Development and Validation of an In Vitro Pharmacokinetic/Pharmacodynamic Model To Test the Antibacterial Efficacy of Antibiotic Polymer Conjugates

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This study describes the use of a novel, two-compartment, static dialysis bag model to study the release, diffusion, and antibacterial activity of a novel, bioreponsive dextrin-colistin polymer conjugate against multidrug resistant (MDR) wild-type Acinetobacter baumannii. In this model, colistin sulfate, at its MIC, produced a rapid and extensive drop in viable bacterial counts (<2 \log_{10} CFU/ml at 4 h); however, a marked recovery was observed thereafter, with regrowth equivalent to that of control by 48 h. In contrast, dextrin-colistin conjugate, at its MIC, suppressed bacterial growth for up to 48 h, with 3 \log_{10} CFU/ml lower bacterial counts after 48 h than those of controls. Doubling the concentration of dextrin-colistin conjugate (to 2× MIC) led to an initial bacterial killing of 3 \log_{10} CFU/ml at 8 h, with a similar regrowth profile to 1× MIC treatment thereafter. The addition of colistin sulfate (1× MIC) to dextrin-colistin conjugate (1× MIC) resulted in undetectable bacterial counts after 4 h, followed by suppressed bacterial growth (3.5 \log_{10} CFU/ml lower than that of control at 48 h). Incubation of dextrin-colistin conjugates with infected wound exudate from a series of burn patients (n = 6) revealed an increasing concentration of unmasked colistin in the outer compartment (OC) over time (up to 86.3% of the initial dose at 48 h), confirming that colistin would be liberated from the conjugate by endogenous α-amylase within the wound environment. These studies confirm the utility of this model system to simulate the pharmacokinetics of colistin formation in humans administered dextrin-colistin conjugates and further supports the development of antibiotic polymer conjugates in the treatment of MDR infections.
models may be used to produce concentration gradients, although they require considerable technical expertise and specialist software and are associated with high costs (18). In the development and screening of nanoantibiotics, a need thus exists for a suitable in vitro model to define the PK/PD profiles and optimize the characteristics of these novel agents prior to in vivo testing.

This study employed dextrin-colistin conjugates as prototypical nanoantibiotics to develop a model system which would afford the opportunity to study the in vitro degradation, diffusion, and activation of the conjugate alone and in the presence of amylase or infected wound fluid. The study sought to define an in vitro model in which the PK/PD parameters of biodegradable antibiotic polymer conjugates can be reliably assessed.

**MATERIALS AND METHODS**

**Materials.** Colistin sulfate, ethanol, human salivary α-amylase, 4-dimethylaminopropylidene (DMAP), anhydrous N,N-dimethylformamide (DMF), succinic anhydride, diethyl ether, type 1 dextrin from cornstarch (molecular weight, ~8,100 g/mol), and carboxi anhydrase (molecular weight, 29,000 g/mol) were purchased from Sigma-Aldrich (Poole, United Kingdom). Sodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, potassium chloride, sodium hydroxide (NaOH), copper (II) sulfate pentahydrate, bicinchoninic acid (BCA) assay kit, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Thermo Fisher Scientific (Loughborough, United Kingdom). Sterile 0.9% sodium chloride solution was obtained from Baxter (Berkshire, United Kingdom). N-hydroxysulfosuccinimide (sulfo-NHS) was purchased from Fluka (Buchs, Switzerland). Trypticase soy agar (TSA), Trypticase soy broth (TSB), and cation-adjusted Mueller-Hinton broth (CAMHB, lot 43145) were purchased from Oxoid (Basingstoke, United Kingdom). Polyvinylidene fluoride sterile membrane syringe filters (0.22 μm) were from Fluka (Buchs, Switzerland). Escherichia coli (E. coli) National Collection of Type Cultures (NCTC) 10418 and Acinetobacter baumannii 7789 (v19) from clinically infected wounds (19) were donated by R. Howe (Public Health Wales Microbiology Laboratory, University Hospital of Wales, Cardiff, United Kingdom). The sensitivities of these strains were reported previously (19). MIC values of A. baumannii 7789 were previously reported as 0.0625 and 8 μg/ml for colistin sulfate and dextrin-colistin conjugate (plus amylase), respectively (20).

