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Fluorescent Labelling of Protein Analytes

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J. E. Noble and E. Cerasoli

Quality of Life Division

ABSTRACT

This report compares fluorescent labelling methods to tag an antibody of an important cardiac biomarker. We have defined the characteristics of an ideal fluorescent labelling tag as one that retains the biological activity, compared to the unlabelled protein and generates a bright fluorescent tag that can be detected at low concentrations. Depending on the end application that the fluorescently labelled protein is required for will determine the number of positional requirement of the label. The four labelling chemistries analysed included amine, thiol and oligosaccharide-directed and biotinylation, all with the Cy3 dye. These labelling chemistries have been assessed in term of binding kinetics, relative quantum yield and labelling ratio, with discussion based on suitability for specific applications.

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Approved on behalf of the Managing Director, NPL
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Contents

Abbreviations

D/P	dye/protein
DTT	Dithiothreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
Fc	Flow cell
FIU	Fluorescence Intensity Units
Gal	Galactose
GlcNac	N-acetylglucosamine
HABA	4-Hyroxyazobenzene-2-carboxylic acid
Man	Mannose
MAb	Monoclonal antibody
NHS	N-hydroxysuccinimide ester
NSB	Non-specific binding
pAb	Polyclonal antibody
pNPP	p-nitrophenyl phosphate
R6G	Rhodamine 6G
RU	Response unit
Sla	Sialic acid
TCEP	tris-(2-carboxyethyl)phosphine
TFP	Tetrafluorophenyl

Fluorescent Labelling of Protein Analytes

1 Introduction

1.1 Project Outline - An Ideal Fluorescent Tag

Fluorescent tagging of protein molecules to aid quantitation and determine the biological activity of important biomarkers is a ubiquitous technique among many assay platforms. Platforms employing fluorescently tagged proteins include; microarrays, immunoassays, flow cytometry and FRET and polarisation screening assays. This project assessed the suitability of 4 fluorescent labelling strategies to label an antibody of an important cardiac biomarker. The suitability of each labelling strategy was compared by looking at the following parameters:

1. *Retention of biological activity of the labelled antibody*: For any labelling reaction it is important that the biological activity (for example, that an antibody still binds its epitope with good affinity) is retained so the labelled protein will function in an analogous manner to the unlabelled protein. This is especially important for competitive immunoassays where the labelled and unlabelled analytes compete for the same binding site. The biological activity of the antibody will be assessed by measuring the binding kinetics to myoglobin, with the equilibrium dissociation constant (K_D) used to directly compare labelling strategies. The Biacore system uses surface plasmon resonance to measure binding reactions in proximity to a gold surface and was therefore used to assess the binding kinetics of the labelled antibodies.
2. *Fluorescent Label Brightness*: How well a fluorescently labelled protein can be detected depends on the number factors including:
    a. Dye labelling ratio – the number of dye molecules per antibody.
    b. The environment of the fluorophore, for example proximity to other fluorophores, or labelling chemistry can affect the brightness of the fluorophore. The brightness of the fluorescent label can be determined by measuring the relative quantum yield (Φ). Analysis of the fluorescent spectra from the antibody dye conjugates also provides details about the environment of the dye, for example aggregation.

   The brightness of the dye contributes to the lowest amount of analyte that can be detected by the chosen fluorescent analytical technique. For many techniques the limit of detection and the dynamic range of the assay will be determined by the brightness of the labelled probe, especially where the analyte is present at limiting amounts and in a complex biological matrix.

1.2 Using Fluorescence for Protein Tagging

Measurements of protein-protein/antibody interactions are important for many areas of drug discovery including compound screening, target discovery, diagnostics and target validation. For applications that require high sensitivity, traditionally radiotracers were used for labelling, however due to cost and safety concerns these have superseded by fluorescence as the preferred read-out mechanism.

Unlike the labelling of nucleic acids, protein labelling is more complicated due to the heterogeneous nature of the reactive groups in the target, in terms of number and

position. Various labelling chemistries have been employed to label specific functional groups (typically specific amino acid bases) within the protein, however unless the protein has been specifically engineered this can lead to a heterogeneous mixture of labelled products. Depending on the end application the approach taken can be to specifically label a protein at a defined site (fluorescent polarization studies), or if sensitivity is important label at multiple random sites (protein microarrays).

1.3 Model System used to Assess Fluorescent Tags

Rather then compare different fluorophores for suitability as protein tags we sought to compare the chemical labelling methods, as the spectral properties of fluorophores tend to be well characterised by the manufacturer. The cyanine series of dyes display many properties making them suitable for biomolecule labelling including: high water solubility, pH insensitivity, good photostability and low dye aggregation [1], compared to other commercially available dyes. The dye Cy3 is a cyanine-based fluorophore that is extensively used in microarray formats, including antibody and functional protein arrays. For multiplexed analysis Cy3 is often used with Cy5, as they are spectrally distinct when excited using 533 and 633 nm laser lines that are common in many fluorescent scanning and flow cytometry systems.

The antigen-antibody pair selected contains human myoglobin represents an important cardiac biomarker for acute myocardial infarction and a specific diagnostic antibody. A monoclonal antibody was chosen so to limit the number of different sequences and possible labelled variants produced when fluorescently labelling the antibody. Assays for this interaction pair have been fully validated in the 'Measurements for Emerging Technologies' Program[1]. We have sought to compare 4-different fluorescent labelling strategies, specifically looking at the retention of protein binding activity and overall brightness. This project is analogous to another MfB project where the activity of an antibody using various immobilisation chemistries/surfaces was assessed in a microarray format with the same human myoglobin antibody/antigen pair.

1.4 Labelling Chemistries

1.4.1 Amine Directed Labelling Chemistry

Various chemical reactive groups can be used to selectively label secondary amines and have been reviewed [2], those analysed for Cy3 dyes include isothiocyanates [3] and succinimidyl esters [1, 4]. Amine directed probes tend to produce a hetereogeneous mixture of labelled proteins as they target lysines residues and the N-terminus. Due to the relative high abundance (average 7 % of residues) of lysine in proteins and their predominance to be surface exposed, labelling often results in a heterogeneous mixture of products. Depending on the nature of the N-terminal amino acid the N-terminus can be targeted by lowering the labelling reaction buffer pH, which can enhance the probability that the N-terminus becomes labelled. Site specific labelling of the N-terminus can be beneficial for various applications including; polarization or

[1] Medical Technologies Theme: PROJECT 1.5: Sensor Techniques for Drug Discovery and Diagnostics (including in-vitro Diagnostics)
http://www.metprog.org.uk/medical/medical_projects/point_of_care.html

FRET studies were a single fluorophore is required in a defined position producing a homogeneous pool of labelled proteins, when labelling proteins that contain lysine residues critical for biological function, or where no lysine are present/reactive in the molecule. Reaction chemistries to specifically label secondary amines include succinimidyl esters, isothiocyanates, sulfonyl chlorides and tetrafluorophenyl (TFP) esters. The selection of which labelling chemistry to use depends on the required pH of the reaction, or to reduce the chance of side reaction with other amino acids within the protein (tyrosine, histidine and cysteine). For this study we choose to use Cy3 with mono-succinimidyl ester-labelling chemistry as it is commercially available and is reported to generate higher fluorescent intensities when compared to isothiocyanate coupling chemistries [4]. The basic reaction for amine modification is shown in Figure 1.

