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The suppression of IgE-mediated histamine release from mast cells following exocytic exclusion of biodegradable polymeric nanoparticles

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ABSTRACT

The objective of this study is to evaluate the effect of polymeric nanoparticles (NPs) on the allergic response of mast cells that release inflammatory mediators such as histamine through exocytosis. Submicron-sized biodegradable poly(DL-lactide-co-glycolide) (PLGA) NPs were prepared by the emulsion solvent diffusion method. Here, we examined the interactions of the mast cells with two types of PLGA NPs, unmodified NPs and NPs modified with chitosan (CS), a biodegradable cationic polymer. The cellular uptake of NPs increased by CS modification due to electrostatic interactions with the plasma membrane. NPs were taken up by mast cells through an endocytic pathway (endocytic phase) and then the cellular uptake was saturated and maintained plateau level by the exclusion of NPs through exocytosis (exocytic phase). Antigen-induced histamine release from mast cells was inhibited during the exocytic phase. The extent of histamine release inhibition was related to the amount of excluded NPs. Exocytic exclusion of NPs competitively antagonize the antigen-induced exocytotic release of histamine by hijacking exocytosis machinery such as SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, since histamine release was recovered in mast cells that overexpress SNAP-23. The inhibitory effect of the allergic response by PLGA NPs was also evaluated *in vivo* using the mouse model for systemic anaphylaxis. The administration of NPs suppressed the antigen-induced systemic allergic response *in vivo*. In conclusion, PLGA NP itself has actions to inhibit the allergic responses mediated by mast cells.

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1. Introduction

Biodegradable polymeric nanoparticles (NPs) have been developed as unique drug carrier systems because of their characteristic colloid-like behavior. In general, polymeric NPs can be used to deliver medicinal compounds because of their high stability and ease of cellular uptake through endocytosis [1,2]. We have successfully developed submicron-sized poly(DL-lactide-co-glycolide) (PLGA) NPs as cellular drug delivery systems for use in delivering anticancer drugs and genes [3–5]. PLGA has been widely accepted as a biocompatible and biodegradable material for drug delivery systems and has been approved by the US Food and Drug Administration for certain clinical uses in humans [6]. We have previously reported that the cellular uptake of PLGA NPs increases

as the diameter decreases to the submicron level and when used in coating with chitosan (CS), which is a biodegradable cationic polymer that mediates surface modification [7]. CS modified-PLGA NPs (CS-PLGA NPs) have a positive zeta potential, and thus, could be associated with negatively charged cell membranes through electrostatic interactions. The intracellular trafficking of PLGA NPs after their uptake into cells has been considered as a typical endocytic–exocytic route in previously published studies [8–12]. Furthermore, the cellular uptake and intracellular distribution of NPs depends on the surface properties of NPs [8].

Mast cells play significant roles in allergic diseases secreting various mediators. In mast cells, the cross-linking of high-affinity IgE (Fcε-RI) receptors by multivalent antigens causes the activation of an intracellular signaling cascade that leads to the exocytic release of inflammatory mediators in secretory granules (degranulation), which results in allergic responses [13–15]. Several groups including us have shown the involvement of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins during the degranulation process [16–23]. Recent studies have suggested that the vesicle-associated membrane proteins

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VAMP-2, VAMP-7, and VAMP-8 are possible v-SNAREs (vesicle SNARE) proteins that are responsible for degranulation of mast cells [16–21]. As for t-SNARE (target SNARE) proteins that reside on the plasma membrane, syntaxin-3, -4 and synaptosomal-associated protein of 23 kDa (SNAP-23) are thought to be involved in degranulation [16,17,22,23].

The aim of this study is to examine the effect of PLGA NP itself on the allergic response mediated by mast cells without loading drug. There are some reports on the interactions between mast cells and nanomaterials, such as fullerene and gold, suggesting their negative and positive effects on the activation of mast cells [24,25]. Furthermore it has been reported that diesel exhaust particles (DEP) deteriorate allergic responses [26,27]. Therefore it is likely that the allergic responses mediated by mast cells could be altered through the interactions with PLGA NPs. Considering the clinical application of PLGA NPs, therefore, it is important to examine their effects on mast cells. In this study, the rat basophilic leukemia cell line, RBL-2H3, which possesses the phenotypic properties of mucosal mast cells, was used [28]. To evaluate the effects of PLGA NPs uptake on the antigen-induced activation of mast cells, the antigen-induced release of β -hexosaminidase and histamine were examined in mast cells. Furthermore, the effect of PLGA NPs on allergic responses *in vivo* was investigated using a mouse model of systemic anaphylaxis.

2. Materials and methods

2.1. Materials

PLGA (lactide:glycolide = 75:25; MW = 20 000) was purchased from Wako (Osaka, Japan). Polyvinyl alcohol (PVA; MW = 25 000; hydrolyzation degree = 88.0%; polymerization degree = 500) was purchased from Kuraray (Osaka, Japan). Hydroxypropyltrimonium CS was obtained from Katakurachikkarin (Tokyo, Japan). The laser grade fluorescent dye 6-coumarin, [3-(2-benzothiazolyl)-7-(diethylamine) coumarin], was purchased from MP Biomedicals (Solon, OH, USA). All other chemicals were of the highest grade commercially available.

2.2. Preparation of PLGA NPs by the emulsion solvent diffusion (ESD) method

PLGA NPs were loaded with 6-coumarin as the fluorescent label and prepared using the previously reported ESD method in an aqueous solution [29]. PLGA (100 mg) and 6-coumarin (1 mg) were dissolved in 3 mL of a mixture of acetone and ethanol (acetone:ethanol = 2:1). The resulting organic solution was poured into 25 mL of an aqueous PVA solution (2% w/v, in distilled water) and stirred at 400 rpm at room temperature using a propeller-type agitator with three blades. The entire dispersed system was then centrifuged (43,400 $\times$ g for 10 min), and the sediment was resuspended in distilled water. This process was repeated and the resulting dispersion was freeze-dried. To prepare CS-modified PLGA NPs, CS (0.25% w/v, in distilled water)-PVA (1% w/v) was used during the dispersing phase of the ESD process.

2.3. Analysis of the physicochemical properties of NPs

The particle size and zeta potential were determined using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK). Particle size was measured using photon correlation spectroscopy. The zeta potential was determined based on the electrophoretic mobility of NPs in an aqueous medium.

2.4. Cell culture

The RBL-2H3 cells were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) containing 10% FBS (Invitrogen) at 37 °C in an atmosphere of 5% CO₂.

