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Response enhancement in SPR using lipophilic antibodies

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Abstract

The study of small biological molecules is sometimes not possible due to the low concentrations of the analyte to be studied, or the small mass of the analyte. The possibility to enhance the SPR-response in a sandwich assay using lipid-conjugated antibodies was studied. A response enhancing reagent composed of mixed micelles was examined as well as the possibility to use liposomes to increase the signal. The results indicate that response enhancements are possible to obtain, but that the system needs to be refined.

Keywords

Biacore, Surface plasmon resonance, SPR, response, signal enhancement, sandwich assay, micelle, liposome

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Response enhancement in SPR using lipophilic antibodies

Ulrika Wiberg

Sammanfattning

Det finns idag ett stort intresse av att studera olika biologiska interaktioner i realtid. Ytplasmonresonansbaserade tekniker (SPR-tekniker) har utvecklats för att möjliggöra dessa studier. Ytplasmonresonans är ett elektromagnetiskt fenomen som uppstår vid totalreflektion i tunna metallfilmer. SPR-tekniker ger responser baserade på massförändringar vid en bioaktiv yta eftersom ytplasmonen påverkas av dessa förändringar. Interaktioner mellan olika biologiska molekyler på ytan kan studeras, t ex bindningen mellan antikropp och antigen. Dock finns det en begränsning för detektion av analysobjektet, vilket innebär att mycket små molekyler i låga koncentrationen är svåra att detektera. Därför är en bra signalförstärkning önskvärd.

En signalförstärkning kan erhållas genom att en antikropp som binder till analysobjektet injiceras över ytan. Bindningen av antikropparna medför en signalförstärkning som kan korreleras till massan av analysobjektet. Detta är dock inte alltid tillräckligt. Med en kemisk koppling av fettsvansar (lipider) till antikroppen borde signalen kunna förstärkas ytterligare då ett reagens som binder till fettsvansen injiceras.

I det här examensarbetet har lipidkoppling till antikropp utförts och möjligheterna till signalförstärkning har undersökts. Som förstärkande reagens har både blandade miceller och liposomer testats. Resultaten visar att signalförstärkning med miceller är möjlig, men att metoden behöver optimeras.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet februari 2004

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1 Introduction

Nowadays it is possible to examine the smallest parts of life in different kinds of assays. Biotechnology has taken us far, and different methods of analysis help us to reach new goals. Studying biomolecular interactions is one of the most interesting areas of biotechnology. How fast, in what order and under which conditions do different interactions take place? One request has been to be able to study biological interactions as they happen, in real time. Several methods have been developed for this purpose, and some of these are using the SPR-technique.

1.1 SPR

Surface Plasmon Resonance, SPR, is a phenomenon arising in thin metal films at conditions of total internal reflection. When light strikes the interface between two optically different media, coming from the media of higher refractive index, total reflection can be observed. These reflections create a non-propagating electromagnetic wave, the evanescent wave [1], which travels a short distance into the media on the side with lower refractive index. The evanescent wave field decreases exponentially from the surface, but reaches far enough (in the order of one wavelength) to detect optical changes close to the surface. If the light is polarized and monochromatic, and if there is a metal film at the interface, the energy loss associated with the surface plasmon creates a measurable decrease in intensity for certain angles of the reflected light. This is what is known as SPR [2]. The angle at which a change in intensity is observed is affected by several different parameters. One of these is the refractive index of the non-reflecting side. This implies that changing the density at the non-reflecting side should produce measurable changes in the so-called SPR angle, that is, the angle of depletion caused by the SPR effect. Figure 1 shows an overview of the SPR-technique.

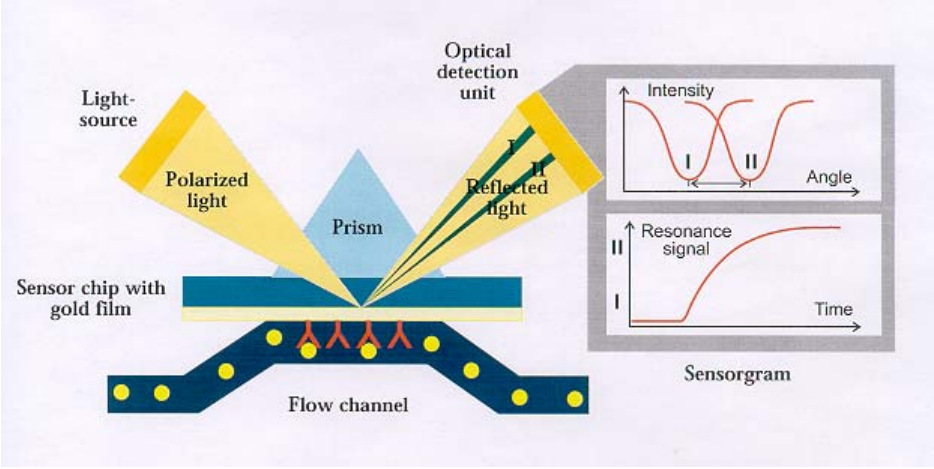

Figure 1 Overview. Mass changes at the non-reflecting side (here illustrated with antibody-antigen binding) produce a shift in the SPR angle. The shift can be converted to a signal in a sensorgram. The illustration was used with permission from Anders Sjödin at Biacore AB.

The SPR-technique can be used to study biological interactions at a surface. Mass changes at the surface induce changes in the refractive index and thus change the intensity of the reflected light. Since the changes in reflected light can be measured continuously, different biological interactions can be studied in real time.

1.2 The Biacore instrument

In the Biacore AB instruments, several different analyses can be performed using the SPR technology: kinetic constants can be determined; concentrations can be measured; analysis of binding, epitope mapping and multiple binding can be examined; and the function of different complexes can be studied. These analyses are performed at a biochip surface and are detected by an optical system.

1.2.1 Chip

In the Biacore chip, the metal film necessary for SPR enhancement is composed of gold. Silver actually generates a stronger signal, but gold is more durable and chemically inert. These qualities are preferred, and gold is therefore the choice of metal in the Biacore chip.

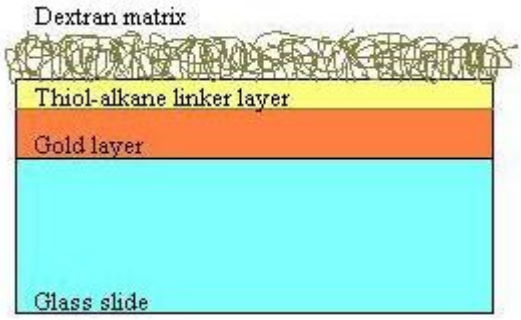

Figure 2 Overview of a Biacore sensor chip. The ligands are linked to the dextran matrix.

The Biacore chip is thus composed of a thin gold layer on a supporting glass slide [2]. To the gold film, a linker layer is coupled. This layer both protects the gold film and acts as a handle to attach the bioactive material (Figure 2). The linker layer is usually a thiol-alkane layer onto which different matrices can be linked. The most commonly used sensor chip, CM5, has a matrix consisting of carboxy-methylated dextran that makes it possible to covalently link different ligands, e.g. antibodies, to the surface.

To determine the conditions for ligand coupling to the surface, a pH scouting can be performed. The pH scouting examines how the pre-concentration of the ligand changes with pH. The pre-concentration is a result of the electrostatic effect on the ligands from the surface. This effect can, under the right conditions, increase the ligand concentration at the surface to well above the ligand concentration in the solution, thereby facilitate a high immobilisation degree even for ligands in low concentrations. The pH scouting does not activate the surface; therefore the surface can be used for immobilisation immediately after the pH scouting.

Ligands can be coupled to the surface of the CM5-chip in different ways [3]. One useful method is thiol disulphide exchange. If the ligand contains a thiol group it can be coupled to disulphides that are created on the surface (the ligand thiol procedure). Alternative, disulphides can be introduced onto the ligand. Thereafter the ligands can be coupled to a thiolated surface (the surface thiol procedure). Aldehyde and streptavidin-biotin couplings are other methods frequently used to immobilise ligands on a CM5 surface.

Commonly, ligands are coupled to the surface of the CM5-chip with an amine coupling procedure. The carboxylic acids at the surface are activated with a mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N'-(dimethylaminopropyl)-carbodiimide (EDC). In this reaction an NHS-ester is formed. When the ligand is applied, NHS acts as a leaving group and the ligand is covalently attached to the matrix. The remaining NHS-esters are deactivated with an injection of ethanolamine.

There are also other kinds of chips. One of these is the L1 chip, which has a lipophilic dextran matrix, and therefore is suitable for analysis of lipophilic compounds. The choice of chip is based on the analyses that will be performed. Different applications make use of different chip.

In the Biacore 3000 instrument, there are four flow cells (other Biacore systems, such as S51, has two flow cells, but four detection spots). This means that each chip can be used with four identical or different ligands. Usually one flow cell is used as reference, that is, activated and deactivated without ligand immobilisation. The reference cell can be used to correct for unspecific interactions with the matrix itself. Ideally no such interactions occur.

