6.3	8.8	4.8	14.4

^aAlmost all PSCs (>99 %) were excluded in the void volume.

Table S3. Particle sizes of Nb-AP, Nb-AP-Cb and PSCs prepared with Nb-AP or Nb-AP-Cb.

Protein	Mean particle diameter [nm]	Polydispersity index	Distribution width [nm]
Nb-AP	11.0 ± 1.1	0.12-0.23	3.36 ± 0.7
PSC with Nb-AP	16.0 ± 2.0	0.13-0.35	4.63 ± 0.9
Nb-AP-Cb	10.0 ± 1.5	0.15-0.28	2.32 ± 0.4
PSC with Nb-AP-Cb	31.9 ± 2.9	0.18-0.42	14.4 ± 3.2

Mean ± SD, n = 5.

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SDS-PAGE analysis of purified proteins

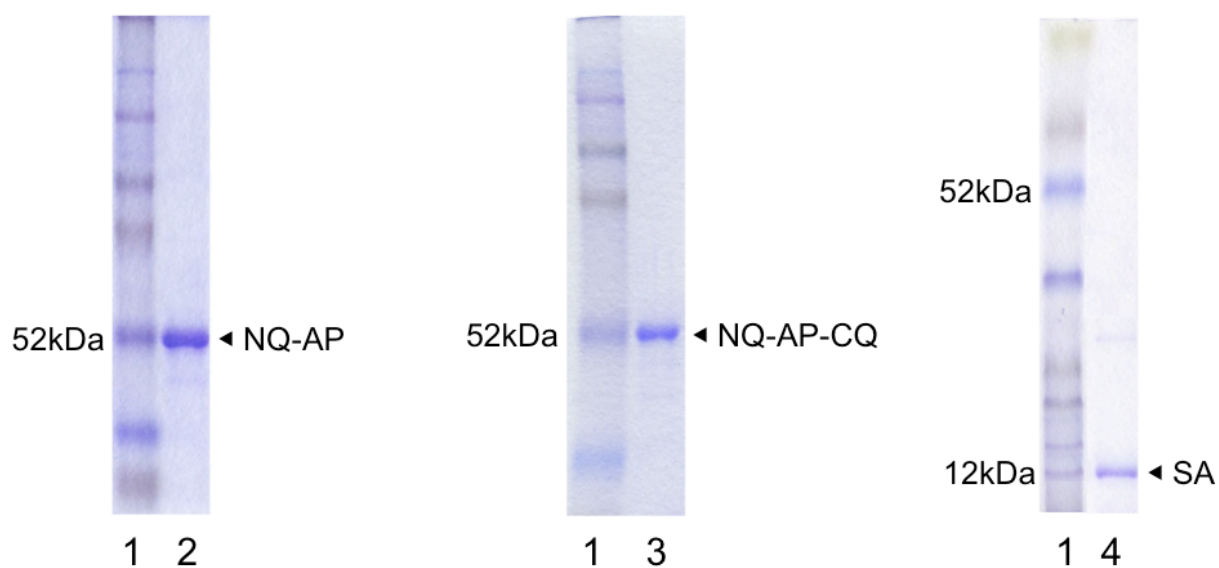


Fig. S1. SDS-PAGE analysis of purified proteins employed in this study. NQ-AP (lane 2), NQ-AP-CQ (lane 3), SA (lane 4) and molecular weight markers (lane 1).

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DLS data of chemically biotinylated APs

Dynamic light scattering data of self-assembled chemically biotinylated AP with SA. The concentration of the biotinylation substrate was 10 (Fig. S-1.), 100 (Fig. S-2) and 1000 μM (Fig. S-3). Blue curves: chemically biotinylated AP; green, red and purple curves represent the ratio of the concentration of SA to AP as 1/4, 1/2 and 1, respectively.

