



OPEN Preparation and antibacterial activities of silver nanoparticle-loaded hydroxyapatite/collagen bone-like nanocomposite

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A hydroxyapatite/collagen bone-like nanocomposite (HAp/Col) loaded with silver nanoparticles (AgNPs) was prepared by immersion of HAp/Col powder in the AgNPs solution. The adsorption-desorption properties on the preparation process were investigated, followed by the evaluation of antibacterial activities and cytotoxicity. Adsorption of AgNPs on HAp/Col powder was almost finished at 240 min after mixing. Adsorption isotherm revealed that maximum adsorption amount was 1621.9 mg/g with the best fitted model, Dubinin-Radushkevich adsorption model. The AgNPs-loaded HAp/Col membrane prepared by immersion into 1.6 µg/mL and 6.3 µg/mL AgNPs colloidal solution demonstrated no survival of *Escherichia coli* and *Staphylococcus aureus*, respectively. Using human osteoblastic cell line, MG-63, no significant cytotoxicity was observed for the AgNPs-loaded HAp/Col membrane prepared with 6.3–12.5 µg/mL AgNPs colloidal solution. These results suggested that AgNPs-loaded HAp/Col would be a good candidate for antibacterial bone void fillers and coating materials for orthopedic and dental implants.

Implant-associated infection is one of the serious problems on surgical implantation such as joint replacements in orthopedic surgery, tooth roots implantation in dentistry, and bone repair using bone void filler in many surgical fields^{1,2}. Implant-associated infection is generally prevented by prophylactic medication of antibiotics as well as clean environment during surgical operations. The prophylactic medication of antibiotics is effective and is specified by the surgical/treatment guidelines in many countries. However, antibiotics administration in orthopedic surgery does not make wide consensus by clinical studies with the side effects of killing useful microorganisms and rising of the risk for antimicrobial resistance in bacteria. In some cases, the presence of the implant provides a hotspot of infection³. In these cases, if the implant itself has anti-infective properties for a necessary period, and if it disappears when it becomes unnecessary, theoretically it prevents severe infection without antibiotics administration.

This kind of function would be possible to install for some bone void fillers by the incorporation of antibiotics into implant materials. Many attempts have been performed for dense and porous bodies of calcium phosphates and their composites in combination with various antibiotics and antimicrobial materials^{4–12}. For example, Niab et al. synthesized submicron particles of hydroxyapatite decorated with silver nanoparticles (HA@Ag nanocomposite) and reported their good antibacterial activities for *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (*S. aureus*)¹¹. However, most of the researches targeted on HAp. Hydroxyapatite is a main inorganic component of bone, has good osteoconductivity, and is widely used artificial bone void fillers; however, its sintered bodies, generally used as bone void fillers, has limited biodegradability, i.e., it remains lifetime of patients¹³. Type-I collagen, a main organic component of bone, has biodegradability but has no bone formation property without cells/cytokines; therefore, collagen itself has not been used as bone void fillers. A half volume of bone is “non-biodegradable” HAp; however, bone is resorbed by osteoclasts. Therefore, we had hypothesized that this resorption property was based on their nanostructure (and chemical composition). Based on this hypothesis the hydroxyapatite/collagen bone-like nanocomposite (HAp/Col) had been prepared using the simultaneous titration method¹⁴. The HAp/Col has a similar nanostructure to the natural bone formed via heterogenous nucleation of HAp nanocrystals on carboxy groups on collagen

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molecules followed by epitaxial growth of HAp nanocrystals on collagen molecules as shown in Fig. 1., The HAp/Col dense body¹⁴, porous body¹⁵, and injectable self-setting paste¹⁶ have a benefit of being incorporated into bone remodeling metabolism. Thus, the HAp/Col does not have risks that remaining bone void fillers, such as sintered HAp, work as an infection focus and necessary period of antibacterial property for the HAp/Col is very initial stage, because the HAp/Col start to resorb in 5 days after implantation¹⁷. Further, we found a possibility that orientation of HAp and collagen allows selected cell recruitments without cytokines and/or drugs¹⁸. We already reported the successful preparation of gentamicin-loaded HAp/Col and its antibacterial

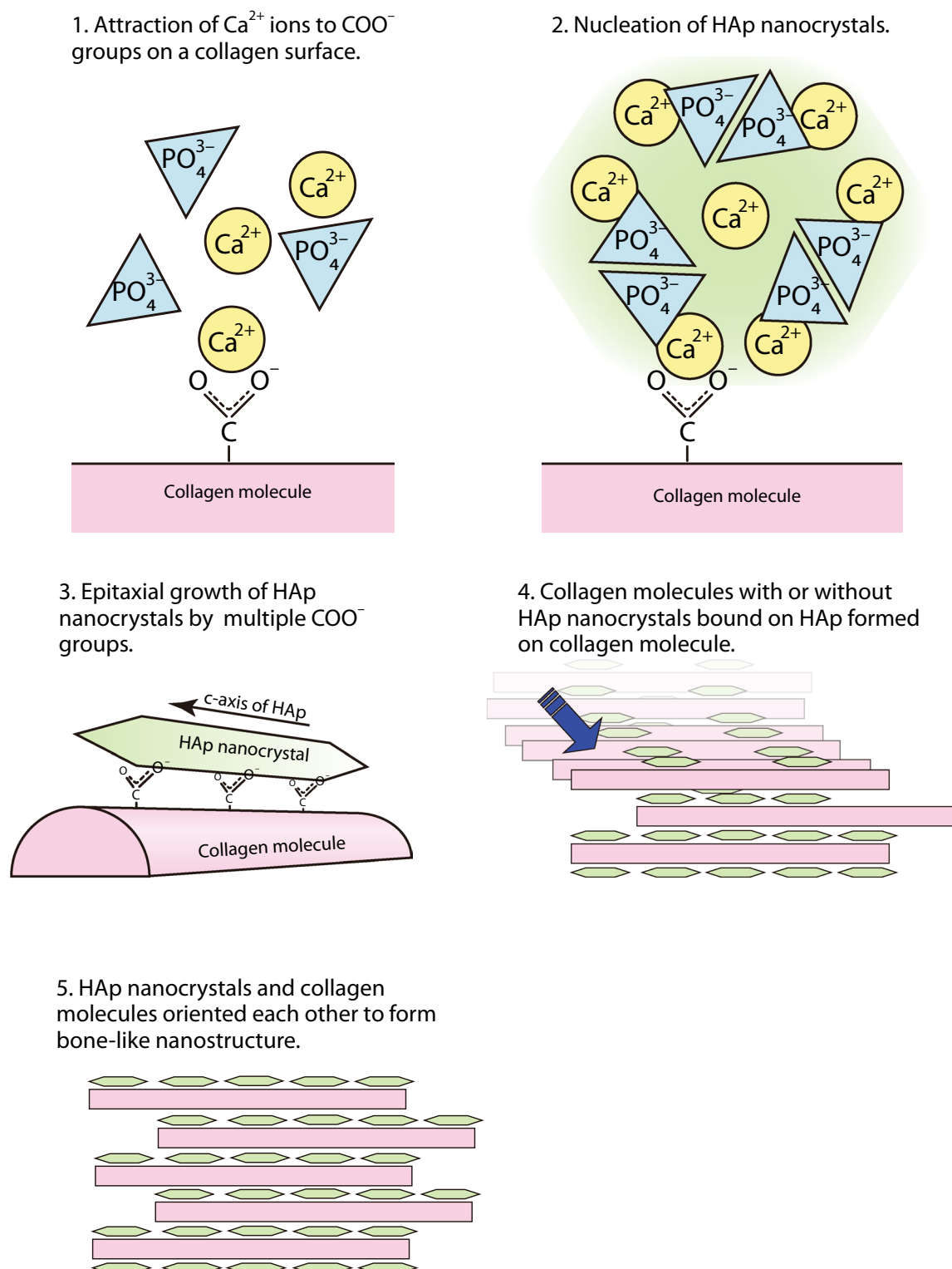


Fig. 1. Schematic illustration of HAp/Col formation.

activity for *E. coli*¹⁹. Gentamicin is a comparatively cheap antibiotic, which is effective for gram-negative bacteria but not for gram-positive bacteria. Therefore, we focus on silver nanoparticles. Silver has a wider antibacterial spectrum both gram-negative and gram-positive bacteria. Further, silver has less risk of antimicrobial resistance and low toxicity for human beings²⁰. In this study, silver nanoparticles (AgNPs) were synthesized according to the literature²¹ and were adsorbed onto the HAp/Col. Adsorption and desorption profiles, antibacterial activities for *E. coli* and *S. aureus*, and cytotoxicity of AgNPs-loaded HAp/Col were investigated.

