

# Structure-dependent immunostimulatory effect of CpG oligodeoxynucleotides and their delivery system

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**Abstract:** Unmethylated cytosine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODNs) are recognized by Toll-like receptor 9 (TLR9) found in antigen-presenting cells and B cells and can activate the immune system. Using CpG ODNs as an adjuvant has been found to be effective for treating infectious diseases, cancers, and allergies. Because natural ODNs with only a phosphodiester backbone are easily degraded by nuclease (deoxyribonuclease [DNase]) in serum, CpG ODNs with a phosphorothioate backbone have been studied for clinical application. CpG ODNs with a phosphorothioate backbone have raised concern regarding undesirable side effects; however, several CpG ODNs with only a phosphodiester backbone have been reported to be stable in serum and to show an immunostimulatory effect. In recent years, research has been conducted on delivery systems for CpG ODNs using nanoparticles (NPs). The advantages of NP-based delivery of CpG ODN include (1) it can protect CpG ODN from DNase, (2) it can retain CpG ODN inside the body for a long period of time, (3) it can improve the cellular uptake efficiency of CpG ODN, and (4) it can deliver CpG ODN to the target tissues. Because the target cells of CpG ODN are cells of the immune system and TLR9, the receptor of CpG ODN is localized in endolysosomes, CpG ODN delivery systems are required to have qualities different from other nucleic acid drugs such as antisense DNA and small interfering RNA. Studies until now have reported various NPs as carriers for CpG ODN delivery. This review presents DNase-resistant CpG ODNs with various structures and their immunostimulatory effects and also focuses on delivery systems of CpG ODNs that utilize NPs. Because CpG ODNs interact with TLR9 and activate both the innate and the adaptive immune system, the application of CpG ODNs for the treatment of cancers, infectious diseases, and allergies holds great promise.

**Keywords:** Toll-like receptor 9 (TLR9), immunostimulation, higher-order nanostructure of DNA, delivery system, nanoparticles

## Introduction

Unmethylated cytosine-phosphate-guanosine (CpG) dinucleotide is recognized by Toll-like receptor 9 (TLR9) and induces immune response. Immune activity in DNA was first discovered when a DNA fraction of Bacille Calmette-Guérin was found to produce type I interferon (IFN), leading to the activation of natural killer cells; the antitumor effect of this induction was recognized.<sup>1</sup> Krieg et al<sup>2</sup> elucidated that immune response is caused only when CpG is included in the DNA, and that immune response is inactivated when the cytosine residue is methylated. This CpG sequence is found with high frequency in bacterial DNA, and only occasionally in mammalian DNA. Because CpG in mammalian DNA is methylated, it is believed that the recognition of unmethylated CpG sequence is an action by the immune system to recognize the DNA

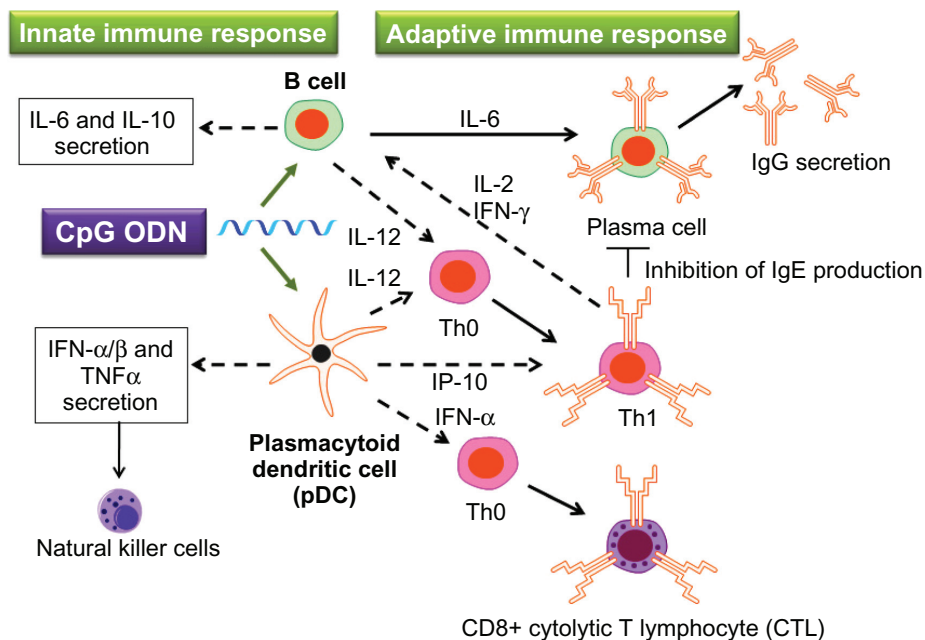
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of bacteria and eliminate it. It was then discovered that TLR9 is the receptor of DNA containing unmethylated CpG.<sup>3</sup>

In human beings, TLR9 is mainly expressed by B cells and plasmacytoid dendritic cells (pDCs).<sup>4</sup> CpG stimulates these cells and induces innate and adaptive immune responses (Figure 1). B cells whose TLR9 is activated by CpG secrete cytokines important to the innate immune system – including IL-6, IL-10, and IL-12 – using nuclear factor-kappa B and other signal transduction pathways.<sup>2,5,6</sup> IL-6 and IL-12 secreted from B cells are also involved in adaptive immune response. IL-6 promotes the multiplication and activation of B cells; as a result, the production of antibodies is enhanced.<sup>7,8</sup>

pDCs whose TLR9 is activated by CpG secrete cytokines involved in innate immune response, including type I IFNs and tumor necrosis factor-alpha (TNF $\alpha$ ).<sup>9</sup> These pDCs also activate natural killer cells.<sup>10</sup> Furthermore, pDCs with activated TLR9 secrete IL-12 and promote the differentiation of T helper (Th) 0 into Th1,<sup>11–13</sup> as well as inducing Th1 to migrate to B cells through the actions of IFN- $\gamma$ -inducible protein of 10 kDa (IP10).<sup>14,15</sup> B cells that interact with Th1 differentiate into plasma cells, which possess the ability to produce antibodies, playing a central role in adaptive immunity. Also, IFN- $\alpha$  promotes CD8-positive cytotoxic T lymphocyte response.<sup>16,17</sup>

Because immune response mediated by the activation of TLR9 induces not only the innate immune system but also the adaptive immune system, its application for treating illnesses including infectious diseases, cancers, allergies, and asthma holds great promise.<sup>18–21</sup> Until now, various CpG oligodeoxynucleotides (ODNs) have been developed to induce immune response via the activation of TLR9. What is most important in the clinical application of CpG ODN is protecting CpG ODN from DNase and delivering CpG ODN to the TLR9 of pDCs. Chemical modification of CpG ODN is an effective technique to protect against degradation by DNase. However, several severe side effects caused by the modification of DNA backbone have been reported. For example, repeated administration of backbone-modified CpG ODNs has resulted in reduced immune responses, lymphoid follicle destruction, and organ enlargement.<sup>22</sup> DNase-resistant natural CpG ODNs consisting entirely of phosphodiester backbone are, therefore, desirable for clinical application, but so far most clinical trials have been conducted using backbone-modified CpG ODNs. Encapsulating CpG ODN and sealing it inside nanoparticles (NPs) is also an effective method to protect ODN against break down by DNase. NPs may make it possible to use naturally occurring CpG ODNs in clinical applications.



