Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Synthesis and Characterization of a Cleavable Fluorescent Biotin Derivative

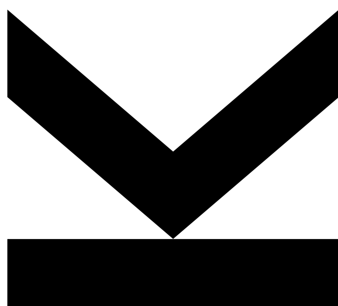
Submitted by
Lakmi L. Pitigala, BSc

Submitted at
Institute of Biophysics

Supervisor
A.Univ.-Prof.Dr. Hermann Gruber

Co-Supervisor
Dr. Felix Faschinger

February 2018



Master Thesis

to obtain the academic degree of

Master of Science

in the Master's Program

Joint Master Programme Biological Chemistry

UNIVERSITY OF SOUTH BOHEMIA IN ČESKÉ BUDĚJOVICE
Branišovská 1645/31a
370 05 České Budějovice,
Česká Republika
www.jcu.cz

JOHANNES KEPLER UNIVERSITY LINZ
Altenberger Str. 69
4040 Linz, Austria
www.jku.at

STATUTORY DECLARATION

I hereby declare that the thesis submitted is my own unaided work, that I have not used other than the sources indicated, and that all direct and indirect sources are acknowledged as references.
This printed thesis is identical with the electronic version submitted.

Place, Date

Signature

ACKNOWLEDGEMENTS

First, I would to thank my supervisor A. Univ.-Prof. Dr. Hermann Gruber for his leadership and giving me the chance to join his research group. Special gratitude goes to my co-supervisors Dr. Felix Faschinger and Dr. Maria Wiesauer for every-day guidance and never-ending patience. Great thanks also belong to Assist. Prof. Dr. Andreas Horner for his assistance with confocal fluorescence microscopy. Thanks to Luise Herrmann Bsc. for all the titration work. Further thanks to all laboratory members for their helpfulness and good atmosphere. I would also like to acknowledge Univ. Prof. Dr. Norbert Müller and Prof. RNDr. Libor Grubhoffer, CSc. for the establishment and management of the Biological Chemistry study program. Finally, I would like to thank my family and friends for their constant support and understanding.

ABSTRACT

Biotin (strept)avidin interaction is one of the strongest non-covalent interaction, which is exemplified by countless different biotechnological applications. In my master thesis, I synthesized a novel biotin analogue, biotin-disulphide-fluorescein (BSSF), in sufficient quantities for a thorough chemical and functional characterisation. This unique molecule has biotin group in one end, a fluorescein group present in the other end, and a cleavable cystamine core with two glycine residues in the centre. According to a preliminary previous study (B. Spitzbart, master thesis at JKU Linz), the two end groups (biotin and fluorescein) are supposed to bind to two adjacent pockets of (strept)avidin by forming a strong interaction. This means that only two BSSF molecules were bound per streptavidin tetramer. When the disulphide bond was cleaved, the binding energy which held fluorescein group inside the biotin-binding pocket was greatly reduced, causing rapid loss of the fluorescein group within 2 minutes, while the biotin group remained bound to the adjacent pocket. In this way two pockets per streptavidin tetramer were found to become available for binding of other biotinylated molecules. These recovered binding sites were quantified by binding of commercial biotin-4-fluorescein.

A major drawback of the above cited study was the minute quantity of the newly synthesized BSSF which could only be characterized by low-resolution mass spectrometry but not by NMR spectroscopy. A second weakness was the narrow evidence for the recovery of new biotin-binding sites after disulphide reduction for re-examination of the above study, I synthesised the BSSF molecule in large quantities which allowed for extensive characterization by NMR spectroscopy, and I used both the homogeneous and heterogeneous fluorescence assays to study the effect of disulphide cleavage. The latter was carried out with commercial streptavidin-agarose beads, as well as with Sephadex G-15 beads to which streptavidin was coupled by a newly developed and optimized procedure. Unfortunately, all results of the homogeneous and heterogeneous assays were in contradiction with the previous literature. According to my results BSSF was bound to (strept)avidin tetramers by forming a simple 4:1 complex, not the unusual 2: 1 complex proposed by the above cited study.

TABLE OF CONTENTS

ABSTRACT **4**
1.Introduction **8**
1.1 (Strept)avidin-biotin interaction 8
1.1.1 Avidin 8
1.1.2 Streptavidin 8
1.1.3 Structure of avidin and streptavidin 9
1.1.4 Biotin and biotin analogues 11
1.2 Binding modes of bivalent biotin analogues 12
1.3 The unusual binding mode of biotin-disulphide-fluorescein (BSSF) 14
1.4 HABA assay 16
1.4.1 HABA complex 16
1.4.2 Experimental conditions for the HABA titrations 17
1.5 Tryptophan quenching 18
1.6 Fluorescence correlation spectroscopy (FCS) 19
1.6.1 Instrumentation of confocal fluorescence microscope 19
1.6.2 Theory behind the fluorescence measurements 20
1.7 Objectives/ aims 23

2. Results and discussion **24**
2.1 Linker chemical synthesis 24
2.2 Protein labelling with biotin and/ or fluorophores for bead experiments 27
2.2.1 Formation of double labelling of goat IgG with biotin-Ahx-NHS and Alexa-fluoro-647-NHS 27
2.2.2 Labelling of BSA with Alexa-fluoro-647-NHS 28
2.2.3 Labelling of streptavidin (SAV) with Alexa-fluoro-647-NHS 29
2.3 Functional experiments with streptavidin beads 30
2.3.1 Test of streptavidin-agarose for its capacity to bind biotinylated biotin-IgG* 30
2.3.2 Test of streptavidin-agarose for its capacity to bind biotinylated B4F 31
2.3.3 Time cause of binding of biotin-IgG* to SAV agarose 32
2.3.4 Test for specific and nonspecific binding of biotin-IgG* to SAV agarose with optimized incubation protocol 33
2.3.5 Test for the amount of BSSF required for the completion of block of SAV-agarose 37
2.3.6 Test for the comparison of BSSF block of SAV-agarose with and without cleavage of disulphides by TCEP 40
2.4 Covalent immobilization of streptavidin on Sephadex G-15 beads and further couplings 41
2.4.1 Activation of Sephadex G-15 with N, N'-disuccinimidyl carbonate (DSC) and coupling of BSA* 41
2.4.2 Activation of Sephadex G-15 with cyanogen bromide (CNBr) and coupling of BSA* 43
2.4.3 Coupling of unlabelled streptavidin and biotin-IgG* to CNBr-activated Sephadex G-15 44

2.4.4 Test for the comparison of biotin-IgG* binding of SAV-CNBr-Sephadex G-15 in absence and presence of BSSF 46
2.4.5 Test for the comparison of biotin-IgG* binding to SAV-CNBr-Sephadex-G-15 beads in presence of BSSF, with and without disulphide cleavage by TCEP 47
2.5 Coupling of 8-arm-PEG-nitrophenyl ester with N-biotinyl-propylamine 50
2.6 Biotin and BSSF titrations against (strept)avidin 51
2.6.1 Biotin vs. avidin titration using tryptophan quenching 51
2.6.2 Biotin vs. streptavidin titration using tryptophan quenching 51
2.6.3 BSSF vs. avidin titration using tryptophan quenching 52
2.6.4 BSSF vs. streptavidin titration using tryptophan quenching 53
2.7 Summary and conclusions 54

3. Experimental section 55
3.1 General methods 55
3.1.1 Materials 55
3.1.2 Chemical syntheses 55
3.1.3 Thin layer chromatography (TLC) 55
3.1.4 Reversed phase (RP) chromatography 55
3.1.5 Size-exclusion chromatography 56
3.1.6 Nuclear magnetic Resonance spectroscopy (NMR) 56
3.1.7 Mass spectrometry (MS) 56
3.2 Linker chemical synthesis 57
3.2.1 Synthesis of NH_2-gly-cystamine-gly-boc 57
3.2.2 Synthesis of biotin-gly-cystamine-cly-boc 60
3.2.3 Synthesis of biotin-gly-cystamine-gly-NH_2 63
3.2.4 Synthesis of biotin-gly-cystamine-gly-fluorescein 65
3.3 Protein labelling with biotin and/or fluorophores for bead experiments 71
3.3.1 Formation of doubled labelling of goat IgG with biotin-Ahx-NHS and Alexa-fluoro-647-NHS 71
3.3.2 Labelling of BSA with Alexa-fluoro-647-NHS 73
3.3.3 Labelling of streptavidin (SAV) with Alexa-fluoro-647-NHS 74
3.4 Functional experiments with streptavidin in bead suspension 76
3.4.1 Test of streptavidin-agarose for its capacity to bind biotinylated biotin-IgG* 76
3.4.2 Test of streptavidin-agarose for its capacity to bind biotinylated B4F 76
3.4.3 Time cause of binding of biotin-IgG* to SAV agarose 78
3.4.4 Test for specific and nonspecific binding of biotin-IgG* to SAV agarose with optimized incubation protocol 78
3.4.5 Test for the amount of BSSF required for the completion of block of SAV-agarose 79
3.4.6 Test for the comparison of BSSF block of SAV-agarose with and without cleavage of disulphides by TCEP 80
3.5 Covalent immobilization of streptavidin on Sephadex G-15 beads and further couplings 81
3.5.1 Method 1: Activation of Sephadex G-15 with N, N'-disuccinimidyl carbonate (DSC) and coupling of BSA* 81
3.5.2 Method 2: Activation of Sephadex G-15 by cyanogen bromide (CNBr) 83

3.5.3 Coupling of unlabelled streptavidin and biotin-IgG* to CNBr-activated Sephadex G-15 84
3.5.4 Test for the comparison of biotin-IgG* binding of SAV-CNBr-Sephadex G-15 in absence and presence of BSSF 85
3.5.5 Test for the comparison of biotin-IgG* binding to SAV-CNBr-Sephadex-G-15 beads in presence of BSSF, with and without disulphide cleavage by TCEP 85
3.6 Coupling of 8-arm-PEG-nitrophenyl ester with N-biotinyl-propylamine 87
3.7 Biotin and BSSF titrations against (strept)avidin 91
3.7.1 BSSF concertation determination by UV-Vis absorption 91
3.7.2 Biotin vs. avidin titration using tryptophan quenching 91
3.7.3 Biotin vs. streptavidin titration using tryptophan quenching 91
3.7.4 BSSF vs. avidin titration using tryptophan quenching 91
3.7.5 BSSF vs. streptavidin titration using tryptophan quenching 92
3.8 Buffers and special reagent recipes 92
3.8.1 PBS 7.3 buffer 92
3.8.2 Buffer 8.6 92
3.8.3 Buffer 9.1 92
3.8.4 Glycine 92
3.8.5 Buffer A 92
3.8.6 Hepes 9.6 92
3.8.7 TCEP – Hydrochloride (Tris – carboxyethyl phosphine hydrochloride) 93
3.8.8 Coupling buffer 93
3.8.9 Deactivation buffer 93
3.8.10 Acetate buffer 93
3.8.11 Tris buffer 93
3.8.12 Preservation buffer 93

4. Abbreviations 94

5. References 95

1. Introduction

1.1 (Strept)avidin-biotin interaction

1.1.1 Avidin

Avidin protein was discovered in 1930s and 1940s, when conducting nutritional investigations on vitamin B [Gyorgy, 1954]. De Lange and Huang were determined the primary avidin structure [De Lange & Huang, 1971]. Avidin is present in egg white with 0.05% of the protein content and it is a water-soluble glycoprotein unit. It has four identical subunits (homotetramer), which are arranged in 222 symmetry. One subunit is consisting with four to five mannoses and three glucosamine residues which gives the total weight of 14,600 Daltons [Green et al., 1971]. Each subunit is folded into eight-stranded antiparallel β-barrel to achieve more stable conformation with the minimum of energy [Livnah et al., 1993]. And, each of these subunits can interact with biotin or biotin analogue with a dissociation constant (K_D) of 10^{-15} M. This is one of the strongest non-covalent natural interactions known in the nature. The dissociation half time of the interaction is about 200 days at the pH of 7 in 25 °C [Green, 1963a]. The isoelectric point of avidin is around pH 10.5 and when it is bound to biotin it can be stable even in the range of pH 2-13.7 [Green 1963b].

The binding pockets are located at the end of β-barrel and each pocket contains tryptophan residues as the biotin binding sites. These tryptophan molecules are present at the bottom of the binding pocket, which enhance the biotin molecules slip very deep into the pockets. Avidin-biotin interactions are formed mainly in three different ways. One is by forming hydrogen bonds, where the urea part of the biotin (NH-CO-NH) gets attracted to the bottom of the binding pocket and another way is by forming hydrophobic interactions between hydrophobic residues and the pocket. When the biotin molecule slips deep into the bottom of the pocket, it gets covered by a loop in the each avidin subunit. A positively charged group of histidine present in avidin pocket forms an ion pair with the COO^- group of the biotin molecule to make avidin-biotin interaction even more strong [Livnah et al., 1993]. Avidin tetramer creates free energy of about 330 KJ/mol by biotin binding [Green, 1990].

Denaturing of avidin subunits is possible with guanidinium chloride (6 M) or HCl (0.1 M), without cleaving disulphide bonds [Green & Toms, 1972]. The avidin-biotin complex is stable and resistant to the digestive tract enzymes which can proteolyze many other complexes. Trypsin or pronase cannot inactivate free avidin [Green, 1972].

1.1.2 Streptavidin

Avidin and Streptavidin are homologous proteins. Streptavidin was first isolated from the bacterium species called *Streptomyces avidinii,* which has higher binding affinity ($K_D = 10^{-14}$ M) towards biotin or biotin analogues [Chaiet et al., 1963]. Avidin and streptavidin has 33% sequence identity, such as both are present in nature as tetramers and they bind to biotin or biotin residues in a similar way. Streptavidin also formed with a β barrel structure like avidin [González et al., 1999].

When comparing streptavidin towards avidin, streptavidin shows non-glycosylated form and it has slightly acidic isoelectric point at pH 5-6, which reduces the water solubility. Due to the nearly neutral pI of streptavidin, non-specific absorption towards charged surfaces are reduced. Streptavidin is more stable than avidin in extreme pH environments, in denaturing agents such as guanidium chloride and in enzyme degradation [Green 1975] but Streptavidin is slightly less thermostable than avidin [González et al., 1999]. Streptavidin has relatively closely paired binding

sites, with approximately 10 Å distance. This fact was proved by investigating the spatial relations of binding sites of avidin and streptavidin using bifunctional biotins [Green et al., 1971].
All the above features of avidin and streptavidin are important in biotechnological experiments. Avidin-biotin experiments became highly applicable since 1980s, when the ''Avidin-Biotin Technology'' book by Wilchek & Bayer [1988] was published. Since then, the avidin-biotin system has become a large topic and still much research is going on in this field with different aspects and applications.

1.1.3 Structure of avidin and streptavidin

Avidin and streptavidin structural analysis were mainly done by using X-ray crystallography method [Livnah et al., 1993]. Most of the structural features of both complexes were similar according to the images. When biotins are bound to adjacent pockets of avidin and streptavidin, carboxyl groups of biotins have very similar distances like 20.2 and 20.3 Å. The structural information obtained on avidin and streptavidin were based on the biotin interactions with each compound [Livnah et al., 1993].
When comparing structures of the two proteins, in avidin the terminal groups of arginine side chains point into the grove which connects the neighbouring binding pocket. One arginine group is present for one monomer; therefore, two arginine groups are present in one such groove. In streptavidin, arginine end group is flat on the groove and most of the times heteroatoms are not present in that. This is clearly visible in Figure 1.
When two biotin residues are bound to adjacent sites of (strept)avidin, the distance between the COOH groups of the valeric acid chains of the two biotins is 20.2 and 20.3 Å. The middle planes, the surface of two proteins are illustrated in 2nd model (carbons in yellow, nitrogen in blue and oxygens in red). When biotin bound to adjacent sites of avidin, it creates a pointed groove of nitrogen and in streptavidin a flat groove is created.

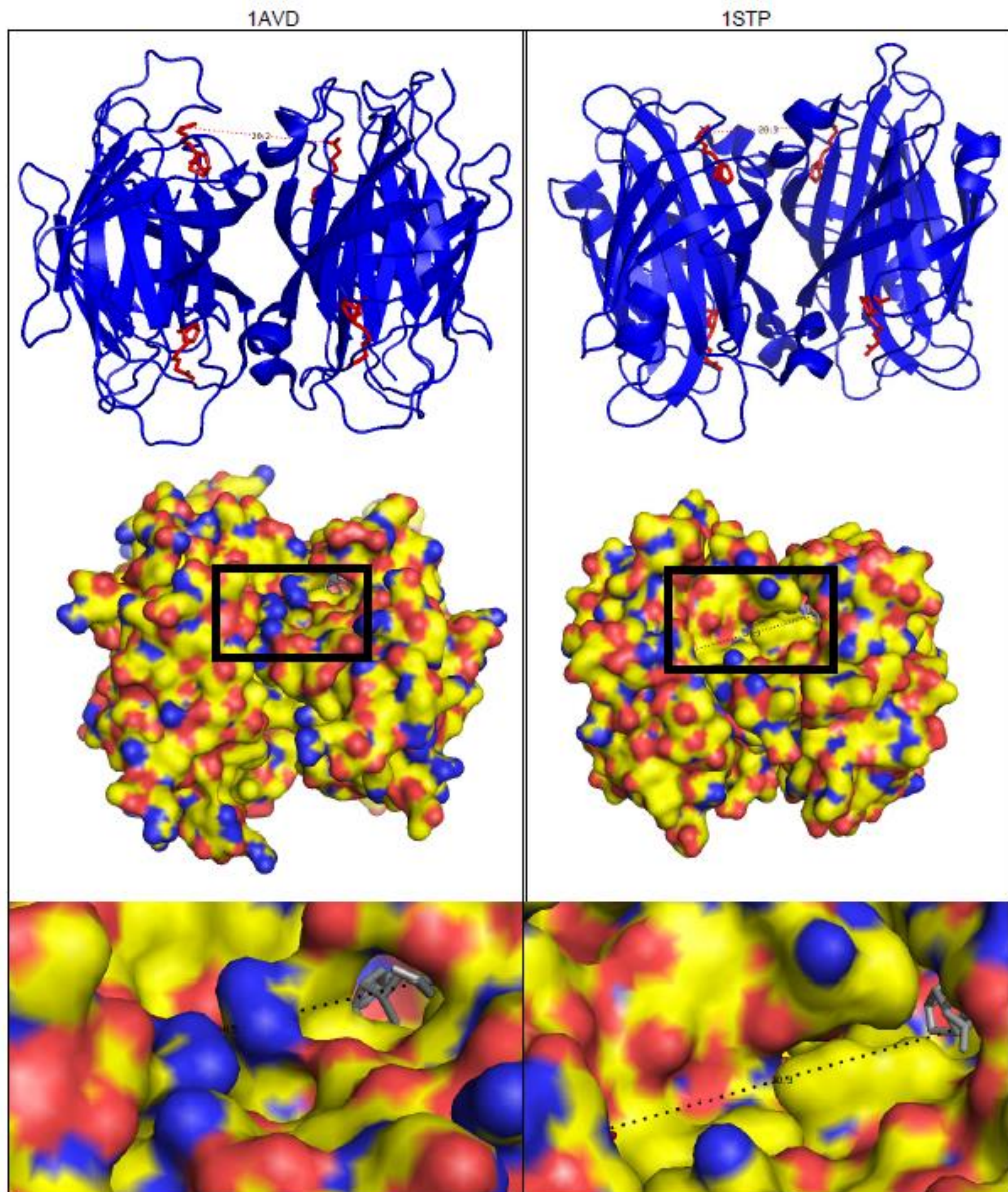


Figure 1: Structures of avidin (left, 1AVD), streptavidin (right, 1SAD). The graph was generated by Maria Wiesauer, using the coordinates from the protein data bank [Wiesauer, 2017].

1.1.4. Biotin and biotin analogues

Biotin is a small molecule with a molecular weight of 244.3 g/mol. Biotin is commonly known as vitamin H or vitamin B_7. It is water soluble in neutral and alkaline pH. Biotin molecule is formed with an ureido group, which is fused with a tetrahydrothiophene ring. Valeric acid is attached to the tetrahydrothiophene ring by creating a long tail (figure 2). Biotin acts as a coenzyme for carboxylation reactions. When biotin creates the interaction with (strept)avidin mainly hydrogen bonding and hydrophobic interactions are involved. While forming this interaction, the whole ring part of the biotin molecule get slipped into the (strept)avidin pocket up to about 6-7 Å in depth [Green et al., 1971].

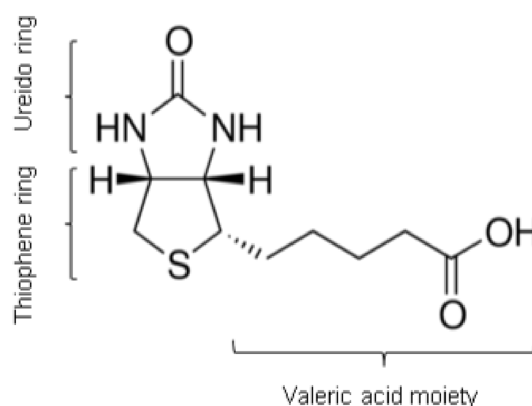


Figure 2: Structure of D-biotin

When biotin forms the interactions with (strept)avidin, initially biotin repulses solvent molecules out of the binding site of the pocket. Then a tight hydrophobic box is created around the binding site with aromatic acids. The heteroatoms of the ureido ring form five hydrogen bond interactions with the side chains of polar amino acid residues present in the binding pocket. The formation of hydrogen bond interactions is similar in both avidin and streptavidin, but the number of hydrogen bonds formed, and the side chains involved are different in two proteins. The oxygen present in the ureido forms three hydrogen bonds, with the side chains of Asn-12, Ser-16, and Tyr-33 in avidin and Asn-23, Ser-27, and Tyr-43 in streptavidin. The two ureido nitrogen forms a single hydrogen-bond interaction with Thr-35 and Asn-118 in avidin and Ser-45 and Asp-128 in streptavidin. Finally, the sulphur atom present in the thiophene ring interacts with Thr-77 in avidin and Thr-90 in streptavidin. In addition to these ring interactions, valeric acid forms five hydrogen bonds with avidin and three bonds with streptavidin [Livnah et al., 1993a].

A wide variety of biotin analogues were experimented with (strept)avidin to find out the strength of the biotin-(strept)avidin interaction under different pH systems. Biocytin is a naturally occurring biotin analogue, where an amide of biotin is formed with the ε-NH_2 group of L-lysine.

The K_D measurements reported in Tables 1 and 2 were performed in borate or ammonia buffers. The spectrophotometric titration curves were used to calculate the dissociation curves.

Table 1: Dissociation constants for biotin and biotin analogous to avidin [Wiesauer, 2017].

Compound	*p*H	K_D [M]
Biotin	6.8	10^{-15}
Biotin-ε-aminohexanoate	6.8	10^{-8}
HABA[1]	4.7	6×10^{-6}
Lipoate	6.8	7×10^{-7}
Hexanoate	6.8	3×10^{-4}
2-iminobiotin	9.0	5×10^{-8}
	free base	3.5×10^{-11}
Desthiobiotin	7.0	5×10^{-13}

[1]2-(4'-hydroxyphenylazo)benzoic acid

Table 2: Dissociation constants for biotin and biotin analogous to streptavidin [Wiesauer, 2017]

Compound	*p*H	K_D [M]
Biotin		10^{-14}
2-iminobiotin	7-11	10^{-5}
*N*3'-ethyl-biotin[1]	7.4	8×10^{-10}
Biotin carbonate[2]	7.4	6.7×10^{-6}
Biotin carbamate[2]	7.4	1.7×10^{-10}

[1] covalently modified with 1,8-diaminotriethyleneglycol
[2] covalently modified with 6-aminohexanoic acid

1.2 Binding modes of bivalent biotin analogues

Multivalency is the capability of ligands to bind more than one binding site of the acceptor molecule. To form stronger bindings, it is necessary to have greater surface for molecular interactions. Bivalent bindings open a wide path for numerous scientific experiments and discoveries. In bivalent approaches, it is possible to control the number of binding sites which is helpful analysing the results quantitatively. The structures synthesised can vary systematically and the linkers connecting to binding sites can also possible to vary [Fasting et al., 2012]. When two ligands are trying to approach the two binding sites of the same receipting molecule, there can be tree different binding approaches. In positively cooperative (synergistic) approach, when the first ligand is bound it increases the affinity of other binding site which helps the binding of the other ligand. Anti-cooperative (interfering) binding is the opposite of cooperative binding. First ligand binding decreases the affinity of the second binding site. Non-cooperative (additive) binding does not show any affinity change of the second binding site [Bertini et al., 2007].

When synthesising multivalent ligand compounds, it is necessary to consider all the necessary facts which affects the binding such as, affinities of all ligands and the length of the molecule. By forming multivalence interactions, the conformation of the ligand molecule changes. If the designed linker molecule is rigid and less flexible, it is conformationally impossible to interact with binding sites, unless the rigid structure is perfectly matching the binding site.

Biotin-fluorophore conjugates ("fluorescent biotin derivatives") are the perfect bivalent ligands to analyse the biotin-(strept)avidin interactions.

Wide range of fluorescent biotins are commercially available and by the fluorometric ligand-binding assay it is possible to quantify (strept)avidin-bivalent biotin interactions.

In the laboratory of my advisor, the properties of biotin-fluorescein conjugates were extensively studied. Initially only "fluorescein-biotin" was available which contains a long 14-atom linker between biotin and fluorescein (Figure 4). This ligand can bivalently bind to fluorescein [Gruber et al., 1998; Kada et al., 1999a], since the fluorescein residue has a significant affinity for the biotin-binding site, and that in streptavidin the protein surface between the two-binding site is less bulky than in avidin (see Figure 3). To eliminate this undesired behaviour, a previous master student of this lab (Gerald Kada) synthesized two new biotin-fluorescein conjugates with a very short 4-atom linker (see Figure 5) [Kada et al., 1999a; Kada et al., 1999b].

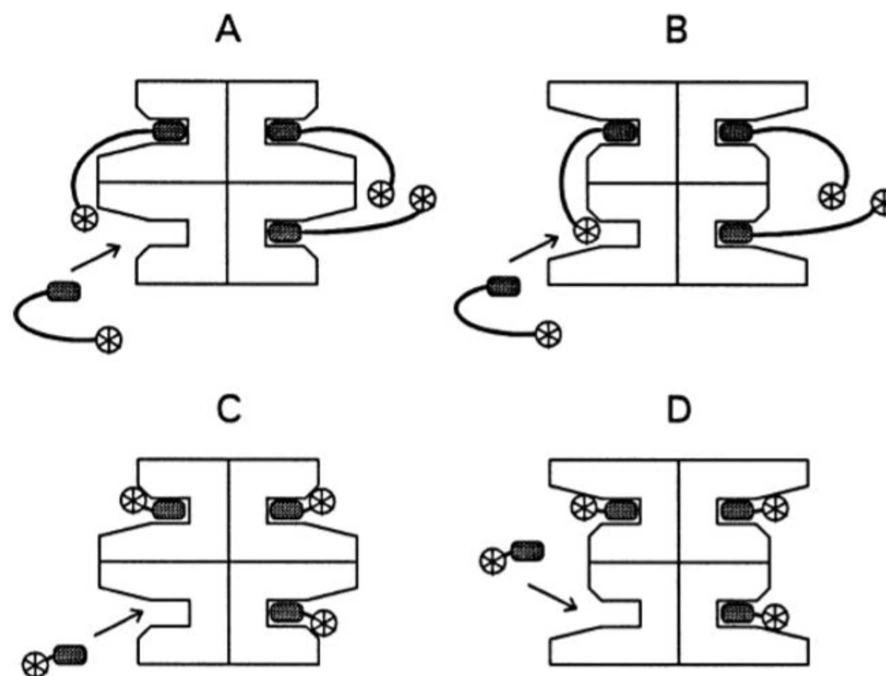


Figure 3: Model for the interaction of avidin (A, C) and streptavidin tetramers (B, D) with different biotin-fluorescein conjugates [Kada et al., 1999a].

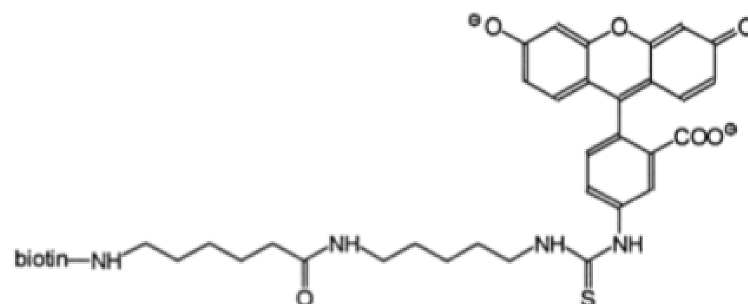


Figure 4: Structure of commercial ''Fluorescein-biotin'' [Kada et al., 1999a].

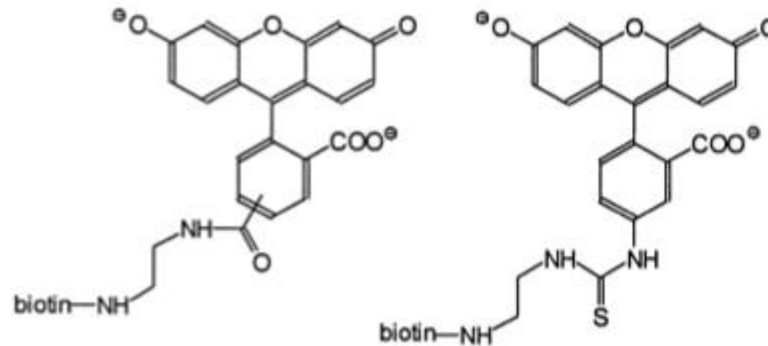


Figure 5: Left is the structure of biotin-4-fluorescein (B4F), right is the structure of biotin-4-FITC [Kada et al., 1999a].

Shorter bivalent linkers show faster association, high affinity and non-cooperative binding to (strept)avidin. Among the two short versions (Figure 5), "biotin-4-fluorescein" (B4F) showed the better performance [Kada et al., 1999a; Kada et al., 1999b]. Four molecules of B4F can easily bind to (strept)avidin and no bivalent binding were observed with it (Figure 3, C and D) [Kada et al., 1999a].

1.3 The unusual binding mode of biotin-disulphide-fluorescein (BSSF)

Several years ago, my advisor had the idea to take advantage of the unusual binding of conventional "fluorescein-biotin" to streptavidin (as depicted in Figure 3B). The idea was to synthesize an analogue of "fluorescein-biotin" (Figure 4) with the same long linker but with a disulphide in the linker chain. This "biotin-disulphide-fluorescein" (BSSF) was expected to bivalently bind to a pair of biotin-binding sites (as in Figure 3B). Then the disulphide should be cleaved by a reducing agent and this was expected to release the fluorescein group from the biotin-binding site. This system was intended to serve as an example for "caged streptavidin". The idea was successfully implemented by the master student Bianca Spitzbart, as explained in detail below.

BSSF is a unique compound with a molecular weight of 850 g/mol. BSSF has a biotin group in one end and a fluorescence group in the other end of the molecule. Biotin and florescence groups are connected via a long carbon chain, which contains a disulphide bond in the middle (gly-cystamine-gly). Speciality of this compound is that both ends of the molecule (biotin and fluorescein groups) can interact with the adjacent binding pockets of (strept)avidin simultaneously by forming a bridge like structure. The fluorescence residues can bind to empty binding pockets of (strept)avidin with a certain affinity. This effect is only visible when the biotin part of the BSSF is bound to the binding pocket [Gruber et al., 1998] or directed to the binding pocket [Barbarakis et al., 1993], [Rao et al., 1997] but not with free fluorescein groups.

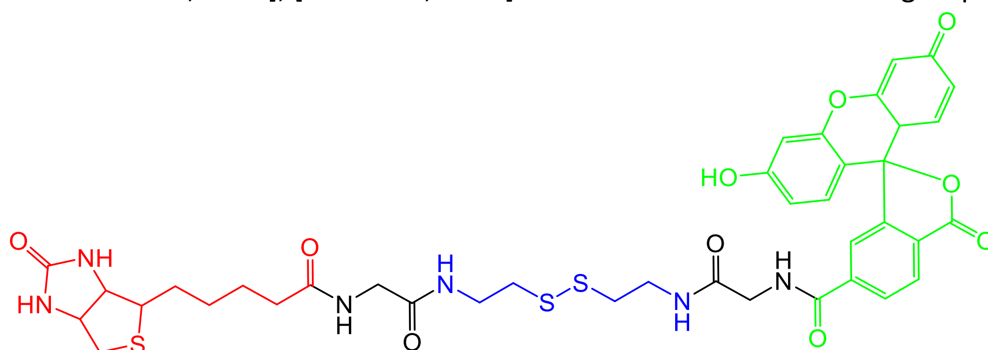


Figure 6: Structure of BSSF (red: structure of biotin, black: glycine linkers, blue: cystamine part, green: fluorescein group)

The speciality of this compound is that the disulphide linkage can be easily cleaved by using TCEP (and Hepes-Na for compensation of the strongly acidic TCEP hydrochloride). Immediately when the disulphide linkage is cleaved, the fluorescein group will lose the affinity towards the binding pocket of (strept)avidin and fluorescence group will come out of the pocket. When the fluorescence group is bound inside the pocket, it has very reduced fluorescence but after it comes out of the pocket it strongly emits fluorescence. This fluorescence can be measured by using fluorescence spectrophotometer. This effect has been shown by fluorescence titrations in Bianca Spitzbart in her master thesis [Spitzbart, 2014].

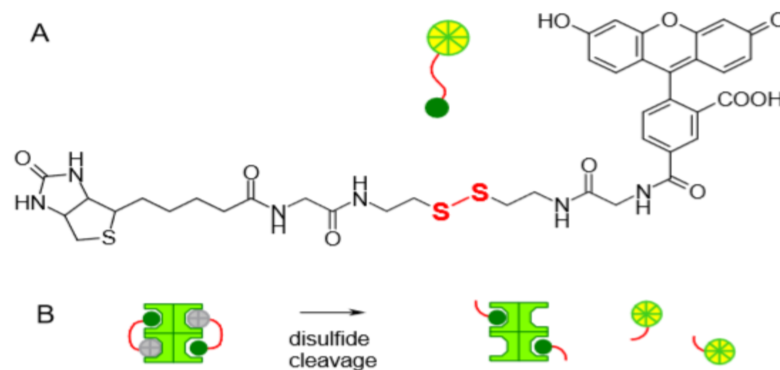


Figure 7: The structure of BSSF (A). Before and after the disulphide cleavage of BSSF and the generation of semi vacant (strept)avidin

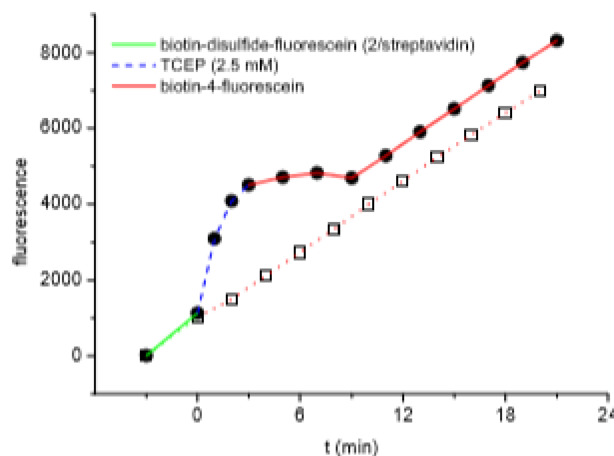


Figure 8: The titration curve of streptavidin with BSSF and B4F (fluorescence vs time) [Spitzbart, 2014].

Initially streptavidin was fully saturated with BSSF (green line). At the time zero TCEP was inserted to the system to cleave disulphide bonds. A sudden increase of fluorescence can see from 0 minutes because of the cleaved free fluorescein (blue dashed line). At 2 minutes by assuming all the disulphide bonds have been cleaved, streptavidin was titrated with B4F. Fluorescence gets diminished when B4F get bind into the free binding pockets (up to 8 minutes). Then fluorescence increases with a constant slope because of free B4F present in the system (red line).

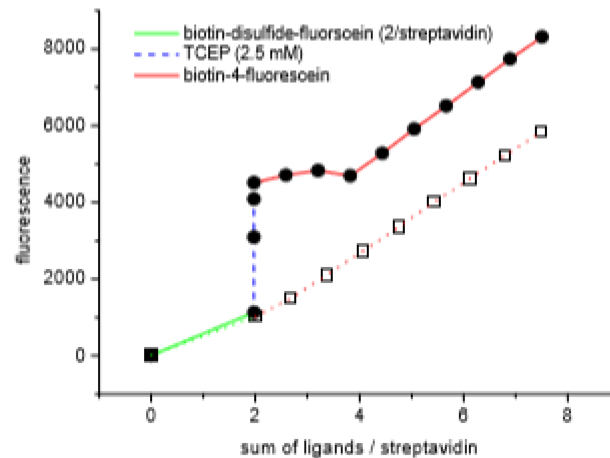


Figure 9: The titration curve of streptavidin with BFFS and B4F (fluorescence vs sum of ligands) [Spitzbart, 2014].

In this graph it is clearly visible that streptavidin gets saturated with 2 ligands, after the formation of 2:1 complex with BSSF (green line). The disulphide cleavage occurs (blue dashed line) with a concomitant increase of fluorescence. In the subsequent titration with B4F, the two vacant sites of streptavidin get filled with B4F until the 4:1 complex is reached (red line). Until the vacant sites get filled, the fluorescence remains constant; subsequently the fluorescence strongly increases with a constant slope, reflecting the further addition of B4F which is not binding to streptavidin any more.

1.4 HABA assay

1.4.1 HABA complex

2-(4'-hydroxyazobenzene) benzoic acid (HABA) is an important reporter molecule in avidin-biotin technology, which is used to determine the biotin binding sites in avidin molecules. Affinity of HABA biding is low compared to biotin binding towards (strept)avidin. Binding affinity of HABA towards streptavidin is $K_D = 1 \times 10^{-4}$, where towards avidin is $K_D = 6 \times 10^{-6}$.

HABA is a planer molecule in nature, but when the nitrogen of HABA binds to avidin it changes the conformation to its hydrazone tautomer by changing its planner sp^2 conformation to tetrahedral sp^3 confirmation. HABA bind to (strept)avidin in the same way as biotin by displacing five water molecules from the binding pocket [Livnah et al., 1993b].

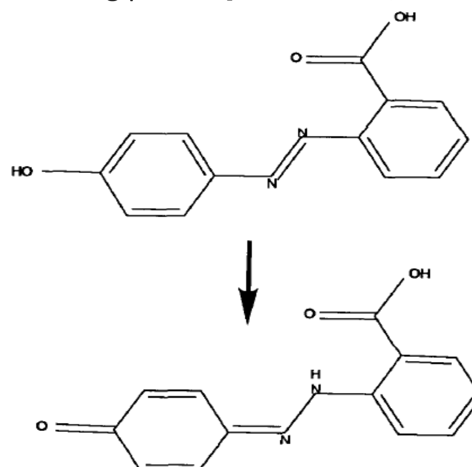


Figure 10: The two possible tautomers of HABA. The top form is the free HABA dye and the bottom form of HABA is bound to (strept)avidin [Livnah et al., 1993b].

