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研究課題名（和文）Antibody-nucleic acid conjugates - linking siRNAs to antibody

研究課題名（英文）Antibody-nucleic acid conjugates - linking siRNAs to antibody

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研究成果の概要（和文）：siRNAの標的薬剤送達システムとしてantibody-nucleic acid conjugate (ANAC)の設計や合成を行うこととした。抗体に核酸を搭載するために毒性の少ないデンドリマーであるポリアミドアミン（PAMAM）を検討した。PAMAMにsiRNAを静電的結合し抗体とのコンジュゲートを目指した。しかし、得られANACはsurface plasmon resonance (SPR)や細胞assayではノンスペシフィックな結合が主であった。今後PAMAMの表面にあるアミノ基の保護基の導入やPAMAM1分子当たりのsiRNAの数などの検討が重要であると考えられた。

研究成果の概要（英文）：In this research, an approach to use antibody-polycationic compound as a delivery vehicle for therapeutic oligonucleotides like siRNA has been proposed. Due to its high loading capacity and well-defined structure, polyamidoamine (PAMAM) was selected as a polycationic compound, which can efficiently carry siRNAs and help release of the payload in target cells. As a target specific ligand, Herceptin was used which is a humanized monoclonal antibody designed to target HER 2 found in breast cancer. Three strategies were attempted to conjugate the antibody and PAMAM. Attempts were also made to synthesize homogenous bioconjugates with defined ratio of antibody and PAMAM. Preliminary cell assay indicated possibility of non-specific interaction of the bioconjugate toward target cells. Further consideration is needed to avoid non-specific interaction towards healthy cells along with controlled loading of siRNA and to improve delivery potential towards target cancer cells.

研究分野：　Nucleic acid chemistry

キーワード：　Antibody drug conjugate　Dendrimer　Bioconjugation　click conjugation　endosomal escape　scFv antibody fragment　siRNA　薬学

１．研究開始当初の背景

RNA interference (RNAi) has been an attractive tool in gene therapy due to its high specificity and potency. RNAi is triggered by double-stranded RNA. Generally, small interfering RNAs (siRNAs) having 21-23 nucleotides are used to modulate gene expression. Despite their huge potential, RNA therapeutics like siRNAs have only little success in their application largely due to the problem in their cell- and tissue-type-specific delivery besides insufficient cellular uptake as well as rapid renal clearance. As a promising candidate, polycationic compounds have exhibited their potential as an efficient carrier for siRNAs through ionic interactions between those polymers and the negatively charged phosphate backbone of nucleic acids. Therefore, much interest has been attracted towards the polycationic carriers equipped with target specific ligands as a delivery system for siRNAs and further improvements are needed for their useful application.

２．研究の目的

The purpose of this research is to develop a promising delivery system for therapeutic oligonucleotides such as siRNAs. As a plausible candidate, a bioconjugate between antibody and oligonucleotide is designed for this research and termed as Antibody Nucleic Acid Conjugate (ANAC). An antibody is used as a targeting ligand for the conjugate due to its high site specificity. Direct conjugation of oligonucleotides to an antibody is not favorable because such strong bond would cause the bioconjugate to end up in lysosome after cell internalization, leading to the degradation of the oligonucleotides due to the abundance of aggressive nucleases. Therefore, there is need of a promising linker between the antibody and the oligonucleotide that would efficiently bind the oligonucleotide payload during blood circulation, and release it only after internalization in target cells and promote endosomal escape. In this research, a dendrimer, polyamidoamine (PAMAM) is proposed as a potential linker candidate, as it can exhibit most of the desired properties and help an oligonucleotide payload to escape efficiently from the endosome through the phenomenon of proton sponge effect. Moreover, a number of oligonucleotides can be loaded on a single PAMAM molecule through electrostatic interaction.

３．研究の方法

PAMAM has a defined structure with many free amine groups on its surface that can be utilized to introduce desired functionality to the structure. In this research, generation 4 and 5 PAMAM were taken as polycationic carrier that contain 64 and 128 free amine groups respectively on their surfaces. The surface of the dendrimers was decorated with various modifications such as fluorescent marker, masking groups, and functional moiety for bioconjugation. Herceptin was used as an antibody to target breast cancer cells and obtained from Gene techno sciences co. ltd. A conjugation site was introduced in the antibody by reacting crosslinkers to lysine or glutamine residues. Moreover, single-chain antibody fragment (scFv) was also used to reduce the bulkiness of the antibody that was obtained from laboratory of biomolecular science,

Hokkaido University. For the conjugation of the antibody and PAMAM, following three methods were employed (Fig 1).

