Supporting Information for

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The Formation of Biodegradable Micelles from a Therapeutic Initiator for Enzyme-Mediated Drug Delivery

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Materials

Triphosgene (98%), anhydrous ethyl acetate (99.8%), anhydrous tetrahydrofuran (THF) (≥99.9%), n-hexane (98%), anhydrous *N,N*-dimethylformamide (DMF) (99.8%), diethyl ether (99.8%) and the amino acids used were all acquired from Sigma Aldrich, U. K. α-Pinene (98%) and phosphate buffered saline (PBS) buffer (Dulbecco 'A' tablets) were supplied by Thermo Fisher Scientific Laboratories. Dopamine Hydrochloride (99%) was supplied by Alfa Aesar. HPLC grade water was supplied by VWR International.

Instrumentation

^{1}H NMR spectra were recorded at 500 MHz on a Brucker Avance 500 spectrometer, in DMSO-d_6 at 25 °C, and analysed using the MestreNova® Research Lab software. The melting range of the NCAs were recorded using a Griffin Edulab 12/04/082 melting point apparatus. pH measurements were done using a Thermo Scientific pH/ mV/temperature meter UY-58800-04 and studies of the dopamine release from micelles were performed on an Agilent Infinity 1260 Series HPLC equipped with a C18 column and photodiode array UV detector. Scanning electron microscopy (SEM) studies were carried out using a JEOL JSM-6610LV microscope. Dynamic light scattering studies (DLS) were performed using Zetasizer Nano ZS series instrument equipped with a 4 mW He-Ne laser operated at a wavelength of 633 nm. The non-invasive back scatter optic arrangement was used to collect the light scattered by the particles, at an angle of 173 °C. The samples were analysed in a quartz cuvette, at 25 °C. A Thermo Scientific Ultimate 3000 mass spectrometer equipped with an electrospray ionisation source was utilised to conduct mass spectrometry analysis.

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Synthesis of Amino Acid *N*-Carboxyanhydrides (NCAs)

All NCAs were synthesised by following an established literature procedure.[1] A representative procedure for the synthesis of the NCA of L-phenylalanine is outlined below:

L-phenylalanine (5.00 g, 30.3 mmol) was weighed into a three neck round bottom flask that was previously dried, evacuated and purged with nitrogen. α-pinene (4.02 g, 29.5 mmol) and anhydrous THF (80 mL) were added and the suspension was refluxed for 30 minutes under a constant flow of nitrogen. Triphosgene (3.81 g, 12.8 mmol) was dissolved in 20 mL anhydrous THF and added dropwise to the refluxing reaction mixture *via* a dropping funnel. The reaction was allowed to reflux for 3.5 hours, under nitrogen, until the suspension lacked turbidity. The reaction mixture was then filtered and the filtrate was concentrated by reducing it to a third of its initial volume by THF removal by rotary evaporation. The concentrated solution was added drop-wise to cold n-hexane (300 mL), sealed and left to stand at -18 °C for 5 hours. It was then refrigerated (at -5 °C) for 12 hours to enhance the precipitation of the crude product which was obtained by vacuum filtration as a white crystal residue. The product was recrystallised twice in THF/n-hexane (1:9 v/v), washed several times with fresh n-hexane and dried in a vacuum oven.

L-phenylalanine NCA (Phe NCA)

Yield: 4.33 g, 22.7 mmol, 74.9 %.

Melting range: 94 °C - 96 °C

^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm): 9.08 (s, 1H, NH), 7.33 - 7.17 (m, 5H, ArH, *J* = 80 Hz), 4.79 - 4.77 (t, 1H, α-CH, *J* = 10 Hz), 3.03 - 3.02 (d, 2H, CH_2, *J* = 5 Hz).

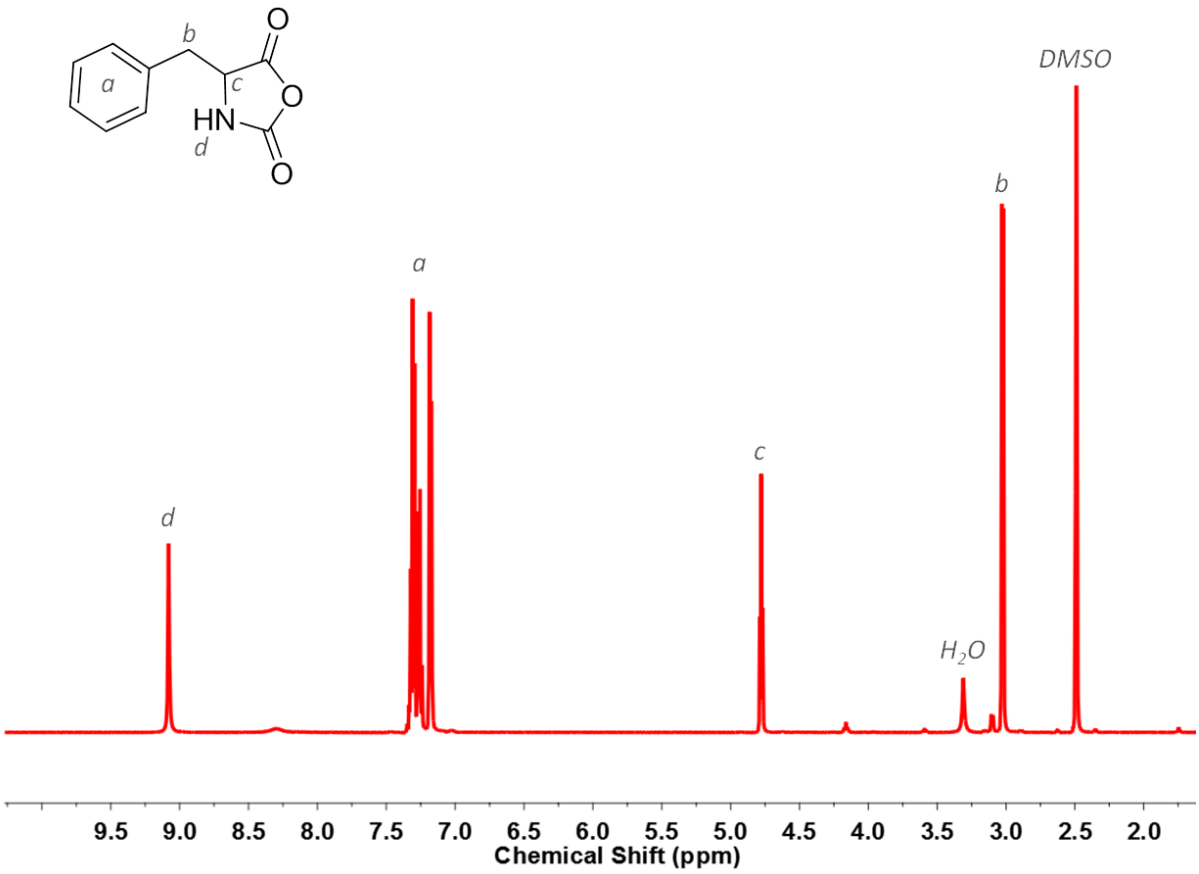

Figure S1. ^{1}H-NMR of Phe NCA in DMSO-d_6.

L-Alanine NCA (Ala NCA)

Yield: 2.28 g, 19.8 mmol, 31.9%.

Melting range: 89 °C - 91 °C

^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm): 8.97 (s, 1H, NH), 4.49 - 4.45 (q, 1H, αCH, *J* = 20 Hz), 1.33 - 1.32 (d, 3H, CH_3, *J* = 5 Hz).

