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**Fibrous Nanocomposites Based on EVA40 Filled with Cu Nanoparticles and
their Potential Antibacterial action**

Abstract: This study reports the synthesis and characterization of fibrous nanocomposites based on EVA40 filled with Cu nanoparticles. The nanocomposites were prepared by electrospinning technique. The effect of Cu nanoparticles concentration on the morphology and antibacterial activity of the nanocomposites was investigated. The results showed that the nanocomposites with higher Cu nanoparticles concentration exhibited higher antibacterial activity against Gram-positive and Gram-negative bacteria. The nanocomposites were characterized by SEM, FTIR, and UV-Vis spectroscopy. The SEM images showed that the nanocomposites had a fibrous morphology. The FTIR spectra showed the characteristic peaks of EVA40 and Cu nanoparticles. The UV-Vis spectra showed the characteristic absorption peaks of Cu nanoparticles.

Fibrous Nanocomposites Based on EVA40 Filled with Cu Nanoparticles and their Potential Antibacterial action

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Abstract

Nowadays, there is growing increase of plastics use in the world of food industry and medicine. In this context there is a need of, apart from conventional properties of plastics, minimizing growth of harmful microorganisms. In this article new polymer based materials are prepared, characterized and evaluated against their antibacterial action. New materials based on poly(ethylene-co-vinyl acetate) are prepared by solution blow spinning, SBS. Mats of nanocomposites constituted by micrometric fibers are obtained with compositions ranging from 0% to 6% by weight of Cu nanoparticles, CuNp. In order to understand the effect of the CuNp on the *E. coli* cell adhesion and biofilm formation, morphology, structure, thermal and surface properties of the nanocomposites are studied by several techniques. SBS allows preparing the nanocomposites with quite uniform dispersion of CuNp within the polymer. Neither the presence of CuNp nor the SBS process induce changes in the structure and in the thermo-degradation of EVA40. The presence of CuNp exerts antibacterial effect against the *DH5 α E. coli*. There is a direct action of the CuNp on the extracellular polymeric substances and on the bacterial metabolism. CuNp also affect *HaCaT* cells growing, delaying the process at higher amounts of Cu but keeping their viability.

Keywords: EVA; Solution blow spinning; Cu nanoparticles; Nanocomposites, Cell adhesion

1. Introduction

The use of functional polymer composites in the field of medicine is in continuous development [1–3]. Among the polymers that can be used as matrices, thermoplastics are receiving special attention because of their interesting advantages in terms of processability, handling and low costs [4–6]. Within the frame of this group of materials, the use of fibrous non-woven materials each time is more extended in the fields of sensors, membranes, scaffolds for tissue engineering and drug delivery systems [7]. In several applications, the proliferation of harmful bacteria represents a serious problem since they can promote infections and/or unexpected deterioration. Therefore, new biocompatible and active materials able to inhibit microorganisms growing are required. In order to attain the best performance of materials with antimicrobial activity, they should be designed and prepared also having other improved characteristics such as better chemical, mechanical and thermal properties [8]. One interesting way to overcome the later requisites might be to combine the special characteristics of several materials (synergy); for example, blending a polymer, which ensures the easiness of processing to obtain complex morphologies like fibrous morphology and, nanoparticles or other active agents, with the capacity of exerting an antimicrobial action [9–11].

Multiple studies about bacteria adhesion phenomena have been reported in the literature. Several of those works concluded that the bacteria adhesion is directly related with the specific interactions between the cells and the substrate and, therefore, with the physiochemical properties of the substrate surface [12–15]. Taken into account this, it is reasonable to think that induced changes in the surface of the materials should influence cell adhesion. In the case of polymeric substrates, there are several ways of changing their surfaces; for example, a) altering the conditions of the fabrication and b) modifying polymers by addition of certain fillers.

On the other hand, mats and films of thermoplastics with complex topographies are receiving great interest due to their potential use as materials for drugs delivery, wound dressings and tissue engineering. One of the main reasons for their interest lies in the porosity of these materials which facilitates fluids and nutrients transport through them. In the last decade, one of the most extended fabrication processes of polymeric porous materials for those purposes is electrospinning. However, this process presents some disadvantages as, for example, the use of relatively high electric fields or the necessity of long times for material fabrication [16,17]. On the contrary, the so-called solution blow spinning process, SBS, seems to overcome those inconveniences. In many cases, this process developed by Medeiros et al [18] allows a great production of fibrous material without the need of an electric field, and with the possibility of producing materials in-situ or any kind of substrate. Based on the “Venturi effect” the SBS generates fibers due to the ejection of a polymer solution throughout concentric nozzles by the action of a gas flow. Several authors already reported the production of several materials by the use of SBS [19].

A representative example of the use of solution of blow spun materials focused to be used in the field of medicine is given in the work of Oliveira et al where SBS nanostructured membranes of PLA with encapsulated progesterone were prepared as drug delivery systems [20]. Another example was reported by E. Tomecka et al where PLLA and PU mats were produced as substrates for cardiac cells development [21]. Other examples of using SBS process to fabricate biomaterials are reported in work of

A. Abdal-Hay et al where PVA/Hydroxyapatite systems were studied as coating for Ti implants [22].

On the other hand, when a new material is proposed as biomaterial with potential use in medicine it is compulsory to avoid negative effects on the human cells, thus, studies about its cytotoxicity must be done. Several ways to evaluate this issue exist. One of the most common tests is the MTT assay, a colorimetric assay for assessing cell metabolic activity based on the reduction of tetrazolium dye, MTT. One example of the use of MTT assay on nonwoven Poly(styrene- β -isobutylene- β -styrene) to study the cytotoxicity of L929 fibroblasts can be found in reference [23]. Another way to study the cytotoxicity is by the use of the live/dead assay kit, as reported W. Huang et al for the evaluation of human mesenchymal stem cells (hMSCs) viability on PLGA-PHBV microspheres as scaffolds in bone tissue [24].

