Hydrolyzable Poly(PEGA) Colistin Prodrugs through Copper-mediated Photo-induced Living Radical Polymerization

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ABSTRACT: Through the recently developed copper-mediated photo-induced living radical polymerization (CP-LRP), a novel and well-defined polymeric prodrug of the antimicrobial lipopeptide colistin has been developed. A colistin initiator (Boc_5-col-Br_2) was synthesized through the modification of colistin on both of its threonine residues using a cleavable initiator linker, 2-(2-bromo-2-methylpropanoyloxy) acetic acid (BMPAA) and used for the polymerization of acrylates via CP-LRP. Polymerization proceeds from both sites of the colistin initiator, and through the polymerization of $PEGA_{480}$, three water-soluble polymer-colistin conjugates (col-PPEGA, DP = 5, 10, 20) were achieved with high yield (conv. ≥ 93%) and narrow dispersities ($Đ$ < 1.3) in 2 – 4 hours. Little or no effect on the structure/activity of the colistin was observed during the synthesis and most of the active colistin can be recovered from the conjugates in vitro within 2 days. Furthermore, in vitro biological analyses including disk diffusion, broth microdilution and time-kill studies suggested all the conjugates have the ability to inhibit the growth of multi-drug resistant (MDR) Gram-negative bacteria, of which col-PPEGA DP5 and DP10 showed similar or better antibacterial performance compared to the clinically relevant colistin prodrug CMS, indicating their potential as an alternative antimicrobial therapy. Moreover, considering the control over the polymerization, CP-LRP technique has the potential to provide an alternative platform for the development of polymer bioconjugates.

INTRODUCTION

The development of new antimicrobial agents against bacterial infections is difficult and time/cost consuming.[1-4] With the increasing amount of drug-resistant infections and the emergence of multi-drug resistant (MDR) Gram-negative bacteria, aka 'superbugs', a lack of effective drugs and treatments is threatening humans all over the world and might have severe consequences if no action is taken.[5,6] Antibiotic resistance currently accounts for an estimated 50,000 deaths in the US and Europe, with estimates that the actual current death toll is ca. 700,000 worldwide. If antibiotic resistance were allowed to grow unabated the number of deaths per year would dramatically increase to an estimated 10 million by 2050.[7] To combat these 'superbugs', colistin, an antibiotic from the polymyxin family has recently re-attracted the attention of researchers.[8-11] As one of the most potent peptide-based antimicrobial agents against MDR Gram-negative bacteria, colistin is a double-edged sword in clinical treatments due to its potential toxicity to human kidneys and the nervous system at high dose and sometimes required long treatment regimens.[12-15] To date, several approaches have been developed for localized infection treatments, including inhaled colistin for lung infections[16] or colistin patches for wound infections.[17] However due to their intrinsic toxicity, intravenous applications have remained challenging.

The only FDA-approved colistin formulations available for systematic administration is the prodrug colistin methanesulfonate (CMS).[18] Modified on the five colistin amines, this inactive prodrug is a less toxic compound that can be converted to active colistin *in vivo*.[18,19] However it is widely acknowledged that the clinical application of CMS still suffers from a number of important drawbacks. For example, its pharmacokinetic properties are far from ideal due to the slow release rate, approximately 60% of the dosed CMS is excreted in the urine in the first 24 hours administration before being hydrolyzed to active colistin.[20] Also importantly, as there are 5 amines capable of undergoing modification during the preparation of CMS, there are potentially 32 (= 2^5) chemical variations, including unmodified colistin, that can be present in CMS formulations, leading to both structural and therefore dose variation from batch to batch, which can have a significant negative impact on clinical outcomes.[19,21]

In an effort to find a replacement for CMS, a more structurally controlled polymeric colistin prodrug (col-aaPEG) has been previously developed.[22] In contrast to the amine modifi-

cation in CMS, an acetic acid terminal poly(ethylene glycol) methyl ether (aaPEG) was attached onto colistin threonine (Thr) residues to minimize the diversity of the modification site and to increase its bioavailability and improve its pharmacokinetics/pharmacodynamics performance. This hydrolyzable PEGylated colistin showed an encouraging *in vitro* and *in vivo* performance similar to CMS and revealed no systematic toxicity or nephrotoxicity. However, there remain two potential modification sites for the more active col-aaPEG monoconjugate and it is not easy to control selectivity between the two Thr residues. Conversely, col-aaPEG$_2$ with well-defined modification at both Thr residues showed a slower release rate and lower activity compared to its monoconjugate analogue. Thus, a colistin prodrug with well-defined site modification, i.e. modification at both Thr residues, with a suitable colistin release profile may be of significant advantage.

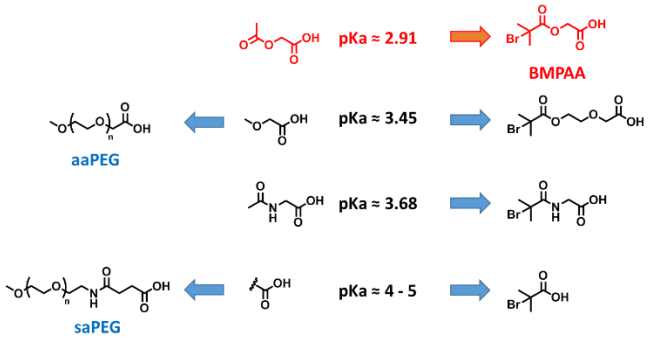
As an alternative to our previous 'grafting-to' strategy for colistin modification, the 'grafting-from' approach to protein/peptide modification confers a number of advantages. The addition of functional small molecules, unhindered by bulky polymer chains, facilitates complete and specific modification of the residues of interest on the target protein/peptide.[23,24] Typically, using this approach moieties capable of mediating polymerization such as α-halo ester/amide initiators or thiocarbonylthio chain transfer agents are initially conjugated to proteins/peptides. The resulting modified structures can be fully and precisely characterized by conventional methods before being employed in polymerizations to afford polymer conjugates. Furthermore, the purification of the resulting conjugates is much less intensive than for 'grafting-to' techniques, as relatively large conjugates are easily separated from small molecule impurities, which include unreacted monomer and other necessary reagents in solution.

