CLOT BUSTER BULLETS:
THE CREATION OF TPA COATED NANOPARTICLES

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Abstract

This research presents a way to attach tissue plasminogen activator (tPA) to spherical nanoparticles, called dendrimers. Attaching dendrimers to drugs creates a drug delivery system. This drug delivery system should require lower dosages, resulting in less severe side effects such as internal bleeding that could require surgery. Specifically, using biocompatible dendrimers, diverse drugs can be attached using covalent bonds because dendrimers incorporate surface- active organic functional groups including carboxylic acids, amines, and alcohol groups. Tissue plasminogen activators, a clot busting drug given to stroke victims, was bonded to three different surface group dendrimers using different chemistry synthesis methods including fischer esterification and an EDC (1-ethyl- 3- [3-dimethylaminopropyl]carbodiimide hydrochloride) coupling reaction. All the reactions happened with a total 50 microliters of reagents. Each generation four dendrimer that was used has 64 bonding locations on the surface, ideally allowing for 64 drugs to attach to each nanoparticle. However, since tPA is about triple the size of the dendrimer, this could create crowding on the dendrimer, potentially limiting the number of tPA molecules attached to each dendrimer. After completing the synthesis, the product was separated from the reagents through centrifugation and filtration. These tPA- dendrimer complexes were tested *in vitro* with an UV-Visable spectrophotometer assay. With this assay, or analytic test, the absorption was measured after every hour and recorded and the data was analyzed with respect to change in time, not total time. The results show that the succinamic acid functionalized dendrimer yielded a higher concentration of tPA- dendrimer complex throughout a wide range of dilution ratios. The higher concentrations can mean that the new complex is quicker reacting and more effective than the current standard of tPA used in stroke, pulmonary embolism and heart attack patients.

Keywords: tPA, dendrimer, nanoparticle

1. Introduction

Dendrimers are spherical shaped nanoparticles that have been studied as ways to delivery pharmaceutical drugs for disease such as cancer[1]. There are two ways of utilizing dendrimers. One way is to encapsulating the drug that would be released when reaching the targeted molecule or cell. The other way is to covalently attach drugs to the surface active functional groups that are on dendrimers[2]. This dendrimer-drug complex can form single molecules that will be more potent than an individual drug molecule, allowing for more direct application with less drug necessary per dose. Each dendrimer has different organic functional groups on the surface, and since the dendrimer is generation four, there are 64 surface groups per dendrimer used.

Tissue plasminogen activator (tPA) is a clot busting drug given to stroke, pulmonary embolism and heart attack patients that consists of a 527 amino acid chain. This drug, however, can cause potential complications, the most

commonly of which are internal bleeding, and superficial or surface bleeding[3]. The ends of proteins consist of a carboxylic acid and an amine that can be reacted to covalently bond to the dendrimer surface groups.

Blood clots are held together by fibrin which traps the red blood cells. On these fibrin is an inactive protein, plasminogen, that when active converts into plasmin. This plasmin dissolves the fibrin, reducing the blood clot and allowing the red blood cells to recirculate into the blood[4]. As a serine protease enzyme, tPA activates plasminogen into plasmin, as shown in figure 1.

By attaching the tPA to dendrimers through organic chemistry synthesis,, each molecule may potentially activate more plasminogen, creating more plasmin. In the end these molecules will dissolve the fibrin, and effectively the blood clot, quicker.

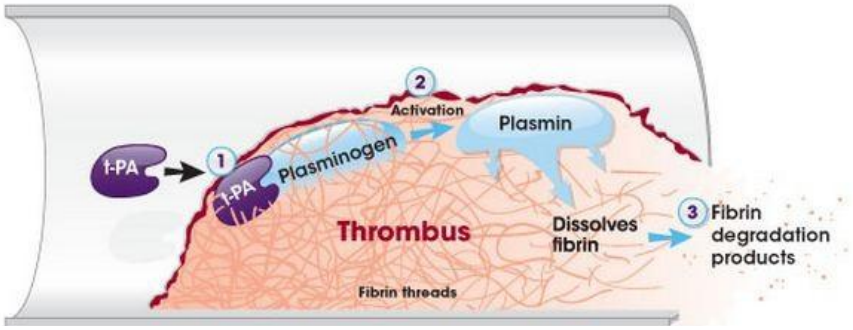

Figure 1: tPA enters the thrombus and connects to the fibrin and plasminogen at step 1. Next in step 2, the plasminogen gets activated into plasmin, which will dissolve the fibrin. Step 3 is the dissolved fibrin degrading away, allowing the red blood cells to re-enter the blood stream [5]

2. Methods & Materials

A kit of hydrophilic generation four dendrimers was purchased from Sigma-Aldrich of Milwaukee, WI (Product 683507). These poly(amido-amine), PAMAM, dendrimer compounds included amine, succinamic acid, and amidoethanol surface active functional groups as shown in figure 2.

