

Accepted Manuscript

A traceless reversible polymeric colistin prodrug to combat multidrug-resistant (MDR) gram-negative bacteria

Chongyu Zhu, Elena K. Schneider, Jiping Wang, Kristian Kempe, Paul Wilson, Tony Velkov, Jian Li, Thomas P. Davis, Michael R. Whittaker, David M. Haddleton



PII: S0168-3659(17)30058-5
DOI: doi: [10.1016/j.jconrel.2017.02.005](https://doi.org/10.1016/j.jconrel.2017.02.005)
Reference: COREL 8655

To appear in: *Journal of Controlled Release*

Received date: 5 December 2016

Revised date: 27 January 2017

Accepted date: 2 February 2017

Please cite this article as: Chongyu Zhu, Elena K. Schneider, Jiping Wang, Kristian Kempe, Paul Wilson, Tony Velkov, Jian Li, Thomas P. Davis, Michael R. Whittaker, David M. Haddleton, A traceless reversible polymeric colistin prodrug to combat multidrug-resistant (MDR) gram-negative bacteria. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Corel(2016), doi: [10.1016/j.jconrel.2017.02.005](https://doi.org/10.1016/j.jconrel.2017.02.005)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

A Traceless Reversible Polymeric Colistin Prodrug to Combat Multidrug-resistant (MDR) Gram-negative bacteria

Chongyu Zhu,^a Elena K. Schneider,^b Jiping Wang,^{b,d} Kristian Kempe,^{c,a} Paul Wilson,^{a,c} Tony Velkov,^b Jian Li,^d Thomas P. Davis,^c Michael R. Whittaker^c and David M. Haddleton^{a,c*}

^aDepartment of Chemistry, University of Warwick, CV4 7AL, Coventry, UK

^bDrug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences; Monash University, Parkville, Victoria 3052, Australia.

^cARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia

^dMonash Biomedicine Discovery Institute, Department of Microbiology; Monash University, Clayton, Victoria 3800, Australia

*Author to whom correspondence should be addressed:

Prof. Dr. David M. Haddleton

Department of Chemistry, University of Warwick, Coventry, CV4 7AL, Phone: 44 2476 523256, Fax: 44 2476 528267, Email: D.M.Haddleton@warwick.ac.uk

Keywords: colistin; MDR Gram-negative bacteria; polymeric prodrug; traceless linker; systemic administration.

1. Introduction

Bacterial infections, especially drug-resistant infections are a major global health issue [1]. The emergence of multidrug-resistant (MDR) strains of bacteria and the lack of new antibiotics is a worrying prospect for all of humanity [2-4]. A recent report suggests failing to control drug-resistant infections may cause in excess of 10 million deaths per year and cost up to US\$ 100 trillion by 2050 [5]. MDR Gram-negative bacteria, are particularly difficult to treat due the nature of their robust outer membrane [6]. Colistin, a lipopeptide and member of the polymyxin family, is currently used as a last-line drug for the treatment of MDR Gram-negative bacterial infections [7-10].

Colistin is a cyclic lipopeptide with an intramolecular hydrophilic loop with a *N*-terminal fatty acyl group. The commercial colistin source contains two major components, colistin A and colistin B, of which the only difference is on the *N*-terminal fatty acyl group; a 6-methyloctanoic acid for colistin A and isooctanoic acid for colistin B (Fig. 1) [11]. The unique structure of colistin allows it to bind electrostatically with the bacterial outer membrane and then directly interact with the lipid A component of the lipopolysaccharide (LPS), the major constituent of the outer membrane of Gram-negative bacteria [12, 13]. The insertion of colistin disrupts the barrier function of the Gram-negative outer membrane that ultimately results in cell death.

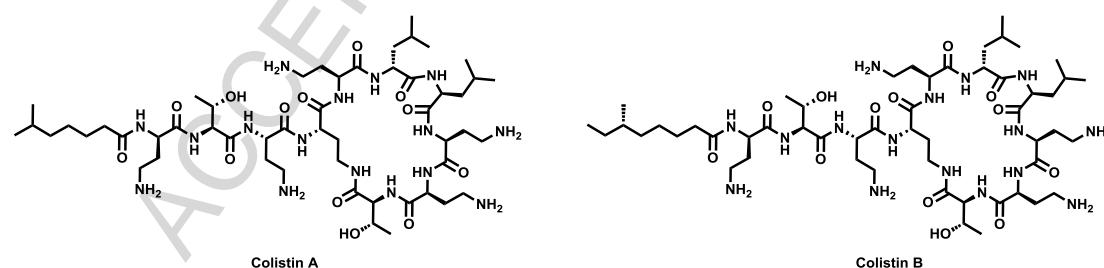


Fig. 1 The chemical structure of the two subgroups of colistin.

The five primary amine groups on the 2,4-diaminobutyric acid (Dab) residues of colistin are essential to this process [13, 14]. The protonated amine groups can recognise and interact with the phosphorylated sugars on lipid A [13, 15, 16]. Unfortunately, while often used as a last defence for drug resistant infections this treatment is not allowed for systematic administration due to its associated

nephrotoxicity [7, 17].

One avenue to reduce the systemic toxicity of polymyxin is to formulate it into a prodrug form [18]. CMS is the only current FDA approved prodrug of colistin that can be used intravenously in the clinic [19]. After the modification on five primary amines to methanesulfonate groups, CMS is less toxic compared to the native colistin. However, the slow and variable colistin release rates from the prodrug form complicate its pharmacokinetics, resulting in variable treatment outcomes for patients [19-21]. A further concerning feature of the prodrug is the heterogeneity of the methanesulfonate modification which leads to supplier to supplier and batch to batch structural variation in the prodrug form [22]. Therefore, an alternative prodrug to CMS is highly desirable.

In polypeptide therapeutics, one favoured approach to improve the pharmacokinetic and pharmacodynamic properties of proteins/peptides is their conjugation to polymers [23-28]. Amongst all polymers available, FDA approved biocompatible poly (ethylene glycol) (PEG) and its derivatives such as poly(ethylene glycol) methyl ether (mPEG) have been best studied and characterised [23, 27]. The attachment of PEG, known as *PEGylation*, onto those proteins/peptide drugs will help them bypass the enzymatic recognition, thus prevent them from antigenic/immunogenic recognition and proteolytic degradation [27, 29, 30]. PEGylation of protein/peptide drugs can also increase the hydrodynamic volume of the drugs, provide anti-fouling properties, prolonging their half-life, which can be important to decrease their toxicity to the kidney and improve patient compliance through longer dosing intervals [27].

However, a significant (or sometimes complete) loss of the protein/peptide activity has often been reported for many polymer-protein/peptide conjugates following such modifications [25, 31, 32]. This has mainly been attributed to the blockage of the active site(s) or the altered structure of the protein/peptide caused by the polymer. Thus, prodrugs based on traceless and/ or reversible conjugations have attracted significant attention [33-36]. These types of prodrugs usually stay as an

inactive or less active derivative under normal (storage) conditions, but can be converted back to the active drug at the targeted site/ environment *in vitro/ in vivo*. Several dynamic covalent bonds, especially disulphide bonds have been exploited to build up these releasable linkers [31, 35, 37, 38]. However, a non-releasable thiol containing spacer was required to be introduced onto the peptides/proteins that lack free cysteine residue(s). The remaining portion of the linker will sometimes modify the protein/peptide properties (such as the surface charge or solubility) and as such, has the potential to result in unwanted side effects, especially with small peptides. Recently, some other well-designed fully degradable or cleavable linkers, including 1,6-elimination [34], thioester [35], bicine [36], and azidomethyl-methylmaleic anhydride (AzMMMan) [39], were developed to target the more universal amine containing residues (i.e. lysine residues or a terminal amine) on the protein/peptide to achieve a traceless recovery of the native protein/peptide.

