EFFECT OF PEG COMPOSITION ON THE DISSOCIATION RATE OF CAMPTOTHECIN-PEG CONJUGATES

A Thesis

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Master of Science

by

Ashlee Greene

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ABSTRACT

Camptothecin (CPT) is an anti-cancer drug that inhibits the enzyme DNA Topoisomerase 1, leading to the apoptosis of cancerous cells. Although several analogs of CPT have been synthesized as chemotherapy agents, a number issues remain that hinder its success including lack of stability of the active form, poor solubility and limited circulation time. To overcome these challenges, conjugation of the CPT drug to polymers such as polyethylene glycol (PEG), has been shown to improve CPT solubility and circulation time, thus leading to an increase in drug bioavailabity. However there is still limited understanding of how the properties of the polymer influence the dissociation of the drug from the drug-polymer complex.

In this study, PEG polymers of varying molecular weights (MW) and polydispersities were conjugated to CPT and characterized via ^{1}H NMR and mass spectrometry (MS). The extent of CPT dissociation from the CPT-PEG conjugate was monitored via liquid chromatography over two days at a variety of temperatures and pH. The data acquired from these studies showed distinct molecular weight effects on CPT-PEG dissociation rates. In addition, we also observed an increase in the rate and extent of dissociation as a function of hydroxide ion concentration. Future areas of investigation will include performing the dissociation studies with additional PEGs of different MW to further confirm the observed MW dependency as well as using PEGs with different end groups to study possible end group effects. The results of this work should lay the foundation for developing controlled-release CPT-PEG conjugates for a wide variety of drug delivery applications.

BIOGRAPHICAL SKETCH

Ashlee Greene was born in Washington, D.C. and raised in Prince George's County, Maryland. She received her Bachelor of Science degree in Chemical-Biological Engineering from MIT. She then continued on to Cornell University to earn her Master of Engineering degree in Chemical Engineering as well. Following the receipt of her Master of Science degree, Ashlee will start at the University of Pittsburgh for her doctorate.

Dedicated to my late grandmother, Margaret Sydnor

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LIST OF ABBREVIATIONS

CPT- Camptothecin

PEG- Polyethylene glycol

1kDa, poly- Polydisperse 1kDa PEG

1kDa, mono- Monodisperse 1kDa PEG

5kDa, poly- Polydisperse 5kDa PEG

CHAPTER 1

Introduction: Camptothecin and PEG Conjugation

1.1 Camptothecin

Camptothecin(CPT) is a natural, plant-based small molecule anti-cancer drug. Derived from the bark and stem of the *Camptotheca acuminata* tree [1]. CPT acts by inhibiting DNA topoisomerase 1; a nuclear enzyme that creates single strand breaks in DNA [2]. Once the single strand breaks have been made by DNA topoisomerase 1, CPT prevents re-ligation of the DNA ultimately leading to apoptosis of the cell [2]. Conclusively, CPT has the potential to be an extremely effective anti-cancer drug. In a 1991 study by Giovanella et al. [3], immunodeficient mice with 13 different human cancer xenograft lines were given CPT orally and by intramuscular and intravenous injection. CPT was found to suppress tumor growth and induce regression in colon, lung, breast, stomach and ovarian cancers [3]. However, there are several issues with CPT that hinder its wide spread clinical use. One of the primary problems is poor aqueous solubility; which lead to the 1991 study by Kingsbury et al. of various water-soluble CPT analogs [4]. Using a variety of chemical modifications, several CPT analogs were produced. The products were first evaluated for their antitumor activity, cytotoxicity and topoisomerase 1 inhibition, followed by a solubility screen to determine the most active and water-soluble analog. This approach led to the analog now known as the anticancer agent, Topotecan (Fig.1).

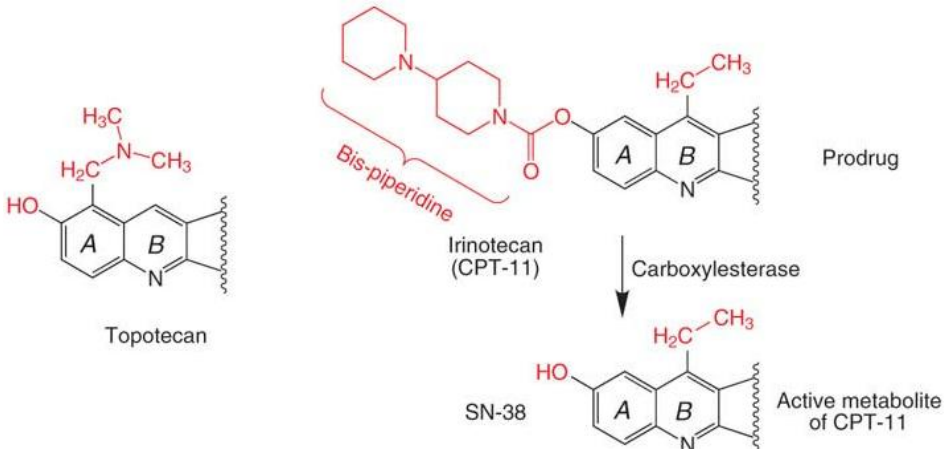


Fig.1 Clinically approved water-soluble Camptothecin derivatives [5].
(Left) Topotecan is active in its delivered form and has been approved by the FDA for use in ovarian and lung cancers. (Right) Irinotecan is a prodrug and requires cleavage by carboxyesterase to become SN-38, its active form. Irinotecan is FDA approved for the treatment of colorectal cancer [6].

Yet, additional problems for CPT still persist including low bioavailability, toxicity and instability of the active form. The structure of CPT in solution is pH dependent and can fluctuate between an active, lactone form and an inactive, carboxylate form [7] (Fig. 2) . Although the carboxylate form is favored at physiological pH, it is inactive and can be highly toxic [7]. Therefore, there has been significant research into what conditions give rise to the carboxylate formation and how it can be circumvented [8].It has been theorized that the 20-OH functional group of CPT either stabilizes the transition state or is involved in one of the proton-transfer steps for lactone hydrolysis [8]. Furthermore, substitutions at the 20-OH of CPT have been shown to reduce the occurrence of lactone ring opening [9].

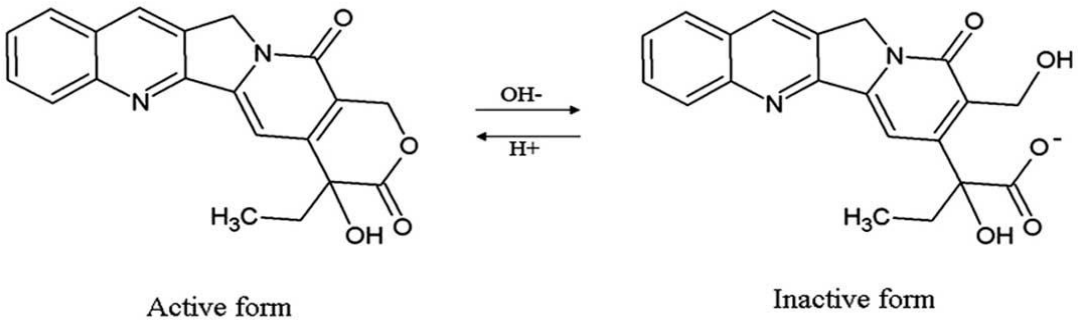


Fig. 2 CPT: the lactone and carboxylate forms[7].
The active, lactone CPT form is favored at low pH, high [H+] while the inactive, carboxylate CPT form is favored at high pH, high [OH-].

1.2 CPT-PEG Conjugation

PEG is a prevalent polymer used in drug delivery for the modification of biomolecules and small molecules alike. Its low toxicity and high aqueous solubility render it an ideal polymer candidate. Although PEG is relatively non-biodegradeable, it displays good biodistribution behavior and is excreteable after administration [10]. The PEG chemistry is also versatile; it is available in a wide range of molecular weights and polydispersities. As a result of the many desirable properties of PEG, advances in drug delivery have been made through PEG-drug conjugation. PEG has been previously conjugated to several therapeutics including granulocyte-colony stimulating factor (G-CSF), interferon (IFN) and erythropoietin (EPO) and has been shown to increase their therapeutic efficacy [11]. By conjugating PEG to CPT, the aqueous solubility and ease of administration greatly increases along with the stability of the active form and bioavailability [11].

There have been many developments in the area of CPT-PEG conjugation to address several of the clinical limitations. However, investigations into the dissociation of CPT from its PEG conjugate have left many parameters yet to be studied.

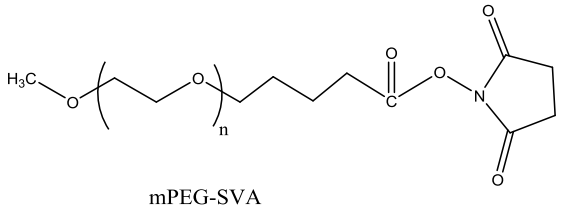
In this project, we hope to gain insight on the kinetics of CPT-PEG hydrolysis. Dissociation studies will be performed on CPT-PEG conjugates made from PEGs of varying lengths and polydispersities. Experiments will be performed at room temperature and 37°C as well as various pH's. The results of this work should help to elucidate characteristics that would aid in the future design of controlled release CPT-PEG conjugates.

CHAPTER 2

Materials and Methods

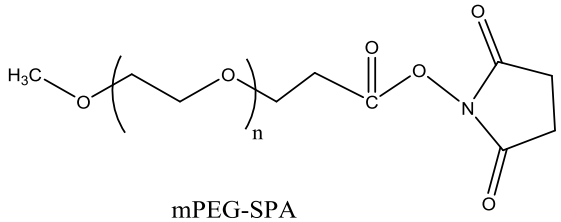
2.1 Materials:

Camptothecin and t-boc glycine was purchased from Alfa Aesar (Ward Hill, MA). Polydisperse PEGs (mPEG-NHS MW= 1kDa, poly and mPEG-NHS MW=5kDa,poly) were purchased from Laysan Bio, Inc. (Arab, AL). Monodisperse PEG (mPEG-NHS MW=1kDa,mono) was purchased from Quanta BioDesign, Ltd. (Plain city, OH).



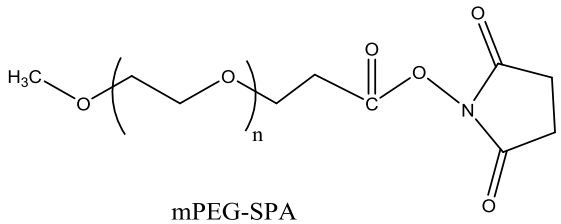
mPEG-SVA

5kDa poly, n=112



mPEG-SPA

1kDa poly, n=23



mPEG-SPA

1kDa poly, n=24

Fig. 3 Structures of mPEG-NHS used, n= number of PEG units.