2.5. Cytotoxicity assay

Cytotoxicity was assessed using the CellTiter 96® Aqueous One Solution assay (Promega, Madison, WI, USA). The solution reagent contained 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate, an electron coupling reagent. The RBL-2H3 cells were seeded in a 96-well plate (2.0 $\times$ 10⁴ cells/well). After confluent growth, different concentrations of NPs in RPMI 1640 were added to the wells. After 2 or 20 h of incubation, the cells were washed three times with RPMI 1640. Twenty microliters of the Aqueous One Solution reagent and 100 μ L of the culture medium were added directly to the culture wells. After 1 h of incubation, absorbance was measured using

a Model DTX880 Multimode Detector (Beckman Coulter, Fullerton, CA, USA) at a test wavelength of 490 nm and a reference wavelength of 660 nm. The quantity of the formazan product formed, as determined by absorbance at 490 nm, is directly proportional to the number of viable cells. The cell viability (%) relative to the control wells that contained the cell culture medium without any test samples was calculated using the following equation: $[A]_{\text{test}}/[A]_{\text{control}} \times 100$, where $[A]_{\text{test}}$ is the absorbance of the test sample and $[A]_{\text{control}}$ is the absorbance of the control sample.

2.6. Cellular uptake of PLGA NPs by the RBL-2H3 cells

The RBL-2H3 cells were seeded in 24-well plates (1.0 $\times$ 10⁵ cells/well). After confluent growth, the medium was replaced with the 6-coumarin-labeled NPs suspension in RPMI 1640 and incubated for 0.5–20 h at 37 °C. For the uptake experiments that were performed at 4 °C, the cells were preincubated with serum-free RPMI 1640 for 30 min before incubation at the respective NPs dispersion temperatures. Uptake was terminated by washing the cells three times with ice-cold phosphate-buffered saline (PBS) and solubilizing the cells with 0.1 mL of the reporter cell lysis reagent (Promega). The 6-coumarin fluorescent dye was extracted from NPs by mixing each sample with 3 mL of a methanol/chloroform mixture (1:1). The samples were centrifuged at 1400 $\times$ g for 10 min and the 6-coumarin concentration was determined by fluorescent spectrophotometry (Hitachi F-2500, 490 nm excitation wavelength, 520 nm emission wavelength; Hitachi Instruments, Tokyo, Japan). The protein content of the cell lysates was measured using the Pierce BCA protein assay kit (Rockford, IL). Uptake was expressed as the amount (μ g) of NPs associated with each unit weight (mg) of cellular protein.

2.7. Observation of cells exposed to PLGA NPs using confocal laser scanning microscopy

The RBL-2H3 cells were seeded in a Lab-Tek® II Chamber #1.5 German Coverglass System (Nalge Nunc International, Naperville, IL, USA) at a concentration of 1.0 $\times$ 10⁵ cells/well. After 1 day, in order to stain the endolysosomal compartments, the cells were incubated for 30 min in NPs suspension (1 mg/mL) of RPMI 1640 that contained 75 nm LysoTracker Red DND-99 (Molecular Probes, Eugene, OR, USA). Cellular uptake was terminated by washing the cells three times with ice-cold PBS and the cell monolayers were fixed using 0.5 mL of a 4% paraformaldehyde solution. After washing with PBS, the fixed cells were observed using an LSM 510 META microscope (Carl Zeiss, Gottingen, Germany) that was equipped with a Zeiss Plan-Apochromat 63 $\times$ /1.4 oil objective lens.

2.8. Assays for β -hexosaminidase and histamine secretions

The RBL-2H3 cells were seeded in 24-well plates (1.0 $\times$ 10⁵ cells/well). After confluent growth, the medium was replaced with the NPs suspension in RPMI 1640 (2.5 mg/mL) and incubated for 2 or 20 h at 37 °C. Uptake was terminated by washing the cells three times with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.6 mM MgCl₂, 0.1% glucose, 0.1% BSA, and 10 mM HEPES; pH 7.4). Cells were sensitized by incubation with anti-dinitrophenyl (DNP)-IgE (200 ng/mL) for 20 min, and then further incubated with DNP groups that had been conjugated with bovine serum albumin (DNP-BSA) in 200 μ L of HEPES-buffered saline for 30 min at 37 °C [30]. Degranulation of the RBL-2H3 cells was monitored by measuring the activity of β -hexosaminidase, a granule-stored enzyme that was secreted into the cell supernatant. Aliquots of the supernatant were incubated with a substrate solution (2 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide in 100 mM citrate; pH 4.5) for 45 min at 37 °C. After terminating the reaction with a Na₂CO₃–NaHCO₃ buffer, we measured the absorbance at 405 nm using a Model DTX880 Multimode Detector (Beckman Coulter). The supernatant was also used to measure the release of histamine by following the manufacturer's protocol for the enzyme immunoassay (EIA) system (Oxford Biomedical Research, Oxford, MI, USA).

2.9. Plasmid construction and generation of stable cell lines

Poly(A)⁺ RNA was obtained using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech) from 1 $\times$ 10⁷ RBL-2H3 cells; the poly(A)⁺ RNA obtained served as the template for cDNA synthesis with SuperScript II RT (Gibco BRL), as reported previously [17]. The primer pair for rat SNAP-23 was 5'-GAATT-CAATGGATGATCATACCA-3' (sense; the *EcoRI* site is underlined) and 5'-GTCCAGCTTAGCTGCAATGAGTTC-3' (antisense; the *Sal I* site is underlined). The PCR products were subcloned into pCR2.1-TOPO and confirmed by sequencing. Verified cDNA was ligated with pEYFP-C1 (CLONTECH, Palo Alto, CA, USA). To verify that the RBL-2H3 cells were able to stably express the yellow fluorescent protein (YFP)-tagged SNAP-23 protein, the RBL-2H3 cells were transfected with the plasmid DNA encoding YFP-SNAP-23. The RBL-2H3 cells were then electroporated in cold PBS with 40 μ g of plasmid DNA at 250 V and 950 μ F using the Gene Pulser II (Bio-Rad, Hercules, CA, USA). Stable transfectants were selected using G418 (Geneticin, Sigma, St. Louis, MO, USA). The localization of YFP-SNAP-23 into the RBL-2H3 cells was observed using fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan).

