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Tulane/Xavier Vaccine Peptide Program

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14. ABSTRACT The objective of this project is to develop novel formulation strategies for delivery of Flufirvitide-3 and other therapeutic peptides. Therapeutic peptides can be delivered in a non-invasive manner through the nasal mucosa and through the lungs. A wide range of factors, including mode of inhalation and particle size, influence the deposition of aerosols within the respiratory tract. Chemical modification of the Flufirvitide-3 will be evaluated to optimize its functional activity. The use of micro- and nano-technology will also be explored through the fabrication of particles encapsulating the peptide that are specifically suited for nasal and pulmonary delivery. The micro- and nano-particle carriers to be considered include a dry powder formulation, microemulsions, nonspherical liposomes, ceramic shell vesicles, and nanometer-sized silk particles. Nasal administration of soluble Flufirvitide-3 both pre- and post-exposure to influenza virus has been shown to be effective in preventing infection in an <i>in vivo</i> animal model. However, multiple doses, pre- and post-exposure were required for efficacy. We hypothesize that the proposed techniques will enhance the efficacy of the therapeutic peptide itself, thereby reducing the required dose, number of doses, and thus the cost of treatment, and improve distribution and release within the upper respiratory tract, thus expanding the duration of bioavailability and efficiency of peptide delivery.					
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INTRODUCTION

Influenza virus infections are among the most prevalent of all viral infections in man with between 10% and 20% of the United States' population suffering from influenza each year. Most individuals recover from influenza in one to two weeks. However, the very young, the elderly and persons with chronic medical conditions can develop post-flu pneumonia and other fatal complications. On average, more than 100,000 people in the United States per year are hospitalized as a result of influenza, with more than 35,000 deaths.

It is possible for the genomic segments of Influenza virus to mix and reassort in co-infected cells to produce a new virus with most segments from the human virus, but with segments encoding the HA and/or NA from the virus of a different animal species (i.e., avian, porcine). Because it contains gene segments encoding proteins for effective replication in humans, the reassorted virus may possess the ability to spread from person-to-person, but would have surface proteins to which people had not been previously exposed. Thus, most individuals will have little or no immune protection against the reassorted virus. The currently circulating strain of avian influenza is an H5N1 strain capable of directly infecting humans and other mammals, resulting in a mortality rate that exceeds 60% among nearly 400 confirmed human cases. This new H5N1 avian influenza A virus is of great concern because it possesses some potential for human-to-human transmission. A novel influenza A (H1N1) emerged in April 2009, and that strain also demonstrated efficient person-to-person transmission. CDC estimates that there were between 43 million and 89 million cases of 2009 H1N1, between 195,000 and 403,000 H1N1-related hospitalizations, and between 8,870 and 18,300 2009 H1N1-related deaths in the U.S. during the period April 2009 and April 10, 2010. Clearly, both seasonal influenza and novel reassortments have the ability to impact both public health and military readiness.

Because of the uncertainty that an antigenically-matched protective vaccine can be made rapidly available, antiviral drugs offer the best method to prevent the spread of a newly emerging strain of influenza A virus in the early stage of a pandemic or after intentional release. Only two classes of drugs are currently available for influenza, adamantanes and NA inhibitors (NAI, oseltamivir and zanamivir). Drug-resistant variants of both of these drug classes have been shown to arise in both seasonal and pandemic strains of influenza viruses, including the novel influenza A (H1N1).

We have recently identified a candidate anti-viral therapeutic peptide, designated Flufirvitide-3. We found that Flufirvitide-3 is a potent inhibitor of infections by divergent influenza viruses *in vitro*, including H1, H3 and H5 subtypes and influenza B viruses. When administered to the nasal cavities of ferrets, Flufirvitide-3 effectively blocked development of influenza. Flufirvitide-3 and other peptide inhibitors of viral fusion have a number of potential applications including pre- and post-exposure prophylaxis against seasonal or pandemic influenza.

BODY

The objective of this project is to develop novel formulation strategies for delivery of Flufirvitide-3 and other therapeutic peptides. Therapeutic peptides can be delivered in a non-invasive manner through the nasal mucosa and through the lungs. A wide range of factors, including mode of inhalation and particle size, influence the deposition of aerosols within the respiratory tract. Chemical modification of the Flufirvitide-3 will be evaluated to optimize its functional activity. The use of micro- and nano-technology will also be explored through the fabrication of particles encapsulating the peptide that are specifically suited for nasal and pulmonary delivery. The micro- and nano-particle carriers to be considered include a dry powder formulation, microemulsions, nonspherical liposomes, ceramic shell vesicles, and nanometer-sized silk particles. Nasal administration of soluble Flufirvitide-3 both pre- and post-exposure to influenza virus has been shown to be effective in preventing infection in an *in vivo* animal model. However, multiple doses, pre- and post-exposure were required for efficacy. We hypothesize that the proposed techniques will enhance the efficacy of the therapeutic peptide itself, thereby reducing the required dose, number of doses, and thus the cost of treatment, and improve

distribution and release within the upper respiratory tract, thus expanding the duration of bioavailability and efficiency of peptide delivery.

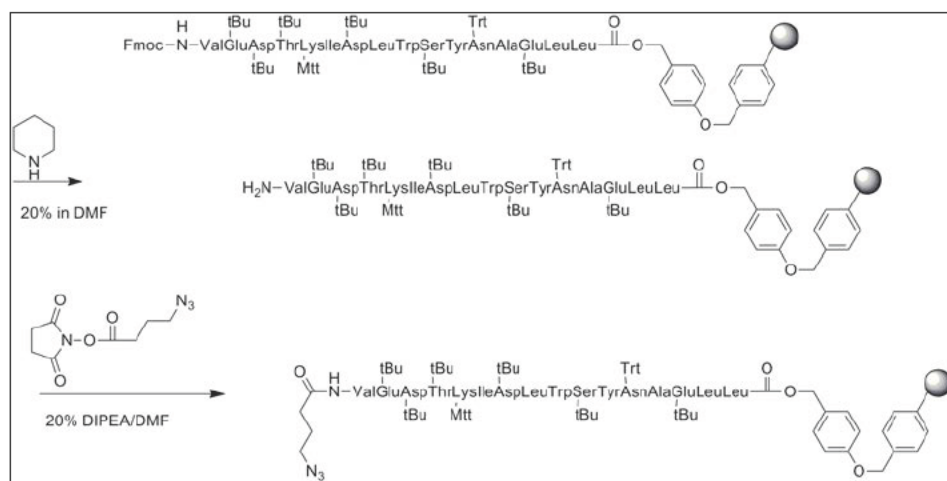
In the first funding cycle, we explored three different approaches: 1) N-terminal modification of Flufirvitide-3 *via* conjugation of an NHS-ester modified 4-azido butanoic acid, 2) Polymeric modification of Flufirvitide-3 - PCL to investigate increasing hydrophobicity, PEG to investigate increasing hydrophilicity, and a variety of multiarm cores to investigate synergistic effect of multiple peptides that are covalently tethered together, and 3) Analysis of different excipients for stable lyophilization and dry powder pulmonary delivery.

N-terminal modification of Flufirvitide-3 *via* conjugation of an NHS-ester modified 4-azido butanoic acid

Our first goal was the N-terminal modification of Flufirvitide-3 peptide *via* conjugation of an NHS-ester modified 4-azido butanoic acid. The peptide was purchased from Anaspec with side chain protecting groups intact, on a solid support.

