Isolation of highly selective IgNAR variable single-domains against a human therapeutic Fc scaffold and their application as tailor-made bioprocessing reagents.

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The adaptive immune system of cartilaginous fish (*Elasmobranchii*), comprising of classical hetero-tetrameric antibodies, is enhanced through the presence of a naturally occurring homodimeric antibody-like immunoglobulin - the new antigen receptor (IgNAR). The binding-site of the IgNAR variable single-domain (VNAR) offers advantages of reduced size (<1/10th of classical immunoglobulin) and extended binding topographies, making it an ideal candidate for accessing cryptic epitopes otherwise intractable to conventional antibodies. These attributes, coupled with high physicochemical stability and amenability to phage display, facilitates selection of VNAR binders to challenging targets. Here, we explored the unique attributes of these single-domains for potential application as bioprocessing reagents in the development of the SEED-Fc platform, designed to generate therapeutic bispecific antibodies. A panel of unique VNARs specific to the SEED homodimeric (monospecific) "by-products" were isolated from a shark semi-synthetic VNAR library via phage display. The lead VNAR candidate exhibited low nanomolar affinity and superior selectivity to SEED homodimer, with functionality being retained upon exposure to extreme physicochemical conditions that mimic their applicability as purification agents. Ultimately, this work exemplifies the robustness of the semi-synthetic VNAR platform, the predisposition of the VNAR paratope to recognise novel epitopes, and the potential for routine generation of tailor-made VNAR-based bioprocessing reagents.

Key words: affinity ligand, SEED bispecific antibody platform, shark IgNAR, phage display, variable new antigen receptor.

Introduction

The plethora of bispecific antibody (bsAb) formats currently in development (Spiess *et al*. 2015; Brinkmann and Kontermann 2017) typifies the heightened interest in these next-generation biotherapeutics. In turn, this expanding multitude of bsAbs necessitates robust manufacturing platforms for their unhindered progress into clinical use and on hopefully to deliver both patient and commercial benefits (Liu *et al*. 2017; Shukla *et al*. 2017). High expression yield is not necessarily the single most important factor for these formats, but rather the efficiency with which the bsAbs form, and the corresponding presence of closely related but contaminating by-products of the processes employed (e.g. monospecific binders) (Brinkmann and Kontermann 2017). One molecular route to bsAbs is based on a heterodimeric Fc engineering approach, to generate asymmetric mutations in each of two parental mAb CH3 domains (Ha *et al*. 2016). This is employed in the strand-exchange engineered domain (SEED) Fc platform (Gross *et al*. 2013), and resolves the majority of the heavy chain mispairing seen for other systems (Davis *et al*. 2010; Krah *et al*. 2017). This increase in efficiency is in part the result of the SEED design rationale, incorporating complementary human IgG/IgA Fc hybrids that are "encouraged" to preferentially heterodimerise, forming an analogue Fc scaffold (Gross *et al*. 2013). Whilst the early efficacy data looks very promising, indicating a favourable pharmacokinetic profile and retention of antibody-like effector functions (Muda *et al*. 2011; Kelton *et al*. 2012), the GA chain can form low-levels of contaminating GA-GA SEED homodimers (Davis *et al*. 2010). Thus far, none of the Fc heterodimeric approaches have achieved 100% heterodimerisation, with low-levels (1–10%) of mispaired homodimerisation being seemingly inevitable in large-scale production (Sampei *et al*. 2013; Ha *et al*. 2016; Skegro *et al*. 2017). This mixed population of therapeutic scale product requires, therefore, to be better characterised (or even polished) and quantifiable batch consistency determined to facilitate efficient downstream bioprocessing and regulatory approvals (Kufer *et al*. 2004; Ha *et al*. 2016).

To date, the main efforts employed to resolve this bioprocessing bottleneck have been focussed on improving the manufacturability of bsAbs by further rounds of molecular engineering (Sampei *et al*. 2013; Skegro *et al*. 2017). At the same time, advances in upstream monoclonal antibody (mAb) processing has led to significant improvements in production yields, placing even greater cost pressures on finding a suitable solution to the presence of monospecific binders in the bispecific therapeutic programs (Girard *et al*. 2015).

For the generation of a generic approach that would work for all SEED systems the recognition and/or purification challenge is not straightforward because of the close structural

and physicochemical resemblance of the desired heterodimeric bsAb with the by-product homodimers. With only a small number of subtle differences between them in their Fc regions (Shukla and Norman 2017) these changes cannot be distinguished by conventional protein A capture, resulting in their co-elution (Liu *et al*. 2017). Alternative purification methods could use anti-idiotypic ligands specific for intact antibody paratopes (Godar *et al*. 2015; Könning, Rhiel *et al*. 2017). However elegant, such a dual anti-idiotypic purification process lacks universal application, requiring an individual selection campaign for each bsAb arm.

Shark-derived single-domains provide opportunities as alternative affinity capture reagents, sharing many similar properties to VHH domains (Wesolowski *et al*. 2009; Könning, Zielonka *et al*. 2017) but superior when one considers size (up to 20% smaller), stability, and density of binding loops per domain (with four regions of hypervariability versus VHH's three) (Kovaleva *et al*. 2014). VNAR's distinct structural paratope (Diaz *et al*. 2002; Stanfield *et al*. 2004), inherent stability (Griffiths *et al*. 2013, Ubah *et al*. 2017), amenability to display technologies (Ubah *et al*. 2016) and site-specific covalent coupling to crystalline nanocellulose (Uth *et al*. 2014), reinforces VNAR's attractiveness.

VNARs were isolated from a semi-synthetic phage display library through a solution-phase selection technique using biotinylated SEED Fc-based protein [N-terminal IgG1 hinge region-IgG1 CH2-(SEED CH3)] in the form of 'the contaminant' SEED homodimer (Fc-GA/GA-Fc) protein. A successful "candidate" affinity ligand had to demonstrate superior selectivity to the target protein present at <1% of the total protein yield (~20–100 µg/mL) (Davis *et al*. 2010), with the ability to differentiate between closely related mispaired formats, and also show efficient elution from the target/ligand complex. In addition, the VNAR affinity ligand had to be sufficiently stable to retain activity under harsh bioprocessing conditions, enabling reusability without compromising performance.

Materials and methods

Phage display selection

A shark VNAR synthetic library (ELSS1, Elasmogen Ltd., patent WO/2014/173959) of combined size $1x10^{11}$, was selected *via* a solution-phase method (Hawkins *et al*. 1992) with biotinylated SEED Fc homodimer protein: GA-GA Fc (Merck KGaA, Darmstadt Germany) captured on streptavidin magnetic beads (Dynabeads M-280, Invitrogen). Clearly the purity of the starting antigens is a key consideration influencing the selection of specific binders. The homodimer purity (GA-GA Fc protein) was confirmed with both analytical HPLC, and isoelectric focusing gel analysis that allows the amino acid composition of the AG and GA

SEED chains to be distinguished using different isoelectric point (pI) values (theoretical pI values: AG=8.2; GA=6.4; AG/GA=7.3) (Gross *et al*. 2013). Using these approaches in combination the protein was determined to be pure. For the purpose of solution-phase selection, the SEED protein was biotinylated *via* EZ-Link Sulfo-NHS-LC-Biotin kit (Thermo Scientific). Phage display library selections were performed using $>10^{13}$ phage/mL. Library phage and Dynabeads (2x 100 µL) were blocked with 3% (w/v) Marvel milk proteins in 1x PBS (MPBS) for 1 h, rotating at room temperature. Phage were deselected on blocked beads, while the remaining beads were incubated with biotinylated-SEED homodimer (200 nM) for 1 h rotating at room temperature. SEED pre-decorated beads were then incubated with deselected phage for a further 1 h. Wash stringency was maintained at five times 1x PBS-0.05% (v/v) Tween20 (PBST) and five 1x PBS, prior to an 8 min elution with 200 µL of 100 mM triethylamine, and neutralisation with 100 µL of 1 M Tris-HCl, pH 7.5. Eluted phage output was amplified through infection of 10 mL mid-log phase *Escherichia coli* (*E. coli)* TG1 cells (Lucigen) for 30 min (static), at 37°C. Following infection, the cells were concentrated by centrifugation, and plated on TYE agar bioassay plate containing 2% (w/v) glucose and 100 µg/mL ampicillin. The phage selection output was rescued according to a previously described protocol by Dooley *et al*. 2003. In brief, the selection output was scraped-off the bioassay agar plate and seeded into 50 mL of 2xTY, 2% (w/v) glucose, 100 µg/mL ampicillin media at OD600 of 0.1, and grown at 37^{o}C 250 rpm to OD600 of ~0.5. The selected phage were rescued by infecting the culture with M13K07 helper phage (New England Biolabs) at 20:1 ratio (phage:cells) and incubated for 30 min (static) at 37°C, followed by 1 h shaking at 150 rpm. The cells were pelleted and re-suspended in 50 mL of 2xTY media containing 100 µg/mL ampicillin and 50 µg/mL kanamycin prior to overnight incubation at 25°C, 250 rpm shaking. Phage-VNAR particles were concentrated from the culture supernatant by two successive precipitations with 1/5th volume of ice-cold 20% (w/v) polyethylene glycol/2.5 M NaCl with 30 min incubation on ice. The resulting precipitated phage were used as the input phage for the next round of selection, with two further rounds of selection being carried out and stringency being increased by a successive 2-fold reduction in biotinylated-SEED homodimer concentration.

Screening of SEED homodimer specific binders

The enrichment of antigen-binding clones was evaluated *via* monoclonal phage enzyme-linked immunosorbent assay (ELISA). Ninety-two individual colonies were picked from each selection round and screened for binding specificity to SEED protein: GA-GA Fc homodimer,

AG-GA Fc heterodimer, and an unrelated human serum albumin (HSA) protein control (Sigma-Aldrich), coated onto MaxiSorp ELISA plates (Thermo Fisher Scientific) at 1 µg/mL in 1x PBS, 4°C overnight. Monoclonal phages were inoculated from overnight starter cultures into 96-deep-well plates (Greiner Bio-One) with 800 µL/well 2xTY, 2% (w/v) glucose, 100 µg/mL ampicillin media, and grown with shaking at 37°C for 4 h. The phage-VNAR particles were rescued by infection with M13K07 helper phage and two successive precipitations with polyethylene glycol/2.5 M NaCl, as described above. Precipitated phage was added to ELISA plates pre-blocked with 3% MPBS and incubated for 1 h at room temperature. Phage signals were detected *via* horseradish peroxidase (HRP)-conjugated anti-M13 antibody (GE Healthcare, 27-9421-01) diluted 1/1,000 in 3% MPBS for 1 h at room temperature. ELISA was developed with 3,3',5,5'-tetramethylbenzidine substrate (Thermo Fisher Scientific) for 10 min at room temperature and stopped with 0.5 volume of 1 M H_2SO_4, and the optical density (OD) was measured at 450 nm. Plasmid DNA was prepared from antigen binding clones *via* Wizard® Plus SV Minipreps DNA Purification System (Promega) for sequencing (GATC Biotech).

Preparation of soluble VNAR proteins

Anti-SEED VNAR binders were rescued with M13K07 helper phage, and the supernatant was used to infect mid-log phase HB2151 cells (a kind gift from Scotia Biologics Ltd.) for 30 min at 37°C static. VNAR proteins were expressed in the cytoplasm of HB2151 cells at 1 litre scale. Day cultures (TB media with 2% glucose and 100 µg/mL ampicillin) were seeded from overnights at 1:50 dilution and grown for 7 h at 37°C, pellets were re-suspended in the same culture volume (minus glucose) with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) at 15°C overnight, with shaking. Cells were harvested, and pellets treated with a 10% culture volume of ice-cold osmotic shock buffer: 200 mM Tris-HCl, 1 mM EDTA, 20% (w/v) sucrose, pH 7.4, shaking on ice for 15 min, prior to an equal volume of ice-cold 5 mM $MgSO_4$ then added with continued shaking for a further 15 min. The supernatant was recovered containing the periplasmic extract. The VNAR soluble protein was purified *via* its hexa-histidine tail using nickel ion charged immobilised metal affinity chromatography agarose (Qiagen), and eluted with 500 mM imidazole, pH 8.0. Protein samples were dialysed against 1x PBS, pH 7.4, before use. Protein concentration and purity was determined by SDS-PAGE.

