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Divalent Forms of CC49 Single-Chain Antibody Constructs in *Pichia pastoris*: Expression, Purification, and Characterization[1]

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Single-chain variable fragments (scFvs) are tumor-recognition units that hold enormous potential in antibody-based therapeutics. Their clinical applications, however, require the large scale production and purification of biologically active recombinant scFvs. In the present study, we engineered and expressed divalent non-covalent [$(scFv)_2$-His_6] and covalent [$sc(Fv)_2$-His_6] scFvs of a tumor-associated monoclonal antibody (MAb) CC49 in *Pichia pastoris*. The purity and immunoreactivity of the scFvs were analyzed by SDS-PAGE, HPLC, and competitive ELISA. The binding affinity constant (K_A), determined by surface plasmon resonance analysis using BIAcore, was 4.28×10^7, 2.75×10^7, and 1.14×10^8 M^{-1} for $(scFv)_2$-His_6, $sc(Fv)_2$-His_6, and CC49 IgG, respectively. The expression of scFvs in *P. pastoris* was 30 to 40-fold higher than in *Escherichia coli*. Biodistribution studies in athymic mice bearing LS-174T human colon carcinoma xenografts showed equivalent tumor-targeting of CC49 dimers generated in yeast $(scFv)_2$-His_6 and bacteria $(scFv)_2$ with 12.52% injected dose/gram (%ID/g) and 11.42%ID/g, respectively, at 6 h post-injection. Interestingly, the pharmacokinetic pattern of dimeric scFvs in xenografted mice exhibited a slower clearance of His-tagged scFvs from the blood pool than scFvs lacking the His-tag ($0.1 \geq p \geq 0.05$). In conclusion, improved yields of divalent scFvs were achieved using the *P. pastoris* expression/secretion system. The *in vitro* and *in vivo* properties of these scFvs suggest possible therapeutic applications.

Key words: colon carcinoma xenografts, pharmacokinetics, single-chain Fv, valency, yeast.

Single-chain antibody fragments (scFvs) are recombinant proteins consisting of covalently-linked variable domains of heavy (V_H) and light chain (V_L) immunoglobulins (*1*). ScFvs provide building blocks for novel engineered antibody-based molecules and are important as immunodiagnostic and therapeutic agents (*2–9*). Several anti-carcinoma scFvs have been evaluated *in vivo* as tumor-targeting agents (*3, 4, 6*). As compared to intact Ig, $F(ab')_2$, and Fab, the monovalent scFvs exhibit excellent tumor specificity and penetration characteristics but reveal poor quantitative tumor retention, probably due to their monovalent nature and small size (*3, 5*). Recently, divalent scFvs have emerged as more promising therapeutic agents, revealing significantly increased tumor retention in xenograft tumor models in mice (*4–7, 9*). These multimeric antibody fragments gain advantage by having a longer biological half-life and increased avidity (*10*). Besides showing a proven efficacy in model animals, the clinical application of scFv-based therapeutics also requires the generation of large amounts of stable, clinical-grade-purified scFv (*8*).

Attempts have been made to maximize the yields of scFvs by changing the expression system and purification regimen (*11–13*). Yeasts are preferred for the expression of heterologous proteins as they portray eukaryote-specific post-translational protein modifications such as proteolytic processing, disulfide bridge formation, *in vivo* folding, and glycosylation besides being as easy as *Escherichia coli* to genetically manipulate (*14*). The expression of human glycoproteins in yeast systems, however, requires careful consideration. Yeasts mainly add oligosaccharide chains composed of mannose units (Man_{8-14} for *P. pastoris* and Man_{50-150} for *Saccharomyces cerevisiae*) to proteins at *O*-serine/threonine- and *N*-asparagine–linked sites, unlike higher eukaryotes in which complex oligosaccharides are added at these sites (*15*). It is known that antibodies are glycosylated at conserved positions in the constant regions

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Abbreviations scFv, single chain Fv; $(scFv)_2$, non-covalent dimeric scFv; $sc(Fv)_2$, covalent dimeric scFv; His-tag, hexahistidine tag; IMAC, immobilized metal affinity chromatography, HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography

of the heavy chains as in IgG, in which a carbohydrate chain is attached to Asn297 in the C_H2 domain (*16*). As scFvs contain only the V_L and V_H regions of the antibody, yeast expression systems can be utilized to generate recombinant scFvs of therapeutic relevance. Moreover, yeasts are free of pyrogens and viruses and can be used to make proteins for clinical applications (*15*, *17*, *18*). The methylotrophic yeast *Pichia pastoris* uses the powerful alcohol oxidase 1 (AOX1) promoter to produce high levels of recombinant proteins and its fermentation can be scaled-up to meet greater demands (*14*).