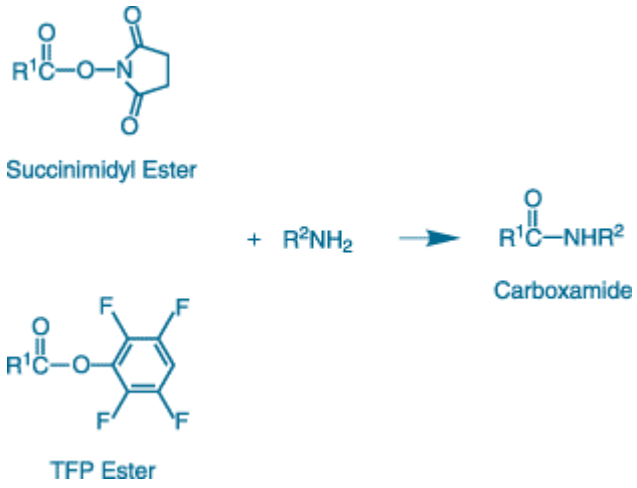

Figure 1: Amine Labelling Chemistries

1.4.2 Thiol Directed Labelling Chemistry

In a similar manner to amine labelling, there are many thiol-directed labelling chemistries that can be used to label proteins [5]. Free thiols (those in the reduced form, not oxidised or in a disulphide bridge) typically in the form of cysteine residues can provided a more targeted labelling of proteins, due to the relative lower abundance compared to lysine residues (relative abundance 2.8 %). This abundance is reduced when cysteines form disulphide bonds in non-reducing (oxidising) environments, or are not surface exposed. If present, cysteine residues can be used for labelling or are often genetically engineered into a protein (Ser→Cys) to facilitate site-specific labelling. Reversible oxidation of cysteine residues can be overcome by using reducing agents, for example dithiothreitol (DTT), or tris-(2-carboxyethyl)phosphine (TCEP), however these often require removal before labelling as they can compete with the thiol labelling reagents. The reaction chemistries used to specifically label thiols include; iodoacetamides, maleimides (Figure 2), alkyl halides and arylating agents. For this project we chose to use maleimide-labelling chemistry over iodoacetamides and other agents due to the stability of the active agent in aqueous solutions and the specificity of maleimide labelling for cysteine residues, compared to the off-labelling of methionine, histidine and tyrosine with iodoacetamides [5].

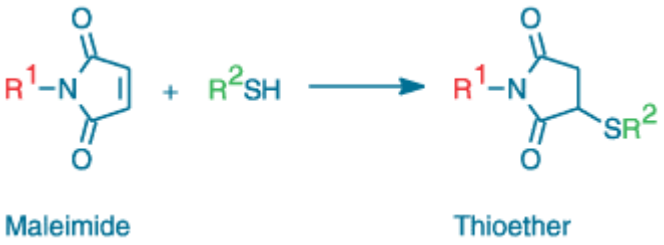

Figure 2: Thiol Labelling Chemistry

1.4.3 Oligosaccharide Directed Labelling Chemistry

In contrast to amine and thiol directed labelling, the labelling of oligosaccharides is typically limited to hydrazine and hydroxylamine derivatives [6]. We have chosen hydazide labelling, as Cy3 derivatives are commercially available. Many proteins including antibodies are glycosylated, this post-translational modification is thought to be important in regulating the structure/stability of the parent protein and key molecular interactions with other biological molecules. The carbohydrate itself contains a unique chemistry, when compared with the parent protein that allows selective labelling of the terminal carbohydrates through the formation of a ketone. Oxidation of the glycoprotein is performed using sodium periodate, which is subsequently removed from the reaction mixture, before the addition of the hydrazide bearing dye molecule (Figure 3). The reactive terminal carbohydrates will vary depending on the protein, species and expression system. For human antibodies the terminal carbohydrates will include: galactose (Gal), sialic acid (Sla), N-acetylglucosamine (Glc Nac), and mannose (Man).

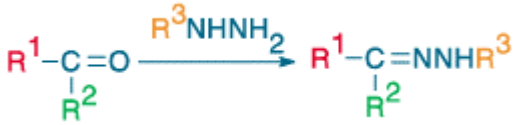

Figure 3: Hydrazide Labelling Chemistry

1.4.4 Biotin-Streptavidin Interaction

Due to the strength of the non-covalent biotin-streptavidin interaction ($K_D \sim 10^{-15}$ M), these molecules have been used extensively in immunoassay technology [7]. Direct labelling of the analyte with biotin, then subsequent incubation with fluorescently labelled streptavidin produces a bright and stable conjugate. This indirect way of labelling an analyte can increase the sensitivity as each streptavidin can be labelled with multiple fluorophores. This technique can be used to label microarrays post-hybridisation, also minimising exposure of the fluorescent label until the last stage. For this project we used standard succinimidyl chemistry to attach biotin groups, separated from the protein by a 14-atom spacer (Figure 4).

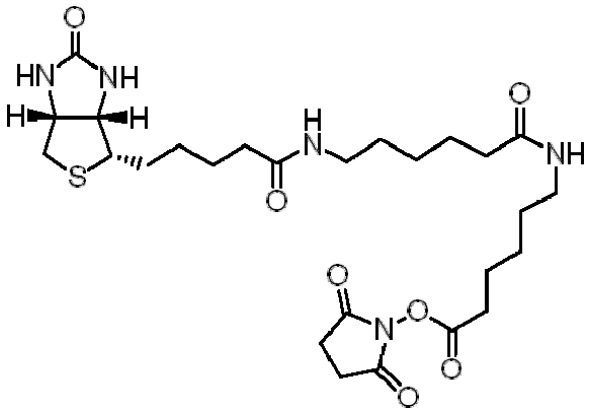

Figure 4: Biotin-Succinimidyl Ester

2 Materials and Methods

2.1 Materials[2]

The antigen-antibody model system chosen was fully validated in our laboratory. The antigen 'human myoglobin' Cat#8M50 (source human heart tissue, with a purity of more than 98 %) and monoclonal mouse anti-human cardiac myoglobin, clone 4E2 (Catalogue #4M23), were both from HyTest (Turku, Finland). The Cy3 dye, with three unique reaction chemistries, or conjugated to streptavidin were obtained from GE Healthcare (Little Chalfont, UK). Cy3 antibody labelling kit (PA33001) consisted of Cy3-maleimide (mono-reactive), Cy3-hydrazide and Cy3-N-hydroxysuccinimide ester (mono-reactive). Cy3-Streptavidin (PA34001) from *Streptomyces Avinidii* was conjugated with an average of 2.7 fluorophores per molecule of streptavidin (supplemented with 1% (w/v) BSA). Biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester 'Biotin-SE' (B3295) and the 4-Hyroxyazobenzene-2-carboxylic acid (HABA) reagent to detect biotinylation were purchased from Sigma (Poole, UK). Unless stated all buffer reagents were purchased from Sigma at analytical grade at the highest available purity.

Binding measurements were performed on a Biacore T100 and all reagents used were purchased from Biacore (Uppsala, Finland) unless stated. Binding isotherms from the Biacore were analysed using Biacore T100 Evaluation Software V1.1. Non-competitive sandwich ELISA's were performed using MAb Clone 4E2 monoclonal mouse anti-human cardiac myoglobin, clone 7C3 as a capture antibody and biotinylated mouse anti-human myoglobin, clone 4M23b as a detection antibody, both from HyTest. High binding immunoassay plates and Superblock T20® blocking buffer were purchased from Fisher (Leicester, UK) and both the amplification reagent 'streptavidin-alkaline phosphatase' and detection reagent p-nitrophenyl phosphate (pNPP) were purchased from Sigma.

[2] Certain commercial materials, instruments, and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply recommendation or endorsement by the National Physical Laboratory, nor does it imply that the material, instrument or equipment identified is necessarily the best available for the purpose.

