PHOTOTHERMAL THERAPY USING AN INTRACELLULAR PHOTOSENSITIZER: CROCONAINE DYE

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ANNEXES

ANNEX I

Croconaine synthesis

An ice-salt water bath at -5°C was prepared for the 5 min stirring of the 2,3,3-Trimethylindolenine (2.0 mL, 12.5 mmol). Then, 1.09 g (13 mmol) of $NaNO_3$ were added at once and 31 mL (591 mmol) of H_2SO_4 were added drop wise for 10 min, watching over the temperature. The mixture was left stirring for 1h in the same cold bath (-5°C). It was then neutralized with 45 g of NaOH (approximately) adding it slowly along some distilled water checking the pH of the mixture. The crude formed was cooled and dissolved in 50 mL of EtOAc. The biphasic solution was separated, and the organic layer was washed three times with 40mL of distilled water. The final product was dried with $MgSO_4$, filtered and concentrated under reduced pressure.

This final product (205.3 mg, 1 mmol) and croconic acid (69.5 mg, 0.49 mmol) were mixed and dissolved in 8 mL of 1-Butanol and 8 mL of Toluene. The solution was heated at 95°C to reflux for 18 h. The mixture was then cooled at -23°C for 2h. The precipitated was filtered and washed with ether three times and finally dried under reduced pressure until the solvents were removed. A dark green-grey solid was obtained [16] (150 mg, 58% efficiency). H NMR (300MHz, $CDCl_3$): 8.19-8.3 (m,2H), 8.08-8.17 (m, 2H), 7.3-7.42 (m, 2H), 6.0-6.27 (m, 2H), 1.53-1.55 (m, 12H) ppm.

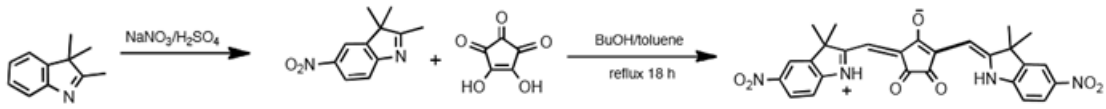

Figure 32. Synthesis scheme of croconaine [16]

Synthesis of 5-(tert-Butyl)isophthaloyl dichloride

5-tert-butylisophthalic acid (10 mg, 56 mmol) was dissolved in 40 mL of benzene and a single drop of DMF. 30 mL of $SOCl_2$ were carefully added in the mixture placed in an ice bath (0°C). The mixture was then refluxed for 5h in an oil bath at 70°C. The $SOCl_2$ was removed by distillation, and the remaining benzene was evaporated, obtaining a white solid [36] (11 g). ^1H NMR (300 MHz, $CDCl_3$): 8.63 (s, 1H), 8.34 (s, 2H), 1.34 (s, 9H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): 167, 154, 134.2, 134, 131.5, 35.5, 31 ppm.

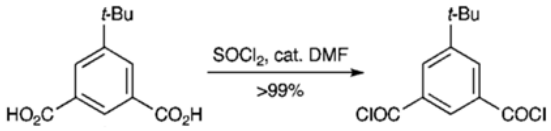

Figure 33. Synthesis scheme of 5-(tert-butyl)isophthaloyl dichloride [36]

9,10-bis(aminomethyl)anthracene synthesis

Anthracene (5 g, 28 mmol) was dissolved in 25 mL of glacial acetic acid and 100 mL of hydrobromic acid. 1,3,5-trioxane (5 g, 56 mmol) and TTA (0.2 g) were added and stirred at 75°C under a reflux condenser sealed with an argon balloon for 24h. The mixture was then cooled, filtered and washed with water. The crude was dried and dissolved with toluene in a 70°C bath, and then recrystallized in an ice bath. Three more washes were needed with hot ethanol at 60°C to remove the remaining anthracene. A green-yellow 9,10-bis(bromomethyl)anthracene solid was obtained [37] (1.3g, 60% efficiency). ^{1}H NMR (300MHz, $CDCl_3$): 8.4 (dd, 4H), 7.7 (dd, 4H), 5.53 (s,4H); C NMR (75MHz, $CDCl_3$): 130.2, 129.6, 126.7, 124.4, 26.6.

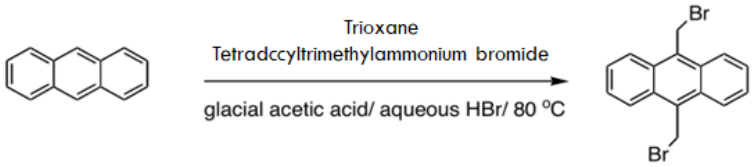

Figure 34. Synthesis scheme of 9,10-bis(bromomethyl)anthracene [37]

Hexamethylenetetramine (0.43 g, 3.04 mmol) was dissolved in 100 mL of Chloroform. Then 9,10-bis(bromomethyl)anthracene (0.5 g, 1.37 mmol) was added and heated at 50°C to reflux overnight, sealed with an argon balloon. The mixture was allowed to cool down to precipitate, then filtered and washed with chloroform. The dried solid was dissolved with a mixture of ethanol and HCl (80mL ethanol and 10 mL pure HCl) and heated at 70°C to vigorously reflux for 48h, sealed again with an argon balloon. The mixture was cooled in an ice bath at 0°C and the precipitate formed was filtered, washed with cold ethanol and dried in the open air. The crude was dispersed and stirred into 50 mL of a 10% Na_2CO_3 solution. 20 mL of chloroform were added into the solution and all of it separated by liquid-liquid extraction, washing three times the aqueous layer with 30 mL of chloroform to extract the product. The final chloroform solution was dried with $MgSO_4$, filtered and dried under high vacuum, obtaining a yellow solid of 9,10-bis(aminomethyl)anthracene [38] (117mg, 50% efficiency). ^{1}H NMR (300MHz, $CDCl_3$): 8.34 (dd, 4H), 7.49 (dd, 4H), 4.76 (s, 4H).

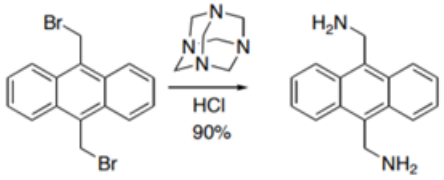

Figure 35. Synthesis scheme of 9,10-bis(aminomethyl)anthracene [38]

Croconaine-macrocycle synthesis

Briefly, croconaine (24.35 mg, 0.16 mmol) was dissolved in 20 mL of chloroform and mixed with 5-(tert-Butyl)isophthaloyl dichloride (128.35 mg, 0.64 mmol) dissolved in 40 mL of chloroform and 9,10-bis(aminomethyl)anthracene (101.95 mg, 0.64 mmol), dissolved in 40 mL of chloroform and 1 mL of triethylamine, using a mechanical syringe pump for 4 hours. The mixture was then left stirring overnight and concentrated under reduced pressure. The final crude product obtained was then purified via silica gel column chromatography. In order to find the adequate concentration of eluent (acetone and chloroform) and find out the polarity of the precursors to separate them, thin-layer chromatography was performed. Two columns were needed, a first one using a solution of chloroform and acetone (98% - 2%), and a second one using chloroform (100%)[16] (18mg, 10% efficiency). ^{1}H NMR (300MHz, $CDCl_3$): 9.32-9.51 (m, 2H), 8.44-8.55 (m, 4H), 8.34-8.39 (m, 2H), 8.15-8.2 (d, 2H), 7.74-8.03 (m, 8H), 7.58-7.72 (m, 4H), 7.08-7.13 (m, 2H), 6.52-7.06 (m, 8H), 5.1-5.39 (m, 8H), 4.55-4.99 (m, 2H), 1.44-1.73 (m, 18H), 0.73-0.83 (m, 12H).

