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Preparation of nanofibrous mat using eco-friendly, biocompatible and biodegradable material

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A novel blend of pectin/ chitosan (CS)/polyvinyl alcohol (PVA)has been prepared as a nanofibrous scaffold for skin tissue engineering and wound healing applications. Pectin has been used as an eco-friendly, non-toxic and water-soluble polymer to improve nanofibre properties. Pectin as a poly-anion polymer, in combination with chitosan polycationic polymers, creates a cross-linked poly-electrolyte complex with biocompatible properties. The CS/PVA solutions containing various concentrations of pectin are electrospun to form a 3D pectin/ CS/ PVA nano fibrous scaffold. Mechanical and antibacterial properties of the nanofibrous scaffolds are evaluated by various instrumental techniques, such as Attenuated Total Reflection-Fourier Transform Infrared, Scanning Electron Microscopy, nitrogen content and Differential Scanning Calorimetry. Mechanical properties are found to be improved and the strong antibacterial activity of the pectin/CS/PVA nano fibrous scaffold is confirmed against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* and *Candida albicans*.

Keywords: Antibacterial properties, Biocompatible fibre, Biodegradable fibre, Chitosan, Mechanical properties, Nanofibrous, Pectin, Polyvinyl alcohol

1 Introduction

Producing polymeric nano fibres is one of the substantial fields in nanotechnology science. The most prominent characteristic of nano fibres is having a high surface area (area per unit mass)[1,2]. The use of biocompatible nanofibrous scaffolds in tissue engineering, wound dressing, and drug delivery system has revolutionized the therapeutic systems and pharmaceutical industry[3-5]. Tissue engineering is always looking for ways to make living components and replace artificial tissues with diseased or injured tissues. It is the technology to encourage damaged cells[6-9] to repair themselves through cells, biomolecules and mechanical support structures, and its main purpose is to restore memory from damaged tissue. Therefore, the role of the main source of artificial tissue production is to replace the function of wound healing and recovery[10,11]. Today, tissue-engineering technology is used to repair and regenerate tissues and organs, such as skin, bone, cartilage, vessels, heart valves, kidneys, and nerve[12-17]. Annually, many people need skin transplantation due to skin wounds caused by burns, skin diseases, or injuries[18-20]. Therefore, tissue engineering is one of the most important strategies for the treatment of skin injuries. In the past, skin grafts like allografts, Xenografts, and autografts have been used for wound healing and tissue repair. These methods are extremely expensive and have significant disadvantages[21,22]. It is likely that they will transmit the disease from one person to another's body and give them irreparable damage reflects.

Nanofibre substrates are suitable substitutes for damaged tissues[23-26]. A wide variety of biomaterials including synthetic polymers, such as poly(ε-caprolactone) and poly-(lactic-co-glycolic acid), and natural polymers, such as chitosan, silk, and collagen, are used in skin tissue regeneration studies[27-29]. The primary function of fibroblasts is the secretion of extracellular matrix precursors to maintain the structural integrity of the connective tissue[30, 31]. Among polymers used for the production of nanofibres, biocompatible polymers are good candidates for biological applications[32-35].

Chitosan(CS) has been considered a biopolymer according to its remarkable properties, such as

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antimicrobial activity, biocompatibility, biodegradability, non-toxicity, morphological similarity to the extracellular matrix (ECM)[26, 27] and oxygen permeability for cell growth. It is extensively used in medicine, biotechnology, and pharmaceutics[28,35]. The antimicrobial activity of CS is well known, and it has a polycationic charge which enables it to bind with anionic sites in microbe's proteins[36,37]. This polysaccharide has a positive charge in acidic media[38,39]. Mimicking the microstructure of ECM is one of the most pivotal elements in skin tissue engineering. Therefore, CS has the ability to become nanofibres, which mimic extracellular matrix ECMs[40]. Recently, the electro spinning process and the nanofibrous matrices achieved remarkable interest, essentially due to the structural mimicking of the ECM and the processing accessibility to a wide range of materials. Moreover, besides conventional two-dimensional (2D) nanofibrous structures, electrospinning is powerful in constructing 3D nanofibrous structures, especially for skin tissue engineering[41]**.** Electro spinning is an easy and impressive way of manufacturing ongoing nanofibres with diameters below 10nm[2,6]. The electro spinning procedure is applied high voltage in order to charge the fluid in a syringe electrically[7-9,42]. When the electric force presented to the polymer fluid dominates the surface among the solution and the needle, a slim polymer jet is excreted and entrusted on a collector as an accidental nanofibre layer[10,12].

