Mimicking Charged Host-Defence Peptides to Tune Antifungal Activity and Biocompatibility of Amphiphilic Polymers

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Abstract

Invasive fungal infections impose a substantial global health burden. They cause more than 1.5 million deaths annually and are insufficiently met by the currently approved antifungal drugs. Antifungal peptides are a promising alternative to existing antifungal drugs, however, they can be challenging to synthesise, and are often susceptible to proteases *in vivo*. Synthetic polymers which mimic the properties of natural antifungal peptides can circumvent these limitations. In this study, we developed a library of 29 amphiphilic polyacrylamides with different charged units, namely amines, guanidinium, imidazole, and carboxylic acid groups – representative of the natural amino acids lysine, arginine, histidine, and glutamic acid. Ternary polymers incorporating primary ammonium (lysine-like) or imidazole (histidine-like) groups demonstrated superior activity against *Candida albicans* and biocompatibility with mammalian cells compared to the polymers containing the other charged groups. Furthermore, a combination of primary ammonium, imidazole, and guanidinium (arginine-like) within the same polymer outperformed the antifungal drug amphotericin B in terms of therapeutic index and exhibited fast *C. albicans*-killing activity. The most promising polymer compositions showed synergistic effects in combination with caspofungin and fluconazole against *C. albicans* and additionally demonstrated activity against other clinically relevant fungi. Collectively, these results indicate the strong potential of these easily producible polymers to be used as antifungals.

Introduction

Invasive fungal infections cause more than 1.5 million deaths annually.[1] People most at-risk of these infections are those with underlying health problems or a weakened immune system.[1, 2] As access to modern medical advancements (like organ transplantation or immunotherapies) increases, at-risk populations continue to expand and new vulnerable clusters are identified, for example hospitalised patients undergoing treatment against COVID-19 during the recent pandemic.[1-3] Bloodstream infections by *Candida* species (called candidaemia) are the leading cause of invasive fungal infections in humans, with approximately 400,000 cases globally per year;[1, 4, 5] *Candida albicans* is the species responsible for causing over 40% of these cases.[1, 5] The mortality rates associated with systemic *Candida* infections exceed 40% even with antifungal treatment.[1, 6] Consequently, the World Health Organization (WHO) classified *C. albicans,* together with *Candida auris*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, as fungal pathogens requiring critical and most urgent attention.[7]

Only three out of the four classes of antifungal drugs approved for use in the clinic are effective against candidaemia.[2, 8] These are polyenes, such as amphotericin B (AmpB), echinocandins, like caspofungin, and azoles, such as fluconazole.[2, 8] The use of these antifungal drugs is complicated by off-target toxicity, as in the case of polyenes, and unfavourable drug-drug interactions, as with the application of azoles.[2, 8] Moreover, azoles and echinocandins are susceptible to increasing rates of antifungal resistance, which hampers the clinical use of these drugs.[2, 8, 9]

The development of new antifungal therapeutics is impeded by the biological similarity of fungi and humans, which are both eukaryotes, limiting unique fungal targets for new drugs.[2, 10] In the last 20 years, no new classes of antifungal drugs have been approved for use against candidaemia, and those undergoing clinical trials mostly belong to existing classes.[2, 8, 11] It is therefore likely that similar issues relating to the off-target toxicity and emergence of resistance will arise, highlighting the need for the development of novel antifungal formulations.[2, 8, 10, 11]

The potential of natural compounds as antifungals is known. For example, polyenes and echinocandins were derived from naturally occurring structures, and natural products continue to be explored as new sources for

antifungal drugs. Antifungal peptides or host-defence peptides are an example of natural products that have aroused interest.[12, 13] Antifungal peptides are produced by almost all domains of life to prevent infection by fungi.[12, 13] However, their clinical use is limited due to their susceptibility to proteases once introduced to patients, and because they are challenging to synthesise.[12, 13] Nonetheless, a few short, synthetic antifungal peptides are under investigation or undergoing clinical trials (for example the cyclic polyarginine NP213),[14] mainly for topical application to treat superficial fungal infections.[12, 13]

To circumvent the aforementioned issues with antifungal peptides, controlled polymerisation techniques have been used to prepare synthetic macromolecules that mimic the overall properties of antifungal peptides.[15] As controlled polymerisation techniques are highly tolerant to different functional groups, they are especially well suited to mimic antifungal peptides, which contain a mixture of various charged and uncharged amino acids with defined chain lengths.[12, 13] Zhang and coworkers[16] showed that the combination of a positively charged ammonium group and hydrophobic groups in a synthetic polymer increased antifungal activity compared to the respective homopolymers.[16] Additional work has demonstrated that the incorporation of an uncharged functional group, such as hydroxyl groups, into amphiphilic polymers decreased the haemolytic activity and protein aggregation, in turn increasing bioavailability in antibacterial studies.[17] Recently, our group studied the influence of the hydrophobicity and molecular weight of ternary, amphiphilic polyacrylamides on their antifungal activity and biocompatibility.[18] With regard to the charged groups of synthetic polymers, a few studies have demonstrated the antifungal activity of different mainly positively charged groups, such as ammonium, guanidinium, and imidazole, which are inspired by amino acids commonly found in antifungal peptides such as LL-37 or histatin 5 (**Figure 1**).[12, 18-21, 22, 23]

Despite these previous works, to our knowledge, a comparison of the antifungal effects of different charged groups in the same amphiphilic polymer system has not been carried out; this was investigated in the present study using acrylamides. After assessing their activity against *C. albicans* and biocompatibility with red blood cells and murine embryonic fibroblasts, we found primary ammonium groups (lysine-like) to be optimal in our amphiphilic polymer library and also demonstrated that polymers containing guanidinium (arginine-like) or imidazole (histidine-like) had antifungal activity. Our most promising polymers were either composed of

primary ammonium or a combination of primary ammonium, guanidinium, and imidazole as charged moieties. These outperformed AmpB in terms of their therapeutic index and showed faster fungicidal activity against *C. albicans*. These most promising polymer compositions were also synergistic with caspofungin and fluconazole *in vitro*, and effectively inhibited the growth of other fungal species.

