Supplementary Information

Self-assembled Fusion Hydrophobins-Coated Nanodiamond System for Synergistic Targeted Therapy of Colorectal Cancer

*Haotian Li,[a] Yupeng Wang,[a] Hui Qiao,[b] Fengming Liu,[a] Zhuoyu Li,[a] Zhiqiang Han,[c] Zhihua Guo,[a] Yingqi Li[*bd] and Mingqiang Qiao[*ae]*

[a]*Laboratory of Resource Microbiology and Engineering, School of Life Sciences, Shanxi University, Taiyuan 030006, P. R. China*

[b]*Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Molecular Science, Shanxi University, Taiyuan 030006, P. R. China*

[c]*Key Laboratory for Biorheological Science and Technology of Ministry of Education, State and Local Joint Engineering Laboratory for Vascular Implants, Bioengineering College of Chongqing University, Chongqing, 400030 China*

[d]*Department of Chemistry, College of Chemistry and Chemical Engineering, Shanxi University, Taiyuan 030006, P. R. China*

[e]*The Key Laboratory of Molecular and Microbiology Technology, Ministry of Education, College of Life Sciences, Nankai University, Tianjin 300071, P. R. China*

**Correspondence Author: Mingqiang Qiao and Yingqi Li*

E-mail address : qmq@sxu.edu.cn and wkyqli@sxu.edu.cn

Experimental section

Materials and methods

Materials. The strains used in this study were *Pichia Pastoris* GS115 (Mut^+, His^-) and *Escherichia coli* DH5α-pPIC9K-*ge*11-*hgf*I containing the target protein gene. *P. Pastoris* GS115-pPIC9K-*ge*11-*hgf*I for methanol induced fermentation to be prepared according to our previous method with slight modifications.[30] Two kinds of nanodiamonds with particle sizes of 5 nm and 100 nm were respectively loaded with DOX according our previous methods with slight modifications, resulting in the nanodiamond drug systems ND-1 and ND-2.[32] The DOX loading capacities of ND-1 and ND-2 were 204 μg/mg and 200 μg/mg, respectively. They were provided by Professor Yingqi Li, School of chemistry and chemical engineering, Shanxi University. Human colorectal cancer cells HCT-116 and SW620 were provided by Professor Shuhua Shan, Institute of biotechnology, Shanxi University. RPMI 1640 medium and DMEM high glucose basal medium were purchased from Gibco (Suzhou, China). QuickCut *Sac*I and DNA purification kits were purchased from TakaRa company (Beijing, China). Easy Taq DNA Polymerase was purchased from TransGen (Beijing, China). Tris-Tricine-SDS-PAGE gel kits were purchased from Solarbio (Beijing, China). The HRP conjugated anti his tag mouse monoclonal antibody (5C3) was purchased from Abbkine (Wuhan, China). Primers were synthesized and the DNA fragments of interest were sequenced by Biologics (Shanghai, China).

Methods.

Fermentation expression of GE11-HGFI. Gradually activate the prepared strain using MD agar plates, YPD tubes, and BMG conical flasks. Subsequently, the BMG secondary seed solution can be transferred to shake flasks or fermentation tanks for 96 h of fermentation to achieve protein expression. During this period, trace elements such as PTM1, kanamycin, and biotin can be added to the culture medium to ensure sufficient nutrition. The DO value gradually decreases during the consumption of glycerol and rapidly increases until glycerol is consumed; During the process of supplementing glycerol, cells grow rapidly and the pH value changes significantly,

consuming a large amount of ammonia at this stage; During the methanol induction process, continuous induction fermentation was carried out for 96 h. Select fermentation broth samples at different time points during the period, measure their OD_{600} and wet weight to draw growth curves.

Preparation and stability detection of GE11-HGFI@ND-DOX. The nanodiamond drug systems ND-1 and ND-2 (500 μL, 1 mg/mL, in ddH_2O) were mixed with the fusion hydrophobin GE11-HGFI (500 μL, 1 mg/mL, in ddH_2O), respectively. At room temperature, the final mass ratio of GE11-HGFI to ND-1 and ND-2 was maintained at 1:1, and GE11-HGFI@ND-DOX were synthesized by vortex mixing and ultrasonic treatment, which was described as Mix-1 and Mix-2. The vortex condition is set to a power of 135 W, a frequency of 50-60 Hz, a duration of 10 minutes, and intermittent oscillation every 2 seconds; The ultrasound conditions are a power of 220 W, a frequency of 40 ± 2 KHz, and a duration of 20 minutes. The composite nanodrug particle system with mass ratios of 3:1 and 1:3 were also prepared by the same method, and the final concentration of DOX in the solution of these systems was maintained at 100 μg/mL. Photos were taken to observe the dispersion state of the composite nanodrug particle suspension, and the best mass ratio system was selected for subsequent experiments. After determining the appropriate mass ratio, samples of the suspension of each system was taken every 24 h, and the retained content of DOX in the supernatant of each system was measured by an enzyme-linked immunosorbent analyzer. The detection wavelength was set at 480 nm, and the detection period lasted for 96 hours. Samples were centrifuged for 2 min at 8000 r/min before detection, and each sample was analyzed in triplicate. The stability of nanoparticles was evaluated based on the variation in the DOX concentration at different time.

The hydrodynamic diameter and potential analysis of GE11-HGFI@ND-DOX. The particle size distribution and zeta potential of Mix-1 and Mix-2 were determined by dynamic light scattering (DLS; Zeta Sizer Nano ZS90, Malvern Instruments, Malvern, UK). Meanwhile, GE11-HGFI, ND-1 and ND-2 were measured for particle size and zeta potential as controls. To ensure thermal stability, the polystyrene size

pool was used in all measurements. The data were visualized with Graphpad Prism 8.

WCA measurement of GE11-HGFI@ND-DOX. Using hydrophilic material mica flakes and hydrophobic material parafilm to study the surface properties of GE11-HGFI, ND-1, ND-2, Mix-1 and Mix-2. Each surface with coated with 100 μg/mL solution (50 μL) and incubated at room temperature overnight. Unmodified hydrophilic material mica flakes and hydrophobic material parafilm were used as controls. After removing excess solution, rinse the surface three times and dry overnight. Measure WCA using a contact angle measurement system (JC 2000 DM) by dropping 5 μL droplets onto the surface, then measure each sample three times in different areas and calculate the average value.

TEM characterization of GE11-HGFI@ND-DOX. The microstructure of GE11-HGFI, ND-1, ND-2, Mix-1 and Mix-2 was observed using transmission electron microscopy (TEM; JEOL JEM 2100 Flash, AEMC, Japan). Adsorb 50 μL of each sample (100 μg/mL) onto a copper grid for 2 minutes, then immerse it in in Milli-Q water for 3 washes. The protein-containing nano-suspensions were respectively immersed in phosphotungstic acid dye solution for negative staining operation for 30 s. Excess liquid was gently absorbed with filter paper, and the grids were left to air dry naturally.

AFM characterization of GE11-HGFI@ND-DOX. The surface morphology of mica coated with different system solutions of GE11-HGFI, ND-1, ND-2, Mix-1 and Mix-2 was characterized by atomic force microscopy (AFM, Bruker, Frankfurt, Germany). Add 50 μL of different solutions (100 μg/mL) droplets onto a clean and dry mica surface, and incubate at room temperature for 5 minutes. Filter paper to remove excess solution, then gently rinse the mica three times with Milli-Q water and let the surface dry naturally. The AFM parameter settings are as follows: scan size of 400 nm, resolution of 704 × 576 pixels, aspect ratio of 1.0.

XPS detection of GE11-HGFI@ND-DOX. The elemental changes of GE11-HGFI, ND-1, ND-2, Mix-1 and Mix-2 incubated silicon wafers was characterized by X-ray photoelectron spectroscopy (XPS; Escalab 250Xi, USA) measurement. Drop 10 μL of different solutions (100 μg/mL) onto a 3 × 3 mm silicon wafer for 3 minutes, remove

excess solution with filter paper, and air dry overnight. Select conventional XPS projects to detect changes in surface element types for each sample. The data were visualized with Avantage software.

In vitro DOX release. The in vitro release of DOX was performed according to the previously reported method.[30] Firstly, 3 mg of ND-DOX or GE11-HGFI@ND-DOX powder was dispersed in individual 30 mL 0.01M PBS buffer at pH 7.4 with 10 μM GSH or at pH 5.0 with 10 mM GSH, and then take 1 mL of each solution and divide it into Eppendorf tubes. Incubate these tubes at 37℃ while oscillating at 100 rpm. At the predetermined time (2, 4, 6, 8, 10, 12, 24, 48 and 72 h), centrifuge the tube at 2500 rpm for 10 min, and the supernatant was collected. The absorbance intensity at 480 nm of the supernatant was measured using an enzyme-linked immunosorbent assay analyzer to analyze the release of DOX.

In vitro cytotoxicity assay. The cytotoxicity of DOX, ND-1, ND-2, Mix-1 and Mix-2 was evaluated using the MTT assay. HCT-116 cells with overexpressed EGFR receptors, and SW620 cells expressed low or no EGFR receptors, were seeded in 96-well plates with a density of 5000 cells per well in DMEM and 1640 cell culture media for 24 h, respectively. Next, various DOX, ND-1, ND-2, Mix-1 and Mix-2 containing different drug concentration were added to each well and further cultured for 24 h, respectively. Then the supernatant from each well was discarded and replaced with 90 μL fresh medium and 10 μL MTT. After incubation for 4 h, the MTT solution was replaced with 110 μL formazan lysis solution. The 96-well plate was shaken in a vulgar shaker for 10 min, and cell viability was assessed at 490 nm using a microplate reader. Untreated cells served as controls.