For simplifying the electrospinning process and improving the mechanical properties of CS, polyvinyl alcohol (PVA) was used as a guest polymer[43-47]. PVA is a nontoxic and hydrophilic polymer[48-50]that has many applications in tissue engineering due to its biocompatibility and adequate physical and chemical properties[51,52]. In addition, it has good compatibility with biopolymers, such as CS and has chemical and thermal resistance properties[53,54]. Polyvinyl alcohol has the nature of the polar and is obtained from the saponify reaction of polyvinyl acetate. The CS/PVA blended polymers have a remarkable impression mainly on their biological behavior[55-57]. The combination of CS with PVA is reported to be better than each polymer on its own[55] and it improves the physico-chemical features and production cost[35,58].

On the other hand, pectin is a natural and water-soluble polysaccharide polymer that has many applications in the pharmaceutical industry[59-64]. As an additive for the preparation of marmalades, ice cream, jelly, and jam as a condenser, pectin is used[65-67]. It contains the benefits of reducing blood cholesterol, digesting food, fixing blood pressure, deleting weighty metal ions from the body, and improving bowel function[68,69]. Pectin has a negative surface charge and the ability to form polyelectrolyte complexes with poly-cations, such as CS[70,71]. For improving the performance of the combination, pectin is added to the blend. The main objective of this study is to design a nanofibrous scaffold based on the three components. In the present study, CS and pectin are prepared in a dilute acetic acid solvent and PVA nanofibres are acquired by the electrospinning process.

2 Materials and Methods

2.1 Materials

Chitosan powder with an average molecular weight (M_w = medium molecular weight) and a high degree of deacetylation (DD = 75-85%), was purchased from Sigma-Aldrich. Polyvinyl alcohol powder with a degree of hydrolysis 87-89% and an average M_W = 125000, was purchased from BDH Chemicals, England. Pectin with molecular weight 30.000-10.000 and a high degree of esterification (DE= high methoxyl pectin), was obtained from Sigma-Aldrich. Acetic acid 90% and distillated water were used as a solvent to prepare the solution of pectin, chitosan, and polyvinyl alcohol.

2.2 Preparation of Pectin/CS/PVA ElectrospunNanofibres

In order to prepare the solutions, CS was dissolved in acetic acid at a concentration of 3% by weight under magnetic stirring for 4 h with 400 rpm at 23°C.To prepare the blend solution, PVA was separately dissolved in distilled water at a concentration of 5% by weight. Different concentrations of pectin (1, 2, and 3%) were added separately to the blend solution of CS/PVA. For the electrospinning process, 5 mL of solutions was fed into the syringe pump using the applied voltage of 20 kV; the tip of the collector distance and the feeding rate were set at 15 cm and 0.02 mm/min respectively.

When the electric force extended to the polymer fluid control the surface tension among the solution and the needle, a slim polymer jet effused and accumulated on a collector as a nanofibre layer.

2.3 Characterizations

For making the electrospinning process simpler and improving the mechanical properties of chitosan, PVA and pectin were used as guest polymers.

Different properties of the chitosan/ PVA/pectin prepared nanofibrous are studied here under.