1.2.2 Detection

The measurements in the Biacore system are monitored by an array of diodes, measuring the light continuously as it is reflected from the surface [2]. Light, in the near-infrared range (760 nm), is focused onto the Biacore chip in a spectrum of different angles. The reflected light is monitored by a detector array of diodes placed at fixed positions. When interactions occur at the surface, the SPR angle, i.e. the angle of reflection depletion, changes. This change is detected by the diode array and transferred to

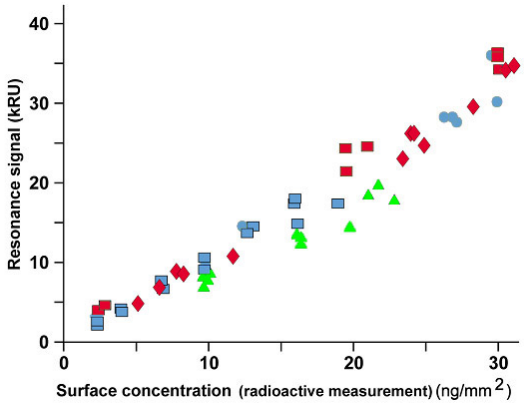

Figure 3 Correlation between SPR and surface concentration for Chymotrypsinogen A (blue), anti-Transferrin (red) and Transferrin (green). The illustration was used with permission from Hans Sjöbom at Biacore AB.

a sensorgram, that is, a plot showing how the SPR angle is changed with time (Figure 1). The change is recorded in response units (RU). One RU corresponds to an angle change of 0.0001 degrees. The response can also be correlated to surface concentration, where 1000 RU corresponds to 1 ng mm^{-2} (Figure 3).

1.2.3 Fluidics

To immobilise ligands and apply samples to be measured, the system has an integrated micro-fluidic system [2]. The Biacore system includes pumps and automatic sample handling, and the integrated µ-fluidic cartridge (IFC) delivers the different liquids to the sensor chip. Together with the sensor chip, the IFC creates a flow cell in which bio-interactions can take place.

1.2.4 Software

For control of the system, the Biacore software is used [2]. The software includes different wizards for easy set up of some common tasks, such as guides to immobilisation of ligands and regeneration scouting. In addition to the wizards, it is possible to write methods that control the assays. It is also possible to run the system manually – which facilitate choosing how to proceed depending on the previously obtained results.

1.3 Antibodies and their use in SPR

Antibodies are widely utilised biological molecules; they can be used and examined in a wide variety of assays. In the Biacore system they can either be immobilised on the surface, as capturing molecules, or be the molecules of interest to examine. In the latter case it might be the antigen that is immobilised on the surface, and the goal of the assay can be to decide whether a sample contains the antibody of interest or not. Antibodies have a molecular mass of about 150,000 Da, a mass that is easily detected by the Biacore system, where the limit of detection is about 200 Da [2].

Since antibodies are very specific in how they bind to certain compounds, they are often used in the Biacore system. One way to use antibodies is in sandwich assays (Figure 4). In a sandwich assay, one antibody, often referred to as the capturing molecule, is immobilised on the surface. Then the analyte, an antigen, is injected over the surface and is allowed to interact with the capturing molecule. As a last step a secondary antibody, the enhancer molecule, is injected. This antibody binds to another epitope on the analyte (antigen). If the analyte is small, the mass increase caused by the second antibody

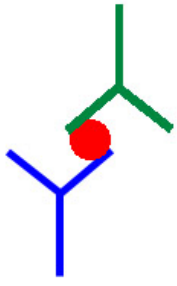

Figure 4 Illustration of a sandwich composed of two different antibodies (blue and green) and an antigen (red).

enhances the signal, and makes it possible to detect even small concentrations of analyte.

Antibodies can thus be used to enhance the signals when used in sandwich assays. In cases where the analytes are present only in very small concentrations, or if they have very low molecular masses, the signal from analyte binding, as well as from the secondary antibody, might be lost in the noise.

1.4 Signal enhancement

By injection of a third component that binds the secondary antibody, the response signal might be amplified even more. Another antibody, binding to the secondary antibody, might be used. It is also possible to bind different kinds of proteins to the secondary antibody to increase the response.

Coupling fatty acids to the secondary antibody and then applying a regent that stick to the lipids, might be another way of increasing the response. The fatty acids might bind to components of mixed micelles or to liposomes. If this is the case, a great signal enhancement should be the result.

Liposomes are vesicles with walls composed of a double layer of lipids [4]. The size of liposomes varies between 25 nm and 2500 nm, depending on how they are prepared and the composition of the lipids. To begin with, they were widely used as a model system of the cell membrane, but nowadays they have found many other uses as well. The double lipid layer allow certain substances to pass the membrane and liposomes can thus be used as carriers to deliver these substances, e.g. drugs or cosmetics. They can also be used to transport different kinds of biomolecules *in vivo*, and to anchor membrane proteins as well as antibodies to their surface. In addition to the liposomes, there are micelles. The micelles are composed of lipids, detergents or mixtures of both, but here no double layers are formed. Instead, the micelles most often consist of spheres of lipids with their tails pointing into the highly hydrophobic core. The composition of micelles can be widely varied, producing micelles with different qualities.

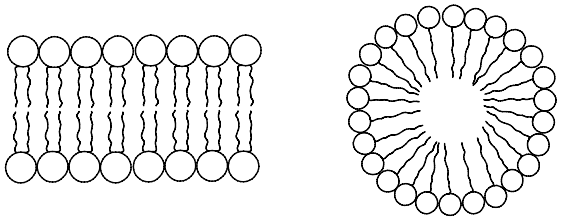

Figure 5 Schematic pictures of a lipid bilayer and a micelle. The lipid bilayers can form large vesicles, liposomes, with diameters between 25 nm and 2.5 μm.

The On-Surface Reconstitution (OSR) reagent consists of mixed micelles with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC; Avanti Polar Lipids Inc) and n-octyl β-D-glucopyranoside (OG; Sigma), and has

previously been used to anchor membrane proteins at a sensor surface [5]. Applying the OSR reagent to an L1 chip (a Biacore chip with lipophilic dextran) and then eluting the detergent covers the L1-surface with lipids that make it possible to "capture" membrane proteins. The captured proteins have been shown to have normal function. On the L1 chip, the OSR reagent forms a lipid bilayer. This bilayer gives a response in the order of 5000 RU. If such a bilayer, or parts of it, could be formed by binding of the OSR reagent to lipids on the antibodies, the response in a sandwich assay should be greatly enhanced. The question is whether such bilayers will cover the whole surface, or only islands on the surface, if it binds to the lipids at all. If the former is true, it might not be possible to determine concentrations of the analyte since different concentrations will give the same response. This work is focused on coupling of lipids to antibodies and analysis of the use of the resulting conjugates to enhance the signal in sandwich assays together with the OSR-reagent.

Since liposomes also bind to other lipophilic compounds, e.g. an L1-surface, they should be able to bind to lipids conjugated to antibodies as well. If they do, liposomes can be used to enhance the signal further in the sandwich assay described above, being an alternative to the OSR reagent.

2 Methods

2.1 Assay

In this work, a Biacore 3000 instrument was used. In the sandwich assay, a monoclonal mouse human anti-myoglobin antibody (here referred to as 2F9.1) was used as the capturing molecule immobilised on the sensor chip surface. Myoglobin (17,500 Da, from human heart, Sigma) was used as analyte. A polyclonal antibody (polyclonal rabbit anti-human myoglobin, DAKO; poly-α-myo) has been used as the amplifying reagent, and, as such, was the target for lipid tagging. On top of the sandwich, the OSR reagent was applied. As running buffers in the Biacore system and for some other applications, HBS-N (10 mM HEPES pH 7.4, 150 mM NaCl) and HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20; both from Biacore) have been used.

2.1.1 OSR reagent

The OSR reagent was prepared as previously described [5]: glass vials were washed with chloroform. Then POPC dissolved in chloroform were transferred to the vials. After drying with a stream of nitrogen gas, which creates a lipid film on the walls of the vials, the vials were put under reduced pressure for at least two hours or overnight to remove all traces of chloroform. To redissolve the lipids, a mixture of HBS-N buffer and OG solution was prepared. This solution was transferred to the vials after drying to give final concentrations of 3.75 mM and 27.5 mM for POPC and OG respectively. (These concentrations were used since they are

optimally fine-tuned for the purpose [Olof Karlsson, personal communication].) The vials were shaken every 10 minutes for at least 45 minutes to dissolve the POPC completely. After this the vials contained mixed micelles: the OSR reagent.

The prepared OSR reagent was tested on an L1-chip. The surface was first conditioned with five 30 seconds injections of 20 mM 3-cholamidopropyl-dimethyl-ammonio-1-propane sulfonate (CHAPS; Sigma) at a flow of 10 µl min^{-1}, and then the OSR-reagent was injected for 1 min at a flow of 5 µl min^{-1}. The surface was regenerated with 50 mM OG (Sigma) in HBS-N buffer. On an L1-chip, the OSR reagent should give a response between 4000 and 6000 RU.

2.1.2 Preliminary work

Before conjugation of lipids to the antibody, the different parts of the assay system were tested in the Biacore instrument to assure that the antibody-antigen interaction worked as intended, as well as that no unspecific interactions took place. The immobilisation conditions were examined and regeneration scouting was performed for poly-α-myo and myoglobin, using the Biacore software wizards.

