International Journal of Microstructure and Materials Properties

ISSN online: 1741-8429 - ISSN print: 1741-8410
https://www.inderscience.com/ijmmp

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Hamzah Hashim Kzar, Saade Abdalkareem Jasim, Sanobar Y. Kurbanova, Hasan S. Al-Ghamdi, Forqan Ali Hussein Al-Khafaji, Moaed Emran Al-Gazally, Acim Heri Iswanto, Surendar Aravindhan, Yasser Fakri Mustafa, Mohammad A. Alghamdi

DOI: 10.1504/IJMMP.2022.10052620

Article History:

Received:	25 March 2022
Accepted:	12 October 2022
Published online:	20 January 2023

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Hamzah Hashim Kzar

Veterinary Medicine College,
Al-Qasim Green University,
Al-Qasim, 51013, Iraq
Email: hamza.hashim@vet.uoqasim.edu.iq

Saade Abdalkareem Jasim*

Al-Maarif University College,
Medical Laboratory Techniques Department,
Al-Anbar-Ramadi, Iraq
Email: saade.a.j@uoa.edu.iq

Sanobar Y. Kurbanova*

Department of Microbiology and Pharmacology,
Tashkent State Dental Institute,
Tashkent, Uzbekistan
Email: b.abdullaev@editory.org

and

Department of Scientific Affairs,
Samarkand State Medical Institute,
Amir Temur Street 18, Samarkand, Uzbekistan
Email: sanobar.kurbanova.70@mail.ru
*Corresponding authors

Hasan S. Al-Ghamdi

Faculty of Medicine,
Internal Medicine Department,
Division of Dermatology,
Albaha University,
Albah City, Kingdom of Saudi Arabia
Email: H.saeed@bu.edu.sa

Forqan Ali Hussein Al-Khafaji

Department of Media,
Al-Mustaqbal University College,
51001, Babylon, Hillah, Iraq
Email: forqan.ali@mustaqbal-college.edu.iq

Moaed Emran Al-Gazally

College of Medicine,
University of Al-Ameed,
Karbala, 56001, Iraq
Email: moaedalgazaly@gmail.com

Acim Heri Iswanto

Faculty of Health Science,
Public Health Department,
University of Pembangunan Nasional Veteran Jakarta,
Jakarta, Indonesia
Email: h.iswanto@upnvj.ac.id

Surendar Aravindhan

Department of Pharmacology,
Saveetha Dental College and Hospital,
Saveetha Institute of Medical and Technical Sciences,
Chennai, India
Email: Surendararavindhan@ieee.org

Yasser Fakri Mustafa

Department of Pharmaceutical Chemistry,
College of Pharmacy,
University of Mosul,
Mosul–41001, Iraq
Email: Dr.yassermustafa@uomosul.edu.iq

Mohammad A. Alghamdi

Faculty of Medicine,
Internal Medicine Department,
Albaha University,
Kingdom of Saudi Arabia
Email: maahmad@bu.edu.sa

Abstract: Wound dressing is one of the most well-known and challenging issues all over the world. Recently, in the field of biological applications, polymeric scaffolds incorporating metal oxide nanoparticles has been extremely important, especially in focusing on wound healing. In this project, we have successfully fabricated a nano-fibrous scaffold using the electrospinning method prepared by poly(ε-caprolactone) (PCL) polymer. Titanium dioxide (TiO_2) is incorporated into the PCL solution as a strong antibacterial element and as a tensile strength enhancer. The characterisation of scaffolds was done by transmission electron microscopy (TEM), scanning electron microscopy (SEM), mechanical analysis, and water vapour transmission rate (WVTR), porosity. The nanofibers' biocompatibility and capacity for cell attachment were demonstrated by MTT and 4′,6-diamidino-2-phenylindole (DAPI) staining. By using the optical density method and coming into direct touch with gram-positive and gram-negative bacteria, the PCL/TiO_2 scaffold exhibits antibacterial action. These data suggest that engineered nanocomposite scaffold has a superior potential for wound healing applications which improves elasticity, strength, and antibacterial properties.

