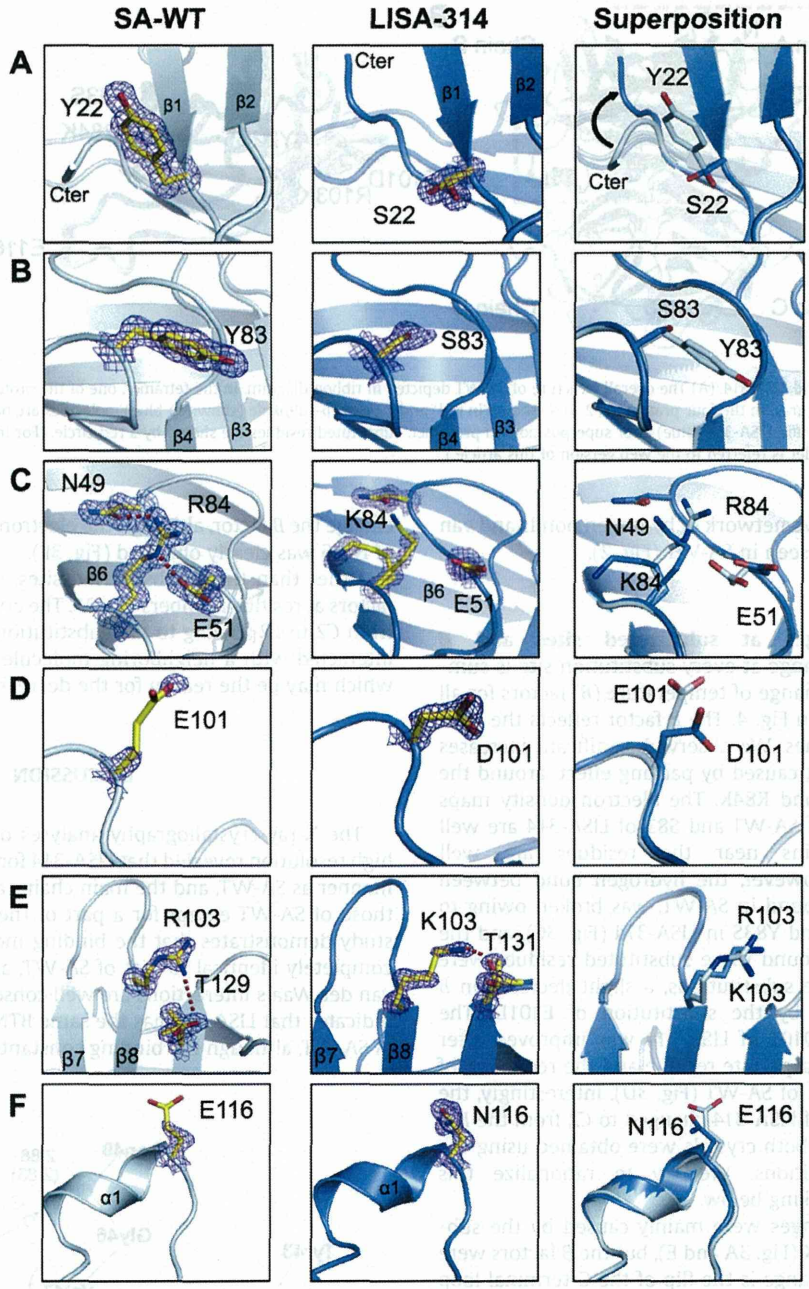

FIG. 3. Structural change of SA induced by six amino-acid substitutions. The electron density maps of SA-WT (cyan) and LISA-314 (blue) are shown in the first and the second lanes, respectively. Superimposed structures between SA-WT and LISA-314 are shown in the third lane. The panels are displayed around (A) Y22S, (B) Y83S, (C) R84K, (D) E101D, (E) R103K, and (F) E116N, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

titration calorimetry (ITC) in previous work (25) exceeded the measurement limit of ITC.

Generally, the higher the *B* factor value, the more flexible the corresponding region in the protein, and vice versa. This attribute has been used to predict linear B-cell epitopes (38). However, we did not observe any large conformational changes corresponding to the large reduction of *B* factors around the substituted sites. Even higher *B* factors were measured because of the substitutions. The effect of substitutions is restricted mainly to the changes of side chains at the substituted sites. From these results, if the lowered immunogenicity of LISA-314 is caused by the conformational changes, we presume that it is not caused by the conformational change of the whole structure, but by the local conformational changes at the substituted sites. In this study, we have proven that LISA-314 has both low immunogenicity and high biotin-binding affinity without a striking change in the whole structure, despite introducing six amino-acid substitutions. Practically, we expect LISA-314 to be most useful in pre-targeting cancer therapy.