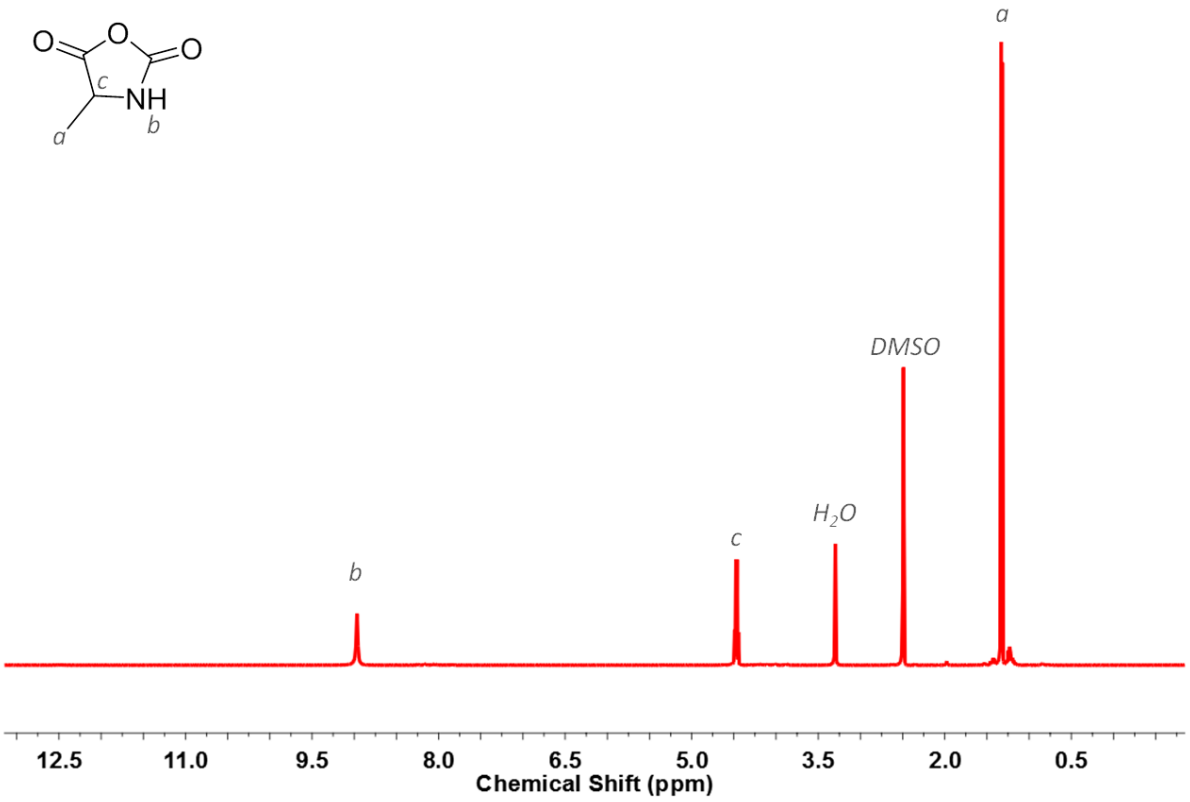

Figure S2. ^{1}H-NMR of Ala NCA in DMSO-d_6.

Synthesis of Sarcosine NCA (Sar NCA)

A synthetic procedure reported previously was used to produce sarcosine NCA.[2]

Yield: 2.94 g 25.8 mmol, 45.1%.

Melting range: 100 °C – 103 °C

^{1}H NMR (500 MHz; DMSO-d6; δ; ppm): *4.21 (s, 2H, CH_2CO), 2.86 (s, 3H, CH_3).*

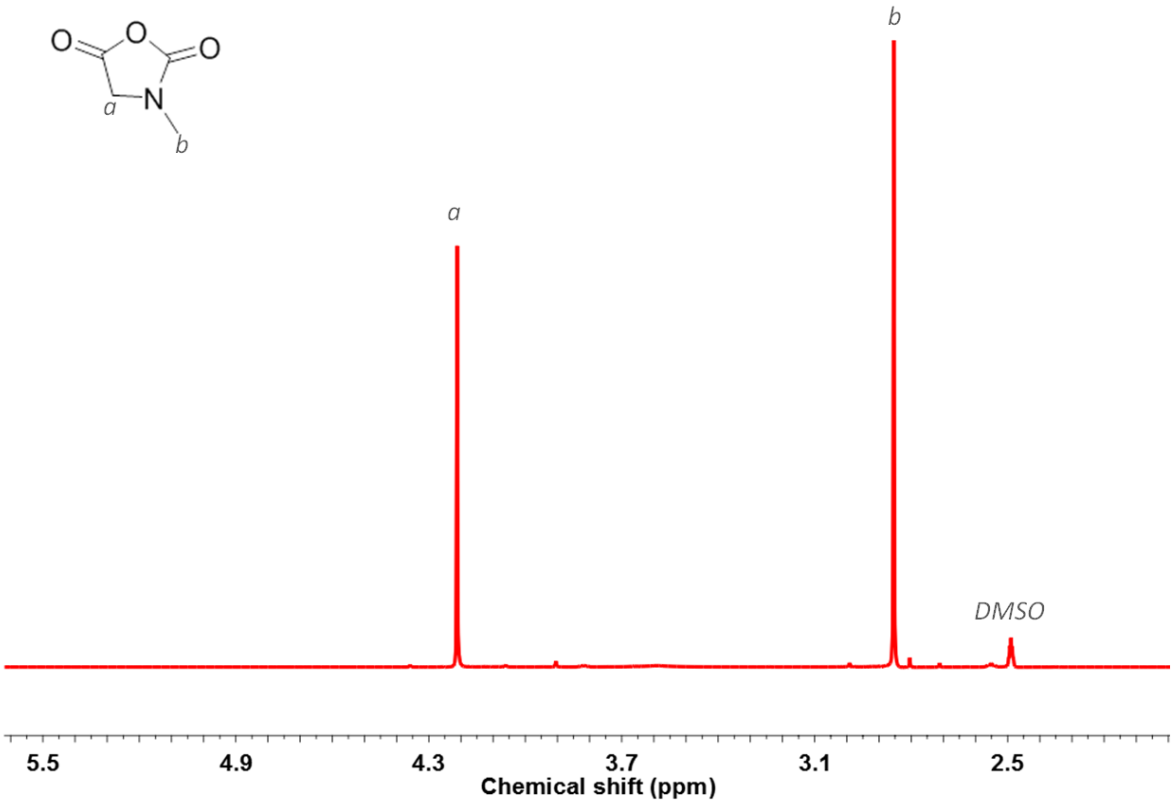

Figure S3. ^{1}H-NMR of Sar NCA in DMSO-d_6

Dopamine-Mediated NCA ROP to Generate Dopamine-(peptide)-*b*-(peptoid) Nanoconjugates

All polymerisations were carried out using the same procedure, which is represented by the dopamine-initiated sequential polymerisation of L-alanine NCA followed by sarcosine NCA:

Ala NCA (60 mg, 0.527 mmol) was dissolved in 20 mL anhydrous DMF. This solution was then injected into a dry and nitrogen-purged Schlenk tube sealed with a rubber septum and equipped with a magnetic stirrer bar. Dopamine hydrochloride (20 mg, 0.106 mmol) was dried under a stream of nitrogen gas for 30 minutes, dissolved in 10 mL anhydrous DMF and injected into the Schlenk tube under a constant flow of nitrogen and with constant stirring.

The reaction was left to stir at room temperature for 96 hours, at which point complete monomer consumption had occurred. After 96 hours, the equivalent of 15 units of the NCA of Sarcosine (180.3 mg, 1.582) mmol) was dissolved in 10 mL anhydrous DMF and injected into the Schlenk tube. The reaction was left to proceed for a further 96 hours under a constant stream of nitrogen.

The polymer was precipitated into cold diethyl ether (300 mL). The obtained precipitate was poured into 50 mL falcon tubes and the polymer isolated from the supernatant by centrifugation (6000 rpm, 20 min, -5 °C). The obtained polymer was then suspended in diethyl ether (45 mL) and isolated by centrifugation. This cycle was repeated three times to enhance the purity of the polymer. The obtained polymer was dried in a vacuum oven maintained at 40 °C for 24 hours and then lyophilised for 48 hours.

The progress of the ROP of Ala NCA and Phe NCA was monitored by ^{1}H NMR in order to determine the point at which the monomer was exhausted before grafting the sarcosine block. 3 mL aliquots were extracted at selected time intervals and precipitated in cold diethyl ether, centrifuged to isolate the polymer and dried for 24 hours in a vacuum oven. The 500 MHz ^{1}H NMR spectra were normalised to the dopamine aromatic ring. Monomer conversions were then determined by comparing the integral the dopamine aromatic ring with the integral of the α-proton for L-alanine and the integral of the aromatic ring of L-phenylalanine on the 500 MHz ^{1}H NMR spectra of the respective oligomers.

Dop-(Ala)$_5$-(Sar)$_{15}$

Yield: 121 mg, 72.6 wt%.

^{1}H-NMR (500 MHz, DMSO-d$_6$, δ, ppm): 8.25 (s, 1H, $(CH_2)_2NH$), 8.15 - 7.80 (m, 9H, NH), 6.65 - 6.42 (m, 3H, Ar-H), 4.37 (m, 10H, αCH), 4.30 – 4.23 (t, 27.5H, CH_2CO), 4.23 – 4.13 (m, 44.5H, $CO(NH)CH_3$), 1.23 – 1.10 (t, 15.82H, $αCH(CH_3)$).