Keywords: nanofibrous scaffold; poly(ε-caprolactone); wound healing; antibacterial activity; titanium dioxide; nanoparticles; elasticity; biocompatibility; cell attachment; engineered nanocomposite.

Reference to this paper should be made as follows: Kzar, H.H., Jasim, S.A., Kurbanova, S.Y., Al-Ghamdi, H.S., Al-Khafaji, F.A.H., Al-Gazally, M.E., Iswanto, A.H., Aravindhan, S., Mustafa, Y.F. and Alghamdi, M.A. (2023) 'The biomedical potential of polycaprolactone nanofibrous scaffold containing titanium oxide for wound healing applications', *Int. J. Microstructure and Materials Properties*, Vol. 16, No. 4, pp.278–291.

Biographical notes: Hamzah Hashim Kzar studied at the College of Medicine, Babylon University, Babylon, Iraq. He works as an Associate Professor in the Department of Biochemistry, College of Veterinary Medicine, Al-Qasim Green University, Iraq.

Saade Abdalkareem Jasim took a Master's degree from the Education for Pure Science College Tikrit University, specialising in microbiology. He is working to take a Doctoral degree from AL-Azhar University in Egypt republic and his thesis is about Hepatitis C. He is Lecturer at Al-maarif University College and his interest in most branches of microbiology (bacteriology and virology) and molecular biology.

Sanobar Y. Kurbanova is a Professor and Head of the Department of Microbiology and Pharmacology at Tashkent State Dental Institute and also works as a researcher in the Department of Scientific Affairs at Samarkand State Medical Institute.

Hasan S. Al-Ghamdi is an Associate Professor of Dermatology and Venereology in the Department of Internal Medicine at Albaha University, Kingdom of Saudi Arabia. He teaches two important modules to medical students at the student level in the medical college. The two modules are the integumentary module and the dermatology and plastic surgery module. He earned his Saudi board of dermatology and venereology at the Saudi Commission for Health Specialties in 2013, having previously earned his MBBS at Taibah University in 2008. He worked as a consultant in dermatology and venereology at the governmental hospital. He is an author of much research in the field of dermatology and its branches.

Forqan Ali Hussein Al-Khafaji is Administrator of the Department at Al-Mustaqbal University College, has obtained an LLM at the Yanka Kupala State University of Grodno. In addition, he has published several scientific research in different journals.

Moaed Emran Al-Gazally studied at the College of Medicine, Babylon University, Babylon, Iraq. He works as a Professor in the Department of Biochemistry, College of Medicine, Al-Ameed University, Karbala, Iraq.

Acim Heri Iswanto is a Professor of Health Economics at Public Health Department, Faculty of Health Science, Universitas Pembangunan Nasional Veteran Jakarta. He received his Doctorate of Economic Science, Master, and Bachelor in Hospital Management. He conducted training in Singapore, Japan, the USA, Taiwan (Rep. of China), IR Iran, Pakistan, Thailand, Malaysia, China, Philippines, Turkiye, and Vietnam. Certified in Lean, Lean Six Sigma Black Belt, Knowledge Management, Human Centered Productivity, and Performance Management Improvement System, Innovation Management System. He has more than 15+ intellectual property rights and author for 150+ publications including journals, books, proceedings, policy briefs, and 43 indexing in Scopus.

Surendar Aravindhan works as an Assistant Professor in the Department of Pharmacology at Saveetha Institute of Medical and Technical Sciences of Chennai, India.

