Supporting Information

Liang et al. 10.1073/pnas.1407808111

SI Text

Preparation and Characterization of H-Ferritin and Doxorubicin-Loaded H-Ferritin. Human heavy-chain ferritin (HFn) was produced in *Escherichia coli* and purified as previously described (1). The loading of doxorubicin (Dox) into the cavity of HFn was carried out in the following procedures. Briefly, HFn at 1 mg/mL was dissolved in 8 M urea (Sinopharm Chemical Reagent Co., Ltd.) and gently vortexed for 30 min at room temperature to ensure complete dissociation. The Dox (Sangon Biotech) was then added into the solution with a final concentration of 1 mg/mL. After incubation for 10 min in the dark, the mixture was transferred to dialysis bags (molecular weight cut-off 3,000 Da; Thermo Scientific) and dialyzed against gradient concentrations of urea buffer (7, 5, 3, 2, 1, and 0 M, each for 4 h) containing 1 mg/mL of Dox at 4 °C to slowly reassemble HFn protein cages. The resulting solution was then dialyzed against saline overnight to remove the free Dox. The final prepared Dox-containing HFn (HFn-Dox) was analyzed on a Superdex 200 10/300 GL column (GE Healthcare) connected to a size-exclusion chromatography (SEC) system (Amersham Pharmacia Biotech). The results showed a single absorbance peak in the UV trace at 280 nm (HFn protein cage) corresponding to a single absorbance peak in the visible at 485 nm (Dox) (Fig. S1*A*), demonstrating the successful loading of Dox into the HFn nanocage. The Dox concentration was determined by measuring the absorbance at 485 nm and the concentration of HFn was determined using a Bradford assay kit (Bio-Rad). The Dox:HFn molar ratio was calculated to be 33.1:1.

The prepared HFn-Dox nanoparticles were further characterized using cryoelectron transmission microscopy (cryo-EM), dynamic light scattering (DLS), and CD. For cryo-EM observation, an aliquot of 3.5 μL purified HFn-Dox or HFn samples was applied to a holey grid and frozen by FEI Vitrobot. The cryo samples were imaged in an FEI 300-kV Titan Krios electron microscope (FEI) operated at 300 kV with a Gatan UltraScan4000 (model 895) CCD camera at the magnification of 96,000 and dose of 20–25 $e^-/Å^2$. DLS was used to measure the particle radius of HFn and HFn-Dox using a DynaPro Titan system DLS instrument (Wyatt Technology). The analysis was performed at 25 °C and the concentration of the samples used was 0.25 mg/mL in PBS buffer. CD spectra were performed on a Chirascan-plus CD Spectrometer (Applied Photophysics) at 25 °C. The sample concentrations used were 0.25 mg/mL in PBS buffer. The spectra were measured from 260 to 200 nm with 0.1-nm resolution in a quartz cell with a 1-cm path length.

In Vitro Drug Release Studies. To assay for the release of Dox, HFn-Dox samples (500 μM Dox equivalents, 500 μL) were placed in a D-tube (molecular weight cut-off 6–8 kDa, D-Tube Dialyzer midi; Novagen) and incubated with gentle stirring in either PBS buffer (0.1 M, pH 7.4) or acetate buffer (0.1 M, pH 5.0) at 37 °C in the dark. The released free Dox at different incubation times was determined by HPLC (Shimadzu Scientific Instruments) with a C18 column. HFn-Dox was incubated with normal mouse serum (ImmunoReagents, Inc.) for HFn-Dox nanoparticle stability evaluation.

Antibody Blocking Assay. An antibody blocking assay was performed to confirm that transferrin receptor 1 (TfR1) is the binding receptor of HFn-Dox to tumor cells. Briefly, 1 μM of HFn-Dox was incubated with HT-29 cancer cells in the presence or absence of a 10-fold molar excess of anti-TfR1 mAbs (clone M-A712; BD Bioscience). After incubation for 1 h at 37 °C, the cells were washed in cold PBS and examined under a confocal laser scanning microscope (Olympus) to observe the fluorescence signal of Dox.

