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Biodegradation of three-layer laminate films based on gelatin under indoor soil conditions

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ABSTRACT

Three-layer gelatin films, composed of sodium montmorillonite (MMt) – plasticized gelatin (Ge-5MMt) (inner layer) and dialdehyde starch (DAS) – cross-linked and plasticized gelatin films (Ge-10DAS) (outer layers), obtained by heat-compression molding, were submitted to degradation under indoor soil burial conditions for 14 days. Biodegradation of multilayer film as well as individual components and control gelatin films was evaluated by monitoring water absorption and weight loss. It was established that technological treatments performed on gelatin, such as cross-linking, compounding with clay and heat-compression molding have a major impact on the biodegradation rate and extent. The possible reasons are discussed. Weight loss results revealed that the susceptibility to microbial attack during soil burial varied in the order: Ge-10DAS < multilayer < Ge-5MMt < gelatin control film. The intermediate behaviour of the multilayer was associated with the presence of hydrogen-bonding interactions between layers, induced by processing. Scanning electron microscopy revealed that the multilayer was preferentially biodegraded by filamentous microorganisms and even larvae in the later stages of the process. The presence of holes and pits on the multilayer surface was more likely attributed to the preferential removal of glycerol and DAS as shown by thermogravimetric analysis.

1. Introduction

In recent years biopolymers have been seen as potential environmentally friendly and sustainable alternatives to petroleum-based plastics, particularly in those applications where biodegradability and derivatisation of natural resources give added value [1]. Gelatin is an edible, biodegradable and renewable biopolymer produced from the partial denaturation of collagen, a by-product of meat processing [2] or from the pharmaceutical industry [3], with film-forming properties appropriate for the production of bio-packaging materials [4]. Like most protein-based materials, gelatin films show excellent oxygen and aroma barrier properties at low to intermediate relative humidity when compared with films derived from conventional polymers, as well as fairly good mechanical properties [4,5]. However, these films tend to be brittle and moisture sensitive [6]. At high relative humidity gelatin films swell due to their hydrophilic nature, lose their dimensional stability and decrease their mechanical and barrier properties, limiting their application in direct contact with foodstuffs with high water content [7,8]. These disadvantages have limited the industrial applications of gelatin-based films [9].

Several attempts have been undertaken to overcome these limitations including blending [10–12], cross-linking [2,9,13–15], compounding with natural fibers [3] or with nanosized clay dispersed in the biopolymer matrix [16–18]. Another approach to solve this problem is creating synergies by putting together films with specific properties or from different sources into multilayer flexible films [8,9,18–21]. In most applications the outer layers are made of water barrier polymers with good mechanical properties meanwhile the inner layers usually consist of materials which offer good gas-barrier properties [21]. Unfortunately, despite many advantages of such materials most of the commercially available multilayers or laminate films currently on the market are based on non-biodegradable polymers [19–21].

Our current work is focused on developing new biodegradable multilayer films based on modified-gelatin layers with no adhesives in their formulations and produced by compression molding. The three-layer film is composed of two outer moisture and mechanical resistant layers made of dialdehyde starch – cross-linked gelatin film plasticized with 30 wt.% glycerol (Ge-10DAS). DAS was selected as non-toxic [23], biodegradable and efficient cross-linking reagent of gelatin according to our previous results [15] and those reported for other proteinaceous materials [24–26].

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The inner layer was obtained by blending non-toxic sodium montmorillonite (MMt) with glycerol-plasticized gelatin in order to produce exfoliated bio-nanocomposite films with enhanced mechanical and vapour and oxygen barrier properties [17,18,22]. The multilayer film produced showed better moisture resistance and oxygen barrier properties than individual components and quite good mechanical properties [22]. However it is important to be sure that modifications and lamination do not compromise the inherent biodegradability of gelatin.

