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Supplementary Information

Novel pH-responsive nanovectors for controlled release of ionisable drugs

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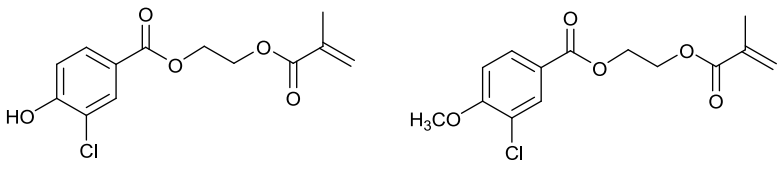
1. Instruments

^{1}H NMR spectra were recorded at room temperature on a 400 MHz spectrometer (Bruker DPX400 Ultrashield). All chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal reference. pH measurement were performed on a Seven Easy S20-K (Mettler Toledo) pH-meter operated with the Inlab 413 electrode (Mettler Toledo). UV-Vis analysis was carried out using a λ25 UV/Vis spectrometer (Perkin Elmer). Fluorescence measurements were performed on a FP 6500 spectrofluorometer (Jasco). Particles size measurements were carried out by Dynamic Light Scattering (DLS) at 25°C using a Zetasizer Nano spectrometer (Malvern Instruments Ltd), equipped with a 633 nm laser at a fixed angle (173°). Transmission electron microscopy (TEM) analyses were carried out using a Tecnai G2 microscope (FEI). Plates reading were performed with a EL311SK microplate autoreader (Bio-Tek Instruments, Winooski, VT-USA).

2. Materials

Visking dialysis tubing, tube diameter 16 mm, flat 25 mm, were furnished by Delchimica Scientific Glassware S.r.l., Naples, Italy. 0.45 and 0.22 μm cellulose membrane were purchased from Millipore, MA, USA. The synthesis of MCH and MCM monomers has been described elsewhere[1] and is reported here for the sake of clarity.



MCH *MCM*

Chart S1 Chemical structure of 2-(methacryloyloxy)ethyl-3-chloro-4-hydroxybenzoate (MCH) (left) and 2-(methacryloyloxy)ethyl-3-chloro-4-methoxyoxybenzoate (MCM) (right).

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3. Methods

3.1 Polymer characterization by gel permeation chromatography and ^{1}H NMR. The polymer molecular weights were determined by gel permeation chromatography (GPC) using a PL50 apparatus (Polymer Laboratories) equipped with two columns (Agilent PLgel 5 µm Mixed D, 7,5 x 300 mm) connected in series and an RI detector, eluting with DMF/0.1 M LiBr (vol/vol) as the mobile phase, at flow rate of 1 mL/min, at 30°C. Narrow PPMA standards (162-371,000 g mol^{-1}) were used to calibrate the SEC. Data processing was carried out using Cirrus GPC/SEC 3.0 software.

Polymer theoretical number-average molecular weights were calculate according to the following equation: [(MW_{MCH} x $C_{Monomer}$/C PEGMA macro-CTA) x Conversion + MW PEGMA macro-CTA].

3.2 Particle size analysis and zeta potential measurements. Particles size measurements were carried out at 25°C on pPEGMA-*b*-pMCH and pPEGMA-*b*-pMCM block copolymer solutions in a 0.5-1 mg/mL concentration range in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4. Zeta potential was determined under the same conditions using a ten-fold diluted PBS buffer (ionic strength <0.02 M). Three measurements were performed for each sample. Micellar dispersions were filtered with a 0.45 µm cellulose disposable membrane filter prior to measurement. Results are reported as z-average.

3.3 Trasmission Electron Microscopy (TEM) Analysis. TEM analysis was performed on both tamoxifen-loaded pPEGMA-*b*-pMCH and unloaded pPEGMA-*b*-pMCH or pPEGMA-*b*-pMCM assemblies prepared by the dialysis method described in section "Preparation of micelles and polymersomes" at 1.5 mg/mL polymer concentration, in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4. A drop of micelles or polymersomes suspension was deposited on a copper grid. The excess volume was removed with filter paper. The polymers were negatively stained with 1% uranyl acetate dissolved in distilled water.