Some groups have discussed the problem of competing endogenous BTN in blood and tissues (39,40). Specifically, the endogenous BTN can effectively block the BTN-binding pocket of administrated SA, impairing the efficiency of the pre-targeting method. To solve this problem, the binding site of LISA-314 has to

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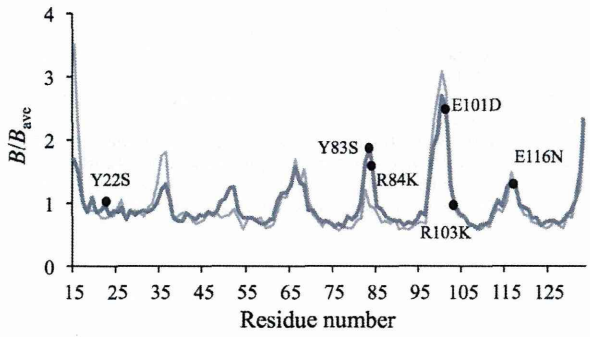

FIG. 4. Comparison of relative temperature (B) factors between SA-WT (thin line) and LISA-314 (thick line). The B factor for Cα atom of each amino acid is standardized by the equation: B factor/B_{ave} (B_{ave}: the average B factor of the whole Cα atoms).

be modified to selectively bind to the non-natural BTN analog without interference of endogenous BTN. Detailed structural information of the present study will be useful for future improvement of LISA-314.

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Note
Running title: A bivalent ligand for a streptavidin mutant

Structure-based design and synthesis of a bivalent iminobiotin analogue showing strong affinity toward a low immunogenic streptavidin mutant

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Abbreviations: SA, streptavidin; BTN, biotin; V212, Variant No. 212; LISA-314, low immunogenic streptavidin mutant No. 314; IMNtail, iminobiotin long tail; IPTG, isopropyl-β-D-thiogalactopyranoside; SPR, surface plasmon resonance; RU, resonance unit

The streptavidin/biotin interaction has been widely used as a useful tool in research fields. For application to a pre-targeting system, we previously developed a streptavidin mutant that binds to an iminobiotin analogue while abolishing affinity for natural biocytin. Here, we design a bivalent iminobiotin analogue that shows 1000-fold higher affinity than before, and determine its crystal structure complexed with the mutant protein.

Key words: streptavidin; iminobiotin; bivalency; X-ray structural analysis; pre-targeting

Streptavidin (SA) is a tetrameric protein that selectively binds biotin (BTN) with an affinity that is amongst the highest displayed for noncovalent bonds between a ligand and protein (K_d = 10^{-14}–10^{-15} M).[1, 2)] For this reason, the SA/BTN system has been put to practical use in molecular biology and biotechnology.[3-6)] This system has also attracted a lot of interest for medical applications, especially as a drug delivery system. One possible use is in a pre-targeting system, conjugated with a monoclonal antibody.[7-15)] In this pre-targeting application, a monoclonal antibody-SA conjugate is first injected into a patient and allowed to localize at

the tumor. The excess reagent is then cleared and the biotinylated radiation carriers are administered. This multistep pre-targeting method can improve the efficiency of the present direct-radiolabeled antibody method.

Despite many trials, its clinical application has not been successful so far for two main reasons. First, the immunogenicity of a bacterium-derived SA precludes its long-term use.[8, 16-18] The second issue is the presence of endogenous BTN species in the serum at sufficient concentrations, which can effectively block the BTN-binding sites of SA before it can bind the exogenous radiolabeled BTN derivative as a drug.[19, 20]

In recent studies described elsewhere, we developed a novel SA mutant, Variant No. 212 (V212), that overcame the two problems outlined above. Briefly, we first developed a low immunogenic streptavidin mutant, LISA-314 by carrying out six amino acid substitutions (Y22S/Y83S/R84K/E101D/R103K/E116N) to residues in wild-type SA (SA-WT) in its immune recognition site[21], and determined its crystal structure (Fig. S1A). We then introduced another three amino acid substitutions (N23D/S27D/S45N) to the binding pocket of LISA-314, and succeeded in creating the mutant V212 (Fig. S1B). This bound to a non-natural biotin analogue, iminobiotin long tail (IMNtail; Fig. S1C), while showing no affinity for an endogenous BTN species, biocytin. However, the binding affinity of V212 for IMNtail was relatively low (K_d= 5.2×10^{-7} M) compared with the original binding affinity of SA/BTN, and further modifications are necessary before it can be used practically in a pre-targeting system.