Determination of binding selectivity and affinity

The binding selectivity of purified VNARs was determined on ELISA plates coated with a range of proteins at 1 µg/mL: both forms of SEED protein, HSA, analogue Fc scaffolds (Sigma-Aldrich) in 1x PBS, 100 µL/well, overnight at 4°C, followed by 1 h blocking at room temperature in 3% MPBS, 200 µL/well. Purified VNARs were added at a top concentration of 2.5 µg/mL and serial dilutions performed, incubated at room temperature for 1 h. The bound VNARs were detected using an anti-c-Myc-HRP antibody (Roche, 11814150001).

Binding affinities were determined by surface plasmon resonance on a BIAcore™ T200 (GE Healthcare). SEED protein was immobilised onto a CM5 Series S chip (GE Healthcare) in 10 mM sodium acetate pH 5.5 *via* an amine coupling kit (GE Healthcare) giving a coating density of approximately 1,000 RU. A blank immobilisation was carried out for reference subtraction. The purified VNAR protein was diluted in running buffer (10 mM HEPES, 500 mM NaCl, 0.05% Tween20, pH 7.4) to 60, 30, 15, 8, 4 and 2 nM. Each concentration was injected over the immobilised ligand for 5 min at a flow rate of 30 µL/min, 20°C, and allowed to dissociate for 10 min, followed by a 0.5 min regeneration with 20 mM glycine-HCl, pH 1.7. The resultant sensorgrams were analysed using BIAcore™ evaluation software.

For the selectivity assessment on BIAcore™ T200, the VNAR F3 monomer was immobilised *via* amine coupling on the chip surface Fc2, giving a coating density of 200 RU. The flow channel Fc1 was used as a reference flow channel. In the interaction assay, different homodimer and heterodimer concentrations present in solution were injected over the immobilised ligand, and regenerated with glycine-HCl, pH 1.5.

Determination of VNAR stability

Thermal stability analysis was conducted using an ELISA format following published methods (Dooley *et al*. 2003; Shao *et al*. 2007). To assess VNAR's ability to withstand irreversible thermal denaturation, soluble VNAR protein samples at 10 µg/mL working concentration were incubated for 1 h at a range of temperatures (25–100°C) on a gradient thermocycler. Following heat treatment samples were allowed to cool to room temperature before analysis of binding activity in an ELISA coated with SEED homodimer at 1 µg/mL. ELISA was performed using 1 µg/mL final concentration of VNAR protein, which was serially diluted. Residual binding activity was detected with an anti-c-Myc-HRP antibody (Roche, 11814150001) and expressed as a percentage of the OD value of untreated controls.

Similarly, pH stability of VNAR domains was determined by ELISA plates coated with SEED homodimer at 1 µg/mL. VNAR proteins were prepared at a working stock of 20 µg/mL in a final volume of 50 µL in 0.1 M glycine-HCl (G-HCl) at designated pH values ranging from

1.5-8.0. Following overnight incubation at room temperature, aliquots were withdrawn and neutralised in 1x PBS (pH 7.4) to a final concentration of 1 µg/mL directly in an ELISA plate and serially diluted. Active protein was detected as above.

A thermal shift assay, to determine VNAR's melting temperature (Tm) and structural stability of the domains, was conducted (Liu *et al*. 2014). This fluorescence-based melting assay made use of a StepOnePlus™ Thermocycler (Applied Biosystems) and SYPRO® Orange fluorophore (Sigma-Aldrich Corp.) at a final dilution of 1:1,000 and a total of 10 µg VNAR protein in a 50 µL reaction volume triplicate, prepared in 1x PBS, pH 7.4. The temperature was increased from 25°C to 80°C at ~2°C/min. Tm values were obtained from melting curves using the corresponding Protein Thermal Shift analysis software.

Assessing VNAR's applicability as a detection reagent

A sandwich ELISA format was applied to test the ability of lead VNAR F3 to detect SEED homodimer present in solution, mimicking intended application as a bioprocessing reagent. ELISA plate was coated overnight at 4°C with the VNAR protein as a capture ligand at 2.5 µg/mL in 1x PBS, 100 µL/well. SEED protein was added at top concentration of 10 µg/mL and serially diluted in 1x PBS, incubated for 1 h at room temperature. Binding was detected indirectly with 1/1,000 dilution of goat anti-human IgG (Fc specific) antibody (Sigma-Aldrich, 2136) and rabbit anti-goat IgG-HRP (Sigma-Aldrich, A8919).

The VNAR proteins were further evaluated for their applicability as detection reagents capturing target from a heterogeneous mix of SEED proteins in solution. VNARs were immobilised on an ELISA plate at a sub-saturating concentration in 1x PBS, 100 µL/well, incubated overnight at 4°C. SEED proteins were pre-mixed at different proportions of homodimer and heterodimer in a final combined concentration of 5 µg/mL prior to their addition to a blocked ELISA plate at 100 µL/well in duplicate, then incubated for 1 h at room temperature and washed as standard. Binding signal was generated following standard protocols and *via* detection of bound SEED protein with a 1/200 dilution of anti-human IgG (Fc specific)-HRP antibody (Sigma-Aldrich, A0170).

Bio-layer interferometry analysis

Determination of VNAR's applicability as a detection reagent to screen and quantify contaminating levels of SEED homodimer (GA/GA) protein was performed on the Octet® QK System (Pall FortéBio) with Octet Data Acquisition software 7.0. Binding association was measured in 100 µL/well 1x PBS at 30°C with 1,000 rpm orbital agitation using 96-well half-

area black microplates (Greiner Bio-One). Anti-mouse IgG Fc capture (AMC) Dip and Read™ biosensors (Pall FortéBio) were pre-equilibrated in 1x PBS, pH 7.4, for 10 min. Subsequently, recombinant VNAR protein containing a C-terminal-mouse IgG2a Fc fusion was loaded onto biosensor tips at a concentration of 80 µg/mL for 200 s. Followed by 60 s wash in 1x PBS. Association was performed using SEED homodimer (GA/GA) Fc protein at 150 µg/mL and 75 µg/mL for 100 s.

pH elution ELISA

An ELISA-elution assay was adapted from (Kummer and Li-Chan 1998), as a screening tool to test the dissociation of the VNAR-SEED complex and to compare the effectiveness of eluents. ELISA plates were coated with F3 VNAR protein at 2.5 µg/mL in 1x PBS, 100 µL/well overnight at 4°C. A total of 100 µL of SEED homodimer protein at a concentration of 5 µg/mL in 1x PBS was applied to the wells and incubated at room temperature for 1 h. The eluent used in this study was 0.1 M glycine-HCl (G-HCl), which is a standard eluent for IgG purification, and the effectiveness of a range of pH 1.5–8.0 on dissociation was assessed. Each elution buffer was applied at a volume of 100 µL/well: once, twice or three times, with incubation periods at room temperature of 20 min each. The SEED protein remaining after elution was detected indirectly with anti-human IgG (Fc specific) (Sigma-Aldrich, I2136) and anti-goat-HRP (Sigma-Aldrich, A8919) conjugate diluted 1/1,000 in 3% MPBS. The potential denaturing effects of the eluents were also monitored, where the integrity of the VNAR was examined by testing subsequent re-binding to SEED homodimer protein.

Site-directed biotinylation of VNAR domains

For the purpose of site-directed biotinylation of VNAR F3 monomer, commercially available Expresso® Biotin Cloning and Expression System (Lucigen) was used as per manufacturer's instructions. To evaluate the capture of SEED protein from solution, biotinylated VNAR F3 protein was immobilised on streptavidin magnetic Dynabeads M-280 for 30 min at room temperature, rotating at 20 rpm. The depleted supernatant was collected through magnetic rack pull-down and the beads were washed with 1 mL 1x PBS, upon which 50 µL of 100 µg/mL SEED protein either homodimer or heterodimer was added to the pelleted beads for re-suspension and incubated for 1 h at room temperature, rotating at 20 rpm. Finally, the supernatant was collected by pelleting the beads on a magnetic rack, and re-suspending the beads in 50 µL 1x PBS. Both supernatant and beads were reduced and analysed on SDS-PAGE,

and Western blot *via* anti-human IgG (Fc specific)-HRP (Sigma-Aldrich, A0170) (1:1,000 dilution) to detect captured SEED protein.

Reformatting and transient expression of VNAR as Fc fusion

Plasmid DNA encoding VNAR F3 was isolated *via* Wizard® Plus SV Minipreps DNA Purification System (Promega), cloned into pEEE2A mouse IgG2a Fc vector (Elasmogen Ltd.) through restriction *Bss*HII and *Bsi*WI cloning and transformed into electrocompetent *E. coli* XL1-Blue cells (Agilent Technologies). Once the DNA sequence was confirmed, plasmid DNA from individual clones was extracted *via* Qiagen Plasmid Midi Kit for PEI-mediated transfection and transient expression in HEK293 host cells (Huh *et al*. 2007; Backliwal *et al*. 2008) using serum free FreeStyle™ 293 media (Invitrogen). Purification of expressed protein was achieved by protein A affinity chromatography (ProSep Ultra Plus Protein A, Millipore). To the harvested supernatant, $1/10^{th}$ volume of 1 M Tris-HCl buffer (pH 8.0) and 5 M NaCl was added and mixed thoroughly before adding 1.5 mL protein A resin per 100 mL culture. The suspension was kept rotating at 15 rpm for 3 h at room temperature. The resulting resin was washed 3x with 1x PBS and loaded onto gravity flow Poly-Prep® columns (Bio-Rad Laboratories). VNAR-Fc fusion protein was eluted using 8 mL 0.1 M G-HCl buffer, pH 3.0, and the eluate neutralised with 2 mL 1 M Tris-HCl buffer, pH 8.0. The eluate was buffer exchanged into 1x PBS through dialysis (Snake Skin® dialysis tubing 7 kDa MWCO, Thermo Fisher Scientific) against 1 L 1x PBS/1 mL eluted protein, pH 7.4, overnight at 15ºC, followed by further 5 h dialysis in fresh 1x PBS and finally concentrated using Amicon® Ultra-15 Centrifugal Filters (MWCO 10 kDa). Protein quality was assessed *via* SDS-PAGE and quantified using Ultrospec™ 6300 pro UV/Visible Spectrophotometer (Amersham Bioscience) taking an absorbance reading at 280 nm wavelength.

For kinetic analysis of VNAR fusions, SEED ligand was immobilised at approximately 1,000 RU on a CM5 series S chip in 10 mM sodium acetate, pH 5.5. The purified F3-Fc fusion protein and the F3 monomer were diluted separately in running buffer to final concentrations of 250, 125, 62.5, 31.25, 15.63, 7.82, 3.91, 1.95 and 0 nM at 25°C. Each concentration was injected over immobilised ligand for 5 min at a constant flow rate of 30 µL/min and allowed to dissociate for 10 min, followed by a 30 s regeneration pulse with 20 mM glycine, pH 1.7, at 30 µL/min, 240 s stabilisation period and a 50 mM NaOH wash. Reference subtracted sensorgrams for each concentration were analysed.

Results

Selection of single-domains specific to SEED homodimer

With the aim of isolating antigen-specific shark-derived single-domain binders *via* phage display, a synthetic VNAR phage library ELSS1 (Elasmogen Ltd., patent WO/2014/173959) was panned against biotinylated SEED target captured on streptavidin magnetic beads. The method of antigen presentation was a key influencer on the selection outcome. To more closely mimic natural target presentation (Verheesen *et al*. 2003) this solution-based method biased outcomes towards the isolation of binders to soluble forms of the target proteins. Three rounds of affinity selections were carried out against SEED homodimer target, with a 2-fold reduction in antigen concentration from round 2 included, to help drive the isolation of target specific high-affinity binders. Enrichment was evaluated *via* monoclonal phage ELISA against the selection target: SEED homodimer and a control protein, HSA. A high proportion of ELISA-positive clones with $OD_{450\ nm} \geq 0.5$ (as determined by ELISA cut-off) were isolated after the third round of selection (Figure 1a). No binding to HSA was observed. Furthermore, individual clones from round 3 phage were assessed for SEED selectivity by ELISA (Figure 1b), and specifically their ability to distinguish between the two forms of the SEED protein, homodimer and heterodimer formats.