Confocal imaging. The cellular uptake and distribution of ND-1, ND-2, Mix-1 and Mix-2 were observed by laser confocal scanning microscope (LCSM). First, HCT-116 and SW620 cells were seeded into 24-well plates at a density of 3×10^5 cells per well and incubated at 37 ℃ for 24 h. The cells were incubated with ND-1, ND-2, Mix-1 and Mix-2 (6 μg/mL of DOX equivalent) for 4 h. After washing twice in PBS, the cells were fixed with 4% paraformaldehyde for 30 min and stained with DAPI for 10 min. The cells were then fixed and observed under a confocal laser scanning

microscope (ZEISS-LSM880, Germany), and the fluorescence imaging of ND-1, ND-2, Mix-1, and Mix-2 originating from dissociated DOX (excitation at 480 nm and detection through a 560 nm filter). For DAPI, excitation was set at 350 nm and detection through a 460 nm filter.

Supplementary Figures

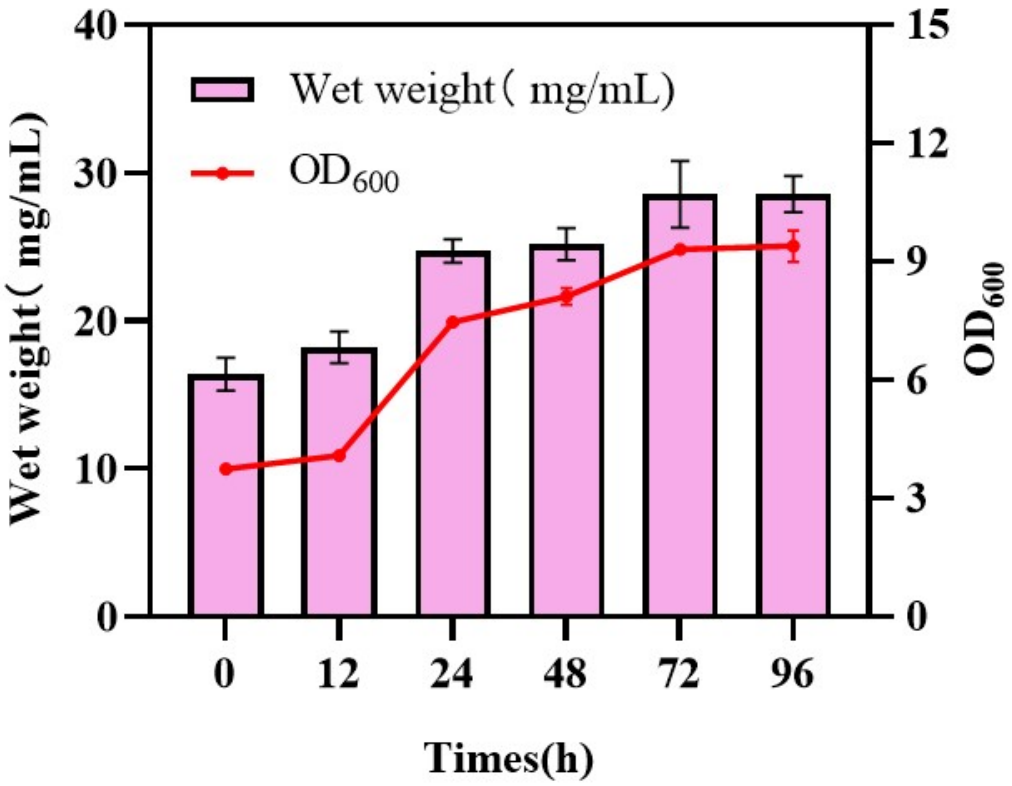

Fig. S1. Growth curves of *Pichia pastoris* cells at different time during shake flask fermentation.

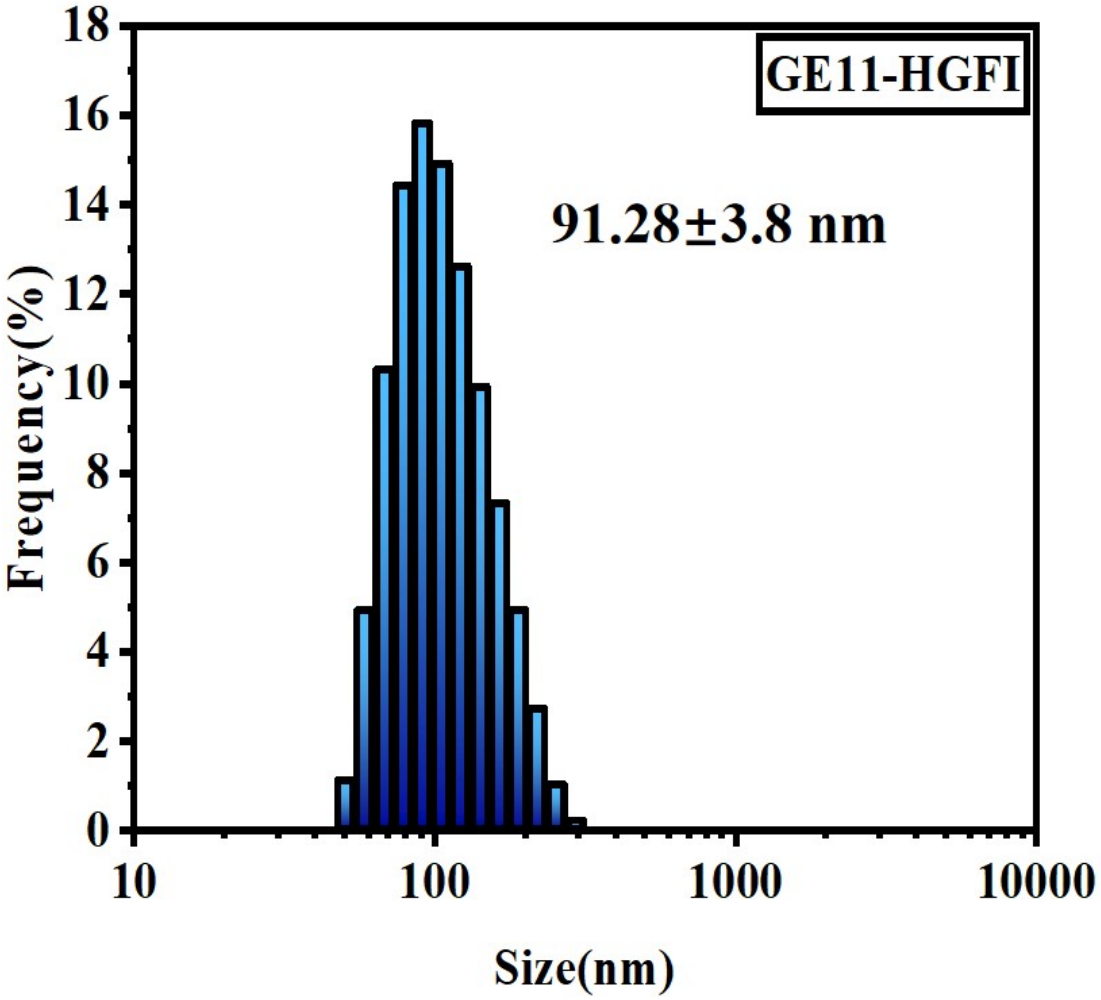

Fig. S2. Hydrodynamic diameter distribution of GE11-HGFI.

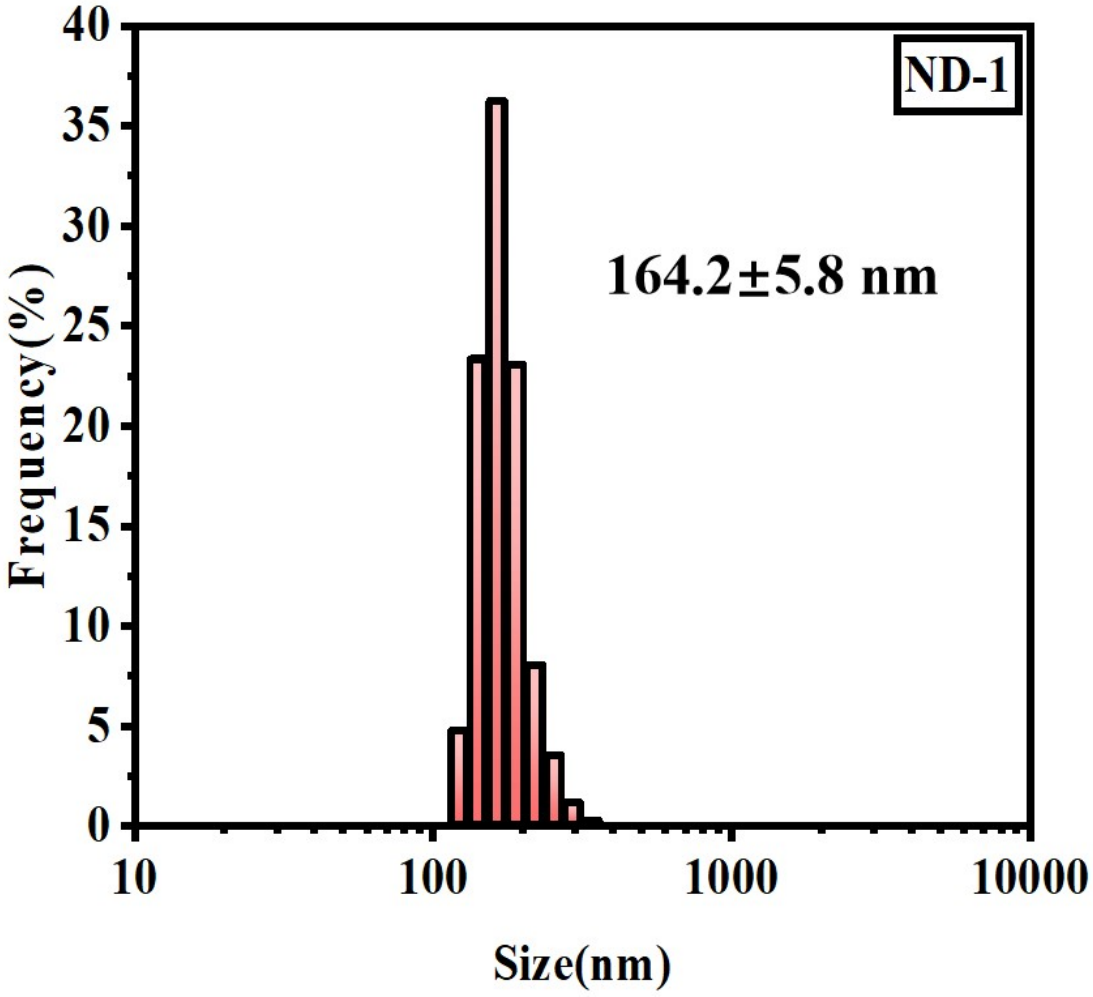

Fig. S3. Hydrodynamic diameter distribution of ND-1.

