Electronic Supplementary Material (ESI) for RSC Advances.

Supplementary information

Supermolecular Peptide Amphiphiles based Nanocarrier for pH-triggered Release, Overcoming Drug Resistance

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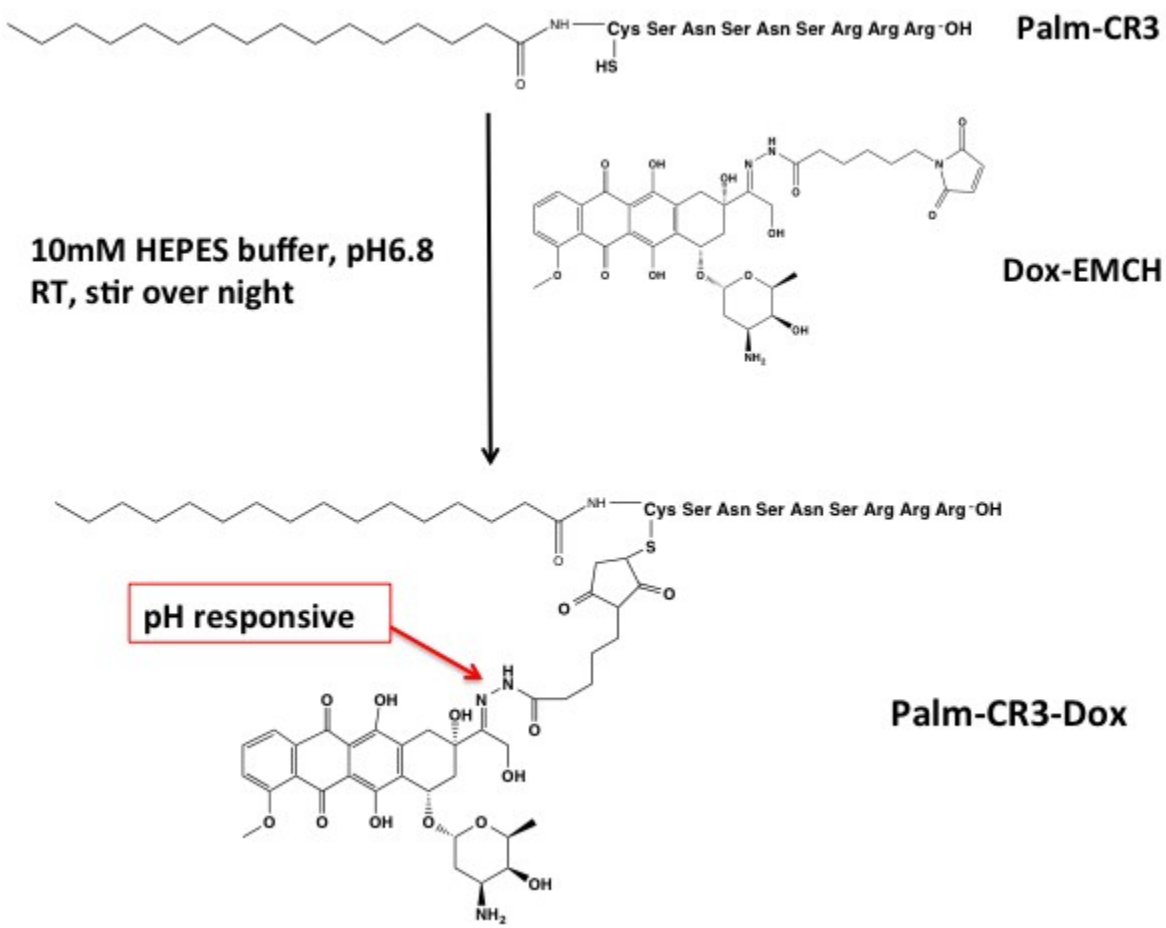
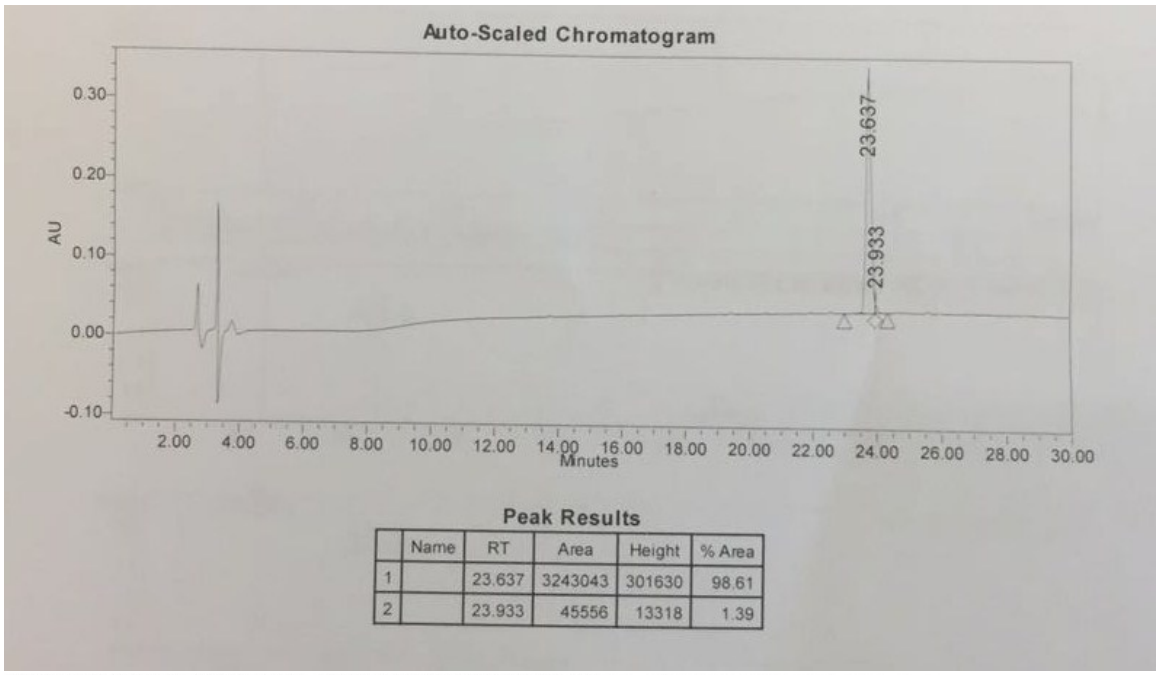