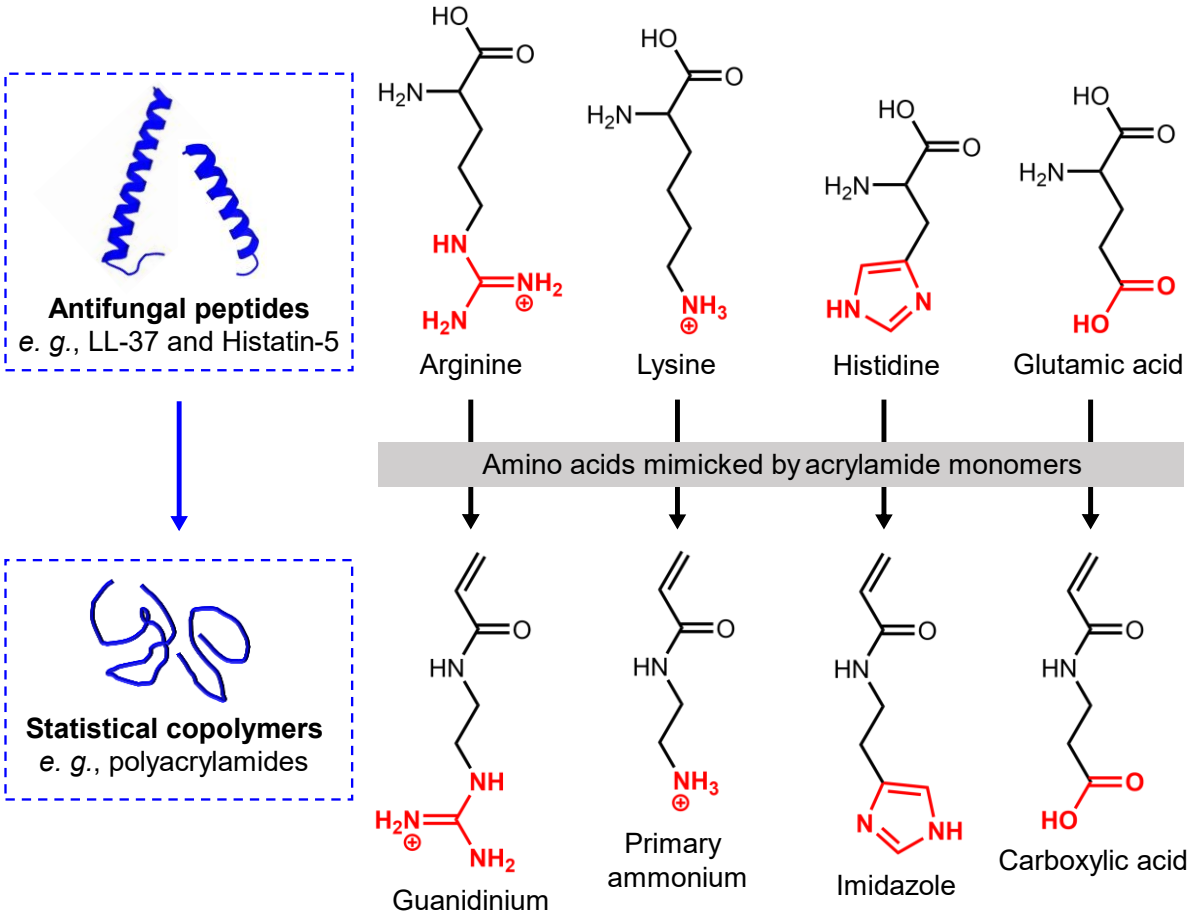

Figure 1. Synthetic acrylamide monomers employed in this study (bottom) mimic natural amino acids (top) commonly found in antifungal peptides. The left blue panels exemplarily represent the predicted structures of the antifungal peptides LL-37 and histatin-5 (predicted by PEP-FOLD 3)[24] and random coil structures of polymers with statistically distributed monomers.

Materials and methods

Materials

Ethylenediamine (Sigma-Aldrich, ≥99%), *N*-[2-(Dimethylamino)ethyl]acrylamide (ChemSupply, >98%), 1,3-Bis(*tert*-butoxycarbonyl)2-methyl-2-thiopseudourea (Sigma-Aldrich, >98%), 1-Ethyl-3-(3-

dimethylaminopropyl)carbodiimide (EDC) hydrochloride (AmBeed, 99%), *N*-heptylamine (Sigma-Aldrich, 99%), Di-tert-butyl dicarbonate (Sigma-Aldrich, 99%), histamine dihydrochloride (Sigma-Aldrich, 98%) iodomethane (Sigma-Aldrich, >99%), *N*-hydroxyethyl acrylamide (Sigma-Aldrich, 97%), beta-alanine *tert*-butylester hydrochloride (Combi-Blocks, 98%), triethylamine (TEA) (Scharlau, 99%), trifluoroacetic acid (TFA) (Sigma-Aldrich, 99%), chloroform (Merck), dichloromethane (DCM) (Merck), tetrahydrofuran (THF) (Merck), diethyl ether (Merck), ethyl acetate (Merck), hexane (Merck), dimethyl sulfoxide (DMSO) (Merck), acetone (ChemSupply), methanol (ChemSupply), isopropanol (ChemSupply), dimethylacetamide (DMAc) (Sigma-Aldrich), acrylic acid (Sigma-Aldrich), deuterated solvents for NMR (Cambridge Isotope Laboratories, Inc.), 2-(butylthiocarbonothioylthio)propanoic acid (BTPA, Boron Molecular), and 5,10,15,20-tetraphenyl-21H,23H-porphine zinc (ZnTPP) (Sigma-Aldrich) were used as received.