**In vitro PK/PD model.** The unmasking of dextrin-colistin conjugates was simulated using a two-compartment static dialysis bag model under sink conditions (total volume, 20 ml; inner-compartment volume, 5 ml), as shown schematically in Fig. 1. Dialysis membrane (10,000 g/mol molecular weight cutoff [MWCO]) was presoaked for 15 to 30 min in distilled water (dH2O), secured with dialysis clips, and suspended from an injection port to separate the inner compartment (IC) from the outer compartment (OC) in a sterilized 25-ml beaker sealed with a sterile medical-grade polyurethane membrane (Tegaderm). The model system was prepared under aseptic conditions in a class 2 laminar airflow cabinet and transferred to a shaking incubator set at 37°C in ambient air and constant orbital agitation at 70 rpm for 48 h.

**Validation of in vitro PK/PD model.** To validate the ability of the dialysis membrane to retain intact dextrin-colistin conjugate in the IC while permitting unrestricted diffusion of unmasked colistin to the OC, the protein content in both compartments was measured over time. Briefly, the IC was spiked with dextrin-colistin conjugate (equivalent to 10 μg/ml colistin) in phosphate-buffered saline (PBS; pH 7.4, 37°C) in the absence and presence of α-amylase (100 IU/liter), while the OC contained sterile PBS (15 ml). The sealed beaker was incubated as described previously. Samples (150 μl) were extracted from each compartment at various intervals (0, 4, 8, 12, 24, 36, and 48 h) using sterile, medical-grade, single-use vascular catheters and immediately frozen on dry ice and stored at −20°C prior to analysis. Protein content was determined using a standard BCA assay, and the antimicrobial susceptibility of E. coli NCTC 10418 isolates was assessed using an MIC assay (see below). At the end of the 48

**FIG 1** Schematic illustration of the two-compartment static dialysis bag PK/PD model under infinite sink conditions (a) and the hypothesized distribution of dextrin-colistin conjugates (b).
h of incubation, FPLC was used to estimate the proportion of unmasked colistin in both compartments by calculating the area under the chromatographic curve. At the end of the experiment, the integrity of the dialysis membrane to α-amylase was confirmed using carbonic anhydrase as a molecular weight marker. The contents of both the IC and OC were removed. The dialysis membrane was flushed with several volumes of sterile D₂H₂O and then resuspended in sterile PBS as described previously. The IC was spiked with carbonic anhydrase (2 μg/ml) in PBS. After a further 17 h of incubation with PBS in the OC, the ratio of carbonic anhydrase in both compartments was quantified by UV-visible (UV-vis) spectrophotometry (n = 3).

Susceptibility testing. Antimicrobial activity in the OC was measured in E. coli NCTC 10418 isolates in a standard MIC assay (21). Isolates were grown overnight in TSB and then diluted in sterile PBS to an optical density at 625 nm (OD₆₂₅) of between 0.08 and 0.10 (equivalent to 0.5 McFarland standard; ~10⁶ CFU/ml). Serial 2-fold dilutions were prepared for each sample in flat-bottom 96-well microtiter plates (100 μl/well). The diluted bacterial cultures were then diluted 10-fold in CAMHB, and 5 μl was added to each well of the microtiter plate. Plates were incubated at 37°C in ambient air for 18 to 20 h, and the lowest concentration at which there was no visible growth was taken as the MIC. In addition, on each plate, triplicate wells were prepared containing no antibiotic and no bacterial inoculum as growth and sterility controls, respectively.