Sequence analysis of selected clones

Sequence analysis identified only four unique homodimer-specific leads which may indicate the rarity of binders capable of distinguishing between two such closely related antigens. Whilst several hundred clones were screened, only 20 clones (less than 1%) were sequenced based on their binding profile meeting the following criteria: (i) a "strong" positive (above 0.5 O.D) binding signal *via* phage ELISA, (ii) confirmatory binding of the same clones after soluble protein was released (but not purified) from the bacterial periplasm and (iii) negative on the control heterodimer antigen. Out of these 20 clones; 4 had frameshifts when sequenced, F3 appeared 3 times out of the remaining 16 sequences, and C3; 5 out of 16, E4 and A4 appeared once. In total 8 unique clones were identified however the remaining 4 clones were considered positive but weak binders as soluble protein.

Unique binding sequences were aligned using the clustalW method in MegAlign DNASTAR Lasergene12 software and deduced amino acid sequences (Figure 2a) with variations limited to within the CDR1 and CDR3 regions. Interestingly, despite the theoretical sequence diversity of the synthetic library (100 billion unique clones), two homodimer specific binders, E4 and C3, shared the same CDR1 sequence. Sequence analysis also established that these selected VNARs were either of type IIb or IIIb IgNAR genes that lacked non-canonical

cysteines residues and maintained a tryptophan (W) residue in CDR1, typical of type IIIb isotypes (Kovaleva *et al*. 2014), and which is a significant proportion of the ELSS1 library. Canonical cysteines were maintained in frameworks 1 and 3b (Streltsov *et al*. 2004) to stabilise the VNAR fold (ribbon representation, Figure 2b).

VNAR characterisation: specificity and affinity

To demonstrate that the lead VNAR domains were capable of selective binding to SEED homodimer, and also lacked cross-reactivity to analogues Fc scaffolds, the VNAR leads were expressed as soluble protein to allow functional characterisation in the absence of phage particles. Recombinant VNARs were harvested from *E. coli* cell periplasm and purified by nickel affinity chromatography *via* the hexa-histidine tail. The specificity of antigen binding was determined by ELISA *via* anti-c-Myc-HRP detection. All four clones showed superior binding to SEED homodimer with no reactivity to other related Fc domains including: SEED heterodimer, IgG or IgA from human sera or IgG from sera of other species: mouse, rat, sheep, rabbit (Figure 3).

The choice of a lead candidate was prioritised on the basis of the greatest affinity and optimal binding kinetics for the SEED homodimer. Using these criteria, clone F3 was selected as it exhibited the most favourable binding kinetics, quantified with surface plasmon resonance analysis by BIAcore^{TM} (Figure 4), with overall affinity in the low nM range (Table 1).

Evaluation of VNAR domain applicability as diagnostic and purification reagents

SEED selectivity in solution

Recombinant VNARs were tested for their ability to capture SEED in a sandwich ELISA (Figure 5), with the ELISA set-up mimicking affinity purification (i.e. where VNAR is immobilised and SEED is present in solution). The F3 clone performed well as a capture reagent, demonstrating its ability to bind SEED homodimer (Figure 5a) whilst maintaining no reactivity to the heterodimer analogue (Figure 5b).

VNAR F3 characterisation: stability assessment

A key deliverable from this study was to assess the utility of isolated SEED binders as (reusable) affinity purification reagents using pH changes to modify elution conditions. VNAR F3 could tolerate a wide range of pH 1.5-8.0 using 0.1 M G-HCl eluent (Figure 6a), a crucial characteristic for the reusability of any purification resin. The ability of purified F3 VNAR protein to withstand irreversible thermal denaturation during a short (1 h) incubation period at elevated temperature was also investigated. Upon heat exposure to a range of temperatures, F3

protein was serially diluted and assessed for binding functionality in ELISA. The percentage binding activity in comparison to controls held at room temperature was calculated and the values were plotted in Figure 6b. Approximately 90% activity was retained at the top VNAR concentration up to 50^{o}C, with a progressive drop in activity observed at higher temperatures. This correlated well with F3's melting temperature of ~50°C as determined by the thermal shift assay (Figure 6c).

pH elution efficacy

An ELISA-elution assay was devised as a screening tool for quantifying dissociation efficiency of the VNAR-antigen complex. The effect of one, increasing to three, washes with 0.1 M G-HCl as eluent, at a range of pHs from 1.5–8.0 on the dissociation of VNAR F3 bound to SEED homodimer protein was quantified (Figure 7a). As a control these ELISA absorbance values were compared to washes with 1x phosphate buffered saline (1x PBS) (pH 7.4). G-HCl eluent at pH 1.5 resulted in the most effective elution profile even after a single treatment exposure, with dissociation also observed at pH 2.0 and 3.0. The integrity of the VNAR protein and its re-binding functionality to SEED homodimer following exposure to eluents was tested by reapplying the homodimer protein post elution (Figure 7b).

VNAR F3 functionalised streptavidin beads as SEED homodimer-specific capturing agents

To further assess the potential application of VNAR F3 as a purification reagent, the lead candidate was biotinylated to facilitate immobilisation on a solid support. In order to avoid generation of heterogeneous products that may occur through chemical biotinylation, a site-directed approach with a C-terminal Avi tag sequence, was used facilitating *in vivo* site-directed biotinylation exploiting the *E. coli* biotin ligase (BirA).

The ability to recover either SEED homodimer or heterodimer proteins from solution was determined using capture by streptavidin magnetic beads pre-decorated with biotinylated VNAR F3, and analysed *via* SDS-PAGE (Figure 8a). The streptavidin beads were heated in loading buffer to release the biotinylated protein from streptavidin prior to gel analysis. Consequently, some streptavidin was also released from the resin yielding a protein band migrating between 10 and 15 kDa. Specific capture of SEED homodimer from solution *via* bead-immobilised biotinylated-VNAR F3 was also evaluated by Western blot through detection of captured protein using an anti-human IgG (Fc specific)-HRP conjugate (Figure 8b).

Affinity enhancement through Fc reformatting

One could imagine a situation where the lead F3 domain is used in certain instances as both an affinity capture reagent and a diagnostic (ELISA) reagent. In an attempt to increase the functional affinity, and in turn the utility of the domain as a diagnostic tool, the VNAR F3 monomer was reformatted as a mouse IgG2a Fc fusion construct, to generate a bivalent binder that should benefit from an avidity effect whilst at the same time offering an alternative immobilisation approach *via* Fc-capture. The VNAR F3-Fc was expressed transiently in a eukaryotic HEK293 system. Purification of the VNAR-fusion protein was achieved *via* protein A affinity chromatography using a suitable gravity-flow column. Protein quality was assessed *via* SDS-PAGE (Figure 9a) and indicated a highly purified VNAR F3-Fc with bands migrating between 40 kDa and 50 kDa, consistent with the predicted molecular weight. The concentration of the expressed protein was determined on an Ultrospec™ 6300 pro UV/Visible spectrophotometer (Amersham Bioscience, GE Healthcare) and confirmed a yield of approximately 4.5 mg per litre of cell culture. A small but noteworthy increase in apparent binding affinity, through an uplift in avidity (decreased off-rate), was achieved as kinetic assessment on the BIAcore™ of the F3 monomer versus F3-Fc fusion resulted in a decrease in K_D from 1.5 nM to 0.6 nM, using the SEED homodimer as an immobilised ligand (Table 2). No binding to heterodimer protein being observed (Figure 9b).

The ability of the F3 lead to differentiate between SEED homodimer and heterodimer proteins in a heterogeneous solution mix was tested to determine the approximate limit of detection for the homodimer contaminant. The sandwich-based ELISA employed, demonstrated VNAR's ability to specifically capture SEED homodimer in a concentration-dependent manner from a heterogeneous mix of SEED proteins (Figure 10). Increasing the heterodimer protein % did not contribute to the binding signal. The modest increase in affinity measure for the F3-Fc format did indeed appear to increase the sensitivity of this new reagent in an ELISA format with the bivalent F3-Fc capable of quantifying SEED homodimer contaminants in the mixture when they are only 2.5% of the total SEED protein, corresponding to 125 ng/mL.

Applicability of VNAR F3 for target detection using bio-layer interferometry and surface plasmon resonance

The VNAR F3-Fc fusion was assessed *via* bio-layer interferometry on an Octet® QK System (Pall FortéBio), testing its possible applicability as an "in-line" biosensor detection reagent with signal generated using anti-mouse IgG Fc capture. The immobilised VNAR-Fc construct

demonstrated a concentration-dependent binding to SEED homodimer protein present in solution (Figure 11a). Surface plasmon resonance studies further validated the overall superior selectivity of the parental VNAR ligand as it was also able to detect and quantify the contaminating presence of SEED homodimer in a heterogeneous mixture of SEED proteins (Figure 11b).

Discussion

The central aim of this project was to explore the utility of shark-derived single domain binders as bioprocessing reagents, and in particular determine their ability to remove an important processing bottleneck in the development of "scaled-up" SEED bispecific therapeutics. The combination of shark-derived VNAR domains and phage-display is a well-trodden route to the successful isolation of specific binders to an ever-growing list of targets (Nuttall *et al*. 2001; Nuttall *et al*. 2002; Dooley *et al*. 2003; Liu *et al*. 2007; Liu, Anderson and Goldman 2007; Shao *et al*. 2007; Ubah *et al*. 2017). The potential applicability of camelid VHH single-domains as bioprocessing and affinity capture reagents (Detmers *et al*. 2010) and biosensors (Bever *et al*. 2016) have been previously reviewed. Of particular relevance to this program of work, Klooster and colleagues (2007) isolated two IgG specific VHHs from an immune phage display library. These specifically recognised all four human IgG isotypes with nanomolar affinities (3–6 nM) through binding to the relatively conserved CH1 domain within the Fab region. This technology was subsequently commercialised under the name CaptureSelect™ IgG-CH1 affinity matrix (Thermo Fischer Scientific) for human IgG purification, with a binding capacity of 25−30 g IgG/L (Kruljec and Bratkovič 2017). GE Healthcare commercialised another camelid-based affinity ligand directed this time against the human constant domain of the λ light chain. This LambdaFabSelect affinity matrix was designed for Fab antibody fragment purification with a binding capacity of 12 g Fab/L.

The growing list of commercially available VHH-based affinity resins sets the scene for the, as yet, unexplored application of shark-derived single-domains as bioprocessing reagents. Whilst they share many similar properties with VHH domains at ~11 kDa, VNARs are the smallest naturally occurring binding domains in the vertebrate kingdom (Stanfield *et al.* 2004), and therefore possess a much higher density of binding loops by size, with four regions of hypervariability contributing to specificity (Kovaleva *et al.* 2014). Their small size combined with their amenability to modular reformatting and biomaterials conjugation (Kovaleva *et al*. 2014; Barelle and Porter 2015; Nogueira *et al*. 2019) offers the potential of

multivalent arrangements for enhanced avidity as well as promoting high surface density formats leading to increased binding capacity.

Interestingly, previous screens with scFv libraries failed to isolate binders capable of distinguishing between hetero and homodimer forms of the SEED protein (pers. commun., results not shown). VHH libraries have not been screened, but their success with other IgG domains would suggest that they may also provide a source of discriminatory clones.

The VNAR phage display library used as the starting material for this project was designed to contain extended CDR3 loops, ranging from 9–26 amino acids and typically longer than those found in the variable domains of mice or humans (Diaz *et al*. 2002). In addition, the paratope of VNARs containing these extended CDRs appear pre-disposed to access pockets, grooves and protein clefts present on the surface of target proteins. These cryptic epitopes (such as enzyme binding sites) (Stanfield *et al*. 2004), are rarely "seen" by more conventional antibodies which have a much more planar binding site, made up from variable heavy and light domains.

Two requirements essential for the usefulness of an affinity ligand are: (i) binding specificity to target protein and (ii) effective elution of target from the ligand complex (with the possible added bonus of reusability) (Verheesen *et al*. 2003; Tu *et al*. 2015). VNARs were isolated using solution-phase selection techniques with biotinylated SEED homodimer protein captured on magnetic streptavidin beads. This solution-phase strategy was chosen to encourage the recognition of the soluble form of the SEED protein. Four leads were specific for SEED homodimer and lacked reactivity to other Fc scaffolds (human, mouse, rat) including the SEED heterodimer.

