Published in final edited form as:

J Pharm Sci. 2014 August ; 103(8): 2231–2239. doi:10.1002/jps.24022.

Aerosol Stable Peptide-Coated Liposome Nanoparticles: A Proof-of-Concept Study with Opioid Fentanyl in Enhancing Analgesic Effects and Reducing Plasma Drug Exposure

JOHN D HOEKMAN¹, PRAMOD SRIVASTAVA, and RODNEY J Y HO^{1,*}

¹Department of Pharmaceutics, University of Washington, Seattle, WA, USA 98195-7610

Abstract

Previous we reported a novel pressurized olfactory drug (POD) delivery device that deposit aerosolized drug preferentially to upper nasal cavity. This POD device provided sustained CNS levels of soluble morphine analgesic effects. However, analgesic onset of less soluble fentanyl was more rapid but brief, likely due to hydrophobic fentanyl redistribution readily back to blood. To determine whether fentanyl incorporated into an aerosol stable liposome that binds to nasal epithelial cells will enhance CNS drug exposure and analgesic effects and reduce plasma exposure, we constructed *RGD* liposomes anchored with acylated integrin binding peptides (palmitoyl-*GRGDS*). The *RGD* liposomes, which assume gel-phase membrane structure at 25°C were stable under the stress of aerosolization as only 2.2 ± 0.5 % calcein leakage detected. The *RGD* mediated integrin binding of liposome is also verified to be unaffected by aerosolization. Rats treated with fentanyl in *RGD*-liposome and POD device exhibited greater analgesic effect, compared to the free drug counterpart ($AUC_{\text{effect}} = 1387.1$ vs. 760.1 %MPE*min); while ~20% reduced plasma drug exposure was noted ($AUC_{0-120} = 208.2$ vs 284.8 ng*min/ml). Collectively, fentanyl incorporated in *RGD*-liposomes are physically and biologically stable under aerosolization, enhanced the overall analgesic effects and reduced plasma drug exposure for the first 2 hours.

Keywords

Aerosols; Liposomes; Fentanyl; Targeted Drug Delivery; Integrin; Aerosolized liposomes; Aerosolized Nanoparticles

INTRODUCTION

Intranasal administration of aerosolized drugs to potentiate neurological impact in general has made significant clinical impact and are believed to reduce first pass metabolism of orally administered drug as well as avoiding more invasive injection dosage forms.¹⁻³ In addition, studies have shown that drugs applied to the upper nasal cavity may bypass the blood stream and directly distribute into the brain in their first pass.⁴ Previously, we reported the first pass distribution of two opioid drugs, fentanyl and morphine, when preferentially

*Correspondence to: Rodney J Y Ho, Ph.D., Department of Pharmaceutics, University of Washington, Seattle, WA 98195-7610, Phone: 206-543-3796, Fax: 206-221-2689, rodneyho@u.washington.edu.

delivered to the upper olfactory region of the nasal cavity with a novel aerosol device called pressurized olfactory drug delivery device (POD).^{5, 6} Compared to typical nasal drop, pump or mucosal atomization device (MAD), this device provides a preferential deposition of aerosol to olfactory tissues at levels and exposure beyond that achievable by MAD. While intranasal POD delivery of more water soluble morphine led to a rapid analgesic onset and low systemic exposure, parallel experiments with more hydrophobic fentanyl exhibited a very rapid and intense onset. However, fentanyl at a typical dose, fentanyl's analgesic effect is relatively short (< 30 minutes), partly due to the rapid redistribution of fentanyl to plasma.⁵ A slower and sustained release of fentanyl may provide longer analgesic coverage and lower plasma exposure often related to reduced bowel movement as well as in severe cases respiratory depression.

Liposomes or lipid vesicles are nano-drug particles may overcome some of these limitations of aerosolized drug delivery to potentiate neurological impacts. Liposomes are nanoparticles consisting of a phospholipid bilayer with an aqueous compartment, which may allow carrying and delivering drugs with a wide range of lipophilicities. With a stable and robust enclosed membrane (outer layer), liposome can carry water soluble hydrophilic small molecule drugs as well as protein, RNA, and DNA drugs within the aqueous compartment⁷; whereas lipophilic drugs such as indinavir and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) were demonstrated to embed stably in the lipid bilayers of lipid nanoparticles.^{8, 9} In addition, the liposome membrane is able to protect many drugs from degradative enzymes located at the epithelium and within the circulation, which can improve their absorption and half-life.

Surface decoration of liposomes with targeting peptides could further improve the absorption properties of aerosol drugs at the nasal epithelium. Fatty acid-linked targeting peptides can be inserted into the liposome membrane facing the external environment to improve drug accumulation at the site of interest.¹⁰ The integrin targeting ligand Arg-Gly-Asp (*RGD*) peptide sequence is a well-established motif mainly used to target tumor cells for therapy and imaging.¹¹⁻¹³ *RGD*-expressed liposomes have been shown to preferentially concentrate at cells over expressing alpha(v)beta(3) integrins. In addition to many types of cancer cells, alpha(v)beta(3) integrins are expressed by most epithelial cells. An *RGD*-expressing liposome could increase binding to the nasal epithelial cells to increase the residence time and absorption of aerosol delivered drugs. An *RGD*-targeting peptide may also increase the ability of liposomes to penetrate through epithelial layers. Several studies have shown *RGD* liposomes to possess increased membrane permeability through integrin-mediated transcytosis.^{14, 15} For lipophilic drugs such as the opioid pain medication fentanyl, targeted liposomes could cause a slower release of drug, which upon transfer to the brain could provide a sustained analgesic effect as well as increase the overall analgesic effects or AUC_{effect} for each dose.

However, applications of liposomes in aerosol delivery have been reported to pose stability problems.¹⁶ Many liposomal formulations have undergone physical structural changes during the aerosolization process.¹⁶ The atomization process involves strong shear force that could potentially disrupt the liposome membrane.¹⁷ This disruption could result in release of

the liposomal drug, which would lower the stability and efficacy of the drug. For *RGD*-liposomes, a change in stability would result in a loss of target binding recognition ability.

