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Inhalable, bioresponsive microparticles for targeted drug delivery in the lungs.

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Title: Inhalable, bioresponsive microparticles for targeted drug delivery in the lungs

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Abstract:

There is a growing interest in developing bioresponsive drug delivery systems to achieve greater control over drug release than can be achieved with the conventional diffusion controlled polymeric delivery systems. While a number of such systems have been studied for oral or parenteral delivery, little or no work has been done on bioresponsive delivery systems for inhalation. Using the raised elastase levels present at sites of lung inflammation as a proof-of-concept model, we endeavoured to develop a prototype of inhalable elastase sensitive microparticles (ESMs). Microparticles degradable by the enzyme elastase were formed by crosslinking the polymer alginate in the presence of an elastase substrate, elastin, using Ca^{+2} ions and subsequent spray drying. The bioresponsive release of a protein cargo in the presence of elastase demonstrated the enzyme-specific degradability of the particles. The microparticles showed favorable properties such as high drug encapsulation and good powder dispersibility. Potential polymer toxicity in the lungs was assessed by impinging the microparticles on Calu-3 cell monolayers and assessing changes in transepithelial permeability and induction of cytokine release. The microparticles displayed no toxic or immunogenic effects. With a manufacturing method that is amenable to scale-up, the ability to be aerosolised efficiently from a first-generation inhaler device, enzyme-specific degradability and lack of toxicity, the ESMs show significant promise as pulmonary drug carriers.

Keywords: Elastin, Alginate, Pulmonary drug delivery

1. Introduction

Drug release from traditional polymeric drug delivery systems (DDS) occurs when the polymer degrades in the body *via* non-specific chemical reactions such as hydrolysis of ester linkages in the polymer. These DDS have many disadvantages such as inconsistent drug release kinetics, lack of response to physiological changes occuring in the body and significant inter and intra-patient variability[1]. To overcome these limitations, research in the past decade has focused on the development of bioresponsive polymers that can respond to various physiological stimuli in the local environment to release their drug payload. Enzyme-sensitive DDS are of growing interest. Certain disease processes e.g. cancer, infection and inflammation, are characterised by high levels of activity of specific extracellular and/or intracellular proteases. Bioresponsive DDS harnessing these increased enzyme levels to target drugs to the diseased site are being explored[2]. Enzyme-controlled drug release in tumors by tumor-associated proteases (*e.g.*, plasmin) and in the gastro-intestinal tract by digestive enzymes has been reported [3-4]. An advantage with enzymatic cleavage is that it breaks down polymers faster than the normal hydrolytic mechanism. This prevents accumulation of the carrier at the site of delivery. Secondly, drug release from such systems occurs only in the presence of the disease-specific enzyme and not at other sites. This allows spatial control of drug release (i.e, releasing drug only where it is required) to be achieved.

Enzyme-responsive DDS can be particularly useful to achieve local drug delivery at sites of inflammation. Inflammation is an immune response that is characteristic of many disease states. It is implicated in wound healing and many diseases such as cancer, rheumatoid arthritis, diabetes, infection etc [5] . During this process, immune cells, especially neutrophils infiltrate the site of injury and secrete a number of proteases (e.g. neutrophil elastase (NE)) in an attempt to remove the harmful stimuli. Inflammatory lung conditions such as chronic bronchitis and emphysema show high levels of NE[6]. By developing an inhalable elastase-sensitive DDS, it could be possible to restrict drug release at sites of lung inflammation, thereby providing a combination of temporal and spatial control of drug delivery to the patient[5]. By delivering their therapeutic payload directly to the site of inflammation, such DDS not only provide enhanced targeting but also minimize drug

associated side-effects through a reduction of the dose that needs to be delivered. Inflammation being a common feature of many disease conditions, such systems can also potentially be used to target anti-inflammatory drugs to other sites in the body.

In situations where the use of a single polymeric material does not satisfy all drug delivery requirements (e.g. resistance against rapid dissolution in aqueous media), a combination of two different polymers have been used to produce a new material that displays the combined advantages of the two individual polymers[7]. Such a system is termed an IPN (Inter-penetrating network). An IPN is a material which comprises two or more networks which are fully or partially interlaced on a molecular scale but not covalently bonded to each other and cannot be separated unless chemical bonds are broken. Enzymatically degradable IPNs consisting of a protein and a polysaccharide have been reported. For example, IPN hydrogels made of gelatin and dextran have been studied as materials for biodegradable implants and as carriers for drug delivery[8].