In this work, we designed a PEGylated colistin prodrug with a site-specific traceless releasable linker exhibiting low toxicity. This gave similar or better antibacterial activity to the commercial CMS prodrug. Although it might be considered that it would be more valuable to investigate reconversion rates under physiological conditions as here there would be esterases present previous work has looked at colistin and colistin methanesulfonate in both aqueous media and plasma it was found that the stability of and release of colistin from the CMS prodrug were comparable in both PBS buffer and plasma with no evidence of a role for esterases [22]. Instead of modifying the five Dab residues of colistin as in case of CMS, the two threonine (Thr) residues were targeted here as it can greatly reduce the site-diversity of the modification products. A relatively labile ester linkage was chosen to build up the colistin prodrug using an acetic acid terminated mPEG (aaPEG) considering the ester is a base and enzyme (i.e. lipase) hydrolysable bond that can be cleaved easily in the human body. *In vitro* degradation studies were performed to investigate colistin release rate. The effect upon the number of mPEG attachments and different linkages on colistin were also

evaluated through the degradation studies. Moreover, the antibacterial activity of the PEGylate colistin prodrug was systematically studied through disk diffusion, broth dilution and time-kill experiments. In addition, a mouse thigh infection model was used to compare the activity performance and toxicity of the PEGylated colistin prodrug with colistin *in vivo*.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased from Sigma-Aldrich and used directly unless otherwise stated. HPLC solvents are obtained from VWR international, LLC. The acetic acid functional mPEG (aaPEG, $M_n \sim 2\text{kDa}$) was synthesised based on the previous report [40]. The succinic acid functional mPEG (saPEG, $M_n \sim 2\text{kDa}$; commercial name: CH₃O-PEG-NHCO-C₂H₄-COOH; reference number: 122000-3) was purchased from Rapp Polymere GmbH.

2.1.2 Bacterial strains

Bacterial strains of *P. aeruginosa* ATCC 27853 and *A. baumannii* ATCC 19606 (American Type Culture Collection, Manassas, VA) were used in this study. The strains were stored at -80 °C in a cryovial storage container (Simport Plastics, Quebec, Canada). Fresh isolates were subcultured on Nutrient agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) and incubated at 37 °C for 24 h prior to each experiment. Cation-adjusted Mueller-Hinton broth (CAMHB; Oxoid, Hampshire, England) was used.

2.2 Methods

2.2.1 Disk diffusion assay

According to the EUCAST guidelines [41], inoculum was standardised in saline to the density of a McFarland 0.50±0.02 standard, corresponding to $\sim 10^8$ CFU/mL of each isolate. Freshly prepared suspension was evenly inoculated onto plates and disks were applied within 15 min and incubated for 18 h at 37 °C. Plates were examined for satisfactory streaked suspension and evenly distributed growth to achieve uniformly circular inhibition zones within the quality control limits.

2.2.2 Minimum inhibitory concentration (MIC) test

MICs were determined in accordance to the recommendations of the Clinical and Laboratory Standards Institute [42]. *P. aeruginosa* ATCC 27853 and *A. baumannii* ATCC 19606 were used for the tests. Experiments were performed with CAMHB in 96-well polystyrene microtiter plates. Wells were inoculated with 100 μ L of bacterial suspension prepared in CAMHB (containing $\sim 10^6$ CFU/mL) and 100 μ L of CAMHB containing increasing concentrations of colistin, CMS and PEGylated colistin prodrugs (0 – 128 mg/L). The MIC measurements were carried out in duplicates with the MIC being defined as the lowest concentration at which visible growth was inhibited following 18 – 20 h of incubation at 37 °C.

2.2.3 Time-kill test

The time-kill kinetics of the PEGylated colistin prodrugs at 0.5, 1, and 2 x MIC were examined against *A. baumannii* ATCC 19606. Briefly, each test sample was added into a 50 mL Eppendorf tube loaded with 20 mL of a logarithmic-phase broth culture of approximately 10^6 CFU/mL to yield concentrations of 0, 0.5, 1, and 2 x MIC (8 x MIC was also tested for col-aaPEG₂) of the isolate. The tubes were incubated in a shaking water bath at 37 °C. The samples were taken at 0 min, 30 min and 1, 2, 4, 6 and 24 h. Subcultures for viable counts were performed on nutrient agar (Oxoid) using WASP2 Spiral Plater (Don Whitley Scientific) and incubated at 37 °C for 24 h (48 h for plates with small colonies). Viable counts were determined by either manual counting or using ProtoCol3 Colony Counter (Don Whitley Scientific).

2.2.4 *In vivo* efficacy study using a neutropenic mouse thigh infection model

All animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee (ID: MIPS.2010.35) and were in conductance with the Australian Code for Care and Use of Animals for Scientific Purposes (8th Edition 2013).

The polymyxin-susceptible strain *P. aeruginosa* ATCC 27853 was cultured on

nutrient agar plates. One colony was randomly selected and incubated overnight at 37 °C. An aliquot of 0.2 mL of the overnight culture was dispersed in 20 mL CAMHB and incubated for the production of an early log-phase culture. Bacteria suspension was concentrated *via* centrifugation ($3220 \times g$ for 10 min) and the resuspended in 0.9% sterile saline for inoculation onto mice. The bacterial concentration of 2×10^5 CFU/50 μ L per mouse was estimated by determining the optical density (OD) at 600 nm and confirmed *via* plating onto agar.

Swiss mice were rendered neutropenic *via* two intraperitoneally i.p. doses of cyclophosphamide, 4 days (150 mg/kg) and 1 day (100 mg/kg) prior to infection. Thigh infection was established by injecting i.v. bolus of 50 μ L of the bacterial suspension (2×10^5 CFU/50 μ L per mouse). Solutions for colistin, col-aaPEG and col-aaPEG₂ were prepared at 1 mg/mL in sterile saline solution (0.9%). At 2 h after inoculation, the mice were treated with 40 mg colistin base/kg of each compound or the same volume of saline, respectively, for the control group. At 0 h and 18 h after administration of either the compound or the control, the animals were sacrificed by an inhalation of isoflurane. The skin was disinfected with 70% ethanol and blood was collected *via* cardia puncture using a 1 mL syringe rinsed with 5,000 IU/mL heparin, diluted serially in sterile 0.9% saline and plated on nutrient agar plates using WASP2 Spiral Plater (Don Whitley Scientific) and incubated at 37 °C for 24 h (48 h for plates with small colonies). Viable counts were determined by either manual counting or using ProtoCol3 Colony Counter (Don Whitley Scientific). The bacterial load (\log_{10} CFU/mL) was calculated for each mouse. Experiments were conducted in duplicates and a mean and standard deviation (SD) was calculated.

2.2.5 Measurement of nephrotoxicity in mice

All animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. Mice were subcutaneously administered with either colistin or PEGylated colistin prodrugs at an accumulated dose of 40 mg colistin base/kg was achieved. At 20 h after the last dose, mice were euthanized by inhalation of

an overdose of isoflurane. Immediately after sampling the blood by cardiac puncture (above) the right kidney of each mouse was collected and placed into 10% buffered formalin at pH 7.4 (Sigma, Australia) and stored in a 5 mL plastic tube. The formalin-fixed kidneys were subjected to histological examination at the Australian Phenomics Network, Histopathology and Organ Pathology (The University of Melbourne, Parkville, Australia). Samples were examined by a pathologist who was blind to the treatment groups. The nature and severity of the histological changes was graded as follows: (i) grade 1, mild acute tubular damage with tubular dilation, prominent nuclei and a few pale tubular casts; (ii) grade 2, severe acute tubular damage with necrosis; (iii) grade 3, acute necrosis/infarction.