Figure S1 The schematic illustration of the reaction between Palm-CR3 and Dox-EMCH in HEPES buffer.



Peak Results

	Name	RT	Area	Height	% Area
1		23.637	3243043	301630	98.61
2		23.933	45556	13318	1.39

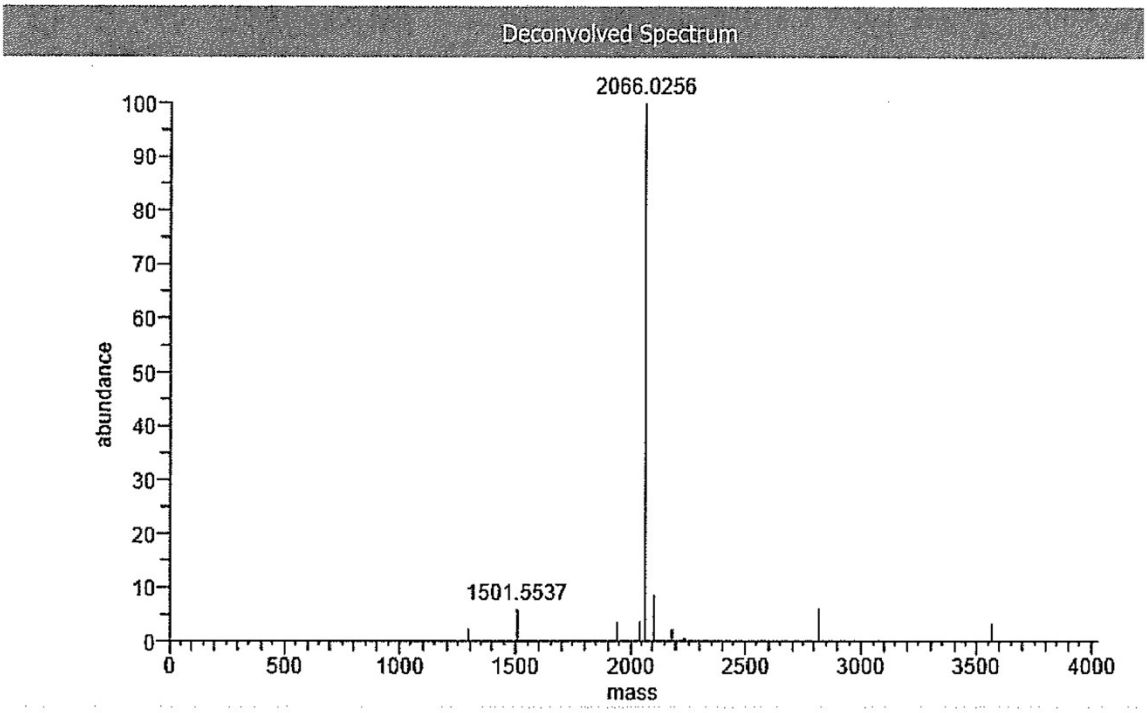


