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Green synthesis of a new gelatin-based antimicrobial scaffold for tissue engineering

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ABSTRACT

With the aim of developing appropriate scaffolds for tissue engineering to suppress the formation of biofilms, an effective one-pot process was applied in this study to produce scaffolds with inherent antibacterial activity. A new method to synthesize genipin-crosslinked gelatin/nanosilver scaffolds with "green" in situ formation of silver nanoparticles by heat treatment is presented in this paper. In this procedure, toxic solvents, reducing agents, and stabilizing agents are avoided. UV–visible absorption spectra of the synthesized gelatin/nanosilver solutions were obtained immediately and three months after the synthesis revealing the presence and high stability of the silver nanoparticles. The TEM of gelatin/nanosilver solutions showed silver particles with spherical shapes that were less than 5 nm in size. Interestingly, contact angle was found to increase from 80° to 125° with the increase in concentration of nanosilver in gelatin. All gelatin/nanosilver solutions showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. However, only the highest concentration showed antifungal effects against *Candida albicans* pathogens. Scaffolds were prepared by a lyophilization technique from this solution and their antimicrobial activities were examined. Introducing this facile green one-pot process of synthesizing scaffolds with antimicrobial and anti-biofilm properties may lead to key applications in tissue engineering techniques.

1. Introduction

During recent years, the application of antimicrobial materials has increased in many areas such as surgical devices, wound dressings, implants and artificial organs to counter disease transmission and cross-infection by microorganisms. Microorganisms can form biofilms on the surface of any health-related product causing morbidity and mortality. It has been estimated that about 60% of the infections in hospitals are related to biofilms [1]. There has also been an emergence of multi-drug resistant bacterial strains, which show remarkable resistance to conventional antibiotics and biocides. Appearance of drug-resistant microorganisms is due to excessive use of antibiotics and constantly evolving microorganisms, which efficiently adapt to new environments. Antibiotic resistant bacteria can cause many difficulties in the process of medical treatment and can endanger the lives of the patients. Hence, developing modified or new antimicrobials has attracted great attention. Among the different kinds of anti-infective materials, metallic nanoparticles with antibacterial activity have garnered prominent consideration due to their chemical stability, long life, and heat resistance. Among the various metallic nanoparticles, silver nanoparticles have attracted intensive research interest since they possess high intrinsic effective antibacterial activity against a broad spectrum of pathogenic microorganisms based on the benefit of their surface plasmon resonance [1–7]. Furthermore, silver nanoparticles are biocompatible and do not easily provoke microbial resistance. It has been reported that at moderate concentrations, silver nanoparticles are nontoxic and have no side effects [7–10]. These exceptional antibacterial and biological properties have led to wide application of silver nanoparticles in wound dressings [11], contraceptive devices, surgical instruments [12], bone prostheses [13], implants [14], water disinfection [15], antibacterial coatings [16], and targeted therapy [17]. Chemical residuals from synthesis steps can be highly cytotoxic, which can restrict their potential applications in the biomedical field. To meet the increasing demand for application

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of silver nanoparticles in biomedicine, silver nanoparticles must be synthesized through "green" methods with low cytotoxic chemical residuals. It is essential to develop efficient green synthesis methods of silver nanoparticles since the most frequently applied methods involve organic solvents, low materials conversion, toxic reducing agents, and difficult, wasteful purifications. The green method for silver nanoparticle preparation is differentiated from other methods based on its use of an appropriate solvent, a reducing agent, and stabilizing agents [18–20]. Polysaccharide [21], Tollens [22], irradiation [23], biological [24] and polyoxometalates methods [25] are some of the previously developed green methods. Recently, Darroudi et al. [26] reported a facile and eco-friendly method of silver nanoparticles preparation by reducing Ag + ions in aqueous gelatin media. Gelatin acts as both reducing and stabilizing agent and plays an important role in the formation, stabilization, homogenous distribution, and uniform dispersion of nanoparticles.

Tissue engineering scaffolds have recently received much attention in regenerative medicine [27–29]. Biofilm formation on scaffolds may lead to complications such as implant failure, hospitalization, and sometimes mortality of the patient [30]. Incorporation of silver nanoparticles into the tissue engineering scaffolds can generate antimicrobial activity hindering bacterial infections [31].

In this work, we studied the green synthesis of nanosilver particles inside the matrix of gelatin scaffolds. Antibacterial gelatin/nanosilver macroporous hybrid scaffolds were prepared for tissue engineering. These scaffolds are expected to combine the beneficial properties of silver nanoparticles with the biocompatibility, biodegradability and low-levels of immunogenicity of gelatin. The effects of incorporating silver nanoparticles in different concentrations in the scaffolds were evaluated with respect to the antimicrobial activities towards two species of prokaryotic microorganisms (*Escherichia coli*, *Staphylococcus aureus*), and one eukaryotic species, the opportunistic fungal pathogen *Candida albicans*.

2. Materials and methods

2.1. Green in situ preparation of nanosilver/gelatin solution and scaffolds

Silver nanoparticles were synthesized in situ during the scaffold fabrication. For this purpose, five types of scaffolds were fabricated and were designated as follows: Gelatin (GA0), Gelatin/Ag 5 mM (GA5), Gelatin/Ag 10 mM (GA10), Gelatin/Ag 20 mM (GA20) and Gelatin/Ag 40 mM (GA40). Different concentrations of silver nitrate (Alfa Aesar) were mixed with 10 wt.% porcine gelatin (Type A, Sigma-Aldrich) in deionized water and stirred for 48 h at 50 °C. All solutions were kept in the dark to avoid any photochemical reactions during the experiment.