Research data on the biodegradability of gelatin-based materials, dialdehyde starch and bio-nanocomposites have been reported [2,9,13,15,27–30]. Gelatin is susceptible to the action of enzymes (proteases) which are present in a variety of microorganisms [27,28]. Some studies highlighted the effect of chemical modifications on the rate and extension of gelatin biodegradation. It was found that blends of gelatin with phenolic resins enhanced gelatin stability against microbial attack in soil mainly due to the biocide activity of phenol [29]. Biodegradation of chemically modified-gelatin films in different environments such as lake and river waters [2] and soil [13,15] under laboratory conditions was also analyzed. Results revealed that the rate and extension of biodegradation depended on the type of cross-linking reagent and on the cross-linking density. Despite numerous studies on gelatin, there are few reports on laminate films based on gelatin. Apostolov et al. [9] developed laminates based on cross-linked or un-cross-linked gelatin reinforced with linen and silk fabrics with enhanced mechanical and impact properties than neat gelatin. These materials suffered enzymatic degradation similarly to gelatin or gelatin-based materials [30]. On the other hand, the effect of clay on the biodegradation in soil of bio-nanocomposites based on gelatin has been poorly explored and it is also the object of the present study.

The main objective of this work was to evaluate the biodegradability of the novel gelatin-based multilayer films exposed to natural mixed microflora present in soil during indoor burial experiments. Water absorption (WA%) and weight loss (WL%) during soil burial were evaluated gravimetrically. Qualitative changes in composition with exposure time were analyzed by thermogravimetry (TG) and morphological characterization of the biodegraded specimens was carried out by scanning electron microscopy (SEM).

2. Experimental

2.1. Materials

Bovine hide gelatin (Ge) type B was kindly supplied by Rousselot (Argentina), Bloom 150, isoionic point (I_p) 5.3. Sodium montmorillonite (MMt) nanoclay was purchased from Southern Clay Products Inc. (Texas, USA); under the trade name Cloisite Na^+. The cation-exchange capacity (CEC) was 92.6 meq/100 mg clay and the interlayer distance was 1.2 nm (as it was determined by X-ray diffraction on the dry powder) [31]. Dialdehyde starch (DAS, 81.8% oxidation degree) was supplied by Sigma–Aldrich (St. Louis, MO, USA) and used as received. Glycerol analytical grade (Gly, 98%) was purchased from DEM Chemicals (Mar del Plata, Argentina).

2.2. Methods

2.2.1. Preparation of multilayer films by compression molding

Multilayer films were prepared by combining the cross-linked gelatin films as the outer layers and nanocomposite film as the inner layer. Multilayer films were obtained through a two-step process. In the first step, the individual layers were obtained separately. Subsequently, they were stacked together and heat-compressed.

Chemical cross-linking combined with plasticising was used to produce gelatin-based films with improved water resistance and fairly good mechanical properties as reported elsewhere [15,22]. Basically, plasticized and cross-linked gelatin films were obtained by adding 10 wt.% of DAS as cross-linking agent and glycerol (30 wt.% on dry gelatin basis) as plasticiser. The Ge/DAS/Gly blend was compression-moulded at 120 °C under a pressure of 50 kg/cm^2 ("EMS", San Justo, Argentina), applied for 10 min followed by a cooling step until room temperature. The brownish colour of the films was attributed to the formation of conjugated Schiff's bases between gelatin and aldehyde functionality of DAS which are intermediate products of the Maillard reaction [15,25]. The obtained films were named Ge-10DAS. Un-cross-linked and plasticized gelatin control films were produced in similar conditions and were named Ge.

Bio-nanocomposite films were obtained by the solution-intercalation method according to the procedure reported elsewhere [16–18,22]. Pure gelatin powder was hydrated in distilled water in 1:10 by weight ratio at 50 °C with 30 wt.% of glycerol and under constant stirring until it was completely dissolved. In parallel, unmodified montmorillonite (5 wt.% on dry gelatin basis) was dispersed in distilled water and pre-treated ultrasonically at 50 °C for 20 min. Before mixing, the pH of gelatin solution was adjusted to 7 (higher than the $I_p = 5.3$) with NaOH 0.1 M. Subsequently, aqueous gelatin solution was added drop-wise into ultrasonically pre-treated clay suspension and mixed together at 50 °C under vigorous stirring for 15 min. The resulting aqueous suspensions were then cast in Teflon-coated moulds and dried at 50 °C in a convection oven. Films were transparent and light yellow due to MMt. These samples were labelled as Ge-5MMt.

