J. Microbiol. Biotechnol. (2017), 27(4), 718–724
https://doi.org/10.4014/jmb.1611.11062

jmb

Fusion Peptide Improves Stability and Bioactivity of Single Chain Antibody against Rabies Virus

Hualong Xi[1], Kaixin Zhang[1], Yanchun Yin[1], Tiejun Gu[1,2], Qing Sun[1], Linqing Shi[1], Renxia Zhang[1], Chunlai Jiang[1,2], Wei Kong[1,2], and Yongge Wu[1,2]*

[1]*National Engineering Laboratory for AIDS Vaccine, Jilin University, Changchun 130012, P.R. China*
[2]*Key Laboratory for Molecular Enzymology and Engineering of the Ministry of Education, Jilin University, Changchun 130012, P.R. China*

Received: November 23, 2016
Revised: December 24, 2016
Accepted: January 5, 2017

First published online
January 9, 2017

**Corresponding author*
Phone: +86-431-85167829;
E-mail: ygwu@jlu.edu.cn

pISSN 1017-7825, eISSN 1738-8872

The combination of rabies immunoglobulin (RIG) with a vaccine is currently effective against rabies infections, but improvements are needed. Genetic engineering antibody technology is an attractive approach for developing novel antibodies to replace RIG. In our previous study, a single-chain variable fragment, scFv57R, against rabies virus glycoprotein was constructed. However, its inherent weak stability and short half-life compared with the parent RIG may limit its diagnostic and therapeutic application. Therefore, an acidic tail of synuclein (ATS) derived from the C-terminal acidic tail of human alpha-synuclein protein was fused to the C-terminus of scFv57R in order to help it resist adverse stress and improve the stability and half-life. The tail showed no apparent effect on the preparation procedure and affinity of the protein, nor did it change the neutralizing potency in vitro. In the ELISA test of molecular stability, the ATS fusion form of the protein, scFv57R-ATS, showed an increase in thermal stability and longer half-life in serum than scFv57R. The protection against fatal rabies virus challenge improved after fusing the tail to the scFv, which may be attributed to the improved stability. Thus, the ATS fusion approach presented here is easily implemented and can be used as a new strategy to improve the stability and half-life of engineered antibody proteins for practical applications.

Keywords: Rabies, scFv, rapid fluorescent focus inhibition test (RFFIT), acidic tail of synuclein (ATS), mouse challenge model

Introduction

Rabies is an ancient and highly lethal disease caused by the rabies virus (RV), infecting both humans and animals [1]. With a global distribution, it has been reported in more than 87 countries worldwide [2]. According to the World Health Organization, more than 50,000 people die of rabies each year, and about one million people require post-exposure prophylaxis (PEP) against RV after exposure [3]. PEP, which involves injection with a vaccine combined with rabies immunoglobulin (RIG), is the most effective method for preventing rabies infection. Although equine rabies immunoglobulin (ERIG) and human rabies immunoglobulin (HRIG) are widely applied in protective measures currently [4, 5], the former is accompanied by side effects, and the latter has the potential risk of transferring pathogens, as well as high cost. Therefore, an inexpensive and safe alternative is greatly needed [1, 5].

In recent years, with the continuous development of genetically engineered antibody technology, a large number of monoclonal antibodies (mAbs) against RV have emerged and gradually gained attention as potential clinical substitutes for HRIG and ERIG owing to their high specificity, good safety, and low production cost [6]. In particular, single chain antibodies (scFvs), derived from the variable domains of the immunoglobulin heavy (VH) and light (VL) chains linked through a flexible polypeptide linker [7], are easily and economically produced in an *Escherichia coli* expression system as the size is only 1/6 of the whole IgG. Because they have a small molecular weight and are easy to

genetically engineer, scFvs have shown greater advantages than full-length mAbs and are commonly studied at present [8]. However, scFvs generally have weak stability and a short half-life in vivo compared with the full-length Ig, rendering them unfavorable for certain clinical applications.

