Radical-Medicated End-Group Transformation of Amphiphilic Methacrylate Random Copolymers for Modulation of Antimicrobial and Hemolytic Activities

Enrico T. Nadres,[†] Haruko Takahashi,[†] Kenichi Kuroda

Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan 48109
Correspondence to: K. Kuroda (E-mail: kkuroda@umich.edu)

Received 10 August 2016; accepted 8 September 2016; published online 12 October 2016
DOI: 10.1002/pola.28384

ABSTRACT: This work describes synthesis of antimicrobial methacrylate copolymers by reversible addition-fragmentation chain transfer (RAFT) polymerization and examines the versatility of this approach for improving chemical optimization to create potent, non-toxic antimicrobial polymers. Specifically, this study focuses on the radical-mediated transformation of end group of antimicrobial peptide-mimetic polymer. RAFT polymerization using 2-cyano-2-yl-dithiobenzoate provided a statistical methacrylate copolymer consisting of aminobutyl and ethyl groups in the side chains. The following radical-mediated modification using free radical initiators successfully transformed the ω-end group of parent copolymer from dithiobenzoate to a cyanoisobutyl or aminoethyl cyanopentanoate group without any significant changes to the polymer molecular weight. In general, the parent polymer and variants showed a broad spectrum of activity against a panel of bacteria, but low hemolytic activity against human red blood cells. The parent copolymer with the dithiobenzoate end-group showed highest antimicrobial and hemolytic activities as compared with other copolymers. The copolymers caused membrane depolarization in *Staphylococcus aureus*, while the ability of copolymers for membrane disruption is not dependent on the end-group structures. The synthetic route reported in this study will be useful for further study of the role of polymer end-groups in the antimicrobial activity of copolymers. © 2016 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2017**, *55*, 304–312

KEYWORDS: antimicrobial; biomaterials; copolymerization; membranes; reversible addition-fragmentation chain transfer (RAFT)

INTRODUCTION The therapeutic potential of membrane-active antimicrobial polymers has been explored in the development of new antimicrobials owing to their potency against antibiotic-resistant bacteria. To that end, we have previously demonstrated that methacrylate random copolymers with cationic amphiphilicity are a promising platform to mimic the biological functions of antimicrobial peptides (AMPs) found in the innate immune system.[1–3] In the polymer design, the cationic functionality of such polymers facilitates selective binding to anionic bacterial membranes. On the other hand, the hydrophobic groups of polymers are inserted into the cell membranes and cause membrane disruption, ultimately resulting in bacterial cell death. However, excess hydrophobicity of polymers drives the polymers to non-specifically bind to human cell membranes, causing undesired toxicity to human cells. Therefore, the design principle of antimicrobial random copolymers is to obtain the adequate balance between hydrophobicity and cationic functionality to maximize the antimicrobial activity and minimize the toxicity to human cells. Toward the further development of polymer-based antimicrobials for therapeutic use, viable and robust chemical optimization methods are necessary to fill the translational gap between the material development and biomedical applications of antimicrobial polymers.

Our laboratory previously developed a "snorkel" design strategy to improve the antimicrobial activity of amphiphilic methacrylate copolymers (Fig. 1: Snorkel vs. end-group).[4] In the previous work, the chain length of cationic side chains of copolymers was increased such that the cationic groups can bind to anionic phosphate lipid-head groups while the polymer chains are inserted into the hydrophobic domains of cell membranes. The elongated cationic side chains allow the polymer chains inserted more deeply into the membranes as compared with counter copolymers with shorter side chains,

[†]These two authors contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

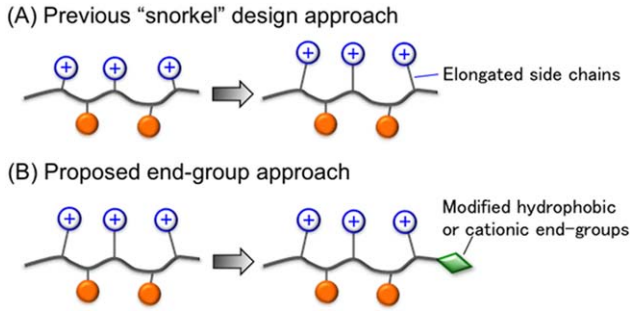


FIGURE 1 Design approaches for antimicrobial polymers. (A) Snorkel design. We have previously design cationic amphiphilic copolymers with elongated cationic side chains to enhance the insertion of polymer chains into bacterial membranes.[4] (B) End-group approach. In this work, we propose to modulate the polymer end groups to tune their antimicrobial activity. [Color figure can be viewed at wileyonlinelibrary.com]

and therefore, this snorkel effect leads to higher antimicrobial activity. In our continuous endeavor to develop efficacious antimicrobial polymers, our primary interest in this study presented here is to identify a chemical method which can derive the end-group functionality of polymers to modulate antimicrobial efficacy and bacterial selectivity. The hydrophobicity of end-groups has been reported to impact the antimicrobial activity and selectivity.[5–7] A class of lipopeptides have acyl groups at the N-terminus, which are involved in the antimicrobial mechanism associated with bacterial membranes.[8] The end-group functionality of copolymers would serve as one of the structural determinants in their interactions with bacterial membranes for antimicrobial activity (Fig. 1).

