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Effect of linkers on immobilization of scFvs with biotin-streptavidin interaction

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Abstract

Single-chain variable fragment antibodies (scFvs) are attractive for use in applications that require high specificity and binding to a target, such as biosensors. Previously, we demonstrated that a variety of scFvs can be immobilized onto a streptavidin surface through *in vivo* biotinylation of the biotin carboxyl carrier protein (BCCP) or smaller AviTag fused to the scFvs. However, the BCCP constructs showed better immobilization than the AviTag constructs. In this work, we investigated whether the discrepancy between the biotinylation tags could be alleviated by incorporating flexible (G₄S)_n linker of varying lengths or a rigid (EA₃K)₃ linker between the biotinylation tags and the scFvs scFv13R4 and scFv5. Fusion of the (G₄S)₅ linker or the (G₄S)₃ linker to the AviTag construct of scFv13R4 or scFv5, respectively, and the (EA₃K)₃ linkers to the AviTag constructs of both scFvs was enhanced by the linkers. Meanwhile, the robust immobilization of the BCCP construct of the scFv constructs remained unaffected. The positive to neutral effect of the linkers, with no adverse effects, make them beneficial tools to incorporate into fusion proteins that show poor immobilization without a linker.

Keywords

biotinylation tags; flexible; rigid; immobilization; peptide linkers; scFv

1. Introduction

Fusion of proteins can lead to enhancement in functionality, solubility, and binding affinity [1-3], which can expand their applications in biotechnology. A common method to create a fusion protein is with peptide linkers, and the linker length has been shown to affect the activity and expression of the fused proteins [4-6]. Two common classes of peptide linkers are flexible and rigid linkers. Flexible linkers such as (G₄S)_n, where *n* denotes the number of repeats of the sequence, are used as a method to separate domains [1] or improve their interaction [7]. This type of linker is typically used to connect the V_H and V_L domains of single-chain variable fragment antibodies (scFvs). While the distance between two fusion partners may fluctuate with the flexible linkers, a more fixed distance can be maintained with rigid linkers such as the α -helix forming (EA₃K)_n linker [8]. A fixed distance prevents

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one partner from interfering with the folding or the activity of the other partner. The type of linker that is used depends on the desired role of the linker in the fusion protein design.

In addition to linking protein domains, another possible application of a linker is to expand the distance between the protein and the point of immobilization for immobilized proteins, thus reducing steric interference. We previously developed a simple, purification-free scFv immobilization method that uses the biotin-streptavidin interaction. Biotinylation tags—the 87-residue biotin carboxyl carrier protein (BCCP) [9, 10] and the smaller 15-residue AviTag [11]—were directly fused to scFvs for *in vivo* biotinylation. *In vivo* biotinylation tags allow site-specific biotinylation of a protein, which, in addition to facilitating immobilization, makes the biotinylated protein more versatile for applications such as formation of nanoassemblies and sensitive detection within cells [12]. We used the versatility of the *in vivo* biotinylated scFvs to immobilize them on a streptavidin-coated surface without a separate purification step [13]. In most cases, the BCCP fusion of an scFv immobilized better than the corresponding AviTag fusion of the same scFv. Co-expression of the biotin ligase BirA, which catalyzes the biotinylation of BCCP and the AviTag [14], with the scFvs enhanced the biotinylation efficiency of the AviTag constructs and improved their immobilization, but the effect was scFv-dependent. For example, the constructs of scFv13R4 still showed a significant difference in immobilization between the biotinylation tags, despite the enhanced biotinylation efficiency of the AviTag construct [13].

