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Poly(ε-Caprolactone)/Poly(Ethylene Glycol)Dithiol Electrospun Nanofibers As a Carrier for the Potent Phytomedicine Bromelain

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Bromelain, Nanofiber, Poly(ε-caprolactone), Poly(ethylene glycol)dithiol

Abstract: Bromelain (Bro), known for its anticancer, immunomodulatory, and anti-inflammatory properties, is a mixture of cysteine proteinases widely utilized in cosmetics and burn debridement. Its effects on wound healing are linked to enhance tissue reconstitution. In this study, we introduced a blend of poly(ethylene glycol)dithiol (PEGdt) an poly(ε-caprolactone) (PCL) electrospun nanofibers (ENs) for the delivery of the potent phytomedicine Bro. Following the determination of the optimum concentration of PEGdt, we observed the enhancing effect of Bro addition on both the physical and chemical characteristics of ENs. An increased concentration of Bro was found to enhance the hydrophilicity of PCL/PEGdt surfaces, as indicated by contact angle analysis. The addition of Bro also created a favourable surface for potential applications on wound healing. Overall, the proven positive effect on cell viability and wettability of Bro-added PCL/PEGdt ENs reveal their potential to address problems of existing cosmetic solutions in wound dressings, such as insufficient ability to absorb excess wound exudate.

Güçlü Bir Bitkisel İlaç Olan Bromelainin Taşınması İçin Elektroeğirilmiş Poli(E-Kaprolakton)/Poli(Etilen Glikol)-Ditiol Nanolifleri

Anahtar Kelimeler
Bromelain, Nanolif, Poli(ε-kaprolakton), Ditiyol-poli(etilen glikol)

Öz: Bromelain (Bro), antikanser, immünomodülatör ve antiinflamatuar özellikleriyle bilinen ve kozmetik ile yanık debridmanı alanlarında yaygın olarak kullanılan sistein proteazlarının bir karışımıdır. Yara iyileşmesi üzerindeki etkileri, doku yenilenmesini desteklemesiyle ilişkilidir. Bu çalışmada, güçlü bir fitoterapötik ajan olan bromelainin kontrollü salımını sağlamak amacıyla, poli(ε-kaprolakton) (PCL) ve poli(etilen glikol) ditiyol (PEGdt) elektroeğrilmiş nanofiberlerden (ENs) oluşan bir karışım geliştirilmiştir. Optimum PEGdt konsantrasyonunun belirlenmesinin ardından, Bro eklenmesinin elektroeğirme ile üretilen ENlerin fiziksel ve kimyasal özellikleri üzerindeki olumlu etkileri gözlemlenmiştir. Temas açısı analizi ile gösterildiği üzere, artan Bro konsantrasyonu PCL/PEGdt'nin yüzeyinin hidrofilikliğini artırmıştır. Ayrıca yara iyileşmesi için potansiyel uygulamalara uygun bir yüzey oluşturmuştur. Genel olarak, Bro-eklenmiş PCL/PEGdt ENlerin hücre canlılığı üzerindeki pozitif etkileri ve ıslanabilirliği kanıtlanmıştır, mevcut yara örtülerinde sıkça karşılaşılan fazla yara eksüdasını emme yetersizliği gibi problemlere çözüm sunma potansiyeline sahip olduğu gösterilmiştir.

1. INTRODUCTION

Engineered polymeric biomaterials, meticulously designed to respond to environmental stimuli, have garnered considerable attention within the fields of biosensors [1], drug delivery [2] and tissue engineering [3]. Along with the transition from macro- to micro-scale in theragnostic approaches, significant advancements in material sciences have also covered the personalized medicine. This heightened interest attributed to their remarkable adaptability, which is contingent upon precise modulation of parameters such as temperature [4], pH [5], electrical/magnetic conductivity [6,7] in addition to their aptitudeıı for reacting to chemical [8] and biological [9] stimuli. Especially the combinations of drug delivery systems with tissue engineering approaches have paved the way for theragnostic applications in the form of wearable sensors. The non-invasive structures of these systems, which allow direct drug delivery into the bloodstream through the layers of the skin without undergoing first-pass metabolism in the liver, have been proven advanced treatment systems through both preclinical and clinical research.

