Supporting information

Concurrent targeted delivery of doxorubicin and curcumin to the cancer cells using ligand installed multifaceted chitosan-based simple and versatile nanoconjugates

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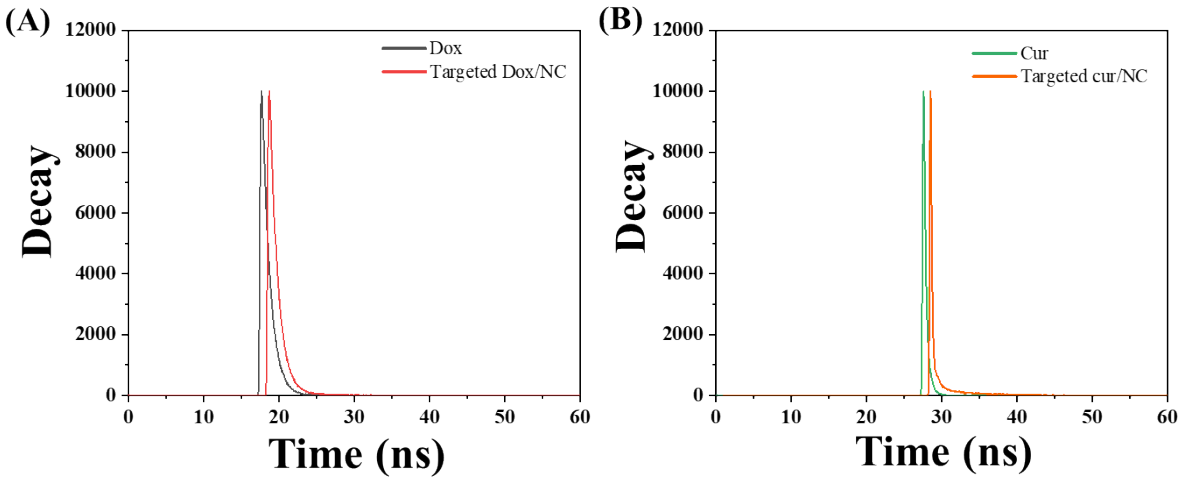
Table S1. Represents the data used for calculation of drug loading %. The term initial indicates the concentration before drug loading as taken by weight; final means the concentration after the drug loading reaction and removal of any free drug molecules using extensive dialysis in different solvents as mentioned in experimental section and measured by using Beer lambert law.

Samples	Curcumin concentration (mg/ml)	Doxorubicin concentration (mg/ml)
Targeted cur/NC	Initial = 10.17 mg/ml, Abs = 0.527 (1000 times dilute) Final = 4.16 mg/ml 41% loaded curcumin Abs = 0.215 (1000 times dilute)	---
Targeted dox/NC	----	Initial = 1.6 mg/ml Abs = 0.52 (100 times dilute) Final = 0.72 mg/ml Abs = 0.23 (100 times dilute) 45 % loaded
Targeted cur+dox/NC	Initial = 2 mg/ml Abs = 0.103 (1000 times dilute) Final = 0.69 mg/ml Abs = 0.357 (100 times dilute) 34.5% loaded curcumin	Initial = 0.83 mg/ml Abs = 0.27 (100 dilute) Final = 0.25 mg/ml Abs = 0.81 (10 times dilute) 31% loaded
Non-targeted cur/NC	Initial = 10.68 mg/ml Abs = 0.553 (1000 times dilute) Final = 3.73 mg/ml Abs = 0.193 (1000 times dilute) 35 % loaded curcumin	---
Non-targeted dox/NC	----	Initial = 1.25 mg/ml Abs = 0.40 (100 times dilute) Final = 0.47 mg/ml Abs = 0.15 (10 times dilute) 38 % loaded
Non-targeted cur+dox/NC	Initial = 1.5 mg/ml Abs = 0.777 (100 times dilute) Final = 0.49 mg/ml Abs = 0.254 (100 times dilute) 33.2 % loaded curcumin	Initial = 1 mg/ml Abs = 0.32 (10 times dilute) Final = 0.3 mg/ml Abs = 0.97 (10 times dilute) 30 % loaded

Figure S1. Representative sample images as prepared. (A) Targeted cur/NC, (B) Targeted dox/NC, (C) Targeted cur+dox/NC, (D) Non-targeted cur/NC, (E) Non-targeted dox/NC, (F) Non-targeted cur+dox/NC. All the samples were dispersed in water.

Samples	Curcumin	Doxorubicin	Curcumin + doxorubicin
Targeted NCs	 (A) Targeted cur/NC	 (B) Targeted dox/NC	 (C) Targeted cur+dox/NC
Non-targeted NCs	 (D) Non-targeted cur/NC	 (E) Non-targeted dox/NC	 (F) Non-targeted cur+dox/NC

Figure S2. Time-correlated single-photon counting (TCSPC): (A) Targeted dox/NC and (B) Targeted cur/NC. The free doxorubicin and targeted dox/NC both emission wavelength was 500 nm, the average PL decay lifetime was increased from 17.75 to 18.71 ns because of doxorubicin was encapsulated in targeted dox/NC; similarly, the curcumin and targeted cur/NC both emission wavelength 425 nm, the average PL decay lifetime was increased from 27.53 to 28.48 ns because

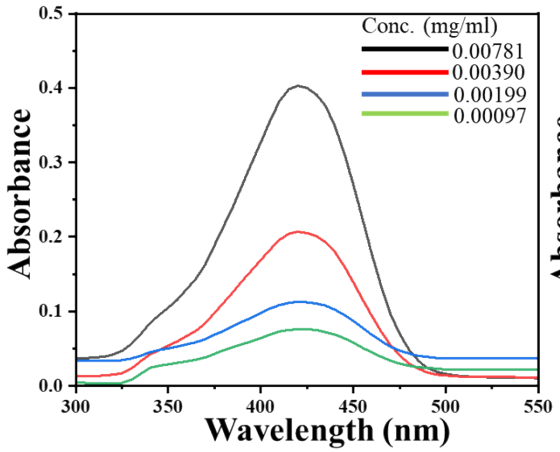


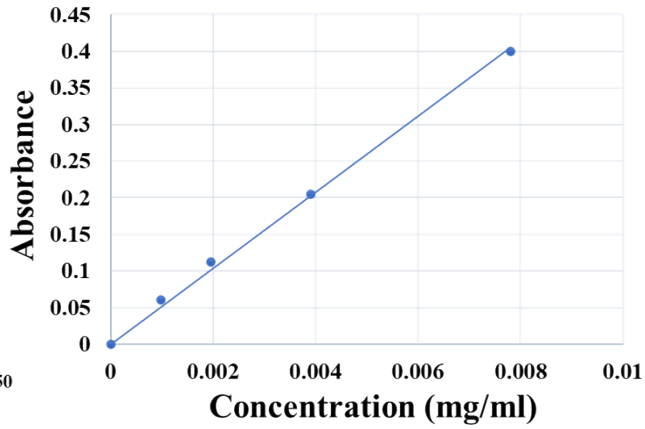
of curcumin was encapsulated in targeted cur/NC.

Figure S3. The calibration curve of curcumin. A series of dilution of curcumin was taken and their absorbance values were measured at 425 nm wavelength. The drug loading percentage was calculated by obtaining the equation Absorbance = 51.86*concentration. The concentration of loaded curcumin was 4.16 mg/ml in targeted cur/NC and 0.69 mg/ml in targeted cur+dox/NC (at absorbance value 0.215 with 1000 times diluted to the stock sample and absorbance value 0.357 with 100 times diluted to the stock sample respectively). Similarly, the concentration of loaded curcumin was 3.73 mg/ml for non-targeted cur/NC and 0.49 mg/ml for non-targeted cur+dox/NC (absorbance value 0.193 with 1000 times diluted to the stock sample and absorbance value 0.254 with 100 times diluted to the stock samples respectively).

Drug loading percentage = (final concentration of drug / initial concentration of drug) × 100%

From the calculations, drug loading efficiency of curcumin was 41% and 34.5% for targeted cur/NC and targeted cur+dox/NC; 35% and 33.2% for non-targeted cur/NC and non-targeted cur+dox/NC respectively.





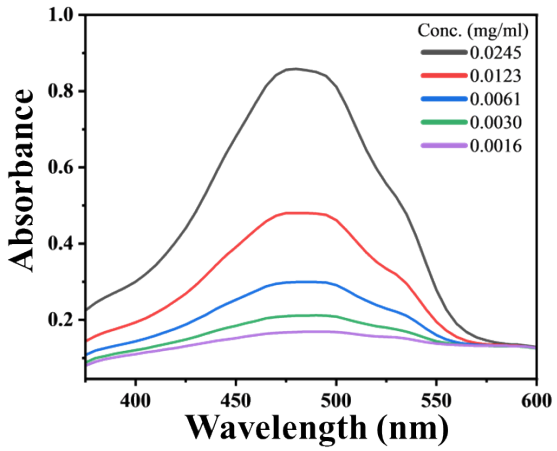
Calibration curve for curcumin
Abs. = 51.863 Conc.

