Supporting Information

pH-Sensitive Multi-ligand Gold Nanoplatform Targeting Carbonic Anhydrase IX Enhances the Delivery of Doxorubicin to Hypoxic Tumor Spheroids and Overcomes the Hypoxia-Induced Chemoresistance

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Summary

S1. Preparation of CAI and Dox ligands and conjugates

A. Preparation of CAI targeting ligands

4-oxo-4-((5-sulfamoyl-1,3,4-thiadiazol-2-yl)amino)butanoic acid 2[1]

In a flame dried 20-mL reaction vial, 2-amino-5-sulfonamido-1,3,4-thiadiazole **1** (0.45 g, 2.5 mmol)[1-2] was dissolved in 2 mL of dry DMF. Succinic anhydride (275 mg, 0.275 mmol) was added over it in small portions and the reaction mixture was stirred for 4 days at 25 ^{o}C. After the reaction reached completion, DMF was removed by rotavap under vacuum, then 10 mL of water was added over the mix, when the product precipitated. The precipitate was filtered, rinsed with cold methanol and dried under vacuum. Yield: 83%; mp. 232-236^{o}C; lit.[1] m.p. 232-236^{o}C. ^{1}H-NMR (400 MHz, DMSO-d^{6}, δ, ppm): 13.06 (br s, 1H, -NH), 12.27 (br s, 1H , NH), 8.32 (s, 2H, -SO_2NH_2), 2.76 (t, J = 6.5 Hz, 2H, CH_2COOH), 2.60 (t, J = 6.5 Hz, 2H, CH_2CONH) ; ^{13}C-NMR (100.6 MHz, DMSO-d^{6}, δ, ppm): 173.3 (CONH), 171.4 (COOH), 164.1 (C_5 TDA), 161.0 (C_2 TDA), 29.9 (-$\underline{C}H_2COOH$), 28.2 (-$\underline{C}H_2CONH$-); LC-MS (> 98%): $C_6H_8N_4O_5S_2$ exact mass: 280.0; Found: 281.0 (MH^+).

N1-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-N4-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)succinamide 3

In a flame dried 20 mL reaction vial, the succinyl derivative **2** (0.536 g, 1.92 mmol) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, 0.37g, 2.11 mmol) were dissolved in 2 mL DMF. The flask was cooled in an ice bath at 0˚C, N-methylmorpholine (NMM, 235 μL, 0.213 g, 2.11 mmol) was added and the reaction mixture was allowed to react at 0˚C for 45 min under nitrogen atmosphere. Ethylenedioxy-bisethylamine (0.312 g, 2.11 mmol, dissolved in 1.5 mL dry DMF) was added to reaction mix in one aliquot. The reaction mixture was stirred at room temperature for two days. The DMF was evaporated via rotavap, under vacuum, and the crude product was

absorbed on silica. Flash chromatography was performed with DCM/MeOH gradients, on SiO_2 column. Useful fractions (determined by TLC, performed with DCM/ methanol (80/20 v/v), by NMR and by LCMS) were grouped and evaporated to dryness to yield 180 mg of crude amine **3**, which was used without further purification in the next step.

Amino-PEG2000-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)succinamide 4

In a flame dried 20 mL reaction vial, the succinyl derivative **2** (0.084 g, 0.3 mmol) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (0.053 g,0.3 mmol) was dissolved in 3 mL DMF. The flask was cooled with an ice bath at 0˚C and N-methylmorpholine (35 μL, 0.031 g, 0.3 mmol) was added. The mix was kept at 0˚C for 45 min under nitrogen, then treated with $H_2N\text{-}PEG_{2K}NH_2$ (0.500 g, 0.250 mmol, dissolved in 1.5 mL dry DMF). The reaction was subsequently stirred at room temperature for two days. The DMF was evaporated via rotavap under high vacuum, and the crude compound was absorbed on SiO_2. Flash chromatography was performed with $CHCl_3$/MeOH gradients on a SiO_2 column. Useful pure fractions (determined by TLC, performed with DCM/MeOH (75/25 v/v), by NMR and by MALDI) were grouped and evaporated to dryness to yield 327 mg of crude compound, which was used without further purification in the next step.

Yield: 66 %; ^{1}H-NMR (400 MHz, D_2O, δ, ppm): 3.80-3.58 (m, 176 H, 44 -OCH_2CH_2O-), 3.53 (t, J = 5.8 Hz, 4H, -O$\underline{CH_2}CH_2NH$-), 3.31 (t, J = 5.4 Hz, 4H, -$OCH_2\underline{CH_2}NH$-), 2.83 (t, J = 6.6 Hz, 4H, -CO$\underline{CH_2}CH_2CO$-), 2.60 (t, J = 6.6 Hz, 4H, -$OCCH_2\underline{CH_2}CO$-).

N1-(2-(2-(2-(5-(1,2-dithiolan-3-yl)pentanamido)ethoxy)ethoxy)ethyl)-N4-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)succinamide 5

In a flame dried 20 mL reaction vial, lipoic acid (25 mg, 0.145 mmol) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (21 mg, 0.145 mmol) were dissolved in 1 mL DMF. The flask was cooled in an ice bath at 0˚C and N-methylmorpholine (15 µL, 13 mg, 0.145 mmol) was added. The reaction mixture was kept at 0˚C for 45 minutes under nitrogen, and was subsequently treated with compound **3** (49 mg, 0.12 mmol, dissolved in 1mL dry DMF). The reaction mixture was then stirred at room temperature for two days. The DMF was evaporated via rotavap, under high vacuum, and the crude product was absorbed on silica. Flash chromatography was performed with chloroform and methanol gradients using a 12g Silica column. Useful pure fractions (determined by TLC, performed with DCM/MeOH (80/20), by NMR and by LCMS) were grouped and evaporated to dryness to yield 20 mg of pure compound. Yield: 27%;

^{1}H-NMR (400 MHz, DMSO-d^{6}, δ, ppm): 11.16 (s, 2H, -CONH-), 8.29 (s, 1H, -SO_2NH_2), 7.99 (t, J = 5.27 Hz, 1H, -CONH-), 7.83 (t, J = 6.08 Hz, -CONH-), 3.55 (m, 4H, 2-NHCH$_2$<u>CH$_2$</u>O-), 3.5 (s, 4H, 2-O<u>CH$_2$CH$_2$</u>O-), 3.39 (t, 4H, 2-NH<u>CH$_2$</u>CH$_2$O-), 3.18 (m, 2H, -S<u>CH$_2$</u>CH$_2$- and 1H, -SCH$_2$<u>CH$_2$</u>-), 2.73 (t, J = 6.8 Hz, 4H, -CO<u>CH$_2$</u>CH$_2$CO-), 2.5 (t, J = 6.8 Hz, 4H, -OCCH$_2$<u>CH$_2$</u>CO-), 2.39 (m, 1H, -S<u>CH</u>CH$_2$-), 2.06 (t, 2H, J = 6.6 Hz, -SCHCH$_2$CH$_2$CH$_2$<u>CH$_2$</u>CO-), 1.84 (m, 1H, -SCH$_2$<u>CH$_2$</u>-), 1.64 (m, 1H, -SCHCH$_2$CH$_2$<u>CH$_2$</u>CH$_2$CO-), 1.51(m, 2H, -SCH<u>CH$_2$</u>CH$_2$CH$_2$CH$_2$CO- and 1H, -SCHCH$_2$CH$_2$<u>CH$_2$</u>CH$_2$CO-), 1.33 (m, 2H, -SCHCH$_2$<u>CH$_2$</u>CH$_2$CH$_2$CO-). LC-MS:

$C_{20}H_{34}N_6O_7S_4$, exact mass: 598.14; Found: 599.2 (MH^+); HRMS: $C_{20}H_{34}N_6O_7S_4$, exact mass: 598.1372; found 598.1376.

Lipoylamido-PEG2000-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)succinamide 6

In a flame dried 20 mL reaction vial, lipoic acid (5.12 mg, 0.0251 mmol) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (4.41 mg, 0.0251 mmol) were dissolved in 1 mL DMF. The flask was cooled in an ice bath at 0˚C, then N-methylmorpholine (2.5 µL, 2.25 mg, 0.0251 mmol) was added. The mix was kept at 0˚C for 45 min under nitrogen, then treated with the crude monoconjugate **4** (51.8 mg, 0.023 mmol, dissolved in 1 mL dry DMF). The reaction was subsequently stirred at room temperature for two days. The DMF was evaporated via rotavap, under high vacuum, and the crude product was absorbed on silica. Flash chromatography was performed with $CHCl_3$/MeOH gradients on a SiO_2 column. Useful fractions (determined by TLC, performed with DCM/MeOH (80/20), by NMR and by MALDI) were grouped and evaporated to dryness to yield 21 mg of pure product. Yield: 37.5 %;

^{1}H-NMR (400 MHz, D_2O, δ, ppm): 3.62 (s, 176 H, 44 -OCH_2CH_2O-), 3.32 (m, 2H, -S$\underline{CH_2}CH_2$- and 1H, -$SCH_2\underline{CH_2}$-), 2.88 (t, J = 6.6 Hz, 4H, -CO$\underline{CH_2}CH_2CO$-), 2.62 (t, J = 6.6 Hz, 4H, -$OCCH_2\underline{CH_2}CO$-), 2.44 (m, 1H, -S$\underline{CH}CH_2$-), 2.19 (t, 2H, J = 6.6 Hz, -$SCHCH_2CH_2CH_2\underline{CH_2}CO$-), 1.66 (m, 1H, -$SCHCH_2CH_2\underline{CH_2}CH_2CO$-), 1.55(m, 2H, -SCH$\underline{CH_2}CH_2CH_2CH_2CO$- and 1H, -$SCHCH_2CH_2\underline{CH_2}CH_2CO$-), 1.34 (m, 2H, -$SCHCH_2\underline{CH_2}CH_2CH_2CO$-). GPC: 96%+, t_R = 15.13 min, PDI = 1.18; MALDI-TOF: $C_{106}H_{206}N_6O_{50}S_4$, exact mass: 2491.3; Found: 2447.4.

