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

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Biodegradable polymers have emerged as highly effective drug delivery vehicles. We combime *N*-carboxyanhydride and *O*-carboxyanhydride ring opening polymerisations to synthesise a poly(amino acid)-polyester graft copolymer capable of encapsulating, and subsequently releasing doxorubicin *via* acid-mediated hydrolysis. Consequently, the nanoparticles detailed are extremely promising vehicles for the controlled delivery of chemotherapeutic agents.

The pharmaceutical industry is in a constant state of flux, with the past 30 years seeing the rise of multiple, paradigm-shifting scientific advances such as high-throughput screening, combinatorial chemistry, genomics, proteomics and computational chemistry.[1] This has resulted in an exponential increase in the number of viable targets and chemical substrates available, often in the form of vast compound libraries and databases.[2] In the past decade alone, the number of therapeutic compounds in development has increased by 62%.[3] However, somewhat counter-intuitively, the number of new drugs approved by the USA Food Drug Administration (FDA) has been in decline since the 1990s, with only 25% approved in 2000-2010 compared with 1990-2000.[4] Poor compound pharmacokinetics are frequently discussed as the most significant contributor to preventing effective therapeutic treatment.

Utilising polymeric carriers for targeted drug delivery enables the distribution of hydrophobic drug molecules in vivo, prior to drug release and deployment at a target site.[5] In addition, the polymer restricts undesired and non-selective drug-receptor interactions, an imperative requirement for the delivery of toxic anti-cancer drugs to minimise the extremely unpleasant side effects that are associated with prolonged chemotherapy.[6] In particular, polyanhydrides,[8] poly(amino acid)s,[9] poly(alkyl cyanoacrylate)s[10] and polyorthoesters[11] have been highlighted as promising drug delivery vehicles. Such polymers are designed undergo programmed hydrolysis at a target side to liberate the therapeutic agent following polymer degradation.

Poly(amino acid)s synthesised by *N*-carboxyanhydride (NCA) ring-opening polymerisation (ROP) are attractive candidates for utilisation as drug delivery vehicles as they can readily self-assemble in aqueous solution to form discrete nanostructures.[12] Additionally, poly(amino acid)s possess a diverse chemical identity with 20 canonical amino acid varieties available, not to mention the multitude of non-classical amino acids. However, poly(amino acid)s are not readily susceptible to hydrolysis at physiologically relevant pH levels, and as such are of limited use for acid-mediated controlled drug delivery. To overcome this obstacle, a pH-responsive element may be incorporated into the polymer that is susceptible to acid-mediated hydrolysis, which prompts polymer degradation, and payload release.

Polyesters are susceptible to hydrolysis at pH levels comparable to that of the cancerous tumour microenvironment.[13] Measured tumour extracellular pH values of many solid tumours range from pH 6.5 to pH 7.2, and the pH within cancerous cells may be between pH 5.0 and pH 6.0 in endosomes, and between pH 4.0 and pH 5.0 in lysosomes.[14] The synthesis of pendant-functionalised polyesters may be achieved by utilising *O*-carboxyanhydride (OCA) ROP. Such functionality may be used to promote polymeric self-assembly and/or be exploited for further (bio)chemical modification. Combining NCA ROP and OCA ROP enables the production of polymers capable of self-assembly to form nanoparticles in aqueous solution, but crucially also yields polymers that can readily partake in acid-mediated hydrolysis to enable payload release.

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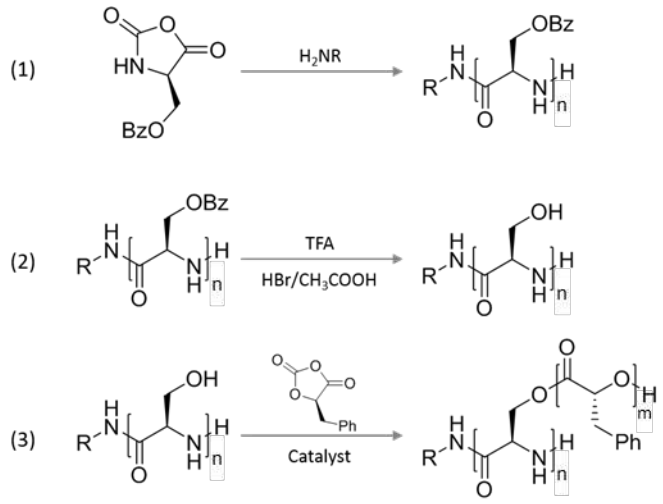

