Supplementary Information

A Simple Add-and-Display Method for Immobilisation of Cancer Drug on His-tagged Virus-like Nanoparticles for Controlled Drug Delivery

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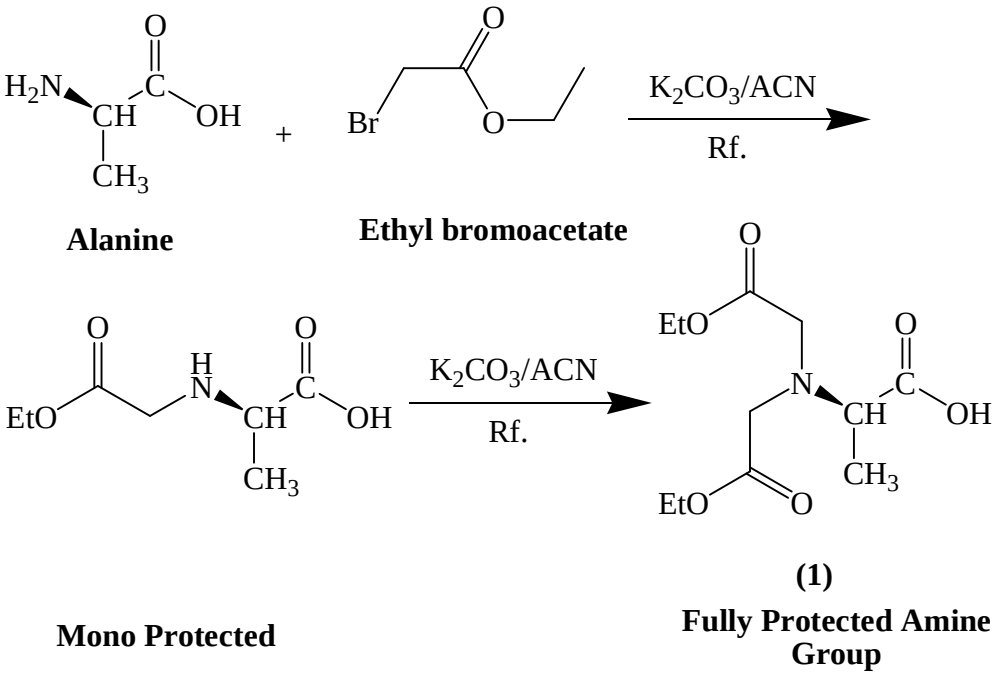
Synthesis and Analysis of NTA-DOX

1. Synthesis of compound 1

Alanine (0.025 mmol; Sigma-Aldrich) in acetonitrile (ACN, 30 mL) was added with K_2CO_3 (2 g) and ethylbromoacetate (0.03 mmol). The mixture was refluxed for 20 h and the reaction is shown in Supplementary Figure S1. The reaction progress was monitored using thin-layer chromatography (TLC; Et:n-Hex, 1:1) on silica gel plates (60F-254) and visualized using $KMnO_4$. The reaction was completed after 20 h. Then, the solvent was evaporated under vacuum using a rotary evaporator. The product was extracted and purified using EtOAc (2×30 mL) and water (30 mL). The organic layer was then washed with brine and water. The organic layer was

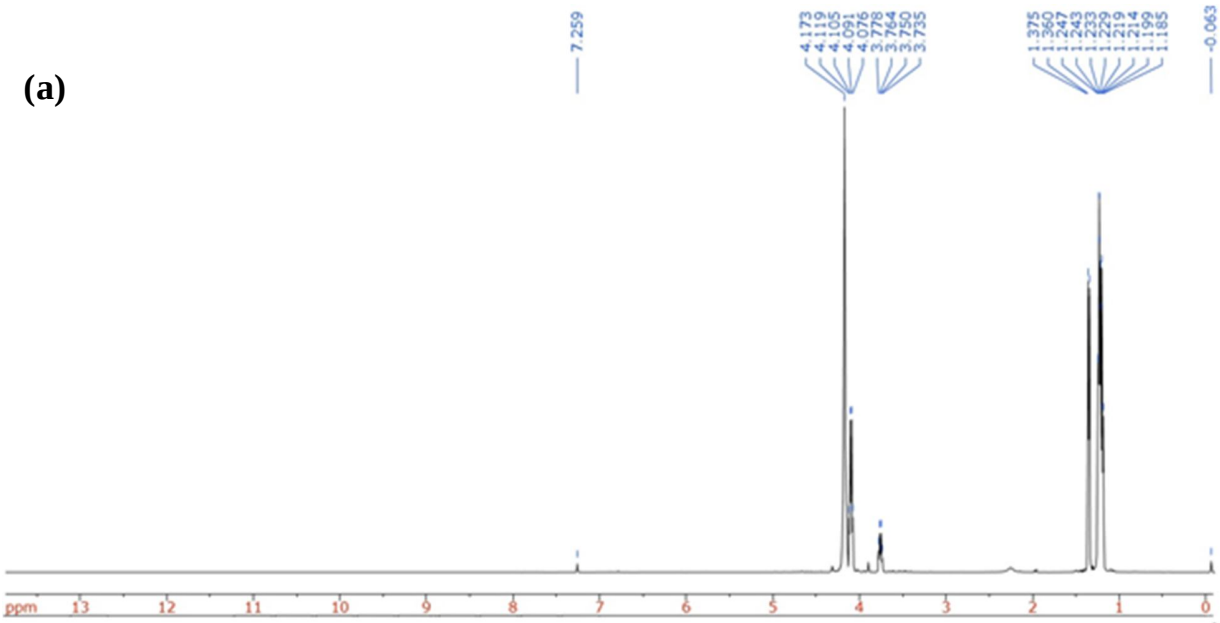
dried with Na_2SO_4 and evaporated to obtain precipitates of compound **1**. The yield of the product was 95%.