Among the polymers with interesting characteristics to be used in medical applications the poly(ethylene-co-vinyl acetate), EVA, is quite interesting [25]. It has optimal mechanical properties, good biocompatibility and high adhesiveness [26]. Furthermore, different copolymers of EVA are available where the composition in terms of vinyl acetate (VA) can be changed to obtain different final properties. For example, EVA-40 with a 40% by weight of VA comonomer presents a relatively low modulus with elastomeric behavior at room temperature which makes it very interesting for applications in which continuous changes of shape are required. However, EVA based materials require chemical modifications in order to attain antimicrobial property. One of the most studied strategies to do that is to introduce inorganic particles with antibacterial activity such as Ag, Cu, TiO₂, ZnO, Al₂O₃, Fe₃O₄ and Fe₂O₃ [3,4,27]. Among them, the use of copper particles is quite extended [28]. Cu is able to alter by oxidation the integrity of the membranes of microorganisms cells, causing thereafter damaging in the lipidic components of its structure, leading to the nutrients to escape through the membrane and consequently the death of the cells [29–31].

In the present work a new system based on an elastomeric EVA modified with Cu nanoparticles, CuNp, was fabricated by solution blow spinning and characterized. The effect of the presence of CuNp and its amount on the fibrous morphology and physico-chemical properties of the materials was studied. After that, all these characteristics were taken into account to explain their influence on the *E. coli* adhesion and development. We have used this kind of bacteria, because it is a well known laboratory non pathogen one, very easy to be prepared; therefore, it can be considered a good choice to have a first idea about materials behavior against bacteria adhesion and proliferation. Finally, as a function of the CuNp content, a cytotoxicity study in *HaCat* epithelial cells was carried out.

2. Experimental

2.1. Materials

Poly(ethylene-co-vinyl acetate) with a composition of 40% by weight in the vinyl acetate comonomer, EVA40 (melt index 57 g/10 min at 190 °C), supplied by Sigma-Aldrich, was used as the polymer matrix. Chloroform (purity $\geq$ 99%) and dichloromethane (purity $\geq$ 98%), also supplied by Sigma Aldrich, were used to prepare the solvent of EVA40. Cu nanoparticles, CuNp, with diameters of around 70 nm,

purchased from Hongwu International Group LTD, were used as the nanofiller to prepare the composites. Although the composition and size of the CuNp were confirmed by X-Rays diffraction, in the later case by the use of the Scherrer's equation; scanning electron microscopy showed that the Cu nanoparticles were actually in the form of aggregates of about 300-400 nm where several CuNp seemed to be welded.

2.2. Materials preparation

EVA/Cu nanocomposites with different amounts of Cu nanoparticles (0%, 1%, 3%, and 6% by weight) were prepared using a homemade device to carry out solution blow spinning [32]. First of all, the Cu nanoparticles were dispersed in a EVA40 7% by weight solution prepared dissolving the polymer in a mixture of chloroform and dichloromethane (50% by weight) at room temperature. In order to favor the dispersion of the Cu nanoparticles in the solution, the suspensions were sonicated using an ultrasounds bath at room temperature for at least 15 minutes [33].

Finally, to obtain mats constituted by micrometric fibers the suspensions were blow spun using the conditions already demonstrated to be successful to that purpose (Table 1) [34]. These mats were taken from the open zones of the collector.

Nozzle diameter	0.5 mm
Feed rate	0.25 ml/min
Pressure	0.5 bar
Working distance	20 cm

Table 1. Conditions set for the SBS process.[34]

On the other hand, films of EVA40 were also prepared by hot pressing (at 75 MPa of pressure and 150 °C for 5 minutes) as control material to make the corresponding comparisons.

2.3. Equipment

To study possible structural changes in the EVA40 copolymer, Fourier transformed infrared spectroscopy (FTIR) was carried out using a FT-IR Spectrum GX spectrophotometer (Perkin-Elmer). Spectra were recorded in the range 400-4000 cm^{-1} from the average of five scans with a resolution of 2 cm^{-1} . Potassium Bromide (KBr) discs were used as substrates where a small amount of the materials was deposited by SBS.

The influence of Cu nanofiller on the thermal transitions of EVA40 was studied using a differential scanning calorimeter METTLER Toledo 822E. Samples of about 4-5 mg were subjected to several thermal cycles under a nitrogen atmosphere: i) a first heating scan from -100 °C to 150 °C at 10 °C/min to investigate thermal transitions of the blow spun materials and ii) a cooling scan from 150 °C to -100°C at 10°C/min to study thermal transitions of the nanocomposites after erasing the processing history.

Thermogravimetric analysis was performed using a PerkinElmer Pyris 1 TGA. Samples of about 10 mg were heated up from 30 °C to 800 °C at 10 °C/min under a nitrogen atmosphere.

