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Evaluation of the antibacterial effects of Ag-Tio$_2$ nanoparticles and optimization of its migration to sturgeon caviar (Beluga)

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Abstract

Effect of nano composite films to prolong the shelf life of Iranian beluga caviar was investigated at different concentrations of silver nanoparticles. In this study 38 caviar packs each containing 5 g of caviar were divided into 6 treatments with 3 replicates. The concentrations of 1000, 2000, 3000, 4000, 5000 and 6000 ppm of nanoparticles as well as a nano free pack as control were used. The size of nanoparticles was less than 50 nm in treatments number one to four and was less than 10 nm in treatment number five. Packed samples were inoculated with bacteria and fungi and microbiological tests were performed for each sample after 24 hours. Results of gram test and detecting the gram positive bacteria showed the considerable decreases in *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus flavus* and *Penicillium* strains. Moreover, there was a considerable decrease in fungi and bacterial growth in 5000 and 6000 ppm nano-silver packages (p<0.05). The amount of silver nanoparticle released into the caviar samples were also measured by titration method and application of titrazol with concentrated sulphuric acid. There was no silver residual in different concentrations of silver nanoparticle packages. These results suggest using silver nanoparticles in accurate concentrations could be considered as one of the main solutions to further inhibit spoilage caused by pathogenic microorganisms, and to extend the shelf life of the valuable food products.

Keywords: Sturgeon, Beluga caviar, Silver, Nano composite, Titanium dioxide, Titration

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Introduction

There is constant research to introduce more innovative and intelligent packaging techniques with the goal of extending shelf life for foods and increasing the period of time that food stays in high quality. Innovative food packaging provides a wide range of functional properties such as preserving and protecting food, protecting the sensory qualities, communicating information to consumers and are generally recognized as safe to be in contact with food (Bumbudsanpharoke and Ko, 2015).

Metal nano particles such as gold, silver and copper are of the bactericidal category. Silver reacts with thiol groups of microorganism enzymes and leads to the denaturation of enzymes and ultimately cell death. Packaging containing silver nanoparticles releases silver ions which reduce, prevent or delay microbial growth and thereby increase shelf life and maintain the quality of the food products. Therefore, advanced packaging techniques are based on the smart nano-materials which could respond to the environmental condition and contain an external and internal indicator to show any contamination, temperature and humidity changes and water penetration, as well as detect spoilage and pathogenic microorganisms. Lin *et al*. (2014), investigated the application of titanium dioxide and found that silver nano-packaging with antimicrobial properties and persistency to the diffusion of gases especially oxygen considerably increased the shelf life and decreased the fungal flora of bread during the storage (Lin *et al*., 2014).

Nano-packaging is among the novel packaging technologies used for increasing the quality and safety of food materials. Nano-coating used in nano-packaging is a great advancement for food products for export purposes. The transparent plastic film “dortan” contains nano-clay that covers the plastic and is able to block the oxygen, carbon dioxide and humidity, which helps protect the packaged food for much longer periods of time. This compound creates more clear colored plastics that are heat resistant. In the food packaging industry there are other useful types of plastics that are produced using Nano-based technology. Oxygen is the most problematic compound in packaging of food because it causes oxidation of fats and fatty portions of foods, as well as quality loss and changes in the food color. In these state of the art plastic materials, nano-particles are placed in a “tortuous pathway” (ziczac form) in order to prevent oxygen penetration. In other words, the tortuous pathway increases the mean gas diffusion length, and thus, the shelf life of spoilable food. Therefore packed food products stay fresh, longer.

Caviar is one of the most expensive foods in the world. The Caspian Sea is the largest source of caviar fish in the world and provides more than 90 percentage of the world caviar. Caviar is a high energy nutritious food with a very pleasant taste and smell. The proteins present in caviar consist of amino acids including, argenine,

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hystamine, isoleucine, lysine and methionine (Bumbudsanpharoke and Ko, 2015; Hubert, Nesvorna *et al*., 2015).