Absorbance measurements were performed using a Lambda 850 spectrophotometer, using UV-WiNLab software both from Perkin Elmer (MA, USA). An internal calibration cycle was performed before each analysis and where possible all measurements were performed using matched quartz cuvettes from Starna Scientific Ltd (Hainault, UK), one containing the solvent blank, typically PBS.

Fluorescence measurements were performed on an LS55 spectrofluorimeter using FL WinLab Software both from PerkinElmer, whose relative spectral responsivity had been calibrated using BAM fluorescent standards (a kind gift from Dr Resch-Genger, BAM)[8]. The fluorescent standards used were CRM's BAM-F001, BAM-F002, BAM-F003, BAM-F004 and BAM-F005. Correction curves were prepared using the program LINKCORR from BAM (a kind gift from Dr Resch-Genger). To validate the correction curve the fluorescent standard quinine sulphate SRM936a (from NIST MA, US) was measured to confirm correct spectral response. All measurements were performed at 25ºC using a cuvette water-bath adaptation to maintain a constant temperature (± 0.1ºC).

2.2 Methods

2.2.1 Protein Labelling

Protein labelling reactions were carried out as detailed in the suppliers instructions, unless where stated. A brief overview of the labelling methods are given below:

2.2.1.1 Amine Directed Labelling

The lysine residues of the MAb were targeted using a labelling buffer of pH 8.5, so to bias the labelling of lysine as opposed to the N-terminus. Briefly MAb was diluted to 1 mg/ml in 'amine labelling buffer' (100 mM sodium bicarbonate pH 8.5). Cy3-N-hydroxysuccinimide ester (NHS) was dissolved into anhydrous DMSO and stored at -20°C. Aliquots were defrosted rapidly and the dye added drop-wise to a stirred MAb solution, excess dye was discarded. The reaction was allowed to proceed for 30 minutes at room temperature; the reaction was then quenched by the addition of 100 µl of 1.5 M n-hydroxlamine pH 8.5. This quenching step inactivates the remaining Cy3-NHS and is reported to remove side reactions of the dye with other 'less stable' modified amino acid residues, for example histidine.

Removal of unreacted dye and buffer exchange into 'immobilisation buffer' (sodium phosphate buffer supplemented with 0.01% sodium azide) was performed using centrifugal filter columns (regenerated cellulose) from Millipore (MA, USA) with a 10 kDa cut-off. Typically the columns were run at 3000 rcf at 4°C, until no dye absorbance could be detected in the eluant.

The labelling ratio of each antibody was estimated using the absorbance ratio of the dye/protein $Abs_{552/280\ nm}$ using Equation 1:

Equation 1: $$DP = \frac{(1.13 \times A_{552})}{(A_{280} - (0.08 \times A_{552}))}$$

Where 1.13 is the ratio of the extinction coefficients of the protein (Abs_{280} =170000 $M^{-1}cm^{-1}$)/dye (Abs_{552} 150000 $M^{-1}cm^{-1}$) and 0.08 represents the % contribution of the Cy3 dye to the Abs_{280}. Absorbance measurements were performed in matched low

volume (130 µl) quartz cuvettes, using the last wash from the centrifugal filter columns as the blank, to correct for incomplete dye removal.

2.2.1.2 Thiol Directed Labelling

IgG antibodies consist of 4 chains (two light and two heavy) that are covalently bound together through disulphide bridges. Labelling experiments in the presence and absence of the reducing agent TCEP were performed to increase the number of potential reactive groups. Labelling of cysteines residues could disrupt the disulphide bridges in the antibody leading to disulphide scrambling and loss of structure/activity. The antibody quaternary structure was therefore assessed both pre and post-labelling with non-reducing SDS-PAGE.

For the labelling reaction the MAb was diluted to 1 mg/ml in 'thiol labelling buffer' (10 mM HEPES pH 7.5 and 0.1 mM TCEP), flushed with N_2 and incubated for 30 minutes. If the TCEP is formulated as a salt with HCl, the resulting solution needs to be neutralised prior to its addition to thiol labelling reactions. Cy3-maleimide was dissolved at 10 mg/ml in DMSO and 5 µl of this solution added drop-wise to the reaction. The vial was then flushed with N_2 and incubated overnight at 4ºC. The dye was quenched by the addition of a 100-fold excess (over dye) of β-mercaptoethanol and allowed to react at room temperature for 30 minutes. Removal of quenched dye, β-mercaptoethanol and buffer exchange into 'immobilisation buffer' was performed using a 10 kDa centrifugal filter column as described in Section 2.2.1.1.

2.2.1.3 Hydrazide Labelling

Antibodies contain varying amounts of glycosylated amino acids, allowing labelling to be directed to sites distinct from the antigen binding sites. The MAb was diluted to 1 mg/ml in PBS, to which was added 150 µl of 0.1 M sodium periodate solution. The resulting mixture was flushed with N_2 and incubated for 30 minutes in the dark. Sodium periodate was removed from the solution using a 10 kDa centrifugal filter as described in Section 2.2.1.1, then to 200 µl of this solution was added 10 µl of Cy3-hydrazide prepared at 1 mg/ml in DMSO. In the modified method after oxidation sodium periodate was removed as above and buffer exchanged into a labelling buffer of 100 mM sodium acetate pH 5.5 before addition of the hydrazide dye. The reaction was left overnight, protected from light at 4ºC, then the excess dye was removed by filtration using a 10 kDa cut-off centrifugal filter as described in Section 2.2.1.1.

2.2.1.4 Biotinylation

Biotinylation of the MAb used the same NHS labelling chemistry as the amine modification, therefore the methods was essentially the same as that described in Section 2.2.1.1, except that the Biotin-SE was prepared at 25 mg/ml in DMF and used at a 20-fold molar excess over the MAb. The degree of biotinylation was determined by either the HABA assay (Sigma Cat# H2153) or a gel sift assay. The HABA reagent was prepared and the assay performed as detailed in the manufacturers instructions, except the volumes used were scaled down to a microplate format using 180 µl of HABA reagent and 20 µl of biotinylated sample. Gel-shift assays were performed using reducing SDS-PAGE for which the samples were not boiled before application to the gel. SDS-PAGE apparatus, gels, Mw standards and buffers were purchased from Invitrogen (Paisley, UK), and samples were run and analysed using the

manufactures instructions. Images were acquired on GS800 Calibrated Densitometer from BioRad (Hemel Hempstead, UK).

Prior to analysis Cy3-Streptavidin was added to the biotinylated MAb, typically at 10-fold molar excess and allowed to incubate for 30 minutes at room temperature. For the fluorescent measurements excess Cy3-streptavidin that was not bound to the antibody was removed by incubating the sample with biotinylated agarose (Sigma, Cat# B6885) prepared in 1 ml PBS supplemented with 1M NaCl to reduce NSB. The purification was performed in a 1.5 ml microcentrifuge tube with constant agitation for 30 minutes, using 40 µl of the biotinylated agarose. The agarose was pelleted by centrifugation, the eluant collected and then concentrated/buffer exchanged into PBS. Purification was confirmed by SDS-PAGE, with a control incubation of streptavidin being completely adsorbed to the streptavidin agarose.

2.2.2 Antibody Binding Studies

2.2.2.1 Biacore Binding Kinetics

A Biacore T100 was used to look at the binding kinetics of the antibody 'analyte' (including labelled variants) to the immobilised ligand 'myoglobin'. The kinetics of the interaction between immobilised myoglobin and the antibody variants were analysed by titrating the antibody over the sensor surface and monitoring the change in SPR signal. The methods used to prepare the immobilised sensor surface, run the binding experiments and analyse the data are based on prescribed Biacore methods unless stated.