The overall kidney histology score was calculated as a product of percentage score and grade score [43]. The SQR score (range: 0-5) was assigned as follows: (0): no significant change; (1): mild damage; (2): mild to moderate damage; (3): moderate damage; (4): moderate to severe damage; (5): severe damage.

3. Results and discussion

3.1 Synthesis of reversibly PEGylated colistin

For the site-specific modification of polymyxin we targeted the Thr residues. However, since the amines of the Dab residues in colistin are more chemically active than the hydroxyl groups of Thr residues, it was necessary to first protect each of the amine groups prior to the attachment of the polymer. A well-developed amine-protection approach using tert-butyloxycarbonyl (Boc) protecting groups was therefore applied (SFig. 1a). In order to ensure that all the five amines were fully protected, an excess of di-*tert*-butyl dicarbonate (Boc₂O) was used with the addition of potassium carbonate (K₂CO₃) during the modification. The reaction was carried out in a water/ tetrahydrofuran (THF) mixed solvent system to prevent further reaction of Boc₂O with the Thr residues. As commercial colistin has two major components (colistin A and colistin B), a double-peak pattern was observed during high performance liquid chromatography (HPLC) analysis of the native lipopeptide (SFig. 1b, black trace). An identical pattern, but at a higher retention time, was found following reaction with Boc₂O, indicating the protecting groups were successfully introduced onto the amines, rendering the colistin much more hydrophobic (SFig. 1b, red trace). Further, a careful comparison by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) of the modified polymyxin to the double-peak pattern of native colistin measurements (SFig. 1c), revealed a 500 Da molecular weight increase confirmed that all five amines were modified whilst both hydroxyl groups of the Thr residues remained intact.

The Thr residues on Boc-protected colistin were then PEGylated using aaPEG *via* Steglich esterification (Fig. 2a). As hydrolysis was to be used as the mechanism to release the native polymyxin from the prodrug form, aaPEG was chosen since the electron-withdrawing nature of the oxygen bonded to the α -carbon of the carboxyl group makes it a better leaving group thus rendering the ester bond formed less stable and more hydrolytically labile. Since there are two hydroxyl groups on the

Boc₅-colistin, two products with different molecular weight distributions, a mono-mPEG adduct (Boc₅-col-aaPEG, M_n Theoretical ~ 3.7 kDa, M_n MALDI ~ 3.6 kDa, Fig. 2b) and a bis-mPEG adduct (Boc₅-col-aaPEG₂, M_n Theoretical ~ 5.7 kDa, M_n MALDI ~ 5.5 kDa, Fig. 2c), were obtained and isolated by HPLC from the reaction mixture at 20-22 min (SFig. 2b, black trace) and 18-20 min (SFig. 2b, green trace), respectively. A polymeric distribution with a typical PEG repeating unit of 44 Da and the double-peak pattern of colistin A and B can be clearly observed from the MALDI-ToF MS data of both adducts (Fig. 2d-e), suggesting the successful attachment of aaPEG onto colistin.

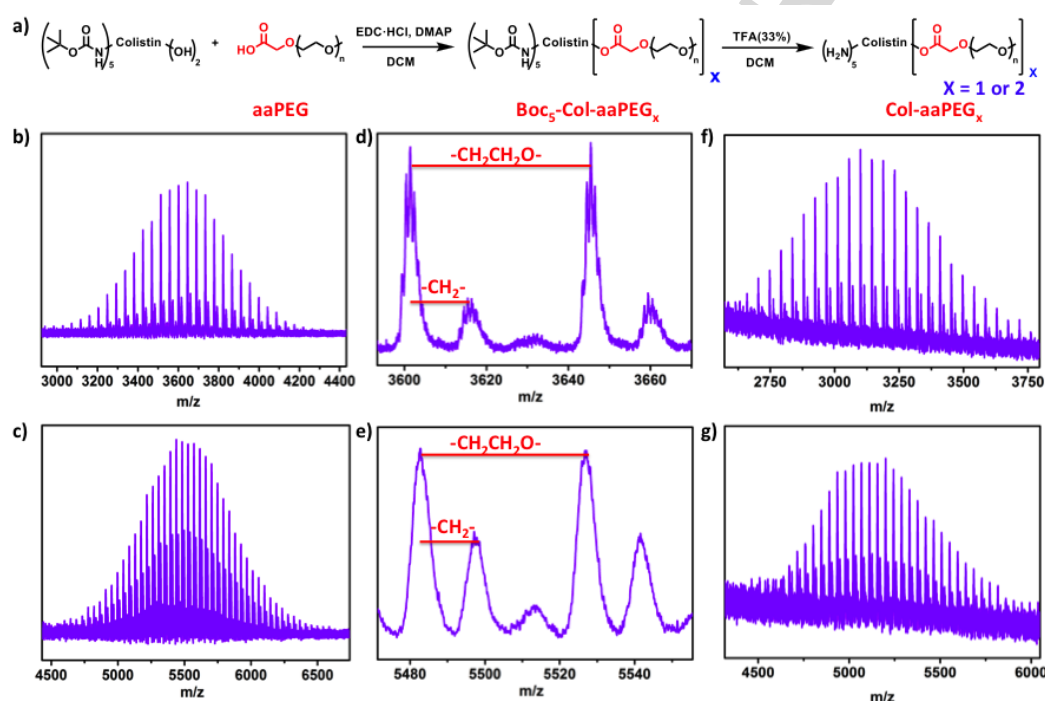


Fig. 2 a) The two-step synthesis of the PEGylated colistin conjugates. b)-e) MALDI-ToF MS results of the purified Boc protected conjugation products, Boc₅-col-aaPEG (b) and Boc₅-col-aaPEG₂ (c) and the related zoom-in data (d) and (e). f)-g) MALDI-ToF MS of the deprotection products of the mono-modification col-aaPEG (f) and double-modification col-aaPEG₂ (g).

The regeneration of amines on colistin was conducted under routine conditions for the cleavage of the Boc-protecting groups (Fig. 2a). After the treatment with 33% TFA in DCM, the more hydrophilic deprotected products (col-aaPEG and col-aaPEG₂) were obtained. A successful deprotection was indicated by the clear shift to a shorter retention time during HPLC characterisation when c.f. the protected form (SFig. 2b). Moreover, only a slight decrease in the molecular weight was observed upon GPC

characterisation (SFig. 2a), suggesting the ester bond between colistin and aaPEG remained unaffected during deprotection of both conjugates. Both col-aaPEG ($M_{n\text{ Theory}} \sim 3.2$ kDa, $M_{n\text{ MALDI}} \sim 3.2$ kDa, Fig. 2f) and col-aaPEG₂ ($M_{n\text{ Theory}} \sim 5.2$ kDa, $M_{n\text{ MALDI}} \sim 5.2$ kDa, Fig. 2g) retained the PEG polymeric distribution along with the colistin double-peak pattern through the MALDI-ToF MS data. About 500 Da molecular weight decrease was observed for the deprotected products, further confirming a full recovery of all the amines on the colistin conjugates.