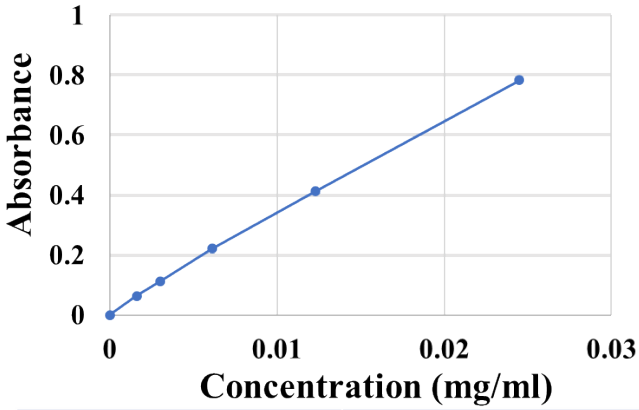
Absorbance	Concentration (mg/ml)
0	0
0.4	0.007813
0.205	0.003906
0.112	0.001953
0.061	0.000977

Figure S4. The calibration curve of doxorubicin: A series of dilution of doxorubicin was taken and their absorbance values at 500 nm wavelength were measured. Doxorubicin loading % was calculated by obtaining the equation Absorbance = 32.54*concentration. The concentration of loaded doxorubicin was 0.72 mg/ml in targeted dox/NC and 0.25 mg/ml in targeted cur+dox/NC (at absorbance value 0.23 with 100 times diluted to the stock sample and absorbance value 0.81 with 100 times diluted to the stock sample respectively). Similarly, the concentration of loaded curcumin was 0.47 mg/ml for non-targeted dox/NC and 0.30 mg/ml for non-targeted cur+dox/NC (absorbance value 0.15 with 10 times diluted to the stock sample and absorbance value 0.97 with 10 times diluted to the stock samples respectively).

Drug loading percentage = (final concentration of drug / initial concentration of drug) × 100%

From the calculations, drug loading efficiency of doxorubicin was 45% and 31% for targeted dox/NC and targeted cur+dox/NC; 38% and 30% for non-targeted dox/NC and non-targeted cur+dox/NC respectively.



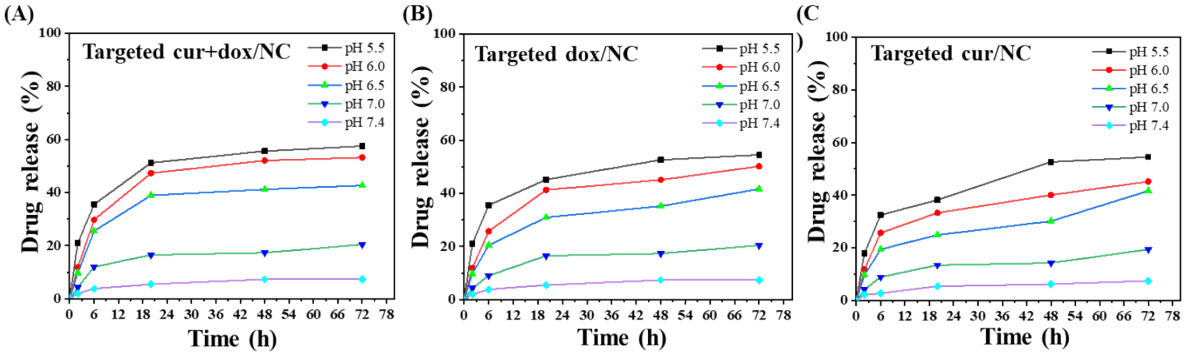


Calibration curve for doxorubicin

Abs. = 32.54 Conc.

Absorbance	Concentration (mg/ml)
0	0
0.064	0.001618
0.112	0.003047
0.221	0.00611
0.4125	0.0123
0.7832	0.0245

Figure S5. Doxorubicin and Curcumin release study of (A) Targeted cur+dox/NC (B) Targeted dox/NC (C) Targeted cur/NC. The doxorubicin and curcumin release was studied in different pH (7.4, 7.0, 6.5, 6.0, 5.5) at different time intervals (2, 6, 20, 48 and 72h). More drugs



are released from the nanoconjugates under more acidic environment.

Figure S6: Particle size determination of nanoconjugates using HRTEM. The average diameter was 40-60 nm of nanoconjugates.

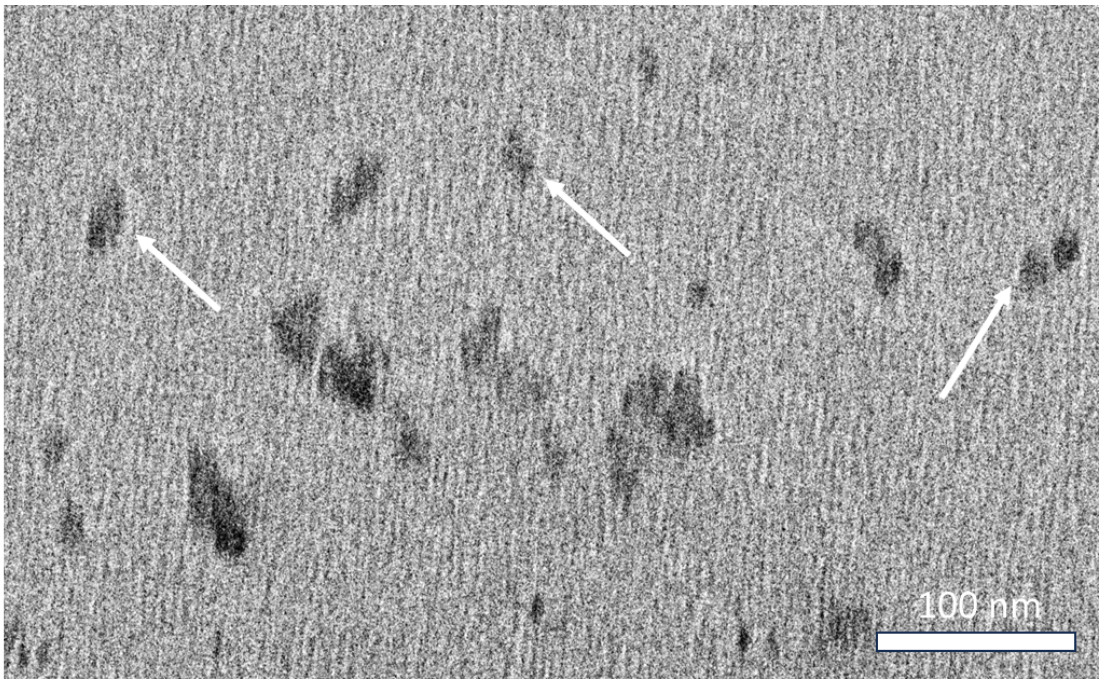
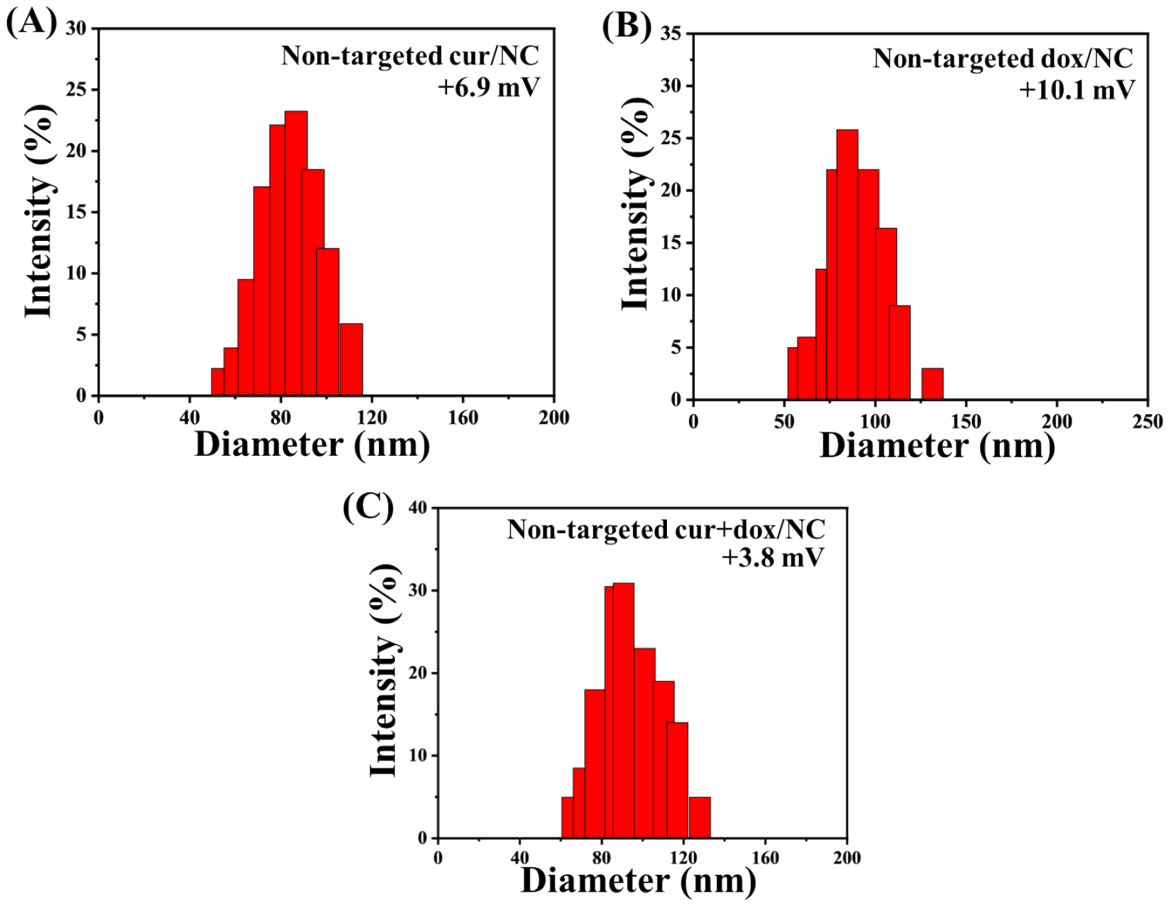
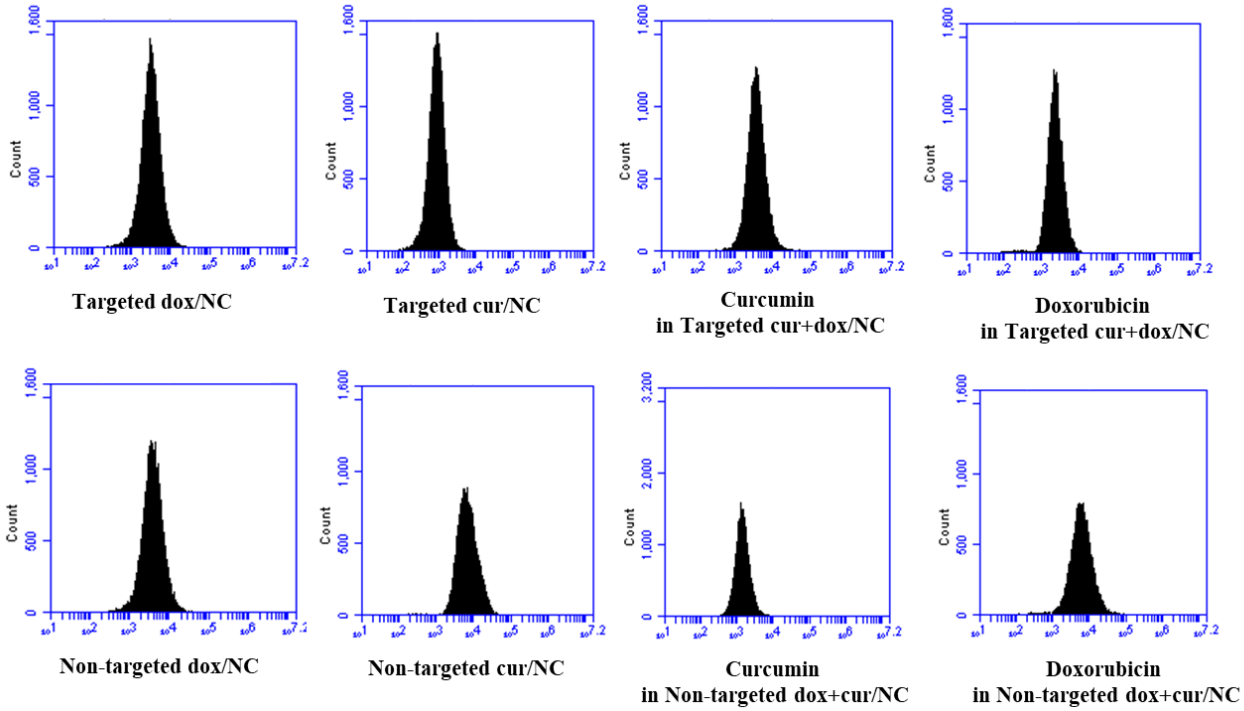


