Biophysical Chemistry 152 (2010) 170–177

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Optimisation of a multivalent Strep tag for protein detection

Michael Busby [a,1], Lukas Kurt Josef Stadler [b,1], Paul Ko Ferrigno [b], Jason J. Davis [a,*]

[a] Department of Chemistry, University of Oxford, Mansfield Road, Oxford OX1 3QR, United Kingdom
[b] Section of Experimental Therapeutics, Leeds Institute of Molecular Medicine, St James's University Hospital, Beckett St, Leeds LS9 7TF, United Kingdom

ARTICLE INFO

Article history:
Received 2 August 2010
Received in revised form 20 September 2010
Accepted 20 September 2010
Available online 29 September 2010

Keywords:
Strep tag
Multivalency
Microarray
Surface plasmon resonance
FRET

ABSTRACT

The Strep tag is a peptide sequence that is able to mimic biotin's ability to bind to streptavidin. Sequences of Strep tags from 0 to 5 have been appended to the N-terminus of a model protein, the Stefin A Quadruple Mutant (SQM) peptide aptamer scaffold, and the recombinant fusion proteins expressed. The affinities of the proteins for streptavidin have been assessed as a function of the number of tags inserted using a variety of labelled and label-free bioanalytical and surface based methods (Western blots, microarray assays and surface plasmon resonance spectroscopy). The binding affinity increases with the number of tags across all assays, reaching nanomolar levels with 5 inserts, an observation assigned to a progressive increase in the probability of a binding interaction occurring. In addition a novel interfacial FRET based assay has been developed for generic Strep tag interactions, which utilises a conventional microarray scanner and bypasses the requirement for expensive lifetime imaging equipment. By labelling both the tagged StrepX-SQM_2 and streptavidin targets, the conjugate is primed for label-free FRET based displacement assays.

1. Introduction

The use of protein affinity tags to facilitate the purification and detection of recombinant proteins has become indispensable to the field of protein chemistry and beyond. The most common classes of tags include antibody epitope tags, such as Myc and haemagglutinin (HA) [1,2], fusion protein tags such as gluthatione *S*-transferase (GST) and maltose binding protein [3,4], and short affinity tags such as the 6×histidine tag and poly-arginine tag [5,6]. The interaction between streptavidin and biotin possesses one of the lowest dissociation constants in biology and is of particular interest because it occurs even when the biotin is covalently attached to proteins [7]. Each streptavidin monomer binds one biotin molecule with femtomolar affinity, one of the strongest non-covalent interactions observed in nature [8]. Over the past 15 years several groups have developed peptide binders to the tetrameric streptavidin protein [9–13] and a variety of protein engineering approaches have been applied to tailor affinity for specific applications [14–16]. This provides a simple and clean system for protein purification as a streptavidin binding peptide can be selectively eluted from a streptavidin affinity column using appropriate concentrations of biotin. A further advantage of streptavidin as an affinity reagent is its relative low production cost. This in turn has led to the commercialisation of a wide variety of streptavidin-derived materials such as enzymes, plates, microarray slides, beads and more. Schmidt and Skerra were among the first to develop a streptavidin binding peptide when they identified the Strep-tag in a peptide library screen [11]. The Strep-tag, although useful for the one-step purification of recombinant proteins [17], could only be used in the C-terminus of the protein, as a free carboxy-terminus is required for binding. This deficit motivated the development of the StrepII-tag, which is similar in length and can be included anywhere within the protein sequence [10]. Both these tags contain the conserved HPQ amino acid motif, which is found in many streptavidin binding peptides and is chiefly involved in mediating the interaction with streptavidin. Although the shortness of the Strep-tags makes them very attractive as biochemical tools, both bind streptavidin with relatively low affinity (37 μM and 72 μM, respectively [10]). This poses a problem in cases where higher affinity is required, e.g. in recombinant protein microarrays, or in the detection of Strep-tagged proteins in Western blots. Consequently, several groups have set out to find higher affinity alternatives to the Strep-tags [9,13]. Using an mRNA display library, Wilson and coworkers, have identified a 38 amino acid long peptide (streptavidin-binding peptide, SBP), which binds with nanomolar affinity [13].

Juntilla and co-workers have explored the possibility of Strep-tag multivalency by using two tandem StrepII-tags separated by a 12 amino acid linker, a configuration named a StrepIII-tag, in the purification of protein complexes with streptavidin coated beads [18]. An improved detection of the relevant protein complex when using the StrepIII-tag compared to the StrepII-tag was observed on a Western blot assay. However, it remains to be shown whether the increased effectiveness of multiple combinations of StrepII-tags results in multivalency effects, where the measured binding affinity is determined by a combination of stable interactions, or simply by a probability effect, where low affinity

* Corresponding author.
E-mail address: Jason.davis@chem.ox.ac.uk (J.J. Davis).
[1] LKJS and MB made equal contributions to this work.

0301-4622/$ – see front matter.
doi:10.1016/j.bpc.2010.09.005

interactions occur at high frequency because the binding sites are present in excess. Multivalency [19] effects are commonly observed in nature, where they have evolved to strengthen a diverse array of biochemical interactions. These include polyvalent antibodies binding to an array of antigens on a pathogenic microorganism [20,21], or indeed the attachment of a virus particle coated in binding proteins (e.g. haemagglutinin) to multiple receptors on a host cell [22]. In the laboratory, recent exploitations of polyvalency include the development of a self-assembling peptide polymer, used to improve the attachment rate of nanoparticles to human cells [23], or the development of a dual 6×histidine tag (two 6×His tags in tandem) in order to improve binding of recombinant proteins to Ni^{2+} coated microarray slides [24].

