Synthesis of a Dual-triggered Polymer-hydrogel System with pH and Near Infrared (NIR) Triggers for Controlled Drug Release

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ABSTRACT

In this study, the effectiveness of a dual trigger system for controlling drug released is investigated. The amphiphilic copolymer poly[(D,L-lactic acid)-co-(glycolic acid)]-poly(ethylene glycol)-poly[(D,L-lactic acid)-co-(glycolic acid)] capped with N-Boc-histidine (his-PLGA-PEG-PLGA-his), and a near infrared (NIR) trigger, Carbon Dot (CD) hydrogel were combined into a dual-triggered polymer-hydrogel system. The his-PLGA-PEG-PLGA-his has shown promising results in the tests, as it was able to effectively retain dye in the absence of the pH trigger (pH neutral), while releasing dye rapidly in the presence of the pH trigger (pH 6.0). In the presence of the NIR laser, the CDs had minimal to no heat emission. The CDs were characterised using Fourier Transform Infrared Spectroscopy (FTIR), while the copolymers were characterised using Proton Nuclear Magnetic Resonance (H-NMR) as well as Fourier Transform Infrared Spectroscopy (FTIR). The FTIR of the CDs showed that CDs were successfully synthesised, while the H-NMR and FTIR of the copolymer (his-PLGA-PEG-PLGA-his) showed that it was successfully synthesised. This study showed that while the copolymer is successful in retaining dye, the CDs are unable to create sufficient heat to cause the hydrogel to melt, and hence the dual-triggered hydrogel system was unable to be synthesised.

1. INTRODUCTION

Despite cancer diagnosis and treatment benchmarking great progress over the last few decades, cancer still remains as one of the leading causes of death each year. (Siegel RL et al., 2015) Cancer is a wildly proliferating and lethal disease, with 18.1 million new cases and 9.6 million deaths in 2018 alone.

The most commonly applied method to treat cancer is chemotherapy. However, patients treated with conventional methods of chemotherapy, using drugs such as Doxorubicin, often suffer from adverse side effects. Despite having treatment coverage for the widest spectrum of

cancers, ranging from lymphoma to thyroid cancer (Arcamone F et al., 1969), its non-specific targeting and high dose requirements harms healthy cells as well. It features apoptosis in cardiomyocytes, resulting in irreversible heart injuries like cardiomyopathy leading to death. (Sudipta Senapati et al., 2018; Zhang, S *et al.,* 2012) Its acute cardiotoxicity therefore greatly reduces the chemotherapeutic efficacy of the drug, often making chemotherapy treatment counterproductive.

Hence, in order to counter the adverse side effects induced by such drugs, yet continue taking full advantage of its chemotherapeutic efficacy, the concept of controlled drug delivery has been increasingly researched. Vehicular drug carriers which passively or actively target cancer cells in a selective drug delivery process were synthesized for controlled drug delivery. The selective drug delivery process is dictated by a "trigger", where the drug is only released into the surrounding environment if a certain stimulus (the "trigger") is present. This concept is otherwise known as triggered drug release. The system potentially reduces adverse side effects, improves drug stability and effectiveness in vivo, and allows for less frequent dosage, which lowers costs and increases patient compliance. It can also prolong the time during which the drug is effective. (Esser-Khan *et al.,* 2011; Maeda, H *et al*., 2000; Koo, H *et al.*, 2011) These novel drug delivery systems are also useful in combating chemoresistance of cancer cells towards conventional chemotherapeutic drugs. (Sudipta Senapati *et al.,* 2018)

While many new advances have been made in the development of new triggered release systems for new stimuli, little research has been done to combine trigger systems to create a double delivery system which only releases a drug in the presence of two stimuli, rather than just one (Esser-Kahn et al., 2011). Yet, dual-triggered release systems are more secure than single trigger systems, and thus promise to better minimize the drug harming healthy cells. (Hong et al., 2014).

Thus, we synthesized a dual-triggered polymer-hydrogel system with pH and Near Infrared (NIR) triggers for controlled drug release. pH acts as a highly effective trigger as both the intracellular and extracellular pH of tumor cells (pH 5.0 and pH 6.0 respectively) is lower than the pH of normal tissue (pH 7.4). (Chang, G et al., 2010) The amphiphilic copolymer PLGA-PEG-PLGA was capped with N-Boc-histidine to form his-PLGA-PEG-PLGA-his

(copolymer) as the pH trigger. The copolymer self-assembles into micelles in water, and the drug can be loaded into the micelle. The acid dissassociable N-boc-histidine caps allows the release of the drug by the copolymer at acidic pH. (Chang, G et al., 2010) According to past in vivo and in vitro studies, his-PLGA-PEG-PLGA-his is also biodegradable and biocompatible, proving to be an ideal pH trigger for the drug delivery system. (Chen et al., 2017)

