Supporting Information for:

Poly(amino acid)-Polyester Graft Copolymer Nanoparticles for the Acid-Mediated Release of Doxorubicin

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Synthesis of *O*-benzyl-L-serine NCA

A previously reported protocol was followed for the synthesis of *O*-benzyl-L-serine NCA [1]. *O*-benzyl-L-serine (5.0 g, 25.61 mmol, 1 eqv) was dissolved in ethyl acetate (60 mL). α-Pinene was added (6.98 g, 51.22 mmol, 2 eqv) and the reaction maintained under nitrogen whilst heated under reflux for 30 minutes. Triphosgene (9.0 g, 30.73 mmol, 1.2 eqv) in ethyl acetate (20 mL) was added dropwise and left to reflux for 5 hours, after which no solid remained in solution. The volume was then reduced *ca.* 30% using rotary evaporation. The solution was then added dropwise to ice-cold hexane (200 mL) to obtain the crude NCA which was then filtered under vacuum. The crude product was then recrystallised twice from ethyl acetate: n-hexane (1:6 v/v), yielding the pure NCA as cream plate-like crystals. Yield: 4.20 g, 85%. FTIR: 3273 cm^{-1} (NH), 2937 cm^{-1} (C-H, Ar), 1825 cm^{-1} (C=O), 1425 cm^{-1} (C=C, Ar), 1259 cm^{-1} (O-C=O). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm) 9.12 (s, 1H, NH), 7.3 - 7.5 (m, 5H, Ar), 4.6 (s, 1H, CH), 4.5 (d, 2H, CH).

Synthesis of 2-hydroxy-3-phenyl Propanoic Acid

L-phenylalanine (5 g, 30.3 mmol, 1 eqv) in 1M H_2SO_4/Acetone (100 mL; 1/1, v/v) was cooled to 0 °C in an ice bath. Sodium nitrite (6.27 g, 90.9 mmol, 3 eqv) in water (10 mL) was added

dropwise over 30 minutes and the reaction maintained at 0 °C for 2 hours before stirring at room temperature for 18 hours. Deionised water (500 mL) was added and the mixture extracted three times with ethyl acetate (300 mL). The organic layers were then combined and washed three times with deionised water (500 mL), followed by saturated brine (500 mL) and dried over magnesium sulphate for one hour. The dried sample was then filtered under vacuum and ethyl acetate was removed under vacuum, yielding the hydroxyl acid as creamy-yellow needles (4.60 g, 91.2%). ESI-MS (189.1, M + Na^+). ^{1}H NMR (500 MHz, $(CD_3)_2CO$, δ, ppm): 7.21 - 7.05 (m, 5H, Ph, *J* = 7.13 Hz), 4.27 - 4.25 (dd, 1H, αC<u>H</u>, *J* = 4.26 Hz), 3.01 - 2.97 (dd, 1H, Ph-C<u>H</u>, *J* = 2.99 Hz) 2.81 - 2.76 (dd, 1H, Ph-C<u>H</u>, *J* = 2.78 Hz).

Synthesis of L-phenylalanine OCA

Activated charcoal (0.34, 16.3 mmol, 1 eqv) was added to a solution of diphosgene (10.28 g, 32.6 mmol, 2 eqv) and 2-hydroxy-3-phenyl propanoic acid (4.6 g, 16.3 mmol, 1 eqv) in anhydrous THF (50 mL) and stirred at room temperature, under nitrogen for 18 hours. Activated charcoal was removed under vacuum and the solvent was reduced by approximately 75% under vacuum. The concentrated solution was then added dropwise to cold anhydrous THF/pentane (1/9, v/v) to crystallise the OCA. The OCA was collected and dried under vacuum, yielding the dry OCA as brown crystals. Yield: 3.51 g, 55%. FTIR: 3080 cm^{-1} (C-H, Ar), 2899 cm^{-1} (C-H), 1850 cm^{-1} (C=O), 1460 cm^{-1} (O-C=O). ESI-MS (206.1, M + NH). ^{1}H NMR (500 MHz, Methanol-d_4, δ, ppm): 7.20 - 7.08 (m, 5H, Ph), 4.27 - 4.25 (ddd, 1H, αC<u>H</u>, *J* = 4.24 Hz), 2.97 - 2.79 (m, 2H, Ph-C<u>H_2</u>).

Synthesis of Poly(*O*-benzyl-L-serine)

O-benzyl-L-serine NCA was dissolved in anhydrous DMF (10 mL) and added to a Schlenk tube which was previously evacuated and purged with nitrogen. Benzylamine (1 equivalent) in anhydrous DMF (2 mL) was injected into the reaction solution and the solution was degassed. The reaction was then stirred under nitrogen for 96 hours, at room temperature. The polymer was precipitated in diethyl ether (250 mL) and left overnight in the freezer to facilitate greater yields. The obtained polymer was isolated *via* centrifugation and dried under vacuum. Yield: 74%. FTIR: 3200 cm^{-1} (C-H, Ar), 2850 cm^{-1} (C-H), 1800 cm^{-1} (C=O). ^{1}H NMR (500 MHz, TFA-d, δ, ppm): 8.51 (s, NH), 7.50 ppm (m, Ar), 4.51 - 5.49 (m, CH).

Deprotection of Poly(*O*-benzyl-L-serine)

Poly(*O*-benzyl-L-serine)$_n$ was dissolved in trifluoroacetic acid (10 mL) and then added to a round bottom flask. HBr in Acetic Acid (33 wt. %, 3 mL) was added dropwise to the polymer solution. The round bottom flask was stoppered loosely and then the solution was stirred at room temperature for 18 hours. The de-protected polymer was precipitated out of solution in cold diethyl ether (200 mL) and then isolated by centrifugation. Yields: 84 wt. % - 95 wt. %. IR: 3185 cm^{-1} (C-H Ar), 2870 cm^{-1} (C-H), 1830 cm^{-1} (C=O). ^{1}H NMR (500 MHz, TFA-d, δ, ppm): 8.51 ppm (s, NH), δ 7.50 (m, Ar (initiator)), 4.50 - 5.52 (m, CH).