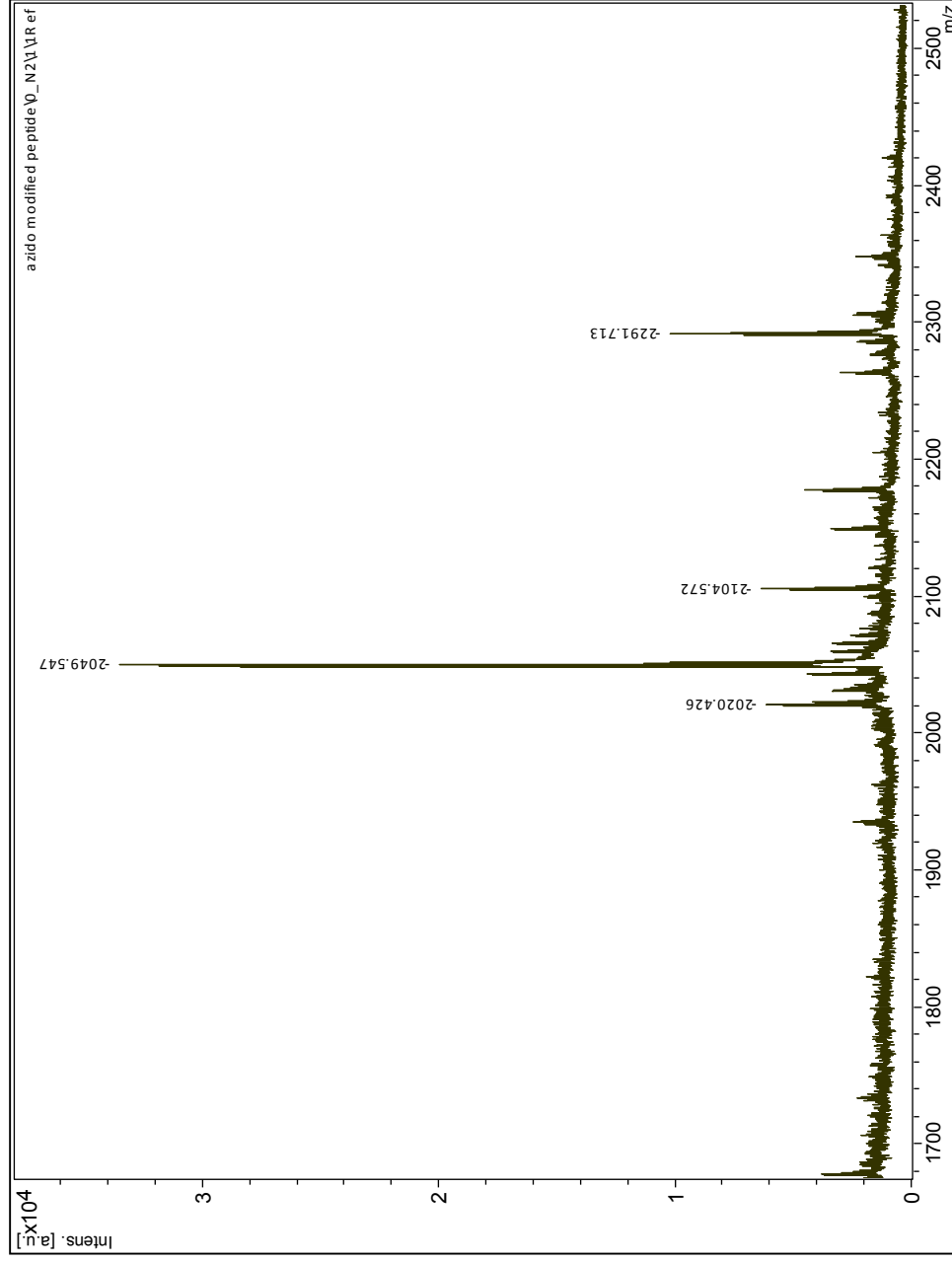
N-Terminal Modification:

The overall modification process is shown below. First, 50 mg of beads were washed twice with 1 mL of DMF, followed by a 30 minute incubation in a solution of 20% piperidine in DMF. The reaction mixture was filtered and washed twice with 1 mL of DMF, followed by the addition of a solution of the NHS-ester modified 4-azido butanoate with 20% DIPEA in DMF. The reaction was allowed to progress overnight with continuous shaking, after which it was filtered and washed twice with 1 mL of DMF.



Analysis:

To determine the success of the reaction, 5 mg of beads were incubated in 50 μ L of a 95% aqueous TFA solution for 20 minutes to effect both global deprotection and cleavage from the resin. The resulting product was washed twice with the 95% TFA solution, followed by removal of the solvent *in vacuo*. The resulting resin was dissolved in 20 μ L of DI water, and analyzed by MALDI-TOF mass spec using α -cyano-4-hydroxy cinnamic acid as a matrix. The resulting spectrum (Figure 1, below) shows no starting material (Calc. mass = 1907.95) and shows a product peak ($M+H^+$) at 2020.426 (Calc. mass = 2018.99) and a possible $M+Na^+$ peak at 2049.547.

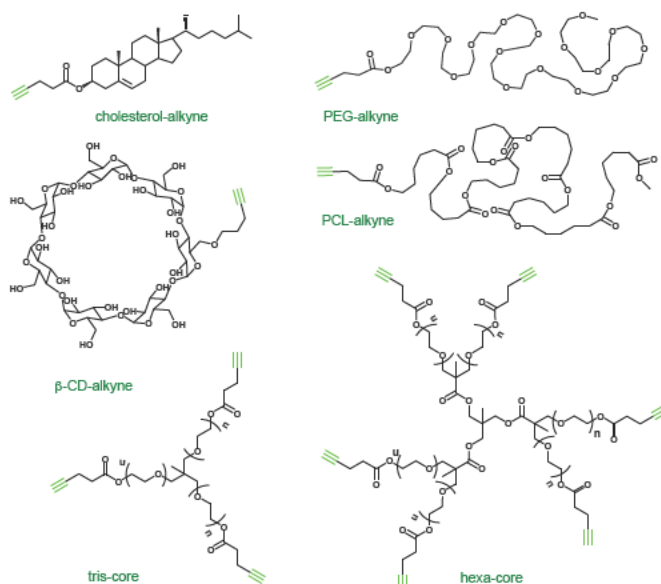


MALDI-TOF MS analysis of crude N-terminal modified Flufirvitide-3 peptide.

Polymeric modification of flufurvitide-3

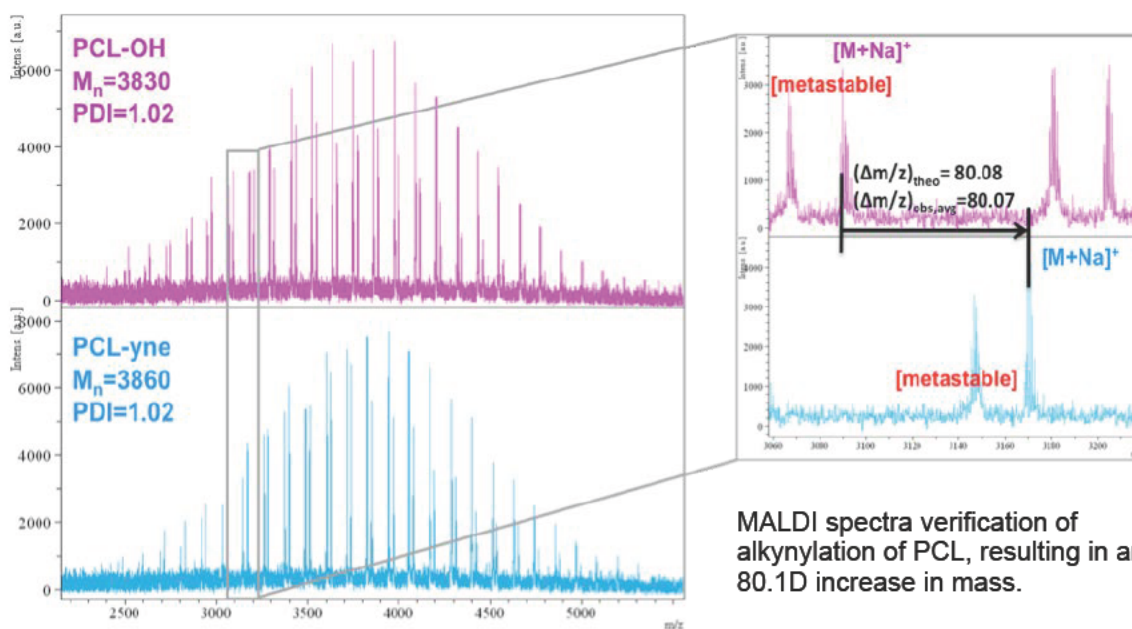
The conjugation chemistry of multiple polymers to flufurvitide-3 was proposed, and three initial targets were investigated:

Targets for conjugation of the peptide includes at least 3 macromolecular targets: PCL to investigate increasing hydrophobicity, PEG to investigate increasing hydrophilicity, and a variety of multiarm cores to investigate synergistic effect of multiple peptides that are covalently tethered together.