Pharmacokinetics Study. To determine the pharmacokinetics, HFn-Dox [10 mg/kg body weight (BW), Dox equivalents], or free Dox (10 mg/kg BW) was i.v. injected into BALB/c healthy mice ($n = 6$ for each group). At various times after injection blood was collected from the tail vein and the plasma was separated and analyzed for Dox concentration by the following procedure. Briefly, the obtained plasma samples (10 μL) were incubated with 490 μL of acidified isopropanol (0.75 M HCl in isopropanol) overnight at −20 °C in the dark to extract Dox. The mixture was then centrifuged at 15,000 × *g* for 20 min and the supernatant was loaded onto a 96-well plate (Corning). Dox concentration was determined by measuring the fluorescence at 485 nm excitation and 590 nm emission, using a Varioskan Flash Spectral Scanning Multimode Reader (ThermoFisher Scientific). To correct for nonspecific background, the fluorescence of blood samples from untreated mice was determined. After deducting the nonspecific fluorescence, the Dox concentration of blood samples was quantified by comparing to a linear standard curve that was created between the fluorescence values and standard Dox concentration from 1 to 3,000 nM.

Biodistribution Study. Female BALB/c mice bearing HT-29 tumors were injected with HFn-Dox (10 mg/kg BW, Dox equivalents), Doxil (10 mg/kg BW, Dox equivalents), or free Dox (10 mg/kg BW) via tail vein to quantify Dox tissue distribution. At 1, 4, and 24 h postinjection, mice were killed. Tumors and the major tissues were collected, weighed, and homogenized with an addition of 1:10 (wt/vol) acidified isopropanol in a tissue homogenizer on ice and extracted overnight at −20 °C in the dark. All homogenate samples were then centrifuged at 4 °C for 10 min at 18,000 × *g*. The supernatant was removed and the fluorescence intensity was measured by a Varioskan Flash Spectral Scanning Multimode Reader. The tissue autofluorescence was corrected by subtracting the background fluorescence of tissues from untreated control mice. The remaining fluorescence values were then converted to drug concentration using the Dox fluorescence standard curve as described above. The results were presented as percentage of injected dose (%ID) per gram of tissue. Values are expressed as means ± SD for a group of four animals ($n = 4$ per group).

Clearance Study. To evaluate the total body clearance, a volume of 200 μL of HFn-Dox (10 mg/kg BW, Dox equivalents) or free Dox (10 mg/kg BW) was administered i.v. into healthy or HT-29 tumor-bearing (~500 mm^3 in volume) BALB/c mice. Mice were housed in special cages to allow for the collection of urine and feces. At 96 h postinjection, mice were killed and the Dox contents in urine or feces were measured by the fluorescence assay, as described for the biodistribution study. The major organs from the remaining carcass were excised and homogenized. Dox was extracted by acidified isopropanol and quantified by fluorescence assay as described above. The retained Dox in the carcass was determined by adding all of the Dox contents in the major organs together.

Tolerability of HFn-Dox in Healthy Mice. Forty-two female BALB/c healthy mice (6–8 wk of age) were randomly assigned to one of the following seven treatment groups ($n = 6$ per group): PBS, free Dox (5 mg/kg BW), free Dox (10 mg/kg BW), free HFn nanocage (480 mg/kg BW, a dose of free HFn equivalent to the 20 mg/kg Dox dose), HFn-Dox (5 mg/kg BW, Dox equivalents), HFn-Dox (10 mg/kg BW, Dox equivalents), or HFn-Dox (20 mg/kg

BW, Dox equivalents). All of the treatments were administrated via tail vein injection, and all drugs were diluted with PBS and injected in a single dose. Body weight and clinical observations were monitored daily until 14 d after injection. Following killing on day 14, blood was collected by heart puncture for blood chemistry analysis. Simultaneously, to identify the cardiac toxicity of the treatment, hearts were harvested for histological analysis. After they were fixed with 10% formalin overnight and sectioned at 5 μm thickness, heart sections were stained with H&E and examined under an Olympus BX51 microscope.

