Temperature controlled electrospinning of EVOH nanofiber mats encapsulated with Ag, CuO and ZnO particles for skin wound dressing

Dandan Liu[1], Chuanwei Zhang[1], Chao Xu[1]*, Dianzi Liu[1,3], Yuan Lv[1], Bin Zhong[1], Bin Wang[1,2]

[1]*School of Mechanical Engineering, Xi'an University of Science and Technology, No.58 Yanta Rd, Xi'an, Shaanxi, P.R. China, 710054*

[2] *School of Engineering and Design, Brunel University, UK*

[3] *School of Engineering and Mathematics, University of East Anglia, UK*

Abstract

Aiming to the treatment of skin burns, a new wound dressing, nanofiber mats with metal or metal oxide nanoparticles (Ag, CuO and ZnO) , was fabricated by the electrospinning technique. During the therapeutic process, the antibacterial ability and bio-compatibility of a new dressing material are of major concerns. To expound the characteristics of EVOH nanofiber mats encapsulated with the metal or metal oxide nanoparticles, notated as Ag-EVOH, CuO-EVOH and ZnO-EVOH, respectively, for possible use as the new wound dressing materials, we have investigated the suitable processing parameters to fabricate these materials, such as the voltage, the tip-to-collector distance, the concentration of the solution, and the effect of environmental temperature. The antibacterial abilities and the bio-compatibilities of Ag-EVOH, CuO-EVOH and ZnO-EVOH were then tested and quantified. The outcomes show that the most suitable temperature for fabrication of the materials is 40℃(±3℃). Antibacterial experimental results indicate that 0.08g/ml of metal/metallic oxide shows the highest antibacterial ability on Staphylococcus aureus. And the largest diameters of bacteriastatic loops of the three kinds of nanofiber mats, ie., Ag-EVOH, CuO-EVOH and ZnO-EVOH, are 5.89mm, 5.21mm and 4.12mm, respectively. Fially, the cell proliferation of the three nanofiber mats show a similar growth trend.

Key words: electrospinning, temperature, nanoparticles, antibacterial ability, cell proliferation

1. Introduction

Skin, the largest organ of the human body, is mainly responsible for protecting the tissues and organs of the body from physical, mechanical, chemical and pathogenic microbiological invasions[1-2]. However, in life, skin is subject to more damage than other organs by many reasons, such as burn, leprosy, scabies, mycosis, etc[3-4]. Broadly, the most common treatment is medication method when skin is damaged[5-6]. Dressing materials are often required to assist during the healing process. There are many kinds of wound dressing, such as hydrogel, film, oil yarn, etc. Antimicrobial dressing is the most widely used medical material at present, which involves not only the antibacterial ability, but also the mechanical strength, bio-compatibility and other properties of the dressing materials. Many new bio-materials have been gradually introduced to replace traditional medicine for skin wound treatment as the result of constant effort of research[9-11]. Electrospinning is a convenient and efficient method to fabricate nanofiber mats encapsulated with antibacterial substance used as antiseptic dressing[7-8]. However the inflammation and suppuration still are the major problems which hinder treatment[12-13].

Hence, one of the key functions of a wound dressing material is to provide a bacteriostatic healing environment for the wounds. In previous studies, a new type dressing material, Ag-EVOH nanofiber mat, with self-antibacterial ability was fabricated by electrospinning technology[14-15]. EVOH (ethylene vinyl alcohol) is a kind of high polymer resin materials which only consist of three elements, carbon, hydrogen and oxygen so that it is really environmental. Besides, the mechanical strength, modulus of elasticity and flexural properties of EVOH are good because of its small flexibility of molecular chains[16]. This dressing material uses nano silver particles as antibacterial substances to ensure its antimicrobial capacity. The antibacterial effect of silver was also proved in the subsequent antibacterial experiments[15, 17]. Also, the mechanical property of the Ag-EVOH nanofiber mats was tested for the requirements in the clinical uses[18].

However, the duration for human body to discharge excessive nano silver particles is long, often taking months, yielding in toxicity to the human body[19-20]. Researches show that the toxicity of the copper and zinc is less than that of silver at the same quality[21]. Thus, EVOH nanofiber mats encapsulated nano-CuO or nano-ZnO particles are considered in this research. On the one hand, copper and zinc are essential trace elements of the human body[22]. On the other hand, antibacterial abilities of nano-CuO or nano-ZnO particles have been proven[23-25]. It is also found that during the fabrication process, nozzle blockages occurred repeatedly. Therefore, in this study, the influence of

the environmental temperature on the electrospinning process was studied, and suitable settings of the fabrication parameters were identified. In addition, the antibacterial properties of the three kinds of nanofiber mats were tested with different concentration of metal or metal oxide supplements. Moreover, planted cell growths were observed and monitored for the attachment and reproduce abilities on the fabricated mats.

This paper is arranged in the following sections: first of all, the materials and approaches used in this study were introduced, the fabrication methods of electrospinning at different temperatures were described emphatically. Secondly, the micro morphologies of the three kinds of nanofiber mats (Ag-EVOH, CuO-EVOH and ZnO-EVOH) were observed by SEM (Scanning Electron Microscopey). Finally, the antibacterial ability and the biocompatibility of the three nanofiber mats were tested, respectively.

2. Materials and Methods

2.1 Materials

The Poly (ethylene-co-vinyl alcohol) (EVOH) was used to make nanofibers, its solvent is made of isopropanol and deionized water. The supplements of Ag particles, CuO and ZnO nano particles were supplied by $AgNO_3$, $Cu(NO_3)_2$, $Zn(CH_3OOH)_2$ and diethanolamine ($C_4H_{11}NO_2$). The materials were bought from Shaanxi Huaxing Experimental Technology Co., Ltd. except EVOH which was purchased from Sigma-Aldrich (Batch number: 12822PE) in a granular form.

