DOI: 10.1002/

Full Paper

Synthesis and Bioactivity of Polymer-based Synthetic Mimics of Antimicrobial Peptides (SMAMPs) Made From Asymmetrically Disubstituted Itaconates

David Boschert,[#] Alexandra Schneider-Chaabane,[#] Andreas Himmelsbach, Alice Eickenscheidt, Karen Lienkamp*

[#] contributed equally

_____

D. Boschert, A. Schneider-Chaabane, A. Himmelsbach, Dr. A. Eickenscheidt, Dr. K. Lienkamp

Freiburg Center for Interactive Materials and Bioinspired Technologies (FIT) and Department of Microsystems Engineering (IMTEK), Albert-Ludwigs-Universität, Georges-Köhler-Allee 105, 79110 Freiburg, Germany; *E-Mail: lienkamp@imtek.uni-freiburg.de

_____

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.2018xxxxx.

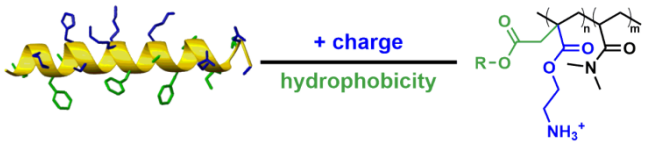
Abstract:

A series of asymmetrically disubstituted diitaconate monomers is presented. Starting from itaconic anhydride, functional groups could be placed selectively at the two non-equivalent carbonyl groups. Using 2D NMR, it was shown that the first functionalization step occurred at the carbonyl in β-position to the double bond. These monomers were copolymerized with dimethylacrylamide (DMAA) to yield polymer-based synthetic mimics of antimicrobial peptides (SMAMPs). They were obtained by free radical polymerization, a metal free process, and still maintained the facial amphiphilicity at the repeat unit level. This eliminates the need for laborious metal removal and is advantageous from a regulatory and product safety perspective.

The poly(diitaconate-co-DMAA) copolymers obtained were statistical to alternating, and the monomer feed ratio roughly matched the repeat unit content of the copolymers. Investigations of varied R group hydrophobicity, repeat unit ratio and molecular mass on the antimicrobial activity against *E. coli* and on the compatibility with human keratinocytes showed that the polymers with the longest R groups and the lowest DMAA content were the most antimicrobially and the most hemolytic ones. This is in line with the biological activity of previously reported SMAMPs. Thus, the design concept of facial amphiphilicity was successfully transferred, yet, the selectivity of these polymers for bacteria over mammalian cells still need to be optimized.

Keywords: antimicrobial polymer, bioactive polymer, copolymerization parameters, itaconic acid, monomer synthesis.

FIGURE FOR ToC_ABSTRACT



TEXT FOR ToC_ABSTRACT

Radically facially amphiphilic. Poly(diitaconates) copy the natural blueprint for antimicrobial peptides, yet can be obtained by simple free radical polymerization and still maintain the desired facial amphiphilicity of the parent peptides.

1. Introduction

Due to the continuously increasing incidence of bacterial infections caused by antibiotics-resistant bacteria, the need for new drugs and materials that inhibit bacterial growth is more pressing than ever.[1] Very few new antibiotic drug classes have been developed in the past decades. However, it was estimated that by 2050, 10 million people will die every year from infections with antibiotics-resistant bacteria - making antimicrobial resistance more deadly than cancer.[2]

Synthetic Mimics of Antimicrobial Peptides (SMAMPs) are a promising substance class that could help relieve this problem; consequently, research on small molecule SMAMPs or polymer-based SMAMPs has strongly increased over the last decade.[3] The SMAMP approach was derived from nature. SMAMPs copy the design of natural antimicrobial peptides (AMPs, Figure 1a).[4] AMPs can be found in most organisms as part of the innate immune system and are crucial for the immediate response of these organisms to pathogens including bacteria; additionally, they activate other parts of the adaptive immune system.[5] The antimicrobial activity of AMPs results from so-called facial amphiphilicity: as a result of the secondary structure of the AMP backbone, the residues of the hydrophobic and cationic amino acids fall onto opposite faces of the molecule (Figure 1a).[3i, 4, 6] This enables AMPs to attach with their cationic face to the negatively charged outer envelope of bacteria, where they aggregate and eventually permeate the cell membrane using their hydrophobic groups.[4a, 6] At the same time, there is no such electrostatic driving force for the interaction of AMPs with overall charge-neutral mammalian cells, which is why AMPs selectively target bacteria and not the cells of their host organism.[4a, 6] For the development of SMAMPs, the concept of facial amphiphilicity was successfully transferred to the design of antimicrobial synthetic molecules.[3d, 3h, i, 3l, 7] In these studies, it became clear that the most important parameter to be controlled in SMAMPs was the regular distribution of the hydrophilic, cationic groups and the hydrophobic groups along the molecular backbone, as this led to SMAMPs which also had a

high activity against bacteria, and selectivity for bacteria over mammalian cells.[3d, 3i, 3l, 8] Small molecule-based SMAMPs are obtained in step-by-step organic synthesis, thus there is excellent control over structural precision.[9] However, such syntheses are laborious and expensive. Polymer-based SMAMPs, on the other hand, can be synthesized in fewer synthetic steps,[3f, g, 8b, 10] yet often at the expense of molecular precision. This is due to lack of sequence-control during the polymerization step. How regular two different monomers are attached to a growing SMAMP chain depends solely on their relative reactivity, i.e. on their copolymerization parameters. Unless these dictate alternating copolymerization, the polymerization outcome is random or statistical. In SMAMPs, this may lead to a local imbalance in the distribution of hydrophobicity, and thus to increased toxicity.[3l, 11] Another factor that reduces structural precision of synthetic polymers compared to peptides is that they are typically lacking a secondary structure that pre-defines the orientation of their functional groups. In spite of this, some polymer-based SMAMPs showed excellent antimicrobial activity and cell selectivity in vitro.[3f, 8c] It was hypothesized that the amphiphilic polymer-based SMAMPs could self-assemble at the liquid-bacteria interface into an appropriate configuration, and thereby become membrane-active.[10c] This hypothesis was substantiated by several studies that confirm membrane-activity of SMAMPs.[12]

A regular distribution of functional groups and a better bioactivity profile of polymer-based SMAMPs are more easily achieved with facially amphiphilic monomers, where the hydrophobic and the cationic, hydrophilic group are attached to the same polymerizable unit. This avoids runs of hydrophobic or hydrophilic repeat units in the polymer, which are often observed when copolymerizing two monomers carrying a hydrophilic and a hydrophobic group each ('segregated units').[3g] For example, Gabriel et al. compared poly(norbornenes) with facially amphiphilic repeat units[8b] to the corresponding segregated polymers,[3g] and found that the cell selectivity of the facially amphiphilic polymers against Gram-positive and Gram-negative bacteria was by far higher.[3g]

However, the abundance of monomers that can be independently functionalized with two groups without becoming unpolymerizable (or of monomers that form strictly alternating copolymers) is rather limited. Norbornenes are such monomers, and facially amphiphilic poly(norbornene) SMAMPs can be obtained fast and efficiently by ring-opening metathesis polymerization (ROMP).[8b, 8d, 13] However, the quantitative removal of the transition-metal based ROMP initiator is often difficult, expensive or just not possible.[14] For medical applications (drugs or biomaterials), this can become a regulatory issue and a product safety problem. While fascinating new concepts that use the ROMP initiator in catalytic amounts are emerging,[15] these as yet lack the precision and control over molecular weight of ROMP with stoichiometric amounts, so that alternatives to the ROMP-based platform for SMAMPs are desirable.

The aim of this work was thus to develop a metal-free platform for the synthesis of SMAMP polymers from facially amphiphilic itaconic acid derivatives. Itaconic acid was chosen because it can carry two functional groups, because it is cheap and can be obtained from sustainable resources, and because it can be polymerized by radical polymerization.[16] Considering that it is side product in the citric acid cycle,[16] itaconic acid is a promising, non-toxic starting point for the development of drugs and biomaterials. Unfortunately, homopolymerization of itaconic acid derivatives by a radical mechanism is rather slow compared to acrylic or methacrylic acid derivatives because itaconates have a high chain transfer constant.[17] Diitaconate esters, particularly those with short substituents, can be driven to quantitative conversion within 48 h, however the molecular weights obtained remain low due to chain transfer.[18] This situation has been improved when controlled radical polymerization, particularly RAFT, was used to polymerize diitaconates, as reported by Barner-Kowolik and also by Kamigaito.[19] In these papers, polymer molecular masses (M_n) as high as 60 000 g mol^{-1} were reported. However, even in controlled radical homopolymerization of diitaconates, high chain transfer was observed.[19b]

However, itaconate derivates can be easily copolymerized, e.g. with styrene, methyl methacrylate or vinyl chloride[20] to obtain poly(itaconates) with varying backbone polarity. To our knowledge, unsymmetrically substituted poly(itaconates) in general have so far only been reported twice, and in these cases the product isolation was rather laborious.[21] We here describe a straight-forward synthesis of a series of facially amphiphilic, unsymmetrically disubstituted diitaconates, and their copolymerizion with with *N,N*-dimethylacrylamide (DMAA). DMAA was chosen as a co-repeat unit because it had a suitable reactivity, and because poly(dimethyacrylamide) (PDMAA), the homopolymer of DMAA, is protein repellent and biologically inert.[22] Thus, the presence of DMAA repeat units in the poly(diitaconate-co-DMAA) SMAMPs would not compromise their biocompatibility. We also determined the copolymerization parameters of the diitaconate monomers in free radical copolymerization with either DMAA or styrene. The results indicate that these copolymerizations yielded statistical to slightly alternating copolymers with tunable antimicrobial activity, as detailed below.

2. Results and Discussion

System Design

To obtain facially amphiphilic SMAMPs by metal-free polymerization, poly(itaconates) derivatives were targeted. The design of the poly(itaconate) SMAMPs here presented is a direct translation of the design concept of the previously reported, highly selective poly(oxonorbornene) SMAMPs:[3f, 3k] (Figure 1). In both polymers, the hydrophilic (blue) and the hydrophobic (green) functional groups are attached via ester groups to the polymer backbone (black). What is particular about the itaconate system is that its two carbonyl groups are not equivalent. As a result, they can be selectively functionalized. This was not possible in the symmetric oxonorbornene monomer, where the direction of the ring opening was random.

