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ORIGINAL RESEARCH

Antigen-Containing Silk Nanoparticles: A New Potential as Oral Vaccine Carriers

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Purpose: Oral vaccines are safer, easier to administer, and more cost-effective than injectable vaccines are. They induce systemic and mucosal immunity, thereby providing broad protection. However, developing an orally administered vaccine that can traverse the stomach and safely reach the intestinal tract to induce antigen-specific immune responses is challenging. Recently, many effective nanoparticle (NP) drug delivery systems have been developed. Various polymeric materials, including synthetic biodegradable and natural polymers, have been used as drug delivery matrices. Silk fibroin, a natural polymer, shows promise as a suitable material for drug delivery due to its biocompatibility, biodegradability, and aqueous processability. In this study, to investigate the potential of silk proteins as oral vaccine carriers, we prepared ovalbumin (OVA)-containing silk NPs through salting-out an aqueous solution of degummed fibers and non-degummed cocoons obtained from silkworms (*Bombyx mori*) and examined whether oral administration could induce OVA-specific antibody production in mice.

Methods: For the OVA-containing silk NPs, OVA was prepared through salting-out a mixture of OVA and an aqueous solution of cocoons or degummed fibers. OVA-specific antibody production in mice orally administrated OVA-containing silk NPs was analyzed using ELISA. Silk NP delivery to the intestinal tract of mice was monitoring using FITC-incorporated silk. The FITC-labeled cocoon and degummed fiber NPs were incubated with peritoneal macrophages, and their internalization observed using fluorescence imaging.

Results: OVA-containing silk NPs can be delivered to the intestinal tract after oral administration to mice and subsequently induce OVA-specific serum IgG and intestinal IgA production without the need for adjuvants. Moreover, internalization of FITC-labeled cocoon or degummed fiber NPs into peritoneal macrophages was observed in vitro.

Conclusion: Overall, silk NPs are promising oral vaccine carriers that can deliver antigens to the intestinal tract while avoiding decomposition by gastric acid and digestive enzymes, thereby facilitating the activation of mucosal and systemic immunity.

Keywords: fibroins, nanoparticles-based drug delivery system, oral administration, antibody production

Introduction

Vaccination is a cost-effective strategy that not only protects vaccinated individuals but also indirectly protects the surrounding community by generating herd immunity. While the majority of licensed vaccines are administered either via subcutaneous or intramuscular injection, these immunizations can induce systemic humoral immunity but cannot provide protection through respiratory and digestive mucosal tissues, which may act as the entry points for pathogens.[1] In contrast, oral vaccines induce humoral and cellular immune responses at both systemic and mucosal sites, potentially establishing broader and longer-lasting protection.[2–5] Further advantages of oral vaccines are that they can be self-administered; do not require sterile needles, syringes, or a medical professional;[6–8] and do not cause pain or fear of injections.[9]

The key requirements for developing an oral vaccine include the ability to pass through the stomach, which is a harsh environment that contains gastric acid and digestive enzymes; safe delivery to the intestinal tract; and efficient induction of antigen-specific immune responses. Recently, various polymeric materials have been used as oral vaccine carriers.

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Particularly, poly(lactic-co-glycolic acid) and chitosan have shown promising results as orally administered vaccine carriers.[10,11] Chitosan, a naturally occurring mucoadhesive cationic polysaccharide, has been intensively investigated as a material for polymeric nanoparticles (NPs) due to its unique properties. Chitosan possesses a positive charge, which allows for strong electrostatic interactions with the negatively charged mucosal surface, prolonging drug residence time and enhancing drug absorption. Furthermore, by opening tight junctions between cells, chitosan also acts as an absorption enhancer for poorly absorbed drugs.[12]

Bombyx mori (silkworm) silk proteins are used as unique biomaterials in medical applications. The *B. mori* silk fibers are composed of two proteins: fibroins (75–80% [w/w]) as the fiber core and sericins (20–25% [w/w]) coated around fibroins as the outer layer.[13] When silk is used as a medical material, it is typically made from fibroin fibers with the sericin layer removed. After removing the outer sericin layer through a degumming process in which the cocoons are soaked in a Na_2CO_3 solution and boiled at 100°C for 30–60 min, the fibroin aqueous solution (dissolved in LiBr or $CaCl_2$) can be fabricated into a variety of formats, including powders, films, gels, and sponges.[14–16] Furthermore, fibroin NPs can be produced from aqueous fibroin solutions via several methods, including desolvation, salting-out, mechanical milling, and electrospraying.[17,18,] Attempts have previously been made to use fibroin NPs as carriers in drug delivery systems.[19–21]

Salting-out is a simple method used to prepare protein-based NPs. Proteins have hydrophilic and hydrophobic regions, where the hydrophobic portion interacts with water molecules, allowing the protein to form hydrogen bonds with surrounding water molecules. As the concentration increases, salt ions attract water molecules, removing the water barrier between protein molecules and increasing protein–protein interactions. Consequently, the protein molecules aggregate by forming hydrophobic interactions with each other and precipitate from the solution. Lammel et al reported that salting-out with potassium phosphate (>0.75M) produced silk fibroin NPs with an average diameter of 486–1200 nm, and that the shape, structure, and size of the particles could be controlled by adjusting the salt concentration and pH.[17]

In this study, to investigate the potential of silk proteins as oral vaccine carriers, we prepared ovalbumin (OVA)-containing silk NPs through salting-out an aqueous solution of non-degummed cocoons, which are anticipated to showcase the adjuvant effect of sericin, or degummed fibers and examined whether they could induce the production of OVA-specific antibodies in orally administered mice. OVA is commonly used as a model protein for studying antigen-specific immune responses in mice. The present study demonstrates the potential of antigen-containing silk NPs as promising oral vaccine carriers.

Materials and Methods

Preparation of the Cocoon and Degummed Fiber Aqueous Solutions

Twenty-four cocoons from the *w1-pnd* silkworm strain[22] were used. The degummed fibers were prepared from raw silk fibers obtained from the commercial Gunma 200 silkworm strain, which were degummed with 0.02 M Na_2CO_3 solution at 98°C for 30 min. The cocoons and degummed fibers were washed with 70% ethanol, followed by 0.1% sodium dodecyl sulfate (SDS) aqueous solution, and thereafter thoroughly rinsed with deionized water. Two grams of the degummed fibers were suspended in 2 mL of 70% ethanol, 18 mL of 10 M LiBr, and 100 mM Tris-HCl (pH 9.0), and then vigorously mixed at 37°C until completely dissolved. One gram of the cocoons was suspended in 4 mL of 70% ethanol, 36 mL of 10 M LiBr, and 100 mM Tris-HCl (pH 9.0), and thereafter vigorously mixed at 37°C until complete dissolution. The resultant silk aqueous solutions were dialyzed against 8 L deionized water for three days at room temperature (25 ± 5°C). The water was refreshed every 12 h. Dialysis proceeded until the electrical conductivity of the external dialysis fluid became ≤0.3 μS. The silk solution after dialysis was collected in a centrifuge tube and centrifuged (13,000 × *g*, 25°C, 10 min) to remove solid matter, and the supernatant then diluted in 10 mg/mL deionized water.