The preparation of the three nanofiber mats' precursor solutions were similar in general. Isopropanol and deionized water were mixed with a 7:3 (v/v) ratio as the solvent to desolve EVOH. Then, 1g EVOH was added to the solvent in a conical flask by water bath heating at 75℃. For Ag-EVOH and CuO-EVOH, adding different masses (0.2g, 0.5g, 0.8g) of $AgNO_3$ and $Cu(NO_3)_2$ into the flask, respectively, stirring for 2-3 minutes until the solution becomes transparent. For ZnO-EVOH, adding different masses (0.2g, 0.5g, 0.8g) of $Zn(CH_3OOH)_2$ which melt at a high temperature about 370℃ and $C_4H_{11}NO_2$ as complexant with a 1:2 (w/w) ratio into EVOH solutions slowly. The conversion of the three solid substance into liquid is shown in Fig.1. After stirring thoroughly, dropping HCl into the solution till it becomes transparent. In total nine kinds of precursor solutions

with different concentrations of solute (0.02g/ml, 0.05g/ml, 0.08g/ml of Ag, CuO, ZnO) were prepared for the process of electrospinning, respectively.

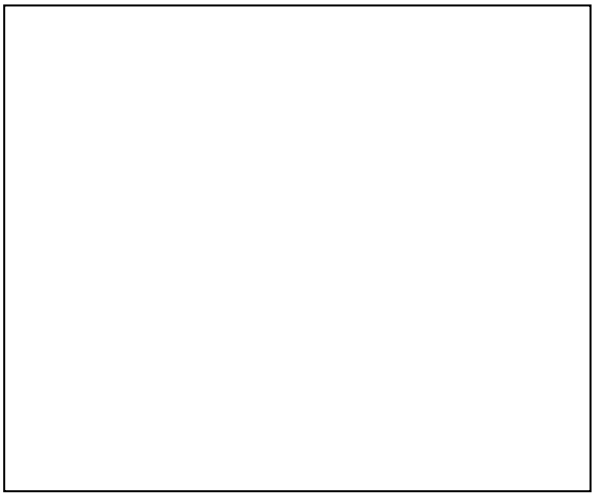


Fig.1 Preparation of three precursor solutions

2.2 Experimental Methods

2.2.1 Electrospinning Process

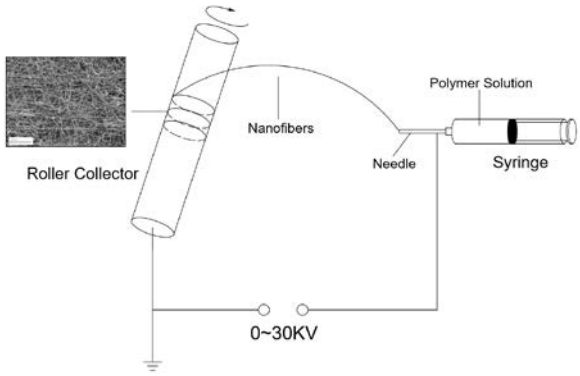


Fig.2 Electrospinning set-up

Fig 2 illustrates the fabrication setup which is consisted of a syringe with affine needle, from which a constant flow of the solution is produced into a static high-voltage electric field which provides the driving force to spin the solution jet into a spiral motion. The jet is stretched and solidified into a continuous fibre of very fine diameter, often down to tens of nano meter. The fibre can be collected in different ways, such as into a mat/sheet with or without fibre orientation control, or being wound into a roller. This process is commonly termed as electrospinning.

There are many factors affecting the final morphology of the nanofibers, such as the voltage of the static electric field, the (needle) tip-to-collector stand-off distance, the concentration of solution,

the environmental temperature, and the flow speed etc. In previous studies, we have fabricated pure EVOH nanofiber mats with 7.5%-12.5% EVOH solutions, used 20kV of voltage, 20-30cm of stand-off distance, 0.2-0.4mm/min of flow speed in ambient room temperature (~ 20℃). It is proven that the pure EVOH nanofiber was fabricated with good mechanical properties in the conditions mentioned above[26]. However, when using the same set-up to produce Ag-EVOH, CuO-EVOH and ZnO-EVOH fibres, needle blockage occurred frequently. After extensive investigation through trial and error, it was found that a higher environmental temperature is needed to produce good quality fibres. The final control parameters used for all sample fabrications were 10% EVOH solution, 0.02g/ml of nanoparticles, 25kV of voltage, 20cm of tip-to-collector distance and 0.2mm/min of flow speed in a heated environment of 40℃.

2.2.2 Micromorphology and Component Detection

The Scanning Electron Microscope (SEM) and Energy Dispersive X Ray Spectrometer (EDX) were used to observe the morphology and the elements of the samples (Fig.3). Fig 3a and 3b show images of pure EVOH nanofibers. The surface of the fibres appears to be fairly smooth. Also, owing to EVOH contains only three elements, C, H and O, it is confirmed that there is no other elements in EVOH nanofiber.

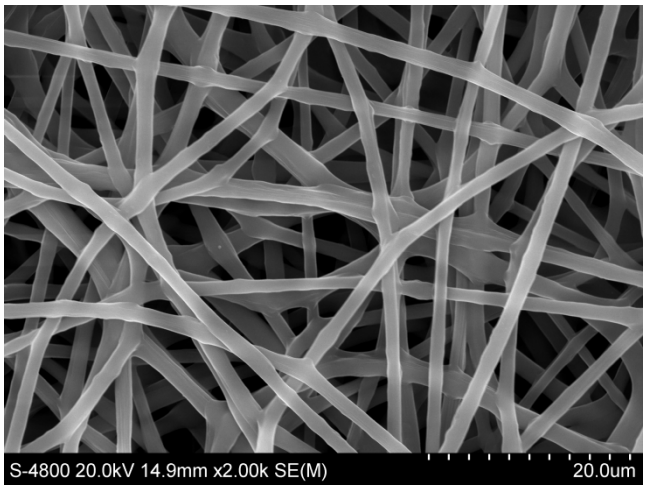
For Ag-EVOH, CuO-EVOH and ZnO-EVOH nanofibers, compared with pure EVOH nanofibers, from the images (Fig.3(c)(d)(e)(f)(g)(h)) and Eqn. (1) to (3), it can be confirmed that the small particles encapsulated on the fibers are Ag, CuO and ZnO nanoparticles, respectively[26-28].