Yasser Fakri Mustafa received his BSc in Pharmacy from the College of Pharmacy at the University of Mosul in 1999. Also, he acquired the MSc and PhD degrees in Pharmacy/Pharmaceutical Chemistry from the College of Pharmacy/University of Mosul, Mosul, Iraq, in 2005 and 2017, respectively. He had various teaching experiences, such as organic pharmaceutical chemistry, inorganic pharmaceutical chemistry, the chemistry of natural products, chemotaxonomy, and heterocyclic compounds. Also, he is an editorial member of several documented journals, such as the *Iraqi Journal of Pharmacy*, the *Mosul Journal of Nursing*, the *Journal of Medicinal and Chemical Sciences*, *Eurasian Chemical Communications*, and the *International Journal of Pharmacy and Chemistry*. The present work of his academic aims to isolate natural coumarins from the seeds of various apple phenotypes and modify their chemical structures for improving biological activities. Also, this project has been extended to another fruit. The other branch of his work is directed toward the chemical modification of curcumin to enhance its physicochemical properties and biological activities to serve better therapeutics.

Mohammad A. Alghamdi is an Assistant Professor of Dermatology and Venereology at Albaha University, Kingdom of Saudi Arabia. He received his medical degree (MBBS) from King Khaled University-Abha Saudi Arabia in 2008. In August 2009, he completed his internship at King Fahad Medical City in Riyadh, Saudi Arabia. He later completed his dermatology residency at a municipal hospital in Karlsruhe, Germany (2012–2017). He is a member of numerous state and national medical associations as well as many dermatological societies.

1 Introduction

Skin is the most exposed tissue to a wide variety of injuries, so protection is a necessity to avoid loss of skin, hair, and glands, and also infection development (Percival, 2002; Velnar et al., 2009). A wound can be caused by a simple or severe break leading to injury to the anatomical or function of skin tissue (Esmaeili et al., 2020). Wound healing is one of the most challenging concerns all over the world which meets two main requirements including cost-effective dressing and enhancement in skin regeneration (Boateng and Catanzano, 2015; Psimadas et al., 2012; Enoch and Leaper, 2005). Nanotechnology has a wide variety of applications in biomedical engineering diagnosis, drug delivery systems, tissue engineering, prostheses, implants, and wound healing (Ramos et al., 2017). Nanomaterials integrate into biomedical devices for the aimed applications since most biological systems are nanostructured (Anil Singare et al., 2018). Wound healing is a complex process composed of five stages including hemostasis, inflammation, proliferation, migration, and remodelling. The ideal wound dressing should be able to avoid infection, provide local moisture to the wound, water absorption, gas exchange, exhibit good biodegradability, and be easy to remove from the skin (Razali et al., 2020; Archana et al., 2013, 2015). Synthetic polymers incorporated with antibacterial elements have the potential to control the release of bioactive materials which are suitable for the treatment of local infectious (Langer, 1980). In addition to serving as an ideal substrate for cell attachment, migration, and proliferation, scaffolds are useful for delivering medicines, genes, and cells into the body (Psimadas et al., 2012). Electrospinning is a flexible technique that creates fibres and nanofibrous structures and has a wide range of applications (Percival, 2002). The benefits of electrospun scaffolds include their fibrous structure, high porosity, and surface area that is similar to the extracellular matrix in nature. They also enable drug delivery to target areas with high loading and efficiency. The electrospinning technique can be applied for biomedical purposes such as vascular grafts, artificial organs, tissue engineering, and wound dressing because of its adaptability.

Poly(ε-caprolactone) (PCL) is a widely practical practical is known as a biodegradable, thermoplastic, and biocompatible polymer which can be easily electrospun using low voltages. This material improves tensile strength and mechanical resistance in a moisture environment making it as good candidate to be used in wound healing dressing (Croisier et al., 2014; Sun et al., 2006; Khalili et al., 2021). PCL nanofibrous scaffold and its composites have gained much success in skin tissue engineering due to mimicking extracellular matrix (ECM) (Joseph et al., 2019).

TiO_2 incorporation into the PCL nanofibrous scaffold can cause superior mechanical strength and introduce antibacterial properties in wound implantation (Wu et al., 2014). The main purpose of this study was to design PCL/TiO_2 nanofibrous film for artificial dressing application which meets the needs of antibacterial and tensile strength properties. The different criteria of the scaffold will be evaluated for wound dressing application.