Supplementary Figure S1. Synthesis of fully protected amine group of alanine. ACN, acetonitrile; K_2CO_3, potassium carbonate; Rf., reflux.

Compound 1 was analysed using a nuclear magnetic resonance (NMR) spectrophotometer (Varian, 500 MHz). All ^{1}H and ^{13}C NMR spectra (Supplementary Figure S2) were recorded at 25°C in methanol-d4. Chemical shifts (δ) were reported in parts per million (ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

(a)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4.119
4.105
4.091
4.076
3.778
3.764
3.750
3.735
1.375
1.360
1.247
1.243
1.233
1.229
1.219
1.214
1.199
1.185
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11
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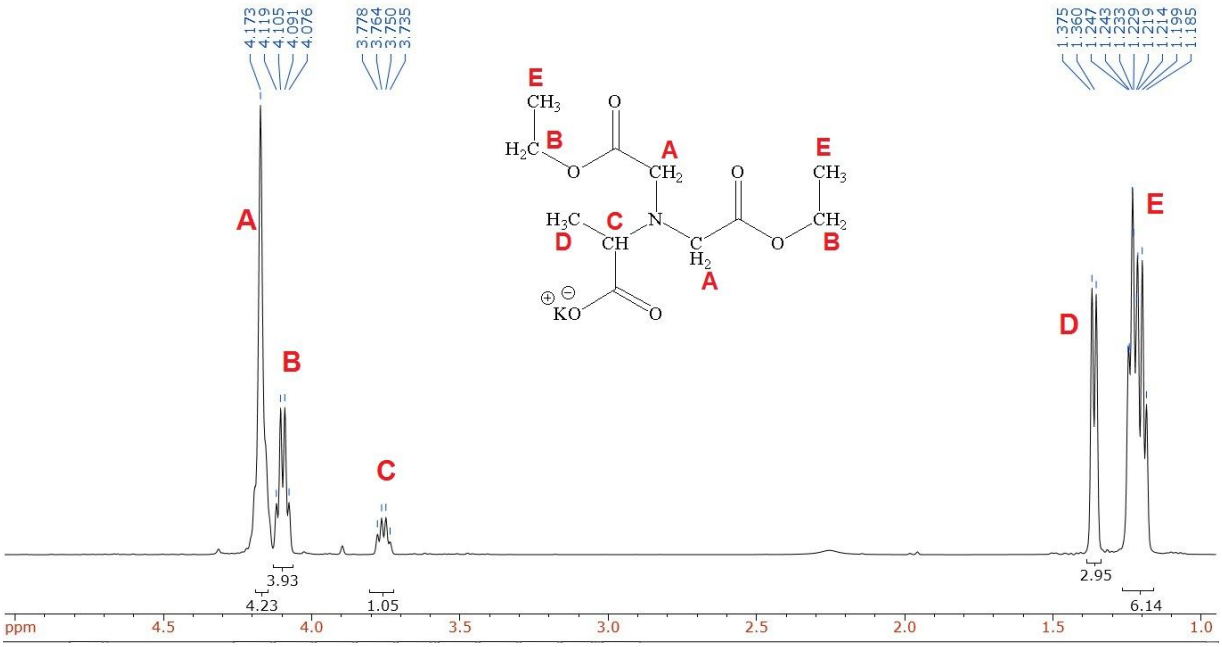
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3.750
3.735
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1.233
1.229
1.219
1.214
1.199
1.185
A
B
C
D
E
3.93
4.23
1.05
2.95
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ppm
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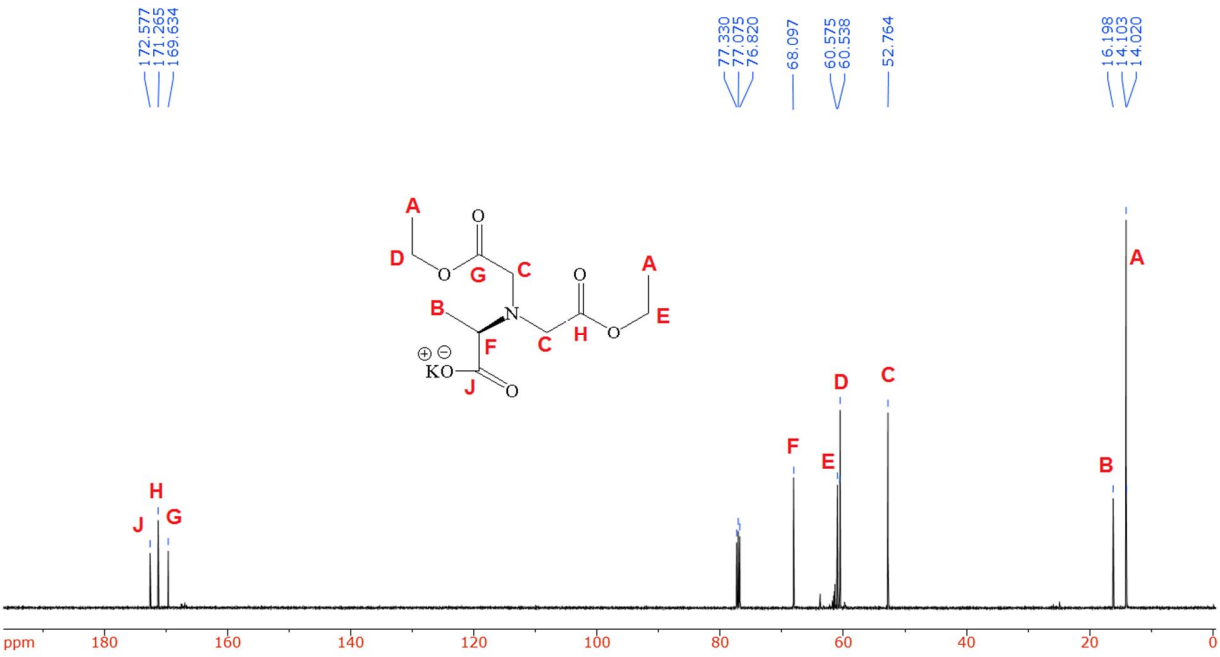
3.0
2.5
2.0
1.5
1.0