In the present study we have taken further the development of the StrepIII-tag [18] in setting out to investigate the role of binding affinity effects in the performance of the StrepII-tag. We have combined up to 5 StrepII-tags (each of sequence WSHPGFEK) separated by three copies of a regular spacer peptide(GGGS) (Fig. 1A). The tags herein have been appended to the N-terminus of a model protein using the Stefin A Quadruple Mutant (SQM) peptide aptamer scaffold protein [25]. Peptide aptamers are engineered proteins designed to possess the binding specificities and affinities of antibodies, without the requirement for a host animal or complex post-translational modifications for their production. In our system, the SQM protein is expressed in *E. coli* with an amino-terminal tail designed to contain a variety of functional domains that enable subsequent purification or modification of the expressed protein. One of these domains introduces a unique cysteine residue [26] that allows the dimerisation of the SQM proteins under oxidising conditions. We use a subscript "2" to indicate where the SQM protein (and any attached Strep tags) are observed to be largely dimerised (Fig 1C). The StrepII-tag, which for simplicity will from now on only be referred to as the Strep-tag, was introduced either once (strep1-SQM) or up to 5 times (strep5-SQM_2) into the N-terminal tail of the SQM scaffold, with each tag separated by the 12 amino acid $(GGGS)_3$ linker. The enhanced binding efficacies afforded by multiple Strep-tags have been assessed by Western blot, surface plasmon resonance and fluorescent microarrays. In addition to obvious application to chromatographic purification, the basic format of a surface microarray based FRET assay [27] is also described for the peptide aptamer scaffold. The advantages of a FRET assay over a single (target labelled) fluorophore equivalent are the inherent reductions in artefactual noise (such as protein autofluorescence) and the confirmation that the two binding partners are in an intimate sub 10 nm binding sphere [28,29]. Such a platform is also inherently "label free", as has shown to be useful for DNA microarrays [30,31].

2. Materials and methods

2.1. Plasmid and DNA manipulation for bacterial expression

The streptavidin binding tags were cloned into a modified pET30a+vector (Novagen, USA; residue [26]), amino-terminal to the SQM peptide aptamer scaffold (pET30a+-SQM, [25]). The Strep-tag (WSHPGFEK) was generated by annealing the primers

F: 5′-CTGGAGCCACCCGCAGTTCGAAAAG-3′-
R: 5′-CTTTTCGAACTGCGGGTGGCTCCAGGTAC-3′,

followed by ligation into *Kpn*I cut pET30a+-SQM vector. Strep-GGGS (x3)-Strep (Strep2) was generated with flanking *Kpn*I sites by PCR-amplifying the Strep containing pET30a+vector using the following primers:

F: 5′-GATCTGGGTACCTGGAGCCACC-3′
R: 5′-ACGCGGTACCCTTTTCGAACTGCGGGTGGCTCCAAGAACCGC-CACCAGAACCGCCACCAGAACCGCCACCCTTTTCGAACTGCGGGTG-3′.

The PCR product was purified, digested with *Kpn*I and ligated into the *Kpn*I cut pET30a+-SQM vector.

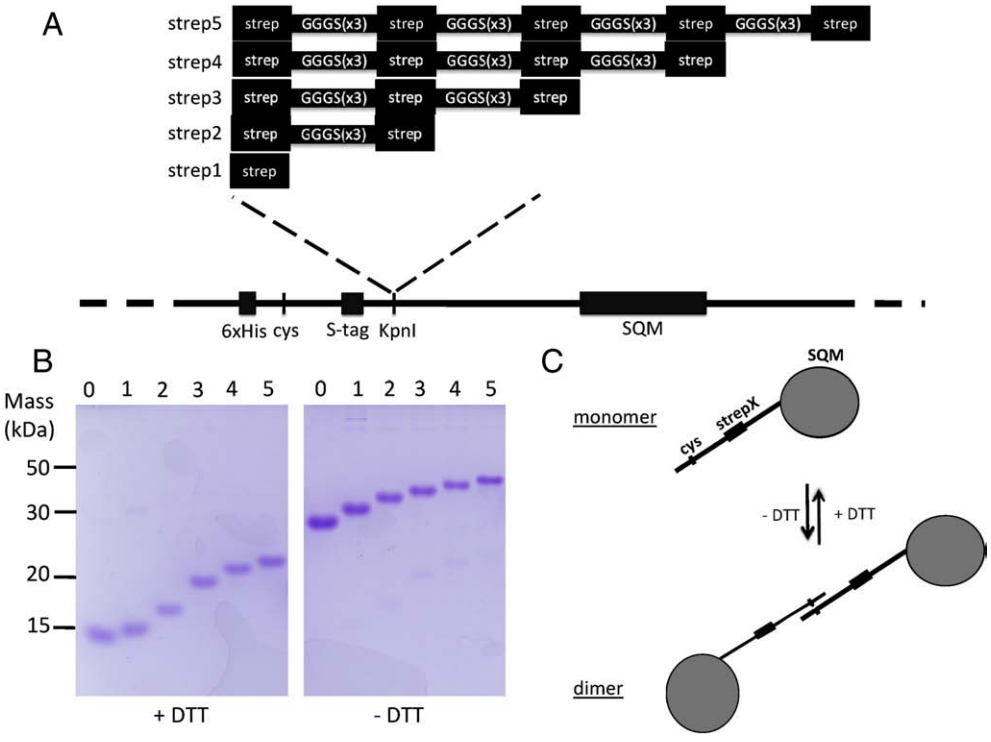


Fig. 1. (A) Multiple cloning site region of the pET30a+-SQM construct used in this study. All streptavidin binding tags were inserted into the *Kpn*I restriction site. Approximate location of the cysteine residue (cys) is indicated. (B) Purified samples of StrepX-SQM separated on SDS-PAGE with and without DTT followed by staining with Coomassie blue. Dimer formation mediated by the cysteine residue is readily observed as a molecular weight shift (increase) in the oxidised state (-DTT). Numbers 1–5 signify number of Strep-tags (X) in the monomer. (C) Schematic representation of dimer formation by StrepX-SQM.

