2021 43rd Annual International Conference of the
IEEE Engineering in Medicine & Biology Society (EMBC)
Oct 31 - Nov 4, 2021. Virtual Conference

Poly (vinyl alcohol)/Silk Fibroin/Ag NPs composite nanofibers for bone tissue engineering

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Abstract— **In this work, electrospinning was used for the preparation of composite nanofibrous scaffold, of polyvinyl alcohol (PVA), silk fibroin (SF) extract of *Bombyx mori* cocoons and silver nanoparticles (Ag NPs), as a substrate for bone tissue engineering. The PVA pristine was prepared at a concentration of 10% wt. The composite nanofibers scaffolds of PVA was prepared with silk fibroin and silver nanoparticles, in relation of PVA: SF (90:10) (v/v) respectively. The formation and presence of AgNPs was confirmed by ultraviolet-visible spectroscopy (Uv-vis). The diameter distribution of the nanofibers was narrow by SEM using Image J software. The chemical composition was determined by FTIR spectra. The wettability was determined using water contact angle. The results showed the average nanofiber diameter of PVA10 pristine was 108.18 nm and to PVA10/SF/Ag NPs was 106.62 nm, no significant changes were noted in the mean diameter, but there were changes in its morphology. The average nanofiber diameter increase with the concentration of PVA at PVA15/SF/Ag NPs was 189.12 nm to PVA18/SF/Ag NPs was 224,23 nm. FTIR spectra indicated characteristic absorption peaks related to the chemical structure of PVA, fibroin and Ag NPs, it demonstrated good interactions between them, caused by strong intermolecular hydrogen bonds. The contact angle of the scaffolds PVA 10%wt decrease with the incorporation of fibroin and show hydrophilic characteristics. The achievements indicate the potential of the nanofibers of PVA15/SF/Ag NPs as a possible substitute for bone tissue engineering.**

Clinical Relevance—**This establishes a possible substrate of PVA/SF/Ag NPs that exhibit desired properties such as porosity and high surface area to volume ratio for bone tissue engineering.**

Keywords: **Silver nanoparticles; Bone tissue engineering; composite nanofibers; Polyvinyl alcohol (PVA); silk fibroin.**

I. Introduction

The bone is a rigid tissue that protects internal organs, supports body weight, supplies blood cells and stores minerals. It is composed of a phase organic of mineralized collagen type I fibrils matrix, which provides flexibility and resilience, and the inorganic phase is composed of the mineral hydroxyapatite (HA) nanocrystals, responsible for the stiffness and strength [1].

The bone has self-renewal and healing capabilities, in normal physiological conditions. However, severe cases of bone defects such as fractures, tumors, and skeletal diseases (e.g., osteoporosis) limit the natural bone self-healing capacity and it is require the use of bone substitutes [1].

Traditional biological substitutes such as autografts, allografts, and xenografts have been used to regenerate bone critical defect problems. So far, autografts are the goal standard as a bone substitute for repairing a critical defect. However, it has several limitations, including the necessity to operate another site, which increase the morbidity and costs associated with surgery [2], whereas allografts and xenografts can present difficulties because of donor scarcity and the risk of infection, immune rejection and disease transfer [2]. Therefore, tissue engineering has emerged as a strategy for bone regeneration using a variety of biomaterials and techniques [2].

Bone tissue engineering (BTE) has been considered as an alternative to regenerate bone critical defects. The goal of BTE is to create 3D bone tissues by combining implanted cells, scaffolds and bioactive molecules such as growth factors [3]. An ideal bone scaffold should be biocompatible, biodegradable, mechanically robust and mimic the extracellular matrix (ECM) to regulate cell adhesion, growth, and proliferation [1].

For BTE has been used different method to produce nanofibers scaffolds as gas foaming, fiber meshes sintering, solvent casting, polymerization in solution, porogen leaching method, freeze-drying, 3D printing and electrospinning. Electrospinning has gained interest due to the morphology of micro/nanofibers mimic the natural extracellular matrix (EMC), as well it has structure with high porosity, high surface area to volume ratio and small interconnect fibrous pore. These features promote the cell migration, adhesion and proliferation to create new bone tissue [4], [5]. In electrospinning, from a polymer solution to which a high voltage is applied to generate aligned or random scaffolds. The solutions can be natural or synthetic polymers [6].

