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Pulmonary sustained release of insulin from microparticles composed of polyelectrolyte layer-by-layer assembly

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ABSTRACT

The present study tests the hypothesis that layer-by-layer (LbL) nanoassembly of thin polyelectrolyte films on insulin particles provides sustained release of the drug after pulmonary delivery. LbL insulin microparticles were formulated using cationic and anionic polyelectrolytes. The microparticles were characterized for particle size, particle morphology, zeta potential and *in vitro* release. The pharmacokinetics and pharmacodynamics of drug were assessed by measuring serum insulin and glucose levels after intrapulmonary administration in rats. Bronchoalveolar lavage (BAL) and evans blue (EB) extravasation studies were performed to investigate the cellular or biochemical changes in the lungs caused by formulation administration. The mass median aerodynamic diameter (MMAD) of the insulin microparticles was 2.7 μm. Confocal image of the formulation particles confirmed the polyelectrolyte deposition around the insulin particles. Zeta potential measurements showed that there was charge reversal after each layering. Pulmonary administered LbL insulin formulation resulted in sustained serum insulin levels and concomitant decrease in serum glucose levels. The BAL and EB extravasation studies showed that the LbL insulin formulation did not elicit significant increase in marker enzymes activities compared to control group. These results demonstrate that the sustained release of insulin could be achieved using LbL nanoassembly around the insulin particles.

1. Introduction

Protein and peptide drugs are frequently administered through intravenous and subcutaneous injections and are rapidly cleared from the body. These routes of drug administration are painful and, as many of the protein drugs are indicated to treat chronic conditions such as diabetes, chronic administration results in patient non-compliance (Todo et al., 2001). Systemic delivery of protein and peptide drugs through pulmonary route has distinct advantages compared to other routes, such as low protease activity, large absorptive surface area, thin alveolar epithelium, high perfusion capacities, no first-pass metabolism and high bioavailability (Adjei and Gupta, 1994; Komada et al., 1994). Pulmonary delivery of sustained release formulations of a protein drug could protect the protein over a prolonged period from degradation or elimination, reduce the frequency of administration, increase the systemic availability, and improve patient compliance. However, until now, there is no pulmonary insulin dosage form available in the market.

The types of current insulin delivery systems used in the management of diabetes include: 1. Short acting insulin formulations deliver insulin at a rate that reaches into the bloodstream in 30 min after injection and stays effective till 3–6 h (Humulin®). 2. Rapid acting insulin formulations deliver insulin at a rate that begins to work after about 5 min and work for 2–4 h (Novolog®). 3. Intermediate acting insulin formulations reaches bloodstream in 2–4 h and stay effective for 12–18 h (Novolin N®, NPH®). 4. Long-acting insulin formulations deliver the drug at a rate that reaches bloodstream in 6–10 h and stays effective for 20–26 h (Lantus®). All of these dosage forms are administered *via* injections. The chronic use of these dosage forms for the treatment of diabetes could decrease the patient compliance.

Insulin dosage forms for pulmonary administration are yet to reach the market. Sustained release formulations for pulmonary delivery in the form of liposomes, solid-lipid nanoparticles, nanospheres, microcapsules and other types of dosage forms have been under

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investigation for many years (Chono et al., 2009; Kawashima et al., 1999; Takenaga et al., 2002). Liposomes provide the stability and prevent the degradation of encapsulated protein drugs. These delivery vehicles are well established and extensively investigated particulate carrier systems that have been successfully employed for the site specific drug delivery (Wang, 1999). However, drug leakage and binding of proteins with the phospholipid molecules are the disadvantages of these systems. Proteins are hydrophilic molecules and encapsulation of these drugs in the core of solid-lipid nanoparticles and nanospheres could be difficult due to the partition of these molecules into the aqueous compartment (Antonio and Eliana, 2007). Protein drugs can be loaded into microcapsules in order to provide the stability of the encapsulated drugs. However, drug leakage is the main problem associated with this type of drug delivery system (Qi et al., 2008). In addition, a more recent layer-by-layer (LBL) self-assembly technique for preparing ultra-thin films over the drug particles has begun to receive much attention in sustained release delivery systems.

LbL approach relies on the electrostatic attraction between oppositely charged polyions and this approach can be used to prepare nanoshell assembly around the drug particles (Qiu et al., 2001). In this methodology, cationic and anionic polyelectrolytes are alternatively adsorbed on a charged drug particle, resulting in multi-layer assemblies on the solid substrate (Ibarz et al., 2001). Unlike liposomes and micelles, the multilayer assembly over the drug particles is usually tough and homogeneous, and can precisely control the release of the drug. The prerequisites to coat substrate particles with these multilayers include the presence of charge on the substrate molecule and that the substrate must not dissolve completely in the coating solution. The rate of drug release generally depends on the thickness of the nanoassembly around the drug particles which in turn depends on the type, concentration, molecular weight and adsorption capacity of polyelectrolyte used for the coating. Varying the number of layers on drug particles could result in a formulation with optimal release characteristics (Suzuki et al., 2002). In one study, fluorescein dye microcrystals were used as core material and coated with anionic polystyrene sulfonate (PSS) and cationic poly (allylamine) hydrochloride (PAH). This process resulted in the sustained release of the dye. The authors found that increasing the number of layers in the shell around the drug core resulted in decreased shell permeability and prolonged core dissolution (Sui et al., 2003). In another study, multi-layer assembly on furosemide drug microcrystals resulted in controlled release of drug depending on the number of layers, thickness of LbL assembly, and type of polyions used in the process of coating. In this study, it was shown that gelatin/PDDA/PSS coating over the furosemide crystals reduced the drug release up to 300 times compared to uncoated drug microcrystals (Ai et al., 2003). Dexamethasone, a potent synthetic member of steroid hormones, was formulated using LbL technique. In this study, drug particles coated with gelatin A/PDDA/PSS resulted in controlled release of the drug (Pargaonkar et al., 2005).