In this report, we have engineered and expressed divalent scFvs of MAb CC49, which recognizes a unique Sialyl-Tn antigen expressed on tumor-associated glycoprotein, TAG-72. Expression in yeast and purification by Immobilized Metal Affinity Chromatography (IMAC) increased the yield significantly as compared to the *E. coli* expression system (*9*, *19*). The *in vitro* binding characteristics of the divalent scFvs were found to be similar to those reported earlier (*9*, *19*). The tumor-targeting properties of scFvs were evaluated *in vivo* using athymic mice bearing LS-174T xenografts. Comparative biodistribution studies involving $(scFv)_2$ with- or without- a His-tag revealed similar tumor uptakes; however, the blood clearance of $(scFv)_2$-His_6 was found to be slower than $(scFv)_2$. The longer biological half-life of His-tagged scFv in blood, along with its bivalent nature may be significant in scFv-based cancer radioimmunotherapy.

MATERIALS AND METHODS

Vector Construction—The CC49 scFv gene (V_L-Linker-V_H) was constructed as previously described (*20*) using the linker designated as 205C (*21*). The bacterial scFv construct was used as the template DNA for the expression of the scFvs (monovalent and divalent) in *P. pastoris*. Table I includes a complete list of oligonucleotide primers (P) used in the PCR reactions. The V_L domain was amplified using P1 (forward) and P2 (reverse), cloned into vector pSE380 (Stratagene, La Jolla, CA) and designated Product I (Fig. 1A). Amplification of the V_H region was done using P3 (forward) and P4 (reverse) and ligated with Product I to obtain the monovalent scFv (scFv-His_6; Fig. 1B).

For construction of the covalent dimeric scFv, Product II was first obtained by amplifying V_H with P3 (forward) and P5 (reverse) and ligating it with Product I (Fig. 1C). The second single chain motif (Linker-V_L-Linker-V_H) was added by amplifying the V_L region with P6 (forward) and P2 (reverse) and ligating the product with Product II. The resultant clone was Product III (Fig. 1D). PCR was carried out with P3 (forward) and P4 (reverse) for the addition of the final V_H region with His-tag (Fig. 1D). The DNA sequence of the resultant constructs was verified. The constructs were cloned into the *P. pastoris* expression vector, pPICZαA (Invitrogen, Carlsbad, CA). Homologous recombination at the 5′ end of the AOX1 gene was achieved by transforming the KM71 strain (*his4 arg4 aox1Δ::ARG4*) of *P. pastoris* with *Sac*I-linearized constructs using the Easy-Comp™ kit (Invitrogen, Carlsbad, CA), and selected on YPDS plates with 100 μg/ml zeocin. The colonies were screened for expression levels by solid-phase competition ELISA.

Protein Expression and Purification—Yeast were grown at 30°C in buffered glycerol-complex (BMGY) medium containing 100 μg/ml zeocin. Incubation with maximum aeration was continued until the culture reached an A_{600} of 2–6. To induce expression, the cells were centrifuged and re-suspended in 1/10 of the original culture volume using buffered methanol-complex (BMMY) medium. Methanol was added to a final concentration of 0.5% every 24 h. The time-course of protein expression was followed and a peak at 72 h was observed The culture was centrifuged and the supernatant containing scFv was dialyzed against 50 mM sodium phosphate (pH 7.2), 300 mM NaCl Purification of the monovalent and divalent forms of scFv was performed by IMAC using the chelating absorbent Ni-Nitrilotriacetic acid (NTA) Superflow (Qiagen, Valencia, CA). Bound fractions were eluted with 250 mM imidazole. The protein was analyzed by SDS-PAGE and by solid phase competitive ELISA. A Superdex 75 pg column (Pharmacia, 1.6 × 70 cm) was used to separate the dimeric forms. Protein concentrations were determined by the method of Lowry *et al.* (*22*).

SDS–Polyacrylamide Gel Electrophoresis—All preparations were analyzed by SDS-PAGE under reducing and non-reducing conditions on 10% polyacrylamide gels according to the method of Laemmli (*23*). Bands were visualized using Coomassie Blue R-250. For ^{125}I- and/or ^{131}I-labeled protein preparations, gels were exposed to a phosphor screen and analyzed using the ImageQuant software of the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Radiolabeling of CC49 scFvs—The scFv forms were labeled with $Na^{125}I$ or $Na^{131}I$ using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodo-Gen, Pierce Chemical, Rockford, IL) as the oxidant (*24*). Briefly, 20–100 μg of protein was adjusted to 0.1 M sodium phosphate buffer (pH 7.2) and transferred into a 12 × 75 mm glass tube coated with 20 μg of Iodo-Gen. The protein was incubated for 3 min with 0.5–1 mCi of $Na^{125}I$ or $Na^{131}I$ (DuPont-NEN, Boston, MA). The unincorporated iodine was separated from labeled scFv forms using a Sephadex G-25 column (Phar-