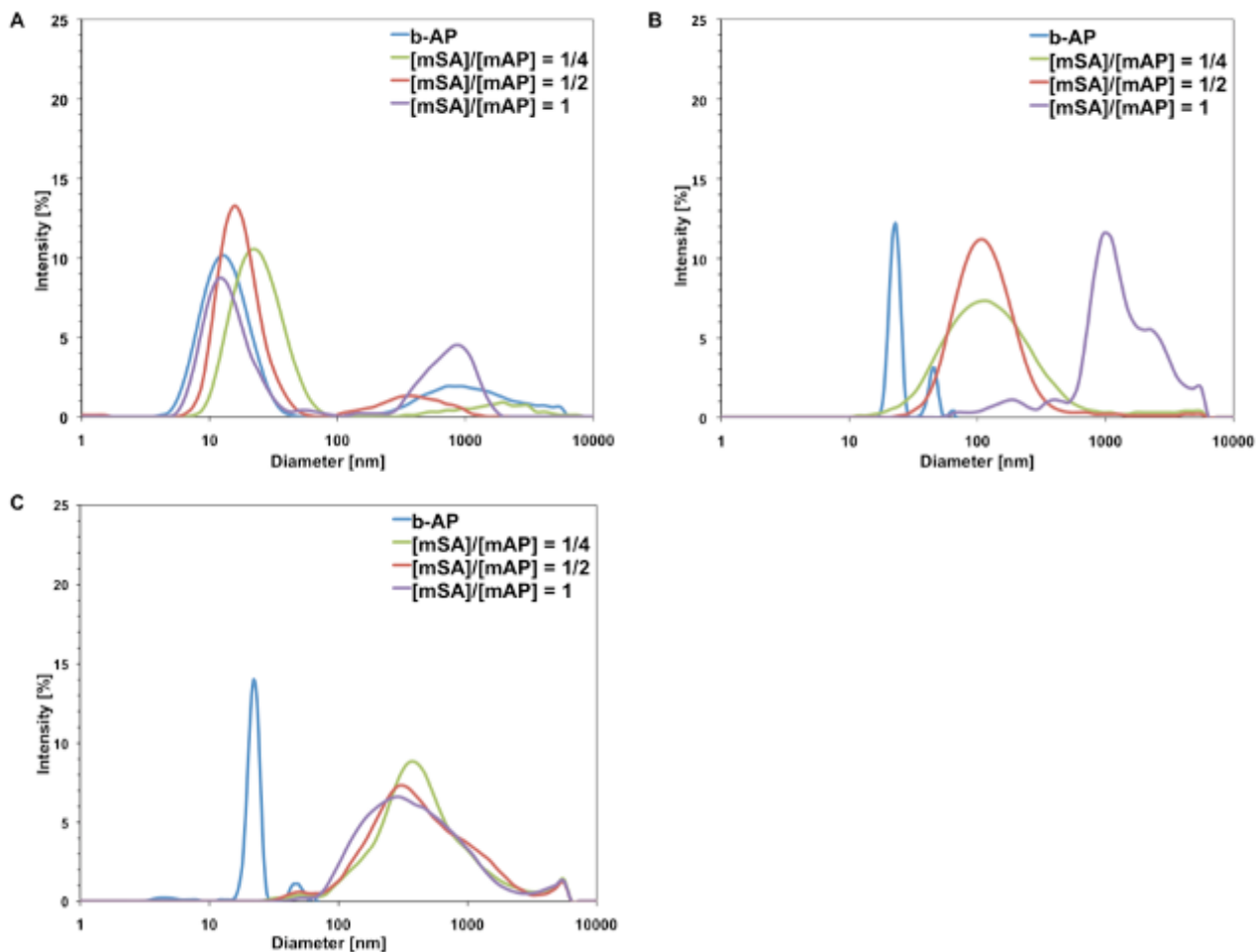


Fig. S2. DLS data of self-assembled chemically modified AP (concentration of the biotinylation substrate was (A) 10, (B) 100 and (C) 1000 μM) with SA.

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SEC chromatogram of the molecular markers and the resulting standard curve