NIR is another potential trigger as it allows external human control of the drug release. 808nm NIR can penetrate human body tissue very well without harmful side effects caused by higher energy radiation, making it particularly useful for cancer treatment. (Bao et al., 2018) By coupling the NIR trigger with a photothermal agent, substances capable of converting electromagnetic radiation to heat, which in this study is Carbon Dots (CD), a low melting point hydrogel system for controlled drug delivery was synthesised. CDs theoretically has good NIR photothermal conversion, causing the temperature of water containing 0.05 mg/mL CDs to increase by ~ 20 °C within 5 min when exposed to 200mW, 808nm NIR irradiation. (Hui Wang et al., 2017) Past cytotoxicity and in vivo studies have also proven the non-toxicity and high biocompatibility of CDs compared to other photothermal agents. (Zhu Lian Wu et al., 2017) By suspending CDs in the hydrogel system, when NIR irradiation is shone on it, the CDs will convert the NIR to heat and melt the agarose hydrogel at 60 ℃, hence releasing the drug to the desired cancer site.

We aim to combine the copolymer and CD hydrogel to synthesize an integrated dual-triggered polymer-hydrogel system for controlled drug release. However, as the drug Doxorubicin cannot be used for trials, a model dye Reactive Blue 19 was used instead (Refer to appendix 2A). The dye will be loaded into the his-PLGA-PEG-PLGA-his copolymer to form a dye loaded micelle which will then be suspended within the CD hydrogel, forming the dual-triggered drug delivery system. Hence, only when the pH and NIR stimulus are both present, then can the drug be released into the body.

1.1 Objectives

We aim to synthesize the copolymer, and CD hydrogel, thereafter creating the dual-triggered polymer-hydrogel system. We would then investigate the effect of the presence of an acidic pH trigger on the dye release of the copolymer system and the effect of the presence of an NIR

trigger on the temperature change of the CD hydrogel system. Lastly, we aim to investigate and compare their control over dye release with and without pH and NIR trigger, and with both triggers.

1.2 Hypothesis

We hypothesize that the copolymer will minimise dye release when the acidic pH trigger is absent, and release dye when the acidic pH trigger is present. The CD hydrogel will heat up to a temperature adequate enough to melt the agarose hydrogel (60 ℃). The dual triggered polymer-hydrogel system will release dye if and only if both triggers are present, and will be just as effective as the individual trigger systems at releasing dye when all its triggers are present.

2. MATERIALS AND METHODS

2.1 Materials

L-Cysteine, Amylodextrin, Agarose, PLGA-PEG-PLGA, Boc-His-OH, Tert-butyl methyl ether, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC HCl), 4-dimethylaminopyridine (DMAP), and Reactive Blue 19 was procured from Sigma Aldrich.

Citric acid, Hydrochloric acid, and ethyl acetate were obtained from GCE Chemicals.

2.2 Synthesis of Carbon Dot (CD) Hydrogel

0.19 g citric acid, 0.11 g L-cysteine and 0.30 g dextrin are dissolved in 15 mL ultra-pure water. The mixture solution was then heated in a domestic microwave oven (power 800 W) for 3 min where the colour of the solution changes from white to brown. After cooling to room temperature, the crude product was mixed with 5 ml ultra-pure water. The large particles were removed from the mixture by filtering it through a 0.22 μm membrane filter. The filtered solution was further purified by dialysis in deionized water for two days. A clear and yellow aqueous solution was lyophilized to obtain the dry CDs product. The CDs were characterized with FTIR. The CDs were then mixed with a low melting point agarose at 60°C in water with concentration of 2.6667mg/mL of Carbon Dots and 1% concentration of agarose. The mixture is heated in a microwave at 800W power. The mixture was then cooled in the refrigerator at 4℃ to form the CD hydrogel matrix.

2.3 Synthesis of Dye Loaded PLGA-PEG-PLGA Micelles

1g of PLGA-PEG-PLGA, 0.22g of N-boc-histidine, 0.23g of EDC HCl, 0.173g of DMAP, and 8mL of ethyl acetate was placed into a two-necked round bottom flask. The reaction was allowed to occur under a nitrogen atmosphere for 48 hours at room temperature. The solvent (ethyl acetate) is partially evaporated and any polymers are precipitated using tert-butyl methyl ether and dissolved again in 1M hydrochloric acid. The polymer was re-precipitated in water at 80°C to rinse leftover EDC·HCL, DMAP, and N-boc-histidine that was not used in the reaction. The mixture was freeze dried for 48 hours (Chang, Ding, Li, & Lu, 2010) and the resulting copolymer was characterized using FTIR and H-NMR. The copolymer was dissolved into 2mL of ethyl acetate at 5mg/mL. The Reactive Blue 19 dye was then added into the solution. The solvent is evaporated using SpeedVac Concentrator and Venturi pump. The resulting thin film was dispersed with 1mL of water followed by 2h of stirring and 15 min of sonication. The resulting micelles were passed through a syringe filter (0.45 micrometers) and put into a dialysis bag (3500 Mw) to dialyze against pure water overnight to remove any free dye.

