Spatiotemporal control of photothermal heating using pH sensitive near-infrared croconaine-based dyes

*Teresa Alejo^{a,b,*}, Clara Font^b, Miriam Abad^c, Vanesa Andreu^b, Victor Sebastian^{b,d},
Gracia Mendoza^b, Manuel Arruebo^{b,d}*

^aFoundation Institute of Nanoscience of Aragón (FINA), 50018- Zaragoza, Spain.

^bDepartment of Chemical and Environmental Engineering. Aragon Institute of Nanoscience (INA), University of Zaragoza, Campus Río Ebro - Edificio I+D, C/ Poeta Mariano Esquillor S/N, 50018-Zaragoza, Spain; Aragon Health Research Institute (IIS Aragón), 50009 Zaragoza, Spain.

^cDepartment of Organic Chemistry, Faculty of Science, Aragon Institute of Material Science (ICMA), University of Zaragoza-CSIC, 50009 Zaragoza, Spain

^dNetworking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, 28029-Madrid, Spain.

* Corresponding author: Teresa Alejo: teresaal@unizar.es

ABSTRACT

Near-infrared (NIR) absorbing dyes sensitive to pH are promising small molecules that can be used in photothermal therapy for intracellular cancer treatment. In this study, a croconaine dye molecule protected within a macrocycle has been prepared to be tested as photothermic agent. This pH sensitive croconaine dye allowed a selective NIR-light absorption only when the molecule was located in an acidic environment like the one found in intracellular vesicles; meanwhile it remained inactivated under physiological conditions. Results showed that photothermic heating produced upon 808 nm laser irradiation on mouse melanoma cells treated with this croconaine-based dye resulted in

cell death which may be attributed to a dye uptake into acidic intracellular vesicles (i.e., endosomal-lysosomal pathway). Cell damage was exclusively produced on irradiated cells, without any damage observed in neighboring cells. Negligible reactive oxygen species release was detected, thus the effect was attributed to a pure photothermal process. Croconaine dye presented a subcytotoxic concentration of 100 μ M on macrophages and tumoral cells. From the results obtained it can be concluded that these types of near-infrared dyes have shown great promise as photothermal agents, providing a great opportunity for achieving a spatiotemporal control on photothermal cancer therapy.

Keywords: Croconaine dye, photothermal therapy, cancer treatment, NIR-light heating, cytotoxicity.

1. Introduction

Currently there are many lines of research concerning advanced therapies applied to cancer treatment, and some of them are under investigation in a clinical level. Some examples include tissue, cell and gene or RNA interference (RNAi) therapies, other treatments use light to eliminate solid tumors including photodynamic (PDT) or photothermal (PTT) therapies, among others [1-6]. Regarding this last one, photothermal therapy basis consists on an agent (usually a nanoparticle or a dye) capable of absorbing light at a specific wavelength and release vibrational energy producing a local heat increase. When this heat rises up above 43°C, the denaturation of proteins is induced and therefore the apoptosis of tumoral cells occurs [7, 8]. Along all the thermal therapies, PTT is known as a minimal invasive technique with a high specificity and efficacy and low toxicity to healthy tissues, that it can be combined with existing cancer therapies [9, 10]. These advantages are achieved by using laser irradiation at near-infrared (NIR) wavelengths (700 - 1100 nm). Compared to ultraviolet (UV) or visible (VIS) light, NIR light is able to penetrate deeply into the tissues without harming healthy cells as neither water, blood cells nor biological tissues can strongly absorb NIR wavelengths [7, 10, 11]. Regarding to the NIR light absorbing agents used in PTT, they should be non-toxic and biocompatible at the doses used and include inorganic nanoparticles (gold nanostructures, carbon materials, copper sulfide nanoparticles, etc.) or organic dyes (namely

