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Inhalable large porous microspheres of low molecular weight heparin: In vitro and in vivo evaluation

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ABSTRACT

This study tests the feasibility of large porous particles as long-acting carriers for pulmonary delivery of low molecular weight heparin (LMWH). Microspheres were prepared with a biodegradable polymer, poly(lactic-co-glycolic acid) (PLGA), by a double-emulsion–solvent-evaporation technique. The drug entrapment efficiencies of the microspheres were increased by modifying them with three different additives – polyethyleneimine (PEI), Span 60 and stearylamine. The resulting microspheres were evaluated for morphology, size, zeta potential, density, in vitro drug-release properties, cytotoxicity, and for pulmonary absorption in vivo. Scanning electron microscopic examination suggests that the porosity of the particles increased with the increase in aqueous volume fraction. The amount of aqueous volume fraction and the type of core-modifying agent added to the aqueous interior had varying degrees of effect on the size, density and aerodynamic diameter of the particles. When PEI was incorporated in the internal aqueous phase, the entrapment efficiency was increased from 16.22 ± 1.32% to 54.82 ± 2.79%. The amount of drug released in the initial burst phase and the release-rate constant for the core-modified microspheres were greater than those for the plain microspheres. After pulmonary administration, the half-life of the drug from the PEI- and stearylamine-modified microspheres was increased by 5- to 6-fold compared to the drug entrapped in plain microspheres. The viability of Calu-3 cells was not adversely affected when incubated with the microspheres. Overall, the data presented here suggest that the newly developed porous microspheres of LMWH have the potential to be used in a form deliverable by dry-powder inhaler as an alternative to multiple parenteral administrations of LMWH.

1. Introduction

The pulmonary route has recently emerged as a viable alternative to the needle-based route of administration for an expanding array of biotechnology-derived drugs with superior therapeutic activity. However, the vast majority of currently available pulmonary drug delivery systems, including recently approved inhaled insulin—Exubera, which was later withdrawn from the market—are designed as immediate-release formulations to produce local and systemic effects for a short period of time [1]. Little has been done in the development of viable, inhaled formulations of therapeutic agents, especially biopharmaceuticals that can be administered via the lungs to release drugs for a prolonged period. In fact, inhaled long-acting formulations remain elusive because of the lack of efficient delivery devices and optimal drug carriers. The factors that have been major barriers to the development of long-acting pulmonary formulations are (i) suboptimal size and shape of the drug substance or the encapsulating carriers within the respirable fraction, (ii) short residence time of the inhaled drug particles or formulations in the respiratory tract, and (iii) poor loading of drugs into the particulate carriers frequently used to prepare inhaled formulations [2–4].

The recent advances in the dry-powder inhalation (DPI) technology have addressed some of the limitations associated with inhaled formulations, including unwanted loss of drug due to oropharyngeal deposition [5,6]. However, the limitations associated with poor deposition of particles larger than 5 μm have yet to be overcome. In a seminal paper, Edwards et al. first proposed that particles with mass densities <0.4 g/cm^3 and geometric diameter >5 μm could be used to ease respirability as well as to enhance residence time in the lungs [3]. Indeed, the report of Edwards et al. spurred significant growth in studies on PLGA-based large porous particles for delivery of biopharmaceuticals. However, suboptimal pore size of the microspheres and poor encapsulation efficiency of many drugs remain major impediments to the widespread use of large porous PLGA microspheres as carriers for inhaled long-acting formulations [7]. In order to achieve sustained release properties and increase drug load, attempts have been made to modify particulate carriers by using polymer blends [8],

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porosity-altering or surface-active agents [9], and by means of the cyclodextrin-mediated double-entrapment technique [10–13].

Over the past few years, the large porous particle technology has been used for a number of biopharmaceuticals and conventional therapeutic agents, including insulin, testosterone, estradiol, deslorelin, tobramycin, ciprofloxacin and para-aminosalicylic acid [3,13–17]. However, currently it is not known if this technology can be used for pulmonary delivery of hydrophilic macromolecules carrying a negative surface charge. Low molecular weight heparins (LMWHs) are negatively charged oligosaccharides used in the treatment of deep vein thrombosis and pulmonary embolism [18]. Because of their relatively high molecular weight, negative surface charge and short half-lives, LMWHs are administered by subcutaneous injection multiple times a day. Recent studies in our laboratory showed that pulmonary administration of LMWH is feasible [19,20]. But the proposed formulation of the drug with absorption enhancers and dendrimers failed to produce a prolonged release of the drug. In this study, we propose that long-acting pulmonary formulations of LMWH can address the limitations imposed by the short duration of action of the drug.

Very recently, PLGA-based microspheres and nanoparticles have been used for oral and nasal delivery of unfractionated heparin and LMWH, although not for pulmonary delivery of LMWH. Moreover, the drug load and release profiles of the proposed formulations were not that encouraging [21–24]. We believe that the poor entrapment efficiency of the formulations was because of the excessive hydrophilicity of the drug, and that the poor release profiles were the result of the use of nonporous particulate carriers. Thus, it is reasonable to assume that the entrapment efficiency of the carriers can be increased by complexing the drug with cationic polymers or surfactants, and that a refined controlled release can be achieved by using microspheres with optimal porosity. This study, therefore, tests the hypothesis that core-modified PLGA-based large porous microspheres can enhance the entrapment efficiency of LMWH and facilitate release of the drug for an extended period after pulmonary administration.