One hypothesis for the difference in immobilization is that the streptavidin on the surface has more difficulty accessing the biotin bound to the short AviTag. The significantly larger BCCP may act as a linker that distances the scFv from the biotinylation site and enhances the accessibility of the bound biotin. To test this hypothesis, a flexible peptide linker of various lengths $[(G_4S)_n]$ and a rigid linker $[(EA_3K)_3]$ were inserted between scFv13R4 and the biotinylation tags, and the effect on immobilization and expression of the scFvs was evaluated. scFv5, which previously showed poor expression and immobilization of its AviTag construct [13] was also tested to determine if the effect of linkers is dependent on the structure of the scFv or its expression level. We found that while addition of the linkers had a limited effect on the scFv constructs that reached saturated immobilization levels at the conditions tested, the linkers improved the immobilization of the AviTag-fusion of scFv5, which previously showed a low immobilization level [13], illustrating that linkers can be used effectively to enhance immobilization of a protein if the initial immobilization is poor.

2. Materials and Methods

2.1. Linker plasmid construction

To test the effect of linkers on expression and immobilization, flexible $(G_4S)_n$ linkers—with variable lengths of $n = 1, 3$, and 5 —and one rigid linker $(EA_3K)_3$ were incorporated into the fusion of scFv13R4 to the BCCP or AviTag biotinylation tag between the scFv and the tag. Pairs of complementary primers (Table 1) that encode for each linker were designed and inserted between the *SaI* and *EcoRI* cut-sites of pSPI02B and pSPI02A plasmid backbones (containing BCCP and the AviTag, respectively) [13] to create new backbones with coding sequences for the linkers N-terminal to BCCP and the AviTag (Table 2). DNA encoding for scFv13R4 was amplified by polymerase chain reaction (PCR) with a C-terminal FLAG tag

and inserted between the *NdeI* and *SaII* cut-sites N-terminal to the linker or, if no linker was present, N-terminal to the biotinylation tag. To test whether the effect of the linkers was scFv specific, scFv5, whose AviTag construct previously showed low immobilization [13], was amplified and inserted into the backbones with (G₄S)₃ and (EA₃K)₃ linkers. scFv5 has an N-terminal FLAG tag, as it was present in the original source plasmid [15].

2.2. scFv expression

The scFv constructs with or without linkers were produced in *Escherichia coli* BL21 cells. Overnight cultures were subcultured and grown at 37 °C with 20 µg/mL chloramphenicol for plasmid maintenance. Once the cultures reached mid-log phase, isopropyl β-D-1-thiogalactopyranoside (to 100 µM) and biotin (to 5 µM) were added to induce expression and allow biotinylation [11, 16]. Expression was induced overnight at 20 °C. Cells were then harvested at 4 °C, and the cell pellets were frozen at -20 °C until needed. To extract the biotinylated scFvs, cell pellets were thawed and lysed with BugBuster Master Mix, following the manufacturer's protocol. To remove excess biotin and exchange the buffer to phosphate-buffered saline (PBS) for each lysate, 5 kDa molecular weight cut-off columns (GE) were used. The total protein concentrations in the cell lysates before and after buffer exchange were quantified by measuring absorbance at 280 nm on a Nanodrop 2000 (Thermo Scientific), with the assumption of 1 absorbance unit equaling 1 mg total protein/mL.

2.3. Western blot for expression analysis

Western blot analysis was used to quantify the effect of the linkers on the expression of the scFvs. After cell lysis with the BugBuster Master Mix, the lysates were mixed with equal volumes of 2X sodium dodecyl sulfate (SDS) gel-loading buffer (with β-mercaptoethanol) [17] and boiled for 5 min at 98 °C. Samples were then run on Any kD Mini-PROTEAN TGX gels (Bio-Rad) to separate the proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The separated scFvs were detected using Western blotting. After SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, and the membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) and 5% non-fat dry milk. To detect the expression level of the scFvs, the membranes were washed and stained with horseradish peroxidase (HRP)-conjugated anti-FLAG antibody (Sigma). Antibody-stained PVDF membranes were developed with the Clarity Western ECL substrate (Bio-Rad), and the resulting chemiluminescence was detected on a ChemiDoc MP imaging system (Bio-Rad). The level of expression was quantified through densitometric analysis on ImageLab software (Bio-Rad). The densitometric values on individual blots were normalized to the total signal from all lanes. Three biological replicates were prepared and run on different days. Two-way ANOVA tests ($p < 0.05$) with the Bonferroni correction for multiple comparison tests were done for statistical analysis. One asterisk indicates a level of statistical significance of $p < 0.05$.