3.2 The stability and the degradation of the mPEG-colistin conjugates

In order to study the cleavable nature of the ester bond, the degradation of both PEGylated conjugates was performed *in vitro* and monitored by HPLC (Fig. 3). To mimic the physiological condition, the test was conducted at 37 °C in phosphate buffered saline (PBS, pH = 7.4). For the col-aaPEG form, the signal of the conjugate decreased continuously with a co-committed appearance and increase in the typical colistin double-peaks up to 48 h (Fig. 3b). Noticeably, we found that more than 75% colistin can be released from col-aaPEG within 6 h whilst it normally required > 24 h for CMS to achieve a similar extent of colistin release under similar conditions [22]. The two colistin peaks were then collected and analysed *via* MALDI-ToF MS confirming their identity as native colistin A and B (Fig. 3d-f). This suggested that the released colistin was chemically unchanged and therefore potentially still active. As for col-aaPEG₂, although the hydrolysis rate is almost twice as slow compared to col-aaPEG in the first 6 h, a similar pattern was observed when following the reaction by HPLC with the colistin peaks appearing and increasing as a function of time, leading to full release of colistin within 48 h (Fig. 3c). Interestingly, during degradation a broad peak appeared at 12-13.5 min, which coincided with the col-aaPEG conjugate. The intensity of this peak initially increased during the first 4 h and then decreased along with the full consumption of col-aaPEG₂ to furnish the native lipopeptides. This indicates that the release of colistin from col-aaPEG₂ undergoes a stepwise degradation process, proceeding *via* a col-aaPEG as an intermediate product (Fig. 3a). Regardless,

this also confirmed that the two aaPEGs which are attached onto the different Thr residues can both be hydrolysed during the degradation process to fully liberate native polymyxins.

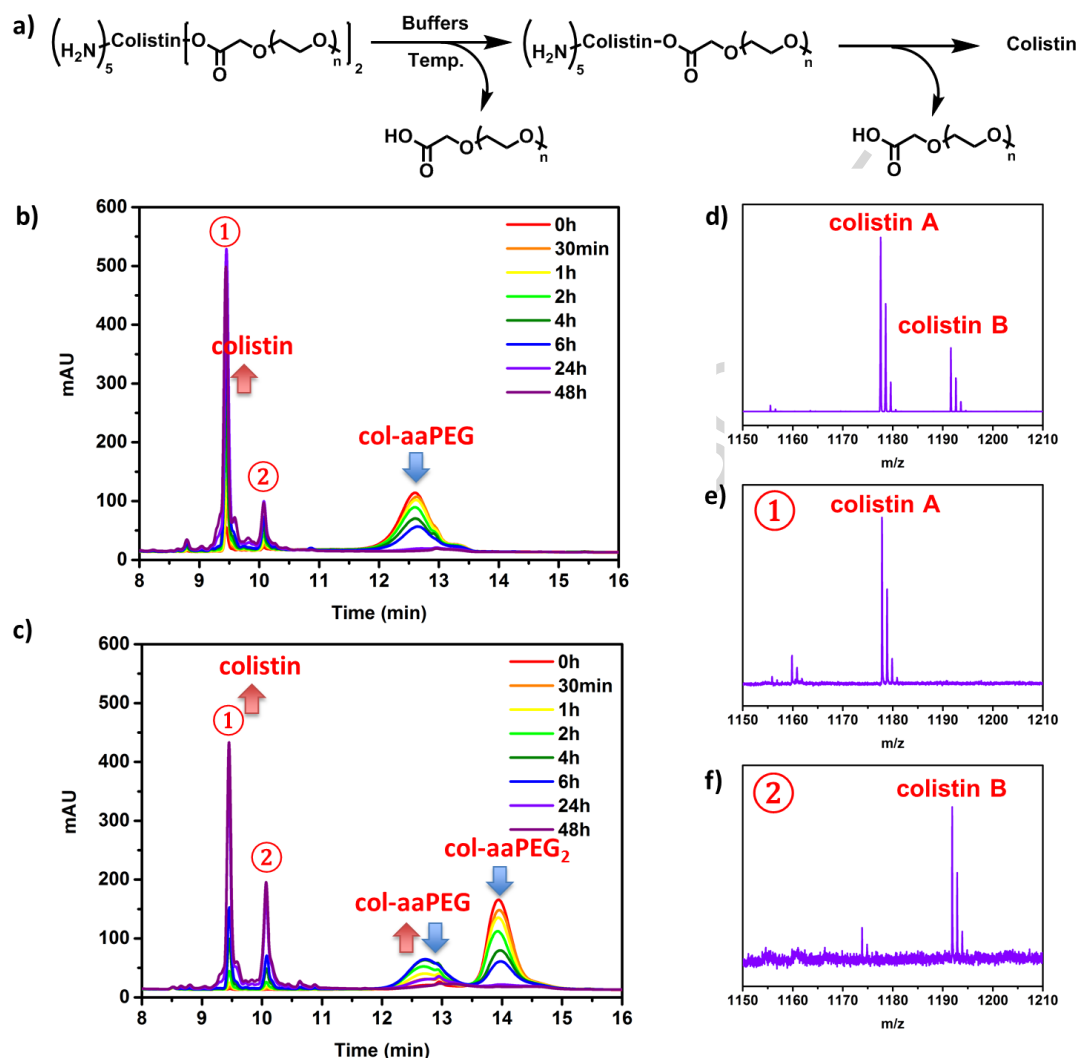


Fig. 3 a) The proposed mechanism of the colistin release from the col-aaPEG₂ conjugate. The ability to release the col-aaPEG (b) and col-aaPEG₂ (c) conjugate at 37 °C in PBS (1X) buffer was monitored by HPLC. The MALDI-ToF MS data of the commercial colistin (d) and the isolated colistin peaks (e and f) from the col-aaPEG prodrug solution after the incubation for 2 days.

To investigate the effect of the traceless linker between mPEG and colistin, a second PEGylated colistin with a different ester bond was synthesized by the same protocol using a commercial available succinic acid terminated mPEG (saPEG) (Fig. 4a). Lacking an electron-withdrawing oxygen bonded to the α -carbon, saPEG was expected to form a more stable conjugate. It was observed that colistin can still be released from both mono- and bis-saPEG modified products through the same

hydrolysis protocol although the release rate is nearly twice slower than from the corresponding colistin aaPEG conjugates in the first 6 h (Fig. 4b-c).

A similar degradation study was conducted at ambient temperature to evaluate the influence of temperature on the conjugate release (Fig. 4b-c). As expected, the rate of hydrolysis for all the colistin conjugates was retarded compared to those at body temperature. In particular, the double-modified conjugates showed much slower release compared to the mono-adducts due to the stepwise nature of the hydrolysis process. All of the results implied that both ester linkers were temperature sensitive with regards to stability.

