

# Triggered release of inhaled insulin from the agglomerated vesicles: Pharmacodynamic studies in rats

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## Abstract

An aerosol insulin carrier based on the agglomerated vesicle technology that the authors have previously advanced [E. Karathanasis et al. *J. Control. Release* 103 (2005) 159–175] was evaluated *in vivo*. The carrier consisted of insulin-loaded liposomes cross-linked via chemical bridges cleavable by cysteine. It was speculated that the cleavage of the cross links released internal surface area and possibly resulted in the disruption of the liposomal walls. The result was a rapid release of encapsulated insulin upon contact of the insulin carrier with cysteine. The particles exhibited a small aerodynamic diameter within the respirable range suggesting deposition into the deep lung of humans along with a large geometrical diameter, consistent with long residence time. Indeed the endotracheal instillation of the particles into hyperglycemic rats decreased the glucose levels rapidly while delivery of cysteine triggered a further drop of glucose implying acceleration of insulin release from the carrier. Euglycemic clamp studies verified the accelerated insulin release upon application of cysteine.

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## 1. Introduction

### 1.1. Pulmonary delivery of insulin

Even though subcutaneous insulin cannot replicate the rapid action of pancreas to glucose changes, substantial progress has been achieved in controlling blood glucose level. Short-acting injectable insulin analogues (lispro and aspart) are absorbed and cleared rapidly reducing the risk of hypoglycemia after a meal [1,2], while the development of long-acting injectable analogues (glargine, NPH, lente and ultralente) maintains basal insulin levels [3]. Therefore, improved management of diabetes can be achieved by a dose of lispro or aspart just before mealtime coupled to a daily dose of glargine insulin. The self-monitoring

of blood glucose with fingerstick devices is an additional tool to control diabetes better.

Yet, while patients suffering from diabetes live longer due to these remarkable advances, they have shorter lives [4], and a lower quality of life than non-diabetics [5]. One reason for this reduction in the overall duration and quality of life is that periodic insulin injections have a poor control of glucose levels close to normoglycemia. The increased incidences of heart disease, hypertension and renal failure in diabetics have motivated studies into the role of insulin dose and frequency. A long-term study [6] investigated the effect of an intensive insulin therapy involving 5 or more daily injections or continuous subcutaneous infusion via a pump. It was demonstrated that the prevalence of hypertension is twice as high in the groups of diabetics treated under the traditional injection frequency compared to groups being treated with an intensive insulin therapy. The outcome of this 10-year study was that a much tighter range of blood glucose levels [7–9] achieved by a more frequent insulin administration benefited diabetics resulting in a significant reduction in cardiovascular diseases,

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retinopathies and nephropathies. Therefore, there has been an increased effort to develop an insulin delivery system that will be able to mimic the function of healthy pancreas.

While the traditional route for the delivery of insulin injection has itself undergone substantial changes, the need for a more intensive insulin therapy of diabetes has resulted in the quest for improved methods for the delivery of insulin. In order to better mimic physiological insulin secretion, subcutaneous insulin infusion or implantable pump has been developed [10]. However, the invasive nature of the pumps and multiple subcutaneous injections limits patients' compliance. Other strategies utilized subcutaneous implants of glucose-sensitive insulin–polymer complexes [11–13]. Thus, improvement of insulin delivery via a non-invasive, more convenient administration route is sought. In addition to the oral and the inhalation routes, there have been numerous efforts on transdermal and even on the ocular delivery of insulin [14].

It has long been recognized that insulin has a high permeability through the alveolar membrane and consequently, the release of free insulin in the region of the alveoli is a suitable method to transport significant amounts of the drug into the blood stream [15–18]. A number of inhaled insulin programs are underway today, in various stages of clinical trials [17]. An inhalable insulin formulation is close to commercialization in the U.S. and European Union [19], but is a short-acting insulin, requiring an injection of a long-acting insulin to control overnight glycemic episodes. However, a breakthrough discovery by Edwards et al. [20] in the late 1990s showed that large porous particles with aerodynamic diameter suitable for deep lung delivery, but geometric diameter too large for macrophage uptake, can facilitate extended release in the lung. This remarkable advancement motivated many investigators to fabricate porous microspheres for pulmonary insulin delivery exhibiting sustained release [21,22]. Extended release of insulin from the lung therefore appears to be very feasible.

### 1.2. Triggered release of inhaled insulin

While these remarkable advances permit controlled release of insulin at some pre-programmed rate, they do not permit modulation of this rate on demand. To address this need, a new technology [23,24] based on chemically agglomerated vesicles (the AVT technology) has been advanced that allows the modulation of the release rate from long-residence time particles deposited in the lung. The AVT particle consists of a microparticle agglomerate of nano-sized liposomal particles, with the agglomeration achieved by chemical cross links that are capable of cleavage. The cleavage of the cross links releases internal surface area, as well as possibly results in the disruption of the bilayer. The result is a rapid release of encapsulated drug upon contact of the agglomerates with the cleaving agent. In previous work [23], cascade impaction studies showed the AVT particles were within the respirable range since the physically large particles (10–40  $\mu\text{m}$ ) had aerodynamic diameters below 5  $\mu\text{m}$  due to the very low density of the structure (<0.1 g/mL). At the same time, this airborne drug carrier maintained its drug content and structure integrity upon aerosolization. However, modulation of the release

was demonstrated by the addition of dithiothreitol (DTT) which is not a suitable cleaver for *in vivo* use.

#### 1.2.1. *In vivo* modulation of the release rate

With the goal of *in vivo* post-inhalation modulation of drug release rate, a dithiobenzyl-urethane (DTB) conjugate cleavable by an *in vivo* acceptable compound such as cysteine has been recently synthesized [24]. Cysteine and its derivatives are currently used therapeutically as mucolytic agents for inhalation (Mucomyst®) which requires a maintenance dose of 6 g for an adult with a body weight of 80 kg. The dose of cysteine needed to cleave the AVT particle is much lower. The DSPE-DTB-PEG-NH<sub>2</sub> conjugate was successfully incorporated into the AVT particles and the preparation of micron-sized particles was achieved. In recently published *in vitro* studies [21], the release of the antibiotic ciprofloxacin from the AVT particle showed a controlled release rate predetermined by the formulation. However, the addition of cysteine cleaved the disulfide bond of the DTB-urethane linkages resulting in accelerated drug release. Hence, cysteine or any other free thiols (e.g. glutathione) can serve as a cleaving agent triggering acceleration of the release. This was consistent with the size reduction observed upon the exposure of the agglomerate to cysteine.

In this work, the performance of the DTB-urethane linked AVT particles was tested *in vivo* using a rat model.

