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SYNTHESIS, CHARACTERIZATION AND SOME BIOLOGICAL PROPERTIES OF PVA/PVP/PN HYDROGEL NANOCOMPOSITES: ANTIBACTERIAL AND BIOCOMPATIBILITY

ABSTRACT

In this study, it was aimed to synthesize hydrogel based antibacterial, biocompatible and non-toxic wound dressing materials by solvent removal method usingpoly(vinylalcohol) (PVA), poly(vinylpyrolidone) (PVP) and nano pomegranate seed (PN).The morphology, swelling capacity, contact angle, antibacterial activity, biocompatibility and cytotoxicity of the synthesized films were determined. From the experimental findings, it was found that the PN particles were nano-sized, showed homogeneous and spherical distribution and improved the hydrophobic properties of the materials obtained by the addition of PN. And also, their swelling capacities were decreased with increased PN amount and all of the materials showed similar antibacterial activity, hemocompatibility and cytotoxicity.

Keywords: *Pomegranate seed; hydrogel nanocomposite; wound dressing material; antibacterial activity*

INTRODUCTION

The largest parts of wound treatment products, which have a billions of dollars of market share each year in the world, form the products used for long-lasting wound healing, such as pressure ulcers, foot ulcers and advanced burn injuries. The most basic purpose of wound dressing materials is to speed up the wound healing process and provide the highest level of comfort for the patients during this process. The wound dressing materials should keep the wound moist, accelerate healing and they should be antibacterial, biocompatible, non-toxic, cheap and easy to use. One of the important factors delaying wound healing is an infection, which can occur in the wound. For this reason, the use of antibacterial wound dressings has become important in recent years and studies in this area have increased [1,2]. Especially nowadays it is seen that the use of nutrients rich in natural active substances is increased rather than the use of synthetic drugs in the field of medicine.

According to literature research, it was seen that the most common wound dressing materials used today were alginate dressings, film dressings, hydrogel dressings, hydrocolloid dressings, foam dressings, reticulated wound dressings obtained from nanofibers and

cellulosic dressings obtained from bacteria. These dressings have been found to have various advantages and disadvantages depending on the type of wound used for. As a result of many studies made in recent years, wound dressings with different characteristics have been developed. In general, the main purposes of these products are to accelerate the wound healing process and reduce the risk of infection [3,4,5,6].

Hydrogels are often preferred as wound dressing materials due to their 3-dimensional and porous structures [7]. Poly(vinylalcohol) (PVA) hydrogels are highly suitable for use as wound dressing materials due to their high water retention capacities, non-toxic, non-carcinogenic, bioactive and biodegradable properties. PVA hydrogels can be prepared by chemical and physical cross-linking. The freeze-thawing method used for physical cross-linking allows the preparation of non-toxic hydrogels without the use of toxic chemical substances such as initiators and cross linkers. PVA hydrogels prepared by this method are particularly noted in biomedical applications. In order to recover weak mechanical strengths, biocompatibility and biodegradability of hydrogels, nanocomposite hydrogels prepared by the addition of nanocrystals and biocompatible polymers to the hydrogel matrix structure offer significant potential as wound dressing materials [8]. Poly(vinylpyrrolidone) (PVP) is a water-soluble, biocompatible, non-toxic, chemically inert, pH-stable and heat resistant, non-ionic polymer approved by the FDA for its wide range of uses. Because its structure resembles proteins, PVP has a great biological importance and is a simple model for scientific studies. PVP is a polymer used in biomedical applications and is used as a binder in pharmaceutical medicines, hydrogels for wound dressing and replacement of blood plasma [9,10]. Wound dressings produced from a blend of poly(caprolactone) and poly(vinylpyrrolidone) are used both in the healing phase of the wound and after the wound is closed [11].

