

Supplementary Information (ESI)

For

Biosensing made easy with PEG-targeted Bi-specific antibodies

Lyndon J. Raftery, Yadveer S. Grewal, Christopher B. Howard, Martina Jones, Muhammad J. A. Shiddiky, Laura G. Carrascosa, Kristopher J. Thurecht, Stephen M. Mahler*, Matt Trau*.

Chemicals

All chemicals purchased from the Australian supplier's branch unless otherwise stated.

Potassium ferrocyanide, potassium ferricyanide, and potassium chloride, methoxy PEG thiol (mPEG), 6-mercaptohexanol (MCH) from Sigma Aldrich.

Generation of bi-specific antibodies (bsAbs)

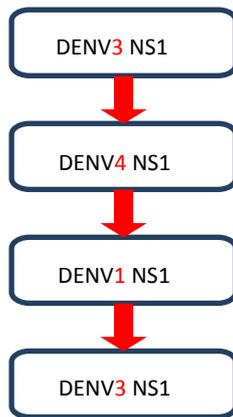
Anti-PEG/anti-EGFR DNA construct was synthesised by GeneArt™ (ThermoFisher Scientific, USA) utilising ABX-EGF and anti-PEG scFv sequences^{1, 2}. The bsAb construct contains a glycine-serine (G4S) linker to enable flexible movement of scFvs, a 6 x histidine affinity tag for immobilised metal ion chromatography (IMAC) purification, an N-terminal signal peptide to ensure adequate secretion and a Myc-tag for western blot analysis. This construct was cloned, expressed and purified in the same manner as described below.

Anti-NS1 scFv was generated through phage display panning of an in-house generated, immunised mouse library. Dengue virus (DENV) non-structural protein 1 (NS1) antigen was changed for each successive round of phage panning in order to maximise the chance of selecting cross-reactive binders (i.e. capable of recognising DENV1, 2, 3 & 4 NS1 serotypes) (Fig. 1a). Briefly, scFv phage library was added to immunotubes containing immobilised - NS1. Unbound phages were washed away with phosphate buffer saline (PBS), whilst bound phages were eluted with 2M glycine. Bound phages were then used to infect *E. coli* cells, thus propagating the phage clone scFv DNA against target antigen. This phage pool was subsequently used in the next round of phage panning.

Following 4 rounds of phage panning, enrichment against target antigen was assessed via polyclonal ELISA. Briefly, DENV NS1 was immobilised on Nunc Maxisorp 96-well ELISA plates

(affymetrix eBioscience, USA). Phage particles from rounds 1, 2, 3 & 4 are added to NS1-coated plates to determine if enrichment towards target antigen had occurred (Fig. 2b).

(A)



(B)

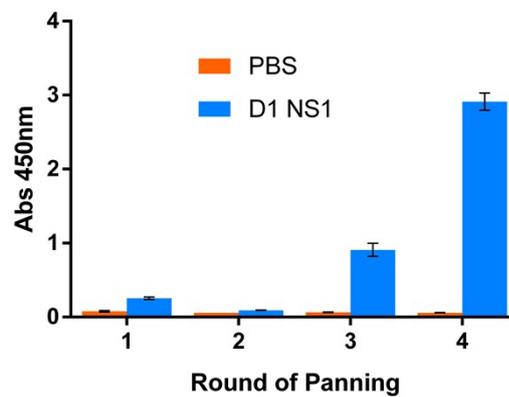


Fig. S1 (A) DENV NS1 serotype used for each respective phage panning round. (B) Polyclonal phage ELISA against DENV1 NS1. ELISA plates were coated with antigens indicated in the graph legends. PBS was used as a negative control. Bound phages were detected using Anti-M13 phage HRP antibody and read at 450nm. Error bars represent standard error of the mean absorbance from duplicate analysis ($n = 2$).

Individual phage clones were then picked and infected into *E. coli* cells. Once phage particles were generated, individual clones were sequenced and assessed for their ability bind all four DENV serotypes (DENV1, 2, 3 & 4 NS1). Of the 16 unique scFv phagemid sequences, 4 clones displaying anti-NS1 scFvs that were capable of binding all four serotypes were identified (Fig. S2).