When HABA is bound to (strept)avidin with an intense red colour, there is high UV-Vis absorption at 500 nm. When biotin replaces HABA at the binding pocket, it adopts a pale-yellow colour which has a weak absorption at 500 nm. This concept is used for HABA titrations with biotin [Livnah et al., 1993b].

1.4.2 Experimental conditions for the HABA titrations

HABA is a cumulative titration method, by which can find out the amount of biotin or (strept)avidin present in the system. HABA assay was experimentally showed by using the biotinylated target specific agent (a biotinylated peptide with a gadolinium label) molecule, which contains a biotin group (Figure 11). The stoichiometric binding was verified between this compound and avidin. HABA assay was performed by measuring UV-Vis absorption at 500 nm [Dirksen et al., 2005].

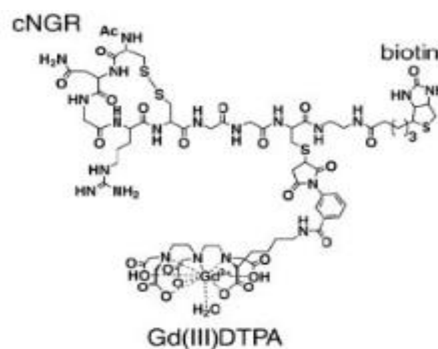


Figure 11: Gadolinium labelled biotinylated peptide with biotin group [Dirksen et al., 2005].

This titration was done by adding Gd(iii)DTPA to a solution of avidin in PBS buffer, where 5 equivalents of HABA per avidin tetramer was present. Initially excess of HABA was weakly bound to biotin binding pockets of avidin. When all the avidin binding pockets were saturated with HABA, the absorption was high. With the addition of Gd(iii)DTPA, biotin quickly replace the HABA binding at avidin pockets by resulting a decrease in the absorption at 500 nm of HABA bound avidin. When all the pockets are saturated with biotin (3.8 equivalents of Gd(iii)DTPA) there was no change of the absorption, which created a constant absorption at 500 nm. [Dirksen et al., 2005].

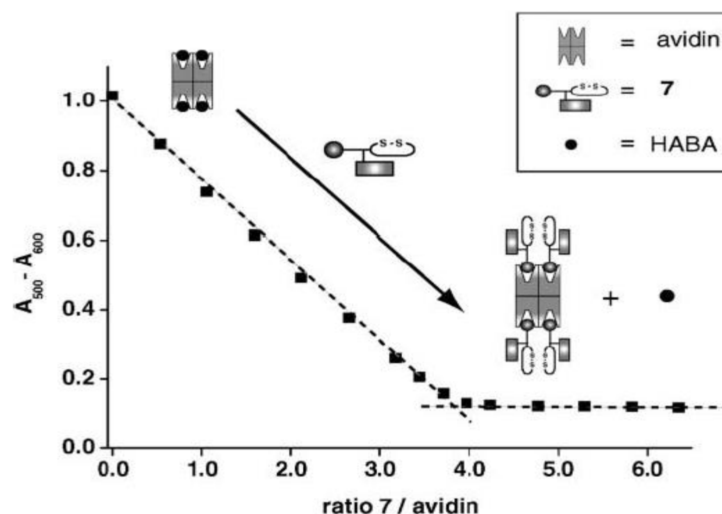


Figure 12: Graph of the HABA assay with avidin (10 µM, 0.5 mL) and Gd(iii)DTPA (0.26 mM) in PBS buffer at pH 7.4 [Dirksen et al., 2005].

1.5 Tryptophan quenching

This method is typically used to find out the amount of biotin which can bind to (strept)avidin. Tryptophan groups present in the (strept)avidin pocket are quenched by binding of biotin, which can be measured by the fluorescence changes. Amount of (strept)avidin present in solutions can be determined by titrating with biotin having a known concentration. When biotin binds in the (strept)avidin pockets, we can observe decrease in fluorescence at 350 nm, with the excitation at 290 nm. Fluorescence decrease is visible until all the pockets are filled with biotin analogues. By this method up to 20 ng of free biotin can be determined [Lin et al., 1977].

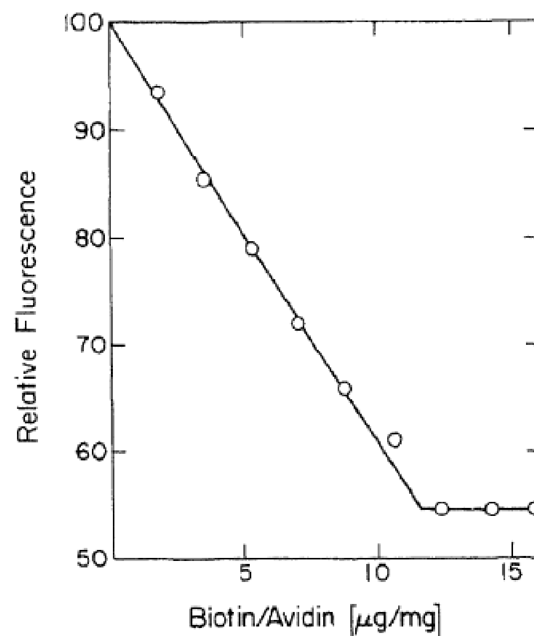


Figure 13: Avidin-biotin titration graph. 25 µg of avidin present in 2 mL of (0.15M) sodium phosphate buffer at pH 7 was titrated with 90 µM solution of biotin. Fluorescence was measured at 350 nm with the excitation of 290 nm [Lin et al., 1977].

Addition of biotin to avidin reduces Tryptophan fluorescence almost by 50%. After all the sites are occupied, fluorescence does not decrease further.

1.6 Fluorescence correlation spectroscopy (FCS)

FCS is a method by which can analyse the special resolution at very low concentrations (nanomolar concentration) of the molecules and by this can determine the local concentrations, inter/ intramolecular reactions of the molecules which have being labelled with fluorescents. FSC was developed in early seventies to analyse molecular relaxations, which influenced by temperature or pressure changes in a reaction system. Now with FCS, the minute spontaneous changes due to physical parameters can be observed by measuring the fluorescence emission of the molecules. For FCS measurements, less than femtoliter of the sample solution is needed, which is an aqueous buffer solution with the fluorescence labelled molecules [Schwille & Haustein 2004].

1.6.1 Instrumentation of confocal fluorescence microscope

The excitation radiation was created by using a laser beam (argon or argon-krypton laser), which is directed and focused to the sample by the help of a dichroic mirror and travels through the objective. The fluorescence light emits from the sample molecules also collected by the same objective and allowed to pass through the dichroic mirror and the emission filter towered the lens. The lens will focus the light to the image plane. The pinhole will block any fluorescence light, which was not originated from the sample at the focal region. Emitting unwanted radiations is called the axial resolution. After the filtration, light is focused to the detector which is a photodiode or a photomultiplier [Schwille & Haustein 2004].

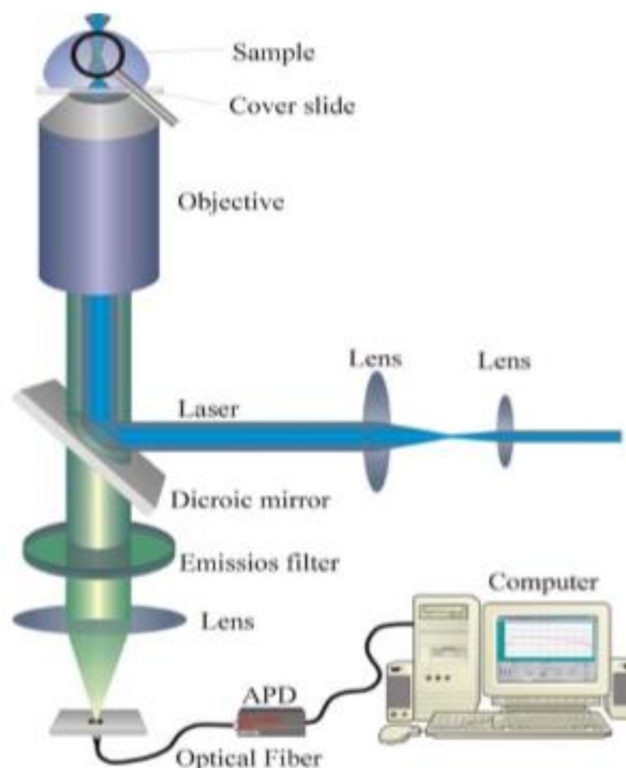


Figure 14: Schematical diagram for an FCS setup

1.6.2 Theory behind the fluorescence measurements

The fluorescence from a tiny volume of the liquid sample is constantly monitors over the time and the amount of the light reaches the detector is controlled by the pinhole. The fluorescence time course F(t) is recorded by the computer. The fluctuations are visible in the spectrum only when the partial number in the imaged volume is low enough to contain different number of particles at different times [Ries & Schwille 2012].

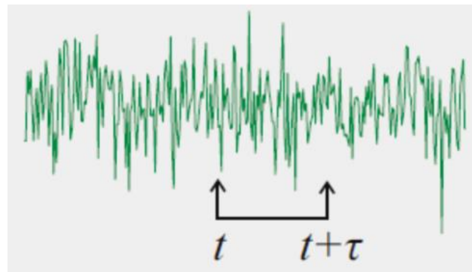


Figure 15: Fluorescence spectrum fluctuations due to difference of the number of particles in different times

The below graph (Figure 16) shows the ideal situation, where one particle is diffuse through the fluorescence image volume (a). The fluorescence fluctuation due to one particle movement F(t), gives a Gaussian shaped curve for with the time difference has showed in (b). As shown in the panel (c), the autocorrelation function G(τ) is generated by shifting a copy of the graph F(t) along the time axis. G(τ) can be calculate by multiplying the product of the shifted function F(t+τ) with the original function F(t) and divide it by the square of the function F(t). When F(t) move along the time axis, graphs create an overlap. The product corresponds to the overlap of the two functions has being indicated by different colours in the panel (d). The total function G(τ) is plotted against a logarithmic time axis, by creating a sigmoidal shape (e). The points for this curve was taken from highest fluorescence value of the peaks, which were created by the overlap of two functions [Schwille & Haustein 2004].

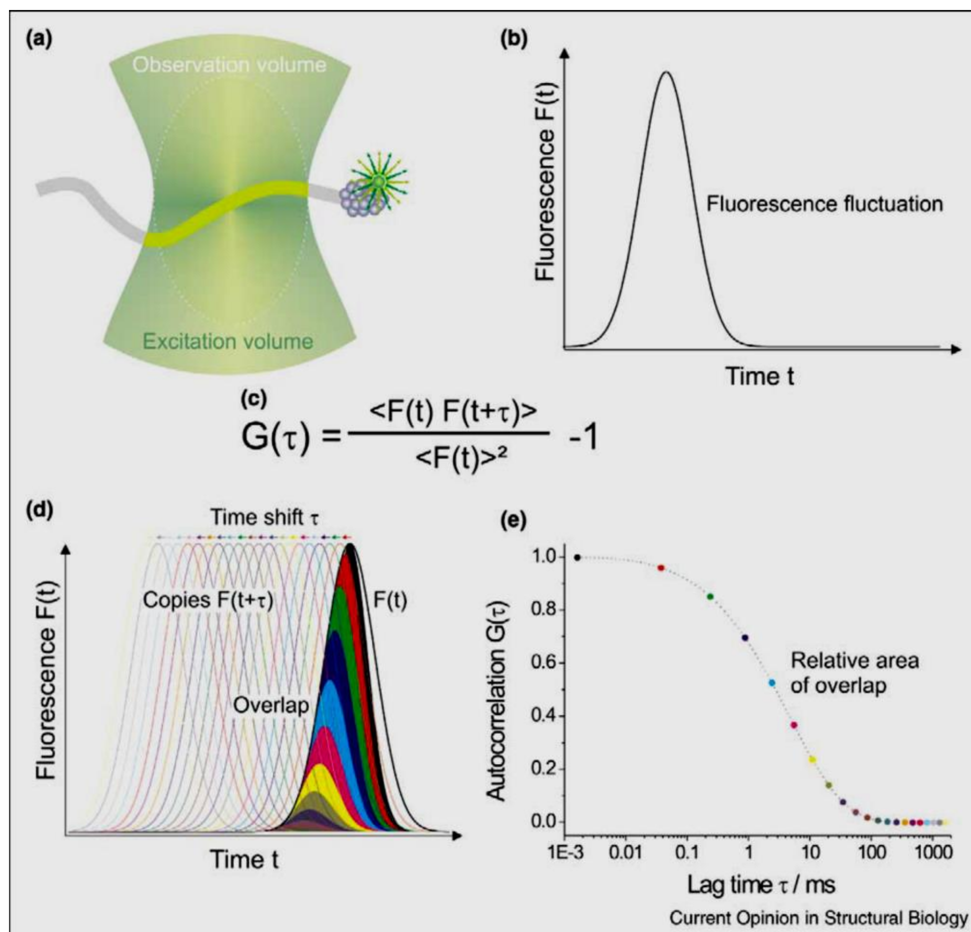


Figure 16: The idealized representation of one particle diffusion in a confocal microscope and explanation of the autocorrelation function [Schwille & Haustein 2004].

The τ value where, $G(\tau) = G(\tau=0)/e$ is called the correlation time. The spherical shaped molecules have the correlation time which is proportional to the square root of the molecular weight.

The effect of the different molecular weight of the different particles is illustrated below in the figure 17 (a-d). When the molecular weight of the particle gets higher, the values of the correlation times shift to higher by increasing the diffusion time. The molecular weight increment by a factor 2 gives very little change in the correlation time, which is far too small for a measurement.

The G_0 value is inversely proportional to the average number of particles in the imaged volume.

$G(\tau \to 0) = 1 / N$

N = particle number in the focus

The effect of increasing the number of particles per image (concentration increment) is demonstrated in panels (e-h). The panel (e) illustrates how few particles lead to high amplitudes of the relative fluctuations and in panel (h) a high G_0 value in the correction function. When the concentration of the image increases it leads to a low G_0 value in the correction function.

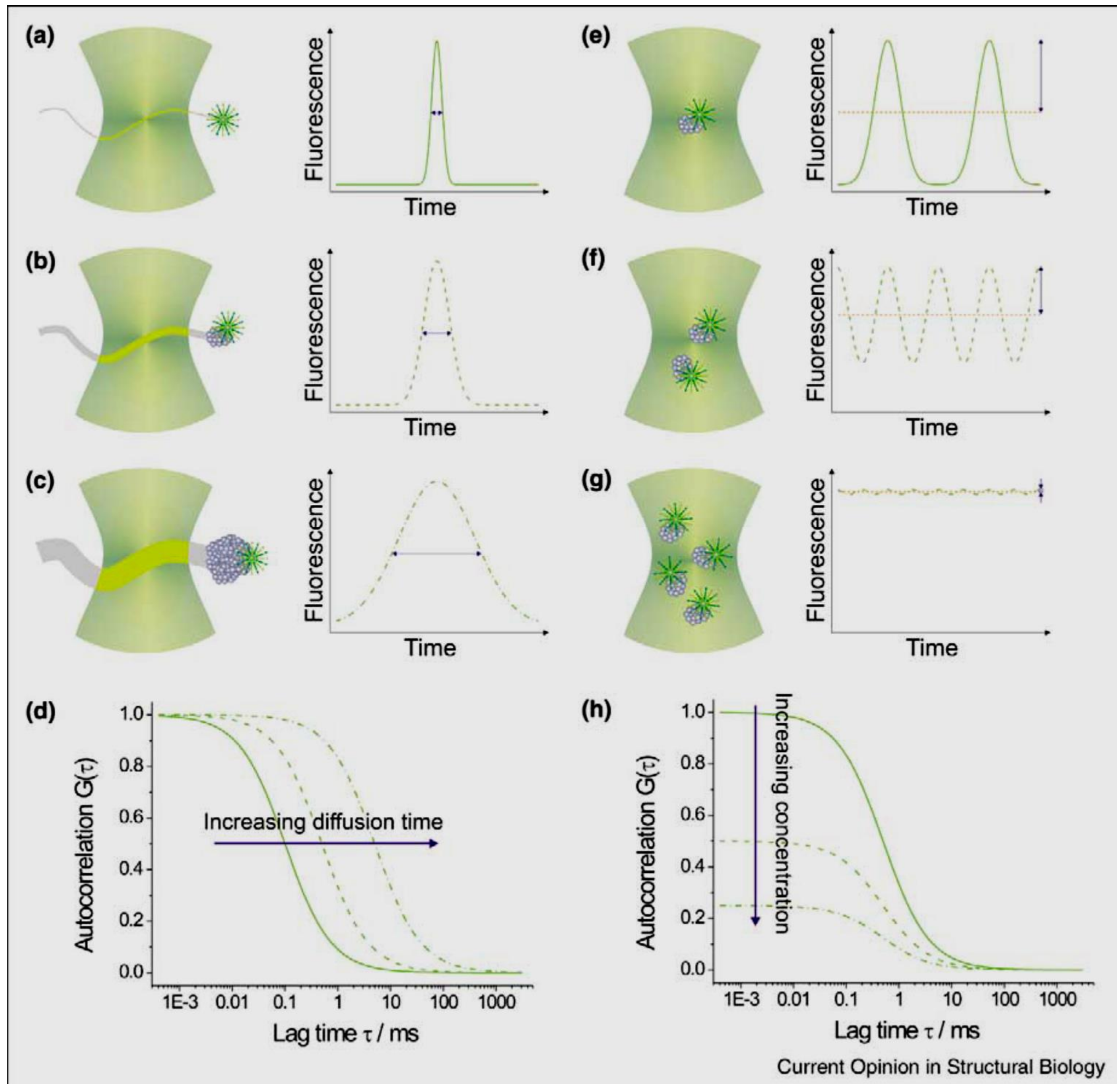


Figure 17: The effect of different molecular weights in FCS imaging [Schwille & Haustein 2004]

1.7 Objectives / aims

The first task of my master thesis was the synthesis of the BSSF ligand which contains biotin in one end and fluorescein in the other end. This ligand contains disulphide bridge in the middle which can cleave easily when it is necessary. Synthesis of biotin-gly-cystamine-gly-fluorescein had 4 reaction steps staring from the compound boc-gly-cystamine-gly-boc, which was previously synthesised by a co-lab worker. After each step, product confirmation was done by using MS and NMR methods.

The second task was to find a model system where the uncaging of BSSF-blocked streptavidin can be demonstrated in a fluorescence microscope. The idea was to immobilize streptavidin on a surface, to block it with BSSF, and to wash away excess of BSSF. In this state, no biotinylated antibody (carrying a fluorescent label) was expected to bind to streptavidin. After cleavage of the disulphide in bond BSSF, the fluorescein groups were expected to dissociate and biotinylated antibody (carrying a fluorophore) should be bound.
To demonstrate the above concept, we did not want to do surface chemistry on glass slides. Instead, we wanted to use commercial streptavidin-agarose for this purpose. When we found out that the high amount of streptavidin bound on each bead made it impossible to use these beads, we decide to activate Sephadex G-15 because here streptavidin can only couple to the outer surface.

The third task was taken over by my colleague Luise Herrman. She repeated the titrations of avidin and streptavidin with my BSSF which Bianca Spitzbart had reported in her master thesis. After doing the titration, we have found out that there is a large difference of the amount of BSSF bound to (strept)avidin which I made and which Bianca Spitzbart had shown in her thesis. According to Bianca Spitzbart's thesis only two BSSF molecules were bound to (strept)avidin but with the BSSF I made four molecules got bound to (strept)avidin.

The final task was to show the uncaging of BSSF-blocked streptavidin in FCS experiments. For this purpose, streptavidin had to be labeled with Alexa647. The idea was to block this streptavidin with 2 molecules of BSSF. In this state it should not be able to bind to 8-arm PEG molecules carrying biotin groups on all arms. Addition of TCEP was expected to cause cleavage of the disulphide and release of fluorescein. Now the Alexa647-labeled streptavidin was expected to be dramatically aggregated by the 8-arm PEG carrying the 7-8 biotin groups. But after seeing the titration results, we have decided that there is no point of doing FCS experiments to show the uncaging of BSSF-blocked (strept)avidin.

2. Results and discussions

2.1 Linker chemical synthesis

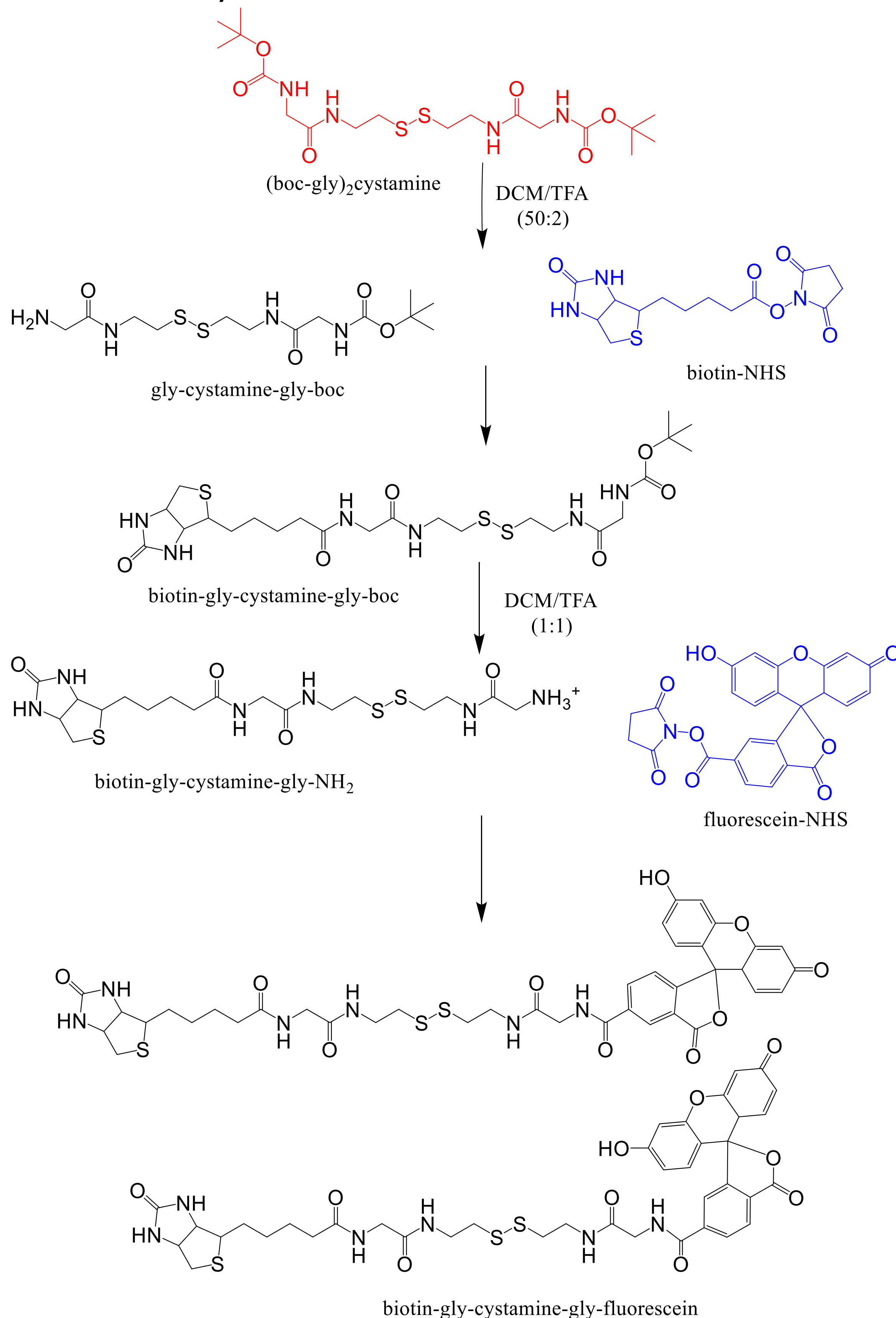


Figure 18: Synthesis pathway of the biotin-gly-cystamine-gly-fluorescein

Boc-gly-cystamine-gly-boc which was used for this chain synthesis was available from Bianca Spitzbart. The detailed procedure for the synthesis of biotin-gly-cystamine-gly-fluorescein from boc-gly-cystamine-gly-boc is described in chapter 3.2 as linker chemical synthesis.
Reaction was conducted under the flow of argon and every 15 minutes checked with TLC to identify the formation of the desired product (NH_2-gly-cystamine-gly-boc) and the side product (NH_2-gly-cystamine-gly-NH_2). For all the synthesis experiments, TLCs were run in the solvent system of CME (80:20:2).
When 50% of boc-gly-cystamine-gly-boc was reacted according to the colour brightness of the boc-gly-cystamine-gly-boc spot on TLC, the reaction was stopped by adding excess of toluene. Then all the solvents were evaporated by using rotary evaporator.
Purification of the desired product (NH_2-gly-cystamine-gly-boc) from the side product (NH_2-gly-cystamine-gly-NH_2) and unreacted boc-gly-cystamine-gly-boc was done by using a tricky form of multistep extraction. To carry out the extraction, solid sample was dissolved by using a mixture of dilute acetic acid (pH 4) with 50% sodium chloride saturation. The extraction scheme was based on the fact that the di-boc molecule (Figure 19) partitions into chloroform at all pH values, that the diamine always remains in water at any pH value, while the mono-boc-mono-amine partitions into water at low pH and into chloroform at high pH. Hereby it was essential to use vigorous argon bubbling instead of shaking to avoid oxidation of the deprotonated amino groups at high pH.
Unreacted boc-gly-cystamine-gly-boc was removed by extracting it into chloroform (× 3). Then the aqueous layer was basified by using 10% sodium carbonate (pH 9) to deprotonate the -NH_2 groups. Then the extraction was carried out with chloroform (× 3) to extract NH_2-gly-cystamine-gly-boc into it. Side product NH_2-gly-cystamine-gly-NH_2 was remained in the aqueous layer and it was discarded. To get rid of citric acid present in the product (NH_2-gly-cystamine-gly-boc), chloroform layer with NH_2-gly-cystamine-gly-boc was washed by using 10% sodium carbonate under the flow of argon. Finally, the chloroform layer with the deprotonated NH_2-gly-cystamine-gly-boc was drained into an excess of toluene with a low concentration of TFA before all solvents were removed on the rotavap.
Maximum obtainable yield was 17.17% for this reaction. Initially few test reactions were carried out by using small scales like 5 mg portions and after the proper procedure was identified, the reaction was carried out with a larger batch of 400 mg. It was hard to obtain a better yield because, “boc” groups of the boc-gly-cystamine-gly-boc is easily converting to the di-amine groups. My task was to stop the reaction where maximum mono anime product was formed. It was hard to decide the maximum yield point by analysing the brightness of the product spots on TLCs.

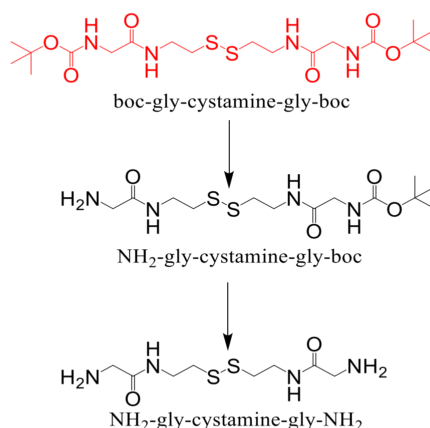


Figure 19: Conversion of di-boc groups to mono-amine and di-amine groups

The next step of the chain reaction series was to produce biotin-gly-cystamine-gly-boc by using NH_2-gly-cystamine-gly-boc. For this, the product from the previous reaction was used. Biotin-NHS and DIPEA were used according to the molar ratio of 2:6 in the excess of DMSO solution. Reaction was carried out overnight under the flow of argon and reaction completion was monitored by using TLCs. When the NH_2-gly-cystamine-gly-boc spot is no longer present in the TLC, the reaction was stopped.

The unreacted excess of biotin-NHS was removed by adding 10 equivalents of 6-aminohexanoic acid with the excess of acetonitrile, DIPEA and water. Reaction was carried out for half an hour at the pH of 8. Acetonitrile was evaporated at rotavap and other solvents were evaporated in high vacuum cool trap. In this way the nonpolar biotin-NHS was converted into biotin-Ahx-COOH plus free NHS which could be extracted into water at high pH.

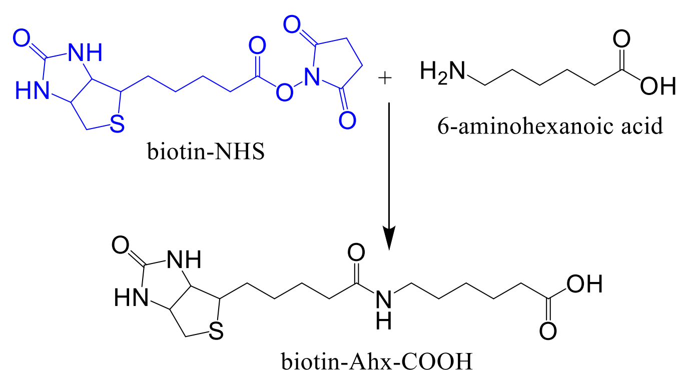


Figure 20: Biotin-NHS with 6-aminohexanic acid reaction

Residue was dissolved chloroform and was extracted with 10% sodium carbonate solution (× 2) to separate out biotin-Ahx-COOH into the aqueous layer. The chloroform layer was extracted with 0.1% phosphoric acid in half brine solution (× 2), with 0.1% phosphoric acid in full brine solution (× 2) and was dried it out by using rotary evaporator. The obtainable maximum yield was 54.24%.

The “boc” deprotection was the next step, which was done by using biotin-gly-cystamine-gly-boc and formed biotin-gly-cystamine-gly-NH_2. Biotin-gly-cystamine-gly-boc was stirred for 3 hours under the flow of argon in the presence of DCM and TFA in 1:1 ratio. After 3 hours, biotin-gly-cystamine-gly-boc spot was no longer in TLC, therefore the reaction was stopped.

Biotin-gly-cystamine-gly-$NH_2$was directly used (without any extractions) for the last step of the chain reaction series to form the final product of biotin-gly-cystamine-gly-fluorescein. Biotin-gly-cystamine-gly-$NH_2$was mixed with fluorescein-NHS and DIPEA in the molar ratio of 1.2:10 and was dissolved by using excess of DMSO. Fluorescein-NHS is a light sensitive compound, due to that flasks were always covered with the aluminium foil. Reaction was stirred overnight under the flow of argon. Next morning 5 equivalents of ethanol amine were added into this and was stirred for 3 hours. All the solvents were fully evaporated by using high vacuum cool trap. For product separation, reverse phase HPLC was used. The obtainable maximum yield was 51.85%.

For all the steps, product analysis and quantification were done by using mass spectroscopy, ^{1}H NMR and ^{13}C NMR.

2.2 Protein labelling with biotin and/ or fluorophores for bead experiments

2.2.1 Formation of doubled labelling of goat IgG with biotin-Ahx-NHS and Alexa-fluoro-647-NHS

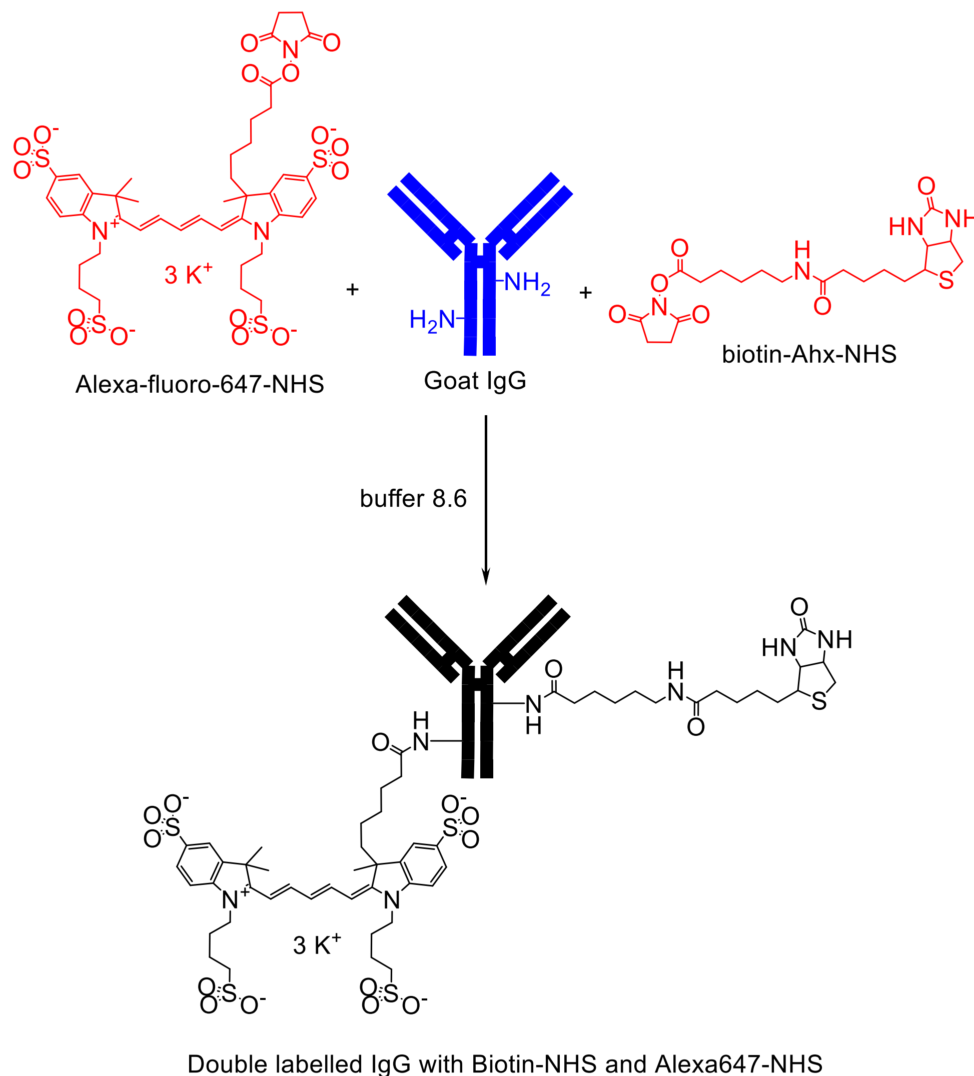


Figure 21: Labelling of goat IgG with biotin-NHS and Alexa-fluor-647-NHS

The detailed labelling procedure for the goat IgG with biotin-Ahx-NHS and Alexa-fluoro-647-NHS is described in chapter 3.3.1.

Goat IgG was mixed with biotin-Ahx-NHS and Alexa-fluoro-647-NHS according to the ratio of 1:4:6. All the reactants were dissolved by using 0.1 M glycine (pH 8.5). Doubly labelled product was separated by size exclusion chromatography. The number of Alexa-fluoro-647-NHS molecules attached per IgG was calculated by measuring UV-Vis absorbance at 652nm for Alexa647 dye and 280nm for IgG. Absorbances were subtracted by the correction factor and divided from molar absorptivity (ξ) for each. According to the calculations 2.6 Alexa-fluoro-647 dyes were attached to one IgG molecule.

2.2.2 Labelling of BSA with Alexa-fluoro-647-NHS

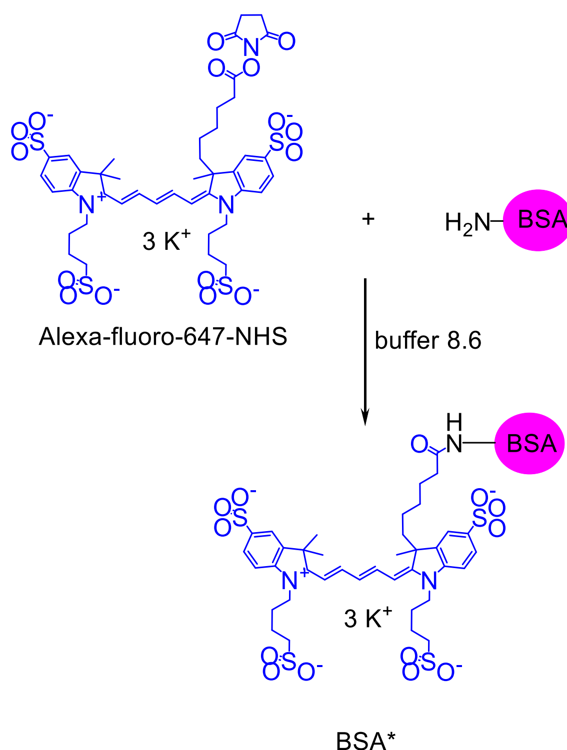


Figure 22: BSA labelling with Alexa-fluoro-647-NHS

The detailed labelling procedure for the BSA with Alexa-fluoro-647-NHS is described in chapter 3.3.2.

Formation of BSA labelling with Alexa-fluoro-647-NHS dye was done according to the ratio of 1:2 and with 0.1 M glycine (pH 8.5) the reactants were fully dissolved. Gel filtration was used to separate out the specific labelling of BSA and binding ratio of the dye was calculated measuring UV-Vis absorbance for the dye and BSA. 1.14 dyes per BSA molecule was attached according to the calculations.

2.2.3 Labelling of streptavidin (SAV) with Alexa-fluoro-647-NHS

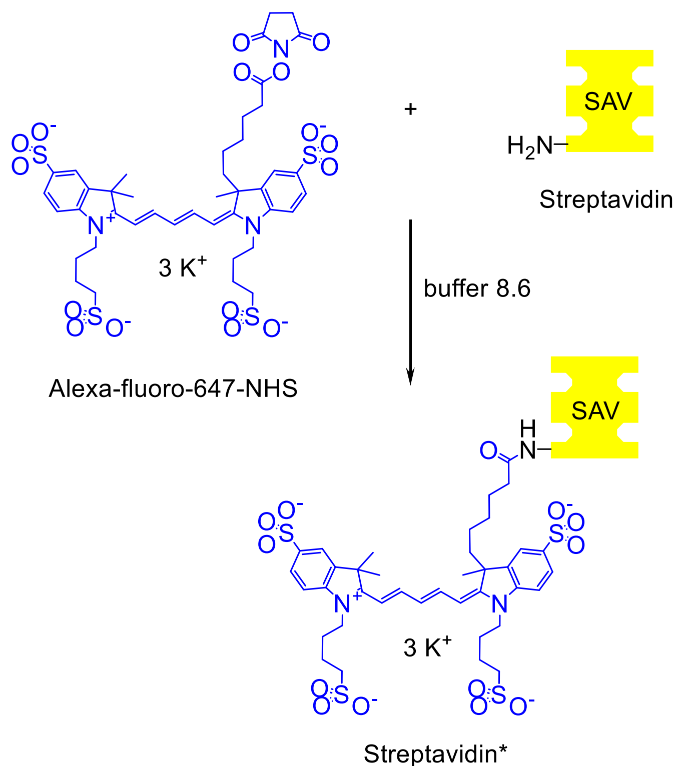


Figure 23: SAV labelling with Alexa-fluoro-647-NHS

The detailed labelling procedure for the Streptavidin with Alexa-Fluoro-647-NHS is described in chapter 3.3.3. Formation of SAV labelling with Alexa-Fluoro-647-NHS dye was done according to the ratio of 1:2 with SAV and dissolved in the excess of 0.1M Glycine (pH 8.5). Dialysis tubing was used to separate out the specific labelling of SAV in coupling buffer at pH = 8.5. The binding ratio was calculated by using UV-Vis absorption. The results have shown that per one BSA molecule, 2.3 Alexa647 dyes were attached.