2.4 Dye Release Studies

2.4.1 Investigation of pH triggered drug release system in controlling drug release

0.5 mL of copolymer was suspended in an aqueous solution at a concentration of 1mg/mL in the Pur-A-Lyzer. Another 0.25 mL of PBS was added into the Pur-A-Lyzer. The Pur-A-Lyzer with the system were placed into 10.5ml of PBS buffer (pH 7.4). 5 replicates with exposure to no triggers and 5 replicates with exposure to the pH trigger were prepared. The replicates were put into an orbital shaker at 200 rpm. If the acidic pH trigger is needed, the PBS added into the Pur-A-Lyzer should be of pH 6.0. There will be 6 intervals of 10 minutes, where 9mL of the buffer must be removed and immediately replaced with the same amount of fresh buffer after the end of every interval. The concentration of the dye inside the sampled buffer solution was then measured using a UV-Vis spectrophotometer. The percentage dye released will be calculated with the formula below.

$R(\%)=100 \times \frac{C_f}{C_i}$ C_i = initial concentration mg/l; C_f = concentration released mg/l

2.4.2 Investigation of NIR trigger on the change in temperature of the NIR triggered system (CD hydrogel).

4 ml of liquid CD hydrogel was placed into a glass container and allowed to cool at room temperature. A heat-sensing data-logger probe was inserted into the CD hydrogel. 5 replicates with and without exposure to NIR trigger were prepared respectively. If NIR trigger is needed, a laser of 15 mW was used to shine light with a wavelength of 808nm onto the hydrogel. (This is 185 mW less than the originally required power due to laboratory restrictions) There will be 6 intervals of 10 minutes, where the temperature of the CD hydrogel at every interval will be recorded.

2.4.3 Investigation of dual-triggered polymer-hydrogel system in controlling drug release

CD hydrogel and dye-loaded copolymer micelles of a concentration of 1mg/10mL each are mixed in an aqueous solution at 60℃. 0.5mL of the hydrogel-polymer mixture and 0.25 mL of PBS is dropped into the Pur-A-Lyzer and cooled in a refrigerator at 4°C to form the polymer-hydrogel system. The Pur-A-Lyzer with the system are placed into 3.5 mL of PBS buffer (pH 7.4). 5 replicates with no exposure to either triggers, 5 replicates with exposure to only the NIR trigger, 5 replicates with exposure to only the pH trigger, and 5 replicates with exposure to both triggers are prepared. Procedures for activating triggers and obtaining results are the same as mentioned above.

3. RESULTS AND DISCUSSIONS

3.1 Characterisation of Carbon Dots (CD)

3.1.1 By FTIR

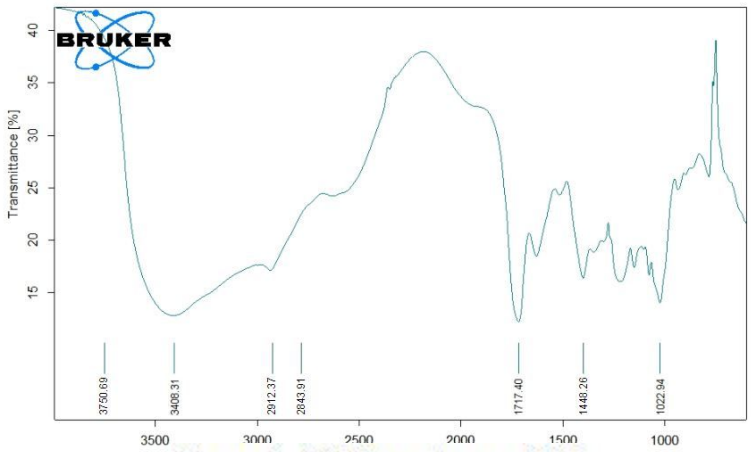

Figure 1: FTIR spectrum of CDs

The FTIR spectrum reflects peaks at wavelength 1022.94 cm^{-1}, representing the ether C-O-C stretch; 1448.26 cm^{-1}, representing the aromatic C=C stretch; 1717.40 cm^{-1}, representing the carboxylic group C=O stretch; 2912.37 and 2843.91 cm^{-1}, representing alkane C-H stretch; and 3408.31 cm^{-1}

representing the carboxylic group O-H bond stretch. These bond stretches are all characteristic of CDs (Demchenko *et al.,* 2013; Thakur, M *et al.,* 2014).

