Supporting information

New N-acyl amino acid-functionalized biodegradable polyesters for pharmaceutical and biomedical applications.

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Experimental section

Materials and Methods

Novozym 435 lipase ([9001-62-1], immobilized on an acrylic resin, anhydrous THF, N,N'-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich, divinyl adipate (DVA) [4074-90-2] was purchased from TCI America. Acetone (HPLC grade) was purchased from Fisher. N-acetyl-tyrosine (Tyr)-phenylalanine (Phe) and –tryptophan (Trp) were purchased from VWR International. All chemicals were used as received without further purification steps. Pancreatin 8x was purchased from Sigma-Aldrich.

^{1}H NMR analysis

^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer employing acetone-d_6. Chemical shifts are expressed in parts per million (δ).

(**PGA** (400 MHz, Acetone- d_6; δ, ppm): 5.31 (m, 1H), 5.10 (m, 1H), 4.90–3.50 (m, 6H), 2.39 (m, 4H), 1.66 (m, 4H).

(**PGATrp50** (400 MHz, Acetone- d_6; δ, ppm): 7.66 (d, 1H), 7.44 (d, 1H), 7.82 (s, 1H), 7.17 (t, 1H), 7.10 (t, 1H), 5.35 (m, 1H), 4.87 (m, 1H), 4.62-4.04 (m, 6H), 3.36 (m, 1H), 3.24 (m, 1H), 2.39 (s, 4H), 1.96 (m, 3H), 1.67 (s, 4H).

(**PGAPhe50** (400 MHz, Acetone- d_6; δ, ppm): 7.49 (s, 1H), 7.34 (m, 4H), 5.35 (m, 1H), 4.75 (m, 1H), 4.61-4.03 (m, 6H), 3.18 (m, 1H), 3.07 (m, 1H), 2.43 (s, 4H), 1.94 (m, 3H), 1.70 (s, 4H).

(**PGATyr50** (400 MHz, Acetone- d_6; δ, ppm): 7.12 (m, 2H), 6.83 (m, 2H), 5.36 (s, 1H), 4.70 (m, 1H), 4.56-4.06 (m, 6H), 3.07 (m, 1H), 2.97 (m, 1H), 2.44 (s, 4H), 1.97 (m, 3H), 1.72 (s, 4H).

GPC Analysis

Gel permeation chromatography (GPC) was performed by using a PL50 Plus Polymer Laboratories system. 2 mixed bed (D) columns at 50° C were employed, using DMF containing 0.1% LiBr as the mobile phase, flow rate 1 mL min^{-1}, equipped with a refractive index detector. Poly (methyl - methacrylate) standards (Mn range: 1,810,000e505 g/mol) were used to calibrate the SEC**.**

Water Contact Angle (WCA) measurements

WCA values were recorded at room temperature by employing a KSV Cam 200 (KSV Instruments Ltd., Helsinki, Finland) equipped with a dedicated software (CAM200). Samples were prepared by coating cover slips with polymer films by evaporating dispensed polymer acetone solutions (3 mg/ml) overnight. Three measurements were repeated for each sample.

Differential Scanning Analysis (DSC)

Polymer glass transitions were investigated by DSC (Q2000, TA Instruments, Leatherhead, UK) at a constant heating rate of 10 °C/min. Crimped hermetic aluminium pans were used for the analysis of the samples under nitrogen flow at rate of 50 mL/min. Glass transitions were analysed by performing three heating/cooling cycles from -90 up to 120 °C.

Dynamic Light Scattering (DLS)

Particle size analyses were performed by DLS utilizing a Zetasizer Nano spectrometer (Malvern Instruments Ltd) equipped with a 633 nm laser at a fixed angle of 173°. All experiments were performed in triplicate on the same sample.

Typical synthesis of linear PGA

PGA is routinely synthesized (Scheme 1) by enzymatic polymerization of divinyl adipate (DVA) and glycerol[1]. Glycerol and DVA (equimolar amount) were poured into a 250 ml 3-neck round bottomed flask and dissolved in anhydrous THF (50 ml). The mixture was allowed to homogenize and a precise amount of Novozym 435 was added under mechanical stirring for 24 h at 40°C.
The reaction was stopped by filtration of the immobilized enzyme, followed by evaporation of the solvent under reduced pressure. The resulting residue was then heated at 90-95 °C for 1 hour to deactivate any residual enzyme which may have leached out of the resin. The residue was kept under vacuum at 40 C until a stable mass was reached. PGA presents as a highly viscous yellow liquid.