Thus, at the monomer level, there is even higher structural control in the diitaconate monomer (Figure 1b) than in the oxonorbornene diester monomer (Figure 1c).

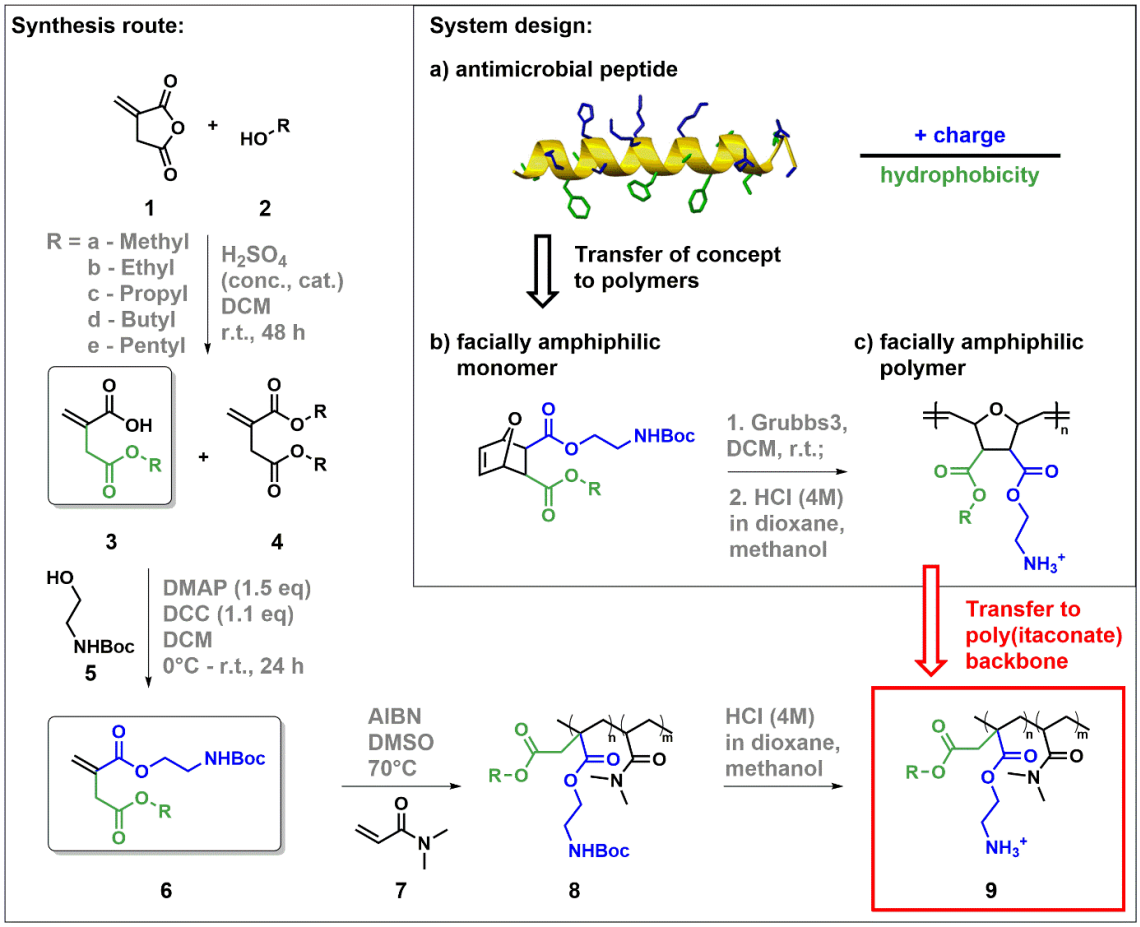


Figure 1. Synthesis route (**1** to **9**) and design concept (**a** to **c**) for poly(itaconate) SMAMPs. The molecular structure of the highly selective poly(norbornene) SMAMPs (c) was transferred to the poly(diitaconate) system (**9**). Both polymers carry a hydrophobic (green) and a hydrophilic (blue) group, which are connected to the polymer backbone (black) via ester bonds.

To obtain polymers with tunable amphiphilicity, the R group was varied from R = methyl to R = pentyl. Since diitaconates do not homopolymerize well, the target polymers were copolymers with dimethyl methacrylate (DMAA). While the diitaconate repeat units contain both functional groups required for bioactivity, their bioactivity profile and tunability will still depend on the ability to control the distribution of the diitaconate and DMAA repeat units along the polymer chain, i.e. on their copolymerization parameters r_1 and r_2. For this reason, the copolymerization parameters for the diitaconates with DMAA (and for comparison also

those of the diitaconates with styrene) were determined. Additionally, the molar ratio of diitaconate to DMAA was used as a further variable to tune the overall polymer hydrophilicity, and the molar fractions of diitaconate (with R = methyl to butyl) was varied from 40 mol% to 60 mol%. The effect of these variations on the bioactivity of the polymers was determined by testing their antimicrobial activity and cell compatibility.

Synthesis of Asymmetrically Disubstituted Itaconates

The asymmetrically disubstituted diitaconates were synthesized in a two-step process (Figure 1). First, itaconic anhydride **1** was ring-opened with the appropriate alcohol **2** (R = methyl to pentyl). The ring-opening of the anhydride **1** took place exclusively at the carbonyl in β-position to the double bond, so that mono-itaconate **3** was obtained. This was confirmed by 2D-NMR spectroscopy (Heteronuclear Multiple Quantum Correlation, HMQC, and Heteronuclear Multiple Bond Correlation, HMBC; the 2D-spectra of compound **3**, with R = propyl, are shown in the Supporting Information as Figures S1 and S2). The side product of the reaction was the symmetrically substituted diester **4**. Compounds **3** and **4** could be separated easily during work-up by washing and extraction steps (see Experimental section). Interestingly, the product ratio of **3** and **4** could be tuned by adding different amounts of acid. The more acid was used, the more diester **4** was obtained. When the acid quantity was reduced to catalytic amounts, a yield of up to 80% of product **3** was obtained (Table 1). For the esterification in the second step, at first Steglich conditions (dicyclohexylcarbodiimide, DCC and a catalytic amount of *N*,*N*-dimethylaminopyridine, DMAP) were applied. However, these conditions only lead to low yields of the disubstituted diitaconates and a large amount of a side product (*N*-acylurea). It is a well-known problem that DCC-activated acids (*O*-acylurea) rearranges intramolecularly to the corresponding *N*-acylurea, which then cannot react further to the desired product. However, this rearrangement is slow and could therefore be suppressed by adding stoichiometric amounts of DMAP, which then transfered the *O*-

acylurea quantitatively to the reactive DMAP-amide. This intermediate reacted rapidly with the alcohol **5** and gave the desired asymmetrically substituted diitaconates esters **6** in good yiels (up to 94%, Table 1). Again, the work-up of the product consisted of simple extraction and filtration steps, and avoided column chromatography.

The structures of all compounds obtained were confirmed by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. As an example, the ^{1}H-NMR spectra of the mono-itaconate **3c** and the disubstituted monomer **6c** (R = propyl in both cases) are shown in Figure 2. All the peaks in these spectra could be assigned to the target compounds (Figure 2). When comparing the spectrum of compound **3c** to **6c** (Figures 2a and 2b) a slight shift of the double bond signals (H5 and H6) to lower field strengths was observed, while the methylene peak (H4) was unaffected. The signal positions and intensities of the other peaks (H1 to H3 and H7 to H9, respectively) confirmed the assumed structures and thus the attachment of the hydrophobic and the hydrophilic groups in each reaction step. The ^{1}H- NMR, ^{13}C-NMR spectra and mass spectra of the other mono- and diitaconates can be found in the Supporting Information (mono-itaconates: Figures S3 to S8 - NMR spectra, Figures S9 to S13 - mass spectra; diitaconates: Figures S14 to S19 - NMR spectra, Figures S20 to S24 - mass spectra).

Table 1. Reaction yields for the synthesis of monoitaconates **3a** to **3e**, the symmetrically substituted diitaconates **4a** to **4e**, and the asymmetrically substituted diitaconates **6a** to **6e**.

R group	**mono-itaconate 3 / %**	**diester 4 / %**	**diitaconate 6 / %**
Methyl **a**	30	25	61
Ethyl **b**	75	2	94
Propyl **c**	80	7	91
Butyl **d**	75	14	70
Pentyl **e**	28	9	78

Copolymerization of Diitaconates with DMAA

To obtain the facially amphiphilic target polymers, the asymmetrically substituted diitaconates **6a-d** were copolymerized by free radical polymerization with DMAA (**7**) at different monomer ratios using standard free radical polymerization conditions (Figure 1). The reaction temperature needed to be carefully adjusted, as higher temperatures (80°C and more) led to polymer cross-linking, and too low polymerization temperatures resulted in long reaction times and low monomer conversion. The optimum temperature range was 65-70°C. The polymer was recovered by precipitation from dichloromethane into *n*-hexane.