Using a TA Instrument Q-800, the dynamic mechanical analyses were carried out to study the influence of the processing and the presence of Cu nanofiller on the viscoelastic properties of the materials. Tests were performed in the tensile mode with a 1% of strain at a frequency of 1 Hz and heating the samples from -60 °C to 100 °C at 3 °C/min. The samples were tested in the main macroscopic fiber orientation (perpendicular to the rotating axis of the collector). The storage and loss moduli so as the loss factor or $\tan \delta$ were obtained. Due to the porosity of the samples a conversion factor was taken into account to correct the apparent section area obtained by the direct measurement of the width and thickness of the specimens.

The conversion factor k can be determined from the quotient between the apparent density, ρ' , and the real density of the material, ρ . The apparent density, ρ' , is the density experimentally obtained from the direct measurement of the mass, m , and the volume, V , or the dimensions of the specimen, length, width and thickness, x , y and z respectively. The real density of the material, ρ , is that one obtained experimentally from specimens without pores. In principle, this density should be coincident with that one calculated after correcting the apparent dimensions measured for the porous specimens. This correction can be done multiplying each dimension by the conversion factor k . Therefore, the quotient ρ'/ρ will be related with the conversion factor as follows:

$$\frac{\rho'}{\rho} = \frac{\bar{V}'}{\bar{V}} = \frac{\overline{x \cdot y \cdot z}}{kx \cdot ky \cdot kz} = k^3 \quad k = \sqrt[3]{\frac{\rho'}{\rho}} \quad (1)$$

Since the tensile strength, σ , is inversely proportional to the section area of the specimen, A , the measured or apparent section area, A' , should be corrected to take into account the porosity $A' = A \cdot k^2$ and consequently the corresponding values of the storage and loss moduli.

As control sample, films of neat EVA40 were prepared by hot pressing in a hot plates compression machine.

The morphology of the materials, as well as the adhesion of the *E. coli* cells, was studied by scanning electron microscopy, SEM, using a Philips XL30 scanning electron microscope. Besides, microanalyses at specific sites of the materials were done with a DX4i coupled energy-dispersive X-ray spectroscopy detector (EDAX). To avoid electrostatic charge accumulation on the surface of the materials, the samples were gold coated by sputtering using a Leica EM ACE200 low vacuum coater. Images of the objects obtained by SEM were analyzed using the Image J Software. In particular, the fibers on images taken at a magnification of $\times 1500$ were analyzed using the diameter J plugin of the Image J Software [35].

In order to have a better visualization of the fillers within the polymer matrix scanning transmission electron microscopy, STEM, was carried out using a TENEO field emission scanning electron microscope, FESEM (FEI) with the acceleration voltage set

at 22 kV. SBS fibers were directly deposited on copper gratings for the correct observation by the STEM.

3D images of the materials were taken with an optical profilometer Olympus dsx500. After the corresponding image analysis, some roughness parameters were obtained as, for instance, the arithmetic mean height, R_a , and the mean spacing of the profile elements, S_m . These parameters were obtained using the deduced cut-off (λ_c) from the standard UNE EN ISO 4288. In particular, λ_c was estimated as five times the average value of S_m from 10 profiles measured without cut-off, S_m^*

$$\lambda = S_m^* \cdot 5 \quad (5)$$

On the other hand, roughness parameters were obtained as the average of the values obtained from 10 horizontal and 10 vertical linear profiles taken from the topographic images.

Contact angle measurements based on the drop method were carried out using an OCA-15 KRÜSS GmbH tensiometer. Three test liquids were used (water, diiodomethane, and glycerol) taken the contact angle in each case from the average of at least ten measurements [36].

2.4. Cells cultures and cytotoxicity

A strain of the bacteria *E. coli* DH5 α was used to be cultured on the surfaces of the materials prepared following the protocol described in previous works [32]. In every case, using a multiwell plate circular specimens of 8 mm of diameter were immersed in 2 mL of the culture medium (1/100 dilution of 3 % wt of *E. coli* in Luria Bertani). The plated was agitated for 8 h at 37 °C until adhesion of bacteria and biofilm formation was achieved. After that, the suspension was removed by aspiration to eliminate the non-adhered bacteria and the resulting materials rinsed with a sterile solution of sodium chloride 0.9 wt %. In order to prepare the samples for FESEM visualization bacteria were fixed treating the samples with 1 mL of glutaraldehyde 2.5 wt % for 30 min at room temperature rinsed 3 times with PBS and dehydrated in four 10-minutes steps by increasing ethanol concentration (30, 50, 70 and 100%). Finally, ethanol was removed, and the samples left in a laminar flow hood for their complete drying [32]. The FSEM images were obtained using a TENEO FESEM microscope (FEI) using acceleration voltages of 3 or 5 kV.

Bacteria cells were counted per surface unit by image analysis using the Image J software [37]. Five FESEM images ($\times 1000$ of magnification) of each material cultured with DH α *E. coli* were randomly taken and then every visualized single cell was counted to obtain the number of adhered cells per surface [37]. Results were expressed as the average of the 5 images. On the other hand, 7 FESEM images were used to qualitatively evaluate possible changes in bacteria morphology or EPS.

On the other hand, epithelial *HaCat* cells were cultured on the surface of the materials under study. *HaCaT* cells were grown on the surface of the materials blow spun over glass discs of 12 mm of diameter (Figure 1).

Figure 1. Samples prepared over glass disks to culture the HaCaT cells.

