Dextrin-Colistin Conjugates as a Model Bioresponsive Treatment for Multi-drug Resistant Bacterial Infections.

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Abstract: Polymer therapeutics offer potential benefits in the treatment of multidrug resistant (MDR) infections; affording targeted delivery of biologically active agents to the site of inflammation, potential decreases in systemic toxicity and the retention of antimicrobial activity at the target site. As a prototype model, these studies developed and characterized a library of dextrin-colistin conjugates (dextrin molecular weight: 7,500 - 48,000 g/mol) as a means of targeting the delivery of colistin. Optimum colistin release kinetics (following dextrin degradation by physiological concentrations of amylase (100 IU/L)) were observed in conjugates containing low molecular weight (~7,500 g/mol) dextrin with ~1 mol% succinoylation (~80% drug release within 48 h, compared to ~33% from sodium colistin methanesulfonate (CMS, Colomycin®)). These conjugates exhibited comparable antimicrobial activity to CMS in conventional MIC assays against a range of Gram-negative pathogens, but with significantly reduced *in vitro* toxicity towards kidney (IC_{50} = CMS, 15.4 μg/mL; dextrin-colistin, 63.9 μg/mL) and macrophage (IC_{50} = CMS, 111.3 μg/mL; dextrin-colistin, 303.9 μg/mL) cells. *In vivo* dose-escalation studies in rats demonstrated improved pharmacokinetics of the conjugates, with prolonged plasma levels of colistin ($t_{1/2}$ 135-1271 min vs. 53 min) and decreased toxicity, compared to colistin sulfate. These studies highlight the potential utility of 'nanoantibiotic' polymer therapeutics to aid the safe, effective and targeted delivery of colistin in the management of MDR infections.

Introduction

The prevalence of infections by Gram-negative, multi-drug resistant organisms, including *Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae,* represents a world health problem and a significant clinical challenge in patients with chronic disease e.g. cystic fibrosis, in whom aminoglycoside and fluoroquinolone resistance is common[1]. Increasing resistance to currently-available antibiotics has been mirrored by decreases in the design/development of new antibiotic entities[1]. To overcome these issues clinicians are employing antimicrobial agents (e.g. colistin and polymyxin B) which are effective against Gram-negative organisms, but have previously been restricted in clinical use due to the potential for asociated toxicity[2].

Polymer therapeutics are being increasingly investigated for the treatment of cancer, arthritis, viral infections[3,4], and, more recently, tissue repair[5-8]. Indeed, two of the US top 10 selling drugs in 2013 were polymer therapeutics (Neulasta® and Copaxone®)[9]. Whilst this technique has been extensively employed to reduce toxicity in the delivery of cytotoxic therapies in the treatment of malignancy[3], surprisingly, little attention has been directed at their use in the treatment of bacterial infection. The conjugation of antibiotics to polymers offers the ability to improve biodistribution, enhance stability and reduce toxicity[10,11]. Polymer masked-unmasked protein therapy' (PUMPT) is a technique, which employs conjugation of a biodegradable polymer to "mask" a protein or peptide's activity in the circulation[12]. The circulating conjugate accumulates at the site of inflammation (or infection) due to the enhanced permeability and retention (EPR) effect[11] and "locally-triggered" degradation of the polymer regenerates bioactivity. The application of PUMPT, to safely deliver antibiotic agents otherwise associated with toxicity or pharmacokinetic problems (e.g. colistin) may, therefore, offer not only the ability to design and deliver novel targeted therapies, but also to improve the safe treatment of patients with Gram-negative MDR infections.

In this study we sought to develop a novel, nanomedicine-based, delivery system to target the delivery of colistin. Colistin was chosen as a model antibiotic since it is an amphiphilic peptide antibiotic (Mw ~1,400 g/mol) which is highly active towards multi-resistant Gram-negative bacteria, but is not readily absorbed orally and the free drug is nephro- and neurotoxic[13]. Consequently, it is commonly administered intravenously as sodium colistin methanesulfonate (CMS), a less toxic pro-drug, that is readily hydrolyzed to partially sulfomethylated derivatives and colistin in aqueous solution. This hydrolysis is however, neither controlled or triggered, and may even occur before patient administeration, which has resulted in fatality.

We hypothesized that polymer conjugation would not only limit systemic toxicity, but passively target the protein to sites of infection/inflammation by the effect, which has been widely observed in cancer and, more recently, to a lesser extent, in inflammation[11] (Figure 1). Dextrin was employed here as the model polymeric carrier as we have previously shown its suitability as a partner polymer in the context of PUMPT[12], as it is rapidly degraded by α-amylase[14,15]. We, and others, have shown how the conjugation can be tailored (employing dextrins of different molecular weights and degrees of succinoylation) to optimize the release and activity of the protein for particular clinical applications e.g. wound healing[12,14,15].

In the present work, we hypothesize that conjugation of dextrin to colistin will improve disease-specific targeting and reduce the toxic effects of colistin, thus re-establishing its value in Gram-negative infections and supporting the development of further polymer therapeutic nanoantibiotics. Here, the synthesis and characterization of a series of dextrin-colistin conjugates is reported and the ability of the conjugates to release colistin in the presence and absence of amylase at human physiological concentrations, their toxicity towards mammalian cells (erythrocytes, human kidney cells) and their *in vitro* antimicrobial activity is described. As dextrin conjugation is hypothesized to extend the serum half-life of colistin, an *in vivo* assessment of plasma drug concentration following a single bolus injection was performed in a dose-escalating study.