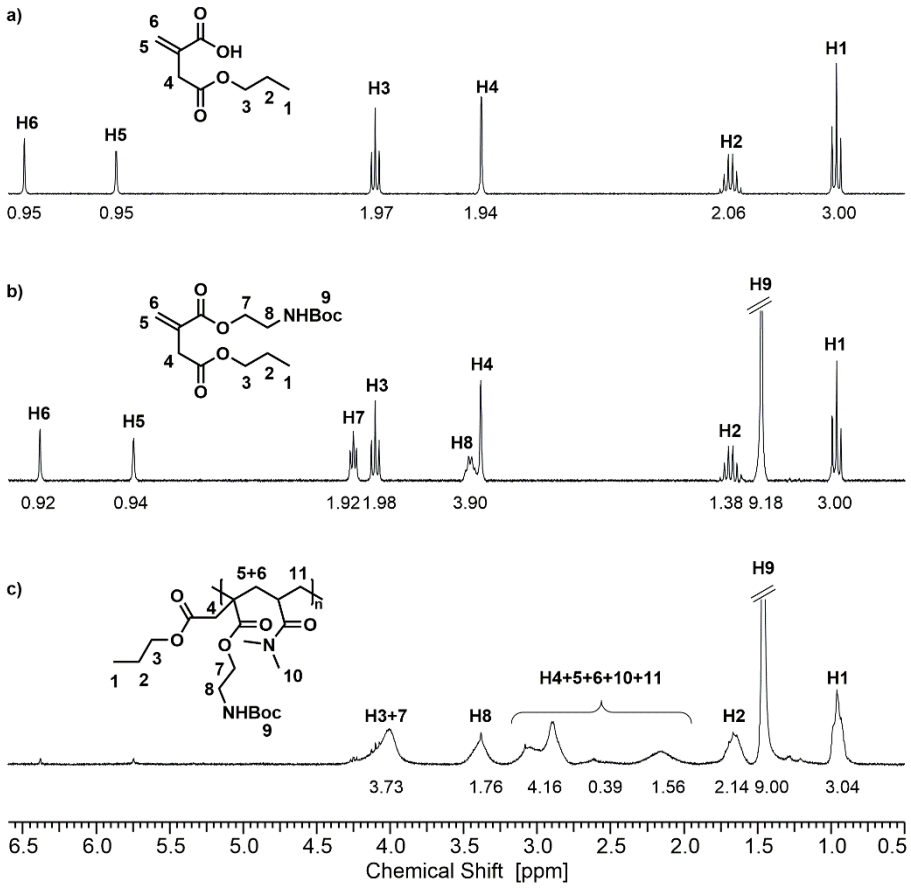


Figure 2. ^{1}H-NMR spectra (in $CDCl_3$) of mono-itaconate **3c** (a), the asymmetrically disubstituted diitaconate **6c** (b), and the poly(diitaconate-co-DMMA) **8c** (c) with a diitaconate:DMAA ratio of 1:1. The NMR signals (H1 to H11) of each spectrum could be assigned to the expected protons of the target structures. The signal intensities (numbers underneath each peak) closely matched the expected proton numbers.

The ^{1}H-NMR spectrum of copolymer **8c** with a diitaconate:DMAA ratio of 1:1 is shown in Figure 2c. Further ^{1}H-NMR spectra of the copolymers **8a-d**, with 40-60 mol% diitaconate content, can be found in the Supporting Information (Figures S25 to S28). The signals of H1 to H3 and H7 to H9 in the diitaconate repeat unit remained at similar chemical shifts as the corresponding signals of monomer **6c** (Figure 2b), albeit with the typical polymer peak broadening. The signal H4 of the methyl group of the DMAA repeat unit appeared slightly above 3 ppm. The polymer backbone protons are, expectedly, even broader than the side chain signals and fall into the typical regions for such aliphatic backbone protons. Gel permeation chromatography (GPC, calibrated with poly(methyl methacrylate) (PMMA) standards) was used to determine the molecular weight (number average molar mass, M_n) and polydispersity index (PDI) of the copolymers **8a-d** with varying diitaconate content (40-60 mol% diitaconate). A typical GPC elugram is shown in Figure3a**.** The GPC elugrams of the other polymers can be found in the Supporting Information (Figure S30); the data thus obtained is summarized in Table 2.

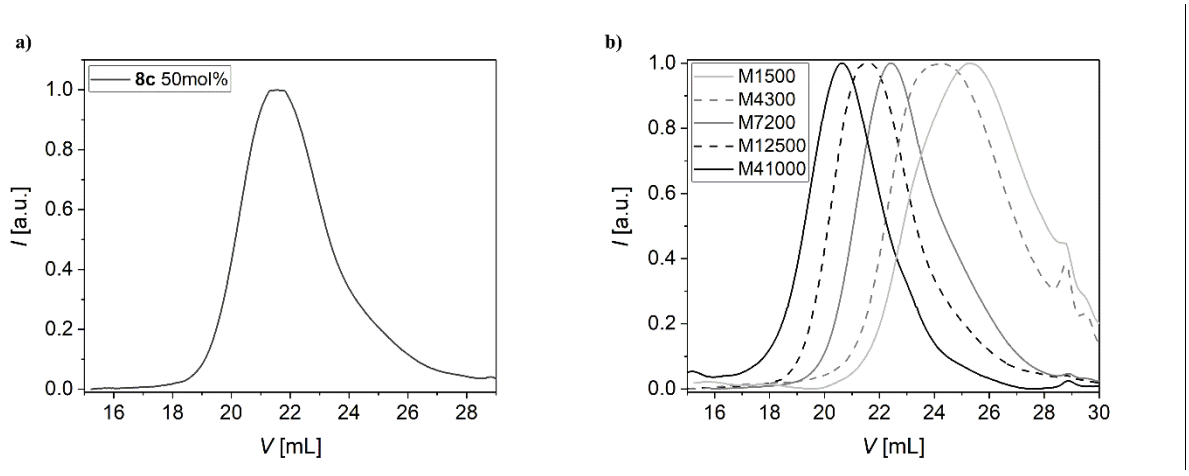


Figure 3. Gel permeation chromatography (GPC) elugrams (in chloroform, flow rate: 1 mL min^{-1}, SDV columns, PMMA standards, refractive index detector intensity *I* vs. elution volume *V*)) of a) poly(diitaconate-co-DMAA) **8c**, diitaconate:DMAA ratio 1:1, and b) poly(diitaconate-co-DMAA) **8c**, diitaconate:DMAA ratio 1:1, with different molecular weights.

Table 2. Analytical data for polymers **8** and **9**. The number average molecular mass M_n and polydispersity index PDI ($=\frac{M_w}{M_n}$) of the N-Boc-protected polymers **8** were determined by gel permeation chromatography (GPC, in chloroform, SDV columns, calibrated with PMMA standards). The minimum inhibitory concentration (MIC_{90}) against *E. coli* and the hemolytic concentration (HC_{50}) of the corresponding deprotected copolymers **9a-d** were determined as described in the Experimental; * = value out of experimental range, n.d. = not determined.

Diitaconate content	**Polymer**	M_n **/ g mol^{-1}**	**PDI**	**Polymer**	**MIC_{90} / µg mL^{-1}**	**HC_{50} / µg mL^{-1}**	**selectivity**
40 mol%		19,100	3.0		*, >400	*, >8000	n.d.
50 mol%	**8a**	11,900	2.6	**9a**	*, >400	*, >8000	n.d.
60 mol%		12,500	2.7		*, >400	*, >4000	n.d.
40 mol%		19,500	2.1		*, >400	*, >8000	n.d.
50 mol%	**8b**	7,300	2.6	**9b**	*, >400	1050	n.d.
60 mol%		6,900	3.3		*, >400	6100	n.d.
40 mol%		11,200	2.5		200	n.d.	n.d.
50 mol%	**8c**	12,500	2.9	**9c**	200	300	1.5
60 mol%		6,600	3.5		100	160	1.6
40 mol%		24,000	3.9		50	9	0.2
50 mol%	**8d**	17,700	2.8	**9d**	25	5	0.2
60 mol%		11,800	2.7		50	3	0.06
50 mol%		1,600	3.4		*, >400	1,260	3.2
50 mol%		4,300	1.6		200	350	1.8
50 mol%	**8c**	7,200	2.8	**9c**	50	230	4.6
50 mol%		12,500	2.9		50	300	6
50 mol%		41,000	1.8		12.5	70	0.6

Reaction Conversion and Copolymerization Parameters of Diitaconate 6c and DMAA

^{1}H-NMR spectroscopy was used to determine the monomer conversion during the copolymerization of diitaconate **6c** with DMAA. For this purpose, nine reaction batches with varying ratios of diitaconate **6c** (10-90 mol%) and DMAA (90-10 mol%, respectively) were prepared. Following a literature method,[23] the conversion of each monomer over time was determined by calculating the relative intensity of the respective monomer peaks for each time point (signal at 6.26 ppm for the diitaconate, signal at 5.62 ppm for the DMAA, normalized to the constant signal of the added *N,N*-dimethylformamid standard at 8.02 ppm). The results are summarized in Figure 4. Figure 4a and Figure 4b show the time dependent consumption of the diitaconate monomer **6c** and the total monomer conversion (**6c** + DMAA), respectively, for the nine different batches. From one time point with low reaction conversion each, a plot of the molar fraction of the diitaconate repeat units (**X**) in the nine copolymers vs. the molar fraction **x** of diitaconate in the monomer mixture was composed (Figure 4c). The curve is slightly S-shaped and bends around the diagonal line on which the repeat unit composition of the polymer corresponds to the composition of the monomer mixture. Thus, the polymerization is almost statistical. This is important for the SMAMPs synthesis, as it means that the monomer feed ratio approximately matches the repeat unit composition of the resulting polymer over a wide range of compositions. Thus, the monomer feed ratio can be used to tune the overall hydrophilicity of the resulting polymer. Figure 4a and Figure 4b also show that the monomer conversion increased with increasing amount of DMAA. This observation matches the results of the GPC analysis (Figure 4d and Table 3), which indicates that the isolated polymers also had higher average molecular masses with increasing DMAA content. This was expected, since DMAA homopolymerizes more easily at 70°C than the diitaconate. In the case of polymer **8c** with 10 mol% diitaconate repeat units, the polydispersity index was extremely high, most likely due to formation of inter-chain cross

links, as the chosen polymerization time (24 h) was too long for this particular composition. For the other compositions, the polydispersity index ranged between 1.7 to 3.8 (Figure 4d).