As a result, the development of antibacterial, biocompatible, non-toxic, easy-to-use and cheap wound dressing materials that accelerate wound healing has become important today. Therefore, in this study, hydrogel-based antibacterial, biocompatible and non-toxic wound dressing materials were synthesized by adding of nano-sized pomegranate seeds (PNs) which are natural nutrient sources, into poly(vinylalcohol) and poly(vinylpyrrolidone) hydrogel mixture and their hydrophilic properties, swelling capacities, antibacterial activities, biocompatibilities and cytotoxicities were investigated. The pomegranate, which has a very rich content, is briefly summarized in the literature as antifungal, antibacterial and anti-inflammatory substance having superior properties used in venous wounds including burn wounds.Morgan and Nigam (2013) reported that the wound healing properties of PNs originated from the presence of tannins and polyphenols.In addition, the researchers indicated that PN extracts induced fibroblast migration, fibroblast proliferation, collagen formation and angiogenesis [12]. Nema et al. (2013) reported that methanol extract of pomegranate accelerated wound healing [13]. In recent years, due to the development of microbial resistance against antibiotics, the use of plants with protective and therapeutic effects has increased the interests of phytotherapy and consumers are aware of the negative effects on traditional antibiotics. Pomegranate, known as "the strong fruit of nature", is regarded as a phytochemical warehouse with an inventive medical value. Generally, freeze-thawing is used for the synthesis of wound dressings. One of the innovations of this study is the use of solvent removal method in the synthesis of wound dressing films. The second originality is the introduction of PNs from the pomegranate factories into the industry and final originality is the use of nanoparticles in the synthesis of wound dressing materials for the first time. In this study, it was aimed to develop antibacterial wound dressing materials which keep the wound moist for wound healing, allow gas exchange, accelerate wound healing, and are easy to use, biocompatible, non-toxic, cheap and easily prepared.

MATERIALS AND METHODS

Pomegranate seed (PN) was provided at the regional boundaries of Balıkesir. PVA (MW: 86,000 g.mol^{-1}) and PVP (MW: 130,000 g.mol^{-1})were purchased from Across Organicsin analytical grade and used without further purification. Ultra distilled water was used as a solvent for prepare the nanocomposites.

Preparation of PN extracts and their grinding

PN was obtained from pomegranate fruit. For this purpose, pomegranate fruit taken from the market was thoroughly washed with tap water and distilled water, and then manually removed from the shell and other non-edible parts. The extracted pomegranates were passed through the juicer (Philips), resulting in a PN pulp. The pulp was washed several times with distilled water and filtered [14]. The obtained PNs were dried at 40 °C for 48 hours. Dried PN pulp was grinded in Fritsch Pulverisette 7 model high energy tungsten carbide ball mill and reduced to nano-size. The mass ratio of ball and PN pulp was fixed at 100:1.

Determination of the particle size

Particle size analysis of PN was performed in automatic mode using a Malvern Nanozetasizer Nano Series Nano-S device and quartz cuvette. In these measurements, 0.1 g of ground PNs were suspended in 100 mL of distilled water. Suspended samples were held in an ultrasonic bath for 10 min in order to separate the inner PNs from each other. After resting the suspension for a few minutes, a certain amount of sample was taken to the quartz measuring cell of the computer-controlled nanozetasizer and the measurements were performed automatically.

Synthesis of wound dressing materials

Wound dressing films were synthesized according to the solvent removal method using PVP, PVAand nano sized PN particles. The chemical structures of PVP and PVA used in wound dressing material synthesis were given in Figure 1. The ratio of wound dressing material compounds (PVP/PVA/PN) were 48.5/48.5/3; 48/48/4; 47.5/47.5/5 and 40/40/20.In the synthesis of wound dressing materials (PVP/PVA/PN), the total of the mixture was kept constant as 1 g. To synthesize wound dressing materials at the mentioned ratios, a certain amount of PVP was added firstly into 50 mL of distilled water. A certain amount of PVA and 50 mL of distilled water were added into another erlenmeyer flask. Both mixtures were stirred in a magnetic stirrer for 24 h to allow complete dissolution of the polymers. These blends were then combined into a single erlenmeyer flask. A certain amount of PNs were added to the combined PVP/PVA solution and mixed again on the magnetic stirrer for 24 h. These mixtures were then poured into petri dishes to remove solvents by vacuum oven and the wound dressing materials were synthesized in film form [15].

a. PVP b. PVA

Fig. 1. The chemical structures of a. PVP and b. PVA

PVP/PVA (50/50) hydrogel was also synthesized without PN using the same method. The synthesized PVP/PVA hydrogel and wound dressing materials were shown in Figure 2 and it is clear that all of the wound dressing materials synthesized are in film form.