2.10. Western blot analysis

The RBL-2H3 cells that stably expressed YFP-SNAP-23 were harvested, and the cell pellets were resuspended in a radioimmunoprecipitation assay (RIPA) buffer that was supplemented with a protease inhibitor mixture (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cell debris was removed, and the protein concentration was determined using the Pierce BCA protein assay. Samples of the cell extracts that contained 25 µg of protein were analyzed by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The SNAP-23 protein was detected using a polyclonal antibody against SNAP-23 (Synaptic Systems, Goettingen, Germany) and horseradish peroxidase (HRP)-conjugated secondary antibody (R&D Systems, Minneapolis, MN, USA). The antigen-antibody complexes on the membranes were visualized using the Enhanced Chemiluminescence Western blotting detection System (Amersham Biosciences, Buckinghamshire, UK). The images were captured using a cooled charge-coupled-device (CCD) camera (Fuji LAS-3000 imaging system, Fuji Film, Tokyo, Japan).

2.11. Cellular uptake of PLGA NPs by mouse peritoneal mast cells (PMC)

All of the animal experimental procedures described here were approved by the Experimental Animal Research Committee of Aichi Gakuin University (Nagoya, Japan). Two hundred microliters of 6-coumarin-labeled PLGA NPs as suspended in PBS (100 g/l) and administered through i.p. injection to C57BL/6 mice (10–12 weeks old, male). Mouse peritoneal mast cells (PMC) were obtained by peritoneal lavage of mice with PBS [31]. The cells were sedimented by centrifugation at 150 ×g for 5 min. For use as unfractionated peritoneal cells, the pellets were resuspended in PBS. For preparation of purified PMC, pelleted peritoneal cells were resuspended in 7.5 mL of 72.5% isotonic Percoll (Pharmacia, Uppsala, Sweden) and centrifuged at 300 ×g for 7 min. The upper cell layer was discarded, and the pelleted cells were washed and resuspended in PBS. The resultant cell pellets were dissolved in a cell lysis buffer (Promega). The fluorescent dye (6-coumarin) was extracted from NPs by mixing 0.5 mL of the cell lysate with 5 mL of a methanol/chloroform mixture (1:1). The samples were centrifuged (1400 ×g for 10 min) and the concentration of 6-coumarin was measured using a fluorescence spectrophotometer (F-3010, Hitachi). Mast cells were determined by metachromatic staining using 0.01% toluidine blue. The stained mast cells were observed using fluorescence microscopy (BZ-9000, Keyence).

2.12. Passive systemic anaphylaxis

C57BL/6 mice were administered i.p. injections of 50 µg of DNP-specific mouse IgE (in 100 µL PBS) and subsequently injected with 20 mg PLGA NPs (suspended in 200 µL PBS). The mice were also administered i.p. injections of 100 µg DNP-BSA (in 100 µL PBS) 20 h later. Body temperature was measured every 10 min for 30 min using a rectal probe. After 30 min, the mice were sacrificed, and blood was collected by cardiac puncture for serum analysis. Serum histamine measurements were determined by EIA system.

2.13. Statistical analysis

One-way analysis of variance (ANOVA) followed by the Tukey test was applied to study the significance of difference.

3. Results

3.1. Physicochemical properties of PLGA NPs

PLGA NPs were prepared by the ESD method [29] and modified using CS, a biodegradable cationic polymer. The average particle diameters of the unmodified NPs (plain-NP) and CS-modified NP were 240 ± 4.7 nm and 338 ± 14.4 nm, respectively. The particle size of CS-PLGA NPs increased because of CS molecular layers formed on the surface of PLGA NPs. Freeze-dried NPs readily dispersed in an aqueous medium by shaking with hand, which resulted in nearly the same particle diameter as that obtained before drying. Plain-PLGA NPs, which were prepared using only PVA, a nonionic polymer, had a negative zeta potential (-28.6 ± 1.4 mV) due to the dissociation of the PLGA carboxyl group in distilled water. CS-PLGA NP had a highly positive zeta potential ($+22.8 \pm 0.8$ mV) in distilled water due to the protonation of the amino group.

3.2. Effects of PLGA NPs on the viability of mast cells

PLGA and CS are well tolerated by cells because they are biodegradable and biocompatible polymers. Cytotoxicity studies using

the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay showed that no adverse effects on cell viability are observed within the dose range used in uptake studies (<2.5 mg/mL) as shown in Fig. 1. None of the NP formulations were toxic to the cells that were incubated for 2 or 20 h at the tested dose ranges, irrespective of the surface modifier used.

3.3. Quantitative assessment of the uptake of PLGA NPs by mast cells

PLGA NPs were fluorescence labeled with 6-coumarin. The NPs were labeled uniformly by adding 6-coumarin to the organic solution containing PLGA to prepare the uniform solution that is subsequently poured into aqueous PVA solution. 6-coumarin-labeled NPs have been well characterized and are stable in the cells [11]. We also reported that 6-coumarin is hardly released from NS in both neutral (pH 7.4) and acidic (pH 5.0) conditions [5], indicating that the fluorescence detected in the cells is associated with the NSs [11]. The amount of PLGA NPs taken up into cells was estimated from fluorescence intensity of 6-coumarin extracted from the cells lysate sample with methanol/chloroform. As shown in Fig. 2A, the uptake of fluorescence-labeled PLGA NPs increased in a concentration-dependent manner during the incubation at 37 °C for 2 h. The cellular uptake of PLGA NPs increased by the surface modification with CS. The uptake of PLGA NPs occurred by endocytosis, based on the reduction in uptake that occurred at lower incubation temperatures (4 °C), irrespective of the surface properties. The uptake of PLGA NPs was dependent on the incubation time (Fig. 2B) and gradually increased with incubation time. The cellular uptake of plain-PLGA NPs was saturated at about 4 h and maintained the plateau level, while the uptake of CS-PLGA NPs showed an initial steep increase followed by decrease at 4 h and then reached the plateau level at 12 h.

3.4. Confocal imaging of PLGA NPs in mast cells

The cellular uptake of PLGA NPs was visually confirmed by a confocal laser scanning microscopy (CLSM), as shown in Fig. 2C and D. Confocal fluorescence images of the mast cells exposed to

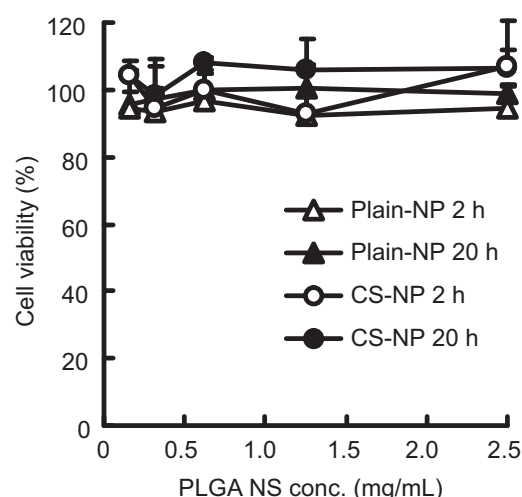


Fig. 1. Cytotoxic side effects of PLGA NP at concentrations ranging from 0 to 2.5 mg/mL in RBL-2H3 cells at 37 °C. PLGA NP were incubated for 2 or 20 h. The viability of the treated cells was determined using an MTS assay. The symbols represent mean $\pm$ SD ($n = 6$).

