Bioconjugate Chemistry

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Enhanced Immunostimulating Activity of Lactobacilli-Mimicking Materials by Controlling Size

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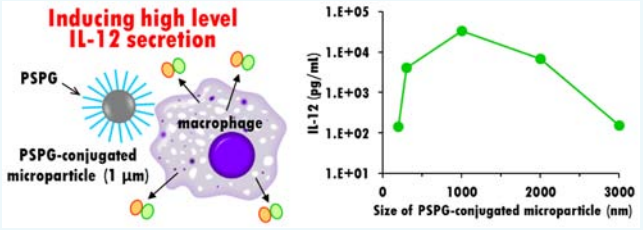
Supporting Information

ABSTRACT: The design and synthesis of materials capable of activating the immune system in a safe manner is of great interest in immunology and related fields. Lactobacilli activate the innate immune system of a host when acting as probiotics. Here, we constructed lactobacilli-mimicking materials in which polysaccharide−peptidoglycan complexes (PS−PGs) derived from lactobacilli were covalently conjugated to the surfaces of polymeric microparticles with a wide variety of sizes, ranging from 200 nm to 3 μm. The artificial lactobacilli successfully stimulated macrophages without cytotoxicity. Importantly, we found that the size of artificial lactobacilli strongly influenced their immunostimulating activities, and that artificial lactobacilli of 1 μm exhibited 10-fold higher activity than natural lactobacilli. One major advantage of the artificial lactobacilli is facile control of size, which cannot be changed in natural lactobacilli. These findings provide new insights into the design of materials for immunology as well as the molecular biology of lactobacillus.



INTRODUCTION

The design and synthesis of safe immunostimulating materials is of great interest in immunology and related fields, for example, for the development of vaccines, adjuvants, functional foods, and cosmetics, because of their ability to tune up the innate immune defenses of the host.[1−4] A bioconjugation approach, in which biologically derived agents are conjugated with synthetic materials, has been applied for the development of immunostimulating materials. One of the most utilized bioderived immunostimulatory agents is cytosine-guanosine oligodeoxynucleotide (CpG ODN), which is widely present in the genomic DNA of bacteria and viruses; CpG ODNs are normally conjugated with various kinds of nanoparticles (NPs) and microparticles (MPs).[5−12] Nembrini et al. reported the conjugation of CpG ODNs with Pluronic-stabilized poly(propylene sulfide) NPs and their enhanced T-cell responses.[7] Wei et al. reported the decoration of gold NP surfaces with CpG ODNs and their highly efficient immunostimulation.[9] Although CpG ODNs effectively activate Th1-like innate and adaptive immune responses through the induction of proinflammatory cytokines from macrophages, their specific cytotoxicity to T-cells and neurons has been reported.[13−15] Hence, novel bioderived immunostimulatory agents capable of both conjugating with synthetic nano/microsized platforms and activating the immune system in a safe manner are still sought after.

Lactobacilli are a major constituent of normal human indigenous flora, and some specific strains activate the innate immune system of the host as probiotics.[16−18] In general, these strains stimulate macrophages to secrete interleukin-12 (IL-12), which plays a key role in activating the innate immunity of the host, such as by augmenting the functions of macrophages and NK cells.[19,20] In particular, *L. casei* strain Shirota (LcS) effectively induces IL-12 secretion.[21] Polysaccharide−peptidoglycan complexes (PS−PGs), which are the main components of the cell wall, are understood as an agent to activate the immune system.

Recently, we have reported the synthesis of novel lactobacilli-mimicking materials in which LcS-derived PS−PG chains were covalently conjugated with polymeric microparticles, approximately 3 μm in size, by using simple surface modification chemistry.[22] These lactobacilli-mimicking materials successfully induced IL-12 secretion from macrophages without cytotoxicity, and the inducing ability was equivalent with that of the original LcS, although the mechanism is not clear. Cell uptake of particulate immunostimulatory agents through phagocytosis by macrophage and subsequent signaling events is a key aspect of its IL-12 secretion level.[23] In principle, phagocytosis was regulated through a combination of physical cues, such as particle size, as well as biological cues, such as recognition of molecular structure and density of immunostimulatory agents by certain receptors.[24] Therefore, it is likely that both physical and biological mechanisms are significant to regulating IL-12 secretion level. Understanding the physical and biological cues