Recent developments in reversible-deactivation radical polymerizations (RDRP), such as reversible addition−fragmentation chain transfer polymerization (RAFT)[25,26] and Cu-mediated polymerizations; atom transfer radical polymerization (ATRP),[23,27] and single-electron transfer living radical polymerization (SET-LRP),[28,29] bestow excellent control over the molecular weight and dispersity ($Đ$) of polymers maintaining high end-group fidelity. The use of external stimuli (i.e., pressure, electricity and light) enables polymerizations to be performed under relatively mild reaction conditions, even mimicking physiological environments, whilst maintaining and even accelerating the rate of reaction and retaining control over the polymerization.[30-32]

One such technique, Cu-mediated photo-induced polymerization (CP-LRP),[33,34] has been studied using visible/UV light, employing a $Cu^{II}(Me_6\text{-Tren})Br_2$ complex in the presence of an excess of tris[2-(dimethylamino)ethyl]amine (Me_6Tren), allows polymerization to take place at and below ambient temperature with near perfect temporal control over the molecular weight, dispersity and chain-end fidelity at relatively low catalyst loadings (2 mol% w.r.t. initiator) in the absence of conventional photoinitiators or dye-sensitizers. To date there have been no reports of this novel technique being employed for the preparation of protein/peptide-polymer conjugates via either the 'grafting-to' or 'grafting-from' methods. The versatility of the approach and its demonstrated use in the polymerization of a wide range of monomer types expands the potential polymer compositions that can be attached to peptides/proteins through the simple variation of the (co)monomer feed, which importantly includes easy access to poly[poly(ethylene glycol) methyl ether (meth)acrylate] (PPEG(M)A) grafted materials which are well established as an alternative to linear PEG.[23, 24] Synthetic issues associated with the attachment of linear PEG to colistin are exacerbated by increasing molecular weight.[35] Similarly, the viscosity and crystallinity of linear PEG increases with molecular weight, as does the propensity for organ accumulation and vacuolization, making it less suitable for intravenous administration.[36-38] The comb-like architecture of PPEG(M)A materials, with their PEG side chains impart a rigid rod-like structure in aqueous solution, which reduces the viscosity compared to linear PEG of the same molecular weight.[38] More importantly, due to its non-crystalline properties, PPEG(M)A does not demonstrate the accumulation in, or damage of, human tissues that has been attributed to linear PEG.[36]

To address these problems, we have investigated the use of CP-LRP to prepare well-defined peptide-polymer conjugates using a 'grafting-from' approach for the first time. To achieve both site specificity and efficient release of colistin we have used a new hydrolytically labile α-halo ester initiating group to modify the two Thr residues. The linker has been specifically selected to provide an enhanced release rate relative to the acetic acid linker (aaPEG), which has been used previously. Conjugates have been prepared with good control of the grafted polymer molecular weight and narrow dispersities, as confirmed by full characterization of the polymers released from the conjugates. The effect of the degree of polymerization on the colistin release rate and bioactivity has been thoroughly evaluated by *in vitro* disk diffusion, broth dilution and time-kill assays. We demonstrate that, subject to efficient release, the polymeric colistin prodrugs have little or no observable effect on the structure or antimicrobial activity of the colistin.

Scheme 1 Acid dissociation constant at logarithmic scale of the acids with different side groups and their relative initiator linker.



RESULTS AND DISCUSSIONS

Design of the Initiator Linker for Colistin Conjugation. The colistin release rate from the polymeric prodrug would greatly affect the final antimicrobial activity and this phenomena would be more significant on the double modified colistin conjugate due to a two-step hydrolysis profile.[22] Thus the design of the linker between colistin and the initiating group is important to the proposed 'grafting from' approach for the formation of a suitable prodrug candidate. In order to obtain an improved performance and a faster colistin release rate, a labile linker comparable to that present in the linear aaPEG is a required design prerequisite in order to afford a suitable hydrolysis whilst counteracting the two-step hydrolysis effect.[22]

The stability of an ester is influenced by the neighboring groups of the carboxyl group. A more acidic group (quantified by the acid dissociation constant K_a) which normally has a stronger electron withdrawing effect, can act as a better leaving group, leading to a faster ester hydrolysis rate. Due to the same reason, the linear PEG-conjugate col-saPEG hydrolyzed slower than col-aaPEG.[22] Thus, based on pKa values, 2-(2-bromo-2-methylpropanoyloxy)acetic acid (BMPAA) was synthesized and chosen as the initiator linker (Scheme 1).

Prior to the attachment of this initiator linker to colistin, all colistin amine residues were protected by *tert*-butyloxycarbonyl (Boc) groups (Boc_5-col).[22] The initiator linker was subsequently attached onto both colistin Thr residues using Steglich esterification (Figure 1a). As the commercial colistin contains two major components (colistin A and B), both colistin peaks with a clear molecular weight increase (414 Da) were observed from the modified product (Boc_5-col-Br_2) compared to Boc_5-col through matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-ToF MS), indicative of the attachment of two initiator groups (Figure 1b). The isotope pattern of Boc_5-col-Br_2 also suggested that this compound contains two bromine atoms (Figure 1c), further confirming the successful attachment of two initiators on Boc_5-colistin and the stability of both bromines of the initiators under the reaction conditions.

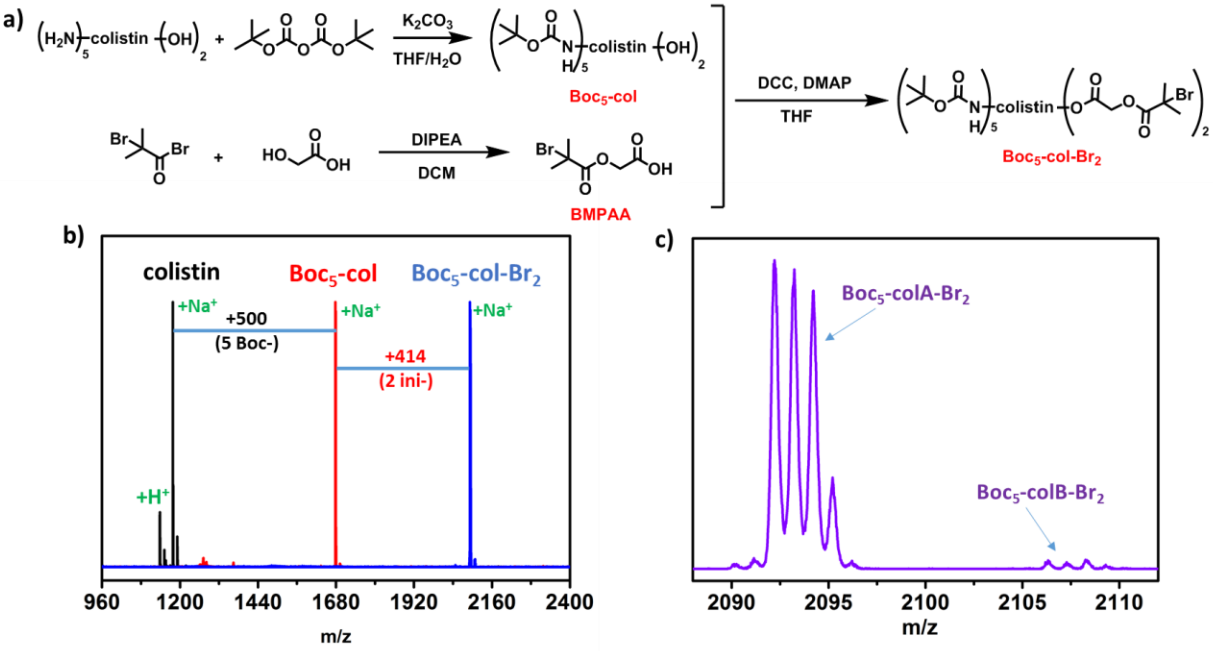


Figure 1 a) The Boc-protection reaction on colistin Dab amines and the subsequent modification of the labile BMPAA initiator on colistin Thr groups, b) MALDI-ToF MS data of native colistin (black), Boc_5-col (red), and Boc_5-col-Br_2 (blue) and c) The zoom-in data of Boc_5-col-Br_2.