6-coumarin-labeled PLGA NPs for 30 min were shown. The images shown in Fig. 2C are optical slices with the nuclei, which indicate that PLGA NPs reside within the cells and were hardly adsorbed on the cell surface. NPs were found in the cytoplasm as early as 30 min after exposure to NPs. Consistent with the quantitative analysis described above (Fig. 2A), the cellular uptake of PLGA NPs was increased by the modification of NPs with CS. For further observation of the localization of NPs, the cells were incubated with NPs in the presence of LysoTracker Red DND-99, which visualize endosomes and lysosomes. 6-Coumarin-labeled PLGA NPs were colocalized with the lysosomal marker, which suggests that PLGA NPs were taken up through an endocytic pathway.

3.5. Effect of PLGA NPs on the exocytosis of mast cells

To evaluate the antigen-induced exocytic activity, β -hexosaminidase and histamine released into the supernatant were quantified. After the incubation with PLGA NPs for 2 or 20 h, the cells were stimulated with DNP-conjugated BSA, because the uptake levels of NPs were same at 2 and 20 h (Fig. 2B). The release of β -hexosaminidase was not affected by the incubation time nor the surface properties of NPs as shown in Fig. 3A. Histamine release was not changed by the incubation with NPs for 2 h. In contrast, when cells were incubated with NPs for 20 h and stimulated with DNP-BSA, histamine release was impaired. The inhibitory effects of plain NPs was greater than that of CS-NP (Fig. 3). The total amounts of β -hexosaminidase and histamine in mast cells were not affected

by the incubation with PLGA NPs, irrespective of the exposure time (Supplementary Fig. S1).

3.6. Exocytic exclusion of PLGA NPs from mast cells

To elucidate the mechanism of the inhibitory effect of PLGA NPs on the antigen-induced histamine release, we observed the exocytic release of NPs taken up by endocytosis. After exposure of mast cells to PLGA NPs for 2 or 20 h, the cells were incubated with DNP-IgE for 20 min. Then, the medium was then changed to HEPES buffer without DNP-BSA antigen. The amount of excluded PLGA NPs to the extracellular medium for 20 min was estimated by measuring the amount of PLGA NPs retained within the cells at 20 min after the medium change to HEPES buffer (Fig. 4A). Most of PLGA NPs taken up by the cells exposed for 2 h were not excluded from the cells. On the other hand, in the cells exposed for 20 h approximately 20%–40% of internalized NPs were exocytosed for 20 min after the medium change. Exocytic release of plain-NP from the cells was greater compared to that of CS-NP (Fig. 4A).

3.7. Co-release of β -hexosaminidase together with NPs from mast cells

During the exocytic exclusion of PLGA NPs (exocytic phase), there is a possibility that the secretory vesicles carrying PLGA NPs contain β -hexosaminidase and histamine, and co-release them without antigen stimulation. To examine this notion, exocytic

