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Facile method for the deposition of volatile natural compound-based nanoparticles on biodegradable polymer surfaces
A. Buslovich, a, b B. Horev, a Y. Shebis, c,a V. Rodov, a A. Gedanken, b E. Poverenov a*

a Institute of Postharvest and Food Sciences, Agriculture Research Organization, The Volcani Center, Rishon LeZion 7505101, Israel
b Department of Chemistry and Kanbar Laboratory for Nanomaterials, Institute for Nanotechnology and Advanced Materials, Bar-Ilan University, Ramat Gan 5290002, Israel
c Institute of Biochemistry, Food Sciences and Nutrition, Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel.

* Corresponding author, E-mail: elenap@volcani.agri.gov.il

Abstract

In the current work, stable nanoparticles (NPs) of vanillin are formed in situ from an aqueous/ethanol solution and deposited on a surface of chitosan, a natural polymer, using a high-intensity ultrasonic method. The spectroscopic, physical, mechanical and morphological properties of the coated chitosan films are examined by helium-ion microscopy (HIM), atomic-force microscopy (AFM), Fourier-transform infrared spectroscopy (FTIR), UV-vis spectroscopy, X-ray diffractometry (XRD), thermal gravimetric analysis (TGA), differential scanning calorimetry (DSC) and texture analysis, and compared with the original film properties. Vanillin NPs were detected on the film surface. It was also found that the sonochemical deposition method does not affect the bulk properties of the chitosan films. All the chitosan films demonstrated antimicrobial activity against Gram-negative and Gram-positive bacteria. The deposition of vanillin NPs on the chitosan film also leads to significant antibiofilm activity, especially against the biofilm formation of Escherichia coli bacteria. The in vivo antimicrobial effect of the modified chitosan films was examined on fresh-cut watermelon, melon and strawberry. Vanillin NPs-coated chitosan films led to a inhibition of total microbial growth and the substantial inhibition of molds and yeast on the fruit. This research can serve as a platform for the development of a mild and effective method for the activation and modification of natural polymers for...
their future application in biomedical devices and biodegradable active packaging materials.

**Introduction**

Natural polymers are safe biodegradable materials that have various applications in medicine, food and cosmetics. \(^{1,2,3}\) In addition, in numerous fields of modern industry current trends require conversion from synthetic to natural polymers to produce environmentally friendly products. For instance, shift from synthetic to biodegradable raw materials can be seen in the production of all kinds of green packages, disposable wares, filling and separating materials, capsules for agricultural fertilizers and more.

Providing natural polymers with valuable activity is a topic of great scientific and practical interest. Raw materials based on active natural polymers may have a wide spectra of advanced applications, such as tissue engineering, drug delivery, medical devices and active intelligent packaging.\(^ {4,5,6,7}\) In this study, we attempt to apply the potential of natural biopolymers for developing active packaging materials that improve storability of food products without affecting their safety to consumer. The food spoilage is an acute problem, in particular with fresh and especially fresh-cut fruits and vegetables. These products are extremely perishable due to the attacks of numerous microorganisms such as spoilage bacteria, fungi and yeast. New biodegradable and bioactive plant-based packaging materials offer prospects to reduce food losses, maintain quality, preserve agricultural produce and ensure food security in a sustainable way.