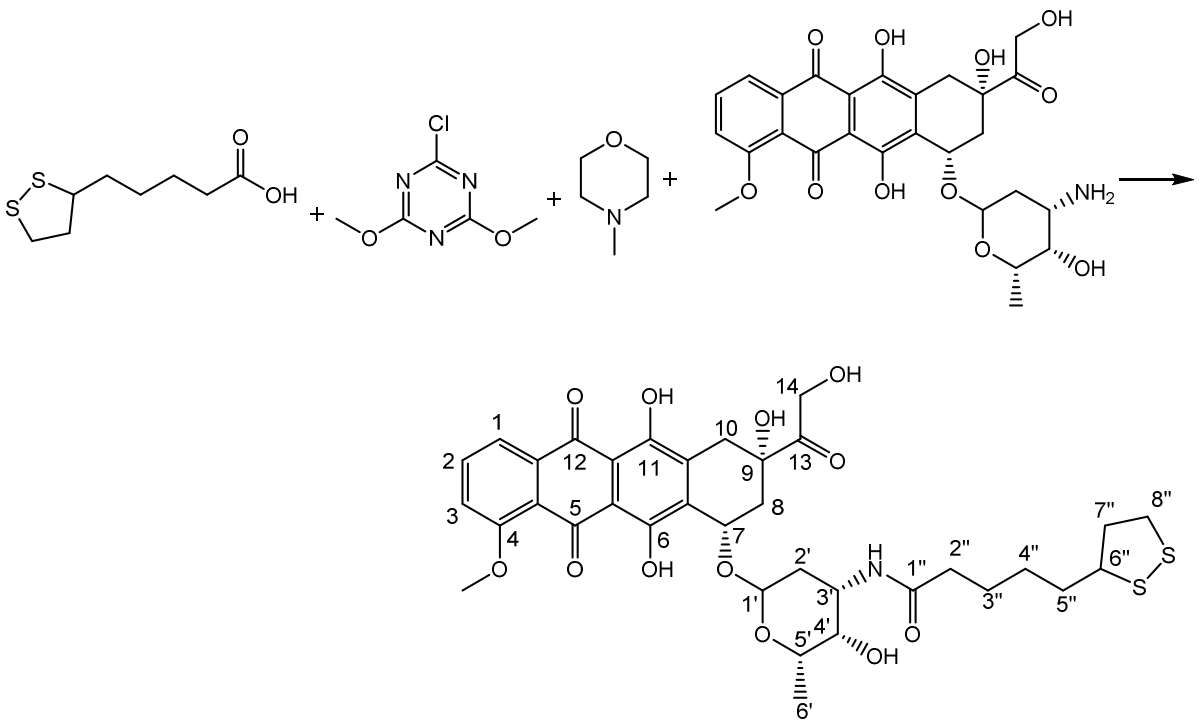
Lipoylamido-PEG2000-OMe 8

In a flame dried 20 ml reaction vial, lipoic acid (28.3 mg, 0.1375 mmol) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (24.1mg, 0.1375 mmol) were dissolved in 1 ml DMF. The flask was cooled in a water-ice bath to 0˚C and N-methylmorpholine (15.3 µL, 13.9 mg, 0.1375 mmol) was added. The homogeneous mixture was allowed to react at 0˚C for 45 min under nitrogen and was subsequently treated dropwise with $CH_3OPEG_{2K}NH_2$ **7** (250 mg, 0.125 mmol) dissolved in 1 mL dry DMF. The reaction was allowed to warm at room temperature and was stirred under inert atmosphere for 2 days. The solvent was evaporated under reduced pressure and the residue was subjected to flash chromatography on SiO_2 using $CHCl_3$/MeOH gradients. Pure fractions (determined by TLC, performed with DCM/MeOH (80/20), by NMR and by MALDI) were grouped and evaporated to dryness to yield 164 mg (63%) conjugate as colorless sticky compound.

^{1}H-NMR (400 MHz, D_2O, δ, ppm): 3.62 (s, 176 H, 44 -OCH_2CH_2O-), 3.32 (m, 2H, -S$\underline{CH_2}CH_2$- and 1H, -$SCH_2\underline{CH_2}$-), 3.29 (s, 3H, CH_3O-), 2.44 (m, 1H, -S$\underline{CH}CH_2$-), 2.19 (t, 2H, J = 6.6 Hz, -$SCHCH_2CH_2CH_2\underline{CH_2}CO$-), 1.66 (m, 1H, -$SCHCH_2CH_2\underline{CH_2}CH_2CO$-), 1.55 (m, 2H, -$SCH\underline{CH_2}CH_2CH_2CH_2CO$- and 1H, -$SCHCH_2CH_2\underline{CH_2}CH_2CO$-), 1.34 (m, 2H, -$SCHCH_2\underline{CH_2}CH_2CH_2CO$-). GPC: 96%+, t_R = 15.21 min, PDI = 1.15; MALDI-TOF: $C_{101}H_{201}NO_{47}S_2$, exact mass: 2244.3; Found: 2288.4.

B. Preparation of Dox conjugates

DOX-lipoic acid amide conjugate (Dox-AM-LA)



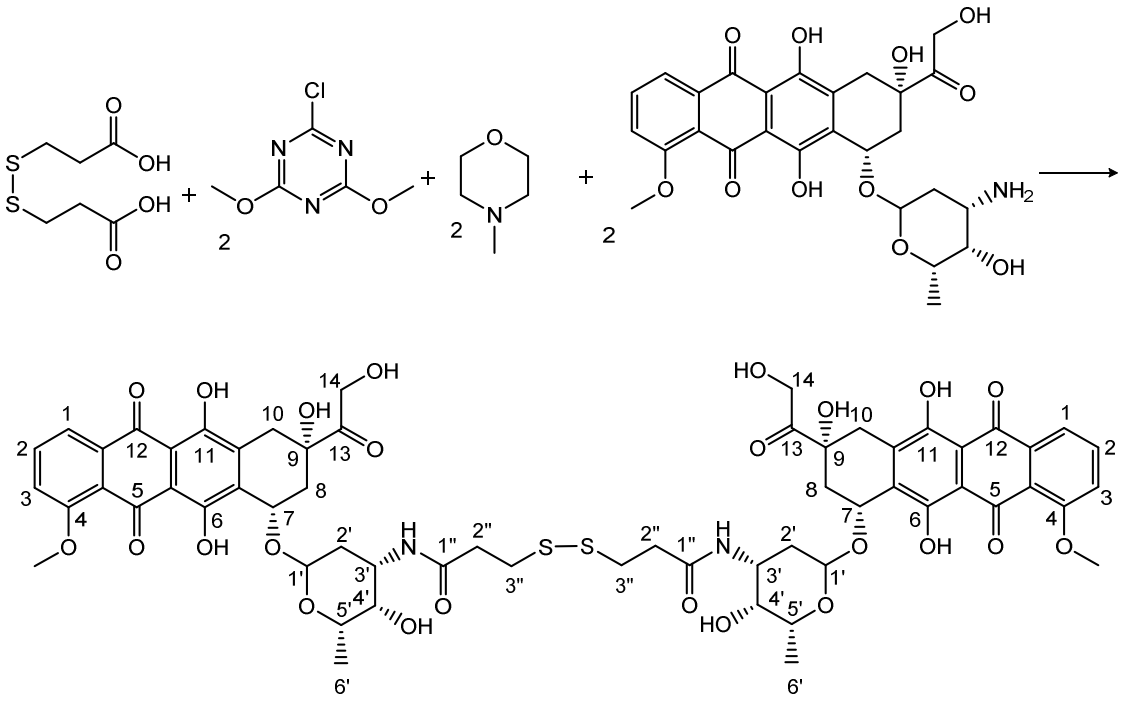
Scheme S1. Synthesis of DOX-lipoic acid amide conjugate (Dox-AM-LA)

In a flame dried 20 mL reaction vial, lipoic acid (17 mg, 0.0825 mmol) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (15 mg, 0.0825 mmol) were dissolved in 2 mL DMF. The flask was cooled in an ice bath at 0°C and N-methylmorpholine (9.2 µL, 8.5 mg, 0.0825 mmol) was added dropwise. The reaction mixture was stirred at 0°C for 45 min under nitrogen and subsequently treated with a solution of doxorubicin hydrochloride (43 mg, 0.0725 mmol) and N-methylmorpholine (9.2 µL, 8.5 mg, 0.0825 mmol) dissolved in 2 mL DMF. Stirring was continued for two days at 25^{o}C, then the reaction was quenched with 10% citric acid solution (15 mL) and the useful product was extracted with DCM (5 times x 15 mL). The DCM layer was

dried on anhydrous Na_2SO_4 and evaporated to dryness to yield the crude product. The crude conjugate was adsorbed onto silica and flash chromatography was performed with DCM/MeOH gradients. Useful fractions (determined by TLC, in DCM: MeOH (95:5), by LCMS and NMR) were grouped and evaporated to dryness to yield the purified product (35 mg, yield 66%).

Dox-AM-LA conjugate. Red amorphous solid, ^{1}H-NMR (400 MHz, DMSO-d^{6}, δ, ppm): 14.01 (s, 1H, 6-OH), 13.25 (s, 1H, 11-OH), 7.89 (dd, J = 6, 2.4 Hz, 2H, H2, H3), 7.62 (dd, J = 6, 3.6 Hz, 1H, H1), 7.49 (d, J = 7.6 Hz, 1H, 3'-NH), 5.43 (br s, 1H, 14-OH), 5.22 (td, J= 19.7, 3 Hz, 4'-OH), 4.92 (t, J= 4.8 Hz, 1H, H7), 4.84 (t, J= 6 Hz, 1H, H4', 4.72 (d, J = 6 Hz, 1H, H1'), 4.58 (d, J= 6 Hz, 2H, H14), 4.18-4.15 (m, 1H, H5'), 4.09 (q, J=5.3 Hz, 1H, H3'), 3.97 (s, 3H, OCH_3), 3.58-3.51 (m, 1H, H6'), 3.23-3.11 (m, 2H, H5'), 2.95 (d, 1H, H10), 2.89 (brs, 1H, H10), 2.40-2.32 (m, 1H, H8"), 2.18-2.03 (m, 4H, H8, H2"), 1.86-1.79 (m, 1H, H8"), 1.65-1.59 (m, 1H, H7"), 1.56-1.42 (m, 4H, H3", H2'), 1.30-1.23 (m, 4H, H4", H7", H9), 1.13 (d, J = 6.5 Hz, 3H, H6'). ^{13}C-NMR (100.6 MHz, DMSO-d^{6}, δ, ppm): 213.7, 186.5, 186.4, 171.3, 160.7, 156.1, 154.5, 136.2, 135.5, 134.6, 134.0, 120.0, 119.7, 118.9, 110.7, 110.6, 100.4, 74.9, 69.9, 68.1, 66.7, 63.6, 56.5, 56.0, 48.6, 44.8, 38.0, 36.6, 35.1, 34.1, 32.1, 29.8, 29.0, 28.2, 25.0, 17.0 LC-MS (>97%): $C_{35}H_{41}NO_{12}S_2$, exact mass: 731.2; Found: 732.2 (MH^+); HRMS: $C_{35}H_{41}NO_{12}S_2$, exact mass: 731.2070; found 731.2076.