From the structural information of the V212-IMNtail complex described elsewhere, it is found that each protomer of tetrameric V212 binds one IMNtail molecule. We focused on the distance and orientation between the carboxylates of two IMNtail molecules bound in neighboring subunits. They seemed to be close enough (2.96 Å) to be linked by two or three additional covalent bonds (Fig. S2). In previous reports,[22, 23] a bivalent BTN analogue (two BTN units connected with a linker) was shown to dramatically increase the binding affinity for SA. It is thought that the dual binding of the bivalent analogue induces a striking avidity effect that leads to higher affinity, although there is no structural evidence for this hypothesis.

In the present study, based on this idea, we synthesized another bivalent analogue by connecting two IMNtail molecules (Bis-IMNtail; Fig. 1A). Considering the linear distance (2.96 Å) between two IMNtails, we connected them with a -N-C-C-N- linker (the N-N distance in *trans* configuration is 3.7 Å) rather than a -N-C-N- linker (the N-N distance 2.4 Å), for the linker flexibility. A surface plasmon resonance (SPR) assay showed that the Bis-IMNtail has a K_d value of over 8.3×10^{-10} M toward V212. This is a much higher affinity than that for the monovalent IMNtail (K_d = 5.9×10^{-7} M). Note that this K_d value was over the detection limit of the SPR assay, thus the actual affinity of V212 for Bis-IMNtail will be higher. The SPR sensorgram also showed that the bound Bis-IMNtail did not dissociate in the running buffer (Fig. S3). This property is advantageous for future applications as a drug delivery tool.

We then solved the crystal structure of V212 in complex with Bis-IMNtail at 1.3 Å resolution to investigate the binding mode of Bis-IMNtail (Table S1). This is the first structure of SA in complex with a bivalent ligand. V212 forms a tetramer composed of a dimer of dimers (Fig. 1B), the same as SA structures solved previously. As expected, two Bis-IMNtail molecules bind to the tetramer, one binding to the pocket of two neighboring protomers. The electron density map of Bis-IMNtail was clearly observed (Fig. 1C). The binding mode of Bis-IMNtail is well preserved in that of IMNtail complexed with V212, including the area around the connecting positions of the Bis-IMNtail (Fig. 1D, Fig. S4). In brief, Asp23 and Asp27 form hydrogen bond with the guanidino nitrogen of Bis-IMNtail in 2.9 Å distance, but Tyr43 residues in both complexes are not located in significant hydrogen-bond distance to their ligands (both are more than 3.5 Å). As for the interaction between Asp128 and the guanidino nitogen, it is altered from interaction *via* a water molecule in the IMNtail complex to a direct hydrogen bond in the Bis-IMNtail complex. The binding loop composed of 45–52 residues forms the flexible open conformation, with no interaction with ligand, in the same way as that

of V212 complexed with monovalent IMNtail. The binding loop usually forms a closed conformation in ligand-bound SA-WT.[24] From structural analyses, we cannot find any newly-formed interactions or conformational changes that may contribute to the increased binding affinity of Bis-IMNtail. Thus, we conclude that the main reason for the dramatically increased affinity of Bis-IMNtail for V212 is ligand bivalency and concomitant avidity.

In this report, we succeeded in increasing the binding affinity of IMNtail for V212, by connecting two IMNtail molecules to give bivalent ligand Bis-IMNtail. Bis-IMNtail demonstrated much higher affinity toward V212 than monovalent IMNtail. Our V212/Bis-IMNtail system displays potential for use in a pre-targeting method as a novel drug delivery tool.

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Figure Legends

Fig. 1. Binding mode of Bis-IMNtail in V212

A. Structural formula of Bis-IMNtail. B. Structure of the V212 tetramer (ribbon model) in complex with Bis-IMNtail (stick model). C. The 2*F*o-*F*c electron density map (blue mesh; contoured at 1.0 σ) of Bis-IMNtail bound to V212. D. Superimposition of Bis-IMNtail-bound V212 (red) and IMNtail-bound V212 (yellow) structures.

