



## Research article

# Generation of dimeric single-chain antibodies neutralizing the cytolytic activity of vaginolysin



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## ARTICLE INFO

## Article history:

Received 11 January 2017

Accepted 16 May 2017

Available online 19 May 2017

## Keywords:

Circulation half-life

ELISA

*Gardnerella vaginalis*

Genetic fusion

Hemolysis

Medical biotechnology

Pore-forming toxin

Recombinant antibodies

Therapeutic agent

Vaginal bacterium

Virulence factor

## ABSTRACT

**Background:** *Gardnerella vaginalis* is a bacterial vaginosis (BV)-associated vaginal bacterium that produces the toxin vaginolysin (VLY). VLY is a pore-forming toxin that is suggested to be the main virulence factor of *G. vaginalis*. The high recurrence rate of BV and the emergence of antibiotic-resistant bacterial species demonstrate the need for the development of recombinant antibodies as novel therapeutic agents for disease treatment. Single-chain variable fragments (scFvs) generated against VLY exhibited reduced efficacy to neutralize VLY activity compared to the respective full-length antibodies. To improve the properties of scFvs, monospecific dimeric scFvs were generated by the genetic fusion of two anti-VLY scFv molecules connected by an alpha-helix-forming peptide linker.

**Results:** N-terminal hexahistidine-tagged dimeric scFvs were constructed and produced in *Escherichia coli* and purified using metal chelate affinity chromatography. Inhibition of VLY-mediated human erythrocyte lysis by dimeric and monomeric scFvs was detected by *in vitro* hemolytic assay. The circulating half-life of purified scFvs in the blood plasma of mice was determined by ELISA. Dimeric anti-VLY scFvs showed higher neutralizing potency and extended circulating half-life than parental monomeric scFv.

**Conclusions:** The protein obtained by the genetic fusion of two anti-VLY scFvs into a dimeric molecule exhibited improved properties in comparison with monomeric scFv. This new recombinant antibody might implement new possibilities for the prophylaxis and treatment of the diseases caused by the bacteria *G. vaginalis*.

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## 1. Introduction

Bacterial vaginosis (BV) is a common vaginal condition that affects women of reproductive age [1]. By causing an abnormal malodorous vaginal discharge, BV can develop serious sequelae for the health of women. BV is linked with increased susceptibility to sexually transmitted bacterial and viral infections, including herpes simplex, HPV, and HIV [2,3]. Bacteria associated with BV have been related to an increased risk of premature delivery [4] and adverse neonatal outcomes [5]. BV is characterized by the depletion of dominant vaginal *Lactobacillus* species and an overgrowth of anaerobic bacteria [6]. Although BV is recognized as a polymicrobial disease, *Gardnerella vaginalis* has been recovered from the vaginal samples of almost all women with this microbial shift condition [6,7]. *G. vaginalis* is suggested as the main player in the development of BV as this bacterium is equipped with a number of virulence factors [8]. The

main virulence factor secreted by *G. vaginalis* is the protein toxin vaginolysin (VLY). VLY is a pore-forming toxin and a member of a family of cholesterol-dependent cytolysins [9]. These toxins form holes in the plasma membrane of eukaryotic cells that lead to cell death. VLY is specific to human cells, and its activity depends on cell membrane cholesterol and human complement glycoprotein CD59 [10,11]. The VLY-coding gene has been found in the vast majority of *G. vaginalis* clinical isolates, while the expression level of the toxin varies among isolates [12]. The disparity in the VLY production level may be related to the different cytotoxicity of *G. vaginalis* strains [13].

The high recurrence rate of BV and the emergence of antibiotic-resistant vaginal bacterial species [14,15] prompt the development and use of recombinant antibodies as novel therapeutic agents for disease treatment. The effectiveness of neutralizing recombinant antibodies against bacterial toxins such as botulinum neurotoxin, *Clostridium difficile* Toxin B, and Shiga toxin has been demonstrated [16,17,18].

