Sensitive protein detection using site-specifically oligonucleotide-conjugated nanobodies

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ABSTRACT

High-quality affinity probes are critical for sensitive and specific protein detection, in particular to detect protein biomarkers at early phases of disease development. Clonal affinity reagents can offer advantages over the commonly used polyclonal antibodies (pAbs) in terms of reproducibility and standardization of such assays. In particular, clonal reagents offer opportunities for site-directed attachment of exactly one modification per affinity reagent at a site designed not to interfere with target binding to help standardize assays. The proximity extension assays (PEA) is a widely used protein assay where pairs of protein-binding reagents are modified with oligonucleotides (oligos), so that their proximal binding to a target protein generates a reporter DNA strand for DNA-assisted readout. The assays have been used for high-throughput multiplexed protein detection of up to a few thousand different proteins in one or a few microliters of plasma. Here we explore nanobodies (Nb) as an alternative to polyclonal antibodies pAbs as affinity reagents for PEA. We describe an efficient site-specific approach for preparing high-quality oligo-conjugated Nb probes via Sortase A (SrtA) enzyme coupling. The procedure allows convenient removal of unconjugated affinity reagents after conjugation. The purified high-grade Nb probes were used in PEA and the reactions provided an efficient means to select optimal pairs of binding reagents from a group of affinity reagents. We demonstrate that Nb-based PEA for interleukin-6 (IL6) detection can augment assay performance, compared to the use of pAb probes. We identify and validate Nb combinations capable of binding in pairs without competition for IL6 antigen detection by PEA.

KEYWORDS: protein detection, PEA, nanobodies, site-specific labeling

INTRODUCTION

Progress in technologies for protein detection and analysis enables studies that go beyond investigations of genetic predisposition at the level of DNA. Most drugs act by interfering with protein functions and proteins are therefore the most relevant molecules as targets for theraoy. Also, for diagnostic purposes protein analyses can provide insights in dynamic states by monitoring protein levels in samples collected at successive intervals. RNA analyses can also provide insights in activity states, but they typically require access to the tissues where the corresponding genes are expressed. Moreover, protein levels and activities often depend on protein degradation rates, post-translational modifications, and their involvement in forming complexes that profoundly affect their activities, neither of which can be predicted from the levels of the corresponding transcripts. Immunoassays are the most commonly used methods for clinical proteomic analysis, widely applied to diagnose disease and to monitor therapeutic effects clinically and during drug development. The success of affinity-based immunoassays depends on the availability of a large repertoire of Abs against most clinically relevant protein targets (1). Molecular protein detection assays with improved proofreading are however needed for efficient detection of target molecules at high efficiency and with minimal nonspecific background to ensure highly specific and sensitive detection. Oligo-assisted proximity-based immunoassays (2) have been adapted for different proteomic applications, such as for high-throughput plasma proteomics (3), visualization of protein and their complexes *in situ* (4), detection of drug-target engagement and extracellular vesicles (5-6), infectious diagnostics (7), flow cytometry (6) and western blotting (8). Variants of proximity-based assays have been developed where targets must be recognized by three Abs, for enhanced detection in solution-phase assays (9) or on solid supports (10) and prostate derived microvesicles called prostasomes have been detected at elevated levels in plasma from prostate cancer patients using sets of five different Abs (11). The proximity extension assay (PEA) is a homogenous immunoassay based on pairs of Abs conjugated to oligos with free, complementary 3' ends (3). When a pair of Ab conjugates bind their target protein, the attached oligos are brought in proximity, permitting extension by a polymerase to produce a DNA reporter molecule for detection by real time PCR or sequencing (3). Around a hundred or even more different proteins can be measured in 1 µl sample aliquots using this technique, but access to suitable affinity reagents represents a limiting factor. Recombinant protein-binding reagents have the advantages over pAbs that they can be produced in any desired amount, and they can be engineered for site-directed conjugation of precisely one oligo per molecule, simplifying standardization (12-14). Examples of such affinity reagents include nanobodies (Nbs), single-chain variable fragments (scFvs), monobodies and designed ankyrin repeat proteins (DARPins) to replace Abs (14). Nbs are single-domain Ab fragments of 120-130 amino acids containing a single conserved disulphide bridge and they are derived from the variable regions of atypical immunoglobulins from *Camelidae* (15-16). Nbs have proven useful as high-affinity reagents for research, diagnostics and therapeutics owing to their high specificity, small size (~15 kDa) and straightforward bacterial expression (15-18). These minimal protein domains can exhibit high-affinity binding for protein targets, and they may be site-directedly modified in convenient sortase-mediated coupling reactions (19).

Here, a set of Nbs were modified with sortase tags and site-specifically conjugated to suitably modified oligos by SrtA-coupling. These reagents were explored as substitutes for, or complements to, pAbs in PEA (Figure 3A). We established assays combining pairs of Nb reagents, and compared the efficiency of protein detection by the different pairs of reagents. The Nb reagents allowed sensitive protein detection by PEA with real-time qPCR readout.

MATERIALS AND METHODS

Identification and selection of IL6-specific Nbs (NbSORIL6s): Briefly, a dromedary (*Camelus dromedarius*, housed in the Central Veterinary Research Laboratory, Dubai, U.A.E.) was immunized six times, at weekly intervals, with 100 µg of recombinant human IL6 (kindly provided by the NNF Center for Protein Research, University of Copenhagen, Denmark as partner of the Affinomics Consortium - EU FP7 Grant Agreement 241481). Four days after the last immunization, lymphocytes were extracted from the anti-coagulated blood of the animal, and cDNA was prepared and used as a template in a two-step, nested PCR that amplified the gene fragments encoding the variable domain of the heavy-chain-only Abs. The amplified DNA fragments encoding Nbs were ligated into a pMECS phagemid vector and transformed into *Escherichia coli* TG1 cells (Lucigen), resulting into a cloned Nb-library of 7.7×10^8 transformants of which 80% contained a phagemid with the expected insert size for a Nb. After three rounds of phage selection, 24 individual clones were cultured from each round of panning, and their periplasmic proteins were tested for specificity against recombinant human IL6 protein in an ELISA (Supporting Information Methods Section). The ELISA-positive clones were selected for DNA sequencing (VIB Genetic Service Facility, Antwerp, Belgium) of their phagemid insert. The inserted DNA sequences were *in silico* translated into amino acid sequences and aligned according to the international ImMunoGeneTics database (http://imgt.cines.fr, accessed on 25 October 2019), and manually annotated (20). The different Nb clones investigated herein were NbSORIL6_xx, NbSORIL6_yy, NbSORIL6_zz and NbSORIL6_qq.

Surface plasmon resonance (SPR) biosensor analysis: The affinity of the six SORIL6 Nbs to human IL6 was determined by SPR, using the BIACORE-T200 (Cytiva, Freiburg, Germany). The experiments were performed by direct immobilization of the IL6 recombinant protein (Cat. No. Z03034, Genscript), on a CM5 biosensor chip surface (Cytiva) by a standard amine coupling procedure using NHS (N-hydroxysuccinimide)/EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) chemistry, resulting in a final change of Response Units (RU) of 300 RU [Rmax (200RU) = MW analyte (15,000) x immobilized ligand/MW ligand (21,041)]. The SPR measurements were performed at 25°C with HBS (10 mM of HEPES pH 7.4, 150 mM of NaCl, 0.005% Tween-20, and 3.4 mM EDTA) as running buffer. For affinity measurements, the purified Nbs were injected sequentially in 2-fold serial dilutions, from 500 to 1.95 nM, at a flow rate of 30 µl/min. The association step was followed for 120 s, the dissociation step was 600 s, and a regeneration of 60 s at 30 µl/min using 100 mM glycine (pH 2.7) was performed. After the regeneration step, an additional stabilization step of 180 s was included. The kinetic rate constants were determined by global fitting of a 1:1 binding model with drift to the sensorgrams using the BIACORE Evaluation software (Cytiva, Freiburg, Germany), and the rate constants k_{off}/k_{on} ratio was used to determine the equilibrium dissociation constant (K_D). For the epitope binning experiments, measurements were performed at 10 µl/min in HBS buffer. The first sample consisting of an excess of a first Nb (NbX), at a concentration of 200 times its K_D value, was initially injected for 300 s to saturate all its available epitopes on the IL6 protein. This step was followed by a second injection under the same conditions for 300 s, but with a mixture of e.g., NbX+NbY (both at a concentration of 200 times their respective K_D values, to guarantee equal competition of all Nbs). The RU was then monitored for 600 s, followed by a regeneration step of 90 s at 30 µl/min, using 100 mM of glycine (pH 2.7). The next cycle was initiated after a stabilization time of 180 s. All Nb-pairs

were evaluated in all possible combinations, and for each pairwise combination 4 cycles were performed:

1) NbX + NbX
2) NbX + Nb(X and Y)
3) NbY + Nb(X and Y) and
4) NbY + NbY

Nb-oligo conjugation, identification and purification: Hundred µL reactions were prepared with 24, 60 and 120 µM Nbs (2-, 5- and 10-fold molar excess of NbSORIL6 over oligos), 150 µM SrtA enzyme (3x molar excess; ~22 kDa) and 12 µM oligo modified at the 5' end with three glycines (GGG-oligo) in SrtA coupling buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$; pH7.5-8.0). The GGG-modified oligos F1 and R1 or H1 and H2 were conjugated at stoichiometric ratio to Nb. The reactions were incubated at 37°C overnight with gentle shaking on a rotating platform. Following incubation, the remaining his-tagged SrtA enzyme, acyl-intermediate, unconjugated Nb and cleaved-off his tags were removed by binding to Dynabeads® Magnetic Beads (ThermoScientific) overnight at 4°C with continuous shaking in binding buffer (100 mM sodium-phosphate, pH 8.0, 600 mM NaCl, 0.02% Tween®-20). Conjugates were validated on 4-12% NuPAGE Bis-Tris Gel (ThermoScientific) with NuPAGE™ LDS Sample Buffer (4X) **(**ThermoScientific) loading sample buffer + 50 mM DTT heated at 80°C for 3 min after electrophoresis in 1x MES-SDS buffer for 60 min at room temperature. The conjugates were visualized by staining using the PlusOne DNA Silver Staining Kit (GE Healthcare, Uppsala) and Gel Doc XR+ (Bio-Rad) for imaging (Figure 1B and Supplementary Figure 6). To ensure thorough purification, the purification process was repeated up to three times on new beads. The conjugates were purified from unreacted oligos and free Nb by HPLC using a proFIRE instrument (Dynamic Biosensors, Planegg, Germany) according to manufacturer's instructions. In brief, the ion exchange column was pre-equilibrated with buffer A (50 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 150 mM NaCl) followed by injection of 160 µl of beads purified Nb-oligo conjugates. The conjugates were eluted by a pre-defined salt gradient using buffer B (50 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 1 M NaCl) at a flow rate of 1 ml/min. Subsequently, the assay-specific 89-mer oligo HF1, 73-mer oligo HR1 or 107-mer oligo RHD, 112-mer oligo RHD comprising 37-nt and 9-nt segments complementary to oligos F1 and R1 were mixed together respectively to prepare a hybrid.