TABLE I **Oligonucleotide PCR primers used in molecular cloning.**

P1 (forward)	CATCG**GAATTC**GACATTGTGATGTCACAG
P2 (reverse)	TAAAGCTAGCACCAAGCGCTTAGTTTCAGCACCAGCTTGGTCCCAG
P3 (forward)	TAAATGCGCA*GATGACGCAAAGAAAGACGCAGCTAAAAAAGACGATGCCAAAAAGGATGACGCCAAGAAAGATCTT*G-AGGTTCAGTTGCAGCAGTCTG
P4 (reverse)	TACCCGGGGCGGCCGCTTATTAATGGTGATGATGGTGATGTGAGGAGACGGTGACTGA
P5 (reverse)	TAAAGCTAGCACCAAGCGCTTAGTGAGGAGACGGTGACTGAGGT
P6 (forward)	TAAATGCGCA*GATGACGCAAAGAAAGACGCAGCTAAAAAAGACGATGCCAAAAAGGATGACGCCAAGAAAGATCTT*-GACATTGTGATGTCACAGTCTCC

All primers are shown 5′ to 3′ Nucleotide sequence of CC49 scFv is shown in bold whereas 205C linker is italicized. Restriction sites are underlined and the His-tag is double underlined. Asterisks denote stop codons.

macia Biotech., Piscataway, NJ). The typical iodination yielded scFvs with a specific activity of 3–9 mCi/mg.

HPLC Analyses—Gel filtration on HPLC was used to analyze the radiolabeled scFvs. Samples were injected onto TSK G2000SW and TSK G3000SW columns (Toso Haas, Tokyo) connected in series using 67 mM sodium phosphate buffer (pH 6.8), 100 mM KCl as the mobile phase at a flow rate of 0.5 ml/min. The eluate was monitored with an in-line UV detector at 280 nm and the radioactivity was determined in a Packard Minaxi Auto-Gamma 5000 gamma counter (Meriden, CT).

Binding Analyses—The immunoreactivity was assessed by solid phase competition ELISA using bovine submaxillary gland mucin (BSM; Sigma, St. Louis, MO) as the antigen (*20*). Test samples were incubated for 2 h at room temperature (RT) in 3 fold serial dilutions with 6 ng of biotinylated CC49 IgG, followed by incubation with alkaline phosphatase–conjugated streptavidin (Jackson ImmunoResearch Lab, West Grove, PA) for 1.5 h at RT. *p*-Nitrophenyl phosphate was used as the substrate and the absorbance was measured at 410 nm using a Dynatech MR 5000 automatic 96-well microtiter reader (Chantilly, VA).

The quality control test of radiolabeled CC49 scFv forms was performed by solid phase RIA in which BSM or BSA (positive and negative controls, respectively) was attached to a solid-phase matrix (Reacti-Gel HW-65F; Pierce, Rockford, IL) (*20*, *21*). Binding was allowed to proceed for 1 h at RT. The unbound radiolabeled protein was removed by repeated centrifugation and washing with PBS containing 1% BSA and 0.1% Tween 20. The radioactivity in the pellet was counted in a gamma scintillation counter and the total percent bound to the BSM beads was calculated.

The affinities of CC49 IgG, $(scFv)_2$-His_6 and $sc(Fv)_2$-His_6 for BSM were determined using surface plasmon resonance in a BIAcore instrument (Pharmacia Biosensor, Uppsala, Sweden). Approximately 400 resonance units (RU) of BSM or BSA were coupled to a CM5 sensor chip as described earlier (*19*, *24*). Binding analyses were performed in HBS buffer (10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) at a flow rate of 75 μl/min at 25°C. The surface was regenerated with 6 M guanidine, 0.2 M acetic acid, at a flow rate of 5 μl/min with no loss of activity. The IgG used for binding analysis was purified on a protein G Sepharose Fast Flow column (Pharmacia Biotech., Uppsala, Sweden) and dialyzed in HBS buffer. The kinetic rate constants (k_{on} and k_{off}), as well as the equilibrium association (K_A) and dissociation (K_D) constants were determined using BIAevaluation 3.0.2 software supplied by the manufacturer where the experimental design correlates with the Langmuir 1:1 interaction model (*25*).

Biodistribution and Pharmacokinetics Studies—Female athymic mice (*nu/nu*) were obtained from Charles River (Wilmington, MA) at 4–6 weeks of age and injected subcutaneously with 4×10^6 human colon carcinoma cells (LS-174T) (*26*). Tumor xenograft bearing animals were used for the biodistribution study 10 days after injection of cells. Dual-label biodistribution studies were performed after simultaneous *i.v.* injections *via* the tail vein of: ^{125}I-$(scFv)_2$-His_6 (5 μCi) and ^{131}I-$(scFv)_2$ (2.5 μCi) or ^{125}I-$sc(Fv)_2$-His_6 (5 μCi) and ^{131}I-$(scFv)_2$-His_6 (2.5 μCi). At designated times, groups of six mice were euthanized, dissected and the major organs were weighed and counted in a gamma scintillation counter. The percentage of the injected dose per gram of tissue (% ID/g) was calculated For whole body retention studies, mice bearing the LS-174T xenografts (three/group) were injected *via* the tail vein with 1.5 μCi of radiolabeled scFvs. Each scFv construct was evaluated separately. The whole body radioactivity was determined at various times after injection in a custom built NaI crystal.