2.3.1 Attenuated Total Reflection-Fourier Transform Infrared Study

Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) (Bruker spectrum Model 27 tensor) analysis was used to characterize the presence of specific chemical groups of CS, PVA, and pectin nanofibres. In addition, it showed the reaction between CS/PVA and pectin in the range of 400-4000 cm^{-1}.

2.3.2 Scanning Electron Microscopic Study

The morphology and microstructure of nanofibres were evaluated by Scanning Electron Microscopy (SEM)at an acceleration voltage of 15 kV (KYKY EM-3200). The samples were first gold coated to avoid charging the specimen. This also increases the secondary electrons that can be detected from the surface of the sample in the SEM and improves the signal-to-noise ratio. The average diameter of nanofibres (average of 15 nanofibres ± SD) was determined.

2.3.3 Differential Scanning Calorimetry (DSC)

The thermal properties of the samples were measured by differential scanning calorimetry (TA Instruments, 2010 model, made in USA). The samples were scanned under an N_2 atmosphere and reciprocating cycle at a constant heating rate of 10º/min from 0 °C to 300°C.

2.3.4 Antibacterial Activity

Luria Bertani (LB) media broth, as a growing medium for *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* and *Candida albicans* for bacteria counting test, was used. Colony of bacteria growth on a nutrient medium can be counted. Bacteria were dripped in 10 mL of LB broth to reach a cell concentration of 1×10^8 (CFU)/mL and finally diluted to a cell concentration of 1×10^5 (CFU)/mL. Samples were incubated at 37ºC for 24 h. The 100 µL of solution was taken from each incubated sample, and distributed in a MHA agar plate. All plates were incubated for 24 h and colonies formed were counted. When calculating the amount of the change in cell number, logarithmic scale (log scale) is used. Log reduction was also calculated using the following equation:

$$\text{Log reduction} = \log_{10} C/A \quad \ldots(1)$$

where C and A are the bacteria colonies counted from the plate of the blank and treated sample srespectively[72].

2.3.5 Nitrogen Content Analysis

The nitrogen contents of the untreated and treated samples were examined by the Kjeldahl method. The treated fabrics were preserved under ambient conditions of 25 ºC and 40 % RH for 24 h before determining.

2.3.6 Mechanical Properties

Additionally, to evaluate the effect of CS, PVA and pectin on the mechanical properties of the electrospun fibre samples, the tensile strength was tested using an Instronmade in USA, with a gauge length of 7 cm and extension rate of 70 mm min^{-1} for three measurements, and the average value was noted. Stress−strain and load−deformation curve data were studied. For each sample, the tensile stress at maximal load was characterized. To examine the tensile properties of the nanofibres, several rectangular samples were prepared for each nanofibre layer. Young's modulus (E) and toughness values were also calculated for all the samples.

2.3.7 Contact Angle

There are different techniques used to calculate contact angles. Here, contact angles were measured at ambient humidity and temperature using a Jikan CAG-20 contact angle goniometer. For each test, 5 µL distilled water was deposited drop wise. The test was repeated for at least five measurements for each sample, with a standard deviation of about 2–3% and the average value was recorded.

3 Results and Discussion

3.1 ATR-FTIR Analysis

The interaction of PVA/CS and CS /PVA/PEC with different percentages of pectin has been analyzed by ATR-FTIR (Fig. 1). The main bands appearing in CS/PVA spectrum are due to stretching vibrations of NH_2 and OH groups at 3356cm^{-1} [Fig.1(a)]. The peaks are observed at 1423 cm^{-1} and 1084 cm^{-1} corresponding to CH_2 and first alcohol type respectively. In addition, the absorption peaks at 1220, 1804 and 2922 cm^{-1}have been reported as a phenol group, C – O groups, and C-H groups respectively. Based on the ATR-FTIR analysis, the absorption band at 3000-3500 cm^{-1} is corresponding to the overlapping of O-H and N-H stretching vibration bands in CS and PVA. Moreover, it can also

