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Conjugated linolenic acid polymer dressings impregnated with silver nano-crystals: Fabrication and dual inhibition functions assessment on tumor cells and microorganisms

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HIGHLIGHTS

- CLn polymer dressings impregnated with silver nano-crystals was fabricated.
- CLn/Ag dressings inhibited the viability of tumor cells and bacterial.
- The biological activity of CLn/Ag dressings was surface feature-dependent.
- CLn/Ag dressings hold promise to inhibit hamartoplasia and bacterial infection.

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ABSTRACT

In this study, a combination route of the solvent volatilization-in situ cross-linking method and ascorbic acid in situ reduction method was developed to fabricate conjugated linolenic acid polymer dressings impregnated silver nano-crystals (CLn/Ag dressings). Scanning electron microscopy, high-resolution transmission electron microscopy, X-ray powder diffraction, Energy Dispersive X-ray and atomic absorption spectroscopy provided a qualitative and quantitative support to the formation of silver nano-crystals on the hill-structured conjugated linolenic acid polymer dressings. The size of hill-shaped structure and silver nano-crystals, and the loading efficiency of silver could be controlled by adjusting the ratio of conjugated linolenic acid to silver source. The cells viability analysis and morphological evidences displayed that the inhibition of CLn/Ag dressings on PC 12 tumor cells were closely related with the conjugated linolenic acid polymers rather than silver nano-crystals. The highest inhibition ratio got to 82.3 %. The antibacterial activity towards *Bacillus subtilis* and *Staphyloccocus aureus* was investigated for different CLn/Ag dressings by using disc diffusion method. The results revealed that the dressings had significant bactericidal effect on both bacteria in a silver loading efficiency-dependent mode. This research supports the potential value of CLn/Ag dressings as coating for tissue engineering substitutes to simultaneously inhibit hamartoplasia and bacterial infection during operation.

1. Introduction

Tissue engineering substitutes are being accepted by more and more people due to their magical and promising effects at improving human life, e.g. applications of the replacement or repair for decaying teeth, burned skin, blocked blood vessels [1]. However, it does not mean that we could neglect the complications arising from the use of currently available tissue-engineered replacement grafts, such as tissue hyperplasia, neoplastic formation and infections [2–7]. With the development of biomaterials and interface biology, all these pressing problems point to the physical and chemical control on the surface/interface of grafts [8,9]. Furthermore, surface modification is becoming a very important aspect that brings tissue engineering technology to clinical fruition [10,11].

Of the available surface modification techniques, antibacterial coatings with silver have attracted much attention due to their

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outstanding and dual effects [4,12–16]. On the one hand, coatings could endow their excellent physical, chemical or biological properties to tissue engineering substitutes. In this way, coatings will guide tissue engineering substitutes to generate a well interaction with the surrounded cells or tissue [17]. On the other hand, silver is famous for its killing ability to bacteria. The existence of silver in coatings will make substitutes avoid microbial infections after the implantation *in vivo* [6,18]. For example, Prabhakar et al. improved the cyto-compatibility of medical grade polyurethane *in vitro* and reduced the inflammatory response *in vivo* by the coating treatment with polyaniline-silver nano-particle composite [19]. Besides, the coated polyurethane also showed high inhibition activity on the attachment of *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Bacillus subtilis* (*B. subtilis*).

Conjugated polyunsaturated fatty acids are currently a focus of attention, linked to human health. Moreover, they have exhibited great potential as fluorescent membrane probes in photophysical and biophysical studies [20–24]. Previously, we fabricated a series of parinaric acid methyl ester polymer (PnA-Me) films and firstly demonstrated their application value as coating materials in the field of tissue engineering [25]. The experimental evidences displayed the selective sensitivity of PnA-Me films to primary and tumorous cells. In the present study, we constructed a combination route of the solvent volatilization-in situ cross-linking method and ascorbic acid in situ reduction method to fabricate a series of CLn polymer dressings impregnated silver nano-crystals (CLn/Ag dressings). Their anti-proliferation effects on tumor cells were assessed by the morphological observation and cells viability analysis. The bacterial killing efficiencies of CLn/Ag dressings were investigated against *Bacillus subtilis* (*B. subtilis*) and *Staphyloccocus aureus* (*S. aureus*). All the experimental data support the conclusion that CLn/Ag dressings have the great potential to act as coating materials simultaneously reducing the growth of tumor cells and microbial contamination from implants.