In this study, the PCL and PCL/TiO_2 nanofibrous scaffolds were fabricated by the electrospinning method. The morphology of fibres and incorporation of nanoparticles into the nanofibers was assessed by SEM and transmission electron microscopy (TEM). Cell toxicity and cell attachment were investigated by biocompatibility and DAPI staining analysis using fibroblast cells seeded on the scaffolds. To evaluate antibacterial

properties, the optical density method was used to evaluate the antibacterial activity of the scaffolds. The results showed that the incorporation of TiO_2 nanoparticles into the PCL scaffold lead to the better physical and biological properties needed for wound dressing applications.

2 Experimental part

2.1 Materials

In this project, poly (ε-caprolactone) (PCL) was used as a matrix and was purchased from Sigma-Aldrich (St. Louis, MO). The organic solvents used were dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and chloroform prepared from Merck (Darmstadt, Germany). TiO_2 nanopowder, 21 nm particle size, was used as fillers and supplied by PlasmaChem Gmbh. 4′,6-diamidino-2-phenylindole (DAPI), and 3-[4,5-dimethylthiazol–2-yl]–2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification.

2.2 Synthesis of PCL and PCL/TiO_2 nanofibers by electrospinning method

Electrospinning was used to create PCL nanofibers and PCL/TiO_2 composite nanofibers. The polymer was dissolved in a chloroform/DMF mixture (4:1 v/v) at room temperature for an overnight period to produce a PCL solution with a polymer content of 14% (w/v). TiO_2 nanoparticles were added to the solution for the composite PCL/TiO_2 and further homogenised by stirrer and ultrasonic. A typical electrospinning machine (Nanoazma, Tehran, Iran) was utilised to create a nanofibrous film utilising a solution flow rate of 0.5 ml/h, electrical voltage of 18 kV, and needle tip to collector distance of 15 cm.

2.3 Characterisation techniques

The morphology of the electrospun nanofibers was studied using a scanning electron microscope (SEM; AIS2100 Model; Seron Technologies, Korea). Using a sputtering device (SC7620, Quorum Technologies, UK) and an accelerating voltage of 20 kV, the samples were coated with gold.

The integration of TiO_2 nanoparticles into composite PCL/TiO_2 nanofibers was investigated using a transmission electron microscope, or TEM, Philips CM–30.

The mechanical characteristics of the constructed scaffolds were investigated using tensile measuring equipment (STM–20, SANTAM, Iran). Plotting the stress-strain curve of each scaffold required stretching the nanofibrous films at a loading velocity of 5 mm/min while they were at ambient temperature.

2.4 The analysis of the membranes' porosity

Using liquid displacement method. The porosity of the scaffolds was determined (Esmaeili et al., 2020). Ethanol does not induce swelling and shrinkage as a non-solvent of the polymer and penetrates easily into the pores of the nanofibrous scaffold, so is used as a displacement liquid. The scaffold samples were immersed in the known amount of

ethanol and kept for 1 h. The membranes' porosity was estimated using the total amount of the ethanol impregnated into the scaffold by the following equation:

$$porosity(\%) = \frac{W_W - W_d}{D_{ethanol} \times V_{membrane}} \times 100$$

where W_d and W_w are the weight of the scaffold before and after immersing into the ethanol. V_{membrane} is the volume of the scaffold after immersing into the ethanol and D_{ethanol} is the density of the ethanol.

2.5 Water vapour transmission rate (WVTR) examination of the scaffolds

nanofibrous scaffold is used for sealing of glass tube containing 10 ml ultrapure water . The membrane was fixed on the glass tube by parafilm. Then, the tubes were placed in an oven at 37°C for 24 h. the WVTR has been estimated by the following equation:

$$Water\ Vapor\ Transmission\ Rate = \frac{W_{loss}}{A}$$

where W_{loss} represents the water vapourisation and A shows the surface area of the open part of the glass tube (1.77 cm^2).