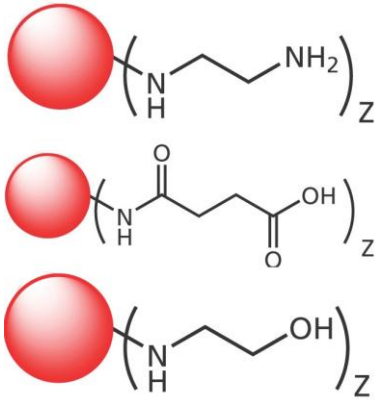

Figure 2. The surface groups of the dendrimers used to conjugate. At the top is the amine functional group. In the middle is the succinamic acid functional group, and at the bottom is the amidoethanol functional group. The z, because the dendrimers are generation four, is 64, for the amount of present surface groups for each dendrimer [6,7,8]

The tPA-dendrimer complexes were prepared as follows; first the Eppendorf tubes and filters were treated with a 5% solution of Triton X 100 to prevent the plastic of the tubes from absorbing tPa. Next the dendrimers were

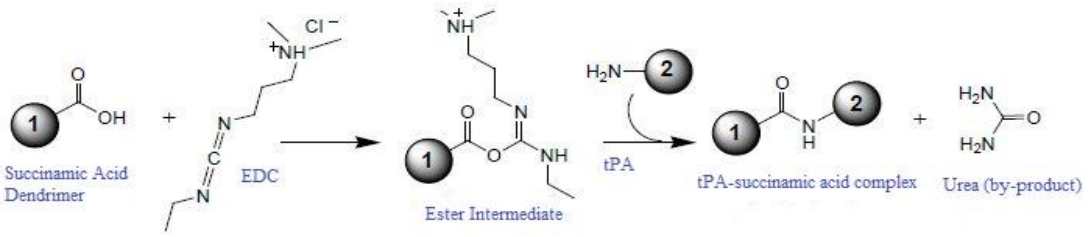

Figure 3: The example reaction, where EDC and the carboxylic acid end create an intermediate. This intermediate, when in contact with the primary amine group of the tPA, creates a stable amine bond that binds the tPA to the dendrimer [9]

serially diluted tenfold in water to achieve the necessary stiochiometric ratios. These solutions were combined with EDC and were set at room temperature for 2 hours to yield the tPA dendrimer complexes.

Upon completion of the synthesis process the products were separated from the remaining reactants via centrifugal filtration. A Pall 100 kilodalton (kDa) molecular weight cut off filter was used to isolate the compounds. This molecular weight cut off was ideal for this extractions because the molecular weight of the dendrimers was approximately 21 kDa and the tPA was 59 kDa. The isolated tPA-dendrimer complex remaining unfiltered, then, should contain at least 2 tPA molecules per nanoparticle. The filters were centrifuged at 5000 x g for 10 minutes and the amount of conjugate unfiltered was checked. If the filter had about 10-20 microliters left unfiltered, it was taken out, and the conjugate was retrieved. If the filter was dry, 15 microliters of water was added and centrifuged for one more minute. If nothing seem filtered, the filter was then centrifuged at 3 minute intervals and checked.

The reaction produced two products, the tPA dendrimer complex and urea, a by-product that was filtered out. The new complexes were made up with, for the amine and succinamic acid dendrimers, amide bonds between the tPA and the dendrimer. For the Amidoethanol, the new bond between the dendrimer and tPA was an ester bond. The new tPA-dendrimer complexes were tested with a chromomeric assay purchased from AssayPro in St Charles, MO (CT1001). Once mixed with the assay reactants and the tPA samples, it incubated in a water bath at 37^0C for 24 hours. Readings were taken at 405 nm with a Genesys 2 UV-Visable spectrophotometer.