Monomer and polymer synthesis section

Monomer synthesis

tert-butyl (2-acrylamidoethyl)carbamate – Boc-primary amine

tert-Butyl (2-acrylamidoethyl)carbamate (Boc-AEAm) was prepared according to the previously reported procedure with slight modifications.[25] Ethylenediamine (0.25 mol) was dissolved in chloroform (250 mL) and cooled down on ice. Di-*tert*-butyl dicarbonate (0.025 mol) was dissolved in 50 mL of chloroform and was added dropwise to the ethylenediamine solution over 4 h on ice while stirring. The reaction was continued overnight at room temperature. After filtering the white precipitate, the organic phase was washed with 200 mL of Milli-Q water six times and then dried using $MgSO_4$. Solids were separated by filtration, and chloroform was evaporated, resulting in a pale-yellow oil product, which was used in the next step.

DCM (50 mL) was added to dissolve the obtained oil and 2 equiv TEA. EDC hydrochloride and acrylic acid (1.2 equiv each) were dissolved in ~15 mL DCM. The EDC/acrylic acid solution in DCM was added dropwise to the DCM solution containing the intermediate and TEA on ice while stirring. The reaction was continued at room temperature overnight. The crude product was concentrated *in vacuo* by rotary evaporation and

washed thrice against 0.1 M HCl, twice against saturated $NaHCO_3$, and brine. The organic phase was dried with $MgSO_4$ and filtered, and the remaining solvent was removed by rotary evaporation. The product was then further purified by eight repeated precipitations in hexane to yield the Boc-protected monomer as a fine white powder, which was dried *in vacuo*.

2-[1,3-Bis(tert-butoxycarbonyl)guanidine]ethyl acrylamide – Di-Boc guanidine

2-[1,3-Bis(*tert*-butoxycarbonyl)guanidine]ethyl acrylamide was prepared according to a previously reported procedure with slight modifications.[26] 1,3-Bis(*tert*-butoxycarbonyl)2-methyl-2-thiopseudourea (7 mmol) was dissolved in 14 mL DCM and added dropwise to 17.5 mmol ethylenediamine in 17.5 mL DCM on ice in a round-bottom glass flask while stirring. The solution was incubated for 4 h at room temperature while stirring. Afterwards, the DCM solution was washed 4 times against 40 mL Milli-Q water and twice against 40 mL brine. The organic DCM phase was dried over $MgSO_4$, solids were separated by filtration, and DCM was removed *in vacuo* with a rotary evaporator.

The crude white product was dissolved in 20 mL chloroform and 1.3 equiv of TEA (with respect to the initial precursor) were added in a round bottom glass flask equipped with a magnetic stir bar and placed in ice. EDC hydrochloride and acrylic acid (1.2 equiv each) were taken up in ~5 mL chloroform and added dropwise to the intermediate, crude product and TEA on ice while stirring. The reaction mixture was incubated over night at room temperature while stirring. The organic phase was washed thrice with 0.1 M HCl, thrice with saturated $NaHCO_3$, and twice with brine (~15 mL each). The organic phase was dried over $MgSO_4$, solids were separated by filtration, and chloroform was removed *in vacuo* with a rotary evaporator. The crude product was redissolved in a small volume of DCM and separated from side products by silica-flash chromatography (hexane/ethyl acetate gradient from 7/3 to 3/7). Fractions containing 1,3-Di-Boc-guanidinoethyl acrylamide (assured by thin layer chromatography) were combined and the solvent was evaporated *in vacuo* to yield a white salt.

Histamine acrylamide - imidazole

Histamine acrylamide was prepared following previously reported procedures with slight modifications.[27] Histamine dihydrochloride (30 mmol) and 4 equiv of NaOH were dissolved in Milli-Q water (2 mL/mmol) in a round bottom glass flask and cooled down on a salted ice bath. EDC hydrochloride and acrylic acid (1.3 equiv each) were dissolved in ~30 mL DCM, cooled down on ice, and added dropwise to the histamine/NaOH solution while stirring. The mixture was incubated for 30 min on ice and 4 h at room temperature. DCM was separated and subsequently evaporated by rotary evaporation, followed by freeze-drying to remove water. The resulting crude product was redissolved in 50 mL isopropanol and 1 equiv of TEA. The redissolved crude was passed through a silica column with isopropanol as an eluent. The fractions containing histamine acrylamide (assured by thin layer chromatography) were dried over $MgSO_4$, solids were filtered, followed by removal of isopropanol *in vacuo* to yield a white powder.

tert-butyl 3-acrylamidopropanoate – carboxylic acid monomer

tert-butyl 3-acrylamidopropanoate was prepared by EDC coupling. Beta-alanine *tert*-butylester hydrochloride (7 mmol) and 2 equiv of TEA were dissolved in ~14 mL DCM and cooled on ice. EDC hydrochloride and acrylic acid (1.2 equiv each) were dissolved in ~5 mL DCM and added dropwise to the amine while stirring on ice. The reaction was stirred over night at room temperature. The crude product was concentrated in DCM and washed thrice against 0.1 M HCl, thrice against saturated $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, the solids were filtrated, and the solvent was evaporated by rotary evaporation and *in vacuo*.

