In vitro evaluation of the structural and physical interaction of 'nanoantibiotic' dextrin-colistin conjugates with bacterial endotoxins.

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ABSTRACT: Dextrin-colistin conjugates have been developed with the aim of reducing clinical toxicity associated with colistin and improving targeting to sites of bacterial infection. This study investigated the in vitro ability of these dextrin-colistin conjugates to bind and modulate bacterial lipopolysaccharide (LPS), and how this binding affects its biological activity. These results

showed that colistin, and 'amylase-activated' dextrin-colistin conjugate to a lesser extent, bound to LPS and induced significant conformational changes to its structure. In biological studies, both colistin and dextrin-colistin conjugate effectively inhibited LPS-induced hemolysis and TNFα secretion in a concentration-dependent manner, but only dextrin-colistin conjugate did not cause additive toxicity at higher concentrations. This study provides the first direct structural experimental evidence for the binding of dextrin-colistin conjugates and LPS, providing insight into the mode of action of dextrin-colistin conjugates.

INTRODUCTION

Infectious diseases account for millions of deaths worldwide annually, with high associated costs.[1] Antibiotic chemotherapy is successfully used to treat the majority of Gram-negative infections, but antibiotic-induced shedding of endotoxic lipopolysaccharides (LPS) may result in systemic sepsis, with an associated mortality of 30-50%. Thus, eradication of bacterial infection does not always lead to a positive clinical outcome. We have previously described the first bioresponsive polymer conjugates to target the delivery of antimicrobials to sites of inflammation, using dextrin-colistin conjugates as a prototypical model.[2] Colistin is an amphiphilic antibiotic which binds to Gram-negative bacterial cell membranes, resulting in cytoplasmic leakage and cell death. Colistin may, however, be associated with considerable nephro- and neurotoxicity in the immunocompromized patient, which limits its clinical use. These 'nanoantibiotic' polymer therapeutics are administered centrally but are locally activated at sites of infection by amylase, an enzyme found throughout the body (typical serum concentration 60-120 IU/L) but present at elevated concentrations at sites of bacterial infection/inflammation.[3] This local activation improves antibiotic targeting to sites of infection, enables the release of free colistin (using

Polymer masked-UnMasked Protein Therapy (PUMPT))[4] and reduces clinical toxicity.

An important property of colistin is its ability to bind bacterial LPS and block its toxic biological activities[5] (Figure 1). The phase structure, conformation and aggregation behavior of bacterial LPS and its components (e.g. lipid A) have been extensively characterized by various physical methods.[6-8] LPS is biologically active in its micellar form, and colistin disaggregation effectively neutralizes its endotoxic effects by disrupting the regular spacing of LPS fatty acid chains and binding in a 1:1 complex.[9] Whilst the toxicity profile and antibacterial activity of the dextrin-colistin conjugates has been described, nothing was known of its structural and functional interactions with LPS. In these studies, colistin-LPS complex formation, and potential LPS neutralization, was studied using small-angle neutron scattering (SANS), circular dichroism (CD) spectroscopy and turbidity assays to characterize the *in vitro* ability of the conjugates to bind and modulate bacterial LPS from *Escherichia coli* (*E. coli*), and determine how the degree of unmasking affected LPS binding. Parallel studies investigated *in vitro* inhibition of LPS-induced erythrocyte hemolysis and TNFα release from human kidney (HK-2) cells as a marker of LPS complex formation with colistin sulfate, colistimethate sodium (CMS) and dextrin-colistin conjugate.

