Article:
Yu, H, Ingram, N orcid.org/0000-0001-5274-8502, Rowley, JV orcid.org/0000-0001-6646-1676 et al. (2 more authors) (2020) Meticulous Doxorubicin Release from pH-responsive Nanoparticles Entrapped within an Injectable Thermoresponsive Depot. Chemistry – A European Journal, 26 (59). pp. 13352-13358. ISSN 0947-6539

https://doi.org/10.1002/chem.202000389

Supporting Information:

Meticulous Doxorubicin Release from pH-responsive Nanoparticles Entrapped within an Injectable Thermoresponsive Depot.

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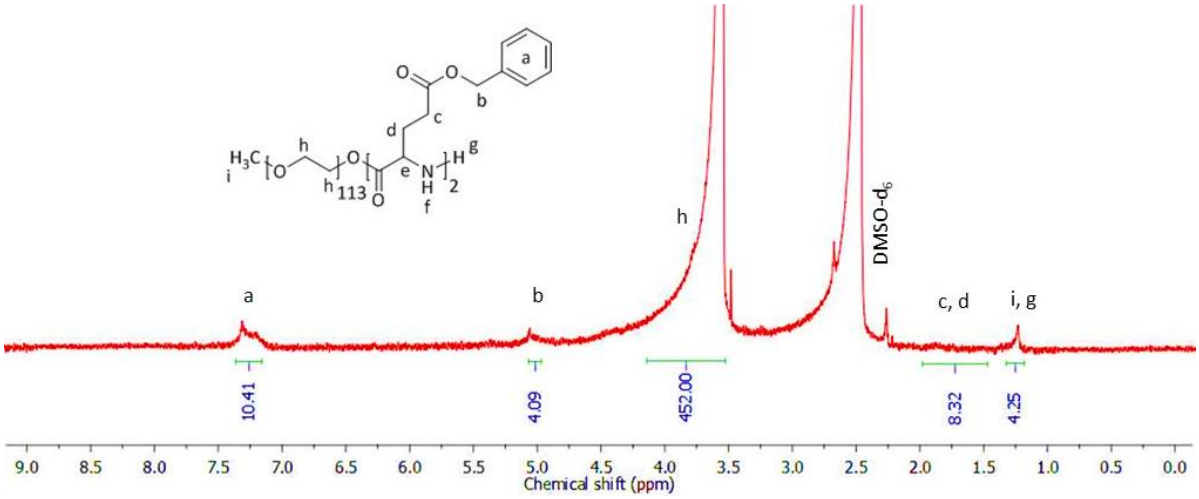

Figure S1. The 500 MHz ^{1}H-NMR of the dialysed PBLG$_2$-*b*-PEG$_{113}$ in DMSO-d$_6$ at 25 °C.

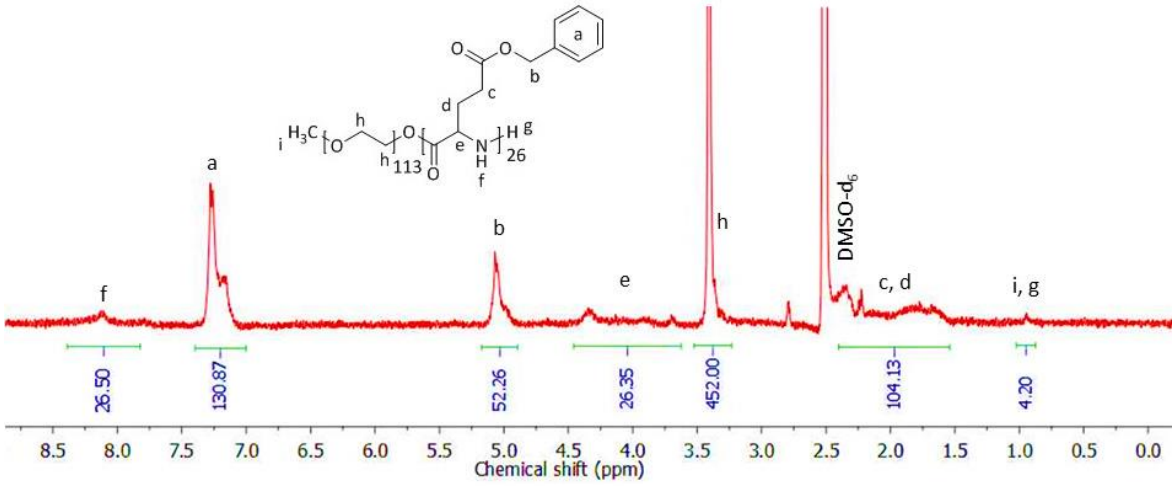

Figure S2. The 500 MHz ^{1}H-NMR of the dialysed PBLG$_{26}$-*b*-PEG$_{113}$ in DMSO-d$_6$ at 25 °C.

