RESEARCH ARTICLE

Antimicrobial properties and permeability of Poly lactic Acid nanocomposite films containing Zinc Oxide

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ABSTRACT

Objective(s): Since microbial contamination can reduce the shelf life of the foodstuff and there is a potential for the growth of some pathogen microorganisms, films containing antimicrobial agents were produced, which are also biodegradable. In this study, the effect of 1, 3 and 5% nano-zinc oxide on antimicrobial properties and permeability of poly lactic acid film was investigated.

Material and methods: the sample was contaminated with standard strains of gram-negative (Escherichia *coli* –code of 1399 (ATCC 25992)) and bacteria gram-positive (Staphylococcus *aureus*–code of 1431 (ATCC 25923)) provided. Diameters of inhibition zones were measured after 24 h incubation of plates at 37 °C, by using Digital Caliper. Also, the permeability to water vapor according to ASTM E96 and oxygen standards according to ASTM D3985 standard was investigated from film surface.

Results: The study of antimicrobial properties of films on Escherichia *coli* and Staphylococcus *aureus* showed that all three percent of "ZnO" in this study had inhibitory effects and increased the percentage of nano-zinc oxide significantly ($P < 0.05$) increased the inhibitory effects.In this test, the diameter of the control film inhibition zone was zero, which indicates that pure poly lactic acid films do not have antimicrobial activity ($P > 0.05$). In the study of Water Vapor Permeability (WVP), bio-composite films with 1% and 3% nano-zinc oxide showed a 19% water vapor Permeability enhancement compared to the pure poly-lactic acid. Moreover, adding 3% nano-zinc oxide had an impact on the reduction of permeability to oxygen.

Conclusion: Poly lactic acid films containing nano-zinc oxide have a high potential for antimicrobial food packaging applications to enhance the safety of food products.

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INTRODUCTION

During recent years, attention to biopolymers because of increasing environmental awareness, the price of crude oil and the challenges related to global warming has drastically increased. Since the biopolymers are obtained from renewable resources and they are biodegradable, therefore using them compared with petroleum-based polymers have the least negative effect on environment. Todays, these compounds are used in different field such as physiotherapy, pharmacy, medicine, coating, and food processing and packaging material [1]. One of the biodegradable compounds is Poly-Lactic Acid. PLA has a great potential for replacing petroleum based polymers due to its thermoplastic nature, biodegradability, biocompatibility, mechanical strength, high elastic modulus and easy processing [2, 3]. Poly lactic acid is a linear aliphatic polyester that can be extracted 100% from renewable resources such as corn [4]. Also, it is authorized by FDA to use as a substance in contact with foodstuffs [5]. Nanotechnology has brought solutions to increase

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the shelf life of food in the food packaging industry [6]. Nanocomposites are thought to be 21st Century materials and expected to be utilized more in the future. Industries tend to produce considerably thin films not only to reduce the material and production costs but also the environmental impacts of packaging. This result-oriented research helps the development of new active packaging based on the composite materials that prevents rancidity, change or loss of color, loss of nutrients, dehydration, gas production, creating odors and dehydration. As regards to the increment of the consumer demands and production of sustainable environmental products, nanocomposites based on the bio-polymers like Poly-Lactic Acid (PLA) have gained a peculiar attention in the field of foodstuff packaging [7, 8]. Generally, active packaging has features beyond the preventing properties and they are obtained by adding active elements and compounds in packaging system [9]. Antimicrobial active packaging made of metal nanocomposites are new generation of nano structures packaging that are produced from direct combinations of metallic nanoparticles with a polymer [10]. The antimicrobial activity of a polymer is usually achieved by adding metal particles, metal oxides and organic compounds. Metals and oxidized particles are the most commonly used particles in the development of antimicrobial activity [11, 12]. Silver, copper, and zinc oxide nanoparticles are the most widely used metal nanoparticles in the production of antimicrobial films [13]. Zinc is one of the nanoparticles which is a kind of metal widely distributed in the nature and imperative for the function of many metalloproteinase. Nanoparticles of zinc oxide have some advantages over silver nanoparticles, including the low cost, white appearance and the ability to block ultraviolet radiation [14]. The antimicrobial mechanism of metal nanoparticle is not specified yet. According to researchers, the antimicrobial activity of these nanoparticles may be related to induction of oxidative stress due to generation of reactive oxygen species (ROS) which may cause the degradation of the membrane structure of the cell [15-17] release of ions from the surface of nanoparticles that has been reported to cause bacterial death due to binding to cell membrane [18, 19]. Antimicrobial effect of nano-zinc oxide nanoparticles against a broad spectrum of gram-positive and gram-negative bacteria such as Staphylococcus *aureus*, Enterococcus *faecalis*, Salmonella *typhimurium*, and enterobacter *aorogenesis* are investigated [20]. The aim of this study was to investigate the antimicrobial properties on E. *coli* and Staphylococcus *aureus* and permeability properties of poly lactic acid film containing nano-zinc oxide with different percentages.

