SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

PREPARATION OF DYE-ANTIBODY CONJUGATES FOR STUDYING THE PLASMA MEMBRANE DISTRIBUTION OF CD1d PROTEINS IN B CELLS

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INTRODUCTION

Antibody conjugation strategies

Antibodies are the functional manifestation of the adaptive immune system against threats that endanger the homeostasis of our defense mechanism. They were the first elements to be identified in the immune system due to its curative powers. Theoretically, any molecule can induce the generation of antibodies. Owing to the target specificity conferred by antibodies, that can discriminate even the smallest of differences in the epitopes of an antigen, it is now widely used as a probe in biological, analytical and therapeutic research domains. However, the use of antibodies underwent a quantum increase only with the discovery of bioconjugation methodologies. The purpose of bioconjugation is to combine the best features of molecules participating in splicing event. Thus, they make them functionally more potent, stable or multimodal. Although several molecules, practically everything with appropriate functional groups, can be conjugated to antibodies, fluorophores are used as an example in the first part of this thesis to explain the chemical reactions that establish the covalent linkage between reactants and an antibody. Like all proteins, antibodies are a polymer of amino acids which give rise to polypeptide chains. Each amino acids has its own identifiable side-chain groups rendering particular chemical structure, charge, hydrogen bonding capability, polarity, and reactivity. These side-chain groups do not participate in peptide bond formation thus provide the functional groups available for modifications in proteins. Hence, the structural features of antibody provide a number of choices in functional groups for modifications and conjugation schemes.

Structural features of an antibody

Most mammalian antibodies have molecular weight (MW) ~ 150 kilodaltons (kDa) and are made up of four polypeptide chains -two identical heavy chains (H) and two identical light chains (L). The polypeptide chains are linked together by covalent and non-covalent forces. Both intra-chain disulfide bonds within each of the polypeptide chains and inter-chain disulfide bonds between ‘H’ and ‘L’ chains and ‘H’ and ‘H’ chains are important for maintaining the shape of an antibody. The pairing of ‘H’ and ‘L’ chains gives rise to two identical antigen binding sites (ABS); thus, a typical antibody is divalent in nature demonstrating “Y” conformation. Fab fragments are prepared by digestion of an antibody with papain enzyme which cuts an Immunoglobulin (Ig) molecule in the hinge region just before the H-H inter-chain disulfide bonds. An antibody produces two identical Fab fragments that contain the whole ‘L’ chain and a part of ‘H’ chain, thus, is still capable of

binding antigen. The remainder of the fragments after papain digestion of an antibody, excluding Fab, is called as Fc region. Both the class and sub-classes of Ig molecules are determined based on their 'H' chain identity. The larger 'H' chain is variable and structurally distinct for each class with a MW in the range of 50000-77000 Da, whereas, the smaller light chain is very similar in all classes and has a MW of 25000 Da. In humans, Igs are grouped into five major classes, also called isotypes: IgG, γ 'H'chain, IgA, α 'H'chain, IgM, μ 'H'chain, IgD, δ 'H'chain and IgE, ε 'H'chain.

Principle side-chain functional groups for coupling in an antibody

Any coupling events should not affect the characteristic features of the coupled proteins. Therefore, careful selection of a functional group is critical to circumvent any unwanted alteration. Depending on the abundance, distribution -whether it is on the surface or inner core of protein- and ionization potential of side-chain functional groups at near neutral pH of amino acids, there are three functional groups in any proteins, including antibodies, which are the primary sites for modifications and covalent linkage: amines ($-NH_2$), thiol groups (-SH) and carbohydrate residues (hereon, it would be denoted as -CHO since aldehyde is formed after oxidation of sugars).

Amines ($-NH_2$)

Owing to the abundance of lysine residues in antibodies and existence of various methods to selectively modify primary amines, the favorite reactive group has always been amino groups in lysine species. Furthermore, lysines are usually on the outside surface of a native protein at physiological pH due to its positive charge. Lysine contains both α-amine and ε-amine which differ in pKa values thus offering selective modifications. Several classes of reactive groups having a specific propensity for amine residues have been described, however, N-hydroxysuccinimidyl (NHS) esters are the ones which are the preferred choice for targeting $-NH_2$ moieties. NHS esters form stable amide bonds with aliphatic amines of protein and achieve conjugation at pH 7.5-8.5. Though, these reactive moieties ensure easy conjugation, it leads to coupling of proteins at unspecified regions.

Thiols (-SH)

The thiolate ion is the most reactive functional group in proteins. However, cysteine is the only sulfur-containing amino acid therefore it is less prevalent in proteins. Cysteine in its natural form is often present in its oxidized form cystine, a dimer of cysteines joined by a

disulfide bridge (-S-S-) thus is the least exposed amino acid on the surface of proteins. In antibody, cysteine residues are responsible for the formation of disulfide bonds between the polypeptide chains. However, only free or reduced -SH groups can react with thiol-reactive fluorophores, therefore to access the -SH groups an antibody must be cleaved with reducing agents. Since disulfide bonds are only present at specific sites of antibody, -SH targeted method provides greater certainty about the preservation of ABS and the uniformity in the density of labels in antibody after conjugation. Among the categories of sulfhydryl-reactive chemical groups, maleimides are preferentially used for its reactivity toward -SH groups in the pH range of 6.5-7.5. In addition, at pH 7.0, the reaction of maleimides to sulfhydryls is about 1000 times greater than its reactions with amines, allowing specificity in modifications by controlling the reaction with the pH of a conjugation buffer.