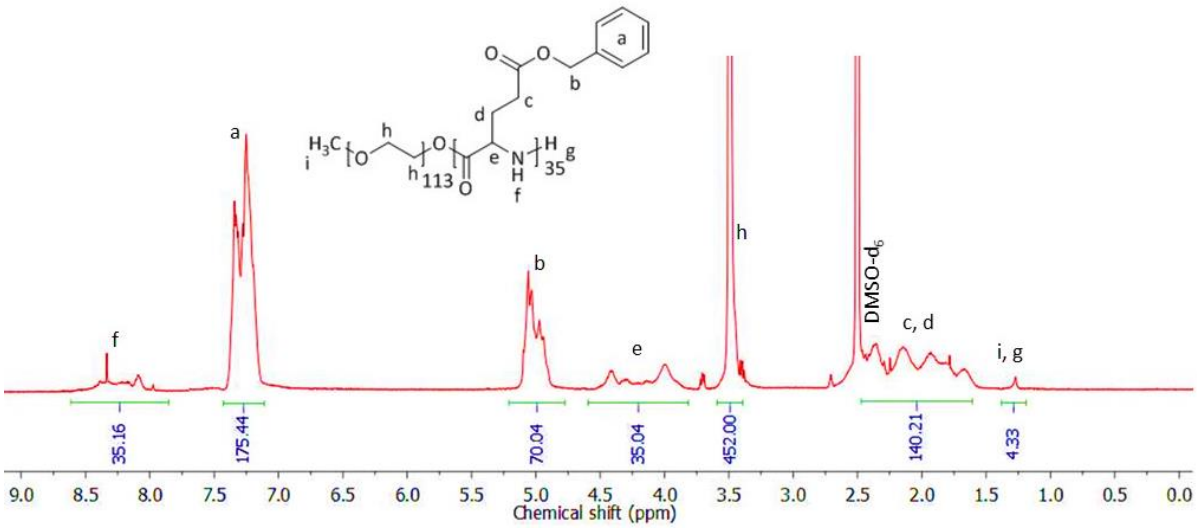

Figure S3. The 500 MHz ^{1}H-NMR of the dialysed PBLG$_{35}$-*b*-PEG$_{113}$ in DMSO-d$_6$ at 25 °C.

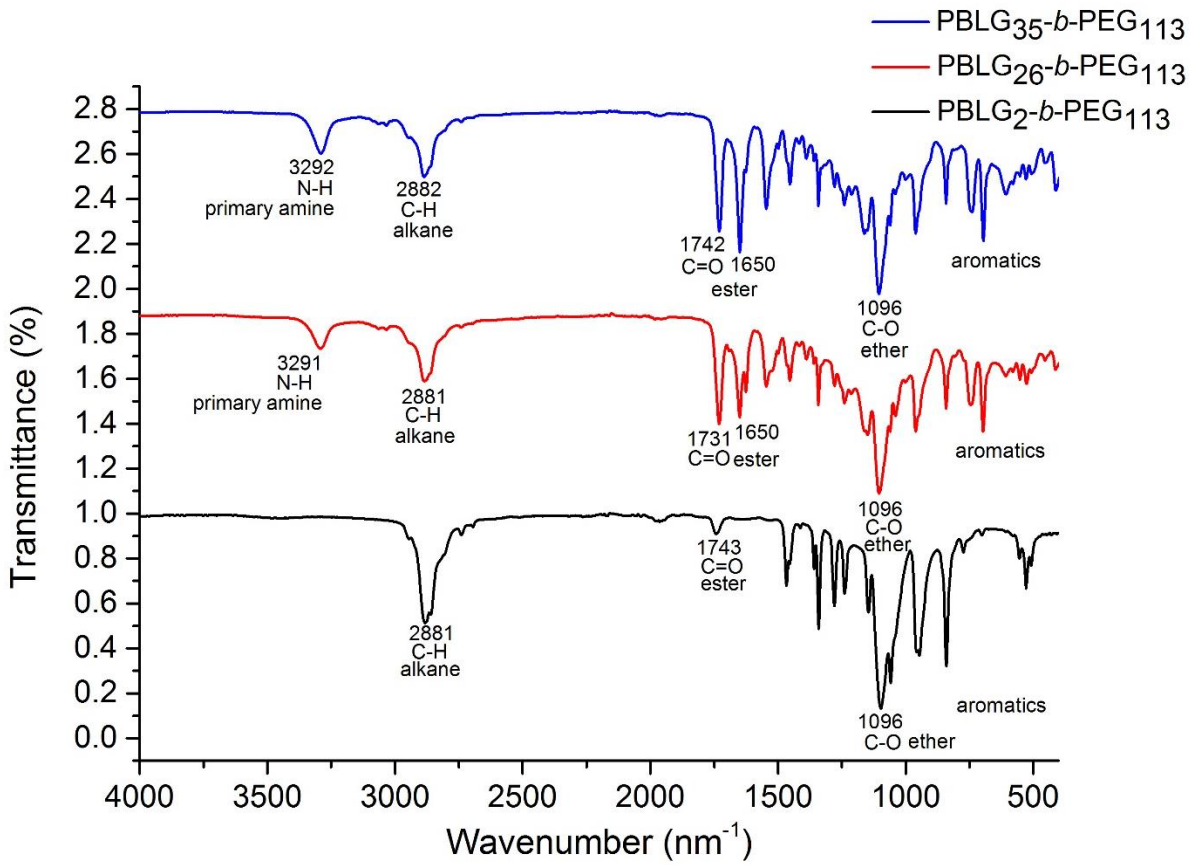

Figure S4. FTIR spectra of dialysed PBLG$_{35}$-*b*-PEG$_{113}$, dialysed PBLG$_{26}$-*b*-PEG$_{113}$ and dialysed PBLG$_{2}$-*b*-PEG$_{113}$.