3.2 Characterisation of n-boc histidine capped PLGA-PEG-PLGA micelles

3.2.1 By FTIR

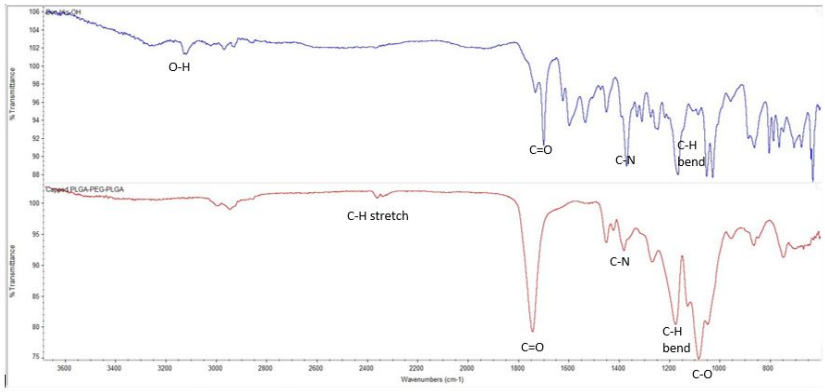

Figure 2: FTIR spectrum of n-boc histidine capped PLGA-PEG-PLGA

The FTIR spectrum reflects peaks at wavelength 1083 cm^{-1}, representing the ether C-O-C symmetric stretching; 1276 cm^{-1}, representing the C-H bend; 1374 cm^{-1}, representing the amine group C-N stretch; 1752 cm^{-1}, representing ketone C=O bond stretch; 2389 cm^{-1} representing the C-H bond stretch; and 3124 cm^{-1} representing carboxylic O-H stretch. These bond stretches are all characteristic of PLGA-PEG-PLGA (Chang, G *et al.,* 2010). In addition, the presence of amine group C-N stretching and carboxylic group C=O and O-H stretches in the copolymer which are characteristic of n-boc-histidine suggests the successful conjugation of n-boc-histidine onto PLGA-PEG-PLGA.

3.2.2 By H-NMR

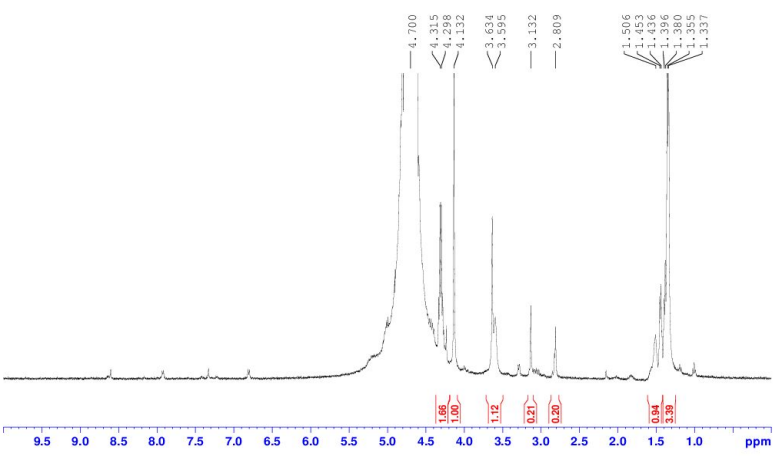

Figure 3: NMR spectrum of his-PLGA-PEG-PLGA-his

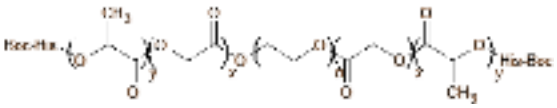

Figure 4: Structural Formula of his-PLGA-PEG-PLGA-his

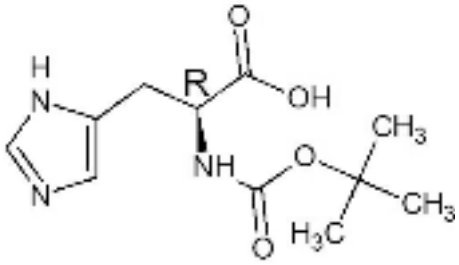

Figure 5: Structure of N-Boc-Histidine

The NMR spectrum of the copolymer reflects chemical shift values (δ ppm) at 4.700 ppm, reflecting a vinylic proton (H is attached to alkene C). The δ value at 1.506 shows the alkyl (methine) proton in the PLGA; In addition, the δ values ranging from 1.453 ppm to 1.337 ppm suggests the existence of alkyl (methylene) proton within the n-boc-histidine, affirming the successful synthesis of n-boc-histidine capped PLGA-PEG-PLGA copolymer (Chang, G *et al.,* 2010).