Time-to-kill assay. A modified TTK assay was used to investigate the concentration- and time-dependent antimicrobial activity of colistin sulfate, dextrin-colistin conjugate, and a mixture of colistin sulfate plus dextrin-colistin conjugate against A. baumannii 7789. In these experiments, the OC contained ~5 log₁₀ CFU/ml of A. baumannii 7789 in MHB, and the IC contained α-amylase (100 IU/liter) in sterile PBS in combination with (i) colistin sulfate (0.25 μg/ml, 1X MIC), (ii) dextrin-colistin conjugate (32 μg/ml colistin equivalent, 1X MIC), (iii) dextrin-colistin conjugate (64 μg/ml colistin equivalent, 2X MIC), or (iv) a mixture of colistin sulfate (0.25 μg/ml, 1X MIC) and dextrin-colistin conjugate (32 μg/ml colistin equivalent, 1X MIC). Colony counts (CFU/ml) from samples taken as described previously were performed in triplicate according to the method of Miles et al. (22). Briefly, samples (50 μl) were diluted 10-fold in PBS and centrifuged. Pellets were resuspended in an equivalent volume of fresh PBS. Serial 10-fold dilutions were performed in triplicate across a 96-well plate. Samples (5 μl) from each dilution were dropped onto the surface of the dried TSA plates in triplicate. The TSA plates were left undisturbed for 30 min, inverted, incubated for 18 to 20 h (37°C in ambient air), and then visually inspected for growth. The drop position left undisturbed for 30 min, inverted, incubated for 18 to 20 h (37°C in ambient air), and then visually inspected for growth. The drop position left undisturbed for 30 min, inverted, incubated for 18 to 20 h (37°C in ambient air), and then visually inspected for growth. The drop position left undisturbed for 30 min, inverted, incubated for 18 to 20 h (37°C in ambient air), and then visually inspected for growth.

Ex vivo sample collection. Following research ethics committee approval and informed consent, wound fluid samples were collected from adult infected burn wounds (n = 6) being treated at the Welsh Burns Centre (Swansea, United Kingdom). Burn wound infection was diagnosed according to the American Burn Association diagnostic criteria (23). Patients with recorded pancreatic or salivary disease were excluded. After dressing removal, the wounds were lightly washed with sterile 0.9% sodium chloride solution, and a sterile Teak gauze pad was used to collect the exudate. Samples were then transferred to a sterile syringe, sealed, frozen on dry ice, and stored at −80°C until required. Samples were centrifuged (15,000 × g for 5 min at 4°C), and then the α-amylase content was determined in triplicate using a Phadebas amylase test according to the manufacturer’s instructions.

In vitro modeling of dextrin-colistin conjugate unmasking in infected wound fluid. The same PK/PD setup was used to evaluate the feasibility of dextrin-colistin conjugate unmasking in infected burn wound samples. Each wound fluid sample was diluted 2-fold in PBS to make up the IC volume (5 ml), and dextrin-colistin conjugate (64 μg/ml colistin equivalent, 2X MIC) was added. For each wound sample, the procedure was repeated in triplicate. Total colistin content in the OC was quantified by ELISA according to the manufacturer’s instructions. Absorbance was measured at 450 nm using a microplate reader, and these values were used to calculate the colistin concentration in the OC (mean ± standard deviation [SD], n = 3) according to the calibration curve assay detection range, 0.5 to 50 ng/ml). The proportion of unmasked conjugate in the OC was calculated using FPLC, as previously described.

**RESULTS**

Method validation. Protein concentration in the IC decreased in a logarithmic fashion over time, while protein concentration in the OC increased reciprocally (Fig. 2). A corresponding increase in antimicrobial activity of the OC over the 48 h of incubation was also observed (Fig. 2). FPLC demonstrated that by 48 h, significant unmasking had occurred (P < 0.05), with unmasked conjugate accounting for ~68% of total colistin content in the system (Fig. 3). At 48 h, there was no significant difference in the distribution of unmasked conjugate between the two compartments (P > 0.05). Correspondingly, there was a significantly higher proportion of high-molecular-weight (masked) dextrin-colistin conjugate in the IC (~80%; P < 0.05) than in the OC (Fig. 3b). When the experiment was conducted using dextrin-colistin conjugate in the absence of α-amylase, the OC protein content was <10% after 17 h. Membrane integrity testing confirmed that the presence of carbonic anhydrase in the OC was always below the lower limit of quantification (data not shown).
PK/PD modeling against A. baumannii 7789 isolates. Colistin sulfate, at its MIC (0.0625 μg/ml), showed rapid initial killing, and viable bacterial counts were below the assay’s lower limit of quantification at 4 h. However, early and significant bacterial regrowth, to the level seen for controls, was observed from 8 h on (Fig. 4). Dextrin-colistin conjugate was bactericidal at 2× MIC, where the maximum reduction in viable bacterial counts (~3 log10 CFU/ml) occurred at 8 h. Thereafter, regrowth was slower than in the presence of colistin sulfate. In contrast, the colistin sulfate plus dextrin-colistin conjugate combination retained optimal characteristics of both components, with rapid initial bacterial killing, maximum reduction in viable bacterial counts at 4 h, and sustained reduction in viable bacteria throughout the experiment. The control growth curves confirmed that bacterial growth can be maintained under the experimental conditions, while sterility controls verified that sterility conditions were maintained throughout. Culture onto TSA plates at the end of each experiment confirmed the presence of a homogeneous culture.