Figure S7: Particle size determination of non-targeted NCs. The average hydrodynamic diameter of was 80 nm, 85 nm, and 100 nm respectively for non-targeted cur/NC, non-targeted dox/NC, and non-targeted cur+dox/NC (A, B and C). The surface charge of all targeted NCs were



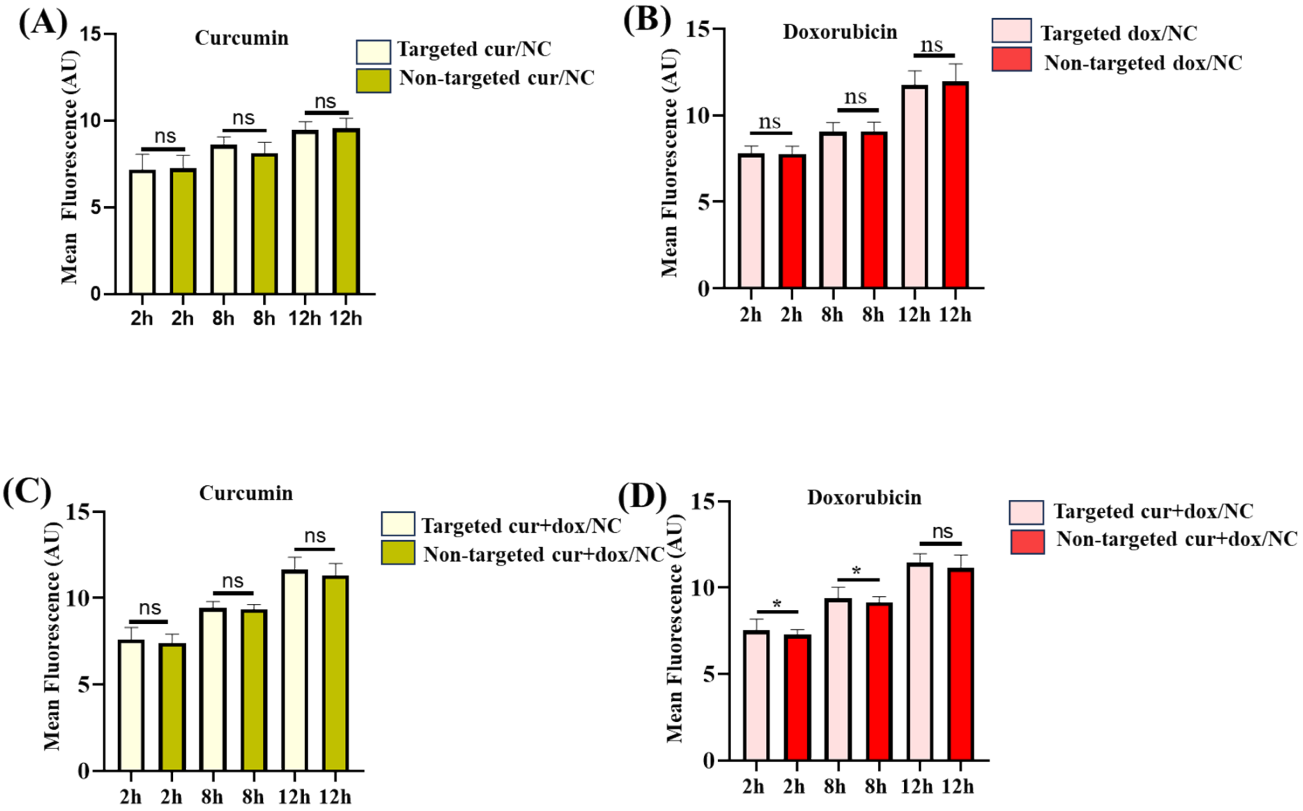
positive

Figure S8. Fluorescence intensity analysis by flow cytometry: Cellular uptake of targeted NCs compared with non-targeted NCs following 4h incubation in KB cells. Fluorescence intensity of doxorubicin and curcumin for targeted NCs is higher than non-targeted NCs confirms more



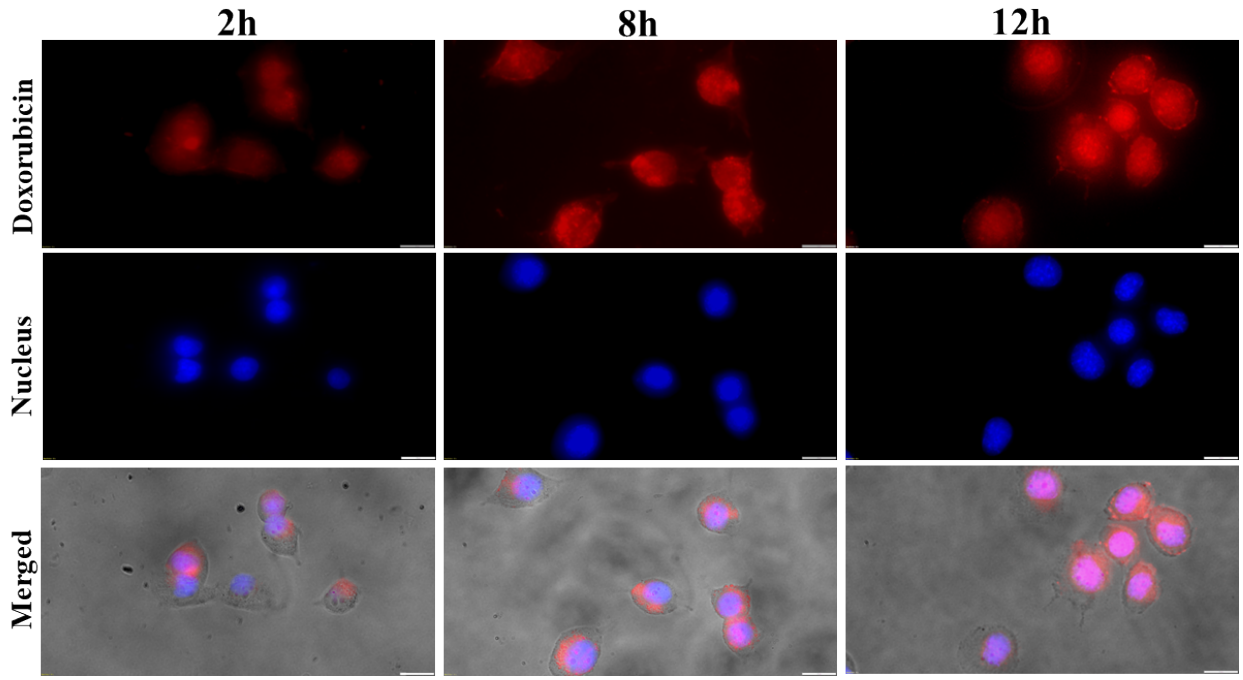
cellular uptake of targeted NCs than non-targeted NCs.