Validation of Nb-oligo probes and comparison via SPR: The binding of 4 different Nbs, and Nb-oligo conjugates of two of them, to IL6 was investigated using two approaches: 1) IL6 was directly immobilized on a CM5 biosensor chip surface (GE Healthcare, Uppsala, Sweden) by a standard amine coupling, resulting in immobilization of 700-900 RU in 10 mM sodium acetate buffer, pH 4.5. The Nbs were injected over the IL6-coated chip surface in a series of concentrations from 15.6 nM to 250 nM in the running buffer of 20 mM Tris-HCl pH 8.0, 125 mM NaCl, 0.05% Tween-20, at a flow rate of 30 µl/min. 2) IL6 was captured to a level of 1300-2800 RU on an anti-IL6 monoclonal antibody (mAb) coupled to the chip surface and the interaction analysis of 4 selected Nbs was performed similarly as described above. Anti-IL6 mAb was immobilized on the chip surface by amine coupling in 10 mM sodium acetate buffer (pH 5.0), resulting in an immobilization level of 15000-19000 RU. Reference surface and blank were subtracted from the sensorgrams. The data were analyzed using Biacore T200 Evaluation software v. 3.0 (GE Healthcare, Uppsala, Sweden).

Preparation of PEA Ab probes: Anti-human IL6 pAbs were diluted in PBS at 2 µg/µl and stored at -20°C. Anti-human IL6 Abs (2 µg/µl in PBS) were activated with a 20-fold molar excess of the cross linker dibenzylcyclooctyne-NHS (DBCO-NHS) ester (CLK-A102N, Jena Bioscience), diluted in dimethyl sulfoxide (DMSO) at 4 mM, and incubated at RT for 30 min. The reaction was stopped by adding 3 µl of 1 M Tris-HCl, pH 8.0 and incubating at RT for 5 min. The excess unreacted DBCO-NHS ester was removed from the activated Ab with an equilibrated Zeba Spin Desalting Column (7k MWCO, Thermo Scientific). After purification, the DBCO-labeled Abs were mixed with a 2.5-fold molar excess of 5' azide-modified forward oligo F1 or reverse oligo R1 and incubated overnight at 4°C. Anti-human IL6 Ab concentrations were quantified using the Quant-it protein assay kit (Life Technologies). Ab conjugates were validated on 10% TBE urea denaturing gels and validated on an agarose gel (Supplementary Figure 6). Then, an assay-specific 89-mer oligo HF1 and 73-mer oligo HR1 comprising 37-nt and 9-nt complementary segments to oligos F1 and R1, respectively, were mixed respectively to prepare DNA hybrids with the pAb_conjugated oligos F1 or R1. In addition, goat IgG was conjugated with both GGG-modified oligos F1 or R1 and used as extension and PCR control. The PEA probes were stored at 250 nM in PBS with 0.1% BSA and 0.05% NaN_3 and the probes were diluted to a 1.33 nM stock concentration in PEA buffer before use.

PEA reactions and data analysis: Recombinant human IL6 protein was diluted in assay diluent (PBS with 5 mM EDTA, 100 µg/ml single-stranded salmon sperm DNA (Sigma Aldrich), 0.1% BSA, 1 mM biotin, 100 nM goat IgG, 0.05% Tween20 solution). Pairs of oligo-conjugated Nb or anti-human IL6 Ab probes were mixed in assay diluent at a final concentration of 100 pM each, and 3 µl of the probe mixture was added per microtiter well, followed by addition of 2 µl IL6 dilutions. After incubation at 37°C for 1 hour, 46 µl extension solution was added, containing 1X Hypernova buffer (BLIRT S.A.), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of each FEP (forward extension primer) and REP (reverse extension primer), 0.2 U/ml DNA Polymerase (Invitrogen) and 1 U/ml Hypernova DNA polymerase. The extension reactions were run at 50°C for 20 min, followed by a 5-min heat-activation step at 95°C and 17 cycles of pre-PCR of 30 sec at 95°C, 1 min at 54° C, and 1 min at 60° C. For the subsequent qPCR detection, 2.5 µl of extension/pre-PCR products were transferred to a 96 or 384-well plate and combined with 7.5 µl qPCR mix containing 1X PCR buffer (Invitrogen), 0.1 µM of each FEP (forward extension primer) and REP (reverse extension primer) PCR primers, 2.5 mM $MgC1_2$, 0.2 µM TaqMan probe (or 0.5x SYBER green I), 0.25 mM dNTPs (including dUTP instead of dTTP), 0.02 U/µl Klenow exo-, 0.02 U/µl uracil-N-glycosylase, 1.5 U/µl Taq polymerase and 1.33 µM ROX (ROXTTTTTTT, Biomers). Quantitative real-time PCR was performed with an initial incubation at 25°C for 30 min, denaturation at 95°C for 5 min, followed by 40 cycles of 15 sec denaturation at 95°C, and 1 min annealing/extension at 60°C. ROX was used as reference fluorescence and SYBR green as detection fluorescence. The qPCR data were recorded as Ct (cycle threshold) values. The data were analyzed with Microsoft Excel. The plots were generated on Microsoft Excel and using an in-house script developed in 'Rstudio' (http://www.R-project.org/). The Ct values were plotted along the y-axis against the concentration of target protein in the reactions (x-axis). The data was analyzed by linear regression to determine the LOD (limit of detection). The LOD was defined as the concentration of protein corresponding to CtLOD=CtN − (2 x SN), where CtN is the average Ct acquired for the background noise, and SN is the standard deviation of that value. 4-parametric linear regression was used to determine the LOD as well as extrapolating the sample concentrations using the Image J image processing software curve fitting function.

RESULTS AND DISCUSSION

Site-specific conjugation of oligos to NbSORIL6, validation and two-step purification of conjugates: Typically, conventional sandwich immunoassays require chemical modification of the Abs in order to attach reporter groups, but random conjugation sites risk compromizing target binding. In our model experiments we used four recombinant Nbs directed against the IL6 protein, site-specifically conjugating exactly one oligo per Nb molecule by SrtA coupling (Figure 1A-B and Supplementary Figure 4). The SrtA enzyme reacts with the consensus sequence LPETGG of the Nb and cleaves the peptide bond between threonine and glycine, subsequently joining the threonine by a peptide bond to the N-terminal glycine on the oligo to give rise to the sequence LPETGGG (Figure 1A) (19). This established site-directed conjugation technique has been shown to allow construction and purification of well-defined conjugates. Conjugation of NbSORIL6 to the glycine-oligos results in removal of the his-tag. Any remaining unconjugated Nbs as well as the SrtA retain his-tags, allowing their convenient removal through incubation with anti-his magnetic Ni-beads (Figure 1A). The pure Nb-oligos conjugates were then isolated from unreacted oligos and excess free Nbs using a proFIRE instrument. The Nb conjugates were well separated from free Nb and oligos yielding 68% and 87% pure conjugates for NbSORIL6_15-oligos H1 and NbSORIL6_16-oligos H2, respectively (Figure 1C-D). Each Nb was conjugated to either of two oligos to which secondary oligos were then hybridized to create reagents for PEA reactions (Supplementary Figure 1). Polyclonal Ab-based PEA probes were prepared through click chemistry conjugation in a non-site-specific manner. The oligos are coupled to primary amines on the Ab via heterobifunctional crosslinkers. The Ab conjugates were validated by agarose gel separation, showing the presence of variable numbers of oligos per Ab (Supplementary Figure 6).

