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Article

Mechanical and Biological Potentials of AgNPs-fortified Gelatin Film Derived from Fish Skin

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Abstract: Biodegradable films were prepared using fish skins treated with silver nanoparticles and gelatin as biodegradable polymers. Different volume of silver nanoparticle solution (15, 10, and 5 ml) were used in producing the edible films. the films were evaluated for their inhibitory effectiveness against some types of bacteria that cause food spoilage. The results showed that the gelatin films containing 10 ml of silver nanoparticle solution gave effective activity against Bacteria for all strains tested. The results also showed an improvement in the mechanical properties of the coating supported with silver nanoparticles, as the thickness increased to 0.9 mm compared to the regular control gelatin film of 0.5 mm. Therefore, the tensile strength and elongation rate of the nano-silver treated films increased compared to the control regular coatings. The results also showed that the solubility of the regular gelatin film was 45.2% which significantly diffed from the solubility of the Nano-silver gelatin treated film, which recorded approximately half the solubility (23.3%).

Keywords: AgNPs-fortified gelatin film, fish skin, bioactive packaging

1. Introduction

Bioactive packaging for food is one of the innovative modern technologies in smart packaging technology that combines the food environment, the packaging and their interaction in order to ensure the preservation of quality and increase the shelf life of food with the presence of inorganic nanoparticles in natural polymers that ensure protection of the consumer and the environment to preserve food from pathogenic and food-destroying microbes. In addition to their edibility, biodegradability, and environmental friendliness [1], natural polymers such as starch, agar, carrageenan, gelatin, chitosan, and others are used for mixing with other synthetic polymers or inorganic nanoparticles in order to expand their applications and enhance the properties of polymeric materials [2]. Many nanoparticles, including nanosheets, nanometal, and metal oxide, have been used to improve polymer properties. Recently, the use of nanometal and metal oxides, such as silver, gold, copper, titanium oxide, and zinc oxide, has emerged as reinforcement materials in polymers [3].

Nanoparticles of a material have better properties than the same material in its natural form, based on basic properties such as size, distribution, and morphology, which has increased the number of new applications for NPs and nanomaterials significantly [4]. The use of silver nanoparticles (AgNPs) in food packaging. Antimicrobial packaging or active packaging has an important impact in prolonging the shelf life of the product and reducing

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the risk of associated pathogens. Natural biopolymers have been blended with nanomaterials to improve the polymer's antimicrobial, confinement, and mechanical properties [2], [5]. Biodegradable polymers represent an alternative option in food packaging because they are low-cost, can be obtained from renewable sources, and are environmentally friendly. This has made biodegradable polymers extensively used in many fields and have attracted much attention as alternatives to non-biodegradable polymers [6]. Therefore, the aim of this study is to prepare edible gelatin covers from fish skins and study the effect of treating the covers with silver nanoparticles compared to the unfortified cover on prolonging the shelf life of foods.

2. Materials and Methods

2.1. Preparing gelatin from fish skin

Gelatin was prepared from fish skins. The skins were washed to get rid of suspended impurities, cut into small pieces (3-2) cm, then weighed 100 grams and soaked in a 2.0 molar sodium hydroxide solution at a ratio of 10:1 (v/w) for a period of time. Half an hour at a refrigerator temperature of 4°C with gentle stirring. Replace the solution every 30 minutes and repeat this process three times. Then the skins were washed with running water, then with distilled water until neutral, then they were soaked in 0.05 molar acetic acid, with a ratio of 10:1 (v/w), for 3 hours and at a temperature of 25°C with gentle stirring. Then they were washed with running water, then in distilled water until neutral, and soaked in Distilled water at a ratio of 10:1 (v/w) at a temperature of 45°C for 10 hours with continuous stirring to obtain the extract. The product was filtered through two plates of tumbler cloth, the filtrate was cooled, then the accumulated fat was removed from the surface of the gel. The extract was dried in a freeze dryer and the product was ground with a ceramic mortar to obtain gelatin powder, then the percentage of the yield was calculated [7].

2.2. Preparing the gelatin film solution

The methods by Chowdhury and Das (2012) [8] were followed for preparing a gelatin film solution. Fish skin gelatin powder was weighed at 2, 3, and 4 g, dissolved it in distilled water with continuous stirring in a water bath at 60 and 90°C, then sorbitol and glycerol were added in proportions of 20, 30, 40% by weight of the gelatin powder separately for total volume of 100 ml with distilled water for 30 minutes and adjusted pH to 7 using 1 standard sodium hydroxide solution.