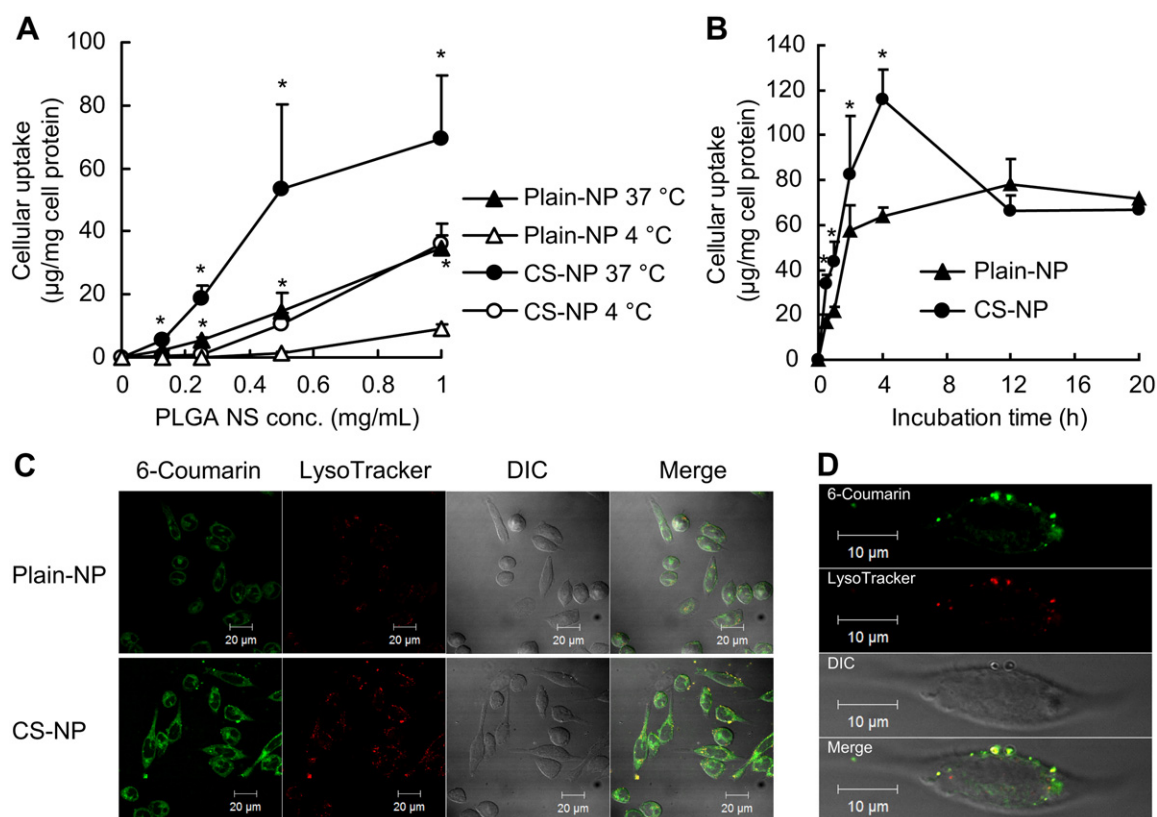


Fig. 2. Cellular uptake of PLGA NP by the RBL-2H3 cells. (A) Effects of NP concentration and incubation temperature (4 °C or 37 °C) on the cellular uptake of PLGA NP. NP concentrations ranged from 0 to 1 mg/mL for 2 h. Data are shown as mean $\pm$ SD ($n = 3$); * $P < 0.05$ compared with 4 °C. (B) Effects of incubation time on the cellular uptake of PLGA NP at 37 °C. The concentration of PLGA NP was 2.5 mg/mL. Data are shown as mean $\pm$ SD ($n = 3$); * $P < 0.05$ compared with plain-PLGA NP. (C) Confocal laser microscopy images of the RBL-2H3 cells following 0.5 h of incubation with PLGA NP containing 6-coumarin (1 mg/mL). (D) Enlarged images of (C) CS-NP. The RBL-2H3 cells that took up PLGA NP were co-incubated with LysoTracker Red DND-99 in order to label the endolysosomes.

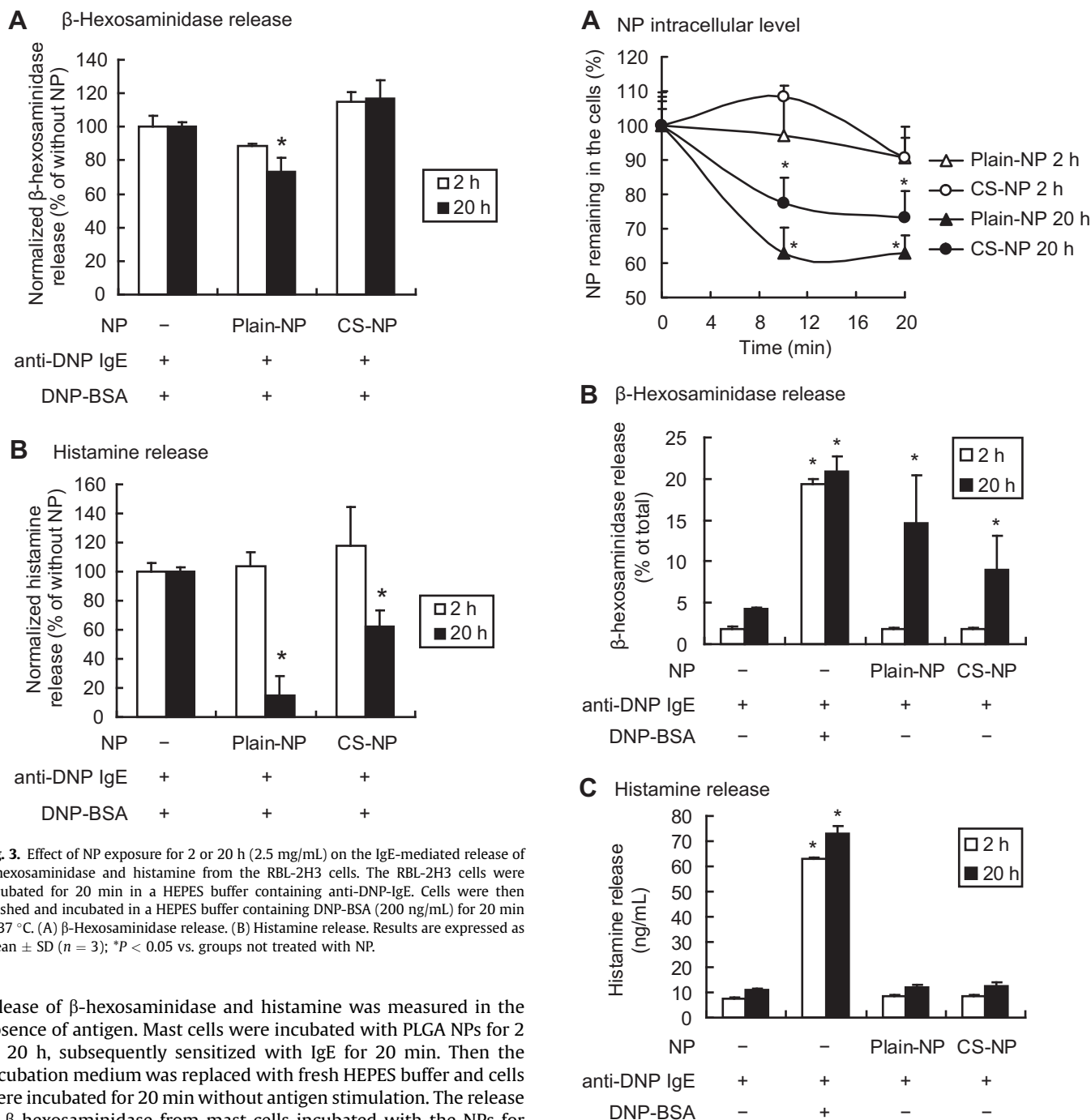


Fig. 3. Effect of NP exposure for 2 or 20 h (2.5 mg/mL) on the IgE-mediated release of β -hexosaminidase and histamine from the RBL-2H3 cells. The RBL-2H3 cells were incubated for 20 min in a HEPES buffer containing anti-DNP-IgE. Cells were then washed and incubated in a HEPES buffer containing DNP-BSA (200 ng/mL) for 20 min at 37 °C. (A) β -Hexosaminidase release. (B) Histamine release. Results are expressed as mean $\pm$ SD ($n = 3$); * $P < 0.05$ vs. groups not treated with NP.

release of β -hexosaminidase and histamine was measured in the absence of antigen. Mast cells were incubated with PLGA NPs for 2 or 20 h, subsequently sensitized with IgE for 20 min. Then the incubation medium was replaced with fresh HEPES buffer and cells were incubated for 20 min without antigen stimulation. The release of β -hexosaminidase from mast cells incubated with the NPs for 20 h was detected as shown in Fig. 4B. In contrast, the release of histamine was not detected under the same experimental conditions (Fig. 4C).