$$2AgNO_3 \rightarrow 2Ag\square 2NO_2\uparrow\square O_2\uparrow \quad (1)$$

$$2Cu(NO_3)_2 \rightarrow 2CuO\square 4NO_2\uparrow\square O_2\uparrow \quad (2)$$

$$Zn(CH_3OOH)_2 \xrightarrow{370℃} CH_3COCH_3 + ZnO + CO_2\uparrow \quad (3)$$

From Figs. 3a, 3c and 3e, it is clear seen that the dispersity of Ag nanoparticles is better than the other two. Also, the morphologies of Ag-EVOH, CuO-EVOH nanofibers are more regular than that of ZnO-EVOH although their diameters are similar. These characters will also influence their microscopic performance to some extent, such as tensile strength, antibacterial ability, etc.


S-4800 20.0kV 14.9mm x2.00k SE(M)
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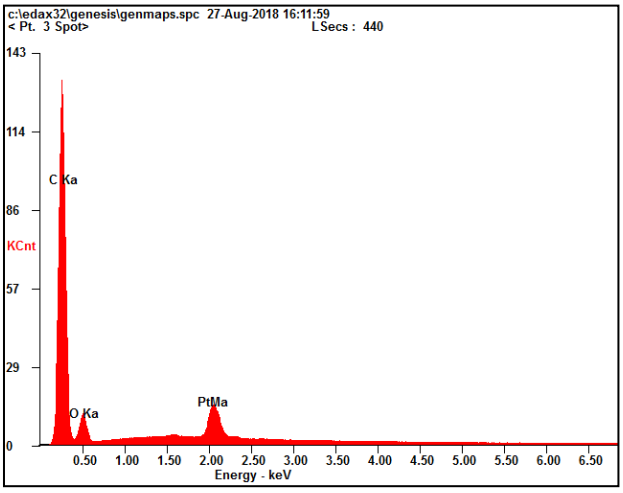
(a)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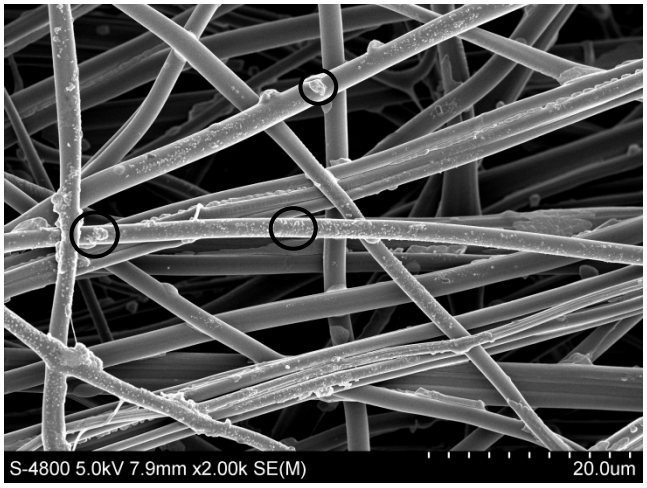
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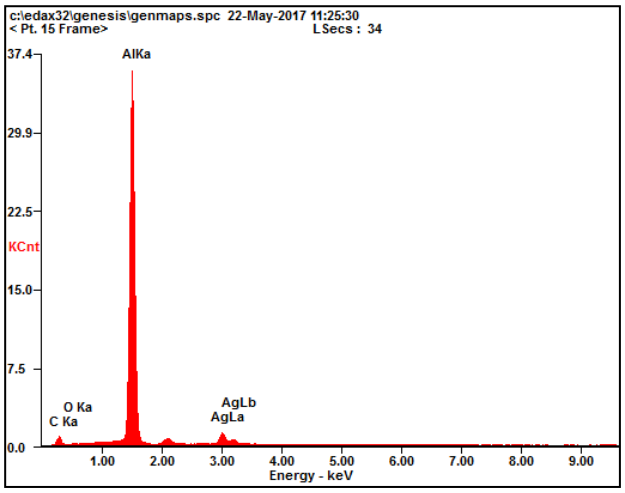
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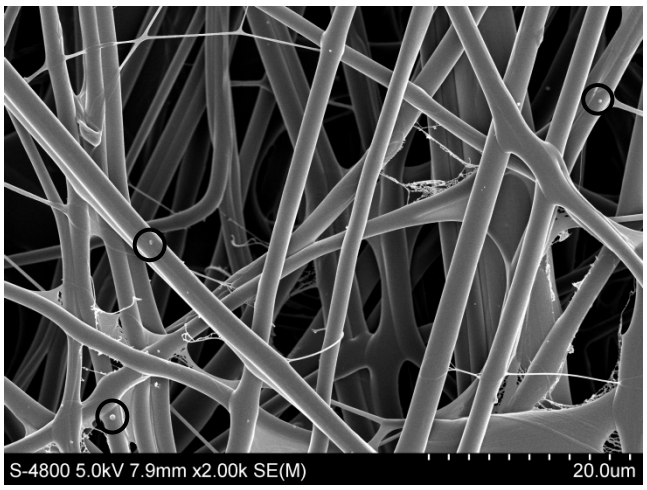
(d)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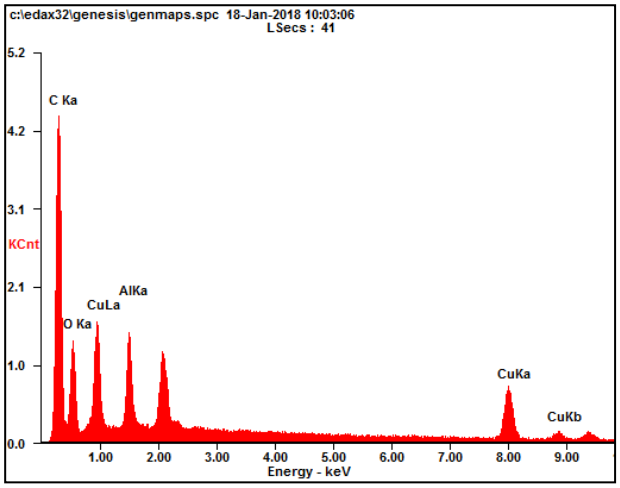
(e)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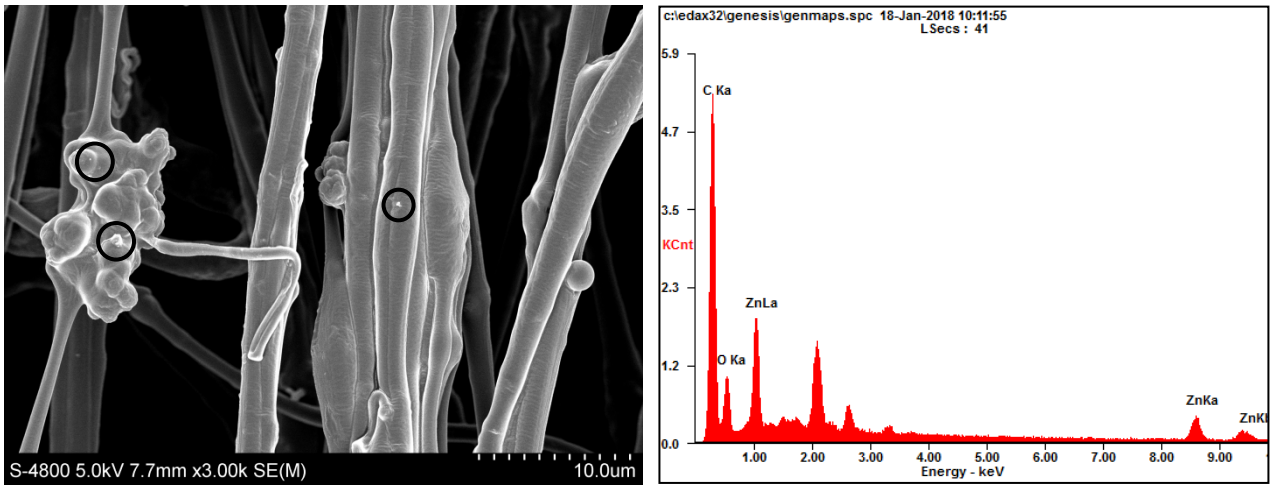
(f)