Doxorubicin-dithiodipropionic acid amide conjugate (Dox-AM-DTDP)



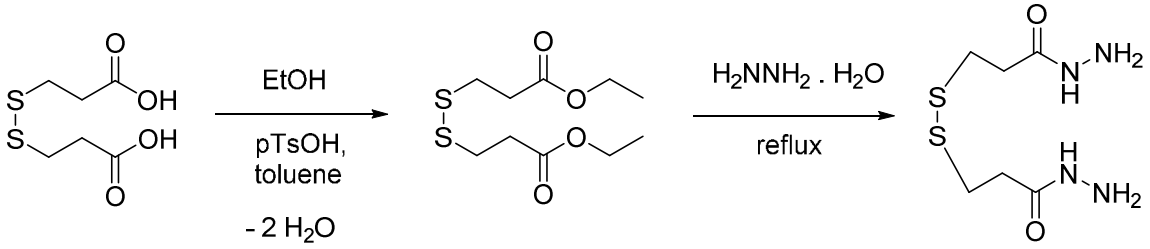
Scheme S2. Synthesis of Doxorubicin-dithiodipropionic acid amide conjugate (Dox-AM-DTDP)

In a flame dried 20 mL reaction vial, 3,3'-dithiodipropionic acid (4.5 mg, 0.0201 mmol) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (7.5 mg, 0.04125 mmol) were dissolved in 2 mL DMF. The flask cooled at 0^{o}C with an ice bath and N-methylmorpholine (4.7 µL, 4.25 mg, 0.04125 mmol) was added dropwise. The reaction mixture was stirred at 0°C for 45 min under nitrogen and was subsequently treated with a solution obtained by dissolving doxorubicin hydrochloride (23 mg, 0.04125 mmol) and N-methylmorpholine (4.7 µL, 4.25 mg, 0.04125 mmol) in 2 mL DMF. Stirring was continued for two days at 25 ^{o}C. The reaction was quenched through dilution with 10% citric acid solution (15 mL) and extracted with DCM (5 times x 15 mL). The DCM layer was dried on anhydrous Na_2SO_4, then evaporated to dryness to yield the crude product. The crude conjugate was absorbed onto SiO_2 and flash chromatography was

performed with DCM/MeOH gradients. Useful fractions (determined by TLC, in DCM: MeOH (95:5), by LCMS and NMR) were grouped and evaporated to dryness to yield the purified product (17 mg, yield 67%).

Dox-AM-DTDP Red amorphous solid. ^{1}H-NMR (400 MHz, DMSO-d^{6}, δ, ppm): 14.02 (brs, 2H, 6-OH), 13.25 (brs, 2H, 11-OH), 7.90 (br d, J = 6, 2.4 Hz, 4H, H2, H3), 7.72 (br d, J = 6, 3.6 Hz, 2H, H1), 7.64 (br t, J = 7.6 Hz, 1H, 3'-NH), 5.45 (brs, 2H, 14-OH), 5.22 (brs, 3H), 5.09-4.80 (m, 3H), 4.62-4.52 (m, 5H), 2.97 (d, 5H), 2.89-2.81 (m, 14H), 262-2.54 (m, 9H), 2.22-2.09 (m, 4H), 1.87-1.75 (m, 2H), 1.44-1.41 (m, 2H), 1.23 (brs, 2H), 1.12 (d, J = 6.5 Hz, 6H, H6′). ^{13}C-NMR (100.6 MHz, DMSO-d^{6}, δ, ppm): 213.7, 186.5, 186.5, 172.6, 169.3, 160.8, 156.1, 154.5, 136.2, 135.5, 134.7, 134.1, 120.0, 119.7, 119.0, 110.8, 110.6, 100.3, 74.9, 69.9, 68.0, 66.7, 63.6, 56.6, 45.0, 40.1, 36.7, 34.9, 34.0, 33.6, 33.1, 33.0, 32.1, 29.7, 17.0. LC-MS (>96%): $C_{60}H_{64}N_2O_{24}S_2$, exact mass: 1260.3; Found: 631.2 ($MH_2^{/2+}$); MALDI-TOF: $C_{60}H_{64}N_2O_{24}S_2$, exact mass: 1260.3; Found 1260.3.

Dithiodipropionic acid hydrazide (HZD-DTDP) – adapted from reference[2]



Scheme S3. Synthesis of dithiodipropionic acid hydrazide (HZD-DTDP)

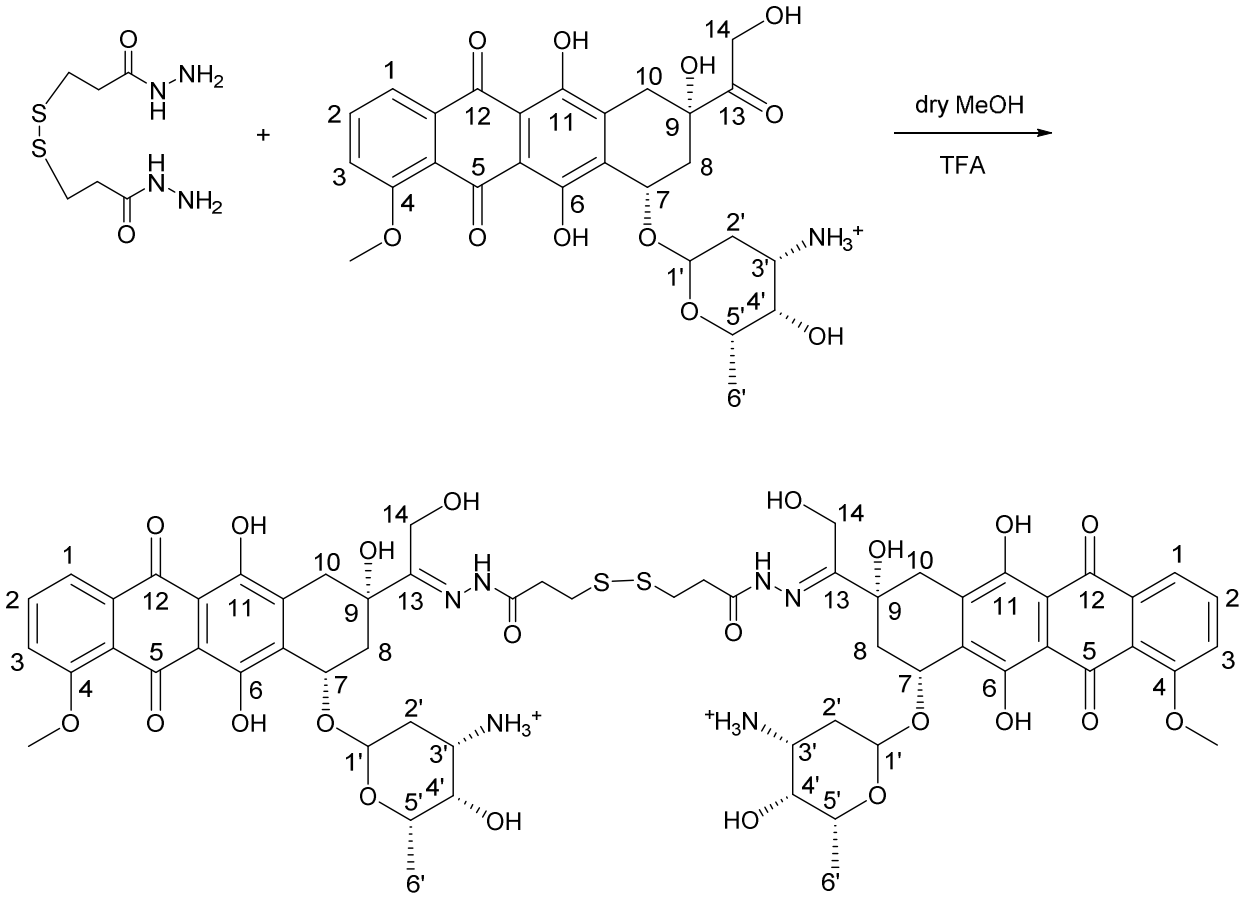
In a flame dried 50 mL round bottom flask, 3,3'-dithiodipropionic acid (5 g, 23.8 mmol) was suspended in ethanol (11.05 g, 240 mmol) and p-toluenesulfonic acid (0.2 g, 1.05 mmol) was added slowly. Then toluene (30 mL) was added over it. The reaction mixture was refluxed for 24 h using a Dean–Stark trap, then it was evaporated to dryness to yield crude ethyl dithiodipropionate followed by washing with $NaHCO_3$ solution and water to pH 7-8. Evaporation to dryness, followed by crystallization from cold ethanol yielded the pure product.

Ethyl dithiodipropionate. ^{1}H-NMR (400 MHz, DMSO-d^6, δ, ppm): 4.16-4.01 (m, 4H), 2.98-2.85 (m, 4H), 2.76-2.63 (m, 4H), 1.26-1.17 (m, 6H). ^{13}C-NMR (100.6 MHz, DMSO-d^6, δ, ppm): 171.0, 60.1, 33.4, 32.6, 13.0.

In the subsequent reaction, ethyl dithiodipropionate (4 g, 15 mmol) was dissolved in ethanol (20 mL) in a 50 mL round bottom flask and hydrazine hydrate (4.8 g, 96 mmol) was added dropwise, under stirring. The reaction mixture was refluxed overnight at 90°C, then cooled at room temperature and bulk solvent evaporated under vacuum. The obtained crude dithiodipropionic acid hydrazide (HZD-DTDP) was recrystallized from (5% v/v) ethanol–water solution three times to yield pure HZD-DTDP.

Dithiodipropionic acid hydrazide (HZD-DTDP) Yield: 60%, mp 128–129°C. Lit. mp 131-132°C. ^{1}H-NMR (400 MHz, DMSO-d^6, δ, ppm): 9.1 (s, -NH), 4.2 (s, -NH_2), 2.89 (t, J= 7.2 Hz, -SCH_2CH_2), 2.41 (t, J= 7.2 Hz, -SCH_2CH_2). ^{13}C-NMR (100.6 MHz, DMSO-d^6, δ, ppm): 169.4, 33.5, 33.2. LC-MS (>99%): $C_6H_{14}N_4O_2S_2$, exact mass: 238.1; Found: 239.2 (MH^+).

Dox-dithiodipropionic hydrazone conjugate (Dox-HZN-DTDP) – adapted after ref [3]



Scheme S4. Synthesis of doxorubicin-dithiodipropionic acid hydrazone conjugate (Dox-HZN-DTDP)

In a flame dried 20 mL reaction vial, doxorubicin hydrochloride (25 mg, 0.0431 mmol) was dissolved in dry MeOH (5 mL) and treated dropwise, under stirring with a solution obtained by dissolving dithiodipropionic hydrazide (5 mg, 0.201 mmol) in dry methanol (3 mL). Trifluoroacetic acid (10 µL) was subsequently added and the reaction mixture was stirred under inert atmosphere for 2 days. Evaporation of solvent yielded the product in pure form, as assessed by TLC and LC-MS.

Dox-HZN-DTDP. Red solid, ^{1}H-NMR (400 MHz, DMSO-d^{6}, δ, ppm): 13.93 (brs, 2H, 6-OH), 13.12 (brs, 2H, 11-OH), 10.40 (s, 2H, 1''-NH) 7.64-7.62 (m, 2H, H1), 7.61-7.58 (m, 4H, H2, H3), 5.25 (brs, 2H, 14-OH), 5.17 (s, 2H, 4'-OH), 4.92 (brs, 2H, H7), 4.72 (brs, 1H, H1'), 4.51 (s, 2H, H4'), 4.46-4.37 (m, 4H, H14), 4.14-4.12 (m, 2H, H5'), 4.09-4.07 (m, 2H, H3'), 4.04-4.02 (m, 2H, 9-OH), 3.92 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 2.92-2.87 (m, 4H, H10), 2.72 (brs, 4H, H3''), 2.22-2.18 (m, 4H, H8, H2''), 1.57-1.55 (m, 4H, H2'), 1.46-1.44 (m, 4H, 3''-NH_2), 0.81 (d, J = 6.5 Hz, 6H, H6'). LC-MS (>98%): $C_{60}H_{70}N_6O_{22}S_2^{2+}$, exact mass: 1290.4; Found: 645.2 (M+/2).

S2 Characterization of citrate-stabilized gold nanoparticles.