2.3 Functional experiments with streptavidin beads

2.3.1 Test of streptavidin-agarose for its capacity to bind biotinylated biotin-IgG-Alexa647 (biotin-IgG*)

The detailed experimental procedure is described in the chapter 3.4.1. One Eppendorf of 50% SAV slurry was incubated with free biotin (10 nmol). A series of biotin-IgG* (16,32,64,128 pmol) in 50% SAV slurry was prepared. Another Eppendorf was prepared with 10 nmol of free biotin and with 16 pmol of biotin-IgG*. After the incubation and sedimentation, supernatants were diluted with PBS buffer to obtain the correct concentration for the fluorescence measurements. The measurements were done at the excitation of 630 (10) nm and the emission of 670 (10) nm, at 700 volts. The parameters for the fluorescent measurements and dilution with buffer is same for all the experiments with biotin-IgG* described below.

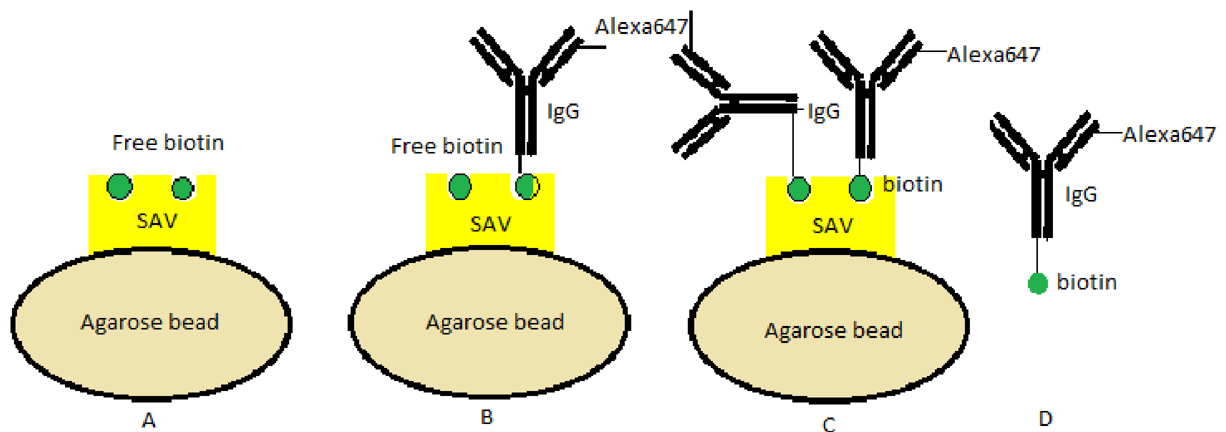


Figure 24: Pictorial description for the experiment

A is the SAV beads which was incubated with free biotin. B is the SAV beads incubated first with biotin-IgG* and then with free-biotin. C is the fully saturated SAV beads with biotin-IgG*. D is the excess (unbound) biotin-IgG* which present in the supernatants.

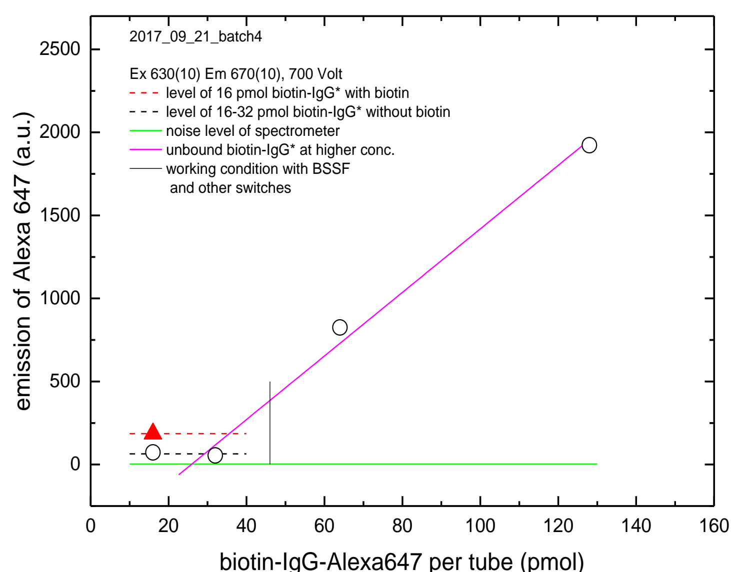


Figure 25: Supernatants fluorescence measurements for the excess (unbound) of biotin-IgG-Alexa647

When beads are saturated with free-biotin and if even there are free-biotins in the supernatant, there will be no fluorescence signals. Because free-biotins have no florescence active dyes attached to it self.

The red triangle is the fluorescence emission level where the beads were incubated with free-biotin and then with biotin-IgG*. First all the free-biotin which have being added, will attach to the SAV pockets. Then with the biotin-IgG* addition rest of the available pockets will be filled. The amount of emission will be obtained with the excess of biotin-IgG* present in the supernatant.

The black outline oval points are the emission measurements of the supernatants from the biotin-IgG* series with SAV beads. The green line is the noise level for the measurements and purple line shows the unbound excess of the biotin-IgG*.

According to the graph, all the streptavidin gets saturated at the presence of around 30 pmol of biotin-IgG*. At the presence of 46 pmol (black straight line) biotin-IgG*, streptavidin is at more than 50% saturation point. The conclusion that made from this experiment was to add 46 pmol of biotin-IgG* in further experiments, for the fully saturation of 50% streptavidin (use the same volume of SAV bead slurry as in this experiment).

2.3.2 Test of streptavidin-agarose for its capacity to bind biotinylated biotin-4-fluorescein (B4F)

The detailed experimental procedure is described in the chapter 3.4.2. A reference series was made by using 16, 32, 64, 128, 512, 1024 pmol of B4F without 50% SAV slurry. Another series of B4F was prepared with 50% SAV slurry with the same amount of BSF. A one special Eppendorf was incubated with 10 nmol free- biotin and then with 16 pmol B4F. All the mixtures were allowed to incubate (15 minutes) and sediment. The fluorescence emission was measured with all the sample supernatants at excitation 490 (10) nm and emission 525 (10) nm with suitable dilutions. Always fluorescence measurements with B4F were done with same parameters.

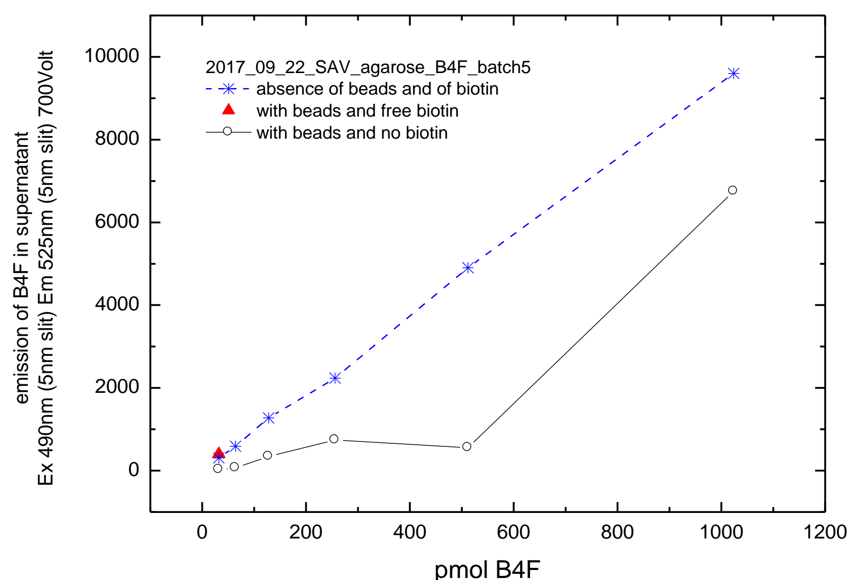


Figure 26: Emission measurements for the free biotin and biotin-4-fluorescein (B4F) binding to streptavidin agarose

The blue dash line of the above graph (Figure 26) is for the fluorescence emission of the B4F (supernatant) without SAV beads. The black line illustrates the B4F incubation with SAV beads. The red triangle is the fluorescence emission for the supernatant of the beads with free-biotin and B4F incubation.
According to the black line graph, packed beads can apparently bind up to 500 pmol B4F and higher amounts remain unbound. Because of the unbound B4F, the emission increases rapidly.
The vertical difference from 512 to 1024 pmol B4F, with and without beads corresponds to the amount of binding.
By analysing the results from the previous experiment (2.3.1), the estimation was that with 46 pmol biotin-IgG*, streptavidin is at more than 50% saturation point. But in this experiment, SAV consumes 500 pmol to obtain the saturation. There is a large experimental uncertainty between two experiments.
In 2.3.1, repeated vortexing was done for 15 minutes but in 2.3.2 frequent vortexing was done to the samples. According to the large difference of the binding amount, can conclude that time of vortexing affects the amount of binding.

2.3.3 Time course of binding of biotin-IgG* to SAV agarose

The detailed experimental procedure for the time course effect of binding is described in the chapter 3.4.3. Sextuplicate were prepared with SAV 50% slurry with 46 pmol biotin-IgG*. The samples were incubated according to the different time scales, such as 5, 10, 20, 30, 40, 60 minutes with frequent vortexing. Fluorescence emission of each supernatant was measure after incubation and sedimentation of beads. The test was repeated once more to get the best results.

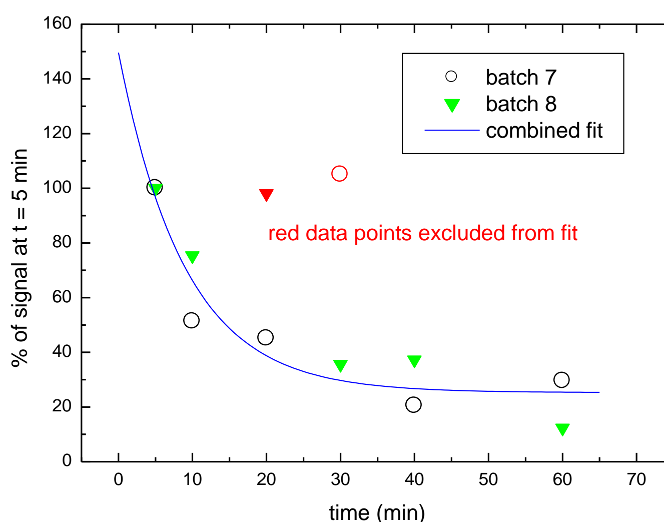


Figure 27: Normalization curve for the initial fluorescence value (=100)

In the above graph (Figure 27), experimental values from duplicate test 2.3.3 (green triangles and black outline ovals) were combined as one data set and 2 outliers were masked in red. The combined data was fitted by monoexponentially decay. According to the graph, after 35 minutes of incubation percentage of the signal gets almost constant. At 60 minutes we can assume that all the bindings are completed. From here onwards, all the other tests were carried out with the incubation time of 60 minutes for SAV beads with biotin analogues.

2.3.4 Test for specific and nonspecific binding of biotin-IgG* to SAV agarose with optimized incubation protocol

The detailed experimental procedure is described in the chapter 3.4.4. 10 nmol of free-biotin were incubated for 15 minutes and then 46 pmol biotin-IgG* were incubated for 60 minutes with SAV beads in quadruplicates. Another set of quadruplets were prepared only with biotin-IgG* incubation. Fluorescence measurements were done with all the supernatants.
The pallets were prepared with the SAV beads for the scanning in the confocal laser microscope with 1% bead concentration.

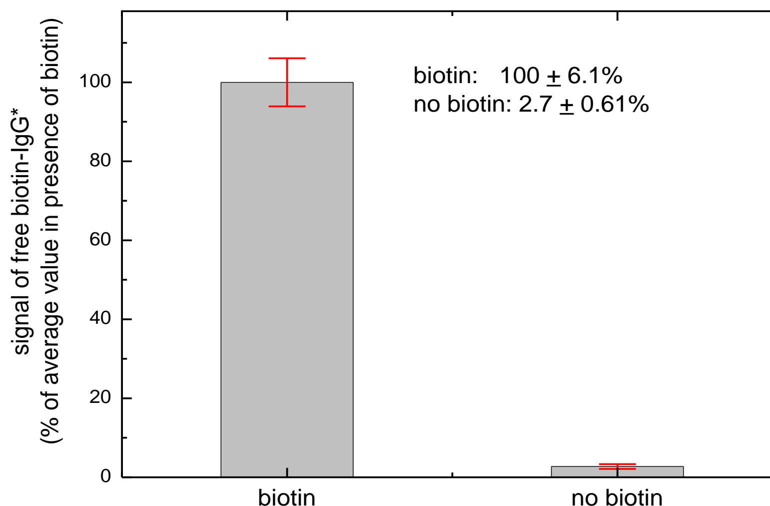


Figure 28: Block/ unblock with free-biotin and biotin-IgG* binding to SAV

The above graph shows the signal (in percentage) from excess unbound biotin-IgG* which was found in the supernatant of streptavidin-agarose. The left bar values were measured from the supernatants, which were pre-blocked with 10 mM free biotin and right bar values were measured from the unblock beads.
When free-biotin was added first (biotin bar graph), biotin was easily bound to SAV pockets and when biotin-IgG* was added to it, there were no free binding pockets it to get bind. Therefore biotin-IgG* was remain in the supernatant and gave a larger signal. But when free-biotin was not present (no biotin bar graph), biotin-IgG* was directly get attached to the pockets and there was very little amount of unbound biotin-IgG* was present in the supernatants.
When free-biotin was present, biotin-IgG* present in the supernatant is 100% (an assumption) and without free-biotin, the percentage of biotin-IgG* in the supernatant was 2.7%. The vortexing for 60 minutes was much efficient while 2.7% of biotin-IgG* was remained in the supernatant in the beads without free-biotin.

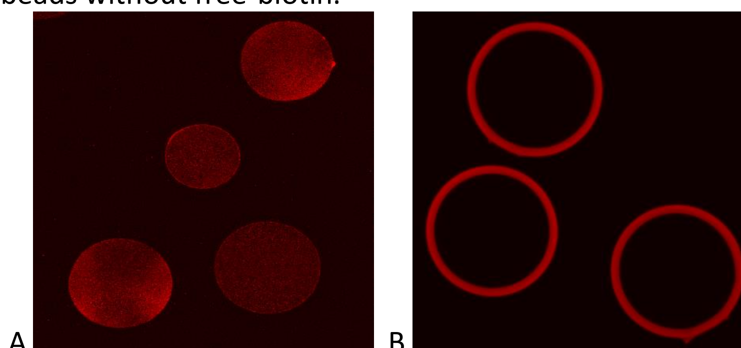


Figure 29: Confocal laser microscope images for samples with free-biotin block (A) and samples without free-biotin block (B)

In the microscopy data can see that beads with free-biotin has no fluorescence compared to the beads without free-biotin. When free-biotin was not present, biotin-IgG* was attached to SAV molecules which were on the surface of the beads, by creating a very intense fluorescent shell over the beads.
Analysis of the bead images were done according to the procedure described below and the same procedure was used for the further experimental image analysis as well.

Microscope image analysis

Figure 30: Cross section of the bead with bound biotin-IgG* (prepared in absence of free-biotin)

For each sample 3 different beads were chosen, and cross-sectioning was done by using the program 'Image J'. These cross sections were plotted with the program 'Origin'. Linear fitting from the left segment was done at the distance from 0 to 80 and Y value was taken (3.321E6).

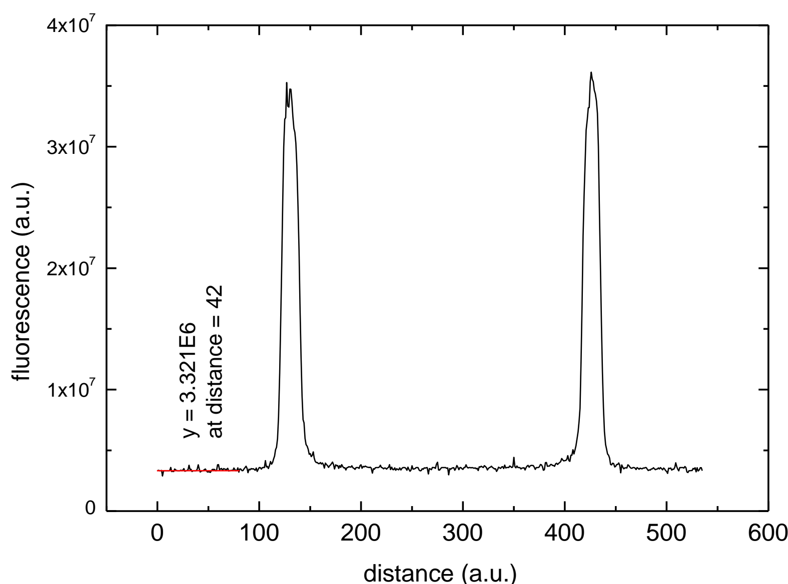


Figure 31: Cross section diagram for the beads without free-biotin with the distance in arbitrary units (a.u.). Y value was subtracted from all data point, to set the base line at 0 fluorescence emission.

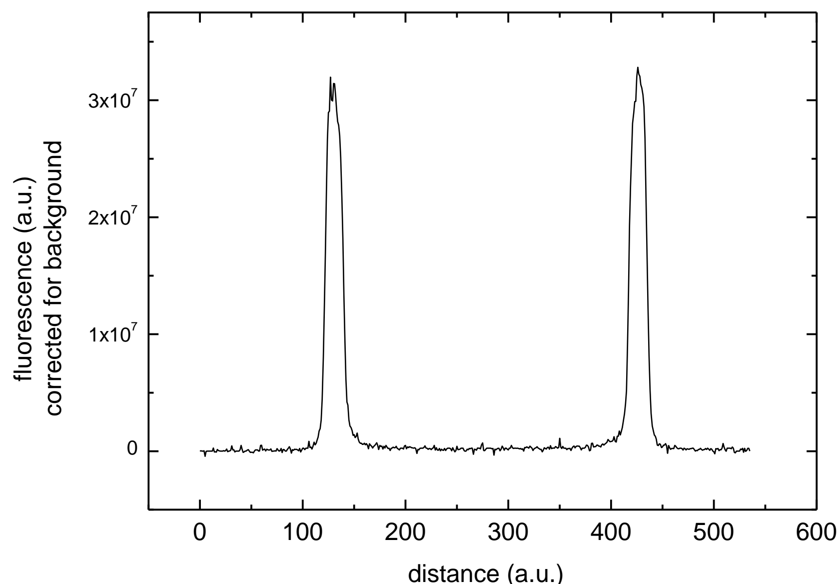


Figure 32: Cross section diagram for the beads without free-biotin with the distance in percentage and base-line correction

X value of left border was set to 0 and right border was set to 100, where Y values were almost 50% of the maximal value (vertical green lines). The fluorescence intensity of the core was determined by a liner fit from 20 to 80% (horizontal red line). At the 50% distance, the intensity was determined (173E3). The fluorescence intensity of the shell was estimated by the eyesight, as the average value of the left peak intensity (horizontal blue line).

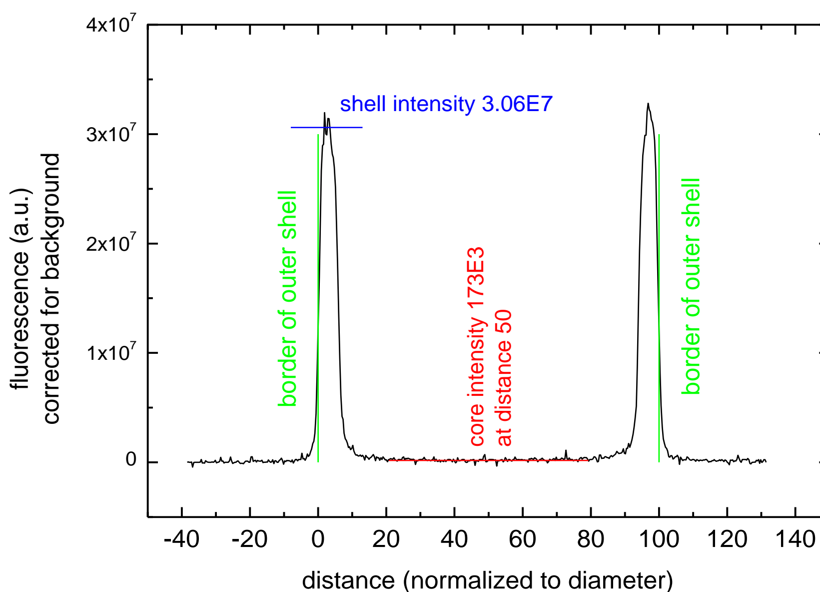


Figure 33: Cross section diagram for the beads without free-biotin with shell and core intensities.

Table 3: The ratio of shell / core was calculated for all the samples.

Image	**Bead**	**Shell intensity at 2**	**Core intensity at 50**	**average core intensity**	**ratio shell/core**	**Average**	**stdev**	**Biotin**
A	1	9,10E+06	7,80E+06		1,17	1,51	0,41	Yes
A	2	9,70E+06	9,90E+06		0,98			Yes
A	3	1,32E+07	1,18E+07		1,12			Yes
B	1	8,60E+06	8,30E+06		1,04			Yes
B	2	1,10E+07	7,20E+06		1,53			Yes
B	3	9,70E+06	8,20E+06		1,18			Yes
C	1	9,90E+06	4,70E+06		2,11			Yes
C	2	1,17E+07	5,80E+06		2,02			Yes
C	3	1,67E+07	9,20E+06		1,82			Yes
D	1	1,67E+07	8,60E+06		1,94			Yes
D	2	8,10E+06	5,90E+06		1,37			Yes
D	3	1,18E+07	6,50E+06		1,82			Yes
E	1	4,96E+07	5,90E+04	1,18E+05	840,68	587,29	123,34	No
E	2	4,01E+07	4,90E+04		679,66			No
E	3	3,91E+07	1,50E+05		662,71			No
F	1	2,50E+07	1,30E+05		423,73			No
F	2	2,80E+07	1,80E+05		474,58			No
F	3	2,50E+07	1,75E+05		423,73			No
G	1	3,70E+07	4,50E+04		627,12			No
G	2	3,80E+07	8,90E+04		644,07			No
G	3	2,80E+07	2,70E+05		474,58			No
H	1	3,70E+07	8,60E+04		627,12			No
H	2	3,20E+07	8,20E+04		542,37			No
H	3	3,70E+07	9,60E+04		627,12			No

The core intensity of all biotin-free beads was averaged and divided by shell intensities to get the ratio.
In the presence of free-biotin, the shell intensity is essentially same as the core intensity (ratio of shell / core is close to 1). In the absence of free-biotin, the shell intensity is more than 500-fold higher than the background intensity of the beads. By this can estimate that, biotin-IgG* binds to streptavidin agarose with a high efficiency (Figure 33).

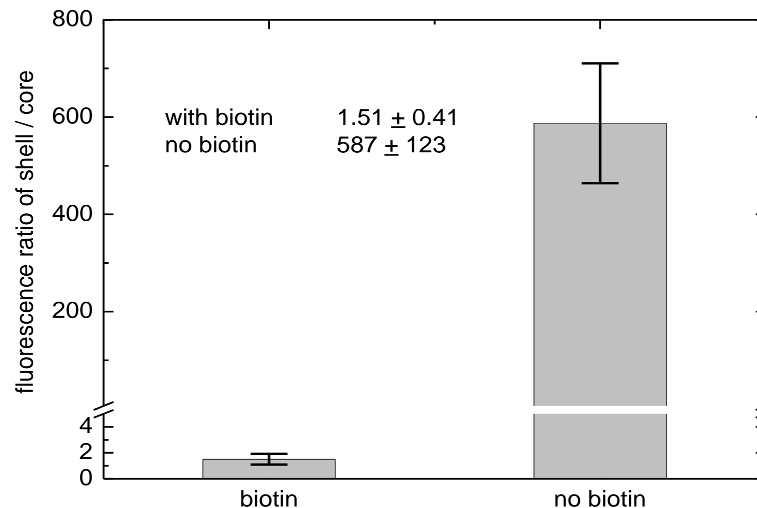
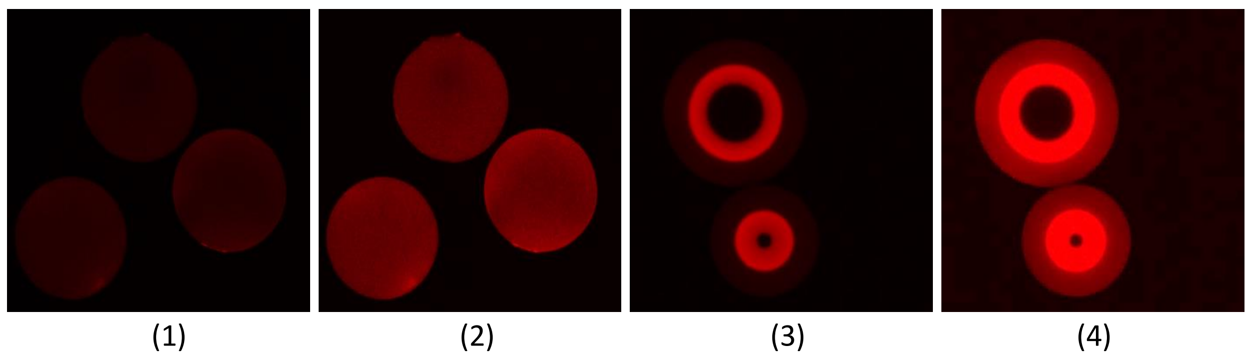


Figure 34: Statistical representation for shell / core ratios

2.3.5 Test for the amount of BSSF required for the completion of block of SAV-agarose

The detailed experimental procedure is described in the chapter 3.4.5. Duplicates of BSSF (1000, 500, 250, 125 µmol) were incubated with SAV-agarose beads for 5 minutes and 46 pmol of biotin-IgG* was incubated for 60 minutes. After the incubation and sedimentation, fluorescence measurements were done with supernatants and beads were imaged in the confocal microscope with 1% concentration



(1) (2) (3) (4)

Figure 35: Confocal laser microscope images for BSSF and biotin-IgG* incubation with SAV-agarose

1. BSSF 1000, 500, 250 µmol incubation (original image)
2. BSSF 1000, 500, 250 µmol incubation (with higher brightness)
3. BSSF 125 µmol (original image)
4. BSSF 125 µmol (with higher brightness)

In image (1), with higher concentrations of BSSF all the SAV present in beads get saturated. There were no free SAV for biotin-IgG* binding. With 125 µM BSSF, SAV were not saturated and biotin-IgG* was bound to the remaining SAV present in the beads.

BSSF can emit fluorescence only when fluorescein is not bound to the SAV pocket. In the image (1), fluorescence emission is very low, by that we can assume that all the fluorescein parts were bound to SAV pockets. In the image (3), outer rim has low fluorescence emission that the inner rim of the beads. The only assumption can make from this difference is that BSSF molecules bind to the SAV present in outer rim of the beads and biotin-IgG* can go into deeper with longer incubation time and emit brighter fluorescence. With the different sizes of the beads, the travelling distance of the biotin-IgG* differ. This is clearly visible in the small bead (in image 3), which has much wider rim filled with biotin-IgG*.

Table 4: Detailed results from image analysis and fluorescence measurements of the supernatants

Image	Bead	Shell intensity at 2	Core intensity at 50	ratio shell over core	average	stdev	(µl) BSSF	(µM) BSSF	fluoresc. in supernat.	av.	stdev
A	1	1,02E+07	6,20E+06	1,65	1,63	0,12	4	1000	1047	1034	18
A	2	7,10E+06	4,80E+06	1,48			4	1000	1021		
A	3	1,00E+07	5,60E+06	1,79			4	1000			
B	1	1,01E+07	6,40E+06	1,58			4	1000			
B	2	7,90E+06	4,50E+06	1,76			4	1000			
B	3	1,01E+07	6,50E+06	1,55			4	1000			
C	1	1,79E+07	1,44E+07	1,24	1,33	0,07	4	500	1091	1019	102
C	2	1,91E+07	1,44E+07	1,33			4	500	946,1		
C	3	2,13E+07	1,47E+07	1,45			4	500			
D	1	1,91E+07	1,43E+07	1,34			4	500			
D	2	1,78E+07	1,38E+07	1,29			4	500			
D	3	1,85E+07	1,38E+07	1,34			4	500			
E	1	1,67E+07	1,23E+07	1,36	1,22	0,13	4	250	1185	1148	52
E	2	1,65E+07	1,46E+07	1,13			4	250	1111		
E	3	1,86E+07	1,33E+07	1,40			4	250			
F	1	1,53E+07	1,39E+07	1,10			4	250			
F	2	1,41E+07	1,20E+07	1,18			4	250			
F	3	1,37E+07	1,20E+07	1,14			4	250			
G	1	3,30E+07	4,60E+05	71,74	102,00	50,64	4	125	379,7	426	65
G	2	3,40E+07	4,80E+05	70,83			4	125	471,6		
G	3	3,30E+07	4,80E+05	68,75			4	125			
H	1	3,50E+07	5,30E+05	66,04			4	125			
H	2	4,00E+07	2,40E+05	166,67			4	125			
H	3	4,20E+07	2,50E+05	168,00			4	125			

Table 5: Results of fluorescence and shell / core for the graph preparation

Image	µmol BSSF	shell/core	stdev	supernat.	stdev
A and B	1000	1,63	0,12	1034	18
C and D	500	1,33	0,07	1019	102
E and F	250	1,29	0,15	1148	52
G and H	125	102,00	50,64	426	65

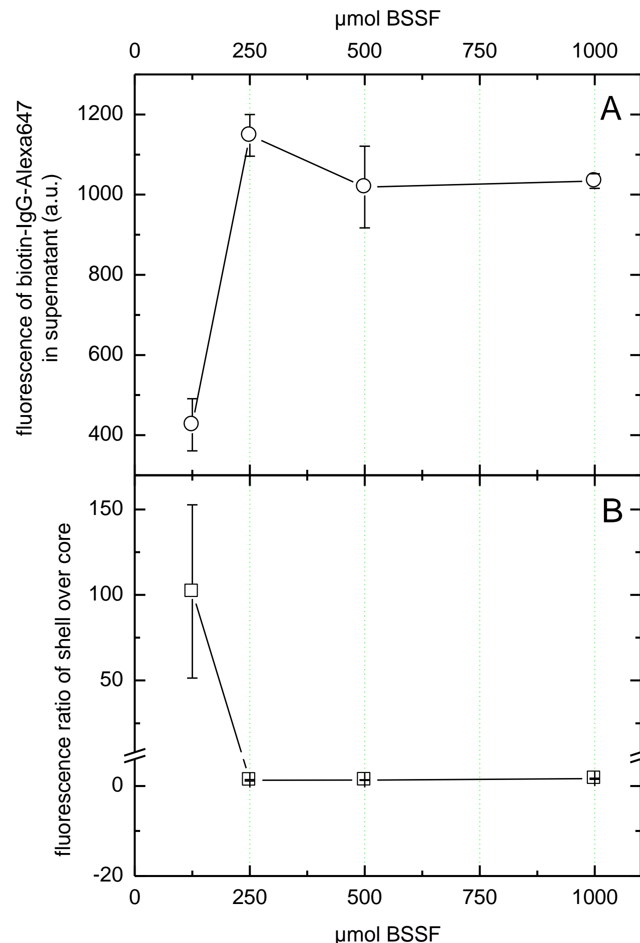


Figure 36: Fluorescence of biotin-IgG* in the supernatant depending on the different BSSF amounts (A) and fluorescence shell / core ratio according to the different BSSF amount (B)

With 125 µmol of BSSF, biotin-IgG* get bound to the remaining SAV beads. Therefore, the fluorescence of the supernatant is about 400 a.u. With 250, 500, 1000 µmol of BSSF, biotin-IgG* was not bound to SAV pockets and remained in the supernatant. Due to that the fluorescence of the supernatants was about 1000 a.u.

In the beads with 125 µmol of BSSF, shell / core ration is higher (about 100) when comparing to 250, 500, 1000 µmol of BSSF. Because with 125 µmol of BSSF, biotin-IgG* got bound to SAV present in the bead shell and emitted higher fluorescence comparing to no fluorescence emissions from the core. The shell / core ratio for 250, 500, 1000 µmol of BSSF is about 1, when the ratio for 125 µmol of BSSF is about 100.

By analysing all the results, minimum BSSF concentration to saturate all the SAV present in the beads is 250 µM. By this concentration, with minimum washing can achieve the fully BSSF bound SAV beads.

2.3.6 Test for the comparison of BSSF block of SAV-agarose with and without cleavage of disulphides by TCEP

The detailed procedure is described in the chapter 3.4.6. SAV-agarose was incubated with 250 µmol of BSSF in two triplicate groups. After the incubation, excess of BSSF present was washed away by using PBS buffer. When the washing was completed, first group was refrigerated until the BSSF of the second group was treated with TCEP and Hepes. TCEP was used to cleave the disulphide bonds of BSSF and Hepes was used to maintain a constant pH of the system. After the cleavage of disulphide bonds of BSSF in the second group, both groups were incubated with biotin-IgG* to fill up the vacant SAV pockets in the beads. After the incubation and sedimentation was completed, beads were imaged by using confocal laser microscope.

Then to all 6 tubes 6 µL of biotin-IgG* was added and continuously vortexed for 60 minutes. Then 400 µL of PBS were added into each tube and the supernatant was used for the fluorescence measurements. The bead pellets were used for the confocal laser imaging.

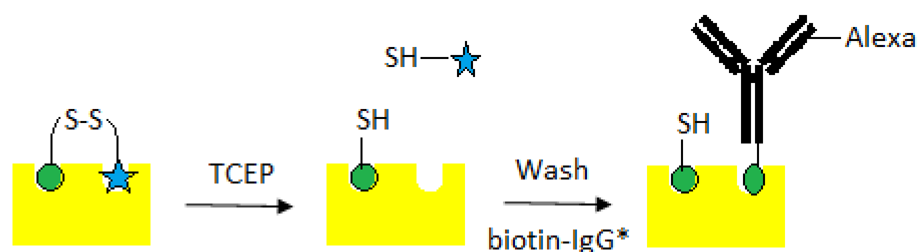


Figure 37: Pictorial description for the experiment. Green oval is biotin and blue star is the fluorescein group.

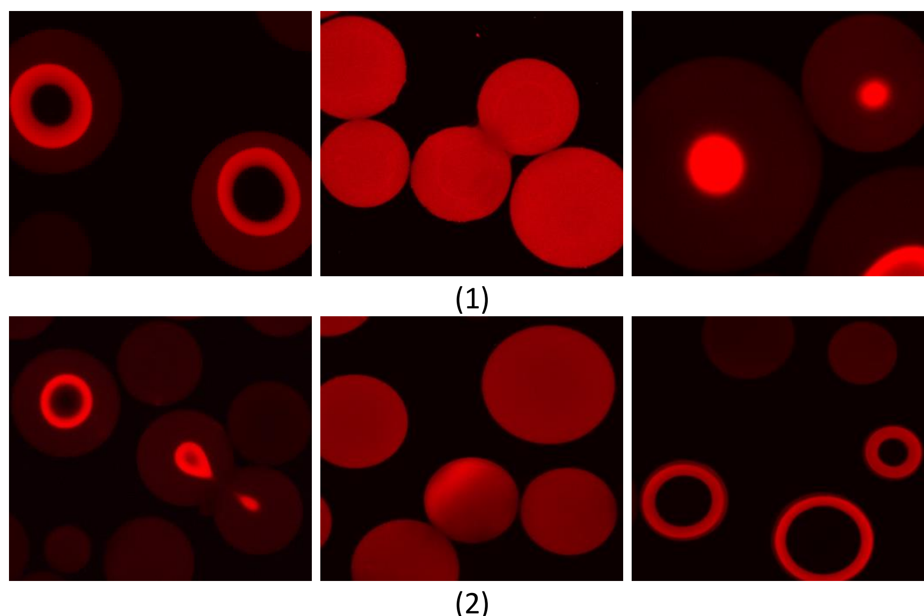


Figure 38: Confocal laser microscope images for group 1 and 2

According to the images, fluorescence emissions show different patterns. Some biotin-IgG* molecules diffuse more into the core of the beads and some remain in the shell area. BSSF molecules were mostly bound in the shell but in some beads BSSF were bound in the whole bead. According to the images, there no difference is visible between two groups. If BSSF disulphide bond was cleaved with TCEP, fluorescein groups are not supposed to be bound in the SAV pockets.

If the test was worked according to the plan, first group should get only BSSF bound beads without fluorescence emissions. If the disulphides of the BSSF were cleaved and the fluorescein groups were washed out, it must create empty SAV pockets. Into this empty pockets biotin-IgG* was supposed to bind and emit fluorescence. Due to that, only in the second group, fluorescence emission should be visible. But with these results it is hard to give an explanation. Because of these complications, final decision was to make beads with streptavidin only bound onto the surface of the beads. This type of beads was not available to purchase. Due to that making surface bound streptavidin beads was the next option. By studying literature, two methods were found to create surface bound streptavidin beads.

2.4 Covalent immobilization of streptavidin on Sephadex G-15 beads and further couplings

Surface activation of Sephadex G-15 beads with SAV were done according to two different methods. First method was the Activation of Sephadex G-15 with N, N'-Disuccinimidyl Carbonate (DSC) [Wilchek & Miron 1985] and the second method was the activation of Sephadex G-15 by using Cyanogen bromide (CNBr) [Kohn & Wilchek 1982].