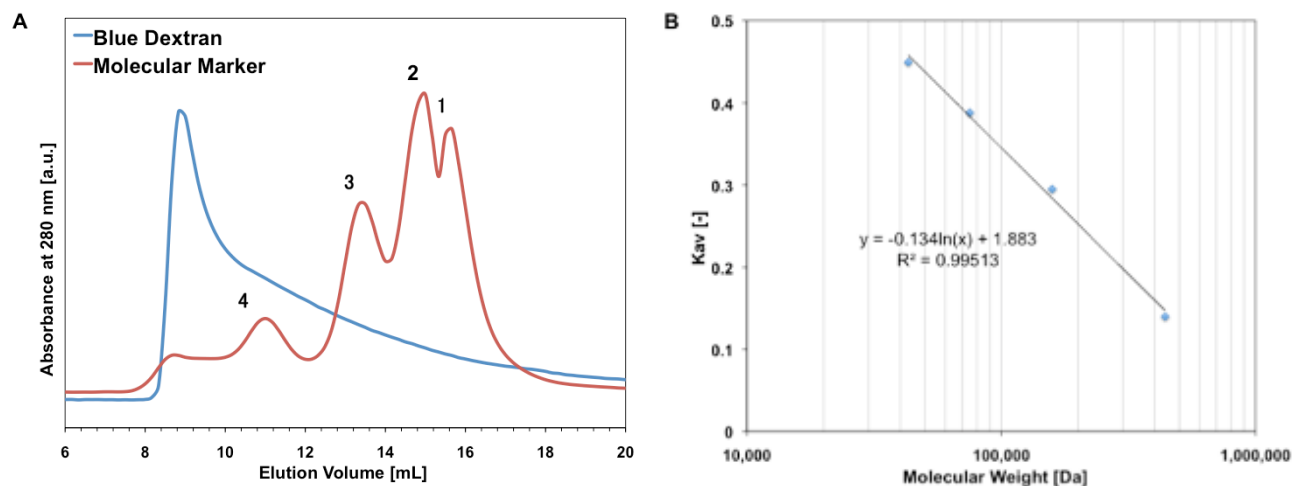


Fig. S3. Calibration of the molecular weight using the calibration results of the SEC column. (A) SEC chromatogram for the molecular weight markers and blue dextran eluting at the void volume. 1: ferritin, 440 kDa; 2: aldolase, 158 kDa; 3: conalbumin, 75 kDa; 4: ovalbumin, 43 kDa (Table S1). (B) The standard curve for the molecular weight was based on the results of the SEC analysis of the molecular weight markers and blue dextran.

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Trihedral figure of the AP crystal structure (1ALK)

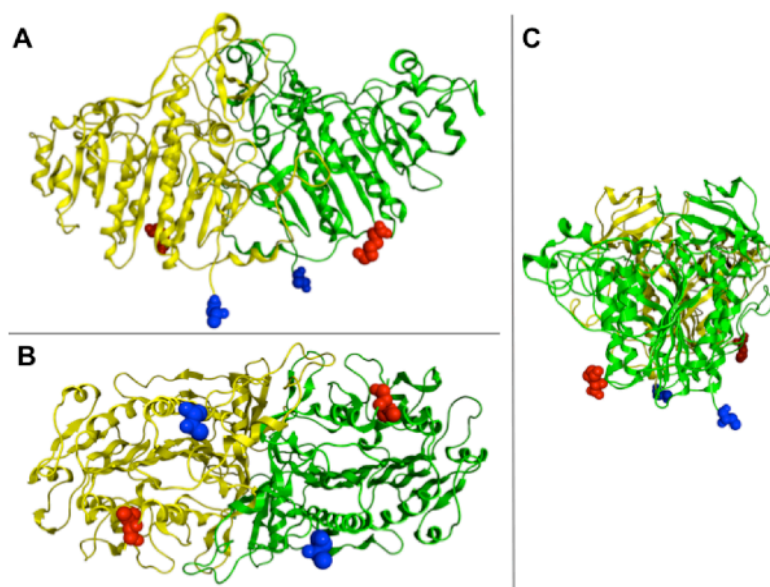


Fig. S4. A trihedral figure of the crystal structure (1ALK). (A) Front, (B) plane, and (C) side views. Molecular image of the wild-type AP with the N- and C- terminal residues highlighted and coloured (blue and red) as CPK models was produced with the Molecular Operating Environment (MOE, v 2009.10) software which was developed by the Chemical Computing Group Inc. (Montreal, Canada).

A front figure of biotinylated APs

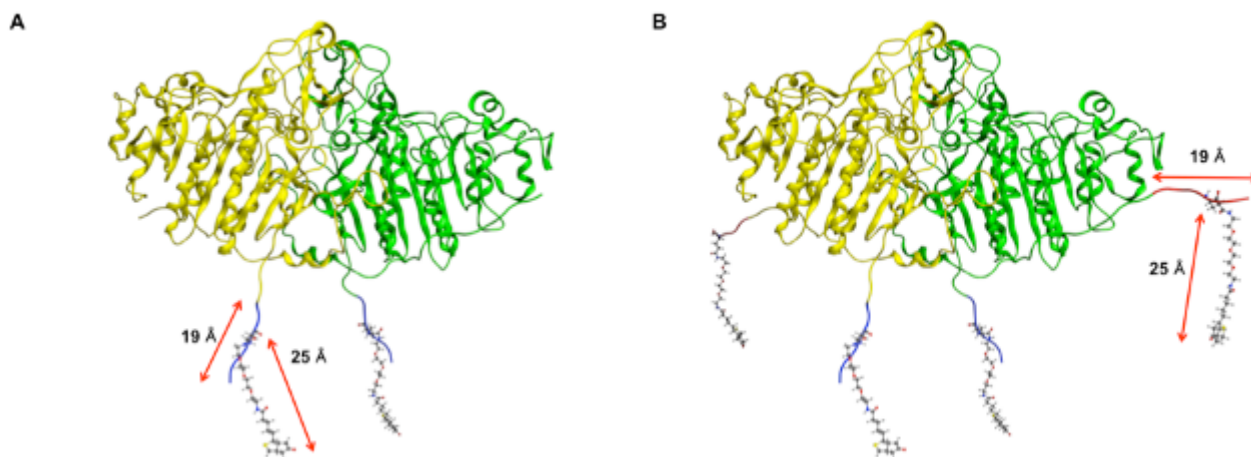


Fig. S5. The front figures of biotinylated APs, (A) Nb-AP, and (B) Nb-AP-Cb. Red double-headed arrows show one line lengths. Molecular image of the wild-type AP with the N- and C- terminal residues of the Q-tags coloured (blue and red) as CPK models was produced with the Molecular Operating Environment (MOE, v 2009.10) software which was developed by the Chemical Computing Group Inc. (Montreal, Canada).