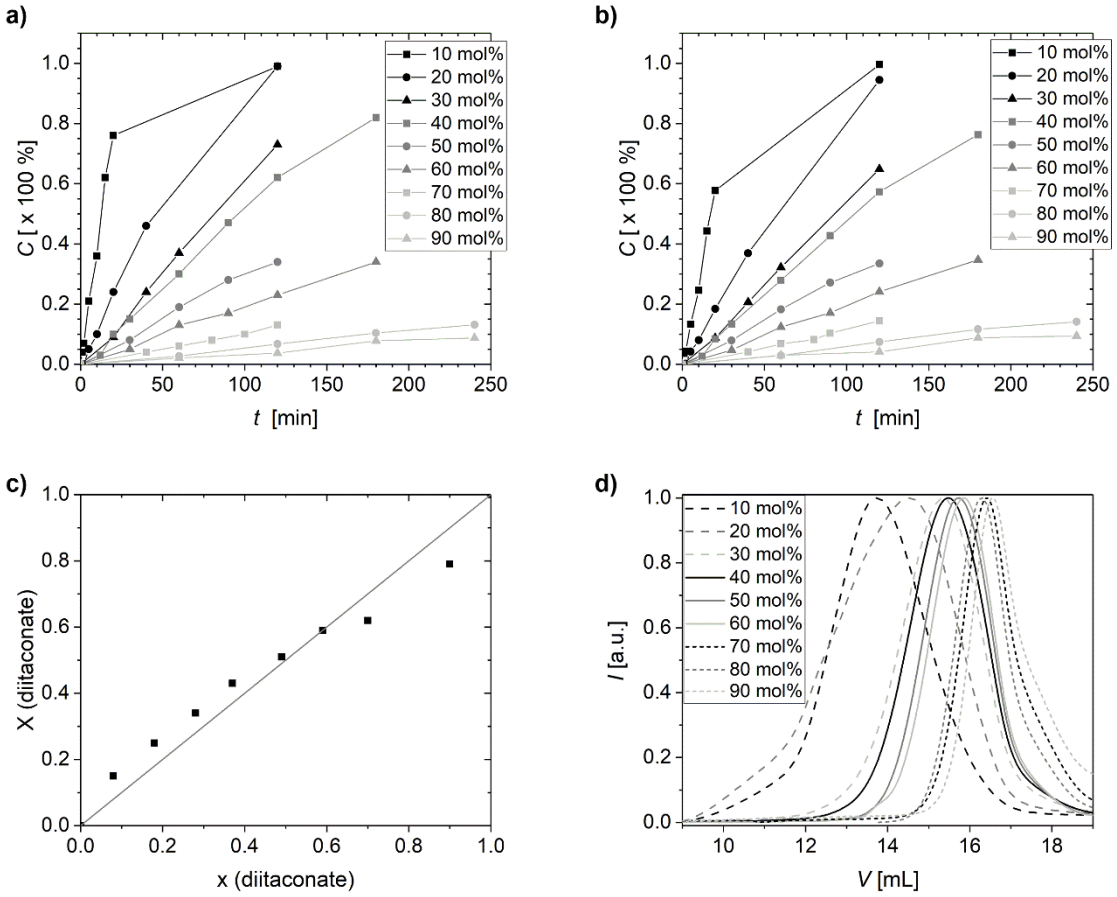


Figure 4. Copolymerization of diitaconate **6c** with DMAA. The monomer composition was varied from 10% diitaconate and 90% DMAA (= 10 mol% in the figure) to 90% diitaconate and 10% DMMA (= 90 mol% in the figure). a) Conversion of the diitaconate monomer vs. time and b) total monomer conversion vs. time. Both data sets were determined by ^{1}H-NMR spectroscopy. c) Plot of molar fraction **X** of diitaconate in the polymer vs. the molar fraction **x** of diitaconate in the monomer mixture at 12-21% total conversion. On the diagonal line, the repeat unit composition of the polymer corresponds to the composition of the monomer mixture. d) Gel permeation chromatography (GPC) elugrams (refractive index detector intensity *I* vs. elution volume *V*) of copolymers **8c** with different monomer ratios of diitaconate and DMAA that were isolated at the end point of the reactions (Figure 4a).

Table 3. Gel permeation chromatography (GPC) data (in chloroform, SDV columns, calibrated with PMMA standards) for polymers **8c** (obtained by copolymerization of **6c** with DMAA), and for copolymers obtained by copolymerization of diitaconate **4c** with styrene. M_n = number average molecular weight, $\frac{M_w}{M_n}$ = polydispersity index (PDI). The polymer composition was varied from 10% diitaconate and 90% DMAA (=10 mol% in the table) to 90% diitaconate and 10% DMMA (= 90 mol% in the table).

Polymer 8c	**M_n / g mol^{-1}**	**PDI**	**Polymer 4c/styrene**	**M_n / g mol^{-1}**	**PDI**
10 mol%	124,100	12.5	10 mol%	23,400	2.3
20 mol%	92,000	2.2	20 mol%	21,000	2.2
30 mol%	32,000	3.8	30 mol%	20,200	1.6
40 mol%	26,700	2.8	40 mol%	17,700	1.6
50 mol%	22,100	2.3	50 mol%	11,200	1.8
60 mol%	20,600	2.2	60 mol%	12,100	1.8
70 mol%	10,100	2.0	70 mol%	8,100	1.8
80 mol%	12,000	1.9	80 mol%	5,200	1.6
90 mol%	8,800	1.7	90 mol%	3,800	1.4

The copolymerization parameters (r-parameters), i.e. the relative reactivity of the two monomers, were calculated from the individual ^{1}H-NMR spectra using the Fineman-Ross plot, the Inverted-Fineman-Ross plot and the Kelen-Tudos plot.[23-24] These plots linearize the Mayo-Lewis equation, which is used to describe copolymerization kinetics, so that the copolymerization parameters can be obtained (see Supporting information, Figures S37 to S38). The r-parameters thus obtained are summarized in Table 4. Copolymerization parameters are different for each comonomer pair A and B and also temperature-dependent, as they are the ratios of two propagation rate constants ($r_1 = \frac{k_{AA}}{k_{AB}}$ and $r_2 = \frac{k_{BB}}{k_{BA}}$, respectively, where k_{BA} (for example) is the rate constant of the addition of monomer A to a polymer with a chain end radical B), which are temperature-dependent themselves. Besides the copolymerization parameters for the reaction of **6c** with DMAA, we also determined the

copolymerization parameters for the reaction of **4c** with styrene to have a reference data set for comparison with literature data (Figure S39) For the system **4c**/styrene (polymerized at 80°C), both copolymerization parameters were well below one, which indicates that the propagation rate of the reaction of each monomer with a like chain end (k_{AA}, k_{BB}, corresponding to a 'homopolymerization' step) was slower than the propagation rate with an unlike chain end (k_{AB}, k_{BA}, corresponding to a 'copolymerization' step). This hints at a tendency to form alternating copolymers rather than strictly statistical copolymers. For the system **6c**/DMMA the polymerization temperature was set to 70°C. This was due to the higher overall reactivity of DMAA compared to styrene. Nevertheless, the copolymerization parameters for the system **6c**/DMAA were similar to those of the system **4c**/styrene. In both cases, the copolymerization parameters were below one, showing that both monomers slightly favored alternating copolymerization rather than forming statistical copolymers.

Table 4. Copolymerization parameters r_1 and r_2 for the reaction of diitaconates **4c** and **6c** with styrene and DMMA respectively.

Comonomer 1	**Comonomer 2**	**Reaction Temperature / °C**	**Method**	**r_1**	**r_2**
4c	styrene	80	Fineman-Ross	0.22	0.46
		80	Inverted Fineman-Ross	0.32	0.45
		80	Kelen-Tudos	0.38	0.58
6c	DMAA	70	Fineman-Ross	0.47	0.53
		70	Inverted Fineman-Ross	0.49	0.49
		70	Kelen-Tudos	0.55	0.58

Tate et al.[20] found copolymerization parameters for the reactions of various itaconic acid derivatives with different comonomers (Table 5). The r-parameters determined in this work were in good agreement with this data: the r_1 values of the diitaconates were below 1 when

copolymerized with either styrene or methyl methacrylate. The r-parameters r_2 of styrene in these systems were also below 1, indicating a statistical to alternating incorporation of both monomers. The literature values of r_2 for reactions of diitaconates with methyl methacrylate were mostly above 1, which indicates a preference of methyl methacrylate for the homopolymerization step in these systems. For the copolymerization of (di)itaconates with DMAA, no copolymerization parameters were reported so far, yet the copolymerization parameters found for the comonomer pair **8c** and DMAA in this work are reasonable. In the context of SMAMPs, the fact that r_1 and r_2 were below 1 for the investigated diitaconate-comonomer systems, which indicates a statistical to alternating monomer incorporation, is an important finding. It means that there is no intrinsic tendency to an irregular repeat unit distribution in these polymers. Thus, there is sufficient reaction control in the polymerization step, and the global as well as the local molecular amphiphilicity and charge density of the SMAMP copolymers can be tuned by varying the R groups on the facially amphiphilic repeat units, and by adjusting the molar fractions of the repeat units.

Table 5. Copolymerization parameters r_1 and r_2 for the reaction of various itaconic acid derivatives with different comonomers.

Comonomer 1	Comonomer 2	r_1	r_2	Lit.
4c	styrene	0.41	0.25	[25]
4a	*n*-butylacrylate	0.94	0.40	[20]
4a	methyl methacrylate	0.3	1.3	[20]
4d	methyl methacrylate	0.4	0.8	[20]

Copolymer Activation and Bioactivity

The protected poly(diitaconate-co-DMAA) **8** copolymers were activated by removing their *tert*-butyloxycarbonyl (Boc) protective group with hydrochloric acid. This gave poly(diitaconate-co-DMAA) SMAMPs **9** with primary ammonium groups. They were

precipitated from methanol into diethyl ether and then dried. The removal of the Boc groups was confirmed by ^{1}H-NMR spectroscopy (see Experimental section and Figure S35). The antimicrobial activities of the copolymers **9a-d** with varying DMAA content and of polymers **9c** with varying molecular mass (at constant diitaconate:DMAA ratio of 1:1) were determined using the Minimum Inhibitory Concentration (MIC) assay. In this assay (described in the Experimental section), the MIC_{90} value is determined, which corresponds to the SMAMP concentration at which 90% bacterial growth is inhibited - the smaller this value, the more active the polymer. The toxicity of the copolymers was estimated by determining their propensity to lyse human red blood cells using and the Hemolysis assay (described in the Experimental section). In this assay, the hemolytic concentration HC_{50} (the concentration at which 50% of human red blood cells are lysed by the polymer) was determined - the higher this value, the less hemolytic the polymer. The curves thus obtained are shown in Figure 5, and the corresponding MIC and HC_{50} values are listed in Table 2. In line with the results obtained for the parent poly(oxanorbornene) system,[3f] the highest antimicrobial activity was found for the more hydrophobic copolymers (with longer alkyl side chains at R, Figure 5a). The impact of hydrophobicity on antimicrobial activity was also seen in the series of polymers which had the same R groups, but different molar ratios of diitaconate and DMAA. As all diitaconate repeat units were more hydrophobic than DMAA, the antimicrobial activity of these polymers also increased with increasing diitaconate content. This was particularly apparent for polymer **9c** (Figure 5a). A similar effect was observed in the HC_{50} data (Figure 5b), which also increased with increasing hydrophobicity. These results are consistent with antimicrobial data of many other polymer series, where a general increase in antimicrobial activity with increased molecular hydrophobicity (at constant overall molecular weight) has been observed.[3h, 8c, 26] Polymers that deviate from this trend are typically found to be less soluble in aqueous media, and thus less bioavailable than their more hydrophilic counterparts.[3h, 8c, 26] The most hydrophobic copolymers (particularly **9d**, with R = butyl)

were also the most toxic ones. The shorter the alkyl group R and the higher the DMAA content (at constant molecular mass), the lower was their hemolytic activity. This is also consistent with the trends observed for previously reported polymers.[3h, 8c, 26] Thus, the design concept of the facially amphiphilic poly(oxonorbornenes) was successfully translated to poly(diitacoante-co-DMAA) copolymers mit facially amphiphilic repeat units, which were antimicrobially active and had tunable properties.

