B3(Fab)-streptavidin Tetramer Has Higher Binding Avidity than B3(scFv)-streptavidin Tetramer

Jae Seon Won, Hye Won Kang, Pil Won Nam, and Mu Hyeon Choe*

Collegeof Life Sciences and Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
**E-mail: choemh@korea.ac.kr*
Received January 12, 2009, Accepted March 24, 2009

Multivalent and multi-specific antibodies can provide valuable tools for bio-medical research, diagnosis and therapy. In antigen-antibody interactions, the avidity of antibodies depends on the affinity and the number of binding sites.[1] As artificial multivalent antibody agents, single chain Fv-streptavidin fusion tetramer proteins $(scFv\text{-}SA)_4$ have been previously tested.[1,2] Although, the Fab domain is known to be more stable than scFv in animal models,[3,4] it has never been used to make a multivalent agent with a streptavidin fusion. In this study, we prepared tetra-valent $(Fab\text{-}cSA)_4$ by fusing Fab with core streptavidin (cSA). This molecule was made using inclusion body production, refolding and chromatography purification. Affinities of the Fab-cSA tetramer and a scFv-cSA tetramer to a cell surface antigen were compared by ELISA using biotin-HRP. The Fab-cSA tetramer showed higher binding avidity than the scFv-cSA tetramer. The higher binding avidity of the Fab-cSA tetramer demonstrates its potential as a therapeutic agent for target-specific antibody therapy.

Key Words: Recombinant antibody, Refolding, Fab, Homo-tetramer, Antibody therapy

Introduction

Antibody fragments and recombinant antibodies are the major paradigm for the design of high-affinity, protein-based binding reagents.[5,6] In recent years, antibodies have been reduced in size, dissected into minimal binding fragments and rebuilt into multivalent,high-avidity reagents.[7,8]

The smallest functional antibody module required for antigen binding is the Fv fragment, which is a hetero-dimer comprised of the variable domain of the heavy chain (V_H) and the variable domain of the light chain (V_L). Single chain Fv antibody fragments (scFvs) are covalently interconnected by various peptide linkers between V_H and V_L to avoid dissociation of the two domains.[9,10] Fab is a larger antibody fragment than scFv, which contains the Fd chain (V_H and C_{H1}) and the light chain (V_L and C_L). The Fd chain and light chain are interconnected by a disulfide bond between cysteines at the end of each chain.

Previously studied antibody-toxins, scFv-PE38 and Fab-PE38, were obtained by fusing the fragments to 38 kDa truncated forms of *Pseudomonas* exotoxin A.[10,11] Fab-toxin has two advantages over scFv-toxin. First, the refolding yields of Fab-toxin are about 10-fold higher than those of scFv-toxin.[12,13] Second, the Fab-toxin shows a longer half-life in animal plasma, which is close to the half-life of whole IgG-toxin.[4,14,15] This indicates an advantage of Fab over scFv as an antibody-fused therapeutic agent.

In this study, we have produced for the first time a tetravalent moleculeby fusing Fab to monomeric core streptavidin, Fab-cSA. As mentioned before, the Fab domain is more stable than scFv in animal plasma. Thus, a Fab-streptavidin fusion protein would be expected to have higher stability and a longer half-life in blood, and it may make an excellent tumor-specific targeting agent for clinical trials. Monomeric streptavidin can form a stable homo-tetramer, and it can bind to a biotin molecule.[16] Fab-cSA used in this study formed a stable tetramer $(Fab\text{-}cSA)_4$ due to the formation of a streptavidin tetramer.

Streptavidin is generally isolated from *Streptomyces avidinii* culture media.[17] However, natural streptavidin usually has truncated terminal sequences due to post-secretory cleavage of the terminal regions that are highly susceptible to proteolysis.[18-20] Thus, we used core streptavidin, which is a natural proteolysis form of full-length streptavidin at both ends (159 residues, 16.5 kDa). It is free from aggregate formation and shows high solubility.[17]

Here, we used Fab and scFv from our model monoclonal antibody B3 for fusion protein construction. B3 is a murine antibody directed against a carbohydrate antigen of the Le^Y family. When examined by peroxidase immunohistochemical techniques using frozen human tumors, the antigen was found on many mucin-containing carcinomas, including those from the colon, stomach, ovary, breast and esophagus.[21] B3 is one of the most widely studied antibodies, as it has very strong, characteristic specific binding to target cell lines, while showing very little binding to non-target cell lines.[10,22-25]

In this study, we describe the construction of a Fab-monomeric core streptavidin fusion, Fab-cSA molecule. The fusion protein was obtained as an inclusion body from *E. coli*. After refolding of the recombinant proteins, Fab-cSA tetramers were successfully purified by affinity chromatography. The final preparation yield was 0.15%, which was nearly the same yield as scFv-cSA tetramer (0.16%). Cell-binding assays

Abbreviations: scFv, single chain Fv; Fab, antigen binding fragment; V_H, heavy chain variable domain; V_L, light chain variable domain; SA, Streptavidin; ELISA, Enzyme-Linked ImmunoSorbent Assay; IMAC, Immobilized Metal Affinity Chromatography

showed that the Fab-cSA tetramer had higher binding avidity than the scFv-cSA tetramer for all antigen positive cell lines. Thus, the Fab-cSA tetramer with high binding avidity may be a useful therapeutic agent for target-specific antibody therapy.