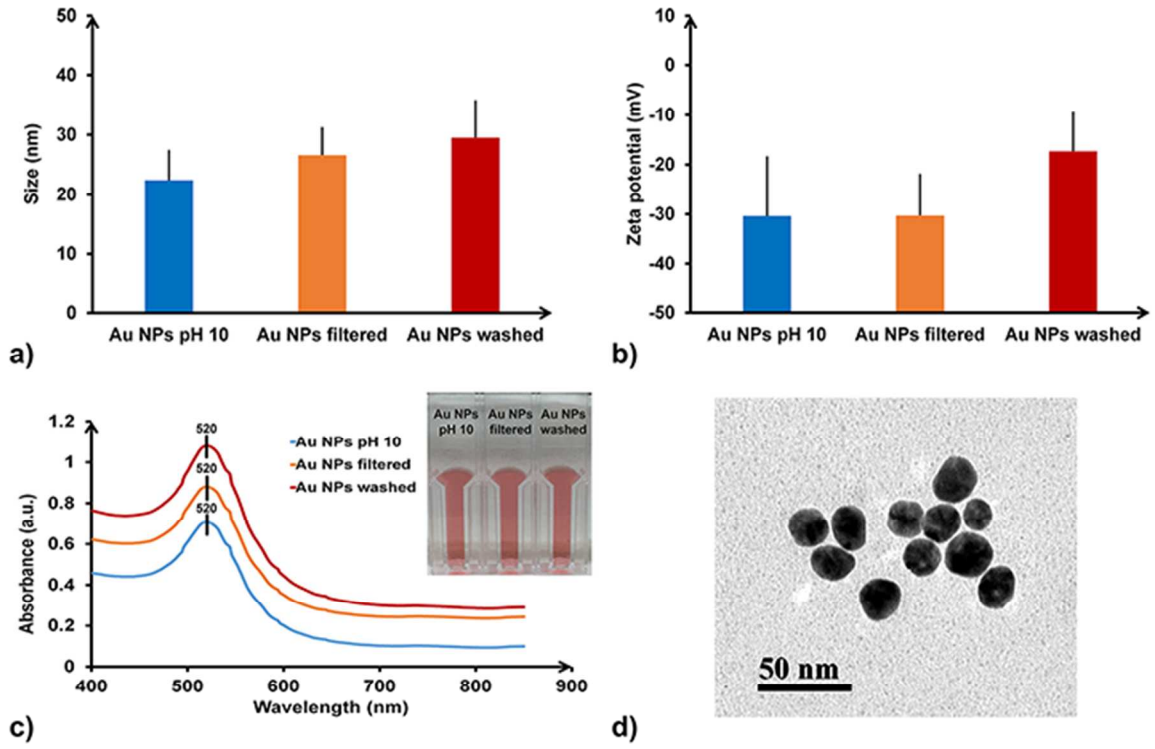


Figure S1. Characterization of citrate-stabilized Au nanoparticles (Au NPs), revealing their size (a), zeta potential (b), plasmon spectrum (c) and aspect (TEM image, d).

S3. Carbonic anhydrase inhibition assay of functionalized gold nanoplatforms

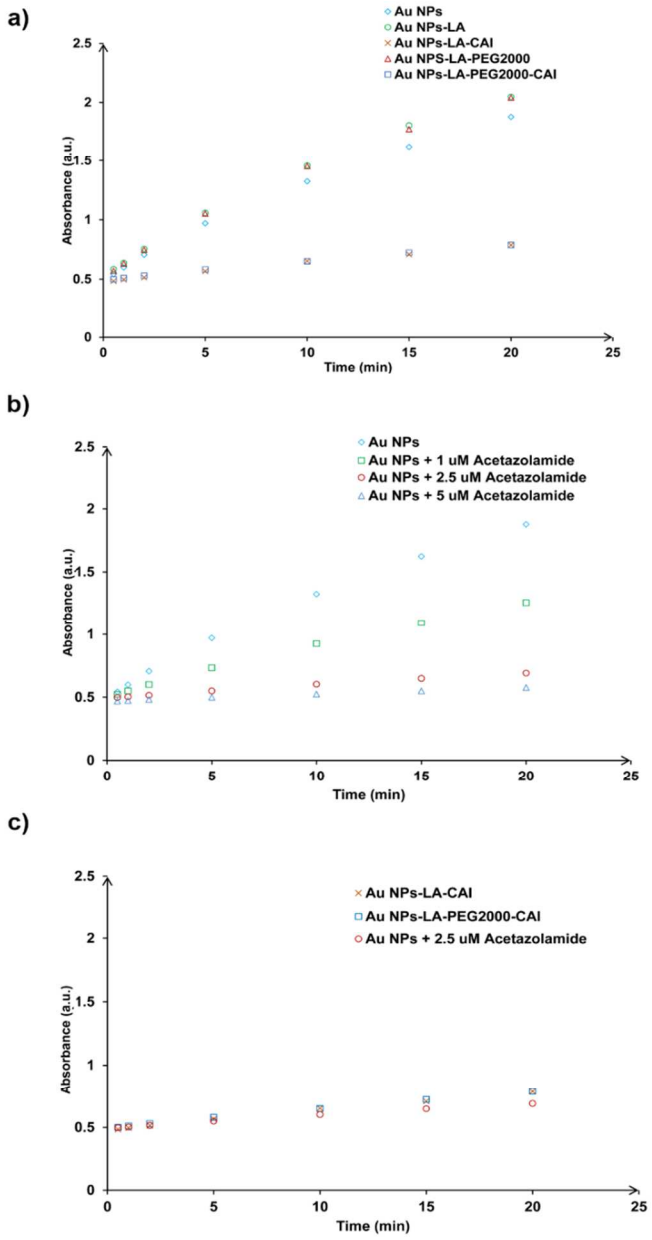
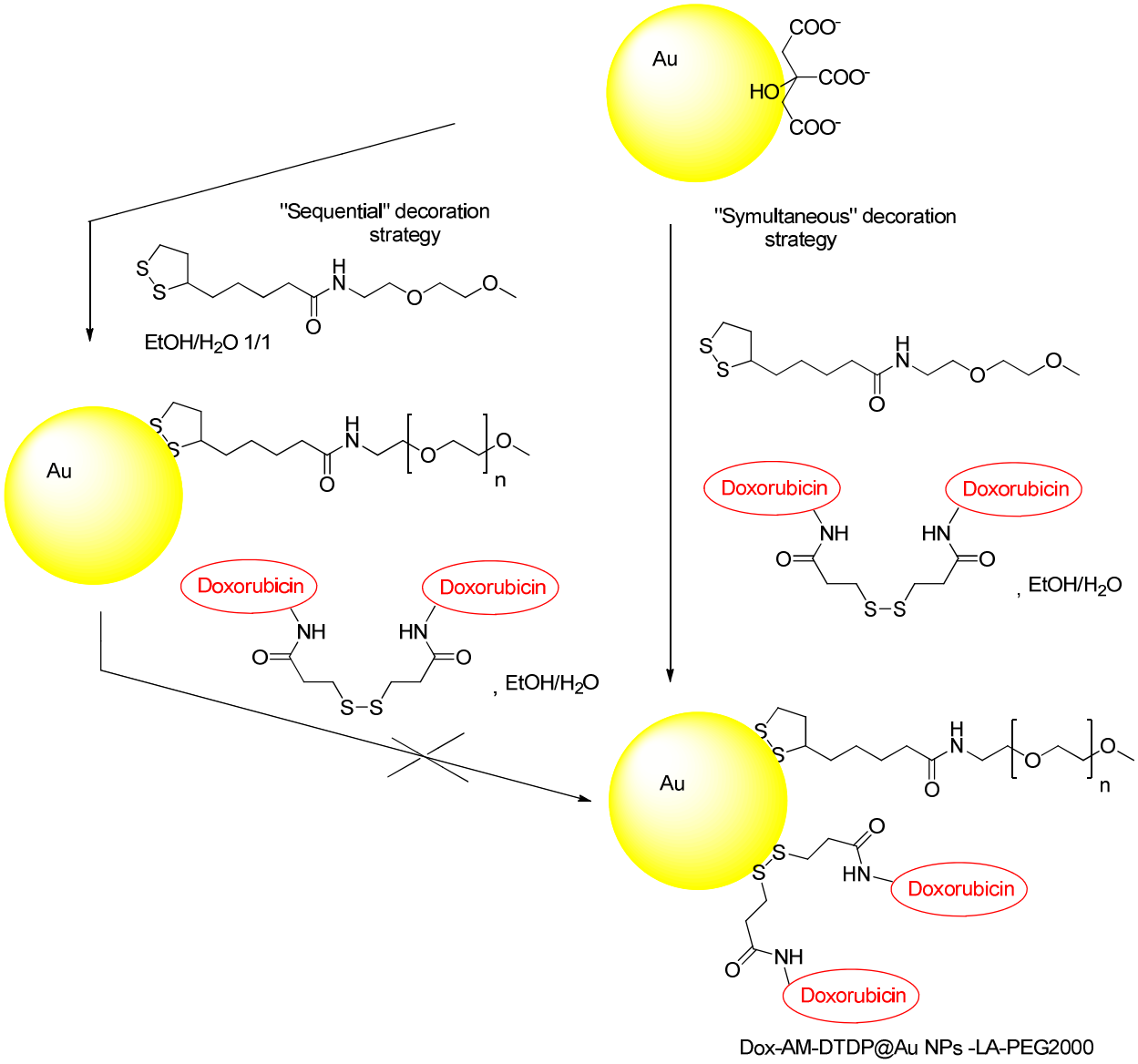


Figure S2. Inhibition of carbonic anhydrase by CAI-decorated AuNPs as compared with standard small inhibitor acetazolamide: CA inhibitory activity of different Au nanoplatforms (a), of classic CAI acetazolamide, at different concentrations (b) and direct comparison of CAI-targeted Au nanoplatforms with acetazolamide at c = 2.5 μM (c)

S4. Preparation of Dox-AM-DTDP @ Au NPs-LA-PEG2000



Scheme S5. Strategies used to generate Dox-loaded Au NPs with amide (AM) –based conjugates. Although both strategies yielded stable Au NPs, the simultaneous strategy generated more homogenous Au NPs with less aggregates as compared with the sequential strategy (see also Supporting Figure 3 below).

S4.1 Sequential Au NPs ligand decoration strategy

In a typical experiment, 100 µL of citrate Au NPs were first treated with 10 µL of 0.02 M LA-PEG2000 stock solution in 50% ethanol, sonicated for 10 min then incubated overnight to complete the Au NPs surface decoration. The next day the Au NPs were pelleted down at 10000 rpm for 15 min to remove excess ligand, supernatant was discarded and the pellet of Au NPs-LA-PEG2000 was resuspended in DI water. The cleaning procedure was repeated, then 20 µL of 0.02 M Dox-AM-DTDP conjugate stock solution in absolute ethanol was added to the PEG decorated Au NPs, preparation was sonicated again for 10 min, then left overnight to complete the process. The next day, the resulted Au NPs (Dox-AM-DTDP @ Au NPs-LA-PEG2000) were centrifuged at 10000 rpm for 15 min, excess ligand was discarded and the Au NPs were resuspended in 100 µL of DI water. The cleaning procedure was repeated once, leaving the Au NPs in 100 µL DI water.