For the copolymer series with different molecular weights (**9c**, diitaconate:DMAA ratio 1:1, M_n = 1,500 - 41,000 g mol^{-1}), the antimicrobial activity increased significantly with increasing molecular mass of the samples tested (Figure 5c). A similar trend was also found in their hemolytic activity (Figure 5d). This was somewhat unexpected, as an increase of antimicrobial activity in this molecular weight range is typically not observed. For example, for amphiphilic poly(norbornene) SMAMPs, antimicrobial activity was either unaffected by molecular weight, or was found to decrease.[8b] Likewise, the hemolytic activity of these polymers was independent of their molecular mass.[8b] For another poly(oxonorbornene) SMAMP, both antimicrobial activity and hemolytic activity decrease with increasing M_n.[3h, 12c] However, the poly(methacrylates) reported by of Kuroda and DeGrado were consistently more hemolytic with increasing molecular weight, although only a narrow molecular range from 1,600 g mol^{-1} to 8,700 g mol^{-1} was investigated in these studies.[26] For the same polymer series, antimicrobial activity decreased with increasing M_n for the more hydrophobic polymers (R = propyl, butyl, benzyl), while it slightly increased for with increasing M_n for the corresponding methyl and ethyl copolymers. For the nylon-3 random copolymers described by Gellman, hemolysis strongly increased with increasing polymer chain length.[8c] Thus, the molecular weight effect on these properties, in contrast to the hydrophobicity effect, is not yet fully understood.

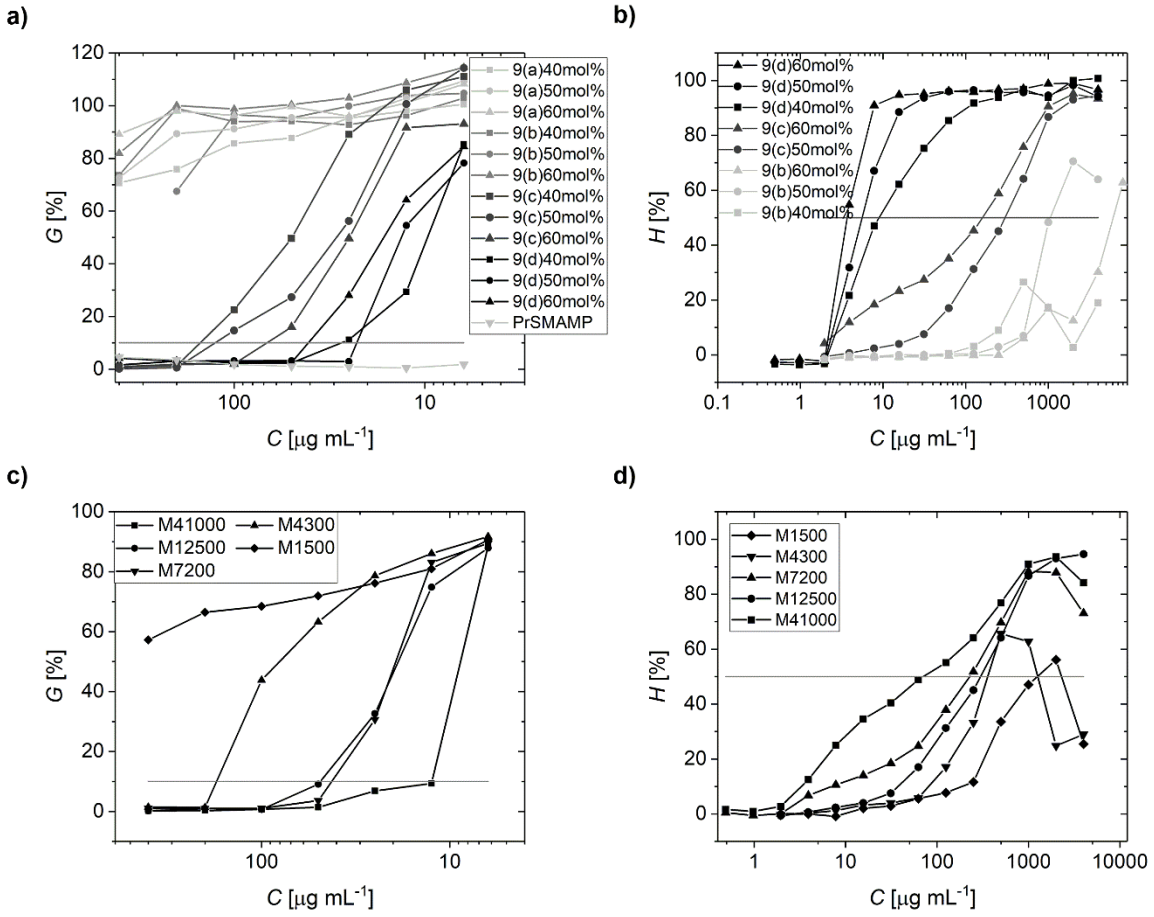


Figure 5. Bioactivity of poly(diitaconate-co-DMAA) SMAMPS. a) Antimicrobial activity against *E. coli* bacteria and b) hemolytic activity of copolymers **9a-d** with different ratios of diitaconate to DMAA; c) antimicrobial activity against *E. coli* bacteria and d) hemolytic activity of copolymers **9c** (diitaconate:DMAA ratio 1:1) with different molecular masses. The data point with a growth percentage below the straight lines in a) and c) indicates the MIC_{90} value; the intercept of the curves and the straight lines in b) and d) the location of the HC_{50} value.

3. Conclusions

We here presented the synthesis and copolymerization of a series of diitaconate monomers from which facially amphiphilic, antimicrobial synthetic mimics of antimicrobial peptides (SMAMPs) could be obtained. Their design concept was adapted from facially amphiphilic poly(oxonorbornenes), yet they had the advantage that they could be obtained by free radical

polymerization, a metal free polymerization technique, and still maintained the facial amphiphilicity at the repeat unit level.

Starting from itaconic anhydride, two distinct functional groups could be placed at the two non-equivalent carbonyl groups of that building block. Using 2D NMR techniques, it was shown that the functionalization of the itaconic anhydride with the first functional group selectively occurred at the carbonyl in β-position to the double bond. By determining the copolymerization parameters of the diitaconate monomers with DMAA, it was shown that the poly(diitaconate-co-DMAA) copolymers obtained were statistical to alternating, and that the monomer feed ratio roughly matched the repeat unit content of the copolymers. By varying the overall hydrophobicity of the polymers using the length of the alkyl chain R and the DMAA content as parameters, it could be shown that the polymers with the longest R groups and the lowest DMAA content were the most antimicrobially active ones, yet they were also the most hemolytic ones. This is in line with the biological activity of the previously reported poly(oxonorbornes) and other polymer series.[3h, 3l] Thus, the design concept of the facially amphiphilic poly(oxonorbornenes) was successfully transfered to the facially amphiphilic poly(diitaconate-co-DMAA) copolymers. So far, however, the selectivity of these polymers for bacteria over mammalian cells (Table 2) remains low due to the multitude of parameters that need to be further optimized in this polymer system. However, the data shows that once the design rules for one facially amphiphilic SMAMP system are known, these design rules can be transferred from one polymer system to another, provided that each system is synthesized with sufficient structural control. Importantly, the here described diitaconate-based SMAMPs can be synthesized by metal free initiator systems. This eliminates the need for laborious metal removal during work-up and is thus advantageous for drug and biomaterials applications from a regulatory and product safety perspective. Additionally, it makes these polymers much cheaper than the corresponding poly(norbornenes), which is of particular importance for materials applications, where larger polymer amounts might be

needed. However, to be truly useful as drugs and materials, their hemolytic activity has still to be optimized. This can be achieved by further structural optimization, particularly by using other co-repeat units than DMAA. In future work we will strive for even more control in the polymerization of these systems. In the light of the molecular weight dependency of the biological activities of these polymers, the next variable to be optimized will be the polydispersity, using controlled radical polymerization. We will report on our progress in this project in due course.

4. Experimental Section

General

All chemicals, e.g. itaconic anhydride, methanol, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, 4-dimethylaminopyridine (DMAP), *N,N'*-dicyclohexylcarbodiimide (DCC), *N*-Boc-ethanolamine, 2,2'-azobis(2-methylpropionitrile) (AIBN) and HPLC grade solvents, e.g. dichloromethane (DCM), acetone, chloroform, hexane or diethyl ether, were obtained as reagent grade from Sigma Aldrich (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany) and used as received, unless otherwise indicated. *N,N*-dimethylacrylamide (DMAA), DMF and styrene were freshly distilled before use. AIBN was recrystallized from methanol. Gel permeation chromatography (GPC, in chloroform, flow rate 1 ml min^{-1}, calibrated with poly(methyl methacrylate) (PMMA) standards) was measured on a non-polar GPC column set (PSS SDV analytical (5 µm, 100 Å and 10.000 Å), PSS, Mainz, Germany). NMR spectra were recorded on a Bruker 250MHz spectrometer (Bruker, Madison, WI, USA).

Synthesis of Asymmetrical Diitaconates

Synthesis of 4-Alkyl Itaconates **3a** to **3e**

To obtain the 4-alkyl itaconates **3a** to **3e** with R = methyl to pentyl, itaconic anhydride (4.3 g, 38 mmol) was dissolved in DCM (10 mL) and the required primary alcohol with the appropriate R group (40 mL, excess) was added. Next, H_2SO_4 (conc., 0.5 mL) was added, and the solution was stirred overnight at room temperature. DCM (50 mL) was added to the reaction mixture to facilitate phase separation. The organic phase was then extracted with aqueous K_2CO_3 (10 w%, 3 x 50 mL) and the aqueous phase was washed with DCM (3 x 50 mL). The combined organic phases were dried over Na_2SO_4. The solvent was evaporated under reduced pressure to yield the side product **4** as a yellow oil. To the aqueous phase, HCl (conc., 40-50 mL) was added until the pH value was 2. The aqueous phase was then extracted with DCM (3 x 100 mL). The organic phases were combined and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the product was dried at high vacuum overnight. The product was obtained as a colorless solid.

