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# Bimetallic Zn/Ag doped polyurethane spider net composite nanofibers: A novel multipurpose electrospun mat

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**Abstract**

The objective of this study was to develop a new class of bimetallic ZnO/Ag embedded polyurethane multi-functional nanocomposite by a straightforward approach. Bimetallic nanomaterials, composed of two unlike metal elements, are of greater interest than the monometallic materials because of their improved characteristics. In the present study the bimetallic composite was prepared using sol–gel via the facile electrospinning technique. The utilized sol–gel was composed of zinc oxide, silver and poly(urethane). The physicochemical properties of as-spun composite mats were determined by X-ray diffraction pattern, field emission scanning electron microscopy, energy dispersive X-ray spectroscopy and transmission electron microscopy. The antibacterial activity was tested using *Escherichia coli* as model organism. The antibacterial test showed that ZnO:Ag/polyurethane composite possesses superior antimicrobial activity than pristine PU and ZnO/PU hybrids. Furthermore, our results illustrate that the synergistic effect of ZnO and Ag resulted in the advanced antimicrobial action of bimetallic ZnO/Ag composite mat. The viability and proliferation properties of NIH 3T3 mouse fibroblast cells on the ZnO:Ag/polyurethane composite nanofibers were analyzed by *in vitro* cell compatibility test. Our results indicated the non-cytotoxic behavior of bimetallic ZnO:Ag/polyurethane nanofibers towards the fibroblast cell culture. In summary, novel ZnO:Ag/polyurethane composite nanofibers which possess large surface to volume ratio with excellent antimicrobial activity were fabricated. The unique combination of ZnO and Ag nanoparticles displayed potent bactericidal effect due to a synergism. Hence the electrospun bimetallic composite indicates the huge potential in water filtration, clinical and biomedical applications.


*Keywords:* Electrospinning; Bimetallic ZnO:Ag/PU composite; Antibacterial mechanism; *Escherichia coli*

## 1. Introduction

Nano-materials exhibit distinctive and appealing characteristics as compared to the bulk materials due to the small dimensions and large surface to volume ratios [1]. With the emergence of nanotechnology, scientists in all over the globe are paying extra attention in studying the unique properties of nanoscale materials. Modern trends in nanomaterials synthesis allocate preparation of hybrid nano-constructs with a variety of architectures such as nanowires, nanoflower, nanofibers and so on, through various synthetic routes. Electrospinning, an electrostatic fiber manufacture practice, has substantiated intense notice and attention during recent years due to its flexibility and potential for applications in diverse fields. The noteworthy applications include tissue engineering, biosensors, filtration, wound dressings, drug delivery, enzyme immobilization [2–5] and so on. Polyurethane (PU) is a thermoplastic polymer having excellent mechanical properties and water

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insolubility [6,7]. Boretos and Pierce first suggested the use of PU elastomers as biomaterials [8]. Since then this family of polymers has found a wide range of biomedical applications due to a number of useful properties such as biocompatibiliy, good hydrolytic and oxidative biostability and processability. Recently, spider net-like structure mats containing interlinked nanowires with ultrathin diameter have been identified. Ding and co-workers firstly reported the formation of nylon 6 and poly(acrylic acid) nano-nets, and concluded that nano-nets can be obtained by adjusting the solution properties and several parameters of the electrospinning process [9]. PU nanofibrous mats containing net like structures have been synthesized by the addition of multiwalled carbon nanotube [10]. Furthermore, this net formation pattern had also been observed in PU nanofibers when different blends of ionic salts complement to PU sol–gel [11]. In recent years, antibacterial nanomaterials have also attracted great interests. In fact, with the appearance and increase of microbial strain resistants to multiple antibiotics, many research scientists have tried to develop new antibiotics. Generally speaking, a great deal of work has been performed on the synthesis and development of metal-based nanocomposite materials. Especially, bimetallic nanoparticles composed of two different metal elements are of greater interest than the monometallic ones for the enhancement of the desired specific properties of metal particles. This is because bimetallization can improve the properties of the original single-metal and create a novel hybrid property, which may not be achieved by monometallic materials. Earlier workers vigorously studied inorganic oxide-supported bimetallic nanoparticles for catalysis [12]. In contrast, relatively very lesser number of studies are conducted on the colloidal dispersions of bimetallic nanoparticles embedded into polymer nanofibers for antibacterial purpose [13,14].