photosensitizers including indocyanine green (ICG), IR780, IR-808, etc.) [8-11]. Small molecular organic dyes show relevant advantages, including a good reproducibility in their effects, a very well defined molecular structure, strong absorption at NIR wavelengths and enhanced biodegradability [9, 10]. However, even if ICG has been approved by the Food and Drug Administration (FDA) and it is widely used in PTT, it shows poor photostability, high fluorescence quenching, reduced tumor accumulation and its chemical modification is not straightforward [12, 13]. Hence, recent studies have developed new synthetic chromophores with absorption in the NIR region to address these issues. In this regard, croconaine dyes are croconaine acid based-molecules with the ability to alter their structure depending on the local chemical environment [14, 15]. They present an intense NIR absorption and high photothermal conversion efficiency [16]. Croconaine dyes actually have a dual behavior depending on the environmental pH, switching their structure and absorption profile from an alkaline form (absorption peak in the visible region under slightly alkaline conditions, i.e., pH 7.4) to an acidic form (high absorption peak in the NIR region) [14]. Moreover, croconaine dyes have a strong resistance to photobleaching, good chemical and thermal stability and show a short-excited state lifetime which underlies highly efficient relaxation to the ground state [17]. They also have very little fluorescence emission and reduced singlet oxygen generation; thus, they constitute ideal dyes to decouple the photodynamic from the photothermal effects [18]. This structural shift determined by the pH of the medium can be very useful for achieving a controlled, effective and safe therapy. The extracellular pH, under physiological conditions, is about 7.3-7.4 [19], keeping the croconaine molecule on its alkaline structure. On the other hand, since the uptake of molecules occurs mainly via endocytosis, more specifically following the non-specific pinocytic pathway (for molecules smaller than 0.15 μm) [20], the pH inside the endosome-lysosome would reach 5.5, meaning that under those conditions an acidic structure of the croconaine molecules would be acquired and their consequent high absorption around 800 nm when located inside intracellular vesicles [21]. This fact not only reinforces the hypothesis that this therapy will not cause harm to neighboring healthy cells having a reduced dye uptake, but it also suggests that cell-death would be exclusively triggered upon light illumination only when the dye was located inside the target cell [21, 22], proving to be promising candidates for PTT. It is important to point out that several NIR dyes have demonstrated preferential uptake by cultured, circulating and disseminated tumor cell lines over normal cells [23-27].

ICG has been used as photothermal agent and also as photosensitizer to produce cytotoxic reactive oxygen species (ROS) upon irradiation with NIR laser on hepatocellular carcinoma cells [28]. However, the combination of PTT and PDT presents some drawbacks, as the ROS produced leads to bleaching of the dye reducing its effect, and also chemotherapy drugs used in synergistic therapies can be decomposed by the ROS generated [18]. Therefore, it is interesting to produce exclusively PTT without simultaneous ROS generation that could damage adjacent cells or inactivate drugs. Croconaine-based molecules have been previously used as photothermal agents in a mixed population of adherent CHO-K1 cells and non-adherent Jurkat cells, where only CHO-K1 cells were incubated with the croconaine dye. Results showed a high selectivity in cell destruction after irradiation only on dye-labeled cells without any damage to adjacent cells [21]. Those authors tested three different croconaine dye molecules, all of them based on croconic acid, but with different flanking units to adjust the degree of lipophilicity. They showed that the highly lipophilic croconaine dyes permeated into the cells and accumulated into organelle membranes and lipophilic intracellular vesicles, whereas the hydrophilic ones were impermeable to cells and thus had no effect on cell viability [21]. Consequently, a high lipophilicity of the dye is desired for achieving cell penetration to be able to effectively inactivate cells intracellularly. Smith and coworkers [14, 29] reported a modification of croconaine dyes trapping the molecule within a macrocycle to form a croconaine rotaxane, **Croc-cycle**, in order to avoid self-aggregation and electronic coupling of the dye. The dye protected with the macrocycle was incorporated into the membrane of liposomes to carry out ratiometric photoacoustic imaging and photothermal heating in the acidic peritoneal cavity [14]. Nevertheless, this **Croc-cycle** dye has not been previously used as photothermic agent against cancer cells. Herein, we have prepared and characterized the free lipophilic croconaine dye protected with a macrocycle to be used as photothermal agent. ROS release produced by this **Croc-cycle** dye was analyzed and its cytotoxicity evaluated in murine melanoma cells (B16F1 cell line) and macrophages. The *in vitro* PTT effect of **Croc-cycle** was tested for both cell lines. The pH-dependent absorption behavior of the dye was used to selectively induce cell death only when irradiating with the NIR laser. This molecule could be internalized within the cells and in their interior it was activated and efficiently absorbed the NIR light to generate heat producing selective and local cell death.