Fig. 1A

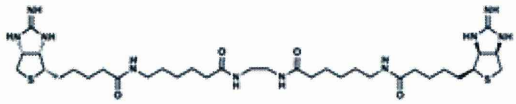


Fig. 1B

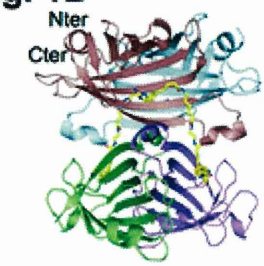


Fig.1C

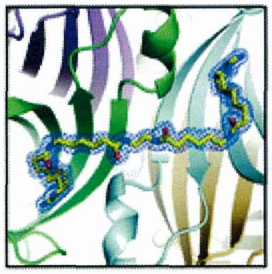
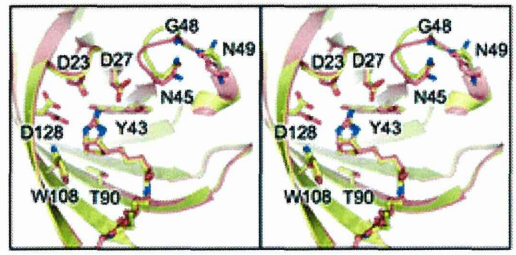


Fig. 1D



Supplementary data

Table S1. Data collection and refinement statistics.

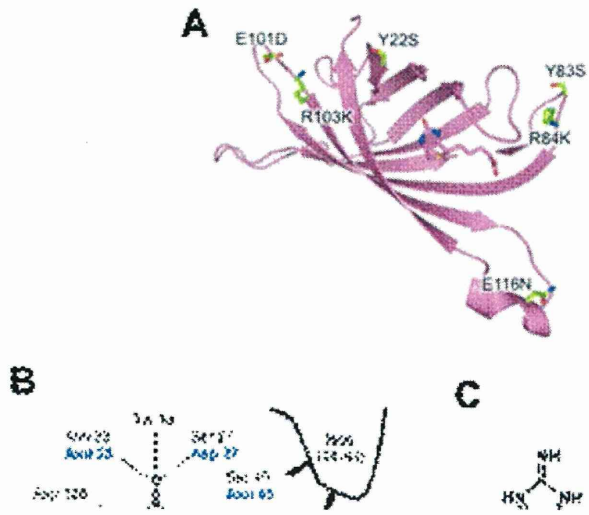
	V212-Bis-IMNtail
Data collection	
Space group	$C222_1$
Unit-cell parameters (Å, °)	a = 77.35, b = 77.38, c = 174.12
Wavelength (Å)	0.90000
Resolution (Å)	50–1.30 (1.35–1.30)[a]
R_{sym} (%)[b]	5.6 (30.4)
Total reflections	1,015,993
Unique reflections	127,145 (12,529)
$I/\sigma(I)$	35.8 (3.9)
Completeness (%)	99.6 (99.6)
Redundancy	8.0 (5.8)
Refinement	
Resolution	1.30
No. of reflections	120,725
R_{work} (%)[c]/R_{free} (%)[d]	14.6/18.8
No. of atoms	
Protein	3,863
Ligand/ion	100
Water	442
B factors	
Protein	22.0
Ligand/ion	23.7
Water	38.5
R.m.s deviations	
Bond length (Å)	0.022
Bond angles (°)	2.23
Ramachandran plot	
Favored (%)	95.48
Allowed (%)	3.85
Outliers (%)	0.68
PDB ID	3X00

[a] Values in parentheses are for the highest-resolution shell.

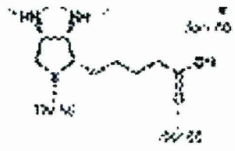
[b] R_{sym} is calculated as $\Sigma_{hkl}\Sigma_i|I_i(\text{hkl}) - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices hkl and $\langle I(hkl)\rangle$ is the average intensity from multiple observations.

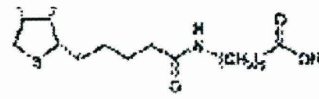
[c] $R_{work} = \Sigma_{hkl}||F_{obs}| - |F_{calc}||/\Sigma_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively.

[d] R_{free} is calculated in the same manner as R_{work} but using only a small set (5%) of randomly chosen intensities that were not used in the refinement of the model.

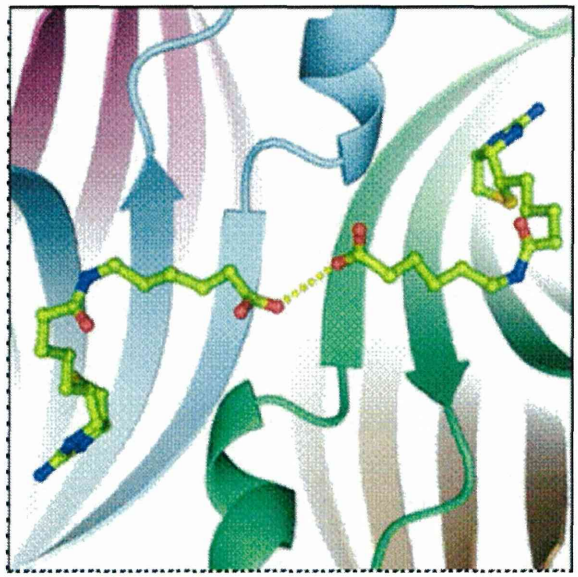


Supplementary Fig. S1. Substituted residues of LISA-314 and V212, and the BTN analogue.
A. The structure of LISA-314 (PDB ID: 3YWQ) is shown as a monomer in the ribbon model. The substituted residues are indicated in stick models.