We have recently developed monoclonal antibodies and single-chain variable fragments (scFvs) against VLY and demonstrated their ability to neutralize VLY catalytic activity *in vitro* [19,20]. Monovalent hybridoma-derived scFvs showed reduced affinity and neutralizing

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

potency as compared to the respective full-length antibodies [20]. The affinity and stability of scFvs could be increased using structure-based engineering [21]. Oligomerization of antibody fragments may result in an increased avidity and half-life extension, allowing the generation of specific and multivalent scFvs [21,22].

In this paper, we describe the development of anti-VLY scFvs generated by the covalent fusion of two scFv molecules connected by an alpha-helix-forming peptide linker. The monospecific dimeric scFv proteins were purified, characterized, and compared with monomeric scFv. The dimeric scFv that showed the highest neutralizing potency compared to monomeric scFv also exhibited extended circulating half-life in mice.

## 2. Materials and methods

### 2.1. Generation of dimeric scFvs and their expression in *Escherichia coli*

For the assembly of dimeric scFvs, two monomeric scFv variants [20] derived from the parental 9B4 monoclonal antibody were used: VL-(G<sub>4</sub>S)<sub>4</sub>-VH (named VL-L4-VH) and VH-(G<sub>4</sub>S)<sub>4</sub>-VL (named VH-L4-VL). The two scFv molecules were connected by an SL linker having amino acid sequence SGLA (EAAAK)<sub>4</sub> ALEA (EAAAK)<sub>4</sub> ALEGS [23]. The DNA fragments coding for VH-L4-VL-SL and VL-L4-VH-SL were amplified using the following primers:

VHVL-fw: AATGGATCCCAGGTTTCAGCTGGAGCAG

VHVL-rev: ATAAAGCTTATTACCGTATTTCAGCTTGGTCC

VLVH-fw: AATGGATCCGATATTGTGATGACACAGTCTACATCCC

VLVH-rev: ATCAAGCTTATTAGGAGGAGACGGTGACTGAGG

*Escherichia coli* DH10B (Invitrogen) was used for all cloning steps. The amplified DNA fragments were verified by sequencing. Two variants of N-terminal hexahistidine-tagged dimeric scFvs were generated: His-tag-(VL-L4-VH)-SL-(VL-L4-VH) (variant AI) and His-tag-(VL-L4-VH)-SL-(VH-L4-VL) (variant AII). The resulting constructions coding for dimeric scFvs were cloned into the expression vector pET-28a(+).

Expression plasmids bearing the genes coding for dimeric scFvs and the plasmid with the gene coding for monomeric scFv (VH-L4-VL) were transformed into *E. coli* BL21(DE3) (Merck). The scFvs synthesis was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific). After induction, the cell pellet was disrupted by sonication and centrifuged. The supernatant (soluble fraction) and the cell pellet (insoluble fraction) were then analyzed by 12.5% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

### 2.2. Purification of dimeric scFvs

Inclusion bodies in the cell lysate were pelleted by centrifugation at 18000 × g for 30 min at 4°C. The pellet fraction was washed twice with 20 mM Tris-HCl (pH 7.0), 1 M NaCl, and 0.1% Tween 80 and once with 20 mM Tris-HCl (pH 7.0). The pellet containing the inclusion bodies was solubilized in 7 M guanidine-HCl and refolded by slow dilution in the presence of oxidation–reduction pair dithiothreitol (DTT) to oxidized glutathione at a ratio of 1 to 5. The target proteins were purified using Ni<sup>2+</sup> IMAC Sepharose 6 Fast Flow column equilibrated with 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 10 mM imidazole. The proteins were eluted stepwise with 500 mM imidazole in the same buffer. The eluted proteins were dialyzed against 20 mM Tris-HCl (pH 8.0) and 300 mM NaCl and centrifuged at 29000 × g for 30 min at 4°C. The concentrations of purified monomeric anti-VLY and dimeric scFvs were determined by the Bradford method [24].