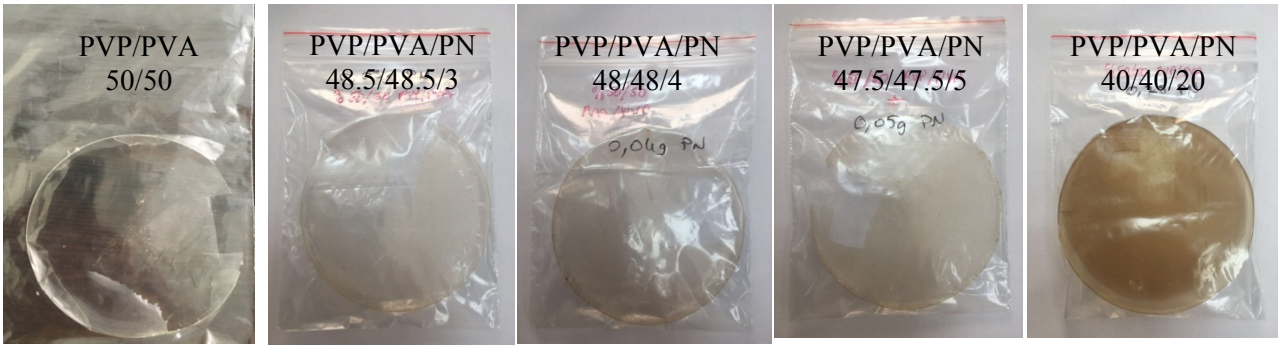


Fig. 2. The photo of the synthesized PVP/PVA hydrogel and their wound dressing materials

Determination of the antibacterial activity

In order to determine the antibacterial activity, the disc diffusion method was performed [16,17]. Samples were prepared by cutting a diameter of 15 mm using a caliper rule device and sterilized by UV light during 15 min. After the samples were prepared, inoculation was carried out with *Escherichia coli* and *Staphylacoccus aureus* cultures at 10^6CFU/mL concentration in tryptic soy agar medium. Samples prepared in a short time were appropriately placed on the labeled parts. Petri dishes were incubated for 24 h at 37 °C. At the end of 24 h, the zones around the materials were measured and the antibacterial activity was determined.

Cytotoxicity test (MTS assay)

The pipette tips and centrifuge tubes used for cytotoxicity test were sterilized by autoclave at 121 °C for 20 min(1.02 atm) and biosafety cabinet were sterilized with UV lampbefore being used in experiments. Any material taken from the outside cabinet was cleaned with 70% (v/v) ethyl alcohol. 3 mL EDTA blood was added into 7 mL Ficoll-Paqueto form two separate layers. The blood-containing tube was centrifuged at room temperature for 30 min at 1500 rpm. As a result of the centrifugation, four layers were observed. The lymphocytes in the second layer from the top were removed by pipetting, transferred to a clean centrifuge tube and dissolved in 1 mL medium. UV sterilized hydrogels (15cm^2) were incubated with 5mL culture media for 24 h. After 24 h of lymphocyte isolation, the culture medium was removed and replaced with the extract medium for each of the samples. Cells were then incubated for 48 h at 37°C and 5% CO_2. At the end of the each 24 h, 20µL of MTS reagent was added into 100 µL of cells(2x10^6 cells/mL). The cell viabilities were analyzed measuring the absorbance at 490 nm after 4 h of incubation at 37°C [18,19].

Hemocompatibility test

The hemocompatibility test was performed by modifying the method of Motlagh et al. [20]. The wound dressing material was used as 0.5 cm^2. 400 µL of anticoagulated (heparin) blood was diluted in 20 mL of 0.9% NaCl solution. 1 mL diluted blood was added onto UV-sterilized wound dressing materials and negative control was prepared without any materials. For positive control, 200 µL of anticoagulated (heparin) blood was diluted in 10 mL of sterile ultrapure water. All tubes were incubated for 2 h at 37 °C. At the end of the period, samples

taken from the incubator were centrifuged for 10 min at 1000g. The supernatant was removed and 200 μL was added to each well of a 96-well plate. Absorbance measurements were made at 545 nm in a microplate reader spectrophotometer. Hemolysis % was calculated using equation (1) [21]:

$$Hemolysis\ \% = \frac{[\text{Absorbance}_{\text{Test sample}} - \text{Absorbance}_{\text{Negative control}}]}{[\text{Absorbance}_{\text{Positive control}}]} x100 \quad (1)$$

Swelling test

Samples were appropriately cut and transferred to a tube containing isotonic solution (0.9% NaCl) after measuring the dry weight. Samples were left in isotonic solution for 30 min, then the droplets on them were taken with a filter paper and their weights were measured again with the aid of a precision scales. The liquid capture capacities (swelling capacities) of both dry weight (M_1) and wet weight (M_2) measured samples were calculated using equation (2) [22].

$$Capture\ Capacity = \frac{M2 - M1}{M1} x100 \quad (2)$$

Measurement of optical contact angle

The contact angle measurements of the samples were made with Attension Theta Lite by taking 15-20 recordings per second for a single drop with ±1° sensitivity in normal mode and dropping distilled water in the micro syringe to the sample surface.

