



Particle-size-dependent toxicity and immunogenic activity of mesoporous silica-based adjuvants for tumor immunotherapy



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ABSTRACT

Conventionally used adjuvants alone are insufficient for triggering cell-mediated immunity, although they have been successfully developed to elicit protective antibody responses in some vaccines. Here, with the aim of eliciting cell-mediated immunity, pathogen-associated molecular patterns (PAMPs) were immobilized with apatite within the pores and on the surface of mesoporous silica (MS) with particle sizes from 30 to 200 nm to prepare novel MS-Ap-PAMP adjuvants, which showed cell-mediated anti-tumor immunity that was markedly improved compared to commercial alum adjuvant *in vitro* and *in vivo*. The toxicity and antitumor immunity of the MS-Ap-PAMP adjuvants were evaluated *in vitro* and *in vivo*. MS with a particle size of 200 nm showed minimum *in vitro* cytotoxicity to NIH3T3 cells, particularly at concentrations no higher than 100 $\mu\text{g ml}^{-1}$. In particular, apatite precipitation within the pores and on the surface of MS decreased the *in vitro* cytotoxicity of MS particles. The MS-Ap-PAMP adjuvants showed the maximum *in vitro* immunogenic activity among original culture medium, PAMP and alum-PAMP. Moreover, injection of the MS-Ap-PAMP adjuvant in combination with liquid-nitrogen-treated tumor tissue (derived from Lewis lung carcinoma cells) into C57BL/6 mice markedly inhibited *in vivo* tumor recurrence and the development of rechallenged tumor compared to those with commercial alum adjuvant. The MS-Ap-PAMP adjuvant contributed to the elicitation of a potent systemic antitumor immunity without obvious toxicity *in vivo*.

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1. Introduction

Immunotherapy is an efficient approach to controlling tumor growth, as the immune system is clearly capable of recognizing and eliminating tumor cells [1]. The poor immunogenicity of traditional tumor therapies may cause the serious problem of tumor recurrence. The immune system requires an external boost from immunotherapy to be meaningfully effective in eliminating tumor cells, because tumor cells (i) show reduced antigen presentation and (ii) down-regulate the immune responses of the body.

Tumor vaccination is one of the most promising external boosters of the immune system, specifically recognizing and destroying tumor cells without harming normal cells [1]. The key elements of effective tumor vaccines are (i) tumor-associated antigens that elicit adaptive immune responses and (ii) adjuvants that stimulate the immune system and ensure the delivery of vaccine to the right place at the right time [2–4].

Tumor antigens may qualitatively and/or quantitatively differ among individual patients. Deriving such antigens from autologous

tumor tissue (autologous tumor antigens) would obviate this problem, given that there are distinct tumor-associated antigens expressed by patients' tumor cells, which are either absent or present at low concentrations on normal cells [5–8]. However, autologous tumor antigens have limited immunogenicity and require adjuvants to boost the desired immune responses.

Adjuvants enhance the magnitude, breadth, quality and longevity of specific immune responses to antigens, and reduce the amount of an antigen and/or the number of immunizations required to achieve the desired immune responses [9,10]. Adjuvants have limited or no efficacy unless properly formulated; therefore, both adjuvant components and formulation are crucial for increasing vaccine potency [2]. Conventionally used adjuvants, such as alum, alone are insufficient to trigger cell-mediated immunity, although they have been successfully developed to elicit protective antibody responses in some vaccines. The next generation of adjuvants will require not only very strong and long-lasting antibody responses, but also cell-mediated immunity [2]. In the case of adjuvants for tumor vaccines, triggering cell-mediated immunity is crucial because antitumor immunity consists of operations orchestrated by innate effector cells, antigen-presenting cells (APCs) and adaptive effector cells. It has been confirmed and accepted that

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cytotoxic T lymphocytes (CTLs) are one of the major effector cells against tumors. APCs, particularly dendritic cells (DCs), activate effector cells. The APCs are activated by various immune potentiators including pathogen-associated molecular patterns (PAMPs), cytokines and bacterial toxins.

Mesoporous silica (MS) and apatite are not only promising delivery vehicles of PAMPs but also have favorable immunogenic functions. MS is a very effective delivery system for inducing immune responses owing to its uniform pore structure, high surface areas and effective adsorption ability [11]. MS materials have been considered to be excellent candidates as carriers for molecules. The texture, morphology, size, surface properties and interactions between molecules and the walls of MS materials are crucial for molecule loading, controlled drug release and delivery, and multifunctionalization [12–20]. Encapsulating poorly soluble drugs in MS improves drug dissolution, thus encapsulation in MS can be applied as a dissolution-enhancing formulation approach for a very wide variety of poorly soluble drugs [21]. The role of residual water inside the pores of MS on the molecular organization of hydrophobic guest molecules was studied. The co-adsorbed water influenced the molecule adsorption, hydrophobic aggregation and migration in MS pores [22]. Moreover, MS can induce DCs to produce interleukin-12 [23], which is important for the generation of interferon γ -expressing CD8⁺ T lymphocyte-mediated immune responses [24]. Apatite is used for immobilizing various biological molecules by either adsorption or coprecipitation [25–28]. A hydroxyapatite adjuvant can also promote cell-mediated immune responses by attracting antigen-presenting cells to the vaccination site and accelerating T-cell-mediated immune responses [29]. MS loaded with the immunopotentiator-tuberculin purified protein derivative and apatite is an effective adjuvant for cancer immunotherapy [30]. The particle size of particles is crucial to their adjuvant activities [31–34]. Because particles with proper size can promote their uptake by immune cells, antigen loading, permeating into biological barriers [35–37]. The size of the particulate adjuvants is related to both the strength of the immune responses induced and their type [31]. Here, MS with particle sizes from 30 to 200 nm were used to immobilize PAMPs with apatite. Particle-size-dependent toxicity and immunogenic activity of mesoporous silica-based adjuvants were studied.