3.8. Effects of overexpression of SNAP-23 on exocytic release

We attempted to examine whether SNAP-23 (one of t-SNAREs involved in regulated exocytosis of mast cells) affects the exocytic exclusion of NPs and antigen-induced exocytosis. Mast cells were transfected with an expression plasmid for YFP-tagged SNAP-23. The stable transfectants were selected with G418. The expression of YFP-SNAP-23 was confirmed by a fluorescence microscopy (Fig. 5A) and western blotting (Fig. 5B). The overexpression of SNAP-23 did not affect the degranulation induced by antigen compared to wild type (Supplementary Fig. S2). The antigen-induced releases of β -

Fig. 4. Effect of PLGA NP exocytosis on the release of β -hexosaminidase and histamine from the RBL-2H3 cells without the use of a stimulating antigen. After exposure to NP for 2 or 20 h (2.5 mg/mL), the RBL-2H3 cells were incubated for 20 min in the HEPES buffer containing anti-DNP-IgE. Cells were then washed and incubated in the HEPES buffer without DNP-BSA for 20 min at 37 °C. (A) Time course of the intracellular level of PLGA NP. * $P < 0.05$ vs. plain-NP groups. Release of (B) β -Hexosaminidase and (C) histamine 20 min after the IgE solution was replaced with fresh HEPES buffer. * $P < 0.05$ vs. groups not treated with NP. All results are expressed as mean $\pm$ SD ($n = 3$).

hexosaminidase and histamine from mast cells that overexpress SNAP-23 were examined after exposure to NPs for 20 h. The release of β -hexosaminidase was not affected regardless of incubation with the NPs (Fig. 5C) as in the case of wild type mast cells (Fig. 3A). On the other hand, the inhibition of histamine release by NPs (Fig. 3B) was not observed in the cells overexpressing SNAP-23 (Fig. 5D).

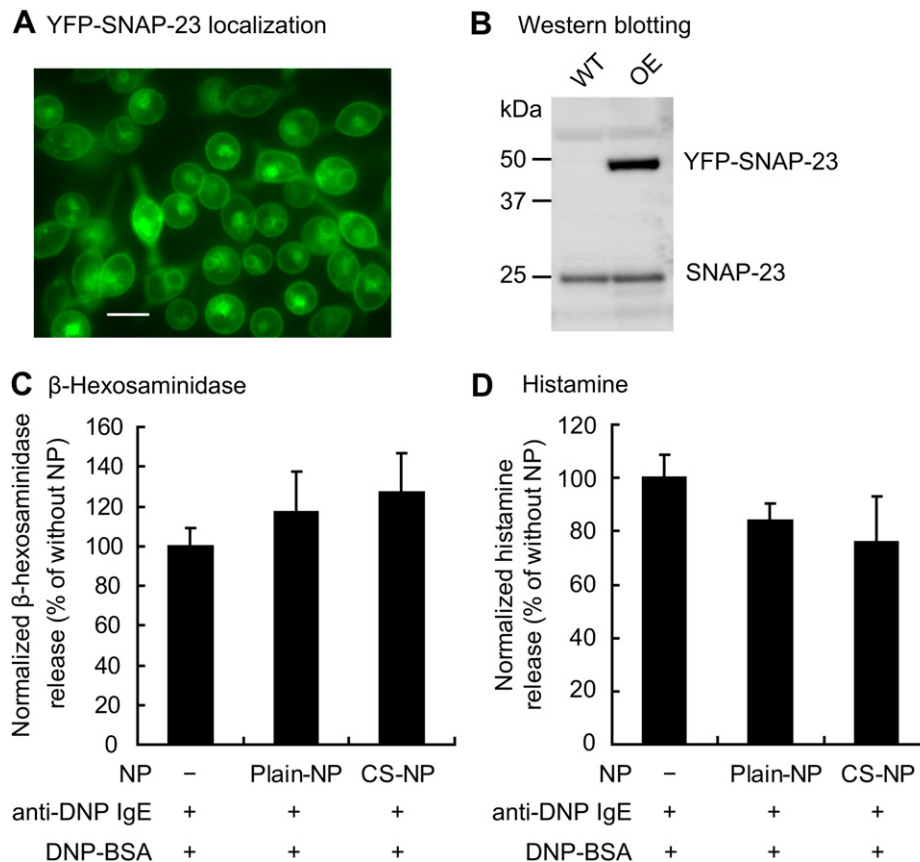


Fig. 5. Effect of the overexpression of YFP-SNAP-23 mutants of the RBL-2H3 cells on the inhibition of histamine release following NP exposure. (A) Fluorescent image of the RBL-2H3 cells expressing YFP-tagged SNAP-23. Scale bar = 20 μ m. (B) The expression of endo- and exogenous SNAP-23 in wild type (WT) and YFP-SNAP-23-overexpressing (OE) The RBL-2H3 cells were confirmed by western blot analysis. The cells were sensitized to DNP-specific IgE for 20 min after NP (2.5 mg/mL) exposure for 20 h and then stimulated using DNP-BSA. Cell supernatants were collected after 20 min and the extent of degranulation was determined by measuring the amount of β -hexosaminidase (C) and histamine (D) released. The results are expressed as mean $\pm$ SD ($n = 3$).

These results suggest that the overexpression of SNAP-23 rescued the impaired histamine release from cells exposed to NPs for 20 h.

not treated with NPs. In contrast, plain-PLGA NPs did not affect the serum histamine level.

3.9. Effect of PLGA NPs on systemic anaphylaxis

Our results mentioned above suggest the anti-allergic activity of PLGA NPs. Thus we investigated their anti-allergic actions *in vivo* using mouse PMC. First, we examined the cellular uptake of 6-coumarin-labeled NPs by PMC at 20 h after an intra peritoneal (i.p.) injection of NPs. PMC were collected by peritoneal lavage of a mouse and identified by staining with 0.01% toluidine blue. The quantitative uptake analysis revealed that the uptake of 6-coumarin-labeled NPs in PMC dramatically increased by the surface modification with CS (Fig. 6A). This is confirmed by imaging analysis (Fig. 6B).

Since systemic anaphylaxis is predominately driven by the activation of mast cell and peripheral blood basophil, we observed the effects of PLGA NPs on the allergic responses in a mouse model of anaphylaxis. An i.p. injection of the antigen to C57BL/6 mice induced systemic anaphylaxis with a rapid drop in rectal temperature, approximately 4 $^{\circ}$ C within 30 min (Fig. 6C). The i.p. injection of CS-PLGA NPs 20 h before the systemic antigen challenge attenuated the anaphylactic drop in core body temperature. As shown in Fig. 6D, mice treated with CS-PLGA NPs through an i.p. injection 20 h before the systemic antigen challenge showed a significant decrease in serum histamine level compared with mice that were

4. Discussion

Polymeric NPs are easily taken up by the cells through the endocytic pathway. The physicochemical properties of PLGA NPs have significant effects on their uptake level into cells and the intracellular fate of NPs [7,8]. The aim of this study was to investigate the effect of PLGA NPs on the allergic response of mast cells after exposure to NPs without loading drug. PLGA NPs and CS-PLGA NPs were prepared using the ESD method. The zeta potential of NP shifted from negative to positive when a cationic polymer (i.e., CS) as a surface modifier was used. This proved that the surface of PLGA NPs can be modified by adding a CS solution to the outer phase with a PVA solution.

