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Antifungal effects of 3D scaffold type gelatin/Ag nanoparticles biocomposite prepared by solution plasma processing

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ABSTRACT

The 3D scaffold type biocomposites of gelatin/silver nanoparticles were prepared through the silver nanoparticles (Ag NPs) formation in gelatin solution using solution plasma process (SPP) and their antifungal activity was evaluated. The mixture of 3% gelatin solution and silver precursor ($AgNO_3$; 1–10 mM) was subject to discharge at high voltage (1600 V) under the controlled conditions to form the suspension of Ag NPs in the gelatin matrix. The freeze-drying process of lyophilization was employed to fabricate the 3D scaffold type biocomposite of gelatin/Ag NPs from the suspension. The water-insoluble property was improved by cross-linking using UV-irradiation ($\lambda = 254$ nm for 15 min). The physical and chemical characteristics of the biocomposite were investigated using UV–vis spectroscopy, EDS, FE-SEM, and TEM. The results indicated that the 3D scaffold biocomposite of gelatin/Ag NPs had spherical shape with approximately 11–12 nm of diameter. The antifungal activity analysis suggested that the biocomposite with Ag NPs could inhibit the growth of *Candida albicans* as well as that of hyphae and spores of *Aspergillus parasiticus* significantly. MIC of the biocomposite for *C. albicans* and *A. parasiticus* was determined as 80 μg/ml and 240 μg/ml of Ag NPs, respectively. The growth inhibition of 92.8% was observed in the biocomposite with 10 mM Ag against *C. albicans*.

1. Introduction

Solution plasma process (SPP) is one of the recently introduced methods used for the synthesis of nanoparticles. SPP is performed through direct discharge occurrence in solution [1]. Plasma is generated in solution by applying high voltage, highly active species such as highly energetic ions and radicals, electrons and UV radiation are generated. This processing has an advantage in that it can be performed in ambient temperature and pressure. Also, it is a green strategy that does not employ any chemical agent. SPP has many application potentials such as nanoparticle synthesis, sterilization, organic decomposition, and polymerization [2,3].

Conventionally so-called two-step method is used to fabricate a biocomposite in that nanoparticles were made separately by various methods such as atomization, mechanical alloying, and so on, and then were mixed with the base materials such as gelatin or alginate acid to fabricate a biocomposite containing nanoparticles. It is, however very difficult to fabricate a biocomposite with uniformly distributed nanoparticles because aggregation or agglomeration of the nanoparticles occurred during mixing process. In this study, we have focused on the advantage of solution plasma in fabricating biocomposites. Using SPP, *in-situ* synthesis of nanoparticles in the gelatin-based solution occurs so that one-step method to fabricate a biocomposite of the uniformly distributed silver nanoparticles (Ag NPs) in gelatin matrix is possible [4]. Also for the synthesis of metallic nanoparticles, properties of the nanoparticles such as size, morphology, and dispersibility should be controllable in this process [5].

Gelatin has superior properties of biodegradation and biocompatibility since it is derived from animal bones [6]. Silver has been known as the most effective and safe metal with antimicrobial activity that can be applied in the fields of pharmaceutics and medicine [7,8]. Bactericidal activities of Ag NPs against many bacterial species such as *Escherichia coli*, *Staphylococcus aureus*, and methicillin resistant *S. aureus* (MRSA) have been reported by numerous research groups [8,9]. On the other hand, not many reports have been made regarding antifungal activity of Ag NPs against the fungi, except a few publications against fungi such as *Fusarium oxysporum* or *Aspergillus niger* [10–12]. Especially for fungi such as *Candida albicans* and *Aspergillus parasiticus*, very

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limited reports on the antifungal activity have been reported so far to our knowledge.

Herein, we report the synthesis of 3D scaffold type biocomposites using various concentrations of gelatin and $AgNO_3$ by SPP. The physical and chemical properties of fabricated biocomposites were investigated by spectroscopic and electron microscopic analyses and their antifungal activities against two fungi, *C. albicans* and *A. parasiticus*, were assessed. *C. albicans* is a species of yeasts pathogenic to human by causing disease such as candida vaginitis [13]. *A. parasiticus* is an animal pathogenic fungus that produces aflatoxin, a potential carcinogen [14]. The results obtained in this study were supposed to provide a basis for development of an efficient method to produce antifungal agents against pathogenic fungi.

2. Experimental procedure

2.1. Synthesizing the antifungal gelatin/Ag NPs solution and fabrication of the 3D scaffold type biocomposite

In order to synthesize biocomposites of gelatin/Ag NPs using SPP, gelatin granule (3%, w/w; Samchun, Korea) and various concentrations of $AgNO_3$ (1–10 mM; Junsei, Japan) were dissolved in deionized water. The suspension (100 ml) was mixed in a beaker with electrodes connected through pores on the wall and stirred with a magnetic bar. The solution plasma was created at the gap between electrodes under controlled plasma conditions using a bipolar pulsed power supply (Kurita-Nagoya MPS-06K06C). The typical parameters including voltage, frequency, discharge time, and electrode gap were set as 1600 V, 20 kHz, 3 min, and 1.0 mm, respectively. Gelatin concentration was fixed as 3% (w/w). Upon generation of plasma, Ag NPs were synthesized in the gelatin matrix and the resulting gelatin/Ag NPs solutions were stored in capped vials.