These results demonstrate VNAR's exquisite ability to access and recognise differences between closely related and sterically restricted conformational epitopes most probably within the CH3-CH3 interface. In the absence of a co-crystal structure, we can only speculate where this homodimer specific domain binds, but it is unlikely to be a linear peptide as this would tend to promote cross-reactivity with the heterodimeric (AG-GA) construct sharing the GA CH3 domain. It is also unlikely that it binds outside of the SEED CH3 domain, such as the hinge region or the CH2 domain, as here again it is hard to see how discrimination between the two closely related proteins could be achieved. It seems sensible to postulate that a combination of their reduced size and protruding CDR3 loop (average loop length of lead clones was 11 residues) enabled them to recognise the target molecule's surface topology. Indeed, these characteristics of a VNAR paratope appears to predispose access to recessed epitopes otherwise intractable to the relatively flat binding surfaces of conventional mAbs

(Stanfield *et al*. 2004; Stanfield *et al*. 2007; Henderson *et al*. 2007) as well as discontinuous anti-idiotypic epitopes (Könning, Rhiel *et al*. 2017). With an average length of 11 amino acids the VNAR CDR3 loops of the lead clones are well within the CDR3 lengths seen in mammals, and so it might be sensible to conclude that the "finger-projection" positioning of the VNAR CDR3 may be even more important than simple loop length alone (Stanfield *et al*. 2004; Kovalenko *et al*. 2013).

The affinity of this interaction was determined *via* surface plasmon resonance analysis on BIAcore™ and one clone in particular, F3, had a single digit nanomolar affinity of 1.6 nM for homodimer and without measurable cross-reactivity to the closely related heterodimer protein. Whilst there are a few absolute affinities of VNARs reported in the literature, this high affinity was actually better than others previously published from a naïve spiny dogfish semi-synthetic CDR3 mutagenised library of 10.2 ± 7.2 nM for staphylococcal enterotoxin B target (Liu, Anderson and Goldman 2007) and was closer to the sub-nanomolar affinity (0.7–0.9 nM) reported for a VNAR isolated from an immunised spiny dogfish-derived library against human serum albumin (Müller *et al*. 2012). This further emphasises the robustness of this non-immunised synthetic VNAR phage display (combinatorial) platform, which combines the isolation of high affinity binders with a predisposition to recognise cryptic epitopes.

One additional characteristic of the VNAR domains is their inherent ability to tolerate non-physiological (denaturing) conditions and again highlights their utility as a possible (and reusable) immunoaffinity reagent. IgNAR's naturally reside in the harsh environment of shark serum consisting of high salt (1 osmolar) and high urea (350–500 mM) (Dooley *et al*. 2006; Stanfield *et al*. 2007; Trischitta *et al*. 2012). The F3 clone, in particular, retained functionality after overnight exposure to extremes of pH (pH 1.5–8.0) typically used for elution and regeneration of affinity resins (Verheesen *et al.* 2003; Wang *et al*. 2013), with retention of binding activity of 99.9 ± 1.4% and 92.4 ± 2% respectively.

Similarly, clone F3 exhibited high thermal stability, retaining 55.6 ± 0.1% binding activity following 1 h incubation at 80°C and correlated well with previous stability assessments of VNARs (Dooley *et al*. 2003; Liu, Anderson and Goldman 2007; Goodchild *et al*. 2011), and VHH's which has achieved <60% binding recovery after heat-induced unfolding in the range of 65–84°C (Dumoulin *et al.* 2002), and contrasted with conventional murine-derived variable fragments and mAbs which were fully inactivated (irreversibly denatured) following 50°C and 70°C incubations, respectively (Goodchild *et al*. 2011). Even after boiling at 100°C for 1 h, VNAR F3 retained 14.1 ± 1% binding activity to the SEED protein. VNAR protein architecture appears to encourage rapid refolding when returned to physiological

conditions facilitated by their simple single-chain Ig structure, which unlike conventional antibodies presents a considerable number of exposed hydrophilic surface residues, as well as stabilising intra-molecular disulphide bridges (Stanfield *et al*. 2004; Streltsov *et al*. 2004).

The VNAR's apparent ability to selectively recognise SEED protein in solution indicates its potential as a sensitive and specific reagent for on-line diagnostic applications. A capture assay using VNAR F3-functionalised magnetic beads showed VNAR's applicability in this format. VNAR-Fc capture *via* anti-mouse IgG Fc capture Dip and Read™ biosensors in conjunction with bio-layer interferometry on the Octet® QK System further confirmed the functional interaction with the GA/GA SEED homodimer present in solution. This setup being an optimal Octet® format for screening cell culture supernatants with SEED protein free in solution, and functioning as a proof of concept of VNAR's intended on-line application as a SEED homodimer detection reagent.

Enhancement of binding reactivity of the single binding domain was achieved through multimerisation (Ubah *et al*. 2017), with dimerisation in the literature often resulting in as much as a 10-fold higher affinity than monomeric domains (Simmons *et al*. 2006). The homodimer specific VNAR F3 was reformatted as a mouse IgG2a Fc fusion, which allowed for dimerisation through Fc-mediated association whilst at the same time offering an alternative immobilisation approach *via* Fc-capture. The reformatted VNAR F3 domain demonstrated an enhanced affinity (K_D) to 0.6 nM, and this was attributed to a modest avidity effect. This 2.5-fold improvement over the parental monomeric construct was brought about by a 7.5-fold slower dissociation rate constant (k_d). The enhanced affinity of the bivalent VNAR-reagent also translated into an improved sensitivity to target in a sandwich-based ELISA, and so whilst this delivered a relatively small enhancement in assay sensitivity from 500 ng/mL to 125 ng/mL for SEED homodimer protein detection, this could eventually be an important format for on-line diagnostic applications.

The VNAR F3 clone could also be used for affinity purification with regeneration of the reagent being an important and desirable property, and ideally comparable to protein A resin reusability of >100 purification cycles (Wang *et al*. 2013; Tu *et al*. 2015). The stability assessment data from these studies certainly confirmed VNAR's ability to withstand overnight exposure to pH 1.5 without any detrimental effects in its binding capacity. The effect of eluent treatment on the VNAR's integrity and ability to rebind SEED was assessed in an ELISA-based elution assay, with a good stability profile observed following the first elution cycle. However, F3's binding capacity was slowly diminished following consecutive cycles of elution at low pH range (1.5–2.0), but importantly stability was retained at pH 3.0, which is a typical

elution condition used in affinity purification. No stripping of immobilised VNAR from the ELISA wells was detected, suggesting that its robustness and biological inactivity could make the VNAR ligand a superior and safer alternative to protein A, which has been associated with safety concerns related to stripping of protein A from columns and resulting risk of product contamination (Low *et al*. 2007). These initial findings imply that further optimisation, for instance by means of engineering pH-dependant binding through the generation of a histidine-doped F3 sub-library (Könning *et al*. 2016) or the addition of a stress engineering step during the selection process for enhanced stability (Pershad and Kay 2013) may be desirable in a final product. In a pH stability test designed to mimic the environment of the gut, comparing anti-hTNF-α VNAR and VHH binding activity following exposure to pH 3.0 over extended periods, concluded that both domain scaffolds retained activity (around 80%) after 28 days. This compared with a mAb which retained only 1.6% following 21 days exposure, when the experiment was stopped (Ubah *et al.* 2017).

In conclusion, we have explored the utility of shark-derived single-domain VNARs as diagnostic and affinity reagents for targets that have proved intractable to more typical antibody-based technologies. Phage display selection was successful in delivering SEED homodimer specific VNAR domains with low nanomolar affinity, despite only subtle interface differences between SEED GA/GA and AG/GA heterodimer protein, with further affinity and sensitivity enhancement achieved upon dimerisation. In addition, VNAR's exceptional stability and resistance to irreversible denaturation marks VNAR domains as promising affinity capturing agents for a range of purification applications.

Acknowledgements

The authors would like to thank Iris Willenbücher and Kerstin Hallstein for the BIAcore™ analysis, and Nadine Barron for the bio-layer interferometry work. This work was supported by the Industrial Biotechnology Innovation Centre, and Merck KGaA.

References

Backliwal,G., Hildinger,M., Hasija,V. and Wurm,F.,M. (2008) *Biotechnol. Bioeng*., **99**, 721-727.

Barelle,C. and Porter,A. (2015) *Antibodies*, **4**, 240-258.

Bever,C.S., Dong,JX., Vasylieva, N., Barnych,B., Cui,Y., Xu,ZL., Hammock,B.D. and Gee,S.J. (2016) *Anal. Bioanal. Chem*., **408**, 5985–6002.

Brinkmann,U. and Kontermann,R.E. (2017) *mAbs*, **9**, 182-212.

Choe,W., Durgannavar,T.A. and Chung,S.J. (2016) *Materials*, **9**, 94.

Davis,J.H., Aperlo,C., Li,Y.,Kurosawa,E., Lan,Y., Lo,K.M. and Huston,J.S. (2010) *Protein Eng. Des. Sel.*, **23**, 195-202.

Detmers,F., Hermans,P., Jiao,J. and and McCue,J. (2010) *Bioprocess Int.*, **8**, 50-54.

Diaz,M., Stanfield,R., Greenberg,A. and Flajnik,M. (2002) *Immunogenetics*, **54**, 501-512.

Dolk,E., van der Vaart,M., Hulsik,D., Vriend,G., de Haard,H., Spinelli,S., Cambillau,C., Frenken,L. and Verrips,T. (2005) *Appl. Environ. Microbiol*., **71**, 442-450.

Dooley,H., Flajnik,M. and Porter,A. (2003) *Mol. Immunol*., **40**, 25-33.

Dooley,H., Stanfield,R.L., Brady,R.A. and Flajnik,M.F. (2006) *Proc. Natl. Acad. Sci. U. S. A*., **103**, 1846-1851.

Dumoulin,M., Conrath,K., Van Meirhaeghe,A., Meersman,F., Heremans,K., Frenken,L., Muyldermans,S., Wyns,L. and Matagne,A. (2002) *Protein Sci.*, **11**, 500-515.

Eifler,N., Medaglia,G., Anderka,O., Laurin,L.and Hermans,P. (2014) *Biotechnol. Prog*., **30**, 1311-1318.

Girard,V., Hilbold,N.J,. Ng,C.K., Pegon,L., Chahim,W., Rousset,F. and Monchois,V. (2015) *J. Biotechnol*., **213**, 65-73.

Godar,M., Morello,V., Sadi,A., Hultberg,A., De Jonge,N., Basilico,C., Hanssens,V., Saunders,M., Lambrecht,B.N., El Khattabi,M., de Haard,H., Michieli,P. and Blanchetot,C. (2016) *Sci. Rep.*, **6**, 31621.

Goodchild,S.A., Dooley,H., Schoepp,R.J., Flajnik,M. and Lonsdale,S.G. (2011) *Mol. Immunol*., **48**, 2027-2037.

Griffiths,K., Dolezal,O., Parisi,K., Angerosa,J., Dogovski,C., Barraclough,M., Sanalla,A., Casey,J.L., González,I., Perugini,M.A., Nuttall,S. and Foley,M. (2013) *Antibodies*, **2**, 66-81.

Gross,A.W., Dawson,J.P., Muda,M., Kelton,C., McKenna,S.D. and Hock,B. (2013) In Schmidt,S.R. (ed), *Fusion Protein Technologies for Biopharmaceuticals: Applications and Challenges*. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 557-569.

Ha,J., Kim,J. and Kim,Y. (2016) *Front. Immunol*., **7**, 394.

Hawkins,R., Russell,S. and Winter,G. (1992) *J. Mol. Biol.*, **226**, 889-896.

Henderson,K.A., StreltsovV.A., Coley,A.M., Doleza,O., Hudson,P.J., Batchelor,A.H., Gupta,A., Bai,T., Murphy,V.J., Anders,R.F., Foley,M. and Nuttall,S.D. (2007) *Structure*, **15**, 1452-1466.

649 Huh,S.H., Do,H.J., Lim,H.Y., Kim,D.K., Choi,S.J., Song,H., Kim,N.H., Park,J.K.,
650 Chang,W.K., Chung,H.M. and Kim,J.H. (2007) *Biologicals*, **35**, 165-171.

651 Kelton,C., Wesolowski,J.S., Soloviev,M., Schweickhardt,R., Fischer,D., Kurosawa,E.,
652 McKenna,S.D. and Gross,A.W. (2012) *Arch. Biochem. Biophys*., **526**, 219-225.

653 Klooster,R., Maassen,B.T.H., Stam,J.C., Hermans,P.W., ten Haaft,M.R., Detmers,F.J.M., de
654 Haard,H.J., Post,J.A. and Verrips, C.T. (2007) *J. Immunol. Methods*, **324**, 1-12.

