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学位論文題目	Synthesis of ABG (avidin-biotin-GlcNAc) glycocluster as an attractive bioprobe & its multivalent recognition with WGA by PL & SPR methods （魅力的なバイオプローブとしての ABG (avidin-biotin-GlcNAc) 糖鎖クラスターの合成と PL 法と SPR 法による WGA との多価認識）
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論文の内容の要旨

Chapter 1 of this thesis describes the main factors, which made us to choose such research. Interactions between carbohydrates and lectins are subjected to intensive studies due to their roles in cellular events like fertilization, cell signaling, immune function, genomic activities, and host-pathogen recognition or cancer metastasis. Carbohydrates present on the cell surface to detect and work as a receptor for any foreign substances like pathogen, bacteria, viruses and toxins, after getting the information from the outside world these carbohydrates soon transfer these signals to protein. This is the reason most of the scientists highly focused towards the study of carbohydrate-protein interactions. Usually, the lectin-carbohydrate interaction is not so strong, but can be enhanced *via* several modifications in carbohydrates, which we did in this research. However, to the best of our knowledge there are surprisingly few reports on the linear linkage of GlcNAc *via* biotin-avidin interactions. In this paper we described the synthesis of biotinylated GlcNAc monomer-using 6-chlorohexanol as linker and further modifications *via* organic synthesis leads to a formation of unique bio-probe. Driven by the high demand for sensitive and specific tools for optical sensing and imaging, bio-probes with various working mechanisms and advanced functionalities are flourishing at an incredible speed. Synthesis of GlcNAc derivative and its biological applications by PL and SPR methods are the main objective of this research.

Chapter 2 describes the synthesis portion of this research. The main goal of this project was to synthesize divergent type of avidinbiotin-GlcNAc (ABG) tetrameric complex using various techniques of organic synthesis. Organic synthesis requires protection, deprotection and glycosylation type of reaction to carry forward the reactions to get targeted compounds in a good yield. Firstly we used D(+)glucosaminehydrochloride as a starting material to get an α-anomeric acetate compound **2** by the acetylation reaction followed by the formation of *oxazoline* **3** intermediate using the reference of *Nakabayashi et al*, and soon applied for the glycosylation step to attach a C_6 length spacer. Spacer is required to use as it provide flexibility and stability to the artificial carbohydrate and basically help to suppress the non-

specific interactions between protein and carbohydrate, as the distance between particular protein and carbohydrate is an important point to avoid any non-specific intimation. The linker we used here is 6-chlorohexanol, which leads to the synthesis of compound **4**, after attachment of such linker we chose displacement of chlorine to azide by displacement reaction to get the compound **5**, followed by the deprotection of all acetate group *via Zemplen`s* method of deacetylation to serve compound 6. After synthesis of compound **6**, the aim was turned towards the reduction of azide to amide, so we chose reduction reaction to make compound **7**. After finishing acetylation, glycosylation, displacement, deacetylation and reduction reactions, we went for the coupling reaction to make a conjugate of GlcNAc and biotin. Two kind of coupling reaction has been chosen based on the reagents use, one is DMAP/DIC coupling and another is DMT-MM coupling. In our study the DMT-MM proved to be a good coupling reagent to make a couple of GlcNAc-biotin with quite good yield (63 %) but if we talk about the DMAP/DIC, it rarely showed any target compound (almost 0 %). Such methods helped to develop the final material 10.

Chapter 3 describes the complete chemical and physical analysis of synthetic compounds. After complete synthesis the chemical and physical analysis has been performed using analytical techniques of IR, NMR, MS and elemental analysis. Proton NMR suggested the coupling constants of anomeric proton (δ 4.96 ppm, d, 1 H, $J_{1,2}$= 8.48 Hz, H-1) for compound **9** and (δ 4.63 ppm, d, 1 H, $J_{1,2}$= 8.28 Hz, H-1) for compound **10**. Carbon NMR showed the anomeric signal in the range of (δ 100.3 ppm, C-1) for compound **9** and the same with compound 10 (δ 101.5 ppm, C-1). IR made the good agreement to explain the structure of compound **9** and **10**. MS showed the molecular mass of both the synthetic compounds same as the theoretical values. To make sure all the synthesized compounds are pure and can be further use for biological assaying and making of ABG tetramer, these analytical techniques explained the level of purity of each and every synthesized compound and helped use to go to the next step.