## 2. Materials and methods

### 2.1. Materials

The phospholipids 1,2-Dipalmitoyl-sn-Glycero-3-Phosphatidylcholine (DPPC) and 1,2-Distearoyl-sn-Glycero-3-Phosphatidylethanolamine (DSPE) were purchased from Genzyme Pharmaceuticals (Cambridge, MA). Cholesterol was purchased from Sigma (St. Louis, MO). Boc-PEG-SPA was obtained from Nektar (San Carlos, CA). The cross-linker Dithiobis[succinimidylpropionate] (DTSSP) was purchased from Pierce (Rockford, IL). Insulin USP (human recombinant-crystalline) was purchased from Serologicals (Milford, MA). Ketamine and xylazine were purchased from Butler Animal Health Supply (Richmond, VA). A Harvard small animal ventilator model 683 and Harvard infusion pump 11-plus were purchased from Harvard Apparatus (Holliston, MA). Spectra/Por biotech cellulose ester membranes (100,000 Da MWCO), Exel International IV Catheter 16G  $\times$  2 in., Hypoguard Assure 3 glucometer, extra dry chloroform, methanol and acetone (water <50 ppm) were obtained from Fisher Scientific (Houston, TX). All the rest of the reagents were also purchased from Fisher Scientific.

### 2.2. Synthesis of the conjugate

The DSPE-DTB-PEG-NH<sub>2</sub> conjugate was prepared according to the previously published procedure [24]. Final purification of the conjugate was achieved by crystallization from warm acetone (30 °C).

### 2.3. Preparation of agglomerated liposomes

#### 2.3.1. Fabrication of parent liposomes encapsulating insulin

Insulin was passively loaded into the liposomes. The liposomes were made by extruding a suspension of hydrated lipids through a single 400 nm Whatman Nuclepore polycarbonate track-etch membrane in a Lipex Biomembranes Extruder. A lipid composition of 57 mol% DPPC, 40 mol% cholesterol, and 3 mol% DSPE-DTB-PEG-NH<sub>2</sub> or DSPE-PEG-NH<sub>2</sub> conjugate was used. Insulin was dissolved in citrate buffer (100 mM) at pH 2.5. The lipids were dissolved in ethanol at 53 °C and then hydrated with the insulin solution, the lipid concentration in the final mixture being 25 mM. Under these conditions, the lipids were rapidly hydrated forming liposomes of varying sizes and lamellarity. The suspension was then passed 5 times through the extruder at 53 °C and a pressure of approximately 100 psi. It was previously found [25] this procedure results in liposomes that are roughly 200–300 nm in diameter, with a wall thickness that is close to unilamellar. This liposome size was chosen to increase the encapsulated volume per mole of lipid. Note that this size is twice what is used in intravenous liposome preparations, and consequently results in roughly twice the encapsulation volume encountered in i.v. preparations. The pH was then raised to 5.3, the isoelectric point for insulin, where its solubility is at a minimum. The majority of the insulin dropped out of solution both inside and outside the liposomes. Coarse filtration through a large pore diameter membrane (1–2 µm) was employed to remove the majority of the unencapsulated insulin. Inside the liposomes, the precipitated insulin probably formed a gel phase with low chemical potential, and therefore did not leak from the liposomes. 5 mL of liposomes were then dialyzed (using 100,000 MWCO dialysis tubing) for 3 h against 1000 mL of citrate buffer at pH of 5.3 in order to remove ethanol and free insulin from the external phase of the liposomes. The external phase was replaced by fresh citrate buffer of equal volume and dialysis was carried out for 3 more hours.

Two preparations of liposomes (both loaded with insulin) were made: 1) liposomes bearing the cysteine-cleavable DSPE-DTB-PEG-NH<sub>2</sub> conjugate (CC-Lips) and 2) liposomes bearing the non-cleavable DSPE-PEG-NH<sub>2</sub> conjugate (NC-Lips). The composition and method for both formulations were identical except the use of different PEG conjugates.

The liposomes were characterized by Dynamic Light Scattering (DLS) using a Brookhaven Instruments BI-9000AT Digital Autocorrelator, a BI-200SM goniometer and a Hamamatsu photomultiplier. The light source was a 532 nm, Ti:sapphire, frequency doubled laser. For the DLS measurement, the liposomal suspension was appropriately diluted in citrate buffer.

#### 2.3.2. Determination of insulin concentration

The concentration of insulin was determined by HPLC. The HPLC system consisted of a Shimadzu SCL-10Avp liquid chromatograph, SPD-10Avp UV–Vis detector (214 nm) and SIL-10Advp auto-injector. The USP assay (official monographs USP 24, 2000) was modified as follows: chromatography was carried out with a Phenomenex Luna 5 µm C18 column (150 ×

2.0 mm); the column temperature was maintained at 40 °C and the flow rate was 0.25 mL/min; the mobile phase was a mixture of 27% (v/v) of acetonitrile and 73% (v/v) of aqueous sodium sulfate (28.4 mg/mL); the sample injection volume was 20 µL.

#### 2.3.3. Determination of entrapped insulin

Dialysis (using 100,000 Da MWCO tubing) was used to evaluate the encapsulated fraction of insulin. The insulin-loaded formulations (5 mL) were dialyzed against 1000 mL of citrate buffer at pH 5.3. Samples were taken from the external phase and insulin content was determined by HPLC. Complete removal of the unencapsulated insulin was assumed to have happened when the insulin content of the external phase did not increase for 2 h. The formulation was then removed from the dialysis tubing and was lysed with methanol (25% total volume). The measured drug by HPLC was the encapsulated fraction inside the formulation.

#### 2.3.4. Agglomeration process

Both insulin-loaded liposomal formulations, CC-Lips and NC-Lips, were agglomerated with the cross-linker DTSSP. DTSSP is a water-soluble, homobifunctional *N*-hydroxysuccinimide (NHS) ester [26]. These cross-linkers are active towards the primary amines on the distal end of the synthesized conjugate resulting in a covalent amide bond and the release of *N*-hydroxysuccinimide. The coupling reaction of the PEG-amines with DTSSP was carried out at pH 7.2 in citrate buffer. The amount of DTSSP used was 20-fold molar excess over the NH<sub>2</sub> groups on the PEG. The pH was kept at 7.2 for 45 min and then was returned to 5.3, where insulin was stably encapsulated. This pH range is optimal for the activity of the cross-linker resulting in fully agglomerated liposomes. Based on the parent liposomes, cysteine-cleavable AVT (CC-AVT) and non-cleavable AVT (NC-AVT) particles were prepared.

The size distribution of the AVT particles was determined using the Fraunhofer diffraction technique (Malvern Mastersizer with 100 mm lens). The particles were measured at different time points in a period of three weeks. The morphology of the agglomerated liposomes was examined by electron microscopy (EM). Copper grids coated with collodion-carbon and freshly glow discharged were used for sample adsorption. Each grid was floated on a drop of sample for 2 min. Excess fluid was removed by blotting with a filter paper, and the grid was washed for 2 s on a drop of water, floated on a drop of 1% uranium acetate for a few seconds, and air dried. All electron micrographs were taken with a JEOL JEM 1230 electron microscope by exposing the samples for 0.2 s under a beam of 80 kV energy and 56 µA current.

#### 2.3.5. Aerodynamic properties of agglomerates

The aerodynamic properties of the agglomerated liposomes containing insulin were evaluated by nebulizing 2–3 mL of the liposomal formulation into an Andersen Cascade Impactor (Series 20-800, 1 ACFM non-viable sampler). A Parijet LC nebulizer was used for the cascade impaction studies.