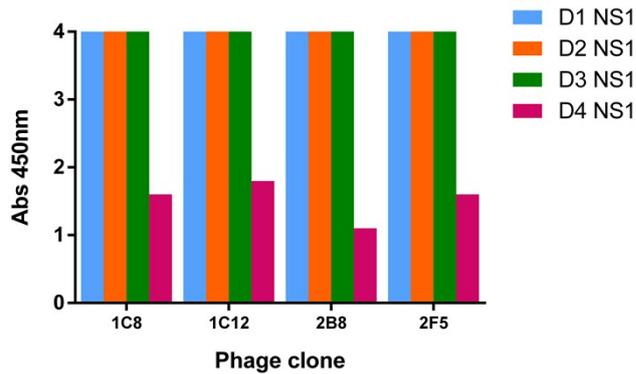


Fig. S2 Anti-NS1 phage particle ELISA screen. ELISA plates were coated with antigens indicated in the graph legends. Bound phages were detected using Anti-M13 phage HRP antibody and read at 450nm.

Phagemid scFv DNA was then cloned into an in-house generated expression vector containing the anti-PEG scFv sequence. Once anti-PEG/anti-NS1 sequence identity was confirmed, bsAb construct DNA was isolated with PureYield™ Plasmid Midiprep system (Promega). This DNA was then transfected into Chinese hamster ovary (CHO) cells via PEI-mediated transfection. After 12 days of 32°C shaking incubation the cells were pelleted by centrifugation for 30 mins at 5250 relative centrifugal force (RCF). The resulting supernatant was collected and filtered using a 0.22µm membrane (Sartorius). The secreted bsAbs were purified via IMAC using a 5mL HisTrap™ excel column (GE Healthcare). The protein was eluted with 20mM sodium phosphate, 500mM sodium chloride and 500mM imidazole (pH 7.4) buffer. Following elution the protein fractions were buffer exchanged into PBS (pH 7.4) using a HiPrep 26/10 column (GE Healthcare). The final product was run through a 0.22µm membrane filter and the 280nm absorbance was determined utilising a Nanodrop 1000 instrument (ThermoFisher Scientific, USA). Following concentration determination, analysis was conducted using a 4-12% Bis-Tris SDS-PAGE gel (Invitrogen). A ~55kDa band on SDS-PAGE gels for anti-PEG/anti-EGFR and ~62kDa apparent molecular weight for anti-PEG/anti-NS1 bsAb was observed (Fig. S3). Furthermore, size exclusion high performance liquid chromatography (HPLC) was performed using TSK gel G3000SW column (Tosoh). HPLC analysis confirmed >90% of purified bsAb existing as a monomeric species. The presence of protein was also determined via western blot.

The specificity of bsAbs were assessed via enzyme-linked immunosorbent assay (ELISA), testing binding of both anti-EGFR and anti-NS1 bsAbs to their respective antigen targets. Significant absorbance at 450nm indicating binding of bsAb to cognate antigen was observed. Negative control antigens (VEGFR2 and Japanese encephalitis NS1) showed negligible signal in the same assay.

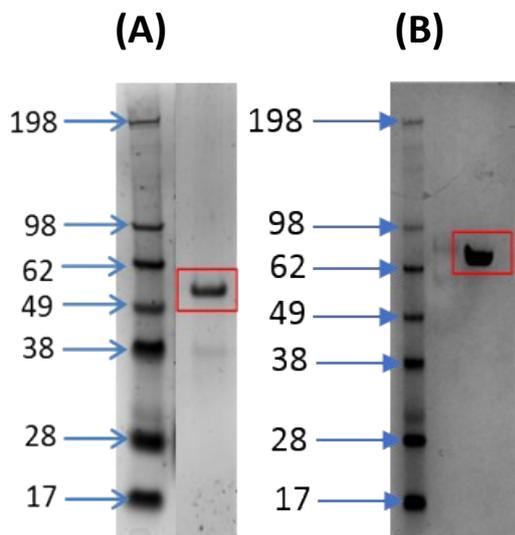


Fig. S3 4-12% Bis-Tris Protein Gels (A) SDS-PAGE gel of anti-mPEG/anti-EGFR bsAb. (B) SDS-PAGE gel of anti-mPEG/anti-NS1 bsAb. Both gels run under reducing conditions. Protein standard is SeeBlue Plus2® (ThermoFisher Scientific, USA). Respective bsAbs are boxed in red.