Multilayer films were prepared by stacking together an outer Ge-10DAS layer, a Ge-5MMt inner layer and a second outer Ge-10DAS layer followed by heat compression. Processing conditions were 50 kg/cm^2 applied for 10 min at 70 °C with a further cooling step at room temperature and pressure [21]. The obtained multilayer films were transparent and light brown and they were stored under controlled temperature and humidity conditions prior to further tests (65 ± 2% HR; 25 ± 2 °C).

2.2.2. Film characterization

Film thickness was measured by using a micrometer (Vernier, China). The average thickness value was 0.60 ± 0.06 mm. Before testing, films were vacuum dried at 25 °C until constant weight. Fracture surfaces were observed on a Phillips 505 microscope (Eindhoven, Netherlands) with an operating voltage of 15 kV. All specimens were previously sputter-coated with gold. Thermogravimetric measurements were performed in a Shimadzu (Shimadzu Corp., Japan) thermogravimetric analyzer. Dynamic tests were carried out from ambient temperature up to 900 °C at a heating rate of 10 °C/min and under nitrogen atmosphere in order to avoid thermo-oxidative reactions (gas flow rate 15 mL/min).

2.2.3. Indoor soil degradation

Experiments were carried out in a series of plastic boxes (80 × 15 × 10 cm^3), each containing 5300 g (dry basis) of characterized soil (Pinocha type = typical Argentinean Argiudol + pine litter). This soil showed pH 6.1 (H_2O), organic matter content (OMC), 7.2%; total nitrogen, 0.19%; NO_3^-, 18 ppm; P, 10.5 ppm; Ca, 12.5 meq/100 g; Mg, 2.1 meq/100 g; Na, 0.3 meq/100 g; K, 1.5 meq/100 g [22]. Samples were cut into rectangular shape (2 × 3 cm^2) and then dried until constant weight in an oven to remove the moisture before testing (m_0). Specimens were put into cups made of an aluminium mesh to permit the access of microorganisms and moisture and the easy retrieval of the degraded samples. The specimens into the holders were buried at 8 cm depth from the soil

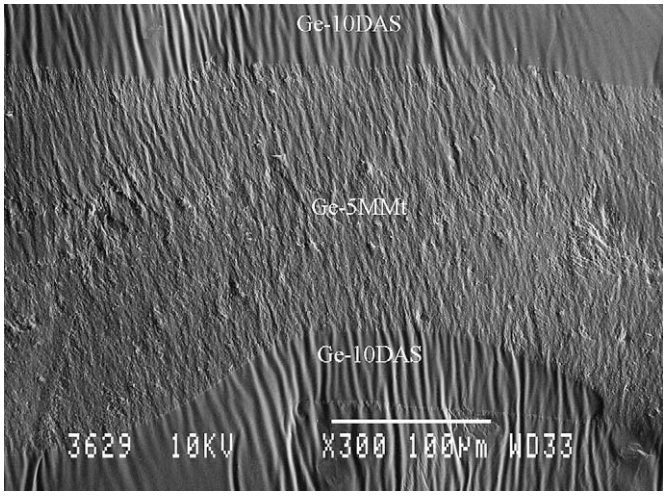

Fig. 1. Scanning electron micrograph (SEM) of multilayer film (magnification: 300×).

surface in order to ensure aerobic conditions of degradation. The relative humidity was kept around 40% by adding water periodically and the temperature was 20 ± 2 °C. Soil moisture fluctuation was followed gravimetrically by using the oven drying method (ASTM D2216).

Water sorption during soil burial was determined gravimetrically. Samples were removed from the soil at specific intervals (t), carefully cleansed with distilled water, superficially dried with a tissue paper and weighed (m_h). Water uptake (%WS) was quantified by the following equation:

$$\%WS = \frac{m_h - m_t}{m_0} \times 100 \quad (1)$$

where m_0 and m_t are the initial and the residual mass at time = t, respectively, and m_h is the humid mass of the specimens after wiping with a tissue paper. The reported values are the average of two measurements.

After water sorption determination, samples were dried under vacuum and at room temperature to constant weight. The specimens were weighed on an analytical balance in order to determine the average weight loss (%WL):

$$\%WL = \frac{m_t - m_0}{m_0} \times 100 \quad (2)$$

where m_0 is the initial mass, m_t is the remaining mass (after drying) after an incubation time, t. All results are the average of two replicates.