RESULTS

Endotoxin binding. LPS caused significant scattering of neutrons, which was altered in the presence of colistin or unmasked dextrin-colistin conjugate at ≥10 mg/mL (Figure 2). The emergence of 2 peaks at Q = 0.06 and 0.11 $Å^{-1}$ and a pronounced increase in scattering intensity at low Q was apparent when LPS was combined with colistin. These peaks did not change position with different colistin concentrations, although the intensity of the peaks varied, and was greatest

with 10 mg/mL colistin. The two characteristic peaks were absent when LPS was pre-incubated with dextrin-colistin conjugate and there was a pronounced decrease in the data at low Q. Pre-incubation of dextrin-colistin conjugate with amylase, however, induced a time-dependent increase in scattering intensity at low Q and the appearance of a peak at Q = 0.06 $Å^{-1}$ at 50 mg/mL. Clearly the peaks are a result of the action of the colistin on the LPS structure.

CD spectroscopy showed that colistin has a rigid cyclic secondary structure that was not altered by conjugation of dextrin or subsequent unmasking by amylase (Figure 3). When LPS was added to the antibiotic solutions, the CD spectra appeared to be equivalent to the sum of the CD signal of the individual components. Succinoylated dextrin had an unstructured conformation, which was unchanged in the presence of LPS (data not shown).

Increasing turbidity was used as a marker of LPS aggregate formation with colistin, CMS or dextrin-colistin conjugate. When compared at equivalent colistin concentration, significant differences between the turbidity of colistin and CMS, dextrin-colistin (with or without amylase) or control were observed (Figure 3d). LPS binding of the dextrin-colistin conjugate was reduced to ~ 50% compared to free colistin; turbidity remained unaltered even after pre-incubation of the conjugate with amylase.

Colistin, and CMS to a lesser extent, were able to inhibit the cleavage of chromogenic substrate by *E. coli* LPS (Figure 4). Neutralization of LPS by colistin required 20-fold more LPS to trigger *Limulus* amebocyte lysate gelation when compared to LPS in water, and was significantly greater than CMS (1 vs. 0.05 ng/mL, respectively). All dextrin-colistin conjugate samples gelled *Limulus* amebocyte lysate without the addition of LPS. Nevertheless, at the lower concentrations of LPS, unmasked conjugate was capable of binding more LPS than masked conjugate and there was a trend for increasing endotoxin binding after unmasking by amylase (6 h > 24 h > 0 h) (data not

shown).

***In Vitro* Inhibition of Endotoxin Activity.** When HK-2 cells were treated with LPS alone or in the presence of colistin sulfate, CMS or 'unmasked' dextrin-colistin conjugate, there was little effect on LPS-induced TNFα release at low concentrations, but increased cytokine release was observed when colistin molar concentration exceeded that of LPS (Figure 5). However, at both LPS concentrations (10 and 100 ng/mL), dextrin-colistin conjugate inhibited LPS-induced TNFα release in a concentration-dependent manner above 5 and 50 μg/mL, respectively.

Similarly, addition of colistin sulfate, CMS and dextrin-colistin conjugate inhibited endotoxin-induced hemolysis of rat erythrocytes up to ~20 μg/mL (Figure 6). However, while higher concentrations of colistin and CMS caused increasing erythrocyte lysis, dextrin-colistin conjugate continued to inhibit hemolysis in a concentration-dependent manner. 'Unmasked' dextrin-colistin conjugate was unable to inhibit endotoxin-induced hemolysis and caused equivalent toxicity to colistin and CMS at higher concentrations.

DISCUSSION

A detailed understanding of the physicochemical properties and structure-activity relationships is vital to predict the behavior of polymer therapeutics in biological systems and ensure the design of safe and efficacious nanomedicines. The ability of colistin to bind and neutralize LPS is well documented, however little is known about how polymer conjugation affects the structural and biological properties of colistin. Whilst the antimicrobial activity of dextrin-colistin conjugates has been demonstrated *in vitro*,[2] antibiotic treatment may induce LPS release from lysed bacteria, which may induce overreaction of the immune system and endotoxic shock. Therefore, these studies investigated whether dextrin-colistin conjugates (before and after amylase-'unmasking')

could bind and neutralize released endotoxins. Dextrin-colistin conjugates used in these studies contained ~10% w/w colistin, of which < 2% was unbound, indicating multiple (typically 2) dextrin chains per colistin with several (typically 3) binding sites. The dextrin-colistin concentrations used in the SANS and CD spectroscopy studies refer to total conjugate; thus conjugate samples contained ~10x less colistin than the equivalent free drug samples. While this may affect the biological activity of the conjugates, it was deemed the most appropriate means of comparing the samples in these structural studies.