Scheme 1: (1) ROP of Serine (Ser) NCA may be conducted from a primary amine-presenting molecue to produce OBz-protected poly(Ser). In this instance benzylamine was utilised as the initiator. (2) Deprotection of OBz-protected poly(Ser), using trifluoroacetic acid and HBr in ethanoic acid solvent, to yield poly(Ser). (3) Poly(Ser) (-OH) initiated ROP of Phenylalanine (Phe) OCA, to yield poly(Ser)-*graft*-poly(Phe α-hydroxyacid). 4-Dimethylaminopyridine (DMAP) was utilised as the catalyst in this instance.

We report the synthesis of a novel poly(amino acid)-*graft*-polyester coploymer by sequential NCA and OCA ROP. Poly(Ser) was created by NCA ROP as the polymer backbone onto which the OCA of phenylalanine was subsequently grafted. Combining NCA ROP and OCA ROP in this manner permits the production of a polymer that possesses the capability to self-assemble to form nanoparticles in aqueous solution, entrap doxorubicin, and undergo degradation to release the therapeutic payload in response to acidic media. It is envisaged that the copolymers produced may be exploited to create materials that are of significance as novel, highly-effective, controlled release systems for the delivery of chemotherapeutic agents to acidic cancerous tissue.

The proposed macromolecular system is detailed in Scheme 1. Firstly, the monomers *O*-Benzyl-L-Ser NCA and Phe OCA were synthesised before poly(*O*-benzyl-L-Ser) was produced by benzylamine-initiated NCA ROP. Upon the cleavage of the benzyl protecting groups, poly(Ser) was yielded and used to initiate the ROP of phenylalanine OCA in the presence of DMAP, to afford the desired graft copolymer. The base activation of the monomer with multiple hydrogen bonds from DMAP is reported to be more energetically favourable than the standard nucleophilic activation achieved by the monomer alone.[15] In addition, it was hypothesised that DMAP may act via a 'two-pronged' activation approach, through its basic nitrogen centre and acidic *O*-hydrogens, making it a bifunctional catalyst. A range of polymers were produced that possessed varying ratios of poly(amino acid) and polyester segments within their composition (ESI Tables S1 and Table S2).

The capability of the polymers produced to self-assemble and form nanoparticles in aqueous medium was then assessed. Poly(Ser) consisting of 9.6, 18.7 and 19.6 amino acid monomer repeat units, as determined by ^{1}H NMR, was used to graft varying degrees of poly(Phe α-hydroxyacid) from the pendant hydroxyl groups present (Table 1). In all cases, nanoparticles were produced following the 'dropping-in' method of self-assembly, in which the polymer is expelled from DMF into PBS solution (pH 7.4). The extent of Phe OCA grafting was relatively low to ensure that polymer precipitation did not occur from DMF, but sufficiently high to ensure nanoparticle formation. The polydispersity index values obtained for the nanoparticlees created ranged from having a narrow distribution (PDI ≤ 0.1) to possessing a moderately polydisperse distribution (0.1 > PDI < 0.4).

Table 1: Mean particle size and PDI values for a series of graft copolymers, as determined by DLS. Data is presented as mean ± SD (n = 3).