In this study, an acidic tail of synuclein (ATS) was fused to the C-terminus of the anti-RV scFv57R, which was derived from the clinical mAb CR57 prepared previously [9, 10], in order to improve the stability and half-life. The ATS originated from the acidic tail of human alpha-synuclein, which functions to prevent the protein from aggregating during biochemical stress conditions within the cell [11, 12]. Some previous studies have shown that a C-terminal protein fusion could be an effective means of preventing target proteins from aggregation by heat treatment and repetitive freeze-thaw cycles. Moreover, in previous reports, the ATS showed a tendency to protect fusion proteins from aggregating in adverse pH or metal ion environment stress [11, 13]. However, whether fusing the tail to recombinant active proteins or antibodies would change their activities or affinities remains unknown.

The scFv57R-ATS fusion protein we prepared demonstrated strong resistance to aggregation when heated at high temperatures compared with the wild-type scFv57R, as well as stronger stability in vitro. Importantly, we found that the tail showed no effect on the affinity of the scFv to RV. We also demonstrated that it endowed the scFv with a higher protection rate against virus challenge in vivo. This study revealed implications for the improvement of proteins or enzymes with inherently poor stability and/or activity. It also suggests further development of the ATS fusion protein in areas of treatment and diagnosis.

Materials and Methods

Construction of Recombinant Vector Expressing scFv57R-ATS

The anti-rabies scFv57R has been described in a previous report [9]. The fusion protein, which was designated as scFv57R-ATS, is composed of scFv57R and the ATS (DPDNEAYEMPSEEGYQDY EPEA) [13]. The purified scFv57R-ATS gene was cloned into a pET20-b (+) vector by T4 DNA ligase to generate the recombinant vector. After transformation of the vector into *E. coli* BL21 (DE3), positive clones with the correct inserts were identified by sequencing.

Expression and Purification of Recombinant scFv57R-ATS

For the expression of scFvs, 10 ml of overnight seed culture was inoculated into 1 L of fresh LB medium and incubated at 37°C and 220 rpm. Cells were induced with 1 mM isopropyl-beta-D-thio galactopyranoside (IPTG) when the cell density reached 0.6–0.8 OD_{600}. After an additional 18 h of induction, cells were harvested and resuspended in TE buffer (50 mM Tris-HCl, pH 8.0). After being lysed by ultrasonication, the supernatants were discarded after centrifugation at 8,000 ×*g* for 20 min, and the inclusion bodies were harvested and resuspended into solubilization buffer (8 M urea, 50 mM Tris-HCl, pH 8.0).

The overnight dissolved proteins were then centrifuged at 14,000 ×*g* for 30 min, and the supernatant was purified by Ni-NTA affinity chromatography. After the sample was completely loaded, the column was washed with 50 mM imidazole and the target proteins were eluted at 300 mM imidazole. To identify scFv57R and scFv57R-ATS, SDS-PAGE and western blot analysis were performed. Detection was performed with a mouse anti-His mAb, followed by an anti-mouse antibody conjugated with alkaline phosphatase [14].

Protein Refolding by Dialysis and Separation of Monomers and Polymers

The purified solution was then diluted with 8 M urea to the final concentration of 0.1 mg/ml. Renaturation was performed by dialysis, as described in our former study [15]. After refolding and concentration, gel filtration chromatography was performed with a HiLoad XK16/60 Superdex 75 prep grade column (GE Healthcare, USA) for further purification. Finally, the monomers and polymers were separated. The preparation of scFv57R was exactly the same as that for scFv57R-ATS.

Relative Affinity Assay of scFv57R and scFv57R-ATS

Proteins were diluted to 0.1 mg/ml in PBS and added to 96-well ELISA plates pre-coated with the inactivated RV aG strain and blocked. After being incubated at 37°C for 1 h and washed with PBS five times, the proteins were then eluted with NH_4SCN at concentrations ranging from 0 to 5 M by shaking at 500 rpm at 37°C [16]. One hour later, NH_4SCN was removed, and the plates were washed. The proteins remaining bound to RV were detected by an anti-scFv57R rabbit antibody and HRP-conjugated goat anti-rabbit IgG, as previously reported [14].