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3.4 Encapsulation studies. pPEGMA-*b*-pMCH and pPEGMA-*b*-pMCM nanovectors loading capacity (LC) was assessed by RP-HPLC using an apparatus equipped with a C_{18} column (Luna, 5 µm, 250 x 46 mm, Phenomenex), using milliQ water/0.05% TFA (eluent A), MeCN/0.05% TFA (eluent B) as the mobile phase (gradient from 40% to 90% of eluent B in 10 minutes), at a flow rate of 1 mL/min. The system was equipped with an UV detector (Jasco UV 2077 Plus) set at λ=275 nm. In a typical experiment 75 µL of a suspension of tamoxifen loaded micelles (polymer concentration range 1.0-2.5 mg/mL) in 0.02 M phosphate buffer, 0.15 M NaCl pH 7.4 were centrifuged at 10000 rpm for 5 minutes and filtered through Corning Costar® Spin-X® centrifuge tube equipped with cellulose acetate membrane filters with pore size of 0.45 µm to remove unloaded drug. 50 µL of filtrate was diluted with 950 µL of MeOH and centrifuged at 1000 rpm for 5 minutes twice to remove any residual traces of precipitated drug. Finally, 20 µL of supernatant were analyzed by RP-HPLC. The amount of tamoxifen in each sample was calculated using a standard curve obtained by eluting solutions of tamoxifen at different concentrations [y = 41020×(tamoxifen µg/mL) + 315.44 R^2 = 0.9996, detection limit 10 ng/mL].

The concentration of paclitaxel in methanol solution was calculated from its molar extinction coefficient (29800 M^{-1} cm^{-1} at λ=227 nm). RP-HPLC was used to quantify the amount of paclitaxel within the pPEGMA-*b*-pMCH or pPEGMA-*b*-pMCM tamoxifen-loaded micelles, using the same stationary and mobile phases reported above, under in isocratic conditions with eluent A/eluent B 45/55 (v/v). The amount of loaded drug was calculated using the calibration curve [y =37529×(paclitaxel µg/mL) – 3198.4 R^2 = 0.9995, detection limit 1 µg/mL].

Spectrophotometric quantitation of doxorubicin HCl solutions was performed using a molar extinction coefficient of 11500 M^{-1} cm^{-1} at λ=480 nm. The amount of doxorubicin HCl encapsulated in the aggregates was performed via fluorimetric analysis of micelle/polymersome samples diluted in DMSO to disassemble the nanovectors and then further diluted in 0.02 M phosphate buffer, 0.15 M NaCl pH 7.4. The amount of loaded drug was calculated from the peak area referring to a calibration curve obtained in 0.02 M phosphate buffer, 0.15 M NaCl pH 7.4 [y = 3.9678x(doxorubicin HCl ng/mL) + 38.219 R^2 = 0.9929, detection limit 5 ng/mL].

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4. Nanovector stability studies

4.1 $PEGMA_{11}$-*b*-MCH_{21} and $PEGMA_{11}$-*b*-MCH_{38} empty nanovectors. pPEGMA-*b*-pMCH and pPEGMA-*b*-pMCM copolymer assemblies were prepared according to the procedure described in the section "Preparation of micelles and polymersomes", and diluted with PBS pH 7.4 or PBS pH 6.8 or 6.5 added of 10% FBS at a final polymers concentration of 0.8-1 mg/mL. At regular intervals of time, the size of the colloidal systems was measured by DLS (Fig. S1).