Therefore we have designed an integrin targeted liposomal formulation composed of two well-defined phospholipids—1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC); 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) lipids, and evaluated aerosol stability essential for use as a delivery vehicle for both hydrophobic and hydrophilic drugs.^{8, 9} In this study we have incorporated an integrin targeting peptide into the bilayer and tested the effects of aerosolization on retention of liposome encapsulated aqueous marker, calcein, and target binding activity of *RGD* expressed on liposomes. When a more hydrophobic compound fentanyl was incorporated into the *RGD* liposomes, effective analgesic duration was increased and the total AUC_{effect} was enhanced with reduction in plasma drug exposure.

MATERIALS AND METHODS

Materials

The phospholipids, DMPC and DMPG (both > 99% purity) were purchased from Sygena, Inc. (Cambridge, MA). Palmitoylated peptide Gly-Arg-Gly-Asp-Ser (*GRGDS*) (> 99.9% purity) was custom synthesized by United Biochemical Research, Inc. 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl); NBD-PE (> 99% purity) was purchased from Avanti Polar Lipids (Alabaster, AL). Fentanyl citrate was purchased from Sigma-Aldrich (St. Louis, MO). Beuthanasia-D (Schering-Plough Animal Health Corp, North Chicago, IL) was used for euthanasia at the end of the distribution studies. Fentanyl was dissolved in 0.9% saline solution (Hospira Inc, Lake Forest, IL). All other chemicals used were of analytical grade.

Integrin Targeted and Non-Targeted Liposomal Preparation

Routinely, lipid-associated fentanyl (1:10; drug: lipid molar ratio) was prepared by first dissolving 20 mg of DMPC and DMPG (1:1, mol/mol) in 1 ml of chloroform in a test tube and evaporating off the solvent with a stream of N₂ gas to form a dry film, followed by vacuum desiccation overnight to remove residual solvent. In the targeted liposomes, 0-1% mol/mol *RGD* was added to the organic phase. Where needed, 1% mol/mol of NBD-PE, (Avanti Polar Lipids, Alabaster, AL) a fluorescent lipid marker, was added to the organic phase. The dry film was then vacuum desiccated for at least 30 minutes. To prepare desired lipid concentrations, a 1-ml volume of 0.9% saline solution with 0.375 mg/ml fentanyl was then added to create a 20 mg/ml suspension. The mixture was then sonicated at 27°C in a (water) bath type sonicator (Laboratory Supplies, Inc., Hicksville, NY) until a uniform translucent suspension of small unilamellar vesicles (SUVs) was obtained. Under these conditions, we found approximately 80% of fentanyl associated with liposomes and used without removal of unbound drug in the preparation. In the case of the drug release study, the PBS added prior to sonication contained 50 mM calcein (Sigma, St. Louis, MO). After sonication the free calcein was removed from the solution by dialysis. Free fentanyl dosages were prepared just prior to drug administration and consisted of fentanyl citrate in sterile 0.9% saline solution.

Liposome and Aerosol Particle Characterization

The size of liposomes was determined by photon correlation spectroscopy (PCS) (Malvern Zetasizer 5000, Southborough, MA). The liposomes and free drug solution were aerosolized by spraying the solution through a pressure driven aerosol nozzle. The liposome containing aerosol droplet size distributions were determined by a Phase Doppler Particle Analyzer (PDPA) (TSI, Shoreview, MN) using 200 mW argon laser emitting beams of 488 and 514.5 nm wavelength (Ion Laser Technology, model # 5500A-00). The measurements were taken 2.75 cm from the tip of the nozzle, as this represents the distance from the proximal opening of the nasal vestibule to the center of the respiratory epithelium. Initially, the measurement volume was moved across the aerosol stream to determine the edges of the spray. Then, sizing measurements were determined at 1mm intervals across the width of the spray, taking 30,000 measurements at each interval. Sizing data is presented as a volume weighted mean

and span, defined as $Span = \frac{D_{v90} - D_{v10}}{D_{v50}}$, where D_v is droplet frequency distribution. For determination of a change in liposome particle size due to aerosolization, we determined liposome size by PCS before and after the solution was collected in a 25 ml conical tube at a 5° angle to the container.

Liposome Membrane Stability

The amount of liposome leakage due to aerosolization was determined according to the method of Piperoudi et. al.¹⁸ The liposomes were sonicated in a solution of PBS containing 50 mM calcein. The liposomes were then dialyzed overnight to remove the non-encapsulated calcein. The fluorescence signal from the encapsulated calcein at 50mM is quenched and has no fluorescence signal, but as the liposomes leak calcein into the surrounding buffer the fluorescence signal increases. A fraction of the liposomes were aerosolized and collected in a 5 ml conical tube and measured for fluorescence signal.¹⁹ The percent leakage was calculated from the difference in fluorescence signal before and after aerosolization compared to the total encapsulated calcein (determined by adding 1% Tween 20 to liposomes to release all encapsulated calcein and measuring fluorescence signal). The fluorescence of the liposome solution was measured on a PerkinElmer 1420 multivariable fluorescence plate reader (PerkinElmer, Waltham, MA).

Cell Lines

Human Umbilical Vein Endothelial Cell (HUVEC) and Porcine Kidney Proximal Tubule Cell (LLC-PK1) are well validated cell lines to study *RGD*-integrin interactions and were purchased from American Tissue Culture Collection (ATCC) (Manassas, VA). LLC-PK1 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 0.1 mg/ml streptomycin). HUVEC cells were cultured in F12-K media supplemented with 100 µg/ml endothelial cell growth supplement (BD Biosciences, Franklin Lakes, NJ), 10% FBS, and antibiotics. All cells were grown at 37 °C in a 5% CO₂ and 95% air humidified atmosphere.