Table S1. Advanced Polymer Chromatography for polymers created for use as nanoparticles

Polymer	M_n (g.mol^{-1})	Dispersity
PBLG$_2$-*b*-PEG$_{113}$	5317	1.18
PBLG$_{26}$-*b*-PEG$_{113}$	9142	1.13
PBLG$_{35}$-*b*-PEG$_{113}$	12008	1.16

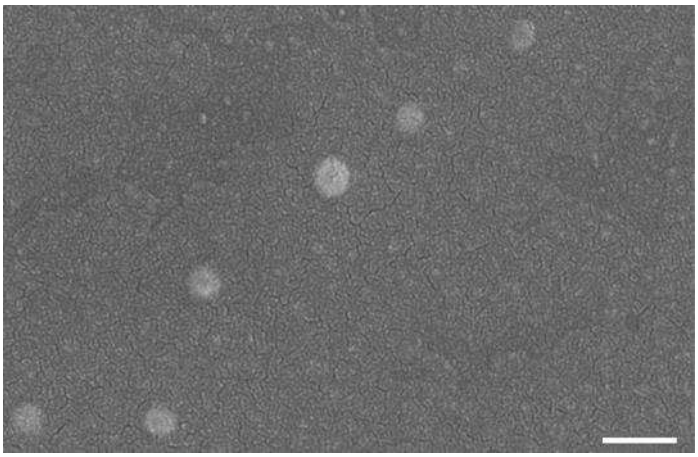

Figure S5. SEM images of PBLG$_{26}$-*b*-PEG$_{113}$; scale bars represent 200 nm.

Dox loading of PBLG$_2$-*b*-PEG$_{113}$ nanoparticles

12.0 mg of Dox was dissolved in 20 μL of trimethylamine and 3.0 mL of chloroform, and stirred for 4 hours in dark (bright red solution). 2.0 mg of PBLG$_2$-*b*-PEG$_{113}$ was dissolved in 1.0 mL of DMF (colourless solution). The polymer solution was then added dropwise into 35.0 mL of PBS buffer or acetate buffer (pH 6.5) (colourless solution). Dox solution was added dropwise into the polymer solution to yield a final volume of 36.0 mL (red solution). PBLG$_2$-*b*-PEG$_{113}$ only has two repeat units of PBLG so more Dox was used for loading in because only a small amount of Dox can be loaded in theoretically.

The Dox concentration for each sample was

$$\frac{12.0\ mg}{36.0\ mL} \approx 0.3333\ mg\ mL^{-1}$$

After three days dialysis for each sample, the concentration for PBLG$_{26}$-*b*-PEG$_{113}$ in PBS buffer was

0.0165 mg mL^{-1}, as measured by UV-vis spectroscopy.

Therefore, the percentage that was encapsulated by the polymer PBLG$_{26}$-*b*-PEG$_{113}$ was,

At pH 7.4, $\frac{0.0165}{0.3333} \times 100\% \approx 4.95\ \%$,

The mass of Dox in the above sample was 0.0165 mg mL^{-1} × 36.0 mL = 0.594 mg

Dox loading of PBLG$_{26}$-*b*-PEG$_{113}$ nanoparticles

1.0 mg of Dox was dissolved in 20 μL of trimethylamine and 3.0 mL of chloroform, and stirred for 4 hours in dark (bright red solution). 2.0 mg of PBLG$_{26}$-*b*-PEG$_{113}$ was dissolved in 1.0 mL of DMF (colourless solution). The polymer solution was then added dropwise into 35.0 mL mL of PBS buffer or acetate buffer (pH 6.5) (colourless solution). Dox solution was added dropwise into the polymer solution to yield a final volume of 36.0 mL (red solution).

The Dox concentration for each sample was

$$\frac{1.0\ mg}{36.0\ mL} \approx 0.0278\ mg\ mL^{-1}$$

After three days dialysis for each sample, the concentration for PBLG$_{26}$-*b*-PEG$_{113}$ in PBS buffer was

0.0122 mg mL^{-1}, as measured by UV-vis spectroscopy.

Therefore, the percentage that was encapsulated by the polymer PBLG$_{26}$-*b*-PEG$_{113}$ was,

At pH 7.4, $\frac{0.0122}{0.0278} \times 100\% \approx 43.9\ \%$,

The mass of Dox in the above sample was 0.0122 mg mL^{-1} × 36.0 mL = 0.4392 mg

All the Dox release samples were prepared in PBS buffer.

Dox release from the PBLG$_2$-*b*-PEG$_{113}$ and PBLG$_{26}$-*b*-PEG$_{113}$ nanoparticles:

2.0 mL of solution containing Dox loaded PBLG$_2$-*b*-PEG$_{113}$ was added to dialysis tubes (MWCO – 2,000 Da). The dialysis tubes were independently immersed in a beaker containing either 70.0 mL PBS buffer solution or pH 6.5 acetate buffer solution. The beakers were covered with aluminium foil and maintained at 37 °C in a water bath before the temperature was increased to 41 °C. 2.0 mL of buffer solution was periodically removed for analysis by UV-vis spectroscopy, before being returned to the beaker. The same procedure was conducted to measure Dox release from PBLG$_{26}$-*b*-PEG$_{113}$ nanoparticles.