Caviar fats contain two main groups consisting of 25% cholestrol and 75% lecithin. Caviar consumption can prevent depression as well as, heart and cardiovascular diseases; this is due to presence of the high omega 3 fat content in this sea food. Caviar is rich in a compound called "Ctacosand" which is a fatty alcohol with long molecular chain that turns into fatty acids in the body. In countries that sea food and caviar are consumed regularly, the rate of depression is greatly reduced. Omega 3 Fatty acids present in caviar prevent the elevation of cholesterol levels in the blood stream and reduce heart and cardiovascular diseases (SaravananNethala *et al*., 2011; Becaro *et al*., 2015).

The purpose of this study was to introduce specific ways of using silver nanoparticles in packaging caviar with the aim of prolonging shelf life as well as providing a safe food.

Materials and methods

Bacterial suspensions including *Staphylococcus aureus* and *Escherichia coli* were obtained from Sigma-Aldrich. DTB culture, BHI Broth, SC culture (Sabouraud Glucose Agar containing chloramphenicol), Muller Hinton Broth and MckFarland standard No. 0.5 were purchased from Merck Chemical Co.

Sputter Coater in Scanning Electron Microscopy was used to cover a polyamide film with a thin layer of conducting Ag.

All the materials used in this experiment were of analytical grade and distilled water was used in all the experiments.

Effect of (N.P) on the bacteria inoculated caviar samples

In this research 38 caviar samples were obtained from the Fisheries Research Institute of Iran. In order to examine the microbial condition of the samples, total count test was performed. To ensure that all the samples were transported in a consistent manner, 12 packs were separated and based on standard No. 2326 were covered with nano polyimide films, obtained from Sharif Nano Pigment Ltd., Tehran, Iran. Samples were then grouped into two treatments including Treatment one (T_1), 10 grams of caviar covered with 100 ppm nanosilver film, and Treatment two (T_2), 10 grams of caviar covered with 150 ppm nanosilver film. Then 5 grams of each treatment was taken to be examined in triplicate under the laminated sterile microbial hood for probability of the presence of mesophile and thermophile, aerobic and anaerobic bacteria due to improper handling of the packs from the Institute to the Laboratory. The probability of the presence of aerobic thermopile and mesophile bacteria was evaluated with Dextrose Tripton Broth (DTB) as the presumptive test medium at 31 and 55°C, respectively for 3 to 5 days. The probabilities of the presence of anaerobic bacteria were evaluated by using (PE2) and Lactose Broth (LB) mediums, incubated in an anaerobic incubator at 31°C for 3 to 5 days. At the

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end of the 5 days of cultivation color and opacity were not changed, and also no gas productions were detected in the two treatments.

All the nano polyimide films were prepared by cutting them into exact sizes of 5×5 cm in the required number and then were sterilized in an autoclave. In order to protect the packs from sticking together, filter paper was placed between them. Different dilutions were made from specific bacterial species which were prepared by using the McFarland 0.5 standard with turbidity of 1.5×10^8. Dilutions in the range of 10^2 to 10^8 were utilized. Seven pieces from each packaging cover were selected and 5 grams of caviar was placed inside each cover. 100 µL portions of seven dilutions were made based on McFarland 0.5 standard from *S. aureus* and *E. coli* were added in to the covered caviar samples. Packed samples were stored at 4^{o}C in a refrigerator for 48 hours. After 48 hours, content of each nano -silver packaging caviar was placed into seven test tubes. Each of the seven test tubes containing caviar with different dilutions of inoculated bacteria was used to prepare a suspension of 0.1%. Then 50 µL of the prepared suspension was cultivated on Muller Hinton Broth and was incubated in 37^{o}C for 48 hours. After testing the samples for probable contaminations, the rest of the 24 packs of caviar were divided into seven treatments (Table 1). The samples were contaminated manually by *S. aureus*, *E. coli* and *Clostridium*.

Each treatment contained 10 grams of caviar. The treatment T1 was covered with 500 ppm of nanosilver filmsT2 was covered with 1000 ppm; T3 was covered with 2000 ppm; T4 was covered with 4000 ppm; T5 was covered with Japanese plastic and a control group consisting of 10 grams of coverless caviar. The six mentioned treatments were divided into three thermal groups and incubated for 2, 5, and 7 days at 7, 22 and 30 ^{o}C, respectively.

