Peptidoglycan (PGN) is a component of bacterial cell wall consisting of glycans and peptide chains forming a three-dimensional mesh-like structure outside the plasma membrane. PGN has been known as a stimulating component of innate immune system. PGN activates sensor proteins, nucleotide-binding oligomerization domain protein 1 (Nod1) and 2 (Nod2), which belong to Nod-like receptor (NLR) family, one of the major pathogen-recognizing receptor (PRR) families. Peptidoglycan recognition proteins (PGRP) are the other important protein families that recognize PGN. In addition, various kinds of enzymes and lectins have been proven to recognize PGN. However, the comprehensive analysis of the substrate structures of recognizing proteins has been not really conducted, because of the lack of pure PGN fragments. Herein, we report the chemical synthesis of the PGN fragment library in order to analyze various ligand/protein interactions. The PGN-fragments microarray was also developed for the rapid and quantitative analysis of the interactions, leading to understanding of the defense system against infection of bacteria.

1. Synthesis of PGN fragments and their glycan sequence-dependent Nod2 activation

Our previous studies revealed that MDP (MurNAC-L-Ala-γ-D-isoGln) showed the most potent activity in Nod2 stimulation. We also found that the activity was decreased as the glycan chain length and peptide chain length increased by using synthesized tetrasaccharide and octasaccharide fragments that contain GlcNAc-MurNAc (GM) repeating units (Figure 1, 2a–2d).

These fragments with GM units are considered to be produced by the lysozyme in host organisms which cleaves 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in PGN. On the other hand, PGN fragments having more than two MurNAc-GlcNAc (MG) repeating units, which are expected to be produced by bacterial N-acetylgalcosaminidase and release to the environment, had not been synthesized. Thus, we synthesized the library of PGN fragments (Figure 1) to explore their biological functions, especially the Nod2 activation.

1-1. Synthetic strategy of PGN fragments

The syntheses of disaccharide analogues were carried out via the intermediate 3 as illustrated in scheme 1. Introduction of appropriate peptides to the liberated carboxylic acid
6 and hydrogenation gave the disaccharide fragments 1a, 1c and 1e.

Scheme 1. Synthesis of PGN disaccharide fragments 1a~1e.

Tetrasaccharide 9 was then synthesized by using 3 as common synthetic intermediate for both glycosyl donor and the acceptor (Scheme 2). Disaccharide donors 7a and 7b were prepared via cleavage of the allyl glycoside and subsequent conversion to the imidate forms (7a: trichloroacetimidate, 7b: N-phenyltrifluoroacetimidate). For the preparation of the tetrasaccharide 9, trichloroacetimidate 7a was first used as the glycosyl donor to couple with the glycosyl acceptor 8. However, the glycosylation between 7a and 8 in the presence of TMSOTf gave the desired tetrasaccharide 9 only in 16% yield, accompanied with 62% yield of recovered 8. The low yield was probably due to the low reactivity of 4-OH group of the disaccharide acceptor 8 caused by steric hindrance of 3-O-lactyl moiety in muramic acid.

Scheme 2. Synthesis of PGN tetrasaccharide fragments.
moiety. We then used the N-phenyltrifluoroacetimidate 7b as the glycosyl donor which has similar high reactivity but improved stability in the corresponding trichloroacetimidates. Excess acceptor 8 was used (ratio of donor : acceptor was 1 : 1.5) in order to promote the reaction. The yield of glycosylation was dramatically improved to give tetrascarbohydrate 9 in 61% yield. The glycan backbone 10 was then coupled with peptides to give the corresponding protected intermediates. All benzyl and benzylidene groups were then removed to obtain tetrascarbohydrate PGN fragments with MGMG sequence having di-, tri-, tetra-, and pentapeptides (1b, 1d, 1f and 1g, respectively). In order to compare the biological activities with the two kinds of glycan sequence, we also prepared the PGN fragments having the GM repeating units as shown in Figure 1, according to the previously reported methods.

1-2. Human Nod2 stimulation with synthetic PGN fragments

Human Nod2 stimulating activity of each synthetic peptidoglycan fragment was then evaluated by using hNod2 transfected HEK293T cells with indicating NF-κB activation. In Figure 2, hNod2 activities of MDP, disaccharide and tetrascarbohydrate fragments were obtained at the concentration of 1 ng/mL. Among these PGN fragments, disaccharide with dipeptide 1a and 2a showed the same level of activities as MDP, which is consistent with the previous results. Among the tetrascarbohydrate fragments, a notable difference between MGMG and GMGM sequence of PGN fragments was observed for the first time. MGMG sequence exhibited higher hNod2 activation than compounds containing the GMGM sequence, all other aspects of the peptide structures are being the same: MGMG2 (1b) had an approximately 10-fold higher activity than GMGM2 (2b); MGMG3 (1d) had an approximately 65 fold higher than GMGM3 (2c); and MGMG4 (1f) had an approximately 26 fold higher activity than GMGM4 (2d). The results suggest that differences in the bacterial PGN degradation or construction enzymes (glycan cleaving enzymes or the peptidases) may affect immunomodulation in humans.