Myoglobin was chemically coupled to the CM5 chip using standard EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide) Biacore protocol. The CM5 chip has a carboxyl modified dextran layer to which the ligand is covalently immobilised typically through free amine groups (N-terminus, or lysine residues)[9]. To increase the efficiency of immobilisation the pH of the immobilisation buffer is optimised so the dextran surface carries a net negative charge and the ligand a net positive charge, attracting each other therefore facilitating pre-concentration at the surface of the chip. Using the pre-concentration method in the Biacore software the optimum immobilization buffer was found to be 10 mM acetate pH 5.0.

For kinetic studies a low level of ligand is preferable on the chip surface, in as such when saturation of the surface is achieved the increase in response units (RU's) should be in the range of 100-200 RU's. With higher ligand densities the surface can be mass transport limited, this results in limited transfer of the analyte from the bulk phase to the chip surface and will result in a poor estimation of the true kinetic parameters for the protein interaction. Based on the respective Mw of the antibody (150 kDa) and myoglobin (17.3 kDa) an ideal amount of ligand to immobilise would be ~ 27 RU's (assuming each antibody can bind bivalently i.e. binds to two immobilised myoglobins), see Equation 3. To obtain an immobilisation level of ~30 RU's myoglobin was diluted to 0.5 µg/ml in acetate pH 5.0 and three aliquots of 12 µl of this ligand at a flow rate of 10 µl/min were injected over the EDC/NHS (10 minute injection at 10 µl/min) activated chip surface 'Fc_4' (Flow cell). To remove un-modified coupling agent 'EDC/NHS' from the chip the surface was blocked with ethanolamine, using multiple 60 seconds injections until a stable baseline was obtained. To correct for changes in the refractive index of the solutions injected into the Biacore a reference channel 'Fc_1' was prepared. The reference channel contained

immobilised lysozyme that did not bind to the anti-human myoglobin antibody, this reference channel was prepared in the same way as the active myoglobin channel.

The activity of the surface was determined using a large excess 'saturating' amount of analyte to determine maximum binding capability 'R_{max}' of the surface and subsequently the immobilised ligands relative immunogenic activity. Buffer optimisation was performed to determine the optimum buffer to perform the myoglobin binding experiment, in terms of generating a stable binding response and minimising non-specific binding. HBS-P (10 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl and 0.005% (v/v) P20 surfactant) was found to give the best results and was therefore used as the running buffer for all the Biacore experiments (data not shown). Controls to assess surface performance, mass transfer and linked reactions were performed prior to kinetic analysis and are detailed in Section 3.2.1.

Kinetic experiments were performed at 25°C with a flow rate of 30 µl/min using HBS-P as the running buffer. The antibody concentrations were determined by UV-Vis spectroscopy before dilution into the HBS-P (minimum of 4-fold dilution to minimise bulk solvent effects). Typically three 'dummy' binding runs using 10 nM unlabelled MAb were performed prior to kinetic runs to 'condition' the surface. Injection of the analyte 'association' was performed for 300 seconds and dissociation was monitored for 600 seconds. Using the Regeneration buffer scouting kit' from Biacore the optimum regeneration conditions were found to be 4M $MgCl_2$ injected over a 45 second burst. The chip was then left to stabilise for 240 seconds before initiation of the next binding cycle.

For all sample runs the blank Fc1 was subtracted from Fc4 the myoglobin immobilised flow cell. For kinetic runs the dilution series spread over at least 2-orders of magnitude around the protein-antibody interaction K_D were performed in duplicate, and each run contained a zero concentration sample from which all the cycles were subtracted.

2.2.3 Relative Fluorescence Quantum Yield Measurements

2.2.3.1 Fluorimeter Calibration

To determine relative fluorescence quantum yields it is vital that the fluorimeter is fully calibrated with respect to spectral responsivity. A fluorimeters spectral response can drift over time requiring the need to update the emission correction file of the instrument through the use of fluorescent standards. The application of fluorescent standards for specific applications and the selection of standards has been reviewed [10] The relative spectral responsivity of the fluorimeter was determined using the BAM calibration kit; using the LINKCORR program [8], an emission correction curve was generated for the fluorimeter so to correct the fluorescence intensity for wavelength dependent variation. The BAM spectral fluorescent standards from the calibration kit [8] were prepared in the designated solvents and used within 1 month of preparation. Measurements and data analysis were performed as detailed in the calibration kit SOP, using the following parameters. Fluorescent measurements were performed in 4 ml capped fluorescent quartz cuvettes from Starna Scientific Ltd. For all measurements the excitation and emission filters were set to 5 and 2.5 nm respectively, so to ensure that fluorescence measurements were recorded in the linear region of the detector (determined to be less than 200 fluorescence intensity units).

Spectra were recorded at scan speeds of 100 nm/min and scans were repeated 20 times to remove noise from each spectrum. Background scans were performed between each sample to confirm no carry-over from the previous fluorescent standard.

2.2.3.2 Relative Quantum Yield Measurements

Relative quantum yield measurements for the labelled MAb were made in relation to the fluorescent standard rhodamine 6G dye, whose fluorescent quantum yield has been evaluated extensively [11]. Fluorescent quantum yield measurements have been reviewed and the method was adapted from Demas and Crosby [12]. R6G was dissolved at ~10 mg/ml in spectroscopic grade ethanol from which subsequent dilutions were prepared in the same solvent. The concentrations of these dilutions were determined using absorbance (R6G $\varepsilon_{529.75\ nm}$ 116000 $M^{-1}cm^{-1}$), which matches the Cy3 major absorption peak ~552 nm. The slits in the spectrophotometer were matched with those used in the fluorescence measurements. To increase the accuracy of the absorbance measurements quartz cells with a path-length of 10 cm were used to determine the concentration of the R6G fluorescent standard. Before spectroscopic measurements the antibody samples were centrifuged at 10000 g for 10 minutes to pellet any solids/precipitates. The absorbance of the Cy3-labelled MAb's were measured in low volume matched quartz cuvettes, directly after this solutions were diluted in PBS and the fluorescent spectra acquired. The R6G $Abs_{490\ nm}$ was also measured, as this was the excitation wavelength used for the fluorescent measurements.

Both the Cy3-labelled antibodies and R6G standard solutions were measured in low volume capped fluorescence quartz cuvette from Starna. Samples were excited at 490 nm and emission was recorded at 100 nm/min from 500-650 nm, with the excitation/emission slits set to 5.0/5.0 nm. Typically 5 spectra were obtained per sample and the blank 'PBS' then subtracted. The resulting spectra within the linear range of the detector were transformed using the correction curve generated with the calibration kit (Section 2.2.3.1). Relative quantum yields were calculating using Equation 2, where X and ST denote the unknown (Cy3-MAb) and standard (R6G) dyes respectively; F is the integrated fluorescence intensity/absorbance and η is the refractive index of the solvent (1.33 for water and 1.35 for ethanol).

Equation 2: $$\Phi_X = \Phi_{ST}\left(\frac{F_X}{F_{ST}}\right)\left(\frac{\eta_X^2}{\eta_{ST}^2}\right)$$

3 Results & Discussion

3.1 Fluorescent Labelling

Rather than optimise the labelling for each technique standard methods (recommended by the dye supplier) were employed so to best represent industrial practice. Amine labelling produced an average dye/protein (D/P) ratio of 5 Cy3 molecules per MAb, as shown from the SDS-PAGE structural integrity was maintained, when compared with the native MAb (data not shown).