Materials and Methods

Construction of plasmids. The plasmids used in this study are described in Table 1. Plasmid pUC57-cSA coding for core streptavidin was a kind gift from Dr. Se-Ho Park, Korea University, Seoul, Korea. pUC57-cSA carries a DNA sequence encoding amino acid residues 13-139 of mature streptavidin,[17,19] which is the natural core streptavidin (127 residues, 13.3 kDa). The core streptavidin regions were PCR-amplified from pUC57-cSA using prSA1 (5'-GGC CAA AGC TTC CGG AGG TCC CGA GGC TGA AGC TGG TAT CAC A-3') and prSA2 (5'-CCG GAA TTC ATT AGT GAT GGT GAT GGT GAT GAG ACC CAC CGC CAC CGA TAG AAG CAG C-3') primers. PCR fragments of 458 bp were obtained. After double digestion with *Hin*dIII and *Eco*RI, these fragments (445 bp) were purified using electrophoresis on low-melting agarose gels, and ligated with *Hin*dIII and *Eco*RI double cut T7 expression vector plasmid containing the scFv sequence. The resulting plasmid, pSA2H, encodes scFv-KASGGPE-cSA-GGGGS-H_6.

The antibody light chain sequence from pMC76,[26] which has a light chain-PE38 (38 kDa, *Pseudomonas* exotoxin) fusion, was used for the construction of plasmid pSA76 for antibody light chain-cSA fusion chain. A *Hin*dIII-*Eco*RI fragment containing the cSA fragment from pSA2H was inserted into *Hin*dIII-*Eco*RI-digested pMC76. The resulting plasmid, pSA76, encodes Light chain-KASGGPE-cSA-GGGGS-H_6. Plasmid pMC73 was used for the expression of the Fd (V_H and C_{H1}) fragment. The Fd chain was from pULI30.[4]

pCSA1 was constructed to express the streptavidin control molecule cSA-GGGGS-H_6 by PCR. The primer prSA3 (5'-GGG AAT TCC ATA TGG CTG AAG CTG GTA TCA CA-3') containing *Nde*I site and prSA2 were used. PCR fragments were digested with *Nde*I/*Eco*RI and cloned into the *Nde*I/*Eco*RI-digested pMC73.

Expression, purification and refolding of antibody-cSA. *Escherichia coli* BL21 (λDE3) cells were transformed with the plasmids pSA76 and pMC73 for the production of Light chain-cSA and Fd chain, respectively. The isolation of inclusion bodies was done as described previously.[4] Inclusion bodies were solubilized in buffer containing 6 M guanidine-HCl, 0.1 M Tris-Cl and 2 mM EDTA (pH 8.0). The amounts of fusion proteins were determined by Bradford assay with Coomasie Plus protein assay reagent (Pierce). Purity of each polypeptide chain was analyzed by SDS-PAGE and densitometry (TINA ver. 2.0). The purity was obtained by averaging the measurements of three different samples from serial dilutions. Solubilized Light-cSA and Fd chain inclusion bodies were combined in a 1:1 molar ratio for a total of 20 mg/2.5 mL. The mixture was reduced by the addition of 0.6 mM DTT and shaking for 2 h at RT. Refolding was done by 100-fold rapid dilution into theredox-shuffling buffer with aggregation-preventing additive (0.1 M Tris-Cl, 0.5 M L-Arginine-Cl, 1.6 mM oxidized Glutathione, 2 mM EDTA, pH 8.7 at 10 °C). The refolding solution was incubated for 2 days. The solution was dialyzed and the proteins were purified using Protein G HP chromatography and Superdex 200 chromatography (Amersham, UK).