*4-Methyl itaconate **3a***

^{1}H-NMR (250 MHz, $CDCl_3$, δ): 6.50 (s, 1H), 5.88 (s, 1H), 3.74 (s, 3H, CH_3), 3.39 ppm (s, 2H, CH_2); ^{13}C NMR (63 MHz, $CDCl_3$, δ): 171.97 (C=O), 171.51 (C=O), 133.60 (s, C=C), 131.33 (s, C=C), 52.56 (C-O), 37.43 ppm (C3); MS (APCI) *m/z*: $[M + H]^+$ calcd. for $C_6H_8O_4$, 143.13; found, 143.03.

*4-Ethyl itaconate **3b***

^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.49 (s, 1H), 5.86 (s, 1H), 4.20 (td, J = 7.10 Hz, 2H, CH_2), 3.37 (s, 2H, CH_2), 1.29 ppm (t, J = 7.11 Hz, 3H, CH_3); ^{13}C NMR (63 MHz, $CDCl_3$, δ): 172.03 (s, C=O), 171.07 (C=O), 133.76 (C=C), 131.10 (C=C), 61.44 (C-O), 37.70 (C3), 14.43 ppm (s, C6); MS (APCI) *m/z*: $[M + H]^+$ calcd. for $C_7H_{10}O_4$, 157.15; found, 157.05.

*4-Propyl itaconate **3c***

^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.49 (s, 1H), 5.87 (s, 1H), 4.10 (t, J = 6.71 Hz, 2H, CH_2), 3.38 (s, 2H, CH_2), 1.68 (tq, J = 7.10 Hz, 2H, CH_2), 0.96 ppm (t, J = 7.42 Hz, 3H, CH_3);

^{13}C NMR (63 MHz, $CDCl_3$, δ): 171.98 (C=O), 171.14 (C=O), 133.81 (C=C), 130.99 (C=C), 67.04 (C-O), 37.71 (C3), 22.24 (C6), 10.63 ppm (C7). MS (APCI) *m/z*: $[M + H]^+$ calcd for $C_8H_{12}O_4$, 171.18; found, 171.06. Anal. calcd. for $C_8H_{12}O_4$: C 55.81, H 7.02; found: C 55.47, H 6.58.

4-Butyl itaconate ***3d***

^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.48 (1H), 5.86 (1H), 4.14 (t, *J* = 6.56 Hz, 2H, CH_2), 3.37 (s, 2H, CH_2), 1.54 - 1.76 (m, 2H, CH_2), 1.40 (tq, *J* = 7.30 Hz, 2H, CH_2), 0.95 ppm (t, *J* = 7.27 Hz, 3H, CH_3); ^{13}C NMR (63 MHz, $CDCl_3$, δ): 172.05 (C=O), 171.10 (C=O), 133.81 (C=C), 130.98 (C=C), 65.30 (C5), 37.71 (C3), 30.90 (C6), 19.40 (C7), 13.97 ppm (C8); MS (APCI) *m/z*: $[M + H]^+$ calcd. for $C_9H_{14}O_4$, 185.21; found, 185.08.

4-Pentylitaconate ***3e***

^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.49 (1H), 5.86 (1H), 4.13 (t, *J* = 6.56 Hz, 2H, CH_2), 3.37 (s, 2H, CH_2), 1.60 - 1.71 (m, 2H, CH_2), 1.28 – 1.37 (m, 4H, CH_2, CH_2), 0.92 ppm (t, *J* = 7.27 Hz, 3H, CH_3); ^{13}C NMR (63 MHz, $CDCl_3$, δ): 170.60 (C=O), 171.13 (C=O), 133.84 (C=C), 130.71 (C=C), 65.48 (C5), 37.61 (C3), 28.49 (C6), 28.27 (C7), 22.55 (C8), 14.14 ppm (C9); MS (APCI) *m/z*: $[M + H]^+$ calcd. for $C_{10}H_{16}O_4$, 200.23; found, 200.11.

Synthesis of 1-(*N*-Boc-2'-aminoethyl) 4-alkyl diitaconate **6a** to **6e**

The reaction was performed under nitrogen atmosphere. *N*-Boc-ethanolamine (1.3 g, 7.9 mmol, 1.2 eq) and DMAP (1.2 g, 9.9 mmol, 1.5 eq) were added to a solution of the appropriate 4-alkylitaconate **3a** to **3e** (6.6 mol, 1 eq) in DCM (10 mL). The solution was cooled with ice for 10 min, then DCC (1.5 g, 7.3 mmol, 1.5 eq) in DCM (10 mL) was added dropwise. The reaction vessel was stirred overnight at room temperature. The reaction

mixture was then filtered and washed with aqueous $KHSO_4$ (10 w%, 3 × 100 mL) and aqueous $NaHCO_3$ (saturated, 2 × 100 mL). The organic phase was dried over Na_2SO_4, and the solvent was removed under reduced pressure. The resulting product was diluted in DCM (5 mL) and stored in the freezer overnight to get rid of left-over urea side product, which precipitated. After the cold liquid phase was filtered to remove the precipitate, the solvent was evaporated under reduced pressure and the product was dried at high vacuum overnight.

1-(N-Boc-2'-aminoethyl) 4-methyl diitaconate ***6a***

^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.35 (s, 1H), 5.72 (s, 1H), 4.94 (br. s, 1H), 4.22 (t, J = 5.21 Hz, 2H, CH_2), 3.71 (s, 3H, CH_3), 3.41 (tq, J = 5.20 Hz, 2H, CH_2), 3.35 (s, 2H, CH_2), 1.44 ppm (s, 9H, $(CH_3)_3$); ^{13}C NMR (63 MHz, $CDCl_3$, δ): 171.68 (s, C=O), 166.23 (C=O), 156.15 (C=O), 133.92 (s, C=C), 129.47 (C=C), 79.83 (C-$(CH_3)_3$), 64.74 (C4'), 52.45 (C5), 39.92 (C5'), 37.96 (C3), 28.72 ppm (C-(CH$_3$)$_3$); MS (ESI) m/z: $[M + Na]^+$ calcd. for $C_{13}H_{21}NO_6$, 310.31; found 310.12.

1-(N-Boc-2'-aminoethyl) 4-ethyl diitaconate ***6b***

^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.39 (s, 1H), 5.75 (s, 1H), 4.92 (br. s., 1H), 4.26 (t, J = 5.30 Hz, 2H, CH_2), 4.20 (q, J = 7.20 Hz, 2H, CH_2), 3.46 (td, J = 5.30 Hz, 2H, CH_2), 3.37 (s, 2H, CH_2), 1.47 (s, 9H, $(CH_3)_3$), 1.30 ppm (t, J = 7.19 Hz, 3H, CH_3); ^{13}C NMR (63 MHz, $CDCl_3$, δ): 171.25 (C=O), 166.30 (C=O), 156.16 (C=O), 134.06 (C=C), 129.31 (C=C), 79.81 (C-$(CH_3)_3$), 64.76 (C4'), 61.37 (C5), 39.94 (C5'), 38.23 (C3), 28.73 (C (CH$_3$)$_3$), 14.51 ppm (C6); MS (ESI): m/z = $[M+Na]^+$ calcd. for $C_{14}H_{23}NO_6$, 324.34; found 324.14.

1-(N-Boc-2'-aminoethyl) 4-propyl diitaconate ***6c***

^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.38 (s, 1H), 5.75 (s, 1H), 4.92 (br. s., 1H), 4.25 (t, *J* = 5.20 Hz, 2H, CH_2), 4.10 (t, *J* = 6.70 Hz, 2H, CH_2), 3.46 (td, *J* = 5.20 Hz, 2H, CH_2), 3.39 (s, 2H, CH_2), 1.69 (tq, *J*=7.10 Hz, 2H, CH_2), 1.47 (s, 9H, $(CH_3)_3$), 0.96 ppm (t, *J* = 7.35 Hz, 3H, CH_3);

^{13}C NMR (63 MHz, $CDCl_3$, δ): 171.35 (C=O), 166.33 (C=O), 156.18 (C=O), 134.08 (C=C), 129.32 (C=C), 79.84 (*C*-$(CH_3)_3$), 66.99 (C5), 64.81 (C4'), 39.93 (C5'), 38.24 (C3), 28.73 (s, C-(*C*$H_3)_3$), 22.27 (C6), 10.69 ppm (C7); MS (APCI) *m/z*: $[M + H]^+$ calcd. for $C_{15}H_{25}NO_6$, 316.37; found, 316.17. Anal. calcd for $C_{15}H_{25}NO_6$: C 57.13, H 7.99, N 4.44; found: C 57.16, H 7.45, N 4.43.

1-(N-Boc-2'-aminoethyl) 4-butyl diitaconate ***6d***

^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.26 (s, 1H), 5.64 (s, 1H), 5.05 (br. s., 1H), 4.13 (t, *J* = 5.29 Hz, 2H, CH_2), 4.03 (t, *J* = 6.63 Hz, 2H, CH_2), 3.33 (td, *J* = 5.20 Hz, 2H, CH_2), 3.27 (s, 2H, CH_2), 1.45 - 1.62 (m, 2H, CH_2), 1.36 (s, 9H, $(CH_3)_3$), 1.19 - 1.34 (m, 2H, CH_2), 0.84 ppm (t, *J* = 7.20 Hz, 3H, CH_3); ^{13}C NMR (63 MHz, $CDCl_3$, δ): 171.20 (C=O), 166.24 (C=O), 156.15 (C=O), 134.08 (C=C), 129.09 (C=C), 79.62 (*C*-$(CH_3)_3$), 65.13 (C5), 64.65 (C4'), 39.84 (C5'), 38.10 (C3), 30.87 (C6), 28.65 (C-(*C*$H_3)_3$), 19.34 (C7), 13.93 ppm (C8); MS (ESI): *m/z* = $[M+Na]^+$ calcd. for $C_{16}H_{27}NO_6$, 352.39; found 352.17.