N-Heptyl acrylamide - hydrophobic monomer

A standard procedure, as previously reported [28], was employed for the synthesis of the hydrophobic monomer *N*-heptyl acrylamide with slight modifications. *N*-heptylamine (20 mmol) was dissolved in chloroform with a ratio of 5 mL of chloroform per 1 mmol amine and cooled on ice. EDC hydrochloride and acrylic acid (1.2 equiv each) were dissolved in ~20 mL chloroform and were then added dropwise to the amine-containing chloroform solution while stirring. The mixture was stirred over night at room temperature. The crude product

in chloroform was subsequently washed sequentially with 0.1 M HCl (thrice), saturated $NaHCO_3$ (thrice), water, and brine using half of the chloroform volume for each wash. The organic phase was dried with $MgSO_4$ and filtered to remove solids. Finally, the solvent was removed by rotary evaporation and dried *in vacuo* to yield the monomer.

Random copolymerisation by photoinduced electron/energy transfer-reversible addition-fragmentation chain transfer (PET-RAFT)

The linear, random copolymers were synthesised using a slight modification of the general one-pot protocol reported previously.[29] Briefly, stock solutions of the monomers were prepared with a concentration between 20% and 33% (w/w) in DMSO. ZnTPP was dissolved in DMSO at a concentration of 1 mg/mL. The RAFT agent BTPA was added to a 4 mL glass vial in an amount corresponding to the targeted X_n of 20 and dissolved in DMSO. Monomer stock solutions were added into the vial to a final monomer concentration of 25% (w/w) in DMSO, corresponding to the targeted ratios. A ZnTPP photocatalyst was added at 100 ppm relative to the monomers. The vial was sealed with a rubber septum, and the headspace was degassed with N_2 for 10 min. The vial was then placed under a green light-emitting diode light (λ = 530 nm) for 16-20 h to produce the copolymers. Finally, the copolymers were analysed with size-exclusion chromatography (SEC) and nuclear magnetic resonance (NMR) to examine the monomer conversion, polymer composition, and molecular weight distribution. Then, the polymer was purified by precipitating in a diethyl ether/hexane mixture (3:7), followed by centrifugation (9000 rpm for 5 min, 0 °C). The precipitate was dissolved in acetone or methanol and reprecipitated twice more. The polymer was then dried *in vacuo* prior to further post modification.

Polymer deprotection and post-polymerisation modification

TFA was used to remove Boc- and tert-butyl protecting groups based on our group's previously reported protocol.[29] Briefly, the polymer was dissolved in DCM [~7% (w/w) polymer], followed by the addition of TFA (20 mol equivalent with respect to Boc groups). The mixture was stirred at room temperature for 3 h and precipitated into diethyl ether. The precipitate was isolated by centrifugation, dissolved in acetone or methanol, and reprecipitated twice more. The polymer was then dried *in vacuo*, and ^{1}H NMR analysis was used to determine the removal of Boc-protective groups and to examine the targeted X_n.

To yield the quaternary ammonium from the tertiary amine functional group after polymerisation, the purified polymer was dissolved in acetone (~5% [w/w]) and 20 equiv iodomethane with respect to tertiary amine functional groups were added dropwise on ice while stirring. The reaction was continued for 6 h at room temperature, the product was concentrated in acetone, and precipitated three times in diethyl ether to yield the polymer with quaternary amine groups.

Polymer characterisation

^{1}H NMR spectra were obtained using a Bruker AVANCE III spectrometer (300 MHz, 5 mm BBFO probe) or a Bruker AVANCE III 400 spectrometer (400 MHz, 5 mm BBFO probe). Deuterated DMSO or deuterium oxide were used as solvents to determine the polymer composition and conversion at concentrations of ~10-20 mg/mL. All experiments were run with a gas flow across the probes at 535 L/h with sample spinning and at a temperature of 25 °C. All chemical shifts were stated in parts per million (ppm) relative to tetramethyl silane.

Size exclusion chromatography (SEC) was performed using a Shimadzu liquid chromatography system equipped with a Shimadzu refractive index detector and three MIX C columns operating at 50 °C. DMAc [containing 0.03% (w/v) LiBr and 0.05% (w/v) 2,6-dibutyl-4-methylphenol] was used as the eluent at a flow rate of 1 mL/min measuring filtered polymer samples in DMAc (3-4 mg/mL). The system was calibrated using poly(methyl methacrylate) (PMMA) standards with molecular weights from 200 to 10^6 g/mol.