Antimicrobial properties are among the most frequently desired features for all active materials. Microbial contaminations and biofilm-formation phenomena are serious global problems that result in injury to health and environment, and cause significant industrial damage. In biofilms, microorganisms are protected by extracellular matrix that makes them resistant to antibiotic and a persistent source of infection and cross-contamination in water-treatment systems, biomedical devices, medical implants and in food contacting materials.\(^ {8,9}\) Microbial attachment and biofilm formation occur on the material surfaces, and surface grafting of antimicrobial agents was reported to effectively prevent microbial growth and biofilm formation.\(^ {10,11}\) For instance, zinc-doped copper oxide and copper-oxide NPs deposited on teeth prostheses show significant reduction in biofilm (plaque) formation.\(^ {12}\) Silver
NPs grafted onto a silicon surface inhibit microbial adhesion on the inserted catheters. Surface grafted chitosan, ZnO and TiO$_2$ have been reported as promising alternatives to traditional chemical disinfectants against biofilm formation on water-filtration membrane surfaces. TiO$_2$ NPs grafted onto stainless steel diminish the risks associated with biofilms of *Listeria monocytogenes* on food-contact surfaces. Quaternary ammonium compounds attached to polyethylene, polypropylene and stainless steel surfaces were found to result in significant biocidal activity including activity against multidrug resistant bacteria. Antimicrobial peptides and enzymes have also been used for a formation of highly effective antimicrobial surfaces.

Grafting of active agents on the surfaces of natural polymers is more challenging since biopolymers are usually less robust than synthetic polymers or surfaces of silicon, glass or metals. Numerous approaches that are used in more robust materials are too vigorous for natural polymers. In addition, the antimicrobial agents that are grafted on biopolymers are usually obtained from natural sources such as essential oils, plants, spices or peptides and enzymes. Such natural agents are generally more sensitive and costly than synthetic ones. Therefore, a mild grafting method that also allows minimizing an amount of the used active agents is required to bestow antimicrobial properties on natural polymers.

In previous studies, Gedanken’s group used the ultrasonic method for surface deposition of metal-oxide-based inorganic NPs. Then, the sonochemical method was extended to deposit organic NPs on various substrates. For example, enzyme nanoparticles such as amylase and pepsin were sonochemically deposited on polyethylene and polycarbonate films and their catalytic activity was determined. In the present study, we explore the possibility of using the ultrasonic approach to graft organic NPs of natural active agent onto a biopolymer surface in order to develop a sustainable active antimicrobial food packaging material. Vanillin was chosen as a model of natural antimicrobial agent. Vanillin’s grafting is challenging because of its volatile nature and low water solubility. Vanillin is approved as a food additive by FDA and other authorities worldwide and is used during many years without any confirmed report of adverse events. Chitosan, a biodegradable, edible and biocompatible polysaccharide was used as a model of natural polymer. Chitosan itself possesses numerous valuable features and is considered as one of the most promising and applicative biopolymers. Chitosan is widely regarded as being a non-toxic, biologically compatible polymer for biomedical
applications. In several countries, it is also approved for dietary applications.\textsuperscript{29,30} Embedding the vanillin NP in chitosan film matrix rather than applying them directly onto a product surface answers the safety concerns about the use of nanoparticles in food.

In this work, structural, physico-mechanical and morphological properties of the new active polymer films were comprehensively studied. Their antimicrobial and anti-biofilm activities were examined on Gram-negative \textit{Escherichia coli} and Gram-positive \textit{Staphylococcus aureus} and \textit{Listeria innocua} bacteria. In addition, the performance of the prepared films was studied in storage trials with a highly perishable food products fresh-cut watermelon, melon and strawberry.

**Experimental details**

**Materials**

99.9\% Vanillin and ethanol were supplied by Sigma-Aldrich. Chitosan (93\%) of deacetylation was purchased from Molekula. Plate count agar (PCA) and potato dextrose agar (PDA) were supplied by Becton, Dickinson and company. Chloramphenicol was supplied by Sigma-Aldrich. Deionized water was used for preparation of all the solutions.

**Preparation of chitosan films**

A chitosan solution, 1.5 w/v, was prepared by dissolving chitosan in an (0.7\% v/v) aqueous acetic acid solution. The solution was stirred at room temperature for 1 h, then cast into petri dishes 5 cm in diameter (5 mL of the solution for every petri dish) and air-dried at room temperature under a hood for 48 hours.