Preparation of Silk NPs

OVA (50 mL of 1 mg/mL [w/w]) (012–09885; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) or 50 mL deionized water was added to 50 mL of the 10 mg/mL silk aqueous solution of cocoons or degummed fibers and then mixed, whereafter the samples were left stationary at room temperature for 20 min. Then, 90 mL of each prepared solution was added to 360 mL of 1.25 M K_2HPO_4, mixed, and left stationary at 4°C for two days to form NPs. Following this, the silk

NPs were collected using centrifugation (2,100 × *g*, 25°C, 30 min), washed twice with 500 mL deionized water, and washed twice more with 50 mL deionized water. Finally, the silk NPs were suspended in deionized water at 30 mg/mL.

Observation of Silk NPs with Scanning Electron Microscopy

The silk NP suspensions were diluted to 1 mg/mL with deionized water, and 1 μL of the diluted solution dropped onto a silicon wafer, which was then thinly coated with Au (Quick Coater SC-701; Sanyu Denshi, Tokyo, Japan). Observations were performed using scanning electron microscopy (SEM) (TM4000Plus; Hitachi High-Tech Corporation, Tokyo, Japan) at 5 kV and a 2500× magnification. After SEM imaging, more than 100 particles/image were randomly selected, approximated as ellipses, and their diameter calculated based on their areas using ImageJ software v.1.53t (National Institutes of Health, Bethesda, MD, USA).

Characterization of Silk NPs with Dynamic Light Scattering

The diameter and polydispersity index (PDI) of silk NPs (0.5 mg/mL) were evaluated via dynamic light scattering (DLS) using a Zetasizer Nano S system (Malvern Panalytical Ltd., Malvern, Worcestershire, UK). Measurements were repeated five times, whereafter the average particle diameter, particle appearance, and PDI were calculated using Zetasizer Nano software v.8.02 (Malvern Panalytical Ltd).

Immunoblotting

The four types of silk NPs were dissolved in 9 M LiBr and 90 mM Tris-HCl (pH 9.0), and the concentration of each silk solution adjusted to 2 mg/mL in 2 M LiBr and 20 mM Tris-HCl (pH 9.0). The resulting silk solutions were treated with 2× SDS sample buffer, separated using 12% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked with Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature and incubated with anti-OVA polyclonal antibody (1:2000) (ab186717; Abcam, Cambridge, UK) and anti-fibroin-L-chain (FibL) polyclonal antibody[23] (1:2000), followed by alkaline phosphatase-conjugated anti-rabbit IgG H&L (1:10000) (ab97048; Abcam). Immunoreactive proteins were detected using a BCIP-NBT Solution Kit for alkaline phosphatase staining (Nacalai Tesque).

Oral Administration to Mice

Female Slc:ICR mice (6–7 weeks old, weighing 25 ± 3 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed under pathogen-free conditions at the National Institute of Agrobiological Sciences Animal Facility (National Agriculture and Food Research Organization, Ibaraki, Japan). The four types of silk NPs were orally administered to mice using disposable feeding needles (code no. 493–18GS; Natsume Seisakusho Co., Ltd., Tokyo, Japan) at 10 mL/kg (mouse body weight) per administration. An equal volume of normal saline and OVA solution, which contained an amount of OVA equivalent to that in the OVA-containing silk NPs, were orally administered as controls. After three consecutive days of oral administration, the silk NPs were orally administered three times at weekly intervals from the first administration. On the day after final administration, the mice are euthanized via cardiac exsanguination under isoflurane (099–06571; FUJIFILM Wako Pure Chemical Corporation) inhalation (4–5% concentration). The intestinal tract was individually collected from euthanized mice, and the insides of the intestinal tract washed with phosphate-buffered saline (PBS). The washings were then centrifuged (4,000 × *g*, 4°C, 10 min), and the supernatant collected as intestinal lavage fluid. All animal procedures were approved by the Institutional Animal Care and Use Committee of the National Agriculture and Food Research Organization (approval ID: R5-M11-NIAS-2).

ELISA

To assess antibody production, degummed fibers and the cocoons obtained from the Sericin Hope[24] strain, consisting of only sericin and no fibroin, were dissolved in 8 M LiBr and 80 mM Tris-HCl (pH 9.0), whereafter they were diluted with 1 mM Tris-HCl (pH 8.0) to a concentration of 0.125 mg/mL. One hundred microliters of the diluted silk and OVA solutions (0.125 mg/mL) were applied to 96-well plates and incubated overnight at 4°C. After four washes with PBS and Tween 20 (PBS-T), each well was blocked with ELISA Assay Diluent (BioLegend, San Diego, CA, USA) at room temperature for

60 min. After four washes with PBS-T, the blood serum and intestinal lavage fluid diluted 200-fold and 20-fold with ELISA Assay Diluent, respectively, were added to the wells and incubated at room temperature for 90 min. Binding was detected using horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (1:2500) (code no. P0447; Dako, Glostrup, Denmark), HRP-conjugated anti-mouse IgA alpha chain (1:10000) (ab97235; Abcam,), and finally incubated with the ELISA POD Substrate TMB solution (Nacalai Tesque). To quantify the amount of OVA contained in silk NPs, the aqueous solutions of OVA-containing silk NPs and serially diluted OVA solutions were added to 96-well plates and incubated overnight at 4°C. After four washes with PBS-T and blocking with ELISA Assay Diluent, an anti-OVA polyclonal antibody (ab186717; Abcam) was added to the wells and incubated at room temperature for 90 min. Binding was detected using HRP-conjugated anti-rabbit immunoglobulin (1:4000) (code no. P0399; Dako) antibodies and finally incubated with the ELISA POD Substrate TMB solution. After color development, the reaction was terminated with 2N H_2SO_4, and the absorbance read at 450 nm using a microplate reader (Infinite F50 Plus; TECAN, Männedorf, Switzerland).