Dynamic light scattering (DLS) and zeta-potential measurements were performed using a Malvern Zetasizer Nano ZS apparatus equipped with a He-Ne laser operated at λ = 633 nm and a scattering angle of 173°. All samples were prepared at a concentration ~1-2 mg/mL in Milli-Q water and filtered with a 400 nm filter before measurement. All Im-based polymers were prepared in acidic phosphate buffer (5.04 g/L disodium hydrogen phosphate, 3.01 g/L of potassium dihydrogen phosphate, pH adjusted to 4.5 with glacial acetic acid) due to their poor solubility in Milli-Q water. In addition to Milli-Q water, the polymers were also prepared equally in PBS and modified RPMI medium (pH 4.0 and 7.4) and analysed by DLS.

cLogP and cLogD calculations

To rationally design our polymer library based on hydrophobicity, the software Marvin (version 22.9, Chemaxon) was used. cLogP was calculated by drawing the respective structures and the Chemaxon cLogP calculation tool was used to receive the cLogP values (settings: Chemaxon method, considering tautomerisation and resonance, non-ionic species). The cLogD was equally calculated with the Chemaxon cLogD calculation tool (settings: Chemaxon method, considering tautomerisation and resonance) and reference pH was set to 4.0 and 7.4. Partition coefficient calculations were carried out considering the polymer structure including the end groups originating from the RAFT agent BTPA.

Biological experiments section

Media and buffers

Phosphate-buffered saline (pH 7.4)

Phosphate-buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer) was prepared by dissolving one PBS tablet (Oxoid) in 100 mL of Milli-Q water, which results in 1×PBS. Subsequently, the solution was autoclaved for sterilisation.

Yeast extract peptone dextrose medium

The yeast extract peptone dextrose (YEPD) [1% (w/v) yeast extract, 2% (w/v) mycological peptone, and 2% (w/v) D-glucose] broth was prepared by dissolving 4 g of yeast extract (Oxoid) and 8 g of mycological peptone (Oxoid) in Milli-Q water up to a total volume of 380 mL. After autoclaving, 20 mL of filter-sterilised 40% (w/v) D-glucose (ChemSupply) was added. For YEPD plates, 8 g of agar no. 2 (Oxoid) was added to the solution before autoclaving.

Sabouraud dextrose agar

Sabouraud dextrose agar (SDA) [1% (w/v) mycological peptone, 4% (w/v) D-glucose, and 2% (w/v) agar no. 2] plates were prepared by dissolving 4 g of mycological peptone and 8 g of agar no. 2 in 360 mL of Milli-Q water. The pH was adjusted to 5.6 ± 0.2. After autoclaving, 40 mL of filter-sterilised 40% (w/v) D-glucose (ChemSupply) was added before the plates were poured.

Modified Roswell Park Memorial Institute (RPMI)-1640 medium

To RPMI-1640 medium powder (with L-glutamine and phenol red, without bicarbonate, suitable to prepare 1 L; Sigma-Aldrich), 18 g D-glucose and 34.53 g 3-(*N*-morpholino)propane-1-sulfonic acid (MOPS; Sigma-Aldrich) were added and dissolved in 900 mL Milli-Q water. Afterward, the pH was adjusted with HCl and NaOH to 4.0 or 7.4, adjusted volume to 1 L, and filter sterilised. The sterile medium was stored at 4 °C.

Polymer and antifungal stock solutions

Polymer stock solutions were prepared at a concentration of 10 mg/mL in Milli-Q water or PBS and stored at 4 °C. Before use, the stock solutions were sonicated for ~3 min.

Antifungals were prepared at different stock solutions in sterile Milli-Q water, PBS or DMSO, as summarised in the following table (**Table 1**).

Table 1. Concentrations, medium and storage temperature of antifungal stocks.

Antifungal	**Stock concentration (mg/mL)**	**Medium**	**Storage temperature**
Amphotericin B (AmpB)	1 or 10	DMSO	4 °C
Caspofungin	1 or 10	Distilled water or PBS	-20 °C
Fluconazole	1 or 10	DMSO	4 °C

Culture conditions of fungal strains

The yeasts were routinely streaked on YEPD agar or SDA and incubated for 2 days at 30 °C. Cultures on agar plates were stored for up to two weeks at 4 °C. Overnight cultures were prepared by inoculating colonies from a plate in YEPD broth and shaking overnight at 30 °C at 200 rpm. Long-term stocks were stored at -80 °C in 50% (v/v) sterile glycerol from an overnight YEPD broth culture.

Fungal strains

For most assays, the *C. albicans* reference strain SC5314[30] was used, unless otherwise indicated. A complete list of used fungal strains is summarised in **Table 2**.

Table 2. Fungal strains used in this study.

Fungal species	**Strain name**	**Reference/Source**	**Notes**
Candida albicans	SC5314	[30]	Lab strain, wild type
	AM2003/0191	[31]	Clinical isolate – bloodstream infection
	b30708/5	[31]	Clinical isolate – bloodstream infection
	s20122.073	[31]	Clinical isolate – bloodstream infection
Candida glabrata (*Nakaseomyces glabrata*)	ATCC® 2001	*C. glabrata* (Anderson) Meyer et Yarrow	Lab strain, wild type
	SCSBB415201	[32]	Clinical isolate – bloodstream infection
Candida dubliniensis	SCS171549690	[32]	Clinical isolate – bloodstream infection
Candida parapsilosis	SCSXM67507	[32]	Clinical isolate – bloodstream infection
Candida tropicalis	SCS501148	[32]	Clinical isolate – bloodstream infection
Candida krusei (*Pichia kudriavzevii*)	AMR29-01	[33]	Clinical isolate – bloodstream infection

Cryptococcus neoformans var. *grubii*	ATCC® 208821	*C. neoformans var. grubii* Franzot et al.	Lab strain, wild type