After 6 and 12 months of covering the caviar with nanosilver films 10 grams of each treatment was sampled to measure the amount of released nanosilver particles. The samples were dissolved in 30% sulphuric acid and then the solution was transferred to a Titrino analyzer at 25˚C and the concentration of silver was measured in 10 seconds (Costantino *et al.,* 2012).

To reduce the microbial contamination during the sampling, caviar packs were quickly cleaned by slightly wiping with 70% ethanol prior to testing and were opened in sterile conditions next to the flame. Packaging materials used for chemical and microbial tests of samples were made from nano polyimide film and obtained from Pars Nano Nasb Co., Tehran, Iran.

Incubating days and the temperature wwere same for all the examined samples and samples were tested in duplicates (Becaro *et al*., 2015; Pezzuto *et al*., 2015).

Effect of (N.P) on the fungi inoculated caviar samples

Initially the nano polyimide film was cut into an exact size of 5×5 cm in the required numbers and then sterilized in

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an autoclave. About of 1 gram caviar was then placed in each sterilized packaging cover. After 4 days of incubation at 4°C, one gram of each nanosilver packaged caviar sample was placed separately in a test tube containing 9 mL of distilled water and mixed well on a vortex. Then 100 µL of the above suspension was cultured in SCA (Sabouraud Glucose Agar containing Chloramphenicol) for the extraction of the cultivated fungi. Cultured plates were then incubated at 25-30°C and after 4 days, the cultivated fungi was determined to be *Aspergillus flavus* and *Penicillium*. The results showed that the content of extracted colonies were different in the different silver nanoparticle concentrations. In other words, colony growth was determined to be lower from 1000 to 6000 ppm nano polyimide films. Moreover, *A. flavus* growth was not reported in 6000 ppm nanosilver packaging.

Particles morphology study using FESEM

Field Emission Scanning Electron Microscope was applied to determine the characteristics of the nanoparticle polymers and to measure the particle sizes.

In order to prepare the electron microscope samples, initially particles with different silver nano -coating percentages were prepared in falcon tubes by suspending them in to acetonitrile. Then 3 mL of the prepared sample was fixed on the stand and the solvent was evaporated in an ambient condition. In order to stabilize the gold plating cover on the fixed samples, sputter deposition was performed by "Sputter Coater" with argon gas. After ten minutes, specimens were transferred to the microscope chamber. By adjusting the SEM on 10X magnification, image of the target sample appeared on the connected monitor.

Evaluation of particles residue by titration method

The accurate determination of the released silver in samples was investigated by titration using the titrino instrument. The coatings of opened packages and the caviar samples inside were disposed and the covers were prepared for the test. The nanosilver packaging with the different nanoparticle percentages were prepared by cutting them into exact sizes of 2×2 cm in the required number and transferred to the Titrino analyzer. Samples were prepared in the following manner: After preparing a 40% sulphuric acid suspension, 5 mL of the prepared solution was mixed with distilled water in sufficient volume to completely immerse the electrode and potassium chloride inlet pipe inside the solution. Auto sampler was then installed to take the proper volume of the sample and set the testing method to detect the amount of silver in ppm scale. The instrument provides the proper conditions for the Cl and Ag ions to react. This reaction depends on the concentration of Ag and the result is recorded in ppm scale.

To increase the accuracy of Ag measurements, the chemical reaction

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curve was drawn to show the amount of consumed potassium chloride. The normality of the solution was measured using the following formula (Kate *et al*., 2011).

$m_1v_1=m_2v_2$

The shelf life of the nanosilver packaged caviar samples was tested in dilutions of 1000, 2000, 4000, 5000 and 6000 ppm of nano polyimide films. To determine the amount of silver residue in the packed caviar, samples were transferred to the food chemical laboratory.

Analytical and statistical validations were performed using Excel 2007 and SPSS 21. In order to check the normal distribution of data, Kolmogrov-Smirnov test which is a nonparametric method was applied. The result of Kolmogrov-Smirnov test indicated that the achieved data were normal and then by using Face Center Response Surface Methodology using design expert 8.

Results

Result of the fungi growth on plates

The amount of the separated colonies of each packaging cover was different. The colony growth in nano polyimide films was reduced from 1000 to 6000 ppm, moreover, in 6000 ppm nanosilver film; growth of *A. flavus* was not detected.