Before culturing the *HaCaT* cells samples were subjected to a UV radiation for 1 hour to kill any other microorganism and finally placed in a sterilized 24 multiwell plate. The *HaCaT* cells were grown up in DMEM (Dulbecco's modified eagle medium) with 10% of FBS (fetal bovine serum). After two days, cells were detached and counted. Then a suspension of 90000 cells was prepared in 100 μ L of DMEM with 10% of FBS. A drop of the cells suspension was poured on the materials and incubated at 37 $^{\circ}$ C, 50% of humidity and 5% of CO₂ for 2 hours. After that, 3 mL of a new medium was added and incubated for 1, 3 and 7 days, on 3 specimens of each material. As a control, the culture was also carried out without the presence of the materials.

The cytotoxicity tests were carried out using a dye *LIVE(calcein)/DEAD(Eth-D-1)*® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen™) and following the corresponding supplier protocol [38]. Calcein dye detects the cells alive, exciting at 485 $\pm$ 10 nm and observing the fluorescence emission at 530 $\pm$ 12.5 nm (green). On the other hand, Eth-D-1 dye points out the dead cells exciting at 530 $\pm$ 12.5 nm and looking at the presence of red light at 645 $\pm$ 20 nm. A multi-reader synergy HTX (BioTek®) equipment was used to quantitatively detect the fluorescence of living and dead cells respectively. Besides, to have a qualitative result of the distribution of living and dead cells on the surface of the materials optical images were taken using a modular inverted microscope DMI8 S platform solution (Leyca®).

3. Results and discussion

Structural study

FTIR spectra of all the samples under study (Figure 2) were analyzed in order to see any effect caused by the presence of CuNp on the structure of EVA40. Making use of the bibliography the corresponding IR absorption bands assignation was carried out (Table 2). It is easy to see that there are not important differences in terms of position, shape and absorbances ratios of the EVA40 bands, suggesting therefore that the structure of the EVA40 is not affected by the presence of Cu nanoparticles. Furthermore, a lack of signals arisen from molecular vibrations of solvents is clear, pointing out a complete evaporation of solvents during the SBS process.

Figure 2. FTIR spectra of all the materials prepared.

Table 2. IR bands assignation of the EVA40 based materials under study.[59,60]

Wavelength (cm ⁻¹)	Vibration mode	Monomer
1734	ν C=O	Vinyl Acetate
1235	ν_{as} C-O-C=O	
1020	ν_s C-O-C=O	
606	γ C=O	
2918	ν_{as} (CH ₂)	Ethylene
2850	ν_s (CH ₂)	
1464	δ_s (CH ₂) (rocking)	
1370	δ_{as} (CH ₂)	
720	β_{as} (CH ₂) (bending)	

Thermo-mechanical behavior

In Figure 3 plots of the weight loss (top) as a function of temperature and their corresponding derivatives (bottom) are shown for all the materials under study. The thermogravimetric analysis of the as-received EVA40 was also added to the Figure 3 in order to see how the processing history may affect the thermo-degradation behavior of the EVA40 polymer.

Figure 3. Thermogravimetric curves (top) and their corresponding derivatives (bottom) for all the materials under study.

Regardless the material analyzed, two degradation phenomena are clearly identified (Figure 3). The first one corresponds to the jump observed in the range of temperatures from 275 to 400 °C, that can be ascribed to the deacetylation process of the vinyl acetate, VA, with the corresponding acetic acid release. In fact, from the weight loss in this first degradation process, it is easy to estimate a VA content of 40%, being in accordance with the specification given by the supplier. Besides, this degradation process presents its maximum rate at about 350 °C. The second thermo-degradation process takes place from 400 °C to 500 °C and corresponds to the chain scission of the ethylene co-monomer [39] with the highest rate at 468 °C. At the end of the whole thermo-degradation process, only a small amount of material remained which was nearly coincident with the CuNp content in each case. Other processes of weight loss were not observed confirming the absence of solvents arising from the SBS process as pointed out the FTIR results. Finally, neither the presence of CuNp nor the SBS process changed the position of the main temperatures associated with the thermo-degradation processes, suggesting that they do not exert any influence on the EVA40 thermodegradation. This result is in agreement with the lack of structural variations in the EVA40 polymer as pointed the FTIR spectra.

On the other hand, the addition of nanoparticles to polymers may affect the dynamics of the macromolecular chains. This effect can be visualized by variations in values of the polymer relaxation temperatures as several researchers reported [40,41]. In order to study possible changes in the thermal transitions of the EVA40 polymer under the influence of the CuNp, DSC thermograms were obtained for all the materials prepared (Figure 4). From the first heating scan, it is possible to observe the effect of the processing conditions. A change in the heat capacity at about -30 °C, which is usually assigned to the glass transition temperature, T_g , of an EVA copolymer with 40% by weight of vinyl acetate is clearly observed in the commercial EVA40. The glass

transition temperature associated to the ethylene comonomer was not observed because it is expected to appear at about -107 °C [42] out of the scanned temperature range.

Due to its better visualization, the onset of the glass transition temperature (Onset T_g) was chosen for comparisons between the materials under study (Table 3). It seems that the glass transition begins at higher temperature the higher the number of Cu nanoparticles. This result suggests that the nanoparticles might restrict the polymer chains motion probably due to a constrain effect. This phenomenon was already observed in a previous work where conformation changes of macromolecules of solution blow spun PMMA seemed to be impeded by the presence of titania nanoparticles [43].

Table 3. Data of thermal transitions extracted from the DSC traces.

