Development of Nanostructured DNA Aimed at Enhancing the Stability and Antigen-presenting Cell Targetability of CpG Oligodeoxynucleotide

（CpG オリゴデオキシヌクレオチドの安定性および抗原提示細胞指向化の増強を目指したナノ構造化 DNA の開発）

要約

２０１９

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Contents

Preface

Nucleic acid medicine is expected as a next generation medicine because of its diverse targets and high specificity. The oligodeoxynucleotide containing unmethylated cytosine-phosphate-guanine, or CpG ODN, is derived from viruses and bacteria, and is a danger signal to stimulate innate immune cells[1]. There are mainly three types of CpG ODNs, A, B, and C types, having different immune activities. The receptor for CpG ODN is Toll-like receptor 9, which is an endosomal receptor in antigen presenting cells like dendric cells and macrophages. Therefore, the CpG ODN can stimulate and enhance the innate immunity so that it has been found as the therapeutic agonist against cancers, allergic or infectious diseases, and so on. For example, a licensed vaccine for B-type hepatitis uses CpG ODN as an adjuvant. Therefore, many drug delivery systems, or DDS, for CpG ODN have been developed[2].

Recent advances on DNA nanotechnology have greatly widened the potential applications for nucleic acid drug delivery. Therefore, many studies reported that delivery systems like liposomes, micelles, gold nanoparticles and DNA nanoparticles are crucial carriers to achieve efficient delivery of CpG ODN to target cells[3-5]. For example, Department of Biopharmaceutics and Drug Metabolism has developed the polypod-like structured nucleic acid, or polypodna, a DNA nanostructure constructed with 3 or more ODNs for efficient delivery of CpG ODNs to antigen presenting cells[6]. In particular, the relationship between the structure and the immunostimulatory activity has been studied. It suggested that the polypodna led to higher cellular uptake and immune activity compared with the conventional, single stranded CpG ODN.

However, two improvable points are deserved to be considered in practical application (Figure Pre.1). On the one hand, the ideal DNA nanocarrier for CpG ODN delivery should be constructed by only CpG sequences with necessary numbers of nucleotides, so that leads to a simple design with low

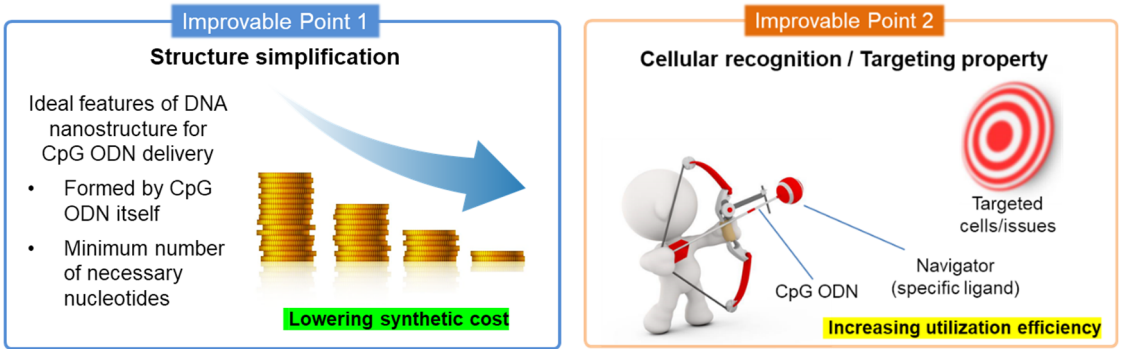

Figure Pre.1. Improvable points in practical application of CpG ODN.

synthetic cost. On the other hand, cellular uptake of DNA nanostructures by immune cells just relies on the concentration and stereochemical structures, therefore, endowing them cellular targeting property can increase their utilization efficiency.

In this thesis, I developed simple strategies to deliver the CpG ODN (Figure Pre.2) by nanostructuration using G-quadruplex just introducing several guanines into the sequence of CpG ODN to realize the structure simplification for delivery system of CpG ODN (Chapter 1), and to modify the DNA nanostructures including polypod-like structured nucleic acid (Chapter 2) and G-quadruplex structured CpG ODN (Chapter 3) by mannose to increase the delivery efficiency of CpG ODN to antigen-presenting cells.