**Figure 1** Immunostimulatory effect of cytosine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODNs).

**Notes:** The immunomodulatory cascade triggered by CpG ODNs includes the activation of T helper 1 (Th1) cells and secretion of proinflammatory cytokines such as interleukin (IL)-6, IL-12, and interferon gamma (IFN- $\gamma$ ). The CpG motifs in either bacterial DNA or synthetic CpG ODNs act as "danger signals" to the innate immune system, triggering a protective immune response against the pathogen. In addition, the adaptive immune response mounted by the host afterward will maintain an immunologic memory and provide long-lasting protection.

**Abbreviation:** TNF $\alpha$ , tumor necrosis factor-alpha.

Delivery systems for CpG ODNs using NPs as carriers differ greatly from delivery systems for anticancer drugs and nucleic acid drugs such as antisense DNA and small interfering RNA (siRNA). For delivery of anticancer drugs using NPs, the NPs must be delivered from the bloodstream to the cancerous tissues through vascular walls. Therefore, NPs of around 100 nm in size are required. Also, manipulation is required so that immune cells do not capture the NPs before they arrive at cancerous tissues. The target cells for the delivery of CpG ODN, on the other hand, are antigen-presenting cells (APCs) and B cells. These immune cells easily take up relatively large particles, greater than 100 nm in size.<sup>23</sup> For delivery of antisense DNA and siRNA, after they have been taken up by cells as a result of endocytosis, their nucleic acids must move from the endosome to the nucleus. However, with the delivery of CpG ODN, because the receptor TLR9 is localized in the endosome, CpG ODNs must be retained in the endosome for a long period of time. Therefore, delivery systems using CpG ODNs require a design strategy different from that of conventional drug delivery systems.

This review summarizes the structural features that depend on base sequences of CpG ODNs consisting of phosphorothioate and phosphodiester backbones and considers their relationship to the capacity of immune mediator cytokine induction. In addition, this review also considers the advantages and disadvantages in a delivery system of these CpG ODNs using various NPs as carriers

and describes the possible future direction of studies on CpG ODNs.

## Synthetic CpG ODNs with various structures and their immunostimulatory effect DNase-resistant CpG ODNs for TLR9 activation

Because ODNs that contain CpG motifs are quickly degraded by DNase, research has been conducted on CpG ODNs resistant to DNase.<sup>24-30</sup> DNase-resistant CpG ODNs consisting of a phosphorothioate backbone have been developed by replacing the oxygen in the phosphate group of the nucleic acid targeted by DNase.<sup>24,25,31</sup> These chemically modified synthetic CpG ODNs are divided into at least four classes (Table 1 and Figure 2).

Class A (also known as type D) CpG ODN has a naturally occurring phosphodiester backbone and palindromic CpG motifs at the center of its sequence. Poly(G) sequences on phosphorothioate backbones are attached to the 3' and 5' ends.<sup>32-34</sup> This class of CpG ODN activates the TLR9 of pDCs and induces IFN- $\alpha$ . However, it almost never induces the multiplication of B cells. The entire sequence of class B (type K) CpG ODN consists of a phosphorothioate backbone.<sup>33-36</sup> This class of CpG ODN induces the proliferation and activation of B cells. However, its ability to induce IFN- $\alpha$  with pDCs is low.<sup>37</sup> Class C includes

**Table 1** Features of each class of cytosine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODNs)

	Class A (type D)	Class B (type K)	Class C	Class P
ODN structure	Central phosphodiester region containing one or more CpG motifs in a palindrome and 5' and/or 3' ends consisting of poly(G) motifs with phosphorothioate backbone	Completely phosphorothioate backbone	One or more 5' CpG motif(s) and a 3' palindrome	Two palindromes consisting of phosphorothioate backbone
Examples	ODN2216 (for human) ODN2336 (for human) ODN1585 (for mouse)	ODN2006 (also known as PF-3512676 and CpG7909, for human) ODN1668 (for mouse) ODN1826 (for mouse)	ODN2395 (for human and mouse) ODN M362 (for human and mouse)	ODN21798
Mainly stimulated cell types	pDCs	B cells	pDCs and B cells	pDCs
Actions	Innate immune responses: IFN- $\alpha$ , TNF $\alpha$ , and IL-12 secretion  Adaptive immune responses: IL-12 and IP10 secretion	Innate immune responses: IL-6, IL-10, and IL-12 secretion  Adaptive immune responses: antibody production; IL-6 and IL-12 secretion	Intermediate between the A and B classes	Potency for IFN- $\alpha$ secretion is higher than that of CpG ODN in class C

**Abbreviations:** IFN $\alpha$ , interferon-alpha; IL, interleukin; IP10, interferon-gamma-inducible protein of 10 kDa; pDC, plasmacytoid dendritic cell; TNF $\alpha$ , tumor necrosis factor-alpha.

## Class-A ODN2216

5'-G-G-G-G-G-A-C-G-A-T-C-G-T-C-G-G-G-G-G-G-3'

## Class-A ODN2336

5'-G-G-G-G-A-C-G-A-C-G-T-C-G-T-G-G-G-G-G-G-3'

## Class-B ODN2006

5'-T-C-G-T-C-G-T-T-T-T-G-T-C-G-T-T-T-T-G-T-C-G-T-T-3'

## Class-C ODN2395

5'-T-C-G-T-C-G-T-T-T-T-C-G-G-C-G-C-G-C-G-C-G-3'

## Class-P ODN21798

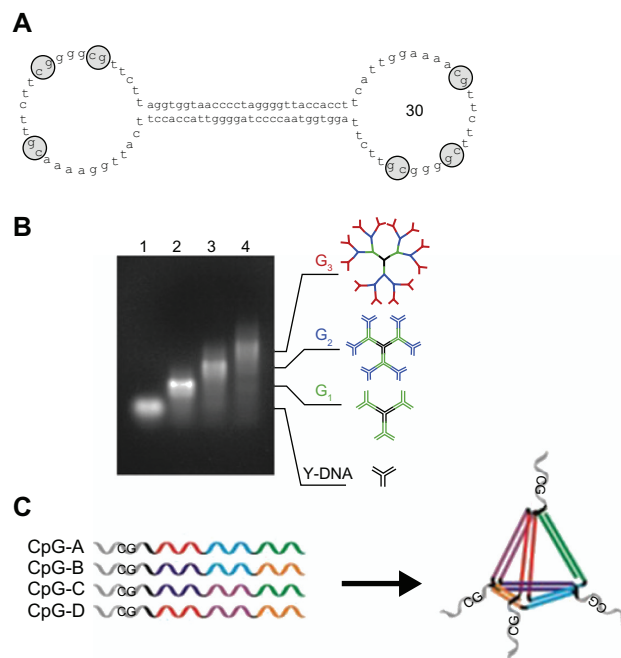
5'-T-C-G-T-C-G-A-C-G-A-T-C-G-G-C-G-C-G-C-G-C-G-3'

**Figure 2** Features of cytosine-phosphate-guanosine oligodeoxynucleotide (ODN) sequences in each class.

**Note:** Underlining indicates palindromic sequence; black and red hyphens indicate phosphodiester and phosphorothioate bonds, respectively.

one or two CpG motif(s) with a phosphodiester backbone at the 5' end, and contains a palindromic sequence on a phosphorothioate backbone at the 3' end. This class of CpG ODN has the capacity to induce the proliferation of B cells and the production of IFN- $\alpha$  via pDCs; it has a quality between classes A and B.<sup>38-41</sup> Also, class P CpG ODN has two palindromic motifs on phosphorothioate backbones. It displays a high capacity for producing IFN- $\alpha$  and the ability to activate nuclear factor-kappa B.<sup>42</sup>