Polyvinyl alcohol (PVA) is FDA approved biomedical synthetic polymer made from the hydrolysis of polyvinyl acetate. It contains a large number of hydroxyl groups. PVA has favorable properties such as non-toxicity, no

*Research supported by Metropolitan Technological Institute (ITM).

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carcinogenicity, biocompatibility, biodegradability, transparency, water-solublility, hydrophilic, good swelling properties in aqueous mediums, suitable chemical, thermal stability, and mechanical strength related to good flexibility [7]. PVA has been used commonly for wound dressings and skin tissue engineering [8], and it has been explored in BTE due to their anabolic effect on bone formation [9]. PVA dissolves easily in water due to numerous polar alcohol groups which form hydrogen bonds [10]. The good electrospinning ability of PVA in aqueous solution, due to their electroconductivity, makes it suitable to obtain continuous and bead-free nanofibers [6], [7]. The main problem with PVA scaffolds is their bioinert nature, which causes poor cell attachment. Another problem is their fast hydrolysis and high degradability, which hinder its application as a pure polymer [4], [6].

Fibroin (SF) is a natural fibrous protein, extracted from the coccon of silkworm. SF has been used to form a variety of biomaterials, such as gels, sponges, membranes and films for biomedical applications [8], [11]. SF has been investigated in drug delivery and tissue engineering as cell culture substrates, because it presents good biocompatibility, biodegradability at a slow rate, microbial resistance, low inflammatory response, good cellular response, good oxygen and water vapor permeability and moisture [1], [4]–[7]. However, fibroin is brittle, so it can be easily broken in the dry state, but combinations with polymers can improve its mechanical properties [8]. Nevertheless, SF nanofibers have low osteoinductivity, therefore a wide range of osteogenic agents have been incorporated in order to improve cell-matrix interactions and osteogenicity [1].

Fibroin has been used in combination with PVA, becasuse it contains amide and hydroxyl groups, which are potentially miscible with PVA through the formation of hydrogen bonds [12]. Fibroin obtained of non-Morera species (*Bombyx mori*) cocoons, such as (*Tussak* or *Samia Cynthia ricini*), is composed of amino acid repeating sequences (Gly-Ala-Gly-Ala-Gly-Ser) responsible for integrin and therefore it can facilitate cell attachment and proliferation, which is a property not owned by PVA [4], [5]. While, PVA could be improve mechanical property of flexibility of SF [13].

So far, fibroin and PVA have been electropun for analyze blend of the solution, using organic solvents such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), trifluoroacetic acid, dichloromethane and formic acid, which can be toxic for cell culture, while the use of water has been proposed as an ideal solvent for biomedical applications, because of avoiding the toxicity [8], [13].

PVA/Fibroin scaffolds has been explored for their antibacterial activity, using silver nanoparticles (Ag NPs), against *E. coli* and *S. aureus*. Ag NPs have a diameter range between 1-100 nm and they have a large relative surface area, thus increasing their contact with various microorganisms, they are even effective at low concentrations [14]. Concentrations >1 of $AgNO_3$ support growth and proliferation of osteoblasts [15], [16]. These nanoparticles can be obtained by the chemical reduction method using the silver nitrate ($AgNO_3$) precursor or by direct synthesis with addition of $AgNO_3$ into the polymer solution prior to electrospinning, to avoid their aggregation. Then, it is usual the post treatments of UV radiation or heat in order to obtain the reduction [17].

Due to the bone exhibit piezoelectric properties are expected that silver nanoparticles immersed in the PVA/ fibroin scaffold promotes osteoinduction of MG63 osteoblasts, when cell culture is stimulated by magnetic field in our future study.