We herein communicate the fabrication of bimetallic (Zn/Ag) doped PU nanofibers with distinctive spider-net morphology. Nevertheless, silver among inorganic nanoparticles have been employed more extensively as antibacterial agents [13,15,16]. Ag nanoparticles have proved to have strong inhibitory and bactericidal effect [17,18]. Many Ag composites have been used as bactericidal materials [19–21]. On the other hand, some studies have also exposed the antibacterial effect of zinc oxide containing nanostructured material [22–24]. Despite a number of investigations on the antibacterial effect of nanostructured silver and zinc oxide materials, to the best of our knowledge there is no published report about the synergistic antibacterial effect of electrospun bimetallic Zn/Ag doped polyurethane composite spider net nanofibers hitherto. In this study, for the first time, a novel Zn/Ag doped PU nanocomposite mat with high antibacterial activities was successfully prepared via sol–gel electrospinning process. Besides, various physicochemical properties of electrospun nanocomposites were investigated. Finally, the cytocompatibility of fabricated nanocomposites was studied using MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide) assay. Thus on the basis of present results we can conclude that the prepared nanocomposites with unique spider-net morphology containing zinc and silver NPs could be employed for a variety of biological applications, such as an ideal wound dressing (which provide the best environment for healing and reduction of bacterial resistance simultaneously), scaffolds for tissue engineering, water and air filtration process and so on.

## 2. Materials and methods

### 2.1. Chemicals, bacterial culture and cell line system

Polyurethane (PU, MW = 110,000, medical grade) was purchased from Cardio Technology Internship, Japan. Zinc oxide and silver powder (99% purity) were purchased from Sigma-Aldrich. Tetrahydrofuran (THF) and *N*,*N*-dimethylformamide (DMF) (analytical grade; Showa Chemicals Ltd., Japan) were used as solvents without further purification. Tryptone soy broth from Torlak, Belgrade; BD Diagnostic, Becton, Dickinson & Co., USA was used as a growth medium. For checking antimicrobial activity *Escherichia coli* ATCC 52922 strain was purchased from American Type Culture Collection (ATCC). Dulbecco's modified Eagle's Medium (DMEM, ATCC) supplemented with a 10% fetal calf serum was purchased from Invitrogen, Grand Island, NY. NIH 3T3 mouse fibroblast cells were purchased from ATCC (CRL-1658$^{TM}$). Penicillin/streptomycin (Gibco$^{TM}$), 0.4% Trypan Blue dye (Invitrogen), 96 wells microplate (Corning, NY), MTT reagent (Sigma, St. Louis, MO), $\geq$ 99.9% DMSO (Sigma-Aldrich), glycine buffer (0.1 M glycine from Bio-Rad plus 0.1 M NaCl from sigma and equilibrated to pH 10.5 with 0.1 N NaOH) were used as received.

### 2.2. Synthesis of bimetallic nanocomposites via electrospinning

The fabrication of bimetallic nanocomposites was carried out by the simple electrospinning process. Briefly, PU (10 wt%) solution was prepared by dissolving PU in DMF:THF (1:1 w/w) under magnetic stirring overnight at room temperature. 1.0 wt% of ZnO powder was added separately into the polymer solution under vigorous stirring for synthesis of ZnO doped PU fiber mats. For the synthesis of ZnO:Ag/polyurethane composite solution, 0.5 wt% of ZnO and 0.5 wt% of Ag were added together in the polymer solution under vigorous stirring. The obtained sol–gel was transferred into a 10 ml syringe. A copper pin connected to a high voltage generator was inserted in the solution as a positive terminal whereas a ground iron drum covered by a polyethylene sheet served as counter electrode. The solution was kept in the capillary by adjusting the inclination angle. A voltage of 15 kV was applied to this solution. The distance between the tip of the needle and the collector was fixed at 15 cm. The as-spun composite nanofibers were dried at 60 °C for 24 h under vacuum.