Typical PGA N-Acyl-Tyrosine (PGATyr) coupling procedure

In order to couple the three N-acyl Aromatic Amino Acids a simple and consistent Steglich esterification was adopted by applying the same reaction conditions regardless the nature of the lateral chain and the polymer/ NAA ratio. Thus the reported example (PGATyr50) can be applied to all the different PGA-NAA modifications. Typically, PGA (4.95 mmol) and DMAP (0.15 mmol) were added to anhydrous THF (20 ml) at room temperature in a round bottom flask under magnetic stirring until complete dissolution. A second solution was prepared by dissolving 1.20 mmol of DCC and 2.48 mmol of N-acyl Tyr in THF (20 ml). The DCC-NAA solution was poured into the organic polymer solution under N_2 flow. The reaction was allowed to stir over night. The resulting dicyclohexylurea was removed by centrifugation and the supernatant solvent was removed under reduced pressure. The modified polymer was re-dissolved in THF and precipitated in a solution 0.1 M NaOH and twice in cold MeOH respectively. The residual material was dried under reduced pressure to a stable weight.

$LogP_{(calc)}$ calculation

The ALOGPS 2.1 used, is an extremely fast online platform that practically combines information from different methods/programs to provide an average LogP. obtained by various methods such as the following: miLogP, ProLogP, ALogP, MlogP, XLogP, etc.

Nanoparticle preparation

The polymeric nanoparticles (NPs) were formed via the nanoprecipitation method. More precisely, 1ml of a 2.5mg/ml PGA-AAs stock solution in acetone was placed in a glass syringe. This organic phase was added dropwise into a vial containing 5ml of HPLC filtered water via a syringe pump with a flow rate of 1 ml/min. The water/acetone uncapped solutions were gently stirred over-night in order to facilitate acetone evaporation. Samples were prepared in triplicate in order to ensure reproducibility of the method.

Hemolysis Assay

Blood was collected into heparinized tubes and erythrocytes harvested and washed in saline then PBS as described[2]. NAAs were diluted in PBS (100 µl) and added to 48-well plates followed by 150 µl erythrocyte suspension and incubated for 1 h at 37°C, before centrifugation at 500 rpm for 5 min. Supernatant (100 µl) was carefully transferred to a clear 96-well plate and release of hemoglobin determined using a TECAN Spark 10M plate reader at 450 nm. PBS was used as the negative (no lysis) control and 0.2% Triton X-100 used as the positive (complete lysis) control, and percentage hemolysis was calculated relative to these controls (%hemolysis = ((Abs test – Abs PBS)/ (Abs TX – Abs PBS))*100%).

Enzyme degradation assay

To evaluate the biodegradability of PGA and PGAPhe30, NPs samples were prepared by nanoprecipitation at a final NPs concentration of 5 mg/ml in presence of Tween 80 at 0.1% w/v. The NP emulsions were diluted with PBS up to a final concentration of 1 mg/ml in 0.95ml. Pancreatin was used at 10µg/ml[3]. Pancreatin (8xUSP) was made up to a 1% solution and 50 µl added to the assay. This amount of enzyme activity equates to 40% of the activity specified in the US Pharmacopeia for simulated intestinal fluid with enzymes. DLS measurements were carried out at defined time frame of 30 mins in order to evaluate the swelling of the NPs in degradative conditions. The samples were stored at 37 °C after each measurement point.