RESULTS AND DISCUSSION

The experimental data of the wound dressing materials synthesized using PN, PVA and PVP have been given below.

Particle size analysis of PN

The graph of the nanozetasizer measurement of PN suspension has been given in Fig. 3.

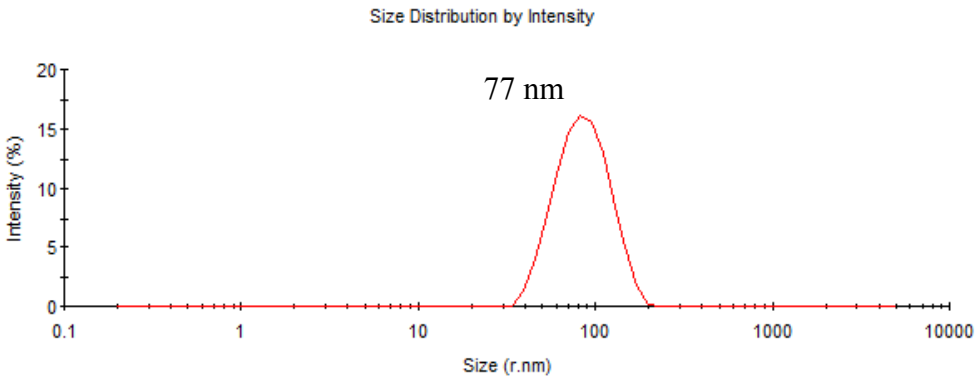


Fig. 3. The result of the nanozetasizer measurement of PN

The single peak lead us to conclude that PNs have a homogeneous particle size range. It

was also observed that the particle size of PN was around 77 nm. The most important reason for making nano-sized PNs was to increase surface area so that the particles penetrate the chains of the polymers more easily and thus increase the interaction. The use of nano-sized antibacterial fillers in wound dressing materials will provide a smooth and homogeneous appearance of the dressing materials.

The measurement of optical contact angle

The wound dressing materials should be antibacterial, biocompatible, non-toxic, inexpensive and easy to use [23]. Generally the hydrophilic properties of the polymers are low. In order to improve these properties, a variety of materials such as clays, oxides etc. are added to the polymers. Because polymers with hydrophobic properties do not like and take water into their structures and therefore do not keep the wound moist. Hydrophilic materials can shorten the healing process by keeping the wound moist as they absorb water. For this reason, it is preferable to use materials with low optical contact angle as wound dressing material. It obviously demonstrates that PVA is a kind of hydrophilic polymer with polar hydroxyl groups on the molecular chain and shows water droplet contact angle of 61-79°, as shown in the literature [24]. The contact angle of PVP was indicated in the literature as 44° and was a hydrophilic polymer [25]. There was no PVA/PVP mixture in the literature. However, by mixing the two hydrophilic polymers, a hydrophilic product was obtained and the contact angle of this nanocomposite was measured as 58.7°. By adding pomegranate peel to the PVP/PVA mixture, the surface can be expected to be more hydrophobic, and the measured optical contact angle values are important in discussing the usability of this nanocomposite as a wound dressing material. Figure 4 shows the photographs of water droplets on the nanocomposite surfaces and Table 1 defines the contact angle values of the wound dressing materials synthesized in four different compositions. When the figure is examined, it can be observed that the hydrophobic property is increased and the hydrophilic property is decreasing with increased amount of PNs. It can be said that PN particles caused an increase in hydrophobic properties of the polymers. Researchers have shown that surfaces with moderate water wettability can hold cells at a maximum rate compared to surfaces with high or low waterproofing properties. The optimum wettability of a substrate was reported to be 55-75° for culturing fibroblasts, and 45-75° for epithelial cells [26-28]. These values are important in the preparation of a wound dressing material. The wettability of the nanocomposites synthesized is well within these ranges and therefore exhibits moderately hydrophilicity suitable for cell attachment. The wound dressing material with the lowest contact angle was the one prepared in 48.5/48.5/3 ratio and in this case, it can be said that the best material for wound dressing material was the one prepared in 48.5/8.5/3 ratio.