Figure S9. Cellular uptake analysis in KB cells cultured with folic acid in the medium. Quantitative analysis of cellular uptake of (A) targeted cur/NC, (B) Targeted dox/NC and targeted cur+dox/NC, (C and D) as compared with their respective non-targeted NCs following 2h, 8h and 12h incubation with KB cells cultured with folic acid in the medium. Targeted cur/NC did not show significant difference in cellular uptake of drugs compared to non-targeted NCs, with P value



summary: ns - not significant, * - significant.

Figure S10. Representative images of cellular uptake of targeted dox/NC in KB cells cultured without folic acid in the medium: Cellular uptake of targeted dox/NC following 2h, 8h and 12h incubation in KB cells cultured without folic acid in the medium. Cellular uptake increased



gradually when incubated for a longer period (scale bar: 20μm).

Figure S11. Representative images of cellular uptake of non-targeted dox/NC in KB cells cultured without folic acid in the medium: Cellular uptake of non-targeted dox/NC following 2h, 8h and 12h incubation in KB cells cultured without folic acid in the medium. Cellular uptake is low in all incubation time (scale bar: 20μm).

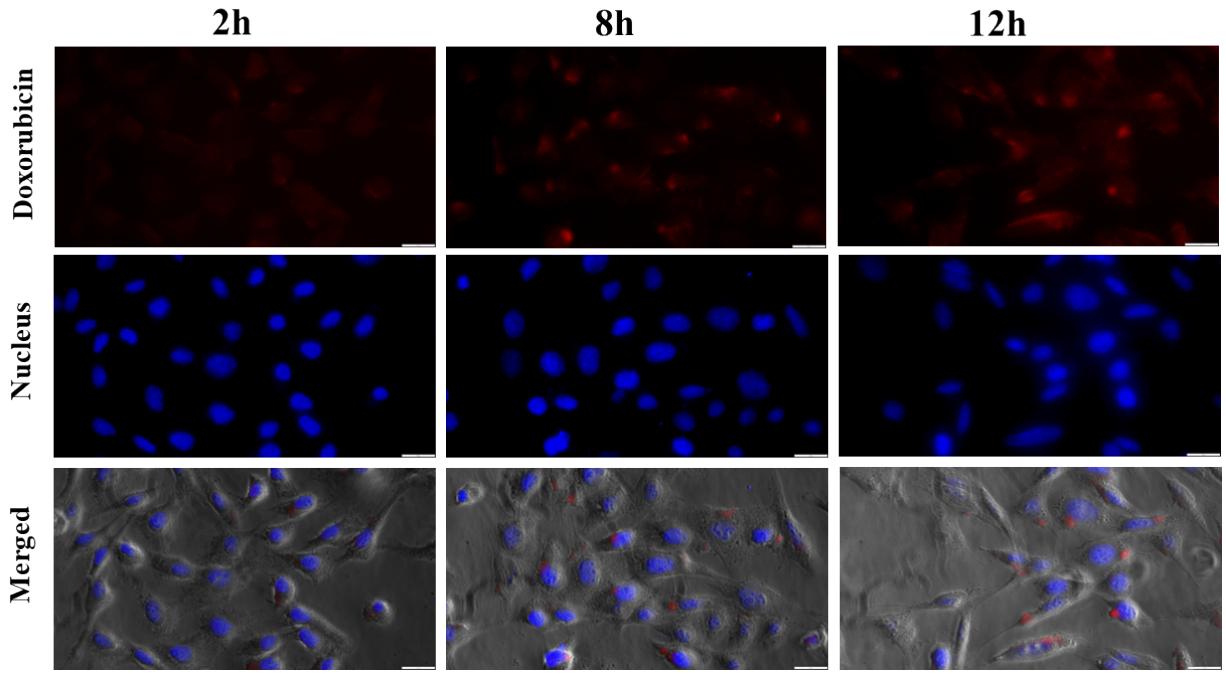
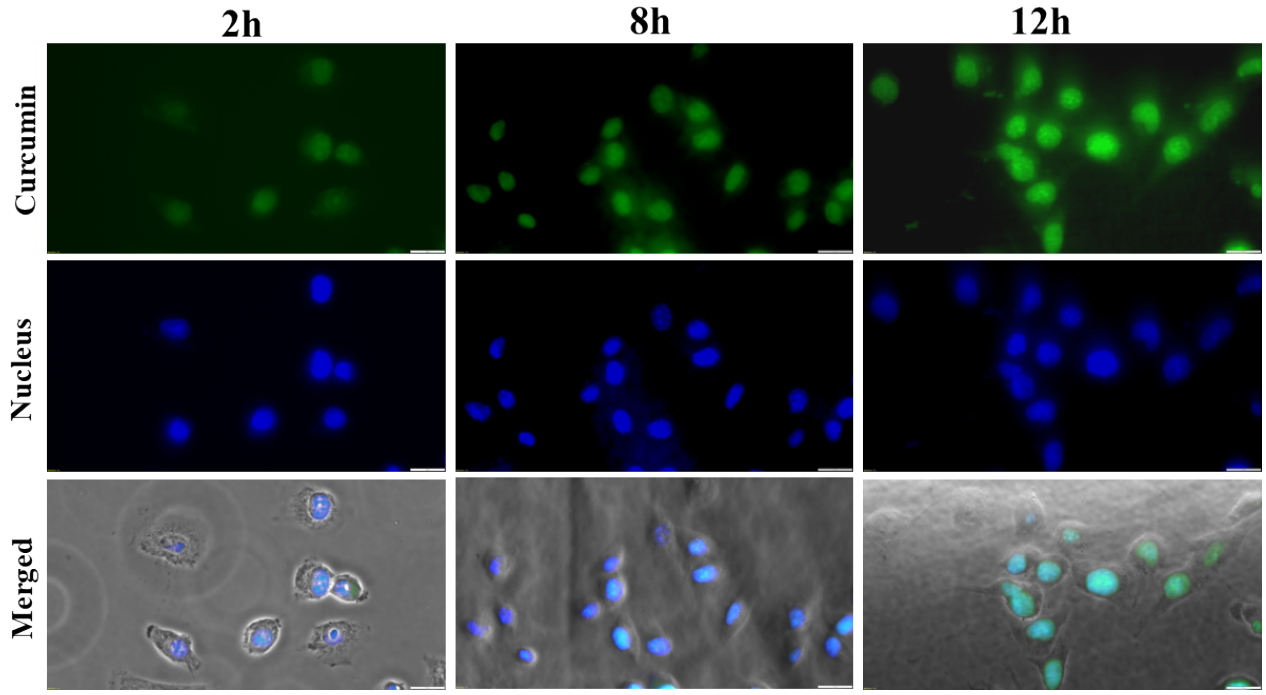
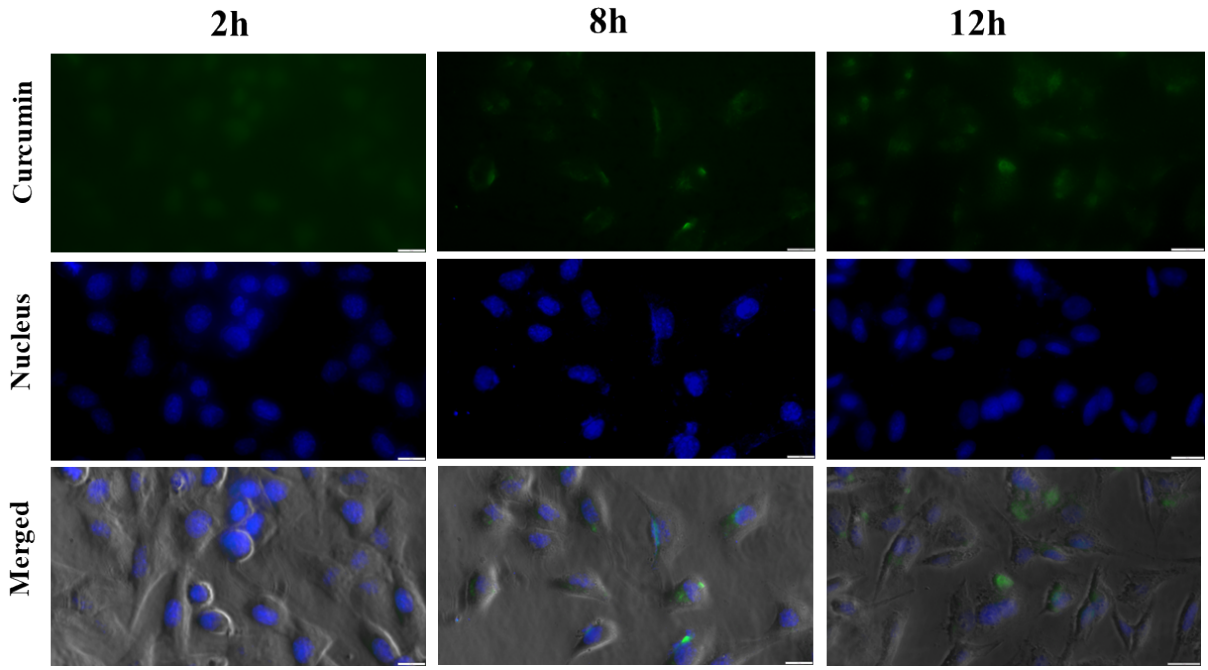