Preparation of FITC-Inner-Labeled Silk NPs and Oral Administration to Mice

Twenty milliliters of the degummed fiber aqueous solution adjusted to 5 mg/mL and 80 mL K_2HPO_4 (1.25 M) and FITC-I (Dojindo, Kumamoto, Japan) adjusted to 1 μg/mL were mixed and left stationary at 23°C for four days to form NPs. After four washes with ultrapure water, the FITC-inner-labeled silk NPs were suspended in 2 mL ultrapure water. Based on previous salting-out tests, which showed that at least 80% of the input silk protein could be recovered, the protein concentration of the suspension was estimated to be 40 mg/mL. The FITC-inner-labeled silk NPs (250 μL) were orally administered to mice using disposable feeding needles. Intestinal tracts were collected from the mice 1, 2, and 4 h after oral administration and photographed under UV light.

Preparation of FITC-Labeled OVA-Containing Silk NPs and Phagocytic Activity of Peritoneal Macrophages

Previously prepared cocoon and degummed fiber silk NPs with or without OVA were suspended in 2.5 mL of 0.1 M $NaCO_3$ buffer (pH 9.0) and incubated with 100 μL FITC-I adjusted to 1 mg/mL in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for three days. After four washes with ultrapure water, the FITC-labeled silk NPs were suspended in ultrapure water at a concentration of 50 mg/mL. Mouse peritoneal cells were seeded at a density of 5×10^5 cells/well in 8-well chamber slides. After the cells adhered to slides, 20 μg/mL FITC-labeled silk NPs were added to the cells and incubated for 2 h. After washing with PBS, the cells were fixed with 10% formalin in PBS for 30 min at room temperature. The cells were then washed with ultrapure water and mounted onto coverslips using the VECTASHIELD Mounting Medium (Vector Laboratories Inc., Newark, NJ, USA). Images were captured using a fluorescence microscope (BZ-X710; KEYENCE, Osaka, Japan).

FACS Analysis

Intestinal lavage fluid from the mice orally administrated FITC-labeled silk NPs were collected by washing with PBS and filtered using an EASYstrainer (item no. 542040; Greiner Bio-one, Frickenhausen, Germany). The intestinal lavage fluid samples were then analyzed using flow cytometry (Accuri C6 Plus; BD Bioscience, Franklin Lakes, NJ, USA). Peritoneal cells were seeded at a density of 2×10^6 cells/well in 6-well cell culture plates and incubated for 2 h. Once the cells adhered to the plates, 100 μg/mL FITC-labeled silk NPs was added to the cells and incubated for 2 h. After washing with PBS, the cells were harvested and analyzed using flow cytometry.

Statistical Analysis

Statistical significance was evaluated using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Student's *t*-test was used for paired samples, and one-way ANOVA with Tukey's test used for multiple samples. A P-value <0.05 was considered statistically significant.

Results

Formation of OVA-Containing Silk NPs via Salting-Out

To prepare the OVA-containing silk NPs, OVA equivalent to 10% of the total silk protein was added to an aqueous solution of cocoons or degummed fibers and mixed with 1.25 M K_2HPO_4.[17] The obtained silk NPs were observed using SEM, and the particle size distribution of silk NPs evaluated via DLS. In the SEM image measurements, particles prepared from the aqueous solution of cocoons had small diameters (<320 nm), with or without OVA. In contrast, particles prepared from an aqueous solution of the degummed fibers had slightly larger diameters, ranging from 417–789 nm, with or without OVA (Figure 1A–D, top panel; Table 1). The presence of OVA did not significantly affect particle size distribution. However, in the DLS measurements, silk NPs prepared from each of the cocoons and degummed fibers had an average diameter of approximately 1000 nm (Figure 1A–D, bottom panel; Table 1). Furthermore, the PDI of each NP was large, exceeding 0.3, indicating their