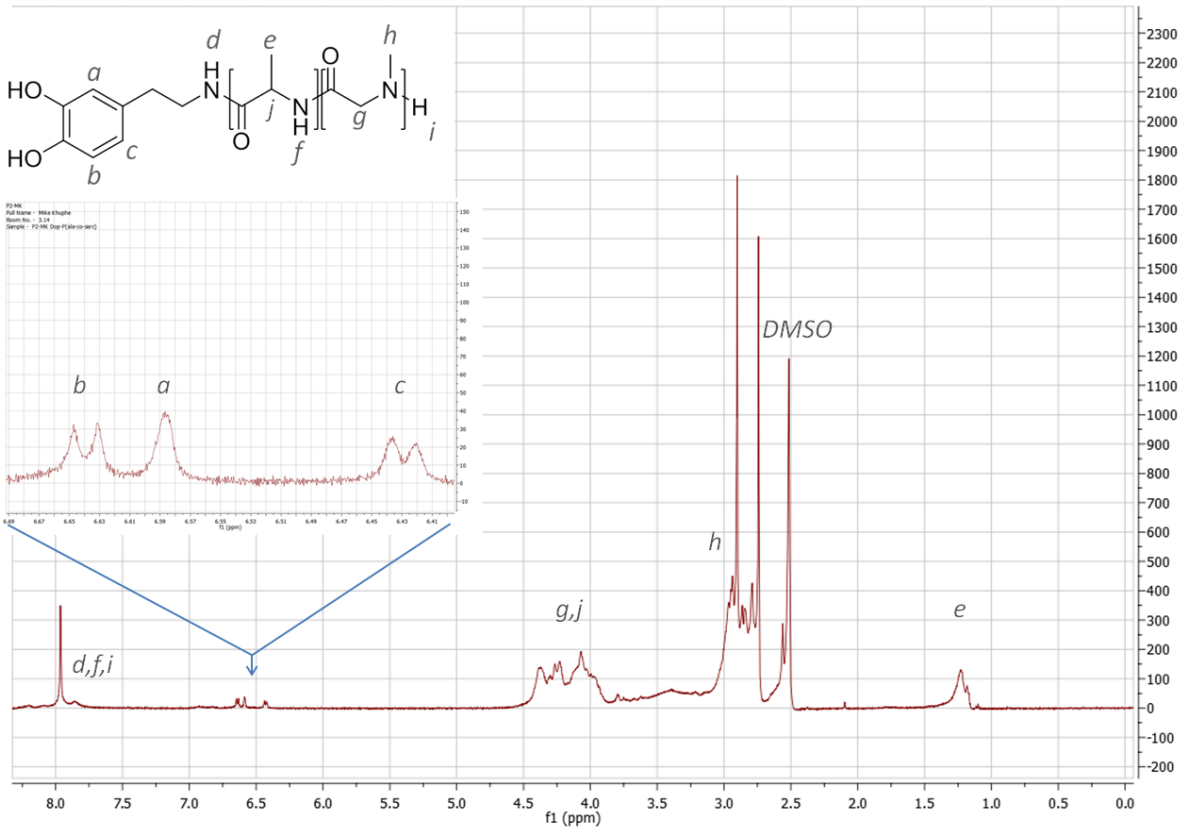

Figure S4: ^{1}H NMR spectrum of Dop-(Ala)-(Sar) in DMSO-*d*$_6$.

Monomer conversion of Ala NCA was monitored to determine reaction completion (Figure S5).

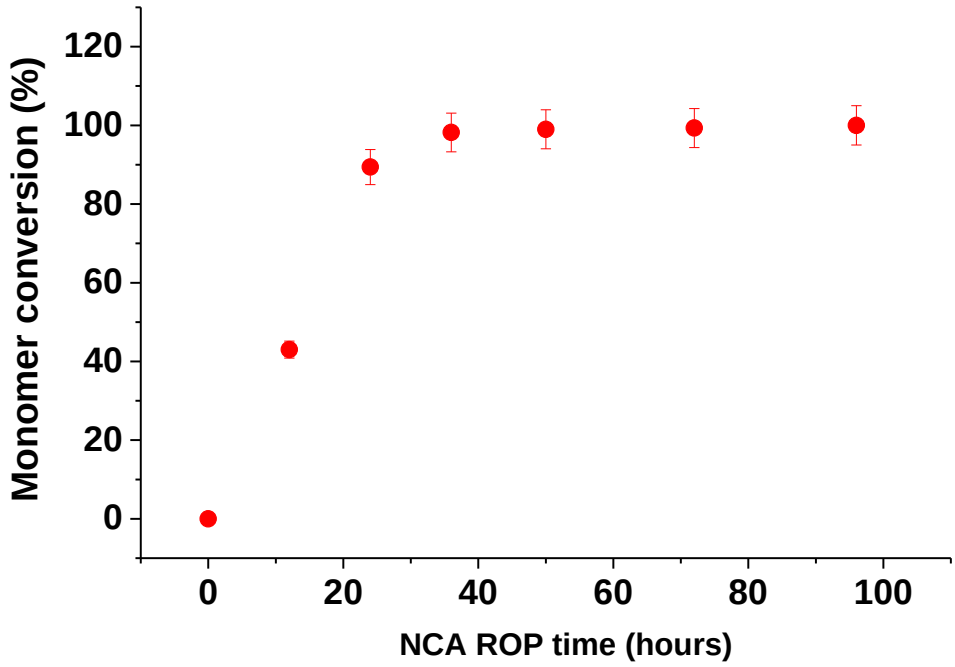

Figure S5: The kinetics of the dopamine-initiated ROP of Ala NCA.

Dop-(Phe)$_4$-(Sar)$_{18}$

Yield: 198 mg, 89.2 wt%.

^{1}H-NMR (500 MHz, DMSO-d$_6$, δ, ppm): 7.97 (s, 1.51H, ArCH$_2$CH$_2$NH), 7.25 - 7.23 (m, 19.65H, ArH), 6.63 – 6.42 (m, 3H, ArH), 4.08 - 4.01 (m, 8.10H, αCH), 3.42 – 3.38 (q, 36.67H, ArCH$_2$), 3.03 - 2.97 (m, 4.88H, ArCH$_2$CH$_2$), 2.10 (s, 47.32H, ArCH$_2$CH), 1.12 - 1.09 (t, 52.86H, CH$_2$NCH$_3$).

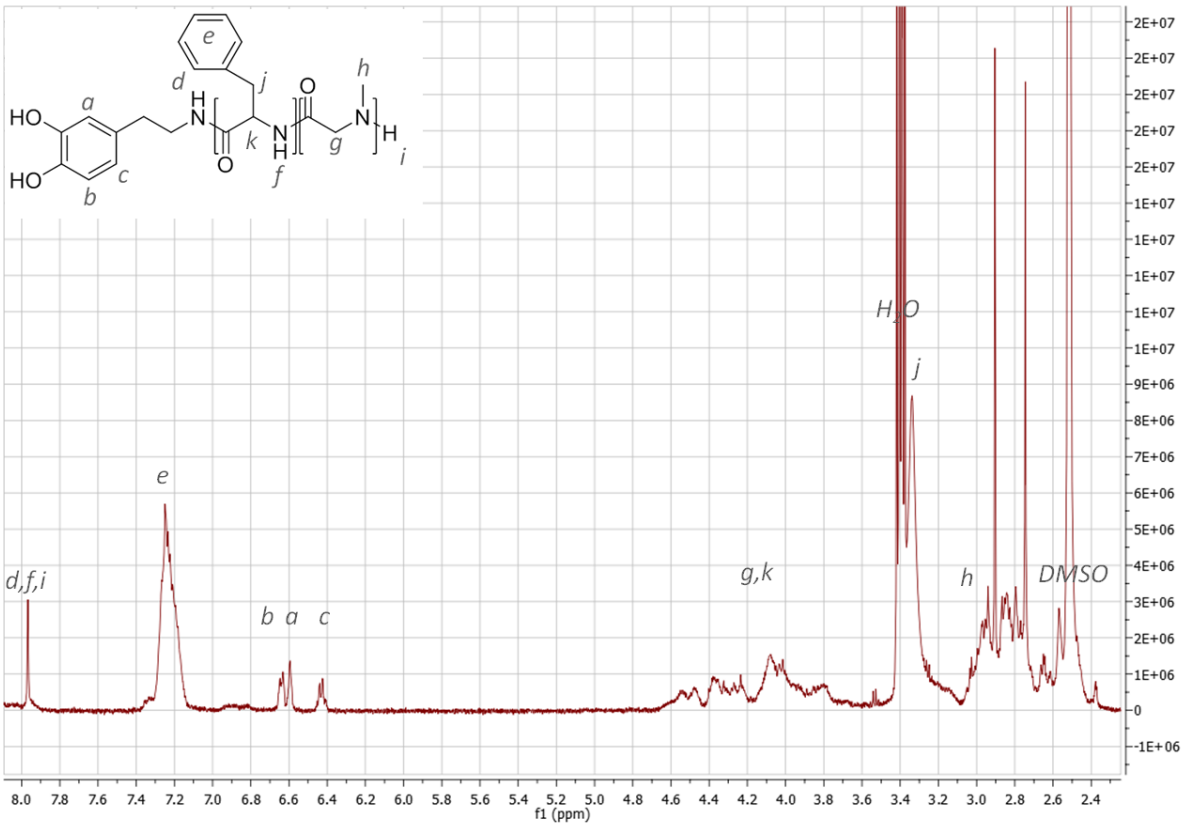

Figure S6. ^{1}H NMR spectrum of Dop-(Phe)-(Sar) in DMSO-d_6.