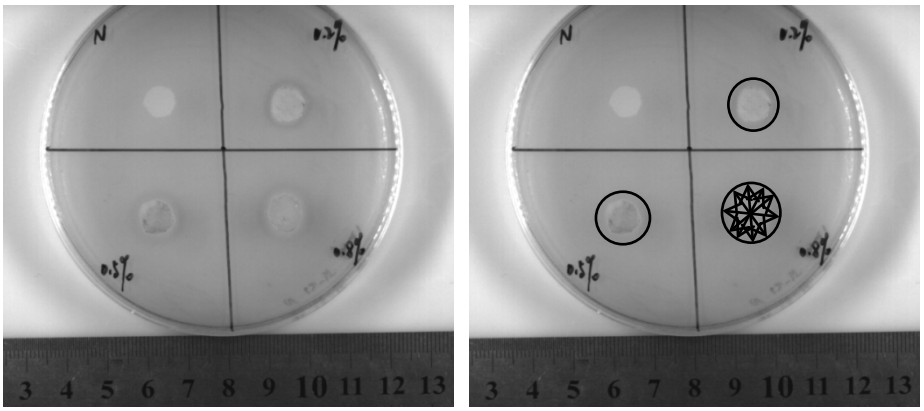
(g) (h)

Fig.3 the images of Morphology ((a), (c), (e), (g)) and Energy Spectrum ((b), (d), (f), (h)) of pure EVOH, Ag-EVOH, CuO-EVOH and ZnO-EVOH nanofibers

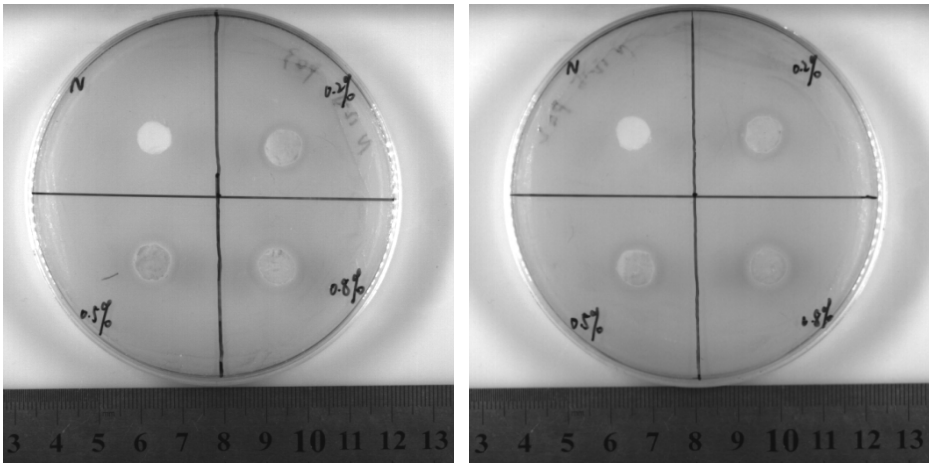
2.2.3 Antibacterial Test

For comparison of the antibacterial abilities of Ag-EVOH, CuO-EVOH and ZnO-EVOH nanofiber mats, the three samples were cut into small circular disks of diameter 8mm. And Staphylococcus aureus, as the most common bacteria on the wound surface, was chosen in this study. Sample mats with different concentrations of their nanoparticle and the pure EVOH one were put into culture dishes coated with bacteria, respectively. The diameter of the bacterialstatic loops forming around the sample mat disks were measured for judging the antibacterial performance[29]. Fig 4 shows the results 24 hours into the antibacterial test. M

easured diameters of the bacterialstatic loops and their standard deviations (σ) at different time points are shown in Table. 1.