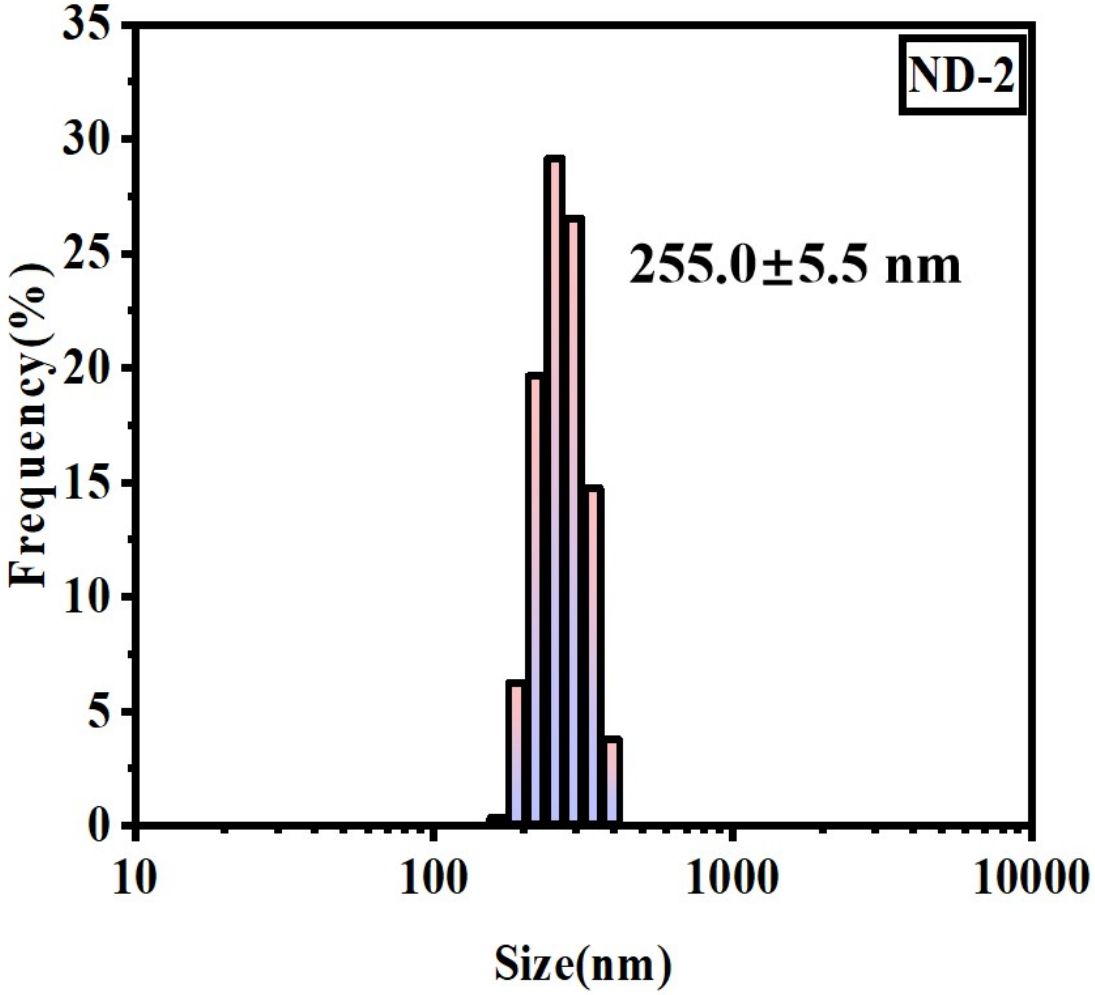

Fig. S4. Hydrodynamic diameter distribution of ND-2.

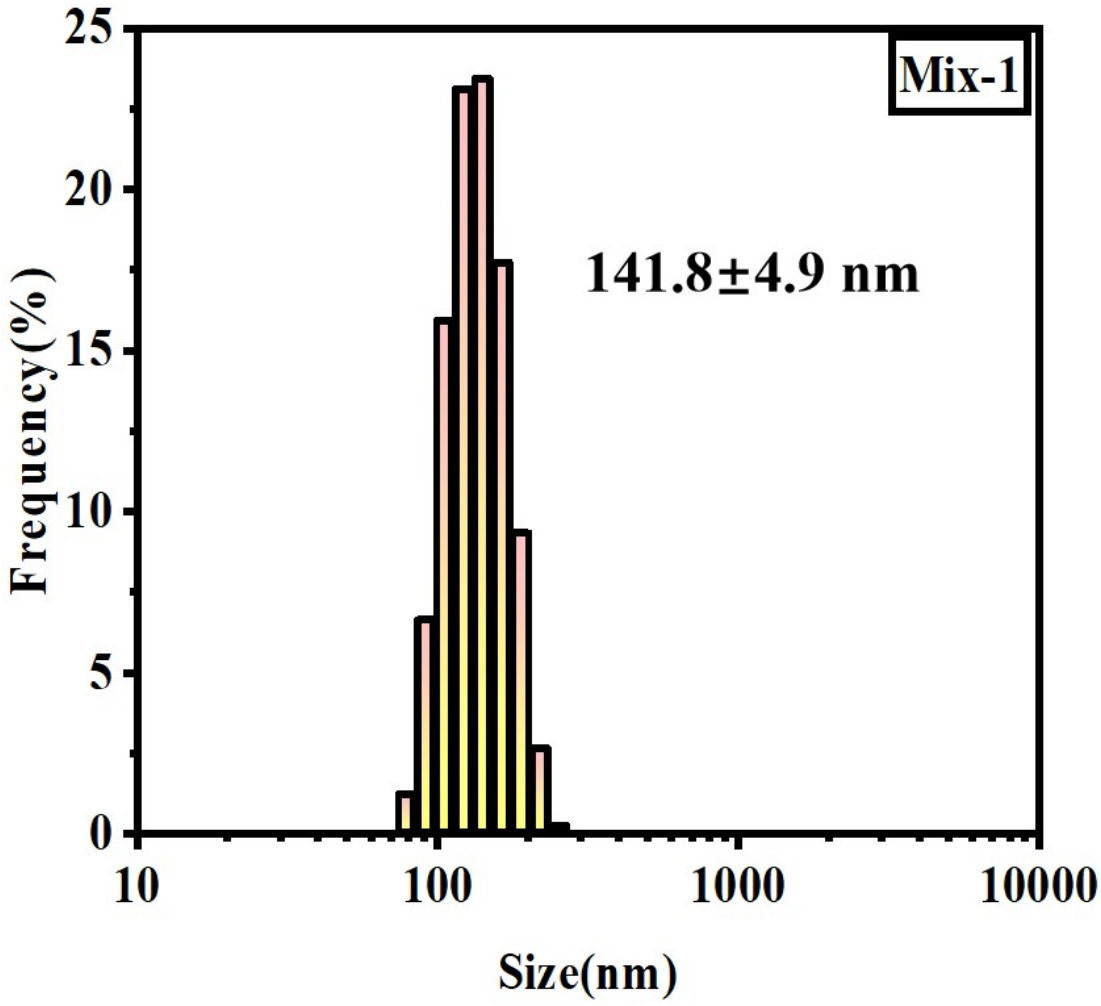

Fig. S5. Hydrodynamic diameter distribution of Mix-1.