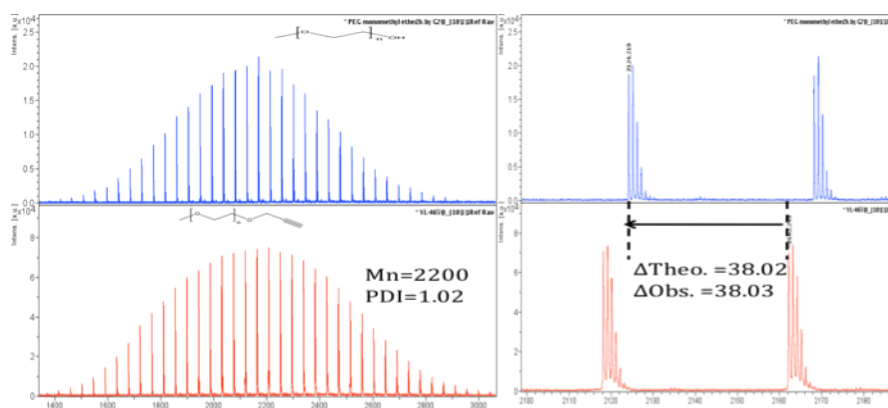
Generation of PCL-alkyne: The generation poly(caprolactone) (PCL) with a single alkyne group can follow one of two routes, the first involves the synthesis of a PCL chain, followed by an esterification with pentynoic anhydride and the second involves polymerization from an alkyne functionalized initiator. Because of the potential instabilities of the triple bond during polymerization conditions and previous experience with this former route, the post-polymerization functionalization was examined.

MALDI-TOF MS is a particularly powerful tool for verifying transformations on polymers because the enhanced signal and high-mass resolution leads to rapid qualitative verification of macromolecular structure, as well as structural determination of impurities. Initial results verified the rapid synthesis of PCL-alkyne, and the ability to tailor the polymer to a range of molecular weights.



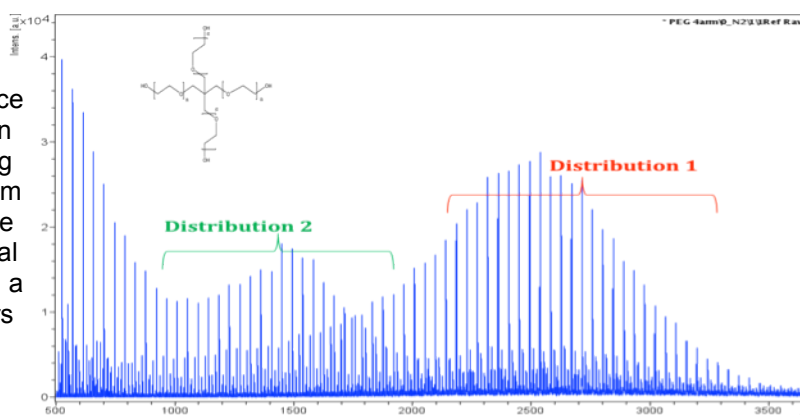
MALDI spectra verification of alkylation of PCL, resulting in an 80.1D increase in mass.

Generation of PEG-alkyne: Likewise poly(ethylene glycol) (PEG) with a single alkyne group was set as an initial goal. Because the backbone consists of ether linkages (as opposed to the ester linkages of PCL) the polymer exhibits vastly improved stability under both acidic and basic conditions. In order to provide an equally hearty linker, an alkyne was attached via a Williamson Ether Synthesis reaction. By reaction of allyl bromide with PEG that has only one free hydroxyl group on the terminal end, the alkynylated product can be generated and easily isolated by precipitation. The purity of the product was verified by a shift of 38.0D in the MALDI-TOF mass spectrum, as well as the lack of any signal for unreacted starting material or byproducts.



MALDI spectra of PEG mono-methyl ether starting material and the alkynylated product verify near-quantitative transformation to the PEG-alkyne, but with a hearty ether linkage as opposed to an ester linkage.

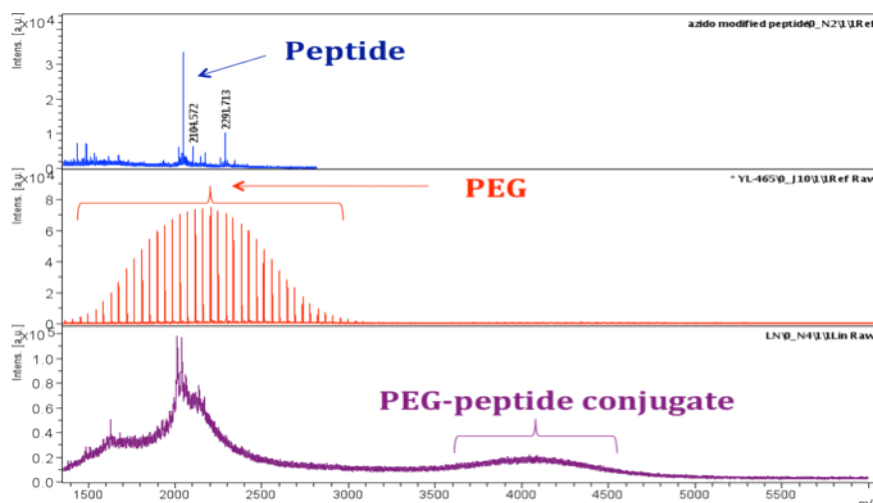
Examination of multiarm PEG: Although multi-arm PEGs are commercially available, in order to provide meaningful results, high purity multi-arm PEG must be used. Therefore MALDI-TOF MS was used to verify the purity of a number of commercial samples. An initial examination verified the presence of substantial impurities in commercial samples, exhibiting two distinct distributions from different core structures. The identity of the impurities, potential methods of purification, and a source of more pure PEG stars were investigated.



Multiple distributions observed in MALDI-TOF MS of commercial PEG-star polymers

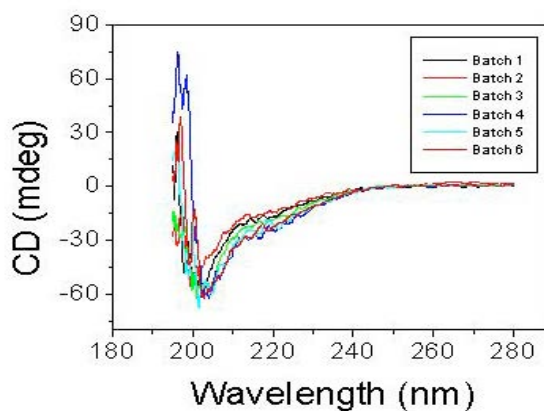
Synthesis of PEG conjugated flufirvitide

We also successfully synthesized resin-bound Flufirvitide-3 peptide that contains an azide functional group on the N-terminus. The first critical step in order to prepare peptide-polymer conjugates is to verify the conjugation chemistry between the two. In particular, click chemistry (using copper (I) catalyst) was used to tether the Flufirvitide-3 peptide bearing the azide ($MW = 2018.99$) and a poly(ethylene glycol) (PEG) chain ($MW = \sim 2000$) with a terminal alkyne. The coupling reaction could be verified by MALDI-TOF MS characterization of the crude product, which exhibits a distinct signal for the new polymer-peptide conjugate ($MW = \sim 4100$) as well as residual starting material. Studies were then conducted to optimize the coupling and purification techniques so that initial bioassays can be investigated in regards to activity of the polymer-peptide conjugate.