With the help of an immobilisation wizard, the monoclonal and polyclonal antibodies (2F9.1 and poly-α-myo) and myoglobin were immobilised in different levels on three separate CM5-chips, using the amine coupling method. The levels the wizard aimed for was 1000, 5000 and 10,000 RU for the two antibody solutions, and 500, 2000 and 5000 RU for myoglobin, which is a much smaller molecule. The immobilisation concentrations were 10 µg ml^{-1} of both antibodies and antigen. All chips also contained a reference cell. The antibody surfaces were tested with myoglobin (5 µg ml^{-1}) as analyte. Myoglobin was tested with 2F9.1 (5 µg ml^{-1}) as analyte. OSR reagent and OG (30 mM) dissolved in HBS-N were injected over all surfaces. HBS-N was used as flow buffer.

The total assay system was tested as well. 2F9.1 (10 µg ml^{-1}) was immobilised on a new CM5-surface. This surface was then subjected to a myoglobin injection (5 µg ml^{-1}) directly followed by injections of poly-α-myo in different concentrations (100, 75, 50, 25 and 10 µg ml^{-1}). At the top of the sandwich, the OSR reagent was injected to see whether unspecific interactions occurred or not.

2.2 Antibodies

There are several different approaches when it comes to coupling of lipids to antibodies. This could for instance be done biosynthetically, that is using cloned genes and bacteria to produce large amounts of antibody-lipid conjugates [6]. In this work chemical modifications and couplings have been used. Two different lipids have been tested, each with its own coupling mechanism.

2.2.1 NHS-palmitate coupling

N-hydroxysuccinimide (NHS) esters are molecules that are widely used in coupling chemistry since NHS acts as a very good leaving group. By using NHS-esters of a fatty acid, in this case palmitic acid, antibodies can be linked to the fatty acid [4, 7]. In the reaction, the ester bond of the NHS-palmitate breaks as an amine of the antibody reacts with the ester, and a stable amide bond is formed instead (Figure 6).

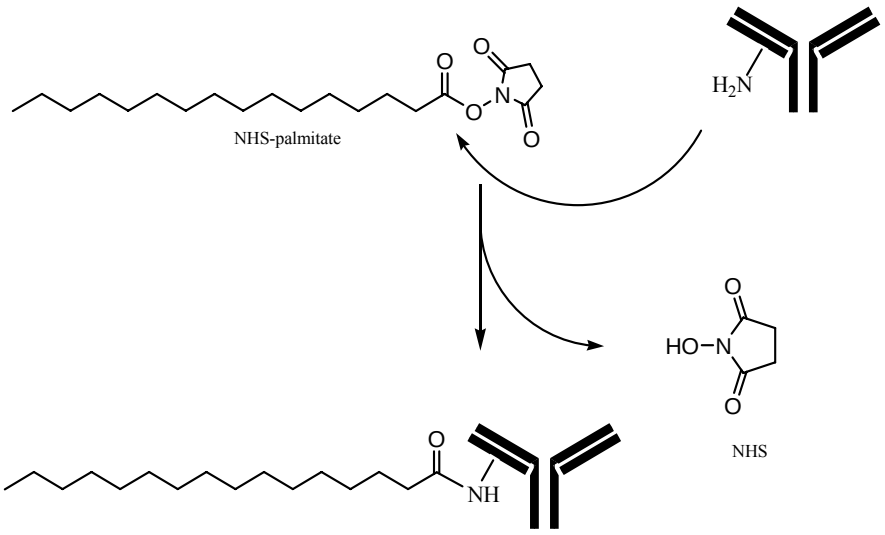

Figure 6 The coupling reaction of NHS-palmitate with an amine group of an antibody. NHS acts as a leaving group, giving place for the antibody. Note that the position of the amine group on the antibody is only schematic.

There are protocols on how to prepare NHS-palmitate [4, 7], but here, pre-prepared NHS-palmitate (Sigma) was used. To start with, dissolving NHS-palmitate in PBS buffer (0.01 M phosphate buffer, 2.7 mM KCl, 0.137 M NaCl; Sigma) containing 30 mM OG was attempted. OG is the detergent in the OSR reagent and was therefore the first choice in this reaction to get a stable assay system. Several other detergents and solvents were also tried, both with and without PBS-buffer: CHAPS; cholic acid (cholate); deoxycholic acid (deoxycholate, all three from Sigma); Zwittergent 3-12 (Calbiochem); dimetylsulfoxid (DMSO, Riedel-de Haën); ethanol (Kemetyl); acetonitrile; N,N-dimetylformamide (DMF, both from Fluka); and chloroform (Merck). Deoxycholate has previously been used successfully [7]. The detergents were dissolved in PBS to a concentration of 2%. The solvents were used both alone and in different concentrations of PBS. NHS-palmitate dissolved in chloroform was dried under reduced pressure in the same way as the OSR reagent (see above), and then subjected to detergent.

Since the NHS-ester immediately reacts with e.g. water, it is important that the dissolved NHS-palmitate is added to the antibody solution as soon as possible when dissolved. Thus the antibodies were prepared first. Poly-α-myo was diluted in buffer (see Table 1). This dilution was subjected to buffer exchange to the same buffer on a NAP-column (Amersham Biosciences), to remove traces of sodium azide. The eluate was diluted to give a final concentration of 1 mg polyclonal antibody ml^{-1}. Different molar equivalents of newly prepared NHS-palmitate mixture was then added to the polyclonal antibodies and allowed to react over night at room temperature (RT), or the polyclonal antibody solution containing a detergent was added directly to vials with dried films of NHS-palmitate and allowed to react over night. For procedures, see Table 1.

Table 1 Buffers and conditions used for the NHS-palmitate coupling. Note that the NHS-palmitate mixtures were not clear solutions of NHS-palmitate.

	Antibody buffer	NHS-palmitate	Procedure
1.	30 mM OG in PBS	a. Mixture with 30 mM OG in PBS b. Mixture with 30 mM OG in PBS and 5% DMSO	2, 5 and 10 molar equivalents of NHS-palmitate. Incubation over night, RT
2.	2% cholate in PBS	a. Mixture with 2% cholate in PBS b. Lipid films of NHS-palmitate	20, 50 100 molar equivalents of NHS-palmitate. Incubation 1 h, RT
3.	2% deoxycholate in PBS	Lipid films of NHS-palmitate	10 and 100 molar equivalents of NHS-palmitate. Incubation 1.5 h, RT

After incubation, the polyclonal antibodies were ready for purification to separate coupled antibodies from non-coupled. Prior to purification, small amounts of the conjugation mixtures were tested in the Biacore system to get an indication of whether the OSR reagent bound to the antibodies or not, that is, if the coupling was successful.

2.2.2 N-PDP-PE coupling

Disulfide bonds are useful for covalent linkage of antibodies to fatty acids. 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)-propionate] (N-PDP-PE, Figure 7) is a lipid conjugate that readily reacts with thiol groups forming stable disulfide bonds.

A buffer exchange was performed on poly-α-myo on a NAP-column to a thiolation buffer (0.1 M PBS, 5 mM EDTA, pH 8,0). Then Traut's reagent was prepared for the thiolation of the antibody: 2-iminothiolane (Pierce) was dissolved in the buffer above (~ 40 mM). 30 or 15 molar equivalents

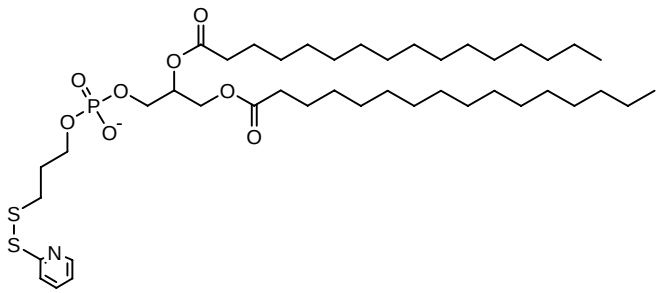

Figure 7 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)-propionate] (N-PDP-PE)

of Traut's reagent were added to the poly-α-myo and the mixture was allowed to react in the dark at RT. The first part of Figure 8 shows this step.

After 45 minutes of incubation, the antibody-reagent mixture was subjected to another buffer exchange to 2% OG in PBS, pH 8,0. The eluate was diluted with the same buffer to give a concentration of 1 or 5 mg poly-α-myo ml^{-1}. The higher concentration was used in the latter conjugations to increase the antibody response at the higher myoglobin concentrations in the sandwich assays.

To get a measure of how much of the antibodies that had been thiolated, Ellman's assay was performed. Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) reacts with thiols, and in the reaction 5-thio-2-nitrobenzoic acid (TNB) is released. The amount of TNB can be spectroscopically measured [4, Anders Sjödin, personal communication]. Ellman's reagent was dissolved in 0.1 M sodium phosphate, 5 mM EDTA, pH 8.0 at a concentration of 4 mg ml^{-1}, and 100 µl of this solution was added to 500 µl sample and reference (2% OG-buffer). After 15 minutes incubation at RT, the absorbance at 412 nm was measured for sample and reference, and the sample value was reference subtracted (A_{412}). The thiol concentration was calculated according to

$$C_{thiol} = \frac{A_{412}}{l \cdot \varepsilon} = \frac{A_{412}}{l \cdot 14500} \qquad (1)$$

where l is the path length and ε is the extinction coefficient of Ellman's reagent. Then the grade of thiolated antibody was calculated:

$$Thiolation\ degree = \frac{C_{thiol}}{C_{poly-\alpha-myo}} \qquad (2)$$

A stock solution was prepared from dried N-PDP-PE (Avanti Polar Lipids Inc) in chloroform. Different volumes of this stock solution were then transferred to glass vials where the chloroform was evaporated (the same procedure as with the OSR reagent above). The thiolated poly-α-myo was added to these vials and allowed to react in RT over night. To get an estimate of how many lipids each antibody had bound, the absorbance at

343 nm was measured both before and after incubation. The difference between the latter and former, A_{343}, gave a measure of how much pyridine-2-thione there was after the reaction was completed:

$$C_{pyridyl-2-thione} = \frac{A_{343}}{l \cdot \varepsilon} = \frac{A_{343}}{l \cdot 8080} \qquad (3)$$

Here, ε is the extinction coefficient of pyridine-2-thione.

