Electronic Supporting Information

Nanodiamonds mediated co-delivery of doxorubicin and malaridine to maximize synergistic anti-tumor effects on multi-drug resistant MCF-7/ADR cells

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1. Materials and methods

1.1 Materials

Doxorubicin hydrochloride was purchased from Beijing Huafeng Lianbo Technology Co., Ltd. (China). Dimethyl sulfoxide (DMSO) was purchased from Sigma Co., Ltd. (USA). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl), N-Hydroxysuccinimide (NHS), succinic anhydride (SA) and folic acid were purchased from Aladdin Industrial Co., Ltd. (China). 1,4-dioxane, triethylamine (TEA), sodium hydroxide and hydrochloride acid were analytical grade and purchased from Shanghai Ling Feng Chemical Reagent Co., Ltd. (China). NH_2-PEG-NH_2 (Mw: 2000) were purchased from Shanghai Yare Biotech Co., Ltd. Dialysis bag (MWCO: 1000) were purchased from Shanghai Genestar Technology Co., Ltd.

1.2 Synthesis and characterization of FA-PEG-DOX

1.2.1Synthesis of FA-PEG-NH_2

Folic acid (13.2 mg, 0.03 mmol), EDC (46.6 mg, 0.24 mmol), NHS (27.6 mg, 0.24 mmol) were dissolved in 1.8 mL anhydrous DMSO, the reaction was lasted for 4 h. NH_2-PEG-NH_2 (60 mg, 0.03 mmol), previously dissolved in 3mL anhydrous DMSO and 33.7 μL TEA were added to the mixture and further reacted for 48 h. The whole reaction was

under the protection of N_2 and in the dark. After that, the mixture was transferred to the dialysis bag (MWCO: 1000) and dialyze against ddH_2O for 2 days, freeze dried to obtain FA-PEG-NH_2. The strcture was confirmed by ^{1}H-NMR spectra recorded on a Varian Mercury Plus-400 NMR spectrometer (Varian, USA).

1.2.2 Synthesis of SA-DOX

DOX·HCl (5 mg, 8.52 μmol) was dissolved in 0℃ 0.8 mL carbonate buffer solution (0.1 M, pH=9) and stirred in the dark. SA (4.43 mg, 44.3 μmol) was dissolved in 0.06 mL 1,4-dioxane and added to the mixture dropwise. Adjust the pH to 8~9 with 0.5 M NaOH, reacted at 0℃ for 20 min and at 25℃ for 20 min. The mixture was cooled on ice and cold HCl (1 M, 8 mL) was added until large amount of precipitate was formed. After centrifugation, the precipitate was freeze dried and SA-DOX was obtained.

1.2.3 Synthesis of FA-PEG-DOX

SA-DOX (3.45 mg, 5.36 μmol), EDC (8.22 mg, 42.88 μmol), NHS (4.94 mg, 42.88 μmol) were dissolved in 0.5mL anhydrous DMSO and reacted for 4h. FA-PEG-NH_2 (7.14 mg, 3.57 μmol), previously dissloved in 0.4 mL anhydrous DMSO, and TEA (0.004 mL, 28.56 μmol) were added to the mixture slowly and reacted for 48 h. The whole reaction was under the protection of N_2 and in the dark. After that, the mixture was transferred to the dialysis bag and dialyze against ddH_2O for 48 h, freeze dried to obtain FA-PEG-DOX. The structure was confirmed by ^{1}H-NMR. The conjugation ratio of DOX on FA-PEG-DOX was measured by the UV-vis absorption at wavelength of 480 nm and calculated according to the standard calibration curve of DOX in DMSO. The conjugation ratio of FA on FA-PEG-NH_2 was measured by the UV-vis absorption at wavelength of 363 nm and calculated according to the standard calibration curve of FA in DMSO.