(a) (b)



(c) (d)

Fig.4 Antibacterial test of Ag-EVOH (a)(b), CuO-EVOH (c) and ZnO-EVOH (d) nanofiber mats for 24h

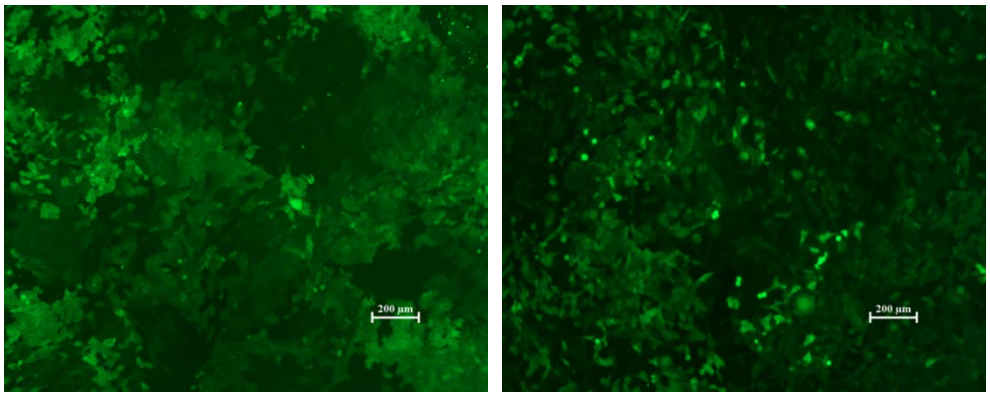
Table.1 bacterialstatic loops' diameters of Ag-EVOH, CuO-EVOH and ZnO-EVOH nanofiber mats at different time points

Concentration Time(h) bacterialstatic loops' diameters(mm) samples		Ag-EVOH	σ	CuO-EVOH	σ	ZnO-EVOH	σ
0.02g/ml	0	0	0	0	0	0	0
	6	2.65	0.19	1.67	0.21	1.95	0.12
	12	3.82	0.20	2.17	0.17	2.65	0.17
	18	3.95	0.22	2.26	0.18	2.80	0.15
	24	4.02	0.17	2.33	0.19	2.91	0.18
0.05g/ml	0	0	0	0	0	0	0
	6	3.42	0.15	2.46	0.14	2.69	0.19
	12	4.58	0.19	3.26	0.16	4.06	0.2
	18	4.86	0.16	3.49	0.11	4.38	0.17
	24	5.01	0.21	3.55	0.15	4.53	0.14
0.08g/ml	0	0	0	0	0	0	0
	6	3.86	0.17	2.95	0.17	3.16	0.18
	12	5.03	0.15	3.75	0.14	4.75	0.16
	18	5.62	0.18	4.01	0.16	5.07	0.15
	24	5.89	0.14	4.12	0.16	5.21	0.13

2.2.4 Cell Proliferation experiments

In order to examine sample mats' capacity to stimulate cell growths during the healing process, cell proliferation tests on the nanofiber mats were carried out. The cell used in this experiment was HUVEC-GFP and the culture medium was consisted of a growth medium and a trypsin digested

solution. The growth medium was mixed with a high glucose medium (H-DMEM), 10% of Gibco, 1% of Penicillin and streptomycin sulfate. The trypsin digested solution was consisted of three parts including PBS and 0.25% of trypsin and 0.02% of EDTA. After HUVEC-GFP cell were cultured for 2 days by the growth medium, inoculating them to 96 orifice plates (2000/hole), onto which a small piece of mat samples pre-treated by hydrophile were placed. The morphology and quantity of cells were observed by inverted fluorescence microscope after having been planted for 1 to 7 days under 37^{o}C. In addition, the absorbance (OD) of 450nm, which is a measurement of cell proliferation, was detected and assessed. Fig.5 shows the images of proliferation of HUVEC cells after culturing 7 days with pure EVOH nanofiber samples (5a) and 0.08g/ml Ag-EVOH nanofiber sample (5b).



(a) Pure EVOH nanofiber mat (b) EVOH nanofiber mat of 0.08g/ml Ag

Fig.5 the proliferation of HUVEC cells after culturing 7 days

3. Discussion

3.1 Influence of Temperature

When electrospinning was carried out for EVOH with metal or metal oxide at room temperature (20℃±2℃), there is a tendency of substance attachment to the outlet of the nozzle which hindered the formation of the Taylor Cone[XX] . Hence, different temperatures (30℃, 40℃, 50℃) were tried with other parameters kept consistent. We found substance attachment is at minimum at 40℃(±3℃). It can be explained in two reasons for this phenomenon. First, the solution in the syringe is prepared at 75℃, its temperature will drop rapidly at the room temperature resulting in the decrease of solubility and fluidity. Besides, the concentration of solute in the Taylor Cone at nozzle is high. Thus, solute would separate out easily at nozzle with lower temperature. This problem was solved effectively by increasing temperature properly. However when temperature was further increased, eg.

close to 60°C, blockages occurred frequently again. This is primarily due to the acceleration of solvent volatilization. The solvent that we used in this study is isopropanol with a boiling point of 82.45℃. Once the environmental temperature is sufficiently high, the solvent of the solution that forming the Taylor Cone would volatilize before ejecting, yielding in attachment to the nozzle. Also, the agglomeration of nanoparticles will be increasing. This is not conducive to the overall antibacterial ability of the nanofiber mat when it is used as wound dressing.

Fig.6 shows the morphology of 0.02g/ml nanofibers fabricated at 40℃ and the measurement of nanofibers' diameter. Each sample were measured for at least 5 parts' diameters and the trimmed mean was taken as the average value of this sample. Comparison of the results of the three samples under different temperatures (30℃, 40℃, 50℃) is given in Fig.7, showing a decreasing trend in nanofibers' diameter in terms of the fabrication temperature.