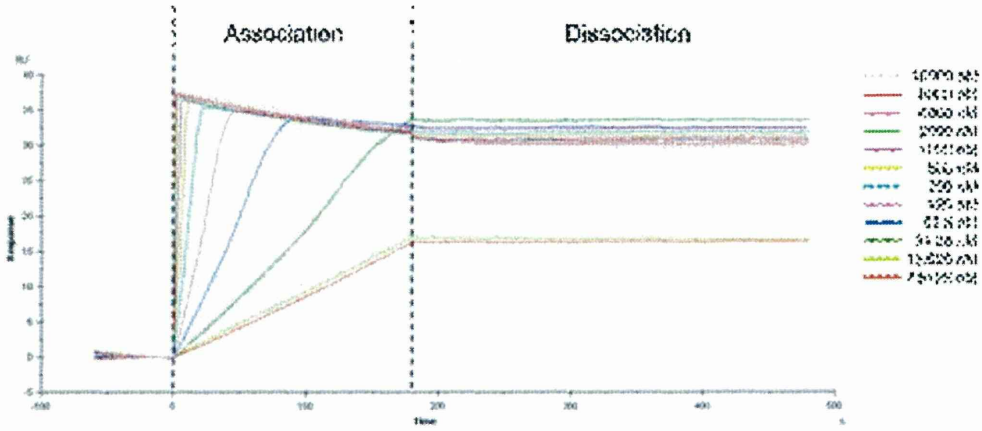




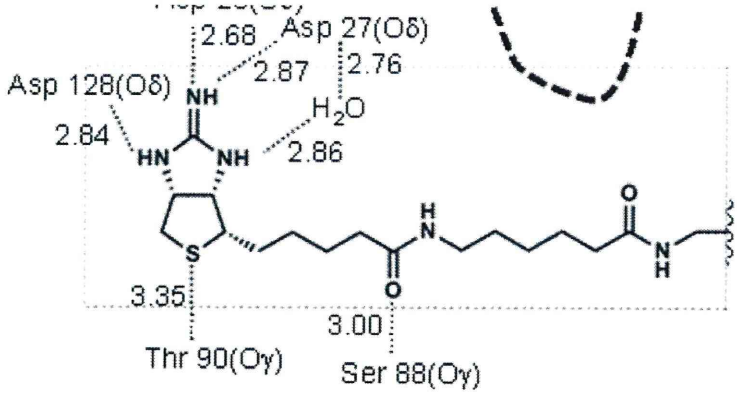
B. Networks of hydrogen bonds between BTN and LISA-314 or V212 are shown by the black dotted lines. Residues of LISA-314 are indicated in black, and three additional substituted residues of V212 are in blue. C. Structural formula of IMNtail, a BTN analogue for V212.



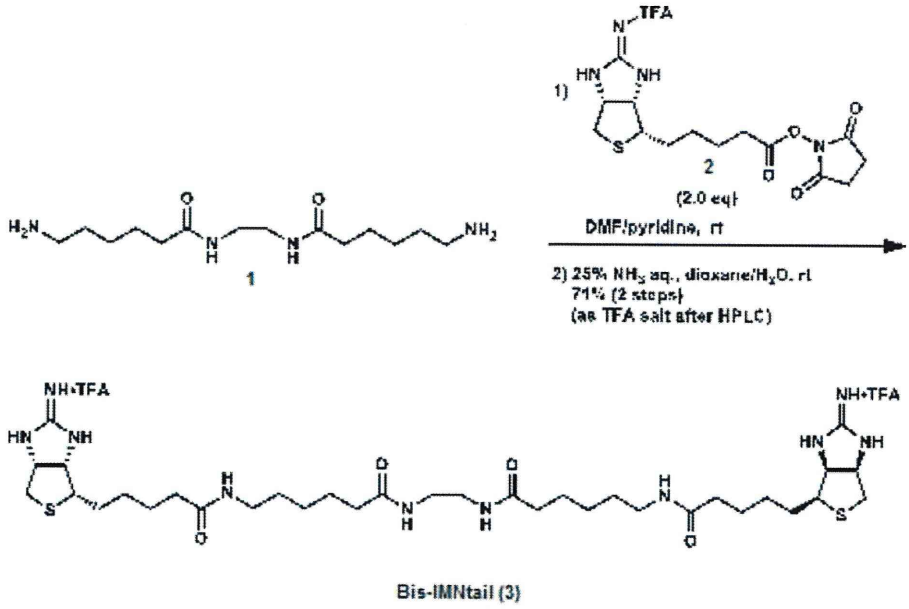
Supplementary Fig. S2. Distance of two neighboring IMNtail molecules in V212. Two IMNtail molecules are shown in stick models, and V212 subunits are drawn as ribbon models. The averaged distance between the carboxylates of two IMNtail molecules is 2.96 Å.