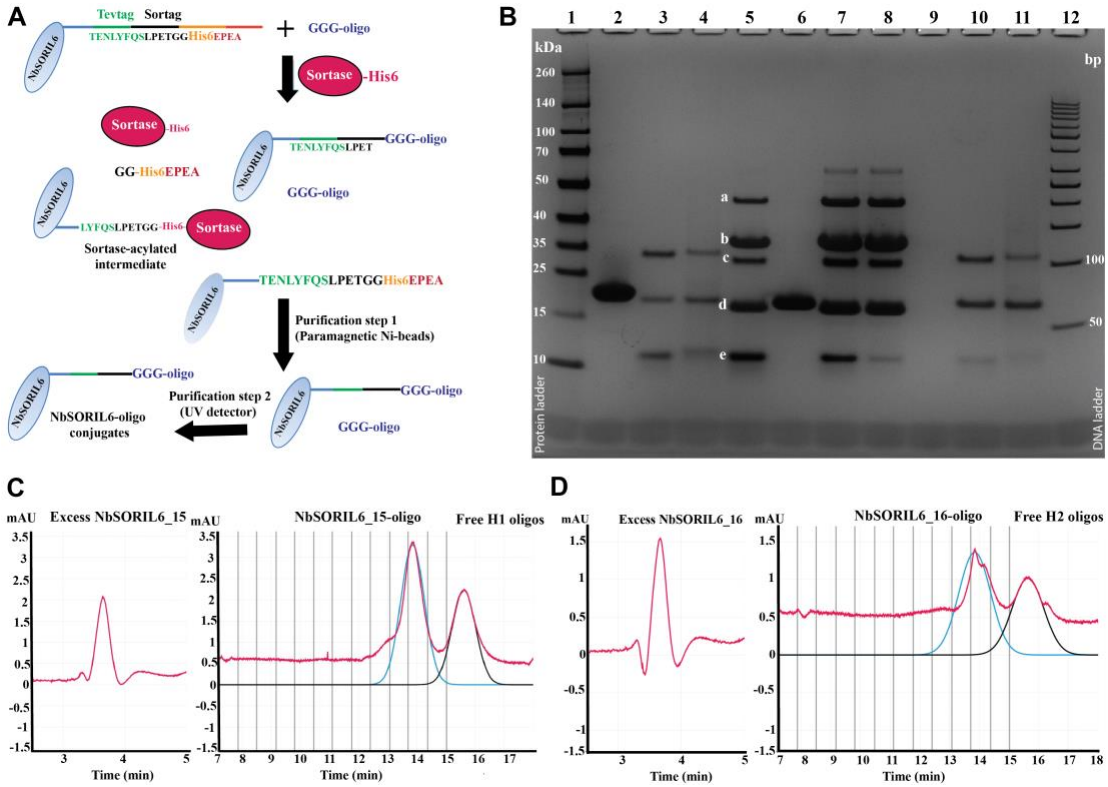

Figure 1. Schematic illustration of oligo conjugation to anti-IL6 Nbs (NbSORIL6), with two-step purification, validation and purification of the NbSORIL6-oligo conjugates. **(A)** Schematic illustration of the site-specific conjugation of PEA oligos to the Nbs. The Nbs were expressed

with a sortase tag sequence (LPETGG) recognized by SrtA enzyme, followed at the end by a his-tag at the C-terminus, and the oligos to be conjugated had three glycines at their 5' ends as required for joining to the Nb by SrtA. The SrtA enzyme also included a sacrificial his-tag at the C-terminus, which allowed for removal of any unconjugated reactants remaining in the solution by reverse nickel affinity pull down with paramagnetic Ni-beads. After the SrtA reaction all his-tagged material in the solution were collected on paramagnetic Ni-beads and discarded, while conjugates and free oligos were obtained in solution. This was followed by separation using a proFIRE instrument (Dynamic Biosensors) monitored via UV detection to isolate pure conjugates. **(B)** Gel electrophoresis of sortase-mediated oligo-Nb conjugation reaction products and samples undergoing two-step purification. Lane 1: protein ladder, lane 12: gene ruler 50 bp DNA ladder, lanes 2 and 6: free NbSORIL6_16 and NbSORIL6_15 respectively, lane 9: negative control (only loading buffer). Unpurified reaction products with a 2, 5 or 10-fold molar excess of NbSORIL6_15 over oligo H1 is seen in lanes 5, 7 and 8, respectively. The species identified in order of increasing migration in lane 5 are a) acylated product, b) free SrtA enzyme, c) Nb-oligo conjugate, d) excess unreacted NbSORIL6_16, and e) free oligo H1. Lanes 3 and 4 show the products NbSORIL6_16-oligo H1 and NbSORIL6_16-oligo H2, respectively, after a single incubation with Ni-beads, while lanes 10 and 11 demonstrate products from NbSORIL6_16-oligo H1 and NbSORIL6_16-oligo H2 reactions, respectively remaining in solution after two incubations with the beads. **(C-D)** The products shown in lanes 10 and 11 of the gel were separated from free Nb and unreacted oligos using the proFIRE instrument, yielding the pure Nb conjugates NbSORIL6_15 **(C)** and NbSORIL6_16 **(D)**.

Characterization of binding affinity using SPR biosensor analysis: The binding affinity of the 4 different NbSORIL6 directed against human recombinant IL6 antigen and their oligo conjugates were validated by SPR by directly immobilizing IL6 on the chip surface by standard amine coupling (Figure 2 and Supplementary Figure 2). The NbSORIL6_21 and _15 and their respective conjugates all showed an interaction with the target IL6 antigen (Figure 2 and Supplementary Figure 2). A good fit was obtained with a 1:1 interaction model for NbSORIL6_5 ($K_D = 6.9 \times 10^{-10}$ M), NbSORIL6_15 ($K_D = 1.1 \times 10^{-8}$ M), NbSORIL6_21 ($K_D = 7.4 \times 10^{-9}$ M), NbSORIL6_21-oligo H2 conjugates ($K_D = 4.4 \times 10^{-8}$ M) and NbSORIL6_16 ($K_D = 4.7 \times 10^{-9}$ M) (Figure 2, Supplementary Figure 2 and Supplementary Table 4). The NbSORIL6_21-oligo conjugates showed a different kinetic of interaction with both slower association and dissociation compared to unconjugated NbSORIL6_21 (Figure 2B). In addition, a steady-state analysis was performed to estimate approximate affinity values. Comparing the affinity constants obtained from 1:1 and steady-state interaction models indicated that NbSORIL6_15 is the weakest binder to the IL6 of the different NbSORIL6 (Supplementary Tables 4 and 5). We included an anti-human IL6-specific mAb as a positive control in our analysis which showed that the mAb binds much stronger than all Nbs tested with almost no observable dissociation rate from the target IL6 protein, preventing an accurate measurement of its affinity (Supplementary Figure 2). It is to be expected that bivalent mAbs via avidity bind with higher (apparent) affinity than monovalent Nbs. We further investigated the binding affinities of all four NbSORIL6s to IL6 captured on mAbs, immobilized on the sensor surface. Under these conditions, the binding of NbSORIL6_15 and _21 was significantly reduced (Supplementary Figure 3), probably due to competition for the binding site with this mAb. However, the affinity of NbSORIL6_5 was only slightly reduced (Supplementary Tables 4 and 5). As for NbSORIL6_16 the affinity as well as the binding level increased significantly when IL6 antigen was captured on mAb rather than via direct immobilization. This could be due to improved orientation or epitope exposure of IL6 for binding by NbSORIL6_16

(Supplementary Tables 4 and 5).

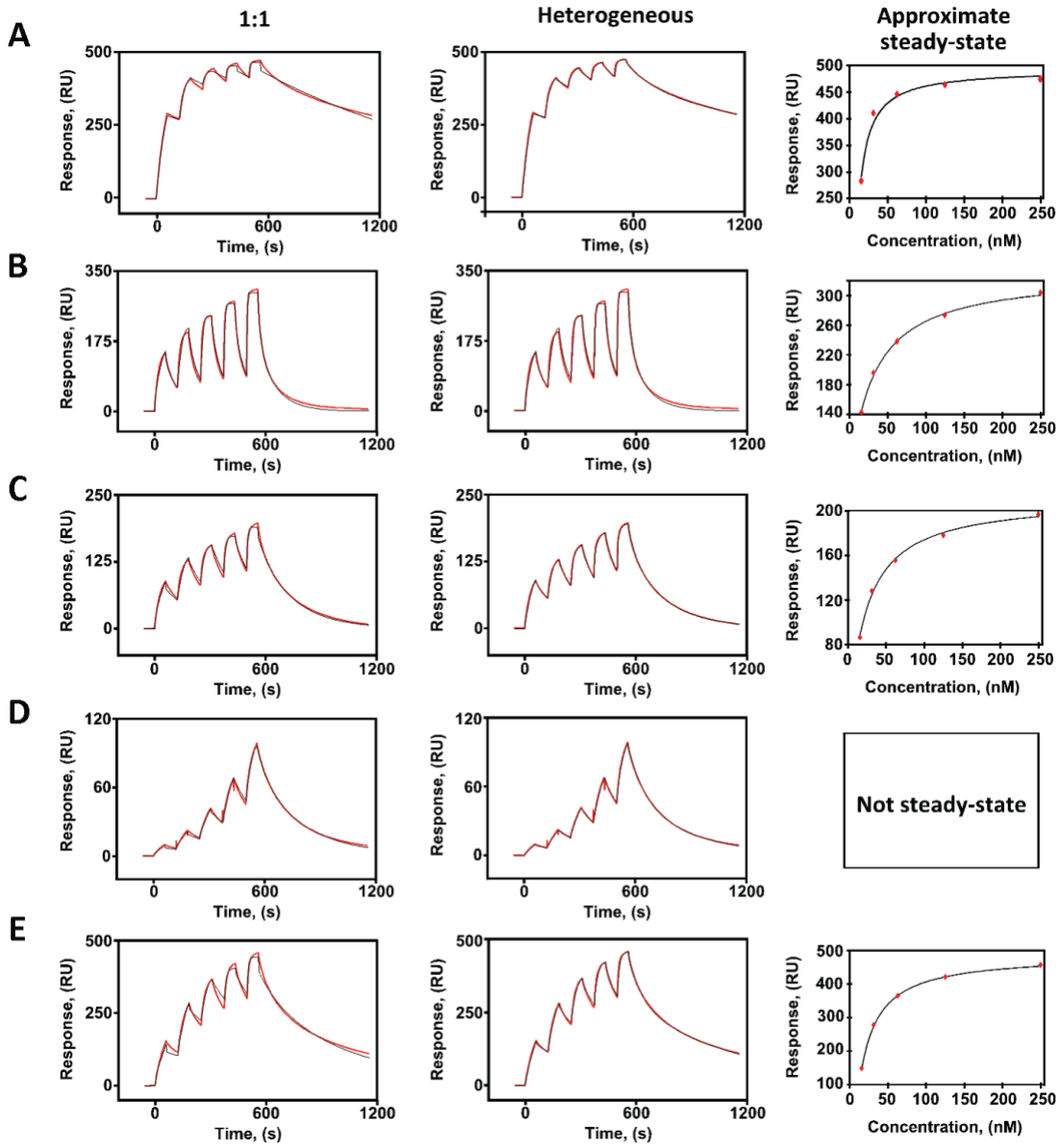

Figure 2. SPR biosensor analysis of IL6 binding by NbSORIL6 and NbSORIL6-oligo conjugates. Interactions between Nbs and IL6 directly immobilized on the chip surface. The Nbs and Nb-oligo conjugates were injected over the IL6-coated chip surface in a series of concentrations from 15.6 nM to 250 nM. The sensorgrams for the Nbs were fitted to the different interaction models (black lines), left column represents fitting to a 1:1 interaction mode, middle to a heteregeneous model (fitted best) and left - steady state analysis. The corresponding equilibrium dissociation constants (K_D) determined by various models are presented in Supplementary Table 4. SPR sensorgrams representing the interaction between NbSORIL6_5 **(A)**, NbSORIL6_15 **(B)**, NbSORIL6_21 **(C)**, NbSORIL6_21-oligos conjugates **(D)**, and NbSORIL6_16 **(E)** to the target IL6 antigen. A pseudo steady-state analysis of the graphs was obtained by fitting a 1:1, heterogeneous ligand model to report points taken at the end of the injection.