Table 1. Plasmids and peptide chains used in this study

Plasmid Name	Name of peptide chain and its amino acid sequence	Ref
pUC57-SA	cSA	Provided by Dr. Se-Ho Park
pCSA1	cSA-G_4S-H_6	This work
pSA2H	scFv-cSA=scFv-KASGGPE-cSA-G_4S-H_6	This work
pSA76	L-cSA=Light-KASGGPE-cSA-G_4S-H_6	This work
pMC73	Fd	Previous work (unpublished)
pMC76	L-PE38=Light-KASGGPE-PE38	26
pMC77	L-m=Lght-m	Previous work (unpublished)

cSA: Monomeric core Streptavidin, scFv: single-chain Fv is covalently connected by $(G_4S)_3$ linker between the V_H (variable domain of heavy chain) and the V_L (variable domain of light chain), L: Light chain (V_L-C_L), KASGGPE: Connecting amino acid sequences are Lys(K)-C3 connecter (Ala-Ser-Gly-Gly-Pro-Glu), Fd: Heavy chain (V_H-C_{H1}) without C_{H2} and C_{H3} domains, Light-m: Light chain with modified end.

The recombinant fusion chain scFv-cSA and cSA were expressed in *Escherichia coli* BL21 (λDE3) that contained pSA2H and pCSA1. Isolation of inclusion bodies and refolding were done as described above. The refolded proteins were purified using IMAC (Immobilized Metal Affinity Chromatography) and Superdex 200 chromatography (Amersham, UK).

After purification, the amounts of properly folded antibody-cSA tetramers were measured by the BCA (Bicinchoninic Acid) Protein Assay Kit (Pierce). Quantitative protein purity analysis was done by SDS-PAGE band intensity measurement using TINA 2.0 software program.

Enzyme-linked immunosorbent assay (ELISA). The purified tetramers, $(Fab\text{-}cSA)_4$ and $(scFv\text{-}cSA)_4$, were analyzed for their ability to bind $Lewis^Y$ antigen by Fab or scFv domain in ELISA. The bound molecules were detected by biotin binding to cSA domain. Four different human cell lines were used in this assay: A431, CRL1739 and MCF7 cell lines are $Lewis^Y$-positive, and KB3-1 cell line is $Lewis^Y$-negative. The ELISA experiments were performed as previously described.[1,27] Briefly, each cancer cell line was plated at 2×10^4 cells/well in a 96-well plate. Cells were cultured for 24 hr at 37 °C and the plate was washed 3 times with PBST (PBS with 0.05% Tween-20). After fixation with fixing solution (methanol/acetone solution, v/v = 1:1) for 30 min, the plate was washed 5 times with PBST. 1% BSA/PBS was used for blocking. Serially diluted antibody-cSA fusion proteins in 1% BSA/PBS were added to each well and incubated for 2 hr at 37 °C. The plate was washed 5 times with PBST. The Biotinylated Horseradish peroxidase (1:2000 diluted by 1% BSA/PBS) or a Protein L-horseradish peroxidase conjugate reagent (1:2000 diluted by 1% BSA/PBS) was added and incubated for 1 hr at 37 °C. TMB substrate

was used for visualizing bound enzyme, and a spectrophotometric reading was taken at 450 nm after the reaction was stopped by the addition of 2 M H_2SO_4.

Results

Construction of a plasmid encoding antibody-cSA and its expression. A schematic for the structure of antibody-cSA is shown in Figure 1. A polypeptide connector (K-ASGGPE) is inserted between the antibody fragment and the cSA, and it helps each domain to fold properly with high yield.[28] The DNA sequences of constructed plasmids were confirmed by sequencing analysis.

An inclusion body for each polypeptide chain (Table 1) was prepared from *E. coli* BL21 (λDE3). Purities of the inclusion bodies were analyzed by SDS-PAGE and TINA 2.0 integration program. The inclusion body yields were 49-103 mg per 1L of cell culture. The range of purities for these polypeptides was about 24-34%.

Refolding and purification of antibody-cSA tetramer. The polypeptide chain scFv-cSA was refolded using a redox shuffling method.[11] scFv-cSA has a hexa-histidinyl tail that enables purification of the proteins by IMAC (Immobilized Metal Affinity Chromatography). The refolded proteins were purified by IMAC and Superdex 200 size exclusion chromatography (Fig. 2). The purified $(scFv\text{-}cSA)_4$ was analyzed by SDS-PAGE either after 10 min incubation at RT or 10 min boiling at 100 °C. The calculated molecular weight of the scFv-cSA tetramer was 166.8 kDa. SDS-PAGE analysis showed that the non-reduced, non-boiled scFv-cSA tetramer migrated with the band of the 205 kDa size marker, which is larger than the calculated molecular weight (Fig. 2B). This was expected because of the branched configuration of the scFv-cSA tetramer. The boiled sample showed only one band for a 41.7 kDa calculated molecular weight monomer (Fig. 2C). This result indicated that the scFv-cSA tetramer is dissociable into homogeneous scFv-cSA monomers. Also, the cSA moiety in the scFv-cSA fusion protein retained its self-affinity to form a homo-tetramer.