### 2.3. Antibacterial assessment of nanocomposites

The antibacterial efficacies of pristine PU, ZnO doped PU and ZnO:Ag/polyurethane composite spider-net nanofibrous mats were studied using the growth inhibition studies as described by Li et al. [25] with suitable modifications against model organism *E. coli*. Briefly, the inoculum was prepared from fresh overnight broth culture grown in Tryptone soy broth (pH—7.3) supplemented with yeast extract (0.6%) that were incubated at 37 °C. For antibacterial assay, the bacterial strains were first grown in solid nutrient medium and from the agar plates fresh colonies were inoculated into the broth (100 ml). Growth was monitored at every 3 h by UV–visible spectrophotometer (UV-2550, Shimadzu, Japan), till the optical density (OD) reaches 0.1 at 600 nm (OD of 0.1 corresponded to a concentration of $10^8$ CFU/ml of medium). Subsequently, 1 ml inocula from the above were further added to 100 ml of freshly prepared broth supplemented with pristine PU, ZnO doped PU and ZnO:Ag/polyurethane composite mats. All the flasks were incubated in a rotary shaker (rpm—150 and temperature 37 °C). Uninoculated and inoculated controls (without nanocomposite material) were also kept in parallel. The growth rates were monitored by measuring the OD as described above at an interval of 3 h for 15 h. Agar (18% w/v) was added to the nutrient solution to make plates. The zinc oxide/silver doped polyurethane electrospun nanofiber solution was mixed with the bacterial culture and spread (approximately, 100 μl culture broth was taken from the cultures) on the plates. All the inoculated petri dishes were incubated at 37 °C for 24 h and the number of colony forming units (CFUs) was counted.

### 2.4. Cell culture studies

In brief, the fibroblast population was efficiently raised from available cryogenic vial to 25 ml culture flask in DMEM medium, this was supplemented with 10% fetal calf serum and 1% penicillin and streptomycin solution and incubated in a humidified incubator with 5% $CO_2$ environment at 37 °C temperature. After receiving the 90% confluent growth, the cell population was subcultured to reach the cell number 25,000 cells/ml; this cell number was maintained for further cell seeding to check the effect of nanocomposites. 160 μl of the 25,000 cell/ml solution was added to experimental microplate wells and allowed to grow for 24 h to create a favorable environment before the synthesized composite nanofibers were introduced. After the 24 h incubation of initial cell seeding, the media were taken out and 80 μl of fresh media was added to the wells. At this point, the nanofibers (which were previously sterilized by exposure of UV light) were added to the 96 wells microplates (in triplicates) and 80 μl of fresh media was added to each experimental well in order to have a final volume of 160 μl. For each sample, a control was added in order to evaluate cell growth, so that a direct comparison could be attained. Finally, the microplates were incubated at 37 °C under the same conditions as aforementioned for 2, 3 and 5 days. During the incubation time, exhausted medium was replaced with fresh medium.

### 2.5. Cell viability test

The MTT assay was used to check the cell viability in the presence of pristine PU, ZnO doped PU, and ZnO:Ag/polyurethane composite spider-web nanofibrous mats by simple production of reducing cell enzymes, which can react with MTT salts to form formazan dyes, giving absorbance under UV spectrophotometer. In order to use this helpful tool, MTT reagent (5 mg/ml) in PBS was made by following stepwise methodology. In brief, media from the microplates, after 2, 3 and 5 days incubation time, were taken out and replaced with a 160 μl of fresh media without phenol red; to these wells 40 μl of the MTT reagent was added, and the microplates were incubated for 4 h. After incubation, the microplates were centrifuged at 3500 rpm to get cell debris, which was separated by decanting the supernatant with the help of aspirator. Further on, these plates were subjected to vacuum drying which was followed by the addition of 160 μl of $\geq$ 99.9% DMSO and 20 μl of glycine buffer (0.1 M glycine plus 0.1 M NaCl and equilibrated to pH 10.5 with 0.1 N NaOH). These microplates were placed in a shaker for 5 min to let the formazan crystals dissolve followed by the nanofiber mats removal from each well. Subsequently, cell viability was checked using a microplate reader (Model 680) at the wave number of 595 nm.

### 2.6. Characterization

The XRD patterns of the synthesized mats were recorded on a Rigaku/Max-3A X-ray diffractometer (XRD, Rigaku Co., Japan) with Cu Kα radiation ($\lambda = 1.540$ Å) over the Bragg angles ranging from 20° to 60° and the operating voltage and current were maintained at 30 kV and 40 mA, respectively. The morphology of as-spun composite nanofibers was observed by field-emission scanning electron microscopy (FESEM, S-7400, Hitachi, Japan). The samples were uniformly sprayed on carbon tape, Pt coating was applied for 10 s onto the synthesized nanofibers and the images were acquired at various magnifications. The fiber diameter was measured directly from SEM images. The microscopic features and elemental composition of composite fibers were examined by transmission electron microscopy (TEM, H-7650, Hitachi, Japan) equipped with energy dispersive X-ray (EDX) analyzer.

