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Preparation of a milk spoilage indicator adsorbed to a modified polypropylene film as an attempt to build a smart packaging

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ABSTRACT

A colorimetric sensor of milk spoilage was built from adsorption of methylene blue (MB) onto a modified polypropylene film (PP). First, acrylic acid (AA) was grafted onto PP surface by photograft polymerization reaction, and then either MB or chitosan chains (Cs) was attached to polyacrylic acid (PAA). Three syntheses were researched in order to fix MB to PP-g-PAA–Cs into different levels of PAA–Cs layers. The growth of microorganisms and the generation of reducing substances take place during milk decomposition, where MB is reduced to its colourless form. Binding stability of dye on the film and its redox activity were confirmed by UV–vis spectrophotometry. Reduction kinetics of MB shows a response fast enough to work as a spoilage indicator against samples of liquid milk in different preservation states. This performance also describes these devices as suitable sensors to be used in the development of smart packagings.

1. Introduction

Over the last decades, researchers have shown a growing interest in developing different food safety measures to ensure the security and quality of food (Smigic et al., 2012). The food industry is constantly trying to enhance product safety by acquiring new technologies (Jung et al., 2012; Duncan, 2011). Microbial growth in food products results in a shelf-life reduction of food and an increase in the risk of food-borne diseases. Therefore, there is a special interest among the food industry, retailers, consumers, and their stakeholders in developing a device that is simple, low-cost, rapid, reliable, non-invasive and non-destructive to evaluate real-time freshness of food products (Kuswandi et al., 2012). An alternative concept to address this requirement is the development of smart packaging in the form of a food spoilage indicator to monitor freshness status of food products (Potyrailo et al., 2012). In the particular case of milk, historical data show that its pasteurization has contributed to public health and more recent data on occasional raw milk consumption indicate the hazard of bacterial infections, which could be avoided by heat treatment (Claeysa et al., 2013). Although milk and dairy foods represent a group characterized by a high nutritional value, most of them are highly perishable and cannot be controlled by using of preservatives due to current regulations (Valbuena et al., 2005). It is known that a natural bacterial growth takes place in pasteurized milk once its packaging is opened despite being kept in the refrigerator. Therefore, the development of a smart packaging to check its state of milk preservation is a topic that might prove a suitable tool for the quality control of safer foods (Maciel et al., 2012; Ensafi and Amini, 2010; Rastegarzadeh et al., 2009). Several colorimetric indicators implanted in the packaging have been used for food applications in recent years (Jung et al., 2012; Maciel et al., 2012; Ensafi and Amini, 2010; Zajko and Klimant, 2013). Usually these indicators are organic molecules whose structure is affected by external stimuli. In addition, MB is a thiazidic dye widely used in biomedical study and considered a leader compound in clinical areas, including therapy for malaria and schizophrenia, as well as photodynamic therapy cancer and, more recently, from microbial infection (photodynamic antimicrobial chemotherapy) (Buchholz et al., 2008; Wainwright et al., 2007). Moreover, this dye has low human toxicity and is able to exhibit efficient properties as a fotosensibilizator (Wainright, 2005). MB also shows good electrochemical properties, therefore, it has been widely used for electrochemical studies, i.e., electrocatalysis, solar cells, and biosensors (Xiao et al., 2011; Barou et al., 2012; Zhang et al., 2010). This dye also has redox activity; it acts as a hydrogen acceptor against a reducing substance in an anaerobic environment and takes the form of a leucobase. On the other hand, the kind of bacteria ordinarily found in milk consumes oxygen during its growth and multiplication. Here, many germs will quickly use up all the oxygen, while a small number will require much longer time.

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The addition of MB to a milk sample with a certain content of oxygen dissolved will reveal a blue colour until all the gas is consumed by bacteria; the sample will then almost immediately recover its white colour (Scheme 1) (Anderson et al., 2011). Therefore, the application of MB has become a helpful test to estimate the number of bacteria presents into liquid milk samples (Bapat et al., 2006; Lee et al., 2009). In this study a sensor device was built and its performance as a milk spoilage indicator was researched. First, MB was attached together with Cs onto the surface of polyacrylic acid-grafted PP films (PP-g-PAA) through electrostatic interaction with carboxyl groups of PAA (Cavallo et al., 2010). In order to avoid the diffusion of MB within food, PAA chains were crosslinked with Cs backbones, leading to formation of a mesh that held the molecule sensor (Scheme 2). Three synthesis techniques were compared, where the dye was deposited into different levels of PAA–Cs layers. Finally, the sensor performance was investigated from colour change of MB attached to film surface as a time function against samples of milk at different preservation states.

2. Experimental section

2.1. Reagents and equipment

Several reagents were used as purchased; chitosan (Cs), medium molecular weight and 85% of deacetilation degree, Aldrich (USA). Acetic acid p.a., Cicarelli (Argentina), methylene blue (MB) p.a., Anedra (Argentina). Instant full cream milk powder, Nestle Nido (Argentina). 20 μm thickness isotactic PP film was kindly supplied by Converflex S.A. (Argentina). Crystallinity degree (48%) was determined from the melting endotherm of the polymer measured with a Perkin–Elmer Pyris DSC calorimeter (USA) at a heating rate of 10 °C min^{-1}, assuming that the enthalpy of 100% crystalline isotactic PP was 138 J g^{-1}. Acrylic acid (AA) p.a., Merck (Germany), was purified by distillation under reduced pressure. Benzophenone (BP), p.a., Mallinckrodt (USA), was recrystallized by decreasing the temperature from a methanolic solution. Spectrophotometric measurements were recorded on a MultiSpec-1501 Shimadzu spectrophotometer (Japan). Fourier-transform infrared (FTIR) spectra of the samples were recorded on a Nicolet 5-SXC spectrometer (USA). pH determinations were carried out using a digital pH Altronix meter, TPXI 1584 (Argentina).