Monomer conversion of Phe NCA was monitored to determine reaction completion (Figure S7).

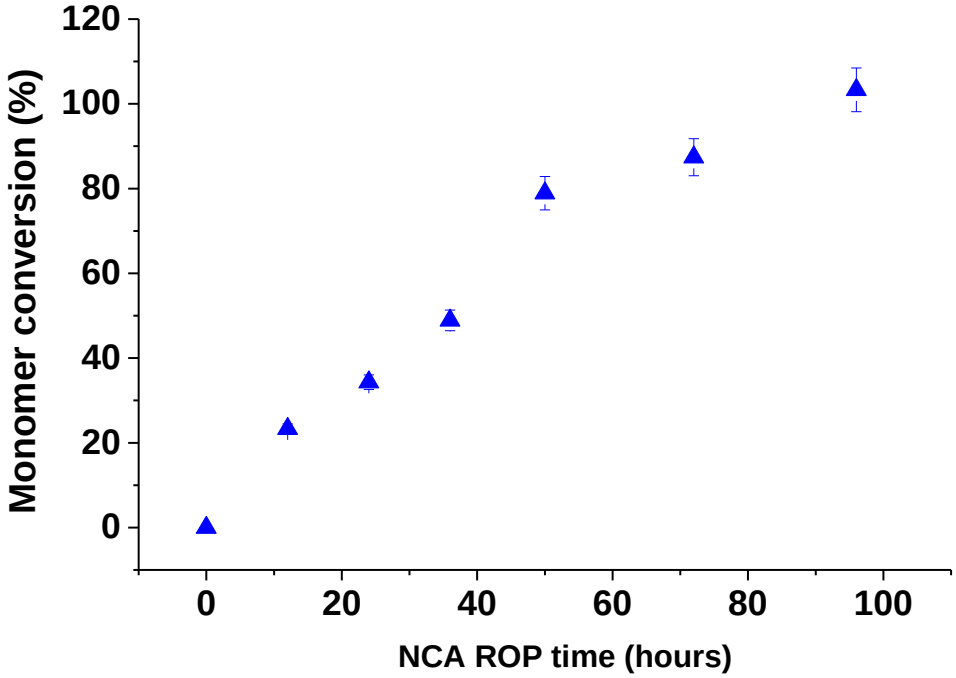

Figure S7: The kinetics of the dopamine-initiated ROP of Phe NCA.

Mass Spectrometry

The ESI-MS data obtained from Dop-(Ala)-(Sar) and Dop-(Phe)-(Sar) are shown in Figure S8 and Figure S9, respectively.

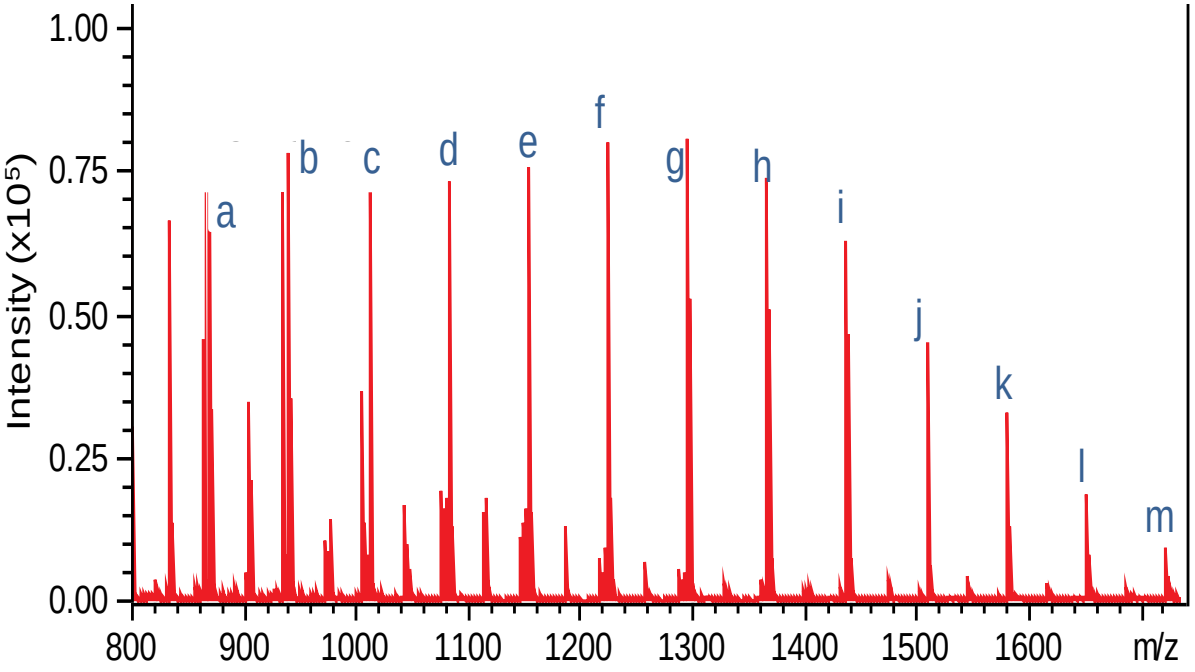

Figure S8. The ESI-MS spectrum of Dop-(Ala)-(Sar). The lettered peaks may be assigned to the various fragments of the macromolecule; a: Dop-$(Ala)_5$-$(Sar)_5$, b: Dop-$(Ala)_5$-$(Sarc)_6$, c: Dop-$(Ala)_5$-$(Sar)_7$, d: Dop-$(Ala)_5$-$(Sar)_8$, e: Dop-$(Ala)_5$-$(Sar)_9$, f: Dop-$(Ala)_5$-$(Sar)_{10}$, g: Dop-$(Ala)_5$-$(Sar)_{11}$, h: Dop-$(ala)_5$-$(sar)_{12}$, i: Dop-$(Ala)_5$-$(Sar)_{13}$, j: Dop-$(Ala)_5$-$(Sar)_{14}$, k: Dop-$(Ala)_5$-$(Sar)_{15}$, l: Dop-$(Ala)_5$-$(Sar)_{16}$, m: Dop-$(Ala)_5$-$(Sar)_{17}$