MATERIALS AND METHODS

In this study, Poly (lactic acid) (PLA) films with 0%, 1%, 3% and 5% silicon dioxide nanocomposite were a commercial grade supplied by NatureWorks LLC, Minneapolis, USA by extrusion method which were made of (PLA (with M_n (PLA) = 88 500, index of polydispersity, M_w/M_n = 1.8, D isomer <2% and a specific gravity of 1.25 g/cm^3 (210 °C, 2.16 kg) and a melting temperature of 170 °C and the ZnO from Wacker-Chemie GmbH (Hydrophilic Wacker HDK® T40, with particle size of about 30 nm, bulk density: 280 g/L, specific surface area (BET): 25 - 35 m^2/g, ZnO content ~99.5%.

Desired microorganisms

Escherichia coli (gram negative) with code 1399 (ATCC 25992) and Staphylococcus Aureus (gram positive) with code 1431 (ATCC 25923) from the collection center of industrial Iranian fungi and bacteria are used.

Microbial test

Activation method of used bacteria

In order to activate Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923), the bacterium was cultured in a nutrient broth and incubated for 24 hours at incubator 37 ℃ (Pars Teb Novin, Iran). After 24 hours, the bacterial strain was cultured into the 4 quadrant streak method nutrient agar and incubated for 24 hours at incubator 37 °C. Then, a colony of bacteria was added to sterile distilled water and compared to 0.5 McFarland standard until its turbidity become equal 0.5 McFarland.

Preparation of 0.5 McFarland microbial suspension

McFarland standards are prepared by adding a specific volume of 1% sulfuric acid solution and 1.175% barium chloride to obtain a barium sulfate solution with specific optical density. Usually 0.5 McFarland standards that contain 9.95 ml sulfuric acid 1% and 0.05 ml barium chloride 1.175% are most commonly used. The McFarland standard provides an equivalent turbidity to a bacterial suspension containing 1.5×10^8 cfu / ml. To prepare a microbial suspension, 24 hours of culture was required from each bacterium. Therefore, 24 hours before the experiment, inoculum from storage culture was carried out in a sloped culture medium of nutrient agar, and then the culture's surface was washed with a ringer's solution and the microbial concentrated suspension was diluted with ringer until the turbidity of the solution was equal with turbidity of 0.5 McFarland solution (optical absorption of 0.132 and an optical transmission rate of 74.3 at wavelength of 600 nm).

The study of antimicrobial effect of films

In order to study the antimicrobial effect of poly lactic acid film containing nano-zinc oxide on the studied bacteria, surface plate method and spherical slices of the film were used. For this purpose, 0.1 ml of microbial suspension of each bacterium equal to 0.5 McFarland is prepared under sterile conditions and under the hood and while transferring to the Muller Hinton Agar culture medium by L- shaped rod, is distributed at the surface of the culture medium. Then, three spherical slices of films with concentrations of 1, 3 and 5% nano-zinc oxide with a diameter of 5 mm were placed on the culture medium. Culture media (3 replicates for each bacterium) with spherical slices of films (1 slice in each culture medium) were incubated at 37 ° C and after 24 hours, the diameter of inhibition halo of growth as an indicator of antimicrobial activity of films were measured using digital caliper with a precision of 0.01 millimeters. To ensure the uniform growth of bacteria on a plate surface, a cultured plate without film was considered for each of the tested bacteria. Also, a plate without bacterium was used to ensure non contamination of the culture media.