Figure S12. Representative images of cellular uptake of targeted cur/NC in KB cells cultured without folic acid in the medium: Cellular uptake of targeted cur/NC following 2h, 8h and 12h incubation in KB cells cultured without folic acid in the medium. Cellular uptake increased



gradually when incubated for a longer period (scale bar: 20μm).

Figure S13. Representative images of cellular uptake of non-targeted cur/NC in KB cells cultured without folic acid in the medium: Cellular uptake of non-targeted cur/NC following 2h, 8h and 12h incubation in KB cells cultured without folic acid in the medium. Cellular uptake



is low in all incubation time (scale bar: 20μm).

Figure S14. Representative images of cellular uptake of targeted cur+dox/NC in KB cells cultured with folic acid in the medium: Cellular uptake of targeted cur+dox/NC following 2h, 8h and 12h incubation in KB cells cultured with folic acid in the medium. Cellular uptake is low at different incubation time (scale bar: 20μm).

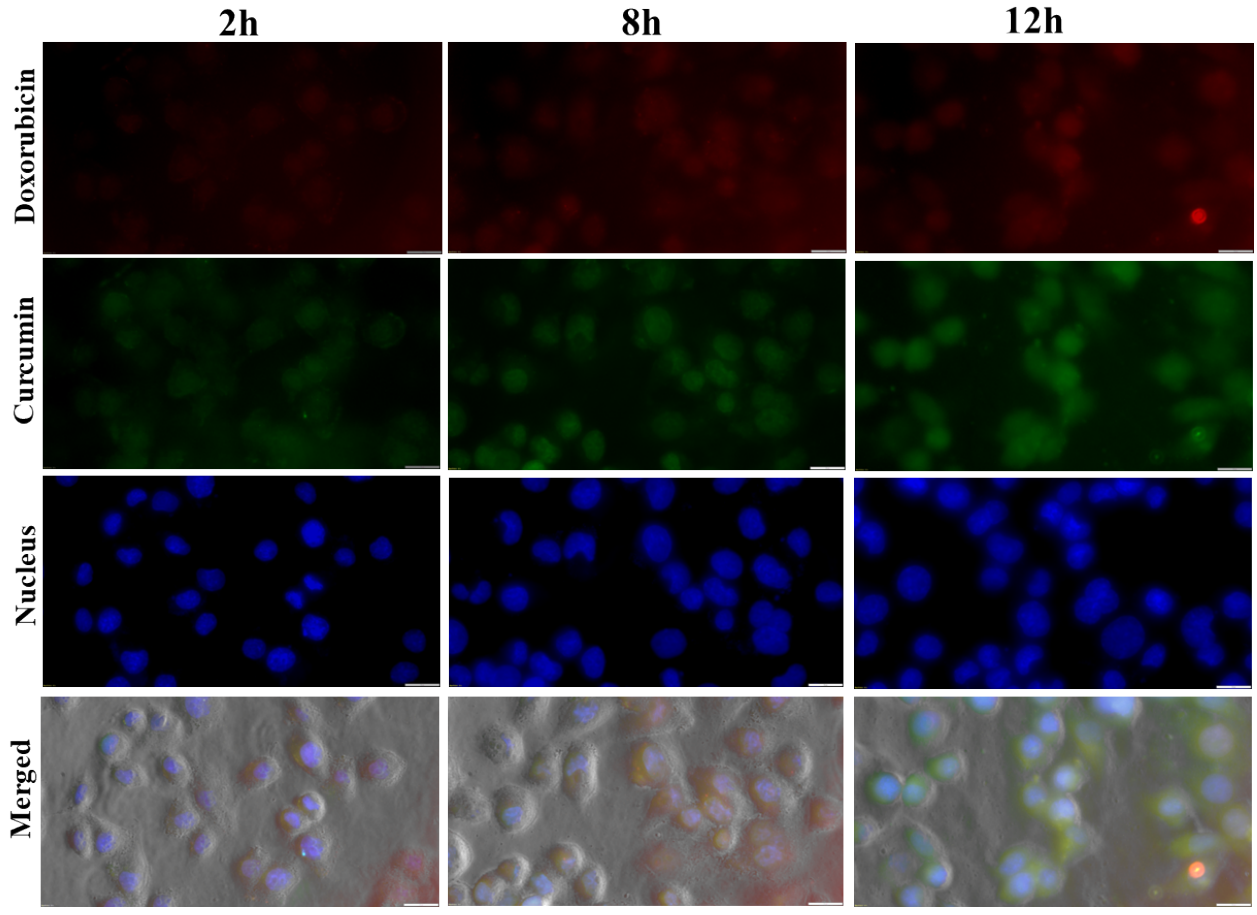
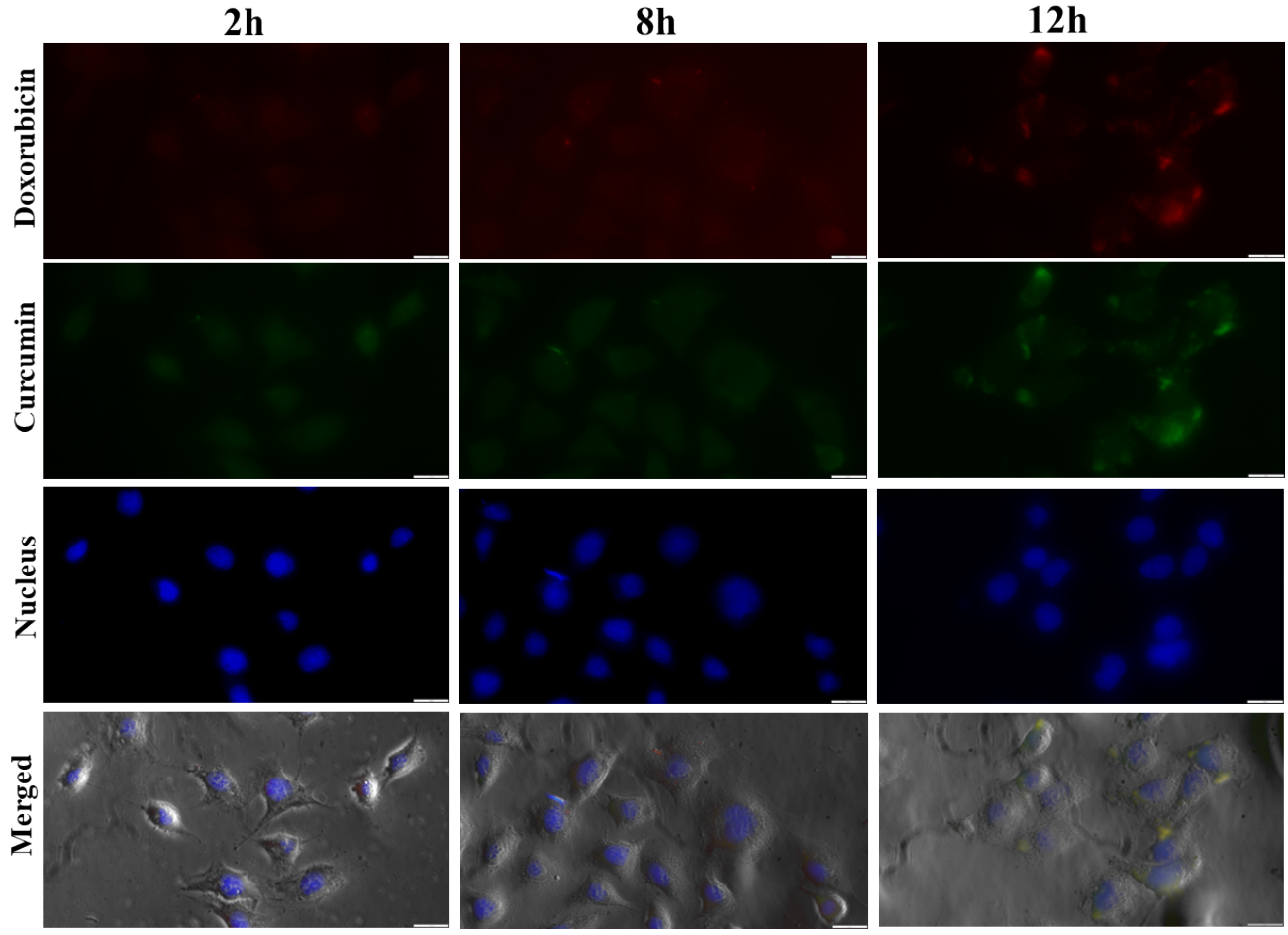
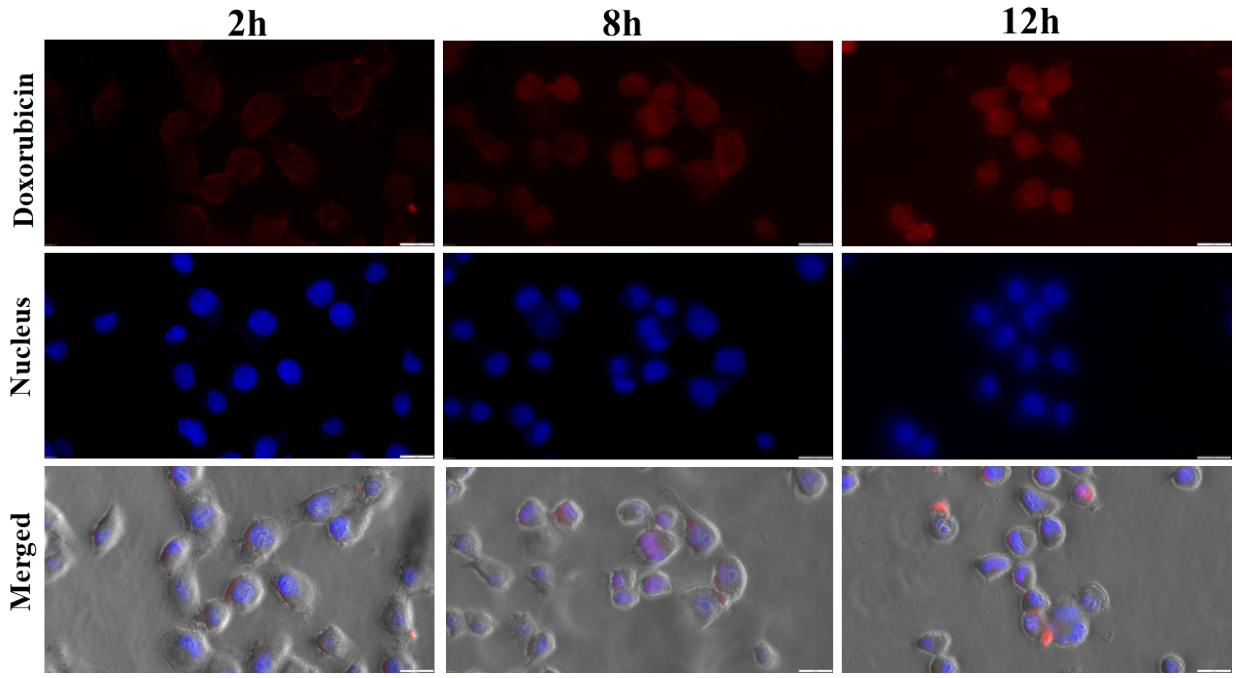