Carbohydrates (-CHO)

IgGs are glycoproteins and they display heterogeneous groups of terminal sugars. IgG molecules have a highly conserved N-linked glycosylation site within the C_H2 domain at Asn297 of the Fc region; however, carbohydrates might also be present in other locations. The sugar constituents of glycoproteins contain vicinal hydroxyl groups that are prone to easy oxidation by treatment with periodic acids or its salts. Once oxidized to dialdehydes, these sites can be specifically targeted with amine containing reagents, including hydrazides and the ε-amino group of lysines of another protein. The reaction rate can also be enhanced by using a catalyst like aniline.

Applications of dye-antibody conjugates

As a reagent, no other material has contributed to this degree of success directly or indirectly to such a vast array of scientific discoveries than antibodies. In consideration of the level of complexity presented by the millions of molecules working together in an integrated system like in a cell or a more sophisticated organism, it is indispensable to have an approach that can help in understanding the spatio-temporal interplay of these molecules. The combination of fluorescence and antibody aptly serves this purpose. Fluorescence methods are very versatile, can be applied to any system, and are easy to score at unimaginable efficiency. Therefore, as an application of antibody conjugates, we have used dye conjugated fluorescent antibodies in determining the two dimensional topological distribution of Cluster of Differentiation 1d (CD1d) on the plasma membrane of B lymphocyte. The main reasons for choosing this protein was the paucity of data regarding the distribution features of CD1d in the plasma membrane, our interest in major histocompatibility complex (MHC) membrane biology and the importance of CD1d expression in B lymphoid lineages.

B cells

B cells are professional antigen presenting cells (APCs). They are the most focused group among APCs. APCs, like B cells, decorate their cell surface with antigen-carrier molecules, MHC I, MHC II and CD1d, which is recognized by T cell receptors (TCRs) in T cells.

Lipid-based antigen presentation

For a long time, only peptides were considered antigenic and effective in initiating humoral immune responses, however, in the last two decades, the roles of lipids in immunity are slowly gaining prominence. Primarily, MHC proteins perform an antigen presentation. Another set of proteins termed CD1 was also later discovered to function as antigen presenters to T cells. However, MHC proteins use peptides as antigens whereas CD1 proteins use lipids as antigens.

CD1 proteins

CD1 was the first human differentiation antigens to be identified by Mab (NA1/34; CD1a) on human thymocytes. In humans, CD1 proteins are sub-grouped into Group I (CD1a, CD1b and CD1c), Group 2 (CD1d) and Group 3 (CD1e). The classification is based on the degree of amino acid and genomic sequence homology and immunological functions; however, it also reflects their variation in tissue distribution. Unlike humans, mice and rat express only

CD1d proteins. CD1 proteins are also structurally similar to MHC I heavy chain (MHC I-HC) and consist of three extracellular domains (α1, α2 and α3), a transmembrane domain and a short cytoplasmic tail necessary for receptor-mediated endocytosis (except for CD1a which lacks any internalization motif). Requirement of chaperones, calnexin, calreticulin, ERp57, etc., for maturation have also been synonymously described for these proteins. Besides, β_2m is found to be very important in the surface expression of CD1 -a, -b, -c and -d proteins. However, β_2m independent isoforms, which is not bound to β_2m, have also been described for CD1d proteins, though immunological functions of these β_2m free CD1d heavy chains are not well understood. Interestingly, the biological behavior of CD1 is similar to that of MHC II instead of MHC I-HC proteins. Therefore, CD1 possesses an admixture of features which have led to the hypothesis that these molecules have diverged from a common ancestral gene. Distribution of MHC I and MHC II proteins in the plasma membrane of APCs and the functional implications of such supramolecular organizations have been explored in great length, however, very limited information is found regarding the topological arrangement of CD1 proteins on the cell surface. Earlier studies primarily demonstrated the trafficking behavior of CD1 from the plasma membrane to the intracellular endosomal membrane compartment and vice versa. B cells play a central role in humoral immunity and is essential for maintaining peripheral blood invariant natural killer T (iNKT) cells, a CD1d restricted T cell subset. iNKT cells are considered to play significant roles in tumors, infectious disease, autoimmune disease and humoral responses. Therefore, we sought to explore the topological features of CD1d in B cells.

Biochemically identified proteins that can interact physically with CD1d proteins

Plasma membrane functions are controlled by the lateral homotypic and heterotypic cis-interactions between proteins and lipids over different scales of time and space, therefore, it is important to determine these dynamic yet structured assemblies of proteins and lipids. Based on biochemical methods, several proteins have been found to interact physically with CD1 proteins and such associations have also been shown to influence biological functions. The identified proteins demonstrating CD1 association are listed in Table 1. Similarly, studies -mainly in murine cell lines- also indicated that CD1d is localized in lipid-based dynamic nano-/micro- assemblies termed rafts in the plasma membrane. In the case of CD1a and CD1d, lipid-raft restricted localization was found to be critical in efficient signal transduction to the target T cells especially at low ligand densities. Though, similar kinetics of

lipid loading as in mice have been observed for human CD1d molecules, systematic investigation of its plasma membrane features has not been done yet.