Data from our experiments on mast cells confirmed that PLGA NPs can be taken up by endocytic processes, although a smaller uptake was observed at 4 $^{\circ}$ C in high doses of NPs probably due to a passive process such as diffusion (Fig. 2A). As shown in Fig. 2B, mast cells readily endocytosed NPs in first 4 h after exposure to NPs. However, the amount of intracellular NPs reached the plateau level around 12 h, suggesting that NPs are excluded from the cells by exocytosis (exocytic phase) and balanced with uptake of NPs.

We found that antigen-induced histamine release from mast cells exposed to NPs for 20 h, or in the exocytic phase was inhibited (Fig. 3B). This raises two interesting questions. First, why the

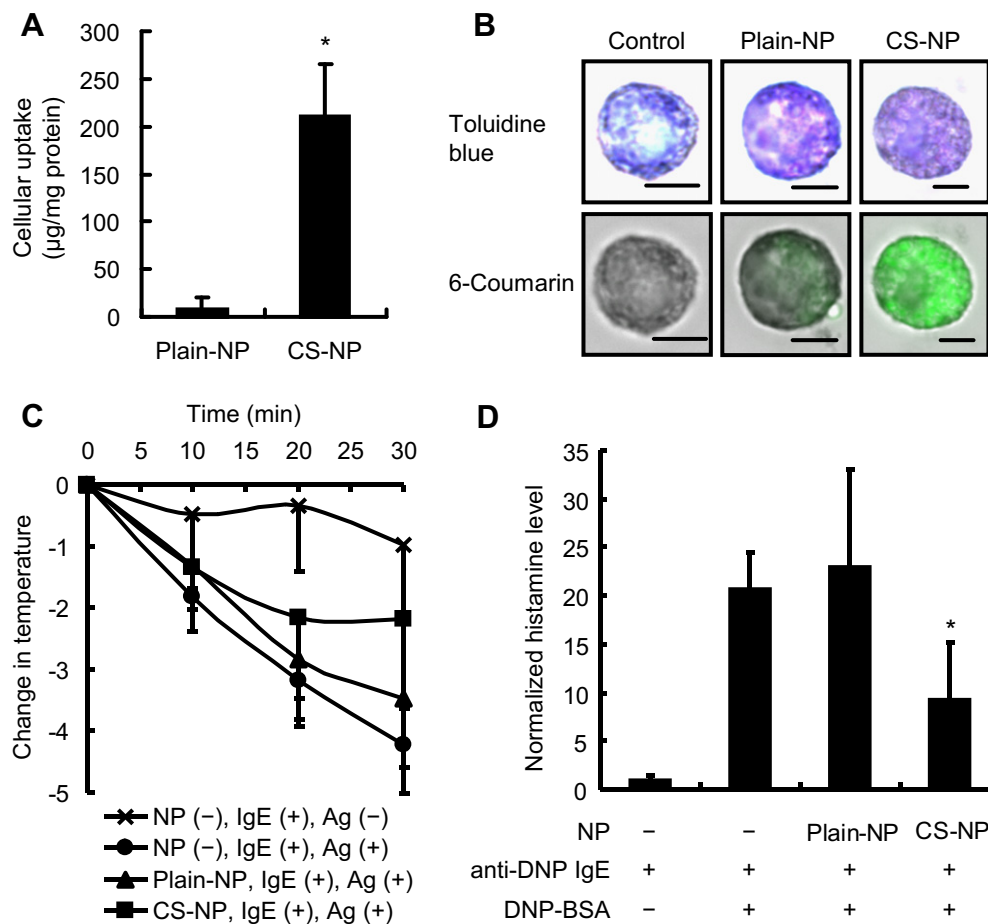


Fig. 6. Effect of an i.p. injection of NP on the cellular uptake of NP by mouse PMC and inhibition of IgE-antigen (Ag)-induced systemic anaphylaxis reactions. (A) Cellular uptake of NP by PMC obtained by peritoneal lavage of C57/BL6 mice; * $P < 0.05$ vs. plain-NP groups. (B) Fluorescence microscopy images of PMC 20 h after administration of NP. Scale bar = 10 μm . (C) C57/BL6 mice were administered an i.p. injection of NP and their rectal temperatures were measured at the indicated times for up to 30 min. (D) Serum histamine levels were measured by enzyme immunoassay; * $P < 0.05$ vs. groups not treated with NP. All results are expressed as mean $\pm$ SD ($n = 6$).

inhibition of histamine was observed in the cells exposed to NPs for 20 h but not for 2 h. Second, why the inhibition of antigen-induced exocytotic release was observed in histamine but not in β -hexosaminidase. The cell viability and the total content of histamine and β -hexosaminidase in the cells were not affected by the exposure to NPs in any experimental conditions (Fig. 1, Supplementary Fig. S1). It could be possible that NPs adsorbed to the plasma membrane inhibited the binding of DNP-IgE to Fc ϵ -RI receptor, or NPs taken up into the cells impaired mechanism involved in antigen-induced exocytosis. If so, however, both histamine and β -hexosaminidase releases should have been inhibited, and it is difficult to explain why the inhibition of histamine release was not observed in the cells exposed for 2 h. To address the first question, we hypothesized that secretory vesicles carrying NPs in the cells with the exposure of 20 h competitively antagonize exocytic machinery for antigen-induced exocytic release of histamine (degranulation), but those in the cells with the exposure for 2 h do not (Fig. 7). It has been suggested that there are at least two kinds of intracellular transport system for internalized NPs, one is like a recycling endosome that turns over rapidly and the other is more complex vesicle transport system that turns over relatively slowly [9] as shown in Fig. 7. When cells are exposed for 2 h, most of NPs taken up by the cells are thought to be present in the recycling endosomes. On the other hand, when cells are exposed for 20 h, a substantial fraction of NP-containing vesicles escaped from the recycling endosome pathway and subsequently interact with the cytoplasmic compartments

