Cooperative crystallization effect in the formation of sonochemically grafted active materials based on polysaccharides

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ARTICLE INFO

Keywords:
Co-crystallization
Polysaccharide
Ultrasonic grafting
Biodegradable
Curcumin
Vanillin

ABSTRACT

The current study explores the formation of active eco-friendly materials capable of preventing microbial contamination using \textit{in situ} ultrasonic grafting of vanillin, curcumin and a curcumin-vanillin mixture on the surfaces of carboxymethylcellulose (CMC) and chitosan films. Spectroscopic, microscopic, physical and mechanical studies revealed that the films grafted with curcumin-vanillin mixture demonstrate improved mechanical properties and higher degree of order. The bioactivity of the prepared films was tested on food model, fresh-cut melons and films with a deposited curcumin-vanillin mixture showed superior antibacterial properties. For instance, this mixture-grafted on CMC films demonstrated a total inhibition of yeast/mold proliferation during 12 days. The HR-SEM studies of the mixture-grafted films revealed the presence of crystalline structures. Cooperative crystallization effect between the curcumin (the crystal maker) and the volatile vanillin is suggested to be responsible for the observed effects. According to our knowledge, this is the first usage of co-crystallization method in surface deposition. The results point out to a general strategy of combining a crystal maker agent with a volatile active agent during \textit{in situ} sonochemical deposition to form bioactive materials that can be further used for food packaging, agriculture, pharmacology and more.

1. Introduction

Active films based on biodegradable polymers and nature-sourced bioactive agents are highly desired since they can be used as advanced eco-compatible and safe packages, patches, covers and coatings for applications in food, pharmacology, agriculture and more \cite{1-3}. However, the incorporation of nature-sourced active agents in the biodegradable polymer matrices is more challenging than the incorporation of synthetic active agents in synthetic polymers, where addition during extrusion \cite{4} bulk polymer modification \cite{5}, nanocomposites \cite{6} and surface grafting \cite{7} are regularly applied. Biodegradable polymers are usually less robust than the synthetic ones, while nature-sourced active agents are often more sensitive, sometimes volatile and have limited solubility. Therefore, the approaches that are used with synthetic components are often too vigorous for nature-sourced materials. Specially designed nanotubes \cite{8}, nanosuspensions \cite{9} and nanogels \cite{10} were reported to be successfully used for incorporation, protection and controlled release of nature-sourced active agents. For the functionalization of biodegradable polymers blending followed by co-drying processes \cite{11}, non-thermal plasma modifications \cite{12} and surface deposition \cite{13} have been utilized. Recently, sonochemical surface deposition technique was reported as a promising method allowing successful adding of active agents to the surfaces of various materials \cite{14,15}.

Co-crystallization is a method used in the pharmaceutical applications for enhancing substance solubility and improve its availability and functionality. Co-crystals are usually formed by two or more different molecular entities where the intermolecular interactions are promoted by hydrogen bonding and $\pi-\pi$ stacking \cite{16,17}. Co-crystallization was also utilized to improve availability of numerous nature-sourced active agents, such as haloprogin \cite{18}, quercetin \cite{19}, fumaric acid \cite{20} and more. However, to the best of our knowledge, co-crystallization processes have yet to be thoroughly studied in the regard of sonochemical deposition.

In the current study, we have utilized the sonochemical grafting method for the deposition of natural bioactive agents on biodegradable
polysaccharide-based films. Polysaccharides are safe, non-expensive, hypoallergenic materials that are being used in drug delivery, medical devices, agricultural and cosmetic industries. A vast array of applications of polysaccharides was reported as biodegradable active packages capable to extend shelf-life, preserve the quality and provide microbial safety of food products [21,22]. Two polysaccharides with excellent film forming ability, chitosan [23] and carboxymethyl cellulose (CMC) [24] were utilized. Vanillin, a volatile antimicrobial aromatic phenolic aldehyde, capable on preventing yeast, mold and bacterial infections [25,26] and curcumin, the non-volatile water insoluble compound demonstrating antimicrobial, antiviral, antioxidant and anti-inflammatory activity [27] were used as the active agents. These compounds were in situ deposited, separately or as a mixture, in order to examine the option of co-crystallization between the curcumin and vanillin to prolong activity of a volatile vanillin. Spectroscopic, microscopic, morphological, physico-mechanical properties as well as the bioactivity of the new active films were comprehensively studied.

