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Molecular interactions, characterization and photoactivity of Chlorophyll *a*/chitosan/2-HP-β-cyclodextrin composite films as functional and active surfaces for ROS production

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ABSTRACT

Novel photosensitizing film based on the natural hybrid polymer Chitosan/2-hydroxy-propyl-β-Cyclodextrin (CH/CD) is synthesized introducing Chlorophyll *a* (CH/CD/Chl*a*) as a photoactive agent for possible application in antimicrobial photodynamic therapy (PDT). The polymer absorbs visible light, in turn able to generate reactive oxygen species (ROS) and, therefore it can be used as environmental friendly and biodegradable polymeric photosensitizer (PS). The modified film is characterized by means of different spectroscopic, calorimetric, diffraction techniques and microscopic imaging methods including time-resolved absorption spectroscopy. UV–Vis, FTIR-ATR and X-ray Photoelectron Spectroscopy (XPS) analyses suggest that Chl*a* shows a strong affinity toward Chitosan introducing interactions with amino groups present on the polymer chains. Nanosecond laser flash photolysis technique provides evidence for the population of the excited triplet state of Chl*a*. Photogeneration of singlet oxygen is demonstrated by both direct detection by using infrared luminescence spectroscopy and chemical methods based on the use of suitable traps. Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) and Differential Scanning Calorimetry (DSC) analyses confirm also the occurrence of structural changes both on the film surface and within the film layer induced by the insertion of the pigment. Moreover, X-ray Diffraction data (XRD) shows the existence of an amorphous phase for the chitosan films in all the compared conditions.

1. Introduction

Since several years, the problem involving the emergence of antibiotic resistance among pathogenic bacteria (Hamblin & Hasan, 2004; Yoshikawa, 2002), seems to lead to the end. Indeed, in several institutions around the world a more effective alternative for antibacterial treatments have been developed (Cerveny, De Paola, Duckworth, & Gulig, 2002; Sajjan et al., 2001; Wainwright, 1998). Among them, Photodynamic Therapy (PDT), *i.e.* the combination of a light source with a photosensitizing agent (PS) and endogenous molecular oxygen is considered as a practical therapy for these diseases (Fu, Jordan, & Samson, 2013; Rizzi et al., 2014a). In fact, the rapid growth and mutations of bacteria, able to facilitate microbes surviving in the presence of an antibiotic drug, will quickly become predominant throughout the microbial population. Additionally, since the indiscriminate and inappropriate use of antibiotics, the problem get worse (Yoshikawa, 2002). Interestingly, for recent years PDT has appeared useful for treatments of several problems as for example those related to food safety, in which a significant number of man-made activities could induce the contamination of food products (Balali, Grelier, Benaissa & Coma,

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2008). As a consequence, the concept of Active Packaging becomes an interesting alternative to the traditional use of the conventional package, *i.e.* a passive barrier protecting the food. In European Community such new innovative concept has been defined as "a type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food" (Gomez-Estaca, Lopez-de-Dicastillo, Hernandez-Munoz, Catala, & Gavara, 2014; Vermeiren, Devlieghere, Van Beest, De Kruijf, & Debevere, 1999). In general, active food packaging provides additional functions that do not exist in conventional packaging systems and not only protect food passively and physically against environmental agents, but also inhibits or retards bacteria growth. Among different active packaging approaches (Gomez-Estaca et al., 2014; Vermeiren et al., 1999), the incorporation of active substances to the packaging material is an attractive development. Not surprisingly, the quality of packaged foods can be improved by controlling the release of these active agents reducing the growth rate of dangerous micro-organisms or inactivating it by contact (Quintavalla & Vicini, 2002). On the other hand, due to the decrease of fossil resources, the food packaging materials based on natural macromolecules from renewable resources have received great attention (Coma, 2013; Pedersen, Stæhr Wernberg, &Thomsen, 2005). In fact, in recent years and in different fields, the main attention has been focused on the Biomass, occurring to be a renewable energy source (Coma, 2013; Pedersen, Stæhr, Wernberg, & Thomsen, 2005). In such context, the attention of this paper has been focused on Chitosan (CH) as inexpensive, biodegradable, biocompatible, nontoxic and environmentally friendly linear amino polysaccharide derived from chitin, a major component of insects and crustacean shells. CH was chosen among biopolymers for the high-quality film forming properties and antimicrobial activity (Bordenave, Grelier, Pichavant, & Coma, 2005; Bordenave, Grelier, & Coma, 2010) useful for several applications (Moczek & Nowakowska, 2007; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). CH contains more than 5000 glucosamine units and it is obtained commercially from shrimp and crab shell chitin (a N-acetylglucosamine polymer) by alkaline deacetylation. For several years, bioactive CH matrices have been used in food preservation (Davies, Elson, & Hayes, 1989; El Ghaouth, Arul, Grenier, & Asselin, 1992; El Ghaouth, Arul, Ponnampalam, & Boulet, 1991; Jiang & Li, 2001; Muzzarelli & Rocchetti, 1986; Rabea et al., 2003). More specifically, in recent years, the CH chemical modification by inserting photoactive groups acting as potential PS for application in photosensitized oxidation reactions in water has been developed (Moczek & Nowakowska, 2007).