### 2.3. Large-scale biosynthesis of scFvs

Biosynthesis of hexahistidine-tagged monomeric and dimeric scFvs was performed in a 10-L Biostat B bioreactor (Sartorius Stedim). The growing flasks and fermentation in fed-batch mode were conducted at 37°C and pH 6.8 in a chemically defined media [25]. For inoculum preparation, bacteria were grown in chemically defined media in a shake flask overnight. The bacterial suspension was inoculated in a 10-L fermentation unit for further growth. The synthesis of the target proteins was induced with 1 mM of IPTG after 12 h of growth. During the process, the pO<sub>2</sub> value in the bioreactor was maintained at 30% by modulating the stirring and the airflow values. The pH was maintained at 6.8 by the regulated addition of NH<sub>4</sub>OH (25% v/v). The biomass was harvested after 3 h of induction with IPTG by centrifugation at 3500 × g for 30 min at 4°C, and the sample was then frozen at -20°C.

### 2.4. Hemolytic assay

Hemolytic assay was performed as described earlier [19]. Briefly, a human blood specimen was anticoagulated with heparin. The specimen was obtained from a healthy adult volunteer by venipuncture after their written informed consent was approved by the Council of the Institute of Biotechnology of Vilnius University (Protocol no. 54 of 20/11/2013). Erythrocytes were isolated by centrifugation at 1300 × g rpm for 5 min and resuspended in sterile phosphate-buffered saline (PBS) stored at room temperature. Red blood cells were washed with PBS twice. Purified full-length recombinant VLY [20] was added to 1 mL of 0.5% erythrocyte suspension in PBS to obtain a final concentration of 3 ng/mL. After 15 min of incubation at 22°C, the cells were pelleted by centrifugation, and the released hemoglobin was measured at 415 nm wavelength in a microplate reader (Muliskan GO, Thermo Fisher Scientific). Complete cell lysis was obtained by the preparation of the erythrocyte suspension in water (positive control). Cell lysis was not detected when the erythrocyte suspension was prepared in PBS (PBS control).

The level of hemolysis was calculated using the following equation:

$$\frac{\text{OD}_{\text{vly sample}} - \text{OD}_{\text{pbs}}}{\text{OD}_{\text{water control}} - \text{OD}_{\text{pbs}}} \times 100 = \% \text{hemolysis}$$

To verify VLY hemolytic activity before performing the VLY neutralization assays with recombinant scFvs, the erythrocyte lysis curve *versus* the toxin concentration was generated and the amount of VLY that produces 50% hemolysis (HD<sub>50</sub>) under the stated conditions was calculated. The test was performed in triplicate with three technical replicates, and the mean value of HD<sub>50</sub> was 0.56 ng ± 0.05 (10 pM ± 1.0) [11].

For the scFv hemolytic activity assay, VLY was pre-incubated with serial dilutions of scFvs for 30 min at 22°C. The obtained mixture was added to 0.5% erythrocyte suspension in PBS. After 15 min of incubation, the released hemoglobin was measured. The assays were repeated three times with three technical replicates. The data were represented as mean ± standard deviation (SD) calculated using Microsoft Excel. The IC<sub>50</sub> for each scFv was defined as the concentration of the respective recombinant antibody that reduces VLY hemolytic activity by 50%. The differences in blood specimens taken from different volunteers had no effect on the results of the assay.

### 2.5. Detection of circulating half-life of scFvs *in vivo*

The circulating half-life of dimeric and monomeric scFvs was determined using two groups of three female BALB/c mice (5–6 weeks old; weight 18.1 ± 0.4 g). The study using laboratory animals was approved by the State Food and Veterinary Service of the Republic of Lithuania (permission No. 12 of 11-06-2014). Each group of three mice received a single intraperitoneal injection of either