Received: June 8, 2015
Revised: July 8, 2015
Published: July 23, 2015

DOI: 10.1021/acs.bioconjchem.5b00319
Bioconjugate Chem. 2015, 26, 1775−1781

of lactobacilli-mimicking materials which trigger macrophage phagocytosis and resulting IL-12 secretion is important to improve their potential as immunostimulatory biocompatible materials. Herein, we therefore constructed a series of lactobacilli-mimicking materials with a wide variety of sizes ranging from 200 nm to 3 μm by a simple coupling reaction using LcS-derived PS−PGs with polymeric NPs/MPs. Moreover, we synthesized lactobacilli-mimicking materials having varied PS−PGs density using polymer microparticles with the same size (3 μm). To clarify the effects of their size and PS−PGs density on their immunostimulating activities, we investigated their cell uptake by macrophage, IL-12 secretion-inducing properties, and cytotoxicity.

RESULTS AND DISCUSSION

Synthesis and Characteristics of NP/MP-PSPGs. Cell wall components, including PS−PGs, were extracted from heat-killed LcS by high-dose Mutanolysin treatment based on a modified published method, as illustrated in Figure S1 (Supporting Information).[26] The PS−PGs were purified by gel filtration on a column of Sephacryl S-200 HR. We have previously identified the structures of PS−PGs of LcS, including the amino acid sequences in the PG region, and the monosaccharide sequences and reducing end monosaccharide residue in the PS region.[27] Prior to the synthesis of NP/MP-PSPG, free amino groups of lysine residues in the PG region were acetylated to avoid a coupling reaction between them and the reducing end monosaccharide of the PS, *N*-acetylglucosamine. Then, reducing end monosaccharide residues of acetylated PS−PGs were conjugated with amino groups of the surfaces of polystyrene NP/MPs with different sizes (diameter: ca. 200, 300, 1000, 2000, 3000 nm) through a reductive amination reaction to give NP/MP-PSPG (Figure 1). The feed molar ratios of PS−PGs to surface amino groups of NP/MPs were fixed in all coupling reactions. We also synthesized MP-PSPGs having different numbers of PS−PGs conjugated by varying the feed molar ratios of PS−PGs to surface amino groups of MP (3000 nm) during the coupling reaction, as described in our previous report.[22] The conjugations of PS−PGs to NP/MPs were confirmed by FTIR measurements of the obtained NP/MP-PSPGs as described in our previous report.[22] We use abbreviations for NP/MP-PSPGs according to the size of the polystyrene platform, e.g., NP_{200}-PSPG has a polystyrene platform of ca. 200 nm. We analyzed the amounts of PS−PGs conjugated to polystyrene NP/MPs. In this study, the number of PS−PGs per NP/MP was estimated by gravimetric measurement because there is no characteristic photochemical property available for detecting PS−PGs. The number of PS−PGs on NP/MP surfaces increased with the increase in the size of the polymeric platform (Table 1). These numbers were used to estimate the density of PS−PGs on the surface of NP/MPs. We found that NP_{200}-PSPG and NP_{300}-PSPG possessed similar PS−PG densities, while the densities of MP_{1000}-PSPG, MP_{2000}-PSPG, and MP_{3000}-PSPG were almost 3-fold higher than those of NP_{200}-PSPG and NP_{300}-PSPG. Hydrodynamic diameters (D_h) of the NP/MP-PSPGs in pure water were analyzed by DLS. NP/MP-PSPGs showed larger D_h values than the corresponding bare NP/MPs, and the increased values were largely consistent with the size of the PS−PGs.