In order to observe cells attached to the surfaces of composite nanofibers, chemical fixation of cells was carried out in each sample after 5 days of incubation. Nanofiber samples were rinsed twice with phosphate buffer saline (PBS) and subsequently fixed in 2.5% glutaraldehyde for 1 h. After cell fixation, samples were rinsed with distilled water and then dehydrated with graded concentrations

(i.e., 20%, 30%, 50%, 70% and 100%) of ethanol for 10 min each. Finally, the samples were kept in a vacuum oven and then observed for the cell morphology.

## 3. Results and discussion

Fig. 1 shows the FE–SEM micrographs of pristine PU, ZnO doped PU, and ZnO:Ag doped PU composite nanofibrous mats. Pristine PU (Fig. 1a) nanofibers had a bead free, smooth surface with uniform diameters along their lengths. The plain and doped PU samples have diameters in the range of 200–300 nm with Ag/Zn in/or on the PU nanofibers encompassing unique interconnected spider-net like structures (Fig. 1b, c). The formation of the net like structure can be interpreted due to the ionization of the polymer solution in the presence of metal oxides (Ag and ZnO) and the phase separation of charged droplets created during electrospinning. The increase of ions in the composite solution (ZnO:Ag/PU) can initiate the splitting of main nanofibers into sub-nanofibers, forming the spider-net morphology during electrospinning as previously reported [26]. Other workers have also observed the formation of highly interconnected spider-net like structure in PU nanofibrous mats with the supplementation of multiwalled carbon nanotubes [10]. Moreover, the spider-net has also been attained with different polymers and salts [11]. Additionally, net like structures have also been observed in nylon 6 mats with the addition of a small amount of $TiO_2$ nanoparticles [13]. Interestingly, our observations are consistent with the previous results. This harbored ultrafine nanofiber networks and produces an enhanced surface area and assists in the diffusion of analytes into the mats, which can be potentially useful for numerous applications. The presence of ZnO and Ag NPs in the dispersed composite was further confirmed by XRD, as shown in Fig. 2. In case of pristine PU (Fig. 2a), no peak appeared in spectrum indicating the amorphous nature of the polymer [27]. On the other hand in the spectrum of Zn doped PU nanofibers (Fig. 1b), two strong bands appeared with maximum intensity at 31.2° and 35.8° representing Bragg's reflection from (100) and (101) planes of ZnO (JCPDS no. 891397), implies the presence of crystalline zinc oxide. Whereas in ZnO:Ag doped PU composite, the peak at 38.858° and 43.77° representing Bragg's reflection from (100) and (101) planes of Ag (JCPDS no. 893722), implies the presence of Ag (Fig. 1c). Thus, the XRD spectrum clearly indicated the presence of ZnO and Ag particles in the composite nanofibrous mats. The EDX spectrum (Fig. 3) of the ZnO:Ag/polyurethane composite nanofibers contains C, Zn, Ag and O; no other element is detected, indicating the final product is free of impurity and composed only of

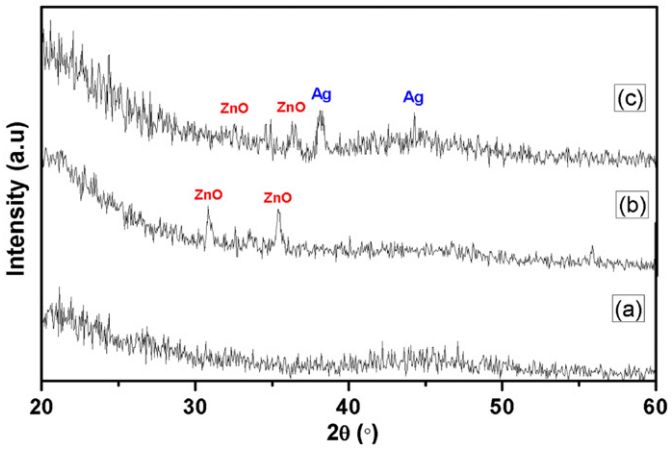



Fig. 2. XRD pattern of (a) Pristine PU (b) ZnO doped PU and (c) ZnO/Ag doped PU composite nanofibers.