Reactivity of Human HFn to Murine T-Cell Immunoglobulin and Mucin Domain Protein-2 Receptors. T-cell immunoglobulin and mucin domain protein-2 (TIM-2) expression in mouse cells was confirmed by flow cytometric analysis. Briefly, mouse EL4 cells were incubated at 4 °C for 2 h with 10 μg/mL of rat anti-mouse TIM-2 mAbs (RMT2-1; Santa Cruz) or rat IgG isotype control (Abcam), followed by incubation with the secondary antibodies anti–rat-AlexaFluor 488 (Invitrogen). After three washes in cold PBS, cells were analyzed immediately using a FACSCalibur flow cytometry system (Becton Dickinson).

The binding activity of human HFn to murine cell surface receptor TIM-2 was assessed using mouse EL4 cells by flow cytometric analysis. Briefly, 2 μg/mL of FITC-conjugated human HFn was added to cell suspensions (1×10^6 cells per milliliter) in the presence or absence of a 10-fold molar excess of anti-murine TIM-2 mAbs. Cell-bound fluorescence was measured by flow cytometry.

1. Fan K, et al. (2012) Magnetoferritin nanoparticles for targeting and visualizing tumour tissues. *Nat Nanotechnol* 7:459–464.

Fig. S1. (*A*) SEC of HFn and HFn-Dox by in-line UV detection at 280 nm (HFn protein cage) and 485 nm (Dox). (*B*) CD spectra of HFn and HFn-Dox showing no secondary structure change to the HFn protein cage after Dox loading.

Fig. S2. HFn-Dox nanoparticles (NPs) bind to TfR1 in tumor cells. Fluorescence staining of HT-29 cancer cells using HFn-Dox in the presence (*Right*) or absence (*Left*) of a 10-fold molar excess of anti-TfR1 mAbs. (Scale bars, 20 μm.)

Fig. S3. Biodistribution of HFn-Dox, Doxil, and free Dox in BALB/c mice bearing HT-29 tumors at 1, 4, and 24 h postinjection. Data are expressed as %ID/g. Student *t* test, $^{*}P < 0.01$, $^{**}P < 0.001$ versus HFn-Dox-treated mice ($n = 4$, bars represent means ± SD).

Fig. S4. HFn-Dox–treated mice showed no observable cardiotoxicity. Hearts were harvested at day 14 after i.v. injection of HFn-Dox (20 mg/kg Dox equivalents), free Dox (10 mg/kg), HFn protein (480 mg/kg), or PBS. (*A*) Mean heart weight of mice after treatment ($n = 4$, mean ± SD, one-way ANOVA, followed by Bonferroni posttest, $^{**}P < 0.001$ versus PBS-treated mice; NS, not significant versus PBS-treated mice). (*B*) H&E-stained heart sections indicated the presence of cardiomyopathy in free Dox-treated groups, which was notably absent in HFn-Dox–, HFn-, or PBS-treated mice. (Scale bars, 100 μm.)

Fig. S5. Total body clearance of HFn-Dox NPs within 96 h post i.v. injection into HT-29 tumor-bearing BALB/c mice (n = 4, bars represent means ± SD).

Fig. S6. Maximum tolerated dose study of HFn-Dox NPs. Healthy female BALB/c mice (n = 6 per group) were administered i.v. on day 0 different concentrations of PBS, HFn protein cage (480 mg/kg), free Dox (5 or 10 mg/kg), or HFn-Dox (5, 10, or 20 mg/kg Dox equivalents) (n = 4, bars represent means ± SD).

Fig. S7. (*A*) Flow cytometric analysis of TIM-2 receptor expression in mouse EL4 cells. (*B*) Flow cytometric analysis of the binding of human HFn to murine TIM-2 receptors in mouse EL4 cells with or without a 10-fold molar excess of anti–TIM-2 mAbs.

Fig. S8. Antitumor activity and toxicity of HFn-Dox in A375 and MDA-MB-231 tumor models. Tumor cells (A375 and MDA-MB-231) were implanted s.c. on day zero. (*A*) Mice with s.c. A375 tumors were treated with HFn-Dox (20 mg/kg Dox equivalents; $n = 7$), Doxil (20 mg/kg Dox equivalents; $n = 10$), free Dox (5 mg/kg; $n = 6$), or PBS ($n = 5$) on day 5 (↑). Asterisk indicates $P = 0.0002$ for HFn-Dox versus Doxil (Kaplan–Meier). (*B*) Mice with s.c. MDA-MB-231 tumors were treated with HFn-Dox (20 mg/kg Dox equivalents; $n = 6$), Doxil (20 mg/kg Dox equivalents; $n = 8$), free Dox (5 mg/kg; $n = 5$), or PBS ($n = 5$) on day 5 (↑). Asterisk indicates $P = 0.0006$ for HFn-Dox versus Doxil (Kaplan–Meier). Bars represent means ± SD; $n = 4$–10.