Neutralization Activity Assay by Rapid Fluorescent Focus Inhibition Test (RFFIT)

The RFFIT was carried out here as reported before [15]. Serial 3-fold dilutions of scFv57R, scFv57R-ATS, HRIG, and standard serum with verified potency (21.4 IU/ml) were added to the wells. Virus CVS-11 that could infect 80% of the BSR cells after 24 h of incubation was added, and the antibody/virus mixture was then incubated for 1 h at 37°C. Before the plates were incubated for a further 24 h, 4×10^4 BSR cells were added at the interval. Subsequently, the plates were fixed with 80% acetone for staining at 4°C, followed by incubation with a mouse anti-NP mAb conjugated with FITC at 0.05 ml/well at 37°C for 1 h. The neutralization potency of the test scFvs was calculated against the standard serum by the Reed and Muench method [17].

Molecular Stability Assay of scFv57R and scFv57R-ATS

To investigate their stabilities, protein samples at 0.5 mg/ml in 20 mM PBS (pH 7.0) were heated for 10 min at various temperatures (45°C, 55°C, 65°C, 75°C, and 85°C) and then cooled to room temperature. After centrifugation at 13,523 ×*g* for 10 min, the supernatants were analyzed by 13.5% SDS-PAGE with 5 μg loaded. The proteins (0.1 mg/ml) were also diluted in PBS and added to ELISA plates after various periods of incubation at 37°C. After four PBS washes, proteins remaining bound to the plate were detected with a rabbit anti-scFv57R antibody and HRP-conjugated goat anti-rabbit IgG as mentioned above. The binding activity at 0 h was set as 100%.

To test the stability of scFvs in serum in vitro, they were diluted to 0.1 mg/ml with mouse serum and then incubated at 37°C for various times. The samples at different times were centrifuged at 13,523 ×*g* for 10 min to remove the precipitated proteins, and the supernatants were added to ELISA plates pre-coated with RV for incubation at 37°C for 1 h. The washing procedure and detection were carried out as above. The binding activity at 0 h was set as 100%.

Mouse Challenge Model In Vivo

Sixty Kunming mice (each 17–20 g, female) were divided into six groups of 10 each. In the positive group, mice were injected with HRIG (20 IU/kg) mixed with 2,500 LD_{50}/0.05 ml of RV CVS-24 in the left quadricep muscles [18]. Mice were injected with only the virus as a negative control. In the other four groups, scFv57R and scFv57R-ATS were used at the doses of 10 and 80 IU/kg mixed with challenge virus. Mice were immediately isolated when symptoms of the disease were initially found during a period of up to 28 days.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD) of values obtained from at least three independent experiments.

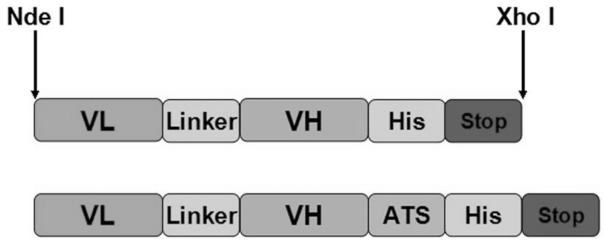

Fig. 1. Graphs of the gene sequence of scFv57R and fusion scFv57R-ATS.

An acidic tail of synuclein (ATS) was connected to the C-terminal of the VH domain. Restriction enzymes NdeI and XhoI were separately introduced at the 5′ and 3′ terminals. The His-tag coding sequence at the C-terminal can help in the purification and identification.

Results

Construction of Recombinant Vector

As depicted in Fig. 1, an ATS was cloned to the C-terminus of scFv57R. For convenient purification, each scFv also contained a His tail at the C-terminus. After sequencing, the correct plasmid was finally transformed into *E. coli* BL21 (DE3) for expression.