After 4 days, the cultivated fungi were detected on the entire sample plates as *A. flavus* and *Penicillium* for 1000, 2000 and 4000 ppm of nanosilver coated packages. In the 5000 ppm nano-covered sample, fungi colony growth on 80% of the entire sample plates was observed. In the 6000 ppm nanocoated sample the growth was observed on only 20% of the sample plates (Fig. 1).

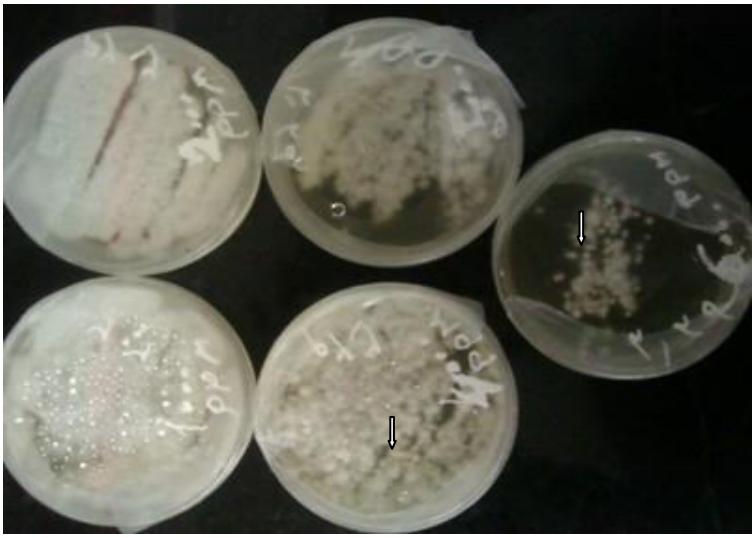


Figure 1: *Aspergillus flavus* and *Penicillium* growth on the surface of the nano silver packaging.

Result of the bacterial growth on the plates

There were no significant differences in the number of colonies in samples covered with 1000 to 6000 ppm silver nanoparticles in both *E. coli* and *S. aureus* (p=0.142). Therefore in different groups with different nanocoated

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particles, type of bacteria had no significant effect on the number of colonies, in other words there was no relationship between the type of bacteria and the number of colonies for each nano coated dilution. Comparing the number of colonies at various nanocoated dilutions, significant differences between the 1000, 2000, 3000 and 4000 with 5000 ppm nanocoated covers (p=0.031) were observed. In other words with an increase in the nanocoated dilution, number of colonies reduced significantly. It should be mentioned that the most effective dose of applied nanofilm is more than 1000 ppm for processed beluga caviar, and there is no difference between the usage of nanosilver films in concentrations more than 1000 ppm for protecting caviar and Japanese plastic coverage.

Table 1: Various diluted coverings with respect to *Escherichia coli* growth.

Nano film	10^2	10^3	10^4	10^5	10^6	10^7	10^8
1000	….	….	….	….	….	….	….
2000	880	1050	….	….	….	….	….
3000	560	1100	1280	….	….	….	….
4000	322	628	890	….	….	….	….
5000	189	290	….	….	….	….	….
6000	0	28	286	….	….	….	….

Table 2: Various diluted coverings with respect to *Staphylococcus aureus* growth

Nano film	10^2	10^3	10^4	10^5	10^6	10^7	10^8
1000	470	620	750	1250	….	….	….
2000	301	502	810	976	….	….	….
3000	203	313	976	….	….	….	….
4000	322	700	880	….	….	….	….
5000	40	170	350	500	….	….	….
6000	0	15	122	268	….	….	….

It should be mentioned that the concentration of silver residue in treatments five and six are significantly higher than treatments one to four (p<0.05); and there was no significant difference between treatments five and six (p>0.7). Moreover, through the oxidation process because of light penetration after 62 days of packing procedure the color of nano-films in all treatments had changed to yellow-brown, which is a sign of quality loss.

Results of the scanning electron microscopy

As be seen in electron micrographs, particle distribution with 15 kV and with magnification of 1.00 KX within the specified range of 1 to 10 micrometers implies that the average number of silver nanoparticles in the coating is 48-68 nm. The accumulation of dark colors in the images prior to the dissolution of the coating in the acetonitrile solvent can be seen in electron micrographs.