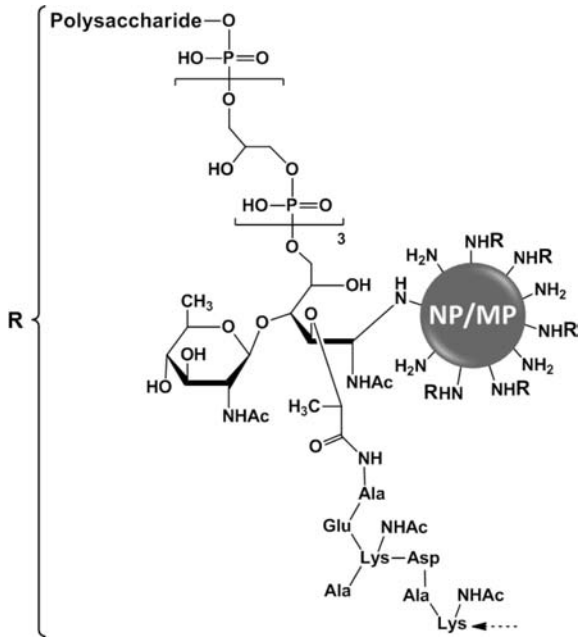


Figure 1. Structures of PS−PG-conjugated polymeric nano- or microparticles (NP/MP-PSPG).

Table 1. Characteristics of NP/MP-PSPGs

sample	# of PS−PGs[a]	Density of PS−PGs[b] (# of PS−PGs/μm^2)	D_h[c] (nm)
NP_{200}-PSPG	3.85×10^4	3.38×10^5	320 ± 50
NP_{300}-PSPG	1.30×10^5	3.70×10^5	410 ± 60
MP_{1000}-PSPG	3.87×10^6	1.26×10^6	1170 ± 110
MP_{2000}-PSPG	1.58×10^7	1.31×10^6	2250 ± 150
MP_{3000}-PSPG	2.85×10^7	1.06×10^6	3100 ± 260
PS−PG	——	——	130 ± 80

[a]Number of PS−PG chains attached to the surface of a NP or a MP. [b]Density of PS−PG chains attached to the surface of a NP or a MP. [c]Hydrodynamic diameter of NP/MP-PSPGs.

IL-12 Secretion-Inducing Activity of NP/MP-PSPGs. We examined the IL-12 secretion-inducing activity of a series of NP/MP-PSPGs using the macrophage-like cell line J774.1 by ELISA. Moreover, the cell compatibility of the NP/MP-PSPGs was investigated by LDH assay. J774.1 cells used in this study secreted a certain amount of IL-12 by LPS stimuli as positive control (Figure S2, Supporting Information). Obvious IL-12 secretion from J774.1 cells was observed for a series of NP/MP-PSPGs (Figure 2a). In contrast, IL-12 secretion was not observed by treatment of bare NP/MP with different sizes as negative controls (Figure S3, Supporting Information). The amounts of IL-12 secreted by stimulation with NP/MP-PSPGs were increased by increasing the concentrations, and the curve showed a threshold. The threshold concentration shifted to lower concentrations with the increase in the size of NP/MP-PSPGs up to 1000 nm (MP_{1000}-PSPG), but the threshold concentration obviously shifted to higher concentrations for the largest sizes (MP_{2000}-PSPG and MP_{3000}-PSPG). Importantly, we found that the amounts of secreted IL-12 were strongly influenced by the sizes of NP/MP-PSPGs (Figure 2b); the amount was obviously increased by increasing the size up to 1000 nm, but then decreased by further increases in size