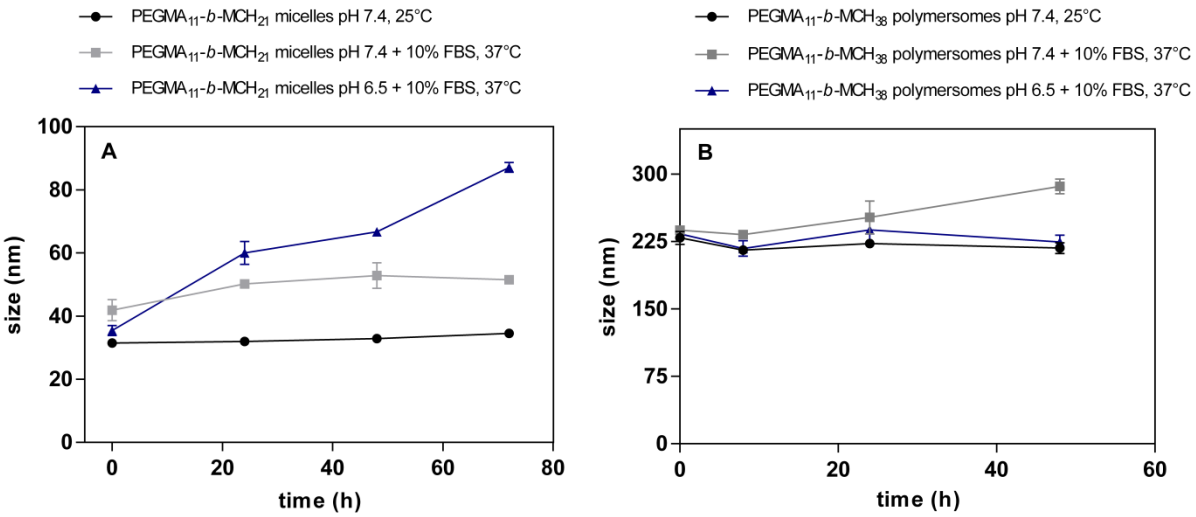

Fig. S1 Kinetic stability profile of A) $PEGMA_{11}$-*b*-MCH_{21} empty micelles, and B) $PEGMA_{11}$-*b*-MCH_{38} empty polymersomes incubated in: (●) 0.02 M phosphate buffer, 0.15 M NaCl pH 7.4, 25°C; (■) 0.02 M phosphate buffer, 0.15 NaCl pH 7.4 + 10% FBS, 37°C; (▲) 0.02 M phosphate buffer, 0.15 NaCl pH 6.5 + 10% FBS, 37°C.

4.2 Tamoxifen-loaded $PEGMA_{11}$-*b*-MCH_{21} micelles 1.0 mL of tamoxifen-loaded polymeric assemblies (1.0 mg/mL polymers concentration) in 0.02 M phosphate, 0.15 M NaCl, pH 7.4 with or without 10% of FBS were incubated at 37°C or 4°C. Similarly, 1.0 mL of tamoxifen-loaded polymeric assemblies (3.0 mg/mL polymer concentration) in 0.02 M phosphate, 0.15 M NaCl pH 7.4 and 6.5 were added of 1 mL of rat plasma to give a final concentration of tamoxifen of 150 µg/mL, and incubated at 37°C. The size of the colloidal aggregates was measured by DLS at regular intervals of time. 100 µL aliquots were

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withdrawn from samples and the concentration of tamoxifen was estimated by RP-HPLC as described in section 3.4.

Table S1. Decrease of tamoxifen titer incorporated in $PEGMA_{11}$-*b*-MCH_{21} micelles after 24 hours of incubation in various conditions (initial loading content 9% w/w).

Incubating media	Loading Capacity % after24 h[a]	Loading Decrease % in 24 h
PBS pH 7.4 4°C	8.6	8.2
PBS pH 7.4 RT	7.8	16.0
PBS pH 7.4 + 10% FBS a 37°C	8.2	12.6
PBS pH 6.5 + 10% FBS a 37°C	8.3	10.5

[a] tamoxifen loading content/decrease was estimated by RP-HPLC

Table S1 reports the stability of tamoxifen-loaded $PEGMA_{11}$-*b*-MCH_{21} micelle formulation in term of drug content.

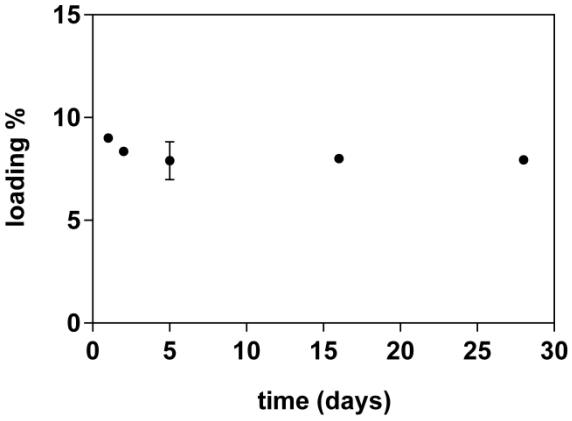

Fig. S2. Drug content of $PEGMA_{11}$-*b*-MCH_{21} micellar nanocarriers initially loaded with 9% w/w of tamoxifen and stored at 4°C for 28 days. Physical stability was also measured by DLS analysis (data not shown).