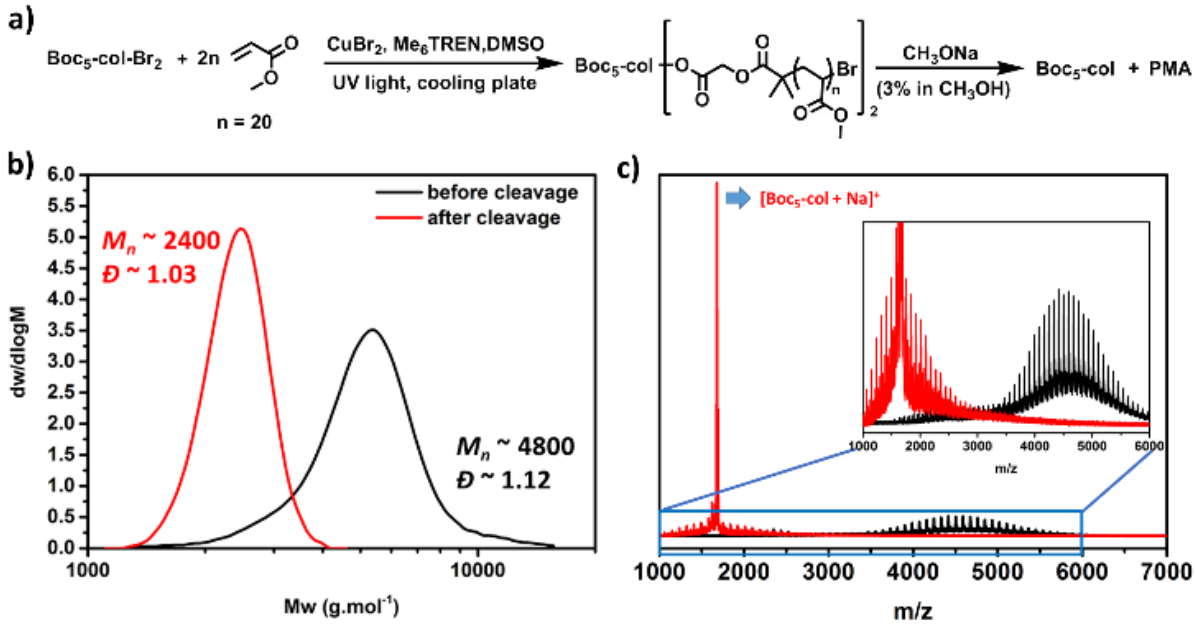


Figure 2 a) Scheme of the polymerization of MA using the colistin initiator *via* CP-LRP and the further cleavage of PMA colistin conjugate using NaOMe (3% in MeOH), b) GPC and c) MALDI-ToF MS of the Boc_5-col-PMA conjugate before (black traces) and after (red traces) treating with NaOMe.

Polymerization of $PEGA_{480}$ from the Colistin Initiator. Due to both the hydrolytic and heat sensitive features of both colistin and the labile initiator linker, compatible polymerization conditions, such as low temperature and a non-aqueous solvent were required. Thus, CP-LRP was explored to achieve polymerization from the colistin initiator.[33] Although this technique has shown to be a versatile and robust platform, to the best of our knowledge, a peptide-based initiator has not yet been investigated. Moreover, it was not certain whether the UV light would have an undesirable effect on the peptide under these conditions. Firstly, methyl acrylate (MA) was used as a model monomer to study the feasibility of the polymerization (Figure 2a).

The total degree of polymerization (DP) was set to 40 (20 for each initiating site) to achieve a better resolution from both nuclear magnetic resonance (NMR) and MALDI-ToF MS analyses. The reaction was carried out under UV light (λ_{max} ~ 360 nm) with copper(II) bromide ($CuBr_2$) and Me_6Tren as the ligand at a ratio of 0.02:0.12:1 relative to the colistin initiator. Anhydrous DMSO was used so as to prevent initiator hydrolysis during the polymerization. A cooling plate was also applied to maintain the reaction temperature (~ 15 °C), further lowering the propensity of any side reactions. The conversion of MA was measured through NMR analysis of the reaction mixture, comparing the vinyl groups (δ = 5.70 - 6.35 ppm, Figure S1b, peak b-d) against the signal of methyl group on both the unreacted monomer and the formed polymer (δ = 3.48 -3.68 ppm, Figure S1b, peak a and a'). This revealed that around 96% of the methyl acrylate was converted into polymer after 4 h polymerization. A monomodal peak with narrow dispersity ($Đ$ ~ 1.12) was observed through gel permeation chromatography (GPC) analysis (Figure 2b, black trace), suggesting that the MA monomer polymerized successfully from the colistin initiator and that the polymerization was controlled. This also indicated that the colistin peptide structure did not disrupt the polymerization process.

As there are two initiating sites in the colistin initiator, a further cleavage experiment was performed on the PMA colistin conjugate (Boc_5-col-PMA) under basic conditions (3 wt% sodium methoxide, NaOMe) in order to investigate whether the polymerization had occurred from both sites (Figure 2a). The cleaved product was found to be monomodal with approximately half the molecular weight of the initial polymer colistin conjugate through both GPC and MALDI-ToF MS analyses (Figure 2b-c), confirming that well-defined polymer chains were grown in a controlled way at both initiator sites in the colistin. Moreover, the peaks belonging to Boc_5-col were also observed *via* MALDI-ToF MS after the cleavage of the PMA chains (Figure 2c, red trace), indicating that the colistin structure was not affected under the polymerization conditions chosen.

Based on these positive results from the MA polymerization, $PEGA_{480}$ was polymerized at three different DPs (DP = 5, 10 and 20 per initiating site) under similar conditions (Figure 3). As expected, most of the $PEGA_{480}$ was consumed after 2-4 h (monomer conversion ≥ 93%, Table 1) and all the final products retained narrow dispersities ($Đ$ ~ 1.1-1.2, Figure 3b-d and Table 1), suggesting the successful synthesis of all the polymers in a controlled manner.