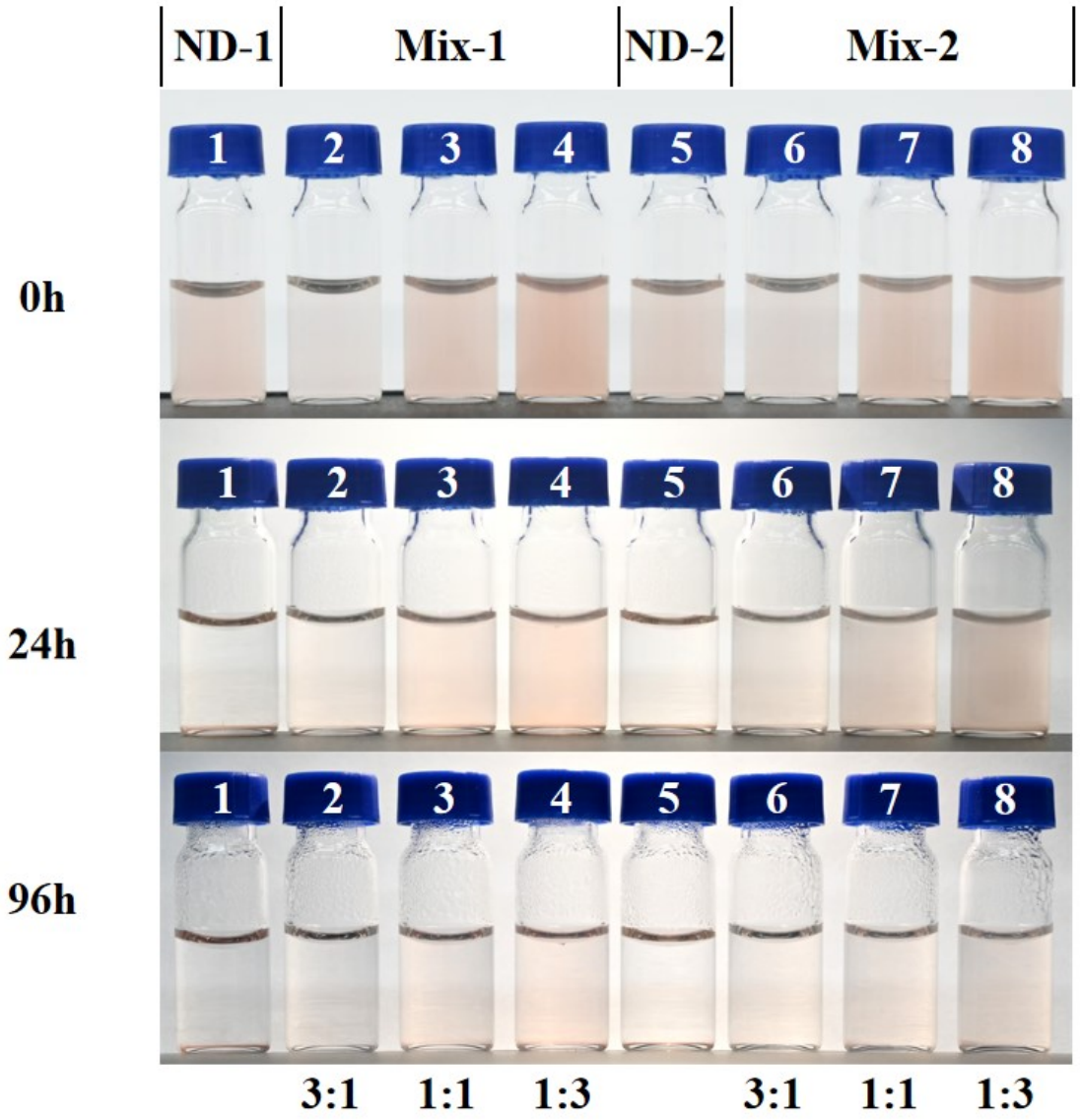

Fig. S6. Dispersion diagram of composite system solution stored for different time under different mass ratios.

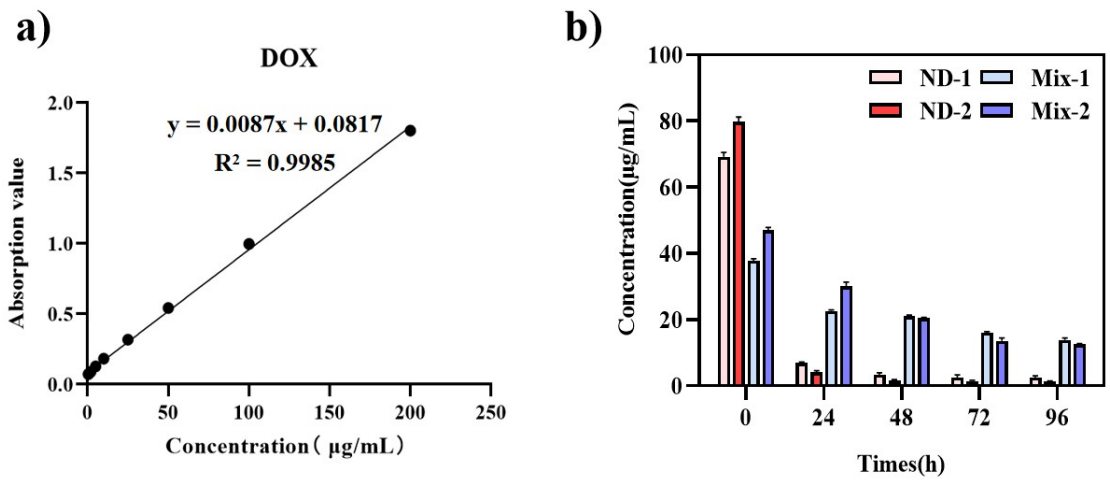

Fig. S7. (a) Standard curve of DOX. (b) Samples were taken every 24 h to measure the retention of DOX in different system solutions at a mass ratio of 1:1.

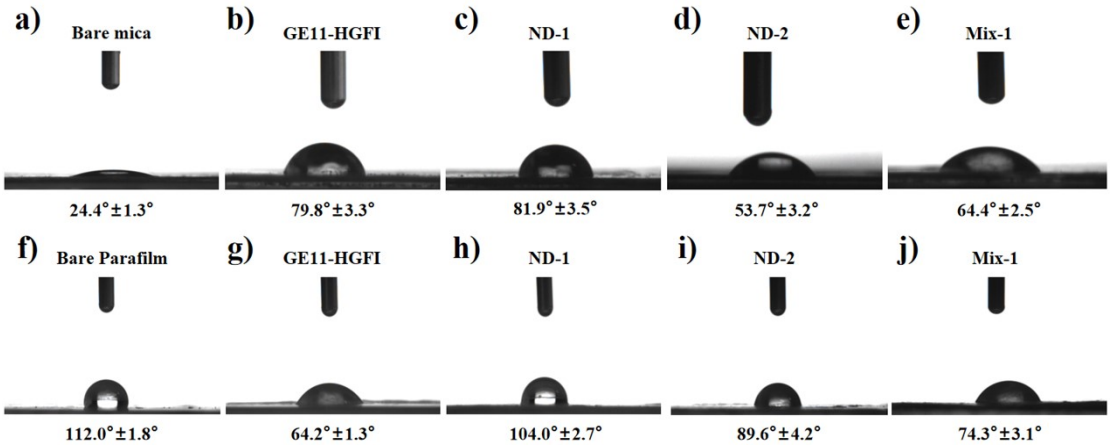

Fig. S8. (a) The WCA of the hydrophilic material bare mica. (b) The WCA of the hydrophilic material bare mica by GE11-HGFI. (c) The WCA of the hydrophilic material bare mica by ND-1. (d) The WCA of the hydrophilic material bare mica by ND-2. (e) The WCA of the hydrophilic material bare mica by Mix-1. (f) The WCA of the hydrophobic material bare parafilm. (g) The WCA of the hydrophobic material bare parafilm modified by GE11-HGFI. (h) The WCA of the hydrophobic material bare parafilm modified by ND-1. (i) The WCA of the hydrophobic material bare parafilm modified by ND-2. (j) The WCA of the hydrophobic material bare parafilm modified by Mix-1.

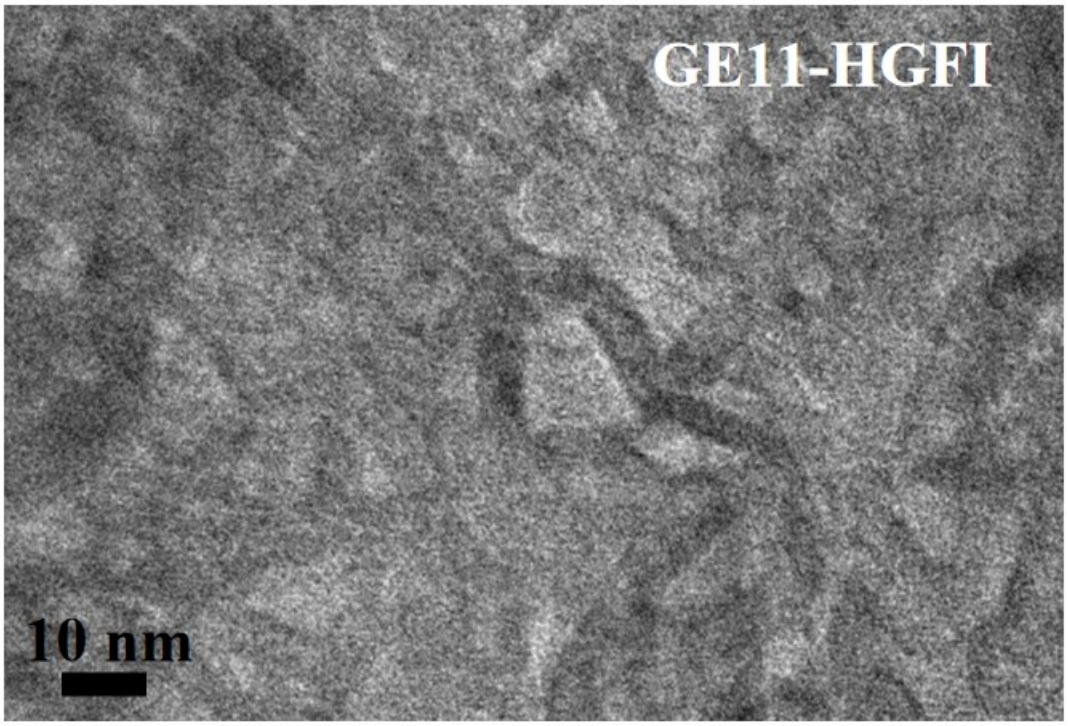

Fig. S9. TEM characterization of GE11-HGFI.