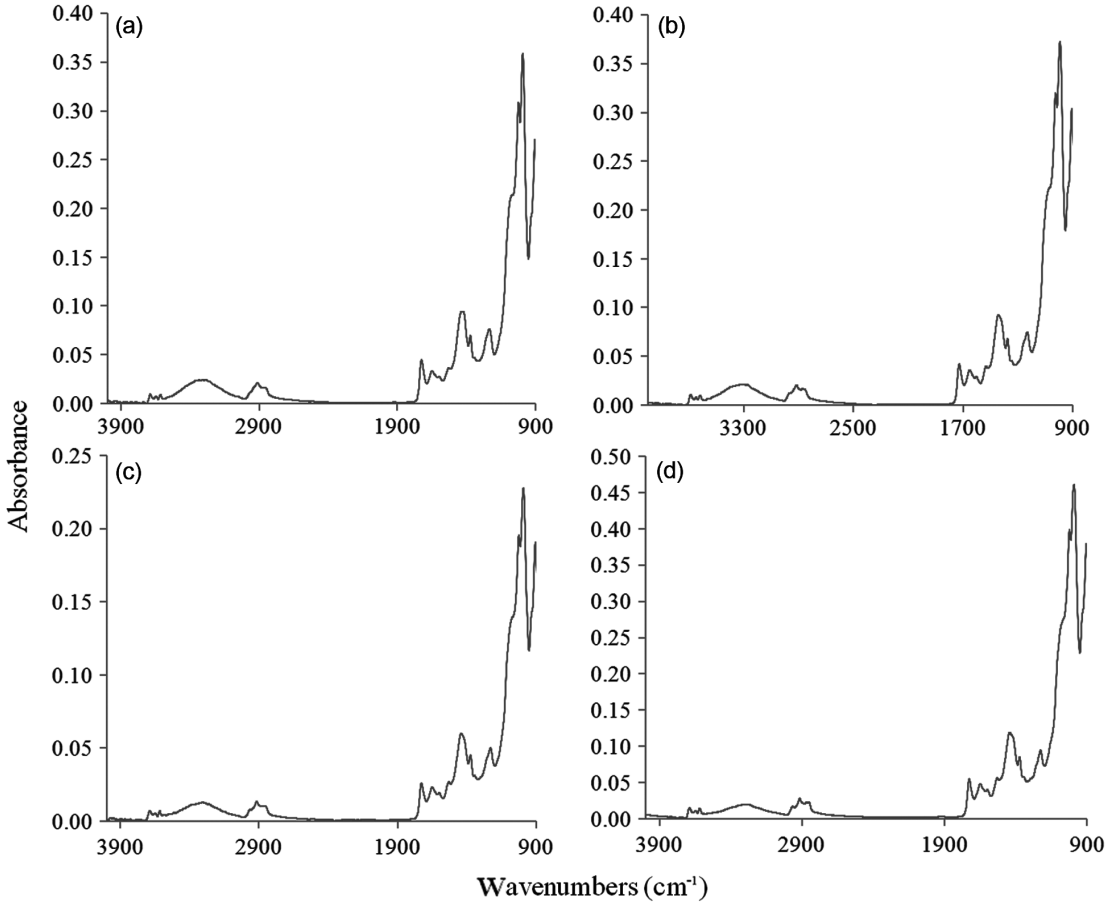

Fig. 1 — ATR-FTIR spectra of (a) PVA/CS, (b) PVA/CS/pectin 1%, (c) PVA/CS/pectin 2% and (d) PVA/CS/pectin 3%

be concluded that no destructive chemical interaction occurs between the two polymers PVA & CS, and only physical interactions happen. In the pectin spectra, the band between 3500cm^{-1} and 3000 cm^{-1}is dedicated to free hydroxyl [Fig.1(b)]. The peaks at two areas of 2922.24cm^{-1} and 2854.01cm^{-1} are related to the stretching band of (C-H) aliphatic and (C-H) aldehyde respectively. In addition, the bands also appeared at 1730.95cm^{-1} and 1623.13cm^{-1} due to the first amino groups and (C-C). Furthermore, the intensity of the absorption bands at 1623cm^{-1}and 1623cm^{-1} is reduced to the first amino group and the C = O respectively that confirms the interaction between CS/PVA and pectin [Figs 1 (c) and (d)][73].