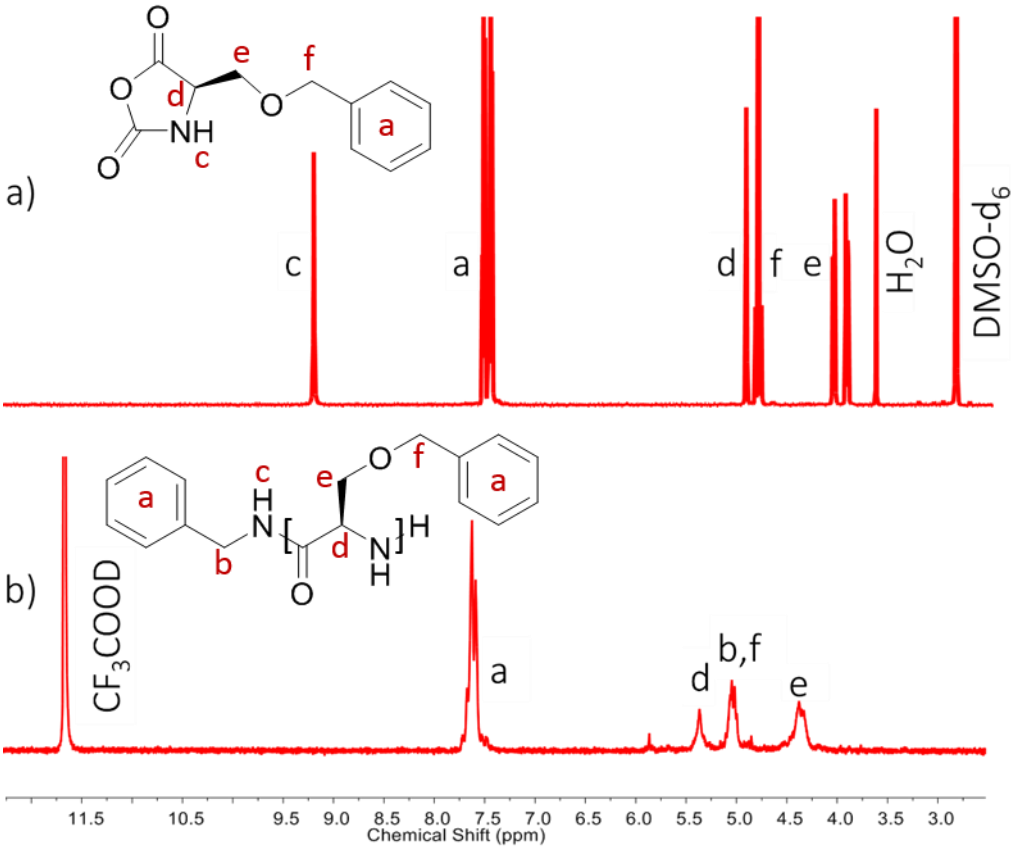

Figure S1. ^{1}H NMR spectrum for *O*-benzyl-L-serine NCA (a) and ^{1}H NMR spectrum for poly(*O*-benzyl-L-serine) (b).

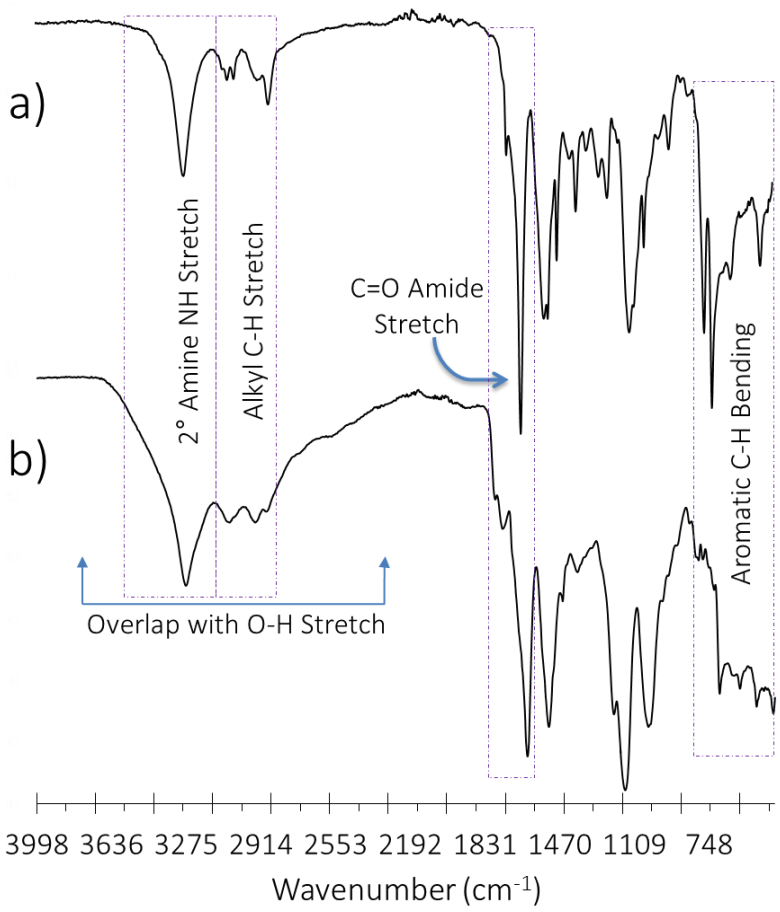

Figure S2. Representative FTIR spectra for poly(*O*-benzyl-L-serine) (a) and polyserine, confirming the emergence of the OH signals and the disappearance of the aromatic C-H bends after the removal of benzyl ester-protecting groups (b).

Table S1. ^{1}H NMR Calculated Masses of four variants of polyserine

Theoretical # of Repeat Units	Theoretical Mass (Da)	Actual Mass (Da)	Actual # of Repeat Units	PDI
20	1847	1792.6	19.6	1.07
15	1412	1715.2	18.7	1.12
10	977	932.6	9.6	1.08

OCA-ROP from Polyserine to yield Poly(serine)-*graft*-(phenylalanine α-hydroxyacid)

Phe-OCA was dissolved in anhydrous DMF (5 mL) and injected into a suba-sealed Schlenk tube which was previously evacuated, nitrogen-purged and equipped with a magnetic stirrer bar. Then, 4-Dimethylaminopyridine (10wt. % of polyserine) was dissolved in anhydrous DMF and injected into the Schlenk tube. Polyserine was dissolved in anhydrous DMF (20 mL) and added to the reaction solution. The reaction was degassed and then stirred under nitrogen for 168 hours. The graft copolymer was precipitated in ice-cold diethyl ether and collected *via* centrifugation and dried under vacuum. Yields: 61 wt. % - 78 wt. %. FTIR: 3150 cm^{-1} (C-H, Ar), 2850 cm^{-1} (C-H), 1850 cm^{-1} (C=O amide), 1765 cm^{-1} (C=O ester). ^{1}H NMR (500 MHz, TFA-d, δ, ppm): 8.52 (s, NH), 7.00 - 7.51 (m, Ar), 4.50 -5.51 (m, CH), 3.09 - 3.52 (m, CH).