2.2. Photograft polymerization of AA onto PP films

The surface of the PP film was initially modified with AA using photograft polymerization at room temperature, BP as a radical initiator, and different reaction times (Scheme 3). A PP film with a surface area of 64 cm^2 was weighed and immersed into a Petri capsule with 1.00 mL of AAc/H_2O 50% by volume solution. This mixture was then irradiated under ultraviolet–visible light using a medium pressure UV lamp (Engenlhard, Hanovia – UK) and a nitrogen atmosphere at room temperature. Subsequently, the modified film was washed once with a NaOH aqueous solution at pH 8 in order to remove the PAA homopolymer and by-products. Finally, the grafted film was washed exhaustively with distilled water and dried under reduced pressure. The reaction yield is expressed as the grafting degree of PAA (G), which is calculated using Eq. (1) (Cavallo et al., 2010).

$$G/\text{wt.}\% = \left(1 - \frac{\text{PP}}{\text{PP-g-PAA}}\right) \times 100 \quad (1)$$

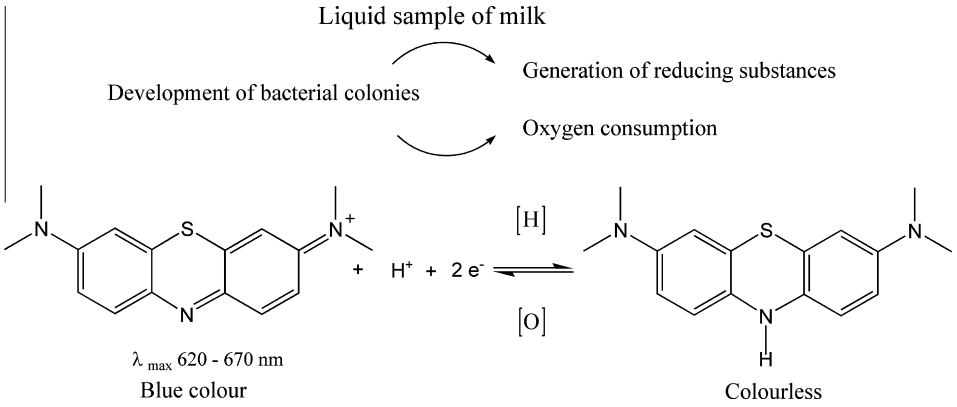
2.3. Chitosan immobilization onto PAA-grafted films

A sample of Cs was solubilized by addition of a 2 wt.% acetic acid aqueous solution under stirring at room temperature, using a 1:1 mol ratio of amino to carboxyl group (Cs/acetic acid). The final concentration of Cs aqueous solution with a pH value close to 5.0 was adjusted at 1 wt.% with distilled water. This polymer was immobilized mostly by electrostatic interaction between its ammonium groups and carboxylate groups of PP-g-PAA, when this film was left in contact with 45.0 mL of a Cs solution for 4 h at room temperature (Scheme 2). PP-g-PAA–Cs films were afterwards washed exhaustively in a beaker with distilled water under stirring at room temperature, and then dried under reduced pressure. Adsorption reaction of Cs took place on films with different G values and temperature reactions. Immobilization degree of Cs (I) was calculated according to Eq. (2) (Cavallo et al., 2010).

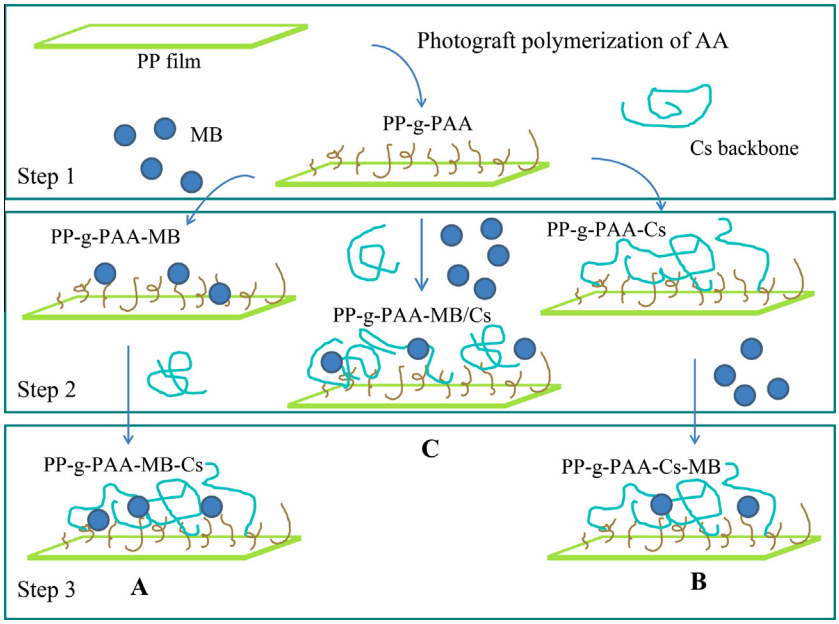
$$I/\text{wt.}\% = \left(1 - \frac{\text{PP-g-PAA}}{\text{PP-g-PAA} - \text{Cs}}\right) \times 100 \quad (2)$$