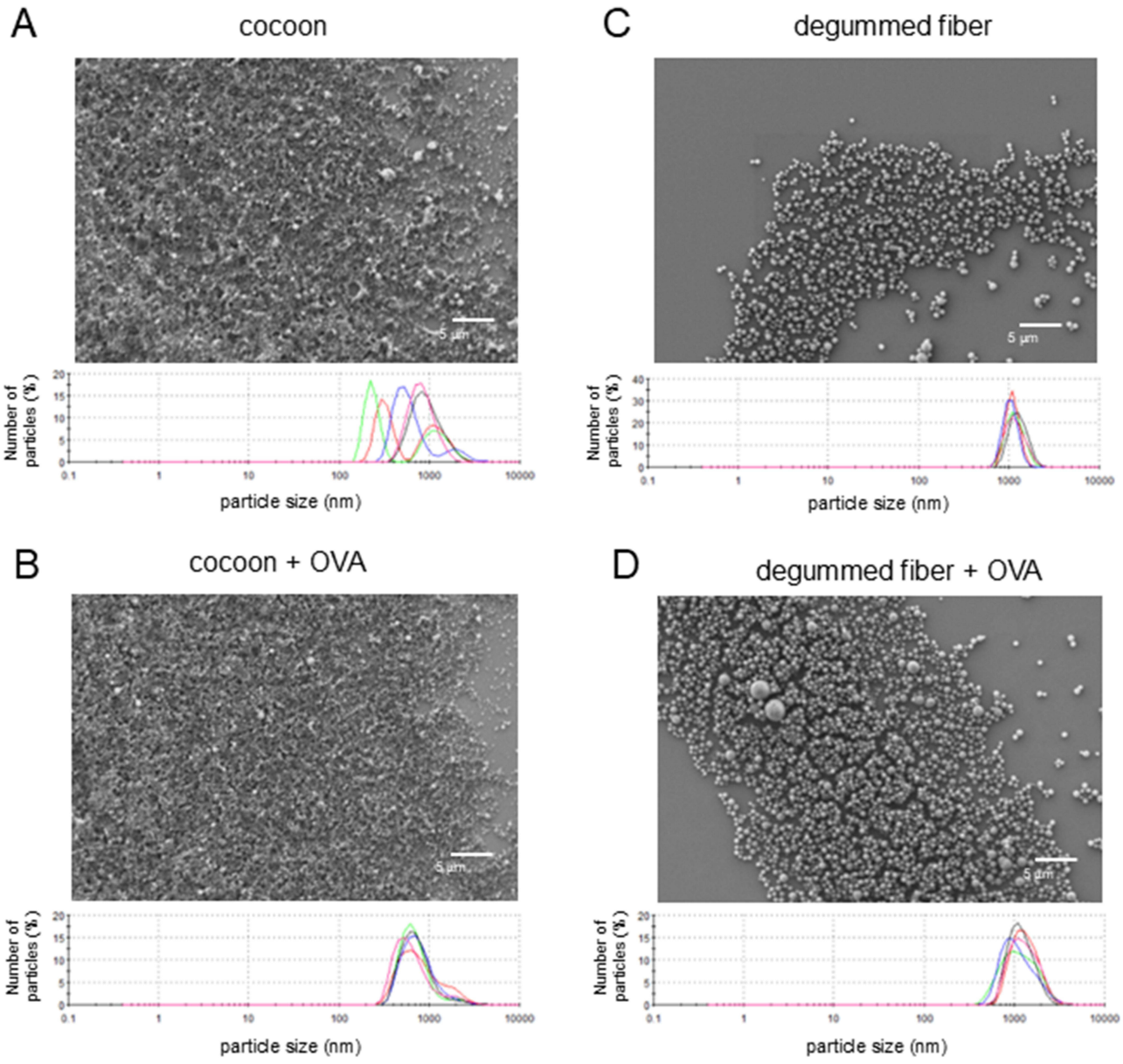

Figure 1 SEM images and particle size distribution of OVA-containing silk nanoparticles. (**A**) Cocoon, (**B**) cocoon with OVA, (**C**) degummed fiber, and (**D**) degummed fiber with OVA nanoparticles were observed via SEM (top). Scale bar = 5 μm. The particle size distribution of each silk nanoparticle was evaluated via DLS. Measurements were repeated five times (bottom).

Abbreviation: OVA, ovalbumin.

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Table 1 Characterization of Silk NPs Based on DLS and SEM Assessments

NP	Composition			DLS Measurement		SEM Measurement
	Fibroin	Sericin	OVA	Average Size (nm)	PDI	Average Size (nm)
Cocoon	~75%	~25%	—	1238 ± 392.8* 322 ± 73.3*	0.403	<320.0
Cocoon + OVA	~75%	~25%	0.710%	911.6 ± 566.2	0.340	<320.0
Degummed fiber	~100%	—	—	1112 ± 179.6	0.580	579.5 ± 131.8
Degummed fiber + OVA	~99.5%	—	0.437%	1353 ± 447.3	0.304	603.0 ± 186.0

Notes: Data are presented as mean ± standard deviation. *Shows data for two particle size distributions detected via DLS analysis.
Abbreviations: DLS, dynamic light scattering; NP, nanoparticle; OVA, ovalbumin; PDI, polydispersity index; SEM, scanning electron microscopy.

heterogeneity (Table 1). In particular, cocoon NPs had strong interparticle aggregation, which might explain why the particle size distribution was estimated to be larger than the actual measured value. Further analysis of the particle size distribution and zeta potential will be necessary to better understand the properties of silk NPs.

To examine whether OVA was incorporated into the silk NPs, the prepared silk NPs were once again dissolved in 9 M LiBr, and equal amounts of protein from the dissolving solutions subjected to SDS-PAGE and immunoblotting using an anti-OVA antibody. OVA was highly incorporated into NPs prepared from the aqueous solution of cocoons rather than from the degummed fibers (Figure 2A). Immunoblotting with an anti-FibL antibody indicated that equal amounts of protein were loaded in each aqueous solution of OVA-containing or -non-containing silk NPs (Figure 2A).

To determine the amount of OVA incorporated into the silk NPs, dissolved solutions of OVA-containing cocoon or degummed fiber NPs were analyzed via ELISA using an anti-OVA antibody. Results showed that the percentages of OVA in total protein of the silk NPs were 0.710% and 0.437% in cocoon and degummed fiber NPs after two days of NP formation, respectively (Figure 2B). Furthermore, to investigate whether OVA could be included in the silk NPs for a long period, OVA-containing cocoon or degummed fiber NPs were stored at 4°C for eight months, dissolved in 9 M LiBr, and then analyzed using ELISA. Results showed that OVA was incorporated in the cocoon and degummed fiber NPs at 0.475% and 0.161% of total protein in the silk NPs, respectively (Figure 2B). OVA released into the supernatant of the OVA-containing silk NP suspension could be observed two days after preparation (Figure 2C). These results suggest that the OVA incorporated into silk NPs was released or degraded to some extent over the course of eight months.

Although OVA was added to aqueous solutions of cocoons or degummed fibers in an amount equivalent to 10% of the silk protein, the content of OVA accounted for approximately 0.710–0.437% of total protein in the silk NPs. These results suggest that the amount of OVA added exceeds the capacity of the silk NPs, or that OVA incorporated in the NPs is released from these silk NPs during the manufacturing process. Therefore, determining the optimal amount of foreign protein to be added to the silk solution is necessary to avoid wastage.

Antibody Production in Mice Orally Administrated OVA-Containing Silk NPs

To evaluate the production of antibodies against OVA or silk components, the blood serum and intestinal lavage fluids of mice subjected to oral administration of silk NPs were collected (Figure 3A) and analyzed using ELISA. A substantial production of antibodies against OVA in blood serum (anti-OVA IgG) was detected in mice orally administered OVA-containing silk NPs, especially OVA-containing degummed fiber NPs, which significantly induced OVA-specific antibody production. However, sufficient antibody production was not induced in mice orally administered OVA alone (assuming that the OVA contained in silk NPs accounts for 1% of the total protein, mice were administered 75 μg OVA/dose) (Figure 3B). For the production of antibodies against silk components, anti-sericin IgG was highly induced in mice orally administered cocoon NPs with or without OVA. In contrast, antibody production against fibroin was low in all mice subjected to oral administration (Figure 3B). The OVA dose used (75 μg/dose) was similar to that reported previously (100 μg/dose)[5] and, therefore, considered appropriate.

