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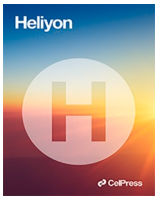

Research article

Fabrication and evaluation of a bi-layered electrospun PCL/PVA patch for wound healing: Release of vitamins and silver nanoparticle

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ABSTRACT

There is still little research on the co-delivery of vitamins and AgNPs to accelerate wound healing. In this study, a bi-layered electrospun PCL/PVA patch loaded with Vitamin C, Vitamin B12, and AgNPs was fabricated using a co-spinning technique. SEM, FTIR, degradation, swelling, tensile strength, disk diffusion, and MTT assay were studied. Nine rats were placed in three groups (control: no treatment, G1: without agents, and G2: with agents) for 14 days in an in-vivo study. H&E and Masson Trichrome staining were employed for histological analysis. Results showed that the final electrospun wound dressings depicted nanofibers with diameters ranging from 100 to 500 nm. The presence of AgNP enhanced the mechanical strength (40–50 MPs). An appropriate swelling (100 %) and degradation (50 %) rate was observed for groups with no significant difference ($P > 0.05$). G1 and G2 did not show a significant difference in terms of porosity (65 % vs. 69 %). Regarding WVTR, G2 demonstrated higher WVTR (88 vs. 95 g/m^2. h). G2 showed a vitamin release of more than 90 % after 48 h. Compared to G1, G2 demonstrated good antibacterial activity (>3 cm) against E. Coli and S. aureous ($P < 0.01$), with cell viability of more than 93 % ($P > 0.05$). Furthermore, the in-vivo study approved that G2 accelerated wound healing in full-thickness wounds, compared to the control groups, with notable wound size reduction (8 mm), epithelialization, and collagen formation. The findings support the use of this simple but potent electrospun wound dressing for the healing of full-thickness wounds.

1. Introduction

Wound healing is a complex process that involves various stages, including inflammation, proliferation, and remodeling. Novel wound dressings are developed by loading therapeutic agents like vitamins, herbal extracts, drugs, growth factors, etc. [1–3]. Vitamins

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play an essential role in wound healing by facilitating these stages. Besides, vitamins showed they can improve signaling in tissue reconstruction [2]. Co-delivery of vitamins and antibacterial agents has attracted the attention of scientists in developing wound dressings [4].

Vitamin C (VitC) is a potent antioxidant that plays a crucial role in collagen synthesis [5]. Collagen is a protein that is essential for wound healing as it provides structural support to tissues. VitC also promotes the proliferation of fibroblasts, which are cells responsible for producing collagen. Studies have shown that individuals with VitC deficiency have delayed wound healing [6]. Therefore, it is crucial to ensure an adequate intake of VitC to promote wound healing. Such an idea has been demonstrated by Esmaeilzadeh and his colleagues by encapsulation of VitC in electrospun polycaprolactone (PCL)/Gelatin [4]. Vitamin B (VitB) is a complex of water-soluble vitamins that play an essential role in the human body. One of the most well-known functions of VitB is its role in energy metabolism, but it also has other important functions, such as supporting the nervous system, promoting healthy skin, and aiding in the formation of red blood cells. In particular, vitamin B12 (VitB12) is important in the healing of skin wounds. One study found that patients with low levels of VitB12 had a slower rate of wound healing than those with normal levels [7]. Vitamin B6 is another vitamin that may be important for wound healing. It has been found to help with the formation of collagen, a protein that is essential for the structure and strength of skin, tendons, and other tissues. In addition, vitamin B6 has been found to have anti-inflammatory properties, which may help to reduce swelling and promote healing [8].

Vitamin A (VitA) is another essential nutrient that plays a vital role in wound healing by controlling the inflammatory response. It promotes the proliferation of epithelial cells, which are the cells responsible for covering the wound. VitA also plays a crucial role in the inflammatory phase of wound healing by enhancing the function of immune cells [9]. Studies have shown that individuals with VitA deficiency have delayed wound healing. Vitamin E (VitE) is a potent antioxidant that protects cell membranes from oxidative damage. It also promotes collagen synthesis and enhances the function of immune cells. Studies have shown that individuals with vitE deficiency have impaired wound healing [10]. Therefore, it is crucial to ensure an adequate intake of vitE to promote wound healing.

Embedding vitamins in wound dressing is a promising approach to enhance wound healing. Vitamin-enriched dressings may have antioxidant properties, which are desired in the management of chronic wounds characterized by excessive reactive oxygen species generation [11]. Studies showed that a nanofiber-based wound dressing integrated with vitamin D held promise for reducing the risk of surgical site infections [12]. In a different study, it was discovered that hydrogel containing 3000 IU of vitamin D3 performed best and caused the greatest amounts of re-epithelialization and granular tissue formation [13]. Atila and his colleagues fabricated a VitC-loaded Pullulan hydrogel-immobilized bacterial cellulose membranes for wound healing purposes. It was discovered that VitC & VtE had a synergistic effect on collagen synthesis, wound healing, and antioxidant activity [14]. Farzanfar et al. loaded VitB12 in a PCL/gelatin nanofibrous scaffold. Results depicted that comparing VitB12-containing dressings to VitB12-free scaffolds, the findings demonstrated that after 14 days, the former could significantly improve wound closure [15].

The claimed information above confirms the efficacy of the delivery of vitamins for better wound healing. However, the lack of antibacterial agents and co-delivery of both vitamins and antibacterial agents seem to reduce the potential of a novel wound dressing. Silver nanoparticles (AgNPs) have been shown to have several beneficial effects in wound healing, including antimicrobial properties and promotion of wound healing. They can penetrate bacterial cell membranes and cause disruption, leading to the release of intracellular metabolites [16]. AgNPs can also release silver ions to generate reactive oxygen species and induce oxidative stress, which can further damage bacterial cells [17]. AgNPs can improve the proliferation and migration of keratinocytes, which are important cells in the process of wound healing [18]. They can also promote angiogenesis, which is the formation of new blood vessels, and stimulate the production of collagen, a protein that helps to strengthen and repair damaged tissue. AgNPs can also reduce inflammation and scarring in wounds [19].

There are different strategies to fabricate scaffolds based on the electrospinning methods [20]. Shahverdi et al. utilized a two-nozzle electrospinning method to create hybrid nanofibers for wound dressing. These nanofibers consisted of chitosan/polyvinyl alcohol (CS/PVA) and PCL, with a 50:50 ratio, and were enhanced with $CeAlO_3$ NPs. The researchers concluded that these nanofibers have the potential to be an effective and cost-efficient wound-dressing option [21]. In another study, Saeed et al. focused on the development of a novel multilayer active wound dressing using the electro-spun process. The dressing consists of PCL, PVA, and curcumin as a biologically active compound. The main objective of this dressing was to manage wound exudates and provide anti-bacterial properties. The multilayer dressing was designed to have water absorbability, anti-bacterial activity, and biocompatibility. The results showed that the dressing with PCL/curcumin and a PVA layer had optimized thickness and was able to absorb three times more exudates compared to a standard dressing [22]. Ali Kamali et al. discussed a bilayer structure for a skin scaffold, composed of a co-electrospun PCL/PVA layer and a CS/gelatin hydrogel layer. The results showed that the combination of the hydrogel and electrospun layers significantly increased tensile strength and elastic modulus values compared to the single-layer hydrogel sample. Although there were no significant differences in swelling and cell proliferation between the two groups, the bilayer scaffold performed better in the in vivo analysis [23].

Research studies have demonstrated that a bilayer mat configuration containing layers of both hydrophilic and hydrophobic materials can efficiently channel wound exudates (i.e., water seeps through the hydrophobic layer and advances towards the hydrophilic layer) while also providing remarkable antibacterial features [24]. Additionally, it can prevent any further damage to the wound (for example, the bonding of hydrophobic fibers to the surface of the wound) [25].

Based on our survey, co-delivery of VtB, VitC, and AgNPs from a bilayered PCL/PVA Electrospun patch has not been under study. To achieve this, it was tried to fabricate a bi-layered electrospun PCL/PVA wound dressing in which the first layer (made of PCL) plays a critical role in controlling the mechanical properties of the final wound dressing. The PCL layer did not contain any drugs, but the second layer (made of PVA), contained distinct concentrations of the aforementioned vitamins and AgNPs. This study was performed in in-vitro and in-vivo sections. The main purposes can be justified by the synergistic impact of the vitamins and AgNPs on the final

wound healing quality.

