Nisin as an Antibacterial Substance in Active Packaging: 2. Use of Ethylene Methyl Acrylate and Co-Polyamide to Enhance Its Effectiveness

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Abstract

This work deals with flexible thermoplastic films incorporating nisin for antibacterial active packaging purposes. A novel approach was used to gain control over nisin release profile from a thermoplastic film with the aim of enhancing nisin antibacterial efficiency. The release profile of nisin from active packaging to foodstuff is a key factor concerning the enhancement of its efficiency. In our last study, polymer blends of EVA and co-polyamide were used to control nisin migration from film to foodstuff simulant. In this part, EVA was replaced by EMA due to applicable reasons. Samples of 400μm thick were produced by using a laboratory twin screw compounder and a laboratory hot press (same as part 1). Release kinetics and antibacterial tests were done in order to characterize different target bacteria response to different migration profiles. Listeria ATCC 33090 was used as target bacteria. Nisin migration profile to foodstuff simulant was determined by Lowry’s protocol. Osmotic pressure driven release mechanism appears to be the migration mechanism and diffusion kinetics was dominant.

Results show that polymer blend continues phase determines the diffusion coefficient. Furthermore, films were characterized for their elastic modulus properties as supplementary data. Elastic modulus and nisin concentration in foodstuff stimulant show an inverse proportion. It is concluded that this proportion is related to the formation of more surface area that is exposed for migration.

Keywords

Active Packaging; Antibacterial Film; Controlled Release; Nisaplin; Nisin; Polymer Blends

Introduction

One of the most relevant fields concerning bacterial contamination is daily consumed ready-to-eat foodstuffs. Food-borne pathogens are a growing concern worldwide because people are exposed to it on a daily basis. The World Health Organization (WHO) and the European Food Safety Authority (EFSA) report in recent years that there has been an increase in food-borne illnesses. Products that are animal origin such as milk, eggs and meat are especially a concern because they acquire the microflora from their environment. Animal origin foodstuffs have a history of more than 50% of total food-borne illnesses in the 1990’s. Listeria monocytogenes and other pathogens are distributed in the environment and are parts of the microflora of humans. Food contamination can occur as a result of hygienic failures during the processing of packaged foodstuff [1].

Bacterial contamination occurs primarily at the surface of foodstuffs due to post processing handling. Attempts have been made to disinfect the surface of contaminated foodstuffs by using direct formulated antibacterial sprays or dips. This direct application had little effect due to antibacterial activity exhaustion [2].

Flexible polymer packaging is increasingly used for foodstuff packaging purposes as a physical barrier from the surroundings among other reasons. Active packaging is a sophisticated approach for the preservation of fresh foodstuffs, control over food-borne pathogens and extension of shelf life [3]. This sophisticated approach actively involves the foodstuff’s polymeric packaging in the preservation of its content, besides being a physical barrier. The objective of an antibacterial active packaging is controlled over bacterial prosperity of non-sterile foodstuffs, maintaining the pasteurized stability for sterile foodstuffs and extended shelf life [4].
Nisin is a potent polypeptide bacteriocin produced by the lactic acid bacteria. Nisin is water soluble, exhibits greatest stability under acidic conditions and is known to inhibit the growth of gram-positive bacteria. It has been used by humans for decades and is considered safe for consumption [5] [6] [7] [8]. Controlling over the release kinetics of nisin is of essence. Its antibacterial effectiveness increases when control is released to the surface of foodstuff, and is compared with traditional nisin instant addition [9] [10] [11]. Release kinetics control technology is used in many fields particularly in drug delivery devices. One method of obtaining control over drug release kinetics is using polymer blends [12]. Each polymer has its own effect on drug release kinetics and by changing the ratio of the polymers in the blend – drug release kinetics control is achieved.

This work aims to study the enhancement of antibacterial effectiveness of nisin incorporated polymer blend films.

**Experimental**

**Methodology**

In order to obtain nisin incorporated polymer films that controls nisin release kinetics and possess antibacterial activity, suitable polymer components were selected according to thermal, rheological and hygroscopic properties:

Since nisin is susceptible to thermal conditions [7] [13], high processing temperature polymers were screened. Hygroscopic properties were of interest since nisin migration occurs on a liquid water medium basis.

Deionized Water (DI water) was used as a migration medium. In order to verify nisin migration from film to DI water, preliminary water swelling tests were done. Polymers that did not increase their water swelling extent as Nisaplin incorporation were screened. Preliminary bacterial tests determined that nisin antibacterial activity was retained in the determined processing conditions and that nisin migration occurred to DI water (data not shown).

This work has applicable purposes, therefore, films containing 4wt% of Nisaplin were of interest.