These classes of CpG ODN include a complete or partial phosphorothioate backbone, so that they are resistant to DNase. However, side effects are a cause for concern. Reports of side effects include the prolongation of coagulation time due to the inhibition of the tenase complex,<sup>43</sup> distribution of cell signaling due to nonspecific adsorption of proteins including transcription factors,<sup>44</sup> and acute toxicity due to complement activation.<sup>45,46</sup> These classes of CpG ODN have also been reported to cause renal damage.<sup>47,48</sup> Therefore, ideally, naturally occurring CpG ODNs consisting only of phosphodiester backbone is desired. Because DNA is mainly decomposed by exonucleases, it is believed that a ring structure like a plasmid is difficult to break down. When DNA with only a phosphodiester backbone was formed into a dumbbell-like structure, it certainly displayed resistance to DNase.<sup>49-51</sup> Schmidt et al<sup>52</sup> synthesized an ODN with a phosphodiester backbone in a dumbbell-like structure designed to include CpG motifs in hairpin-loops (Figure 3A). By utilizing the actions of peripheral blood mononuclear cells, immunostimulatory effects similar to linear-structure CpG ODNs with phosphorothioate backbones at both ends were obtained. Immunostimulatory activity that induces TNF- $\alpha$  and IL-6 is found in Y-shaped DNA (Y-DNA) itself, formed



**Figure 3** Structures of cytosine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODNs) consisting of entirely phosphodiester backbone. **(A)** Sequence and structure of CpG ODN with a dumbbell-like structure: the CpG ODN with a dumbbell-like structure has 30 nucleotides in both loops that contain three CpG dinucleotide motifs.<sup>a</sup> **(B)** Structure of dendrimer-like DNA: Y-shaped DNA consists of three single-stranded DNA with 30 nucleotides containing CpG dinucleotide motifs; G1, G2, and G3 dendrimer-like structures were synthesized by ligation of Y-shaped DNA; the sizes of CpG ODNs with G1, G2, and G3 dendrimer-like structures were about 12, 20, and 36 nm, respectively.<sup>b</sup> **(C)** Assembly of CpG bearing DNA tetrahedral nanostructure: the core tetrahedral nanostructure consists of assembly with four 55-mer ODNs. The CpG motif is linked to each ODN via a 7-mer oligothiamine spacer.<sup>c</sup>

<sup>a</sup>Reproduced with permission from Schmidt et al;<sup>52</sup> <sup>b</sup>reproduced with permission from Rattanakiat et al<sup>30</sup>; <sup>c</sup>reproduced with permission from Li et al.<sup>55</sup>

from three single-strand DNA having naturally occurring phosphodiester backbones.<sup>53</sup> DNA with branch structures, like Y-DNA, has outstanding cellular uptake efficiency. However, it has no resistance to DNase. Rattanakiat et al<sup>30</sup> discovered that dendrimer-like DNA (DL-DNA) (Figure 3B) with phosphodiester backbones, formed by linking Y-DNA containing CpG motif, has high immunostimulatory activity. One of the causes of this high activity is believed to be DL-DNA's resistance to DNase. Recently, Nishikawa et al<sup>54</sup> observed that TNF- $\alpha$  release from RAW264.7 cells at 8 hours after stimulation by CpG motifs contained X-shaped DNA consisting entirely of phosphodiester backbone. This suggests that X-shaped DNA was stable for 8 hours at least, although Y-DNA has no resistance to DNase. Li et al<sup>55</sup> synthesized the CpG-bearing DNA tetrahedral nanostructure with only a phosphodiester backbone (Figure 3C). The core tetrahedral structure comprised four 55-mer ODNs self-assembled with one another by an annealing process. CpG motif sequence was linked to each ODN via a 7-mer oligothiamine spacer.

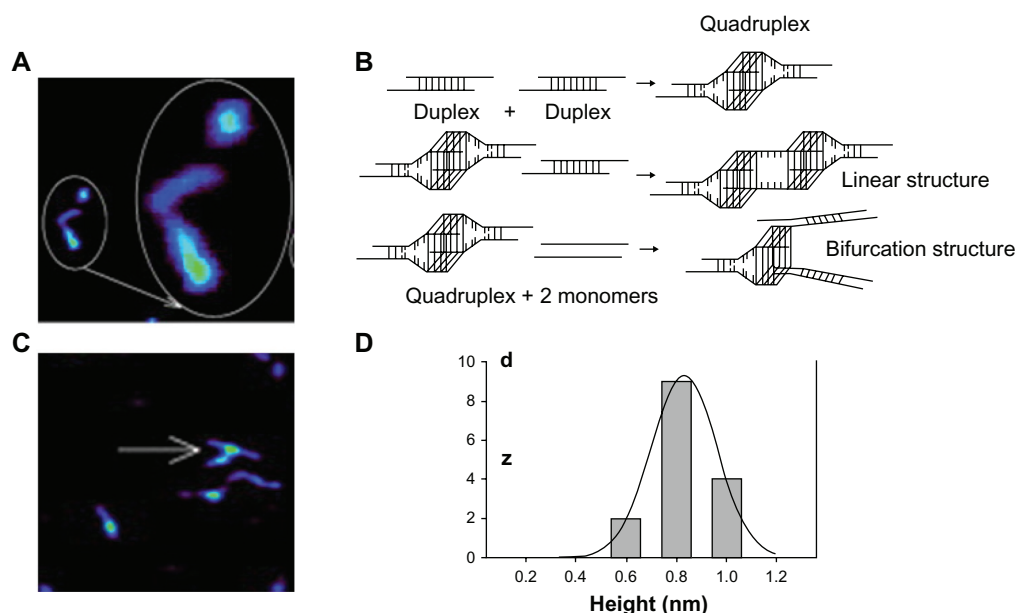
The CpG-bearing DNA tetrahedral nanostructure was efficiently taken up into macrophage-like cells, and induced various pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, and IL-12 through TLR9 activation. Li et al<sup>55</sup> also suggested that the stimulatory effect of the tetrahedral nanostructure is due to resistance to DNase, because the tetrahedral nanostructure DNA was stable in serum for 8 hours.

Furthermore, Meng et al<sup>56</sup> discovered that linear-structured CpG ODNs consisting only of a phosphodiester backbone possess high TLR9 activation capacity. TLR9 activation by class B ODNs is most optimal when there are two to four CpG motifs. When four or more CpG motifs were linked, ODN consisting only of a phosphodiester backbone remarkably improved its resistance against DNase. The ODN contained nine or more CpG motifs, remained largely intact in serum for more than 24 hours, and possessed high TLR9 activation capacity even in low concentrations. Because DNA administered inside the body is cleaved from the 3' end by exonuclease, when the 3' end of CpG ODN with only a phosphodiester backbone was modified, its resistance to DNase increased.<sup>56</sup> When multiple CpG motifs are linked, even when the 3' end is cleaved by exonuclease, the activation capacity of TLR9 can be maintained when the cell is acted on because many CpG motifs still remain.

## Structure-dependent immunostimulatory effect of synthetic CpG ODNs

Class A CpG ODNs induce the production of IFN- $\alpha$  by activating the TLR9 of pDC. However, class B ODNs do not induce the production of IFN- $\alpha$  via pDCs. This shows that the action of CpG ODNs is dependent on base sequence and structure.