Each sample was nebulized for 5 min. At the end of the nebulization run, the filter papers from each plate of the impactor, and the filter paper at the end of the last stage were

lyophilized in order to remove the water content. Additionally, the elbow was washed with citrate buffer which was also collected and lyophilized. Upon lyophilization, the collected dry powder deposited on each filter was evaluated by weighing the filter paper and comparing this reading to the one prior to nebulization. In addition, a sample of the liposomal formulation was weighed upon lyophilization in order to evaluate the density of the suspension. According to the manual for the Andersen Cascade Impactor, each stage has a range of particle sizes. The amount of dry material collected on each stage was then correlated to the corresponding size of each stage to determine the size distribution.

The weight of the drug preparation in the nebulizer was recorded before and at the end of the nebulization in order to complete a mass balance calculation and determine any losses upon nebulization.

#### 2.4. Pharmacodynamic studies in rats

Male Sprague–Dawley rats 75 days old (Charles River Laboratories) weighing 250–280 g were housed in a 12-h light/12-h dark cycle and a constant temperature environment of 21 °C. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Houston. A standard diet with water was supplied during the period of acclimatization. 6 groups were used in order to evaluate the pharmacodynamics of the AVT particles during 24 h. Table 1 summarizes the dose and time schedule of the administered compounds.

Blood glucose concentration was measured by a commercial glucose meter (Hypoguard Assure 3) which required 20 µL of blood and 10 s to provide the reading. The small volume of blood required for each reading allowed very frequent sampling (every 5–10 min). Each blood sample was analyzed for glucose 2–4 times and the average was recorded.

Each data point was represented by the mean and standard deviation of the group (3–5 rats per group). The difference of two means was verified to be significant by variance analysis (Student's *t*-test). A *p*-value less than 0.05 was used to confirm significant differences at the 95% confidence level.

##### 2.4.1. Control group 1

Upon restraining the rat using a Plexiglas restrainer, the animal was anesthetized with a mixture of ketamine (80 mg/kg)

and xylazine (10 mg/kg) administered intraperitoneally (i.p.). The rat was then placed on a surgical table. Temperature was maintained stable by placing a temperature-controlled pad below the animal. 40 and 90 min after the initial cocktail of anesthetic drugs, the animal was injected i.p. with a maintenance dose of a cocktail of ketamine (20 mg/kg) and xylazine (3 mg/kg). The animal fully recovered after 3–4 h and was returned to its cage. Blood samples were taken from the tail veins under anesthesia or restraint to monitor the glucose concentration in the blood. Food was not available to the animal during the first 8 h of the experiment's duration. After the initial 8 h, the animal was returned to its cage where standard food and water were provided. The animal was euthanized after the end of the 24-h study by a pentobarbital overdose (100 mg/kg) administered i.p.

##### 2.4.2. Control group 2

This control group was injected with the cocktail of anesthetic drugs with the same doses and frequency. Additionally, a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) was injected i.p. 345 min after the initial dose of the anesthetics. All the rest of the procedures were followed as described previously.

##### 2.4.3. AVT group 1

On the day of the study each animal went under oral endotracheal intubation. After restraining the rat using a Plexiglas restrainer, it was anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) administered i.p. On the establishment of anesthesia indicated by the absence of reflexes, the rat was endotracheally intubated. The endotracheal tube was connected to the ventilator and the rat remained under forced ventilation while still being under anesthesia. Successful intubation was verified if the chest expanded with the same rate as the frequency of the ventilator. The ventilator was then removed and a maintenance dose of ketamine (20 mg/kg) and xylazine (3 mg/kg) was injected i.p. 40 min after the initial cocktail of anesthetics. 5 min later, 40 µL of CC-AVT particles was instilled using a pipette while the animal was placed vertically. The animal was then placed on the surgical table and the endotracheal tube was connected to the ventilator which was operated at 2.5 mL tidal volume and 90 strokes/min frequency. The forced ventilation was used to ensure the particle suspension was well mixed in the lungs. After 5 min, the

Table 1  
Timetable of dosing of anesthetics, cysteine, and insulin formulations

Time (min)	-45	-5	0	45	50	300	375
Control group 1	Ket/Xyl A	Ket/Xyl B		Ket/Xyl B			
Control group 2	Ket/Xyl A	Ket/Xyl B		Ket/Xyl B		Ket/Xyl C	
AVT group 1	Ket/Xyl A	Ket/Xyl B	CC-AVT	Ket/Xyl B			
AVT group 2	Ket/Xyl A	Ket/Xyl B	CC-AVT	Ket/Xyl B	CYS		
AVT group 3	Ket/Xyl A	Ket/Xyl B	CC-AVT	Ket/Xyl B		Ket/Xyl C	CYS
AVT group 4	Ket/Xyl A	Ket/Xyl B	NC-AVT	Ket/Xyl B			

Ket/Xyl A: i.p. injection of ketamine (80 mg/kg) and xylazine (10 mg/kg); Ket/Xyl B: i.p. injection of ketamine (20 mg/kg) and xylazine (3 mg/kg); Ket/Xyl C: i.p. injection of ketamine (50 mg/kg) and xylazine (5 mg/kg); CC-AVT: instillation of 40 µL of AVT particles (cysteine-cleavable) with 1.420 U/kg of insulin; NC-AVT: instillation of 40 µL of AVT particles (cysteine-noncleavable) with 1.512 U/kg of insulin; CYS: instillation of 50 µL of PBS containing 5 mg of cysteine.

forced ventilation was terminated and the animal was left to breathe freely. The duration of the entire process of insertion and instillation was 10 min. 90 min after the initial cocktail of anesthetic drugs, the animal was injected i.p. once more with a cocktail of ketamine (40 mg/kg) and xylazine (4 mg/kg). The endotracheal tube was then removed. The rest of the procedures were identical to the ones described for the control group 1.

#### 2.4.4. AVT group 2

This group differed from the previous one because a cysteine solution was instilled 95 min after the initial anesthetic cocktail. The volume of the solution was 50  $\mu$ L containing 5 mg of cysteine. The animal was then ventilated for 5 min under forced ventilation at tidal volume of 2.5 mL and frequency of 90 strokes/min to ensure thorough mixing of cysteine with the lung surfactant. The rest of the procedures followed for this group were identical to the ones described for the AVT group 1.

#### 2.4.5. AVT group 3

The procedures were identical to the ones of the AVT group 2 with the major difference being the time when cysteine was administered. 345 min after the initial anesthetic cocktail, the rat was injected i.p. with a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg). Endotracheal intubation was carried once more as described previously. Cysteine was instilled 420 min after the initial anesthetic cocktail. The volume of the solution was 50  $\mu$ L containing 5 mg of cysteine. The animal was then put for 5 min under forced ventilation at tidal volume of 2.5 mL and 90 strokes/min. The rest of the procedures followed for this group were identical to those of the other groups.

#### 2.4.6. AVT group 4

The procedures followed for this group were identical to the ones of the AVT group 1 with the only difference being that NC-AVT particles were instilled.

### 2.5. Euglycemic clamp studies in rats

The measured quantity was the glucose infusion rate required to maintain the clamp, a macroscopic number that was easy to measure. The glucose clamp technique is the standard technique in the evaluation of insulin delivery systems. Two groups of animals were used: 1) rats treated with the CC-AVT formulation (Clamp group 1) and 2) rats treated with the CC-AVT formulation followed by cysteine (Clamp group 2).

