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Bglbrick strategy for the construction of single domain antibody fusions

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Abstract

Single domain antibodies, recombinantly expressed variable domains derived from camelid heavy chain antibodies, are often expressed as multimers for detection and therapeutic applications. Constructs in which several single domain antibodies are genetically fused serially, as well as those in which single domain antibodies are genetically linked with domains that naturally form multimers, yield improvement in apparent binding affinity due to avidity. Here, using a single domain antibody that binds envelope protein from the Dengue virus, we demonstrated the construction of single domain antibody dimers using the Bglbrick cloning strategy. Constructing single domain antibodies and multimerization domains as Bglbrick parts enables the easy mixing and matching of parts. The dimeric constructs provided enhanced fluorescent signal in assays for detection of Dengue virus like particles over the monomeric single domain antibody.

Keywords: Biochemistry, Bioengineering

1. Introduction

Single domain antibodies (sdAbs), the binding domains derived from camelid heavy chain antibodies, are attractive alternatives to conventional immunoreagents for therapeutic, detection, and diagnostic applications (Muyldermans, 2013). They

provide small, highly soluble, domains that are amenable to protein engineering to both improve their characteristics and tailor them to specific applications. For example, expressing sdAbs in a multimer format is a proven method to increase apparent affinity (Zhang et al., 2004; Herrera et al., 2015). In addition, sdAb multimers have shown orders of magnitude improvement in their ability to neutralize virus (Hultberg et al., 2011), and multimeric sdAbs have also been utilized to tune the serum half-life of sdAbs (Coppieters et al., 2006). Methods to multimerize sdAbs include genetically linking two or more sdAbs with flexible peptide linkers (Conrath et al., 2001; Glaven et al., 2012) and expressing sdAbs as genetic fusions with a multimerization domain (Zhang et al., 2004).

In the field of synthetic biology there has been a push to create libraries of standard “parts” that offer a uniform restriction enzyme assembly standard for assembling parts into larger composites. As part of this effort, the Bglbrick standard was developed to provide a facile method to produce fusions of proteins without altering the reading frame or introducing stop codons (Anderson et al., 2010). While originally developed for the construction of larger synthetic biology circuits, the Bglbrick formulation is convenient for the production of sdAb multimers and fusions of sdAbs with other proteins. Herein we describe the modification of the commercial pET22b expression vector for Bglbrick cloning, and the construction of sdAb multimers using Bglbrick parts. Starting with an sdAb specific for the envelope protein (E) of the serotype 1 Dengue virus (DENV1), we configured the sdAb as a Bglbrick part and showed that dimer constructs are much more effective than monomers at recognizing DENV1 virus like particles (VLPs).

2. Materials and methods

2.1. Reagent and buffers

The CC9 sdAb was isolated from a llama immunized with a DNA vaccine (Porter and Raviprakash, 2015) using standard library construction and panning techniques (Walper et al., 2014). Rhizavidin has been shown previously to act as an efficient dimerization domain (Liu et al., 2014). Enzymes used for cloning were from New England Biolabs. Reagents used in PCR were from ThermoFisher. Oligonucleotide synthesis and DNA sequencing services were provided by Eurofins Genomics. Unless otherwise specified, chemical reagents were from Sigma Aldrich, Fisher Scientific, or VWR International.

2.2. Modification of pET22b vector

The pET22b vector for periplasmic expression was subjected to site directed mutagenesis using the quick change kit (Agilent) to remove a single BglIII site at the start of the cloning/expression region. Primers were designed that changed the “AGATCT” restriction enzyme site to “AGGTCT”. Mutagenesis was

accomplished using the manufacturer's protocols. The modified pET22b vector was termed pET22b-BglII.

2.3. Construction of Bglbricks

We used PCR to amplify the sequence of each “part”. The primers used for amplification coded for NcoI, EcoRI, and BglII restriction enzyme sites on the N terminus of each “part” as well as BamHI and XhoI restriction enzyme sites on the C terminus (Fig. 1A). Similar PCR was also used to construct a CC9 version which contained a 10 amino acid gly-ser linker (GSGGSGSGGS) followed by the BamHI and XhoI sites. Parts constructed by PCR were cloned as NcoI – XhoI fragments into the pET22b-BglII expression vector. In cases where a sAb sequence includes an internal NcoI site, sAb parts can easily be cloned into the pET22b-BglII expression vector as EcoRI – XhoI or BglII – XhoI fragments.