The pioneering works of Krausz *and co-workers* (Mbakidi et al., 2013), and related references, highlight the interest in this field of research, also in the recent past. For example, Krouit *and co-workers* (Krouit, Granet, Branland, Verneuil, Krausz, 2006) showed their new photoantimicrobial films composed of porphyrinated lipophilic cellulose esters and also the photobactericidal films from porphyrins grafted to alkylated cellulose (Krouit, Granet, Krausz, 2009).

Many kind of applications can be listed, however among them it is worth mentioning the work of Ringot *and co-workers* (Ringot et al., 2011) highlighting that porphyrins maintain their properties as PSs when grafted to polysaccharides, *i.e.* chitosan or cellulose, obtaining modified polymers as photobactericidal membranes or films for various applications.

Starting from results obtained by Krausz, and thanks to our experience on a natural chlorine, Chl*a*, solubilized in different systems (Agostiano, Catucci, Cosma, & Fini, 2003; Agostiano, Cosma, Trotta, Monsù-Scolaro, & Micali, 2002; Dentuto et al., 2007), the development of CH film containing such pigment, for potential application as bioactive antimicrobial packaging material, is presented for the first time in this paper as a novel photoactive system. Nonetheless the excellent CH properties, the system Chl*a*/CH presents several limitations due to the acid pH condition necessary for the preparation of CH hydrogel. In fact, CH is insoluble in water, but soluble in dilute organic acids which induce the protonation of CH free amino groups (Rabea et al., 2003), with pH condition not suitable for the chemical stability of Chl*a*. Hence the standard procedure used in literature to prepare CH films from hydrogel was modified in order to optimize the condition for introducing Chl*a*.

It is ascertained in literature that CH films (indicated in the text as CH STD) were generally prepared by the method described by B. Krajewska (Krajewska, Leszko, & Zaborska, 1990; Krajewska, 1991) in which a 1% (v/v) solution of CH is dissolved in 0.8% (v/v) aqueous acetic acid solution. The acidity of the medium is too high and induces the chemical degradation of Chl*a* with the release of the central Mg atom, inducing the loss of the chemical properties of the pigment. In our proposed procedure the pH of CH hydrogel is maintained at about 6 units, value at which Chl*a* is chemically stable. 2-HP-β-CD (or CD) has been used to promote chitosan polymer chains association (Burns et al., 2015). As for the photodynamic properties of PS-modified CH film, recent literature has shown that PSs conjugated with CH chains (Shrestha & Kishen, 2012) or saccharide like-structures (Cellamare, Fini, Agostiano, Sortino, & Cosma, 2013) retained their photoactive properties making it a possible option for PDT applications. On the other hand, only one paper by P. Mandal and co-workers (Mandal, Manna, Das, & Mitra, 2015), was known in literature related to Chl*a* molecules and chitosan hydrogel as scaffold, for application in artificial light harvesting antenna. In this paper, stacked Chl*a* molecules were entrapped within the chitosan matrix, then the pigment is not in its monomeric form and it is not photoactive (Mandal et al., 2015).

Starting from these considerations a comprehensive investigation on the CH/CD/Chl*a* film properties has been thus undertaken in our laboratories using several complementary techniques, namely spectroscopic, calorimetric, X-ray diffraction analyses and microscope imaging methods. A comparison of Chitosan biofilms arisen from our innovative procedure (with and without CD) with those well characterized in literature and arisen from a well-known procedure have been also performed in order to strengthens similarity and differences between them. In addition, for the first time, in order to show the photoactivity of the hybrid system CH/Chl*a*, among ROS, Singlet Oxygen is searched for. Nanosecond laser flash photolysis technique provides evidence for the population of the excited triplet state of Chl*a* and the photogeneration of singlet oxygen is demonstrated by both direct detection by using infrared luminescence spectroscopy and chemical methods based on the use of suitable traps. The main results arising from such characterization involving Chl*a* in solid state device will be described in the present paper opening new horizons in an enhanced antimicrobial activity of chitosan film for possible applications in PDT.

2. Experimental Section

2.1. Materials

All the chemicals used were of analytical grade and samples were prepared using double-distilled water. Commercial grade Chitosan powder (CH, from crab shells, with a molecular weight of 150,000, highly viscous, with a hypothetical deacetylation degree $\geq$ 75%), Acetic acid (99,9%), EtOH (99,9%) and glycerol (99,9%) were purchased from Sigma Aldrich.

The deacetylation degree has been experimentally estimated. Among several methods proposed for measuring the real degree of