(b)

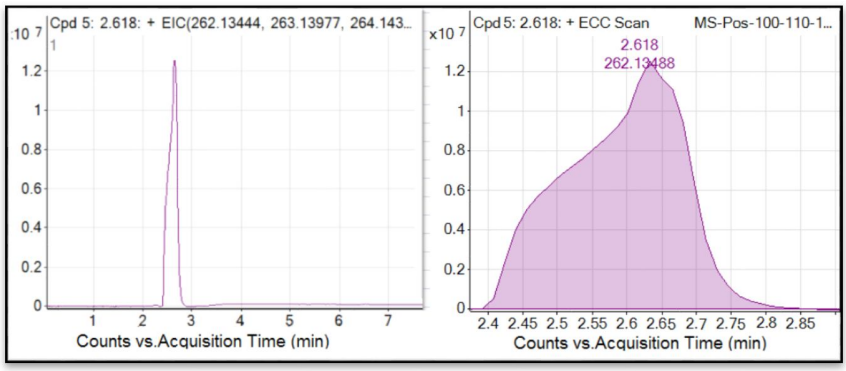


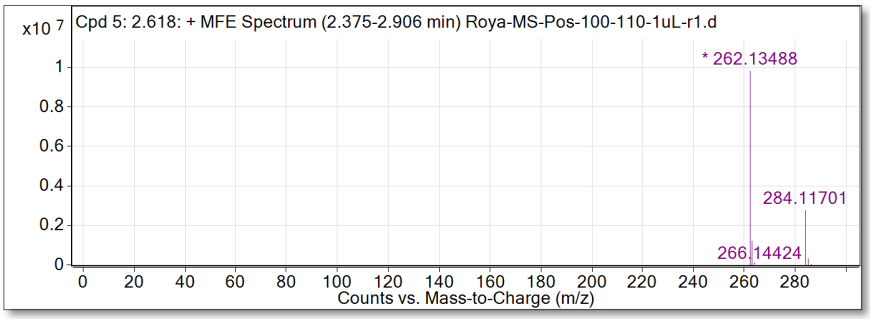
Supplementary Figure S2. NMR spectroscopy of compound 1. (**a**) ^{1}HNMR, (**b**)^{13}CNMR spectra of compound 1.

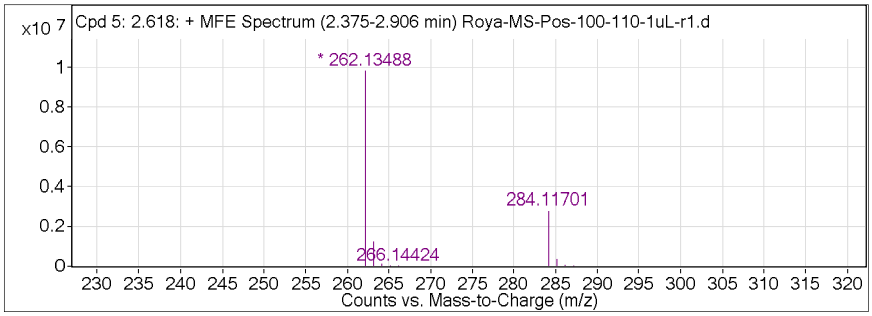
^{1}HNMR (500 MHz, $CDCl_3$), δ (ppm) = 4.17(s, 4H, A), 4.09(q, 4H, J = 7.14 Hz, B), 3.75(q, 1H, J = 7.0 Hz, C), 1.36(d, 3H, J = 7.5 Hz, D), 1.20 – 1.22(m, 6H, E).

^{13}CNMR (100 MHz, $CDCl_3$), δ (ppm) = 14.02, 14.10, 16.19, 52.79, 60.53, 60.57, 68.09, 169.63, 171.26, 172.57.

Compound 1 was analysed using a LC-MS (Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source) and the data are shown in Supplementary Figure S3.