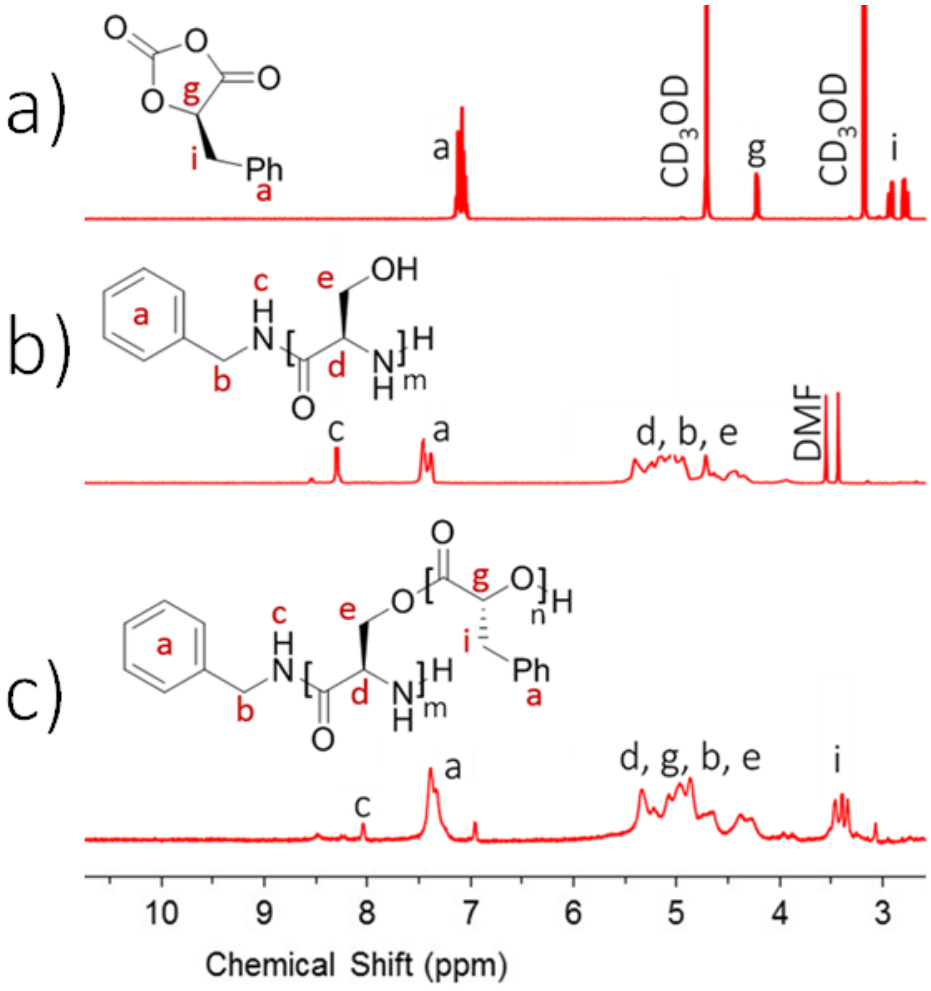

Figure S3. ^{1}H NMR spectrum for L-phenylalanine OCA (a) and representative ^{1}H NMR spectra of polyserine before polyester grafting from the pendant OH groups of polyserine (b) and poly[(serine)-*graft*-(phenylalanine α-hydroxyacid)] after the grafting of hydroxyl-acid repeat units using OCA ROP (c).

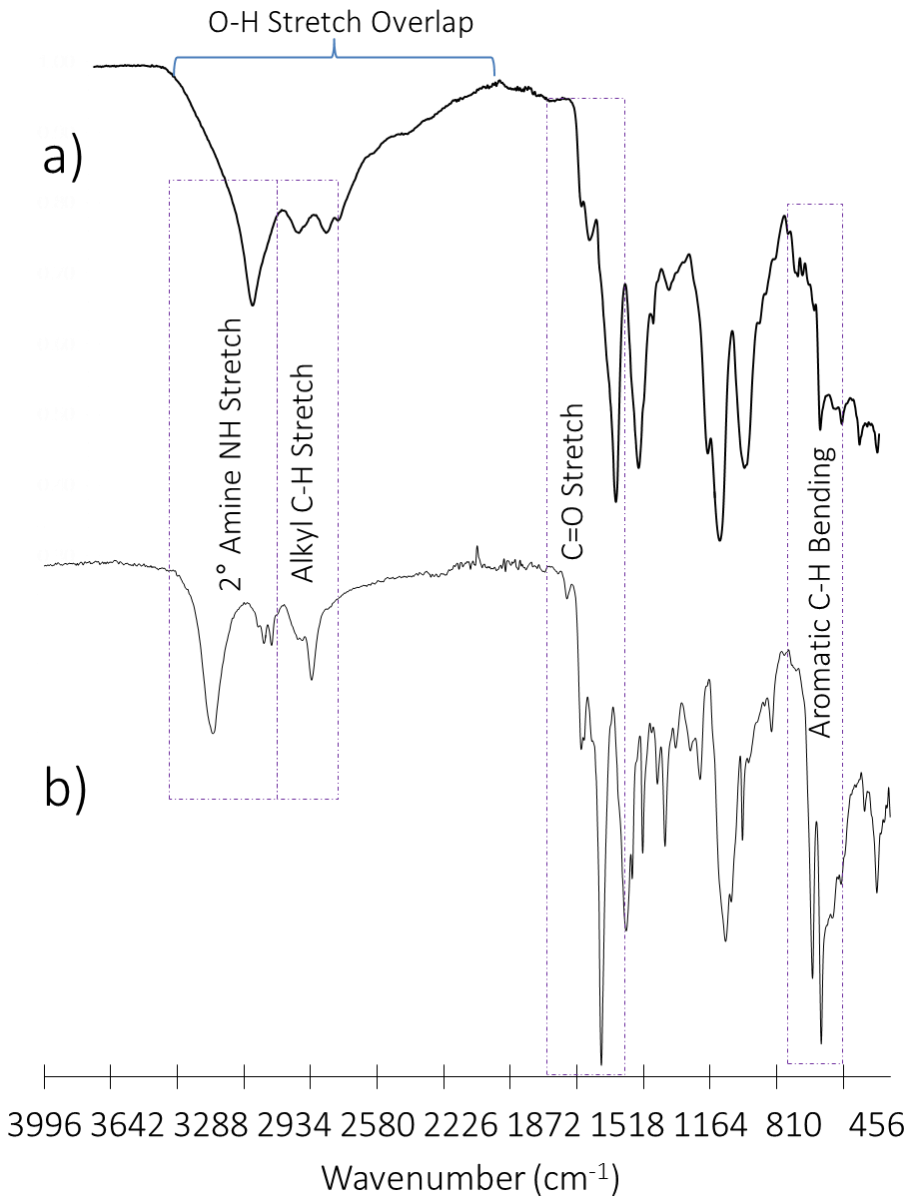

Figure S4. Representative FTIR spectra for polyserine (a) and poly(serine)-*graft*-(phenylalanine α-hydroxyacid), confirming the increase in the intensity of the peaks that represent aromatic C-H bends, and a decrease in the broad OH-signal that previously overlapped with the CH and NH peaks prior to the grafting of phenylalanine hydroxyl-acid groups (b).

Table S2. The poly(phenylalanine α-hydroxyacid) content of the various copolymers

Copolymer	Theoretical AHA Repeat Units	Actual AHA Repeat Units	PDI
Poly[$(Ser)_{19.6}$-*g*-$(Phe\ AHA)_5$]	5	6.0	1.13
Poly[$(Ser)_{19.6}$-*g*-$(Phe\ AHA)_{2.5}$]	2.5	3.1	1.17
Poly[$(Ser)_{18.7}$-*g*-$(Phe\ AHA)_5$]	5	3.0	1.13
Poly[$(Ser)_{18.7}$-*g*-$(Phe\ AHA)_{2.5}$]	2.5	3.0	1.13
Poly[$(Ser)_{9.6}$-*g*-$(Phe\ AHA)_5$]	5	5.2	1.14
Poly[$(Ser)_{9.6}$-*g*-$(Phe\ AHA)_{2.5}$]	2.5	1.8	1.14

Polymer Nanoprecipitation

A previously reported technique was followed for the preparation of nanoparticles (NPs) [2]. Briefly, a solution of poly(serine)-*graft*-(phenylalanine α-hydroxyacid) in DMF (2 mg/mL, 2 mL) was added dropwise to PBS solution (pH 7.4, 10 mL) under vigorous stirring. The dispersion was dialysed against PBS to remove DMF and then the resultant nanoparticles were collected into clean glass vials.

