Supporting Information

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A Novel Virus-Mimetic Nanogel Vehicle

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Experimental Methods

Polymer synthesis

The poly(His_{32}-co-Phe_6)·HCl copolymer consisting of L-histidine (His) (32 repeating units) and L-phenylalanine (Phe) (6 repeating units), was synthesized by ring-opening co-polymerization of His N-carboxyanhydride (NCA)·HCl and Phe NCA as previously described[4]. Briefly, 1-Benzyl-N-carbobenzoxy-L-histidine·HCl (2.5 g) (Sigma) suspended in anhydrous 1,4-dioxane (30 ml) (Sigma) was transformed to 1-benzyl-L-histidine NCA·HCl (His (Bz) NCA·HCl) by phosphorous pentachloride (1.8 g) (Sigma). L-phenylalanine (2.5 g) (Sigma) suspended in anhydrous 1,4-dioxane (30 ml) was transformed to Phe NCA by triphosgene (1.8 g) (Sigma). These reactions were performed in 20 min. His (Bz) NCA·HCl (0.95 g) and Phe NCA (0.05 g) were then polymerized in dimethylformamide (20 ml) at room temperature using isopropylamine (Sigma) (20 μl) as an initiator. The degree of polymerization (DP) and the composition of purified polymers was analyzed by ^{1}H NMR (DMSO-*d*6 with TMS)[4]. The copolymer block was then coupled with monocarboxylated PEG (M_w ~ 2000)[4] via the conventional carbodiimide reaction[4,9]. Next, the protecting (Bz) group of His was deprotected using metallic sodium and anhydrous liquid ammonia[4]. The yields of the final copolymer (poly(His_{32}-co-Phe_6)-*b*-PEG) ranged from 70.0 and 85.0 wt.-%.

Modification of the open end (-OH terminal group) of PEG (in poly(His_{32}-co-Phe_6)-*b*-PEG) by succinyl ester was performed with *N*,*N*¢disuccinimidyl carbonate (2.5 mol) (Sigma) and poly(His_{32}-co-Phe_6)-*b*-PEG (1 mol)[9,13] in dimethylformamide (50 ml) (Sigma) at room temperature for 1 day. The solution (50 ml) was mixed with excess diethyl ether (400 ml) (Sigma). After 1 hour, the precipitate was filtered and was dried *in vacuo* for 2 days. This modification was confirmed by the transfer of ^{1}H NMR peak at δ 3.56 (-C**H**$_2$-OH, at the open end of PEG) to δ 4.11 (-C**H**$_2$-O-CO-, at the modified site of PEG) (percentage conversion: 96.6±3.2 wt.%).

VM-nanogel synthesis

Triethylamine-treated DOX preparation: Triethylamine-treated DOX[9,13] was prepared as stock solution after stirring DOX (10 mg) and triethylamine (0.1 ml) in dichloromethane (0.5 ml) for 4 hours at room temperature. The appropriate amount of this triethylamine-treated DOX stock solution was used for loading to VM-nanogels.

VM-nanogel synthesis: Poly(His_{32}-co-Phe_6)-*b*-PEG-succinyl ester (4 mg) was dissolved in dichloromethane (0.1 ml) (Sigma) with/without triethylamine (Sigma)-treated DOX (1 mg: optimized amount (see Supporting Table 1) (Simga)[9,13] and BSA (1 mg: optimized amount (see Supporting Figure 1)) (Sigma) was dissolved in phosphate buffered saline (PBS) (pH 7.4, 20 mM) (0.9 ml). Two solutions were vigorously stirred at 10,000 rpm for 10 min followed by gentle stirring at 1,000 rpm for 20 min. Residual dichloromethane was evaporated at 30 °C for 12 hours. Furthermore, the BSA shell (Figure 1) was further conjugated, when necessary, with fluorescent probe molecules to allow confocal imaging within cells.