In order to make a Fab-cSA tetramer, the solubilized polypeptide chains, Light chain-cSA of 39.5 kDa and Fd chain of 25 kDa, were refolded by mixing in a 1 : 1 molar ratio to a final total protein concentration of 20 mg/250 mL. Refolding was performed as described previously. The refolded proteins were purified by Protein G HP affinity chromatography. Protein G selectively binds to the Fc region of IgG.[29] After Protein G HP, proteins were applied to Superdex 200 size exclusion chromatography (Fig. 3).

The purified $(Fab\text{-}cSA)_4$ was analyzed by SDS-PAGE after 10 min incubation at RT under non-reducing conditions and 10 min boiling at 100 °C under reducing conditions. The Fab-cSA tetramer had a calculated molecular weight of 258 kDa. After incubation at RT under non-reducing conditions, it migrated slower than the 250 kDa size marker band (Fig. 3B). The Fab-cSA tetramer has a bulky branched molecular shape, which causes drag in SDS-PAGE resulting in an apparently higher molecular weight. When the same samples were analyzed under reducing conditions with 10 min boiling at 100 °C, two

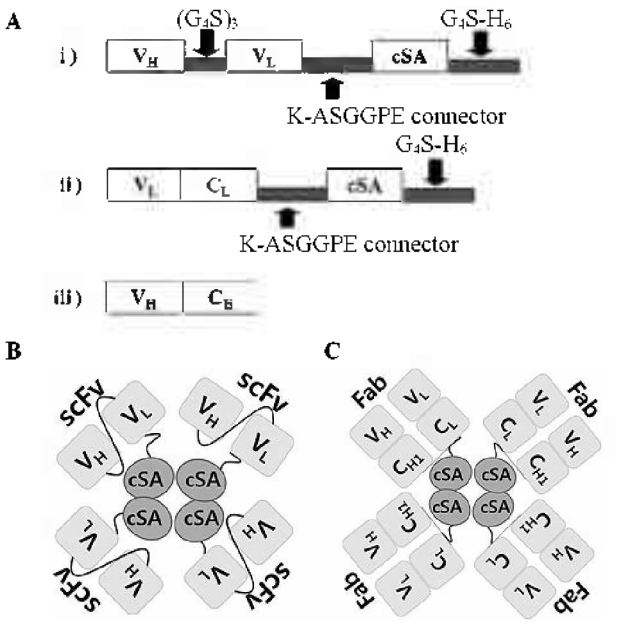

Figure 1. Structure of the antibody-cSA protein used in this study. A. Overview of the peptide chain construct for the $(scFv\text{-}cSA)_4$, and $(Fab\text{-}cSA)_4$. i) scFv-cSA, ii)Light chain-cSA and iii) Fd (V_H and C_{H1}) chain. B. Schematic structure of homo-tetramer protein $(scFv\text{-}cSA)_4$ and C. $(Fab\text{-}cSA)_4$. Each monomeric antibody-cSA was obtained as an inclusion body from *E. coli*. After refolding, the tetravalent antibody-cSA protein results from the spontaneous tetramer formation with a fused core Streptavidin.

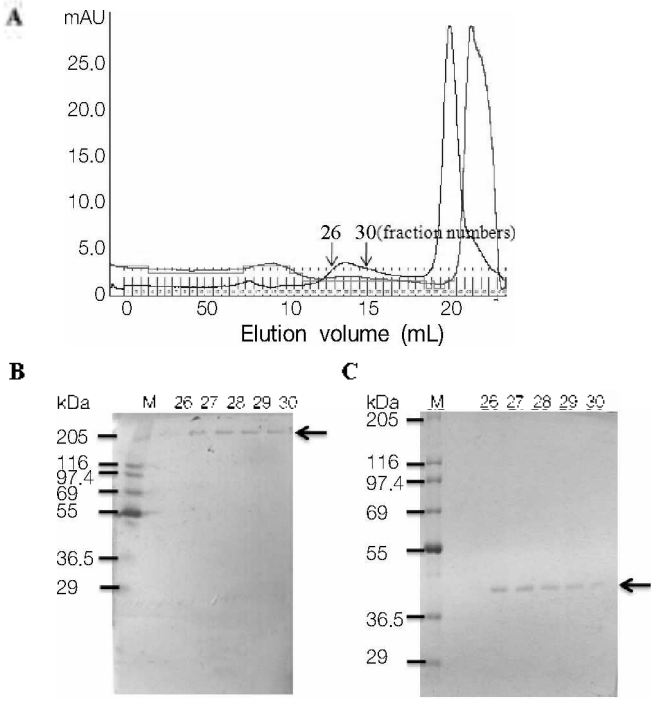

Figure 2. HPLC size exclusion chromatography purification of $(scFv\text{-}cSA)_4$. A. Superdex 200 profile. B. 10% SDS-PAGE gel. $(scFv\text{-}cSA)_4$ purified by superdex 200 and incubated at RT for 10 min. The arrow indicates the position of scFv-cSA tetramer. C. 12% SDS-PAGE reducing gel. The same sample as in (B) but boiled at 100 °C for 10 min. Under these conditions, the tetramer of cSA dissociate into monomers. The arrow indicates the position of scFv-cSA monomers (41.7 kDa). M: size marker (kDa), 26-30: fraction numbers.