Holding a significant promise in the field of cosmetics, electrospun nanofibers (ENs) are fibrous nanostructures that possess a notable feature of having a substantial surface area-to-volume ratio, enabling the efficient delivery of both hydrophilic and hydrophobic active molecules and drugs [10]. Moreover, the fibrous structures found in blends of polymers reveal remarkable characteristics, including a 3D- structural network, high porosity and permeability, which make them useful tools for tissue engineering applications. Hence, prior research endeavors sought to create structures by blending or layering polymers with significantly diverse characteristics, to harness the various attributes offered by different materials in nanofiber fabrication [11]. Being one of the most advantageous polymers, poly(ethylene glycol) (PEG) is a good example due to their permeable and biodegradable features, and their resistance to protein adsorption makes them suitable as plasticizer to blend or incorporate to other materials[12,13]. As such, the PEG incorporated graphene oxide structure has been proved to reduce protein adsorption by creating an interface for protein interaction through PEGylation [14]. Besides, PEG blended polylactide (PLA)[12] , poly(vinyl alcohol) (PVA) [13] or poly(ε-caprolactone) (PCL) [15] polymers have been reported to be changed their structural and physicochemical features such as printability, biocompatibility, and biodegradability, proving that the PEG can be use when a faster degradation is required. Notably, for targeted drug delivery, thiol functionalized PEG polymers are widely used due to their enhanced biocompatibility, and antifouling properties.

In this study, we introduced the utilization of poly(ethylene glycol)dithiol (PEGdt) and PCL blend for the delivery of the potent phytomedicine bromelain (Bro) [16]. Being a proteolytic enzyme extracted from pineapple stems [17], Bro has attracted significant attention due to its multiple physiological effects, including antioxidant [18], anti-inflammatory [19], and anticancer [20] properties. In addition to these versatile biological activities, Bro exhibits a well-documented positive effect on wound healing, making it a promising candidate for advanced wound care applications [21]. Remarkably, the integration of Bro into nanostructured materials *via* electrospinning has further enhanced its therapeutic potential by improving its bioavailability and facilitating controlled delivery. Recent studies have consistently demonstrated that Bro-loaded ENs not only promote tissue regeneration but also significantly accelerate the wound healing process [22,23,24]. Herein this study, along with the well-defined physical features, the proven biocompatibility of Bro-loaded PCL/PEGdt (PCL/PEGdt/Bro) ENs have been shown their potential to be used as wound healing materials in the field of cosmetics and tissue engineering applications.

2. MATERIAL AND METHOD

2.1 Materials

PCL (Mn: 80000), PEGdt (Mn:1000) and Bro were purchased from Sigma-Aldrich. Formic acid (FA) and acetone (Ac) were obtained from Merck. Human keratinocytes cell line (HaCaT) was supplied by CLS. Dulbecco's Modified Eagle Medium (DMEM) cell culture media and fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin supplements were purchased from Gibco. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) used for cell viability/cytotoxicity testing was obtained from Sigma-Aldrich.

2.2 Instrumentation

NanoWeb Electrospin 103 (MaviTech, Turkey) was used to obtain PCL/PEGdt ENs. The morphological structure of the ENs were examined through scanning electron microscopy (SEM; Carl Zeiss 300 VP). The contact angles of PCL/PEGdt ENs were measured with the Attention Theta Goniometer device. The Fourier-Transform Infrared Spectroscopy (FTIR) spectra of PCL, PCL/PEGdt and PCL/PEGdt/Bro ENs were determined by PerkinElmer Spectrum 100 instrument between the wavelengths of 400–4000 cm^{-1}. Multimode microplate reader (Thermo Varioscan Flash) was used to measure MTT absorbance.

2.3 Preparation of PCL/PEGdt and PCL/PEGdt/Bro ENs

10% (w:v) PCL polymer was dissolved in FA:Ac (at the ratio of 3:7; v:v) solvents and stirred at 350 rpm, 24 °C overnight. Followed by, 2.5-12.5% (w:v) PEGdt was added to blend the optimized PCL solution. The prepared PCL/PEGdt solution was transferred to a 21 G syringe at 2 mL volume and placed to syringe pump (ATABA AC–DC Adapter AT-511). Electrospinning was performed using an applied voltage of 16 kV, a solution flow rate of 0.3 $mL.h^{-1}$, and maintaining 15-20 cm between the syringe tip and the collector plate. The electrospinning occurred at a temperature range of 21–24 °C and a humidity level of 63–70%. As for the preparation of PCL/PEGdt/Bro

ENs, bromelain was added in certain amounts of (0.25-5% (w:v)) to the PCL/PEGdt polymer blend.