VM-nanogel characterization

In order to characterize BSA content in VM-nanogel, VM-nanogel solution was ultra-centrifuged at 25,000 rpm (precipitating the nanogels) for 20 min and a supernatant was extracted. A BCA protein assay kit (Pierce) was used to analyze a supernatant because non-reacted BSA is present in supernatant. BCA protein assay protocol is well described in Pierce homepage (http://www.piercenet.com/products/browse.cfm?fldID=02020101). As a result, 85.1±5.0 wt.% (n=3) of the feed BSA was found to react with poly(His_{32}-co-Phe_6)-*b*-PEG-succinyl ester. This indicates that the BSA content was approximately 180 μg for 1 mg of VM-nanogel.

Loaded DOX content in the VM-nanogels was determined by measuring the UV absorbance at 481 nm of DOX-loaded VM-nanogels dissolved in a dimethylsulfoxide-0.1 M HCl mixture (4: 1, vol/vol%)[9,13]. As shown in Supporting Table 1, the DOX feed was optimized.

FITC or folate labeling

A small amount of FITC (0.2 mg) (Sigma) or folate-succinyl ester (0.2 mg) was coupled with the

VM-nanogels (4 mg) at room temperature for 3 hours[9,13]. Herein, folate-succinyl ester was prepared by the conventional carbodiimide reaction; folate (1 mmol) in dimethylsulfoxide (30 ml) was reacted with *N*, *N*¢dicyclohexylcarbodiimide (1.2 mmol) and *N*-hydroxy-succinimide (2 mmol) as previously described[9]. In order to determine FITC or folate content reacted with BSA, the VM-nanogel solution was ultra-centrifuged at 25,000 rpm for 20 min and a supernatant was extracted. A supernatant includes non-reacted FITC or folate. Concentration in the supernatant was measured by high performance liquid chromatography (HPLC). From standard curves of FITC or folate obtained from HPLC, 85.1±4.6 wt.% (n=3) of the feed FITC and 80.2±4.3 wt.% (n=3) of the feed folate were found to react with the VM-nanogels.

Transmittance

The transmittance changes of the VM-nanogel solution with pH were measured using a UV/Visible spectrophotometer (wavelength of 500 nm)[9,13]. Before testing, the pH of the VM-nanogel solution (0.01 wt.%, ionic strength: 0.15) was reversibly changed between pH 7.4 and 5.5 and was stabilized at 37 °C for 1 hour at each pH. Relative transmittance of the VM-nanogel solution was measured at the selected pH change with respect to transmittance at pH 7.4.

Particle size analysis

Dynamic light scattering (Malvern Instrument)[9,13] was used to determine the particle size of the VM-nanogels with pH. For evaluating each result, the particle size of BSA (as a control) was measured as a function of bulk pH. BSA showed no significant difference in particle size with pH, ranging from 5 nm and 7 nm (see Supporting Figure 2).

Drug release

DOX-loaded VM-nanogels in PBS pH 7.4 solution (1 ml, ionic strength: 0.15) in a dialysis membrane tube (Spectra/Por MWCO 15K) were immersed in a vial containing fresh PBS (20 ml) with different pHs (pH 7.4, 6.8 and 6.4) for 1 hour or 24 hours (including mechanical shaking

(100 *rev.* /min) at each pH and 37 °C. DOX concentration was determined with a UV/Visible spectrophotometer[9,13].

Cell culture

Human ovarian carcinoma A2780 cells (from ATCC), A2780/AD cells (kindly supplied by the Kopecek research group, Utah)[12], and human non-small lung carcinoma cells A549 cells (from ATCC) were maintained in RPMI-1640/PBS medium (Gibco) with 0.5M PBS, 2 mM` L-glutamine (Sigma), 5 % penicillin–streptomycin (Gibco), 10 % fetal bovine serum (Gibco) in a humidified incubator at 37 °C and 5 % CO_2 atmosphere. Before testing, cultured cell (1×10^6 cells /ml) monolayers were harvested by 0.25 % (w/v) trypsin–0.03 % (w/v) EDTA solution (Gibco).