Materials and methods

Materials

Water-soluble starch (First grade, mean molecular weight 1,000,000), D(+)-glucose (C₆H₁₂O₆, Reagent grade) were supplied by NACALAI TESQUE, INC. AgNO₃ (Reagent grade), CaCO₃ (Alkaline analysis grade), and H₃PO₄ (Reagent grade) were supplied by FUJIFILM Wako Pure Chemical Co. Porcine dermal atelocollagen (Biomaterial grade) was supplied by Nitta Gelatin Inc. Calcium- and magnesium-free phosphate buffered saline, PBS(−) was supplied by Thermo Fisher Scientific, Inc. *E. coli* (ATCC 8739) and *S. aureus* (ATCC6538) were employed as EZ-PEC kits which supplied by Microbiologics, Inc. (0483-PEC, and 0485-PEC, respectively). The Nutrient Broth 'Eiken' (abbreviated as NB) was supplied by Eiken Chemical Co. Ltd. MG-63 (RCB1890) was supplied by RIKEN BRC Cell bank. The Cell Counting Kit-8 (CKK-8) was supplied by Dojindo Science Institute, Inc. Dulbecco's modified Eagle's minimum essential medium (D-MEM, high-glucose), fetal bovine serum, and penicillin-streptomycin solution (10,000 units for penicillin and 10 mg/mL for streptomycin) were supplied by Sigma-Aldrich.

Preparation of silver nanoparticles colloidal solution

Silver nanoparticles were synthesized according to Raveendran's method²¹. Briefly, 8 mL of water-soluble starch aqueous solution at a mass ratio of 0.2% were prepared in a screw-cap bottle, followed by the addition of 40 mL of 0.1 M AgNO₃ aqueous solution. The solution in the bottle was mixed by shaking. Then, 100 mL of 1.0 M D(+)-glucose aqueous solution was added to the bottle and mixed well by shaking. The solution in the bottle was maintained at 33 °C in a water bath for 24 h to allow redox reaction. Silver nanoparticles in the resulting colloidal solution (AgNPsCS) were observed with a scanning electron microscope (SEM, JSM-5600, JEOL, Japan). The particle size distribution and zeta-potential of AgNPs were measured with a Zeta-potential and Particle Size Analyzer (ELSZ-2000ZS, Otsuka Electronics Co., Ltd., Japan).

Preparation of hydroxyapatite/collagen bone-like nanocomposite

The HAp/Col having the HAp: Col ratio of 4:1 was synthesized by the simultaneous titration method¹⁴. Briefly, pure Ca(OH)₂ was synthesized by hydration of CaO prepared by thermal decomposition of CaCO₃. A 199.1 mL portion of 400 mM Ca(OH)₂ aqueous suspension and 398.2 mL of 120 mM of H₃PO₄ aqueous solution containing 2.0 g of porcine dermal atelocollagen were simultaneously titrated into a 199.1 mL portion of pure water in a reaction vessel. Temperature of the reaction vessel was maintained in a water bath at 40 °C, and reaction pH was maintained at 9.0 ± 0.1 with auto titration units controlled with a pH controller. For HAp/Col powder (HCP) preparation, the synthesized HAp/Col was vacuum-filtered, frozen at −20 °C overnight and freeze-dried at −20 °C for 24 h followed by at 0 °C for 48 h. The dried HAp/Col was then crushed with an alumina pestle in a mortar and classified into 212 μm or smaller with a sieve. Specific surface area of the HCP was measured with BELSORP-MINI II (Microtrac Retsch GmbH, Germany).

For antibacterial and cytotoxicity tests, ring-shaped HAp/Col membranes (HCM) were prepared in the following procedure. A 50 mL portion of the fibrous HAp/Col in the reaction vessel were vacuum filtrated for 15 min and frozen overnight. The frozen membrane-shaped HAp/Col was lyophilized at −20 °C for 2 days and pressed at 30 MPa for 5 min. The HAp/Col membrane was then punched out to be a circle of 6 mm in diameter having a hole in 4 mm diameter in its center as shown in supplementary Fig. S1. The HCM was then hydrothermally crosslinked at 140 °C for 12 h under vacuum. To allow saturated adsorption of Ca²⁺ and Mg²⁺ on the HCM, the HCM was soaked into each culture medium, described in 2.6 and 2.7, for 7 days in prior to the antibacterial or cytotoxicity tests, while replacing the medium on every day. After the immersion, the HCM was collected and vacuum dried.

Adsorption profile measurements of AgNPs on HCP

Adsorption equilibrium time

An adsorption equilibrium time of AgNPs on HCP was measured by the following procedure. A 50 mg portion of HCP and a 10 mL portion of 2800 mg/L AgNPsCS were prepared in 7 Erlenmeyer flasks with stoppers and shaken at 4.2 rad/s by a rocking shaker for 5, 10, 20, 40, 80, 120 and 240 min. After each period, the contents of each flask were filtrated to obtain the filtrate. The amount of AgNPs adsorbed on the HCP at each period was calculated from the amount of silver in the filtrate quantified using an inductively coupled plasma optical emission spectrometer (ICP-OES, model 720, Agilent Technologies Inc., CA, USA). The experiment was performed in triplicate.

The results were analyzed using either the pseudo-first order model (PFO model)²² or the pseudo-second order model (PSO model)²³. The details of both models are described in the supporting material.

Adsorption isotherm

Generally, adsorption isotherm is measured using serially diluted adsorbate solutions. However, serial dilution of the AgNPsCS could change the dispersion state and surface properties (e.g., zeta potential) of AgNPs. Thus, the adsorption isotherm of AgNPs on HCP was measured by serially increase the amounts of HCP to a certain

concentration of AgNPsCS²⁴, i.e., the HCP of 10 to 120 mg were added to a 20 mL portion of 2880 mg/L AgNPsCS in each Erlenmeyer flask with stopper and shaken at 4.2 rad/s by the rocking shaker. The shaking (adsorption) time was determined based on the result of adsorption equilibrium time measurement. After the shaking, adsorption amount of AgNPs on the HCP was measured by the same procedure as described in 2.4.1. The experiment was performed in triplicate.

The data were analyzed by 4 major, two-parameter adsorption isotherm models: Langmuir, Freundlich, Dubinin-Radushkevich (D-R), and Temkin. Each equation and its transformed one for data plotting²⁵ are described in the supporting material. The experimental data were plotted with the parameters for these models to obtain linear fitting curves by the least square method.

Desorption profile measurement of AgNPs

The AgNPs-loaded HCP (AgNPs-HCP) used in the desorption test was prepared by soaking 500 mg of HCP into 100 mL of 3340 mg/L AgNPsCS in an Erlenmeyer flask with stopper and shaken at the speed of 4.2 rad/s for 2 h. After the shaking, the solid phase was collected by filtration, freeze-dried at −20 °C, crushed thoroughly with the alumina pestle in the mortar, and classified to a particle size of 212 µm or less with the sieve. The amount of AgNPs adsorbed on HCP was considered as the maximum adsorption amount determined in the result of 2.4.1.

A 100 mg portion of the AgNPs-HCP was added to 10 mL of PBS(−) in a screw-top test tube and then, incubated at 37 °C for 1 to 9 days. During the incubation, a 1 mL portion of the supernatant was collected every 2 days, followed by the replenishment with fresh PBS (−). Silver amounts in the collected supernatants were quantified by the ICP-OES to calculate the released silver amount. The obtained data was analyzed in the following equation:

$$A_{total} = 10 \times \sum_{i=1}^n (C_i - C_{i-1}) \quad (1)$$

where A_{total} and C_i indicates the accumulated desorption amount of silver (mg) and concentration of silver in the collected solution at day i (mg/mL), respectively. The ratio of cumulative desorption amount (%) was decided by the dividing A_{total} by the adsorbed amount of the AgNPs-HCP, which is obtained by the ICP-OES analysis of the silver in the filtrate from the preparation process of AgNPs-HCP.

In addition, the particle size distribution of released AgNPs after 9 days of incubation was measured using the ELSZ-2000ZS. The experiment was performed in three times.

Antibacterial test

In the present study, antibacterial tests were carried out using two types of bacteria specified in the ISO 22916:2011²⁶ and Japanese Industrial Standard JIS Z2801:2012²⁷, i.e., a gram-negative bacterium *E. coli* and a gram-positive bacterium *S. aureus*. These bacteria were prepared following the protocol supplied with the kits. The Nutrient Broth 'Eiken' (abbreviated as NB) containing 5.0 g/L NaCl, 10.0 g/L peptone, and 3.0 g/L meat extract was sterilized by autoclaving at 121 °C for 30 min in prior to use. Water-soluble tetrazolium salts-8 [WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 *H*-tetrazolium, monosodium salt] was employed as a colorimetric reagent to estimate the viable number of bacteria in the solution of the interest^{28,29}. The CCK-8, which contains WST-8 and an electric mediator, 1-methoxy-PMS (1-methoxy-5-methylphenazinium methylsulfate) in appropriate concentrations, was used for the experiment.