Materials and Methods

Materials. Type 1 dextrin from corn (Mw ~ 48,500 g/mol) was from ML laboratories (Keele, UK). Type I dextrin from corn (Mw ~ 7,500 g/mol), dextrin from maize starch (Mw ~28,000 g/mol), colistin sulfate, α-amylase from human saliva, N-hydroxysulfosuccinimide (sulfo-NHS), copper (II) sulfate pentahydrate 4% w/v solution, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), bicinchoninic acid solution (BCA), ethanolamine, ninhydrin, hydrindantin, lithium acetate dihydrate, acetic acid and dimethyl sulfoxide (DMSO) were all from Sigma-Aldrich (Poole, UK). Dextrin from potato starch (Mw ~10,500 g/mol) was from Fluka (Gillingham, UK). 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC) was from Pierce (Rockford, USA). Disodium hydrogen phosphate, potassium dihydrogen phosphate, potassium chloride, 4-dimethylaminopyridine (DMAP) and sodium chloride were from Fisher Scientific (Loughborough, UK). Pullulan gel filtration standards (Mw = 11,800–210,000 g/mol) were from Polymer Laboratories (Church Stretton, UK). Lactate dehydrogenase (LDH)-cytotoxicity assay kit was from abcam (Cambridge, UK). MaxSignal® colistin ELISA test kit was from Bioo Scientific Corp. (Austin, USA). Unless otherwise stated, all chemicals were of analytical grade. All solvents were of general reagent grade (unless stated) and were from Fisher Scientific (Loughborough, UK).

Cell culture. Mouse macrophage cells (RAW 264.7) and human kidney proximal tubule cells (HK-2) were from ATCC (Manassas, USA). Cells were screened and found to be free of mycoplasma contamination before use. Dulbecco's Modified Eagle's medium (DMEM) with GlutaMAX™, fetal calf serum (FCS), keratinocyte serum-free medium (K-SFM) with L-glutamine, epidermal growth factor (EGF), bovine pituitary extract (BPE) and 0.05% w/v trypsin-0.53 mM EDTA were obtained from Invitrogen Life Technologies (Paisley, UK).

Bacterial culture. The strains used for susceptibility testing include both culture collection

strains and clinical isolates (Table 1). Their known relevant genotypes and origin have been described by Khan et al.[16]. Bacterial colonies were grown on tryptone soya agar (TSA) and liquid cultures were suspended in tryptone soya broth (TSB) for overnight culture or Mueller-Hinton broth (MHB) for MIC determination (from Oxoid; Basingstoke, UK).

Synthesis and purification of dextrin-colistin conjugates. First, succinoylated dextrins (1-60 mol%) were synthesized, as previously described[15] (Figure 2(a)). Briefly, with dextrin of molecular weight 7,500 g/mol, 5 mol% succinoylation was achieved as follows. Dextrin (1000 mg, 6.17×10^{-3} mol) was dissolved in anhydrous DMF (10 mL) in a 50 mL round-bottomed flask. Succinic anhydride (61.7 mg, 6.17×10^{-4} mol) and DMAP (28.6 mg, 2.34×10^{-4} mol) were subsequently added, the reaction purged with nitrogen, sealed and left to stir at 50 ^{o}C for 18 h. Then, the reaction mixture was poured into vigorously stirring diethyl ether (250 mL) and stirred overnight. The ether was removed by filtration under vacuum and the residual solid was dissolved in minimal distilled water (dH_2O), poured into a dialysis membrane (molecular weight cut-off 2,000 g/mol) and dialysed against 4 x 5 L dH_2O. The resultant solution was freeze-dried to yield succinoylated dextrin. The degree of succinoylation was quantified by titration against a standard solution of NaOH (5×10^{-4} M) with bromophenol blue as indicator, and the product was further characterized by FT-IR (Avatar 360 ESP spectrometer with EZ OMNIC ESP 5.2 software; Thermo Nicolet, Loughborough, UK) to confirm identity and by gel permeation chromatography (GPC) (TSK G4000PW_{XL} and G3000PW_{XL} columns (Polymer Laboratories, Church Stretton, UK) in series, mobile phase PBS (pH 7.4), flow rate of 1 mL/min) to measure approximate molecular weight and polydispersity (compared to pullulan standards). Samples for GPC were prepared in PBS (3 mg/mL) and the eluate was monitored using a differential refractometer (Gilson 153). PL Caliber Instrument software, version 7.0.4, from Polymer Laboratories (Church Stretton, UK) was used for data analysis.

Succinoylated dextrins (1-30 mol% succinoylation) were then conjugated to colistin (Figure 2(b)). Briefly, for 10 mol% succinoylated dextrin, succinoylated dextrin (200 mg, 1.11×10^{-4} mol

COOH; 2.50 x 10^{-5} mol dextrin) was dissolved under stirring in dH_2O (1 mL) in a 10 mL round-bottomed flask. To this, EDC (21.3 mg, 1.11 x 10^{-4} mol) and sulfo-NHS (24.1 mg, 1.11 x 10^{-4} mol) were added, and the mixture was left stirring for 30 min. Subsequently, colistin (35 mg, 2.50 x 10^{-5} mol) dissolved in dH_2O (1 mL) was added, followed by NaOH (0.5 M) drop-wise to raise the pH to ~8.0. The reaction mixture was left stirring for up to 18 h, typically for 2 h. The conjugate was then purified from the reaction mixture by fast protein liquid chromatography (FPLC) (ÄKTA FPLC; Amersham Pharmacia Biotech, UK) using a pre-packed Superdex 75 10/300 GL column with a UV detector and data analysis using Unicorn 4.0 software (Amersham Pharmacia Biotech, UK). Samples of the reaction mixture (0.5 mL) were injected into a 500 μL loop using PBS (pH 7.4), pH 7.4 at 0.5 mL/min as a mobile phase. Fractions (1 mL) were collected, desalted using Vivaspin tubes (5,000 g/mol cut-off) and assayed for protein content (BCA assay) before pooling fractions containing conjugate (typically fractions 6-13). The final conjugate was lyophilised and stored at -20 ^{o}C.