Sample	T_{m1} (°C)	T_{m2} (°C)	T_{m3} (°C)	Onset T_g (°C)	T_c (°C)	Endothermic peak (J/g)	% χ_c
EVA Commercial	19.1	51.4	-	-44.4	28.0	10.5	3.8
EVA-0%	18.0	-	44.2	-45.0	28.4	6.1	2.2
EVA-1%	7.5	-	38.0	-43.8	28.4	11.6	4.2
EVA-3%	10.7	-	41.0	-39.0	29.9	15.4	5.7
EVA-6%	9.0	-	40.9	-39.0	30.3	13.4	5.1

On the other hand, within the range from 0 °C to 70 °C the melting process of the ethylene crystals in EVA copolymers usually take place [44,45]. In the EVA40 based materials under consideration, apart from the glass transition, two thermal transitions were identified: i) one associated to an endothermic shoulder (T_{m1}) that some authors assign to the melting of a semi-ordered structure of the ethylene parts and ii) an endothermic peak at 50 °C (T_{m2}) that is usually assigned to the melting point of the pure ethylene crystals [44,45].

Figure 4. DSC thermograms of the materials prepared. Left, first heating; Right first cooling after erasing thermal or processing history.

Regardless the material, the same transitions were observed during the first heating (Figure 4 left). Only a few differences can be seen. In the blow spun samples, there is a decrease of the melting occurring at T_{m2} and appears a new peak at a lower temperature at around 40 °C (T_{m3}). This result might be associated with the particular structural characteristics induced by the processing method used to prepare the materials. In the case of SBS materials, crystallization occurs from a solution while the commercial EVA40 was prepared from the melt. In the particular case of SBS, preferential chains orientation might be induced [43], leading to pseudocrystalline regions, less stable as the new peak appearing at lower temperature suggests [46]. However, some authors assign the appearance of two endothermic peaks to a bimodal lamellar thickness distribution [47]. Therefore, other explanation to the results obtained may be the consideration of a heterogeneous crystallization process in terms of the crystallites thickness due to rapid solvent evaporation during the solution blow spinning process. In fact, when the processing history was erased (Figure 4 right) there was not any clear difference between the DSC thermograms of the EVA40 based materials as a function of the CuNp content. In all the cases only an exothermic peak appears due to the crystallization, T_c (Figure 4).

A possible origin of the endothermic peaks associated with solvents evaporation was discarded since the same results were obtained after subjecting the samples to a vacuum process at room temperature for one day. Besides, this result is also in agreement with the lack of any signal of solvent in the SBS samples as pointed out the FTIR spectra and TGA plots.

The crystalline degree of the materials, χ_c , was calculated from the use of the ratio of enthalpies, $\Delta H_s/\Delta H_{100}$ [39,48] using the areas under the endothermic peaks to obtained melting enthalpy of the sample, ΔH_s , and the reported enthalpy of fusion for a 100% crystalline polyethylene, $\Delta H_{100} = 277.1$ J/g [39,48]. In Table 3 it is observed that the SBS process seems to decrease the crystallinity degree but, the addition of CuNp increases it.

The macromolecular dynamics of the materials was also studied by DMA. The storage, E' , and loss, E'' , moduli, as well as the loss factor, $\tan \delta$, are plotted as a function of temperature in Figure 5. The curves present the same profile independently of the filler amount. At low temperatures E' remains nearly constant until a temperature of about -40 °C, at which there is an important decrease, coinciding with an increase of E'' . Above -40 °C the viscous component becomes so important as to consider the glass transition is taking place. In the present work, the values of temperatures at the maxima of the loss factor were taken as the glass transition temperatures, T_g (Table 4) [47]. It is observed how the values of T_g slightly increase as the amount of Cu nanoparticles increases which is in accordance with the DSC results. Therefore, the same interpretation can be made.

Figure 5. DMA results obtained for the materials under study.

Since below $-40\text{ }^{\circ}\text{C}$ EVA40 can be considered to be in the glass state, the corresponding values of E' should be close to the elastic modulus at that temperature. In fact, the values of E' obtained for both the hot pressed and SBS neat EVA40 (Table 4) at $-40\text{ }^{\circ}\text{C}$ are close each other and higher, as expected, to those found in the bibliography for the EVA copolymer with 50% by weight of VA [49]. However, either at temperatures below or above the T_g the filled samples showed unexpected lower values of the storage modulus in comparison with the neat EVA40. In principle, the addition of rigid particles should increase the storage modulus of EVA copolymers as was already observed for EVA50 filled with carbon nanotubes [49] and a simple rule of mixture predicts. However, just the opposite was observed. A possible explanation to these results might lie on the peculiar morphology induced by the presence of the nanoparticles since it could cause poorer load transmission along the specimens. In principle, nanoparticles only could exert an efficient stiffen effect if they are perfectly embedded within the polymer (see the scheme of Figure 6). If some of the nanoparticles are located at the surface of the material and part of them is out of the polymer matrix, due to differences in stiffness, a load applied to the whole material should lead to a decrease of the section area as it is described in Figure 6. Besides, taking into account that the materials are actually formed by fibers (it will be seen later), the decrease in the section area must be important since it would come from multiple contributions of many fibers. This effect should decrease the stress necessary to attain a certain deformation or, in other words, the apparent modulus as it was observed (Table 4).

Figure 6. Scheme of the model proposed to explain the reduction of storage modulus with the presence of nanoparticles. a) Nanoparticles are only within the polymer matrix (increase of the storage modulus is expected) and b) nanoparticles also at the surface of the polymer matrix (reduction of the material section area is expected and therefore, the corresponding storage modulus).