Thiol labelling of the native MAb did not result in significant labelling, whereas when the MAb was reduced with TCEP prior to labelling a D/P ratio of 1.4 was obtained. The quaternary structure of the thiol-labelled MAb was analysed using non-reducing SDS-PAGE. When compared to the migration of the native MAb, the thiol labelled

MAb displayed multiple bands (data not shown), which suggest that its quaternary structure may have been changed. However the altered band pattern could also indicate that labelling had altered the surface charge of the antibody, or induced a partially aggregated state. When analysed using reducing SDS-PAGE both the native and thiol modified MAb's showed a similar band pattern suggesting that thiol modification had not altered the primary sequence (data not shown).

Multiple hydrazide labelling methods were attempted due to the low yields (D/P ratio's from 0.24 –0.38 Cy3 molecules per MAb) obtained with the recommended method. The method was then optimised to favour the labelling of aldehydes, by using a modified reaction buffer of 100 mM sodium acetate pH 5.5. The D/P ratio was improved to 1.4, therefore in the sample the majority of the MAb's should be labelled with at least a single dye molecule. It is reported that the source of the antibody can affect its degree of glycosylation and subsequently the success of hydrazide labelling. Polyclonal antibodies (PAb) are reported to be more heavily glycosylated than MAb's, however in two control reactions a MAb from a goat source (anti-human interferon, Peirce), and a PAb from a rabbit source (anti-mouse interferon α, 20 KU, Cat# 407291, Calbiohem) generated D/P ratios of 2.4 and 1 respectively. This data suggests that labelling oligosaccharides can give various levels of modification dependent on the antibody source and species. When analysed on both reducing and non-reducing SDS-PAGE the hydrazide labelled and native MAb migrated together suggesting retention of structural integrity (data not shown).

Two methods were used to quantify the degree of biotinylation of the MAb, the colorimetric HABA assay and SDS-PAGE gel-shift analysis. A microtitre plate modified HABA assay was not sensitive enough to detect biotinylation in the antibody samples using up to 2.5 μg/ml of biotinylated protein. Gel shift assays measure the change in migration of the MAb when streptavidin is bound. The samples when run in these assays are not heated before loading so to preserve the biotin-streptavidin interaction. For buffers, which were used for the streptavidin-Cy3, the MAb-streptavidin complex appeared to form multimers on reducing SDS-PAGE gels, generating a smear at ~116 kDa. When heated at 100ºC for 5 minutes streptavidin migrates as a sharp band at 12 kDa (which is assumed to be the monomer) Figure 5. It is reported that native streptavidin forms tetramers with a Mw of ~53 kDa, which is lower than was observed above. The difference in Mw observed with streptavidin could be due to aggregation, induced by Cy3 dyes, which is reported to be a problem in specific Cyanine dye-labelled systems. On reducing SDS-PAGE the MAb sample ran as 28 and 55 kDa bands, titration with excess streptavidin-Cy3 reduced the intensity of the 28 and 55 kDa bands and introduced a higher Mw smear. As a control the biotinylated commercial antibody (anti-human myoglobin 4M23b from mouse, HyTest) was also titrated with excess streptavidin, producing a similar response to MAb Clone 4E2 (data shown). Due to the smearing of the streptavidin-MAb multimer an accurate assessment of the degree of biotinylation was not possible, however from the titration experiments an estimate of >4 biotin sites was made.

A method to determine the number of biotin sites on a molecule through the fluorescence quenching of streptavidin has been documented [13]. To a defined concentration of streptavidin, with 1-2 sites occupied with the biotinylated protein of interest biotin was titrated into the mixture and the decrease in fluorescence due to streptavidin quenching was used to estimate the degree of biotinylation. This would probably be the best method to determine the degree of biotinylation of the anti myoglobin MAb generated in this project.

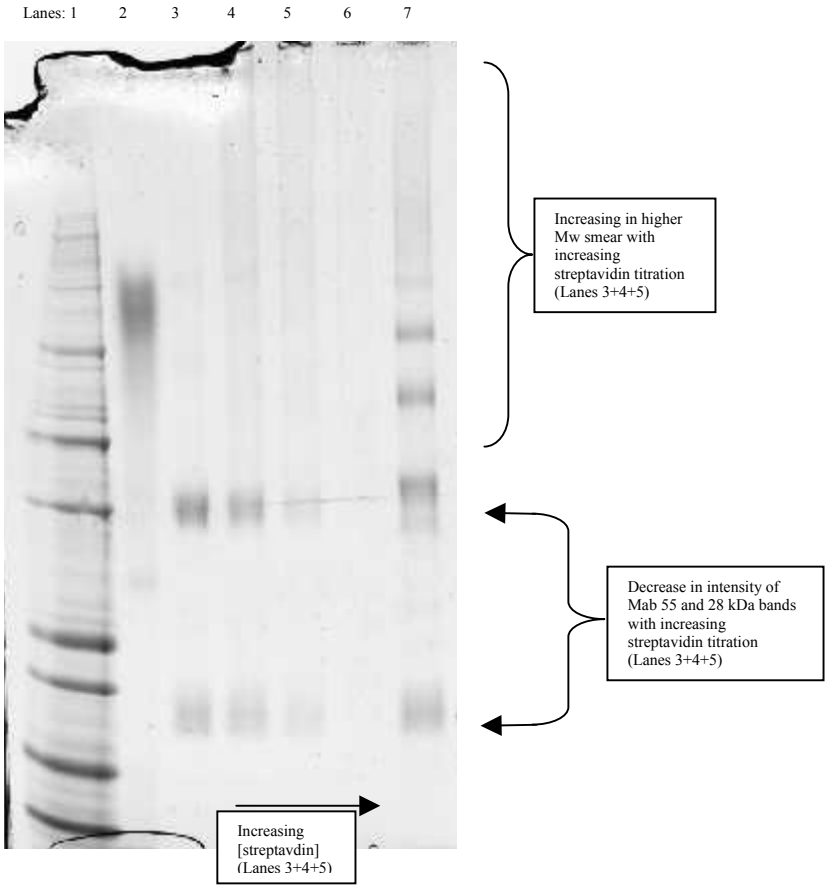

Figure 5: Gel-shift Analysis of the Biotinylated MAb. Samples were run on a 4-12% Bis-Tris gel in MES running buffer, in reducing conditions. All the samples where prepared without heating except Lane#7. Lane numbering from right to left; Lane #1: Mark 12 Ladder (bands 14, 21, 31, 36, 55, 66, 97, 116 and 200 kDa), Lane #2: streptavidin 3 μg, Lane #3: MAb 0.2 μg, Lane #4: MAb 0.2 μg + streptavidin 0.5 μg, Lane #5: MAb 0.2 μg + streptavidin 1.5 μg, Lane #6: MAb 0.2 μg + streptavidin 3μg and Lane #7: MAb 0.2 μg + streptavidin 2.5 μg heat denatured.

3.2 Biacore Binding Studies

3.2.1 Preparation and Characterisation of the Myoglobin Chip Surface

For Biacore experiments it is important to optimise the system so to avoid non-specific binding, retain ligand activity after immobilisation and obtain a stable chip that can be analysed and regenerated over multiple cycles. For kinetic experiments it is important to minimise mass transport limitations to and from the sensor surface. By immobilising limiting amounts of ligand onto the surface mass transport can be minimised, an ideal surface would give an R_{max} level of 100-200 RU's upon saturation binding to the analyte.