Binding Studies

The cell lines used for the binding studies were plated into 96-well flat-bottomed cell sterile culture plates (BD Biosciences, Franklin Lakes, NJ) at a density of 1.0×10^5 cells/well. Once the cells were attached, the media was removed and the cells were incubated with 200 μ l of the various liposome preparations for 30 minutes at 37 °C in a 5% CO₂ and 95% air humidified atmosphere. For the competitive binding experiment, the cells were pre-incubated for 20 minutes with 25 $\times$ concentration of soluble cyclo Arg-Gly-Asp-D-Phe-Lys (cRGD) peptide (Peptides International, Louisville, KY). The cells were then gently washed 3 times with PBS, pH 7.4, covered with 200 μ l of PBS, pH 7.4, and then analyzed with either a fluorescent plate reader or a Zeiss Axiovert 200 fluorescent microscope (Carl Zeiss Inc., Jena, Germany).

Fentanyl Studies in Rats

All animal experiments were performed under the approval of the institutional animal care and use committee. For each experiment, the animals were dosed free drug or liposome encapsulated fentanyl while under isoflurane anesthesia. For the tail-flick and distribution study, each animal was briefly anesthetized with 5% isoflurane in an induction chamber, the animal was removed from the induction chamber and the drug was administered with a pressurized olfactory delivery (POD) device. This device and the administration technique are more fully described in a previous publication.⁵ After dose administration, the animal was turned onto its side and then the animal was allowed to recover from the anesthesia. After a 30 minute resting period under anesthesia after surgery, the rats were administered a single dose of 15 μ g/kg fentanyl.

Each animal remained anesthetized with 2% isoflurane throughout the pharmacokinetic study. First the femoral vein was cannulated in order to draw blood during the experiment. After surgery, the animal was allowed to remain under anesthesia for 20 minutes. Then, for the olfactory nasal drug delivery, the anesthesia nose cone was removed, the dose was given quickly (< 45 seconds), and the nose cone was replaced. After drug administration, each animal was placed on its side on a heat pad.

In the CNS distribution experiment, animals were initially anesthetized with 5% isoflurane. Once they were unconscious, they were quickly removed from the induction box and dosed as described for the tail-flick experiment. They were allowed to naturally recover from the anesthesia.

Analgesic Tail-Flick Test

Each group of animals first went through three days of placebo testing to get a baseline reading for the tail flick test as well as to acclimate the animals to handling. Each animal (N=8) was exposed to 5% isoflurane in an induction box. Once anesthetized, the animal was removed and given a 10 μ l dose of 0.9% saline solution, pH 7.4 via POD device. Once the placebo dose was administered, the animals were allowed to fully wake from the isoflurane in a padded tray. Then at 5, 10, 30, 45, 60, 90, and 120 minutes, each animal was wrapped gently in a towel, had their tails placed in room temperature water ($18^\circ \pm 0.5^\circ$ C) for 5

seconds, the tail was quickly dried, and then the distal 3 cm of the tail was placed in $55^{\circ} \pm 0.5^{\circ}\text{C}$ water. The time until tail removal was measured with a digital stopwatch.

After the initial placebo trials, the same procedure was repeated three times, every other day over 5 days, with each rat receiving a single dose of fentanyl by POD spray with either free drug fentanyl or fentanyl incorporated into *RGD*-liposomes. The cutoff time, at which the tail would be removed from the water to prevent tissue damage, was set at 10 seconds for all tail-flick trials.

Tissue and Plasma Sampling

In the PK experiments, 300 μl of blood was drawn from the rats ($N=4$) femoral vein catheter at 5, 10, 30, 45, 60, 90, and 120 minutes after drug administration. The blood was collected in a 1 ml syringe (Becton Dickinson, Franklin Lakes, NJ) and transferred to a microcentrifuge tube for blood/plasma separation. The tubes were immediately centrifuged at 8,000g for 5 minutes. Then the plasma was removed and frozen on dry ice. At the end of the experiment 1.0 ml of sterile 0.9% saline solution was slowly injected via the femoral vein catheter to replace the removed blood volume.

In the tissue distribution experiments, the animals were sacrificed at various time points after drug administration with an overdose of Beuthanasia-D. Immediately after death, the animal was decapitated. The base of the skull and the parietal bones were quickly removed. The brain was removed within 2 minutes of sacrifice. The brain was dissected into the cortex, diencephalon, brainstem, cerebellum, and olfactory bulbs. The cervical spinal cord was also collected from the body. The olfactory bulbs were the final brain tissues collected.

Tissue and Plasma Fentanyl Determination Based on LC/MS Assay

A fixed volume (20 μl) of fentanyl d-6 (Cerilliant, Palo Alto, CA) was added into each tissue and plasma sample to act as an internal standard. Fentanyl tissue samples were homogenized in 5-10 times volume of 0.1M potassium phosphate buffer, pH 6.0, and centrifuged for 10 minutes at 1000 g. The fentanyl tissue supernatant and plasma samples were passed over Certify solid phase extraction cartridges (Varian, Palo Alto, CA) and eluted with methylene chloride: isopropanol: ammonium hydroxide (80:20:2). After elution, the samples were evaporated under N_2 gas until dry. The samples were resuspended in 75 μl of mobile phase which consisted of 40% 10 mM ammonium acetate and 60% acetonitrile. An Agilent HPLC/MS series 1100 series B with autosampler (Agilent, Santa Clara, CA) was used for quantification. The injection volume was 5 μl . A Zorbax SB-C8 column (Agilent, Santa Clara, CA) was used for the separation. The flow rate of the column was 0.25 ml/min. The ionization setting was API-ES in positive mode with a capillary voltage of 1400 V.

A standard curve was created on the day of analysis according to the same process described for the samples. Each standard curve was linear with a coefficient of linear regression $R^2 > 0.99$. In addition, two quality control samples with a known amount of drug were processed on the day of analysis in order to ensure day-to-day consistency of the analytical assay.

Statistical Analysis

All tail flick test values are presented as a percentage of maximal possible effect, which is defined as:

$$\frac{\text{Post drug delivery} - \text{baseline latency}}{\text{Cutoff time} - \text{baseline latency}} \times 100$$

AUC values from all experiments were calculated using the trapezoidal rule without extrapolation to infinity. Pharmacokinetic values were obtained from one-compartmental modeling in WinNonlin (Pharsight, Mountain View, CA). $\text{AUC}_{\text{effect}}/\text{AUC}_{\text{plasma}}$ ratios were calculated from individual animals so they could be statistically compared with a one-way ANOVA. Data are presented as the mean $\pm$ SD. Statistical significance was evaluated either by unpaired Student's t-tests (two-sided) or one-way ANOVA (either paired or unpaired) using SigmaPlot software (Systat, San Jose, CA).