2. Experimental Section

2.1. Materials and methods

2,3,3-Trimethylindolenine (98%), sodium nitrate (NaNO_3 , 98%), sulfuric acid (H_2SO_4 , 98%), sodium hydroxide (NaOH , $\geq 98\%$), ethyl acetate (EtOAc), magnesium sulfate (MgSO_4 , $\geq 99.99\%$), croconic acid (98%), 1-butanol (anhydrous, 99.8%), toluene (anhydrous, 99.8%), ether ($\geq 99.7\%$), anthracene, aqueous hydrobromic acid (HBr , 47%), glacial acetic acid (100%), 1,3,5-trioxane ($\geq 99\%$), tetradecyltrimethylammonium bromide (TTA), ethanol (95%), hexamethylenetetramine ($\geq 99\%$), chloroform (CHCl_3 , 99.9%), chloroform (CHCl_3 , anhydrous, $\geq 99\%$) hydrochloric acid (HCl , 37%), sodium carbonate (Na_2CO_3 , $\geq 99\%$), 5-tert-butylisophthalic acid (98%), benzene (99.8%), dimethylformamide (DMF), thionyl chloride (SOCl_2 , $\geq 99\%$), acetone ($\text{C}_3\text{H}_6\text{O}$, $\geq 99\%$), deuterated chloroform (CDCl_3 , 99.96%), dihydrorhodamine 123 (DHR123, $\geq 95\%$) and phosphotungstic acid (PTA) were purchased from Sigma-Aldrich. All of these chemicals were used as received without further purification. Human monocytes (THP-1) (*ATCC*® *TIB-202*™) were obtained from the American Type Culture Collection (ATCC), while B16F1 mouse melanoma cells were kindly donated by Dr Pilar Martin-Duque. Dulbecco's modified Eagle's medium high glucose (DMEM w/stable Glutamine, Biowest), RPMI-1640 medium (w/stable Glutamine, Biowest), fetal bovine serum (FBS) (Gibco), penicillin-streptomycin-amphotericin B (Biowest), non-essential aminoacids (Biowest), sodium pyruvate (Biowest), HEPES (Biowest), 2-mercaptoethanol (Gibco), CellQuanti-Blue reagent (Abnova), phorbol 12-myristate 13-acetate (PMA) and dimethyl sulfoxide (DMSO, $\geq 99.7\%$) (Sigma-Aldrich) were used as reagents for cell culture experiments.

Nuclear magnetic resonance (NMR) spectroscopy was carried out on a 400 MHz Bruker AV-400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) using CDCl_3 as solvent.

UV-vis spectra were recorded using a Jasco V670 spectrophotometer (JASCO Corporation, Tokyo, Japan) in a quartz cuvette with 1 cm path length, to evaluate the characteristic absorption bands of the molecules in the electromagnetic spectrum.

Fluorescence spectra were recorded using a Perkin Elmer Luminescence Spectrometer Model LS-55 (Massachusetts, USA) with a slit width of 10 nm and excitation and emission wavelengths set at 480 nm and 530 nm, respectively.

Croc-cycle dye irradiation was performed using an 808 nm-laser diode (model MDL-III-808-2W, Changchun New Industries Optoelectronics Technology Co., Ltd., Changchun, P.R. China). Samples of 100 μ M **Croc-cycle** concentration in DMEM and RPMI media were irradiated using a laser irradiance of 5.2 W/cm² during 10 minutes. The ROS generation study was performed at 16 μ M **Croc-cycle** concentration in ethanol using an irradiance of 5.2 W/cm².

2.2. Synthetic procedures

2.2.1. Synthesis of croconaine dye **Croc**

Croc was synthesized following the procedure reported by Guha et al. (Scheme S1) [14]. 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 mol) of NaNO₃ were added at once and 31 mL (591 mmol) of H₂SO₄ were added drop wise for 10 min. The mixture was left stirring for 1h in the same cold bath (-5°C). It was then neutralized with the slow addition of NaOH 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 generated was washed three times with 40 mL of distilled water. The final product was dried with MgSO₄, 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 under reflux for 18 h. The mixture was then cooled at -23°C for 2h. The precipitated solid was filtered and washed with ether three times and finally dried under reduced pressure until the solvents were removed. **Croc** was obtained as a dark green-grey solid (150 mg, yield 58%). ¹H NMR (400 MHz, CDCl₃): δ ppm 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).