Antibacterial activities of AgNPsCS

A 10-fold dilution series of bacterial suspension (180 µL) containing 10^6 to 10^{-1} CFU/mL of bacteria with the NB were prepared in the calibration wells of a 96-well microplate. A two-fold dilution series of AgNPsCS with the NB (100 µL) were also prepared in test and blank wells. For the test and blank wells at 0 µg/mL of AgNPs, 100 µL of the NB was dispensed. Then, 80 µL of the bacterial suspension containing 2.3×10^6 CFU/mL was added to each test well, whereas 80 µL of the NB was added to each blank well. Then, 20 µL of the CCK-8 was added to each of the calibration, test, and blank wells. The final concentrations of the AgNPs tested were 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, and 0 µg/mL, with 1×10^6 CFU/mL of bacterial cells. The contents and the total volume of the liquid of each well are summarized in Table 1.

The microplate was sealed by a microplate film and inserted into a microplate reader (Multiscan FC, Thermo Fisher Scientific, USA) which is warmed by a sheet heater to be 35 °C. The absorbance of each well was measured at 450 nm every 20 min up to 16 h.

	Calibration	Test	Blank	Solution volume
Bacterium / CFU•mL ^{−1}	10^{-1} – 10^6	10^6	0	180 µL (in total)
AgNPsCS / µg•mL ^{−1} or	0	0–3.2	0–3.2	
AgNPs-HCM* / µg•mL ^{−1}	0	0–50	0–50	
NB	included	included	included	20 µL
CCK-8	included	included	included	

Table 1. Contents and total solution volume of each well in antibacterial tests. *The concentration of the AgNPs in the AgNPsCS in which the HCM soaked.

The obtained data were analyzed by the following procedure. The absorbance difference between the test well and the blank well (at the same AgNPs concentration) was plotted against the incubation period (h) to find the incubation period when the absorbance difference reached to 1.0 ($T_{abs1.0}$) for *E. coli* and to 1.5 ($T_{abs1.5}$) for *S. aureus*, as shown in supplementary Fig. S2a. For the calibration wells, the absorbance difference between the calibration well and the blank well at the concentration of AgNPs=0 (only NB) was plotted against the incubation period to find $T_{abs1.0}$ or $T_{abs1.5}$ for *E. coli* and *S. aureus*, respectively. Then, the inoculated number of the bacterium (CFU/mL) was plotted against $T_{abs1.0}$ or $T_{abs1.5}$ (h) of the calibration well to obtain a calibration curve, as shown in supplementary Fig. S2b. This curve is utilized to estimate the initial (survived) number of bacterium (CFU/mL) in the test well (at a certain conc. of AgNPs) using the $T_{abs1.0}$ or $T_{abs1.5}$ of the test well. Then, the minimum inhibitory concentration (MIC) was decided using the probit method.

Antibacterial activities of AgNPs-HCM

The HCM was soaked in a 2-fold dilution series of AgNPsCS (50, 25, 12.5, 6.3, 3.2, 1.6, and 0.8 µg/mL) for an appropriate period which was determined in 2.4.1. After the soaking, obtained AgNPs-loaded HCM (AgNPs-HCM) was vacuum-dried and sterilized by ethylene oxide gas. As a control, the HCM as prepared (without soaking to AgNPsCS) was also sterilized in the same manner.

The sterilized AgNPs-HCM was placed in each of the test and blank wells in a 96-well microplate. In the test and blank wells of AgNPs=0, the sterilized HCM was placed. A 180 µL portion of bacterial suspension containing 1×10^6 CFU/mL was dispensed into each test well, whereas 180 µL of NB was dispensed into each blank well. As the same manner to Sect. 2.5.1, the calibration wells were also prepared. Then, 20 µL of CCK-8 was added to each of the calibration, test, and blank wells. The contents and total volume of each well were as shown in Table 1. Each condition was prepared in triplicate. The microplate was sealed to measure the absorbance at 450 nm in the same manner to Sect. 2.6.1. Obtained data were analyzed by the same procedure described in Sect. 2.6.1 as well.

After the incubation and measurement was finished, 100 µL of the solution in each blank well was collected and solutions collected from five wells, that placing the same AgNPs-loaded HCM, was accumulated in one solution to quantify the AgNPs released from the AgNPs-HCM during the test by ICP-OES.

Cytotoxicity test

Osteoblastic cell line, MG-63, originated from human osteosarcoma was used for cytotoxicity tests. D-MEM supplemented with 10 vol% heat-inactivated fetal bovine serum and 1 vol% penicillin-streptomycin solution was employed as “complete” D-MEM. The CCK-8 was used as a colorimetric reagent.

Considering the small sample size ($n=3$), the Steel-Dwass method was used for the significance test. The significance level was set to less than 5%. Static analyses were performed using R (Version 3.6.0 for macOS, R Foundation for Statistical Computing, <https://www.R-project.org/>.)

Cytotoxicity test of AgNPsCS

A two-fold dilution series of AgNPsCS (64, 32, 16, 8, 4, 2, and 1 µg/mL) with complete D-MEM (100 µL) was prepared in test and blank wells of a 96-well microplate. In the test and blank wells of AgNPsCS=0, 100 µL of complete D-MEM was dispensed. Then, 80 µL of cell suspension was added to each test well, whereas 80 µL of complete D-MEM was added to the blank well. Table 2 summarizes the contents and total volume of solution of each well. Four microplates were prepared for each culture period. Two thousand cells were seeded in each well for 1-day experiment, and 200 cells were seeded in each well for 3-, 5-, and 7-day experiments. After the cell seeding, all microplates were incubated at 37 °C under the atmosphere of 5% CO₂ in humidified air. After each culture period, 20 µL of CCK-8 was dispensed into all wells and incubated for additional 2 h at the cell culture conditions to promote WST-8 reaction. After the incubation, absorbance at 450 nm of each well was measured by the microplate reader (GENios microplate reader, Tecan Trading AG, Switzerland). The absorbance difference between the test well and the corresponding blank well was calculated to estimate the number of cells in each well based on a calibration curve prepared in advance. Then, relative cell viability (for 1 day) or relative cell growth (for 3–7 days) was calculated as dividing the cell number of the test well by that of the test well where AgNPs=0. Then, the 50% inhibitive concentration (IC₅₀) was decided using the probit method.

		Test	Blank	Solution amount
Cell number	1 day	2000	0	180 µL (in total)
	3–7 days	200		
AgNPs / µg•mL ⁻¹ or		0–64	0–64	
AgNPs-HCM* / µg•mL ⁻¹		0–50	0–50	
Complete D-MEM		include	include	

Table 2. Contents and total solution volume of each well in cytotoxicity test. *The concentration of the AgNPs in the AgNPsCS in which the HCM soaked.

Cytotoxicity test of AgNPs-HCM

The HCM was soaked in a 2-fold dilution series of AgNPsCS (50, 25, 12.5, and 6.3 $\mu\text{g/mL}$) for an appropriate period which was determined in 2.4.1. After the soaking, obtained AgNPs-HCM was vacuum-dried and sterilized by ethylene oxide gas. The HCM without soaking to AgNPsCS was also sterilized in the same manner.

The sterilized AgNPs-HCM was placed in each of the test and blank wells in a 96-well microplate. In the test and blank wells of AgNPs=0, the sterilized HCM was placed. Then, 180 μL of cell suspension was dispensed into each test well, whereas 180 μL of complete D-MEM was dispensed into each blank well. The contents and total solution volume of each well were shown in Table 2. The subsequent procedures were the same as those in 2.7.1, except medium changes at every 24 h. In case of 7-day experiments, a 100 μL portion of the medium was collected from each blank well at every medium change to quantify the released AgNPs from the AgNPs-HCM by the ICP-OES.

Results and Discussion

Characterization of AgNPs

The scanning electron micrograph of synthesized AgNPs is shown in Fig. 2, which is blurred due to the presence of starch on their surface. The AgNPs seemed to aggregate during drying, but their primary particles had spherical shape with the diameter range of 10–100 nm. The previous report prepared AgNPs in the similar green methods characterized them as face-centered cubic silver^{30–33}. The particle size distribution illustrated in Fig. 3a revealed that 95% of the AgNPs had diameters larger than 55 nm, agreeing with the SEM observation. The zeta-potential of the AgNPs ranged between -3.5 and -1.0 mV, which would be beneficial for their adsorption on the HAp/Col, because HAp nanocrystals were reported to have positive zeta-potential in the pH range of 3–8³⁴.