Chapter 4 describes the synthesis of main material that is divergent type tetrameric complex of avidin-biotin-GlcNAc (ABG) and reason to choose avidin-biotin system. We all know avidin-biotin system, which is famous for its strongest known non-covalent interaction ($K_D \sim 10^{-15}$ M), because of high affinity between biotin and avidin synthetic assembly of the biotin and other bioactive compounds are of great interest in biochemical and biomedical research fields in order to evaluate activities of the bioactive compounds. Therefore we chose such system to make a tetrameric stable complex of ABG. After the fruitful synthesis of ABG complex, the estimated concentration of avidin was measured to be 0.257 μM in the mixture of ABG complex by using absorption coefficient at 282 nm and accordingly the concentration of GlcNAc moiety is 1.028 μM (4 × 0.257 μM).

Chapter 5 describes the biological activity of ABG and compound **10** using fluorometric assay. First is to check the binding affinity of ABG complex with WGA (Wheat Germ Agglutinin) we use fluorometric assay by means of specific excitation of tryptophan at wavelength of 295 nm. Interestingly, high affinity constant has been measured for ABG complex with WGA ($K_A \sim 10^7$ M^{-1}) while its glycoside showed just in the range of ~10^3 M^{-1}. ABG complex shows the binding almost thousand times of glycoside **10**. This huge difference in affinity binding is proved due the phenomenon of glycocluster effect, which is more favorable for ABG as compared to compound **10**. PL method in this research does not required any fluorescence labeling method, because we use the excitation wavelength for tryptophan and it is an automated form of fluorescence spectroscopy which proved to be advantageous to get a result according to our hypothesis.

Chapter 6 describes the biosensor assaying using Surface plasmon resonance (SPR) methodology. In this we used ABG complex and three linear type of GlcNAc polymers named glycopolymer **1**, **2** and **3** as control against ABG. Glycopolymer **1** with monomer and acrylamide ratio of 1:10, polymer **2** with 1:20, polymer **3** with 1:4 and ABG were

used as respective analytes. WGA used as ligand and was immobilized on sensor surface *via* amine coupling method. The efficiency of the method was exhibited in the analysis of the interactions that covered a high affinity range; namely the strong binding ($K_A \sim 10^7\ M^{-1}$) for ABG compared with the polymers **1**, **2** and **3** which shows the binding of ($K_A \sim 10^5\ M^{-1}$). The usefulness of these synthetic glycopolymers as tools in the study of sugar-lectin interactions has been proved due to glycocluster effect and the combination of lectin immobilization with SPR is characterizing specific screening. The lectin-based SPR made it possible to carry out the label-free detection of glycoproteins with a broad concentration range with a good linearity. SPR also provided all the thermodynamical evaluations and all tested glycopolymers showed their own unique lectin-binding pattern.

Chapter 7 The evaluation of binding by a variety of techniques facilitates a better understanding of the molecular basis by which multivalent ligands exert their effects. We compared the kinetic affinity calculated by SPR data with PL data of same sugar conjugates and both the methods are almost in the same agreement, little fluctuation in results may occurred in ABG and glycopolymer **2**. It should be emphasized that the PL method measures the solution-solution carbohydrate and protein interactions, which is different from the solution-solid interactions measured by SPR. Surface plasmon resonance and fluorescence spectrophotometry are the techniques used in this research for identifying the binding between carbohydrate-protein and gave the good results according to our hypothesis. Combination of the lectin panel with surface plasmon resonance and flourospectrophotometry makes it possible to carry out both qualitative screening and quantitative detection of synthesized glycoconjugates of GlcNAc. Additionally, the assays used to evaluate protein-carbohydrate interaction each evaluate a range of different process, either because the assay itself measures a phenomenon that incorporates additional interactions beyond simple binding or because the assay operate under different concentration regimes. In addition, both the techniques offer several unique advantages. These implied techniques uses very small amounts of material, expanding the range of systems amenable to study. It may be worth to say that the unique selectivity, high sensitivity, excellent reproducibility, simplicity, absence of labeling agents, rapidity, automation and small consumption of reagents make these two methods SPR and PL good alternative to the more complicated and time consuming glycoprotein analysis techniques based on structural investigations and quantitative determination of the carbohydrate composition.

We can conclude the protein-glycan interactions are guided by the same principles as other macromolecules interactions, like hydrogen bonding, hydrophobic interactions and electrostatic interactions. Most monovalent interactions between proteins and glycans are low affinity (almost in mM range) and frustrate efforts towards the development of carbohydrate therapeutics, and recognition but in case of modified carbohydrates after clustering and polymerization can leads toward the strong affinity (nM range) due to the glycocluster effect. High specificity can be achieved, but not all interactions are totally selective. Most interactions are multivalent, usually involving oligomerization of the lectin or multiple types of interactions (protein-protein as well protein-carbohydrate). The use of carbohydrate-protein interaction study is worth in many biological functions such as immune function, fertilization, cancer metastasis and many more. The ability to control these events with selective small molecule inhibitors offers enormous potential for the study of biology. The development of strong binding ligands for carbohydrate binding proteins continues to be a demanding sector throughout the carbohydrate chemistry and biology community.