2. Material and methods

2.1. Materials

PCL (Mw: 80,000, Sigma–Aldrich) and PVA (Mw:85,000, Sigma–Aldrich) were purchased from a local supplier "Alborz Shimi Co.", Tehran, Iran. VitB12 and VitC were provided by a local drugstore. AgNPs were purchased from Arminano Co., Pardis, Iran. Chloroform (Merk), Ethanol (Merck), Dimethyl sulfoxide (DMSO, Merck), 3- (4,5-dimethyltiazole-2-yl)- 2,5-diphenyltetrazolium bromide (MTT, Merck), Hematoxylin & Eosin, and Masson Trichrome were purchased from Sigma–Aldrich (local supplier, TamadKala CO., Tehran, Iran). L929 cell lines were purchased from the Pasteur Institute of Iran. All other chemicals were of reagent grade.

2.2. Solution preparation (Grouping (Cont, G1, G2))

Firstly, The PCL solution (12 % w/v) was prepared by dissolving PCL in Chloroform/Ethanol (7:3) solvent (S1). Simultaneously, the PVA solution (10 % w/v) was prepared by dissolving PVA in distilled water (S2). Both solutions were left to mix overnight. Afterward, specific quantities of Vt B12 and VitC were added to the (S2) solution and stirred for 30 min to achieve a uniform distribution. Subsequently, the synthesized AgNPs were mixed into the PVA/Vitamins solution, and the mixture, including PCL and PVA/Vitamins/AgNPs solutions, was stirred for 3 h to ensure proper integration.

2.3. Fabrication of wound dressing (freeze thawing (crosslinking))

The electrospinning process was conducted using the Nanorisan Sepanta electrospinning machine (Tehran, Iran). Two separate 5-ml syringes were used to hold the polymer solutions (S1 and S2), which were connected to the syringe pump. The nanofibers fabrication process involved a two-step process. Initially, the PCL nanofibers were electrospun for 4 h (Voltage = 12 kV, distance = 10 cm, and flow rate = 0.4 ml/h). Afterward, the second layer containing the PVA was electrospun for 1 h simultaneously (Voltage = 10 kV, distance = 10 cm, and flow rate = 0.2 ml/h). Then, the PCl pump was turned off and the PVA electrospinning was continued for 5 h under specific conditions: (Voltage = 20 kV, distance = 15 cm, and flow rate = 0.5 ml/h). Note: when two syringe pumps are working their current field affects each other, so when one of them is off the operational parameter must be adjusted.

2.4. SEM

The morphology, surface roughness of the electrospun mats, and the evaluation of the NaAl NPs were analyzed using a scanning electron microscope (SEM FEI Quanta 200) under a 25 kV accelerated voltage. For this purpose, the surface of the sample was covered with gold coating and then photography was done.

2.5. FTIR

FT-IR spectroscopy (JASCO-4600 spectrophotometer, USA) was used to determine the FTIR spectra of each mat. The corresponding spectrum was captured in the 4000-400 cm^{-1} wavelength region.

2.6. Tensile

The tensile test was performed to investigate the mechanical strength of the produced mats. To check the mechanical behavior, the mat was cut in dimensions of 30 × 5 mm^2 and fixed between the two holders of the machine. The mechanical analysis was performed by the SANTAM tensile machine (Iran) (50 N, rate of 1 mm/min). This test was performed according to the ASTM D882 standard.

2.7. Degradation

The mats were weighed to determine their initial masses (W0). Then, the mats were incubated in PBS solution (10 mM) at pH = 7.4 at 37 °C for 3, 7, 14, and 21 days to obtain the degraded mats. At each interval, the PBS solution was removed from the mats and then weighed again (Wt). The mat degradation percentage was calculated using the following equation:

$$\%\ \text{Degradation} = (W_0 - W_t) \times 100 / W_0 \quad \text{Eq.1}$$

2.8. Swelling

The approach used in the prior study was used to measure the mat swelling behavior [26]. The mats' primary weight (W0) was measured. The mat was thereafter incubated at 37 °C in a 10 mM PBS solution (pH 7.4). The mat was then weighed in different intervals to check for any mass changes after 1, 2, and 3 h. Before weighing, the mat was cleaned of any extra or free water with a Kimwipe. The following equation was used to calculate the mats' swelling:

$$\%\text{Swelling} = (W_t\text{-}W_0) \times 100 / W_0 \quad \text{Eq.2}$$

2.9. Porosity

With the use of the liquid displacement method, the porosity of wound dressing (G1 and G2) was assessed. Since ethanol quickly penetrates the structure without causing the wound dressing to dissolve or change its structure, it was thought to be an alternate liquid. A 10 ml graduated cylinder (V_1) was loaded with 5 ml of ethanol to determine the porosity. The wound dressings (2×5 cm^2) were then submerged for 30 min in pure ethanol (96 %). The final unchanged volume of ethanol in the graduated cylinder was measured after 30 min (V_2). The wound dressings were then taken out of the ethanol without any traces of the substance remaining on its surface. V_3 represents the measured final volume of ethanol in the graduated cylinder. Finally, using equation (3), the porosity value for each wound dressing was determined

$$P = (V_1 - V_3) / (V_2 - V_3) \times 100 \quad \text{Eq. 3}$$

2.10. Water vapor transmission rate (WVTR)

To calculate the Water Vapor Transmission Rate (WVTR), dimensions of 3×3 cm^2 were utilized for each sample of wound dressing (G1 and G2). Subsequently, these wound dressings were positioned onto the lid of a 50-ml centrifuge tube filled with 5 ml of distilled water. The setup was then hermetically sealed and weighed to obtain the initial weight (m1), which was compared to the weight after 24 h (m2). WVTR was calculated using the following formula:

$$WVTR = (m1 - m2) / (t \times A) \quad \text{Eq.4}$$

In which t and An indicate the time and the perforated surface area.

2.11. Disk diffusion (G+, G-)

The antibacterial capacity of the bi-layered film was determined by measuring the disk diffusion technique against *Staphylococcus aureus* and *Escherichia coli*. Bacterial colonies were counted based on the 0.5 McFarland standards. First, 100 μL of the microbial suspension was evenly spread on Mueller-Hinton agar. Then, 10-mm film discs were sterilized under UV radiation for 15 min, and placed on the plate. Finally, all plates were incubated in a biochemical incubator at 37 °C for 24 h before recording their inhibition circle diameters (mm).

2.12. Drug release study

Drug release measurement was performed using the dialysis bag technique [27]. To do this, 2×2 cm^2 G2 was prepared. Each mat was placed in a dialysis bag (cut off 12,000 kD) immersed in 200 ml of distilled water and kept in an incubator at 37 °C for 48 h. At different intervals (after 3, 6, 12, 24, and 48 h), 4 ml of the sample was taken and replaced with 4 ml of fresh water. This study was done in three replications and the mean value was used to plot the graphs. To calculate the concentration of the released vitamins, standard curves were created by preparing standard solutions (0, 10, 20, 50, 100, 150, and 200 μg/ml).

2.13. MTT assay

For 24 h, the mats (1×1 cm^2) were submerged in 70 % ethanol for the sterilization. The mats were disinfected for 1 h using UV light (254 nm). After that, sterile PBS was used to rinse the mats. The mats were then planted with 1×10^4 L929 cells per well using a 96-well plate. The mats were then underwent a 48-h incubation period at 37 °C and 5 % CO_2. Each well received 10 μl of the MTT labeling reagent (0.5 mg/ml) after 48 h, and each well was then incubated for another 4 h. The solubilization solution (DMSO) was then applied in 100 μl portions to each well. Overnight, leave the plate incubating at 37 °C and 5 % CO_2. The positive control was applied to the cells in the wells of the plate without stems. The purple formazan crystals were checked and the absorbance was measured by Wareness Technology Microplate-Reader. All experiments were repeated three times.

2.14. Animal study and wound induction

The National Institutes of Health's "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85-23, updated 1985) was followed while caring for the rats used in the in vivo experiment. According to the "Regulations for using animals in scientific procedures," Research Ethics Committees of Islamic Azad University-Science and Research Branch, Iran, approved this experiment (IR.IAU.SRB.REC.1402.320). N = 3 rats in each group were taken into consideration. Nine male Wistar rats, each weighing 200–250 g, were separated into three groups at random and given injections of xylazine (10 mg/kg body weight) and ketamine hydrochloride (100 mg/kg body weight) to induce anesthesia.