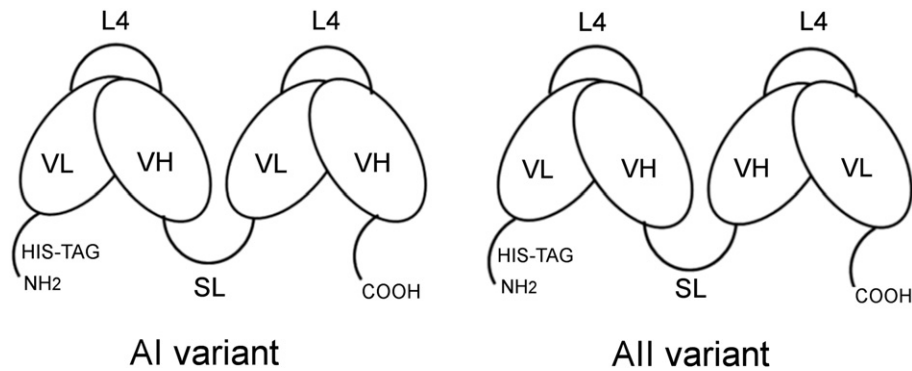


Fig. 1. Schematic representation of dimeric anti-VLY scFvs.

dimeric anti-VLY scFv (variant AI) or monomeric scFv. In total, 50  $\mu\text{g}$  doses of the protein in a volume of 1 mL were injected. Blood samples were drawn from each mouse at selected time points (0.5, 1, 2, 3, 4, 6, 8, and 21 h after injection). Blood samples were diluted 25-fold using PBS containing protease inhibitors (ProteoBlock Protease Inhibitor Cocktail, Thermo Fisher Scientific) and centrifuged at  $2000 \times g$  for 5 min at room temperature. The concentration of scFvs in the blood plasma was determined by enzyme-linked immunosorbent assay (ELISA) as described previously [20]. Blood samples from each mouse were analyzed in triplicate, and the scFv concentration was represented as mean  $\pm$  standard deviation (SD), calculated using Microsoft Excel. Concentration values of scFv proteins in plasma at the selected time intervals after scFv injection were fitted with an exponential function using the GRAFIT software and the coefficient  $k_{\text{app}}$  for each protein was calculated for each of the three mice  $\pm$  standard error of mean (SEM). The circulating half-life of the proteins was calculated according to the function  $T_{1/2} = \ln(2) / k_{\text{app}}$  and was represented as a mean  $\pm$  SEM.

### 3. Results

#### 3.1. Production of dimeric anti-VLY scFvs in *E. coli*

Dimeric anti-VLY scFvs connected by the covalent fusion of two scFv molecules by the 54-aa-long SL linker sequence were constructed. Two variants of dimeric scFvs were obtained: (i) variant AI, where the

DNA fragments coding for monomeric scFv consisting of VL-L4-VH were fused in the same orientation (VL-L4-VH)-SL-(VL-L4-VH), and (ii) variant All, where the DNA fragments coding for monomeric scFvs consisting of VL-L4-VH and VH-L4-VL were fused in opposite orientations (VL-L4-VH)-SL-(VH-L4-VL) (Fig. 1). The SL linker joining the two scFv molecules forms an  $\alpha$ -helix conformation, which could efficiently separate domains of the fusion protein [23,26].

The synthesis of N-terminal hexahistidine-tagged dimeric scFvs was obtained in *E. coli*. The cells were harvested after 1, 2, and 3 h of induction with IPTG, and their lysates were analyzed on SDS-PAGE. The expression level of dimeric scFvs ranged up to 20% of the total cell protein (Fig. 2a). The expression levels of AI and All variants of dimeric scFvs were similar. The analysis of soluble and insoluble fractions of the respective proteins of 57.8 kDa demonstrated that the major part of the proteins was detected in the insoluble fraction of the cell lysate (Fig. 2).

The dimeric scFvs were purified from inclusion bodies by refolding and the subsequent application of nickel chelate affinity chromatography. The purified scFv proteins appeared as a single band on SDS-PAGE under reducing conditions (Fig. 2b), indicating that two molecules of the monomer were fused *via* peptide linkage. The monomeric scFv (VH-L4-VL) used as a control was purified by using the same purification scheme (Fig. 2a, lane 3).