RESULTS

Design and Characterization of Integrin Targeted Liposomes

We first prepared liposomes expressing a well-described integrin ligand, Arg-Gly-Asp (*RGD*) tripeptide. The *RGD* motif is expressed on liposome membrane composed of DMPC and DMPG (1:1 m/m) using palmitoylated pentapeptide (PAL-*GRGDS*; 1 mole%). These liposomes, referred to as *RGD* or integrin targeted liposomes were fluorescently labeled with NBD-PE to characterize their binding selectivity to LLC-PK1 epithelial cells expressing $\alpha V\beta 3$ integrin. As shown in Fig. 1, increasing concentrations of *RGD* liposomes exhibit much higher than the control liposome NBD fluorescent tracer binding when they were exposed to LLC-PK1 epithelial cells. In addition, *RGD* liposome binding to LLC-PK1 cells exhibited a lipid (or liposome) concentration dependent manner. In contrast while the control liposomes did not exhibit dose dependent binding. To further determine the density and selectivity of *RGD* mediated liposome binding to integrin-expressing cells, we repeated cell-binding assay with a fixed liposome concentration while the density of *RGD* on each liposome is varied. The increasing density of *RGD* on liposome is achieved by varying the percentage of palmitoylated *GRGDS* in the lipid from 0.25% to 1.0% of the total lipid. For these experiments, we employed HUVEC cells with higher density of integrin expression. The liposomes with varying ratios of *RGD* on their surface (under a fixed and identical lipid concentration) were incubated with HUVEC epithelial cells that express high levels of integrin. As shown in Fig. 2, when *RGD* density in the liposome was increased from 0.25% to 1.0%, an increase in level of NBD-labeled liposome binding was detected (Fig. 2). While there were some differences in degree of surface versus internalized liposome, fluorescence was noted in LLC-PK1 and HUVEC cells, their significance is not clear.

These data suggests that the liposome binding to cells expressing integrin is dependent on *RGD* density on the liposomal membrane. To further determine selectivity of liposome binding to integrin on cell surface, a competitive binding assay experiment with free cyclic form of *RGD* or *cRGD*, which exhibits a higher affinity to $\alpha V\beta 3$ integrin receptors. When the cells were pre-incubated with 25 $\times$ molar excess of a cyclic *RGD* peptide (which exhibits

much higher affinity than *RGD* for $\alpha V\beta 3$ integrin receptors), binding of the integrin targeted liposomes to LLC-PK1 cells was completely inhibited (Fig. 3). Collectively these data indicate that *RGD* liposomes binding to cells expressing integrin are mediated by *RGD* expressed on liposome surface.

Stability of Liposomes and RGD Ligand Under Aerosolization

As *RGD* liposomes are intended for intranasal aerosol dosage form, it is essential to determine stability of membrane structure and liposome expressed ligand *RGD* as they pass through the aerosol jet under mechanical stress. We evaluated physical stability before and after aerosolization by monitoring liposome diameter and the ability to retain an entrapped self-quenching fluorescence dye calcein. The functional stability of *RGD* was also evaluated based on *RGD* mediated binding to integrin on LLC-PK1 epithelial cells. The *RGD* liposomes (0.1 mM lipid) were tested on a prototype aerosolization device. The performance and characteristics of this prototype device have been reported and please refer to these publications for details.^{5, 6} Operating at 2.0 psi this device produces a pressure similar to that of a typical nasal spray pump. The volumetric mean aerosol diameter of the liposome solution using the aerosol device was 29.18 μm with a span of 0.95 μm and a driving pressure of 2.0 psi. With this robust device, we first examined *RGD* liposome stability by evaluating their diameter before and after aerosolization. The *RGD* liposome size distribution before and after aerosolization were 96.5 ± 6.1 nm (mean $\pm$ SD) versus 104.1 ± 4.9 nm, respectively indicating that there is no significant ($P > 0.05$) impact on the particle size of liposomes as they pass through the aerosol jet under mechanical stress.

To evaluate *RGD* liposome membrane stability after aerosolization, we evaluated the ability of *RGD* liposome to retain a hydrophilic, water soluble fluorescent marker calcein.²⁰ As a self-quenching dye, at high calcein concentration (50 mM), calcein encapsulated stably in the liposomes would be quenched (low fluorescence value). If the calcein released from the liposomes due to membrane instability or leakage during aerosolization, the greatly diluted calcein would be unquenched, and thus liposome lysis is detectable as an increase in calcein fluorescence. By following the gain in fluorescence of 50 mM calcein encapsulated in *RGD* liposome before and after aerosolization, impact of passage liposome through aerosol nozzle mechanical sheering was monitored.²¹ We found a minimal impact of aerosolization on *RGD* liposome membrane stability as increase in calcein fluorescence was minimal and recorded at 2.0–2.2% of total encapsulated calcein after liposome passage through the pressurized aerosol nozzle of the POD delivery system.

To evaluate functional stability of the *RGD* on liposome surface, we determined binding affinity of the *RGD* liposomes binding to LLC-PK1 cells before and after aerosolization. As shown in Fig. 4, the dose-dependent binding for *RGD* liposome before and after subjected to aerosolization was nearly identical suggesting that passage through the nozzle of aerosol device had no impact on *RGD* ability to bind to its receptor, integrin on epithelial cells. Taken together, these data indicate that passage through a typical aerosol nozzle, the *RGD* liposomes composed of DMPC:DMPG and palmitoylated-*GRGDS* is physically and functionally stable for the intended aerosolized intranasal dosage form.

Effects of RGD Liposome Incorporation on the Pharmacokinetics and Analgesic Impacts of an Opioid Fentanyl Intranasal Dosage Form

To evaluate impacts of the *RGD* liposomes binding to epithelial cells *in vivo*, we incorporated an opioid analgesic fentanyl into the liposomes and determined the time-course plasma concentration profiles (Table 1).