A mutagenesis PCR reaction was carried out in order to remove the upstream *Kpn*I restriction site flanking the Strep-tag insert using the primers

F: 5′-CCAGATCTGAGTACCTGG-3′
R: 5′-CCAGGTACTCAGATCTGG-3′

In order to make 3, 4, and 5 Strep tags, we first annealed the primers:

F: 5′-TCTGGGTACCGGTTCTGGTGGCGGTTCTGGTGGCGGTTCTTG-GAGC-3′
R: 5′-CGTCGGTACCCTTTTCGAACTGCGGGTGGCTCCAAGAACCGCC-3′,

followed by a 'fill-in' reaction using Phusion polymerase (NEB, UK). This yields the element GTGSGGGSGGGS-Strep (i.e. spacer plus tag), flanked by *Kpn*I restriction sites. The amino acid sequence GTG instead of GGG at the beginning of the spacer is necessary as the encoding DNA sequence contains the upstream *Kpn*I restriction site. After the PCR reaction the DNA was digested with *Kpn*I, followed by a ligation reaction with *Kpn*I cut Strep-pET30a + -SQM containing a 10-fold excess of the insert. This yields a Strep-pET30a + -SQM vector with varying additional copies of the Strep-tag (i.e. a total of 3, 4, 5 and more Strep-tags). Finally, to reverse the GTGS part of the spacer (generated by the *Kpn*I restriction site) back to the desired GGGS sequence a PCR mutagenesis reaction was performed on the new constructs using the following oligonucleotides:

F: 5′-GCAGTTCGAAAAGGGTGGCGGTTCTG-3′
R: 5′-CAGAACCGCCACCCTTTTCGAACTGC-3′

This reaction will yield a mix of plasmids, some containing the desired reverting mutation to encode the amino acids GGGS, others having some GTGS and some GGGS. Consequently several colonies have to be screened until the desired plasmid encoding all GGGS is found. This was achieved with Strep3- and Strep4-pET30a + -SQM, but not with Strep5-pET30a + -SQM, which consequently remained with the amino acid sequence GTGS at the beginning of 3 of its spacers. A schematic diagram of the constructs used in this study is shown (Fig 1A).

2.2. Gel electrophoresis, gel staining and Western blotting

StrepX-SQM$_1$ was separated on a 15% polyacrylamide SDS gel followed by either transfer onto nitrocellulose membrane (Pierce, UK) for Western blotting, or incubation in Coomassie blue stain (40% ethanol, 10% isopropanol, 10% acetic acid, 0.25% w/v Coomassie brilliant blue) for 1 h at room temperature, followed by incubation in destain (the staining solution without Coomassie blue) overnight at 4 °C. For Western blotting the membrane was blocked in 3% fat-free powdered milk in TBST (Tris buffered saline pH 8.5 with 0.05% Tween-20) at 4 °C overnight and then briefly rinsed in TBST. The membrane was then incubated with 1 μg/ml streptavidin-HRP (Horseradish peroxidase; Invitrogen, USA) for 1 h at room temperature and washed 3 times for 10 min each in TBST. A chemi-luminescent HRP substrate (Perbio, UK) was then applied, excess rinsed off in TBS and the membrane air-dried and exposed to photographic film. For the loading controls the membrane was re-probed using an anti-S-tag antibody (1:5000; Novagen, USA), which recognises a unique S-tag encoded by the pET30 vector upstream of the Strep tags. A secondary anti-mouse antibody (Jackson Immunolabs) was used at 1:12,000 in TBST. Western blots were quantified using the Quantity One Software (Biorad) and data processed using Excel for Macintosh (Microsoft).

2.3. Surface plasmon resonance (SPR)

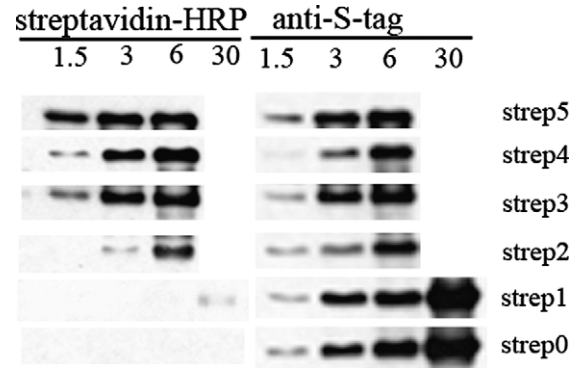
2.3.1. Surface activation

SPR experiments (Autolab ESPRIT, Ecochemie) were performed on gold surfaces initially cleaned in hot piranha solution (warning, piranha solution is a strong oxidant and reacts violently with many organic materials) and then subsequently functionalised overnight with a monolayer of COOH-OEG thiol ($HS(CH_2)_{10}(OCH_2CH_2)_3OCH_2COOH$, Prochimia Surfaces), 625 μM in ethanol. SPR chips were then equilibrated to a stable time dependant signal under 10 mM phosphate buffer, pH 7.4 (running buffer). Terminal acid groups were activated with 100 μl of a 1:1 0.2 M EDC and 0.05 M NHS solution for 15 min. Depending on the required surfaces, the following final immobilisation protocols were used:

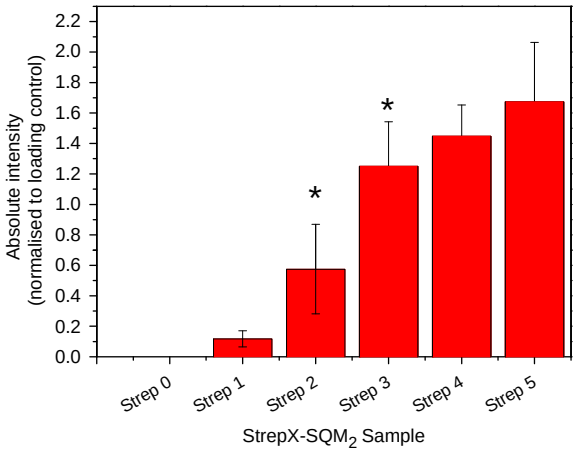
2.3.2. Streptavidin surface

A SAM (Self-Assembling Monolayer) consisting of a biotin termination coupled to an underlying gold surface via three repeating ethylene glycol groups was formed by coupling the commercially available EZ-Link Biotin-PEO-LC-Amine, 0.01 M (Pierce) in 0.1 M Bicarbonate buffer pH 8 with the active NHS ester for 20 min. Subsequently, streptavidin was injected at a concentration of 10 μM in running buffer until the absorption had reached a plateau and the SPR sensorgram indicated a large change in surface mass, within 5 mDeg.

A



B



C

No. Strep-tags	1	2	3	4	5
StrepX/Strep1	1	4.8	12.1	13.7	15.6

Fig. 2. (A) Western blot, probed using streptavidin-HRP and anti-S-tag antibody loading control. Amounts of protein loaded: 1.5, 3, 6, 30 pmoles. The highest amount, 30 pmoles was only used for 0 and 1 Strep-tags, as the signal was too strong at this level with multiple tags. (B) Densitometry analysis using Strep1-SQM as the baseline. Affinity of Strep2-SQM compared to Strep1-SQM is significantly improved (Student's *t*-test $p<0.05$), as well as Strep3-SQM compared to Strep2-SQM ($p<0.05$). Although there is further improvement with 4 and 5 Strep-tags, this is not statistically significant ($p>0.05$). *denotes a statistically significant increase in affinity of StrepX compared to Strep(X-1). (C) Fold increase of the affinity of streptavidin for StrepX-SQM normalised to Strep1-SQM.

2.3.3. StrepX-SQM$_2$ surface

The relevant StrepX-SQM$_2$ protein was injected at a concentration of 10 μM in running buffer until a stable baseline was reached. The complementary target group, either streptavidin or the StrepX-SQM$_2$ was then injected at variable concentrations and absorption/desorption profiles were measured. Surfaces were regenerated using 0.1 M glycine, pH 2.4, for 2 min. Surface coverages in ng mm^{-2} were calculated from the relationship 1mDeg = 122 ng mm^{-2} [32]. Kinetic parameters were calculated with Kinetic Evaluation Software (Ecochemie).

2.4. Microarray and FRET assays

All proteins were labelled using standard protocols supplied by the dye manufacturer, ATTO-TEC Gmbh. Concentrations were checked using a Nanodrop spectrophotometer (Thermo Scientific). Microarray assays were performed by spotting (BioOdessey, Biorad Corp) the StrepX-SQM$_2$ samples on amine coated glass slides (Genetix) using 100 μm capillary pins. Samples were printed at concentrations of 10 μM in print buffer which comprised of PBST (phosphate buffered saline + 3% Tween 20) and 10% glycerol. Array features were printed in repeats of four and the entire array was repeated three times across the slide. Spotted volumes were allowed to incubate on the slide for 60 min prior to a blocking step in 1% BSA in PBST for 60 min. Atto 647 N-NHS ester labelled streptavidin (1:1 labelling ratio) was then incubated at a concentration of 7 nM in 1% BSA in PBST for 40 min using 50 μl volume Lifterslips (Thermo Scientific). Slides were washed in PBST twice for 10 min then twice in deionised water for 2 min. The slides were dried under a stream of nitrogen and scanned at a resolution of 5 μm with 543 nm lasers under the Cy3 detection protocol or 633 nm lasers under the Cy5 detection protocol (ScanArray Express, Perkin Elmer). Spot intensities were analyzed with the integrated ScanArray software. FRET assays were performed under identical conditions to microarray binding assays, *vide supra*, though now both capture and target molecule are labelled, with donor (Atto 550) and acceptor (Atto 647 N) fluorophores, respectively. For these assays, array features were printed in repeats of three and at two concentrations (5 μM and 10 μM).