DOI: 10.1021/acs.bioconjchem.5b00319
Bioconjugate Chem. 2015, 26, 1775−1781

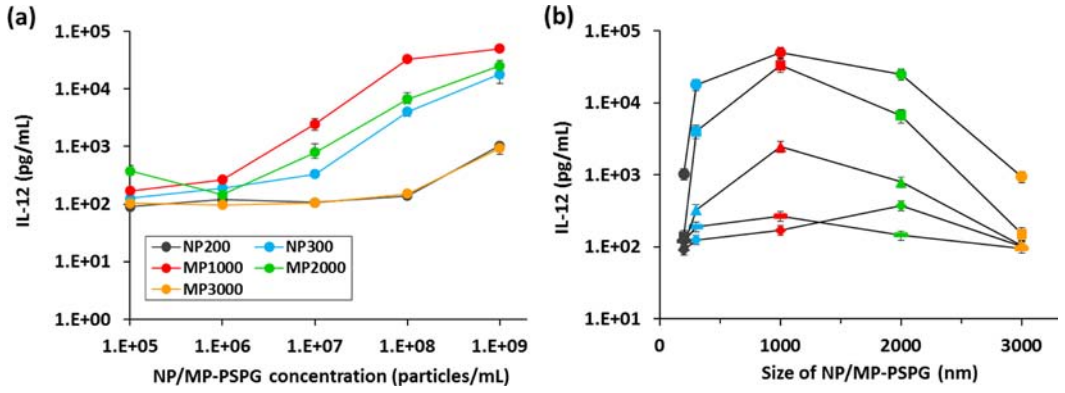


Figure 2. (a) IL-12 secretion from J774.1 cells after treatment with NP/MP-PSPGs of different sizes. (b) Plots of the amount of secreted IL-12 from J774.1 cells after treatment with NP/MP-PSPGs of different concentrations vs the size of NP/MP-PSPGs: 1.0×10^5, 1.0×10^6, 1.0×10^7, 1.0×10^8, and 1.0×10^9 particles/mL. The results are expressed as the means ± SD of three independent measurements.

(MP_{2000}-PSPG and MP_{3000}-PSPG). These phenomena were observed clearly above the threshold concentrations that induced IL-12 secretion in macrophages. These results clearly demonstrate that the size of NP/MP-PSPGs is a critical factor to control their IL-12 secretion-inducing ability, and that MP_{1000}-PSPG possesses the highest IL-12 secretion-inducing activity. Importantly, the amount of IL-12 secretion by MP_{1000}-PSPGs stimulation (3.2×10^4 pg/mL) was about 8-fold higher than that of the original LcS (4.3×10^3 pg/mL) when comparing the activities at the same concentration (1.0×10^8 particles/mL) (Figure S4, Supporting Information). Suzuki et al. investigated the ability of 46 kinds of lactic acid bacteria (LAB) strains to induce production of IL-12. Almost all of these LAB strains induced IL-12 secretion from J774.1 cells, but the average amount is not high (below 5.0×10^3 pg/mL).[28] These results indicate an efficiently high IL-12 secretion-inducing ability of NP/MP-PSPGs with certain sizes. Interestingly, the IL-12 secretion-inducing ability of NP/MP-PSPGs could be roughly controlled by varying the size of the polymer platform used for conjugation with PS−PGs. Because natural lactobacilli cannot change their inherent size, this easily controllable feature of NP/MP-PSPGs is highly advantageous. Notably, NP/MP-PSPGs with size below 1000 μm showed no cytotoxicity to J774.1 cells during IL-12 secretion, while NP/MP-PSPGs with size above 2000 μm showed dose-dependent cytotoxicity (Figure 3a). These tendencies were also seen in the case of bare NP/MPs (Figure 3b), indicating that the cytotoxicity is not derived from PS−PGs. Taken together, NP/MP-PSPGs are a potential material for activating the immune system in a safe manner. Although we used polystyrene NP/MPs as a primitive platform in this study, polystyrene is not optimum because of its nonbiodegradability. Therefore, we will use biocompatible NP/MPs consisting of biodegradable polymers in our future research to develop NP/MP-PSPG-based immunostimulatory biomaterials.

Influences of Various Characteristics of NP/MP-PSPG on Their Immunostimulating Ability. Generally, size is one of the most essential factors for nano/microsized objects, including both synthetic materials such as polymeric particles, liposomes, and inorganic nanoparticles, and natural organisms such as bacteria and viruses, to control their cellular response.[23,24] Phagocytosis is a representative cellular response that is influenced by size. The immunostimulating activity of lactobacilli has been thought to be connected strongly with their cellular uptake by immune-related cells such as macrophages. Therefore, we examined the cellular uptake of the NP/MP-PSPGs by J774.1 cells.[16,29] Figure 4 shows photographs of J774.1 cells treated with NP/MP-PSPGs and changes in the numbers of NP/MP-PSPGs internalized into cells over time. While size dependence of particles has been shown to affect phagocytosis in previous studies,[30,31] this is the first study to look at the effects of particles size with PS−PGs on phagocytosis. At the initial stage (after 2 h), the number of NP/MP-PSPGs internalized increased with a decrease in size. After that, the numbers increased with time for all NP/MP-PSPGs, except NP_{200}-PSPG. The decrease in the number observed for NP_{200}-PSPG can be attributed to extracellular release caused by their relatively small size. The numbers of NP_{1000}-PSPG internalized effectively increased between 2 and 10 h, while NP_{300}-PSPG, MP_{2000}-PSPG, and MP_{3000}-PSPG showed stalling of phagocytosis. Finally, NP_{300}-PSPG and NP_{1000}-PSPG showed the highest cellular uptake, and the numbers internalized were comparable. On the other hand, dependence of the numbers of NP/MP-PSPGs internalized on

