Creating and Orientating HIV-1 Fusion Peptide Samples

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The goal of this project is to determine whether or not and how the presence of the HIV-1 fusion peptide will disrupt the formation of lipid membranes. This paper will discuss the processes involved in orienting the basic lipid samples and then the more complicated problem of making the fusion peptide orient as well.

Introduction

The work cited in this paper actually began in late 1999 and the work conducted this summer was merely a continuation of the work begun in 1999 by this author. The primary goal of the author, which would continue throughout the project, was to purify raw HIV-1 fusion peptide, heretofore FP23, by using an HPLC machine. This machine pumps a diluted liquid sample, which is diluted further by the machine as it pumps two buffers through the system, of what needs to be purified through a column of silica beads. The flow of liquid passes through a laser and a detector system, which can be calibrated for a certain absorption wavelength. The column separates the product, which is pure, from impurities because particles of different sizes pass through the beads at different speeds. The collected sample contains mostly the buffer solution, but that can be evaporated off and the remaining water solution can be freeze-dried until only the pure FP23 is left.

The secondary goal was to achieve a high rate of success in orienting lipid samples on stacked glass plates. Lipids tend to orient into bilayers if they are completely dried and then slowly rehydrated at roughly 40˚ C, even while on flat plates of glass. To this end the lipid 2-Dimyristoyl-sn-Glycero-3-Phosphocoline, DMPC, was the primary lipid used in the early phases. A small amount of DMPC was dissolved in a mixture of organic solvents, chloroform and methanol. The lipid solution would then be spread over several glass plates, and in the early stages plastic, hopefully over the entire plate, until a drop formed over the entire plate. The surface tension of the solvent was used to ensure that the solution did not spill off of the plate. Organic solvents were used initially because of their high volatility. The plates would then be allowed to air-dry overnight before being placed in a desiccator overnight. During the initial phases, roughly 30 glass plates would then be stacked, placed in a round tube, which would then be placed into a small vial with some salt water in the bottom, and placed in an incubator at 42˚ C for up to two weeks. As the technique was improved upon the time of incubation would shorten to a few days, the plastic was abandoned completely, and the singular lipid would be changed to a mixture of several lipids.

The third step, which is the current one, involves getting the FP23 into a homogenous mixture with the lipids. The main purpose of this project is to see if the fusion peptide will orientate at all or whether it will disrupt the formation of the lipid membrane. Both the lipids and the FP23 are soluble in a chloroform/methanol mixture, but have different degrees of solubility. This means that the previous method of applying the solution to the glass plates and letting the solvent evaporate off might not work. The fear was that the peptide would precipitate before the lipids and, thus, not be evenly mixed. A possible solution to this was to freeze dry the lipids and peptide in their organic solvent. A potential problem with this process is that there was no guarantee that the chloroform and methanol would sublime off at temperatures and pressures that are capable in the lab. This is the current state of the project.

The First Step

As stated in the introduction, the first step was to purify raw FP23. The fusion peptide is actually a chain of 23 amino acids: H-Ala-Val-Gly-Ile-Gly-Ala_Leu-Phe-Leu-Gly-Phe-Leu-Gly-Ala-Ala-Gly-Ser-Thr-Met-Gly-Ala-Arg-Ser-NH_2. There are two peptide synthesizers available to the group. One synthesizer is model Applied Biosystems Inc 431A and the other is Applied Biosystems Inc 433A, both utilizing 9-fluorenylmethoxycarbonyl, FMOC, chemistry.[1] In essence FMOC chemistry involves protecting the ends of amino acids with a large molecule, the FMOC molecule, which is cleaved before another amino acid is added. The process continues and in this way chains are formed. Once the material comes out of the synthesizer the peptide is attached to a resin, which initially formed the seed for the chain to start. This resin needs to be cleaved before the material can be purified in the HPLC. The basic HPLC process was described above in the Introduction and further specifics involve only the settings and programming of the machine and will not be discussed.

The Second Phase

Before orienting of the FP23 could be attempted the success of orienting pure lipids needed to be achieved. Ultimately this was achieved with a reasonable rate of success. Roughly 30 samples were made during this phase, each sample taking about 2 weeks from cutting of the glass to sealing the glass tube. The bulk of the time was spent while the samples were incubating and several pairs of samples were incubating simultaneously. The whole process can be broken up into four steps, first involving the setup of the glass plates and application and dehydration of the lipids, the second is the incubation of the stacked glass plates, third being the capping of the glass tubes in which the plates were stacked, and fourth obtaining the NMR results to see whether or not the lipid had oriented in anyway.