AFM

A2780/AD cells were treated in culture medium with VM-nanogels (containing DOX 10 μg/ml equivalent) for 4 hours and in fresh medium (no VM-nanogel) for 20 hours. After 24 hours, culture medium (pH 7.4) was extracted and dropped to the AFM shell (ThermoMicroscopes Explorer with ECU-Plus Electronics) for investigation. On the other hand, the culture medium was readjusted to pH 6.4 using 0.1 M HCl, in order to evaluate swelling properties of VM-nanogels escaped from dead cells. The resulting samples were imaged by AFM (ThermoMicroscopes Explorer with ECU-Plus Electronics). In addition, VM-nanogels (0.01 wt.%) suspended in fresh PBS pH 7.4 or 6.4 solution (ionic strength: 0.15) were also visualized as a control (see Supporting Figure 6).

Laser scanning confocal microscopy

Fluorescence intensity within cells grown on Lab-Tek II chamber slides or slide glass fragments was examined with a Leica TCS NT confocal microscopy using appropriate optical filters for each fluorescent probe. All confocal images were slice images[9,13] to distinguish VM-nanogels internalized from that adherent to the outside cellular membrane.

Repeated cell infection cycles

A2780/AD cells grown on slide glass fragments (dimension 1×1 cm) were pre-treated with free DOX (10 μg/ml) or equivalent DOX-loaded samples (VM-nanogel and control NPs[13]) for 4 hours. The two plates were separated by 1 mm in the same culture dish, continuously sharing fresh culture medium. Pretreated cells (A) were washed with PBS pH 7.4 and then co-cultured with fresh cells (B-0) on new slide glass fragments for 20 hours in fresh culture medium. (B-1) is (B-0) co-cultured with (A). (B-1) was withdrawn and was washed with PBS pH 7.4. (B-1) was co-cultured with fresh cells (B-2) on new slide glass fragment for 20 hours in fresh culture medium. (B-2) was withdrawn and was co-cultured with free cells (B-3) on new slide glass fragment for 20 hours in fresh culture medium. Confocal slice images were obtained to observe fluorescent DOX in cells. To evaluate the DOX effect on VM-nanogel migration, blank VM-nanogels (50 μg/ml) were tested under the same procedure. FITC-labeling (green color) was used to visualize the VM-nanogels.

Cell viability

Cells suspended in RPMI 1640 medium (200 μl) were seeded in a 96-well plate and cultured for 24 hours before testing. Free DOX (5 μg/ml) or equivalent DOX-loaded samples (VM-nanogels and control NPs[21]) dispersed in RPMI 1640/PBS medium with different pHs (pH 7.4 and 6.8) were added to the medium-removed 96-well plate for cell cytotoxicity test. The pH of culture medium was adjusted to each pH for 48 hours during the test as described before[9,13]. Cell viability (%) of treated cells was normalized to cell viability of untreated (negative) cells at each pH. Cell viability (%) was measured using tetrazolium salt MTT assay[9,13]. Briefly, one hundred ml of medium containing 20 μl of MTT solution were added to each well and the plate was incubated for an additional 4 hours, and then 100 μl of dimethylsulfoxide were added to each well. The solution was vigorously mixed to dissolve the reacted dye. The absorbance of each well was read with a microplate reader using a test wavelength of 570 nm and a reference wavelength

of 630 nm.

Statistical analysis

All results were analyzed by student t-test or ANOVA test $p < 0.05$ significance. MINITAB® *release 14* statistical software was used.

References

All references were listed in the main text.

Supporting Table 1. DOX loading content and efficiency with DOX feed (n=3)

Experimental condition	DOX feed (mg)	Loading content (wt.%) [a]	Loading efficiency (wt.%) [b]
(1)	0.5	9.2±0.4	91.6±4.6
(2)	**1**	**15.3±0.7**	**85.1±5.0**
(3)	2	16.7±0.8	53.1±2.8
(4)	3	19.7±1.3	38.6±3.2
(5)	4	21.1±2.8	31.7±5.3
(6)	6	24.3±3.1	25.3±4.1