Minimum inhibitory concentration (MIC) assay and evaluation of drug interactions

The MICs of polymers against different fungal strains (**Table 2**) were determined *via* the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines for fungal susceptibility testing, with slight modifications.[34, 35] Briefly, the fungal strains were grown on SDA for 48 h at 30 °C. One colony was emulsified in 1 mL of sterile Milli-Q water. Cells were counted using a haemocytometer, and the concentration of the cell suspension was adjusted to 5×10^6 cells/mL. The cell suspension was diluted 1:1000 in the modified Roswell Park Memorial Institute (RPMI)-1640 medium (supplemented with D-glucose and MOPS, pH adjusted to 4.0 or 7.4) to obtain the 2× stock suspension for the MIC assay. A two-fold dilution series of the 100 µL polymer solution in the respective modified RPMI-1640 medium was added into 96-well microplates (varying final concentrations between 0.5 and 1024 µg/mL), followed by the addition of 100 µL of cell culture (final cell concentration of $\sim2.5\times10^3$/mL). After resuspension of cells in the wells, the absorbance was measured at 405 nm with a microtiter plate reader after incubation for 24 h (pH 4.0) or 48 h (pH 7.4, *C. albicans* SC5314 only) at 35 °C in a humidified chamber (*C. glabrata* and *C. parapsilosis*: 48 h at pH 4.0; *C. neoformans*: 72 h at pH 4.0). Additionally, AmpB, fluconazole, and caspofungin were tested at varying concentrations between 0.016 and 128 µg/mL. No-polymer and no-cell controls were included in all experiments. The MIC value was defined as the lowest concentration of the respective polymer that showed growth inhibition of >90% compared to the untreated control. Three independent biological replicates were carried out.

To evaluate interactions of selected polymer compositions with the antifungal drugs AmpB, fluconazole and caspofungin, *C. albicans* SC5314 cells were treated and prepared as described above. The drug-dilution plates were prepared separately for each drug before combining them. For that, a four-fold dilution series of the respective antifungal drug was added into a 96-well microplate along the rows. The same was performed with the antifungal polymers, diluting it in a 96-well microplate along the columns. Then, 50 µL of antifungal drug

were combined with 50 µL of polymer, resulting in a two-fold dilution series of the respective compounds in the plate (as exemplarily shown in **Figure 2**). Thereby, one row and one column acted as controls only containing one compound. Afterward, 100 µL of *C. albicans* was added and incubated at MIC assay conditions. After 24 h, drug interactions were classified according to their fractional inhibitory concentration indexes (FICI). The FIC index was calculated as described in the following equation, where $c_{A/B}$ are the concentrations of compounds A or B, respectively, in combination resulting in growth inhibition >90%, and $MIC_{A/B}$ are the MICs of compound A or B, respectively, alone:

$$FICI = \frac{c_A}{MIC_A} + \frac{c_B}{MIC_B}$$

A combination was called synergistic if the FICI was below 0.5, antagonistic for a FICI above 4 and values in between as additive (between 0.5 and 1.0) or indifferent (between 1 and 4). Three independent biological replicates were carried out.

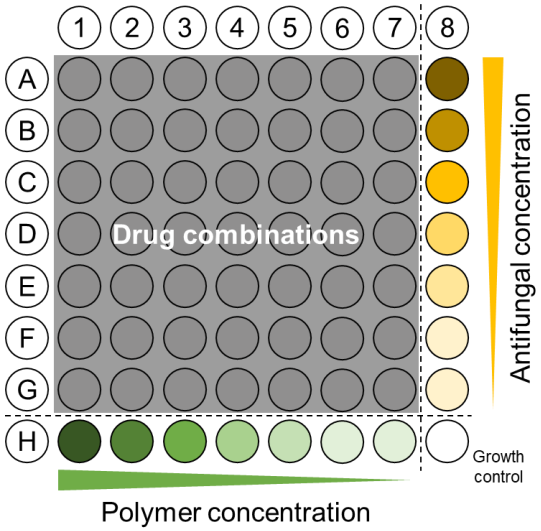

Figure 2. Exemplary plate layout for polymer (green) and antifungal (yellow) interaction studies.

Time-kill kinetic assay

To assess the activity of selected polymers against *C. albicans* SC5314 over time, a time-kill kinetic experiment was carried out at slightly modified MIC assay conditions. A *C. albicans* culture was grown in YEPD overnight at 30 °C and 200 rpm. The overnight culture was diluted 1:50 in YEPD and incubated for 4 h at 30 °C and 200 rpm to obtain a log-phase culture. Polymer and antifungal drug stocks were prepared at 4×MIC in modified RPMI-1640 (pH 4.0 or 7.4) and 100 µL of these 4× solutions were added to the wells of

a sterile, flat-bottom 96-well plate and preheated at 35 °C in a humidified chamber. An aliquot of the *C. albicans* log-phase culture was washed twice with PBS. Cells were counted with a haemocytometer and the cell suspension was adjusted to 4×10^5 cells/mL in preheated, modified RPMI-1640 (pH 4.0 or 7.4). The cell suspension (100 µL) was added to the preheated 96-well plate containing the antifungal polymers and drugs, reaching final concentrations of 2×10^5 cells/mL and 2×MIC of the respective antifungal compound. An inoculum control was serially diluted and plated on YEPD to assure cell viability. The 96-well plate was incubated over 4 h at 35 °C in a humidified chamber. After 0.25, 0.5, 1, 2, and 4 h, the cells in the wells were resuspended, serially diluted and 100 µL were plated on YEPD (30 °C, 48 h) to count colony forming units after treatment. The experiment was carried out in biological triplicates.