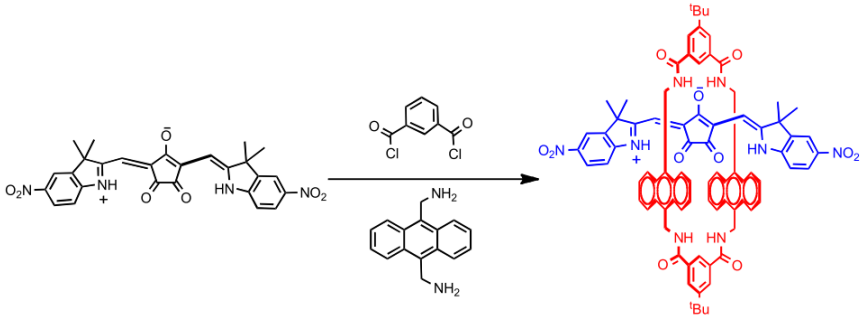

Figure 36. Synthesis scheme of croconaine-macrocycle [16]

ANNEX II

Croconaine:

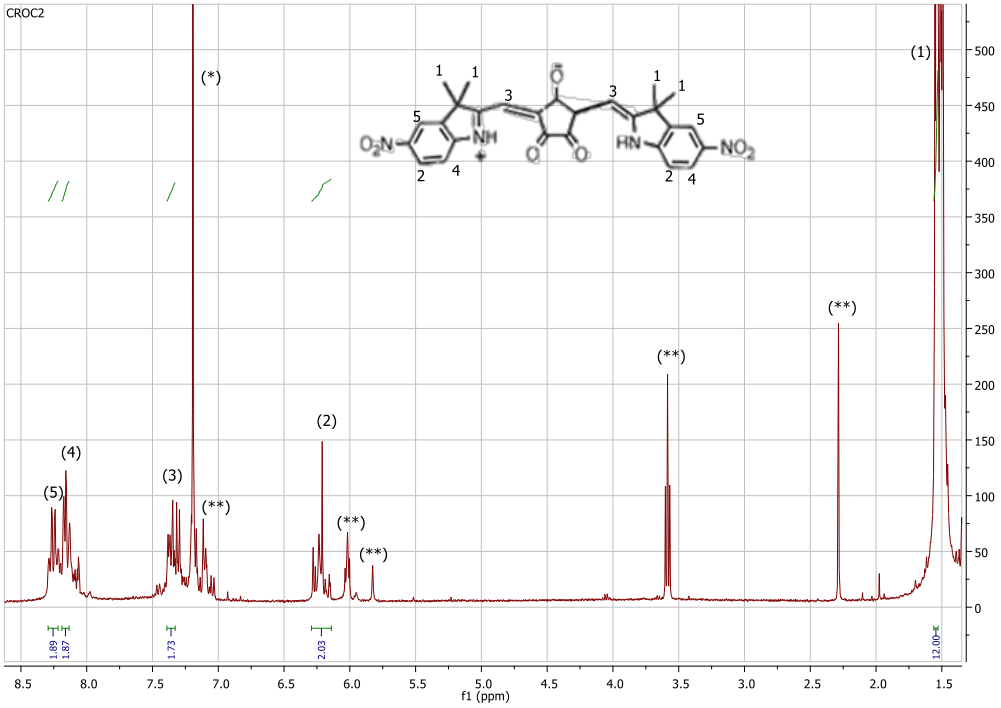

Figure 37. ^{1}H NMR spectrum of croconaine in $CDCl_3$. (*) Is the solvent $CDCl_3$ (7.2 ppm). (**) Is the remaining reagent toluene (2.3, 7.02 ppm), 1-butanol (3.58 ppm) and other contaminants (6, 5.82 ppm).

5-(tert-Butyl)isophthaloyl dichloride:

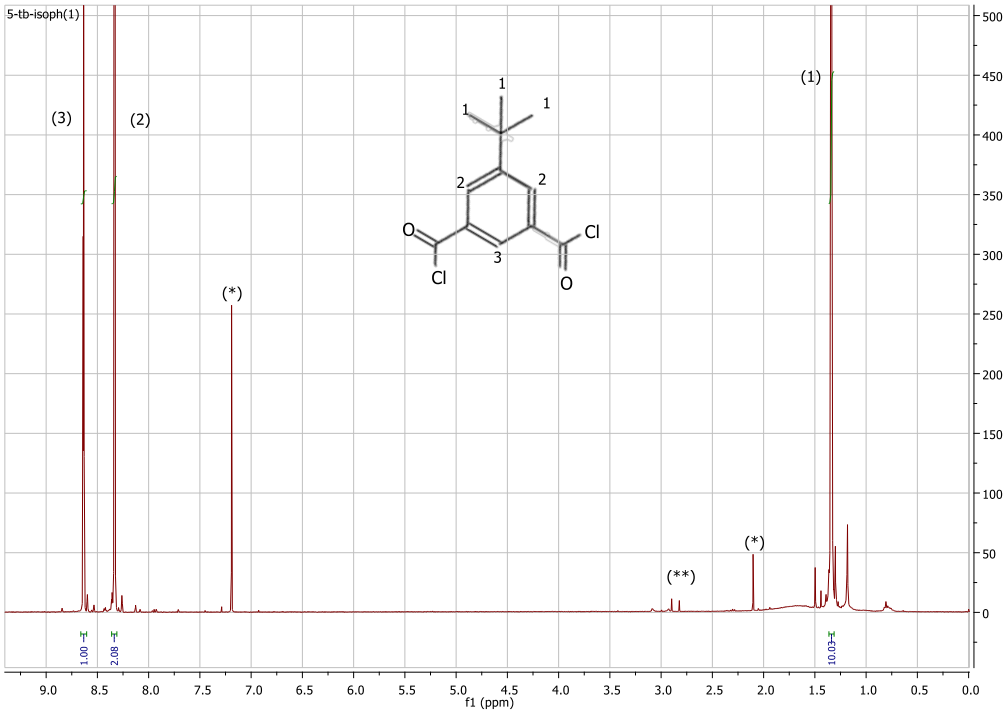

Figure 38. ^{1}H NMR spectrum of 5-(tert-butyl)isophthaloyl dichloride in $CDCl_3$. (*) Are solvents such as $CDCl_3$ (7.2 ppm) and acetone-D (2 ppm). (**) Is remaining reagent DMF (2.9 ppm).