The gelatin/Ag NPs solutions were fabricated to 3D scaffold type biocomposites by freeze drying. The gelatin/Ag NPs solution (5 ml) was placed in a petri dish and frozen at -80 °C in a deep freezer (Deep freezer 907 792, Forma Scientific, U.S.) for 2 h. During the process, the sample was packed in a polystyrene box for stable freezing. Frozen biocomposites were lyophilized in a freeze dryer (FDV-7024, OPERON, Korea) below the pressure of 6.38×10^{-4} MPa (4.6 Torr) and at -40 °C for 24 h. Then, structures of the freeze-dried biocomposites were improved by cross-linking via UV-irradiation ($\lambda = 254$ nm) for 15 min [15].

2.2. Characterization of the 3D scaffold type biocomposite

The physical and chemical properties of the fabricated biocomposites were investigated by UV–visible spectroscopy, transmission electron microscopy (TEM), energy dispersive spectroscopy (EDS), and field emission scanning electron microscopy (FE-SEM). The formation of Ag NPs in the gelatin matrix was confirmed by using an UV–visible spectrophotometer (UV–3600, Shimadzu Scientific Instruments, Japan) by scanning at wavelength between 200 and 1200 nm as well as EDS (JEOL JEM-2010, acceleration voltage: 200 kV, JEOL Co., Ltd., Japan). The size and shape of Ag NPs were observed using a TEM (JEOL JEM-2010, acceleration voltage: 200 kV, JEOL Co., Ltd., Japan). For EDS and TEM, the 3D scaffold type biocomposites (0.002 g in 5 ml C_2H_5OH) were decomposed using an ultrasonic cleaner (NXPC 2010, KODO Technology, Korea) with ultrasonic frequency of 40 kHz and 200 W power for 30 min in a capped vial [16]. Structure of the 3D scaffold type biocomposites made of gelatin was observed by a FE-SEM (JEOL JSM-6700F, acceleration voltage: 5 kV, JEOL Co., Ltd., Japan) after sputtering with Au.

2.3. Antifungal effect of the 3D scaffold type biocomposite

Antifungal activity of the 3D scaffold type biocomposite against *A. parasiticus* KCTC 6598 and *C. albicans* was examined by measuring zone of growth inhibition, determining minimal inhibitory concentration (MIC), and assessing effect on colony forming units (CFU). Inhibition of *A. parasiticus* growth by the biocomposites was examined by inserting a disc of fungal hyphae (6 mm in diameter) on the center of a potato dextrose agar plate (PDA; potato dextrose 2.4%, agar 1.5%) with and without 200 μl of the biocomposite solution, which contains 17 μg Ag and 3% gelatin in distilled water. A disc of fungal hyphae was prepared by punching from the PDA plate with a grown fungi, *A. parasiticus* and the biocomposite solution containing Ag nanoparticles was integrated into PDA plate by spreading. Effect of the biocomposite on the hyphal growth was observed in 150 h incubation at room temperature.

Zone of growth inhibition formed by the biocomposite was measured using the agar diffusion method [17]. Cell culture or spore solution of *C. albicans* and *A. parasiticus* was spread on a yeast peptone dextrose agar (YPD; yeast extract 1%, peptone 2%, dextrose 2%, agar 1.5%) and a PDA agar, respectively. Then, discs of the 3D scaffold type biocomposites (6 mm in diameter) with various concentration of Ag (1, 3, and 5 mM) were placed on the plates. Diameter of the clear zone formed around the discs was measured in 48 h at 30 °C and in 120 h at room temperature of incubation for *C. albicans* and *A. parasiticus*, respectively.

MIC of the gelatin/Ag NPs solution was determined by adding it to the test tubes containing 50 μl of *C. albicans* and *A. parasiticus* spores in 5 ml of YPD and PD broth, respectively. None, 20, 40, 80, 160, 240, or 320 μg/ml of the biocomposite solution containing 5 mM Ag and 3% gelatin was added to each test tube. *C. albicans* was incubated at 30 °C for 48 h with shaking (230 rpm) and *A. parasiticus* was incubated in 25 °C for 120 h with shaking (150 rpm). MIC of the biocomposite for the fungi was determined by turbidity of the test tubes.

Antifungal activity of the biocomposite against *C. albicans* was also examined quantitatively by determining CFU after treatment with the fabricated gelatin/Ag NPs. First, one day old culture of *C. albicans* was diluted in YPD broth by 10^{-7} fold and a same amount (0.018 g) of the 3D scaffold type biocomposites containing various concentrations of Ag (1, 3, 5, 7, and 10 mM) was added. The tubes were kept at room temperature for 1 h and then, the mixture was spread on YPD agar plates and incubated at 30 °C for 48 h to allow the formation of colonies.

3. Results and discussion

3.1. Synthesis of the gelatin/Ag NPs biocomposite

Ag NPs were embedded in gelatin matrix by SPP without addition of any reducing agent. The gelatin solution in light yellow color turned to dark gray upon discharge, suggesting that Ag NPs were formed indeed as shown in Fig. 1a. The 3D scaffold type biocomposite of porous structure was fabricated by lyophilizing the solution for 24 h. The porous structure of lyophilized biocomposite was modified to increase water-insolubility by cross-linking it via UV-irradiation. By UV-irradiation, the biocomposite could also be sterilized as well. Synthesis of Ag NPs embedded in the gelatin matrix was verified by UV–visible spectrophotometer and EDS analysis (Fig. 1b and c).

Results obtained by UV–visible spectrophotometry indicated that Ag NPs were formed and the intensity of UV–visible spectra was dependent on the concentration of Ag in the biocomposite. When the concentration of Ag was 1 mM, the highest peak was found at 422 nm. As the concentration of Ag was increased to 3 mM