Figure S2 HPLC spectra of Palm-CR3 (Top) provided by the manufacture and MS results for Palm-CR3-Dox (bottom). The calculated molecular weight (MW): 2066.92 for Palm-CR3-Dox.

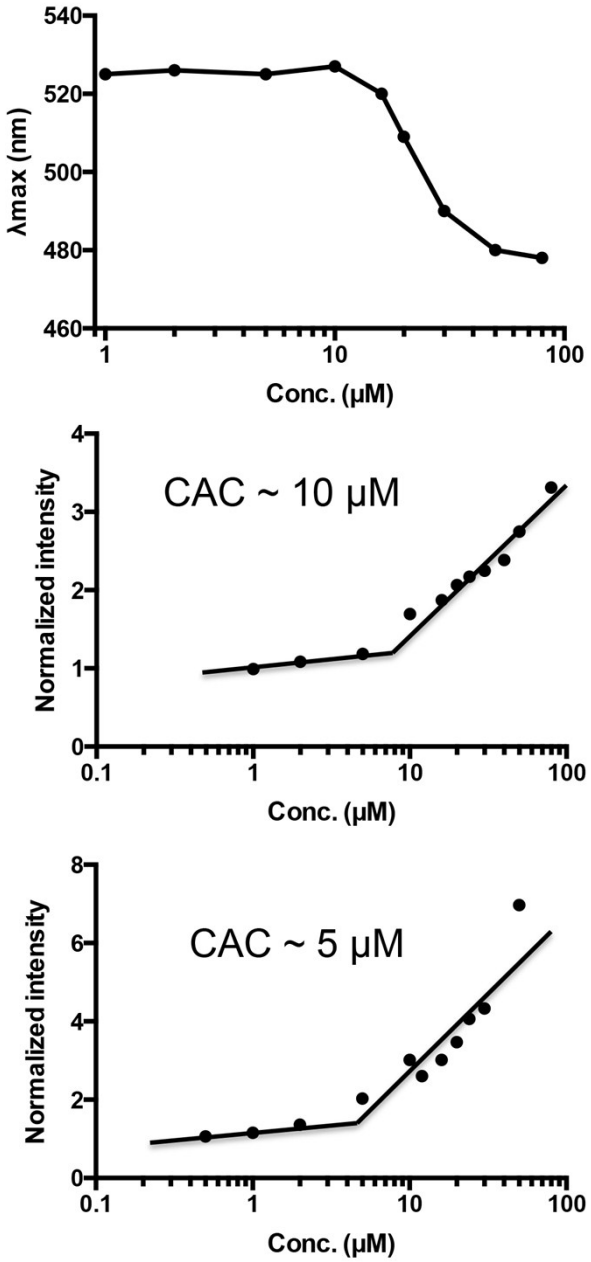
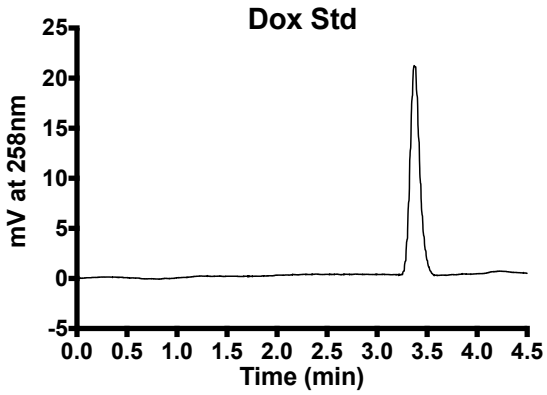
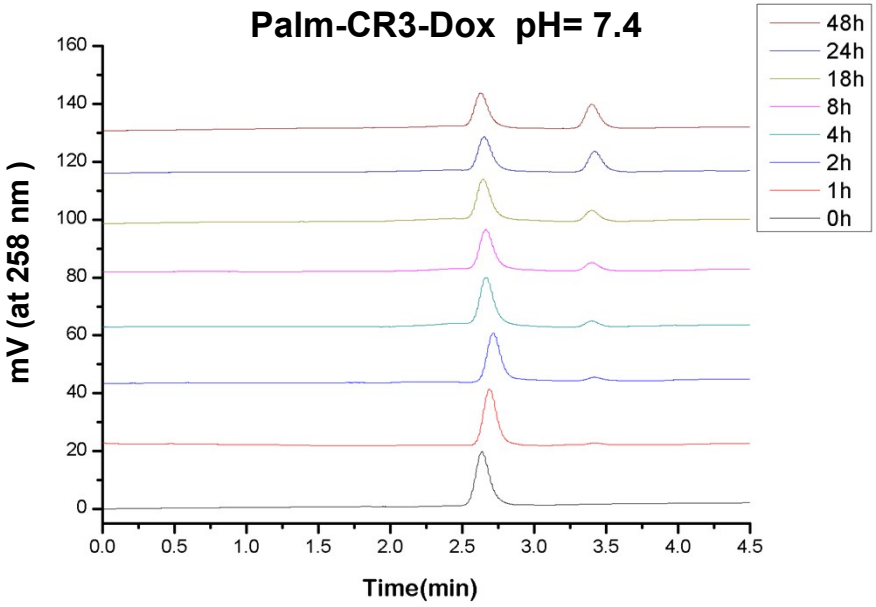