**Deposition of vanillin NPs on chitosan film**

Vanillin (0.5 g) was dissolved in 5 mL of deionized water and 45 mL ethanol. The 5-cm chitosan-film samples were put into the vanillin solution and the mixture was sonicated for 5 min by a high-intensity ultrasonic horn (Ti horn, 20 kHz, 750 W at 30\% efficiency) in a thermostatic (30 °C) sonicator chamber. A cold-water bath was utilized to prevent overheating, and safety rules for applying sonochemical methods in the laboratory and industrial practice\textsuperscript{31} were followed. At the end of the process, the
films were washed with ethanol and water, and air-dried in a chemical hood. The dry films were peeled off and stored at -20 °C.

**Scanning helium-ion microscopy (HIM)**

Scanning HIM was performed using an Orion NanoFab three-ion-beam system (Carl Zeiss Microscopy, Peabody, MA). Images were formed by collecting the secondary electrons produced by the interaction between the helium ion beam and the sample. 30-keV beam energy was used and the current was probed over the range of 1.7–2.7 pA. No conductive coatings were applied to the samples prior to imaging, in order to preserve the sample-surface information. The samples were maintained at a vacuum of 2.25×10⁻⁷ torr during the imaging session. Charge neutralization was maintained through the use of a low-energy electron flood gun (850 eV), which was applied in an integrated way with the imaging beam. Charge neutralization was applied after each individual line pass of the beam. The image signal was acquired in a line-averaging mode, with either 32 or 64 lines integrated into each line in the final image.

**Atomic-Force Microscopy (AFM)**

The roughness of the coated and un-coated surfaces was studied by AFM. Images were acquired with Dimension FastScan AFM (Bruker Corp., Santa Barbara, CA) in Peak Force mode. FESP probes with the nominal spring constant 2.8 N/m were used. The typical scan rate was 3 Hz, the scan size 1 and 3 microns and the resolution 512×512 pixels. The determination of the roughness factor (r) is based on the ratio of the calculated area, comprised of 3 points (X, Y, Z), versus the scanned area.

**UV-vis spectroscopy**

UV-vis measurements were performed on a SHIMADZU 1800 UV/vis spectrophotometer to determine the vanillin concentration and the film light transmittance.

**Quantification of the surface grafted vanillin**

To quantify the vanillin concentration on the chitosan surface, 1 g of the coated film was extracted in 15 mL of ethanol and stirred at room temperature for 24 h in a closed vial. The absorbance of the vanillin solution was measured by a UV-vis spectrophotometer at 200–600 nm and the vanillin concentration was determined in
accordance with a calibration curve. The calibration graph was prepared by measuring the absorbance of a series of ethanol solutions of vanillin of known concentrations at 231 nm.

**Fourier-transform infrared spectroscopy (FTIR)**

The coated and uncoated films were characterized by FTIR using a Thermo Scientific Nicolet iS5 spectrometer. Film samples were placed under the ray with 32 scans for each spectrum. The spectrum was recorded for 500–4,000 cm\(^{-1}\) with a 4-cm\(^{-1}\) resolution.

**Mechanical properties**

The mechanical properties of the film samples were measured according to the standard method of tensile testing. The mechanical properties of tensile stress (TS), percent elongation at break (PE) and Young’s modulus (YM) were measured using an Instron Universal Testing Machine (Instron 3345, Tester, Instron, Norwood, Ma) equipped with a 100-N-load cell. Each film sample was cut into a rectangular strip (4×2.5 cm) and the thickness was measured using a Mitutoyo Digimatic Indicator (Type ID-S112MB; Mitutoyo Manufacturing Co. Ltd, Tokyo, Japan) at three random positions on the film. The tests were performed at a speed of 1 mm/s. Five measurements were carried out for each film and the average values are presented.

**Thermal stability**

The thermal stability of the film samples was tested using a thermogravimetric analyzer (Pyris 1 TGA, PerkinElmer). A film sample (about 5 mg) was placed in a standard aluminum cup and scanned at a heating rate of 10 °C/min over the temperature range of 25–600 °C under a nitrogen flow of 50 cm\(^3\)/min.