2.6 The MTT assay and DAPI staining

The nanofiber scaffolds were plasma treated at 30 w for 3 min to make them hydrophilic which is appropriate for cell attachment. The scaffolds were sterilised by incorporation in a filtered 70% ethanol for 2 h and after 20 min exposure under UV radiation, the scaffolds were incorporated in a culture media for 24 h to facilitate cell attachment on the nanofibrous scaffold.

The foreskin fibroblast cells purchased from the Stem Cell Technology Research Center (Tehran, Iran) were seeded in culture medium (DMEM; Hyclone, Logan, UT, USA) rich with 10% fetal bovine serum (FBS; Hyclone). The cells were seeded on the scaffolds with a density of 1×10^4 cells and kept in the incubator with 5% CO_2 at 37°C. After one and three days of cell seeding, the substance was removed, and also the MTT solution (0.5 mg/ml) was added to every well and incubated in the dark for 3 h at 37°C. Then, the MTT solution was removed and dimethyl sulfoxide (DMSO) was added to dissolve the formazan reaction merchandise. Lastly, the optical density of the purple formazan solution was scanned at 570 nm employing a microplate reader (BioTek, ELX800, USA) to quantify the cell viability.

For DAPI staining, the scaffold seeded with fibroblast cells was fixed with 4% paraformaldehyde at 4°C for 20 min and then at room temperature for 5 min on day 3 after cell seeding. Thereafter, the samples were washed with PBS, incubated with 2 µg/mL DAPI solution for 1 m to stain the nuclei of the cells, and rinsed again with PBS. Using a fluorescence microscope (TE-2000; Nikon), the cell nucleus images on the samples were obtained.

2.7 Antibacterial test

Antibacterial activity was characterised by the optical density method using Escherichia coli, E. coli, as the gram-negative and Staphylococcus aureus, S. Aureas, as the gram-positive test organism. The bacteria were cultured in Mueller–Hinton media at 37°C overnight. The 0.5 McFarland bacterial suspension was diluted to 1:10 and then exposed to the samples for 24 h at 37°C, the turbidness of the media was evaluated at 610 nm employing an ultraviolet photometer (CECIL-CE9200). Negative control serves as the bacterial suspension without any antibacterial element and the media containing Pen-Strep was employed as a positive control. The following equation is used for calculation of the antibacterial rate:

$$antibacterial\ efficacy\,(\%) = \left(1 - \frac{A_{sample}}{A_{negative\ control}}\right) \times 100$$

3 Result and discussion

Poly-α-hydroxy esters including PCL are a good candidate to produce electrospun microstructures for wound dressing applications due to their rate of biodegradability, providing an orderly self-repair for skin cells, and inhibiting to generate scars. This type of dressing can mimic the extracellular matrix, ECM, providing a good environment for cell attachment and development, which leads to an increase in wound healing (Lima et al., 2019). SEM pictures of the PCL and PCL/TiO_2 electrospun membranes have been shown in Figure 1(a)–(b). The produced scaffold showed a continuous cylindrical fibre shape without any bead morphology. The fibre diameters were analysed using Image J software from the SEM micrographs, showing the average diameter of 343±111 and 121±46 for PCL and PCL/TiO_2, respectively. It is evident that fibre diameter and distribution size decreased while incorporating TiO_2 nanoparticles into the PCL nanofibers. The ECM, the natural environment around the cells in human tissues is a porous, three-dimensional matrix with nanostructured units. The ideal scaffold should mimic the ECM. The produced scaffold can simulate the ECM properties such as porosity, three-dimensionality, and nanostructured building blocks. The prepared nanofibrous scaffolds provide interconnected open pores which is a crucial parameter for the cells to exchange gas, nutrients, and wastes inside the scaffold (Mikos and Temenoff, 2000). TEM images of the PCL/TiO_2 nanofibrous scaffold confirm the incorporation of 21 nm TiO_2 nanoparticles into the PCL matrix, Figure 1(c). The TEM image shows a good distribution of TiO_2 nanoparticles into the scaffold which can lead to a monotonous mechanical strength and antibacterial activity throughout the scaffold.