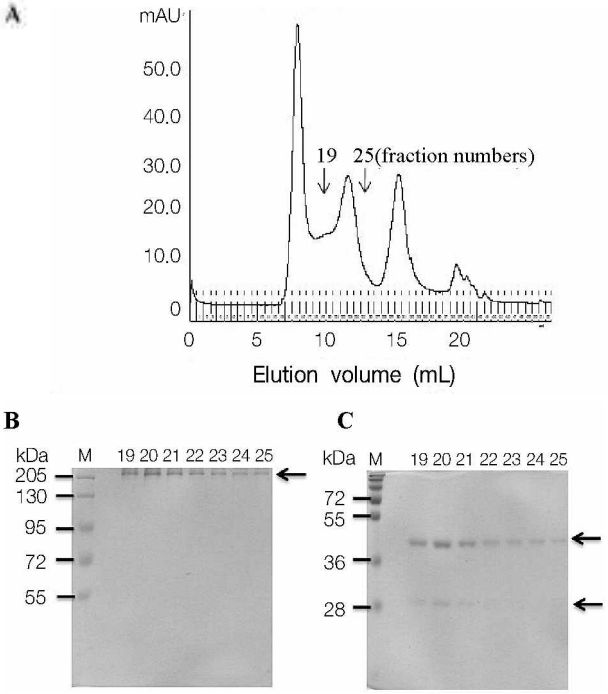

Figure 3. HPLC size exclusion chromatography purification of $(Fab\text{-}cSA)_4$. A. Superdex 200 profile. B. 10% SDS-PAGE gel. $(Fab\text{-}cSA)_4$ purified by superdex 200 and incubated at RT for 10 min. The arrow indicates the position of Fab-cSA tetramer. C. 12% SDS-PAGE reducing gel. The same sample as in (B) but boiled and reduced at 100 °C for 10 min. Under these conditions, the tetramer of cSA dissociates into monomers and the disulfide bond between the Fd chain and L-cSA was reduced. The upper arrow indicates L-cSA (39.5 kDa) and lower arrow indicates Fd chain (25 kDa). M: size marker (kDa), 19-25: fraction numbers.

Table 2. Refolding yields of antibody-cSA tetramers

Refolding[a]	Refolding yield[b]	
	ug	%
$(Fab\text{-}SA)_4$	294	0.15
$(scFv\text{-}SA)_4$	311	0.16

[a]The refolding of different antibody-toxin molecules was performed by 100-fold rapid dilution method. [b]The refolding yields are an average of three repeated test results with 200 mL refolding for each antibody-cSA.

bands were observed. Under this condition, the tetramer dissociates into monomers and DTT reduces the disulfide bond between C_{H1} and C_L of Fab-cSA. These bands had mobilities that matched the 39.5 kDa and 25 kDa for L-cSA and Fd chains, respectively (Fig. 3C). These results indicated that tetramers were made only with these two polypeptide chains.

The refolding yields of antibody-cSA tetramers are summarized in Table 2. These were 0.15% for $(Fab\text{-}cSA)_4$ and 0.16% for $(scFv\text{-}cSA)_4$. The yields of these tetramers were nearly the same.

Binding assays for antigen-expressing cancer cells and biotin by ELISA. The binding avidities of antibody-cSA tetramers were analyzed by ELISA. The antigen-positive cancer cell lines used were A431, CRL1739 and MCF7. The KB3-1 cancer cell line was used as a negative control. The amounts