A majority ~80% of fentanyl was incorporated into *RGD* liposome and these fentanyl *RGD* liposomes were used. For evaluation of pharmacokinetics and analgesic effects, we employ a pressurized olfactory drug (POD) delivery device that previously shown to achieve preferential deposition on the upper nasal cavity including olfactory epithelial cells lining nose and brain interface.^{5, 6} If *RGD* liposome mediated enhanced accumulation of fentanyl binding to olfactory and upper nasal epithelium, a time-delay in appearance and lower plasma fentanyl exposure would be expected. On the other hand, the enhanced olfactory exposure of fentanyl and higher resident time provided by fentanyl in *RGD* liposome dosage form given in POD delivery device should provide a faster analgesic onset and extended coverage. The plasma time course of fentanyl in Fig. 5 showed that at 5 minutes after administration, rats dosed with fentanyl in *RGD* liposomes exhibited significantly lower (by about 27 percentage) plasma concentration than free drug. Over the 120 minutes, rats receiving fentanyl in *RGD* liposome formulation exhibited lower plasma drug concentrations, which reflected in about 20% reduction in total plasma fentanyl exposure ($AUC_{0-120} = 208.2$ vs 284.8 ng*min/ml) (Fig. 5).

The analgesic pharmacodynamics effects of fentanyl in rats were determined with a well-established tail-flick latency test as described in the Materials and Methods. Both free and *RGD* liposome formulated intranasal fentanyl were administered with a POD delivery device intended to deposit in the upper nasal cavity. As shown in Fig. 6, a clear impact of *RGD* liposome effects in the fentanyl analgesic effects was detected at early and throughout the time-course. Animal receiving *RGD* liposome formulation experiencing much higher overall analgesic effects compared to their free drug counterpart ($AUC_{\%MPE} = 1387.1$ vs 760.1 ; Fig. 6). The animals receiving free fentanyl exhibited greater than 2 fold higher analgesic effect at 5 min, compared to those receiving the same dose in *RGD* liposome formulation (Fig. 6). However, the analgesic effects decline faster with time in animals receiving free drug. By 30 minutes, no analgesic effect was detectable for this group. In contrast, animals receiving fentanyl in the *RGD* liposome exhibited a slower analgesic onset with the maximum effects recorded at 10 minutes. Even with a slower onset, animals receiving fentanyl in *RGD* liposomes exhibited a higher analgesic effect at every time point measured after 10 min. As a result, animals receiving fentanyl in the *RGD* liposomes exhibited 1.8 fold higher overall analgesic effects detected in AUC_{effect} ($AUC_{\%MPE*min} = 1387.1$ vs 760.1) than those treated with intranasal free drug in the same POD delivery device. Collectively these data indicate that when intranasal fentanyl is given by POD delivery device that preferentially deposit at olfactory regions, drug incorporated in *RGD* liposome provided a lower overall plasma drug exposure and higher total analgesic effects but slower therapeutic onset than those receiving free drug formulation.

DISCUSSION

Taking advantage of the ability of form aerosol stable integrin targeted *RGD* liposomes with more robust membrane composition, we have demonstrated enhancement in integrin-mediated liposome binding to epithelial cells. The enhanced epithelial cell binding after intranasal administration of fentanyl opioid analgesic in *RGD* liposome form may have contributed to the extended analgesic coverage and lower plasma exposure than that given as free fentanyl dosage form (Figs. 5 and 6). In our gel-phase liposome design, we used two phospholipids with symmetrical C16 saturated fatty acyl chains, di-myristoyl carrying phosphatidylcholine (DMPC, a neutral net charge at pH7) and phosphatidylglycerol (DMPG, a negative net charge at pH7). These liposomes containing DMPC and DMPG (1:1 m/m) carry a surface net negative charge may have reduced liposome aggregation and assume a gel-phase membrane structure under room and physiological temperature. These attributes may be related to retention of encapsulated materials and overall membrane integrity, including size attribute as liposomes undergone sheer force through aerosolization process.

We chose to test the stability and functionality of *RGD* liposomes with a POD intranasal delivery device that produces an aerosol with a well-defined size range ($d = 29.18 \mu\text{m}$). With 2 psi input pressure, this pressure setting is similar to that typically used by other nasal pumps and aerosol devices. As *RGD* liposome diameters are within 97 – 104 nm range, which value is not affected by aerosolization, the size stability of liposomes may be due to the size differential between liposome and aerosol. With an aerosol diameter of $29.18 \mu\text{m}$, this aerosol volume is 2.9×10^6 times larger than the liposome volume (diameter approximately 104 nm). This large volume and size difference has likely avoided disruption of liposome membranes when they are being aerosolized. The stable size distribution before and after aerosolization indicates the *RGD* liposomes can withstand the shear force during aerosolization. With other lipid composition, scientists have reported significant content leakage of liposome encapsulated materials.⁷ Current data demonstrating the near complete retention of liposome encapsulated calcein under aerosolization suggest that little or no disruption and reforming of the liposomes; thus validating the physical stability of the liposome membrane during aerosolization.

In addition to physical stability of liposomal membrane, we found that the integrin targeting function of the *RGD* peptide was unaffected by the aerosolization process. The stability of the liposome membrane is most likely due to the choice of lipids, which form gel-phase liposome membranes at room temperature during aerosolization. In addition, the short *RGD* peptide integrated in palmitoylated peptide palmitoyl-*GRGDS* form was chosen to minimize functional impact due to shear force. The palmitoyl fatty acyl chain allows complete incorporation of lipidic peptide into liposomes in one-step mixing with DMPC, DMPG together in organic phase prior to solvent evaporation and and liposome size reduction after rehydration in aqueous buffer. This simplified process is particularly important for liposome scale up preparation essential for successful clinical translation. With complicated *RGD* and other ligand coupling chemistry after liposome formation, scale up process became a costly and challenging process with respect to cost and reproducibility.⁷ Although some suggested that palmitoylated or fatty acid anchored peptides in liposomes may be unstable in serum, the palmitoylated peptide stability may be liposome composition and peptide specific.⁷ In

our studies, we performed integrin mediated *RGD* liposome binding to integrin in epithelial cells in the presence of serum and thus suggest that these liposomes are serum stable. The overall chemical and physical stability of *RGD* liposomes in blood and their pharmacokinetic fate after IV administration is not known. However such study, while it is of interest, is beyond the scope of this report.