Loading of Dox encapsulated PBLG$_{26}$-*b*-PEG$_{113}$ nanoparticles in PHPMA$_{200}$ depots

0.0007 g of lyophilised and Dox encapsulated PBLG$_{26}$-*b*-PEG$_{113}$ nanoparticles were dissolved in 0.8 mL of DMSO. 0.5585 g of PHPMA$_{200}$ was then dissolved in the DMSO solution. 0.05 mL of the mixture was independently injected into 14.0 mL of PBS solution or pH 6.5 acetate buffer solutions, producing Dox loaded PBLG$_{26}$-*b*-PEG$_{113}$ nanoparticles in the PHPMA depot. The same procedure was used to determine the release of free dox from PHPMA depots.

The mass of Dox within each sample was:

$$\frac{0.7\ \text{mg} \times 44\%}{16} = 0.01925\ \text{mg}$$

Dox release from PBLG$_{26}$-*b*-PEG$_{113}$ nanoparticles embedded within the PHPMA$_{200}$ depot:

Nanoparticle-loaded PHPMA$_{200}$ depot (35 mg) was added to either 2.0 mL PBS buffer solution or pH 6.5 acetate buffer solution within separate dialysis tubes (MWCO – 2,000 Da). The dialysis tubes were independently immersed in a beaker containing either 70.0 mL PBS buffer solution or 70.0 mL pH 6.5 acetate buffer solution. The beakers were covered with aluminium foil and maintained either in a water bath at 37 °C or in a fumehood at 20 °C. 2.0 mL of buffer solution was periodically removed for analysis by UV-vis spectroscopy, before being returned to the beaker.

Cytotoxicity assay: MCF-7, MDA-MB-231 cells were obtained from ECACC and MDA-MB-453, MCF10A and HB2 from ATCC. All were cultured in DMEM (Invitrogen) supplemented with 10 % (v/v) FCS (Sigma) at 37 °C in 5 % CO_2 apart from MCF10A which were cultured in DMEM/F12 supplemented with 5 % (v/v) horse serum, 20 ng/mL epidermal growth factor, 0.5 ug/mL hydrocortisone, 10 ug/mL insulin and 100 ng/mL cholera toxin (all Sigma). The vehicle control is tissue culture media containing 0.1% (v/v) DMSO. The cells were certified mycoplasma-free and were STR profiled for verification. $5x10^3$ MCF-7 cells, $1x10^4$ MDA-MB-231 cells, $2x10^4$ MDA-MB-453, 5×10^3 MCF10A and 4×10^3 HB2 cells were plated per well in 96-well plates. 24 hours later, Dox-loaded polymers were added to the cells in quadruplicate at each concentration. Equivalent concentrations of polymer alone were also added to cells alongside free Doxorubicin. Cells were incubated with the polymers and drug for 72 hours before the medium was replaced with 0.5 mg/mL MTT-containing medium. After incubation for 3 hours at 37 °C, the medium was removed and DMSO was added. The absorbance at 620 nm of each well was read on a plate-reader (BertholdTech Mithras). Each entire experiment was carried out in quadruplicate. To obtain an IC_{50} value, the results were fitted with a three-parameter log(inhibitor) vs. response curve or a log(inhibitor) vs normalised response variable slope curve using GraphPad Prism software version 8.0.0.

Table S2. The IC_{50} values obtained for the cell lines tested.

IC_{50} (ug/mL)		
	Dox loaded (R^2)	**Free dox (R^2)**
MCF-7	14.56 (0.8381)	0.5677 (0.8900)
MDA-MB-231	64.83 (0.8814)	2.039 (0.9294)
MDA-MB-453	ambiguous	4.796 (0.9069)
HB2	4.685 (0.8759)	0.2008 (0.9545)
MCF10A	10.36 (0.7111)	0.3907 (0.8199)

Statistical Tests

Cytotoxicity of $PBLG_{26}$-*b*-PEG_{113} (Figure 2). Using fitted curves, a comparison of Log IC_{50} values between the data sets using an extra Sum-of-Squares F test was performed, where alpha = 0.05. The null hypothesis is that the Log IC_{50} value is the same for all the data sets.

F ratio = F-ratio is the ratio of the between group variance to the within group variance. It can be compared to a critical F-ratio, which is determined by rejecting or accepting the null hypothesis, which determines whether there are no differences between groups.

DFn = degrees of freedom for the numerator of the F ratio

DFd = degrees of freedom for the denominator of the F ratio

MCF-7 F(DFn,DFd) = 21.71 (2,110) $p < 0.0001$, the null hypothesis is rejected

MDA-MB-231 F(DFn,DFd) = 18.10 (2,109) $p < 0.0001$, the null hypothesis is rejected

MDA-MB-453 F(DFn,DFd) = 90.23 (2,116) $p < 0.0001$, the null hypothesis is rejected

In conclusion, the difference in IC_{50} values between the polymer, Dox-loaded nanoparticles, and free Dox are significantly different for each cell line.

Table S3. Two-way ANOVA test with Tukey's multiple comparison. All other comparisons were not statistically significantly different.