3.2 Scanning Electron Microscopy

The morphology and diameters of different electro spun nano fibres are observed by scanning electron microscope (Fig. 2). In CS/ PVA blend (weight ratio 20/80), the average fibre diameter is found to be 67.44±14.86nm with uniform morphologies and minimum beads and defects.

Pectin/CS/PVA nanofibres show almost similar surface morphologies that consist of continuous and randomly oriented nano fibres. The average fibre diameters of various percentages of pectin (1, 2, and 3%)/CS/PVA nano fibrous scaffolds are also shown in Fig. 2. By increasing the percentage of pectin, the average diameter of nano fibres has increased. The average fibre diameters are in the range of 156.5±72.4-105.28±17.40 nm. The unique morphology of pectin/CS/PVA nanofibres with high porosity and surface area makes this mat a promising scaffold for skin tissue engineering applications.

3.3 Differential Scanning Calorimetry

DSC is a thermal analysis device determining how the physical properties of a sample change with temperature in contradiction of time. Thermal methods, such as differential scanning calorimetry are known as great thermos-analytical methods to study and monitor characteristic chemical and physical changes in biopolymers. The DSC thermograms of CS powder, pectin powder, PVA, PVA/CS and pectin/

CS/PVA blended membrane are presented in Fig. 3. Figure shows the DSC curves of the blended CS/PVA membrane together with their pure CS and PVA components. As it can be seen, the DSC curve of CS powder shows a broad endothermic peak at 91.98°C with 126.3J / g enthalpy. The DSC of pectin [(Fig. 3 (e)] indicates that the T_g is 94.42°C. However, PVA nanofibre has a sharp endothermic peak at 52.15℃

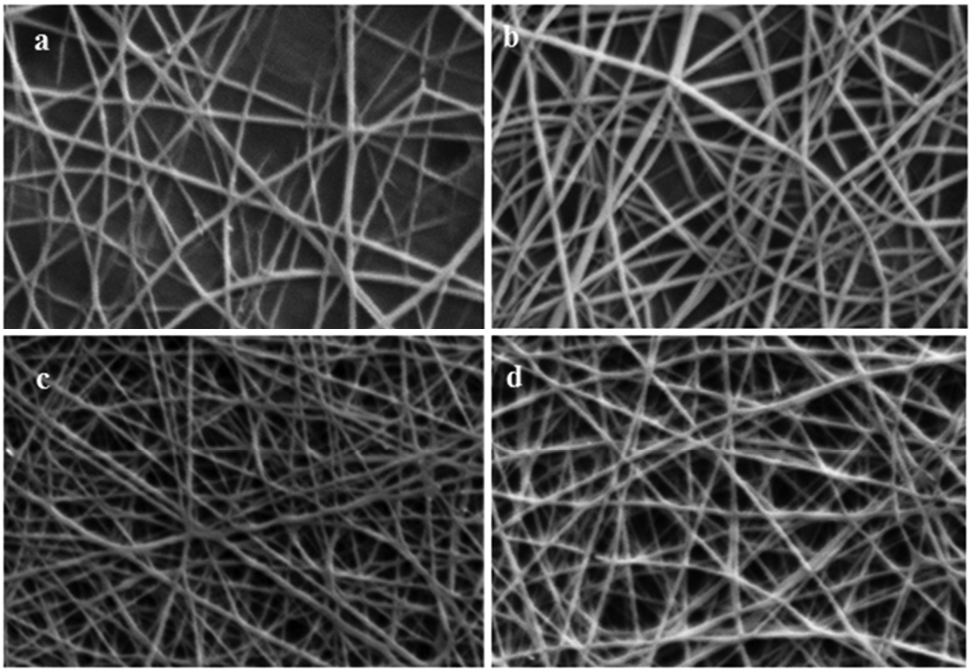

Fig. 2 — SEM micrograph of the electrospunnanofibers (a) PVA/CS (80:20),(b) PVA/CS/PEC (80:20:1), (c) PVA/CS/PEC (80:20:2), and (d) PVA/CS/PEC (80:20:3)