Identifying non-overlapping binding pairs via PEA: The NbSORIL6-oligo conjugates were then explored for their potential to expand the range of reagents used for PEA, complementing Abs. Two aliquots of all NbSORIL6s were conjugated using SrtA to either of two oligos to

which secondary oligos were then hybridized to create reagents for PEA reactions (Supplementary Figure 1A). We combined pairs of Nb probes at 100 pM with IL6 protein ranging from 100 ng/ml to 10 fg/ml and no added protein in PEA reactions (Figure 3B). We investigated three combinations of the reagents to see which would yield the highest sensitivity to detect target protein in PEA reactions. Of the evaluated pairs, the combination of NbSORIL6_5 and NbSORIL6_16 conjugates yielded the assay with the greatest sensitivity and a detection limit below 1 pg/ml (Figures 3B and 4A).

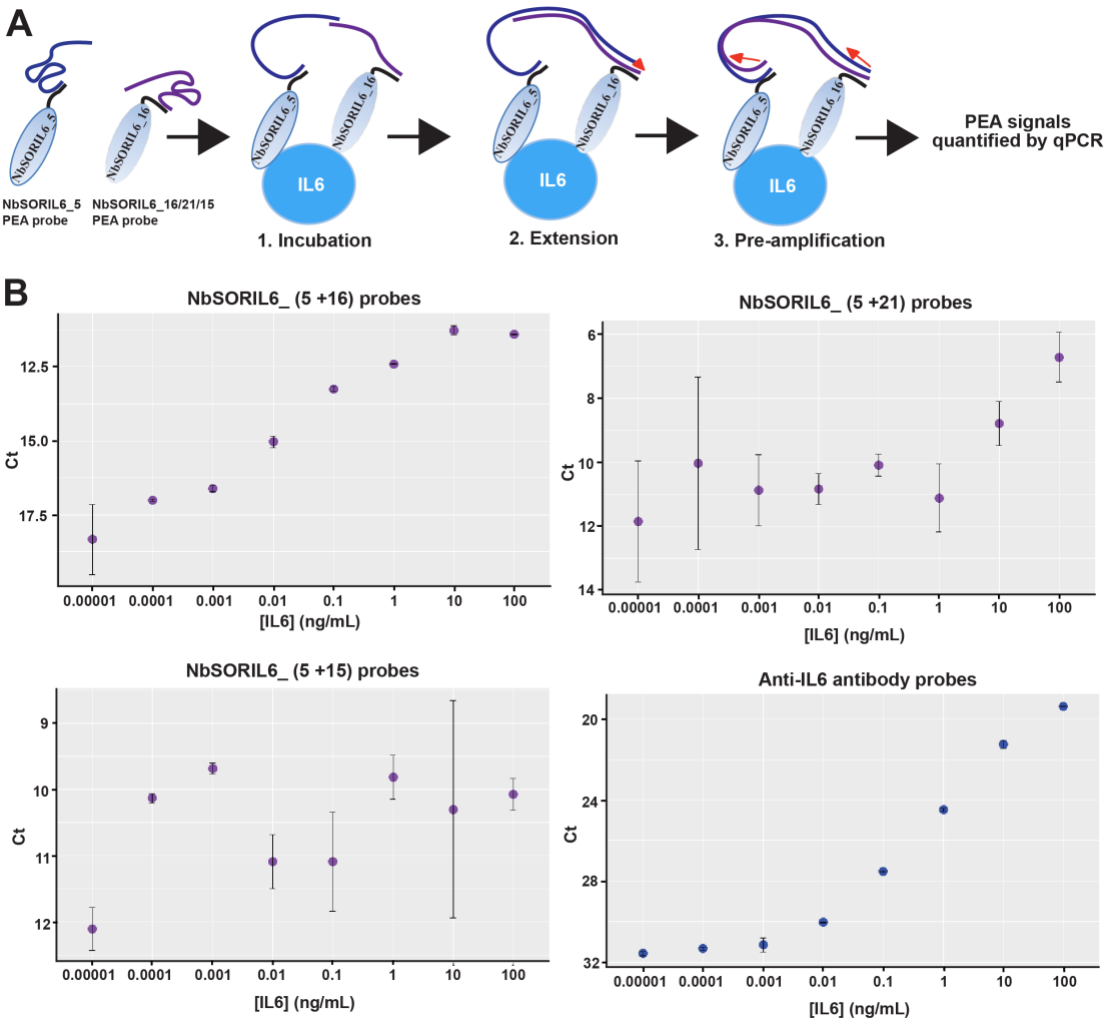

Figure 3. Nb-based detection of IL6 by PEA using Nb-oligo conjugates and regular pAb PEA probes. **(A)** Schematic illustration of Nb-based PEA reactions for protein detection. Proximity Nb probes pairs were incubated with target IL6 proteins, bringing the attached oligos in close proximity so that they can be mutually extended upon dilution and addition of a DNA polymerase. The resulting reporter DNA strands were quantified by real-time PCR (qPCR) as a measure of the amount of IL6 antigen in the buffer. **(B)** Demonstration and verification of different Nb probes combination with serial dilutions of IL6 from 100 ng/ ml to 10 fg/ml in PEA buffer. A pair of pAb-based PEA probes served as a positive control (PC) in a PEA detection. The data were analyzed using an in-house script developed in 'Rstudio' and the Ct values were plotted along the y-axis against the concentration (x-axis).

We performed SPR epitope binning and compared all different pairwise combinations of SORIL6 Nbs from the set investigated herein (data not shown). Our identification of the best optimal Nb probe pair agreed with epitope mapping SPR data that showed that these two Nbs (SORIL6_5 and SORIL6_16) had the most effective binding as well as slower dissociation

rates compared to the other combinations (Figure 4A-B). We did not detect any signal for this combination of two Nbs (SORIL6_5 and SORIL6_16) when the IL6 antigen was absent. As shown, detection of IL6 using pAbs generated significantly higher signals and detected lower concentrations of proteins than Nbs PEA probes (Figure 3B). The limit of detection (LOD) was calculated as described under data analysis in the Materials and Methods section and is represented in Supporting File-2. The optimal Nb PEA reached a plateau at the higher IL6 concentration, while the pAb PEA probes did not reach a plateau even at 100 ng/ml of IL6 (Figures 3B and 4C). But Nb PEA reaction combining the 5 and 16 Nb isolates detected lower concentrations of IL6 compared with the pAb PEA probes, and with a broader dynamic range (Figure 4C). The NbSORIL6 probes demonstrated a LOD of approximately 0.2 fM or 5 fg/ml while PEA using pAbs probes has a LOD of 3 fM or 71.2 fg/ml, which indicates that the performance of NbSORIL6 probes are comparable to Ab probes (Figure 4C and Table 1). To determine whether Nbs probes can be combined with Ab probes we performed PEA with a combination of Nb (NbSORIL6_5) and pAb PEA probes, however this assay performed poorly (Figure 4D). This could be because the probes share a same epitope and compete for binding.

