Chapter 3

Identification and validation of an interaction between SELP and PfMSP7

3.1 Introduction

This chapter describes the screening of *Plasmodium falciparum* merozoite cell surface and secreted proteins for interactions with human platelet proteins, and the subsequent identification and biochemical validation of an interaction between *P. falciparum* merozoite surface protein 7 (PfMSP7) and human P-selectin (SELP).

3.1.1 Rationale for screening

Extracellular host-pathogen protein-protein interactions are vital to the pathogenesis of disease and represent potential anti-malarial drug and vaccination targets (Section 1.2.1.4). High-throughput, unbiased screening approaches such as AVEXIS (Section 1.2.3) are powerful tools to identify novel interactions of this nature[40]. AVEXIS has had proven success with the discovery of, amongst others, the interaction between BSG and PfRH5, which is essential for parasite entry into RBCs and is thus an ideal and specific target for therapeutics[64]. This interaction was discovered via the screening of a library of recombinant *P. falciparum* merozoite cell surface and secreted proteins[65] against a panel of proteins from the RBC surface. In this work I screened a subset of the same merozoite protein library against proteins from the human platelet. A library of almost 200 recombinant platelet proteins had already been created in our laboratory, and within this library there are a large number of potential target proteins with which merozoite proteins could interact during disease. Platelets are implicated in both exacerbating disease and protecting the host, with reports that they are directly capa-

Platelet protein name	Uniprot Accession Number
PECAM1	P16284
SELP	P16109
APLP2	Q06481
BSG	P35613
CD59	P13987
ESAM1	Q96AP7
FURIN	P09958
GP6	Q9HCN6
ICAM2	P13598
PRNP	P04156
SCARF	Q14162
TMED1	Q13445
LAMP2	P13473
MET	P08581

Table 3.1: **Platelet bait proteins selected for AVEXIS screening**

ble of killing merozoites[211, 264] but are also responsible for the pathological clumping of pRBCs[259] and potentially contribute to the development of cerebral malaria[263]. Hence, detecting interactions between parasite and platelet proteins could contribute to our understanding of these processes at a molecular level. Many of the proteins in this platelet library are not restricted to the platelet surface, appearing on many other cell types with which *Plasmodium* parasites interact, for example endothelial cells and leukocytes. A large number are also released into the bloodstream as soluble proteins, many of which are important players in the immune response[346]. Hence interactions between proteins from the platelet and merozoite libraries could form part of a range of processes occurring in malaria, including cellular adhesion, platelet-mediated parasite killing and parasite-mediated immune evasion.

3.1.2 Selection of proteins for screening

For an initial screen I shortlisted high-expressing proteins from each library. These included 14 platelet bait proteins (see Table 3.1), each with evidence linking them to a role in immune functionality. For example, PECAM1, CD59, FURIN and PRNP have been implicated as regulators of T-cell maturation and functionality[146, 148, 168, 331], which *Plasmodium* parasites may suppress[224, 320]. PECAM1 and CD59 are also thought to affect B-cell development[148, 168] and thus have a role in the humoral immune response that protects the host from chronic or repeated infections, commonly observed with *P. falciparum*. APLP2 and

Merozoite protein name	PlasmoDB identifier
MSP1	PF3D7_0930300
MSP2	PF3D7_0206800
MSP4	PF3D7_0207000
MSP5	PF3D7_0206900
MSP10	PF3D7_0620400
Pf12	PF3D7_0612700
Pf38	PF3D7_0508000
ASP	PF3D7_0405900
AMA1	PF3D7_1133400
MTRAP	PF3D7_1028700
MSP3	PF3D7_1035400
MSP7	PF3D7_1335100
Pf41	PF3D7_0404900
Rh5	PF3D7_0424100
PF10_0323	PF3D7_1033200
AARP	PF3D7_0423400
MSP3.4	PF3D7_1035700
Pf12p	PF3D7_0612800
PF11_0373	PF3D7_1136200
PF14_0293	PF3D7_1431400

Table 3.2: **Merozoite prey proteins selected for AVEXIS screening**

LAMP2 are important regulators of antigen presentation[337, 373], FURIN and SCARF participate in signalling pathways that modulate the production of parasite-killing cytokines[305, 331], and CD59 has a well-characterised role in the complement cascade[168]. A subset of the selected proteins (PECAM1, SELP, ESAM, ICAM2, SCARF, MET) can also be found on the surface of endothelial cells, and of these PECAM1, SELP and ICAM2 have been associated with the recruitment of leukocytes to sites of inflammation[111, 148, 206]. Unsurprisingly for a group of platelet proteins, many also have known roles in haemostasis[7, 148, 152, 234], an important process to blood-dwelling pathogens[318]. I included BSG in the shortlist primarily to use as a positive control, known to interact with PfRH5[64]. However, BSG is also an interesting candidate to screen in its own right as it has been implicated as playing roles in cellular adhesion, leukocyte migration and RBC longevity[368], all of which could affect the survival of *Plasmodium* parasites in their host. The initial selection of merozoite prey proteins was more arbitrary; I chose 20 proteins from the library that were known to express at medium to high levels (see Table 3.2).

3.1.3 A brief introduction to SELP and PfMSP7

SELP, also known as GMP-140, PADGEM, CD62(P) and LECAM3, is a 140kDa glycoprotein expressed on the surface of activated platelets and endothelial cells. The protein is comprised of a C-type lectin domain, EGF-like domain and nine short consensus repeats as shown in Figure 4.2. Via its N-terminal C-type lectin domain, SELP binds to glycoprotein ligands such as leukocyte cell surface PSGL1 via sialyl Lewis-X (sLe^X) tetrasaccharide moieties on their surfaces. This property allows SELP to be an endothelial ligand to which leukocytes loosely adhere, or 'roll' in the early stages of inflammation. It thus contributes to the recruitment of leukocytes to vessel walls[71, 229].

The *PfMSP7* gene encodes a 40kDa precursor protein, and it is this full length protein that was used in the original screen. The precursor is believed to be proteolytically processed then exported from the merozoite. PfMSP7 interacts with PfMSP1 and a fragment from the C-terminus is retained on the merozoite surface, as part of the PfMSP1 complex (see Figure 3.1)[258]. The biological roles of these proteins, and those in their broader protein families, will be discussed in detail in Chapter 4.

3.2 Results

3.2.1 PfMSP7 and SELP interacted in an AVEXIS screen

To identify novel host-pathogen receptor-ligand interactions involved in the pathogenesis of malaria, I made use of the *P. falciparum* merozoite and human platelet protein expression constructs that were available in our laboratory. I shortlisted a panel of proteins from each library to screen against each other by AVEXIS, so as to identify interactions that merozoite proteins could be making with host immune effector targets. For this screen I produced 14 high-expressing human proteins with a known role in immune functionality as biotinylated 'baits' and 20 high-expressing merozoite proteins as pentameric β-lactamase-tagged 'preys'. Interactions were identified by observation of a colour change in the nitrocefin substrate; when a merozoite prey protein binds to one of the arrayed baits its β-lactamase enzyme cleaves the yellow substrate to produce a red binding signal, which can be quantified by measuring the solution's absorbance at 485nm (see 2.3 and Figure 1.4A). Of the merozoite prey proteins shortlisted for screening (see Table 3.2), I excluded three from the analysis; PfASP because it produced a binding-like signal when screened against the Cd4 negative control bait, and Pf12p and PF14_0293 as they did not produce any observable colour change when screened against the OX68 positive control bait. The remaining 17 merozoite proteins interacted with