Characterization of dextrin-colistin conjugates. Dextrin-colistin was characterized by FPLC and GPC to assess purity and estimate molecular weight, and the total protein content of the conjugate was determined by the BCA assay using colistin standards.

The FPLC system described above for purification was used again for final conjugate characterization. Samples (200 μL) were dissolved in PBS (pH 7.4) and injected into a 100 μL loop at 0.5 mL/min. The molecular weight was estimated using GPC relative to pullulan standards.

Ninhydrin assay. Prior to conjugation of polymer to protein, a ninhydrin assay was used to confirm the number of available amine groups in colistin for conjugation and how many amine groups were subsequently used for binding to dextrin. First, a 4 M lithium acetate buffer solution was prepared by dissolving lithium acetate dihydrate (40.81 g) in 60 mL dH_2O. Sufficient acetic acid (glacial) was added until pH 5.2 was reached. The volume was made up to a final volume of 100 mL with dH_2O. Next, ninhydrin (0.2 g) and hydrindantin (0.03 g) were dissolved in 7.5 mL DMSO and 2.5 mL lithium

acetate buffer. Buffered ninhydrin reagent (86 μL) was added to an equal quantity of sample/ standard solution (1.5 mL eppendorf) and heated in a water bath at 100 ^{o}C for 15 min. The mixture was subsequently cooled to room temperature and 130 μL of 50% v/v ethanol was added. The solution was mixed before adding 200 μL of the final solution into wells of a 96-well plate. Spectrophotometric analysis was performed at 570 nm. Calibration of the assay was achieved using ethanolamine (0-0.1158 mM).

Degradation of dextrin, succinoylated dextrin and dextrin-colistin conjugates by amylase. To compare the rate of amylase degradation of dextrin, succinoylated dextrin and dextrin-colistin conjugates, solutions (3 mg/mL in PBS, pH 7.4) of each sample was prepared containing amylase (100 IU/L in PBS) and incubated at 37 ^{o}C for up to 48 h. At various time points, samples (300 μL) were taken, immediately snap-frozen in liquid nitrogen to stop the reaction and stored at –20 ^{o}C until analysis by GPC and FPLC (conjugates only). Prior to analysis, samples were placed in a water bath (100 ^{o}C) for 5 min to denature the enzyme activity of the amylase and stop polymer degradation. The supernatant was then analyzed by GPC to determine the change in molecular weight over time and by FPLC to determine the change in free colistin over time.

***In vitro* toxicity assay: MTT assay.** An MTT assay was used to assess cell viability in a mouse macrophage (RAW 264.7) cell line (24 h incubation) and a human kidney (HK-2) cell line (72 h incubation). RAW 264.7 cells were seeded into sterile, 96-well microtiter plates (2 x 10^5 cells/mL) in 0.1 mL/well of media (DMEM) containing heat-inactivated FCS (1% v/v) and HK-2 cells were seeded into sterile 96-well microtiter plates (1 x 10^5 cells/mL) in 0.1 mL/well of media (K-SFM) containing L-glutamine, EGF and BPE. They were allowed to adhere for 24 h. The medium was then removed and test compounds (0.2 μm filter-sterilized) were added to the cells. To study the effect of colistin sulfate, CMS and dextrin-colistin conjugate (7,500 g/mol, 1.1 mol% succinoylation) on cell viability, complete media was supplemented with a range of concentrations of each. To study the effect of 'unmasked'

dextrin-colistin on cell viability, complete media was supplemented with a range of concentrations of conjugate and amylase (100 IU/L). After a further 67 h incubation, MTT (20 μL of a 5 mg/mL solution in PBS) was added to each well and the cells were incubated for a further 5 h. The medium was then removed and the precipitated formazan crystals solubilized by addition of optical grade DMSO (100 μL) over 30 min. Absorbance was measured at 540 nm using a microtiter plate reader. Cell viability was expressed as a percentage of the viability of untreated control cells. The IC_{50} values were expressed as mean ± SEM (n=18).

***In vitro* toxicity assay: LDH assay.** The LDH assay was used to assess cell membrane damage (24 h incubation) in a HK-2 cell line. Cells were seeded into sterile 96-well microtiter plates (1×10^5 cells/ mL) in 0.1 mL/well of media (K-SFM) containing L-glutamine, EGF and BPE. They were allowed to adhere for 24 h. The medium was then removed and test compounds (0.2 μm filter-sterilized) were added to the cells. To study the effect of colistin sulfate, CMS and dextrin-colistin conjugate on cell membrane integrity, complete media was supplemented with a range of concentrations of each. To study the effect of 'unmasked' dextrin-colistin on cell viability, complete media was supplemented with a range of concentrations of conjugate and amylase (100 IU/L). After 24 h, microtiter plates were centrifuged (600 g, 10 min), the supernatant was transferred to a clean 96-well plate and stored at -20 ^{o}C until determination of LDH content. LDH content in the cell supernatant was determined using a commercial LDH-cytotoxicity assay kit following the manufacturer's protocol. Absorbance was measured at 450 nm using a microtiter plate reader. The absorbance values were expressed as mean ± SEM (n=6).