The use of SANS to study evolving biological systems has been previously employed to investigate dextrin-phospholipase A_2-triggered degradation of liposomes.[10] Here, we examined the effect of dextrin conjugation and unmasking by amylase on the interaction of colistin with LPS. With a critical micelle concentration (CMC) of ~14 μg/mL,[8,11] LPS readily micellizes at the concentrations used in these studies. These studies assumed, not unreasonably, that the SANS scattering is dominated by that of the LPS. The data revealed significant conformational rearrangement of LPS following incubation with both colistin sulfate and unmasked dextrin-colistin conjugate (colistin > 24 h unmasked dextrin-colistin > 6 h unmasked dextrin-colistin) at ≥10 mg/mL. This conformational rearrangement was evident in the emergence of the peaks indicative of a regular ordered structure. Similarly, marked differences between the aggregation of colistin and LPS, and that observed with dextrin-colistin conjugates were observed in both the turbidity and LAL assays. CD spectroscopy, however, did not show any substantial change in the secondary structure of colistin or dextrin-colistin conjugates (masked and unmasked) in the presence of LPS, indicating that aggregation did not induce an α-helical structure, presumably due to the rigidity of colistin's cyclic structure. This has also been observed with polymyxin B, while WLBU2, a cationic amphiphilic peptide, became markedly helical in the presence of LPS, which

the authors attribute to the ability of polymyxin B (but not WLBU2) to destabilize and disrupt LPS vesicles.[12]

Molecular modelling has demonstrated that colistin exerts its anti-endotoxic effects by breaking up the supramolecular structure of LPS.[9] The downturn in the SANS from LPS at low Q in the presence of colistin (50 mg/mL) is an indication of this neutralization effect, since LPS and colistin have been shown to bind at an LPS to colistin ratio of 5.2:1 (by weight).[13] The regularity and clarity of the peaks in the SANS induced by colistin are commensurate with a spacing of 5 nm, suggestive of a bilayer stack. The 5 nm structures seen here are in good agreement with the size of lipid A bilayers, a component of LPS, found by Labischinski et al.[6] in their SANS studies, and by Katowsky et al.[14] in their molecular modelling and x-ray powder diffraction experiments of bacterial LPS. Both these studies demonstrated that lipid A and rough LPS (having a short O-specific chain, like *E. coli* O26:B6 used here) are 2.4 nm and 2.8 nm long, respectively. This would indicate that the structures seen in these studies are from the LPS. This is further supported by Wallace et al.,[15] who reported that colistin aggregates formed above 2.1 □g/mL with a diameter of 2.07 ± 0.3 nm.

Binding and structural data using several biophysical techniques, including isothermal titration calorimetry (ITC), fluorescent probes and nuclear magnetic resonance (NMR) spectroscopy, have indicated that the LPS-colistin interaction is a two-stage mechanism.[16] First, the complex is stabilized by electrostatic charges between colistin's positive charges and LPS's negatively-charged head groups. Subsequently, complexation is strengthened by hydrophobic interactions between the hydrophobic domains of the two molecules. Given that dextrin-colistin conjugation uses ~3 of colistin's positively charged amine groups, it is unsurprising that masked conjugates have no effect on LPS structure. While 'unmasked' conjugates induced some conformational

rearrangement of LPS, this was not as obvious as native colistin. This was not surprising since α-amylase is an endoamylase that cleaves dextrin within the polyglucose chain, thus, 'unmasked' dextrin-colistin conjugates retain oligosaccharide fragments attached to colistin's amine groups, which would reduce its cationic charge.