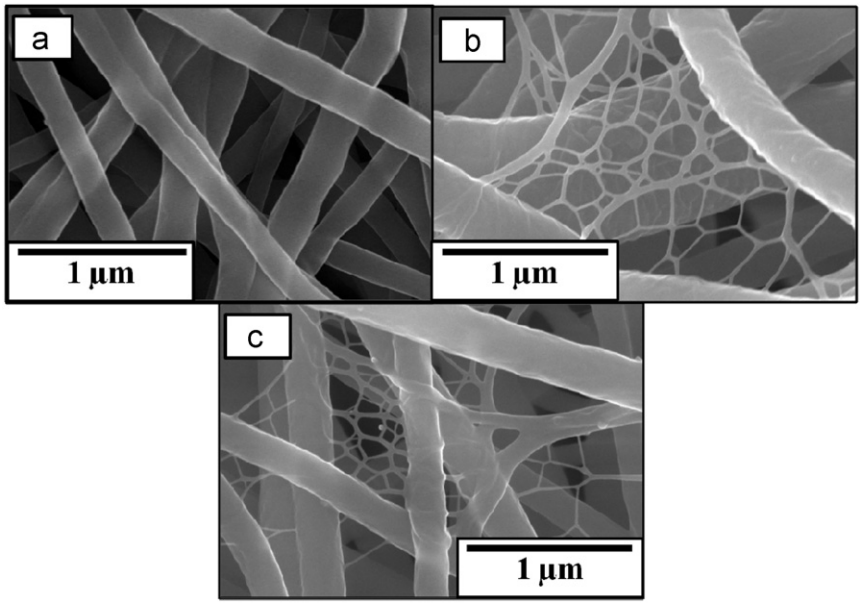



Fig. 1. FE–SEM micrographs of (a) Pristine PU (b) ZnO doped PU and (c) ZnO/Ag doped PU composite nanofibers.

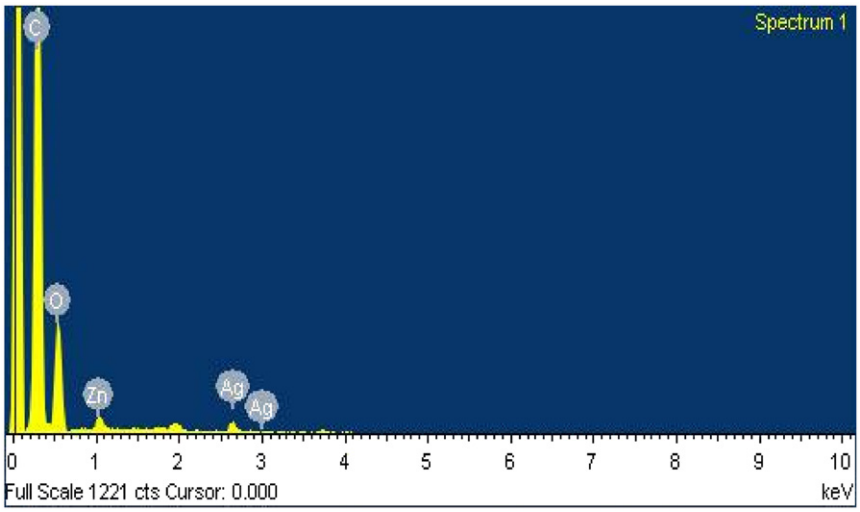



Fig. 3. EDX spectrum of ZnO/Ag doped PU composite nanofibers.