In buffer, after 24 hours at 4°C only about 8.21% of drug was released and after that, both the vesicle size and the drug titer were found to remain stable over 28 days (Fig. S2). The concentration of tamoxifen in the formulation was 100 µg/mL at time zero, which is 286 times higher than the solubility of the free drug under the same conditions. Tamoxifen is poorly soluble in water and tends to aggregate

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and precipitate in aqueous medium, resulting in the titer decrease[2]. Therefore, the drug detected in these studies corresponds to the tamoxifen incorporated in the micellar nanocarriers, whilst the released drug is expected to precipitate in the medium soon after the release from micelles. A blank experiment showed that under the same conditions free tamoxifen titer decreased by 50% in less than 6 hours. Tamoxifen-loaded $PEGMA_{11}$-*b*-MCH_{21} micelle formulation was then tested in 0.02 M phosphate buffer, 0.15 M NaCl, supplemented with fetal bovine serum at pH 7.4 and 6.5, to evaluate the stability of tamoxifen-loaded carrier in presence of serum proteins. At both pH values only about 10% of drug decrease was observed in 24 hours. Preliminary studies showed that the solubility of free tamoxifen in 0.02 M phosphate buffer, 0.15 M NaCl pH 7.4 supplemented of 10% FBS is about 10 μg/mL, which is only the 10% of the total tamoxifen in the $PEGMA_{11}$-*b*-MCH_{21} formulation. This confirms that the drug detected in this assay corresponds to the tamoxifen encapsulated in the micellar hydrophobic lumen (about 80% of initial LC).

The stability of $PEGMA_{11}$-*b*-MCH_{21} tamoxifen-loaded micelles with 9% w/w was also evaluated in rat plasma (Fig. S3). $PEGMA_{11}$-*b*-MCH_{21} micelles incubation in 0.02 M phosphate buffer, 0.15 NaCl pH 7.4/rat plasma 1:1 v/v, 37°C did not result in a significant decrease in the drug titer. Indeed, after 48 hours of contact with plasma, more than 96% of the loaded tamoxifen was found to be still in solution. Preliminary studies showed that under the experimental condition the solubility of tamoxifen in the buffer/plasma mixture was 40 μg/mL. Therefore, even considering the maximal solubility of the drug in the medium, about 70% of the drug was till loaded in the micelles. In addition, RP-HPLC analysis of the entrapped drug revealed the absence of tamoxifene degradation products.

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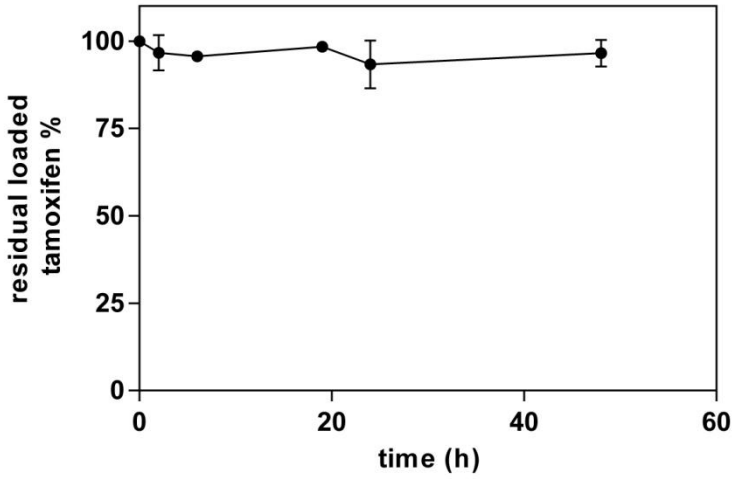

Fig. S3 Drug titer decrease over time of $PEGMA_{11}$-*b*-MCH_{21} tamoxifen loaded micelles (9 % w/w) incubated in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4/rat plasma 1:1, at 37°C.

Micelles are able to prevent drug binding to protein[3] promoting its accumulation in tumor tissue. Drug binding to proteins – mainly albumins - has been reported to be the main cause for the decrease of the concentration of tamoxifen in plasma.[4] These results showed that pPEGMA-*b*-pMCH and pPEGMA-*b*-pMCM micellar systems can at least partially prevent tamoxifen binding to plasma proteins.