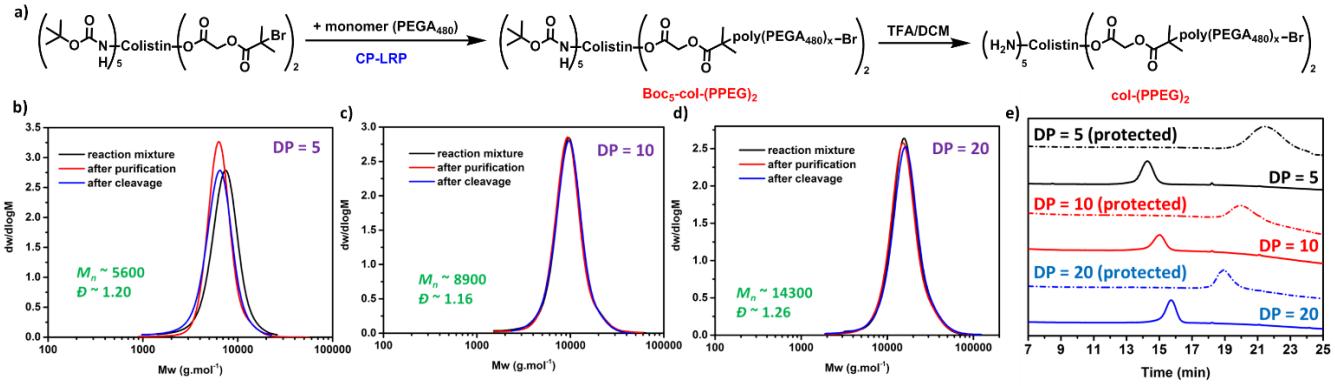


Figure 3 a) Polymerization of $PEGA_{480}$ using CP-LRP and the cleavage of the Boc groups on the polymer, b)-d) the GPC traces of the col-PPEGA conjugates with DP = 5 (b), 10 (c), and 20 (d) in reaction mixture, after purification and after cleavage and e) the HPLC traces of the col-PPEGA conjugates before and after the Boc groups cleavage.

Table 1 Summary of the conversion, molecular weight and dispersities ($Đ$) of each conjugate analyzed from ^{1}H-NMR and GPC.

DP	Conversion (%)	$M_{n\text{Theory}}$ (g·mol^{-1})	$M_{n\text{NMR}}$ (g·mol^{-1})	$M_{n\text{GPC}}$ (g·mol^{-1})	$Đ$
5	99	6875	6800	6100	1.13
10	93	11675	11000	8700	1.20
20	93	21275	19900	14400	1.21

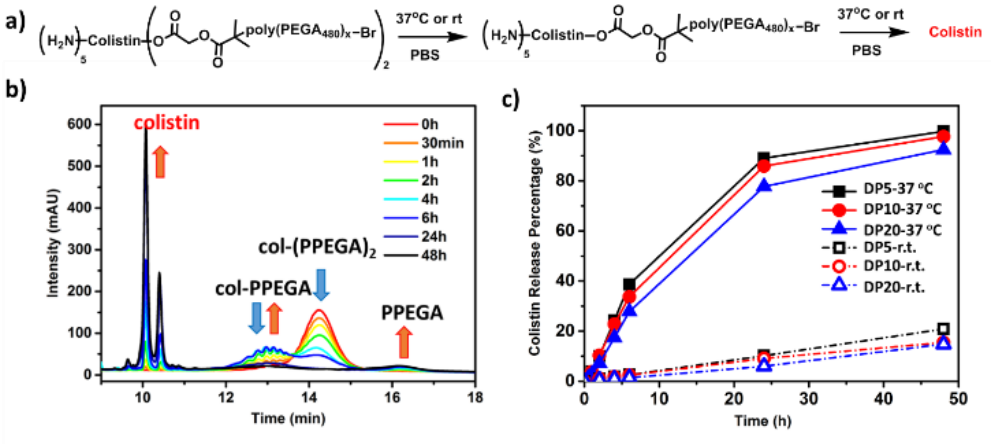


Figure 4 a) Scheme of the hydrolysis process of the col-PPEGA conjugates, b) Degradation of col-PPEGA conjugates (DP = 5) at 37 °C in PBS (1X) monitoring by HPLC and c) Different colistin release profiles obtained from col-PPEGA conjugates with different DPs at 37 °C or ambient temperature.

Table 2 The diameter of zone of inhibition (ZOI) results of the colistin-PPEGA conjugates against two different Gram-negative bacteria through disk diffusion assay (24 h).

	Diameter of Zone of Inhibition (ZOI) (cm)					
	saline	**colistin**	**CMS**	**col-PPEGA DP5**	**col-PPEGA DP10**	**col-PPEGA DP20**
Pa ATCC 27853	0	1.8	1.7	1	0.8	0
Ab ATCC 19606	0	1.8	1.8	1.1	0.9	0.65

Table 3 The minimum inhibitory concentration (MIC) of the conjugates against two different Gram-negative bacteria on a mass basis. (Data with* was acquired from Ref. 21)

Minimum inhibitory concentration against *Ab ATCC* 19606 (MIC) (mg/L)						
colistin	**CMS**	**col-aaPEG**	**col-aaPEG$_2$**	**col-PPEGA DP5**	**col-PPEGA DP10**	**col-PPEGA DP20**
1*	16*	8*	32*	16	16-32	16-32

Subsequently, the polymers were treated with trifluoroacetic acid (TFA, 20% in dichloromethane, DCM), to remove all of the Boc groups on colistin to realise the amine residues. A clear shift to a shorter retention time was observed in all three polymers through high performance liquid chromatography (HPLC) analysis, suggesting quantitative removal of the Boc groups (Figure 3e). Further, GPC analysis revealed similar molecular weights and dispersities before and after the TFA cleavage process (Figure 3b-d), indicating the successful synthesis of colistin-PPEGA conjugates with three different DPs (col-PPEGA DP5, DP10 and DP20) with both ester bonds from the initiator linker being stable under the deprotection conditions.

Hydrolysis Test of col-PPEGA Conjugates. After obtaining the pure col-PPEGA conjugates, the hydrolysis of the linkers was investigated at both body and ambient temperature using the previously established hydrolysis method with phosphate-buffered saline (PBS, 1X, pH = 7.4) used to mimic physiological conditions (Figure 4).[22] The degradation of the col-PPEGA conjugates was monitored by HPLC and revealed a two-step degradation process for all three conjugates (Figure 4b). Typically, the intensity of the col-PPEGA conjugate peak, which varied from 14 - 16 min depending on the DP of polymers, gradually reduced over time with the concomitant appearance of two peaks (9.8 - 10.6 min) of increasing intensity which are assigned to colistin A and B (Figure 4b). An intermediate peak, with a retention time approximately 1.5 min earlier than that of the conjugate peak, was attributed to the mono-cleaved col-PPEGA conjugate. These peaks appeared and continued to increase in intensity over the first 6 h after which they were observed to decrease as the result of further polymer cleavage. The cleaved PPEGA was also observed by HPLC but the signal was relatively low owing to the weak absorbance of PPEGA at 214 nm.

The released colistin peaks were then collected and subsequently analyzed by MALDI-ToF MS, which revealed that colistin was recovered unmodified and the chemical structure remained unaltered further confirming the colistin remained unchanged during both the polymerization and the TFA cleavage process (Figure S2). Moreover, it confirmed that the initiator linkers were cleaved completely from colistin and that the hydrolysis occurred at the ester bond to the colistin rather than the ester bonds within the BMPAA linker or the PPEGA polymer.