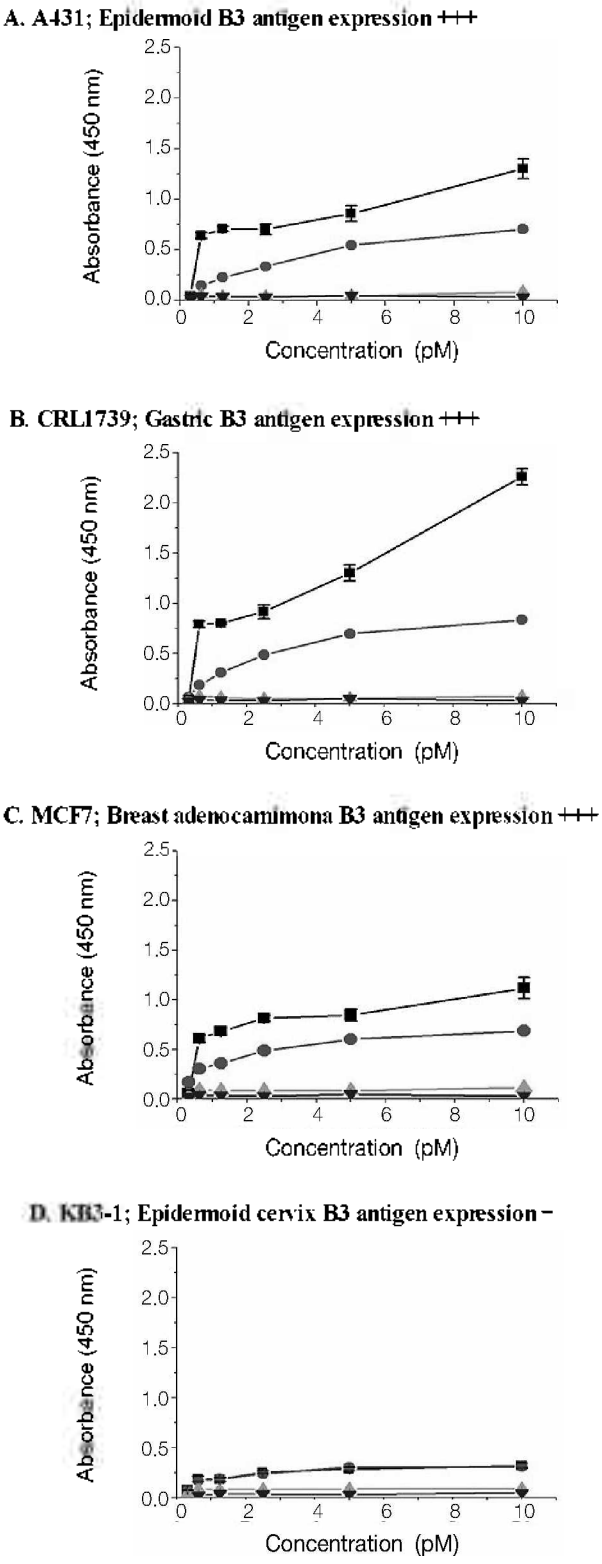

Figure 4. Binding assays for biotin and antigen by antibody-cSA tetramer using cell-ELISA. Cultured human cancer cell lines A431, CRL1739, MCF7 (A, B, C; B3 antigen positive) and KB3-1 (D; B3 antigen negative) were fixed with methanol/acetone solution (v/v = 1:1). Cells were incubated with 2-fold serial dilutions of antibody-cSA tetramer, starting at 10 pM and decreasing to 0.313 pM. Bound $(Fab\text{-}cSA)_4$ (■), $(scFv\text{-}cSA)_4$ (●), Fab-m(▲) or $(cSA)_4$ (▼) were detected using biotin-HRP. Each data point is the average value of triplicate samples, and the assay was repeated three times. Fab-m was used as a streptavidin negative control. Error bars are SD.

of bound antibody-cSA fusion proteins on these cells were detected using biotinylated horseradish peroxidase (HRP). Fab-m, which is a Fab binding domain with a modified end, was used as a negative control for the HRP detection system, as it does not have streptavidin for biotin binding. The end modification of the light chain in Fab-m is by a polypeptide addition that does not influence antigen-antibody interactions. cSA tetramer was also used as a negative control for detecting the non-specific interaction of streptavidin on cell surface antigens. ELISA was performed in triplicate and repeated three times.

ELISA assay showed that the Fab-cSA tetramer had a higher binding avidity than the scFv-cSA tetramer (Fig. 4) for the A431, MCF7 and CRL1739 cells. However, both antibody-cSA tetramers showed low binding on KB3-1 cells. For the Fab-m, no HRP signal was observed, as it does not bind with biotin. cSA tetramer also showed no signals, as it does not interact with any cell surface antigens. To check the binding of all sample molecules to target cells, protein L conjugated with peroxidase was used to detect the antibody fragments. Protein L is an immunoglobulin-binding protein that has the unique ability to bind kappa light chains without interfering with antibody fragment's antigen-binding.[30] All the sample molecules were bound to the target cells (data not shown). To test whether antibody-cSA molecules are stable against any thermal or proteolytic degradation during the experiment, the molecules were left overnight at RT. The SDS-PAGE analysis of the molecules showed no changes in integrities and migrations of them (data not shown), and it guaranteed the stabilities of the molecules during our assays.