2.4. Enzyme-linked immunosorbent assay for immobilization analysis

The effectiveness of immobilization of the biotinylated scFvs was quantified using an enzyme-linked immunosorbent assay (ELISA). High-binding 96-well polystyrene plates (Corning) were coated with streptavidin (NEB), as previously described [13]. Buffer-exchanged cell lysates were serially diluted in PBS with 0.1% Tween 20 (PBST) with 0.5%

milk and loaded onto streptavidin-coated plates (50 μL /well), which had been washed with PBST prior to loading of the samples. After 45 min of incubation, unbound scFvs were washed off with PBST, and the immobilized scFvs were incubated for 1 h with an anti-FLAG antibody (HRP-conjugated, Sigma). Unbound antibody was washed off, and 200 μL of *o*-phenylenediamine dihydrochloride HRP substrate (Sigma) was added to each well to react with the HRP on the antibody. After the reaction proceeded for 30 minutes, 50 μL of 3 M H_2SO_4 was added to quench the reaction. The level of immobilization was quantified using absorbance at 492 nm, measured on a plate reader (Epoch, Bio-Tek). Three separate samples were evaluated on different days, with at least five replicates for each construct. For statistical analysis, two-way ANOVA tests ($p < 0.05$) with the Bonferroni correction for multiple comparison against no linker constructs were done at 1 $\mu\text{g}/\mu\text{L}$, 0.063 $\mu\text{g}/\mu\text{L}$, and 0.008 $\mu\text{g}/\mu\text{L}$ total protein concentrations.

3. Results

This study investigated whether the distance between the antibody fragment and the point of immobilization affects the immobilization of biotinylated scFvs to a streptavidin-coated surface. The distance between the scFv and the biotinylation site was lengthened through addition of flexible linkers of various length— $(\text{G}_4\text{S})_1$, $(\text{G}_4\text{S})_3$, and $(\text{G}_4\text{S})_5$ —and a rigid α -helix forming linker $(\text{EA}_3\text{K})_3$ between the scFv and the C-terminal biotinylation tags, BCCP and AviTag (Figure 1). scFv13R4 (binds to β -galactosidase) was chosen as the first test candidate, as the BCCP fusion and the AviTag fusion of the antibody fragment previously showed a difference in immobilization [13]. The difference was present even after the biotinylation efficiency of the AviTag construct was enhanced with BirA enzyme. To determine if the effects of the linkers extend to other scFvs or are scFv-specific, one of the flexible linkers $(\text{G}_4\text{S})_3$ and the rigid linker $(\text{EA}_3\text{K})_3$ were also tested with another scFv, scFv5 (binds to *Candida albicans* and *Candida dubliniensis*) [15] (Figure 1).