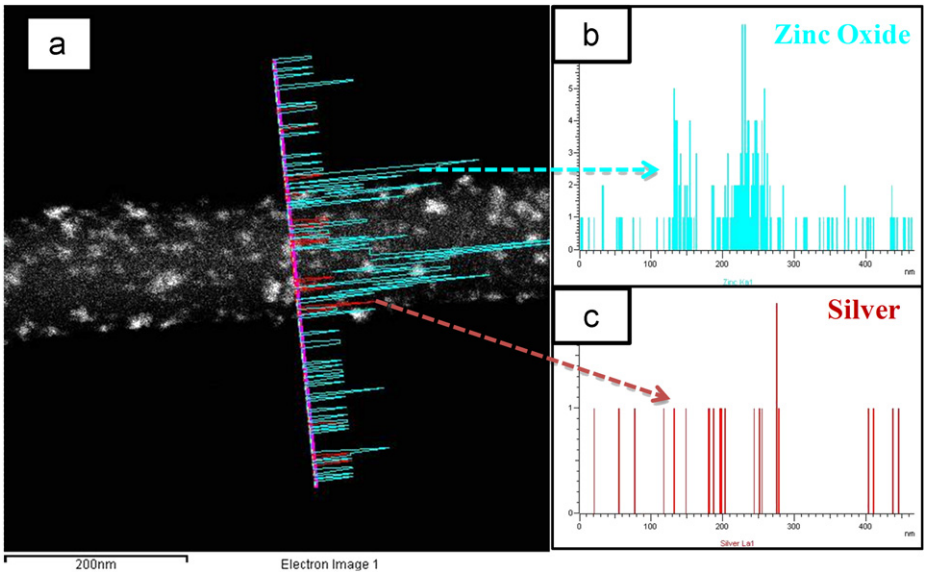



Fig. 4. (a) TEM images of ZnO/Ag doped PU composite nanofibers along with the linear TEM–EDX for (b) ZnO and (c) Ag.

ZnO, Ag and C. To investigate the homogeneous distribution of ZnO and Ag in the produced PU nanocomposite, linear analysis TEM–EDX was utilized. Fig. 4 shows that both ZnO and Ag are found along the selected line, which confirmed that both the oxides are mixed at the crystalline level. It was clearly noted that the signal corresponding to ZnO and Ag was observed by EDX line mapping measurement as shown in Fig. 4(b) and (c).

Antibacterial properties of the ZnO doped PU and ZnO:Ag doped PU electrospun nanofibers were tested using *E. coli* as model organism. For comparison, results for pristine PU nanofibers are also shown (Fig. 5a). PU nanofibers without silver/zinc compounds showed a little antibacterial activity. Conversely, ZnO/PU hybrid nanofibers showed significant activity. Nevertheless, ZnO:Ag doped PU electrospun showed complete inhibition of *E. coli* indicating that the nanofibers are endowed with excellent antibacterial properties due to the introduction of Ag and ZnO nanoparticles. Marked differences in growth rates have been noticed during 3–12 h of incubation period. In case of control samples the logarithmic phase was found to be extended from 3 h to 9 h or more (Fig. 5a). ZnO:Ag doped PU composite nanofibers have shown effective antibacterial activity against *E. coli*. Noticeable inhibition has been observed by ZnO:Ag doped PU nanofibers during 3–15 h of incubation period. The effect of ZnO:Ag doped PU nanofibers on colony forming units (CFUs) of *E. coli* was also observed on solid agar plates (Fig. 5b–d). As expected, the number of CFUs decreased drastically in the presence of ZnO–Ag doped PU nanofibers. Photographs of agar plates plated with the control cell suspension and those exposed to ZnO/PU and