2.2 Methods:

2.2.1 Synthesis of CPT conjugates

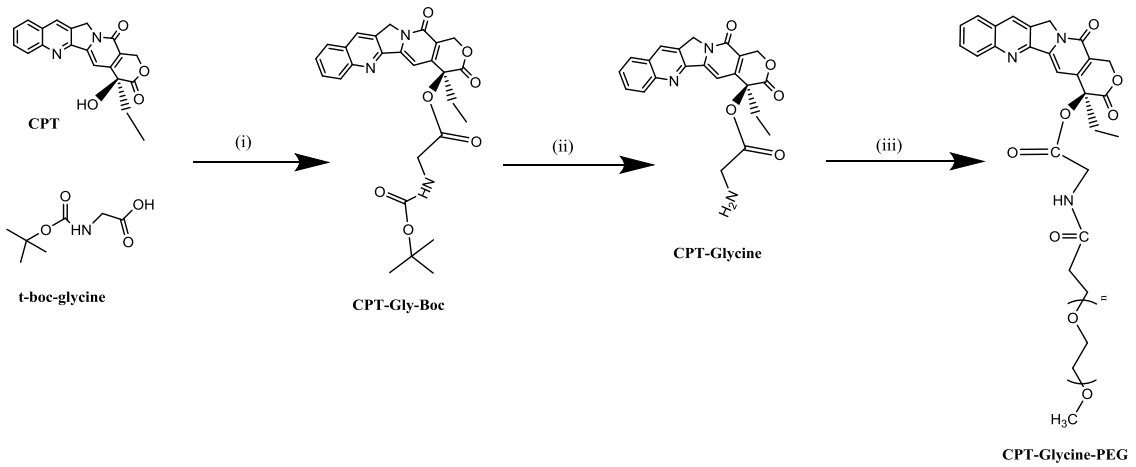


Fig. 4 Scheme for CPT-PEG conjugation.

CPT-PEG conjugates were synthesized in three steps similar to as shown by Paranjpe et al. [12].

Boc protected CPT-Glycine (CPT-Gly-Boc): t-boc-glycine (377mg, 2.1mmol) was dissolved in 200mL of anhydrous dichloromethane (DCM) at room temperature. Then, CPT (250 mg, 0.72 mmol), DCC (444mg, 2.1mmol) and DMAP (87 mg, 0.72 mmol) were added to the dissolved t-boc glycine. This mixture was stirred for 1 hour at 4 °C then left overnight at room temperature. The dicyclohexylurea (DCU) precipitate was removed, the filtrate washed and solvent evaporated by rotary evaporation. The resulting CPT-Gly-boc was characterized via ^{1}H NMR (Appendix A-1).

CPT-Glycine (CPT-Gly): Boc deprotection was then performed with 20mL of TFA:DCM mixture (1:1) while stirring, for 2 hours. The solvent was removed by

rotary evaporation. An excess of HCl (~100uL,1.2mmol) was added, allowed to stand at room temperature for 30min, before the remaining solvent was removed by rotary eveporation. Flash chromatography was performed for purification. The resulting purified CPT-Gly was characterized using ^{1}H NMR (Appendix A-1). Mass spectrometry (MS) gave m/z (M+1)=406 (Appendix A-2).

CPT-Glycine-PEG (CPT-Gly-PEG): In the last step, mPEG-NHS (30mg, 0.03mmol) was dissolved in 5mL of Dimethylformamide (DMF). CPT-Gly (50mg, 0.12 mmol) and 1% DIEA (50uL) were added to the dissolved mPEG-NHS. This reaction was stirred for 3 hours at room temperature. The solvent was removed by high vacuum centrifugal evaporation overnight. The products were purified by reversed-phase high-performace liquid chromatography (RP-HPLC) (Appendix B1-B3). CPT-PEG (1kDa, poly) was characterized by characterized using ^{1}H NMR (Appendix A-3). Mass spectrometry (MS) gave m/z (M+1)=1416 (Appendix A-4). Conjugates CPT-PEG (5kDa,poly) and CPT-PEG (1kDa,mono) were also characterized using ^{1}H NMR (Appendix A-6).

2.2.2 Dynamic light scattering studies (DLS)

DLS studies were performed to study micelle formation in solution. The CPT-PEG (1kDa, poly) was made at concentrations; 10mM, 1mM, 100μM, 10μM, 1μM, 0.1μM and 0.01μM in phosphate-buffered saline (PBS) at pH 7.4. An S-curve was observed and the critical micelle concentration was determined at the inflection point (Appendix C-1).

2.2.3 Hydrolysis studies

The hydrolysis studies were conducted at three different pHs; 5.5, 7.4, 9.0 using a Citric Acid/Sodium Citrate buffer (CA), phosphate-buffered saline (PBS) and borate buffer (BBS) respectively. The CPT-PEG conjugates were made at a concentration of 800μM in each buffer. The samples were left on the benchtop for the room temperature condition and placed on a heating block for the 37°C condition, for the course of 48hours. At measured intervals, an aliquot was taken from each sample and diluted by a factor of 10 into Dimethyl sulfoxide (DMSO) and frozen for later analysis.

2.2.4 HPLC analysis

Frozen samples were first allowed to come to room temperature prior to being run on the HPLC. Free CPT was found to have a retention time of ~4 minutes in all buffers.

Analysis was done by measuring the increase in free CPT (Appendix B-4). Calibration curves were created by injecting varied amounts of CPT to create a correlation between area under the curve (AUC) and amount of CPT in micrograms (μg) (Appendix C-2). Then the AUC, for the peak representing the free CPT, was measured for each sample. Using the buffer appropriate standard curve, the amount of free CPT (μg) was calculated and converted into moles. The percent of dissociated CPT was found (Eq.1) and compared.

$$\% \text{ CPT dissociated} = \frac{\text{moles of CPT released}}{\text{moles of starting CPT}} \times 100 \qquad \textbf{(Eq. 1)}$$

CHAPTER 3

Results and Discussion

3.1 Results

The result of placing CPT-PEG 5kDa, poly in buffers of different pHs is seen in the HPLC trace of Figure 5. Free CPT had a retention time of ~ 4.5 min at pH 5.5, 7.4 and 9.0. The retention time of the conjugate varied; 8, 8.2, 4 min for pH 5.5, 7.4 and 9.0 respectively. The dramatic shift for the conjugate at pH 9.0 is somewhat arguable. It is also possible the "conjugate" peaks at 4 and 4.5 are both free CPT; one the carboxylate form, the other the lactone form. The same trend was seen for the other two conjugates; CPT PEG 1kDa, poly and CPT PEG 1kDa,mono as well.

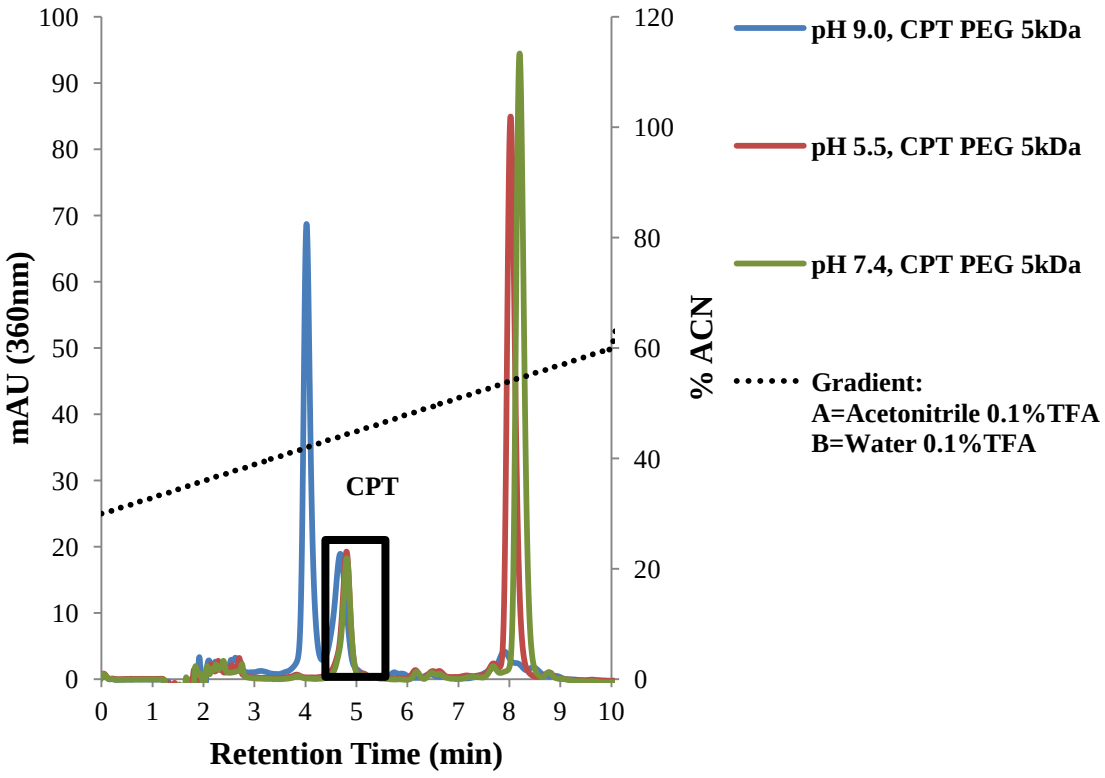


Fig. 5 HPLC at 360nm of CPT-PEG 5kDa,poly t=0 pH 5.5, 7.4, 9.0**.**This shows the shift in retention times of the conjugate.

The dissociation studies showed near negligible amounts of CPT dissociated at pH 5.5. By the end of the 48 hour study at pH 5.5, less than 4% CPT dissociated for all of the conjugates, even at 37°C (Fig. 6). The percentage of CPT dissociated for CPT PEG 1kDa, mono was 2.1% and 3.3% at room temperature and 37°C respectively. The percentage of CPT dissociated for CPT PEG 1kDa,poly was 0.2% and 1.7% at room temperature and 37°C respectively. The percentage of CPT dissociated for CPT PEG 5kDa,poly was 1.4% and 3.8% at room temperature and 37°C respectively.