2.4.1 Activation of Sephadex G-15 with N, N'-Disuccinimidyl Carbonate (DSC) and coupling of BSA*

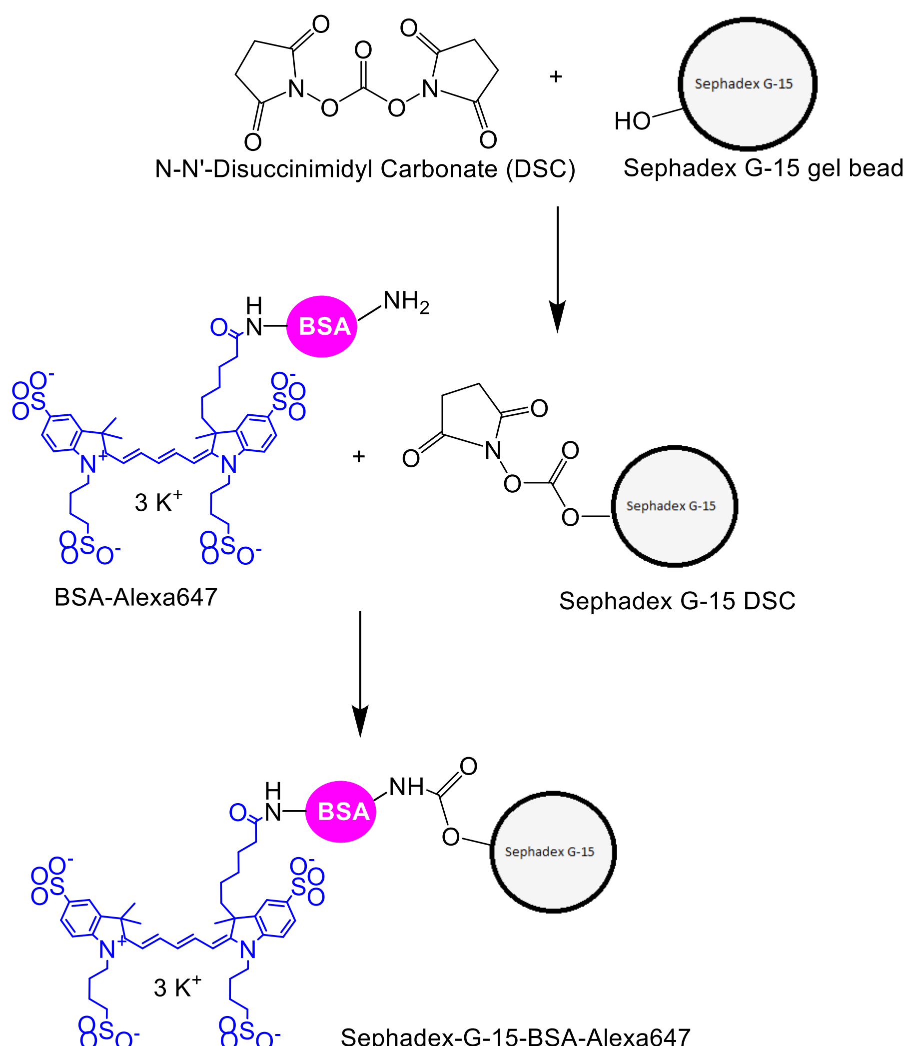


Figure 39: Reaction scheme for Sephadex G-15 activation with DSC and BSA* coupling

The detailed coupling procedure is in the chapter 3.5.1. First, Sephadex G-15 dry beads were boiled in water. Boiled Spehadex G-15 gel was washed several times with water, acetone/water mixtures and finally with pure acetone. This was transferred into a mixture of DSC (3.123 mmol) and dimethylamine pyridine (5.32 mmol) in acetone. The suspension was shaken for one hour at 4°C for bead activation. After the activation step, beads were washed with acetone, TFA with 5% acetic acid, methanol and with isopropanol.
The BSA* coupling to DSC-activated gel was done in the coupling buffer with 3.27 nmol of BSA*. The incubation of DSC activated gel with BSA* was done overnight at room temperature. Deactivation was done by using deactivation buffer and stored in the preservation buffer.
BSA*-DSC activated gel was imaged by using confocal laser microscope.

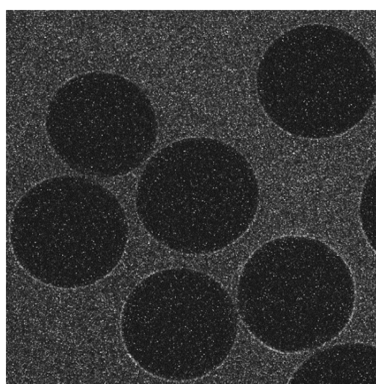


Figure 40: Confocal laser microscope image for BSA*-DSC activated gel beads

According to the bead images, core had low fluorescence than suspension and there was no visible shell activation. DSC Sephadex G-15 activation was not successful, therefore BSA* could not attached to the outer layer of the beads.

2.4.2 Activation of Sephadex G-15 with Cyanogen bromide (CNBr) and coupling of BSA*

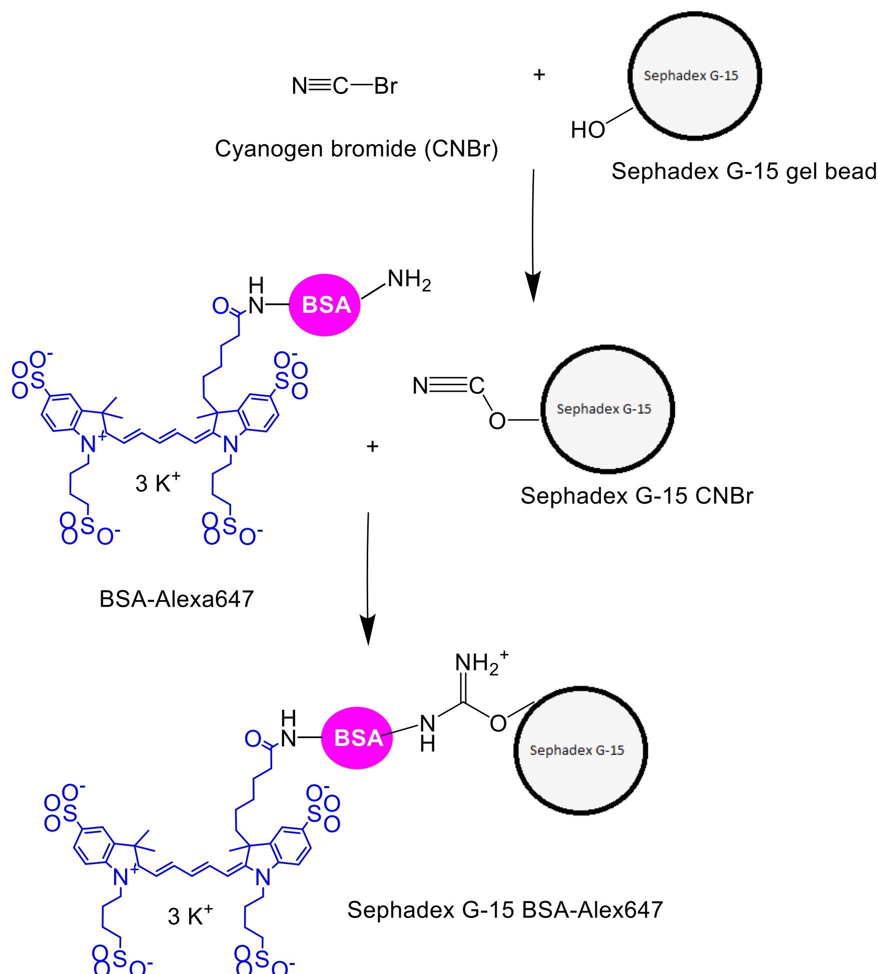


Figure 41: Reaction scheme for Sephadex G-15 activation with CNBr and BSA* coupling

The detailed coupling procedure is in the chapter 3.5.2. Pre-boiled Spehadex G-15 gel was washed several times with water, acetone mixtures. A solution of CNBr (1 mmol) in TEA and acetone was transferred into the washed beads dropwise at the temperature of -15°C and mixed for 3 minutes. Then the gel suspension was transferred into ice cold water. Finally, the beads were preserved by using the preservation medium.

BSA* coupling to the CNBr activated beads were done according to the same procedure as in 2.4.1. After the overnight BSA* coupling, beads were imaged with confocal laser microscope.

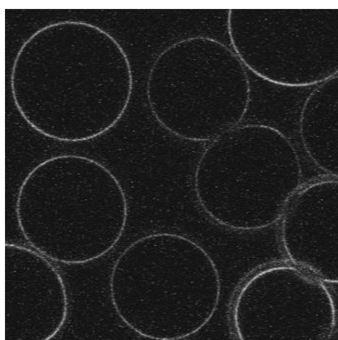


Figure 42: Confocal laser microscope image for BSA*-CNBr activated gel beads

According to the images (Figure 40), Sephadex G-15 outer bead surface activated and the BSA* coupling was successful. Therefore, these beads were used for the further experiments.

2.4.3 Coupling of unlabelled streptavidin and biotin-IgG* to CNBr-activated Sephadex G-15

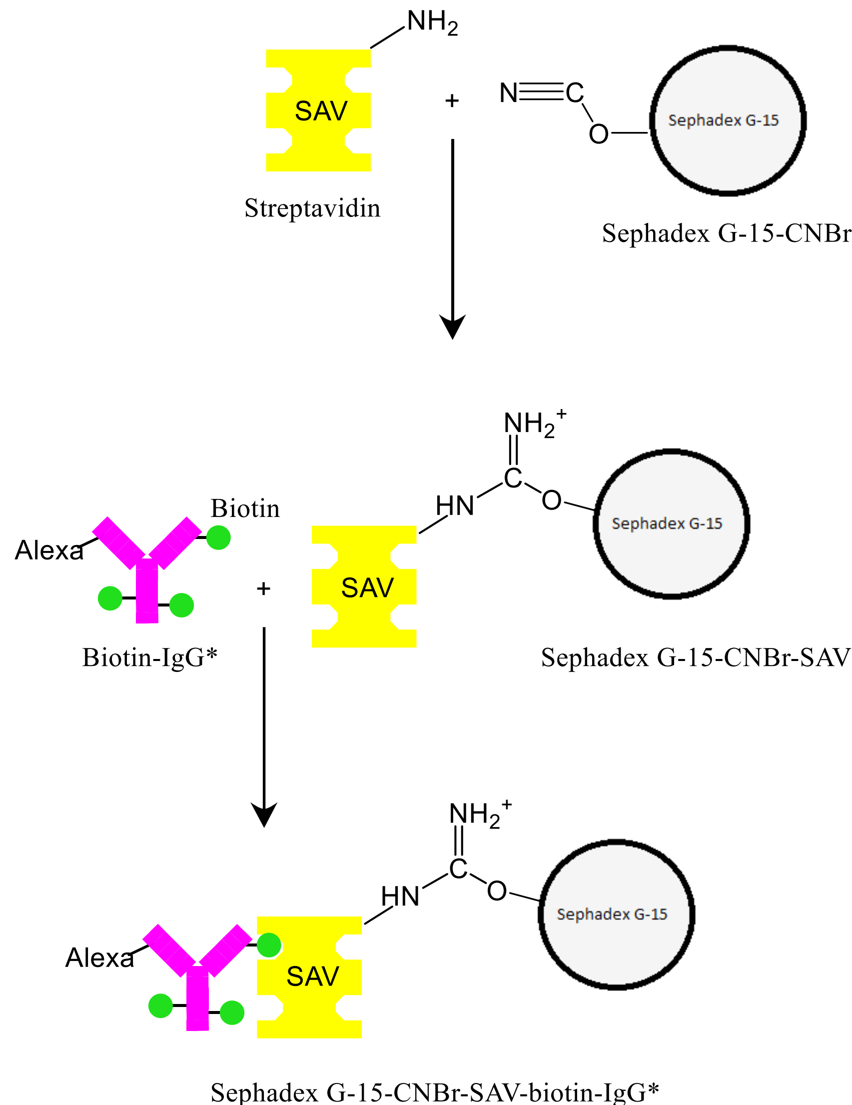
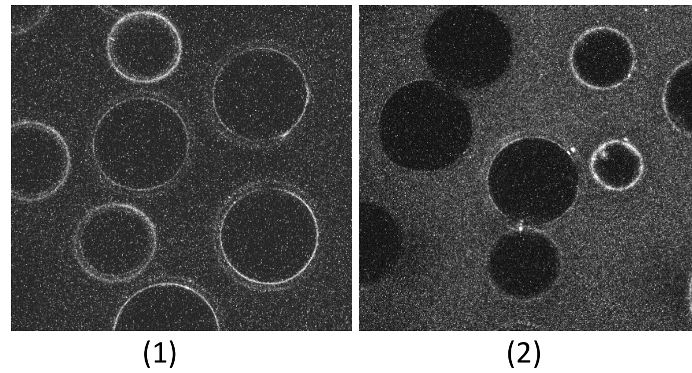


Figure 43: Reaction scheme for CNBr-Sephadex G-15 coupling with SAV and BSA*

First step was bind streptavidin to the CNBr activated Sephadex G-15 gel bead surface. 50% CNBr activated Sephadex G-15 gel was washed with coupling buffer and incubated with 3.6 µmol of SAV overnight in cold room. Then the deactivation was done with deactivation buffer and stored in preservation buffer.

Streptavidin binding was tested by using biotin-IgG*. Two sets of triplicates were prepared with CNBr-Sephadex G-15-SAV beads, one was incubated with free-biotin and with biotin-IgG* (38 pmol). Second set of beads were incubated only with biotin-IgG*. All the bead samples were kept for sedimentation and imaging was done at confocal laser microscope.



(1) (2)

Figure 44: Confocal laser microscope images for free-biotin + biotin-IgG* (1) and biotin-IgG* (2) incubation

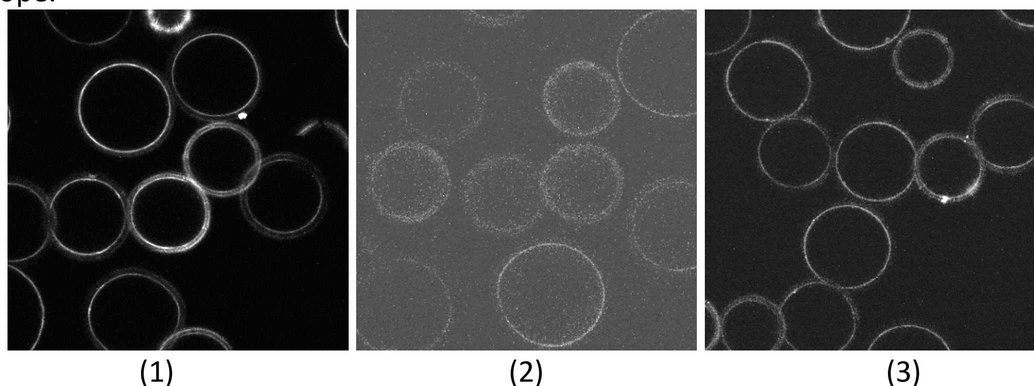
Table 6: Results from the image analysis

Image	Bead	Peak 1	Peak 2	aveg. (y)	x	y-x	z	shell/core
A	1	0	0	0	8,30E+06	0	2,50E+06	0
A	2	0	0	0	8,00E+06	0	2,70E+06	0
A	3	0	0	0	9,00E+06	0	3,10E+06	0
B	1	0	0	0	5,70E+06	0	2,60E+06	0
B	2	0	0	0	7,10E+06	0	3,20E+06	0
B	3	0	0	0	8,20E+06	0	3,10E+06	0
C	1	0	0	0	6,10E+06	0	3,40E+06	0
C	2	0	0	0	6,10E+06	0	2,70E+06	0
C	3	0	0	0	6,30E+06	0	3,00E+06	0
D	1	2,10E+07	2,90E+07	2,50E+07	3,40E+06	2,16E+07	3,30E+06	6,55
D	2	2,20E+07	4,00E+07	3,10E+07	3,40E+06	2,76E+07	3,10E+06	8,90
D	3	2,70E+07	3,00E+07	2,85E+07	3,70E+06	2,48E+07	3,10E+06	8,00
E	1	3,50E+07	1,90E+07	2,70E+07	3,50E+06	2,35E+07	3,30E+06	7,12
E	2	2,60E+07	1,70E+07	2,15E+07	3,30E+06	1,82E+07	3,10E+06	5,87
E	3	3,80E+07	2,60E+07	3,20E+07	3,40E+06	2,86E+07	3,30E+06	8,67
F	1	2,30E+07	3,30E+07	2,80E+07	3,20E+06	2,48E+07	3,30E+06	7,52
F	2	4,10E+07	3,30E+07	3,70E+07	4,00E+06	3,30E+07	3,10E+06	10,65
F	3	1,70E+07	4,30E+07	3,00E+07	2,80E+06	2,72E+07	3,10E+06	8,77

Average of shell / core value for the 2nd set = 8.00
Standard deviation of shell / core for the 2nd set = 1.44
By the results can predict that SAV binding to CNBr activated Sephadex G-15 was successful. In the 1st set of samples almost no fluorescence can be seen in the surface of beads and in the 2nd set of samples fluorescence emission from biotin-IgG* is clearly visible.

2.4.4 Test for the comparison of biotin-IgG* binding of SAV-CNBr-Sephadex G-15 in absence and presence of BSSF

Three sets of 50% SAV-CNBr-Sephadex G-15 beads were used for the test with duplicates. 250 pmol of BSSF was incubated for 5 minutes with 1st two sets of beads. Only the 2nd set of beads were washed with PBS buffer for several times. Then all three bead sets were incubated with 38 pmol of biotin-IgG* for 10 minutes. Finally, all the beads were imaged in confocal laser microscope.



(1) (2) (3)

Figure 45: Confocal laser microscope images

(1) With BSSF
(2) With BSSF and wash
(3) Without BSSF

Table 7: Results from the image analysis

Image	Bead	Peak 1	Peak 2	Aveg. (y)	x	y-x	z	shell/core
A	1	8,10E+05	1,24E+06	1,03E+06	3,20E+05	7,05E+05	3,20E+05	2,20
A	2	7,10E+05	5,60E+05	6,35E+05	3,30E+05	3,05E+05	3,20E+05	0,95
A	3	7,40E+04	1,34E+05	1,04E+05	3,30E+04	7,10E+04	3,30E+04	2,15
B	1	7,90E+05	1,23E+06	1,01E+06	3,30E+05	6,80E+05	3,40E+05	2,00
B	2	7,40E+04	7,30E+04	7,35E+04	3,30E+04	4,05E+04	3,40E+04	1,19
B	3	9,20E+05	7,10E+05	8,15E+05	3,30E+05	4,85E+05	3,60E+05	1,35
C	1	6,60E+05	6,50E+05	6,55E+05	1,32E+05	5,23E+05	1,50E+05	3,49
C	2	6,20E+05	5,70E+05	5,95E+05	1,34E+05	4,61E+05	1,40E+05	3,29
C	3	6,60E+05	4,00E+05	5,30E+05	1,35E+05	3,95E+05	1,40E+05	2,82
D	1	5,90E+05	4,50E+05	5,20E+05	9,60E+04	4,24E+05	9,60E+04	4,42
D	2	5,10E+05	3,60E+05	4,35E+05	9,70E+04	3,38E+05	9,50E+04	3,56
D	3	2,90E+05	2,50E+05	2,70E+05	9,90E+04	1,71E+05	9,80E+04	1,74
E	1	4,60E+07	5,50E+07	5,05E+07	1,30E+06	4,92E+07	1,50E+06	32,80
E	2	4,00E+07	5,90E+07	4,95E+07	1,20E+06	4,83E+07	9,60E+05	50,31
E	3	4,60E+07	5,10E+07	4,85E+07	1,00E+06	4,75E+07	1,50E+06	31,67
F	1	5,50E+07	4,90E+07	5,20E+07	9,60E+05	5,10E+07	9,60E+05	53,17
F	2	3,80E+07	5,10E+07	4,45E+07	7,80E+05	4,37E+07	1,20E+06	36,43
F	3	2,40E+07	3,50E+07	2,95E+07	7,50E+05	2,88E+07	1,10E+06	26,14

Table 8: Average and Standard deviation for different groups of images

Image set	Condition	Average	Stdev.
1	BSSF	1,64	0,54
2	BSSF+wash	3,22	0,89
3	no BSSF	38,42	10,87

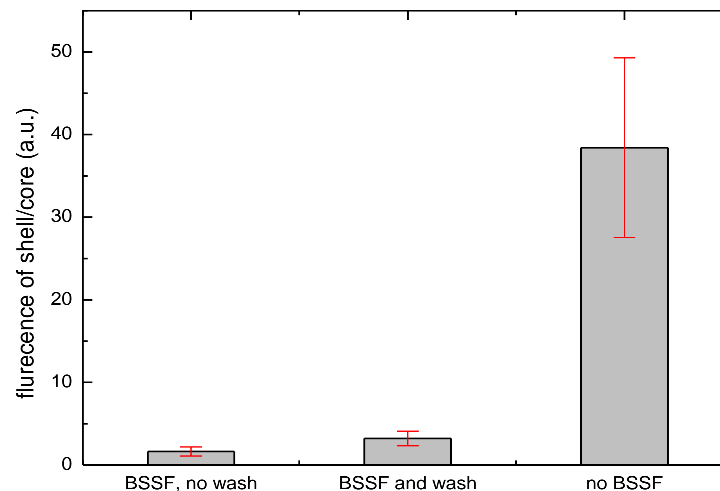


Figure 46: Fluorescence of shell/core for different conditions

By image analysis, we can see that BSSF without wash has the lowest fluorescence shell/ core ratio. When BSSF was washed for several times, fluorescence emission was increased for the BSSF bound beads. Which means some of the BSSF had washed away after the binding to SAV pockets. The difference of no BSSF and BSSF + wash reveals that most of the BSSF molecules were still bund to the beads even after the wash. If most of BSSF were washed away, fluorescence emission would be much higher due to rebound of biotin-IgG* to vacant pockets (which were reopen by BSSF washing) of SAV. What we can conclude from this test is that, after BSSF binding, the washing can be done to beads and proceed with the experiments.

2.4.5 Test for the comparison of biotin-IgG* binding to SAV-CNBr-Sephadex-G-15 beads in presence of BSSF, with and without disulphide cleavage by TCEP

Two sets (with triplicates) of SAV-CNBr-Sephadex G-15 beads were incubated for 5 minutes with 250 µmol of BSSF. All of them were washed for several times with PBS buffer to remove unbound BSSF. For the 2nd set 0.4 µmol of TCEP hydrochloride and Hepes pH 9.6 Were added and incubated for 5 minutes. After the disulphide cleavage, the beads were washed again for several times with PBS buffer.

Then 38 pmol of biotin-IgG* were added to both beads sets and incubated for 10 minutes. Finally, all of them were washed again with PBS and imaged by using confocal laser microscope.

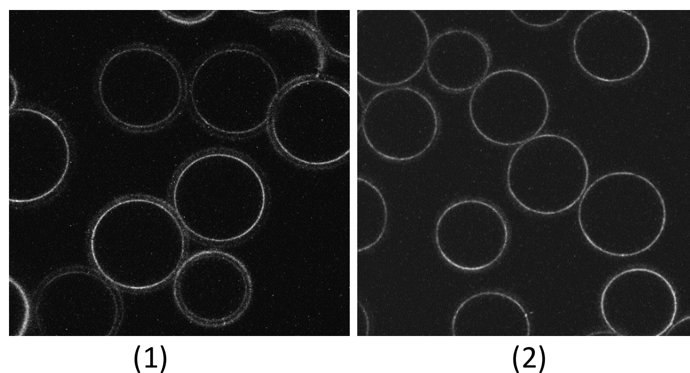


Figure 47: Confocal laser microscope images of BSSF without disulphide cleavage (1) and with cleavage (2)

Table 9: Results from the image analysis

Image	Bead	Peak 1	Peak 2	Avrg. (y)	x	y-x	z	shell/core
A	1	1,80E+05	1,20E+05	1,50E+05	2,80E+04	1,22E+05	2,60E+04	4,69
A	2	1,50E+05	8,20E+04	1,16E+05	2,70E+04	8,90E+04	2,70E+04	3,30
A	3	1,30E+05	8,80E+04	1,09E+05	2,70E+04	8,20E+04	2,70E+04	3,04
B	1	2,40E+05	1,70E+05	2,05E+05	2,90E+04	1,76E+05	2,90E+04	6,07
B	2	1,70E+05	8,60E+04	1,28E+05	2,90E+04	9,90E+04	3,00E+04	3,30
B	3	1,10E+05	1,80E+05	1,45E+05	3,00E+04	1,15E+05	3,00E+04	3,83
C	1	7,90E+04	1,20E+05	9,95E+04	1,40E+04	8,55E+04	1,50E+04	5,70
C	2	7,70E+04	8,60E+04	8,15E+04	1,50E+04	6,65E+04	1,50E+04	4,43
C	3	6,90E+04	9,40E+04	8,15E+04	1,50E+04	6,65E+04	1,40E+04	4,75
D	1	2,40E+05	8,50E+04	1,63E+05	2,80E+04	1,35E+05	2,70E+04	4,98
D	2	2,10E+05	1,50E+05	1,80E+05	2,80E+04	1,52E+05	2,90E+04	5,24
D	3	1,10E+05	1,40E+05	1,25E+05	2,70E+04	9,80E+04	2,90E+04	3,38
E	1	1,80E+05	1,40E+05	1,60E+05	1,10E+04	1,49E+05	1,10E+04	13,55
E	2	1,40E+05	1,70E+04	7,85E+04	1,20E+04	6,65E+04	1,20E+04	5,54
E	3	1,60E+05	9,30E+04	1,27E+05	1,10E+04	1,16E+05	1,20E+04	9,63
F	1	2,50E+05	2,50E+05	2,50E+05	2,80E+04	2,22E+05	3,00E+04	7,40
F	2	2,50E+05	1,90E+05	2,20E+05	3,00E+04	1,90E+05	3,20E+04	5,94
F	3	1,10E+06	1,10E+06	1,10E+06	2,80E+05	8,20E+05	3,10E+05	2,65

Table 10: Average and Standard deviation for different groups of images

Image	Condition	Average	Stdev	%	stdev%
3 (last exp.)	no BSSF	38,42	10,87	100	28,3
2	BSSF+wash+TCEP	6,48	3,35	16,86	8,73
1	BSSF+wash+noTCEP	4,35	1,08	11,31	2,8
2 (last exp.)	BSSF+wash	3,22	0,89	8,4	2,3
1 (last exp.)	BSSF	1,64	0,54	4,3	1,4

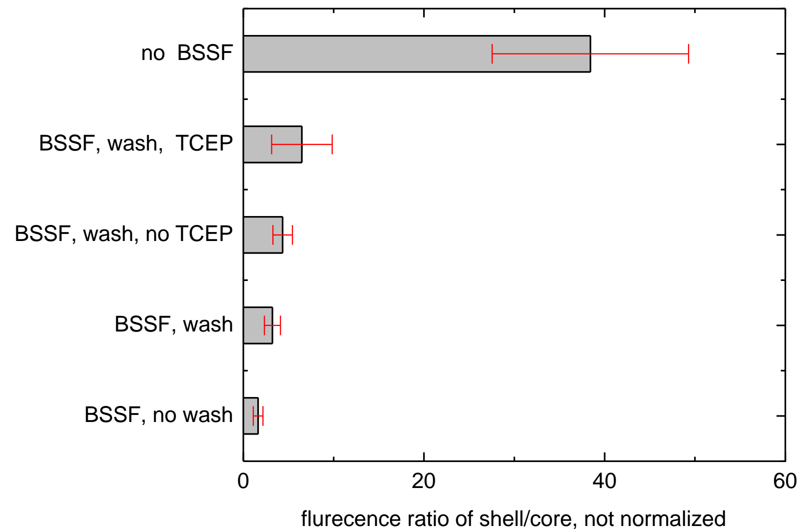


Figure 48: Fluorescence of shell/core for different conditions

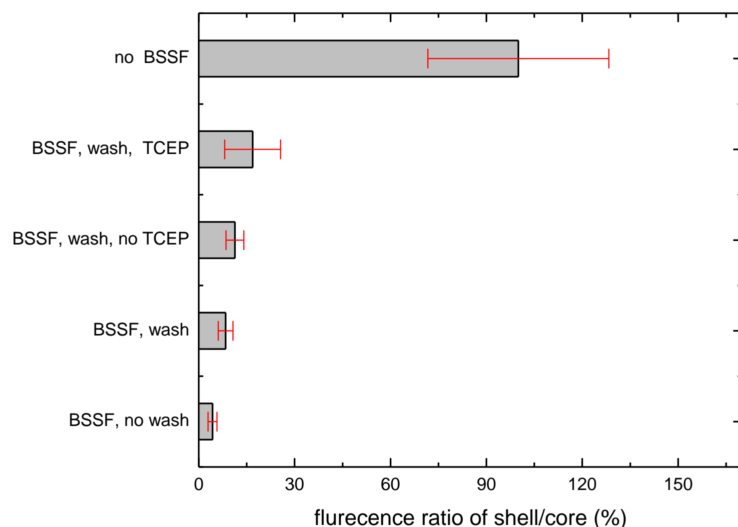


Figure 49: Fluorescence shell/ core (%) for different conditions

The graphs were made by using results from the experiments 2.4.4 and 2.4.5. Figure 48 graph is made in fluorescence emission in a.u and Figure 49 graph is made according to the percentage of emission which makes results clearer.

When no BSSF was used for SAV-CNBr-Sephadex G-15 beads, biotin-IgG* was bound to all the SAV pockets, that emission value was took as the 100% standard. When BSSF disulphides were cleaved with TCEP and washed away the excess, emission percentage was 16.9%, where no TCEP cleavage was 11.3%. The difference between 16.9% and 11.3% is the fluorescein groups, which have been cleaved and washed away and biotin-IgG* was bound to those empty pockets of SAV.

In the experiments 2.4.4 and 2.4.5, BSSF without cleavage of disulphide followed by the washing got two different emission percentages, such as 8.4% and 11.3%. Here for both had the same conditions but the results were quite different, which might lead to a question about the reliability of bindings. The last result was the BSSF without any washings, which shows the lowest fluorescence emission percentage of 4.3%. Where only a very little amount of biotin-IgG* was bound to the beads.

2.5 Coupling of 8-arm-PEG-nitrophenyl ester with N-biotinyl-propylamine [Haizinger, 2012]

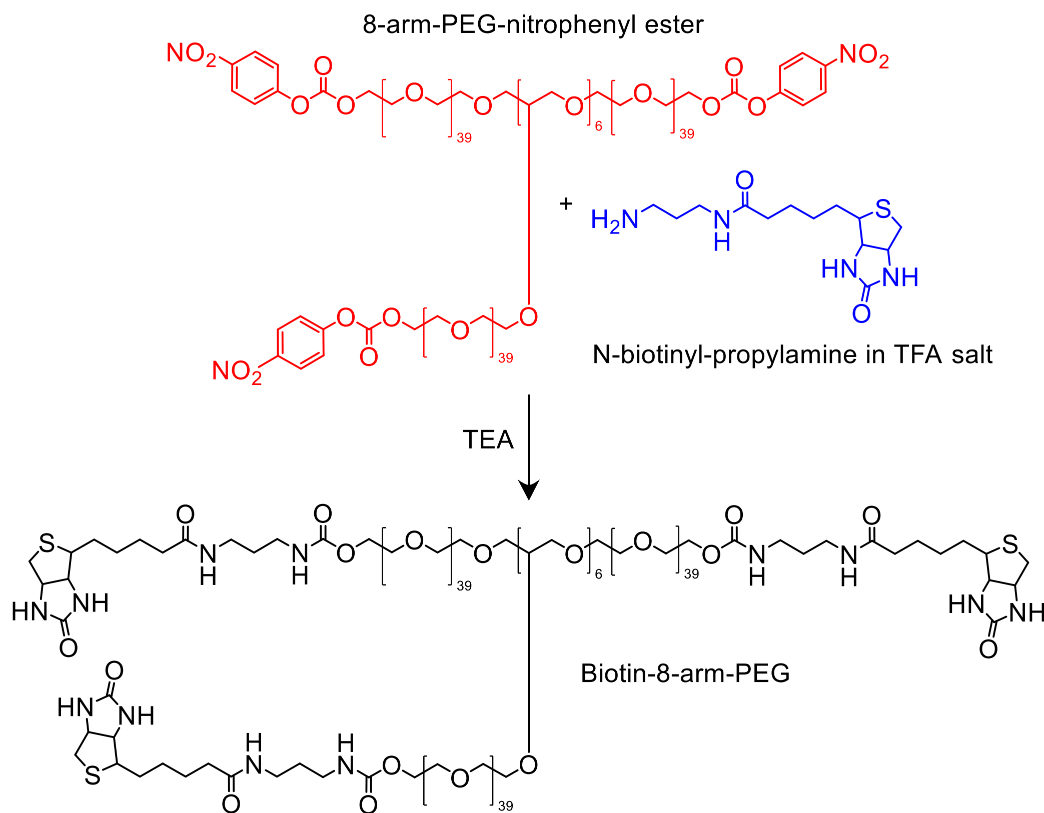


Figure 50: Reaction scheme for 8-arm-PEG-nitrophenyl ester coupling with N-biotinyl-propylamine

N-biotinyl-propylamine and 8-arm-PEG-nitrophenyl ester in the ration of 30:1 (made by Fraynziska Haizinger) were reacted with DMSO and TEA. The product was identified by using NMR.

For the purification of batch 1, first liquid-liquid extraction was done by using 0.1 M phosphoric acid (in 2.5 M NaCl) with chloroform. By the extraction unreacted/ excess of N-biotinyl-propylamine was separated into the aqueous phase. The organic phase (chloroform) was then extracted with 10% Na_2CO_3 to separate out the cleaved p-nitrophenol from biotin-8-arm-PEG. The yield of the first batch was 37.69%.

The purification of batch 2 was done by dialysis tubing method. The first dialysis was done with water, acetic acid mixture to remove excess of N-biotinyl-propylamine and cleaved p-nitrophenol. The second dialysis was done in methanol for the further purification of the product. The yield obtained from the second batch was 84.62%.

By the difference of two yields can identify that, from liquid-liquid extraction method most of the product which was present in the emulsion could not recover and the dialysis method is much more efficient in purification process.

2.6 Biotin and BSSF titrations against (strept)avidin

Titrations were done at Fluorimeter by Luise Hermann.

2.6.1 Biotin vs. avidin titration using tryptophan quenching

80 µL of (1.25 µM, 0.1 pmol) avidin was titrated against 8 µM biotin by measuring the fluorescence emission at the excitation 290 nm (slit 2.5 nm) and emission 340 nm (slit 10 nm).

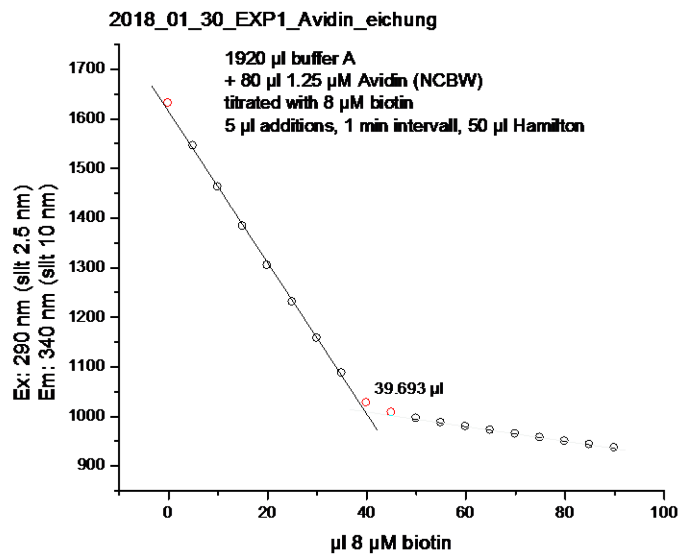


Figure 51: Biotin vs. avidin titration curve (performed by Luise Herrmann in her ongoing master thesis at JKU Linz)

0.1 pmol of avidin has 0.4 pmol of binding pockets. 39.69 µL of 8 µM (0.317 pmol) biotin was consumed to fill all the avidin pockets according to the end-point of the titration. Almost 4 biotin molecules were bound per avidin molecule.

2.6.2 Biotin vs. streptavidin titration using tryptophan quenching

8 µM biotin was titrated against 80 µL (1.25 µM, 0.1 pmol) SAV in the fluorimeter at the excitation 290 nm (slit 2.5 nm) and emission 340 nm (slit 10 nm).

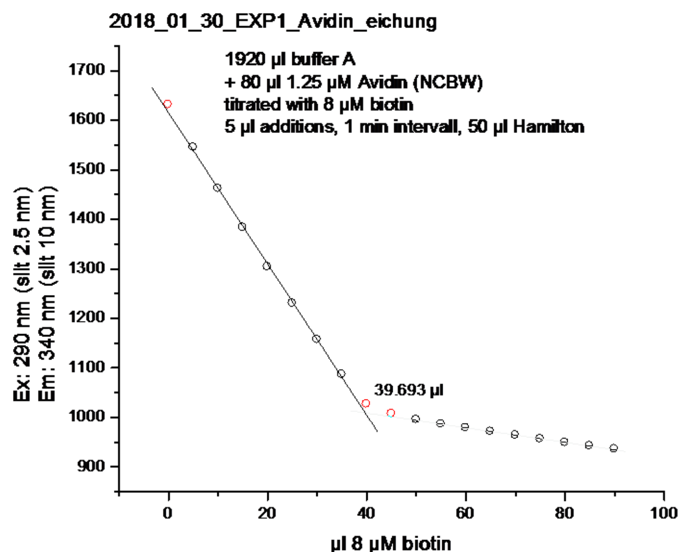


Figure 52: Biotin vs. streptavidin titration curve (performed by Luise Herrmann in her ongoing master thesis at JKU Linz)

The end-point of the titration was reached by consuming 48.01 µL of biotin (0.384 pmol). The amount of biotin consumption also proves the 4:1 (strept)avidin: biotin complex.
When biotin was titrating against (strept)avidin, fluorescence emission gradually decreases due to the biding of biotin to (strept)avidin pockets. Tryptophan present in the (strept)avidin pockets emits fluorescence and when biotin get bound, tryptophan was no longer able to emit fluorescence. When all the pockets were filled with biotin, there was be no more decrease of fluorescence and that was the end-point of the titration.

2.6.3 BSSF vs. avidin titration using tryptophan quenching

8 µM (nominal concentration) BSSF was titrated against 80 µL (1.25 µM, 0.1 pmol) SAV in the fluorimeter at the excitation 490 nm (slit 2.5 nm) and emission 525 nm (slit 5 nm).

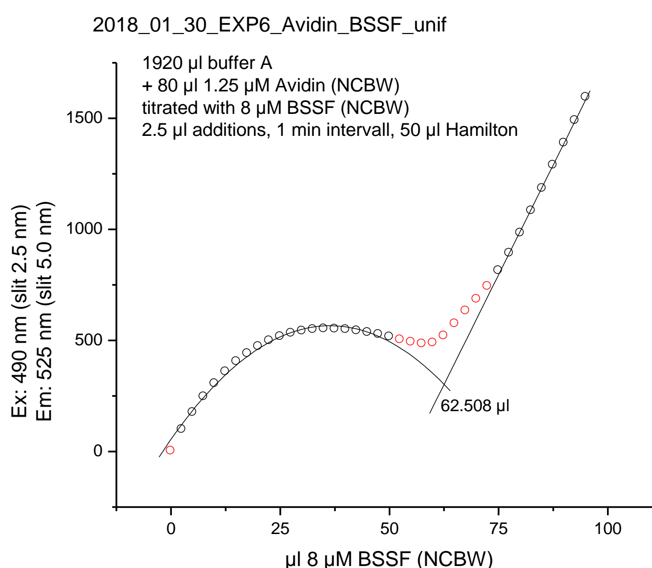


Figure 53: Biotin vs. avidin titration curve (performed by Luise Herrmann in her ongoing master thesis at JKU Linz)

Nominal concentration of BSSF by UV-Vis = 6.77 µM
The end-point BSSF consumption = 62.508 µL
Amount of BSSF bound to avidin = 6.77 µM × 62.508 µL = 0.423 pmol
Almost 4 biotin molecules (4 BSSF molecules) were bound per avidin molecule.