Analysis of excipients for nasal delivery

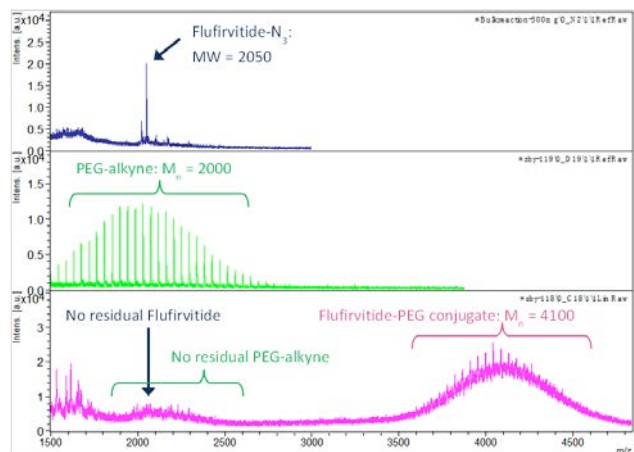
Specific Aim 2 is to formulate the following micro- and nano-carriers for encapsulation of active peptide and suitable for pulmonary delivery: dry powder formulation, microemulsions, nonspherical liposomes, ceramic shell vesicles, and nanometer-sized silk particles. The initial formulation of Flufirvitide-3 is an aqueous solution in a phosphate buffer saline. Our first – formulation approach was a dry powder. We prepared ten lyophilized powder formulations containing Flufirvitide-3 with or without different dry powder excipients. The excipients were β -cyclodextrin, 2-hydroxypropyl β -cyclodextrin, trehalose, tween 20, span 20. The lyophilized powder formulations were analyzed for solubility and any structural changes. The results of the analysis showed that the lyophilized process did not cause any structural or solubility change. However, the formulations containing tween 20 and span 20 showed some flocculation following the reconstitution with deionized water. Addition of trehalose or cyclodextrins significantly increased the aqueous solubility of Flufirvitide-3 from 1 mg/ml to 5 mg/ml. The lyophilized powder formulations were very smooth, spherical, and free-flowing. These lyophilized formulations were later reconstituted with deionized water to use as a nasal spray.



In the second funding cycle, we 1) optimized the conjugation of Flufirvitide-3 to PEG, 2) built a diverse library of azide functionalized polymers, 3) developed novel formulations for parenteral and mucosal delivery of Flufirvitide-3 and other peptides, and 4) prepared additional Flufirvitide-3 lyophilized powder formulations for pulmonary delivery.

Optimization of Flufirvitide-PEG conjugation

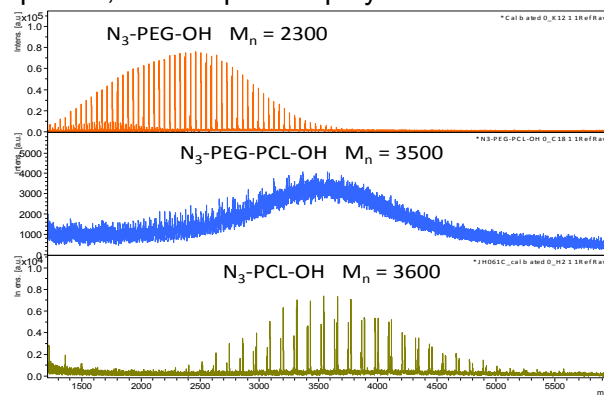
The conjugation of peptides to water soluble polymers, such as poly(ethylene glycol) (PEG) has been demonstrated to enhance water solubility, improve stability, and increase blood-circulation lifetimes relative to the parent peptide. The copper catalyzed azide-alkyne cycloaddition coupling reaction has been demonstrated to yield clean products and rapid reactions, ideal for hindered conjugation reactions such as peptide-PEG conjugates. In the previous funding period, MALDI-TOF MS was used to confirm that the azide modified flufirvitide peptide could be coupled to poly(ethyleneglycol) bearing a single alkyne end group. In order to provide sufficient quantities to evaluate the activity of the modified peptide, the resin-bound peptide was coupled on a larger scale, and the product purified by repeated washing of the resin bound peptide-PEG conjugate to remove copper and uncoupled PEG. After removal from the resin, MALDI-TOF MS analysis of the product exhibited high purity, exhibiting negligible amounts of residual, unmodified peptide, or unreacted PEG chains.



Building a diverse library of azide functionalized polymers

In addition, alkyne modified PEGs with a range of molecular weights were synthesized and with a degradable ester linkage adjacent to the alkyne, as well as a much harder ether linkage. Representatives include hydrophilic biocompatible polymers: azido and ethynyl PEG monomethyl ether and azido PEG alcohol; hydrophobic, biocompatible polymers: azido and ethynyl poly(ϵ -caprolactone) (PCL) alcohol as well as an amphiphilic block copolymer: azido PEG-PCL alcohol block copolymer.

PEG di-azide and dialkynes can also be prepared using the same end group functionalization chemistry that was successfully demonstrated with the mono-azido PEG. Three and four arm PEGs were also purchased, but analysis verified the presence of a substantial amount of impurities, and so an alternative method for synthesizing these multi-arm PEGs is presently being investigated in our labs.

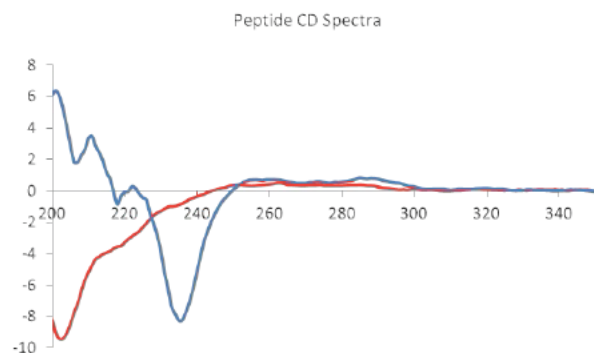


Analysis of PEG-conjugated Flufirvitide, Fluorophore Conjugation and Supramolecular Platforms for Assembly

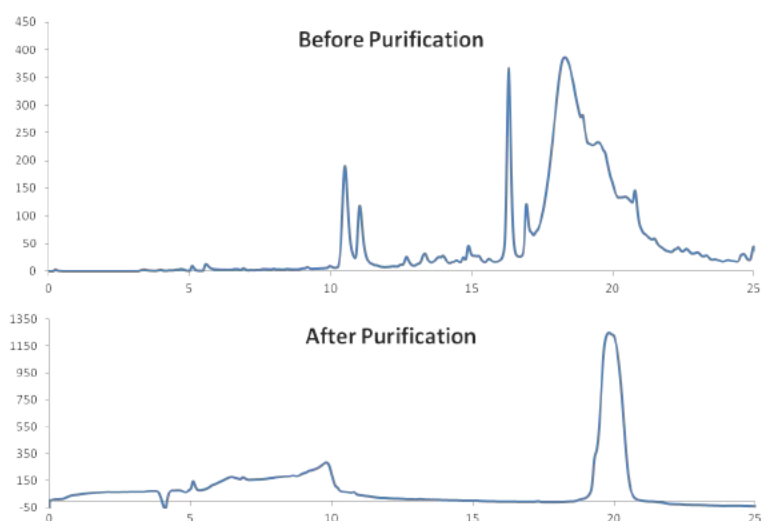
While the conjugation of peptides to solubilizing polymers such as poly(ethylene glycol) has been shown to improve stability and increase blood-circulation times, there is the potential for such a large modification to effect the native conformation of a short peptide. As described previously, a synthetically protected Flufirvitide peptide containing a terminal azide modification (prepared by a bifunctional linker which we have synthesized) was used to further modify the peptide with a singly modified PEG chain bearing a terminal alkyne functionality through a copper-catalyzed azide-alkyne cycloaddition. HPLC-based purification using a C18-silica based column resulted in a pure product, as

evidenced by MALDI-TOF MS analysis.