Figure S3 The critical assembly concentrations of Palm-CR3 and Palm-CR3-Dox determined by ANS fluorescence assay and/or static light scattering technique. The emission peak of ANS incubated with Palm-CR3 starts to shift significantly when Palm-CR3 concentration is above 10 μM (top). Similarly, the static light scattering intensities indicate that Palm-CR3 has a CAC ~10 μM (middle); while Palm-CR3-Dox has a CAC ~5 μM (bottom). Because Dox absorbs the emission of ANS, the CAC of Palm-CR3-Dox was only determined by static light scattering technique.


Dox Std
mV at 258nm
25
20
15
10
5
0
-5
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
Time (min)


Palm-CR3-Dox pH= 7.4
mV (at 258 nm)
160
140
120
100
80
60
40
20
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
Time(min)
48h
24h
18h
8h
4h
2h
1h
0h

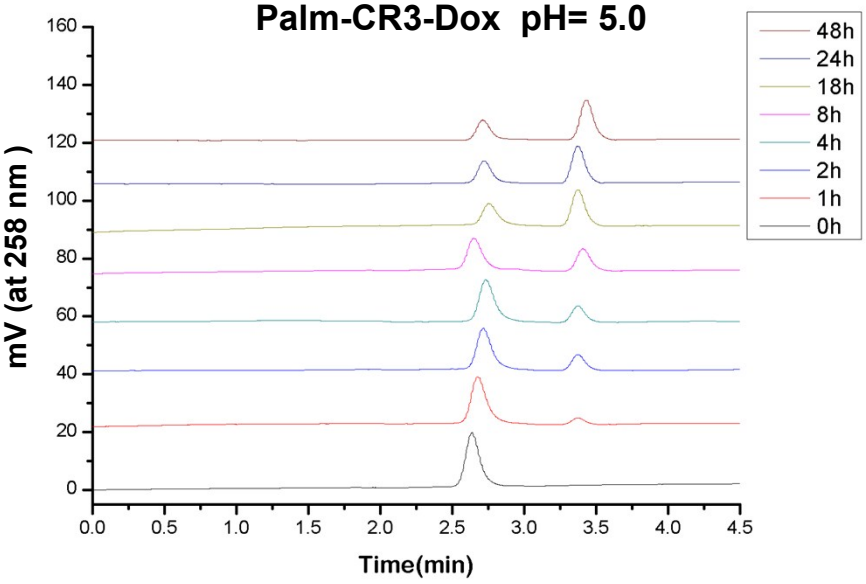

Palm-CR3-Dox pH= 5.0
mV (at 258 nm)
160
140
120
100
80
60
40
20
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
Time(min)
48h
24h
18h
8h
4h
2h
1h
0h

Figure S4 HPLC spectra of Dox standard sample and Palm-CR3-Dox at pH 7.4 or 5.0 over different incubation times. The peaks at ~2.7 min represent Palm-CR3-Dox and the ones at ~3.4 min represent Dox.