MS Spectrum Peak List

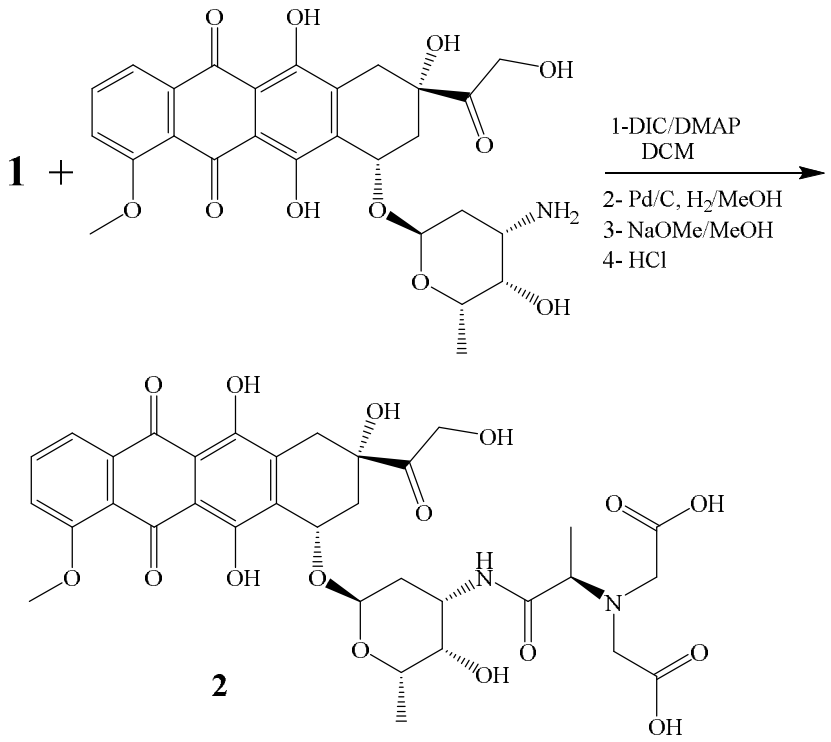
m/z	z	Abundance	Ion
262.13488	1	9841889.00	$(M+H)^+$
263.13813	1	1222952.75	$(M+H)^+$
264.13983	1	111440.54	$(M+H)^+$
265.14235	1	9659.17	$(M+H)^+$
266.14424	1	0	$(M+H)^+$
284.11701	1	2789810.25	$(M+Na)^+$
285.12010	1	364496.28	$(M+Na)^+$
286.12157	1	55474.67	$(M+Na)^+$
287.12385	1	4384.43	$(M+Na)^+$

Supplementary Figure S3. Mass spectroscopy of compound 1. Molecular mass was reported as $(M+H)^+$ and $(M+Na)^+$. The most abundant ions detected were 262.13488 Da $(M+H)^+$ and 284.11701 Da $(M+Na)^+$. The calculated molecular mass by the Chemdraw software was 261.12 Da.

2. Synthesis of compound 2

Compound 1 (4.5 mg) in dichloromethane (DCM, 10 mL) was added with doxorubicin (DOX, 5 mg) and 4-Dimethylaminopyridine (DMAP, 5 mg). The mixture was kept in ice-bath for 30 minutes. Then, a solution of N,N′-Diisopropylcarbodiimide (DIC, 5 mg) in DCM (3 mL) was added to the above mixture and stirred for 3 h at room temperature (RT). Completion of the reaction was checked by TLC ($CHCl_3$:MeOH, 9:1) and visualized under UV light. The reaction completed after an overnight incubation. The mixture was filtered and the filtrate was evaporated under vacuum using a rotary evaporator. The product was extracted and purified using EtOAc (2×30 mL) and water (30 mL). The organic layer was then washed with brine and water. The organic layer was dried with Na_2SO_4 and evaporated. The crude compound was purified using a flash chromatography ($CHCl_3$:MeOH, 8:2) and the yield of the product was 95%.