The importance of *RGD* mediated liposome binding to epithelial cells for the overall improvement in enhanced opioid effects and reduce plasma drug exposure should not be underestimated. The initial experiment (Fig. 1) shows that the *RGD* peptide mediated a concentration-dependent binding to the epithelial cells expressing integrin receptors. The increased binding of liposomes to cells with escalating *RGD* (Fig. 2) density on liposome membrane confirms further that the cell binding is *RGD* density dependent and an optimal binding was achieved with 1 mole percentage *RGD* (in pal-*GRGDS*) in total composition. This value is similar to those reported for *RGD* mediated binding to epithelial cells and integrin mediated cellular uptake of liposomes.^{14, 15} In addition, liposome binding to epithelial cells could be inhibited with excess free *cRGD* verifying specificity of *RGD*-integrin binding (Fig. 3). Whether optimization could be achieved with multiple repeat of *RGD* sequence or with additional spacers between palmitoyl and *RGD* motif is not clear. This and other issues related to binding affinity, specificity and selectivity are under our future investigation and beyond the scope of this report.

While the exact mechanisms and physiological processes leading to fentanyl analgesic effect is not fully understood, it is possible that the fentanyl in *RGD* liposomes binds to integrin proteins on epithelial cells, and that this binding allowed retention of fentanyl for an extended time in nasal and olfactory epithelia. When the *RGD* liposomes are administered with the POD device, it has been demonstrated that drugs in aerosol are preferentially deposited at the olfactory epithelium as depicted in schematic Fig. 7. We envisioned that the *RGD* liposomes bound to the integrin in the olfactory epithelial cells located at the interface of the nose and the brain. Anatomical evidence suggests that the liposomes would not be able to easily cross the epithelium after deposition due to the size limitation. In the current liposome design with *RGD* expressed on surface (Fig. 7), it is unlikely that liposomes bound to the olfactory epithelial will penetrate as a lipid bound fentanyl in the brain. It is likely however that the lipophilic opioid fentanyl is released from liposome either due to degradation of liposomal lipid by lipases or drug dissociation due to proteins and other factors interacting with liposome fentanyl. We hypothesize that mostly free fentanyl is distributed to the brain rapidly and released into blood stream. This hypothesis is consistent with the fentanyl distribution profile in brain (Table 2) where no significant differences between free or *RGD* liposome-associated fentanyl dosed with POD device, which was previously shown to deposit the drug mostly in upper nasal cavity or olfactory regions.^{5, 6} At 5 minute after nasal administration there is no difference in fentanyl levels in the forebrain or MCS (Table 2), even with higher plasma drug levels detected in animals received free fentanyl (Fig. 5).

Evidence in the literature indicates that the olfactory epithelium contains extracellular channels, which make the olfactory epithelium less resistance to extracellular transport compared to the nasal and respiratory epithelium.²¹ These extracellular channels in the

olfactory epithelium are created by the olfactory neurons and their surrounding olfactory ensheathing cells which leave fluid filled pathways that are 10-15 nm in diameter.^{22, 23} These extracellular pathways, which cross into the lamina propria of the epithelium, are too small for the *RGD* liposomes (d~ 100 nm) to pass. This has been confirmed in other studies where particles diameter greater than 50 nm were unable to penetrate the nasal epithelium or via extracellular routes.^{24, 25} Considering the integrin binding properties of the *RGD* liposomes, these liposomes most likely and readily bind to the epithelium after aerosol deposition on the olfactory epithelium; and epithelial bound fentanyl is released locally, acting as a drug depot at the nasal and olfactory epithelium. The fentanyl then passed through the nasal and olfactory epithelium where it was taken up in the blood stream or directly distributed to the brain through olfactory. It is likely that both nasal and olfactory epithelial passage of fentanyl is in operational for *RGD* liposome delivery system. This hypothesis is consistent with a significant enhancement in the overall analgesic coverage without detectable reduction in the initial onset of analgesia compared to free fentanyl treatment (Fig. 6). This effect led to a lower, more stable analgesic effect, which lasted 60 minutes in comparison with 10 minutes with the free drug formulation (both using POD delivery device). While the elucidation of the exact mechanisms remained, our data indicates that fentanyl delivered in the *RGD* liposome formulation also led to a significantly higher and longer lasting analgesic effects (AUC_{effect} compared to the free drug formulation) and reduce plasma drug exposure (over 2 hrs experimental period) that may be related to respiratory and gastrointestinal untoward effects of fentanyl in blood.

CONCLUSIONS

We have developed an aerosol stable *RGD* tripeptide coated liposome formulation suitable for nasal drug delivery that retained structural integrity and integrin targeting ability. This formulation with an appropriate olfactory targeted delivery device could be adapted to express peptides selecting cancers and other pathogenic cells or tissues. As aerosolized drugs are used in nasal, topical, and other applications, such liposome formulations could be widely applicable for these routes of administration as well. When the analgesic drug fentanyl was incorporated into these *RGD* liposomes and administered intranasal with a device targeted to the olfactory region of the nasal cavity, they resulted in a more gradual, longer lasting analgesic effect compared to the free drug formulation. This formulation coupled with an olfactory delivery device have the potential to incorporate other small molecule drugs to extend the release from the nasal cavity into the blood stream and increase the overall effectiveness of the drug.

Acknowledgments

This work was supported in part by NIH grants AI 077390 and MH086351, and University of Washington Technology Innovation grant TGIF-1001. RYJH is also supported by Milo Gibaldi Endowment.