Figure S15. Representative images of cellular uptake of non-targeted cur+dox/NC in KB cells cultured with folic acid in the medium: Cellular uptake of non-targeted cur+dox/NC following 2h, 8h and12h incubation in KB cells cultured with folic acid in the medium. Cellular uptake is



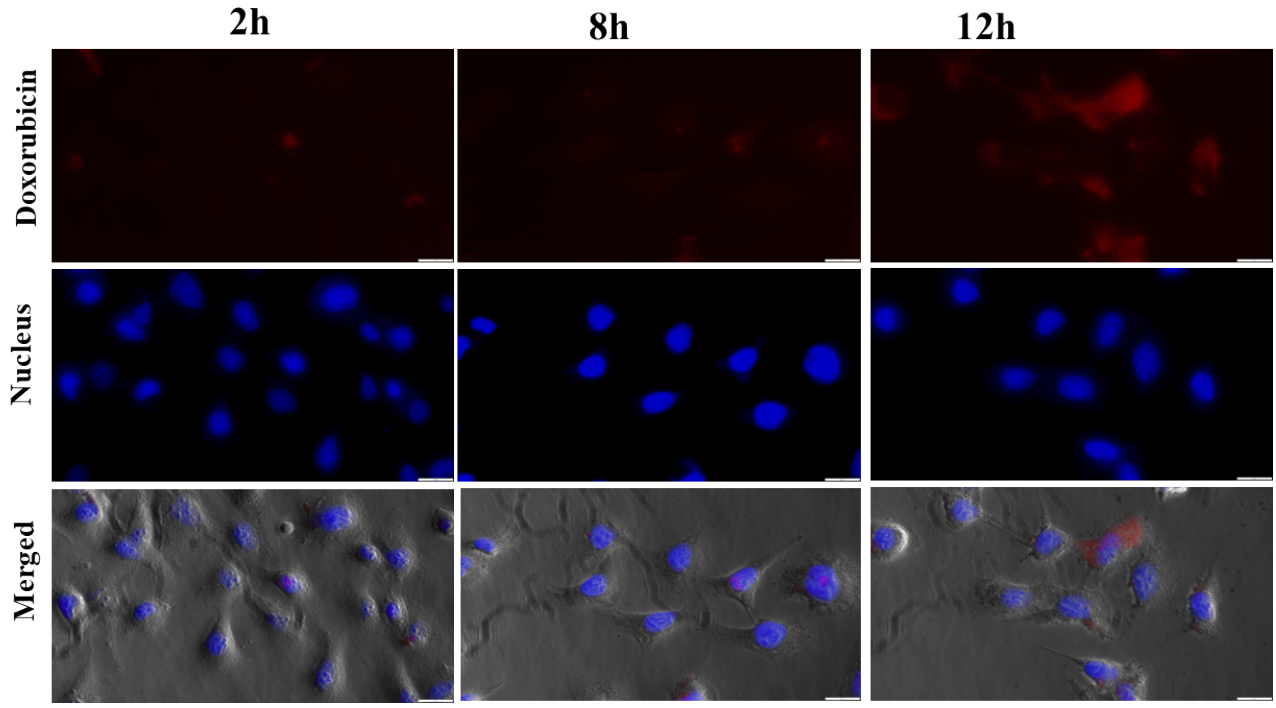
low at different incubation time (scale bar: 20μm).

Figure S16. Representative images of cellular uptake of targeted dox/NC in KB cells cultured with folic acid in the medium: Cellular uptake of targeted dox/NC following 2h, 8h and 12h incubation in KB cells cultured with folic acid in the medium. Cellular uptake is low at different



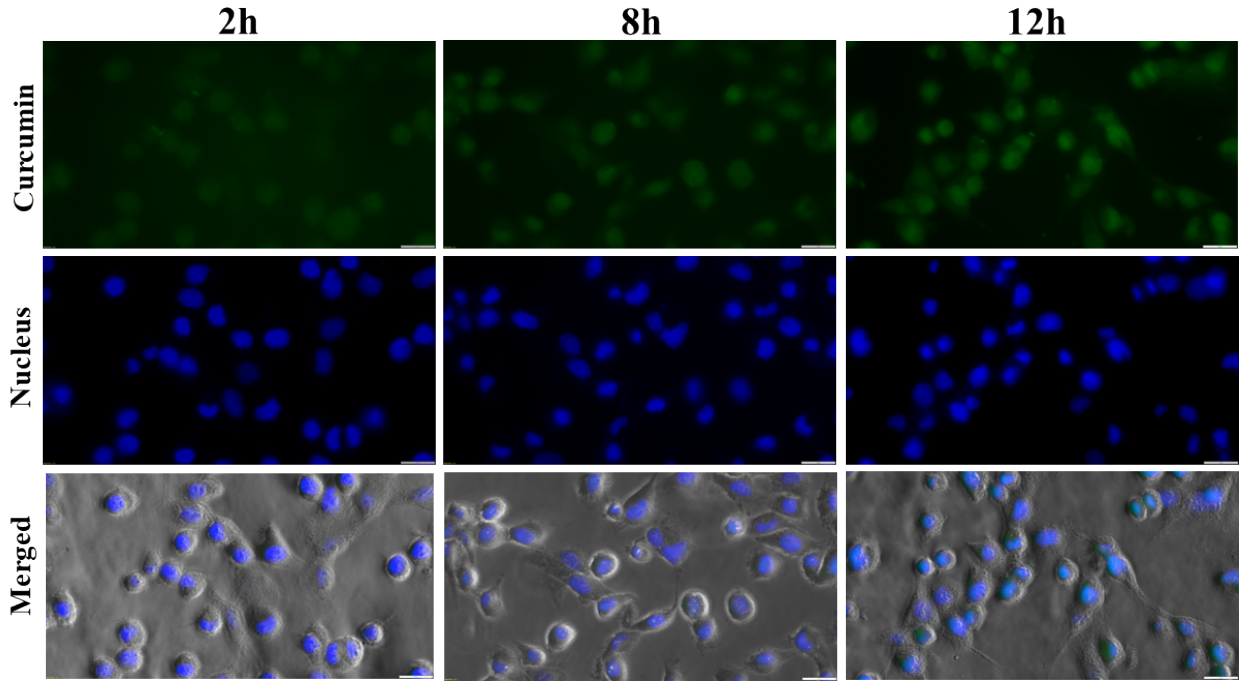
incubation time (scale bar: 20μm).