3. Results and discussion

3.1. Visual aspect and microstructure of the multilayer film

An SEM image of the cross-section of the multilayer film is shown in Fig. 1. The outer Ge-10DAS layers were found to be tightly bonded to the inner one and no delamination was observed, confirming the high compatibility between them. This was mainly attributed to the formation of hydrogen bonds between layers due to the large amounts of amide groups (from gelatin) in all layers [9]. The presence of such interactions does not only affect physical and mechanical properties of the film but may also influence its biodegradability.

3.2. Water absorption and weight loss of multilayer, Ge-5MMt and Ge-10DAS films buried in soil

Soil microflora constitute a mixed microbial population (including bacteria, actinomycetes, fungi and protozoa, among others) which may act synergistically during degradation and may also reproduce naturally occurring conditions. In addition, microorganisms in the soil produce many types of enzymes that are capable of reacting with exposed protein surfaces [27]. The experiment was carried out up to 14 days. However beyond the 10th day, samples could not be easily recovered due to their macroscopic deterioration.

Pictures of the recovered samples of the control, individual components and multilayer films before and after 10 days of exposure to soil burial are shown in Fig. 2. Macroscopic examination revealed that Ge control film was the most sensitive material to the biodegrading medium meanwhile Ge-10DAS was the most resistant to microbial attack. This can be attributed to the hindering effect of the chemical network on the enzymatic degradation of the gelatin matrix [13,15]. It was also visible that during soil burial all materials absorbed water, losing their initial shape. Particularly, Ge-10DAS and the multilayer flim suffered discolouration due to the hydrolysis of Schiff's bases initially present in both materials. From the 10th incubation day all the materials tested lost their structural integrity. Therefore, from the practical point of view, they might be classified as quite rapidly degradable materials [13].

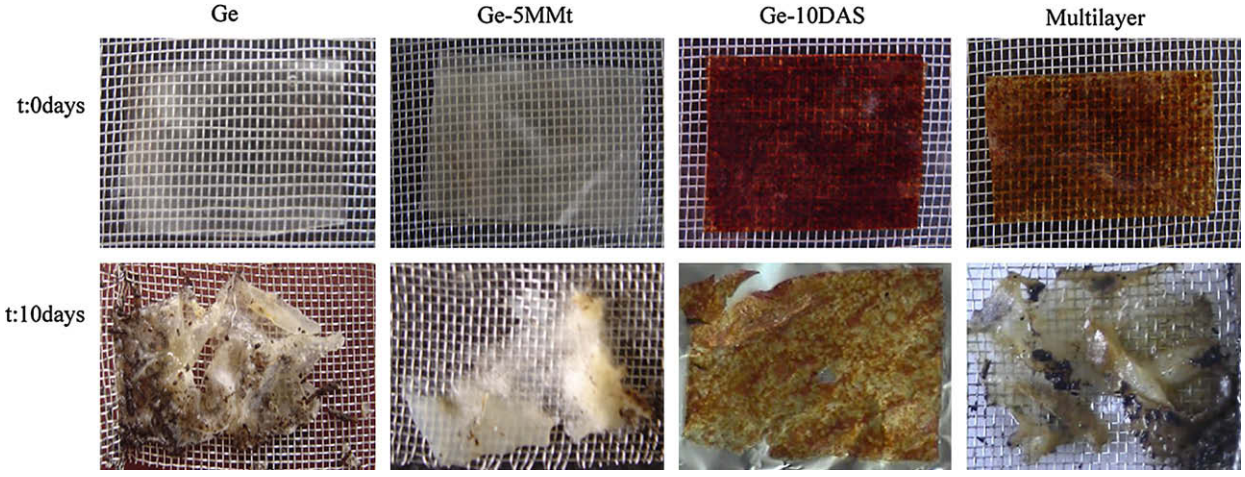

Fig. 2. Macroscopic aspect of the individual and multilayer films prior and after 10 days of incubation in soil.