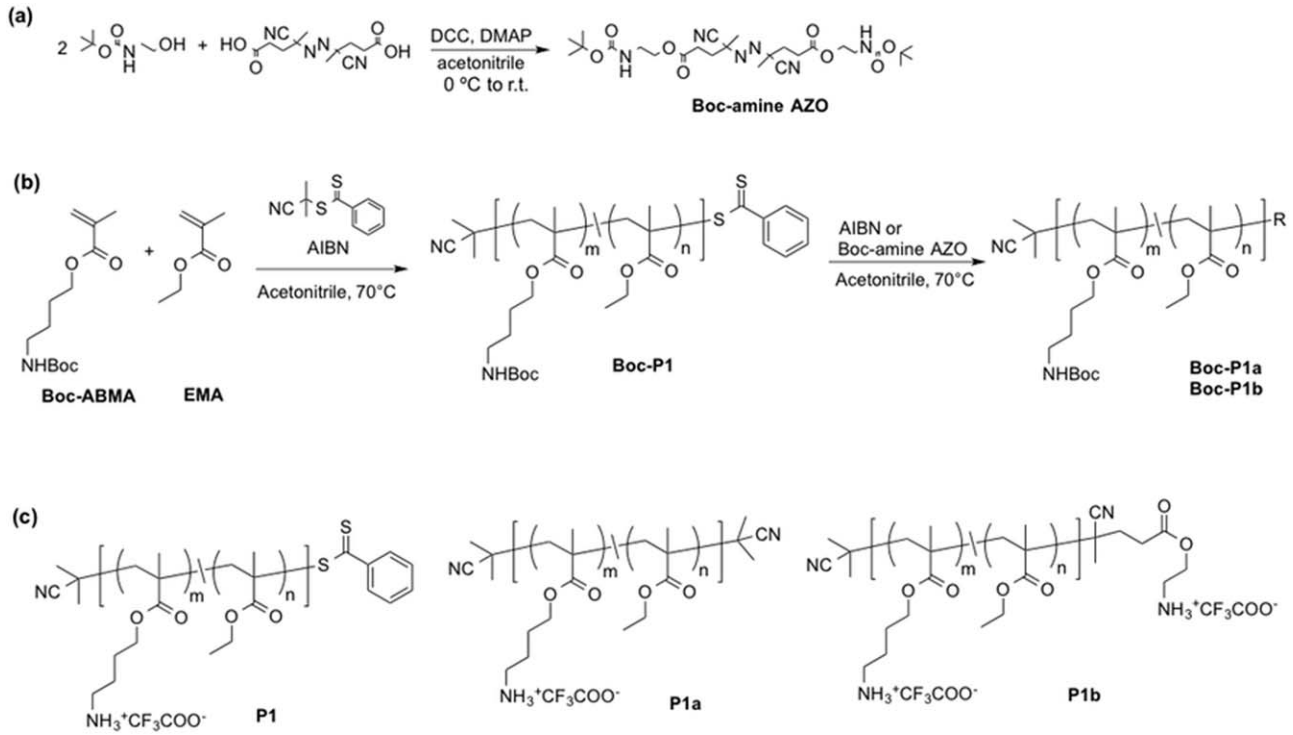
In this study, we extend our synthetic approach to examine the effect of end-groups on the antimicrobial activity of cationic amphiphilic copolymers by taking advantage of controlled radical polymerization. Antimicrobial methacrylate copolymers have been recently prepared by controlled radical polymerization methods including atom transfer radical polymerization (ATRP)[9–12] and reversible addition-fragmentation chain transfer (RAFT).[13,14] Controlled radical polymerizations are robust and scalable synthetic methods to produce precisely controlled mono-disperse polymers with different amphiphilic structures, architectures and functionalities,[15] which facilitates identifying leads for the development of antimicrobial polymers for therapeutic and biomedical applications. Given the potential benefits of controlled radical polymerizations, we exploit the advantages of RAFT polymerization to synthesize methacrylate copolymers. RAFT polymerization was selected because it has been widely used to prepare biomedical materials, and many functional monomers are compatible with this polymerization as compared with other polymerization methods. The RAFT polymerization also provides several synthetic routes to alter the polymer end-groups.[16]

The specific purposes of this study are to (1) examine the monomer distribution of polymers prepared by RAFT polymerization, (2) validate a radical-medicated method to transform the end group of copolymer to other groups, and (3) examine the effect of end-groups on their antimicrobial and hemolytic activities. This study first examines if the monomer composition of polymers is statistically distributed or drifted during the polymerization because the amphiphilic sequence presented by monomer distribution is a critical factor in antimicrobial activity and selectivity,[17,18] but is only explicitly considered in several reports.[4,6] Then, two copolymer variants were derived from a previously developed methacrylate copolymer, but with different end-groups. The RAFT agent at the polymer terminal groups (dithiobenzoate) was replaced by the radical-mediated method to give a cyanoisobutyl or aminoethyl cyanopentanoate group. The antimicrobial activity of these copolymers was examined using selected bacteria, and the hemolytic activity was measured using human red blood cells (RBCs) as a toxicity measure. The ability of copolymers to disrupt bacterial membranes was also examined by a membrane depolarization assay. Cationic amphiphilic methacrylate random copolymers have been previously prepared by RAFT polymerization using chain transfer agents with various lengths of alkyl chains, and the effect of end-group hydrophobicity on their antimicrobial and hemolytic activities has been examined.[7] We intend in this study to examine a new synthetic route to prepare methacrylate copolymers with different end-groups rather than the systematic investigation of effect of end-groups on their activities.

EXPERIMENTAL

Materials

4-Amino-1-butanol, di-*tert*-butyl dicarbonate, *N*,*N*′-dicyclohexylcarbodiimide (DCC), dimethylaminopyridine (DMAP), triethylamine, and methyl 3-mercaptopropionate (MMP) were purchased from Acros Organics. 2-Cyanoprop-2-yl-dithiobenzoate was purchased from Strem Chemicals, Inc. 4,4-azobis(4-cyanovaleric acid) was purchased from MP Biomedicals, LLC. 2,2′-azobis(2-methylpropionitrile) (AIBN) and the bee venom toxin Melittin (purity >85%) were purchased from Sigma-Aldrich Co. LLC. Trifluoroacetic acid (TFA) and solvents were purchased from Thermo Fisher Scientific, Inc. 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3$-(5)) was purchased from AnaSpec, Inc. Most of the chemicals were used without further purification, except for methacryloyl chloride and ethyl methacrylate (EMA), which was purchased from Acros Organics and was freshly distilled before use. ^{1}H NMR was performed using a Varian MR400 (400 MHz) and analyzed using VNMRJ 3.2 and MestReNova. Gel permeation chromatography (GPC) analysis was performed using a Waters 1515 HPLC instrument equipped with Waters Styragel (7.8 mm × 300 mm) HR 0.5, HR 1, and HR 4 columns in sequence and detected by a differential refractometer (RI). *Bacillus subtilis* ATCC®6633™, *Enterococcus faecalis* ATCC®29212™, *Staphylococcus aureus* ATCC®25923™, Community acquired methicillin-resistant *S. aureus* (MRSA) strain LAC BB1263, *Acinetobacter baumannii* ATCC®17978™, *Escherichia coli* ATCC®25922™, *Pseudomonas aeruginosa*



SCHEME 1 (a) Synthesis of amine-functionalized radical initiator; (b) Polymer synthesis involving RAFT polymerization and radical-mediated end-group transformation. (c) Polymers with primary ammonium groups. The boc-protected polymers were treated by TFA to remove the boc groups, yielding the random polymers with cationic and hydrophobic ethyl side chains.