Following the synthesis of a purified Flufirvitide-PEG conjugate, the CD spectra of both the PEG-modified and unmodified peptide were collected to determine whether the conjugation resulted in a change in the native conformation of the peptide. As can be seen below, the unmodified peptide shows a primarily disordered (random coil) structure as evidenced by the strong negative peak at 200 nm, but also shows a trace of a β -sheet structure, as evidenced by the negative peak (shoulder) just before 220 nm. By contrast, the post-modification CD shows two positive peaks at approximately 200 and 210 nm, as well as negative peaks at approximately 219 and 235 nm.



CD Spectra of native (unmodified) Flufirvitide (Red) and PEG-conjugated Flufirvitide (Blue).



HPLC Purification, showing (Top) the trace before purification, and (Bottom) the trace resulting from the collection of the peak(s) at 19-21 minutes post-purification.

While more analysis of the exact structure is necessary, it can definitely be shown that PEGylation induces a shift from a random coil structure to a more structured form showing a mix of β -sheet and α -helix qualities.

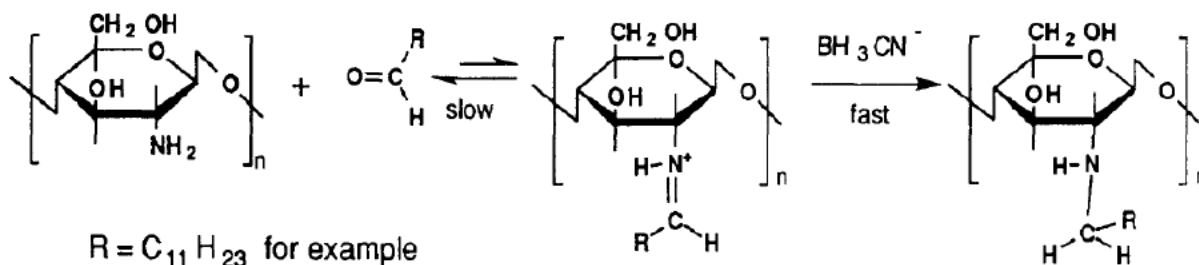
Additionally, there would be a definite benefit to the creation of fluorophore-Flufirvitide conjugates, allowing the distribution of the peptide to be followed, as well as allowing for the facile determination of encapsulation of the peptide for delivery system development. Initial methods involving the direct conjugation of Fluorescein Isothiocyanate (FITC) to the amine terminus of the synthetic peptide through DIPEA mediated thiourea formation

proved ineffective, however, under a variety of reaction conditions.

Novel Formulations for Parenteral and Mucosal Delivery Platforms

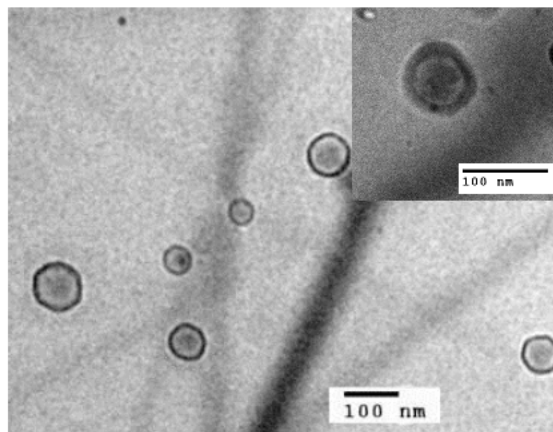
For this aspect of the project, we worked with modified liposomes for both oral and aerosol delivery of Flufirvitide. Our objective in this work is two fold (a) develop liposomal formulations that are robust with increased circulation times in the body (b) develop liposomal formulations that have an improved ability for cell uptake and can be functionalized to be cell specific. Within the framework of these objectives, we developed (a) a class of coated liposomes that have the potential to survive for longer periods without immediate recognition by the immune system (b) a class of tubular liposomes that have the potential to be easily aerosolized and travel deep into lung alveoli.

The coated liposomes are made by attaching a polysaccharide biopolymer, hydrophobically modified chitosan (HMC), to the liposome. The schematic below shows the reductive amination of chitosan to HMC.

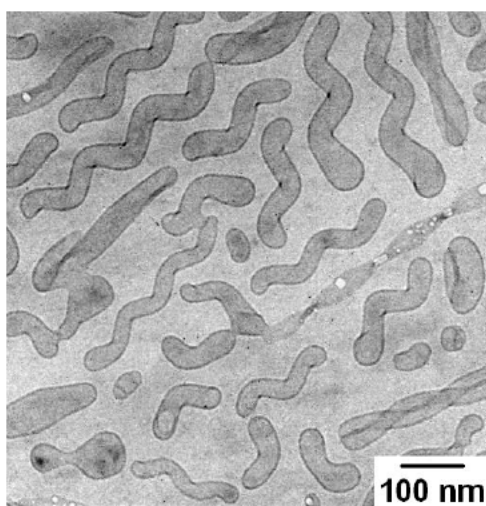


Synthesis of hydrophobically modified chitosan

When contacted with liposomes, the biopolymer inserts the alkyl groups into the liposome bilayer and forms a coating on the liposome, as shown in the cryo transmission electron micrographs in the figure to the right (the inset shows details of a thickened liposome). Chitosan and its variants also have strong mucoadhesive properties and can be integrated into mucosal delivery systems

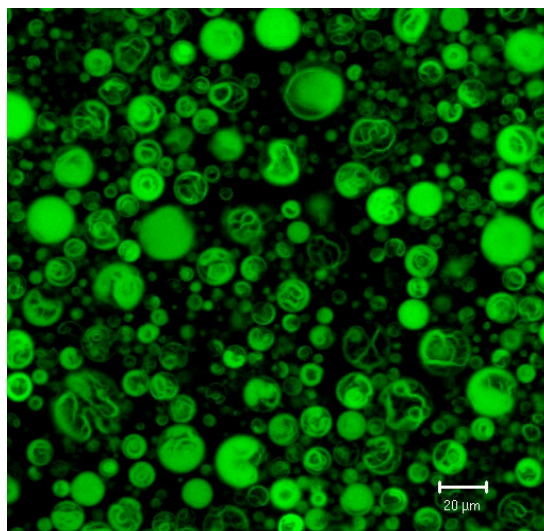


The second system is one where the introduction of small amounts of sphingolipids (e.g., ceramide VI) transforms spherical liposomes into long and undulating tubular liposomes as shown below. Because of their very high aspect ratio and narrow dimensions (<40 nm), these liposomes may have an enhanced potential for cell entry. Again, this system can be coated with hydrophobically modified chitosan to provide additional robustness. Aerosolization of these peptide containing liposomes may lead to an aerodynamic delivery system capable of deep penetration into the lung.



Tubular liposomes obtained by incorporating sphingolipids into phospholipid bilayers.

Finally, in collaboration with Dr. David Kaplan at Tufts University, we developed a novel system of silk fibroin microspheres with liposomal coatings. Such silk microspheres are entirely biocompatible but degrade slowly allowing sustained drug release over extended periods. Additionally they are strongly mucoadhesive. The following Figure illustrates a fluorescence micrograph of FITC tagged phospholipids coating silk microspheres. These materials also have excellent lubrication properties and are being developed for biolubrication.



Fluorescence Micrograph of lipid coated silk microspheres.