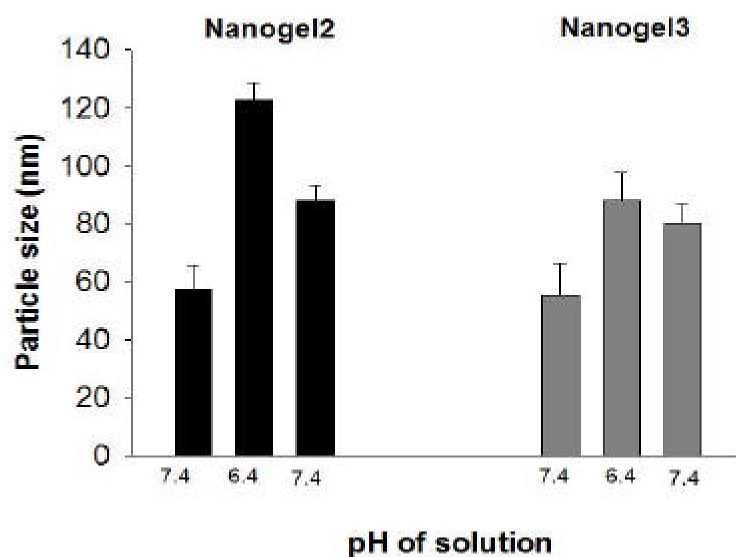
Other experimental conditions were fixed. Briefly, poly(His_{32}-co-Phe_6)-*b*-PEG-succinyl ester (4 mg) and BSA (1 mg) were used for VM-nanogels preparation.

[a]: DOX loading content (wt.%) was measured by following formula (=encapsulated DOX (wt.)/(DOX-loaded VM-nanogel (wt.)) ×100).

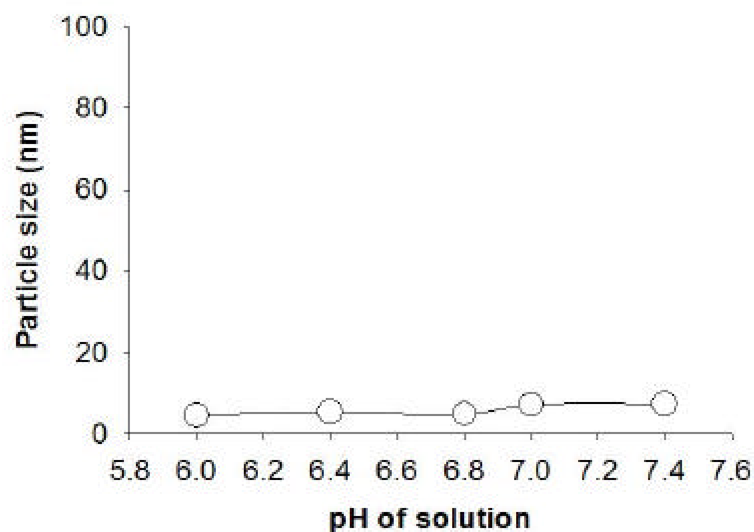
[b]: DOX loading efficiency (wt.%) was measured by following formula (=encapsulated DOX (wt.)/DOX feed (wt.) ×100).

From Supporting Table 1, experimental condition (**2**) was selected because experimental condition (1) showed limited DOX loading content and experimental conditions (3)-(6) lost significant DOX content during DOX loading.

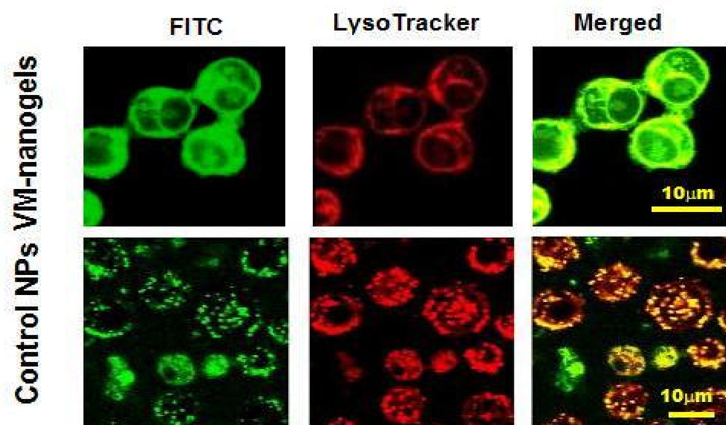
Supporting Figures:



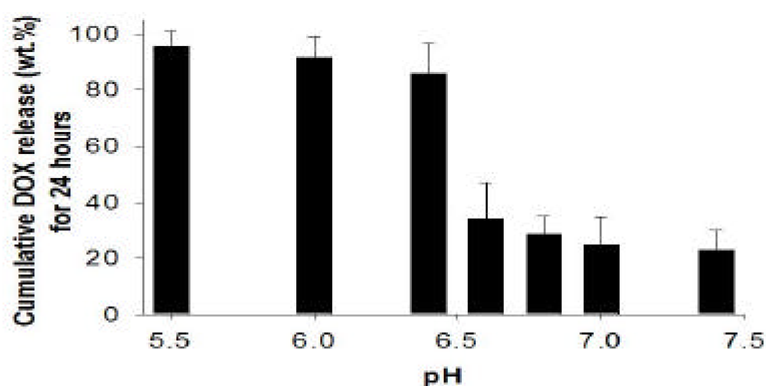
Supporting Figure 1. Particle size changes (from dynamic light scattering) of other nanogels. Poly(His$_{32}$-co-Phe$_{6}$)-*b*-PEG-succinyl ester (4 mg) was reacted with 2 mg of BSA for Nanogel 2, 4 mg for Nanogel 3 in PBS. The pH of the solution is adjusted in the following order: pH 7.4→ 6.4→ 7.4, equilibrating the nanogels for 1 hour at each pH. Each data point represents an average standard deviation (n=3).



Supporting Figure 2. Particle size changes (from dynamic light scattering) of BSA particles with pH. Each data point represents an average standard deviation (n=3).

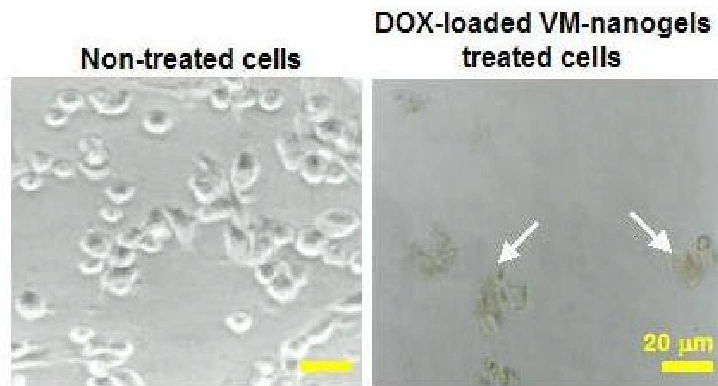


Supporting Figure 3. Endosomal escape activity of VM-nanogels. The endosomal escape activity studies were performed using LysoTracker Red that selectively stains acidic organelles (such as lysosomes and endosomes). A2780 cells were treated with FITC-labeled VM-nanogel (20 μg/ml) or FITC-labeled control NPs (*Ref., G. Mohajer, E. S. Lee, Y. H. Bae, Pharm. Res.* ***2007****, 24, 1618-1627*) for 4 hours and then with LysoTracker Red DND-99 (80nM) for 30 min (*Ref., M. A. Yessine, M. H. Dufresne, C. Meier, H. U. Petereit, J. C. Leroux, Bioconjug. Chem.* ***2007****, 18, 1010-.1014*). If VM-nanogels are trapped in endosomes and lysosomes, co-localization of FITC-labeled VM-nanogel (green) and LysoTracker (red) will result in a yellow color in a merged picture. However, the cells treated with VM-nanogels showed bright green fluorescence in the merged image with some yellow, indicating that a significant fraction of VM-nanogels was located out of the endolysosomal compartments. This is comparable with control NPs that show yellow color in a merged picture (due to no endosomal escaping property in the control NPs).