The percentage of CPT dissociated at pH 7.4 was more pronounced (Fig. 7). At 48 hours, the percentage of CPT dissociated for CPT PEG 1kDa, mono was 5.1% and 27.6% at room temperature and 37°C respectively. The percentage of CPT dissociated for CPT PEG 1kDa,poly was 1.7% and 8.6% at room temperature and 37°C respectively. The percentage of CPT dissociated for CPT PEG 5kDa,poly was 2.7% and 35.0% at room temperature and 37°C respectively.

The highest CPT dissociation was observed at pH 9.0 (Fig.8). The percentage of CPT dissociated for CPT PEG 1kDa, mono was 38.9% and 42.7% at room temperature and 37°C respectively. The percentage of CPT dissociated for CPT PEG 1kDa,poly was 18.2% and 23.3% at room temperature and 37°C respectively. The percentage of CPT dissociated for CPT PEG 5kDa,poly was 61.6% and 76.3% at room temperature and 37°C respectively.

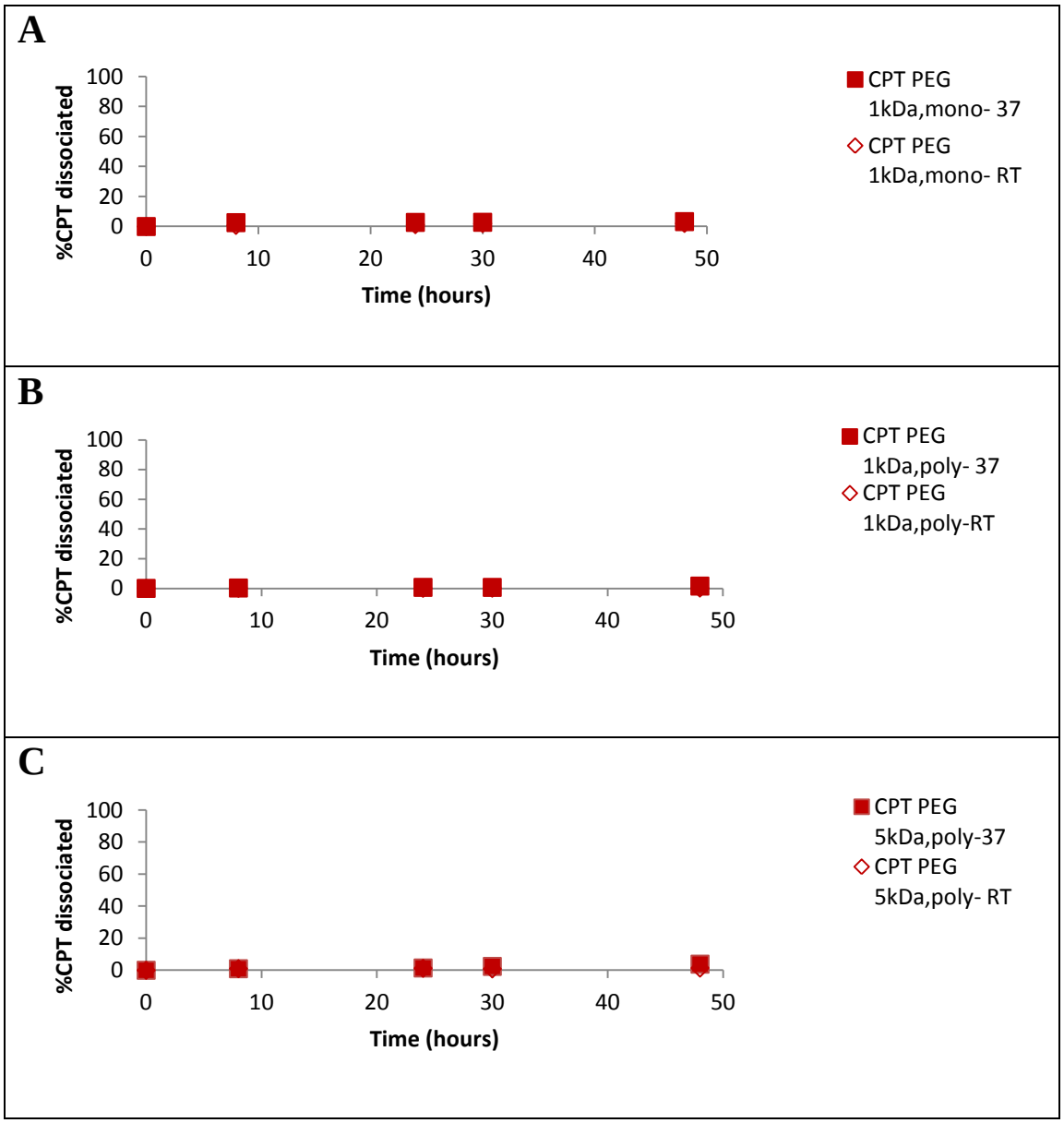


Fig. 6 CPT dissociation over time at pH 5.5. Filled in symbol means Temp= 37°C, open symbol means Temp= room temperature. **A)** CPT PEG 1kDa,mono for Temp= room temperature and 37°C. **B**) CPT PEG 1kDa, poly for Temp= room temperature and 37°C . **C**) CPT PEG 5kDa, poly for Temp= room temperature and 37°C .

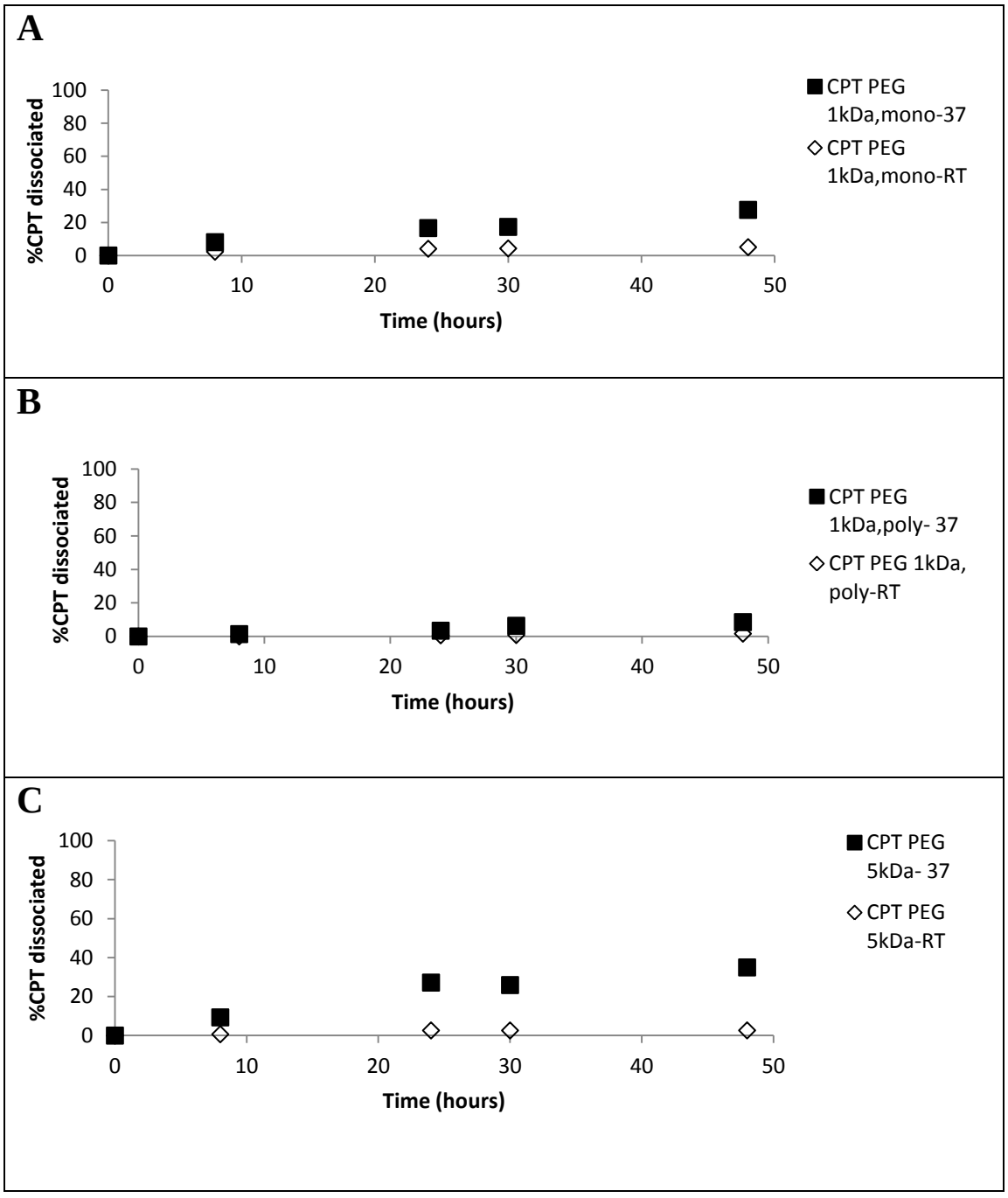


Fig. 7 CPT dissociation over time at pH 7.4. Filled in symbol means Temp= 37°C, open symbol means Temp= room temperature. **A**) CPT PEG 1kDa,mono for Temp= room temperature and 37°C. **B**) CPT PEG 1kDa, poly for Temp= room temperature and 37°C . **C**) CPT PEG 5kDa, poly for Temp= room temperature and 37°C .