2.4. Adsorption of methylene blue to modified films

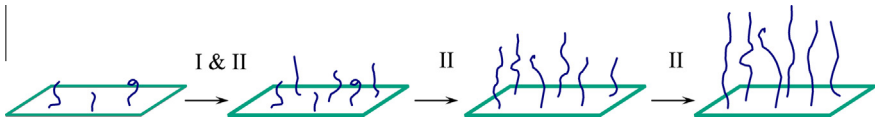
The dye was deposited on PP films with different G values using three synthesis techniques (Scheme 2). Technique A: the dye was mostly adsorbed to PP-g-PAA through electrostatic interaction between carboxylate groups of PAA and MB with a positive charge in its molecular structure. Here, the PP-g-PAA film (near 0.030 g, 16 cm^2) was left in contact with 20.0 mL of a 2.10^{-5} M of MB aqueous solution at pH 5.0 for 4 h at room temperature. The film was then exhaustively washed with distilled water in order to eliminate the dye unattached. Finally, Cs backbones were fixed to the PP-g-PAA–MB film using the procedure detailed in Section 2.3. Technique B: the MB molecule was mostly adsorbed to the more external carboxylate groups of PP-g-PAA–Cs, a 16-cm^2 film, where



Scheme 1. Oxidized and reduced MB species present in redox reactions.



Scheme 2. Modification steps and adsorption of MB onto the surface of the PP film for technique A, B and C.



Scheme 3. Increase in the graft density (I) and length of PAA chain (II) during photograft polymerization of AA onto a PP film.

MB diffused from solution (2.0 mL, 2.10^{-5} M) onto PAA layer. In technique C, the dye was mixed with 20.0 mL of a 1 wt.% Cs aqueous solution at pH 5.0, and then adsorbed on the carboxyl groups of PP-g-PAA (Scheme 2). In this reaction, a 2.10^{-5} M MB solution was used for 4 h at room temperature. Finally, the film was exhaustively washed with distilled water. The adsorption degree of MB (AD) on the films was estimated from Eq. (3).

$$\mathrm{AD/wt.\%} = \left(1 - \frac{\text{PP-g-PAA} - \text{Cs}}{\text{PP-g-PAA} - \text{Cs} - \text{MB}}\right) \times 100 \quad (3)$$

2.5. Stability study of MB immobilized onto films

Desorption degree of MB corresponding to modified films was examined in order to determine the interaction stability between the dye and the film. Then, the latter was left into a beaker with 6.0 mL of a 0.05 M buffer phosphate solution pH 7 under stirring at 37 °C. The content of dye released to the buffer solution against the immersion time was recorded by UV–vis spectroscopy, where the MB concentration in the supernatant was determined from its absorbance value at 664 nm. MB content released into buffer solution was calculated in relation with the total amount of MB adsorbed onto the modified film (4.0 cm^2). In addition, the calibration curve of MB was performed in buffer phosphate solution at pH 7. Other spectral curves of MB solution were attained at pH 1, 4 and 13, where a 0.10 M solution of chlorhydric acid, acetic acid and sodium hydroxide was used as solvent, respectively.

2.6. Performance of MB attached to films as a sensor of the state of milk conservation

Redox activity of MB deposited on the surface of modified films (18 wt.% of *G*) was investigated for liquid milk samples in two states of preservation. A sample of freshly prepared milk (pH 6.7), and a sample whose storage temperature (4 °C) was interrupted for 24 h at room temperature after being made.

Film modified with MB (1 × 2 cm) was immersed in 5.0 mL of a 13 wt.% milk aqueous solution, reaction mixture whose reactor was immediately capped to keep constant the oxygen content in the sample, and left at 37 °C in order to encourage bacterial growth. In addition, a dark glass container was used to protect the milk sample from light. In this way, the gas oxygen level in the reactor is decreased and reducing substances are generated, which lead to reduction of the oxidized form of MB. Therefore, reduction kinetics of attached dye were determined by UV–visible spectroscopy from the absorbance value at 664 nm, since reaction rate is directly proportional to the sanitary quality of the milk analyzed in controlled conditions (Scheme 1) (Atherton and Newlander, 1977; Minj and Behera, 2012; Nandy and Venkatesh, 2010; Lee et al., 2009). The general procedure is as follows: after a given reaction time the film is removed from the reaction mixture and instantly washed with distilled water; its absorbance is determined.

2.7. Indicator response for the homogenous system

An aqueous solution 2×10^{-4} M of MB (10.0 mL) was prepared and mixed with 100.0 mL of a 13 wt.% freshly prepared milk aqueous solution into a dark glass container, which was immediately capped to keep constant the oxygen content in the sample. The reaction mixture was incubated in a water bath at 37 °C. After a given reaction time, an aliquot (3.0 mL) is taken and then precipitated by addition of a 1.7 M of acetic acid aqueous solution (0.10 mL). Next, the sample was centrifuged and the supernatant was characterized by UV–vis spectrophotometry, where its absorbance was record at 664 nm.