The initial step of the process called for the cutting of microscope cover slides into 64 near uniform sized plates of glass with a diamond tipped scriber. Next 32 mg of DMPC would be weighed out and a mixture of 5ml of a 4:1 chloroform:methanol solution would be made. The lipid would be dissolved in the solvent and mixed

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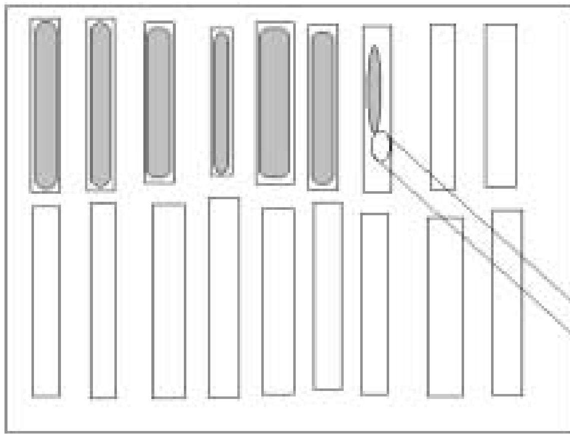
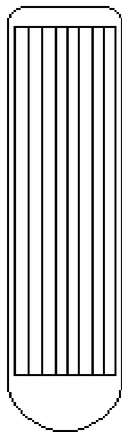
[1] The information on FMOC chemistry was taken from chapter 3 of the ABI 433A peptide synthesizer's user manual.

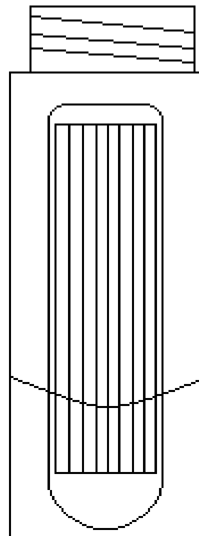
thoroughly. Using a pasture pipet the solution would be applied to the slides in two coatings, each coat filling the glass slide until a bubble of solution formed on top of the plate. The glass plates would then air-dry overnight and then be placed in a desiccator overnight. Below is a diagram that shows how a pipet would be used to apply the lipid solution.



Once the glass plates had been in the desiccator overnight, they were ready to be stacked and placed in tubes to be incubated. Care was taken to handle the plates by they sides and with gloves so sweat and oils would not ruin the purity of the lipids. About 30 plates could be stacked one on top on the other and then were placed into small circular tubes. The tubes were then placed in small glass vials, which were partially filled with salt water. Salt was added to prevent the growth of mold in the vial. The vial would be capped and placed in the incubator for up to two weeks, or until the stack became clear. A problem developed with water condensing on the top of the glass vial and dropping down into the glass tube and ruining the glass slides inside. To prevent this, a piece of cotton was placed in the top of the glass tube, which allowed the humid air through, but liquid would be stopped from entering. The cotton proved to be a successful innovation.



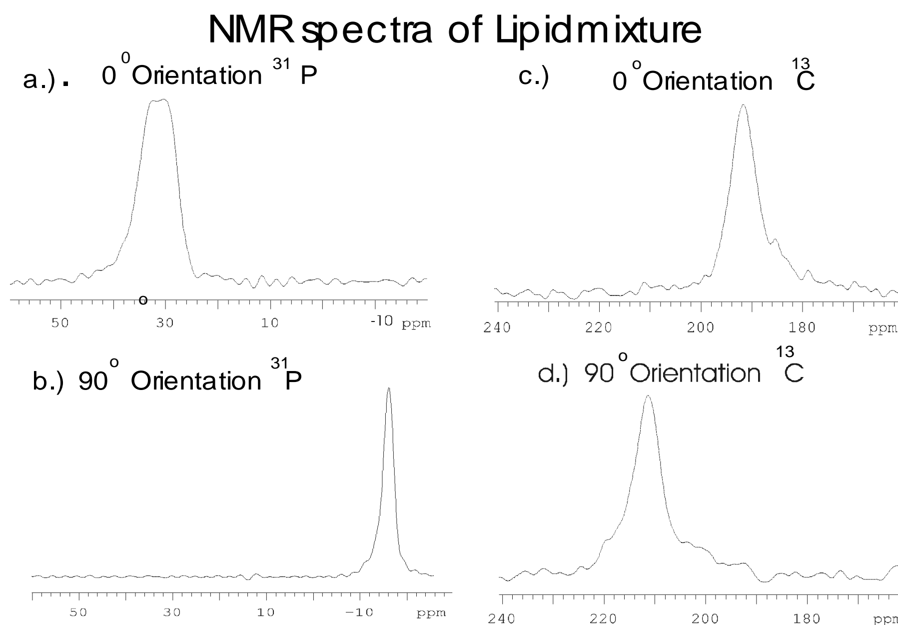
Stacked plates in glass tube.