Class A CpG ODNs form nanometer-sized multimers under certain physiological conditions and take on globular and linear structures (Figure 4A).<sup>57</sup> This globular structure is split into two forks.<sup>57,58</sup> This is because the structure forms a duplex because of the palindromic sequence of base pairs at the center of the ODN. Next, the four poly(G) sequences at the end of these duplexes combine with one another because of Hoogsteen base pairing and become a G-tetrad structure. As a result, they become G-quadruplex structures (Figure 4B). G-quadruplex can form a linear structure when it further forms a G-tetrad with another duplex (Figure 4B). Also, when two strands of monomeric CpG ODN combine with the G-quadruplex, there is a possibility that two-forked NPs can form (Figure 4C). In other words, class A CpG ODNs spontaneously form higher-order structures because of their palindromic sequence and poly(G). For class A CpG ODN2216, in the case of linear structure, its length is more than 100 nm, and in the case of globular structure, the maximum



**Figure 4** Formation of higher-order structure in class A oligodeoxynucleotides (ODNs). **(A)** Globular structure of class A cytosine-phosphate-guanosine (CpG) ODN observed by atomic force microscopy (large circled structure is a close-up view of small circle). **(B)** Possible higher-order structure formation of class A CpG ODNs. Class A CpG ODNs comprise a palindromic sequence in the center and poly(G) sequences at both the 5' and the 3' ends. Two monomer molecules form a duplex that is attributed to palindromic sequences. Two duplexes further form a quadruplex through G-tetrad formation of four poly(G) ends. Association of the quadruplex with another duplex causes a linear structure. Two other CpG monomers replace the original duplex by forming two new duplexes, which leads to formation of a bifurcation with three ends. **(C)** A bifurcation structure of class A CpG ODNs imaged by atomic force microscopy. **(D)** Height histogram of higher-order structure in class A CpG ODNs. Reproduced with permission from Klein et al.<sup>58</sup>

size is 50 nm.<sup>57</sup> Also, class A CpG ODN2336 has an average height of  $0.8 \pm 0.1$  nm (Figure 4D) and length of 30–70 nm.<sup>58</sup> Meanwhile, such higher-order structures are not observed in class B CpG ODNs; they form a linear structure.<sup>58</sup>

Because poly(G) binds with scavenger receptors on the cell surface,<sup>59</sup> ODNs that contain poly(G) sequences have been reported to show an increase in cellular uptake efficiency.<sup>34,60</sup> This suggests that the high cellular uptake efficiency of class A CpG ODNs with poly(G) also means a high TLR9 activation capacity. On the other hand, Kerkmann et al<sup>57</sup> reported that the cellular uptake efficiency of class A and class B CpG ODNs did not change. They suggested that the greater ability of class A CpG ODNs to produce IFN- $\alpha$  than class B CpG ODNs is because of the higher-order structure of class A CpG ODNs. This is because when the palindromic sequence is changed, higher-order structures are not formed, and the ability to produce IFN- $\alpha$  is decreased. Furthermore, it has been observed that when class B CpG ODNs were loaded onto polystyrene NPs 180 nm in diameter, their production capacity of IFN- $\alpha$  was greater than that of class A CpG ODNs.<sup>57</sup> Because the cellular uptake efficiency of free class B CpG ODNs loaded onto polystyrene NPs do not change, it is believed that the high IFN- $\alpha$  production capacity of class A CpG ODNs is due to its spontaneously formed higher-order structure. Other research groups have reported that by artificially causing class B CpG ODNs to form higher-order structures using NPs, an immune profile similar to class A CpG ODNs could be obtained.<sup>61–63</sup> Class C CpG ODNs also form a duplex, because of the palindromic sequence at the 3' end. This structure is believed to affect the activation of TLR9.

Naturally occurring DL-DNA containing CpG motifs synthesized as Y-DNA structural units displayed high immunostimulatory activity.<sup>30</sup> The hydrodynamic size of Y-DNA formed from three-strand 30-base ODN was  $7.0 \pm 0.2$  nm; however, second- and third-generation DL-DNA (Figure 3B) were  $20 \pm 1.2$  nm and  $35.8 \pm 3.2$  nm, respectively. Receptor-mediated endocytosis is dependent on the size of ligands, and the optimal size for uptake is known to be 25–30 nm.<sup>64–67</sup> The size of DL-DNA falls within this range, so its high immunostimulatory activity may have an effect on cellular uptake efficiency in addition to resistance to DNase. What is extremely interesting is that Y-DNA has immunostimulatory activity even if it does not contain CpG motifs.<sup>54</sup> This suggests the importance of the higher-order structure of ODNs on immunostimulation.

Meng et al<sup>56</sup> reported that ODNs consisting only of a phosphodiester backbone and linked with numerous

CpG motifs exhibited high TLR9 production capacity. CpG ODN2006, a class B prototype, includes three CpG motifs. PD-ODN2006 is synthesized with a phosphodiester backbone, not the phosphorothioate backbone of the original ODN2006 sequence, and it has no TLR9 activation capacity because DNase degrades it. PD-ODN2006-2006, composed of two PD-ODN2006s linked together, is not degraded much by DNase and possesses high TLR9 activation capacity (Figure 5). When the size of the DNA is below 250 bases, cellular uptake efficiency increases as the DNA size increases.<sup>68</sup> However, a sequence that indirectly connects PD-ODN2006s using a 14-mer ligand sequence without a CpG dinucleotide sequence (PD-2006-linker-2006) showed lowered TLR9 activation capacity (Figure 5). PD-ODN2006-2006 and PD-ODN2006-linker-2006 both contain six CpG motifs. The difference in TLR9 activation capacity despite this characteristic suggests that aside from the number of CpG motifs and size, TLR9 activation is also dependent on the ODN sequence.

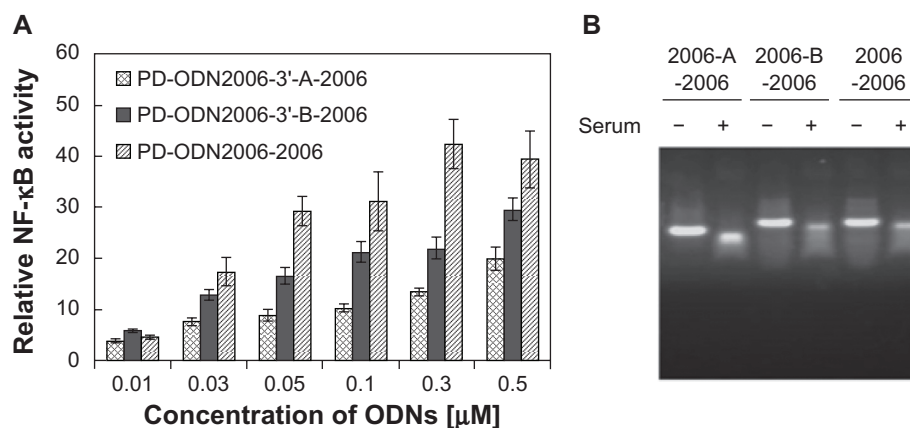
## Delivery of CpG ODNs using NPs

Control of the immune system via TLR9 by CpG ODNs has been shown to be effective for treating infectious diseases, cancers, and allergies. In recent years, various NPs have been developed as carriers of CpG ODNs. The number of papers related to the delivery of CpG ODNs using NPs has increased sharply since 2007.

The advantages of using NPs as CpG ODN carriers include (1) protection from DNase degradation, (2) extension of retention time inside the body, (3) decrease in the amount administered because cellular uptake efficiency is improved, (4) the ability to change the structure of CpG ODNs, (5) the ability to deliver to target tissues, (6) the ability to change localization inside the body, and (7) allow the slow release of CpG ODNs over a long period of time.