Pre-concentration studies indicated that the optimum pH for immobilisation of myoglobin (theoretical pI 7.29) was ~pH 5.0. Immobilisation of myoglobin gave an increase of ~49 RU, saturation analysis using 2000 nM unlabelled MAb generated an R_{max} of 191 RU (though this did not produce a steady state response). Using Equation

3 the theoretical R_{max} value can be calculated (where R_L = immobilization level; RU and S_m = stoichiometric ratio, analyte Mw 150000 and the ligand Mw 17053). Where the theoretical R_{max} deviates from that observed experimentally it indicates that there is either lower surface activity and/or a different stoichometry of binding present in the system.

Equation 3: $$R_{\max} = \left(\frac{analyteMW}{ligandMW} \right) \times R_L \times S_m$$

The activity of the surface can only be estimated accurately if the model of interaction is known; antibodies are bivalent and therefore can bind to two surface bound myoglobin molecules. However bivalent binding depends on the density of the immobilised molecule, with higher surface concentrations promoting bivalent binding. The relatively low surface density obtained (R_{max} ~190 RU's) would favour the 1-2-1 'Langmuir' model, however in reality a mixture of models will be present on the surface. Assuming a 1-2-1 model the activity/accessibility of the surface was calculated to be 51% that of the theoretical R_{max} using Equation 3. That the surface is not 100% active suggests that immobilisation of the myoglobin either reduces its biological activity/blocks access to the myogblobin epitope, or that a fraction of the antibody is binding two myoglobins (bivalent model). If the bivalent model is assumed and the majority of the antibody is binding two myoglobin molecules then the surface displays 100% activity.

Where the k_d is tight (typically less than 1E-03 s^{-1}), for example MAb's, dissociation of the analyte from the surface will not be complete, therefore regeneration between each cycle is required. Regeneration should not change the activity of the ligand on the surface, or alter the dextran layer (for example permanent changes the refractive index). Regeneration scouting assesses the ability of various regeneration reagents at removing bound analyte, while retaining the activity of the chip. The following regeneration reagents were found to be suitable; 4 M $MgCl_2$, 0.2 M NaOH and Glycine pH 1.5; however due to the nature of the strong acidic and alkali regeneration reagents and their effect on the long term stability of the chip 4 M $MgCl_2$ was chosen as the regeneration reagent. This was also confirmed using a 'Surface Performance' test where the binding and regeneration parameters were assessed by performing multiple binding events using a fixed concentration of analyte. The baseline and binding response remained stable over 15 binding cycles indicating the system was suitable for kinetic analysis (data not shown).

Mass transport limitation occurs when either the rate of association, or disassociation contains a significant component due to the rate of transport of the analyte to and from the chip surface. Mass transport limitations can be reduced by lowering the density of immobilised ligand, or increasing the flow rate. Comparing the binding and dissociation rates at various flow rates (10, 30 and 75 μl/min) 'mass transfer control' was performed. The observed binding and dissociation rates were approximately the same for two MAb concentrations (20 and 100 nM) for all three-flow rates analysed (data not shown); this suggests that the surface was not mass transport limited.

Linked reactions describe a model in which in a time dependent manner binding of the analyte is followed by a conformation change in the analyte-ligand complex, resulting in a stabilisation in this complex and a change the dissociation rate. A time dependent dissociation rate can also be observed for bivalent binding reaction, therefore the linked control can be used to identify bivalent binding in model systems.

Linked reactions can be identified by looking at the relationship between association time and the rate of dissociation. Since as the conformational change is time dependent, longer association times will result in a higher degree of complex stabilisation on the surface and therefore slower dissociation rates. Figure 6 shows the adjusted dissociation curves for 100 nM MAb injected for 1, 2 and 10 minutes over the myoglobin surface of the chip. The dissociation rate does not appear to vary with injection time therefore it would appear no linked reactions are occurring on the surface of the chip (the similar time-independent response was observed using 10 nM MAb, data not shown).

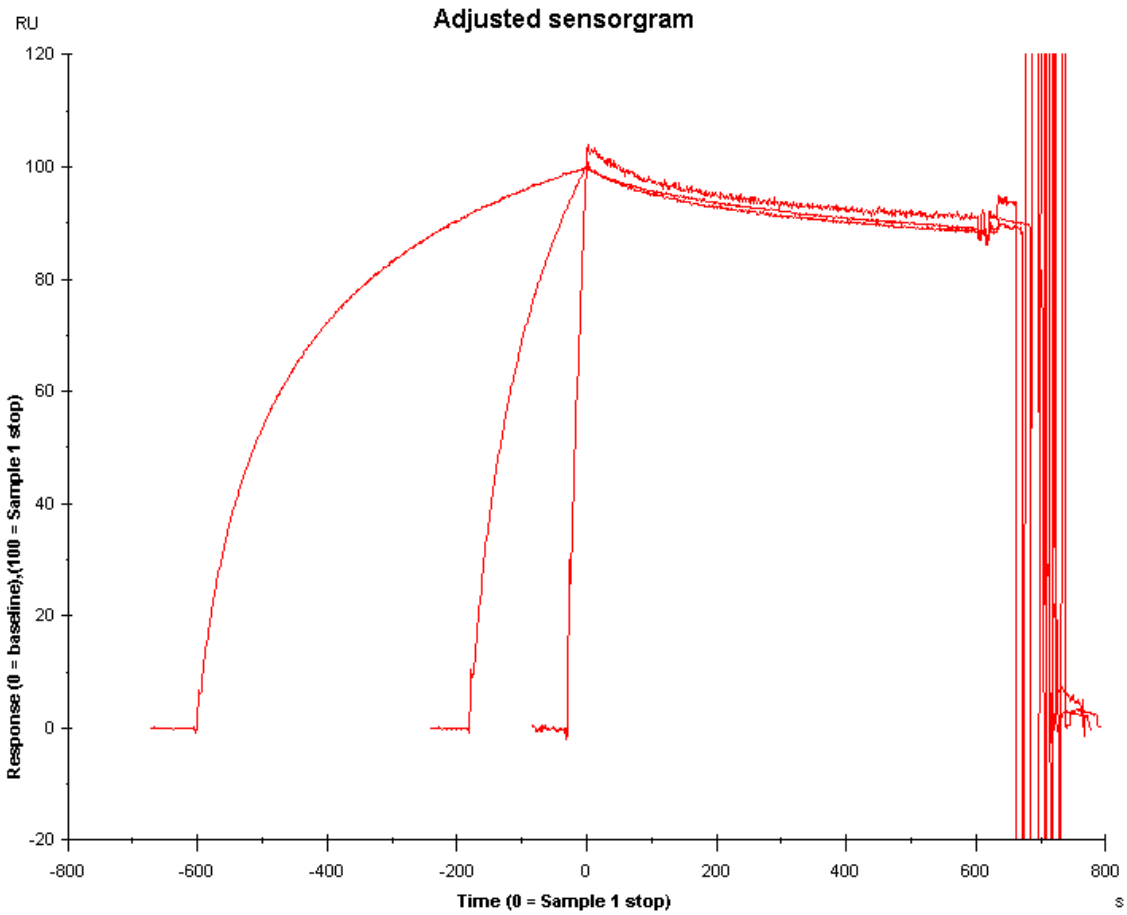

Figure 6: Linked Reaction Sensogram. The adjusted sensogram shows the dissociation rates are not dependent on the length of the sample injection (association time).