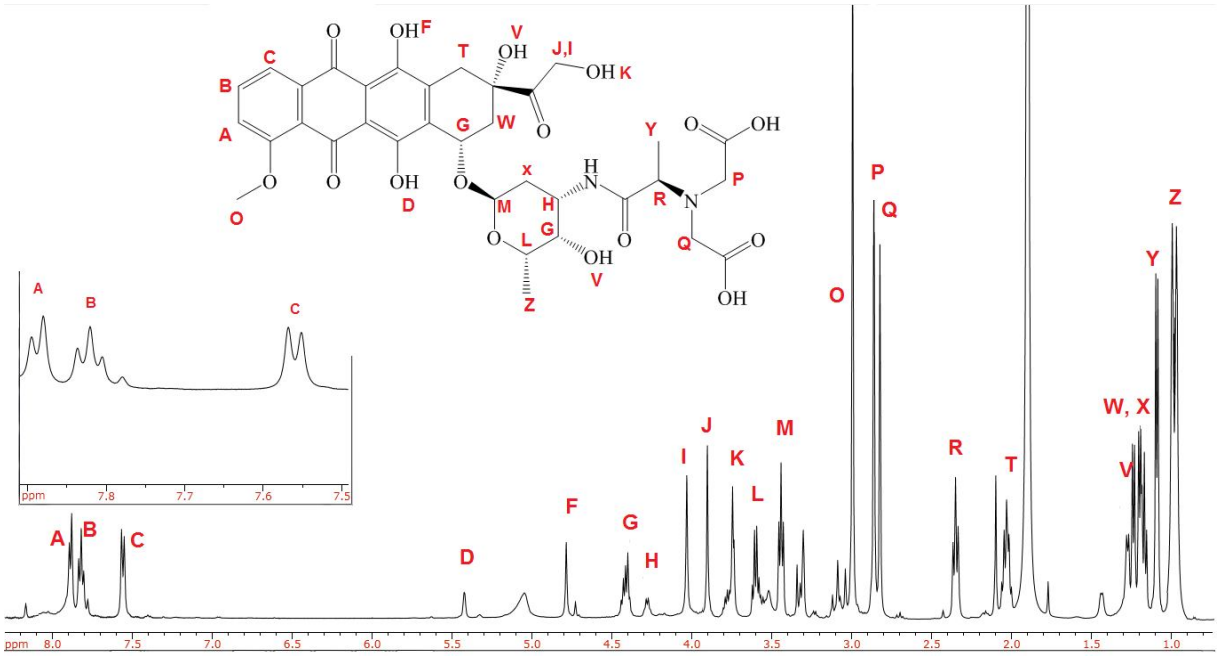
A solution of NaOMe (28%, 20 µL) was added to a solution of above deprotected compound (3 mg) in 60% aqueous CH_3OH (5 mL) and stirred for 1 h at RT. The reaction is depicted in Supplementary Figure S4. When the reaction was completed, the pH of the mixture was adjusted to 6-7 by adding 0.1 M HCl. Then the solvent was desalted using an ion exchange resin (Dowex 50WX8-200). The solvent was evaporated using a rotary evaporator under reduced pressure. The crude product was purified using flash column chromatography on silica gel (CH_3Cl:CH_3OH 8:2) to obtain compound **2** (NTA-DOX) with a yield of 85%.



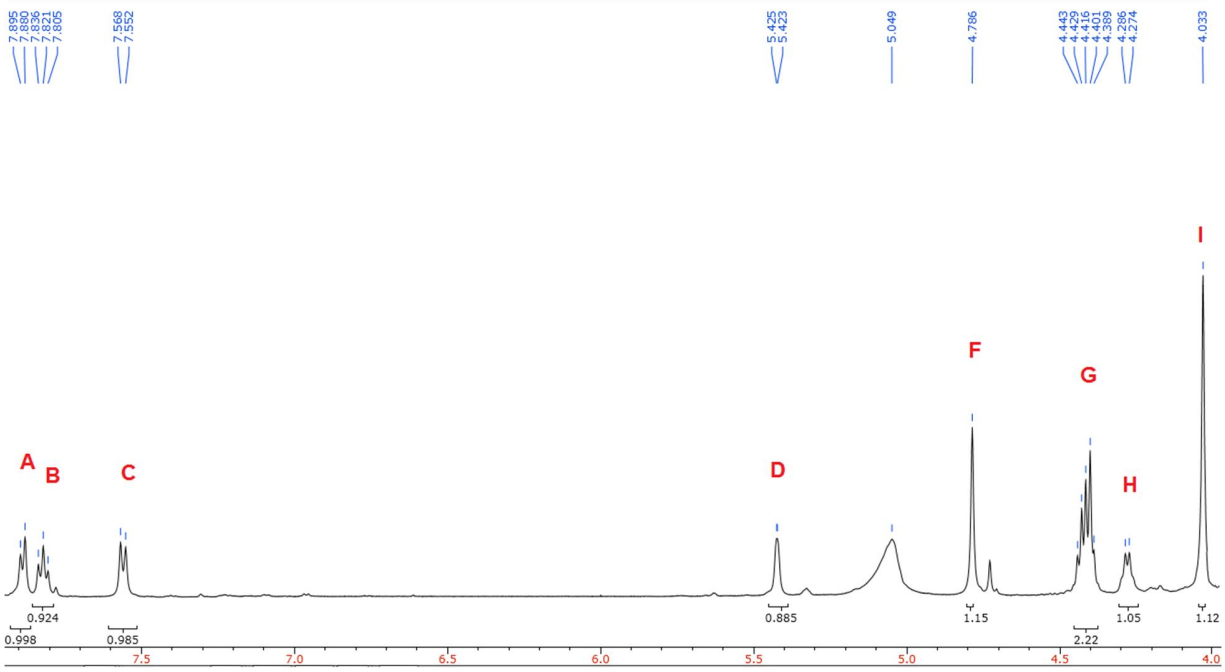
Supplementary Figure S4. Synthesis of NTA-DOX. Compound 1 in dichloromethane (DCM) was mixed with doxorubicin (DOX) in the presence of 4-Dimethylaminopyridine (DMAP) and *N*,*N'*-Diisopropylcarbodiimide (DIC) at RT for 3 h. Then the product was converted to the dicarboxylic-Ala-DOX (NTA-DOX) compound by the hydrolysis method using NaOMe in methanol.