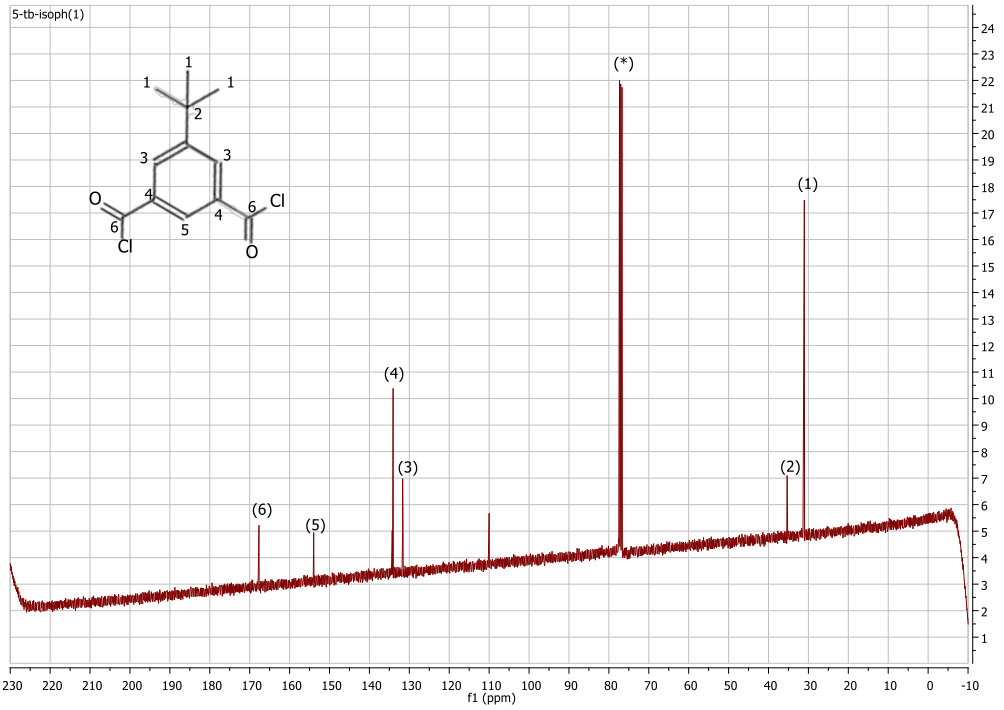

Figure 39. ^{13}C NMR spectrum of 5-(tert-butyl)isophthaloyl dichloride in $CDCl_3$. (*) Is $CDCl_3$ (77 ppm).

9,10-bis(bromomethyl)anthracene:

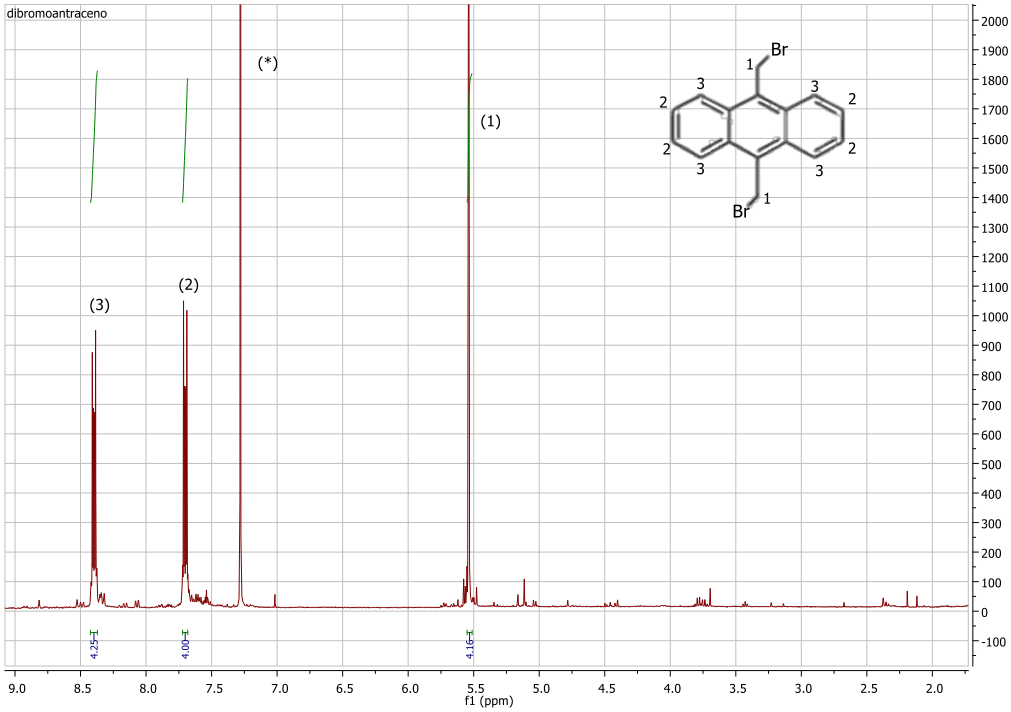

Figure 40. ^{1}H NMR spectrum of 9,10-bis(bromomethyl)anthracene in $CDCl_3$. (*) Is $CDCl_3$ (7.2 ppm).