## Protection of CpG ODNs from degradation by DNase

Concerning the protection of CpG ODNs from DNase by NPs, many research studies use CpG ODNs with a phosphorothioate backbone resistant to DNase, so there is little direct evidence available. Because antisense ODNs encapsulated by cationic lipid NPs or lipid NPs have been reported to be partially or completely protected from DNase degradation in serum,<sup>69,70</sup> CpG ODNs with only phosphodiester backbone are believed to have similar effects when encapsulated by these nanoparticulates. Zhu et al<sup>71</sup> showed that CpG ODNs with only a phosphodiester backbone



**Figure 5** Effect of linker sequences in phosphodiester (PD) cytosine-phosphate-guanosine (CpG) oligodeoxynucleotide 2006 (ODN2006) connection on Toll-like receptor 9 (TLR9) activation.<sup>3</sup> (Original ODN2006 consists entirely of phosphorothioate backbone, but the backbones of all the CpG ODNs used in this experiment were replaced by naturally occurring phosphodiester bonds.) (A) Activation of TLR9 represented by nuclear factor-kappa B (NF-κB) activity: 293XL-hTLR9 cells were stimulated by series connection of PD-ODN2006 with and without linker sequences. PD-ODN2006-2006, composed of two phosphodiester ODN2006s directly linked together, showed higher capacity for TLR9 activation than PD-ODN2006-A-2006 and PD-ODN2006-B-2006, whose sequences consist of indirectly connected phosphodiester ODN2006s using 14-mer linkers (sequence of linker A, CCTTCAGTGGGACC; sequence of linker B, GGTCCCACTGAAGG). (B) Stability of these CpG ODNs consisting of entirely phosphodiester backbone in solution with and without serum imaged by gel electrophoresis: these CpG ODNs consisting entirely of phosphodiester backbone were resistant to deoxyribonuclease in serum.

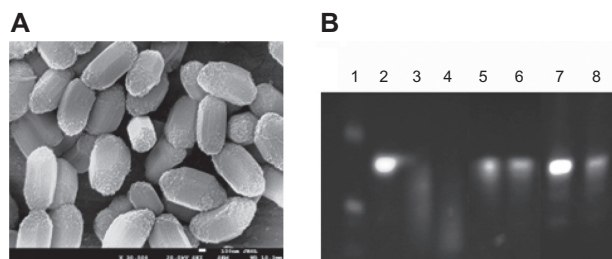
**Notes:** \*The sequence of ODN2006 that contains three CpG motifs is shown in Figure 2. Reproduced with permission from Meng et al.<sup>56</sup>

joined electrostatically to the surface of NPs acquired resistance to DNase degradation. They observed that all molecules of CpG ODNs with a completely phosphodiester backbone were broken down within an hour in 20% serum. However, CpG ODNs attached electrostatically to mesoporous silica NPs, the surface of which were modified with amino groups, remained after 3 hours without being degraded (Figure 6). Furthermore, when the conjugate of these NPs and CpG ODNs was coated with poly(allylamine hydrochloride), the efficiency of protection against DNase was discovered to increase. In vivo mice experiments have

shown that 272 nm cationic poly(D,L-lactic-co-glycolic acid) (PLGA) NPs were taken up into pDCs in less than an hour,<sup>72</sup> so that protection of CpG ODNs against DNase by NPs for several hours is considered to be sufficient. The greatest advantage of using NPs is protection against DNase, as CpG ODNs consisting of chemically unmodified phosphodiester backbone can be used.

### Prolonged circulation time and increased cellular uptake

In vivo experiments have reported that NPs prolong the circulation lifetime of CpG ODNs in the body. Pan et al.<sup>73</sup> included a CG sequence in an 18-mer antisense ODN for controlling the Bcl-2 gene and discovered that this could activate the immune system. When free antisense ODN with phosphorothioate backbone was administered intravenously to mice, only 1% remained in plasma after 24 hours. When this antisense ODN was encapsulated in lipid NPs (89 ± 45 nm in diameter) composed of 3β-[N,N-(dimethylaminoethane)carbamoyl] cholesterol (DC-Chol), egg yolk phosphatidylcholine, distearoylphosphatidylethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG<sub>2000</sub>-DSPE), and protamine, 25% of the ODN remained in plasma after 24 hours. This shows that encapsulation in lipid NPs extends blood circulation time. DC-Chol, a cationic lipid, partially deprotonates in near-neutral pH and decreases the surface electrical charge of NPs. Also, mPEG<sub>2000</sub>-DSPE on the surface of NPs decreases the uptake of NPs by



**Figure 6** Mesoporous silica nanoparticles (NPs) for delivery of natural cytosine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODNs) consisting entirely of phosphodiester backbone. (A) Scanning electron microscopic image of amino-modified mesoporous silica SBA-15 particles. (B) Stability of CpG ODNs consisting entirely of phosphodiester backbone in 20% serum containing medium: lane 1, DNA marker; lane 2, undigested CpG ODN consisting entirely of phosphorothioate backbone; lanes 3 and 4, free natural CpG ODN after incubation for 1 and 3 hours; lanes 5 and 6, natural CpG ODN loaded on mesoporous silica NPs after incubation for 1 and 3 hours; and lanes 7 and 8, natural CpG ODN loaded on mesoporous silica NPs followed by polycation (poly(allylamine hydrochloride)) treatment after incubation for 1 and 3 hours. This indicates that natural CpG ODN consisting entirely of phosphodiester backbone loaded on mesoporous silica NPs was stable in serum. Reproduced with permission from Zhu et al.<sup>71</sup>

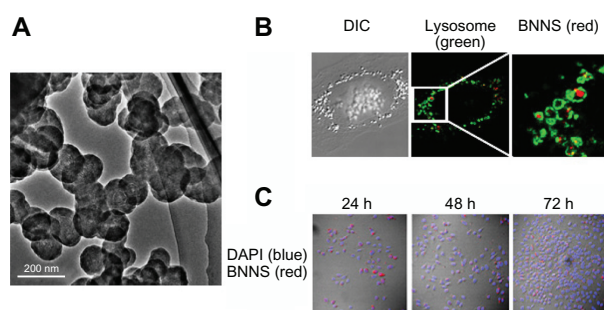
the reticuloendothelial system. These actions are believed to prolong the blood circulation time. When this lipid NP-encapsulated antisense ODN was administered, the cumulative dose to tumorous tissues was about nine times that of free ODN. Traditional cationic lipid NPs are quickly eliminated from the circulatory system,<sup>45</sup> but if the electrical charge on the NPs' surface is reduced, as in the case of DC-Chol, circulation time can be extended. Wilson et al<sup>27</sup> discovered neutral cationic lipid NPs ionizable at physiological pH. When ionizable cationic lipid NPs and ODN are mixed under a low pH condition, the surface of the NPs becomes charged, and the ODN is electrostatically attached. Next, when these NPs are transferred to 40% ethanol, the structure of the NPs becomes unstable and ODN is taken up into the interior of the NPs. Afterwards, when the pH is made neutral, the NPs become neutral NPs. In contrast to the free ODN circulation lifetime of a few minutes, the circulation lifetime of ODN encapsulated in ionizable cationic lipid NPs can be extended from several hours to several days.<sup>74,75</sup>