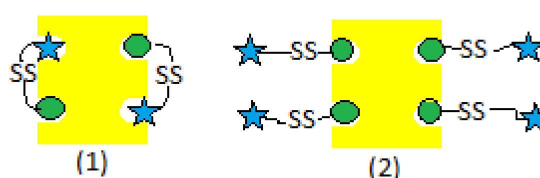


Figure 54: Expected and real binding if BFFS to (strept)avidin

BSSF molecules were supposed to bind like (1), but according to the results they were bound like (2), by forming 4: 1 complex with (strept)avidin. In the Figure 54, green ovals are biotins and blue stars are fluorescein groups connected to biotin with disulphide (SS) bonds.

2.6.4 BSSF vs. streptavidin titration using tryptophan quenching

80 µL (1.25 µM, 0.1 pmol) streptavidin was titrated against 8 µM BSSF and fluorescence measurements were done at the excitation 490 nm nm (slit 2.5 nm) and emission 525 nm (slit 5 nm).

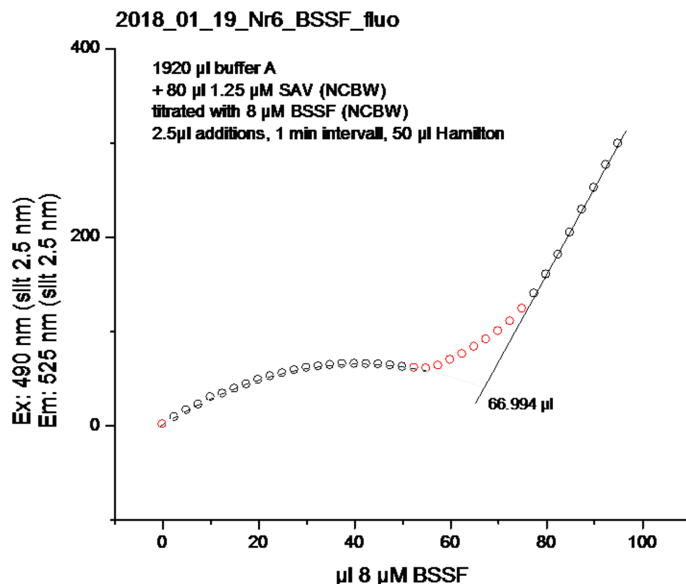


Figure 55: Biotin vs. streptavidin titration curve (performed by Luise Herrmann in her ongoing master thesis at JKU Linz)

The end-point BSSF consumption = 66.994 µL

Amount of BSSF bound to avidin = 6.77 µM × 66.994 µL = 0.454 pmol

4 molecules of BSSF per SAV molecule has being bound according to the results.

According to the titration curve, Figure 9 [Spitzbart, 2014] only 2 molecules of BSSF was supposed to bind one (strept)avidin molecule. But unfortunately, Figure 9 and Figure 53, 55 graphs show a big difference in results. The BSSF molecule which I synthesised did not work according to Spitzbart's results.

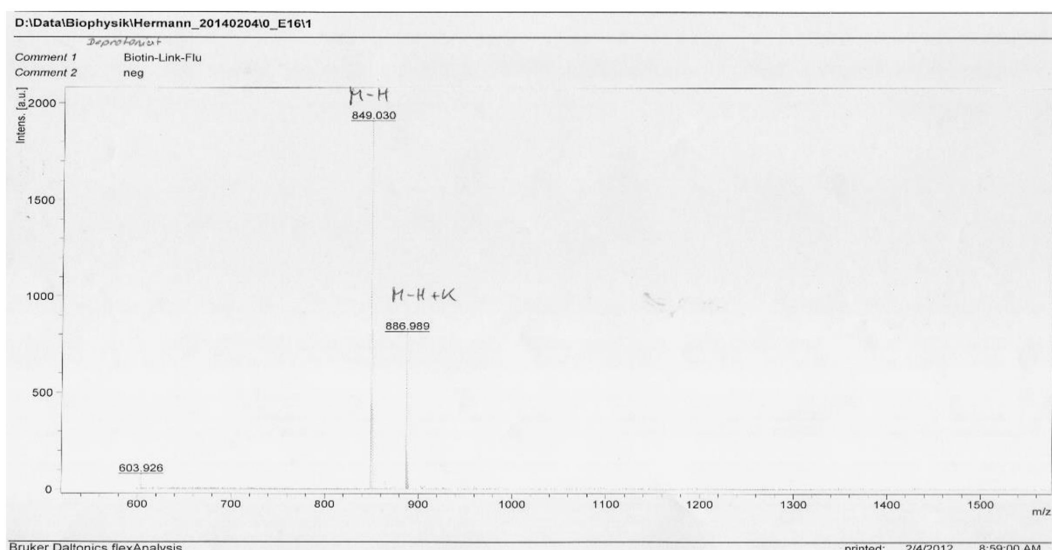


Figure 56: BSSF mass spectrum from Spitzbart, 2014

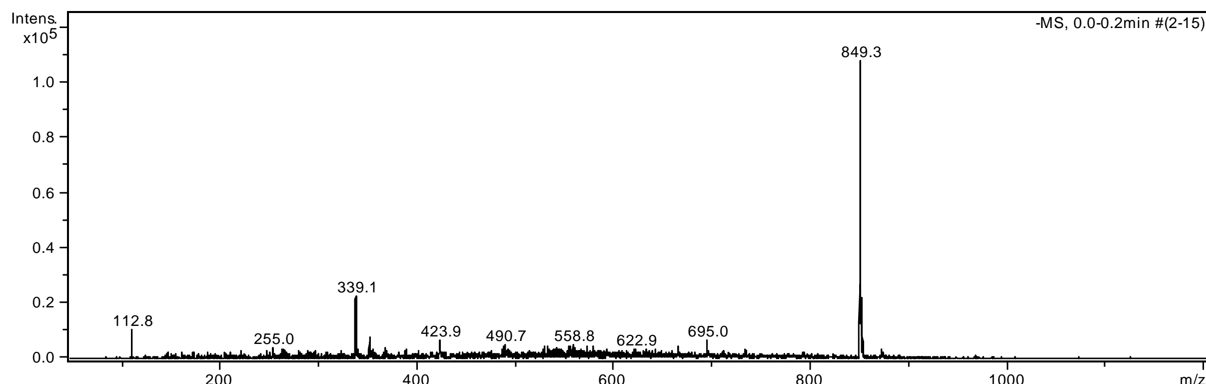


Figure 57: BSSF mass spectrum from my results

According to the mass spectrums, there were no difference of the BSSF we both synthesised. I must conclude that the conflict between the results of Biancy Spitzbart and my results remains unresolved, because it was impossible to find out the reason behind the difference of the results.

2.7 Summary and conclusions

The task of my master thesis was to synthetize and functionally characterized the molecule BSSF. The synthesis part of my master thesis was started from the boc-gly-cystamine-gly-boc molecule. The first step was to deprotect one boc group from the starting material. Then the deprotected molecule (boc-gly-cystamine-gly-NH_2) was used for the second step where the biotin was attached to the amine end of the molecule. The third step was to deprotect the other boc group of the molecule. The final step was to attach fluorescein group to the deprotected end, by forming biotin-gly-cystamine-gly-fluorescein (BSSF). The final product was purified by using reverse phase chromatography. The products from each step were confirmed by using NMR and mass spectroscopy. The synthesising of BSSF molecule was successful according to the results.

The SAV-agarose bead experiments were carried out by using BSSF and other fluorescence active groups. These agarose beads were filled with SAV inside the beads as well as on the bead surface. The diffusion of the different compounds inside the beads was very slow, therefore it took hours until they reached the inner SAV molecules in the centre of beads. This made it impossible to test for reversible blockage of the bead-bound SAV molecules by BSSF and the results were very variable under similar test conditions.

The next step was to synthesis surface-activated Sephadex G-15 beads to overcome the time delay of molecule penetration to the inner core of the beads. Due to the tiny pores of Sephadex G-15, streptavidin could only be bound to the outer surface of the beads. By using a special form of the CNBr activation method (with triethylamine instead of sodium carbonate), the surface of the Sephadex G-15 beads was successfully activated. BSSF functional experiments were carried out with these surface-activated beads. These beads had very low binding capacity for SAV, therefore it was hard to get proper fluorescence measurements with BSSF and after the disulphide cleavage.

In parallel I synthesised a biotin-8arm-PEG to test for reversible blockage of streptavidin by BSSF via fluorescence correlation spectroscopy. Unfortunately, this test became obsolete when all other tests showed that BSSF binds to streptavidin with a simple 4:1 stoichiometry where no binding site was occupied by a fluorescein residue.

3. Experimental section

3.1 General methods

3.1.1 Materials

All the chemicals used for the experiments were purchased from commercial suppliers if possible with analytical grade or with the highest purity and directly used without further purification. Dry DCM was purchased from Merck. The dried solvents (eg: toluene) were dried by using 4 Å activated molecular sleeve for at least one week of time. For all the experiments and for buffer preparations ultrapure deionized water (MilliQ-50 setup from Millipore (Billerica, MA, USA)) was used.

All the molecules in red have been synthesized by my lab colleagues, molecules in blue were directly purchased and black were synthesized by myself in this master thesis project.

3.1.2 Chemical synthesis

All the experiments were performed using clean and dry glassware. Always synthesis reactions were performed under argon gas. All the glassware was filled with argon gas before starting the reactions and well-sealed to avoid gas leaks. Argon gas flow was minimized while the reactions were proceeding. Room temperature (~20°C) was used for the reactions except if it is clearly stated. Teflon joint adaptors were used to fix glass parts instead of grease to avoid gas leaks. All the products and reaction mixture were stored in the freezer (~-20°C).

3.1.3 Thin layer chromatography (TLC)

As the stationary phase, silica gel 60 from Merck (Darmstadt, Germany) TLC plates were used. Mobile phase was prepared according to the polarity of the samples, chloroform/methanol/acetic acid (CME) was the solvent system which I have used for TLC. Solvents of developed plates were evaporated before the plates were stained. Three different methods of staining were used in the synthesis work.

- Iodine – TLC plates were stained with iodine crystals in a closed chamber at room temperature until the spots were clearly visible.
- Ninhydrin – TLC plates were sprayed with ninhydrin solution (50 mg ninhydrin mixed with 50 mL of 1-butanol and 1 mL of acetic acid) and heated on the heating plate (150-200 °C) for specific amine detection.
- DACA reagent - TLC plates were initially sprayed with DACA reagent 1 (1 mL of sulfuric acid (2%) was mixed with 50 mL of ethanol) and then sprayed with DACA reagent 2 (100mg of *p*-Dimethylamine-cinnamaldehyde (0.2 %)was mixed in 50 ml Ethanol) followed by drying for biotin detection.

3.1.4 Reversed phase (RP) chromatography

As the stationary phase in reversed phase chromatography Lichroprep C18-bonded silica self-packed column (d = 1.5 cm, h = 11 cm, 40-63µM particle size, Merck, Darmstadt, Germany) was used. Gradient elution was created according to the product mixture which is needed to separate. The column was stored in absolute ethanol after each run. All the solvents used to create the gradient elution were degassed with helium before each chromatography run. The sample needed to separate was dissolved in 1 mL of the starting solvent or solvent mixture. Before injecting the sample into the column, it was filtered with a cotton plug to remove all the insoluble small particles. Injection valve was connected to automatic pump mixture (KNAUER, Berlin, Germany) with 3 mL injection loop.

Elution solvents were collected with automatic fraction collector (Foxy Jr., Teledyne Isco, Lincoln, NE, USA). Detection was done using UV detector (ERC 7215, ERMA Cr. Inc.) at the absorbance of λ = 214 nm.

3.1.5 Size exclusion chromatography

For size exclusion chromatography Sephadex LH-20 (GE Healthcare) packed column (d = 2.5 cm, h =95 cm, 1.5 cm^2 x 95 cm, Kronlab, Sinsheim, Germany) column was used. Degassed chloroform with helium gas was used as the mobile phase. Sample was dissolved in 5 mL of chloroform and was filtered with a cotton plug before the injection to remove all the insoluble particles. Injection valve was connected to automatic pump (KNAUER, Berlin, Germany) with 15 mL injection loop. Elution flow rate was settled to 0.4 mL/min and the fractions were collected with automatic fraction collector (Foxy Jr., Teledyne Isco, Lincoln, NE, USA).

3.1.6 Nuclear magnetic resonance spectroscopy (NMR)

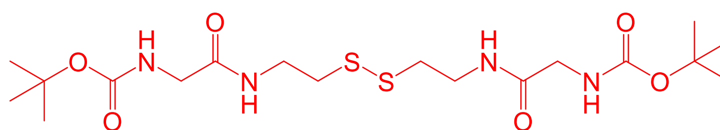
300 MHz Advance III spectrometer (Bruker company) was used for all the Nuclear Magnetic Resonance spectroscopy measurements. Measurements were done at the Institute of Organic Chemistry, Johannes Kepler University Linz. ^{1}H spectra were recorded with the manually specified relaxation delay (d1) 1S - 30s and the number of scans (ns) set to 16 or 32 scans (depending on the amount of the sample). For other complicated spectrums such as, COSY, HSQC, HMBC, ^{13}C the relaxation delay (d1) was assigned automatically and number of scans (ns) were manually specified according to the needed scan time. All the spectrums were analysed using Topspin 3.5pl7 software. NMR tubes were cleaned after each measurement with distilled water, isopropanol, chloroform and dried at room temperature at least for one day before using again.

3.1.7 Mass spectrometry (MS)

All the mass spectroscopic measurements were done at the Institute of Analytical Chemistry, Johannes Kepler University Linz. Agilent 1100 LC system equipped with a vacuum degasser and a quaternary pump coupled to a 6510 QTOF mass spectrometer (Agilent technologies) was used for the measurements. MS parameters were settled as, capillary voltage 3.5 kV, nebulizer pressure 50 psi, dry gas flow 10 L/min at 350°C. Samples were prepared approximately with 0.1 mg/mL in methanol. Positive mode (+MS) of scanning was done using 97% methanol with 10 mM ammonium formate and 3% formic acid makeup flow to enhance ionization efficiency and the negative mode acquisition (-MS) was done using 100% methanol with 10 mM ammonium formate. Image scanning range was specified according to the target mass of the product and the side products.

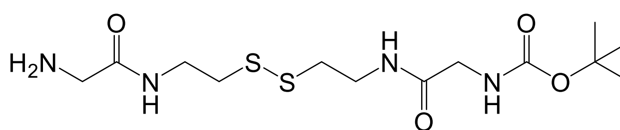
3.2 Linker chemical synthesis

3.2.1 Synthesis of NH_2-gly-cystamine-gly-boc



boc-gly-cystamine-gly-boc
Exact Mass: 466,19g/mol

DCM/TFA
(25:1)



NH_2-gly-cystamine-gly-boc
Exact Mass: 366.14g/mol

Figure 58: Reaction scheme for synthesis of NH_2-gly-cystamine-gly-boc

400 mg (858 µmol, 1 eq, M_W = 466.61 g/mol) of boc-gly-cystamin-gly-boc (which was made by a previous lab co-worker, Bianca Spitzbart) was taken into a 50 mL round bottom flask and 3.0 mL (47 mmol, 50 eq M_W = 84.93 g/mol, ρ = 1.33 g/mL) of DCM and 150 µL of TFA (1.96 mmol, 2 eq, M_W = 114.02 g/mol, ρ = 1.49 g/mL) were added into it. Reaction was continued under the flow of Argon. In every 15 minutes a TLC was run in CME (80: 20: 2) to monitor the progress of the reaction and 30 µL (392 µmol) of TFA was added into the mixture until the reaction was completed. At the total volume of 420 µL (5.5 mmol) TFA, the reaction was stopped by adding 20 mL of toluene into it. All the solvents were fully evaporated by using rotary evaporator at 25^0C temperature.

Liquid-liquid extraction was used to separate out NH_2-gly-cystamine-gly-boc of the reaction. Mixture of 400 mM acetic acid and 50% sodium chloride solution (pH 4) was prepared to dissolve the dried products of the reaction. All the compounds were dissolved by using volume of 50 mL. First extraction was done by using chloroform (50 mL × 3). All the unreacted boc-gly-cystamine-gly-boc was extracted into the chloroform.

Second extraction was done by using liquid layer from the previous extraction. The pH was increased to 9 by using 23 mL of 10% sodium carbonate solution. Then extraction was carried out by using chloroform (50 mL × 2). Second part of the extraction was done under flow of argon. Organic layer was used for the third extraction and aqueous layer was discarded.

The separation funnel was washed with deionized water. Then the organic layer was once extracted with 40 mL of 10% sodium carbonate solution under the flow of argon gas. Finally, the organic layer was collected into a toluene (50 mL) and TFA (420 µL) solution mixture. All the solvents from organic layer were fully evaporated by using rotavap at 25^0C temperature to obtain NH_2-gly-cystamine-gly-boc.

70.72 mg (147.3 µmol, M_W = 480.13 g/mol with TFA) of pure NH_2-gly-cystamine-gly-boc was obtained with a yield of 17.17%.

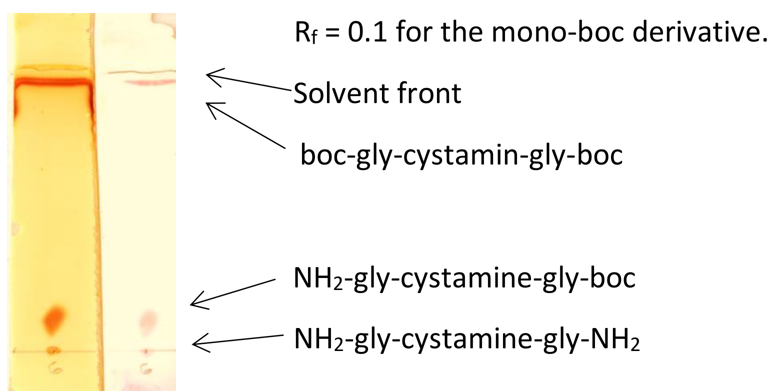


Figure 59: TLCs of the solution mixture after adding total volume of 420 µL of TFA, developed in CME 80: 20: 2 stained in both iodine (left) and ninhydrin (right).

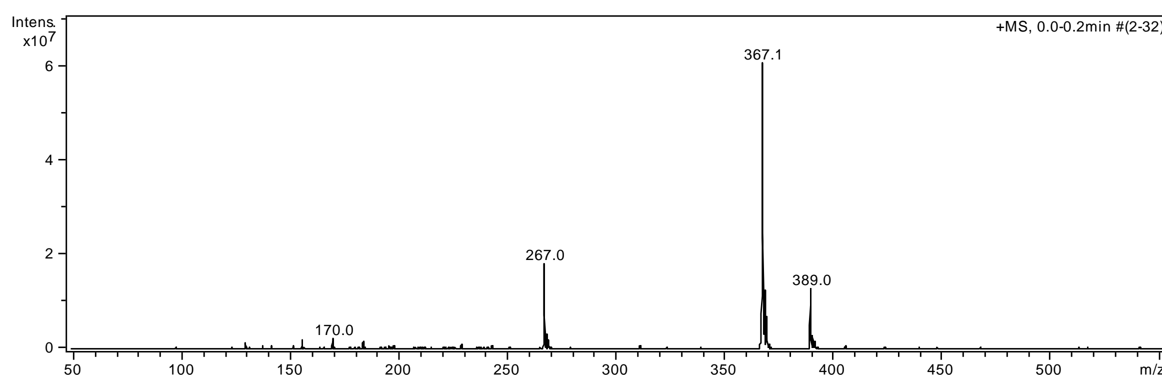


Figure 60: Mass spectrum of NH_2-gly-cystamine-gly-boc. ESI-MS (positive mode) in the acquisition mass range of 50-600 (m/z). Expected 367. Found: 367 $(M+H)^+$, 389 $(M+Na)^+$.

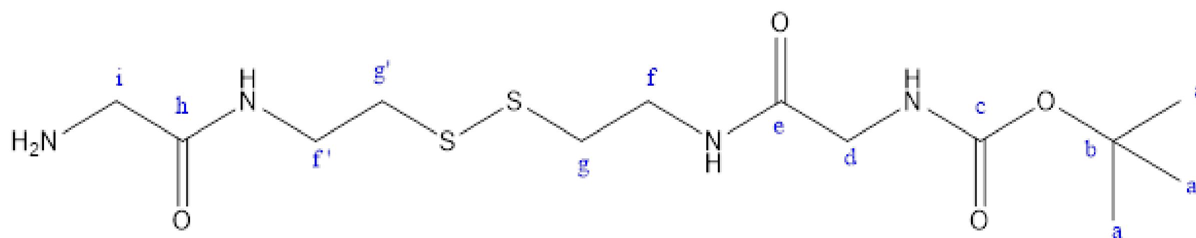


Figure 61: Structure of NH_2-gly-cystamine-gly-boc

^{1}H NMR (300MHz, MeOD) δ (ppm): 1.36 (9 H, s, a-C(**CH_3**)$_3$), 2.72 (2 H, t, J = 6.5 Hz, g-**CH_2**), 2.76 (2 H, t, J = 6.4 Hz, g'-**CH_2**), 3.42 (2 H, t, J = 7.7 Hz, f-**CH_2**), 3.47 (2 H, t, J = 6.7 Hz, f'-**CH_2**), 3.59 (4H, s, d and i-**CH_2**), 4.76 (H_2O).

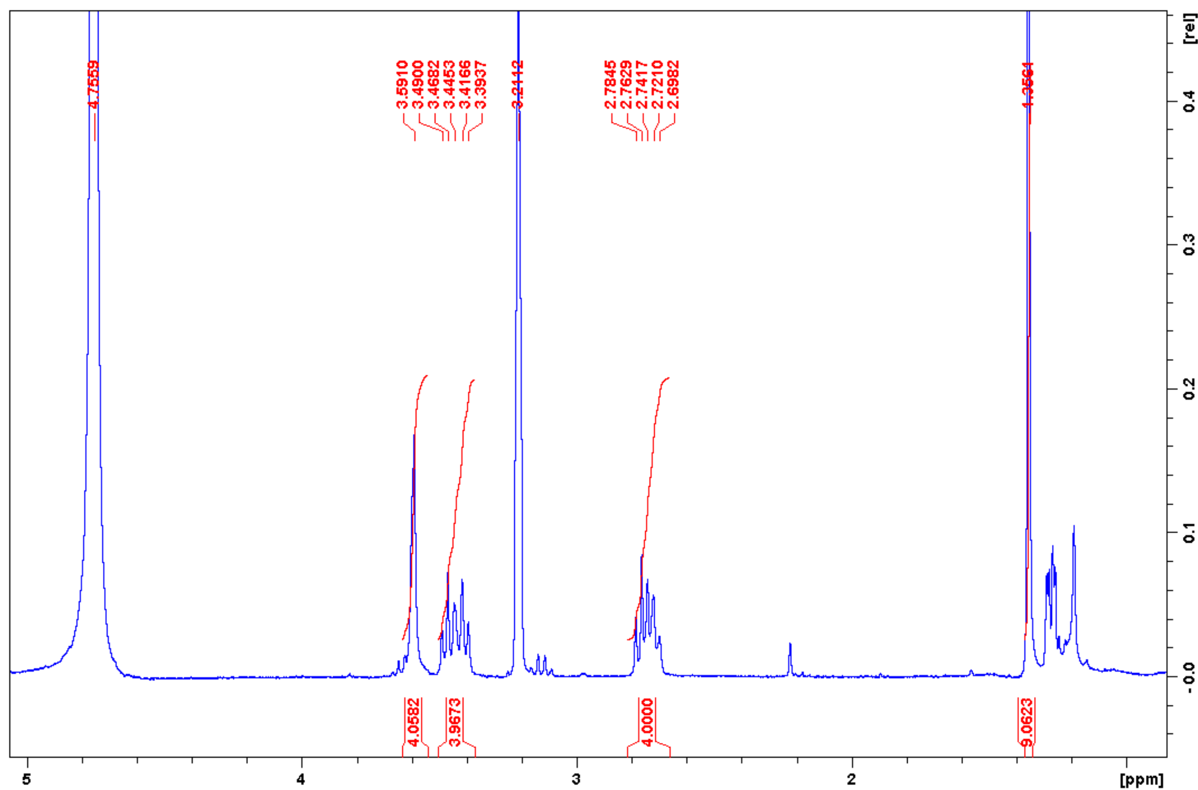


Figure 62: ^{1}H NMR spectrum for NH_2-gly-cystamine-gly-boc

^{13}C NMR (300MHz, MeOD) δ (ppm): 27.29 (3C, a-**CH_3**), 36.81 (1C, g-**CH_2**), 37.01 (1C, g-**CH_2**), 38.12 (1C, f-**CH_2**), 38.23 (1C, f-**CH_2**), 40.08 (1C, i-**CH_2**), 43.26 (1C, d-**CH_2**), 47.61 (MeOD), 79.40 (1C, b-**C $(CH_3)_3$**), 157.05 (1C, c-**C=O**), 166.04 (1C, e/h-**C=O**), 171.40 (1C, e/h-**C=O**).

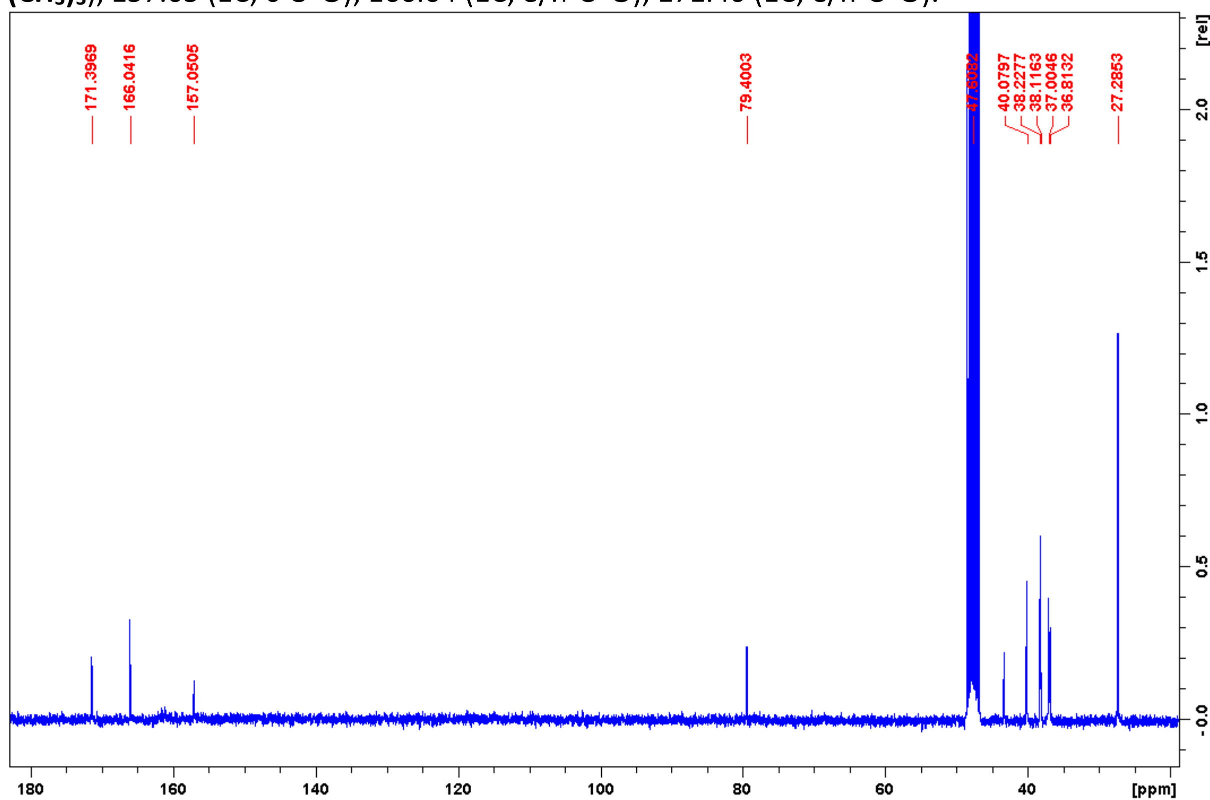


Figure 63: ^{13}C NMR spectrum for NH_2-gly-cystamine-gly-boc

3.2.2 Synthesis of biotin-gly-cystamine-gly-boc

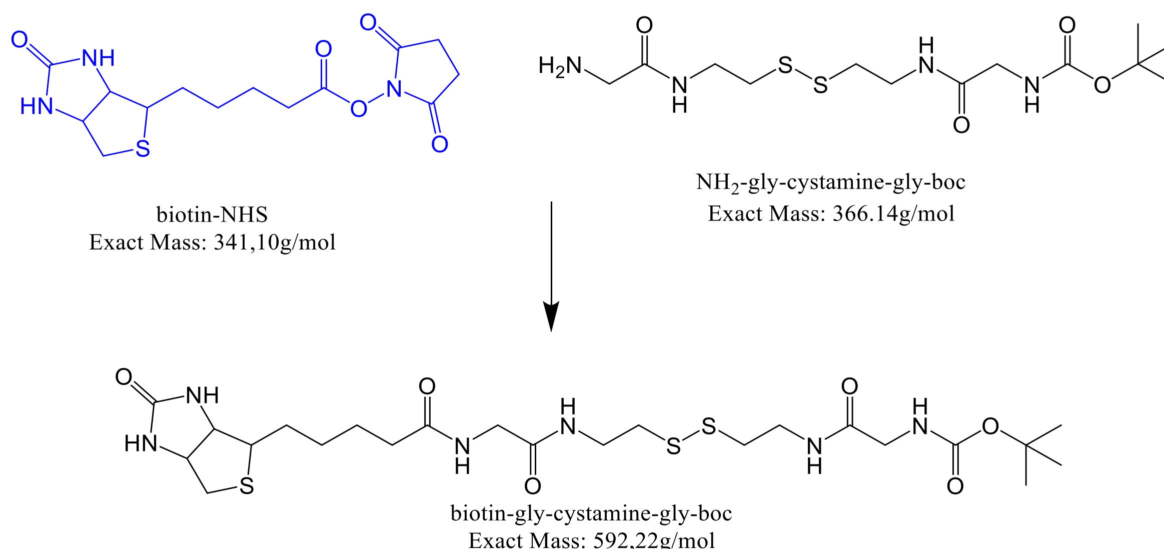
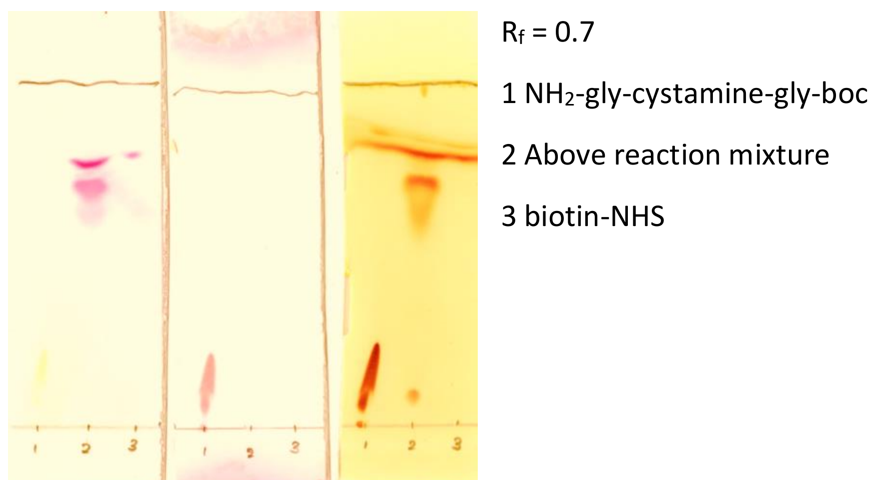


Figure 64: Reaction scheme for synthesis of biotin-gly-cystamine-gly-boc

95 mg (0.198 mmol, 1 eq, M_W = 366.14 g/mol) of NH_2-gly-cystamine-gly-boc was used for the reaction. 170 mg (0.5mmol, 2.5 eq, M_W = 34.10 g/mol) of biotin-NHS was mixed with NH_2-gly-cystamine-gly-boc in a 25ml round bottom flask and was partially dissolved by using 1 mL (M_W = 78.13 g/mol, ρ = 1. 1 g/mL) of DMSO. 203 µL (1.2 mmol, 6 eq, M_W = 129.25 g/mol, ρ = 0.742 g/mL) of diisopropylethylamine (DIPEA) was added into the mixture and minimum amount of DMSO (1 mL) was used to dissolve the mixture completely. Reaction was stirred overnight under the flow of argon.

Completion of the reaction was checked by a TLC in morning.



R_f = 0.7

1 NH_2-gly-cystamine-gly-boc

2 Above reaction mixture

3 biotin-NHS

Figure 65: TLC was developed in CME 80: 20: 2, stained in DACA (left), ninhydrin (middle) and iodine (right).

Removal of excess biotin-NHS was done by using 6-aminohexanoic acid. 262 mg (2 mmol, 10 eq, M_W = 131.17 g/mol) of 6-aminohexanoic acid was dissolved in a 50mL round bottom flask by using 5.5 mL of water, 1.1 mL (15.4 mmol, M_W = 78.13 g/mol, ρ = 1.1 g/mL) of DMSO and 1.1 mL (20.1 mmol, M_W = 41.05 g/mol, ρ = 0.786 g/mL) of acetonitrile under the argon flow. 21.7 µL (124.6 µmol, M_W = 129.25 g/mol, ρ = 0.742 g/mL) of DIPEA was initially added into the mixture to increase the pH up to 8.

Products from the above reaction, unreacted excess of biotin-NHS and biotin-gly-cystamine-gly-boc were added drop wise in to the flask while stirring. Reaction was carried out for 45 minutes while checking the pH of the system. To maintain pH at 8 DIPEA was added in 25 µL portions. Total of 146.7 µL (0.84 mmol, M_W = 129.25 g/mol, ρ = 0.742 g/mL) DIPEA was in the reaction mixture at the end.

Acetonitrile was evaporated using rotavap and other solvents were evaporated using high vacuum cool trap. Residue was dissolved in 90 mL of chloroform and was transferred in to the separation funnel. First extraction was carried out by using10% sodium carbonate solution (60mL × 2). Secondly 0.1% phosphoric acid in half brine solution (60 mL × 2) was used for the extraction. Finally, 0.1% phosphoric acid in full brine solution (60 mL × 2) was used. Chloroform layer was dried out by using anhydrous sodium sulphate and fully evaporated the solvent using rotavap.

63.6 mg (0.1074 mmol, M_W = 592.22 g/mol with) of pure biotin-gly-cystamine-gly-boc was obtained with a yield of 54.24%.

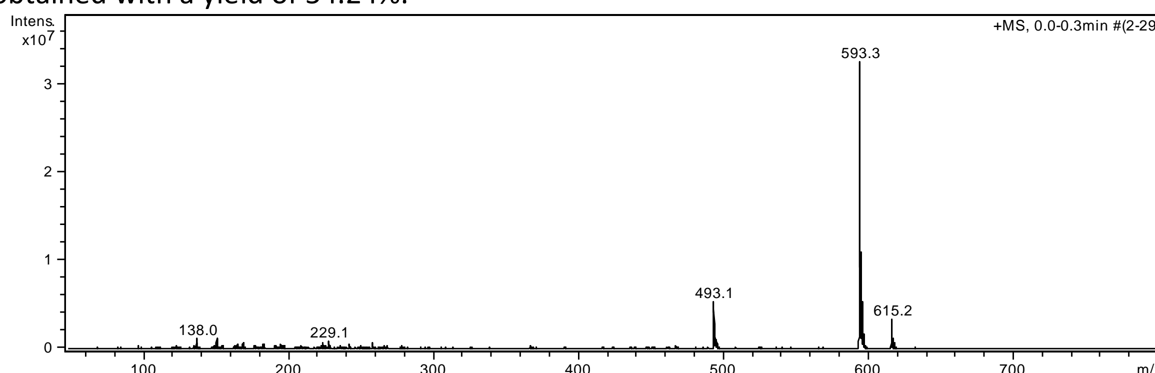


Figure 66: Mass spectrum of biotin-gly-cystamine-gly-boc. ESI-MS (positive mode) in the acquisition mass range of 50-800 (m/z). Expected 593. Found: 593 $(M+H)^+$, 615 $(M+Na)^+$.

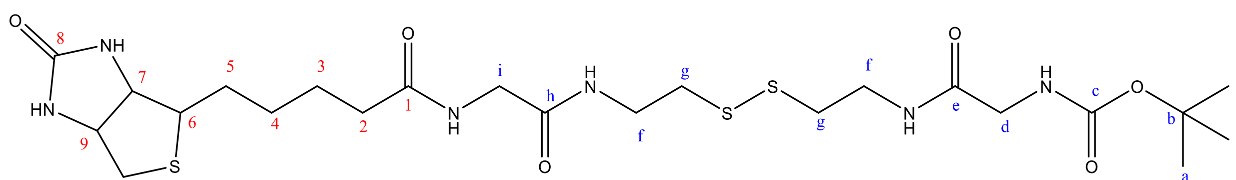


Figure 67: Structure of biotin-gly-cystamine-gly-boc

^{1}H NMR (300MHz, MeOD) δ (ppm): 1.35-1.59 (15 H, m, a-C(**CH₃**)₃, 1.35-1.59 (6 H, m, 3-**CH₂**, 4-**CH₂**, 5-**CH₂**), 2.20 (2 H, t, J = 7.3 Hz, 2-**CH₂**), 2.61 (1 H, d, J = 12.7 Hz, 10-**CH**), 2.73 (4 H, t, J = 6.5 Hz, g-**CH₂**), 2.83 (1 H, dd, J = 12.8 Hz, J=4.9, 10'-**CH₂**), 3.12 (1 H, q, J = 6.2, 6-**CH₂**), 3.21 (MeOH), 3.25 (MeOD), 3.42 (4 H, t, J = 6.7, f-**CH₂**), 3.60 (2 H, s, d-**CH**), 3.74 (1 H, s, i-**CH**), 4.20-2.24 (1 H, m, 7-**CH**), 4.38-4.42 (1 H, m, 9-**CH**), 4.75 (H_2O).