2.2.2. Synthesis of croconaine-macrocycle **Croc-Cycle**

Synthesis of 5-(tert-Butyl)isophthaloyl dichloride 1

5-tert-butylisophthalic acid (10 mg, 56 mmol) was dissolved in 40 mL of benzene and a single drop of DMF. 30 mL of SOCl₂ were carefully added in the mixture placed in an ice bath (0 °C). The mixture was then refluxed for 5 h in an oil bath at 70 °C (Scheme S1). The SOCl₂ was removed by distillation, and the remaining benzene was evaporated,

obtaining a white solid **1** [30] (11 g, yield 94 %). ^1H NMR (400 MHz, CDCl_3): δ ppm 8.63 (s, 1H), 8.34 (s, 2H), 1.34 (s, 9H); ^{13}C NMR (100 MHz, CDCl_3): δ ppm 167, 154, 134.2, 134, 131.5, 35.5, 31.

9,10-bis(aminomethyl)anthracene 3 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 reflux with a 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 with hot ethanol at 60 °C were needed to remove the remaining anthracene. A green-yellow solid of 9,10-bis(bromomethyl)anthracene **2** was obtained [31] (1.3 g, yield 60 %). ^1H NMR (400 MHz, CDCl_3): δ ppm 8.4 (dd, $J = 6.9$ Hz, $J = 3.2$ Hz, 4H), 7.7 (dd, $J = 6.9$ Hz, $J = 3.2$ Hz, 4H), 5.53 (s, 4H); ^{13}C NMR (100 MHz, CDCl_3): δ ppm 130.2, 129.6, 126.7, 124.4, 26.6.

Hexamethylenetetramine (0.43 g, 3.04 mmol) was dissolved in 100 mL of chloroform. Then **2** (0.5 g, 1.37 mmol) was added and heated at 50 °C to reflux overnight while sealed with an argon balloon. The mixture was allowed to cool down to precipitate, then it was filtered and washed with chloroform. The dried solid was dissolved with a mixture of ethanol and HCl (80 mL ethanol and 10 mL of pure HCl) and heated at 70 °C to vigorously reflux for 48 h, 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 bisamine **3** [32] (117 mg, yield 50%). ^1H NMR (400 MHz, CDCl_3): δ ppm 8.34 (dd, $J = 6.9$ Hz, $J = 3.2$ Hz, 4H), 7.49 (dd, $J = 6.9$ Hz, $J = 3.2$ Hz, 4H), 4.76 (s, 4H).

Croconaine-macrocyclic Croc-cycle synthesis

Briefly, **Croc** (24.35 mg, 0.16 mmol) was dissolved in 20 mL of chloroform and mixed with **1** (128.35 mg, 0.64 mmol) dissolved in 40 mL of chloroform and **3** (101.95 mg, 0.64 mmol), dissolved in 40 mL of chloroform and 1 mL of triethylamine injected using a mechanical syringe pump in 4 hours (Scheme S1). 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 proportion of eluents (acetone and chloroform) and find out the polarity of the precursors to separate them, thin-layer chromatography was used. Two columns were needed, a first one using a solution of chloroform and acetone (98% - 2%), and a second one using just chloroform (100%). A dark green-black solid **Croc-cycle** was obtained (18 mg, yield 10%). ¹H NMR (400 MHz, CDCl₃): δ ppm 9.32-9.51 (m, 2H), 8.44-8.55 (m, 4H), 8.34-8.39 (m, 2H), 8.15-8.2 (d, 2H), 7.63-8.13 (m, 8H), 7.38-7.62 (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).

2.3. Cell culture and biological studies

The cytocompatibility of the **Croc-cycle** dye was evaluated in B16F1 mouse melanoma cells and in THP1 human macrophages.

B16F1 cells were grown using DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic in a humidified atmosphere containing 5% CO₂ at 37°C. THP-1 cell line (human acute monocytic leukemia suspension cell) was cultured in RPMI 1640 medium supplemented with 10% FBS, 1% non-essential aminoacids (v/v), 1% sodium pyruvate, 1% HEPES, 0.1% 2-mercaptoethanol and 1% and antibiotic-antimycotic at 37 °C in a 5% CO₂ humidified incubator. Monocytic cells were differentiated into the adherent macrophage-like state in supplemented RPMI 1640 medium containing 1 μM phorbol 12-myristate 13-acetate (PMA) for 72 h.

2.3.1. Cell viability assay

In vitro cytotoxicity of **Croc-cycle** dye was evaluated using the Blue Cell Viability assay according to the manufacturer's instructions. B16F1 cells and macrophages (density of 5000 and 70000 cells/well, respectively) were seeded in a 96-well plate prior to incubation with the corresponding **Croc-cycle** dye doses.