After removing the back hair of rats and disinfecting, a circular wound was generated on the back of each rat by biopsy punch. Each one of the 3 groups of rats was treated with one type of treatment including G0 (no wound dressing), G1 (wound dressing without

vitamins and AgNPs), and G2 (wound dressing loaded with vitamins and AgNPs). To evaluate the appearance of the wounds on days 3rd, 7th, and 14th the grafts were removed and the wound sites were photographed. At intervals, the tissue was removed and kept in 10 % v/v formalin. However, the wound closure rate was measured using the following index:

$$Wound\ closure\ index = d - D \quad \text{Eq. 5}$$

d: the initial diameter of the created wound
D: the diameter of the wound at each interval

2.15. Histological analysis

The histopathological characteristics of the wound tissue including re-epithelialization, inflammation, angiogenesis, and collagen deposition were studied. The sections were stained by hematoxylin-eosin (H&E) staining and Masson's trichrome staining. Histopathological images were prepared using light microscopy (Olympus BX51, Japan). To perform the staining, the tissue was harvested and fixed with 4% formaldehyde, then embedded in paraffin in 5 μm-thick sections.

2.16. Statistical analysis

Prism software and ANOVA statistical tests were employed to analyze the obtained results. The results were expressed as mean ± standard deviation, and $P < 0.05$ was considered as a significant difference.

3. Results and discussion

Wound care is an essential aspect of healthcare, and the development of innovative wound dressings has significantly improved

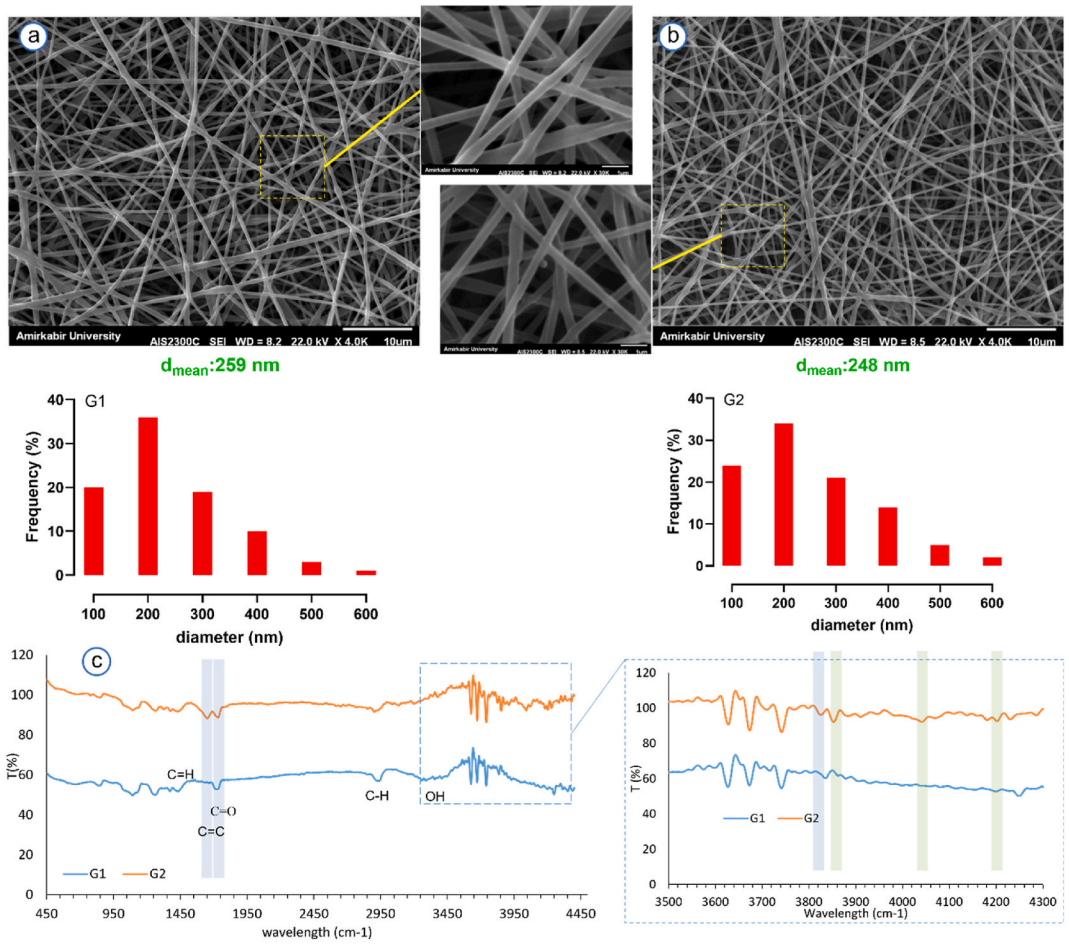

Fig. 1. SEM images and size distribution analysis for **a)** G1 and **b)** G2 groups. **c)** FTIR analysis of the fabricated electrospun patch.

patient outcomes. Nanofibers, with their unique properties and versatile applications, have emerged as a promising material in the field of wound management. Nanofibers are ultrafine fibers with diameters ranging from a few nanometers to several micrometers. They possess an extremely high surface area-to-volume ratio, enabling enhanced drug loading capacity and controlled release. Nanofibers can be fabricated from various materials, including synthetic polymers, natural polymers, or their combinations, offering a wide range of properties such as biocompatibility, biodegradability, and mechanical strength [28].

Fig. 1a and b demonstrates the scanning electron microscopy images of the synthesized bilayered path made of PCL and PVA with and without vitamins and AgNPs. These images are highly valuable for understanding the structural properties and surface morphology of these materials. The uniform distribution of fibers creates a highly porous structure, offering a large surface area for efficient wound-healing processes such as cell adhesion, proliferation, and migration. The SEM image highlights the presence of randomly oriented nanofibers, forming a three-dimensional interconnected network. The nanofibers exhibit a smooth surface, devoid of any visible defects or irregularities. The image reveals a dense network of nanofibers with diameters ranging from 100 to 500 nm. The mean diameter for G1 and G2 was about 259 nm and 246 nm. The size of nanofibers in G1 and G2 mostly ranged from 100 to 300 nm and 100–400 nm respectively. AgNPs seem to be encapsulated within the fiber structure because no AgNP was observed on the surface of nanofibers. Besides, comparing the size of nanofibers, it can be concluded that the presence of AgNPs reduced the size of nanofibers [29]. Such a well-organized structure promotes enhanced moisture management, breathability, and the ability to retain bioactive agents for controlled release [30]. These features play a crucial role in facilitating cell attachment and proliferation. Besides, the unique surface features of these nanofibers also aid in the formation of blood clotting and the promotion of angiogenesis, ultimately accelerating the wound-healing process [31].

Nanofibers also exhibit a porous structure, which allows for efficient gas exchange and moisture management in wound healing. The interconnected pores within the nanofiber network enable the absorption and release of wound exudate, which is crucial for maintaining a moist wound environment. This property prevents the wound from drying out, promotes angiogenesis, and facilitates the removal of waste products and toxins [32].

After confirming the microscopic structure of the prepared nanofibers, chemical analysis of the fibers integrated with and without vitamins and AgNPs via FTIR analysis (Fig. 1c). FTIR spectroscopy measures the interaction of infrared radiation with the molecular vibrations of a material. It provides information on the functional groups and chemical bonds present in the sample. The FTIR spectrum of the PCL/PVA scaffold revealed several characteristic peaks, indicating the presence of specific functional groups. The major peaks observed in the spectrum were as follows. Hydroxyl Group (OH) Stretching: A broad peak in the range of 3200–3400 cm^{-1} indicates the presence of hydroxyl groups, which are characteristic of both PVA and PCL. A strong peak at around 1730 cm^{-1} is observed, which signifies the presence of carbonyl groups (C═O) in both PCL and PVA [33]. Peaks in the range of 2850–3000 cm^{-1} indicate the presence of aliphatic C–H stretching vibrations, which are characteristic of PCL [34]. Some characteristic peaks in the FTIR spectrum of VitB12 were similar to those of PCL. For example, the stretching vibrations of the C═O group in the ester functional group, which typically appear in the region of 1740–1750 cm^{-1} can be attributed to VitB12 too. The presence of the C═C stretching vibrations in the conjugated double bonds can be observed in the range of 1630–1650 cm^{-1} [35]. This peak is indicative of the presence of the double bond in the VitC structure. This peak corresponds to the stretching vibration of the C═C bond. It provides important information about