3.1. Linkers enhance immobilization of AviTag fusions but not BCCP fusions of scFvs

The effect of the linkers on the immobilization of scFv13R4 was assessed on streptavidin coated 96-well plates using ELISAs. The extent of immobilization was quantified by detection of the FLAG tag at the C-terminal end of scFv13R4. In agreement with the previous study [13], the BCCP constructs immobilized better than the AviTag constructs (Figure 2), approaching higher absorbance signals at lower total protein concentrations than the AviTag constructs. The immobilization of BCCP fusions was not significantly affected by the addition of any of the linkers. As more cell lysate was loaded per well, the absorbance signals of BCCP constructs with linkers were at comparable levels to the construct with no linker, and statistical analysis at 1 $\mu\text{g}/\mu\text{L}$, 0.063 $\mu\text{g}/\mu\text{L}$, and 0.008 $\mu\text{g}/\mu\text{L}$ showed no statistically significant difference for BCCP fusions. The AviTag constructs also showed no difference at the low and middle concentrations tested and were particularly robust, with most of the signals overlapping one another, making it difficult to distinguish the individual curves. However, at the highest total protein concentration tested (1 $\mu\text{g}/\mu\text{L}$), the insertion of $(\text{G}_4\text{S})_5$ and $(\text{EA}_3\text{K})_3$ linkers into the AviTag fusion of scFvR4 improved immobilization relative to the construct with no linker ($p < 0.05$). Furthermore, *post hoc* Student's *t*-tests of the improved AviTag fusions against BCCP construct with no linker showed no statistically

significant difference between them. This suggests that at the concentration leading to saturation of immobilization—indicated by the flattening of the curves—these linkers enable the AviTag fusion of scFv13R4 to immobilize at a level similar to the BCCP fusion. Overall, the longer flexible linker and the rigid linkers influence immobilization of the AviTag fusion of scFv13R4 but not the BCCP fusion.

To determine if the effect of the linkers on immobilization is scFv-specific, the flexible (G₄S)₃ linker and the rigid (EA₃K)₃ linker were inserted into the BCCP and AviTag fusions of scFv5. scFv5 was selected since the immobilization levels of its constructs were lower than both of the scFv13R4 constructs in the previous study [13]. As with the scFv13R4 constructs, the level of immobilization was quantified by detection of the FLAG tag fused to the scFv5, which is N-terminal to the scFv in the scFv5 constructs. Similarly to the scFv13R4 fusions, the insertion of the linkers led to no statistically significant difference in immobilization of the BCCP constructs of scFv5 (Figure 3). However, the AviTag constructs with linkers showed significantly higher levels of immobilization than the construct with no linker at the highest concentration tested ($p < 0.001$ for (G₄S)₃ and $p < 0.0001$ for (EA₃K)₃ at 1 µg/µL total protein concentration). Both the flexible and rigid linkers led to a similar level of enhanced immobilization, which suggests that the flexibility of the linker may not play a significant role in immobilization. This result shows that improved immobilization of AviTag fusions of scFvs with linkers is not scFv-specific and that the enhancement can be greater for scFvs that initially have low levels of immobilization.

3.2. Linkers do not enhance expression of the scFvs

Since the immobilization assay indicated that linkers could modify immobilization level, the expression level of the constructs was studied to assess whether it played a role in the improvement. The expression was detected by Western blotting with an anti-FLAG antibody and quantified by densitometric analysis on the developed membranes. The analysis showed no significant enhancement in the expression level of the scFv13R4 constructs due to the presence of the linkers (Figure 4). Similarly, with the exception of the (G₄S)₁ linker, no statistical difference was observed between the BCCP and AviTag constructs (Figure 4B). The lack of difference in expression is in line with the robustness observed with these constructs in the immobilization assay (Figure 2).

Similar to the scFv13R4 constructs, the insertion of a linker did not improve the expressions of the scFv5 constructs (Figure 5). The expression levels between the two biotinylation tags were also comparable (Figure 5B), as they were for scFv13R4. The Western blot analysis indicates that the expression of the scFv constructs fused to BCCP or the AviTag are not enhanced by the addition of peptide linkers between the scFv and the biotinylation tags.

4. Discussion

A number of previous studies have used peptide linkers to enhance activity and expression of fusion proteins [5, 7, 18]; however, limited studies have used them as a tool to enhance binding onto a surface [19] or to functionalized beads [4, 20]. This study investigated the effect of flexible and rigid linkers on immobilization of scFvs using the biotin-streptavidin interaction. A flexible linker (G₄S)_n or a rigid linker (EA₃K)₃ were evaluated with scFv13R4

and scFv5. The immobilization of the AviTag fusions of both scFvs at concentrations leading to near saturation of the surface, were significantly enhanced by the introduction of the linkers (Figure 2 & 3). At the same time, the linkers had no effect on the immobilization of the BCCP constructs of either scFv (Figure 2 & 3).