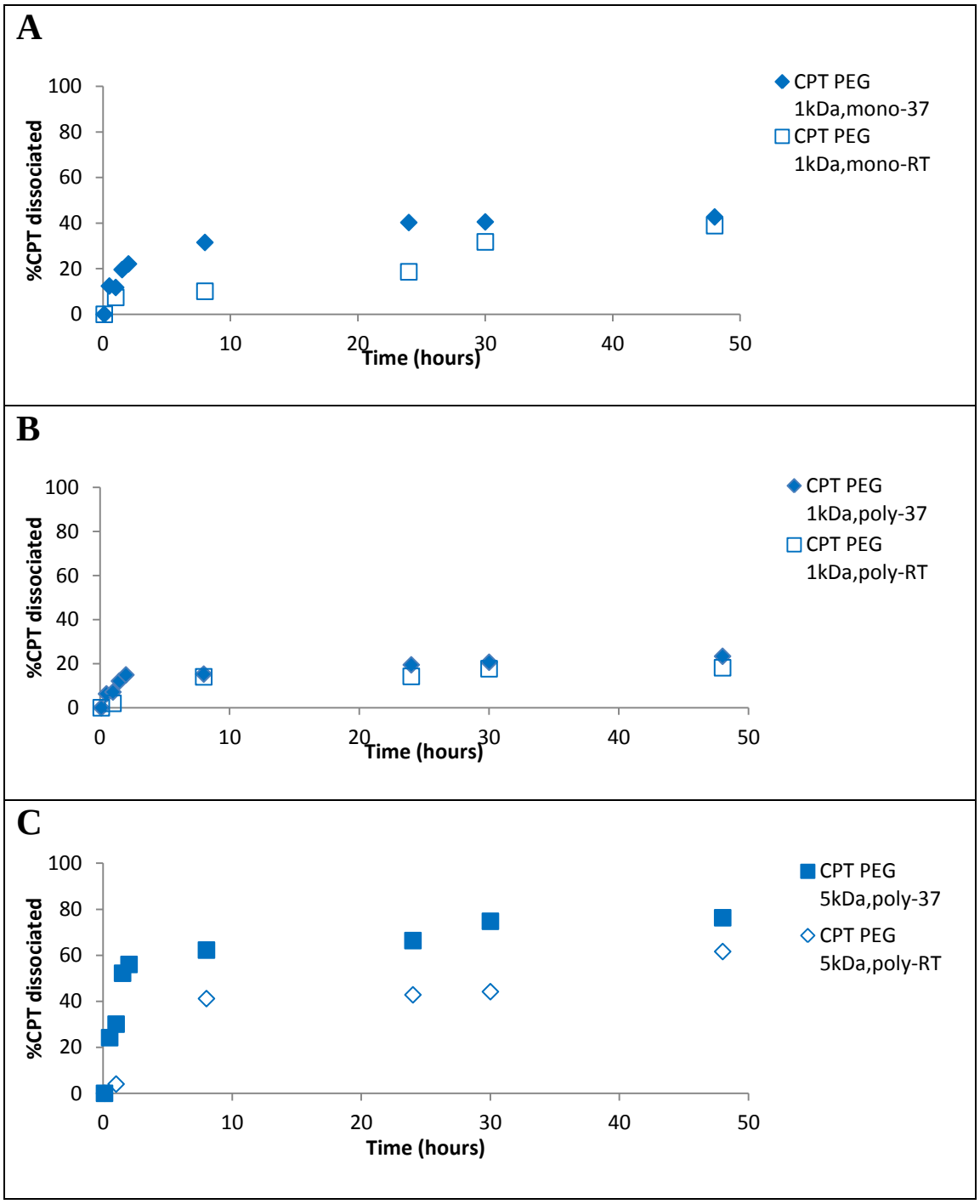


Fig. 8 CPT dissociation over time at pH 9.0. Filled in symbol means Temp= 37°C, open symbol means Temp= room temperature. **A)** CPT PEG 1kDa,mono for Temp= room temperature and 37°C. **B**) CPT PEG 1kDa, poly for Temp= room temperature and 37°C . **C**) CPT PEG 5kDa, poly for Temp= room temperature and 37°C.

3.2 Discussion

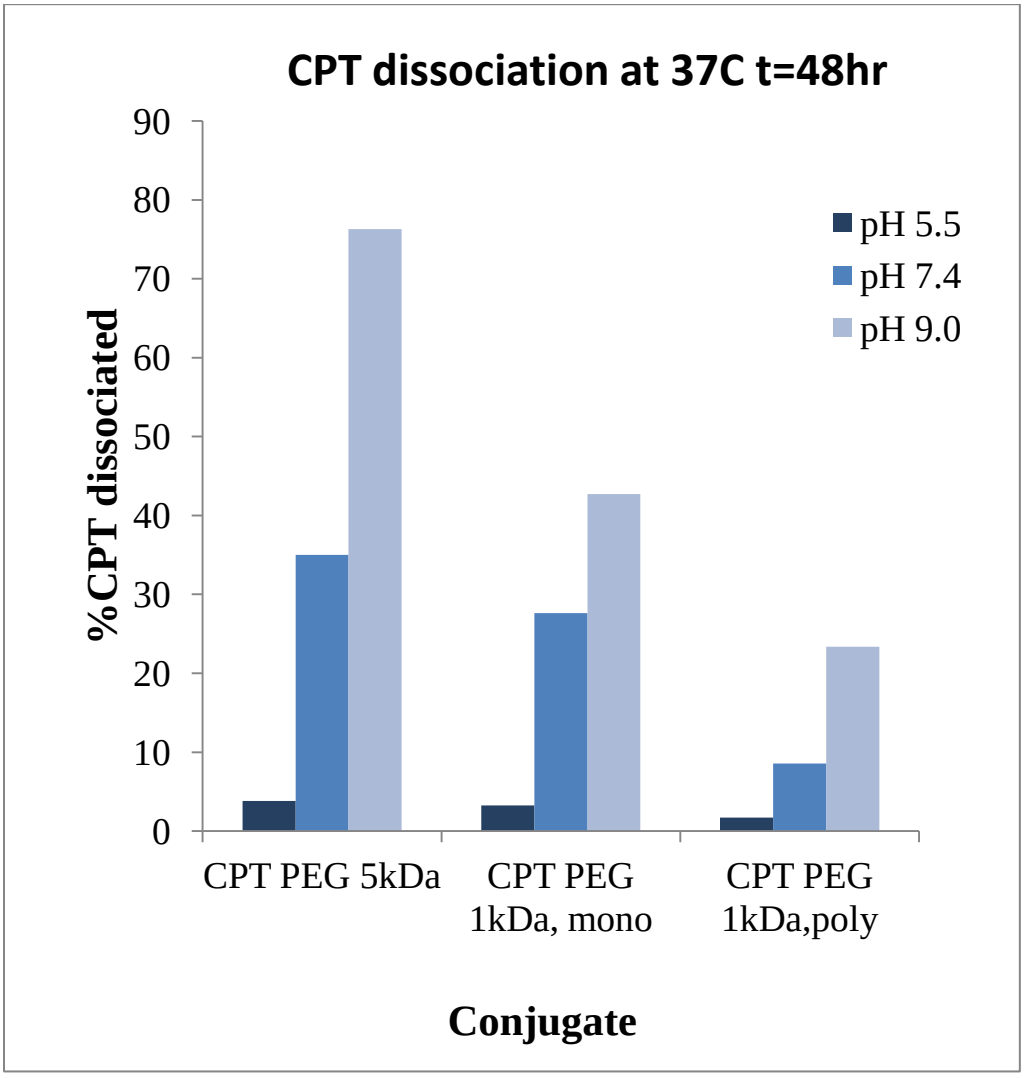


Fig. 9 CPT dissociation by conjugate length at 48hours and 37°C.

3.2.1 Molecular Weight

The differences in dissociation observed based on molecular weight (1kDa vs. 5kDa) and polydispersity (monodisperse vs. polydisperse) is best represented by Figure 9. In a comparison of the conjugates, the CPT PEG 5kDa dissociated the most in 48 hours at 37°C. The 1kDa PEGs were slower but the CPT PEG 1kDa, mono showed a higher percentage of CPT dissociated than the CPT PEG 1kDa, poly. This trend was consistent at pH 5.5, 7.4 and pH 9.0. From this data, we can speculate that hydrolysis if favored for higher molecular weight conjugates: CPT PEG 5kDa = 5321, CPT PEG 1kDa, mono= 1504, CPT PEG 1kDa, poly= 1415.

However, in addition to the different lengths of the PEGs used in the conjugation to CPT, the structure of the ester on the PEG was slightly different. As seen in Figure 3, the PEG 5kDa contained an SVA ester; two additional methylene units in-between the PEG units and the carbonyl, compared to the PEG 1kDa,mono and PEG 1kDa,poly SPA ester. Differences in dissociation rates due to the linker length and type between the CPT and PEG components have been measured by Greenwald et al. [13]. As seen in Table 1, the SVA ester is proven to have a longer half-life and slower hydrolysis kinetics than the SPA ester. This means that if a 5kDa, SPA ester had been used rather than the 5kDa, SVA ester, even faster kinetics of the CPT PEG 5kDa could have been observed.

There are two additional factors to take note of that could be skewing the results. In the ^{1}H NMRs of A-6, there is DMF present in the samples at 2.5-3ppm. The amount of DMF is highest in the CPT PEG 5kDa sample and least in the CPT PEG 1kDa sample;

following the trend of dissociation rates. It could be that due to the DMF, the injection amounts were less than what was calculated, therefore changing the AUC and μg correlation. So while the CPT PEG 5kDa appeared to have given the fastest dissociation rate, it could be a result of having the most DMF and the least amount of sample analyzed. The other factor is the intial amount of CPT in the CPT PEG conjugate samples. In B-1, B-2 and B-3, two peaks were collected as the product peak, with one attributed to free CPT. As seen in B-4, there is free CPT observed in the sample even at the t=0 time. Free CPT is not known to form micelles in solution, but the presence of free CPT in solution could affect the kinetics.

Table 1. Dissociation rates by ester at pH 8, 25°C; an adapted table from Laysan Bio, Inc [14]

PEG NHS Ester	**Ester (Symbol)**	**Half-life (minutes)**
PEG-O-$CH_2CH_2CH_2CH_2$-CO_2-NHS	Succinimidyl Valerate (SVA)	33.6
PEG-O-CH_2 CH_2-CO_2-NHS	Succinimidyl Propionate (SPA)	16.5

3.2.2 pH

The results also show considerable temperature and pH effects. As expected, the samples left at room temperature dissociated much slower than the samples that were at 37°C. However, the pH effects were not expected. For every conjugate, dissociation was fastest at pH 9.0 and slowest at pH 5.5. This is unexpected because ester hydrolysis is both acid and base catalyzed. In both acid and base catalysis, an

acceleration of proton transfer and hydrolysis is observed for an increase in hydrogen ion concentration and hydroxide ion concentration, respectively. Therefore, it would be expected that dissociation would occur fastest at pH 5.5 and pH 9.0 and slowest at pH 7.4.

Yet, in a 1997 study by Foroutan et al. [15], a similar pH dependent hydrolysis curve was identified (Fig. 10). They did not use CPT but instead were interested in the synthesis of hydrocortisone PEG conjugates (H-PEG). With PEGs of different lengths, hydrolysis of the ester bond was studied at varying pHs and 37°C. They found that the minima hydrolysis rate was not at pH 7.4 but rather pH 4. This would also support our findings and explain why hydrolysis at pH 9.0 was faster than pH 7.4 and pH 7.4 faster than pH 5.5. It is possible our CPT conjugates follow the same curve as Figure 10 but the pHs used in our studies were only to the right of the hydrolysis minima. Also in their study, conjugates with PEG MWs of 200, 400, 600, 900 and 2000 Da were used but they found no significant difference in the hydrolysis kinetics.