ATCC®27853™, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC®14028™ were used for model bacteria to evaluate antimicrobial activity of polymers. Human RBCs (leukocytes reduced adenine saline added) were obtained from the American Red Cross Blood Services Southeastern Michigan Region and used prior to the out date indicated on each unit.

Polymer Synthesis

Synthesis of Bis(2-((Tert-Butoxycarbonyl) Amino)Ethyl) (4,4-Azobis(4-Cyanovalerate)) (Boc-Amine AZO)

Tert-butyl (2-hydroxyethyl) carbamate (25 mmol, 4.08 g), DCC (25 mmol, 5.16 g) and DMAP (2.5 mmol, 306 mg) was dissolve in anhydrous acetonitrile (50 mL) at 0 °C [Scheme 1 (a)], followed by the addition of 4,4-azobis(4-cyanovaleric acid) (10 mmol, 2.8 g). The mixture was allowed to warm to room temperature and stirred in the dark at room temperature. After 24 h, the suspension was filtered and the white solid was washed with cold acetonitrile (10 mL). The combined filtrate and washings was evaporated and the residue was dissolved in dichloromethane (50 mL). The resulting solution was washed with water (3 times), saturated $NaHCO_3$ (3 times), and brine (3 times), then dried with $MgSO_4$. The residue was loaded to silica gel column (6:4 hexanes–ethyl acetate solvent) to give purified white solid product (5.40 g, % yield= 95%, $R_f = 0.31$ (6:4 hexanes:ethyl acetate)), which is a mixture of *dl* and *meso* isomers. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.94–4.83 (m, 2H, *g*), 4.20–4.16 (m, 4H, *e*), 3.30–3.40 (m, 4H, *f*), 2.37–2.60 (m, 8H, $c + d$), 1.74 (s, 3H, *b*), 1.69 (s, 3H, *b*), 1.45 (s, 18H, *a*). ^{13}C NMR ($CDCl_3$, 400 MHz) δ 171.3, 171.2, 155.8, 117.5, 117.4, 79.6, 71.7, 64.3, 39.4, 33.1, 33.0, 29.0, 28.9, 28.3, 23.9, 23.7.

Synthesis of Boc-Protected Methacrylate Random Copolymers

4-((*tert*-Butoxycarbonyl) amino)butyl methacrylate (Boc-ABMA) was synthesized according to the previous report.[4] The copolymers were prepared by mixing Boc-ABMA (7.0 mmol, 3.5 mL of 2 M solution in acetonitrile), ethyl methacrylate (EMA) (3.0 mmol, 1.5 mL of 2 M solution in acetonitrile), RAFT chain transfer agent 2-cyanoprop-2-yl-dithiobenzoate (1.0 mmol, 221 mg, 10 mole% relative to total amount of monomers), and radical initiator AIBN (0.1 mmol, 16 mg, 1 mole% relative to total amount of monomers) in a flask [Scheme 1 (b)]. The mixture was flushed with nitrogen gas for 5 min, then stirred at 70 °C. After 16 h, the reaction was stopped by cooling the vial in a dry ice–acetone bath. The copolymer was isolated by evaporating the acetonitrile under reduced pressure, and then dissolving the residue in dichloromethane, followed by precipitation in excess hexanes twice to remove unreacted impurities. The resulting Boc-protected copolymers were characterized by ^{1}H NMR analysis to determine the mole percentage of EMA (MP_{ethyl}), the degree of polymerization (DP), and consecutive the number average molecular weight (M_n). The MP_{ethyl} was determined by comparing integrated peaks of butylene groups of Boc-ABMA and ethylene groups of EMA in the ^{1}H NMR spectra. The DP was calculated by comparing

integrated peaks of phenyl group of chain transfer agent at the polymer ω-end and side chains in the ^{1}H NMR spectra. The copolymers were also characterized by GPC analysis to measure the number average molecular weight (M_n) and the weight average molecular weight (M_w) calculated using a calibration curve based on 10 standard samples of poly(methyl methacrylate), M_W 500–50,000 (Agilent Technologies, M-L-10, no. PL2020-0100). The product was a pink solid copolymer (2.02 g, % yield= 85%). ^{1}H NMR ($CDCl_3$, 400 MHz), DP= 15, MP_{ethyl}= 30.3 mole%, M_n (GPC) = 2800, PDI = 1.12: δ 7.90–7.26 (m), 5.25–4.75 (brs), 4.20–3.83 (m), 3.25–2.92 (m), 2.15–1.77 (m), 1.70–1.45 (m), 1.42 (brs), 1.32–1.17 (m), 1.16–0.80 (m).

Reaction Kinetics

4-((*tert*-Butoxycarbonyl) amino)butyl methacrylate (Boc-ABMA) (2.5 mmol, 1.25 mL of 2 M solution in acetonitrile), ethyl methacrylate (EMA) (2.5 mmol, 1.25 mL of 2 M solution in acetonitrile), RAFT chain transfer agent (0.5 mmol, 10 mole% relative to total amount of monomers), radical initiator AIBN (0.05 mmol, 8.2 mg, 1 mole% relative to total amount of monomers) was mixed in a 10 mL Schlenck flask and flushed with nitrogen gas for 5 min. The mixture was stirred at 70 °C and aliquots were drawn at time intervals up to 24 h. After 24 h, the reaction was stopped by cooling the flask in a dry ice–acetone bath. The drawn aliquots were separated into two portions: one was directly analyzed by ^{1}H NMR to obtain charts of total materials, and the other was mixed with chloroform and the solution was evaporated under reduced pressure to remove volatile EMA monomer. This cycle was repeated thrice before ^{1}H NMR analysis and remaining non-volatile materials including Boc-ABMA monomer were analyzed by ^{1}H NMR. The conversion and ratio of remaining Boc-ABMA monomer were calculated by comparing integrated peaks of phenyl group of chain transfer agent and the vinylic protons of monomers in the ^{1}H NMR spectra and plotted versus time.