Croc-cycle dye was added to the cells in complete growth medium at different concentrations (75 - 400 μM) and, then, cells were maintained in standard culture conditions for 24 h. After incubation, cells were washed with Dulbecco's Phosphate

Buffered Saline (DPBS, Biowest) and incubated with complete growth medium containing 10% (v/v) Blue Cell Viability assay reagent for 4 h (37 °C, 5% CO₂). The fluorescence intensity of the medium was measured in a multi-mode Synergy HT Microplate Reader at excitation and emission wavelengths of 530 nm and 590 nm, respectively (Biotek). Cell viability was calculated by interpolation of the emission data displayed by the treated samples and the control ones (untreated samples = 100% viability). The percentages obtained are depicted as the average of five values.

2.3.2. Photothermic in vitro effects

In order to evaluate the photothermally induced cell death mediated by the **Croc-cycle** dye upon NIR-light irradiation, B16F1 and human macrophages were seeded on 24-well plates at cell densities of 40000 and 500000 cells/well, respectively. Then, cells were treated with **Croc-cycle** dye solutions in DMSO mixed with growth medium to reach the desired concentrations (100 µM final concentration, subcytotoxic concentration obtained from the Blue Cell Viability assay) and no precipitates were observed in any case. After incubation for 30 min, cells were irradiated with a NIR laser (808 nm; 10 min at 5.2 W/cm²). Changes in cell well temperature were also monitored. Then, cell damage was evaluated by fluorescence microscopy just after irradiation by using the LIVE/DEAD Viability/Cytotoxicity Kit (Thermo-Fisher Scientific, USA) following the manufacturer instructions. Briefly, a solution containing 2 µM calcein AM and 4 µM ethidium homodimer-1 was added. After incubation at room temperature (30 min), cells were visualized under an inverted fluorescence microscope Olympus IX81 through the composition of 20 fields on each well acquired at 4X magnification to obtain a mosaic of each well. Control samples (treated and not irradiated, not treated and irradiated, not treated and not irradiated) were also assayed to evaluate the basal status of cells. Quantitative analysis of cell dead area was done from microscopy images using Image J software.

2.3.3 ROS generation using a red-ox sensitive probe.

To analyze the ability of **Croc-cycle** dye to release ROS, and to ensure that cell damage was exclusively produced by a photothermic effect we evaluated the **Croc-cycle** ROS generation using a fluorescence probe. Dihydrorhodamine 123 (DHR123) was used as indicator of ROS presence as it reacts with ROS producing its oxidized and cationic form (i.e., rhodamine 123) [33-35]. DHR123 is nonfluorescent but its oxidized derivative (R123) emits strong green fluorescence at 530 nm (I_{max} 530 nm) [36]. A solution

containing 6.6 μM DHR123 and 16 μM **Croc-cycle** dye in an acidic medium (ethanol-HCl) was tested. The final dispersion was irradiated (808 nm, 5.2 W/cm²) to evaluate the amount of ROS generated. The generation of R123 was monitored by using fluorescence spectroscopy using excitation and emission wavelengths of 485 nm and 530 nm, respectively. As control, the influence of the light and heating caused by the irradiation were also evaluated in order to discriminate the ROS generation attributed exclusively to the **Croc-cycle** dye. Sample fluorescence was quantified before and after 5 and 10 minutes of irradiation or heating.