2. Experimental

2.1. Materials

99.9 % vanillin, 95 % acetic acid and absolute ethanol were supplied by Sigma-Aldrich. Chitosan (100–300 cps, low molecular weight) was purchased from Glentham life sciences. 95 %, curcumin and carboxymethylcellulose sodium salt were supplied by Alfa Aesar. Plate count agar (PCA) was purchased form Merck and potato dextrose agar (PDA) were supplied by Becton, Dickinson and company. Chloramphenicol was supplied by Sigma-Aldrich. Distilled water was used for the preparation of all solutions.

2.2. Film preparation

CMC solution, 1.5 w/v, was prepared by dissolving 1.5 g CMC in 100 ml of distilled water that were preheated to a temperature of 70 °C, stirred and mixed over a hot plate until full dissolution. Chitosan solution, 1.5 w/v, was prepared by dissolving 1.5 g Chitosan in 100 ml of distilled water adding 500 μl of acetic acid solution. The solution was stirred at room temperature for 1 h. After preparation, 5 ml of each of these solutions was cast into 5 cm diameter petri dishes and air-dried at room temperature under a chemical hood for 48 h.

2.3. Sonochemical deposition of the active agents

Different active agent solutions were prepared as follows: Vanillin 3 g was dissolved in 180 ml of ethanol and 20 ml of distilled water for the total concentration of 1.5 % w/v. Curcumin 1.5 g was dissolved in 72 ml of ethanol and 8 ml of distilled water for a total concentration of 1.8 % w/v. Curcumin - vanillin mixture 0.75 g of curcumin dissolved in 72 ml of ethanol and 8 ml of distilled water was mixed with 0.75 g of dissolved vanillin in 72 ml of ethanol and 8 ml of distilled water achieving 0.9 % w/v concentration of each added substance.

Chitosan and CMC films were each soaked in the prepared solutions and the mixture was sonicated for 5 min by a high-intensity ultrasonic horn (Ti horn, 20 kHz, 750 W at 25 % efficiency) in a thermostatic (30 °C) sonicator chamber. An ice water bath was utilized to prevent overheating. At the end of the process, the films were air-dried in a chemical hood.

2.4. Characterisation of the prepared active films

2.4.1. Fourier-transform infrared spectroscopy (FTIR)

All films were characterized by FTIR using a Thermo Scientific Nicolet iS5 spectrometer. Each film sample was placed under the light beam and 32 scans were performed for each spectrum. Each spectrum was recorded between 500–4000 cm⁻¹ with at 4 cm⁻¹ resolution.

2.4.2. Mechanical properties

The mechanical properties including tensile stress (TS), percent elongation at break (PE) and Young’s modulus (YM) were measured using an Instron Universal Testing instrument (Instron 3345, Norwood, MA, USA) equipped with an Instron force transducer load cell. Film thickness was determined by averaging six measurements at different points for three films of each type using a desktop Mitutoyo Digimatic Indicator thickness gauge with an accuracy of ± 0.001 mm (Mitutoyo Corp, Kawasaki, Kanagawa, Japan). The tests were performed at speed of 1 mm/s. All measurements were performed in triplicate for each film type.

2.4.3. Water contact angle

This measurement was performed on the films in order to check intensity of the phase contact between liquid and solid substances to assess hydrophobic or hydrophilic coating changes of the films. Drop shape analysis (KRÜSS DSA100) was conducted: The contact angle was measured using the image of a sessile drop at the points of intersection (three-phase contact points) between the drop contour and the projection of the surface.

2.4.4. X-ray diffractometry (XRD)

X-ray patterns of the films were probed with Brucker D8 advanced X-ray diffractometer. The samples were scanned for 2θ = 10–70° in scanning speed of 2° min⁻¹.

2.4.5. Thermogravimetric analysis

The thermal stability of the bare film or the coated film or both samples was tested using a thermogravimetric analyzer (TGA 8000, PerkinElmer). Film samples (about 2 mg) were placed in a standard TGA ceramic crucible and scanned at a heating rate of 10 °C/min over the temperature range of 50–800 °C under a nitrogen flow of 50 cm³/min.

2.4.6. High resolution scanning electron microscope (HR-SEM)

The morphology and topography analysis were measured by FEI, Magellan 400 L operated at 10 kV. A small piece of thin film was mounted onto a carbon coated SEM sample holder. The carbon coating was performed on the prepared sample to minimize the charging effect. Elemental analysis was performed using Energy Dispersive X-ray Spectroscopy (EDS) connected to the Scanning electron microscope (SEM).

2.4.7. Weight configuration

All films were weighed prior and after the sonication to assess the amount of substance deposited on the film. Films were cut into squares of 3 × 3 cm².