2.3. Preparation of gelatin film fortified with AgNPs

The coatings were prepared according to the method Tao et al. (2018) [9], replacing the enzymatic and chemical substances with a solution of silver nanoparticles. The nanosphere was prepared by placing the required volume of the nanomaterial solution in a water bath for 10 minutes at a temperature of 40°C, then completing the volume to 100 ml with warm distilled water. The preparation was carried out as in the previous steps, where the film solution was prepared by adding different volumes (5-10-15 ml) of the Nano-silver solution [9].

2.4. Mechanical tests

2.4.1. Estimating film thickness

The thickness of the membranes was measured using a digital micrometer with a sensitivity to the nearest 0.01 mm. The measurements were made by taking five random locations from the periphery to the center of the membrane and then calculating the average of the five measurements [10].

2.4.2. Estimating tensile strength and elongation

The tensile strength and elongation ratio were estimated according to the method described by Yuan et al. (2015) [11], using an initial grip separation device. The membranes were cut into strips with a length of 45 mm and a width of 20 mm. Fix the membrane well between the two handles of the device at a sample withdrawal speed of 50 mm/min. The elongation rate at cut was estimated from the stress and ductility curves (Stress-strain Curve). The tensile strength of the membrane sample was calculated by measuring the uniaxial force required to cut the membrane strip (sample) using the equation:

$$T.S = \frac{Fmax}{A}$$

Where:

T.S = Tensile strength (MPa)

Fmax = Maximum force required to cut the sample (Newton)

A = Cross-sectional area of the sample (mm^2)

The percentage of elongation to break (E) was calculated by dividing the elongation value at the moment the sample broke by the initial length of the sample and multiplying the result by 100:

$$\%E=\Delta L/L*100$$

Where:

E = Percentage of elongation at cut

ΔL = Change in sample length (mm)

L = Initial length of the sample (mm)

2.5. Estimating film's solubility

The solubility of regular gelatin films and gelatin fortified with silver nanoparticles were estimated according to the method by Nur-Hanani et al. (2012) [10] for five samples each:

$$\textbf{Solubility in water} = \frac{\textbf{initial sample weight (g)} - \textbf{final weight (g)}}{\textbf{initial weight(g)}} \textbf{ X 100}$$

2.6. Gelatin film coating

The skinless and boneless fish fillets were cut into 15 grams per shell. The treatments included encapsulation with regular gelatin or nano-silver encapsulation, in addition to the negative control treatment without encapsulation. Treatment samples were placed in sterile petri dishes and stored at refrigerator temperature (4-5°C). The samples were evaluated for microbial, chemical, and sensory tests periodically after (1, 3, 6, 9, 12, 15) days.

2.7. Bioassays

Inhibitory activity of AgNPs-treated gelatin films to food spoilage bacteria:

1. Gram-positive and Gram-negative bacteria

The inhibitory effectiveness of gelatin coatings fortified with Ag NPs compared to regular gelatin film were tested against K. pneumonia, S. aureus, and E. coli and P. mirabilis isolated and identified from fresh fish meat (Kumer et al. 2015). The wells spread method was used, and the bacteria were carefully spread on the surface of the Mueller Hinton disc in succession and kept at room temperature for one hour, after which 4 holes were made in each plate using a sterile corck borer of 7 mm. each well was filled with the gelatin films fortified with 5,10 or 15 ml of AgNPs and the regular gelatin using the same diameter corck borer. The agar plates were incubating at 37 °C for 24 hours, then the antibacterial activity was evaluated by measuring the diameter of the inhibition zone formed around the holes.

2. Total number of bacteria

The method was followed by Ben-David and Davidson (2014) [12] in estimating the total bacterial count by using the nutrient medium (Nutrient agar) and using the dilution method. The nutrient medium was prepared, poured it into the dishes, then of 0.1 ml of the nutrient dilution was transferred and spread it on the surface of the medium. Using a diffuser, from the highest to the lowest dilution, then incubating at a temperature of 37°C for 24 hours. The colonies formed were counted using a colony counter, and the bacterial average number was calculated for three plates. The same method was followed in calculating the bacterial numbers in subsequent experiments, taking into account the control to each treatment.

3. Psychrophilic bacteria

In case of the psychrophilic bacteria, the same method described by Ben-David and Davidson (2014) [12] was followed for estimating the number of cold-loving bacteria using the nutrient medium (Nutrient agar) where the plates were incubated at 7±1˚C for 7 to 10 days.