Expression and Purification of scFv57R and scFv57R-ATS

After overnight induction, both scFv57R and scFv57R-ATS were expressed in *E. coli* abundantly. Lysates of BL21 cells were separated by centrifugation and analyzed by SDS-PAGE. The results revealed that the scFvs were mainly expressed in the form of inclusion bodies (Fig. 2), which fortunately were solubilized in 8 M urea easily. Purification was performed using nickel affinity chromatography. After elution with 300 mM imidazole, the target protein showed

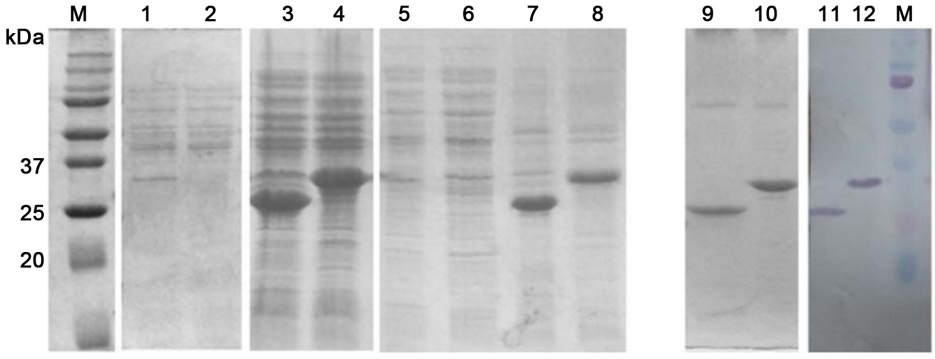

Fig. 2. Expression, purification, and identification of scFv57R and scFv57R-ATS.

Lane M: Molecular weight marker; Lanes 1 and 2: Cells of scFv57R and scFv57R-ATS before induction; Lanes 3 and 4: Cells after IPTG induction; Lanes 5 and 6: Supernatant of scFv57R and scFv57R-ATS cell lysate; Lanes 7 and 8: Inclusion bodies solubilized in 8 M urea; Lanes 9 and 10: Affinity chromatography-purified scFv57R and scFv57R-ATS protein after dialysis; Lanes 11 and 12: Western blotting identification of scFv57R and scFv57R-ATS.

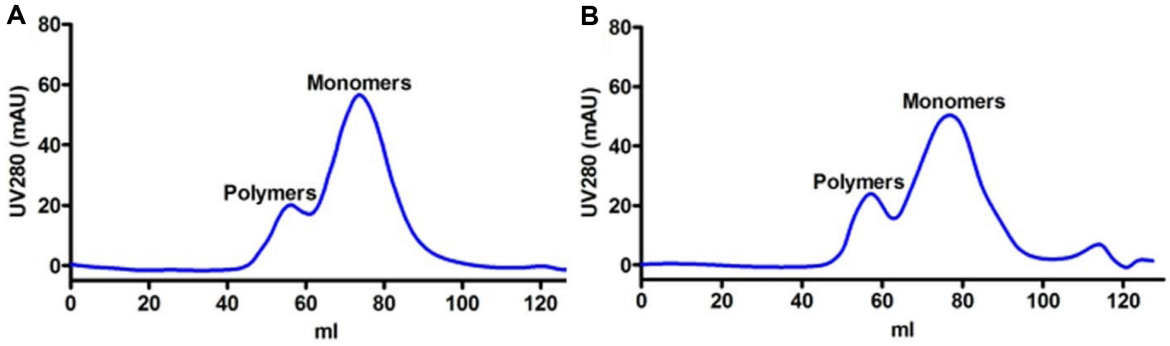

Fig. 3. Further purification by gel filtration chromatography.
(**A**) Profile of gel filtration of scFv57R-ATS. (**B**) Profile of gel filtration of scFv57R. The same amount of proteins were injected to the Superdex75 column to separate monomers and polymers.

a high purity, as identified by both SDS-PAGE and western blot analysis. In reducing conditions, the scFv57R-ATS protein appeared to have a molecular mass of ~2 kDa greater than that of scFv57R and could be detected by an anti-His-tag antibody as expected (Fig. 2).

Inclusion Body Refolding and Separation of Monomers and Polymers

The purified inclusion bodies were dialyzed for refolding into the active form. Before the gel exclusion chromatography, samples were concentrated by ultrafiltration to a concentration above 1 mg/ml. Each peak was collected during gel exclusion chromatography and subsequently analyzed by SDS-PAGE. As shown in Fig. 3, monomers and polymers were successfully separated, and the tail had no apparent effect on their relative amounts.