The mechanical characterisation of the nanofibrous mats has been shown in Figure 2. The mechanical properties of PCL mat are enhanced due to the presence of TiO_2 nanoparticles which act as nanofillers. Tensile strength has increased from 1.044 MPa for PCL to 1. 78 MPa for PCL/TiO_2. It will be explained by the intra and building block element bonding between the TiO_2 and PCL compound molecules that caused the purpose bond structure. Elongation at break has experienced significant growth in the presence of TiO_2 nanoparticles. It can be observed that the mechanical strength of

PCL/TiO_2 nanofiber might meet the essential needs for wound dressing application (Gupta et al., 2012).

Figure 1 SEM images of nanofibrous scaffold: (a) PCL; (b) PCL/TiO_2 and TEM image of (c) PCL-TiO_2 and their histogram profiles (see online version for colours)

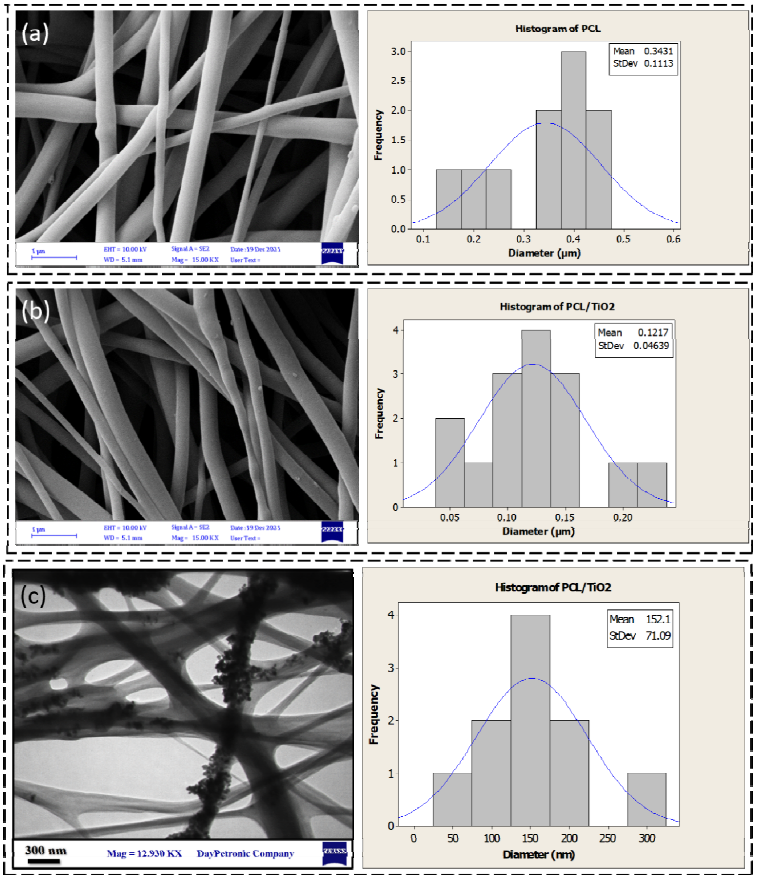
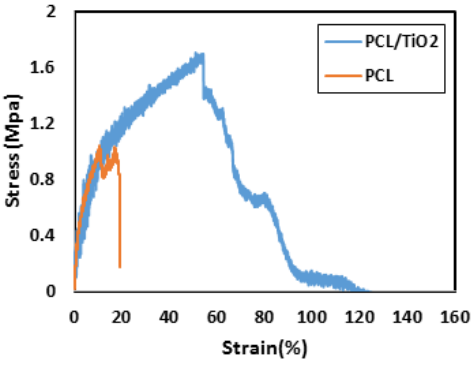


Figure 2 The mechanical properties of the nanofibrous scaffolds resulted from the uniaxial tensile experiment (see online version for colours)



The porosity of the scaffold plays a vital role in the mechanical and biological properties. It affects cell migration and proliferation, and the gas and nutrient exchange, especially when the vascular system has been damaged and does not work properly (Annabi et al., 2010). The porosity values obtained using the liquid displacement method were 187.8 ± 1.8 and 182.1 ± 0.3 for PCL and PCL/TiO_2 nanofibers, respectively, which has no significant difference among the samples, Table 1. It has been presented that the scaffolds with >90% porosities can be suitable for skin tissue engineering providing enough space for cell proliferation and migration and nutrient exchange (Esmaeili et al., 2020). So, the prepared scaffold with more than 180% porosity is a good candidate for wound healing application.