Elastin is one of the primary substrates of NE. It is an extracellular matrix protein found in skin, blood vessels, and tissues of the lung. It imparts elasticity and flexibility to these tissues. Elastin-based biomaterials are being increasingly studied for tissue engineering and drug delivery applications[9]. Elastin particles having diameters between 200nm to 10µm and containing fluorescently-labelled model drugs were prepared as potential drug delivery systems by a novel approach using lyophilisation. The particles underwent degradation in the presence of elastase to release their cargo[10]. No such delivery systems have been studied for treatment of lung diseases. In this study, we sought to determine whether DDS based on elastin could be used as inhalable drug carriers for potential treatment of inflammatory lung diseases. Bovine serum albumin was encapsulated within the particles as a model protein.

2. Materials and methods

2.1 Materials

Bovine Serum Albumin-Fluorescein Isothiocyanate (BSA-FITC), Sodium fluorescein (Na-Flu), low viscosity alginic acid sodium salt from *Macrocystis pyrifera*, and elastin, soluble from bovine neck ligament were purchased from Sigma Chemical Co. (St. Louis, MO,

USA). D-Mannitol (Mannidex®) was a gift from Cerestar, Belgium. Calu-3 cells were obtained from American Type Culture Collection, Rockville, MD, USA. Cell culture media and supplements were purchased from Gibco BRL, Paisley, Scotland. Tissue culture plastics were from Sarstedt AG & Co., Germany and Transwell clear polyester inserts (12mm diameter, pore size 0.4μm) from Corning Costar, Corning, NY.

2.2 Preparation of protein-loaded microparticles

Alginate was dissolved in DI water at 0.3%w/v. Elastin and BSA-FITC were added to this solution (Elastin:alginate ratio = 1:6, BSA-FITC: polymer =1:100) and stirred to dissolve. The solution was homogenized at 6500 rpm for 10 minutes while 10ml of $CaCl_2$ solution (10mM Ca^{+2}) was added at a constant rate dropwise. The resulting mixture was spray dried using a laboratory Buchi 190 spray dryer (Buchi, Flawil, Switzerland). The following conditions were used: Inlet temperature: 170ºC, Outlet temperature: 56 ºC, Pump flow: 22%, Aspirator: 75%. The recovered particles were stored in a desiccator at 4°C until further use.

2.3 Protein loading

To determine the encapsulation efficiency (%EE) of BSA-FITC, 10mg of microparticles were completely dissolved in 5ml of an aqueous solution of 0.1M sodium citrate by magnetic stirring for 4 hours[11]. The protein content was analysed by UV spectrophotometry at 495nm (Biochrom, UK).

2.4 Particle characterisation

Morphological assessment was performed using a Hitachi scanning electron microscope (Model S3500N) after mounting the preparations on studs and sputter–coating with gold (Polaron SC500 Gold Sputter Coater, Quotum technologies, Newhaven, UK). The particle size of the microparticles was determined by laser diffraction (Malvern Mastersizer 2000, Malvern Instruments Ltd, Malvern, UK) following suspension of the microparticles (~25mg) in 5ml ethanol and bath sonication for one minute (320 W, Branson Ultrasonic, Danbury, CT). The density of the microparticles was determined as the tapped density using a tap density tester (Copley Scientific, Nottingham, UK)[12].

2.5 Determination of aerosolisation efficiency

An Andersen Cascade Impactor (ACI) (Copley Ltd., Nottingham, UK) was used to determine the fine particle fraction (FPF) of the microparticles. To avoid particle bounce from the plates and re-entrainment in the flowing air, each plate of the impactor was coated with a solution of Tween 80 in acetone (5%w/v). Acetone was evaporated by placing the plates in an oven at 60°C for 5 minutes[13]. About 5mg of the blend was manually loaded into the Diskhaler® and aerosolised by drawing air at a flow rate of 60 $lmin^{-1}$ for 4 seconds through the ACI. Five such doses were discharged into the apparatus. The apparatus was subsequently dismantled and each stage along with the inhaler device were washed with appropriate volumes of DI water and collected separately. The collected samples were freeze dried to remove water. The freeze dried mass was completely dissolved in an aqueous solution of sodium citrate (0.1M) by magnetic stirring for 4 hours. The solutions were then analysed for protein content using reverse-phase HPLC (Perkin Elmer, Model Series 200). The system was equipped with a Gemini C_{18} column (5μm, 250 x 4.6mm, Phenomenex, UK) and a UV detector (Perkin Elmer, Model Series 200). The following conditions were used: detection wavelength -220nm; mobile phase A: 0.1%v/v trifluoroacetic acid (TFA) in water, mobile phase B: 0.08%v/v TFA in acetonitrile; flow rate: 1ml/min[14]. The emitted dose (ED) is the total drug mass exiting the inhaler. The Fine particle dose (FPD) was calculated as the cumulative amount of drug recovered from Stage 1 to Stage 6 of the device. The fine particle fraction (FPF) is the FPD expressed as a percentage of ED. The experimental mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) of the particles were also calculated[15]. All experiments were carried out in triplicate.