Dynamic Light Scattering (DLS)

DLS analyses were performed on a Malvern Zetasizer Nano ZSP instrument equipped with a 4 mW He-Ne laser operating at a wavelength of 633 nm and an avalanche photodiode (APD) detector. The non-invasive back scatter optic arrangement was used to collect the light scattered, at an angle of 173 °C. Samples were analysed at 37 °C in a disposable 12 mm polystyrene cuvette. Data was processed by cumulative analysis of the experimental correlation function and the diameter of the particles was computed from the diffusion coefficients using Stokes–Einstein's equation. Measurements were carried out in triplicate.

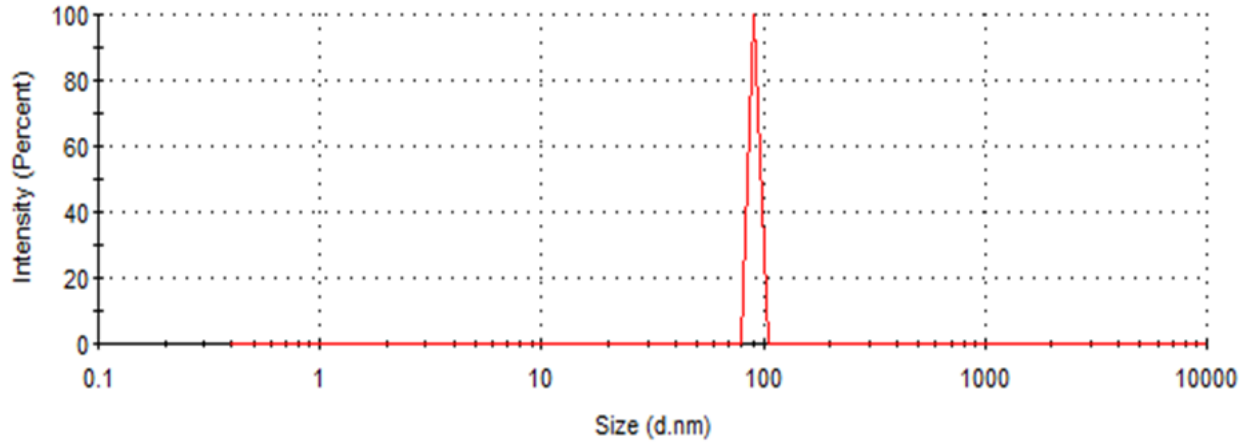

Figure S5. DLS trace depicting the particle size distribution of nanoparticles produced from poly(serine)$_{19.6}$-*graft*-(phenylalanine α-hydroxyacid)$_{6}$

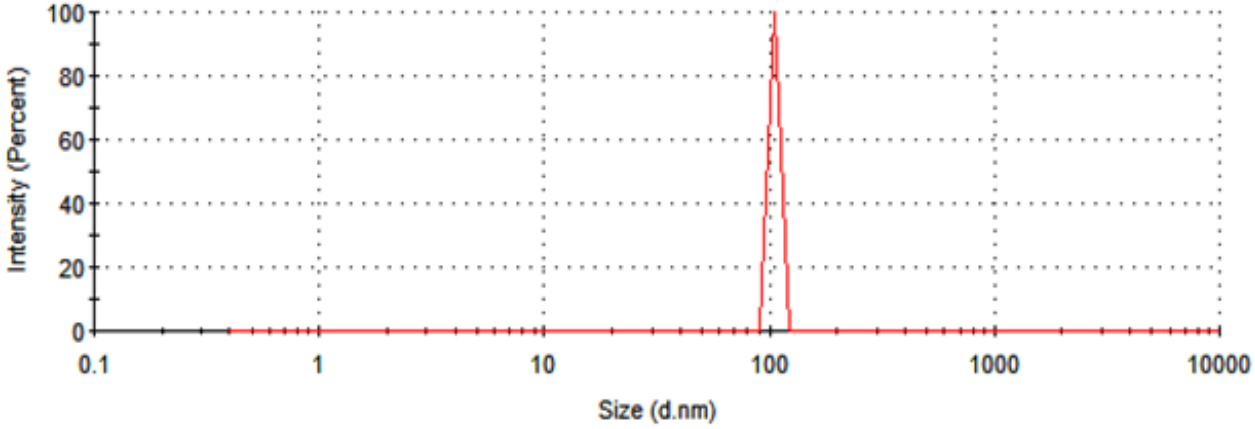

Figure S6. DLS trace depicting the particle size distribution of nanoparticles produced from poly (serine)$_{19.6}$-*graft*-(phenylalanine α-hydroxyacid)$_{3.1}$

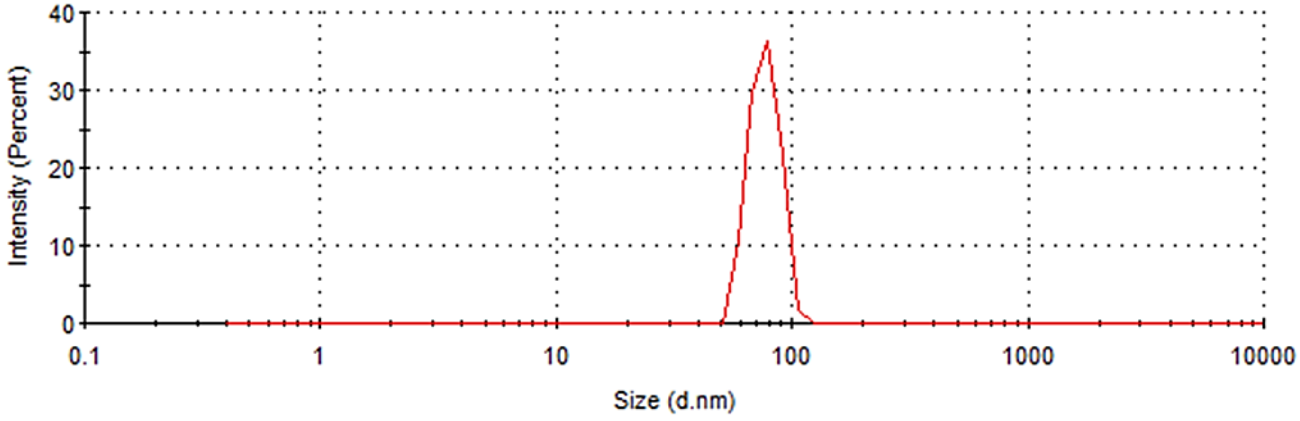

Figure S7. DLS trace depicting the particle size distribution of nanoparticles produced from poly(serine)$_{18.7}$-*graft*-(phenylalanine α-hydroxyacid)$_{3.0}$