3. Results and discussion

In an attempt to develop a smart packaging, the preparation of a sensor device based on MB attached to PP modified films and its performance as a indicator of milk decomposition state were evaluated against a homogeneous system. This dye was adsorbed onto several levels of film surface, while samples of milk with two degrees of conservation were researched.

3.1. Photograft polymerization of AA onto PP films

Surface of PP films was modified by AA photograft polymerization under nitrogen atmosphere at room temperature, where reaction conditions used were those described in a previous study (Cavallo et al., 2010). Chemical composition of PP films was analyzed using FTIR spectroscopy, where the chemical modification of the film in each reaction pathway could be demonstrated. Most relevant bands found in the initial PP film are attributed to C—H stretching vibration at 2925 cm^{-1}, and to C—H deformation vibration at 147 and 125 cm^{-1} (Fig. 1). The film of PP-g-PAA shows a new band at 1710 cm^{-1} corresponding to the C=O stretching vibration of the carboxyl group. Finally, the adsorption of Cs onto the film modified was confirmed through the presence of a new band at 1560 cm^{-1}, corresponding to N—H deformation of the amino group in the PP-g-PAA–Cs film. Moreover, data of absorption (FTIR) and reflection spectroscopy (ATR-IR) corresponding to films with different *G* values were evaluated from the band ratio of stretching C=O of carboxylic acid (1710 cm^{-1}) in relation to the deformation vibration C—H (1470 cm^{-1}) of PP. Fig. 2 shows that the curve obtained from ATR-IR depicts a 10 *G*% and then describes a plateau for further *G* values. This behaviour is attributed to the fact that this technique monitors only the film surface. Grafting density of PAA remains constant once the film surface is completely covered by PAA chains despite fact that the carboxyl group content increases in the film bulk (Scheme 3). FTIR spectroscopy reveals that carboxyl group content increases with *G* value (Fig. 2), which is related to the development of the length of PAA chain. This behaviour is also supported by the gravimetric method. Therefore, the ATR-IR spectroscopy only scans functional groups present on the surface film, while FTIR is a successful tool to determine the carboxyl group content found in the bulk.

3.2. Preparation of the sensor device

Three synthesis techniques were used to attain these sensors (Scheme 2), which were named A (PP-g-PAA–MB–Cs), B (PP-g-PAA–Cs–MB) and C (PP-g-PAA–Cs/MB). For technique A Fig. 3 shows that the AD curve describes a behaviour similar to that found in the carboxyl group content on film surface (Fig. 2). This phenomenon supports the fact that carboxylate groups of PAA layer near surface are neutralized mostly through electrostatic interaction with the dye. In addition, the performance of MB attaching depends directly on carboxyl groups present on the external surface of the PAA layer. On the other hand, curves corresponding to techniques B and C show AD values lower than those found in A, and an almost negligible increase in MB attaching along the *G* axis (Fig. 3). In method B the dye diffuses through the Cs layer to interact with the external surface of PAA (Scheme 2). At the PAA/Cs interface crosslinking points are generated from chain interpenetration and electrostatic interactions, which regulate dye diffusion and content of carboxylate group available in this place.

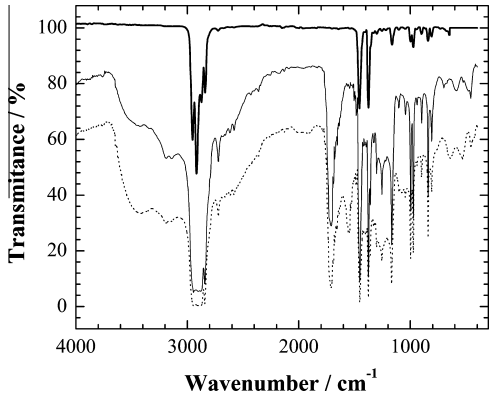


Fig. 1. FTIR spectra corresponding to different PP films. Initial PP (continuous line), PP-g-PAAc with *G* 16% (dash line) and PP-g-PAAc–Cs with *G* 16% and *I* 2.7% (dotted line).

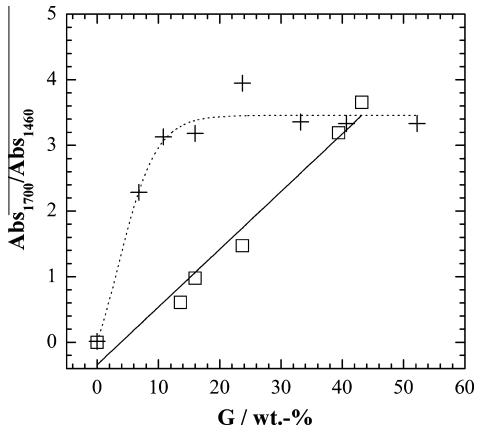


Fig. 2. Ratio of absorption bands belonging to PAA (1700 cm^{-1}) and PP (1460 cm^{-1}) as a function of grafting degree through FTIR (square) and ATR-IR (cross).