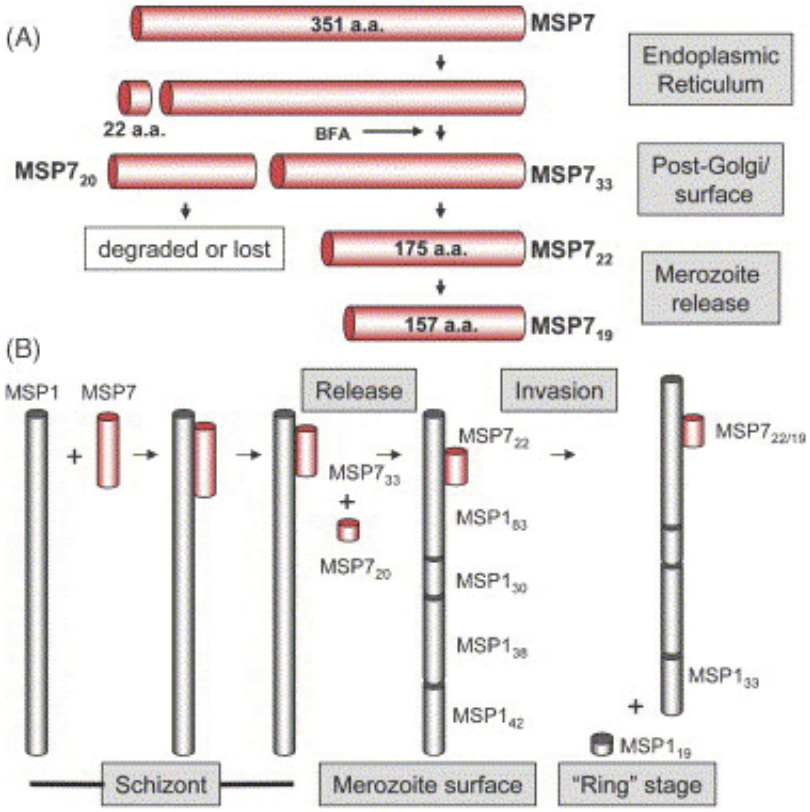

Figure 3.1: **PfMSP7 is proteolytically processed**
Figure from *Extensive proteolytic processing of the malaria parasite merozoite surface protein 7 during biosynthesis and parasite release from erythrocytes* by Pachebat *et al.* (2007)[258]. Reproduced with permission.
A. The PfMSP7 precursor protein is proteolytically processed in multiple steps. PfMSP7 is initially cleaved to release a 20kDa fragment from its N-terminus, then the 33kDa fragment remaining from the C-terminus of the precursor is N-terminally cleaved to leave a 22kDa fragment. This 22kDa fragment from the C-terminus of PfMSP7 is displayed on the surface of the merozoite as part of the MSP1 complex. In isolates containing glutamine at position 194 this fragment is cleaved again at the merozoite surface to leave a 19kDa fragment in this complex
B. The full-length PfMSP7 precursor protein is thought to associate with PfMSP1 soon after translation, after which PfMSP7 and PfMSP1 are processed concurrently.

the OX68 bait and not with the Cd4 tag bait, and were thereby judged to have been appropriately standardised for screening (Figure 3.2A). This original small-scale screen yielded two hits: the known interaction between PfRH5 and BSG (Figure 3.2C) and a potentially novel interaction between PfMSP7 and SELP (Figure 3.2B).

To further validate interactions discovered by AVEXIS we aim to demonstrate the interactions in both bait-prey orientations. PfMSP7 bait protein and SELP prey were seen to interact by AVEXIS, although the colour change observed was significantly slower than that seen when the assay was performed using in the reciprocal bait:prey orientation (where PfMSP7 is used as the prey, see Figure 3.3A). The PfMSP7 bait was not able to capture and retain sufficient β-lactamase-tagged SELP prey to saturate nitrocefin hydrolysis within one hour. Whist AVEXIS is not a quantitative assay, this result implies that the SELP prey/PfMSP7 bait interaction is not as strong as the PfMSP7 prey/SELP bait interaction. This could mean that the arrangement of proteins where SELP is pentamerised and PfMSP7 is arrayed on a surface is not optimal for the observation of the interaction[1]. SELP is thought to dimerise on the surface of platelets and endothelial cells, and this dimerisation has been shown to be important in increasing the avidity of the interactions SELP makes with its known ligands[16, 274]. It is possible that this SELP arrangement is replicated more accurately when the proteins are arrayed (as baits) as opposed to pentamerised via their COMP sequence (as preys).

3.2.2 The recombinant SELP and PfMSP7 proteins were biologically active

When working with recombinant proteins, especially those produced in heterologous expression systems, it is important to ensure that they are correctly folded so as to be biologically active. Fortunately, both SELP and PfMSP7 have previously-identified interacting partners which can be utilised to assay the functionality of our recombinant proteins. I was able to demonstrate that PfMSP7 prey binds to a recombinant PfMSP1 bait by AVEXIS (Figure 3.3A) and that SELP bait binds an alkaline phosphatase (AP)-conjugated sLe^X reagent[2] (Figure 3.3B), indicating that both recombinant proteins are functional and active. SELP was unable to bind to a recombinant PSGL1 in either bait:prey orientation, though this may be explained by a lack of essential post-translational modifications on the PSGL1 surface, since

[1]This is not an unusual phenomenon for AVEXIS. In fact in a large scale screen for interactions between zebrafish receptors only 56 of the 100 of heterophilic interactions identified could be observed in both bait-prey orientations[205]. These included a handful of known interactions, including those between Robo and Slit proteins involved in *Drosophila* development[34, 167].

[2]This sLe^X-AP reagent was created by saturating streptavidin-AP (Sigma) with biotinylated sLe^X (Glycotech)

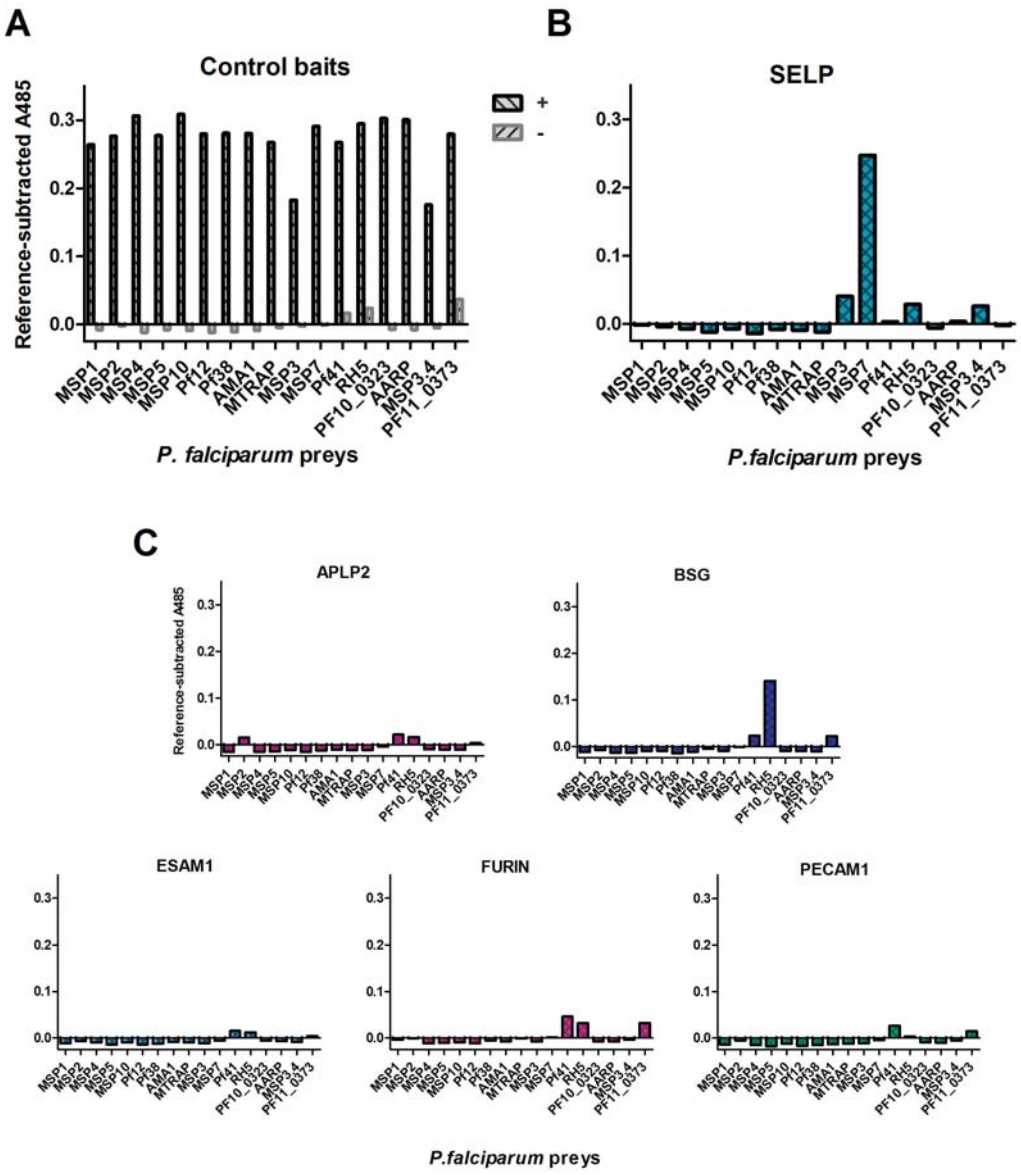

Figure 3.2: **SELP bait interacted with PfMSP7 prey**
All bars represent reference-subtracted AVEXIS signals as described in Section 2.3.1.3
A. AVEXIS signals when 17 *P. falciparum* merozoite prey proteins were screened against controls. Each prey protein included in the analysis produced a strong signal upon screening against positive control bait (OX68 antibody) and minimal signal when screened against the negative control (rat Cd4 tag region)
B. AVEXIS signals when SELP bait was screened against merozoite preys. SELP bait interacted with PfMSP7, but not with any other merozoite preys.
C. AVEXIS signals when additional platelet bait proteins were screened against the merozoite proteins. The signals observed for five of the 14 baits are shown. The only other significant signal observed was when BSG bait was incubated with PfRh5 prey. This is a known interaction.

