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Collagen-based silver nanoparticles: Study on cell viability, skin permeation, and swelling inhibition

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ABSTRACT

Collagen is considered the most abundant protein in the animal kingdom, comprising 30% of the total amount of proteins and 6% of the human body by weight. Studies that examine the interaction between silver nanoparticles and proteins have been highlighted in the literature in order to understand the stability of the nanoparticle system, the effects observed in biological systems, and the appearance of new chemical pharmaceutical products. The objective of this study was to analyze the behavior of silver nanoparticles stabilized with collagen (AgNPcol) and to check the skin permeation capacity and action in paw edema induced by carrageenan. AgNPcol synthesis was carried out using solutions of reducing agent sodium borohydride ($NaBH_4$), silver nitrate ($AgNO_3$) and collagen. Characterization was done by using dynamic light scattering (DLS) and X-ray diffraction (XRD) and AFM. Cellular viability testing was performed by using flow cytometry in human melanoma cancer (MV3) and murine fibroblast (L929) cells. The skin permeation study was conducted using a Franz diffusion cell, and the efficiency of AgNPcol against the formation of paw edema in mice was evaluated. The hydrodynamic diameter and zeta potential of AgNPcol were 140.7 ± 7.8 nm and 20.1 ± 0.7 mV, respectively. AgNPcol failed to induce early apoptosis, late apoptosis, and necrosis in L929 cells; however, it exhibited enhanced toxicity in cancer cells (MV3) compared to normal cells (L929). AgNPcol demonstrated increased toxicological effects in cancer MV3 cells, promoting skin permeation, and preventing paw edema.

1. Introduction

Collagen is a fibrous protein found in skin, tendons, bones, teeth, blood vessels, intestines, and cartilage, comprising 30% of the total amount of proteins and 6% of the human body by weight [1]. Thus, it is considered the most abundant protein in the animal kingdom [2,3] and has various functions in the body that range from supporting organs and tissues to storing energy [4].

There is growing interest in the use of collagen owing to its diverse properties. Collagen has low allergenicity, low antigenicity, and high biocompatibility [5]. It is bioabsorbable, hemostatic [6,7], biodegradable, non-toxic [3], synergistic with bioactive components, and compatible with natural and synthetic polymers [6]. Additionally, it possesses high tensile strength and exhibits high affinity for water [6,8].

Until the 1980s, the importance of collagen as a biomaterial was restricted to the production of surgical sutures. Currently, its applications range from coating large caliber vascular prosthesis to supporting the orientation of growing nerve cells. The increase in the application of collagen as a biomaterial is due to its natural abundance [4] and the diversity of ways by which it can be molded [6,7].

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In the 1990s, up to 26 types of collagen were identified owing to the complexity and diversity in structure, additional presence of non-helical domains, structure of assembly, and function of collagen. Collagen is divided into fibril-forming collagen (types I, II, III, V, and XI), collagen associated with fibrils (types IX, XII, XIV, XVI, XIX, and XX), and other types of collagen (types IV, VI, VII, VIII, and X). The most abundant are the fibril-forming collagen, comprising 90% of collagen present in living and are able to form structures with highly organized fibers [9].

Type I collagen is the most studied and most abundant type of collagen. It corresponds to >90% of the organic bone mass and is found in tendons, skin, ligaments, and cornea. Type I collagen is the major component that provides rigidity to surrounding structures [9]. All of these features suggest that type I collagen is a good candidate in studies associated with other materials.

In general, studies involving the different types of collagen are extremely important because the differences between their structures and properties may be beneficial in therapeutic applications such as drug delivery systems, growth factor and cell formation anchoring systems, and tissue repair [10].

The interest in conducting research using proteins and silver nanoparticles (AgNPs) extends beyond the field of health and biotechnology. Data on the antimicrobial activity, biocompatibility, and adsorption of AgNPs have been shown in the literature [11–13]. Studies involving medical devices for drug delivery and the interactions between AgNPs and proteins in order to modify or discontinue cellular activities are of paramount importance to the scientific community with applications of interest to the general population [13–15]. Studying the interaction of AgNPs with proteins can be valuable for understanding the stability of nanoparticle systems, the effects of discovery in biological systems, and the development of new pharmaceutical products [16,17].

Alarcon et al. [18] and Cardoso et al. [19] used collagen type I, to stabilize the AgNPs starting from a silver nitrate solution. The authors conducted a study to test the biocompatibility and the antibacterial properties of AgNPs stabilized with collagen. This study was performed biological tests with AgNPcol checking the skin permeation; action against inflammation and flow cytometry. The aim of the current study was to analyze the behavior of the stabilized AgNPs with collagen by assessing cell viability using flow cytometry, permeation front (*in vitro*), and paw edema induced by carrageenan.