2.5. Antimicrobial activity of the prepared films

For each experiment, two melons (“Galia” type) were purchased at a nearby convenience store. The melons were cleaned in a four-step program: washed with tap water, decontaminated with sodium hypochlorite solution (100 ppm active chlorine), rinsed with distilled water and air-dried. Cylindrical plugs (diameter 2.5 cm, length ca. 3 cm, weight 3.5 g) were excised from the melons pulp at the circumference of the fruit using a sterile cork borer and a scalpel. The prepared films were placed in polyethylene terephthalate (PET) clamshell containers commonly used in delis and supermarkets for fresh-cut fruit packaging that were preliminarily sterilized with ethanol and UV light. An equal number of melon plugs were mounted onto the film pieces and were covered by films on top to imitate an industrial packaging. A control test was performed during all experiments that included freshly cut melon with no films. Three containers (nine pulp plugs) were prepared with each type of film. All containers were then stored for a period of twelve days overall at 8 °C, simulating storage conditions of fresh-cut...
fruit marketing on a refrigerated shelf.

The plugs were sampled for microbiological analysis at t₀ and after 5, 8 and 12 days. The samples were transferred into centrifuge tubes containing 15 ml of sterile saline solution (0.9 % NaCl). The tubes were vigorously stirred with a vortex for 2 min. 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 the plate count agar PCA. 

A mixture of vanillin-curcumin were aerially decimally diluted with sterile saline and the aerobic plate counts were determined by surface inoculation of PDA (PDA + A), and the number of CFU per gram of fruit was calculated after 48 h of 30 °C incubation. The reported values are average of three replications.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey honestly significant difference (HSD) pairwise comparison tests at p ≤ 0.05 were applied by means of the JMP statistical software program, version 7 (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Formation, characterization and physico-mechanical studies of the active films

CMC and chitosan films were casted from film forming aqueous solutions and then volatile vanillin, non-volatile curcumin, and the mixture of vanillin-curcumin were in situ deposited on the film surfaces from ethanol/aqueous solution using ultrasonic irradiation. The High-Resolution Scanning Electron Microscope (HR-SEM) revealed grafting of active agents on the polymer substrates was observed. For the prepared films, the deposition of curcumin led to a larger angles shift, while curcumin-vanillin behave oppositely shifting this peak to lower angles. Instead, quite ordered structures (similar to that of the curcumin alone) were observed suggesting interactions between curcumin and vanillin (Fig. 1).

The prepared films were characterized by FTIR spectroscopy verifying the addition of curcumin, vanillin and curcumin-vanillin mixture by appearance of new peaks at the CMC and chitosan films’ spectra (Fig. 2). These new peaks were observed in C=O aromatic, C=O and phenolic O–H bond area and are in accordance with previously reported characteristic peaks for vanillin and curcumin [28,29].

X-ray diffraction studies were performed (Fig. 3). The diffraction peaks of the deposited materials are not observed because of the small amounts of the coating (less than 1% wt.), only the effect of the deposited materials on the polymer substrates was observed. For the pristine and the modified films similar diffraction peaks were found at 30° and 40° region, indicating that a parallelism of the polymeric chains remains uninfluenced by the deposited material. On the other hand, in the 20° region that indicates an arrangement of the polymer in the unit cell, notable differences were detected. For the CMC, sonocochemical deposition of curcumin and vanillin-curcumin mixture lead to a smaller angle shift, indicating the growth of the unit cell. For chitosan, the deposition of curcumin led to a larger angles shift, while curcumin-vanillin behave oppositely shifting this peak to lower angles.

The interaction between the film and the deposited material can lead to the increase or the shrinkage of the unit cell. A possible explanation is that the presence of curcumin enhances the vanillin grafting and its interactions with chitosan. Such enhanced interactions may cause Schiff-base reactivity between the amine groups of chitosan and the aldehyde groups of vanillin effecting the order in of the chains [30].