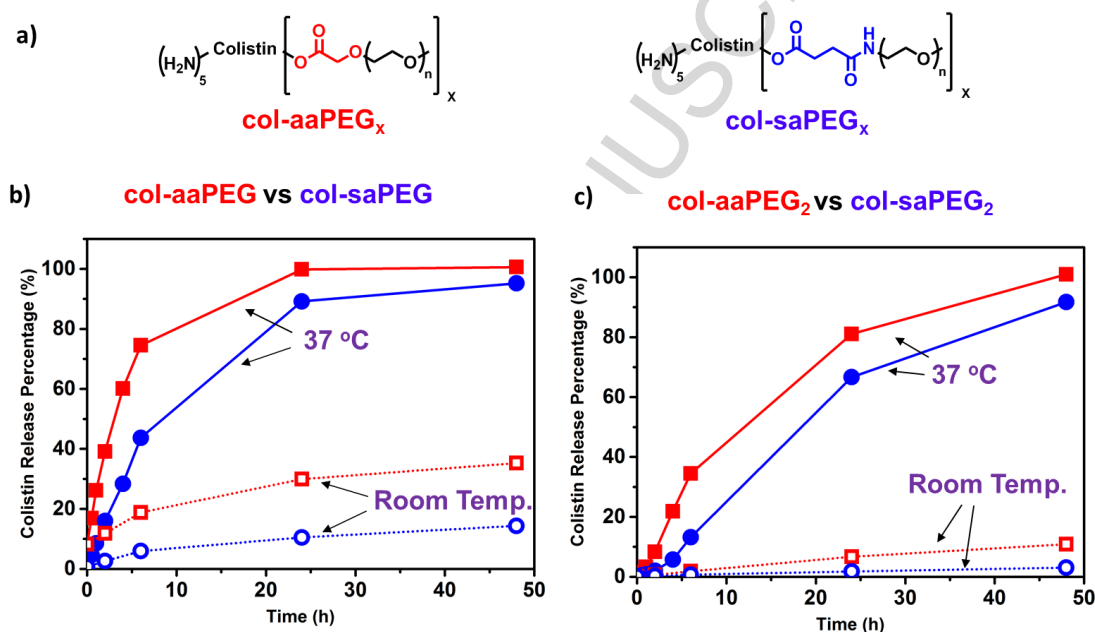


Fig. 4 a) The chemical structures of the aaPEG modified colistin prodrugs (left) and saPEG modified ones (right). The colistin release profiles of the mono PEGylated prodrugs (b) and double modified prodrugs (c) from PBS solution. Red lines: aaPEG modified prodrugs (left: col-aaPEG and right: col-aaPEG₂); blue lines: saPEG modified prodrugs (left: col-saPEG and right: col-saPEG₂); solid lines: 37 °C and dotted lines: ambient temperature.

3.3 *In vitro* antimicrobial activity test of the colistin-aaPEG conjugates

The antibiotic activity of the PEGylated conjugates was subsequently tested against two different MDR Gram-negative bacteria strains (*P. aeruginosa* ATCC 27853 and *A. baumannii* ATCC 19606). A disk diffusion assay was first conducted using colistin and CMS as positive controls. The diameter of zone of inhibition (ZoI) of each

conjugate is shown in Table 1. Overall, aaPEG alone showed no antibiotic activity whereas both aaPEG modified colistin conjugates showed a clear ZoI. This indicated that the colistin conjugates exhibited antimicrobial activity and crucially that the activity did not occur as a result of the cleaved polymer but rather, from the released native colistin. Owing to the larger molecular weight and a slower colistin release rate, col-aaPEG₂ showed a smaller ZOI compared to col-aaPEG at the same loading amount (20 µg). Conversely, both saPEG modified colistin conjugates showed a smaller ZOI compared to the corresponding aaPEG modified colistin conjugates which is in agreement with the observed colistin release profiles. Notably, col-saPEG₂ revealed no activity through the disk diffusion assay, indicating the attachment of PEG inhibited the activity of colistin and highlighting the need for a more labile, cleavable linker for the desired prodrug to maintain its activity over a time scale relevant to microbial proliferation.

Table 1 The diameter of zone of inhibition (ZOI) results of the conjugates against two different Gram-negative bacteria through disk diffusion assay (20 µg, 24 h).

	Diameter of Zone of Inhibition (ZOI) (mm)							
	colistin	CMS aaPEG	col-aaPEG	col-aaPEG ₂	col-saPEG	col-saPEG ₂	milliQ H ₂ O	
<i>Pa</i> ATCC 27853	19.0	17.5	0	14.5	9.0	12.5	0	0
<i>Ab</i> ATCC 19606	20.5	19.5	0	13.0	9.5	11.5	0	0

To obtain a more accurate comparison of the relative activities of the prepared conjugates, minimum inhibitory concentration (MIC) assays were performed (Table 2). The results revealed that the col-aaPEG conjugate behaved similarly to the native colistin in terms of the molar activity. This suggests that nearly full cleavage can be achieved from this colistin conjugate. Furthermore, the released colistin was chemically identical to native colistin, as confirmed during chemical characterisation (Fig. 3), and crucially that activity of the released polymyxin was retained. More interestingly, even though the molecular weight of the col-aaPEG are nearly twice the

commercial prodrug CMS ($M_{n\text{CMS}} \sim 1743 \text{ g mol}^{-1}$), less material was required to inhibit the bacteria, indicating that the colistin release profile of the col-aaPEG prodrug is more efficient than of CMS in the selected bacteria growth media. In agreement with the colistin release profiles (Fig. 4b) and disk diffusion assay (Table 1), the col-saPEG conjugate showed a lower activity (2-4 times) than the aaPEG conjugate, highlighting the need for faster colistin release to inhibit the growth of bacteria. Conversely, both double PEGylated prodrugs exhibited less activity against both bacteria strains on both mass and molar basis due to the molecular weight of the prodrugs as well as the slower degradation process. Especially col-saPEG₂ showed no activity in the selected concentration range, confirming the inhibition of the bacteria growth results from the release of colistin rather than from the prodrug itself.

Table 2 The minimum inhibitory concentration of the conjugates against two different Gram-negative bacteria on a weight and molar basis.

	Minimum inhibitory concentration (MIC) (mg/L; $\mu\text{mmol/L}$)						
	colistin	CMS	aaPEG	col-aaPEG	col-aaPEG ₂	col-saPEG	col-saPEG ₂
<i>Pa ATCC 27853</i>	2; 1.4	8; 4.6	> 128; > 64	4; 1.3	64; 12	16; 5.0	> 128; > 25
<i>Ab ATCC 19606</i>	1; 0.7	16; 9.2	> 128; > 64	8; 2.5	32; 6.1	16; 5.0	> 128; > 25

In order to determine a better understanding of the killing kinetics of the prodrug conjugates, time-kill studies were subsequently conducted by incubating *A. baumannii* ATCC 19606 bacteria strain as a model with colistin, CMS and colistin-aaPEG conjugates. The time-kill curves for selected dosage regimen are shown in Fig. 5. Three different doses (0.5 x MIC, 1 x MIC, and 2 x MIC, referring to mass based MICs of each compound) were initially tested. At the lower dose (0.5 x MIC), neither CMS nor aaPEG prodrugs exhibited inhibition with the killing curves essentially indistinguishable from those of the control, whereas colistin showed antibacterial killing in the first 6 h (Fig. 5a). By increasing the dose to 1 x MIC and 2 x MIC of both CMS and col-aaPEG, antimicrobial activity was observed with col-aaPEG performing

better at both doses than CMS (Fig. 5b-c). Particularly, col-aaPEG could inhibit the growth of bacteria to below the detection limit within 30 min at 2 x MIC, whilst it takes 4 h for CMS to control the bacteria growth close to the detection limit. At all three dosage regimens, col-aaPEG₂ did not show any inhibition against the bacteria which is possibly due to the slower degradation rate compared to the bacteria growth rate. However, it still showed an antibacterial killing at 30 min up to 4 h with regrowth occurring at 24 h when using a higher dose (8 x MIC) (Fig. 5d, purple trace).