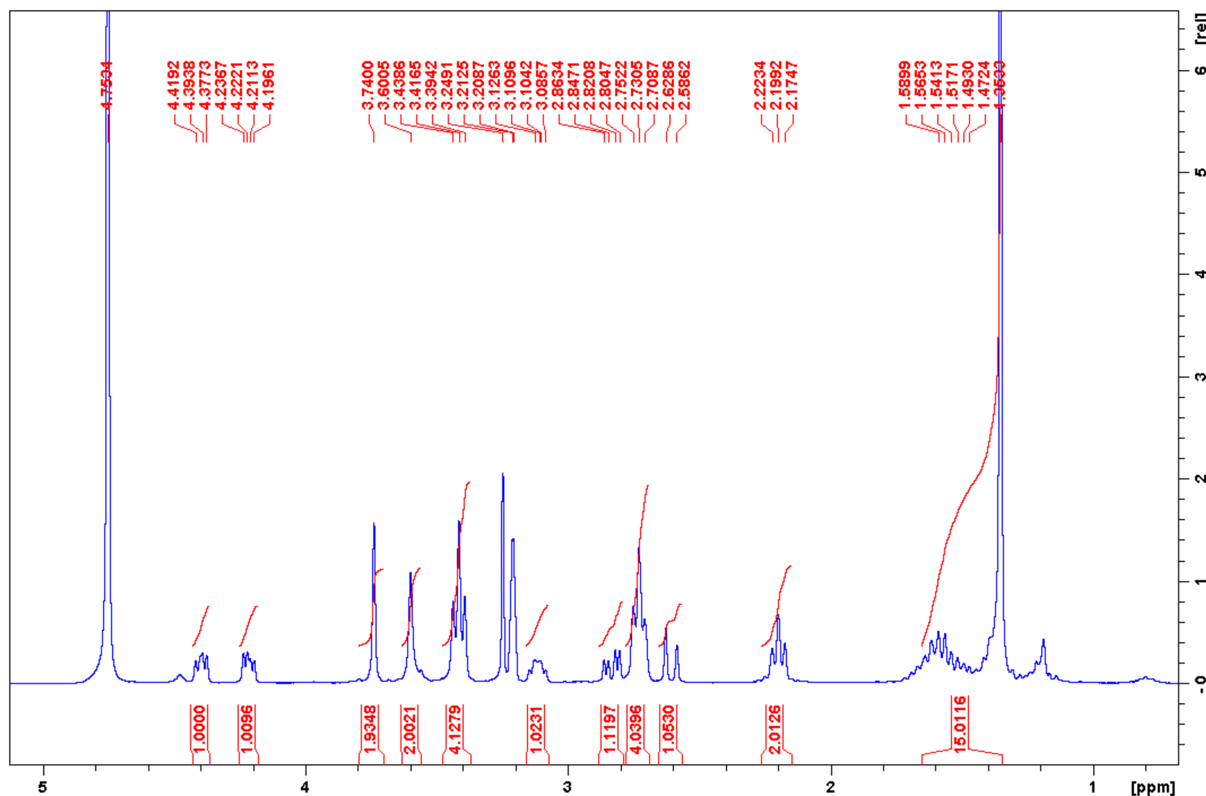


Figure 68: ^{1}H NMR spectrum for biotin-gly-cystamine-gly-boc

^{13}C NMR (300MHz, MeOD) δ (ppm): 25.19 (1C, 3/4/5-**CH_2**), 27.32 (3C, a-**CH_3**), 28.03 (1C, 3/4/5-**CH_2**), 28.27 (1C, 3/4/5-**CH_2**), 35.10 (1C, 2-**CH_2**), 36.95 (1C, g-**CH_2**), 37.07 (1C, f-**CH_2**), 38.20 (1C, f-**CH_2**), 39.66 (1C, 10-**CH_2**), 42.10 (1C, i-**CH_2**), 43.25 (1C, d-**CH_2**), 47.61 (MeOD), 55.56 (1C, 6-**CH**), 60.26 (1C, 9-**C (CH_2)**), 61.87 (1C, 7- **C (CH_2)**), 79.35 (1C, b-**C (CH_3)$_3$**), 157.01 (1C, c-**C=O**), 164.75 (1C, 8-**C=O**), 170.47 (1C, h-**C=O**), 171.31 (1C, e-**C=O**), 175.18 (1C, 1-**C=O**).

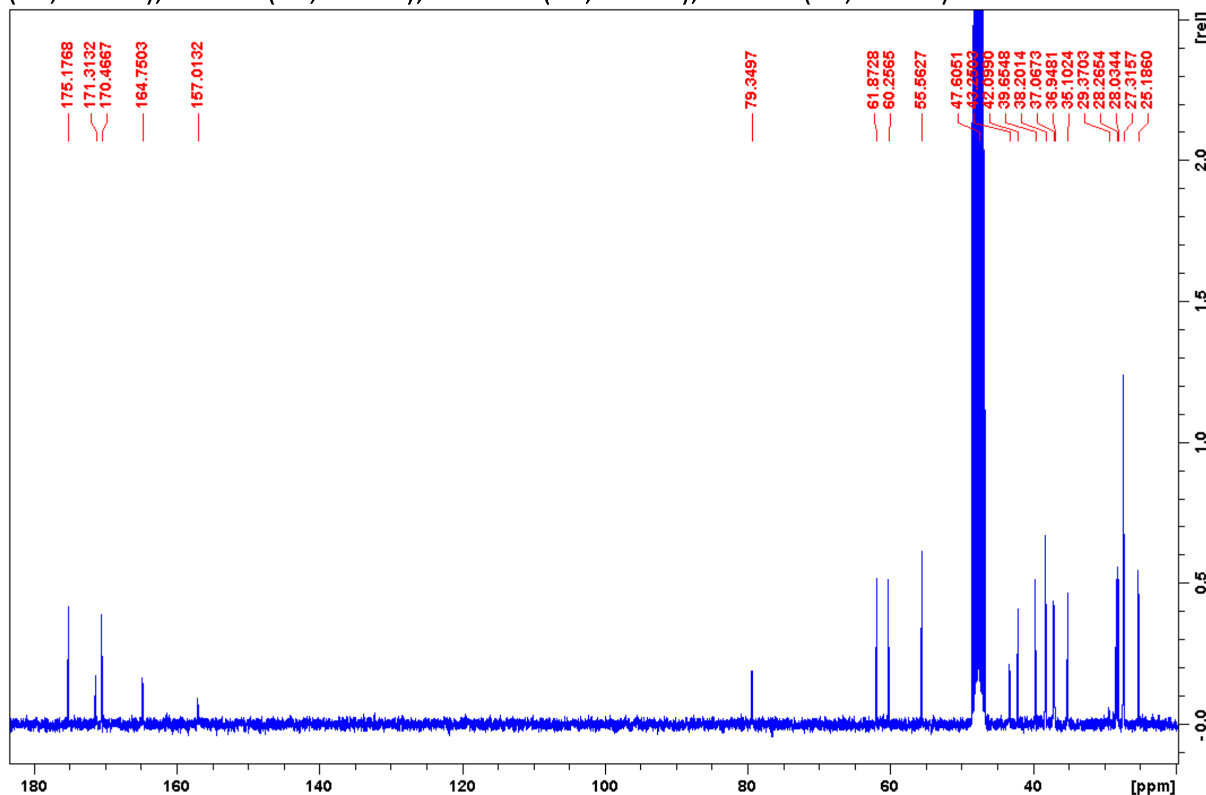


Figure 69: ^{13}C NMR spectrum for biotin-gly-cystamine-gly-boc

3.2.3 Synthesis of biotin-gly-cystamine-gly-NH_2

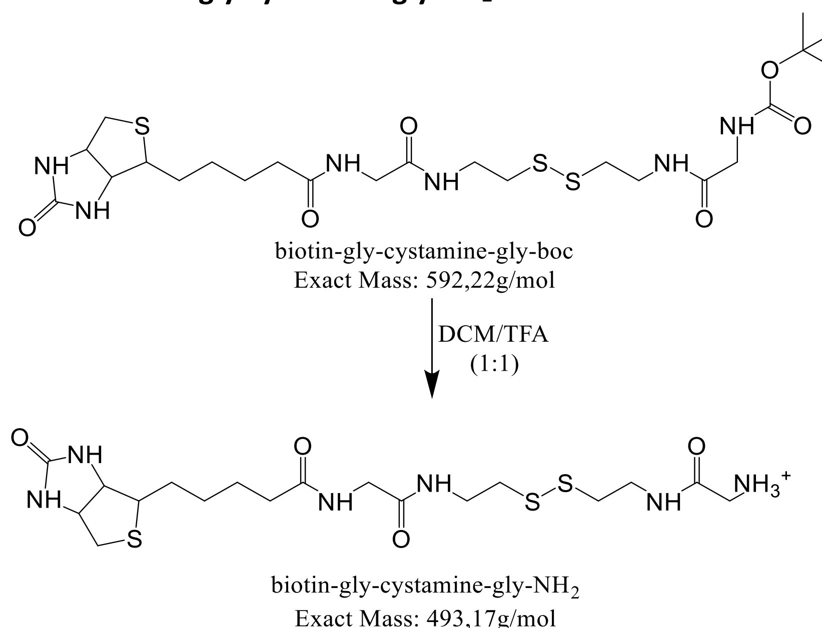


Figure 70: Reaction scheme for synthesis of biotin-gly-cystamine-gly-NH_2

30.7 mg (51.8 µmol, 1 eq, M_W = 592.22 g/mol) of biotin-gly-cystamine-gly-boc was mixed with 1.5 mL of DCM and 1.5 mL of TFA in a 25 mL round bottom flask and stirred for 3 hours under the argon flow.

31.5 mg (51.8 µmol, M_W = 607.17 g/mol with TFA) of pure biotin-gly-cystamine-gly-NH_2 was obtained with a yield of 100%.

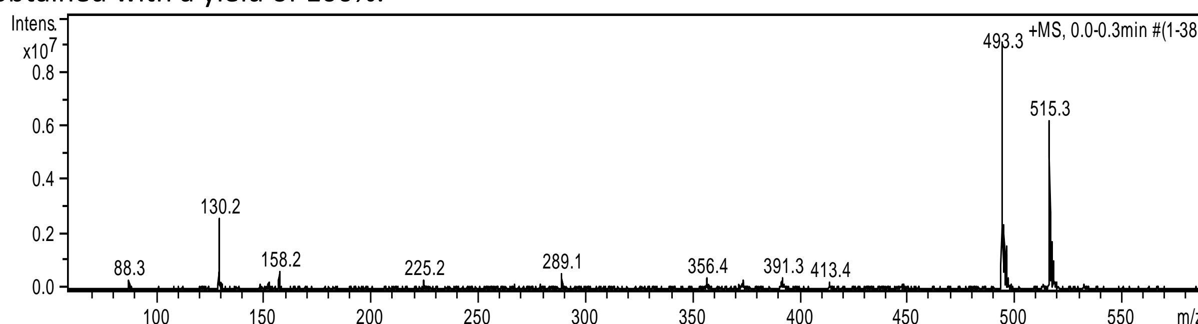


Figure 71: Mass spectrum of biotin-gly-cystamine-gly-NH_2. ESI-MS (positive mode) in the acquisition mass range of 50-600 (m/z). Expected 493. Found: 493 $(M+H)^+$, 515 $(M+Na)^+$.

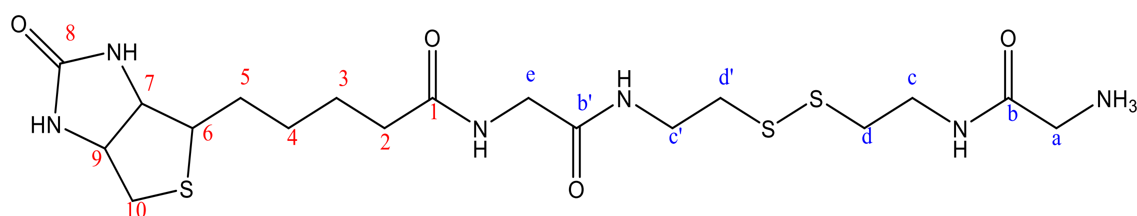


Figure 72: Structure of biotin-gly-cystamine-gly-NH_2

^{1}H NMR (300MHz, MeOD) δ (ppm): 1.38-1.62 (6 H, m, 3-**CH$_2$**, 4-**CH$_2$**, 5-**CH$_2$**), 2.21 (2 H, t, J = 7.2 Hz, 2-**CH$_2$**), 2.61 (1 H, d, J = 12.7 Hz, 10-**CH**), 2.74-2.76 (2 H, m, d/d'-**CH$_2$**), 2.84 (1 H, dd, J = 12.8 Hz, J=4.8 Hz, 10/10'-**CH$_2$**), 3.12 (1 H, q, J = 6.1, 6-**CH$_2$**), 3.22 (MeOH), 3.25 (MeOD), 3.44 (2 H, m, c/c'-**CH$_2$**), 3.60 (2 H, s, a-**CH**), 3.74 (1 H, s, e-**CH**), 4.21-2.25 (1 H, m, 7-**CH**), 4.39-4.43 (1 H, m, 9-**CH**).

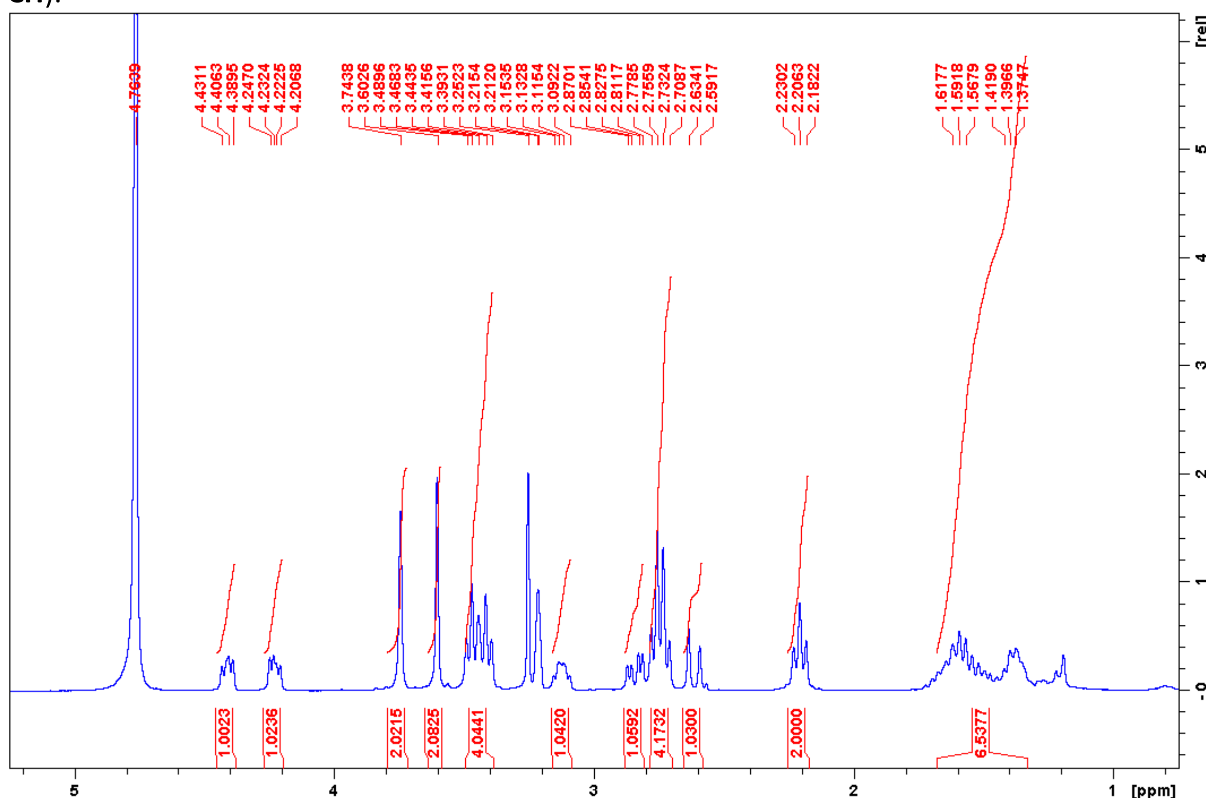


Figure 73: ^{1}H NMR spectrum for biotin-gly-cystamine-gly-NH$_2$

^{13}C NMR (300MHz, MeOD) δ (ppm): 25.22 (1C, 3/4/5-**CH$_2$**),28.05 (1C, 3/4/5-**CH$_2$**), 28.26 (1C, 3/4/5-**CH$_2$**), 35.09 (1C, 2-**CH$_2$**), 36.94 (1C, d/d'-**CH$_2$**), 37.02 (1C, d/d'-**CH$_2$**), 38.18 (1C, c/c'-**CH$_2$**), 38.30 (1C, c/c'), 39.68 (1C, 10-**CH$_2$**), 40.12 (1C, e-**CH$_2$**), 42.16 (1C, a-**CH$_2$**), 47.63 (MeOD), 55.57 (1C, 6-**CH**), 60.28 (1C, 9-**C (CH$_2$)**), 61.89 (1C, 7- **C (CH$_2$)**), 164.82 (1C, 8-**C=O**), 166.10 (1C, b-**C=O**), 170.56 (1C, b'-**C=O**), 175.28 (1C, 1-**C=O**).

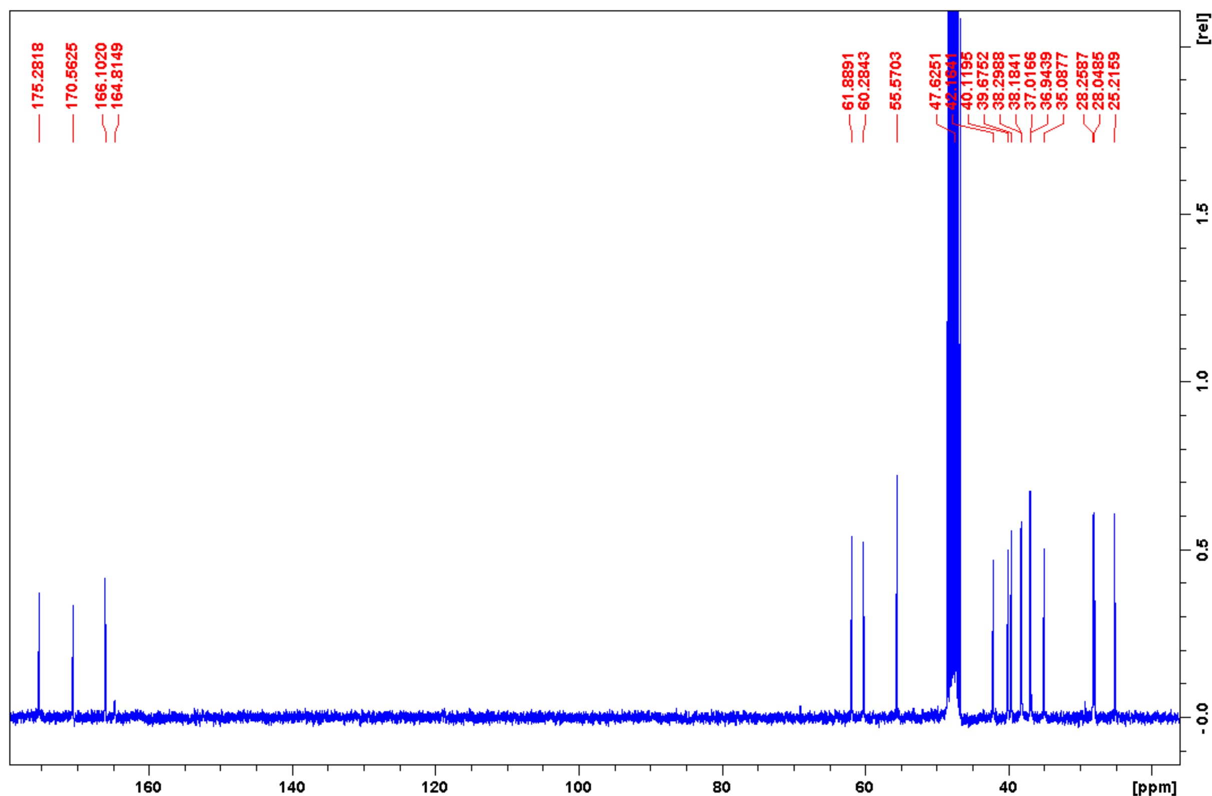


Figure 74: ^{13}C NMR spectrum for biotin-gly-cystamine-gly-NH_2

3.2.4 Synthesis of biotin-gly-cystamine-gly-fluorescein

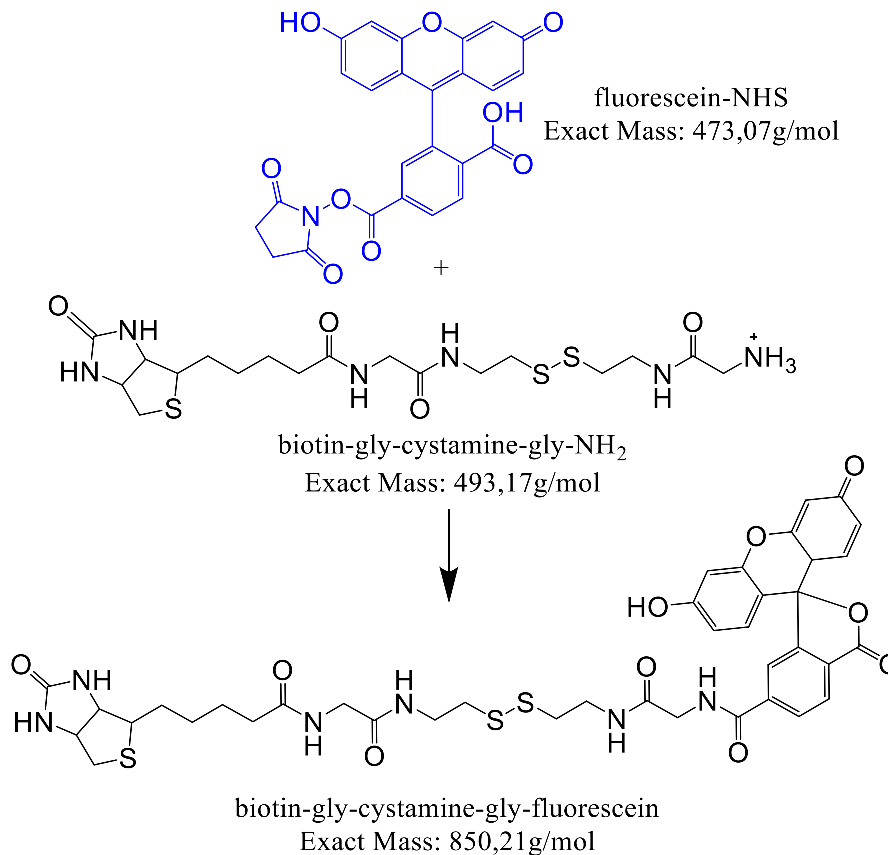


Figure 75: Reaction scheme for synthesis of biotin-gly-cystamine-gly-fluorescein

40 mg of biotin-gly-cystamine-gly-NH_2 (81 µmol, 1 eq, M_W = 493.17 g/mol) was mixed with fluorescein-NHS 46 mg (97.2 µmol, 1.2 eq, 473.07 g/mol) in a 25 mL round bottom flask. Initially 750 µL of DMSO and 141.1 µL (0.81 mmol, 10 eq, M_W = 129.25 g/mol, ρ = 0.742 g/mL) of DIPEA was added into the mixture. Then the minimum amount of DMSO (1.5 mL) was added to dissolve the mixture completely. Round bottom flask was covered with aluminium foil to avoid light and was stirred under the flow of argon overnight. A TLC was run in next morning using the solvent system CME (80: 20: 2) to check the reaction completion. Then 24.8 µL (0.41 mmol, 5 eq, M_W = 61.08 g/mol, ρ = 1.01 g/mL) of ethanol amine was added to the mixture and let it react for 3 more hours. All the solvents were fully evaporated using high vacuum cool trap. Fully dried product was dissolved using 50 mM Tris-base (pH = 7.5) and was injected into the HPLC to separate out biotin-gly-cystamine-gly-fluorescein.

Product purification by RP-HPLC

RP-HPLC method was used to obtain the highest purify of the product by analysing the chromatogram. For the total run 3 different eluent solutions were prepared.

Eluent A - 3% Acetonitrile in water
Eluent B - Pure Acetonitrile (HPLC grade)
Eluent C - 1% Acetic acid in water

Table 11: Gradient elution programme for the chromatogram run

Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)
0	100	0	0
5	100	0	0
6	90	0	10
15	90	0	10
50	45	45	10
55	0	90	10
65	0	90	10
67	90	0	10
77	90	0	10
87	90	0	10
107	0	0	0

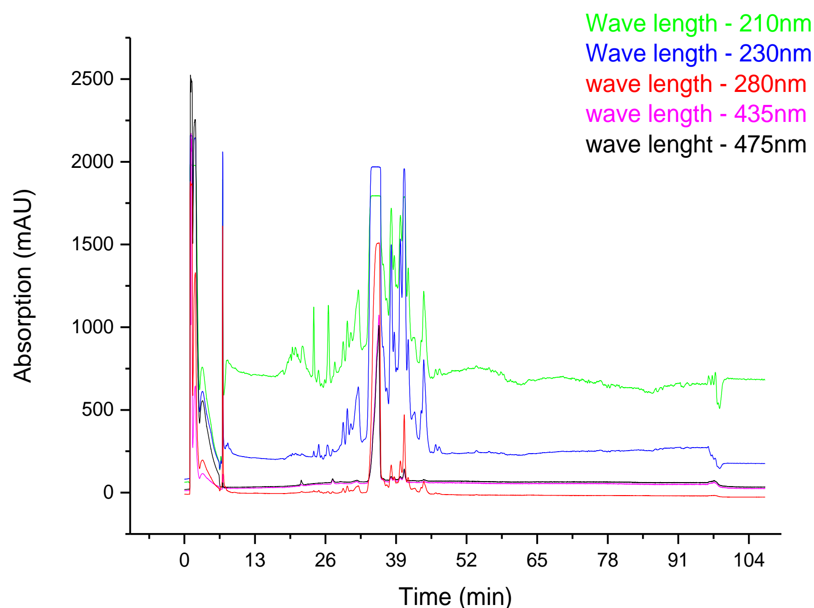


Figure 76: Chromatogram with all the wave lengths

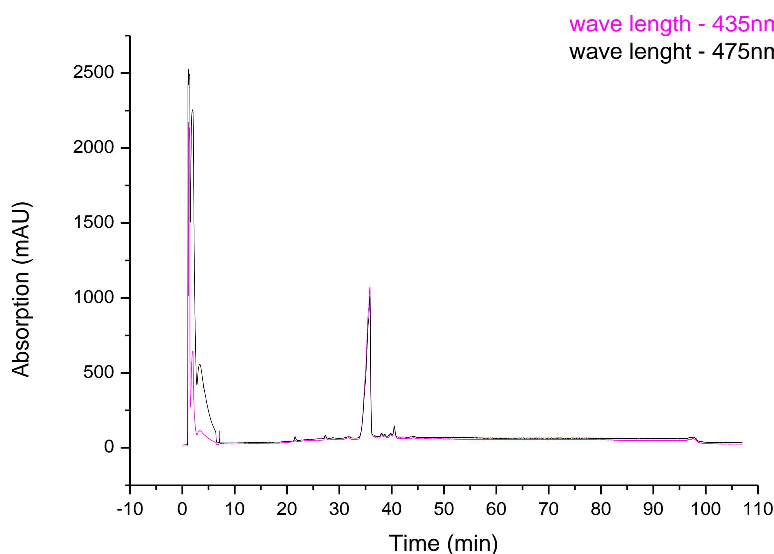


Figure 77: Chromatogram at higher wave lengths which correspond to the biotin-gly-cystamine-gly-fluorescein

Peak fractions were combined into a weighted pear-shaped flask and 3 mL of 1% TFA was added into that. Solvents were fully evaporated by rotavap.

35.7 mg (42 µmol) of pure biotin-gly-cystamine-gly-fluorescein product was obtained with a yield of 51.85%. The verification of the product was done by mass spectroscopy and NMR.

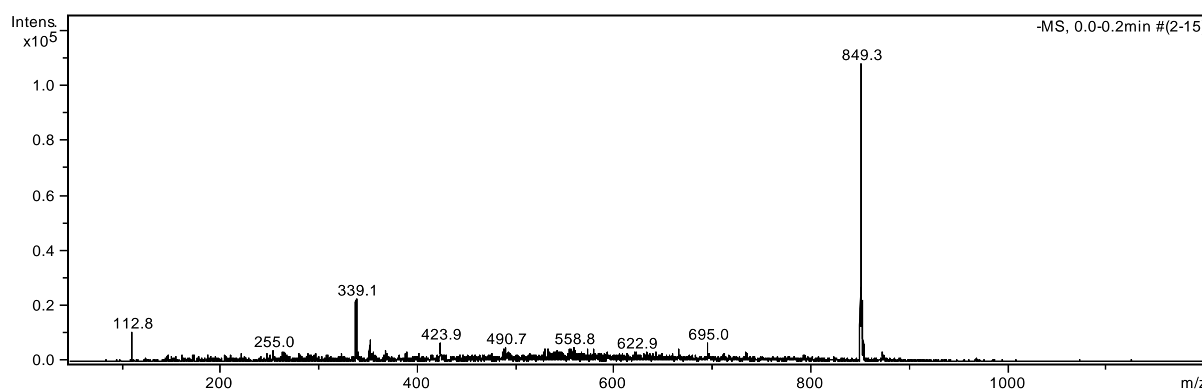


Figure 78: Mass spectrum of biotin-gly-cystamine-gly-fluorescein. ESI-MS (negative mode) in the acquisition mass range of 50-1200 (m/z). Expected 850. Found: 850 $(M+H)^+$.

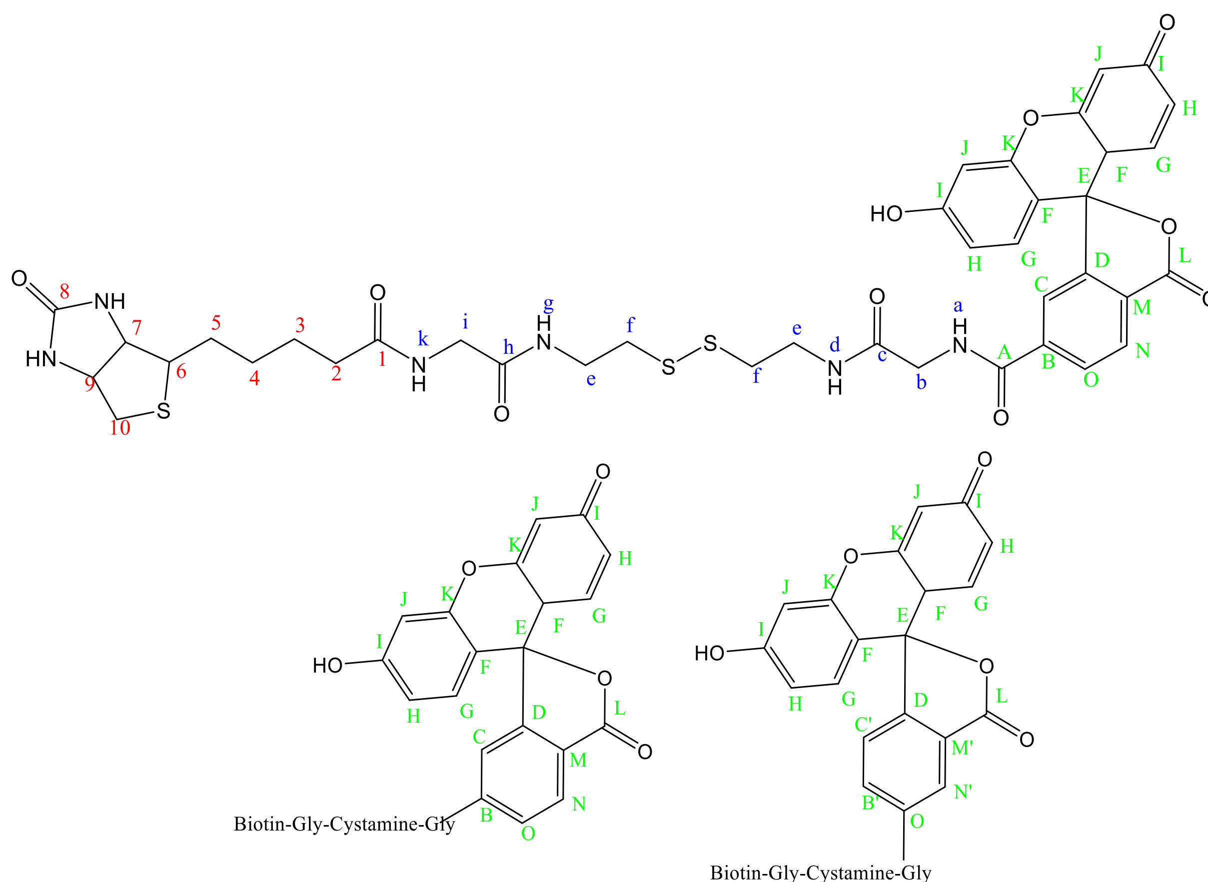


Figure 79: Structure of biotin-gly-cystamine-gly-fluorescein

^{1}H NMR (300MHz, DMSO) δ (ppm): 1.40-1.68 (6 H, m, 3-**CH₂**, 4-**CH₂**, 5-**CH₂**), 2.13 (2 H, t, J = 7.1 Hz, 2-**CH₂**), 2.51 (DMSO), 2.58 (1 H, d, J = 12.7 Hz, 12’-**CH**), 2.72-2.85 (5 H, m, f-**CH₂**, 12-**CH**), 3.09 (1 H, q, J = 6.2, 6-**CH₂**), 3.32-3.41 (4 H, m, e-**CH₂**), 3.65 (2 H, t, J = 6.4, i-**CH₂**), 3.79 (2 H, t, J = 6.4, b-**CH₂**), 3.91 (H_2O), 4.11-4.15 (1 H, m, 7-**CH**), 4.29-4.33 (1 H, m, 11-**CH**), 6.55-6.63 (4 H, m, G-**CH**, H-**CH**), 6.70 (2 H, s, J-**CH**), 7.40 (0.5 H, d, J = 8.0, N-**CH**), 7.72 (0.5 H, s, N’-**CH**), 8.08-8.15 (0.5 H, m, B’-**CH**), 8.18-8.21 (0.5 H, m, C’-**CH**), 8.28 (0.5 H, d, J = 8.1, O-**CH**), 8.52 (0.5 H, s, C-**CH**), 8.98 (0.5 H, t, J = 5.7, a’-**NH**), 9.15 (0.5 H, t, J = 5.7, a-**NH**).

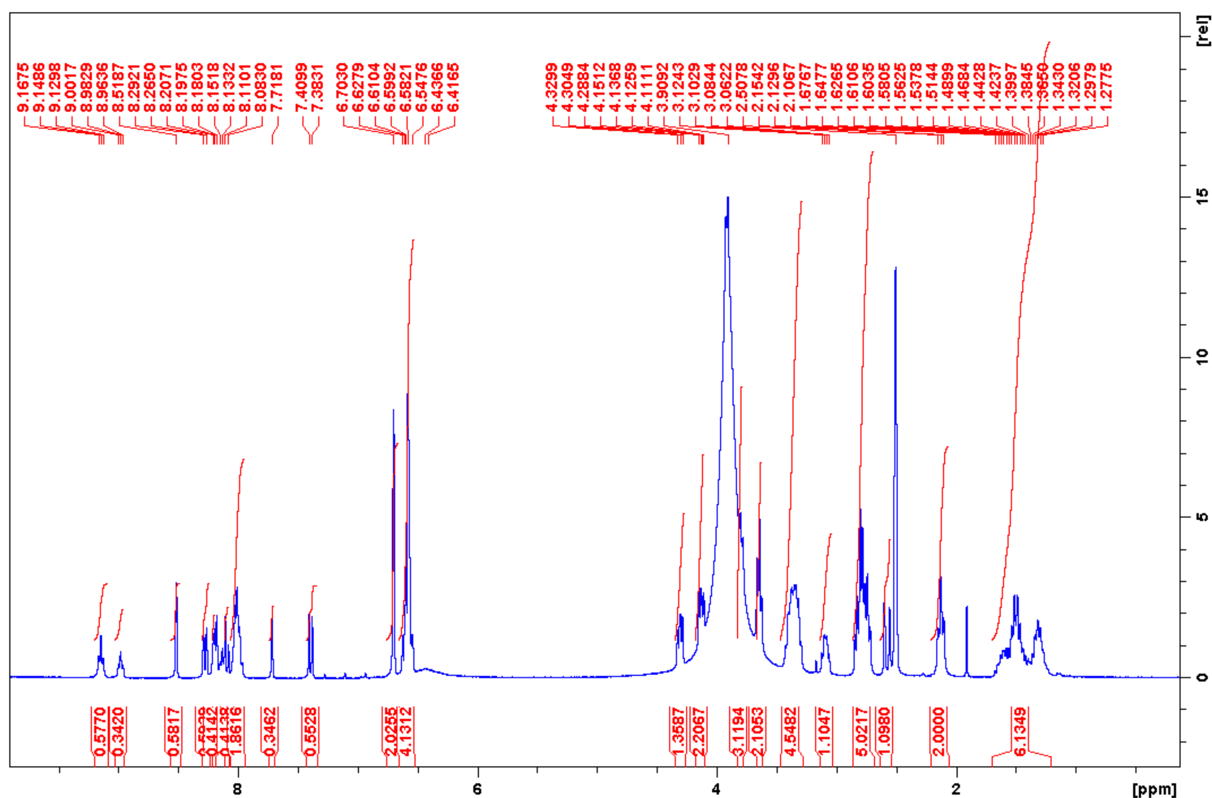


Figure 80: ^{1}H NMR spectrum for biotin-gly-cystamine-gly-fluorescein

^{13}C NMR (300MHz, MeOD) δ (ppm): 25.254(1C, 3/4/5-**CH₂**), 28.48 (1C, 3/4/5-**CH₂**), 28.65 (1C, 3/4/5-**CH₂**), 35.41 (1C, 2-**CH₂**), 37.53 (1C, f-**CH₂**), 38.45 (1C, e/e'-**CH₂**), 38.61(1C, e/e'-**CH₂**), 39.93 (MeOD), 42.39 (1C, i-**CH₂**), 43.10 (1C, b/b'-**CH₂**), 43.26 (1C, b/b'-**CH₂**), 55.86 (1C, 6-**CH**), 59.69 (1C, 11-**C (CH₂)**), 61.50 (1C, 7- **C (CH₂)**), 83.89 (1C, E-**C**), 102.76 (1C, J-**CH**), 109.55 (1C, F-**C**), 113.20 (1C, H-**CH**), 117.64 (TFA), 124.10 (1C, N'-**CH**), 124.72 (1C, N-**CH**), 125.34 (1C, B'-**CH₂**), 126.87 (1C, M'-**C**), 128.69 (1C, D'-**C**), 128.59 (1C, M-**C**), 129.73 (1C, G-**CH**), 135.32(1C, D-**C**), 136.31 (1C, O-**CH**), 140.73 (1C, C'-**CH**), 152.29 (1C, K-**C**), 155.28 (1C, B-**C**), 164.82 (1C, 8-**C=O**), 160.11 (TFA), 163.23 (1C, I-**C=O**), 165.52 (1C, 9-**C=O**), 168.65 (1C, A-**C=O**), 169.18 (1C, L-**C=O**), 168.34 (1C, c-**C=O**), 169.70 (1C, h-**C=O**), 173.00(1C, 1-**C=O**).