Since pyridine-2-thione is the leaving group of the coupling reaction, the amount of pyridine-2-thione is directly correlated to the number of coupled lipids. This could then be used to calculate the grade of modification, that is, the average number of conjugations for each antibody:

$$Modification\ degree = \frac{C_{pyridyl-2-thione}}{C_{poly-\alpha-myo}} \qquad (4)$$

Pyridine-2-thione is the leaving group of the reaction. For an overview of the thiolation reaction and lipid coupling, see Figure 8.

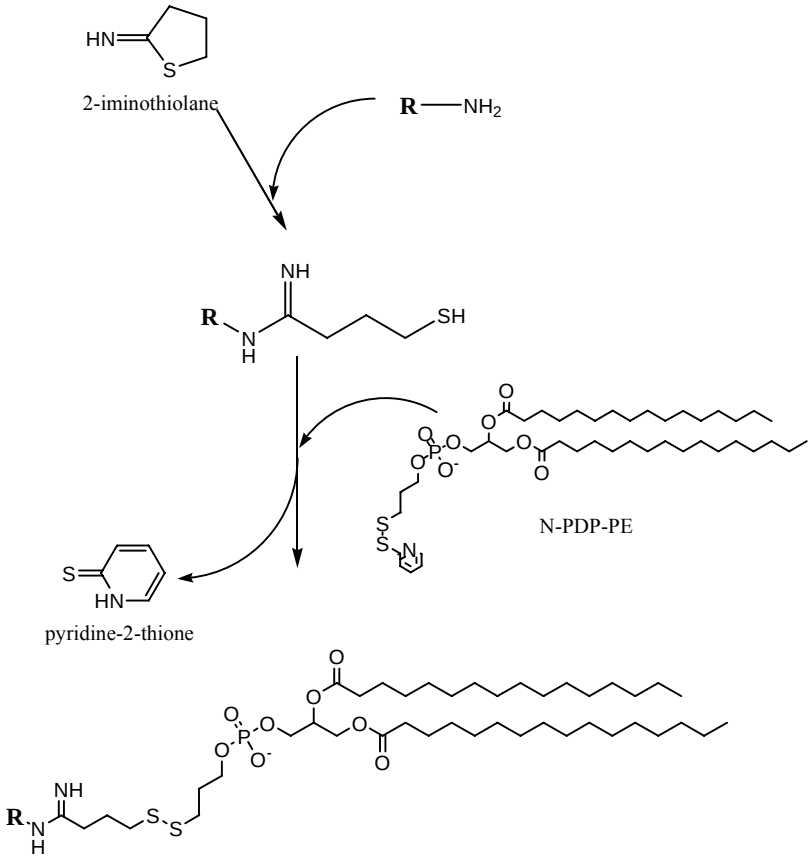

Figure 8 Thiolation of antibody (**R**) followed by disulfide bonding of N-PDP-PE to the antibody. Upon the latter reaction, pyridine-2-thione is the leaving group.

2.2.3 HIC

In hydrophobic interaction chromatography (HIC), molecules are separated according to hydrophobicity. The separation begins at a rather high salt concentration, a condition that promotes hydrophobic interactions. Different compounds are then separated using either a decreasing salt gradient or a stepwise elution. In some cases a combination of these two modes of elution is the best choice to find the optimal conditions to get as good a separation as possible. Several trial purifications usually have to be run. Here, a salt gradient was used for elution.

There are many different columns to use in HIC. Each one has its own advantages and disadvantages. The differences can e.g. be grade of substitution or hydrophobicity. Here, three different 1 ml Sepharose™ columns have been used: Octyl 4 FF, Butyl 4 FF and Phenyl HP (all from Amersham Biotech). Phenyl HP is used when high resolution is needed. Butyl FF has a hydrophobicity that is low to medium and the butyl ligand has different mechanisms of adsorption and desorption. Octyl FF has somewhat different hydrophobic qualities than the other two [8]. The columns were used in an FPLC® system. 250 µl of the sample was loaded onto the columns, and the used flow rate was 1 ml min^{-1}. Fractions of 1 ml were collected for further analysis in the Biacore system. Buffers with varying composition and pH have been used (Table 2).

The first HIC purification was carried out on samples of NHS-palmitate conjugated antibodies. These samples were prepared according to conditions 2 in Table 1. Solid ammonium salt ($(NH_3)_2SO_4$) was added to the samples, giving them the initial concentration 1 M, and after filtration the samples were ready for the HIC column. The Octyl FF column was used with corresponding buffers (A, Table 2) at pH 7.0, with the initial salt concentration 1 M.

Table 2 Columns and buffers used for HIC. Note that each buffer has been used with two different pH values. The start buffer with 1.7 M $(NH_3)_2SO_4$ was mixed with the corresponding elution buffer to give start concentrations of $(NH_3)_2SO_4$ ranging from 1.0 to 1.7 M.

	Columns used	Start buffer	Elution buffer
A.	Octyl FF, Butyl FF	0.02 mM KH_2PO_4 0.6 mM CHAPS 1.7 M $(NH_3)_2SO_4$ pH 7.0 / pH 7.4	0.02 mM KH_2PO_4 0.6 mM CHAPS pH 7.0 / pH 7.4
B.	Butyl FF, Phenyl HP	50 mM Na_2PO_4 1.0 M $(NH_3)_2SO_4$ pH 7.0 /pH 8.0	50 mM Na_2PO_4 pH 7.0 / pH 8.0

Since the FPLC-system allows mixing of the start and elution buffers, the initial salt concentration can be varied. This was used to examine the effects of different salt levels (1.7, 1.5, 1.2 and 1.0 M) in the samples as

well as in the start buffer. Buffers A (Table 2), pH 7.4, and the Butyl FF column were used for this application. The significance of pH was studied using the Phenyl HP column and corresponding buffers (Table 2), pH 7.0 and 8.0.

To examine how the buffer exchanges prior to purification affected the samples, non-conjugated poly-α-myo was subjected to different buffer exchanges: either directly to the start- or elution buffer or to the elution buffer only after an exchange to the conjugation buffer. Two different columns, Butyl FF and Phenyl HP (pH 8.0), were tested to find the best purification strategy of the conjugated antibodies.

Another purification was performed on NHS-palmitate conjugated antibodies according to 3 in Table 1. Here the Butyl FF column was used and the buffer pH was 8.0. The salt gradient started at 1 M $(NH_3)_2SO_4$.

HIC purification was also attempted on the N-PDP-PE conjugation, using the Butyl FF column and buffers according to Table 2, pH 8.0

2.2.4 Gel filtration

Another mode of column separation of proteins is gel filtration. Gel filtration separates protein according to size. Therefore there is no need of gradients in the elution buffers and there is also no binding of analyte to the column. The sample is applied on the top of the column and then eluted with a steady flow of buffer. The largest molecules come out first since they cannot enter the polymer network inside the gel. Here a Superdex™ 200 column from Pharmacia was used together with an FPLC® system. 500 µl of the sample was loaded on the column, the flow rate was 0.5 ml min^{-1} and fractions of 1 ml were collected.

HBS-EP was used as the buffer in the gel filtration. The buffer was used both without any additives and with extra P20 (Tween, Biacore). P20 is already present as a detergent in the HBS-EP buffer. Additional P20 was added to exceed the critical micelle concentration (CMC) of the detergent. The final concentration of P20 after extra addition was 0.012% (approximately 0.1 mM).

2.3 Analyses

2.3.1 Analysis of antibody conjugates

The antibody conjugates from the NHS-palmitate coupling were tested in the Biacore instrument prior to HIC, to see if they still bound to myoglobin and if the OSR reagent was binding to the conjugates or not. The conjugates were tested either in a sandwich assay, where 2F9.1 was immobilised on the surface and the conjugate was injected after an injection of myoglobin, or directly on a myoglobin surface. The response of the OSR reagent was studied for different concentrations of myoglobin and conjugated antibody.

After HIC or gel filtration, the fractions of antibody from both coupling reactions were tested in a Biacore assay to identify the fractions containing the conjugated antibody as well as the native antibody (that is, fractions with components that bind to myoglobin) and other complexes, by comparing the Biacore result with the chromatogram from the FPLC. The assays were performed as above, with a 2F9.1 surface and myoglobin or directly on a myoglobin surface, with or without the OSR reagent at the top. The fractions were diluted four times in flow buffer (HBS-N or HBS-EP; Biacore), to reduce the concentration of salt (only after HIC) and to use only a small volume of the fractions.

2.3.2 Sandwich assay

The purified antibody conjugates were examined in a sandwich assay. 2F9.1 was immobilised on the CM-5 surface and myoglobin was injected over the surface in known concentrations, as well as the antibody conjugate, diluted in flow buffer (HBS-N or HBS-EP). A few different dilutions of the antibody containing fractions were tested to see what worked best.

