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Evaluation of a combined drug-delivery system for proteins assembled with polymeric nanoparticles and porous microspheres; characterization and protein integrity studies

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ABSTRACT

This work presents an evaluation of the adsorption/infiltration process in relation to the loading of a model protein, α-amylase, into an assembled biodegradable polymeric system, free of organic solvents and made up of poly(D,L-lactide-*co*-glycolide) acid (PLGA). Systems were assembled in a friendly aqueous medium by adsorbing and infiltrating polymeric nanoparticles into porous microspheres. These assembled systems are able to load therapeutic amounts of the drug through adsorption of the protein onto the large surface area characteristic of polymeric nanoparticles. The subsequent infiltration of nanoparticles adsorbed with the protein into porous microspheres enabled the controlled release of the protein as a function of the amount of infiltrated nanoparticles, since the surface area available on the porous structure is saturated at different levels, thus modifying the protein release rate. Findings were confirmed by both the BET technique (N_2 isotherms) and *in vitro* release studies. During the adsorption process, the pH of the medium plays an important role by creating an environment that favors adsorption between the surfaces of the micro- and nano-structures and the protein. Finally, assays of α-amylase activity using 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNP-G3) as the substrate and the circular dichroism technique confirmed that when this new approach was used no conformational changes were observed in the protein after release.

1. Introduction

Protein formulation has constituted a significant challenge for the pharmaceutical scientists because the therapeutic activity of these drugs is closely-related to their conformational structure and integrity, and because their physical and chemical instability must be dealt with. Recent advances in biotechnological techniques like fermentation and cloning have made it possible to produce large quantities of biologics (*i.e.*, biotherapeutic proteins) for pharmaceutical applications whose benefits present an opportunity to improve current treatments for disorders such as cancer, genetic and enzymatic deficiencies, autoimmune diseases and infections like HIV (Frokjaer and Otzen, 2005). More than 324 biomacromolecules – mostly proteins – are currently undergoing clinical trials. A general trend is to use drugs produced by recombinant DNA techniques because it is expected that biologics will be less toxic and more predictable regarding their behavior *in vivo* (Pavlou and Reichert, 2010). However, factors such as pH, temperature, high shear forces, interfaces, storage, handling, formulation and administration of such proteins can create undesirable conditions that lead to physical and chemical instability in the form of denaturation, deamidation, aggregation, oxidation, peptide bond hydrolysis, thiol–disulfide exchange, crosslinking, intramolecular conformational scrambling and precipitation processes, all of which can alter their activity (Fu et al., 2000; Krishnamurthy and Manning, 2002; Manning et al., 2010). Thus, the success in formulating proteins requires knowledge and understanding of

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their physical, chemical and biological properties, as well as the use of adequate materials and carriers (Manning et al., 2010).

To facilitate the administration of biologics and to maintain protein integrity during formulation and storage, different drug delivery systems have been proposed. Polymeric and lipid-based nanoparticles (*e.g.,* liposomes), nanocapsules, hydrogels, injectable implants, microemulsions and microspheres are common examples of systems developed to improve protein delivery. Most of these systems are based on entrapment and microencapsulation principles, whose stages basically include emulsification processes and posterior solvent evaporation. Unfortunately, these common manufacturing methods generate a microenvironment that can compromise the protein integrity during drug-loading, microcarrier formation and drying because molecules are exposed to organic solvents, aqueous/organic interfaces, elevated temperatures, vigorous agitation, hydrophobic surfaces and detergents (Van der Weert et al., 2000). Hence, this physicochemical stress affects proteins during the entire life of the drug-delivery system; including formulation, storage and protein release *in vivo* (Dai et al., 2005; Degim and Çelebi, 2007; Sun et al., 2009; Tan et al., 2010; Van der Weert et al., 2000).

Our understanding of protein instability has improved greatly since 1989 and several alternatives, such as stabilization by ligand-binding to the native state (surfactants, polymers, cyclodextrins, metal ions, anion binding, *etc.*), colloidal and interfacial stability, drying, chemical modifications, site-directed mutagenesis and the development of novel drug delivery systems, have been described to protect the integrity of proteins during their life-cycle (Fu et al., 2000; Manning et al., 2010; Wang, 1999). Because research into protein pharmaceutical technology has focused on proposing strategies that will prevent protein damage during formulation, various experiments have been conducted to better understand the critical steps involved in the obtaining of protein drug-delivery systems, instability, and degradation mechanisms (Fu et al., 2000). Polymeric nanoparticles and injectable parenteral depots loaded with peptides and proteins and designed with biodegradable materials like PLGA (polylactide-*co*-glycolide acid) are important in this field, because the protein drug-delivery systems approved by the FDA are based on polymeric microspheres, some of which are already available in the pharmaceutical market (Dai et al., 2005; Degim and Çelebi, 2007; Putney, 1998; Spada et al., 2011). Other approaches, such as incorporating of protein–biopolymers complexes which are then mixed with porous microspheres to obtain self-healing PLGA microspheres, have been proposed to enhance protein loading efficiency and prevent drug instability during encapsulation and release by protein immobilization. The biopolymers used are members of the glycosaminoglycan (GAG) family, or they have similar structural moieties (*e.g.*, dextran sulfate and chitosan) (Shah and Schwendeman, 2014).