Discussion

We have designed and produced a tetrameric Fab-core streptavidin fusion molecule, $(Fab\text{-}cSA)_4$, which is comprised of the Fab region of mAb B3 and core streptavidin. The recombinant protein was expressed as inclusion bodies in *E. coli*, and the active form of the molecule was obtained by refolding of the reduced protein. In previous reports, scFv-cSA tetramer, $(scFv\text{-}cSA)_4$, formed larger amounts of aggregates during the refolding process.[1,31] In our initial trials, the refolding yields of antibody-cSA tetramer were also very low (< 0.03% for scFv-cSA tetramer and < 0.01% for Fab-cSA tetramer). To improve the refolding yield, we tried to find the optimal pH for the refolding buffer.

Reduced and denatured proteins were renatured at pH 8.0, 8.7 or 9.4. Renaturation at pH 8.0 resulted in very large amounts of aggregatesat the initial stage of the refolding process. At pH 8.7, we produced a better yield due to less aggregation, and it was nearly the same at pH 9.4. Hence, the optimal pH for renaturation was pH 8.7. We also tried small and large scale refolding to get higher yields. We found that the optimal condition was a protein concentration of 20 mg/250 mL in refolding buffer. The yield of tetramer was 0.15 - 0.16%, which is about 5-15 fold higher than the initial trials (data not shown).

Previously reported multivalent fusion proteins were usually made using a scFv fragment.[1,5] For the first time, we madea tetravalent molecule by fusing a Fab fragment to improve the avidity and stability.

To quantitatively examine the enhancement of binding avidity, the binding avidities for each tetramer were determined by cell-ELISA.[32] ELISA assay with biotin-HRP showed that the Fab-cSA tetramer binds with significantly higher avidity than the scFv-cSA tetramer on antigen-positive cell lines. These results show that the Fab binding domain may have an advantage over scFv for cell surface antigen binding, as it is larger and has a longer shape and, thus, can reach multiple antigens in its tetrameric form for multi-valent binding.

Further, the maximum difference for binding avidities was observed for CRL1739 cells. This suggests that the activity of the Fab-cSA tetramer may be dependent on the antigen environment on the cell surface. If the antigens are on a long, flexible structure and can come close enough to permit multiple binding at the same time, a tetramer will show a higher avidity. If the antigen is on a rigid structure, and the distances between antigens are too far to be reached at the same time, a tetramer will show the same avidity as a dimer or a monomer. It is not clear which is the most favorable structure for multiple-binding to antigen, and it remains to be studied.

In this study, we used a Fab fragment to increase the stability. In a Fab fragment, the C_{H1} and the C_L domains have hydrophobic patches on their surfaces that bring, and fit, the Fd and light domains together. A disulfide bond between them provides locking and stabilizing effects on their conformations. However, a single-chain Fv is made of V_H and V_L by connecting them with various kinds of polypeptide linkers.[33] The quaternary structures of scFv molecules are unstable, as the V_H and V_L do not stay strongly associated, and the hydrophobic residues of the V_H and $V_L$domains are exposed to water. As a result, they have a tendency to aggregate and to be easily attacked by proteases.[34,35] Although the lower stability of scFv limits its clinical application, a Fab fusion protein is expected to have higher stability and a longer half-life in blood and has potential as a therapeutic agent for wide applications.

In recent years, mAbs coupled to a variety of agents, including radioisotopes, protein toxins and cytokines, have been shown to be valuable tools for cancer therapy. In this study, we made a Fab-cSA tetramer, which has a core SA that binds biotin with a very high affinity ($K_d \sim 10^{-15}$M).[17] If used with radio-labeled biotin, a Fab-cSA tetramer may make a very useful tool for radio-immunotherapy for cancer treatment.

In this report, we describe for the first time the genetic engineering, expression, purification and in vitro testing of a tetrameric Fab-cSA fusion protein, and showits potential for future clinical applications.

Acknowledgments. We thank Dr. Se-Ho Park for kindly providing plasmid pUC57-cSA. This work was supported by a Korea University Grant and the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. R01-2006-000-10125-0).