Panel B of Fig. 1 shows an overview of the method we used to join two parts. The vector containing the part which will be first in the fusion is digested with BamHI and XhoI, phosphatased (we used CIP) and purified using the Qiaquick PCR cleanup kit (Qiagen). The second part is digested with BglII and XhoI and gel purified (Qiaquick gel cleanup kit; Qiagen). Finally, the parts are ligated to form the multimer construction (Fig. 1B). Using this method, the final fusion product is in the Bglbrick format enabling further fusions. The scar left by joining the strands

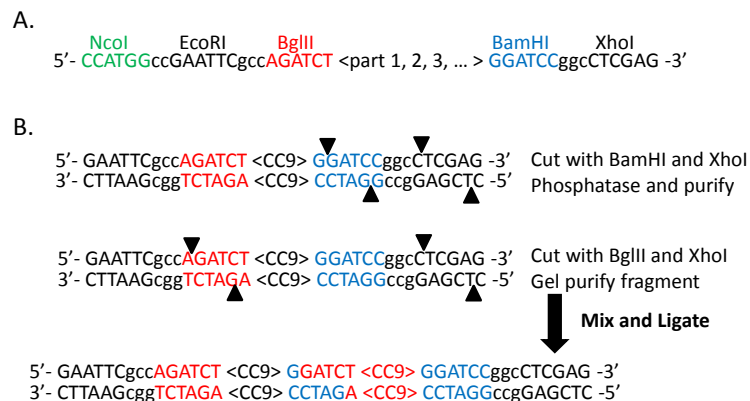


Fig. 1. Bglbrick cloning scheme. DNA sequences of the cloning area and an overview of the cloning for dimer formation are presented. Restriction sites, as well as the product from ligating BglII and BamHI digested DNA are shown in capital letters. Panel A shows the regions N and C terminal to the part within pET22b-BglII, the pET22b Bglbrick compatible vector. The pel B leader sequence is located immediately before the sequence shown and includes the “CC” of the NcoI site. A 6-histidine tail followed by a stop codon are located just after the XhoI site. Panel B shows the scheme used to construct the CC9 dimers. A vector containing the CC9 part is digested with BamHI and XhoI and then treated with phosphatase to prevent re-ligation. Likewise a CC9 fragment is generated by digesting with BglII and XhoI and then purified from a gel. The pieces are ligated, forming a fusion with a 6-base sequence that codes for Gly-Ser inbetween the two CC9 parts shown in the illustration.

cut by BglII and BamHI keeps the fusion protein in frame and adds two amino acids (GS) between the parts.

2.4. Protein production and evaluation (SPR and MAGPIX)

The sdAb and fusion constructs were expressed and purified as previously described (Glaven et al., 2012; Walper et al., 2014; Liu et al., 2014). Briefly, the pET22b-Bglbrick plasmid carrying the sdAb construct was transformed into Tuner (DE3) for expression. Single colonies were transferred to 50 mL in Terrific Broth (TB) containing ampicillin (100 µg/mL) and grown overnight at 25 °C. The 50 mL cultures were used to inoculate 0.5 L of TB which was grown for 2 h at 25 °C and then induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown a further 2 h. Cultures were centrifuged and the cell pellets subject to an osmotic shock protocol. Protein was purified by Ni-NTA followed by size exclusion chromatography and was quantified by absorbance at 280 nm.

Evaluation by surface plasmon resonance (SPR) and MagPlex fluid array immunoassay was performed as described previously (Glaven et al., 2012; Walper et al., 2014). SPR was carried out on a ProteOn XPR36 (BioRad), and we examined binding of the sdAb constructs to recombinantly produced DENV 1 E protein (CTKbiotech). The MagPlex fluid array immunoassay was carried out on the MAGPIX instrument (Luminex). MagPlex assays were performed in a sandwich format with beads and biotinylated (Bt) reporter prepared as described previously (Walper et al., 2014; Liu et al., 2014). The sdAb constructs were evaluated for their ability to detect DENV1 virus like particles (VLPs; The Native Antigen Company).

3. Results and discussion

Our research has focused on the development of sdAb reagents directed against a variety of toxin, bacterial, and viral targets. SdAbs, also known as nanobodies, are the recombinantly expressed variable domains derived from the unique heavy-chain only antibodies found in camelids. These binding domains can easily be engineered and generally offer good protein production. A study of 22 toxin binding sdAbs showed production averaging 9 ± 4 mg/mL (Shriver-Lake et al., 2017); in other studies, several sdAbs averaged production of 20 mg/L or higher in *E. coli* (Liu et al., 2015). The solubility of sdAbs enables their use as modular building blocks for the generation of bivalent and bispecific constructs in which two of the same sdAb or two different sdAbs are genetically linked (Conrath et al., 2001; Coppieters et al., 2006; Hultberg et al., 2011; Glaven et al., 2012; Movahedi et al., 2012). Similarly trivalent sdAbs and fusions with other protein can be produced (Zhang et al., 2004; Desmyter et al., 2017). Our work has included the construction of various sdAb-based fusion constructs that introduce beneficial

properties such as directional immobilization, enzyme functionality, and/or improved apparent affinity (Glaven et al., 2012; Liu et al., 2013; Liu et al., 2014).