**Differential scanning calorimetry (DSC) analysis**

DSC analysis was performed with a NETZSCH DSC 200 F3 Maia. The film sample (about 5 mg) was placed in a standard aluminum cup and scanned at a heating rate of 10 °C/min over the temperature range 25–350 °C under a nitrogen flow of 50 cm\(^3\)/min.

**Water-vapor permeability (WVP)**

The film WVP was measured gravimetrically using the ASTM E-96 method (2005) adapted to hydrophilic films. The test tubes were filled with 5 mL of DDW,
sealed by film samples using carbon tape and placed in a desiccator containing dry silica gel (50% RH; 23 °C). The tubes were allowed to stand for 2 h to reach an initial equilibrium and then weighed at 3-h intervals for 48 h. Water-loss weight was recorded on an analytic scale (±0.0001 g). The water-vapor transmission rate (WVTR, g/h m²) was obtained by plotting the weight loss versus time in a linear regression (r≥0.99) and dividing the slope by the exposed film area (m²). The water-vapor permeability was calculated according to the WVP equation

\[ WVP = \frac{WVTR}{P(R1-R2)} \times X, \]

where P is the partial water-vapor pressure difference (kPa) across the two sides of the film, R1 and R2 are the moisture inside the test tube and inside the desiccator, respectively, and X is the film thickness.

**X-ray diffractometry (XRD)**

X-ray patterns of chitosan and chitosan coated with VNP films were analyzed using a Brucker D8 advanced X-ray diffractometer. The samples were scanned for 2θ = 5–40° with a scanning speed of 2° min⁻¹.

**Antimicrobial activity studies**

The antimicrobial activity of the prepared films was tested on two bacterial species, Gram-negative *Escherichia coli* ATCC 25922 and Gram-positive *Staphylococcus aureus* ATCC 25923. Each bacterial culture was grown aerobically in a shaking incubator at 37 °C under an agitation of 250 rpm for 20 h in nutrient-broth (NB) medium (Conda, Madrid, Spain) containing (g L⁻¹): meat extract, 1; yeast extract, 2; peptone, 5; sodium chloride, 5; pH 6.8. For preparing the inoculum, the culture was diluted into fresh NB to obtain 10⁵ colony-forming units per milliliter (CFU mL⁻¹). The inocula of each bacterial strain (1.5 mL) were pipetted into the wells of 6-well tissue culture plates containing discs of chitosan film (1.2 cm in diameter), either coated or non-coated with vanillin NP. The content of vanillin in the vanillin-grafted chitosan film was calculated as 0.56 mg g⁻¹ based on the spectrophotometric analysis. Thus, each coated 1.2-cm chitosan film disc contained 0.67 µmol (ca. 0.1 mg) of vanillin nanoparticles. The samples were incubated for 24 h at 37 °C under an agitation of 100 rpm. Aliquots of 100 µL were taken from each well, diluted seven-
fold with sterile NB and plated in duplicate onto nutrient agar, 20 µL per plate. The plates were incubated at 37 °C overnight, and then counted for viable bacteria.

Biofilm control studies

The activity of the prepared active biopolymer-based films against bacterial biofilms development was determined via the static biofilm formation assay using *Escherichia coli* ATCC 25922 and *Listeria innocua* ATCC 33090. Bacteria were grown overnight in Mueller Hinton (MH, DIFCO) medium. On the following day, *E. coli* and *Listeria* cultures were diluted 1:100 in fresh MH to obtain working bacterial cell suspensions with an OD<sub>595</sub> of 0.03. The bacterial suspensions were pipetted into the wells of 24-well plates (DE-GROOT), 1 mL per well, containing discs (1.2 cm in diameter) of the films tested. The plates were then incubated for 20 h at 37°C. On the day after, the films were rinsed 3 times with distilled water to remove the unattached bacteria and subsequently the attached cells were scraped from the films using 250 µl of MH 1% and cell scrapers (Greiner Bio-one). 200 µl out of the 250 µl, used for scrapping the cells, were transferred into the first line of a 96-well plate (Greiner Bio-One), while the rest of the lines were filled with 180 µl of MH. Serial dilutions were carried out and the cells spotted onto LB agar plates, which were then incubated at 37°C for 20 h. Cell growth was monitored and determined by a viable cell count.