In general, the colistin release rates and release profiles of each conjugate were similar (Figure 4c), suggesting the hydrolysis of the cleavable linker was not hindered greatly by the steric constraints imposed by the large comb-like structure of the attached polymers. However it should be noted that a slightly lower release rate (5-10%) for higher DP and hence molecular weight was observed.

Disk Diffusion Assay for In Vitro Antimicrobial Activity Evaluation of the Col-PPEGA Conjugates with Various DPs. In order to evaluate the antibiotic activity of each col-

PPEGA conjugate, two MDR Gram-negative bacteria strains, *P. aeruginosa* ATCC 27853 and *A. baumannii* ATCC 19606, were first tested through disk diffusion assay. As a facile and economic standard, disk diffusion assay provide the visualization of the relative antimicrobial activity of each compound via a bacteria-free zone (zone of inhibition (ZoI)) from the bacteria-cultured agar plate. To this end, 20 μg of each conjugate were applied onto a blank disc (Ø = 0.6 cm) before being placed on a bacterium inoculated agar plate. Colistin and CMS were also tested as positive controls and saline as a negative control. The potency of each conjugate could be estimated through the measurement of the diameter of ZoI (Table 2).

As PEG itself has been shown to exhibit no antibiotic activity and the molecular weight of each col-PPEGA conjugate (Table S1) is at least 5-10 times larger than colistin and CMS, it was expected that they would demonstrate a lower activity with the same mass. We found that indeed col-PPEGA DP20 did not show a significant activity during the test, however, the other two col-PPEGA conjugates still showed a clear ZoI, indicating the potency of the released colistin from these colistin conjugates. Although the ZoIs are smaller than the ones obtained from colistin and CMS, the observed antimicrobial activity obtained from col-PPEGA DP5 and DP10 suggested the cleavable linker can be effectively hydrolyzed under these model physiological conditions, and importantly that this 'grafting-from' approach for colistin conjugation did not alter the biological activity of the released colistin.

Quantitative In Vitro Antimicrobial Activity Evaluation of the Col-PPEGA Conjugates with Various DPs via Broth Microdilution Method. A further examination for the minimum inhibitory concentration (MIC) of each conjugate was performed through a broth microdilution method to achieve a more accurate quantitative comparison. Although all the obtained polymer colistin conjugates have a narrow dispersity, an accurate comparison through the molar basis is not possible. Thus, the MIC test was conducted on a mass basis.

All of the col-PPEGA conjugates have shown antibacterial activity against *Ab ATCC* 19606, indicating active colistin can be released from these colistin conjugates (Table 3). Particularly, all these conjugates have comparable or better antimicrobial activity than the previous reported doubly functionalized col-aaPEG_2 even though the molecular weight of these conjugates are all larger than col-aaPEG_2. This suggests that the PPEGA polymer with the BMPAA linker released colistin faster than aaPEG_2 and that the molecular weight of the comb-like PPEGA did not greatly hinder the linker hydrolysis which is in agreement with the colistin release profile from previous studies. Furthermore, col-PPEGA DP5 has a similar activity to the commercial prodrug CMS although the average molecular weight is around five times larger, highlighting its potential as a more efficacious alternative to CMS.

Kinetics of In Vitro Antimicrobial Activity of the Col-PPEGA Conjugates with Various DPs. To study the effects of the colistin release rate and the DP of the polymer employed in the col-PPEGA conjugates upon their antibacterial kinetics, time-kill studies of the conjugates were performed against the *A. baumannii* ATCC 19606 at three different doses referenced to their MICs (0.5 x MIC, 1 x MIC, and 4 x MIC, referring to mass based MICs of each compound). Note, if a range of MICs was obtained e.g. col-PPEGA DP10 (Table 3), calculations were based on the higher MIC value.

All conjugates showed no significant inhibition towards the bacteria at the lower doses of 0.5 x MIC or 1 x MIC. When applying a higher dose (4 x MIC), both the DP5 (Figure 5a) and DP10 (Figure 5b) polymer-colistin conjugates were found to have a similar antibacterial performance when compared to the commercial prodrug CMS (Figure S4b), causing a significant decrease of the amount of live bacteria in the broth. Notably, it took 3h for DP5 conjugates (MIC 4x) to inhibit the growth of bacteria to levels approaching the detection limit, while around 6h was needed for either DP10 conjugate or CMS, suggesting the colistin release rate from the conjugates played an important role for their bacteria inhibition profiles. Although the DP20 polymer conjugate (Figure 5c) did not produce a similar effect found from the DP5 and DP10 conjugates, an inhibition of bacteria growth was still observed through the experiment, suggesting antibacterial activity caused by the successful release of colistin.

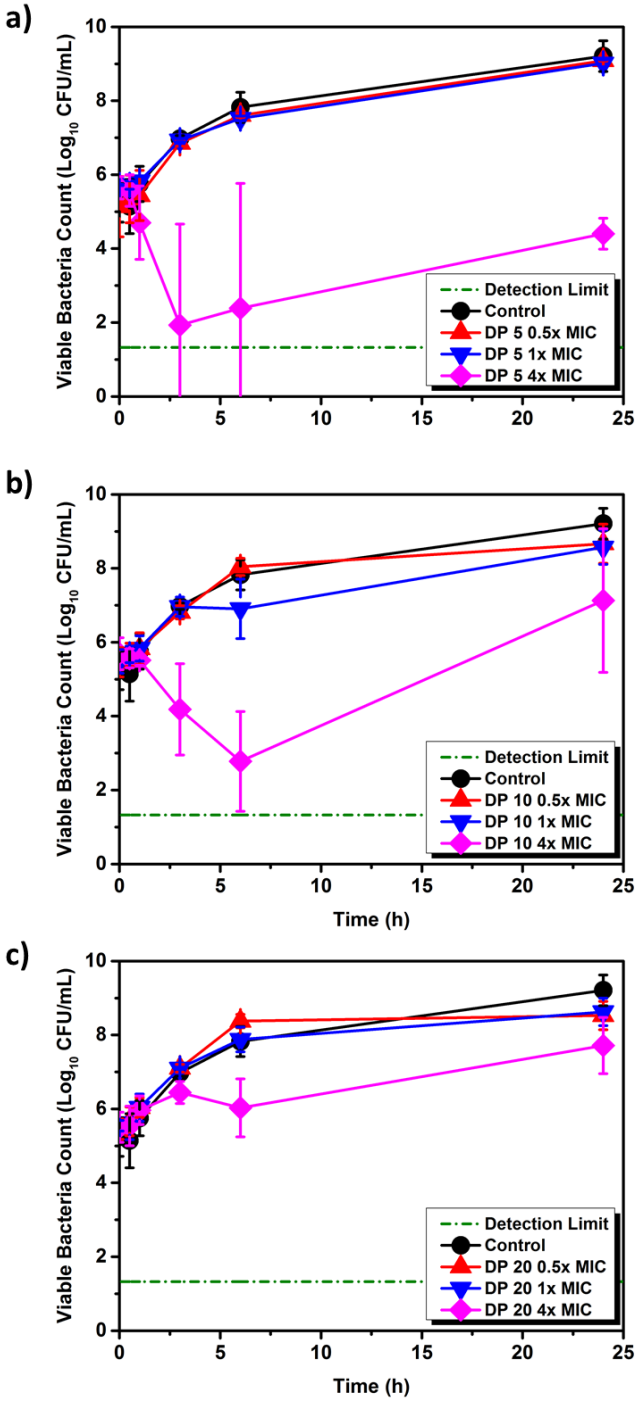


Figure 5 The 'time-kill' kinetics with different amount for the col-PPEGA conjugates with a) DP = 5 , b) DP = 10 and c) DP = 20 (c).