2.6 In-vitro protein release studies

A Franz diffusion cell was used to carry out in-vitro protein release studies. The apparatus consists of two chambers, the donor and the receptor, which were separated by a cellulose acetate (CA) membrane. The receptor chamber of the cell was filled to contain 10ml of pH 7.4 phosphate buffer. 5mg of the microparticles were uniformly deposited on the CA membrane. 50µl of 0.1M PBS (pH 7.4) with or without porcine pancreatic elastase

(100μg/ml) was added to the donor chamber containing the particles to evaluate elastase-induced degradation of the microparticles and its effect on the release of BSA-FITC. Pure cross-linked alginate microparticles (without elastin) were also manufactured and subjected to the same treatment to verify whether alginate had any effect on elastase-mediated degradation. Specificity of elastin-alginate microparticles to elastase was verified by exposing them to another serine protease, trypsin at the same concentration (100μg/ml). Samples (250μl) were withdrawn from the receptor compartment at regular time intervals and analysed spectrophotometrically at 495nm for the content of BSA-FITC. The release study was also done in the presence of 'mock' sputum to determine it's effect on elastase activity. 5%w/w gastric mucin was dispersed evenly in DI water[16]. Elastase solution was mixed with the mucin suspension such that 100μl of the suspension contained 5μg of Elastase. This suspension (100μl) was evenly spread over the CA membrane. 5mg of microparticles were then deposited uniformly on the mucin layer.

The release profiles of the untreated and elastase-treated microparticles from all formulations were compared for similarity using the Fit Factor, f_2 described by Moore and Flanner[17]. This test has also been adopted by the US Food and Drug administration for comparison of the dissolution profiles of immediate release solid oral dosage forms[18]. In this test, the average dissolution value from each time point for each formulation is compared and the overall similarity is calculated. When two release profiles are similar, f_2 has a value between 50-100[18].

2.7 Cytotoxicity studies

Calu-3 cells, an adenocarcinoma cell line derived from a 25 year old Caucasian male were purchased from the American Type Culture Collection (Rockville, Md., USA) at passage 14 and used between passages 20 and 50.

2.7.1 MTT assay

For viability studies, the cells were plated at a density of 3 x 10^4/well in a 96-well format at 37°C in 5% CO_2. After 24 hours of culture, a suspension containing 1mg of microparticles per ml of growth media was added to each well and incubated for 24 hours at 37°C and 5% CO_2. Cellular viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide) colorimetric assay[19]. Untreated cells were used as controls and the OD value at 570 nm for untreated cells were considered as 100% viability.

2.7.2 Sodium Fluorescein transport

An in-house developed device was used to impinge the microparticles on to Calu-3 monolayers cultured on Transwell® inserts[20]. To determine the quantity of microparticles deposited on the cell monolayer, deposition reproducibility studies were conducted for each formulation. 1mg of spray dried microparticles containing Na-flu were loaded into the inhaler and aerosolised onto slightly wetted Transwell filter inserts containing no cells (n=5). The powder deposited on the filters was analysed for the content of Na-flu using a fluorescence plate reader (Wallac Victor, Perkin Elmer, Cambridge, United Kingdom) at excitation and emission wavelengths of 488 and 530nm respectively.

After quantifying deposition, blank microparticles of each formulation were aerosolised onto the cell monolayers. The filter inserts were then placed into new wells containing 1.5ml of bicarbonated Krebs-Ringer (KRB) solution in the basolateral compartment. 0.5ml of a 50μM Sodium fluorescein (Na-flu) solution in KRB was added to the apical compartment of each well. 100μl samples were taken at predetermined intervals up to 4hours from the basolateral compartment and replaced with an equal amount of fresh buffer. The fluorescence of Na-flu was measured in 96 well plates using a fluorescence plate reader as above.