S4.2 Simultaneous Au NPs ligand decoration strategy

In a typical experiment, 10 µL 0.02 M of LA-PEG2000 stock solution in 50% ethanol and 20 µl 0.02 M of Dox-AM-DTDP stock solution in absolute ethanol were pre-mixed then added to 100 µL of citrate Au NPs. The suspension was sonicated for 10 min, then left overnight. The next day, the Au NPs preparation was centrifuged at 10000 rpm for 15 min to remove excess ligand, supernatant was discarded and the Au NPs resuspended in 100 µL DI water. The cleaning procedure was repeated, leaving the Au NPs suspended in 100 µL DI water. Size, zeta potential and UV spectrum of Au NPs were determined as described above.

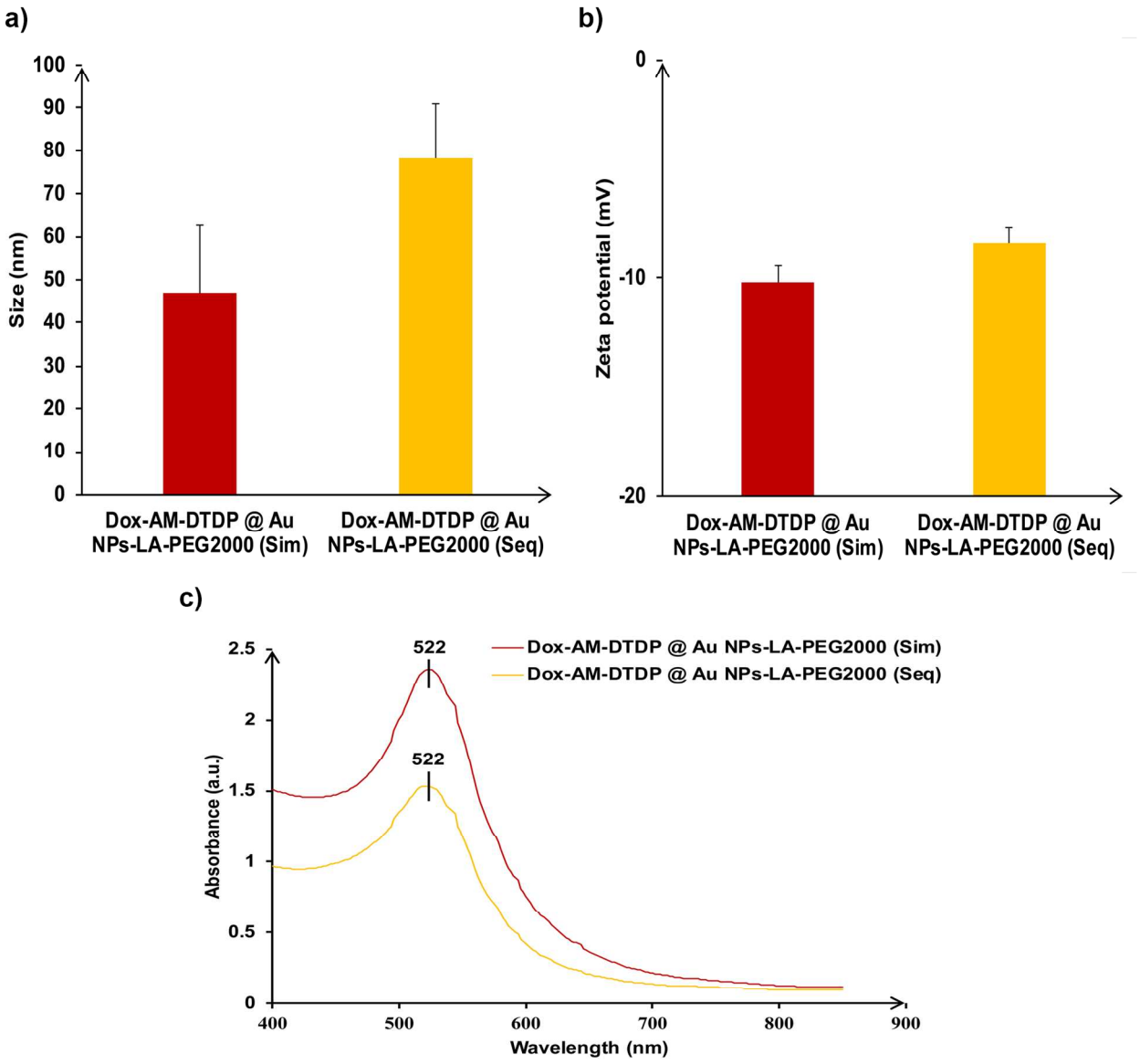
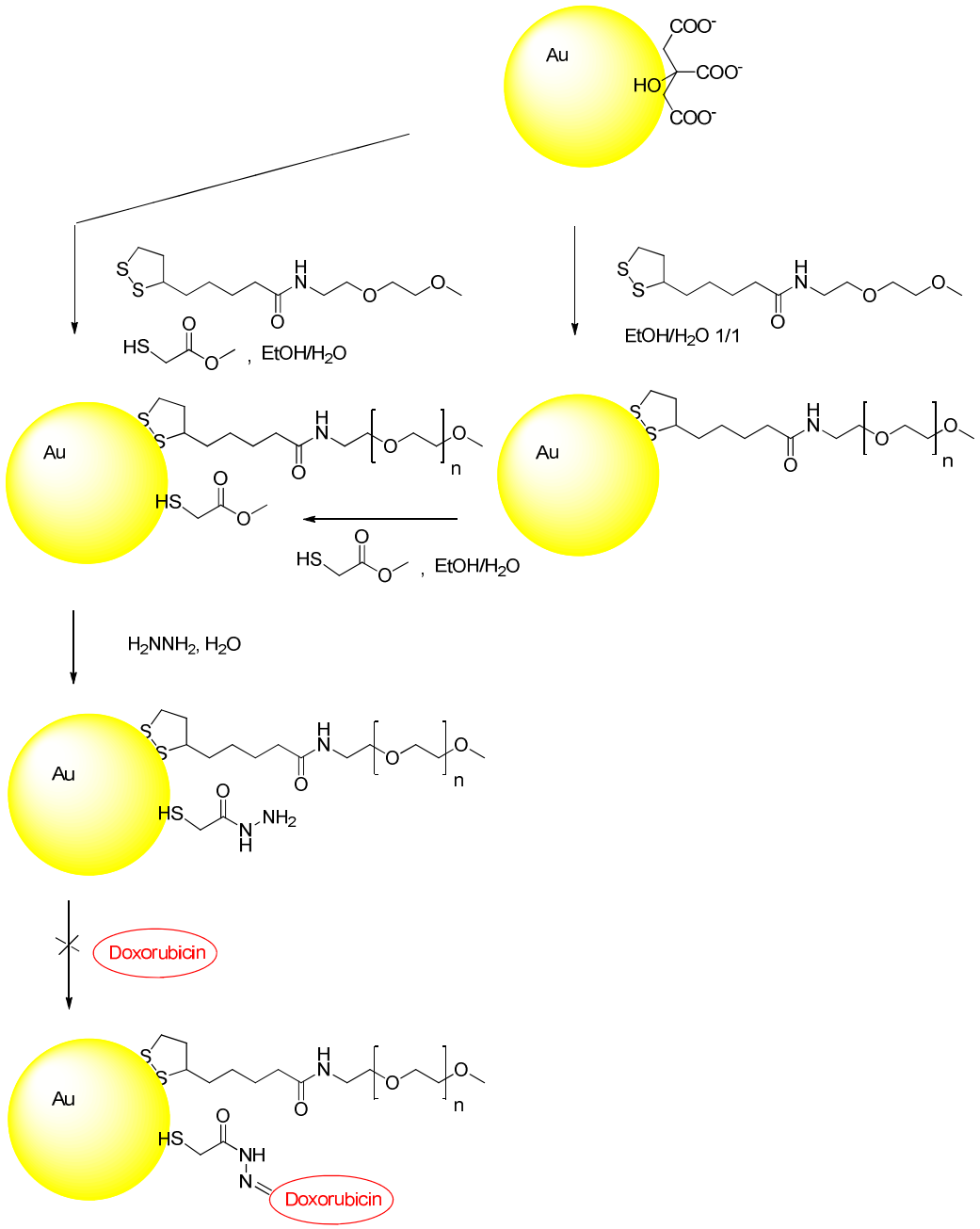


Figure S3. Characterization of Dox-AM-DTDP @ Au NPs-LA-PEG2000 generated through a simultaneous or sequential mixing strategies revealing their size (a), zeta potential (b) and plasmon spectrum (c)

S5. Preparation of MeTG @ Au NPs-LA-PEG2000



Scheme S6. Dox loading strategy on the Au NPs via a pH-sensitive, hydrolysable hydrazone linker generated in situ from thioglycolic acid hydrazide (also made in situ) and doxorubicin

S5.1 Sequential Au NPs ligand decoration strategy for MeTG @ Au NPs-LA-PEG2000

In a typical experiment, 100 µL of citrate Au NPs were first treated with 10 µL of 0.02 M LA-PEG2000 stock solution in 50% ethanol, sonicated for 10 min, then incubated overnight to complete the Au NPs surface decoration. The next day the Au NPs were pelleted down at 10000 rpm for 15 min to remove excess ligand, supernatant was discarded and the pellet of Au NPs-LA-PEG2000 was resuspended in 100 µL DI water. The cleaning procedure was repeated, then 20 µL of 0.02 M methylthioglycolate (MeTG) stock solution in absolute ethanol was added to the PEG decorated Au NPs, preparation was sonicated again for 10 min, then left overnight to complete the process. The next day, the resulted Au NPs (MeTG @ Au NPs-LA-PEG2000) were centrifuged at 10000 rpm for 15 min, excess ligand was discarded and the Au NPs were resuspended in 100 µL of Di water. The cleaning procedure was repeated once, leaving the MeTG @ Au NPs-LA-PEG2000 suspended in 100 µL DI water.

S5.2 Simultaneous Au NPs ligand decoration strategy for MeTG @ Au NPs-LA-PEG2000

In a typical experiment, 10 µL 0.02 M of LA-PEG2000 stock solution in 50% ethanol and 20 µl 0.02 M of methylthioglycolate stock solution in absolute ethanol were pre-mixed then added to 100 µL of citrate Au NPs. The preparation was sonicated for 10 min then left overnight. The next day, the Au NPs preparation was centrifuged at 10000 rpm for 15 min to remove excess ligand, supernatant was discarded and the Au NPs resuspended in 100 µL Di water. The cleaning procedure was repeated, leaving the MeTG @ Au NPs-LA-PEG2000 suspended in 100 µL DI water. Size, zeta potential and UV spectrum of Au NPs were determined as described above.