Blood clearance studies were done as described previously (*9*, *19*). Blood samples were obtained from the tail vein at various times after injection of 10 μCi of the individual radioiodinated dimers. Data from two independent studies of 6 mice/study are presented. The half-lives were calculated using a numerical module of the SAAM II computer program (SAAM Institute, University of Washington) for kinetic analysis. The data were fitted to a bi-exponential equation with a bolus injection as an experimental model. The clearance rates were compared by two-samples Student's *t*-test for differences between means.

RESULTS

Construction, Expression, and Purification of Divalent CC49 scFvs—The scFv constructs were engineered from the variable regions of murine MAb CC49. For monomeric scFv, the variable regions of the light chain (V_L) and heavy chain (V_H) were connected using a 25-amino-acid linker (Fig. 1B). In the covalently-linked divalent form, the monomeric units were associated in tandem using the 205C linker (Fig. 1D). A His-tag was introduced at the 3′ end of the constructs to facilitate purification by IMAC. The scFvs were secreted into the medium by cloning the recombinant gene in-frame to an export signal (secretion signal sequence from the *S. cerevisiae* α-factor prepro-peptide) (*14*). The monomer scFv construct expressed in *P. pastoris* yielded a non-covalent dimer $(scFv)_2$-His_6 that represented 60–70% of the total scFv. The divalent scFvs were separated from the aggregated and/or monovalent forms by size-exclusion chromatography.

Characterization of $(scFv)_2$-His_6 and $sc(Fv)_2$-His_6—The single chain antibody forms obtained after IMAC and size-exclusion chromatography were routinely greater than 90–95% pure on SDS-PAGE (Fig. 2). The non-covalent dimer migrated as a 30 kDa band (size of the monovalent scFv) under reducing and non-reducing conditions (Fig. 2; lanes 1 and 3) due to the dissociation of the monomer subunits by the ionic detergent. The $sc(Fv)_2$-His_6 appeared as a major band of 58 kDa with accompanying minor bands of 45 and 30 kDa (Fig. 2; lanes 2 and 4). The purity of the scFv preparation was also confirmed by HPLC size-exclusion chromatography (data not shown). Purified scFvs were radioiodinated to evaluate the effect of labeling on the immunoreactivity of the molecule. HPLC analysis indicated that nearly all ^{125}I/^{131}I (≥95%) was associated with the scFv peak suggesting that radioiodination does not alter the overall structure of scFvs (Fig. 3). This was confirmed in a series of binding studies. The immunoreactivity of the radiolabeled form was determined by solid phase RIA using antigens attached to Reacti-Gel beads. BSM, which contains the epitope recognized by MAb CC49, was used to determine the specific binding whereas BSA was used to evaluate control binding. The $(scFv)_2$-His_6, $sc(Fv)_2$-His_6, and intact CC49 IgG showed specific binding of 82–85, 78–81, and 87–90%, respectively. Non-specific binding to BSA-coated beads was ≤0.5%.

Surface plasmon resonance studies using BIAcore were performed to detect the real-time interaction between scFvs and BSM and to measure the kinetic parameters of the interaction. As shown in Fig. 4, the k_{on} rates for $(scFv)_2$-His_6 and CC49 IgG were similar (1.84×10^5 M^{-1} s^{-1} and 2.37×10^5 M^{-1} s^{-1}, respectively) and about 10 fold higher as compared to $sc(Fv)_2$-His_6 (2.22×10^4 M^{-1} s^{-1}). However, the k_{off} rate for $sc(Fv)_2$-His_6 (8×10^{-4} s^{-1}) was approximately 5- and 2.5-fold slower than for $(scFv)_2$-His_6 (4.31×10^{-3} s^{-1}) and CC49 IgG (2.07×10^{-3} s^{-1}), respectively. The slower k_{off} of $sc(Fv)_2$-His_6 may be due to the formation of stable divalent complexes in comparison with $(scFv)_2$-His_6. The association constants (K_A: k_{on}/k_{off}) as determined by BIAcore analysis for $(scFv)_2$-His_6 and $sc(Fv)_2$-His_6 obtained from yeast were 4.28×10^7 and 2.75×10^7 M^{-1}, respectively, and were comparable to those of scFvs obtained from *E. coli* (*9*, *19*). The K_A for the CC49 IgG was 1.14×10^8 M^{-1} and was therefore 2.5- to 4-fold higher than the values obtained for the divalent scFvs.

Pharmacokinetic Studies with $(scFv)_2$-His_6 and $(scFv)_2$—Pharmacokinetic studies with non-covalent dimer produced in *P. pastoris* and *E. coli*, *i.e.* $(scFv)_2$-His_6 and $(scFv)_2$, showed a rapid clearance from the whole body with 50% being cleared by 4 h (data not shown). However, a much slower blood clearance was observed for $(scFv)_2$-His_6 as compared with $(scFv)_2$ (Fig. 5). The $T_{1/2}$ α values for $(scFv)_2$-His_6 and $(scFv)_2$ were 10 and 8 min with $T_{1/2}$ β values of 76 and 59 min, respectively (Table II). The data were analyzed using a bi-exponential model showing an alpha phase $T_{1/2}$ (the clearance of molecules from the blood to the extravascular space) and a beta phase $T_{1/2}$ (the clearance of molecules from blood to the non-extravascular space or out of the body). However, the overall clearance of radioactivity from the blood pool was observed to be tri-phasic with the third phase (24 and 48 h) being essentially the clearance of free radioiodide. The differences in the blood clearance pattern of the two $(scFv)_2$ were statistically significant ($0.1 \geq p \geq .05$) after 5 min (Fig. 5). The blood clearance data for $sc(Fv)_2$ with- or without- a His-tag was similarly analyzed (*27*). We found a faster clearance for $sc(Fv)_2$ without the