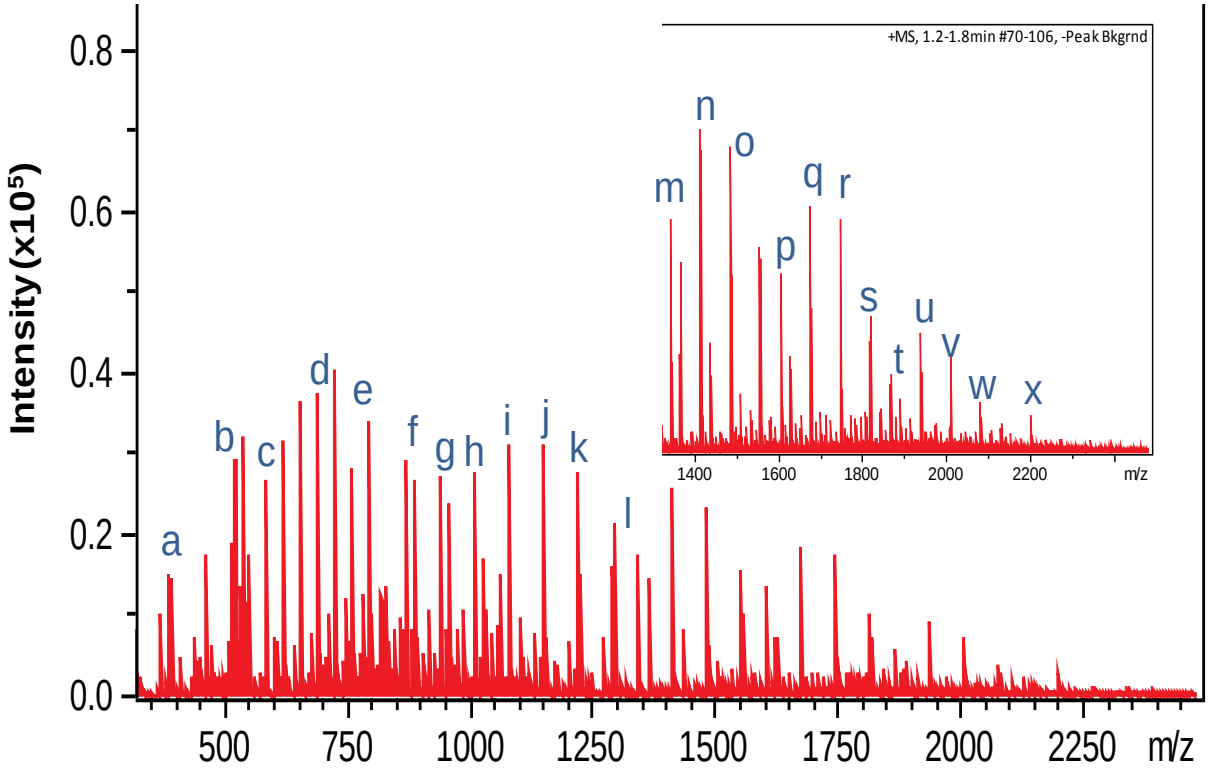

Figure S9. The ESI-MS obtained from the Dop-(Phe)-(Sar). The lettered peaks may be assigned to the various fragments of the macromolecule ; a) Dop-$(Phe)_2$, b) Dop-$(Phe)_2$-(Sar), c) Dop-$(Phe)_3$, d) Dop-$(Phe)_4$, e) Dop-$(phe)_4$-(Sar), f) Dop-$(Phe)_4$-$(Sar)_2$, g) Dop-$(Phe)_4$-$(Sar)_3$, h) Dop-$(Phe)_4$-$(Sar)_4$, i) Dop-$(phe)_4$-$(Sar)_5$, j) Dop-$(Phe)_4$-$(Sar)_6$, k) Dop-$(Phe)_4$-$(Sar)_7$, l) Dop-$(Phe)_4$-$(Sar)_8$, m) Dop-$(phe)_4$-$(Sar)_9$, n) Dop-$(Phe)_4$-$(Sar)_{10}$, o) Dop-$(Phe)_4$-$(Sar)_{11}$, p) Dop-$(Phe)_4$-$(Sar)_{12}$, q) Dop-$(Phe)_4$-$(Sar)_{13}$, r) Dop-$(Phe)_4$-$(Sar)_{14}$, s) Dop-$(Phe)_4$-$(Sar)_{15}$, t) Dop-$(Phe)_4$-$(Sar)_{16}$, u) Dop-$(Phe)_4$-$(Sar)_{17}$, v) Dop-$(Phe)_4$-$(Sar)_{18}$, w) Dop-$(Phe)_4$-$(Sar)_{19}$, x) Dop-$(Phe)_4$-$(Sar)_{20}$.

Preparation of polymer samples and PBS buffer for nanoprecipitation

1 mg/mL solutions of each polymer in DMF were prepared. Homogenous solutions were obtained upon vigorous stirring using a vortex mixer and sonication for at least 30 minutes. The solutions were then centrifuged (6000 rpm, 5 °C, 10 min) and the supernatant was collected and passed through a 0.45 µm Millipore polytetrafluoroethylene (PTFE) syringe filter.

10 mM PBS buffer solution (pH 7.4) was prepared using ultrapure water (18.2 megohms-cm). The solution was autoclaved at 115 °C for 10 minutes and then allowed to cool to room temperature. The obtained buffer was passed through a 0.45 µm Millipore PTFE filter.

Nanoprecipitation, Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM) Studies

Micelle nanoparticles were generated using the 'dropping-in' method of micelle formation. 20 µL of polymer solution (1 mg/mL) was added dropwise to vigorously stirred PBS buffer (2 mL) and the solution was left to equilibrate for 1 minute. The samples produced contained polymer concentrations of 10 µg/mL and were used for both DLS and SEM analysis. Particle size and particle size distribution analysis was performed twice using a Zetasizer Nano ZS series DLS instrument, at 25 °C. The nanoparticle solutions were retained. These solutions were re-analysed twice by DLS after 14 days (Figures S10 and S11). For SEM studies, a glass pipette was used to extract a droplet of the nanoparticle solution. The sample was deposited onto a SEM glass cover slip and allowed to air-dry. The cover slip was then mounted on a SEM stub using conductive tape and the dry samples were sputter-coated with a gold film on a Quorum Q150RS sputter coater. The coated samples were analysed for particles size and morphology using a JEOL JSM-6610LV scanning electron microscope, at an accelerating voltage of 15 kV and a working distance of 11 mm.

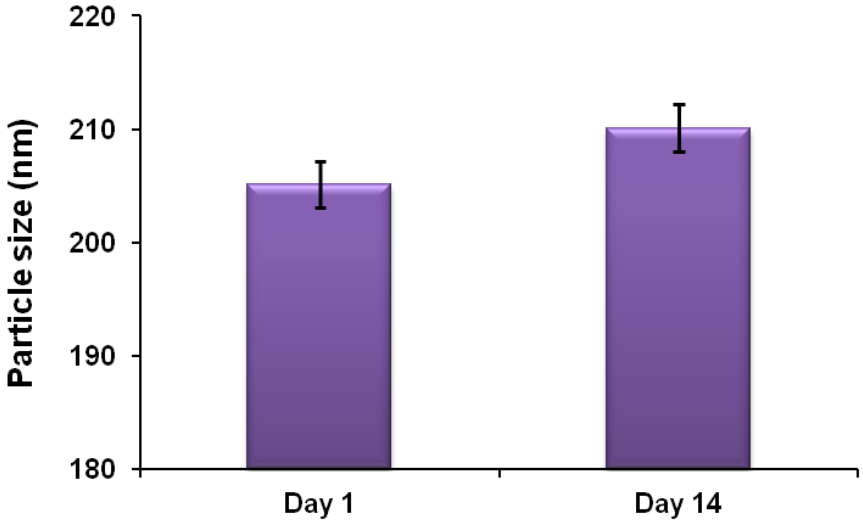

Figure S10. Stability assessment of Dop-(Ala)-(Sar) nanoparticles in PBS buffer. The particles remained stable for 14 days.

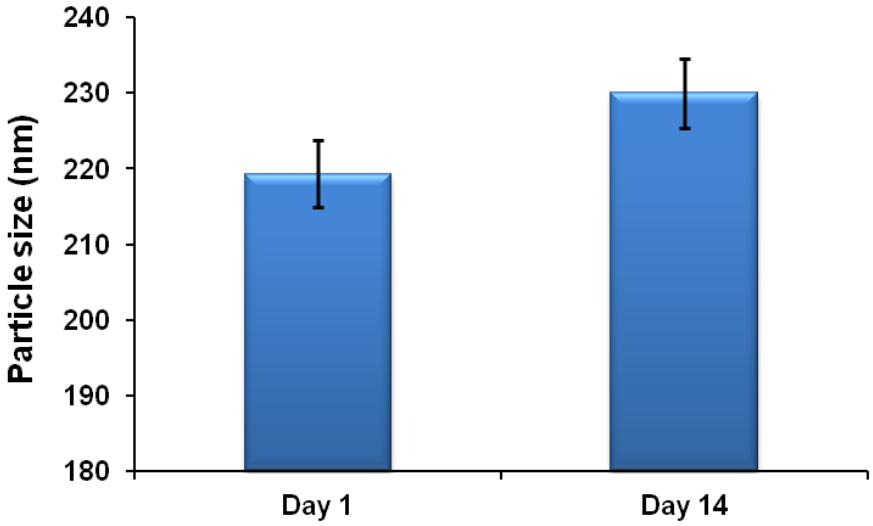

Figure S11. Stability assessment of Dop-(Phe)-(00 Sar) nanoparticles in PBS buffer. The particles remained stable for 14 days.

Critical Micelle Concentration (CMC)

The CMC of the materials produced was determined by following a DLS method,[3] utilising a Malvern ZetaSizer, Nano Series ZS instrument. The block copolymer sample was dissolved in DMF to prepare a 10 mg/mL stock solution. Micellisation was then performed by independently dropping varying volumes of the stock solution into vigorously stirred nanopure water to yield a series of samples with varying polymer concentrations (ranging from 10^{-4} mg/mL to 5 mg/ mL). The obtained samples were analysed by DLS, with changes in the intensity of the scattered light (in kilo counts per second (kcps)) in response to the concentration of the polymers being monitored. The variation in the intercept of the correlation function was also monitored so as to substantiate the results obtained from the analysis of the intensity of scattered light. In this study, the CMC of Dop-Ala_5-Sar_{15} was found to be 4.01×10^{-6} mol/L (i.e. 5.99 µg/mL) and the CMC of Dop-Phe_4-Sar_{18} was found to be 3.58×10^{-7} mol/L (i.e. 0.724 µg/mL), as shown in Figures S12 and S13 respectively.