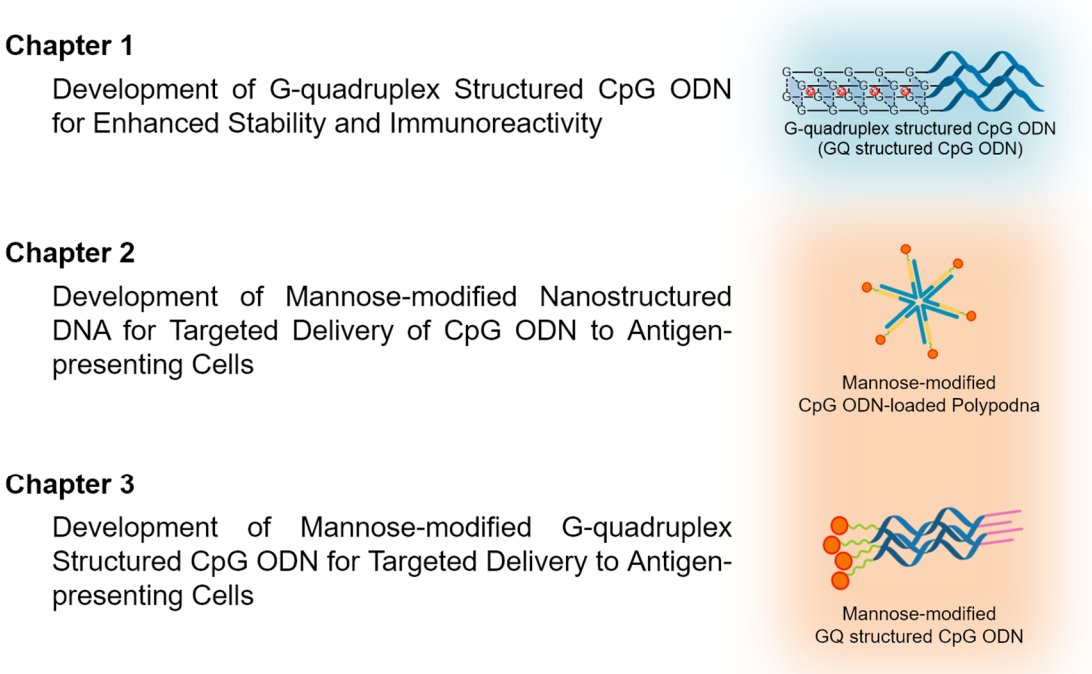

Figure Pre.2. Constitution of the thesis. The blue background is corresponding to improvable point 1, and the orange background is corresponding to improvable point 2.

Chapter 1
Development of G-quadruplex Structured CpG ODN for Enhanced Stability and Immunoreactivity

G-quadruplex (GQ) structure, a stable helical secondary nucleic acid structure, is formed by stacking of G-quartets, a guanine tetrad tightened by Hoogsteen interaction with coordinating metal cations (e.g. Na^+, K^+)[7]. GQ structures exist naturally at the 3′-end of chromosomes, which is well known as telomeric region protecting chromosomes from degradation by nucleases. Taking the advantage of its unique structural property and stability, GQ structure has been widely studied as an important component part in nucleic acid nanotechnology including nanocarrier design for drug delivery[8]. Nevertheless, only a few applications of GQ structures to nucleic acid delivery have been reported. Recently, Hoshi *et al.* reported that the stability in serum, immunostimulatory activity and cellular uptake of a phosphodiester class B CpG ODN were improved by addition of several G-tracts to the ODN[9]. This result provides a new approach for class B CpG ODN delivery, and the increased stability could avoid the use of phosphorothioate linkages which are associated with renal toxicity[10, 11]. Despite its potential usefulness in the delivery of CpG ODN, several G-tracts in one sequence are required, which is linked with an increased cost[12]. More importantly, it is quite difficult to obtain GQ-structured CpG ODN with precise structure[7].

In this chapter, I focused on a phosphodiester-backboned CpG ODN (CpG1668) with the same sequence as CpG ODN 1668, a class B CpG ODN, simply added 5 guanines (G-tract) to either 5′-end [1668(5′-G_5)], 3′-end [1668(3′-G_5)], or internally within the sequence [1668(mid-G_5)], and investigated their structural properties and immunostimulatory activity. Addition of only one G-tract was sufficient for the formation of parallel GQ structured CpG ODN in 150 mM KCl. The CpG ODN with a 5′-end G-tract [1668(5′-G_5)] formed a dimeric GQ structure, but monomeric GQ structures were formed when 5′-end G-tract was shaded by thymine or shifted away from the 5′-end. GQ structured CpG ODN, especially 1668(3′-G_5), showed significantly increased stability, cellular uptake and induced high secretion of tumor necrosis factor-α and interleukin-6 from mouse macrophage-like RAW264.7 cells compared with CpG1668-loaded tetrapod-like nucleic acid, which was previously developed in Department of Biopharmaceutics and Drug Metabolism[6, 13]. Further investigation revealed that CG sequence but not GQ structure activated Toll-like receptor 9 and 22 nucleotides was necessary to keep 1668(3′-G_5) a high immunostimulatory activity. Thus, this study provided a simple and economical strategy for the delivery of CpG ODN to TLR9-positive immune cells by GQ nanostructuration.