Microbiological analysis

Watermelon

A watermelon was purchased from a local store. The watermelon was washed with water, decontaminated with sodium hypochlorite solution (100 ppm active chlorine), rinsed with water, air-dried and cut. Cylindrical plugs (diameter 2.5 cm, length ca. 3 cm) were excised from the watermelon pulp at the circumference of the fruit using a sterile cork borer and a scalpel. Chitosan and chitosan–vanillin NP films were prepared as described above. Square film pieces (5×5 cm) were placed in polyethylene terephthalate (PET) clamshell containers commonly used for fresh-cut fruit packaging, three pieces of the same type per container. Watermelon plugs were mounted onto the film pieces, with one of their flat surfaces facing the film. A control test included freshly cut watermelon with no films. All clamshell containers were preliminarily sterilized with ethanol and UV light. Three containers (nine pulp plugs) were prepared with each film type. The freshly cut watermelon plugs were stored for
nine days at 10 °C, the conditions simulating fresh-cut fruit marketing on a refrigerated shelf. The plugs were sampled for microbiological analysis by aseptic excision of the surface layer from the side that was in contact with the treated films (weight 1.5 g).

**Melon**

The melons were purchased from a local store, decontaminated with sodium hypochlorite solution (100 ppm active chlorine), rinsed with water, air-dried and cut. Cylindrical plugs (diameter 2.5 cm) were excised from different melons pulp at the circumference of the fruit using a sterile cork borer and a scalpel. Chitosan and chitosan–vanillin NP films were prepared as described above. Square film pieces (5×5 cm) were placed in polyethylene terephthalate (PET) clamshell containers commonly used for fresh-cut fruit packaging, three pieces of the same type per container. Melon plugs were mounted onto the film pieces, with both of their flat surfaces facing the film. A control test included freshly cut melon with no films. All clamshell containers were preliminarily sterilized with ethanol and UV light. Three containers (nine pulp plugs) were prepared with each film type. The fresh-cut melon plugs were stored for overall 12 days at 8 °C and were tested for microbiological analysis at three time periods after 5, 8 & 12 days.

**Strawberries**

Strawberries were purchased from a local store, washed with water and cut. Cylindrical plugs (diameter 2.5 cm) were excised. Chitosan and chitosan–vanillin NP films were prepared as described above. Square film pieces (5×5 cm) were placed in polyethylene terephthalate (PET) clamshell containers commonly used for fresh-cut fruit packaging, three pieces of the same type per container. Strawberry plugs were mounted onto the film pieces, with both of their flat surfaces facing the film. A control test included freshly cut strawberries with no films. All clamshell containers were preliminarily sterilized with ethanol and UV light. Three containers (nine pulp plugs) were prepared with each film type. The freshly cut strawberry plugs were stored for overall 12 days at 8 °C and were tested for microbiological analysis at three time periods after 5, 8 & 12 days.

The fruit plugs (weight 3.5gr) were transferred into centrifuge tubes containing 15 mL of sterile saline solution (0.9% W/W NaCl). The tubes were vigorously stirred with a vortex for 2 minutes. The tube contents were serially decimally diluted with sterile saline and the aerobic plate counts were determined by surface inoculation of
the plate count agar PCA. Mold and yeast counts were determined by surface inoculation of PDA supplemented with 100 ppm chloramphenicol for controlling bacterial growth (PDA+A), and the number of CFU per gram of plant material was calculated.

Statistical Analysis

All biological experiments were conducted at least in triplicate. Three independent repetitions of each trial were performed. One-way analysis of variance (ANOVA) and Tukey’s HSD pairwise comparison tests were applied by means of the JMP statistical software program, version 7 (SAS Institute Inc., Cary, NC, USA).