1. Maleimide-thiol conjugation
2. Disulfide conjugation
3. Strain promoted azide alkyne cycloaddition (SPAAC)

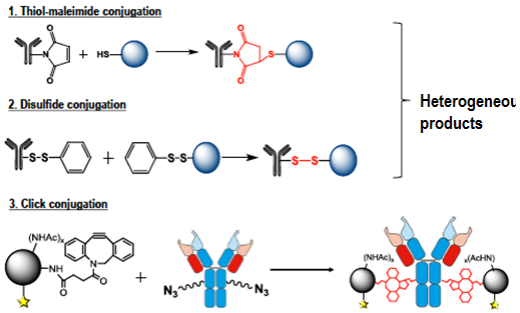

Fig. 1 Conjugation reaction trials

For maleimide-thiol conjugation, maleimide group was introduced into lysine residues of the antibody using SMCC crosslinker and thiol group was introduced into PAMAM using SPDP crosslinker. For Disulfide conjugation, SPDP crosslinker was introduced into both antibody and PAMAM. For SPAAC, azide moieties were introduced into site-specific glutamine residue of antibody following the procedure reported by Denner *et al.* (2014, Bioconjugate Chemistry, 25: 569-578). Briefly, the glycan moieties at N295 residue of the antibody was removed by peptide-N-glycosidase and azide-PEG-amine was incorporated using microbial transglutaminase.

The structure of PAMAM after modification was identified and confirmed by UV spectroscopy, NMR spectroscopy, and MALDI-tof mass. The formation of antibody-PAMAM conjugate was confirmed by SDS-PAGE, fluorescent imaging, and MALDI-tof mass (Fig 2 and 3). The binding affinity of the conjugate towards target protein i.e. Her-2 was examined by Surface plasmon resonance (SPR) and cell ELISA assay (Fig 4 and 5). Cell assay of the conjugates were conducted by Gene techno science co. ltd.

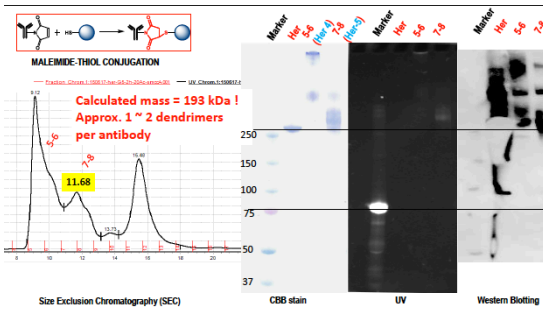

Fig 2. Synthesis of antibody-dendrimer conjugate through maleimide-thiol conjugation reaction. The conjugate was separated by size exclusion chromatography (SEC) and confirmed by SDS-PAGE, UV, and western blotting.

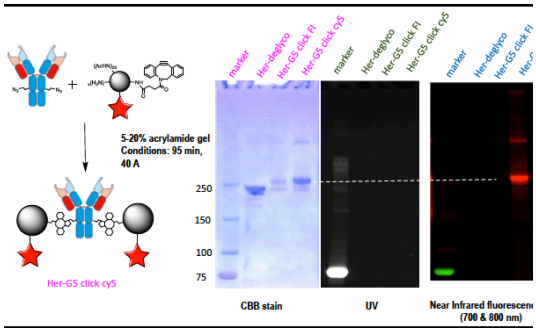

Fig. 3 Synthesis of Antibody-dendrimer conjugate through strain-promoted azide-alkyne cycloaddition (SPAAC) reaction. The formation of the conjugate was detected by SDS-PAGE, UV, and fluorescence imaging.

4．研究成果

The preliminary conjugation attempts with maleimide-thiol as well disulfide conjugation resulted in the formation of heterogeneous products with the uncertain ratio of antibody to dendrimer. With

controlled molar equivalents of dendrimer with respect to antibody, an antibody-PAMAM conjugate was synthesized and SPR study was carried out. From the SPR study, very high association of the conjugate towards Her2 was observed along with non-specific binding towards BSA (Fig 4). Similar result was observed in cell ELISA where Her2 nonspecific IgG conjugated PAMAM also showed significant binding to Her2 (Fig 5), indicating the possibility of non-specific interaction of the conjugate as. It was anticipated that reducing the size of PAMAM would reduce the molecular weight and free amine groups. Therefore, antibody conjugate with