One of the reasons that CpG ODNs adsorbed into NPs or with encapsulated phosphorothioate backbone show greater TLR9 activity than free CpG ODNs is that the cellular uptake efficiency of CpG ODNs is improved. The cellular uptake efficiency of CpG ODNs is being studied using fluorescently labeled CpG ODNs. When fluorescently labeled free CpG ODN was added to cultured 239XL-TLR9 cells, fluorescence was not observed inside the cells. In contrast, strong fluorescence was observed in CpG ODN adsorbed by mesoporous silica NPs and boron nitride NPs (Figure 7).<sup>71,76</sup> In vivo studies have shown the method of delivering CpG ODN using lipid NPs significantly increased uptake efficiency

compared with free CpG ODN when it was administered to mice and the uptake into APCs in the spleen and lymph nodes was observed after 24 hours.<sup>27</sup> Also, when CpG ODNs were loaded onto cationized gelatin NPs with an average diameter of  $272 \pm 33.3$  nm, after 2 days DCs in draining lymph nodes were 30% CpG positive and B cells were 20% positive.<sup>77</sup> The cause is believed to be the increase in the delivery efficiency of CpG ODNs by NPs, with many CpG ODNs being adsorbed or encapsulated per NP. Klier et al<sup>78</sup> also reported that class A CpG ODN delivered by gelatin NPs showed significant immunomodulation effect (Th2/Th1 shift) on equine bronchoalveolar lavage cells. This effect is believed to be an enhancement of cellular uptake of CpG ODNs by gelatin NPs.

Clathrin-mediated endocytosis, caveolae-mediated uptake, phagocytosis, macropinocytosis, and clathrin- and caveolae-independent endocytosis are all possible methods for the uptake of lipid NPs into cells. The specific method depends on the type of cells.<sup>27</sup> When free CpG ODNs were charged negatively, it was difficult for them to attach to a negatively charged cell surface. This electrostatic repulsion is believed to limit the efficiency of free CpG ODN uptake.

The size of NPs affects both cellular uptake and TLR9 activation. Foged et al<sup>23</sup> investigated phagocytic activity on DCs by polystyrene NPs of various sizes from 0.04 to 15  $\mu\text{m}$ , and reported that the size of 500 nm was optimal for uptake into DCs. Also, when the NP size was greater than 200 nm, it was reported that the retention time in the endosome was long. However, NPs form aggregates or agglomerates in solution. When NPs form aggregates or agglomerates, cells take in not only NPs by themselves but also NPs in aggregates or agglomerates. Therefore, not the primary size of NPs but their hydrodynamic size should be used as the indicator of the effects of the size of NPs on cellular uptake.



**Figure 7** Boron nitride nanospheres (BNNSs) as carrier for cytosine-phosphate-guanosine oligodeoxynucleotide delivery: **(A)** transmission electron microscopic image of BNNSs; **(B)** localization of BNNSs taken up into cell (BNNSs [red] are localized in endolysosome [green]); **(C)** distribution of BNNSs during cell proliferation. Cells and BNNS were stained with DAPI (blue) and rhodamine B (red), respectively. BNNSs (red) were observed even in divided cells that were incubated for 48 and 72 hours (h).

Reproduced with permission from Zhi et al.<sup>76</sup>

**Abbreviation:** DIC, differential interference contrast.

## Retention of NPs loaded with CpG ODNs in endolysosome

TLR9 joins with CpG ODNs in the endolysosome; therefore, it is important to retain the CpG ODN in the endolysosome. Vectors and siRNA delivery systems must deliver to the nucleus; therefore, breaking out from the endolysosome is necessary. However, for the delivery systems of CpG ODNs, because TLR9 exists in the endolysosome, breaking out from the endolysosome is not necessary but staying in is. Chen et al<sup>79</sup> coated the surface of polystyrene NPs with four types of cationic polymers – poly(ethylenimine), chitosan, poly(2-dimethyl-amino)ethyl methacrylate, and poly(L-lysine) – to cause CpG ODN to bind. They reported that



with poly(L-lysine), there was least escape of NPs from the endolysosome. This means that the type of polycation has an effect on the retention of NPs in the endosome. When Zhi et al<sup>76</sup> added boron nitride NPs to 293XL-TLR9 cells, it was localized in the lysosome after 24 hours. This localization was maintained even after the cell division (Figure 7).

## Sustained release of CpG ODNs

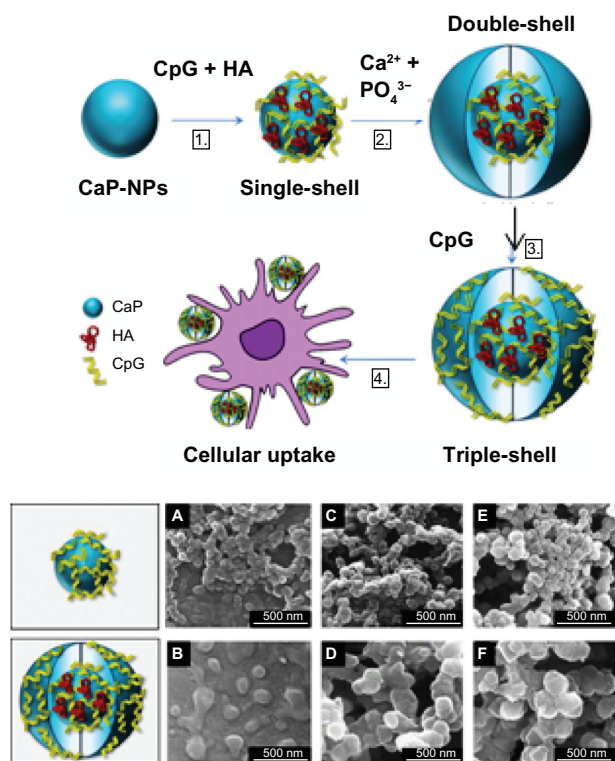
The increase in uptake efficiency of CpG ODNs and in their efficiency of delivery means that the dose of CpG ODN can be decreased. Furthermore, the retention of NPs in the endolysosome and lysosome leads to continuous effects by CpG ODN. Furthermore, the sustained release of CpG ODN by NPs also results in decreased dosage and continuous effects. Sokolova et al<sup>80</sup> prepared multishell NPs that causes CpG ODN to be adsorbed to calcium phosphate (Figure 8). By using a multishell structure, CpG ODN can be protected from DNase. At the same time, calcium phosphate gradually dissolves in the acidic environment of the lysosome's interior, so the slow release of CpG ODN can be expected. Zhu et al<sup>81</sup> have

reported on the enzyme-triggered sustainable release of CpG ODNs. They caused naturally occurring CpG ODN to be adsorbed by hollow mesoporous silica NPs with a laminated surface. Furthermore, they coated the surface with poly(L-lysine). By repeating the adsorption of this CpG ODN and poly(L-lysine), a layer-by-layer adsorption can be formed. By dissolving the NP surface of poly(L-lysine) with  $\alpha$ -chymotrypsin, CpG ODN together with low-molecular-weight compounds loaded in the hollow NPs can be slowly released (Figure 9). Demento et al<sup>72</sup> controlled the rate of release of CpG ODN from NPs by attaching biotinylated CpG ODN to the surface of PLGA NPs modified with avidin-palmitate. Usually 100  $\mu$ g of CpG ODN is administered per mouse, but with NPs, effects were observed with administration of 0.5  $\mu$ g. By minimizing the release of CpG ODN, side effects including autoimmunity and lymphoid architectural damage that occur when CpG ODN is given in large amounts can be decreased.<sup>22</sup>

