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# The in vivo characteristics of genetically engineered divalent and tetravalent single-chain antibody constructs☆

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**Abstract**

Engineered multivalent single-chain Fv (scFv) constructs have been demonstrated to exhibit rapid blood clearance and better tumor penetration. To understand the short plasma half-life of multivalent single-chain antibody fragments, the pharmacokinetic properties of covalent dimeric scFv [$sc(Fv)_2$], noncovalent tetrameric scFv {$[sc(Fv)_2]_2$} and IgG of MAb CC49 were examined. The scFvs displayed an ability to form higher molecular aggregates in vivo. A specific proteolytic cleavage of the linker sequence of the covalent dimeric or a deterioration of the noncovalent association of the dimeric scFv into tetravalent scFv constructs was not observed. In conclusion, $sc(Fv)_2$ and $[sc(Fv)_2]_2$ are stable in vivo and have significant potential for diagnostic and therapeutic applications.


*Keywords:* Monoclonal antibodies; Immunotherapy; Pharmacokinetics; Cancer therapy

## 1. Introduction

Antibody-based radio-immunotherapy has been more successful in the management of hematological malignancies than solid tumors [1–5] because of the easier access of the therapeutic antibody to its target antigen on tumor cells. In solid tumors, however, the antigens are well "protected" due to poor vascular supply and high tumor interstitial pressures [6]. Additionally, the therapeutic antibody has to overcome large transport distances on its way to its target in solid tumors [7]. To overcome these obstacles, great effort has been undertaken to increase the diffusion of therapeutic antibodies into the tumor.

A common approach for increasing tumor diffusion is the generation of antibodies with lower molecular weights [8]. Several strategies resulting in low molecular weight antibodies with unaltered binding of the antigen have been described [9]. We have developed several single-chain derivatives of CC49, a murine MAb directed against the cancer-associated glycoprotein TAG-72 [10]. These include monovalent (scFv) and divalent $sc(Fv)_2$ forms as well [11,12]. One of the characteristics of these proteins was the formation of noncovalent dimers resulting in noncovalent dimeric (divalent) $sc(Fv)_2$ and tetrameric (tetravalent) $[sc(Fv)_2]_2$ forms, respectively [11,12]. These noncovalent dimeric and tetrameric forms exhibited an increased biological half-life and higher tumor uptake than their monomeric counterparts [11,13,14].

With the decrease in molecular weight, however, these new molecules were subjected to a rapid in vivo clearance with a dramatic reduction of the biological half-life when compared to whole IgG [14,15]. The shortened in vivo half-life of the antibody fragments opened a whole new avenue for tumor imaging and cancer therapy with a lower delivery of the radioconjugate to healthy organs, while the delivery of the antibody to the tumor is characterized by an improved capability of diffusion [16,17].

The mechanisms underlying the rapid clearance of these antibody fragments, leading to their pharmacological superiority, are not fully understood. While the plasma half-life appears to correlate with the molecular weight from

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monovalent to tetravalent scFvs [18], very little is known about the possible proteolytic cleavage and/or aggregate formation of these antibody fragments in vivo. The multivalent constructs, like the $sc(Fv)_2$ engineered with three linker sequences within the molecule, could be targets for rapid in vivo proteolytic cleavage followed by a fast renal elimination [9]. In the current study, we have examined the in vivo stability and pharmacological properties of the covalent dimeric scFv and the noncovalent tetrameric scFv derived from CC49 MAb as members of multivalent scFvs.

## 2. Materials and methods

### 2.1. Antibody synthesis and purification

The engineering and purification of divalent scFv construct $V_L$-linker-$V_H$-linker-$V_L$-linker-$V_H$-$His_6$ of murine CC49 has previously been described [12]. Upon expression, the covalent dimeric scFv spontaneously formed the noncovalent tetrameric $[sc(Fv)_2]_2$. The scFvs were first purified by nickel affinity chromatography ($Ni^{2+}$-nitrilo triacetic acid Superflow, Quiagen, Valencia, CA), and dimeric and tetrameric forms were further purified by size fractionation on a Superdex 200 column (1.6×60 cm, Pharmacia, Upsala, Sweden), concentrated (Cententricons Y-10, Millipore, Bedford, MA) and stored at −70°C. Protein concentrations were assessed with the Bio-Rad $D_C$ protein assay (Bio-Rad Laboratories, Hercules, CA) using the BSA standard curve.

