

## **A tumor responsive self healing prodrug hydrogel enables synergistic action of doxorubicin and miltefosin for focal combination chemotherapy.**

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### **1. Materials and methods**

#### **1.1. Materials**

Carboxymethyl cellulose (high viscosity, viscosity of 1% at 25°C-3000 cps, degree of carboxymethylation 0.5), Dulbecco's Modified Eagle's medium, antibiotic/antimycotic solution, fetal bovine serum was procured from Hi Media laboratories Ltd, India. Gelatin (Type A, Bloom 300), sodium metaperiodate, borax (sodium tetraborate decahydrate), TNBS (2, 4, 6-trinitro benzene sulphonic acid), Doxorubicin hydrochloride was gift from RPG lifesciences, India. Miltefosine was procured from Avanti polar lipids, USA. GFP transfected MD AMB-231 triple negative breast cancer cell line was gift from ACTREC, Mumbai, India. Dialysis tubing (Spectra/Por®, M.W.C.O 3500) was from Spectrum Laboratories Inc., CA, USA. All other reagents were of analytical or equivalent grade. Milli Q water was employed throughout.

#### **1.2. Periodate oxidation of carboxymethyl cellulose**

Carboxymethyl cellulose (CMC) (20 gm) dispersed in 200 mL of ethanol was treated with aqueous solution of sodium metaperiodate (7.12 gm in 200 mL) under dark and stirring condition for 6 h at 25 °C. The reaction mixture was then dialyzed against MilliQ water for 48 h until the dialyzate is free of unreacted periodate. Thereafter solution was then kept at -80°C and lyophilized. The yield of product is 70-80%. IR spectra were recorded with a Magna 550 FTIR spectrometer (Nicolet Instruments Corporation USA) in the range of 4000–400 cm<sup>-1</sup> using KBr pellets. <sup>1</sup>H NMR spectrum of CDA was recorded with a Plus 300 MHz spectrometer (Varian, USA) in D<sub>2</sub>O at 25 °C. Degree of oxidation and dialdehyde content were estimated by iodometric titration and hydroxylamine hydrochloride titration, respectively.

#### **1.3. <sup>11</sup>B NMR analysis**

Solid state <sup>11</sup>B NMR experiments on lyophilized hydrogels were performed on a Bruker Avance II+ spectrometer at a spinning speed of 10 kHz for all the samples. <sup>11</sup>B basic frequency is 128.335 MHz. To see the effect of hydrogel in different media, gels were incubated with PBS (pH 7.4), PBS (pH 6.5) and PBS + 25 mM glucose (pH 6.5) for 24 and 48 h. <sup>11</sup>B NMR spectra of medium was then recorded with Plus 300 MHz spectrometer (Varian, USA) in H<sub>2</sub>O at 25°C at 96.32 MHz for <sup>11</sup>B nuclei. <sup>11</sup>B chemical shifts were measured relative to 0.1 M boric acid which is having a chemical shift at  $\delta$  value 36 ppm.

#### **1.4. Hydrogel preparation and characterization**

One mL of varying concentration of CDA (5% and 10%), dissolved in 0.1 M borax with or without drug molecules (40  $\mu$ L each of dox and mf (both 2.5 mg/mL)) was stirred magnetically with 1 mL of varying concentration of gelatin (Type A, bloom 300) (10, 15 and 20%) at 37 °C using teflon coated stir bead of dimension 10 mm length and 5 mm diameter in a 10 mL glass beaker (20 mm diameter). The time required by the stir bead to stop was noted as the gelation time