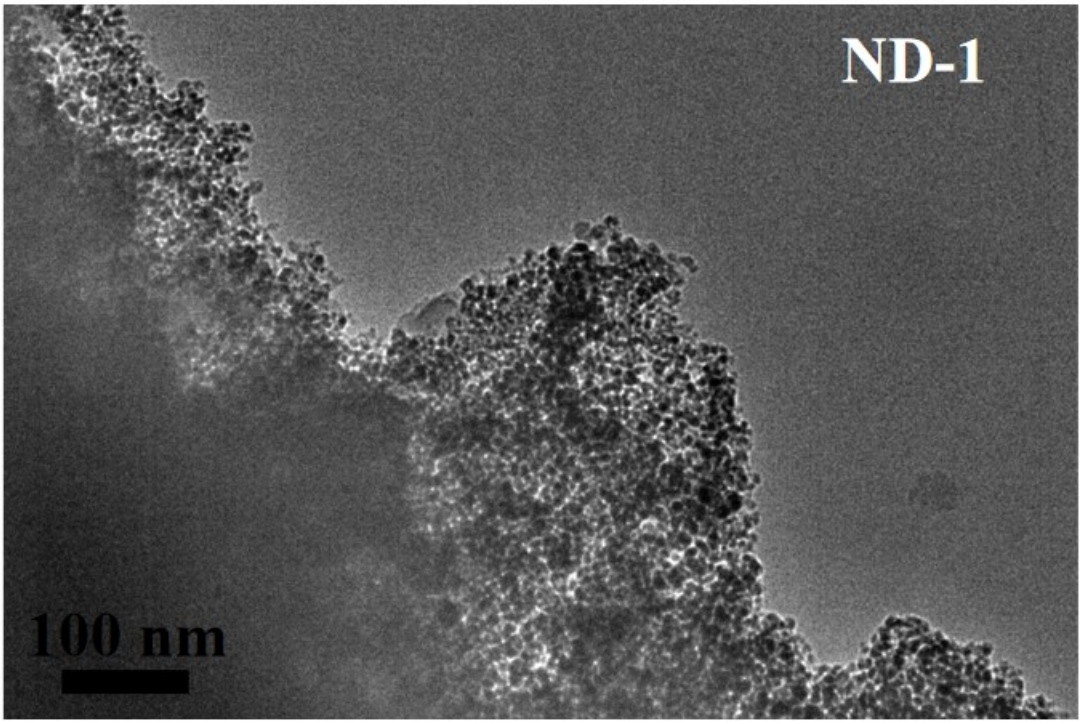

Fig. S10. TEM characterization of ND-1.

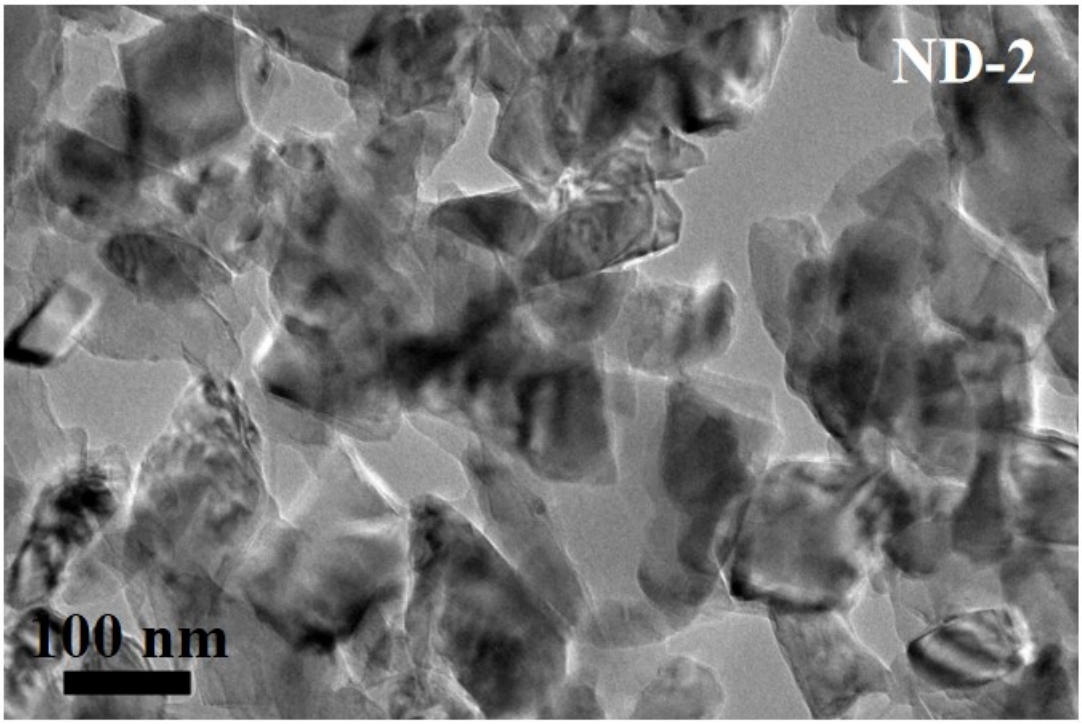

Fig. S11. TEM characterization of ND-2.

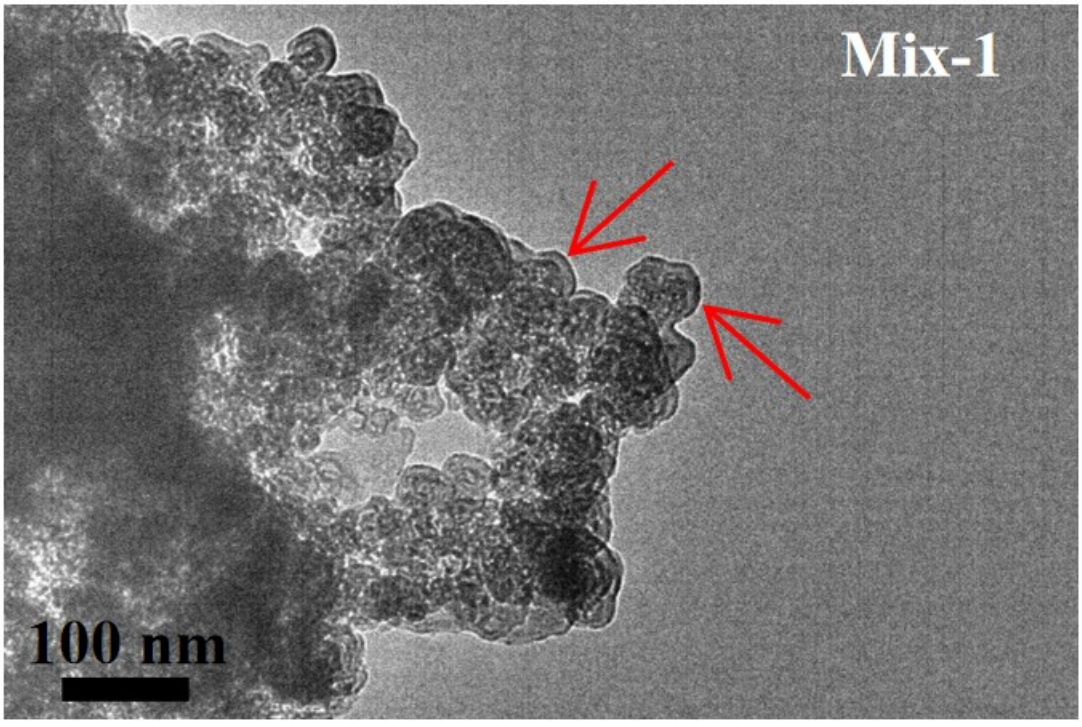

Fig. S12. TEM characterization of Mix-1. The red arrow indicates the coating of hydrophobin layer.

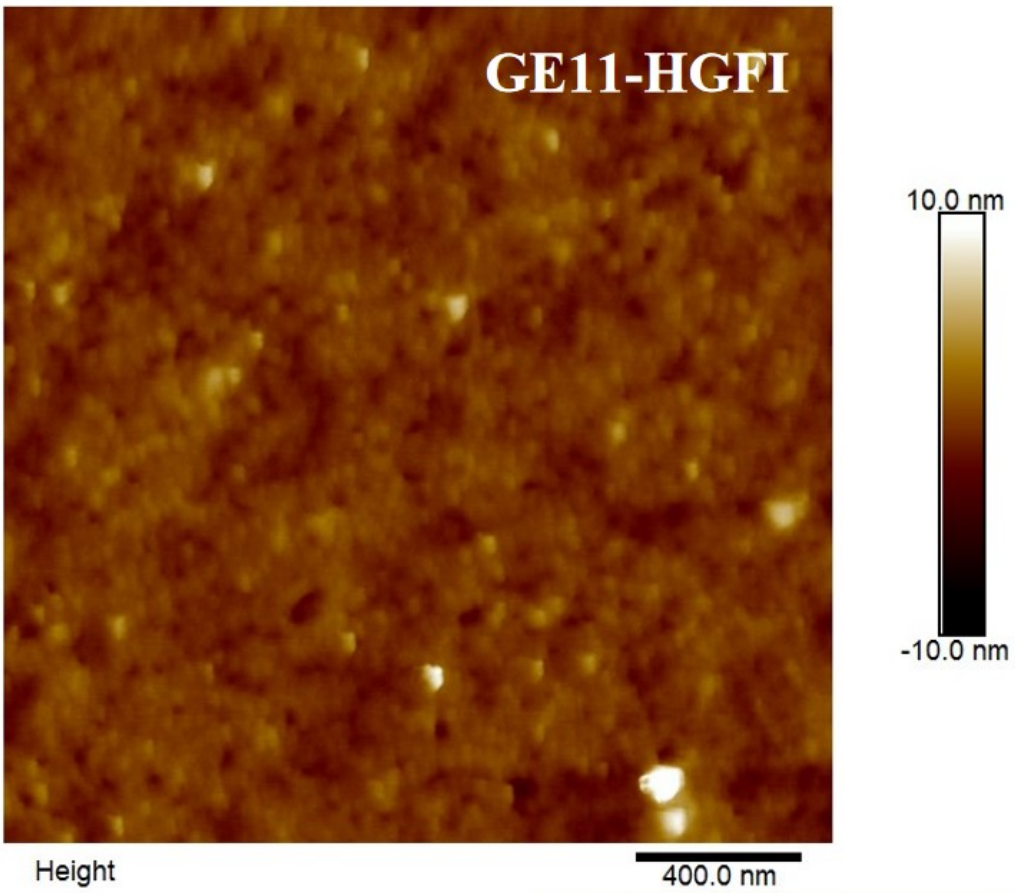

Fig. S13. AFM characterization of GE11-HGFI.