**Materials**

Two polymers were used as polymer matrix components: Elvaloy ac1820 supplied by DuPont (EMA). This ethylene methyl acrylate copolymer is designed for flexible packaging and complies with the rules and complies with the U.S food and drug administration regulations for use in contact with all types of food.

Some properties include: melting point of 92°C, 20% of methyl acrylate content, MFR = 8cm^3/10min (ASTM D1238) and density of 0.942 gr/cm^3.

Grilon CF 6 S was generously donated by EMS (PA). This Co-polyamide(6,12) is designed for packaging applications and complies with EU requirements and FDA regarding food contact. Some properties include: melting point of 130°C, MVR = 180cm^3/10min (ISO 1133) and density of 1.05gr/cm^3.

Nisaplin, supplied by Handary SA, Brussels, Belgium (Nis) is a commercial product. Nisaplin appearance is a light brown fine powder and is composed of 2.5wt% nisin, 95wt% sodium chloride and remains of milk solids asreported by the manufacturer.

**Sample Preparation**

**Compound Preparation**

EMA, PA and Nisaplin were compounded using a twin screw extruder (Prism, EuroLab). Screw speed was 250 RPM and temperature profile was 160°C for all five heating zones. Samples of 4wt% Nisaplin were produced for biological tests and samples of 12wt% Nisaplin for migration tests. Composition ratios are specified in table 1.

<table>
<thead>
<tr>
<th>Sample notation</th>
<th>Matrix composition EMA/PA [wt%]</th>
<th>Nisaplin (Nisin) [wt%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMA/Nis4</td>
<td>100/0</td>
<td>4 (0.1)</td>
</tr>
<tr>
<td>EMA/PA-30/Nis4</td>
<td>70/30</td>
<td>4 (0.1)</td>
</tr>
<tr>
<td>EMA/PA-50/Nis4</td>
<td>50/50</td>
<td>4 (0.1)</td>
</tr>
</tbody>
</table>
**Film Preparation**

Films were prepared using a laboratory hot press and a rectangular shaped mold. The Laboratory press was heated to 160°C and samples were incubated for 5 minutes. Pressure of 15,000psi were applied for 10 seconds and the resulting film thickness was about 400μm.

**Characterization**

**Thermal Analysis (DSC)**

DSC (TA instruments, Q200) was used (temperature ramp of 10°C/min) in order to evaluate the degree of polymer blend interactions by monitoring the change of characteristic transition temperatures with composition.

In order to eliminate the thermal history, two runs were performed within a temperature range of (-40) – 200°C.

**Scanning Electron Microscopy (SEM)**

SEM (Aspex, explorer) was used in order to characterize polymer blend morphology and evaluation of Nisaplin responding to water activity at the time nisin migration occurs. Samples were immersed for 24 hours in DI water in order to witness film state. Samples were let to dry, a cryogenic fracture was done and the cross section was examined. Samples were coated with a thin layer of gold and images were captured at a magnification of 750X.

**Nisin Migration Profile**

The effect of polymer blend composition on nisin migration profile to DI water was evaluated using migration cells at room temperature. Migration cells were composed of a glass cell equipped with a removable 60mm diameter lid. 10ml of DI water was introduced to the cell as migration medium. Nisaplin incorporated films were cut to fit the cell’s lid. Each container was positioned so that DI water and film contact took place. Films were let to reach equilibrium for one hour. Sink conditions were verified [14]. At specific time intervals, water samples were taken and characterized for their nisin concentration by Lowry’s protocol [15]. A U.V visible spectrophotometer (SHIMADZU, UV-1650PC) was used to evaluate the absorbance peak at 750nm. Nisaplin was used as standard. Samples were tested using triplicates.

**Mechanical Properties**

A tensile test was conducted in order to evaluate the influence of mechanical properties on the migration extent [16]. The tensile test was conducted, according to ASTM D882, at room temperature and samples were let to condition for more than 40 hours prior to the test. Test conditions were initial grip separation of 50mm and rate of grip separation was 500mm/min. Each result consists of five tests.

**Antibacterial Activity**

In order to evaluate the antibacterial activity of the films, a Gram-positive target bacteria (Listeria ATCC 33090) was used. Antibacterial activity tests were done at 50% nutritional broth (NB) at room temperature. Samples were tested using duplicates. Corresponding control films were tested and no significant influence on target bacteria was seen (data were not shown). Films containing 4wt% Nisaplin were tested, as noted earlier, due to an applicative approach and the high sensitivity of this test.
Results and Discussion

Thermal Analysis (DSC)

A DSC diagram of EMA/PA blends is shown in figure 1. Melting temperatures of 130°C and 92°C can be seen for PA and EMA blends respectively. EMA/PA-70 sample does not show the EMA melting point, due to the existence of a Tcc peak at 78°C which is attributed to PA. The PA glass transition temperature shown is about 29°C. EMA glass transition temperature is not evident at this DSC diagram and not reported by the manufacturer. All EMA/PA blends show approximately these glass transition temperatures without any significant change with composition. It is concluded that no significant physical interactions exist in these blend compositions.