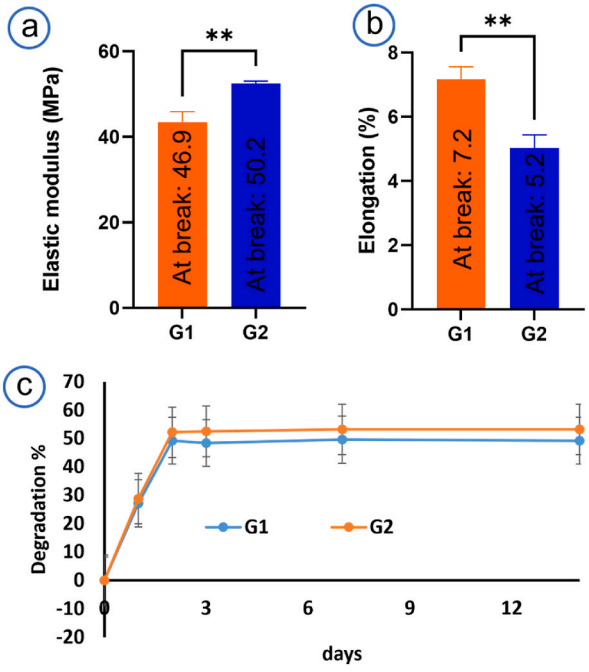

Fig. 2. Results of **a)** elastic modulus, **b)** elongation, and c) degradation studies about groups G1 and G2.

the conjugated system present in ascorbic acid. The broad peak (1400 cm^{-1}) is associated with the stretching vibrations of the C–H bonds and various functional groups present in the molecule [34].

Wound dressings should possess adequate mechanical strength to protect the wound site and withstand external forces. Nanofibers, despite their ultrafine nature, can be engineered to have high tensile strength and flexibility, providing structural integrity and conformability to the wound. This allows the dressing to adapt to different wound shapes and contours, promoting optimal wound coverage and protection.

Fig. 2a depicts the elastic modulus (EM) values for wound dressings without (G1) and with vitamins and AgNPs. Elasticity refers to the ability of a material to regain its original shape after deformation. Nanofiber-based wound dressings typically possess excellent elasticity, enabling them to conform to the contours of the wound and maintain close contact with the skin. This property facilitates optimal wound coverage and prevents the entry of foreign particles, reducing the risk of infection [36]. Results showed that the EM for G1 was around 42 MPa, while for G2 was around 52 Mpa with a significant difference ($P < 0.05$). In contrast, Fig. 2b confirms the reduction in elongation of G2 (~5 %) compared to G1 (~7 %) ($P < 0.05$). It is worth to mention that PCL inherently has stronger mechanical properties than PVA and it can be concluded that the final mechanical strength depends on PCL.

Several studies have investigated the effect of AgNPs on the elastic modulus of electrospun wound dressings. One study conducted by Madivoli et al. examined the incorporation of AgNPs into the PVA electrospun matrix. The results demonstrated that increasing the AgNP concentration led to a significant increase in the elastic modulus of the wound dressing. This finding suggests that AgNPs can enhance the mechanical properties of electrospun wound dressings, potentially improving their stability and ability to withstand external forces [36]. Another study by Thanh and her colleague explored the impact of AgNPs on the elastic modulus of a blend of PCL (PCL) and gelatin electrospun fibers [37]. The findings revealed that the addition of AgNPs resulted in a slight decrease in the elastic modulus compared to the control group. This reduction in stiffness could be attributed to the interaction between AgNPs and the polymer matrix, altering the overall mechanical properties of the wound dressing. However, further investigations are required to fully understand the underlying mechanisms and optimize the AgNP concentration for optimal elastic modulus. This contradiction can be justified by the optimization of the AgNP concentration. Higher NP concentrations within the electrospun fibers have been found to significantly enhance the mechanical properties of wound dressings [38]. These NPs act as reinforcement agents within the fibers, improving their tensile strength, elongation at break, and overall durability. NPs act as reinforcing agents, reinforcing the polymer matrix and preventing fiber fracture under mechanical stress. Additionally, NPs can enhance the interfacial bonding between the polymer fibers, resulting in improved tensile strength and elongation. Furthermore, the presence of NPs can alter the viscoelastic properties of the dressing, leading to better flexibility and stretchability [39].

The mechanical characteristics of PCL/PVA wound dressings have been studied. Due to its excellent mechanical characteristics, absorption, swelling, and degradation, PVA has been employed in past investigations for medical applications [40]. An active wound dressing should have ideal water absorption, acceptable flexibility, and adequate mechanical qualities [22]. Due to its advantageous biocompatible and mechanical features, PCL is frequently used in biomedical applications, including as wound dressings [41]. However, one of the main disadvantages that limit PCL's usage as a substrate for wound dressings is its lack of hydrophilicity. The ability to absorb wound exudate and sustain a sufficiently moist wound environment is a benefit of scaffolds with good hydrophilicity and hygroscopicity. This promotes cell adhesion and proliferation [41].

The degradation of polymeric materials used in wound dressings is a critical factor to consider when designing effective and efficient wound healing products. PCL and PVA are commonly used polymers in electrospun wound dressings due to their biocompatibility and tunable properties. Fig. 2c shows the degradation trend of G1 and G2. There are two significant points about the degradation. Firstly, both groups demonstrated a similar and consistent trend of degradation, and secondly, the rate of degradation decreased nearly to nil starting on day three. It can be inferred that the presence of vitamins and AgNPs had no appreciable impact on the degradation rate. It can also be concluded that PVA was degraded completely (100 %) after three days and the remains mass can be attributed to the PCL. This statement can be justified by the low degradation rate of PCL compared to PVA.

The degradation of PCL/PVA electrospun wound dressings can occur through various mechanisms, including hydrolysis, enzymatic degradation, and oxidative degradation [42]. Hydrolysis is the primary mechanism responsible for the degradation of PVA, which involves the cleavage of ester bonds by water molecules [43]. PCL, on the other hand, undergoes both hydrolytic and oxidative degradation, where ester bonds are broken down by water molecules and reactive oxygen species, respectively. Enzymatic degradation, catalyzed by specific enzymes present in the wound environment, can also contribute to the degradation of PCL/PVA wound dressings [44].

The degradation behavior of PCL/PVA electrospun wound dressings is influenced by various factors, including polymer composition, fiber morphology, and environmental conditions. The inclusion of PVA in a distinct layer amplifies the hydrophilic properties of the electrospun fibers, consequently expediting the degradation process. This rate of degradation is subject to additional regulation through modifications in the PCL/PVA ratio, polymer chain length, and fiber size. Additionally, the presence of additives, such as vitamins and AgNPs in this study, can modify the degradation behavior of the wound dressings and extend their functional lifespan [45].

For applications in wound healing, the controlled degradation of PCL/PVA electrospun wound dressings offers several benefits. By releasing bioactive chemicals and breakdown byproducts as it breaks down, the wound dressing fosters cell migration, proliferation, and tissue regeneration. Additionally, the slow deterioration lessens the requirement for frequent dressing changes, reducing patient discomfort and enhancing wound healing outcomes. Additionally, the creation of extremely porous and linked structures is made possible by the use of electrospinning techniques, which support effective fluid absorption and moisture control in the wound bed [46].

Several studies have investigated the degradation of PCL/PVA wound dressings. In a study on drug-loaded PVA/PCL fibers for wound dressing applications, the degradation rate of the fibers was measured and found that by increasing the PVA more degradation

happens and vice versa [47]. The degradation of PCL and PVA was explored in research on a nanofibrous matrix based on gum tragacanth-PCL-PVA for use in diabetic wound healing. It was discovered that the final wound dressing had a quick deterioration because of the presence of PVA and gum tragacanth [48].

The efficacy of wound dressings in promoting wound healing as a therapeutic intervention is greatly determined by their swelling characteristics. The capacity of wound dressings to soak up exudate, uphold a moist wound environment, and facilitate wound healing are all distinctly affected by their tendency to expand. Fig. 3a contains results from the swelling study for both G1 and G2. As can be seen, no significant difference was observed between G1 (90 %) and G2 (102 %) ($P > 0.05$). It should be noted that the presence of vitamins and AgNPs did not affect how the wound dressings reacted to edema. However, several variables, such as the polymer composition, crosslinking, porosity, and the inclusion of additives or functionalization, affect the swelling and water uptake behavior of electrospun wound dressings. These elements impact the dressings' general morphology and structure, which in turn affects how well they can hold and absorb water [49]. As can be seen in Fig. 3b, both G1 and G2 showed a porosity of around 70 % and based on the statistical analysis no significant difference was observed ($P > 0.05$). These results confirm that the swelling and water uptake (and porosity) behavior of the wound dressings produced by electrospinning is significantly influenced by polymer selection. Hydrophilic polymers like gelatin, CS, and PVA exhibit greater swelling ratios in comparison to hydrophobic polymers like PCL. This phenomenon enables dressings to enhance their fluid absorption and retention capacity due to the inherent stronger attraction of hydrophilic polymers towards water molecules.