Cell line	Time Point (h)	Treatment 1	Treatment 2	P value
MDA-MB-231	48	Control	PHPMA/free dox	<0.0001
MDA-MB-231	48	PHPMA	PHPMA/free dox	0.0004
MDA-MB-231	48	PHPMA/NPs	PHPMA/free dox	0.0003
MDA-MB-231	48	PHPMA/Dox NPs	PHPMA/free dox	0.0005
HFFF2	24	Control	PHPMA/free dox	0.0137
HFFF2	48	Control	PHPMA/free dox	0.0005
HFFF2	48	PHPMA	PHPMA/free dox	0.0132
HFFF2	48	PHPMA/NPs	PHPMA/free dox	0.0084
HFFF2	48	PHPMA/Dox NPs	PHPMA/free dox	0.013

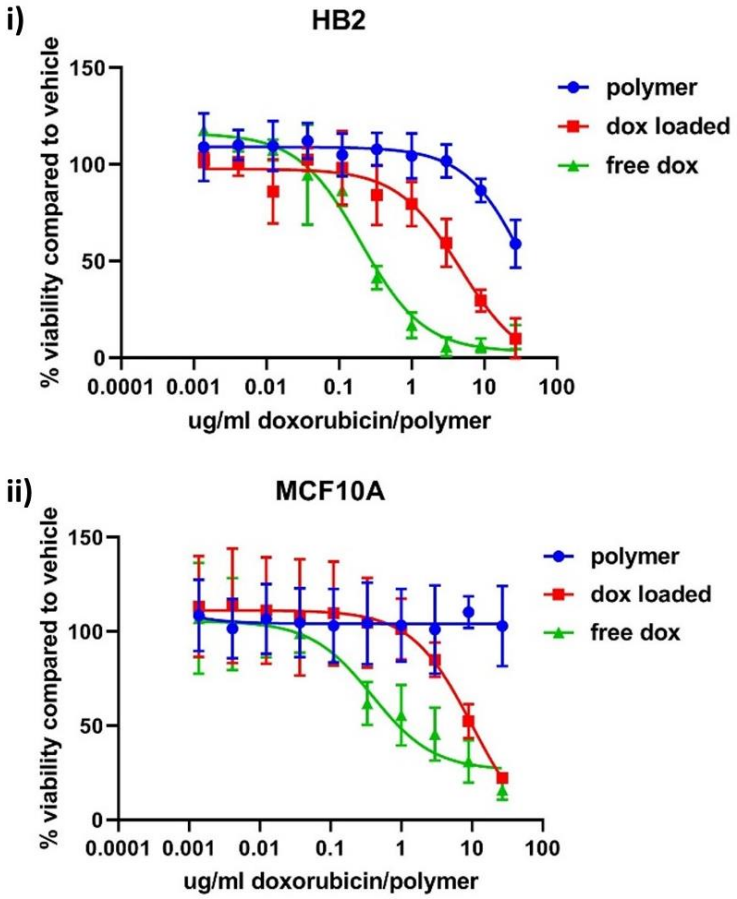

Figure S6. Cytotoxicity of $PBLG_{26}$-*b*-PEG_{113} particles either empty (polymer only) or loaded with doxorubicin (dox loaded) against two normal breast cell lines. Serial dilutions of polymer particles or dox loaded polymer particles were incubated with i) HB2 and ii) MCF10A cell lines.