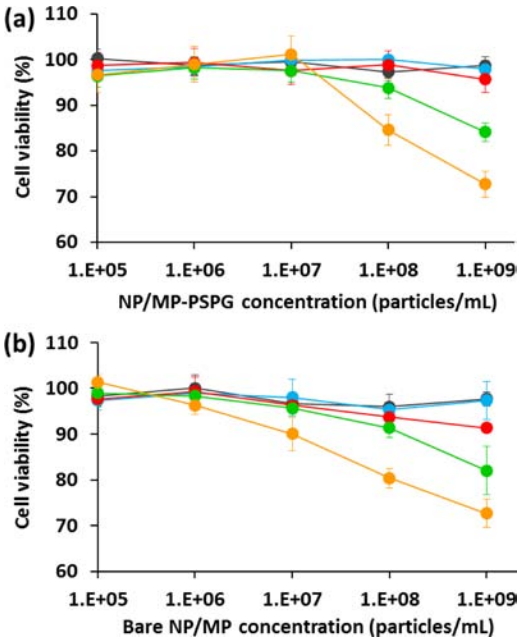


Figure 3. Cell viability after stimulation by (a) NP/MP-PSPGs and (b) bare-NP/MPs. Refer to Figure 2 for legend. The results are expressed as the means ± SD of three independent measurements.

DOI: 10.1021/acs.bioconjchem.5b00319
Bioconjugate Chem. 2015, 26, 1775−1781

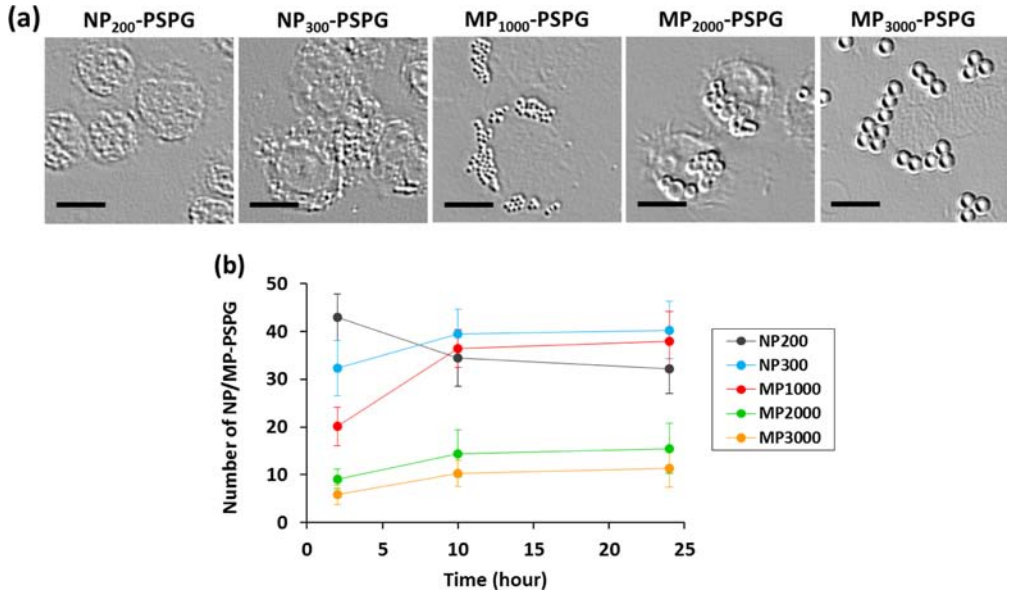


Figure 4. (a) Phase contrast microscopic images of J774.1 cells treated with NP/MP-PSPGs. Scale bar: 10 μm. (b) Changes in the numbers of MP/MP-PSPGs internalized into J774.1 cells with time.