The synthesised NTA-DOX was analysed using a nuclear magnetic resonance (NMR) spectrophotometer (Varian, 500 MHz). All ^{1}H and ^{13}C NMR spectra (Supplementary Figure S5) were recorded at 25℃ in methanol-d4. Chemical shifts (δ) were reported in parts per million (ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

(a)


A
B
C
D
F
G
H
I
J
K
L
M
O
P
Q
R
T
V
W, X
Y
Z
ppm 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
ppm 7.8 7.7 7.6 7.5

(b)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4.401
4.389
4.286
4.274
4.033
A
B
C
D
F
G
H
I
0.924
0.998
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1.05
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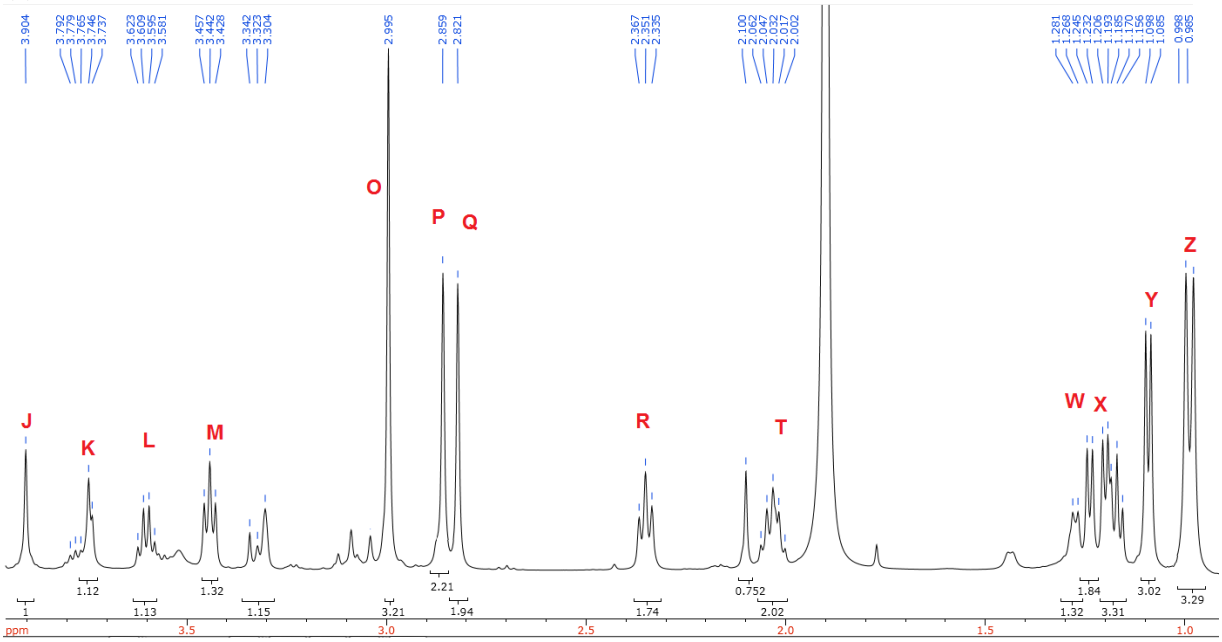
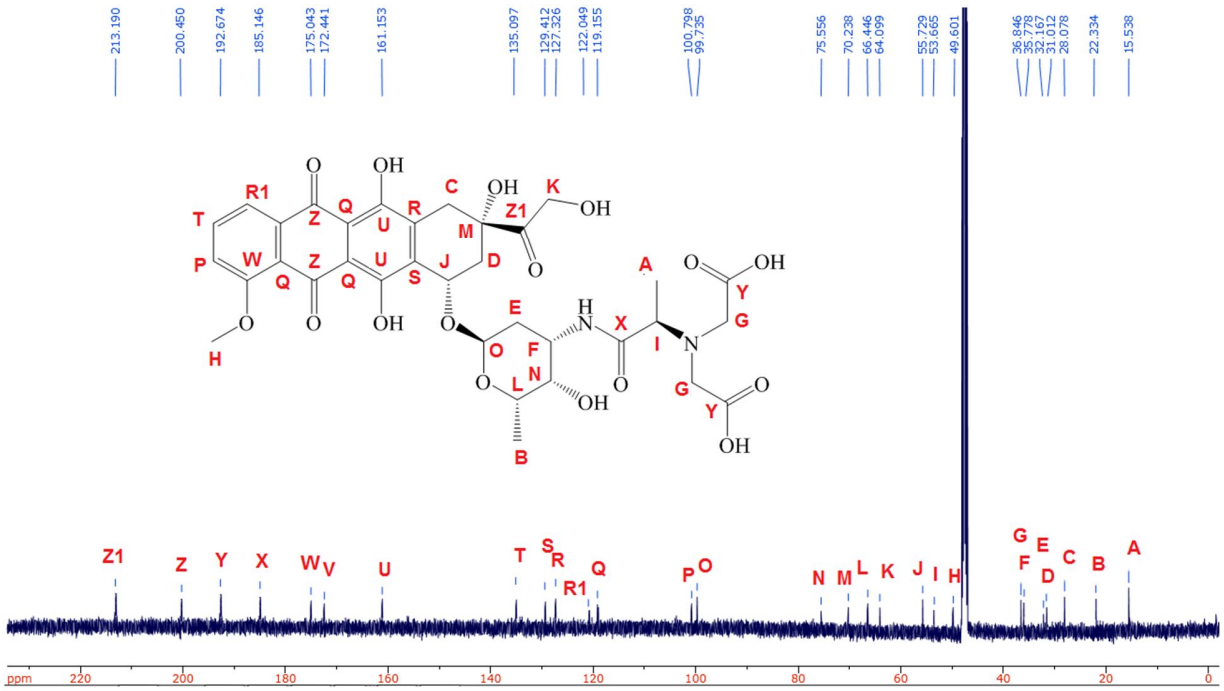
1.12
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0