The swelling behavior of a PCL/PVA scaffold was examined using several solvent solutions [40]. The researchers noticed that as

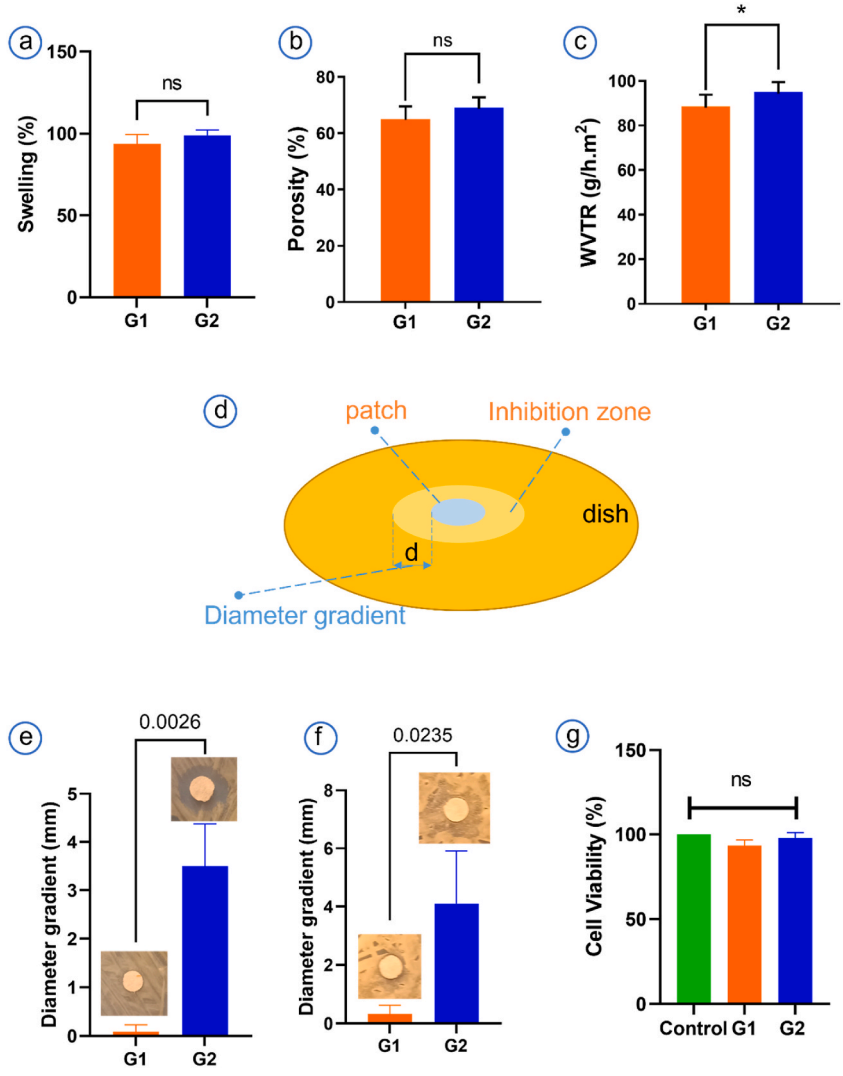

Fig. 3. a) swelling behavior in PBS at 37 °C, **b)** results from porosity analysis using solvent displacement technique, **c)** results from WVTR measurement at room temperature, **d)** the strategy to measure the antibacterial activity of the fabricated patch based on the diameter gradients. Antibacterial results from disk diffusion assay for G1 and G2 against **e)** *S. aureus* (p-value = 0.0026), and **f)** *E. coli* (p-value = 0.0235). **g)** measurement of the cytotoxicity of G1 and G2 under MTT assay against L929 cell line (ns: non-significance).

PVA concentration increased, the scaffold's swelling ratio grew as well. Additionally, it was shown that the swelling behavior was pH-sensitive, with larger swelling ratios being seen in acidic environments [40]. According to the findings, the PCL/PVA scaffold had better swelling characteristics and a larger water absorption capacity. The hydrophilic properties of PVA and the scaffold's interconnected porous structure are responsible, according to the researchers, for this improved swelling behavior. It is important to find out how crosslinking affected the swelling behavior of PCL/PVA wound dressings. Crosslinking the PCL/PVA matrix, the researchers discovered, reduced swelling and increased mechanical strength. The controlled swelling behavior of the crosslinked dressings proved helpful for preserving a moist wound environment and accelerating wound healing [50].

Swelling is influenced by knowledge of the mechanical characteristics, absorption, and degradation of the PVA/PCL fibers. In research, Khorshidi et al. created hybrid nanofibers for use as dressings for wounds using biopolymers and PVA/PCL. The nanofibers were discovered to help facilitate wound healing and to have high mechanical and biocompatibility qualities. When compared to a patch made of free PVA nanofibers and nanofibers that had been loaded with medication, the nanofibers' swelling analysis revealed a significant swelling (200 %) [51]. The mechanical characteristics of the dressing, such as its flexibility and ability to adhere to the location of the wound, can also be impacted by swelling. Swelling can be affected by elements including the amount of PCL present in the fibers and the presence of additional components in PVA/PCL wound dressings. Although no study particularly addresses the importance of swelling in PVA/PCL wound dressings, the behavior of the fibers as they swell is frequently stated as part of a description of the dressing's qualities.

The Water Vapor Transmission Rate (WVTR) plays a crucial role in determining the efficacy of electrospun wound dressings. Various studies have illustrated that the WVTR values of these dressings align closely with those of commercially accessible products. Specifically, the WVTR of PLCL-5BA (poly(ι-lactide-*co*-ε-caprolactone enriched with 5 % Boric Acid) fibers was recorded at 557 ± 20.9 g/m^2. day after 24 h, showcasing similarity to existing commercial items and published literature [52]. Furthermore, wound dressings constructed from PCL/Aloe Vera_CS nanofibrous displayed a WVTR of 1252.3 ± 21.2 g/m^2 day, whereas a wound dressing based on electrospun PVA/CS/starch nanofibrous mats exhibited a WVTR of around 90–140 g/m^2. h [53]. These outcomes suggest that electrospun wound dressings can effectively regulate moisture levels at the wound site, thereby fostering an environment conducive to optimal healing. According to Fig. 3c, G1 and G2 showed a WVTR equal to 88.6 and 95 g/m^2. h respectively ($P < 0.05$). It confirms that the presence of AgNPs increased the WVTR of the final wound dressing. Besides the impact of nanofiber size and also its distribution can be considered as other effective factors.

In the context of healthcare facilities, wound infections pose a significant challenge due to their association with delayed wound healing, increased morbidity, and elevated healthcare costs. The emergence of antibiotic-resistant pathogens has significantly complicated the management of these conditions. Consequently, there is a growing inclination towards exploring alternative strategies for addressing wound infections. Fig. 3d shows how the antibacterial activity of the final wound dressing was measured to evaluate the efficacy of the wound dressing loaded with vitamins and AgNPs. The parameter "d" shows the variance between the radius of the patch and the inhibition zone. Fig. 3e depicts the antibacterial activity of the G1 and G2 wound dressing against *S. aureus*. It was concluded that the wound dressing without the vitamins and AgNPs showed no antibacterial activity ($d = 0.25$ mm), whereas the G2 wound dressing loaded with the vitamins and AgNPs depicted inhibition ($d = 3.5$ mm) ($P < 0.05$). Similarly, G2 ($d = 4.5$ mm) also showed a stronger inhibition against *E. coli* compared to G1 ($d = 0.33$ mm) (Fig. 3f).

Silver has been utilized in the field of wound care owing to its antibacterial properties, and products containing silver have a rich historical background of application. AgNPs exhibit remarkable potency against a broad spectrum of pathogens, encompassing both Gram-positive and Gram-negative bacteria. Ongoing investigations are being conducted to elucidate the specific mechanism by which AgNPs display their antibacterial efficacy. It is hypothesized that AgNPs adhere to the surfaces of microbial cells, disrupt membranes, cause DNA damage, and impede biological processes, ultimately resulting in bacterial eradication [54].