Haemolysis assay

The haemolytic activity of polymers was determined using fresh ovine red blood cells (RBCs) (Serum Australis), as previously reported.[36] The fresh, defibrinated ovine blood was diluted 1:20 in PBS (pH 7.4), pelleted by centrifugation (1000×g, 10 min), and washed three times in PBS. RBCs were then resuspended to a concentration of 5% (v/v) in PBS. Different concentrations of polymers (150 µL) were prepared in sterilised tubes, followed by the addition of the 5% (v/v) RBC suspension (150 µL). Polymer concentrations tested were 2, 1, 0.5, and 0.25 mg/mL. Additionally, the antifungal controls AmpB (8-128 µg/mL), fluconazole, and caspofungin (both 16-128 µg/mL) were tested for their haemolytic activity. The PBS buffer was used as a negative control, while Triton-X 100 [Sigma-Aldrich, 1% (v/v) in PBS] was used as a positive haemolysis control. Tubes were incubated at 37 °C for 2 h with shaking at 150 rpm. Samples were then centrifuged (1000×g, 8 min), and 100 µL aliquots of supernatants were transferred into a sterile 96-well microplate where absorbance values were read at 485 nm using a microtiter plate reader (FLUOstar Omega, BMG Labtech). The percentage of haemolysis of the polymer- or drug-treated samples was calculated by relating the absorbance values to the ones of the positive control (set to 100% haemolysis) and the negative control (set to 0% haemolysis). All experiments were performed in triplicate.

Cytotoxicity assay

The cytotoxicity of the polymers was tested using the alamarBlue assay (Thermo Fisher Scientific) as published previously.[18] The cytotoxicity was assessed using murine embryonic fibroblasts (MEFs) CF-1 (ATCC® SCRC-1040), kindly provided by the Cell Culture Facility of the Mark Wainwright Analytical Centre (UNSW). MEFs were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with L-glutamine (2 mM, Sigma-Aldrich) and foetal bovine serum [FBS, 10% (v/v), Sigma-Aldrich], until reaching sub-confluency at 37 °C and 5% CO_2 in a cell culture incubator (Eppendorf CellXpert C170i). MEFs were used at passage numbers below 25 and were tested for mycoplasma contamination regularly. For the cytotoxicity assay, MEFs were subcultured twice and diluted to a concentration of 5×10^4 cells/mL in DMEM (without FBS). The cell suspension (100 µL) was added to each well of a flat-bottom, cell-culture-grade 96-well plate. The plates were incubated for 16-20 h at 37 °C and 5% CO_2 to let the cells attach to the surface. A separate drug dilution plate was prepared containing polymers in DMEM (without serum) at concentrations of 8-1024 µg/mL, and antifungal drug controls (AmpB, fluconazole and caspofungin, 10 mg/mL stocks) in DMEM at concentrations of 2-256 µg/mL. The supernatant in the plate containing MEFs was aspirated, and 100 µL of solution of the preheated drug dilution plate was added. Subsequently, the plates were incubated for 24 h at 37 °C and 5% CO_2. According to the manufacturer's instructions, 10 µL of the alamarBlue assay reagent (Thermo Fisher Scientific) was added to each well and incubated for 4 h at 37 °C. The fluorescence was measured (excitation 550 nm and emission 590 nm) using a microtiter plate reader (CLARIOstar Plus, BMG Labtech). DMEM, DMEM plus cells, and DMEM with the respective tested compounds without cells were included as controls. The 50% inhibitory concentration was defined as the concentration above the one at which the fluorescent signal of the sample decreased below 50% compared to the untreated control. All experiments were performed in independent triplicates.

Results and discussion

Design, synthesis, and characterisation of polyacrylamides with different charged functionalities

To mimic the physicochemical properties of antifungal peptides, our group previously synthesised ternary acrylamide polymers with statistically distributed monomers using a photoinduced electron/energy transfer-reversible addition fragmentation chain transfer (PET-RAFT) polymerisation.[18] We established the effect of hydrophobic balance and molecular weight of these polymers on their antifungal activity and cytotoxicity.[18] Although positively charged moieties have demonstrated promising antifungal properties in different synthetic peptides and polymers,[14, 16, 19-21, 37, 38] studies comparing the individual groups within the same structural, amphiphilic polymer system have not been performed. Only Jiang *et al.*[19] reported that poly(2-oxazoline) featuring guanidinium pendent groups showed superior antifungal activity when compared to homopolymers featuring primary ammonium groups.[19]

In the present study, we investigated the influence of charged groups which mimic specific charged amino acids on the antifungal activity and biocompatibility of the resulting synthetic polymers. A ternary polyacrylamide structure (**Figure 3**) was selected to mimic the amphiphilic properties of antifungal peptides. The polyacrylamides were composed of charged groups (positive or negative); hydrophilic units (hydroxyethyl acrylamide (HEA)) – resembling uncharged hydrophilic amino acids; and hydrophobic heptyl groups (*N*-heptyl acrylamide) – mimicking hydrophobic amino acids.