Table S1. Clinical chemistry values for healthy mice ($n = 6$ per group) treated with HFn-Dox, free Dox, HFn protein, or PBS

Clinical chemistry parameters	PBS	HFn (480 mg/kg)	Free Dox (10 mg/kg)	HFn-Dox (5 mg/kg)	HFn-Dox (10 mg/kg)	HFn-Dox (20 mg/kg)
ALT, U/L	71.67 ± 20	76 ± 22	55 ± 3.5	56 ± 8.16	65.33 ± 1.70	112.34 ± 41
AST, U/L	147 ± 7	154.5 ± 44.5	116.5 ± 11.5	124.67 ± 23.70	127.33 ± 4.64	152.5 ± 18.5
TP, g/L	74.85 ± 0.35	71.55 ± 3.15	45.3 ± 3.6*	77.5 ± 1.35	81.1 ± 4.10	80.6 ± 3
ALB, g/L	38.47 ± 1.85	38.3 ± 1.13	20.2 ± 1.4*	39.07 ± 0.21	36.63 ± 0.82	37.23 ± 0.54
GGT, U/L	(−)8.67 ± 1.35	(−)7.5 ± 2.5	(−)7 ± 3	(−)5.67 ± 0.47	(−)4.67 ± 1.70	(−)6 ± 3.55
TBIL, μM	1.73 ± 0.15	1.87 ± 0.37	0.95 ± 0.25*	1.7 ± 0.08	1.4 ± 0.14	1.67 ± 0.09
CREA, μM	18.37 ± 2.12	17.27 ± 1.58	25.05 ± 2.75*	19.03 ± 1.18	15.6 ± 0.64	19.9 ± 3.39
BUN, U/L	10.68 ± 1.48	10.70 ± 0.75	15.31 ± 4.3	9.84 ± 0.64	10.14 ± 0.87	11.34 ± 0.77
ALP, U/L	191.33 ± 9.33	210.67 ± 24	74 ± 10*	195 ± 14.44	193 ± 28.71	230.33 ± 19.29
TCHO, U/L	2.45 ± 0.05	2.34 ± 0.12	19.69 ± 2.87*	2.47 ± 0.04	2.49 ± 0.17	2.49 ± 0.15
TG, mM	3.16 ± 0.41	2.62 ± 0.19	7.2 ± 0.93*	2.34 ± 0.38	2.56 ± 0.42	2.39 ± 0.42
GLU, mM	5.66 ± 0.11	4.92 ± 0.61	2.19 ± 0.35	5.17 ± 0.18	5.36 ± 0.01	5.68 ± 0.55
LDH, U/L	679.5 ± 76.5	711 ± 50	901.5 ± 45.5*	739 ± 22.91	612.33 ± 38.58	763.5 ± 3.5
Na, mM	151.23 ± 2.25	153 ± 0.4	150.5 ± 0.8	152.67 ± 0.66	152.43 ± 0.19	150.9 ± 1.15
K, mM	9.03 ± 0.26	9.97 ± 0.06	10.05 ± 0.46	8.94 ± 0.17	9.57 ± 0.27	9.9 ± 0.36
Cl, mM	122.8 ± 1.92	121 ± 1.3	116.1 ± 1.9*	120.43 ± 0.26	120.8 ± 0.36	120.13 ± 1.58

Plasma samples were obtained 14 d after treatment. ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cl, chlorine; CREA, creatinine; GGT, gamma glutanyl transferase; GLU, glucose; K, potassium; LDH, lactate dehydrogenase; Na, sodium; TBIL, total bilirubin; TCHO, total cholesterol; TG, triglyceride; TP, total protein. *$P < 0.05$ versus PBS (one-way ANOVA, followed by Bonferroni posttest).