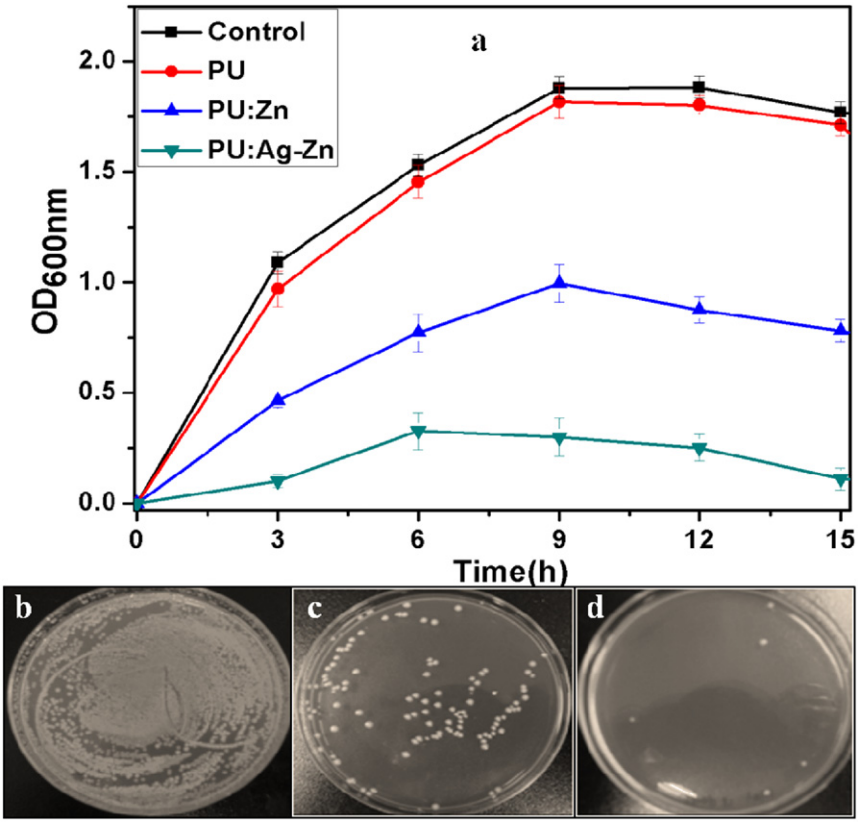
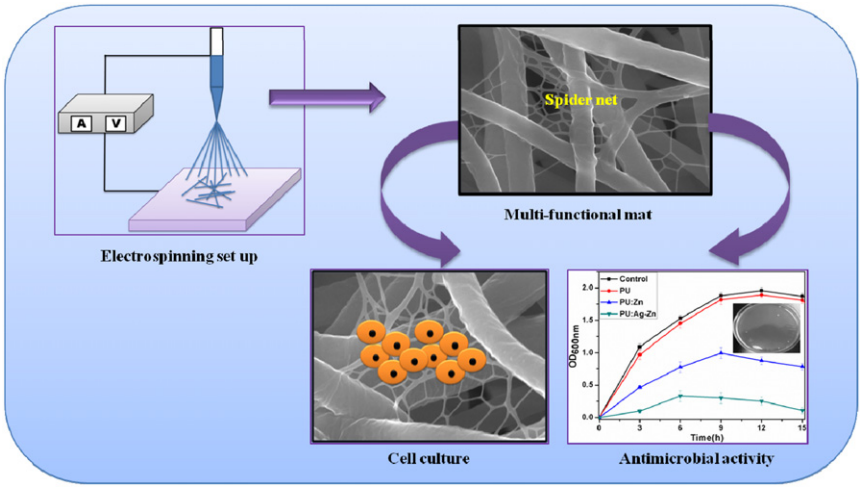



Fig. 5. (a) Growth curve of *E. coli* in the presence of pristine PU, ZnO doped PU and ZnO/Ag doped PU composite nanofibers as a function of contact time. Representative digital Plate images of (b) unexposed conrol (c) exposed to ZnO doped PU and (d) exposed to bimatellic ZnO:Ag composite nanofibers.

ZnO:Ag doped PU hybrid nanofibers are shown in Fig. 5(b–d). The absence of colony-forming units on the plates exposed to ZnO:Ag doped PU hybrid nanofibers suggests a complete kill effect. Antibacterial nanofibers have attracted huge interests in recent years. In fact, with the emergence and increase of microbial resistant to multiple antibiotics, many researchers/scientists have tried to develop new antibiotics. The aim of this research is to compare the antibacterial activity of mono-metallic with bimetallic composite nanofibers using *E. coli* as a model organism. There are few reports on the antimicrobial properties of ZnO nanoparticles against *Staphylococcus aureus* and *E. coli* [23,24]. Nevertheless, the antibacterial activity of the silver is well known; therefore, it is expected that the electrospun nanofiberous mats will have fine antibacterial action. Earlier workers have also reported the antibacterial effect of ZnO [22]. Silver is a widely used and recognized broad spectrum biocidal agent that is effective against bacteria, fungi and viruses but is non-toxic to human cells [28–32]. For silver-based antibacterial materials, the most critical factor is the silver release behavior, which can inhibit the growth of bacteria. It has been reported that a steady and prolonged release of silver at a concentration level as low as 0.1 parts per billion can render effective antimicrobial activity [33]. However the exact mechanism is not fully clear but some researchers [34,35] share the opinion that Ag+ hinders DNA replication and inhibits the expression of ribosomal proteins and enzymes for ATP hydrolysis. It is believed that Ag nanoparticles display the same mechanism as Ag+ and create a redox imbalance, which causes extensive bacterial death. In the present investigation, the bimetallic ZnO:Ag doped PU composite nanofibers demonstrated excellent bactericidal effect (Scheme 1).