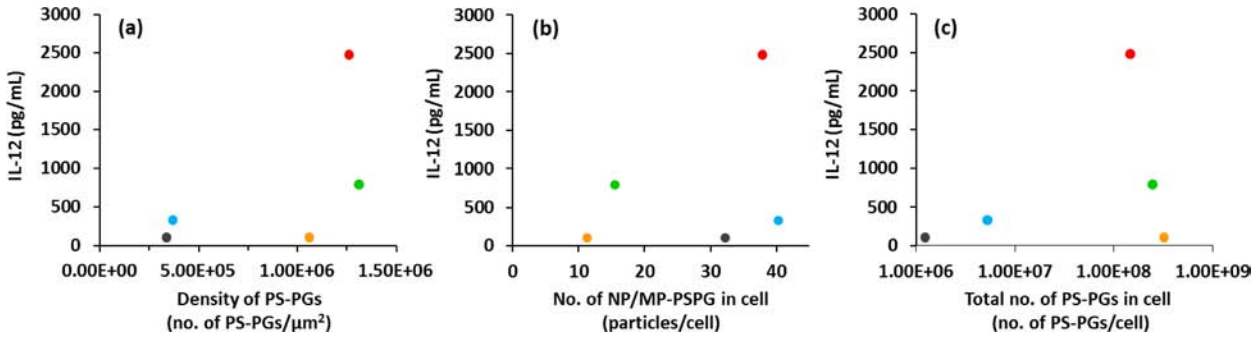


Figure 5. Influence of various properties of NP/MP-PSPGs on their ability to induce IL-12 secretion from J774.1 cells. Plots of IL-12 secretion from J774.1 cells after treatment with NP/MP-PSPGs as a function of (a) the surface density of PS−PG chains, (b) the amount of NP/MP-PSPG internalized in J774.1 cells, and (c) the total numbers of PS−PG chains internalized in J774.1 cell. NP200 (black), NP300 (blue), MP1000 (red), MP2000 (green), MP3000 (yellow).

the PS−PGs density was not observed, indicating that phagocytosis of NP/MP-PSPGs is strongly influenced by the size. A possible explanation for the size dependence in the initial and ongoing processes of phagocytosis could be posited to result from the dynamics of cell signaling components recruited in the process. A theoretical model suggests that larger particles will require the cell membrane receptors to diffuse over larger distances resulting in the cell membrane taking longer to envelop the particle. Particle size can also lead to stalling of the phagocytosis process and the low phagocytosis rates we found for the MP_{2000}-PSPG and MP_{3000}-PSPG.[32] In contrast, smaller particles require the recruitment of fewer signaling components before their phagocytosis, providing less time to be internalized. Moreover, it has been suggested that there is a specific size threshold of 500 nm to 1 μm whereby the macrophage phagocytosis transitions from a coated vesicle-mediated uptake into an actin-mediated uptake.[31] Considering these transitions, cell uptake of NP/MP-PSPGs by macrophages would be generally governed by the rule found in typical polymeric particles.

Based on the results and characteristics of NP/MP-PSPGs listed in Table 1, the influence of possible characteristics, such as the density of PS−PG chains conjugated to NP/MP-PSPGs, the number of NP/MP-PSPGs internalized in single cells, and the total number of PS−PGs internalized in single cells, on their IL-12 secretion-inducing ability were analyzed (Figure 5). However, no significant correlation was observed between these three characteristics. The NP_{1000}-PSPG showed the highest IL-12 secretion-inducing ability in all three cases, but the NP_{1000}-PSPG possessed the second highest PS−PGs density, the second highest number of NP/MP-PSPGs internalized in single cells, and the third highest total number of PS−PGs internalized in single cells, indicating that the size of NP/MP-PSPGs is the main factor controlling the induction of IL-12 secretion. In contrast, a good correlation was observed between the surface PS−PG density of MP_{3000}-PSPGs and their IL-12 secretion-inducing ability (Table S1 and Figure S5, Supporting Information). Taken together, these results indicate that the surface PS−PG density is also an important factor controlling the induction of IL-12 secretion. However, the surface PS−PG density may be an important factor only when NP/MP-PSPGs possess the same size and not the primary factor when they have different sizes. Consequently, it can be concluded that both the size and the surface PS−PGs density are important factors for immunostimulation, but size is a higher priority than the surface PS−PG density. Interestingly,