Figure S17. Representative images of cellular uptake of non-targeted dox/NC in KB cells cultured with folic acid in the medium: Cellular uptake of non-targeted dox/NC following 2h, 8h and 12h incubation in KB cells cultured with folic acid in the medium. Cellular uptake is low



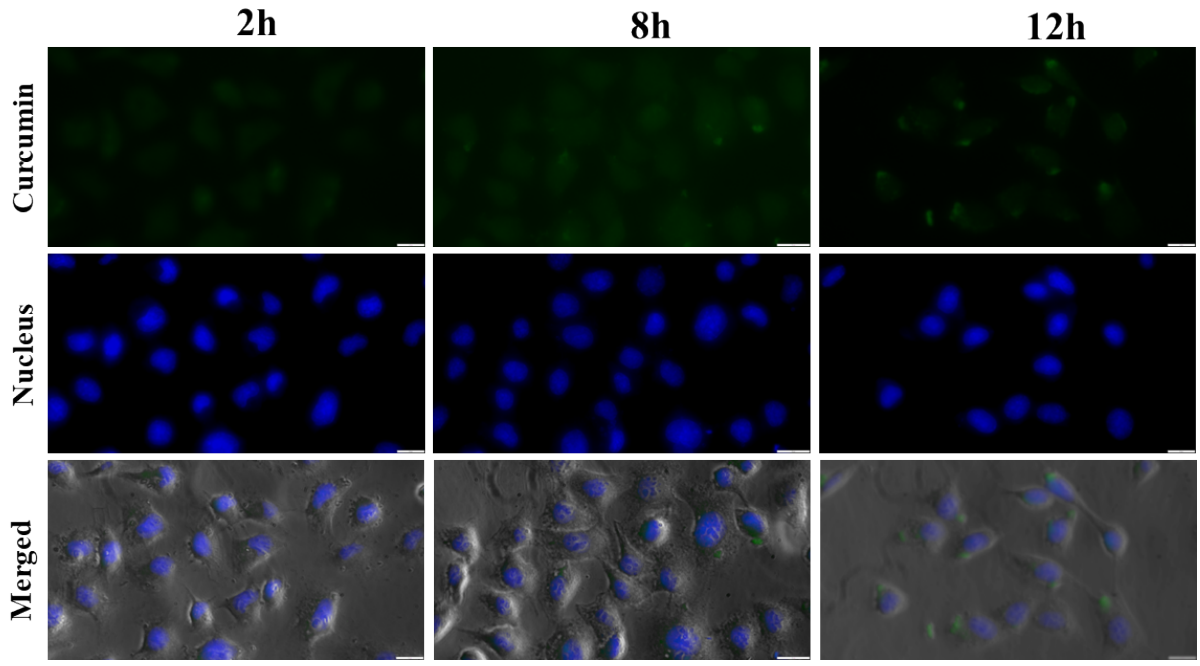
at different incubation time (scale bar: 20μm).

Figure S18. Representative images of cellular uptake of targeted cur/NC in KB cells cultured with folic acid in the medium: Cellular uptake of targeted cur/NC following 2h, 8h and 12h incubation in KB cells cultured with folic acid in the medium. Cellular uptake is low at different



incubation time (scale bar: 20μm).

Figure S19. Representative images of cellular uptake of non-targeted cur/NC in KB cells cultured with folic acid in the medium: Cellular uptake of non-targeted cur/NC following 2h, 8h and 12h incubation in KB cells cultured with folic acid in the medium. Cellular uptake is low



at different incubation time (scale bar: 20μm).

Figure S20. Cellular uptake analysis in CHO cells. Quantitative analysis of cellular uptake of (A) targeted cur/NC vs non-targeted cur/NC, (B) targeted dox/NC vs non-targeted dox/NC, (C) curcumin in targeted cur+dox/NC vs non-targeted cur+dox, (D) doxorubicin in targeted cur+dox/NC vs non-targeted cur+dox as compared with following at 2h, 8h incubation periods in CHO cells. Targeted NC did not show significant difference in cellular uptake of drugs compared to non-targeted NCs, with P value summary: ns - not significant.

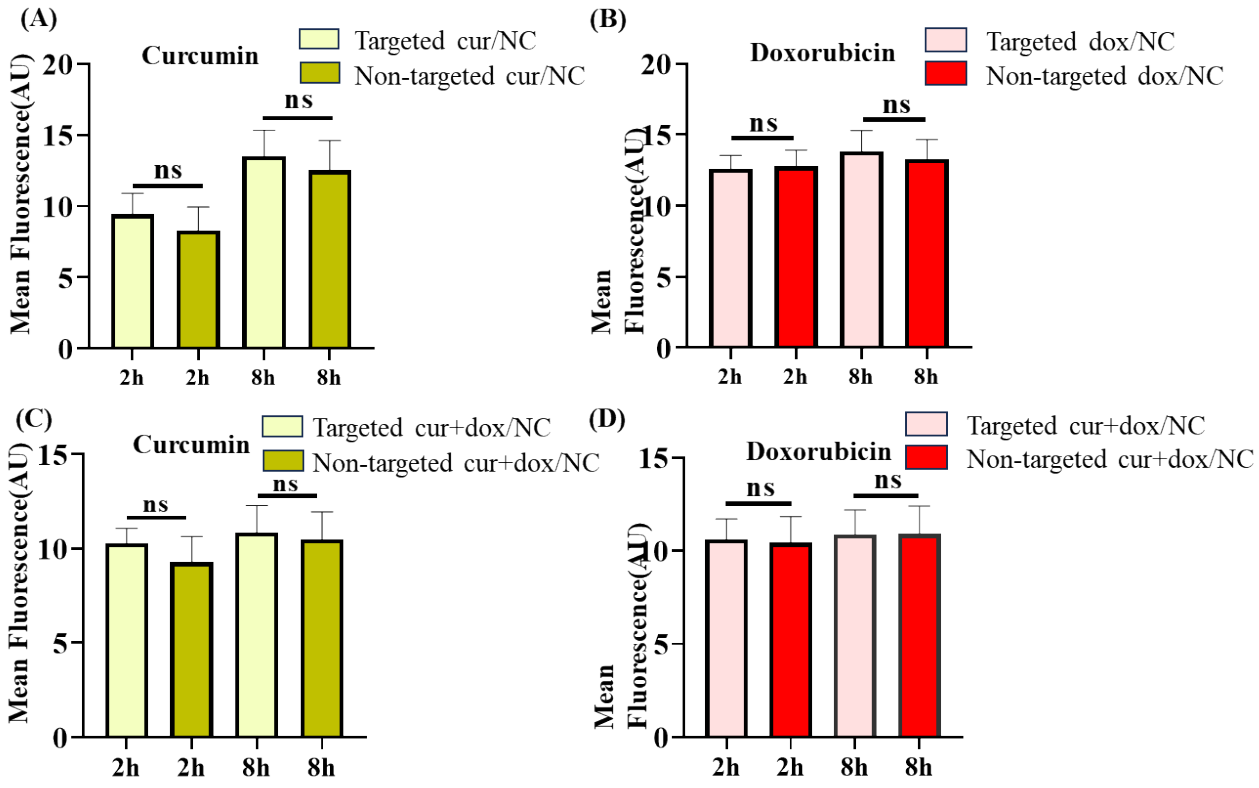
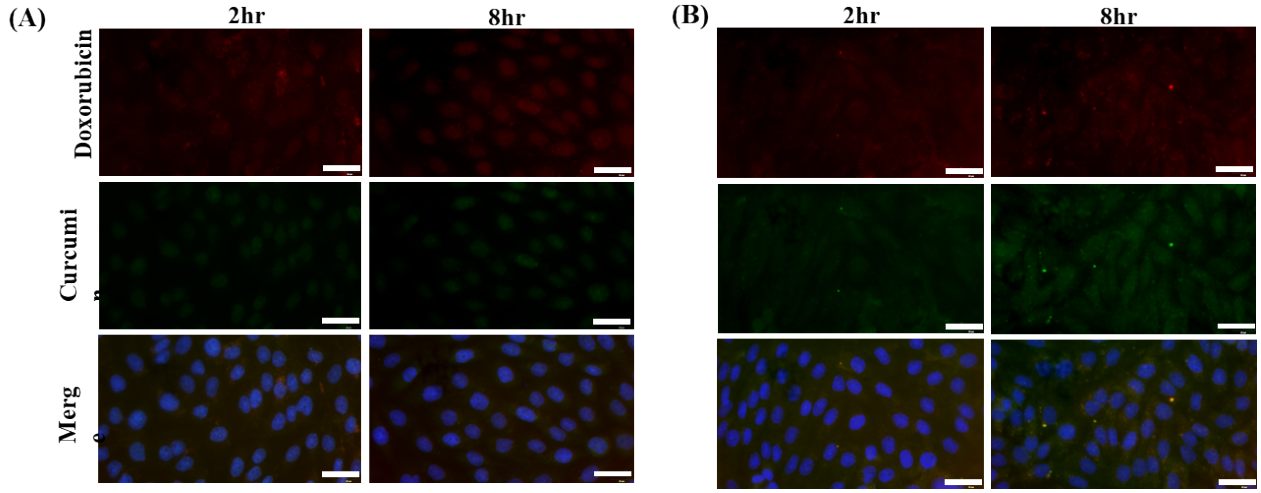
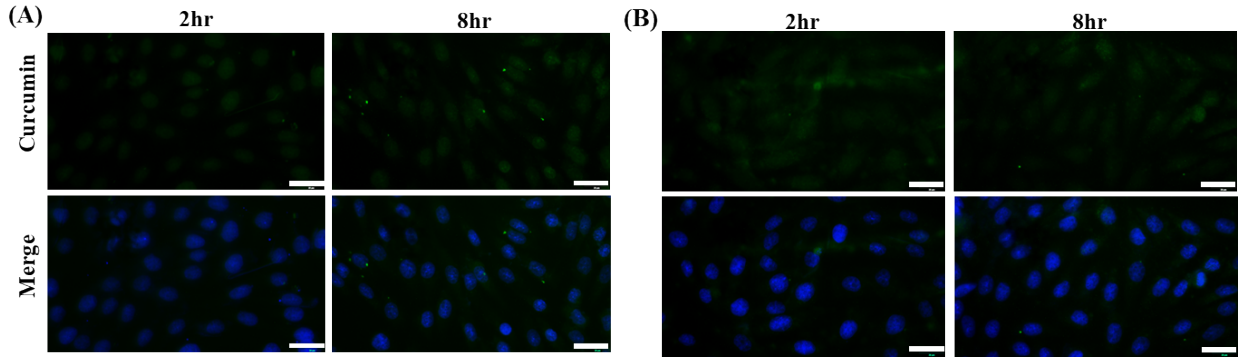