Table 1. *Optic contact angle (%) values of the wound dressing materials*

Samples	Optical Contact Angle (□)
PVP/PVA/PN (50/50/0)	58.7 ± 1.8
PVP/PVA/PN (48.5/48.5/3)	68.9 ± 0.9
PVP/PVA/PN (48/48/4)	73.5 ± 0.4
PVP/PVA/PN (47.5/47.5/5)	75.2 ± 0.5
PVP/PVA/PN (40/40/20)	83.7 ± 0.6

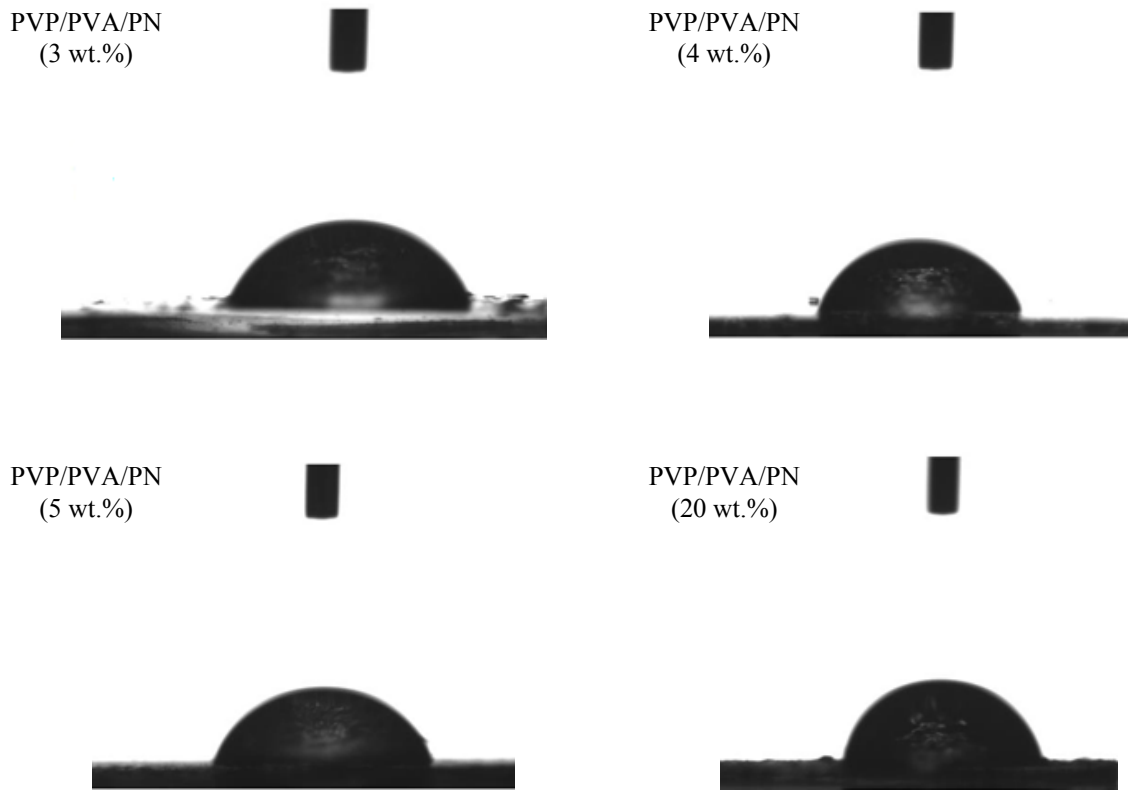


Fig. 4. The optical contact angle images and values of the wound dressing materials

The swelling tests

Biomaterials or polymer films based on hydrophilic polymers are generally susceptible to water and environmental moisture. Such materials are hygroscopic since they contain a large number of hydrophilic groups in the macromoleculechains [29,30]. Knowing the swelling properties of polymer films has great importance on drug release systems, wound dressing materials, adsorption of chemical materials and various applications in contact lenses. The rate of swelling is usually used to characterize the swelling behavior of films or hydrogels [31]. Gravimetric measurement is commonly used to determine the swelling rate of films due to its simplicity and easy application [31-33]. This method was also used in this study. The swelling rates of the synthesized wound dressing materials were calculated using the equation (1) and the results obtained were given in Figure 5 and Table 2. It was found that the wound dressing material containing 3% (w/w) of PN had a relatively high liquid capture capacity compared to the PVP/PVA hydrogel and this ratio was approximately 150%. Swelling rates of the materials containing 4, 5 and 20% (w/w) of PNs were calculated as 135%, 107% and 46%, respectively. According to the results, swelling rates of wound dressing materials were different from each other and the rates were decreased with increasing amount of nano PN particles. Swelling is a penetration process carried out with the pressure or concentration gradient between the inner and outer parts of the film. Transport of the water in the film takes place under a moisture concentration gradient between the surface layer and interior part of the film. The water molecules penetrate into the inner parts of the wound dressing material by the diffusion so the wound dressing material swells and absorbs the water in the inner parts [34].