On the other hand, adsorption of hydrophilic drugs is a strategy that has been used to avoid high-energy processes in drug entrapment. This approach has been applied successfully to adsorb biomacromolecules onto nanoparticle surfaces (Vrignaud et al., 2011). Many papers have discussed the use of PLGA to adsorb proteins onto micro- and nano-particles in order to avoid the risk of protein degradation caused by the interaction with organic solvents, interfaces, homogenization and drying (Kim et al., 2006a; Sun et al., 2009). The option of using adsorption as an alternative to load drugs takes into account the interaction between the surface of polymeric structures and proteins by establishing and controlling certain variables such as pH, temperature, protein and polymer properties (*e.g.*, isoelectric point, pKa, end functional group, etc.) in an aqueous medium. Recently, the use of porous microspheres with a large pore surface area and interconnecting pores has drawn attention for loading therapeutic drugs (including peptides and proteins) by adsorption in an immersion medium (Kilpeläinen et al., 2011; Rodríguez-Cruz et al., 2009; Sun et al., 2009). The adsorption process and the large surface area of porous microspheres and nanoparticles form the basis of the strategy proposed in earlier research (Alcalá-Alcalá et al., 2013), in which a peptide was formulated using the adsorption/infiltration process. This technique produced assembled systems that load drugs which are sensitive to physicochemical stress with high adsorption efficiencies and a controlled release that are dependent on the continuity of a nanoparticle film that forms on the microsphere surface. The aim of this work was to formulate a globular molecule, a protein (enzyme α-amylase), using the adsorption/infiltration process with biodegradable, PLGA micro- and nano-particles in order to obtain an injectable assembled system with the capacity to release proteins during a week or more. Additionally, the assembled system was characterized and the integrity of the protein after release was evaluated in order to demonstrate the efficacy of this novel protein drug-delivery system.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-*co*-glycolide) acid (PLGA 50:50, DLG 4A, molecular weight 38,000), was obtained from Lakeshore Biomaterials (Birmingham, AL, USA). α-Amylase (from *Aspergillus oryzae*, 10065-50 G), poly(vinyl alcohol) (PVAL: Mowiol® 4–88, molecular weight 58,000), the Bicinchoninic acid kit for protein determination (BCA1) and 2-chloro-4-nitrophenyl-α-D-maltotrioside (93834 CNP-G3) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methylene chloride, ethyl acetate, acetonitrile, sodium chloride, calcium chloride, potassium thiocyanate and mannitol were provided by J.T. Baker® Chemicals (Avantor Performance Materials, Center Valley, PA, USA). Ammonium carbonate, monobasic potassium phosphate, sodium citrate, citric acid, acetic acid, sodium acetate and sodium hydroxide were all supplied by Productos Quimicos Monterrey, SA (Monterrey, NL, Mexico). Distilled water was obtained from a RiOs™ distiller (EMD Millipore, Billerica, MA, USA).

2.2. Preparation of the components of the assembled systems

The protein drug-delivery systems were assembled using polymeric nanoparticles and porous polymeric microspheres of PLGA, following well-known and reproducible methods.

2.2.1. PLGA nanoparticles

The emulsification-solvent diffusion method was used to manufacture polymeric nanoparticles (Quintanar-Guerrero et al., 1996). Two phases were prepared after saturation between distilled water (phase W) and ethyl acetate (phase O). A solution of 5% w/v of polyvinyl alcohol (PVAL) was obtained in phase W. Then, 400 mg of PLGA were dissolved in 20 mL of phase O and emulsified with 40 mL of PVAL solution by using a homogenizator (ULTRA-TURRAX®; IKA® Works Inc., Wilmington, NC, USA) at 11,000 rpm for 10 min. Afterwards, 160 mL of water without saturation was added to the emulsion in order to allow the diffusion of the organic solvent. After diffusion, the nanoparticles remained in suspension and the solvent was evaporated under reduced pressure at 90 rpm and 30 °C. Nanoparticles were recovered by centrifugation (Optima® LE-80 K; Beckman Coulter Inc., Fullerton, CA, USA) at 25,000 rpm for 20 min, and then washed three times. Finally, they were frozen and lyophilized at 50×10^{-3} mbar and −40 °C for 24 h (FreeZone 6; Labconco®, Kansas City, MO, USA), using mannitol as the cryoprotectant at 1 mg/mL of nanoparticle suspension.