The CC9 sdAb clone was derived from an immune library and recognizes the E protein from DENV1. While sdAbs from immune libraries generally have excellent affinity for target, CC9's K_D was calculated as 10 nM (Table 1), thus less than ideal for detection assays. Functionally the CC9 could be used in a sandwich format to detect DENV1 VLPs, however the assay only showed reliable signal at the highest VLP concentrations. To rectify this limitation we turned to the proven strategy of multimerization in an effort to increase the apparent affinity and utility of this sdAb. Our aim was to generate a reagent which could more effectively detect DENV1 VLPs.

Generally sdAb multimers are made through traditional cloning. To produce genetically linked sdAbs, the sdAb components are flanked by specific restriction enzyme sites and cloned on either side of a linker (Conrath et al., 2001; Glaven et al., 2012). This can be more complicated when expanding to linked trimer constructs as the sites flanking the sdAb must be unique. Some of the sites that have been used in cloning, such as PstI, can naturally occur within sdAbs and require removal via mutagenesis in order to construct the linked sdAb (Harmsen et al., 2008). Multimeric sdAbs have also been assembled through PCR and mobilized into expression vectors (Hultberg et al., 2011). Constructs in which sdAbs are fused with a multimerization domain usually include unique cloning sites for sdAb insertion N or C terminal to the multimerization domain (Zhang et al., 2004; Liu et al., 2014).

The Bglbrick method allows the facile mixing and matching of parts which can consist of sdAbs, multimerization domains, enzymes, or domains with other functionalities. A series of 96 BglBrick-compatible plasmids with a combination of replication origins, antibiotic resistance genes, and inducible promoters have been produced and characterized (Lee et al., 2011). This provides a valuable tool for those interested in synthetic biology and metabolic engineering. Although these

Table 1. Yield and binding kinetics.

Clone name	Yield (mg/L) ^a	k_a (1/Ms)	k_d (1/s)	K_D (nM)
CC9 ^b	2.6	4.6 E + 05	4.8 E-03	1.1 E-08
CC9 (Bglbrick) ^c	2.6	4.5 E + 05	4.7 E-03	1.1 E-08
CC9-CC9 ^c	1.5	1.4 E + 06	9.7 E-04	6.7 E-10
CC9-L10-RZ	2.0	3.1 E + 05	7.7E-05	2.5 E-10

^a average from at least 2 growth trials; ^b in unaltered pet22b vector without incorporation of the Bglbrick restriction sites; ^c in pet22b-BglIII vector incorporating the Bglbrick restriction sites.

vectors could be utilized for the production of sdAb parts, we chose to modify the commercial pET22b periplasmic expression vector to accommodate Bglbrick parts. We have found robust production of sdAb and fusions using pET22b based expression vectors. Typically we clone sdAbs as NcoI-NotI fragments, and often insert fusion partners such as multimerization domains as NotI-XhoI fragments.

Formatting an sdAb as a Bglbrick compatible part is accomplished by inserting the required restriction sites at the N and C terminal (Fig. 1). The same can be done for multimerization domains or other desired fusion domains. However, first we needed to use site directed mutagenesis to remove the single BglIII site that sits at the start of the pET22b cloning/expression region. Next, we used PCR to modify CC9 into the Bglbrick format. After eliminating the BglIII site from the expression vector and adding flanking restriction sites to CC9, we expressed the CC9 sdAb and confirmed that the changes did not compromise protein production or functionality (Table 1).

Using the Bglbrick strategy outlined in Fig. 1, we constructed genetically linked homodimers of CC9 which we refer to as CC9-CC9. In addition we made fusions of CC9 with the biotin binding protein rhizavidin, which serves as a dimerization domain; this construct was termed CC9-L10-RZ. Rhizavidin is a dimeric biotin binding protein derived from the symbiotic nitrogen-fixing bacterium *Rhizobium etli* CFN42 (Helppolainen et al., 2007; Meir et al., 2009). Previously, we demonstrated the first genetic fusions of sdAbs with rhizavidin; we showed ricin binding sdAbs prepared as fusions with rhizavidin provided improved capture reagents and could be used to functionalize biotin-conjugated quantum dots (Liu et al., 2014; Liu et al., 2016). Because the anti-ricin sdAb monomers have sub nM affinity, we attributed the better toxin detection using rhizavidin fusions to oriented immobilization. In the current work rhizavidin fusions are utilized as dimerization domains to provide reporter reagents with an effective affinity superior to the monomer sdAb.