Studies with other protein fusions have demonstrated enhanced expression of protein fusions due to linkers [6, 18]. However, Western blot analysis with an anti-FLAG antibody showed that the linkers did not increase the expression of either scFv fused to a biotinylation tag (Figure 4 and Figure 5). The lack of improved expression due to linkers indicates the immobilization improvements observed with the scFv constructs are not due to a presence of a higher level of soluble scFv-biotinylation tag fusion in the cell lysate. Instead, the improvement could be due to the spatial separation of the biotin from the scFvs allowing biotin to interact more freely with streptavidin or due to changes in the orientation of the immobilization allowing more scFv molecules to access the surface. The size of the biotinylation tags may explain the more prominent effect of the linkers on immobilization of scFvs fused to the AviTag compared to BCCP. With 15 residues, the AviTag biotinylation tag [11] is much smaller than the 87 residues of BCCP [21]. Therefore, the addition of 15 residues from the three repeats of the linker sequences likely has a larger impact on the AviTag constructs by more greatly increasing the distance between the surface and the scFv. Even when there is no improvement in immobilization, the linker has no negative effect, though, so it could be included as a default part of a construct without the potential to decrease immobilization.

5. Conclusion

This study demonstrated that peptide linkers have a positive or neutral effect on immobilization. The linkers can enhance immobilization of AviTag fusions of scFvs, especially those that show poor immobilization without a linker, while having no adverse effect on BCCP fusions of scFvs, demonstrating their potential for application that utilize scFv immobilization, such as biosensors. Further work to optimize linker properties—for example, using a longer linker—could lead to an AviTag construct of scFv5 that immobilizes at similar level to the BCCP construct as observed with scFv13R4. Additionally, extending this work to evaluate other linker properties and additional scFvs could give more insight into whether the observations in this study can be extended to a broader set of scFvs and linkers.

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Abbreviations

BCCP	biotin carboxyl carrier protein
scFv	single-chain variable fragment antibody

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Figure 1.

Schematic of each scFv construct in this work. Linkers of $(G_4S)_1$, $(G_4S)_3$, $(G_4S)_5$, $(EA_3K)_3$ were used in scFv13R4 constructs and linkers $(G_4S)_3$ and $(EA_3K)_3$ were used for scFv5 constructs. The lengths of components are not to scale.

Figure 2.

Immobilization of scFv13R4-linker-BCCP constructs and scFv13R4-linker-AviTag constructs onto streptavidin-coated plates. Serial dilutions of cell lysates were loaded onto the coated 96-well plates, and the immobilization was detected with an anti-FLAG antibody. Data are normalized to the signal from the highest concentration of scFv13R4-BCCP with no linker for each biological replicate ($n = 5$). The error bars represent the standard error of the mean.

Figure 3.

Immobilization of scFv5- linker-BCCP constructs and scFv5-linker-AviTag constructs onto streptavidin-coated plates. Serial dilutions of cell lysates were loaded onto the coated 96-well plates, and the immobilization was detected with an anti-FLAG antibody. Data are normalized to the signal from the highest concentration of scFv5-BCCP with no linker for each biological replicate ($n = 6$). The error bars represent the standard error of the mean.

Figure 4.

The expression of scFv13R4 constructs by (A) Western blotting and (B) densitometric analysis. The constructs were expressed in BL21 *E. coli* cells, and Western blotting was performed on the soluble cell lysates ($n = 3$). Expression of each scFv13R4 construct was detected with an anti-FLAG antibody. For densitometric analysis, the signals were normalized to the total protein signal on each blot. Error bars are standard error of the mean. The asterisk indicates statistical significance with $p < 0.05$.