The scaffold synthesis process starts by molding the solution and freezing at −20 °C for 4 h followed by lyophilization at −53 °C and 0.05 mbar for 36 h to form the three dimensionally macroporous scaffolds. For crosslinking, the scaffolds were immersed in a 0.5% w/v genipin (Challenge Bioproducts, 98%) ethanolic solution (British Drug Houses) bath at room temperature for 48 h. Subsequently, the scaffolds were submerged in 1% (w/v) glycine (Alfa Aesar) solution at 25 °C for 24 h followed by rinsing with deionized water. The crosslinked scaffolds were kept in a freezer at −20 °C for 4 h and lyophilized under the same conditions.

2.2. Characterization

The ultraviolet to visible spectral measurements of nanosilver/gelatin were performed using a NanoDrop 1000 Spectrophotometer over a wavelength range of 300 to 800 nm. Transmission electron microscopy (TEM) imaging for the characterization of prepared silver nanoparticles in the gelatin solution was carried out using a JEOL JEM-2100 TEM operated at 200 kV. In order to prepare the samples for TEM imaging, gelatin/nanosilver colloids were diluted 50 fold and a drop of the colloid was placed on a carbon-coated copper grid, followed by solvent evaporation at room temperature. Analyzing the TEM images by ImageJ software, we measured the average size and distribution of silver nanoparticles. Scanning Electron Microscopy (SEM) of the synthesized nanocomposite scaffolds, before and after cell seeding, was performed using a Hitachi S-4800 microscope. Prior to SEM imaging of the microbial cells grown in the scaffolds, samples were dehydrated in a series of ethanol solutions (10, 30, 50, 70, 80, 90 vol.%) for 30 min each. X-ray diffraction (XRD) patterns were collected using a Bruker D8 Discover system operated with 0.8 mm beam size at a generator voltage of 40 kV, tube current of 40 mA and Cu-Kα radiation.

The static contact angle was measured with the sessile drop method under static conditions by imaging a carefully settled deionized water droplet of 2 μl on the surfaces. In order to compare the hydrophilicity of gelatin/nanosilver colloids with different silver concentrations, films of gelatin/nanosilver were prepared on the surface of freshly cleaned glass slides. Glass slides were cleaned by ultrasonication in acetone, ethanol and deionized water (five minutes in each solvent). Then gelatin/nanosilver colloid films were deposited at room temperature on glass slides by spin-coating (Smart Coater SC100, 50 μl sample, spin-speed 200 rpm, 60 s). Subsequently, the films were dried at 40 °C. Contact angles were measured at five different regions on the surface of each sample and the average value was calculated. It is presumed that the droplet is axisymmetric and its shape is dominated by interfacial tensions [32,33].

^{13}C NMR spectra were taken on a 600 MHz Varian Inova NMR spectrometer, using an Agilent solid-state triple resonance HXY 3.2 mm magic-angle spinning (MAS) probe. The probe was tuned to $^{1}H/^{13}C/^{2}D$, and the ^{2}D channel was used for field-frequency locking. MAS rotors from Revolution NMR, LLC with O-ring spacers and a silicone disk were used for containment of liquid in the hydrated samples. Disk-shaped pieces of GA0 and GA40 scaffold were ground in a mortar using a pestle after dipping the sample piece in liquid nitrogen. Additional liquid nitrogen was added into the mortar to grind the sample into smaller pieces. D_2O was added to dry ground samples already packed in rotors. For GA0, 14.2 mg of D_2O was added to 13.9 mg of dry sample, and for GA40, 13.5 mg of D2O was added to 13.1 mg of dry sample, giving a hydration level of roughly 50%. The hydrated samples were spun at about 6 kHz without speed control. ^{13}C HR-MAS NMR one-pulse spectra with ^{1}H decoupling were taken with a 90-degree pulse of 5 microseconds, delay between scans of 5 s, and 3000 scans, while controlling the temperature at 25 °C. Spectra were referenced externally by setting the methyl peak of DSS (4,4-Dimethyl-4-silapentane-1-sulfonic acid) in a spectrum of a sample of 5% DSS in D_2O to −1.927 ppm, following Morcombe and Zilm [34].

2.3. Antimicrobial performance

2.3.1. Strains and culture conditions

The *Candida albicans* and bacterial strains used in this study are listed in Table 1. Stock cultures of all strains were stored at −80 °C with 15% v/v glycerol as cryopreservative. For agar plate assays, *C. albicans* was grown on YPD (10 g/l yeast extract, 20 g/l tryptone, 20 g/l dextrose) whereas *S. aureus* and *E. coli* were grown on Mueller Hinton Agar (MHA) and LB agar (all Fisher Scientific), respectively. For liquid culture, the bacterial strains were grown in LB. Standard incubation conditions for *Candida* and bacterial cultures were 30 °C and 37 °C, respectively.

Table 1
List of bacterial and fungal strains used in this study.

Species	Strain(s)	Origin	Reference
Candida albicans	ATCC 44808	Clinical isolate	[31]
Candida albicans	SC5314	Clinical isolate	[32]
Escherichia coli	NovaBlue Singles	EMD Millipore	www.emdmillipore.com
Staphylococcus aureus	ATCC 25923	Clinical isolate	www.atcc.org