655 Könning,D., Zielonka,S., Grzeschik,J., Empting,M., Valldorf,B., Krah,S., Schröter,C.,
656 Sellmann,C., Hock,B. and Kolmar,H. (2017) *Curr. Opin. Struct. Biol*., **45**, 10-16.

657 Könning,D., Zielonka,S., Sellmann,C., Schröter,C., Grzeschik,J., Becker,S. and Kolmar,H.
658 (2016) *Mar. Biotechnol*., **18**, 161-167.

659 Könning,D., Rhiel,L., Empting,M., Grzeschik,J., Sellmann,C., Schröter,C., Zielonka,S.,
660 Dickgießer,S., Pirzer,T., Yanakieva,D., Becker,S. and Kolmar,H. (2017) *Sci. Rep.*, **7**, 9676.

661 Kovalenko,O.V., Olland,A., Piché-Nicholas,N., Godbole,A., King,D., Svenson,K.,
662 Calabro,V., Müller,M.R., Barelle,C.J., Somers,W., Gill,D.S., Mosyak,L. and Tchistiakova,L.
663 (2013) *J. Biol. Chem*., **288**, 17408-17419.

664 Kovaleva,M., Ferguson,L., Steven,J., Porter,A. and Barelle,C. (2014) *Expert Opin. Biol. Ther*.,
665 **14**, 1527-1539.

666 Krah,S., Sellmann,C., Rhiel,L., Schröter,C., Dickgiesser,S., Beck,J., Zielonka,S., Toleikis,L.,
667 Hock,B., Kolmar,H. and Becker,S. (2017) *N. Biotechnol*., **29**, 167-173.

668 Kruljec,N. and Bratkovič,T. (2017) *Bioconjug. Chem*., **28**, 2009-2030.

669 Kufer,P., Lutterbuse,R. and Baeuerle,P.A. (2004) *Trends Biotechnol*., **22**, 238-244.

670 Kummer,A. and Li-Chan,E. (1998) *J. Immunol. Methods*, **211**, 125-137.

671 Ladenson,R.C., Crimmins,D.L., Landt,Y. and Ladenson,J.H. (2006) *Anal. Chem*., **78**, 4501-
672 4508.

673 Liu,H., Saxena,A., Sidhu,S.S. and Wu,D. (2017) *Front. Immunol.*, **8**, 38.

674 Liu,J.L., Anderson,G.P., Delehanty,J.B., Baumann,R., Hayhurst,A. and Goldman,E.R. (2007)
675 *Mol. Immunol.*, **44**, 1775-1783.

676 Liu,J.L., Anderson,G.P. and Goldman,E.R. (2007) *BMC Biotechnol*., **7**, 78.

677 Low,D., O'Leary,R. and Pujar,N.S. (2007) *J. Chromatogr. B Analyt. Technol. Biomed. Life*
678 *Sci.*, **848**, 48-63.

679 Muda,M., Gross,A.W., Dawson,J.P., He,C., Kurosawa,E., Schweickhardt,R., Dugas,M.,
680 Soloviev,M., Bernhardt,A., Fischer,D., Wesolowski,J.S., Kelton,C., Neuteboom,B. and
681 Hock,B. (2011) *Protein Eng. Des. Sel*., **24**, 447-454.

682 Müller,M.R., Saunders,K., Grace,C., Jin,M., Piche-Nicholas,N., Steven,J., O'Dwyer,R.,
683 Wu,L., Khetemenee,L., Vugmeyster,Y., Hickling,T.P., Tchistiakova,L., Olland,S., Gill,D.,
684 Jensen,A. and Barelle,C.J. (2012) *mAbs*, **4**, 673-685.

685 Nogueira,J.C.F., Greene,M.K., Richards,D.A., Furby,A.O., Steven,J., Porter,A., Barelle,C.,
686 Scott,C.J and Vijay Chudasama. (2019) *Chem. Commun*., **55**, 7671-7674.

687 Nuttall,S.D., Krishnan,U.V., Doughty,L., Nathanielsz,A., Ally,N., Pike,R.N., Hudson,P.J.,
688 Kortt,A.A. and Irving,R.A. (2002) *FEBS Lett*., **516**, 80-86.

689 Nuttall,S.D., Krishnan,U.V., Hattarki,M., De Gori,R., Irving,R.A. and Hudson,P.J. (2001) *Mol.*
690 *Immunol*., **38**, 313-326.

691 Pershad.K. and Kay,B.K. (2013) *Methods*, **60**, 38-45.

692 Sampei,Z., Igawa,T., Soeda,T., Okuyama-Nishida,Y., Moriyama,C., Wakabayashi,T.,
693 Tanaka,E., Muto,A., Kojima,T., Kitazawa,T., Yoshihashi,K., Harada,A., Funaki,M.,
694 Haraya,K., Tachibana,T., Suzuki,S., Esaki,K., Nabuchi,Y. and Hattori,K. (2013) *PLoS One*, **8**,
695 e57479. doi:10.1371/journal.pone.0057479.

696 Shao,C.Y., Secombes,C.J. and Porter,A.J. (2007) *Mol. Immunol.*, **44**, 656-665.

697 Shukla,A. and Norman,C. (2017) In Gottschalk,U. (ed), *Process scale purification of*
698 *antibodies*. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 559-595.

699 Shukla,A., Wolfe,L., Mostafa,S. and Norman,C. (2017) *Bioeng. Transl. Med*., **2**, 58-69.

700 Simmons,D.P., Abregu,F.A., Krishnan,U.V., Proll,D.F., Streltsov,V.A., Doughty,L.,
701 Hattarki,M.K. and Nuttall,S.D. (2006) *J. Immunol. Methods*, **315**, 171-184.

702 Skegro,D., Stutz,C., Ollier,R., Svensson,E., Wassmann,P., Bourquin,F., Monney,T., Gn,S. and
703 Blein,S. (2017) *J. Biol. Chem*., **292**, 9745-9759.

704 Spiess,C., Zhai,Q. and Carter,P.J. (2015) *Mol. Immunol.*, **67**, 95-106.

705 Stanfield,R.L., Dooley,H., Verdino,P., Flajnik,M.F. and Wilson,I.A. (2007) *J. Mol. Biol*., **367**,
706 358-372.

707 Stanfield,R., Dooley,H., Flajnik,M. and Wilson,I. (2004) *Science*, **305**, 1770-1773.

708 Streltsov,V.A., Varghese,J.N., Carmichael,J.A., Irving,R.A., Hudson,P.J. and Nuttall,S.D.
709 (2004) *Proc. Natl. Acad. Sci. U.S.A*., **101**, 12444-12449.

710 Trischitta,F., Faggio,C. and Torre,A. (2012) *OJAS*., **2**, 32-40.

711 Tu,Z., Xu,Y., Fu,J., Huang,Z., Wang,Y., Liu,B. and Tao,Y. (2015) *J. Chromatogr. B Analyt.*
712 *Technol. Biomed. Life Sci*., **983**, 26-31.

713 Ubah,O.C., Steven,J., Kovaleva,M., Ferguson,L., Barelle,C., Porter,A.J.R. and Barelle,C.J.
714 (2017) *Front. Immunol*., **8**, 1780.

715 Ubah,O.C., Barelle,C.J., Buschhaus,M.J. and Porter,A.J. (2016) *Curr. Pharm. Des.*, **22**, 6519-
716 6526.

717 Uth,C., Zielonka,S., Hörner,S., Rasche,N., Plog,A., Orelma,H., Avrutina,O., Zhang,K. and
718 Kolmar,H. (2014) *Angew. Chem. Int. Ed. Engl.*, **53**, 12618-12623.

719 Verheesen,P., Ten Haaft,M.R., Lindner,N., Verrips,C.T. and De Haard,J.J.W. (2003) *Biochim.*
720 *Biophys. Acta*, **1624**, 21-28.

721 Wang,L., Dembecki,J., Jaffe,N.E., O'Mara,B.W., Cai,H., Sparks,C.N., Zhang,J., Laino,S.G.,
722 Russell,R.J. and Wang,M. (2013) *J. Chromatogr. A*, **1308**, 86-95.

723 Wesolowski,J., Alzogaray,V., Reyelt,J., Unger,M., Juarez,K., Urrutia,M., Cauerhff,A.,
724 Danquah,W., Rissiek,B., Scheuplein,F., Schwarz,N., Adriouch,S., Boyer,O., Seman,M.,
725 Licea,A., Serreze,D.V., Goldbaum,F.A., Haag,F. and Koch-Nolte,F. (2009) *Med. Microbiol.*
726 *Immunol.*, **198**, 157-174.

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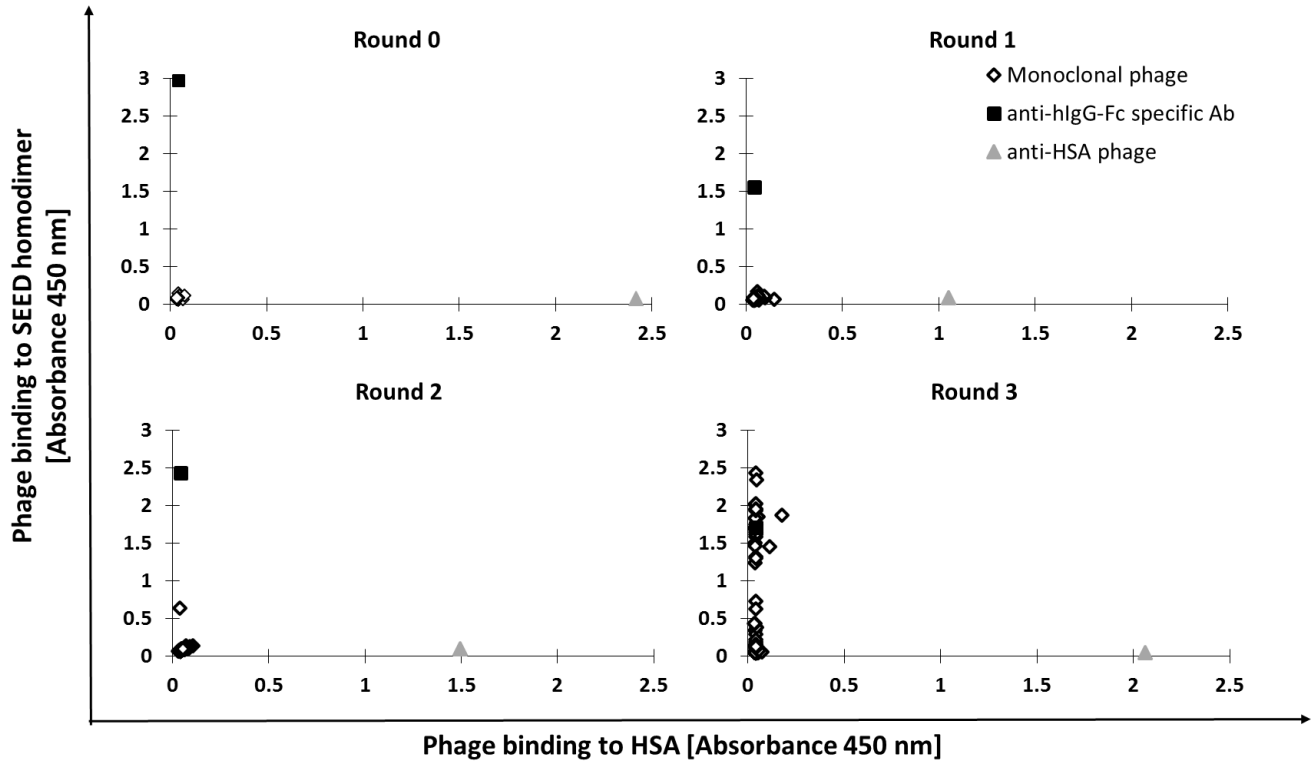