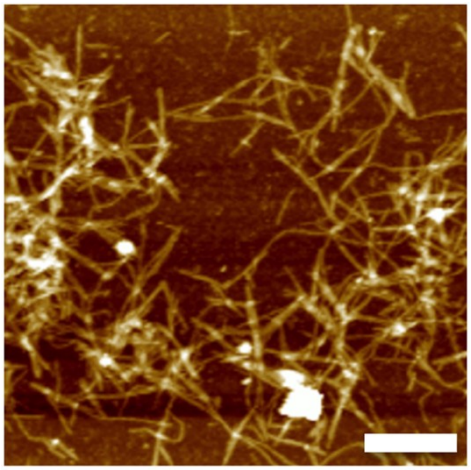


Figure S5 AFM image of Palm-CR3-Dox incubated in pH 5.0 buffer solution for 48 h. The scale bar is 200 nm.

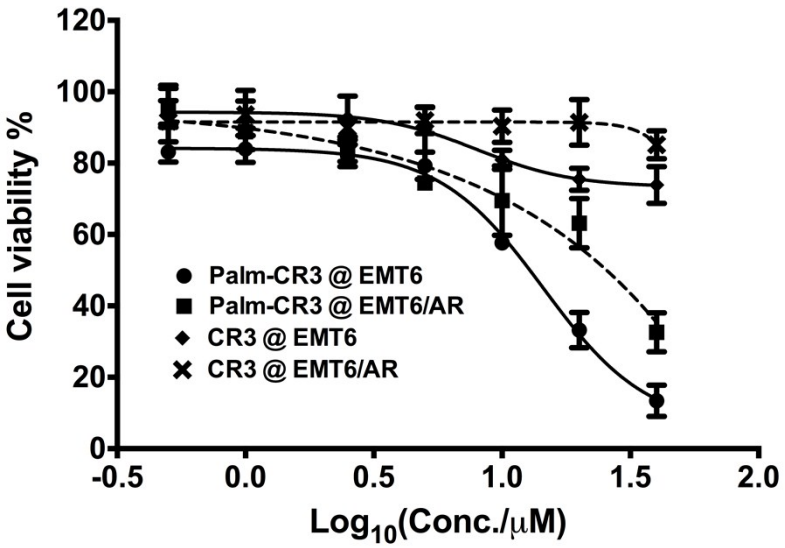


Figure S6 Cytotoxicity of Palm-CR3 and CR3 against EMT6 or EMT6/AR cells. IC_{50} values of Palm-CR3 against EMT6 and EMT6/AR was ~14.45 and 29.8μM, respectively.

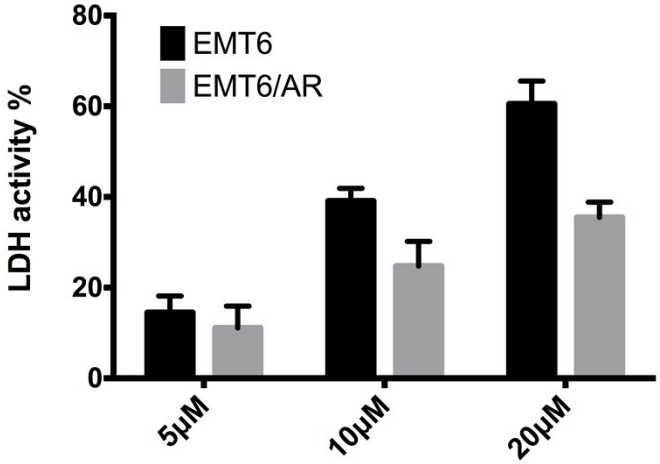


Figure S7 LDH activity of Palm-CR3 against EMT6, EMT6/AR cells.