1-(N-Boc-2'-aminoethyl) 4-pentyl diitaconate ***6e***

^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.37 (s, 1H), 5.74 (s, 1H), 4.94 (br. s., 1H, NH), 4.24 (t, *J* = 5.29 Hz, 2H, CH_2), 4.12 (t, *J* = 6.63 Hz, 2H, CH_2), 3.46 (td, *J* = 5.20 Hz, 2H, CH_2), 3.37 (s, 2H, CH_2), 1.46 (s, 9H, $(CH_3)_3$), 1.28 - 1.36 (m, 6H, 3 × CH_2), 0.92 ppm (t, *J* = 7.20 Hz, 3H, CH_3); ^{13}C NMR (63 MHz, $CDCl_3$, δ): 171.34 (C=O), 166.33 (C=O), 156.18 (C=O), 134.08 (C=C), 129.29 (C=C), 79.89 (*C*-$(CH_3)_3$), 65.59 (C5), 64.83 (C4'), 38.25 (C5'), 35.30 (C3),

28.75 (C6), 28.36 (C-(CH_3)$_3$), 22.65 (C7), 14.68 (C8), 14.31 ppm (C9); MS (ESI): *m/z* = $[M+Na]^+$ calcd. for $C_{17}H_{29}NO_6$, 366.42; found 366.19.

Copolymerizations

Copolymerization of 1-(N-Boc-2'-aminoethyl) 4-alkyl diitaconates) **6a** to **6e** with DMAA at different ratios.

The itaconate monomers were copolymerized under nitrogen atmosphere. The different reagent amounts for the synthesis of each polymer with different comonomer ratios or different molecular weights are listed in Table S1. In a typical reaction, the diitaconate monomer **6**, e.g. **6c**, and DMMA were dissolved in DMSO to obtain a total monomer concentration of 0.5 g mL^{-1}. Next, the initiator AIBN was added. The reaction mixture was degassed by three freeze-pump-thaw cycles and placed into a pre-heated oil bath (bath temperature: 70°C) for 24 hours. The polymerized mixture was then precipitated into n-hexane (150 mL). The precipitate was re-dissolved in DCM (15 mL) and again precipitated into n-hexane (150 mL). This was repeated until no more monomer peaks were observed in the ^{1}H-NMR spectrum. The thus obtained copolymer **8** (**8c** in this case) was dried under high vacuum.

Poly(1-(N-Boc-2'-aminoethyl) 4-propyl diitaconate) ***8c*** *(50 mol%* ***6c****, 50 mol% DMAA):* ^{1}H NMR (250 MHz, $CDCl_3$, δ): 4.01 (br. s, 4H, CH_2, CH_2), 3.39 (br. s, 2H, CH_2), 3.08 (br. s, 2H, CH_2), 2.23 (br. s, 2H, CH_2), 2.90 (br. s, 3H, CH_2, CH), 1.70 (s, 2H, CH_2), 1.46 (s, 9H, (CH_3)$_3$), 0.95 ppm (br. s, 3H, CH_3).

For the other molar ratios, the chemical shifts of the peaks in the ^{1}H-NMR spectra were identical, only the peak ratios were different. The ^{1}H-NMR spectra of these polymers are shown in Figures S25 to S28 of the Supporting Information. The chemical shifts of the peaks

in the ^{1}H-NMR spectra of copolymer **8c** with different molecular weight were identical to copolymer **8c** which is shown in Figure S27. The Gel permeation chromatography elugrams for these polymers are summarized in Table 2 and shown in Figures S30, S33).

Deprotection of the Copolymers

The removal of the *tert*-Butyloxycarbonyl protecting group was carried out under nitrogen atmosphere. The respective polymer was diluted in MeOH (anhydrous, 3 mL). Then HCl (4M in dioxane, 3 mL) was added. The solution was stirred overnight, and the solvent was removed under reduced pressure. The copolymer **8** was dissolved in DCM (10 mL) and repeatedly precipitated into *n*-hexane (100 mL). The copolymer was then dried in high vacuum overnight to yield the deprotected copolymers **9a-d**.

^{1}H-NMR (250 MHz, $CDCl_3$, δ): 8.63 (br. s., 1 H), 4.01 (br. s., 1 H), 2.92 (br. s., 7 H), 2.20 (s, 1 H), 1.99 (br. s., 4 H), 1.66 (br. s., 3 H), 1.28 (s, 2 H), 0.95 ppm (br. s., 3 H). The ^{1}H-NMR spectra of the deprotected polymers **9a-d** with 50 mol% diitaconate are shown in Figure S29 of the Supporting Information. For the other molar ratios, the position of the peaks in the ^{1}H-NMR spectra were identical, only the peak ratios differ. The chemical shifts of the peaks in the ^{1}H-NMR spectra of copolymer **8c** with different molecular weight were identical to copolymer **8c** which is shown in Figure S29.

Determination of the r-parameters

To determine the r-parameters, the itaconate monomers were copolymerized under nitrogen atmosphere in DMSO-d_6, so that the crude reaction mixture could be directly used to determine the monomer conversion by ^{1}H-NMR spectroscopy. DMF was added as a standard to determine the conversion of the comonomers at different time points. The different amounts of reagents used for these polymerizations can be found in Tables S2 and S3 in the

Supporting Information. For a typical copolymerization, all reagents were dissolved in DMSO-d_6 at a total monomer concentration of 4 mmol mL^{-1}, to which the initiator AIBN was added. The monomer ratio was varied from 10 mol% diitaconate to 90 mol% diitaconate. The reaction mixture was degassed by three freeze-pump-thaw cycles. 0.5 mL of the reaction mixture was then filled into the required number of NMR tubes, which had been kept under inert atmosphere (one tube per time point). One NMR spectrum of each sample was measured before heating. The samples were then immersed into a pre-heated oil bath at the required temperature. After the required reaction times, one tube per monomer composition was removed and the reaction was quenched by cooling and exposing the reaction mixture to atmospheric oxygen. After that, the NMR spectrum of the sample was measured. After 24 hours, the samples were precipitated first from DMSO, then from ethanol or acetone into n-hexane several times. The thus purified copolymer was analyzed by ^{1}H-NMR spectroscopy and gel permeation chromatography.

Minimum Inhibitory Concentration (MIC)

The MIC is defined as the lowest concentration of a substance that can fully inhibit the growth of bacteria under defined conditions. For practical reasons, we here report the MIC_{90}, i.e. the concentration at which 90% of bacterial growth was inhibited by the SMAMP polymers. This was tested the Gram-negative *E. coli* bacteria (ATCC25922) as reported previously.[12d] In short, an overnight culture of the bacteria in Mueller-Hinton broth medium (MHB) was prepared and adjusted to a bacterial cell density of 10^6 colony forming units (CFU) per mL. The respective volumes of each MHB bacterial culture were placed in a 96-well microtiter plate with a multi-channel pipette (Eppendorf, Wesseling-Berzdorf, Germany). The tested polymers were diluted with dimethyl sulfoxide (DMSO, Sigma, Steinheim,

Germany) to the desired concentration, and added to the appropriate wells. These were filled to a total volume 200 µL with MHB medium, so that a polymer concentration series from 400 µg mL^{-1} to 6.25 µg mL^{-1} in MHB was obtained. A negative control ("no growth" control, bacteria in MHB + isopropanol), a positive control ("growth control", bacteria with MHB medium only), and a black well were also prepared. The 96-well plates were incubated for 18 h at 37 ºC in an aerobic atmosphere with 5% CO_2 and no agitation. Afterwards, the optical density (OD) of each well at 595 nm was measured using a Tecan Infinite 200 plate-reader (Tecan, Crailsheim, Germany).[12d, 27] The results of the MIC assay (bacterial growth versus polymer concentration) are shown in Figure 5; the MIC_{90} values are listed in Table 2.

Hemolytic Assay (HC)

Human red blood cells (erythrocytes) were obtained from the full blood of volunteers who have given their informed written consent in accordance to the guidelines of the World Medical Association Declaration of Helsinki; this was approved by the institutional ethics committee (Ethik-Kommission der Albert-Ludwigs-Universität Freiburg, Germany, vote number 381/15). In the hemolysis assay, lysis of human red blood cells caused by various concentrations of the test polymer is measured to determine the hemolytic concentration HC_{50}, where 50% of the blood cells are lysed by the polymer. The haemolysis experiments were performed as described by Mowery et al. with minor modifications.[12d, 28] In short, human erythrocytes (RBCs) were isolated from fresh blood to obtain a solution of 2% v/v RBCs in Tris-buffered saline (TBS). SMAMP solutions DMSO (80 mg mL^{-1}) were diluted with TBS in a 96-well plate to obtain a concentration series of 8000 µg mL^{-1} to 40 µg mL^{-1} SMAMP in DMSO/TSB. Melittin was used as a positive control (100% hemolysis), and 50 µL TBS as control (blank). 50 µL of the 2% RBC solution were added to each well containing 50 µL polymer solution or control. As another positive control, TritonX-100, another known hemolytic agent, was used.[29] The plate was incubated for 1 hour at 37°C, and the supernatant

was pipetted into a new 96-well plate to measure its optical density (OD) at 414 nm on a Tecan Infinite 200 plate-reader (Tecan, Crailsheim, Germany). The relative percentage of RBC lysis was plotted vs. concentration, as shown in Figure 5. The HC_{50} values are listed in Table 2.

Acknowledgement

Funding of this work by the German Research Foundation (Emmy-Noether-Program, LI1714/5-1) and the European Research Council (ERC-StG REGENERATE) is gratefully acknowledged. Diana Lorena Guevara-Solarte and Prof. Dr. Ali Al-Ahmad, University of Freiburg Medical Center, Department of Operative Dentistry and Periodontology, are gratefully acknowledged for their help with the antimicrobial assays.

References.