Parallel studies investigated *in vitro* inhibition of LPS-induced erythrocyte lysis and TNFα release from human kidney cells as a marker of LPS aggregate formation with colistin or dextrin-colistin conjugate. Following systemic administration, dextrin-colistin conjugates are expected to remain in the bloodstream for an extended period, due to their macromolecular size, but would ultimately be excreted via the kidneys following amylase unmasking. Despite the negligible binding to bacterial LPS, the ability of dextrin-colistin conjugates to exert a protective effect from endotoxins on erythrocytes and human kidney cells was in keeping with previous studies showing that dextrin is able to effectively impede the hemolytic activity of drugs, such as zidovudine,[17] and proteins, such as phospholipase A_2.[18] In these previous studies the effect was evident when zidovudine was covalently linked to dextrin or physically mixed with dextrin, providing further proof that dextrin acts by a cytoprotective mechanism. Moreover, dextrin conjugates (iodine-lithium-α-dextrin, ILαD) have been described with anti-endotoxin activity.[19,20] These studies demonstrated that ILαD suppresses CD14 receptor expression, inhibits LPS-induced pro-inflammatory activation of monocytes and neutrophils, and increases tolerance to endotoxins.

Polymyxin B-dextran 70 conjugates have been described that effectively inhibit LPS-induced TNF□α production *in vivo*, Lake et al.[21] proposed that polymyxin B-dextran 70 conjugates neutralize the pathogenic pharmacophore of endotoxin, citing unpublished studies corroborating its ability to bind to radiolabeled endotoxin. Interestingly, parallel studies employed polymyxin B conjugated to a 70,000 g/mol, dextran; yielding conjugates containing 3.8% w/w polymyxin B

(equivalent to 2.1 polymyxin B per /dextran); with a markedly reduced antibiotic activity.[22] In contrast, our dextrin-colistin conjugates contain 8,000 g/mol dextrin and 10.1% w/w colistin, equivalent to 1.6 dextrin chains per colistin, which may explain the difference in antibacterial activity.

CONCLUSIONS

These data clearly demonstrate physical differences in the LPS interaction of colistin and dextrin-colistin conjugates pre- and post-amylase unmasking. No change in the LPS structure was observed for dextrin-colistin conjugates, however, degradation of conjugates showed time-dependent changes in LPS complex formation, that were more pronounced after prolonged incubation with amylase. Despite the inability of dextrin-colistin conjugates to bind to bacterial LPS, concentration-dependent inhibition of LPS-induced toxicity was evident, indicating that dextrin-colistin conjugates could represent effective neutralizers of endotoxin with application in the treatment of sepsis.

EXPERIMENTAL SECTION

Dextrin-colistin conjugate synthesis. Dextrin-colistin conjugate was synthesized and characterized using methods previously described.[2] The dextrin-colistin conjugate used in these studies contained dextrin with 1.1 mol% succinoylation and had a molecular weight of approximately 9,000 g/mol (gel permeation chromatography (GPC) with pullulan standards) and a colistin content of approximately 10.1% w/w (bicinchoninic acid (BCA) assay) with < 3% free colistin (fast protein liquid chromatography (FPLC)). Purity of dextrin-colistin conjugates was ≥ 95%.

Small-angle neutron scattering measurements. To assess the colistin-induced disruption of LPS aggregates, LPS from *E. coli* 026:B6 (5) (10 mg/mL) (Sigma-Aldrich, U.K.) was incubated with succinoylated dextrin, colistin sulfate (Sigma-Aldrich, U.K.), dextrin-colistin conjugate or unmasked dextrin-colistin conjugate (0.01, 0.1, 1, 10, 50 mg/mL) in D_2O (Sigma-Aldrich, U.K.) containing phosphate buffer (PBS) (Oxoid, U.K.) (3 h at 37 ^oC, pH 7.4) prior to analysis by SANS. 'Unmasked' dextrin-colistin conjugates were prepared by incubation of dextrin-colistin conjugate (50 mg/mL in PBS) with α-amylase from human saliva (100 IU/L) (Sigma-Aldrich, U.K.) for 6 or 24 h at 37 ^oC, then lyophilized and stored at -20 ^oC before reconstitution in D_2O prior to analysis by SANS.