Table 1

CD1 protein	Associated Protein	Cell Types	Related Biological Functions
CD1-a,-b, -c, and -d	β_2m	APCs	Functional maturation and cell surface expression of CD1d
CD1d	MHC II	B cell	MHC II controls intracellular trafficking of CD1d
CD1d	Invariant chain	B cell	Intracellular trafficking of CD1d
CD1d	gp180	Epithelial cells	Activation of CD8+ T cells / Mucosal tolerance
CD1d	ILT4	Epithelial cells	Inhibits NKT cell activation due to CD1d
CD1d	Prolyl-4-hydroxylase	Epithelial cells	Post-translational modification of CD1d
CD1a	CD9	Monocytes	Undefined
CD1a	Invariant Chain	Monocytes	Endocytosis of CD1a proteins from plasma membrane
CD1a	CD1b and CD1c	Normal thymocytes	Undefined / probably controls the T cell activation function of each other
CD1a	MHC I-HC	Normal thymocytes	Undefined / probably controls the T cell activation function of each other
CD1a	CD8	Normal thymocytes	Undefined

Statin and antigen presentation

Statins are a class of drugs that can lower cellular cholesterol by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the cholesterol biosynthesis pathway. Notably, statin is also shown to have pleiotropic effects which are independent of its cholesterol reduction feature. It is also found to decrease both MHC II and CD1d antigen presentation by hindering isoprenylation in B cells.

Fluorescence Resonance Energy Transfer (FRET)

FRET is a photophysical process where energy is transferred by an excited donor molecule to a neighboring acceptor molecule in a non-radiative fashion. Such process of radiationless transfer of energy occurs when the molecular states of donor and acceptor are in resonance and the distance separating these molecules are only a few nanometers, typically from 1 nm and up to 10 nm. The rate of energy transfer efficiency is inversely proportional to the sixth power of the distance separating the donor and acceptor molecules. The energy transfer efficiency thus shows strong distance dependence between the donor and acceptor (FRET pair) and therefore, it is also termed "spectroscopic ruler".

The energy transfer efficiency is illustrated by the following equation:

$$E_{FRET} = \frac{R_0^6}{R_0^6 + R^6}$$

where R_0, Förster radius or critical distance, is the characteristic distance at which FRET efficiency is 50%. FRET efficiency is closer to maximum at distances less than R_0, and minimum for distances greater than R_0.

AIMS OF THE THESIS

Our primary aim of the thesis was to prepare dye-antibody conjugates suitable for characterizing the organization and distribution of CD1d proteins on the surface of B cells.

To summarize, we had the following set of goals:

Dye-antibody conjugation strategies

We performed dye-antibody conjugations using three different strategies which are common in most laboratories these days. We also compared the efficiency of antigen recognition by the obtained antibody conjugates. The goal was to provide an informative guidance for choosing and optimizing critical conditions necessary for dye-protein conjugation.

1. To optimize dye conjugation strategies targeting amine, sulfhydryl and carbohydrate functional groups in antibodies.
2. To lay out guidelines in performing site-specific antibody conjugations.
3. To determine the efficiency, yield of conjugation, the ease in performing the dye-antibody conjugations and financial cost involved in each procedure.
4. To illustrate the advantages and disadvantages related to each of the dye-antibody conjugation methodologies.

Membrane distribution of CD1d proteins

Though considered as an important element of the innate and adaptive immunity, plasma membrane topological features of CD1d proteins at quiescent state have not been described yet, especially in human cells. Therefore, in this study we have attempted to unravel the cell surface features of CD1d in human B cells.

1. General features of CD1d distribution in the plasma membrane of B lymphocytes.
2. Relationship between CD1d and MHC proteins in the plasma membrane.
3. Association of CD1d with GM_1 gangliosides in the plasma membrane.
4. To characterize CD1d enriched regions in the plasma membrane of B cells.

MATERIALS AND METHODS

Antibodies and dyes

Various functional derivatives –succinimidyl ester, maleimide or hydrazide – of Alexa -546, -555 or -647 were purchased from Life Technologies/Invitrogen (Budapest, Hungary). The monoclonal antibodies (Mabs) used in this thesis were W6/32 (recognizes a complex of MHC I-HC and β_2m), HC-10 (recognizes free MHC I-HC), L368 (anti-β_2m), L243 (anti-MHC-DR), P2A4 (anti-intercellular adhesion molecule 1(ICAM1), IgG1), OKT9 (anti-transferrin receptor (TfR)), 51.1.3 (anti- CD1d) and 27.1.9 (anti-CD1d). Antibodies were purified from supernatants of the hybridoma cell lines by sepharose A affinity chromatography.

Chemicals

All reagents including $NaIO_4$, sodium azide, ethylenediaminetetraacetic acid (EDTA), MEA, DTT and N-ethylmaleimide (NEM) dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Kft (Budapest, Hungary) unless otherwise stated.

Dye-antibody conjugation protocol

Succinimidyl ester-amine reaction to form carboxamide Bond

Conjugations at amine regions of antibody were performed by preparing a mixture of NHS ester dyes and antibodies. The mixture was incubated at room temperature with gentle mixing in a rotator. pH of the mixture was ~9.1. After incubation, the free dyes were separated by gel filtration using sephadex G25 columns. Various fluorophore to protein (F/P) variants were obtained by changing the molar concentration of dye to antibody.