Measurement of antimicrobial activity. Antimicrobial activity was measured using broth micro-dilution in a standard MIC assay[17]. Test organisms were suspended in Mueller Hinton cation-adjusted broth (100 μL, $1 - 5 \times 10^4$ CFU/mL) and incubated in 96-well microtiter plates in serial two-fold dilutions of the test compounds.

The antimicrobial activity of dextrin-colistin conjugates was similarly determined following incubation (3 mg/mL colistin equiv.) with amylase (100 IU/L for up to 48 h at 37 °C in PBS buffer (pH 8.2)).

***In vivo* dose escalating studies.** Sprague-Dawley rats (390-470 g) (Charles River (UK) Limited) with a jugular vein cannulation were housed in an animal care facility and acclimatised for 1 week prior to experimentation. During the on-study period, rats were housed individually in a metabolism cage with food and water *ad libitum*.

On the day of the experiment, colistin sulfate, dextrin-colistin conjugate (1.4 mol% succinoylated 7,500 g/mol dextrin) and dextrin-colistin conjugate (7.2 mol% succinoylated 7,500 g/mol dextrin) were dissolved in 0.9% w/v sterile saline (0.1 mg/mL colistin equiv.), filtered (0.22 μm) and injected via the tail vein as a bolus to 2 rats per test item (0.1 mg/kg colistin equiv.). Following administration, blood was collected (5 and 30 min, 1, 4, 8 and 24 h post-dose). Blood samples were placed into tubes containing lithium heparin and centrifuged (3000 rpm, for 10 min at 4 ^{o}C) immediately, before storing the resulting plasma samples at -20 ^{o}C prior to assay. The concentration of "total" colistin in plasma was assessed using a commercial colistin ELISA test kit, according to the manufacturer's instructions.

Pharmacokinetic Analysis. Non-compartmental analysis of the pharmacokinetics of colistin sulphate and dextrin-colistin conjugates was performed using GraphPad Prism, version 6.0d for Macintosh, 2014. The following pharmacokinetic parameters were calculated: peak plasma colistin concentration after administration (C_{max}), time to reach (t_{max}), plasma half-life ($t_{1/2}$), area under the concentration-time curve to 24 h ($AUC_{0\text{-}24\ h}$), volume of distribution (V_d) and total body clearance (CL). Plasma half-life was calculated following linear regression analysis of log-transformed plasma concentration–time points. The $AUC_{0–24\ h}$ of colistin was calculated with the presumption of the initial concentration being zero.

Statistical Analysis. Data are expressed as mean ± the error, calculated as either standard

deviation (SD) where n = 3, or standard error of the mean (SEM) where n > 3. Statistical significance was set at $p < 0.05$ (indicated by *). Evaluation of significance was achieved using a one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests that correct for multiple comparisons. All statistical calculations were performed using GraphPad Prism, version 6.0d for Macintosh, 2014.

Results

Synthesis and characterization of dextrin-colistin conjugates. The characteristics of the library of succinoylated dextrins synthesized are summarized in Table 1. Succinoylated dextrin intermediates were synthesized having a degree of modification of 1-29 mol% and showed increased FT-IR signal strength of the ester peak (~1,720 cm^{-1}) relative to degree of modification (Figure 3(a) and Figure SI 1(a, c, e)). GPC using pullulan standards suggested an increase in dextrin molecular weight following succinoylation with little change in polydispersity.

Using theses succinoylated dextrin intermediates, a series of dextrin-colistin conjugates were prepared (Table 1). Typical FPLC elution profiles of the purified conjugates and free colistin are shown (Figure 3(b)), with the conjugate typically eluting in the void volume of the column, enabling the separation of free and bound colistin. The protein content was 3-23% w/w. FPLC analysis confirmed the presence of a high molecular weight conjugate, however, the free colistin content was always < 4%. The ninhydrin assay indicated that colistin has 4.8 NH_2 groups per molecule and that dextrin typically bound to ~3 of these groups in dextrin-colistin conjugates.

Degradation of dextrin and dextrin-colistin conjugate by amylase. GPC analysis of dextrin degradation by amylase revealed a decrease in molecular weight with time (Figure 4(a) and Figure SI 1(b,d,f)). While dextrin degraded rapidly ($t_{1/2}$<30 min) in the presence of amylase, chemical modification by succinoylation slowed the rate of degradation ($t_{1/2} \geq 4$ h).

When degraded dextrin-colistin conjugates were analyzed by FPLC, a peak corresponding to

free colistin appeared (~16 mL), which increased in intensity with time (Figure 4(c) and Figure SI 2). In parallel, the peak corresponding to dextrin-colistin conjugate (~7.5 mL) decreased and GPC analysis revealed a reduction in conjugate molecular weight over the incubation timecourse (Figure 4(b)). This corresponds to an increasing concentration of free colistin in parallel to reducing concentrations of dextrin-colistin conjugate; indicative of release of colistin from the conjugate due to amylase degradation of dextrin. In these experiments, dextrin-colistin conjugates containing low molecular weight dextrin (7,500 g/mol) with a low level of polymer modification (1.1 mol%) released the most free colistin (~80%) after 48 h incubation (Table SI 1). Amylase-triggered dextrin-colistin conjugates synthesized using low molecular weight (7,500 g/mol) dextrin released more than twice as much free colistin within 48 h than the commercially available CMS (Figure 4(d)).