Supplementary Fig. S3. An SPR sensorgram showing the kinetics of the interaction between V212 and Bis-IMNtail.



Supplementary Fig. S4. Binding mode of Bis-IMNtail in V212. Schematic representation of the interactions between Bis-IMNtail and V212 is shown. The loop in V212 is presented with the dash-dot lines, because it forms an open conformation and does not interact with the ligand. The distances (Å)between amino-acid residues and Bis-IMNtail are represented by dotted lines.



Supplementary Fig. S5. Synthetic route of Bis-IMNtail

Supplemental Methods

General methods

^{1}H NMR spectra were recorded on JEOL ECS400 (400 MHz for ^{1}H NMR) spectrometer. Chemical shifts were reported relative to the solvent used as an internal reference for ^{1}H (δ = 3.31 ppm for CD_3OD). ESI-mass spectra were measured on a Waters ZQ4000 spectrometer (for LRMS). HPLC purification was conducted using a JASCO HPLC system (pump: PU-2086Plus; detector: UV-2075Plus, measured at 254 nm; column: YMC-Pack ODS-AM (150×4.6 mL); mobile phase: acetonitrile/0.1% TFA MQ solution).

EZ-Link® NHS-Iminobiotin was purchased from Thermo Scientific. Other reagents were purchased from Aldrich, Tokyo Chemical Industry Co., Ltd. (TCI), Kanto Chemical Co., Inc., and Wako Pure Chemical Industries Ltd. and used without further purification.

Synthesis of (S,R,S)-N,N'-(Ethane-1,2-diyl)bis(6-(5-((3aS,4S,6aR)-2-iminohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanamide) (Bis-IMNtail)

Synthetic route of bis-iminobiotin long tail (Bis-IMNtail) is shown in Fig. S5. To a solution of known diamine **1** (3.3 mg, 0.012 mmol) in DMF/pyridine (0.4 mL/0.1 mL) was added EZ-Link® NHS-Iminobiotin **2** (10 mg, 0.023 mmol) at room temperature and the mixture was stirred for 6 h at the same temperature. After removing the solvent by evaporation, the resulting residue was dissolved in dioxane (0.5 mL), and 25% aq. ammonia (2 mL) was added. After stirring for a further 6 h at room temperature, the aqueous phase was washed with diethyl ether and the aqueous layer was concentrated to give crude product. The crude residue was purified by reverse phase HPLC (YMC-Pack ODS-AM, gradient: 0–10–11–36–37–50 min; 0 –0–17–42–100–100% CH_3CN in 0.1% TFA MQ, ramp time 25 min (17–42%), t_R = 26.7 min) to give Bis-IMNtail (**3**) (7.9 mg, 71% over 2 steps, colorless amorphous solid).

^{1}H NMR (400 MHz, CD_3OD) δ: 1.31–1.38 (m, 4H), 1.42–1.55 (m, 8H), 1.56–1.70 (m, 10H), 1.78 (sext., 2H, J = 8.0 Hz), 2.20 (q, 8H, J = 7.2 Hz), 2.83 (d, 2H, J = 13.4 Hz), 3.01 (dd, 2H, J = 13.4, 4.5 Hz), 3.17 (t, 4H, J = 8.0 Hz), 3.27 (s, 4H), 3.30–3.33 (m, 2H), 4.54 (dd, 2H, J = 8.0, 4.5 Hz), 4.73 (dd, 2H, J = 7.6, 4.5 Hz); LRMS (ESI): m/z 369 $[M+2H]^{2+}$.