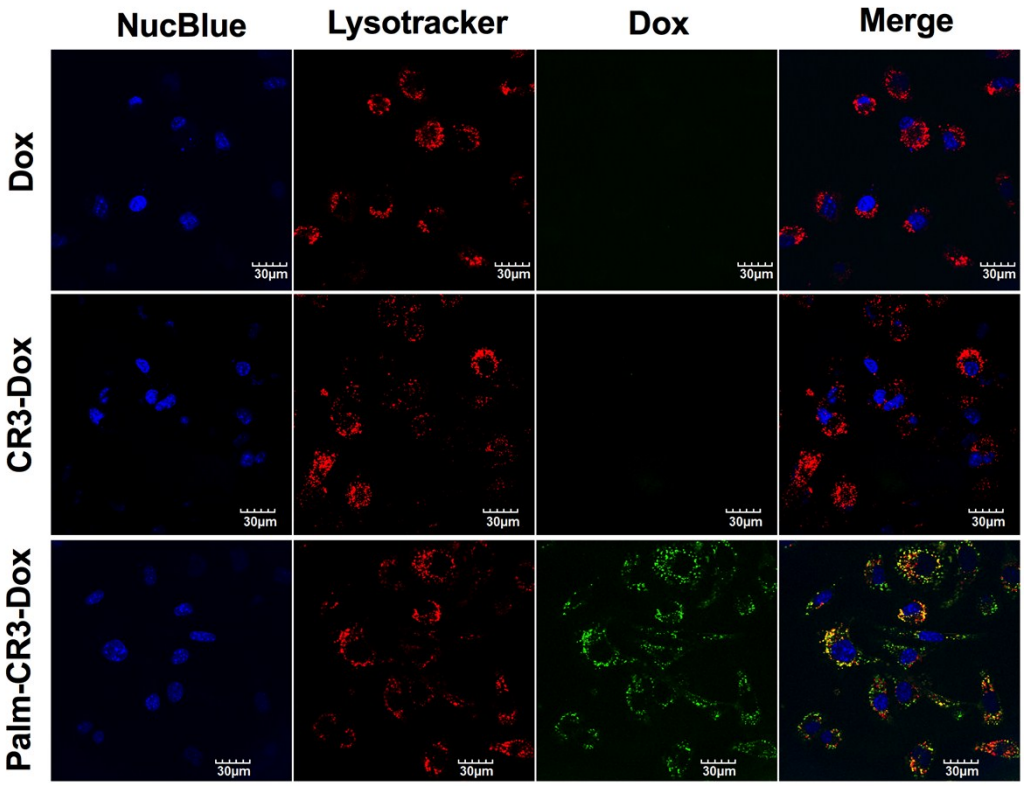


Figure S8 CLSM images of EMT6/AR cells that were treated with Dox, CR3-Dox or Palm-CR3-Dox for 2h. Prior to observation on confocal microscopy, nuclei were stained with Nucblue, acidic compartments with Lysotracker deep red. The scale bars correspond to 30µm.

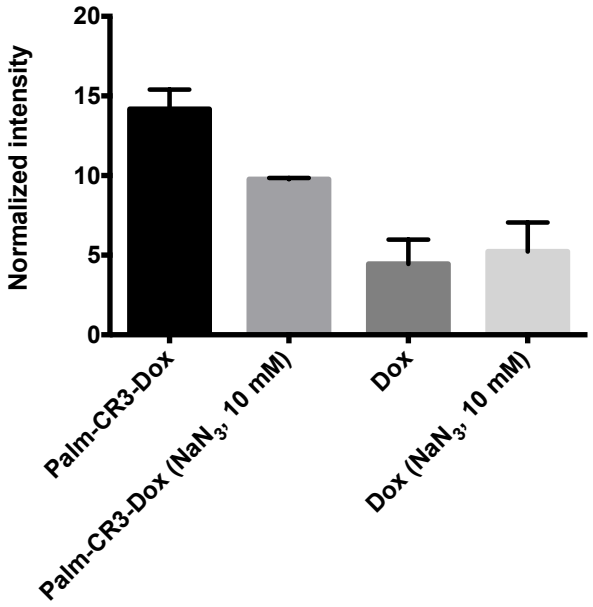


Figure S9 The cellular uptake of Dox and Plam-CR3-Dox into EMT6/AR cells. The endocytic activity was inhibited by pre-incubating cells with 10 mM sodium azide for 30 min.[1] The fluorescence intensity of Dox was normalized to non-treated cells.