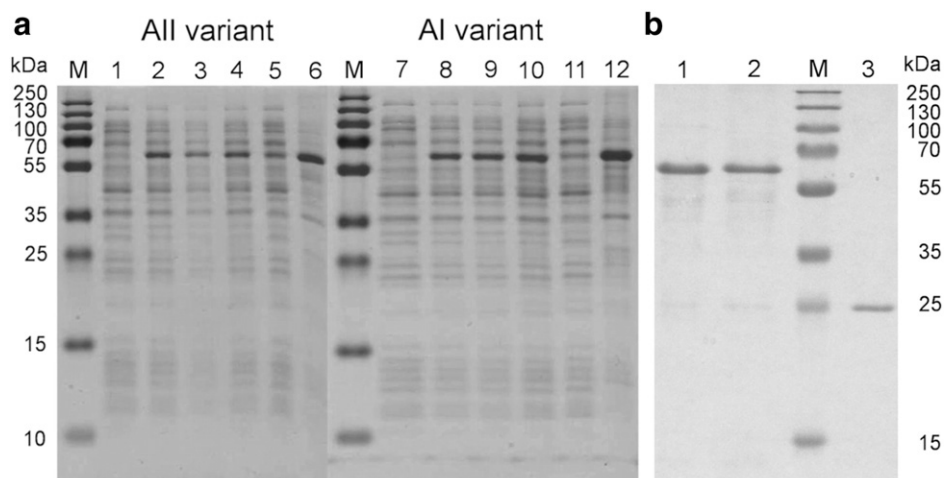
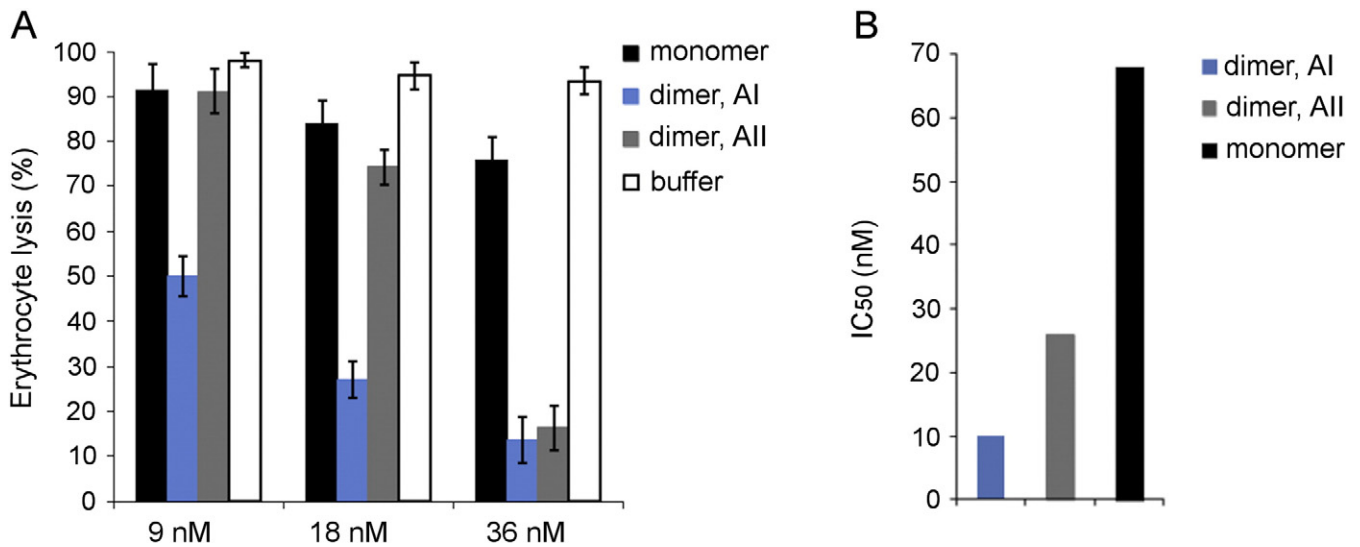


Fig. 2. (a) SDS-PAGE of dimeric anti-VLY scFv (variants AI and All) in different fractions of *E. coli* cell lysate. Lane M: molecular weight marker (Thermo Fischer Scientific); lanes 1 and 7: cell lysate before induction; lanes 2–4 and 8–10: cell lysate after 1, 2, and 3 h of induction; lanes 5 and 11: soluble fraction of the cell lysate; lane 6 and 12: insoluble fraction of the cell lysate. (b) SDS-PAGE of purified scFvs. Lane 1: dimeric scFv, variant AI; lane 2: dimeric scFv, variant All; lane 3: monomeric scFv; lane M: molecular weight marker.