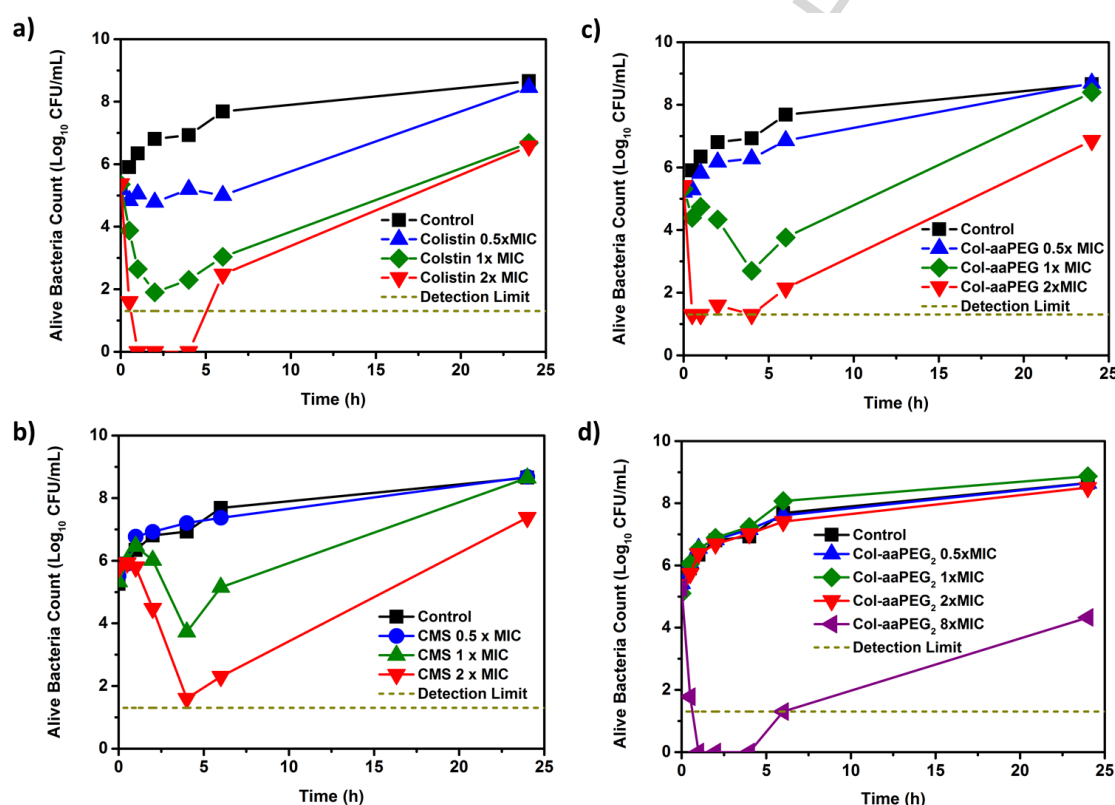


Fig. 5 *In vitro* time-kill studies of colistin (a), col-aaPEG (b), CMS (c), col-aaPEG₂ (d). The dashed line indicates the lower limit of detection of bacterial growth.

3.4 *In vivo* antimicrobial activity and toxicity evaluation of the colistin-aaPEG conjugates

Due to the encouraging *in vitro* performance from the col-aaPEG prodrugs, the *in vivo* efficacy of these two labile ester based conjugates (col-aaPEG and col-aaPEG₂) was investigated in comparison to native colistin treatment against *P. aeruginosa* ATCC 27853 in a neutropenic mouse thigh infection model, the gold standard method

was employed [49]. Colistin was employed as the comparator as the highly variable release and inter-batch heterogeneity of CMS yields large data fluctuations. After 18 h administration of colistin and the aaPEG prodrugs (40 mg colistin base/kg), a significant reduction in the bacterial burden was observed for both colistin (mean \log_{10} CFU/thigh 0.73 ± 1.46) and col-aaPEG (mean \log_{10} CFU/thigh 4.77 ± 0.89) compared to the 0 h (mean \log_{10} CFU/thigh 5.85 ± 0.06 , > 91.6% bacteria kill). However, col-aaPEG₂ failed to produce sufficient bacterial killing *in vivo* (mean \log_{10} CFU/thigh 8.75 ± 0.12). (Fig. 6a)

Nephrotoxicity remains a dose-limiting issue with the broader application of polymyxin therapy as it impacts the ability of clinicians to increase the dose [44-50]. Therefore, in the present study, the *in vivo* apoptotic effect of col-aaPEG and col-aaPEG₂ was examined through the histological examination of the kidneys from mice subcutaneously administered either colistin or PEGylated colistin prodrugs at an accumulated dose of 40 mg colistin base/kg (Fig. 6b). The kidneys of mice administered with the saline control had no observable histological damage (semi quantitative SQR score of 0, Fig. 6c). In comparison, the histological examination of the kidneys from the mice treated with colistin, col-aaPEG₂ showed comparable mild histological damage with tubule damage, i.e. tubular dilation and degeneration (semi quantitative SQR score of 1, Fig. 6d, f). Encouragingly, the kidneys of mice treated with col-aaPEG had no significant histological damage and were essentially comparable to the saline control (grade 0 histology kidney scoring; Fig. 6e). These results demonstrate that colistin and col-aaPEG₂ (both grade 1 histology kidney scoring) appears to be more nephrotoxic than col-aaPEG (Fig. 6). The observation that the doubly PEGylated prodrug generates more nephrotoxicity than the mono-PEGylated prodrug is interesting. As the histology assay is well validated it is obviously not as simple as correlating nephrotoxicity with rates of colistin release, the observed differences more likely reflects the complex and different renal handling

mechanism at play for the mono-PEGylated vs the doubly PEGylated prodrug [13].

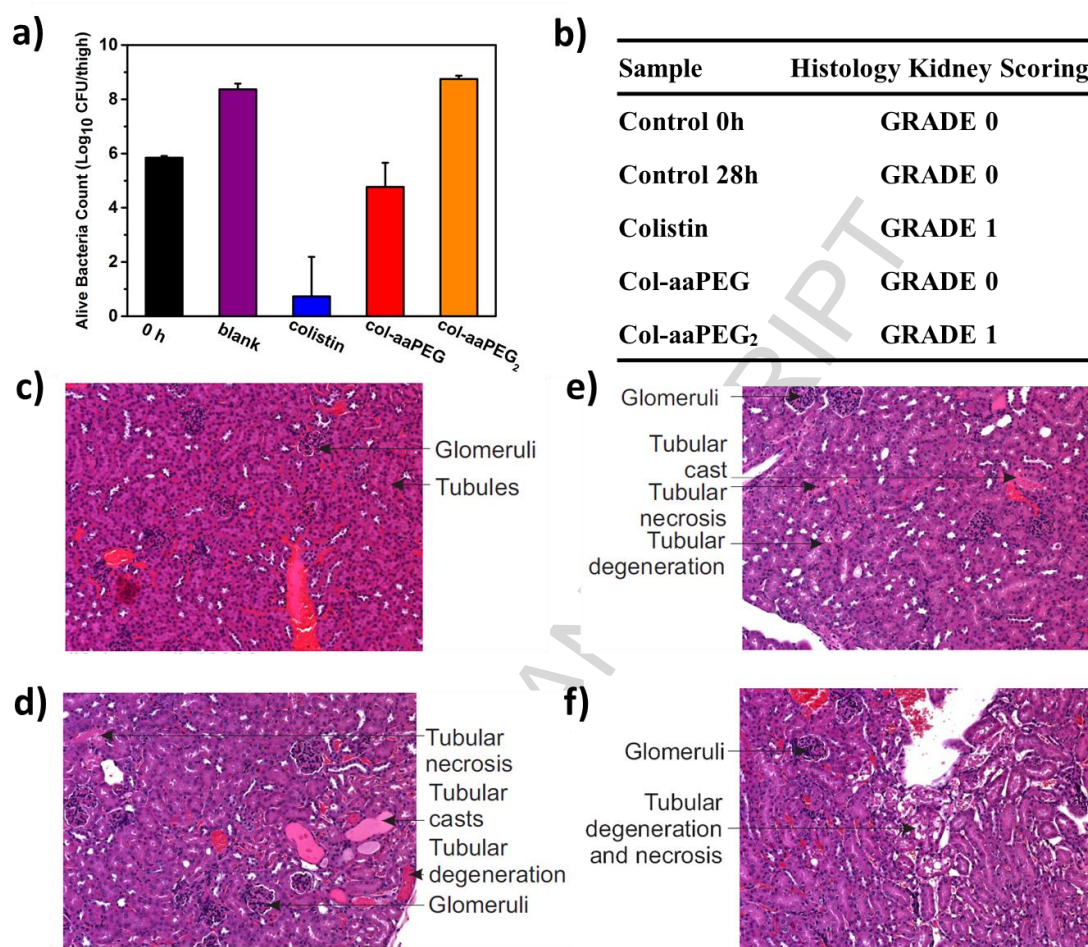


Fig. 6 a) *In vivo* activity of colistin and prodrugs (col-aaPEG and col-aaPEG₂) against *P. aeruginosa* ATCC 27853 using a mouse thigh infection model. (n = 4). b) Summary of histology kidney scoring from each sample. c)-f) Microscopic image of the cortex section of the kidneys of mice treated with saline control, colistin, prodrugs col-aaPEG and col-aaPEG₂ (accumulated dose 40 mg colistin base/kg). c) control (0 h) (SQR score 0); d) colistin (SQR score 1); e) col-aaPEG (SQR score 0), f) col-aaPEG₂ (SQR score 1).