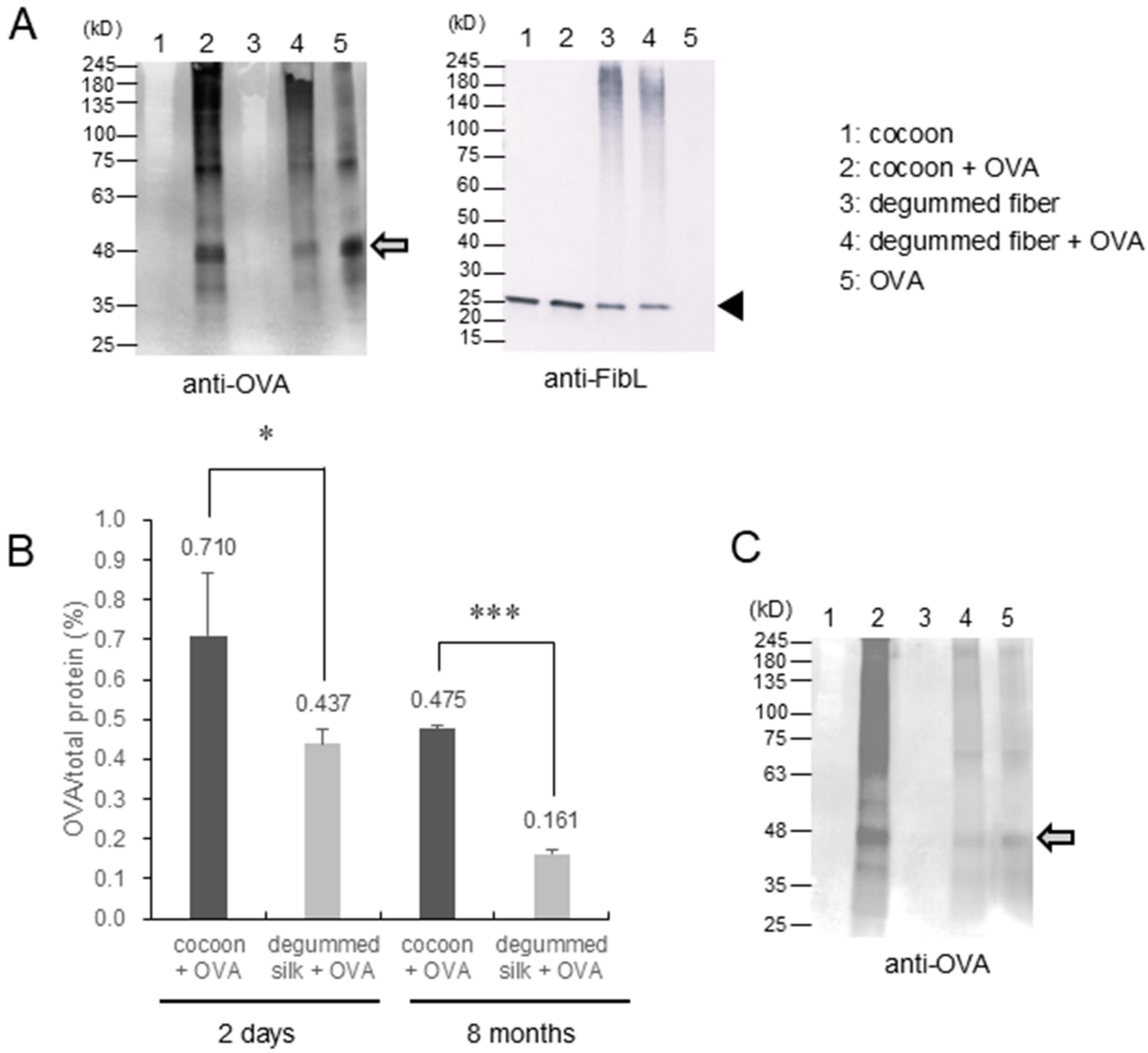

Figure 2 Detection of OVA incorporated into silk nanoparticles. (**A**) Western blotting analysis of the OVA incorporated into cocoon or degummed fiber nanoparticles. Each silk nanoparticle solution dissolved with LiBr was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-OVA or anti-FibL polyclonal antibody. Lane numbers indicate each silk nanoparticle or OVA solution. The arrow indicates OVA, and the arrowhead FibL. (**B**) Quantification of OVA contained in the silk nanoparticles. Two days or eight months after the formation of OVA-containing cocoon or degummed fiber nanoparticles, they were dissolved in LiBr and analyzed via ELISA using an anti-OVA antibody. The percentages of OVA in total protein of the silk nanoparticles are indicated. **P* <0.05; ****P* <0.001. Error bars represent the SE (n = 3). (**C**) Detection of OVA released from the silk nanoparticles. Supernatant of the OVA-containing silk nanoparticle suspension two days after formation was immunoblotted with anti-OVA polyclonal antibody. The arrow indicates OVA. Immunoblots are representative of three independent experiments.
Abbreviations: OVA, ovalbumin; FibL, fibroin-L-chain.

To examine the production of IgA against OVA or silk components, the intestinal lavage fluids of mice subjected to oral administration of silk NPs were analyzed using ELISA. Similar to the production of anti-OVA IgG in serum, the production of intestinal anti-OVA IgA was highly induced in mice orally administered OVA-containing degummed fiber NPs, but only a slight production of anti-OVA IgA was observed in mice orally administered OVA-containing cocoon NPs. In contrast, the production of anti-OVA IgA was not induced in mice orally administered OVA alone (Figure 3C). Furthermore, the production of anti-sericin IgA was highly induced in mice orally administered cocoon NPs, but the production of anti-fibroin IgA was hardly detected in the intestinal lavage fluid of mice used in these experiments. These results suggest that OVA-specific antibody production can be successfully induced by the oral administration of OVA-containing silk NPs without the use of adjuvants. Moreover, the production of anti-sericin antibodies was markedly induced in mice that were orally administered cocoon NPs, indicating that