[1] a) W. H. O. (WHO) in *Report on global surveillance of antimicrobial resistance*, *Vol. 2014* World Health Organization (WHO), **2014**; b) J. O'Neill in *The Review on Antimicrobial Resistance - Tackling Drug-resistant Infections Globally: Final Report and Recommendations*, *Vol.* **2016**.
[2] J. O'Neill in *Antimicrobial resistance: tackling a crisis for the health and wealth of nations.*, *Vol.* **2014**.
[3] a) E.-R. Kenawy, S. D. Worley and R. Broughton, *Biomacromolecules* **2007**, *8*, 1359-1384; b) E. K. Riga, M. Vöhringer, V. T. Widyaya and K. Lienkamp, *Macromolecular Rapid Communications* **2017**, accepted; c) A. E. Madkour and G. N. Tew, *Polym. Int.* **2008**, *57*, 6-10; d) R. W. Scott, W. F. DeGrado and G. N. Tew, *Curr. Opin. Biotechnol.* **2008**, *19*, 620-627; e) A. Som, S. Vemparala, I. Ivanov and G. N. Tew, *Biopolymers* **2008**, *90*, 83-93; f) K. Lienkamp, A. E. Madkour, A. Musante, C. F. Nelson, K. Nusslein and G. N. Tew, *J. Am. Chem. Soc.* **2008**, *130*, 9836-9843; g) G. J. Gabriel, J. A. Maegerlein, C. F. Nelson, J. M. Dabkowski, T. Eren, K. Nusslein and G. N. Tew, *Chem. Eur. J.* **2009**, *15*, 433-439; h) K. Lienkamp and G. N. Tew, *Chem. Eur. J.* **2009**, *15*, 11784-11800; i) G. N. Tew, R. W. Scott, M. L. Klein and W. F. De Grado, *Acc. Chem. Res.* **2010**, *43*, 30-39; j) H. D. Thaker, F. Sgolastra, D. Clements, R. W. Scott and G. N. Tew, *J. Med. Chem.* **2011**, *54*, 2241-2254; k) K. Lienkamp, A. E. Madkour and G. N. Tew, *Adv. Polym. Sci.* **2013**, *251*, 141-172; l) F. Dorner and K. Lienkamp in *CHAPTER 5 Polymer-Based Synthetic Mimics of Antimicrobial Peptides (SMAMPs) - A New Class of Nature-Inspired Antimicrobial Agents with Low Bacterial Resistance Formation Potential*, *Vol.* The Royal Society of Chemistry, **2014**, pp. 97-138.
[4] a) M. Zasloff, *Nature* **2002**, *415*, 389-395; b) K. A. Brogden, M. Ackermann, P. B. McCray and B. F. Tack, *Int. J. Antimicrob. Agents* **2003**, *22*, 465-478.

[5] a) L. T. Nguyen, E. F. Haney and H. J. Vogel, *Trends in Biotechnology* **2011**, *29*, 464-472; b) N. J. Afacan, A. T. Y. Yeung, O. M. Pena and R. E. W. Hancock, *Current Pharmaceutical Design* **2012**, *18*, 807-819.
[6] K. A. Brogden, *Nature Reviews Microbiology* **2005**, *3*, 238-250.
[7] a) Y. Hamuro, J. P. Schneider and W. F. DeGrado, *Journal of the American Chemical Society* **1999**, *121*, 12200-12201; b) D. H. Liu and W. F. DeGrado, *Journal of the American Chemical Society* **2001**, *123*, 7553-7559; c) G. N. Tew, D. Liu, B. Chen, R. J. Doerksen, J. Kaplan, P. J. Carroll, M. L. Klein and W. F. DeGrado, *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 5110-5114.
[8] a) L. Timofeeva and N. Kleshcheva, *Appl. Microbiol. Biotechnol.* **2011**, *89*, 475-492; b) M. F. Ilker, K. Nusslein, G. N. Tew and E. B. Coughlin, *Journal of the American Chemical Society* **2004**, *126*, 15870-15875; c) B. P. Mowery, A. H. Lindner, B. Weisblum, S. S. Stahl and S. H. Gellman, *J. Am. Chem. Soc.* **2009**, *131*, 9735-9745; d) K. Lienkamp, A. E. Madkour, A. Musante, C. F. Nelson, K. Nüsslein and G. N. Tew, *Journal of the American Chemical Society* **2008**, *130*, 9836-9843.
[9] a) H. Tang, R. J. Doerksen, T. V. Jones, M. L. Klein and G. N. Tew, *Chemistry & Biology* **2006**, *13*, 427-435; b) H. D. Thaker, A. Som, F. Ayaz, D. Lui, W. Pan, R. W. Scott, J. Anguita and G. N. Tew, *J. Am. Chem. Soc.* **2012**, *134*, 11088-11091.
[10] a) K. Kuroda and W. F. DeGrado, *J. Am. Chem. Soc.* **2005**, *127*, 4128-4129; b) V. Sambhy, B. R. Peterson and A. Sen, *Angewandte Chemie* **2008**, *120*, 1270-1274; c) B. P. Mowery, S. E. Lee, D. A. Kissounko, R. F. Epand, R. M. Epand, B. Weisblum, S. S. Stahl and S. H. Gellman, *Journal of the American Chemical Society* **2007**, *129*, 15474; d) M.-R. Lee, S. S. Stahl, S. H. Gellman and K. S. Masters, *J. Am. Chem. Soc.* **2009**, *131*, 16779-16789.
[11] G. J. Gabriel, J. A. Maegerlein, C. F. Nelson, J. M. Dabkowski, T. Eren, K. Nüsslein and G. N. Tew, *Chemistry – A European Journal* **2009**, *15*, 433-439.
[12] a) K. Lienkamp and G. N. Tew, *Chemistry – A European Journal* **2009**, *15*, 11784-11800; b) A. E. Madkour, A. H. R. Koch, K. Lienkamp and G. N. Tew, *Macromolecules* **2010**, *43*, 4557-4561; c) K. Lienkamp, K.-N. Kumar, A. Som, K. Nuesslein and G. N. Tew, *Chem. Eur. J.* **2009**, *15*, 11710-11714, S11710/11711-S11710/11713; d) A. Al-Ahmad, D. Laird, P. Zou, P. Tomakidi, T. Steinberg and K. Lienkamp, *PLoS ONE* **2013**, *8*, e73812; e) A. Stulz, W. Römer, K. Lienkamp, H. Heerklotz and M. Hoernke, *Biophysical Journal* **2017**, *112*, 381a; f) A. Stulz, L. Akil, K. Lienkamp and M. Hoernke, *Biophysical Journal* **2018**, *114*, 377a.
[13] J. A. Love, J. P. Morgan, T. M. Trnka and R. H. Grubbs, *Angewandte Chemie International Edition* **2002**, *41*, 4035-4037.
[14] a) L. A. Paquette, J. D. Schloss, I. Efremov, F. Fabris, F. Gallou, J. Méndez-Andino and J. Yang, *Organic Letters* **2000**, *2*, 1259-1261; b) S. H. Hong and R. H. Grubbs, *Organic Letters* **2007**, *9*, 1955-1957; c) H. Wang, H. Matsuhashi, B. D. Doan, S. N. Goodman, X. Ouyang and W. M. Clark, *Tetrahedron* **2009**, *65*, 6291-6303.
[15] A. A. Nagarkar and A. F. M. Kilbinger, *Nat. Chem.* **2015**, *7*, 718-723.
[16] W. M. W. Y. Helia Hajian, *Current Research Journal of Biological Sciences* **2015**, *7*, 37-42.
[17] S. L. Tomić, J. M. Filipović, J. S. Velicković, L. Katsikas and I. G. Popović, *Macromolecular Chemistry and Physics* **1999**, *200*, 2421-2427.
[18] B. E. Tate, *Fortschr. Hochpolym.-Forsch.* **1967**, *5*, 214-232.
[19] a) Z. Szablan, A. A. Toy, T. P. Davis, X. Hao, M. H. Stenzel and C. Barner-Kowollik, *J. Polym. Sci., Part A Polym. Chem.* **2004**, *42*, 2432-2443; b) Z. Szablan, A. A. Toy, A. Terrenoire, T. P. Davis, M. H. Stenzel, A. H. E. Muller and C. Barner-Kowollik, *J. Polym. Sci., Part A Polym. Chem.* **2006**, *44*, 3692-3710; c) K. Satoh, D. H. Lee, K. Nagai and M. Kamigaito, *Macromolecular Rapid Communications* **2013**, *35*, 161-167.

[20] B. Tate in *Polymerization of itaconic acid and derivatives*, *Vol. 5/2* Springer Berlin Heidelberg, **1967**, pp. 214-232.
[21] a) W. Lang and Y. C. Lai in *Novel polymerizable surface active monomers with both fluorine-containing groups and hydrophilic groups*, *Vol.* Google Patents, **2009**; b) T. Otsu, K. Yamagishi, A. Matsumoto, M. Yoshioka and H. Watanabe, *Macromolecules* **1993**, *26*, 3026-3029.
[22] a) C. K. Pandiyarajan, O. Prucker, B. Zieger and J. Ruhe, *Macromol. Biosci.* **2013**, *13*, 873-884; b) A. Worz, B. Berchtold, K. Moosmann, O. Prucker and J. Ruhe, *Journal of Materials Chemistry* **2012**, *22*, 19547-19561.
[23] a) A. R. Mahdavian, M. Abdollahi, L. Mokhtabad, H. Reza Bijanzadeh and F. Ziaee, *Journal of Applied Polymer Science* **2006**, *101*, 2062-2069; b) C. Barner-Kowollik, J. P. A. Heuts and T. P. Davis, *Journal of Polymer Science Part A: Polymer Chemistry* **2001**, *39*, 656-664.
[24] a) T. Kelen and F. TÜds, *Journal of Macromolecular Science: Part A - Chemistry* **1975**, *9*, 1-27; b) M. Fineman and S. D. Ross, *Journal of Polymer Science* **1950**, *5*, 259-262.
[25] D. Braun and T.-O. Ahn, *Kolloid-Zeitschrift und Zeitschrift für Polymere* **1963**, *188*, 1-4.
[26] K. Kuroda, G. A. Caputo and W. F. DeGrado, *Chem. Eur. J.* **2009**, *15*, 1123-1133.
[27] B. E. Kirsop and A. Doyle, *Maintenance of microorganisms and cultured cells : a manual of laboratory methods*, Academic Press, London; San Diego, **1991**, p.
[28] B. P. Mowery, A. H. Lindner, B. Weisblum, S. S. Stahl and S. H. Gellman, *J. Am. Chem. Soc.* **2009**, *131*, 9735-9745.
[29] G. E. Deibler, M. S. Holmes, P. L. Campbell and J. Gans, *Journal of Applied Physiology* **1959**, *14*, 133.