CONCLUSION

In conclusion, we have demonstrated a novel and facile approach for the synthesis of colisitin polymeric prodrugs with enhanced properties. A range of hydrolyzable polymer colistin conjugates have been successfully synthesized using a colistin initiator, which has been site selectively modified with two cleavable initiator precursors (BMPAA) at both Thr residues, and a novel 'grafting-from' approach *via* CP-LRP. Through a model PMA system, it was confirmed that initiator sites resulted in similar chain growth in a controlled manner and the structural integrity of colistin during the polymerization process. Site specific colistin-PPEGA conjugates (col-PPEGA) with different DPs have been successfully synthesized with narrow dispersities ($Đ$ ~ 1.1-1.2) using $PEGA_{480}$ monomer and fully characterized. Moreover, degradation studies under simulated physiological conditions suggested that most of the native colistin can be recovered from all three conjugates within 48 hours. The colistin release rate from the three PPEGA conjugates were similar though it slightly decreased with increasing DP of the attached polymers. *In vitro* antimicrobial activity tests revealed that all three conjugates remained active against the tested MDR Gram-negative bacteria. In particular, col-PPEGA DP5 and DP10 showed similar or better antibacterial activities to CMS. In summary, the combination of state-of-the-art CP-LRP and traceless polymer-peptide conjugation methods enabled the synthesis of well-defined col-PPEGA systems and highlighted their potential as prodrug alternatives to CMS. Moreover, it was demonstrated that this CP-LRP methodology represents a powerful approach for the 'grafting-from' polymer modification of biological entities and opens new opportunities for future polymer-peptide conjugation strategies.

EXPERIMENTAL PROCEDURES

Synthesis of initiator precursor, 2-((2-bromo-2-methylpropanoyl)oxy)acetic acid (BMPAA)

Glycolic acid (3.00 g, 40 mmol, 1.0 equiv.) was dissolved with DCM (60 mL) and *N,N*-diisopropylethylamine (DIPEA, 8 mL, 45 mmol, 1.125 equiv.) in a 250 mL 3-neck round bottom flask under N_2. The reaction mixture was kept stirring in an ice bath for 30 min before the dropwise addition of α-bromoisobutyryl bromide (6 mL, 50 mmol, 1.25 equiv.) along with DCM (30 mL) through a dropping funnel. After the addition of all the chemicals, the system was gradually warmed up to room temperature and left overnight. The solvent was subsequently removed under vacuum and the crude product was extracted by diethyl ether/0.2 M hydrochloric acid (HCl) solution to remove the remaining DIPEA. After the removal of solvent, the product was then purified by Kugelrohr Distillation. The product is normally obtained as a white solid with a slight amount of an acid impurity (which we suspected it to be α-bromoisobutyric acid) at 50 °C to 100 °C under $2.2x10^{-2}$ mbar. The purity of the product can be analyzed by TLC plate (diethyl ether: AcOH = 400: 1 v/v). A further purification can be performed by the recrystallization from hot hexane. The final product consists of white flaky crystals with a yield of 3.6 g (16 mmol, 40%). ^{1}H-NMR (300 MHz, $CDCl_3$, 298 K) δ (ppm) = 1.99 (-$C(CH_3)_2$Br), 4.76 (-OCH_2CO-) and 7.83 (br, -COOH).^{13}C-NMR (75.5 MHz, $CDCl_3$, 298 K) δ (ppm) = 30.73 (-$C(CH_3)_2$Br), 54.56 (-$C(CH_3)_2$Br), 61.12 (-O<u>C</u>H_2CO-), 171.21 (-<u>C</u>OOCH_2CO-), 172.74 (-OCH_2<u>C</u>O-).IR (neat) ν/cm^{-1}: 3300-2300 (O-H), 2975, 2865 (CH_2, CH_3), 1724, 1706 (C=O), 1421, 1248, 1147.HR-MS (ESI, negative mode): m/z (found) 222.9607 (M-H), m/z (calculated) 222.9611 (M-H).

Synthesis of Boc_5-colistin-Br_2 (Boc_5-col-Br_2)

The synthesis of Boc_5-colistin-Br_2 was modified from the standard Steglich esterification. 1.00 g of colistin sulphate (0.789 mmol, 1 equiv.) was converted into Boc_5-colistin based on the previous report.[22] The crude Boc_5-colistin product (~ 1.10 g) was then dissolved in anhydrous THF (50 mL), following the addition of 2-((2-bromo-2-methylpropanoyl)oxy)acetic

acid (885.0 mg, 3.95 mmol, 5 equiv.) and DMAP (48.0 mg, 0.393 mmol, 0.5 equiv.). After the chemicals completely dissolved, *N,N′*-dicyclohexylcarbodiimide (DCC, 977.0 mg, 4.74 mmol, 6 equiv.) was added afterwards and the reaction mixture was kept stirring overnight. The reaction mixture was then centrifuged to remove most of insoluble compounds. The solvent was then removed under vacuum and a further purification was performed by the flash column (0% -10% MeOH in DCM). The final product is a fine pale yellow powder with a total yield of 0.96 g (0.463 mmol, 59%).

Procedure for the polymerization of MA using colistin initiator Boc$_5$-colistin-Br$_2$

The polymerization condition of MA was modified from previous report.[39] A nail gel curing box with four 9 Watt bulbs was used as a UV source. MA was passed through a short basic aluminium column to remove the inhibitor prior to use. A stock solution of $CuBr_2$ (1 mg/mL in DMSO) and the ligand Me_6TREN (6.5 µL/mL in DMSO) was freshly prepared before the reaction. MA (73 µL, 0.80 mmol, 40 equiv., 20 equiv. per arm), Boc$_5$-colistin-Br$_2$ (42 mg, 20 µmol, 1 equiv.), $CuBr_2$ stock solution (90 µL, 0.4 µmol, 0.02 equiv.), Me_6TREN stock solution (100 µL, 2.4 µmol, 0.12 equiv.) were added to a 2 mL vial. 102 µL DMSO was then added to make the monomer/solvent ratio to around 1: 4. The reaction mixture was then stirred until all the compounds were dissolved. The system was then sealed and carefully degassed by purging with N_2 for 15 min (make sure the monomer is not blown off by the N_2). The polymerization was performed under UV with a cooling plate (CAMLAB, KP283) to maintain the reaction temperature. The reaction was stopped at 4h and the conversion were measured using ^{1}H-NMR (Figure S1). MALDI-ToF MS and GPC analyses were also conducted to evaluate the molecular weight and dispersity of the polymer (Figure 2).