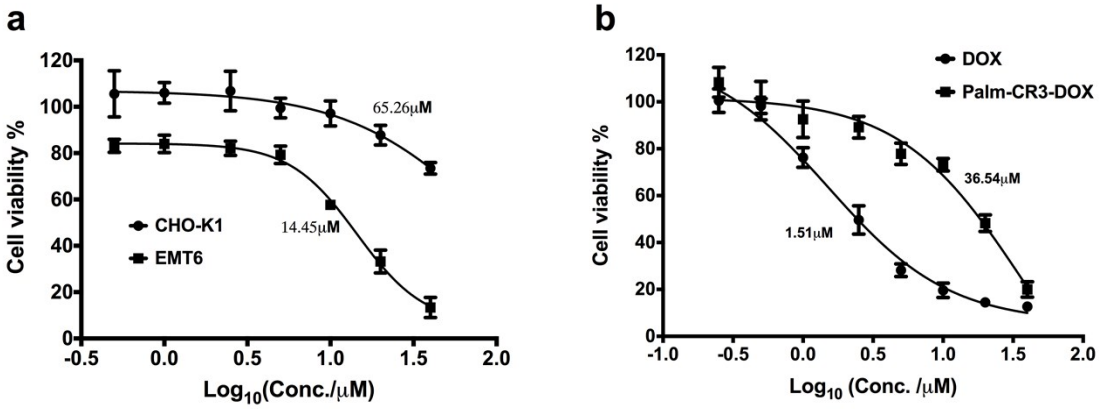


Figure S10 The cytotoxicity of Palm-CR3 on CHO-K1 and EMT6 with the incubation of 24h (a); and cytotoxicity of Dox or Palm-CR3-Dox towards CHO-K (b).

Experimental

Materials

The peptides were purchased from CanPeptide (Montreal, Canada), which were further treated with VariPure IPE column (Agilent, Canada) to remove remaining trifluoroacetic acid (TFA), followed with lyophilization in lab prior to use. α-Minimum Essential Medium (MEM) was purchased from Life Technologies, while fetal bovine serum (FBS) was purchased from Sigma (Oakvile, Canada). Dye used in samples preparation for confocal microscope: Lysotracker Deep red, Nucblue Live Ready probes reagent (Life technology).

Cell culture

The EMT6 mouse breast cancer cell line and its doxorubicin resistant counterpart (EMT6/AR) were gifts from Dr. Shirley Wu' lab in University of Toronto. EMT6 cells were cultured in α-MEM medium supplemented with 10% fetal bovine serum (FBS), while Doxorubicin (Dox) was added to the culture medium at 1μg/mL for EMT6/AR. CHO-K1 (Chinese hamster ovary) cells were purchased from American Type Culture Collection (ATCC, Manassas, USA). All of the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Synthesis of peptide-drug conjugates

The peptide Palm-CR3 and Dox-EMCH•HCl were dissolved in 10mM HEPES buffer (pH 6.8) at 0.50mM respectively. After the sonication of 0.5h for complete dissolution, the two solutions were mixed upon the volume ratio at 1:1.2 (peptide over drug), and stirred overnight. Then the purification was conducted on a Waters 600 reverse-phase high performance liquid chromatography (RP-HPLC) with a Vydac C18 column, using 0.1% TFA water and 0.1% TFA acetonitrile as eluents. The effluents of Palm-CR3-Dox were treated with Vari Pure IPE column (Agilent, Canada) to remove TFA, and concentrated with rotating evaporator. The white powder was obtained after lyophilization, and the molecular weight was confirmed with ESI-MS on Waters Q-TOF Ultima mass spectrometer. CR3-Dox was synthesized with the same method.

Morphology characterization with atomic force microscopy (AFM) and transmission electron microscopy (TEM)

The nano-structure of the aggregates of Palm-CR3 or Palm-CR3-Dox was observed with atomic force microscopy. Briefly, 100μL sample solution was dropped on the surface of a freshly cleaved mica sheet for 5mins. Then free peptide was removed by washing with 300μL pure water. After air-drying, AFM imaging was taken at room temperature using the Peak Force tapping mold on a Dimension Icon AFM (Bruker, Santa Barbara, CA). To confirm the structures acquired from AFM, TEM was also utilized. 10μL solution was applied to a 400 mesh Formvar coated copper grid for 5 minutes, which was followed by negative staining with 10μL 2% uranyl acetate, blotted drying, and analysis on TEM (Philips CM10 TEM, operating at 60KeV).

Circular dichroism (CD) spectra

Far-UV circular dichroism spectra of the samples were measured at room temperature using a Jasco J-815 CD spectrometer (Tokyo, Japan). 150μL solution was scanned in a 0.1cm quartz cell from 190 to 260nm. Solvent spectrum was used as the baseline for data collection. The final spectrums of all samples shown here were an average of three independent measurements.

Particle size measured by dynamic light scattering (DLS)

The hydrodynamic diameter of the aggregates was measured using DLS on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a 4mW He-Ne laser operating at 633nm. 50μL solution was added in low volume disposable polystyrene cuvette and the scattered light intensities were collected at an angle of 173°. Three independent measurements were performed to generate the intensity based size distribution profile.