Thermal gravimetric analysis (TGA) of the films was performed (Fig. 4). The CMC film exhibits two distinct weight loss patterns at ∼280 and ∼620 °C (Fig. 4a). At 280 °C area the behavior of the pristine and the modified CMC films is very similar. On the other hand, at 620 °C the films act very differently, CMC shows sharp weight loss peak, the decomposition of the CMC vanillin film is less pronounced and CMC curcumin and curcumin-vanillin films barely have weight loss at these temperature. At the highest temperature of 800 °C, the total weight loss of the films is 84 % (pristine CMC film), 85 % (vanillin films), 72 % (curcumin-vanillin) films and 70 % (curcumin-vanillin films). Notably, the curcumin and curcumin-vanillin deposited films behave very similarly, without any significant effect of vanillin component pointing on the dominant effect of the “crystal maker”, curcumin. In case of the chitosan films (Fig. 4b), the total weight loss of the pristine chitosan films at 800 °C was found to be 78 %, vanillin addition resulted in the total weight loss (76 %), while curcumin and curcumin-vanillin addition showed a bigger change in the weight loss (70 % and 71 %). Concerning the decomposition pattern, here the effect of crystal maker, curcumin, in the chitosan-vanillin mixture is less dominant, probably because of the competitive effect of Schiff-base interactions of vanillin with chitosan [30].

Contact angle measurements were carried out to quantify the
Fig. 2. Fourier-transform infrared spectroscopy (FTIR) of pristine CMC films vs. CMC film grafted with active materials and pristine chitosan films vs. chitosan films grafted with active materials.

Fig. 3. X-ray diffractograms of (left) CMC- and (right) chitosan-based films.
**Table 1**

Water contact angle, elongation at a break, Max tensile stress and Young modulus of films. Values represent means of five replications followed by ± as standard deviations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water contact angle (°)</th>
<th>Max tensile stress (MPa)</th>
<th>Elongation at break (%)</th>
<th>Young Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td>57.48 ± 0.53</td>
<td>31.54 ± 1.10</td>
<td>20.37 ± 1.24</td>
<td>1407.21 ± 322.23</td>
</tr>
<tr>
<td>CMC vanillin</td>
<td>58.47 ± 1.33</td>
<td>74.14 ± 2.21</td>
<td>29 ± 1.5</td>
<td>1501 ± 39.1</td>
</tr>
<tr>
<td>CMC curcumin</td>
<td>70.84 ± 0.43</td>
<td>79.3 ± 0.5</td>
<td>25 ± 1.1</td>
<td>1701 ± 83.5</td>
</tr>
<tr>
<td>CMC-curcumin vanillin</td>
<td>78.5 ± 1.02</td>
<td>23.85 ± 0.88</td>
<td>25.74 ± 0.35</td>
<td>1335.77 ± 75.06</td>
</tr>
<tr>
<td>Chitosan</td>
<td>77.8 ± 1.29</td>
<td>54.90 ± 0.59</td>
<td>27.19 ± 1.51</td>
<td>745.39 ± 97.70</td>
</tr>
<tr>
<td>Chitosan vanillin</td>
<td>76.6 ± 0.85</td>
<td>58.6 ± 0.22</td>
<td>24 ± 0.7</td>
<td>1535 ± 40.29</td>
</tr>
<tr>
<td>Chitosan-curcumin vanillin</td>
<td>107.1 ± 0.98</td>
<td>57.4 ± 0.32</td>
<td>28.6 ± 0.83</td>
<td>1410 ± 53.4</td>
</tr>
</tbody>
</table>

Fig. 4. (A) Total microbial count on PCA plates and (B) mold and yeast count on PDA + A plates of fresh-cut melon after 5, 8 & 12 days of storage at 8 °C wrapped with CMC-based films. * = no microbial growth was observed. Values followed by a different letter within the same sampling time are significantly different according to Tukey HSD test at p ≤ 0.05.

Fig. 5. (A) Total microbial count on PCA plates and (B) mold and yeast count on PDA + A plates of fresh-cut melon after 5, 8 & 12 days of storage at 8 °C wrapped with chitosan-based films. * = no microbial growth was observed. Values followed by a different letter within the same sampling time are significantly different according to Tukey HSD test at p ≤ 0.05.
hydropobicity of the surface before and after the grafting of active agents (Table 1). Being originally quite hydrophobic, chitosan films did not demonstrate substantial changes upon grafting. On the other hand, in the case of CMC surface grafting of active agents on the led to significantly increased hydropobicity. Moreover, when the vanillin-curcumin mixture is grafted on CMC almost super hydropobic surfaces are formed [31].

The mechanical properties, such as maximum tensile stress at break, elongation at break and Young modulus, of the original and the modified films were also examined (Table 1). Overall it can be seen that grafting of curcumin alone or mixed with vanillin enhances the mechanical properties of films, while grafting of vanillin alone decreases the mechanical characteristics. In the case of CMC, the films grafted with a curcumin-vanillin mixture result in the best mechanical properties, better than that of the curcumin alone. In the case of chitosan, adding of vanillin to curcumin does not provide notable advantages additional to that provided by curcumin alone.