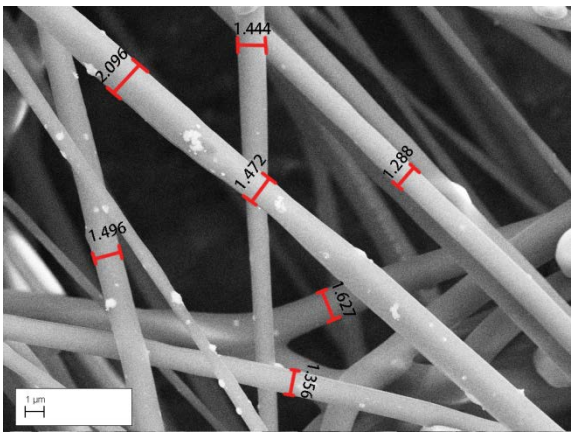


Fig.6 the SEM image of 0.02g/ml nanofibers fabricating at 40℃

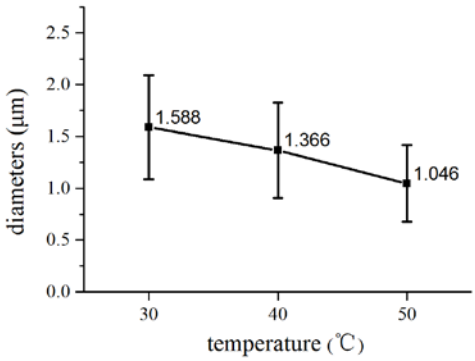
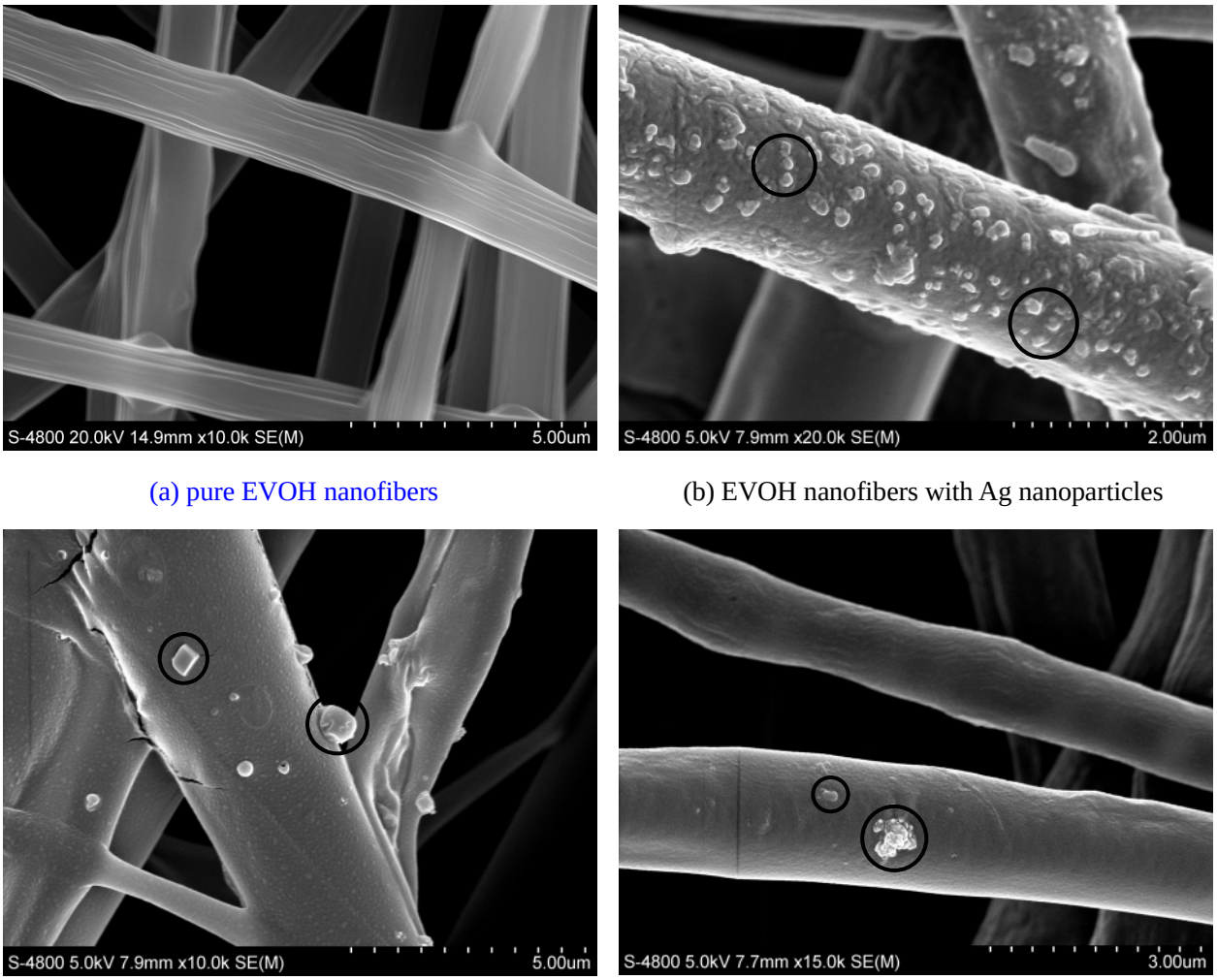


Fig.7 the influence of temperature on diameter of nanofibers

3.2 Antibacterial Ability

Fig.8 (b) to (d) shows the morphology of the Ag, CuO and ZnO nanoparitcles attached to the

nanofibers mats, respectively. Most of the particle size are less than 100nm, leading in their antibacterial abilities. Comparing the three images, it is clear that Ag particles are distributed most evenly. There is a little agglomeration of CuO and ZnO nanoparticles. The micro-characteristics will also have some effect on the antibacterial properties of nanofiber mats.