Lyophilized Powder Formulations for Aerosol delivery

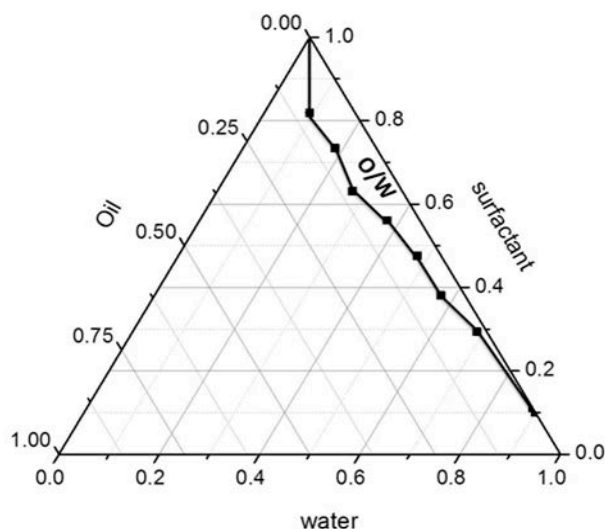
Finally, we prepared ten more Flufirvitide lyophilized powder formulations using four different sugars (trehalose, sucrose, mannitol, and lactose) in pH 7.4 potassium phosphate buffer. The lyophilized formulations were easy to reconstitute instantly with de-ionized water. We also prepared twenty batches of spray dried Flufirvitide/sugar formulations using mannitol, sucrose, and lactose. A 10% solution of sugar in pH 7.4 potassium phosphate buffer premixed with 4 mg Flufirvitide was sprayed through a Buchi Laboratory Spray dryer B290. The formulations were analyzed for Flufirvitide content, degradation, particle size and morphology. There was no sign of degradation of Flufirvitide due to the processing conditions. The particles were smooth and spherical with average sizes between 3 and 6 μm . However, the formulations with lactose showed some interaction with the peptide at 40°C. This observation indicated that a lactose/Flufirvitide formulation might trigger stability concern during the storage.

In the third funding cycle, we transitioned to Specific Aim 4: Evaluate the effect of encapsulation and chemical modification of Flufirvitide-3 on viral inhibition using an in vitro immunoplaque assay. Below is an example of one of the microemulsions we have developed for this Specific Aim.

Flufirvitide-3 Microemulsion Formulation

These microemulsions are oil-in-water (O/W) type, with thermodynamically stable, transparent and low viscous properties. Since Flufirvitide-3 has hydrophobic and hydrophilic regions, high local administration concentration is expected due to the adsorption of Flufirvitide-3 on the interface of O/W interface, facilitating efficient administration.

These O/W microemulsions are composed of an aqueous phase (PBS buffer, pH=7.4), oil phase (isopropyl myristate, IPM), surfactant (polysorbate 80, Tween 80) and cosurfactant (propylene glycol, PG). During the fabrication of these microemulsions, the surfactant Tween 80 and cosurfactant PG were weighed at the ratio of 4:1 (w/w), and vortexed vigorously to make the surfactant mixture (S_{mix}). Surfactant



Pseudo-ternary phase diagram of PBS/Tween 80/PG/IPM. The ratio of surfactant Tween 80 to cosurfactant PG is 4:1.

mixture was then mixed with the oil phase IPM, and the resulting mixture was finally vortexed with the required amount of PBS buffer. Samples were kept at room temperature to equilibrate overnight. Clear and isotropic samples were determined to be within the oil-in-water microemulsion region.

The pseudo-ternary phase diagram was constructed for PBS buffer, IPM oil and surfactant mixture to determine the microemulsion region. In the figure above, the region to the right of the boundary line is transparent O/W microemulsions with lower viscosity. The final formulation identified to use in our experiments contained Flufirvitide-3 in PBS buffer as aqueous phase, surfactant mixture and IMP oil phase at the ratio of 6:4:0.3 respectively, with the final concentration of Flufirvitide-3 at 10 mM.

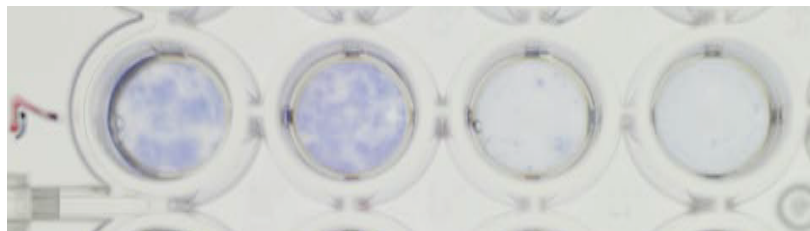
The figure to the right illustrates that the microemulsions are still transparent and stable after adding Flufirvitide 3 in the aqueous phase. Cryo-TEM image (B) confirms that oil droplets in microemulsions have quite uniform size with the diameter ~100 nm.

We have begun evaluation of the effect of encapsulation of Flufirvitide-3 within phospholipid liposomal vesicles and are currently in the process of having the modified Flufirvitide-3 synthesized in sufficient quantity/purity for evaluation.

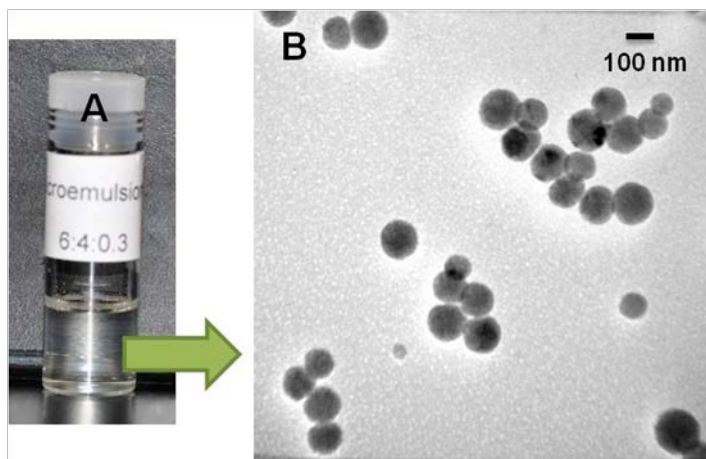
In order to confirm that the Flufirvitide-3 peptide and derivatives maintain activity through the various formulation processes and chemical modification techniques, we established an *in vitro* immunoplaque assay. Influenza

A/H1N1 was used since we have previously demonstrated the effectiveness of Flufirvitide-3 against this strain. In a typical assay, virus is pretreated with Flufirvitide-3 for one hour and subsequently used to infect a cell monolayer. One-hour after infection of the monolayer, the virus inoculum is removed and a low-viscosity Avicel overlay media with or without active peptide is added to cover the monolayer. After incubation, the cells are fixed and stained to detect influenza virus nucleoprotein and indicate viral plaque formation.

Shown below is a representative result. Untreated virus was added to the first two wells for infection of MDCK cells. In the last two wells, virus was incubated with 10 μ M Flufirvitide-3 for 1 hour prior to infection of the cell monolayer. As seen below, we observed a reduction in the number and the size of viral plaques when virus was incubated with Flufirvitide-3 prior to infection of cell monolayers. This resulted in an 86.25% reduction in viral PFUs.



This assay allowed us to compare the activity of Flufirvitide-3 in its native form (above) with the various formulations obtained in years 1 and 2 to identify those exhibiting enhanced antiviral activity. Pretreatment of the cell monolayer with our different micro- and nano-carrier

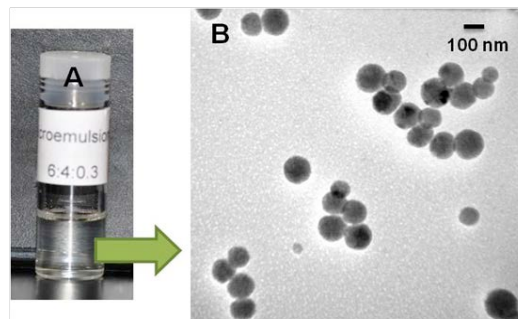


A: visual observation of O/W microemulsions with the compositions of Flufirvitide 3 aqueous solution, surfactant mixture and IMP oil phase at the ratio of 6:4:0.3; B: cryo-TEM image of O/W microemulsions.

formulations for various time points in addition to extended incubation post-infection could also be included. This would allow us to assess whether the extended release of peptide from these specifically-tailored vehicles will provide sustained release of active protein to increase protection of the monolayer pre- and post-infection.