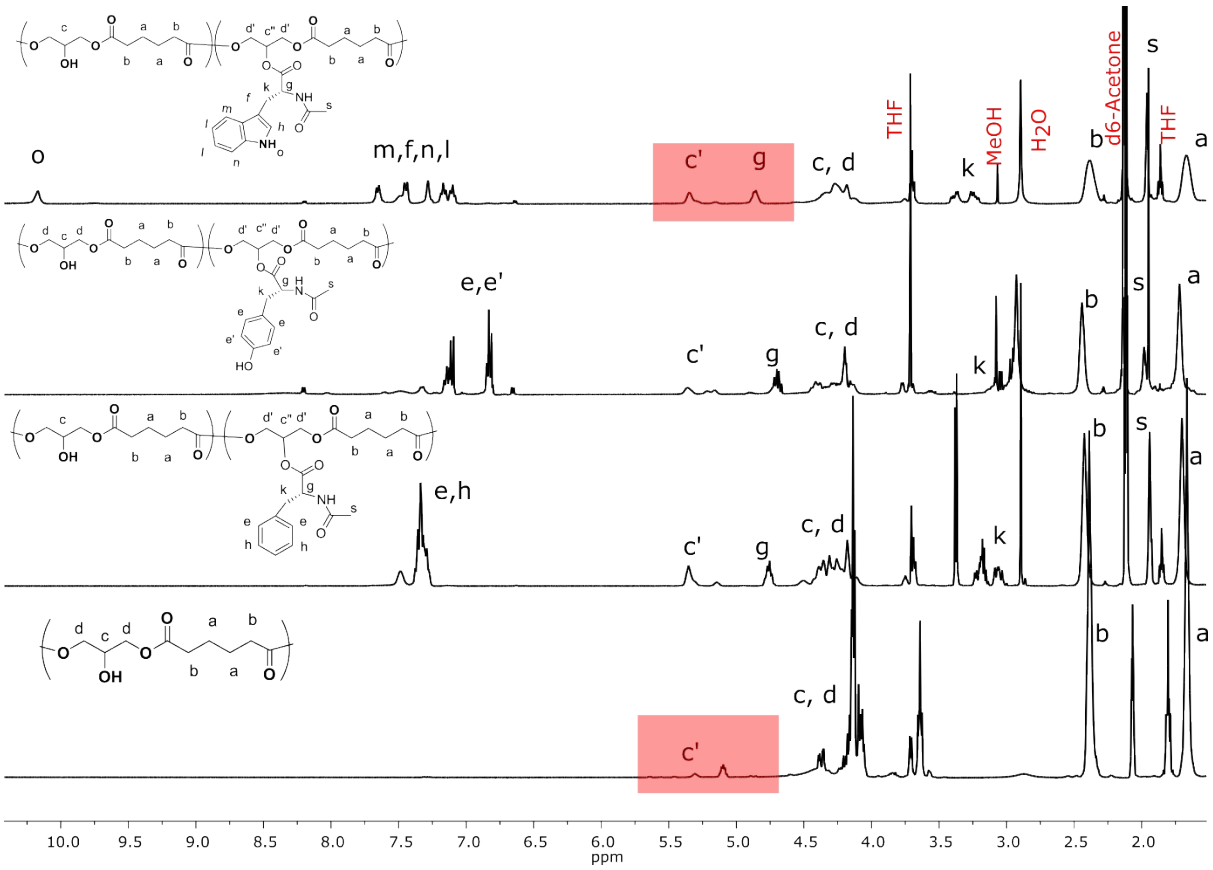
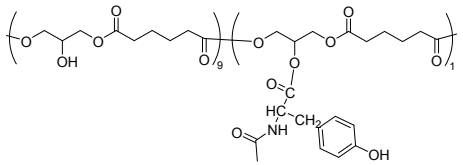
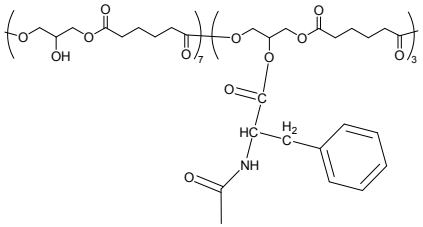
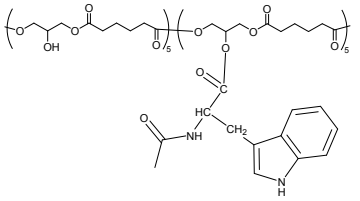


Figure SI1. From the bottom to the top stacked PGA, PGAPhe50, PGATyr50 and PGATrp50 ^{1}HNMR spectra. The two red rectangles between 5.7 and 4.7 ppm highlight the chemical shift range represented in Figure 1.