Stability of dextrin-colistin and CMS *in vitro*. Dextrin-colistin conjugates (7,500 g/mol conjugates at 1.1, 2.5, 4.7 and 8.3 mol%) were stable in water and PBS at 4 ^{o}C (Figure 5, Table SI 1). After 48 h, the percentage of liberated colistin was typically <5%. Dextrin-colistin conjugates were less stable in water at 37^{o}C in the absence of amylase, and increased colistin was evident in PBS at 37^{o}C in the absence of amylase. Release of colistin by hydrolysis was also greatest for conjugates containing low degrees of succinoylation. CMS was less stable in PBS than water, releasing 33.0% free colistin after 48 h in PBS at 37^{o}C. After incubation of CMS in PBS or water at 4^{o}C for 48 h, more free colistin was detected than for any of the dextrin-colistin conjugates.

***In vitro* toxicity assay.** The concentration-dependent cytotoxicity of colistin sulfate, CMS and dextrin-colistin conjugates (with and without amylase) is shown in Figure 6. The dextrin-colistin conjugate caused less metabolic changes and membrane damage than free colistin and CMS in RAW 264.7 and HK-2 cells. Cell viability, measured by MTT assay, was greatest in HK-2 (kidney) cells (IC_{50} = 11.0 ± 1.0 μg/mL (colistin sulfate) > 15.4 ± 1.0 μg/mL (CMS) > 35.5 ± 1.3 μg/mL (dextrin-colistin with amylase) > 63.9 ± 1.6 μg/mL (dextrin-colistin)), compared to RAW 264.7 (macrophage)

cells (IC_{50} = 111.3 ± 1.1 μg/mL (CMS) > 180.5 ± 1.3 μg/mL (dextrin-colistin with amylase) > 187.4 ± 1.1 μg/mL (colistin sulfate) > 303.9 ± 1.4 μg/mL (dextrin-colistin)) (Figure 6(a,b)). Similarly, the LDH assay in HK-2 cells demonstrated that dextrin-colistin conjugate caused significantly less (~50%) membrane damage than colistin sulfate or CMS (Figure 6(c)), however, amylase unmasking of dextrin-colistin conjugate restored the membrane permeabilization activity of colistin. Likewise, dextrin-colistin conjugates induced significantly less erythrocyte lysis after a 24 h incubation than colistin sulfate or CMS, even in the presence of amylase (Figure 6(d)).

Measurement of antimicrobial activity. Conjugation of dextrin masked the antimicrobial activity of colistin in a panel of Gram-negative bacteria to some extent, typically increasing the MIC to 2-fold that of CMS. Pre-incubation with amylase (at physiological concentrations) generally caused a 1-fold reduction in MIC value, compared to untreated conjugate (Tables 2, SI 2). Antimicrobial activity was greatest for conjugates containing low molecular weight dextrin (7,500 g/mol) with a low degree of succinoylation (1.1 mol%).

***In vivo* pharmacokinetics.** Figure 7 shows the mean concentrations of colistin in plasma as a function of time in 2 rats after an IV dose of 0.1 mg/kg colistin sulfate or dextrin-colistin conjugates. In all cases, there was a short distribution phase (5-60 min) followed by an elimination phase. After 8 h, the concentration of colistin sulfate was below the limit of quantification, but for both conjugates, drug was detectable in the plasma 48 h post-dose. Colistin sulfate had a significantly shorter half-life than the dextrin-colistin conjugates (53.2 min vs. 135.3 min (1.1 mol%) and 1,270.9 min (7.2 mol%)) (Table 3).

Discussion

While previous applications of PUMPT, in cancer and wound repair[5,18], require prolonged release of the bioactive protein over several days, acute bacterial infections need much more rapid and

307 complete release of the antibiotic at sites of infection. Here we have optimized the release of colistin by
308 varying the degree of dextrin functionalization. It has previously been shown that dextrin's degradation
309 rate, and consequent drug release, can be extended by increasing its molecular weight and degree of
310 succinoylation[12]. In these studies, dextrin-colistin conjugates were synthesized using dextrins with
311 molecular weights between 7,500 and 48,500 g/mol and 1-29 mol% succinoylation. Typically, dextrin-
312 colistin conjugates contained 1-2 dextrin chains per colistin molecule, bound via ~3 primary amine
313 groups. Dextrin degradation by amylase breaks the glycosidic bonds within the polymer chain, leaving
314 oligosaccharides and/or maltose linked to colistin. These cationic amine groups play an important role
315 in the interaction of colistin with bacterial lipopolysaccharides, as well as permeabilization of the
316 kidney's proximal tubule, so the number of binding sites was optimized to sufficiently 'mask' colistin
317 during transit but allow sufficient antimicrobial activity to be regenerated at the target site.

318 Couet et al.[19] recently reported that only 30% of the CMS dose administered intravenously to
319 healthy volunteers was converted into colistin after 24 h. Here, we demonstrated, using FPLC, that *in*
320 *vitro* hydrolysis of CMS in PBS at 37 ^{o}C released just 23.6% free colistin after 24 h. In contrast,
321 amylase-triggered release of colistin from dextrin-colistin conjugates released up to 80% free drug in
322 the same period. While amylase triggered significant release of free colistin from conjugates, stability
323 experiments showed that drug may also be released at a slower rate in the absence of amylase. This is
324 important as some patients, such as those with cystic fibrosis, display reduced physiological levels of
325 amylase[20], and 'enzyme-free' liberation of drug may be an advantage in these patients. In all cases,
326 however, the release of free colistin from dextrin-colistin conjugates was less than from amylase-
327 activated conjugates. While *in vivo* liberation of colistin from dextrin-colistin conjugates is important
328 for antibacterial activity, release of drug during storage may be harmful. Storage of the dextrin-colistin
329 conjugates in solution at 4^{o}C resulted in minimal (≤5.3% after 48 h) colistin release, however, up to
330 13.2% colistin was liberated from CMS under the same conditions.