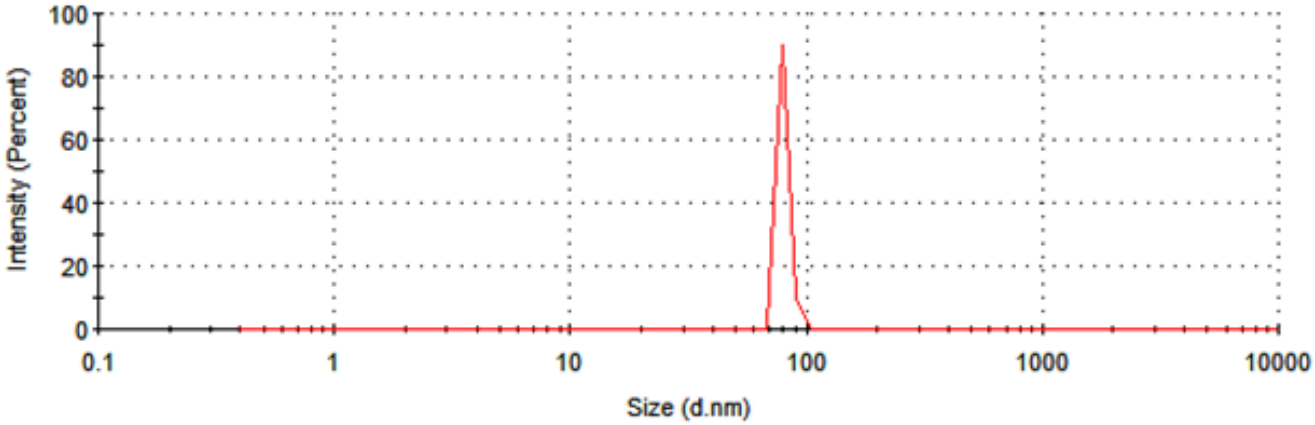

Figure S8. DLS trace depicting the particle size distribution of nanoparticles produced from poly(serine)$_{18.7}$-*graft*-(phenylalanine α-hydroxyacid)$_{3.0}$

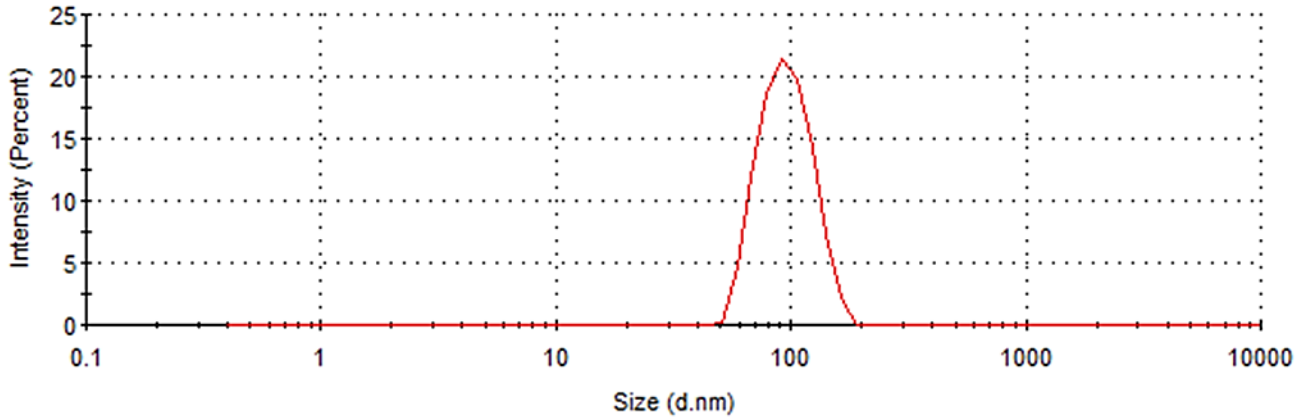

Figure S9. DLS trace depicting the particle size distribution of nanoparticles produced from poly(serine)$_{9.6}$-*graft*-(phenylalanine α-hydroxyacid)$_{5.2}$

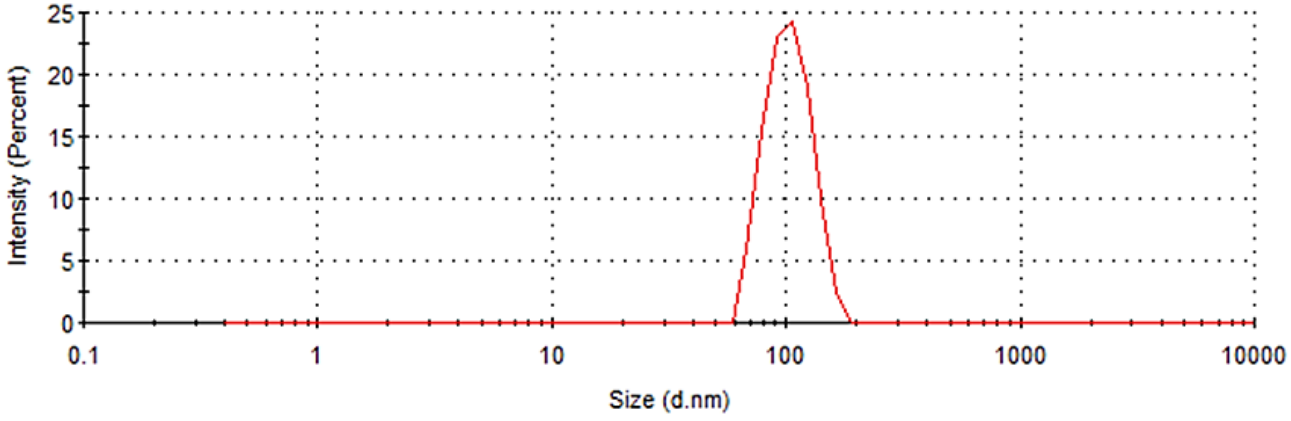

Figure S10. DLS trace depicting the particle size distribution of nanoparticles produced from poly(serine)$_{9.6}$-*graft*-(phenylalanine α-hydroxyacid)$_{1.8}$

Scanning Electron Microscopy (SEM)

Clean, dust-free pipettes were used to independently extract a droplet from the respective nanoparticle solutions. The respective drops were deposited onto SEM glass cover slips and allowed to air-dry in a fume hood. The cover slips were then mounted on individual SEM stubs using conductive tape. The samples were then sputter-coated with a thin layer of gold in a Quorum Q150RS sputter-coater. Particle size and morphology were assessed using a JEOL JSM-6610LV Scanning Electron Microscope, utilising an accelerating voltage that varied between 5 kV and 15 kV and an average working distance of 11 mm.