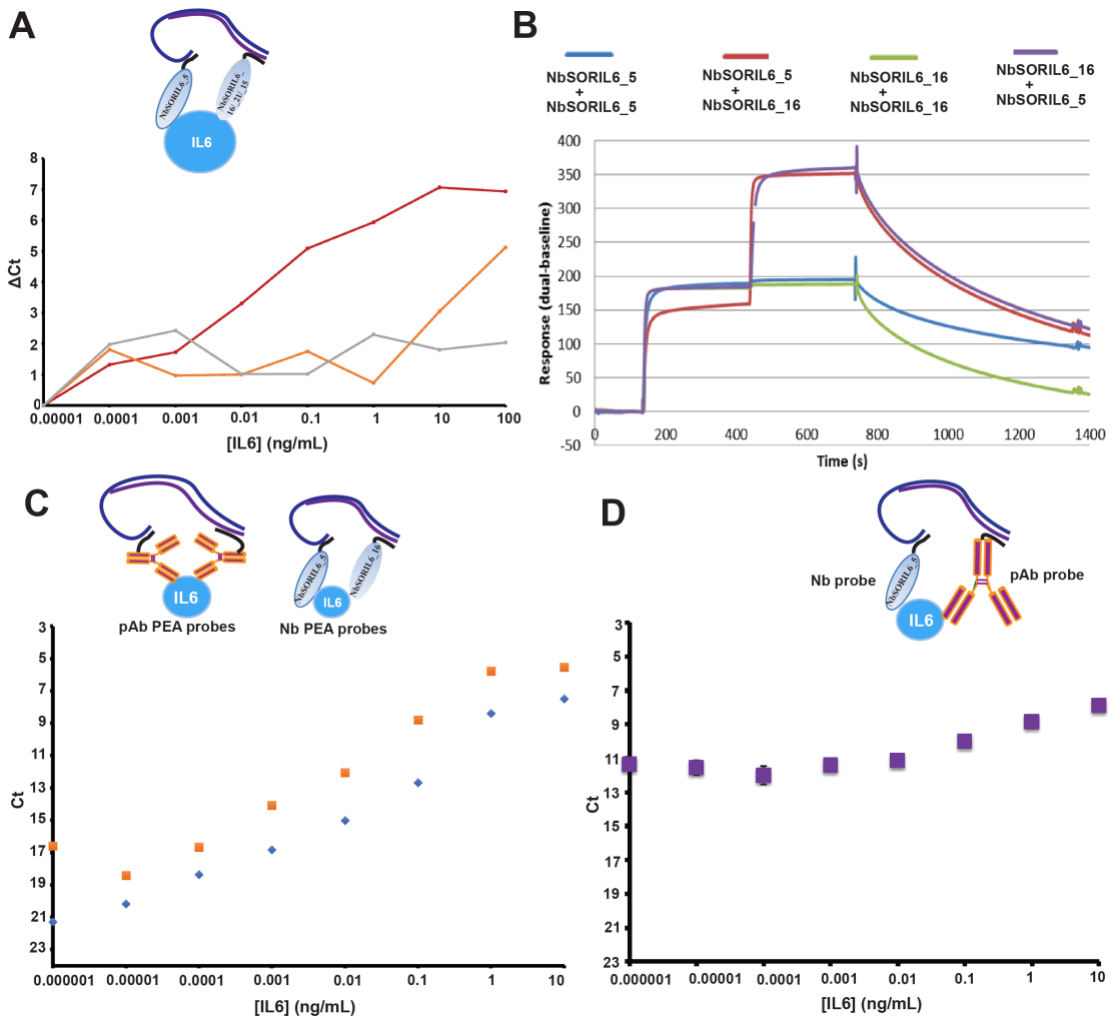

Figure 4. Comparison between Ab and Nb reagents for PEA. Detection of IL6 by PEA using NbSORIL6 pairs, pAb pairs or a combination of pAb and Nb probes. **(A)** Comparison of PEA results using NbSORIL6_5+ NbSORIL6_16 (dark red), NbSORIL6_5+ NbSORIL6_21 (orange) and NbSORIL6_5+ NbSORIL6_15 (gray). The x-axes show the concentration of IL6 antigen, while the y-axes show the cycle threshold values with background subtracted (delta Ct). **(B)** SPR epitope binning of NbSORIL6 with IL6 directly immobilized on the chip surface. Comparison of

four pairwise combination of two Nbs (NbSORIL6_5 and NbSORIL6_16). Sensorgrams representing homo or hetero combinations interactions between NbSORIL6_5 and NbSORIL6_16. **(C)** Assessment of IL6 detection using either NbSORIL6_5 and NbSORIL6_16 (blue) or pAb pairs (orange) as PEA probes. The y-axes show the cycle threshold values (Ct) while the x-axes show the concentration of IL6 antigen. **(D)** Detection of IL6 antigen using a combination of NbSORIL6_5 probe and pAb.

Table 1. Analytical characteristic of pAb-based and Nb-based PEA assays, values are means of three separate experiments

IL6 PEA assays	LOD_{mean} (pM)	LOD_{mean} (pg/ml)	R^2_{mean}	Inter assay variation $_{mean}$ (CV %)
pAb probes	0.002924	0.07115	0.999	7%
Nb probes	0.000208	0.00493	0.979	7.37%

Dimerization of Nb reagents: Dimerization of affinity reagents with an appropriate oligo design can be expected to demonstrate increased avidity and may therefore improve assay sensitivity. We designed and evaluated the possibility to dimerize NbSORIL6-oligo conjugates in the PEA experiments with the best performing combination of NbSORIL6_5 and NbSORIL6_16 probes (Supplementary Figure 5A). From the selected combination homodimers of the conjugates were prepared through complementary oligos hybridization (Supporting Information Table 3 and Supplementary Figure 1). to form PEA probes in a dimer format, which were validated on gel (Supplementary Figure 5B and Supplementary Figure 1). We established assays combining pairs of reagents and then compared the performance of the homo-dimers to their monomeric counterparts. However, under the investigated conditions we observed no improved performance with the use of homodimers of NbSORIL6_5+5 and NbSORIL6_16+16 conjugates over single Nbs combination of NbSORIL6_5 and NbSORIL6_16 (Supplementary Figure 5C).

CONCLUSIONS

Abs randomly modified with DNA by chemical coupling can yield inconsistent results, affecting assay reproducibility (21). The site-directed attachment of exactly one oligo per Nb opens interesting possibilities where binders against different epitopes on the same protein molecule can be brought together by hybridization between their attached oligos for proximity ligation (PLA) or extension (PEA) assays. In this manner both efficiency and specificity of binding might be enhanced (22-23). Immunoassays that require dual recognition by binders provide higher specificity and can reduce the background compared to single binder-assays by reducing risks of cross reactivity for irrelevant target molecules (24). However, identifying non-competing pairs of binders remains a challenging task. Here we used PEA to identify optimized non-overlapping nanobody binding pairs. We compared different combinations of Nb pairs from 4 selected Nbs and identified a pair that provided assay performance comparable to that of pAbs. The Nb-based PEA assay is a simple assay to establish that may be promising for future diagnostic multiplex assays that require highly specific and sensitive detection of sets of targets over wide ranges of protein concentrations (25).

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AUTHOR CONTRIBUTIONS: R.A.A.-A., C.V., E.A. and P.M. performed most of the experimental work. C.V. produced and together S.M. validated the Nbs results. R.A.A.-A. conjugated with DNA and analyzed results. C.V., E.A. and R.A.A.-A. performed SPR validation and H.D. helped to analysis data. P.M., R.A.A.-A., and U.L. designed and analyzed the PEA experiment. R.A.A.-A. wrote the manuscript and all authors contributed to the final paper. *Conflict of interest statement.* U.L. is a co-founder and shareholder of Olink Proteomics, having rights to the PEA technology.

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Supplementary Information and Results

Title: Sensitive protein detection using site-specifically oligonucleotide-conjugated nanobodies

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[5]These P.M.M., E.A. and C.V. authors contributed equally.

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Supporting Information

Materials: Recombinant human IL6 protein (206-IL-010 &), anti-human IL6 mAb (#6708) and human IL6 affinity purified polyclonal Ab AF-206-NA were obtained from R&D System Inc (USA), human IL6 recombinant protein (Cat. No. Z03034, Genscript) and anti-human IL6 mAb 13A5 were from MABTECH. Buffers used include PEA buffer (1xPBS with 1% BSA (New England Biolabs; NEB), 100 µg/ml salmon sperm DNA (Invitrogen), 100 µg/ml goat serum (Sigma-Aldrich), and 0.05% Tween-20 (Sigma-Aldrich)), probe storage buffer (1xPBS, 0.1% BSA, 0.05% NaN3), and wash buffer (1xPBS with 0.1% BSA and 0.05% Tween-20). All buffers were prepared in-house and their pHs were calibrated to 7.2 and buffers including water, TBS, PBS were purified with Nalgene Rapid-Flow Filters (Thermo Scientific). All enzymes, dUTPs and dNTPs were purchased from NEB. The sequence compositions and modifications of the DNA oligonucleotides (oligos) are reported in Supplementary Table 2. All oligos were purchased from integrated DNA technologies (IDT). Their concentrations were measured by UV spectroscopy using a Nanodrop ND1000 spectrophotometer and the oligos were analyzed by PAGE (15% TEB-urea gel (Invitrogen).

Methods

Expression of IL6-specific Nbs (NbSORIL6): Nbs were cloned into the pTEVSOR expression vector, containing a TEV-cleavage site (TENLYFQS), a sortase recognition site (LPETGG), a His6-tag and a C-tag (EPEA) downstream of the Nb. The Nb genes were amplified by PCR, and *PstI* and *Eco91I* (ThermoScientific) digestion fragments were ligated into the pTEVSOR vector cut with the same restriction enzymes and transformed in *Escherichia coli* WK6 cells for periplasmic expression of Nbs. Following extraction from the periplasm by osmotic shock, the SORIL6 Nbs (IL6-specific Nbs containing the sortase recognition site at the C-terminus) were purified by IMAC and SEC (1).

Reference: 1. Vincke, C., Gutiérrez, C., Wernery, U., Devoogdt, N., Hassanzadeh-Ghassabeh, G., & Muyldermans, S. (2012). Generation of single domain antibody fragments derived from camelids and generation of manifold constructs. *Methods in Molecular Biology* (Clifton, N.J.), **907**, 145–176.

NbIL6s clones' selection via enzyme-linked immunosorbent assays (ELISA): Selection of the original four Nbs (NbIL6_5, 15, 16 and 21) clones against recombinant human IL6. ELISA was performed to test the capacity of wild-type NbIL6 (containing HA and His6 tags) to bind its cognate IL6 antigen. A 96-well microplate (Thermo scientific Nunc Maxisorp™) was coated with 100 µl/well of IL6 antigen (at 2 µg/ml in ELISA coating buffer (0.1 M $NaHCO_3$, pH 8.2)) and incubated overnight at 4°C. Next, the wells were rinsed 5 times using PBST20 buffer (PBS + 0.05% Tween-20), blocked for 120 min at room temperature (RT) with 200 µl skimmed milk powder in PBS (1% w/v) and again washed 5 times using PBST20 buffer. The NbIL6-HAHis6 were then added to the wells in a 4-fold serial dilution starting from a concentration of 8 µM to 30.5 pM in PBS. After 60 min incubation at RT, the plate was washed 10 times with PBST20 buffer. Detection of attached Nb was performed with a monoclonal mouse anti-HA Ab (Biolegend; 1 µg/ml) followed by a 2000-fold diluted goat anti-mouse IgG-alkaline phosphatase conjugated Ab, each for 60 min at RT. Finally, the plate was rinsed 5 times with PBST20 and the color was developed with 4-phenylphosphate disodium substrate (Sigma-Aldrich) (2 mg/ml) and read at 405 nm after 30 min of reaction. The affinities (K_D) of the six IL6-specific Nbs for NbIL6_5 (0.86 nM), NbIL6_15 (55.91 nM), NbIL6_16 (15.44 nM), NbIL6_21 (27.98 nM), NbIL6_25 (2.81) and NbIL6_95 (3.01 nM) were estimated via ELISA against recombinant human IL6.

Supplementary Table 1. Summary of Nbs and tag sequences.