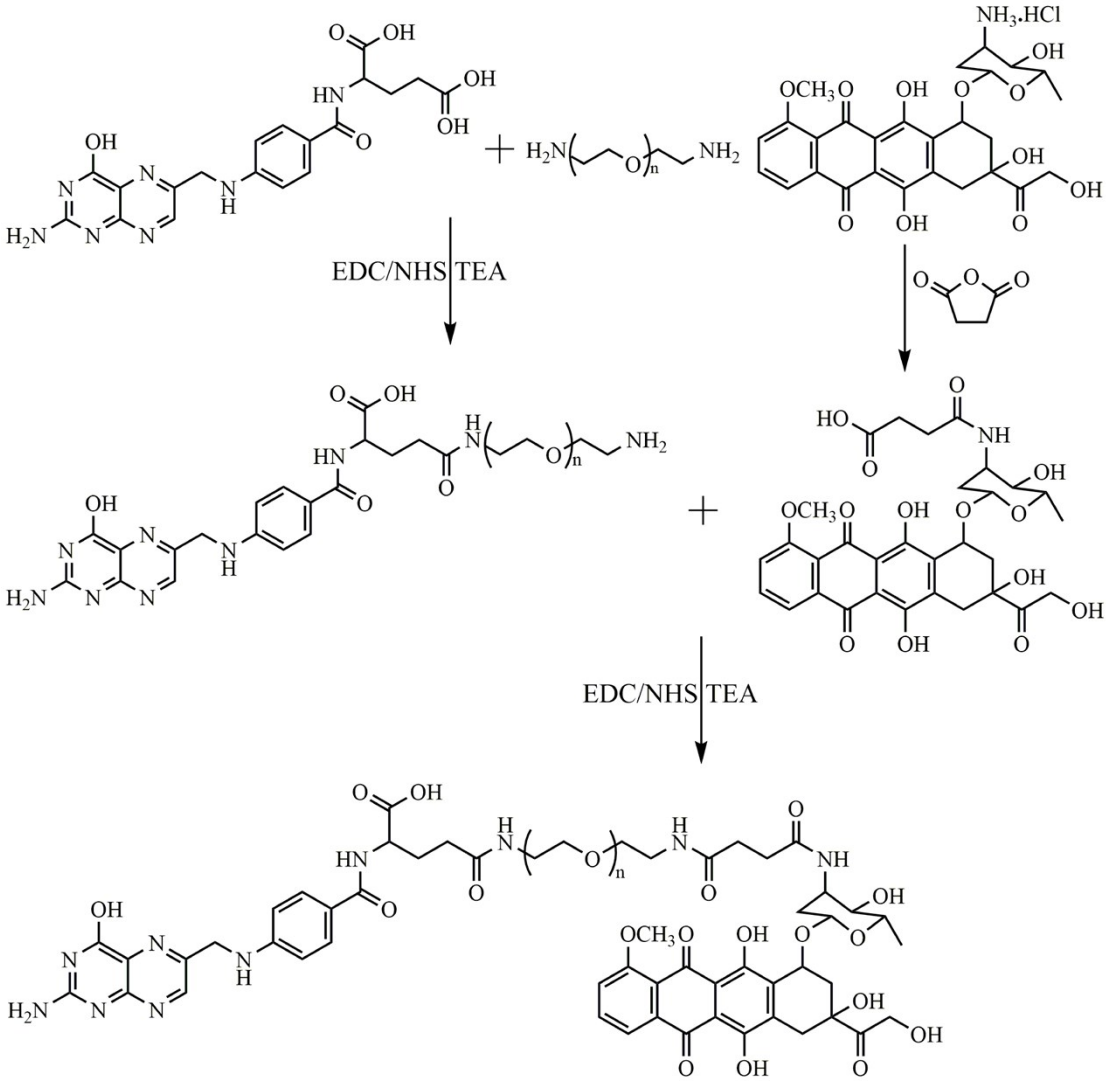

Fig. S1 The synthetic route of FA-PEG-DOX

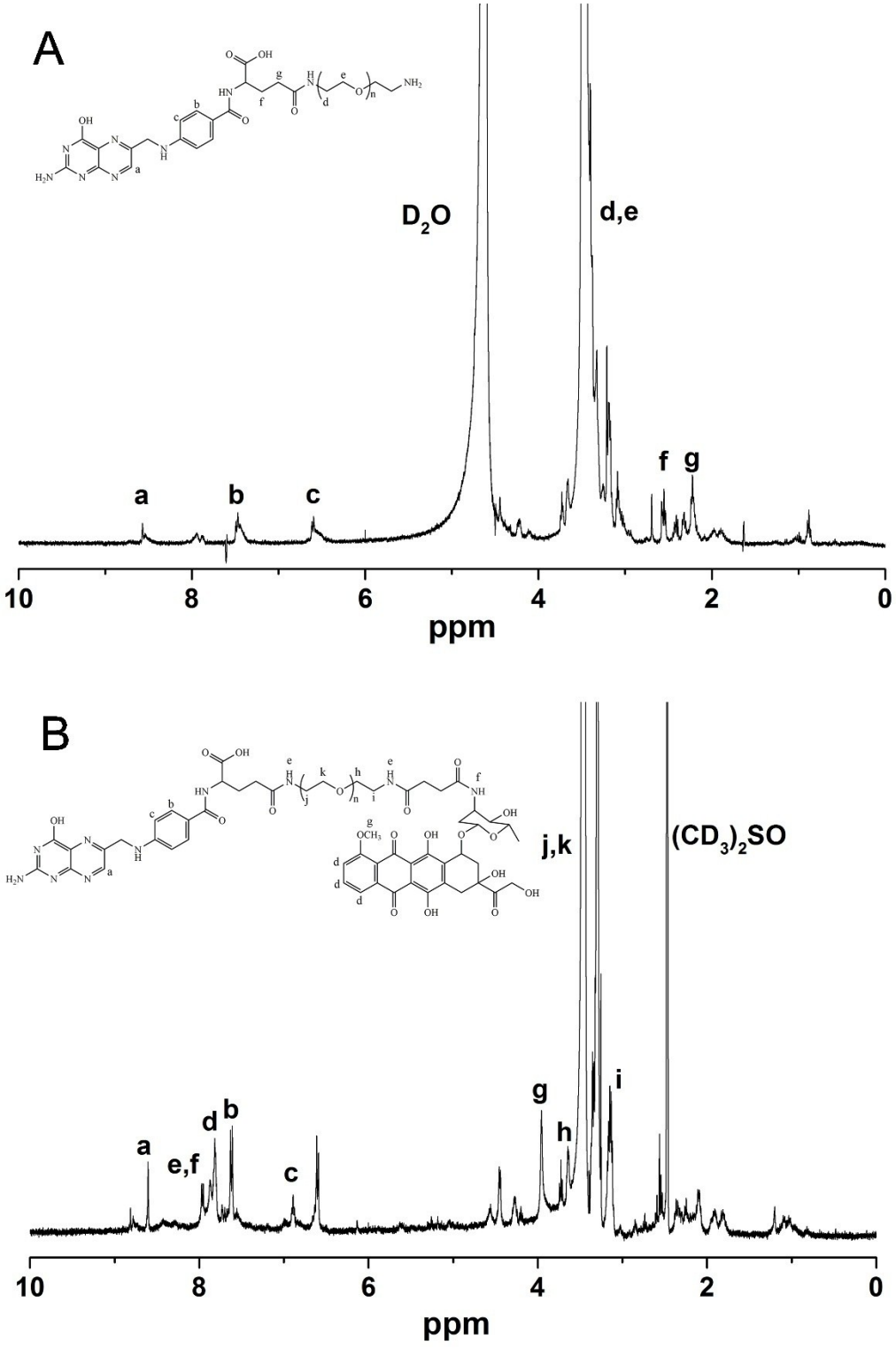

Fig. S2 ^{1}H-NMR spectra of (A) FA-PEG-NH_2 and (B) FA-PEG-DOX

2. Results and discussion

2.1 Characterization of FA-PEG-DOX

The synthetic route of FA-PEG-DOX was shown in Fig. S1. The ^{1}H-NMR spectra of FA-PEG-NH_2 and FA-PEG-DOX were shown in Fig. S2 and the characteristic peaks were marked and assigned to the specific protons of the structure. In brief, folic acid was grafted onto NH_2-PEG-NH_2 through the amidation reaction and the peaks at (a) 8.57 ppm, (b) 7.49 ppm, (c) 6.62 ppm belonged to the characteristic protons on the aromatic rings of folic acid, which suggested the successful conjugation of folic acid onto PEG. Doxorubicin was first linked with succinic anhydride via amido bond, and then SA-DOX was conjugated to FA-PEG-NH_2 through the amidation reaction. Apart from the characteristic signals of FA-PEG, the peaks at (d) 7.8 ppm and (g) 3.9 ppm could be attributed to the peaks of doxorubin, which proved the successful synthesis of FA-PEG-DOX. The detailed affiliations of the peaks were shown in Fig. S2.

The conjugation ratio of FA and DOX were calculated to be 7.59% and 19.13%, respectively.

Table S1 The concentrations of DOX and MAL at a 50% inhibition rate against MCF-7/ADR cells at different DOX and MAL mass ratios and the corresponding combination index (CI). Data are presented as mean ± SD (n=3).