Sprague–Dawley male rats having both jugular veins catheterized with body weights of 330–350 g were obtained from Charles River Laboratories. The double jugular vein catheterization provided stable catheterization for extended periods of time, and allowed simultaneous blood sampling and infusion without cross-contamination of the two streams. The animal was under anesthesia during the entire duration of the study (not more than 7 h). A sterile glucose solution of 100 mg/mL was infused continuously during the experiment by a continuously variable infusion pump (Harvard infusion pump 11-plus). The maximum total glucose solution that was infused

was less than 5 mL whereas the total amount of blood that had to be withdrawn was around 0.5 mL.

#### 2.5.1. Clamp group 1

The animals were restrained in a Plexiglas restrainer and anesthetized with ketamine/xylazine 80/10 mg/kg i.p., and endotracheally intubated (as described earlier). Upon intubation, the animal was placed on the surgical table and the temperature was maintained stable using a temperature-controlled pad. The endotracheal tube was connected to the ventilator which provided oxygen at 0.5 L/min under forced ventilation (tidal volume of 2.5 mL and frequency of 65 strokes/min). After 1 h, and before the animal recovered from anesthesia, the vaporizer was turned on and adjusted at 1–2.5% isoflurane. The animal was maintained under anesthesia with isoflurane during the rest of the study. Blood was sampled ( $\sim$ 20  $\mu$ L) every 2–10 min, and the glucose level was measured. Glucose infusion was then adjusted to maintain a blood glucose level of  $\sim$ 200 mg/dL. 2 h after the injection of the anesthetic drugs, 50  $\mu$ L of the CC-AVT was administered endotracheally. The glucose infusion rate (GIR) was continuously recorded during the next 5–6 h, and the response to the insulin administration was recorded. (Increased GIR corresponded to increased levels of insulin in the blood). The experiment was then stopped, and the animal was euthanized.

#### 2.5.2. Clamp group 2

The procedures were identical to the ones of Clamp group 1. The only difference was the instillation of cysteine twice (70 and 125 min after the administration of the AVT particles). Briefly, the animal was removed from the ventilator, kept at vertical position and 50  $\mu$ L of PBS containing 5 mg of cysteine was instilled. The whole instillation process lasted for less than a minute. The animal was then connected again to the ventilator.

## 3. Results and discussion

### 3.1. Characterization of parent liposomes

Extruded liposomes showed narrow size distribution exhibiting a mean diameter of 300 nm as measured by DLS. Both liposomal preparations with the cysteine-cleavable and the non-cleavable conjugate (CC-Lips and NC-Lips) showed very similar sizes. In the size distribution, particles in the micron-range were observed. It is speculated that traces of unencapsulated insulin aggregated due to the pH of 5.3 which is the isoelectric point of insulin.

After removal of unencapsulated insulin, the concentration of the encapsulated drug, determined by HPLC analysis, was 2.1 and 2.3 mg/mL for the CC-Lips and NC-Lips respectively. The insulin content of the suspension was measured before and after each step of the fabrication and the results are shown in Fig. 1. It is speculated that the significant losses of insulin during extrusion were due to high pressures and temperatures. It was also observed that the coarse filtration upon raising the pH to 5.3 was very efficient. The complete removal of the unencapsulated insulin was verified by dialysis since no

leakage of insulin from the liposomes was detected after an hour.

Both liposome formulations had diameters of around 300 nm and 25 mM lipid content encapsulating  $\sim 2.2$  mg/mL of insulin. Assuming a bioavailability of 25% of inhaled insulin [18] and a daily dose of insulin for an adult type-I diabetic patient of 50 IU (1 mg  $\approx 27.6$  IU), the total lipid excipient load for a daily dose is  $\sim 60$  mg. This is reasonable compared to the total lipid already present in the lung as constituents of lung surfactant [27].

### 3.2. Size of the agglomerates before and upon cleavage

Upon completion of the agglomeration, the insulin content was measured to be 0.92 and 0.98 mg/mL for the CC-AVT and NC-AVT respectively while the lipid content of both preparations was 15 mM. As shown in Fig. 1, agglomeration caused a small degradation of insulin. Since DTSSP is membrane impermeable and cannot penetrate the liposomal bilayer, it is suspected that the amine-termini of the remaining unencapsulated insulin were cross-linked. However, the bioactivity of the resulting cross-linked insulin is unknown.

Fig. 2 shows the size distributions measured by Fraunhofer diffraction, during the formation of CC-AVT particles by the agglomeration of  $-\text{NH}_2$  terminated liposomes using the DTSSP cross-linker. The large agglomerates exhibited a size distribution between 2 and 70  $\mu\text{m}$  with a mean diameter of 14.7  $\mu\text{m}$  and a standard deviation of 1.4  $\mu\text{m}$ . The DTSSP linkage reaction proceeds optimally around pH 7.2 and therefore particles  $< 1.5$   $\mu\text{m}$  were not seen, indicating that all the parent liposomes were consumed in the cross-linking reaction. In comparison, the size distribution of the parent liposomes had its mode at 1.3  $\mu\text{m}$  (shown in the same figure). It should be noted that the diameter of parent liposomes indicated by Fraunhofer diffraction is substantially different from that indicated by dynamic light scattering (DLS). This is due to the inaccuracy of Fraunhofer diffraction in this low size range. Therefore this peak is only used as an indication of the presence of small particles, akin to

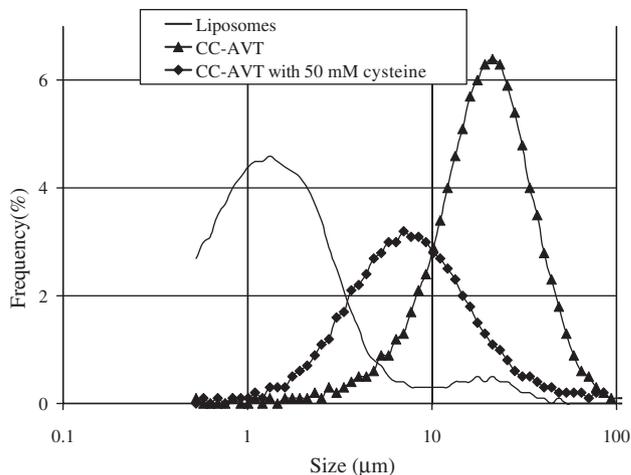


Fig. 2. Size distributions measured by Fraunhofer Diffraction of liposomes, and cysteine-cleavable AVT particles before and upon cleavage.

parent liposomes, but separate DLS measurements determined the size of the parent liposomes accurately.