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Copolymer	Particle Size (d.nm)	PDI
Poly[$(Ser)_{19.6}$-*g*-$(Phe\ AHA)_6$]	90 ± 6	0.07 ± 0.01
Poly[$(Ser)_{19.6}$-*g*-$(Phe\ AHA)_{2.5}$]	100 ± 8	0.11 ± 0.03
Poly[$(Ser)_{18.7}$-*g*-$(Phe\ AHA)_5$]	96 ± 8	0.28 ± 0.04
Poly[$(Ser)_{18.7}$-*g*-$(Phe\ AHA)_{2.5}$]	95 ± 5	0.10 ± 0.02
Poly[$(Ser)_{9.6}$-*g*-$(Phe\ AHA)_5$]	97 ± 6	0.14 ± 0.03
Poly[$(Ser)_{9.6}$-*g*-$(Phe\ AHA)_{2.5}$]	104 ± 7	0.23 ± 0.04

From these results, poly$(Ser)_{19.6}$-*graft*-poly(Phe α-hydroxyacid)$_6$ (Table 1, Entry 1) was deemed to be the most attractive candidate for use as a drug delivery vehicle due to the average size and the narrow polydispersity of the nanoparticles produced. As a result, this polymer was progressed into proof-of-concept studies, which aimed to demonstrate successful encapsulation and subsequent release of the chemotherapeutic doxorubicin from the formed nanoparticles. After successful conversion to the free-base, doxorubicin solution (chloroform) was added to the nanoprecipitation solution of the polymer in pH 7.4 PBS solution, and the mixture left overnight. It was observed that after 12 hours of stirring at room temperature, the chloroform layer had completely disappeared and the aqueous portion of the mixture had developed a purple-red hue, indicating that the nanoparticles had successfully encapsulated the doxorubicin (ESI Figure S11, inset). This solution was then dialysed to allow any un-encapsulated doxorubicin to be isolated from the nanoparticles, which were subsequently lyophilised. Post-dialysis, the colour remained and the dialysis supernatant was shown by Uv-vis spectroscopy to have no doxorubicin present, suggesting complete encapsulation within the polymer nanoparticles. The drug-encapsulation efficiency of poly$(Ser)_{19.6}$-*graft*-poly(Phe α-hydroxyacid)$_6$ nanoparticles was 52.3% ± 6.2% and the drug loading content of the nanoparticles was 2.90% ± 1.3% (w/w). The particle size and morphology of a sample of doxorubicin-loaded polymer was then assessed by DLS and SEM. It was observed that after the encapsulation of doxorubicin, nanoparticles of 118 nm mean diameter formed (ESI Figure S11). This increased swelling from the initial 90 nm adds further confidence to confirm the successful encapsulation of doxorubicin within the nanoparticles.

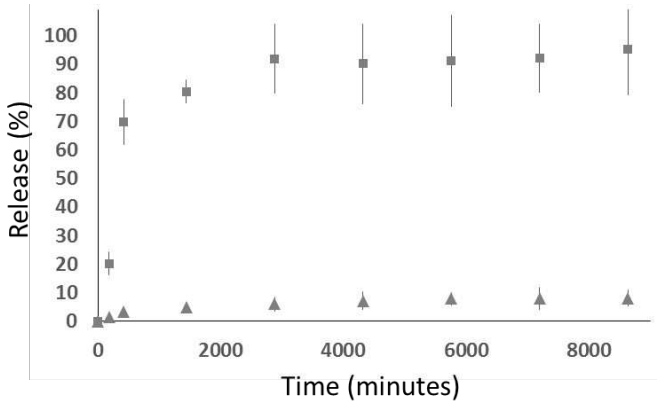

Figure 1: Doxorubicin release from nanoparticles consisting of that are maintained in solution of pH 5.0 (■) and pH 7.4 (▲). Release % has been adjusted to account for the assumed equal concentration of doxorubicin that could not be analysed (n=3).