Relative Affinity Assay

In order to detect whether the ATS affected the scFv binding affinity to RV, a relative affinity assay was performed by an ELISA method. As the concentration of NH_4SCN increased, the OD_{450} absorption value decreased. The concentration of NH_4SCN was 2.1 M when the OD_{450} of the scFv57R sample was decreased to half of the initial value with no NH_4SCN, whereas that of the scFv57R-ATS sample was 2.3 M (Fig. 4). The results demonstrated that the added tag had no effect on the affinity against RV.

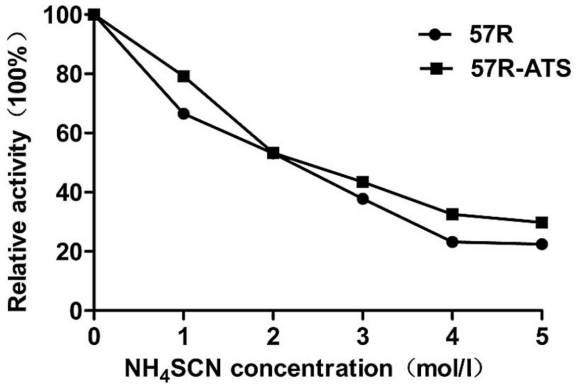

Fig. 4. Relative affinities of scFv57R and scFv57R-ATS measured by ELISA.
The scFvs at the same concentration were incubated and then eluted with various concentrations of NH4SCN (0–5 M). Proteins that remained bound to rabies virus were detected by rabbit anti-scFv57R mAb and HRP-conjugated goat anti-rabbit IgG at the absorbance OD_{450}.

Neutralizing Potency Test In Vitro

RFFIT was used to test the neutralizing potency of scFvs against RV. The protein concentrations were evaluated by the BCA method. The activities of scFvs were calculated and compared with that of the reference standard. HRIG was also used in the test to demonstrate the experimental reliability. Low levels of scFv57R and scFv57R-ATS polymers were detected at 11.7 IU/mg and 7.6 IU/mg, whereas those of scFv57R and scFv57R-ATS monomers were 780 IU/mg

Table 1. Neutralizing potency test.

	scFv57R polymer	scFv57R monomer	scFv57R-ATS polymer	scFv57R-ATS monomer	HRIG
Neutralizing potency (IU/ml)	2.5 ± 0.4	83.8 ± 9.4	2.9 ± 0.5	73.6 ± 10.1	105.2 ± 11.3
Neutralizing potency (IU/mg)	11.7	780.6	7.6	681.1	/

HRIG is polyclonal serum product and the unit 'IU/mg' was meaningless.

and 681 IU/mg, respectively (Table 1). Thus, both monomers were highly active against RV.

Molecular Stability

To investigate the ability of the ATS to suppress aggregation of scFv57R-ATS at high temperatures compared with scFv57R, the thermal behaviors were first detected using SDS-PAGE. As expected, scFv57R-ATS did not precipitate even up to 85°C, whereas scFv57R completely precipitated at 65°C (Fig. 5A). As scFv57R-ATS did not precipitate at high temperatures, its binding ability was measured by ELISA. The half-lives of scFv57R and scFv57R-ATS were determined to be 14.1 h and 33.9 h, respectively (Fig. 5B). Because it would be difficult to detect and evaluate the clearance of scFv57R and scFv57R-ATS in vivo, an in vitro analysis was carried out in serum by ELISA. The half-life of scFv57R-ATS in serum was 15.6 h, which was 4 times higher than that of scFv57R (3.8 h) (Fig. 5C). Results of the stability assay indicated that the tail endowed the protein with improved thermal stability and resistance to metabolism.