α-Amylase activity in clinical samples. Wound fluid was collected from a consecutive series of six burn patients (Table 1). The total protein content in the supernatant was 50.7 ± 15.1 mg/ml (mean ± SD). These studies revealed that α-amylase activity was significantly increased in infected wound fluid (mean ± SD, 408.4 ± 168.3 IU/liter) compared to that in patient-matched serum (mean ± SD, 60.0 ± 25.3 IU/liter; P < 0.05) (Fig. 5).

Unmasking of dextrin-colistin conjugate in infected wound fluid. A time-dependent increase in colistin concentrations in the OC was observed in all cases (Fig. 6). Similarly, concentrations of colistin in the OC also increased in the presence of higher α-amylase activity, equating to a mean OC colistin content of 68.1% to 86.3% of the theoretical concentration of the original dose concentration at equilibrium (16 μg/ml) at 48 h.

DISCUSSION
We hypothesized that a static, two-compartment dialysis bag model under infinite sink conditions would offer a simple and cost-effective method for the initial PK/PD characterization of biodegradable antibiotic polymer conjugates. Using dextrin-colistin conjugate, we showed that the model can be employed to study degradation, diffusion, and activity across a biological membrane. Moreover, we demonstrated how these processes may be modified by local enzyme activity at sites of infection.

The use of a two-compartment system permitted the discrete
sampling and detailed analysis of the individual compartments over time. Furthermore, this model enabled the composition of the two compartments to be modified to represent the environmental conditions of the disease of interest, as demonstrated here using simulated and real biological fluids containing physiological concentrations of α-amylase. The ex vivo model described here can easily be adapted to allow modeling of disease- and target site-specific levels of enzymatic activity in the assessment of enzyme-triggered pharmacokinetics and pharmacodynamics using alternative biological fluids (e.g., wound fluid or bronchoalveolar lavage fluid). The model utilized Spectra/POR 7 membrane to prevent nonspecific drug binding to the membrane and heavy metal contamination (24–26). The MWCO of the dialysis membrane serves as a size exclusion barrier and can be selected according to the molecular weight of the drug carrier, antibiotic, or activating enzyme. Thus, the dialysis membrane can also effectively prevent premature denaturation of the activating enzyme by artificial media in the OC. The dextrin-colistin conjugates studied here were activated by α-amylase–mediated degradation of dextrin, resulting in a reduction in its molecular weight and diffusion of the unmasked conjugate along a concentration gradient to the OC (Fig. 1), where bioactivity can be assayed under various conditions; constant agitation rendered microcompartmentalization of antibiotic activity (4, 41). A potential limitation of this model is its inability to replicate the potential benefits of the EPR effect, representing an avenue for future development.

