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In vitro Sustained Release of alpha 1-antitrypsin from Poly (D,L-lactide-co-glycolide) Nanoparticles

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***Abstract*— Alpha 1-antitrypsin is a member of serine protease inhibitors which mainly inactivates neutrophil elastase and prevents the destruction of the pulmonary extracellular matrix. The main goal of the present study was to investigate ability of poly (D, L-lactide-co-glycolide) nanoparticles to carry this protein as a potential therapeutic agent for the protection of lung tissue against free proteolytic activity. Poly (D, L-lactide-co-glycolide nanoparticles with an average size of 550 nm were produced by the "oil-in-oil emulsification solvent evaporation method" and successfully loaded with the Alpha 1-antitrypsin protein during the production of the nanoparticles. The constructed nanoparticles were then characterized in terms of size; morphology and fourier transform infrared spectroscopy which was subsequently followed by studying the *in vitro* release of the protein.**

Keywords-Nanoparticles; Poly (D, L-lactide-co-glycolide); Alpha 1-antitrypsin; Oil-in-oil emulsification solvent evaporation

I. INTRODUCTION

Human alpha 1-antitrypsin (AAT) is a 56 KD glycoprotein which is secreted mainly by hepatocytes and alveolar macrophages and to a lesser extent by other tissues [1; 2]. This diffusible molecule is present in high concentrations in most tissues and is a major protein component of the lung epithelial lining fluid [3]. AAT has a wide spectrum of anti protease activity inhibiting several serine proteases, but neutrophil elastase is its primary protease target. AAT deficiency predisposes patients to emphysema, juvenile cirrhosis and hepatocellular carcinoma [4; 5]. The only existing treatment for AAT deficiency is augmentation therapy by intermittent intravenous administration of plasma derived AAT [6; 7]. Although this kind of therapy is effective and has little side effects [8], but it is highly expensive and because of AAT's short half-life in the bloodstream-approximately 6 days-patients need frequent administrations to maintain the therapeutic levels (~1.3 g/l) [9]. Exogenous doses of AAT such as 60 mg kg- every 7 days are recommended in patients with severe AAT deficiency [10]. However, long-term administration of weekly doses is not well-accepted by patients [11].

Recently, therapeutic agents like pharmaceutical proteins, nanoencapsulated in a polymeric matrix or absorbed or conjugated onto the surface, and that are administered orally or injected locally, have been used [12; 13; 14; 15]. These nanoencapsulated particles are useful for drug delivery in a convenient and controlled manner with smaller quantities of drugs and longer intervals when compared with non-encapsulated ones [16; 17; 18]. Nanoparticles (NPs) can release the embedded, absorbed or conjugated protein in a sustainable manner [19].

One of the most common types of NPs is made of poly lactic acid (PLA), poly glycolic acid (PGA), and their copolymer poly (D, L-lactide-co-glycolide) (PLGA). This polymer has been approved by the USA Food and Drug Administration (FDA) and has notable characteristics like excellent biocompatibility, controllable biodegradability and high safety besides its nontoxic nature [20; 21; 22]. The emulsification solvent evaporation method is used routinely for nano/microencapsulation [23; 24; 25; 26]. In the present work, we have explored the potential of a modified version of this method for the preparation of AAT-loaded PLGA NPs and subsequent characterization of the fabricated NPs.

II. MATERIALS & METHODS

A. Preparation of AAT

In this study we used recombinant AAT that is produced by the methylotrophic yeast *Pichia pastoris.* This recombinant protein is expressed under the control of the inducible alcohol oxids 1 (AOX 1) promoter, and is produced in a secretary manner. The secreted protein was purified from the other components of the medium by nickel-affinity chromatography and subsequently lyophilized (unpublished data).

B. Preparation of PLGA nanoparticles

AAT-loaded NPs were prepared by the oil in oil (o/o) emulsification solvent evaporation technique. For this purpose the polymer solution (3 mL) was initially prepared by dissolving 5 mg of PLGA copolymer (RG 504H® with a monomer ratio of 50:50, Boehringer Ingelheim, Germany) in acetonitrile. One miligram of AAT was then dissolved in 250

µl of deionized water and slowly added in a drop-wise manner to 3 mL of the polymer solution. The resulting mixture was added to 40 mL of viscous liquid paraffin (Merck, Germany) containing Span 80 (Fluka, Switzerland) and stirred for two hours to ensure complete evaporation of acetonitrile. Nanospheres were then collected by centrifugation at 20000 g for 30 minutes at 15 ºC and washed twice with n-hexane to remove mineral oil. Particles were filtered, dried and stored under refrigeration in desiccators until further used. Blank NPs were prepared according to the same procedure omitting protein.

C. *Study of the surface morphology and particle size of NPs by scanning electron microscopy (SEM)*

Microscopic characteristics of AAT-loaded PLGA NPs were investigated by SEM (Philips, XL30, Netherland). The samples were placed on a double stick tape over aluminum stubs to get a uniform layer of particles. They were then gold-coated using a sputter gold coater at a current of 40 mA and 10-3 Torr pressure for 400 seconds at a thickness of 400 Å. Gold-coated particles were subsequently cooled over dry ice to avoid being melted under high magnification due to exposure by electron beam.