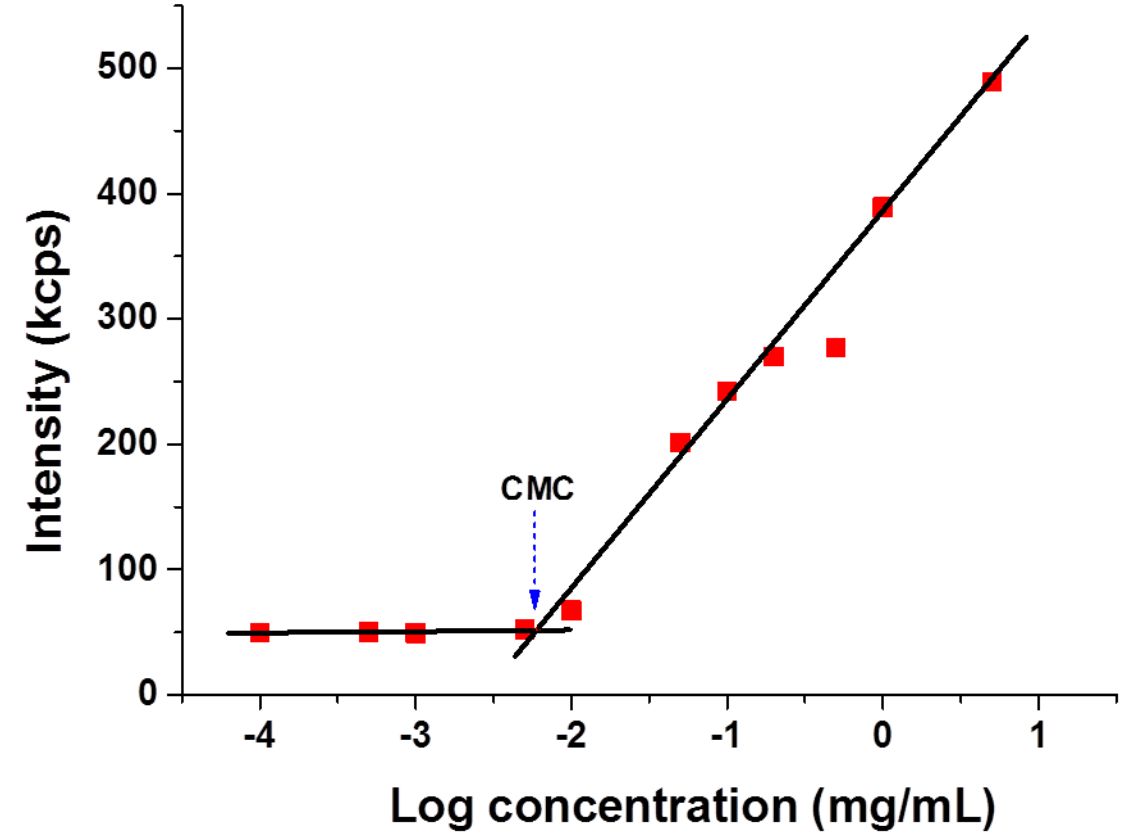

Figure S12. The intensity of the scattered light at various concentrations of Dop-$(Ala)_5$-$(Sar)_{15}$. The CMC is the point of intersection of the two linear plots.

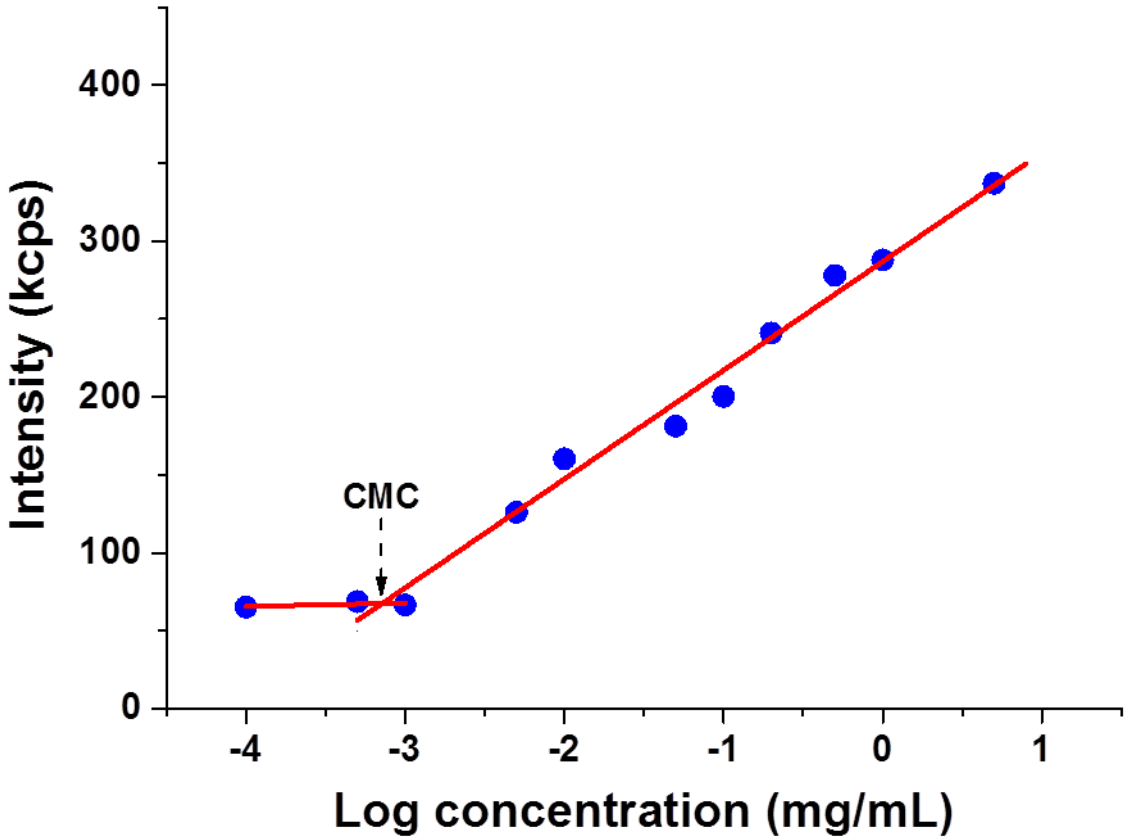

Figure S13. The intensity of the scattered light at various concentrations of Dop-$(Phe)_4$-$(Sar)_{18}$. The CMC is the point of intersection of the two linear plots.

Enzymatically-Induced Dopamine Release Assessment by HPLC Analysis

Stock solutions of varying concentrations of dopamine hydrochloride, 0 µg/mL – 100 µg/mL (0 mM - 0.528 mM), were prepared by serial dilution of a solution of dopamine hydrochloride in HPLC grade water (18.2 megohms-cm). Table S1 gives the optimised experimental conditions that were used to study the release of dopamine from the micelles. A calibration curve was generated by analysing a series of solutions of dopamine hydrochloride made to different concentrations in PBS (Figure S14).

Table S1: HPLC conditions used to study the release of dopamine from Dop-(peptide)-b-(peptoid) micelle nanoparticles.

Column details	Ascentis Express C18 5 cm x 2.1 mm, 2.7 µm particle size
Mobile phase	A: Water + 0.1% TFA B: Acetonitrile + 0.1% TFA
Flow rate	0.5 ml/min
Sample injection volume:	10µL
Gradient	Time (min) A% B% 0 100 0 5 100 0 10 97 3 11.5 0 100 13.0 0 100 13.5 100 0 15.0 100 0
Column compartment/ autosampler temperature	5°C
Detector	Photodiode array UV detector

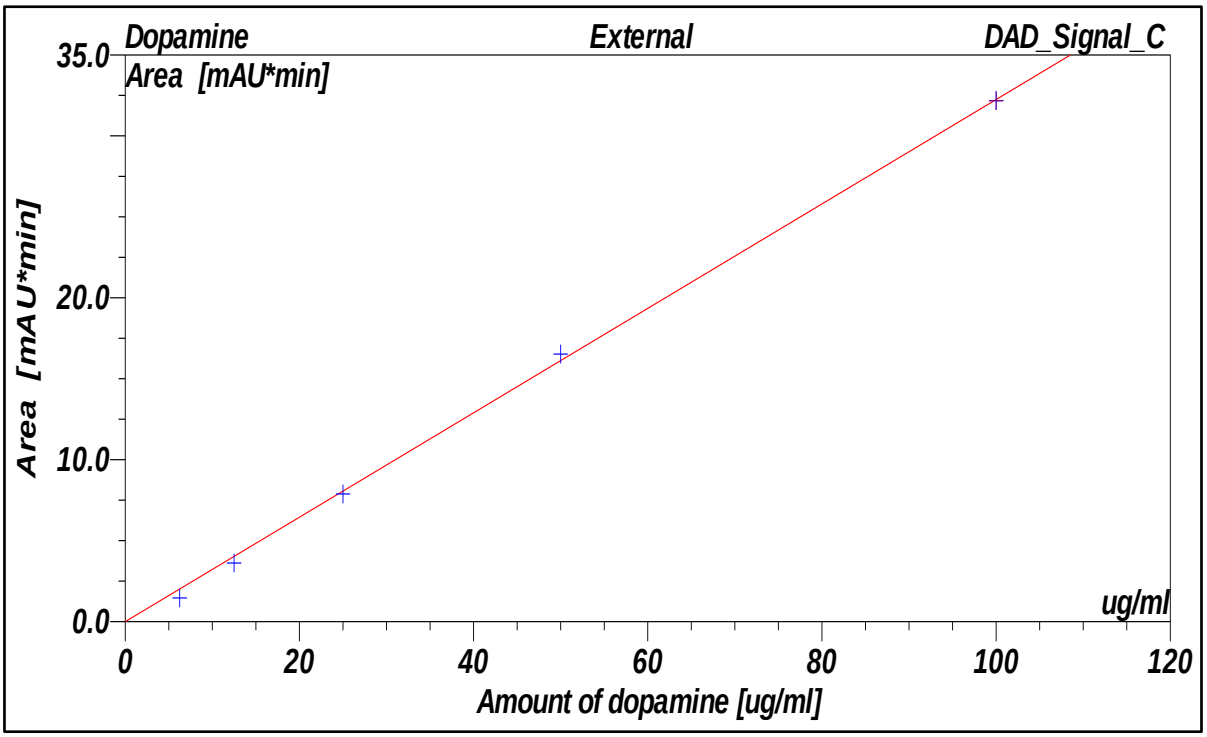

Figure S14: The calibration graph for HPLC studies of dopamine release from the dopamine-conjugated nanoparticles