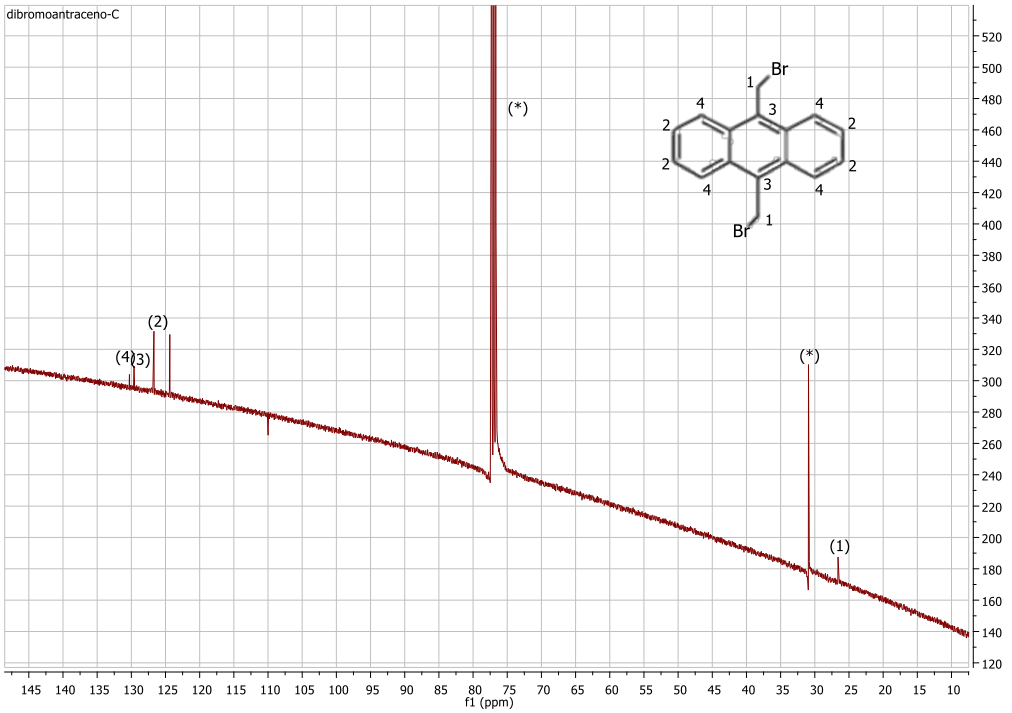

Figure 41. ^{13}C NMR spectrum of 9,10-bis(bromomethyl)anthracene in $CDCl_3$. (*) Is $CDCl_3$ (77 ppm).

9,10-bis(aminomethyl)anthracene:

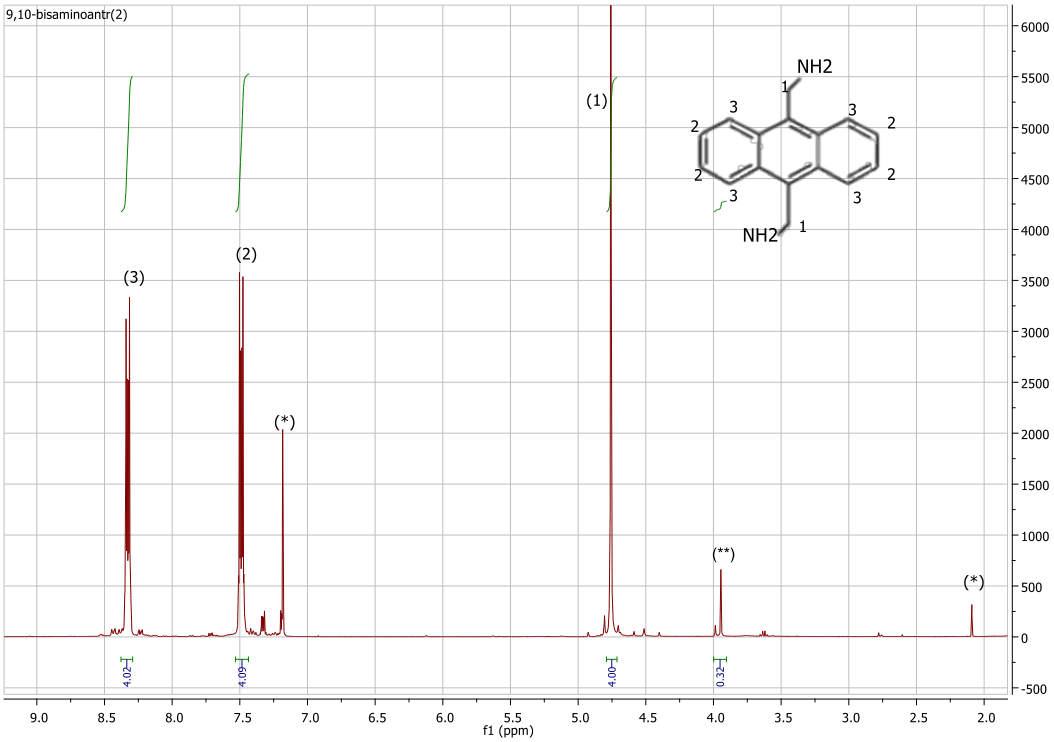

Figure 42. ^{1}H NMR spectrum of 9,10-bis(aminomethyl)anthracene in $CDCl_3$. (*) Are solvents such as $CDCl_3$ (7.2 ppm) and acetone-D (2 ppm). (**) Is remaining reagent hexamethylenetetramine (3.9 ppm, 8%).

Croconaine-macrocycle:

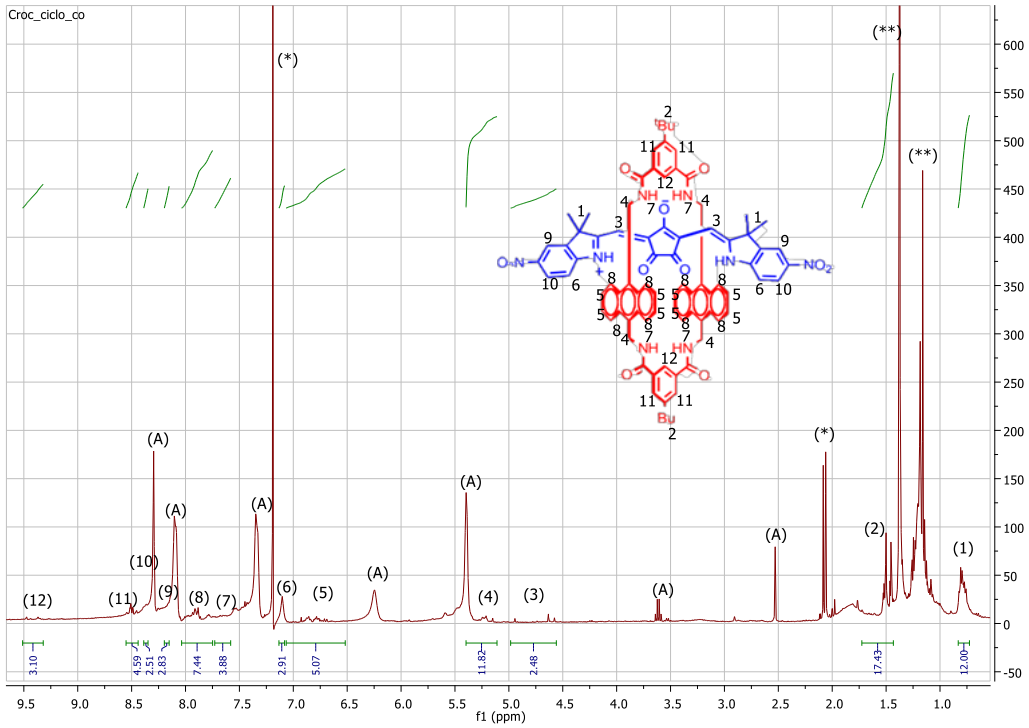

Figure 43. ^{1}H NMR spectrum of croconaine-macrocycle in $CDCl_3$. (*) Are solvents such as $CDCl_3$ (7.2 ppm) and acetone-D (2 ppm). (**) Are contaminations of the process. (A) Is remaining empty macrocycle (40%).