Nbs	C-terminal tags				MW (Da)
	TeV-tag	**Sor-tag**	**His6-tag**	**C-tag**	
NbSORIL6_5	TENLYFQS	LPETGG	HHHHHH	EPEA	16912
NbSORIL6_15	TENLYFQS	LPETGG	HHHHHH	EPEA	15823
NbSORIL6_16	TENLYFQS	LPETGG	HHHHHH	EPEA	16444
NbSORIL6_21	TENLYFQS	LPETGG	HHHHHH	EPEA	15459

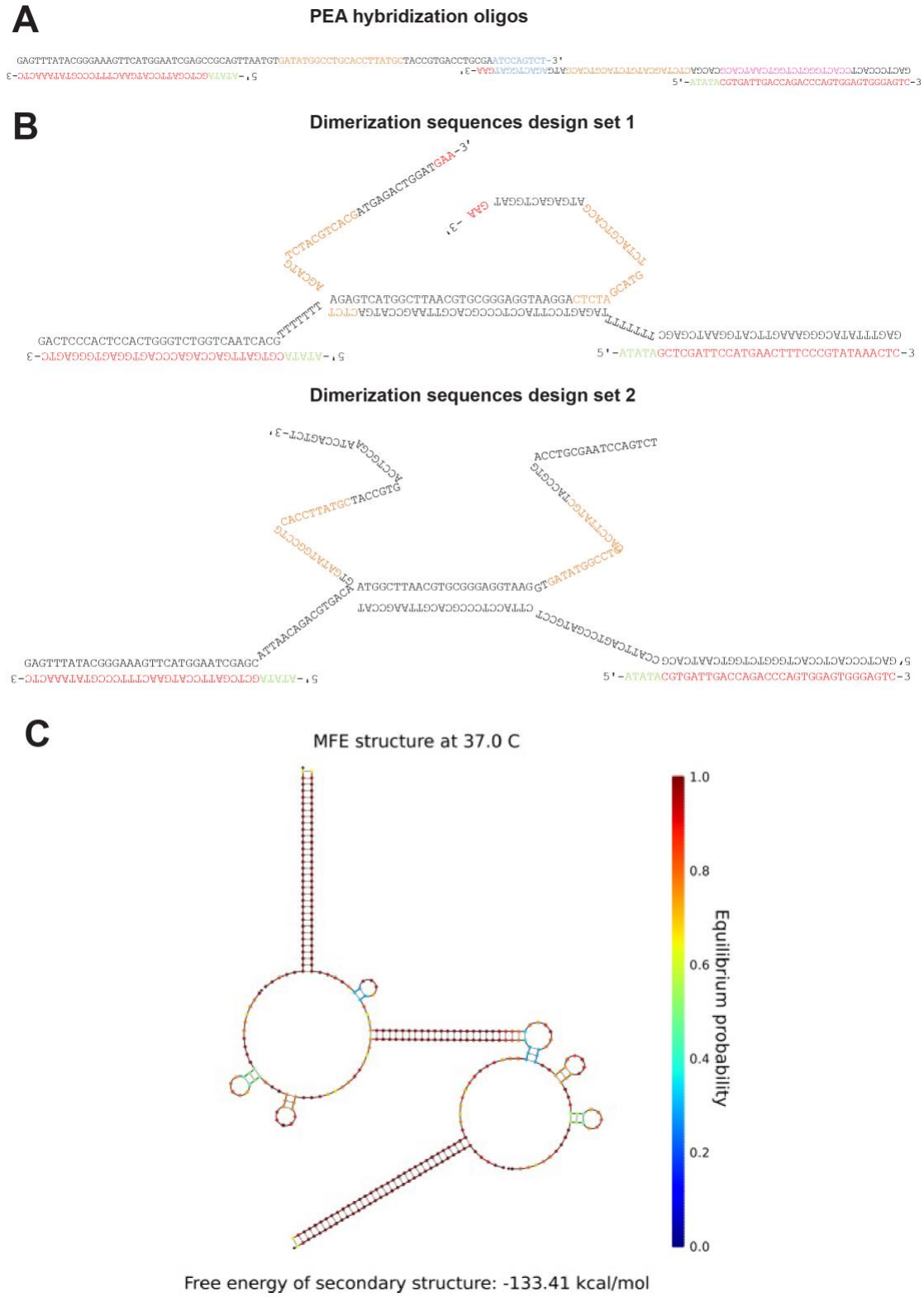
Expression, isolation and affinity measurement of NbSORIL6: Recombinant protein binding reagents may be modified for convenient site-directed conjugation, without risking inactivation of the affinity reagent. We expressed IL6-specific Nbs and performed enzyme-linked immunosorbent assays (ELISA) to select the most suitable IL6 binders. To allow coupling of the oligos required for PEA to the Nbs via a SrtA reaction, four IL6-specific Nbs (NbSORIL6s) were recloned to include a C-terminal sortase tag. The affinities for IL6 of these Nbs with or without sortase tags, were measured via SPR and are reported in the Supplementary Table 2 below. The analyses revealed no major differences in binding kinetics when comparing the Nbs in their original form with C-terminal HA-his6-tag (hexa-histidine tag) versus the sortase tag-modified variants (Supplementary Table 2).

Supplementary Table 2. The affinity for the IL6-specific Nbs (NbIL6) with C-terminal HA-His6tags were compared to the corresponding sortase-tagged anti-IL6 Nbs (NbSORIL6).

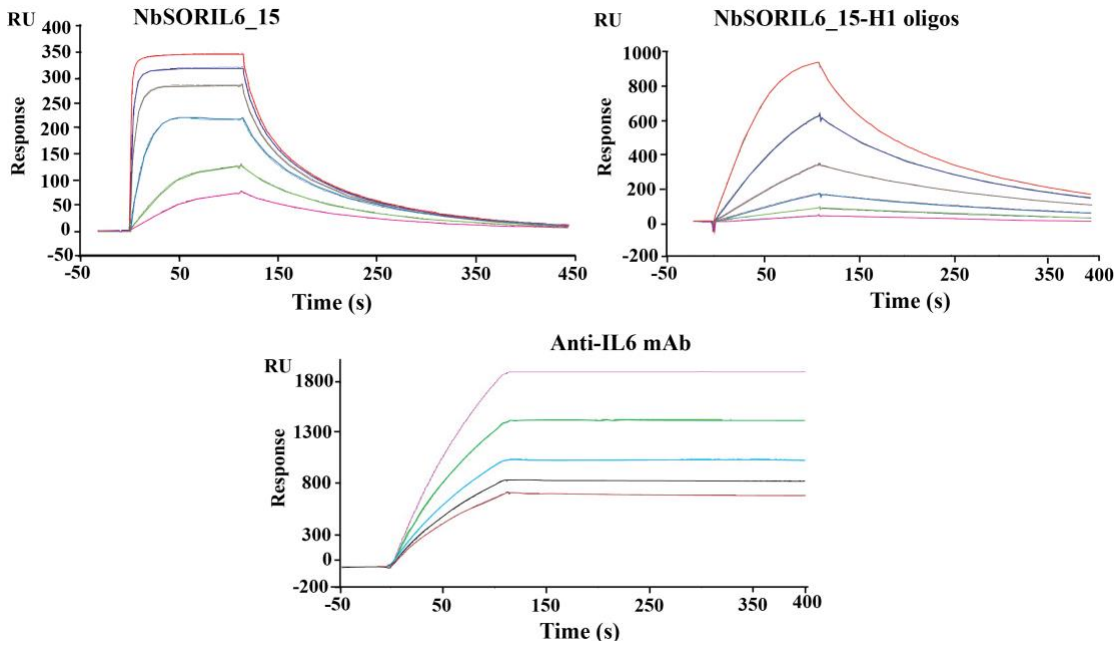
Nbs (NbIL6)	K_D **(M) 1:1**	**Nb (NbSORIL6)**	K_D **(M) 1:1**
NbIL6_5	1.71×10^{-9}	NbSORIL6_5	2.03×10^{-9}
NbIL6_15	6.47×10^{-9}	NbSORIL6_15	8.14×10^{-9}
NbIL6_16	6.96×10^{-9}	NbSORIL6_16	7.41×10^{-9}
NbIL6_21	1.37×10^{-8}	NbSORIL6_21	1.0×10^{-8}

Supplementary Table 3. Summary of oligos sequences composition, length (nt) and modifications, conjugated to the Ab and Nb used in PEA.

Oligos name, length (nt)	Sequence (5´-> 3´) and modifications	Suppliers
H1, 33	5'-phosphate-TCGTGTCTAAAGTCCGTTACCTTGATTTCTCTC-linker-3'-Gly-Gly-Gly	Biosynthesis
H2, 35	Gly-Gly-Gly-linker-5'-TTTTTTCATCGCCCTTGGACTACGACTAAATCGTG-3'	Biosynthesis
F1, 37	Gly-Gly-Gly-linker-5'-ATATACGTGATTGACCAGACCCAGTGGAGTGGGAGTC-3´	Biosynthesis
R1, 37	Gly-Gly-Gly-linker-5'-ATATAGCTCGATTCCATGAACTTTCCCGTATAAACTC-3´	Biosynthesis
HF1, 89	GAGTTTATACGGGAAAGTTCATGGAATCGAGCCGCAGTTA ATGTGATATGGCCTGCACCTTATGCTACCGTGACCTGCGAA TCCAGTCT	IDT
HR1, 73	GACTCCCACTCCACTGGGTCTGGTCAATCACGCACGACTCT AGCATGTCTACGTCACGATGAGACTGGATGAA	IDT
DimerS1-F, 111	AGTTTATACGGGAAAGTTCATGGAATCGAGCCCATTCAGT CCGATGCCTCTTACCTCCCGCACGTTAAGCCATGACTCTAG CATGTCTACGTCACGATGAGACTGGATGAA	IDT
DimerS1-R, 121	GACTCCCACTCCACTGGGTCTGGTCAATCACGCCATTCAGT CCGATGCCTCTTACCTCCCGCACGTTAAGCCATGTGATATG GCCTGCACCTTATGCTACCGTGACCTGCGAATCCAGTCT	IDT
DimerS2-F, 121	GACTCCCACTCCACTGGGTCTGGTCAATCACGCCATTCAGT CCGATGCCTCTTACCTCCCGCACGTTAAGCCATGTGATATG GCCTGCACCTTATGCTACCGTGACCTGCGAATCCAGTCT	IDT
DimerS2-R, 119	GAGTTTATACGGGAAAGTTCATGGAATCGAGCATTAACAG ACGTGACAATGGCTTAACGTGCGGGAGGTAAGGTGATATG GCCTGCACCTTATGCTACCGTGACCTGCGAATCCAGTCT	IDT
FEP, 21	CTCTAGCATGTCTACGTCACG	IDT
REP, 21	GATATGGCCTGCACCTTATGC	IDT
F qPCR primer, 22	CACGACTCTAGCATG TCTACG	IDT
R qPCR primer, 22	CGCAGTTAATGT GATATGGCC	IDT
TaqMan probes, 17	FAM-5'-TAGGTCAGAGTAGCACT-3'-MGB	IDT