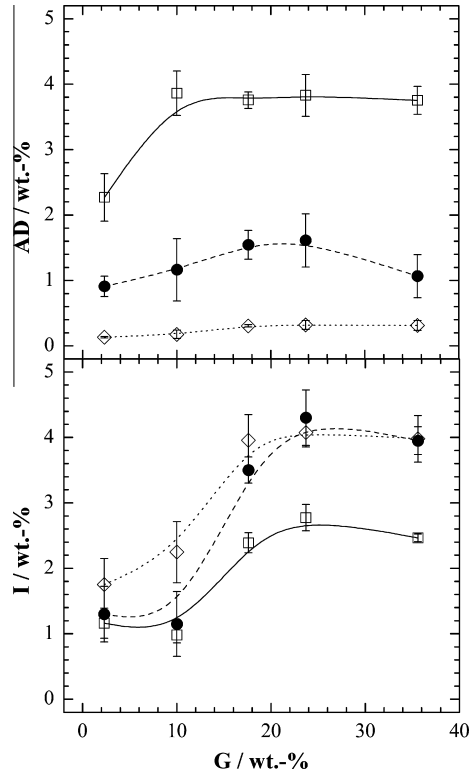


Fig. 3. Adsorption process of MB (top) and Cs (bottom) carried out onto modified films with several grafting degree.

Moreover, technique C shows the lowest AD value since MB adsorption is poor as a result of a high competition between the dye solubilized in the polysaccharide solution and ammonium groups of Cs by PAA carboxylate groups (Fig. 3, Scheme 2).

On the other hand, the degree of Cs immobilization (I) corresponding to three techniques exhibits an behaviour analogous to that found for dye attachment, in which Cs adsorption depends on the carboxyl group content (Fig. 3). This phenomenon is related to the fact that once surface is completely covered with the first polysaccharide chains, the charge reversion of the surface takes place, due to the predominance of the ammonium group, which avoids further binding of new Cs chains. Fig. 3 also shows that a higher amount of dye-attached leads to a lower I value for films containing MB. This confirms the fact that the interaction of the carboxylate group with either the dye or the Cs chain is electrostatic in the PAA/Cs interface. Here, the cationic dye competes with the ammonium group of Cs by the PAA carboxylate group. For technique C, the dye solubilized in the Cs solution masks positive charges onto polysaccharide and reduces the hydrodynamic volume of polymer coil conformation (Scheme 4), favouring the immobilization of a higher amount of Cs (Fig. 3).

On the other hand, the stability of MB immobilized onto modified films was examined at 37 °C through UV–visible spectroscopy. The pH effect was then analyzed on the change of bands in the spectral curve of the dye solution. Fig. 4 shows no variation in the spectral curve of MB in the 1–7 pH range, from which a pH 7 has been used in this study. Therefore, modified films containing MB were immersed in a buffer pH 7, a 0.05 M sodium phosphate solution under stirring. Supernatant absorbance at 664 nm was recorded after a given time, and dye concentration was determined using a $(6.50 \pm 0.02) \times 10^4\ cm^{-1}\ M^{-1}$ molar absorption coefficient. Fig. 5 shows that the content of dye released into the solution is almost zero for the three samples, which highlighting the fact that the electrostatic interaction between the dye and carboxylate groups of PAA is stable enough against to the desorption conditions used. The highest MB content released corresponds to the film obtained from technique C with a value near 0.06 wt.% of dye after 30 h. This behaviour supports the fact that there is a high competition between the dye and the ammonium groups of Cs by carboxyl groups of PAA. This is also the reason for which this technique exhibits the lower content of dye adsorbed. Taking into account these results, sensor A seams to exhibit better properties than those found in other devices since MB is first attached to PAA-modified film (Table 2). For this technique, the dye has a greater supply of carboxyl groups, resulting in a higher loaded MB (Scheme 2). Furthermore, the dye placed in the most inner layer is held more strongly by the mesh formed by the interaction between PAA and Cs chains (Scheme 5). However, the device A shows a performance similar to that found for sensor B and C,

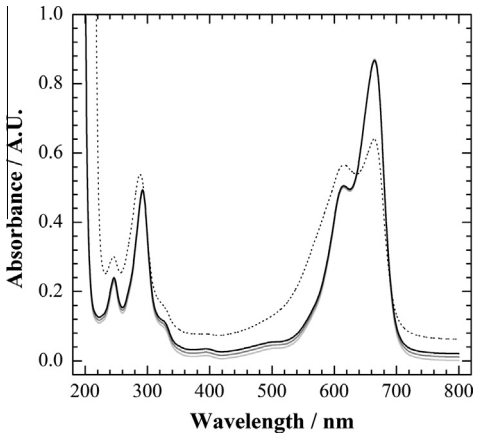


Fig. 4. Spectral curve corresponding to a 1.4×10^{-5} M MB aqueous solution recorded at different pH values such as 1 (light grey line), 4 (grey line), 7 (black line) and 13 (dot line).