Results and discussion

Ultrasonic irradiation of ethanol/aqueous vanillin solution resulted in the in situ deposition of vanillin NPs on the surface of the chitosan film. The vanillin content was quantified spectrophotometrically and found to be 3.7 µmol per gram of film, i.e. 562 µg g⁻¹. This value far exceeded the minimal content of vanillin needed for manifestation of antibacterial effect defined as 80 µg g⁻¹ for polyhydroxybutyrate film.³³

Scanning helium-ion microscopy (HIM) allows direct imaging of the polymer surface without vaporizing of the conductive coating. HIM method is very useful to follow surface modifications of delicate biopolymers. The untreated chitosan films exhibited smooth morphology, and after ultrasonic deposition, the homogeneous distribution of the vanillin particles on the chitosan surfaces was observed. The HIM revealed near-spherical or ellipsoidal structures (Figure 1).
AFM studies showed that the deposition of vanillin NPs increased the film-surface roughness. An Rq of the original chitosan films was found to be 5.73 nm and the vanillin NPs-modified films demonstrated an Rq of 8.58 nm (Figure 2). This observation is different from that measured for synthetic polymers, where NPs predominantly settle in the polymer-surface cavities, resulting in a decrease of the polymer roughness upon ultrasonic deposition.\(^{24}\) In the current case, probably because of the very smooth surface of chitosan, the NPs deposited on the surface and not in the cavities. Therefore, the overall roughness increased.
The pristine chitosan films and vanillin NP-coated chitosan films were characterized by FTIR (Figure 3). For the vanillin NP-coated films, new peaks at 1649 cm\(^{-1}\) attributed to the vanillin aldehyde, at 1552, 1420 cm\(^{-1}\) attributed to the aromatic (C=C bonds) and at 3517 cm\(^{-1}\) attributed to the phenolic hydroxyl group, were found.\(^{34}\) The film transmission in the UV (250–350 nm) and visible (350–700 nm) ranges was examined (Figure 3). The deposition of the vanillin NPs led to a reduction in the film transmittance, especially in the UV and blue ranges. The reduction of UV light transmittance can be a good applicative feature since, in numerous cases, the ability of the material to provide UV protection is desired.\(^{35}\)

![Figure 2. 3D AFM images of (left) chitosan and (right) vanillin NP chitosan films.](image)

![Figure 3. (left) FTIR and (right) UV-vis spectra of chitosan and vanillin NP-coated chitosan films.](image)

Thermal gravimetric analysis (TGA) of the pristine and vanillin NP-grafted chitosan films was performed (Figure 4). The first weight loss peak was observed
between 30–125 °C. This weight loss is probably caused by the evaporation of water solvent residues, since chitosan films contain 11–15% of water. The weight loss related to polymer decomposition (dehydration and depolymerization) was detected after 200 °C. The total weight loss of the original chitosan films at 600 °C was found to be 65%. The TGA thermogram of the modified chitosan film represents a combination of chitosan and vanillin-NP thermograms. There is a small shift in the total weight loss with the addition of vanillin NPs (59%). This shift indicates the formation of intermolecular interaction between the two components.

The DSC thermogram for the original chitosan films and chitosan coated with vanillin NPs was also measured (Figure 4). The original chitosan films exhibit a broad endothermic peak at ~130 °C, which is attributed, as mentioned above, to the evaporation of solvent residues while, in the vanillin NP-treated films, this peak is shifted to a higher temperature and centered around 170 °C. Interestingly, the addition of vanillin NPs makes the loss of the solvents more difficult. This observation is further supported by our XRD studies. An exothermic peak at ~300 °C is attributed to polymer decomposition and is similar for the original and modified films.

![Figure 4](left) TGA and (right) DSC of original and vanillin NP-coated chitosan.