Table 2. *Parameters of nanocomposites and theirs % swelling capacity*

Samples	Swelling Capacity (%)
PVP/PVA/PN (50/50/0)	787.1 ± 11.1
PVP/PVA/PN (48.5/48.5/3)	1222.3 ± 65.8
PVP/PVA/PN (48/48/4)	1075.1 ± 23.4
PVP/PVA/PN (47.5/47.5/5)	859.1 ± 28.5
PVP/PVA/PN (40/40/20)	374.0 ± 35.7

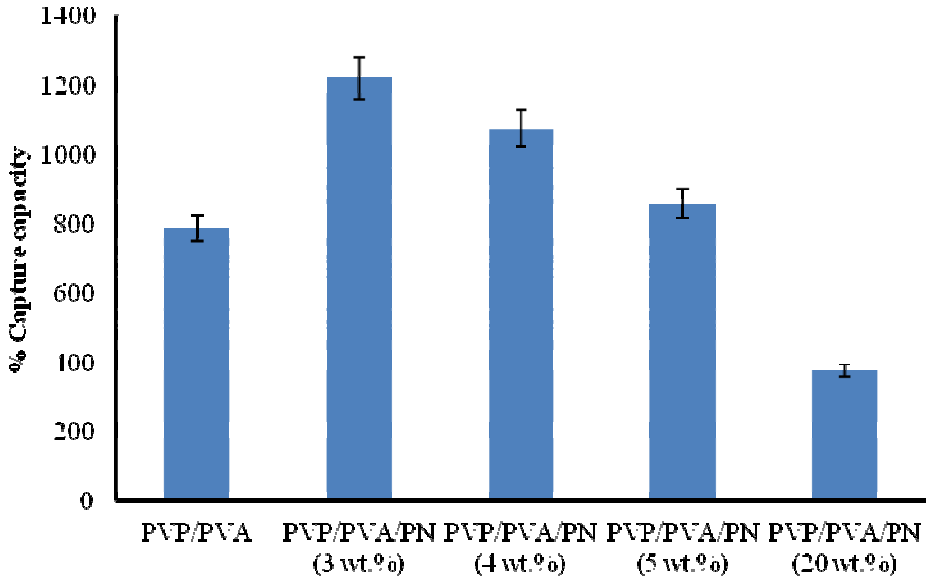


Fig. 5. The swelling test results of PVP/PVA hydrogel and wound dressing materials

If the wound dressing material is not sufficiently absorbent, the wound area will be at risk of softening due to intense moisture. According to the results, the absorbency properties of the wound dressings synthesized in this study were very high. If these materials are used as wound dressing materials, they do not need to be changed frequently and the covers keep the underlying microbial fluid for a long time because of their high absorbency properties [35]. Therefore, the wounds covered with dressings will be healed faster than the ones contacting the air [36]. From the results of optical contact angle, it can be concluded that the best material was the one prepared in 48.5/48.5/3 ratio with the highest swelling capacity.

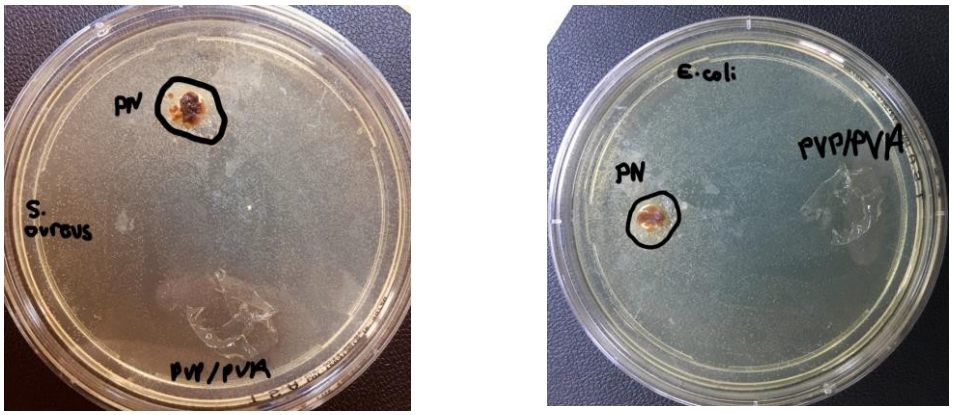
The antibacterial activity tests

The main purpose of wound dressing materials is generally to protect the wound against bacterial contamination, which is an important source of post-operation diseases. Therefore, the materials used as wound dressings should have antibacterial properties [37,38]. Antibacterial activities of PN, PVP/PVA hydrogel and synthesized wound dressing materials were tested against gram negative *Escherichia coli* and gram positive *Staphylacoccus aureus* using disk diffusion method (Figures 6 and 7). In fact, the most important reason of the use of