End-Group Reaction of Copolymers

The Boc-protected copolymers and AIBN or Boc-amine AZO (20 eq) was dissolved in acetonitrile [Scheme 1 (b)]. The solution was bubbled with nitrogen gas for 1 h and stirred at 70 °C for 16 h. The reaction mixture was cooled to room temperature and acetonitrile was evaporated under reduced pressure. The copolymers were purified either by precipitation in hexanes or loaded to a Sephadex LH-20 column (GE Healthcare Life Sciences, Ltd) with methanol as solvent. Products after purification step were colorless to cream substances.

Boc-P1a: **Boc-P1** (150 mg), **AIBN** (0.87 mmol, 142 mg), and acetonitrile (90 mL) were used. After the reaction and evaporation of solvent, the residue was dissolved in dichloromethane and precipitated in excess hexanes giving a cream colored copolymer (143 mg, % yield= 98%). ^{1}H NMR ($CDCl_3$, 400 MHz), MP_{ethyl}= 29.8 mole%, M_n (GPC) = 3100, PDI = 1.13: δ 5.22–4.60 (brs), 4.08–3.80 (m), 3.21–2.92 (m), 2.15–1.73 (m), 1.72–1.48 (m), 1.41 (brs), 1.35–1.17 (m), 1.10–0.80 (m).

Boc-P1b: **Boc-P1** (150 mg), **Boc-amine AZO** (0.87 mmol, 492 mg), and acetonitrile (90 mL) were used. After the reaction and evaporation of solvent, the residue was loaded to a Sephadex LH-20 column to give a cream colored copolymer (155 mg, % yield= 99%). ^{1}H NMR ($CDCl_3$, 400 MHz), MP_{ethyl}= 28.1mole%, M_n (GPC) = 2800, PDI = 1.16: δ 5.25–4.80 (brs), 4.09–3.85 (m), 3.41–3.25 (brs), 3.21–3.01 (m), 2.70–2.40 (m), 2.15–1.76 (m), 1.75–1.50 (m), 1.44 (brs), 1.35–1.16 (m), 1.14–0.80 (m).

Deprotection of Copolymers

Boc-protected copolymers (**Boc-P1**, **Boc-P1a**, and **Boc-P1b**) were mixed with methyl 3-mercaptopropionate (MMP), followed by the addition of trifluoroacetic acid (TFA). After stirring for 30 min, the TFA was removed by blowing with nitrogen gas. The residue left was dissolved in methanol and the deprotected copolymers were obtained by precipitating in excess diethyl ether. Subsequently, the copolymer precipitates were dissolved in distilled water and lyophilized to yield light, fluffy fibrous copolymers (**P1**, **P1a**, and **P1b**) [Scheme 1 (c)].

P1: **Boc-P1** (1.00 g), MMP (500 μL), TFA (5 mL) was used. After lyophilization, a light pink fluffy product was obtained (0. 94 g, % yield= 90%). ^{1}H NMR (methanol-d_4, 400 MHz), DP = 16, MP_{ethyl} = 30.6: δ 8.18–7.35 (m), 4.25–3.83 (m), 3.09–2.84 (m), 2.16–1.82 (m), 1.80–1.65 (m), 1.46–1.17 (m), 1.13–0.80 (m).

P1a: **Boc-P1a** (100 mg), MMP (50 μL), TFA (1 mL) was used. After lyophilization, a light white fluffy product was obtained (101 mg, % yield= 97%). ^{1}H NMR (methanol-d_4, 400 MHz), MP_{ethyl} = 29.6: δ 4.23–3.80 (m), 3.08–2.82 (m), 2.18–1.82 (m), 1.81–1.67 (m), 1.54–1.17 (m), 1.14–0.77 (m).

P1b: **Boc-P1b** (100 mg), MMP (50 μL), TFA (1 mL) was used. After lyophilization, a light white fluffy product was obtained (94 mg, % yield= 89%). ^{1}H NMR (methanol-d_4, 400 MHz), MP_{ethyl}= 28.3: δ 4.22–3.78 (m), 3.25–3.16 (m), 3.07–2.85 (m), 2.73–2.54 (m), 2.18–1.82 (m), 1.81–1.58 (m), 1.57–1.18 (m), 1.16–0.77 (m).

Antimicrobial and Hemolytic Assays and Membrane Depolarization Study

The antimicrobial activity of polymers was evaluated against *B. subtilis*, *E. faecalis*, *S. aureus*, CA-MRSA, *A. baumannii*, *E. coli*, *P. aeruginosa*, and *S. enterica* using a turbidity-based micro-dilution assay reported previously.[19,20] The minimum inhibitory concentration of polymers (MIC) was determined as the polymer concentration to completely inhibit bacterial growth. The polymer-induced lysis of human RBCs (hemolysis) was evaluated by monitoring release of hemoglobin upon lysis of cells according to our previous report.[4,21] The polymer concentration necessary for 50% hemolysis (HC_{50}) was measured from dose–response curves. Each MIC and hemolysis assay was independently repeated at least three

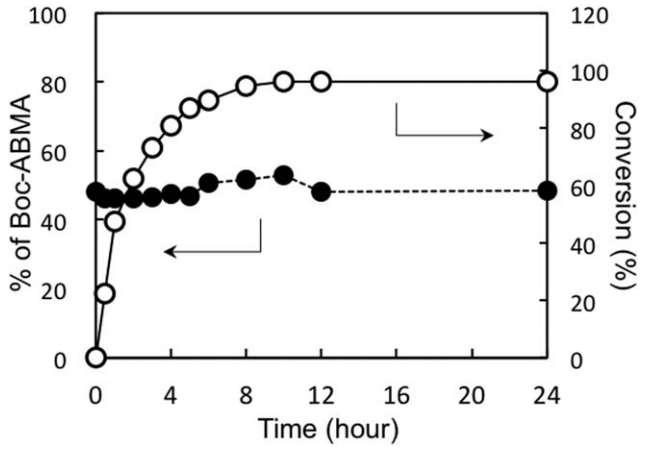


FIGURE 2 Conversion-time plots for the copolymerization of Boc-ABMA with EMA. The monomer ratio of Boc-ABMA to EMA is 1:1. Left axis: mole percent of Boc-ABMA monomer relative to total monomers in copolymerization. Right axis: conversion of copolymerization.

times using different polymer stock solutions in triplicate on different days. The MIC and HC_{50} values were reported as an average of three or more independent assays. To determine the membrane-lytic activity of polymers, polymer-induced membrane disruption was evaluated against *S. aureus* using the membrane potential-sensitive dye $DiSC_3$-(5). See the Supporting Information for the experimental details.