Nevertheless, PVA/fibroin scaffold have been little explored for bone tissue engineering. M. Kalani, et. al. (2019), blended PVA (1 g), *Bombyx mori* fibroin at a concentration of (13%wt), using formic acid as a solvent with Rosuvastatin (RSV) (50 mg) and cultured the scaffold with human adipose-derived stem cells (MG63). This improved cell proliferation and assist osteogenic differentiation [1], T. Kobori et. al. (2007) used PVA at a concentration of (10%wt) and *Antheraea mylitta* fibroin at a concentration of (2 – 4% wt) using water, as solvent. The scaffold was cultured with human osteoblast cells showing their osteoconductivity [18].

In our best knowledge, the solution of *Bombyx mori* fibroin with PVA dissolved in water and silver nanoparticles, has not yet been used for bone tissue engineering yet. In this study, PVA/Fibroin/Ag NPs composite nanofibers are prepared at different solution concentrations by electrospinning process. Ag NPs was characterized by UV-vis. The morphological characteristics, chemical composition and weattability were explored by composite nanofibers, as a possible substrate for future research as a culture MG63 osteoblast cells that promotes adhesion, growth and proliferation that allows bone regeneration.

II. Materials and Methods

A. Materials

Silk cocoons from *Bombyx mori* silkworms were purchased to Corseda (Colombia). Sodium carbonate (Na_2CO_3), cellulose dialysis tube (12 kDa, MWCO), calcium chloride ($CaCl_2$), distillate water, absolute ethanol 99.7% were obtained from Merck. Polyvinyl alcohol (Medium molecular weight Mw=89000 to 98000 Da) 99% hydrolyzed, was provided by Sigma-Aldrich. Silver nitrate ($AgNO_3$) 100% pure, Trisodium citrate dihydrate at 99% purity were obtained from PanReac. All reagents were analytical grade.

B. Fibroin extraction

Bombyx mori silk cocoons were cut into small pieces (5 g) were degummed in 0.5% (w/v) of sodium carbonate (Na_2CO_3) aqueous solution (500 ml) for 1 hour at 100 °C, and then washed with distilled water several times to remove sericin. Fibroin degummed was dried overnight at 37℃, and then dissolved in (calcium chloride/ethanol/water) in a molar ratio of (1:2:8) respectively, at 60 °C for 1 h to simplify the fibroin structure. Fibroin solution was dialyzed with a food cellulose membrane against deionized water for 3 days [19], with water replacement every 2-6 hours to remove salt residue. The

solution was centrifuged 2 times at 3000 rpm for 10 min to remove silk aggregates as well as debris from original silk. Then, fibroin solution was filtered to remove impurities. The fibroin solution was stored at 5 °C prior to use. The final concentration of the fibroin aqueous solution was about 2% wt.

C. Ag NPs synthesis

The silver nanoparticles were synthesized by chemical reduction, making modifications to the protocol implemented by Cuervo-Osorio et. al. 2020 [20]. Distilled water was heated to a temperature of 100 ° C and then 0.1 M $AgNO_3$ was added as a precursor agent and stirred for 10 minutes. Then, 1% wt trisodium citrate dihydrate was slowly dropped as reducing agent, maintaining stirring until the solution turned yellow and allowed to cool to room temperature. PVA at 1% wt was used as dispersing agent for Ag NPs, in a 1: 1 ratio (PVA / Ag NPs) for 30 minutes with constant stirring.

D. Preparation of electrospinning solutions

PVA pristine solution was prepared at 10% wt concentration as reported by P. Bhattacharjee et. al. 2015 [18]. PVA/SF/Ag NPs solutions were prepared with distilled water as a solvent, in concentration of PVA/SF (90:10) (v/v) at a concentration of PVA (10%wt, 15%wt, 18% wt), all samples with fibroin (2%wt) and silver nanoparticles (0.5%wt) in which it complies with its antibacterial characteristics, without compromising cell behaviour [15], [16]. They were mixed together and stirred for 1 h at 80°C, the samples were labelled as PVA10/SF/Ag NPs, PVA15/SF/Ag NPs and PVA18/SF/Ag NPs, as shown in TABLE *1*.