At this point, the award went into no-cost extension and we had to prioritize our remaining studies based on the funds remaining. We first assessed different carrier formulations encapsulating Flufirvitide-3 peptide for their effect on viral inhibition in the immunoplaque assay. These include water-in-oil microemulsions, DPPC/DMPG/cholesterol liposomes, and hydrophobically modified chitosan (HMC) coated liposomes.

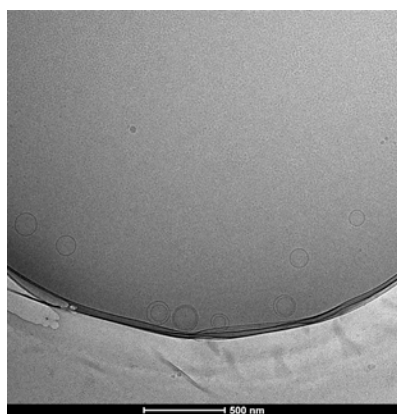
The oil-in-water (O/W) microemulsions are composed of isopropyl myristate dispersed in an aqueous solution containing the flufirvitide peptide using Tween 80 and propylene glycol co-surfactants. This emulsion preparation is stable at room temperature or at 4°C for over 6 months. Images of the O/W microemulsion are seen at right.



A: visual observation of O/W microemulsions with the compositions of Flufirvitide 3 aqueous solution, surfactant mixture and IMP oil phase at the ratio of 6:4:0.3; B: cryo-TEM image of O/W microemulsions.

The DPPC/DMPG/cholesterol liposomes are composed of dipalmitoyl-phosphocholine, dimyristoyl-phosphoglycerol and cholesterol. For each liposome preparation, the lipids are hydrated in the presence of an aqueous solution containing flufirvitide-3 peptide to facilitate the peptide's encapsulation within the aqueous core of the vesicles. To prepare the hydrophobically modified chitosan (HMC) coated liposomes, chitosan is added drop by drop to the liposome dispersion with continuous stirring for hours to ensure chitosan has been evenly attached on the liposome surface. A representative electron micrograph of the hydrophobically modified chitosan (HMC) coated liposomes is shown below.

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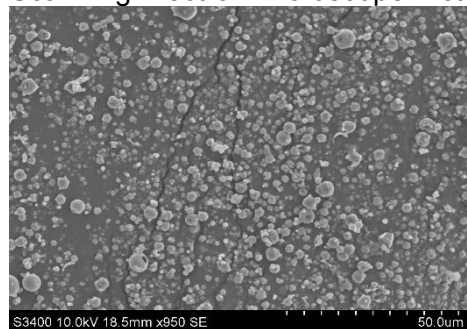


We also continued our work on spray dried formulations for aerosol delivery

For these studies, four control spray dried sugar (sucrose and mannitol) formulations were prepared. None of these formulations contained FF-3. The spray dried mannitol and sucrose particles were evaluated for particle size and morphology. The particles were smooth and spherical with an average size between 6 and 10 µm. Ten batches of spray dried FF3 formulations were then prepared and analyzed for FF-3 content using UPLC/MS. All our testing confirmed no degradation of FF-3 during the processing.

Unlike previous observations with lyophilized powders, the spray dried powders containing FF-3 did not go into solution instantly when the excipients were either sucrose or mannitol. However, sucrose was slightly better than mannitol. The mannitol containing FF-3 formulation did not go into solution instantly when added to deionized water. This will not be an issue in the future if the formulation is delivered as a dry powder; however, if powder is reconstituted before delivery, other sugars would be preferable.

Scanning Electron Microscope Picture of FF3 formulation



Preparation of FF3 Spray Dried Powder with Instant Solubility

The initial formulation of Flufirvitide-3 was an aqueous solution in a phosphate buffer saline. Our first formulation approach was a lyophilized powder. We prepared ten lyophilized powder formulations containing Flufirvitide-3 with or without excipients. The excipients were β -cyclodextrin, 2-hydroxypropyl β -cyclodextrin, trehalose, tween 20, span 20. The lyophilized powder formulations were analyzed for solubility and any structural changes. The lyophilized powder formulations were very smooth. These lyophilized formulations were later reconstituted with deionized water to use as a nasal spray.

During the second stage of the project (above), twenty-five different formulations were prepared using a laboratory spray dryer. The purpose of the spray dried powder was to formulate FF-3 for pulmonary delivery. FF-3 was either spray dried alone or dissolved with three different sugars: lactose, mannitol, and sucrose. The average size of these spray dried particles was between 2 and 4 μm . Thermal analysis of these formulations showed average T_g around 43°C. These results confirm the stability of the formulations at room temperature. The average particle size confirms the suitability of the formulations via inhalation route. Since the size of the particles are less than 5 μm size, it is most likely that the particles will be distributed evenly to the deep lung region.

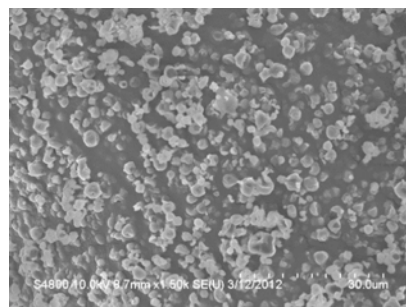
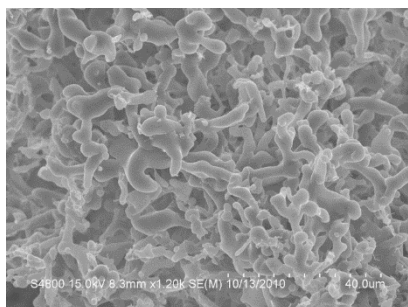
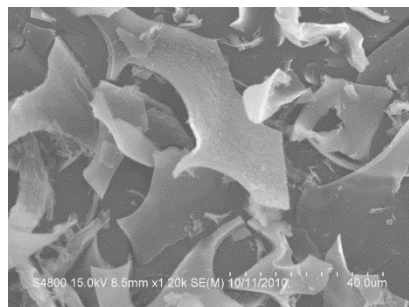


Figure 1. SEM picture of FF-3.

Figure 3. SEM picture of spray dried FF-3 and sucrose particles

Figure 2. SEM picture of lyophilized FF-3 and sucrose particles

Microcarriers for Flufirvitide-3 Encapsulation

For this final phase of the project, we assessed various microemulsion formulations to optimize stability. Biocompatible oils used for comparison included soybean oil, sesame oil, squalene, and isopropyl myristate. Surfactants considered included Tween 20, Tween 80 and propylene glycol. The water:oil:surfactant ratio was varied for each combination. The formulation that exhibited optimal stability with a clear appearance, an indication of microemulsion formation, was Tween 80 with propylene glycol as a co-surfactant at a 4:1 volume ratio, isopropyl myristate as the oil phase, and water. Based on a ternary phase diagram for this combination, the volumetric ratio for water:surfactant:oil for optimal stability was 6:4:0.2. This emulsion preparation is stable at room temperature or at 4°C for over 6 months with either phosphate buffer or Flufirvitide-3 dissolved in phosphate buffer as the aqueous phase.

The DPPC/DMPG/cholesterol liposomes are composed of dipalmitoyl-phosphocholine, dimyristoyl-phosphoglycerol and cholesterol. For each liposome preparation, the lipids are hydrated in the presence of an aqueous solution containing Flufirvitide-3 peptide to facilitate the peptide's encapsulation within the aqueous core of the vesicles. To prepare the hydrophobically modified chitosan (HMC) coated liposomes, chitosan is added drop by drop to the liposome dispersion with continuous stirring for 2 hours to ensure chitosan has been evenly attached on the liposome surface.