(c)



(d)

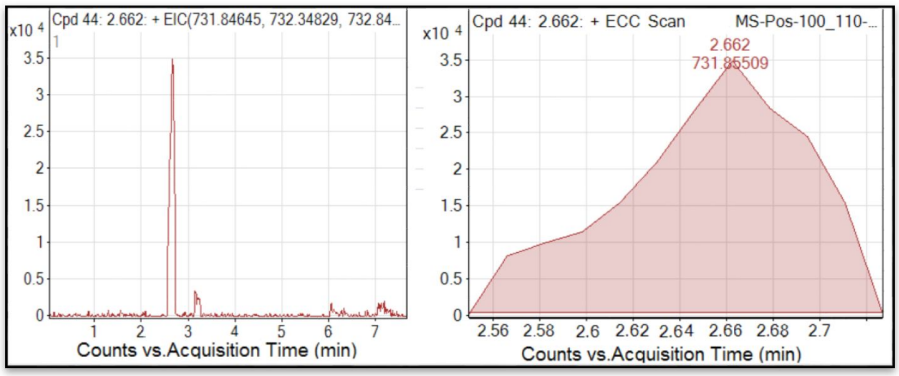


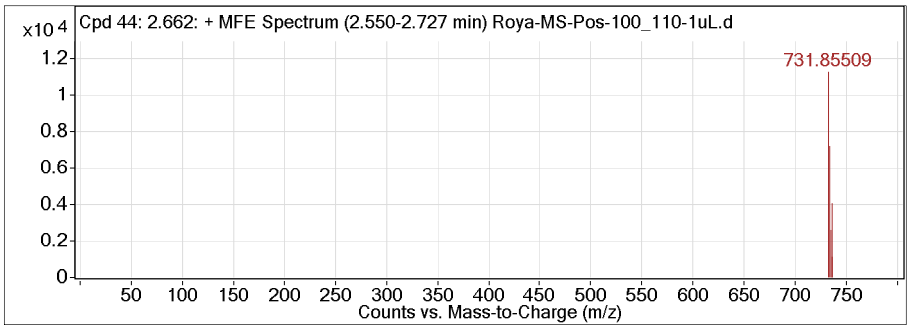
Supplementary Figure S5. ^{1}HNMR and ^{13}CNMR spectra of NTA-DOX. (**a**, **b**, and **c**) ^{1}HNMR spectra of NTA-DOX, (**d**) ^{13}CNMR of NTA-DOX.

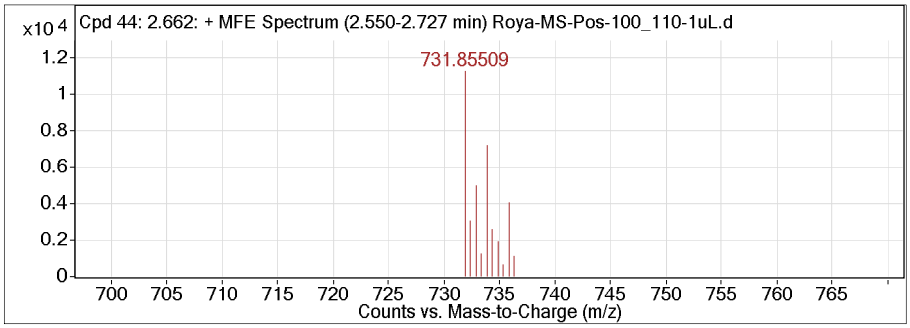
^{1}HNMR (500 MHz, CD_3OD), δ 7. 8(d, 1H, J = 7.38 Hz, A), 7.82(t, 1H, J = 7.87 Hz, B), 7.56(d, 1H, J = 8.25 Hz, C), 5.42(d, 1H, J = 1.1 Hz, D), 4.78(s, 1H, F), 4.41(dt, 2H, J = 13.67, 6.67 Hz, G), 4.27(d, 1H, J =6.43 Hz, H), 4.03(s, 3H, I) 3.90(s, 1H, J), 3.76(s, 1H, K), 3.60(q, 1H, J = 7.00 Hz, L), 3.44(t, 1H, J = 7.06 Hz, M), 3.06(s, 3H, O), 2.99(s, 2H, P), 2.82(s, 2H, Q), 2.35(t, 2H, J = 8.04 Hz, R), 2.03(dt, 2H, J = 15.04, 7.54 Hz, T), 1.43(d, J = 5.59 Hz, U), 1.27(d, 1H, J = 6.59Hz, V), 1.23-1.18(m, 3H, W), 1.09(d, 3H, J = 6.47Hz, X), 0.99(d, 3H, J = 8.56Hz, Y)

^{13}CNMR (100 MHz, CD_3OD), 15.53, 22.33, 28.07, 31.01, 32.16, 35.77, 36.84, 49.60, 53.66, 55.72, 64.09, 66.44, 70.23, 75.55, 99.73, 100.79, 119.15, 122.04, 127.32, 129.41, 135.09, 161.15, 172.44, 175.04, 185.14, 192.67, 200.45, 213.19.

NTA-DOX was analysed using a LC-MS (Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source) and the data are shown in Supplementary Figure S6.