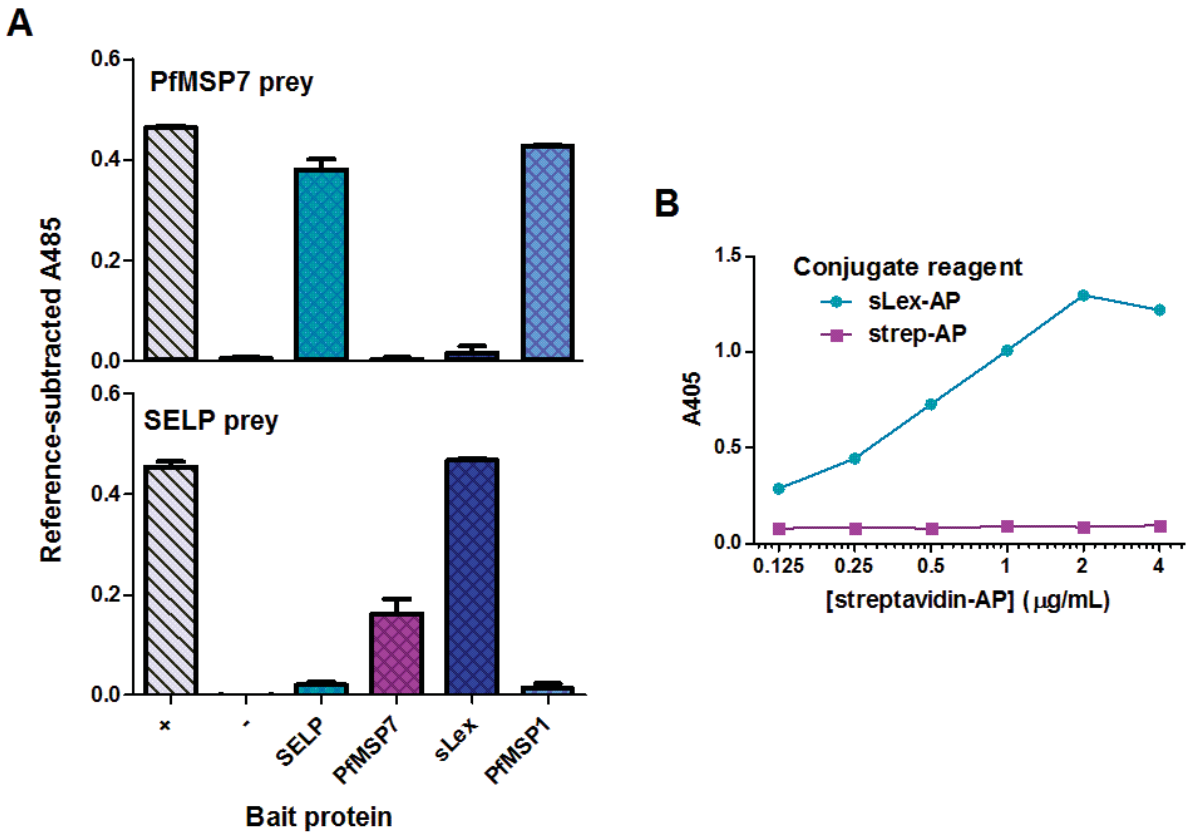

Figure 3.3: **Recombinant, soluble SELP and PfMSP7 preparations were biochemically active**

A. AVEXIS signals when PfMSP7 and SELP preys were screened against known ligands. PfMSP7 prey bound to PfMSP1, as well as SELP, bait. SELP prey bound to sLe^X and PfMSP7. Both prey proteins were also screened against OX68 positive control bait (+) rat Cd4 tag region negative control bait (-). Bars represent means +/- SD, n=3.

B. Alkaline phosphatase(AP) mediated substrate activity when streptavidin-AP conjugates were incubated with SELP bait. A sLe^X conjugate bound to SELP bait. The bait did not bind the streptavidin-AP used to create the conjugate.

co-transfection of a fucosyltransferase may be required to ensure that sLe^X is incorporated into the molecule[291]. It is also worth noting that both PfMSP7 and SELP proteins were expressed at high levels, which is in itself a good indicator that the proteins are correctly folded prior to secretion.

3.2.3 The SELP/PfMSP7 interaction could be blocked by an anti-SELP mAB

To provide further evidence for the correct folding of SELP, I tested the binding of the recombinant protein to a commerically-available anti-SELP monoclonal antibody (mAB). The antibody bound to recombinant SELP by ELISA. However the binding of this CLB-thromb/6 antibody was not affected by denaturation of the recombinant SELP bait (Figure 3.4B), indicating that the CLB-thromb/6-binding epitope is not conformation-sensitive. This means that the binding of the antibody cannot be used as an indicator of correct protein folding. To provide more evidence for the specificity of the SELP/PfMSP7 interaction, I showed that the binding of the antibody to immobilised SELP bait blocks the binding of PfMSP7 prey (Figure 3.4A). This result may indicate that there is some overlap between the binding sites for CLB-thromb/6 and PfMSP7 on SELP, and/or may result from the steric effect of the antibody, impeding access of PfMSP7 pentamers to their binding sites. Additionally, this result means that this particular antibody clone could be a useful reagent to block the interaction in further studies.

3.2.4 PfMSP7 bound to recombinant SELP at the cell surface

To demonstrate that PfMSP7 can bind to SELP at the cell surface, and to verify the interaction in a different experimental system, I developed a flow cytometry-based binding assay (described in Figure 2.4). By transfecting HEK293F cells with plasmids encoding protein ectodomains fused to a transmembrane domain and green fluorescent protein (GFP), I was able to over-express receptors at the cell surface with a cytoplasmic GFP tag (Figures 2.1C & 3.5A). I incubated the transfected cells with pentamerised, FLAG-tagged proteins and then with a Cy3-conjugated anti-FLAG antibody. I then used flow cytometery to distinguish fluorescent populations. I used the interaction between rat Cd200 and Cd200R proteins to optimise the experimental parameters of the assay, expressing Cd200R at the cell surface and incubating the cells with Cd200 pentamer. I observed a strong correlation between GFP signal and Cy3 fluorescence, indicating that Cd200 protein bound specifically to transfected cells, at levels

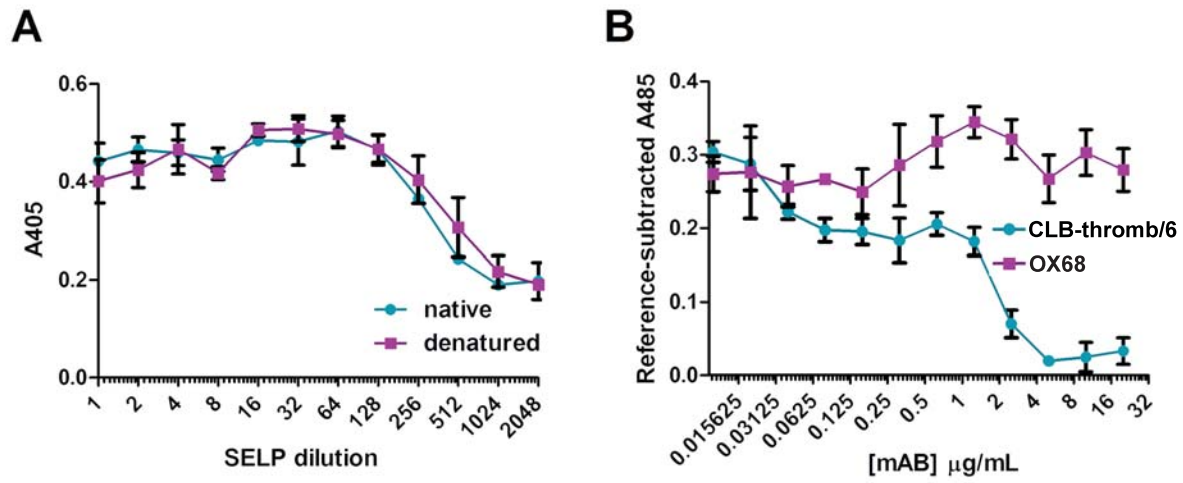

Figure 3.4: **An anti-SELP mAB blocked the SELP/PfMSP7 interaction**
A. ELISA using CLB-thromb/6 to detect recombinant SELP. The CLB-thromb/6 binding epitope on SELP is not conformation-sensitive, since boiling the protein for ten minutes with 1% SDS prior to capture did not affect the result.
B AVEXIS signals resulting from the interaction between SELP bait and PfMSP7 prey, blocked by incubating the bait protein with CLB-thromb/6, a mAB against SELP. The interaction was not blocked with OX68 mAB, which binds to the Cd4 tag region of SELP.
Error bars represent means +/- SD, *n*=3.

proportionate to the receptor concentration (Figure 3.5B). The interaction could be blocked by pre-incubating cells with OX102 anti-Cd200R monoclonal antibody, indicating that the Cd200 pentamers are binding to the cell surface specifically via their interaction with the transfected Cd200R.