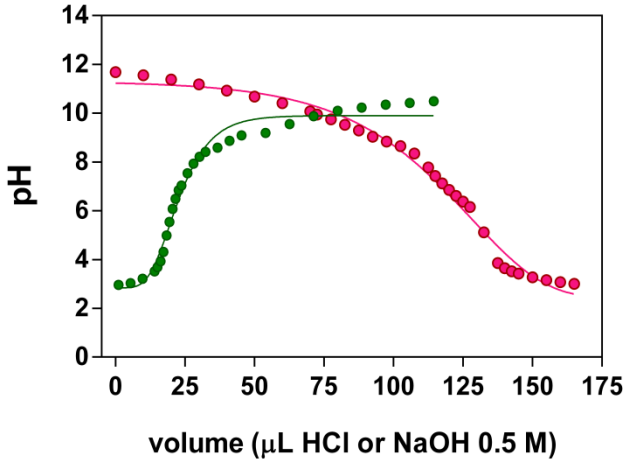

Fig. S4 $PEGMA_{11}$-*b*-MCH_{21} (●) titration and (●) back-titration curves. The pH of a 1 mg/mL polymer solution in milliQ water was adjusted to pH 12 by addition of 1N NaOH and titrated to pH 3 with 0.5 N HCl. The same solution underwent back-titration using 0.5 N NaOH.

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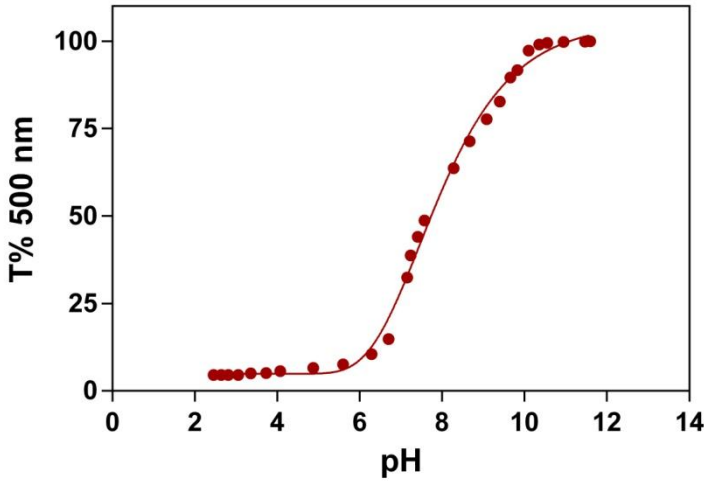

Fig. S5 $PEGMA_{11}$-*b*-MCH_{38} turbidimetric assay. The pH of a 1.0 mg/mL polymer solution in milliQ water was adjusted to pH 12 by addition of 5N NaOH. The transmittance (T%) at λ=500 nm was recorded after sequential additions of 1 N HCl until pH 2 was reached.

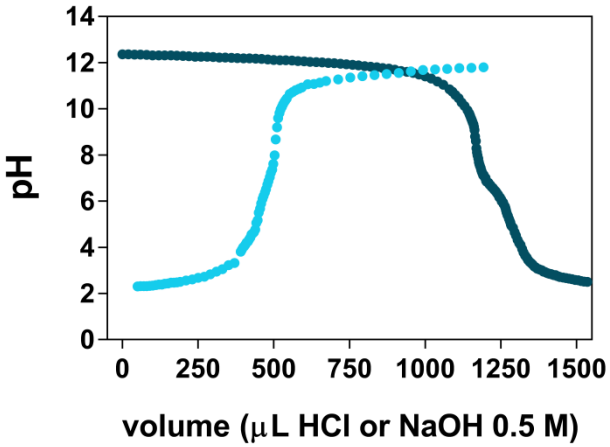

Fig. S6 (●) titration and (●) back-titration of MCH monomer. The pH of a 0.25 mg/mL monomer solution in milliQ water was adjusted to pH 12 by addition of 1N NaOH and titrated to pH 3 with 0.5 N HCl. The same solution was then back-titrated with 0.5 N NaOH.

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Table S2 Tamoxifen-loaded polymeric nanocarriers prepared at increasing drug/polymer wt%. Loading Capacity (LC), Encapsulation Efficiency (EE), size and polydispersity (PDI). All samples are prepared in 0.02 M phosphate buffer, 0.15 M NaCl at pH 7.4, room temperature.