SANS experiments were performed on the steady-state reactor source on the D22 diffractometer at the ILL, Grenoble. A $Q = (4\pi/\lambda) \sin(\theta/2)$ range between 0.005 and 0.5 $Å^{-1}$ was obtained by choosing two instrument settings at a constant neutron wavelength (λ) of 6 Å, the two sample-detector distances were 2 m and 14 m respectively, both working with the detector being offset by 40 cm with respect to the direct beam position on the detector. The samples were contained in 2 mm path length, UV-spectrophotometer grade, quartz cuvettes (Hellma, U.K.) and mounted in aluminium holders on top of an enclosed, computer-controlled, sample chamber. Sample volumes were around 0.4 cm^3. All experiments were conducted at 37 ^oC. Temperature control was achieved by using a thermostatic circulating bath pumping fluid through the base of the sample chamber, achieving a temperature stability of ± 0.2 ^oC. Experimental measuring times were approximately 40 min.

All scattering data were (a) normalized for the sample transmission, (b) background corrected using a quartz cell filled with D_2O, and (c) corrected for the linearity and efficiency of the detector response using the instrument specific software package. Data was analyzed using SasView 2.2.0

software.

Circular Dichroism Spectroscopy. CD spectra were obtained using a Model 215 spectrometer (AVIV® Instrument Inc.) with a 1 mm path length quartz cell at 4 °C. Samples were dissolved in sodium fluoride (NaF) (100 mM) and potassium phosphate (KH_2PO_4) (20 mM) buffer at pH 7.4. Spectra of succinoylated dextrin, colistin sulfate, dextrin-colistin conjugate or 'unmasked' dextrin-colistin conjugate (0.5 mg/mL) were obtained alone and following incubation with LPS (0.1 mg/mL) (3 h at 37 °C, pH 7.4). 'Unmasked' dextrin-colistin conjugates were prepared by incubation of dextrin-colistin conjugate (10 mg/mL in NaF/KH_2PO_4 buffer) with amylase (100 IU/L) for 24 h at 37 °C. The CD spectra were then obtained as described above.

Turbidity Assay. A turbidimetric assay technique was used in which the binding of LPS to colistin results in precipitation of aggregates and increased turbidity. LPS was dissolved in pre-warmed PBS buffer (1 mg/mL, 37 °C, pH 7.4) containing colistin sulfate, CMS (Colomycin® injection, Forest Laboratories UK Limited, U.K.), dextrin-colistin conjugate or 'unmasked' dextrin-colistin conjugate (4 mg/mL colistin equiv., 200 μL per well) and added to the wells of a 96-well microtiter plate. Control samples contained only LPS dissolved in PBS. Plates were incubated at 37 °C throughout the experiment and absorbance was read at 625 nm at various timepoints (0, 15, 30, 60, 90, 120 min). Samples were assayed in triplicate and experiments were repeated once, and means of the results were calculated.

'Unmasked' dextrin-colistin conjugates were prepared by incubation of dextrin-colistin conjugate (10 mg/mL in PBS buffer) with amylase (100 IU/L) for 24 h at 37 °C. The turbidity assay was then followed as described above.