S6. Preparation of Dox-HZN-TG @ Au NPs-LA-PEG2000

Loading was performed only on MeTG @ Au NPs-LA-PEG2000 prepared by the simultaneous decoration strategy described above. The similar formulation made through sequential decoration had large aggregates and was useless. In a typical experiment, 100 µL of MeTG @ Au NPs-LA-PEG2000 was treated with 400 µL of hydrazine hydrate. The preparation was left on a rocker for 24 h total incubation time. The next day, the Au NPs preparation was centrifuged at 10000 rpm for 15 min to remove excess ligand, supernatant was discarded and the Au NPs resuspended in 100 µL Di water. After performing the same cleaning procedure, the formulation HZD-TG @ Au NPs-LA-PEG2000 thus generated, was treated with 40 µL of doxorubicin stock solution in water (1 mg/ml) at 25 ^{0}C for 24 h. The next day, the Au NPs preparation was centrifuged at 10000 rpm for 15 min to remove excess drug, supernatant was discarded and the Au NPs resuspended in 100 µL DI water. The cleaning procedure was repeated leaving the Au NPs suspended in 100 µL DI water. Size, zeta potential and UV spectrum of Au NPs were determined as described above.

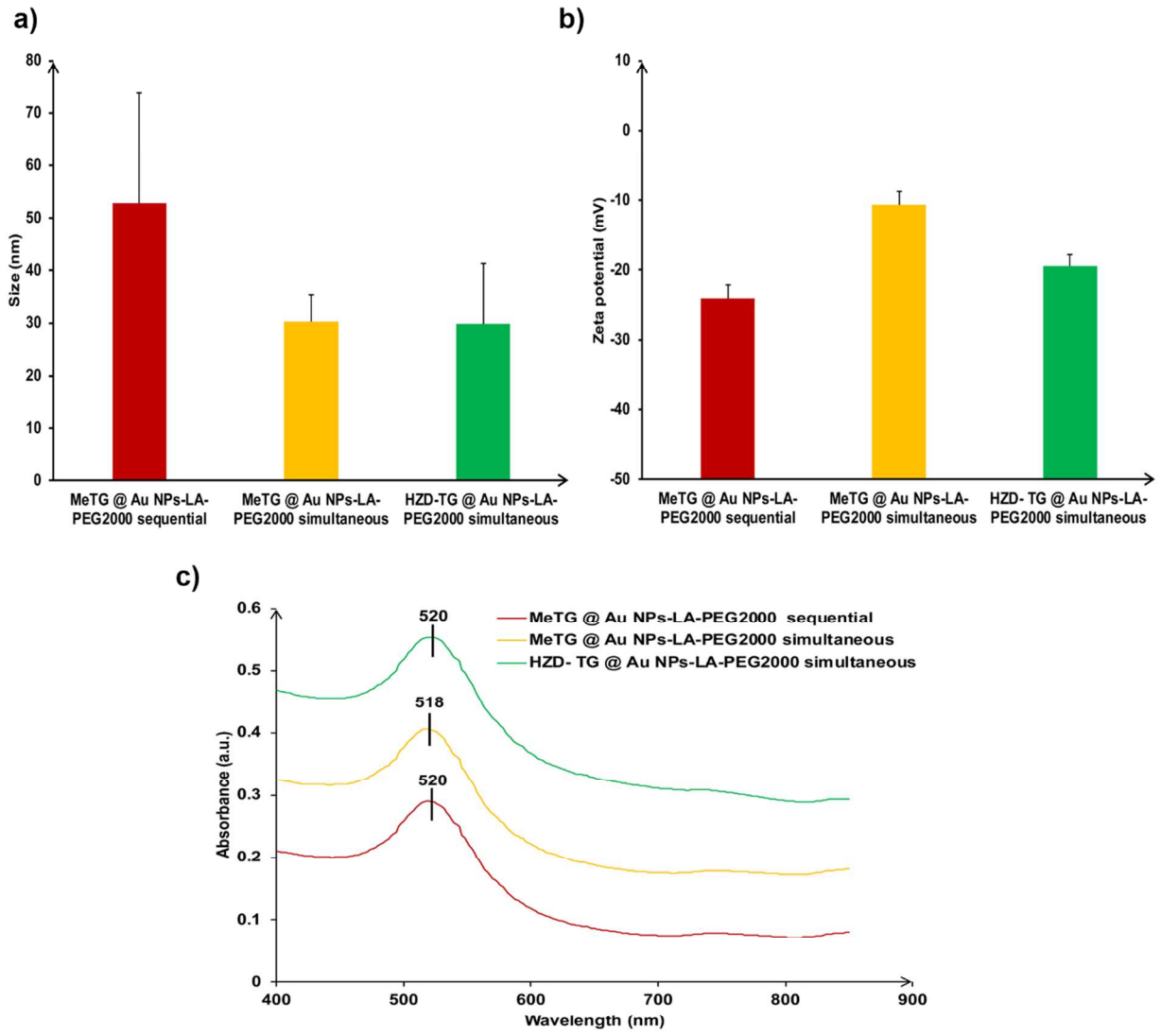
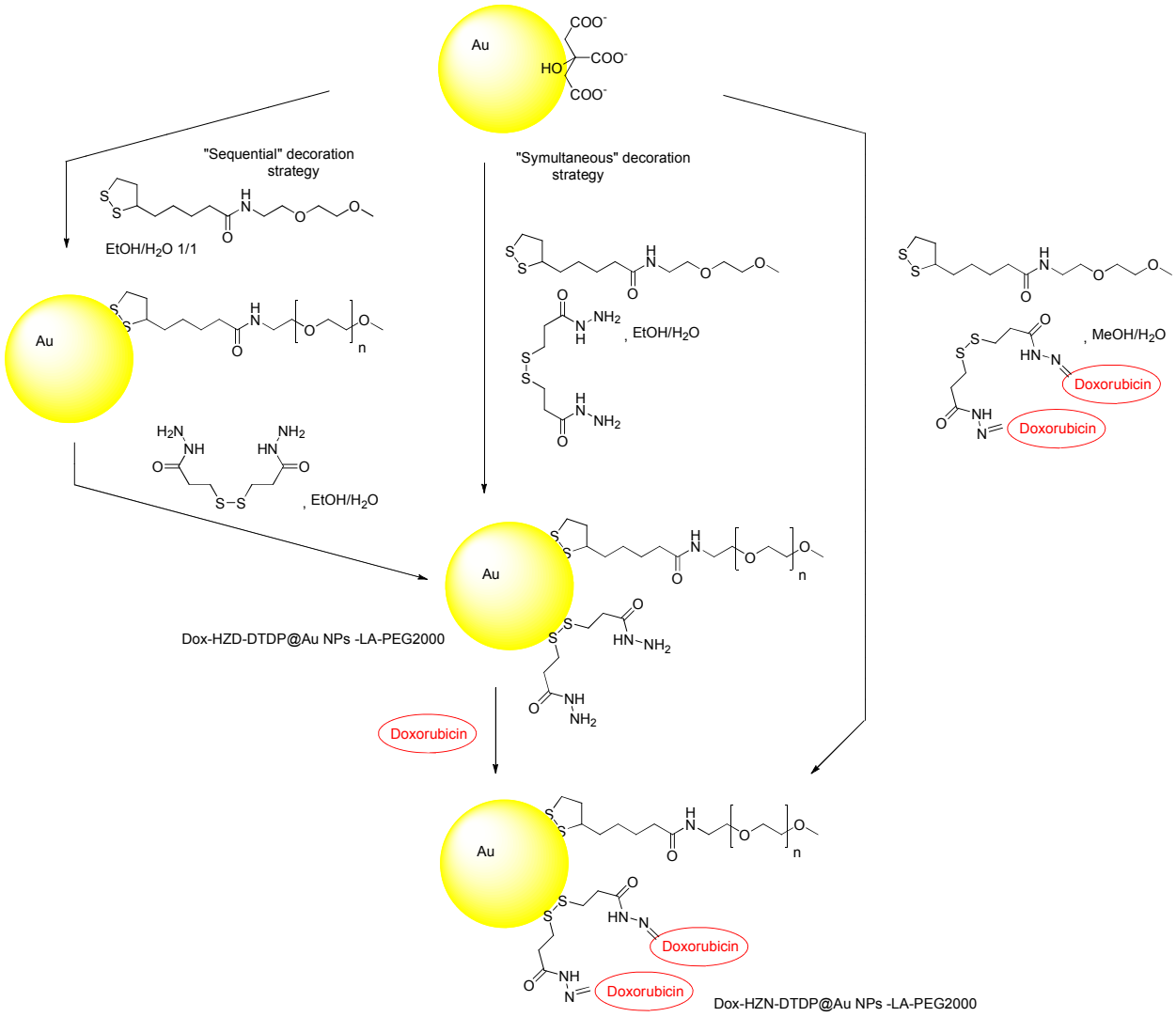


Figure S4. Characterization of different Au NPs used in the generation of Dox-HZN-TG @ Au NPs-LA-PEG2000 in terms of their size (a), zeta potential (b) and plasmon spectrum (c)

S7. Preparation of Dox-HZN-DTDP @ Au NPs-LA-PEG2000



Scheme S7. Dox loading strategy on the Au NPs via a pH-sensitive, hydrolysable hydrazone linker generated in situ from DTDP hydrazide (HZD-DTDP) and doxorubicin or via a pre-formed Dox-hydrazone-DTDP (Dox-HZN-DTDP)

S7.1 Method A. Doxorubicin loading onto HZD-DTDP @ Au NPs-LA-PEG2000

S7.1.1 Preparation of HZD-DTDP @ Au NPs-LA-PEG2000

Stock solutions of 0.02 M of LA-PEG2000 in 50% ethanol and 0.02 M of DTDP-hydrazide (DTDP-HZD) conjugate in absolute ethanol were pre-mixed together in two different ratios (1:1 and 1:3 respectively) then added to 100 µL of citrate Au NPs. The preparations were sonicated for 10 min then left overnight. The next day, the preparations were centrifuged at 10000 rpm for 15 min, supernatant was discarded and Au NPs resuspended in 100 µL of DI water. The cleaning procedure was repeated once and final preparations were made in 100 µL DI water. The resulted HZD-DTDP @ Au NPs-LA-PEG2000 preparations were characterized for size, zeta potential and UV spectrum.

S7.1.2 Loading of HZD-DTDP @ Au NPs-LA-PEG2000 with doxorubicin

In a typical experiment, 100 µL of HZD-DTDP @ Au NPs-LA-PEG2000 was treated with 40 µL of doxorubicin stock solution in water (1 mg/ml). The preparation was left on a rocker for 24 h incubation time. The next day, the Au NPs preparation was centrifuged at 10000 rpm for 15 min to remove excess drug, supernatant was discarded and the Au NPs resuspended in 100 µL Di water. After performing the same cleaning procedure, the formulation (Dox-HZN-DTDP @ Au NPs-LA-PEG2000) was characterized for size, zeta potential and UV spectrum as described above.

S.7.2 Method B. Preparation of Dox-HZN-DTDP @ Au NPs-LA-PEG2000 using the Dox-HZN-DTDP conjugate

Stock solutions of 0.02 M of LA-PEG2000 in 50% ethanol and 0.02 M of Dox-HZN-DTDP conjugate in methanol were pre-mixed in four different ratios (1:1, 1:2, 1:3 and 1:4 respectively) then added to 100 µL of citrate Au NPs. The preparations were sonicated for 10 min then left overnight. The next day, the Au NPs preparations were centrifuged at 10000 rpm for 15 min to remove excess ligand, supernatant was discarded and the Au NPs resuspended in 100 µL DI water. After performing the same cleaning procedure, the formulation (Dox-HZN-DTDP @ Au NPs-LA-PEG2000) was characterized for size, zeta potential and UV spectrum as described above.