References

1. Kipriyanov, S. M.; Little, M.; Kropshofer, H.; Breitling, F.;

Gotter, S.; Dübel, S. *Protein Eng.* **1996**, *9*, 203.
2. Lin, Y.; Pagel, J. M.; Axworthy, D.; Pantelias, A.; Hedin, N.; Press, O. W. *Cancer Res.* **2006**, *66*, 3884.
3. Wu, A. M.; Senter, P. D. *Nat. Biotechnol.* **2005**, *23*, 1137.
4. Choe, M.; Webber, K. O.; Pastan, I. *Cancer Res.* **1994**, *54*, 3460.
5. Hudson, P. J.; Kortt, A. A. *J. Immunol. Methods* **1999**, *231*, 177.
6. Dall'acqua, W.; Carter, P. *Curr. Opin. Struct. Biol.* **1998**, *8*, 443.
7. Pluckthun, A.; Pack, P. *Immunotechnology* **1997**, *3*, 83.
8. Kortt, A. A.; Lah, M.; Oddie, G. W.; Gruen, C. L.; Burns, J. E.; Pearce, L. A.; Atwell, J. L.; Mccoy, A. J.; Howlett, G. J.; Metzger, D. W.; Webster, R. G.; Hudson, P. J. *Protein Eng.* **1997**, *10*, 423.
9. Huston, J. S.; Levinson, D.; Mudgett-Hunter, M.; Tai, M. S.; Novotny, J.; Margolies, M. N.; Ridge, R. J.; Bruccoleri, R. E.; Haber, E.; Crea, R.; Al, E. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 5879.
10. Brinkmann, U.; Pai, L. H.; Fitzgerald, D. J.; Willingham, M.; Pastan, I. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 8616.
11. Buchner, J.; Pastan, I.; Brinkmann, U. *Anal. Biochem.* **1992**, *205*, 263.
12. Benhar, I.; Pastan, I. *Clin. Cancer Res.* **1995**, *1*, 1023.
13. Buchner, J.; Rudolph, R. *Biotechnology (N Y)* **1991**, *9*, 157.
14. Derocq, J. M.; Casellas, P.; Laurent, G.; Ravel, S. Vidal, H.; Jansen, F. *J. Immunol.* **1988**, *141*, 2837.
15. Saito, T.; Kreitman, R. J.; Hanada, S.; Makino, T.; Utsunomiya, A.; Sumizawa, T.; Arima, T.; Chang, C. N.; Hudson, D.; Pastan, I. *et al. Cancer Res.* **1994**, *54*, 1059.
16. Green, N. M. *Methods Enzymol.* **1990**, *184*, 51.
17. Sano, T.; Pandori, M. W.; Chen, X.; Smith, C. L.; Cantor, C. R. *J. Biol. Chem.* **1995**, *270*, 28204.
18. Bayer, E. A.; Ben-Hur, H.; Hiller, Y.; Wilchek, M. *Biochem. J.* **1989**, *259*, 369.
19. Argaraña, C. E.; Kuntz, I. D.; Birken, S.; Axel, R.; Cantor, C. R. *Nucleic Acids Res.* **1986**, *14*, 1871.
20. Pähler, A.; Hendrickson, W. A.; Kolks, M. A.; Argaraña, C. E.; Cantor, C. R. *J. Biol. Chem.* **1987**, *262*, 13933.
21. Pastan, I.; Lovelace, E. T.; Gallo, M. G.; Rutherford, A. V.; Magnani, J. L.; Willingham, M. C. *Cancer Res.* **1991**, *51*, 3781.
22. Pai, L. H.; Batra, J. K.; Fitzgerald, D. J.; Willingham, M. C.; Pastan, I. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 3358.
23. Pai, L. H.; Batra, J. K.; Fitzgerald, D. J.; Willingham, M. C.; Pastan, I. *Cancer Res.* **1992**, *52*, 3189.
24. Brinkmann, U.; Lee, B. K.; Pastan, I. *J. Immunol.* **1993**, *150*, 2774.
25. Jung, S. H.; Pastan, I.; Lee, B. *Proteins* **1994**, *19*, 35.
26. Choi, S. H.; Kim, J. E.; Lee, Y. C.; Jang, Y. J.; Pastan, I.; Choe, M. H. *Bull. Kor. Chem. Soc.* **2001**, *22*, 1361.
27. Wang, J. L.; Zheng, Y. L.; Ma, R.; Wang, B. L.; Guo, A. G.; Jiang, Y. Q. *World J. Gastroenterol* **2005**, *11*, 4899.
28. Brinkmann, U.; Buchner, J.; Pastan, I. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 3075.
29. Björck, L.; Kronvall, G. *J. Immunol.* **1984**, *133*, 969.
30. Björck, L. *J. Immunol.* **1988**, *140*, 1194.
31. Schultz, J.; Lin, Y.; Sanderson, J.; Zuo, Y.; Stone, D.; Mallett, R.; Wilbert, S.; Axworthy, D. *Cancer Res.* **2000**, *60*, 6663.
32. An, S. A. A.; Suh, I. *Bull. Kor. Chem. Soc.* **2008**, *29*, 2209.
33. Colcher, D.; Bird, R.; Roselli, M.; Hardman, K. D.; Johnson, S.; Pope, S.; Dodd, S. W.; Pantoliano, M. W.; Milenic, D. E.; Schlom, J. *J. Natl. Cancer Inst.* **1990**, *82*, 1191.
34. Bera, T. K.; Pastan, I. *Bioconjug. Chem.* **1998**, *9*, 736.
35. Lee, D. Y.; Kim, M. K.; Kim, M. J.; Bhattarai, B. R.; Kafle, B.; Lee, K. H.; Kang, J. S.; Cho, H. J. *Bull. Kor. Chem. Soc.* **2008**, *29*, 342.