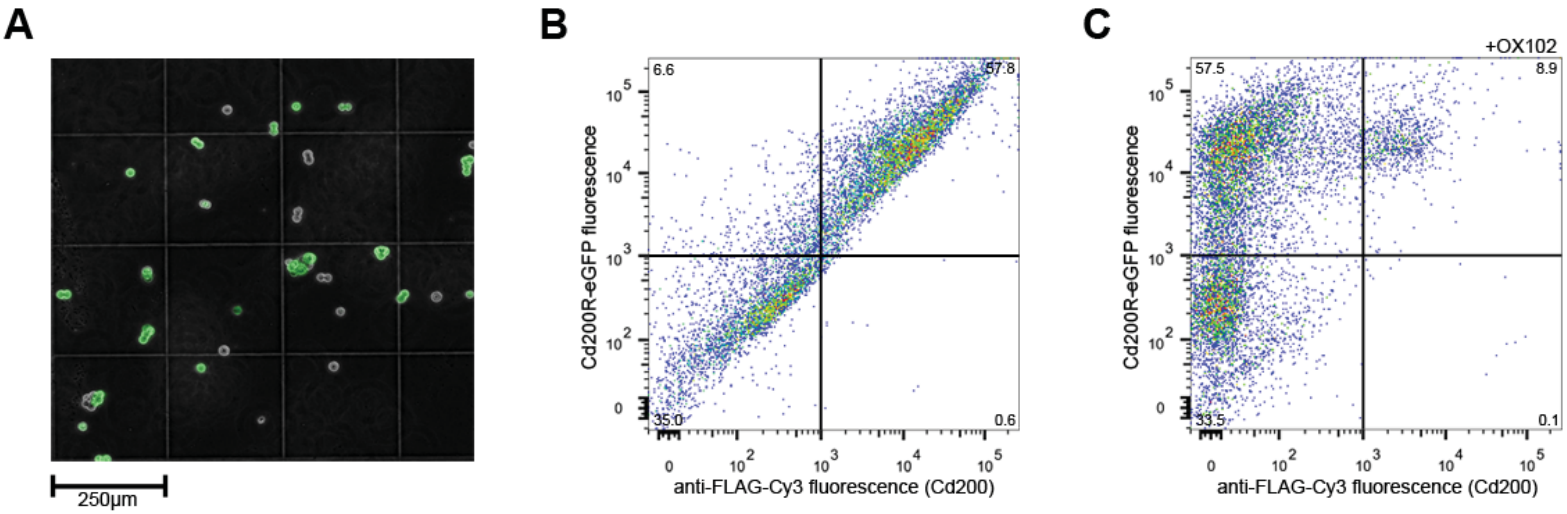

Figure 3.5: **Development of flow cytometry-based assay to measure protein binding to HEK cells**
A. Fluorescence microscopy on HEK293F cells 24 hours after transfection with a GFP-tagged Cd200R expression construct. Over half of the population of cells were fluorescent.
B. Flow cytometry dot-plots when Cd200R-eGFP transfected cells were incubated with Cd200. Cd200-FLAG pentamers bound specifically to Cd200R-eGFP transfected cells, as observed by the correlation of eGFP and Cy3 fluorescence intensity.
C. Flow cytometry dot-plots when OX102-blocked Cd200R-eGFP transfected cells were incubated with Cd200. The shift in the eGFP-positive cells from the double positive quadrant (top right) of the dot plot (in B) to the eGFP-positive quadrant (top left), shows that OX102 blocked the interaction between transfected cells and the Cd200 ligand.
5,000 events within forward and side-scatter parameters that facilitate counting of HEK cells are displayed on the above dot plots.

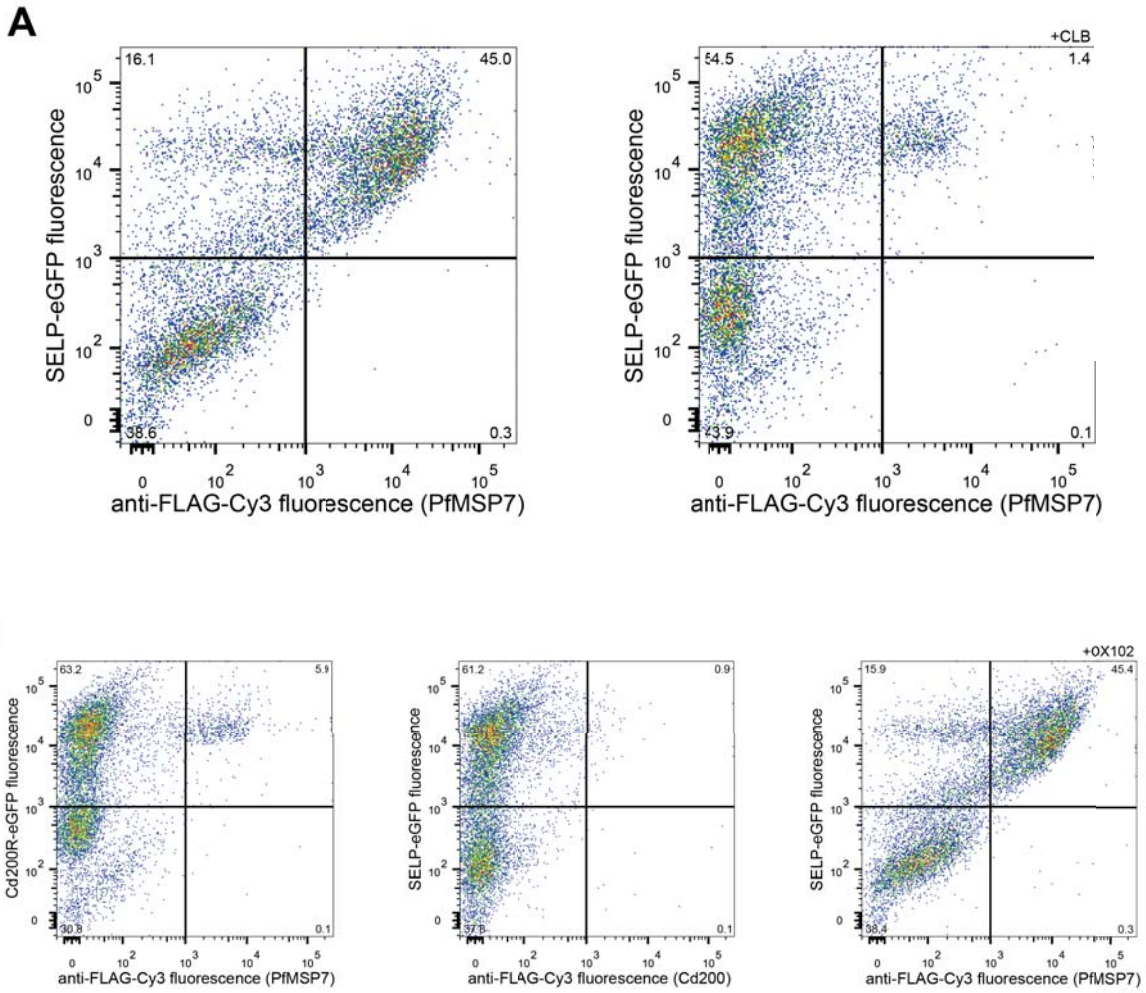

Figure 3.6: **PfMSP7 pentamers bound specifically to SELP at the cell surface**

A. Flow cytometry dot-plots when SELP-eGFP transfected cells were incubated with pentameric FLAG-tagged PfMSP7 bound to cells expressing GFP-tagged SELP on the cell surface. FLAG-tagged PfMSP7 interacted with eGFP-positive cells (left), but not those that were blocked by an anti-SELP mAB (right).

B. Dot plots of three negative controls. All negative control samples behaved as expected; Cd200R positive cells did not bind the PfMSP7 pentamers (left), SELP-transfected cells did not interact with Cd200 (centre) and mABs not directed against SELP could not block the SELP-PfMSP7 interaction (right).

5,000 events within forward and side-scatter parameters that facilitate counting of HEK cells are displayed.

Similarly, I could detect a specific interaction between SELP receptors and PfMSP7 pentamers (Figure 3.6). PfMSP7 prey did not bind to untransfected cells, or cells transfected with Cd200R-GFP. The CLB-thromb/6 anti-SELP monoclonal antibody that I earlier found to block the interaction (Section 3.2.3) could also block the interaction between the PfMSP7 and SELP-transfected cells. Antibodies against Cd4 or BSG (which is present on the HEK cell surface) did not block the interaction (Figure 3.6B). This further demonstrates that PfMSP7 bound to the cell surface specifically via its interaction with SELP. I was unable to express sufficient PfMSP7 at the cell surface to perform the assay using SELP prey.