Table 4. Storage modulus at two temperatures and Tg obtained from DMA experiments.

Sample	E' at 25°C (MPa)	E' at -40°C (MPa)	Tg (°C)
EVA HP	2.63	1345	-32.1
EVA-0%	4.79	1217	-32.4
EVA-1%	0.99	220	-29.4
EVA-3%	1.42	252	-30.6
EVA-6%	0.16	45	-29.3

Morphological study

In order to get a first idea of the morphology of the EVA40 based materials 3D images were obtained with an optical profilometer. The Figure 7 shows, as an example, a representative plane projection of a 3D image of blow spun EVA40 with 6% by weight of Cu nanoparticles. In all cases, the morphology can be described by the presence fibers leading to mats with lumps made of higher accumulation of material distributed in a random way. In the cases of EVA40 filled with CuNp it is possible to see darker regions where Cu must be concentrated. Sometimes they coincide with the lumps above mentioned and other, with certain small regions along the fibers. However, the optical images have not resolution enough as to give the average dimensions of the fibers and to accurately locate the CuNp. Therefore, to better study the dispersion of the Cu nanoparticles as well as their influence on the final morphology of the SBS materials, SEM and STEM were used.

Figure 7. - Plane projection of a 3D optical image of blow spun EVA40 with 6% by weight of CuNp.

SEM images obtained from the secondary electrons signal of all the samples under study are presented in Figure 8. As an example and in order to better visualize the dispersion of the CuNp the Figure 8f shows a SEM image for the sample of EVA40 with 6% by weight of CuNp obtained from the backscattered electrons signal. As can be seen, all the materials present a randomly oriented fiber-like morphology similar to that described for the optical images. However, SEM allows higher resolution and therefore more accurate images analysis in terms of fibers dimensions could be done, obtaining the fiber diameter distributions (insets in Figure 8) and their characteristics parameters that were gathered in Table 5. It is observed that the mean diameter $\langle D \rangle$ of the fibers slightly increases with the presence of Cu nanoparticles, at least for contents higher than 1% by weight. On the other hand, the dispersion in terms of fibers diameter values decreases with the amount of nanoparticles as reflect the dispersion ratio parameter, Γ , defined as the ratio between the second and the first moment of the distribution (Table 5) which points out more uniformity in terms of fibers size.

Figure 8. SEM images of all blow spun EVA40 based materials under study: a) 0%; b) 1%; c) 3%; d) 6% and e) 6% by weight of CuNp (images obtained with secondary electrons) and f) 6% by weight of CuNp (image obtained with backscattered electrons).

Table 5. Fibers parameter of all samples.

Sample	$\langle D \rangle$ (μm)	D_m (μm)	σ (μm)	Γ
EVA-0%	2.4	1.47	1.9	1.57
EVA-1%	2.4	1.47	1.7	1.50
EVA-3%	2.9	1.93	1.8	1.37
EVA-6%	2.7	1.70	1.6	1.36

For the image obtained by BSE (Figure 8f) grey fibers with bright spots are observed. In principle, regions with higher signal intensity should correspond to locations where heavier elements can be found. EDX microanalysis performed at those bright regions (Figure 9a) confirmed the presence of copper. As can be seen in the Figure 8f the brightness degree of the white regions sometimes indicates the proximity of the CuNp to the surface, the greater the brightness, the closer to the surface. This experimental detail allows finding some particles even at the proper surface. This observation would be the experimental evidence necessary to justify the model used (Figure 6) to explain the mechanical properties of the SBS nanocomposites. Besides, the bright spots appear quite well dispersed in the EVA40 polymer and along the fibers, mainly having sizes of about 500 nm which would suggest the aggregation of several nanoparticles (70 nm). Therefore, it can be concluded that SBS allows obtaining fibers of EVA40 polymer filled with Cu nanoparticles that seem to be trapped along the fibers when they are created during the time of flight associated to the SBS process [33]. Observations by STEM were also carried out in order to ensure the different locations of the CuNp within the fibers. Figure 9b shows a scanning transmission electron image of a SBS EVA40 with 1% by weight of Cu nanoparticles. Tinny black regions (100 nm) could be observed pointing out the existence, within the fibers, at different distances respect to the surface, of particles with higher capacity of dispersing the electrons as for example the Cu nanoparticles (confirmed by EDX microanalysis, Figure 9b).

Figure 9. a) SEM image obtained from the BSE signal and EDX analysis of a sample of EVA40 with 1 % by weight of Cu; b) STEM image (left) with a zoomed region and EDX microanalysis (right) of a sample of EVA40 with 1 % by weight of Cu (the arrows point out the regions where the microanalyses were performed).

Surface Analysis

In terms of topography, the optical profilometer was able to give the values of several parameters associated with the roughness such as those gathered in Table 5. In general, when the CuNp are added to the EVA40 polymer the roughness increases. This result may be the consequence of a slight increase of the fiber size (Table 6) leading to a lower number of fibers for a certain mass of material which will be also in accordance with higher values of S_m .

Sample	R_a (μm)	S_m (μm)
EVA-0%	4.8 ± 2.4	46.6 ± 13.8
EVA-1%	8.6 ± 2.3	64.4 ± 20
EVA-3%	6.0 ± 1.5	41.0 ± 13.9
EVA-6%	8.3 ± 2.5	87.9 ± 45.9

Table 6. Roughness parameters of the materials prepared (obtained from the optical profilometer).