3. Results

3.1. Strep-tag/streptavidin interaction in a Western blot format

Initially the relative changes in binding levels between one and multiple Strep-tags for streptavidin were assessed in a Western blot format. Heat denaturation and SDS stabilisation of the StrepX-SQM proteins in the presence of DTT produce linear, monomeric forms of the proteins containing 0–5 Strep-tags that are separated electrophoretically and transferred onto nitrocellulose. Therefore in this assay, the interactions occur between reduced monomeric StrepX-SQM immobilised on a membrane and soluble HRP-conjugated streptavidin, although some re-folding can occur during the transfer step. HRP metabolises a chemi-luminescent substrate and relative binding levels are measured via signal intensity on photographic film (Fig. 2A). The use of 2 Strep-tags increases binding to streptavidin by approximately 5-fold compared to a single Strep tag according to this method. A further significant increase in affinity is observed when 3 tags are used (12-fold compared to a single Strep-tag and 2.5 fold

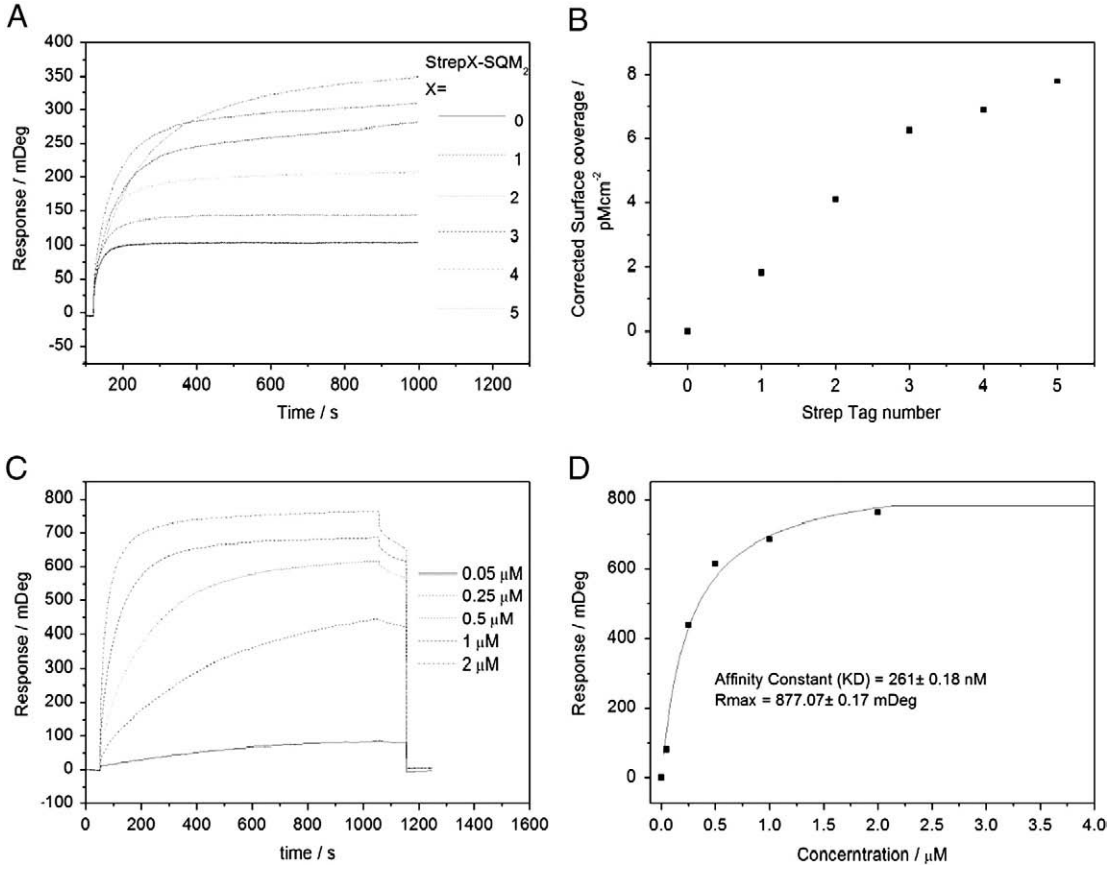


Fig. 3. (A) Surface plasmon resonance response of the target StrepX-SQM$_2$ (X = 0–5) injected at equal concentrations over a streptavidin-biotin-SAM. (B) Plot of corrected molar surface coverage of the target StrepX-SQM$_2$ (X = 0–5) obtained from A in comparing molar surface coverages. A trend in increasing interaction is clearly seen from Strep 1 to 5. All values have been corrected for molecular mass differences, and nonspecific (Strep0-SQM$_2$) binding. (C) Variable concentrations of the target streptavidin injected over a Strep5-SQM$_2$-OEG-SAM immobilised surface. (D) Equilibrium analysis over the variable concentrations of the target streptavidin obtained from C, showing the calculated K_d = 260 ± 0.18 nM and R_{max} = 877.07 ± 0.17 mDeg.

compared to Strep2). Further small improvements are observed with Strep4 and Strep5 (Fig. 2B, $p>0.05$; Student's t-test used).

3.2. Surface plasmon resonance measurements

A more quantitative analysis of relative binding levels of the StrepX-SQM_2 systems in their native state (oxidised, dimeric, with no denaturation) was sought through surface plasmon resonance analysis. The series of StrepX-SQM_2 samples were flowed at 1 μM over a pre-formed streptavidin-Biotin-SAM (Fig. 3A). The maximum equilibrium coverage taken from sensorgrams was plotted after subtraction of any non-specific binding contributions measured using Strep0-SQM_2 and the binding levels were displayed as pmol cm^{-2}. Consistent with the Western blot data, binding is observed to increase with additional Strep-tags from 1 to 5, though the rate of increase is observed to progressively fall. The percentage increase in the number of moles of streptavidin immobilised between Strep1-SQM_2 and Strep2-SQM_2 was 124% whereas between Strep4-SQM_2 and Strep5-SQM_2 the increase was much smaller at 13%. This observation is consistent with the onset of steric saturation as the surface density of Strep tags increases (Fig. 3B) [33]. Analyses repeated under reducing conditions were equivalent (data not shown).