DOI: 10.1021/acs.bioconjchem.5b00319
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the size of NP_{1000}-PSPG is similar to the cell size of natural LcS, which exhibits the highest IL-12 secretion-inducing ability known among lactobacilli, implying that cell size is an important factor for controlling immunostimulation. At present, some specific strains of lactobacilli are known to activate the immune system of the host as probiotics. Moreover, it is also known that the immunostimulating activity is an inherent property of each strain.[16−18] However, the reasons the immunostimulating activity is significantly different among strains is not yet clear. According to our findings, analyses that account for the size of lactobacillus strains may provide new insights into their immunostimulating abilities.

CONCLUSIONS

In summary, we demonstrated the successful fabrication of lactobacilli-mimicking materials with a wide variety of sizes ranging from 200 nm to 3 μm by using simple surface modification chemistry. These lactobacilli-mimicking materials induced high levels of IL-12 production from macrophages without cytotoxicity. Among them, the MP_{1000}-PSPG with 1 μm in size exhibited the highest IL-12 secretion-inducing ability, which was 10-fold higher than that of the natural lactobacillus strain. In this study, we discovered that the size of the lactobacilli-mimicking material plays a critical role in its immunostimulating activity. Taken together, these materials have the potential to activate the immune system in a safe manner. Moreover, lactobacilli-mimicking materials could be used for basic research into probiotics so as to clarify the mechanism of their immune system stimulation. Thus, our findings could lead to new insights into the design of available materials in immunology and related fields as well as into the molecular biology of lactobacillus.

MATERIALS AND METHODS

Materials. Acetic anhydride, sodium cyanoborohydride, bovine serum albumin (BSA), and all organic solvents were purchased from Wako Pure Chemicals. Amino-modified polystyrene nano- and microparticles with different sizes were purchased from Invitrogen. DNase and RNase were purchased from Roche Diagnostics. Mutanolysin from *Streptomyces globisporus* ATCC 21553 was obtained from Sigma-Aldrich. Bacteria: The probiotic strain LcS was originally isolated at the Yakult Central Institute (Tokyo, Japan), based on acid and bile tolerance and survival in the gastrointestinal passage.

Isolation of PS−PGs. Isolation of PS−PGs was conducted based on a modified published method.[25] In brief, heat-killed LcS (500 mg) was exhaustively digested with Mutanolysin (9000 U) for 24 h at 37 °C. The digest was then centrifuged (10 000 × *g*, 45 min), and the supernatant was dialyzed against 10 mM phosphate buffer containing 0.25 M NaCl. The nondialyzable material was lyophilized and then dissolved in a small volume of aqueous solution of pure water. The solution was then subjected to gel filtration on a column of Sephacryl S-200 HR (50 mm × 600 mm) with the same eluent. Fractions were collected and assayed for hexose. The hexose-containing fractions were used as PS−PGs (M_w: 30 000).

Synthesis of NP/MP-PSPG. Isolated PS−PGs (18.0 mg) were dissolved in 3.3 mL of phosphate buffer (50 mM, pH 8.5), and 420 μL of acetic anhydride was added and stirred at room temperature for 6 h. The reaction solution was dialyzed (MWCO: 3500) against excess amounts of pure water for 2 days to remove unreacted acetic anhydride, and then lyophilized to give acetylated PS−PGs as a white powder (11.6 mg). Then, PS−PG-conjugated polystyrene NP/MPs (NP/MP-PSPGs) were synthesized through a reductive amination reaction between surface amino groups of NP/MPs and the reducing ends of acetylated PS−PGs. In brief, acetylated PS−PGs (4.7 mg) were dissolved in 200 μL phosphate buffer (50 mM, pH 8.5). 30 μL of MP_{3000} dispersion (8.4×10^{10} particles) and 90 μL of sodium cyanoborohydride aqueous solution (100 mM) were added to the acetylated PS−PG solution and stirred at 45 °C for 72 h. The reaction solution was centrifuged (5000 rpm, 10 min, 4 °C) and the supernatant was removed. The precipitate was washed seven times each by 300 μL of pure water. Finally, the precipitate was lyophilized to give MP_{3000}-PSPG.