Table SI1.Summary of unmodified PGA and its NAA modifications thermal behaviour.

POLYMER	Tg (°C)
PGA	-33
PGATyr10	-19
PGATyr30	7
PGATyr50	11
PGAPhe10	-14
PGAPhe30	4
PGAPhe50	12
PGATrp10	-8
PGATrp30	13
PGATrp50	49

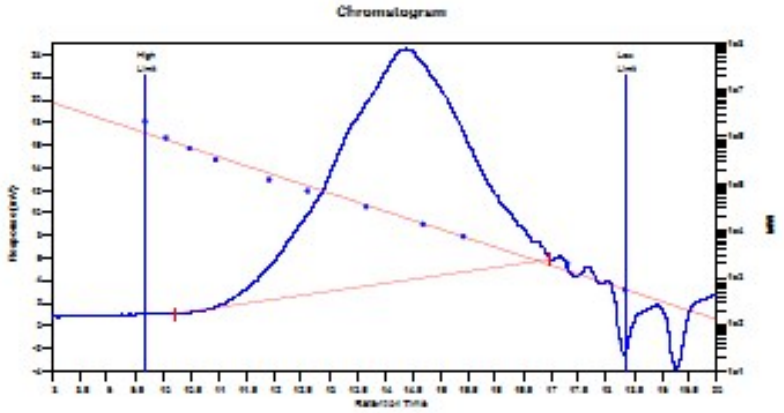
Table SI2. Calculated LogP of PGA, free N-acetyl Amino acids and PGA-conjugated with examples of the modifications between the different moieties and AAs

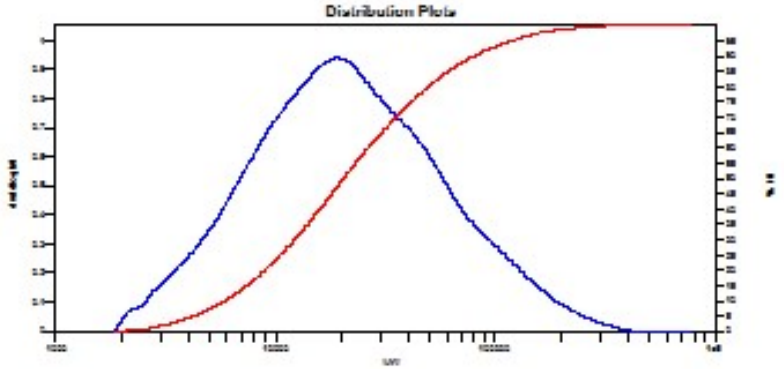
	0% mol/mol	10% mol/mol	30% mol/mol	50% mol/mol
PGA	1.173			
Tyrosine	1.030	2.060 	2.540	2.820
Phenylalanine	1.110	2.070	2.800 	3.300
Tryptophan	1.450	2.280	3.130	3.600 

From the Table SI1 it is possible to observe that the free NAAs show a LogP comparable with the bare PGA, while the different modifications show LogP values up to three fold higher than the unreacted polymer.