In the case of the NC-AVT, the agglomerated liposomes were larger but comparable to the size of the CC-AVT. They exhibited a size distribution with a mean diameter of 22.7  $\mu\text{m}$  and a standard deviation of 2.3  $\mu\text{m}$ . Several factors affect the size of the AVT particles formed during the agglomeration process [25]. These include pH, the linker:lipid ratio, the lipid molarity and the number of linking ligands present on the surface of the parent liposomes. Each of these parameters affects the reaction rate of the linking ligands with the linker and thus controls the rate of formation of the interliposome cross links resulting in different nanostructures of the microparticle. Obviously, the nanostructure of the agglomerate determines the release of drug from them. Specifically, a tight, compact structure would not promote diffusive transport, but would represent a high concentration of drug-bearing nanoparticles. On the other hand, a more porous, open structure would represent a lower concentration of drug-bearing particles, but a higher diffusive transport rate. The correlation of the release rate to the agglomerate structure is a subject of ongoing investigation. Fig. 3 shows electron microscope images of parent liposomes with diameters of  $\sim 200$  nm (no insulin encapsulated) and agglomerated liposomes. Fig. 3d is the EM image of the parent liposomes with diameters narrowly distributed around 200 nm which is consistent with extrusion through a membrane with pores of 200 nm. Since the length of the PEG anchor ( $\sim 7$  nm) is very small compared to the size of the liposome, linked liposomes are in close proximity as shown in Fig. 3a–c. The agglomerates clearly consisted of clusters of cross-linked liposomes including high amounts of the negative stain suggesting high accessibility of the aqueous phase to the internal liposomal compartment. A heterogeneous structure can be seen including highly porous as well as compact domains within the agglomerate. As the compact structures were nucleated rapidly, diffusion-limitations dominated strongly resulting in formation of porous structures of the compact “nuclei”.

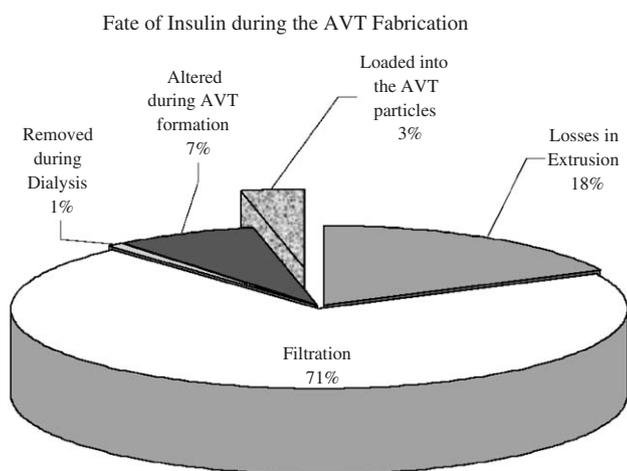


Fig. 1. The final insulin content of the AVT particle and losses during each fabrication step.

Upon induction of cleavage, the CC-AVT cleaved rapidly. A comparison of size distributions of cleaved and non-cleaved CC-AVT is shown in Fig. 2. Agglomerates of 0.5 mM lipid content were incubated with 50 mM cysteine at 37 °C and measured by Fraunhofer Diffraction. These concentrations of both the liposomal preparation and cysteine were similar to those expected in the lungs in the *in vivo* studies described later in this paper. The size of the NC-AVT was not altered in the presence of cysteine. A more detailed study of the cleavage of the CC-AVT particles by cysteine is reported elsewhere [24].

The insulin content and the size distribution of each of the particle preparations used in this study were monitored throughout the duration of this study (~3 weeks) and found to be insignificantly changed. These results suggest that cysteine-mediated cleavage of the DTB-urethane conjugate broke the interliposomal linkages resulting in looser agglomerate structure as well as liberation of free liposomes whereas the NC-AVT were unaffected by cysteine.

### 3.3. Aerodynamic properties of agglomerates

Fig. 4 shows the results of the cascade impactor studies done with CC-AVT containing insulin. The results suggest that the aerodynamic diameter of the agglomerates made them highly respirable. Approximately 40% of the agglomerates had aerodynamic diameters ( $D_{\text{aer}}$ ) in the respiratory range (between 1 and 5  $\mu\text{m}$ ). Specifically, the CC-AVT exhibited a mass mean

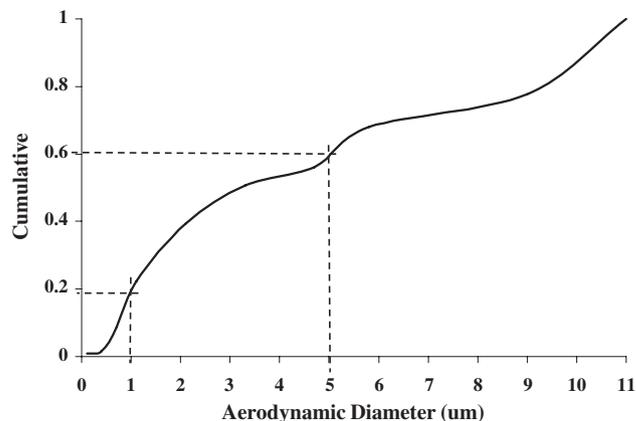


Fig. 4. Aerodynamic characterization of the cysteine-cleavable AVT particles using cascade impaction.

aerodynamic diameter of 3.9  $\mu\text{m}$  with a standard deviation of 3.6  $\mu\text{m}$ . These diameters are clearly much lower than the geometrical diameters ( $D_{\text{geo}}$ ) measured by the Fraunhofer technique. This data would be consistent with either fragmentation of the agglomerates in the nebulizer or low density of the porous agglomerates resulting in lowered aerodynamic diameter. In previous studies [23], we verified that the agglomerate maintains its structural integrity upon nebulization. Applying  $D_{\text{aer}} = \rho^{1/2} D_{\text{geo}}$  (derived from Stokes Law) and assuming that

