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Construction of antibacterial poly(ethylene terephthalate) films via layer by layer assembly of chitosan and hyaluronic acid

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ABSTRACT

Polyelectrolytic multilayers (PEMs) with enhanced antibacterial properties were built up onto commercial poly(ethylene terephthalate) (PET) films based on the layer by layer assembling of bacterial contact killing chitosan and bacterial repelling highly hydrated hyaluronic acid. The optimization of the aminolysis modification reaction of PET was carried out by the study of the mechanical properties and the surface characterization of the modified polymers. The layer by layer assembly was successfully monitored by TEM microscopy, surface zeta-potential, contact angle measurements and, after labeling with fluorescein isothiocyanate (FTIC) by absorption spectroscopy and confocal fluorescent microscopy. Beside, the stability of the PEMs was studied at physiological conditions in absence and in the presence of lysozyme and hyaluronidase enzymes. Antibacterial properties of the obtained PEMs against *Escherichia coli* were compared with original commercial PET.

1. Introduction

Poly(ethylene terephthalate) (PET) is a low cost polymer that has shown excellent mechanical properties and moderate biocompatibility (Fu, Ji, Yuan, & Shen, 2005). These features have led to be broadly used as material for vascular catheters and implants, urinary catheters, heart-valves, and sutures, which nowadays are crucial in clinical applications. However, the high rate of infection, and the significant mortality arising from its use limit its applicability as biomaterial. These infections are consequence of the bacterial adhesion to the implanted surface before tissue integration (Hetrick & Schoenfisch, 2006) that results of its hydrophobic nature. Certainly, a huge number of publications have aimed to functionalize surface of PET in order to get good wettability, adhesion, adsorption, lubrication, permeability and biocompatibility and in this way overcome the limitations arising from its hydrophobic nature (Indest, Laine, Kleinschek, & Zemljič, 2010; Liu, Sheng, Gao, Li, & Yang, 2013).

Polyelectrolyte multilayers (PEMs) are considered as a versatile technique in thin films coating for a wide range of antibacterial surface applications (Lichter, Van Vlietpa, & Rubner, 2009). PEMs are easily built up by the layer-by-layer method (LBL), where alternated cationic and anionic layers of polyelectrolytes are adsorbed onto a solid substrate from dilute aqueous solutions (Decher, 1997). The resulting films are highly interpenetrated by ionic crosslinks (Baur, Rubner, Reynolds, & Kirn, 1999) that have demonstrated be stables at specific ranges of external pHs and ionic strength (Kujawa, Sanchez, Badia, & Winnik, 2006). This method generally led to coatings with a thickness that varies from tens to hundreds of nanometers depending on the characteristics of the polyelectrolytes employed and the conditions of the multilayer construction (Johnell, Larsson, & Siegbahn, 2005).

Since PET is an inert polymer it must be modify when it is used as substrate for PEMs. This modification consists in obtaining charged groups that allow the electrostatic binding to the polyions. Several papers have reported physical and chemical protocols to add ionizable functional groups such as carboxylic acid, sulfonic acid, amine, acrylate, onto PET surfaces (Fadeev & Mccarthy, 1998; Morent, De Geyter, & Leys, 2008). Plasma methods are the physical techniques that have been most intensively used to modify PET with carboxyl and amino groups. But these techniques are expensive, tedious and sometimes have resulted to be toxic and consequently

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inappropriate for biomedical applications (Siow, Britcher, Kumar, & Griesser, 2006). Besides, the aging effects observed on plasma modified polymers (e.g., post-plasma oxidation) (Yang & Gupta, 2004), and the vast array of functional groups formed during the treatment (Siow et al., 2006), which difficult the control of surface modification, have led to the development of alternative via, like wet chemistry. Wet chemistry is referred to a variety of chemical modifications such as, hydrolysis and aminolysis reactions that allow adding carboxyl and amino groups respectively onto PET surface. These chemical techniques are common to immobilize bioactive agents onto PET, (Aflori et al., 2013) as well as to get charged substrate for construction of PEMs. However, these lysis reactions can dramatically diminish mechanical properties of PET because they lead to chain scissions that could reach the bulk material (Sheikhzadeh, Ghaeli, Pirzadeh, & Bateni, 2010). For this reason, the optimization of lysis reaction conditions is a crucial issue before PET modification, and several authors have focused on this (Rosmaninho et al., 2006; Ng, Zhang, Liu, & Yang, 2009), but, up to now, the effect on the mechanical properties has not been deeply studied. In the last decade these chemical techniques have been common to immobilize bioactive agents onto PET (Aflori et al., 2013), as well as to get charged substrate for construction of PEMs by LBL assembly.

In this sense, biopolymers have been extensively studied as polyions in LbL assemblies due to their biocompatibility and abundance (Boudou, Crouzier, Ren, Blin, & Picart, 2010). The combination of CHI and HA has been extensively explored for fabrication of multilayers using LBL assembly as a promising approach for designing natural antibacterial coatings.