More recently, a glucose sensitive multilayer film was fabricated on to tablet core by the LbL assembly method (Chen et al., 2011). In this method positively charged poly [2-(dimethylamino) ethyl methacrylate] polymer (star PDMAEMA polymer) and negatively charged insulin and glucose oxidase were used. The authors found that, in response to stepwise *in vitro* glucose challenge, the multilayer film showed an on–off regulation of insulin release. Furthermore, the multilayer film continuously released insulin after being subcutaneously implanted in streptozocin induced diabetic rats and reduced the blood glucose level for at least two weeks. In a separate study, the arrangement sequence of the LbL components was altered. Subcutaneous administration of this altered LbL system prolonged the hypoglycemic effect from 17 days to 36 days (Chen et al., 2012). Moreover, Luo et al. (2012) prepared supramolecular assembly of porcine insulin (P-SIA) and then fabricated a glucose sensitive LbL film using a star polymer, glucose oxidase, catalase and P-SIA. A single subcutaneous dose of this LbL formulation resulted in effective glycemic control in diabetic rats for up to 295 days without hypoglycemia (Luo et al., 2012). Based on these studies, the LbL encapsulation method can be used to formulate protein drugs and control the release of these macromolecules following pulmonary delivery. Also, to the best of our knowledge there are no published reports available on pulmonary efficacy and toxicity evaluation of LbL drug microparticles.

The objective of the present study was to formulate insulin, utilizing LbL nanoassembly technique and to test the *in vivo* efficacy of the formulation after pulmonary administration in rats. The effect of number of layers on drug release profiles was studied. Pulmonary toxicity due to short-term and chronic administration of LbL insulin formulation was also investigated.

2. Materials and methods

2.1. Materials

Recombinant human insulin (28.8 U/mg), poly (dimethyldiallyl ammonium chloride) (PDDA: MW 100,000; cationic polymer), sodium (polystyrenesulfonate) (PSS: MW 70,000; anionic polymer), *N*-Acetylglucosaminidase (NAG) assay kit, sodium monododecyl sulfate (SDS) and evans blue (EB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Reagents for lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) determination were obtained from Pointe Scientific Inc. (Canton, MI, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific (Rockford, IL, USA). Fluorescein-5-isothiocyanate (FITC; Sigma–Aldrich) was used for labeling LbL insulin microparticles. Human insulin-specific radioimmunoassay (RIA) kit and glucose (Hexokinase) assay kit were purchased from Linco Research Inc. (St. Charles, MO, USA) and Pointe Scientific Inc. (Canton, MI, USA), respectively. Anesthetics, ketamine and xylazine were purchased from Henry Schein, Inc. (Melville, NY, USA).

2.2. Preparation of pulmonary LbL insulin formulations

The procedure used to introduce LbL assembly on insulin particles was described in previous reports (Pargaonkar et al., 2005). Briefly, based on the negative charge of the suspended insulin particles at pH above 5.3 (isoelectric point of insulin), 5 mg of insulin was suspended in 1 ml of PDDA solution (2 mg/ml in PBS, pH 7.4). The suspension was sonicated for 10 s, stirred for 10 min and centrifuged (5700 × *g* at 4 °C) (Eppendorf, Model 5804 R, VWR, NY, USA). The supernatant polyelectrolyte solution was discarded and the separated drug particles were washed two times with PBS (pH 7.4) to remove unadsorbed polyelectrolyte (PDDA). This resulted in the formation of first layer of polyions around insulin particles. These particles were resuspended in a 1 ml of PSS solution (2 mg/ml in PBS, pH 5.8) and stirred for 10 min to ensure coating. The suspension was centrifuged (5700 × *g* at 4 °C), and separated particles were washed 2 times to remove unadsorbed PSS. Alternate layers of PDDA and PSS were subsequently deposited until the desired number of layers was achieved. In addition, insulin solution for subcutaneous administration was prepared by dissolving the drug in acidic PBS (pH 2). The pH of the solution was adjusted to a physiological level (pH 7.2–7.4) with NaOH. The concentration of insulin in the final formulations was measured by BCA protein assay kit. For this, few drops of 0.1 N HCl was added to formulation while mixing until all the particles are dissolved. The pH of the solution was adjusted to 7.4 with the addition of 0.1 N NaOH and the insulin concentration was determined.