![DSC Diagram of EMA/PA Blends](image)

Scanning Electron Microscopy (SEM)

SEM images of 12wt% Nisaplin incorporated EMA/PA blends are shown in figure 2. Image A shows dry swollen capsules that did not rupture, probably due to the depth of about 150μm and the distance between capsules. Furthermore, the capsule's inner surface seems rough because of dried Nisaplin solution which contains sodium chloride. Image B, C and D shows droplet morphology and swollen capsules at varying depths. The inner surface of the swollen capsules seems smooth, compared with the rough droplet morphology, this is due to the peripheral tensile stress that osmotic pressure generates as a result of exposure to water. Image E shows a ruptured capsule, probably due to the proximity to the surface and a few swollen capsules are seen nearby.

These images indicate that an osmotic pressure driven release is the main Nisaplin release mechanism [17] [18].

![SEM Images of Cross Section Area](image)
Nisin Migration Profile and Diffusion Coefficient Determination

Samples containing 12wt% of Nisaplin were characterized so that reliable data were obtained. The migration profiles of samples that contain 4wt% of Nisaplin can be derived from 12wt% ones. This assumption can be made due to Nisaplin loading of 5.5vol% for 12wt% samples. This extent of loading is below the critical volumetric loading (about 33vol%) [17] [18]. Figure 3 illustrates plots of nisin concentration versus time for various matrix compositions. An initial steep raise of nisin concentration is seen within the first hour probably due to Nisaplin particles that are easily accessible to the simulant at the surface of the sample [19].

This steep raise is followed by some decrease, seen well in EMA/PA-30/Nis12 and EMA/PA-70/Nis12 samples. These samples may experience a tendency towards the influx of DI water containing nisin that was released in the first burst, rather than a balance or a tendency towards nisin migration, like EMA/PA-50/Nis12 and PA/Nis12.

It can be seen that different matrix compositions give different migration profiles with time. Furthermore, after 24 hours of exposure, the final concentration of different samples is spanned over a range of 2–6μg/ml that correspond to 0.6–2.0wt% of total nisin content released.

The corresponding diffusion coefficients, specified in table 2, were determined by the trend line slopes of fractional release vs. square root of time plots (1-24 hours), figure 4, according to Fick’s second law – equation 1 [20] [21]:

\[
\frac{M_t}{M_\infty} = \frac{2}{\pi} \left(\frac{D t}{\lambda}\right)^{0.5}
\]

Where \( t \) is time [hours], \( \frac{M_t}{M_\infty} \) is the fractional release at time \( t \), \( D \) is the diffusion coefficient \( \frac{m^2}{h} \) and \( L_p \) is the sample thickness \( m \). It should be noted that equation 1 is valid for \( \frac{M_t}{M_\infty} \leq 0.6 \).

![Figure 3. Migration curves of EMA/Nis12, EMA/PA-30/Nis12, EMA/PA-50/Nis12, EMA/PA-70/Nis12, and PA/Nis12 at 20°C. Error bars refer to ±2Σ.](image)

![Figure 4. Plots of fractional release versus square root of time for the migration of nisin from different polymer matrix blend compositions at 20°C. Error bars refer to ±2Σ.](image)
TABLE 2. CALCULATED DIFFUSION COEFFICIENTS AND LEAST-SQUARES FIT FOR DIFFERENT POLYMER BLEND COMPOSITIONS.

<table>
<thead>
<tr>
<th>Sample Notation</th>
<th>$D [m^2/h]$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMA/Nis12</td>
<td>2.12∙10⁻²³</td>
<td>0.8123</td>
</tr>
<tr>
<td>EMA/PA-30/Nis12</td>
<td>1.81∙10⁻¹³</td>
<td>0.9076</td>
</tr>
<tr>
<td>EMA/PA-50/Nis12</td>
<td>2.01∙10⁻¹⁴</td>
<td>0.9663</td>
</tr>
<tr>
<td>EMA/PA-70/Nis12</td>
<td>3.14∙10⁻¹⁴</td>
<td>0.9624</td>
</tr>
<tr>
<td>PA/Nis12</td>
<td>1.13∙10⁻¹⁴</td>
<td>0.9777</td>
</tr>
</tbody>
</table>

Figure 5 illustrates the change in the diffusion coefficients as a function of weight percent of PA in the polymer blend matrix. The diffusion coefficient decreases slightly when 30wt% of PA are introduced to neat EMA matrix. Considering further addition of PA, it can be seen that a sharp decrease is seen and further addition has no significant effect. SEM images reveal that droplet morphology exists differing in the inversion of component’s role (figure 2). It is concluded that the diffusion coefficient is governed by the continuous phase.