Advanced Polymer Chromatography (APC)

Advanced Polymer Chromatography (APC) analyses (DMF eluent, 1 g/L LiBr) were carried out using an ACQUITY APC AQ (200Å, 2.5 µm) column packed with bridged ethylene hybrid particles, on a Waters ACQUITY APC system equipped with an ACQUITY refractive index (ACQ-RI) detector. Column temperature was maintained at 40 °C and the flow rate at 0.5 mL/minute. System calibration was done using poly(methyl methacrylate) standards and data was processed using Empower 3 software to provide PDI values.

Ultraviolet-Visible (UV-Vis) Spectrophotometry

UV-Vis Spectrophotometry S4 Absorbance readings (190 - 750 nm) were performed on a dual beam Varian Cary 50 UV0902M112 UV-Vis spectrophotometer (Agilent Technologies) equipped with a xenon pulse lamp and Varian Cary WinUV 3.0 software. Samples were analysed in UV micro quartz cuvettes (10 mm, 700 µL and 1700 µL, black wall).

Preparation of Doxorubicin Free-Base

Doxorubicin was supplied by Sigma-Aldrich in its hydrochloride salt form. In order to appropriately screen the encapsulating and release potential of the prepared nanoparticles it was essential to convert the doxorubicin to its free-base form. In order to achieve this, doxorubicin hydrochloride (3.00 mg, 5.50 µmol, 1 equivalent) was added to a solution of triethylamine (20 uL, 55 µmol, 10 eqv.) in chloroform (3 mL) and left to stir at room temperature for 4 hours [3, 4].

Preparation of Doxorubicin-Loaded Nanoparticles

poly(serine)$_{19.6}$-*graft*-(phenylalanine α-hydroxyacid)$_6$ (6 mg) was dissolved in DMF (3 mL). Doxorubicin was then encapsulated by adopting an established protocol [3, 4]. Briefly, the poly(serine)$_{19.6}$-*graft*-(phenylalanine α-hydroxyacid)$_6$ solution and doxorubicin-free base solution (3 mg/mL, 5.5 µmol) were added simultaneously to vigorously-stirred PBS buffer (pH 7.4, 10 mL). The NPs were dialysed against PBS buffer and obtained *via* lyophilisation.

Doxorubicin encapsulation resulted in a change in colour of the nanoparticle solution from clear to purple (Figure S11, inset). Furthermore, DLS analysis of doxorubicin-loaded nanoparticles showed a slight swelling in size to around 118 nm, further confirming a successful encapsulation.

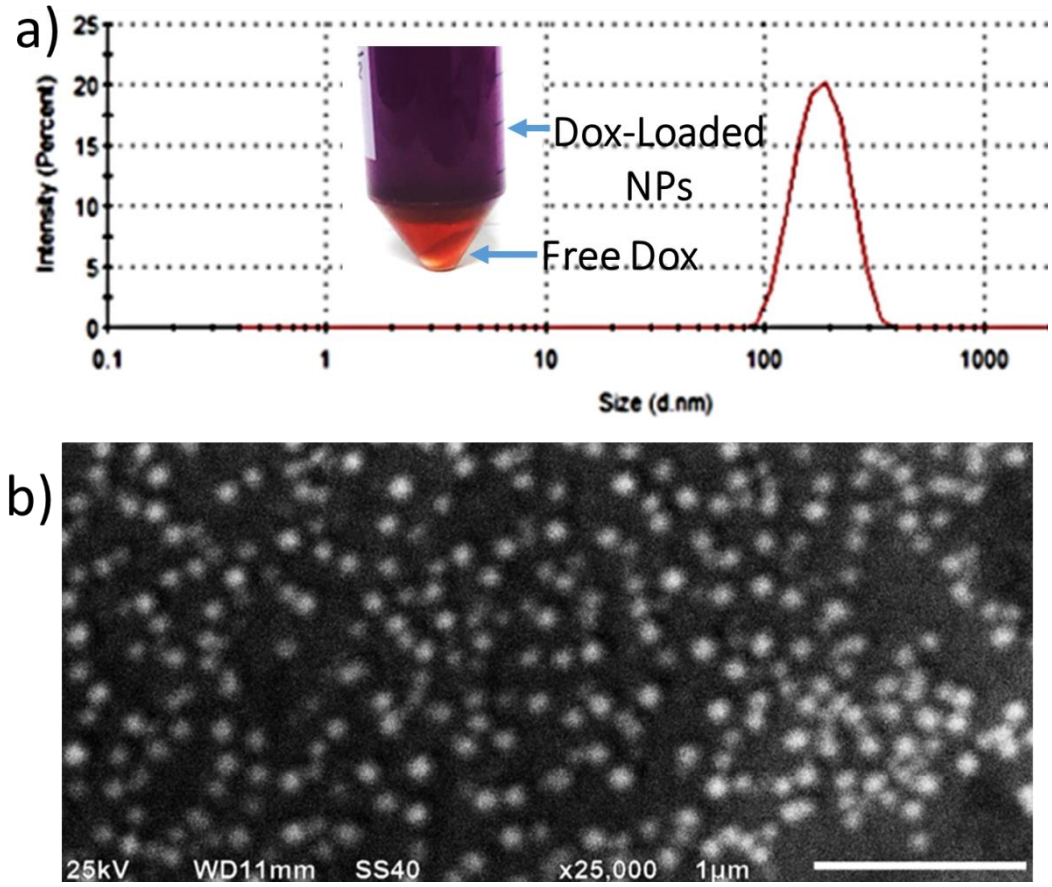

Figure S11. Doxorubicin loading within the nanoparticles resulted in the formation of a solution of purple hue (inset) and an increase in nanoparticle diameter, as revealed by using DLS (a) and SEM (b).

Release of Doxorubicin from poly(serine)$_{19.6}$-*graft*-(phenylalanine α-hydroxyacid)$_6$ Nanoparticles

Doxorubicin-loaded NPs were reconstituted in an acetate buffer solution (pH 5.0) only, and in a PBS buffer solution (pH 7.4) only. NPs that were reconstituted in an acetate buffer solution were collected into a dialysis tubing membrane (2000 Da MWCO) and dialysed against an acetate buffer (pH 5.0). Similarly, NPs that were were reconstituted in PBS buffer were collected into a dialysis tubing membrane (2000 Da MWCO) and dialysed against a PBS buffer solution (pH 7.4). The set-ups were incubated in the dark, under constant agitation, at 37 °C. Then, 1 mL samples were extracted from the dialysate at predetermined time intervals and analysed by UV-Vis spectrophotometry. The concentration of released doxorubicin was evaluated using UV-Vis spectrophotometry at 483 nm. The amount of doxorubicin released at each time point was then quantified using a pre-prepared standard calibration curve obtained by performing measurements of samples containing different concentrations of doxorubicin at 483 nm (Figure S12).