Characterization of HCP

Powder X-ray diffraction pattern and Fourier-transformed infrared spectrum of the HAp/Col are shown in Figs S3 and S4. Details of the HAp/Col are described in our previous study¹⁴. The adsorption-desorption isotherm of N_2 on the HCP was shown in Fig. 3b. In comparison to adsorption-desorption isotherm in the literature³⁴, the types of isotherms and hysteresis loop were respectively determined as types IV and H3. The type IV isotherm is a typical for gas adsorption/desorption on a specimen with mesopores, and the type H3 isotherm is a typical hysteresis loop for gas adsorption/desorption on a specimen with a slit-like pores. Thus, the HCP was considered to have slit-shaped mesopores, which is very reasonable, because the HAp/Col is fibrous nanocomposite that aggregates to form a macrostructure having pores between fibers, i.e., slit-like pores. The primary structure of the HAp/Col fibers is the oriented adsorption of HAp nanocrystals at approximately 40 nm in length on to collagen molecules of 300 nm in length. Therefore, a dominant pore size formed between the HAp/Col fibers is presumably less than 40 nm, as is in the range of mesopores, formed between the HAp nanocrystals. However, these mesopores had no or less electrostatic force compared with the force between the HAp nanocrystals and collagen molecules. The specific surface area of the HCP was determined as 42.2 m^2/g by calculation based on its Brunauer-Emmett-Teller (BET) plot.

Adsorption profile measurement of AgNPs

Adsorption equilibrium time

The time-dependent adsorption isotherm for the AgNPs on to the HCP with PSO and PFO model plots were shown in Fig. 4a. Coefficients of determinations, R^2 , for the linear fitting curves for the PFO and the PSO models were calculated respectively as 0.8372 and 0.9999; therefore, the adsorption kinetics of the AgNPs on the HCP agreed to the PSO model. The equilibrium adsorption amount, Q_e was 526.31 $\text{mg}\cdot\text{g}^{-1}$ and the adsorption rate constant, k_2 was 0.0006 $\text{g}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$. In fact, the calculated PSO plot almost completely fitted to the experimental plot. No comparable references are available for Q_e value of the AgNPs on the HAp or other calcium phosphate bioceramics, but those are reported as 91.9 $\text{mg}\cdot\text{g}^{-1}$ for large pore mesoporous silica³⁶ and 3.198 $\text{mg}\cdot\text{g}^{-1}$ for dolomitic limestone³⁷. Thus, HCP indicated superior Q_e value than other materials because of

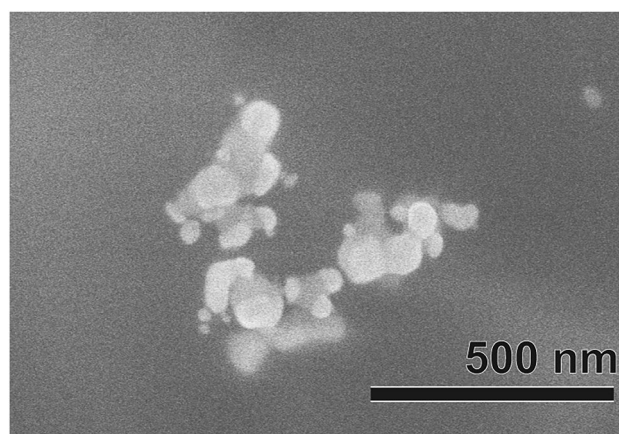


Fig. 2. Scanning electron micrograph of AgNPs.

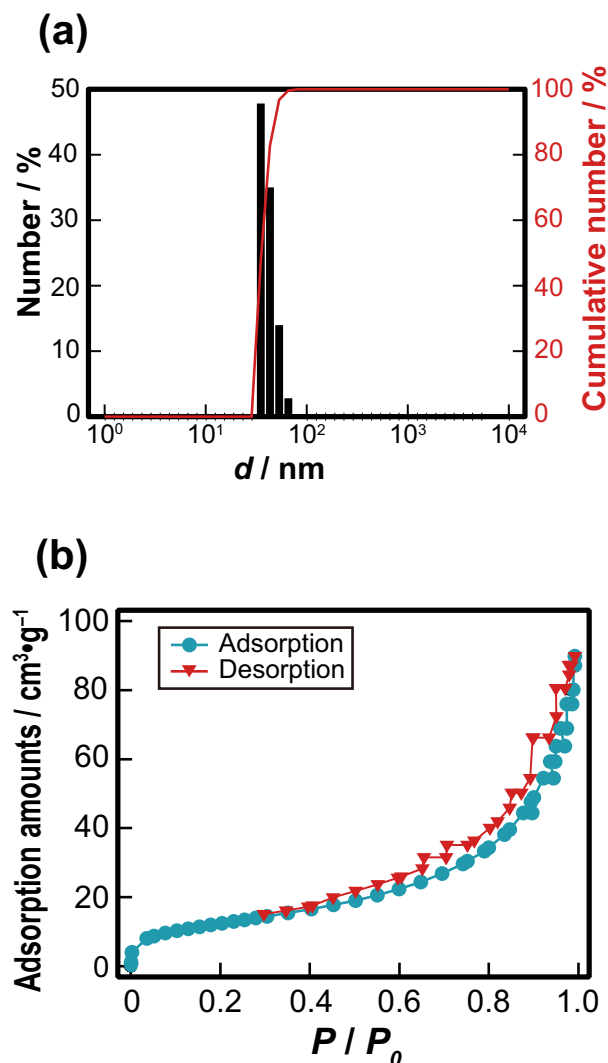


Fig. 3. Particle size distribution of AgNPs. Bars are numbers of particles (left axis) and red line is cumulative number of particles (right axis).

its unique nanostructure and chemical composition; HAp nanocrystals less than 40 nm in size¹⁴ are oriented to collagen molecules 300 nm in length.

Under the PSO model, soaking time necessary to reach the calculated equilibrium adsorption amount was impractically long to use for further experiments as 60 days. However, the adsorption amounts at 4 h as practical time was calculated to be 519.48 mg/g, which was approximately 99% of the equilibrium adsorption amount and was very close to the maximum adsorption amount of this experiment as 516 ± 0.8 mg/g. Therefore, in the subsequent experiments, 4 h was used as the adsorption equilibrium time, except further noticed.

Adsorption isotherm

The adsorption isotherm for the AgNPs on to the HCP with calculated Langmuir, Freundlich, D-R, and Temkin model plots were illustrated in Fig. 4b, indicating that D-R and Temkin models had better fit to the experimental plot. Based on the linear fitting curves, R^2 for Langmuir, Freundlich, D-R, and Temkin models were 0.6349, 0.6227, 0.9112, and 0.7598, respectively; therefore, the best fit was the D-R isotherm model. The D-R constant, K_{dr} , was $0.0114 \text{ mol}^2 \cdot \text{kJ}^{-2}$ and the theoretical saturation capacity, Q_s , was $1621.9 \text{ mg} \cdot \text{g}^{-1}$.

The D-R model describes the adsorption mechanism on heterogeneous surfaces by the adsorption energy distribution^{38,39}. Using the model, the average adsorption energy of the AgNPs on to the HCP was calculated as $9.37 \text{ kJ} \cdot \text{mol}^{-1}$. This value was in the range of $8 \leq E \leq 16$, indicating the dominancy of chemical interaction⁴⁰ in the adsorption process of the AgNPs on the HCP. Since chemical interaction is generally stronger than physical interaction, the HCP is a stable carrier for the AgNPs. No literatures were found describing detailed adsorption mechanisms for AgNPs on HAp or other calcium phosphate bioceramics; however, Marcin et al.³⁷ reported chemical adsorption of AgNPs on dolomitic calcite with the Freundlich isotherm. Thus, chemical adsorption of AgNPs is possible mechanism, at least, for calcium-salt ceramics.