(A)
Curcumin
Targeted cur/NC
Non-targeted cur/NC
Mean Fluorescence(AU)
ns
ns
2h
2h
8h
8h
(B)
Doxorubicin
Targeted dox/NC
Non-targeted dox/NC
Mean Fluorescence(AU)
(C)
Curcumin
Targeted cur+dox/NC
Non-targeted cur+dox/NC
Mean Fluorescence(AU)
(D)
Doxorubicin
Targeted cur+dox/NC
Non-targeted cur+dox/NC
Mean Fluorescence(AU)

Figure S21. Representative images of cellular uptake of (A) targeted cur+dox/NC (B) non-targeted cur+dox/NC in CHO cells. Cellular uptake of targeted cur+dox/NC and non-targeted cur+dox/NC followed at 2h, 8h incubation in CHO cells. Cellular uptake is very low for both



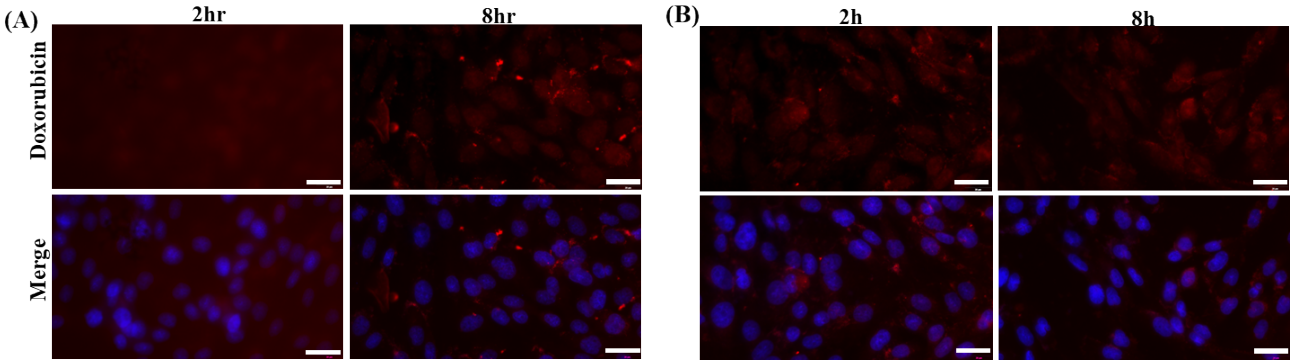
targeted and non-targeted sample at different incubation time. (scale bar: 20μm).

Figure S22. Representative images of cellular uptake of (A) targeted cur/NC (B) non-targeted cur/NC in CHO cells. Cellular uptake of targeted cur/NC and non-targeted cur/NC followed at 2h, 8h incubation time in CHO cells. Cellular uptake is very low for both targeted and non-targeted



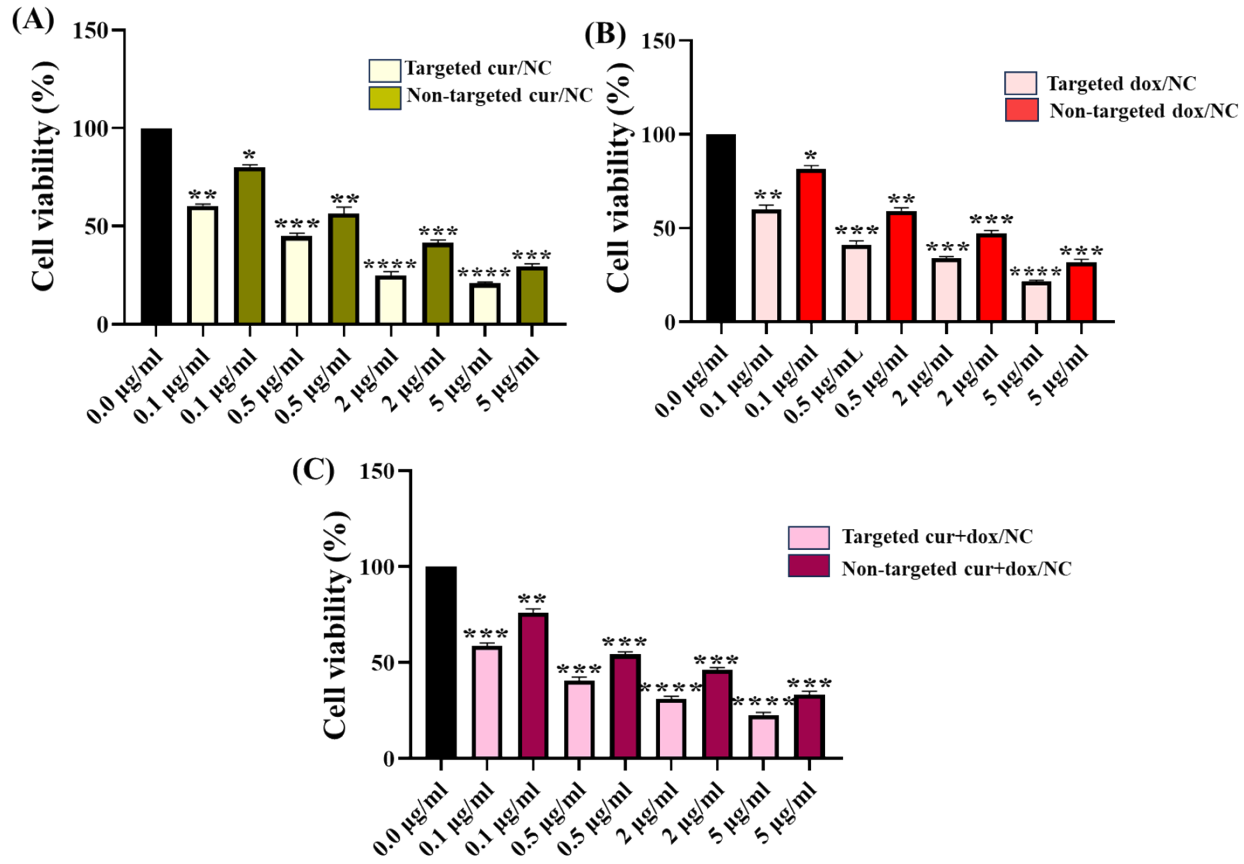
NC at different incubation time. (scale bar: 20μm).

Figure S23. Representative images of cellular uptake of (A) targeted dox/NC (B) non-targeted dox/NC in CHO cells. Cellular uptake of targeted dox/NC and non-targeted dox/NC following 2h, 8h incubation in CHO cells. Cellular uptake is very low for both targeted and non-



targeted NC at different incubation time. (scale bar: 20μm).

Figure S24. Cytotoxic effect of drug/s incubated with KB cells for 48h with targeted NCs and non-targeted NCs measured using MTT assay. Higher concentrations and longer incubation time intervals show toxicity in KB cells. Cell culture media was used as the control (100% viability). (A) the P value summary of targeted cur/NC and non-targeted cur/NC at 0.1 µg/ml, 0.2 µg/ml, 2 µg/ml, 5 µg/ml were *-significantly different, ns (non-significant), ***, **, ***, **, ***, ** -significantly different. (B) the P value summary of targeted dox/NC and non-targeted dox/NC at 0.1 µg/ml, 0.2 µg/ml, 2 µg/ml, 5 µg/ml were **, **, **, **, ***, **, ****, *** -significantly different. (C) the P value summary of targeted Cur+Dox/NC and non-targeted Cur+Dox/NC at 0.1



µg/ml, 0.2 µg/ml, 2 µg/ml, 5 µg/ml were **, *, ***, **, ***, **, ****, *** -significantly different.