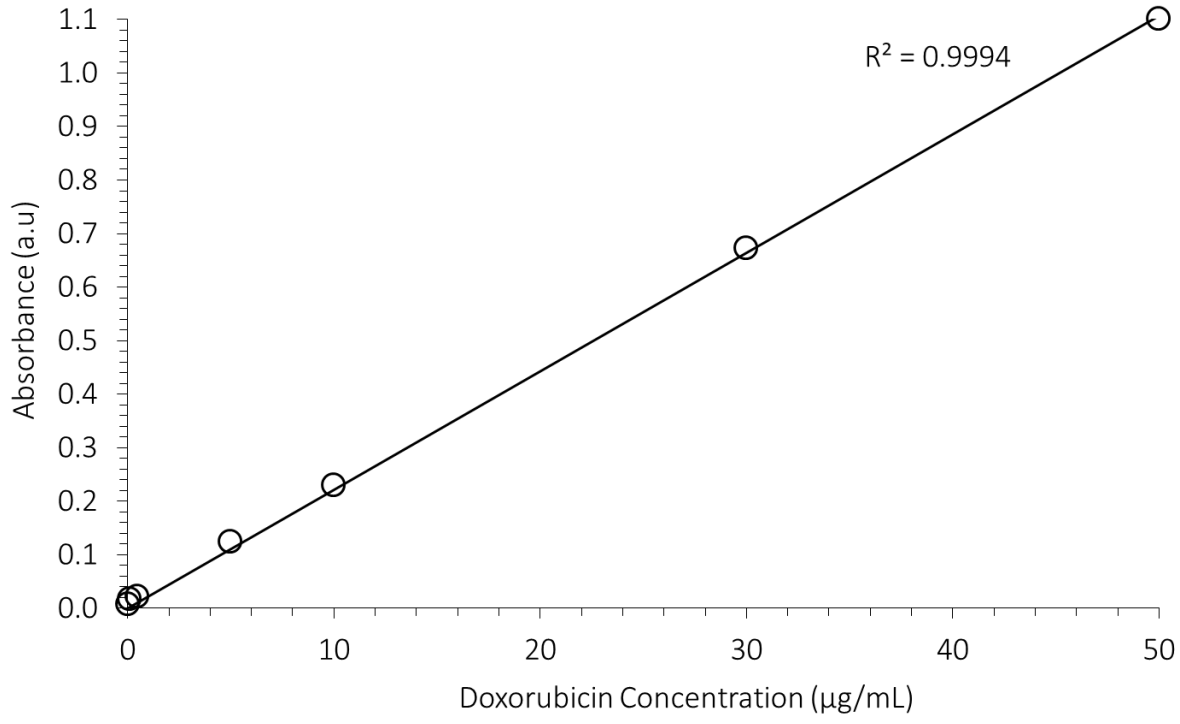

Figure S12. The calibration linear graph that was utilised for computing the quantities of doxorubicin released at respective time intervals during the pH-mediated doxorubicin-release from poly(serine)$_{19.6}$-*graft*-(phenylalanine α-hydroxyacid)$_6$ nanoparticles.

Acid-Mediated Nanoparticle Degradation

0.25 mg/mL (5 mL) solutions of poly(serine)$_{19.6}$-*graft*-(phenylalanine α-hydroxyacid)$_6$ nanoparticles prepared in PBS buffer (pH 7.4) and acetate buffer (pH 5.0) were independently incubated at 37 °C for 48 hours. A sample was isolated from the respective media and the samples were analysed using DLS and SEM.

A sample of poly(serine)$_{19.6}$-*graft*-(phenylalanine α-hydroxyacid)$_6$ nanoparticles was independently incubated for 48 hours in acetate buffer (pH 5.0) before the polymer sample was diluted with DI water and dialysed initially against DI water using regenerated cellulose dialysis tubing (MWCO 1,000 kDa) for four days with multiple solvent changes. The polymer was recovered by lyophilisation and re-dialysed using the same type of dialysis tubing against chloroform for four days with multiple solvent changes. The solvent that remained within the dialysis tubing was removed *in vacuo* to leave the polymer that had remained within the dialysis tubing throughout the dialysis process. The ^{1}H NMR spectrum of this polymer is provided in Figure S13 and confirms polyester cleavage from the poly(amino acid) backbone.

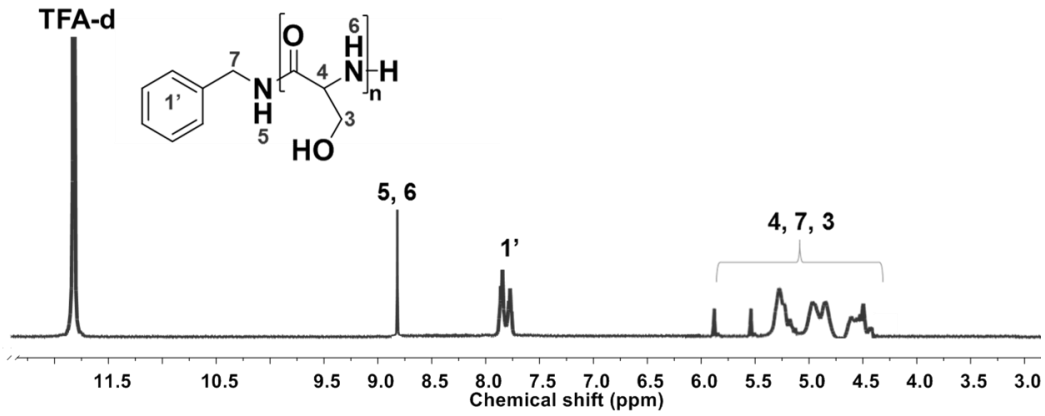

Figure S13. ^{1}H NMR spectrum of this polymer that was too large to pass through the dialysis tubing following incubation of Poly(serine)$_{19.6}$-*graft*-poly(phenylalanine α-hydroxyacid)$_6$ in acidic solution.

Poly(serine)$_{19.6}$-*graft*-poly(phenylalanine α-hydroxyacid)$_6$ steralisation

Both DOX loaded and unloaded poly(serine)$_{19.6}$-*graft*-poly(phenylalanine α-hydroxyacid)$_6$ nano particles were sterilised using 2.5 MRad gamma irradiation with a cell 1000 Elite irradiator.

In vitro Cytotoxicity Assessment of Blank Nanoparticles and Doxorubicin-Loaded Nanoparticles (L929Cells)

L929 Murine fibroblasts cultured using Dulbecco's-modified Eagle's medium (DMEM) in the presence of; fetal calf serum (10% v/v), L-glutamine (2 mM), and penicillin (100 $IU.mL^{-1}$ and streptomycin (100 $mg.mL^{-1}$) at 37 ^{o}C in 5% (v/v) CO_2 in air. Cells were harvested and suspended in cell culture medium. L929 cells were seeded in 24 well plates at a cell density of 10,000 cells/well. After 24 hours both the dox loaded and unloaded nanoparticles were suspended in cell culture medium with 10% Alamar Blue (Invitrogen) and added separately to the cells at concentrations of 0.5 µg/mL, 0.25 µg/mL, 0.125 µg/mL and 0.0125 µg/mL with a negative and positive control (in cell culture medium and in cell culture medium with 40% DMSO, respectively). The experimental set ups were incubated for 12, 24, 48 and 72 hours at 37 ^{o}C in 5% (v/v) CO_2 in air. Cells were 70-80% confluent after 72 hours. Fluorescence readings were measured according to the manufactures guidelines using Thermo Scientific Varioskan Flash. Cell viabilities were then normalised to the negative control.