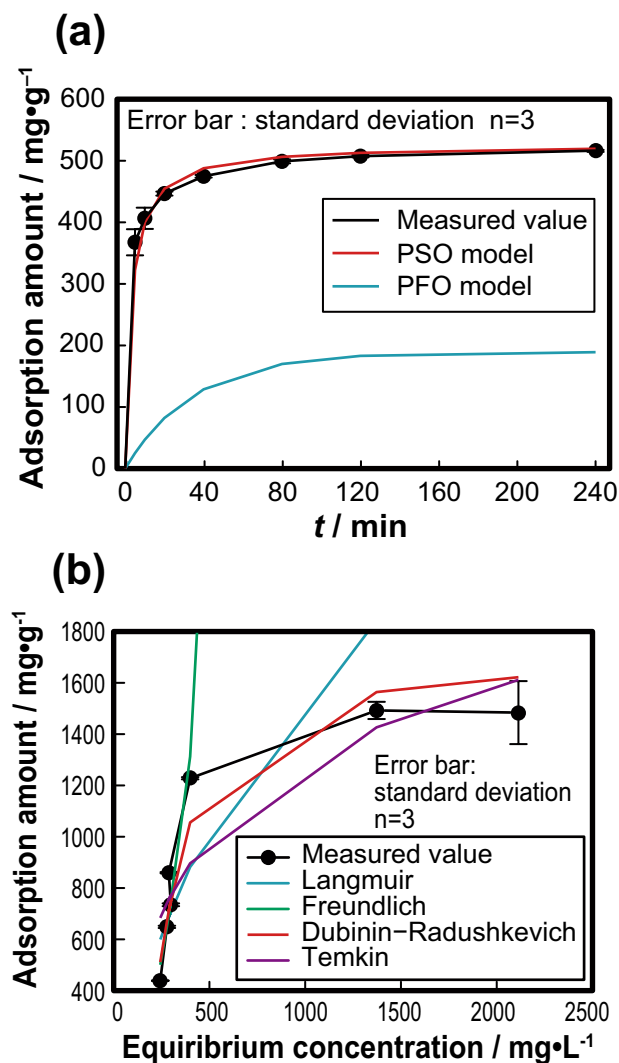


Fig. 4. Time-dependent adsorption isotherm for AgNPs on to HCP plotted with calculated PSO and PFO models (a) and adsorption isotherm for AgNPs on to the HCP plotted with calculated Langmuir, Freundlich, D-R, and Temkin models (b).

Desorption profile measurement of AgNPs

Figure 5a shows the cumulative desorption amounts of the AgNPs from the AgNPs-HCP in PBS(–) during first 9 days, indicating the rapid increase in desorption amount up to day 3, followed by the constant, slow desorption after day 5. In this test, 10 vol% of the fluid was replaced by the fresh one every two days, which is much slower than that by in vivo bone blood flow ($0.37\text{--}12.33\text{ mL}\cdot\text{min}^{-1} = 532.8\text{--}17755.2\text{ mL}\cdot\text{day}^{-1}$, depended on bone)⁴¹ and lymphatic circulation ($10\text{--}40\text{ mL}\cdot\text{h}^{-1} = 240\text{--}960\text{ mL}\cdot\text{day}^{-1}$)⁴². Therefore, in vivo desorption amounts of the AgNPs per day should be larger than that of the first day in the test. Based on the above assumption, in vivo releasing period of the AgNPs was estimated to be two weeks at longest, since the desorption amount in the first day was approximately 6% of the total carrying amount of the AgNPs on the HCP, and it decreased to 4 to 5 days.

The AgNPs concentrations in the collected solution at each immersion period and the particle size distribution of the released AgNPs in the supernatant after 9-day immersion are shown in Figs. 5b–c. The AgNPs concentration reached to 100 $\mu\text{g}/\text{mL}$ on the first day, and thereafter, it fluctuated between 125 and 145 $\mu\text{g}/\text{mL}$. After 9 days, more than 90% of the released AgNPs had the particle sizes of 50 nm or less. According to a report by Castañón et al.⁴³, AgNPs with a mean particle size of 89 nm had the minimum inhibitory concentrations (MICs) for *E. coli* and *S. aureus* as 11.79 and 33.71 $\mu\text{g}/\text{mL}$, respectively. Literatures^{44–46} reported that the smaller AgNPs in their particle sizes tend to have the stronger antibacterial activity. Therefore, the particle size and concentration of the released AgNPs obtained in this study can be sufficient for initial antibacterial activity. Contrarily, Singh et al.⁴⁷ reported that the 50% inhibitory concentration (IC_{50}) of AgNPs with a mean particle size of 102 nm against MG-63 was 60.42 $\mu\text{g}/\text{mL}$; the particle size and concentration of the released AgNPs in this test may be cytotoxic for osteoblastic cells. Therefore, the adsorption amount of AgNPs on the HAp/Col should be controlled to be an appropriate level to meet the requirement for both antibacterial and non-toxic properties.

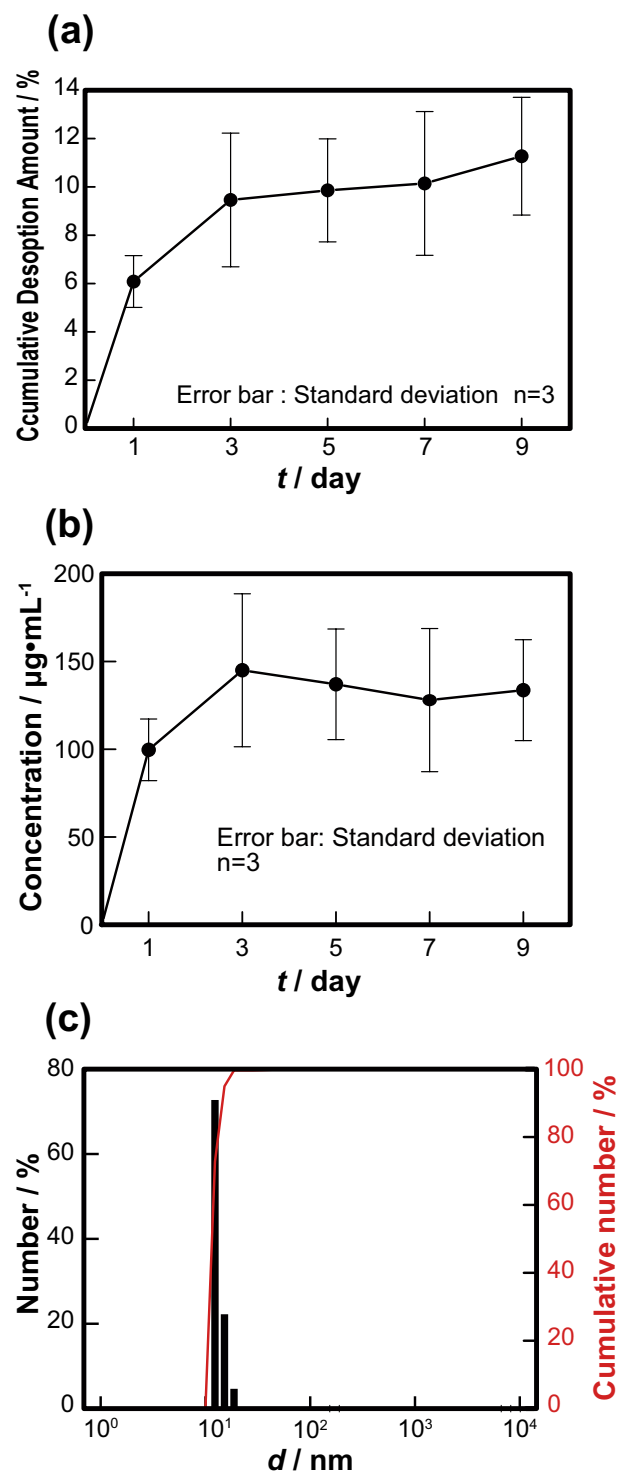


Fig. 5. Cumulative desorption amounts of the AgNPs from HCP in PBS(–) (a), concentration of AgNPs at each time point (b), and the particle size distribution of the released AgNPs in test vessels (c). Bars are numbers of particles (left axis) and red lines are cumulative number of particles (right axis).

Antibacterial activity

Antibacterial activities of AgNPsCS

The absorbance changes in the test wells and the dose-response curves of AgNPs for *E. coli* and *S. aureus* are demonstrated in Fig. 6. The MICs (mean $\pm$ sd) of AgNPs against *E. coli* and *S. aureus* were determined as 0.96 ± 0.02 and 1.76 ± 0.06 $\mu\text{g}/\text{mL}$, respectively, by the probit method. These results were also confirmed by no logarithmic increase in the absorbance curves of the corresponding wells applied with the AgNPsCS of greater concentrations than MICs, indicating no bacterial growth. These results also conclude the sufficient AgNPs