Water Vapor Permeability Test

The water vapor permeability was measured by the ASTM E96 standard. The glass cups with an inner diameter of 3 cm and the height of 3.5 cm were used to accomplish this test. Then, 10 cc deionized water was poured in each cup which could create a 100% moisture in the interior space of the cup in such a way that the moisture inside the cup became more than the moisture of out. First, the pieces of films were cut from the intact parts of the film as much as the outer mouth of the cup. Then, 10 cc deionized water was poured in each cup and films are placed on the outer mouth of the cup. saeling was performed by the help of Para-film and film was placed on the cup in a fixed way. There was a gap between the films and the surface of the water inside the cup. Then the cups were located inside a desiccator containing activated silica gel. Initial weighing was carried out prior to locating the cups inside the desiccator and recorded as the initial weight. Then, cups were located inside a desiccator and weighed every 2 hours. Thus, 7 weights were recorded for each cup. After these procedures, the thickness of each film was accurately determined at 5 points and used to perform the calculations. In fact, the amount of cup weight loss is equal to the amount of water passing through the film. To calculate the water vapor permeability, the curve of the water passing through the film was first plotted in a time unit and the slope of the curve was measured in the linear part of the curve ($\Delta m/\Delta t$) Equation 1. Then, the obtained amount was divided into the area of the film which in fact is the amount of Water Vapor Transmission Rate (WVTR) Equation 2.

Equation 1 defines the curve slope in the linear section. The curve slope is identical to the Water Vapor Transmission Rate.

$$(\Delta m/\Delta t) \quad \text{(curve slope)} \tag{1}$$

Then the WVTR value was calculated by equation 2.

$$\text{WVTR} = (\text{Curve slope/Film area})=(\Delta m/\Delta t)/A \tag{2}$$

According to equation 3, to obtain the WVP, the WVTR value is multiplied by the mean film thickness (Ɣ) and the resulting value is divided by the difference in water vapor pressure on the two sides of the film.

$$\text{WVP} = (\text{WVTR}\times \text{Ɣ})/\Delta p \tag{3}$$

In other words, equation 4 can determine the value of WVP.

$$\text{WVP} =[(\Delta m/\Delta t)/A\times \text{Ɣ})]/\Delta p \tag{4}$$

Oxygen Permeability test

To perform the oxygen permeability test, the samples were cut in three replications with the desired sizes according to the device. Oxygen permeability was measured according to ASTM D3985 standard. The rate of oxygen permeability was calculated by multiplying the amount of

Oxygen Transfer Rate (OTR) in uniform flow mode to the average film thickness and its division into the pressure difference between two levels. The oxygen transfer rate is the amount of oxygen gas that passes through the thickness of the film at a specified time. Before the experiment, the films were immersed in a desiccator containing a saturated solution of magnesium nitrate at 25 °C (55% relative humidity). The test sample is installed in a way that separates the two sides of the test chamber. One of the surfaces of the film is in contact with the nitrogen atmosphere and the other one is in contact with oxygen. The amount of oxygen passing through the nitrogen atmosphere is measured by a barometer sensor which is mounted on the side of the nitrogen atmosphere. The experiment is completed when the oxygen concentration at the nitrogen atmosphere side reaches a constant limit.

Statistical analysis

The software used to analyze the data are SPSS software package and Windows-based Excel spreadsheet. Significant differences ($P<0.05$) were assessed by variance analysis (ANOVA). Also, Tukey test was applied to confirm the existence of a significant difference between the means.