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The Gaussian peak deconvolution of the SEC trace

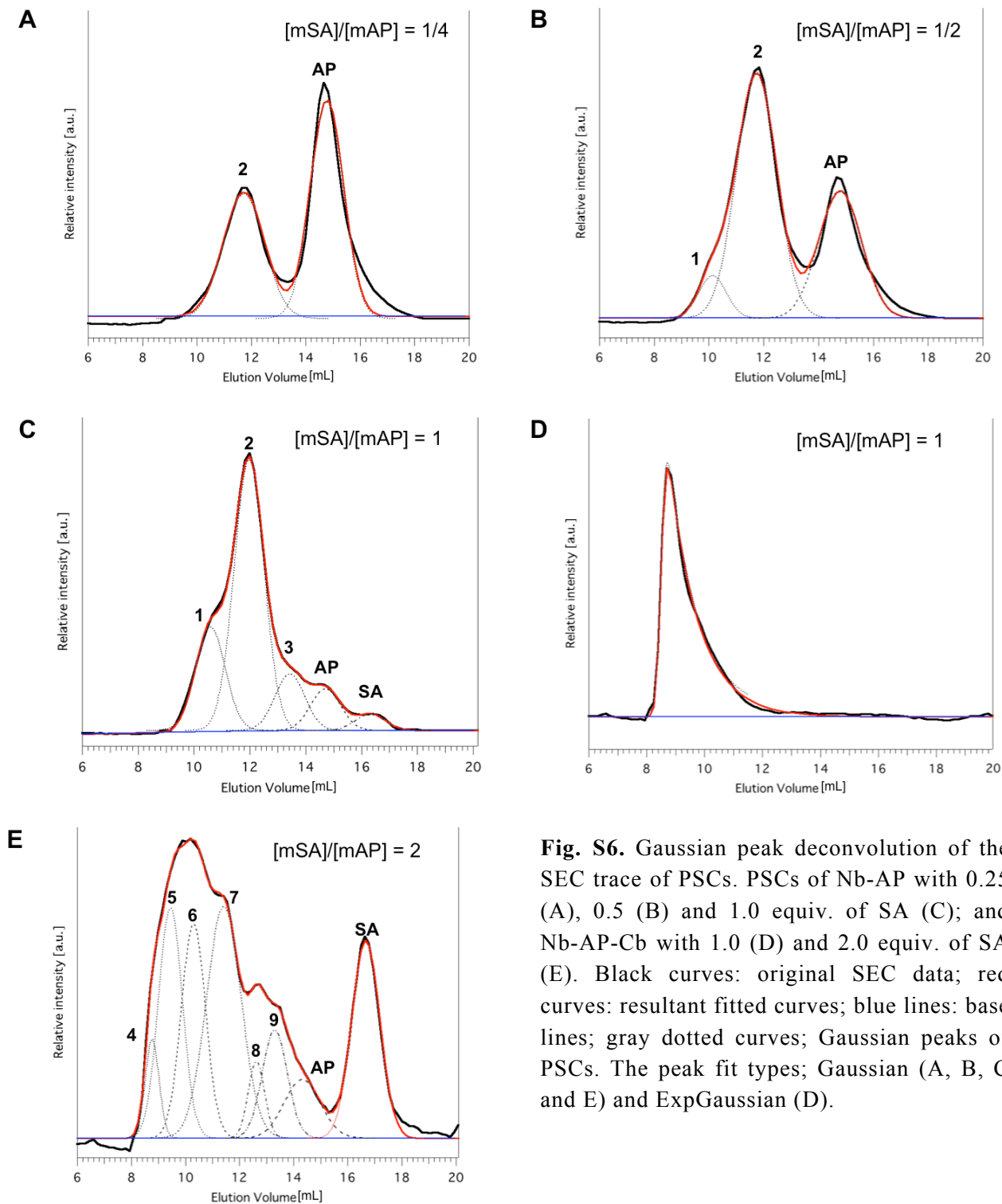


Fig. S6. Gaussian peak deconvolution of the SEC trace of PSCs. PSCs of Nb-AP with 0.25 (A), 0.5 (B) and 1.0 equiv. of SA (C); and Nb-AP-Cb with 1.0 (D) and 2.0 equiv. of SA (E). Black curves: original SEC data; red curves: resultant fitted curves; blue lines: base lines; gray dotted curves: Gaussian peaks of PSCs. The peak fit types; Gaussian (A, B, C and E) and ExpGaussian (D).