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Polymeric nanoparticles for pulmonary protein and DNA delivery

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ABSTRACT

Polymeric nanoparticles (NPs) are promising carriers of biological agents to the lung due to advantages including biocompatibility, ease of surface modification, localized action and reduced systemic toxicity. However, there have been no studies extensively characterizing and comparing the behavior of polymeric NPs for pulmonary protein/DNA delivery both *in vitro* and *in vitro*. We screened six polymeric NPs: gelatin, chitosan, alginate, poly(lactic-co-glycolic) acid (PLGA), PLGA–chitosan and PLGA–poly(ethylene glycol) (PEG), for inhalational protein/DNA delivery. All NPs except PLGA–PEG and alginate were <300 nm in size with a bi-phasic core compound release profile. Gelatin, PLGA NPs and PLGA–PEG NPs remained stable in deionized water, serum, saline and simulated lung fluid (Gamble's solution) over 5 days. PLGA-based NPs and natural polymer NPs exhibited the highest cytocompatibility and dose-dependent *in vitro* uptake, respectively, by human alveolar type-1 epithelial cells. Based on these profiles, gelatin and PLGA NPs were used to encapsulate plasmid DNA encoding yellow fluorescent protein (YFP) or rhodamine-conjugated erythropoietin (EPO) for inhalational delivery to rats. Following a single inhalation, widespread pulmonary EPO distribution persisted for up to 10 days while increasing YFP expression was observed for at least 7 days for both NPs. The overall results support both PLGA and gelatin NPs as promising carriers for pulmonary protein/DNA delivery.

1. Introduction

Nanomedicine in the area of pulmonary protein/DNA delivery has emerged as a cutting edge technology combining nanotechnology and pharmacotherapeutics for drug delivery and tissue remodeling. Conventional methods of delivering proteins and DNA are limited by low bioavailability, denaturing/instability of the product and variation between doses [1]. Delivery of nanoparticles (NPs) loaded with therapeutic agents via inhalation takes advantage of the ease and non-invasive nature of administration, the large alveolar surface area for rapid uptake, prolonged local action and a lower effective dose, resulting in lower risk of toxicity compared to systemic drug delivery [2]. For instance, Terzano et al. [3] recently developed non-phospholipid vesicles encapsulating beclomethasone dipropionate for the treatment of chronic obstructive pulmonary disease that can enhance penetration through the mucus layer and provide localized therapy. In addition, NPs under ~200 nm could theoretically escape detection by alveolar macrophages [4], leading to more effective uptake and action.

Different types of nanocarriers such as liposomes, lipid- or polymer-based micelles, dendrimers and polymeric NPs have been used for encapsulation and delivery of therapeutic agents to the lung [5]. Polymeric NPs are of growing interest, as the polymers can be co-polymerized, surface-modified or bioconjugated for better targeting capability and delivery of the encapsulated agents. The commonly used nanocarriers in pulmonary drug delivery include natural polymers such as gelatin, chitosan, alginate and synthetic polymers like poloxamer, poly(lactic-co-glycolic) acid (PLGA) and poly(ethylene glycol) (PEG) [6]. Gelatin is a biocompatible, biodegradable protein that covalently binds the active compound [7], resulting in greater loading efficiency. Chitosan, a polysaccharide, is a mucoadhesive and permeation enhancer that facilitates NP retention in the lung following administration [6]. Alginate is another highly biocompatible natural polymer with a

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hydrophilic matrix for efficient protein loading [8]. PLGA has been established as a biocompatible and biodegradable synthetic polymer approved by the US Food and Drug Administration that allows sustained drug release over a period of weeks to months, depending on the ratio of monomers used [9]. Further, there is growing literature to support the use of PLGA-based nano-/micro-particles for pulmonary delivery due to their biocompatibility and the option of tailoring their rate of drug release and biodegradation based on the intended applications [10,11] without causing tissue damage in the lung [12]. For example, *in vitro* studies by Tahara et al. [13] demonstrated that PLGA NPs with and without chitosan coating are cytocompatible with A549 lung epithelial cells up to a high concentration of 5 mg ml^{-1}. Recent studies on PLGA NPs prepared with poly(vinyl alcohol) (PVA) surfactant also demonstrated minimal inflammatory reaction and good cytocompatibility at <1 mg ml^{-1} concentration with A549 cells [14]. Further, histological examination of lung tissue sections following PLGA NP administration by intratracheal instillation have shown that these particles do not cause lung tissue damage [15]. PEG is known to improve the hydrophilicity, aerodynamic characteristics and retention time of NPs [16,17].

Due to the small size of NPs, they tend to remain suspended in air, making direct delivery to and deposition in the deep lung difficult. Therefore, the mode of pulmonary delivery also plays a crucial role in facilitating NP deposition and distribution in distal lung tissue. Use of a metered dose inhaler or a dry powder inhaler could result in significant oropharyngeal NP deposition and variation in dosage when the device is not shaken correctly [18]. The use of a nebulizer on the other hand could maintain a relatively constant size of aerosol droplets in the range (4–6 μm diameter) that easily allows the suspended NPs to reach the distal lung. For example, the celecoxib-loaded lipid nanocarriers developed by Patlolla et al. [19] (~217 nm size) were shown to deposit in the alveolar region of murine lungs following nebulization. A recent study demonstrated that aerosol droplets containing 5(6)-carboxyfluorescein-loaded NPs (195 nm) generated by an Aeroneb™ nebulizer possessed aerodynamic properties suitable for alveolar deposition [20].