These quantitative trends were observed to be fully consistent on inverting the interfacial configuration, that is on exposing immobilised StrepX-SQM_2 on a COOH-OEG terminated SAM to solution phase streptavidin (0.05 to 2 μM). The SPR response for the surface coverage of Strep1-SQM_2 experiment was 33.59 mDeg, and 377.14 mDeg for Strep5-SQM_2. Association and disassociation curves gave clear visual indications that a surface binding equilibrium was occurring. Equilibrium dissociation constants, K_D, obtained for Strep1-SQM_2 and Strep5-SQM_2 (Fig. 3C) were 1.49 ± 0.01 μM and 260 ± 0.18 nM, respectively (Fig. 3D). The associated R_{max} values for the interaction were 186.61 ± 0.70 mDeg for the Strep1-SQM_2 surface and 877.07 ± 0.17 mDeg for that presented by Strep5-SQM_2. When comparing the R_{max} for the initial StrepX-SQM_2 surface and the R_{max} for the saturated binding data it becomes apparent that, although capture ratios scale with the number of Strep-tags, they remain below 1:1 for both conditions. Thus, the capture ratio for Strep1-SQM_2 = 0.18:1 while that for Strep5-SQM_2 = 0.43:1. This is consistent with both expected steric limitations and observed electrophoretic mobility shift assays carried out in solution where only 1:1 complexation was observed (data not shown). These analyses, of course, do not shed light on how many tags per SQM molecule are involved in the binding to any one streptavidin but prior work, at typical surface presentations of biotin, have confirmed that streptavidin binds predominantly through two of its sites [34–36], a configuration demonstrably useful in building up surface structures through accessing the initially unused streptavidin sites pointing away from the surface.

3.3. Affinity measurements in a protein microarray format

In order to further study the binding behaviour of the multiple Strep-tags and pave the way for a surface based FRET sensor, the binding efficiencies of the Strep-tag systems were investigated in a microarray format. StrepX-SQM_2 samples were spotted from 10 μM solutions onto amine modified glass slides in repeated arrays to compensate for potential slide inhomogeneities. An excessive concentration was used to ensure that all the active surface binding sites were saturated with the StrepX-SQM_2 protein. Incubation with fluorescently labelled streptavidin and subsequent fluorescence intensity analysis confirmed a broadly linear increase in binding affinity with tag number (Fig. 4B), tailing off slightly at higher tag numbers. Interestingly, in this format, the strep0-SQM_2 gave a zero background response, indicating that the microarray surface was entirely specific.

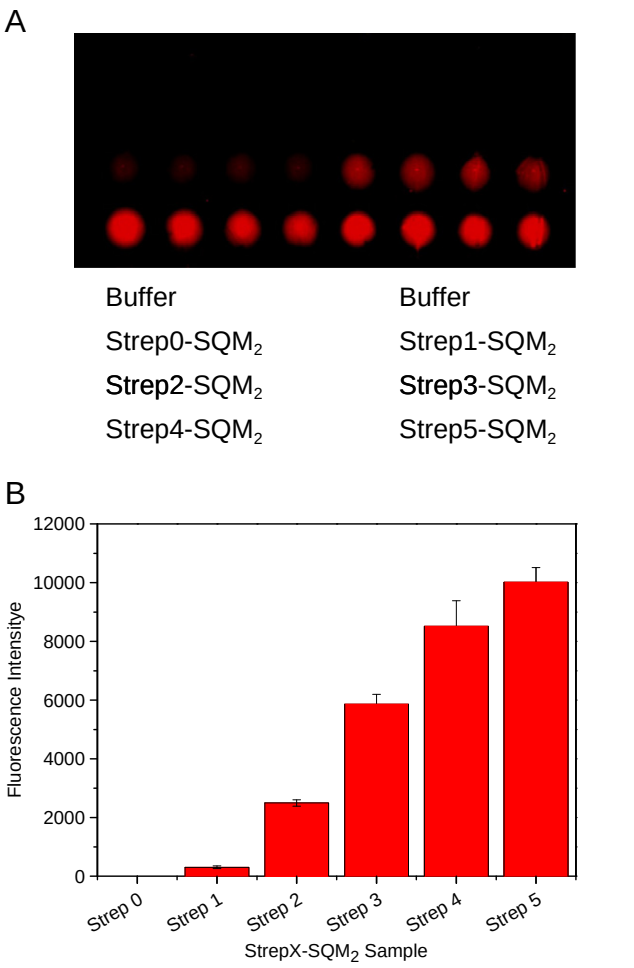


Fig. 4. (A) Microarray spotting of StrepX-SQM_2 sample capture of Atto-647 N labelled streptavidin. The microarray consists of seven spotted samples: a buffer control (spotted twice) and six StrepX-SQM_2 samples ($X=0$–5). Each line contains two samples each, spotted in repeats of four. There are four lines in total, the first two lines cannot be seen due to no signal (buffer control, Strep0-SQM_2) or low signal (Strep1-SQM_2). (B) Relative average intensities with standard deviations over four samples of captured streptavidin emission.