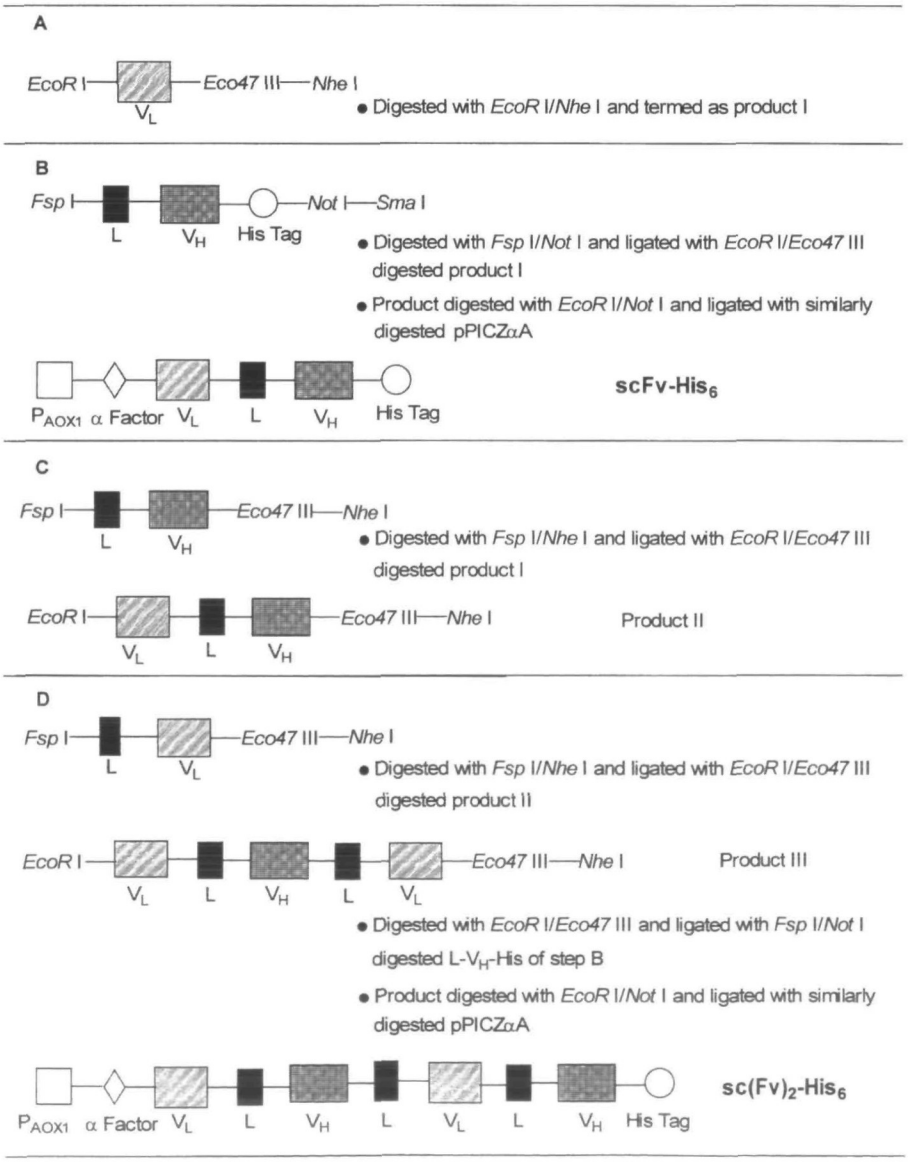

Fig 1. **Schematic representation of the construction of CC49 scFvs (monomer and covalent dimer) for the expression in *Pichia pastoris*.** P_{AOX1}, promoter for the alcohol oxidase gene; V_L, light chain variable region; V_H, heavy chain variable region and L, 205C linker.

His-tag as reflected in the differences in the $T_{1/2}\beta$ values (Table II).