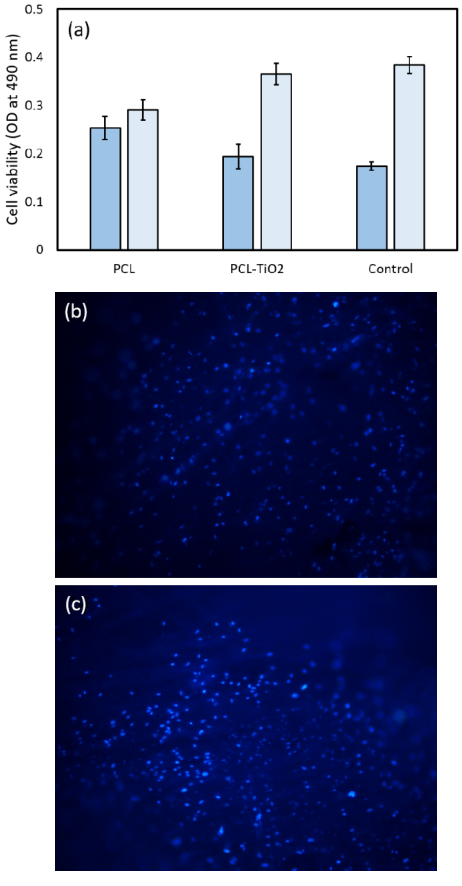
The optimal wound dressing should maintain an appropriate humidity of the wound environment, which affects cellular granulation and epithelisation. Higher WVTR is related to rapid wound dehydration, conversely, extremely low WVTR is related to exudate accumulation, leading to delay in the healing process, and an enhanced risk of infection. It is reported the WVTR of 204 ± 12 g/m^2/day for normal skin, 279–5138 g/m^2/day for the injured skin, and 2028.3 ± 237.8 g/m^2/day for optimal wound dressing to enhance epithelialisation and healing process (Yuan et al., 2018). WVTR of PCL and PCL/TiO_2 scaffolds were 828.9 ± 1.6 and 883.8 ± 0.6, respectively, Table 1. The enhanced water vapour transmission rate (WVTR) of PCL/TiO_2 nanofibers are often explained by the chance of higher molecular hydrogen bonding between TiO_2 and water molecules. Although the WVTR of the current scaffolds is smaller amount than the perfect worth, it is comparable with business ones (Op Site, Smith & Nephew, London, UK) (Miguel et al., 2017).

Table 1 The porosity and WVTR evaluation of the scaffolds

	WVTR	*Porosity*
PCL	828.9 ± 1.6	187.8 ± 1.8
PCL-TiO_2	883.8 ± 0.6	182.1 ± 0.3

The toxicity of the pure PCL and PCL/TiO_2 composite scaffolds has been analysed using the MTT assay. To do this end, the MEFs were seeded on the different scaffolds to assess the potential of the PCL and PCL/TiO_2 composite scaffold in tissue engineering. Figure 3(a) shows the viability and proliferation of fibroblast cells cultured on the nanofibrous mats of PCL and PCL/TiO_2 on days 1 and 3. The samples show good biocompatibility, confirming the non-toxicity of the samples for cell activity. Also, the samples show superior cell proliferation, making them a good applicant for wound dressing applications. The up going trend of cell growth seeded on the scaffolds represented that the PCL scaffold TiO_2 incorporation displayed no toxicity against this kind of cells, so it can be concluded that the scaffolds are biocompatible with cells. The DAPI staining results, Figure 3(b) and (c), show good cell attachment and location spreading of the cells on the samples, which are according to the MTT assay regarding the biosafety of the nanofibrous scaffolds.