2.4 Assessment of the cytotoxicity of PCL/PEGdt/Bro ENs on HaCaT cells

HaCaT cells were used to test the effect of PCL/PEGdt/Bro ENs on cell viability. For the cultivation of HaCaT cells, DMEM medium containing 10 % fetal bovine serum (FBS), 1 mM sodium pyruvate, and 2 mM glutamine were used. The cell viability test of PCL/PEGdt/Bro ENs was assessed *via* MTT assay. Briefly, HaCaT cells were suspended in DMEM cell culture media at $2x10^4$ cell/well, seeded in 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h. Following, the medium was removed and UV-sterilized ENs (2x2 cm) were fitted to each well as test groups within the fresh culture media. Only PCL ENs extract added group was considered as the positive controls. After 24 h of incubation, MTT assay was performed and the cell viability (%) was calculated.

2.5 Statistical analysis

Statistical analyses were carried out by Student's t-test cells were used in cell viability studies.

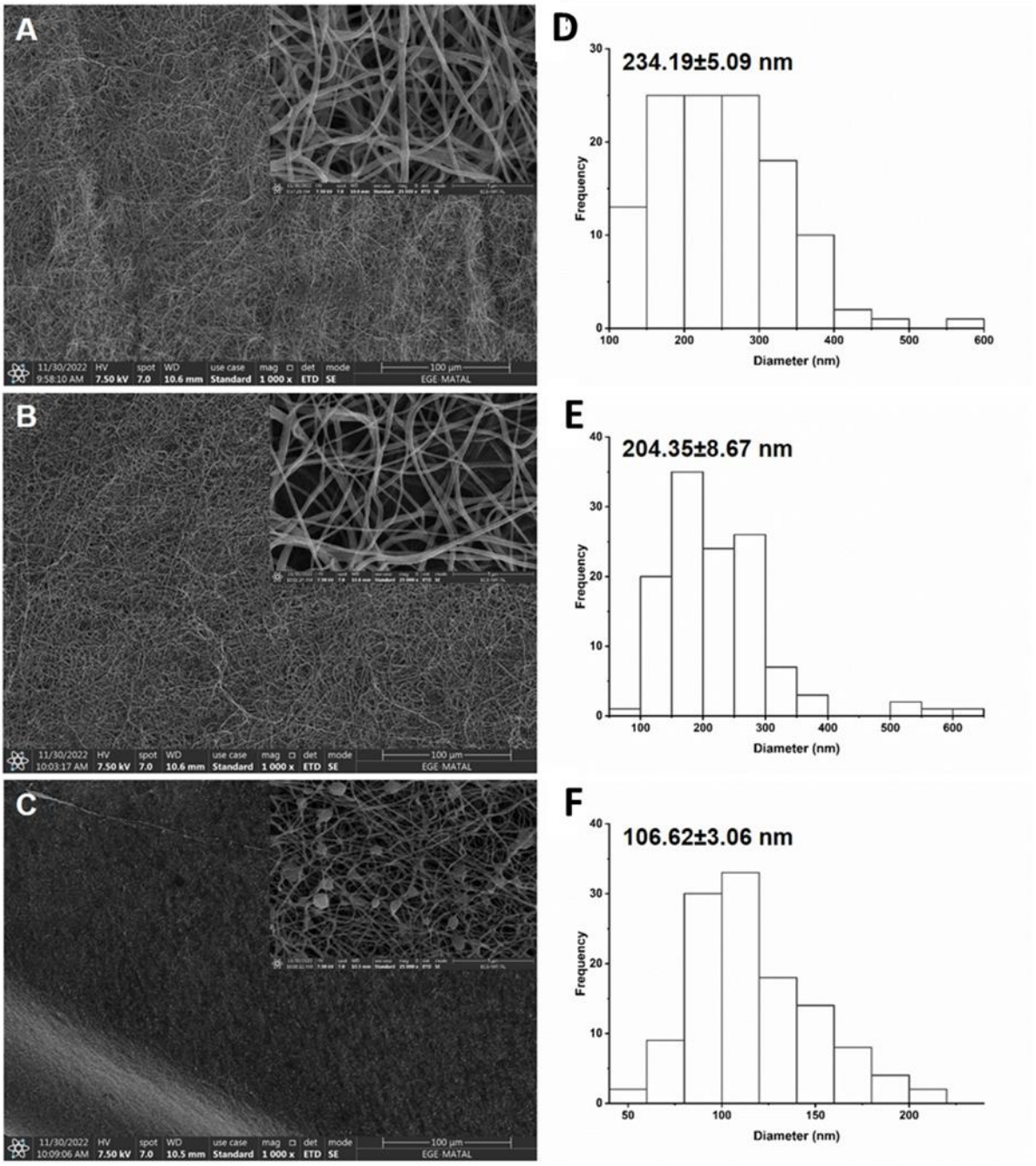

Figure 1. SEM micrographs of **A)** PCL (10%; w:v), **B)** PCL/PEGdt ([PCL]: 10%; w:v and [PEGdt]: 2.5%; w:v), **C)** PCL/PEGdt ENs ([PCL]: 10%; w:v and [PEGdt]: 5.0%; w:v) (Magnification 1000X, scale bar 100 µm and for inset figures, magnification 25000X, scale bar 5 µm). Histogram for ENs diameter distribution and average diameters of **D)** PCL (10%; w:v), **E)** PCL/PEGdt ([PCL]: 10%; w:v and [PEGdt]: 2.5%; w:v), **F)** PCL/PEGdt ENs ([PCL]: 10%; w:v and [PEGdt]: 5.0%; w:v).