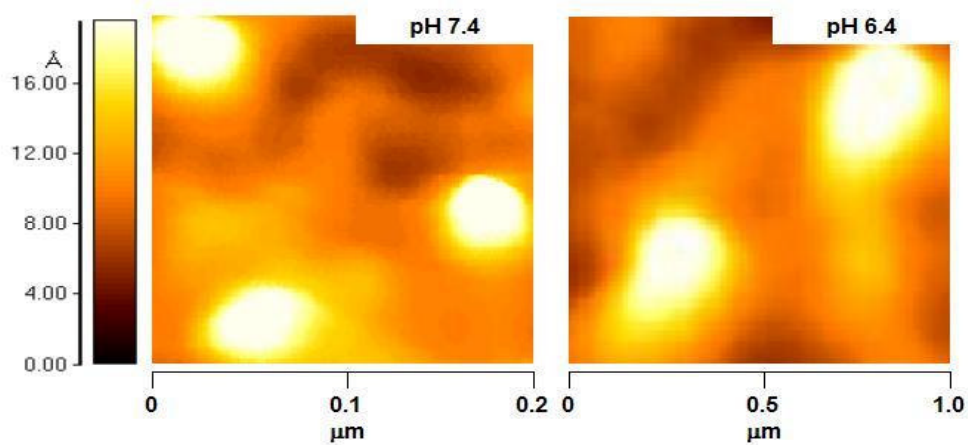


Supporting Figure 4. Cumulate release behavior of DOX from DOX-loaded VM-nanogels for 24 hours. The pH of the solution is adjusted from pH 7.4-5.5. Each data point represents an average with standard deviation (n=3).

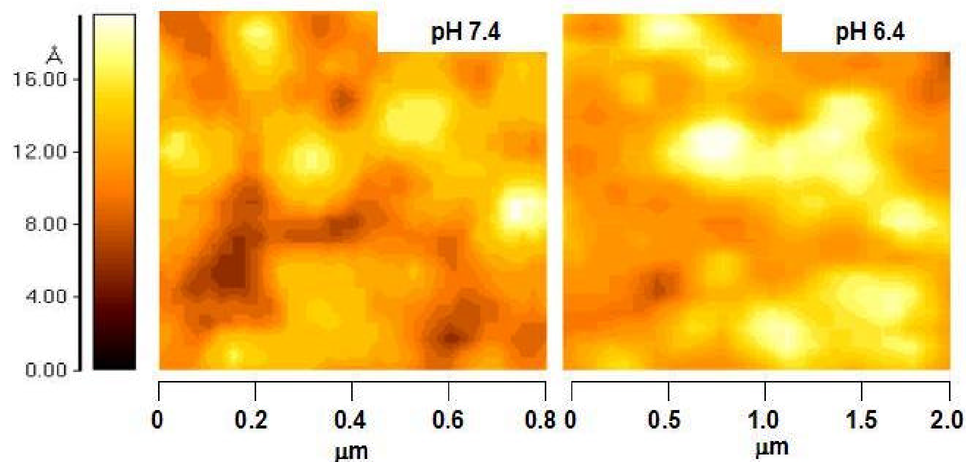
The VM-nanogels released a significant amount of DOX at endosomal pH (*e.g*., pH 6.4), while reducing DOX release rate at cytosolic or extracellular pH (*e*.g., pH 7.4-6.8).



Supporting Figure 5. Phase contrast microscopy images of A2780/AD cells treated with DOX-loaded VM-nanogels for 4 hours and subsequent incubation in fresh culture medium for 20 hours. A2780/AD cells showed cell death after this treatment (see white arrows): before treatment (left panel) and after treatment (right panel).