3.2.5 Recombinant PfMSP7 formed metastable oligometric complexes in solution

To isolate monomeric PfMSP7 for use in surface plasmon resonance studies, I purified the 6xHis-tagged protein by immobilised metal ion affinity chromatography (IMAC) and then separated the elutant by size-exclusion chromatography (SEC). I observed high batch-to-batch variability in the elution profiles from each SEC experiment and observed multiple peaks in the majority of profiles (see Figure 3.7A). SDS-PAGE analysis on the eluted fractions confirmed that each peak contained only PfMSP7 proteins, indicating that PfMSP7 exists in multiple oligometric states (Figure 3.7B). The gel filtration column is calibrated using a series of globular protein standards, allowing the estimation of the molecular mass of proteins based on their elution volume. However, the volume at which a protein elutes is dependent on its size and conformation, not its molecular mass alone, such that elution volume more accurately reflects the hydrodynamic volume (V_{H}) or radius of gyration (R_{g}) of the eluting species[319]. In most cases the slowest-eluting fractions had a V_{H} equivalent to a globular protein with a molecular mass of at least 100kDa; if these proteins correspond to PfMSP7 monomers this would imply that PfMSP7 adopts a more extended conformation than globular proteins of an equivalent (~60kDa) mass. The earliest-eluting PfMSP7 fractions, with V_{H}s equivalent to a globular protein hundreds of kDa in mass, are certainly higher-order PfMSP7 complexes. The degree to which the PfMSP7 preparations oligomerise did not appear to be related to protein concentration or the length of time the protein had been stored.

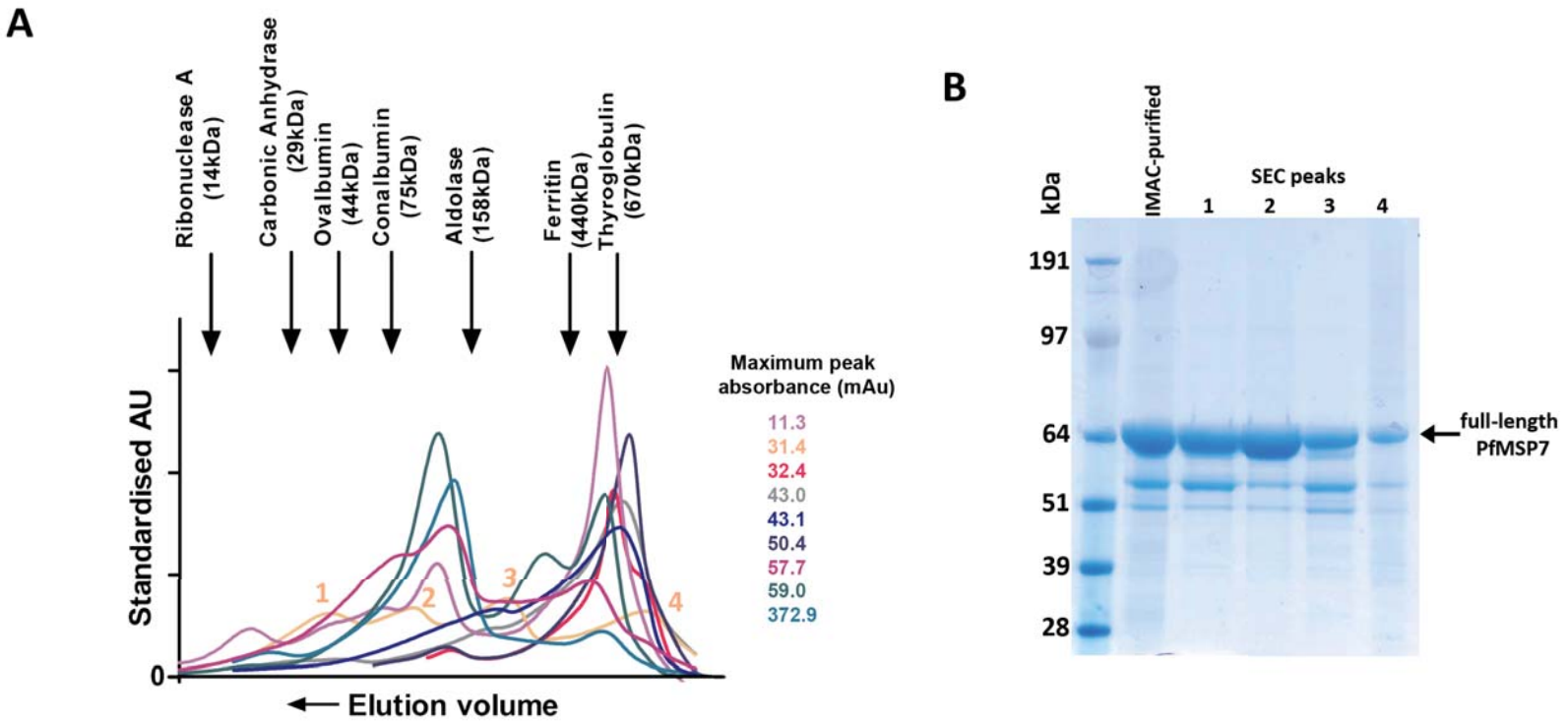

Figure 3.7: **PfMSP7 formed oligomers *in vitro***

A. Nine independent PfMSP7 gel filtration (SEC) profiles. The profiles were highly variable but reliably contained complexes containing multiple PfMSP7 molecules. As two different columns were used in the course of this work I have used the calibration data to convert elution volumes to an estimate of molecular mass, so as to more accurately compare profiles between columns. The traces shown are standardised for input protein amount by equalising the area under the curve for each protein. The recorded peak absorbance value for the highest peak in each trace is indicated.

B.SDS-PAGE analysis confirmed that each peak is comprised of PfMSP7 proteins. 15µL of the four 3µM SEC eluate fractions from the indicated peaks in A were resolved alongside 2µL of 25µM IMAC purified PfMSP7.

3.2.5.1 The flexible N-terminus of PfMSP7 was responsible for self-oligomerisation

PfMSP7 undergoes proteolytic cleavage to leave a 22 or 19kDa fragment from the C-terminus on the surface of the merozoite and fragments as part of the MSP1 complex, and N-terminal fragments that are thought to be degraded or lost from the merozoite (Figure 3.1)[158, 258]. To determine whether a particular portion of the molecule is responsible for the formation of metastable oligomers, I expressed and purified the 22kDa fragment of MSP7 ($MSP7_{22}$) and the remaining portion from the N-terminus (referred to hereafter as MSP7-N). and analysed both proteins by size-exclusion chromatography (SEC) (Figure 3.8A & C). Each of three independent PfMSP7-N preparations exhibited very different SEC elution profiles. This was similar to the behaviour I observed when analysing full-length PfMSP7 (Figure 3.7) and suggested that the N-terminus was the source of this potential oligomerisation activity and variability in the SEC elution traces. Corroborating this, three SEC profiles of $PfMSP7_{22}$ preparations were very similar to one another (Figure 3.8B & C). They showed a single predominant peak, which could theoretically correspond to a monomeric protein, and a smaller peak comprising oligometric protein, indicating that this C-terminal region is not the source of the variable oligomerisation behaviour observed in PfMSP7.

3.2.5.2 'Intrinsic disorder' in PfMSP7

Oligomerisation of *P. falciparum* merozoite surface proteins has been reported previously, with SEC elution profiles for PfMSP2, PfMSP3 and PfMSP3 fragments yielding multiple peaks with large predicated hydrodynamic radii[2, 118, 144]. It is thought that both PfMSP2 and PfMSP3 proteins self-assemble into amyloid like filaments, and it has been hypothesised that these assemblies promote binding to RBC surfaces prior to invasion[144, 372]. To investigate whether the same sort of oligometric self assembly may be occurring in PfMSP7, I looked for amino acid sequences that might promote this behaviour. I did not identify any regions of similarity to the leucine-zipper region or aggregation motif that are speculated to be involved in PfMSP3 self-assembly, or with the N-terminal region of PfMSP2 which is thought to be responsible for its oligomerisation[144, 366]. The formation of amyloid fibrils, similar in their structure to those seen in PfMSP2 and PfMSP3, is associated with flexible regions in component protein monomers[370]. By running the PfMSP7 amino acid sequence through the several algorithms that predict flexible or 'disordered' regions in protein structure, I identified that the PfMSP7 sequence is very likely to contain disordered regions, particularly towards its N-terminus (Figure 3.8D)[95]. The four algorithms PrDOS[147], Disprot[363], Disopred3[157] and IUPred[73] predict 44-75% of the amino acid residues comprising MSP7-N to be part

of disordered regions, whereas the percentage for $MSP7_{22}$ is only 18-22% (see Figure 6.1C). This suggests that the N-terminus of PfMSP7 is intrinsically disordered.