All samples, except EMA/Nis12, have a least square fit above 0.90 and that diffusion is the dominant release mechanism. EMA/Nis12sample has a lower least square fit (0.81). This can indicate that diffusion is not the only release mechanism in action at this case.

**Mechanical Properties**

Samples consisting of neat EMA/PA polymer blends were characterized in order to evaluate the influence of the mechanical properties on the migration extent.

Figure 6 illustrates nisin concentration post migration of 24 hours to deionized water (data from figure 3) and elastic modulus as a function of PA content (numerical values are listed in table 3).
TABLE 3. CORRESPONDING NUMERICAL VALUES FOR FIGURE 6.

<table>
<thead>
<tr>
<th>PA[wt%]</th>
<th>Nisin Concentration [μg/ml]</th>
<th>Modulus [Mpa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.3±0.3</td>
<td>19±1</td>
</tr>
<tr>
<td>30</td>
<td>5.4±0.2</td>
<td>35±2</td>
</tr>
<tr>
<td>50</td>
<td>4.5±0.2</td>
<td>79±8</td>
</tr>
<tr>
<td>70</td>
<td>2.9±0.2</td>
<td>210±15</td>
</tr>
<tr>
<td>100</td>
<td>1.8±0.2</td>
<td>364±9</td>
</tr>
</tbody>
</table>

It can be seen that an inverse proportion exists. This inverse proportion can be related to the swelling extent of Nisaplin capsules and by that, nisin migration extent. As the water front enters a capsule, an osmotic driven release mechanism takes place (discussed earlier). This mechanism induces tensile stress at the perimeter of the capsule and as a result the capsule strains. It is known that the elastic modulus is inversely proportional to strain. As the polymer matrix modulus decreases, more capsule strain occurs and more surface area of Nisaplin solution is exposed for migration.

Similar results were seen when different sizes of BSA and sodium chloride granules were incorporated into silicone rubber. Results show that larger granules produce higher release profiles. It can be understood that when the capsule’s size is larger, more surface area is exposed for migration [22].

Similar results were shown when sodium iodide was incorporated into silicone rubber with varying grain size. It was concluded that coarser granules lead to faster salt release [23].

**Antibacterial Activity**

Antibacterial activity tests were done in order to evaluate target bacteria response to different nisin migration profiles. The antibacterial activity of the different films was evaluated after 24 hours of exposure to different films.

It can be seen from figure 1 that all active films reduced target bacterial count compared with the natural growth of target bacteria (control). These results indicate that films of EMA/Nis4 and EMA/PA-30/Nis4 reduce bacterial count 4 fold, film of EMA/PA-50/Nis4 reduce bacterial count 7 fold and film of EMA/PA-70/Nis4 and PA/Nis4 reduce bacterial count 1-2 fold. Concerning figure 3, EMA/PA-50/Nis4 migration profile shows a lower concentration profile with time compared with EMA/Nis4 sample. It is known that target bacteria are susceptible to specific migration profiles more than others. In this case, migration profiles were determined by the polymer matrix composition. This result is coherent with studies that dealt with inhibition significance of target bacteria exposure to different nisin migration profiles [9] [10].

**Conclusions**

This study investigated different EMA/PA blends and its induced nisin migration profiles with the aim of enhancing the effectiveness of Nisaplin incorporated antibacterial active packaging. Nisin retained its antibacterial
activity when subjected to temperature of 160°C for 5.5 minutes. The diffusion coefficient was mainly determined by the continuous phase of the polymer blend matrix. The use of an immiscible polymer blend, as different composition ratios, altered the matrix modulus. This induced different surface areas ready for migration. It is concluded that by controlling over the elastic modulus of the polymer matrix "fine tunes" the release profile, nisin incorporated active packaging effectiveness is enhanced. Antibacterial activity tests show that target bacteria response depends on nisin delivery profile. Comparing these results with our previous study (EVA/PA blends incorporating Nisaplin), the migration profiles and diffusion coefficient induced are different at this case. This means that EMA has different influence on nisin release kinetics. This can be related to the relatively higher polarity that EMA contributes to EMA/PA blend than EVA dose for EVA/PA blend. Antibacterial activity tests show that the induced migration profiles in this study are more effective than the ones shown by EVA/PA blend. Higher antibacterial activity is seen at higher NB concentration.

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REFERENCES