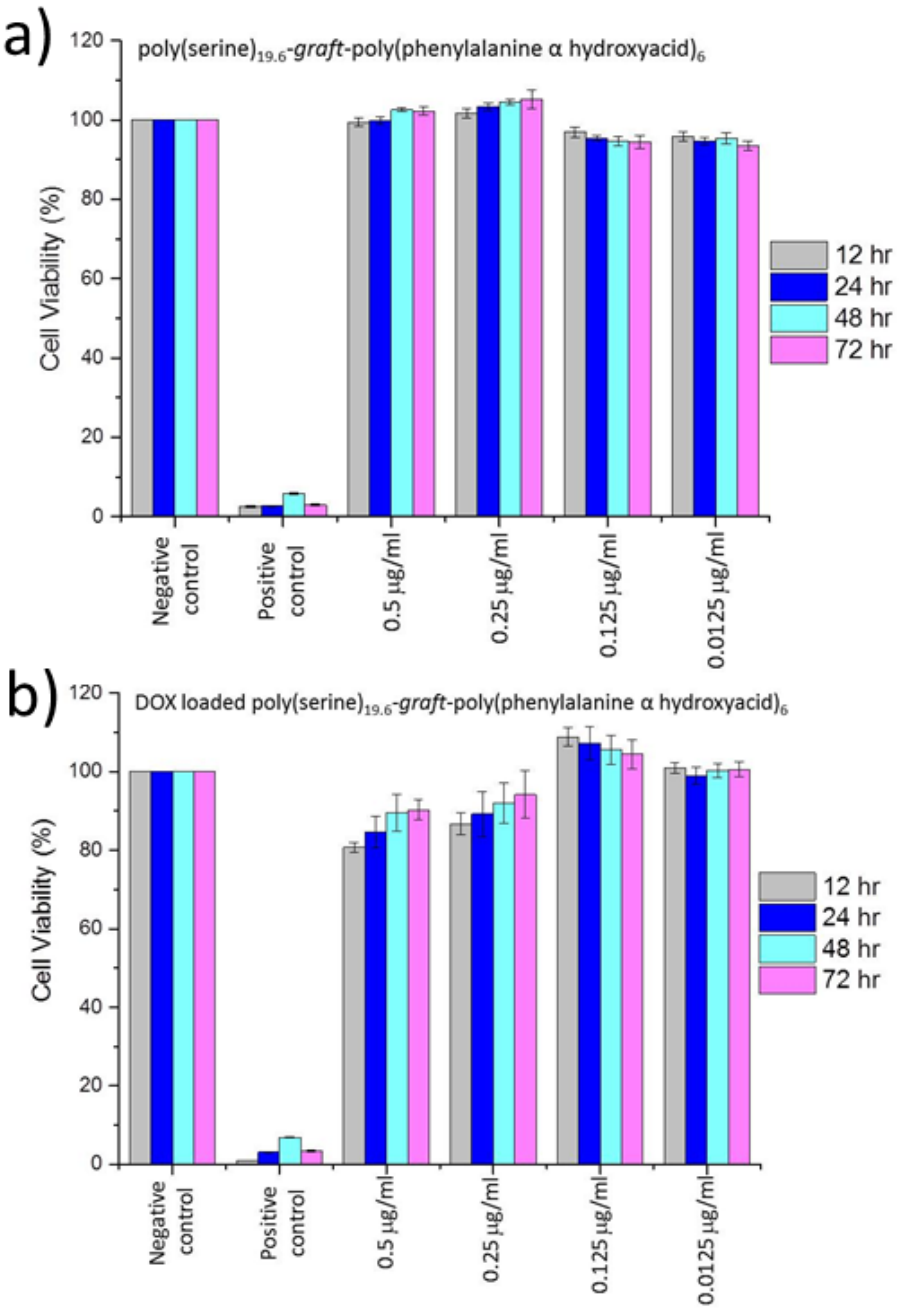

Figure S14. Normalised Cell viability of L929 cells a) blank poly(serine)$_{19.6}$-*graft*-poly(phenylalanine α-hydroxyacid)$_6$ nanoparticles b) Dox loaded poly(serine)$_{19.6}$-*graft*-poly(phenylalanine α-hydroxyacid)$_6$ nanoparticles (n = 3).

In vitro Cytotoxicity Assessment of Blank Nanoparticles and Doxorubicin-Loaded Nanoparticles (T47D Cells)

T47D cells were supplied by the European Collection of Authenticated Cell Cultures (ECACC) and were cultured in DMEM (Invitrogen), supplemented with 10% (v/v) FCS (Sigma) at 37 °C in 5% CO_2. The cells were certified mycoplasma-free and were short tandem, repeat-profiled for

verification. 1000 T47D cells were plated in quadruplicate per well, in 96-well microplates. After 24 hours, the doxorubicin-loaded NPs were added to the cells at varying dox concentrations. In addition, equivalent loadings of blank NPs (polymer) only, and free doxorubicin only were added to cells in independent wells. The experimental sets-up were incubated for 72 hours, at 37 °C, in a humidified 5% CO_2-containing atmosphere. Then, the culture medium was replaced with a 0.5 mg/mL MTT-containing medium and the sets-up were incubated further for 3 hours, at 37 °C. The medium was replaced with DMSO. Then absorbance readings were recorded at 620 nm, using a Mithras LB 940 plate-reader. To obtain IC_{50} values, data were fitted to the four-parameter log (inhibitor) versus response curve in GraphPad Prism (software version 7.02).

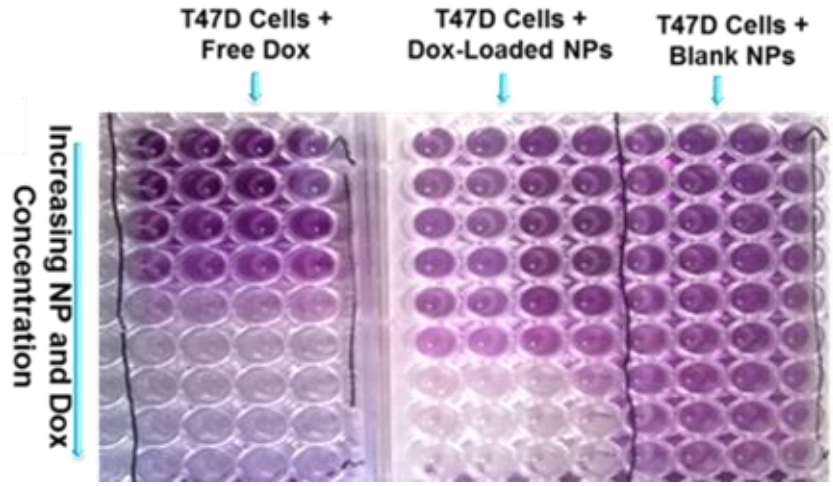

Figure S15. Representative macro images obtained from the colorimetric MTT assays in which the purple colouring indicates viable cells.

References

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4. S. Li; W. Wu; K. Xiu; F. Xu; Z. Li; J. Li, *J. Biomed. Nanotechnol.,* 2014, 10, 1480-1489.

Graphical Abstract