Maleimide-thiol group reaction to give thioether bond

Reduction of antibodies was firstly carried out to generate thiol groups with MEA in 5 mM EDTA containing phosphate buffer (pH 7) for 90 min at 37°C. After the treatment, the solution was thoroughly washed. Thereafter, the mixture of maleimide dyes and MEA free antibody solution was incubated for 1 hr at room temperature. After incubation, separation of the free dyes was performed by gel filtration. Importantly, inert atmosphere is maintained throughout the procedure using N_2 gas.

Hydrazide-aldehyde reaction to form hydrazone bond

Aldehydes were created in antibodies by oxidation with $NaIO_4$ for 3 h at 37 °C in a phosphate buffer (pH 5.6). To remove the excess $NaIO_4$, oxidized antibodies were passed through sephadex G25 gel filtration column followed by washing using 50 kDa MWCO amicon concentrator tubes. Thereafter, hydrazide dyes were mixed with antibodies (0.5 mg/ml) at the ratio of 20:1 along with aniline (100 mM). Incubation was performed overnight at room temperature with gentle rotation. Lastly, separation of free dyes from the dye-antibody conjugates was done as described above.

Fluorophore per protein (F/P) calculation

F/P ratios of dye-antibody conjugates were calculated from the absorbance taken at 280 nm and at the maximum absorption wavelength of the respective dyes using spectrophotometry (Nanodrop, Wilmington, USA).

The following formula was used to calculate the F/P:

$$\text{Concentration of antibody (mg/ml)} = \frac{A_{280} - (A_{dye} \times CF)}{1.4} \text{ mg/ml}$$

$$\text{Concentration of antibody (Molar)} = \frac{\text{Concentration of antibody (mg/ml)}}{\text{Molecular weight of antibody}}$$

$$\text{Concentration of dye (Molar)} = \frac{A_{dye}}{\varepsilon}$$

$$\text{F/P} = \frac{\text{Concentration of the dye (Molar)}}{\text{Concentration of antibody (Molar)}}$$

(A_{dye} – Absorbance of Alexa dye at a maximum absorption wavelength; Correction factor (CF) for Alexa dyes at 280 nm; A_{280} – Absorbance of protein at 280 nm, ε – Molar extinction coefficient of Alexa dyes at maximum absorption wave length; MW of antibody – 150 kDa (112.5 kDa as MW was used for sulfhydryl labeling).

Cell line

A CD1d expressing stable cell line, C1R-CD1d, a generous gift from Mark A. Exley, Harvard Medical School, Boston, USA, was used in these studies. For our purpose, we sorted these cells using 27.1.9 CD1d antibody and pan anti-mouse secondary antibody conjugated magnetic dynabeads (Life Technologies/Invitrogen, Budapest, Hungary). The proteins of interest for us have been found to be expressed at good levels in the plasma membrane of this cell line: MHC I, β_2m, MHC II, ICAM1, and CD1d. C1R-CD1d cells were sub-cultured every second day in 10% newborn calf serum (NCS) RPMI media containing 300 μg/ml G418 unless stated otherwise.

Cell labeling

Cells ($1x10^6$) were labeled in 50 µl volume of PBS buffer (pH 7.4) containing 1 mg/ml BSA and 0.01% sodium azide. Dye-conjugated/or unconjugated antibodies were added to these cells at specific concentrations depending on the requirement (for FRET experiments predetermined saturating concentration of the dye-antibody conjugates were used). Following antibody addition, the mixture was incubated in a dark environment for 30 min on ice. Subsequently, samples were washed twice with ice-cold PBS buffer in order to remove unbound antibodies. If unconjugated primary antibody was used, then a secondary $F(ab')_2$ conjugated to Alexa Fluor 647 dyes 10 µg/ml) was added to the cells and was reincubated on ice and dark for another 20 min. Next, the samples were washed and the cell pellets were suspended in 1% formaldehyde solution. It was stored at $4^{\circ}C$ until measurements were performed.

Flow cytometry and data analysis

Depending on the type of studies, fluorescence intensities were measured using FACScan or FACSCalibur or FACSArray flow cytometers (Becton Dickinson, Franklin Lakes, NJ). The instruments are equipped with various combinations of lasers: FACScan (Blue, 488 nm), FACSCalibur (Blue, 488 nm and Red, 635 nm) and FACSArray (Green, 532 nm and Red, 633 nm). FACSArray was used for the study involving the analysis of dye-antibody conjugation methods. A functional derivative of Alexa 647 dye was specifically used for this purpose to minimize the contribution of autofluorescence. For the topological study of CD1d proteins, all three FACS instruments were used. Flow-cytometric FRET experiments (FCET) were performed in FACSArray flow cytometer. Cells were labeled with Alexa 546/ Alexa 555 and/or -647 dye conjugated probes/antibodies. Fluorescence signals were collected using 575+/-25 BP filter for donor, 650 LP for transfer and 650+/-10 BP for acceptor molecules. The FCS data files were evaluated using ReFleX software.

Antibody saturation curve determination

The concentration dependence of fluorescence intensities was used to determine the saturation curves for each antibody. C1R-CD1d cells were labeled with various concentrations of antibodies (conjugated/ non-conjugated) as described above. The data were analyzed using ReFleX and the graphs of fluorescence intensity versus concentration of antibody (conjugated/non-conjugated) were created using Sigma Plot Version 10. A single

ligand-binding module was used to fit the data thus generating equilibrium dissociation constant (Kd).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For SDS-PAGE, a mixture was prepared from 10 µg of sample and 5X sample buffer (0.25 M Tris–HCl, pH 6.8, 8% SDS, 40% glycerol, and 0.01% bromophenol blue). When desired, either 100 mM DTT or 25 mM NEM was also included. For loading, (3-5 µg) of each sample was used in each well of an 8% poly-acrylamide gel. The gel was then run accordingly.