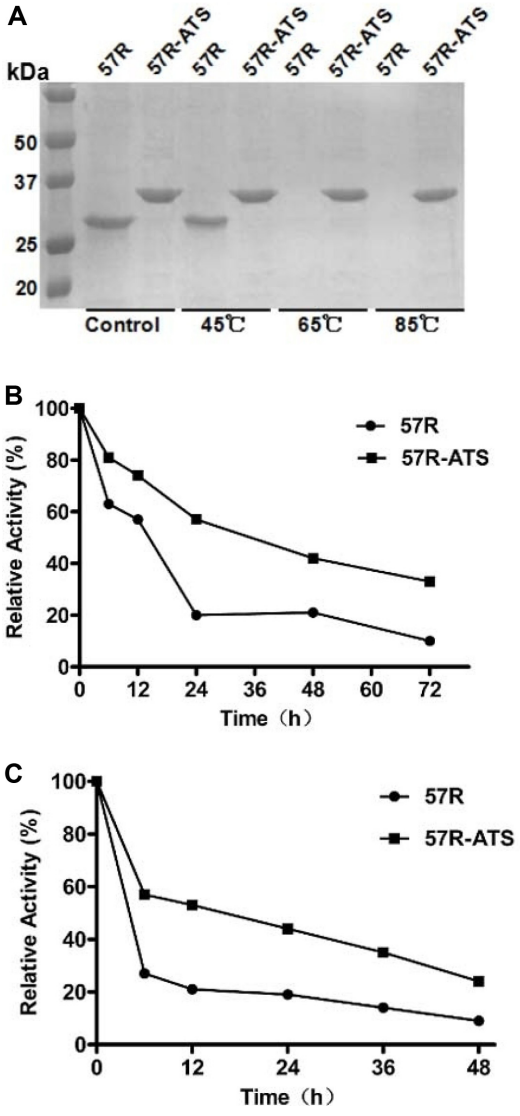

Fig. 5. Molecular stability of scFv57R and scFv57R-ATS.

(**A**) SDS-PAGE analysis of scFv57R and scFv57R-ATS before and after heat treatment at various temperatures for 10 min. (**B**) Short-term storage stabilities measured by ELISA. The scFvs diluted in PBS were incubated at 37°C for 0–72 h and then added to ELISA plates and detected. $p = 0.0106$ in a comparison of the two group. (**C**) Stability from in vitro serum studies. The scFvs diluted in mouse serum were incubated at 37°C for various times and then added to ELISA plates and detected. $p = 0.0080$ in a comparison of the two group.

Challenge Model in Kunming Mice

The in vivo lethal challenge model was established in Kunming mice, and the survival rates against CVS infection are shown in Fig. 6. Statistical analysis showed that the survival rates of groups administered 20 IU/kg HRIG, 80 IU/kg scFv57R-ATS, and 80 IU/kg scFv57R were significantly higher than that of the control group injected with virus only. The scFv57R-ATS provided a protection rate of 90%, which was 10% higher than that conferred by scFv57R. Furthermore, the survival rate of the 10 IU/kg scFv57R-ATS group was 50%, whereas that of the 10 IU/kg scFv57R group was only 20% (Fig. 6). The results indicated that 80 IU/kg scFv57R or scFv57R-ATS provided effective protection against rabies comparable with the positive control group (20 IU/kg HRIG).

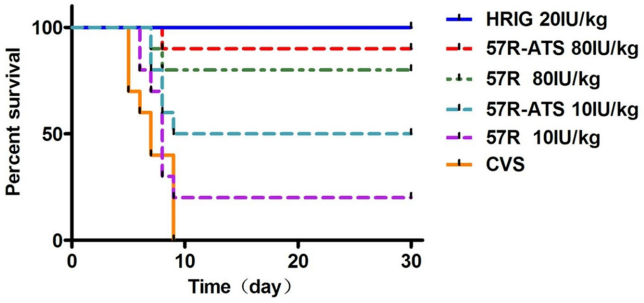

Fig. 6. Mice challenge model in vivo.

Mice (10 mice each group) were challenged with 2,500 LD_{50} CVS mixed with scFvs on day 0. CVS mixed with 20 IU/kg HRIG was the positive control, and CVS only was the negative control. All mice were monitored daily for a period of 30 days. No significant differences (ns) were observed in a comparison of the groups that received 10 IU/kg 57R with the CVS group; $p = 0.0191$ in a comparison of mice that received 10 IU/kg 57R-ATS and CVS.