In addition to having antibacterial effects, AgNPs have been discovered to have several additional qualities that aid in wound healing. The likelihood of negative responses is greatly reduced by the exceptional biocompatibility these NPs have with human tissues. Additionally, AgNPs have anti-inflammatory qualities that aid in reducing inflammation at the location of the wound and speeding up the healing process. Additionally, it has been demonstrated that AgNPs encourage angiogenesis, and the growth of new blood vessels, which is essential for the transport of nutrients and oxygen to the wound bed. A persistent antibacterial impact is provided by the release of silver ions from the NPs, ensuring ongoing defense against bacterial colonization. AgNPs' carefully regulated release reduces the possibility of systemic silver poisoning [16].

The antimicrobial action of vitamins B and C has recently garnered attention due to their potential therapeutic benefits. VitB, a water-soluble vitamin category vital for various physiological processes such as wound healing, has been found to possess antibacterial properties. Research has identified multiple forms of VitB, including niacin (vitamin B3), pantothenic acid (vitamin B5), and pyridoxine (vitamin B6), that exhibit antibacterial characteristics against bacteria [15]. Ascorbic acid, another name for VitC, is a well-known antioxidant that is essential for collagen formation, immune system health, and wound healing. VitC has also been shown to have antibacterial action against a variety of bacteria in addition to these functions. Several processes explain how VitC fights germs: i) Disruption of Bacterial Biofilms, ii) Enhancement of Immune Response, and iii) Generation of Reactive Oxygen Species [5].

Electrospun PCL/PVA wound dressing was also confirmed to be a good carrier to deliver antibacterial drugs. For instance, A study developed drug-loaded PVA/PCL fibers for wound dressing applications and found that the developed fibers exhibited good antibacterial activity against *S. aureus* bacteria [47]. Zhang et al.'s study examined the antibacterial efficacy of PCL/PVA composite dressings that contained AgNPs [55]. According to the findings, *Staphylococcus aureus* and *Escherichia coli* were two bacterium types that the dressings had high antibacterial action against. AgNPs were added, which greatly reduced bacterial growth and accelerated wound healing.

Apart from physical, chemical, mechanical, and antimicrobial properties, having good cell viability and good biological properties

is another critical feature of wound dressing. The concentration of AgNPs and also vitamins may cause side effects. Therefore, ensuring the safety and biocompatibility of AgNPs is essential before their application in wound dressing. So, it was tried to assess the cytotoxicity of the final wound dressing. Fig. 3g depicts the cell viability results for G1 and G2 against the L929 cell line after 48 h. Compared to the control group, G1 and G2 had a cell viability of around 93.5 % and 97.9 % with no significant difference ($P > 0.05$). However, G2 showed a bit better results in contrast with G1, which confirms a small improvement in biological properties. In a study, human dermal fibroblast cells were cultured and seeded onto the prepared wound dressings integrated with AgNPs [56]. The integration of AgNPs into wound dressings did not significantly impair the vitality of human dermal fibroblast cells, according to preliminary data from cell viability studies. Comparable cell viability (more than 85 %) was found in the MTT experiment between the control group and the AgNP-containing wound dressings, indicating no detrimental effects on cell growth. These findings are encouraging because they imply that AgNPs may be used in wound dressings without risking cytotoxicity or cell proliferation side effects. Because of their biocompatibility and antibacterial qualities, AgNPs are a promising contender for accelerating wound healing and lowering the risk of wound infections. Our study confirmed that the presence of vitamins increased cell viability to more than 93 %. However, the concentration of the AgNPs must be considered.

AgNPs have negligible cytotoxicity at lower doses, allowing cells to continue to be viable and proliferative. However, when AgNP concentration rises, the cytotoxic effects become more apparent, impairing wound healing and decreasing cell viability. The antibacterial effectiveness of AgNPs and their potential cytotoxicity must be balanced to get the ideal concentration for wound healing applications [57]. Fig. 3e confirms that the employed AgNP concentration along with vitamins showed a reliable and rational cell viability.

In a study, the authors investigated the effect of PCL/PVA nanofiber dressings on fibroblast viability [58]. The results demonstrated enhanced cell proliferation and adhesion, indicating the biocompatibility of the dressing. Similarly, Wang et al. [59]evaluated the cell viability of PCL/PVA hydrogel dressings and observed improved cell attachment and viability compared to traditional wound dressings. In a study that was published in 2021, hybrid nanofibers made of PCL/PVA and biopolymers were created for use in wound dressings. When the nanofibers were treated with L929 fibroblast cells during the in vitro cytotoxicity assay, high cell viability of more than 90 % was observed [60].

To ensure the release of both VitC and VitB12, the release assay was performed and the results are presented in Fig. 4. Fig. 4a and b depict the standard curve for both VitC and VitB12 respectively. As it can be seen, R^2 for both vitamins is nearly equal to 1 which confirms the accuracy of the final model and calculated equation. (Regarding Vitamin B12, data for the sample with the concentration of 200 μg/ml was removed due to its non-reasonable value to make the model much more reliable.) According to the data, both

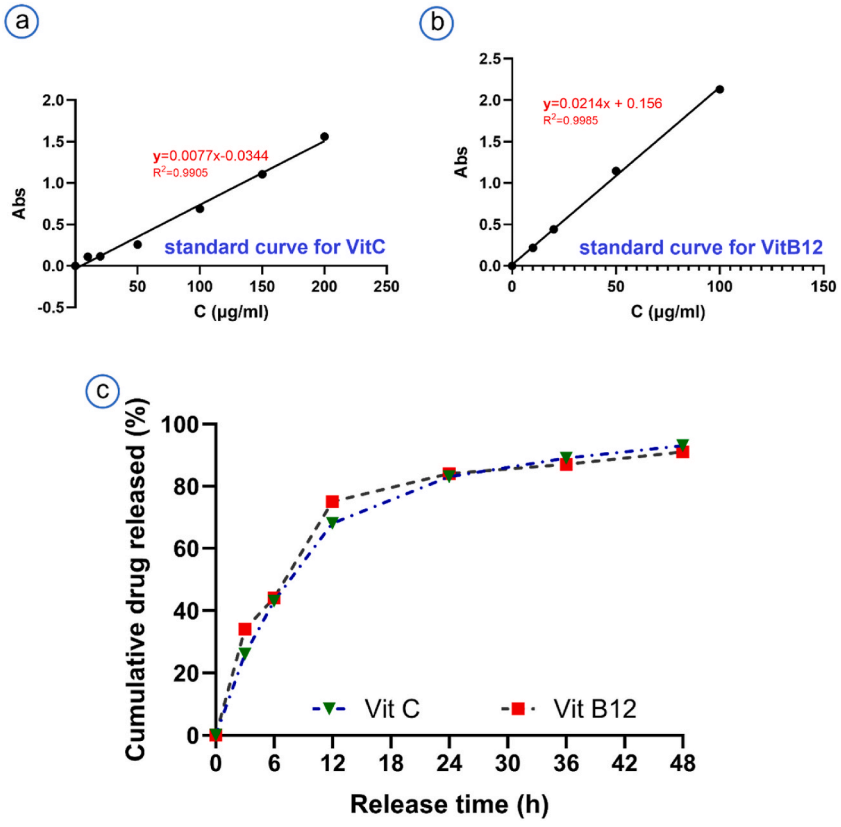

Fig. 4. a) standard curve for VitC, **b)** standard curve for VitB12, and **c)** the release profile for both VitC and VitB12.

vitamins showed a similar release trend. After 12 h, nearly 70 % of the loaded drug was released, and after 24 h and 48 h around 80 % and 90 % of the encapsulated vitamins were released. This issue can be justified by the degradation rate and swelling rate. The deterioration of drug delivery systems can have a significant impact on the effectiveness of drug release [61]. When drug carriers degrade prematurely or lose their protective coating, it can impede the delivery of the drug to the specific target tissue. Research indicates that certain nanocarriers are engineered to degrade gradually via hydrolysis, resulting in the release of drugs contained within them [62].

Considering this issue, it can be claimed that in a drug delivery system with a degradation rate of m%, it can be hypothesized that the release trend can be similar for all loaded therapeutic agents. This phenomenon is independent of the release measurement assay. Thus, the degradation of drug delivery systems plays a critical role in determining the efficiency and success of drug release mechanisms. In this regard, the type of employed biomaterials (natural or synthetic biomaterials) with distinct degradation rates plays a critical role in controlling the final release trend (slow or burst release) [63].