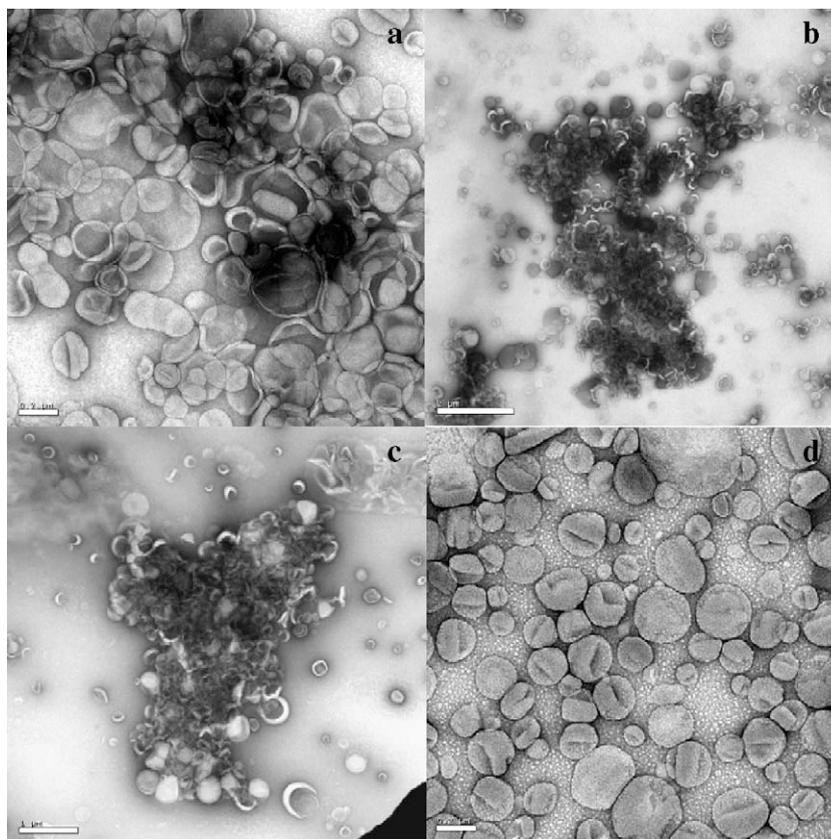


Fig. 3. Negative stain electron microscope images (1% uranyl acetate) taken on a JEOL JEM 1230. (a) Agglomerate at 10 K magnification, scale bar 0.2  $\mu\text{m}$ ; (b) agglomerate at 2 K magnification, scale bar 2  $\mu\text{m}$ ; (c) agglomerate at 3 k magnification, scale bar 1  $\mu\text{m}$ ; (d) liposomes at 10 K magnification, scale bar 0.2  $\mu\text{m}$ .

the AVT particles are spherical, the density of the particle was calculated to be 0.07 g/mL.

### 3.4. Pharmacodynamic studies in rats

Hyperglycemia is induced by the ketamine/xylazine cocktail of anesthetics. Hindlycke and Jansson compared the effect of different anesthetics on glucose tolerance [28]. They observed a marked decrease in the pancreatic blood flow of rats injected i.p. with ketamine and xylazine which resulted in elevated glucose levels for 5 h. Upon this observation, this cocktail of anesthetics has been used as a hyperglycemic agent in similar studies [29]. The use of this anesthetic therefore allows testing over a wide range of blood glucose levels, and temporary diabetic characteristics.

Fig. 5 compares the blood glucose profiles obtained from the control group 1 and the two groups treated with the non-cleavable (NC-AVT) and the cysteine-cleavable (CC-AVT) particles. As expected, upon multiple injections of the anesthetics the control group 1 showed dramatically high glucose concentrations reaching the levels of diabetic rats (>400 mg/dL for ~2 h). At  $t=0$  the AVT formulations were instilled. The release pattern of insulin from the endotracheally instilled NC-AVT formulation (AVT group 4 of rats) exhibited a short-acting ( $\alpha$  phase) and a long-acting behavior ( $\beta$  phase). During the  $\alpha$  phase, a dramatic drop of glucose (-120 mg/dL) was observed within 20 min implying that initially the formulation was releasing insulin rapidly. The release rate of insulin continued to be high and the glucose drop reached a minimum of -400 mg/dL at  $t=100$  min. Thereafter, the NC-AVT particle exhibited a much slower release rate resulting in the maintenance of the glucose levels at subnormal levels for rats of 70 mg/dL for the next 4 h. A controlled release pattern of insulin was observed even after 20 h.

Similarly, the CC-AVT treated group (AVT group 1 of rats) exhibited an initial rapid glucose decrease. However, the

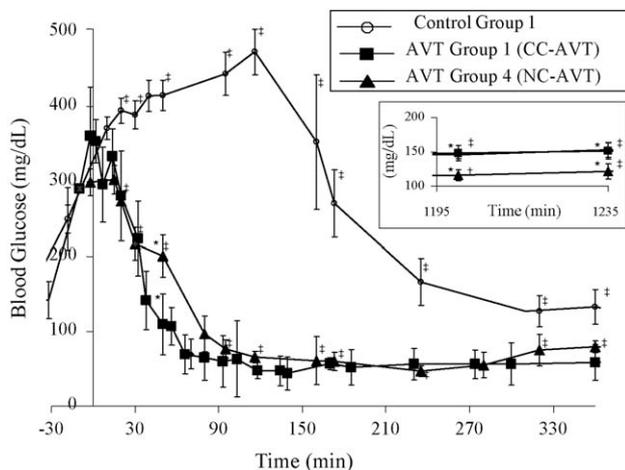


Fig. 5. Blood glucose concentration over the period of 21 h of the control group 1 ( $n=5$ ), the AVT group 1 treated with the CC-AVT ( $n=4$ ), and the AVT group 4 treated with the NC-AVT ( $n=3$ ). The \* and ‡ indicate significant statistical difference ( $p<0.05$ ) between AVT-1 and AVT-4 and between AVT-4 and control 1 respectively.

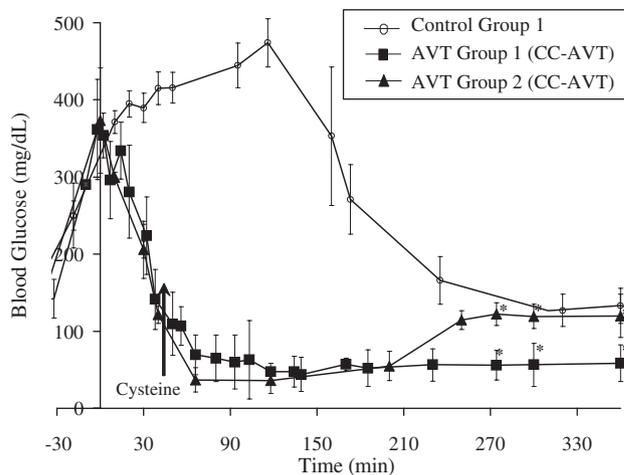


Fig. 6. Blood glucose concentration over the period of 6 h of the control group 1 ( $n=5$ ), the AVT group 1 treated with the CC-AVT ( $n=4$ ), and the AVT group 2 treated with the CC-AVT followed by “early” cysteine ( $n=3$ ). The \* indicates significant statistical difference ( $p<0.05$ ) between AVT-1 and AVT-2.

glucose drop after 50 min was -306 mg/dL comparing to -215 mg/dL for the case of the NC-AVT. This was expected since in previous in vitro studies in lung surfactant medium [24] an accelerated release from cysteine-cleavable particles was observed which was attributed to the action of the free thiols present in the lung surfactant. The CC-AVT formulation was therefore expected to release insulin faster since the free thiols in the lungs constantly cleaved the agglomerate resulting in a larger glucose drop. The fact that the CC-AVT particles had faster release capabilities than the NC-AVT particles was supported by the observation that after 20 h the NC-AVT formulation was still releasing insulin while the CC-AVT stopped doing so resulting in elevated glucose levels compared to the NC-AVT. This is consistent with the CC-AVT particle releasing its content faster.

Fig. 6 compares the effect of CC-AVT and CC-AVT particles cleaved by cysteine (AVT group 1 vs. AVT group 2 of rats). Upon introduction of cysteine shortly after instillation of the particles (50 min later), the glucose level of AVT group 2 was slightly lower glucose than AVT group 1 (no statistical difference). The particles’ insulin reservoir was empty after 5 h as it is indicated by the fact that the glucose levels coincided with that of the untreated animals.