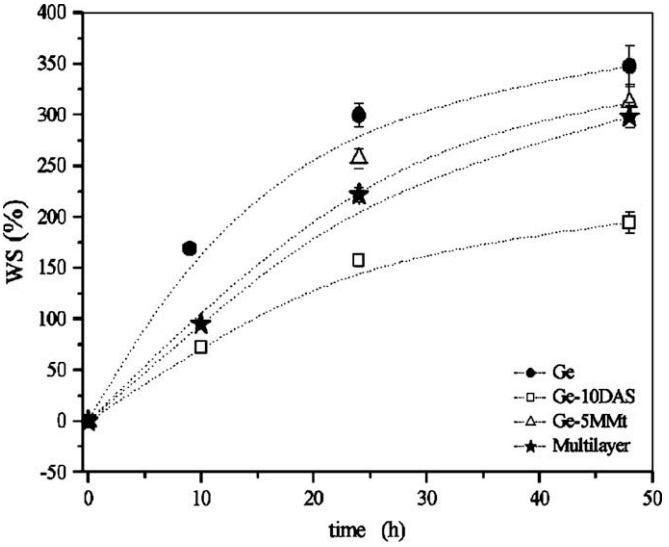

Fig. 3. Water absorption of control gelatin (•) Ge, (□) Ge-10DAS, (△) Ge-5MMt and (★) multilayer film.

Fig. 3 shows the results of water absorption of control, multilayer, Ge-5MMt and Ge-10DAS films during the first two days of soil burial. Estimation at longer times is difficult and could lead to wrong results because gravimetrical measurement is prone to errors due to the loss of degradation products [32–34]. All materials absorbed enough water during the first two days to ensure the water bio-availability which favour the microbial attack and the beginning of the hydrolysis of the matrix components. Results in Fig. 3 clearly indicate the different water absorption capacity of the investigated materials. Control gelatin film displayed the highest initial absorption rate while Ge-10DAS film showed the lowest one. This result is in accordance to the restricted water binding ability due to cross-linking induced by DAS and agrees well with the water absorption behaviour previously reported for different proteinaceous films cross-linked with DAS [15,24–26]. For Ge-5MMt film the rate of water absorption was faster than that of the cross-linked counterpart due to the hydrophilic character of Na^{+}MMt [35]. This observation shows that the tortuous path offered by silicate layers and the physical interactions between gelatin, glycerol and clay

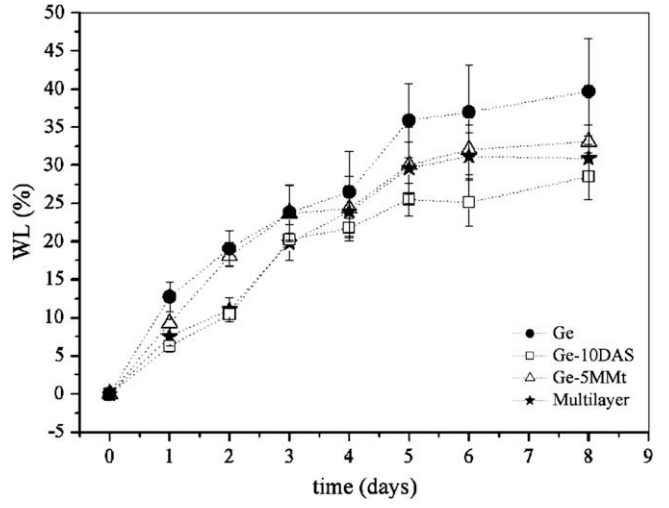

Fig. 4. Relative weight loss curves as a function of the exposure time in soil burial for control (•) Ge, (□) Ge-10DAS, (△) Ge-5MMt and (★) multilayer films.

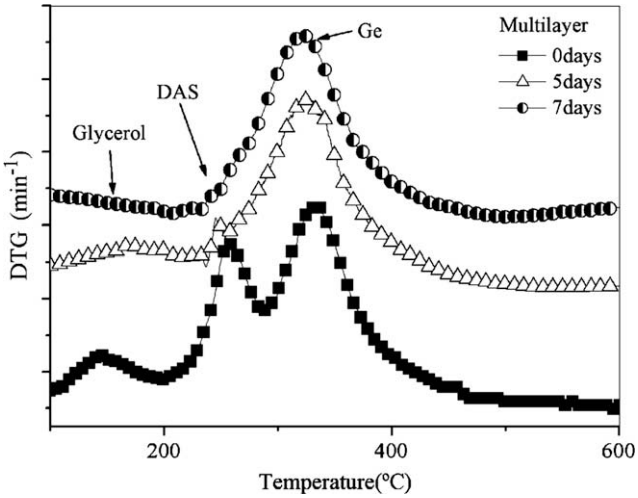

Fig. 5. DTG of the normalized weight loss curves of multilayer film prior and after different exposure times to soil burial.

[22] are less effective in reducing gelatin water affinity than is cross-linking [36,37]. The multilayer film exhibited an intermediate behaviour between those of its individual components (Fig. 3). Most of the absorbed water probably entered from the cut edges of the multilayer film specimen due to the absence of a protective layer of Ge-10DAS when film was cut for soil burial experiments. Similar results were reported by Rhim et al. [20], for three-layer laminates based on poly(lactic acid) (PLA) as outer layers and soybean protein isolate as the inner one. The slightly lower water absorption of the multilayer compared to that of Ge-5MMt film could be attributed to the fact that water molecules have to break hydrogen bonds between layers induced during processing to reach the more hydrophilic Ge-5MMt inner layer.

Weight loss data as a function of the exposure time to soil burial of control, individual and multilayer specimens are shown in Fig. 4. The average values representing the weight losses of the films buried in soil were probably underestimated as soil debris and occluded biomass was difficult to remove without damaging the samples [32–34]. Despite these potential problems, weight loss data were used herein only to qualitatively analyze the effect of microbial attack on the gelatin-based materials under study. During the first day of soil burial all samples lost between 5 and 9% of their initial weight due to the loss of low molecular weight compounds [38]. These could have been mainly glycerol and oligomers leaching out of the polymer as was previously observed on gelatin–glycerol films [15], starch-based polymers and their composites with sorbitol [33,34] and soy protein–glycerol polymers [39]. Glycerol would eventually be adsorbed by soil or passed through the cell membrane, being metabolized by microbes. The relative biodegradation rates (see the slope on each curve in Fig. 4) observed for multilayer and individual films were slower than those of the control gelatin film, giving some evidence on the dependence between weight loss and modifications performed on gelatin.

After 10 days incubation, the maximum WL showed the following tendency Ge-10DAS < multilayer < Ge-5MMt < control film. These results are more likely related to the water absorption capacity of the different films since biodegradation depends on transporting water from the surface to the bulk of the film [13,38]. Ge-5MMt film degraded faster and suffered higher weight loss than Ge-10DAS, showing that the formation of a network could have a greater effect on prolonging biodegradation of gelatin films than the addition of MMt. Similar behaviour was already reported for other MMt-containing biodegradable polymers such as methyl

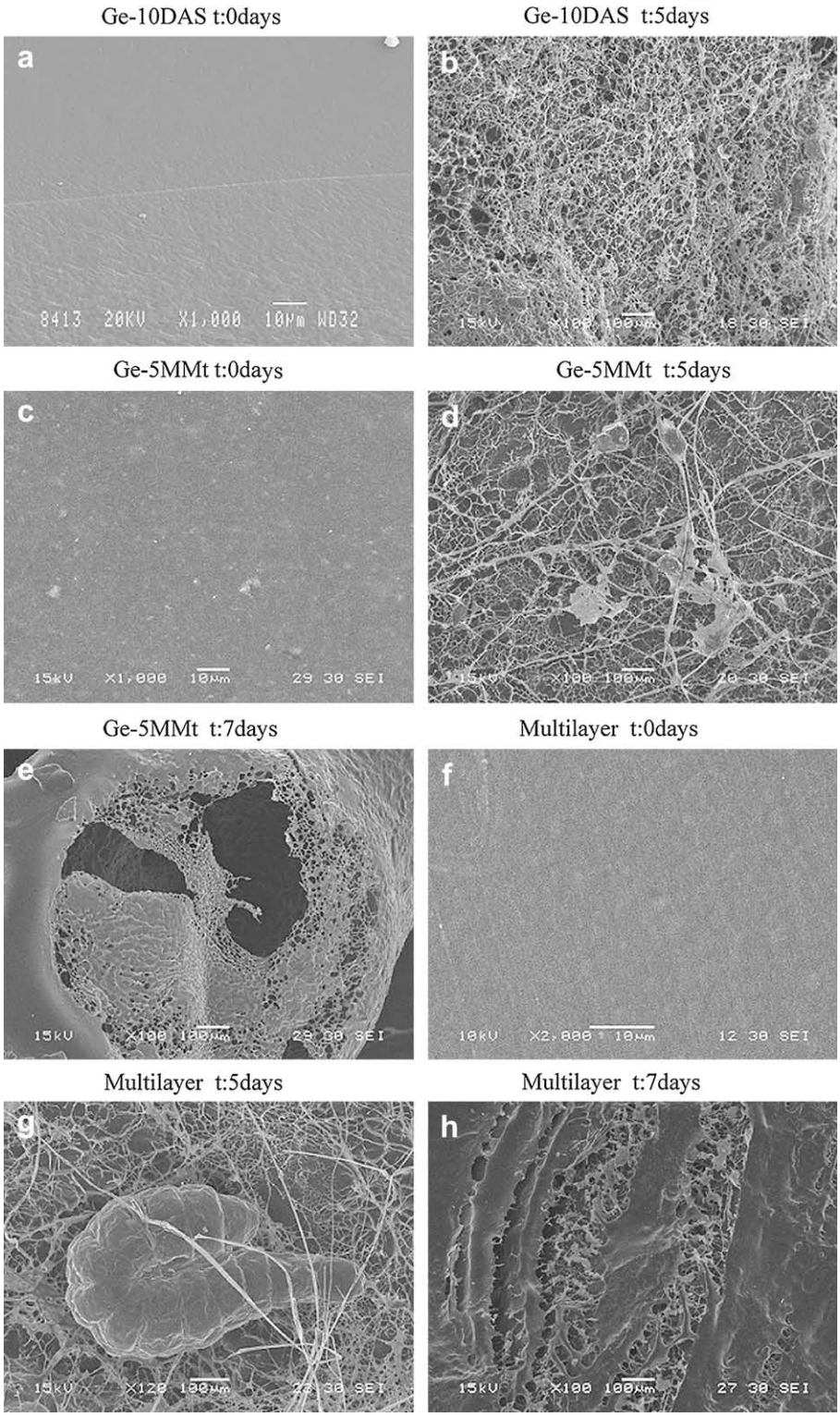

Fig. 6. SEM micrographs of the outer surfaces of the studied materials after and before different exposure times to soil burial. (a) Ge-10DAS: prior exposure and after 7 days; (b) Ge-5MMt: before, 5 days and 7 days of exposure; (c) multilayer: prior, 5 days and 7 days of incubation.

cellulose (MC) [36] and aliphatic polyesters [37]. The WL curve of the multilayer practically superimposed that of the Ge-10DAS film during the first 3–4 days of incubation (Fig. 4). This observation suggests that the most bio-susceptible material should be the component present in both layers, i.e. gelatinized starch from DAS. After 4 days of incubation, the rate of weight loss increased again and the curve of the multilayer film superimposed that of Ge-5MMt film. It was assumed that during this latter phase, delamination may probably take place promoting the microbial access to the inner Ge-5MMt layer where MMt may offer a surface prompting the microbial attack [38], and consequently the biodegradation.

3.3. Thermogravimetric analysis

Fig. 5 shows the differential degradation curves (DTG) of the normalized weight loss curves for multilayer film prior and after different times of exposure to soil burial. The decomposition peaks have been resolved into the different components which can be assigned to the volatilization of glycerol, gelatin and DAS based on the DTG thermograms of the neat components. Before biodegradation tests, the multilayer film showed a first peak in the range 120–150 °C, which was attributed to the loss of low molecular weight compounds, such as water, glycerol and oligomers [15,40]; a second one with a maximum degradation rate (T_{max}) at 260 °C associated to the decomposition of the polymeric chain of DAS [15,41], and a third peak centred at 332 °C assigned to the thermal breakage of peptide bonds in the main chain of gelatin [17,40]. The peak corresponding to montmorillonite ($T_{max\ MMt} = 634$ °C [17]) was not visible within the range of temperatures analyzed. After 5 days, it was verified that the peak assigned to glycerol and low molecular weight compounds decreased practically to zero. As degradation proceeded, the intensity of the DAS peak diminished significantly being almost zero at the 7th day. This observation confirms the preferential consumption of the polymeric chain of DAS by microorganisms. The reduction in DAS was accompanied by a widening and slight decrease in intensity of the gelatin peak, which could be associated with a broadening in the molecular weight distributions of gelatin. Water and microbial attack produce the scission of gelatin chains, but the fragments are not small enough to diffuse from the material into the surrounding environment. Additionally, the T_{max} values associated with the decomposition of gelatin suffered a slight shift toward lower temperatures as incubation time increased (i.e. from about 332 °C to 324 °C after 7 days of incubation), implying that gelatin might be degraded under soil burial conditions.

3.4. Microscopic analysis

SEM micrographs of the outer surfaces of Ge-10DAS, Ge-5MMt and multilayer films after different exposure times to soil burial are shown in Fig. 6.

As can be seen in Fig. 6a, prior to soil burial ($t = 0$ days) Ge-10DAS film showed a relatively smooth surface with some irregularities and scratches associated with processing operations. By the 5th day of incubation (Fig. 6b), Ge-10DAS film displayed higher porosity, holes and cracks with random shape characteristics of polymeric materials degraded in soil [42] and a reduced colonization by filamentous microorganisms was developed on the film surface. The increment in porosity could be attributable to the diffusion of water-soluble compounds (glycerol, gelatinized starch from DAS). Even if starch and gelatin are degraded by either bacterial and fungal strains [28,43], the presence of filaments of microorganisms on the film surface agrees well with the fact that fungi are ubiquitous and extremely effective in their biodegradation effect at different moisture regimes [44] and extreme temperatures [28]. The apparent absence of larvae or insects on the Ge-10DAS surface could be due to the fact that organisms higher in the food chain require moisture levels higher than 60% [44].

Fig. 6c–e shows the sequence of biodegradation for Ge-5MMt. Before exposing to the degrading medium (Fig. 6c) the surface of Ge-5MMt displayed some heterogeneities due to the solvent evaporation as a consequence of the casting process. Beyond the 5th day of incubation (Fig. 6d), the material showed an irregular surface with channels as a consequence of the leaching of water-soluble compounds produced by the microbial metabolism. Ge-5MMt surface showed a significant microbial activity, showing that MMt indirectly promotes the biodegradation. As biodegradation proceeded (7th day, Fig. 6e) the microorganisms became very active and even the presence of larvae was evident. Furthermore, the different filament sizes seem to suggest that fungi and actinomycetes were prevalent at the early stages of biodegradation [43], since the presence of bacterial cells was not observed.

The analysis of Fig. 6f indicates that the multilayer film's surface was smooth before exposing to soil burial. On the 5th day (Fig. 6g), filaments not only invaded the multilayer surface but also penetrated into the bulk and the presence of larvae between layers was observed. This could be an indirect evidence that at later stages delamination may enhance moisture uptake and consequently, microbial attack. Beyond this time, the film surface appeared highly eroded and completely covered by filamentous microorganisms (Fig. 6h). These observations may suggest that this material could be bio-assimilated by mixed soil consortia which may act in a synergetic way promoting the degradation.

4. Conclusions

Indoor soil burial testing demonstrated that the three-layer laminate film based on modified-gelatin individual layers was more resistant to attack by microorganisms than was the gelatin control film, but it still could be classified as a rapidly degradable material.

The susceptibility to microbial attack seems to depend on the modifications performed on gelatin which in turn determine the water uptake of the resulting materials during soil burial. Moisture absorption capacity of the different materials plays a key role in the vulnerability of the specimens to soil microflora. Both, cross-linking and the compounding with un-modified clay can delay the overall extent of gelatin biodegradation. In comparison, cross-linking with DAS had higher effect in depressing biodegradation due to the restriction in water and/or microorganism diffusion through the bulk imposed by cross-linking. The multilayer film exhibited behaviour intermediate between those of Ge-10DAS and Ge-5MMt individual layers. The main reason is that water molecules have to break hydrogen-bond interactions between layers to eventually reach the bulk of the film where the presence of MMt favours microbial attachment and growth.

Results reported here indicate that the association of gelatin-based layers subjected to different technological treatments such as cross-linking, compounding with clay and heat-compression molding can provide a new multilayer material with modulated biodegradability under soil burial conditions. In addition, if environmentally safe reagents such as DAS, glycerol and Na^{+}MMt are used to modify gelatin, the degradation products will not have any eco-toxicological effect, and therefore it is anticipated that gelatin-multilayer films could be potentially degradable in natural environments or landfills.

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