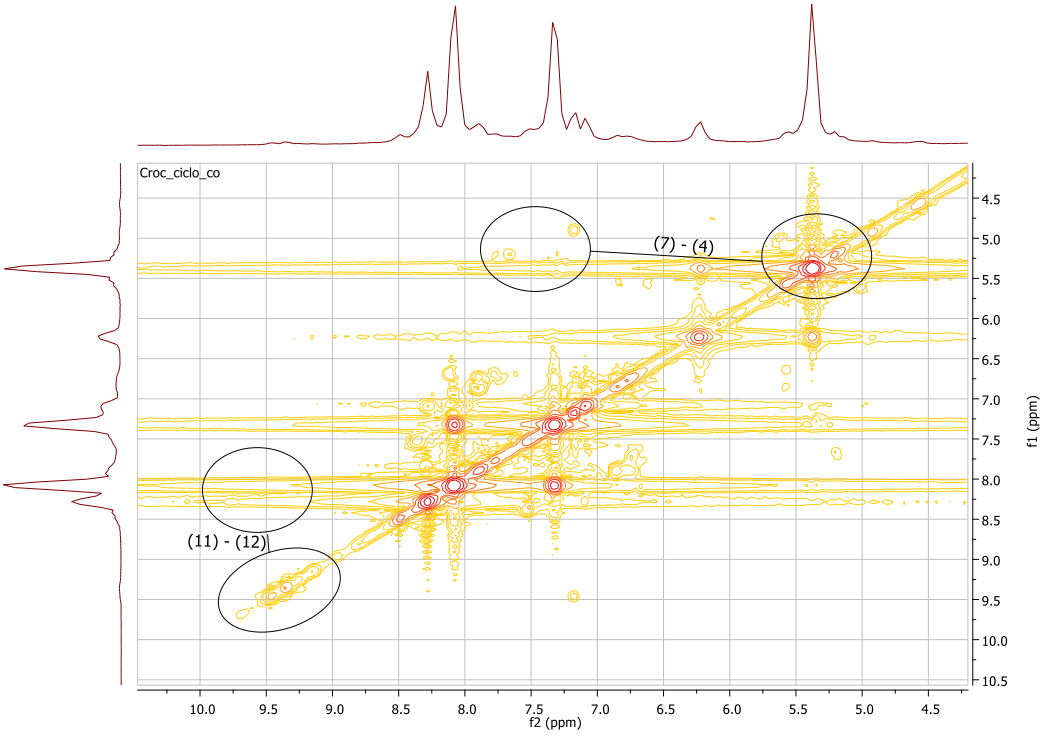

Figure 44. Selected region of ^{1}H-^{1}H COSY NMR spectrum of croconaine-macrocycle in $CDCl_3$. Interest couples are pointed, highlighting the interaction created between them and only present when croconaine is trapped within the macrocycle.

ANNEX III

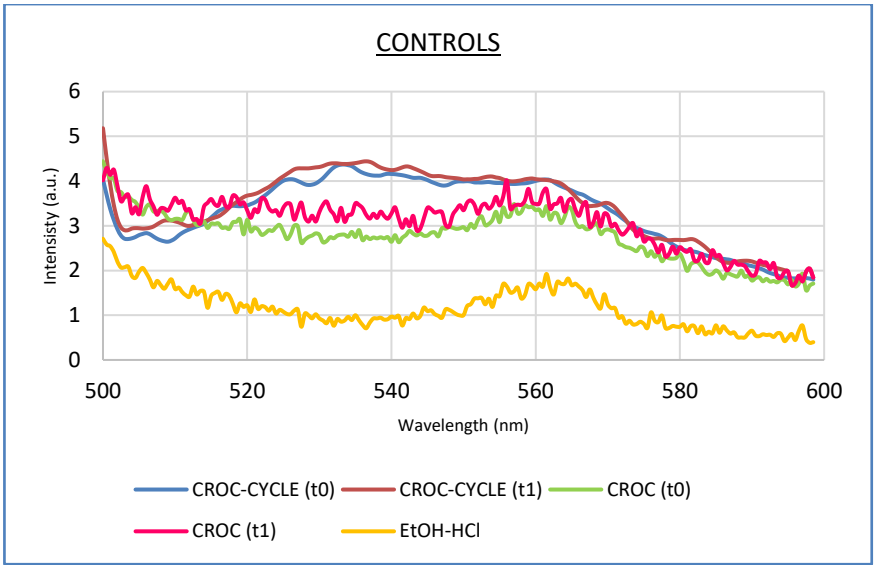

Figure 45. Fluorescence emission plot for croconaine-macrocycle solution (acidic ethanol solution), after 5 min irradiation (CROC-CYCLE (t1)), simple croconaine after 5 min irradiation (CROC (t1)) at 808 nm and 2 W/cm², and ethanol-HCl solvent control.

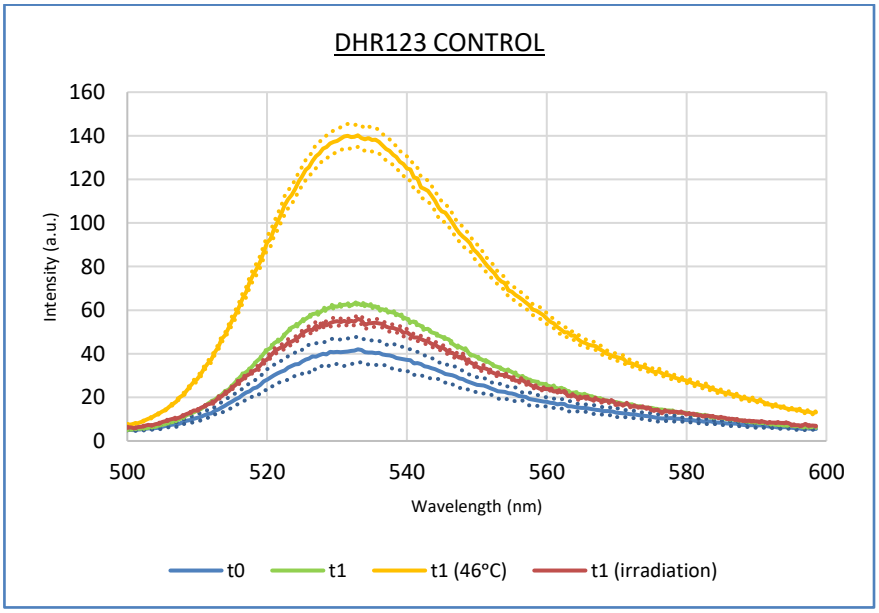

Figure 46. Fluorescence emission plots for DHR123 solution (acidic ethanol solution), after 5 min stirring (t1), 5 min stirring in a water bath at 46°C (t1 (46°C)) and 5min irradiation (5 min stirring (t1 (irradiation)) at 808 nm and 2 W/cm².