2.8 Immunogenicity

Basolateral media collected after 4 hours exposure to the microparticles were analysed for IL-8 levels using ELISA MAX™ Kit (Biolegend, Inc., San Diego, CA, USA).

2.9 Statistical analysis

Data for each experiment was expressed as mean ± standard deviation. The results were analysed for statistical significance using unpaired Student's *t*-test. A probability level of <0.05 was considered to be statistically significant.

3.0 Results

3.1 Physico-chemical characterisation of microparticles

The geometric diameter of the particles as determined by laser diffraction was 4.12±0.65µm. The tapped density of the particles was 0.09±0.02g/cm^3. Using the two data, the calculated aerodynamic diameter of the particles was 1.24µm. The MMAD of the particles as determined using the ACI was 3.65±0.26µm with a GSD of 1.84±0.06. SEM studies showed that alginate-elastin particles had a raisin-like or corrugated morphology (Figure 1, A to C) possibly due to adsorption of elastin at the air/liquid interface of the droplets in the spray[21]. Crystals of the crosslinking agent, $CaCl_2$ were also seen embedded to the surface of these particles.

The encapsulation efficiency of BSA-FITC in the particles was 96.8±5.7%. Aerosolisation studies using the ACI showed the fine particle fraction of the microparticles to be 31.3±7.5%. The quantity of protein deposited at each stage of the impactor is shown in Figure 2, expressed as % of the loading dose.

3.2 Bioresponsive protein release *in-vitro*

Protein release studies for alginate-elastin particles were carried out using Franz diffusion cells in the presence and absence of elastase. The concentration of elastase used for the studies was within the reported range for sputum samples from infected bronchitic and cystic fibrosis patients, which is 26-100µg/ml[22]. Complete release of BSA-FITC was seen with the alginate-elastin microparticles that were exposed to elastase while less than 10% of the protein was released with the controls i.e. non-elastase exposed particles (Figure 3A). The two release profiles were compared for similarity using the Fit factor (f_2) model. The value of f_2 was 15.6 indicating a significant difference between the 2 release profiles. Trypsin had no effect on the rate of degradation of the microparticles with the level of protein release being similar to the untreated controls (Figure 3A). Cross-linked alginate particles (without elastin) that were treated with elastase also showed a protein release profile that was similar to the controls.

Elastase treated alginate-elastin particles showed similar drug release profiles in the presence and absence of ‘mock’ sputum indicating that sputum had no effect on elastase

activity (Figure 3B).Comparing the two release profiles using the Fit factor model gave a value of 78.7 for f_2. In the presence of mock sputum, alginate-elastin microparticles that were exposed to elastase showed significantly higher release of BSA-FITC as compared to the control samples (not exposed to elastase) (Figure 3B). Using the Fit factor model gave a value of 18.9 for f_2 indicating a significant difference between the two release profiles.

3.3 Cytotoxicity and immunogenicity

With the MTT assay, after 24 hours exposure to the microparticles, no significant difference ($p<0.05$) in viability was observed between the treated cells and the control (Table 1). Deposition reproducibility studies using blank filters (without cells) showed that, of the 1mg of microparticles that were initially loaded in the inhaler, 564±122μg was reproducibly delivered on to each insert using the in-house device. The apparent permeability coefficient (P_{app}) of Sodium Fluorescein for cell monolayers that were impinged with the microparticles was similar to the control ($p<0.05$) (Table 1) and no permeation enhancement effects were therefore evident. Basolateral media collected after 4 hours exposure to the microparticles was analyzed for the inflammatory marker, Interleukin (IL) -8. No significant increase in the level of secretion was observed ($p < 0.05$) (Table 1).

4.0 Discussion

While site-specific drug delivery after oral administration of drugs using polymers has become common-place e.g. enteric coated tablets, little work has been done to-date on harnessing polymeric carriers for site-specific delivery in the lungs after inhalation. Given that airway inflammation in asthma and COPD is associated with high levels of active neutrophil elastase[6], polymeric carriers that undergo degradation in the presence of this enzyme could be used to achieve targeted delivery of anti-inflammatory drugs to the lungs.