2. Materials and methods

2.1. Materials

Poly(DL-lactide-co-glycolide) (50:50) PLGA (inherent viscosity 0.15–0.25 dl/g; weight average molecular weight = 10.6 kDa) was purchased from Boehringer Ingelheim (Lactel Absorbable Polymers, Pelham, AL). LMWH (average molecular weight and anti-factor Xa activity, 4493 Da and 61 U/mg, respectively) was obtained from Celsus laboratories (Cincinnati, OH). Poly vinyl alcohol (PVA), Span 60 (SP), polyethyleneimine (PEI) and stearylamine (SA) were purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO).

2.2. Preparation of LMWH-loaded porous microspheres

The LMWH-loaded PLGA microspheres were prepared by the water-in-oil-in-water (w/o/w) emulsion and evaporation method [3]. Briefly, an aqueous solution of LMWH (internal aqueous phase, IAP) was first emulsified in 5.0 ml of dichloromethane (organic phase, OP) containing (0.25 g) PLGA polymer by homogenization (Ultra-Turrex T25 basic, IKA, Wilmington, DE) at 15,000 rpm for 3 min. The volume of the IAP was varied from 0.25 to 1 ml to obtain microspheres of varying porosity (Table 1). To prepare core-modified microspheres, three different core-modifying agents – Span 60, stearylamine and polyethyleneimine – were added to the IAP. As both Span 60 and stearylamine are hydrophobic in nature, they were dispersed in water by heating at 85 °C for 15 min. The concentrations of the core-modifying agents used were 1.25 and 2.5%. The resulting water-in-oil (w/o) emulsion was then poured into 25 ml of 1.0% w/v PVA aqueous solution (external aqueous phase, EAP) and emulsified by homogenization at 8000 rpm for 5 min. The w/o/w emulsion thus obtained, the secondary emulsion, was stirred overnight at room temperature for evaporation of dichloromethane. The polymeric particles were then washed thrice and lyophilized to get free-flowing powder. A blank microsphere formulation without LMWH was also prepared according to the same procedure. Each batch was prepared in triplicate.

Table 1
Composition and entrapment efficiency of the microspheric formulations

Formulations	IAP:OP:EAP (v/v/v)	IAP composition (w/v)	Entrapment efficiency of LMWH (%)
PM-1	0.25:5:25	–	17.87 ± 1.79
PM-2	0.5:5:25	–	16.22 ± 1.32
PM-3	1:5:25	–	9.39 ± 1.21
PM-SP-1	0.5:5:25	1.25% Span 60	36.7 ± 2.08
PM-SP-2	0.5:5:25	2.5% Span 60	47.34 ± 1.98
PM-SA-1	0.5:5:25	1.25% SA	40.2 ± 2.77
PM-SA-2	0.5:5:25	2.5% SA	54.44 ± 2.02
PM-PEI-1	0.5:5:25	1.25% PEI	43.5 ± 1.32
PM-PEI-2	0.5:5:25	2.5% PEI	54.82 ± 2.79

SP: Span 60; SA: Stearylamine; PEI: Polyethyleneimine.

2.3. Particle characterization

Microspheres were characterized for their morphology, size, zeta potential, tapped density, and aerodynamic diameter. The morphology of the formulations was studied under a scanning electron microscope. The samples for SEM were prepared on a conductive, double-sided adhesive tape and then sputter-coated with gold under argon (Emitech K550X, Kent, UK). The microphotographs were taken using a Hitachi S-3400N (Freehold, NJ) scanning electron microscope. The mean volume-based diameter and size distribution of the particles were determined by a Microtrac® S3500 (North Largo, FL) particle size analyzer after dispersing the freeze-dried microspheres in a 0.2% w/v aqueous solution of PVA. The polydispersity indices of all formulations were calculated as the ratio of volume-averaged mean particle size to number-averaged mean particle size [25]. The zeta potential was measured by Zeta potential analyzer V. 3.40 (Brookhaven Instruments Corp. Holtsville, NY) after dispersing the formulations in PBS buffer. The density of the particles was estimated from the tapped density as described previously [4,12]. An aliquot of 100 mg microspheres was transferred to a 10 (±0.05) ml graduated cylinder and the initial volume was recorded. Tapped density of the particles (ρ) was calculated as the ratio between sample weight (g) and the volume (ml) occupied after 200 tappings. The aerodynamic diameter of the dry-powder formulations was determined in an eight-stage Andersen cascade impactor (Westech Instruments Inc., Marietta, Georgia). The formulations were fired 5–6 times into the cascade impactor at a flow rate of 28.3 L/min. The deposited formulation at each stage was collected by rinsing the impactor and the samples were analyzed for LMWH content. The studies were performed in triplicate. In addition to actual aerodynamic diameter, the theoretical mass mean aerodynamic diameter (MMAD) of the particles was also calculated using the following equation [26]:

$$\mathrm{MMADt} = d(\rho/\rho_0 X)^{1/2}$$

Where d is the geometric mean diameter obtained from particle size analysis, ρ is the tapped density, ρ_0 is the reference density of 1 g/cm^3 and X is the shape factor, which is 1 for a sphere.

2.4. Encapsulation efficiency

The amount of heparin entrapped within microspheres was determined using an azure A colorimetric assay by measuring the amount of unentrapped drug in the external aqueous solution recovered after centrifugation and washing of the microspheres [27]. Typically,