In recent years there has been growing recognition that a large proportion of proteins, particularly those in eukaryotes[350][3], lack the ordered, globular domain structures that are commonly associated with binding or catalytic functions[361]. These proteins are characterised by flexible structures, which have been shown to adopt more ordered conformations upon binding to their (often numerous) ligands. This property has been associated with the ability of proteins to perform multiple functions and to bind multiple targets, and could contribute to the tendency of PfMSP7 to self-associate[332].

3.2.6 PfMSP7 binding to SELP was observed using SPR

Surface plasmon resonance (SPR) is a leading method for determining the kinetic parameters of a protein-protein interaction. I attempted to use this approach to determine equilibrium binding measurements for the interaction between monomeric PfMSP7 and SELP. Upon injecting PfMSP7 analyte over immobilised SELP bait, I observed a clear binding response, indicating that the two proteins interact directly (Figure 3.9). Fitting the association curves to different binding models indicated that complex, multivalent binding of PfMSP7 to SELP took place, which was anticipated given the observed tendency for PfMSP7 to oligomerise (see 3.2.5). Where I observed multiple peaks in SEC elution profiles, I often observed significantly greater binding signals when PfMSP7 complexes of larger molecular mass (but equivalent amount of PfMSP7 molecules) were injected over SELP (Figure 3.9B). The binding of a larger complex will in itself increase the SPR signal which is inherently sensitive to the size of the binding analyte, though given the magnitude of this increase it could be speculated that these larger complexes are also binding more avidly. More detailed kinetic data are required to confirm this. In all cases where PfMSP7 species were injected, dissociation of PfMSP7 analyte appeared to be very slow, suggesting that this is a highly avid interaction (Figure 3.9).

Whilst these data validate the interaction between SELP and PfMSP7, their binding characteristics meant that the interaction was not particularly amenable to kinetic analysis. Firstly, binding did not saturate rapidly, such that a long injection time, and consequently a large volume of PfMSP7 analyte would be required to perform equilibrium binding analyses. Unfortunately I was unable to generate sufficient purified PfMSP7 analyte to perform these analyses.

[3] and as much as half of those in *Plasmodium* species [94]

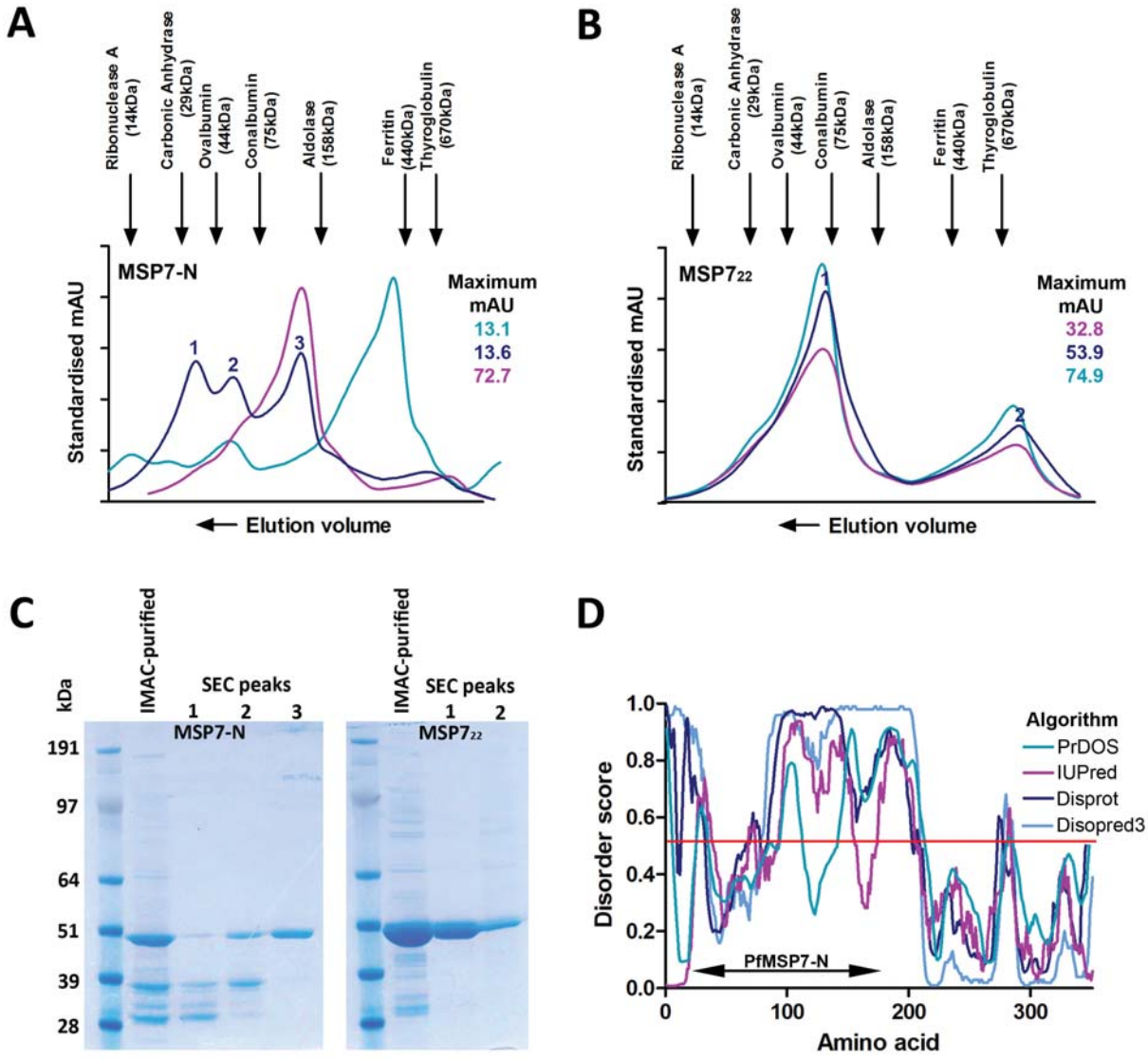

Figure 3.8: **PfMSP7-N showed similar oligomerisation behaviour to full-length PfMSP7**
A. SEC elution profiles from 3 independent PfMSP7-N preparations. As observed with full length PfMSP7 there is great batch-to-batch variabilty, with profiles showing multiple peaks.
B. SEC elution profiles from 3 independent PfMSP7$_{22}$ preparations. These profiles are much more consistent, with one predominant peak and a smaller peak containing higher order complexes.
C. SDS-PAGE analysis of each of the indicated Cd4 domain-tagged PfMSP7-N or PfMSP7$_{22}$ peaks confirms that each contains PfMSP7 fragments of the expected size. It is possible that the peak 1, with the smallest predicted V_H, from the PfMSP7-N SEC elutant did not contain PfMSP7 proteins.
D. Protein disorder scores predicted by four different algorithms IUPred, Protein disorder prediction system (PrDOS), Disprot and Disopred3. The N-terminal region of PfMSP7 is predicted to be significantly disordered, which may account for the formation of metastable oligomers in solution.