3.3 Dye Release Studies

3.3.1 Investigation of pH triggered drug release system in controlling drug release

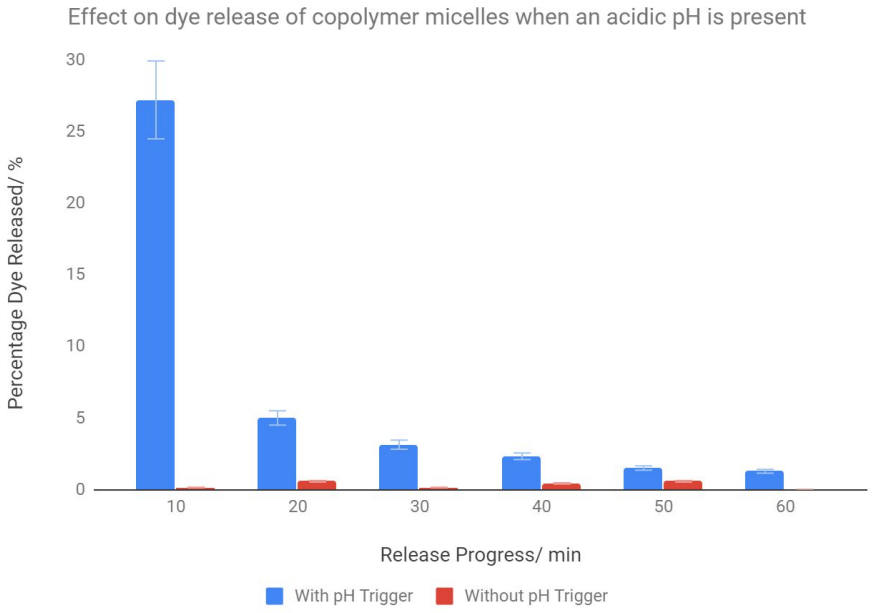

Figure 6: Effect of acidic pH on dye release of copolymer micelles

Figure 6 shows the effect on dye release of the copolymers with and without the presence of the pH trigger. The general trend observed is that the percentage dye released decreases as the experiment progresses (Figure 6), and this could be due to most of the dye being released in the first interval, thus resulting in lesser dye being released in subsequent intervals. The release of dye under the presence of an acidic pH trigger is higher compared to without the trigger. The total percentage dye release when acidic pH trigger is present is 40.7%. This is likely due to the protonation of the imidazole group in N-boc-histidine which causes the copolymer to become more hydrophilic. The protonated copolymer chains thus dissolve into the solution, resulting in the remaining fewer copolymer chains reassembling, shrinking the copolymer. This would force more dye to be released (Chang, G *et al.,* 2010).

The percentage dye release with and without the acidic pH trigger were analysed using Mann

Whitney Test. P values obtained reveal that the difference in the percentage dye release is significant at the 10, 20, 30, 40 and 60 minute interval.

Release Progress/ min	P value	Inference
10	0.0122	Significant difference
20	0.0122	Significant difference
30	0.0367	Significant difference
40	0.0216	Significant difference
50	0.1437	Insignificant difference
60	0.0472	Significant difference

Table 1: Mann Whitney analysis (with and without acidic pH trigger)

As there is an overall significantly higher dye release by the copolymer when acidic pH is present, it suggests that the copolymer is indeed effective at releasing dye when an acidic pH trigger is present. It is also thus capable of withholding dye effectively when acidic pH is absent.