Protein expression and purification

For the preparation of V212, theisolation and refolding protocols were modified from previous reports.[1-4] The V212 gene was constructed in the pET21a(+) vector (Novagen). The T7 tag was fused at the N-terminus of V212, with a 6×His tag at the C-terminus. BL21Star (DE3) cells harboring the mutant plasmid were grown at 37 °C in 2×YT medium containing 100 μg/mL ampicillin to an OD of 0.8, and protein expression induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside and cells grown at 37 °C for 5 h. Cells were harvested by centrifugation at 8000 × *g* for 20 min, resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0) and ruptured by sonication. The lysed cells were centrifuged at 16,500 × *g* for 20 min. The insoluble fraction was washed three times with the lysis buffer containing 2% Triton X-100 and then washed twice with distilled water. The inclusion bodies were dissolved in 6 M guanidine hydrochloride, pH 1.5, and dialyzed against the dissolution buffer at 4 °C overnight. Insoluble material wasremoved by ultracentrifugation at 16,500 × *g* at 4 °C for 30 min, and the supernatant was added to refolding buffer (50 mM Tris-HCl, pH 8.0, 400 mM L-arginine hydrochloride, 200 mM NaCl, and 1 mM EDTA) by the rapid refolding method and left for 2 d. The sample was loaded onto cOmplete His-tag Purification Resin (Roche) and eluted with the refolding buffer containing 400 mM imidazole. The eluted sample was buffer-exchanged into gel-filtration buffer (1×PBS) by dialysis. Further purification was carried out by gel-filtration chromatography using a HiLoad 16/600 Superdex 75 column. Bis-IMNtail was added to V212 at an 8:1 molar ratio. Finally, the purified protein was buffer-exchanged and concentrated to 10 mg/mL in 20 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl using Vivaspin 10-kDa cutoff (GE Healthcare).

Binding assays by SPR

The interactions between V212 and Bis-IMNtail were analyzed by SPR with a Biacore T200 instrument (GE Healthcare). We used sensor chip NTA and NTA reagent kit (GE Healthcare). C-terminal His-tagged V212 was immobilized on sensor chip NTA through His tag. 1× HSB-P+ buffer (GE Healthcare) was used as the running buffer. Each ligand was immobilized between 2400 RU and 3500 RU. Each analyte was dissolved and diluted from 1 nM to 10 nM in the running buffer. Steady-state affinity analysis was done by Biacore evaluation software.

Crystallization

Crystallization was performed by the sitting-drop vapor-diffusion method at 20 °C in Violamo 96-well plates (As One, Osaka, Japan). Sixty microliters of the reservoir solution was added to each well of the 96-well plates. Crystals were obtained by mixing 0.1 μL of protein solution (10 mg/mL Bis-IMNtail-bound V212, 20 mM Tris-HCl, pH 7.5 and 200 mM NaCl) and 0.1 μL of reservoir solution (0.2 M sodium fluoride and 20% PEG3350).

Structure determination

All datasets were collected on the beamline at BL44XU at SPring-8 (Harima, Japan) under −173°C. A crystal of V212 complexed with Bis-IMNtail was cryoprotected by a well solution containing 20% glycerol. Data were indexed and scaled with the programs *DENZO* and *SCALEPACK* from the *HKL2000* program suite (HKL Research). The structure was solved by the molecular replacement with the program *PHASER* [5] from the *CCP4i* [6] package using LISA-314 (PDB ID: 3YWQ) as the search model. The resultant structure was manually modified to fit into the experimental electron density maps, using the program *Coot* [7], then refined with the program *REFMAC5* [8] from the *CCP4i* package. Figures were prepared using *Pymol* (http://www.pymol.org/). The final structure coordinates and structure factor amplitudes were deposited into the Protein Data Bank with ID 3X00.

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Regular Paper, fields: Biotechnology, topics: Protein Engineering

Structure-based design of a streptavidin mutant specific for an artificial biotin analogue

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Running title: Streptavidin mutant specific for a biotin analogue

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Abbreviations: IMNtail. iminobiotin long tail; SA, streptavidin; BTN, biotin; LISA-314, low immunogenic streptavidin mutant No. 314; IPTG, isopropyl-β-D-thiogalactopyranoside; TF, trigger factor; BTNtail, biotin long tail; SPR, surface plasmon resonance; RU, resonance unit; IMN, iminobiotin

Summary

For a multistep pre-targeting method using antibodies, a streptavidin mutant with low immunogenicity, termed LISA-314, was produced previously as a drug delivery tool. However, endogenous biotins with high affinity ($K_d < 10^{-10}$ M) for the binding pocket of LISA-314 prevents access of exogenous biotin-labeled anticancer drugs. In the present study, we improve the binding pocket of LISA-314 to abolish its affinity for endogenous biotin species, therefore ensuring that the newly designed LISA-314 binds only artificial biotin analogue. The replacement of three amino acid residues was performed in two steps to develop a mutant termed V212, which selectively binds to 6-(5-((3a*S*,4*S*,6a*R*)-2-iminohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)hexanoic acid (IMNtail). Surface plasmon resonance results showed that V212 has a K_d value of 5.9×10^{-7} M towards IMNtail, but no binding affinity for endogenous biotin species. This V212/IMNtail system will be useful as a novel delivery tool for anticancer therapy.