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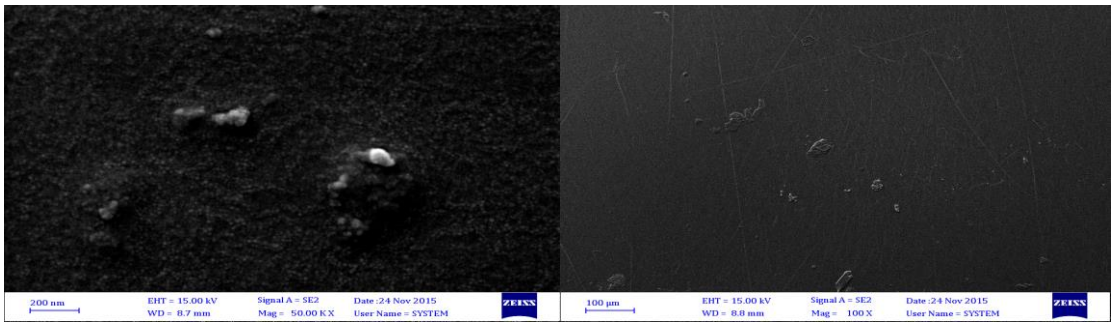


Figure 2: Scanning Electron Microscope (SEM) of nano silver packaging based on TiO_2 shows the average of nano -particles=28nm. a) EHT=15.0, WD=10mm, Mag. =10.0kx b) EHT=15.0, WD=8.7mm, Mag. =50.00 kx c) EHT=15.0, WD=8.8mm, Mag. =100.0 kx d) EHT=15.0, WD=8.7mm, Mag. =50.00 kx.

Particles release by titration method

Tested samples coated with silver nano particles showed that there were no silver residues in caviar.

The following were the samples weight measurements:

Caviar sprayed in 1000 ppm: 26.847 g

Caviar sprayed in 2000 ppm: 27.394 g

Caviar sprayed in 4000 ppm: 26.89 g

Caviar tested by 5000 ppm film: 28.867 g

Caviar tested by 6000 ppm film: 27.738 g

Table 3: Particle release in caviar samples with different percentages.

Tiring	ppm		Tiring	ppm		Tiring	ppm	
0	49.	5000	0	25.	3000	0	**1.**	1000
0	50.	5000	0	26.	3000	0	**2.**	1000
0	51.	5000	0	27.	3000	0	**3.**	1000
0	52.	5000	0	28.	3000	0	**4.**	1000
0	53.	5000	0	29.	3000	0	**5.**	1000
0	54.	5000	0	30.	3000	0	**6.**	1000
3	55.	5000	0	31.	3000	0	**7.**	1000
0	56.	5000	0	32.	3000	0	**8.**	1000
0	57.	5000	0	33.	3000	0	**9.**	1000
0	58.	5000	0	34.	3000	0	**10.**	1000
0	59.	5000	2	35.	4000	0	**11.**	1000
5	60.	5000	0	36.	4000	0	**12.**	2000
0	61.	5000	0	37.	4000	0	**13.**	2000
1	62.	5000	3	38.	4000	0	**14.**	2000
0	63.	6000	0	39.	4000	0	**15.**	2000
0	64.	6000	0	40.	4000	0	**16.**	2000
0	65.	6000	0	41.	4000	0	**17.**	2000
0	66.	6000	0	42.	4000	0	**18.**	2000
4	67.	6000	0	43.	4000	0	**19.**	2000
6	68.	6000	0	44.	4000	0	**20.**	2000
0	69.	6000	2	45.	4000	0	**21.**	2000
6	70.	6000	0	46.	4000	0	**22.**	2000
			2	47.	5000	0	**23.**	2000
			1	48.	5000	0	24.	3000

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The Kolmogorov-Smirnov nonparametric one sample test was used for data distribution studies. Since in this test $p<0.05$, we can conclude that the data distribution does not follow a normal distribution (data are not normally distributed). Therefore, in order to compare particles by Titrino method in 6 groups, Kruskal-Wallis test was applied and by using Response Surface Methodology. One of the techniques response surface methodology (RSM) which is used to explain the release data by titration methods.