The nanoparticles created were dialysed (1,200 Da MWCO) versus PBS buffer solution for 24 hours, with constant solution changes. Three enzyme solutions containing an equivalent of 20 enzyme units in PBS were prepared from thermolysin from *Bacillus thermoproteolyticus rokko*, α-chymotrypsin and elastase from porcine pancreas. The enzymes were allowed to equilibrate in the PBS buffer for 3 hours. A fourth solution contained PBS buffer only.

Each enzyme solution (5 mL) and the blank (PBS only) was added independently to the dialysed nanoparticles (1 mg in 5 mL to give an overall nanoparticle concentration of 0.1 mg/mL). The solutions were incubated at 37 °C, under constant agitation and dialysed against PBS buffer (10 mL, pH 7.4). At selected time intervals, a sample was taken and the dialysate analysed for any dopamine released using HPLC.

A representative chromatogram from the analysis of dopamine released from Dop-$(Ala)_5$-$(Sar)_{15}$ particles is shown in Figure S15.

Released Dopamine Verification

The dialysate sample obtained from the Dop-$(Ala)_5$-$(Sar)_{15}$ micelles incubated with elastase was injected into the HPLC column, and the dopamine released from the micelles verified by collecting the fractions containing dopamine. Multiple injections (20 µL) and collections were done to obtain 5 mL of solution which was then concentrated by freeze-drying. The dry sample was re-dissolved into methanol (50 µL) and analysed by ESI-MS to substantiate its composition (Figure S15).

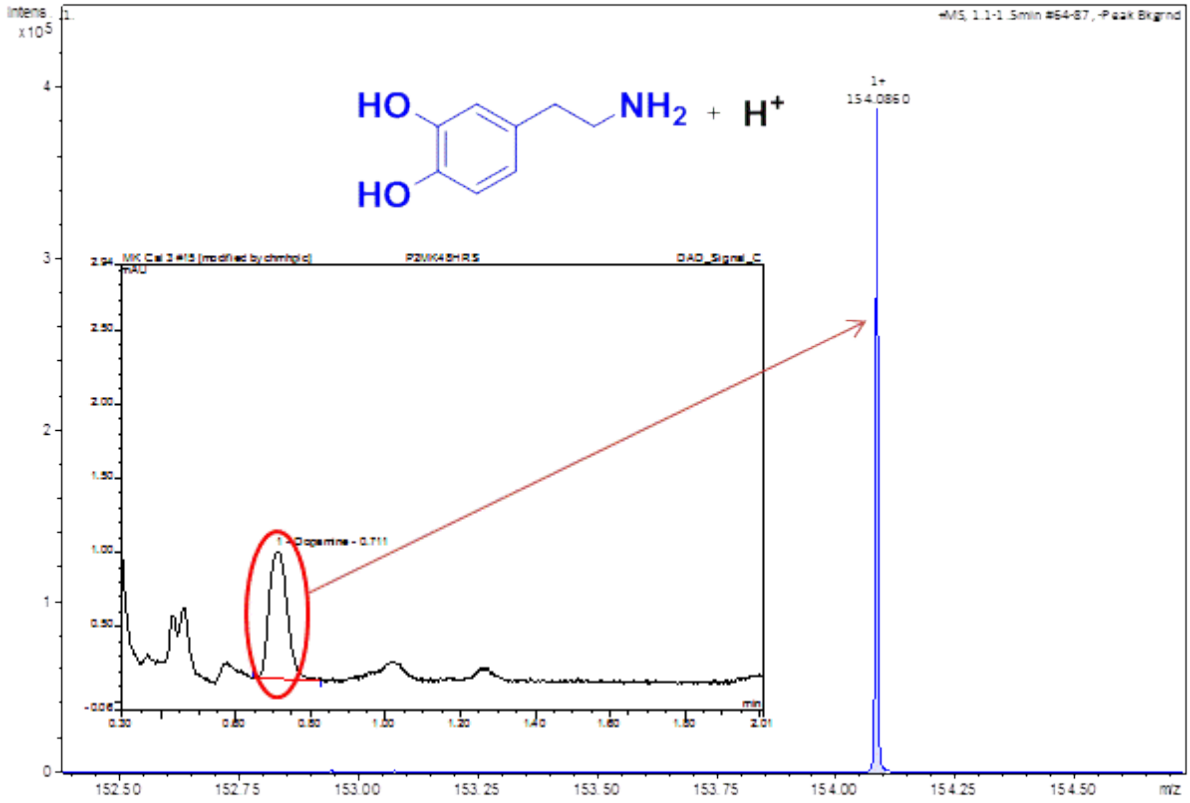

Figure S15. ESI-MS of dopamine released from (Dop-$(Ala)_5$-$(Sar)_{15}$ showing the molecular weight of the released dopamine to correspond to that of free dopamine**.** *Insert* HPLC chromatogram from Dop-Ala-Sar analyte.

Dispersions of Dop-$(Ala)_5$-$(Sar)_{15}$ and Dop-$(Phe)_4$-$(Sar)_{18}$ particles, containing no enzyme (blanks) were analysed by HPLC respectively, in order to verify the appearance of the peak obtained from a dopamine molecule which is still conjugated to the peptide block or an amino acid unit. The chromatograms obtained for unmodified dopamine, Dop-$(Ala)_5$-$(Sar)_{15}$ and Dop-$(Phe)_4$-$(Sar)_{18}$ and provided in Figures S16, S17 and S18 respectively. Splitting of the dopamine peak occurs if the dopamine molecule is covalently conjugated to a peptide unit. No such splitting occurs within the dopamine peak of unmodified dopamine, or the

peaks corresponding to enzyme-mediated release of dopamine from the micelles produced, confirming that the dopamine expelled is in an unbound form.

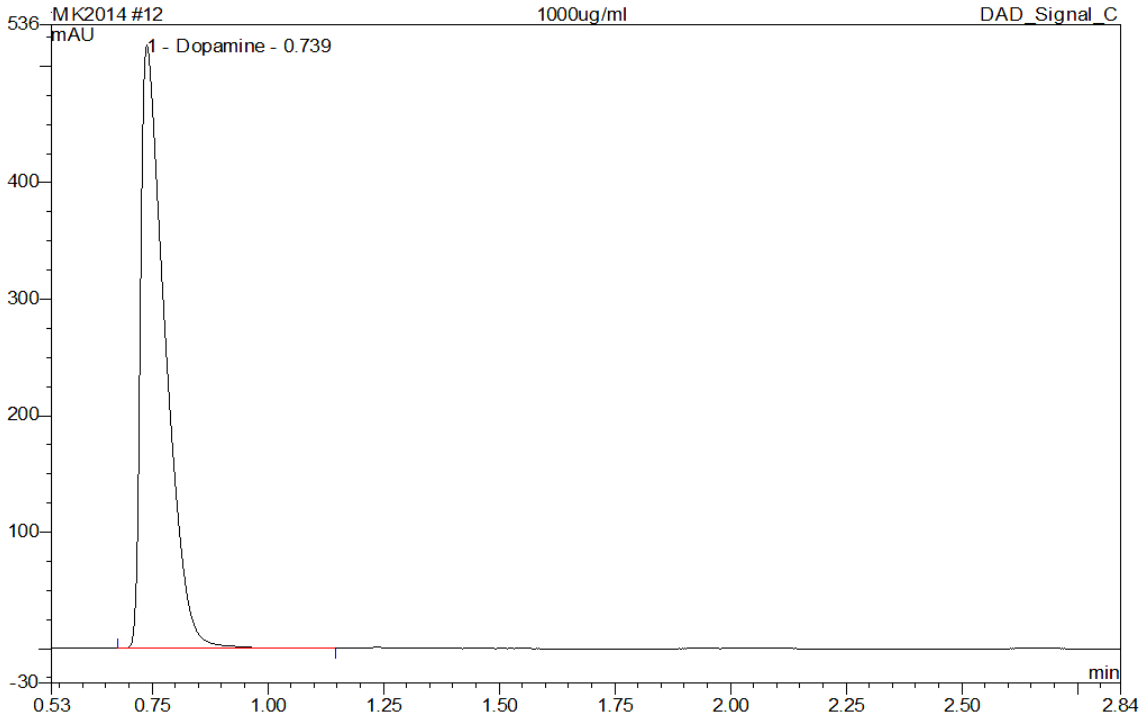

Figure S16. The chromatogram of a sample from free dopamine.