The purity and integrity of the constructs were analyzed by SDS-PAGE according to the method described by Laemmli [19]. The electrophoresis was performed using a 10% acrylamide gel under reducing and nonreducing conditions. Gels were either stained with Coomassie blue R250 or exposed directly to film for autoradiography.

### 2.2. Determination of immunoreactivity

The specific immunoreactivity of the proteins at various points in the purification process was assessed by solid-phase competitive ELISA. As previously published [12], polystyrene plates were coated with 50 ng/well bovine submaxillary gland mucin (Sigma, St. Louis, MO) and blocked with 5% BSA at 37°C for 1 h. Threefold serial dilutions of CC49 IgG and scFvs, prepared with 6 ng of biotinylated CC49 IgG, were added to the wells and incubated for 2 h at room temperature. After washing, the plates were incubated with alkaline phosphatase-conjugated streptavidin for 90 min. The reaction was developed using *p*-nitrophenyl phosphate as a substrate (KPL, Gaithersburg, MD) and the absorption was read at 410 nm using a Dynatech MR 5000 automatic microtiter plate reader.

### 2.3. I and $^{131}I$ labeling and analysis of radiolabeled scFv forms

The scFv constructs and CC49 IgG were labeled with $Na^{125}I$ as described by Colcher et al. [20]. In a tube coated with 20 μg Iodo-Gen (Pierce, Rockford, IL USA), 100 μg protein was mixed in 0.1 M sodium phosphate buffer with 0.1 mCi $Na^{125}I$ or $Na^{131}I$, and incubated for 3 min at room temperature. The samples were subjected to size exclusion chromatography over a Sephadex 25(Sigma) column for the removal of free iodine, and the labeled protein was collected in the void volume. Instant thin-layer chromatography (ITLC) was performed with the purified, labeled product and the reaction mixture with 25% methanol in water as a solvent to determine the percentage of free iodine.

### 2.4. HPLC analysis

The degree of aggregation or degradation following the labeling procedure, as well as in mouse sera for the in vivo stability studies, was monitored by HPLC gel-filtration. Samples were injected onto a TSK G2000 and TSK G3000 (Toso Haas, Tokyo, Japan) size exclusion column connected in a series with 67 mM phosphate and 100 mM KCl buffer (pH 6.8) as the mobile phase at a flow rate of 0.5 ml/min. The absorption was monitored at 280 nm and 0.25-ml fractions were collected. The radioactivity of the fractions was determined with a Packard Minaxi Auto-Gamma 5000 gamma counter (Packard, Meriden, CT).

### 2.5. Assessment of the in vivo stability and blood clearance

For the assessment of the in vivo stability, 5 μCi of radiolabeled scFvs or CC49 whole IgG were injected intravenously in the tail vein of 8-week-old female athymic mice (NIH). After 0.5, 1, 2 and 4 h, animals receiving the $sc(Fv)_2$ and $[sc(Fv)_2]_2$ were euthanized and blood was collected from the left subclavian artery. Due to the longer $t_{1/2}$ of $[sc(Fv)_2]_2$, additional animals were euthanized after 6 and 12 h, while for CC49 IgG, the in vivo stability was determined after 1, 4, 6, 12, 18, 24, 48 and 72 h. The blood was collected for all animals, cells removed and 50 μl of the undiluted serum was applied to the HPLC gel-filtration columns, as described previously. The radioactivity of the fractions was plotted as percentages of the radioactivity loaded on the column.

For the determination of the serum half-lives, 5 μCi of $^{125}I$-labeled $[sc(Fv)_2]_2$ and 2.5 μCi $^{131}I$-labeled $sc(Fv)_2$ were injected intravenously, and 5 μl of blood samples were collected at indicated time intervals. The time point of 1 min was defined as 100%. Regression curves and $t_{1/2}$ were calculated and plotted through the values obtained.

### 2.6. Biodistribution of the scFvs and CC49 MAb

Female athymic mice (8 weeks old), bearing LS174T colon carcinoma xenografts, were used for biodistribution studies. Cells ($3\times10^6$) were implanted subcutaneously, and after 8 to 10 days, when most of the tumors reached a volume of 250–300 $mm^3$, the animals were randomized in groups of five for the biodistribution experiment. Pair-label biodistribution was performed with simultaneous injection of 2.5 μCi of $^{131}I$-labeled $sc(Fv)_2$ and 5 μCi of $^{125}I$-labeled