3.3.2 Investigation of NIR trigger on the change in temperature of the NIR triggered system (CD hydrogel)

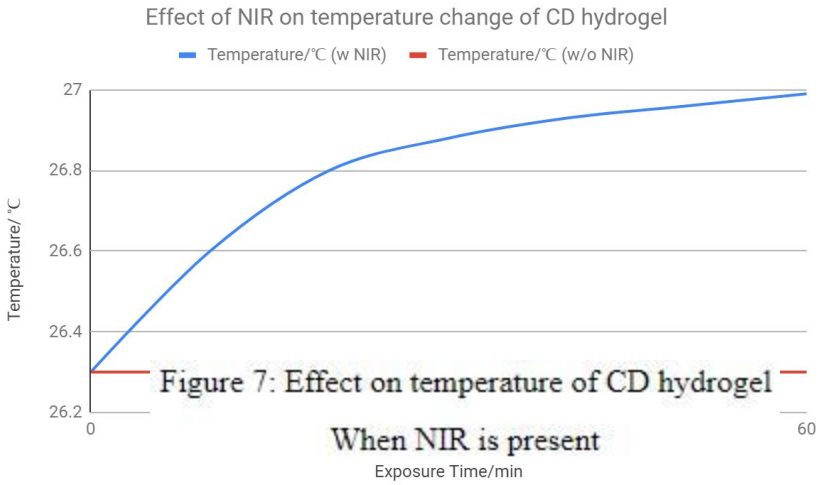

Figure 7: Effect on temperature of CD hydrogel

It can be observed from figure 7 that the temperature of the CD hydrogel increases slightly under the presence of NIR. The initial increase in temperature is the highest, suggested by a steep gradient when exposure time = 0 min. The gradient of the curve gradually decreases, eventually plateauing at a maximum of 27.0 °C. Hence in 60 min, the total temperature increase averages at 0.7°C when a 15mW laser of wavelength 808nm is shone on it. This is a huge disparity from the originally expected 33.7°C increase.

This issue could first be attributed to limitations in the experimental set-up. Due to strict laboratory restrictions, the NIR irradiation available was limited to 15 mW, 185 mW less than the originally required 200 mW. Furthermore, the main absorption bands of CDs are typically in the ultraviolet (UV)-to-green region of the spectrum (Bao, X *et al.,* 2018). As a result, the estimated photothermal conversion efficiency of CDs under NIR is a low 38.7%, (Zheng, M *et al.,* 2016), and thus the power of heat emission is, theoretically, a miniscule 5.805 mW. Coupled with natural energy loss, the heat transferred into the agarose hydrogel is very limited, and hence the temperature increase is impeded.

As the temperature increase is unable to rise to 60.0 ℃, the melting point of the agarose hydrogel, the hydrogel system will not melt to release the suspended dye or copolymer micelles. The NIR trigger is thus incapable of functioning and releasing dye when the NIR trigger is present.

3.3.3 Investigation of dual-triggered polymer-hydrogel system in controlling drug release.

As the CD hydrogel was not capable of being fully optimized and utilised as an NIR trigger as of present, the dual-triggered polymer-hydrogel system cannot be brought to fruition. Tests for the dual-triggered system were thus incapable of being carried out.

4. CONCLUSION & FUTURE WORK

The CDs and copolymer were successfully synthesized. The copolymer micelles were able to retain dye substantially when an acidic pH trigger is absent. When the acidic pH is present, the copolymers were effective at releasing dye with a maximum percentage dye release of 40.7%. However, the CD hydrogel was unsuccessful in heating up under the presence of an NIR trigger. The maximum temperature reached by the CD hydrogel under the NIR trigger is 27.0 ℃. As the maximum temperature reached was not high enough to melt the hydrogel (60 ℃), the dual-triggered system cannot be synthesized and tested. In the future, the CD hydrogel will be replaced with making the copolymer micelle itself responsive to heat, or in other words thermosensitive, for the NIR trigger. The NIR trigger will thus be optimised and the dual-triggered polymer-hydrogel system will be synthesized and tested. The biostability of the dual-triggered system in the human body will also be investigated.

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Appendix:

1A. Calibration Curve of Reactive Blue 19

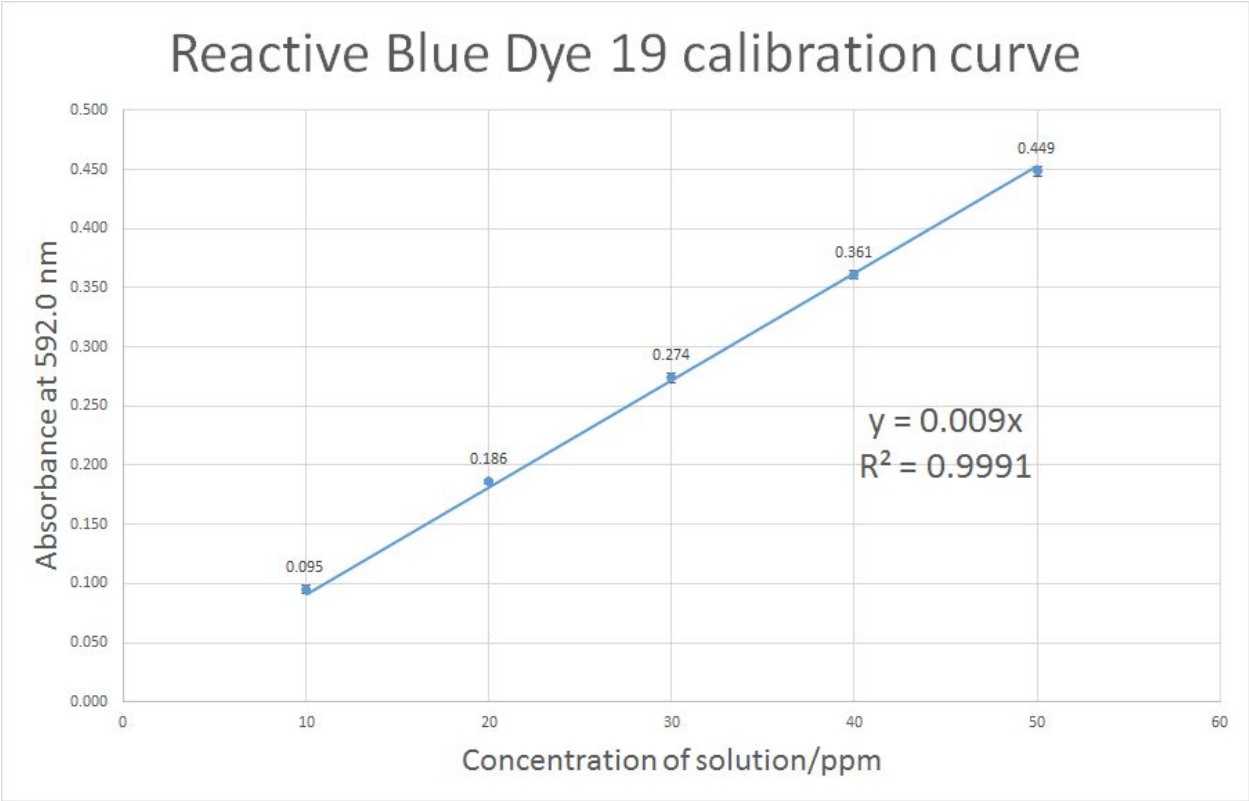

Figure 8: Calibration Curve of Reactive Blue 19

Using the Beer-Lambert Law, the calibration curve for Receive Blue 19 dye in PBS is plotted in Figure 3 as shown.

The Beer-Lambert Law relates the radiant power in a beam of electromagnetic radiation at a suitable wavelength to the length of the path of the beam in absorbing medium and to the concentration of the absorbing species. It assumes that the concentration of the sample is proportional to the light absorbed, wherein the R2 parameter indicates the goodness of the fit. As the calibration curve has a R2 > 0.99, it indicates a good fit.

2A. Reactive Blue 19 as a feasible replacement for doxorubicin

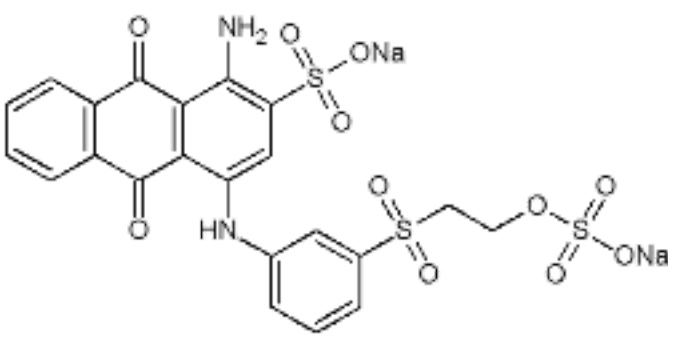

Figure 9: Structural Formula of Reactive Blue 19

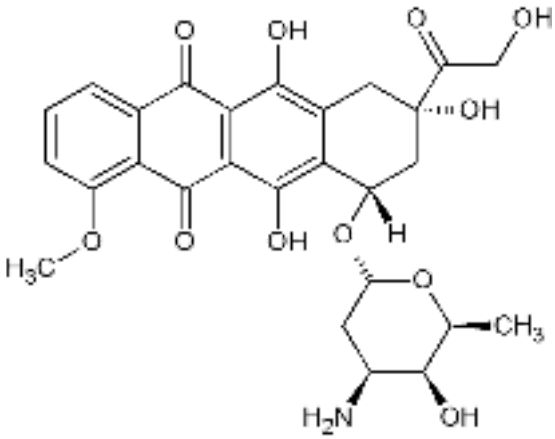

Figure 10: Structural Formula of DOX

As doxorubicin cannot be used in the studies, reactive blue 19 will be used to model doxorubicin. This dye has a sodium sulfonate group (Na_2SO_4), allowing it to be soluble and thus feasible in our investigative model. Furthermore, it has an anthraquinone functional group which doxorubicin also shares, expressing their similarity in structure and dye properties. Reactive Blue 19 is hence a feasible model for the drug.

