**Enhanced Immunostimulatory Activity and Reduced Side Effects of Immunostimulatory Oligonucleotides through Phosphorothioate Modification and Loading into Self-gelatinizable Nucleic Acids**

**Naruhito Kohori1**, Kosuke Kusamori2, Shoko Itakura1, Makiya Nishikawa1.

1Laboratory of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Niijuku, Katsushika, Tokyo, Japan; 2Laboratory of Cellular Drug Discovery and Development, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Niijuku, Katsushika, Tokyo, Japan.

**Background and aims.** CpG oligodeoxynucleotides (ODNs) are attracting attention as adjuvants that activate innate immunity. However, phosphorothioate (PS) modification, commonly used to enhance *in vivo* stability, has been associated with systemic side effects. This study aimed to enhance immunostimulatory activity and reduce side effects by loading CpG1018 with varying numbers of PS modifications into self-gelatinizable nucleic acids.

**Methods.** CpG1018 with PO, PS/PO/PS, or PO-type backbones were prepared. Self-gelatinizable nucleic acids were designed with sequences complementary to CpG1018 and used to construct CpG1018-loaded DNA hydrogels. Hydrogel formation was assessed by polyacrylamide gel electrophoresis (PAGE). The immunostimulatory activity of the hydrogels was assessed by incubating them with antigen-presenting cells and measuring the concentration of tumour necrosis factor (TNF)-α in the culture supernatant using enzyme-linked immunosorbent assay (ELISA). Cellular uptake of CpG1018-loaded DNA hydrogels, prepared with fluorescently labelled CpG1018, was evaluated by flow cytometry. Finally, CpG1018-loaded DNA hydrogels were subcutaneously administered to mice, and interleukin-12 (IL-12) levels in the inguinal lymph nodes and IL-6 levels in plasma were quantified using ELISA.

**Results.** PAGE results showed efficient formation of DNA hydrogels with PO- and PS/PO/PS-type CpG1018, whereas PS-type CpG1018 exhibited poor gelation efficiency. Loading PO and PS/PO/PS-type CpG1018 onto self-gelatinizable nucleic acids significantly enhanced TNF-α production from antigen-presenting cells. When CpG1018-loaded DNA hydrogels were subcutaneously administered to mice, PO-type CpG1018-loaded DNA hydrogel induced significantly higher IL-12 levels in the inguinal lymph nodes. PS/PO/PS-type hydrogel showed a similar trend, though less pronounced. In contrast, administration of free PS-type CpG1018 resulted in marked elevation of plasma IL-6, while CpG1018 delivered via DNA hydrogels maintained low systemic IL-6 levels.

**Conclusion/Discussion.** This study demonstrates that loading PO-type CpG1018 into self-gelatinizable nucleic acids enhances immune activation in the draining lymph nodes while suppressing systemic cytokine production associated with side effects.