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DEVELOPMENT AND CHARACTERIZATION OF BIOMATERIALS FOR BIOMIMETIC TISSUE APPLICATIONS

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ABSTRACT

To create artificial tissues, a matrix is needed as structural support. It must be biodegradable, and it must cause the minimal inflammation response in the human body. However, it is important to consider the cost, which usually is very high, and the materials used: their origin and properties.
In this work, animal waste and plant extracts were used to generate a biomaterial that is part of a multiphase system from type I collagen as a temporary support, taken from rat tails; which can also be used as a nanoparticles distributor. Those nanoparticles were taken from a plant, called Tinospora cordifolia, and also from a waxy resin obtained from bee hives, called propolis, both used as regenerative agents.

It was necessary to prepare and characterize the collagen and nanoparticles using Polyacrylamide gel electrophoresis (SDS- Page), Amplitud Modulated Atomic Force Microscopy (AFM) and Thermogravimetric Analysis (TGA). Skin cells behavior with the nanoparticles was evaluated too.
Collagen obtained has a correct structural conformation, with a 65nm D-periodic and repeated sequences of triplets Gly-Pro-Pro (GPP) and a denaturalization temperature from 75 to 85ºC. Nanoparticles from natural extracts have spherical shapes, in sizes of 13.8 nm in average from tinospora and 15 nm from propolis. They produce positive effects on the regeneration of the cells in study.

The results show that it is possible to produce a biopolymeric prototype that induce cell self-regeneration from natural materials, as a useful solution in the treatment of skin diseases.

Keywords: collagen, propolis, tinospora, nanoparticles, biomaterials, biomimetic tissue.

Introduction

Sustain, restore and enhance tissue and organ functions by delivering the necessary cells and biological environments to regenerate the diseased parts, is one of the main objectives of the tissue engineering [1]. This multidisciplinary field is very recent and since it was first developed, a wide range of biomaterial scaffolds have been fabricated for tissue engineering applications [2]. Biomaterial is a systemically, pharmacologically inert substance designed for implantation within or incorporation with living systems [3] which is able to perform, replace, or enhance a natural function without giving rise to any undesirable toxic reactions to the surrounding tissues/bones [4] and scaffolds are implants or injects, which are used to deliver cells, drugs, and genes into the body [5]. Thus, various factors need to be taken into consideration to select an ideal scaffold, including chemical composition of material, its porous structural design, mechanical as well as degradation properties [4] and water adsorption, considering that water is important for maintaining the resiliency and lubrication of joint in cartilage [2].

Some of the main natural polymers that have been investigated for creating scaffolds or matrices are alginate, proteins, collagens, gelatin, fibrins, and albumin [5].

Collagen has a complex hierarchical conformation. Cell adhesive ligands such as Arginine-Glycine-Aspartic acid (RGD) aminoacid triplets are abundant in this protein and play vital roles for cellular attachment [6]. These, as well as other physical and chemical characteristics, make collagen one of the favorite biomaterials for the development of matrices and motivates the advance of this work that takes those properties, to conform a scaffold which can also be used as a nanoparticles distributor. Nanoparticles are obtained from natural sources: Tinospora and propolis.

Tinospora cordifolia, also commonly known as guduchi, is an important medicinal plant distributed throughout the tropical Indian subcontinent and China [7] and it is widely used as anti-bacterial, analgesic, antipyretic and also for the treatment of jaundice, skin diseases, anemia, etc. [8]. Currently, studies have been carried out using tinospore extracts to differentiate and regenerate the cells of the nervous system [9], or using it against tumor cells [10]. However, the use of the nanoparticles of these extracts in the tissue engineering has not yet been reported in the literature, similar to the propolis.

Propolis is a brownish waxy product collected by the honeybee from plant buds, leaves, and exudates. It is known from ancient times, possesses antimicrobial, anti-cancerous [11], antioxidant, anti-inflammatory, anaesthetic, hepato-protective, immunostimulating and cytostatic properties [12]. It is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris, but it is important to consider that this chemical composition and properties may vary depending of the geographical origin [13] and collection season [14]. In Costa Rica this natural compound is not commercialized, it is not edible, and results have not yet been reported that involve this residue with cellular regeneration. The present investigation is aimed at the obtaining and characterization of nanoparticles of tinospora and propolis, and its evaluation of cytotoxicological properties, to consider its use in natural matrices based on type I collagen, obtaining a viable and lower cost option of scaffold based on biomaterials which stimulate cell proliferation, with applications in the treatment of various dermal pathologies.

Materials and Methods

Collagen type I extraction.

Tendons were extracted from Wistar Hannover male specimens, provided by the Laboratorio de Ensayos Biológicos (LEBI) of the Universidad de Costa Rica (UCR). Approximately 1 g of tendon was solubilized on 200 ml of 3% acetic acid solution with agitation, for 24 h at 4 ºC. The solution was filtered at room temperature with gauzes and centrifuged at 4500 rpm for 30 min. The supernatant was lyophilized at 1.3 mbar, -20 ºC, for 240 h.

Collagen type I gelation.

Lyophilized collagen was dissolved in 3% v/v acetic acid solution while bringing the pH 7.47 by adding dropwise a 1 N solution of sodium hydroxide (NaOH). The solution was incubated overnight at 4 °C for gelation. The gel was centrifuged and washed three times with Type I ultrapure water and was dialyzed with a Spectra/Por 3 membrane (32 mm diameter, 3.2 ml/cm volume) shaking overnight at 4 °C during 4 d. Water was changed every 2 h. For non-dialyzed collagen, these steps were omitted. Whenever dry films of collagen were needed, the gel was dried on an oven at 45 °C during 4 d.

Tinospora nanoparticles extraction.

The stems of the plant from Varanasi, India, were sprayed and the product was added in ethanol, in agitation. Filtration (medium porosity) was performed, and a sample of this alcoholic solution was placed in the oven at 45 °C for 16 hours, obtaining the dried extract. Ultra-purified water was added to that extract. The resulting aqueous suspension was sonicated according to the conditions: power: 4W, time: 5 minutes, amplitude: 40%, probe diameter: 6mm.

Subsequently, three concentrations of the aqueous solution of tnospora nanoparticles of 10 µg/ml, 100 µg/ml and 1000 µg/ml were obtained to perform different tests.

Propolis nanoparticles extraction.

The pure propolis extract was diluted in ethanol under stirring, prior to filtration. A sample of this alcoholic solution was dripped in ultra-purified cold water with stirring during 30 min. To evaporate the ethanol the sample was placed in the oven at 45 °C. The resulting aqueous suspension was cold sonicated according to the conditions: power: 4W, time: 5 min, amplitude: 40%, probe diameter: 6 mm and then it is filtered, pore size 5 µm. Subsequently, three concentrations of the aqueous solution of propolis nanoparticles of 10 µg/ml, 100 µg/ml and 1000 µg/ml were obtained to perform different tests.

Collagen and nanoparticles characterization.

Polyacrylamide gel electrophoresis (SDS- Page). The electrophoresis assay was performed according to the methods of Laemmli [15], using 4% gel stacking and 7.5% gel resolution. Samples were dissolved in 0.5 M Tris-HCl buffer containing 25% (v / v) glycerol, 2% (w / v) SDS, 5% (v / v) β-mercaptoethanol and 0.1% (w / v) bromophenol blue. Thermoscientific molecular weight markers, 100V, were used for 2 hours. After electrophoresis, the gels were visualized with Coomassie-Brilliant Blue R-250.

Amplitude modulated atomic force microscopy (AFM). Dry films of collagen were directly deposited on the sample holders. AFM images were obtained with an MFP-3D Classic system (Asylum Research, CA), using tapping mode at room temperature.

Thermogravimetric Analysis (TGA). Thermal stability anal analysis were performed on a TA Instruments USA brand, model Q500 under inert nitrogen atmosphere, flow of 90.0 ml / min, in platinum crucibles, at a heating rate of 10 °C / min and a temperature ramp of 20 to 800 °C. Approximately 5 mg of each sample was used.

Cytotoxicity assay. For the cytotoxic evaluation, 3T3 fibroblast cell line were used, planted in a 96-well plate and growing in an incubator during 24 h, then the samples provided with the Alamar Blue compound were added, which allowed cell viability measurement. Fluorescence readings were obtained with a gain of 70 at the times: 0, 24, 48 and 72 hours with an emission and excitation of 540, 590 nm. At the mean of the data the fluorescence target was subtracted (supernatant with culture medium in the absence of cells).

Results and Discussion

Collagen characterization

To assure the quality of type I collagen according to its chemical composition the SDS-Page analysis is performed. The profile obtained in Figure 1 shows three bands: A band, on the left, indicates the protein markers with its apparent molecular weights (kDa), B band correspond to 10 µg of collagen, C band designates 15 µg of collagen and D band correspond to 20 µg collagen.

SDS-Page analysis revealed the presence of intense bands according to the molecular weights of these subunits of approximately 140 kDa and 110 kDa. It is also possible to appreciate the presence of

other bands above these subunits, close to 170 kDa. Protein patterns are similar for the different collagen concentrations used.

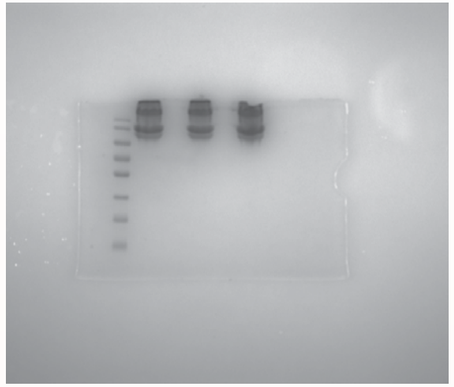

Figure 1. SDS-PAGE profile of collagen isolated from rat tail tendons, Different concentration of collagen was loaded per lane and the A band correspond to protein markers with their apparent molecular weights (kDa) indicated on the left.

The morphology of the self-assembled collagen fibers can be observed in detail, in Figure 2, with the atomic force microscopy (AFM) image.

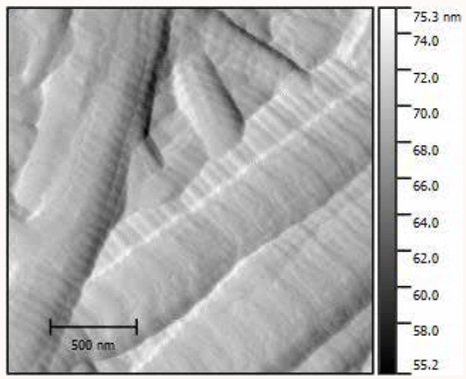

Figure 2. Atomic force microscopy (AFM) self-assembled collagen images in the amplitude mode, 10 µm.

The TGA thermograms of the different collagen samples are shown in Fig. 3. Through this analysis three important thermal processes can be observed for freeze-dried collagen (which has not undergone self-assembly processes), dialyzed (purified) collagen and no dialysis collagen. The first slight weight loss is between 25 and 150 °C, principally visible for the freeze-dried collagen, followed by a weight loss between 250 and 500°C. After reaching the temperature of 500 ° C, a slight weight loss continues, close to 10% by weight for the three samples of collagen.

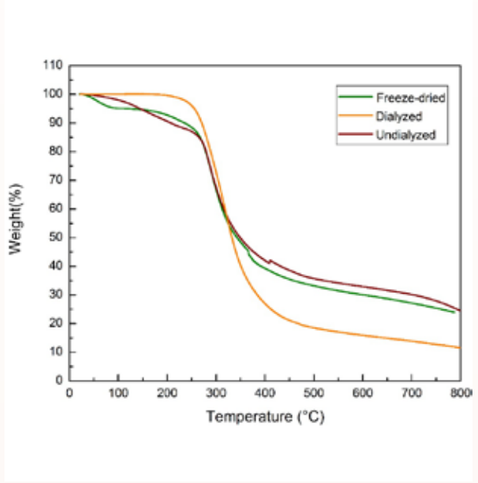

Figure 3. TGA curves of three collagen samples of 5± 0.5 mg: freeze dired, dialyzed and undialyzed, heated at 10°C/min from 25°C to 800°C in a nitrogen atmosphere.

Nanoparticles characterization

The size of the particles and nanoparticles of tinospora can be observed by electron microscopy (AFM) in Fig. 4. Particles from aqueous extract are on the left, with an average size of 42 nm, and nanoparticles after sonicating and filtering on the right, with an average size of 13.8 nm.

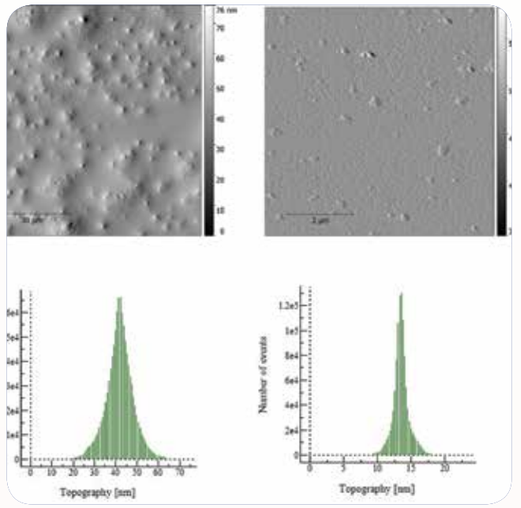

Figure 4. Atomic force microscopy (AFM) tinospora particles images on the left and nanoparticles images on the right, in the amplitude mode, 10 µm; and histogram of the particle size distribution of tinospora.

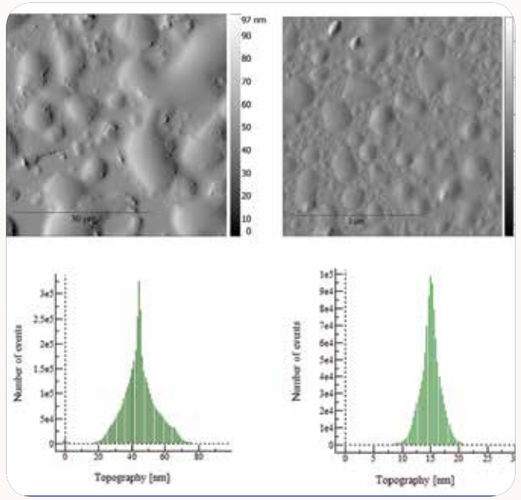

Figure 5. Atomic force microscopy (AFM) propolis particles images on the left and nanoparticles images on the right, in the amplitude mode; and histogram of the particle size distribution of propolis.

Particles and nanoparticles of propolis observed by electron microscopy (AFM) (Fig. 5) showed an average particle size of 45.8 nm and 15 nm respectively. Particles from aqueous extract are on the left and nanoparticles after sonicating and filtering on the right.
Both the tinospora and propolis nanoparticles have a spherical shape.

Cytotoxicity of tinospora and propolis nanoparticles was evaluated using 3T3 fibroblast. Evaluation of tinospora nanoparticles in different solutions (Fig. 6) showed that the nanoparticles at higher concentrations did not show significant increase in cells growth as the lower concentration did. However cell viability increases at all concentrations and times, more than 100%, except for the concentration 100 µg/ml at 72 h. From the 22 h incubation studies, it was observed that the nanoparticles at lower concentration showed the major cell viability.

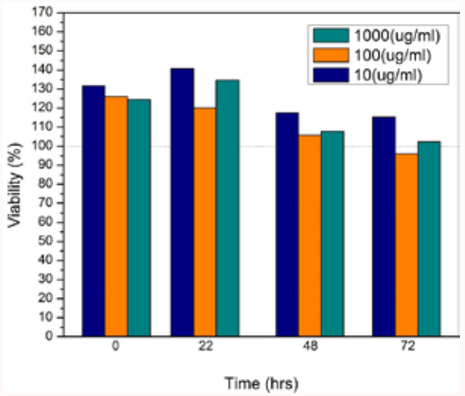

Fig. 6. Tinospora nanoparticles cell viability for three different concentration: 10, 100 and 1000 µg/ml.

In propolis nanoparticles cytotoxicity studies, there is a tendency of increase in cell viability with decreasing concentration, as seen in figure 7. Based on the control 100%, higher values (up to 141%) are obtained near the 22 hours at lowest concentration (10 µg/ml). All concentrations used for all measured times showed a viability greater than 100%.

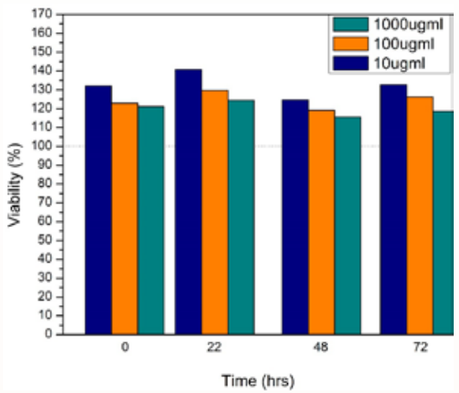

Fig. 7. Popolis nanoparticles cell viability for three different concentration: 10, 100 and 1000 µg/ml.