3. Results and discussion

3.1. Characterization of Croc dye and Croc-cycle dye.

Croc dye (Fig. 1a) was synthesized following a procedure described by Guha et al. (Scheme S1) [14]. The chemical structure of the final compound was confirmed by proton nuclear magnetic resonance spectroscopy (¹H-NMR), as shown Fig. 1a. The macrocycle used for the inclusion of croconaine inside was composed of two synthetic molecules (Scheme S1): 5-(tert-Butyl)isophthaloyl dichloride **1** was obtained from the transformation of a 5-tert-butylisophthalic acid into an acyl chloride as described by Bugarin et al. [30] and on the other side, 9,10-bis(aminomethyl)anthracene **3**, which was synthesized in two steps. First, a 9,10-bis(bromomethyl)anthracene **2** was obtained from a procedure reported by Altava et al. [31] improving the purification process. In order to remove the remaining anthracene, the solid was filtered and washed with warm ethanol (60°C) three times, acquiring a 90% yield. Finally, the crude **2** was reacted with hexamethylenetetramine retrieving 117 mg (50% yield) of **3** as reported by Gassensmith et al. [32]. The chemical structure of each compound obtained was confirmed by magnetic resonance spectroscopy. The croconaine dye trapped within the macrocycle to form a croconaine rotaxane, **Croc-cycle** (Fig. 1b), was synthesized according to the protocol described by Guha et al. (Scheme S1) [14], using a modification in the purification process. Two chromatographic columns were needed to purify the final product, a first one using a solution of chloroform and acetone (98% - 2%) as eluent, and a second one using just chloroform (100%). The adequate portion of solvents (acetone and chloroform), which separates the precursors by their polarity, was previously established using thin-layer chromatography (TLC). To ensure the product obtained was the croconaine trapped into the macrocycle, proton nuclear magnetic resonance spectroscopy

(^1H NMR) (Fig. 1b) and two-dimensional nuclear magnetic resonance spectroscopy (COSY) (Fig. S1) were performed. The product desired was obtained as a result of a 10% yield.

Fig. 1. ^1H NMR spectrum of **Croc** (a) and **Croc-cycle** (b) in CDCl_3 . (*) stands for the solvent CDCl_3 (7.2 ppm). (**) stands for the remaining solvents used including toluene, 1-butanol and ethanol.

Croc and **Croc-cycle** were characterized by UV/vis spectroscopy to evaluate their absorption spectra profile and the influence of pH in their absorption properties. Therefore, acidic and alkaline **Croc** dye and **Croc-cycle** solutions were prepared for UV/vis analysis. **Croc** and **Croc-cycle** were tested in ethanol-HCl (pH 5) and ethanol-NaOH (pH 8) solutions, having a concentration of 16 μM of the dye molecule. Absorbance was measured in the range from 200 nm to 1000 nm. As shown in Fig. 2, both molecules have a specific high absorption in the NIR region around 800 nm in the presence of acidic pH, and a lower one around 600 nm in the presence of alkaline pH. Moreover, the **Croc-cycle** (I_{max} (ethanol-HCl) = 782 nm and I_{max} (ethanol-NaOH) = 640 nm), presents about 10 nm red-shift of both **Croc** absorption maxima (I_{max} (ethanol-HCl) = 778 nm and I_{max} (ethanol-NaOH) = 630 nm), Fig. 2b. A similar red-shift effect was previously observed by Guha et al. due to croconaine encapsulation inside the macrocycle [14].

Fig. 2. UV-Vis spectra of acidic and alkaline **Croc** (a) and **Croc-cycle** (b) in ethanol solutions at 16 μM concentration.

3.2. Reactive oxygen species (ROS) study

A reactive oxygen species (ROS) study was also performed to verify if **Croc-cycle** dye generates ROS, and if consequently the potential therapy based on those organic dyes would be exclusively based on thermal ablation. **Croc-cycle** ROS release was evaluated following a modification of the procedure reported by Brawek et al. [33]. Dihydrorhodamine 123 was used as the indicator given that in the presence of ROS it suffers an oxidation turning into its cationic form (rhodamine 123), a fluorescent dye, therefore, a fluorescence emission reading can be carried out showing an emission maximum at 530 nm [34, 35]. A solution containing 6.6 μM DHR123 and 16 μM **Croc-cycle** in an acidic medium (ethanol-HCl) was tested. The samples' fluorescence was quantified in a fluorescence spectrometer set at 480 nm for excitation, after 5 min and 10 min of laser irradiation (800 nm, 5.2 W/cm²). Controls were performed using a solution of DHR123 (6.6 μM) after 5 min stirring and after 5 min of irradiation, also using a solution of **Croc-cycle** (16 μM) after 5 min stirring and after 5 min of irradiation, using a solution of DHR123 (6.6 μM) and **Croc-cycle** (16 μM) after 5 min and 10 min stirring, and after 5 min warmed up in a water bath at 46°C. As shown in Fig. 3a, there is a correlation between the irradiation time and the increase in fluorescence emission at 530 nm. After 5 minutes of irradiation, the mixture fluorescence increased about 60 units, and after 5 more minutes under irradiation, the fluorescence increased 110 more units. Additionally, several controls were performed in order to assure if the ROS released were just related to the irradiation. First of all, it was measured the fluorescence emitted by the **Croc-cycle** itself after 5 min of irradiation, and the acidic ethanol solution by itself again