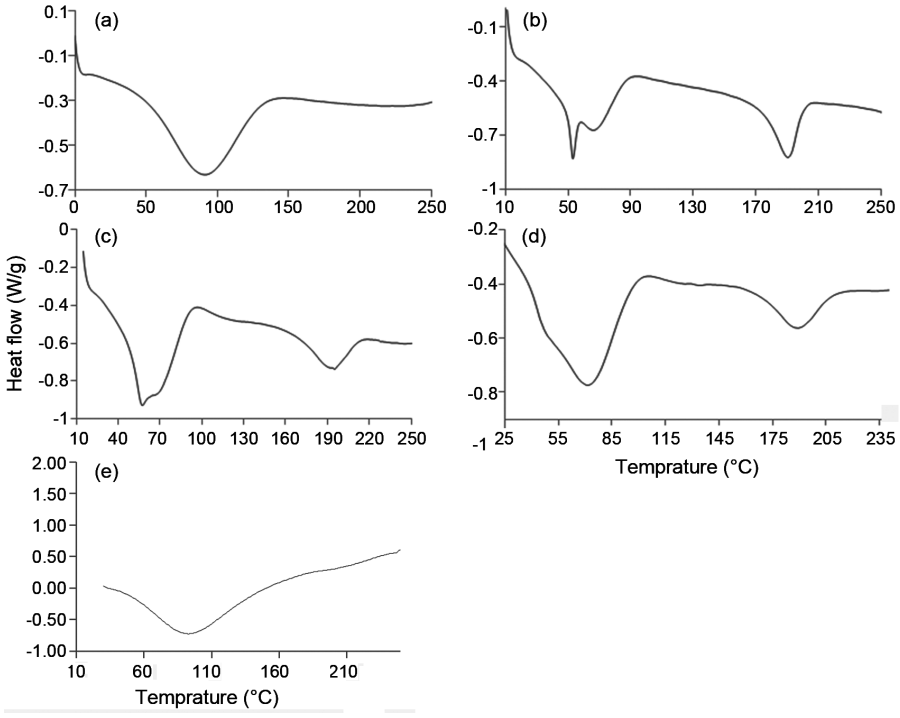

Fig. 3 — DSC curves of (a) CS powder, (b) PVA nanofibres, (c) PVA/CS(80:20)nanofibres, (d) PVA/CS/PEC (80:20:3) nanofibres and (e) pectin powder

with 35.95 J/g enthalpy, which indicates the glass transition temperature. Additionally, broad peak at 190.52º through 35.95 J/g enthalpy are related to PVA nanofibres and designates the melting temperature.

As shown in Table 1, PVA/ CS (80:20) nanofibres have a broad peak at 195.15º with 32.87J/g enthalpy, which represents the melting point which is related to pure PVA nanofibres. Temperature variations show that the interactions occur between the two polymers. The DSC curve of PEC/CS/PVA shows a broad peak at 188.34°C endothermic peak [Fig. 3 (d)], which indicates that the melting point and the enthalpy of the combination are partially reduced. Therefore, by adding pectin to the CS/PVA blend, the growth rate of crystallization is reduced as well. Results obtained from the thermal stability studies reveal that the interaction between CS/PVA and pectin is achieved. The similarity of a peak appeared in PVA/CS/PEC compared to the PVA/C Speak, suggests that pectin does not separate CS from PVA in the combination of these two polymers. This decrease in T_g may be due to the disruption of crystalline structure when grafted, indicating the formation of a new polymer.