The above-discussed features confirm the in-vitro efficacy of the wound dressing. To make it more reliable, an in-vivo study was performed using G1 and G2 wound dressing. Fig. 5a shows the images of the wound site after 3, 7, and 14 days. The wound area was measured and analyzed by Image J software after taking pictures of the wounds at each interval and the statistical results are presented

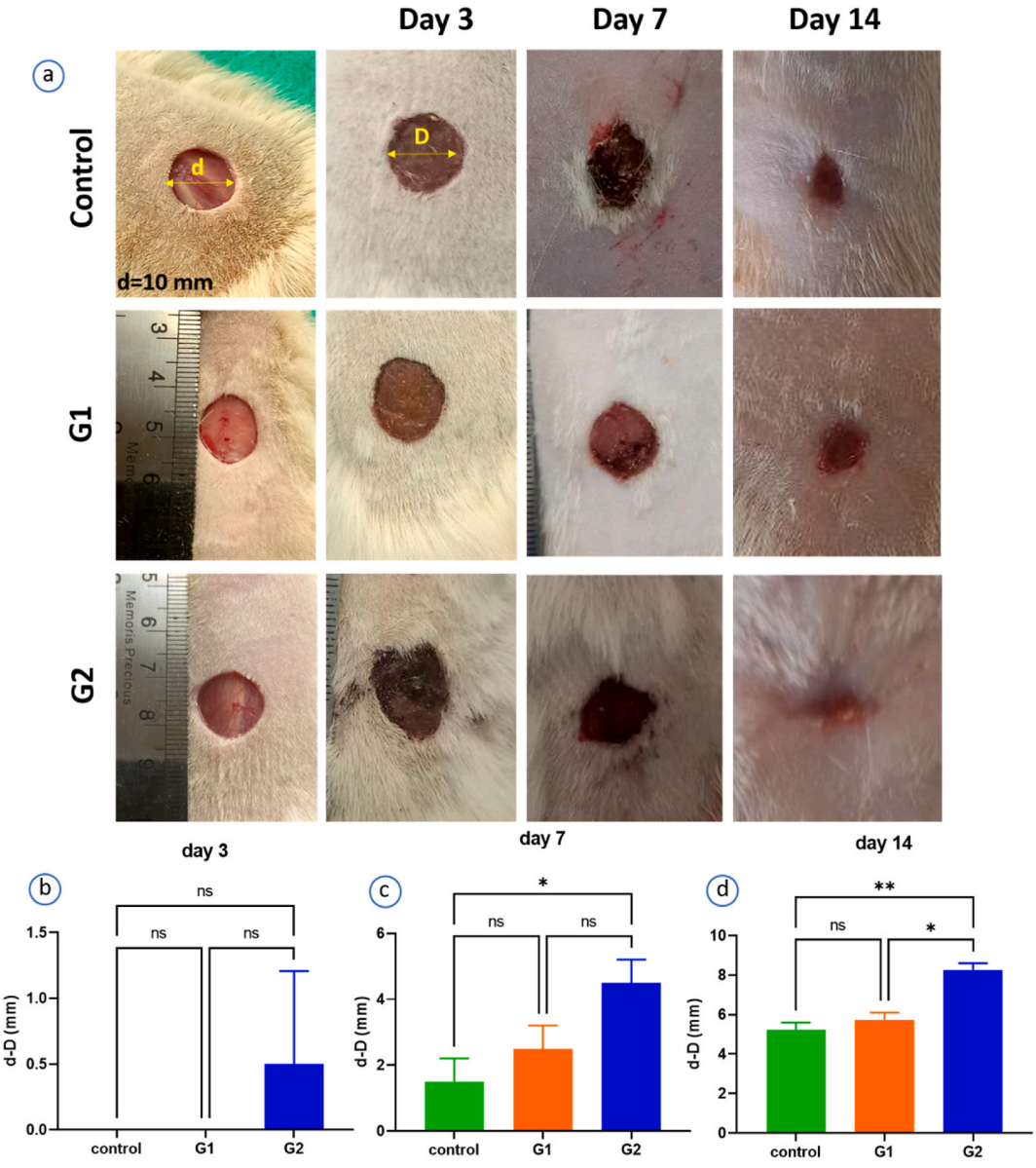

Fig. 5. a) images of the wound site after 0, 3, 7, and 14 days. Comparing the wound size based on the d-D variance after **b)** three days, and **c)** seven days. and **d)** fourteen days.

in Fig. 5b–d separately. The primary indicator used to assess the degree of healing was the difference between the wound diameter on each day and the original wound diameter (on day 0). The wounds that were treated with G2 showed signs of faster wound healing, per the findings compared to the G1. After three days, no reduction in the wound area was observed for the control and G1 groups ($P > 0.05$), while wound treated with G2 wound dressing revealed a reduction in wound diameter d-D>0.5 mm ($P < 0.05$), Fig. 5b. After 7 days, no significant difference was observed between control and G1 ($P > 0.05$), Fig. 5c. Although no significant statistical difference was observed between G1 and G2 ($P > 0.05$), G2 owned a larger "d-D" index. G2 also had a difference compared to the control group ($P < 0.05$). Differences were more distinguished on day 14 (Fig. 5d). interestingly, G2 showed significant difference compared to G1 ($P < 0.05$) and control ($p < 0.01$) groups. However, no significant difference was reported between G1 and the control group.

($P > 0.05$). Comparing these results with a previous one by Zafari et al. [64], showed that the quality of the healed wound on the 14th day in our study was similar to that on the 20th day. This comparison approves the potential of the designed wound dressing (G2).

The wounds underwent histological investigation using Masson's trichrome and H&E staining to assess tissue regeneration, collagen deposition, and angiogenesis in tissue sections. Trichrome-Masson (TM) staining was used to provide the cytoplasm, keratin, muscle fibers, and intracellular components a red color while providing a better microscopic examination of the collagen fibers in blue (Aniline reagent) color. Transverse sections of the wound region were produced at regular intervals to help with the diagnosis of wound healing. Fibroblasts, neutrophils, hair follicles, epithelial layers, and arteries were counted on average. The pathological analysis was explained and discussed by considering both H&E (Fig. 6) and TM stainings (Fig. 7).

Based on the pathological reports, The wound area was repaired without epidermis and the wound surface was covered with fibrinonecrotic exudate with significant infiltration of inflammatory cells. The dermis area was mainly filled with newly formed fleshy bud tissue (control-day3). The wound area was repaired without epidermis, and the wound surface was covered with fibrinous exudate. The dermis area was mainly filled with newly formed fleshy bud tissue along with active fibroblasts (G1-day3). The surface of the wound was filled with significant amounts of fibrinonecrotic and fibrinoplurant exudates, and the regeneration of the epidermis was not done. In the dermis area, the newly formed and edematous tissue of the fleshy bud had filled the area (G2-day3). The epidermal area was filled with significant amounts of fibrinonecrotic and fibrinoplurant exudates. In the dermis area, newly formed edematous flesh bud tissue along with active fibroblasts and infiltration of inflammatory cells (multinucleated and mononucleated) were observed (control-day7). The area of the epidermis without regeneration was significant and the wound surface was filled with exudates. The restoration of the dermis area was better than the previous cases and the density of fibroblastic cells along with newly formed collagen fibers was higher than before (G1-day7). The regeneration of the epidermis area has started, and in the dermis area, the tissue of the fleshy bud was mainly filled with fibroblast cells and newly formed collagen fibers (G2-day7). The regeneration of the epidermis area was not done and the regeneration of the dermis area was more compared to the previous cases (compared to itself). The amount of cell density in the dermis area was reduced and the amount of newly formed fibers was increased (control-day14). Epidermal regeneration was not done. Hypertrophy of the dermis was observed in the regenerated area. In this section, the density of collagen fibers and newly formed vessels was significant, and the pattern of the filaments had become more regular (G1-day7). Areas of the epidermis and dermis