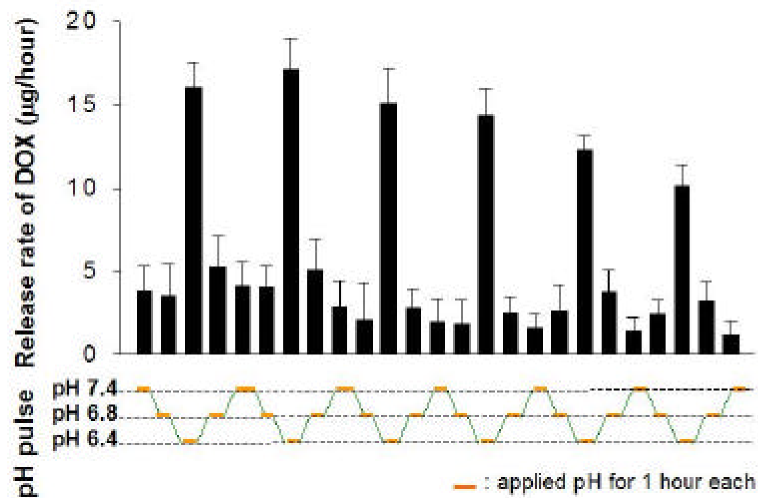


Supporting Figure 6. Atomic force microscopy (AFM) images of VM-nanogels suspended in fresh PBS pH 7.4 or 6.4 solution. This figure shows swollen VM-nanogels at pH 6.4.



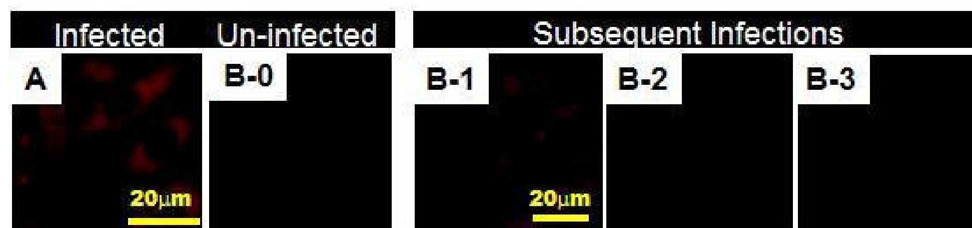
Supporting Figure 7. **A atomic force microscopy (AFM) images.** DOX-loaded VM-nanogels released from apoptosed DOX-resistant A2780/AD cells were exposed to different pH buffer solutions (left: pH 7.4, right: pH 6.4).

Supporting Figure 7 shows an AFM image of DOX-loaded VM-nanogel particles in cell culture medium once freed from multi-drug resistant (MDR) ovarian cells (A2780/AD: host cells) after spontaneous cell death. Due to cell debris, the image is not as sharp as ordinary well-prepared samples (see Supporting Figure 6). However the pictures show that VM-nanogels are still active (able to swell or deswell) with pH change.



Supporting Figure 8. **Release rate of DOX from DOX-loaded VM-nanogels.** 150 μg of DOX was encapsulated into 1 mg of DOX-loaded VM-nanogels. The pH of the solution is stepwise adjusted to pH 7.4, pH 6.8 and pH 6.4 at one-hour intervals. Each data point represents an average with standard deviation (n=3).

The tube (Spectra/Por MWCO 15K) containing DOX-loaded VM-nanogels (1 mg/0.5 ml of PBS 10 mM pH 7.4) were immersed in a vial containing fresh PBS (20 ml, 150 mM) with different pHs (pH 7.4, 6.8 and 6.4) for 1 hour (including mechanical shaking (100 *rev.* /min)) at 37 °C. Supporting Figure 8 shows that the VM-nanogels showed 6 de/swelling cycles before the release of DOX dropped sharply. The DOX release hysteresis is likely due to the short non-equilibrium time frame (1 hour interval) used for changing pH and sampling. DOX concentration was determined with a UV/Visible spectrophotometer[9,13].



Supporting Figure 9. **Migration of DOX-loaded VM-nanogels from infected A549 cells to untreated cells.** A549 cells[16] do not express folate receptor. All confocal images were sliced.

These results demonstrate that VM-nanogels do not infect cells lacking FR. Very limited cellular uptake of VM-nanogels, probably by fluid phase pinocytosis, was observed in A.