331 Colistin exerts its bactericidal activity by permeabilizing bacterial membranes[21,22]. However,

since this mechanism is only partially selective, polymyxins can also increase the permeability of mammalian cells- leading to an increased influx of cations, anions, and water, which eventually result in cell swelling and lysis[23,24]. Permeabilization of renal proximal tubule cells has been attributed to colistin's dose-dependent nephrotoxicity, affecting up to 36% of patients receiving intravenous CMS[25]. Biocompatibility of dextrin-colistin conjugates is essential for safe systemic administration. HK-2 cells were chosen for these preliminary *in vitro* toxicity studies since they are derived from the proximal cells of human kidneys and are known to retain the functional characteristics of proximal tubular epithelium[26]. The MTT and LDH assays demonstrated that dextrin conjugation significantly reduced the cytotoxicity of colistin. Since colistin is only weakly cytotoxic (compared to an anti-cancer agent), and toxicity normally ensues from long-term use, treatment incubation times for the toxicity assays were chosen to replicate the prolonged exposure observed from the conjugates due to extended plasma half-life observed in the *in vivo* studies. Given that hemocompatibility would be critical to ensure safe systemic administration of dextrin-colistin conjugates following IV injection, the finding that the concentration-dependent erythrocyte toxicity exhibited by colistin was effectively inhibited by dextrin conjugation, but only partially restored after amylase unmasking, was also encouraging. In these experiments, dextrin conjugation via colistin's cationic amine groups, and the inability of amylase degradation to restore these positively charged groups, could explain the reduced toxicity of dextrin-colistin conjugates in comparison to colistin and CMS and support the 'masking' effect of polymer conjugation. Previous attempts to reduce the toxicity of polymyxin antibiotics, by eliminating two of the five cationic groups, have been described by Vaara *et al*, resulting in a 6-7-fold lower affinity for the brush border membrane of the renal cortex[27].

Having confirmed that dextrin conjugation could mask colistin's cytotoxicity, it was important to confirm that conjugates retained antimicrobial activity prior to future clinical testing. Drug resistance often occurs when bacteria are exposed to sub-optimal concentrations of antibiotic, while high doses can cause unpleasant or harmful side effects. Masking of antibiotic activity in transit followed by

efficient reinstatement of antibiotic activity after passive localisation at sites of infection/ inflammation by the EPR effect, provides an ideal means of optimising drug dosing to reduce the emergence of resistance. The Clinical and Laboratory Standards Institute (CLSI) susceptibility breakpoints for colistin have been identified in *Acinetobacter baumannii* (susceptible, ≤2 mg/L; resistant, ≥4 mg/L) and *Pseudomonas aeruginosa* (susceptible, ≤2 mg/L; intermediate, 4 mg/L; resistant, ≥8 mg/L)[28], while the general MIC breakpoints for CMS susceptibility are typically higher (susceptible, ≤4 mg/L; resistant, ≥8 mg/L)[29], Despite their higher *in vitro* cytotoxicity, colistin sulfate and CMS were very active against Gram-negative bacteria (with MIC values as low as 0.016 mg/L and 0.25 mg/L, respectively) (Table 2). As expected, dextrin conjugation reduced the antimicrobial activity of colistin, with dextrins of higher molecular weight and degrees of succinoylation causing an almost quantitative decrease in activity. Dextrin-colistin conjugates were not always below the susceptibility breakpoints, even after amylase unmasking with equivalent concentrations of amylase as that found in human serum. However, since dextrin-colistin conjugates are hypothesized to accumulate in areas of inflammation and infection by the EPR effect[11], serum concentration will not predict the drug concentration at disease sites and pharmacokinetic studies will be important to characterize the distribution and local concentration of the conjugates. This will represent an important future series of experiments in pre-clinical studies.

The pharmacokinetics of a single IV bolus dose of two "prototype" conjugates with contrasting degrees of succinoylation (1.4 and 7.2 mol%) was tested in comparison to colistin sulfate, to determine if conjugation increased the plasma half-life of colistin, and to help identify a suitable dosing interval for subsequent repeated-dose studies. These studies employed a commercial colistin ELISA test kit since high-performance liquid chromatography (HPLC), the standard method for colistin detection in plasma and urine[30-33], was not possible as dextrin conjugation inhibits colistin binding to the HPLC column (data not shown). In accordance with our hypothesis, preliminary experiments showed that, after IV administration, the blood clearance of dextrin-colistin conjugates was markedly inhibited by

conjugation. Similarly, conjugation of dextrin (6,600 g/mol) to zidovudine also extended the plasma half-life from 1.3 to 19.3 h and an increase in the area under the plasma concentration-time curve[34]. The effect of degree of dextrin succinoylation on *in vivo* pharmacokinetics has not previously been reported, however, as hypothesized, increasing the degree of modification led to extended plasma half-life of colistin. Given that serum amylase concentration in rats is in excess of 20 times higher than in human serum[35,36], we predict that unmasking of the conjugate in humans would be slower, thereby increasing plasma retention further. Rats in these studies had a plasma amylase concentration of 3,068 ± 119 IU/L (measured by Phadebas® assay), compared to the normal human plasma amylase range of 40-125 IU/L. Interestingly, whilst this study was not designed to measure toxicity, the administration of 0.5 mg/kg colistin sulfate, induced signs of systemic toxicity which prevented sampling until 2 h post-dose, which was not evident at equivalent concentrations of the dextrin-colistin conjugates.