It has been shown by Rhim et al. that for a higher degree of crystallinity, the water permeability of the films is lower [32]. There is a good correlation between water contact angle measurements, mechanical studies, XRD and HR-SEM results. All these results show that the deposition of curcumin-vanillin co-crystals improve the resulting film properties, providing better order, higher hydropobicity and enhanced mechanical features. The effect of curcumin-vanillin co-crystallization is most clearly observed on CMC films. This is because chitosan films undergo a disturbing effect of Schiff-base interactions between vanillin and chitosan [30].

3.2. Antimicrobial activity of the films

Fresh-cut melons were wrapped by the prepared films and stored in a commercial plastic clam-shells typically used for storage of fresh-cut fruit at 8 °C for 12 days. Total microbial count (Fig. 5) and yeast/mold count (Fig. 6) was examined at three time periods, after 5, 8 and 12 days. Pristine CMC films did not show significant antimicrobial activity compared to control. Vanillin-grafted CMC films show minor activity against bacterial growth and considerable activity against yeast/mold growth. Curcumin-grafted CMC films showed higher activity compared to that of vanillin-grafted films in both bacterial and yeast/mold inhibition. Notably, the highest antimicrobial activity was demonstrated by CMC films grafted with curcumin-vanillin mix. These films showed ~2–4 log CFU g⁻¹ inhibition in bacterial growth. Even after 12 days of storage the total microbial count reached by fruits wrapped with these films was only 6.2 log CFU g⁻¹ staying below the allowed threshold of 7 log CFU g⁻¹ [33]. Moreover, 100% inhibition of the yeast/mold growth was observed during all storage period, verifying the notable effect of curcumin-vanillin composite on the film properties (Fig. 6). Regarding, the chitosan films, the pristine films demonstrated antimicrobial activity because of the intrinsic antimicrobial properties of chitosan [34]. Grafting of curcumin increased the antimicrobial properties, grafting of vanillin increase them even more and as in also here the highest activity was observed on vanillin-curcumin grafted films, especially in the case of the yeast/mold growth. In consistence with our previous results, the beneficial effect of curcumin-vanillin co-crystallization was higher in the case of CMC, since chitosan has Schiff-base interactions with vanillin [30].

Thus, the curcumin-vanillin co-crystallization benefit notable prolonged antimicrobial activity, improved mechanical properties and enhances structural order of the prepared biodegradable active films. Previously the co-crystallization method was used to increase the solubility or improve functionality of active agents. For instance, co-crystallization with vanillin was used to increase stability and overcome light sensitivity of Rosuvastatin drug [35], while, the co-crystallization with methylparaben was used to overcome low solubility of curcumin in pharmaceutical applications [36]. In this study, curcumin is used as an auxiliary crystalizing agent to provide stability and prolong activity of the volatile agent vanillin. According to our knowledge, this is the first utilization of co-crystallization in surface grafting deposition.

4. Conclusions

Biodegradable, active films based on nature-sourced polysaccharides CMC and chitosan were prepared using in situ ultrasonic deposition of vanillin, curcumin and curcumin-vanillin mixture. All the prepared films demonstrated good chemico-physical properties and antimicrobial activity. However, films with a deposited curcumin-vanillin mixture showed superior performance. As was confirmed by HR-SEM and XRD, π–π stacking interactions between the aromatic rings of curcumin and vanillin promote their co-crystallization upon deposition that allows reducing vanillin volatility enhancing and prolonging its antimicrobial/antifungal functions.

On the other hand, co-crystallization together with highly ordered curcumin allowed improved film features in terms of thermal stability, crystallinity, hydropobicity and mechanical features. The presented finding may lead to a new general strategy of combining crystal maker agent with volatile active agent during their in situ sonochemical deposition to form advance active materials that can be further used in food packages, agriculture, pharmacology and more.

CRediT authorship contribution statement

Yevgenia Shebis: Conceptualization, Methodology, Software, Writing - original draft, Investigation, Formal analysis. Vijay Bhoooshan Kumar: Methodology, Software, Writing - original draft, Formal analysis. Aharon Gedanken: Conceptualization, Writing - original draft, Supervision, Funding acquisition. Elena Poverenov: Conceptualization, Writing - original draft, Supervision, Investigation, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The research leading to these results has received funding from the Israeli Ministry of Health Grant No. 3-0000-99611 and contribution from the Agricultural Research Organization, The Volcani Center, Rishon Lezion, Israel.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2020.110931.

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