Table 4: the central composite experimental design and result of release extract of nano composite packaging.

STD	ID	RUN	Block	Type	Nano Composite	Release	ppm
7	7	1	Block 1	Axial	3500.00	1.00	0
9	9	2	Block 1	Center	3500.00	3.50	0
10	9	3	Block 1	Center	3500.00	3.50	0
6	6	4	Block 1	Axial	6000.00	3.50	0
12	9	5	Block 1	Center	3500.00	3.50	0
4	4	6	Block 1	Factorial	6000.00	6.00	4
3	3	7	Block 1	Factorial	1000.00	6.00	0
13	9	8	Block 1	Center	3500.00	3.50	0
5	5	9	Block 1	Axial	1000.00	3.50	0
11	9	10	Block 1	Center	3500.00	3.50	0
2	2	11	Block 1	Factorial	6000.00	1.00	6
1	1	12	Block 1	Factorial	1000.00	1.00	0
8	8	13	Block 1	Axial	3500.00	6.00	0

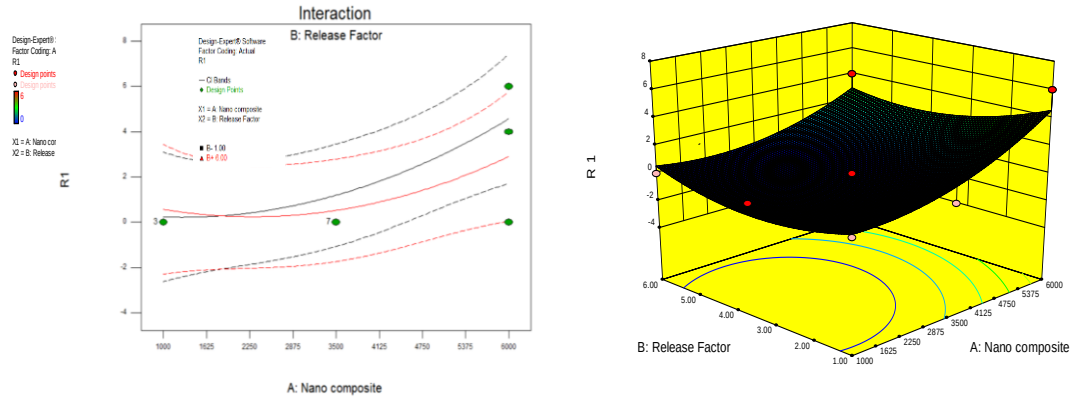


Figure 1: Response surface methodology statistical histogram.

The results shows that there was no significant difference in particle release by using Response Surface Methodology (Face center – RSM) for 6 studied groups (p=0.141).

Silver nanoparticles often show unique and considerably different properties for various bacterial strains. Studies have shown that negatively charged bacterial cells absorb silver nanoparticles onto the bacterial cell. Therefore, the differences in surface charge of various types of bacteria can cause differences in the absorption of silver nanopericles for different bacterial groups (Gallocchio *et al.*, 2016).

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The smaller silver nanoparticles have larger ratio of surface to volume, as well as the high number of metal atoms in the unit of surface. This makes a better contact with microorganisms and considerably increases the potential for silver ions to be released. Therefore, silver nanoparticles are a more effective antimicrobial agent in compression with silver salt (Ahari, 2008). Silver can be a cell-killer by making compounds with the group of enzymes and denature the structure of enzymes. The present study demonstrated that the covers containing silver nano particles prevent fungal and bacterial growth in a given period of time by releasing silver ions. In our previous study on Iranian Saffron Nano packaging by Ahari *et al*. (2014) the results indicated that silver nanoparticles at 4000 ppm are able to reduce the microbial load up to 98%. It was also observed by the Nitrino test that there were zero percent of nanoparticle released in the final packaged product, which represented the effectiveness of applying silver nanoparticles to increase the shelf life of valuable food products *(Vernikov et al*., 2009; Lin *et al*., 2011).