$PEGMA_{11}$-*b*-MCH_{21}	**(drug/polymer wt% in the reaction feed)**	**LC[a]**	**EE[b]**	**Micelle size (nm)[c]**	**PDI[c]**
A	10	8.5	93.2	57.9±1.7	0.095
B	25	14.7	69	39.20±2.71	0.439
C	40	13.8	40	37.58±0.23	0.306
D	50	17.5	42.6	42.56±1.11	0.247
$PEGMA_{11}$-*b*-MCM_{20}				**Micelle size (nm)[b]**	
a[1]	10	0.9	9.5	17.24±0.35	0.235
b[1]	25	0.9	3.5	17.11±0.28	0.294
c[1]	40	1.0	2.5	17.8±0.34	0.356
d[1]	50	1.0	1.9	18.3±0.48	0.482
$PEGMA_{11}$-*b*-MCH_{38}				**Polymersomes size (nm)[b]**	
a[2]	10	8.6	90.4	163±2.83	0.151
b[2]	25	15.8	75.3	161.4±1.2	0.142
c[2]	40	13.5	39	167.4±0.51	0.146
d[2]	50	14.4	33.7	188.6±3.12	0.192

[a] determined by HPLC using the following equation: (amount of loaded drug/amount of drug+amount of polymeric micelles)*100
[b] determined by HPLC using the following equation: (amount of loaded drug/amount of total drug)*100
[c] determined by DLS using 1 mg/mL diblock copolymer solution in PBS pH 7.4

Table S3 Paclitaxel-loaded polymeric nanocarriers prepared at increasing drug/polymer wt%. Loading Capacity (LC), Encapsulation Efficiency (EE), size and polydispersity (PDI). All samples are prepared in 0.02 M phosphate buffer, 0.15 M NaCl at pH 7.4, room temperature.

$PEGMA_{11}$-*b*-MCH_{21}	**(drug/polymer wt% in the reaction feed)**	**LC [a]**	**EE**	**Micelle size (nm)[b]**	**PDI[b]**
e	**10**	4.8	51	69.64±0.54	0.045
f	**25**	5.5	23.2	88.08±1.1	0.141
g	**40**	8.7	24	163.9±1.4	0.181
h	**50**	7.9	17.2	162.2±0.51	0.214
$PEGMA_{11}$-b-MCH_{38}				**Polymersomes size (nm)[b]**	
e’	**10**	Aggregation		-	-
$PEGMA_{11}$-b-MCM_{20}				**Control micelle size (nm)[b]**	
e[2]	**10**	0.9	9.3	20.08±0.4	0.45
f[2]	**25**	1.3	5.0	20.07±0.25	0.86
g[2]	**40**	1.4	3.5	21.25±0.05	0.764
h[2]	**50**	0.9	1.8	20.07±0.1	0.418

[a]determined by HPLC
[b]size and PDI of colloidal systems were determined by DLS

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Table S4. Doxorubicin HCl-loaded polymeric nanocarriers prepared at increasing drug/polymer wt%. Loading Capacity (LC), Encapsulation Efficiency (EE), size and polydispersity (PDI). All samples are prepared in 0.02 M phosphate buffer, 0.15 M NaCl at pH 7.4, room temperature.

$PEGMA_{11}$-*b*-MCH_{21}	**drug/polymer (wt%)**	**LC [a]**	**EE**	**Micelle size (nm)[b]**	**PDI[b]**
i	**10**	1.6	15.6	24.99±0.14	0.239
$PEGMA_{11}$-*b*-MCH_{38}				**Polymersome size (nm)[b]**	
i’	**30**	12.3	45.6	113.3±2.31	0.25
l’	**50**	11.6	25.7	117.4±3.6	0.253
$PEGMA_{11}$-*b*-MCM_{20}				**Control micelle size (nm)[b]**	
i^2	**10**	1.5	15	39.6±1.5	0.155

[a]determined by fluorimetry
[b]size and PDI of colloidal systems were determined by DLS