	Concentration of DOX (μg/mL) (50% inhibition)	Concentration of MAL (μg/mL) (50% inhibition)	CI
Free MAL		9.03	
Free DOX	49.65		
DOX:MAL=20:1	18.90	0.95	0.49
DOX:MAL=15:1	17.96	1.20	0.49
DOX:MAL=12:1	15.34	1.28	0.45
DOX:MAL=10:1	9.57	0.96	0.30
DOX:MAL=8:1	8.46	1.06	0.29
DOX:MAL=5:1	4.10	0.82	0.17
DOX:MAL=3:1	5.22	1.74	0.30
DOX:MAL=1:1	2.65	2.65	0.35
DOX:MAL=1:5	0.69	3.44	0.39
DOX:MAL=1:10	0.40	4.00	0.45
DOX:MAL=1:15	0.26	3.95	0.44
DOX:MAL=1:20	0.25	5.08	0.57

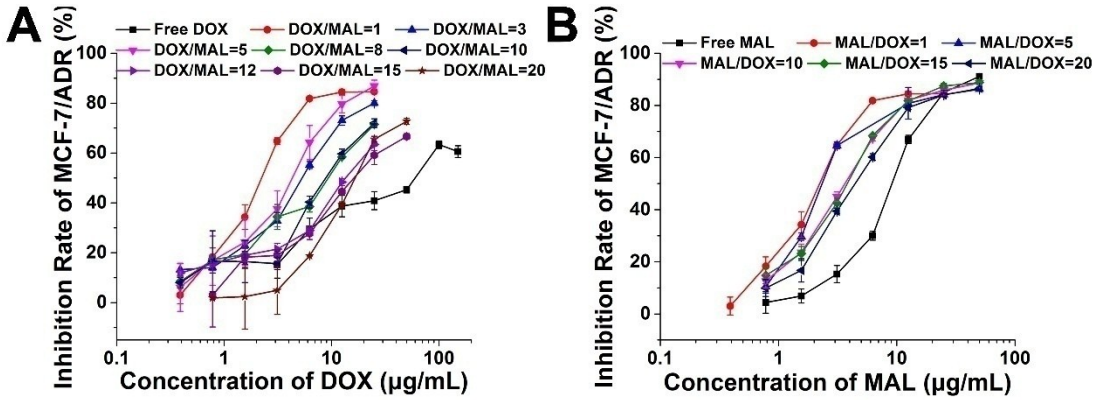

Fig. S3 The cytotoxicity of free DOX, free MAL and their different combination ratios towards MCF-7/ADR (A, B) cells. Data are presented as mean ± SD (n=3).

Table S2 Drug loadings of DOX or MAL single drug under different pH conditions

pH	Drug loading of DOX (%)	Drug loading of MAL (%)
6.0	9.19	9.91
7.0	12.63	12.76
8.0	17.33	13.17
9.0	15.62	13.19

Table S3 The sizes and ζ-potentials of nanoparticles (n=3, data are presented as mean±S.D.)

	DOX:MAL	Z-ave (nm)	PDI	ζ-potential(mV)
Pristine NDs		175.23±3.81	0.34±0.03	-35.27±1.32
DOX@NDs		397.80±9.35	0.34±0.03	3.53±0.19
MAL@NDs		209.67±6.40	0.35±0.01	-8.63±0.42
(DOX+MAL)@NDs	4.3:1.0	338.90±5.01	0.36±0.04	-3.77±0.35
	5.3:1.0	337.90±5.98	0.38±0.02	-6.82±0.19
	6.5:1.0	373.17±9.35	0.36±0.03	-8.66±0.68
FA-PEG-DOX/(DOX+MAL)@NDs	4.0:1.0	214.70±4.36	0.38±0.01	-9.30±0.70
	4.9:1.0	202.50±5.04	0.37±0.01	-8.26±0.43
	5.8:1.0	203.17±5.29	0.37±0.01	-8.90±0.62

Table S4 The IC_{50} and index of drug resistance (IDR) of DOX and MAL in MCF-7 and MCF-7/ADR cells

Cells	IC_{50} of DOX (μg/mL)	IC_{50} of MAL (μg/mL)
MCF-7	0.22	3.78
MCF-7/ADR	49.65	9.03
IDR	229.86	2.39