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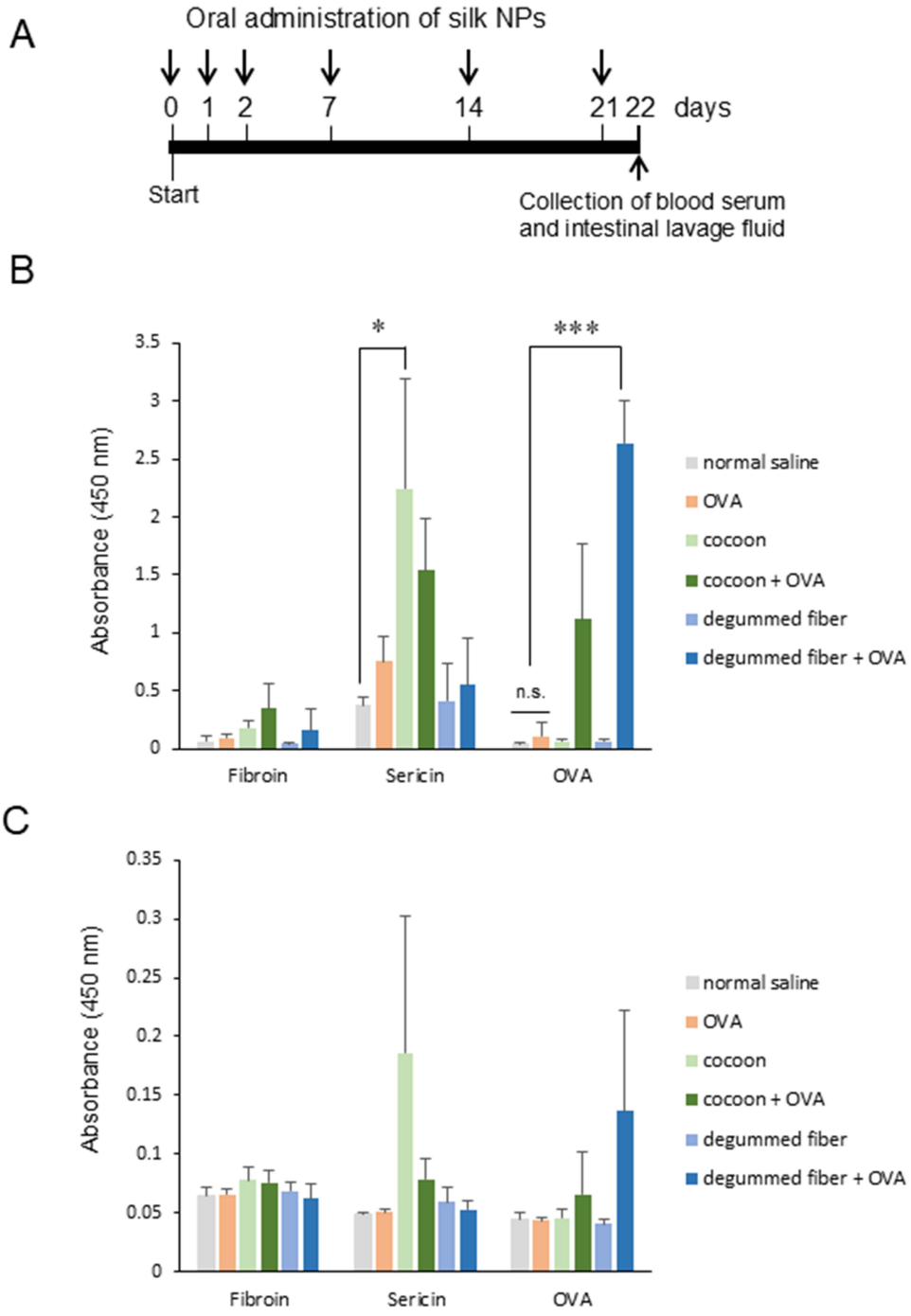

Figure 3 Antibody production induced by oral administration of OVA-containing silk nanoparticles. Slc:ICR mice (three mice per group) were orally administered OVA-containing/non-containing cocoon or degummed fiber nanoparticles. An equal volume of normal saline or OVA solution was orally administrated as controls. (**A**) Oral administration schedule for the experiment. After three consecutive days, oral administration was performed three times at weekly intervals. On the day after final administration, blood serum and intestinal lavage fluid were individually collected from the mice. (**B**) The levels of blood serum IgG and (**C**) intestinal IgA specific to fibroin, sericin, and OVA were measured using ELISA. Values are represented as the means ± SE of each mice within their separate group and representative of three independent experiments. **P* <0.05; ****P* <0.001.

Abbreviations: n.s., not significant. NP, nanoparticle; OVA, ovalbumin.

sericin has a higher immunogenicity than fibroin does. In this experiment, no noticeable harm to the mice orally administered various silk NPs was observed, and they showed similar weight gain as that of control mice orally administered normal saline. For the animal experiments, we only used the minimum number of mice necessary (three female mice per group), in accordance with the relevant guidelines, to strive to reduce the number of experimental animals used. However, the differences between male

and female mice might have an effect on the immune response. We are thus planning future animal experiments using both male and female mice.

Delivery of Silk NPs to the Intestinal Tract

To demonstrate the delivery of silk NPs to the intestinal tract, FITC-inner-labeled silk NPs were orally administered to mice, and the intensity of their fluorescence in the intestinal tract monitored after 1, 2, and 4 h. FITC-inner-labeled silk NPs passed through the stomach without significant digestion and were successfully delivered to the intestinal tract (Figure 4A). Although a strong intensity of FITC fluorescence was observed in the intestinal tract 1 and 2 h after oral