Quantitation of membrane proteins

QIFIKIT (Dako Cytomation, Denmark) was used for quantifying the membrane proteins following the manufacturer's instructions.

Confocal microscopy

Microscopic measurements were performed on a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany) with a Plan-Apochromat 40 X (NA= 1.2) water immersion objective. Horizontal optical slice (512 × 512-pixels) of 1.5 µm thickness, four-times averaged, was recorded in line mode from the top of a cell for co-localization experiments.

Co-localization study of the membrane proteins

Cross-correlation coefficient (C) from the image pairs were determined using a custom written program in LABVIEW platform using the formula described below:

$$C = \frac{\sum_i \sum_j x_{i,j} - x\rangle\,(y_{i,j} - y\rangle}{\sqrt{\sum_i \sum_j x_{i,j} - x\rangle^{2} \sum_i \sum_j y_{i,j} - y\rangle^{2}}}$$

Where $x_{i,j}$ and $y_{i,j}$ represent fluorescence pixel values at co-ordinates i and j in images x and y. The maximum is '1' for 100% co-localization and '−1' indicates anti-correlation.

CD1d and GM_1 ganglioside association study

Cholera toxin subunit B (CTxB, Invitrogen) and 27.1.9 CD1d Mab which are specific for GM_1 ganglioside and CD1d proteins were used for this purpose. The co-localization analysis was performed as described above. FCET experiments between these two probes were performed on FACSArray instrument.

Flow cytometric detergent resistance (FCDR) test

FACScan was used for FCDR experiments. The samples were prepared similarly as described above except the final step where fixation was avoided in this case. For a baseline recording, fluorescence intensities were measured for ~50 s, thereafter, Triton X-100 (TX100) was added at various concentrations, mixed promptly and the measurements were continued for another five minutes. The FCS files were analyzed using ReFleX software and the calculated data were used for plotting the graph of fluorescence intensity versus time using Sigma Plot Version 10.

Treatment of cells with Methyl-β-Cyclodextrin (MβCD) or Simvastatin

MβCD and simvastatin were purchased from Sigma. Specific instructions from the companies were followed to dissolve the reagents. C1R-CD1d cells (4×10^6 to 10×10^6) were treated with simvastatin -μM concentrations- in 1% NCS RPMI media for 48 h at 37°C. MβCD usage was slightly different because it was a short-term treatment. Briefly, C1R-CD1d cells (5×10^6 to 10×10^6) which were grown in 1% NCS, unless otherwise mentioned, were suspended in 1 ml of 0.1% NCS RPMI media and were incubated with 2 mM MβCD for 40 min or 10 mM MβCD for 15 min at 37°C. The cells were thoroughly washed once the incubation was over in both cases. It was followed by the cell labeling protocol as described above.

Isolation of iNKT cells from peripheral blood mononuclear cells (PBMC)

iNKT cells were isolated from PBMC using anti-iNKT magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Expansion of iNKT cells and co-culture assays with C1R-CD1d cells were carried out according to the protocol described by Exley M. A *et al.* (Isolation and functional use of human NKT cells. Curr Protoc Immunol Chapter 14, 2010).

RESULTS AND DISCUSSION

Analysis of dye-antibody conjugation methods

The purpose of this study was to evaluate and compare dye conjugation methods with respect to effect in binding affinity, financial aspects, the yield of conjugates and ability to produce higher dyes per antibody.

Unconjugated antibody: A reference for antibody conjugates

We selected four antibodies of different isotypes for this study. For comparison purpose, the antigen recognition features of an unconjugated antibody was taken as a reference. The binding affinity of an unconjugated antibody was estimated by indirect labeling method using $F(ab')_2$ fragments. Variable binding affinities were noticed among four Mabs, which could be categorized as : High-affinity antibodies: W6/32, L243 and P2A4, and, Low-affinity antibodies: 51.1.3 CD1d antibody. The (Kd ± S.D.)s were found to be (27.7± 6.7) nM, (31.1±9.1) nM, (32.8±16.8) nM and (174.2±21.8) nM for W6/32, L243, P2A4 and 51.1.3 antibodies respectively. Mabs P2A4 and 51.1.3 also showed a larger variation in Kd value, however, it was not related to Mab isotype dependent Fc receptor binding specificities. Thus, the problems associated with P2A4 and 51.1.3 seem to be an intrinsic feature of these Mabs which might be related to its ABS.