PGA

Eluent: DMF 0.1% LiBr



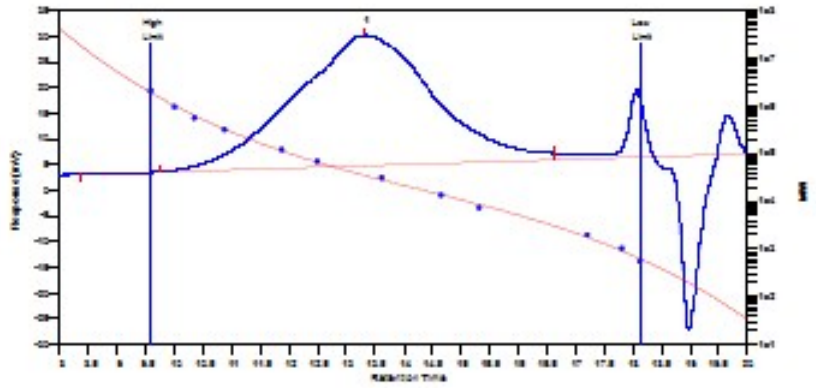


Peak No	Mp	Mn	Mw	Mz	Mz+1	Mv	PD
1	19084	13482	34695	83790	148588	29955	2.57343

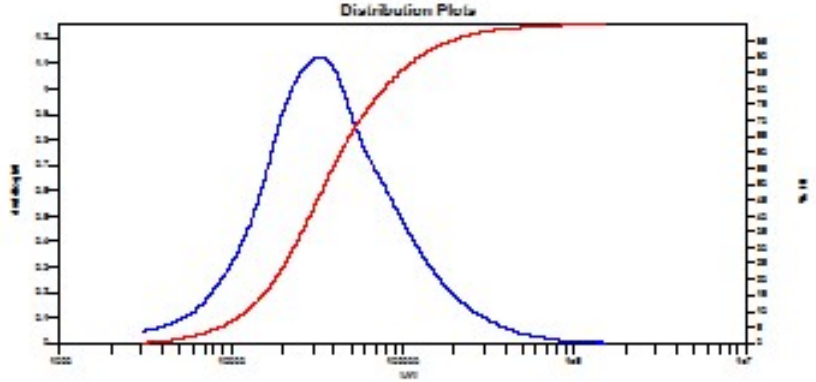
PGAPhe50

Eluent: DMF 0.1% LiBr

Chromatogram



Distribution Plots

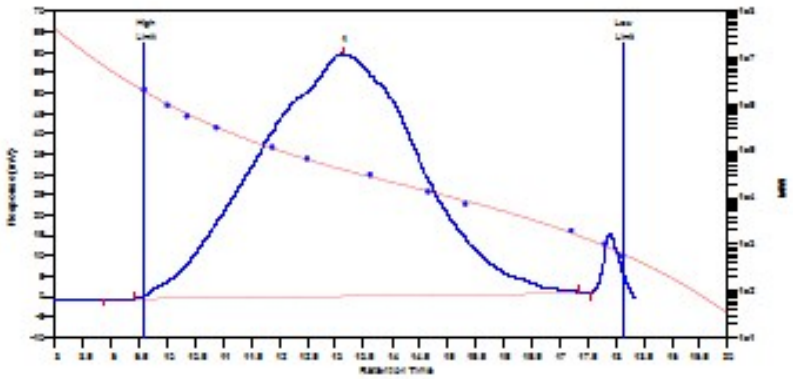


Peak No	Mp	Mn	Mw	Mz	Mz+1	Mv	PD
1	35115	23978	51482	142142	380703	44977	2.14705