Chapter 2
Development of Mannose-modified Nanostructured DNA for Targeted Delivery of CpG ODN to Antigen-presenting Cells

Recent advances on DNA nanotechnology have provided various delivery systems for nucleic acid therapeutics, such as siRNA, miRNA, aptamer and, of course, CpG ODN[14]. Polypod-like structured nucleic acid, or polypodna, is a DNA nanostructure constructed with 3 or more ODNs, which has been developed in Department of Biopharmaceutics and Drug Metabolism, for the efficient delivery of CpG ODNs to APCs[6, 15-19]. Compared with the conventional, single-stranded CpG ODN, CpG ODN-loaded polypodna led to higher cellular uptake and immunostimulatory activity.

A previous study in Department of Biopharmaceutics and Drug Metabolism demonstrated that macrophage scavenger receptor 1 (MSR1), one of the scavenger receptors, was involved in the efficient uptake of polypodna depending on its structural complexity[20]. Further increase in the cell specificity and targeting efficiency of nanostructured DNAs to immune cells could be achieved by the use of receptor-ligand recognition[21]. Mannose receptor (MR), which can specifically recognize saccharides like mannose, is primarily expressed on the surface of macrophages and dendritic cells[22]. Recently, a mannosylated CpG ODN (Man-CpG ODN) has been developed, which showed enhanced both immunostimulatory activity and cellular uptake[23].

In this chapter, I first designed and synthesized a mannose modifier with a 5-carbon alkyl linker, then selected a phosphodiester CpG ODN with the same sequence as CpG ODN 1668, named it as ODN1668 to synthesize a mannosylated ODN1668 (Man-ODN1668). In 2017, a hexapodna (the polypodna with 6 pods) backbone designed to be a versatile carrier for CpG ODNs has been developed in Department of Biopharmaceutics and Drug Metabolism[13]. Using this backbone, I successfully constructed a hexapodna loaded with Man-ODN1668 (Man-ODN1668/hexapodna) and found that loading of ODN and mannose modification hardly influenced thermal stability of hexapodna. The mannose modification only slightly affected the TNF-α release from mannose receptor-positive mouse peritoneal macrophage induced by ODN1668. No significant TNF-α release was observed when hexapodna backbone or mannose was added into cells. Compared with single-stranded CpG ODNs, a higher TNF-α release was observed by ODN1668- or Man-ODN1668-loaded hexapodna. Man-ODN1668/hexapodna induced two-fold higher TNF-α release than ODN1668/hexapodna. However, no difference was detected in RAW264.7 cells which hardly expressed surface mannose receptors. This result indicated that the combination of mannose modification and incorporation into hexapodna is an effective approach for enhancing the immunostimulatory activity of CpG ODN.

Chapter 3
Development of Mannose-modified G-quadruplex Structured CpG ODN for Targeted Delivery to Antigen-presenting Cells

In Chapter 1, the stability, cellular uptake, and immunostimulatory activity of CpG ODNs were successfully improved through G-quadruplex nanostructuration by the introduction of several guanines into the sequence. In Chapter 2, it was established that mannose modification is a useful approach for the further enhancement of the immunostimulatory activity of the CpG ODN-loaded polypodna. In the parallel GQ structure, all ODNs are arranged in the same direction. This structural property gives rise to a situation where modifiers bound to the terminal of GQ structure-forming ODN come in close contact with one another after GQ structuration, which leads to the aggregation of the modifiers. It has been reported that mannose density is an important parameter for ligand recognition by the mannose receptor (MR)[24]. Therefore, mannose modification of the GQ structure-forming CpG ODN might increase the interaction between GQ-structured CpG ODN and the MR, by increasing the efficiency of uptake by the MR.