The gels were analysed for rheology using a Physica MCR 301 rheometer (AntonPaar, Graz, Austria) using a parallel plate geometry (diameter of 2.5 cm, gap of 5 mm) and Rheoplus/32 software version V3.21 (Anton Paar, Graz, Austria). Gels (2.5 mm diameter and 0.5 mm thickness) were incubated with 0.1 M PBS at 37 °C for 1 h and placed on the lower plate of the rheometer. Strain sweep was performed to verify that all the measurements are done within the visco-elastic region of gels such that the storage ( $G'$ ) and loss ( $G''$ ) modulus were independent of strain. Frequency sweep was done by varying the angular frequency from 1 to 100 rad/s at 5% strain. Time sweep was performed to find the gelation time, immediately after mixing equal volume (0.5 mL) each of CDA (10% in 0.1 M borax) with or without drug molecules (20  $\mu$ L each of stock 2.5 mg/mL) and gelatin (15%) on the lower plate of the rheometer at 0.5% strain and angular frequency 1 rad/s. Self healing ability of hydrogels were analysed by exposing gel upto 50 % strain followed by monitoring time dependent recovery for 100 s at 1 % strain and 10 rad/s angular frequency. This was repeated for 500% strain followed by monitoring time dependent recovery for 100 s at 1 % strain and 10 rad/s angular frequency

Cross-linking degree of gels was evaluated by Trinitro benzene sulphonic acid (TNBS) assay. Concisely, equal volumes of varying concentrations of CDA with or without drug molecules and gelatin were mixed to form gels. Within a minute of gel formation, gels were frozen and lyophilized. About 10 mg of lyophilized gel was treated with a mixture of 1 mL each of 0.5 mL 5 % TNBS and 4 % sodium bicarbonate at 40 °C for 4 h to form a soluble complex. In order to hydrolyze the complex, 1 mL of the resultant mixture was treated with 3 mL of 6 N HCl at 60 °C for 2 h and absorbance was determined at 334 nm using UV-visible spectrophotometer (Lamda 25, Perkin Elmer, USA) after dilution. A calibration curve was plotted by treating different concentrations of gelatin with TNBS in a similar way to determine the absorbance of non-crosslinked gel.

$$\text{Cross-linking degree (\%)} = \left\{ 1 - \frac{\text{Absorbance of cross-linked gel}}{\text{Absorbance of non-cross-linked gel}} \right\} \times 100$$

Swelling studies of hydrogels were done by incubating the hydrogels in PBS at 37 °C for 24 h and cross-linking density was calculated. Briefly, 0.5 mL each of CDA (10% solution in 0.1 M borax) with or without drug molecules (20  $\mu$ L each of stock 2.5 mg/mL) and 0.5 mL of gelatin (15%) were mixed to obtain different gels. Five mL of PBS was added to the vial and incubated at 37 °C. After 24 h, medium was then removed; gels were blotted gently with a filter paper and was noted the weight. Swelling ratio ( $Q_m$ ) was calculated as the ratio of weight of absorbed water to that of dried gel. Degree of swelling ( $Q$ ) was defined as the reciprocal of the volume fraction of the polymer ( $v_2$ ) in the hydrogel.

$$Q = v_2^{-1} = \left[ (1/\rho_p) \left[ (Q_m/\rho_s) + (1/\rho_p) \right]^{-1} \right]^{-1}$$

where  $\rho_p$  is the polymer density (0.75 g/cm<sup>3</sup>),  $\rho_s$  is the density of water (0.9971 g/cm<sup>3</sup> at 25 °C) Flory-Rehner equation was used for calculating the cross-linking density ( $v_e$ mol/cm<sup>3</sup>) of the swollen network, which is written as

$$(v_e = -[\ln(1 - v_2) + v_2 + \chi_1 v_2^2] [V_1(v_2^{1/3} - 2 v_2/f)]^{-1})$$

where,  $\chi_1$  is the Flory-Huggins interaction parameter (0.35),  $f$  is the cross-linking functionality (50),  $V_1$  is the molar volume of water (18.062 cm<sup>3</sup>/mol) and  $v_2$  is the volume fraction of polymer in the hydrogel when it reaches the equilibrium swelling state.

The morphology of gels was examined by field emission gun scanning electron microscopy (FEG-SEM). Concisely, lyophilized gels were kept on double sided tape; sputter coated with platinum and porous structure was examined in the FEG SEM (JSM-7600F, JEOL, USA).