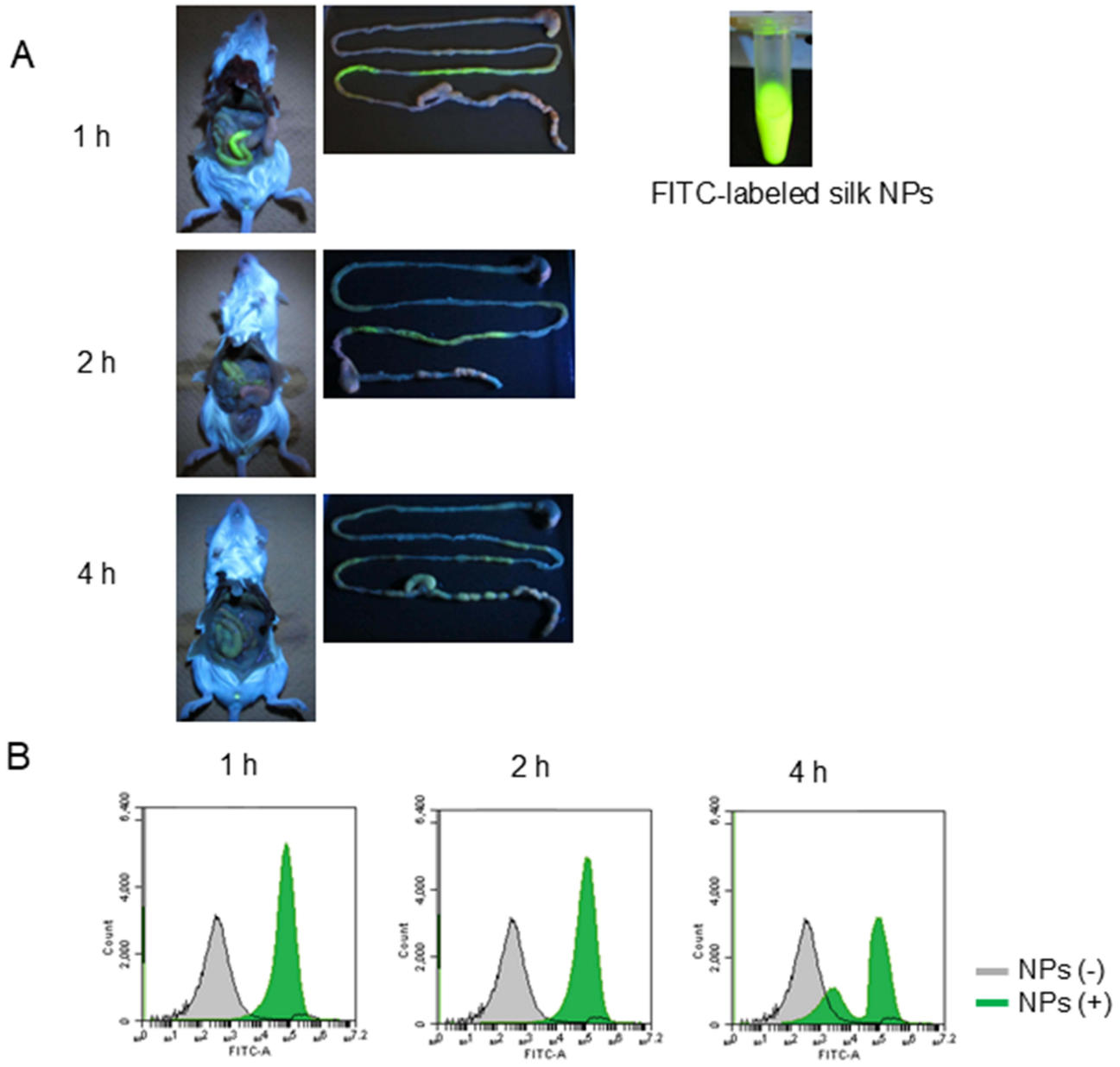

Figure 4 Delivery of FITC-inner-labeled silk nanoparticles to the intestinal tract. (**A**) FITC-inner-labeled degummed fiber nanoparticles were orally administrated to mice. Intestinal tracts were collected from the mice 1, 2, and 4 h after oral administration and photographed under UV light. (**B**) The intestinal lavage fluid from mice orally administered FITC-inner-labeled degummed fiber nanoparticles were collected through washing with phosphate-buffered saline and analyzed using flow cytometry. The histograms filled with green or grey represent the fluorescence intensity of intestinal lavage fluid collected from mice orally administrated the FITC-inner-labeled degummed fiber nanoparticles or untreated mice, respectively.
Abbreviation: NP, nanoparticle.

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administration, the FITC-inner-labeled silk NPs were partially digested after 4 h, and the intensity of FITC fluorescence gradually reduced (Figure 4B). These results indicate that silk NPs are resistant to the highly acidic environment and digestive enzymes in the stomach and can induce antigen-specific immune responses by oral administration of antigen-containing silk NPs.

Internalization of Silk NPs in Peritoneal Macrophages

To investigate whether the cocoon or degummed fiber NPs were taken up by peritoneal macrophages via phagocytosis, FITC-labeled cocoon and degummed fiber NPs, with or without OVA, were incubated with peritoneal macrophages. The internalization of cocoon or degummed fiber NPs into peritoneal macrophages was observed using fluorescence imaging (Figure 5A). Furthermore, FACS analysis revealed a broad range of fluorescence intensities of FITC-labeled silk NPs internalized into peritoneal macrophages, indicating that the size and fluorescence intensity of the FITC-labeled silk NPs were not uniform (Figure 5B). These observations confirm that the sizes of the cocoon or degummed fiber NPs are suitable for phagocytosis in macrophages and the antigen-specific immune responses occur when antigens are processed in macrophages.

Discussion

In this study, we demonstrated that OVA-containing silk NPs, as prepared through salting-out a mixture of OVA and an aqueous solution of silkworm cocoons or degummed fibers, could be delivered to the mouse intestinal tract and induce the production of OVA-specific antibodies through oral administration.

Silk fibroin is a protein-based biomacromolecule widely used in the biomedical field as a biomaterial because of its excellent biocompatibility, biodegradability, and low immunogenicity.[25–28] Silk sericin belongs to a family of glue-like proteins that coat fibroin fibers.[29] Virgin silk, a natural combination of fibroin and sericin structures, frequently induces immune and allergic reactions when used as a suture material.[30] Furthermore, intraperitoneal or intramuscular injection of silk sericin into mice significantly induces the production of silk sericin-specific antibodies, whereas silk fibroin is poorly immunogenic.[31] In the present study, oral administration of non-degummed cocoon fiber NPs strongly induced the production of antibodies against sericin but not fibroin, whereas oral administration of degummed fiber NPs did not induce the production of antibodies against silk proteins in mice (Figure 3). Moreover, in mice orally administered OVA-containing degummed fiber NPs, the production of OVA-specific antibodies was significantly induced, whereas in mice orally administered OVA-containing cocoon fiber NPs, the production of sericin-specific antibodies was higher than that of OVA-specific antibodies, likely because the sericin content was more than 20 times higher than that of OVA (Figure 3). As sericin induces a predominant immune response, degummed fiber NPs are preferable as materials for oral vaccines.

The OVA-containing cocoon and degummed fiber NPs prepared through salting-out had diameters <320 nm and ranged from 417–789 nm in the SEM image measurements, respectively (Figure 1 and Table 1). In comparison to the degummed NPs, numerous small cocoon NPs were formed, even with the same amount of protein (Figure 1). Differences in the amount of OVA incorporated into the silk NPs are thought to reflect the number of silk NPs formed via salting-out (Figure 2A and B). Similarly, the amount of OVA released from each silk NP reflected the quantity of silk NPs (Figure 2C). These results suggest that the cocoon and degummed fiber NPs may take up and release OVA to the same extent. Salting-out would prepare OVA-containing silk NPs, in which the OVA was presumably incorporated through hydrophobic interactions between OVA and silk proteins. However, the detailed mechanism of OVA-containing silk NP formation remains unclear. In vitro release studies of OVA from OVA-loaded silk NPs under conditions simulating the gastrointestinal environment will be conducted in the future.

FITC-inner-labeled silk NPs orally administered to mice were confirmed to be sufficiently delivered through the stomach into the intestinal tract, wherein they were gradually digested approximately 4 h after oral administration (Figure 4A and B). These findings indicate that silk NPs can overcome the harsh environment of the stomach and act as carriers to deliver intact antigens to the intestinal tract. In addition to antigens, therapeutic drugs, including enzymes, proteins, peptides, and genetic materials, can be encapsulated in silk NPs and delivered to the intestinal tract via oral administration.

FITC-labeled cocoon and degummed fiber NPs containing OVA were internalized into peritoneal macrophages (Figure 5A and B), suggesting that the size of these silk NPs was within acceptable limits for phagocytosis by macrophages.