after 5 min irradiation. In both cases, no fluorescence emission was measured (Fig. S2a). Secondly, the fluorescence emitted by the DHR123 was tested under different scenarios: after 5 min stirring, after 5 min irradiation and after 5 min under a 46°C water bath, which is the same temperature reached on the irradiated **Croc-cycle** solution (Fig. S2b). As observed in Fig. S2b, DHR123 suffers a spontaneous oxidation when heated at 46°C (about 80 units increase), which led to a final control to quantify this DHR123 influence on the results previously obtained. Fig. 3b shows that there was a ROS generation increase only when a temperature (46°C) was applied on the sample, **Croc-cycle** with DHR123 for 5 min (20 units increase), and even some increase when only stirring it for 5 min (10 units increase). Hence, the real irradiation influence on the ROS release was measured in about 30 units (Fig. 3b). Spence et al. [17] asserted a low oxygen photosensitization ability of a different croconaine dye with two appended thiophene units, using the singlet oxygen trap 1,3-Diphenylisobenzofuran (DPBF). Herein, our results by means of the ROS sensor DHR123 pointed out that our **Croc-cycle** dye produces a reduced singlet oxygen generation. Therefore, it has been demonstrated that the presence of ROS when the sample containing **Croc-cycle** was irradiated seems to be almost negligible when compared it with the ROS generated by the controls.

Fig. 3. Fluorescence emission spectra for **Croc-cycle** and DHR123 solution (acidic ethanol solution), (a) after 5 min irradiation (t1) and 10 min irradiation (t2) at 808 nm and 5.2 W/cm² (b) after 5 min stirring (t1), 5 min stirring in a water bath at 46°C and 5 min irradiation at 808 nm and 5.2 W/cm².

3.3. Cell viability assay

The cytocompatibility of the **Croc-cycle** dye was studied in the cell lines used for the subsequent laser-induced photothermal assays, being B16F1 melanoma cells and human macrophages, which are the cell models for the proposed biomedical application. The treatment of the cell lines incubated with different **Croc-cycle** concentrations (75-400

μM) involved detrimental effects on viability on a dose dependent manner at the higher concentrations assayed as it was determined by the Blue Cell Viability assay (Fig. 4a). The increase in **Croc-cycle** concentration (Fig. 4a) beyond 100 μM implied a decrease in viability higher than 50% for macrophages, and higher than 70% in the case of B16F1 cells. At the highest concentration assayed (400 μM), the viability percentages obtained were 24% and 66% on macrophages and B16F1 cells, respectively. According to these results and the recommendations of the ISO 10993-5 [37], 100 μM was considered the subcytotoxic concentration for further studies as this concentration displayed viability percentages higher than 70% in all the cell lines assayed. Compared to the existing cytotoxicity tests concerning other croconaine dyes, reported by Harmatys et al. [21] or by Tang et al. [12], which rendered cytotoxicity up to 25-30 μM , herein it has been corroborated that even at higher concentrations (100 μM), this molecule is not cytotoxic. Therefore, we used the subcytotoxic concentration previously obtained, 100 μM , for the following experiments to test the photothermic behavior of the **Croc-cycle** dye on cells.

Fig. 4. (a) Cell viability of **Croc-cycle** dye in the two cell lines assayed after 24 h. Percentages are displayed as mean $\pm$ SD (N = 5). (b) Heating curve of 100 μM **Croc-cycle** dye in DMEM and RPMI media at pH 5.5 upon irradiation at 808 nm and 5.2 W/cm ^{2} .

3.4. Laser-Induced Heat Generation Studies

Photothermal properties of the **Croc-cycle** dye alone were initially studied. **Croc-cycle** dye was dissolved in DMSO and added to DMEM and RPMI media at pH 5.5 to obtain final solutions of 100 μM concentration. 1 mL of the solution was irradiated using an 808 nm-laser diode with the beam passing through it in a plate well, in the same conditions than in the ones used in the subsequent cell assays. The laser irradiance was set at 5.2 W/cm ^{2} . The temperature was monitored by a thermocouple immersed into the

solution, recording the temperature every 0.017 s. When the **Croc-cycle** dye was irradiated with the laser (808 nm, 5.2 W/cm²), a 20 °C temperature increase was achieved using 100 µM **Croc-cycle** solution (Fig. 4b). Laser irradiation assays were carried out in order to monitor the temperature increase when **Croc-cycle** was induced to its acidic structure, reaching a final temperature of ~50 °C in 10 min (Fig. 4b), which can be used for photothermal *in vitro* tests with cells. Controls were performed for the irradiation of DMEM and RPMI media at pH 5.5 in the same experimental conditions, but without **Croc-cycle** dye, presenting the absence of heating (Fig. S3).