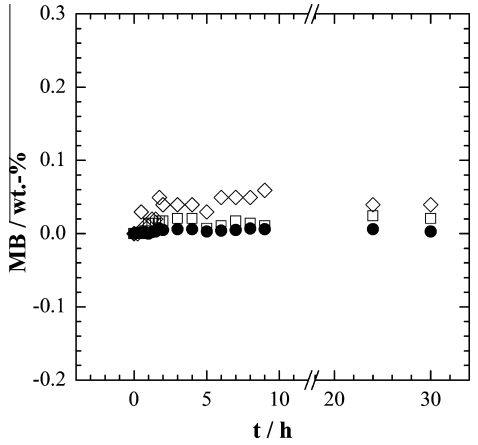
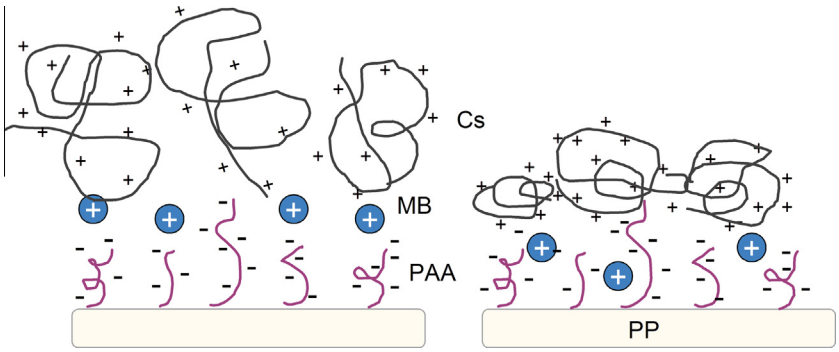
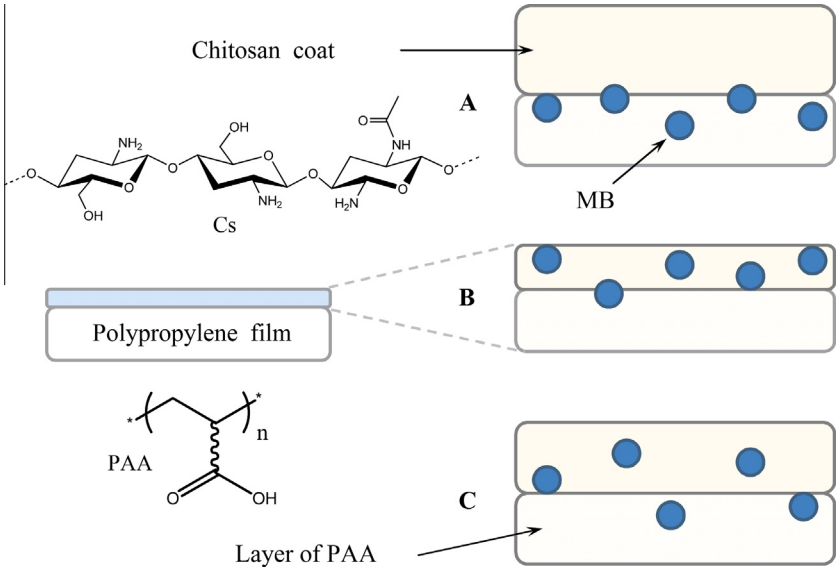


Fig. 5. Stability of MB attached to modified film attained from A (square), B (sphere) and technique C (diamond). The percentage of dye released is calculated in relation to the content of MB attached to film.

when their response of discoloration against milk samples are analyzed. These sensors exhibited no significant lag time, which is related to the fact that the diffusion of reducing substances near MB is faster than their formation rate. Table 2 shows also data corresponding to sensors built from PAA-grafted films with a 18 wt.% of G, since the latter exhibited the best mechanical properties when the G effect on the elastic modulus were examined (Cavallo et al., 2010). Although a longer time of photograft polymerization involves a higher G value and a longer PAA-grafted chain, a G value



Scheme 4. Influence of synthesis conditions on the structural organization of polyelectrolyte chains for techniques B and C.



Scheme 5. Cross-section of the device sensor generated where the dye molecule has been placed at different levels of the surface.

Table 1
State of milk preservation as a function of DD value.

Class of milk sample	Milk state	t_{DD50}/h
I	Excellent	>8
II	Good	6–8
III	Fair	2–6
IV	Bad	<2

higher than 18 wt.% corresponds to films modified with a higher level of degradation.

3.3. Performance of the colorimetric indicator into liquid milk samples

The dissolved oxygen content in the sample of liquid milk decreases during its natural decomposition due to the metabolic consumption of microorganisms and generation of reducing substances. Taking advantage of the MB ability as an oxidizing agent, this dye is immobilized and used as an indicator of the bacterial load in liquid milk samples. It is known that reduction kinetics of MB from its oxidized (blue colour) to the reduced species (colourless) under controlled conditions (Scheme 1), is directly related to the sanitary quality of milk (Nandy and Venkatesh, 2010). Although it is difficult to establish the exact number of microorganisms, Venkatesh et al. found that the kinetics of MB discoloration is directly related to the rate of bacterial growth determined as colony forming unit (CFU). Therefore, the decomposition degree of a milk sample as a function of the time is followed according to the discoloration degree of dye attached (DD), which is calculated from Eq. (4).

$$DD/\% = \left(1 - \frac{Abs_t}{Abs_0}\right) \times 100 \quad (4)$$

The absorbance of MB attached to the film is determined by UV–visible spectrophotometry at a wavelength of 664 nm, before (Abs_0) and after (Abs_t) which is in contact with the milk sample during a specific time. Moreover, the time in which MB decolorizes to a 50% of DD value (t_{DD50}) is attained, parameter from which the milk health status is classified according to data in Table 1 (Atherton and Newlander, 1977; Minj and Behera, 2012; Nandy and Venkatesh, 2010; Lee et al., 2009).