Effect of NHS ester based coupling method on the binding affinity of Mabs

Amino groups are distributed throughout the entire antibody structure. It can also be present in ABS regions. Therefore, we assumed that dye conjugations at amine residues would have negative effects on antibody features. Our results suggested that although dye-coupling seems to decrease the binding affinity of an antibody, this phenomenon is not proportional to F/P ratios. There was no strict correlation between F/Ps and the decrease in binding affinity of an antibody. As expected, higher F/P conjugates were brighter than the lower F/P counterparts, however, there was no linear increase in the fluorescence intensity of the conjugates especially above F/P>3. Interestingly, higher F/Ps showed reduced fluorescence intensities in comparison with their preceding lower F/P variants despite showing intensity saturating effects. We presume such an effect is due to quenching of fluorescence intensity at higher F/Ps. Due to this defined length of arms in antibody (~9 nm in length), most dye conjugation distances are below 10 nm. Therefore, at high F/Ps such a conjugation scheme would offer crowding of dyes in the same antibody. This would lead to complex spatial interactions and

self-quenching processes in the dyes. In addition, dyes will also be positioned closer to aromatic amino acids like tryptophan and tyrosine in the antibody. Aromatic amino acids have been found to quench the fluorescence of organic dyes through a combination of static and dynamic quenching mechanisms. Therefore, the diminished fluorescence intensity at higher F/Ps is due to the increased number of complex photophysical events including homoFRET, photoinduced electron transfer, as well as static and dynamic quenching processes. Besides, a complex structure like cell membrane where membrane proteins exist in cluster also poses its own complexity.

Assessment of dye conjugation after maleimide reduction of an antibody

Disulfide bonds maintain the tertiary structure of antibodies. These bonds are present between "H" and "H" chains and "H" and "L" chains of an antibody. Therefore, appropriate cleaving at the hinge region of an antibody is required to generate functional monovalent halves. Such a fragment would be an ideal replacement for Fabs. Theoretically, labeling of these specific sulfhydryl groups would be of great advantage due to non-interference in the antigen binding activity. In this study, we compared various features of the dye-antibody conjugates prepared by -SH method with that of dye-antibody conjugates prepared from $-NH_2$ method or unconjugated counterparts. We observed a decrease in antigen binding affinity of antibodies after dye conjugation as in the case with amine method. We attribute the decrease in binding affinity to a reduction in the avidity of an antibody instead of the direct effect in binding itself because only functional monovalent halves of the antibody can produce fluorescence intensities. Fluorescence intensities from dye conjugates obtained by -SH method were always lower in comparison with the conjugates generated by $-NH_2$ method at similar F/Ps. With SDS-PAGE experiment, we figured out that it was due to the non-uniform reductive nature of MEA which lead to the generation of a mixture of antibody fragments including non-reduced unconjugated antibodies, inactive fragments of antibodies and functional half antibodies in the mixture. In comparison with $-NH_2$ method, -SH method consists of multiple steps including reduction of antibody, series of washing then co-incubation with dyes. Therefore, it is a cumbersome procedure requiring effective optimization.

Effect on antibody after dye conjugation on carbohydrate residues

IgGs are glycoproteins thus have terminal sugars including sialic acid, galactose, N-acetylglucosamine and fucose residues. Conjugation at sugar units allow site-specific labeling and is considered to have negligible effect on the affinity of an antibody. Such a presumption is made due to the presence of sugar residues at Fc site. Carbohydrates are generally oxidized with the help of oxidizing agents -like sodium periodate ($NaIO_4$)- to aldehydes for coupling purpose, which can then be easily targeted with hydrazide dyes. We performed dye conjugations at carbohydrate residues and compared the obtained conjugates with the unlabeled antibody, -NH_2 conjugates and -SH conjugates. We found that the binding affinity of an antibody dependent on the oxidizing agent, $NaIO_4$. Interestingly, different antibody isotypes and antibody with different binding affinities responded differently to $NaIO_4$. Inclusion of aniline, a catalyst that facilitated dye-antibody conjugation, was critical for dye conjugation. Concentration of $NaIO_4$ (10mM or 2mM) and temperature of oxidation also determined the effects on binding affinity and consequently the F/Ps. Higher temperature -increase from ~20 $^{\circ}$C to 37 $^{\circ}$C- favored efficient oxidation of antibodies and generation of higher F/P conjugates (>1). Similarly, higher concentration of $NaIO_4$ although produced antibody conjugates with higher F/Ps, these conjugates had a lower binding affinity in comparison with the conjugates prepared from lower concentrations of $NaIO_4$. With this procedure, we could not generate F/Ps>3 suggesting either incomplete oxidation or poor glycosylation status of the antibodies. In addition, fluorescence intensities and binding affinities were always lower relative to the conjugates prepared by -NH_2 method, and were comparable or lower relative to the conjugates obtained by -SH method at similar F/Ps. In fact, SDS-PAGE experiment suggested the generation of cross-linked antibodies and smaller fragments of oxidized antibodies. Hence, this procedure seems to either inactivate antibodies or produce lower amounts of functional dye-antibody conjugates. Among the three dye-conjugation methods, -CHO method was the most difficult one requiring rigorous optimization, longer incubation hours, higher financial cost and lower yield of desired products.

Enigma of CD1d protein distribution on the plasma membrane of human B cells

In this study, we used fluorescent dye-antibody conjugates to unravel the topological features of CD1d proteins on the plasma membrane of human B cells employing various biophysical methods - including FRET- based on confocal microscopy and flow cytometer.