TABLE 1. ELECTROSPINNING SOLUTIONS

Solution	PVA	Fibroin	Ag NPs
PVA Pristine	10%wt	--	--
PVA10/SF/Ag NPs	10%wt	2% wt	0.5% wt
PVA15/SF/Ag NPs	15%wt	2% wt	0.5% wt
PVA18/SF/Ag NPs	18%wt	2% wt	0.5% wt

E. Preparation of nanofiber scaffolds

The electrospinning process was performed on a FLUIDNATEKTM LE 100 equipment at room temperature and relative humidity of 42%. The solutions were loaded in a 5 ml plastic syringe, the distance between injector to the collector was 20 cm, the voltage was 25 kV and the flow rate was 0.3 ml/h. It was used a 15 x 15 cm^2 aluminium foil as a collector. The PVA pristine and PVA/SF/Ag NPs nanofiber scaffolds were vacuum dried in an oven for 48 h.

F. Characterization

UV-vis spectrometry

The ultra-violet (UV)-visible spectra of the Ag NPs solution was obtained with 3200 PC MAPADA UV-vis spectrometer in absorbance mode over the range of 300-600 nm.

Morphology of Electrospun Fibers (FE-SEM)

Morphological analyzes were performed in a SEM field emission (FE-SEM) JEOL JSM-7100F. The samples of PVA pristine and PVA/SF/Ag NPs were coated with gold using a sputtering Quorum Q300T for 10 min at 10 mA to supply electrical conductivity. Magnification of 10000x, was performed. The accelerating voltage was 10 kV and the average nanofiber diameter was calculated by measurement of 100 random fibers was analyzed using the software Image J for each nanofibrous scaffolds.

Chemical Interaction (FTIR)

The chemical interaction of the PVA/SF composite nanofibrous scaffolds was determined by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectrophotometer (Shimadzu IR trace-100 model). The ATR-FTIR spectrum of each scaffold was detected in the 4000 to 500 cm^{-1} wavenumber with 32 scans at a resolution of 4 cm^{-1} in transmittance mode, under ambient conditions.

Wettability

The wettability or contact angle measurements were used to analyze the hydrophilicity of the scaffolds. The four kinds of scaffolds were measured using a contact angle goniometer (SCA 20). Briefly, 20 µl of deionized water was dropped onto the surface of each scaffold. Then, the images of the droplets were captured using a camera. Data were taken in triplicate for each scaffold. The contact angle instrument and the surface of scaffolds were calculated based on the recorded images of the water droplets.

III. RESULTS AND DISCUSSION

A. UV-vis spectrometry

The peak of the UV-vis absorption spectrum was located in the surface plasmon resonance absorption band (SPR) in the range of 325 to 550 nm. With a maximum absorption band peak at approximately 435 nm (Figure 1), indicating the formation of small diameter Ag NPs. These results are similar to those obtained by Lee et. al. 2014 [21], Bhui et. al. 2009 [22] and Patil et. al. 2019 [15].

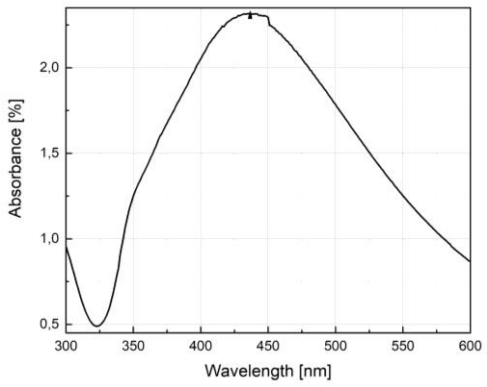

Figure 1. UV-vis of Ag NPs

B. Morphology of the electrospun nanofibers

It can be seen in several research that the solvents used for fibroin have been distilled water and formic acid. Some researchers who lyophilize fibroin then dissolve it in formic acid. Fibroin dissolved in water was used in this research, as several studies suggest that this improves cell adhesion and avoid the toxicity, a desirable property for bone tissue engineering. In this work, randomly oriented, continuous PVA pristine and PVA/SF/Ag NPs, composite nanofibers were produced with a smooth and uniform morphology under controlled parameters. The average nanofiber diameter of

PVA10 pristine was 108.18 nm (Figure 2. A), and the diameter of PVA10/SF/Ag NPs was 106.62 nm (Figure 2. B) no significant changes were noted in the mean diameter, but there were changes in its morphology with the addition of fibroin causing the bead formation, due to, it reduces the viscosity of the solution. The increase in solution concentration of PVA had influence in the average nanofiber diameter for PVA15/SF/Ag NPs at 189.12 nm (Figure 2. C) to PVA18/SF/Ag NPs at 224.23 nm (Figure 2. D), nevertheless the PVA concentration at 18% produced needle plugging, nevertheless, there was less formation of bead defects. The control of the flow rate at 0.3 ml/h, allowed evaporation of water solvent during spinning and drying of the fibers in the collector. PVA15/SF/Ag NPs exhibit suitable porosity and continues fibers that could supports osteoblast growing. The Figure 2 presents the morphology of different electrospun nanofibers mats.

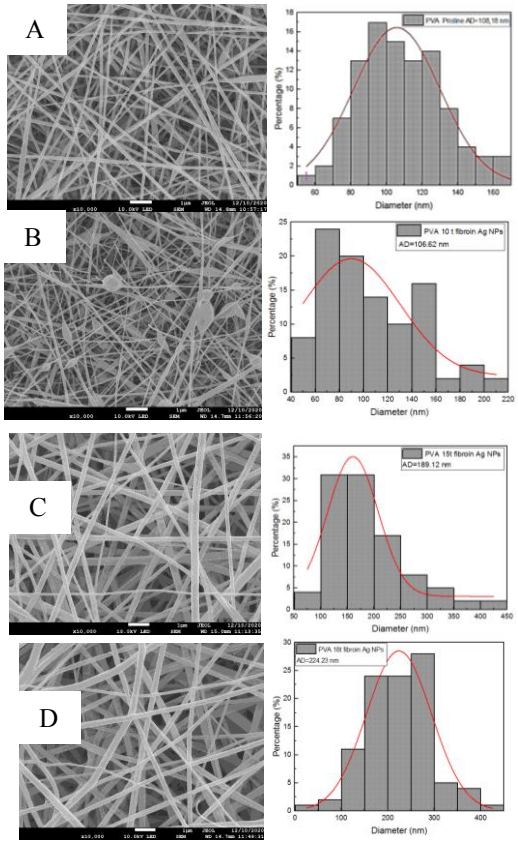

Figure 2. SEM images and histograms of the (A) PVA Pristine, (B) PVA10/SF/Ag NPs, (C) PVA15/SF/Ag NPs (D) PVA18/SF/Ag NPs.

C. *FTIR spectroscopy*

The FTIR spectroscopy analysis, in mode ATR, confirmed blending of PVA, fibroin and Ag NPs of nanofibers scaffolds, it demonstrated good interactions between them, caused by strong intermolecular hydrogen bonds. As can be seen in the Figure 3, PVA pristine, the characteristic peaks were observed at 3500-3000 cm^{-1} (OH stretches from the intermolecular and intramolecular hydrogen bonds), 2934 cm^{-1} (C-H stretching), 1450 cm^{-1} (C- H bending) and 1085 cm^{-1} (C-O-C stretching). There is an increase at 2360 cm^{-1} peak with the increase of PVA concentration, due to the fibroin and Ag NPs concentrations were maintained constant. Fibroin, presented the characteristic peaks of silk I α helix amorphous conformation at 1650 cm^{-1} (amide I C=O stretching). Some research cross-linked fibroin with glutaraldehyde or methanol to obtain β sheets (Silk II), in order to improve more stabilized nanofibers in hydrolytic and thermal properties, but there is a risk of cytotoxicity, in future studies. It is recommended to take these procedures into account to improve water stability, but using the minimum amounts. In relation to silver nanoparticles, the peak at 1277 cm^{-1} related to silver nitrate (NO_3-) is not observed, which indicates that pure nanoparticles were obtained and their reduction was achieved, this corresponds to what was reported by Patil et. al. 2019 [15].