The administration of cysteine during the  $\beta$  phase clearly induced an accelerated release of post-inhaled insulin encapsulated into CC-AVT particles. Fig. 7 shows the blood glucose profiles of the control group 2 and the group treated with the CC-AVT formulation followed by “late” cysteine (AVT group 3 of rats). A cocktail of ketamine and xylazine was injected 5 h after the instillation of the CC-AVT particles. The expected elevated glucose levels ( $\Delta C=+120$  mg/dL) were observed in both the control and the treated group of animals. The administration of cysteine triggered an additional release of insulin resulting in a decrease of glucose by 150 mg/dL within 20 min.

The possibility of this glucose reduction being an effect of the cysteine itself was investigated by administering cysteine to

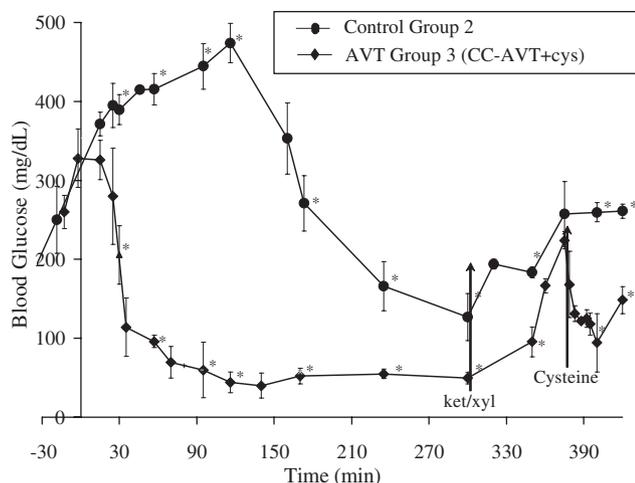


Fig. 7. Blood glucose concentration over the period of 7 h of the control group 2 ( $n=3$ ), and the AVT group 3 treated with the CC-AVT followed by “late” cysteine ( $n=3$ ). The \* indicates significant statistical difference ( $p<0.05$ ) between control 2 and AVT-3.

the lungs of animals both untreated and treated with non-cleavable particles (data not shown). Endotracheal instillation of cysteine had no effect in blood glucose of control group 1 rats and rats treated with NC-AVT (AVT group 4 of rats).

Fig. 8 summarizes the glucose drop caused by each formulation. The glucose drop was obtained by subtracting the glucose profile of the appropriate control from the one of the treated group (zero glucose drop corresponds to the control). This normalization allows a direct visualization of the pharmacodynamic effect of each formulation. The profiles of glucose drop of all formulations reached a maximum at similar time points ( $T_{\max} \sim 115$  min) and the corresponding maximum glucose drop ( $\Delta C_{\max}$ ) was 422, 438, 427 and 404 mg/dL for the AVT group 1, 2, 3 and 4 of rats respectively. Clearly, the CC-AVT followed by early cysteine (AVT group 2) had the largest  $\Delta C_{\max}$  which is attributed to the initial cleavage due to the free thiols already present in the lung lining fluid coupled to the administration of cysteine.

The area under the curve (AUC) of the glucose drop of each formulation was estimated by integrating the glucose drop profiles shown in Fig. 8. Cefalu et al. investigated the inhalation of insulin ( $\sim 1.2$  IU/kg) and the subcutaneous injection of insulin ( $\sim 0.15$  U/kg) in healthy volunteers [30]. The estimated  $AUC^{t=0 \rightarrow 80}$  values of the glucose drop for both treatments were similar and equal to  $-0.8 \cdot 10^4$  mg min  $dL^{-1}$ . This AUC value can be used as a comparative tool to evaluate the AUC values of the glucose drop caused by the AVT formulations with respect to clinical pharmacodynamics. The CC-AVT particle (AVT group 1) exhibited an  $AUC^{t=0 \rightarrow 240}$  value of  $-5.8 \cdot 10^4$  mg min  $dL^{-1}$  which is comparable to the  $AUC^{t=0 \rightarrow 240}$  value observed in the AVT group 4 caused by the NC-AVT particle ( $-5.5 \cdot 10^4$  mg min  $dL^{-1}$ ). As expected, the CC-AVT particle caused a slightly larger glucose drop than the NC-AVT particle during the  $\alpha$  phase ( $t=0 \rightarrow 120$  min). However, the NC-AVT particle appeared to cause a higher glucose drop than the CC-AVT particle during the beginning of  $\beta$  phase ( $t=120 \rightarrow 240$  min) resulting in a similar overall  $AUC^{t=0 \rightarrow 240}$ . After 20 h, the CC-

AVT particle had no effect on the blood glucose whereas the NC-AVT particle sustained a lower glucose level by  $-30$  mg  $dL^{-1}$ . The CC-AVT followed by “early” cysteine at  $t=50$  min (AVT group 2) had an  $AUC^{t=0 \rightarrow 240}$  value of  $-6 \cdot 10^4$  mg min  $dL^{-1}$  which is slightly higher than the value of the CC-AVT without the addition of cysteine (AVT group 1). It is speculated that the small effect of cysteine addition during the  $\alpha$  phase is due to the fact that the release of insulin from the CC-AVT particle was already very fast. The glucose drop reached zero at  $t=420$  min which would be consistent with no insulin left encapsulated in the CC-AVT particle.

The glucose drop caused by the CC-AVT particle followed by “late” cysteine (AVT group 3) for the first 6 h was statistically identical to the one of the CC-AVT particle (AVT group 1). However, the addition of “late” cysteine ( $t=375$  min) triggered clearly a higher glucose drop. The  $AUC^{t=375 \rightarrow 420}$  value of the CC-AVT particle followed by late cysteine was by 88% greater than that of the CC-AVT without the addition of cysteine. Specifically, the addition of “late” cysteine caused a maximum glucose drop of  $-93$  mg  $dL^{-1}$  with respect to the glucose levels achieved by the CC-AVT particle (AVT group 1). This additional glucose drop caused by cysteine is within the range of a glucose excursion observed during mealtime in patients with type I diabetes. It can be concluded that cysteine triggered a significant release of insulin from the CC-AVT particles only when the release pattern was during the  $\beta$  phase.

Clearly, all the AVT formulations showed an initial rapid decrease of glucose followed by maintenance of glucose to low levels. The undesirable hypoglycemia caused by the AVT particles can be addressed by altering/optimizing easily manipulated parameters such as the encapsulated fraction of insulin, dosing, size and number of liposomes within the agglomerate, and number of inter-liposome linkages. It should

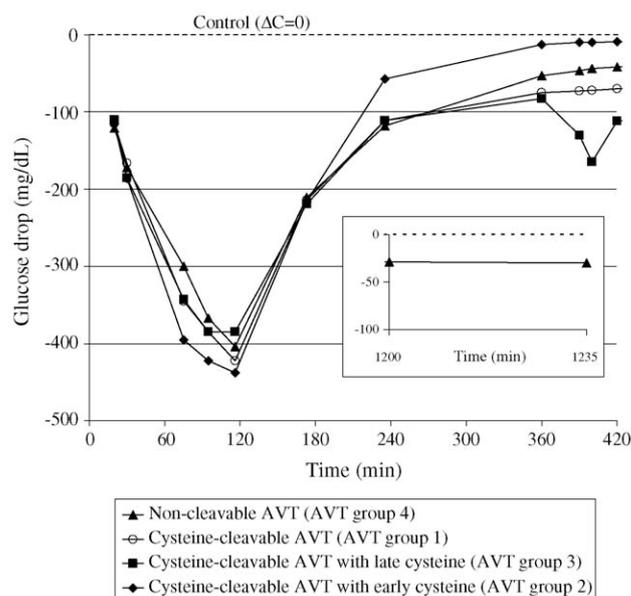


Fig. 8. Comparison of the glucose drop caused by each formulation. The glucose profile of each group treated with AVT particles was normalized by subtracting it from the glucose levels of its control group.