**Fig. 3.** Inhibition of VLY-mediated hemolysis of human erythrocytes by scFvs. (A) Erythrocyte lysis was evaluated after the incubation of VLY with dimeric scFvs (variants AI and AII), monomeric scFv, and PBS. Released hemoglobin was detected at the indicated concentrations (9, 18, and 36 nM) of scFvs. The assays were repeated three times with three technical replicates. The data were represented as mean  $\pm$  standard deviation (SD) calculated using Microsoft Excel. (B) Graphical presentation of IC<sub>50</sub> values of monomeric and dimeric scFvs (variants AI and AII).

### 3.2. Hemolytic activity of dimeric scFvs

The ability of both monomeric and dimeric scFvs to inhibit the hemolytic activity of VLY was detected using human erythrocytes. VLY inactivation by dimeric scFvs was dose dependent, and the dimeric scFvs demonstrated stronger neutralizing potency than monomeric scFv (Fig. 3a). The IC<sub>50</sub> values calculated from concentration-dependent curves were 68 nM for monomeric scFv and 10 nM and 26 nM for dimeric scFvs variants AI and AII, respectively (Table 1, Fig. 3b). Variant AI exhibited stronger neutralizing activity (IC<sub>50</sub> = 10 nM) than variant AII (IC<sub>50</sub> = 26 nM).

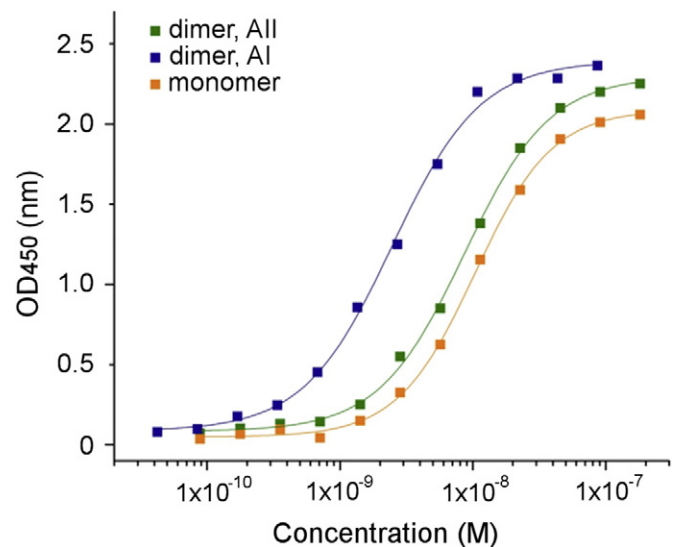
The VLY-binding activities of recombinant purified scFvs were investigated by an indirect ELISA as described previously [20]. Both AI and AII variants of dimeric scFvs recognized VLY immobilized on the plate in a concentration-dependent manner (Fig. 4). To evaluate the affinity of dimeric scFvs, their apparent dissociation constant (K<sub>d</sub>) values were calculated from concentration-dependent curves as described previously [20] and compared to the K<sub>d</sub> values of monomeric scFv. The calculated K<sub>d</sub> values were 10.4 nM for monomeric scFv, and 2.5 nM and 8.6 nM for dimeric scFvs variants AI and AII, respectively (Table 1). The calculated IC<sub>50</sub> and K<sub>d</sub> values demonstrated that dimeric scFvs had higher neutralizing potency and higher affinity to VLY than monomeric scFv. Moreover, dimeric scFv variant AI showed higher VLY-binding capacity and higher neutralizing potency than the dimeric scFv variant AII.