2. Materials and methods

2.1. Mesoporous silica (MS) preparation

MS particles were prepared based on previous reports with modification [38]. In this study, MS particles with four different particle sizes (MS-A, MS-B, MS-C and MS-D) were prepared by adjusting synthesis parameters as listed in Table 1. In a typical synthesis procedure, 0.28 g sodium hydroxide and a certain amount of cetyltrimethylammonium bromide (CTAB) were dissolved into 480 ml ultrapure water under stirring. When the CTAB was completely dissolved, the temperature of the solution was adjusted to 25 or 60 °C. Tetraethoxysilane (TEOS) was then added dropwise to the CTAB solution under vigorous stirring at 600 rpm. The stirring was continued for 2 h. The precipitate was collected from the solution by centrifugal separation, washed with deionized

Table 1
Synthesis parameters for mesoporous silica (MS) particles.

	CTAB (g)	H ₂ O (ml)	NaOH (g)	TEOS (mL)	Temperature (°C)
MS-A	1	480	0.28	5	60
MS-B	1	480	0.28	10	60
MS-C	1	480	0.28	10	25
MS-D	2	480	0.28	10	25

water and ethanol, and dried. Any CTAB remaining in the as-synthesized product was removed by extraction with ethanol/concentrated hydrochloric acid (50 ml/1 ml).

2.2. Immobilization of PAMP with apatite within the pores and on the surface of MS particles

In this study, the selected PAMP was a hydrothermal extract of a human tubercle bacillus (*Mycobacterium tuberculosis*). The source of the hydrothermal extract of human tubercle bacillus was Ancer[®] s.c. injection solution (Zeria Pharma Co. Ltd, Japan), which contains PAMPs, including lipoarabinomannan, as the active component. The amounts of PAMP were tested by a phenol–sulfuric acid method in accordance with the manufacturer's instructions. The phenol–sulfuric acid method is widely used for measuring carbohydrate contents because of its sensitivity and simplicity [39,40]. The PAMP was immobilized with apatite within the pores and on the surface of MS particles by the chemical coprecipitation method. All the solutions used in this study are clinically available in Japan. The merits of using clinically approved pharmaceutical formulations are that they are sterile and endotoxin free, and have a low regulatory barrier for clinical applications [41–43]. Ancer[®] s.c. injection solution was concentrated five times by heating in a water bath at 100 °C before use. The supersaturated calcium phosphate solution, designated as RSM, was prepared by mixing aseptic Ringer's solution (Otsuka Pharmaceutical, 2.25 mM Ca²⁺), Solita[®]-T No. 2 (Ajinomoto Pharmaceuticals, 10 mM PO₄³⁻) and an alkalizer (Meylon[®], Otsuka Pharmaceutical, 833 mM NaHCO₃) at the mixing ratio shown in Table S.1. The chemical compositions in RSM are shown in Table S.2.

To prepare adjuvants consisting of MS, apatite and Ancer-derived PAMPs (MS-Ap-PAMP) for in vitro experiment, 3 mg of MS particles, sterilized at 160 °C for 3 h in a dry sterilizer (SG600, Yamato Scientific, Ltd., Co., Japan), was dispersed in 2 ml of RSM with the presence of 10 vol.% of the five-times-concentrated Ancer solution. The reaction proceeded under a continuous stirring with a stirring speed at 200 rpm under an aseptic condition at 25 °C for 1 day. An adjuvant consisting of MS and apatite (MS-Ap) was prepared in a similar manner without using the five-times-concentrated Ancer solution.

2.3. Characterization of MS, MS-Ap and MS-Ap-PAMP

The morphology of the as-prepared MS particles was observed using a field emission scanning electron microscope (S-4800, Hitachi, Japan) at an accelerating voltage of 10 kV after being coated with platinum. The particles were observed by transmission electron microscopy (TEM; EM-002B, TOPCON, Japan). The phase compositions of MS and MS-Ap-PAMP were analyzed by X-ray diffractometry (XRD) employing Cu K α X-ray at 40 kV and 300 mA using a powder X-ray diffractometer (Model RINT 2400, Rigaku, Japan) and a silicon-zero-background plate. Fourier transform infrared (FTIR) spectra were recorded using an FTIR-350 spectrometer (JASCO Corporation, Japan) by the KBr pellet method.

The nitrogen gas (N₂) adsorption–desorption isotherms were measured at –196 °C using a specific surface area/pore size distribution analyzer (BELSORP 28, BEL Japan) under continuous adsorption conditions. Prior to measurement, all samples were heated at 160 °C for 2 h and then outgassed to 0.133 Pa at room temperature. Brunauer–Emmett–Teller (BET) analysis was used to determine the total specific surface area (S_{BET}). The total pore volume (V_{total}) was calculated by a t -plot analysis. The Barrett–Joyner–Halenda method was used to calculate the mesopore size distribution.

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