### **1.5. Release experiments**

The release studies were done under normal physiological conditions (pH 7.4, 37 °C) for 48 hours under continuous stirring, in the presence of pH and glucose triggers. Gels were prepared by mixing 0.5 mL each of CDA with dox (0.1 mg/mL, 20  $\mu$ L of 2.5 mg/mL stock solution) and gelatin. After 5 min of gel formation, 5 mL of medium was added to the vial containing gel. To study the effect of acidic conditions, release was carried out in PBS adjusted to pH 6.5 and pH 7.4 and the effect of glucose concentration on release profile was studied using 5mM glucose at pH 7.4 and 25 mM at pH 6.5. About 0.5 mL aliquots were taken out from the release medium at predetermined time intervals and replenished with equal volume of fresh medium. The amount of doxorubicin in the withdrawn aliquots was determined spectrofluorometrically using excitation wavelength of 480 nm and emission wavelength of 580 nm. The release experiment was carried out for 48 hours and in triplicates.

### **1.6. Cell culture studies**

#### *(a) Cellular uptake of gel releasate*

Cellular uptake was studied using GFP transfected MDA-MB-231 cell line (human triple negative breast cancer cells). Cells were harvested at 70 -80% confluency and seeded at concentration of 10<sup>5</sup> cells/well in 24 well-plate having a glass coverslip in each well. Then the plate was incubated at 37 °C and 5% CO<sub>2</sub> for cells to adhere. After 24 hours, spent medium was removed from the wells and replaced with fresh serum free media containing inhibitors. Inhibitors used to study the mechanism of cellular internalization of doxorubicin are 0.1 % sodium azide (metabolic inhibitor), 2  $\mu$ g/ml nystatin (inhibitor of caveole mediated pathway), 10 $\mu$ g/ml phenothiazine (inhibitor of clathrin mediated pathway), and 4 $\mu$ /ml colchicine (inhibitor of microtubule formation). Following 1 hour of incubation with inhibitors, the medium was discarded and 1 mL of CDA10doxmf releasate added to each well. Releasate was prepared in medium by incubation of gels for 48 h. After 3 hours of incubation, wells were washed with PBS thrice and 1 mL of 10% formaldehyde was added to each well for fixation of cells on the cover slips. After 20 minutes of fixation, coverslips were removed from the plate and placed over glycerol mount on a glass slide. These slides were observed using confocal laser scanning microscope (CLSM, Olympus Fluoview, FV500, Tokyo, Japan) using red (526 nm excitation and

555 nm emission) and green (490 nm excitation and 520 nm emission) fluorescence. Images were acquired with 60X oil immersion objective using the Fluoview software (Olympus, Tokyo, Japan). To determine the intracellular doxorubicin content at different time points, same procedure was carried out in 24 well-plate without coverslips such that the cells were incubated with CDA10doxmf releasate for 3 different time periods viz. 0.5 hour, 1 hour and 3 hours. After the last washing with PBS, cells were lysed using lysis buffer (1% Triton X-100 and 2mM EDTA) and cellular doxorubicin content was determined by using fluorescence plate reader (Victor 3V Multilabel plate reader, PerkinElmer, USA) using excitation wavelength of 570 nm and emission wavelength of 590 nm. Doxorubicin content was normalized with cellular protein content which was quantified using BCA protein assay kit (Thermo Scientific, Pierce, USA).

*(b) Cellular uptake analysis using FITC tagged polymer*

Cells pretreated with FITC tagged CDA, CDA-dox prodrug and releasate of gel prepared using both FITC-CDA and CDA-dox prodrug with gelatin were viewed using CLSM in order to see whether cells can uptake CDA-dox prodrug that is released to medium upon hydrolysis along with free dox. FITC-CDA was prepared by reacting CDA with fluoresce isocyanate (FITC) for 2 h, precipitated in ethanol, dialysed to remove free FITC and lyophilized. CDA-dox prodrug was prepared by stirring CDA with dox for 2 h, dialysed to remove free dox and lyophilized.