## Delivery of CpG ODNs to target tissues

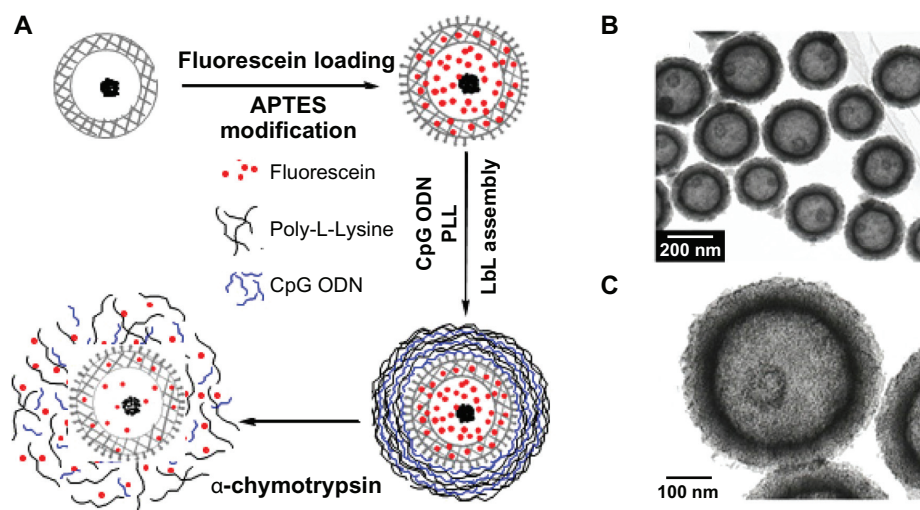
Bourquin et al<sup>77</sup> reported that CpG ODN loaded on cationized gelatin NPs had remarkably high antitumor effects compared with free CpG ODN in a mouse melanoma model. They investigated the localization of CpG ODN in vivo, and revealed that while free CpG ODN accumulated in splenocytes, CpG ODN delivered by NPs was selectively stored in APCs inside draining lymph nodes. The activation of adaptive immune response due to the selective transport to APCs inside draining lymph nodes by NPs is believed to be the cause for the high antitumor effect.

The size of NPs also has an effect on transferability into tissues. The molecular weight of drugs and the size of NPs are known to be factors determining the transferability into lymph nodes. Drugs with a molecular weight less than 5000 injected intramuscularly or subcutaneously are absorbed by capillaries and circulated. However, drugs with a molecular weight greater than 20,000 mainly transfer to lymph nodes.<sup>82</sup> The commonly used class B CpG ODN with phosphorothioate backbone has a molecular weight of about 8000, and its transferability to lymph nodes is low. For therapeutic NPs administered into the abdominal cavity of mice, it has been reported that NPs with sizes of 100–200 nm are excellent for transferring to lymph nodes and in retention.<sup>83</sup> Kuramoto et al<sup>84</sup> prepared liposomes 100–200 nm in size composed of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride, a cationic lipid, and cholesterol, to which CpG ODNs were loaded. When these NPs were administered to



**Figure 8** Multishell calcium phosphate (CaP) nanoparticles (NPs) for cytosine-phosphate-guanosine (CpG) delivery: (A) preparation of multishell CaP-NPs functionalized with CpG oligodeoxynucleotides and antigen (hemagglutinin, HA); (B) scanning electron micrographs of single-shell (top panels) and triple-shell NPs (bottom panels).

Reproduced with permission from Sokolova et al.<sup>80</sup>



**Figure 9** Hollow mesoporous silica/poly(L-lysine) (HMS/PLL) nanoparticles for codelivery of drug and cytosine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODNs): (A) preparation of the fluorescein and CpG ODN-loaded HMS/PLL particles and enzyme triggered release; (B and C) transmission electron microscopic images of HMS nanoparticles.

Reproduced with permission from Zhu et al.<sup>81</sup>

**Abbreviations:** APTES, aminopropyltriethoxysilane; LBL, layer-by-layer.

the abdominal cavity of mice with peritoneal dissemination, a pronounced antitumor effect was observed compared with CpG ODN alone.

By attaching specific ligands to the surface of NPs, it is possible to deliver them to target cells. Because macrophages and pDCs, which are target cells of CpG ODN, have mannose receptors, it is believed that introducing mannose to the surface of NPs can intensify the effects of CpG ODNs. Kuramoto et al<sup>85</sup> loaded CpG ODN on mannose-modified liposome, the surface of which was modified with mannose-modified cholesterol derivative cholesten-5-yloxy-N-(4-((1-amino-2- $\beta$ -D-thiomannosylbutyl)amino)alkyl)formamide, and administered it to the abdominal cavity of mice with peritoneal dissemination. As a result, by targeting the immune cells in the greater omentum and the mesentery, where numerous lymph nodes exist, increased antitumor effect was reported. Also, Chen et al<sup>86</sup> synthesized glycodendrimers with terminals consisting of  $\alpha$ -mannose and made this adsorb to the surface of boron nitride nanotubes. Because  $\alpha$ -mannose binds with the mannose receptor of macrophages, CpG ODN delivered by these NPs is selectively taken up into macrophages. Also, because glycodendrimer is recognized by lectins on the cell surface, cellular uptake efficiency can also be expected to increase.

## Multicomponent delivery system including CpG ODNs

When antigens and CpG ODNs are delivered at the same time, the advantages of both agents can be maximally

drawn out.<sup>87,31</sup> Furthermore, when delivering these agents simultaneously with NPs, it has been reported that using separate NPs to deliver each of the agents results in greater immunostimulatory effect.<sup>88–91</sup> This suggests that delivering antigens and CpG ODN to APCs at the same time is important.

Standley et al<sup>29</sup> encapsulated ovalbumin (OVA), and furthermore prepared acid-degradable NPs (CpG-OVA-NPs) based on a cross-linked polymer network that included CpG ODN. The size of these NPs was 200–500 nm, and the bonding amount of the encapsulated OVA and CpG ODN were 40 and 25  $\mu\text{g}/\text{mg}$  NPs for CpG ODN. In vitro assays showed CpG-OVA-NPs increased the amount of IL-12 secreted by APCs by 45 times compared with NPs encapsulating only OVA. Also, the induction of CD40, CD80, and CD86, indicators of DC activation also increased. However, co-delivery had no effect on major histocompatibility complex (MHC) I and MHC II. It has been reported that for in vivo assays, co-delivery can induce OVA-specific CD8 T-cell response. Lee et al<sup>92</sup> also showed the enhancement of MHC-restricted presentation of antigen by using biodegradable PLGA NPs loaded with CpG ODNs and OVA. Similar profound augmentation of anti-OVA-specific immune response was observed by co-delivery of OVA and CpG ODN using nanoliposomes.<sup>93</sup>

Nasal and intradermal vaccination is an attractive strategy for CpG ODN delivery. For this strategy in previous studies, mucoadhesive *N*-trimethyl chitosan NPs loaded with OVA and CpG ODN were prepared.<sup>94,95</sup> This delivery system induced a significantly higher level of IgG2a and increased the number of

OVA-specific IFN- $\gamma$  producing T cells in the spleen compared with NPs with only OVA, suggesting that the co-delivery of antigen and CpG ODN improves the immunostimulatory effect.