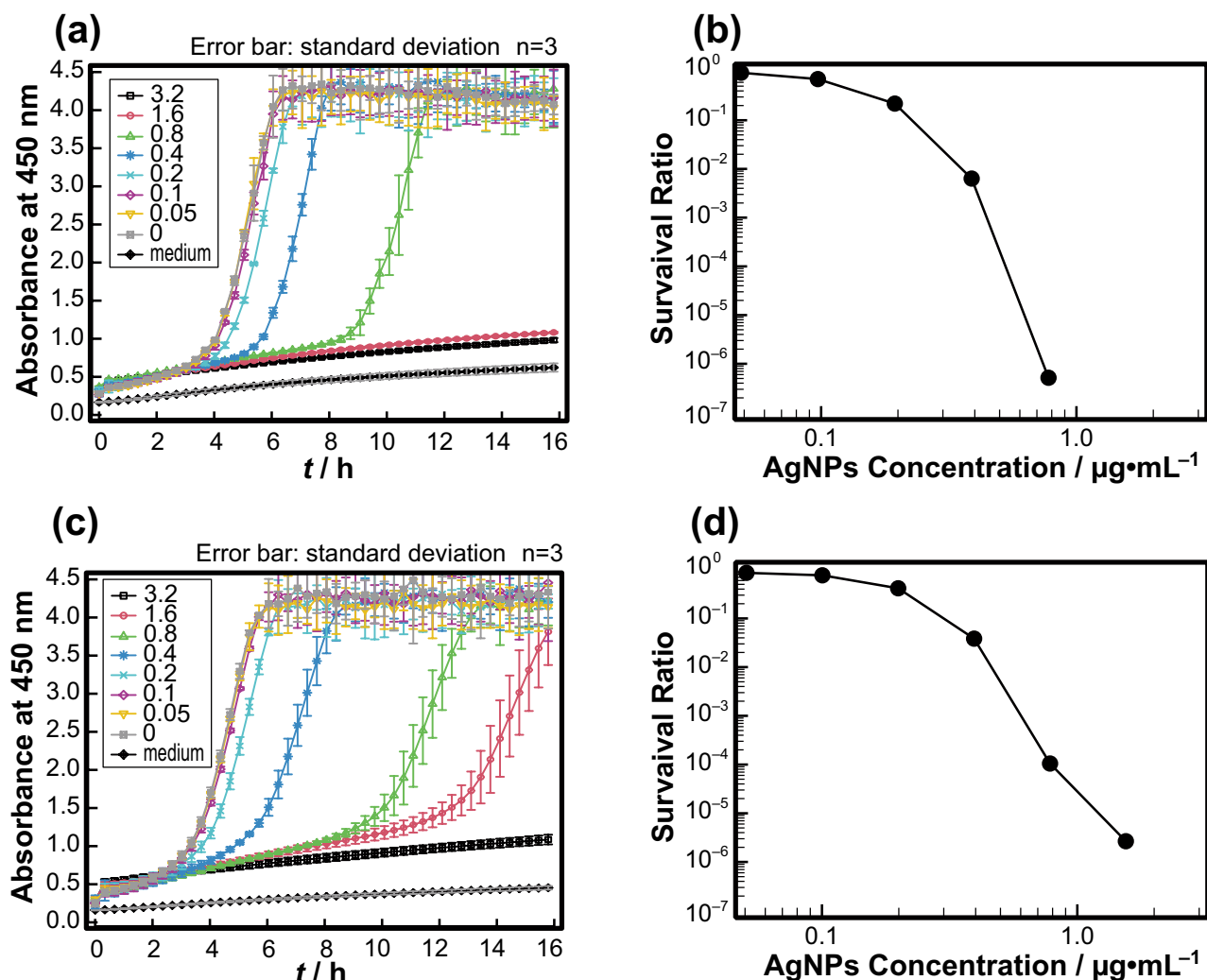


Fig. 6. Absorbance changes in the test wells (a, c) and dose-response curves (b, d) of AgNPs for *E. coli*. (a, b) and for *S. aureus* (c, d). Legends in (a) indicate concentration of AgNPsCS at a unit of $\mu\text{g/mL}$.

concentration for inhibition of both *E. coli* and *S. aureus* as 3.2 $\mu\text{g/mL}$. These MICs were 1/10 to 1/100 times lower than those for the AgNPs in similar particle sizes reported by Castañón et al.⁴³, and Agnihotri et al.⁴⁴. These differences may be attributed to the differences in bacterial strains, testing conditions including cultural media, and synthesis conditions for AgNPs. Therefore, antibacterial application of AgNPs is preferably confirmed for each synthesis batch using target or standard bacterial strains.

Antibacterial activities of AgNPs-HCM

The AgNPs concentrations desorbed from the AgNPs-HCMs in the blank wells after 24-h incubation was plotted against immersing AgNPsCS concentrations as shown in Fig. 7a. The desorbed AgNPs concentration increased almost linearly with increase in the immersing AgNPsCS concentration up to 25 $\mu\text{g/mL}$, reaching to 3.2 $\mu\text{g/mL}$ when the AgNPsCS concentration was 6.3 $\mu\text{g/mL}$ or higher. These AgNPs-HCMs would exhibit antibacterial properties against both *E. coli* and *S. aureus*. Desorption ratio of AgNPs during 24-h immersion, which can be calculated as dividing desorption amount at 24 h by initial adsorption amount, was the highest as 10.8% at immersing concentration of 6.3 $\mu\text{g/mL}$, decreasing to 1.9% at 50 $\mu\text{g/mL}$.

Figures 7b and c show the absorbance curves of the test wells with the AgNPs-HCM for *E. coli* and *S. aureus*, respectively. The AgNPs-HCM prepared at an immersing concentration of 1.6 $\mu\text{g/mL}$ or higher showed no logarithmic increase in absorbance, i.e., no growth of *E. coli* (Fig. 7b). In the case of *S. aureus*, the similar results were obtained for the AgNPs-HCMs at an immersing concentration of 6.3 $\mu\text{g/mL}$ or higher. The MIC (mean $\pm$ sd) of the AgNPsCS concentration in the immersing solution at AgNPs-HCM preparation was 2.07 ± 0.12 $\mu\text{g/mL}$ for *S. aureus* by the probit method whereas that for *E. coli* was decided as 0.78 ± 0.00 $\mu\text{g/mL}$ by linear regression. Based on Fig. 7a, the AgNPs concentrations desorbed from the AgNPs-HCMs prepared at immersing concentration of 0.78 and 2.07 $\mu\text{g/mL}$ were about 0.9 and 1.8 $\mu\text{g/mL}$, respectively. These values are close to the MICs of the AgNPsCS against these bacteria, 0.96 ± 0.02 and 1.76 ± 0.06 $\mu\text{g/mL}$. Therefore, the antibacterial properties of the AgNPs showed no significant changes by the adsorption on and desorption from the HCM.

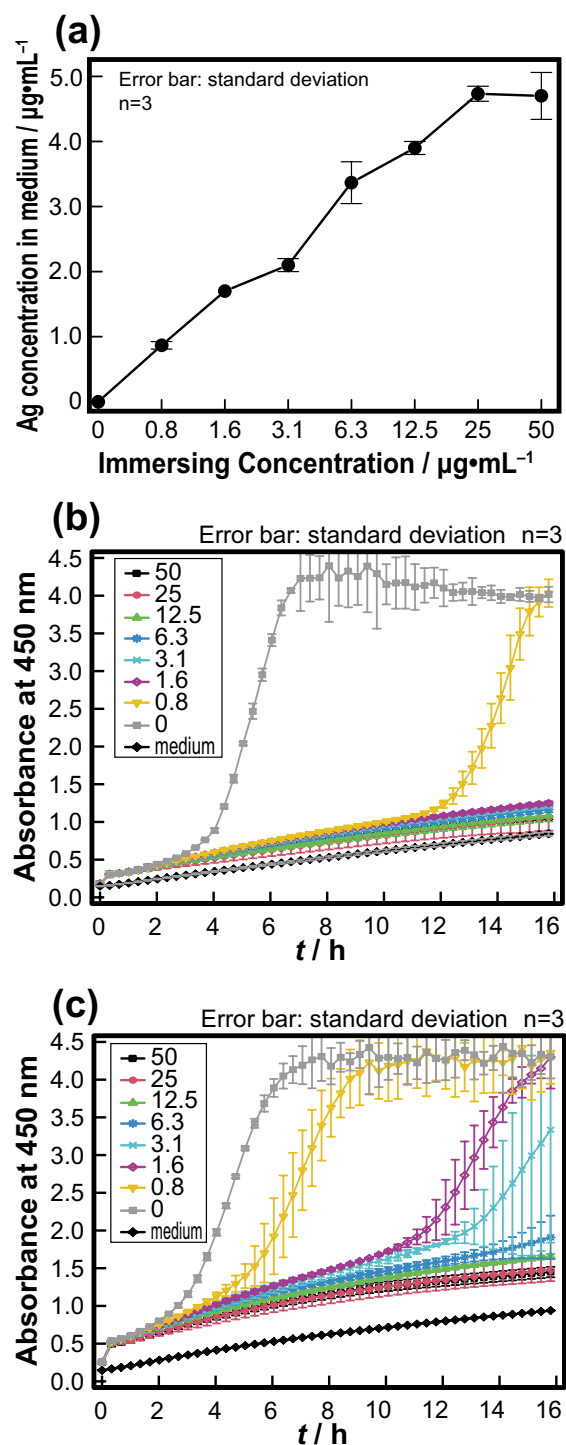


Fig. 7. Concentrations of AgNPs desorbed from AgNPs-HCM in blank wells after 24 h incubation as a function of immersing AgNPsCS concentration of HCM (a), absorbance changes in the test wells for *E. coli* (b) or for *S. aureus* (c) incubated with AgNPs-HCM prepared by immersing in various AgNPsCS. Legends indicate concentration of immersing AgNPsCS at a unit of $\mu\text{g}/\text{mL}$ for preparation of AgNPs-HCM.