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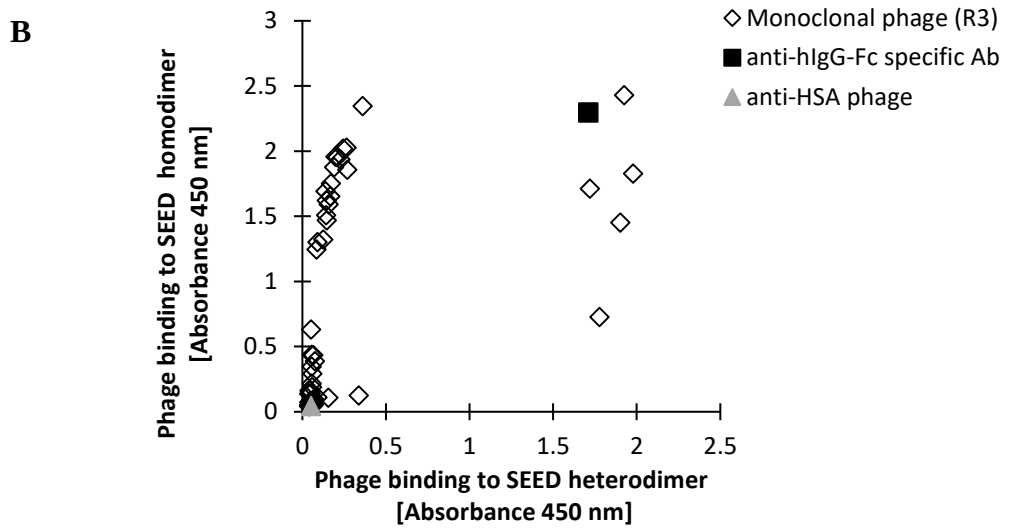
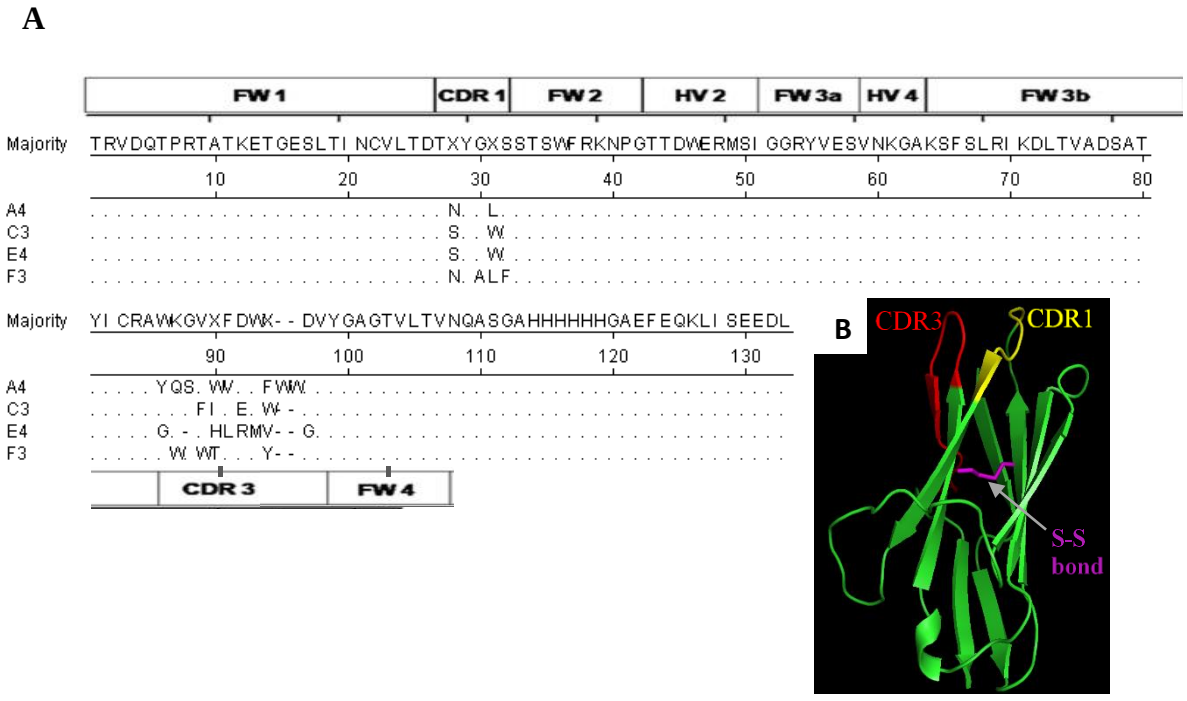


729

730 **Fig. 1.** Round specific monoclonal phage ELISA. **(A)** Monoclonal phage ELISA evaluating 92
731 clones at each round of selection. Each point represents a single clone tested for binding to
732 either the target SEED homodimer, or irrelevant control HSA. **(B)** Round 3 phage monoclonal
733 ELISA assessing binding selectivity to SEED homodimer versus SEED heterodimer. Positive
734 binders are defined as an absorbance 450 nm reading of ≥0.5. Relevant positive controls
735 included: anti-HSA phage, anti-human IgG (Fc specific)-HRP antibody control for SEED
736 coating.



737

738 **Fig. 2.** Sequence analysis of selected VNAR leads. **(A)** Amino acid sequence alignment
739 (clustalW) of selected anti-SEED homodimer VNAR leads, with dots indicating amino acid

identity with the consensus (majority) sequence of the synthetic library backbone. Standard single letter amino acid nomenclature is employed. Relevant tags present: hexa-histidine tag - HHHHHH, c-Myc tag - EQKLISEEDL. **(B)** Ribbon representation of VNAR F3 domain generated *via* homology modelling using SWISS-MODEL and PyMOL software. VNAR F3 domain modelled on PDB entry 4hgk (type IV short CDR3 loop) (Kovalenko *et al*. 2013). CDR1 and CDR3 loops, and the disulphide bond (S-S bond) formed between cysteine residues, are indicated. SWISS-MODEL accessed at http://swissmodel.expasy.org/ and PyMOL software (Schrödinger, Inc.).

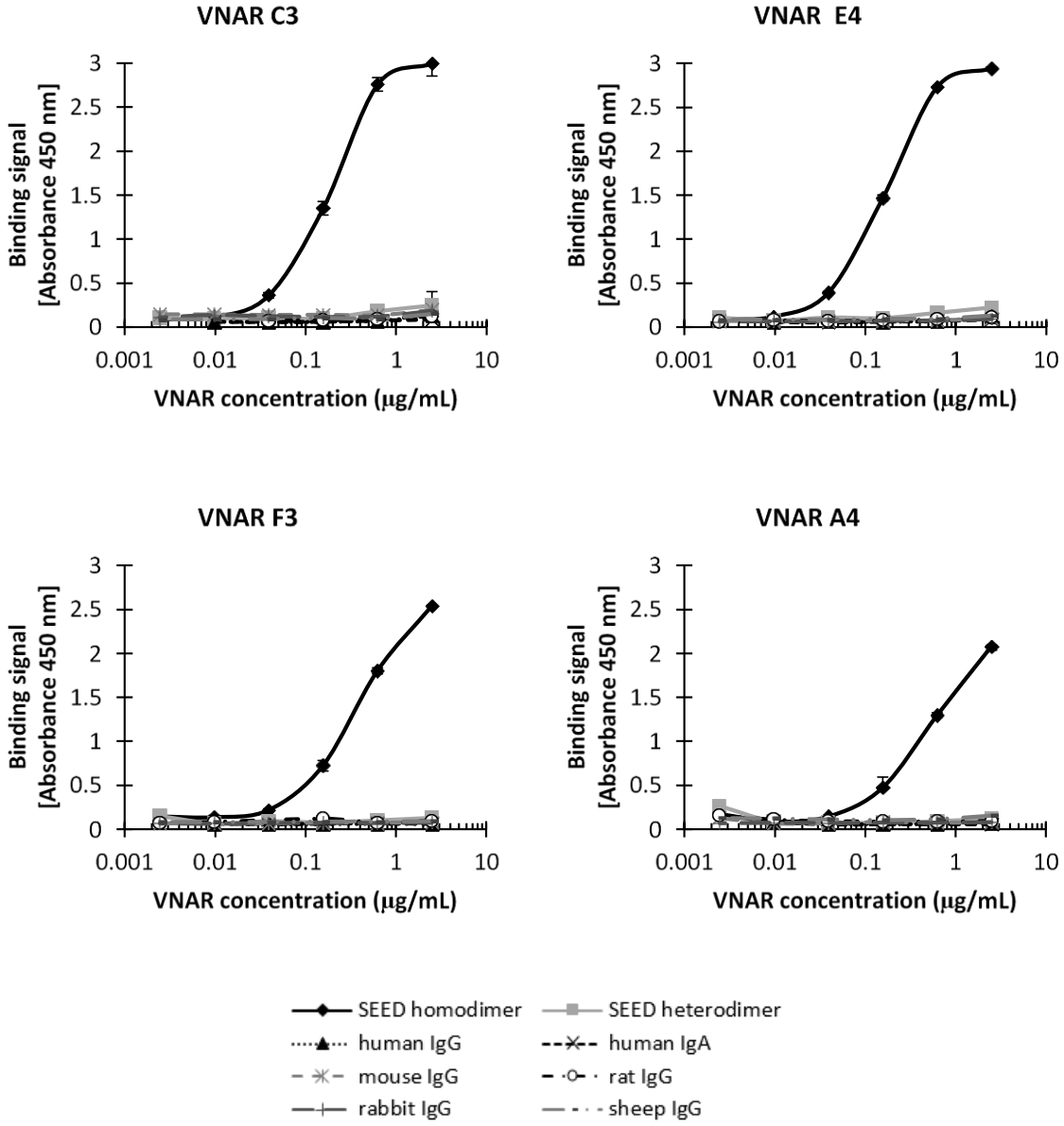


Fig. 3. Antigen-binding analysis of four VNAR proteins assessing selectivity to SEED

homodimer versus binding to analogue Fc scaffolds: SEED heterodimer, human IgG and IgA from sera, and species cross reactivity to IgG from animal sera. Experiments were performed in duplicate, standard deviations are indicated.

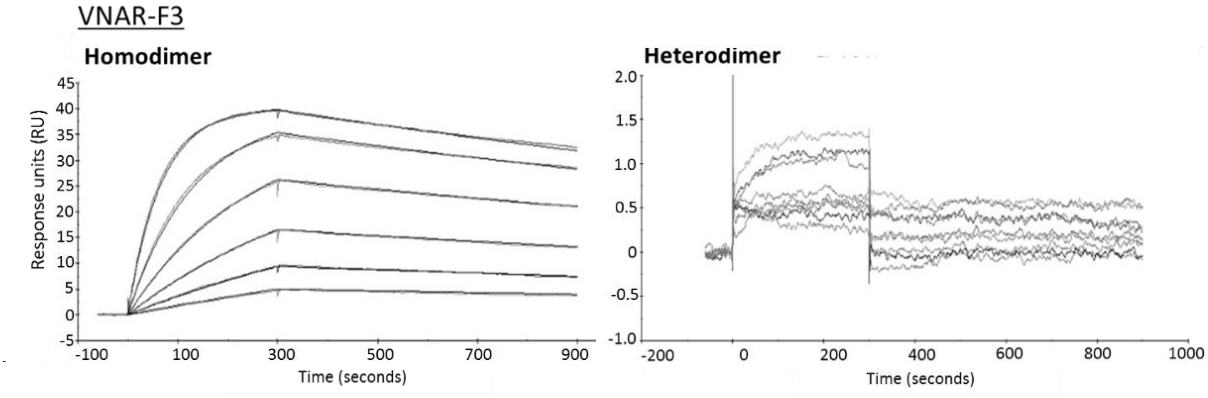


Fig. 4. BIAcore™ analysis of the F3 VNAR protein binding to SEED homodimer or heterodimer coated CM5 chip. See Methods for protocol details.