Table 1. Selection parameters for the PCL/PEGdt blend ratio

PCL/PEGdt	Average diameter (nm)	Bead formation	Comment
PCL (10%; w:v)	234.19±5.09	No	uniform
PCL(10%; w:v)/PEGdt(2.5%; w:v)	204.35±8.67	No	uniform
PCL(10%; w:v)/PEGdt(5.0%; w:v)	106.62±3.06	Yes	non-uniform

3. RESULTS AND DISCUSSION

3.1 Characterization of PCL/PEGdt/Bro ENs

The impact of PEGdt on the ENs structure assessed through a comparison of SEM images, the diameters, and contact angles. Notably, due to its low molecular weight, PEG functions as a plasticizer between PCL chains, leading to a decrease in solution viscosity, and, consequently a reduced diameter [25]. Figure 1A-1C shows the SEM images of PCL and PCL/PEGdt ENs and Figure 1D-1F displays the histogram for the dimeter of the PCL/PEGdt ENs. As shown in SEM images (Figure 1A-1C), the presence of PCL and PCL/PEGdt ENs were observed. With the increase of PEGdt concentration, the formation of beads in PCL/PEGdt ENs was noticed. Therefore, the optimum PEGdt concentration was found to be 2.5%. As such, in contrast to the average diameter of fibers achieved with 10% (v:v) PCL, which is 234.19 ± 5.09 nm (Figure 1D), the diameters of PCL/PEGdt ENs with a 2.5% (v:v) PEGdt incorporated ENs were computed to be an average of 204.35 ± 8.67 nm (Figure 1E). Moreover, thinner fiber diameters (106.62 ± 3.06 nm) along with the bead formation have been observed with the increased PEGdt concentration (Figure 1C, F; Table 1).