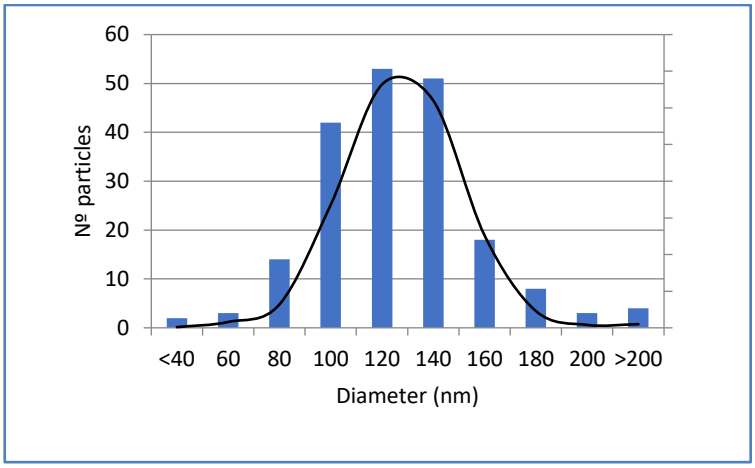

Figure 47. Size distribution histogram of PLGA-PEG nanoparticles loaded with croconaine-macrocycle and synthesized by nanoprecipitation method.

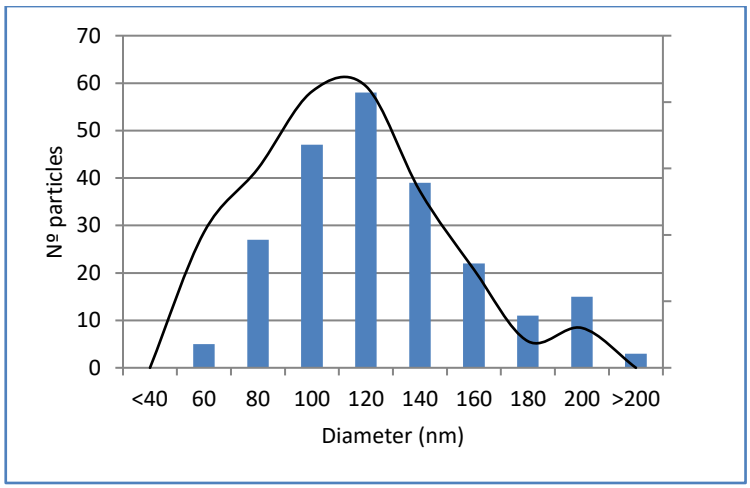

Figure 48. Size distribution histogram of PLGA nanoparticles loaded with croconaine-macrocycle and synthesized by double emulsion method using sodium cholate surfactant.

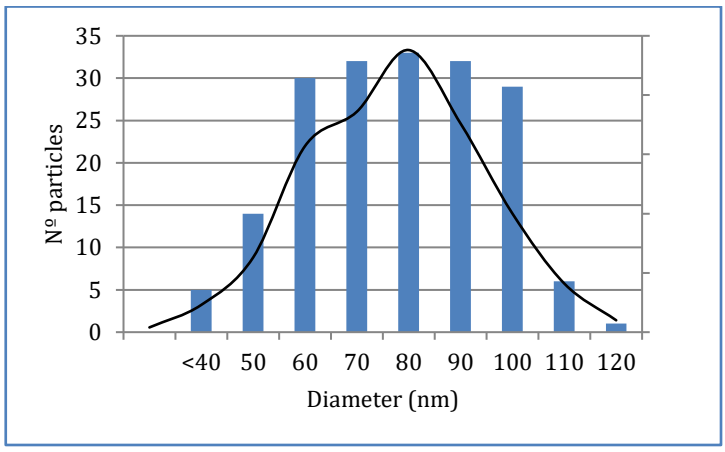

Figure 49. Size distribution histogram of PLGA nanoparticles loaded with croconaine-macrocycle and synthesized by double emulsion method using CTAB surfactant.

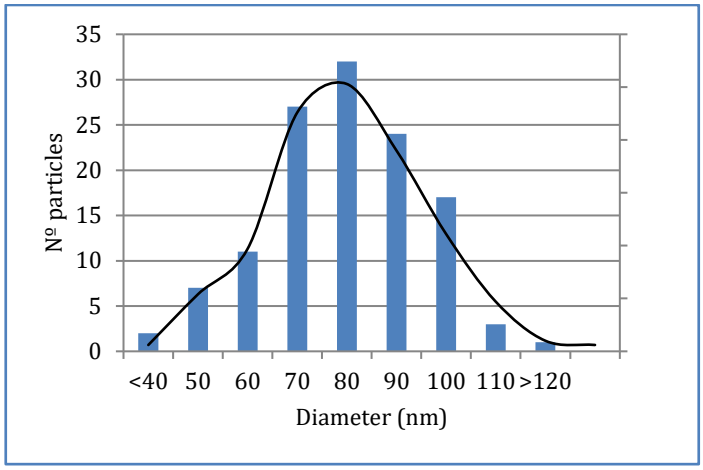

Figure 50. Size distribution histogram of PLGA nanoparticles loaded with croconaine-macrocycle and synthesized by double emulsion method using DC-Cholesterol surfactant.

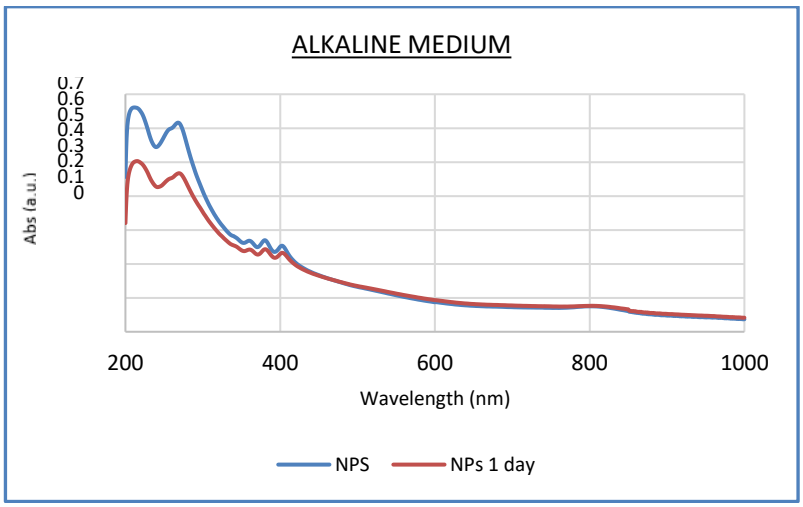

Figure 51. UV/VIS absorbance plot of croconaine-macrocycle loaded NPs (nanoprecipitation method) 24h after synthesis, in alkaline buffer (PBS in water).