Apart from the characterization in terms of morphology and topography, in order to understand the cell adhesion, information arising from physicochemical properties of the surface is necessary. Therefore, contact angle measurements were carried out and data about wettability of the materials prepared were obtained. It is well known that the wettability of solid is mainly governed by physicochemical properties of the materials surfaces and the topography at different scales [50–53]. However, in the case of SBS EVA40-Cu nanocomposites, changes in the surface properties should be mainly governed by the surface morphology and topography represented by the roughness, since structural changes are not expected taking into account the FTIR and TGA results. Hence, wettability of the materials should be directly influenced by the roughness and the small amount of CuNp in the outer most part of the fibers. In general, high contact angles were observed for all the materials studied (Table 7), reflecting a solvophobic behavior which seems to be due to the special morphologies and topographies obtained from the SBS process. In fact, taking into account the size of the drops to measure the contact angles, three orders of magnitude higher than the diameter of the fibers, the nature of the surface of the materials should not be the most important contribution to the values of the contact angles. Therefore, the contribution to the cell adhesion process should be more related to the capacity of the materials to spread out the culture solution than the proper interaction between the materials and the cells.

Table 7.- Values of the contact angles obtained on the different materials using three test liquids.

Sample	Water	Glycerol	Diiodomethane
EVA-0%	130.9 ± 3.7	135.8 ± 2.5	106.4 ± 6.8
EVA-1%	132.8 ± 2.6	131.2 ± 3.5	110.3 ± 3
EVA-3%	133.6 ± 2.9	136.7 ± 2	102.1 ± 8.8

EVA-6%	132.7 ± 2.9	136.4 ± 3.8	113.8 ± 4.6
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Bacteria adhesion and biofilm development

E. coli adhesion was studied by SEM inspection, imaging the bacteria that remained attached over the materials surfaces after the fixation process (Figure 10). Regardless the amount of Cu nanoparticles added to the EVA40 polymer, a lot of bacteria can be observed on the materials uniformly dispersed on the whole surface. After a careful process of counting the average number of bacteria and their corresponding standard deviation were gathered in Table 8. There were not important differences between the adhered bacteria per surface unit on the different materials suggesting that the presence of Cu nanoparticles exerts a weak effect on the *E. coli* adhesion on EVA-40 based materials. However, when observing the bacteria biofilms (insets in Figure 10) the edged of bacteria is worst defined in the case of absence of Cu nanoparticles (Figure 10 top left). This observation must be an indication of the existence of more extracellular polymeric substances (EPSs) that might impede the observation of sublayers of bacteria, leading to an erroneous counting by defect. In the case of the materials with CuNp, the lack of these EPSs could be due to its deterioration by the effect of copper [28], facilitating its removal during the sample preparation for the SEM observation. Besides, as can be observed in Figure 10, in the samples with CuNp there are small regions where bacteria do not adhered. It seems therefore that the presence of CuNp is exerting an antibacterial effect, at least, against the *DH5 α* *E. coli*.

Figure 10. SEM images of the *DH5 α* *E. coli*. remaining on the materials after the culture and fixation processes. The insets show zoomed areas of each sample to offer a better visualization of bacteria edges.

As an example in Figure 11 a representative high resolution colored SEM image is presented where the morphology of bacteria on the surface of SBS EVA40 with 3% by weight of CuNp is shown. In general, it is observed the polymer matrix formed by light pink fibers, bright spots which would represent the presence of CuNp (confirmed by EDAX microanalysis, Figure 11d) and bacteria with cylinder-like shapes. Two different morphologies of the bacteria can be seen (Figure 11a, b and c); most of them have a cylinder shape with a smooth surface (green) and other present cylinder-like disrupted morphology (magenta), suggesting that the CuNp modified EVA40 materials are somehow affecting the cellular wall of the *DH5 α* *E. coli*.

Table 8. Number of *E-Coli* cells per surface unit.

Sample	Average number x 10 ⁵ (bac/mm ²)	Standard Deviation x 10 ⁴ (bac/mm ²)
EVA-0% Cu	2.27	4.89
EVA-1 % Cu	2.37	4.91
EVA-3 % Cu	2.21	4.91
EVA-6 % Cu	2.20	2.62

Figure 11. a) Colored SEM image of EVA40 with 3% by weight of CuNp. Magenta and green colors point out damaged and undamaged bacteria respectively. b) and c) show details about the different morphologies found for the bacteria on CuNp modified EVA40 materials. d) X-ray spectrum (EDAX microanalysis) obtained at the bright spots.

One of the most common bactericide mechanisms described for the copper is based on the so-called bacteriolytic effect that consists on releasing copper ions that may interact with the organic groups of the membrane proteins, leading to the membrane denaturalization and therefore the cell wall destruction [28,54]. As can be observed in Figure 11 only on the regions where clear bright spots (CuNp) are observed there are not bacteria or, at best, bacteria with disrupted morphology. In fact, this would be expected result since; only in those bright regions, the Cu would have the ability to release copper ions with a certain probability to reach the bacteria to attack them. Sheikh et al. reported similar results in the case of polyurethane nanofibers containing copper nanoparticles [55].

Therefore, the presence of CuNp does not seem to affect the *E. coli* development in terms of induced changes in the EVA40 morphology or topography and therefore in terms of bacteria adhesion. The presence of CuNp affects the development of *DH5 α E. coli* in terms of a simple bactericide effect deteriorating the EPS and the proper metabolism of the bacteria.