In addition, a comparative study between heterogeneous and homogeneous systems was carried out in order to examine the sensor response against a freshly prepared milk sample at 37 °C. The latter promotes bacterial growth in the milk sample, which leads to a decrease in the MB content. A closed container was used

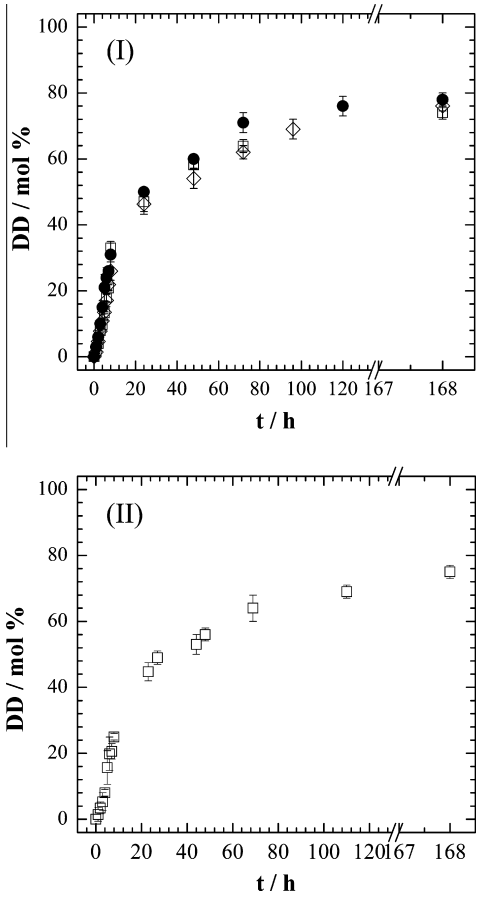


Fig. 6. Reduction kinetics of MB in a freshly milk sample for A (square), B (sphere) and the heterogeneous sensor C (diamond) determined at 37 °C by UV–vis spectrophotometry (I), and also the homogeneous system (II).

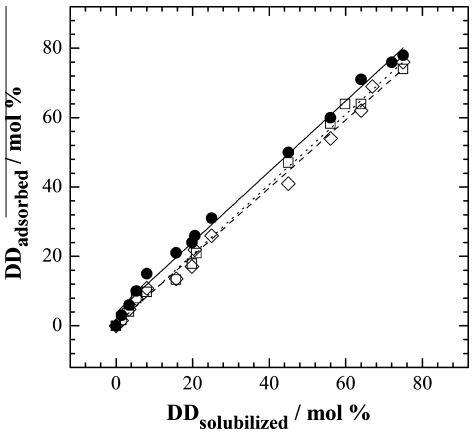


Fig. 7. Correlation between DD values found for A (square), B (sphere) and sensor C (diamond) against those data obtained for methylene blue solubilized in a freshly prepared sample of milk.

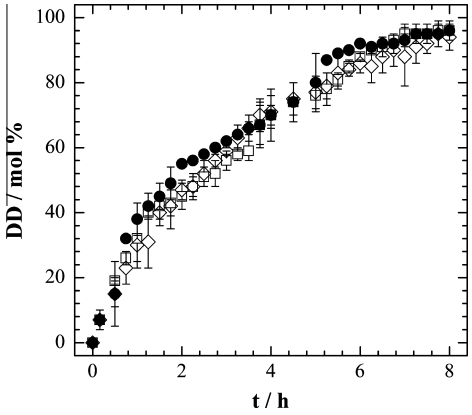


Fig. 8. Reaction kinetics of MB exhibited by A (square), B (sphere) and sensor C (diamond), taking place in a degraded milk sample at 37 °C.

to avoid entry of oxygen into reaction system, which is able to oxidize leucobase and generate MB again (Scheme 1). Fig. 6. I shows DD evolution as a sensor response for the chemical modification by degradation of a milk sample freshly prepared versus time. The performance of DD described by sensors A, B and C agrees with that of sample Class I, which frames this milk as a sample in excellent state (Table 1). A 50% discoloration is reached after 24 h, corroborating the excellent state of the milk sample prepared freshly (Fig. 6). (I). In addition, theses sensors illustrate DD curves with a similar performance, where the DD value increases together with the contact time. This behaviour is also found for the homogeneous system, where methylene blue is solubilized in the milk sample (Fig. 6). (II). This phenomenon exhibits the fact that the growth of the bacterial population is the slow step that determines the rate of MB discoloration. Therefore, these sensors show no differences in the diffusion of reducing agents to the dye deposited on the film, although the latter is present at different levels of the outer layer (Scheme 5). In addition, when collecting data of discoloration kinetics of MB adsorbed to modified films are contrasted with those values obtained for the dye solubilized in milk a direct correlation is attained. Fig. 7 exhibits linear curves corresponding to three sensors, where the average value of slope is close to one and an intercept near zero. This behaviour supports the fact that MB discoloration depends mainly on the formation of reducing substances in milk, process that controls the overall reaction rate.

On the other hand, a similar study was carried out on a degraded sample of milk since its cold chain was interrupted during 24 h at room temperature after preparation. Fig. 8 shows that a 50% of DD is attained after 2.5 h, and a 100% of discoloration is reached after 8 h of reaction for three sensors. This behaviour agrees with that found for sample Class III–IV for human consumption, which supports the fact that the quality of this milk sample can be classified between fair and bad state (Table 1). Exposing milk sample at room temperature for 24 h leads to degradation by increase in bacterial load. In the milk sample this process also generates a decrease in oxygen level and a higher amount of reducing substance, which increases the discoloration rate of MB.