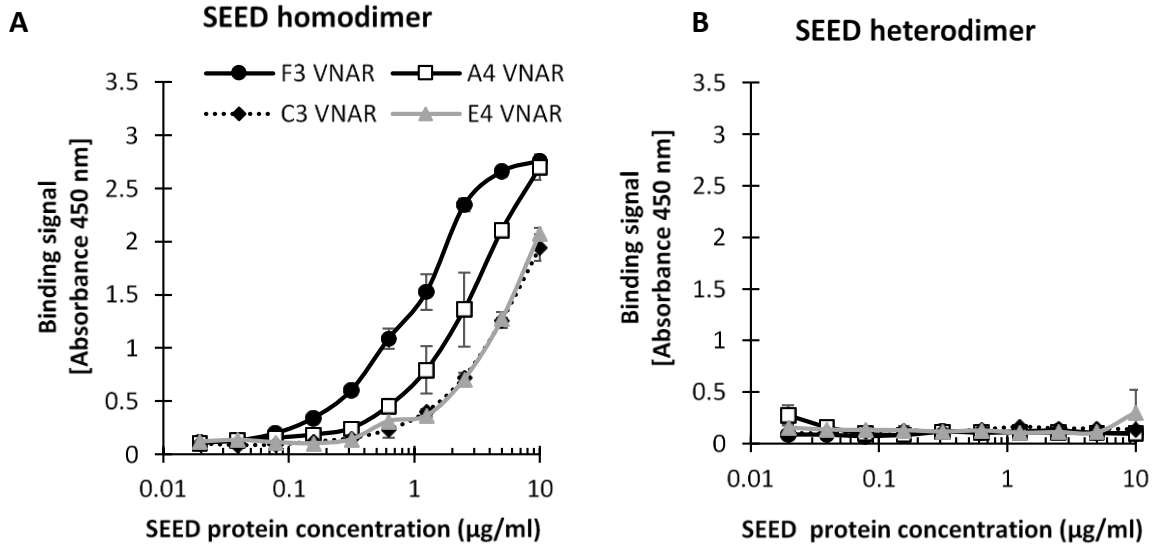


Fig. 5. Sandwich ELISA determining the activity of immobilised VNAR proteins as capture reagents specific for SEED targets in solution. Either SEED homodimer **(A)** or SEED heterodimer **(B)**. Experiments were performed in duplicate, standard deviations are indicated.

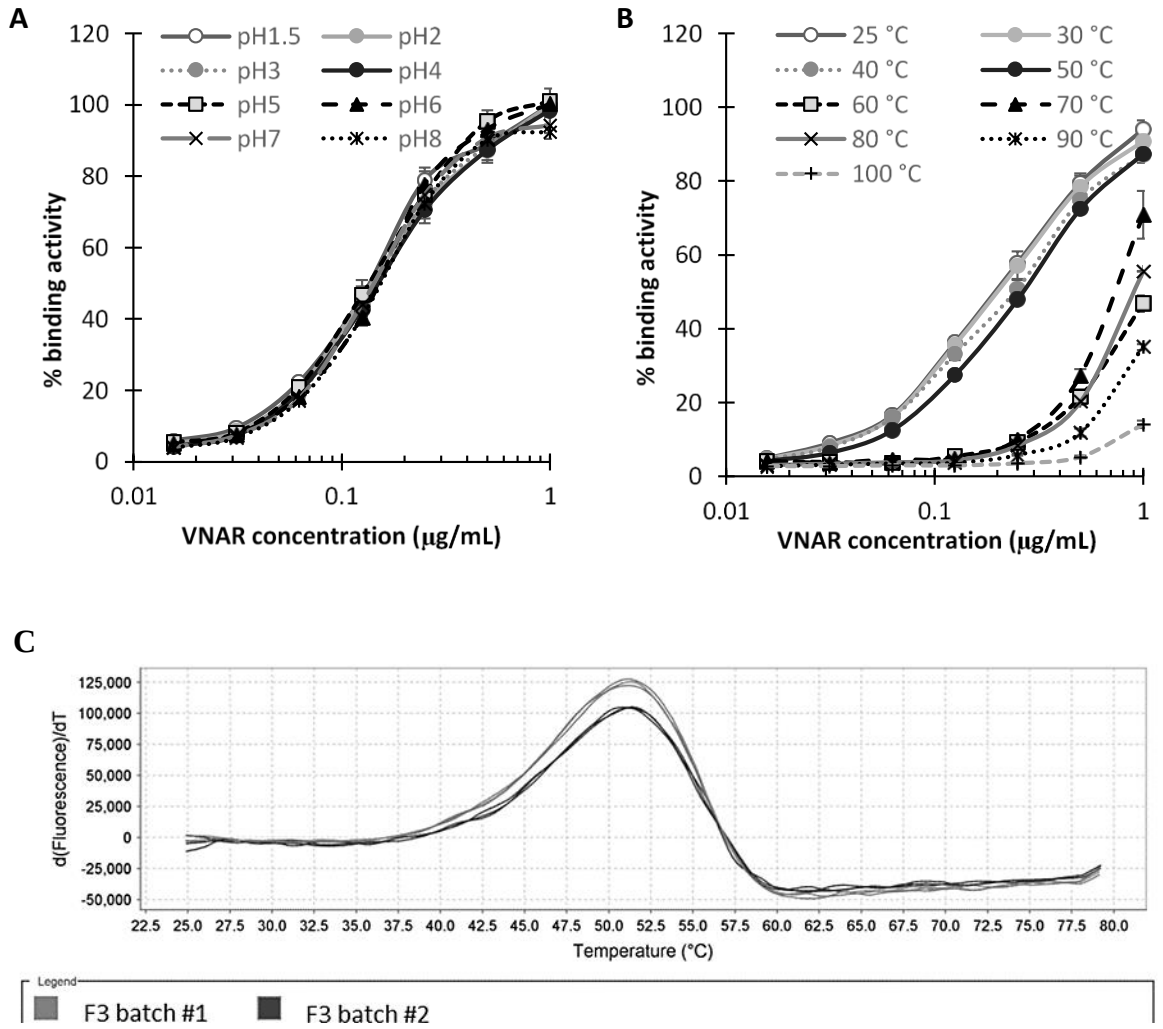


Fig. 6. Stability analysis of VNAR F3 protein to resist irreversible denaturation in a range of physiological and non-physiological conditions. The % binding activity to SEED homodimer, with respect to an untreated homodimer control, was determined following incubation at a range of **(A)** pH and **(B)** temperatures. Experiments were performed in duplicate, standard deviations are indicated. (**C**) Thermal shift assays determining Tm of VNAR F3 (two separate protein expression batches). Melt peaks are shown for each individual VNAR on the first derivative plot of florescence data. Experiments were performed in triplicate.

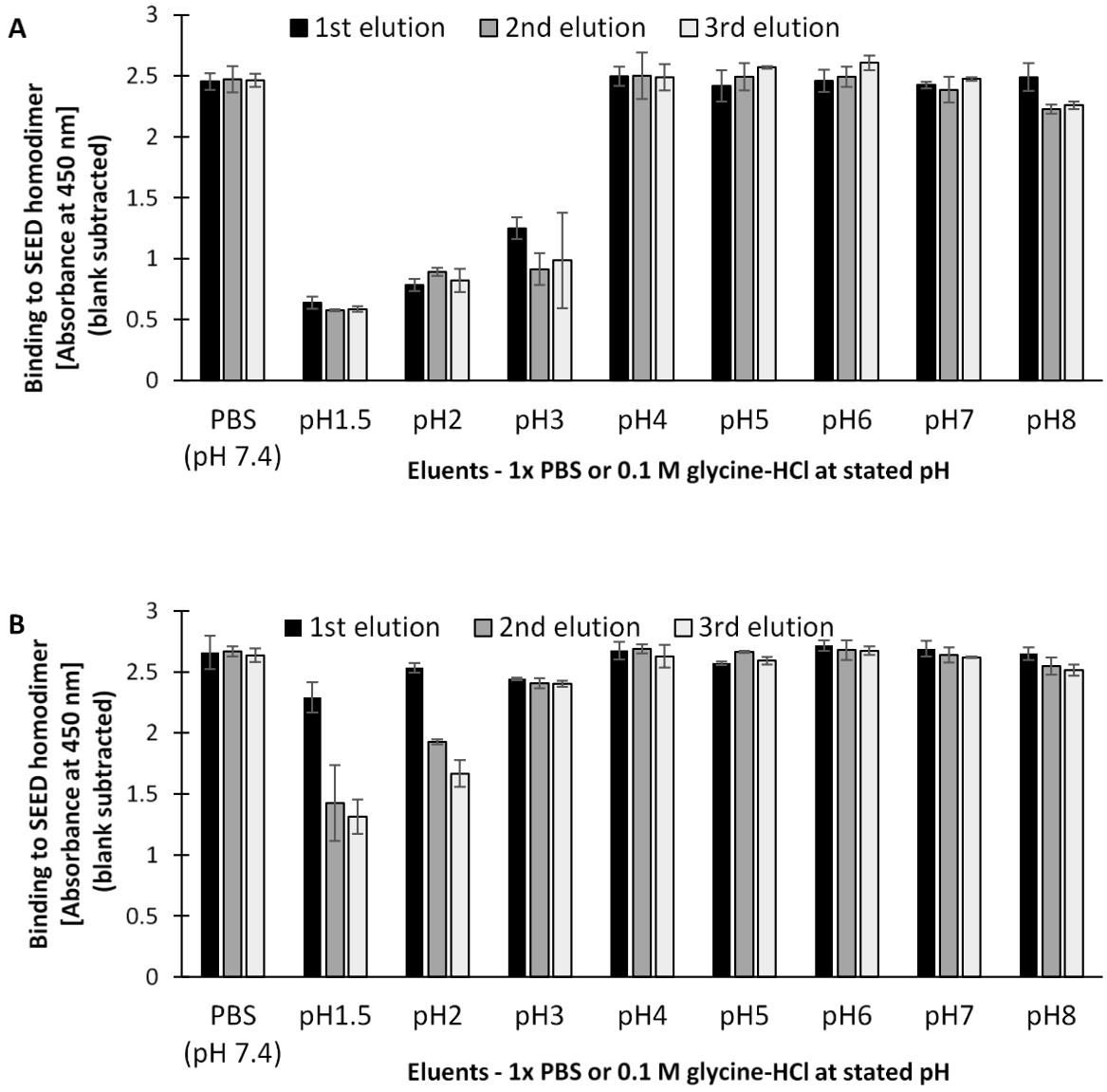


Fig. 7. ELISA-based elution assay comparing the effect of pH on elution efficiency. Absorbance values indicate relative amount of SEED homodimer bound to VNAR F3 immobilised on an ELISA plate **(A)** upon application of eluents at various pH. Eluents were applied once, twice and three times. **(B)** Absorbance values indicate re-binding of immobilised VNAR F3 to SEED homodimer following multiple elutions at various pH. Experiments were performed in duplicate, standard deviations are indicated.