be noted that the control and the treated animals were all fasted for the duration of the first 8 h after the administration of the AVT formulation. This was abnormal for the fast metabolism of a rat and therefore the glucose levels of similar experiments with rats fed normally during the study are expected to be closer to normoglycemic. Even though a typical glucose reduction anticipated in a human with type I diabetes during mealtime is  $\sim 100$  mg/dL, this animal model was used to show a very fast response causing a rapid decrease of glucose followed by maintenance of glucose to constant values for many hours. An optimization of the formulation to achieve normoglycemia within the human physiological levels is considered to be within the characteristics of the AVT platform.

A futuristic scenario deriving from these glucose profiles could be a diabetic patient inhaling an insulin dose encapsulated in AVT particles shortly before lunch. During the meal, the insulin delivery from the AVT particles deposited in the deep lungs would be rapid maintaining normoglycemic levels. Thereafter, the formulation would be releasing constantly low amounts of insulin keeping glucose close to physiological values. In case of an outdoor non-scheduled snack and difficult access to refrigerated insulin, the use of a pocket-sized inhaler containing cysteine (a stable powder at ambient conditions) would trigger extra insulin release to sustain normoglycemia.

### 3.5. Euglycemic clamp studies in rats

The rats used in these studies were fasted overnight in order to decrease blood glucose below 200 mg/dL upon i.p. injection of the anesthetics. Fig. 9 shows the blood glucose of a control group ( $t=0$  on the axis corresponds to the i.p. injection of anesthetics). The anesthetics caused an increase from 100 to 165 mg/dL for 2 h which allowed the application of a clamp to the 200 mg/dL range. The glucose concentration of the fasted animals was much lower compared to the non-fasted ones.

Prior to instillation of the cysteine-cleavable AVT particles, the glucose level of the animals was conveniently adjusted to  $\sim 195$  mg/dL with minor differences in the glucose infusion rate. Upon administration of the AVT formulation, the infusion rate had a sharp increase within 10 min as shown in Fig. 10. As expected, both formulations reached the same maximum infusion rate ( $GIR_{max} = 25.71$  mg min $^{-1}$  kg $^{-1}$ ) at similar time

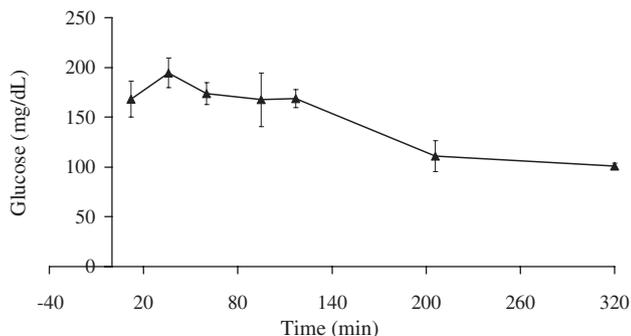


Fig. 9. Blood glucose concentration of animals that were fasted overnight and at  $t=0$  were injected i.p. with ketamine (80 mg/kg) and xylazine (10 mg/kg).

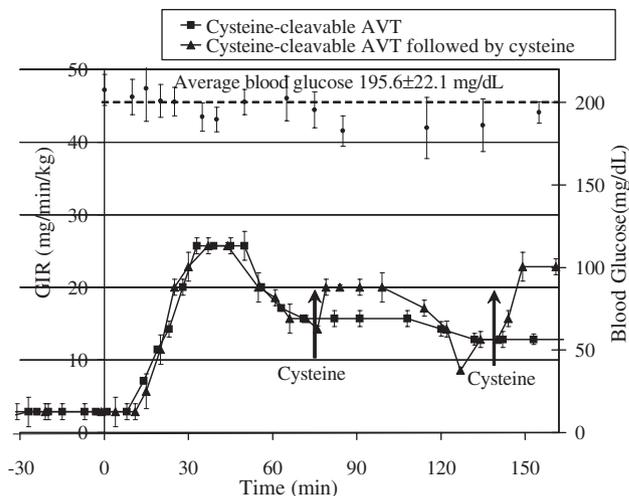


Fig. 10. The Glucose Infusion Rate (GIR) required to maintain constant the blood glucose levels of two treated groups of animals for 3 h. One of the groups was treated with CC-AVT and the other one was treated with CC-AVT particles followed twice by a cysteine dose.

points ( $T_{max} \sim 35$  min). Both profiles then dropped to lower rates ( $GIR = 15.7$  mg min $^{-1}$  kg $^{-1}$ ) which in the case of the non-cleaved CC-AVT remained constant for the next 80 min. However, the application of cysteine caused a significant increase of the GIR. The first addition of cysteine increased the GIR by 6 mg min $^{-1}$  kg $^{-1}$  while the second addition caused an increase of 10 mg min $^{-1}$  kg $^{-1}$ . Overall cysteine triggered a significant acceleration of the insulin's release which is indicated by the total amount of glucose infused over the 160 min after the instillation of the formulations. By integrating the GIR curve, the infused glucose was calculated to be 2467.4 mg/kg for the CC-AVT particles and 2749.3 mg/kg for the twice cysteine-cleaved CC-AVT.

## 4. Conclusions

To the best of our knowledge, this is the first in vivo demonstration of post-inhaled modifiable release of insulin. The insulin carrier caused an initial rapid glucose drop (short-acting) followed by sustaining glucose for a period of  $\sim 20$  h (long-acting). The application of cysteine triggered the release of insulin resulting in an additional reduction of the glucose levels. The cysteine triggered acceleration of insulin release was more applicable when the particle was in its "long-acting" phase. Euglycemic clamp studies verified the triggered acceleration of insulin release upon introduction of cysteine. Additional glucose had to be infused in order to compensate for the effect of cysteine. While the resulting glucose decrease was greater than the physiological demands in humans upon glucose excursions during mealtime, the optimization of the release pattern to meet these needs is within the abilities of the current AVT technology. The release rate can be altered by changing the liposomal wall permeability, the size and density of the microparticle, and the insulin load.

Even though in these studies the particles were instilled and not aerosolized into the lungs of the animals, cascade impaction

studies showed that these particles were highly respirable. Clearly, the *in vivo* fate of the particles is an indicated study. The distribution in the lungs as well as the rate and mechanism of clearance from the lungs is a subject of future studies. In addition, the correlation of the AVT structure to the *in vivo* release pattern and clearance mechanism is under investigation.

The goal of developing a sustained release inhaled insulin formulation capable of post-inhaled modifiable release upon command seems to be very feasible. Especially, the combination of cleavable and non-cleavable inter-liposomal linkages is expected to result in improved *in vivo* behavior.

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