3. Results & Data Analysis

The first conjugation was with the succinamic acid dendrimer. Figure 5 shows the results of the diluted tPA-dendrimer conjugate versus the filtrate counterparts. All of these were then compared with the tPA standard that the conjugation happened with.

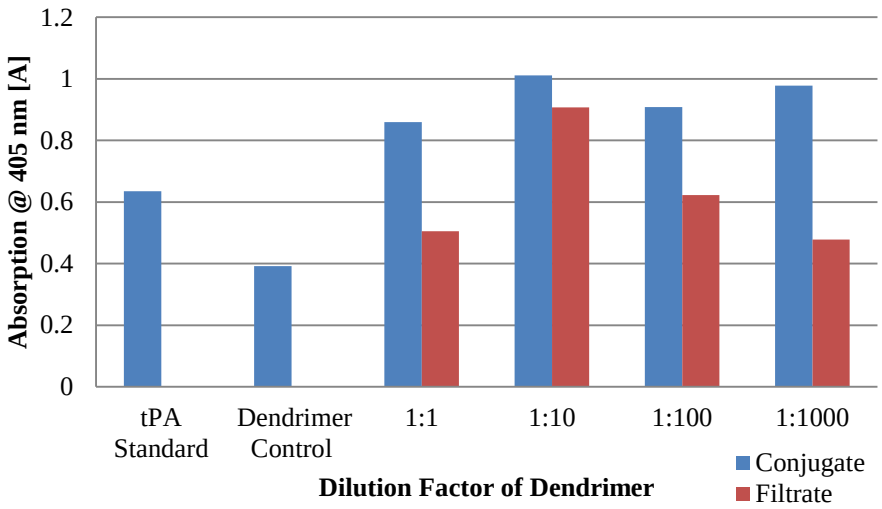

Figure 4: the results of the succinamic acid dendrimer from the assay done at 405 nm

The results show that each of the diluted tPA-dendrimer conjugates was higher than the corresponding filtrates, tPA standard and the dendrimer control. Most notably, each of the conjugates is about 1.5 times more absorbent of the wavelength light than the tPA standard.

Next, the amine dendrimer was conjugated with the graph of the results shown in figure 6. The dendrimer control for the amine was higher than the succinamic acid, but the tPA standard was the same. This could be that the succinamic acid functional dendrimer was packaged in water where as the amine and amidoethanol functional dendrimers were packaged in methanol.

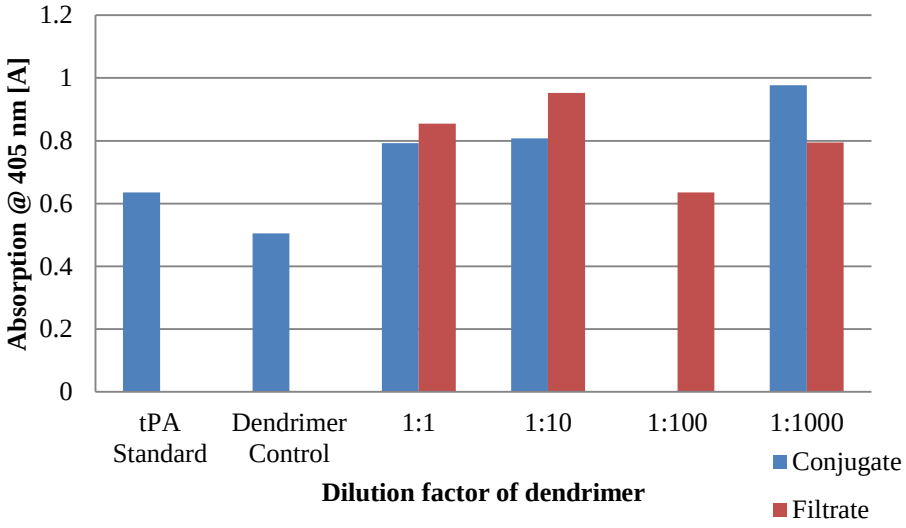

Figure 5: the results of the amine dendrimer conjugation from the assay at 405 nm

This conjugation has an outlier in the 1:100 dilution as it has no conjugate, meaning that the reaction didn't happen and everything was filtered out. Further, the 1:1 and 1:10 dilutions had the filtrates high than the conjugates, but still were higher than the tPA. The last dilution, 1:1000, had about the same results of that of the succinamic dendrimer.