***Limulus* amebocyte lysate (LAL) Assay.** Endotoxin neutralization was evaluated using a ToxinSensor™ chromogenic LAL endotoxin assay kit (GenScript, U.S.A.). LPS was dissolved in

pyrogen free water (0-50 ng/mL) containing colistin sulfate, CMS, dextrin-colistin conjugate or 'unmasked' dextrin-colistin conjugate (4 μg/mL colistin equiv., 100 μL) in pyrogen-free vials. Control samples contained only LPS dissolved in pyrogen-free water. Solutions were mixed well and incubated at 37 ^{o}C for 3 h. Reconstituted LAL (100 μL) was then added and the solution mixed well before incubation at 37 ^{o}C for 45 min. Next, reconstituted substrate solution (100 μL) was added to each vial, mixed well and incubated for a further 6 min. Finally, the reaction was terminated by addition of reconstituted stop solution and color stabilizers. Samples (100 μL) were pipetted into the wells of a 96-well microtiter plate (6 wells per sample) and absorption was read spectrophotometrically at 540 nm. The concentration of LPS producing 50% maximal absorption was taken as the ED_{50}.

'Unmasked' dextrin-colistin conjugates were prepared by incubation of dextrin-colistin conjugate (0.5 mg/mL in pyrogen free water) with amylase from human saliva (100 IU/L) for 6 h at 37 ^{o}C. The LAL assay was then followed as described above.

Cell culture. Human kidney (HK-2) cells (immortalized proximal tubule epithelial cell line from normal adult human kidney) (Institute of Nephrology, Cardiff University, U.K.). were cultured in keratinocyte-serum free medium (K-SFM) medium with L-Glutamine, EGF and BPE (Invitrogen Life Technologies, U.K.) in standard culture conditions (37 ^{o}C, humidified air containing 5% CO_2).

Expression of TNFα HK-2 cells were seeded into sterile 96-well microtiter plates (2 x 10^5 cells/ mL) in 0.1 mL/well media and allowed to adhere for 24 h. The medium was then removed and LPS (10 ng/mL) was added in the absence or presence of colistin sulfate, CMS or dextrin-colistin conjugate (with or without amylase, 100 IU/L) (0.2 μm filter-sterilized) at different concentrations. After 24 h, microtiter plates were centrifuged (600 g, 10 min) and the supernatant

was transferred to a clean 96-well plate and stored at -20 ºC until determination of TNFα content. TNFα content in the cell supernatant was determined using a commercial human TNFα ELISA set (Thermo Scientific, U.K.) 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) and used to calculate TNFα concentration in pg/mL (assay detection range 15-1000 pg/mL).

Erythrocyte Lysis. Rat erythrocytes were prepared as previously described.[18] Briefly, blood was obtained from a male Wistar rat (~250 g body weight) by cardiac puncture and collected in a heparin/lithium blood tube, in PBS (pH 7.4). Following centrifugation (1500 g, 10 min, 4 ºC) and washing (x 2), the final erythrocyte pellet resuspended in PBS to produce a 2% w/v suspension. Subsequently, this erythrocyte suspension (100 μL) was added to a pre-incubated (3 h, 37 ºC) 96-well plate containing LPS (100 μg/mL final concentration) in the absence or presence of colistin sulfate, CMS, dextrin-colistin conjugate or 'unmasked dextrin-colistin conjugate (0-500 μg/mL colistin equiv. final concentration). PBS was used as a negative control, and Triton X-100 (1% v/v) (Sigma-Aldrich, U.K.) was used to achieve 100% hemolysis. The plate was then incubated for 24 h at 37 ºC, and then centrifuged at 1500 g for 10 min at 4 ºC. The supernatant (100 μL) of each well was then carefully removed and placed into a clean 96-well plate and absorbance at 540 nm read using a microtiter plate reader. The background hemolysis (PBS) was subtracted and the results were expressed as percentage hemoglobin released (± SEM).

'Unmasked' dextrin-colistin conjugates were prepared by incubation of dextrin-colistin conjugate (2 mg/mL in PBS buffer) with amylase (100 IU/L) for 24 h at 37 ºC. The hemolysis assay was then followed as described above.

Statistics. The significance of the data was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical significance was set at $p < 0.05$.