These studies demonstrate the clear potential of bioresponsive polymer therapeutics-based "nanoantibiotics", in which colistin is conjugated to dextrin, a clinically approved biodegradable polymer. Having established the optimum composition of dextrin-colistin conjugates which show comparable antimicrobial activity to commercially available CMS, but with reduced toxicity, ongoing work is determining *in vivo* distribution and clinical effectiveness.

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Supporting Information: Additional experimental details as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Table 1 Characteristics of succinoylated dextrins and dextrin-colistin conjugates.

Compound	Dextrins		Dextrin-colistin conjugates				
	Mw[a] (g/mol) (M_w/M_n)	Succinoylation (mol%)	Mw[a] (g/mol) (M_w/M_n)	Protein content (% w/w)	Molar ratio (dextrin:colistin)	Conjugated NH_2 per molecule[b]	Free protein (%)
Colistin	1,408						
Dextrin	7,500 (1.8)						
Succ. dextrin (7,500 g/mol)	8,500 (1.9)	1.1	9,000 (1.5)	10.1	1.6:1	3.3	3.6
Succ. dextrin (7,500 g/mol)	9,000 (1.9)	2.5	14,500 (1.5)	17.0	0.9:1	3.2	3.7
Succ. dextrin (7,500 g/mol)	9,500 (2.0)	4.7	15,500 (1.7)	22.2	0.6:1	3.0	3.0
Succ. dextrin (7,500 g/mol)	12,000 (1.8)	8.3	22,000 (1.6)	21.6	0.6:1	3.5	0.7
Dextrin	10,500 (2.6)						
Succ. dextrin (10,500 g/mol)	13,500 (2.0)	2.2	35,000 (2.7)	10.6	0.8:1	1.4	2.8
Succ. dextrin (10,500 g/mol)	17,500 (2.2)	4.3	165,000 (4.3)	11.5	0.7:1	1.6	2.4
Succ. dextrin (10,500 g/mol)	17,500 (2.0)	7.0	180,000 (6.4)	14.5	0.4:1	1.4	2.7
Dextrin	28,000 (2.6)						
Succ. dextrin (28,000 g/mol)	28,500 (1.9)	2.0	95,000 (4.3)	3.3	1.4:1	2.2	1.3
Succ. dextrin (28,000 g/mol)	39,000 (2.0)	3.4	140,000 (3.8)	3.0	1.5:1	1.4	1.2
Succ. dextrin (28,000 g/mol)	45,000 (1.9)	6.1	270,000 (5.5)	6.6	0.7:1	1.0	0.8
Dextrin	48,500 (2.2)						
Succ. dextrin (48,500 g/mol)	98,000 (1.9)	17.4	55,000 (1.8)	5.1	1:1	1.2	0.1
Succ. dextrin (48,500 g/mol)	97,000 (1.7)	28.6	66,500 (1.7)	7.3	0.7:1	1.3	0.1

[a] M_w was estimated by GPC using pullulan standards. [b] usually 4.81 NH_2 per free colistin.

Table 2 Antimicrobial activity of colistin sulfate, CMS and dextrin-colistin conjugates* (containing 1.1 mol% succinoylated dextrin with and without amylase pre-exposure), measured by MIC assay. Data is expressed as mode (*n*=3). *MIC value represents equivalent colistin concentration of conjugates.

	MIC (□g/L)					
Isolate	Colistin sulfate	CMS	Conjugate at indicated amylase pre-incubation (h)			
			0	3	6	24
V4 A. baumannii MDR ACB	0.125	4	8	8	8	8
V9 A. baumannii	0.25	4	16	16	16	8
V19 A. baumannii 7789	0.25	2	8	16	8	16
V20 A. lwoffi 8065	0.063	1	8	4	4	4
V22 A. lwoffi 6056	0.125	2	16	8	8	8
V5 E. coli AIM-1	0.016	0.25	1	0.5	0.5	0.125
V11 E. coli 5702	0.5	1	8	4	8	2
V24 E. coli 7273	0.25	2	16	32	16	8
V12 K. pneumoniae 5725	0.5	1	4	4	4	2
V6 K. pneumoniae IR25	0.5	2	8	8	8	8
V8 K. pneumoniae K3	0.5	2	8	16	16	8
V3 K. pneumoniae KP05 506	0.25	2	4	16	16	16
V13 P. aeruginosa PA01	0.25	1	64	64	128	64
V1 P. aeruginosa R22	0.5	0.5	128	128	128	128
V2 P. aeruginosa MDR 301	0.5	1	512	512	512	512
V7 P. stuartii IR57	0.25	1	4	16	8	8

Table 3 Mean estimates of pharmacokinetic parameters for colistin sulfate or dextrin-colistin conjugates in rats (n = 2).