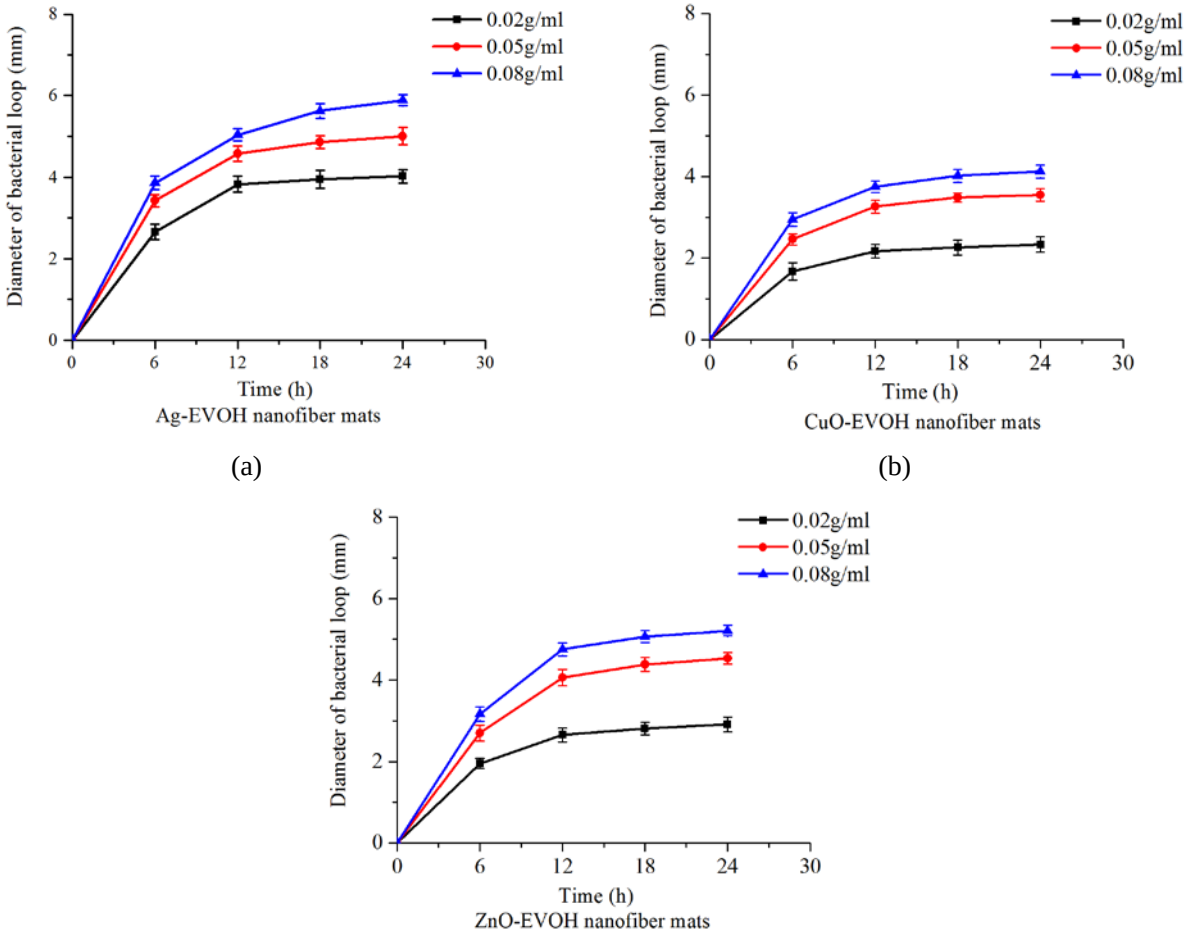
(a) pure EVOH nanofibers (b) EVOH nanofibers with Ag nanoparticles

(c) EVOH nanofibers with CuO nanoparticles (d) EVOH nanofibers with ZnO nanoparticles

Fig.8 morphology of nanoparticles of one single nanofiber

Fig.9 shows the results of antibacterial tests for the three kinds of nanofiber mats. From the results, it can conclude that Ag-EVOH, CuO-EVOH ZnO-EVOH nanofiber mats are all effective in eliminating Staphylococcus aureus. The antibacterial ability increases with the concentration of Ag, CuO and ZnO nanoparticles. However, the antibacterial ability of ZnO-EVOH nanofiber mats is increased inhomogenously compared to the Ag-EVOH and CuO-EVOH nanofiber mats, meanwhile, the antiseptic effect of the ZnO-EVOH nanofiber mats is weaker than Ag-EVOH nanofiber mats in the first 6 hours. The main reason could be the way of adding ZnO to EVOH solution. It is more difficult to produce the ZnO by the $Zn(CH_3OOH)_2$ than producing the Ag and CuO by the $AgNO_3$

and $Cu(NO_3)_2$. The ZnO-EVOH precursor solution was mixed by fully melt Zinc Acetate and dissolved EVOH solution, rather than adding $Zn(CH_3OOH)_2$ to EVOH solution directly like preparing Ag-EVOH and CuO-EVOH solutions. Therefore, the dispersion of ZnO nanoparticles in solution is in a different form comparing with the Ag and CuO nanoparticles. Fig.8 also provides the total surface area of ZnO nanoparticles is lower than the Ag and CuO nanoparticles, this phenomenon requires a longer time to release, therefore, this could be the reason that it works slowly. The antibacterial ability of CuO nanoparticles has been proven by others[23-24]. In this study, EVOH nanofiber mats encapsulated with CuO nanoparticles perform antibacterial ability in the Staphylococcus aureus.