3.4. Streptavidin FRET assay

A microarray streptavidin FRET assay was developed using the microarray surface described previously. In this configuration, the capture and target biomolecules are labelled with complementary (FRET pairing) dye molecules. In the proximity afforded by binding, donor excitation-dependant fluorescence is detected at the acceptor emission wavelength. In a comparative analysis of binding interactions between streptavidin and Strep0-SQM_2 and Strep5-SQM_2 previously discussed, one would expect that "no FRET" and "strong FRET" behaviours will be observed, respectively (if nonbonded dye-dye interactions are negligible, Fig. 5A). The dye pair choice was optimised so as to both maximise spectral overlap at convenient frequencies and generate a Förster radius large enough to accommodate the dimensions of the proteins used herein (Atto550/Atto647N, $R_o = 6.5$ nm, Atto-tec.com). In all experiments, dye-to-protein labelling ratios were maintained at 1:1, so as to remove potential contributions from labelling variance to any observed variations in emission.

The first microarray lines (Fig. 5B) show Strep0-SQM_2-Atto550 spots used as donor only leakage controls, to assess whether the donor contributes fluorescence at the measured emission wavelength of the acceptor. As noted earlier, streptavidin-Atto647N binding is not expected here. The absence of acceptor dye is confirmed by the strong

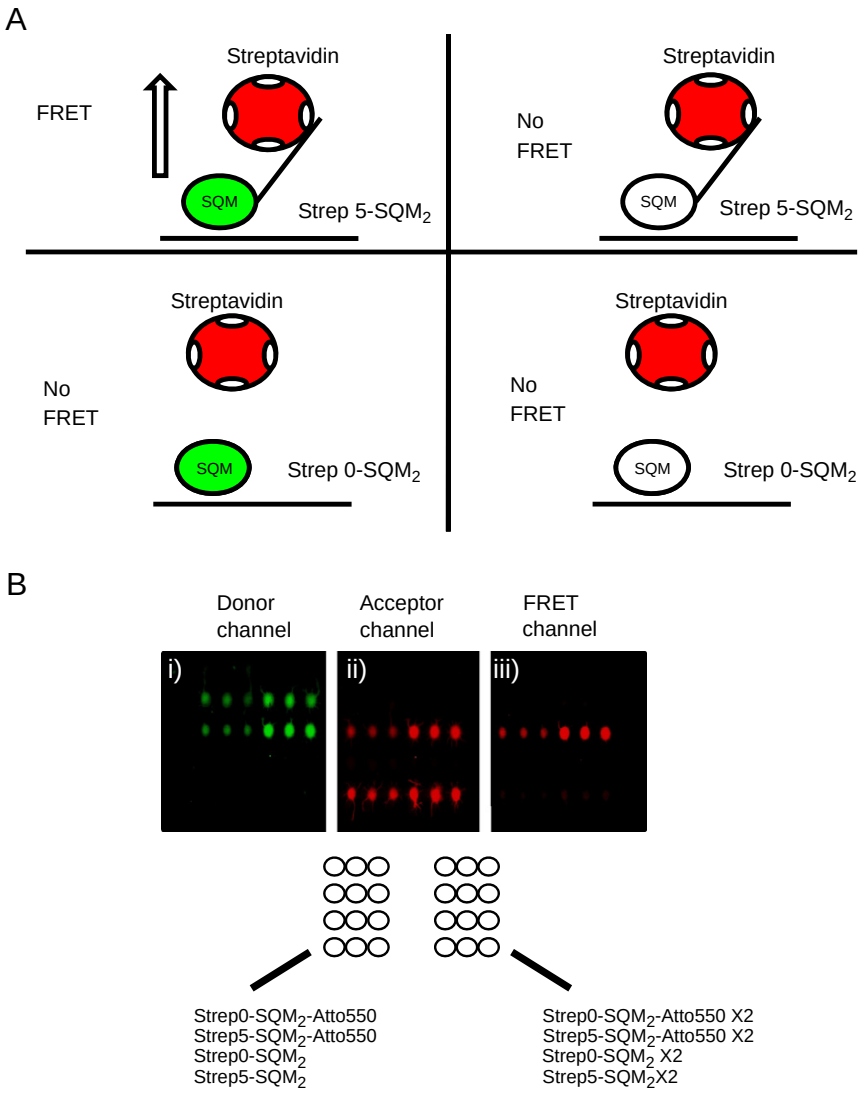


Fig. 5. (A) Experiment and control scenarios which define the specificity of the FRET assay. FRET will only occur when both StrepX-SQM_2 and streptavidin are labelled with donor (green) and acceptor (red) dyes, and when the StrepX-SQM_2 is present to bring the donor and acceptor units together in proximity. StrepX-SQM_2 proteins are in the dimeric state. (B) Microarray FRET assay measured in different emission channels. (i) Donor channel measuring Atto 550 fluorescence, which corresponds to the spotted StrepX-SQM_2 samples. (ii) Acceptor channel measuring Atto 647 N fluorescence, which corresponds to the bound streptavidin-Atto 647 N molecules. iii) FRET channel measuring Atto 647 N fluorescence upon Atto 550 excitation. The sample layout is described beneath different lines correspond to different samples spotted in repeats of three times at two concentrations.

donor signal present, confirming no FRET and no crosstalk. The second line of the array confirms the presence of streptavidin bound acceptor (streptavidin-Atto647N) bound to Strep5-SQM_2-Atto550, as excitation of the SQM bound donor results in emission from Atto647. The bottom microarray line is a Strep5-SQM_2/streptavidin-Atto647N pairing. Here, only the Atto647N dye is present which acts as the acceptor leakage control; again no signal is seen in either the donor or the FRET channel. These results confirm that the dyes are acting orthogonally to each other and the assay is not detecting crosstalk. It also confirms that the FRET signal is due to binding interactions between the two labelled proteins (see discussion for further details).