Table 1 — Results of DSC thermogram of samples

Samples	Glass transfer temperature (T_g), deg	Melting temperature (T_m), deg	Enthalpy (Hm), $(^{j}/_{gr})\Delta$
Chitosan powder	91.98	-	126.3
Pectin powder	94.42	-	357.0
NanofibresPVA	52.15	190.52	35.95
NanofibresPVA/CS	57.47	195.15	32.87
PVA/PEC/CS nanofibres	70.61	188.34	24.97

Table 2 — Nitrogen content of grafted samples using the Kjeldhal method

Sample	CS, %	PEC, %	Nitrogen content, %
PVA	0	0	0
PVA/CS	80:20	0	0.039
PVA/CS	50:50	0	0.594
PVA/CS/PEC	50:50	1	0.570
PVA/CS/PEC	50:50	2	0.591
PVA/CS/PEC	50:50	3	0.543

3.4 Nitrogen Content Analysis

Amino acids contain nitrogen and they are building blocks of proteins. Therefore, the nitrogen content is characteristic of protein content as the percentage of nitrogen present in samples is directly relative to the percentage of proteins. This rate differs significantly, depending on the protein source. Consequently, nitrogen content can be used to conclude the quantity of protein in a sample. Nitrogen content is determined by the Kjeldahl method.

Table 2 shows the nitrogen content of the PVA, PVA/CS and PVA/CS/PEC with different ratios of CS. The nitrogen content of the samples containing chitosan is higher than that of the chitosan-free sample. According to the results, by increasing the ratio of chitosan in the composition, the amount of nitrogen increases. On the other hand, with the addition of pectin, there is no difference in the amount of nitrogen.

3.5 Antibacterial Activity

The antibacterial activity of the samples against four bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* and *Candida albicans*) was determined. To assess the antibacterial activity of PVA/CS/PEC nano fibres along with the control sample and antibacterial activity of this sample, international standard AATCC100 and also I.R.I National Standard with the number of 11070 were used.

The results of the counting test (Table 3) show more than a 99% reduction in all tested bacteria in the case of chitosan-treated samples. This is because of the interaction between chitosan with bacteria, which can change the metabolic activity of bacteria and eventually cause their death. Up to 99% of reduction

Table 3 — Antibacterial properties of CS, PVA/ CS and PVA/CS/PEC nanofibres against four bacteria

Sample	Colony count			
	S. aureus	*E. coli*	*Klebsiella*	*C. albicans*
Blank	2.3×10^5cfu/mL	2.3×10^5cfu/mL	2.3×10^5cfu/mL	2.1×10^5cfu/mL
CS	100 < 4.32≤R. log≤6.41	100 < 5.53≤R. log≤7.38	100 < 4.43≤R. log≤6.43	100 < 5.53≤R. log≤7.53
PVA/CS (50:50)	100 < 4.32≤R. log≤6.41	100 < 5.53≤R. log≤7.38	2×10^2 R. log=3.13	100 < 5.53≤R. log≤7.53
PVA/CS/PEC (50:50/ 3%)	5.4×10^1cfu/mL R. log=3.71	3.7×10^1cfu/mL R. log=3.82	1.3×10^3 R. log=3.31	10^3 R. log=2.36

Table 4 — Mechanical properties of fabricated PVA, PVA/CS, PVA/CS/PEC 1%, PVA/CS/PEC 2% and PVA/CS/PEC 3% nanofibres