3. Results and Discussion

3.1. Mechanical tests

The results (Table 1) indicate that the thickness of the cover supported with silver nanoparticles was 0.9 mm, with a significant difference compared to the regular cover not treated with silver nanoparticles, which recorded a thickness of 0.5 mm. The thickness of the coating treated with nanogel increased despite the fact that both types of gelatin coatings were prepared in the same way and under the same conditions and poured into dishes in the same quantities. This may be attributed to the increase in shell thickness resulting from an increase in the viscosity of the nanocomposite solution [13].

An improvement was also observed in the mechanical properties of the gelatin shell supported with silver nanoparticles. An increase in the tensile strength and elongation percentage by 0.88 Newtons and 35%, respectively, compared to significantly lower values in the ordinary shell, which recorded 0.45 Newtons and 12.0%. This indicates that gelatin has a better electrostatic interaction by forming a hydrogen bond, which led to improved mechanical properties in the presence of silver nanoparticles [14]. The improvement of mechanical properties in gelatin coatings supported by nano-silver is attributed to the increase in height and width caused by the nanomaterial [15]. On the other hand, the percentage of solubility of the films in water decreased when silver nanoparticles were added to the gelatin film. The regular gelatin shell recorded a solubility of 45.2%, while the solubility of the gelatin shell supported with silver nanoparticles did not exceed 23.3%. The presence of binding agents from simple sugars that form covalent bonds with the nanomaterial leads to a decrease in the solubility of the treated membranes [16].

Table 1. Effect of silver nano-particles AgNPs in preparation gelatin coating film (GCF) on film thickness, tensile strength and elongation (%)

Coating type	Film thick-ness (mm)	Tensile strength	Elongation%	Solubility
GCF	0.5	0.45	12	45.2
GCF/AgNPs	0.9	0.88	35	23.3

The results of the mechanical tests were consistent with previous results, as Al-Murshedi (2020) indicated that reinforcing the gelatin covers with nano-silver increased the thickness of the gelatin film to (0.7 mm) compared to the regular control film (0.3 mm). The results of the tensile strength and elongation tests also agreed with the results of Al-Issawi (2021). The use of zinc nanoparticles to fortify food coating gelatin led to an increase in the tensile strength of the film to (0.885 N) and the elongation rate to 38.5% for the reinforced wrapper compared to (0.689 Newton) and (19.0%) for regular coating gelatin. Another study also indicates that strengthening the coatings with liposome nanoparticles reduced the solubility of the coating in water to 23.76%, compared to the untreated gelatin film, which recorded a higher solubility of 46.55% [17].

3.2. Gram-positive and Gram-negative bacteria

Table 2 shows the inhibition zone diameters for gelatin films samples against both Gram-positive bacteria S. aureus and Gram-negative bacteria (E. coli, P. mirabilis, and K. pneumonia). Regular gelatin coatings (not treated with Nano-silver) showed no Inhibition of microbes compared to gelatin covers containing 5, 10, and 15 ml of silver nanoparticles, which showed effective antibacterial activity against all strains tested. Zone diameters of inhibition ranging from 14 to 16 mm in gelatin treated with Nano-silver at the lowest concentration (5 ml), while the diameter of inhibition ranged from 19 to 22 mm in the concentration of 10 ml, which did not differ from the concentration of the most expensive 15 ml, which resulted in diameters of inhibition from 21 to 25 mm. The gelatin coating containing 10 ml of silver nanoparticles was found to be the most suitable and effective for use in active packaging. These results indicated that the inhibitory zone of gelatin coatings containing AgNPs increased with increasing silver nanoparticle concentration. The inhibitory activity of AgNPS coating against bacteria may be due to the rupture of the bacterial cell membrane and the surface activity of the AgNPs when in contact with the bacterial surface.

Table 2. Inhibitory effect of AgNPs fortified gelatin coating film (GCF) on some food spoilage bacteria

AgNPs concentration (mg/L) in the gelatin film	Inhibition zone diameter (mm)			
	K. pneumonia	*P. mirabilis*	*E. coli*	*S. aureus*
5	16mm	15mm	14mm	17mm
10	22mm	19mm	21mm	22mm
15	25mm	21mm	23mm	24mm

The contact between the nanoparticles and the bacterial cell started from the surface charges on the particle and the electrostatic interaction between the surface of the bacteria and the nanoparticles. After contact with the bacterial membrane, the high rate of reactive

oxygen species generated by silver nanoparticles leads to the death of bacteria by chemicals [18].