Many studies have reported on systems that incorporate CpG ODN and antigens in biodegradable NPs.<sup>96-101</sup> These systems co-encapsulate CpG with antigens in biodegradable NPs. Demento et al<sup>72</sup> minimized the release rate of CpG ODN by using biotin-avidin binding to attach CpG ODN to the surface of biodegradable PLGA NPs. CpG ODN-modified antigen-encapsulating PLGA NPs that encapsulated antigen peptides of the West Nile virus envelope protein were prepared. The average size of these NPs was 272 nm, and the encapsulated antigen peptides were about 4.7  $\mu\text{g}/\text{mg}$  NPs. These NPs were administered to mice, and their immunostimulatory effect was compared with Alhydrogel, which includes antigens and aluminum hydroxide (as adjuvant). The results showed that with Alhydrogel, the level of IgG1, associated with Th2-skewed response, was high. With CpG ODN-modified antigen-encapsulating PLGA NPs, the levels of IgG2a and IgG2b, involved in Th1-based response, were high. Concerning infection of the West Nile virus, the test group administered Alhydrogel had a 44% survival rate. The test group administered CpG ODN-modified antigen-encapsulating PLGA NPs had a 94% survival rate.

Intratumoral injection of PEGylated unilamellar liposomes bearing surface-conjugated anti-CD40 antibody and CpG ODN has demonstrated synergistic antitumor effects without dose-limiting inflammatory toxicity.<sup>102</sup> In addition, co-delivery of polyriboinosinic-polyribocytidylic acid (poly(I:C)), an agonist of CpG and TLR3, with CD40 ligand (CD40L), has been reported to intensify the antitumor effect of CD40L.<sup>103</sup> When vector-expressing CD40L (pSP-D-CD40L) was directly administered to mouse tumor together with CpG ODN and poly(I:C), antitumor activity was displayed. When pSP-D-CD40L was loaded onto poly( $\beta$ -amino esters), a cationic polymer, and polyethylenimine NPs, and delivered together with CpG ODN and poly(I:C), even greater antitumor effect was obtained. Wells et al<sup>104</sup> observed high antitumor activity as a result of the delivery of anti-CD40 antibody together with CpG ODN, poly(I:C9), and IFN- $\gamma$  in an emulsion of squalene and Tween 80. These high antitumor activities are believed to be due to the induction of the powerful antitumor response of CD8-positive T cells by the stimulation of CD40 combined with TLR agonists. Also, Sokolova et al<sup>80</sup> reported that CpG ODN and poly(I:C) incorporated with hemagglutinin, a model antigen, into multishell calcium phosphate NPs and administered to DCs resulted in a greater amount of IL-12 produced.

## Disadvantages of delivery system

Meanwhile, the disadvantages of using NPs include not being able to establish the safety of NPs and not being able to clarify the metabolic process. When mixed with DNA, cationic polymers like poly(L-lysine) and polyethyleneimine form polyplexes.<sup>105-107</sup> Also, the method to attach CpG ODN by modifying negatively charged NPs with polycation is the most generally implemented method. However, polycations elicit the nonspecific adsorption of negatively charged molecules, and also promote the formation of NP aggregates.<sup>108-110</sup> These results are believed to be causes of side effects. Furthermore, it has been suggested that polycations may cause damage to liver from complement activation.<sup>45</sup> Research has been conducted to avoid these disadvantages of polycations by using PEG<sup>111</sup> and polyanions.<sup>112,113</sup> Also, it has been reported that ionizable cationic liposome eliminates these disadvantages of polycations.<sup>27</sup> Kim et al<sup>110</sup> prepared amphiphilic NPs composed of hydrophilic poly( $\gamma$ -glutamic acid) ( $\gamma$ -PGA) and hydrophobic L-phenylalanine. These NPs are negatively charged by ionizing the carboxyl group near the surface of  $\gamma$ -PGA.<sup>114</sup> However, it has been reported that free CpG ODN of the same negative charge can be encapsulated. Forming a polyplex of CpG ODN and poly( $\epsilon$ -lysine), the CpG ODN load can be increased by encapsulating the polyplex in  $\gamma$ -PGA and hydrophobic L-phenylalanine.

## Prospects for the future

Recently, a cytosolic DNA-sensing receptor that functions upstream of the TLR was discovered and named the high-mobility group box (HMGB) protein.<sup>115</sup> In addition to being expressed in immune cells, HMGB protein can be found in a variety of other cell types. The protein can recognize DNA and RNA and activate the innate immune system. Furthermore, since HMGB recognizes mammalian DNA, it is thought to be implicated in autoimmune diseases. Class B CpG ODNs with a phosphorothioate backbone demonstrated a high affinity to HMGB,<sup>115</sup> whereas interaction was not seen with a natural CpG ODN with a phosphodiester backbone.<sup>116</sup> These results suggest that CpG ODNs with a phosphorothioate backbone activate innate and adaptive immune systems in B cells and APCs via TLR9, and inhibit the innate immune system in other types of cells. Conversely, natural CpG ODNs with only a phosphodiester backbone activate the innate and adaptive immune systems in B cells and APCs via TLR9 and do not inhibit the innate immune system in other types of cells. From the standpoint of improvement and maintenance of systemic immune activation, the presence of natural CpG ODNs with a phosphodiester backbone is therefore more

advantageous than CpG ODNs with a phosphorothioate backbone.

The number of natural CpG ODNs consisting entirely of phosphodiester backbone developed is significantly smaller than that of CpG ODNs with a phosphorothioate backbone. The development of clinically applicable natural CpG ODNs is therefore expected to grow in the future. On the other hand, since CpG ODNs with a phosphorothioate backbone demonstrate high affinity with HMGB protein, they may be effective in the treatment of HMGB-mediated autoimmune diseases<sup>116</sup> such as rheumatoid arthritis and systemic lupus erythematosus.

There are many benefits of NP-mediated delivery of CpG ODNs. The author found that the binding modes of CpG ODNs to NPs affect the production of immune mediators (cytokines) (unpublished data). That is, the type of the binding mode of class B CpG ODNs to NPs can induce the production of either IL-6 or IFN- $\alpha$ . This ability to control cytokine induction opens the possibility of treatment-specific preparations of CpG ODN carriers. Most CpG ODN NP preparations employ CpG ODNs with a phosphorothioate backbone. As described earlier in this review, natural CpG ODNs do not require encapsulation in NPs such as liposomes to be protected from DNase activity: adsorption to NPs is sufficient. The development of safe delivery systems for natural CpG ODNs in NPs is fast approaching.

## Conclusion

The application of CpG ODNs for the treatment of cancers, infectious diseases, and allergies holds great promise, because CpG ODNs interact with TLR9 and activate both the innate and the adaptive immune system. This review paper summarizes the structural features that depend on base sequences of CpG ODNs consisting of phosphorothioate and phosphodiester backbones, and their relationship to the capacity of immune mediator cytokine induction. In addition, advantages and disadvantages in the delivery system of these CpG ODNs using various NPs and future direction of studies on CpG ODNs are described.

The number of natural CpG ODNs consisting entirely of phosphodiester backbone developed is significantly smaller than that of CpG ODNs with a phosphorothioate backbone. The development of clinically applicable natural CpG ODNs is therefore expected to grow in the future. From the standpoint of improvement and maintenance of systemic immune activation, the presence of natural CpG ODNs with a phosphodiester backbone is therefore more advantageous than CpG ODNs with a phosphorothioate backbone.

However, since CpG ODNs with a phosphorothioate backbone demonstrate high affinity with HMGB protein, they may be effective in the treatment of HMGB-mediated autoimmune diseases<sup>116</sup> such as rheumatoid arthritis and systemic lupus erythematosus.

There are many benefits of NP-mediated delivery of CpG ODNs, and the development of safe delivery systems for natural CpG ODNs in NPs is fast approaching.

## Acknowledgments

This study was funded by a grant-in-aid for scientific research (C-22560777) from the Japan Society for the Promotion of Science and the Ministry of Education, Culture, Sports, Science and Technology.

## Disclosures

The author reports no conflicts of interest in this work.

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