3A. Formula of Condensation reaction between N-Boc-Histidine and PLGA-PEG-PLGA

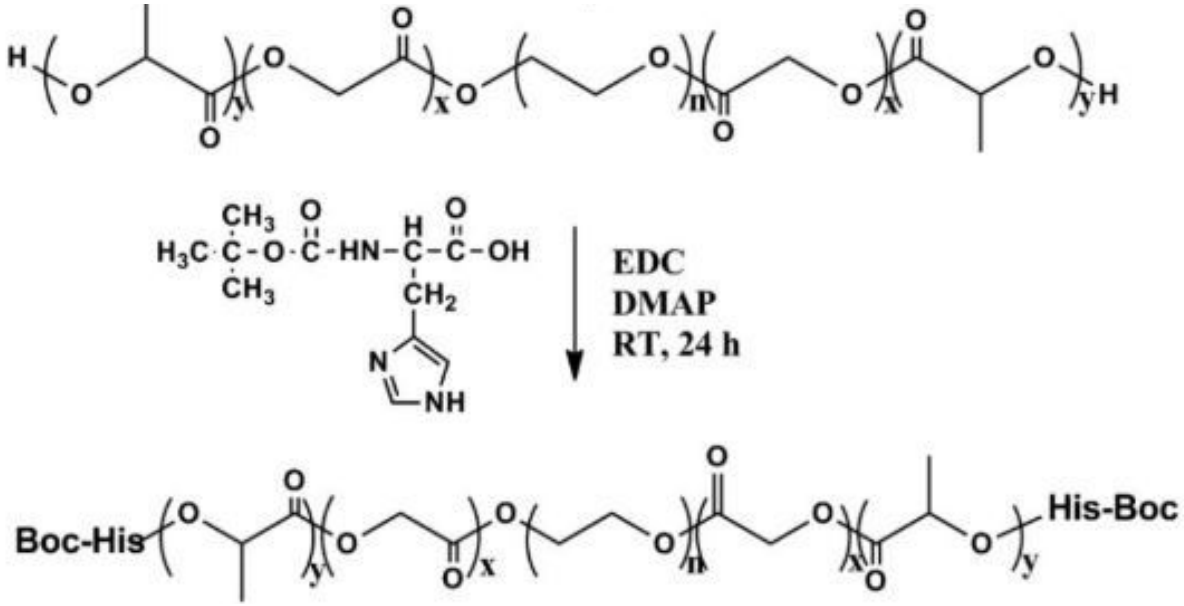

Figure 11: Visual representation of the condensation reaction between N-boc-histidine and PLGA-PEG-PLGA

The figure above illustrates how the PLGA-PEG-PLGA polymer reacts with the n-boc histidine to form the n-boc-histidine capped PLGA-PEG-PLGA copolymer. It involves the vigorous mixing of the 2 chemicals with DMAP acting as a catalyst. It is a condensation reaction where the H of the n-boc-histidine reacts with the OH of the PLGA-PEG-PLGA to form a new C-O single bond between the chemicals. In the process, H_2O is released.

4A. Effect of UV light trigger on the CD hydrogel

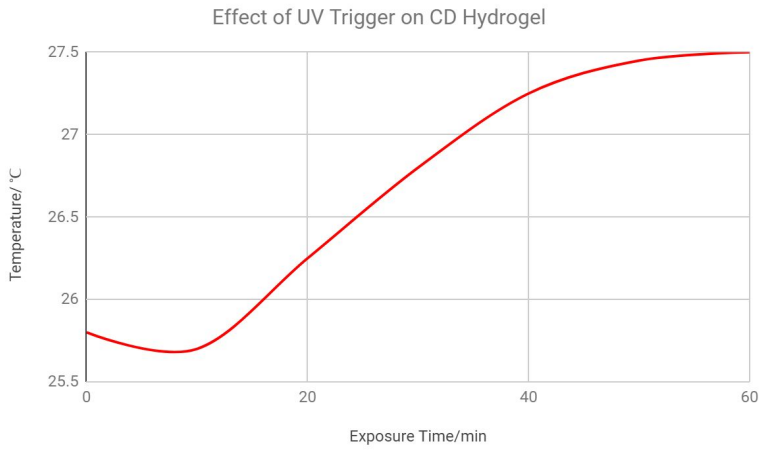

Figure 12: Effect of UV light trigger on CD hydrogel

With the understanding that the main radiation absorption bands of the CDs are in the UV range (Bao, X *et al.,* 2018), a test was conducted to observe the effect of UV on CDs. From figure 10, it can be seen that the UV enabled a total temperature increase of 2.25 ℃, wherein the maximum temperature reached was 27.5 ℃. This performance was slightly better compared to the when NIR was used. This reveals that UV light does indeed illicit a stronger response from the CDs, and that the main absorption bands of the CDs are in the UV range.