Characterization. The amounts of PS−PGs conjugated to polystyrene NP/MPs were estimated by gravimetric measurement using an electronic microbalance (AX26, Mettler Toledo). PS−PGs conjugated to polystyrene NP/MPs (μg) = weight of NP/MP-PSPG after lyophilization (μg) − weight of NP/MPs used for coupling reaction (μg). These values were utilized to estimate the density of PS−PGs on the surfaces of NP/MPs (the number of PS−PG chains per surface area of NP/MP). Sizes of NP/MP-PSPG were measured by dynamic light scattering (DLS) on a Malvern Zetasizer Nano ZS at 25 °C. The lyophilized NP/MP-PSPG was dispersed with pure water to a concentration of 0.1 wt % prior to measurement. The diameters of NP/MP-PSPG were calculated as means ± SD of three independent measurements.

Cell Culture. J774.1 mouse macrophage-like cells were grown in RPMI-1640 medium (Nissui Pharm) supplemented with 10% heat-inactivated FBS, 0.15% $NaHCO_3$, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.05 mM 2-mercaptoethanol at 37 °C in humidified air containing 5% CO_2.

Induction of IL-12 Secretion by NP/MP-PSPG Simulation. J774.1 cells were seeded in a 96-well plate (1.0×10^5 cells/well) with 100 μL of culture medium and incubated for 2 h at 37 °C. Then, certain concentrations of NP/MP-PSPG suspensions (100 μL), bare NP/MP suspensions, or lipopolysaccharide (LPS, Sigma) suspensions were added and incubated for 24 h at 37 °C. Supernatants were collected and filtered (pore size: 450 nm). Filtrates were used for determination of IL-12 concentrations. Concentrations of IL-12 in culture supernatants were determined by sandwich ELISA. Rat anti-mouse IL-12 monoclonal antibody (clone C15.6) was used as the capture antibody. Biotinylated rat anti-mouse IL-12 (clone C17.8) monoclonal antibody was used as the detection antibody. These antibodies and standard recombinant mouse IL-12 were purchased from BD Pharmingen. The concentrations were calculated as means ± SD of three independent measurements.

Cytotoxicity of NP/MP-PSPG. J774.1 cells were seeded in a 96-well plate (1.0×10^4 cells/well) with 100 μL of culture medium and incubated for 2 h at 37 °C. Then, certain concentrations of NP/MP-PSPG suspensions or bare NP/MP suspensions (100 μL) were added and incubated for 24 h at 37 °C. Cell viability was measured by an LDH assay kit (Takara Bio) according to the manufacturer's instructions. The viability was calculated as means ± SD of three independent measurements.

Cellular Uptake of NP/MP-PSPG. J774.1 cells were seeded in a 96-well plate (1.0×10^4 cells/well) with 100 μL of culture medium and incubated for 2 h at 37 °C. Then, 100 μL of NP/

DOI: 10.1021/acs.bioconjchem.5b00319
Bioconjugate Chem. 2015, 26, 1775−1781

MP-PSPG suspensions (1.0×10^7 particles/well) were added and incubated for 12 h at 37 °C. Cells were washed with PBS twice and then microscopic observation was performed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00319.

Schematic illustration of isolation of PS−PGs from cell wall of LcS, IL-12 secretion-inducing property of LPS, heat-killed LcS, bare-MP/NPs with different sizes, and MP_{3000}-PSPGs with different surface PS−PG densities, and characterizations of MP_{3000}-PSPGs with different surface PS−PG densities (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Dr. Masato Nagaoka (Yakult Central Institute) for his technical support.

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