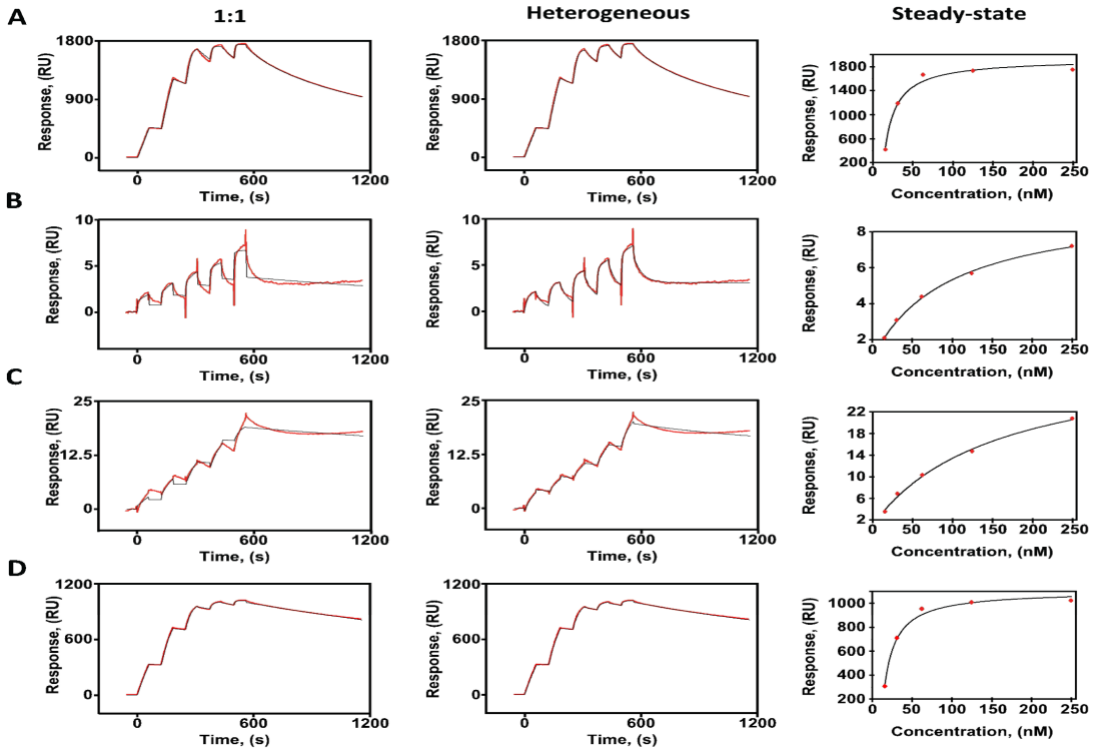
Supplementary Figure 1. (A) Design of oligos for PEA probes. **(B)** Dimerization of oligo sequences. **(C)** Nupack software prediction secondary structure at 37°C of dimerization oligos used to form Nb homodimers through secondary hybridization (http://www.nupack.org/partition/new_from_job/3521912?token=BHLxRYMgrp).



Supplementary Figure 2. Sensorgrams displaying interactions with IL6 of NbSORIL6_15 with or without conjugated oligos and of an anti-IL6 monoclonal Ab (anti-IL6 mAb). Interactions between Nbs and IL6 directly immobilized on the chip surface. The Nbs and Nb-oligo conjugates and the mAb were injected over the IL6-coated chip surface in a series of concentrations from 15.6 nM to 250 nM. A pseudo steady-state analysis of the graphs was done by fitting a 1:1, heterogeneous ligand model to report points taken at the end of the injection.

Supplementary Table 4. Equilibrium dissociation constants obtained from different interaction models between IL6, immobilized on the chip surface, and Nbs. The column K_D (M) 1:1, represents fitting to a 1:1 interaction mode, the middle column K_D (M) to a heteregeneous and the right column to a steady state analysis plot. The corresponding equilibrium dissociation constants (K_D) determined by various models are presented in the table below.

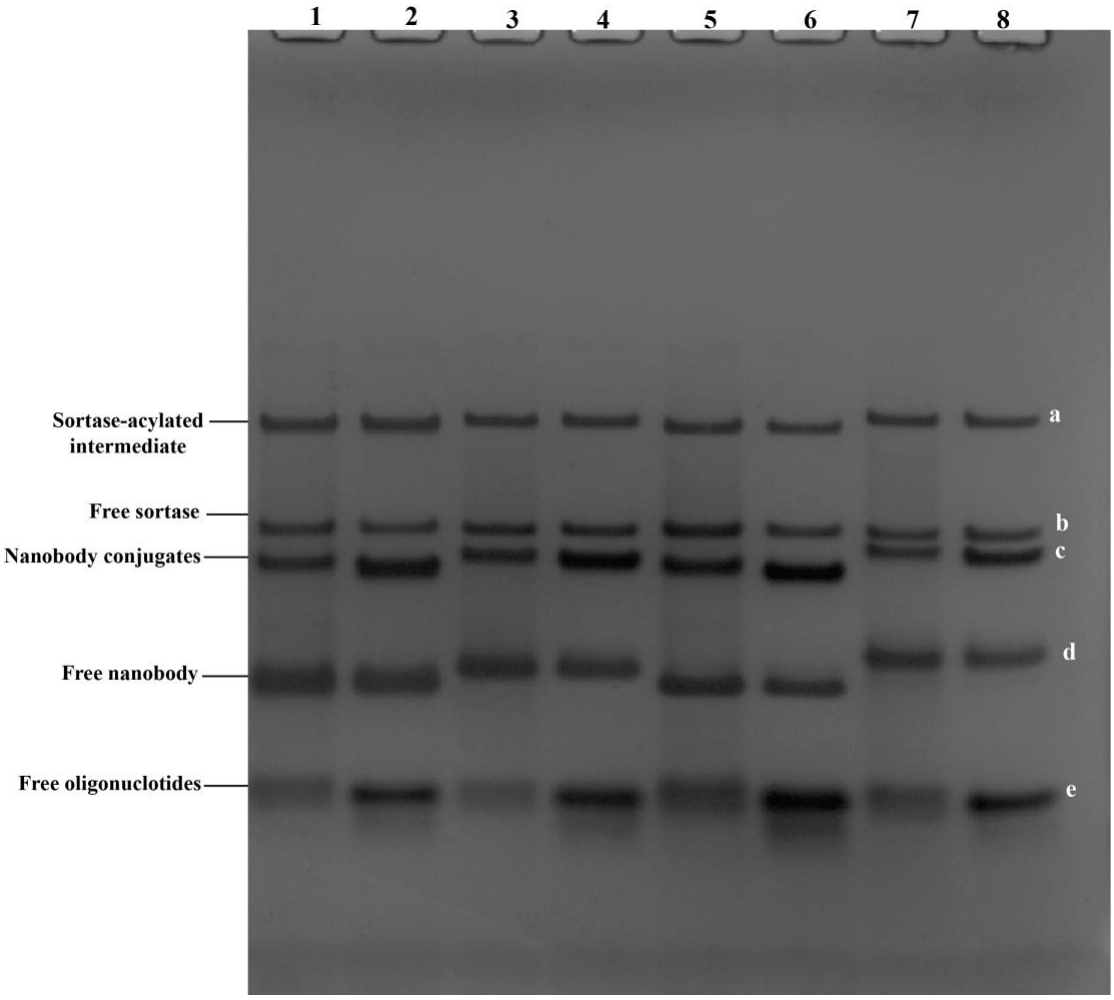
Nbs	**K_D (M) 1:1**	**K_D (M) Heterogeneous**	**K_D (M) Steady-state**
NbSORIL6_5	6.9×10^{-10}	1) 3.4×10^{-10} (R_{max}=378) 2) 2.1×10^{-8} (R_{max}=96)	1.6×10^{-10}
NbSORIL6_15	1.1×10^{-8}	1) 1.1×10^{-8} (R_{max}=271) 2) 6.1×10^{-9} (R_{max}=0,07)	3×10^{-8}
NbSORIL6_16	4.7×10^{-9}	1) 5.7×10^{-8} (R_{max}=187) 2) 2.9×10^{-9} (R_{max}=291)	1.1×10^{-8}
NbSORIL6_21	7.4×10^{-9}	1) 3.8×10^{-9} (R_{max}=114) 2) 3.3×10^{-8} (R_{max}=88)	2.1×10^{-8}
NbSORIL6_21-oligo conjugates	4.4×10^{-8}	1) 2.5×10^{-8} (R_{max}=59) 2) 1.8×10^{-7} (R_{max}=92)	Not determined