The Young modulus, elongation at break, maximum tensile stress at break and WVP of the original and modified chitosan films were examined (Table 1). The vanillin NPs had no significant effect on the mechanical properties and WVP of the chitosan films. Our results match previous studies that report the slight effect of surface modification on the bulk chitosan. This observation is encouraging, since it shows that the proposed method of ultrasonic surface deposition does not affect the mechanical and permeability properties of this quite sensitive natural polymer.
Table 1. Young modulus, elongation at a break, tensile stress at a break and WVP of the original and coated films.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Young modulus (MPa)</th>
<th>Elongation at break (%)</th>
<th>Max tensile stress (MPa)</th>
<th>WVP (g mm kPa⁻¹ h⁻¹ m⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chitosan</td>
<td>1204.75±309.65ₐ</td>
<td>25.63±4.97ₐ</td>
<td>6.51±3.12ₐ</td>
<td>0.51±0.13ₐ</td>
</tr>
<tr>
<td>chitosan +vanillin NPs</td>
<td>1412.10±406.1ₐ</td>
<td>24.25±2.43ₐ</td>
<td>24.24±18.33ₐ</td>
<td>0.29±0.01ₐ</td>
</tr>
</tbody>
</table>

Data represent means of six replications ± SE. The letters indicate that the values for the original and the vanillin NP-coated chitosan are not significantly different (at p≤0.05) according to a Tukey HSD test.

XRD measurements of the original and vanillin NP-grafted chitosan films were performed (Figure 5). For the pristine chitosan films, two diffraction peaks at 2θ = 14.5° and 30° were observed. The sharp peak at 2θ = 14.5° indicated a hydrated crystalline structure, while the broad peak at 2θ = 30° could be assigned to the amorphous structure of the chitosan film.³⁸,³⁹ For the vanillin NP-coated chitosan films, two crystalline peaks with the same 2θ were observed. However, their intensity slightly increased. Khan et al. reported that an increase in the intensity of the diffraction peaks of the chitosan film can be attributed to the transcrystallization effect.⁴⁰ In this case, the transcrystallization can be defined as the perpendicular orientation of the vanillin nanocrystals, leading to a semicrystalline chitosan matrix.⁴¹

Figure 5. X-ray diffraction patterns of original and vanillin NP-coated chitosan films.

The antimicrobial activity of the pristine and vanillin NPs grafted onto chitosan films was tested against Gram-negative *E. coli* and Gram-positive *S. aureus* bacteria. A bacterial solution was contacted directly with the treated films and the samples were
plated after 24 h for CFU quantification. The effect of the films on the bacterial survival was examined. Since chitosan possesses intrinsic antimicrobial activity, the significant reduction (~6-log) of both bacterial populations was found (Figure 6).

![Graph](image)

**Figure 6.** Antibacterial activity of chitosan, vanillin NPs grafted onto chitosan and films against *E. coli* and *S. aureus* after overnight incubation at 37 °C. The data represent the average data and standard errors of four independent experiments with three replicates each. The statistical comparisons tests were applied by means of the JMP statistical software program to all reported data. The values followed by the different letters are significantly different from each other according to Tukey HSD tests at $p \leq 0.05$.

However, no extra antimicrobial effect of vanillin on microbial killing was observed. In contrast, when the biofilm formation was studied, the synergetic effect of vanillin and chitosan was clearly observed against *E. coli*, but not against *Listeria innocua* biofilms (Figure 7).

Our results agree with the previous studies that showed that *Listeria* biofilms were more resistant to natural antimicrobials than *E. coli* biofilms. Listeria is known as a highly adaptable microorganism that can survive in adverse environments. One of the mechanisms of *Listeria* biofilm persistence is the capability of this bacterium to recover after the exposure to antimicrobial agents e.g. peracetic acid or chitosan. At the same time, chitosan types of certain molecular size and deacetylation degree, combined with selected antibiotic, were able to disrupt an established *L. monocytogenes* biofilm.
Figure 7. Effect of chitosan films with or without vanillin NP grafting on the development of *E. coli* and *Listeria innocua* biofilms. The data represent the average data and standard errors of four independent experiments with three replicates each. The statistical comparisons tests were applied by means of the JMP statistical software program. The values followed by the different letters are significantly different from each other according to Tukey HSD tests at $p \leq 0.05$.