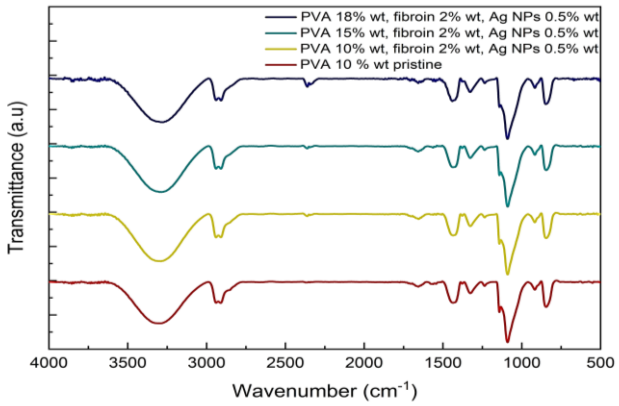

Figure 3. ATR-FTIR spectra of PVA/Fib/Ag NPs composite nanofiber.

D. *Wettability*

PVA and fibroin are hydrophilic in nature. In the range of 40-70°, it has been reported that optimal water contact angle (WCA) needed for cell attachment and proliferation, hydrophilic surfaces, can absorb more serum and growth factors from the medium, which contribute to improving the cell culture of osteoblasts [23]. Bhattacharjee et. al. 2015, reported that contact angles of the nanofibers scaffolds of PVA/SF decreased with increased silk fibroin concentration, increasing the hydrophilicity [18]. While others reported, that the heavy chains in *Bombyx mori* SF structure consist of repetitive hydrophobic domains of amino acids, which are assembled into nano-crystals known as β-sheet, which work as a hydrophobic domain in SF and showed increases in the water contact angle (WCA) with increased SF in PVA/SF scaffolds [1], [4], [6]. In this study, the contact angles of PVA pristine nanofibers scaffolds were 42.5°, we can analyze that the WCA of PVA nanofibers decrease with the incorporation of fibroin and silver nanoparticles to PVA10/SF/Ag NPs at 31.1°, PVA15/SF/Ag NPs at 32°, PVA18/SF/Ag NPs at 31°. The PVA concentration have not influence in the hydrophilic characteristics as shown in the Figure 4.

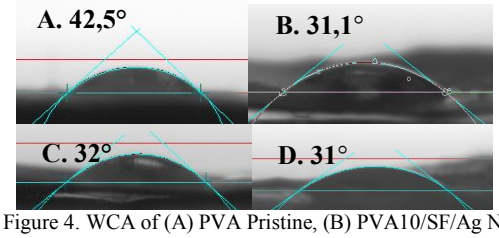

Figure 4. WCA of (A) PVA Pristine, (B) PVA10/SF/Ag NPs, (C) PVA15/SF/Ag NPs (D) PVA18/SF/Ag NPs.

IV. Conclusion

In this study, the blend solutions of PVA, *Bombyx mori* fibroin with silver nanoparticles solved in distilled water, were prepared by electrospinning. UV-vis allowed confirmed the formation of small diameter of Ag NPs. The morphological characteristics, show improved the nanofiber formation at PVA15/SF/Ag NPs with 189.12 nm with bead free formation. FTIR permitted analysed the chemical composition of PVA/SF/Ag NPs nanofibers, and shown good interactions between them. Wettability allowed to know that hydrophilic surfaces between 40-70º are better for osteoblast attachment, so, it is recommended to cross-linked PVA scaffolds to improve water stability and increase hydrophilic characteristics up to 40º, as a possible substrate for bone tissue engineering.

Acknowledgment

The authors thank at research group of advanced materials and energy (MATyER) and Biomaterials Research Group (Biomat) for their help in tracking each of the processes developed in this research.

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