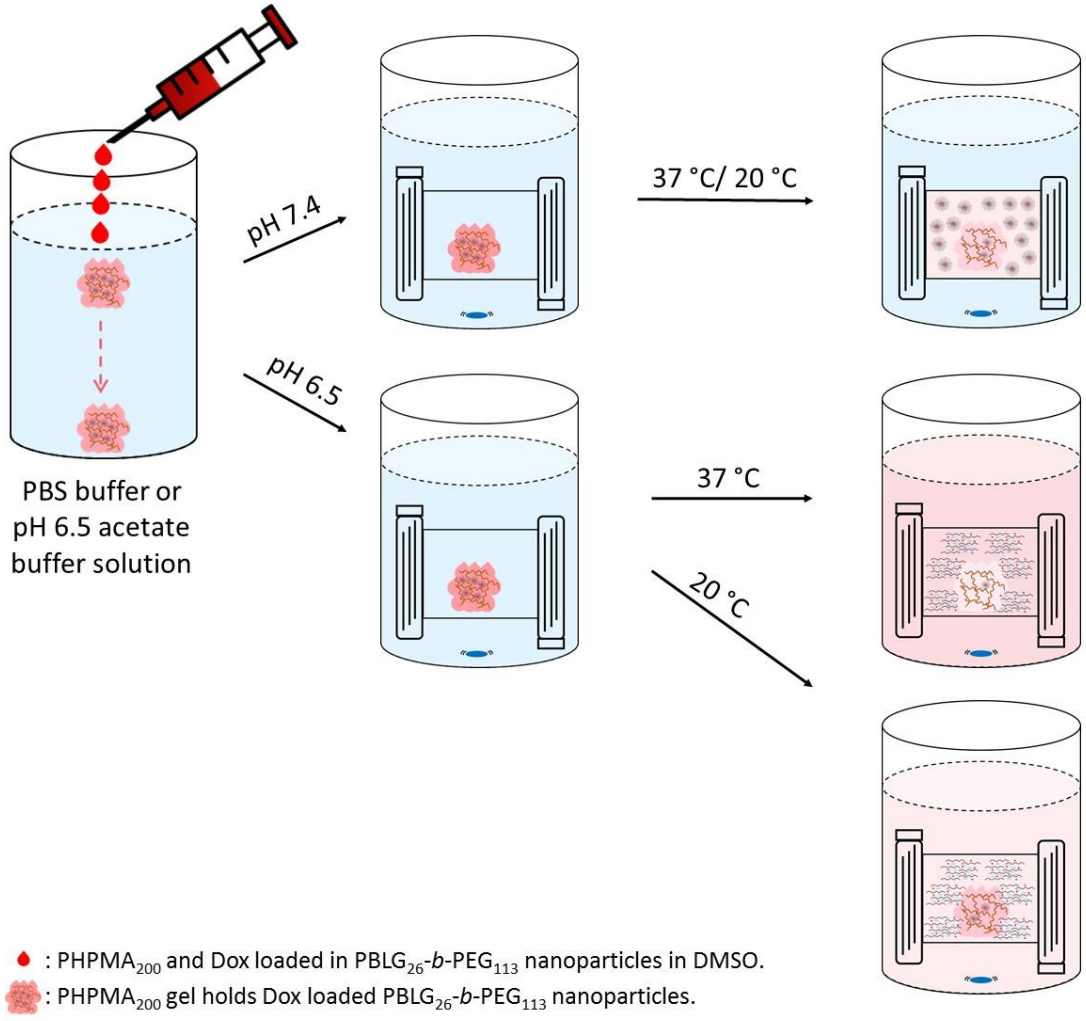

Figure S7. Dox release from $PBLG_{26}$-*b*-PEG_{113} nanoparticles encapsulated in $PHPMA_{200}$ gel in pH 7.4 PBS and pH 6.5 acetate buffer solutions, at 37 °C and at 20 °C.