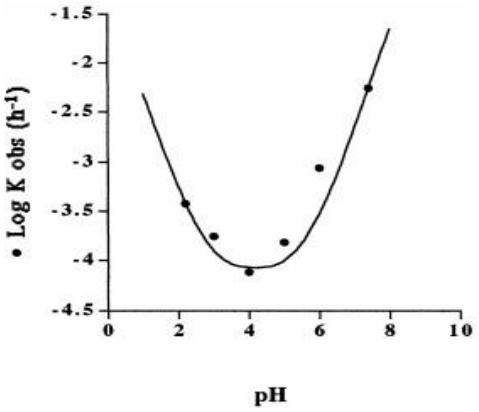


Fig. 10 pH hydrolysis curve of H-PEG400 at 37°C from Foroutan et. al [15]

CHAPTER 4

Conclusion

4.1 Conclusion:

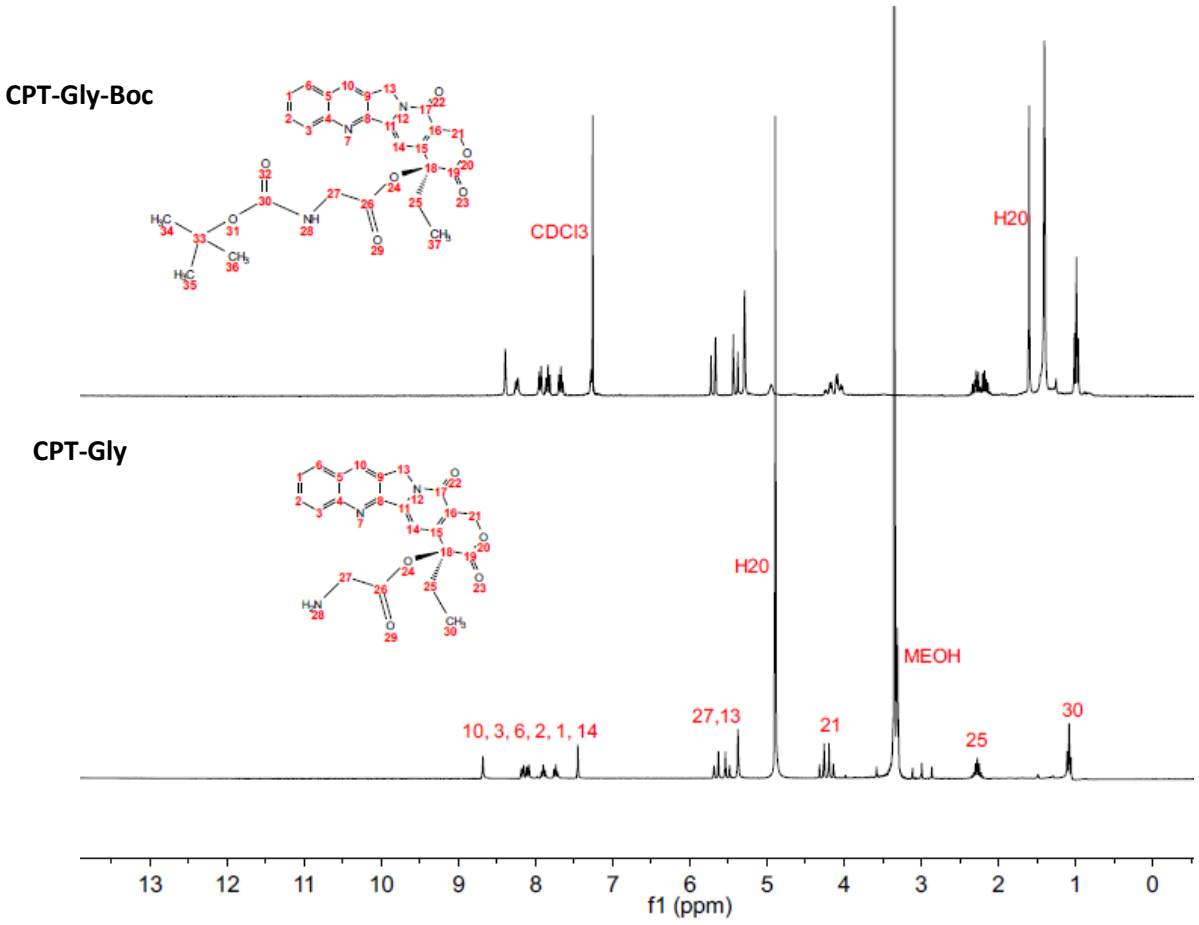
Although the data obtained for CPT dissociation was thought-provoking, it is still inconclusive. These experiments will need to be repeated several times to obtain statistically significant results. The trend of increasing dissociation rate with increasing molecular weight will need to be tested by including more variations in PEG length; for example by using a CPT PEG conjugate made from a 3400Da PEG and 500Da PEG. Dissociation rate was also hypothesized to be an effect of increasing ion concentration. An experiment that includes buffers on the lower spectrum in pH, pH 1- pH 5 should be tested to verify this trend. Nevertheless, one of the stronger points of apprehension involves not knowing the form of the dissociated CPT; whether it is in the lactone or carboxylate form. The shift in retention times seen for the conjugate while in buffer of pH 9.0 to ~4min (Fig. 5) is possibly not the conjugate at all but instead another form of the free CPT observed at ~4.5min . The increase of the peak at ~4.5min as a function of time appears to correspond to the decrease of the peak at ~4min. Therefore, the free CPT at ~4 min could be one form of CPT (carboxylate or lactone) while the peak at ~4.5min could be the other form of CPT and one form dominates the other over time. It is possible that the rate of dissociation of the conjugate at the higher pH is so much faster, there is very little conjugate left in the sample to show up at the expected ~8-8.5 retention time during HPLC analysis even for the t=0 time point. This would skew the results as the peak taken for measurements under the curve would be different. An additional matter of consideration is to revisit DLS and the study of micelle formation. Although the DLS measurements indicated that there would not be micelle formation at the concentration

chosen for these studies, a measurement should be done with free CPT and PEG-Gly in solution. It is possible that the two-phase decay observed at pH 9.0 was due to micelle formation and the dissociated PEG-Gly component in solution could be slowing down the kinetics.

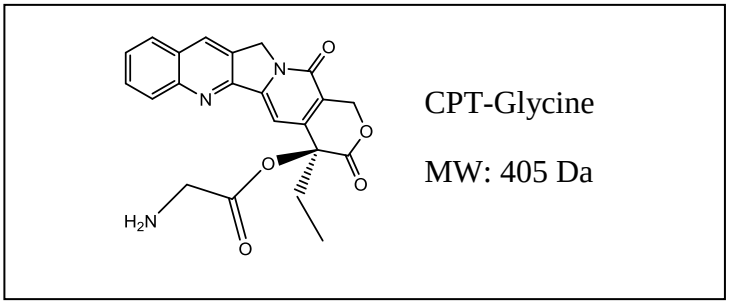
Some additional remarks include that there are several other characteristics that can be studied for the kinetics of CPT-PEG dissociation. The use of PEGs with different end groups can be tested to see how properties such as hyrophobicity/hydrophilicity of the end group effects hydrolysis. Also, the conjugation of CPT and PEG components at other sites besides the CPT 20'OH position could be studied to see the effects of electron stabilization and steric hinderance which we anticipate to slow the rate of hydrolysis.

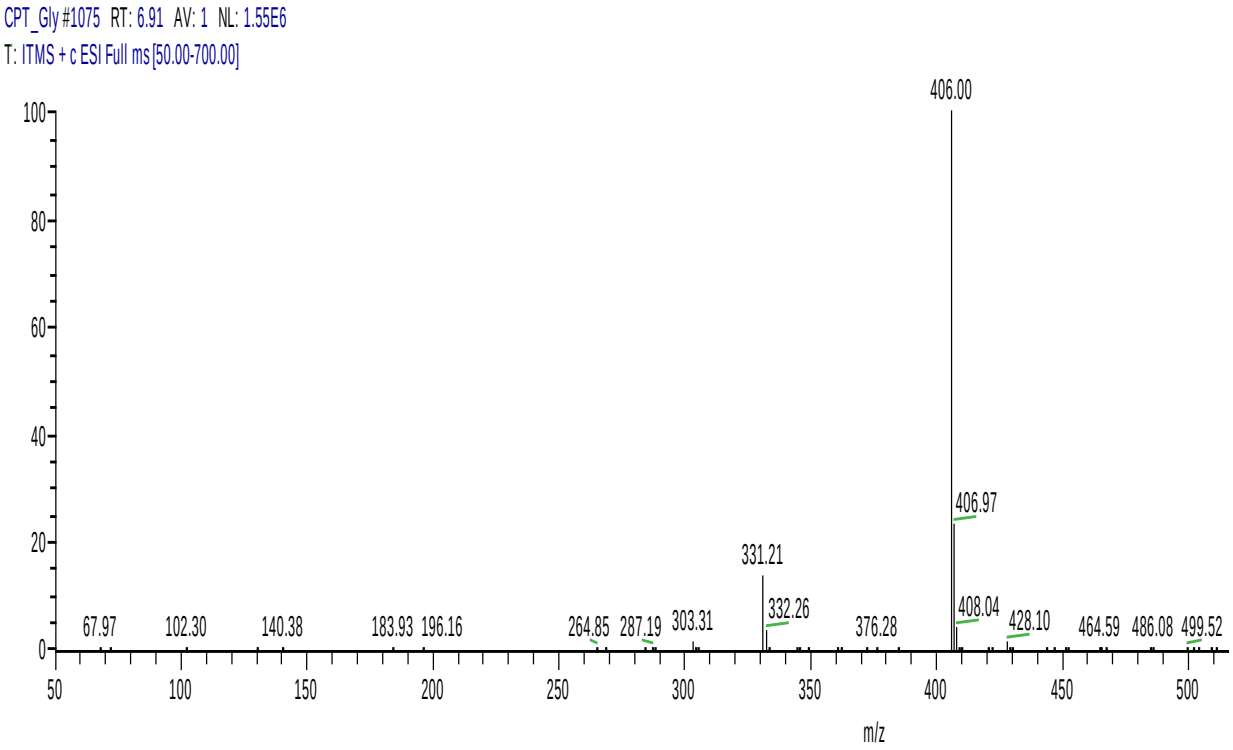
This work lays down the foundation for studying the controlled release of CPT from CPT-PEG conjugates but there still remains much more research to be done.