S8. Preparation of Dox-HZN-DTDP @ Au NPs-LA-PEG2000-CAI using the Dox-HZN-DTDP conjugate

This procedure was identical with method B of doxorubicin loading described in section 2.12.2 above. Stock solutions of 0.02 M of LA-PEG2000-CAI in 50% ethanol and 0.02 M of Dox-HZN-DTDP conjugate in methanol were pre-mixed in ratio 1:1 then added to 100 µL of citrate Au NPs. The preparation was sonicated for 10 min then left overnight. The next day, the Au NPs preparation was centrifuged at 10000 rpm for 15 min to remove excess ligand, supernatant was discarded and the Au NPs resuspended in 100 µL DI water. After performing the same cleaning procedure, the formulation (Dox-HZN-DTDP @ Au NPs-LA-PEG2000-CAI) was checked for size, zeta potential and UV spectrum as described above.

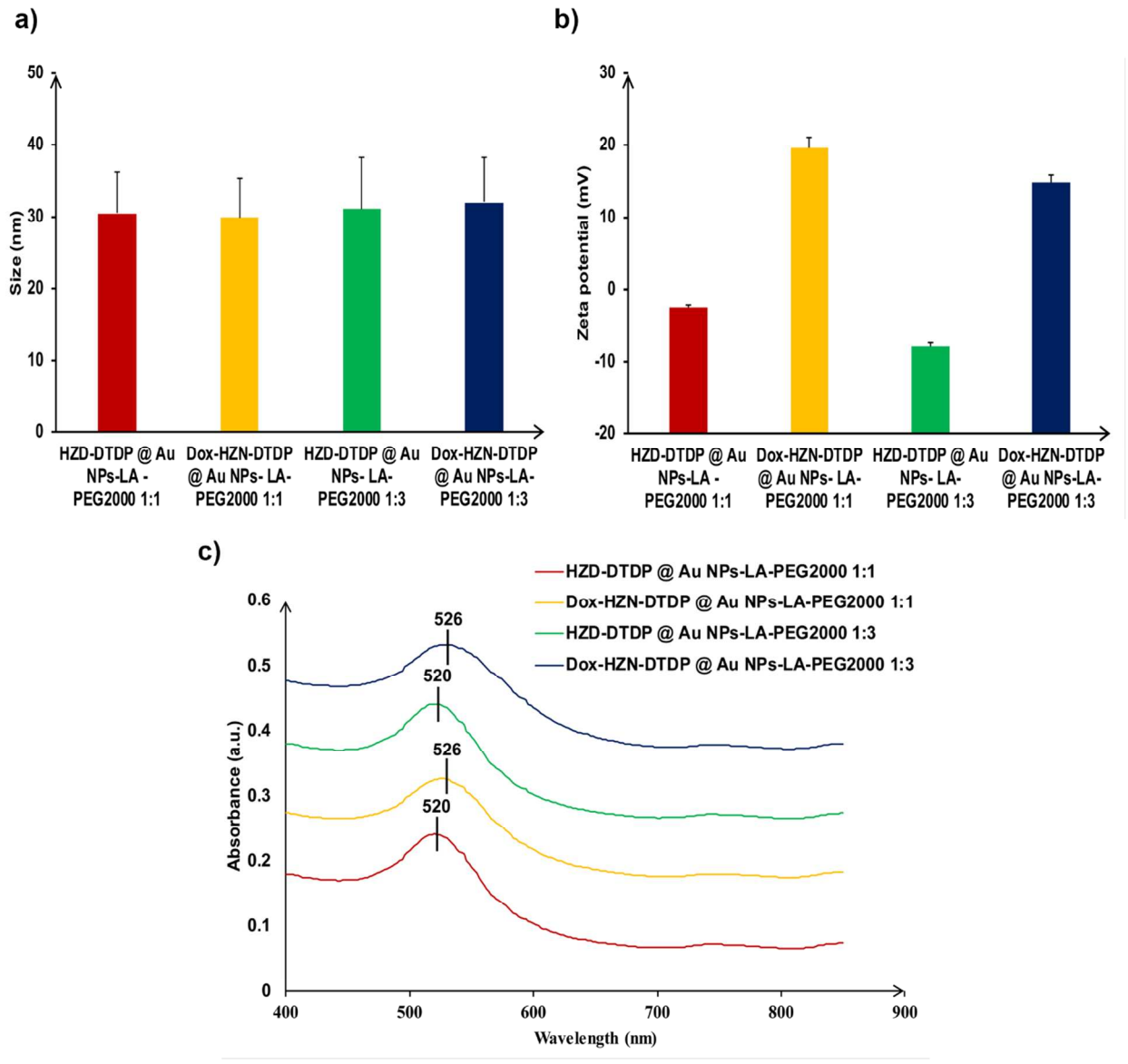


Figure S5. Characterization of HZD-DTDP @ Au NPs-LA-PEG2000 and subsequently generated Dox-HZN-DTDP @ Au NPs-LA-PEG2000, revealing their size (a), zeta potential (b) and plasmon spectrum (c)

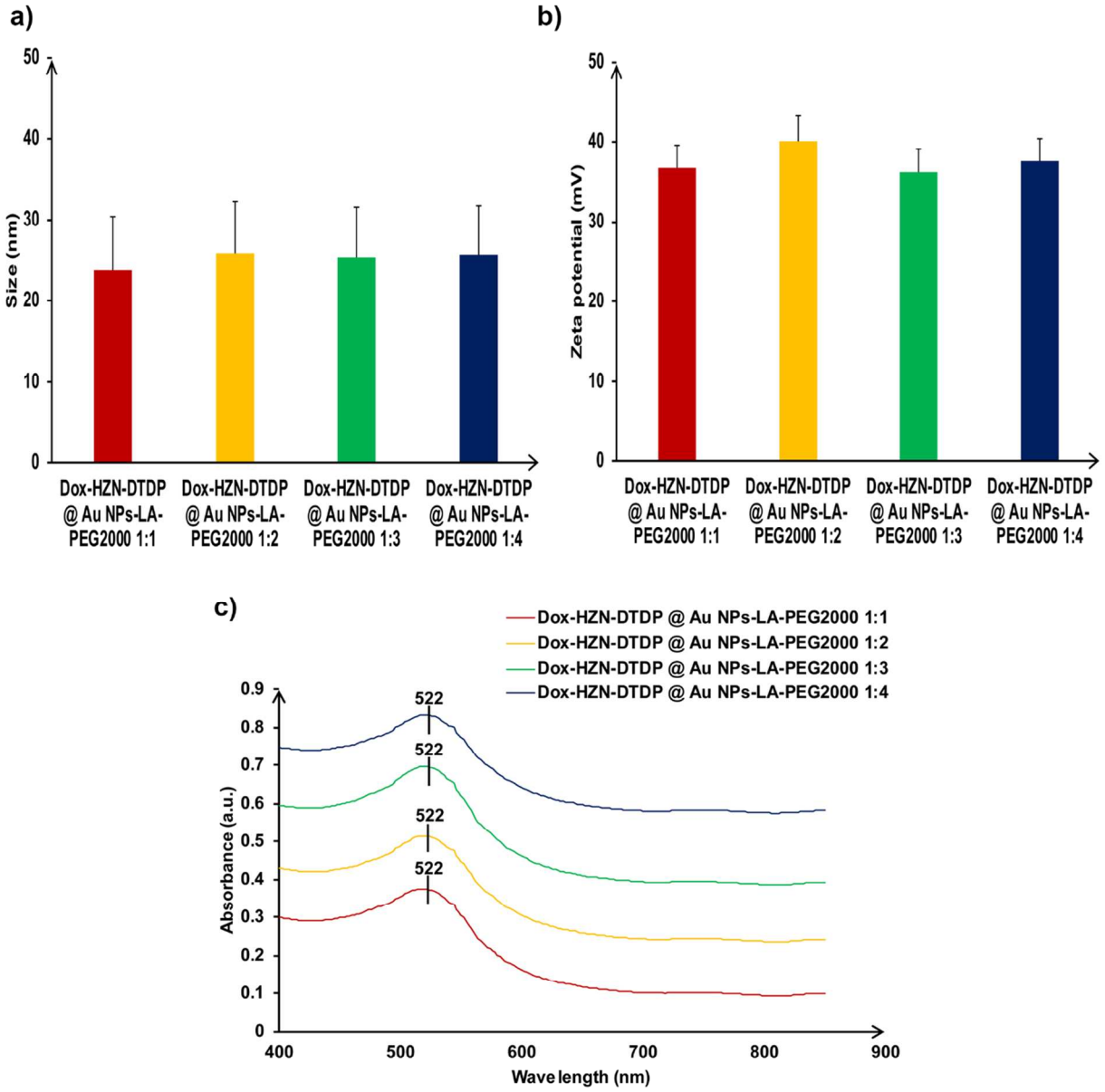


Figure S6. Characterization of Dox-HZN-DTDP @ Au NPs-LA-PEG2000 prepared from simultaneous addition of Dox-HZN_DTDP and LA-PEG2000 premixed ligand, at four different molar ratios revealing their size (a), zeta potential (b) and plasmon spectrum (c)

S9. Direct comparison of optical properties of doxorubicin free conjugate and of doxorubicin-loaded Au NPs

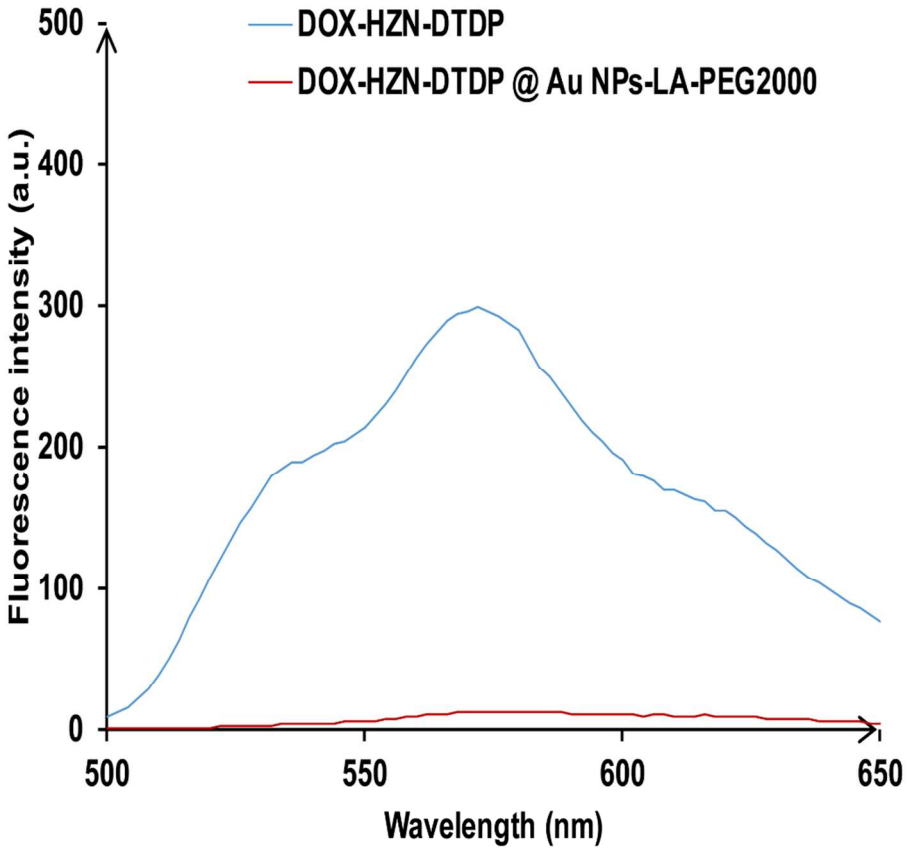


Figure S7. Emission spectrum of DOX-HZN-DTDP conjugate and DOX-HZN-DTDP @ Au NPs-LA-PEG2000. The adsorption of DOX-HZN-DTDP on Au NPs resulted in quenching of its fluorescence signal, confirming the loading of Dox on Au NPs (excitation was done at 496 nm).