2. Experimental section

2.1. Materials

Pomegranate seeds were obtained from a local seed company (Sanhe-Tian, Xinxiang, Henan, China). A natural CLn isomer, punicic acid (c9, t11, t13-18:3; purity $\geqslant$90%), was extracted in hexane, concentrated in a rotary evaporator at 30 °C (RE-52 rotary evaporator, Shanghai, China) and purified according to our previous work [26]. Hexane was HPLC grade. All other solvents and chemicals were analytical grade without further purification.

2.2. Preparation of CLn polymer dressings impregnated with silver nano-crystals

2.2.1. Preparation of CLn polymer dressings

The CLn polymer dressings were firstly fabricated by the solvent volatilization-in situ cross-linking method according to our previous work [25]. Briefly, the CLn monomer was dissolved in ethanol with the final concentration of 0.02, 0.2 and 2 mg/mL. Then 40 μL of CLn monomer alcoholic solution was dropped onto the glass surface with the diameter of 9 mm and dried through volatilization for 1 h at room temperature.

2.2.2. In situ synthesis of silver nano-crystals on polymer dressings

To prepare CLn/Ag dressings, the CLn polymer dressings were immersed into 0.34, 3.4 and 34 mg/mL of $AgNO_3$ aqueous solution for 1 h at room temperature, respectively. Subsequently, the samples were rinsed completely with deionized water. 6 mg/mL ascorbic acid aqueous solution were then applied to immerse the samples and allowed to reduce the silver ions at room temperature for 1 h. After washing with deionized water for three times, the obtained products were dried at 37 °C for 24 h.

2.3. Characteristics of CLn/Ag dressings

2.3.1. Fourier transform infrared (FTIR) spectra

The products were scraped off, mixed with KBr powder, and then pressed into pellets directly for FTIR determination on a Bio-Rad FTS-40 FTIR spectrograph in the wavenumber range of 4000–400 cm^{-1}. Also, the spectra were collected at 2 cm^{-1} resolution with 128 scans.

2.3.2. Morphology observation

The samples were vacuum-coated with a gold layer and then their surface morphologies were observed by a scanning electron microscope (SEM, JSM6390LV, JEOL, Tokyo, Japan). CLn/Ag dressings were also scraped off and high-resolution transmission electron microscopy (HRTEM) observation was carried out in a JEM-2010 electron microscope working at 200 kV for measuring the morphology and microstructure of silver nano-crystals.

2.3.3. Crystal structure analysis

The samples on the glass were scraped off and X-ray powder diffraction (XRD) measurements were performed on a Bruker D&Advance X-ray powder diffractometer with graphite monochromatized Cu Kα (λ = 0.15406 nm). A scanning rate of 0.05 deg/s was applied to record the pattern in the 2θ range of 10–90°.

2.3.4. Silver loading efficiency on CLn polymer dressings

The atomic absorption spectroscopy (AAS) was performed on atomic absorption spectrometer (180-50, Japan) to measure the content of silver in the polymer dressings. The sample was dissolved into nitric acid for 30 min. Then the concentration of the silver ion in HNO_3 solution was measured with AAS.

2.4. Responses of PC 12 cells to CLn/Ag dressings

2.4.1. Cell viability assay

PC12 cells were routinely cultured in tissue culture flasks with a high-glucose Dulbecco's Modified Eagle's medium, containing 10% total bovine serum and incubated at 37 °C in a humidified atmosphere with 95% air and 5% CO_2. When the cells became almost confluent, they were detached by treatment with 0.25% trypsin for 2–3 min at 37 °C. Before cell seeding, the CLn/Ag dressings were placed in a 48-well culture plate, sterilized by UV for 20 min, and then equilibrated with a prewarmed medium for 2 h. After removal of the medium from the wells by pipetting, the cells were counted to 10^4 cells/mL and 500 μL of the cell suspension was poured onto each substrate. 24 h later, the wells were carefully washed with PBS. A total of 20 μL of MTT was added in 180 μL of a culture medium, and cell culture was continued for another 4 h. Then the solution was removed and the wells were washed twice with PBS. A total of 200 μL of dimethyl sulfoxide was pipetted into the wells and OD 490 nm values were read on a microplate reater (Multiskan MK3; Thermo Labsystems, Hudson, NH). The inhibition rate of the CLn/Ag dressings on tumor cells was calculated as follows:

$$\text{Inhibition rate} = (\text{ODc} - \text{ODt})/\text{ODc} \times 100\%$$

where ODc and ODt are the OD 490 nm values of control group and the sample group, respectively.

2.4.2. Cell proliferation and distribution

After cell seeding for 24 h on CLn/Ag dressings, all samples were rinsed in PBS and the cells attached on the surfaces were stained with acridine orange florescent dye in PBS for 5 min. Then the cells