(a) (b)

(c)

Fig.9 the results of antibacterial test for three kinds of nanofiber mats

3.3 Cell Proliferation

Fig.10 shows the values of absorbance OD of different kinds of samples. It indicates that after inoculation for 24 hours, HUVEC cells can be attached to the EVOH film in the shuttle forms. HUVEC cells proliferated on the Ag-EVOH, CuO-EVOH ZnO-EVOH nanofiber mats in a similar

trend. However, the growth of HUVEC cells on nanofiber mats with nanoparticles is slightly lower than that of pure EVOH nanofiber mats. This can also be regarded as a clear evidence of the biocompatibility of EVOH nanofiber mats which had been studied previously [30]. Note that the metals and metal oxides cannot be the base of cell proliferation, and their existence seems to have some, though apparently limited effect on inhibiting the growth of cells.

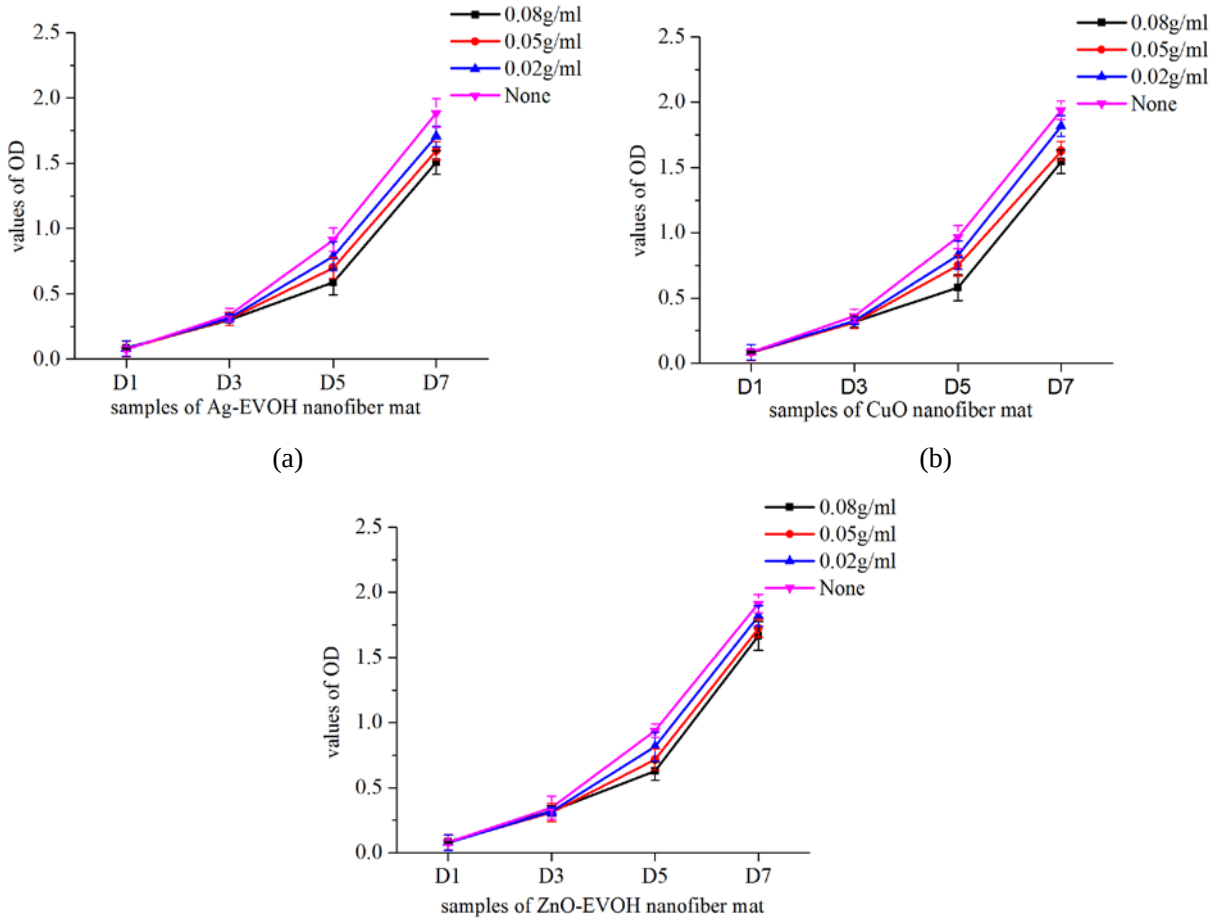


Fig.10 the values of OD of different kinds of samples

4. Conclusions

In this study, Ag-EVOH, CuO-EVOH and ZnO-EVOH nanofiber mats were fabricated by electrospinning. Experimental research was carried out for verifying the effect of temperature and the biological application performance of the mats obtained. These led to the following conclusions.

(1) Environmental temperature has a significant effect on the electrospinning process mainly by the phenomenon of needle blockage. The experimental investigations has led to the conclusion that the most suitable temperature for electrospinning of EVOH nanofiber mats encapsulated with Ag,

CuO and ZnO particles is about 40℃.

(2) In antibacterial tests using Staphylococcus aureus, Ag-EVOH nanofiber mats show the maximum antibacterial capacity while CuO-EVOH nanofiber mat the weakest. The antibacterial mechanisms of Ag, CuO and ZnO are complex[31-33]. These metal or metal oxide particles continuously release metal ions in aqueous media, which can penetrate cell walls of bacteria and destroy the activity of cell synthetase to make them lose their proliferation and division abilities. Metal ions are then freed from the cells after sterilization, and continue to kill other bacteria. This is the mechanism for the persistent antibiosis of metal or metal oxide.

(3) Cell proliferation experiments show that the HUVEC cells proliferate in a similar growth trend amongst all samples. However, the existence of metal and metal oxide particles on the surface of the fibres may hinder cell growth to a small extent compared with pure EVOH fibre mats.

This study shows that EVOH nanofiber mats encapsulated with metal or metal oxide (Ag, CuO and ZnO) nanoparticle by electrospinning have demonstrated antibacterial and cells growth capacities. With studies on other properties such as the mechanical and biodegradable properties, they could potentially be used as wound dressing materials for improved functionality in the wound healing process. Further study and more tests will be carried out on a broader range of samples and conditions.

Acknowledgement

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