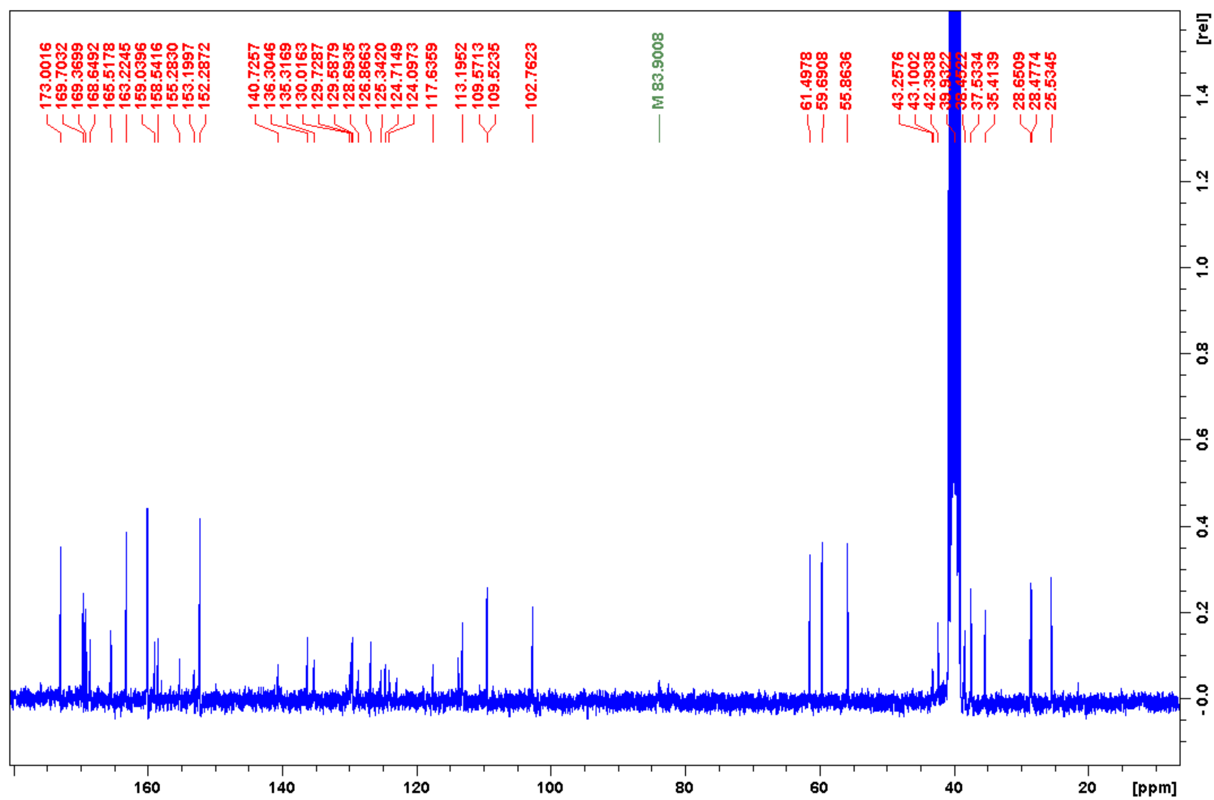


Figure 81: ^{13}C NMR spectrum for biotin-gly-cystamine-gly-fluorescein

3.3 Protein labelling with biotin and/or fluorophores for bead experiments

3.3.1 Formation of doubled labelling of goat IgG with biotin-Ahx-NHS and Alexa-fluoro-647-NHS

Procedure for labelling of goat IgG with biotin-Ahx-NHS and Alexa-fluoro-647-NHS

Goat-IgG was purchased under Sigma I-5256, Alexa-fluoro-647-NHS and biotin-Ahx-NHS were previously synthesised by co-lab workers.

All the materials and buffers were brought into the room temperature. 3 mg of goat IgG (M_w = 150000 g/mol, 1 eq, 20 nmol) was weight into a glass 5 mL vial and was dissolved in 0.5 mL of buffer 8.6 by yielding a protein with the concentration of 6 mg/mL. 0.1 mg (M_w = 1250 g/mol, 4 eq, 80 nmol) of Alexa-fluoro-647-NHS (was stored in a solution of 2 µL DMSO at -80°C) was dissolved in a total volume of 10 µL DMSO and was slowly added into the goat IgG vial while stirring. 12 µL of biotin-Ahx-NHS (M_w = 454.54 g/mol, 6 eq, 120 nmol) was slowly added into the IgG dye mixture under continues stirring. The mixture was stored in dark for half an hour. 50 µL of the glycine solution at the pH of 8.5 was added into the IgG mixture and was stirred for a while. Again, the mixture was stored in dark for another hour for the quenching of all the NHS with glycine.

Mixture was inserted into the column and allowed to run with the buffer PBS 7.3 (60 minutes at 0.5 mL/min). Fractions were collected by analysing the chromatogram.

Gel filtration on column Superdex 200 Tricorn 10/300

The Superdex 200 Tricorn 10/300 column was used for the separation of the product. The column with a diameter of 10 mm and a length of 300 mm. The flow rate should be 0.5 mL/min and one column run takes 60 minutes.

Solvents and buffers were filtered (0.4 µm) under vacuum suction shortly before use, to obtain a dust-free and degassed solution. As an exception, Milli-Q water was already filtered (0.2 µm) and was degassed by vacuum suction without filtering.

The column was stored in 20% ethanol. It was washed with water (overnight at 0.1 mL/min flowrate) and with PBS 7.3 (60 minutes at 0.5 ml/min flowrate)

The loop has a volume of 1.0 mL and was loaded with a sample volume of 0.5 mL. The detector was set to 280 nm.

Table 12: Protocol for the chromatographic run

Time (min)	Text
-15.92	Start of the run
0.00	Strat of the injection (at 0.1 mL/min flow)
~2	Increase the flow rate up to 0.5 mL/min
20.74	Start of fraction 1 (coloured, aggregated antibodies)
23.12	Start of fraction 2 (weakly coloured, mixture of aggregates and monomers)
23.71	Start of fraction 3 (Strongly blue coloured, main peak with antibody monomers)
26.98	Start of fraction 4 (weakly blue, tail of monomeric antibodies)
27.60	Start of fraction 5 (uncoloured)
28.40	Elution goes into waste

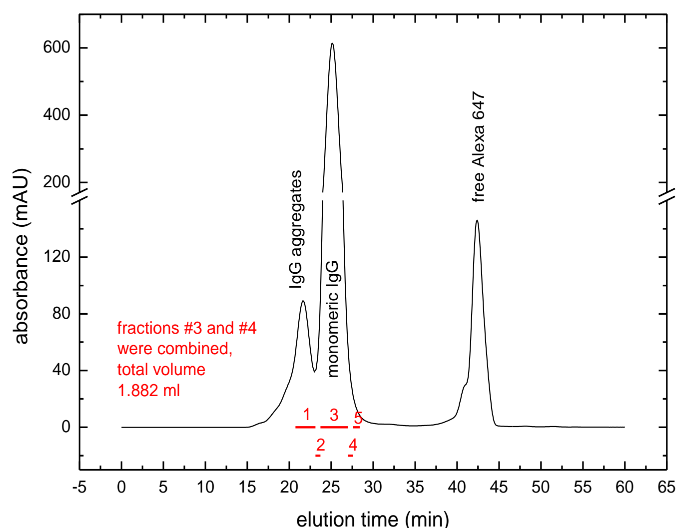


Figure 82: Chromatogram of the product separation from gel filtration

Fractions 3 and 4 were well mixed in a pre-weighed tube, the net weight of the mixed solution was 1.882 g = 1.882 mL.

50 µL aliquots were prepared by using this solution and were frozen at -25°C.

To identify the product UV-Vis spectroscopy was used. Expectation was to have around ~50 nmol from the total of ~80 nmol Alexa-fluoro-647 in the peak which was the volume of 1.882 mL. The Alexa concentration is 50 nmol x 1.882 mL = 26.6 µM. If so the Alexa absorbance should be 26.6 µM x 270000 = 7.18, which is much too high to measure.

If round ~2 mg IgG (13 nmol) is in the peak (1.882 mL), the IgG concentration is 13 nmol / 1.882 mL = 6.9 µM. The IgG absorbance should be 6.9 µM x 150000 = 1.0, which is also rather high for the measurement.

Due to this 50 µL of the combined fraction 3 and 4 was diluted 20 times by adding 950 µL using PBS 7.3 buffer to obtain correct diluted concentration for the UV-Vis spectroscopy. Then IgG will give A280 ~ 0.050 and Alexa-fluoro-647 will give A647 ~ 0.360.

The baseline of the spectrometer was corrected from 800 nm to 200 nm and the absorbance spectrum was recorded from 800 nm to 200 nm at the rate of 300 nm/min.

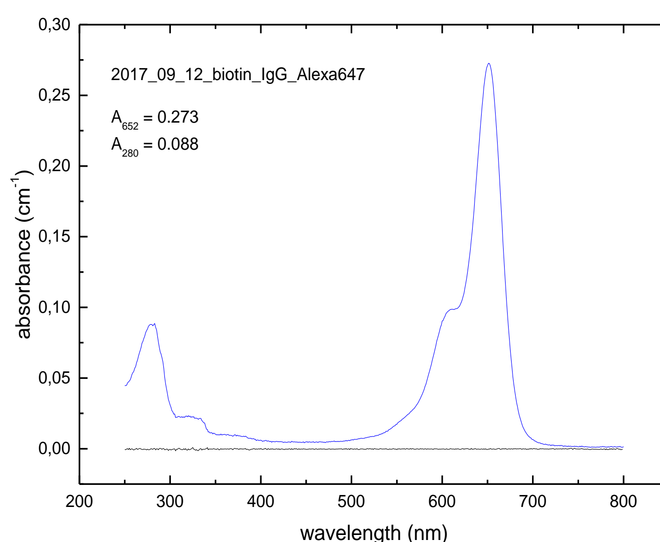


Figure 83: Absorption spectrum for the labelled biotin-IgG*

Table 13: Absorbance at specific wavelengths

Wavelength (nm)	Absorbance (cm^{-1})	Corrected absorbance (cm^{-1})
A_{652} (Alexa647)	0.273	0.265
A_{280} (IgG)	0.088	0.080

[IgG] = 0.080 / 210000 = 0.381 µM in cuvette
[IgG] = 0.381 x 20 = 7.62 µM in undiluted peak
[Alexa] = 0.265 / 270000 = 1.01 µM in cuvette
[Alexa] = 0.98 x 20 = 19.6 µM in undiluted peak
Alexa/IgG = 19.6 / 7.62 = 2.6 dyes per protein
The results have shown that per one IgG molecule, 2.6 Alexa647 dyes were attached.

3.3.2 Labelling of BSA with Alexa-fluoro-647-NHS

<u>Procedure for labelling of BSA with Alexa674-NHS</u>
BSA was purchased from Roche Diagnostics (fraction V, fatty acid free), catalogue number 755 827.
5.3 mg (M_w = 66000 g/mol, 1 eq, 80 nmol) of BSA was weighed in to a 4 mL screw-cap vial and was fully dissolved using 0.5 mL of buffer 8.6. 2 × 0.1 mg of Alexa-Fluoro-647-NHS (M_w = 1250 g/mol, 2 eq, 160 nmol) was dissolved using total of 10 µL DMSO and was added slowly in to pre-dissolved BSA vial while stirring. The vial was kept in a dark place and was allowed to proceed the reaction for 2 hours at room temperature without stirring.
50 µL of glycine HCl (100 mM, pH 8.5) was added in to the reaction mixture while stirring for the quenching of all the NHS esters. Again, the vial was incubated in dark for 1 hour.

<u>Separation of labelled BSA* from unreacted Alexa-fluoro-647-NHS</u>
Gel filtration was done by using PD10 column (prepacked, disposable, 10 mL). The column was first opened on top and then the sealed outlet was cut open. The azide buffer in the upper reservoir was discarded and replaced by water for several times while allowing many drops to fall out from the lower outlet. The outlet was closed and about 2 mL of coupling buffer was added to the top reservoir. The top reservoir was extended with an adaptor and a 25 mL syringe barrel and the syringe barrel on top of the column is filled also with coupling buffer. The outlet was opened and almost all buffer was drained into a waste beaker, except for 1 cm of buffer above the gel whereupon the outlet was closed again.
A 50 mL flask with a 2 mL Eppendorf tube is prepared for collecting the sample.
The mixture with the labelled BSA* and the unreacted deactivated Alexa-fluoro-647 was transferred into the column reservoir and distributed evenly over the frit. This sample had a volume of 0.5 mL. The outlet was opened, and the column was allowed to drain by gravity flow until the flow stops by itself. The effluent was discarded.
1 mL of coupling buffer was added in one step to the top reservoir and was allowed to drain until the flow stops. The effluent was discarded and was repeated this step for one more time.
The flask with the 2 mL Eppendorf vial was placed underneath the column outlet and 1.3 mL coupling buffer was added to the top reservoir. This causes the labelled protein to elute. The flow was stopped before free Alexa-fluoro-647 was elute from the column. Finally, the column was discarded.
BSA* was diluted by the factor of 40 (975 µL of PBS 7.3 with 25 µL of BSA*) for the UV-vis spectroscopy measurement. Concentration of the BSA* was measured by the BSA and Alexa647 absorptions. 50 µL aliquots were prepared, frozen with liquid nitrogen and stored at -25°C for further use.

Table 14: Absorbance at specific wavelengths

Wavelength (nm)	Absorbance (cm^{-1})	Corrected absorbance (cm^{-1})
A_{652} (Alexa647)	0.4183	0.4156
A_{280} (BSA)	0.0749	0.0722

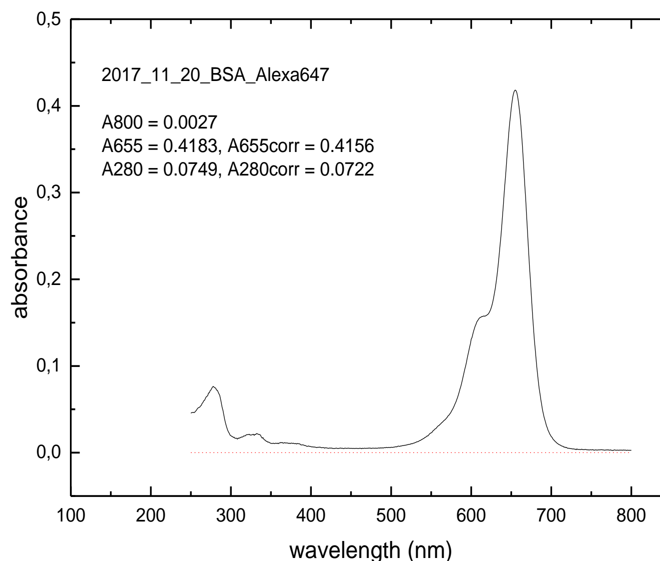


Figure 84: Absorption spectrum for the labelled BSA*

[BSA] = 0.0722 / 44300 = 1.35 µM in cuvette
[BSA] = 1.35 x 40 = 53.9 µM in undiluted peak
[Alexa647] = 0.4156 / 270000 = 1.54 µM in cuvette
[Alexa647] = 1.54 x 40 = 61.6 µM in undiluted peak
Alexa647/BSA = 61.6 / 53.9 = 1.14 dyes per protein
The results have shown that per one BSA molecule, 1.14 Alexa647 dyes were attached.

3.3.3 Labelling of streptavidin (SAV) with Alexa-fluoro-647-NHS

Procedure for labelling of SAV with Alexa-fluoro-674-NHS

Streptavidin (core streptavidin, recombinant) was purchased from Roth in the catalogue number 6073.2

2.2 mg (M_w = 55000 g/mol, 1 eq, 40 nmol) of streptavidin was weighed in to a 4 mL screw-cap vial and was fully dissolved using 0.33 mL of buffer 8.6. 0.1 mg of Alexa-fluoro-647-NHS (M_w = 1250 g/mol, 2 eq, 80 nmol) was dissolved using total of 5 µL DMSO and was added slowly in to pre-dissolved BSA vial while stirring. The vial was kept in a dark place and was allowed to proceed the reaction for 2 hours at room temperature without stirring.

50 µL of glycine HCl (100 mM, pH 8.5) was added in to the reaction mixture with stirring for the quenching of all the NHS esters. Again, the vial was incubated in dark for 1 hour.

Separation of labelled SAV* from unreacted Alexa-fluoro-647-NHS

Separation of the labelled SAV* was done by using a dialysis tube. 2 g of Na_2CO_3 and 0.2 g of EDTA. Na_2CO_3 were weight into a 150 mL beaker. Dialysis tube (7 cm height and 2 cm width) was placed as a coil at the bottom of the beaker. 50 mL Milli-Q water was added in to it and was boiled in the microwave oven. The tubing and the beaker was washed with plenty of water.

The lower end of the tubing was closed with a dialysis clamp and the SAV* sample was transferred into it.

The screw-cap vial where the reaction took place was rinsed with 110 µL PBS 7.3 and this solution was also transferred into the dialysis tubing. Then the dialysis tubing was sealed with a clamp on top, whereby a small air bubble was enclosed inside the tubing.
The closed tubing was placed in a 400 mL beaker containing 250 mL PBS 7.3 and was dialyzed in the cold room with continuous stirring. After 2 hours the dialysis tubing was transferred into a 1 L beaker containing 750 mL PBS 7.3 and dialysis was proceed overnight in the cold room with stirring.
In next morning product was harvested with 1 mL blue pipette tip and transferred it in to 1.5 mL Eppendorf tube. Dialysis tube was washed with 100 µL of PBS 7.3 and that was also transferred in it the Eppendorf tube (total 635 µL of the product was present).
SAV* was diluted by the factor of 40 (975 µL of PBS 7.3 with 25 µL of SAV*) for the UV-vis spectroscopy measurement. Concentration of the SAV* was measured by the SAV and Alexa647 absorptions. 30 µL aliquots were prepared, frozen with liquid nitrogen and stored at -25°C for further use.

Table 15: Absorbance at specific wavelengths

Wavelength (nm)	**Absorbance (cm^{-1})**	**Corrected absorbance (cm^{-1})**
A_{652} (Alexa647)	0.6042	0.602
A_{280} (SAV)	0.1902	0.188

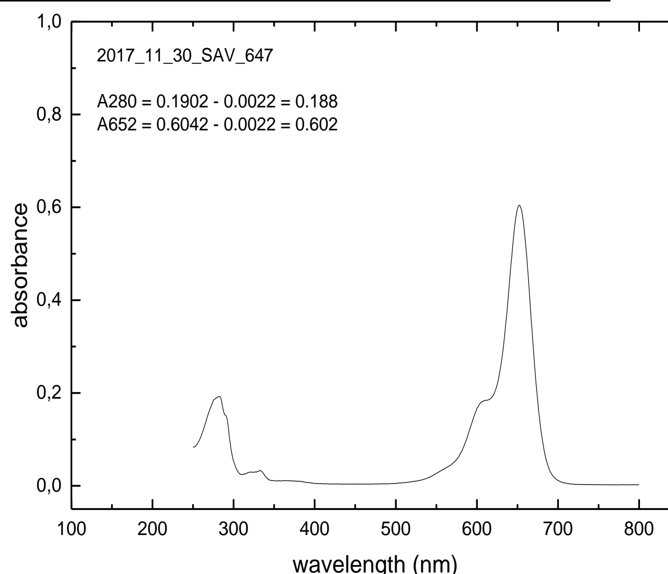


Figure 85: Absorption spectrum for the labelled SAV*

A_{280} (SAV) = 0.188-0.018 = 0.170
[SAV] = 0.170 / 174350 = 0.975 µM in cuvette
[SAV] = 0.975 x 40 = 39.0 µM in undiluted peak
[Alexa647] = 0.6020 / 270000 = 2.23 µM in cuvette
[Alexa647] = 2.23 x 40 = 89.2 µM in undiluted peak
Alexa647/SAV = 89.2 / 39.0 = 2.3 dyes per protein.
The results have shown that per one SAV molecule, 2.3 Alexa647 dyes were attached.

3.4 Functional Experiments with streptavidin in bead suspension

High Capacity Streptavidin Agarose Resin, 2 mL stock suspension. (Thermofisher, catalogue number 20537, 6% beaded agarose, 45-165 µm beads, and 50% slurry with 0.02% Na-Azide).

3.4.1 Test of streptavidin-agarose for its capacity to bind biotinylated biotin-IgG*

500 µL Eppendorf tubes were used for the reaction. Amount of free biotin, PBS buffer, biotin-IgG* and 50% slurry additions to each tube were illustrated in the table below. Eppendorf A was incubated after the addition of free-biotin for 5 minutes with repeated vortexing at 1700 RMP. All the Eppendorf tubes (A-E) were vortexed at 1700 RPM for 15 minutes after the addition of biotin-IgG*. Then the Eppendorf tubes were microfuge for 15 seconds and by adding 400 µL of PBS buffer for the bead sedimentation.

Table 16: Pipetting table for the preparation of different reaction mixtures

Code	PBS 7.3 (µL)	Free Biotin (10mM)		Biotin-IgG* (7.6µM)		50% Slurry	
		Volume (µL)	Amount (nmol)	Volume (µL)	Amount (pmol)	Volume (µL)	Amount (pmol)
A	10	1	10	2	~16	1	~80
B	10	0	0	2	~16	1	~80
C	10	0	0	4	~32	1	~80
D	10	0	0	8	~64	1	~80
E	10	0	0	16	~128	1	~80

Supernatant was recovered with a blue 1 mL tip, which leaves 12-15 µL in the 500 µL Eppendorf tube. Supernatants were transferred into 5 mL PS tubes and 350 µL of each supernatant was mixed with 650 µL of PBS buffer in a new PS tube. Fluorescence emission was measured with the excitation of 630 (10) nm and the emission of 670 (10) nm, at 700 volts.

3.4.2 Test of streptavidin-agarose for its capacity to bind biotinylated B4F

B4F was purchased from Roche Diagnostics under the catalogue number of B-10570. 5 mg batch of B4F was dissolved in 5 ml ethanol and divided into ten 0.5 mL aliquots which were taken to dryness and stored at -80°C. These frozen aliquots were used for the experiments.

500 µL Eppendorf tubes were used to prepare different mixtures. Amount of free-biotin, PBS buffer, B4F and 50% slurry additions to each tube were illustrated in the tables below. The second table shows the reference series, which prepared according to the standard series without the 50% slurry in the tubes. After the addition of free-biotin into Eppendorf tube A, it was incubated for 5 minutes with repeated mixing at 1700 RPM on the vortexer. Then for all tubs B4F was added according to the above table. Eppendorf tubes B'-G' were incubated without slurry, as the reference for the maximal signal.

All the samples were incubated for 15 minutes with repeated mixing on the vortexer at 1700 RPM. Sedimentation of the pallets were done by using Biozym microfuge for 15 seconds and 400 µL of PBS buffer was added into each tube after that. Then again microfuge for 2 minutes until the pallets were seen.

Table 17: Pipetting table for the preparation of different reaction mixtures

Code	PBS 7.3 (µL)	Free Biotin (10mM)		B4F (16µM)		50% Slurry	
		Volume (µL)	Amount (nmol)	Volume (µL)	Amount (pmol)	Volume (µL)	Amount (pmol)
A	63	1	10	2	~16	1	~80
B	62	0	0	2	~16	1	~80
C	60	0	0	4	~32	1	~80
D	56	0	0	8	~64	1	~80
E	48	0	0	16	~128	1	~80
F	32	0	0	32	~512	1	~80
G	0	0	0	64	~1024	1	~80

Table 18: Pipetting table for Preparation of maximal signal reference series

Code	PBS 7.3 (µL)	Free Biotin (10mM)		B4F (16µM)		50% Slurry	
		Volume (µL)	Amount (nmol)	Volume (µL)	Amount (pmol)	Volume (µL)	Amount (pmol)
B’	62	0	0	2	~16	0	0
C’	60	0	0	4	~32	0	0
D’	56	0	0	8	~64	0	0
E’	48	0	0	16	~128	0	0
F’	32	0	0	32	~512	0	0
G’	0	0	0	64	~1024	0	0

Supernatant was recovered with a blue 1 mL tip, which leaves 12-15 µL in the 500 µL Eppendorf tube and were transferred into 5 mL PS tubes. 350 µL of each supernatant was mixed with 650 µL of PBS buffer in a new PS tube and fluorescence emission was measured with the excitation of 490 (10) nm and the emission of 525 (10) nm, at 700 volts.

3.4.3 Time cause of binding of biotin-IgG* to SAV agarose

In the previous experiments incubation was done by repeated vortexing for 15 minutes. Vortexing time was affected the binding according to the results. Due to that, frequent vortexing for longer time was the decision made for further experiments.

6 Eppendorf tubes (500 µL) were prepared according to the table below and frequent vortexing was done at 1700 RPM for different incubation times. After the incubation 400 µL of 7.3 PBS buffer (without BSA) was added into each and was microfuge (Biozym) for 2 minutes to obtain the pallet.

Table 19: Pipetting table for the preparation of different reaction mixtures

Code	BSA (1mg/mL) in PBS (µL)	Biotin-IgG* (7.6µM)		50% Slurry		Incubation time (min)
		Volume (µL)	Amount (pmol)	Volume (µL)	Amount (pmol)	
A	10	6	46	1	~80	5
B	10	6	46	1	~80	10
C	10	6	46	1	~80	20
D	10	6	46	1	~80	30
E	10	6	46	1	~80	40
F	10	6	46	1	~80	60

The supernatants were saved in 5 mL PS tubes by using blue 1 mL tip. 350 µL of each supernatant was mixed with 650 µL of PBS buffer in new PS tubes and fluorescence was measured at the excitation of 630 (10) nm and the emission of 670 (10) nm at 700 volts.

3.4.4 Test for specific and nonspecific binding of biotin-IgG* to SAV agarose with optimized incubation protocol

Amount of free-biotin, BSA in buffer PBS, biotin-IgG* and 50% slurry additions to each tube were illustrated in the tables below. Biotin was incubated with the beads A-D in 500 µL Eppendorf tubes for 5 minutes at 1500 RPM vortexing. Then 6 µL of biotin-IgG* was added into all the tubes (A-H) and were incubated for 60 minutes. For each Eppendorf 400 µL of PBS 7.3 was added and sediment using Biozym microfuge for 2 minutes.

Table 20: Pipetting table for the preparation of different reaction mixtures

Code	BSA (1mg/mL) in PBS (µL)	Biotin (10mM)		Biotin-IgG* (7.6µM)		50% Slurry	
		Volume (µL)	Amount (nmol)	Volume (µL)	Amount (pmol)	Volume (µL)	Amount (pmol)
A	10	1	10	6	46	1	~80
B	10	1	10	6	46	1	~80
C	10	1	10	6	46	1	~80
D	10	1	10	6	46	1	~80
E	10	0	0	6	46	1	~80

F	10	0	0	6	46	1	~80
G	10	0	0	6	46	1	~80
H	10	0	0	6	46	1	~80

Supernatant was recovered by using a blue tip and was saved in 5 mL PS tubes. 350 µL of each supernatant was mixed with 650 µL of PBS buffer and was used for the fluorescence measurements at the excitation 630 (10) and emission 670 (10), 700 volts.
The pallets were mixed with 35 µL of PBS, resulting 50 µL volume (1% bead concentration) for the imaging. Imaging was done by using confocal laser microscope.

3.4.5 Test for the amount of BSSF required for the completion of block of SAV-agarose

A - H mixtures were prepared in 500 µL Eppendorf tubes. Amounts of BSSF, BSA in buffer PBS, biotin-IgG* and 50% slurry additions to each tube were illustrated in the tables below. 4 µL of BSSF solutions with different concentration were incubated with the beads for 5 minutes with continuous vortexing by using the foam pad adaptor at 1500 RPM. Then 6 µL of 7.6 µM biotin-IgG* was incubated with the beads for 60 minutes with continuous vortexing at 1500 RPM.

Table 21: Pipetting table for the preparation of different reaction mixtures

code	BSA 1 mg/mL in PBS	50% slurry	BSSF vol.	BSSF conc.	biotin-IgG* 7.6 µM	Biotin-IgG* offered
	µL	µL	µL	µM	µL	pmol
A	10	1	4	1000	6	46
B	10	1	4	1000	6	46
C	10	1	4	500	6	46
D	10	1	4	500	6	46
E	10	1	4	250	6	46
F	10	1	4	250	6	46
G	10	1	4	125	6	46
H	10	1	4	125	6	46

400 µL of PBS 7.3 was added to each mixture and was sediment by using Biozym microfuge for 2 minutes. The supernatants were recovered with a blue 1 mL and saved in 5 ml PS tubes. 350 µl of each supernatant was mixed with 650 µl PBS in a new PS tube and the fluorescence was measured with Excitation 630 (10) and Emission 670 (10) 700 Volt.
The pellets were mixed with another 35 µl PBS, resulting in a total volume of 50 µl and a bead concentration of 1% (0.5 µl packed beads in 50 µl liquid) for imaging.

3.4.6 Test for the comparison of BSSF block of SAV-agarose with and without cleavage of disulphides by TCEP

Mixtures A -F were prepared in 500 µL Eppendorf tubes. Amount of the different compounds used to make the mixtures in tubes were illustrated below in the table. BSSF was incubated with the beads with BSA in PBS for 5 minutes with continuous vortexing by using the foam pad adaptor at 1500 RPM). Next 400 µL PBS (without BSA) was added into each vial and was sediment for 2 min in Biozyme centrifuge. Supernatant was removed with blue tip so that all beads and about 12-15 µl of buffer remain behind. Washing with PBS was repeated for 2 more times. Bead mixtures A, B and C was refrigerated.

Bead mixtures D, E and F were mixed with TCEP 3 µL (M_w = 286.65 g/mol, 10 mM) and 3 µL of Hepes (M_w = 238.30 g/mol, 100 mM). Then mixtures were incubated with continuous vortexing for 5 min. Bead mixtures D, E and F were washed 3 x with 400 µL PBS to remove the TCEP.

Then biotin-IgG* 6 µL were added to all bead mixtures (A – F) and was incubated for 60 min with continuous vortexing at 1500 RPM. 400 µL of PBS was added into each vial and the sedimentation was done using Biozyme microfuge for 2 min.

Table 22: Pipetting table for the preparation of different reaction mixtures

code	**BSA 1 mg/ml in PBS**	**50% slurry**	**BSSF vol.**	**BSSF conc.**	**BSSF moles**	**biotin-IgG* 7.6 µM**	**IgG offered**	**TCEP 10 mM**	**TCEP moles**	**Hepes "0.1mM"**
	µL	µL	µL	µM	nmol	µL	pmol	µL	nmol	µL
A	10	1	4	250	1	6	46	-	-	-
B	10	1	4	250	1	6	46	-	-	-
C	10	1	4	250	1	6	46	-	-	-
D	10	1	4	250	1	6	46	3	30	3
E	10	1	4	250	1	6	46	3	30	3
F	10	1	4	250	1	6	46	3	30	3

The supernatant was recovered with a blue 1 ml tip which leaves 12-15 µl in the 500 µl Eppendorf tube. The supernatants were saved in 5 ml PS tubes. 350 µL of each supernatant was mixed with 650 µL PBS in a new PS tube and the fluorescence of the unbound antibodies in the supernatant was measured with Excitation 630 (10) and Emission 670 (10) 700 Volt.

The pellets were mixed with another 35 µL PBS, resulting in a total volume of 50 µL and a bead concentration of 1% (0.5 µ packed beads in 50 µL liquid), which were imaged by confocal laser microscope.

3.5 Covalent immobilization of streptavidin on Sephadex G-15 beads and further couplings

3.5.1 Method 1: Activation of Sephadex G-15 with N, N'-disuccinimidyl carbonate (DSC) [Wilchek & Miron, 1985] and coupling of BSA*

Swelling and degassing of Sephadex G-15

7 g of G-15 dry beads were weighed into a 100 mL graduated cylinder and water was added to a level of 30 mL. The cylinder was heated in a beaker with boiling water for 1 h and let it cool to about 40°C The suspension was transferred into a 50 ml Falcon tube and the Falcon tube was inserted in a 500 ml vacuum filter flask for degassing. The gel was adjusted to a 50% suspension by letting the gel settle and removing the liquid which is in excess. The gel was suspended, and enough suspension is removed so that only 20 mL of the 50% suspension are left in the Falcon tube. The rest is saved in another tube and was stored at 4 °C in the refrigerator.

Preparations for gel activation with disuccinimidyl carbonate (DSC)

A 50 mL single-neck round-bottomed flask was mounted above an ice-water bath with a dropping funnel that has a pressure compensation. A thermometer was kept nearby for intermittent temperature measurements. 800 mg DSC (256.17 g/mol, 3.123 mmol) was weighed into the round-bottomed flask and dissolved in 10 mL dry acetone. The flask was immersed in an ice-water bath. Let the temperature of the acetone solution inside cooled down to 4°C.
In a separate 50 mL glass bottle, 650 mg dimethylamine pyridine (122.17 g/mol, 5.32 mmol) was dissolved in 10 mL dry acetone.

Dehydration of the swollen gel with acetone in a sintered glass funnel

The 20 mL of the 50% gel suspension was transferred from the Falcon tube into a sintered glass funnel with inner diameter = 3.5cm, inner height = 5.5 cm. The gel in the funnel was first washed with a mixture of 25 mL acetone and 75 mL water. Secondly with a mixture of 50 mL acetone and 50 mL water and then with a mixture of 75 mL acetone and 25 mL water. Finally, the gel was washed with dry acetone 300 mL and immediately transferred into the round-bottomed flask which already contains the DSC (800 mg) in dry acetone (10 mL).

Activation step with DSC in a round-bottomed flask

The suspension was shaken by hand in the ice/water bath until the suspension temperature was 4°C. The solution of the base dimethylamine pyridine in 10 mL acetone was slowly added with manual shaking. Always thick protection gloves were used.
The suspension was shaken at 4°C for 1 hour and transferred into a sintered glass funnel. First washing was done by using 200 mL cold acetone. Secondly with cold THF with 5% acetic acid (10 mL acetic acid in 200 mL THF). Then the gel was washed with 400 mL of cold methanol and finally with 200 mL of isopropanol. The gel was suspended in cold isopropanol as 50% suspension and stored in the refrigerator at 4°C.

BSA* coupling to DSC activated Sephadex G-15 gel beads

The coupling buffer was pre-cooled in ice. 80 µL of 50% slurry of DSC activated gel was placed into 6 (A-F) glass tubes. 5 mL of water was added into each tube, mixed well and kept for sedimentation on ice. Supernatants of the tubes were removed with a pipette. 1 mL of ice cold coupling buffer was added into each tube, mixed well with blue tip and transferred the gel into new 6 PS tubes (A-F). Transferring step was repeated 3 more times with each 1 mL coupling buffer to get all the beads into PS tubes. Slurry in (A-F) PS tubes were kept in ice for sedimentation and removed the supernatant as much as possible. BSA* and coupling buffer were added according to the table below.

Table 23: Pipetting table for the preparation of different reaction mixtures

Tubes	**Gel beads 50% slurry**	**Coupling buffer**	**3.6mg/mL BSA***	**BSA* wright**	**BSA* amount**
	µL	**µL**	**µL**	**µg**	**nmol**
A	80	0	60	216	3.273
B	80	10	30	108	1.636
C	80	20	20	72	1.091
D	80	30	10	36	0.545
E	80	35	5	18	0.273
F	80	37	3	10.8	0.164

The tubes were inserted into the vortexer and vortexed in the cold room at 400 RPM overnight. In the next morning, 4 mL of deactivation buffer was added into each tube, mixed well and allowed to settle. 3.5 mL of the supernatant was removed from each. PS tubes with beads and 0.5 mL of deactivation buffer were vortexed for 60 minutes at room temperature with a speed of 800 RPM.

After the removal of deactivation buffer, 4 mL of preservation buffer was added into each tube and allowed to settle the beads. The supernatants were transferred into another set of PS tubes (a-f).

2 mL of PBS 7.3 was added into each tube (A-F) with beads, mixed well and allowed to settle. Supernatant was completely removed. This step was repeated 3 more times. Finally, 2 mL of PBS 7.3 and 0.5 mL of 0.5 % SDS were added into each tube (A-F) and was done the imaging of beads in confocal laser microscope.

3.5.2 Method 2 - Activation of Sephadex G-15 by cyanogen bromide (CNBr) [Kohn Wilchek, 1982]

Prewashing of swollen and preserved Sephadex G-15

Settled gel (11 mL, up to 6.2 cm height of the column) from previously swollen sephadex G-15 (in preservation buffer, pH = 8.5) was inserted into an Econo column (width = 1.5 cm, height = 30 cm, area = 1.78 cm^2, volume = 53 mL). Column was equipped with an outlet valve and about 1 m outlet tubing for increasing the gravity flow. The excess of buffer was drained without drying the gel. The gel was washed by filling the 30 cm column with water (5 $\times$ 40 ml), then with 30% acetone (3 $\times$ 30 ml) and finally, with 60% acetone (3 $\times$ 30 ml). The gel in the Econo column is resuspended with 11 mL 60% acetone.

Activation of Spehadex G-15 gel with CNBr

CNBr is an extreme irritant, therefore for the whole experiment thick gloves and gas mask was used. 1.5 M triethylamine (TEA) solution in 60% acetone was prepared by mixing 0.21 mL of 1.5 M TEA with 0.79 mL of 60% acetone in an 8 mL screw-cap vial. 100 mg of CNBr was weighed in to another 8 mL screw-cap vial. All the CNBr was dissolved by using TEA acetone 0.9 mL mixture and screw-cap vials and paster pipettes were cleaned with 1.5 M NaOH. The flask should be held via a long clamp which is mounted on the centre neck. In this way the hand which holds the flask is not in danger of being contaminated with TEA solution in the next step.
A 3-neck 50 ml round-bottomed flask with one wide NS29-neck in the centre and two small NS14-necks on the sides was used to perform the reaction. One of the side necks was equipped with a thermometer, the other small neck was closed with a stopper and both stoppers were well fixed. Prewashed Sephadex G-15 gel was placed in the round bottom flask and the gel was cooled down in an ice bath to -15°C. The ice bath was prepared by using 115g of NaCl with 385g of crushed ice to obtain -20°C in it.
When the temperature of the gel was reached -15°C TEA acetone mixture with CNBr was added dropwise from a Pasteur pipette with swirling over a time of 1-3 min while checking the temperature. Then gel suspension was immediately poured into the 100 mL ice-cold termination medium. The termination medium was prepared by mixing 0.41 mL of concentrated HCl with 50 mL of dry acetone.
Finally, the activated gel was transferred into the precooled preservation medium from the termination medium. The preservation medium was prepared by mixing 180 mL of dry acetone, 105 mL of THF and 15 mL of water.

Preservation of the activated gel

The gel was allowed to settle down and the supernatant was decanted as much as possible. The remaining gel was suspended with a little preservation medium and transferred into a 50 mL glass bottle. More preservation medium was used to suspend all gel in the Erlenmeyer flask and transfer it into the 50-mL glass bottle. The excess of supernatant was removed to obtain 50% suspension of gel and was stored in 4°C for further use.
Procedure for the coupling of BSA* to CNBr activated sephadex-G15 was same as the coupling of BSA* to DSC activated sephadex-G15. Only difference was the method of activation of the gel beads. For this experiment CNBr activated sephadex-G15 beads were used. Finally, beads were imaged by confocal laser microscope.