Figure 5.

The expression of scFv5 constructs by (A) Western blotting and (B) densitometric analysis. The constructs were expressed in BL21 *E. coli* cells, and Western blotting was performed on the soluble cell lysates (n = 3). Expression of each scFv5 construct was detected with an anti-FLAG antibody. For densitometric analysis, the signals were normalized to the total protein signal on each blot. Error bars are standard error of the mean. Statistical analysis showed no difference between constructs with and without linker.

Table 1Complementary primers with *Sa*I at N-terminus and *Eco*RI at C-terminus.

Primer name	Sequence
pGGGS_1-F	5'-TCGACGGTGGCGGAGGGTCTG-3'
pGGGS_1-R	5'-AATTCAGACCCTCCGCCACCG-3'
pGGGS_3-F	5'-TCGACGGAGGCGGTGGGTGAGGCGGTGGAGGGTCCGGTGGCGGAGGGTCTG-3'
pGGGS_3-R	5'-AATTCAGACCCTCCGCCACCGGACCCTCCACCGCCTGACCCACCGCCTCCG-3'
pGGGS_5-F	5'-TCGACGGGGGTGGAGGCTCGGGAGGTGGGGGCTCAGGAGGCGGTGGGTGAGGCGGTGGAGGGTCCGGTGGCGGAGGGTCTG-3'
pGGGS_5-R	5'-AATTCAGACCCTCCGCCACCGGACCCTCCACCGCCTGACCCACCGCCTCCTGAGCCCCACCTCCCGAGCCTCCACCCCG-3'
pEAAK_3-F	5'-TCGACGAGCCGCAGCTAAAGAAGCAGCTGCCAAGGAAGCCGCGGCTAAAG-3'
pEAAK_3-R	5'-AATCTTTAGCCGCGGCTTCCTTGGCAGCTGCTTCTTTAGCTGCGGCCTCG-3'

Table 2

Constructs of scFv13R4 and scFv5 with and without linkers used in this study.

scFv	Linker	Biotinylation Tag	Plasmid backbone	Construct
scFv13R4	None	BCCP	pSPI01B	scFv13R4-BCCP
	(G ₄ S) ₁	BCCP	pSPI03B	scFv13R4-(G ₄ S) ₁ -BCCP
	(G ₄ S) ₃	BCCP	pSPI04B	scFv13R4-(G ₄ S) ₃ -BCCP
	(G ₄ S) ₅	BCCP	pSPI05B	scFv13R4-(G ₄ S) ₅ -BCCP
	(EA ₃ K) ₃	BCCP	pSPI07B	scFv13R4-(EA ₃ K) ₃ -BCCP
	None	AviTag	pSPI01A	scFv13R4-AviTag
	(G ₄ S) ₁	AviTag	pSPI03A	scFv13R4-(G ₄ S) ₁ -AviTag
	(G ₄ S) ₃	AviTag	pSPI04A	scFv13R4-(G ₄ S) ₃ -AviTag
	(G ₄ S) ₅	AviTag	pSPI05A	scFv13R4-(G ₄ S) ₅ -AviTag
	(EA ₃ K) ₃	AviTag	pSPI07A	scFv13R4-(EA ₃ K) ₃ -AviTag
scFv5	None	BCCP	pSPI02B	scFv5-BCCP
	(G ₄ S) ₃	BCCP	pSPI04B	scFv5-(G ₄ S) ₃ -BCCP
	(EA ₃ K) ₃	BCCP	pSPI07B	scFv5-(EA ₃ K) ₃ -BCCP
	None	AviTag	pSPI02A	scFv5-AviTag
	(G ₄ S) ₃	AviTag	pSPI04A	scFv5-(G ₄ S) ₃ -AviTag
	(EA ₃ K) ₃	AviTag	pSPI07A	scFv5-(EA ₃ K) ₃ -AviTag