The cleavage of the linker from PMA-Boc$_5$-colistin conjugate was conducted directly using the crude polymer obtained from previous procedure. 100 µL of the crude polymer was diluted by 1 mL 3% NaOMe in MeOH and the reaction mixture was kept stirring at ambient temperature for 1 day. The solvent was then blown off by N2 and the product were analysis by MALDI-ToF MS and GPC (Figure 2).

Polymerization of PEGA$_{480}$ of different DPs using colistin initiator Boc$_5$-colistin-Br$_2$

The polymerization procedure of PEGA$_{480}$ was similar to that used for MA. The ratio of [initiator]: [$CuBr_2$]: [ligand] was kept the same as the one used in the polymerization of MA (1: 0.02: 0.12). The PEGA$_{480}$ monomer to initiator ratio was set to 10:1, 20:1, 40:1 for the synthesis of DP5, DP10, and DP20 polymer, respectively. For the synthesis of DP10 and DP20 polymer, 21 mg colistin initiator was used and the monomer/DMSO ratio was changed to 1: 2 so that the reaction time was shorten to 2 h instead of the standard 4h to reach high monomer conversion (> 90%) which can be analyzed by ^{1}H-NMR. To minimise the hydrolysis during the purification steps, the further separation of the polymer and the trace PEGA$_{480}$ monomer was done by diluting the crude using ACN and purifying by RP-HPLC (0-12 min 50%-100% B; 12-20 min 100% B, 20-21 min 100%-50% B; 21-30 min 50% B). The polymer peak would be found from 8-15 min depending on the DP. Once collected from RP-HPLC, the product was frozen by liquid N_2 and the solvents were directly removed under high vacuum. The products before and after the purification were performed by GPC and HPLC analysis to observe whether any change was occurred during the purification step.

Deprotection of Boc groups from Boc$_5$-col-PPEGA conjugates

The cleavage of Boc groups on the colistin initiator from the polymer conjugates to regenerate col-PPEG conjugates were performed under the similar conditions described previously using 20% TFA in DCM.[22] Briefly, 5 mL 20 % TFA in DCM was added slowly into the purified polymer conjugates. [Be cautious of the release of gas!] After most gas was released, the reaction mixture was sealed and kept stirring at ambient temperature overnight. After the removal of solvents, the product was frozen by liquid N_2 and all the solvents were directly removed under high vacuum. The products were performed by GPC and HPLC analysis for comparison.

Protocol for *in vitro* releasability of col-PPEGA prodrugs

The degradation of the col-PPEGA conjugates was based on the previous report.[22] Each conjugate was weighed into a 2 mL vial, followed by the addition of a certain amount of PBS (1X) to make the final concentration of 0.4 µmol/mL. 900 µL of each prodrug solution was taken into another vial and incubated in an oil bath with stirring at 37 °C. The rest of each prodrug solution (> 500 µL) was stirred at room temperature. Samples (85 µL each time) were taken periodically and frozen by liquid nitrogen before the analysis. The analysis was conducted by RP-HPLC (injection volume = 70 µL) using the standard analysis condition. The colistin release percentage was calculated based on the reported method.

ASSOCIATED CONTENT

Supporting Information. Experimental details for the synthesis, instruments, protocols and characterizations including NMR, HPLC, and GPC data are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

CP-LRP, copper-mediated photo-induced living radical polymerization; colistin initiator, Boc_5-col-Br_2; BMPAA, 2-(2-bromo-2-methylpropanoyloxy) acetic acid; PPEGA, poly[poly(ethylene glycol) methyl ether acrylate; CMS, colistin methanesulfonate; MA, methyl acrylate; DP, degree of polymerization; *Đ*, dispersities; ZoI, zone of inhibition; MIC, minimum inhibitory concentration; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; DCM, dichloromethane.

REFERENCES

(1) Fair, R. J.; Tor, Y. (2014) Antibiotics and bacterial resistance in the 21st century. *Perspect. Medicin. Chem.* 6, 25-64.

(2) Spellberg, B.; Powers, J. H.; Brass, E. P.; Miller, L. G.; Edwards, J. E. (2004) Trends in antimicrobial drug development: implications for the future. *Clin. Infect. Dis.* 38, 1279-1286.

(3) Coates, A.; Hu, Y.; Bax, R.; Page, C. (2002) The future challenges facing the development of new antimicrobial drugs. *Nat. Rev. Drug Discov.* 1, 895-910.

(4) Norrby, S. R.; Nord, C. E.; Finch, R. (2005) Lack of development of new antimicrobial drugs: a potential serious threat to public health. *Lancet Infect. Dis.* 5, 115-119.

(5) Ventola, C. L. (2015) The antibiotic resistance crisis: part 1: causes and threats. *P&T* 40, 277-283.

(6) Waterer, G. W.; Wunderink, R. G. (2001) Increasing threat of Gram-negative bacteria. *Crit. Care Med.* 29, N75-N81.

(7) O'Neill, J. 2016; Tackling drug-resistant infections globally: final report and recommendations. http://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf Accessed: 10 March, 2017.

(8) Roberts, K. D.; Azad, M. A.; Wang, J.; Horne, A. S.; Thompson, P. E.; Nation, R. L.; Velkov, T.; Li, J. (2015) 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, 568-575.

(9) Falagas, M. E.; Kasiakou, S. K.; Saravolatz, L. D. (2005) Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. *Clin. Infect. Dis.* 40, 1333-1341.

(10) Li, J.; Nation, R. L.; Turnidge, J. D.; Milne, R. W.; Coulthard, K.; Rayner, C. R.; Paterson, D. L. (2006) Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect. Dis.* 6, 589-601.

(11) Li, J.; Nation, R. L.; Milne, R. W.; Turnidge, J. D.; Coulthard, K. (2005) Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. *Int. J. Antimicrob. Agents* 25, 11-25.

(12) Spapen, H.; Jacobs, R.; Van Gorp, V.; Troubleyn, J.; Honoré, P. M. (2011) Renal and neurological side effects of colistin in critically ill patients. *Ann. Intensive Care* 1, 14.

(13) Ordooei Javan, A.; Shokouhi, S.; Sahraei, Z. (2015) A review on colistin nephrotoxicity. *Eur. J. Clin. Pharmacol.* 71, 801-810.

(14) Lim, L. M.; Ly, N.; Anderson, D.; Yang, J. C.; Macander, L.; Jarkowski, A.; Forrest, A.; Bulitta, J. B.; Tsuji, B. T. (2010) Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing. *Pharmacother.* 30, 1279-1291.

(15) Falagas, M. E.; Kasiakou, S. K. (2006) Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Crit. Care* 10, R27-R27.