A survey of literature indicates that although several polymeric NPs have been characterized for pulmonary delivery of different compounds, there have been no studies to the authors' knowledge that corroborated the *in vitro* cellular uptake and retention time of NPs with their behavior *in vivo*. Further, the optimum formulation that facilitates prolonged core compound delivery and release as well as comparatively longer retention in the lung is unknown. Therefore, it is essential that polymeric NPs are thoroughly evaluated in terms of physical and chemical properties and release efficacy of the therapeutic agent to choose the optimum nanocarrier for the specific type of compounds being delivered. Studies have been conducted previously to compare the properties of selected polymeric (e.g. PLGA, chitosan, gelatin) nano-/micro-particles for pulmonary delivery of therapeutic agents like tobramycin and rifampicin [12,21]. Recently, chitosan and PLGA-based NPs have also been developed for pulmonary delivery of proteins/peptides such as insulin and calcitonin [22,23]. However, it is essential to determine the most promising NP formulation that can efficiently deliver these core compounds to the alveoli for treatment of pulmonary ailments. Therefore, the goal of this project was to compare selected naturally and synthetically derived biocompatible polymer-based NPs encapsulating model proteins (bovine serum albumin (BSA) and rhodamine conjugated to recombinant human erythropoietin (EPO)) or plasmid cDNA (encoding yellow fluorescent protein, YFP) in terms of their physical–chemical properties, *in vitro* cell uptake and compatibility with human alveolar epithelial cells and *in vivo* pulmonary uptake following inhalation in rats. Our goal was to determine the most promising formulation(s) for further development as carriers for pulmonary delivery of biological agents.

2. Materials and methods

2.1. Synthesis of natural polymer-based NPs

Gelatin NPs were prepared by the two-step desolvation method described by Shutava et al. [24] Briefly, 0.05% (w/v) gelatin solution was prepared in deionized (DI) water and 25 ml of acetone was rapidly added to it. The gel-like precipitate obtained was re-dissolved in water and 75 ml of acetone was added dropwise at 40 °C to obtain a milky-white solution. 0.2 ml of 25% glutaraldehyde as a cross-linker was then added and stirred overnight, following which the solution was dialyzed and lyophilized to obtain gelatin NPs.

Chitosan NPs were prepared by ionic gelation using sodium tripolyphosphate (TPP) [25]. 0.2% (w/v) chitosan (Polysciences Inc., Warrington, PA) solution in 1% (w/v) acetic acid was adjusted to a pH of 5.5, following which TPP was added dropwise to allow the formation of particles. After 1 h stirring, the particles were dialyzed and freeze-dried.

Alginate NPs were prepared by cation-induced controlled gelification of alginate described by Rajaonarivony et al. [26] with slight modifications [27]. Briefly, 18 mM of calcium chloride was added dropwise to sodium alginate solution (0.06% w/v). Chitosan solution of concentration 0.05% w/v was then added followed by stirring overnight. The NPs were recovered by centrifugation at 19,000 rpm for 30 min, followed by lyophilization to obtain the NPs.

2.2. Fabrication of synthetic polymer-based NPs

The emulsion–solvent evaporation method was used to prepare PLGA NPs. For this procedure, 3% w/v PLGA (Lakeshore Biomaterials, Birmingham, AL) solution was prepared in chloroform. This solution was then added to an aqueous solution of 5% w/v PVA to create the emulsion, and sonicated. This particle suspension was stirred overnight at room temperature, allowing the solvent to evaporate. NPs were recovered by ultracentrifugation at 25,000 rpm for 30 min at 10 °C. For BSA-loaded NPs, 3% BSA solution (30 mg in 300 μl of DI water) was emulsified in PLGA solution, while for cDNA loaded NPs, 0.1% of the cDNA was dispersed in DI water and used for emulsification.

For the preparation of PLGA–CS NPs, carboxymethyl chitosan (CMC) was mixed with PVA solution and allowed to be adsorbed onto the surface of the PLGA NPs. The NP preparation procedure is similar to PLGA NPs except for the addition of 0.5% (w/v) CMC in 12 ml of 4.5% (w/v) PVA.

The copolymer of PLGA–PEG was synthesized by conjugation of COOH–PEG–NH_2 (Laysan Bio Inc., Arab, AL) to the free COOH groups on PLGA using carbodiimide chemistry. PLGA–N-hydroxysuccinimide (NHS) was obtained by the addition of excess NHS and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide to PLGA solution in dichloromethane. The resultant polymer was precipitated by cold methanol and vacuum dried. 1 g of PLGA–NHS was dissolved in 4 ml of chloroform and then 250 mg of COOH–PEG–NH_2 and 28 mg N,N-diisopropylethylamine was added and stirred for 12 h. The copolymer was precipitated with cold methanol and washed three times to remove unreacted PEG. This polymer was dried under vacuum and used further for NP preparation [28]. BSA was used as the protein model while YFP plasmid cDNA was used as the cDNA model for encapsulation within all six NPs. All NPs were lyophilized and stored in powder form at −20 °C when not being used. For all of our *in vitro* and *in vivo* studies, the particles were freshly constituted in either DI water, media or saline.

2.3. Characterization of NPs

The NPs were characterized for their particle size, polydispersity and zeta potential using dynamic light scattering (DLS;