Spray drying was used in this study as a one-step process to produce microparticles of inhalable size. To stabilise the particles, an ionic crosslinking based approach was explored. This involved combining a specific elastase substrate, elastin with a polysaccharide, alginate. Conventionally used chemical crosslinking agents (eg. aldehydes) during particle preparation are considered toxic[23]. An alternative approach was harnessed in this study. Crosslinking of alginate was achieved using Ca^{+2} ions to produce a

matrix in which elastin molecules were interwoven with crosslinked molecules of alginate. With this technique, the integrity of the individual polymers was maintained as no covalent modification was required. $CaCl_2$ is currently used in a marketed inhalable formulation of rhDNase, Pulmozyme® with patients inhaling upto 0.8mg of the salt per day[24]. For the present study, assuming a daily dose of 40mg of the powder and an inhaler delivery efficiency of 30-40%, 12-16mg of the formulation is emitted from the device[25]. About 40% of the emitted dose is considered to deposit in the central and intermediate lung (i. e. 4.8-6.4mg)[26]. The quantity of $CaCl_2$ from this dose works out to be 1.2-1.5mg which is close to the marketed formulation. IPNs also have advantages for enzyme targeted drug delivery systems (DDS) as it is possible to control the rate and extent of enzymatic degradation of the DDS by varying the proportion of the enzyme sensitive polymer within the IPN network[27]. *In vitro* protein release studies showed low non-specific release of BSA-FITC (7%) from the particles over a period of 4 hours. On contact with the small volume of release media present on the apical side of the franz cells (at 37°C), soluble elastin possibly undergoes reversible coacervation to form a mucilaginous precipitate[28]. An insoluble calcium alginate gel layer would also form due to dissolution of the crystals of $CaCl_2$ as they come in contact with the dissolution medium. This combined viscous gel barrier inhibits penetration of the dissolution medium into the particle matrix thereby reducing non-specific release of BSA-FITC from the microparticles. On treatment of alginate-elastin microparticles with elastase, complete release of BSA-FITC was observed within 4 hours. Elastin is rich in hydrophobic amino acids such as alanine, valine, proline and glycine that form a large number of elastase-cleavable sites within the protein[29]. Ca^{+2} ions in this study were used to crosslink alginate while no crosslinking agent was used for elastin. And yet, a dramatic increase in the release of BSA-FITC was observed when the particles were exposed to elastase. This suggested that elastin was important in maintaining the integrity of the particles and preventing non-specific protein release. In studies conducted on enzyme degradable IPNs consisting of gelatin and dextran, Kurisawa et al. reported the presence of intimate physical chain entanglements between the two polymers[8]. Among proteins, elastin is unique in that it can self-assemble at 37ºC via interactions between its hydrophobic amino acids to form cross-links *in-situ*[9]. This coacervation process could also be promoted via ionic interactions between the lysine

residues of elastin and the carboxylate moieties in alginate[30]. Thus even in the absence of an external crosslinking agent, elastin could have reinforced the network through the combined effect of self-association and inter-polymer chain entanglement. Elastase had no effect on protein release from crosslinked alginate microparticles that did not contain elastin. To determine whether alginate-elastin microparticles would undergo degradation by other enzymes, the microparticles were exposed to another protease, trypsin. Trypsin had no effect on the release of BSA-FITC from the particles with the release profile being similar to the controls (untreated microparticles). Trypsin cleaves mainly at sites that contain positively charged amino acids such as arginine or lysine[31]. These amino acids are present in very low amounts in elastin[32].

Mucus accumulation in the airways is a characteristic feature of lung diseases such as cystic fibrosis. Particles carrying drugs to treat such conditions are likely to deposit on lung mucus and interact with its components. For example, interaction of alginate with the glycoproteins present in mucus (mucins) has been reported to induce the formation of a viscoelastic gel[33]. Such a gel formation could inhibit elastase-mediated degradation of the particles. To evaluate this, hydrated gastric mucin, which consists of high molecular weight glycoproteins from the porcine stomach, was used as a model to study possible effects of alginate-mucin interaction on elastase-mediated protein release[16]. As shown in Figure 2B, the presence of mucin had no impact on the rate and extent of elastase mediated degradation of the particles. However, in the absence of elastase, the mucin caused about 24% BSA-FITC to be released from the microparticles non-specifically. Without sputum, non-specific release had been low at 7%. During the release study, the presence of a hydrated mucin layer (sputum) on the surface of the filter membrane meant that these microparticles were wetted to a greater extent than the particles that were deposited on the membrane alone (without sputum). This increased wetting and consequent dissolution or erosion of the polymeric network could be responsible for the higher non-specific protein release observed with the alginate-elastin microparticles in the presence of the sputum.