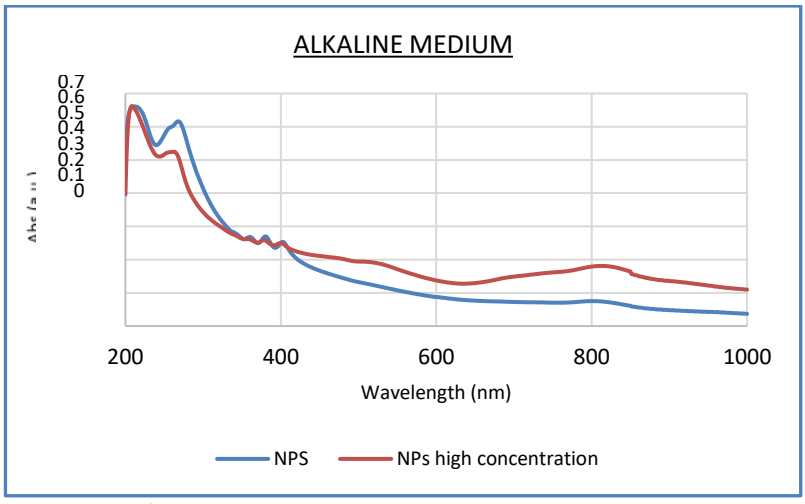

Figure 52. UV/VIS absorbance plot of double concentrated croconaine-macrocycle loaded NPs (nanoprecipitation method) in alkaline buffer (PBS in water).

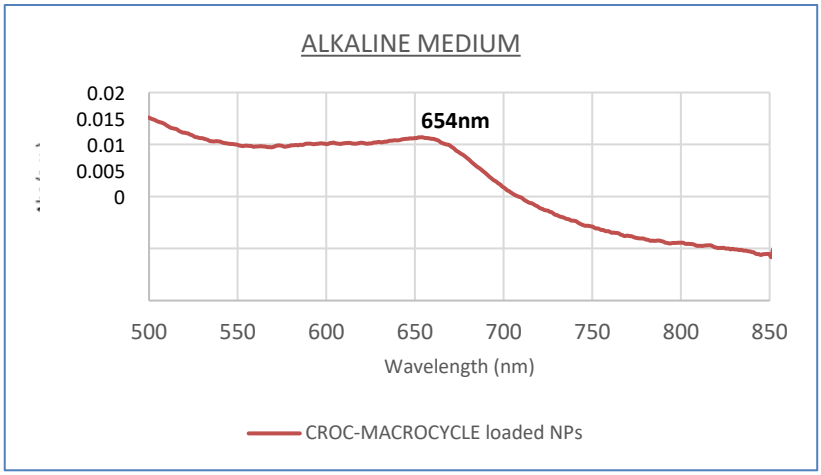

Figure 53. UV/VIS absorbance plot of croconaine-macrocycle loaded PLGA NPs (double emulsion method) in alkaline medium (NaOH in water)

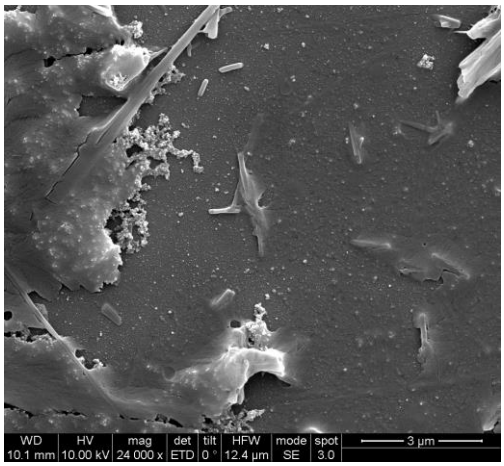

Figure 54. SEM image of croconaine-macrocycle loaded PLGA NPs (double emulsion method) in alkaline buffer (NaOH in water)

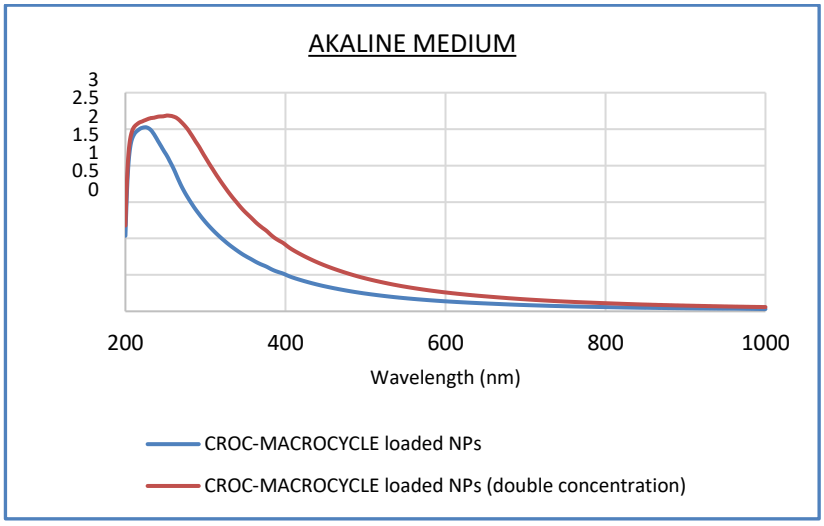

Figure 55. UV/VIS absorption plot of croconaine-macrocycle double concentration loaded PLGA NPs (double emulsion method with CTAB) in acidic and alkaline water media.

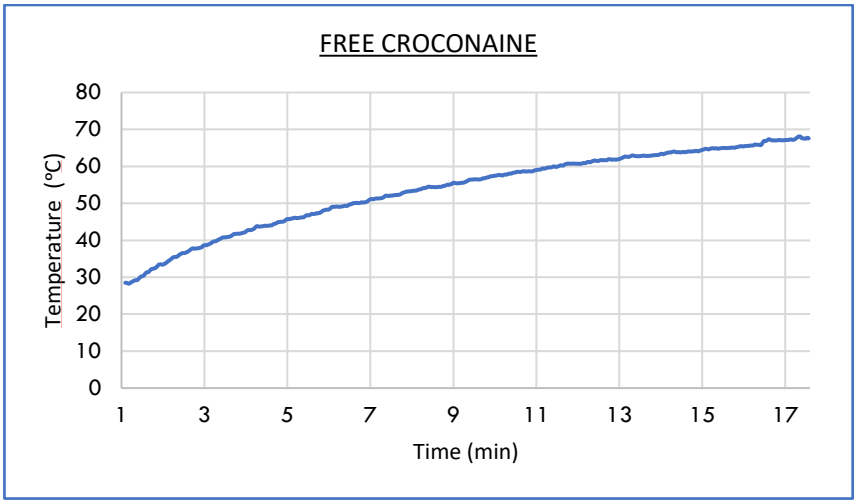

Figure 56. Temperature changes during laser irradiation (808 nm, 2 W/cm^2) of free croconaine in acidic ethanol solution (16 µM).