nano-sized PN particles in the wound dressing material in this study was to induce antibacterial, biocompatible and non-toxic properties to these materials. Bacterial cultures were prepared for antibacterial testing at a concentration of 10^6 CFU/mL. According to the results, PVP/PVA hydrogel did not show any antibacterial activity while PN showed antibacterial activity against bacteria. In Figure 7 it is seen that wound dressing materials inhibited the growth of gram positive and negative bacteria and caused a large inhibitory zone after 24 h. It was found that PNs which had been previously identified as antibacterial [39] were showed same property in a hydrogel film. Wound dressing materials prepared using PNs at different ratios exhibited different antibacterial activities. This can be observed from the data in Table 3. It was determined that the inhibition zones of wound dressing films did notchange significantly with the increasing amount of PNs. This is a desirable condition in wound dressings. When the wound dressing material is to be givenan antibacterial property with an external additive substance, it would be better to add this substance in a minimum amount. This avoids both the cost increase of the wound dressing material and the negative changes that may occur in the mechanical properties. According to the Figure 7 and Table 3, it can be said that wound dressing films showed antibacterial activities against both of the bacterial strains.

Table 3. *Antibacterial activity results of the wound dressing materials against Escherichia coli and Staphylacoccusaureus*

Samples	Bacteria	
	Escherichia coli	*Staphylacoccus aureus*
PN	11 mm	13 mm
PVP/PVA(50/50)	0	0
PVP/PVA/PN (48.5/48.5/3)	21 mm	20 mm
PVP/PVA/PN (48/48/4)	20 mm	21 mm
PVP/PVA/PN(47.5/47.5/5)	23 mm	22 mm
PVP/PVA/PN (40/40/20)	17 mm	19 mm



Staphylacoccus aureus *Escherichia coli*

Fig. 6. Antibacterial activities of PN and PVP/PVA hydrogel

Staphylacoccus aureus *Escherichia coli*

Fig. 7. Antibacterial activities of the synthesized wound dressings. Compositions of the materials are coded as number 1 for 48.5/48.5/3, number 2 for 48/48/4, number 3 for 47.5/47.5/5 and number 4 for 40/40/20

The hemocompatibility tests

Hemocompatibilities of the synthesized wound dressings were measured spectrophotometrically and the obtained results were given in Table 4. Shanthiniet al. classified the materials with hemolysis % less than 5 as hemocompatible, with hemolysis % up to 10 as biocompatibleand with hemolysis more than 20 as non-hemocompatible [40]. When thehemolysis % values in Table 4 were examined, it was found that all of the results were smaller than 5%. According to these results, it can be said that all of the synthesized wound dressings are highly hemocompatible.

Table 4. *Hemolysis % of the wound dressing materials*

Samples	Hemolysis, (%)
PVP/PVA/PN(50/50/0)	0
PVP/PVA/PN(48.5/48.5/3)	0.95
PVP/PVA/PN (48/48/4)	0.46
PVP/PVA/PN(47.5/47.5/5)	0.60
PVP/PVA/PN (40/40/20)	0.85

The cytotoxicity tests (MTS Assay)

The absorbance values measured according to MTS test results of wound dressing materials have been given in Figure 8 (a-b). This test (MTS Assay) is based on the reduction of the tetrazolium compound in the cell to the colored formazan product. This change is thought to be mediated by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells [19]. The high absorbance values seen in the MTS test are directly proportional to cell viabilities. After 24 h of incubation, the highest absorbance was seen in cells treated with

the PVP/PVA/PN (20 wt.%) sample. After 48 h, similar absorbances were observed in PVP/PVA/PN (4 wt.%), PVP/PVA/PN (5wt.%) and PVP/PVA/PN (20 wt.%) samples while the PVP/PVA/PN (3 wt.%) sample caused the lowest absorbance. According to our results, it can be seen that there wasn't any dramatic difference between the samples prepared with different amounts of PN particles. Navarro et al. (2014) also studied the cytotoxicity of PN extract (1–100 µg/mL) by determining its effect on cell viability and found that cell integrity didn't changed with aqueous PN extract (up to 100µg/mL) [41].