APPENDIX A: CHARACTERIZATION OF CPT-PEG

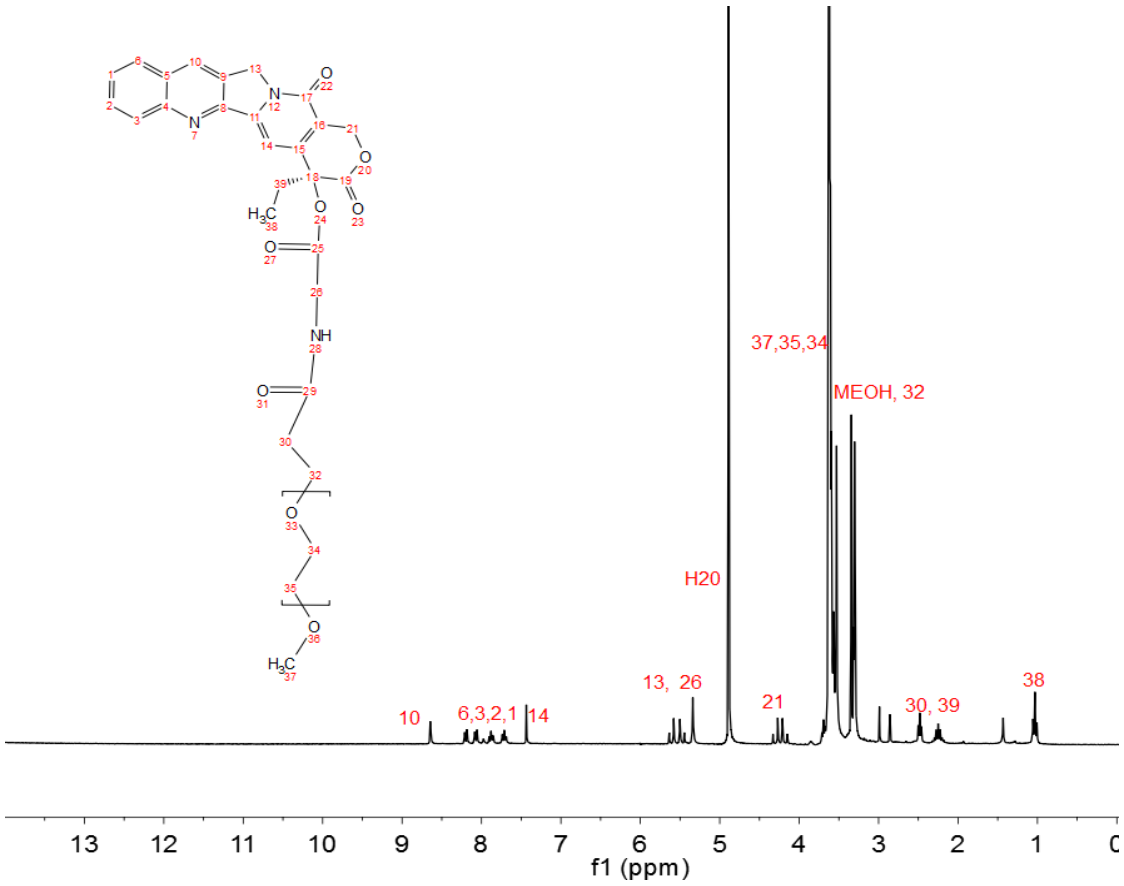


A-1. Stacked ^{1}H NMR of CPT-Gly-Boc vs. CPT-Gly. Due to the deprotection, note the disappearance of the boc protons on the CPT-Gly spectra vs. CPT-Gly-Boc at 1.5ppm. The remaining protons have remained intact and match up on both spectras.

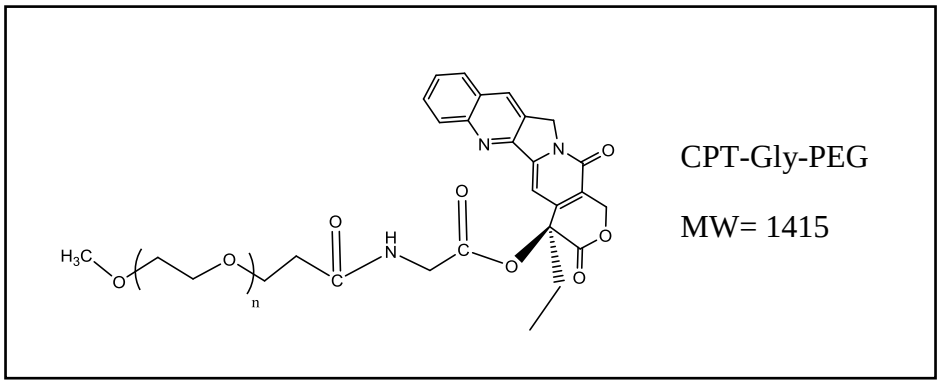


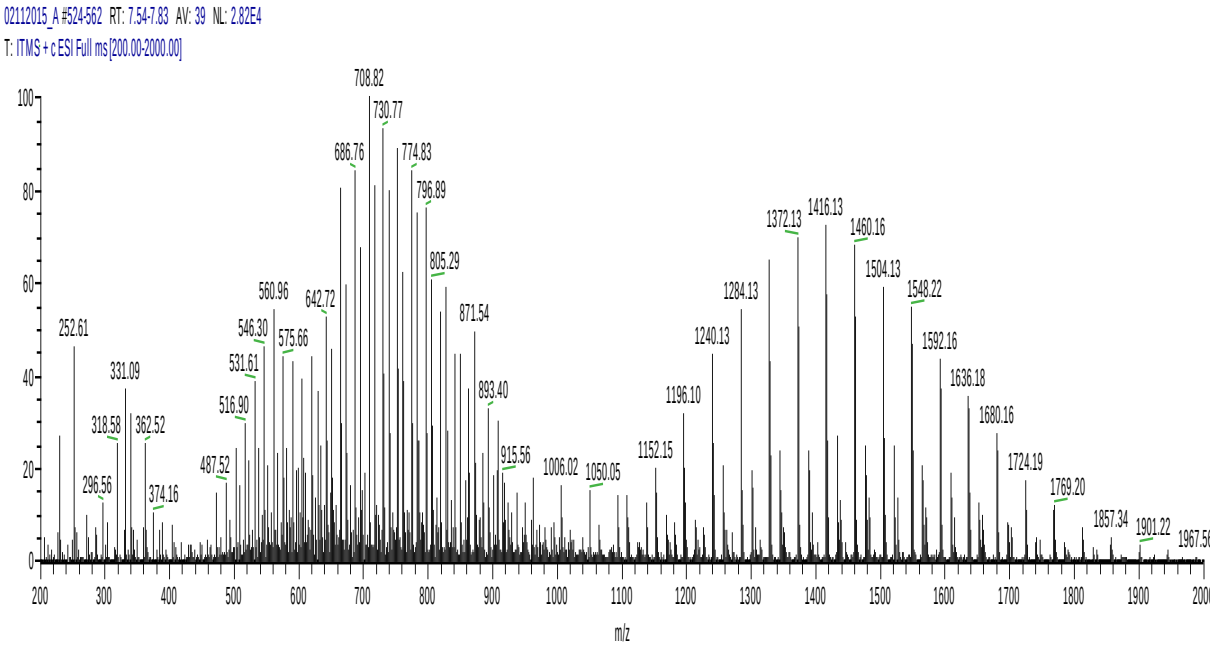


A-2. Mass spectrometry (MS) of CPT-Gly with (M+1) =406.

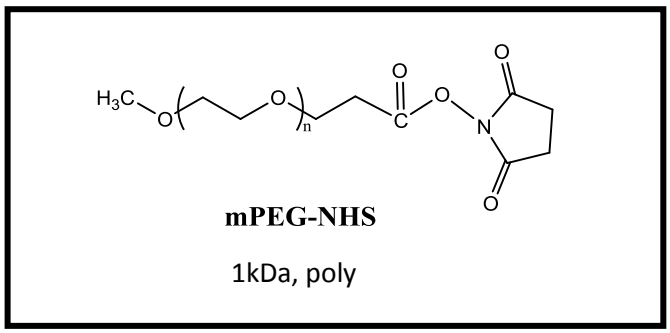


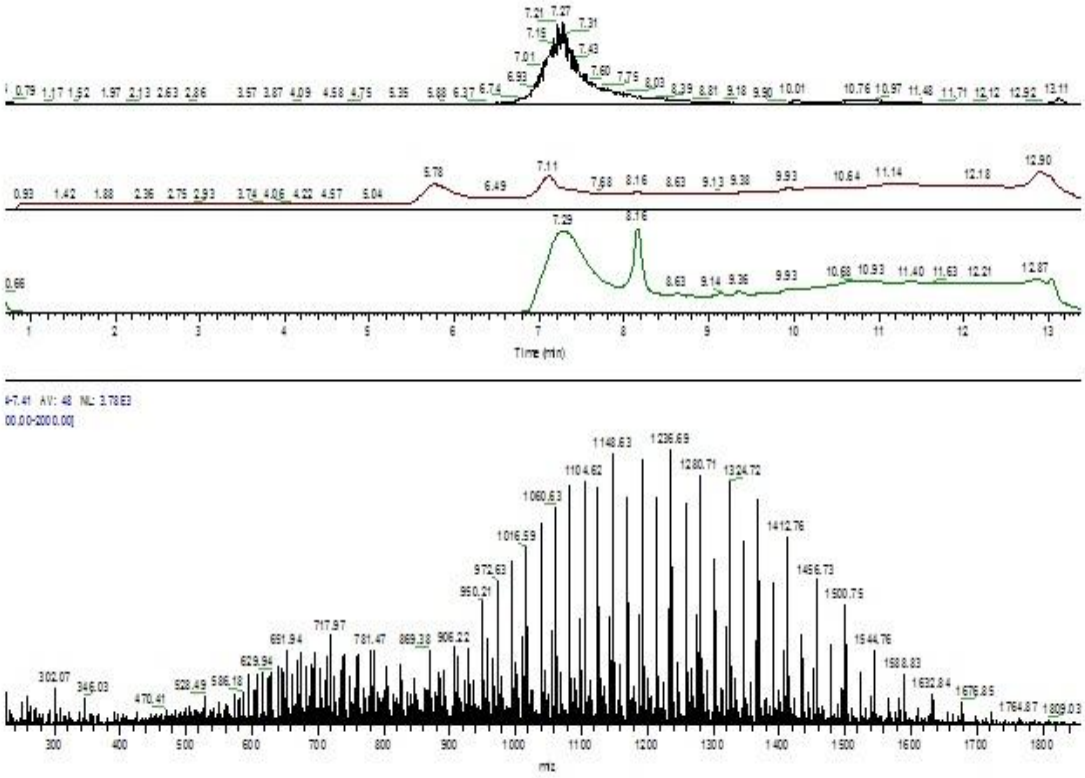
A-3. ^{1}H NMR of CPT-Gly-PEG 1kDa,poly.