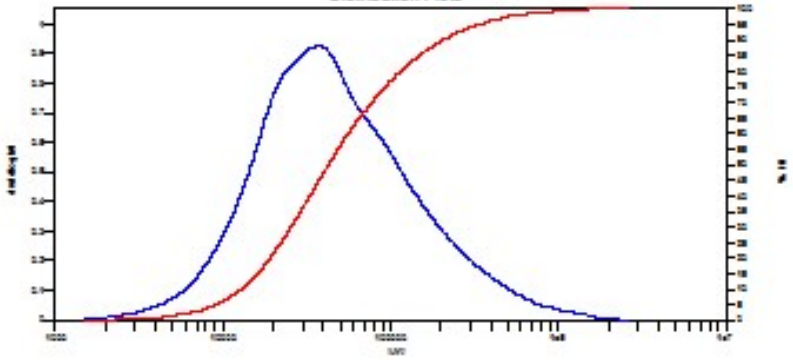
PGATrp50

Eluent: DMF 0.1% LiBr

Chromatogram

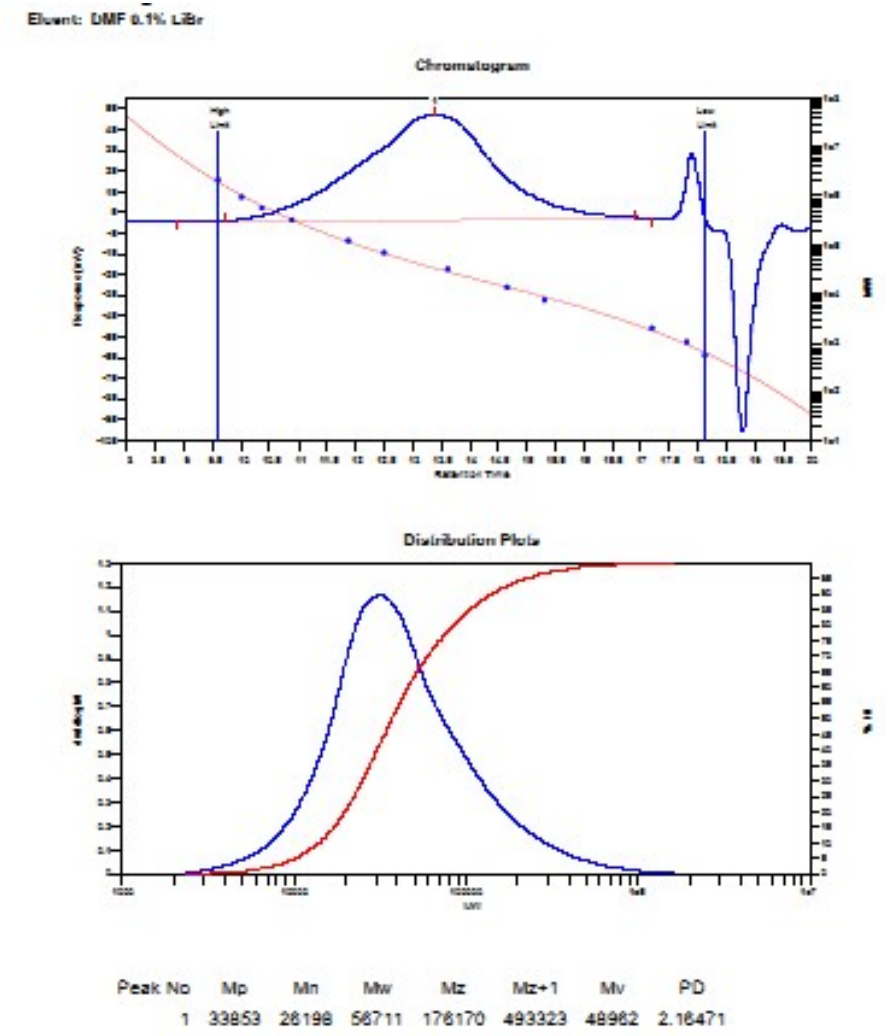


Distribution Plots



Peak No	Mp	Mn	Mw	Mz	Mz+1	Mv	PD
1	40498	25638	72857	275744	722809	59934	2.83396

PGATyr50



Peak No	Mp	Mn	Mw	Mz	Mz+1	Mv	PD
1	33853	28198	58711	176170	493323	48962	2.16471

Figure SI2. GPC traces of PGA, PGAPhe50, PGATrp50 and PGATyr50.