Evaluation of expression level of membrane proteins in C1R-CD1d cells

Our objective was to define the relative distribution of CD1d and MHC proteins on the plasma membrane of B cells. For this purpose, we selected C1R-CD1d cell line which has been used for studying the biological functions of CD1d proteins. As a first step, we analyzed the expression of various membrane proteins in this cell line by flow cytometry. Then, we quantitatively determined the number of these proteins on the plasma membrane by using QifiKit. Based on these data, we found that MHC II was the highest in expression, followed by CD1d, β_2m and MHC I-HC proteins in the plasma membrane of these cells. Numerical evaluation of these proteins also confirmed that the expression of β_2m was higher than that of MHC I-HC proteins in the plasma membrane. This might be due to the ability of both MHC I-HC and CD1d to bind β_2m proteins. Surprisingly, we found that β_2m dependent CD1d comprised only a fraction (~15%) of total membrane CD1d proteins based on conformation sensitive antibodies. The functional roles of these β_2m independent CD1d proteins are still unclear. Nonetheless, these isoforms might have distinctive roles in the activation of T cells.

Exogenous expression of CD1d decreases MHC II expression and increases MHC I-HC and β_2m in the plasma membrane

To understand the effect of exogenous expression of CD1d in MHC proteins, we compared the membrane expression of these proteins in C1R cells lacking or expressing CD1d proteins. We found that the expression of CD1d would decrease the expression of MHC II (down by ~31.8±4.6%) and increase the expression of MHC I-HC (up by ~10±2.6%) and β_2m (up by ~46.7±11.5%) proteins in the plasma membrane. Invariant chain is found to be associated with both MHC II and CD1d. Considering the study in immature dendritic cells which suggested a higher degree of association between CD1a and invariant chain in the plasma membrane, it could be speculated that CD1d might also be associated with invariant chain on the cell surface. Since membrane expression of both CD1d and MHC II was found to require invariant chain, competition seems to have occurred between these proteins for shared chaperones. Likewise, a mild increase in MHC I-HC due to exogenous CD1d

expression might be related to its ability to bind CD1d. However, these areas would need further studies taking into account of the spatial and temporal distribution of CD1d, MHC I, MHC II and invariant chain proteins in the cell.

Association of CD1d, MHC and β_2m proteins in the plasma membrane of C1R-CD1d cells

FRET efficiency results from FCET clearly indicated that both MHC proteins, MHC I-HC and MHC II, were physically bound to CD1d proteins in the plasma membrane of C1R-CD1d cells. Interestingly, high FRET efficiency was also noted between CD1d and β_2m, however, quantitative study of membrane proteins indicated that only ~15% of total CD1d was directly bound to β_2m in the plasma membrane. In addition, homoassociation FRET was also observed between CD1d proteins. Thus, high FRET efficiency observed between β_2m and CD1d is due to direct association between CD1d and β_2m as well as the positioning of β_2m closer to CD1d due to binding of CD1d with MHC I. Furthermore, it also suggests the possibility of multimolecular complexes of MHC I-HC, β_2m, MHC II and CD1d proteins in the plasma membrane. Our study thus confirmed the association of CD1d with MHC II in B cells which was demonstrated earlier by co-immunoprecipitation study. It is also reminiscent of the association between CD1a and MHC I-HC observed in normal thymus cells. Considering the association of CD1a with MHC II and MHC I-HC, it can be postulated that all CD1 isoforms might interact with MHC I-HC since other proteins from tetraspanin family also seem to associate with MHC I-HC, CD1 isoforms and MHC II proteins. In line with such possibility, multimolecular complexes of CD1 isoforms (CD1a, CD1b and CD1c) have also been demonstrated in normal thymus cells.

Endogenous clusters of CD1d proteins in the plasma membrane of B cells

Based on homoassociation FRET analysis, we demonstrated for the first time that at resting state CD1d could exist as oligomers on the plasma membrane of B cells. Most probably, the two isoforms of CD1d (β_2m-dependent and β_2m-independent) can also associate with each other. Oligomeric states of CD1d proteins have also been described previously in the plasma membrane of a monocytic cell line, THP1, using atomic force microscopy (AFM). However, THP1 cells were activated with α-galactosylceramide, a ligand which is known to facilitate the entry of CD1d proteins to detergent resistant membrane (DRMs) regions. In the study based on AFM, TCRs were used to identify CD1d proteins. TCRs would only recognize lipid-loaded CD1d proteins. Therefore, the ligand activated THP1 cells must have

documented the modified patterns of CD1d in the cell surface. We used Mab (27.1.9 clone) that can detect both empty or lipid loaded CD1d proteins. Thus, our study demonstrates the unperturbed distribution state of CD1d in the plasma membrane of B cells..

Mild effect of cholesterol depletion in CD1d association of GM_1 ganglioside in C1R-CD1d cells and concomitant iNKT cell activation

Our co-localization study between CD1d and GM_1 gangliosides suggested that these molecules are in the vicinity of each other in the plasma membrane. This was also confirmed by our FCET experiments between these two molecules. Interestingly, cholesterol depleting agents, MβCD and simvastatin, had mild but non-significant effect on such association features as demonstrated by FCET studies. The modest effect of cholesterol depletion was also noted in a co-culture experiments between MβCD/simvastatin treated C1R-CD1d cells and iNKT cells by measuring secreted cytokines. The secretion of cytokines (IFNγ and IL4) by iNKT cells was only mildly affected by such treatments of C1R-CD1d cells. This supports the notion that cholesterol in the membrane has only a minor role in CD1d mediated iNKT cell activation. Thus it argues that either CD1d is present in the low cholesterol containing regions at the periphery of GM_1 gangliosides or the CD1d enriched membrane microdomains are different from conventional rafts.