4. Discussion

The first experiments on the multivalent effects of multiple Strep-tag units enhancing streptavidin binding affinity came from Hengsakul and Cass, who showed that, when coupling the original Strep-tag [10,11] to the C-terminus of dimerising alkaline phosphatase, a dissociation constant of 0.5 μM was measured [37]. Though K_d values obtained were lower than reported with a single "original" Strep-tag (72 μm) [10], an observation assigned to the presence of two tags in the dimeric phosphatase, direct comparisons cannot be made as experimental conditions in the two studies were distinct. Though the StrepIII-tag, comprised of two StrepII-tags, is reported to engender improved streptavidin binding affinity, reported results are wholly qualitative [18]. We have demonstrated herein that combining 2 StrepII-tags separated by a 12 amino acid linker and fused to an empty peptide aptamer scaffold protein does improve streptavidin binding, by approximately five fold, compared to that of the single tag. Furthermore, the addition of a third, fourth and fifth StrepII-tag leads to further sequential binding increases, though the advantage of this progressively diminishes. These observations have been reproducibly confirmed under carefully controlled conditions across three assay formats. They have also been confirmed in either "inversion" of the surface binding assay (the SQM fusion protein on the surface capturing solution phase streptavidin or *vice versa*).

The physical basis of the increase in target pair binding can be explained in terms of multivalence [19,21]. The increase in tag number results, specifically, in both a higher local concentration of binding points, and a greater receptive surface area at which the large

52.8 kDa streptavidin molecules can be accommodated. The added advantage of streptavidin having four binding pockets increases the potential network of interactions that may occur, though it is likely that only 1–2 binding pockets per streptavidin are bound to any one SQM receptor at any single point in time [34–36]. Though the addition of each strep tag leads to a probabilistic increase in binding affinity (by virtue of the increased surface concentration of binding sites presented to the target), and associated K_d values fall into the nanomolar regime, this stepwise increase becomes progressively smaller through the Strep1–Strep5 series, an observation we deem to be reflective of the steric constraints inherent across the relatively constrained aptamer surface.

The discrepancy observed herein between the Strep1-SQM_2 and original calculated StrepII-tag K_d values may be reflective of interactions involving the scaffold protein. Mammen et al. [38], have presented the relationship between poly and monovalent binding constants (K_{poly} and K_{mono}, respectively) as $K_{poly} = (K_{mono})\alpha^N$, where α quantifies the "degree of cooperativity" and N is the potential number of individual interactions. Applied here to the data presented in Section 3.2 (Strep1-SQM_2 vs Strep5-SQM_2), α is resolved as being very close to 1.0, consistent with these observations being probabilistic rather than cooperative. All experimental formats utilised indicate a progressive reduction in the advantage conferred by adding more tags across the StrepX-SQM_2 series. This observation is entirely consistent with a progressive steric saturation of binding sites presented by the StrepX-SQM_2 protein (the steric footprint of streptavidin covering large portions of the active surface), preventing any further streptavidin molecules from binding and is thus related to similar observations made on increasing biotin surface density at planar monolayer surfaces [33]. Such behaviour has been quantitatively defined herein by SPR, where the maximum molar ratio of streptavidin that may bind to the Strep5-SQM_2 is observed to be 0.43:1. For the Strep1-SQM_2 surface the maximum streptavidin binding is 0.18:1.

Finally, the strong StrepX-SQM_2 streptavidin association can be utilised in establishing robust interfacial FRET interactions. Though numerous examples of the applications of FRET based bioassays exist [39–43] few have translated to solid surfaces [27,44,45] due to the complexity of experiment design. In addition, the majority of interfacial FRET based assays have relied on the use of lifetime imaging instrumentation [27,45], which although convenient and sensitive is also highly specialised. Examples which rely on photoluminescence intensity have utilised quantum dot/gold nanoparticle pairs, which due to their size may interfere with complex protein–protein interactions [44]. The work described herein presents a self consistent methodology for configuring surface based assays between two protein pairs that can be performed on a conventional microarray scanner, with small molecule modification of the capture and target proteins. The data also confirm that the two binding partners are in an intimate sub 10 nm binding sphere. These factors contribute favourably to the potential applications of the assay towards the capture of more complex recombinantly expressed biomolecules with appended Strep-tag tails or towards the development of more sophisticated and sensitive assays based with potential label-free applications [30,31].

5. Conclusion

A progressive improvement in binding affinity between the StrepII-tag and streptavidin can be engineered in association with a StrepX-SQM_2 scaffold. On increasing the number of Strep-tags to 5, nanomolar K_d's are afforded and can be replicated across several interfacial assay formats. A surface based microarray based on FRET between the capture molecule and the target has also been demonstrated, highlighting this new highly sensitive, selective assay platform. Future work should explore the use of different length spacers between the tags, which might further improve affinity. Applications of the FRET assay can be progressed with the introduction of FRET assays that detect more complex and physiologically relevant targets. In addition even higher sensitivity and limits of detection are obtainable based on lifetime dependant analysis [27].

Acknowledgements

This work was funded through BBSRC Grant BB/F011296/1 to JJD and PKF. LKJS and PKF gratefully acknowledge the support of AstraZeneca.

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