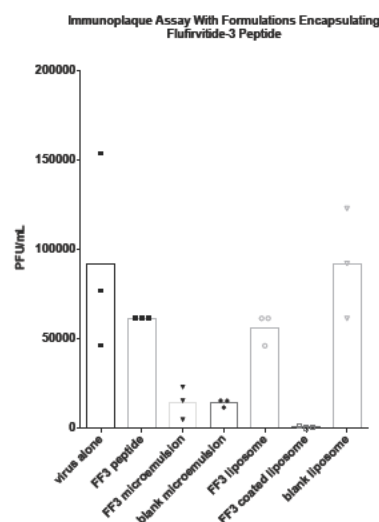
Virus Inhibition with Microcarrier Formulations

Using an immunoplaque assay with Influenza A/PR/8 and MDCK cells, we were able to compare viral inhibition in the presence of Flufirvitide-3 peptide encapsulated in the liposome and microemulsion formulations described above. Virus was pre-treated with peptide alone or peptide encapsulated in a microemulsion, liposomes, or HMC coated liposomes. MDCK cells were then infected with the virus +/- peptide for one hour after which the inoculum was removed and an Avicel overlay was added to the cell monolayer. After a 40-hour incubation, cells were stained for the presence of influenza virus and plaque numbers quantified. All treatments were performed in triplicate.

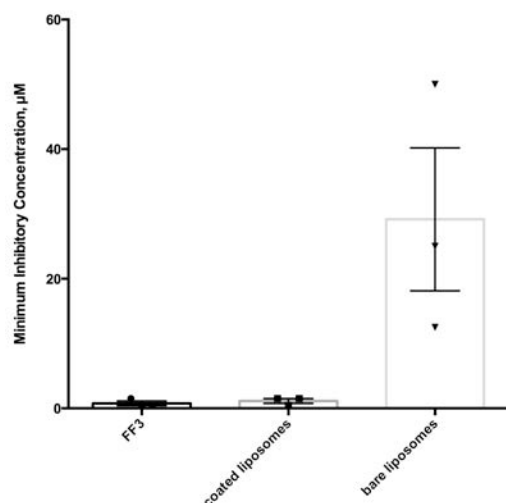
In initial studies, we compared the viral plaque-forming unit concentration following incubation with peptide formulations at a constant peptide concentration of 10 μ M. Results are shown in the figure at right, indicating that oil-in-water microemulsions and hydrophobically modified chitosan (HMC) coated liposome preparations encapsulating Flufirvitide-3 peptide at a concentration of 10 μ M resulted in reduced numbers of viral plaques as compared to either peptide alone or virus alone. However, viral inhibition with the DPPC/DMPG/cholesterol non-coated liposomes was similar to the results seen with peptide alone. Inhibition was also noted with the blank microemulsion.

In subsequent studies we compared the lowest peptide concentration in the various formulations that completely inhibited viral plaque formation at a constant virus concentration of 100 pfu/ml. In these experiments, we saw similar inhibition with microemulsions with and without FF3 peptide in all microemulsion dilutions. This is likely due to interaction between surfactant in the emulsion preparations and the viral particles; at surfactant concentrations as low as 0.14 percent (vol/vol) we noted viral inhibition in our *in vitro* assay. The microemulsions, however, did not appear to have an effect on the integrity of the epithelial cell monolayer following 40 hours of incubation.

With the liposome preparations, results indicated that the peptide alone showed viral inhibition at concentrations as low as 1.56 μ M, as did FF3 encapsulated within coated liposomes, as



Minimum Inhibitory Concentration of FF3 Peptide Encapsulated in Liposome Formulations



shown at left. FF3 peptide encapsulated within non-coated liposomes required a higher concentration of FF3 to induce complete viral inhibition.

The lipid content of the coated vesicle preparations at the inhibitory peptide concentration was 0.0672 percent (wt/vol). Viral plaque formation with blank coated vesicles was noted only at higher lipid concentrations of 0.26 percent or more, indicating some non-specific interaction of the lipid vesicles with the virus and/or the epithelial cells. There was no detection of disruption to the cell monolayer with any of the vesicle preparations.

These results support our hypothesis that hydrophobically modified chitosan (HMC) coated liposome preparations encapsulating Flufirvitide-3 peptide will promote the efficient delivery of FF3 peptide for viral inhibition. We expect that further *in vivo* experiments with these formulations will demonstrate that the particulate nature of

these carriers will improve delivery within the respiratory tract and that the interaction of HMC with the mucosal epithelium will enhance the efficacy of FF3 peptide. *In vivo* studies would allow further analysis of the benefits of microemulsion droplet structure for pulmonary delivery. We expect that the increased concentration of peptide at the oil/water interface on the emulsion droplet surface will also improve the effectiveness of Flufirvitide-3 peptide as a therapeutic against influenza virus.

KEY RESEARCH ACCOMPLISHMENTS

Year 1

- N-terminal modification of Flufirvitide-3 via conjugation of an NHS-ester modified 4-azido butanoic acid
- Polymeric modification of Flufirvitide-3 - PCL to investigate increasing hydrophobicity, PEG to investigate increasing hydrophilicity, and a variety of multiarm cores to investigate synergistic effect of multiple peptides that are covalently tethered together
- Analysis of different excipients for stable lyophilization and nasal delivery
- Encapsulation of Flufirvitide-3 in microemulsions
- Established an in vitro immunoplaque using Influenza A/H1N1 and demonstrated the efficacy of Flufirvitide-3 in this model.

Year 2

- Optimized the conjugation of Flufirvitide-3 to PEG
- Built a diverse library of azide functionalized polymers
- Developed novel formulations for parenteral and mucosal delivery of Flufirvitide-3 and other peptides
- Prepared additional Flufirvitide-3 lyophilized powder formulations for pulmonary delivery.

Year 3

- Encapsulation of Flufirvitide-3 in microemulsions
- Established an in vitro immunoplaque using Influenza A/H1N1 and demonstrated the efficacy of Flufirvitide-3 in this model.

Year 4

- Preparation of FF3 Spray Dried Powder with Instant Solubility
- Demonstrated virus inhibition with microcarrier formulations

REPORTABLE OUTCOMES

Dhule, S.; Penformis, P.; He, J.; Harris, M.; Terry, T.; Pochampally, R.; John, V. "The Combined Effects of Encapsulating Curcumin and C6 Ceramide in Liposomal Nanoparticles against Osteosarcoma", *Molecular Pharmaceutics*, 2014, 11, 417.

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CONSLUSIONS

Flufirvitide-3 and other peptide inhibitors of viral fusion have a number of potential applications:

- be used for pre- or post-exposure prophylaxis in civilians of all ages and in military personnel, including first responders, for seasonal or pandemic influenza viruses or to protect from deliberate release of a new strain of influenza virus.
- be used for treatment of persons of all ages infected with seasonal or pandemic influenza viruses.
- be stock-piled in the event of an emerging pandemic strain of influenza A virus in a stable formulation, with use as pre- or post-exposure treatment and prophylaxis.
- be used to protect personnel working in biodefense laboratories from accidental exposures to highly pathogenic influenza viruses.
- be used to protect persons traveling to locales where influenza is endemic from exposure to novel strains of influenza virus.
- be used to protect hospital personnel from exposure to influenza virus from infected patients.
- serve as a deterrent from using this easily acquired pathogen as a bioweapon.
- pilot a platform technology, which may be useful against other viruses that are potential bioweapons.

Nasal administration of soluble Flufirvitide-3 both pre- and post-exposure to influenza virus has been shown to be effective in preventing infection in an *in vivo* animal model. However, multiple doses, pre- and post-exposure were required for efficacy. We hypothesize that the proposed techniques will enhance the efficacy of the therapeutic peptide itself, thereby reducing the required dose, number of doses, and thus the cost of treatment, and improve distribution and release within the upper respiratory tract, thus expanding the duration of bioavailability and efficiency of peptide delivery.