The binding analysis of the octasaccharide fragments with other PGN binding proteins are now under investigation.

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Figure 2. hNod2 activation by the chemically synthesized disaccharide- and tetrascarbohydrate-containing PGN fragments.

The 1000 mU is the activity of Nod2 activation with indicating the NF-κB at the concentration of 1 ng/mL of MDP, using hNOD2 transfected HEK293T, determined by luciferase reporter assay.
2. PGN fragment library for microarray analysis

For the further comprehensive analysis of PGN recognition in many proteins, we developed PGN arrays by utilizing synthesized PGN fragments on a solid surface of the array platforms (Figure 3). An amorphous carbon chip having free carboxyl acids surface was chosen as the solid interphase (PepTenChip® PTC-CA). PepTenChip® has significant advantages over conventional glass slides, 1) mechanical stability, 2) chemical inertness, 3) no self-fluorescence, 4) low background, and so on.9,10

Mono-, di-, and tetrasaccharide PGN fragments with linkers were synthesized for the later amide bond formation between the chip and ligands. An ester linker having terminal free amino group was chosen. We then set up the microarray platform. The carbon chip (PepTenChip® PTC-CA) has 31.4 pmol of free carboxyl acid moieties on each spot, allowing covered by condensation with 1 μg/spot of PGN ligands at most. Condensation reagents HOOb and WSCD in DMF were firstly loaded on the plate to activate the carboxyl groups. The Boc protections on lysine were then cleaved by dipping the chip in aqueous TFA. The carbon chip has relatively stable linkage at the reacting site, and it made possible to use acidic reagent on the chip.

![Figure 3. Schematic diagram of PGN microarray construction.](image)

We then analyzed the interaction of the PGN fragments microarray with several proteins. The major targets of the analysis are peptidoglycan recognition proteins (PGRPs), which widely exist in insects and mammals. Drosophila and mammals have families of 13 and 4 PGRP genes, respectively. We have analyzed the PGRP recognition using the synthetic PGN fragments.11-14 For example, we showed that human PGRP-S strongly binds to GMGM3 and GMGM4 but weakly binds to GMGM2 by using SPR analysis, where PGRP-S was attached to SPR chip.12

In the present study, human PGRP-S was thus used for evaluation of our array. The result showed that PGRP-S bound to GMGM3 and GMGM4 more tightly than GMGM2, and it was consistent with the SPR previous data. The analysis against (MurNAc-GlcNAc)_2 containing ligands MGMT3 and MGMT4 is also presented in this conference.

3. Conclusion

PGN partial structure library and their microarray were successfully constructed. This work enabled the study of the precise PGN structures responsible for the immunostimulating activity as well as the interactions between PGN structures and PGN-binding proteins.
References
Bacterial Cell Wall Peptidoglycan Fragment Library/Array for Investigation of Their Protein Recognition

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Bacterial cell wall peptidoglycan (PGN) has been known as a potent immunopotentiator. Recently, various proteins that recognize PGN have been reported, such as nucleotide-binding oligomerization domain protein 1 (Nod1) and 2 (Nod2), peptidoglycan recognition proteins (PGRP), and lectins. However, the comprehensive analyses of their substrate structures have not been really conducted because of the lack of pure chemically synthesized PGN fragments. In this work, PGN fragments library including two kinds of sequences of alternating glycans (GlcNAC/MurNAc) with various length of glycan chains were synthesized. The targets covered the compounds having disaccharide, tetrasaccharide, and octasaccharide. The Nod2 stimulating activity of each synthetic peptidoglycan fragment was then evaluated. A notable difference between MGMG and GMGM sequence of PGN fragments was observed for the first time. MGMG sequence fragments showed higher activity than the corresponding GMGM fragments having the same peptides.

Construction of PGN arrays was performed by attaching synthesized PGN fragments on a solid surface of the array platforms. In order to introduce the PGN fragments to the array, several linkage structures were examined, and finally a linker with terminal amine was successfully attached to the PGN fragments. Human PGRP-S with combination of fluorescence labeled antibody was used for evaluation of the array. PGRP-S bound to GMGM3 and GMGM4 more tightly than GMGM2. The result was also reproducible in SPR analysis. PGN partial structure library and their microarray were successfully constructed. This work enabled the study of the precise PGN structures responsible for the interactions with various PGN-binding proteins.