Critical aggregate concentration determination

The critical aggregate concentration (CAC) of Palm-CR3 was determined by monitoring the changes of 8-Anilinonaphthalene-1-sulfonic acid (ANS) fluorescence after mixing with Palm-CR3 solutions. ANS solution (10 μM, in a 10 mM phosphate buffer at pH 6) was mixed with Palm-CR3 solutions at concentrations range from 0.5 to 80 μM at 1:1 volume ratio. The mixtures were vortexed for 10 s, and then tested on the Photon Technology International spectra fluorometer (Type QM4-SE, London, Canada) with excitation wavelength set at 360 nm. The ANS fluorescence spectrum was collected from 420 to 670 nm.

The light scattering experiments were carried out on the same instrument to determine the CACs of Palm-CR3 and Palm-CR3-Dox. The samples were irradiated at 310 nm, and the scattered light was collected from 295 to 325 nm. The light intensity of each sample was averaged from 308 to 312 nm, and normalized with the averaged intensity of solvent.

***In-vitro* drug release**

The release of Dox from Palm-CR3-Dox conjugate was measured at 37°C in two different buffers: acetate buffer, pH 5.0 and HEPES buffer, pH 7.4. The stock solution of Palm-CR3-Dox was prepared at 0.30 mM in Milli-Q water. An aliquot of 50 μL of solution was transferred to 950μL buffer in an eppendorf tube, which was kept at 37°C. At certain time intervals (0, 1, 2, 4, 8, 12, 24, 48h), 10μL sample was taken out and analyzed on HPLC. The analysis was carried out on a Waters 600E HPLC equipped with a UV detector, using 0.1% TFA water and 0.1% TFA acetonitrile as eluents. The retention time for Dox was determined with a standard Doxorubicin hydrochloride solution at 15μM, and the amount of released Dox was calculated by dividing the chromatographic peak area of Dox in buffer solution by the peak area of the one in the standard solution. The experiments were conducted in triplicate.

Cytotoxicity assay

Cytotoxicity of the peptide or peptide-Dox conjugated against EMT6, EMT6/AR or CHO-K1 cells was evaluated using Cell Counting Kit-8 (CCK-8) assay. EMT6/AR cells were seeded in 96-well plated at the density of 10000 cells/well, while EMT6 or CHO-K1 cells were seeded at the density of 7500 cells/well. The seeded cells were incubated overnight prior the treatment. After the medium was removed, 150μL fresh medium was added, and followed by the treatment of 50μL peptide or peptide-drug conjugate solution. With the incubation of 24h, CCK-8 kit assay was performed following the manufactory's protocol. Cell viability was assessed by measuring the absorbance at 450nm with a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany) and expressed as the ratio of the cells with the treatment over the non-treated ones. The results presented are the average from three independent experiments. The error bars are standard deviation.

Fluorescence-activated cell sorting (FACS)

Cellular uptake of Dox in different forms was studied with flow cytometry (BD Biosciences, USA). EMT6 or EMT6/AR cells were treated with the samples at 5μM for 2h, which were then thoroughly washed with PBS, and fixed with 4% paraformaldehyde, prior to the analysis on FACS.

Intracellular distribution of drug or peptide-drug conjugates

The cellular uptake and intracellular release behaviours of Palm-CR3-Dox were monitored with confocal laser scanning microscopy (CLSM). EMT6 cells were seeded in a 4-well Lab-Tek chamber slide with 80000 cells in 450μL medium per well and incubated at 37°C over night. Then the cells were treated with Palm-CR3-Dox (final concentration at 5μM) for 2h, which were thoroughly washed three times with PBS to remove the free conjugates, and re-cultured in fresh medium. After desired time intervals (i.e. 0.5, 4, 12h), the cells were stained with Lysotracker Deep red for the acidic organelles, and NucBlue for the nuclei. Afterwards, Zeiss LS700 microscopy was utilized to visualize the cells. The co-localization of Dox with Lysotracker or Nucblue was expressed by the Mander's overlapping co-efficient, calculated with ImageJ software. The experiments were repeated for two times. Similar procedures were applied to EMT6/AR cells, which were treated with Dox, CR3-Dox, or Palm-CR3-Dox for 2h, in order to investigate the cellular uptake by drug resistant cells.

Reference

1. **H. L. Gao, Z. Yang, S. Zhang, S. J. Cao, S. Shen, Z. Q. Pang and X. G. Jiang, *Scientific reports*, 2014, 4.**