4. Conclusions

In this work, fully characterised mono- and bis-mPEG modified colistin conjugates with two different ester linkages on the Thr residue(s) of colistin were synthesised *via* Boc-protection/deprotection and Steglich esterification. All the prodrug candidates were tested through the *in vitro* degradation studies and revealed native colistin can be

released smoothly from all the colistin conjugates and that the colistin release rate from the PEGylated conjugates can be altered by the number of mPEG attachment and the lability of the linker. It is noted that a full PK study is well beyond the scope of the study and this would require the development of a validated assay that takes into account the stability of the prodrug during storage periods and during assay.

Studies also showed the degradation process is temperature sensitive and the prodrugs are relatively stable at ambient temperature. Among them, the col-aaPEG showed the fastest colistin release rate, achieving near full colistin release within 24 h, which makes it a viable prodrug candidate. All *in vitro* antimicrobial activity tests suggested col-aaPEG performs with a similar or better activity relative to the commercial prodrug CMS or colistin on both molar and mass basis. In comparison to colistin, col-aaPEG showed acceptable bacterial killing in an *in vivo* mouse model against *P. aeruginosa* ATCC 27853 and displayed no nephrotoxicity through the histological examination of the kidneys. Although further evaluation is needed to determine whether this activity profile of col-aaPEG *in vivo* extends to other Gram-negative species, we believe that due to the enhanced specificity confirmed by targeting Thr residues without loss of antibacterial civility, col-aaPEG has the potential candidate to become a potent alternative prodrug to CMS.

Acknowledgements

The authors thank Dr. Lijiang Song for the help on MALDI-ToF MS analysis and gratefully acknowledge Heidi Yu and Jesmin Akter for the help and discussion on the microbiological test. We gratefully acknowledge the financial support from the EPSRC (C. Z.), Leverhulme Trust (P. W.) and Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology (project number CE140100036). K.K. gratefully acknowledges the award of an NHMRC-ARC Dementia Research Development Fellowship (APP1109945).

References:

- [1] D.I. Andersson, D. Hughes, Antibiotic resistance and its cost: is it possible to reverse resistance?, *Nat. Rev. Micro.* 8 (2010) 260-271.
- [2] U.S. Department of Health and Human Services, Antibiotic resistance threats in the United States, 2013, <http://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf>, 2013 (accessed: 9 September, 2016).
- [3] J. O'Neill, Tackling a global health crisis: initial steps, <https://amr-review.org/sites/default/files/Report-52.15.pdf>, 2015 (accessed: 9 September, 2016).
- [4] Australian Government, Department of Health and Department of Agriculture, Responding to the threat of antimicrobial resistance, [https://www.health.gov.au/internet/main/publishing.nsf/Content/1803C433C71415CA CA257C8400121B1F/\\$File/amr-strategy-2015-2019.pdf](https://www.health.gov.au/internet/main/publishing.nsf/Content/1803C433C71415CA CA257C8400121B1F/$File/amr-strategy-2015-2019.pdf), 2015 (accessed: 9 September, 2016).
- [5] J. O'Neill, Tackling drug-resistant infections globally: final report and recommendations - The review on antimicrobial resistance, http://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf 2016 (accessed: 9 September, 2016).
- [6] R.J. Fair, Y. Tor, Antibiotics and bacterial resistance in the 21st century, *Perspect. Medicin. Chem.* 6 (2014) 25-64.
- [7] L.M. Lim, N. Ly, D. Anderson, J.C. Yang, L. Macander, A. Jarkowski, A. Forrest, J.B. Bulitta, B.T. Tsuji, Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing, *Pharmacother.* 30 (2010) 1279-1291.
- [8] J. Li, R.L. Nation, J.D. Turnidge, R.W. Milne, K. Coulthard, C.R. Rayner, D.L. Paterson, Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections, *Lancet Infect. Dis.* 6 (2006) 589-601.
- [9] R.L. Nation, J. Li, Colistin in the 21st century, *Curr. Opin. Infect. Dis.* 22 (2009) 535-543.
- [10] J. Li, R.L. Nation, R.W. Milne, J.D. Turnidge, K. Coulthard, Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria, *Int. J. Antimicrob. Ag.* 25 (2005) 11-25.
- [11] K.D. Roberts, M.A. Azad, J. Wang, A.S. Horne, P.E. Thompson, R.L. Nation, T. Velkov, J. Li, Antimicrobial activity and toxicity of the major lipopeptide components of polymyxin B and colistin: last-line antibiotics against multidrug-resistant gram-negative bacteria, *ACS Infect. Dis.* 1 (2015) 568-575.
- [12] T. Velkov, P.E. Thompson, R.L. Nation, J. Li, Structure– activity relationships of polymyxin antibiotics, *Journal of medicinal chemistry* 53 (2009) 1898-1916.
- [13] T. Velkov, K.D. Roberts, R.L. Nation, P.E. Thompson, J. Li, Pharmacology of polymyxins: new insights into an old class of antibiotics, *Future microbiology* 8 (2013) 711-724.