Interactions between uncharged particles occurs mainly via Van der Waals (VDW) forces. These are attractive forces that arise due to the formation of dipoles in a molecule. The magnitude of the VDW forces (F) between two ideally smooth spheres of diameters d_1 and d_2, separated by a distance r in vacuum is given by the following relation[34],

$F = (A / 12r^2) \times (d_1 d_2) / (d_1 + d_2)$

where A is a constant.

As seen from the above relation, interparticulate forces decrease as a function of increased distance of separation between the particles and hence, any means of increasing this distance would reduce particulate interaction. Particles having small surface protrusions experience reduced VDW attractive forces since the protrusions increase the separation distance between the particles.

Alginate-elastin particles had a highly corrugated surface morphology with prominent grooves and ridges. Crystals of $CaCl_2$ also protruded outwards from their surface. These factors would have increased the distance of separation between the particles thereby reducing cohesive VDW forces. This would explain the high dispersability and respirable fraction observed with these particles.

5.0 Conclusion

The use of an IPN as an enzyme targeted drug delivery system for the inhaled route has not been reported before. In this study, we have shown that IPNs can be readily manufactured by spray drying into inhalable microparticles, a method that is easily scaled-up. The particles provided controlled and enzyme-specific drug release and were easily aerosolised. Even though *in vitro* assays did not reveal any particle induced cytotoxic or immunogenic effects, other factors such as the accumulation of these polymeric carriers within the lungs on prolonged administration must be considered.

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Table legends:

Table 1: Effect of exposure of Calu-3 cells to Alginate-elastin microparticles with respect to cellular viability, apparent permeability (P_{app}) of Sodium-fluorescein and secretion of IL-8 (n=4±SD)

Figure legends:

Figure 1: Scanning electron micrographs of BSA loaded alginate-elastin microparticles: (A) at 30,000X magnification; (B) and (C) at 80,000X magnification

Figure 2: The deposition of BSA-FITC loaded microparticles in different stages of an Andersen cascade impactor operated at 60 l/min (n=3±SD).

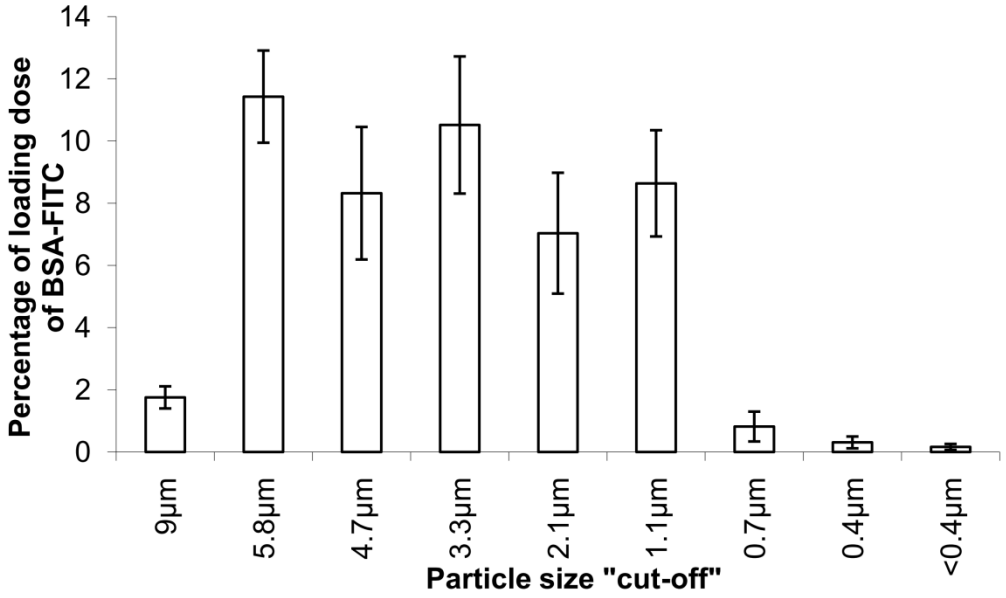
Figure 3A: BSA-FITC release profiles from alginate-elastin microparticles treated with/without elastase or with trypsin (n=3±SD).

Figure 3B: BSA-FITC release profiles from untreated or elastase treated alginate-elastin microparticles in the presence and absence of 'mock' sputum (n=3±SD).

Table 1:

Treatment	P_{app} (x 10^{-7}cm/sec) of Fluorescein Sodium	% cell viability	IL-8 (pg/ml)
Control	1.07±0.11	100±7.6	219±28
Alginate-elastin microparticles	0.67±0.14	111.2±6.7	231±66

477 Figure 2



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