2. Materials and methods

2.1. Synthesis of collagen-based silver nanoparticles (AgNPcol)

A solution of silver nitrate ($AgNO_3$) at a concentration of 108 μg Ag/ml, a solution of collagen type I from rat tail (Santa Cruz Biotechnology, Dallas, TX, USA) at a concentration of 0.1 mg/ml, and a solution of sodium borohydride ($NaBH_4$) at a concentration of 3.78 mg/ml were used to carry out the synthesis of nanoparticles. The collagen was prepared using acetic acid (10 mg/ml) and $AgNO_3$ and $NaBH_4$ were prepared using ultrapure water at 4 °C. The $AgNO_3$ solution was added to an equal volume of collagen, and the mixture was agitated and homogenized for 10 min. The $NaBH_4$ solution was added later and the solution was homogenized for 10 min. Subsequently, the reaction mixture was centrifuged at 3600 rpm for 15 min (temperature 25 °C) and finally separated from the supernatants of the final solution present in the container [19].

2.2. Structural characterization of AgNPcol

Powder X-ray diffraction (XRD) was carried out on dried samples in order to determine the phase, percentage of crystallinity, and crystallite size of the silver present in the nanoparticles. The experiment was performed with a diffractometer (Rigaku Ultima IV, Spring, TX, USA) using CuKα monochromatic radiation in step-scanning mode (0.02° step) and range of 8–60° with an exposure time of 5 s/step. Further investigations were conducted applying the Rietveld refinement, aiming to fit the starting model of the phases, found by searching the previous phase, to the entire powder pattern obtained from the experiment, using the software MAUD [20,21]. The structures were designed using HyperChem and geometrically optimized with Model Building (HyperChemm TM, Professional 8.0.6, Hypercube Inc., Gainesville, FL, USA). Dynamic light scattering (DLS) and zeta potential measurements were conducted on a Zetasizer Nano-ZS (Malvern Instruments, UK) using an acid solution of AgNPcol.

2.3. Morphological characterization of AgNPcol

Atomic force microscopy (AFM) analysis was conducted using a TT-AFM instrument (AFM Workshop, USA) in vibrating (tapping) mode. Therefore 6 μm area scans were conducted after the deposition of 10 μl of AgNPcol onto clean mica substrate and the sample was dried at room temperature. Once dry representative images were performed using ACT-20 cantilevers (AppNano - USA) with a resonant frequency of approximately 319 kHz. Images were analyzed using Gwyddion software 2.40.

2.4. Cell culture

Chemicals and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Penicillin/streptomycin, fetal bovine serum (FBS), and Dulbecco's modified Eagle medium (DMEM) were purchased from Vitrocell (Campinas, SP, Brazil). Sterile pipettes, plates, and flasks for cell culture were purchased from TPP® (Switzerland). All experiments were done in a clean and sterile atmosphere to eliminate the probability of contamination with endotoxin [22].

The human melanoma cancer (MV3) and murine fibroblast (L929) cell lines were purchased from the cell bank at the Federal University of Rio de Janeiro. Cells were maintained at 37 °C in a 5% CO_2/air incubator and cultured in 75-cm^2 flasks in DMEM supplemented with 10% (v/v) FBS, penicillin/streptomycin (50 IU/ml and 50 μg/ml, respectively), and 2 mM L-glutamine. The cells were grown to confluence, which was verified by observation under an inverted microscope (Nikon Eclipse Ti®, Japan). The cell viability after exposure to AgNPs was determined using the trypan blue exclusion assay immediately before *in vitro* assays (data not shown) to verify that the viability was higher than 98%.

2.5. Apoptosis and necrosis assays by flow cytometry

Apoptosis and necrosis were analyzed using fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) staining using a kit (annexin V-FITC apoptosis detection kit, BD®, USA) and flow cytometry. Cells were plated in 6-well culture plates for 24 h to attach. They were then treated with AgNPcol at 4.35, 2.17, 1.08, 0.5, and 0.25 μg Ag/ml, collagen alone at 6.25 μg/ml, or $AgNO_3$ alone at 3.37 μg Ag/ml and 6.25 μg Ag/ml for 24 h at 37 °C in 5% CO_2 before the assays. The untreated cells were considered as negative controls. The annexin V-FITC assay was used to differentiate apoptosis from necrosis induced by AgNPcol after incubation for 24 h. After that, both cells types were harvested, washed twice in cold PBS, and further ressuspended in binding buffer. Next, FITC-labeled annexin V was added and incubated for 15 min in the dark at 25 °C. PI was added and incubated for 5 min in the dark. Cells were immediately analyzed by flow cytometry. Ten thousand cells were analyzed as percentages and the data stored. The excitation wavelength was set at 488 nm. Flow cytometry analysis was performed in a FACSCalibur® (Becton Dickinson, CA, USA) with the BD CellQuest 3.3 software.