ABSTRACT SUMMARY
The objective of this work was to develop a mucoadhesive film containing nanoparticles of lysozyme as a model protein. A recently developed antisolvent co-precipitation process was used to manufacture Lys-loaded submicron particles, before inclusion into polymeric films that were based on either polymethacrylates and/or HPMC. Films were characterized for mechanical properties, mucoadhesion, Lys release kinetics and activity after manufacture. All Eudragit RL films had acceptable mechanical properties, excellent mucoadhesive activity, and excellent remaining Lys activity. Moreover, the use of HPMC allowed for tailoring the lysozyme release rate.

INTRODUCTION
Mucoadhesive films as dosage forms for the buccal route of delivery have been investigated in the past decade but little efforts have been done with regards to the delivery of proteins and peptides in particulate forms [1,2]. From a formulation standpoint, actives are usually added to the film by their inclusion to the casting solution, then allowing it to dry into the solid form. However, in general the polymers utilized in formulations containing proteins are more hydrophobic in comparison to the hydrophilic nature of proteins [3]. This could potentially lead to separation and/or aggregation during storage, or in vivo, leading to possible instability [4].

Recent investigations of enzyme immobilization in organic solvents have opened the door for the manufacture of particulates-containing films with enhanced activity. In particular, the antisolvent co-precipitation method has been shown to produce particles coated with a variety of biologicals including nucleic acids, proteins, enzymes, and other particulate systems. However, most of these investigations lead to particles in the range of 1–5 μm or higher. To guarantee physical stability of the films in terms of mechanical and also mucoadhesive properties, such large particles are undesired due to potential for aggregation and loss in active distribution homogeneity [5]. Our group has recently published on the manufacture of submicron and nanosized particles of lysozyme (Lys) loaded D,L-valine (Val), also known as protein-coated nanoparticles (PCNP), and the advantages of this method of manufacture to provide high yield and enzymatic stability [6].

In this study we investigate the development of mucoadhesive films that are intended to aid the controlled delivery Lys, and maintain high enzyme activity throughout the manufacturing process.

EXPERIMENTAL METHODS
D,L-valine (Val) and lysozyme (Lys) were obtained and used as received (Sigma-Aldrich, St. Louis, MO). The manufacturing method is based in an antisolvent co-precipitation process as previously reported [6]. Eudragit RSPO and RLPO (ERS and ERL) were kindly donated by Evonik (Evonik Industries, Darmstadt, Germany). Carbopol® 974P (C974P) and Noveon® AA-1 Polycarbophil (PCP) were donated by Lubrizol (Lubrizol Advanced Materials, Cleveland, Ohio). Hydroxypropyl methylcellulose (HPMC, Methocel E50 Premium LV) was donated from Colorcon (Colorcon, Harleysville, Pennsylvania). Triethyl citrate (TEC; Vertellus Specialties Inc, Indianapolis, Indiana), mucin (Spectrum Chemical, New Brunswick, New Jersey).

Particle sizing was performed using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Enzymatic activity of lysozyme throughout was determined turbidimetrically based on the Shugar method [7]. Additionally, lysozyme concentrations were determined using a previously published validated HPLC method [6].

Casting solutions were prepared by combining two organic solutions and cast overnight in PTFE molds. Acetone was used to dissolve or suspend the polymer combinations as depicted in Table 1. This solution was combined in a 4:6 acetone to IPA ratio, with suitable amounts of Lys-containing IPA, to yield the final casting solution. After 24 hours, films were peeled off and stored in aluminum foil sachets in a desiccator until characterization. Control films of C974P and PCP were also prepared with no lysozyme.

Mucoadhesion tests were conducted on a TA.XTPlus texture analyzer ( Stable Micro Systems, Godalming, UK) equipped with a 5 Kg load cell. Briefly, films were held in the horizontal position and 5 μL of model mucus (a freshly made 2% w/v mucin solution) was placed on top of the film. A 7 mm diameter stainless steel cylindrical probe was attached to the mobile arm of the texture analyzer and it was brought in contact with the film (1 x 5 cm²) attached to the texture analyzer and mucin solution. The film was held at an applied force of 50 mN for 15 seconds and then withdrawn at a 0.5 mm/second rate. Mucoadhesive force (MAF) and work of adhesion (WoA) were obtained from the peak and the area under the curve in the force versus distance profile, respectively.
Lysozyme release was performed on Franz diffusion cells with phosphate buffer pH 6.8 as media.

A Hitachi S-5500 field emission scanning electron microscope (SEM, Hitachi High-Technologies Corp., Tokyo, Japan) was used for imaging of Pt/Pd-coated particles and cross-sections of films.

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Table 1. Film formulation compositions (as % w/w) that were studied to investigate drug release and uniformity of films containing lysozyme. *Control.

RESULTS AND DISCUSSION

Particles obtained in optimized conditions allowed for a z-average of 439 nm with a yield around 50% and remaining activity of 99%, following the manufacturing process. Nanoparticles with an enzyme load of 40% w/w, were obtained using the processing condition outlined above. SEM observation of cross sections of film formulations obtained by freeze fracture reveals a uniform distribution of the flake-like particles (lysozyme) throughout the polymeric matrix (data not shown).

When ERL was used as the only polymeric material the MAF achieved was higher (but not statistically different, p>0.05) and the WoA was significantly higher than any of the other formulations studied (data not shown). The absence of particulate material in the formulations containing only C974P and PCP leads to their inherent mucoadhesion that results from the capacity of the polymer to absorb water and plasticize the polymer chains to interact with mucin. It should be noted that the addition of HPMC hinders the full extent of mucoadhesion enhancement possibly by capturing the particles (that are more hydrophilic) in HPMC-rich domains. This results in slower hydration and thus a weaker mucoadhesive bond that mostly depends on the mucoadhesion of HPMC. HPMC has been used in the past as a mucoadhesive material but its mucoadhesive power is lower than that observed for PCP and C974P.

ERS exhibited higher MAF than results of ERL films with drug in solid solution. This in interesting considering that ERS is the more hydrophobic material due to its lower content of quaternary ammonium groups. We have previously shown that ERS consistently exhibits lower MAF and WoA in comparison to ERL. However, we believe that the enhancing effect of the water-soluble particulate material discussed earlier is responsible for this higher extent in mucoadhesive properties.

From the drug release profiles we can observe an increase in the release rate and extent of release as the concentration of HPMC increased in the formulations (FPH01–FPH04, Figure 1). HPMC is a water swellable and erodible polymer that will dissolve from the dosage form; therefore, increasing concentrations of HPMC in formulations allow for domains in the film that will release Lys faster than ERL-rich domains. In accordance with the similarity value, F2, FPH03 and FPH04 are the only formulations that render a similar Lys release profile. Therefore, an increase in the HPMC content from 30 to 50% w/w of polymer did not elicit significant differences in the release profile. According to the Korsmeyer-Peppas model, even though FPH04 has a higher constant (k = 0.2800 for FPH04 and 0.2255 for FPH03) contributing to faster release at earlier times, the higher exponential term of FPH03 (n = 0.6604 for FPH03 and 0.4875 for FPH04) allows for faster release at later times.

CONCLUSIONS

Lys incorporated into Eudragit RL containing films demonstrated excellent mucoadhesive properties with sustained the release of Lys over 4 hours. The release rate of Lys was modulated by the use of HPMC.

REFERENCES

7. D. Shugar, Biochimica et Biophysica Acta. (1952) 8, 302–309.