3.2.2 MAb Binding Kinetics to Immobilised Myoglobin

The binding kinetics for the fluorescent-labelled anti-myoglobin MAb samples were compared to that of the unlabelled MAb. To ensure equal concentration series were prepared for each of the labelled and unlabelled antibodies the concentration of antibody in the stock solution was determined via absorbance again, just before the Biacore assay. This was to ensure that no precipitation, or protein adsorption to the microfuge tube has occurred during storage at 4˚C. Figure 7 highlights the choice of model used to fit the kinetic data, for all data sets both the 1-2-1 and bivalent models were analysed, however without sufficient evidence (negative for linked-reaction) for

bivalent binding all data used for comparison of kinetics was fitted using the 1-2-1 model. Looking at the residuals Figure 7 and Chi^2 values for the respective fits (1-2-1 Chi^2 = 0.343 and bivalent Chi^2 = 0.265) there appears to be no significant improvement when fitting with the bivalent model, therefore due to the extra parameters and degrees of freedom associated with the bivalent model, the 1-2-1 fit is the most appropriate model to use.

The stability of the initial preparation of biotinylated MAb was reduced upon addition of a large excess of streptavidin-Cy3 resulting in protein precipitation. The majority of the precipitated protein appeared to be the streptavidin-Cy3, as determined by the decrease in absorbance at 552 nm, compared to the protein absorbance at 280 nm. Due to the low stability of this labelled conjugate another batch of biotinylated MAb was prepared using a lower amount (10 fold molar excess) of biotin-SE to reduce the labelling ratio. When analysed on gel-shift assays the resulting streptavidin-biotinylated MAb conjugate displayed the characteristic smear indicating binding, but again the exact labelling ratio could not be estimated. For the biotinylated samples the 'high ~ 20 fold molar excess' and 'low ~10 fold molar excess' descriptors represent the labelling reaction using high and low amounts of Biotin-SE. Biotinylated samples were also tested in the absence of streptavidin to determine the effect of biotinylation on the binding of the MAb.

Table 1 details the kinetic values derived between the binding of the labelled MAb's and immobilised myoglobin. To assess the activity of the labelled MAb the rates of 'association' and 'dissociation' k_a and k_d along with the global R_{max} and K_D parameters were compared. Both the amine and thiol directed labelling did not significantly alter the interaction with myoglobin. Thiol directed labelling altered the quaternary structure of the MAb, however this did not appear to alter its binding mechanism, or affinity for myoglobin. Thiol reduction and subsequent blocking of the reduced cysteine can produce a mixture of light and heavy chains from the antibody, both of which can bind to the antigen. If thiol-directed labelling blocked the disulphide bridges, the concentration of free epitopes (assuming the native MAb can only bind a single antigen due to the low surface density) might be higher than the native. This was not apparent when the thiol data was compared with the native; therefore it was assumed that thiol labelling had not significantly altered the concentration of free epitope binding sites. That amine and thiol-directed labelling did not alter the binding kinetics of the anti-myoglobin MAb was further supported by sandwich ELISA data where the labelled MAb was immobilised to the plate surface. No change in the titration-binding curve for myoglobin when compared to the wild-type was observed indicating retention of function (data not shown).

Hydrazide directed labelling significantly altered the MAb's binding affinity Table 1. Where the antibody was labelled with a D/P ratio of 1.4, the K_D was altered significantly, indicating a reduced affinity for the myoglobin, another labelling reaction where the labelling ratio was only 0.34 generated similar results to the unlabelled MAb, though with a reduced R_{max}. This suggests that the hydrazide labelling rather than the oxidation (Cys, Met and Trp are prone to oxidation; therefore sodium periodate oxidation as part of the hydrazide labelling process may have damaged the MAb) resulted in the decrease in K_D observed. This reduction in activity due to hydrazide labelling is surprising since the reactive oligosaccharides are at a distinct site from the epitope binding site and are not thought to be involved in antigen binding. Due to the low yield of the Cy3-hydrazide labelled MAb this sample could not be tested in an ELISA format.

Biotinylation either in the presence or absence of bound streptavidin also resulted in a significant decrease in the K_D value that appeared to be independent of the degree of biotinylation Table 1. That the biotinylation chemistry affects the binding is surprising as it uses the same reactive chemistry as the amine-directed Cy3 dye (NHS), which did not display altered binding kinetics. ELISA data to support the change in binding kinetics was not attempted as the ELISA employed a streptavidin-biotin interaction for the enhancement step, which was incompatible when using a biotinylated capture antibody. For many sensor/diagnostic applications the reduction in binding observed for biotinylation would not be classed as significant as it was still had a K_D in the low nM range.

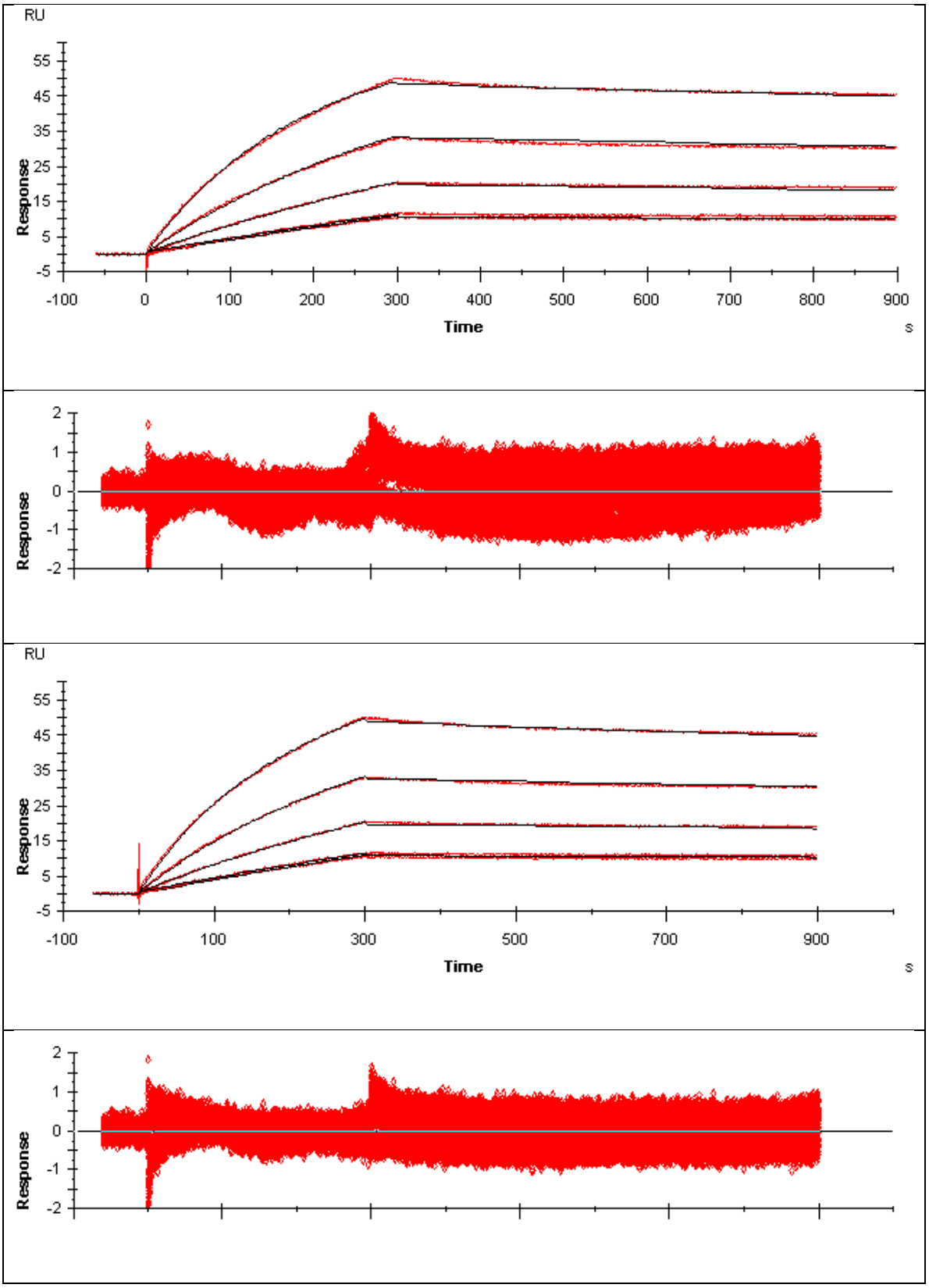

Figure 7: Model Fitting for the Unlabeled MAb. Data was fit with the 1-2-1 (top) and bivalent (lower) models, underneath each fit is a plot of the respective residuals.