RESULTS AND DISCUSSION

The effect of poly lactic acid films containing nano-zinc oxide on E. coli

The inhibitory activity of films was measured based on the diameter of the halo formed around the spherical slice of the films. Whatever, the antimicrobial property of the film is more; the diameter of the halo formed around the film is more. In this test, the diameter of the control film inhibition zone was zero, which indicates that films do not have antimicrobial activity ($P>0.05$). The results of the study of the effect of films containing 1, 3 and 5% nano-zinc oxide on Escherichia *coli* are shown in (Table 1) and (Fig. 1). In this study, all three percent has inhibitory effect and Increasing the percentage of Nano-zinc oxide significantly ($P<0.05$) increased the inhibitory effect on E. *coli* growth.

The effect of nano-zinc oxide poly lactic acid films on S.aureus

Also in this test, the diameter of the control film inhibition zone was zero, which indicates that films do not have antimicrobial activity ($P>0.05$). The results of the study of the effect of films containing 1, 3 and 5% nano-zinc oxide on Staphylococcus *aureus* are shown in (Table 2) and (Fig. 2).

In this study, all three percent has inhibitory effect and Increasing the percentage of Nano-zinc

Table 1. Diameter of inhibition zone (mm) in poly lactic acid films containing nano-zinc oxide on E. *coli*

Sample	Diameter of inhibition zone (mm)
	E.*coli*
PLA	0 ± 0
PLA/ZnO1%	9 ± 0.05
PLA/ZnO3%	10 ± 0.03
PLA/ZnO5%	12± 0.06

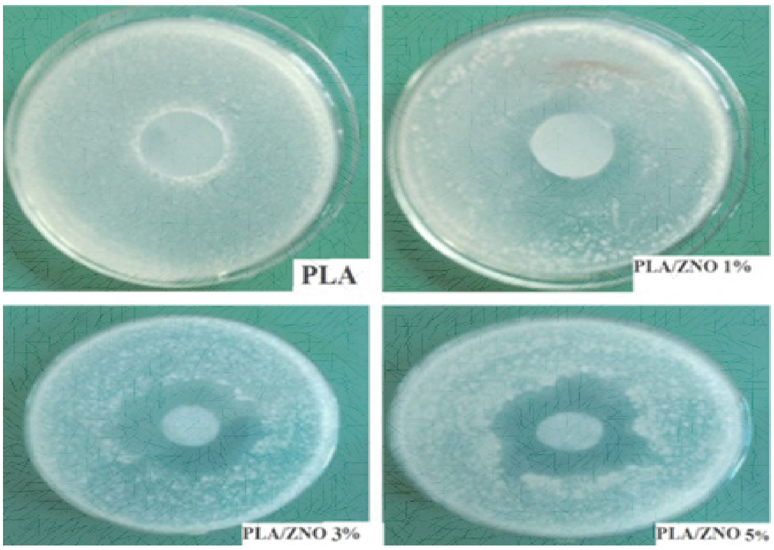

Fig. 1. The inhibitory effect of poly-lactic acids containing nano-zinc oxide E. *coli*

oxide significantly ($P<0.05$) increased the inhibitory effect on Staphylococcus growth. In this study, similar to the previous studies, the highest non growth halo was observed around Staphylococcus *aureus* and the lowest non growth halo was observed around the E. *coli* bacteria. According to these results, the antimicrobial effect of poly lactic acid containing nano-zinc oxide on gram positive bacteria was more than that of gram negative bacteria, due to the presence of outer membrane in gram negative bacteria that contains hydrophilic liposaccharides. The results of research by Hosseini and *et al* showed that gram negative bacteria are more resistant to nano-zinc oxide than gram positive bacteria, due to the structural difference of the cell wall of these two groups of bacteria. The main composition of cell wall of the gram positive bacteria is peptidoglycan with asmall amount of protein, but the cell wall of gram negative bacteria in spite of less thickness have more complexity and in addition to peptidoglycan contain various polysaccharides, protein and lipids [21, 22]. Also, the cell wall of gram negative bacteria has an outer membrane (which covers the outer surface of the wall) and a peri-plasmic space that is not seen in gram positive. The hydrophilic surface, outer membrane of the gram negative bacteria, that rich in lipopolysaccharide molecules, creates a barrier against the release of antibiotic molecules (natural or synthetic), and enzymes in their peri-plasmic space can break down the molecules entered into this space [23]. Which causes their resistance to antibacterial agents. While gram-positive bacteria lack such a membrane and wall, antibacterial agents (such as phenols) easily destroy the cell wall and membrane proteins, interfere with membrane enzymes, cause secrete cellular compositions, cytoplasmic coagulation and damage the bacteria [24]. All these factors increase the resistance of gram negative bacteria to gram positive bacteria.