The myoglobin concentration was varied to examine the variation of the relative responses of conjugated antibodies as well as the successive responses of the OSR reagent.

Since the OSR reagent is somewhat "sticky" (it might adhere to the needle and IFC tubings), by-pass washes with 40% iso-propanol and 30 mM NaOH were introduced in the method both before and after the OSR-injection to assure that no interactions took place in the tubings, but only in the flow cells.

The conjugated antibody and the interactions with the OSR reagent were also tested directly on surfaces with different levels of immobilised myoglobin. In addition to the sandwich assays, the conjugated antibody was immobilised directly onto a new surface and its ability to bind the OSR reagent was examined.

Regeneration conditions were examined during the course of the work to find the best regeneration possible. 10 mM Glycine at pH 1.5, 2.0, 2.5 and 3.0 as well as 4 M $MgCl_2$, 50 mM NaOH, 0.1% TFA and 0.1% SDS were tested to optimise regeneration in the sandwich assay.

2.4 Liposomes

To further study the N-PDP-PE-conjugated antibodies, two different liposome preparations, POPC (for composition, see above) and BLEND (consisting of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dioleoyl-*sn*-glycero-3-(phospho-L-serine) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; Avanti Polar Lipids Inc) were injected over surfaces with antibody immobilised in different levels (blank, 200, 400, 800, 1600, 2000, 4000 and 8000 RU). The liposomes were also injected over an L1 surface. Used concentrations were 0.5 mg ml^{-1} for both POPC and

BLEND. The responses were in all cases compared to the corresponding values of the OSR reagent responses.

3 Results

3.1 Fundamental results

3.1.1 OSR reagent

When the prepared OSR reagent was injected over an activated L1-surface, a response of approximately 5000 RU was observed. This is in accordance with the expected value of 4000-6000 RU.

3.1.2 Monoclonal anti-myoglobin, 2F9.1

Good immobilisation and regeneration conditions for 2F9.1 was previously known [Anders Sjödin, personal communication] as 10 mM Acetate, pH 5.0 and 4 M $MgCl_2$ respectively (Table 3). The immobilisation succeeded with immobilisation levels of approximately 1100, 5000 and 9000 RU (wanted levels were 1000, 5000 and 10,000 RU).

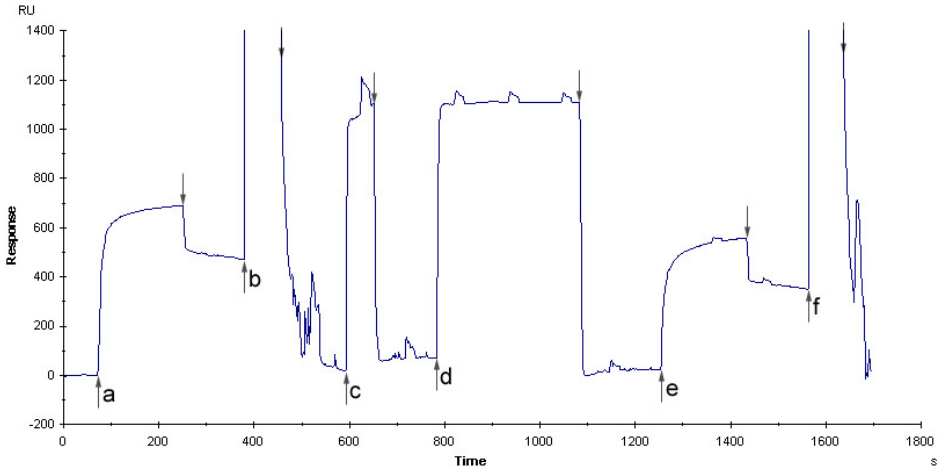

Figure 9 Sensorgram from the 5000 RU-cell with the injections myoglobin (5 µg ml^{-1}, a), $MgCl_2$ (4 M, b), OSR reagent (c), OG (30 mM diluted in HBS-N, d), myoglobin (5 µg ml^{-1}, e) and $MgCl_2$ (f). Arrows indicate injection starts and ends.

According to the analysis of binding and unspecific binding, 2F9.1 showed good binding of myoglobin and no unspecific binding of OSR reagent or OG-solution (Figure 9). The regenerating conditions also seemed to work quite well since the baseline is reached after regeneration (c, Figure 9) and also since myoglobin show good binding also after the first regeneration (e-f, Figure 9).

3.1.3 Polyclonal anti-myoglobin, poly-α-myo

The immobilisation pH scouting showed that 10 mM Acetate buffer with a pH of 5.5, 5.0, 4.5 and 4.0 gave the approximate responses 8300, 12,000, 15,000 and 16,000 RU respectively for poly-α-myo. Acetate pH 4.5 was used in the successive immobilisations. The regeneration scouting showed that 10 mM Glycine, pH 2.0 and pH 1.5 as well as 1% TFA gave good results. To begin with, Glycine pH 2.0 was used (Table 3).

Table 3 Used immobilisation buffers and regeneration solutions for the three different components of the sandwich assay.

	Immobilisation	Regeneration
2F9.1	10 mM Acetate pH 5.0	4 M $MgCl_2$
Poly-α-myo	10 mM Acetate pH 4.5	10 mM Glycine pH 2.0
Myoglobin	10 mM Acetate pH 5.0	4 M $MgCl_2$

The immobilisation of poly-α-myo gave the following levels: 1000, 4000 and 7000 RU, somewhat lower than expected (1000, 5000 and 10,000 RU respectively). This is not surprising realizing the fact that the exact poly-α-myo concentration in the stock solution was not known.

The preliminary tests of poly-α-myo, with sensorgrams similar to that of 2F9.1 (Figure 9), showed that myoglobin binds to poly-α-myo and that only small unspecific binding of the OSR reagent occurred.

3.1.4 Myoglobin

The pH scouting for the appropriate immobilisation buffer for myoglobin, clearly showed that 10 mM Acetate, pH 5.0 was the best choice. It gave a response of nearly 6500 RU compared to the other acetate buffers that gave responses of 3600 RU and less. For regeneration, 4 M $MgCl_2$ was used (Table 3).

The immobilisation succeeded and gave the following approximate immobilisations levels: 440, 2200 and 4000 RU (aiming for 500, 2000 and 5000 RU respectively).

Binding and unspecific binding tests were performed as above using 2F9.1 as analyte. 2F9.1 bound well to the myoglobin surfaces, and the OSR reagent bound only in small amounts (Figure 10).

3.1.5 Sandwich assay

2F9.1 was immobilised to a level of 13,000 RU. Over this surface myoglobin, poly-α-myo and OSR reagent was injected as shown in Figure 11.

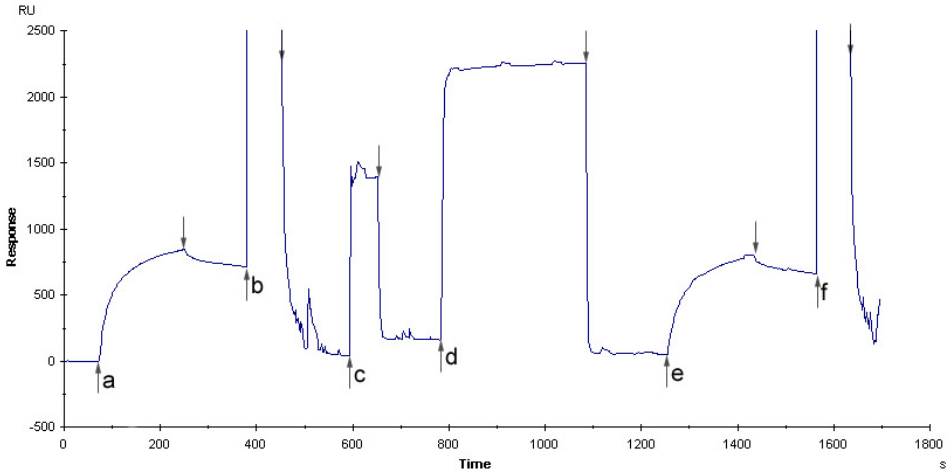

Figure 10 Sensorgram from the 2000 RU-cell with the injections 2F9.1 (5 µg ml^{-1}, a), $MgCl_2$ (4 M, b), OSR reagent (c), OG (30 mM diluted in HBS-N, d). 2F9.1 (5 µg ml^{-1}, e) and $MgCl_2$ (f). Arrows indicate injection starts and ends.

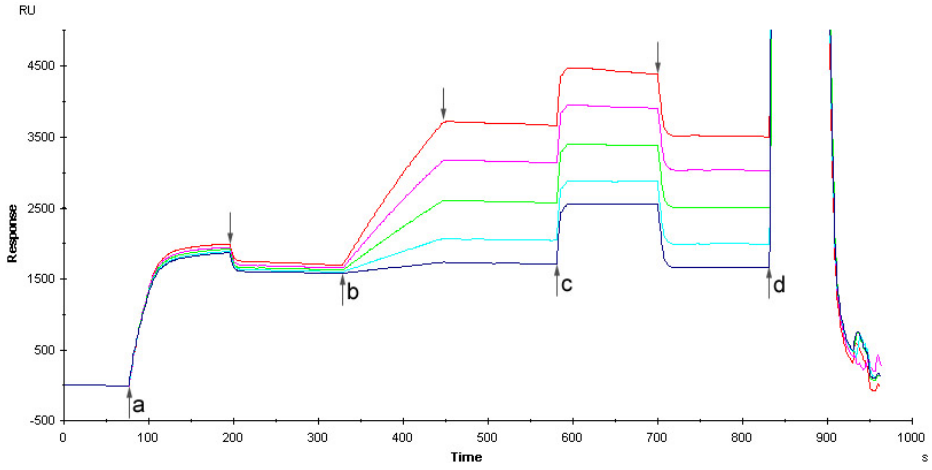

Figure 11 Sensorgram from the sandwich assay. On a 2F9.1 surface, myoglobin (5 µg ml^{-1}, a), poly-a-myo (100, 75, 50, 25 and 10 µg ml^{-1}, b) and OSR reagent (c) were injected. Arrows indicate injection starts and ends. The last injection is the regeneration with $MgCl_2$ (4 M, d).