Furthermore, obtained results confirmed HCM immersed into 3.1 $\mu\text{g}/\text{mL}$ or higher concentration of AgNPsCS can adsorb enough AgNPs and desorb it after 24 h of immersion into NB to demonstrate its antibacterial effect. The concentration of AgNPsCS as 3.1 $\mu\text{g}/\text{mL}$ is relatively low in consideration of the maximum loading; we have a wide range of controlling AgNPs loading on HCM and its desorption profile optimized for a specific target.

Cytotoxicity

Cytotoxicity of AgNPsCS

Figure 8a shows the dose-response curve of AgNPsCS for MG-63 cells after 1-day culture. The relative cell growth was slightly increased by the addition of AgNPsCS up to 4 $\mu\text{g/mL}$, which was probably attributed to the reaction of WST-8 with AgNPsCS. Thereafter, the relative cell growth decreased with increase in AgNPsCs concentration. The IC_{50} of AgNPsCS for 1-day culture was calculated as $13.3 \pm 0.02 \mu\text{g/mL}$ by the probit method. This value is

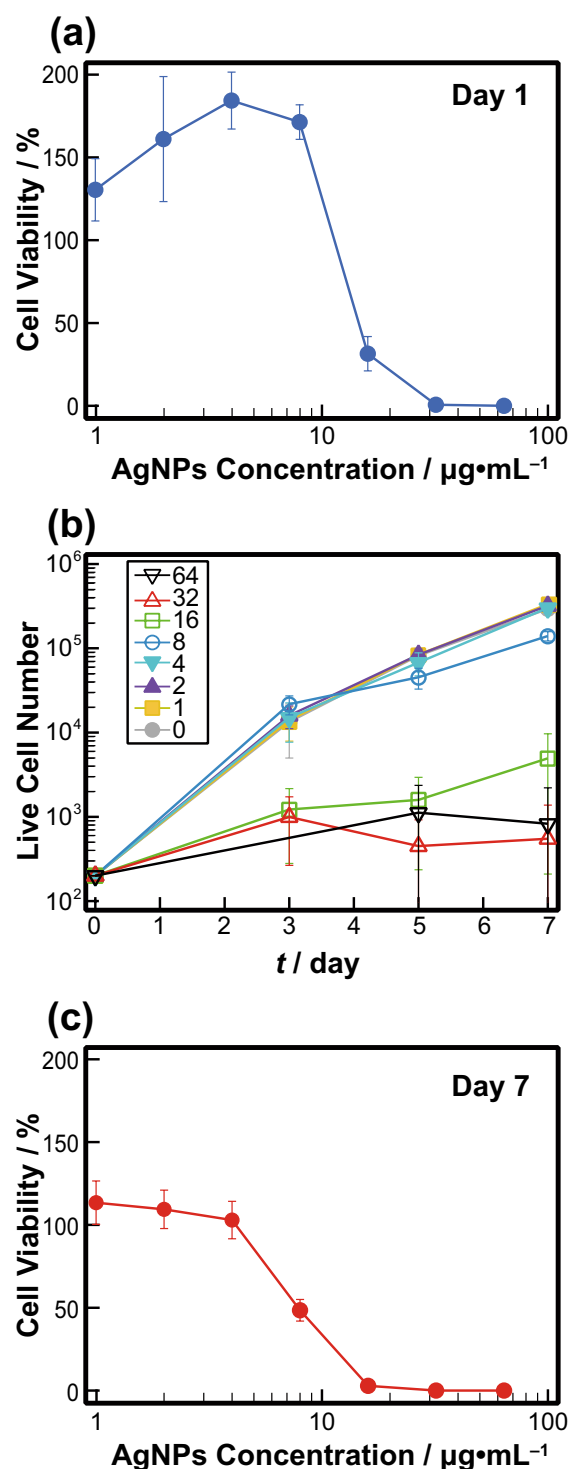


Fig. 8. Dose-response curve of AgNPs after 1-day of culture for MG-63 (a), cell proliferation curves for MG-63 cultured with various AgNPs concentrations up to 7 days (b), and dose-response curve of AgNPs after 7 days of culture for MG-63 (c). Bars are standard deviations.

7.5 ~ 13.8 times higher than the MICs of AgNPsCS for *E.coli* and *S.aureus* (0.96 ± 0.02 and 1.76 ± 0.06 $\mu\text{g/mL}$), suggesting the existence of the concentration window of AgNPsCS which is non-cytotoxic but antibacterial.

Cell proliferation curves for 3 to 7 days were demonstrated in Fig. 8b. Proliferation curves for 1 to 4 $\mu\text{g/mL}$ of AgNPs showed no significant differences in comparison to control (AgNPs=0), but that for 8 $\mu\text{g/mL}$ showed slight reduction in proliferation at day 5 and 7. Contrarily, cells did not proliferate in 32 $\mu\text{g/mL}$ or higher concentration of AgNPs. Interestingly, cells exposed to 16 $\mu\text{g/mL}$ AgNPs showed slight proliferation at 5 and 7 days. The dose-response curve of AgNPsCS after 7-day culture was displayed in Fig. 8c. By the probit method, the IC_{50} for 7-day culture was decided as 7.85 ± 0.38 $\mu\text{g/mL}$.

Cytotoxicity of AgNPs is varied by particle sizes, preparation methods, and cell lines. Gliga et al. reported that the AgNPs of 10 nm in particle size were toxic for BEAS-2B cells derived from human bronchial epithelium, but AgNPs in larger, 40 to 75 nm size had less or no toxicity with or without coating by citrate⁴⁸. They also reported the AgNPs of 50 nm had no cytotoxicity at the concentration of 50 $\mu\text{g/mL}$ after 24-h culture⁴⁸, suggesting the lower toxicity of their AgNPs than those in the present study. Kim et al. examined the cytotoxicity of AgNPs with different particle sizes against 4 kinds of cell lines, MC3T3-E1 (mouse pre-osteoblast), PC12 (rat neuroblastic cell), HeLa (human cervical cancer cell), and CHO (Chinese hamster ovary cell)⁴⁸. The IC_{50} s of AgNPs for cell lines tested are 80–160 $\mu\text{g/mL}$ after 24-h culture, which reduces with increase in the culture period⁴⁸. This range of the AgNPs concentration (80–160 $\mu\text{g/mL}$) are almost 10 times higher than the IC_{50} s for 1- and 7-day culture in the present study. Interestingly, their results show that the AgNPs with larger particle size demonstrate the higher cytotoxicity contrary to the results of Gliga et al. Based on these studies including the present one, the cytotoxicity of AgNPs would vary with the types of cells and testing methods, but also with their preparation methods and batches, as the same as the antibacterial activity. Further systematic research is necessary to elucidate control factors and mechanisms of AgNPs cytotoxicity.