Pharmacokinetic parameter	**Colistin sulfate**	**Dextrin-colistin (1.4 mol%)**	**Dextrin-colistin (7.2 mol%)**
C_{max} *(ng/mL)*	96.1	106.7	101.6
t_{max} *(min)*	5	30	60
$t_{1/2}$ *(min)*	53.2	135.3	1270.9
$AUC_{0\text{-}24\ h}$ *(mg.min/mL)*	0.0082	0.0342	0.0913
V_d *(mL/kg)*	669.7	676.4	925.7
CL (mL/min/kg)	13.4	2.8	1.1

Legends to Figures

Figure 1 Schematic showing the proposed mechanism of action of dextrin-colistin conjugates.

Figure 2 Synthesis of (a) succinoylated dextrin; and (b) dextrin-colistin conjugates.

Figure 3 Characterization of succinoylated dextrin intermediates and dextrin-colistin conjugates. (a) FT-IR spectra showing amplification of peak intensity at 1720 cm^{-1} with increasing incorporation of carboxyl groups; and (b) FPLC chromatogram of dextrin-colistin conjugates containing dextrins (7,500 g/mol) with different degrees of succinoylation (V_0 = void volume (7.7 mL)).

Figure 4 Characterization of the degradation of dextrin, succinoylated dextrin and dextrin-colistin conjugates (3 mg/mL) in the presence of amylase (100 IU/L in PBS at 37 ^oC). Panels (a) and (b) show the change in relative molecular weight in the presence of amylase by GPC of (a) native dextrin (7,500 g/mol) and its succinoylated intermediates; and (b) dextrin-colistin conjugates containing dextrin (7,500 g/mol) with different degrees of succinoylation. Panel (c) shows a typical elution profile of dextrin-colistin conjugate (containing 7,500 g/mol dextrin, 4.7 mol% succinoylation) from a Superdex 75 FPLC column, following incubation with amylase (V_0 = void volume (7.7 mL)), and panel (d) shows the release of colistin from dextrin-colistin conjugates

(containing 7,500 g/mol dextrin; 3 mg/mL) in the presence of amylase (100 IU/L in PBS) and CMS (3 mg/mL) in PBS at 37 ^{o}C (measured by FPLC). Data is expressed as the percentage of total colistin. (*n*=1). Where ◆ = dextrin or dextrin-colistin conjugate; ○ = 1.1 mol% dextrin or dextrin-colistin conjugate; ▼ = 2.5 mol% dextrin or dextrin-colistin conjugate; ✳ = 4.7 mol% dextrin or dextrin-colistin conjugate; ● = 8.3 mol% dextrin or dextrin-colistin conjugate; ■ = 21.3 mol% dextrin or dextrin-colistin conjugate and □ = CMS.

Figure 5 Stability of dextrin-colistin conjugates (containing 7,500 g/mol dextrin; 3 mg/mL) in dH_2O and PBS at pH 7.4 (37 ^{o}C) in the absence of amylase (measured by FPLC), and in comparison with amylase-treated conjugates. Data is expressed as the percentage of total colistin. Panels show conjugates containing (a) 1.1 mol% succinoylation, (b) 2.5 mol% succinoylation, (c) 4.7 mol% succinoylation, and (d) 8.3 mol% succinoylation. Where ● = dextrin-colistin conjugate with amylase (100 IU/L) in PBS at 37 ^{o}C; ▼ = dextrin-colistin conjugate in PBS at 37 ^{o}C; ◆ = dextrin-colistin conjugate in PBS at 4 ^{o}C; ■ = dextrin-colistin conjugate in dH_2O at 37 ^{o}C and △ = dextrin-colistin conjugate in dH_2O at 4 ^{o}C.

Figure 6 *In vitro* cytotoxicity of colistin sulfate, CMS and dextrin-colistin conjugates. Panels (a) and (b) show cell viability by MTT assay of RAW 264.7 (24 h incubation) and HK-2 (72 h incubation) cells, respectively, following incubation with colistin sulfate, CMS or

dextrin-colistin conjugate with and without amylase (100 IU/L) at 37 ^{o}C. Data is expressed as mean % untreated control ± SEM, n=18. Panel (c) shows membrane integrity by LDH assay of HK-2 cells incubated for 24 h with colistin, CMS or dextrin-colistin with and without amylase (100 IU/L) at 37 ^{o}C. Data is expressed as mean ± SEM, n=6. Panel (d) shows hemolysis of rat erythrocytes following incubation for 24 h with colistin, CMS or dextrin-colistin conjugate with and without amylase (100 IU/L) at 37 ^{o}C. Data is expressed as mean % triton X-100 control ± SEM, n=18. Where ● = colistin sulfate; × = CMS; ◇ = dextrin-colistin conjugate and ▲ = dextrin-colistin conjugate with amylase. * indicates significance (p<0.05) compared to colistin sulfate; ** indicates significance (p<0.01) compared to colistin sulfate; *** indicates significance (p<0.001) compared to colistin sulfate.

Figure 7 Mean plasma concentration of colistin following an IV dose of colistin sulfate, dextrin-colistin conjugate (1.4 mol%) and dextrin-colistin conjugate (7.2 mol%) (0.1 mg/kg). Data is expressed as colistin concentration ± S.D. (n=2). Concentrations of colistin following administration of colistin sulfate were not quantifiable beyond 4 h. Where ● = colistin sulfate; □ = dextrin-colistin conjugate (with 1.4 mol% succinoylation) and ◆ = dextrin-colistin conjugate (with 7.2 mol% succinoylation). * indicates significance (p<0.05) compared to colistin sulfate; ** indicates significance (p<0.01) compared to colistin sulfate; *** indicates significance (p<0.001) compared to colistin sulfate.