In order to investigate the toxicity of as-spun composite we selected a mouse fibroblasts cell system. The fibroblasts were cultured *in vitro* to determine the silver effect on morphology and cell distribution. Fig. 6a shows the data originated from the MTT assay after 2, 3 and 5 days of incubation time. From the figure, it is evident that the growth in case of control cell shows the 100% cell growth. Meanwhile, the fibroblasts cultured in the presence of bimetallic composite showed a similar trend as that of the control cells. Overall, the as-spun (ZnO:Ag/PU) composite showed almost confluent growth during the entire culture period. It is also observed that the growth proceeds in an exponential manner, while days of incubation pass on (i.e., 2, 3 and 5 days). These results further clarify that the prepared nanofibers are non-toxic to the cells; therefore, they are used in wound dressing applications. Fig. 6(b, c) shows the results of SEM analyses done after cell fixation. The images were obtained from 3 days of incubation of fibroblasts while growing them in the presence of the composite bimetallic mat. Data originated from these




Scheme 1. Illustration of the steps involved in the fabrication of composite nanofibers by electrospinning, antibacterial test and proliferation of cells on the nanocomposite.

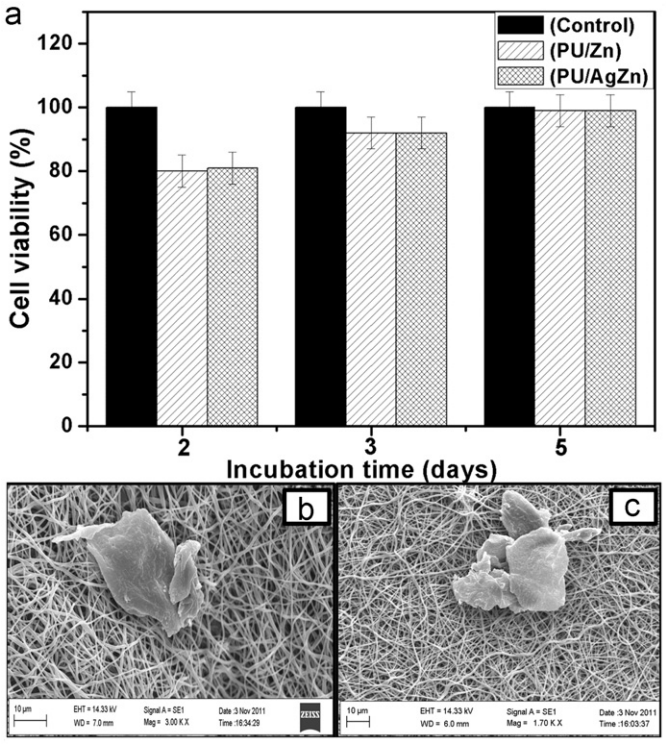



Fig. 6. (a) MTT assay of control and composite nanofibers, the viability of control was set 100%, and viability relative to the control was expressed. The experiments were conducted at least in triplicate. Representative SEM images of the cell fixation test on (b) ZnO doped PU and (c) ZnO/Ag doped PU composite matrix, after 5 days of incubation period.

observations (Fig. 6) indicate that fibroblasts are proliferating in a healthy manner; there are no stress-related cells present on the surfaces of nanofibers. Overall, the treated cells showed no obvious detrimental effects such as cell aggregation, distortion, or lysis when compared with the controls. Thus we can conclude that the prepared bimetallic composites are depicted as a promising candidate to be used as an excellent antibacterial agent and should provide another attractive approach to be utilized in multiple applications such as material for defensive fabrics, water filtration, wound dressing, protective masks for research laboratories, in medical and food packaging fields and so on.

## 4. Conclusions

In conclusion, novel bimetallic ZnO:Ag doped PU composite nanofibers with unique spider nets were synthesized via the facile electrospinning method. The morphology of synthesized nanofibers was characterized by SEM, FE–SEM and TEM whereas the crystallinity was analyzed by XRD pattern. The results of antimicrobial test indicated that the combination of the different ZnO and Ag nanoparticles embedded in PU composite had a synergistic bactericidal effect without any detrimental effects upon normal mouse fibroblast cells, indicating the great potential of synthesized composite mats in relevant clinical, textile, biomedical and filtration applications.

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