Modification of the IC to incubate dextrin-colistin conjugate directly in ex vivo infected wound fluid represented an adaptation of the PK/PD model to a higher level of complexity to enable ex vivo testing. In these studies, the α-amylase concentration in burn wound fluid was markedly higher than that in plasma. These results cannot be simply explained by the effects of thermal injury, as the mean time of sample collection was >2 weeks postburn, when vascular permeability and interstitial fluid leakage have largely delayed the biodistribution of colistin conjugates by sulphate-loaded liposomes. In this study, a combination of colistin and liposomes (37–39), which can readily be tested in our in vitro system. For instance, Wang et al. (40) used a similar two-compartment model to characterize the in vitro release kinetics of colistin sulfate-loaded liposomes. In this study, a combination of colistin sulfate and dextrin-colistin conjugate provided a rapid decline in viable counts, followed by a sustained suppression of bacterial growth rate. The conjugate unmasking by α-amylase was shown to be time dependent, meaning that in clinical use, administration of an initial dose of conventional antibiotic may be required when treating acute disease. In addition to improving the therapeutic efficacy of nanoformulated drugs, incorporating antibiotics into nano-sized structures also converts conventional small-molecule antibiotics into macromolecules, which may benefit from passive size-based targeting and accumulation at sites of inflammation/infection via the enhanced permeability and retention (EPR) effect (4, 41). A potential limitation of this model is its inability to replicate the potential benefits of the EPR effect, representing an avenue for future development.

The ability of the dialysis membrane to retain the intact conjugate within the IC until it is unmasked was confirmed during validation, where, in the absence of α-amylase, OC protein content remained at <10% at 17 h. The addition of α-amylase led to increases in the protein content and antimicrobial activity of the OC, which were mirrored by decreased bacterial counts in the TTK study and were in agreement with previous in vitro physicochemical data (8, 9).

The analysis of bacterial counts in the presence of colistin sulfate was interesting and showed that bacterial recovery occurred earlier than the shortest currently recommended dosing interval for colistin (31, 32), demonstrating the clinical benefit that these models may offer. In contrast, with the dextrin-colistin conjugates, the recovery of bacterial counts was significantly delayed, presumably due to the sustained release of colistin from dextrin-colistin conjugates by α-amylase. Since colistin is a concentration-dependent antibiotic (33) with a modest postantibiotic effect (34), the ability of the polymer conjugates to prolong exposure of bacteria to colistin may be more beneficial to antibacterial efficacy than the achievement of high peak concentrations (25, 35, 36). Several concentration-dependent antibiotics, including azithromycin, amikacin, and ciprofloxacin, have been incorporated into controlled-release polymeric carriers, such as nanoparticles and liposomes (37–39), which can readily be tested in our in vitro system. For instance, Wang et al. (40) used a similar two-compartment model to characterize the in vitro release kinetics of colistin sulfate-loaded liposomes. In this study, a combination of colistin sulfate and dextrin-colistin conjugate provided a rapid decline in viable counts, followed by a sustained suppression of bacterial growth rate. The conjugate unmasking by α-amylase was shown to be time dependent, meaning that in clinical use, administration of an initial dose of conventional antibiotic may be required when treating acute disease. In addition to improving the therapeutic efficacy of nanoformulated drugs, incorporating antibiotics into nano-sized structures also converts conventional small-molecule antibiotics into macromolecules, which may benefit from passive size-based targeting and accumulation at sites of inflammation/infection via the enhanced permeability and retention (EPR) effect (4, 41). A potential limitation of this model is its inability to replicate the potential benefits of the EPR effect, representing an avenue for future development.

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FIG 5 Patient-matched α-amylase activity in infected wound fluid (Phadebas assay) (mean ± SD, n = 3) and plasma from clinical reports. The average data set represents mean α-amylase activity in wound fluid and plasma samples (mean ± SD, n = 6). *, Statistically significant difference (t test, P < 0.05).
subsided (42, 43). Incubation of dextrin-colistin conjugates in infected wound fluid demonstrated that colistin would be readily released from the conjugate locally at infected sites due to local α-amylase activity. The conjugate unmasking rate (68.1% to 86.2% at 48 h) compared favorably to published values for CMS, for which conversion to colistin in ex vivo human plasma samples after 48 h was ~60% (44).

Recent advances in the design and development of nanoantibiotics, with complex drug-release profiles, warrant the establishment of a novel PK/PD model for monitoring their drug release and antibiotic efficacy. This two-compartment model system permits the building in of increasing levels of complexity, providing a versatile tool to describe the effect of bioresponsive dextrin-colistin conjugates by characterization of in vitro drug release (in simulated and ex vivo biological fluids) and bacterial killing over time. It is anticipated that these results will assist in designing optimal dosing strategies for dextrin-colistin conjugates in vivo.

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REFERENCES