Enrichment of CD1d in detergent sensitive membrane regions

Having been surprised by the features of CD1d in the plasma membrane, we decided to apply detergent resistance test to CD1d proteins. Operationally, the hallmark feature of raft and raft-resident proteins is their resistance to high detergent concentration. Detergent sensitivity is considered as a feature of non-raft resident proteins like transferrin receptor (TfR) only. We employed a flow-cytometric approach that was published earlier by Gombos *et al* and realized that the detergent resistance capability of CD1d enriched regions were far lower than that of TfR. Sensitivity to detergents by CD1d rich regions has also been demonstrated in other cell types. Therefore, susceptibility to low concentration of detergents seems to be a specific feature of CD1d enriched regions. Thus to conclude, we propose that either CD1d proteins are weakly associated with GM_1 gangliosides -most probably at the periphery of such rafts- or CD1d is present in mildly cholesterol dependent detergent sensitive membrane regions which could be a new raft subtype. MHC I proteins were also found in low cholesterol containing membrane regions previously. The possible presence of cholesterol dependent and cholesterol independent raft subtypes were also documented using biophysical

methods for the epidermal growth factor receptor. In addition, presence of cholesterol independent sphingolipid domains, ~200nm in diameter, were documented recently in the plasma membrane of fibroblasts. Thus, the nature of CD1d enriched membrane regions needs further characterization especially by comparing it with MHC enriched domains and other raft like structures.

Multimolecular complexes of CD1d, MHC and lipid species on the cell surface

Co-localization and FCET experiments revealed that CD1d, β_2m, MHC and GM_1 would co-exist in the plasma membrane of C1R-CD1d cells. FCET studies also demonstrated that CD1d, β_2m and MHC proteins had a differential propensity of enrichment in GM_1 gangliosides. Therefore, we speculate that multimolecular complexes of MHC I-HC, β_2m, MHC II and CD1d proteins would also be found in the plasma membrane of these cells. In fact, several white pixels were observed when triple co-localization was performed between these proteins or with GM_1 gangliosides indicating co-distribution of these molecules. The likelihood of the existence of multimolecular complexes in the plasma membrane was also suggested by our two-color but three-protein FCET. With this modified scheme, the FRET efficiency should increase between two proteins in comparison with conventional two protein FRET system when a new protein which is generally a part of trimolecular complex is also included in the FRET pair. In accordance with such assumptions, we observed an increase in the FRET efficiency between any two proteins with the inclusion of a third protein mainly with MHC II as an acceptor. Several tetraspanin proteins (CD82 and CD9) and invariant chain proteins are found to be associated with both MHC and CD1 proteins. Therefore, it is highly probable that these molecules as a supramolecular complex would be present in the plasma membrane of these cells although differences might occur in the abundance of these clusters in GM_1 rich or non-GM_1 regions.

SUMMARY

In the first part of the thesis, we generated dye-antibody conjugates by three different strategies which are commonly employed in most research laboratories (amine, sulfhydryl and carbohydrate targeted approaches) so that it could be used for studying membrane protein dynamics. The goal was to obtain conjugates which retained the maximum antigen recognition features and that produced the highest fluorescence intensities. We found that only amine targeted approach could produce higher dye per antibody variants (>3) with a maximum yield of antibody conjugates. Sulfhydryl and carbohydrate targeted approaches are site-specific, however, they led to the generation of low amount of functional dye-antibody conjugates. Therefore, amine targeted approach is the best method of antibody conjugation in comparison with the other two approaches in totality.

In the second part of the thesis, our goal was to unravel the topological features of a membrane protein termed cluster of differentiation 1d (CD1d). These proteins are involved in lipid-based antigen presentation. We wanted to demonstrate the relationship of CD1d with peptide antigen presenters i.e. major histocompatibility complex (MHC) proteins on the cell surface. We documented using fluorescent dye-antibody conjugates and biophysical tools that CD1d harbors a close relationship with MHC I, β_2-microglobulin (β_2m), MHC II and GM_1 gangliosides on the plasma membrane of human B cells at resting state. Surprisingly, β_2m dependent CD1d constituted only ~15% of the total membrane CD1d proteins. In addition, fluorescence resonance energy transfer (FRET) studies revealed only minimal effect of membrane cholesterol depletion on the association between CD1d and GM_1 ganglioside on the cell surface. Instead, CD1d rich regions were highly sensitive to low concentration of Triton X-100. Therefore, CD1d is either located at the periphery of GM_1 gangliosides or is enriched in low cholesterol containing detergent sensitive membrane regions of the plasma membrane which could be a distinct raft subtype.

In summary, we generated dye-antibody conjugates which were suitable for studying the membrane distribution of CD1d proteins. We believe the investigated antibody conjugation approaches can provide researchers with up-to-date information in the field of bioconjugation.

Likewise the membrane topological features of CD1d discovered in our study might be immunologically relevant considering the importance of CD1d mediated lipid antigen presentation in linking the innate and adaptive immunity.

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   Anal. Bioanal. Chem. 404 (5), 1449-1463, 2012.
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List of other publications

3. **Shrestha, D.**, Szöllősi, J., Jenei, A.: Bare lymphocyte syndrome: An opportunity to discover our immune system.
Immunol. Lett. 141 (2), 147-157, 2012.
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Anal. Chim. Acta. 584 (2), 343-349, 2007.
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