(16) Falagas, M. E.; Siempos, I. I.; Rafailidis, P. I.; Korbila, I. P.; Ioannidou, E.; Michalopoulos, A. (2009) Inhaled colistin as monotherapy for multidrug-resistant gram (−) nosocomial pneumonia: A case series. *Respir. Med.* 103, 707-713.

(17) Zhu, C.; Zhao, J.; Kempe, K.; Wilson, P.; Wang, J.; Velkov, T.; Li, J.; Davis, T. P.; Whittaker, M. R.; Haddleton, D. M. (2016) A hydrogel-based localized release of colistin for antimicrobial treatment of burn wound infection. *Macromol. Biosci.* DOI: 10.1002/mabi.201600320.

(18) Bergen, P. J.; Li, J.; Rayner, C. R.; Nation, R. L. (2006) Colistin methanesulfonate is an inactive prodrug of colistin against Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* 50, 1953-1958.

(19) Li, J.; Milne, R. W.; Nation, R. L.; Turnidge, J. D.; Coulthard, K. (2003) Stability of colistin and colistin methanesulfonate in aqueous media and plasma as determined by high-performance liquid chromatography. *Antimicrob. Agents Chemother.* 47, 1364-1370.

(20) Li, J.; Milne, R. W.; Nation, R. L.; Turnidge, J. D.; Smeaton, T. C.; Coulthard, K. (2004) Pharmacokinetics of colistin methanesulphonate and colistin in rats following an intravenous dose of colistin methanesulphonate. *J. Antimicrob. Chemother.* 53, 837-840.

(21) He, H.; Li, J.-C.; Nation, R. L.; Jacob, J.; Chen, G.; Lee, H. J.; Tsuji, B. T.; Thompson, P. E.; Roberts, K.; Velkov, T. (2013) Pharmacokinetics of four different brands of colistimethate and formed colistin in rats. *J. Antimicrob. Chemother.* 68, 2311-2317.

(22) Zhu, C.; Schneider, E. K.; Wang, J.; Kempe, K.; Wilson, P.; Velkov, T.; Li, J.; Davis, T. P.; Whittaker, M. R.; Haddleton, D. M. (2017) A traceless reversible polymeric colistin prodrug to combat multidrug-resistant (MDR) gram-negative bacteria. *J. Control. Release* DOI: 10.1016/j.jconrel.2017.02.005.

(23) Averick, S.; Simakova, A.; Park, S.; Konkolewicz, D.; Magenau, A. J. D.; Mehl, R. A.; Matyjaszewski, K. (2012) ATRP under Biologically Relevant Conditions: Grafting from a Protein. *ACS Macro Letters* 1, 6-10.

(24) Cobo, I.; Li, M.; Sumerlin, B. S.; Perrier, S. (2015) Smart hybrid materials by conjugation of responsive polymers to biomacromolecules. *Nat. Mater.* 14, 143-159.

(25) Boyer, C.; Bulmus, V.; Liu, J.; Davis, T. P.; Stenzel, M. H.; Barner-Kowollik, C. (2007) Well-defined pro-

tein−polymer conjugates via in situ RAFT polymerization. *J. Am. Chem. Soc.* 129, 7145-7154.
(26) Bulmus, V. (2011) RAFT polymerization mediated bioconjugation strategies. *Polym. Chem.* 2, 1463-1472.
(27) Siegwart, D. J.; Oh, J. K.; Matyjaszewski, K. (2012) ATRP in the design of functional materials for biomedical applications. *Prog. Polym. Sci.* 37, 18-37.
(28) Zhang, Q.; Li, M.; Zhu, C.; Nurumbetov, G.; Li, Z.; Wilson, P.; Kempe, K.; Haddleton, D. M. (2015) Well-defined protein/peptide–polymer conjugates by aqueous Cu-LRP: synthesis and controlled self-assembly. *J. Am. Chem. Soc.* 137, 9344-9353.
(29) Zhang, Q.; Wilson, P.; Li, Z.; McHale, R.; Godfrey, J.; Anastasaki, A.; Waldron, C.; Haddleton, D. M. (2013) Aqueous copper-mediated living polymerization: exploiting rapid disproportionation of CuBr with Me6TREN. *J. Am. Chem. Soc.* 135, 7355-7363.
(30) Rzayev, J.; Penelle, J. (2004) HP-RAFT: A free-radical polymerization technique for obtaining living polymers of ultrahigh molecular weights. *Angew. Chem. Int. Ed.* 43, 1691-1694.
(31) Tanabe, M.; Vandermeulen, G. W. M.; Chan, W. Y.; Cyr, P. W.; Vanderark, L.; Rider, D. A.; Manners, I. (2006) Photocontrolled living polymerizations. *Nat Mater* 5, 467-470.
(32) Magenau, A. J. D.; Strandwitz, N. C.; Gennaro, A.; Matyjaszewski, K. (2011) Electrochemically mediated atom transfer radical polymerization. *Science* 332, 81-84.
(33) Anastasaki, A.; Nikolaou, V.; Zhang, Q.; Burns, J.; Samanta, S. R.; Waldron, C.; Haddleton, A. J.; McHale, R.; Fox, D.; Percec, V.; et. al (2014) Copper(II)/tertiary amine synergy in photoinduced living radical polymerization: accelerated synthesis of ω-functional and α,ω-heterofunctional poly(acrylates). *J. Am. Chem. Soc.* 136, 1141-1149.
(34) Jones, G. R.; Whitfield, R.; Anastasaki, A.; Haddleton, D. M. (2016) Aqueous copper(II) photoinduced polymerization of acrylates: low copper concentration and the importance of sodium halide salts. *J. Am. Chem. Soc.* 138, 7346-7352.
(35) Roberts, M. J.; Bentley, M. D.; Harris, J. M. (2012) Chemistry for peptide and protein PEGylation. *Adv. Drug Deliv. Rev.* 64, 116-127.
(36) Ogden, J.; Palmer, R. 2011; Novel polymers for enhancing therapeutic half-life and drug targeting. http://www.ondrugdelivery.com/publications/Injectable%20Formulations%202011/WEP.pdf. Accessed: 1 December, 2017.
(37) Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S. (2010) Poly (ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angew. Chem. Int. Ed.* 49, 6288-6308.
(38) Podobnik, B.; Helk, B.; Smilović, V.; Škrajnar, Š.; Fidler, K.; Jevševar, S.; Godwin, A.; Williams, P. (2015) Conjugation of polyPEG to interferon alpha extends serum half-life while maintaining low viscosity of the conjugate. *Bioconjugate Chem.* 26, 452-459.
(39) Anastasaki, A.; Nikolaou, V.; McCaul, N. W.; Simula, A.; Godfrey, J.; Waldron, C.; Wilson, P.; Kempe, K.; Haddleton, D. M. (2015) Photoinduced synthesis of α,ω-telechelic sequence-controlled multiblock copolymers. *Macromolecules* 48, 1404-1411.