S10. Determination of doxorubicin content of doxorubicin loaded Au NPs

The amount of doxorubicin loaded on Au NPs was determined by fluorescence spectroscopy. In a typical experiment, 100 μL of Dox-containing Au NPs formulation was treated with 20 μL of 0.1 N HCl to liberate the free drug then the doxorubicin amount was determined by excitation at 488 nm and emission at 570 nm against a calibration curve made with known concentration of doxorubicin hydrochloride.

S11. pH-dependent release of doxorubicin loaded Au NPs bearing hydrazone linker

The release of Dox from Au NPs in which the drug was loaded with the hydrazone linker was examined at two different pH values, namely pH = 5.5, mimicking the lysosomal pH and pH = 7.4, mimicking the blood/extracellular fluid pH. Both NTHZN and THZN were incubated with PBS, adjusted either to pH = 5.5, or to pH = 7.4, over a period of 24 h. The fluorescence emission spectrum of Dox was recorded at different time intervals. To quantitate the percentage of Dox released at each time point, the fluorescence intensity of Dox released at 570 nm was compared with the fluorescence intensity of Dox after incubating the Dox loaded Au NPs with 0.1 N HCl (100 % release).

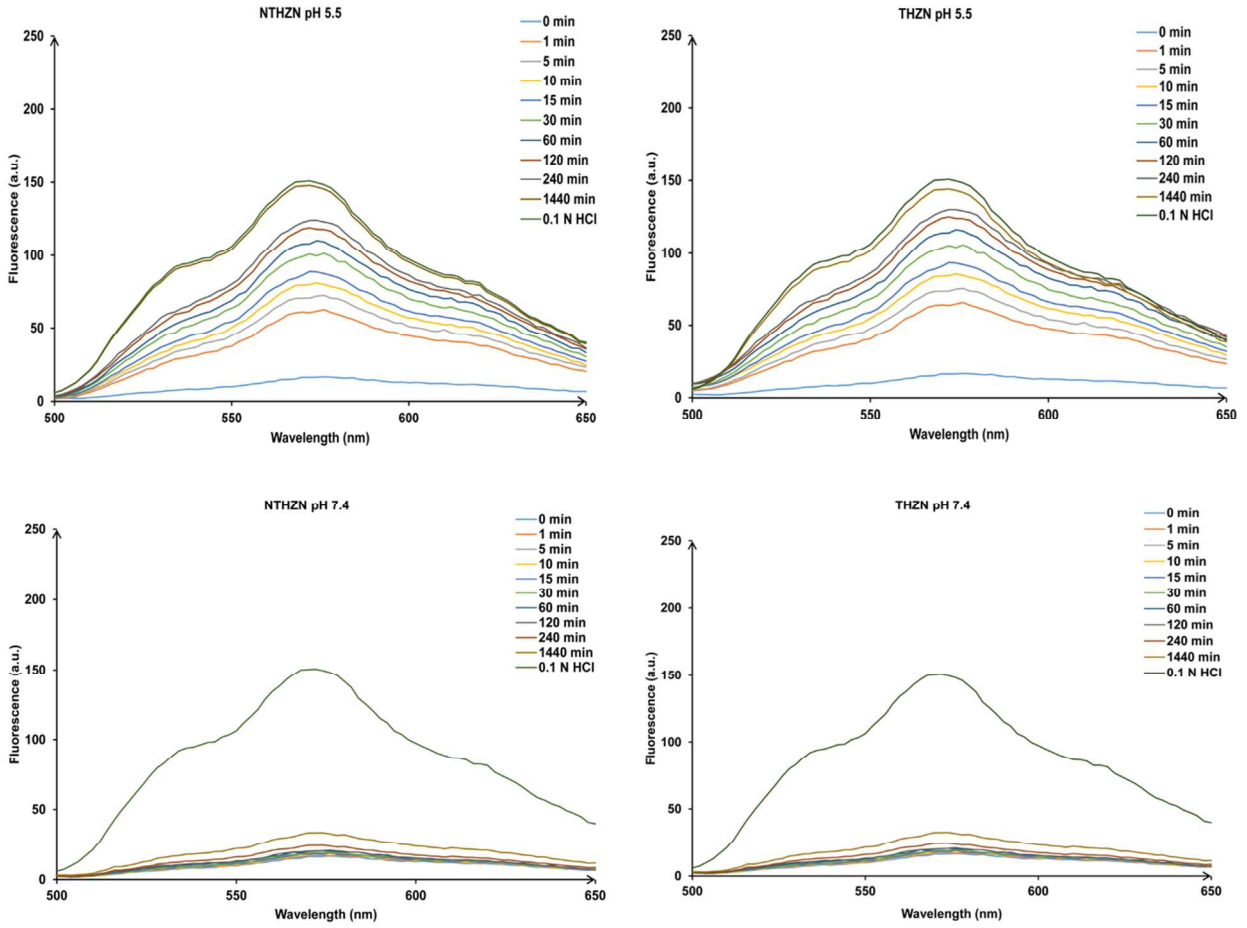


Figure S8. Emission spectrum of NTHZN and THZN after incubation in phosphate buffer saline at either pH 5.5 or pH 7.4, over a period of 24 h. The fluorescence intensity is proportional to the amount of free Dox released, as Dox fluorescence is quenched when bound to Au NPs. Total Dox loaded was determined by separate hydrolysis with 0.1 N HCl, and it is also shown as reference.

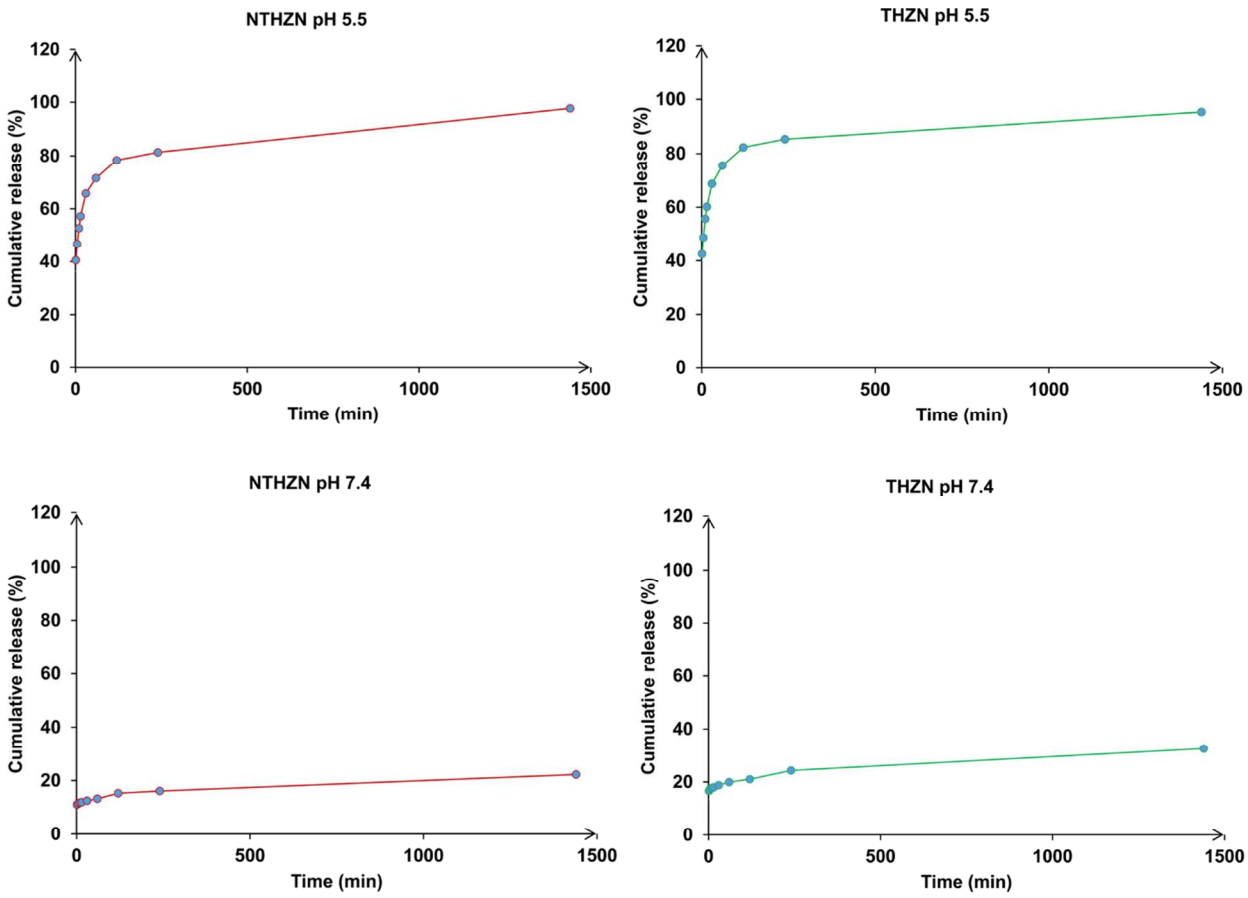


Figure S9. Dox release kinetics from NTHZN and THZN after incubation in phosphate buffer saline at either pH 5.5 or pH 7.4, over a period of 24 h. Results are expressed as percentage of total Dox loaded (determined by separate hydrolysis with 0.1 N HCl)

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(1) Ilies, M. A.; Vullo, D.; Pastorek, J.; Scozzafava, A.; Ilies, M.; Caproiu, M. T.; Pastorekova, S.; Supuran, C. T. Carbonic Anhydrase Inhibitors. Inhibition of Tumor-Associated Isozyme IX by Halogenosulfanilamide and Halogenophenylaminobenzolamide Derivatives. *J. Med. Chem.* **2003,** *46* (11), 2187-2196.

(2) Akocak, S.; Alam, M. R.; Shabana, A. M.; Sanku, R. K.; Vullo, D.; Thompson, H.; Swenson, E. R.; Supuran, C. T.; Ilies, M. A. PEGylated Bis-Sulfonamide Carbonic Anhydrase Inhibitors Can Efficiently Control the Growth of Several Carbonic Anhydrase IX-Expressing Carcinomas. *J. Med. Chem.* **2016,** *59* (10), 5077-5088.