Water Vapor Permeability

The Water Vapor Permeability (WVP) has significant effect on the shelf-life of the foodstuff which is a scale for measuring the moisture transfer through materials. The ability to control the loss of the water molecule from the product is an important feature for the film that influences the quality of the final product [25]. The results of the experiments (WVP) are presented in (Table 3) and (Fig. 3).

Table 2. Diameter of inhibition zone (mm) in poly lactic acid films containing nano-zinc oxide on S.*aureus*

Sample	Diameter of inhibition zone (mm)
	S.*aureus*
PLA	0 ± 0
PLA/ZnO1%	9 ± 0.07
PLA/ZnO3%	11 ± 0.05
PLA/ZnO5%	13 ± 0.05

Table 3.Water vapor permeability rate in poly lactic acid films containing nano-zinc oxide with different percentages

Sample	Water vapor permeability
PLA	0.21 ± 0.01
PLA/ZnO 1%	0.25 ± 0.01
PLA/ZnO 3%	0.25 ± 0.01
PLA/ZnO 5%	0.24 ± 0.02

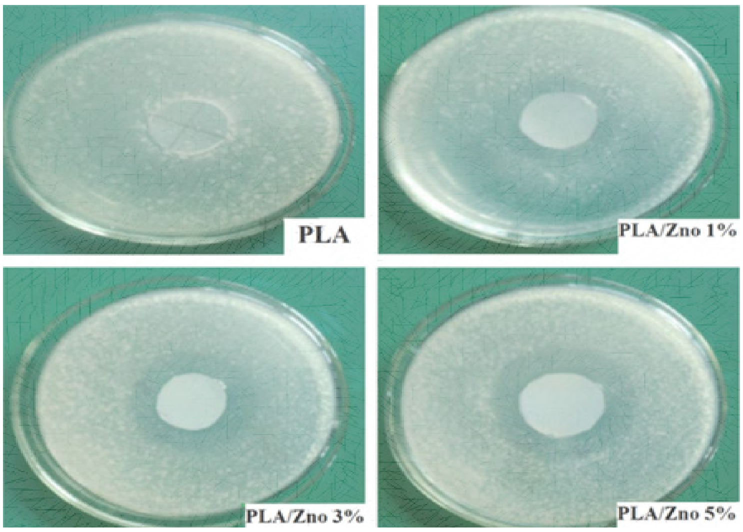

Fig. 2. The inhibitory effect of poly-lactic acids containing nano-zinc oxide on S.*aureus*

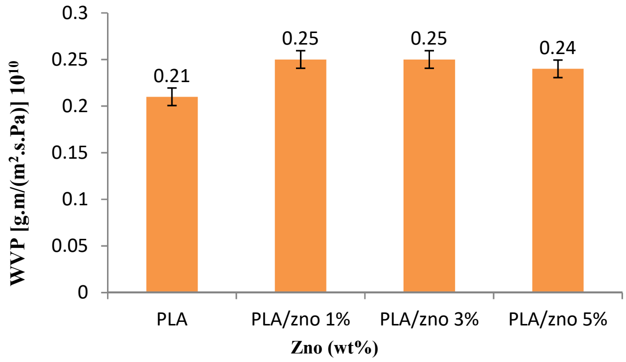

Fig. 3. Comparison of the effect of adding nano-zinc oxide with different percentages on the water vapor permeability in poly-lactic acid film