Biodistribution Studies—The tumor-targeting of $sc(Fv)_2$-His_6, $(scFv)_2$-His_6, and $(scFv)_2$ was analyzed by biodistribution studies in mice bearing LS-174T human colon carcinoma xenografts. As shown in Table III, the %ID/g for various organs studied did not differ appreciably between the non-covalent dimers (with- or without-His-tag) and covalent dimer (with His-tag). At 6 h post injection, the %ID/g values for $sc(Fv)_2$-His_6, $(scFv)_2$-His_6, and $(scFv)_2$ in tumors were 11.78, 12.52, and 11.42, respectively (Table III). The radiolabeled constructs revealed no specific uptake in any of the normal tissues tested. The %ID/g values for $sc(Fv)_2$-His_6, $(scFv)_2$-His_6, and $(scFv)_2$ in kidneys were 21.27, 19.99, and 41.22, respectively, at 30 min post-injection (Table III). At 24 h post-administration, the radiolocalization indices (RI = ratio of the %ID/g in tumor to the %ID/g in normal tissue) for blood, liver and spleen were 34.6, 9.5, and 12.2, respectively, for $sc(Fv)_2$-His_6; 44.9, 11.1, and 18.4, respectively, for $(scFv)_2$-His_6; and 41, 7.7, and 16.2, respectively, for $(scFv)_2$. Overall, divalent scFvs (with- or without-His-tag) showed comparably high RI values.

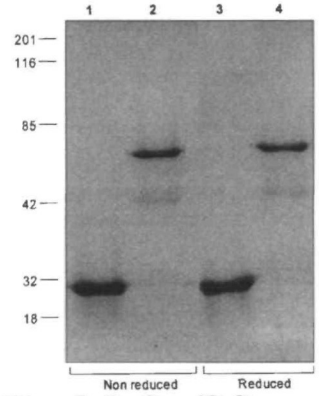

Fig. 2. **SDS-PAGE analysis of purified monovalent and divalent CC49 scFvs under reducing and non-reducing conditions.** Lanes 1 and 3, $(scFv)_2$-His_6; lanes 2 and 4, $sc(Fv)_2$-His_6. Molecular weight markers include myosin (201 kDa), β-galactosidase (122 kDa), bovine serum albumin (85 kDa), carbonic anhydrase (41 8 kDa), soybean trypsin inhibitor (31.8 kDa), and lysozyme (18 kDa).

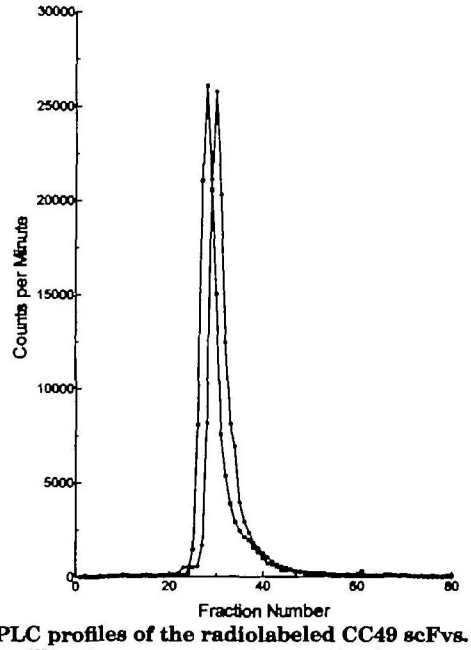

Fig 3. **HPLC profiles of the radiolabeled CC49 scFvs.** After radiolabeling, ^{125}I-scFvs were analyzed using TSK G2000SW and TSK G3000SW size exclusion columns connected in series. The $(scFv)_2$-His_6 (●) and $sc(Fv)_2$-His_6 (■) were eluted as single peaks with no evidence of aggregates or breakdown products.

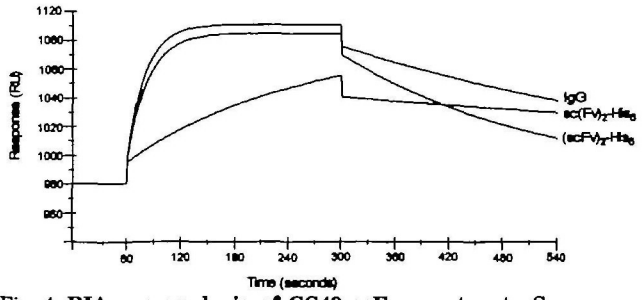

Fig 4 **BIAcore analysis of CC49 scFv constructs.** Sensogram demonstrating the binding and dissociation of MAb CC49 IgG, $(scFv)_2$-His_6, and $sc(Fv)_2$-His_6 to BSM on the biosensor chip.

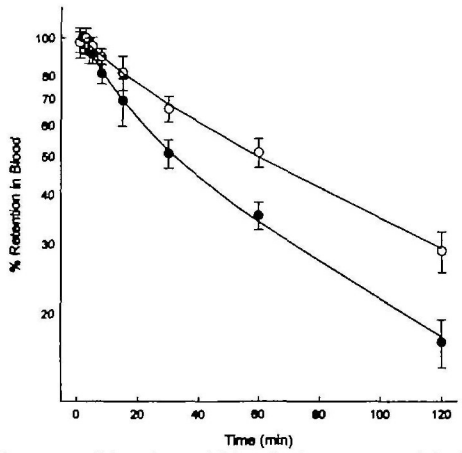

Fig. 5 **Pharmacokinetics of blood clearance of $(scFv)_2$-His_6 (○) and $(scFv)_2$ (●) in athymic mice bearing the LS-174T colon carcinoma xenograft.** ^{125}I-$(scFv)_2$-His_6 and ^{131}I-$(scFv)_2$ were co-injected *i. v.* into mice bearing subcutaneous tumors. Blood samples were obtained at the times indicated. Each data point represents the average of two experiments.