Yields of the wild-type CC9 were poorer than many sdAbs. This was true also for the dimer constructs, however all produced reproducibly at levels of at least 1.5 mg/L, providing sufficient protein for evaluation. If higher protein production is needed, CC9 can be subject to mutagenesis in an effort to improve yield (Shriver-Lake et al., 2017). Alternatively, other strategies to increase yield such as cytoplasmic production can be explored (Zarschler et al., 2013; Djender et al., 2014).

We used SPR to assess the binding of the constructs to commercial recombinantly produced DENV1 E protein. SPR showed that the linked constructs had a better overall affinity as measured by their slower off rate and lower dissociation constant (K_D ; Table 1). The CC9-L10-RZ had the slowest off rate of the constructs.

Both the monomer CC9 and the dimer constructs were incorporated into a MagPlex fluid array immunoassay for the detection of DENV1 VLPs which act as a surrogate for the virus (Fig. 2). The CC9 and CC9-CC9 were both immobilized onto color-coded magnetic beads. The monomer and both versions of the dimer were utilized as reporters. The most effective pair was the CC9-CC9 capture in conjunction with the CC9-L10-RZ reporter.

Although the construct in which two CC9s were genetically linked (CC9-CC9) was more effective than the CC9 monomer, in both SPR and MagPlex assays, the best performing reagent was one in which dimerization of CC9 was achieved through use of the rhizavidin dimerization domain (CC9-L10-RZ). One strategy that might enable better performance from the CC9-CC9 would be to optimize the linker length and/or linker composition (Glaven et al., 2012; Movahedi et al., 2012). Ideally the linker needs to be long enough to enable both component sdAbs to bind their targets simultaneously. Other linker characteristics such as rigidity, hydrophilicity, and charge may also be important. In addition to a glycine-serine based flexible linker, sdAbs can be joined, for example, by peptides derived from the upper hinge sequence from natural camelid or human antibodies.

Both the dimeric forms of CC9 (CC9-CC9 and CC9-L10-RZ) outperformed the monomeric sdAb as reporters for the detection of DENV1 VLPs. This result is consistent with multiple studies showing that multimeric sdAbs can have a clear advantage for detection and therapeutic applications. Configuring protein parts, including sdAbs, in the Bglbrick format offers a facile method to assemble multimeric constructs. By definition, Bglbrick parts are flanked by extra amino acids encoding restriction sites, however for CC9 we found the yield and binding characteristics of traditional and Bglbrick versions were equivalent suggesting that the redundant amino acids did not affect production or binding in this case. Routinely producing sdAbs in the Bglbrick format provides a path to mix and match sdAbs for the generation multimeric sdAbs without the need to introduce or

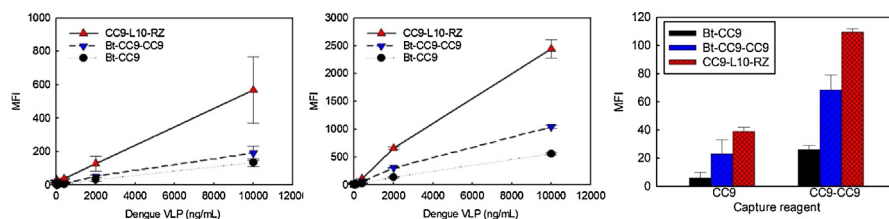


Fig. 2. MagPlex fluid array immunoassay for the detection of DENV1 VLPs. Assays were performed in a sandwich format with either a CC9 or CC9-CC9 capture immobilized on beads. Signal was generated through use of a biotinylated (Bt) CC9 monomer, a Bt CC9-CC9 dimer, or the dimeric CC9-L10-RZ. The left panel and middle panel show dose response curves with the CC9 capture and CC9-CC9 capture respectively. The right panel presents data at 400 ng/ml of DENV1 VLPs and compares the response of both captures paired with all three reporters.

eliminate restriction sites or the need to individually generate sdAb multimers through PCR schemes.

4. Conclusions

We demonstrated that Bglbrick cloning offers a facile way to mix and match sdAb “parts” to create dimers. This strategy can similarly be expanded to create trimers or even longer sdAb multimers. Additionally it can be employed to join sdAbs with other types of parts such as multimerization domains or enzymes. In this work we formed two types of dimers of the CC9 sdAb which binds the DENV1 E protein. As predicted from prior work, the dimer formats enabled improved detection reagents over the monomeric sdAb.

Declarations

Author contribution statement

Ellen R. Goldman, Jinny L. Liu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Aeris Broussard, George P. Anderson: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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