In this chapter, I focused on the GQ-structured CpG ODN, 1668(5′-G_5). I modified its 5′- and 3′-terminal amino-modified derivatives with mannose, and then investigated their structural properties and immunostimulatory activity. 1668(5′-G_5) formed a dimeric GQ structure, and the 5′-end amino-modified 1668(5′-G_5) [5′-NH_2-1668(5′-G_5)] and 3′-end amino-modified 1668(3′-G_5) [1668(5′-G_5)-NH_2-3′] formed a monomeric GQ structure. However, the ODNs modified with the mannose motif synthesized in Chapter 2 resulted in a relatively low conversion of single-stranded CpG ODNs to GQ-structured CpG ODNs, irrespective of the modification site. A recent work has revealed that the terminal lipid modification might lead to a very fast self-aggregation of DNA if no complementary exists[25]. Therefore, α-mannopyranophenyl isocyanate, a mannose motif contains a shorter linker, was used for modification. 5′-end modified CpG ODN [1668(5′-Man, 5′-G_5)] formed a monomeric GQ structure and 3′-end modified one [1668(5′-G_5, 3′-Man)] formed a dimer. 1668(5′-G_5, 5′-Man) induced slightly lower IL-6 release than 5′-NH_2-1668(5′-G_5). 1668(5′-G_5, 3′-Man), i.e., the 5′-end GQ-structured, 3′-mannose modified CpG ODN, induced high IL-6 release from mouse macrophage like J774.1 cells. No significant difference in IL-6 release was detected from RAW264.7 cells after mannose modification relative to that observed before mannose addition. An increased release of TNF-α in response to GQ structured CpG ODN from mouse peritoneal macrophages after mannose modification was also detected. These results indicate that the GQ structure is suitable for the delivery of CpG ODN to antigen-presenting cells by mannose modification.

Conclusion

In this thesis, I focused on the development of simple strategies (1) to deliver the CpG ODN to APCs by nanostructuration using G-quadruplex just introducing several guanines into the sequence of CpG ODN and (2) to modify the DNA nanostructures including polypod-like structured nucleic acid and G-quadruplex structured CpG ODN by mannose to increase the delivery efficiency of CpG ODN to APCs. The detailed summaries are demonstrated below.

Chapter 1 Development of G-quadruplex Structured CpG ODN for Enhanced Stability and Immunoreactivity

The G-quadruplex (GQ) structure has potential applications in nucleic acid drug delivery because of its superior stability. Here, I added one G-tract (5 guanines) into a CpG ODN to construct a GQ structured CpG ODN with precise structural properties, increased biological stability, and efficient delivery to Toll-like receptor 9 (TLR9)-positive immune cells. A G-tract was added to phosphodiester-backboned CpG1668 at the 5′-end [1668(5′-G_5)], 3′-end [1668(3′-G_5)], or within the sequence [1668(mid-G_5)]. Circular dichroism analysis showed that all CpG ODNs with a G-tract formed parallel GQ structures, irrespective of its position. Electrophoresis showed that 1668(5′-G_5) formed a GQ dimer whereas others remained GQ monomers. GQ structured CpG ODNs induced greater TNF-α and IL-6 secretion from TLR9-positive mouse macrophage-like RAW264.7 cells than single-stranded CpG ODNs, with the highest for 1668(3′-G_5). GQ structuration increased CpG ODN uptake by RAW264.7 cells, and 1668(3′-G_5) decomposed more slowly in serum than 1668(5′-G_5). Thus, GQ formation with one G-tract is a simple and efficient strategy for CpG ODN delivery to TLR9-positive cells, and addition of a G-tract to the 3′-end is effective in obtaining monomeric GQ structured CpG ODN with high biological stability and immunostimulatory activity.

Chapter 2 Development of Mannose-modified Nanostructured DNA for Targeted Delivery of CpG ODN to Antigen-presenting Cells

In this study, I selected phosphodiester CpG ODN, ODN1668, that had the identical sequence to CpG1668 and a hexapodna, a polypodna with six pods, and newly designed a hexapodna that harbored ODN1668 or mannosylated CpG ODN (Man-ODN1668), which was synthesized by modification of the 5′-terminal of ODN1668 with a synthesized mannose motif. Mixing of ODN1668 or Man-ODN1668 with a hexapodna resulted in the formation of ODN1668/hexapodna and Man-ODN1668/hexapodna with high yield. The T_m measurement showed the modification hardly

influenced the thermal stability of hexapodna. Man-ODN1668/hexapodna induced greater TNF-α release from TLR9-positive mouse peritoneal macrophages than Man-ODN1668 or ODN1668/hexapodna. In addition, no improvement after mannose modification was detected from RAW264.7 cells which hardly expressed surface mannose receptor. These results indicate that the combination of mannose modification and incorporation into nanostructured DNA is an approach for enhancing the immunostimulatory activity of CpG ODN to mannose receptor positive antigen-presenting cells.