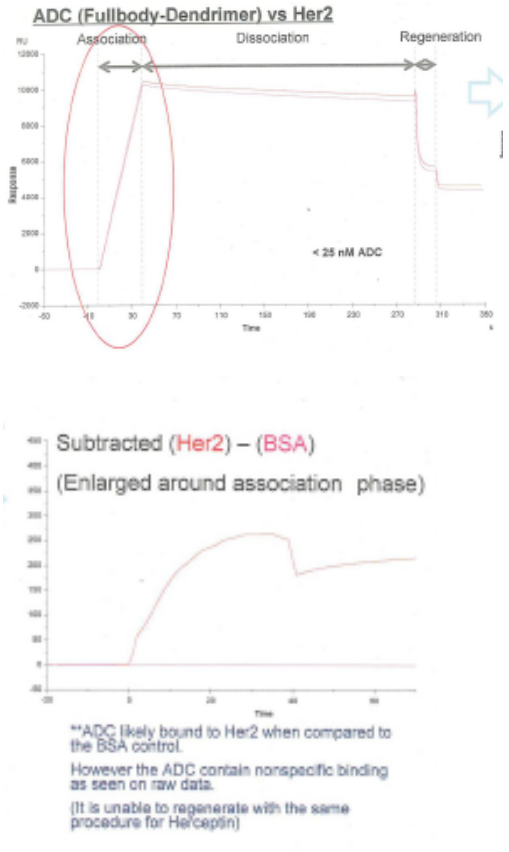

Fig. 4 Surface plasma resonance (SPR) assay. Experiment condition: machine: Biacore 3000; Chip: CM5; Ligand: HER2-ECD-Fc, BSA (control); Flow rate: 30 ml/min; Temperature: 30 ^{o}C

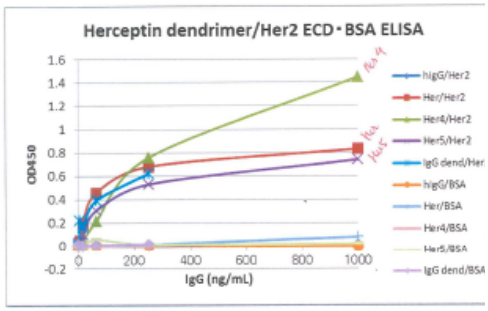

Fig. 5 Cell-ELISA assay. Capture: Her2 ECD; samples: human IgG (hIgG), Herceptin (Her), Herceptin dendrimer fraction 4 (Her4), Herceptin dendrimer fraction 5 (Her5), huIgG dendrimer (IgG dend)

generation-4 PAMAM was synthesized. However, the non-specific interaction was still observed. These results could be attributed to the highly polycationic character due to the availability of free amine moieties on the surface of PAMAM. Proper masking of the surface of PAMAM and loading of siRNA payload is expected to check the formation of non-specific aggregation with protein. Moreover, the non-specific interaction may be due to the formation of heterogeneous products with uncertain antibody to dendrimer ratio. Therefore, to synthesize ANAC with exact ratio of dendrimer to antibody, an enzymatic method was employed using microbial transglutaminase to introduce a conjugation site (azide moiety) in the antibody. In PAMAM, a cyclooctyne moiety was introduced as a conjugation site. Then, the antibody and PAMAM were conjugated via SPAAC reaction. It was expected that the DAR (drug antibody ratio) would be 2, however MALDI-tof mass indicated the possibility of formation of conjugate with DAR equal to 1. Further research is

necessary to optimize the conditions for the synthesis of antibody-PAMAM conjugate with DAR 2. The approach was also employed in scFv antibody fragment as a model. However, cell assay study of scFv antibody-PAMAM conjugate is under progress.

As a future recommendation, careful study is needed to determine DAR of antibody-dendrimer conjugate to confirm the number of payloads per conjugate. Moreover, there is probability of early release of siRNA payload during blood circulation when it is loaded only through ionic interaction. Therefore, covalent conjugation of the siRNA payload to PAMAM with a bioreleasable linker should also be considered. It is expected that careful modification of ANAC could produce an interesting candidate of carrier system for oligonucleotide therapeutics.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　0　件）

〔学会発表〕（計　0　件）

〔図書〕（計　0　件）

〔産業財産権〕
○出願状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
出願年月日：
国内外の別：

○取得状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
取得年月日：
国内外の別：

〔その他〕
ホームページ等

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