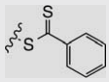
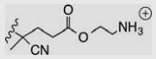
RESULTS AND DISCUSSION

We first characterized the RAFT polymerization of random methacrylate copolymer prior to end-group functionalization to determine the monomer distribution in a polymer chain. A protected amine monomer Boc-ABMA and hydrophobic monomer ethyl methacrylate (EMA) were chosen for model polymers, which have been used in our previous study.[4] To examine the monomer distributions of copolymers, the relative reactivities of these monomers in the RAFT polymerization were measured by monitoring the monomer conversion of monomers or compositional drift during the polymerization. To facilitate the analysis, these monomers were copolymerized with a 1-to-1 ratio using conventional azo-based radical initiator AIBN in the presence of commercially available 2-cyanoprop-2-yl-dithiobenzoate [Scheme 1 (b): RAFT polymerization]. The conversions of monomers reached to >95% after 10 h (Fig. 2). The percentages of Boc-ABMA found in a copolymer chain were ~50% for all time during the polymerization, indicating that these monomers have the same reactivity at this composition, and therefore, the monomers are statically distributed in a polymer chains, giving a statistical copolymer.

We next examined a synthetic method to prepare copolymers with different functionalities at the ω-end of copolymers [Scheme 1 (b)]. Given the purpose of improvement of antimicrobial activity and toxicity profiles of polymers, we used a copolymer with 30% EMA for end-group modulation because this polymer formulation showed most potent antimicrobial activity, with minimal hemolytic activity against human RBCs in our previous studies.[4] RAFT polymerization of Boc-ABMA and EMA yielded the boc-protected polymer **Boc-P1**. These copolymers have narrow molecular weight distribution (dispersity $Đ$= 1.12) (Table 1). The DP values of resultant polymers are larger than the target DP of 10 (the mole ratio of monomers to CTA). This is likely because of selective precipitation of longer polymer chains during the precipitation.

The boc-protected copolymer **Boc-P1** was used as a parent polymer to prepare copolymer variants with chemical mutations in the ω-end groups. To modulate the ω-end groups, we used the radical-medicated modification method, which involves re-initiation of polymerization by free radical to generate active radical at the polymer end upon removal of dithioester RAFT agent, which follows radical coupling with radical initiators [Scheme 1 (b)].[16,22–24] To minimize the potential side reaction of polymer chain radical homocoupling, highly diluted conditions were used, a potential drawback of this approach. However, this method requires less reaction steps as compared with the thiol-mediated modification method in which a thiol group is produced by aminolysis of CTA thio-ester groups and conjugated with other functional molecules via thiol–ene coupling reaction. In addition, using CTAs prepared with desired end-groups for polymerization would be also an attractive option,[7] while this method may require extensive synthesis effort for CTAs. The **Boc-P1** parent polymer was reacted with the excess amount (20 eq) of azo-based radical initiators (**AIBN** and **Boc-amine AZO**) in the high dilution condition (100 mg polymer in 60 mL acetonitrile).[22] **AIBN** was chosen as a conventional radical initiator, which has been used to remove the RAFT agent from the polymer end to avoid potential cytotoxicity.[25] **Boc-amine AZO** contains a chemical structure with a primary amine groups to examine the effect of cationic groups at the ω-end of polymers in the antimicrobial activity and prove the potential use of chemically functional initiators in this method. The ^{1}H NMR spectra of copolymers did not show any signals from the phenyl group of CTA at the ω-end of parent copolymers after the reaction (Supporting Information Fig. S1: ^{1}H NMR spectra of parent and variants). The GPC elution curves of copolymers showed no significant difference between parent and variant copolymer (Supporting Information Fig. S2: GPC elution curves of parent and variants), and the molecular weights are found similar (Table 1). These results suggest that this radical-medicated modification method proceeded quantitatively without any significant polymer−polymer coupling reactions. The boc groups of copolymers were subsequently removed by TFA to give cationic amphiphilic random copolymers [**P1** and variants, Scheme 1 (c)], in the presence of methyl 2-mercaptopropanoate as a scavenger for tert-butyl cations. The resultant copolymers showed no significance changes in DP and MP_{ethyl} from the boc-protected copolymers (Table 1). These results suggest that this chemical approach successfully derives methacrylate copolymer variants with different end-group functionalities.

TABLE 1 Characterization of Boc-Protected and Deprotected Copolymers

	End-Group		Boc-Protected						Deprotected		
	α	ω	MP_{ethyl}[a] (mol. %)	DP[b]	M_n, NMR[c] (g mol^{-1})	M_n, GPC[d] (g mol^{-1})	M_w, GPC[d] (g mol^{-1})	$Đ$[e]	MP_{ethyl}[a] (mol. %)	DP[b]	M_n, NMR[c] (g mol^{-1})
P1			30.3	15	3400	2800	3100	1.12	30.6	16	3800
P1a			29.8	n.d.	n.d.	3100	3500	1.13	29.6	n.d.	n.d.
P1b			28.1	n.d.	n.d.	2800	3200	1.16	28.3	n.d.	n.d.

[a] Mole percentage of ethyl group (MP_{ethyl}) in a polymer chain determimed by ^{1}H NMR.
[b] The number average degree of polymerization (DP) determimed by ^{1}H NMR. The DP and MP_{ethyl} of the boc-protected copolymer **Boc-P1** and deprotected copolymer **P1** were determined by comparing integrated peaks of phenyl group of CTAs at the omega-end and the polymer side chains in the ^{1}H NMR spectra.
[c] The number average molecular weight (M_n) calculated based on the molecular weight of monomers, MP_{ethyl} and DP.
[d] The number average molecular weight (M_n), the weight average molecular weight (M_w) determimed by GPC. The molecular weight calibration was based on poly(methyl methacrylate) standards.
[e] Dispersity ($Đ$) was calculated as M_w/M_n using M_w and M_n values determined by GPC.
n.d., Not determined.