D. *Encapsulation yield and efficiency*

The yield of nanoencapsulation was calculated as follows:

$$\text{Yield (\%)} = [(W2-W1)/W1] \times 100 \quad (1)$$

where W1 is the initial weight of polymer and W2 is the weight of particle collected on the filter surface.

The amount of protein loaded onto the PLGA NPs was determined directly by recovering the protein from the NPs. A ten milligram sample of dried NPs was accurately weighed, transferred to a separator funnel and dissolved in 2 mL of acetone. After adding 4 mL of phosphate buffer to the funnel, it was capped with a stopper and the resulting mixture was agitated in a shaker for two days. The funnel was then left hanging on a metal ring. After complete separation of the two aqueous and organic phases, the aqueous layer was collected for protein assay. A commercial human AAT enzyme-linked immunosorbent assay (ELISA) quantitation kit (GenWay, USA) was used to determine protein concentration in the aqueous extract using phosphate buffer as a mobile phase. The experiments were performed in triplicate. The encapsulation efficiency of the nanosphere was calculated as below:

$$\text{Encapsulation efficiency (\% w/w)} = (\text{actual drug content}/\text{nominal drug content}) \times 100 \quad (2)$$

E. *Study of in vitro protein release*

Two samples of PLGA NPs (10 and 15 mg) were weighed and each immersed in one milliliter of phosphate buffered saline (PBS) (pH 7.2) in 2-mL microtubes. The suspensions were then incubated at 37 ºC with constant shaking (120 rpm). At appropriate time intervals, one mL of the medium was removed from each tube and replaced by the same quantity of fresh PBS solution. The withdrawn media were analyzed using ELISA for quantitative determination of the released protein and its integrity. For all experiments, the samples were diluted as 1:100 in sample/conjugate diluent buffer (50 mM Tris, 0.14 M NaCl, 1% (w/v) bovine serum albumin (BSA), 0.05% (v/v) Tween 20, pH 8.0). Different concentrations of commercial human AAT (Sigma, USA) were used to construct a standard curve. The corresponding protein release-profiles were established by plotting of the cumulative percentage of the released protein (Eq. (3)) versus the time.

$$\text{Cumulative amount of released protein (\%)} = (\Sigma M_t/M_{actual}) \times 100 \quad (3)$$

where M_t is the amount of AAT released at time t, and M_{actual} is the actual amount of AAT- loaded onto the NPs.

F. *Study of protein-NP interaction through fourier transform infrared spectroscopy (FTIR)*

In order to investigate possible molecular interaction between protein and the polymer, FTIRspectroscopy (Perkin-Elmer, USA) of the pure protein, control PLGA NPs and AAT-loaded PLGA NPs was carried out. The FTIR spectra were collected in the range of 4000–400 cm^{-1} at room temperature and 2 cm^{-1} resolution, with a scan number of 128 and an optimized gain for all samples

III. RESULTS

A. *Characterization of AAT-loaded NPs*

The SEM micrographs of PLGA NPs and AAT-loaded PLGA NPs indicated that both types of particle have spherical shapes and relatively smooth surfaces (Fig. 1A & 1B). In comparison to the control NPs, AAT-loaded NPs were of a smaller size (with an average size of 550 nm) and lower yield with about 50% efficiency. The size and yield of NPs are depicted in table I.

TABLE I. PHYSICOCHEMICAL CHARACTERIZATION OF PLGA NPS, WITHOUT (BATCH NO.1) AND WITH AAT (BATCH NO.2)

Batch No.	Polymer Concentration (w/v%)	Stirring Speed (rpm)	Surfactant Conc. (v/v%)	Temp (□C)	Size (µm)	Yield (%)	Encapsulation Efficiency
1	1.25	5500	0.50	25	770	73.5	w/o AAT
2	1.25	5500	0.50	25	550	40.2	~ 50

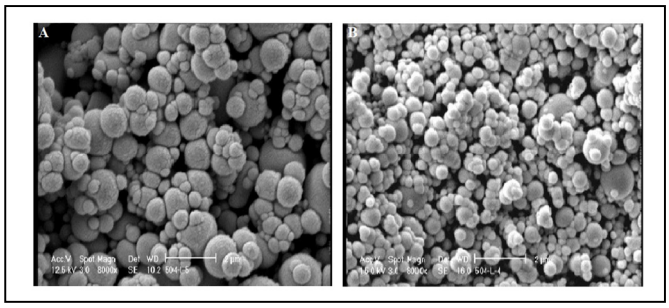

Figure 1. A) SEM images of control NPs, B) SEM images of NPs containing AAT

B. *In vitro release study*

An *in vitro* release study was performed to determine the protein release profile and assess the state of the encapsulated AAT. The resulting AAT release profiles indicated three phases; the first was an initial burst phase (day 1 to 3) with 60% of the AAT released. The second phase was an intermediate one (day 4 to 9) with a low and continuous release including approximately a 36% of release and finally a third phase (till day 36) was detected with a moderate burst (Fig. 2).