Notably, although the addition of PEGdt did not affect the contact angles (CAs) of PCL ENs surfaces solely, the decrease on contact angles were observed along with the increasing amount of Bro. As such, the resulting homogeneous and bead-free PCL/PEGdt/Bro ENs surfaces demonstrated hydrophilic nature, as evidenced by contact angles both before and after the addition of Bro. Contact angles of PCL/PEGdt and PCL/PEGdt/Bro ENs were 123.24°±3.18°, 126.58°±2.77° ([Bro]: 0.25%; w:v), 124.01°±2.77° ([Bro]: 0.5%; w:v), 87.92°±28.85° ([Bro]: 1.25%, w:v), 83.84°±15.25° ([Bro]: 2.5%; w:v), and 66.57°±4.36° ([Bro]: 5.0%; w:v), respectively. Hydrophilicity of the ENs is crucial for wound healing applications, since the recent research have shown that hydrophilic polymers have the potential to address problems of existing wound dressings, such as insufficient ability to absorb excess wound exudations [26]. Moreover, literature findings prove that wounds tend to heal more rapidly in moist environments [27,28]. Hence, the hydrophilic characteristics and its capacity to sustain a moist environment of materials used for wound healing are pivotal as the material not only shields the wound area from potential secondary damage caused by bacteria and external mechanical stress but also promotes a prompt healing process. In addition to their benefits for wound healing, they can potentially serve as a scaffold in tissue engineering applications, providing a substrate for cell adhesion. The formation of PCL/PEGdt/Bro ENs also confirmed by FTIR analysis. In the PCL fibers' spectrum, the prominent peaks at 2900 cm^{-1} and 2869 cm^{-1} correspond to the asymmetric and symmetric stretching of the CH_2 group, respectively. The sharp peak at 1724 cm^{-1} corresponds to the stretching of the carbonyl group. In the spectrum of PEGdt, the peak at 2945 cm^{-1} is attributed to C–H stretching. Confirming the individual spectra of PCL and PCL/PEGdt, similar groups observed within the blended polymer mixture (Figure 2). Moreover, the C=O stretch of the aldehyde and amide group at 1645 in PCL/PEGdt/Bro ENs was attributed to the bromelain addition.

3.2 *in vitro* cytotoxicity test of PCL/PEGdt/Bro ENs

The cytotoxicity assays are practical methods for assessing the biological safety of materials according to standard guidelines. In this context, effects of five different concentrations (0.25, 0.5, 1.25, 2.5, 5.0 % (w:v)) of Bro-containing PCL/PEGdt/Bro ENs on cell viability were evaluated using *in vitro* cytotoxicity test (MTT). The cytotoxic effects of PEGdt and Bro were tested on the HaCaT cell line over a 24 h period. The results indicated that cell viability for ENs cells remained above 80%, showing no significant cytotoxic effect in any of the ENs formulations, whether with or without Bro. Additionally, the presence of Bro in PCL/PEGdt resulted in the highest and most significant proliferative effect, particularly at 0.25% (w:v) of Bro concentration, with cell viability reaching more than 100% and t-test was applied on the results (Figure 3). The observed high cell viability at a 0.25% (w:v) of Bro was thought to be related to the peptidase activity of bromelain [17], which becomes more pronounced at higher concentrations. As is well known, Bro is a proteolytic enzyme that breaks down proteins. At low concentrations, partial degradation of proteins in the cellular environment may lead to a favorable microenvironment that supports cell adhesion and proliferation. However, as the concentration of Bro increases, particularly at high levels such as 5.0 % (w:v) and above, its excessive peptidase activity may lead to the degradation of surface proteins, thereby limiting cell adhesion and reducing cell viability. It is worth noting that Bro is typically formulated at concentrations up to 3% (w/v) in cosmetic application [18], which suggests that concentrations exceeding this range may not be suitable for maintaining cellular compatibility. In our study, we interpret the relatively high cell viability observed at 0.25% (w:v) Bro concentration as a result of moderate enzymatic activity. In line with this, several studies in the literature have explored the use of ENs for wound healing purposes. A comparison of some key properties of these ENs is presented in Table 2.