REFERENCES

1. Aggarwal R, Cardozo A, Homer JJ. The assessment of topical nasal drug distribution. Clin Otolaryngol Allied Sci. 2004; 29:201–205. [PubMed: 15142061]

2. Charlton S, Jones NS, Davis SS, Illum L. Distribution and clearance of bioadhesive formulations from the olfactory region in man: effect of polymer type and nasal delivery device. *Eur J Pharm Sci.* 2007; 30:295–302. [PubMed: 17223022]
3. Illum L. Nasal drug delivery--possibilities, problems and solutions. *J Control Release.* 2003; 87:187–198. [PubMed: 12618035]
4. Dhuria SV, Hanson LR, Frey WH 2ND. Intranasal delivery to the central nervous system: mechanisms and experimental considerations. *J Pharm Sci.* 2010; 99:1654–1673. [PubMed: 19877171]
5. Hoekman JD, Ho RJY. Enhanced analgesic responses after preferential delivery of morphine and fentanyl to the olfactory epithelium in rats. *Anesth Analg.* 2011; 113:641–651. [PubMed: 21709146]
6. Hoekman JD, Ho RJY. Effects of localized hydrophilic mannitol and hydrophobic nelfinavir administration targeted to olfactory epithelium on brain distribution. *AAPS Pharm Sci Tech.* 2011a; 12:534–543.
7. Kraft JC, Freeling J, Wang Z, Ho RJY. Emerging research and clinical development trends of liposome and lipid nanoparticle drug delivery systems. *J Pharm Sci.* 2014; 103:29–52. [PubMed: 24338748]
8. Bethune C, Blum A, Geyer JR, Silver JR, Ho RJ. Lipid association increases the potency against primary medulloblastoma cells and systemic exposure of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) in rats. *Pharm Res.* 1999; 16:896–903. [PubMed: 10397611]
9. Bethune CR, Geyer RJ, Spence AM, Ho RJ. Lipid association improves the therapeutic index of lomustine [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea] to suppress 36B-10 tumor growth in rats. *Cancer Res.* 2001; 61:3669–3674. [PubMed: 11325837]
10. Willis M, Forssen E. Ligand-targeted liposomes. *Adv Drug Deliv Rev.* 1998; 29:249–271. [PubMed: 10837594]
11. Garanger E, Boturyn D, Dumy P. Tumor targeting with RGD peptide ligands-design of new molecular conjugates for imaging and therapy of cancers. *Anticancer Agents Med Chem.* 2007; 7:552–558. [PubMed: 17896915]
12. Dubey PK, Mishra V, Jain S, Mahor S, Vyas SP. Liposomes modified with cyclic RGD peptide for tumor targeting. *J Drug Target.* 2004; 12:257–264. [PubMed: 15512776]
13. Kurohane K, Namba Y, Oku N. Liposomes modified with a synthetic Arg-Gly-Asp mimetic inhibit lung metastasis of B16BL6 melanoma cells. *Life Sci.* 2000; 68:273–281. [PubMed: 11191643]
14. Xiong XB, Huang Y, Lu WL, Zhang X, Zhang H, Nagai T, Zhang Q. Enhanced intracellular delivery and improved antitumor efficacy of doxorubicin by sterically stabilized liposomes modified with a synthetic RGD mimetic. *J Control Release.* 2005; 107:262–275. [PubMed: 16125816]
15. Xiong XB, Mahmud A, Uludag H, Lavasanifar A. Multifunctional polymeric micelles for enhanced intracellular delivery of doxorubicin to metastatic cancer cells. *Pharm Res.* 2008; 25:2555–2566. [PubMed: 18636321]
16. Niven RW, Schreier H. Nebulization of liposomes. I. Effects of lipid composition. *Pharm Res.* 1990; 7:1127–1133. [PubMed: 2293210]
17. Bridges PA, Taylor KM. An investigation of some of the factors influencing the jet nebulisation of liposomes. *Int J Pharm.* 2000; 204:69–79. [PubMed: 11011988]
18. Piperoudi S, Fatouros D, Ioannou VP, Frederik P, Antimisariis SG. Incorporation of PEG-lipids in arsonoliposomes results in formation of highly stable arsenic-containing vesicles. *Chem Phys Lipids.* 2006; 139:96–106. [PubMed: 16405880]
19. Choi S, Bui T, Ho RJY. pH-dependent interactions of indinavir and lipids in nanoparticles and their ability to entrap a solute. *J Phar Sci.* 2008; 97:931–943.
20. Hu LR, Ho RJ, Huang L. Trypsin induced destabilization of liposomes composed of dioleoylphosphatidylethanolamine and glycophorin. *Biochem Biophys Res Commun.* 1986; 141:973–978. [PubMed: 3814129]
21. Jansson B, Bjork E. Visualization of in vivo olfactory uptake and transfer using fluorescein dextran. *J Drug Target.* 2002; 10:379–86. [PubMed: 12442808]

22. De Lorenzo AJ. Electron microscopic observations of the olfactory mucosa and olfactory nerve. *J Biophys Biochem Cytol.* 1957; 3:839–850. [PubMed: 13481018]
23. De Lorenzo AJ. Electron microscopy of the olfactory and gustatory pathways. *Trans. Am Laryngol Assoc.* 1960; 81:39–49. [PubMed: 13720652]
24. Miyamoto M, Natsume H, Satoh I, Ohtake K, Yamaguchi M, Kobayashi D, Sugibayashi K, Morimoto Y. Effect of poly-L-arginine on the nasal absorption of FITC-dextran of different molecular weights and recombinant human granulocyte colony-stimulating factor (rhG-CSF) in rats. *Int J Pharm.* 2001; 226:127–138. [PubMed: 11532576]
25. Huang Y, Donovan MD. Large molecule and particulate uptake in the nasal cavity: the effect of size on nasal absorption. *Adv Drug Deliv Rev.* 1998; 29:147–155. [PubMed: 10837585]

Figure 1. Concentration-dependent binding of integrin targeted DMPC:DMPG liposomes with or without *RGD* coated

LLC-PK1 cells in 96-well flat-bottomed cell sterile plates were incubated with indicated concentrations (20-100 μM) of NBD fluorescently labeled liposome for 30 minutes at 37 °C and the fluorescent intensity and distribution was examined. Panels B1-B5 represent cells exposed to 20, 40, 60, 80 and 100 μM concentrations of liposomes with *RGD* expression. Panels A1-A5 represent cells exposed to 20, 40, 60, 80 and 100 μM concentrations of control liposomes without *RGD*.