C. *Protein-polymer interaction*

The FTIR spectroscopy method was used to investigate the interaction between the functional group of the protein under study and the carrier NPs. The spectra of the PLGA NPs and AAT-loaded PLGA NPs showed two distinct peaks at wave numbers 1300-1450 cm^{-1} and 1720-1800 cm^{-1}, which were assigned to δCH and V(C=O), however, there were no changes in the position of peaks (Fig. 3).

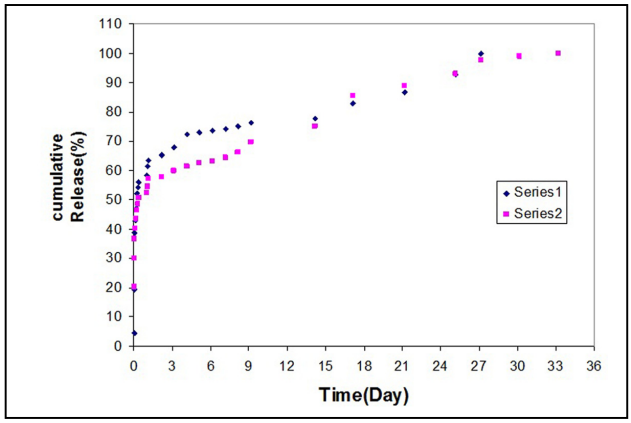

Figure 2. Release profiles of AAT from PLGA NPs (♦ 15 mg NPs, ■ 10 mg NPs).

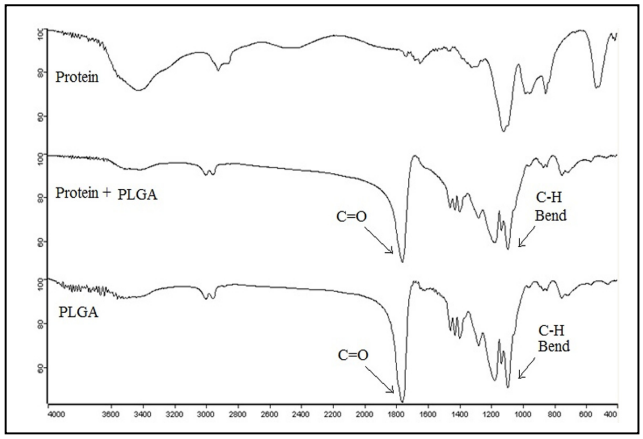

Figure 3. The FTIR spectra of AAT, PLGA+ AAT and PLGA NPs.

IV. Discussion

Pharmaceutical AAT protein is a blood-derived high-cost product and puts financial pressures on many patients in need. Isolating the products of this kind from blood is associated with main obstacles like source limitation and virus contamination [27; 28]. Although recombinant technology can overcome these problems, but it is still not enough and there are difficulties regarding short biological half-life of some proteins like AAT and subsequent need for repeated injections which is undesirable [29; 30; 31; 32]. Improving the manner of delivery could be an effective strategy in this case which makes the products more economical and affordable [33; 34; 35].

In present study, the PLGA polymer which has excellent biocompatibility and biodegradability properties was used for this purpose. The AAT protein was encapsulated in the PLGA polymer by an o/o emulsification solvent evaporation technique with significant efficiency. A drug-excipient interactions study employing FTIR spectroscopy was performed to determine if there was any interaction between the AAT protein and the PLGA polymer. FTIR spectroscopy is a powerful technique that can indicate interactions between the various functional groups of protein and excipient molecules [36]. Our results showed no chemical interactions between the protein and polymer. This suggests that the NPs can be used to formulate and deliver the AAT protein as a drug. The main limitation of this process is that the protein is physically bound to the NPs' surface and as a result, desorption of protein from the NPs' surface can be easily influenced by the environment. However, these facts can also help us to predict the characteristics of protein release from a formulation.

The other important feature of AAT-polymer formulation is protein release behavior. The profiles obtained during the release study show three different phases. The first fast release may be due to the AAT protein sitting on or embedded in the PLGA NPs' surface or it may have arisen from the lack of any chemical interaction between the protein and polymer. The second phase, showing a continuous slow release can be attributed to the solubilization of the nanocapsules. Finally, the third phase demonstrating a faster release could be the result of polymer degradation. In fact during 36 days of the *in vitro* protein release study, over 95% of the loaded protein was found to be released. Hence, the retardation of the burst phase and the slow release characteristics of the PLGA NPs, make this new biodegradable system a good carrier for prolonged protein drug delivery.

V. Conclusion

The present work has demonstrated the fabrication of the AAT protein- loaded PLGA NPs via a simple o/o emulsification solvent evaporation technique. Evaluation of these loaded NPs indicated that they have spherical morphology, high protein incorporation efficiency, and good stability. The protein was effectively protected by the starch environment when entrapped in PLGA NPs, demonstrating sustained release from its encapsulated polymer *in vitro*.

According to our results, this newly designed system can eventually be considered as a potent AAT protein delivery system *in vivo*.

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