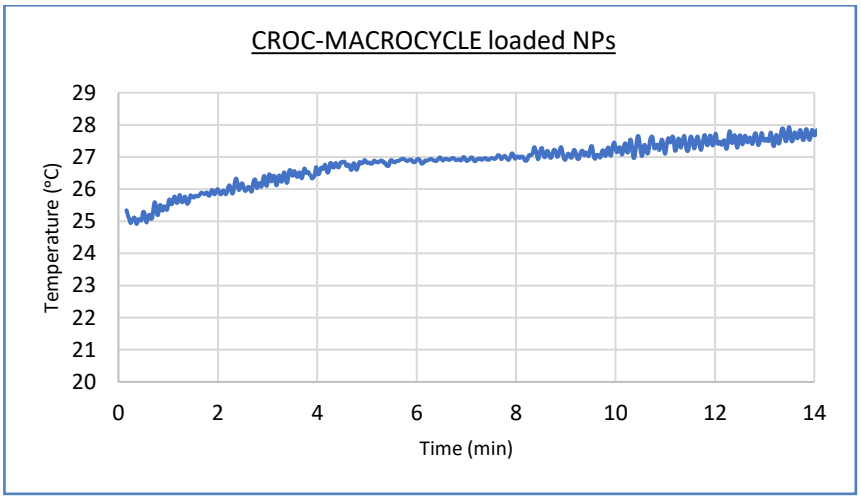

Figure 57. Temperature changes during laser irradiation (808 nm, 2 W/cm^2) of croconaine-macrocycle loaded PLGA NPs samples in acidic water media (double emulsion method with CTAB surfactant)

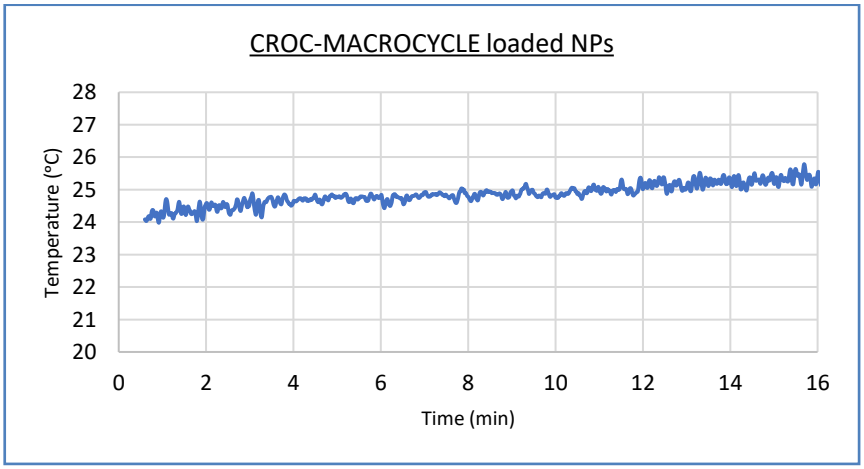

Figure 58. Temperature changes during laser irradiation (808 nm, 2 W/cm^2) of croconaine-macrocycle loaded PLGA NPs samples in acidic water buffer (double emulsion method with DC-Colesterol surfactant)

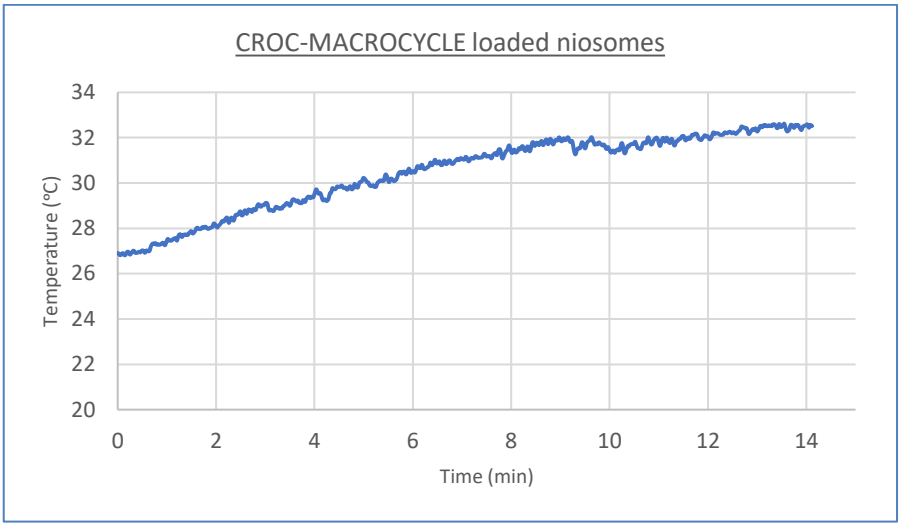

Figure 59. Temperature changes during laser irradiation (808 nm, 2 W/cm^2) of croconaine-macrocycle loaded niosomes samples in acidic water medium.

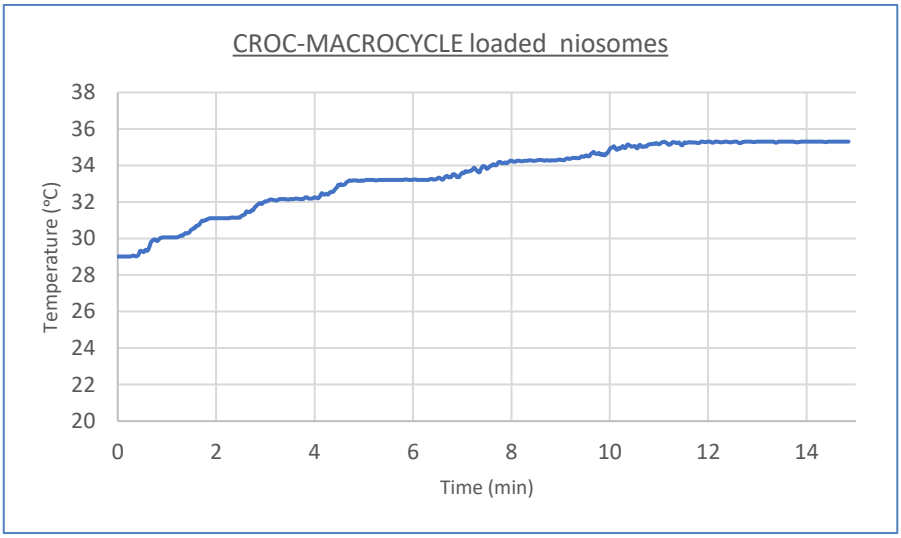

Figure 60. Temperature changes during laser irradiation (808 nm, 2 W/cm^2) of croconaine-macrocycle loaded niosomes samples in alkaline water meidum.