Cytotoxicity of AgNPs-HCM

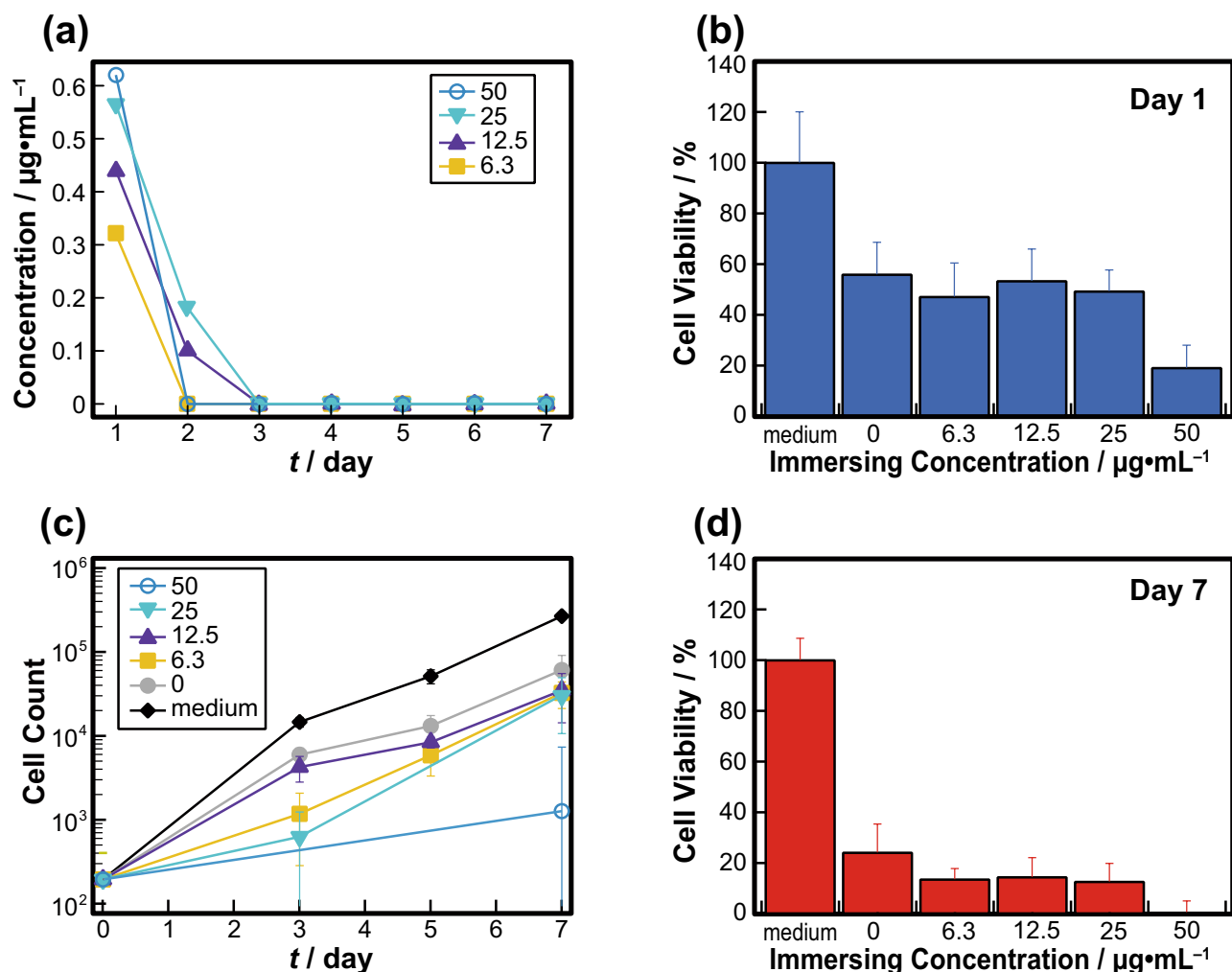
Changes in the concentrations of released AgNPs from the AgNPs-HCM in the complete D-MEM were illustrated in Fig. 9a. The data indicated that the maximum AgNPs concentration in the complete D-MEM was 0.63 $\mu\text{g/mL}$ at the AgNPs-HCM prepared in 50 $\mu\text{g/mL}$ AgNPsCS, which was lower than IC_{50} obtained in 3.6.1. The lower release of AgNPs in the complete D-MEM in comparison to that in NB can be attributed to various ingredients in the complete D-MEM, which may inhibit the desorption of AgNPs from the HCM. The complete D-MEM is supplemented by 10 vol% fetal bovine serum, however interstitial fluid has relatively low concentration of plasma proteins since proteins cannot permeate through the capillary wall. Therefore, the desorption rate of AgNPs in the tissue can be higher than those in complete D-MEM obtained here.

Figure 9b illustrates the cell viability after 1-day culture with AgNPs-HCM prepared in different immersing concentrations of AgNPsCS. Even though the HCM has no cytotoxicity⁵⁰, the number of viable cells was decreased to 57.8% by the present HCM prepared by soaking in D-MEM, suggesting the influence of WST-8 formazan adsorption on to the HCM. Therefore, the cytotoxicity of the AgNPs-HCM was evaluated in comparison to that of the HCM. The IC_{50} decided by the probit method was 42.4 ± 6.0 $\mu\text{g/mL}$ as the immersing AgNPs concentration.

Cell proliferation curves of MG-63 incubated with AgNPs-HCMs with changing medium every 24 h are illustrated in Fig. 9c. Cells cultured with AgNPs-HCM prepared in 12.5 $\mu\text{g/mL}$ AgNPsCS showed similar proliferation to those cultured with the HCM (indicated as 0 in Fig. 9c). In Fig. 9d, the cell viability after 7-day culture with AgNPs-HCM prepared in different immersing concentrations of AgNPsCS. The IC_{50} for 7-day culture was decided as 28.6 ± 12.9 $\mu\text{g/mL}$, which is smaller than IC_{50} for 1-day culture (42.4 ± 6.0 $\mu\text{g/mL}$). These values are slightly higher than the IC_{50} s of AgNPsCS for 1- and 7-day culture (13.3 ± 0.02 $\mu\text{g/mL}$ and 7.85 ± 0.38 $\mu\text{g/mL}$, respectively), but the maximum concentration of the desorbed AgNPs from the AgNPs-HCM in D-MEM was much lower. For example, the maximum concentration was 0.45 $\mu\text{g/mL}$ at day 1 from the AgNPs-HCM prepared in 12.5 $\mu\text{g/mL}$ AgNPsCS. This is quite different from the case of antibacterial tests in which the concentration of desorbed AgNPs was very close to MICs of AgNPsCS. This fact suggests the involvement of the AgNPs absorbed on the HCM surface for inhibition of cell growth, either directly reacting with cells or through the local gradient in AgNPs or released Ag^+ concentration near the AgNPs-HCM surface. This is also related to the anchoring dependance of MG-63 whereas bacterial cells do not require attachment to substrate surface. However, this possible involvement of the AgNPs adsorbed on the HCM surface is also beneficial for the implant application of the AgNPs-loaded HCP. For this point, further assessment of the antibacterial effect of adsorbed AgNPs is important to be performed under the similar environment to in vivo implantation such as the circulation of the body fluid for the mimicking of removal of the desorbed AgNPs by the fluid circulation.

Conclusion

In this paper, AgNPs-HAp/Cols were prepared, and their antimicrobial activities and cytotoxic influences were investigated to assess its possibility for the application as antibacterial bone-void fillers. The HCP had sufficient adsorption-desorption properties of AgNPs; the maximum AgNPs load was calculated as 1621.9 mg/g based on the D-R model. The AgNPs release from the AgNPs-HCM is controllable by the concentration of AgNPsCS where the HCM is soaked. The AgNPs-HCM indicated good antibacterial properties as MICs of 0.78 ± 0.00 and 2.07 ± 0.12 $\mu\text{g/mL}$ of the immersing AgNPsCS concentration for *E.coli* and *S.aureus*, respectively, giving the concentrations of desorbed AgNPs close to MICs of AgNPsCS (0.96 ± 0.02 and 1.76 ± 0.02 $\mu\text{g/mL}$). The IC_{50} of AgNPs-HCM for MG-63 after 1-day culture was 42.4 ± 6.0 $\mu\text{g/mL}$ of the immersing AgNPsCS concentration, releasing much lower amount of AgNPs (0.45 $\mu\text{g/mL}$ as the maximum at day1 from the AgNPs-HCM prepared in 12.5 $\mu\text{g/mL}$ AgNPsCS) than the IC_{50} of the AgNPsCS (13.3 ± 0.02 $\mu\text{g/mL}$). Obtained results suggest the involvement of AgNPs absorbed on the AgNPs-HCM surface for the cytotoxicity expression against MG-63, which can be beneficial property of the AgNPs-HCM for the implant application as bone void fillers and guided



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Fig. 9. Changes in the concentration of AgNPs desorbed from the AgNPs-HCMs into complete D-MEM (a), cell viability of the AgNPs-HCM after 1-day culture plotted against the immersing AgNPsCS concentration (b), cell proliferation curves incubated with the AgNPs-HCM (c), and cell viability of the AgNPs-HCM after 7-day culture plotted against the immersing AgNPsCS concentration (d). Legends for (b, d) demonstrate immersing AgNPsCS concentrations in $\mu\text{g/mL}$ for preparing AgNPs-HCM.

bone tissue membranes. Further investigation is necessary for optimization of AgNPs desorption behavior under simulated environment of targeting tissue.

Funding declaration.

This study was partly funded by a research grant 21im0210221h0203 from AMED, Japan.

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Received: 29 October 2025; Accepted: 20 May 2026

Published online: 01 June 2026

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Author contributions

M.K. planned all investigations, wrote manuscript text, prepared figures and tables. A.Y. conducted antimicrobial test sections and advised and revised related manuscript text, Figs. 5, 6, 7, 8 and 9; Table 1, and 2. Y.S. conducted SEM and specific surface area measurements. T.Y. conducted particle size distribution measurements solely and conducted other experiments with A.Y. and Y.S. T.Y. also performed statistical analyses for all experiments. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Disclosure

Statement of COI.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-026-54878-2>.

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