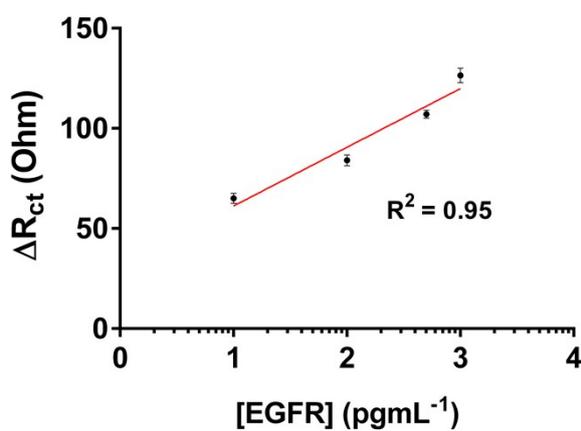


Fig. S4 Calibration curve using EGFR diluted in PBS at 10, 100, 500 & 1000pgmL⁻¹, where percentage change $R_{ct} = \Delta R_{ct} = (R_{ct,after} - R_{ct,before})/R_{ct,before} \times 100$. Error bars indicate the variance for triplicate measurements.

Assay Protocol

DropSens screen-printed gold electrodes (SPGE) were functionalised with 1mM mPEG by incubation at 25°C static for 1.5hrs. 1mM MCH was then incubated for 1hr under the same conditions. Following monolayer formation on the sensor surface, 1µg/mL of bsAb was incubated on the electrode surface for 45mins to maximise surface coverage whilst also allowing for optimal detection of target antigen³. Antigens diluted in PBS were added and incubated for 1hr. SPGEs were washed with PBS following each incubation step. 100µL volume was utilised for each reagent. For neat serum testing, 10µg/mL bsAb were incubated on mPEG/MCH monolayer for ~45mins. Antigens were spiked into neat foetal calf serum and allowed to incubate for 2hrs.

Electrochemical procedure

All electrochemical experiments were conducted at room temperature ($25 \pm 1^\circ\text{C}$) in a standard three-electrode electrochemical cell arrangement using an electrochemical analyser CHI 650D (CH Instruments, Austin, TX), where the electrochemical cell consisted of a Au sensor as a working electrode, a Pt counter electrode, and a Ag/AgCl (3 M NaCl) reference electrode (DropSens, Spain). Electrochemical signals were measured in a 10 mM phosphate buffer solution (pH 7.4) containing 2.5 mM $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ (1:1) and 0.1 M KCl. Differential pulse voltametric (DPV) signals were obtained with a potential step of 5 mV, pulse amplitude of 50 mV, pulse width 50 ms, and a pulse period of 100 ms. The EIS spectra were recorded in 10 mM phosphate buffer solution (pH 7.4) containing 2.5 mM $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ (1:1) and 0.1 M KCl using an alternating current voltage of 10 mV, with the frequency range of 0.1 Hz – 100 kHz.

The Faradaic current generated by the $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}^2[\text{Fe}(\text{CN})_6]$ probe accounts on the presence of a protein.

References

1. X.-D. Yang, X.-C. Jia, J. R. F. Corvalan, P. Wang and C. G. Davis, *Critical Reviews in Oncology/Hematology*, 2001, **38**, 17-23.
2. T. L. Cheng, S. R. Roffler, K. H. Chuang and S. J. Lu, *Journal*, 2012.
3. T. Bryan, X. Luo, P. R. Bueno and J. J. Davis, *Biosensors and Bioelectronics*, 2013, **39**, 94-98.