MS Spectrum Peak List

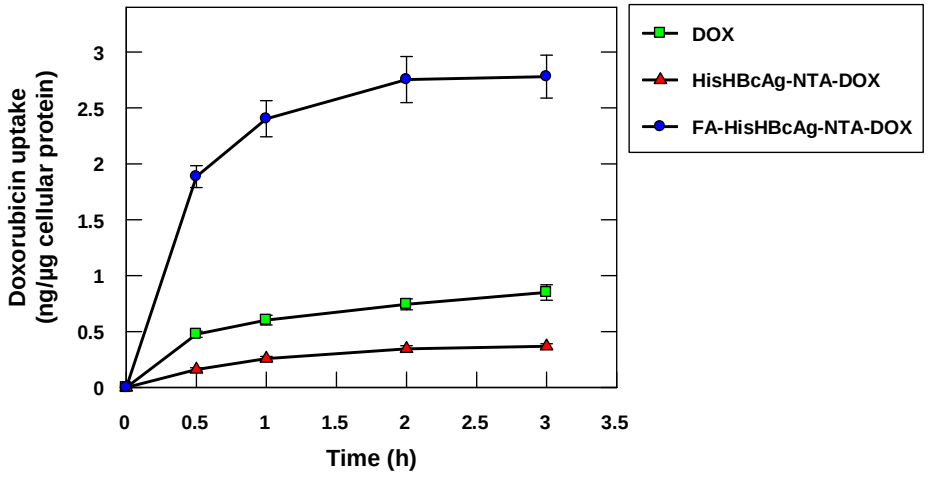
m/z	z	Abundance	Ion
731.85509	2	11223.61	$(M+2H)^{+2}$
732.35531	2	3091.22	$(M+2H)^{+2}$
732.85150	2	4967.72	$(M+2H)^{+2}$
733.35447	2	1258.55	$(M+2H)^{+2}$
733.85727	2	7187.43	$(M+2H)^{+2}$
734.35814	2	2611.45	$(M+2H)^{+2}$
734.84584	2	1935.03	$(M+2H)^{+2}$
735.34231	2	673.28	$(M+2H)^{+2}$
735.85016	2	4069.64	$(M+2H)^{+2}$
736.34987	2	1129.65	$(M+2H)^{+2}$

Supplementary Figure S6. Mass spectroscopy of NTA-DOX. The most abundant ion detected was 731.85509 Da $(M+2H)^{+2}$. The calculated molecular mass by the Chemdraw software was 730.22 Da.

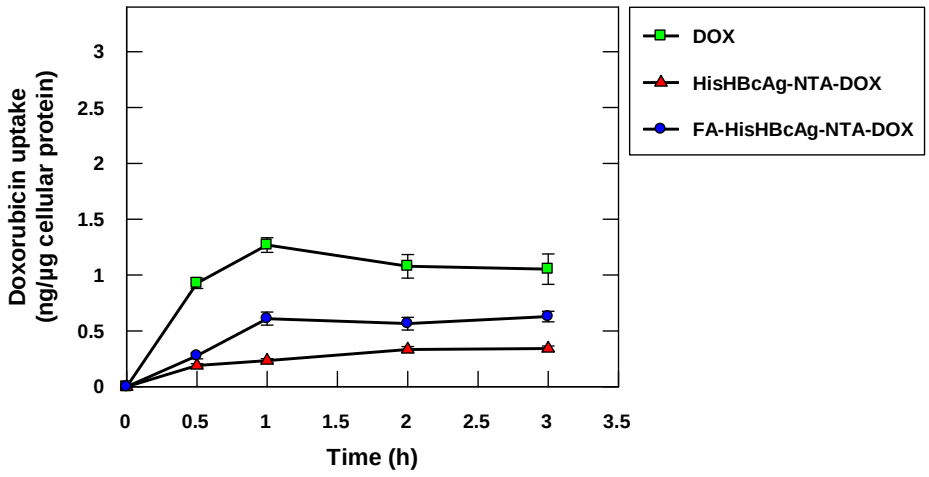
Cellular Uptake of Doxorubicin

OVCAR-3 and 3T3cells (10^5 cells per well) were seeded in six-well plates and incubated for 24 h. Then, free doxorubicin (DOX), HisHBcAg nanoparticles conjugated non-covalently with NTA-DOX (HisHBcAg-NTA-DOX), and HisHBcAg nanoparticles conjugated covalently with folic acid (FA) and non-covalently with NTA-DOX (FA-HisHBcAg-NTA-DOX) were added at equivalent DOX concentration (5 μg/mL). After washing 3 times with ice-cold PBS (pH 7.4), lysis buffer [50 mM Tris (pH 7.4), 0.8% Triton, 0.2% SDS] (0.5 mL per well) was added to lyse the cells. Internalised DOX was measured at A_{490} using a microplate reader (ELX 800; BioTeck Instruments, Winooski, VT, USA) and the results are shown in Supplementary Figure S7.

(**a**)



(**b**)



Supplementary Figure S7. Doxorubicin uptake by ovarian cancer and normal cells. Cellular uptake of doxorubicin by (**a**) cancer OVCAR-3, and (**b**) normal 3T3 cells incubated with free doxorubicin (DOX), HisHBcAg nanoparticles conjugated non-covalently with NTA-DOX (HisHBcAg-NTA-DOX), and HisHBcAg nanoparticles conjugated covalently with folic acid (FA) and non-covalently with NTA-DOX (FA-HisHBcAg-NTA-DOX). Data represent mean ± SD of triplicate determinations.