3.5. Evaluation of laser-induced cell damage

Photothermal effects of **Croc-cycle** dye on melanoma cells (B16F1) and human macrophages were measured using fluorescence microscopy (Fig. 5) after staining live cells with calcein and dead cells with ethidium bromide. B16F1 and human macrophages were incubated with **Croc-cycle** dye (100 µM) during 30 min at 37°C. After incubation, cells were subsequent irradiated for 10 minutes with the NIR-laser operating at 808 nm (5.2 W/cm²). The rise of temperature in the well after irradiation was measured using a near-infrared sensor, showing a final temperature below 44°C in all cases. For both cell lines treated with **Croc-cycle** dye, cell damage was focalized on the irradiated area, Fig. 5, showing a central area of dead cells (red) corresponding with the laser spot surrounded by live cells (green). Furthermore, control samples treated with **Croc-cycle** dye and no irradiated, and cells not treated with **Croc-cycle** and irradiated, did not exert cell damage (Fig. S4). The increase of temperature in the controls cell wells was always < 3 °C, indicating the absence of a thermal effect. With the same laser spot projected area cell death seemed to be more accentuated in melanoma cells (22.3 % cell dead area) than in macrophages (7.7 % cell dead area), which can be attributed to the lower pH of intracellular vesicles of cancer cells compared to the one of normal cells as previously reported [38-40]. These results highlight the selective cellular ablation effect of our **Croc-cycle** dye, showing high viability in neighboring cells not irradiated though displaying a local photothermal cell damage after NIR irradiation when the dye is placed in the acidic intracellular milieu, necessary to activate its absorption in the NIR region of the spectrum. Overall, **Croc-cycle** dye has demonstrated to be a potential agent for PTT, producing no cell death without irradiation and a selective cell destruction when the dye is located inside the cells.

Fig. 5. Photothermal effects on murine melanoma cells (B16F1 cell line) and macrophages treated with **Croc-cycle** dye using the same laser spot projected area on the culture wells. Composition of 20 pictures (4X magnification) obtained by fluorescence microscopy to show the whole culture well when cells were treated for 30 min with **Croc-cycle** dye (at a subcytotoxic concentration, 100 μ M). (a) Macrophages and (b) B16F1 cells laser irradiated (808 nm, 5.2 W/cm²) for 10 min and images acquired immediately after irradiation. Live cells are stained in green while dead cells in red. Scale bar 2 mm.

4. Conclusions

In summary, we have synthesized and characterized a croconaine-based dye trapped within a macrocycle as photothermal therapeutical agent. The **Croc-cycle** dye was capable of heating up a medium in a pH-dependent manner, therefore in the case of acidic pH **Croc-cycle** dye absorbs the irradiation of a laser in the NIR region, efficiently increasing the medium temperature at very low concentrations. **Croc-cycle** dye was internalized within the acidic interior of tumoral cells and macrophages, and only in the intracellular milieu it was activated and efficiently absorbed the NIR light resulting in cell death. Consequently, cell damage was exclusively produced intracellularly on irradiated cells, without damage observed in neighboring cells, which remained alive. The presence of the molecule in the extracellular media did not produce cytotoxic effect at the concentrations tested. ROS generation observed for the **Croc-cycle** dye was negligible so the reported cytotoxic effect was exclusively driven by photothermia. Results demonstrated that **Croc-cycle** dye could have great potential in photothermal therapy, and could be not only potentially used for the selective damage of cancer cells labeled, but also for a wide range of materials science applications in which its pH-responsiveness could be used.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors thank financial support from the ERC Consolidator Grant program (ERC-2013-CoG-614715, NANOHEDONISM). CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008–2011 financed by the Instituto de Salud Carlos III with the assistance of the European Regional Development Fund. T. Alejo thanks the Fundación Institute of Nanoscience for her postdoctoral fellowship. We also acknowledge Dr. Pilar Martín-Duque for donating B16F1 mouse melanoma cells.

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