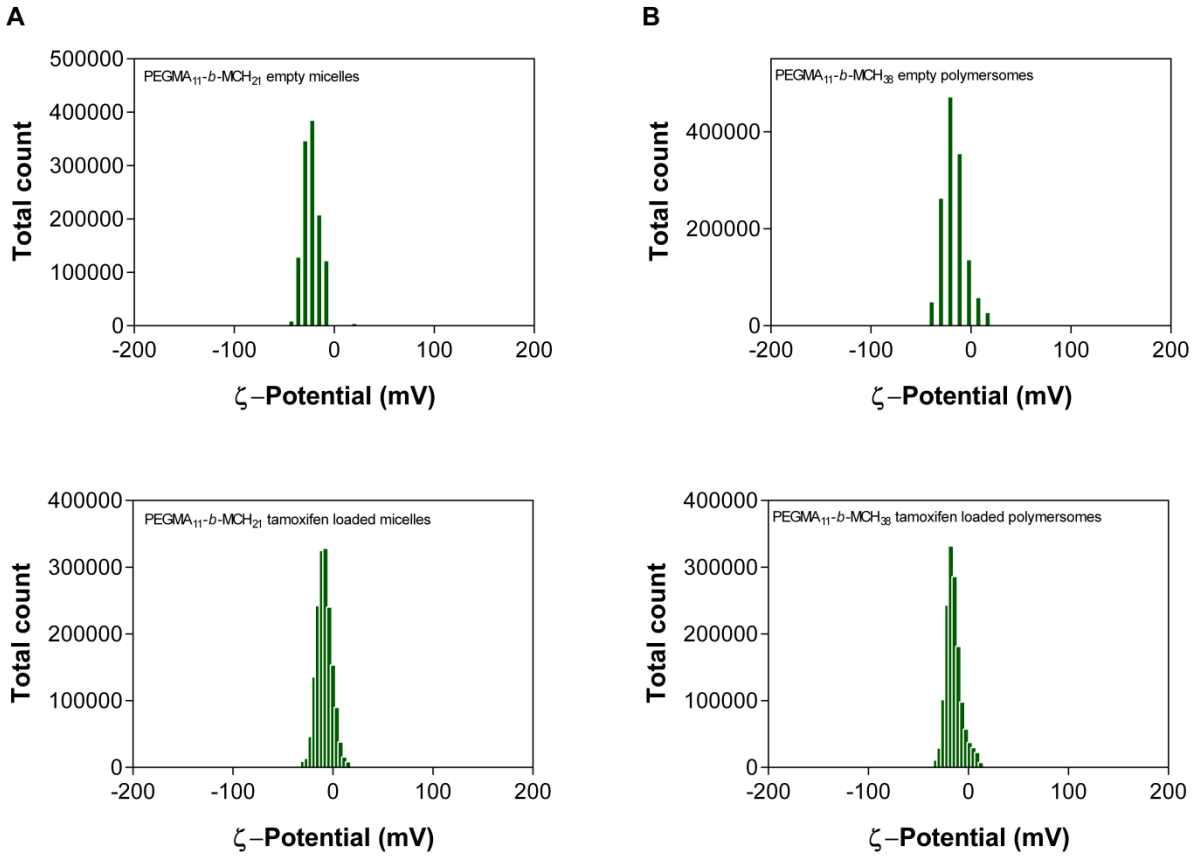

Fig S7 ζ-potential plot of (A) $PEGMA_{11}$-*b*-MCH_{21} micelles and (B) $PEGMA_{11}$-*b*-MCH_{38} polymersomes empty (above) or loaded with tamoxifen (below). Measurements were performed at a polymer concentration of 0.5 mg mL^{-1} in 2 mM PBS, 15 mM NaCl pH 7.4.

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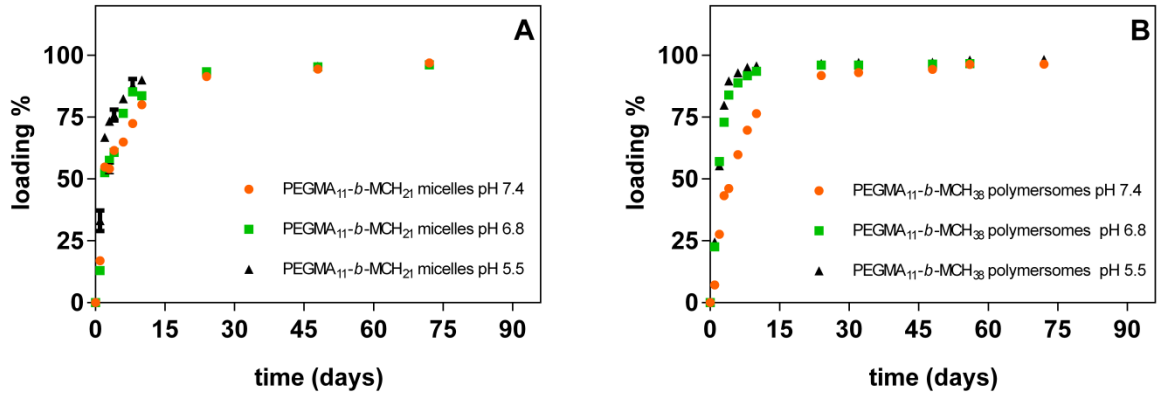

Fig. S8 Release profile of Doxorubicin HCl from (A) pH sensitive $PEGMA_{11}$-*b*-MCH_{21} micelles and (B) $PEGMA_{11}$-*b*-MCH_{38} polymersomes in PBS, 37°C at pH 7.4(■), 6.8 (▲) or 5.5 (•).