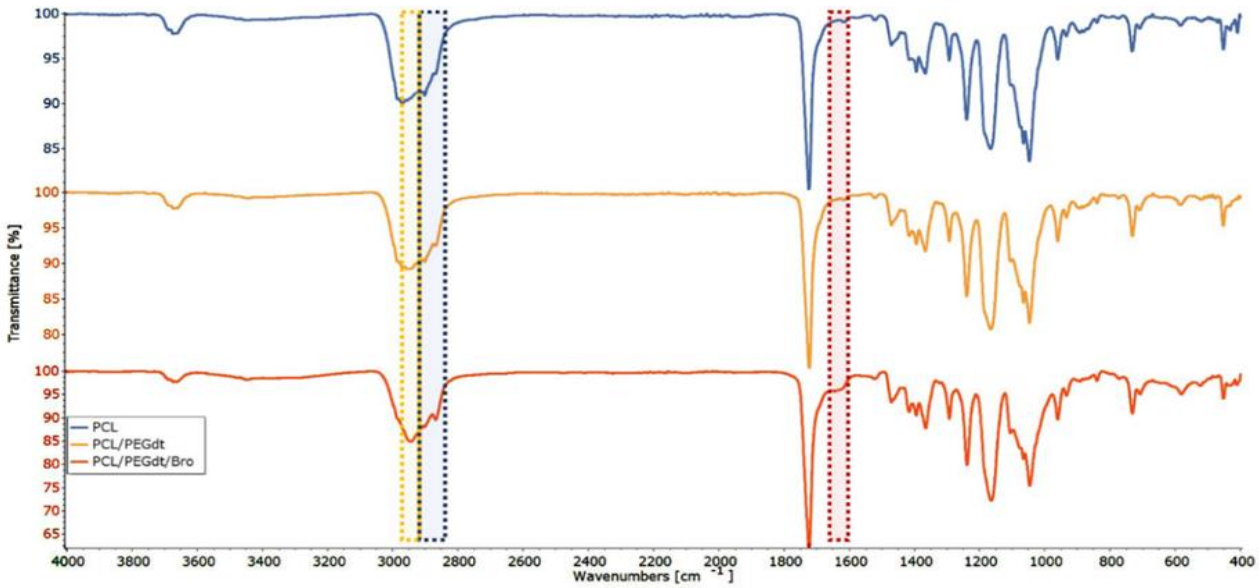

Figure 2. FTIR Spectra of PCL (blue line), PCL/PEGdt (yellow line; [PCL]: 10% (w:v) and [PEGdt]: 2.5% (w:v) and PCL/PEGdt/Bro (orange line; [PCL]: 10% (w:v), [PEGdt]: 2.5% (w:v), and [Bro]: 0.25% (w:v)). Each component's specific regions are highlighted with dotted boxes that match the colors of the lines.