Glass tube inside glass vial filled with some amount of salt water

The third step was to cap the tubes after they had been successfully hydrated. This was done using an epoxy and plastic, Kel-F, caps. A small bead of epoxy was placed around the edges of the cap and the cap partially stuck into the tube, but had a larger outer lip, which prevented the entire thing from falling into the tube.

The NMR readings of the samples, which were primarily done by a graduate student, were the fourth and final step of this process. The primary objective of this step was to make sure that the lipids had oriented. This was done by taking the sealed tube with slides and taking two sets of readings. One set of readings were done with the direction of the magnetic field normal to the plane of the glass, and the second set of readings were done with the magnetic field parallel with the plane of the glass plates. By comparing these two sets of data with each other a determination could be made as to whether or not the lipid had oriented. As it turns out, in a significant number of cases the results were positive, the lipid had oriented. Note the zero and 90° orientation of the samples to the right. The different spectra suggest that the lipids are oriented and not random. If the lipid was arranged randomly, then rotating the plates by 90° would have no affect, but since the spectrum changes for each sample, we know that the lipid is not random, but oriented in some direction. With the feeling that the lipid can be successfully oriented the goal then turned to orienting the lipid in the presence of the FP23.



The Third Phase

This is the phase where the project is currently. The purpose of this stage is to determine how the fusion peptide affects the formation of a lipid membrane. It is possible that the peptide will make orientation impossible due to its "membrane-destabilizing effects."[2] The new goal is to mix the FP23 with several lipids and put them on glass plates in a way similar to before. What is causing a problem is that the FP23 is not very soluble in chloroform and methanol and will precipitate well before the lipids do. If the same process to apply the solution to the plates is used now that was used before then the lipids and peptide will not be homogeneously mixed. A freeze-thaw

[2] Dr. David Weliky's NIH Grant Proposal, page 14.

process should be able to mix the lipids and peptides. For the peptide in this phase, however, two labels were put onto the FP23. Namely a 13C was placed on the number 8 Phe and a 15N was placed on the 9 Leu, heretofore CF8-NL9. Reason being for the labels is that a 13C NMR reading should show that the 13C and 15N would have a spin-spin coupling.

The first order of business was to synthesize new, labeled FP23. The synthesizing was explained above. Once some material had been purified an NMR spectrum had to be taken in order to ensure that the labels were in the right place. A mass chromatography revealed that the labels were present, but they might not be where they were expected. The solution NMR sample was prepared by making a 700μL sample at 2mMolar concentration. That was roughly 2.9mg FP23 in 700μL of water. To increase the solubility of the peptide the pH was lowered to roughly 3. Unfortunately, this was too concentrated and the peptide still precipitated so the precipitate had to be centrifuged and the remaining, hopefully saturated, solution was all that could be tested in the NMR. After nearly 100 scans, which is not much when all things are considered, of the 300MHz NMR the only signal being seen was that from the water. A graduate student tried a similar NMR scan using the 600MHz NMR, but still revealed nothing.

The first plan to freeze-dry the solution was to have a vacuum flask sit in a dry-ice bath and to pump on it until all the organic solvent was pumped off. Dry ice, however, is barely cold enough to keep the solvent frozen, let alone get it to a point where it could easily sublime. The solvent in the dry-ice bath actually showed almost no progress of subliming in roughly 10 days of pumping. The method of using a dry-ice bath still has not been abandoned, however. More trials are needed to make sure that other factors were not disrupting the freeze-drying process. Another idea was to place the solvent in liquid nitrogen and pump on it. Hopefully the nitrogen would be cold enough and the pressure low enough to allow the solvent to sublime. Currently, after two attempts with just solvent, nothing added to the solvent, there is evidence that the lower temperature will work. If the two trials are accurate, then roughly 1.5mL of solvent can be pumped off about every 3 hours. Even if this were successful, this would pose a new problem entirely. Since the nitrogen evaporates so quickly, someone needs to add more every 30 minutes or so, but for 50mL to sublime off that could take more than 4 days. If this works, then clearly a larger reservoir of liquid nitrogen needs to be developed.

Conclusions

Conclusions at this point in the research are a bit premature since the project seems to be stuck on freeze-drying the sample. However, progress has been made at the earlier stages. Once the immediate problem is solved, the research will move forward into the production of samples where the peptide is oriented.

Acknowledgements

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Works Cited

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