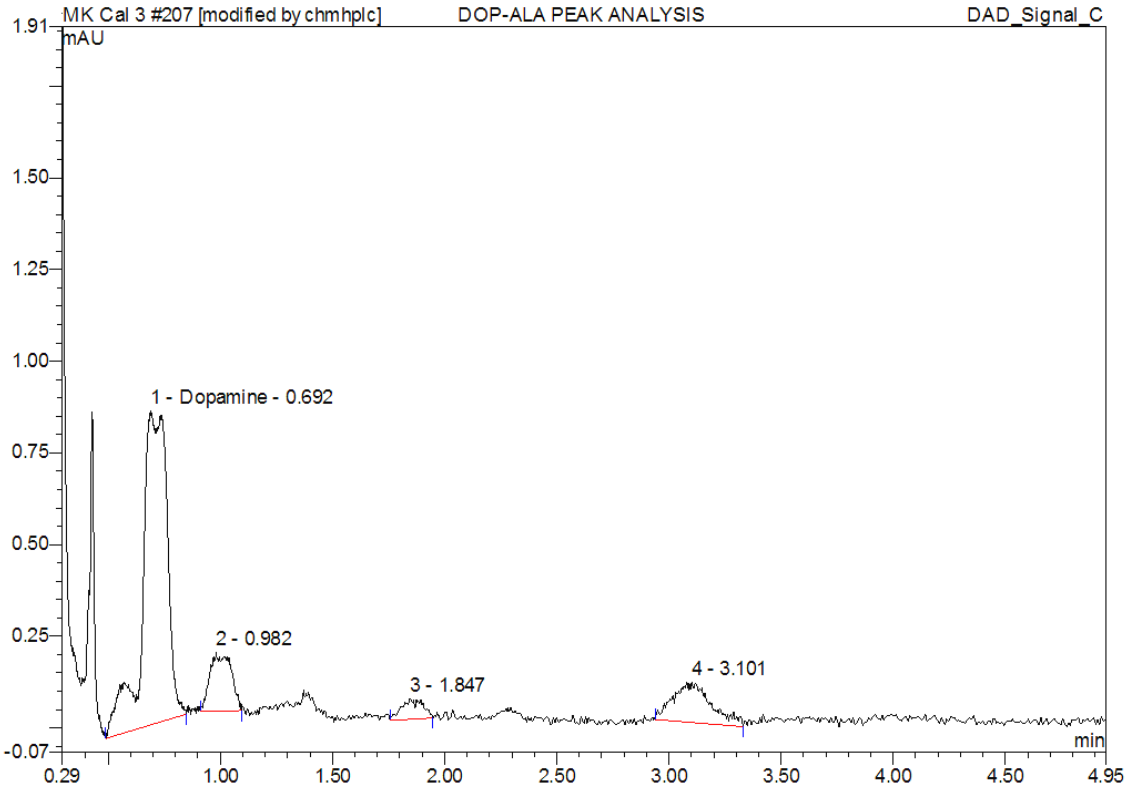

Figure S17. HPLC Chromatogram obtained from the analysis of Dop-$(Ala)_5$-$(Sar)_{15}$. The observed peak-splitting of the dopamine peak can be attributed to the dopamine molecule being conjugated to an alanine unit.

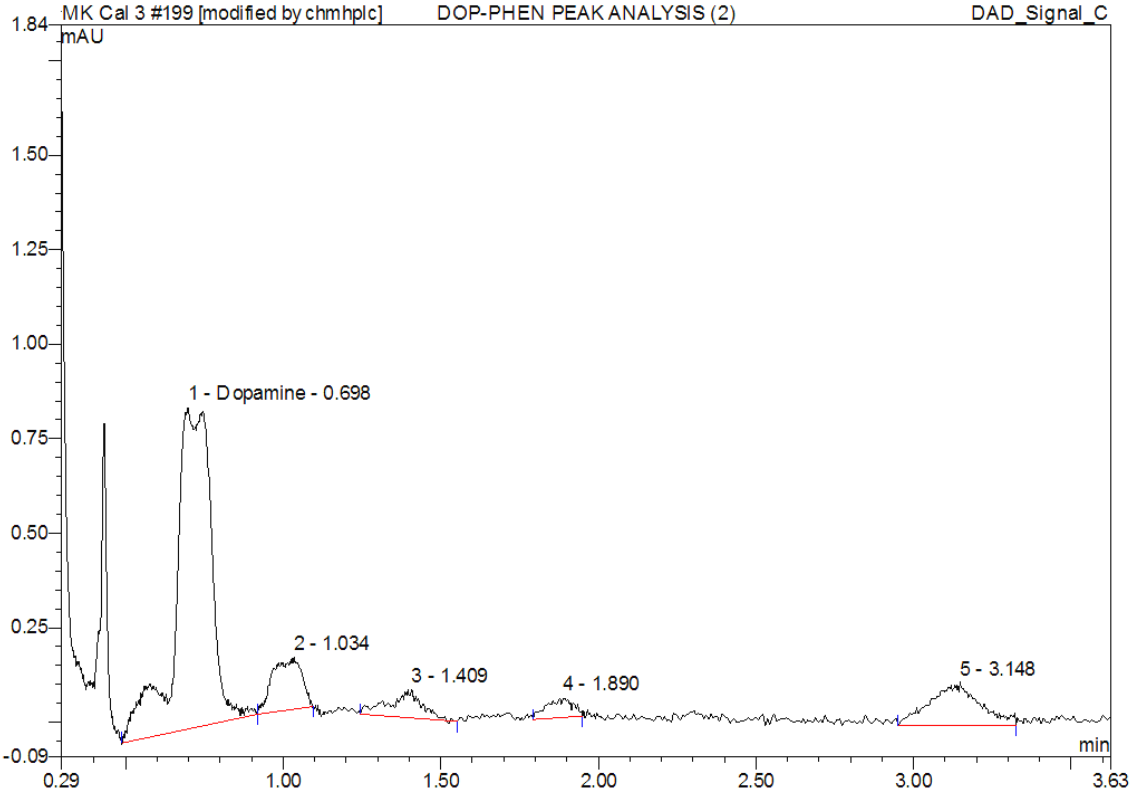

Figure S18. HPLC Chromatogram obtained from the analysis of Dop-$(Phe)_4$-$(Sar)_{18}$, The observed peak-splitting of the dopamine peak can be attributed to the dopamine molecule being conjugated to a phenylalanine unit.

Determination of the Dopamine Loading within the Nanoparticles Formed

The data obtained from the HPLC experiments was used to determine the percentage of dopamine that was detected. The maximum possible dopamine loading per unit volume of the nanoparticles was calculated from the ^{1}H NMR spectra of the dopamine-terminated block copolymers produced. The spectra were normalised to the dopamine aromatic peak and the concentration of dopamine in each sample calculated using the equations given below. The respective dopamine concentrations were used as references (relative to the results obtained from the HPLC studies) to determine the percentage of dopamine released during the individual enzyme-mediated release experiments. Once the results were plotted (Figure 3 in the main manuscript) a line of best fit was applied to the data using Origin 9.0 software.

Dop-(Ala)$_5$-(Sar)$_{15}$:

^{1}H NMR compositional ratios; Dop : Alanine: Sarcosine = 1 : 5 : 15

Dopamine loading by mass = (152) / (152*1 + 5*71 + 15*71) = 9.6692%

Concentration of polymer in nanoparticle sample = 0.1 mg/mL

Therefore, maximum dopamine loading = (9.6692/100) * 0.1 mg/mL

= 9.67 µg/mL

Dop-(Phe)$_4$-(Sar)$_{18}$:

^{1}H NMR compositional ratios; Dopamine: Phenylalanine: Sarcosine = 1 : 4 : 18

Dopamine loading = (152) / (152*1 + 4*147 + 18*71) = 7.5322%

Concentration of polymer in nanoparticle sample = 0.1 mg/mL

Therefore, maximum dopamine loading = (7.5322/100) * 0.1 mg/mL

= 7.53 µg/mL

References

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[2] C. Fetsch, A. Grossmann, L. Holz, J. F. Nawroth and R. Luxenhofer *Macromolecules*, 2011, **44**, 6746–6758.

[3] R. B. Dorshow, C. A. Bunton, and D. F. Nicoli. *J. Phys. Chem*. **1983**, 87, 1409-1416.