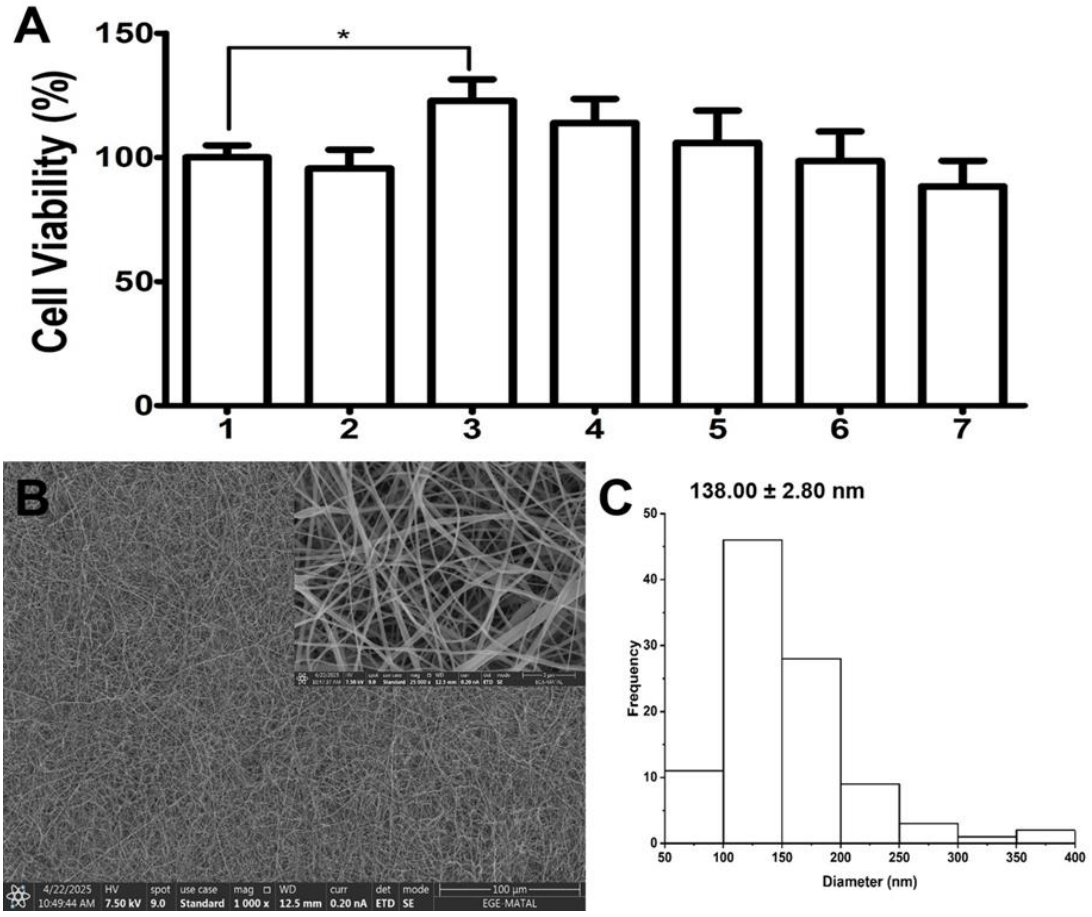

Figure 3. A) Effects of ENs on the cell viability of HaCaT cells after 24 h. **1)** PCL ENs, **2)** PCL/PEGdt ENs ([PCL]: 10%; w:v), [PEGdt]: 2.5%; w:v); **3)** PCL/PEGdt/Bro ([PCL]: 10%; w:v, [PEGdt]: 2.5%; w:v, [Bro]: 0.25%; w:v); **4)** PCL/PEGdt/Bro ([PCL]: 10%; w:v, [PEGdt]: 2.5%; w:v, [Bro]: 0.5%; w:v); **5)** PCL/PEGdt/Bro Bro ([PCL]: 10%; w:v, [PEGdt]: 2.5%; w:v, [Bro]: 1.25%; w:v); **6)** PCL/PEGdt/Bro Bro ([PCL]: 10%; w:v, [PEGdt]: 2.5%; w:v, [Bro]: 2.5%; w:v); **7)** PCL/PEGdt/Bro Bro ([PCL]: 10%; w:v, [PEGdt]: 2.5%; w:v, [Bro]: 5.0%; w:v) (Bars represent standard deviation, n=5, * ($P < 0.05$; t-test). **B)** SEM micrographs of PCL/PEGdt/Bro ([PCL]: 10%; w:v, [PEGdt]: 2.5%; w:v, [Bro]: 0.25%; w:v) (Magnification 1000X, scale bar 100 µm and for inset figures, magnification 25000X, scale bar 3 µm). **C)** Histogram for ENs diameter distribution and average diameters of PCL/PEGdt/Bro ([PCL]: 10%; w:v, [PEGdt]: 2.5%; w:v, [Bro]: 0.25%; w:v).

Table 2. Comparison of some properties of ENs prepared for wound healing in literature

EN material(s)	Bioactive ingredient	Diameter of ENs (nm)	Contact angle of ENs	Cell culture	Reference
Carboxymethyl cellulose/PVA	Colistin	-	78.44°	HFF-1	[29]
PCL/PLA	AgNP	397.23	133.4°	L929 and SK-MEL-30	[30]
PCL/Soy Protein	Tea tree oil	136	-	NIH3T3	[31]
PCL-Collagen/PVA	Collagen	191.8±113.9	35.8°	L929	[32]
PCL/ Chitosan	-	280 ± 89	60.9° ± 4.0°	Mesothelial cells	[33]
PCL/PEGdt	Bro	138.00±2.80	126.58°±2.77°	HaCaT	This study

4. CONCLUSION

In the present study, the functionality of both PCL/PEGdt and PCL/PEGdt/Bro ENs in terms of interpenetration of polymer networks as well as wettability and biocompatibility were assessed. The results revealed a successful utilization of a blend of PEGdt and PCL ENs for Bro incorporation, resulting in notable improvements in both physical characteristics and biological effects. These findings highlight the potential of Bro-loaded PCL/PEGdt ENs to overcome the limitations of existing wound dressings, particularly in their ability to absorb excess wound exudate and promote effective wound healing.

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