Results represented by Mohammadi *et al*. (2013) indicated that increase in the shelf life and decrease on the fungal flora of bread significantly depends on the packaging material. Accordingly use of silver nano particles packages based on titanium dioxide, prevails over the polyethylene packages.

Based on our previous research (Hosseini *et al*., 2017) it was investigated that the diameter zone of inhibition for *E. coli* and *S. aureus* in all the time levels was significantly different (p<0.01) and the silver nano particles packaging also showed the higher inhibition zone diameter than other coatings with exceptions for phosphomycin and penicillin antibiogram disks (p<0.05). Also it was observed that the total silver nanoparticle release was less than the standard level which showed that this coating is suitable to preserve the product.

Results of the research by Shrivastava *et al*. (2007) showed that the differences in the thickness of the membrane peptidoglycan in Gram-positive and Gram-negative bacteria cannot be the main reason to increase or decrease the antibacterial properties of silver nanoparticles on the bacteria. In this case the absence of nanoparticle residue in food materials is a basic concern to assure the safety of food. However, the small size of nanoparticles is extremely important to extend the shelf life. In other words production of the small dimension particles in nano metric scale is the most important innovation of this technology in basic sciences (Pezzuto *et al*., 2015)

Research in the Health and the Aquatic Animal Disease by Soltani *et al*. (2009) on increasing the shelf life of *Oncorhynchus mykiss* showed that shelf life was not significantly changed by the application of films with average particle size of 40 nm. This was completely different from the results of the 5000 ppm ano films which had significantly increased the shelf life and imported films that has also showed a

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sensible shelf life (Soltani *et al*., 2009; Soltani *et al*., 2011).

In this research the number of colonies at various anocoating dilutions in two groups of *E. coli* and *S. aureus* were statistically analyzed. There were no significant differences in the number of colonies covered with 1000 to 6000 ppm silver nanoparticles in both *E. coli* and *S. aureus* (p=0.142). Therefore in different groups with different Nanocoated particles, type of bacteria had no significant effect on the number of colonies, in other words there is no relationship between the type of bacteria and the number of colonies for each ano coated dilution. Comparing the number of colonies at various anocoated dilutions, significant difference between the 1000, 2000, 3000 and 4000 with 5000 ppm anocoated covers (p=0.031) was observed. In other words with an increase in the Nanocoated dilution, the number of colonies reduced significantly. It should be mentioned that the most effective dose of applied anofilm was more than 1000 ppm for processed beluga caviar, and there was no difference between the use of anosilver films in concentration more than 1000 ppm and Japanese plastic coverage to protect caviar.

The SEM pictures represented the homogeneity in the surface of nano polyimide films used for packaging with the evaluated average anoparticle size of 48-68 nm. The accumulation of dark colors in the images prior to the dissolution of the coating in the acetonitrile solvent can be seen in electron micrographs.

Various factors will affect the microbiological properties of the silver nanocoating. This coating is composed of sodium alginate as the main base and silver nano particles as an antibacterial compounds. One of the mechanisms is permeability and disruption of the polyanionic lipopolysaccharide (LPS), which is a major component of the cell wall in the outer membrane of gram-negative bacteria and acts as a strong permeable barrier and inhibitor to oxygen transmission. Another mechanism is the interaction of nanomaterials with anionic groups on the cell surface due to the polycationic nature of cell membrane and formation of an impenetrable layer around the cell wall which protects solvent transmission, and inhibits RNA and protein formation by penetration into cell core. Besides the sodium alginate polysaccharide, silver nano particle was used as an antimicrobial compound. The nano silver solution contained 4000 ppm silver ion incorporated in 1, 3, and 5 percent to sodium alginate thin films. The metallic silver nano particles continuously diffused (released) silver ions in the film and by converting –SH bonds in the microorganisms' cell wall to –SAg bonds through a substitution reaction led to consequent degradation of microorganisms. Therefore, it can be said that the aforementioned mechanisms causes microbial destruction in the fisheries products. Changes in total mesophyll and pschycrophl bacteria were studied. The fish used in this experiment was in good quality since the bacterial total count was low. As the experiment

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progressed, the bacterial count increased and this increase was extremely higher for the fish that was not covered with the nanofilm (Soltani *et al*., 2009; Song *et al*., 2011).

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