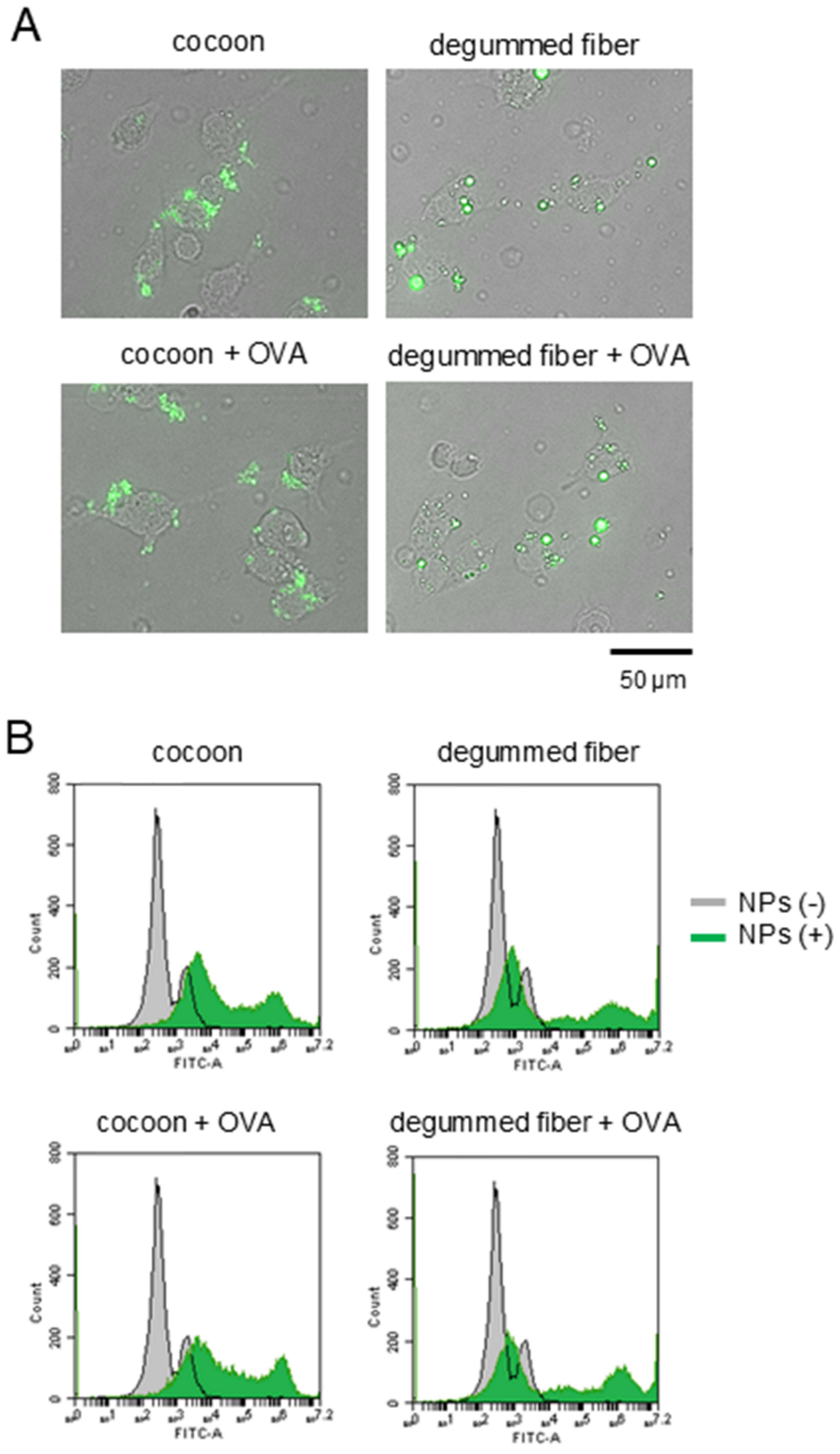

Figure 5 Internalization of silk nanoparticles in peritoneal macrophages. (**A**) Mouse peritoneal cells were seeded in 8-well chamber slides and incubated with the four types of FITC-labeled silk nanoparticles for 2 h. After washing with phosphate-buffered saline (PBS), the cells were fixed and imaged via fluorescence microscopy. Scale bar = 50 μm. (**B**) Mouse peritoneal cells were seeded in a 6-well cell culture plate and incubated with the four types of FITC-labeled silk nanoparticles for 2 h. After washing with PBS, the cells were harvested and analyzed using flow cytometry. Histograms filled with green represent the fluorescence intensity of the FITC-labeled silk nanoparticles incorporated in peritoneal macrophages, and those filled with grey represent the untreated peritoneal macrophages.
Abbreviations: NP, nanoparticle; OVA, ovalbumin.

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Following oral administration, OVA-containing silk NPs are delivered to the small intestine, transported across the mucosal barrier, and taken up and processed by dendritic cells or macrophages that present antigen fragments on their cell surfaces to elicit immune responses. Silk fibroin has mucoadhesive properties and is expected to be useful for drug delivery. For example, liposomes coated with silk fibroin adhere to corneal cells and are rapidly absorbed, making them a promising corneal drug delivery system.[32] Orally administered silk NPs might bind to gastrointestinal epithelial cells, thereby promoting cellular internalization.

Furthermore, oral administration of OVA-containing silk NPs induced the production of OVA-specific IgA and IgG, which activate mucosal and systemic immunity, respectively, without requiring the co-delivery of adjuvants, indicating that silk NPs possess the characteristics of adjuvants. At present, no established technology exists to control uniformity in the particle size of silk NPs or the amount of antigen incorporated into each silk NP. In the future, optimizing the manufacturing conditions for antigen-containing silk NPs will be necessary.

Conclusion

In conclusion, silk NPs are promising oral vaccine carriers that can deliver antigens to the intestinal tract while avoiding their decomposition by the gastric acid and digestive enzymes present, thereby making it possible to activate both mucosal and systemic immunity. Further investigation and verification of silk NPs containing other antigens, such as toxins or the surface proteins of microorganisms, are necessary for the development of next-generation oral vaccines.

Abbreviations

DLS, dynamic light scattering; FibL, fibroin-L-chain; HRP, horseradish peroxidase; NP, nanoparticle; OVA, ovalbumin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline and Tween 20; PDI, polydispersity index; SEM, scanning electron microscopy; SDS, sodium dodecyl sulfate.

Data Sharing Statement

The data supporting the findings of this study are available from the corresponding author, Mitsuru Sato, upon reasonable request.

Ethics Approval

Animal experiments were conducted in strict accordance with the recommendations of the "Standards Relating to the Care and Keeping and Reducing Pain of Laboratory Animals" (Ministry of the Environment, Japan). All animal procedures were approved by the Institutional Animal Care and Use Committee of the National Agriculture and Food Research Organization (approval ID: R5-M11-NIAS-2).

Author Contributions

All authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests in this work.

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