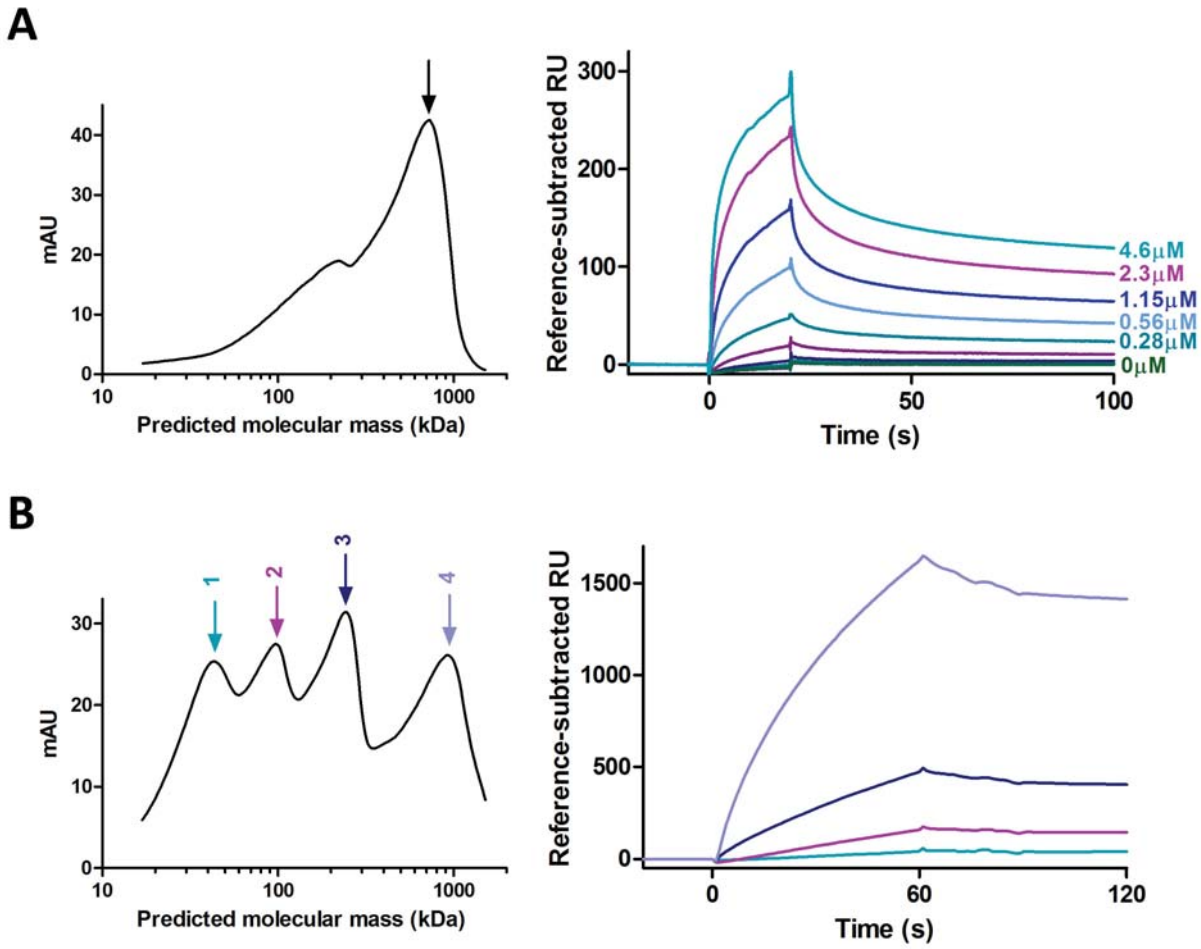

Figure 3.9: **PfMSP7 bound specifically to SELP in SPR experiments**
A. SPR signals when a range of PfMSP7 concentrations from a predominantly monodisperse SEC peak were injected over SELP bait. A representation of the SEC elution profile of the PfMSP7 analyte used in this particular experiment is shown on the left.
B. SPR signals when 3μM PfMSP7 protein from indicated SEC fractions was injected over SELP. Larger binding responses were observed when larger PfMSP7 complexes were injected.

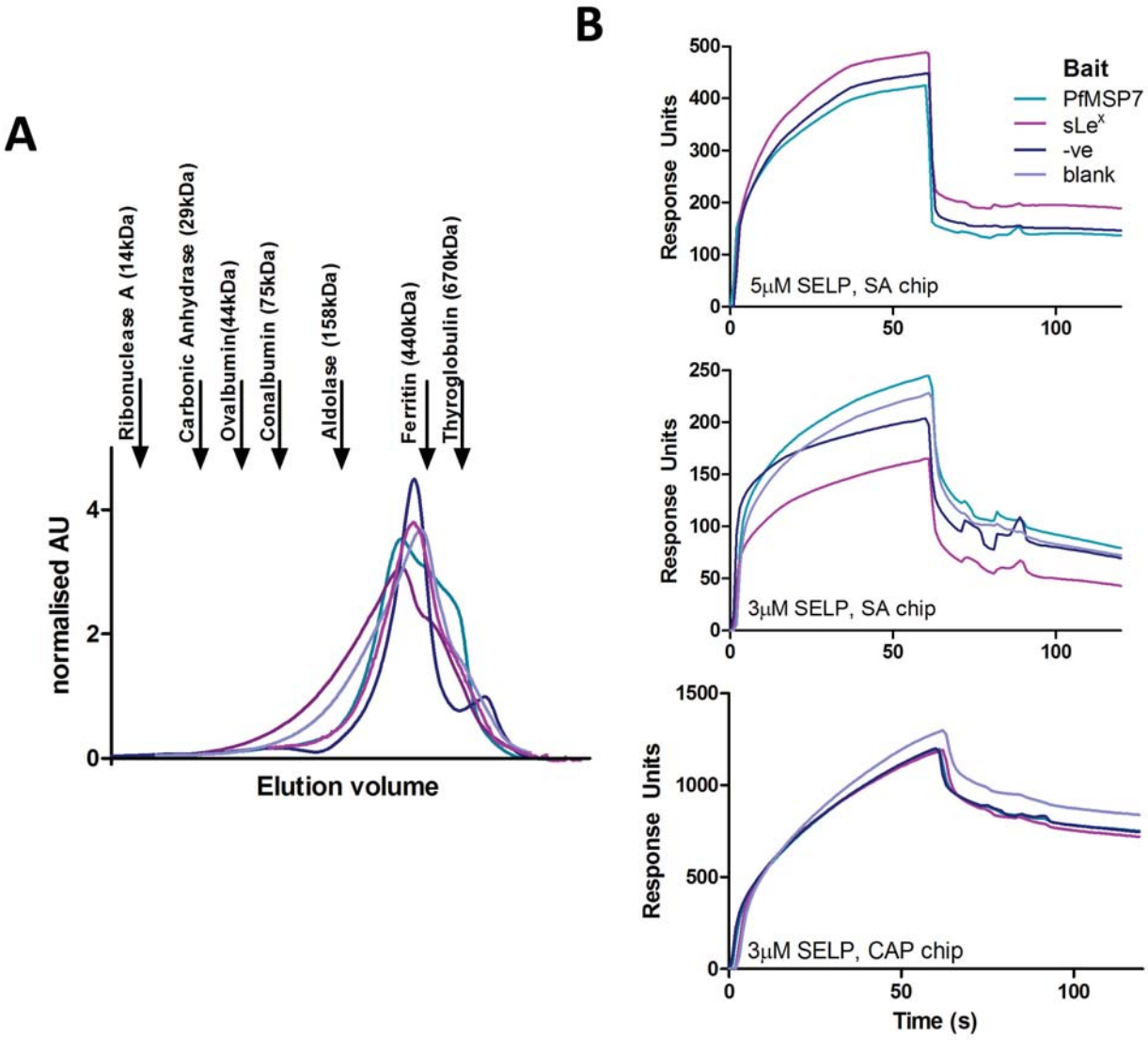

Figure 3.10: **Purified SELP bound to SPR chips**
A. SEC elution profiles from five independent SELP preparation. Predominantly broad, monodisperse peaks were observed.
B. SPR responses recorded when the indicated concentrations of SEC-purified SELP were injected over four baits for 60 seconds. In each case comparable and highly significant binding to positive controls (sLe^X tetrasaccharide), negative controls (rat Cd4 tag), PfMSP7 and even blank flow cells was evident.

Secondly the interaction was fairly resistant to washing with a range of regeneration solutions, including 0.2-1M NaOH, 1-3M Glycine HCl (pH1.5), 5M NaCl and 4M $MgCl_2$. This made it difficult to fully regenerate the SELP-coated surface, which adversely affected the reproducibility and reliability of successive analyte injections.

To overcome the difficulties in analysing multivalent PfMSP7 binding to SELP, I prepared SEC-purified SELP analyte to inject over immobilised PfMSP7. Encouragingly, the SEC elution profiles of SELP proteins were consistent between experiments, traces showing broad but monodisperse peaks (Figure 3.10A). However, injecting SELP analyte in SPR experiments proved problematic as it appeared to bind non-specifically in every flow cell (Figure 3.10B). This suggested that SELP was binding to the carboxymethylated dextran surface of the SPR chip. Selectins have been reported to bind dextrans, but only in a sulphated form[133], so it is currently unclear as to why this binding activity was observed.

3.3 Discussion

3.3.1 Multiple biochemical assays indicate that PfMSP7 and SELP interact, but oligomerisation of PfMSP7 may be required

The work described above shows that recombinant PfMSP7 and SELP proteins interact reproducibly in multiple assay systems (Figures 3.3, 3.9 & 3.6). Pentamerised PfMSP7 interacts with monomeric SELP in AVEXIS, with nitrocefin hydrolysis saturating in just minutes, significantly faster than all positive controls. Flow cytometry-based assays also show that PfMSP7 pentamers can bind specifically to SELP expressed at the cell surface. We can also see a large SPR signal when injecting purified PfMSP7 over SELP. In AVEXIS, PfMSP7 bait binding to SELP prey appears to be significantly weaker (Figure 3.3A), indicating that there may be a requirement for a higher-order spatial arrangement of either protein. SEC experiments show that recombinant PfMSP7 readily self-associates (Figure 3.7) and the higher-order oligomers appear to exhibit increased SELP-binding ability in SPR experiments (Figure 3.9B). Especially given the predicted flexibility of the N-terminus (Figure 3.8D), it is not unlikely that PfMSP7 proteins would oligomerise *in vivo*, and there is a precedent for doing so provided by PfMSP2 and PfMSP3[118, 366], whose oligomerisation is thought to increase the proteins' ability to participate in binding interactions[2, 144].