The antibacterial activity of copolymers was evaluated against a panel of bacteria by determining the minimum inhibitory concentration (MIC) that is necessary for complete inhibition of bacterial growth, using a turbidity-based microdilution assay.[19,20] The toxicity of polymers was also evaluated by determining lytic activity of polymers against human RBCs. It should be noted that all copolymers are soluble to an assay medium and did not cause any precipitation under the assay condition (Supporting Information Fig. S3). In general, all copolymers showed potent antimicrobial activity (MIC = 2.0–31.3 μg mL^{-1}) against a broad spectrum of bacteria (Table 2). Some copolymers showed larger MIC values (MIC = 62.5–125 μg mL^{-1}) for *S. aureus*, methicillin-resistant *S. aureus* (MRSA), and *S. enterica*. In addition, the MIC values of copolymers were increased in the order of **P1** < **P1a** < **P1b**. This indicates that the antimicrobial activity of copolymers was decreased as the end-groups were transformed from the phenyl thio-ester (**P1**) to cyanoisobutyl (**P1a**) and primary ammonium (**P1b**) groups. However, the difference in the MIC values of copolymers studied here varies by only 2- to 4-fold, as compared with orders of magnitude changes reported by Mowery et al.[5] which used longer alkyl groups for the end groups of nylon-3 copolymers. In this study, we used only copolymers with three different

TABLE 2 Antimicrobial Activity Spectra and Hemolytic Activity of Copolymers

	P1	P1a	P1b	Magainin-2	Melittin
MIC (μg mL^{-1})[a]					
Gram (+)					
Bacillus subtilis	2.0	2.0	2.0	63[d]	2[d]
Enterococcus faecalis	3.9	7.8	15.6	>500[d]	6[d]
Staphylococcus aureus	15.6	31.3	62.5	>500[d]	6[d]
CA-MRSA[b]	15.6	31.3	62.5	>500[d]	3[d]
Gram (−)					
Acinetobacter baumannii	3.9	3.9	7.8	n.d.	n.d.
Escherichia coli	7.8	15.6	15.6	125[d]	13[d]
Pseudomonas aeruginosa	7.8	15.6	15.6	500[d]	100[d]
Salmonella enterica	15.6	31.3	31.3	500[d]	50[d]
HC_{50} (μg mL^{-1})[c]					
Human RBCs	>1000 (26.9 ± 9.6%)	>1000 (12.4 ± 4.8%)	>1000 (9.8 ± 4.3%)	>250 (9%)[d]	3.8 ± 1.3

[a] Minimum inhibitory concentration against bacteria in Mueller-Hinton Broth.
[b] Community acquired methicillin-resistant *S. aureus* (CA-MRSA) strain LAC BB1263.
[c] HC_{50} at which concentration causing 50% hemolysis or lysis of RBCs (hemolysis%) at highest concentration used in the assay relative to Trinton-X (positive control, 100%) and PBS (negative control, 0%).
[d] MIC and HC_{50} values were previously reported.[4,21]
n.d., Not determined.

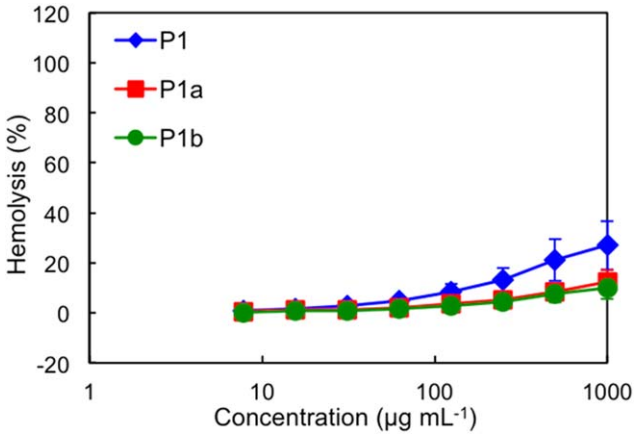


FIGURE 3 Hemolysis dose–response curves of copolymers. [Color figure can be viewed at wileyonlinelibrary.com]

end groups, which appear not to provide the range of hydrophobicity to induce such large variations in their antimicrobial activity. On the other hand, the polymers caused relatively low percent hemolysis at the polymer concentration of 1000 μg mL^{-1}, the highest concentration tested in this study, indicating that the polymers are selective to bacteria over human cells. For comparison, lytic peptide melittin caused 100% hemolysis above 20 μg mL^{-1} (Supporting Information). Similar to the antimicrobial activity, the polymer-induced hemolysis was decreased for **P1a** and **P1b** (Fig. 3 and Supporting Information Fig. S4). These variants with different end-groups are derived from the same precursor polymer **Boc-P1** and thus the inherent antimicrobial activity of parent polymer chains would presumably remain same after the end-group modulation. Therefore, the dependence of MIC values on the end-groups is likely to be resulted from the properties of end-groups. The end-group dependence on the antimicrobial and hemolytic activities appears to reflect the hydrophobicity of end-groups. It has been previously reported that the hydrophobic groups in the side chains enhance the ability of polymers to disrupt membranes, resulting in higher antimicrobial activity and hemolytic activity.[1,3,4,21] Similarly, the hydrophobicity of end-groups would also drive the disruption of cell membranes in bacteria and RBCs. In addition, as described above, the cationic and hydrophobic monomer units are likely to be statistically distributed in these polymers. Therefore, because the end-groups would change only the hydrophobicity of polymer end locally, the changes of antimicrobial activity are likely to reflect the functional role of hydrophobicity or cationic functionality specific to the polymer end. Alternatively, if the monomer composition were drifted during polymerization, the polymer would have gradation in the density of cationic and hydrophobic monomers, or the monomer distribution would be block copolymer-like. The impact of end-group hydrophobicity or functionality to the amphiphilic properties of such a block-like copolymer would be different from that to a statistical/random copolymer, and thus the effect of end-groups on the antimicrobial activity would be different. As Gellman and coworkers previously pointed out,[18] the data to examine the role of end-groups of polymers in biological activities should be interpreted based on not only monomer composition, but also monomer distribution in a polymer chain.