### 3.3. Circulation half-life of scFvs in vivo

Small proteins are rapidly cleared from circulation; therefore, various half-life extension strategies have been proposed for therapeutic

applications [27]. One of the approaches includes the increase of the hydrodynamic radius of the target protein by fusion or conjugation with the protein compound [27]. A covalent fusion of two recombinant antibodies may increase the hydrodynamic radius of the molecule, and that promotes the extension of the half-life of the resulting protein.

To estimate the half-life of monomeric and dimeric scFvs *in vivo*, two groups of mice (n = 3 per group) were injected with either monomeric scFv or dimeric scFv variant AI, which showed the highest VLY-binding activity. Each mouse in the group received a single intraperitoneal injection of the respective protein with a dose of 50  $\mu$ g of the total protein. Blood specimens were collected at selected time intervals after the injection, and plasma concentration of scFvs was detected by an indirect ELISA as described previously [20]. The pharmacokinetic profile demonstrating scFv concentration in blood samples *versus* time was generated (Fig. 5). The scFvs were cleared from circulation

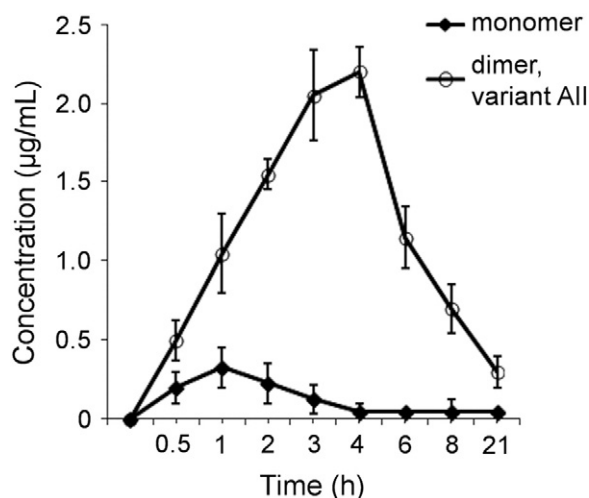


**Fig. 4.** VLY-binding activity of monomeric and dimeric scFvs (variants AI and AII) in indirect ELISA. The assays were performed in triplicate, and the mean value was calculated. K<sub>d</sub> values were calculated using the OriginPro 8.0 program.

**Table 1**  
Neutralizing activity (IC<sub>50</sub>) and affinity (K<sub>d</sub>) of dimeric and monomeric scFvs.

scFvs	IC <sub>50</sub> , nM	K <sub>d</sub> , nM
Dimeric scFv, variant AI	10	2.5
Dimeric scFv, variant AII	26	8.6
Monomeric scFv	68	10.4





**Fig. 5.** Pharmacokinetic profile of intraperitoneally administered monomeric and dimeric (variant AI) scFvs. Mean scFv plasma concentration ( $\mu\text{g/mL}$ ) obtained from three mice per group at different time points is presented. Error bars represent standard deviation of concentration values indicating scFv concentration obtained from three mice per group.

with terminal half-lives of  $1.79 \pm 0.04$  h and  $1.18 \pm 0.09$  h for the dimeric scFv variant AI and monomeric scFv, respectively (Table 2). The obtained data indicate that the clearance of dimeric scFv was reduced 1.5-fold compared to monomeric anti-VLY scFv. Moreover, dimeric scFv reached a higher maximum concentration after injection ( $2.2 \mu\text{g/mL}$  in ELISA compared to monomeric scFv ( $0.4 \mu\text{g/mL}$ )).

#### 4. Discussion

The increasing number of antibiotic-resistant bacteria, the persistence of infections, and their severity upgraded the interest in antibody therapy against toxins associated with the development of disease. Recombinant scFvs are suggested as an alternative to full-length antibodies because of easier and less expensive manufacture in microbial host organisms and the opportunity of genetic engineering to obtain the protein with properties optimized for specific applications [28]. It was demonstrated by *in vitro* and *in vivo* assays that recombinant antibodies neutralize the activity of toxins that are highly diverse in their nature and mode of action [16,17,18,29]. We previously showed that anti-VLY scFvs could inhibit the cytolytic activity of a pore-forming toxin VLY both on human erythrocytes and cervical epithelial HeLa cells [20]. However, scFvs demonstrated decreased neutralizing potency and reduced VLY-binding capacity compared to full-length monoclonal antibodies [20]. This avidity could be increased using several approaches, one of which comprises multimerization of scFvs that results in recombinant antibodies with multivalent binding capacity [30].