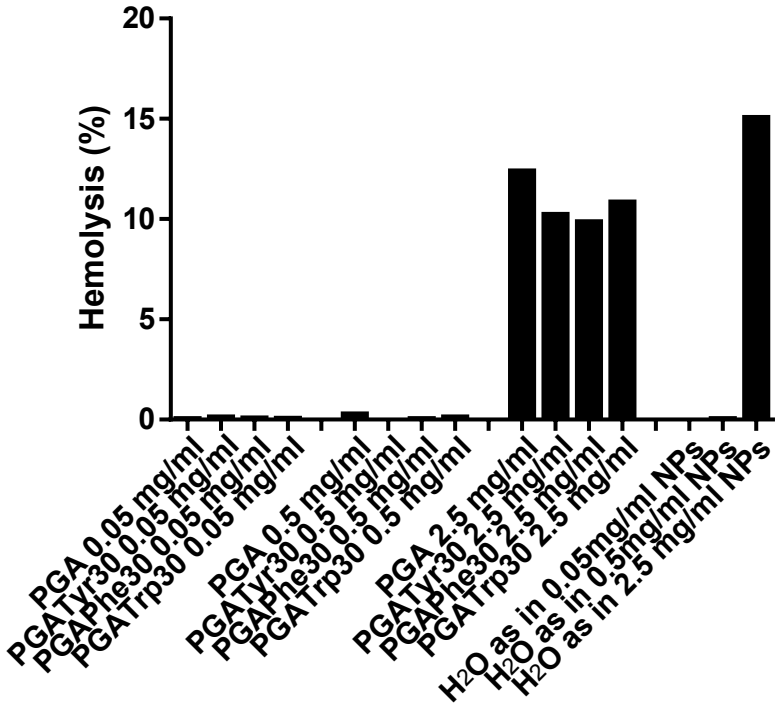


Figure SI3. PGA, PGATyr30, PGAPhe30 and PGATrp30 %hemolysis at 0.05, 0.5 and 2.5 mg/ml polymer concentrations The apparent haemolysis at the higher concentration of polymer is similar to the control showing the % haemolysis expected from the amount of water present in the sample. Thus no haemolysis is seen due to the polymers even at concentrations as high as 2.5mg/ml.

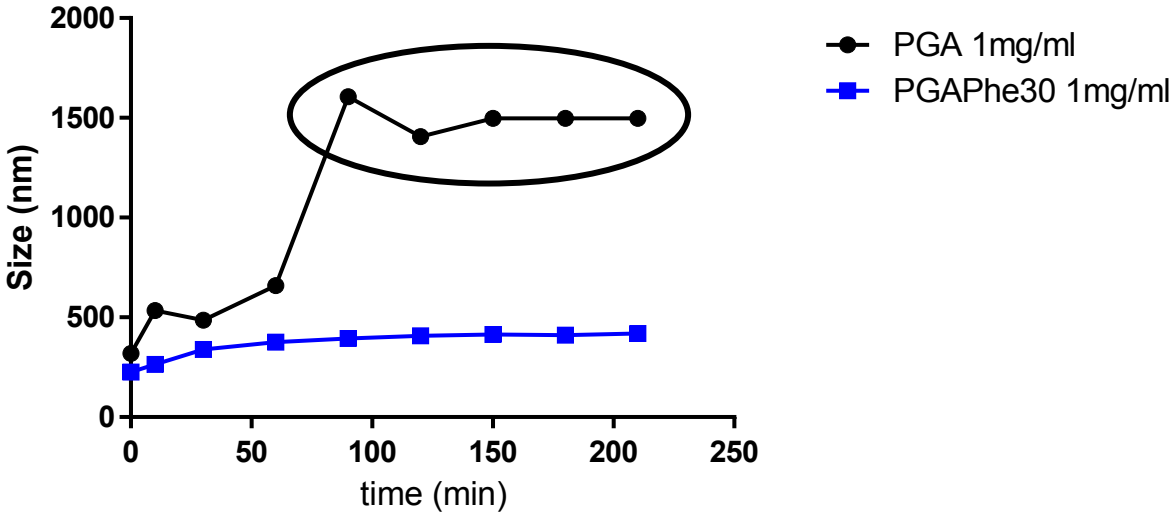


Figure SI4. PGA and PGAPhe30 NPs swelling kinetics in presence of Pancreatin. PGA showed a fast and pronounced swelling which finished with a weak signal from the enzyme (1630 nm) and suggested degradation was complete by 100min. PGAPhe30 showed some initial swelling, but reached a plateau suggesting that a complete degradation had not been reached even at 200min.

1 V. Taresco, R. G. Creasey, J. Kennon, G. Mantovani, C. Alexander, J. C. Burley and M. C. Garnett, *Polymer (Guildf).*, 2016, **89**, 41–49.

2 B. C. Evans, C. E. Nelson, S. S. Yu, K. R. Beavers, A. J. Kim, H. Li, H. M. Nelson, T. D. Giorgio and C. L. Duvall, *J. Vis. Exp.*, 2013, e50166.

3 C. Chen, Y. C. Cheng, C. H. Yu, S. W. Chan, M. K. Cheung and P. H. F. Yu, *J. Biomed. Mater. Res. - Part A*, 2008, **87**, 290–298.