According to the results on the antimicrobial and hemolytic activities, we hypothesize that the hydrophobicity of end-groups would enhance the disruption of bacterial cell membranes, resulting in higher antimicrobial activity. To test this hypothesis, it was first examined if the copolymers are capable of permeabilizing *S. aureus* membrane or not. To that end, we used the membrane potential-sensitive fluorophore $DiSC_3$-(5), the fluorescence of which is quenched upon binding to intact bacterial cell membranes.[26–29] After addition of copolymers at each MIC to *S. aureus* pre-incubated with $DiSC_3$-(5), the fluorescence was recovered (Fig. 4). It should

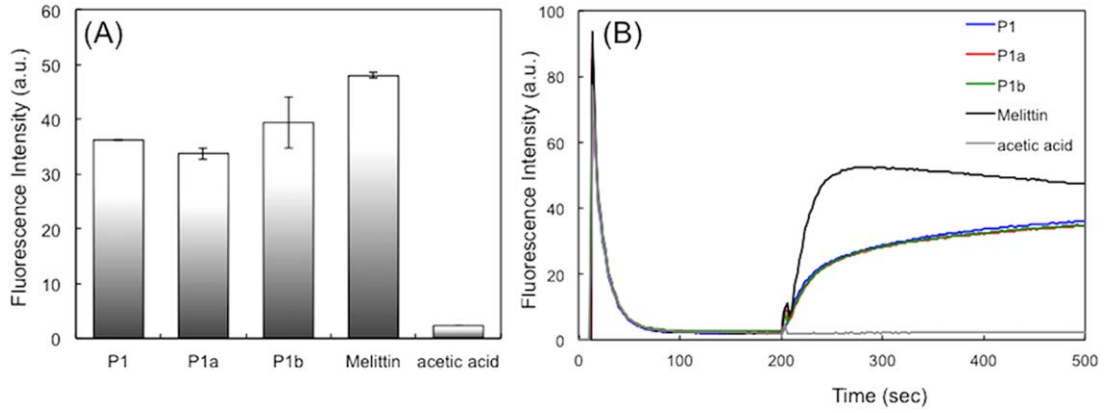


FIGURE 4 *S. aureus* membrane depolarization induced by copolymers. (A) Fluorescence intensity at 5 min after injection of copolymers or melittin to the *S. aureus* suspension. The final concentrations are equal to their MICs determined in M-H Broth. 0.01% acetic acid was tested as solvent control. (B) Time course of *S. aureus* membrane depolarization induced by copolymers. A $DiSC_3$-(5) dye was added to *S. aureus* suspension at 20 s and stirred for 180 s. At 200 s, copolymers or melittin was injected to the suspension to give final concentrations equal to their MICs as determined in M-H Broth (Table 2). 0.01% acetic acid was tested as solvent control. [Color figure can be viewed at wileyonlinelibrary.com]

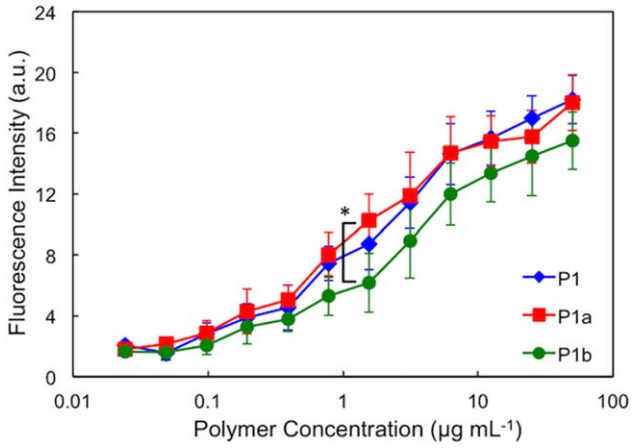


FIGURE 5 Effect of ω-end groups on *S. aureus* membrane depolarization. The fluorescence intensity was measured using a microplate reader. [$DiSC_3$-(5)] = 0.5 μM. Error bars represent the standard deviation from three independent trials, each done in triplicate. *$p < 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

be noted that the fluorescence of dye did not change after incubation with the polymers in solution (Supporting Information Fig. S5), indicating the potential interaction between the dye and polymers do not quench or enhance the fluorescent property of dye. This result supports the notion that these copolymers exert their antibacterial effects by disrupting the *S. aureus* membrane. The recovered fluorescence intensities of all copolymers did not show any statistically significant difference at each MIC, suggesting that the copolymers disrupt the *S. aureus* membrane to the same extent to inhibit *S. aureus* growth.

We further examined the effect of polymer concentration on depolarization of *S. aureus* membrane. The copolymers did not show any statistically significant differences in the recovered fluorescence intensities for all polymer concentrations except the data points for **P1a** and **P1b** at 1.6 μg/mL ($p < 0.05$) (Fig. 5). The little or no difference in the membrane depolarization caused by the copolymers may reflect the fact that the MIC values varied by only 2- to 4-fold among the copolymers as discussed above. This small range of changes in the MIC values may not result in significant difference in their ability to disrupt membrane as we expected. However, it cannot be ruled out that the polymers may also exert other antimicrobial mechanisms along with membrane disruption, which contribute to the activity. Specifically, the RAFT end-groups have been reported to cause cytotoxicity in host cells.[30] Therefore, the dithioester groups may cause cytotoxicity to bacteria although the toxicity mechanism is not clear at this point. It would be necessary to conduct a more systematic approach using a library of copolymers to elucidate the effect of end-groups on their activity as well as their role in the antimicrobial mechanisms.

Regarding the antimicrobial spectrum of polymers, the MIC values against *S. aureus* and *E. faecalis* changed 4-fold when the polymer end-groups were modified, while those against other bacteria are not significant (within 2-fold). It is not clear at this point why *S. aureus* and *E. faecalis* are more sensitive to the chemical structures of end-groups of copolymers than other bacteria. This appears to be specific to these bacteria rather than Gram-specific activity because the copolymers did not show significant differences in MICs against Gram-positive *B. subtilis*. The end-group dependence of MIC could be related to the cell wall properties specific to bacteria and cell physiology of these bacteria, which might control the diffusion of copolymers in the cell wall and their impact to the cellular activities differently. These results also in turn suggest the possibility that bacteria-specific antimicrobial polymers could be designed by tailoring the end-groups to be active against only targeted bacteria without harm to commensal bacteria.

CONCLUSIONS

In summary, the RAFT polymerization provided cationic amphiphilic methacrylate copolymers with statistical distributions of monomers used in this study. The radical-medicated modification method transformed the ω-end groups from the conventional RAFT agent to different functional groups, which modulate their antibacterial and hemolytic activities. In this study, because the polymers with only three different end-groups were tested, the results cannot be extended to the generalization of the role of end-groups in the antimicrobial and hemolytic activities. Polymers with systematic chemical variations at the polymer end should be designed and tested for their biological activities to provide a functional link between the end group modulation and membrane activity of amphiphilic copolymers.