Hyaluronic acid (HA) is a linear glycosaminoglycan composed of repeating disaccharide units of β-(1,4)-D-glucuronic acid and β-(1,3)-*N*-acetyl-D-glucosamine that has proven to be an effective polyanion that has demonstrated to be able to complex cationic polymers such as chitosan (Hartmann et al., 2013), L-polylysine (Picart et al., 2001), poly(allylamine) (Szarpak, Pignot-Paintrand, Nicolas, Picart, & Auzély-Velty, 2008), providing for opportunities to generate PEMs systems. It is found in most connective tissues and particularly in synovial fluid, vitreous fluid of the eye, umbilical cords and chicken combs (Necas, Bartosikova, Brauner, & Kolar, 2008). HA has a crucial biological function in joint lubrication, tissue hydration, and wound healing, among other ones (e.g. receptor-mediated targeting, mitosis, tumor development and metastasis, or inflammation) (Kogan, Šoltés, Stern, & Gemeiner, 2007). It is one of the most hydrophilic molecules in nature and is employed as moisturizer in cosmetic and pharmaceutical applications. As a consequence of its highly hydrated state, HA displays a naturally non adhesive nature (Chen & Abatangelo, 1999).

Chitosan (CHI), is a partly acetylated (1-4)-2-amino-2-deoxy-β-D-glucan, natural and biocompatible. Due to its non-toxicity, biodegradability, biocompatibility, antitumor, antioxidant and mucus adhesiveness properties, CHI, has attracted much attention in food, pharmaceutical, and cosmetic fields (Xia, Liu, Zhang, & Chen, 2011). Additionally, CHI is positively charged in mildly acidic aqueous solutions, and has demonstrated excellent antibacterial "killing contact" properties by disruption of the negatively charged cell membranes of microbes (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003; Savard, Beaulieu, Boucher, & Champagne, 2002). CHI-based materials have thus been used to prevent bacterial contamination and biofilm formation as tissue engineering scaffold, gels, packaging and other formulations (Kong, Chen, Xing, & Park, 2010). Furthermore, as charge density of CHI is high, it can be used as polycation in a LBL assembly on which various polyanions have been successfully adsorbed, such as heparin (Fu, Ji, Fan, & Shen, 2006), HA, alginate (Carneiro-da-Cunha et al., 2010), chondroitin sulfate (Liu, He, & Gao, 2005).

Nevertheless, CHI cannot be considered a suitable biomaterial for in vitro fibroblast cultivation. Jou et al. (2007) grafted CHI onto PET substrates and although they showed the endorsement of antibacterial activity against different bacteria, CHI caused the reduction in the number of fibroblast. Due to this, CHI is usually used in the design of antibacterial surface in combination with other materials. Jou et al. in the same work were able of immobilizing HA covalently onto CHI-grafted PET fibers and observed not only that the prepared system possessed antibacterial activity, but also improved the cell proliferation for fibroblast.

Richert et al. (2004a) observed a decrease in cell adhesion increasing the number of bilayer in CHI/HA multilayer films. Croll, O'Connor, Stevens, and Cooper-White (2006) demonstrated that CHI/HA PEMs are nonadhesive toward most proteins under physiological conditions and their stability against enzymatic degradation can be modulated without losing non-adhesive properties.

CHI/HA multilayers have been built up onto a huge diversity of substrates like, glass (Mulligan, Jakubek, & Johnston, 2011), Ti (Chua, Neoh, Kang, & Wang, 2008), detachable PP (Larkin, Davis, & Rajagopalan, 2010), among other ones. However, a few studies has attempted onto PET. Liu et al. (2006) developed a microchip reactor on the basis of a layer-by-layer deposition of CHI/HA on microstructured PET for digestion of proteins. The multilayers were characterized by AFM, ATR-FTIR and contact angle measurements, however, to the best of our knowledge, the antibacterial properties of the CHI/HA multilayers were not studied.

More recently, Li, Ge, Zhang, Wu, and Chen, (2012a) and Hong et al. (Li et al., 2012b) analyzed in vitro and in vivo PET artificial ligament graft healing in bone tunnel modified by plasma treatment and LBL assembly with CHI/HA. They concluded that polysaccharides coating accelerates artificial graft-to-bone healing after implantation in bone tunnel, promoting rapid recovery after implantation. Nevertheless antibacterial properties were not analyzed and a thorough characterization of the multilayers was not reported.

Taking into account all the above described, this paper aims to prepare, characterize and corroborate the antimicrobial properties of PEMs that combine adhesion resistance and contact killing properties in a poorly study system based on PET, CHI and HA. Additionally, stability of above PEMs against lysozyme and hyalurodinase enzymes is reported for the first time.

2. Experimental part

2.1. Materials

PET films (75 μm) were supplied by HIFI Film Industria. Chitosan (high molecular weight, Sigma-Aldrich) was purified using the procedure previously described (Signini and Campana Filho, 1999). The deacetylation degree of chitosan determined by ^{1}H NMR was 79% which is in good agreement with the value reported by the supplier (75–85%). Hyaluronic acid sodium salt (Sigma Aldrich 0.6–1.1 MDa) was used without further purification and its weight average molecular weight determined by GPC was 254,000 g/mol. The viscosity average molecular weight of chitosan measured by an Ubbelohde capillary viscometer (HAc 0.1 M/NaCl 0.2 M, 25 °C) (Rinaudo & Michel Milas, 1993) was 1080,900 g/mol. Ethylenediamine (ETDA, 99%), ethanol (absolute), methanol (99.5%) and HCl (37%) were purchased by Panreac. The dye Orange II (salt 4-(2-hydroxy-1-naphthylazo) bencenosulfuric sodium salt) (Sigma-Aldrich) was used for the colorimetric method and isothiocyanate of fluorescein (Sigma Aldrich) was used to label chitosan. NaCl (Probus), NaOH (pellets, Panreac). Fluorescein isothiocyanate (FTIC, isomer I, Sigma Aldrich), lysozyme (From chicken egg white,