Gels were prepared using equal volumes of (a) FITC-CDA in 0.1 M borax and gelatin; (b) CDA-dox in 0.1 M borax and gelatin and (c) half volumes of CDA-dox prodrug and FITC-CDA in 0.1 M borax and gelatin. The gels (500  $\mu$ L) were prepared in 24 well plates under sterile conditions and drug was allowed to release in 1 mL of DMEM medium for 24 h. Human breast cancer MDA MB-231 cells were harvested at 70 -80% confluency and seeded at concentration of  $10^5$  cells/well in 24 well-plate having a glass cover slip in each well. Then the plate was incubated at 37 °C and 5% CO<sub>2</sub> for cells to adhere. After 24 hours, spent medium was removed from the wells and cells on the cover slip were treated with releasate medium. In a similar way, cells were also treated with same concentrations of solutions of FITC-CDA and CDA-dox prodrug. After 3 hours of incubation, wells were washed with PBS thrice and fixed with cells with 10% buffered formalin. After 20 minutes of fixation, coverslips were removed from the plate and placed over glycerol mount on a glass slide. These slides were observed using confocal laser scanning microscope (Olympus Fluoview, FV500, Tokyo, Japan) using red (526 nm excitation and 555 nm emission) and green (490 nm excitation and 520 nm emission) fluorescence.

*(c) In vitro cytotoxicity studies*

In vitro cytotoxicity of the system was evaluated using MDA-MB- 231 (triple negative breast cancer cell line). Cells were harvested at 70-80% confluency and seeded in a 96 well-plate (Corning Costar, Sigma-Aldrich) at a concentration of  $10^4$  cells per well. These plates were then kept in saturated humid conditions at 5% CO<sub>2</sub>, 37 °C overnight for cells to adhere. After 24 hours, spent medium from the wells was replaced with serum free medium containing the graded concentrations of releasate of CDA10dox, CDA10mf and CDA10doxmf as well as equivalent amount of free drugs, and the plates are again incubated in the saturated humid

condition. Cells treated with only medium were kept as controls and at the end of 48 hours, cell viability was evaluated using SRB (Sulforhodamine B) assay. Briefly, the media containing the samples was discarded and replaced with fresh medium. Then 50  $\mu\text{L}$  of 10 % trichloroacetic acid was added to each well for fixing the cells and plates were incubated at 4  $^{\circ}\text{C}$  for 1 hour. Subsequently, the plates were washed with double distilled water and air dried. 100  $\mu\text{L}$  of 0.4% SRB prepared in 1% acetic acid solution was added to the fixed cells and kept for 20 minutes at room temperature. Following this, plates were washed with 1% acetic acid solution to remove the unbound dye. The plates were then air dried and 100  $\mu\text{L}$  of 10 mM Tris base was added to the wells for solubilizing the dye. After 20 minutes, plates were shaken and OD at 560 nm was taken using plate reader (Thermo Electron Corporation, USA). The viability was calculated as following:

$$\% \text{ viability} = (\text{Absorbance of sample/absorbance of control}) \times 100$$

The IC<sub>50</sub> values are then calculated using GraphPad Prism 4 software. Combination index for doxorubicin and miltefosine was calculated using isobologram equation.

$$\text{CI} = a/A + b/B$$

where, a is the IC<sub>50</sub> of doxorubicin in combination with dose b of miltefosine as obtained with CDA10doxmf, and A is the IC<sub>50</sub> of free doxorubicin without miltefosine and B is the IC<sub>50</sub> of free miltefosine without doxorubicin as obtained in CDA10mf. In this case if CI<1, the interaction of two agents is synergistic; if CI=1, the interaction is additive; and if CI>1, interaction is antagonistic.

#### *(d) Migration analysis by cell culture wound closure assay*

This assay was performed to see whether the gel can prevent the migration of cancer cells. For this MDA-MB-231 human breast triple negative cancer cells were seeded in a well of a 12-well plate and allowed to reach confluence. After reaching confluence, a gap of approximately 600  $\mu\text{m}$  was made in the central region of the well using a sterile tip. The medium and cellular debris were removed. The drug releasate (1 mL) which is prepared in a similar manner as described in section 2.7 (a) and was added to this well. After 24 h, the cells were stained using calcein AM and viewed using spinning disc confocal laser scanning microscope (Zeiss, USA) using red (526 nm excitation and 555 nm emission) and green (490 nm excitation and 520 nm emission) fluorescence.

#### *(f) Internalization on MDA- MB- 231 spheroids*

Tumor spheroids were prepared using MDA-MB- 231 cells on an agarose coated 96 well plate. The spheroids were allowed to form and then treated with the releasate. After 24 h, the cells were viewed using spinning disc confocal laser scanning microscope (Zeiss, USA) using red (526 nm excitation and 555 nm emission) fluorescence.