The myoglobin response is practically constant in the five different runs. The response flattens out in the end of the injection, meaning that the surface is in equilibrium with the solution.

Poly-α-myo gives a response in proportion to concentration (the lowest response from the lowest concentration etc.). These curves lack the plateau, indicating that the myoglobin on the surface has not bound all antibody it is capable of binding. If the injection time and/or concentration of poly-α-myo would be increased, this response should increase as well and eventually flatten out.

The OSR reagent on the other hand is not increasing the response, that is, it does not show any unspecific binding to the sandwich complex.

Actually, the OSR reagent decreases the signal a little. This decease may be caused by the detergent (OG) present in the reagent solution.

The regeneration brings the response back to its initial value. This, and the fact that equal amounts of myoglobin bind every time, indicates that the regeneration works well for this application.

3.2 NHS-palmitate

3.2.1 Conjugation

It proved to be a great challenge to dissolve the pre-prepared NHS-palmitate. NHS-palmitate did not go into solution in detectable amounts in any of the detergents tried. Reducing the amount of NHS-palmitate did not improve solubility. Even after warming, NHS-palmitate would not be dissolved in 30 mM OG in PBS. After warming, the NHS-palmitate was dissolved in DMSO but precipitated upon addition of PBS-OG. The same happened with ethanol, acetonitrile and DMF. Here no warming was needed though.

Chloroform dissolved the NHS-palmitate and it was therefore possible to carry out lipid film drying. Addition of antibodies in PBS-cholate to the lipid films did not seem to give an NHS-palmitate solution, but since small amounts might have been dissolved anyway, some of the NHS-palmitate might have reacted with the antibody.

Even though NHS-palmitate seemed to withstand the solvation attempts, some trial conjugations were performed. NHS-palmitate/detergent mixtures were added to the antibody solutions and incubated over night. The resulting mixtures were subjected to purification, in some cases preceded by filtration through a 0.2 µm syringe filter (Acrodisc®).

Parts of the conjugated mixtures were tested in the Biacore system prior to purification. These tests did not show any specific enhancement of the antibody signal when subjected to the OSR reagent. Impurities in the samples and an excess of non-conjugated antibodies might be the explanation for this.

3.2.2 Purification

The first HIC purification of the NHS-palmitate coupled antibodies on the Octyl FF column gave no distinct results. The reference sample fractions (non-conjugated antibodies) were analyzed on a myoglobin surface in the Biacore instrument. Several of the first fractions gave responses of 100-150 RU, and a smaller response came in fraction 13 (that is, 13 ml after the sample was loaded onto the column). Since the fractions giving the highest responses were collected prior to the start of the elution gradient, no separation based on hydrophobicity was accomplished on these fractions.

When different salt concentrations were tested, 1.0 M proved to be the best concentration to use. The higher salt levels (1.5 and 1.7 M) gave turbid antibody solutions, an indication of the antibodies precipitating at such high concentrations. When comparing 1.2 and 1.0 M of salt, the yield after HIC was higher for the lower salt level (Figure 12). Therefore, 1.0 M $(NH_3)_2SO_4$ was henceforth used as the start concentration.

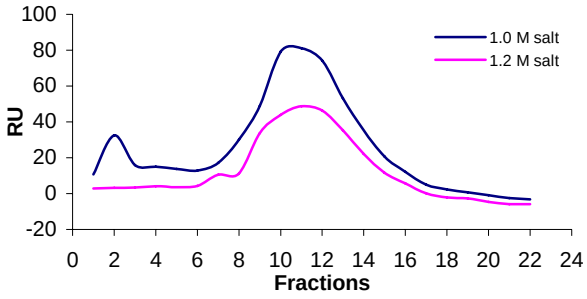

Figure 12 Results from the Biacore tests of the fractions from the salt tests. The recovery of antibody is higher when a lower salt concentration is used.

Comparing the effects of pH, a slightly higher recovery was displayed at pH 8.0, in relation to pH 7.0 (Figure 13). The effect of sample treatment demonstrated that antibodies that only went through one buffer exchange (directly to the elution buffer) had the best recoveries of active antibody. Antibodies first subjected to an exchange into a detergent buffer gave responses significantly lower than those that were not when examined in the Biacore instrument (data not shown). Comparisons between the two columns used in the latter case showed that the Butyl FF column might

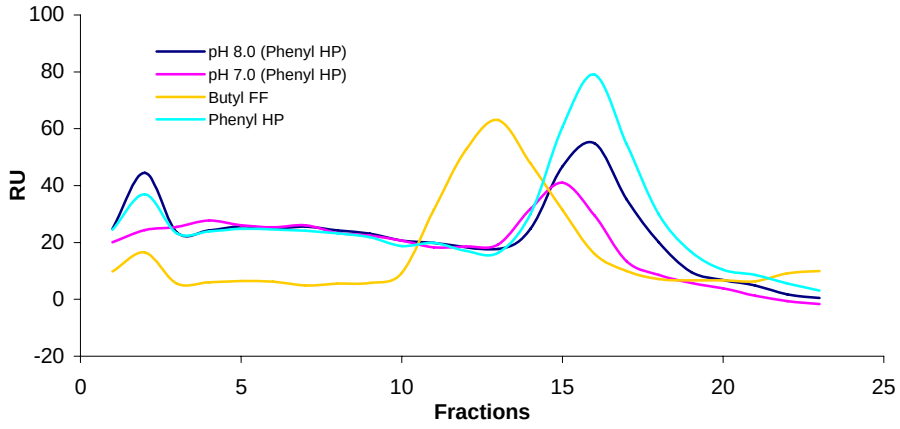

Figure 13 Effects of different pH in the flow buffers and comparisons of the different HIC columns. Using buffers at pH 8.0 results in a higher recovery of antibody after purification than buffers with pH 7.0. The antibody peak from the Butyl column is broader than the peak from the Phenyl column. The Phenyl peak comes rather late in the elution though, indicating that the antibodies bind harder to this column. Note that the pH and column tests are two individual tests using antibodies prepared under different conditions.

work better for the applications in this work than the Phenyl HP column, despite its slightly broader peak (Figure 13). The reason for this is that the Butyl column does not bind the antibodies as hard as the Phenyl column. This is an important factor considering that the lipids, when conjugated to the antibodies, make the antibodies more hydrophobic. The conjugates will therefore elute later, i.e. when the salt level is lower.

In the last purification of NHS-palmitate conjugated antibodies, the results above were used. Due to turbid solutions after addition of salt, the samples were filtered prior to purification. The spectrogram from the HIC displayed a peak around fraction 13 (Figure 14). The Biacore test on a myoglobin surface showed that fractions 12 and 13 of the sample with lower level of NHS-palmitate (10 molar equivalents) gave a small response, indicating that these fractions contained the antibody. The sample with higher levels (100 molar equivalents) gave a response only about half of the lower molar equivalent sample response. The OSR reagent responses for all fractions were less than zero.

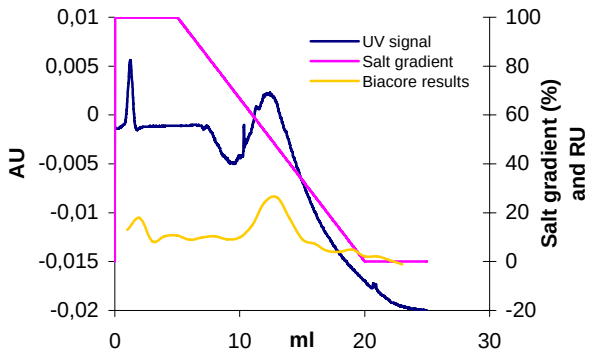

Figure 14 Overlay plots of the spectrogram and the salt gradient from HIC, and the results from the Biacore run of the fractions.

3.3 N-PDP-PE

3.3.1 Conjugation

Several conjugations were carried out. A summary of molar equivalents, thiolation degrees and modification degrees (where existing) can be found in Table 4. Thiolation and modification degrees have been calculated according to equations 1 through 4 above. The amounts of N-PDP-PE were lowered after the successive analyses of the first conjugates to prevent non-conjugated N-PDP-PE from interfering with the antibodies. To get a lower thiolation degree, the amount of Traut's reagent was reduced by a factor two in the two last thiolation reactions. This was based on subsequent gel filtration results, as a means of preventing the conjugates from aggregating.

The thiolation succeeded in all cases (with a small reservation for conjugation number 4 in Table 4). The modification degree for conjugation 4, 5 and 7 (Table 4) show that in these cases literally all, or as

much as possible concerning the grade of thiolation, of the lipids have been used in the coupling reactions.