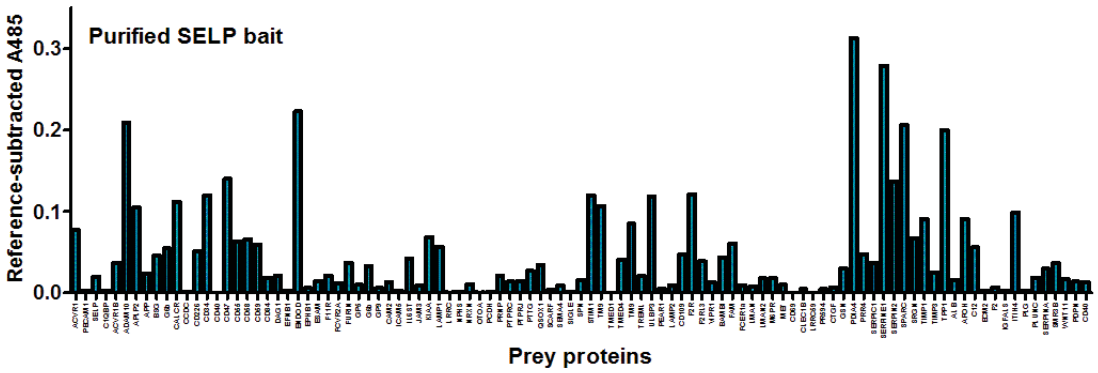

Figure 3.11: **Purified SELP bait interacted non-specifically in another AVEXIS screen** AVEXIS data from a platelet-platelet protein interaction screen showed that SELP bait interacts non-specifically with a range of platelet preys.
Data courtesy of Dr Yi Sun.

3.3.2 Recombinant SELP is prone to non-specific interactions, but multiple lines of evidence support the validity of its interaction with PfMSP7

In the course of this project, unrelated research within the group showed that purified recombinant SELP bait proteins exhibit 'promiscuous' binding behaviour in AVEXIS. In these experiments, purified recombinant bait proteins from a platelet protein library were screened against the same library expressed as purified prey proteins. A large proportion of the platelet prey proteins produced a binding signal when screened against SELP bait, which was amongst the 'noisiest' of the bait library (Figure 3.11). Similar results were observed in a microarray-based AVEXIS-like interaction screen using the same proteins[321].

These AVEXIS data, coupled with the observation that recombinant SELP binds to the supposedly inert SPR chip surface (Figure 3.10B), raised legitimate concerns about the meaningfulness of my biochemical data about the SELP-PfMSP7 interaction. In both instances where this promiscuous binding was observed the recombinant SELP protein was purified by IMAC, so it is possible that purification increases the propensity of SELP to bind non-specifically. In light of these concerning observations, I avoided using purified protein where possible and took measures to ensure that the SELP proteins used in all PfMSP7 interaction assays were correctly folded and functional, for instance by demonstrating that they bind to sLe^X (Figure 3.3). Even though these assays increase our confidence that the recombinant protein is folded and active, the possibility remains that a proportion of the proteins in each preparation are locally unfolded and responsible for non-specific interactions. To ultimately exclude the

possibility that the interaction we observe is the result of non-natively folded SELP proteins, I would ideally demonstrate that the interaction takes place between naturally-occurring proteins, which will be folded and post-translationally modified as they would be *in vivo*. The cell surface expression of SELP can be induced in platelets and endothelial cells, so I attempted to stain the surface of activated platelets and human umbilical vein endothelial cells (HUVECs) with FLAG-tagged PfMSP7 pentamers. Attempts to stain PMA-activated HUVECs with either anti-SELP antibodies or PfMSP7 were unsuccessful, both when using microscopy and flow cytometry as detection methods. It is possible that the surface expression level of SELP in this cell line was insufficient. The activation of platelets leads to a very significant increase in surface SELP, which I was able to detect by flow cytometry (Figure 3.12A). In initial attempts to stain platelets with PfMSP7, I incubated activated platelets with FLAG-tagged PfMSP7 pentamers followed by a fluorescently conjugated anti-FLAG monoclonal antibody. These experiments were ultimately unsuccessful, as the antibody itself bound the platelets at a level that was unaffected by the presence of PfMSP7, positive control or negative control pentamers (Figure 3.12B). Before pursuing *in vivo* experiments to investigate the function of the interaction, it will be important to improve this platelet-based assay or otherwise demonstrate that PfMSP7 can bind to native SELP. Data presented in Chapter 4 support there being an important, conserved role for this interaction in a number of *Plasmodium* species, and provide more evidence for the validity of the interaction discussed here.

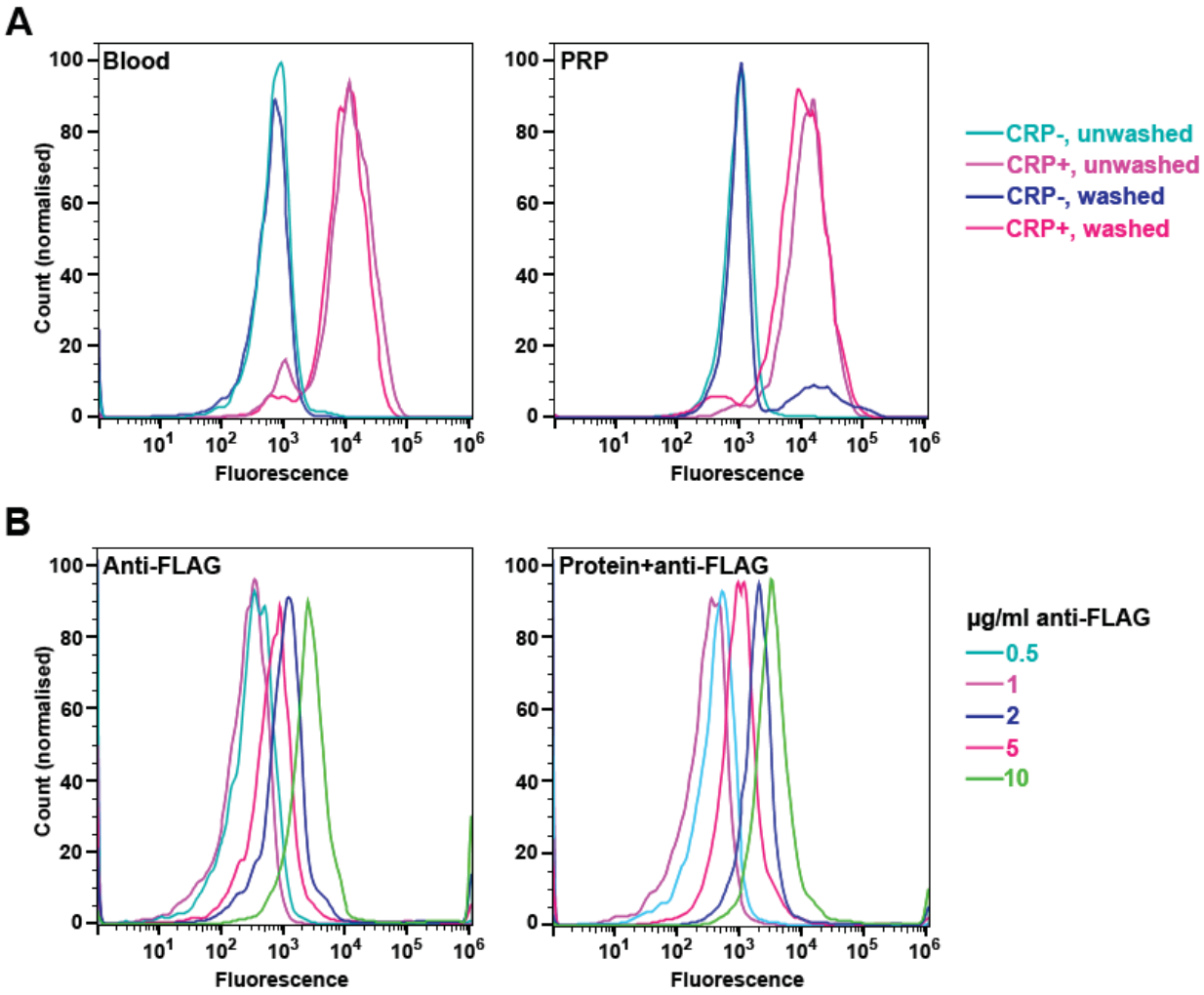

Figure 3.12: **Platelet staining assays were optimised but unsuccessful due to antibody binding to the platelet surface**

A. Flow cytometry histograms of FITC flourescence when platelets were incubated with conjugated anti-SELP antibody. SELP was detected on the surface of activated platelets within whole blood or platelet rich plasma (PRP). Washing the blood or PRP by centrifugation did not impair sample quality.

B. Histograms of FITC fluorescence when platelets were incubated with or without FLAG-tagged pentameric EphrinB2 positive control proteins and then with a FITC-conjugated anti-FLAG antibody. It was not possible to discern EphrinB2 binding to platelets(right) as the anti-FLAG antibody appeared to bind directly to platelets(left).

5,000 events within forward and side-scatter appropriate for counting platelets are displayed.