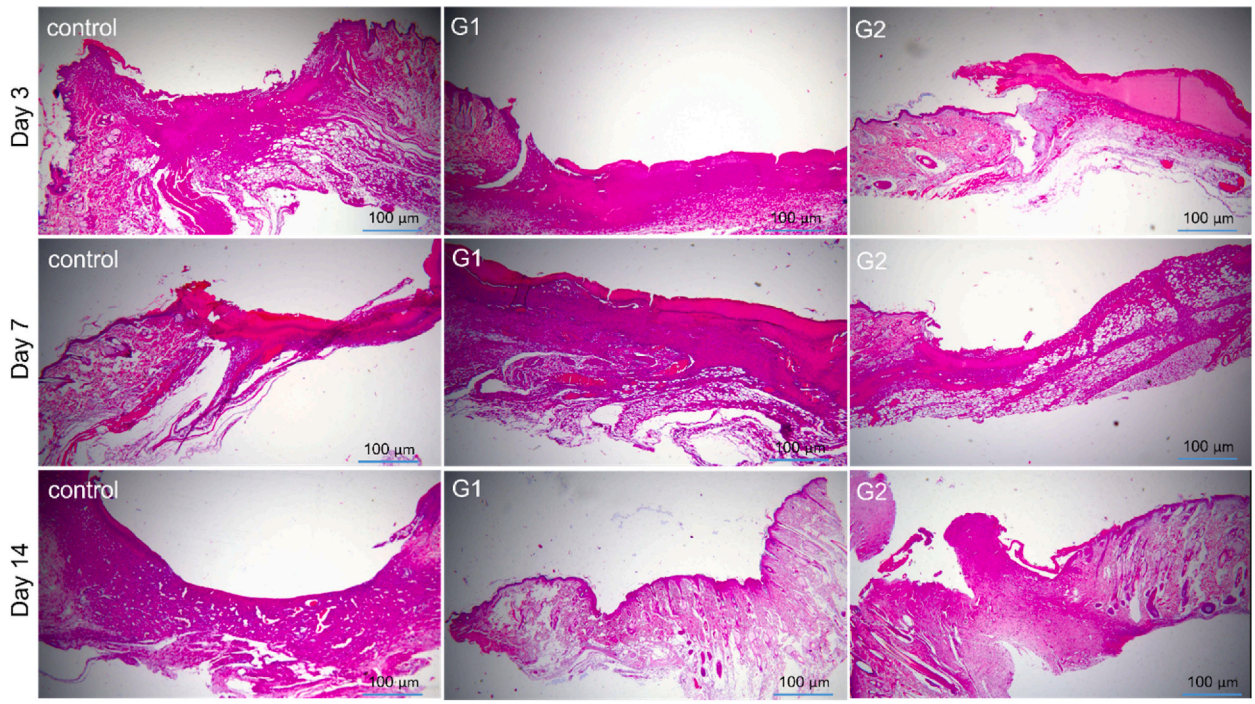

Fig. 6. Results from Hematoxylin and Eosin staining after 3, 7, and 14 days.

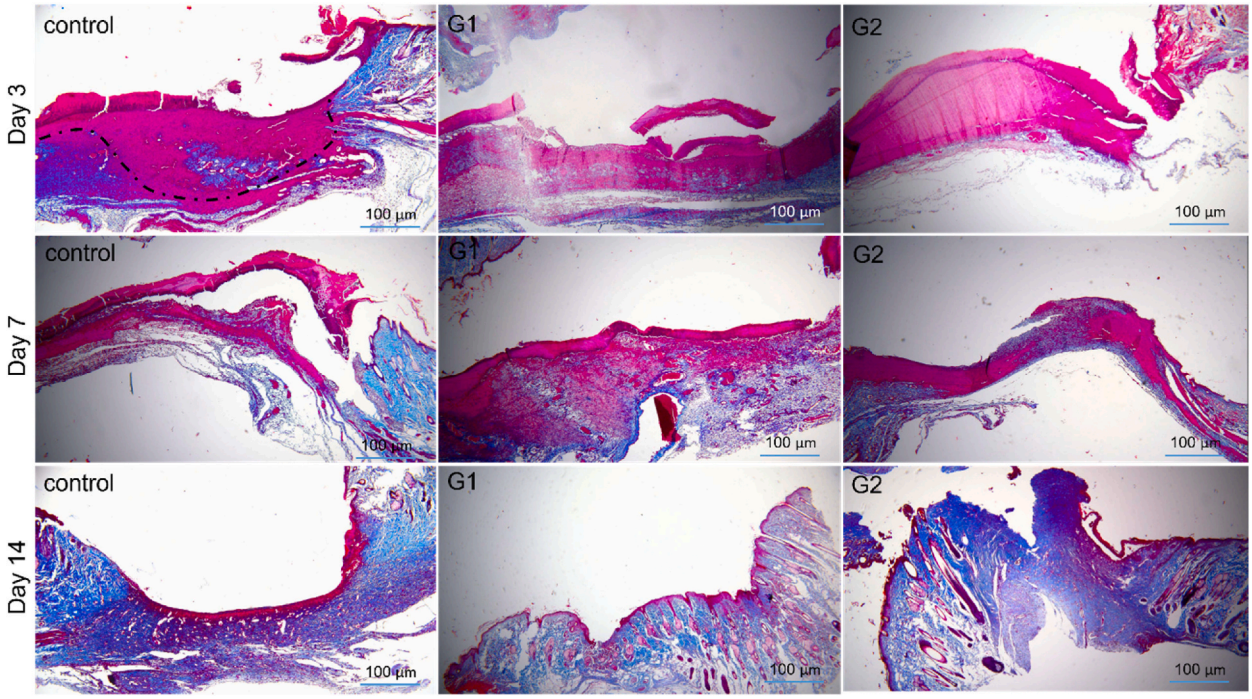

Fig. 7. Results from Trichrome Masson staining after 3, 7, and 14 days.

had a structure similar to normal tissue. The number of newly formed vessels was also higher in this sample than in the previous case (G2-day14).

4. Conclusion and future perspectives

From a general insight towards this study, in addition to having antibacterial qualities, the bilayered mat in this study could provide easy clinical handling, cytocompatibility, and faster wound healing. This study demonstrated that using bilayered strategies to produce wound dressing could improve mechanical and physicochemical properties. PCL was used just to create the first layer as the substrate, and PVA was employed to fabricate the second layer as the carrier layer to deliver vitamins and AgNPs. It is worth mentioning that coating a hydrophilic layer on a hydrophobic layer was challenging and time-consuming. By the way, we employed a strategy that caused a physical interaction (entanglement) between two layers. Simply, the PCL fibers were first created and then the PVA fiber was produced simultaneously for a while. This strategy caused a complicated entanglement between the PCL fibers and PVA fibers. This strategy, based on the results, showed acceptable results. This strategy was the first try with nanofibers and also PCL and PVA. However quick crosslinking was notably important for better interaction (data not provided).

To sum up, the final wound dressing loaded with VitB12, VitC, and AgNPs showed well-linked porosity of the nanofibrous mats and their consistent morphology. The linked functional groups of the components in the nanofibrous mats have been shown by FTIR. The degradation confirmed that drug delivery can be prolonged for 3 days which means after three days dressing replacement is required. Besides, having a swelling rate of 100 % can be a notable feature for drug delivery. Antibacterial activity from disk diffusion assay revealed that the created bilayered wound dressing could prevent the growth of gram-positive and gram-negative microorganisms due to the uniform release of vitamine and AgNPs. The Results from the MTT assay depicted no cytotoxicity. According to the in vivo study, the designed wound dressing has the potential to heal a wound (10 mm in diameter) for 14 days by accelerating healing, collagen formation, and lowering inflammation.

Regarding the main challenges and weaknesses of the present study, it is necessary to highlight that the co-delivery of vitamins and AgNPs might be more effective if the vitamins were encapsulated. One of the main limitations was the selection of vitamins. Choosing the right polymer and also the right vitamin with acceptable solubility made the fabrication process a bit complicated. But more interestingly, these limitations and challenges recommended more hypotheses and ideas for future study. Co-delivery of both soluble-in-water vitamins and soluble-in-fat vitamins raised the idea of co-encapsulation of them in various types of nanocarriers and evaluation of their efficacy in the release of vitamins. Furthermore, regarding the type of nanofibers using coaxial nanofibers can be another idea for encapsulation. However, in this regard, there is a limitation that turns back to the employed materials. For instance, if PCL is used as shell and PVA as core, then a low release rate is predicted due to the low degradation rate of PCL. So, nominating another biomaterial can be the plan B, but it needs optimization and evaluation.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

CRediT authorship contribution statement

Amir Ali Iranpour Mobarakeh: Writing – original draft, Software, Project administration, Funding acquisition. **Ali Shahmoradi Ramsheh:** Project administration, Funding acquisition, Formal analysis. **Ali Khanshan:** Software, Project administration, Funding acquisition, Conceptualization. **Samira Aghaei:** Writing – original draft, Project administration, Funding acquisition. **Mahnaz Mirbagheri:** Writing – original draft, Project administration, Methodology. **Javad Esmaeili:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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