Table 4 Summary of performed N-PDP-PE conjugations. *This value is not reliable since the measurements were performed the day after the thiolation, thus the antibodies probably have conjugated to each other to a certain amount. **Here, half the molar equivalent of Traut's reagent was used.

	Molar equivalents of N-PDP-PE	Thiolation degree of antibody	Modification degree of antibody
1.	10 30 100	2.0	–
2.	100	1.3	–
3.	1 5 10	1.3 1.4 1.9	–
4.	2	0.98*	2.0
5.	2	2.8	2.0
6.	2	1.5**	–
7.	2	1.3**	1.3

3.3.2 Purification

At first, HIC was used to purify the antibody conjugates (conjugation number 1, Table 4). The results can be seen in Figure 15. Analyses in the Biacore instrument of the fractions showed that only the reference sample gave a noticeable signal at and around fraction 13 (Figure 15). Injection of unpurified samples resulted in baseline increases of 1000 RU or more, increases that were not regenerable.

For the remaining conjugations, gel filtration was used for purification. The first gel filtration was performed on samples from conjugation 2 (Table 4). In the FPLC-chromatogram, double peaks could bee seen (Figure 16). The reference sample, containing no N-PDP-PE, gave only one peak, indicating in what fraction the non-conjugated antibodies should be found. Analyses in the Biacore instrument confirmed that fractions 13 contained antibodies alone, and also showed that fractions 9 of the samples bound to the myoglobin surface, indicating where to find the conjugated antibodies.

The double peaks in the chromatogram might be caused by the large amounts of non-conjugated lipids. Therefore, conjugations with lower lipid levels were examined.

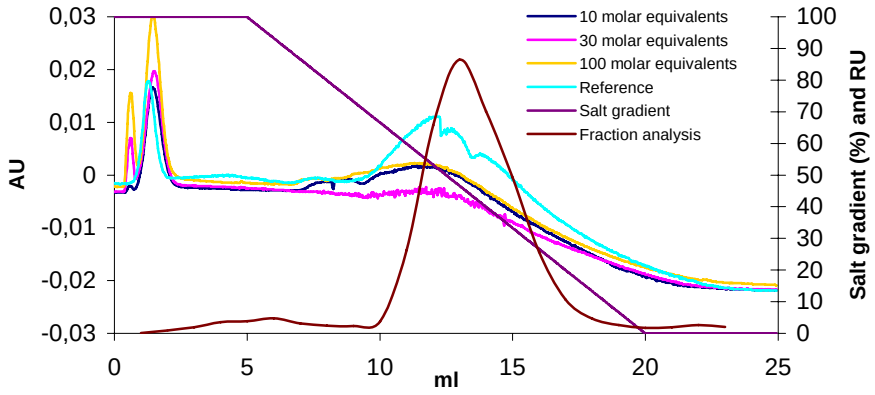

Figure 15 Results from the HIC of the first N-PDP-PE coupling reaction, as well as the corresponding fraction analysis of the reference sample.

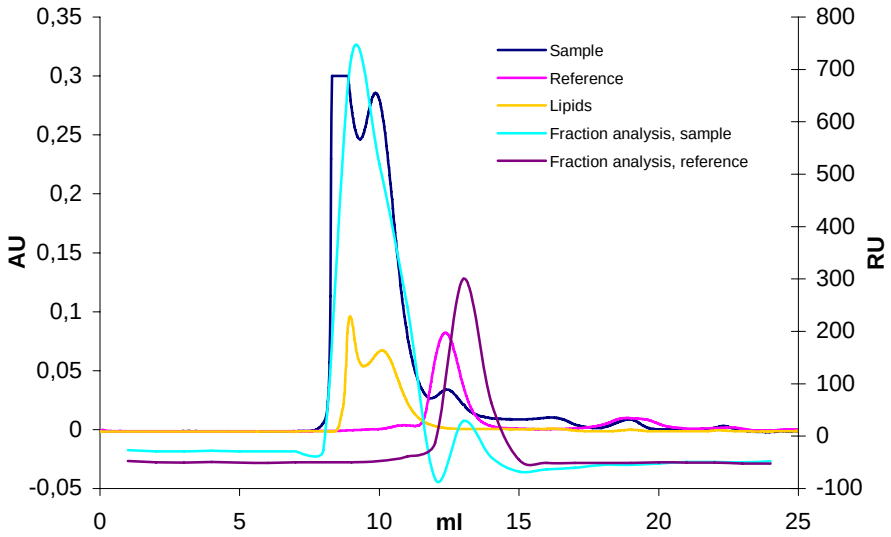

Figure 16 Results from the gel filtration of conjugation with 100 times molar equivalents of N-PDP-PE. Note that the sample chromatogram was recorded on another scale than the reference and lipid. The figure also displays the results from the fraction analyses of sample and reference. Also note that the fraction analysis curves are slightly displaced compared to the chromatograms.

When the level of N-PDP-PE was decreased (Figure 17), to prevent the lipids from aggregating with the conjugated antibodies, and after addition of extra P20 in the gel filtration buffer, more uniform results were obtained. The less N-PDP-PE, the more distinct the peak with conjugated antibody. The fraction analyses showed that antibodies are found in fractions 11 and 13. Only antibodies in fraction 11 showed binding to the OSR reagent in the same analyses. Testing lipids alone also resulted in a distinct peak (Figure 17). Best results were obtained with an amount of N-

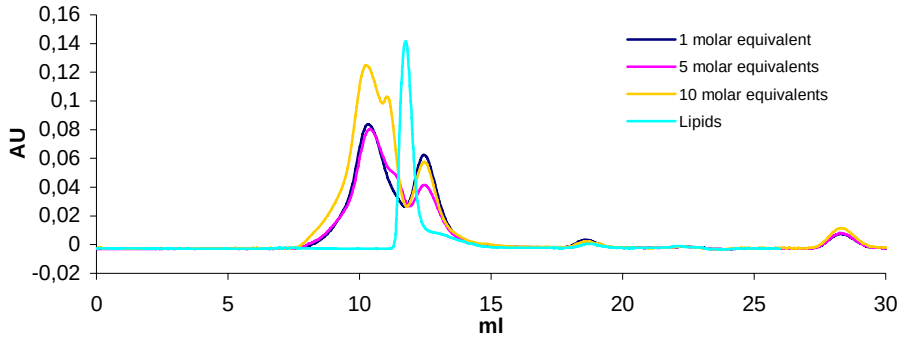

Figure 17 Chromatogram results from purification of conjugated antibodies with different molar equivalents of N-PDP-PE and of lipids alone.

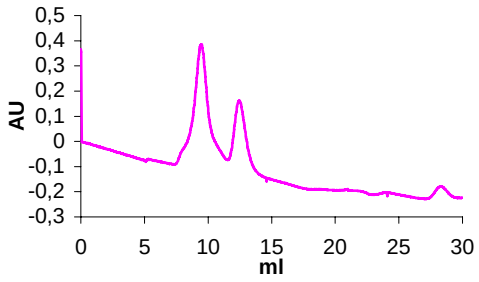

Figure 18 Chromatogram from gel filtration of antibody conjugated with two molar equivalents of N-PDP-PE. The two peaks, corresponding to conjugated and non-conjugated antibody, are well separated and distinct.

PDP-PE corresponding to two moles of N-PDP-PE per mole of antibody, where the two peaks, corresponding to non-conjugated as well as conjugated antibodies, were distinct and well separated (Figure 18).

After gel filtration of the antibodies with the concentration 5 µg ml^{-1}, some of the fractions identified as containing conjugated antibodies, were turbid. Since this is an indication of aggregation, the thiolation level was lowered (see above) to give a smaller modification degree. This resulted in clear solutions with good separations in the gel filtration.

3.4 Sandwich assay

No additional sandwich assays were performed on the NHS-palmitate coupled antibodies. The N-PDP-PE coupled antibodies were examined in several Biacore assays though.

It seemed like a twofold dilution of the antibody solution in HBS-EP worked best, independent of the antibody concentration. When not diluted at all, higher responses were sometimes obtained, but these results also varied to a greater extent than the results from the diluted antibody solutions. More diluted samples gave too low concentrations of antibody,

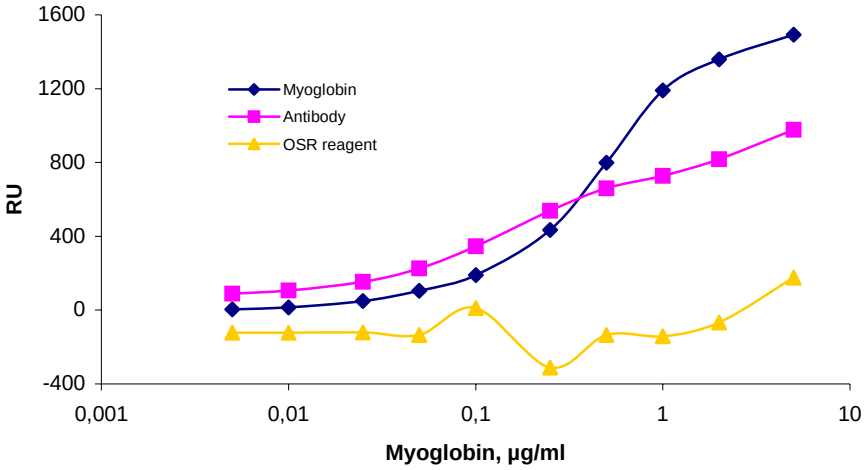

Figure 19 Average values from a sandwich assay with varying concentrations of myoglobin. The myoglobin response is correlated to its concentration. So is the response for the conjugated antibody when the myoglobin concentration is less than 0.25 µg ml^{-1}. The OSR response is not correlated to the antibody responses.

thus the antibody response was not linearly correlated to the myoglobin concentration for higher concentrations of the latter.