The last dendrimer to conjugate, the amidoethanol functional group, was also successful, as shown in Figure 6.

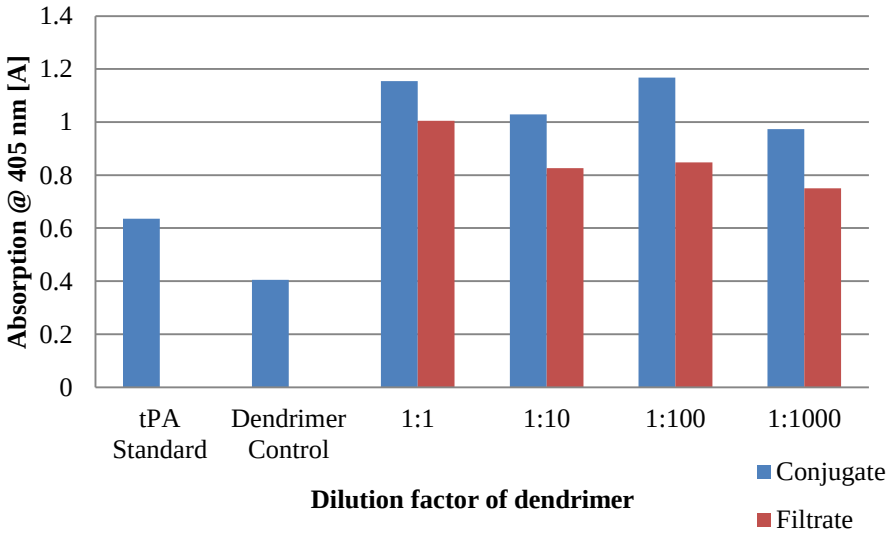

Figure 6: the results of the amidoethanol dendrimer from the assay done at 405 nm

This conjugation shows similar results as the succinamic acid dendrimer. The conjugates were higher than the filtrates, amidoethanol dendrimer, and the tPA standard in all dilution ratios. Further, the filtrates were lower than

the previous for each dilution. This decrease shows that the more diluted dendrimers were more successful conjugates.

4. Conclusions

There is evidence to support that the conjugations happened and that the new tPA-dendrimer complexes are more effective than the tPA standard. Specifically, the succinamic acid and amidoethanol functional dendrimers were the most effective of the dendrimers used. For the succinamic acid functional dendrimer, the conjugates were higher than the filtrates, dendrimer control and the tPA standard for all controls, and the amidoethanol dendrimer had higher absorption levels than the succinamic acid.

The reason the succinamic acid dendrimer was successful could be because it utilized the open amine end of the tPA protein. This openness would allow the tPA to just react at the end and not the other exposed functional groups. Not reacting with the other functional groups would keep the functionality of the protein, and not deactivate the enzymatic activity, which could be what happened in the amine functional dendrimer with the carboxylic acid functional groups.

The amine dendrimer may not have been conjugated, or the reaction may not have happened to completion. As shown in the 1:100 dilution, there was no conjugate, and in the 1:1 and 1:10 dilutions the filtrate was higher than the conjugate. The reason behind this could be that the carboxylic acid end of the tPA that was necessary for the amine conjugation was less accessible than the amine end.

However, since each sample size for all reactions was one with only 50 microliters for each reaction, nothing can be fully concluded from these results. In order to fully conclude anything, more testing would need to be done, see future work.

5. Future Work

In order to move forward with this work, all the results need to be replicated to make sure that they are accurate and precise. By increasing the sample size, there would be more evidence to support to refute the hypothesis and conclusions. Once these results are analyzed, the dendrimers that seem to be most effective would then be used in dilutions to create a curve of absorbance versus dilution of tPA. These curves would then be compared to a standard curve of unbound free tPA. To analyze these curves, linear regressions would be calculated and compared to see if the new tPA-dendrimer complexes were more effective than the unbound free tPA.

Finally, once the conjugation happens, using an atomic force microscope, mass spectroscopy, or infrared spectroscopy, find out where the tPA-dendrimer bond is on the tPA molecule. Finding where the bond is located will give details about change in functionality of the tPA if there is any. The location can be changed by blocking the outside functional groups of the tPA molecule and then performing the conjugations, but a further analysis of the tPA and what functional groups need to be blocked would need to be researched.

6. Acknowledgments

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7. References

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