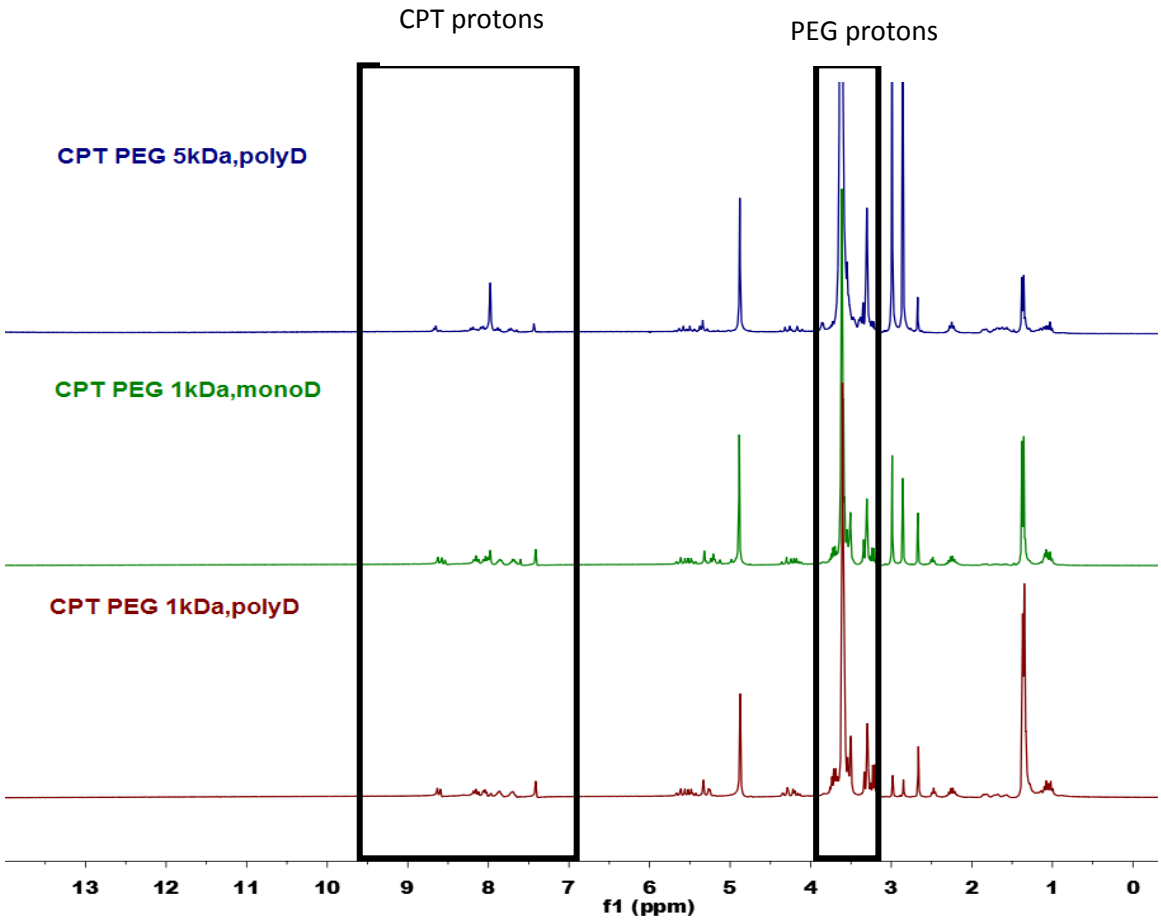


A-4. Mass spectrometry (MS) of CPT-Gly-PEG 1kDa,poly with (M+1) =1416.





A-5. Mass spectrometry (MS) of starting material mPEG-SPA 1kDa,poly .



A-6. Stacked ^{1}H NMR of the three CPT-PEG conjugates. For 7-9ppm, the CPT ring protons are visible while the PEG protons are 3-4ppm. The overlap of the protons in all of the spectra indicate that the CPT-PEG conjugates are all structurally the same.

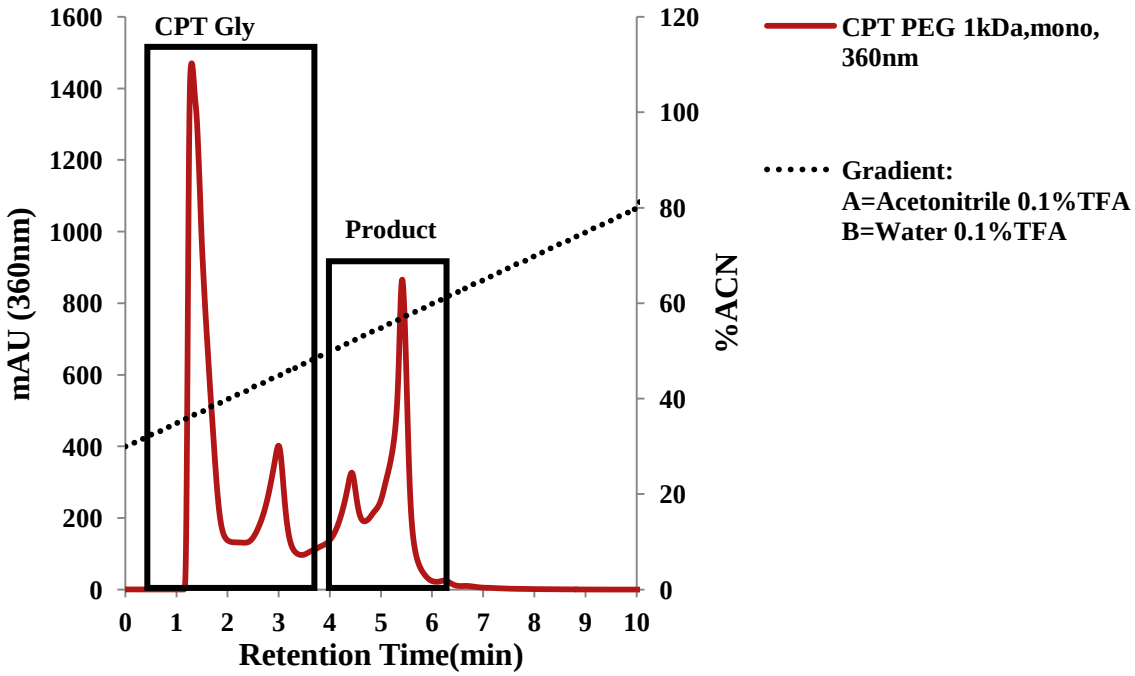
APPENDIX B: HPLC TRACES

HPLC method for CPT-PEG purification:

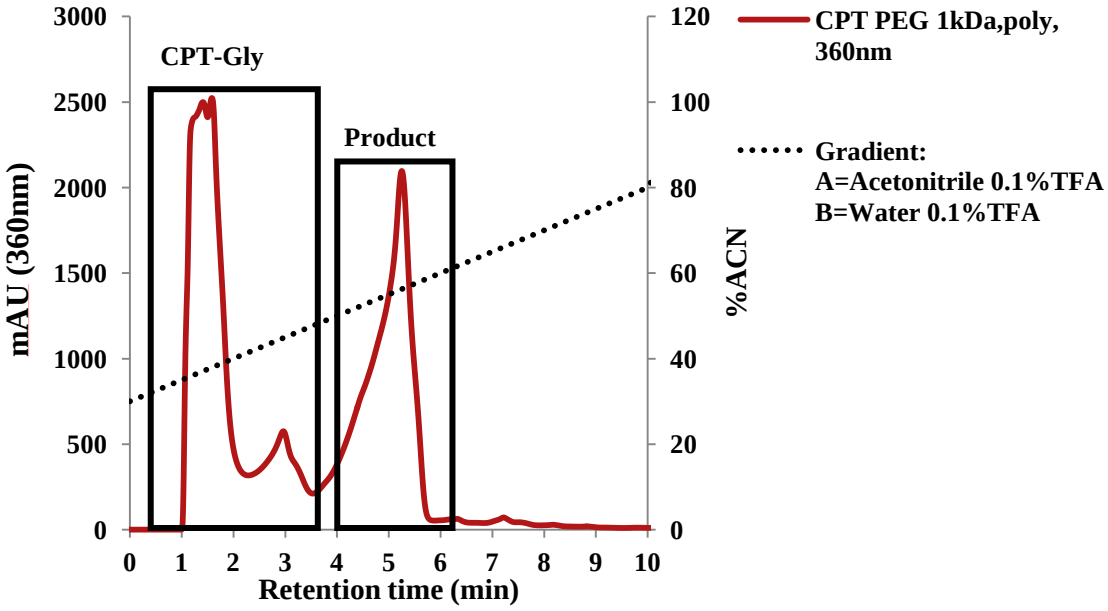
Rate: 1mL/min
30% A to 80%A in 10min

HPLC method for CPT-PEG dissociation analysis:

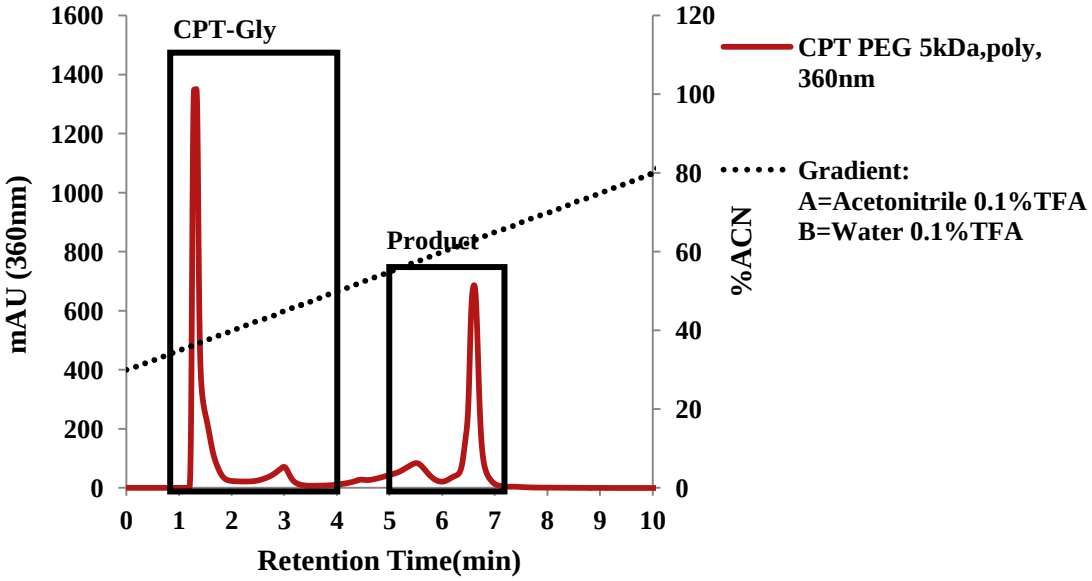
Rate: 1mL/min
30% A to 60%A in 10min



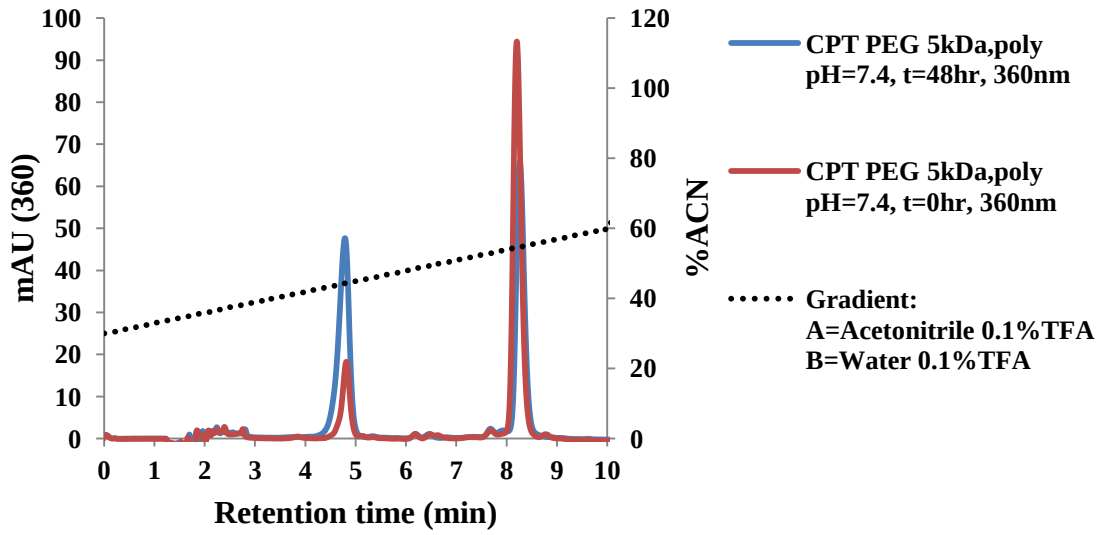
B-1. HPLC purification of CPT-PEG 1kDa,mono at 360nm. The product peak was collected between retention times 4-6min.



B-2. HPLC purification of CPT-PEG 1kDa,poly at 360nm. The product peak was collected between retention times 4-6min.

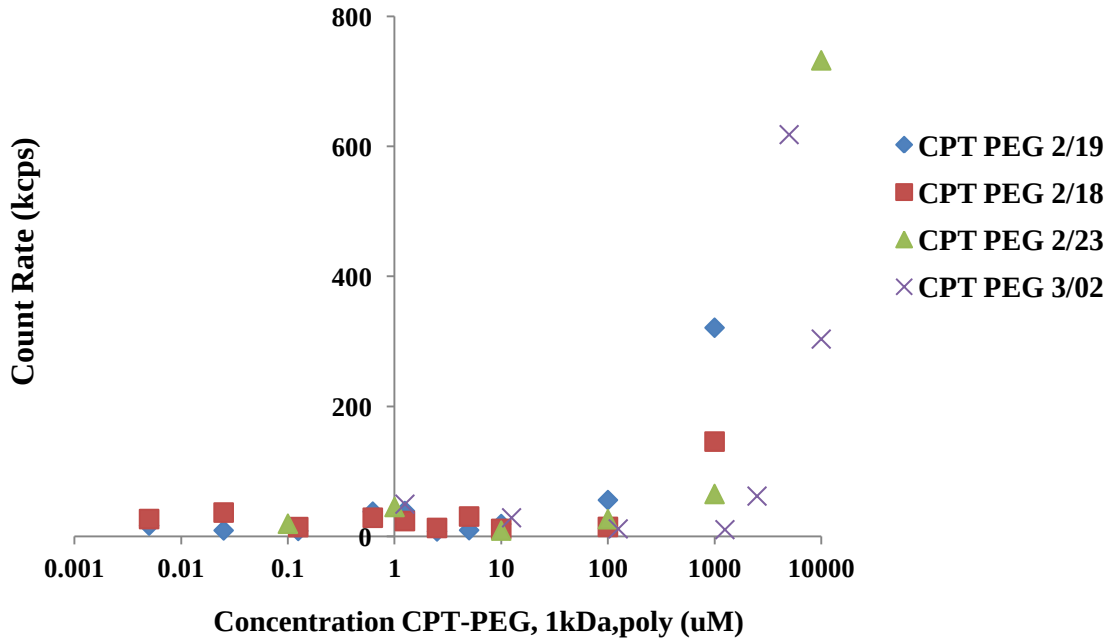


B-3. HPLC purification of CPT-PEG 5kDa,poly at 360nm. The product peak was collected between retention times 5-7min.

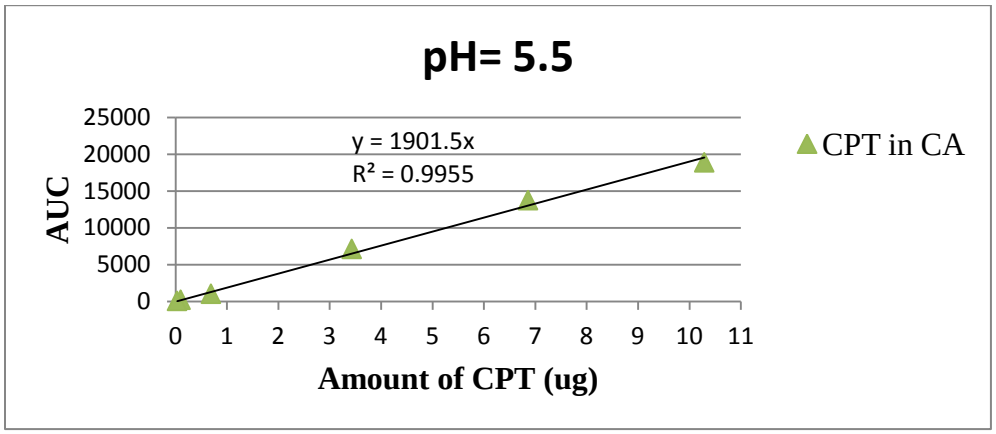


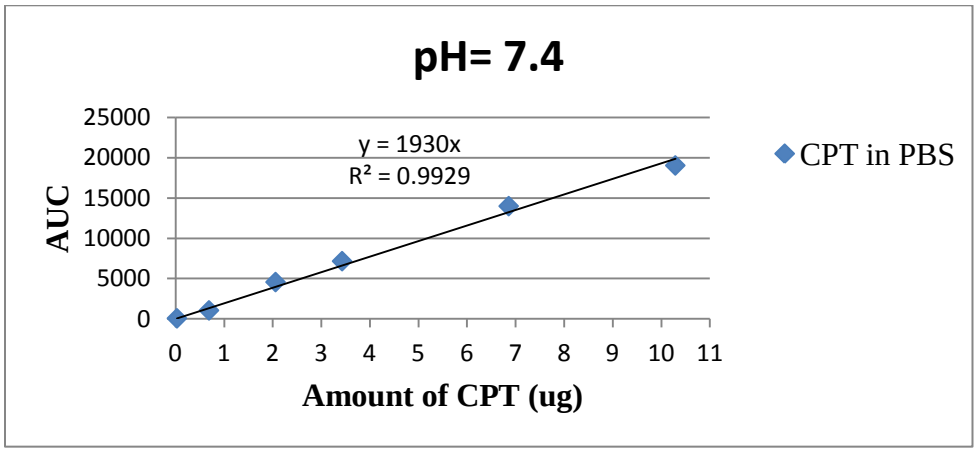
B-4. HPLC at 360nm of CPT-PEG 5kDa,poly at pH 7.4, 37°C. The area under the curve was taken for the peak with a retention time 4-5minutes. With a calibration curve, area under the curve was correlated to ug of product and moles of CPT dissociated.

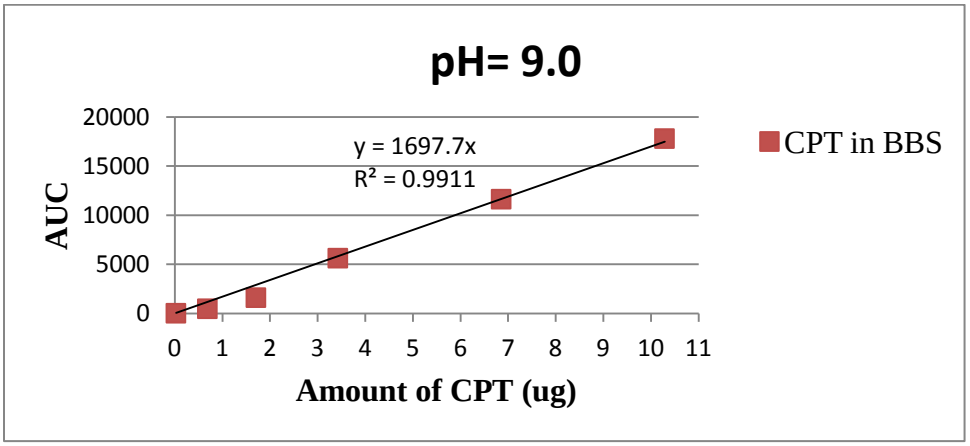
APPENDIX C



C-1. Plot of count rate vs concentration of CPT-PEG 1kDa, poly in uM.







C-2. Plots of AUC and amountof CPT (ug) correlations by pH.

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