Keywords: Streptavidin, endogenous biotin, molecular design, X-ray structural analysis, pre-targeting

Monoclonal antibodies represent an attractive approach for the treatment of a range of diseases, because they can be designed to selectively target disease marker biomolecules. In particular, antibodies conjugated with radionuclides are promising molecules for imaging and therapy in cancer treatment. Direct-radiolabeled antibodies are currently used (*1*). However, their clinical adoption has not fulfilled initial expectations, because of the extended time required for tumor localization by the antibody. In particular, the treatment of solid tumors by antibodies is not effective because the low permeability coefficient does not allow sufficient tumor penetration by the antibody. This results in high levels of background radioactivity and toxicity in normal tissues, because of the high concentrations of direct-radiolabeled antibodies used as therapy (*2-5*). As an alternative strategy for improving efficiency, multistep approaches known as pre-targeting have been a major area of investigation. In the pre-targeting method, treatment is first performed with a high concentration of the antibody vehicle, followed by administration of a small amount of a radionuclide, which displays more favorable tumor-targeting properties. Several preclinical studies have validated the advantages of this approach using the streptavidin/biotin system (*6-14*); although, systems comprising a biospecific monoclonal antibody/hapten and an oligonucleotide/antisense have also been proposed (*8, 15*).

Streptavidin (SA) is a tetrameric protein that selectively binds biotin (BTN) with an affinity that

is amongst the highest displayed for non-covalent bonds between a ligand and protein (K_d = 10^{-14}–10^{-15} M) (*16, 17*). For this reason, the SA/BTN system has been used successfully in molecular biology and biotechnology, such as in labeling and binding experiments (*18-21*). Additionally, this system has attracted great interest for use in medical applications, especially as a drug delivery system. In this pre-targeting application, a monoclonal antibody-SA conjugate is first injected into a patient and allowed to localize at the tumor, followed by the clearing of excess reagent and administration of the radiolabeled BTN derivative.

Despite many trials, its clinical application has not been successful to date. Two major problems must be overcome. The first is that immunogenicity of a bacterium-derived SA precludes its long-term use. This is because of the associated reduction of the SA/BTN system functionality through the production of anti-SA antibodies (*7, 22-24*). The second problem is the presence of endogenous BTN species in the blood and tissues at sufficient concentrations to effectively block the BTN-binding sites of SA (*25, 26*) before it can bind the exogenous radiolabeled BTN derivative as a drug.

Recently, we reported the development of a low immunogenic SA mutant that retains BTN binding affinity, termed LISA-314, by introducing six amino acid substitutions to various charged and aromatic residues of wild-type SA (SA-WT) that were proposed to be involved in its immune

recognition (*27*). LISA-314 was shown to have low immune-reactivity against crab-eating monkey anti-SA-WT-serum. However, the second problem regarding endogenous BTN species remains unresolved. Reznik *et al.* (*28*) developed a SA mutant that shows higher affinity to a non-natural BTN analogue, iminobiotin (IMN) than BTN. Hamblett *et al.* (*29*) prepared a SA mutant that has a reduced affinity for BTN because of the faster dissociation, and a bivalent BTN showed a higher affinity to the mutant than monovalent BTN, allowing the exchange of pre-bound endogenous BTN species with the bivalent BTN molecule (*30*). Although progress has been made, no mutant has been created that has completely lost its binding affinity for natural BTN species.

Here, we present a novel SA-binding system that is not affected by a natural BTN species, biocytin. By improving the binding pocket of LISA-314 through three amino acid substitutions, we have developed a modified LISA-314 that binds only to a newly designed artificial BTN analogue, (K_d = 5.9 × 10^{-7} M) and has no binding affinity for biocytin. This is the first streptavidin binding system without any influence from endogenous BTN species.

Materials and Methods

General method

^{1}H NMR spectra were recorded on JEOL JNM-LA500 and JEOL ECX500 (500 MHz for ^{1}H