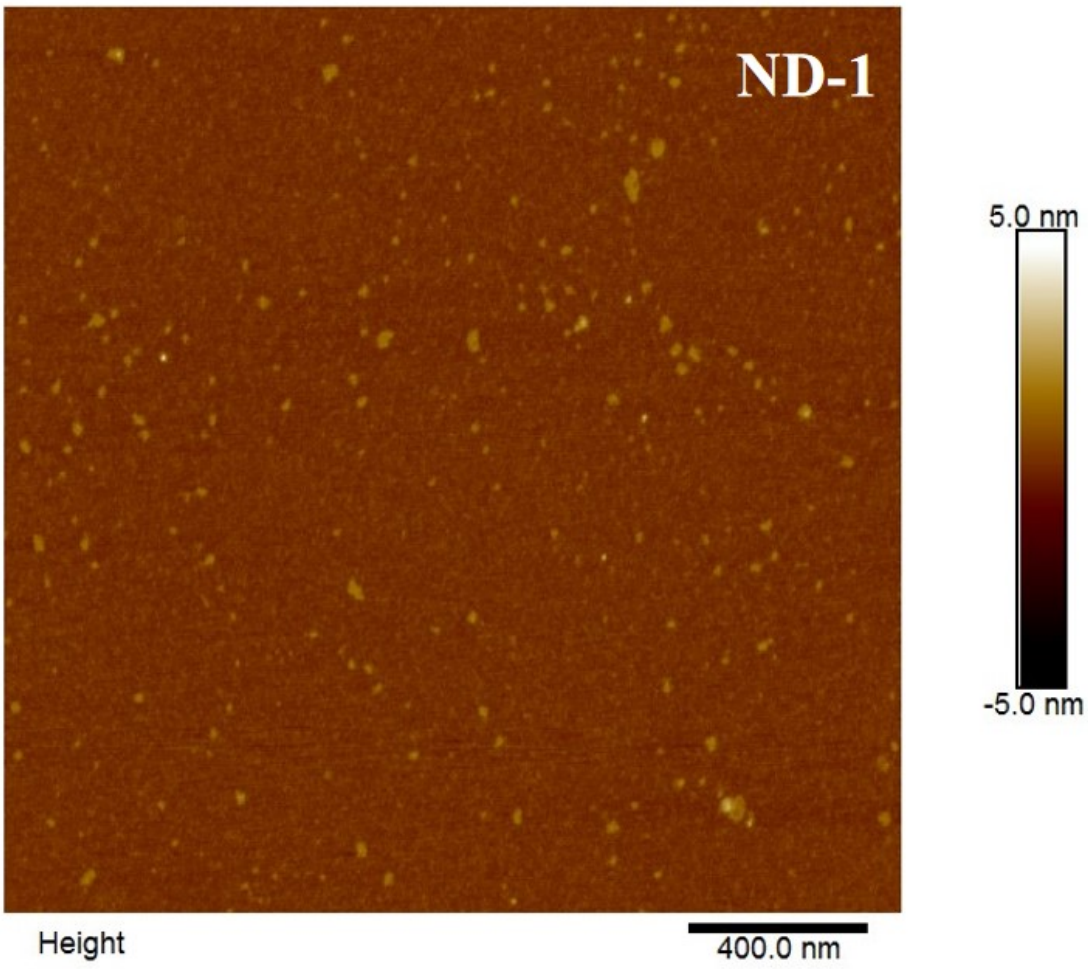

Fig. S14. AFM characterization of ND-1.

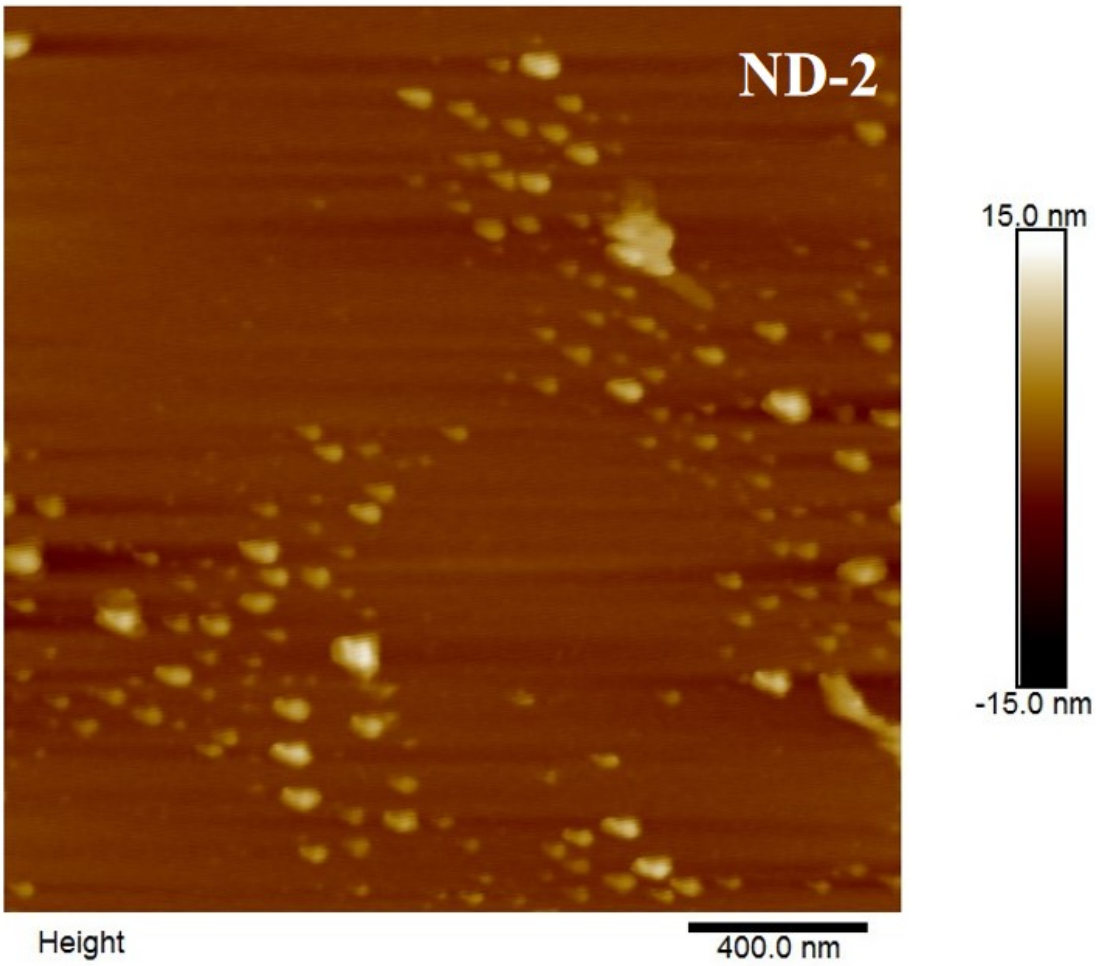

Fig. S15. AFM characterization of ND-2.