Samples	Tensile strength GPa	Initial modulus GPa	Toughness MPa	Elongation-at-break %	Contact angle deg
PVA	1.42 ± 0.10	29.98 ± 0.24	58.29 ± 1.36	7.4 ± 1.3	51 ± 1.7
PVA/CS	1.60 ± 1.05	33.45 ± 0.89	61.02 ± 1.61	7.3 ± 1.1	42 ± 1.3
PVA/CS/PEC1%	1.74 ± 1.07	34.78 ± 0.29	66.92 ± 1.18	6.9 ± 0.9	47 ± 1.7
PVA/CS/PEC2%	1.81 ± 1.38	34.97 ± 0.77	72.36 ± 1.02	7.1 ± 1.5	49 ± 1.2
PVA/CS/PEC3%	1.91 ± 1.53	35.48 ± 0.86	77.81 ± 1.33	7.0 ± 1.5	49 ± 1.9

in survival bacteria means 2 log reduction. However, the reduction logarithm for chitosan/PVA/pectin treated samples is more than 2 and samples are listed as antibacterial fabrics. Adding pectin to the samples reduces the antibacterial efficiency but is negligible and more than 99% reduction is obtained and more than 2 log reduction is achieved. It can be concluded that the addition of pectin to the composition reduces the reduction logarithm, although the treated samples are still listed as antibacterial fabrics.

3.6 Mechanical Properties

Mechanical properties, such as initial modulus, tensile strength, elongation-at-break and toughness, are shown in Table 4. The initial modulus, tensile strength, and toughness for the PVA/CS/PEC fibres improve significantly. For the PVA/CS/PEC 3% fibre, the average tensile strength is 1.91GPa, which is higher than 1.60GPa for PVA/CS fibre sample. Likewise, the average initial modulus for the PVA/CS/PEC3% is 35.48GPa which is higher than that measured for PVA/CS fibre (33.45GPa). In addition, the toughness of the PVA/CS/PEC3% fibre in comparison with PVA/CS fibre is increased. In all samples, elongations-at-break is almost similar and PVA/CS/PEC 3% fibreretains the same ductility as PVA/CS fibre. The mechanical properties, such as tensile strength, initial modulus, and toughness are improved significantly. Results show that the addition of a third phase (pectin) into a PVA/CS composition can enhance its mechanical properties. Chitosan can be coupled with pectin through a polyelectrolyte complex, which increases mechanical stability, biocompatibility, and hydrophobicity properties. This polymer is used in skin tissue engineering, drug delivery, and pharmaceuticals. Pectin can be used in the electrospinning process to prepare nanofibres as a suitable substitute for cell culturing[74-77].

For calculating the wettability difference between pure PVA, PVA/CS and PVA/CS/PEC electrospun samples, contact angle measurements were used and the results are presented in Table 4. The contact angles of pure polymer nanofibres (PVA) are averaged at 51°. Addition of pectin shows modification on the surface wett ability and increases the hydrophilicity on the surface of PVA/CS nanofibres. The increase in contact angle shows that composite nanofibers are gained cause of grafting pectin with chitosan via polyelectrolyte complexes. Hydroxyl groups on the pectin surface increase the hydrophilicity of the composite.

4 Conclusion

In this research, a novel nano fibrous scaffold made from pectin/chitosan/PVA as an excellent alternative for tissue engineering applications has been studied. ATR-FTIR and DSC results show that physical interaction occurs between three polymers. Results of the antibacterial test show that the nanofibre layer is resistant against both Gram-positive and Gram-negative bacteria and the reduction logarithm for chitosan-treated samples is more than 2, and hence, samples are listed as antibacterial fabrics. Adding pectin to the spinning solution improves the physical and mechanical properties of the prepared nano fibres and modification on the surface wettability and increasing the hydrophilicity on the surface of treated nanofibres are observed. In conclusion, without the use of harmful solvents, a novel chitosan/pectin/PVA nanofibrous scaffold with acceptable mechanical properties is made effectively. In the next research, the prepared substrate will test for the growth of skin cells and biophysical characteristics of the prepared nanofibrous scaffolds will be checked, and the effects of the pectin/CS/PVA scaffolds on stem cell growth will be investigated.

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