The fractions containing the conjugated antibodies were tested in assays with varying concentrations of myoglobin. Myoglobin bound to the immobilised 2F9.1 as expected and lipid coupled poly-α-myo bound to the myoglobin. The antibody binding was correlated to the myoglobin levels, but showed somewhat lower levels than expected. After an increase in injection time, the levels reached higher values. However, the OSR reagent did not show any dose dependent response (Figure 19). In several sandwich assays, only the very first OSR injection gave a response.

After introduction of several wash commands in the methods, the OSR reagent still did not show a response correlated to the level of myoglobin/poly-α-myo.

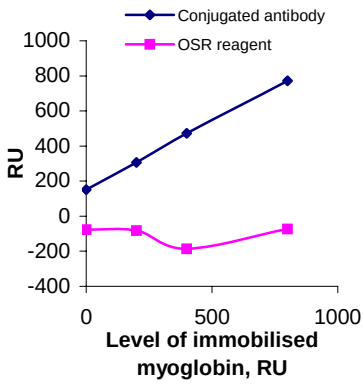

Figure 20 Test of conjugated antibody and OSR reagent on myoglobin surfaces.

When the conjugated antibody was tested on a chip immobilised with different myoglobin levels, the results showed that the antibody bound to myoglobin giving responses correlated to the myoglobin level, but the

OSR reagent did not give the expected response (Figure 20). Only the first cycle, that is, the first injections in each flow cell, gave good antibody responses. This might be related to the regeneration problems.

When the OSR reagent was injected over immobilised N-PDP-PE conjugated antibodies, immobilised to different levels, a clear relation between antibody levels and OSR response could be seen (Figure 21). Note also the fact that if the running buffer contains detergent or not, is of minor importance to the response of the OSR reagent.

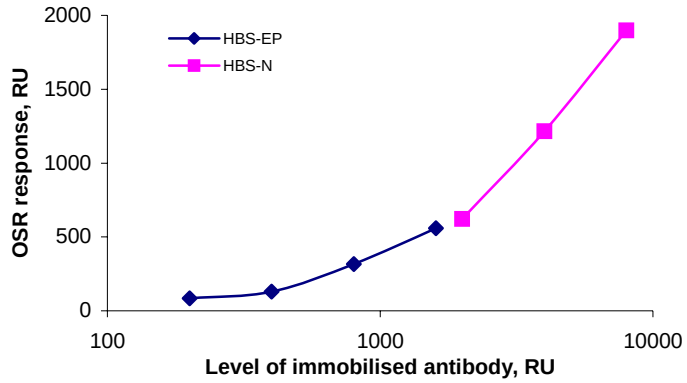

Figure 21 Correlation between level of immobilised antibody and OSR response. Note that two different flow buffers have been used.

Thus, the sandwich assay works as intended concerning the antibodies and myoglobin. When the OSR reagent is applied on top of the sandwich, the expected response increase is not displayed. If the monoclonal antibody is removed from the sandwich and myoglobin is immobilised on the surface, the OSR reagent still does not improve the signal. However, when the lipid-coupled antibody is immobilised directly onto a surface, the OSR reagent gives responses proportional to the levels of conjugated antibody.

3.5 Additional results

Regeneration proved to be a critical moment in the sandwich assays. The regeneration tests showed that Glycine 2.0, previously shown to be a good regeneration solution for the sandwich assay with the unmodified antibody, no longer gave desired results after modification of the antibody. 4 M $MgCl_2$, 0.1% SDS and Glycine 2.5 were tested and used, with varying results, but the perfect regeneration solution remains to be found.

Injections of liposomes did not increase the signal when applied to the chips with immobilised N-PDP-PE conjugated antibodies. Comparisons between the liposome and OSR responses can be found in Figure 22. On an L1 surface the liposomes gave responses around 5000 RU, i.e. in the same range as the OSR reagent.

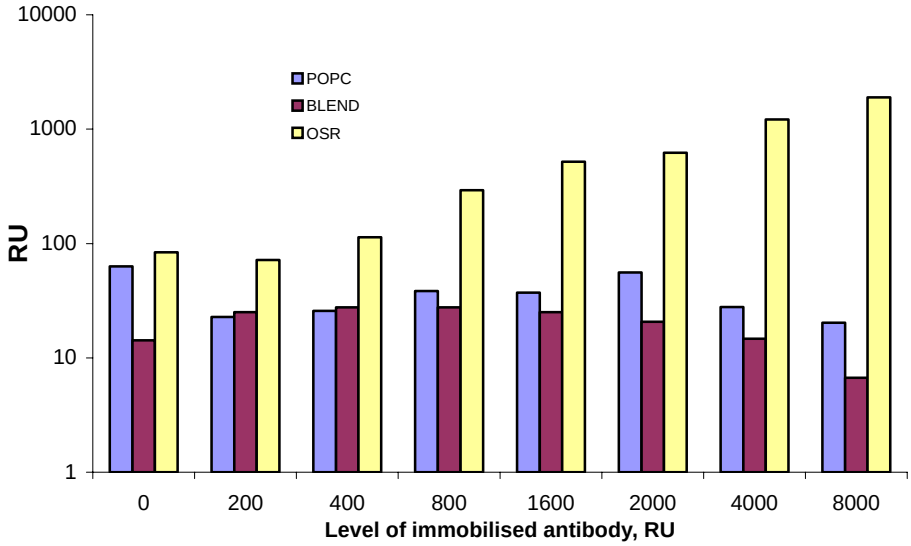

Figure 22 Responses of the two different liposomes, POPC and BLEND, and the OSR reagent when injected over surfaces with conjugated antibody. Note the logarithmic scale on the y-axis.

4 Discussion

Since the conjugation using NHS-palmitate did not turn out to give the desired results, this method was abandoned in this work. The first setback was the difficulties concerning the solubility. It is possible that this moment could be overcome by synthesis of the NHS-palmitate immediately prior to use, instead of using pre-prepared material.

Another difficulty seemed to be the purification. The Biacore responses from the mixtures with conjugated antibody were higher for the antibody containing fractions with a lower molar surplus of NHS-palmitate than the fractions with higher molar surplus. Material in these fractions bound to myoglobin but did not interact with the OSR reagent. This indicates that these peaks contains unmodified antibody, thus material is lost somewhere between the coupling reaction and the end of the purification. If the coupling actually succeeds, even if it is to a limited extent, the difference in responses might reflect the coupling degree. Antibodies with a lipid tail might either aggregate and therefore get caught in the filter used prior to purification, or they might get caught in the HIC column, binding the column matrix too hard to be eluted using the ordinary methods. If this is the case, gel filtration could be a good alternative also for the NHS-palmitate coupled antibodies.

When it comes to the N-PDP-PE coupling the situation is quite different. Here the coupling and purification seem to work well, after changing purification strategy to gel filtration. The HIC purification performed on

the N-PDP-PE coupled antibody further confirmed the theories above. After HIC, the only detectable signals in the Biacore run were from the reference sample as well as from the injections of unpurified samples. The coupled samples gave no responses. This indicates that the coupling reaction might have gone to completion, and that the lipid-coupled antibody was lost in the purification step.

The actual problems with this coupling are of another character. The regeneration proved to be a hard task to deal with. It seemed clear that the modified antibody differed from the unmodified antibody. Trying different regeneration solutions did not improve the results.

This was however not the greatest challenge concerning this part of the work. The individual parts of the sandwich assay seem to work well. The modified antibody binds to myoglobin, and the OSR reagent bind to the modified antibody – as long as these bindings are separated from each other. When the complete sandwich assay is investigated several problems arises. The OSR reagent is not binding to the modified antibody when this itself is bound to myoglobin. There can be many reasons for this; one is that the lipids on the antibodies might aggregate to each other on the surface, protecting themselves from the hydrophilic environment. This could block the lipids from exposure to the OSR reagent, and thereby preventing an interaction between the modified antibody and the OSR reagent. The observed binding when the antibody is immobilised directly onto the surface can be a result of the antibody being either sterically hindered by the binding, or sufficiently far away from each other, not to aggregate. If this is true, the problem is actually a problem concerning the buffers used in the assay system.

The liposomes used in this work did not show any specific binding to the conjugated antibody. Other liposomes might be useful though, as well as other mixtures of micelles. Since liposomes are rather large, they would greatly enhance the signal – if they bind. Further research in this area might be successful.

To proceed with the signal enhancing methods using lipid-conjugated antibody, the assay might have to be modified. Other combinations of antibodies and antigens, or a different model system might work better. The enhancement reagent might have to be improved or changed, as well as the regeneration conditions, to get a stable system that can be used over a longer period of time. The use of detergents in the flow buffers of the assay system needs to be investigated as well. How do the detergents affect the different parts of the system?

This work shows that it is possible to couple lipids to antibodies, purify the conjugates and get an enhancement of the signal from these conjugates using mixed micelles. This work also shows the difficulties associated with hydrophobic materials and the problems that can arise in a model system. However, the results indicate that a signal enhancing method might be at hand, ready to be discovered. Only small modifications might be needed to get a working system.

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