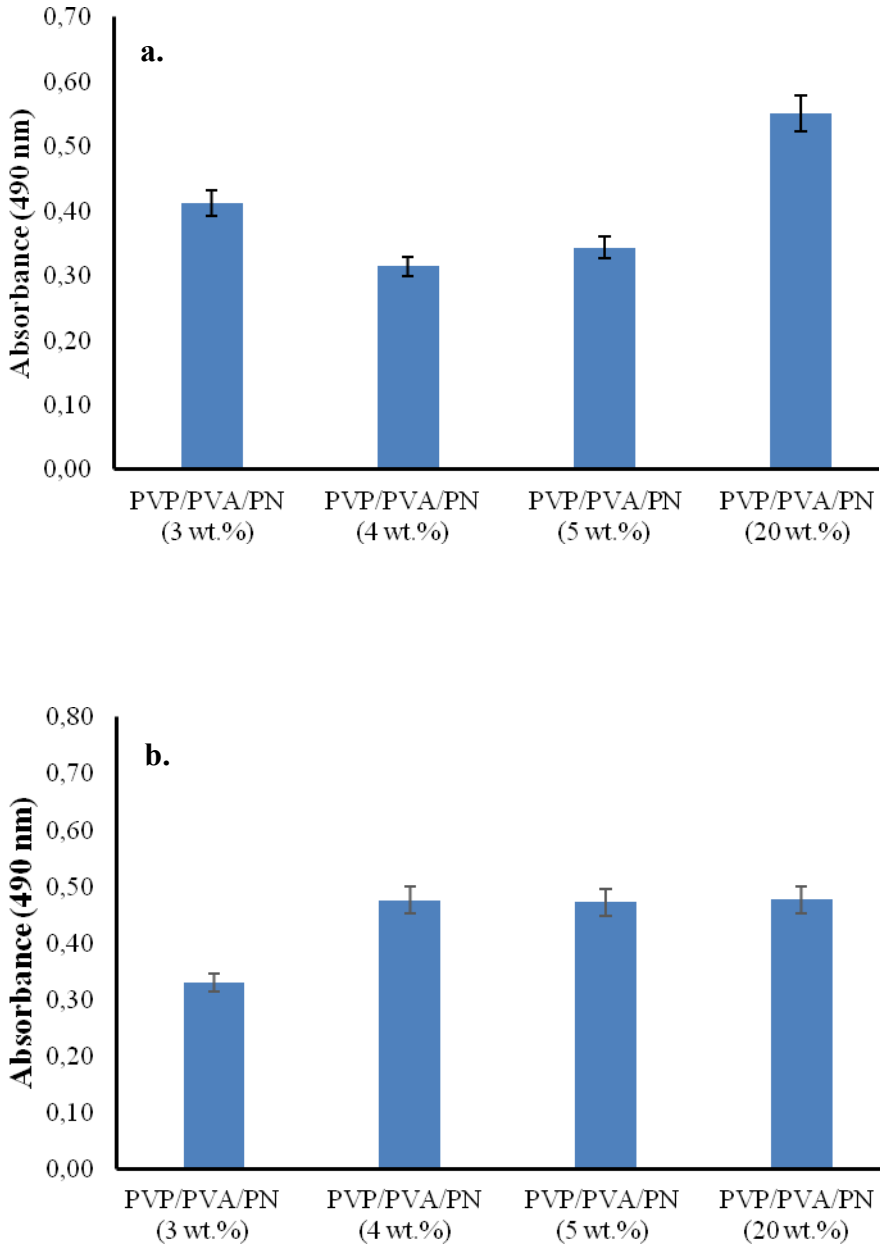


Fig. 8. Cytotoxicity test results of the wound dressing materials after: a. 24 h and b. 48 h of incubations

CONCLUSIONS

In this study, different compositions of wound dressing materials were synthesized according to solvent removal method using PVP and PVA polymers and antibacterial nano PNs. It was concluded that:

- PN particles were obtained in nano size,
- the hydrophobic properties of the wound dressing materials were increased with increasing amount of PN,
- the swelling rates were decreased with increasing amount of PN particles,
- all of the wound dressing materials showed similar antibacterial activities, hemocompatible and non-toxic.

When the results of this study were evaluated collectively, it can be concluded that the material prepared in (48.5/48.5/3) ratio may be considered as a candidate material to be used as a wound dressing material in the health sector with its high hydrophilicity, its capacity to let highly homogeneous and nano-sized distribution of PNs in the polymer matrix, the highest swelling rate and antibacterial, biocompatible and non-toxic properties.

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