$IDR=IC_{50\ (MCF\text{-}7/ADR)} / IC_{50\ (MCF\text{-}7)}$

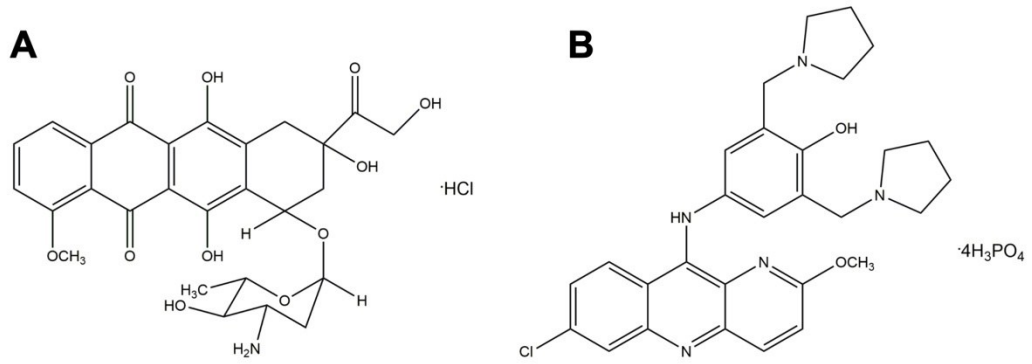

Fig. S4 Chemical structures of DOX (A) and MAL (B)

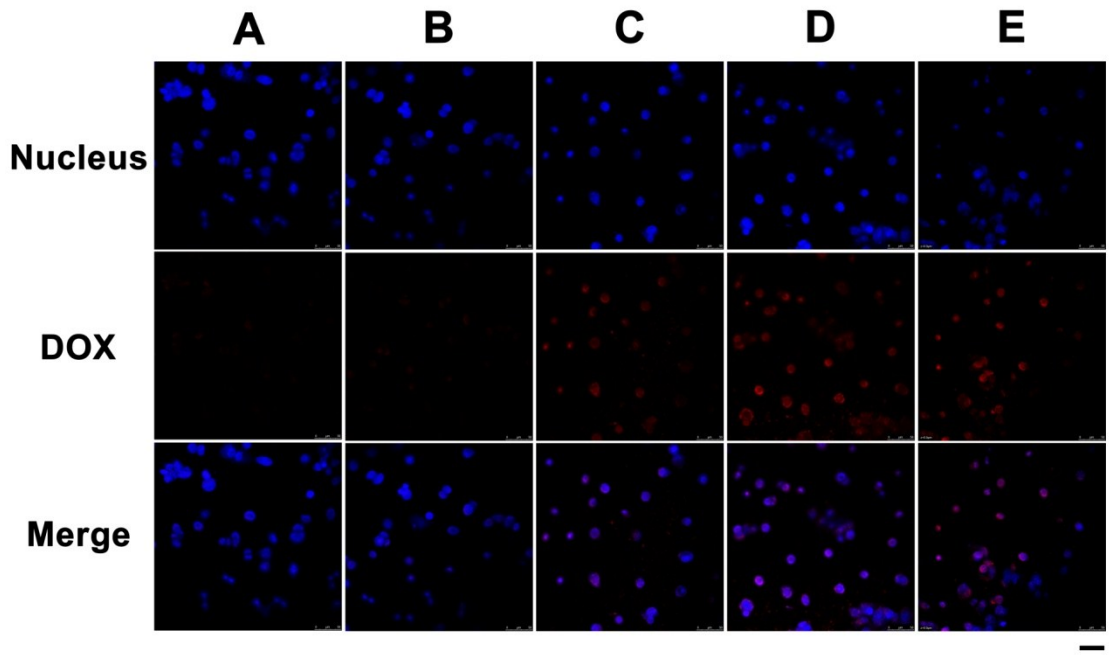

Fig. S5 Confocal fluorescence images of MCF-7/ADR cells after 6 h treatment with different formulations at an equivalent DOX concentration of 5 μg/mL. (A) free DOX, (B) free DOX+MAL (DOX:MAL=5.0:1.0), (C) DOX@NDs, (D) (DOX+MAL)@NDs (DOX:MAL=5.3:1.0), (E) FA-PEG-DOX/(DOX+MAL)@NDs (DOX:MAL=4.9:1.0). Cells nuclei were stained in blue and DOX was in red color. (Scale bar: 50 μm)