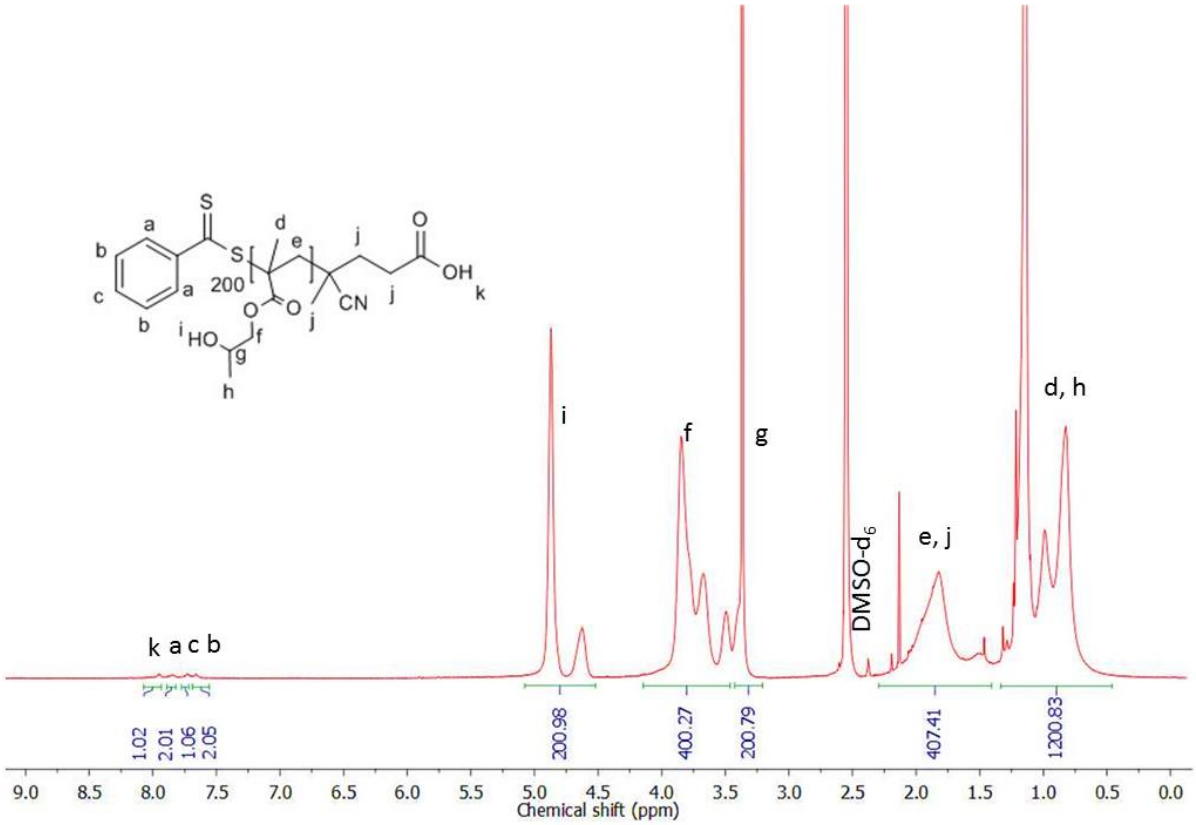

Figure S8. The 500 MHz ^{1}H-NMR of $PHPMA_{200}$ in DMSO-d_6 at 25 °C

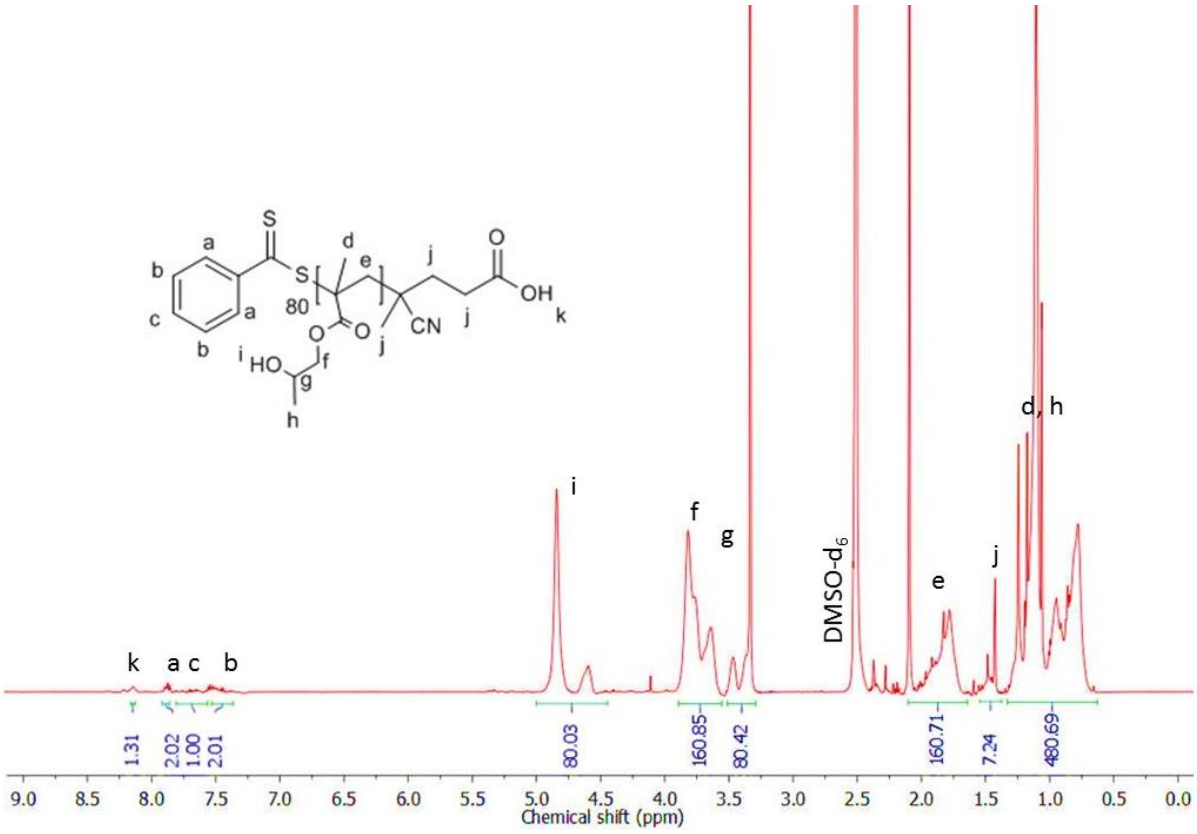

Figure S9. The 500 MHz ^{1}H-NMR of $PHPMA_{80}$ in DMSO-d_6 at 25 °C

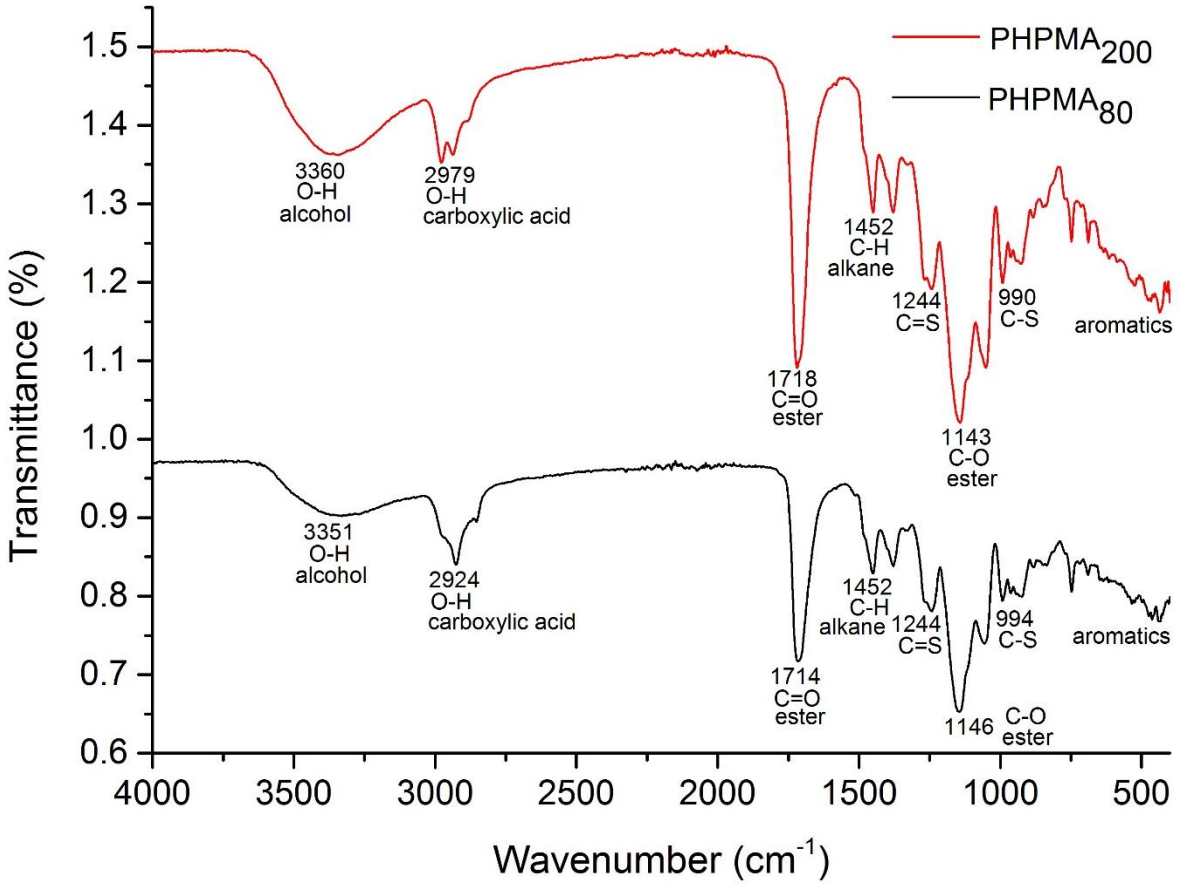

Figure S10. FTIR spectra of $PHPMA_{200}$ and $PHPMA_{80}$.