論文の審査結果の要旨

本学位論文に関する最終試験は、平成２９年１月６日（金曜）に審査委員４名の出席の下、実施された。

バイオプローブとは生体機能を解析するために有用な探り針のことを指し、新薬候補の探索やタンパク質研究に応用が期待される付加価値の高い機能材料である。Y. C. Lee らの先駆的な研究により発見された、糖鎖が集合体構造を取ることでレセプターとの相互作用を増強する「糖鎖クラスター効果」と呼ばれる現象を利用した多価型バイオプローブの合成に興味が持たれている。本研究では、糖鎖クラスター効果の発現が期待できる新しい多価バイオプローブの合成を行い、得られた化合物の機能評価を標的タンパク質由来の蛍光強度変化により実施した。さらに別途調製した高分子型バイオプローブと本学位論文において合成された多価型バイオプローブに対する標的タンパク質との相互作用解析を表面プラズモン共鳴法（SPR 法）により行い、興味深い結果を示した。以下にそれら詳細を述べる。

本学位論文は、10 章から構成される。それぞれの章について、下記に概要を記載する。

第 1 章においては、本学位論文に関する研究背景及び目的について記載されており、糖鎖、レクチン、糖鎖クラスター効果、各種分析方法等について記載され、本学位論文に関連する事項についてまとめられている。

第 2 章においては、新しい糖鎖クラスターを構築するためのビオチン化された糖鎖誘導体に関する合成研究が記載されている。特に、GlcNAc (*N*-acetyl-D-glucosamine) 誘導体の調製法についての記述と biotin との結合に関して、詳細な検討がなされている。

第 3 章においては、本学位論文において合成された化合物に関して、各種分光法を用いた構造解析について記載され、標的化合物である検証が実施されている。

第 4 章においては、アビジンとビオチンに関する相互作用について記載したのち、第 2 章で得られたビオチン化された糖鎖誘導体とアビジンを結合させることにより、ユニークな多価型バイオプローブへ誘導している。また、得られた多価誘導体の精製、分光学的評価を実施している。

第 5 章においては、生物学的評価として、得られた多価型バイオプローブと標的タンパク質となるコムギ胚芽レクチン（WGA）との相互作用解析を検討している。ここでは、レクチン由来の蛍光強度変化を指標として実施し、高い糖鎖クラスター効果の発現を見出している。

第 6 章においては、5 章において実施した評価法とは異なる生物学的評価法として、表面プラズモン共鳴（SPR）による詳細な相互作用解析を行っている。その際、別途調製した直鎖高分子型バイオプローブとの考察も行っている。本学位論文において合成した多価型バイオプローブがデンドリティックなプローブ配置に対して、直鎖高分子型バイオプローブがペンダントトップにプローブを配置した化合物であり、双方の活性はあるものの本学位論文において合成した多価型バイオプローブのほうがより高い活性を発現することを見出している。

第 7 章においては、異なる生物学的評価における評価法の違いと活性についてまとめられている。

第 8 章においては、本学位論文に関する全体のまとめが記されている。さらに、第 9 章においては、本学位論文において合成に用いられた一般的な試薬、その取扱い、構造解析に用いられた機器および生物学的評価に用いられた機器等のまとめが記載されている。最後に、第 10 章として、本学位論文の総括がなされている。

これらの研究成果を最終試験において報告し、質疑応答に関しても充分対応できたと判断した。また、学会やシンポジウムにおいても国内外を問わず積極的に参加し、発表・討論を行っている。その過程において、ポ

スター賞および国際会議の session best paper に選出されている。さらにこれらの研究成果は、英語の査読付学術論文（筆頭著者）2 編に反映され、国際的に権威ある雑誌に公表されている。

以上のように、Amrita KUMARI 氏は、多価型バイオプローブの合成および機能評価を行い、新規プローブの合成法の開拓および活性評価手法の有効性を示し、多価型バイオプローブの新しい活用法を見出した。今後の学術的発展が十分期待できると判断された。以上の結果から、同氏の博士（学術）の学位を受けるに充分な資格を有するものと審査員一同は判断し、最終試験の合格に至った。