such as ER, Golgi apparatus, and secretory granules. This allows vesicles containing NPs to carry β -hexosaminidase and v-SNARE. Then the secretory vesicles with v-SNARE and NPs could compete with secretory granules for t-SNARE, resulting in decrease in antigen-induced histamine release. In fact, the histamine release was recovered in the cells overexpressing SNAP-23, a t-SNARE involved in antigen-induced histamine release in mast cells. It is suggested that the inhibitory mechanism is that the exocytic exclusion of NPs competitively antagonizes the antigen-induced degranulation from mast cells by hijacking exocytosis machinery such as SNARE protein. Furthermore, to examine the effects of NP on the process after elevation of intracellular Ca^{2+} , we stimulated cells with the phorbol ester and calcium ionophore (Supplementary Fig. S3). Since the stimulation with phorbol ester and calcium ionophore bypasses events before the increase in Ca^{2+} , we can see whether NPs uptaken into the cells affects the membrane fusion between the secretory granules and the plasma membrane [30]. When stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL), and A23187 (1 μM) as a calcium ionophore in the presence of 1 mM Ca^{2+} , exocytosis was also inhibited by NP's exposure for 20 h. This suggests that intracellular NPs inhibited the exocytotic membrane fusion itself.

As for the second question, β -hexosaminidase in the secretory vesicles containing NPs might explain. As mentioned above, after exposure to NPs for 20 h, the NP-containing vesicles have β -hexosaminidase inside. This is confirmed by the release of β -

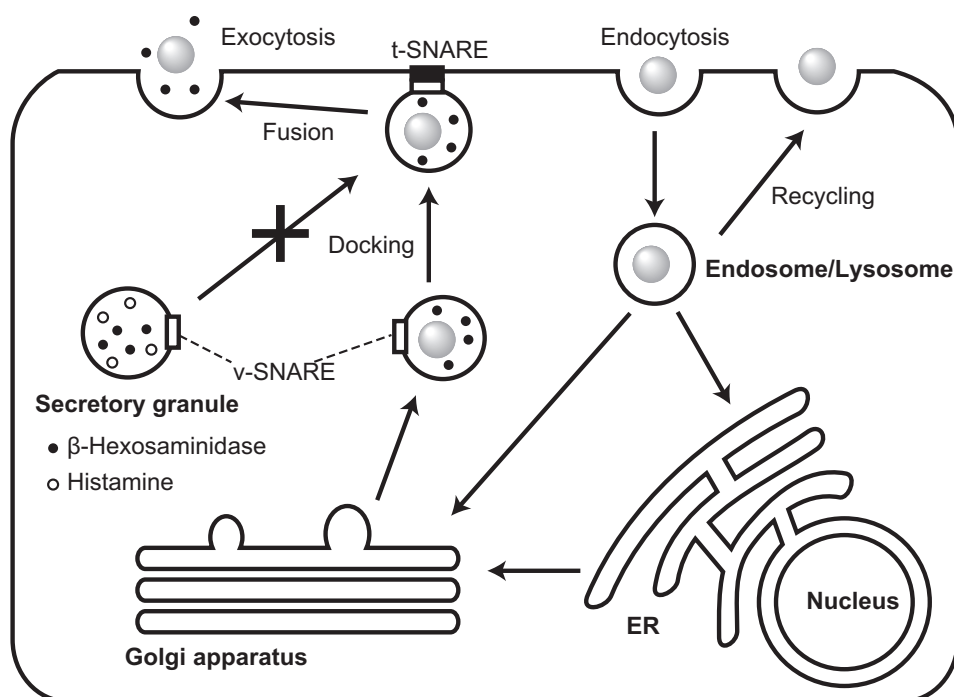


Fig. 7. Schematic representation of the competitive inhibition of degranulation by exocytosis of NP. By the exposure to NPs for 20 h, a substantial fraction of NP-containing vesicles escaped from the recycling endosome pathway. The secretory vesicles that contain NPs and β -hexosaminidase (but not histamine) compete with secretory granules that contain histamine to bind with t-SNARE on the cell membrane, preventing the secretory granules from docking and fusion with the cell membrane (+).

hexosaminidase without antigen stimulation (Fig. 4B). However, histamine was not contained in the vesicles carrying NPs even after exposure to NPs for 20 h, since histamine was not released as shown in Fig. 4C. The antigen stimulation induces secretion through secretory granules that contain histamine and β -hexosaminidase (degranulation). During the degranulation, secretory vesicles that contain NPs and β -hexosaminidase also release their intra-vesicular contents regardless of stimulation. Both types of secretory vesicles compete each other and the release of histamine is inhibited, while the release of β -hexosaminidase was not (Fig. 3A, B), because β -hexosaminidase was contained in both types of vesicles.

The exocytic exclusion of NPs from the cells exposed to plain-NP for 20 h was higher than that in the case of CS-NPs despite of the same levels of cellular uptake (Figs. 2B and 4A). The low efficiency of the exocytic exclusion of CS-NPs might be explained by the positive charge of CS that enhances the interaction between NPs and the cytoplasmic compartments through electrostatic interactions [8].

In the experiments using mouse model of anaphylaxis, CS-NPs effectively reduced anaphylaxis and histamine levels in serum (Fig. 6C,D). In contrast, the histamine level did not decrease by an i.p. injection of plain-NPs. These results are not consistent with the results shown in Fig. 3B in which plain-NPs inhibited the release of histamine more effectively than CS-NPs. One reason for this discrepancy is that the cellular uptake of plain-NPs in PMC was smaller compared with CS-NPs, as shown in Fig. 6A and B. CS is a cationic polymer and has mucoadhesive abilities. Therefore, CS-NPs retained in the abdominal cavity would interact with PMC more effectively compared with plain-NPs.

We have developed the PLGA NPs system to deliver pulmonary drugs, including peptides and nucleic-acid based drugs [3,4,32–35]. Based on the results of this study, the use of PLGA NPs as a carrier of pulmonary drug has little risk of activation of pulmonary mast cells and the release of inflammatory mediators. This suggests that PLGA NPs are suitable for carrying drugs to be delivered through inhalation.

5. Conclusions

PLGA NP itself has an action of inhibiting allergic responses mediated by mast cells *in vitro* and *in vivo*. The inhibitory effect of NPs seems to be explained by the mechanism that the exocytic exclusion of NPs competitively antagonizes the antigen-induced degranulation mediated by SNARE proteins. It would be expected that PLGA NPs works as not only a safe and efficient drug carrier but also an anti-allergic drug.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.09.043.

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