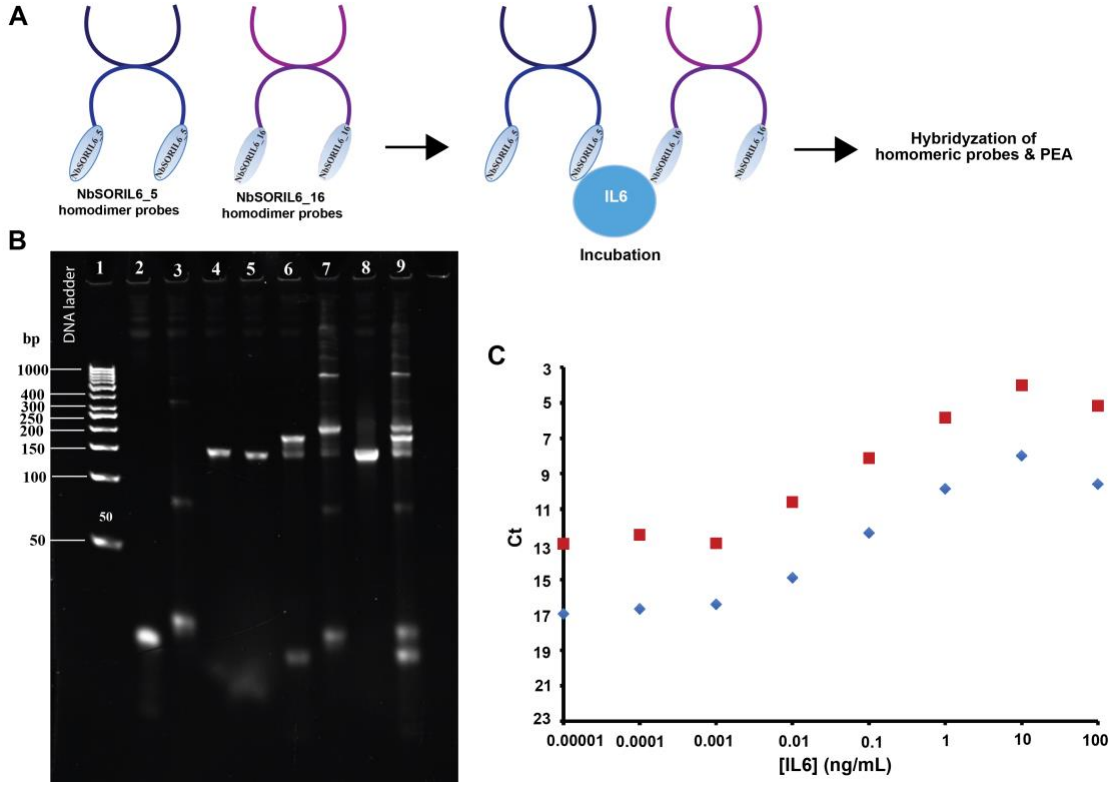
Supplementary Figure 3. Interactions between Nbs and IL6 captured on mAb that has been immobilized on the chip surface. Since the NbSORIL6 showed a slow dissociation from the target protein, a single-cycle kinetics experiment was performed. The sensorgram results, shown in red, were fitted to the different interaction models in black. The data were fitted to 1:1, heterogeneous ligand, and steady-state affinity models. The left column represents fitting to a 1:1 interaction mode, the middle to a heteregeneous model, and the right represents a steady state analysis. For all four NbSORIL6 SPR sensorgrams representing the interaction between NbSORIL6_5 **(A),** NbSORIL6_15 **(B),** NbSORIL6_21 **(C)** and NbSORIL6_16 **(D)** to the target IL6 antigen and the sensorgrams were best fitted to the heterogeneous ligand model reported in Supplementary Table 5.

Supplementary Table 5. The corresponding equilibrium dissociation constants (K_D) determined by various models are presented in below:

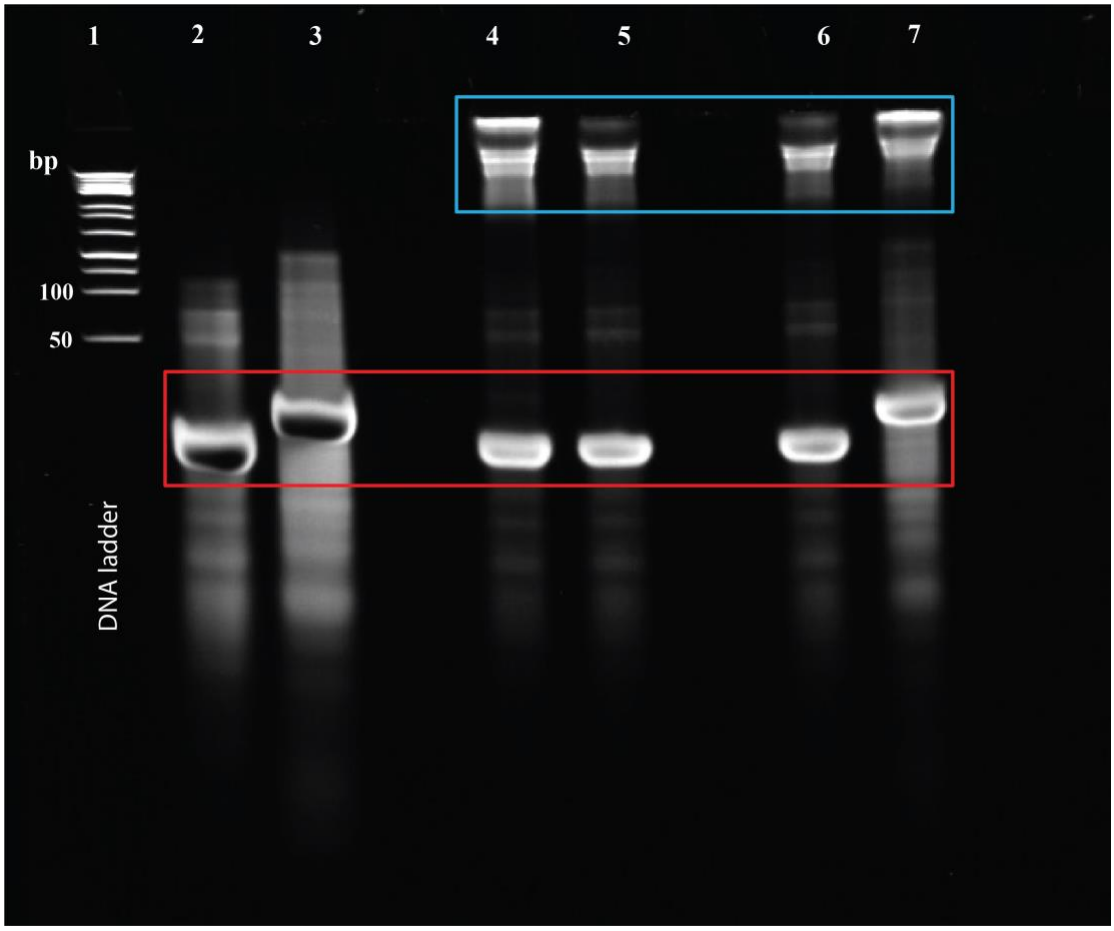
Nb_mAB	**K_D (M) 1:1**	**K_D (M) Heterogeneous**	**K_D (M) Steady-state**
NbSORIL6_5	1.4×10^{-9}	1) 6.9×10^{-9} (R_{max}=137) 2) 1.3×10^{-9} (R_{max}=1635)	2.5×10^{-9}
NbSORIL6_15	1.9×10^{-9}	1) 3.1×10^{-10} (R_{max}=3.8) 2) 1.1×10^{-8} (R_{max}=3.1)	9.6×10^{-8}
NbSORIL6_16	6.6×10^{-9}	1) 8.8×10^{-9} (R_{max}=159) 2) 2.5×10^{-10} (R_{max}=858)	1.9×10^{-9}
NbSORIL6_21	1.4×10^{-9}	1) 1.1×10^{-9} (R_{max}=5.4) 2) 3.5×10^{-11} (R_{max}=19)	1.8×10^{-7}



Supplementary Figure 4. Gel electrophoresis validation of unpurified sortase-mediated conjugation reactions of four different Nbs (NbSORIL6_21 16, 15 and 5) with a 5-fold molar excess of NbSORIL6 over two different oligos (H2 and H1). The species identified on the gel in order of increasing migration were: a) acylated intermediate b) SrtA enzyme c) conjugates d) excess Nb (NbSORIL6) and e) free oligos (H2 & H1). Unpurified reaction products for lanes 1 and 2: NbSORIL6_21-oligos H2 and H1, lanes 3 and 4: NbSORIL6_16-oligos H2 and H1, lane 5 and 6: NbSORIL6_15-oligos H2 and H1, lanes 7 and 8: NbSORIL6_5-oligos H2 and H1.



Supplementary Figure 5. Comparison of the effect of dimerizing Nb probes. **(A)** Illustration of homo-dimerized Nbs in PEA assays for IL6 protein detection. Upon incubation with IL6, homodimer pairs of Nb probes (NbSORIL6_5 and _16) bound to antigen were brought in proximity, allowing their attached oligos to hybridize and combine via DNA polymerization reactions. The resulting DNA extension products were quantified by real-time PCR as a measure of the amount of IL6 antigen in the sample. **(B)** Evaluation of reagents by polyacrylamide gel electrophoresis (PAGE); 50-bp DNA size marker (lane 1), lanes 2 and 3 for primary GGG-modified conjugation oligos, secondary hybridization oligos (lanes 4 and 5), hybridized primary and secondary oligos (lanes 6 and 7), dimerization of the secondary oligos black arrow indicates a circularized padlock probe (lane 8) and all four oligos (lane 9). **(C)** Comparison of IL6 detection by PEA using homodimerized Nb probes or monomeric NbSORIL6_5 and NbSORIL6_16 probes. Results of monomeric NbSORIL6_5 and _16 are indicated with a blue line while the red line corresponds to the homo-dimer pairs of Nb probes.



Supplementary Figure 6. Ab-oligos conjugation by copper free click chemistry. Ab-oligo conjugates were validated by agarose gel electrophoresis, bands in the red box in lanes 2, 3, 4, 5, 6 and 7 are free oligos while the Ab-ssDNA conjugates are in the blue box in lanes 4, 5, 6 and 7. In contrast, Ab-oligo conjugation produces multiple species with varying number of oligos conjugated to the individual Abs at different locations.