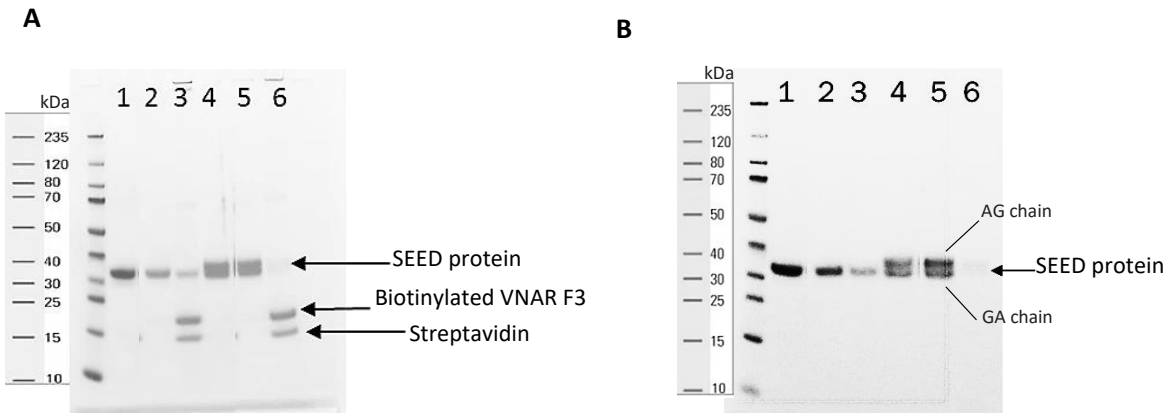
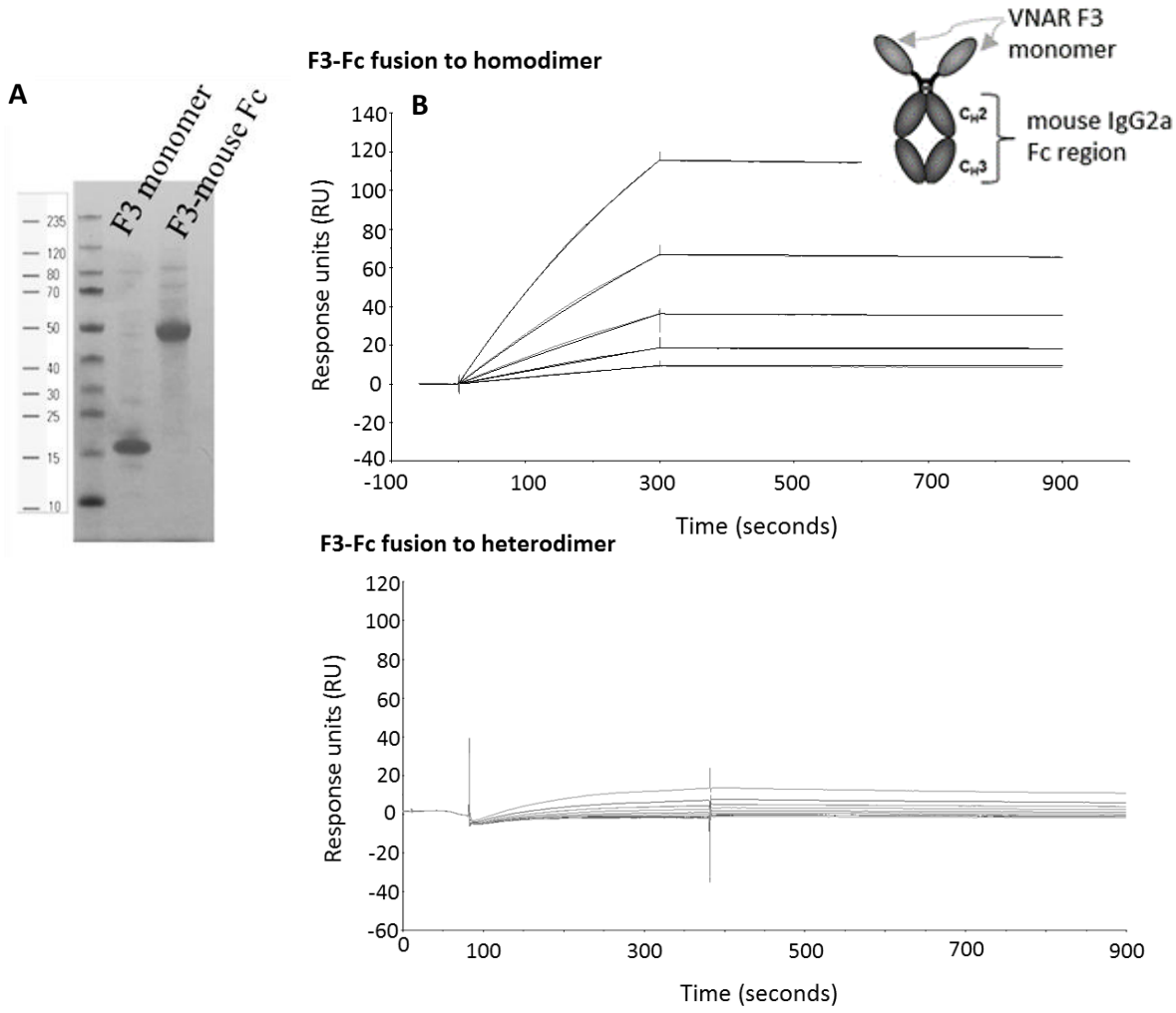
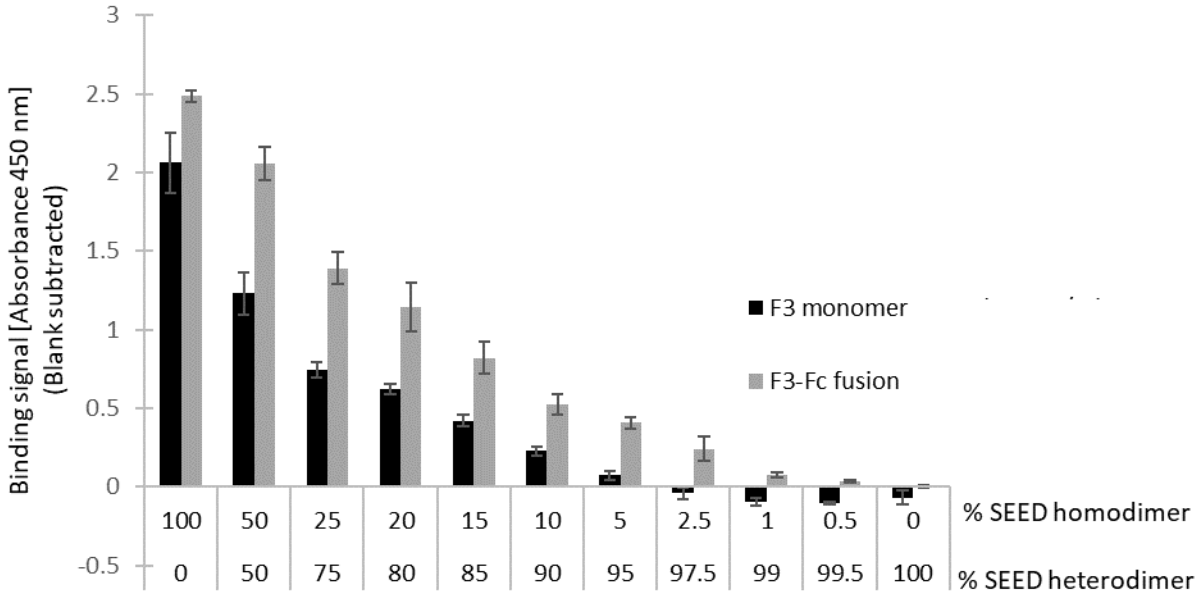


Fig. 8. Assessing capture of SEED protein *via* biotinylated VNAR F3 immobilised on streptavidin magnetic beads. SEED protein - either homodimer (lane 1) or heterodimer (lane 4) prior to depletion (control), depleted supernatant (lane 2 and 5 respectively) and VNAR-functionalised streptavidin beads post-depletion (lane 3 and 6 respectively). **(A)** SDS-PAGE analysis of reduced samples detected with Coomassie staining. **(B)** Western blot analysis of reduced samples detected with anti-human IgG (Fc specific)-HRP conjugate. Protein marker: Spectra^{TM} multicolour broad range protein ladder.



784

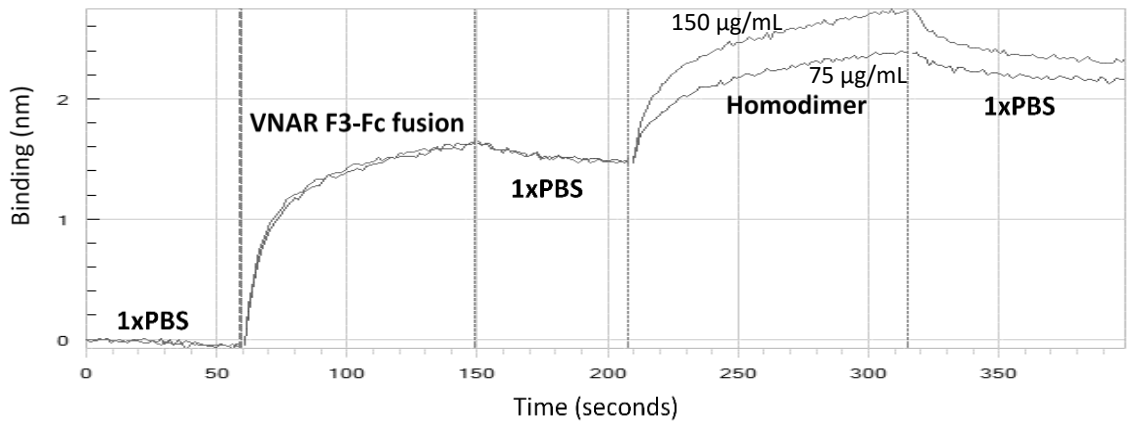
785 **Fig. 9.** Quality assessment of purified VNAR F3-Fc fusion. **(A)** SDS-PAGE of reduced
786 samples, the VNAR F3-Fc fusion and a VNAR F3 monomer for comparison. **(B)** BIAcore™
787 T200 Sensorgrams of the VNAR F3-Fc fusion used to determine K_D values *via* surface plasmon
788 resonance. SEED homodimer or heterodimer protein was immobilised on the CM5 chip
789 surface.



790

791 **Fig. 10.** Sandwich ELISA determining binding selectivity of the VNAR F3 monomer versus
792 the F3-Fc fusion in a heterogeneous mix of SEED proteins in solution. SEED binding was
793 detected with an anti-hIgG Fc-HRP. Experiments were performed in duplicate, standard
794 deviations are indicated.

A



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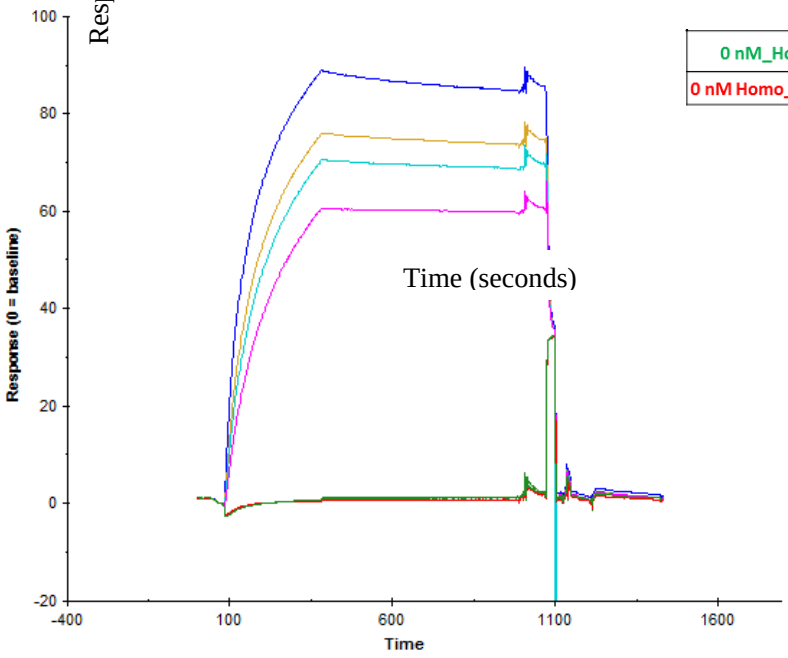
B

796

Analyte	k_a (x 10^5 M^{-1} s^{-1})	k_d (x 10^{-4} s^{-1})	K_D (nM)
F3 VNAR	2.3	3.7	1.6
E4 VNAR	2.6	5.6	2.1
C3 VNAR	1.9	5.6	2.9
A4 VNAR	0.4	7.9	21

797

Sample	Homodimer [nM]	Heterodimer [nM]
125 nM_Homo_Hetero	125	0
83 nM_Homo_Hetero	83	42
62.5 nM_Homo_Hetero	62.5	62.5
42 nM_Homo_Hetero	42	83
0 nM_Homo_Hetero	0	0
0 nM Homo_125 nM Hetero	0	125



798

799 **Fig. 11.** Binding assessment *via* bio-layer interferometry on the Octet® QK System (Pall
800 FortéBio) and surface plasmon resonance on a BIAcore™ T200 System. **(A)** VNAR F3-Fc
801 fusion capture *via* anti-mouse IgG Fc and the detection of SEED homodimer in solution, with
802 concentration-dependent binding observed. **(B)** Surface plasmon resonance sensorgrams
803 depicting binding between amine coupled VNAR F3 on the chip surface (at 200 RU) and its
804 ability to recognise homodimer protein in a range of SEED protein mixtures. Response levels
805 shown are with background values subtracted, and 0 RU marking the baseline.

806 **Table 1**. Kinetic measurements of VNAR protein (analyte) binding to ligand SEED
807 homodimer. Association (k_a) and dissociation (k_d) rate constants, and the equilibrium
808 dissociation constants (K_D) shown. No binding to SEED heterodimer was observed for any of
809 the clones tested.

Analyte	k_a (x 10^5 M^{-1} s^{-1})	k_d (x 10^{-4} s^{-1})	K_D (nM)
F3 VNAR	2.3	3.7	1.6

E4 VNAR	2.6	5.6	2.1
C3 VNAR	1.9	5.6	2.9
A4 VNAR	0.4	7.9	21

Table 2. Kinetic measurements of VNAR F3 monomer versus F3-Fc fusion (dimer) binding to SEED homodimer ligand. Association (k_a) and dissociation (k_d) rate constants, and equilibrium dissociation constants (K_D) shown. No binding to SEED heterodimer was observed (Figure 9).

Analyte	**k_a (x10^5 M^{-1} s^{-1})**	**k_d (x10^{-4} s^{-1})**	**K_D (nM)**
F3 monomer	1.9	3.0	1.5
F3-Fc	0.7	0.4	0.6