From the electrophoretic profiles obtained it was observed that the extracted type I collagen molecule consists mainly of two distinct subunits with molecular weights of approximately 140 kDa and 110 kDa, according to the protein marker used, which can be assigned respectively as the collagen chains α1 (I) and α2 (I), according to previous studies, and which show a polypeptide composition of this type, quite common of the type I collagen molecule between different tissues, mainly mammals [16,17]. Some α-chains were found to remain in cross-linked forms, resulting in appreciable levels of β chains (α-chain dimers) and small amounts of Y chains (α-chain trimer), also found in other type I collagen extracts [18], even in some extracted from fish skins [19]. It is observed that in all the columns, the contents of the chains are very similar to each other. It seems that the difference in the amounts of added collagen is not enough to cause noticeable changes in the intensities of the bands corresponding to the chains α, β, and Y. These data indicate an adequate collagen type I chemical composition, and the acceptable extraction process is ensured.

Collagen microfibrils form a pattern with a period (D-period) of 65 nm, a value that is within the characteristic values of 64-67 nm [20] and corresponds to the displacement of each molecule in the axial direction, with Relative to the adjacent molecule [21]. It is argued that this variation in periodic spacing is intrinsic to type I collagen, its self-assembly, is a reflection of local changes in mechanical stresses and may be the result of the variant in intrafibrillary interactions including hydrophobic, electrostatic, Hydrogen bonds and crosslinks in hydroxylysines and hydroxyproline [22].
The diameter of each fibril has a measurement of between 450 and 550 nm, which coincides with that reported for tendon fibrils that are up to 1 cm long and 500 nm in diameter [23].

About collagen thermal stability, the first decrease in percentage of significant weight that is presented in TGA is related to a structural water loss, between 25 and 150 °C, mainly for freeze-dried collagen, which is highly hygroscopic [24]. The second weight loss observed in a temperature range that is between 250 and 500 °C is due to the decomposition of collagen molecules and depolymerization [25], once all the water bound to the collagen is released; Then, after reaching the temperature of 500 °C, a slight loss close to 10% by weight for the three collagen samples corresponding to the combustion of the residual organic components continues [16,24].
For the latter degradation, close to the temperature of 600 °C, residues were quantified for lyophilized, dialysed and dialysed collagen of approximately 30.08%, 15.97% and 32.91%, respectively. The mass loss of the collagen without dialysis is lower, which shows a greater thermal stability for it, which is associated to the presence of sodium acetate salt residues in the structure of this collagen, that has not been purified; as opposed to dialyzed collagen, for which a loss of 84.03% of mass is reported, the greater with respect to the other collagens, which implies that there is less organic residues in it, thus evidencing the effectiveness of the process of collagen purification employed.

When analyzing the size and morphology of extracts and nanoparticles of tinospora and propolis, it can be observed that in the aqueous extract of both tinospore and propolis, no particles with well-defined shapes are distinguished, whereas once this solution is subjected to the sonication and filtration process the formation of nanoparticles can be clearly seen, with even more defined spherical geometry.

The average size of the nanoparticles of tinospora is approximately 13.8 nm, according to the histogram with a geometric distribution of the data and the average size of the nanoparticles of propolis is 15 nm. The values obtained for the dimensions of the propolis particles agree with values reported in other studies: approximately 50-170 nm, using methods of suspension of the alcoholic extract on water, similar to the methodology used in this study, to obtain the hydroalcoholic extract [11]. In spite of the good results obtained, it is recommended to perform dynamic light scattering studies to corroborate the size of the hydrodynamic diameter of the nanoparticles.
The effectiveness of the nanoparticle synthesis method (sonication and filtration) is evident when comparing the sizes obtained from the aqueous solutions.

3T3 epithelial cells were used to evaluate cytotoxicity of nanoparticles because they reside in the connective tissue and are able to synthesize fibers and maintain the extracellular matrix of the tissue of many animals [26]. The results suggest that cells metabolic activity increases significantly in the presence of the tinospora and propolis nanoparticles, reinforcing some current studies [8,27]. The lowest concentration for all the times increased the cell viability more than 100% in both, tinospora and propolis.
Data obtained also revealed the potential of tinospora and propolis nanoparticles, as regenerative agents in biomimetic tissues.

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