In the current study, we generated and characterized two functionally active monospecific dimeric scFvs obtained by the covalent fusion of two anti-VLY scFv molecules. The SL linker used to fuse

monomers forms an  $\alpha$ -helix conformation, which allows the regulation of the distance between monomers and reduces the interference between the domains [23,26]. Dimeric scFvs with hexahistidine residues at the N-terminus were expressed in *E. coli*, recovered from the inclusion bodies, and purified using metal chelate affinity chromatography. The dimeric anti-VLY scFvs inhibited VLY-mediated hemolysis of human erythrocytes with different efficiencies. The variant AI of dimeric scFv, which consisted of scFv molecules fused in the same orientation, demonstrated higher neutralizing potency and affinity than the variant All, which consisted of scFv molecules fused in opposite orientations. This suggests that the orientation of the VL-VH fragments in the fused monomers has an impact on the antigen-binding capacity of dimeric scFvs. The obtained discrepancy in the activity of dimeric scFvs might be explained by the diverse accessibility of the antigen to different dimeric scFvs. The stepwise binding of VLY to two antigen-binding sites of dimeric scFvs may also be a factor determining the higher affinity and the neutralizing potency of the scFv variant AI. The obtained data on the activity of scFvs demonstrated that multimerization of scFv has an advantage over monomeric forms of scFv.

Our results demonstrate that increased both molecular weight and hydrodynamic radius of the dimeric scFv molecule prolonged its half-life *in vivo*. The half-life of the variant AI of dimeric scFv in mouse plasma was extended to 1.79 h; it was longer than that of monomeric anti-VLY scFv and other unrelated monomeric scFvs described previously [31]. However, the circulating half-life of recombinant dimeric scFv was less pronounced than scFv fused with the immunoglobulin-binding domain (20.8 h) demonstrated in previous studies [31]. Overall, the obtained data are in line with other studies demonstrating the calculated half-lives of small recombinant antibodies: a bispecific single-chain antibody against carcinoembryonic antigen was cleared from circulation with a terminal half-life of 1.3 h, while the half-life of monomeric scFv was 0.6 h [31].

Antibody engineering resulting in improved properties of recombinant proteins opens new possibilities for the neutralization of toxins participating in the development of bacterial infections. It was shown that scFvs and their modifications could inhibit the *in vitro* activity of very complex pore-forming toxins, such as VLY and pneumolysin [32]. The application of recombinant antibodies as an alternative or supplemental tool along with antibiotic treatment can be envisaged for future management of infections.

#### Conflict of interest

The authors declare that they have no competing interests.

#### Financial support

This work was supported by the Research Council of Lithuania (grant number TEC-15015).

#### Acknowledgments

We are grateful to Dr. Virginija Bukelskienė and Rita Lasickiene for their kind help with the *in vivo* experiments.

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**Table 2**  
Estimated circulating half-life of dimeric and monomeric anti-VLY scFvs in mice.

Protein	$k_{app}, h^{-1a}$	$T_{1/2}, h$
<i>Dimeric scFv variant AI</i>		
mouse I	$0.37 \pm 0.02$	1.87
mouse II	$0.39 \pm 0.02$	1.78
mouse III	$0.40 \pm 0.06$	1.73
<i>Monomeric scFv</i>		
mouse I	$0.67 \pm 0.13$	1.00
mouse II	$0.55 \pm 0.16$	1.26
mouse III	$0.54 \pm 0.12$	1.28

<sup>a</sup> Coefficient  $k_{app}$  was calculated for each mouse  $\pm$  standard error of mean (SEM).

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