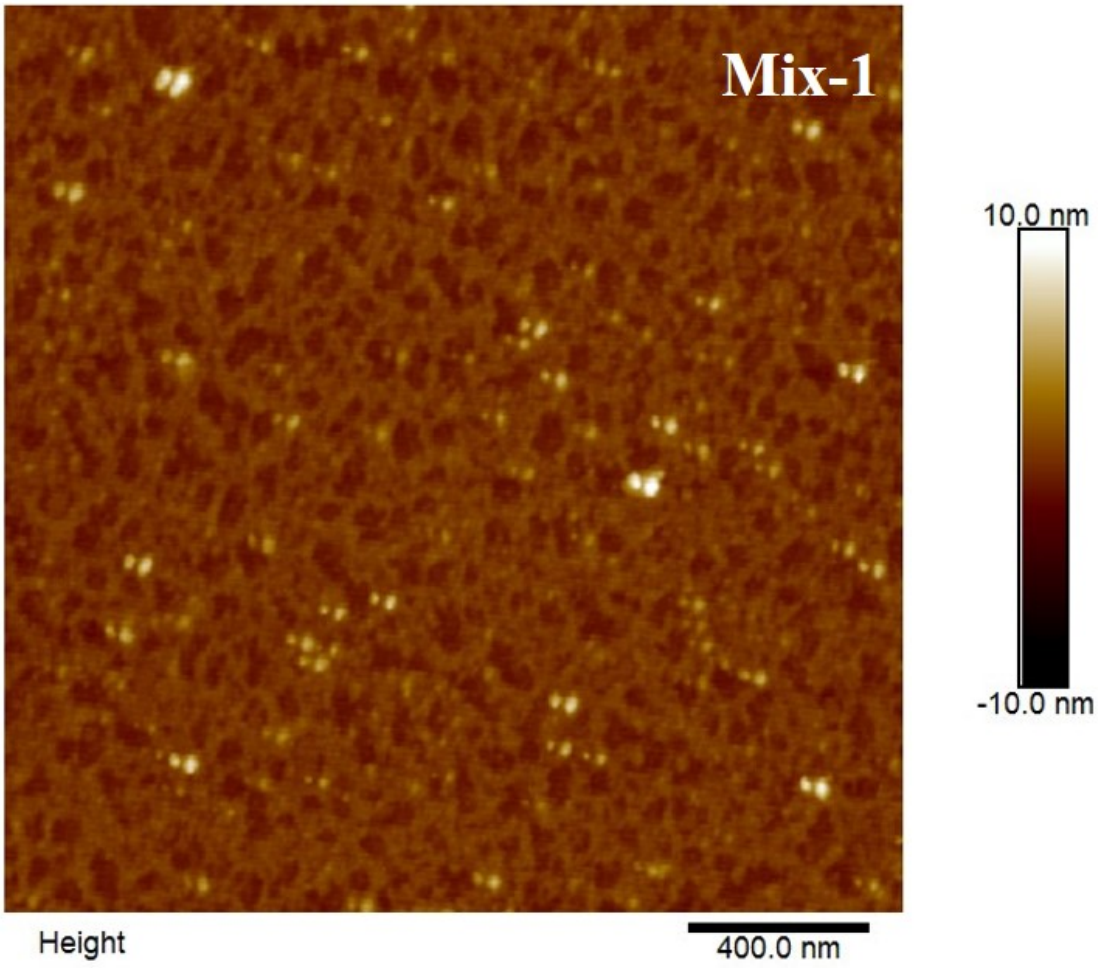

Fig. S16. AFM characterization of Mix-1.

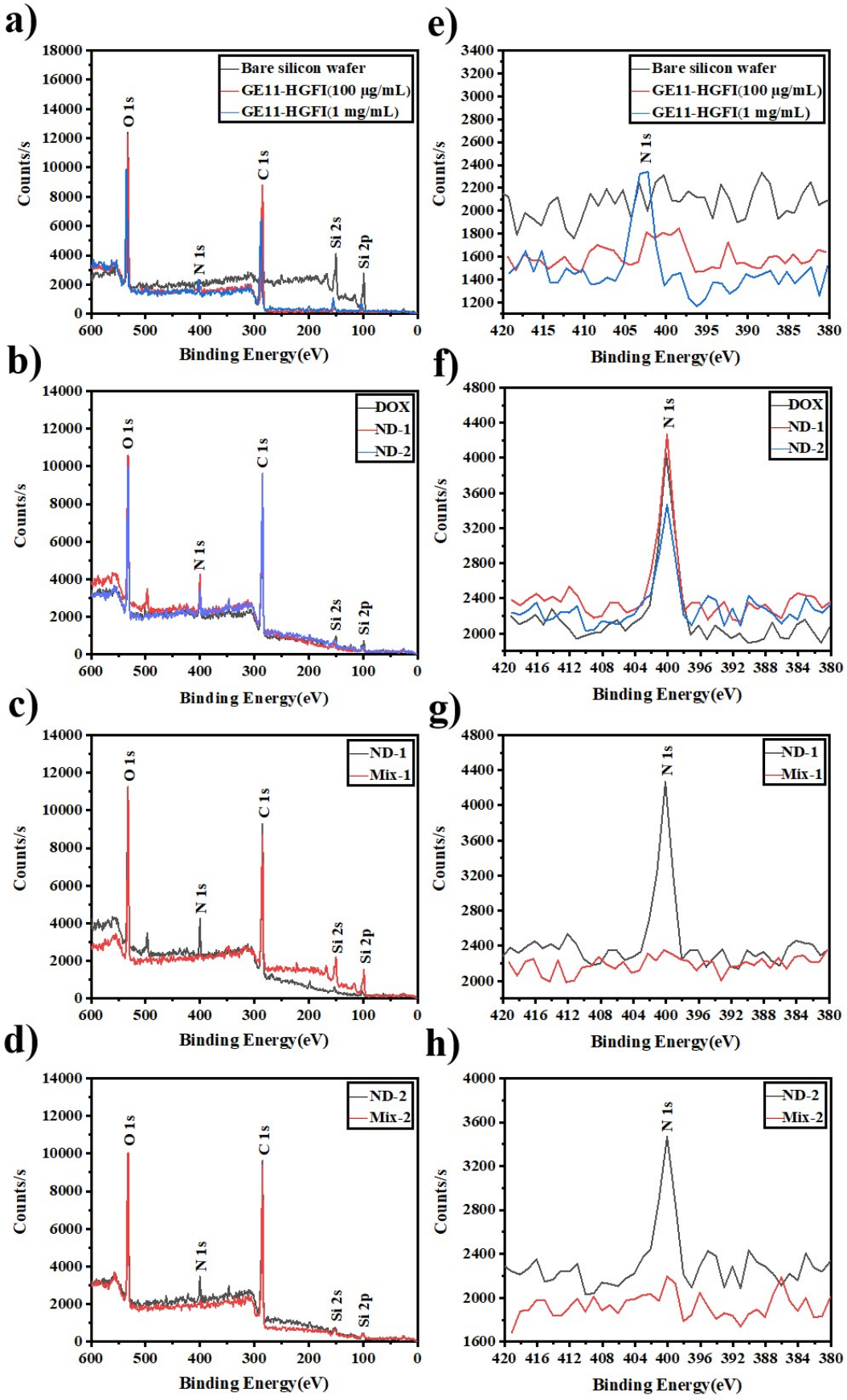
a)
Bare silicon wafer
GE11-HGFI(100 μg/mL)
GE11-HGFI(1 mg/mL)
O 1s
C 1s
N 1s
Si 2s
Si 2p
Counts/s
Binding Energy(eV)
e)
Bare silicon wafer
GE11-HGFI(100 μg/mL)
GE11-HGFI(1 mg/mL)
N 1s
Counts/s
Binding Energy(eV)
b)
DOX
ND-1
ND-2
O 1s
C 1s
N 1s
Si 2s
Si 2p
Counts/s
Binding Energy(eV)
f)
DOX
ND-1
ND-2
N 1s
Counts/s
Binding Energy(eV)
c)
ND-1
Mix-1
O 1s
C 1s
N 1s
Si 2s
Si 2p
Counts/s
Binding Energy(eV)
g)
ND-1
Mix-1
N 1s
Counts/s
Binding Energy(eV)
d)
ND-2
Mix-2
O 1s
C 1s
N 1s
Si 2s
Si 2p
Counts/s
Binding Energy(eV)
h)
ND-2
Mix-2
N 1s
Counts/s
Binding Energy(eV)

Fig. S17. (a) XPS spectrum of untreated silicon wafer and silicon wafer incubated with different concentrations of GE11-HGFI. (b) Comparison of XPS spectra of DOX, ND-1 and ND-2 incubated silicon wafers. (c) Comparison of XPS spectra of ND-1 and Mix-1 incubated silicon wafers. (d) Comparison of XPS spectra of ND-2 and Mix-2 incubated silicon wafers. (e) N1 high-resolution spectra of untreated silicon wafers and silicon wafers incubated with different concentrations of GE11-HGFI. (f) N1 high-resolution spectra of DOX, ND-1 and ND-2 incubated silicon wafers. (g) N1 high-resolution spectra of ND-1 and Mix-1 incubated silicon wafers. (h) N1 high-resolution spectra of ND-2 and Mix-2 incubated silicon wafers.

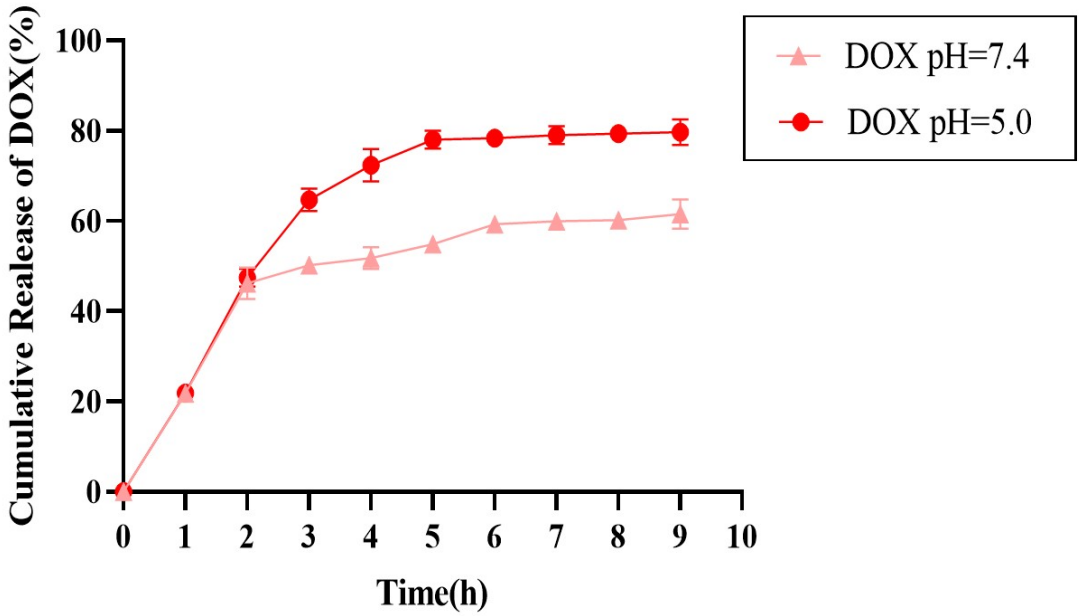

Fig. S18. In vitro release profiles of DOX at different pH in 0.01 M PBS.