Discussion

The scFv recombinant antibody format is popular owing to its ease of genetic manipulation and expression, especially in the cost-effective *E. coli* expression system, not only in the field of rabies but also many other diseases [8, 19]. However, the weak stability and rapid clearance of scFvs are serious problems for a wide range of applications from treatments to diagnostics. In order to overcome these disadvantages, many studies have been carried out in an attempt to improve the stability and half-life of scFvs [8, 20, 21].

Our laboratory group is currently focused on developing scFvs for therapeutic and diagnostic uses. In this study, an ATS was fused to scFv57R with the aim of improving its stability and half-life. In previous studies, point mutations in proteins were adopted to induce disulfide bonds, typically to ensure the stability of therapeutic proteins. However, the mismatch of disulfide bonds during renaturation could cause the reduction of the protein, and the introduction of GSSG/GSH increased the expenses [14, 18]. In addition, chemical modifications such as PEG may affect the virus neutralization activity. Abilities of the ATS to resist stress have been reported in many studies. Moreover, the tail is a small molecule derived from a human source, which minimizes the chance of eliciting an immune response.

In this study, the scFv57R-ATS fusion protein was successfully expressed in *E. coli* as inclusion bodies at similar levels as the scFv57R protein. Although expression in a soluble form would be more ideal generally, these proteins could be easily purified from the inclusion bodies at a high yield [22, 23]. Results of this study indicated that the tail did not affect the purification and refolding of scFv57R-ATS, which were performed in the same manner as that for scFv57R. Interestingly, the ATS fusion protein was not more prone to aggregation than the wild-type protein in the refolding step (data not shown). Moreover, the tail did not disturb the complementary determining region. The relative affinities of scFv57R and scFv57R-ATS were also at the same levels. After further purification, both monomers were highly active against RV, and their neutralizing titers were several times higher than that of the Fab antibody derived from the same complete antibody [24]. Notably, another study reported a disulfide-stabilized three-domain scFv with a titer of only 83.3 IU/mg, nearly 9 times lower than its former type, even though it showed improved stability [20].

To characterize the stability of the scFvs, their thermal behaviors were detected by SDS-PAGE and ELISA. The ability of the ATS fusion to suppress aggregation at high temperatures was basically the same that described previously [25]. When incubated in PBS buffer, the fusion scFv had a 2.4-fold longer half-life than the wild type. In a previous study, nearly no increase in half-life was conferred by introduction of a disulfide bond [14].

Once an individual is infected by RV, PEP must be administered [26]. Direct administration of antibodies against RV is extremely important to neutralize the virus before the development of antibodies can be induced by a vaccine [27, 28]. Therefore, a long half-life is an advantage for scFvs, especially in the first 24 h. In order to estimate the clearance of scFvs in the blood, scFv57R and scFv57R-ATS were incubated in serum at 37°C to simulate the metabolism in the blood [29]. The half-life of scFv57R-ATS in serum was 4 times higher than that for scFv57R. This result suggested that the fusion scFv may have a longer half-life in vivo than the wild type. By reducing the length of the linker between the VH and VL domains, scFvs could be designed as dimers (~60 kDa), trimers (~90 kDa), and tetramers (~120 kDa) to be more stable, but those molecules were mainly connected by non-covalent bonds, and the size was difficult to control [30]. In the mouse challenge model, scFv57R-ATS provided greater protection against RV infection in mice, with a better survival rate, especially in the low-dose 10 IU/kg group, which may be attributed to the longer half-life. The advantage of the increased half-life of scFv57R-ATS in both in vitro conditions and serum demonstrated the feasibility of extending its application. Despite all of these results, a big difference remains when comparing the fusion scFv with the full-length IgG in terms of stability and resistance to metabolism.

In summary, an anti-RV scFv and its ATS fusion form were prepared and evaluated in this study. Without altered affinity, scFv57R-ATS demonstrated a notably improved half-life in vitro and in serum. Both scFvs showed protective efficacy against fatal rabies infection in a mouse model. Although the neutralizing titers of the two scFvs were not significantly different, scFv57R-ATS was more effective. Thus, the ATS fusion may be an effective and widely used modification for stabilizing proteins or enzymes in practical applications.

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