TABLE II. **Estimated values of the half-lives in blood of engineered CC49 divalent scFvs with or without a C-terminal His-tag.**

Ig form	$T_{1/2}$* of scFv constructs expressed in:			
	P pastoris (with His-tag)		*E coli* (without His-tag)	
	$T_{1/2}\alpha$ (min)	$T_{1/2}\beta$ (min)	$T_{1/2}\alpha$ (min)	$T_{1/2}\beta$ (min)
Non-covalent dimer	10	76	8	59
Covalent dimer	6	88	8	55

*The clearance curve was determined as a best fit for a two-compartment clearance model with an α and β phase.

TABLE III **Biodistribution of CC49 sc(Fv)$_2$-His$_6$, (scFv)$_2$-His$_6$, and (scFv)$_2$ (percent injected dose per gram) in athymic mice bearing LS-174T xenografts.**

Tissue	Time (h)						
	0.5	1	4	6	16	24	48
sc(Fv)$_2$-His							
Tumor	9.91	8.49	13 87	11 78	7.49	6.58	3 67
Blood	26.83	17.59	5 62	2.23	0 51	0 26	0.11
Liver	9 05	5 12	2 46	1.98	1 23	0 69	0 21
Spleen	15 19	8.09	4 11	2 29	1 17	0 54	0 16
Kidneys	21.27	16.36	5.38	2.37	0.66	0.32	0.15
Heart	4.26	2 98	1.13	0.82	0.24	0.11	0.04
Lungs	7.17	4.06	2.26	1.52	0.63	0.24	0.09
(scFv)$_2$-His$_6$							
Tumor	10 47	11 85	15.04	12.52	9.81	8.08	3.08
Blood	24.80	17 19	4.31	2.45	0.38	0.18	0.06
Liver	6.48	5.81	3.69	2.54	1.39	0.73	0.13
Spleen	8.84	6 99	3 66	2.59	0.67	0.44	0.20
Kidneys	19.99	14 80	4.24	2.54	0.53	0 45	0.18
Heart	5.27	3 94	1 20	0 76	0 13	0 07	0 06
Lungs	6.73	4 49	2 43	1 45	0.24	0.15	0 05
(scFv)$_2$							
Tumor	10 41	11 32	14.03	11.42	8 72	6.97	2.41
Blood	20 27	11 45	2 82	1.84	0.21	0.17	0.06
Liver	7.20	6 43	3 64	2 75	1 73	0 91	0.18
Spleen	7.82	6.60	2 82	2 24	0 73	0 43	0 22
Kidneys	41 22	24.68	3.79	2.12	0 67	0.48	0 19
Heart	4 42	3 03	0 87	0.58	0 13	0.07	0.07
Lungs	5 42	3.43	1.78	1.19	0 22	0.13	0.05

The SEM for the samples were less than 20% of the corresponding tissue.

DISCUSSION

In antibody-based therapy, recombinant scFvs have been found to have immense potential in clinical applications for diagnostic imaging and designing therapeutic modalities (*2*, *8*, *28*, *29*). Monovalent scFvs, however, have limited utility as carrier vehicles for radionuclides or cytotoxic agents *in vivo* due to their rapid clearance from the circulation and lower quantitative tumor retention than bivalent antibody fragments (*2–4*). For TAG-72, a highly polymeric antigen, a significant increase in binding avidity has been reported for divalent forms such as F(ab')$_2$ and IgG as compared to monovalent fragments such as scFv and Fab (*3*). Stable divalent and multivalent scFvs are being engineered in an effort to improve functional affinity, *in vivo* tumor-targeting and to achieve more selective localization (*10*).

In the present study, we engineered and expressed divalent scFv constructs of the tumor-associated MAb CC49 in *P. pastoris*. The most frequently used prokaryotic vector system for the expression of scFvs has been *E. coli*, in which the scFvs are obtained by either *in vitro* refolding from inclusion bodies or by secretion to the bacterial periplasm (*30*). Much effort has been made to improve the functional yields of scFvs from *E. coli*; however, the methods require extensive optimization at purification levels with low yields of functional scFvs from the "periplasmic inclusion bodies" (*31*). Furthermore, for clinical applications, the removal of bacterial endotoxins is essential (*8*, *17*). Recently, the powerful expression/secretion system of methylotrophic yeast *P. pastoris* has been used to produce large amounts of recombinant proteins and has been extended to the production of bi-functional proteins of scFv with effector domains and to disulfide linked bivalent scFvs (*12*, *32–35*). Some of the recombinant proteins synthesized in *P. pastoris*, such as hepatitis B surface antigen and α-galactosidase, are candidates for Phase I FDA-approved clinical trials (*15*, *18*).