Chapter 3　Development of Mannose-modified G-quadruplex Structured CpG ODN for Targeted Delivery to Antigen-presenting Cells

To further expand the utilization efficiency of GQ structured CpG ODN, the mannose modification of GQ structured CpG ODN was conducted. Initially, 1668(5′-G_5) were successfully modified at 3′-end [1668(5′-G_5, 3′-Man)] and 5′-end [1668(5′-Man, 5′-G_5)] separately by the mannose motif which was synthesized in Chapter 2 but failed to form the GQ structure due to the steric hindrance and repulsion from the long hydrophobic linker. Modification by α-D-mannopyranosylphenyl isothiocyanate, a mannose motif with shorter linker, lead to a success in GQ structuration for both 5′- and 3′-end modification of 1668(5′-G_5). 1668(5′-G_5, 3′-Man) induced greater cytokine release from mannose receptor positive APCs, J774.1 cells and mouse peritoneal macrophages, but no improvement was detected from RAW264.7 cells which hardly expressed surface mannose receptor. These results suggested that the immunostimulatory activity of GQ structured CpG ODN could be further increased by mannose modification and the improvement from 3′-end modification of 5′-end GQ structured CpG ODNs indicated the balance between higher density of mannose motif and its degree of freedom to interact with receptors might be important to increase the interaction of CpG ODN with APCs.

As described above, the applicant clarified that the stability, delivery and immunostimulatory activity of CpG ODN can be improved by introduction of G-quadruplex structure instead of using other nanocarrier. In addition, mannose modification is suitable for enhancement of delivery of CpG ODN loaded nanostructures. The results in this study not only reveal the potential in simplification of delivery system for CpG ODN, but also provide useful information for antigen presenting cell-targeted strategy in practical application of CpG ODN.

List of Publications

Construction of Monomeric and Dimeric G-quadruplex Structured CpG Oligodeoxynucleotides for Enhanced Uptake and Activation in TLR9-positive Macrophages

Wenqing Liao, Mengmeng Tan, Kosuke Kusamori, Yoshinobu Takakura, Makiya Nishikawa

Submitted (under revision)

Enhanced Immunostimulatory Activity of CpG Oligodeoxynucleotide by Combination of Mannose Modification and Incorporation into Nanostructured DNA

Wenqing Liao, Sakiko Akahira, Rintaro Iwata Hara, Takeshi Wada, Kosuke Kusamori, Yoshinobu Takakura, Makiya Nishikawa

Submitted

Development of Mannose-modified G-quadruplex Structured CpG ODN for Targeted Delivery to Antigen-presenting Cells

Wenqing Liao, Kosuke Kusamori, Yoshinobu Takakura, Makiya Nishikawa

Manuscript in preparation

Other Publications

Nanostructured DNA for the delivery of therapeutic agents

Makiya Nishikawa, Mengmeng Tan, Wenqing Liao, Kosuke Kusamori

Adv. Drug Del. Rev., **147**, 29-36 (2019).

Photo-assisted Fixation of CO_2 onto Aryl Bromides Producing Aromatic Esters

Naoki Ishida, Yusuke Masuda, Wenqing Liao, and Masahiro Murakami

Chem. Lett., **48**, 1316-1318 (2019).

Buttressing Salicylaldehydes: A Multipurpose Directing Group for C(sp^3)-H Bond Activation

Akira Yada, Wenqing Liao, Yuta Sato, and Masahiro Murakami

Angew. Chem. Int. Ed., **56**, 1073-1076 (2017).

Synergistic Pd/Enamine Catalysis: A Strategy for the C−H/C−H Oxidative Coupling of Allylarenes with Unactivated Ketones

Shan Tang, Xudong Wu, Wenqing Liao, Kun Liu, Chao Liu, Sanzhong Luo, Aiwen Lei

Org. Lett., **16**, 3584-3587 (2014).

Revealing the metal-like behavior of iodine: an iodide-catalysed radical oxidative alkenylation

Shan Tang, Yong Wu, Wenqing Liao, Ruopeng Bai, Chao Liu, Aiwen Lei

Chem. Comm., **50**, 4496-4499 (2014).

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