- [14] A. Clausell, M. Garcia-Subirats, M. Pujol, M.A. Busquets, F. Rabanal, Y. Cajal, Gram-negative outer and inner membrane models: insertion of cyclic cationic lipopeptides, *J. Phys. Chem. B* 111 (2007) 551-563.
- [15] M.A. Azad, J.X. Huang, M.A. Cooper, K.D. Roberts, P.E. Thompson, R.L. Nation, J. Li, T. Velkov, Structure–activity relationships for the binding of polymyxins with human α -1-acid glycoprotein, *Biochem. Pharmacol.* 84 (2012) 278-291.
- [16] J.X. Huang, M.A. Blaskovich, R. Pelingon, S. Ramu, A. Kavanagh, A.G. Elliott, M.S. Butler, A.B. Montgomery, M.A. Cooper, Mucin Binding Reduces Colistin Antimicrobial Activity, *Antimicrob. Agents Chemother.* 59 (2015) 5925-5931.
- [17] H. Spapen, R. Jacobs, V. Van Gorp, J. Troubleyn, P.M. Honoré, Renal and neurological side effects of colistin in critically ill patients, *Ann. Intensive Care* 1 (2011) 14.
- [18] E. Forde, M. Devocelle, Pro-moieties of antimicrobial peptide prodrugs, *Molecules* 20 (2015) 1210-1227.
- [19] P.J. Bergen, J. Li, C.R. Rayner, R.L. Nation, Colistin methanesulfonate is an inactive prodrug of colistin against *Pseudomonas aeruginosa*, *Antimicrob. Agents Chemother.* 50 (2006) 1953-1958.
- [20] P. Koomanachai, C.B. Landersdorfer, G. Chen, H.J. Lee, A. Jitmuang, S. Wasuwattakul, S. Sritippayawan, J. Li, R.L. Nation, V. Thamlikitkul, Pharmacokinetics of colistin methanesulfonate and formed colistin in end-stage renal disease patients receiving continuous ambulatory peritoneal dialysis, *Antimicrob. Agents Chemother.* 58 (2014) 440-446.
- [21] A.S. Michalopoulos, M.E. Falagas, Colistin: recent data on pharmacodynamics properties and clinical efficacy in critically ill patients, *Ann. Intensive Care* 1 (2011) 30.
- [22] J. Li, R.W. Milne, R.L. Nation, J.D. Turnidge, K. Coulthard, Stability of colistin and colistin methanesulfonate in aqueous media and plasma as determined by high-performance liquid chromatography, *Antimicrob. Agents Chemother.* 47 (2003) 1364-1370.
- [23] J.M. Harris, R.B. Chess, Effect of pegylation on pharmaceuticals, *Nat. Rev. Drug Discov.* 2 (2003) 214-221.
- [24] E.M. Pelegri-O'Day, E.-W. Lin, H.D. Maynard, Therapeutic protein–polymer conjugates: advancing beyond PEGylation, *J. Am. Chem. Soc.* 136 (2014) 14323-14332.
- [25] M.A. Gauthier, H.-A. Klok, Polymer-protein conjugates: an enzymatic activity perspective, *Polym. Chem.* 1 (2010) 1352-1373.
- [26] J.Y. Shu, B. Panganiban, T. Xu, Peptide-polymer conjugates: From fundamental science to application, *Annu. Rev. Phys. Chem.* 64 (2013) 631-657.
- [27] M. Roberts, M. Bentley, J. Harris, Chemistry for peptide and protein PEGylation, *Adv. Drug Deliv. Rev.* 64 (2012) 116-127.
- [28] I.W. Hamley, PEG–peptide conjugates, *Biomacromolecules* 15 (2014) 1543-1559.

- [29] A. Abuchowski, T. Van Es, N. Palczuk, F. Davis, Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol, *J. Biol. Chem.* 252 (1977) 3578-3581.
- [30] J.M. Harris, N.E. Martin, M. Modi, Pegylation, *Clin. Pharmacokinet.* 40 (2001) 539-551.
- [31] L. Tao, J. Liu, J. Xu, T.P. Davis, Synthesis and bioactivity of poly (HPMA)-lysozyme conjugates: the use of novel thiazolidine-2-thione coupling chemistry, *Org. Biomol. Chem.* 7 (2009) 3481-3485.
- [32] R.J. Mancini, J. Lee, H.D. Maynard, Trehalose glycopolymers for stabilization of protein conjugates to environmental stressors, *J. Am. Chem. Soc.* 134 (2012) 8474-8479.
- [33] J. Khandare, T. Minko, Polymer-drug conjugates: progress in polymeric prodrugs, *Prog. Polym. Sci.* 31 (2006) 359-397.
- [34] R.B. Greenwald, K. Yang, H. Zhao, C.D. Conover, S. Lee, D. Filpula, Controlled release of proteins from their poly (ethylene glycol) conjugates: drug delivery systems employing 1, 6-elimination, *Bioconjugate Chem.* 14 (2003) 395-403.
- [35] J. Chen, M. Zhao, F. Feng, A. Sizovs, J. Wang, Tunable thioesters as "reduction" responsive functionality for traceless reversible protein PEGylation, *J. Am. Chem. Soc.* 135 (2013) 10938-10941.
- [36] H. Zhao, K. Yang, A. Martinez, A. Basu, R. Chintala, H.-C. Liu, A. Janjua, M. Wang, D. Filpula, Linear and branched bicin linkers for releasable PEGylation of macromolecules: controlled release in vivo and in vitro from mono-and multi-PEGylated proteins, *Bioconjugate Chem.* 17 (2006) 341-351.
- [37] L. Tao, J. Liu, J. Xu, T.P. Davis, Bio-reversible polyPEGylation, *Chem. Commun.* (2009) 6560-6562.
- [38] G. Saito, J.A. Swanson, K.-D. Lee, Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities, *Adv. Drug Deliv. Rev.* 55 (2003) 199-215.
- [39] X. Liu, P. Zhang, D. He, W. Röhl, T. Preiß, J.O. Rödl, E. Wagner, U. Laichelt, pH-reversible cationic RNase A conjugates for enhanced cellular delivery and tumor cell killing, *Biomacromolecules* 17 (2015) 173-182.
- [40] F.M. Veronese, P. Caliceti, A. Pastorino, O. Schiavon, L. Sartore, L. Banci, L.M. Scolaro, Preparation, physico-chemical and pharmacokinetic characterization of monomethoxypoly (ethylene glycol)-derivatized superoxide dismutase, *J. Control. Release* 10 (1989) 145-154.
- [41] Disk Diffusion Method for Antimicrobial Susceptibility Testing, Version 5.0 (January 2015), in: E.C.o.A.S.T. EUCAST (Ed.), 2015.
- [42] CLSI, *Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement (M100-S20)*. CLSI, Wayne, PA, USA., 2010.
- [43] K.D. Roberts, M.A.K. Azad, J. Wang, A.S. Horne, P.E. Thompson, R.L. Nation, T. Velkov, J. Li, Antimicrobial Activity and Toxicity of the Major Lipopeptide Components of Polymyxin B and Colistin: Last-Line Antibiotics against

Multidrug-Resistant Gram-Negative Bacteria, *ACS Infect. Dis.* 1 (2015) 568-575.

[44] D. Landman, C. Georgescu, D.A. Martin, J. Quale, Polymyxins revisited, *Clin Microbiol Rev.* 21 (2008) 449–465.

[45] J.D. Hartzell, R. Neff, J. Ake, R. Howard, S. Olson, K. Paolino, M. Vishnepolsky, A. Weintrob, G. Wortmann, Nephrotoxicity associated with intravenous colistin (colistimethate sodium) treatment at a tertiary care medical center., *Clin. Infect. Dis.* 48 (2009) 1724-1728.

[46] C.J. Kubin, T.M. Ellman, V. Phadke, L.J. Haynes, D.P. Calfee, M.T. Yin, Incidence and predictors of acute kidney injury associated with intravenous polymyxin B therapy, *J. Infect* 65 (2012) 80-87.

[47] D.S. Akajagbor, S.L. Wilson, K.D. Shere-Wolfe, P. Dakum, M.E. Charurat, B.L. Gilliam, Higher incidence of acute kidney injury with intravenous colistimethate sodium compared with polymyxin B in critically ill patients at a tertiary care medical center., *Clin. Infect. Dis.* 57 (2013) 1300-1303.

[48] M.H. Rigatto, T.F. Behle, D.R. Falci, T. Freitas, N.T. Lopes, M. Nunes, L.W. Costa, A.P. Zavascki, Risk factors for acute kidney injury (AKI) in patients treated with polymyxin B and influence of AKI on mortality: a multicentre prospective cohort study, *The Journal of antimicrobial chemotherapy* 70 (2015) 1552-1557.

[49] R.V. Dudhani, J.D. Turnidge, R.L. Nation, J. Li, fAUC/MIC is the most predictive pharmacokinetic/pharmacodynamic index of colistin against *Acinetobacter baumannii* in murine thigh and lung infection models., *J Antimicrob Chemother* 65 (2010) 1984-1990.

[50] S.M. Garonzik, J. Li, V. Thamlikitkul, D.L. Paterson, S. Shoham, J. Jacob, F.P. Silveira, A. Forrest, R.L. Nation, Population pharmacokinetics of colistin methanesulfonate and formed colistin in critically ill patients from a multicenter study provide dosing suggestions for various categories of patients., *Antimicrob Agents Chemother* 55 (2011) 3284-3294.