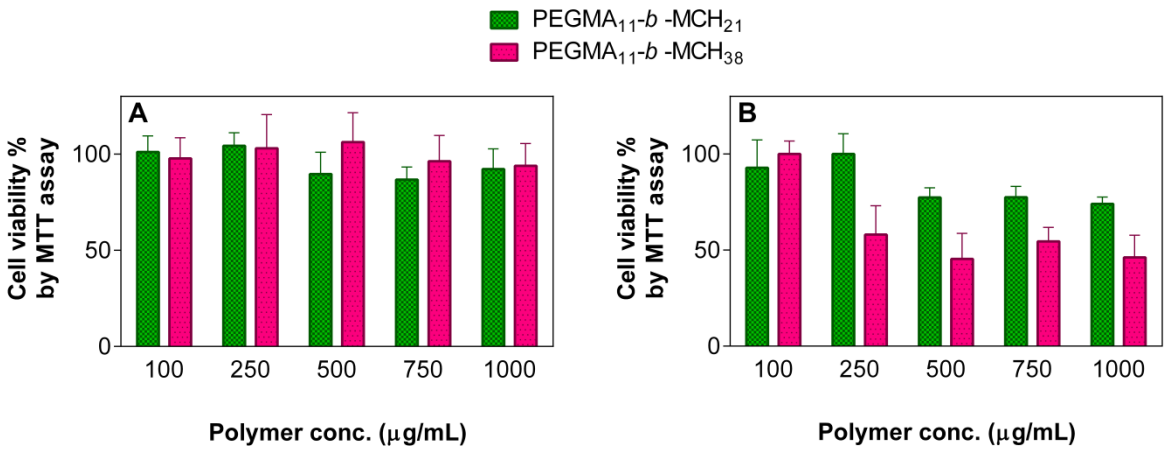

Fig. S9 In vitro cytotoxicity at pH 7.4 of $PEGMA_{11}$-*b*-MCH_{21} micelles (green bars) or $PEGMA_{11}$-*b*-MCH_{38} polymersomes (pink bars) at increasing polymer concentrations (0.1-1 mg/mL). A) 4 h and B) 48 h contact time with MCF-7 cell line.

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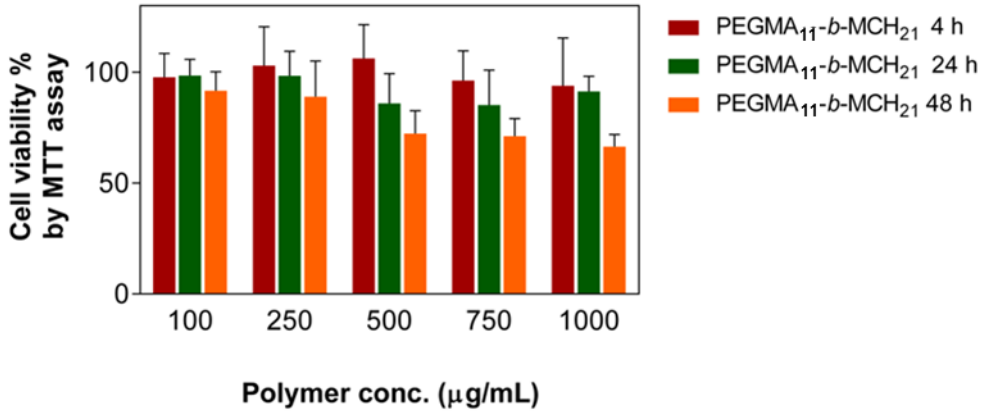

Fig. S10 In vitro cytotoxicity at pH 6.8 of $PEGMA_{11}$-*b*-MCH_{21} blank micelles (0.1-1 mg/mL polymer concentrations). after 4 (red bars), 24 (green bars) and 48 (orange bars) hours of contact time with MCF-7 cell line.

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