3.3. Effect of coating treatments on total number of bacteria

The results in Table 3 indicate that the logarithm of the total number of bacteria did not differ between the treatments on the first day of storage. However, on the third and sixth days, the gelatin wrapping treatment supported with nano-silver differed, with the lowest number of bacteria recorded, significantly different from the regular gelatin wrapper or the uncoated samples. The unwrapped control treatment recorded microbial counts of 8.658 log cycles, exceeding the permissible number in meat of 7 log cycles according to the Iraqi standard for standardization and quality control. In terms of packaging treatment with regular gelatin, it exceeded the permissible limit for the total number of bacteria in meat after the ninth day by 8.575 log cycles, while the gelatin wrappers supported with silver nanoparticles maintained an acceptable microbial load by 7.551 log cycles after 15 days of refrigerated storage. These results reflect the potential of gelatin films supported with silver nanoparticles for fish fillets.

Table 3. Logarithm of total bacteria numbers (colonies/g) for fish fillet treatments after different periods of refrigerated storage at 4°C

Treatments Fish fillet coating	**Days post treatment**					
	1	3	6	9	12	15
Uncoated	3.892 a	6.984 c	8.658 c	--	--	--
Regular gelatin coating	3.780 a	5.791 b	6.664 b	8.575 b	--	--
AgNPs-treated gelatin coating	3.671 a	4.421 a	5.285 a	6.129 a	6.871	7.551

Values are means of three replications, means followed by different letter within a column are significantly different according to Duncan's multiple range tests (P≤0.05)

The apparent increase in the total bacterial count for the negative control treatment is due to the fish meat not being packaged and exposed to oxygen directly, thus increasing the number of aerobic bacteria that cause meat damage and spoilage [19].

3.4. Effect of coating treatments on psychrotrophic bacteria

Regarding the effect of coating treatments on the numbers of psychrotrophic bacteria, no difference was recorded among the three samples on the first day of refrigerated storage. On the third day of storage, the gelatin-coated treatment was superior to those treated with nano-silver with a significant decrease in the total number of psychrotrophic bacteria, while the normal coating treatment did not differ from the uncoated negative control treatment. In general, after six days of storage, the uncoated fish fillets sample recorded the highest number of psychrotrophic bacteria, exceeding the permissible limit (5 log cycles), while the normal gelatin film treatment continued until the ninth day compared to the

nano-silver treated gelatin, which maintained a bacterial count below the permissible limit even after 15 days of storage.

Table 4. Effect of gelatin coating type fish fillet on psychrotrphic bacteria Log. cycle numbers (colonies/g) after different periods of refrigerated storage at 4°C

Treatments Fish fillet coating	Days post treatment					
	1	3	6	9	12	15
Uncoated	3.610 a	4.950 C	6.061 c	--	--	--
Regular gelatin coating	3.582 a	4.128 B	5.206 b	6.105 b	--	--
AgNPs-treated gelatin coating	3.519 a	3.829 A	4.227 a	4 , 720 a	5.089	5.991

Values are means of three replications, means followed by different letter within a column are significantly different according to Duncan's multiple range tests (P≤0.05)

The gelatin film treated silver nanoparticles maintained the permissible limits throughout the experiment under the maximum limits allowed for psychrotrophic bacteria 5 logarithmic cycles which was confirmed by the Iraqi standard for quality control and standardization (ICOSQC, 2006) [20]. The reason for the decrease in psychrotrophic bacteria in a fish meat sample coated with a AgNPs treated gelatin film indicate the effect of nano-silver due to its large surface area. As it penetrates into the microbial cell, it interacts with cellular structures or cellular molecules such as proteins, lipids, and DNA. Because of this interaction, AgNPs lead to the dysfunction of the bacteria and their death in the end. As the interaction between AgNPs silver nanoparticles and ribosomes leads to bacteria cell denaturation, which causes inhibition of translation and protein synthesis [21].

4. Conclusion

The findings of this study showed that the gelatin-base coatings treated with AgNPs showed high properties as valuable antibacterial agents against Gram-negative and Gram-positive bacteria. Fish fillets could be cold-stored for longer period when coated by AgNPs-treated gelatin film as compared to coating with regular gelatin film. This study confirmed that gelatin, especially if fortified with Nano-particles substance, can be widely used for its unique properties of water binding ability, gel formation, water vapor barrier, protective film formation, foaming ability and tendency to emulsify. All these properties make the coating components functional in various food systems or edible packaging materials.

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