### **1.7. Statistical analysis**

Statistical analysis of data was performed by one-way analysis of variance (ANOVA), assuming confidence level of 95 % ( $p > 0.05$ ) and 99 % ( $p > 0.01$ ) for statistical significance. All data were expressed as Mean  $\pm$  standard deviation (S.D).

## 2. Results and discussion (additional figures)

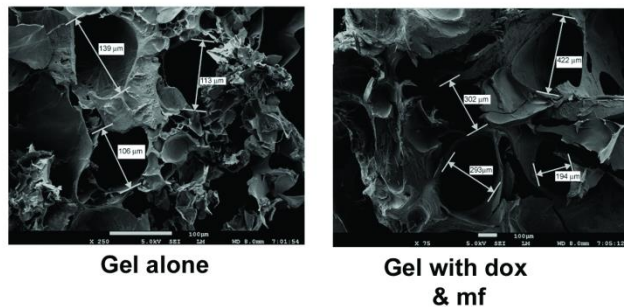


Figure S1. SEM images of gel alone and gel with dox & mf.

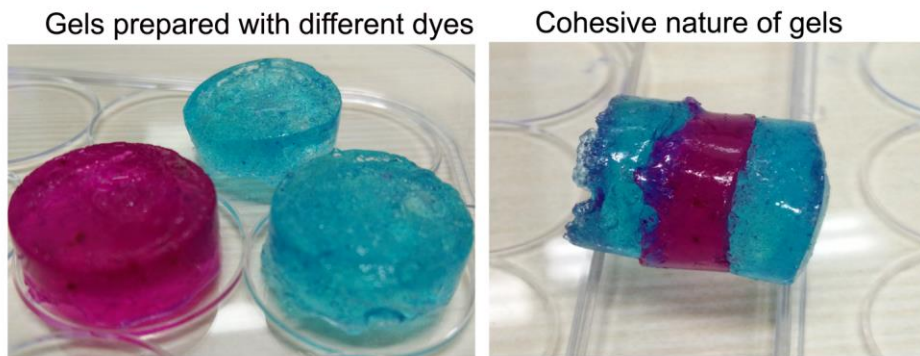
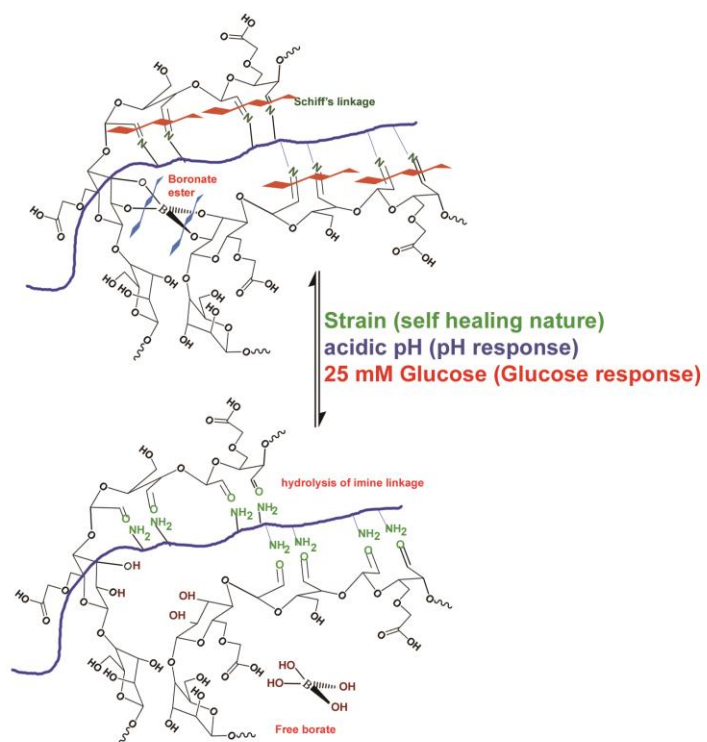
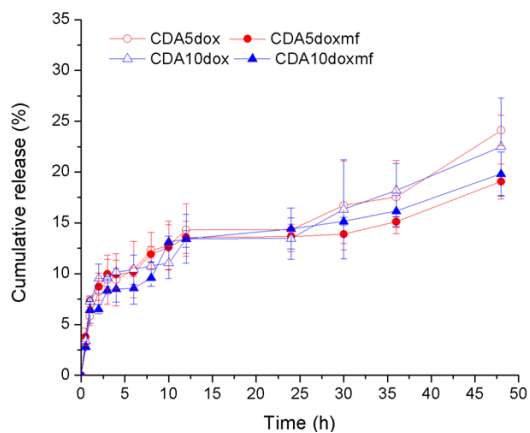