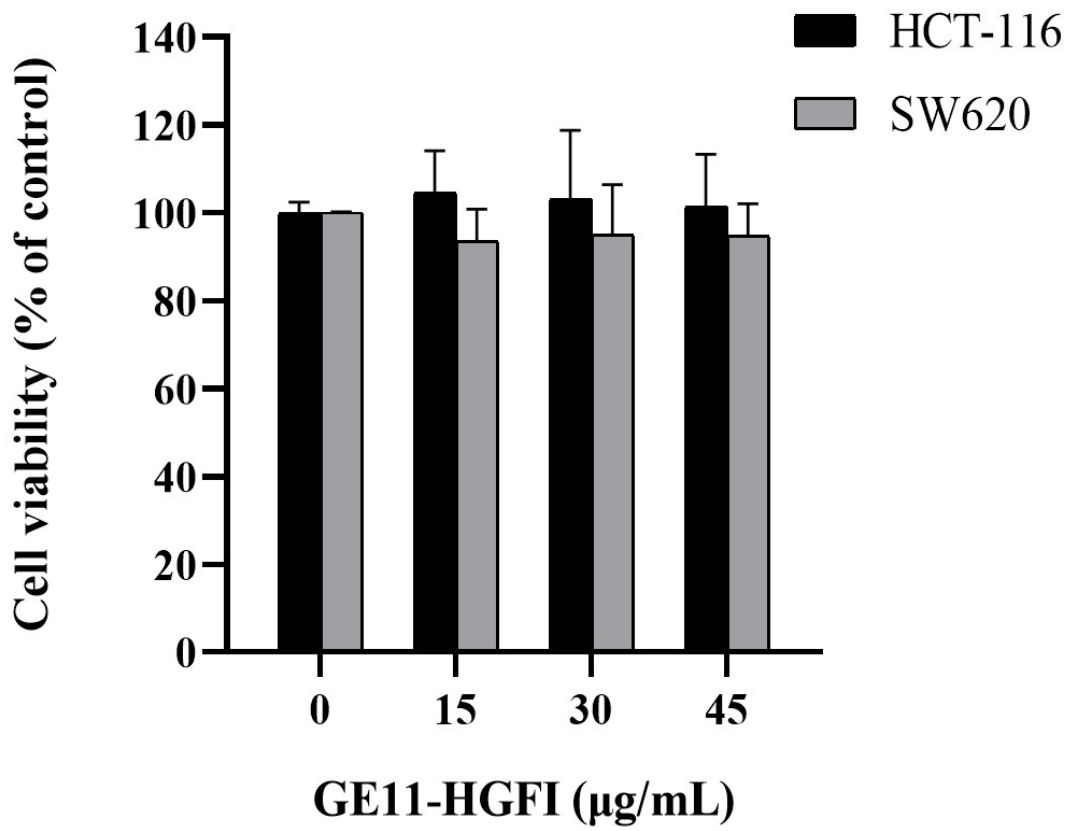

Fig. S19. The cell viability of HCT-116 and SW620 cells incubated with GE11-HGFI at different concentrations of 0, 15, 30 and 45 μg/mL for 24 h (this concentration gradient corresponds to the concentration of DOX of 0, 3, 6 and 9 μg/mL, respectively).

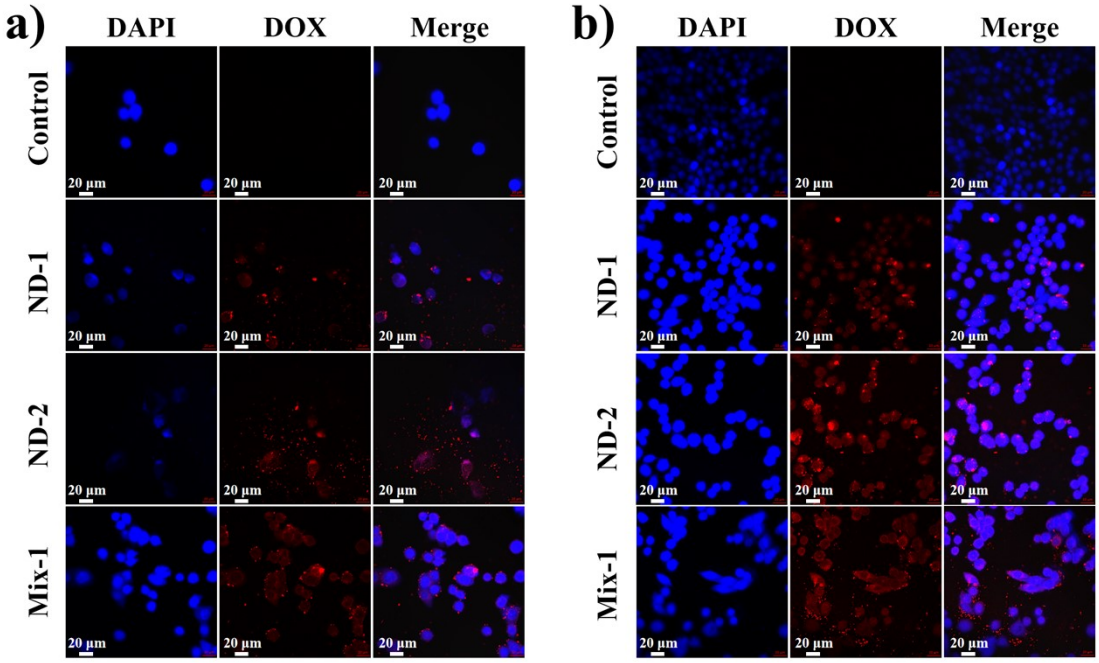

Fig. S20. (a) CLSM images of HCT-116 cells treated with ND-1, ND-2, Mix-1 for 4 h. (b) CLSM images of SW620 cells treated with ND-1, ND-2, Mix-1 for 4 h. The blue fluorescence is the nucleus, the red fluorescence is DOX, and the scale bar is 20 μm.

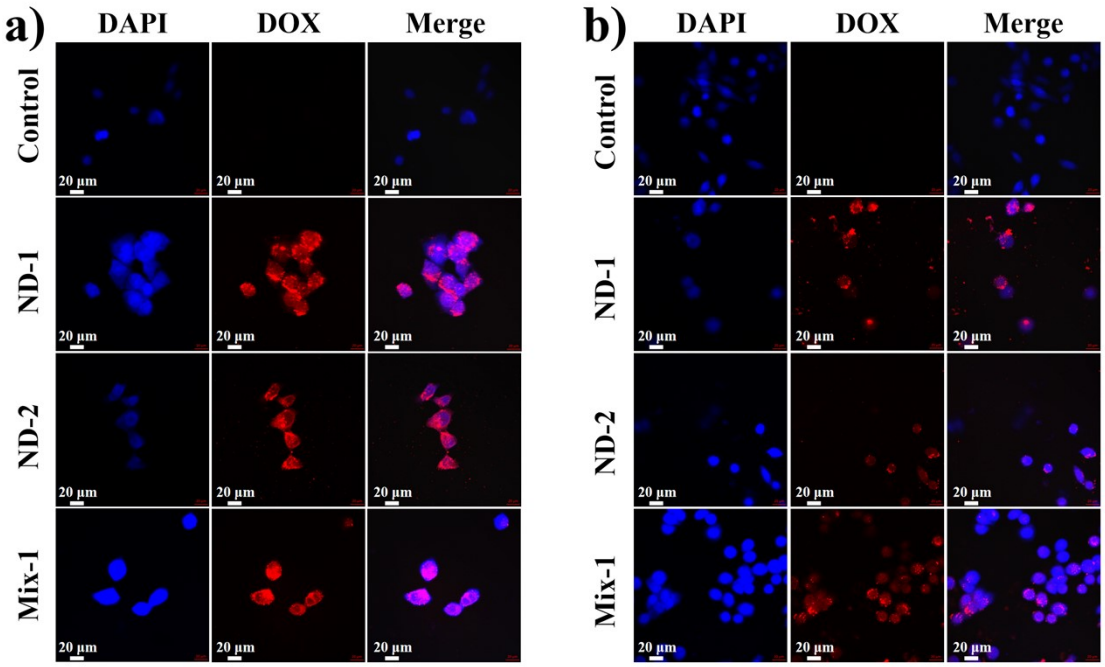

Fig. S21. (a) CLSM images of HCT-116 cells treated with ND-1, ND-2, Mix-1 for 24 h. (b) CLSM images of SW620 cells treated with ND-1, ND-2, Mix-1 for 24 h. The blue fluorescence is the nucleus, the red fluorescence is DOX, and the scale bar is 20 μm.

Table 1. Zeta Potential of GE11-HGFI, ND-1, ND-2, Mix-1 and Mix-2

	Zeta potentials (mV)			Mean (mV)
GE11-HGFI	-11.6	-10.9	-11.2	-11.2 ± 0.4
ND-1	-10.9	-12.1	-11.4	-11.5 ± 0.6
ND-2	-4	-5.4	-6.7	-5.4 ± 1.4
Mix-1	-14	-17.8	-17.9	-16.6 ± 2.2
Mix-2	-13.8	-13.8	-12.7	-13.4 ± 0.6

Table 2. WCA measurement of bare surface and material surface modified with GE11-HGFI, ND-1, ND-2, Mix-1 and Mix-2

Modifier	Mica (°)	Parafilm (°)
Bare surface	24.4 ± 1.3	112.0 ± 1.8
GE11-HGFI	79.8 ± 3.3	64.2 ± 1.3
ND-1	81.9 ± 3.5	104.0 ± 2.7
ND-2	53.7 ± 3.2	89.6 ± 4.2
Mix-1	64.4 ± 2.5	74.3 ± 3.1
Mix-2	39.5 ± 2.1	59.7 ± 3.0