Figure 2. Liposome cell binding with varying density of *RGD*

α V β 3 integrin expressing HUVEC cells in 96-well flat-bottomed cell sterile plates were incubated with liposomes containing fixed and identical lipid concentration (0.4 mM DMPC:DMPG) but with varying concentration of PA-GRGDS for example 0%, 0.25%, 0.5%, or 1.0% (panels **A**, **B**, **C** and **D** respectively) for 30 minutes at 37 °C and the *RGD* peptide density was measured.

Figure 3. Selectivity of *RGD* dependent liposomal binding to HUVEC cells

The integrin expressing HUVEC cells incubated with DMPC:DMPG liposomes with 1% NBD-PE and 1% PA-*GRGDS* were treated with 25 mole excess free *cRGD* for 30 minutes at 37 °C and inhibition of fluorescent intensity was analyzed. Panel **A** represents cells exposed to 25 mole excess free *cRGD* and panel **B** represents cells incubated without *cRGD*.

Figure 4. RGD-liposome quantitative cell binding in vitro

Integrin expressing LLC-PK1 cells in 96-well plates were incubated with varying concentrations (20-100 μM) of control ($\blacktriangledown$, $\triangle$; triangles) or RGD-coated ($\bullet$, $\circ$; circles) liposome for 30 minutes at 37 $^{\circ}\text{C}$ before ($\blacktriangledown$, $\bullet$; closed symbols) and after ($\triangle$, $\circ$; open symbols) aerosolization for the determination of binding affinity of the RGD liposomes to its receptor. The cell binding did not significantly change before and after the aerosolization for either the RGD-liposomes or the liposomes without RGD ($P > 0.05$).

Figure 5. Fentanyl plasma concentration over time after delivery of free fentanyl or fentanyl incorporated in *RGD*-liposome

Rats were delivered 15µg/kg free fentanyl (closed circles) and fentanyl incorporated in *RGD* liposomes (open circles) as described in materials and methods, n= 3) with the pressurized olfactory delivery device targeting the olfactory epithelium of the nasal cavity. Time course plasma drug concentration was monitored over 120 minutes and fentanyl concentration was determined in the plasma by LC/MS. The pharmacokinetic values were estimated based on one compartment analysis of time- course plasma drug concentration with WinNonlin.

Figure 6. Analgesic effect after nasal delivery of either free fentanyl or *RGD*-liposome incorporated fentanyl

Rats were intranasally administered with 15 μ g/kg fentanyl either in free and soluble (closed circles) or *RGD*-liposome encapsulated form (open inverted triangle) while under isoflurane anesthesia. The tail flick test was performed over a 120 minute period and analgesic effects were expressed as %MPE (or AUC_{effect}). The error bar indicates the SE of 7 animals per group tested. Incorporating fentanyl into the *RGD* liposomes resulted in a lower analgesic effect at 5 minutes, significantly higher analgesic levels at 30 and 45 minutes, as well as a significantly higher AUC_{effect} (* = $p < 0.05$).

Figure 7. An overview of the RGD-liposome and interaction with the cell

A The liposome were made with a 1:1 mixture of DMPC:DMPG phospholipids and with palmitic acid (PA) linked GRGDS used as the targeting peptide. B The liposomes formed an enclosed bilayer with PA embedded into the bilayer exposing the GRGDS peptide to the outside environment. Lipophilic drugs such as CCNU or fentanyl can be embedded into the lipid bilayer for drug delivery. C The RGD-liposomes were shown in this study to have an increased binding to integrin expressing cells. These RGD liposomes are suitable for aerosol delivery as aerosolization was shown to have little impact on the structural integrity of the liposome or targeting ability of the RGD-liposomes.

Table 1
Effects of *RGD*-liposomes containing fentanyl on pharmacokinetic and pharmacodynamic parameters after POD administration^a

		Free drug	<i>RGD</i> -liposomes-
Tmax(plasma)	(min)	1.70	4.77
Cmax	(ng/ml)	7.78 ± 0.079	4.95 ± 1.78
AUC (plasma)	(ng*min/ml)	284.79 ± 26.45	208.20 ± 74.88
Tmax(effect)	(min)	5	10
E _{max}	(%MPE)	76.94 ± 32.24	44.1 ± 16.62
AUC (effect)	(%MPE*min)	760.14 ± 425.75	1387.10 ± 234.39
<u>AUC (effect)</u>		1.37	7.58
AUC (plasma)			

Due to limitation in the fixed time drug sampling in the experimental design for all animals, no variation analysis could be done for the Tmax(plasma) and Tmax(effect)

^a *RGD*-liposomes containing fentanyl (15µg/kg) was delivered to rats with the pressurized olfactory delivery device targeting the olfactory epithelium of the nasal cavity. Time course plasma drug concentration and analgesic effects were monitored over 120 minutes. The pharmacokinetic values were estimated based on one compartment analysis of time- course plasma drug concentration with WinNonlin. The effects were analyzed based on E_{max} model to estimate maximum effect as %MPE and time-to-maximum effects or T_{max} (effect). (N=3 in the pharmacokinetic experiments and N=8 in the pharmacodynamics experiments)

Table 2
Effects of *RGD*-liposome on fentanyl localization in the brain at 5 min after intranasal fentanyl administration targeted to the olfactory region with POD device^a

Brain tissue	Fentanyl (ng/g tissue)	
	Soluble form	<i>RGD</i> -liposome
Fore brain	10.9±2.7	7.9±3.6
MCS	8.7±2.7	7.8±2.8

^aRGD-liposomes containing fentanyl or free fentanyl (15 µg/kg) was delivered to rats with the pressurized olfactory delivery (POD) device targeting the olfactory epithelium of the nasal cavity. Fentanyl concentrations in the brain were determined in the fore brain and MCS (midbrain-cerebellum-brain stem-spinal cord) as described in materials and methods. Values are expressed as mean ± SD of 8 animals per group.