The performance of the pristine and vanillin NP-grafted films in controlling the microbial spoilage of food products was tested in storage trials with fresh-cut watermelon, melon & strawberries (Figure 8a-8c). In watermelon after nine days of storage, the total plate counts on the surface of the fresh-cuts kept in regular clamshell packages (control) reached 9 log CFU g$^{-1}$ exceeding the acceptability thresholds of 7$^{46}$ or 8$^{47}$ log CFU g$^{-1}$ that determine the end of the shelf-life of fresh-cut fruits or vegetables. At the same time, the yeast and mold counts in the control exceeded the acceptability threshold of 5 log CFU g$^{-1}$.$^{46,47}$ On the other hand, all microbial counts on the fresh-cut watermelon surfaces contacting the active films were far below the thresholds indicating the acceptable microbiological quality. The vanillin NP-grafted chitosan films demonstrated more significant microbial inhibition than the pristine films, especially with yeast and mold counts that were below the detection limit on the chitosan/vanillin film (Figure 8).

Figure 8. (left) Total microbial count on PCA plates and (right) mold and yeast count on PDA+A plates of fresh-cut watermelon after nine days of storage at 10 °C in contact with chitosan and vanillin
NP-grafted chitosan films. The data represent the average data and standard errors of three independent experiments with three replicates each. The statistical comparisons tests were applied by means of the JMP statistical software program to all reported data. The values followed by the different letters are significantly different from each other according to Tukey HSD tests at $p \leq 0.05$.

In melons, most of the microbial counts on the fresh-cut melon surfaces contacting the active films were below the thresholds indicating the acceptable microbiological quality after eight days of storage. The vanillin NP-grafted chitosan films demonstrated more significant microbial inhibition than the pristine films even after twelve days of storage. Microbiological testing in strawberries has further showed the synergetic effect of the two antibacterial materials (chitosan and vanillin) when even after twelve days of storage the total plate counts on the surface of the fresh-cuts contacting the active films was far below the acceptability threshold (Figure 9). This synergetic effect is a known and desirable phenomenon. In this case, the film polymer bulk and the grafted NPs serve as two antimicrobial agents, resulting in a highly effective active material for safe biodegradable packaging of food products.
Figure 9. (left) Total microbial count on PCA plates and (right) mold and yeast count on PDA+A plates of fresh-cut melon and strawberries after 5, 8 & 12 days of storage at 8 °C in contact with chitosan and vanillin NP-grafted chitosan films. The data represent the average data and standard errors of three independent experiments with three replicates each. The statistical comparisons tests between different treatments in each measurement time were applied by means of the JMP statistical software program. The values followed by the different letters are significantly different from each other according to Tukey HSD tests at $p \leq 0.05$.

Conclusions

Biodegradable active films were prepared by a one-step ultrasonic deposition of vanillin on the surface of the natural polymer chitosan. The proposed method does not cause changes in mechanical and physical properties of the modified bulk polymer. The deposited NPs of the volatile antimicrobial vanillin reveal significant anti-biofilm activity. Moreover, the prepared films demonstrate in vivo microbial growth inhibition on several food models, fresh-cut watermelon, melon and strawberries, controlling its spoilage and extending the shelf-life. The combination of chitosan and vanillin enhances their antibacterial activity, establishing a synergetic effect. The reported method provides a mild approach for the activation of biopolymers with natural active agents to form active food packaging. The reported method may serve as a platform for further development of active natural polymer-based materials for various applications.

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References


7 S. Dai, Natural Cationic Polymers for Advanced Gene and Drug Delivery, RSC Polymer Chemistry Series, 2015, chapter 12.
16 T. Fadida, Y. Kroupitski, U. M. Peiper, T. Bendikov, S. Sela (Saldinger), and E. Poverenov, Air-ozonolysis to generate contact active antimicrobial surfaces:


