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Encapsulation of Lysozyme into halloysite nanotubes and dispersion in PLA: Structural and physical properties and controlled release analysis

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ABSTRACT

Novel biodegradable composites were prepared using PLA and a nano-hybrid composed of halloysite nanotubes (HNTs) filled with Lysozyme, as antimicrobial. The nano-hybrid was characterized from a structural point of view and the Lysozyme content evaluated by thermogravimetric analysis. Several composites were prepared using ball milling, in dry conditions at ambient temperature, varying the nano-hybrid loading (i.e. 3, 5, 10 wt%). The structural organization and physical properties (thermal, mechanical and barrier to water vapor) ware analyzed and correlated to the nano-hybrid content. Controlled Lysozyme release into saline solution was followed using UV spectrophotometry. The release kinetics was found to be dependent on HNTs-Lysozyme relative fractions. The experimental results were analyzed by a modified Gallagher-Corrigan model.

1. Introduction

The possibility to modulate the release of active molecules from a packaging material represents a fundamental issue to ensure continuous replenishment of functional molecules and inhibit bacterial growth to extend product shelf life [1]. The traditional methodologies in which active molecules are directly mixed to the food formulation result in a fast activity loss and overloading of active compounds [2,3]. On the opposite, packaging with controlled release allows the desired level of food protection using smaller amounts of active molecules. The active molecules, incorporated into the packaging, can be released at rates that ensure a sufficient amount on the packaged food surface at any time both during storage [4,5]. Lysozyme is one of the most commonly used natural proteins with antimicrobial activity. It has a great potential in antimicrobial packaging due to its stability over a wide range of temperature and pH values [6–8]. European Union (E 1105) classifies Lysozyme as molecule with bacteriostatic, bacteriolytic, and bactericidal activity, and Food and Drug Administration (FDA) considers such protein as GRAS (Generally Recognized As Safe) substance. Its chemical structure is characterized by single polypeptide chain. The antimicrobial activity is related to the ability to hydrolyze the beta 1–4 glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid. Such bonds are present in peptidoglycans, which comprise 90% of the cell wall of Gram-positive bacteria, making them very susceptible to Lysozyme antimicrobial activity [9]. Literature reports several methods to immobilize Lysozyme on different supports, i.e. adsorption, entrapment, and surface conjugation [10–13], however its use as antimicrobial agent, covalently bonded, is already still limited. Different strategies have also been utilized to control the release rate of lysozyme; most of these are focused on changing the packaging material's

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morphology, such as polymer concentration [7], additive types and amount [14], plasticizer loading [15], degree of crosslinking [16–18] and number of layers [19]. Other approaches are focused on the variation of the pH of the release medium to control the release of Lysozyme from the films [20,21]. In all cases, the complete release time ranges from some hours up to few days. For food packaging applications, next to controlled release, the sustained antimicrobial efficacy of the packaging is required. In the last decade nanotechnology opened new and interesting opportunities for developing packaging materials with controlled release of active molecules. Very recently a new class of inorganic fillers, halloysite nanotubes (HNTs), attracted considerable interest in this context. They are green materials, cheap, and available in thousands of tons from natural deposits. They belong to the alumosilicate clays with a length of about 1000 nm, an internal diameter (lumen) of 10–15 nm, and external diameter of about 50–80 nm. The general chemical formula of HNTs is $Al_2Si_2O_5(OH)_4{\cdot}nH_2O$, which exhibits a predominant form of hollow tubes, and it is similar to kaolin, but its alumosilicate sheets are rolled into tubes [22–24]. The HNTs external surface is composed of Si-O-Si groups, whereas the internal surface consists of a gibbsite-like array of Al-OH groups. HNTs can be dispersed in polymeric matrices without exfoliation, as required for a good dispersion of platy clays, due to the tubular shape and less abundant -OH groups on the surface. Polymeric materials has been filled with these tubular nano-containers [25–28] that release active molecules (antimicrobial, drugs, essential oils, flame retardant, self-healing, anticorrosion, etc…) in specific environments [29–36]. Very recently halloysite nanotubes were also used for enzyme immobilization, to study the enzymatic activity [37–38] also with respect to peculiar environments for anti-biofouling applications [39]. Poly (lactic acid) (PLA) is currently receiving a great attention for various utilizations such as packaging, textile, biomedical, and more recently, engineering applications, because it has a key position on the market of bio-based and biodegradable polymers [40,41]. In competition with the traditional oil derived polymers, PLA is expected to be one of the most promising candidates for further developments. Therefore, following this huge interest, many R & D works are focused on the realization of new PLA grades with improved chemical and physical properties. In response to the demand for new PLA grades, characterized by improved characteristic features, HNTs were evaluated as potential new nanofiller for such bio-polymer [42–44]. Lysozyme was already incorporated into a PLA matrix [45], by melt extrusion, as free molecule. In this paper, we use halloysite nanotubes as nano-containers for Lysozyme, to be introduced into a PLA matrix. The HNTs-Lysozyme nano-hybrid was incorporated into PLA using high energy ball milling (HEBM) at ambient temperature and with no solvent. Such technique has been demonstrated to be an efficient and green methodology to obtain novel polymeric nanocomposites from polymers and active molecules [46,47]. The composites PLA/HNTs-Lysozyme contained 3%, 5% and 10 wt% of nano-hybrid filler. Films were obtained, and structural characterization and physical and barrier properties to water vapor evaluated. Studies of release of Lysozyme, in saline solution, were conducted and correlated to nano-hybrid loading, Lysozyme amount, and film morphologies. The experimental results were then analyzed by a modified Gallagher-Corrigan model.

2. Experimental

2.1. Materials

PLA used in this work is a Polylactic acid with a D content less then 4%, and supplied from Natureworks (2003D). Molecular weight parameters, from GPC, are: Mw = 107,099; Mn = 162,680; Mw/Mn = 1.52. It is a thermoplastic resin derived from annually renewable resources and is specifically designed for use in fresh food packaging and food service ware applications.

Halloysite nanoclay powders (CAS 1332-58-7) and Lysozyme powders (CAS 12650-88-3) were supplied from Sigma Aldrich (Italy) and used as received.

2.2. Preparation of HNTs-Lysozyme nano-hybrid

Lysozyme (3 g) was first dissolved in water (30 ml) at 50 °C for 20 min. The HNTs (3 g) were mixed with the lysozyme solution. Then, ultrasonic processing was performed for 10 min to make HNTs sufficiently dispersed in the lysozyme solution. The solution was heated at 50 °C for 2 min, then vacuum (0.085 MPa) was applied to remove the air between and within the hollow tubules. The vacuum was maintained for 15 min. The solution was then taken out from the vacuum and shaken for 5 min. Vacuum was re-applied, to remove the trapped air, for 15 min. The lysozyme-loaded HNTs were dried in an oven for 16 h at 50 °C to reach a constant weight. The content of lysozyme (wt%) in the HNT-lysozyme hybrid was calculated, using the TGA analysis, according to following equation:

$$\alpha_3 = w{\cdot}\alpha_1 + (1-w){\cdot}\alpha_2$$

where α_1 is the mass loss of Lysozyme (99.1%) at 413.2 °C; α_2 is the mass loss of HNTs (16.9%) at 457.9 °C; α_3 is the mass loss of Lysozyme/HNTs (58.6%) at 416.0 °C (see TGA Fig. 2). Therefore, the content of lysozyme (w) in Lysozyme/HNTs was estimated to be 50.7% and the HNTs content was 49.3%. The Lysozyme content much exceeds the loading capacity of the halloysite nanotubes. This detected amount is then relative either to the molecules inside the nanotubes, either to the molecules external to the nanotubes, that concur in different ways to the release (see Section 3.5).

2.3. Incorporation of HNTs-Lysozyme into PLA matrix

The incorporation of the nano-hybrids into PLA was achieved by High Energy Ball Milling (HEBM) method. Powder mixtures composed of PLA and nano-hybrid (vacuum dried for 24 h) was milled at 3, 5 and 10 wt%, of filler at room temperature in a Retsch (Germany) centrifugal ball mill (model S 100). The milling process occurred in a cylindrical steel jar of 50 cm^3 with 5 steel balls of