As *P. pastoris* secrete negligible amounts of native proteins, a single-step purification by IMAC yields a ≥95% pure scFv population. The typical yield of purified scFvs obtained was 15–20 mg/liter for (scFv)$_2$-His$_6$ and sc(Fv)$_2$-His$_6$ and 12–15 mg/liter for monovalent scFv-His$_6$. We were, therefore, able to increase the yields significantly as compared to the overall scFv production in *E. coli* (0.2–0.4 mg/liter) (*9*, *19*). The optimal desired expression of the recombinant protein, however, involves the adjustment of several factors including growth and induction conditions, vectors/host strain, copy number of the recombinant DNA, and characteristics of the protein being expressed (*15*). It may be possible to improve the yield of CC49 scFvs in *P. pastoris* further by manipulating the above conditions to match those reported by other investigators (*11*).

The monovalent scFvs were observed to associate and form non-covalent dimers. Our previous studies in *E. coli* also showed the non-covalent association of monomers to generate dimers and multimeric aggregates (*19*, *20*). The spontaneous formation of non-covalently associated divalent and multivalent scFvs has been reported (*36–38*). Size-exclusion chromatography was used to separate the divalent scFvs from the aggregated and/or monovalent forms.

The *in vitro* binding characteristics of the divalent scFvs generated in yeast were comparable to the divalent constructs produced earlier in *E. coli* (*9*, *19*). The K_A value for CC49 scFvs in the present study was based on multiple preparations of the protein, alterations in the flow rate and injection length, and different ligand surface densities. The affinity constants (K_A) for (scFv)$_2$-His$_6$ and sc(Fv)$_2$-His$_6$ were similar and approximately 2.5- to 4-fold lower as compared to parental CC49 IgG. When compared to the monovalent CC49 scFv without His-tag ($K_A = 1.4 \times 10^6$ M^{-1}, data not shown), the divalent scFvs (with- or without-His-tag)

showed approximately 20-fold higher affinities. This increased affinity of dimers can be attributed to the divalent nature of the molecules. The binding kinetics of the His-tagged dimers (non-covalent and covalent) was, however, different. $(ScFv)_2$-His_6 showed a faster k_{on} as compared with $sc(Fv)_2$-His_6, probably due to the more flexible nature of the non-covalent dimer. The $sc(Fv)_2$-His_6, however, after undergoing conformational adjustments at the binding interface, revealed a k_{off} about 5-fold slower compared to $(scFv)_2$-His_6, indicating the formation of more stable complexes.

The *in vivo* biodistribution, pharmacology, and tumor-targeting properties of scFvs were evaluated in athymic mice bearing LS-174T human colon carcinoma xenografts. Both $sc(Fv)_2$-His_6 and $(scFv)_2$-His_6 demonstrated efficient tumor-retention with 11.78 and 12.52 %ID/g at 6 h, respectively. Similar *in vivo* studies with monovalent CC49 scFv showed tumor uptake of only 2.01 %ID/g at 6 h (*9*, *19*). This increased tumor-targeting of dimeric scFv was probably due to the increased functional affinity rendered by divalency along with the higher molecular weight providing an increased residence time in the blood. Both $(scFv)_2$-His_6 and $sc(Fv)_2$-His_6 showed rapid blood and whole-body clearance suggesting the elimination of the scFvs from the body, probably through the urine.

In the present study, we added a His-tag at the carboxyl-terminus of the scFvs to facilitate purification by IMAC. In order to investigate the effect of His-tag on the *in vivo* characteristics of scFvs, a comparative biodistribution study involving divalent scFvs with- and without a His-tag was performed. Similar tumor-uptakes for both $(scFv)_2$-His_6 and $(scFv)_2$ were obtained. Casey *et al.* (*12*) also observed no alteration in the biodistribution and tumor localization of His-tagged anti-carcinoembryonic antigen scFv (*12*). Interestingly, the blood clearance of $(scFv)_2$-His_6 was found to be significantly slower than $(scFv)_2$. Also, the %ID/g value for kidneys at 30 min post injection was found to be two fold higher for $(scFv)_2$ as compared to $(scFv)_2$-His_6. The mechanism regulating the reduction of kidney uptake of $(scFv)_2$-His_6 remains speculative at this point. There are reports showing that basic compounds can inhibit the tubular resorption of peptides and proteins thereby lowering the kidney uptake of antibody fragments (*39*).

In summary, non-covalent and covalent divalent CC49 scFvs were engineered, expressed, and purified using the *P. pastoris* expression system with yields 30- to 40- fold higher than those earlier obtained using *E. coli*. The *in vitro* binding affinities of divalent CC49 scFvs to BSM were found to be similar to the divalent constructs engineered and expressed in *E. coli* (*9*, *19*). The *in vivo* studies of divalent His-tagged scFvs generated in *P. pastoris* were found to be comparable in affinity and tumor-targeting properties to those expressed in *E. coli*. A slower blood clearance of $(scFv)_2$-His_6 as compared to $(scFv)_2$ was, however, observed. The longer biological half-life of His-tagged scFvs in blood along with their bivalent nature makes them strong candidates for cancer radioimmunotherapy.

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