	Kinetic Parameters from a 1-2-1 model				
Sample	$k_a \pm SE \times 10^4$ (1/Ms)	$k_d \pm SE \times 10^{-4}$ (1/s)	$R_{max} \pm SE$ (RU)	K_D (nM)	Chi^2
Unlabelled (n=3)	7.89 ± 0.05	1.22 ± 0.06	74.1 ± 0.01	1.6	0.36
Amine-directed	7.65 ± 0.16	1.45 ± 0.01	60.4 ± 0.1	1.89	0.2
Thiol-directed	8.25 ± 0.16	1.79 ± 0.01	69.3 ± 0.1	2.17	0.27
Hydrazide (D/P: 1.4)	2.06 ± 0.012	10.25 ± 0.04	15.1 ± 0.04	49.7	0.135
Hydrazide (D/P: 0.34)	8.13 ± 0.17	2.77 ± 0.02	46.2 ± 0.1	3.4	1.32
Biotin (n=2)	1.69 ± 0.05	3.46 ± 0.03	48.1 ± 0.1	20.5	0.538
Biotin + Streptavidin (n=2)	1.71 ± 0.05	3.62 ± 0.03	46.4 ± 0.1	21.23	0.665

Table 1: Kinetic Parameters for the Interaction of Immobilised Myoglobin and its Respective MAb. Errors expressed as SE, except where a titration experiment has been repeated more than once, then the average SE from the repeated experiments is expressed.

3.3 Fluorescence Characteristics of the Cy3-labelled Probes

The fluorescence quantum yield of the Cy3-labelled MAb's was determined using the fluorescent standard R6G. A linear response ($R^2 = 0.9995$) between total fluorescence (integrated area under the curve) and absorbance for R6G standard dilutions were obtained indicating that from 0-200 FIU the detector was linear. Table 2 details the relative quantum yield (Φ) values obtained for the Cy3-labelled MAb's. The values obtained are low compared to those reported by the dyes manufacturer who report for a D/P ratio of 2 Φ >0.15. Other reports give the Cy3 Φ as 0.04 at 540 nm excitation in PBS [1]. Apart from the thiol-directed labelling all the other reagents displayed similar relative Φ values. Cy3 quenching due to over-labelling was not predicted for any of the conjugates produced as [3]Hahn *et.al.* reported the optimal fluorescence for IgG-labelling was 6-8 cy3 molecules [14] .

The ratio between the two main Cy3 absorbance peaks (around 550/520 nm) is reported to relate information about intramolecular interactions between Cy3 dyes, with values around 1 indicating possible quenching. Quenching is often due to over labelling and results in a decrease in the Φ, all the peak height ratios were greater than 1, however the thiol labelled MAb displayed a low peak height ratio Table 2, which may explain the lower Φ when compared with the other labelling methods. The Cy3-maleimide labelled MAb would not have expected to display quenching due to over labelling due to the low D/P ratio of 1.4. The low Φ for Cy3-maleimide labelled MAb could be due to the close proximity of the Cy3 dyes in the antibody, or that another process that reduces the fluorescence is occurring.

[3] http://www.bphys.uni-linz.ac.at/bioph/download/label.pdf

Cy3-label	D/P	Φ	Peak height ratio: 550/520 nm
Amine-directed	5	0.089	1.64
Thiol-directed	1.4	0.049	1.3
Hydrazide	1.4	0.08	1.56
Hydrazide	0.34	0.083	1.5
Streptavidin	2.7	0.083	1.95

Table 2: Spectral Parameters for the Cy3-labelled MAb's.

4 Conclusions

Four strategies were employed to label a MAb with a fluorescent reporter, the resulting conjugates were then analysed for binding activity and the brightness of the fluorescent signal. The data presented here represent only a single model system; other labelling strategies may be more suitable for other antibodies, classes of antibodies, or proteins depending on the their structure and residues critical for their function. The labelling of proteins through amines (dominated by lysine residues) using NHS chemistry generated a good D/P ratio and resulting fluorescent yield. As with the thiol directed labelling amine-labelled antibodies bound to the antigen in both the Biacore and ELISA format with a similar response to the unlabelled MAb, indicating its function had not been altered. Thiol modification can be more directed due to the relative low abundance of cysteine molecules within proteins, however this can limit detection. The Hydrazide labelling method appeared variable for different classes and species of antibodies and labelling reactions were difficult to reproduce as several attempts were made using the same conditions to obtain D/P ratio's greater than 1, with only a single batch producing a suitable fluorescent reporter. The labelled method could be improved by denaturing the antibody prior to labelling thereby exposing the previously shielded glycosylation sites. The loss of binding activity displayed by this conjugate was surprising as the epitope binding site is distinct from the glycosylation site and hydrazides have previously been used successfully to label antibodies. The low labelling ratio, 1.4 D/P could mean that the activity displayed from the Biacore could be from unlabelled antibodies, which would be difficult to separate from the labelled species. The biotin-streptavidin system is commonly used in applications were fluorescent enhancement is required as the antibody can be labelled with multiple streptavidin molecules each with multiple fluorescent reporters. For many diagnostic applications the amount of detection antibody is limited, this therefore precludes the use of the HABA assay for quantifying the degree of biotinylation. The gel shift assays were difficult to analyse for large molecules with multiple biotinylation sites, due to the apparent oligmerisation of the streptavidin. Biotinylation appeared to alter the binding activity of the MAb, however this change

in activity might not be so significant, and could be compensated for by the increase in the number of fluorescent reporter groups on the molecule of interest.

Biopharmaceutical and imaging applications often require that antibodies are labelled with a detection agent, or drug 'warhead' that may disrupt the parent antibodies function. The effects of labelling MAb's with various chemistries for fluorescent tagging is analogous to the problems faced by tagging with drugs or imaging agents. For *in vivo* applications additional problems from immunogenicity of the linker chemistry may cause additional problems that need to be examined. It is known that the oligosaccharides play an important role in antibody function and pharmacokinetics *in-vivo,* which might preclude the use of hyrazide labelling strategies where the terminal sugars are chemically modified.

To conclude amine directed labelling produced the most suitable fluorescent tag according to the criteria set (Table 3) and appeared to be a good mimetic of the unlabelled antibody. Thiol and hydrazide directed labelling and biotinylation produced fluorescent tags that either displayed alter biological performance, poor reproducible labelling, or a low quantum yield that could limit their use for certain applications.

Cy3-label	Labelling Ratio	Brightness (Φ)	Binding Activity 'K_D' (nM)
Unlabelled	-	-	1.6
Amine-directed	5	0.089	1.89
Thiol-directed	1.4	0.049	2.17
Hydrazide	1.4	0.08	49.7
Streptavidin	2.7	0.083	21

Table 3: Overview of Key Parameters used to determine a Suitable Fluorescent Tag

5 Acknowledgements

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