The potential for acid-mediated release of doxorubicin from the nanoparticles was then assessed. For this purpose, a dialysis-setup was employed, whereby the doxorubicin-loaded nanoparticles were suspended in aqueous medium within a dialysis bag; released doxorubicin passes through the dialysis bag and into the surrounding medium of equal volume to that within the dialysis tubing, whilst unhydrolysed polymer nanoparticles, which are doxorubicin loaded, are retained within the dialysis tubing. Doxorubicin release to the exterior solution only occurs upon acid-mediated polymer degradation, which can then be quantified. Significant and rapid payload release occurred when doxorubicin nanoparticles were suspended in an acidic environment, although only the doxorubicin that passed to the outside of the dialysis bag could be measured. 47.7% of the loaded doxorubicin had passed through the dialysis membrane to the exterior solution after six days of incubation. This value is presented as 95.4% release in Figure 1 as it is assumed that the released doxorubicin concentration outside the dialysis tubing (which could be analysed) is equal to the concentration of released doxorubicin within the dialysis tubing (which could not be analysed), owing to the equal volume of solution internal and external to the dialysis membrane. The release of doxorubicin to the external dialysis medium is theoretically limited *via* simple diffusion kinetics to 50%. Conversely, only 4.0% (presented as 8.0% in the adjusted Figure 1) of loaded doxorubicin passed through the dialysis membrane to the exterior solution from nanoparticles that were maintained within an aqueous solution (pH 7.4) after six days, highlighting the requirement of an acidic environment to instigate substantial payload release.

In order to support the positive release profile of doxorubicin from the nanoparticles stored within an acidic medium, a degradation study was carried out to correlate payload release with nanoparticle degradation. Nanoparticle samples were independently stored in buffer solution maintained at pH 5.0 and buffer solution maintained at pH 7.4, and after 48 hours the size distribution of both samples was determined by DLS, and representative images obtained by SEM (Figure 2). The nanoparticles held in buffer solution of pH 7.4 possessed a mean diameter of 100 nm after 48 hours, and were clearly visible during SEM analysis. This contrasts to the nanoparticles that were maintained within acidic medium; these

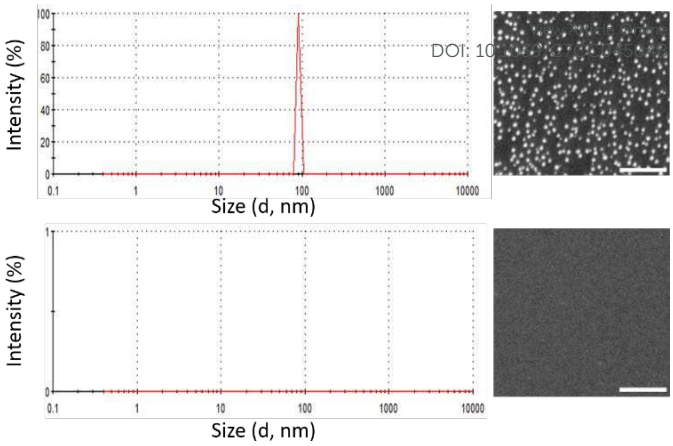

Figure 2: *Top,* DLS and SEM analysis reveal the maintained presence of Poly(Ser)-*graft*-poly(Phe α-hydroxyacid) nanoparticles when they are maintained in PBS solution (pH 7.4). *Bottom,* The presence of nanoparticles was not detected by SLS or DLS analysis when they were incubated in acidic solution (pH 5.0). Scale bars represent 1 μm.

nanoparticles were not evident in either DLS or SEM analyses, suggesting that acid-mediated polymer degradation is responsible for the release of doxorubicin. Additionally, poly(serine)$_{19.6}$-*graft*-poly(phenylalanine α-hydroxyacid)$_6$ nanoparticles were maintained within acetate buffer (pH 5.0) for 48 hours to fully confirm acid-mediated ester hydrolysis as the cause of payload release. Following incubation, the polymer was dialysed (regenerated cellulose, MWCO 1,000 kDa) against DI water and then chloroform to remove all non-polymeric groups from the solution. ^{1}H NMR was utilised to analyse the recovered polymer and confirmed the removal of grafted polyester side chains, as evidenced by the lack of peak at ~3.5 ppm, following graft copolymer incubation in acidic media (Figure S13).