ACKNOWLEDGMENTS

We acknowledge the National Science Foundation for NSF CAREER Award (DMR-0845592 to K.K.) and Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan. This work was also supported by JSPS Postdoctoral Fellowships for Research Abroad (No. 26-774 to H.T.). We thank Dr Blaise R. Boles at University of Iowa Carver College of Medicine for providing the CA-MRSA LAC BB1263 and Dr Robertson D. Davenport at University of Michigan Hospital for providing the RBCs.

REFERENCES AND NOTES

1 K. Kuroda, W. F. DeGrado, *JACS* **2005**, *127*, 4128–4129.

2 K. Kuroda, G. A. Caputo, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2013**, *5*, 49–66.

3 H. Takahashi, E. F. Palermo, K. Yasuhara, G. A. Caputo, K. Kuroda, *Macromol. Biosci.* **2013**, *13*, 1285–99.

4 E. F. Palermo, S. Vemparala, K. Kuroda, *Biomacromolecules* **2012**, *13*, 1632–1641.

5 B. P. Mowery, A. H. Lindner, B. Weisblum, S. S. Stahl, S. H. Gellman, *JACS* **2009**, *131*, 9735–9745.

6 J. Zhang, M. J. Markiewicz, B. P. Mowery, B. Weisblurn, S. S. Stahl, S. H. Gellman, *Biomacromolecules* **2012**, *13*, 323–331.

7 T. D. Michl, K. E. S. Locock, N. E. Stevens, J. D. Hayball, K. Vasilev, A. Postma, Y. Qu, A. Traven, M. Haeussler, L. Meagher, H. J. Griesser, *Polym. Chem.* **2014**, *5*, 5813–5822.

8 D. Jung, J. P. Powers, S. K. Straus, R. E. W. Hancock, *Chem. Phys. Lipids* **2008**, *154*, 120–128.

9 S. Venkataraman, W. L. Ong, Z. Y. Ong, S. C. J. Loo, P. L. R. Ee, Y. Y. Yang, *Biomaterials* **2011**, *32*, 2369–2378.

10 Y. Chen, P. A. Wilbon, Y. P. Chen, J. H. Zhou, M. Nagarkatti, C. P. Wang, F. X. Chu, A. W. Decho, C. B. Tang, *RSC Adv.* **2012**, *2*, 10275–10282.

11 A. Punia, E. He, K. Lee, P. Banerjee, N. L. Yang, *Chem. Commun.* **2014**, *50*, 7071–7074.

12 J. L. Grace, J. X. Huang, S. E. Cheah, N. P. Truong, M. A. Cooper, J. Li, T. P. Davis, J. F. Quinn, T. Velkov, M. R. Whittaker, *RSC Adv.* **2016**, *6*, 15469–15477.

13 K. E. S. Locock, T. D. Michl, J. D. P. Valentin, K. Vasilev, J. D. Hayball, Y. Qu, A. Traven, H. J. Griesser, L. Meagher, M. Haeussler, *Biomacromolecules* **2013**, *14*, 4021–4031.

14 X. Yang, K. Hu, G. T. Hu, D. Y. Shi, Y. J. Jiang, L. W. Hui, R. Zhu, Y. T. Xie, L. H. Yang, *Biomacromolecules* **2014**, *15*, 3267–3277.

15 Matyjaszewski K. In Controlled Radical Polymerization: Mechanisms; Rousseau, E. D., Ed.; ACS Symposium Series 1187; American Chemical Society: Washington, DC, **2015**; p 1–17.

16 H. Willcock, R. K. O'Reilly, *Polym. Chem.* **2010**, *1*, 149–157.

17 Y. Oda, S. Kanaoka, T. Sato, S. Aoshima, K. Kuroda, *Biomacromolecules* **2011**, *12*, 3581–3591.

18 R. H. Liu, X. Y. Chen, S. Chakraborty, J. J. Lemke, Z. Hayouka, C. Chow, R. A. Welch, B. Weisblum, K. S. Masters, S. H. Gellman, *JACS* **2014**, *136*, 4410–4418.

19 R. E. W. Hancock, Hancock Laboratory Methods, Department of Microbiology and Immunology, University of British Columbia, British Columbia, Canada, **2001**, http://www.cmdr.ubc.ca/bobh/methodsall.html

20 A. Giacometti, O. Cirioni, F. Barchiesi, M. S. Del Prete, M. Fortuna, F. Caselli, G. Scalise, *Antimicrob. Agents Chemother.* **2000**, *44*, 1694–1696.

21 I. Sovadinova, E. F. Palermo, M. Urban, P. Mpiga, G. A. Caputo, K. Kuroda, *Polymers* **2011**, *3*, 1512–1532.

22 S. Perrier, P. Takolpuckdee, C. A. Mars, *Macromolecules* **2005**, *38*, 2033–2036.

23 C. Boyer, V. Bulmus, T. P. Davis, V. Ladmiral, J. Liu, S. Perrier, *Chem. Rev.* **2009**, *109*, 5402–5436.

24 G. Moad, E. Rizzardo, S. H. Thang, *Polym. Int.* **2011**, *60*, 9–25.

25 T. H. Nguyen, S. H. Kim, C. G. Decker, D. Y. Wong, J. A. Loo, H. D. Maynard, *Nat. Chem.* **2013**, *5*, 221–227.

26 C. L. Friedrich, D. Moyles, T. J. Beveridge, R. E. W. Hancock, *Antimicrob. Agents Chemother.* **2000**, *44*, 2086–2092.

27 H. Sugiarto, P. L. Yu, *FEMS Microbiol. Lett.* **2007**, *270*, 195–200.

28 R. F. Epand, H. Sarig, A. Mor, R. M. Epand, *Biophys. J.* **2009**, *97*, 2250–2257.

29 K. A. Gibney, I. Sovadinova, A. I. Lopez, M. Urban, Z. Ridgway, G. A. Caputo, K. Kuroda, *Macromol. Biosci.* **2012**, *12*, 1279–1289.

30 C. W. Chang, E. Bays, L. Tao, S. N. S. Alconcel, H. D. Maynard, *Chem. Commun.* **2009**, *24*, 3580–3582.