3.5.3 Coupling of unlabelled streptavidin and biotin-IgG* to CNBr-activated Sephadex G-15

Coupling of unlabelled SAV to CNBr activated Sephadex G-15 beads

According to the images from 3.5.4, CNBr surface activation of Sephadex G-15 beads was successful. Therefore, theses surface activated beads were used for the SAV coupling.
50% CNBr-Sephadex G-15 gel was used for SAV coupling in a 5 mL glass tube. For this 240 µL of bead suspension was washed for several times with water. Then pre-cooled coupling buffer (4mL) was added into it, mixed well and let for sedimentation on ice. All most all the supernatant was removed from the tube. Another 10 µL of coupling buffer and 30 µL of 2.2mg/mL SAV (1.2 µmol) was added into the tube. The SAV bead mixture was vortexed at 400 RPM overnight in cold room.
Next morning beads were deactivated by adding 4 mL deactivation buffer into it and vortexing for 60 minutes at room temperature. After removing deactivation buffer, 2 mL preservation buffer was added into it and let for sedimentation of the beads. The excess of preservation buffer was removed to make 50% Streptavidine-sephadex-G-15 gel medium.

BSA* coupling to CNBr-SAV-Sephadex G-15 gel beads

Mixtures A -F were prepared in 5 mL glass test tubes. Amount of the different compounds used to make the mixtures in tubes were illustrated below in the table. First free-biotin was incubated for 5 minutes with continuous vortexing at 1500 RPM for test tubes A – C. Next biotin-IgG** was incubated with all and was vortexed for 10 minutes at the same speed.

Table 24: Pipetting table for the preparation of different reaction mixtures

code	BSA 1mg/ml in PBS	50% slurry	biotin 10mM	biotin-IgG* 7.6µM	IgG offered
	µl	µl	µl	µl	pmol
A	10	1	1	5	38
B	10	1	1	5	38
C	10	1	1	5	38
D	10	1	0	5	38
E	10	1	0	5	38
F	10	1	0	5	38

400 µL of PBS 7.3 was added into each tube for Biozym sedimentation and the supernatants were removed by using 1 mL blue tip (which leaves around 12 – 15 µL in the Eppendorf). Finally, 35 µL of PBS 7.3 was added into each to obtain 1% packed gel beads for confocal laser microscope imaging.

3.5.4 Test for the comparison of biotin-IgG* binding of SAV-CNBr-Sephadex G-15 in absence and presence of BSSF

A – D samples were prepared according to the below table by using 1 µL 50% SAV-CNBr-Sephadex G-15 slurry, 10 µL BSA (1 mg/mL) in PBS and 1 µL BSSF. E and F were prepared in same way without BSSF.

All the samples were incubated for 5 minutes at 1500 RPM. Only C and D samples were washed with 400 µL of PBS for 3 times. Then 5 µL of biotin-IgG* were added into each tube and incubated for 10 minutes at the same speed. Final wash was done for all the tubes with 400 µL of PBS and repeated the washing for 2 more times. All the time bead sedimentation was done by using Biozym microfuge. Samples were prepared for imaging by adding 35 µL of PBS into each.

Table 25: Pipetting table for the preparation of different reaction mixtures

code	**BSA 1mg/ml bufferA**	**50% slurry**	**BSSF vol.**	**BSSF conc.**	**BSSF amount**	**biotin-IgG* 7.6 µM**	**IgG offered**
	µl	**µl**	**µl**	**µM**	**pmol**	**µl**	**pmol**
A	10	1	1	250	250	5	38
B	10	1	1	250	250	5	38
C	10	1	1	250	250	5	38
D	10	1	1	250	250	5	38
E	10	1	0	0	0	5	38
F	10	1	0	0	0	5	38

3.5.5 Test for the comparison of biotin-IgG* binding to SAV-CNBr-Sephadex-G-15 beads in presence of BSSF, with and without disulphide cleavage by TCEP

Eppendorf tubes A – F were prepared according to the below table with 10 µL of BSA (1 mg/mL) in Buffer A, 1 µL 50% Streptavidin-CNBr-Sephadex G-15 slurry and 1 µL BSSF. All were incubated for 5 minutes at 1500 RPM. All the beads were washed with 400 µL of PBS, sediment with Biozym microfuge and removed the supernatants as much as possible. Washing was repeated for 2 more times. After the washing 10 µL of BSA (1mg/mL) in buffer A was added into each tube and additional 4 µL for tubes A – C. Tubes A – C were kept a side.

2 µL (20 mM) TCEP hydrochloride and 2 µL (0.2 M) Hepes pH 9.6 was added to tube D- F and was incubated for 5 minutes. Tubes D – F were washed according to the same above procedure for 3 times.

The biotin-IgG* was added to all kinds of beads in the tubes A-F and was incubated for 10 minutes with continuous vortexing at 1500 RPM. All the bead tubes were again washed in the same procedure for 3 times and 35 µL of PBS were added into each for imaging.

Table 26: Pipetting table for the preparation of different reaction mixtures

	BSA 1mg/ml bufferA	50% slurry	BSSF vol.	BSSF conc.	BSSF amount	biotin-IgG* 7.6µM	IgG offered	bufferA	TCEP 20mM	Hepes "0.2M"
	µl	µl	µl	µM	pmol	µl	pmol	µl	µl	µl
A	10	1	1	250	250	5	38	4	-	-
B	10	1	1	250	250	5	38	4	-	-
C	10	1	1	250	250	5	38	4	-	-
D	10	1	1	250	250	5	38	-	2	2
E	10	1	1	250	250	5	38	-	2	2
F	10	1	1	250	250	5	38	-	2	2

3.6 Coupling of 8-arm-PEG-nitrophenyl ester with N-biotinyl-propylamine [Haizinger, 2012]

Procedure for the coupling of octopus-nitrophenyl ester with N-biotinyl-propylamine

For this reaction 8-arm-PEG-nitrophenyl ester was used. It was prepared from 8-arm-PEG (M_w = 15000 g/mol, Nektar) by Fraynziska Haizinger for her master thesis in January 2012. N-biotinyl-propylamine compound was bought from Sigma-Aldrich, which is present in TFA salt.

12.1 mg (29.2 µmol, 22.5 eq, M_W = 414.45 g/mol) of N-biotinyl-propylamine was weighted in to a 5mL finger flask with a stir bar. The argon bubbler was attached into it and the flask was flushed with argon for 1 minute. 21 mg (1.3 µmol, 1 eq, M_W = 15810 g/mol) of 8-arm-PEG-nitrophenyl ester was weighed into a separate 4 ml screw-cap glass vial. 8-arm-PEG-nitrophenyl ester was dissolved in 200 µL DMSO and the solution was transferred into flask which contains the N-biotinyl-propylamine. The screw-cap vial is rinsed with 100 µL of more DMSO and this solution was also added to the reaction mixture. The flask was flushed with argon for 2 minutes while stirring. 70 µL (500 µmol, 385 eq, M_W = 101.19 g/mol) triethylamine was added with stirring and the flask was cautiously flushed with argon for 2 minutes while stirring. The stopper was inserted tightly with a Teflon sleeve for optimal seal and the reaction was allowed to proceed 7 hours with stirring under argon bubbling.

Separation of coupled biotin-8-arm-PEG from excess of N-biotinyl-propylamine

I have done this reaction twice to get the pure product. The purification parts were different to 1st and 2nd batches. The purity of the product was determined by analysing the NMR.

Product purification of batch 1

The reaction mixture was transferred from the finger flask into a round-bottomed flask and the finger flask was rinsed with 2 x 0.25 mL DMSO which was also added to reaction mixture in the round-bottomed flask. The liquid in the round-bottomed flask was frozen with liquid nitrogen and attached to a nitrogen-cooled cold trap which was connected to an oil pump. All the DMSO was removed by vacuum. Then the dry residue was dissolved in chloroform (5 mL) and extracted with 0.1 M phosphoric acid (5 ml, containing 2.5 M NaCl). Centrifugation was done at 1000 RPM for 15 minutes to minimize the extent of emulsion. The chloroform extraction was repeated for 3 more times with the aqueous emulsion phase until the emulsion phase was gone. The combined chloroform phase was dried with anhydrous Na_2SO_4 and filtered through paper. The filtrate was taken to dryness in a flask with a known tare weight.

The product weight was 17.9 mg. All the product was dissolved in 650 µL of $CDCl_3$ and ^1H NMR was measured from it.

According to the ^1H NMR spectrum p-nitrophenol, which was cleaved from the 8-arm-PEG was also present in the product. To remove p-nitrophenol basic extraction was done by using 2 x 10 mL of 10% Na_2CO_3 with chloroform. After drying the chloroform layer with anhydrous Na_2SO_4, solvent was fully evaporated using rotary evaporator and weigh the product.

The weight of the product was 8.3 mg and all of it was dissolved in 650 µL of $CDCl_3$ and ^1H NMR was measured using it.

The ^1H NMR spectrum was much better with proper integral proportions and product was pure according to the spectrum. But the yield was less because of the long purification procedure.

8.3 mg (0.49 µmol, M_W = 17101 g/mol) of pure biotin-8-arm-PEG was obtained with a yield of 37.69 %.

Product purification of batch 2

Purification of the product was done by using dialysis in benzylated cellulose tubing. 1 L vacuum filter flask was filled with 1 L of water and 1 mL pure of acetic acid (17.5 M). The dilute solution (0.1%) had a concentration of 17.5 mM and the pH of the solution was 3.2. At this pH, N-biotinyl-propylamine, triethylamine was well protonated and p-nitrophenol was in uncharged form and all the compounds were highly water soluble. The filter flask was connected to the argon flow and filled with argon gas.

The reaction mixture was diluted with 300 µL water and then transferred into a dialysis sack of Benzoylated cellulose tubing (6 cm long) and both ends were closed with dialysis closures, whereby an air bubble was included.

The sample in the dialysis tubing was immersed in the 1 L flask, the argon flow was reduced and stirring was continued for one hour. After one hour the sack was still yellow, which means still the nitrophenolate was still inside the sack. Then 9 ml acetic acid was added to the dialysis buffer, resulting in a final concentration of 1% acetic acid (175 mM) and a pH of 2.7 in the outer solution and the dialysis was continued overnight.

Second dialysis step was done by using 250 mL ethanol in a vacuum filter flask. The dialysis tubing with the sample was transferred into the ethanol flask and continued the dialysis for 5 hours under the flow of argon.

The sample was recovered from the dialysis tubing and transferred into a pre-weighed 10 mL pear-shaped flask. The solvents were evaporated by using rotary evaporator, again dissolved in chloroform and dried down several times to remove ethanol from the sample.

The weight of the product was 18.9 mg and all of it was dissolved in 650 µL of $CDCl_3$ and ^1H, ^{13}C NMR spectrums were measured using it.

18.9 mg (1.11 µmol, M_W = 17101 g/mol) of pure Biotin-octopus was obtained with a yield of 84.62 %.

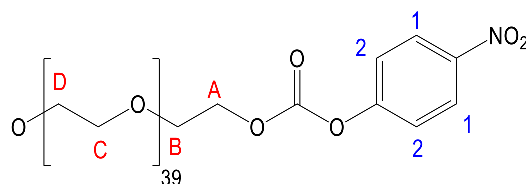


Figure 86: Structure of one arm in 8-arm-PEG-nitrophenyl ester

^1H NMR (300MHz, $CDCl_3$) δ (ppm): 0.00 (TMS), 2.18 (H_2O), 3.39 – 3.89 (160 H, m, B, C, D -**CH_2**), 4.43 – 4.46 (2 H, m, A-**CH_2**), 7.28 ($CDCl_3$), 7.4 (2 H, d, J = 8.97 Hz, 2-**CH**), 8.28 (2 H, d, J = 8.97, 1-**CH_2**).

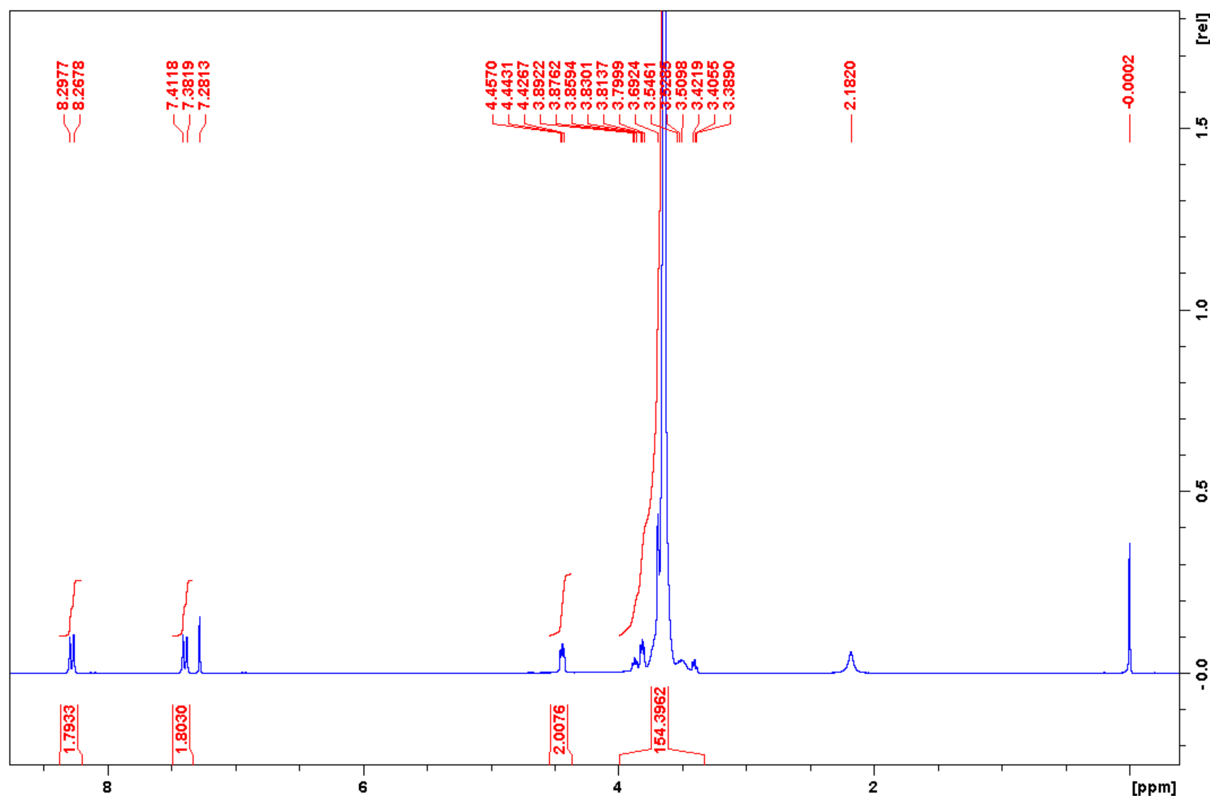


Figure 87: ^{1}H NMR spectrum for 8-arm-PEG-nitrophenyl ester

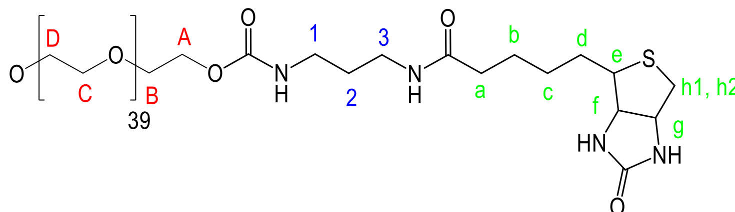


Figure 88: Structure of one arm in 8-arm-PEG-biotin

^{1}H NMR (300MHz, $CDCl_3$) δ (ppm): 0.00 (TMS 2.21 (2 H, t, J = 7.2 Hz, 2-**CH_2**), 1.26 (H grease), 1.45 – 1.69 (6 H, m, b, c, d-**CH_2**), 2.20 – 2.22 (4 H, m, a, 2-**CH_2**), 2.74 (1 H, d, J=12.7, h1-**CH**), 2.91 (1 H, d, J = 9.5 Hz, h2-**CH_2**), 3.39 – 3.42 (4 H, m, 1, 3-**CH_2**), 3.44- 3.89 (160 H, m, B, C, D-**CH_2**), 4.21 (2 H, s, A-**CH_2**), 4.33 (1 H, s, f-**CH**), 4.52 (1 H, s, g-**CH**), 7.28 ($CDCl_3$).

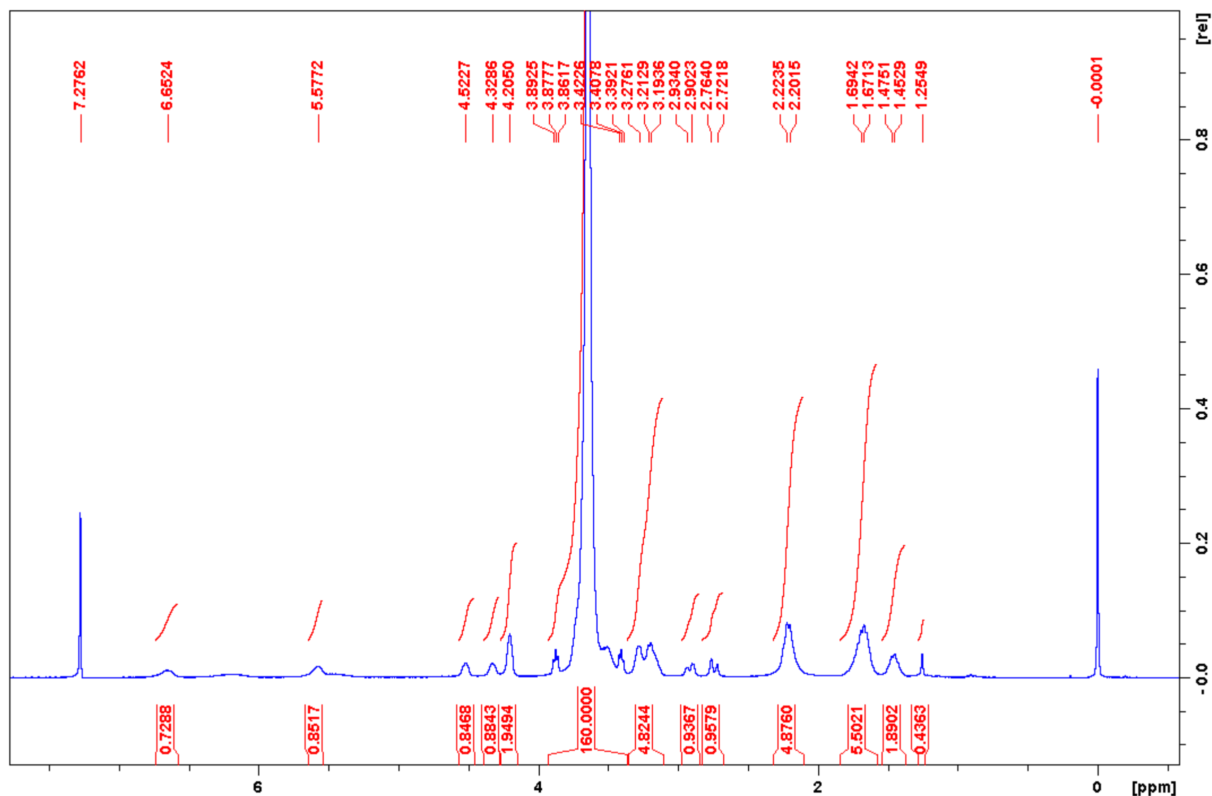


Figure 89: ^{1}H NMR spectrum for 8-arm-PEG-biotin

^{13}C NMR (300MHz, $CDCl_3$) δ (ppm): 0.00 (TMS), 25.59 – 29.88 (3C, b, c, d-**CH_2**), 35.95 (2C, a, 2-**CH_2**), 37.78 (2C, 1, 3-**CH_2**), 40.54 (1C, h-**CH_2**), 55.24 (1C, e-**CH**), 60.21 (1C, g-**CH**), 61.37 (1C, f**-CH**), 63.98 (1C, A-**CH_2**), 69.58 – 70.57 (79C, B, C, D-**CH_2**), 76.63 – 79.77 (1C, e/h-**C=O**), 171.40 ($CDCl_3$).

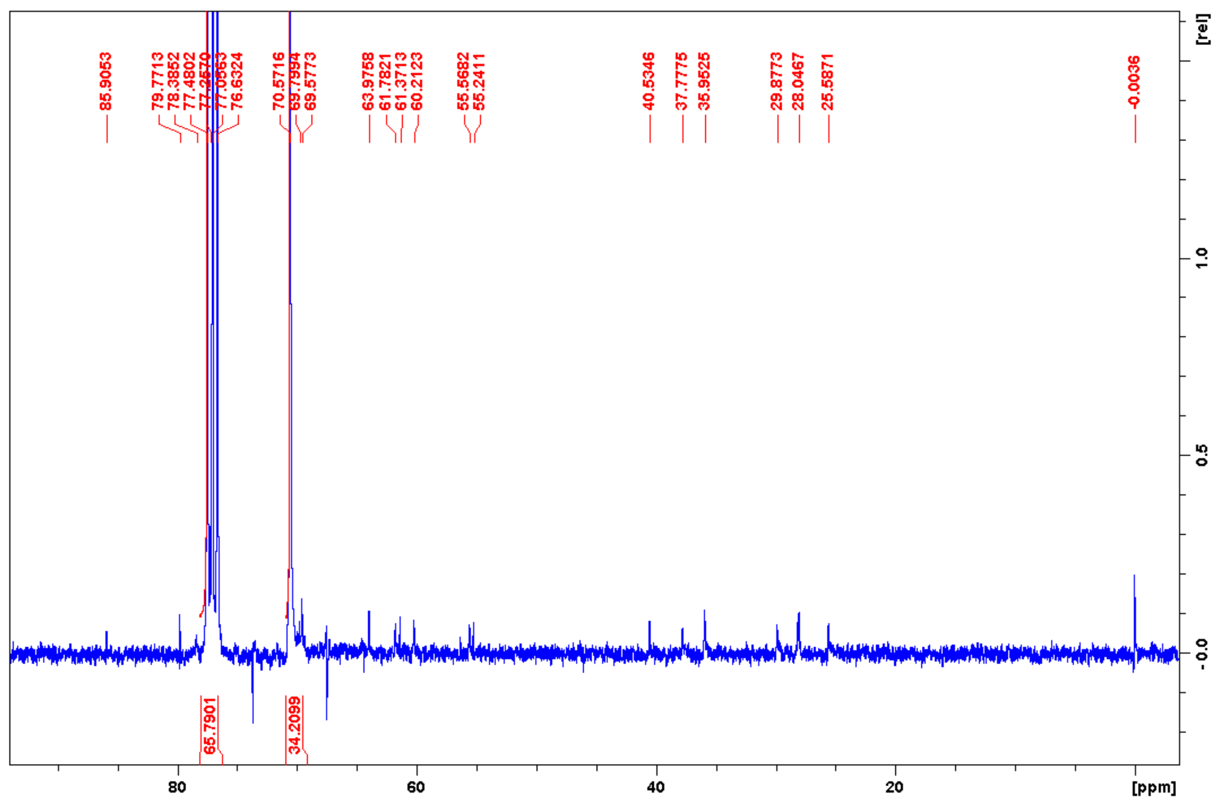


Figure 90: ^{13}C NMR spectrum for 8-arm-PEG-biotin

3.7 Biotin and BSSF titrations against (strept)avidin

Titrations were done at Fluorimeter by Luise Hermann.

3.7.1 BSSF concertation determination by UV-Vis absorption

4960 µL of buffer 9.1 was mixed with 40 µL of BSSF (1 mM) solution to get BSSF solution with the concentration of 8 µM. Baseline of the UV-Vis spectrometer was adjusted by using buffer 9.1 and the spectrum was measured between 300 nm – 700 nm with 10 nm slit.

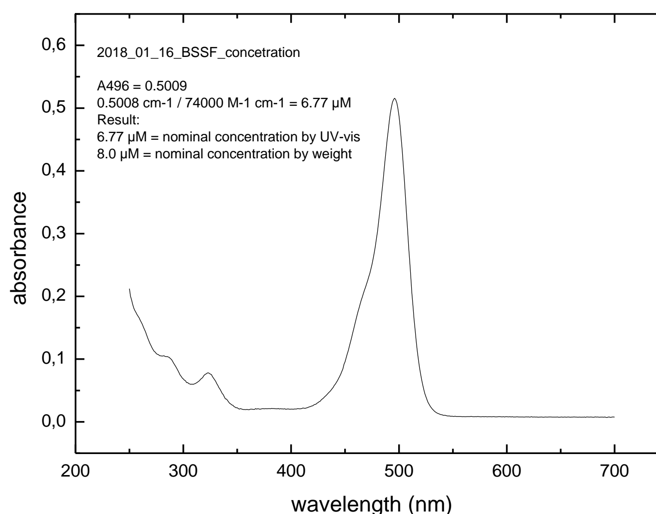


Figure 91: Absorption spectrum for the BSSF to determine the concentration

$A_{496,\ fluorescein}$ = 0.5009

[BSSF] = 0.5009 / 74000 = 6.77 µM

6.77 µM nominal concertation by UV-Vis

8 µM nominal concentration by weight

3.7.2 Biotin vs. avidin titration using tryptophan quenching

8 µM biotin was prepared by using 10 mM D-Biotin, which was purchased from AMRESCO, catalogue number 3435-C-174.

80 µL of (1.25 µM, 0.1 pmol) avidin was mixed with 1920 µL of buffer A in a cuvette. Avidin was titrated against 8 µM biotin by measuring the fluorescence emission at the excitation 290 nm (slit 2.5 nm) and emission 340 nm (slit 10 nm). When all the avidin pockets were filled with biotin, fluorescence emission remained almost constant.

3.7.3 Biotin vs. streptavidin titration using tryptophan quenching

8 µM biotin was titrated against SAV with 5 µL additions for each minute until all the binding sites of SAV were filled with biotin. 80 µL (1.25 µM, 0.1 pmol) SAV was mixed with 1920 µL of buffer in a cuvette. This was titrated with biotin until the fluorescence emission got constant. Tryptophan quenching was measured in the fluorimeter at the excitation 290 nm (slit 2.5 nm) and emission 340 nm (slit 10 nm).

3.7.4 BSSF vs. avidin titration using tryptophan quenching

8 µM (nominal concentration) BSSF was titrated against 80 µL (1.25 µM, 0.1 pmol) SAV with 2.5 µL additions for each minute. 80 µL (1.25 µM) SAV was mixed with 1920 µL of buffer in a cuvette and titrated with BBSF until the fluorescence emission increase linearly with each BSSF addition.

Tryptophan quenching of avidin pockets was measured in the fluorimeter at the excitation 490 nm (slit 2.5 nm) and emission 525 nm (slit 5 nm).

3.7.5 BSSF vs. streptavidin titration using tryptophan quenching

80 µL of 1.25 µM (0.1 pmol) streptavidin was mixed with 1920 µL of buffer A for the titration. 8 µM BSSF was titrated against streptavidin with 2.5 µL additions for each minute until all the binding sites were filled with biotin present in BSSF. Fluorescence measurements were done at the excitation 490 nm nm (slit 2.5 nm) and emission 525 nm (slit 5 nm).

3.8 Buffers and special reagent recipes

3.8.1 PBS 7.3 buffer

PBS 7.3 buffer was prepared by weighing 16.36 g of NaCl (M_w = 58.44 g/mol, 280 mmol), 0.403 g of KCl (M_w = 74.56 g/mol, 5.4 mmol), and 3.12 g of dihydrate NaH_2PO_4 (M_w = 156.01 g/mol, 20 mmol) into a beaker and dissolved with around 200 mL distilled water. Then the pH was adjusted to 7.3 with 1 M KOH. Mixture was transferred into a 2 L volumetric flask and was topped up by adding distilled water. Aliquots were frozen at -25°C.

3.8.2 Buffer 8.6

Buffer 8.6 was prepared by weighing 1.461 g of NaCl (M_w = 58.44 g/mol, 25 mmol) and 0.541 g of boric acid (M_w = 61.83 g/mol, 8.75 mmol) into a beaker and added around 100 mL of distilled water. Then the pH was adjusted to 8.6 by using 1 M NaOH. The mixture was transferred to a 250 mL volumetric flask and was filled up with distilled water. Aliquots were frozen at -25°C.

3.8.3 Buffer 9.1

Buffer 9.1 was prepared by using Boric acid 1.546 g (M_w = 61.83 g/mol, 50 mmol) in 500 mL volumetric flask. pH was adjusted to 9.1 with 2 M NaOH and was filled up with distilled water.

3.8.4 Glycine

375 mg of Glycine (M_w = 75.01 g/mol, 5mmol) was weighted into a beaker and 45 mL of distilled water was added into it. pH was adjusted to 8.5 with 2 M NaOH. Mixture was transferred into a 50 mL measuring cylinder and was filled up with water (C = 0.1 M Glycine solution). Aliquots were frozen at -25 °C.

3.8.5 Buffer A

Buffer A was prepared by weighing 5.84 g of NaCl (M_w = 58.44 g/mol, 100 mmol) and NaH_2PO_4 water free 5.99 g (M_w = 119.98 g/mol, 50 mmol), (or can use $NaH_2PO_4.1H_2O$ 6.89 g (M_w = 137.99 g/mol, 50 mmol), or $NaH_2PO_4.2H_2O$ 7.8 g (M_w = 156.01 g/mol, 50 mmol) as substitutions for NaH_2PO_4 water free). Finally, EDTA.Na_2 0.372 g (M_w = 372.24 g/mol, 1 mmol) were weighted and all the substances were combined in a 1 L volumetric flask. Substances were fully dissolved using around 750 mL of distilled water. pH of the mixture was adjusted to 7.5 with 2 M NaOH and was topped up to 1 L with distilled water.

3.8.6 Hepes 9.6

7.15 g of Hepas (M_w = 238.30 g/mol, 30 mmol) was weighed into a 50 mL Falcon tube and dissolved in 30 mL distilled water. pH was adjusted to 9.6 by using 5 M NaOH to obtain 1 M Hepes 9.6.

3.8.7 TCEP – Hydrochloride (Tris – carboxyethyl phosphine hydrochloride)

100 mg of TCEP – Hydrochloride (M_w = 286.65 g/mol, 100 mmol) was dissolved in 3.47 mL of distilled water and store in different aliquots sizes and store at -20 °C. Large aliquots (1 ml aliquots in 1.5 ml Eppendorf tube), intermediate aliquots (0.1 ml aliquots in 0.5 ml Eppendorf tubes), and small aliquots (15 µl aliquots in 0.5 ml Eppendorf tubes).

3.8.8 Coupling buffer

7.305 g of NaCl (M_w = 58.44 g/mol, 500 mmol) and 4.142 g of $NaHCO_3$ (M_w = 82.834 g/mol, 200 mmol) were dissolved in a 250 mL volumetric flask with 200 mL of distilled water. pH of the mixture was adjusted with 1M NaOH to 8.5. Then was topped up to 250 mL with distilled water.

3.8.9 Deactivation buffer

2.438 g of ethanolamine HCl (M_w = 97.54 g/mol, 100 mmol) was weighed in to a 250 mL volumetric flask. Ethanolamine HCl was completely dissolved with about 200 mL of water. pH of the solution was adjusted to 9.0 by using 1 M NaOH and filled up to 250 mL with water.

3.8.10 Acetate buffer

7.305 g of NaCl (M_w = 58.44 g/mol, 500 mmol) and 2.051 g of Na-acetate (M_w = 82.834 g/mol, 100 mmol) were weighed in to a 250 mL volumetric flask and was adjusted the pH to 4.0 by HCl (1.5 M) after completely dissolving the compounds with about 200 mL of water. System was topped up to 250 mL with water.

3.8.11 Tris buffer

7.305 g of NaCl (M_w = 58.44 g/mol, 500 mmol) and 3.829 g of tris base (M_w = 121.14 g/mol, 100 mmol) were weighed into a 250 mL volumetric flask. pH of the mixture was adjusted to 8.0 by using 1.5 M HCl, after dissolving the compounds completely with ~200 mL of water. Afterwards was filled up to the 250 mL level with water.

3.8.12 Preservation buffer

For the preparation of preservation buffer, 0.081 g of NaN_3 (M_w = 65.01 g/mol, 5 mmol) and 2.100 g of $NaHCO_3$ (M_w = 84.01 g/mol, 100 mmol) were weighed into a 250 mL volumetric flask. Compounds were dissolved with about 200 mL water and pH was adjusted to 8.5 with 1M NaOH. The alkaline pH is important to prevent release of toxic NH_3 from the buffer. By using water mixture was topped up to 250 mL.

4. Abbreviations

*	Alexa-fluoro-647
AVD	avidin
B4F	biotin-4-fluorescein
Boc	tert-butyloxycarbonyl
BSA	bovine serum albumin
BSSF	biotin-gly-cystamine-gly-fluorescein
CME	chloroform/methanol/acetic acid mixture
DCM	dichloromethane
DIPEA	N, N-diisopropyl-N-ethylamine
DMSO	dimethyl sulfoxide
DSC	N, N'-Disuccinimidyl Carbonate
FCS	fluorescence correlation spectroscopy
HABA	2-(4'-hydroxyazobenzene) benzoic acid
HEPES	4-(2-hydroxyethyl) piperazinyl-1-ethanesulfonic acid
IgG	immunoglobulins
K_D	dissociation constant
M	moles
MS	mass spectrometry
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
OtBu	tert-butyl ester
PBS	phosphate buffered saline
PDB	protein data bank
pH	potential of hydrogen
ppm	parts per million
RP	reversed phase
RT	room temperature
SAV	streptavidin
TCEP	tris-(carboxyethyl)phosphine
TEA	triethylamine
TFA	trifluoracetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
UV-Vis	ultraviolet-visible

5. References

Barbarakis, M. S.; Smith-Palmer, T.; Bachas, L. G.; Chen, S. Y.; Van Der Meer, B. W. (1993) Enhancement of the emission intensity of fluorophore-labeled avidin by biotin and biotin derivatives. Evaluation of different fluorophores for improved sensitivity. Talanta, no. 40, pp. 1139-1145.

Bertini I.; Gray H. B.; Stiefel E. I.; Valentine J. S. (2007) Biological Inorganic Chemistry. University science Books, pp. 357 – 360.

Chaiet, L.; Miller, R.; W., Tausig, F.; Wolf, F. J. (1963) Antibiotic MSD-235. II. Separation and Purification of synergic components. Antimicrob. Ag. Chemother, no. 161, pp. 28-32.

De Lange, R. J.; Huang, T. S. (1971) Egg white avidin. III. Sequence of the 78-residue middle cyanogen bromide peptide. Complete amino acid sequence of the protein subunit. J. Biol. Chem., no. 246, pp. 698-709.

Dirksen, A.; Langereis, S.; De Waal, F. M.; Van Genderen, M. H. P.; Hackeng, T. M.; Meijer, E. W., (2005) A supramolecular approach to multivalent target-specific MRI contrast agents for angiogenesis. Chem.Commun, 2811-2813.

Fasting, C.; Schalley, C. A.; Weber, M.; Seitz, O.; Hecht, S.; Koksch, B.; Dernedde, J.; Graf, C.; Knapp, E.-W.; Haag, R. (2012) Multivalency as a Chemical Organization and Action Principle. Angew. Chem. Int. Ed., no. 51, pp. 10472-10498.

González, M.; Argaraña, C. E.; Fidelio, G. D. (1999) Extremely high thermal stability of streptavidin and avidin upon biotin binding. Biomol. Eng., no. 16, p. 67–72.

Green, N. M. (1963) The nature of biotin-binding site. Biochem. J., no. 89, pp. 599-609.

Green, N. M. (1963a) The use of (14-C) biotin for kinetic studies and for assay. Biochem. J., no. 89, pp. 585-591.

Green, N. M. (1963b) Stability at extreme of pH and dissociation into subunits by guanidine hydrochloride. Biochem. J. 89, no. 89, pp. 609-620.

Green, N. M. (1975) Avidin. Adv. Protein Chem., no. 29, pp. 855-133.

Green, N. M. (1990) Avidin and Streptavidin. Methods Enzymol, no. 184, pp. 51-67.

Green, N. M.; Konieczny, L.; Toms, E. J.; Valentine, R. C. (1971) The Use of Bifunctional Biotinyl Compounds to Determine the Arrangement of Subunits in Avidin. Biochem. J., no. 125, pp. 781-791.

Green, N. M.; Toms, E. J. (1972) The dissociation of avidin-biotin complexes by guanidinium chloride. Biochem. J., no. 130, pp. 707-711.

Gruber, H. J.; Kada, G.; Marek, M.; Kaiser, K. (1998) Accurate titration of avidin and streptavidin with biotin-fluorophore conjugates in complex, colored biofluids. Biochim Biophys Acta, no. 1381, pp. 203-212.

Gyorgy, P. (1954) Biotin in The Vitamins. Vol. 1, New York, Academic Press, pp. 527-571.

Haizinger, F. (2012) Master thesis at Johannes Kepler University.

Haustein, E.; Schwille, P. (2004) Single-molecule spectroscopic methods. Sciencedirect, current opinion in structural biology, no. 14; 531-540

Kada, G.; Falk, H.; Gruber, H. J. (1999a) Accurate measurement of avidin and streptavidin in crude biofluids with a new, optimized biotin-fluorescein conjugate. Biochim Biophys Acta, 1427, 33-43.

Kada, G.; Kaiser, K.; Falk, H.; Gruber, H. J. (1999b) Rapid estimation of avidin and streptavidin by fluorescence quenching or fluorescence polarization. Biochim Biophys Acta, 1427, 44-48.

Kohn, J.; Wilchek, M. (1982) A new approach (cyano transfer) for cyanogen bromide activation of sepharose at natural pH*, which yields activated resins, free of interfering nitrogen derivatives. Biochem. Biophys. Res. Commun. 101, 878-884.*

Lin, H. J.; Kirsch, J. F. (1977) A sensitive fluorometric assay for avidin and biotin. Analytical Biochemistry 81, 442-446.

Livnah, O.; Bayer, E. A.; Wilchek, M.; Sussman, J. L. (1993) Three-dimensional structures of avidin and the avidin-biotin complex. Proc. Natl. Acad. Sci., no. 90, pp. 5076-5080.

Livnah, O.; Bayer, E. A.; Wilchek, M.; Sussman, J. L. (1993a) Three-dimensional structures of avidin and the avidin-biotin complex. Proc. Natl. Acad. Sci., no. 90, pp. 5076-5080.

Livnah, O.; Bayer, E. A.; Wilchek, M.; Sussman, J. L. (1993b) The structure of the complex between avidin and the dye, 2-(4'- hydroxyazobenzene) benzoic acid (HABA). FEBS 12654, vol. 328, no. 1,2 165-168.

Rao, S. V.; Anderson, K. W.; Bachas, L. G. (1997) Determination of the extent of protein biotinylation by fluorescence binding assay. Bioconjugate Chem., no. 8, pp. 94-98.

Ries, J.; Schwille, P. (2012) Probing molecular dynamics and interactions, Fluoresence correlation spectroscopy. Bioessays., no. 34; 361-368

Spitzbart, B. (2014) Herstellung von bivalentem Streptavidin mit einem spaltbaren fluoreszierenden Biotin-Derivat. Master thesis at Johannes Kepler University.

Wiesauer, M. (2017) Chemical and photochmcal control of streptavidin-biotin based biological applications. Doctoral thesis at Johannes Kepler University.

Wilchek, M.; Bayer, E. A. (1988) The Avidin-Biotin Complex in Bioanalytical Applications. Anal. Biochem., no. 171, pp. 1-32.

Wilchek, N.; Miron, T.; (1985) Activation of Sepharose with N, N'-Disuccinimidyl Carbonate. Appl. Biochem. Biotechnol. 11, 191-193.