Figure 3 (a) The MTT assay to analyse the cell biocompatibility on the scaffolds. DAPI staining of the fibroblast cells on the mats; (b) PCL and (c) PCL/TiO_2 (see online version for colours)



The antibacterial properties of the dressing's mats should be analysed before being applied in the regeneration of the defected skin. The optimal wound dressing should inhibit bacterial growth to prevent wound infection. A serious problem of the biopolymers such as PCL is the lack of antibacterial properties which limits their biomedical applications. In this study, TiO_2 nanoparticles have been embedded into the PCL scaffold with the aim of introducing the antibacterial activity against pathogenic species to enhance their potential to be used in the medical field (Rezk et al., 2019). Figure 4 and Table 2 illustrates the comparative results of the antibacterial properties of PCL and PCL/TiO_2 nanofibers. An increasing trend of bacterial growth for two kinds of E-coli and S-aureus is observed for the PCL sample, opposite to the PCL/TiO_2 sample which shows inhibition of bacterial growth. Also, E-coli is more inhibited compared to S-aureus, due to the denser peptidoglycan layer of the Gram-positive bacteria which is cocomparableith Gram-negative ones, which endue them more protection and delay apoptosis (Esmaeili et al., 2020).

Figure 4 Antibacterial activities of nanofibrous scaffolds against E.coli and S.aureus measured by optical density method (see online version for colours)

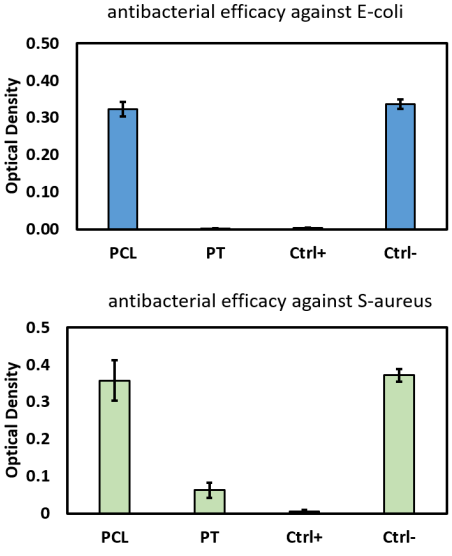


Table 2 Antibacterial activities of nanofibers determined by optical density method

Scaffold	*PCL*	*PT*	*Ctrl+*
Antibacterial efficiency (E-coli)	4.07	99.5	98.81
Antibacterial efficiency (S-aureus)	4.07	83.12	98.56

4 Conclusion

In this study, we aimed to produce an economical wound dressing to simulate the ECM of the skin tissue regarding physical, mechanical, and biological properties. To do this end, electrospun polycaprolactone nanofiber mats embedded with TiO_2 nanoparticles were successfully developed and characterised. TiO_2 nanoparticles endue the scaffold the mechanical strength and antibacterial activity. The results showed that the nanofibers were continuous and bead-free, suitable for biomedical applications. The random morphology of fibres makes it appropriate for skin tissue engineering. The presence of TiO_2 nanoparticles resulted in finer fibre diameter and a narrower distribution size. Also, the mechanical properties including tensile strength and elongation at the break of the PCL scaffold were increased by the incorporation of TiO_2 nanoparticles into the mats, as they can act as a nanofiller. MTT assay and DAPI staining of TiO_2/PCL scaffolds showed superficial biocompatibility and more attached cells rather than pure scaffolds. The fabricated nanocomposite, PCL/TiO_2, showed superior antibacterial properties against both Gram-negative, E-Coli; and Gram-positive, S. aureus, bacteria, suggesting the potential of the fabricated nanocomposite to prevent bacterial colonisation in wound dressing applications. This type of scaffold has superior mechanical strength and antibacterial properties which meets the economic issues.

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