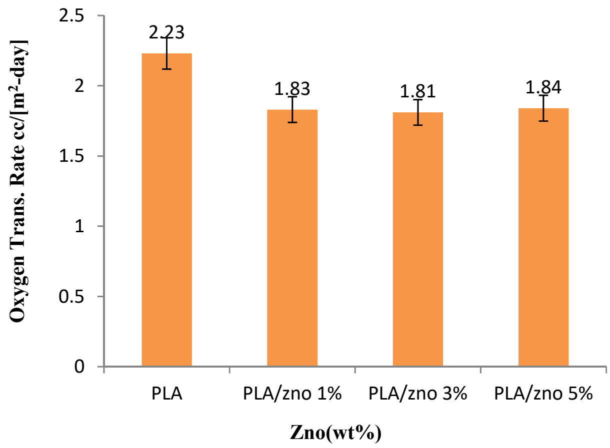

Fig. 4. Comparison of the effect of nano-zinc oxide addition with different percentages on oxygen permeability in poly-lactic acid film

According to (Table 3), the poly-lactic acid film containing 1% and 3% nano-zinc oxide has the highest value of average water vapor permeability which is 0.25 while the pure poly-lactic acid with the average value of 0.21 is the lowest one. Bio-composite films with 1% and 3% nano-zinc oxide demonstrate 19% growth in the water vapor permeability compared to the pure poly-lactic acid and also no change in the permeability is observed for higher percentages of nano-zinc oxide in this test. The permeability for non-polar gas molecules in nanocomposites is reduced while it is increased for polar water molecules [26]. The presence of nano-zinc oxide particles and other internal factors of the poly-lactic acid matrix change the free volume. Therefore, the final result shows an increase in the permeability for polar molecules of water and more increase in the metal oxides do not make any changes in the permeability.

Oxygen Permeability

One of the other considerable properties of foodstuff packaging polymers is oxygen permeability in addition to the water vapor permeability because the presence of oxygen is an important factor to the formation of reactions such as oxidation and rancidity of lipids, micro-organisms growth, etc. In order to determine this indicator, devices designed for this purpose can be used. In this research, the average oxygen permeability for poly-lactic acid film containing 3% nano-zinc oxide is 1.81 which is the lowest value and also pure poly-lactic acid has the highest value of 2.32 as shown in (Fig. 4) and (Table 4). The addition of 3% nano-zinc oxide has impact on decreasing the oxygen permeability and adding more nano-zinc oxide up to 5% does not a notable reduction. This reduction in permeability can probably be attributed to the homogeneous distribution of nano-zinc oxide particles with the

Table 4. Oxygen permeability rate in poly-lactic acid films containing nano-zinc oxide with different percentages

Sample	Oxygen permeability
PLA	2.23 ± 0.22
PLA/ZnO 1%	1.83 ± 0.03
PLA/ZnO 3%	1.81 ± 0.24
PLA/ZnO 5%	1.84 ± 0.06

lowest concentration. Owing to the fact that poly-lactic acid in all four films is in an amorphous phase, the reduction of the oxygen permeability in nanocomposites can be caused by the presence of nano-zinc oxide particles which changed the permeation of molecules on the path [26].

CONCLUSION

The effect of films containing ZnO 1, 3 and 5% on Escherichia *coli* and Staphylococcus *aureus* showed that all three percent of ZnO in this study has inhibitory effect and Increasing the percentage of Nano-zinc oxide significantly ($P<0.05$) increased the inhibitory effect on Escherichia *coli* and Staphylococcus *aureus* growth. In this test, the diameter of the control film inhibition zone was zero, which indicates that these films do not have antimicrobial activity ($P>0.05$). In the evaluation of Water Vapor Permeability (WVP), bio-composite films with 1% and 3% nano-zinc oxide have 19% increment compared to the pure poly-lactic acid ($P<0.05$). It is essential to note that, no change is observed for higher percentages of nano-zinc oxide in this experiment. In this study, 3% nano-zinc oxide has an effect on reducing the oxygen permeability ($P<0.05$) and a further increase in nano-zinc oxide up to 5% has no significant decrement.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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