To determine the *in vitro* cytotoxicity of poly(Ser)$_{19.6}$-*graft*-poly(Phe α-hydroxyacid)$_6$ nanoparticles and doxorubicin-loaded poly(Ser)$_{19.6}$-*graft*-poly(Phe α-hydroxyacid)$_6$ nanoparticles against a non-cancerous and cancerous cell line, Alamar Blue and MTT assays were carried out, respectively. Against the non- cancerous cell line (L929 fibroblasts), all polymer concentrations of both loaded and unloaded nanoparticles indicated a cell viability of 80% and above at 12, 24, 48 and 72 hours (Figure S14). MTT assays were then carried out to determine the *in vitro* cytotoxicity of poly(Ser)$_{19.6}$-*graft*-poly(Phe α-hydroxyacid)$_6$ nanoparticles and doxorubicin-loaded poly(Ser)$_{19.6}$-*graft*-poly(Phe α-hydroxyacid)$_6$ nanoparticles against T47D cells. The blank polymer nanoparticles exhibited nominal cytotoxicity against the cells during a prolonged period of 72 hours, particularly at polymer concentrations ≤ 0.01 μg/mL (Figure 3). At polymer concentrations ≥ 0.1 μg/mL, a significant difference in the cytotoxicity of doxorubicin-loaded nanoparticles and nanoparticles that lack doxorubicin encapsulation was found. Differences between the cell viability of free doxorubicin and doxorubicin-loaded nanoparticles suggest that the polymeric

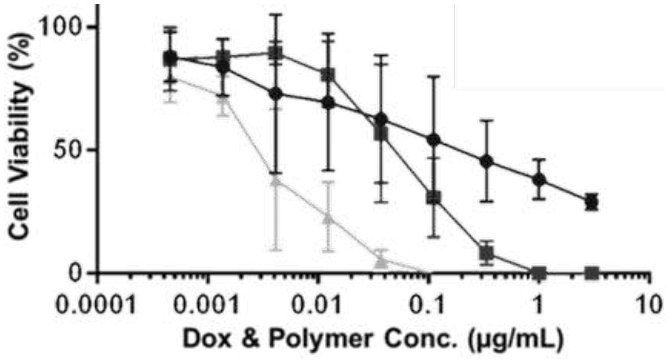

Figure 3: (a) The effect of blank polymer nanoparticles (●), doxorubicin-loaded polymer nanoparticles (■) and free dox (▲) on the viability of T47D cells. Data is presented as mean ± SD (n=4).

carrier inhibits doxorubicin-cell interaction prior to doxorubicin release, or that the process of endosome uptake of nanoparticles, followed by polymer hydrolysis and the endosomal escape of doxorubicin limits the activity of doxorubicin compared to free doxorubicin. Examination of the assay plates show the presence of solubilised formazan crystals, confirming the presence of viable cells whose viability was affected marginally by culturing them in the presence of blank nanoparticles for 72 hours (Figure S15). In contrast, the doxorubicin-loaded nanoparticles exhibited significant cytotoxicity against the cancerous cells (the IC_{50} value of dox-loaded nanoparticles on T47D cells was 0.065 μg/mL). The viability of the cancerous cells reduced to zero after 72 hours of incubation with doxorubicin-loaded nanoparticles (doxorubicin loadings ≥1 μg/mL).

In summary, NCA ROP and OCA ROP were combined to create poly(amino acid)-polyester graft copolymers that were capable of self-assembly in aqueous media to form discrete nanoparticles. The chemotherapeutic doxorubicin was encapsulated within the nanoparticles formed from poly(Ser)$_{19.6}$-*graft*-poly(Phe α-hydroxyacid)$_6$, and remained encapsulated in response to a physiologically neutral environment. However, the polyester functionality of the nanoparticles granted an innate susceptibility to acid-mediated polymer hydrolysis. Consequently, nanoparticle degradation and doxorubicin release occurred when the nanoparticles were maintained within solution of pH 5.0. Both doxorubicin containing and blank nanoparticles were non-cytotoxic against non-cancerous L929 fibroblasts cells, as determined by Alamar Blue assays. Promisingly, MTT assays revealed that poly(Ser)$_{19.6}$-*graft*-poly(Phe α-hydroxyacid)$_6$ nanoparticles have limited cytotoxicity against T47D cancer cells, whilst doxorubicin-loaded nanoparticles exhibited significant anticancer activity against the same cell line. The polymers reported are capable of releasing a chemotherapeutic agent in response to acidic environments, and are thus highly promising candidates to be utilised for controlled therapeutic release at acidic, cancerous, sites.

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