Therefore, this kind of sensor reveals an adequate response time to the system chemical variation, which allows monitoring reduction kinetics of dye as an indirect measure of the population growth of microorganisms in the milk sample.

Usually, bacterial cultures grow exponentially where its growth rate describes the change in the number of cells per minute (Hall et al., 2014). But the instantaneous change is a function of the number of cells that are present at any given moment

$$\frac{dN}{dt} = \alpha N \tag{5}$$

where N is the number of cells at time t and α is the first-order growth rate constant. When Eq. (5) is integrated from $t = 0$ to $t = t$, the relationship is

$$\ln\frac{N_t}{N_0} = \alpha(t - t_0) \tag{6}$$

In practice, α can be estimated from the plot of $\ln Abs_t/Abs_0$ versus t, when the discoloration reaction of MB takes place in the milk sample (Nandy and Venkatesh, 2010). Venkatesh et al. demonstrated that α is related to the discoloration rate constant (k_d) by a factor f whose value is near one ($\alpha = f{\cdot}k_d$).

Table 2 shows α values corresponding to different sensors against samples of freshly prepared milk. Here, a value of α close to 3×10^{-2} h^{-1} was found for sensor A, B and C, and also in the milk sample with MB solubilized. This behaviour supports the fact that the overall reaction rate depends mainly on the formation of reducing substances in milk. In addition, the growth rate constant obtained from sensors for a degraded milk sample was an average value close to 3×10^{-1} h^{-1}, ten times higher than that value found for a freshly prepared milk. All these results demonstrate the ability of MB immobilized to the film as a successful colorimetric sensor of the state of milk decomposition, which may be used for building a smart packaging.

Table 2
Comparative parameters corresponding to synthesized sensors.

Sensor	Location of the dye on the surface layer[a]	AD/wt.%	I/wt.%	α/h^{-1}	
				Freshly prepared milk[b]	Degraded milk sample
A	PP-PAA–MB–Cs	3.8 ± 0.1	2.3 ± 0.1	$(3.1 \pm 0.1) \times 10^{-2}$	$(3.3 \pm 0.2) \times 10^{-1}$
B	PP-PAA–Cs–MB	1.5 ± 0.2	3.5 ± 0.2	$(3.5 \pm 0.2) \times 10^{-2}$	$(3.4 \pm 0.3) \times 10^{-1}$
C	PP-PAA–Cs/MB	0.31 ± 0.04	4.0 ± 0.2	$(3.0 \pm 0.1) \times 10^{-2}$	$(2.9 \pm 0.1) \times 10^{-1}$

[a] The attaching of MB took place onto PAA-grafted films containing a 18 wt.% of G.
[b] The growth rate constant (α) observed for the homogenous system was $(3.2 \pm 0.3)\ 10^{-2}$ h^{-1}.

4. Conclusion

Methylene blue, a thiazidic dye, was immobilized on a PAA-modified PP film and then used as an indicator of the preservation state of liquid milk samples. These sensors with potential application as a smart packaging were built using three techniques, where MB was adsorbed to PP modified into different levels of PAA–Cs layers. It is found that the adsorption degree of MB as well as Cs onto PAA-modified film depends on the carboxyl group content, due to the fact that both reagents exhibit electrostatic interaction with PAA. This bond was stable enough at 25 °C against MB desorption in a 0.05 M buffer phosphate solution pH 7 for at least 30 h. Clearly, the binding capacity of Cs helped to keep MB attached to the film surface. For a limited content of oxygen, the dye exhibits redox activity, despite being adsorbed on film surface as a response to chemical changes in milk sample, where MB becomes from blue to colourless. Dye reduction kinetics allowed indirectly monitoring the population growth of microorganisms present in milk; afterwards, its state of decomposition could be ranked. In addition, the discoloration rate of MB shows that the sensor exhibits a fast response enough against samples of liquid milk in different spoilage states at 37 °C. This behaviour is related to the fact that MB discoloration mainly depends on the formation of reducing substances in milk, process that controls the overall reaction rate. Therefore, film sensors reveal an adequate response time to system chemical variation, where its colour change allows classifying the state of milk preservation. All these results demonstrate the feasibility of the MB-attached film as a successful sensor device to determine the preservation quality of liquid milk. In addition, despite MB having been adsorbed in a layer deeper in the device A, its performance as an indicator has been similar to that found for sensors B and C, where the former showed no significant lag time. This phenomenon is justified due to the fact that the diffusion of reducing substances near MB is faster than their formation rate. This hypothesis is reasonable because the rate of MB decomposition depends on the concentration of the reducing substance generated during the growth of microorganisms in the system. Finally, sensor A (*G* 18 wt.%) seams to exhibit better properties than those found in other devices since MB is first attached to PAA-modified film. This dye has a greater supply of carboxyl groups, resulting in a higher loaded MB. Furthermore, it is expected that the sensor A shows the highest stability since the dye placed in the most inner layer and is held more strongly by the mesh formed by the interaction between PAA and Cs chains.

Taking into account these results obtained in this study, we are able to propose for a future work the development of a smart packaging containing the milk spoilage indicator in order to analyze its performance in a real system. A small window of the sensor on the inner face of the packaging film will determine the level of degradation milk. Based on the fact that the device is into a closed system with a constant oxygen level, the degree of milk preservation may be checked by the customer from colour change shown by the sensor. Reference colours will be arranged around the sensor window in the outer packaging in order to indicate to the customer the spoilage milk state.

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