Table S4. Advanced Polymer Chromatography for the polymers created for use as the depot.

Polymer	Mn (Daltons)	Dispersity
$PHPMA_{80}$	11678	1.15
$PHPMA_{200}$	28455	1.24

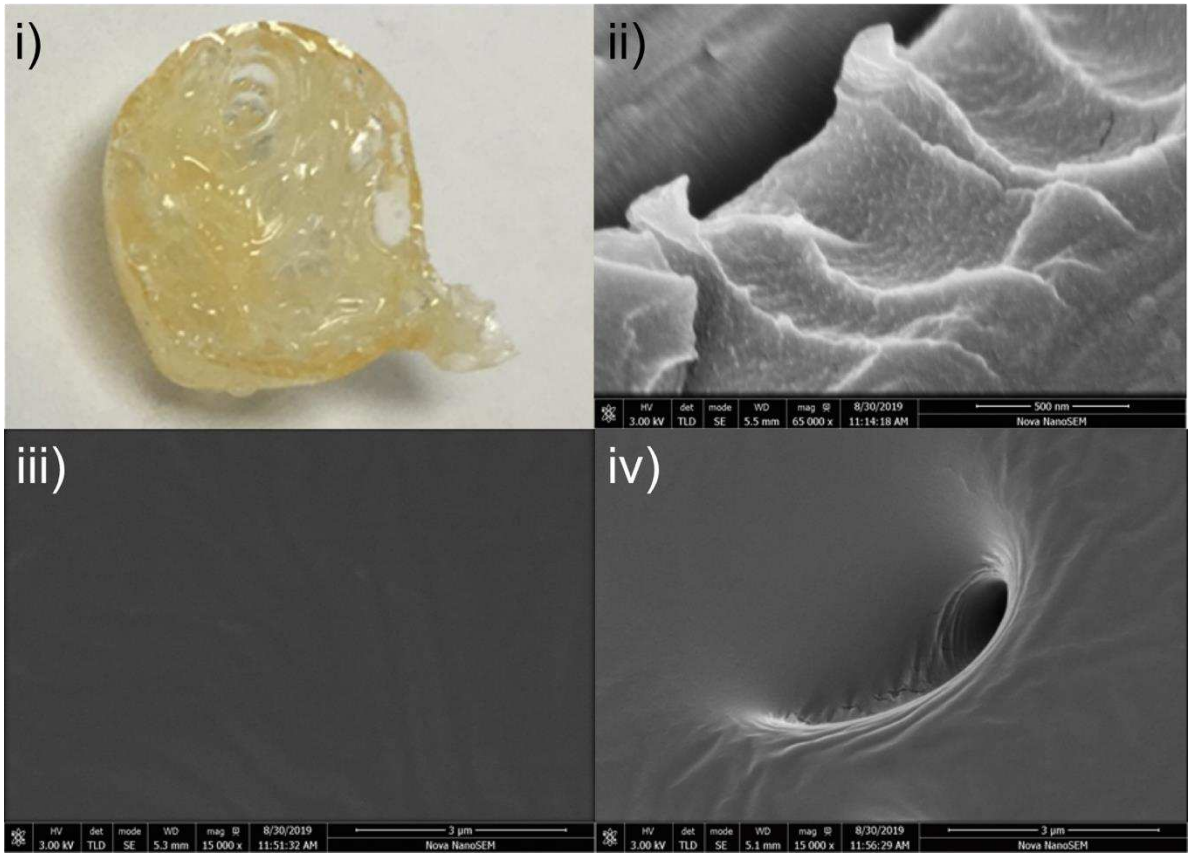

Figure S11. i) Lyophilised PHPMA gels were subject to SEM analysis. ii) The hollow core contained a rough surface. iii) The surface was smooth and largely pristine, iv) although some pores were detected.