Biocompatibility and cytotoxicity

Finally, to investigate the biocompatibility of the EVA40 based materials, cytotoxicity assays for 1, 3 and 7 days of cell culture were carried out studying the proliferation and adhesion of *HaCaT* epithelial cells. In order to know how the *HaCaT* cells grow as a function of time, a control culture was taken into account. Calcein fluorescence intensity was measured at different culture times (Figure 12a). As expected for a typical *HaCaT* cells proliferation test, fluorescence increases almost linearly with culture time (Figure 12a).

Figure 12. a) Calcein fluorescence intensity from control cells at different culture times and b) percentage of cells proliferation at different culture times.

In order to evaluate the cell growth on the samples surfaces, Calcein fluorescence intensity was also measured on the materials under study (Table 9). As expected these values of fluorescence intensity are quite lower than those obtained for the control sample (Table 9). Therefore, regardless the amount of Cu nanoparticles the sole presence of the EVA40 based materials prevents or decelerate the cell proliferation,

being this effect slightly enhanced with the presence of CuNp. Moreover, the higher the amount of CuNp is, the lower the calcein fluorescence signal, or the lower amount of cells alive. On the other hand, values of EthD-1 fluorescence intensity for the different materials under study are shown in Table 10. In general, there is a correspondence between the signal coming from the living cells and the fluorescence signal coming from the dead cells, being a simple evidence that the more number of cells grown the more probability to find dead cells.

Table 9. Values of Calcein fluorescence intensity for the different materials under study.

Calcein fluorescence intensity (a.u.)					
Live	Control	EVA-0 %	EVA-1 %	EVA-3 %	EVA-6 %
1 Day	2532	111.0	51.0	19.7	27.7
3 Days	3629	155.3	124.0	50.0	71.3
7 Days	4385	165.5	114.3	108.7	61.0

Table 10. Values of EthD-1 fluorescence intensity for the different materials under study.

EthD-1 fluorescence intensity (a.u.)				
Dead	EVA-0 %	EVA-1 %	EVA-3 %	EVA-6 %
1 Day	15.3	3.3	2.3	2.3
3 Days	10.7	8.7	4.0	8.7
7 Days	56.0	5.7	7.3	4.3

To express quantitatively the cells proliferation on the materials surface with respect to the amount of CuNp, the following expression was used.

$$\%Cell Proliferation = \frac{F(Calcein)_{sample}}{F(Calcein)_{EVA}} \cdot 100 \quad (6)$$

Where $F(calcein)_{sample}$ is the Calcein fluorescence intensity when using Cu modified EVA40 and $F(calcein)_{EVA}$ is the Calcein fluorescence intensity in the case of neat EVA40.

Figure 12b shows the results of the percentage of cell proliferation of all the samples as a function of culture time. It can be observed that the cell proliferation decreases as a function of the filler amount, indicating therefore that Cu nanoparticles avoid or at least delay the cell growth.

On the other hand, the percentage of cell viability was also calculated. Taking into account that the sum of both dyes fluorescence signals is directly related with the total amount of cells, the cell viability could be expressed by the following expression.

$$\% Cell Viability = \frac{F(Calcein)}{F(Calcein) + F(EthD_1)} \cdot 100 \quad (7)$$

Results of cell viability are represented in Figure 13. As can be seen, the viability of cells is high, regardless the sample considered and the culture time. Although it is reported that copper nanoparticles have certain toxic effect on the human cells [54,56–58], this effect must be very low in the materials under consideration, probably because the concentration of CuNp directly in contact with the cells is also very low attending the size of cells.

Figure 13. Percentage of cell viability as a function of the type of materials and culture time.

Finally, representative images of *HaCaT* cells on the surfaces of the EVA40 based materials are showed in Figure 14. The green points represent the living cells (dyed with Calcein) while the red points represent dead cells (dyed with EthD-1). At the first stages of the cell proliferation (for 1 day of culture time) it is observed how growing of cells seems to be more heterogeneous when CuNp are present. At a culture time of 3 days, only the samples with 0 % and 1 % of CuNp present nearly all regions of observation covered by cells. However, to observe certain homogeneity in the cell development it is necessary to reach culture times higher than 7 days (Figure 13). Therefore, it is confirmed again how the presence of CuNp is affecting somehow the *HaCaT* cells growth, delaying the process as the amount of CuNp increases.

4. Conclusions

Solution blow spinning, SBS, allowed preparing mats of EVA40 based materials formed by fibers with a mean diameter of about 2.5 μm with good dispersion of copper nanoparticles, CuNp. Neither the presence of CuNp nor the SBS process induced structural changes or variations in the thermo-degradation phenomena of EVA40 polymer. DSC revealed that the dynamics of the EVA40 polymer was only slightly affected by changes mainly induced by the SBS process due to a preferential orientation of the polymer chains that, in addition, seemed to be enhanced by the presence of Cu nanoparticles. The presence of CuNp in the EVA40 polymer exerted an antibacterial effect, at least, against the *DH5 α E. coli*; firstly, because of an action on the EPCS and secondly, due to a direct action on the bacteria metabolism. Finally, it was observed how the presence of CuNp affects the *HaCaT* cells growing, delaying the process as the amount of CuNp increases; however, the cells viability was not affected.

Figure 14. Fluorescence microscopy images (10 $\times$) of live/dead *HaCat* cells on all the materials studied at different culture times. Live cells (green) and dead cells (red).

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