FIGURES

Figure 1. Representation of the anti-endotoxin mechanism of dextrin-colistin conjugate, showing the unmasking strategy utilized to control delivery of colistin, and the effect of colistin on LPS aggregates.

Figure 2. Small-angle neutron scattering from LPS (10 mg/mL in D_2O) following incubation (3 h at 37 ^oC) with colistin sulfate, dextrin-colistin conjugate, and 'unmasked' dextrin-colistin conjugates at (a) 10 mg/mL and (b) 50 mg/mL. Panel c) shows the radii of gyration (Rg) calculated from the SANS data.

Figure 3. CD spectra of a) colistin sulfate, b) dextrin-colistin conjugate, and c) 'unmasked' dextrin-colistin conjugate (0.5 mg/mL) in NaF (100 mM) and KH_2PO_4 (20 mM) buffer at pH 7.4, and following incubation (3 h at 37 ^oC) with LPS (0.1 mg/mL). Where sum = LPS signal + colistin signal and dif = LPS-colistin mixture signal – sum. Panel d) shows precipitation of LPS from *E. coli* (1 mg/mL) in the absence and presence of colistin sulfate, CMS, dextrin-colistin conjugate and 'unmasked' dextrin-colistin conjugate (4 mg/mL colistin equiv.). Data represents mean absorbance at 625 nm ± SEM, $n = 6$; * ($p<0.01$) and ** ($p<0.001$) indicate significance compared to LPS alone (control).

Figure 4. Colorimetric detection of endotoxin following incubation of LAL with LPS from *E. coli* (0-50 ng/mL) in pyrogen-free water or pre-incubated for 3 h at 37 ^oC with colistin sulfate, CMS, dextrin-colistin conjugate and 'unmasked' dextrin-colistin conjugate (4 μg/mL colistin equiv.). Data represents mean absorbance at 540 nm ± SEM, $n = 6$; * ($p<0.01$) and ** ($p<0.001$) indicate significance compared to LPS alone (control); $^+$ ($p>0.01$) and $^{++}$ ($p>0.001$) indicate significance compared to dextrin-colistin conjugate.

Figure 5. Inhibition of LPS-induced (a) 10 ng/mL and (b) 100 ng/mL) TNFα release from HK-2 cells following incubation with colistin sulfate, CMS, dextrin-colistin conjugate and dextrin-colistin + amylase at 0-1000 μg/mL colistin equiv. (24 h incubation). Panel a) shows cell response to 10 ng/mL LPS and panel b) shows cell response to 100 ng/mL LPS. Data represents mean percentage TNFα release ± SD, n = 3; * (p<0.05), ** (p<0.01) and *** (p<0.001) indicate significance compared to LPS alone (control).

Figure 6. Inhibition of LPS-induced (*E. coli* 026:B6, 100 μg/mL) hemolysis (24 h incubation) of rat erythrocytes following pre-incubation with colistin sulfate, CMS, dextrin-colistin conjugate and 'unmasked' dextrin-colistin conjugate for 3 h at 37 ^{o}C. Data represents mean percentage hemoglobin release ± SEM, n = 9; * (p<0.05) and ** (p<0.001) indicate significance compared to LPS alone (control).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

ANOVA, one-way analysis of variance; BPE, bovine pituitary extract; CD14, cluster of differentiation; CD spectroscopy, circular dichroism spectroscopy; CMC, critical micelle concentration; CMS, colistimethate sodium; CO_2, carbon dioxide; D_2O, deuterium oxide; EGF, epidermal growth factor; ELISA, Enzyme-linked immunosorbent assay; HK-2, human kidney 2;

ILαD, iodine-lithium-α-dextrin; ITC, isothermal titration calorimetry; K-SFM, keratinocyte-serum-free medium; LAL, *Limulus* amebocyte lysate; LPS, lipopolysaccharide; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PUMPT, polymer masked-unmasked protein therapy; SANS, small-angle neutron scattering; TNFα, tumor necrosis factor alpha

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