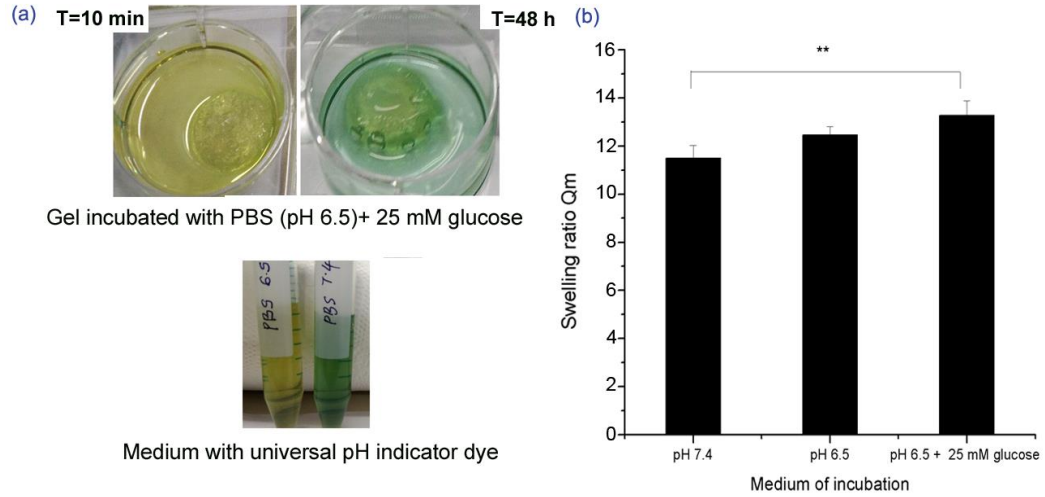
Figure S2. Self healing property gels



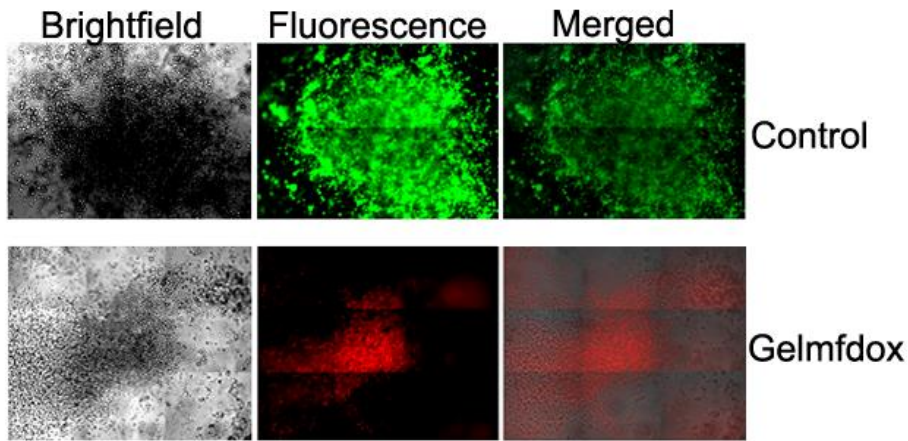
**Figure S3. Schematic representation of changes occur in gel structure upon exposed to strain, reduced pH and glucose**



**Figure S4. Release profile of gels with different concentrations of dox and mf**



**Figure S5.** Change in the pH of medium as illustrated by change in colour of medium having universal pH indicator in which gel is incubated (a); effect of pH and glucose on the swelling ratio of gels (b).



**Figure S6.** Effect of gel releasate on tumor spheroid growth prevention on tumor spheroids.