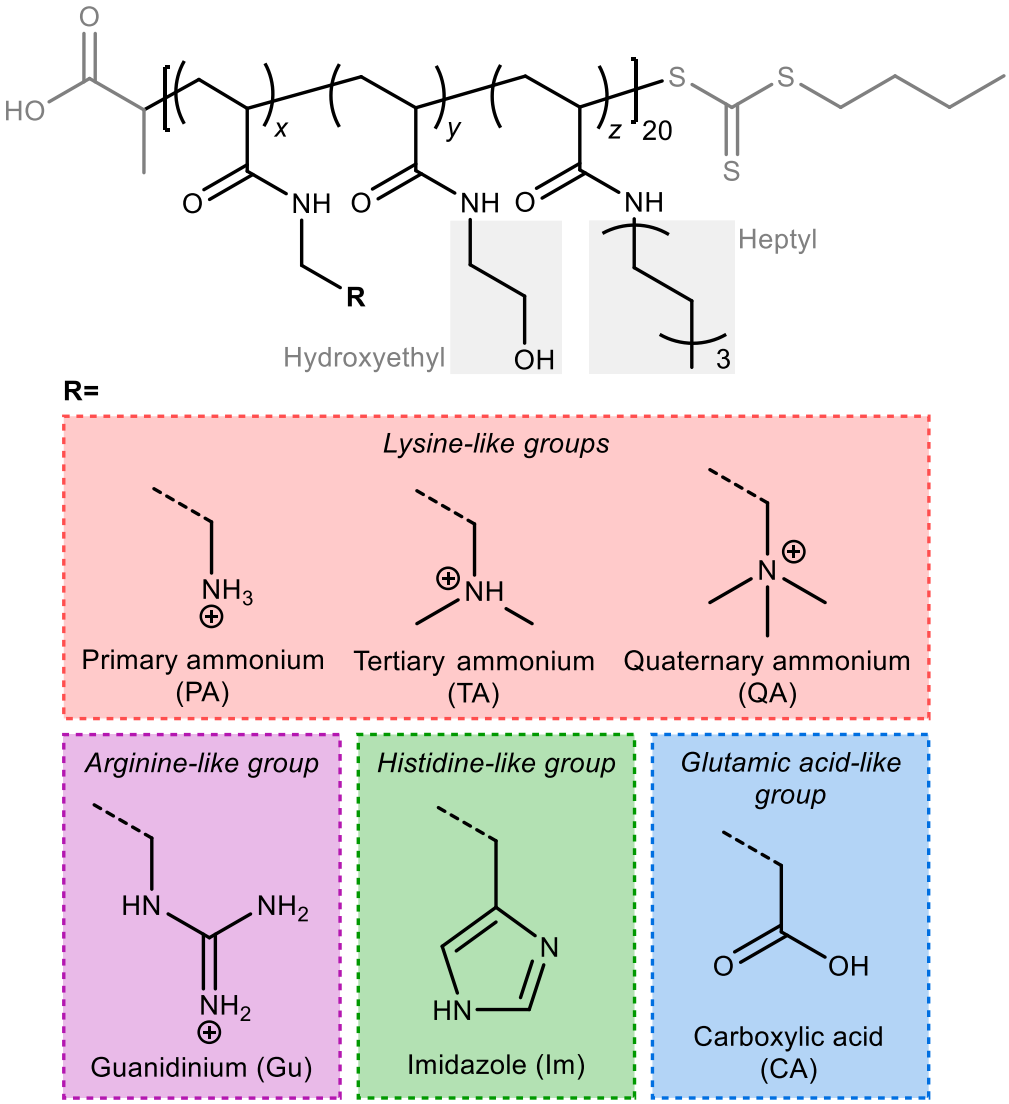

Figure 3. Chemical structures of the polyacrylamides synthesised in this study. The general ternary acrylamide structure is shown at the top. This is comprised of randomly distributed monomers (20 in total in the ratio *x*:*y*:*z*) with: charged groups (R, variants shown in different colours at the bottom); uncharged hydrophilic hydroxyethyl groups, and hydrophobic heptyl groups. The residual RAFT agent end groups are indicated in grey.

The positively or negatively charged acrylamide monomers which were used to synthesise the polymers in this study were inspired by charged amino acids commonly present in antifungal peptides (**Figure 1 and 3**).[12, 23] These included positively charged primary ammonium (PA), tertiary ammonium (TA), and quaternary ammonium (QA) groups (red in **Figure 3**), which mimic lysine; guanidinium (Gu, magenta in **Figure 3**), which mimics arginine; imidazole (Im, green in **Figure 3**) inspired by histidine; and the negatively charged

carboxylic acid (CA, blue in **Figure 3**), which mimics glutamic acid. We obtained monomers from commercial suppliers if available (*e. g.*, *N*-2-(dimethylamino)ethyl acrylamide (TA) and HEA), and synthesised those that were not. Other acrylamide monomers were synthesised by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-mediated coupling of acrylic acid and the respective precursor amine, where the functional groups were Boc-protected (PA, Gu) or *tert*-butyl protected (CA). Monomer synthesis and characterisation are accessible in the supplementary information (**supplementary Figures S1-8**). Boc- and *tert*-butyl protective groups were removed post-polymerisation by trifluoroacetic acid. Quaternisation of polymerised TA was performed with iodomethane post-polymerisation to obtain polymerised QA.

The polymers targeted in this work had a degree of polymerisation (X_n) of 20 and ratios of 50 mol% charged groups, 25 mol% hydroxyl groups, and 25 mol% hydrophobic heptyl groups (**supplementary Table S1**, highlighted in grey). As shown previously, this general polymer makeup showed good activity against *C. albicans* and biocompatibility with mammalian cells, when using PA as positively charged groups.[18] Therefore, this polymer was used as a starting point for the current work and is referred to as PA-50:25:25 – where the first two letters correspond to the type of charged group, and the numbers refer to the monomer ratios in mol% (charged:hydroxyl:heptyl).

We then used computationally derived estimates of hydrophobicity, *i. e.*, cLogD and cLogP, to guide the design of the next polymers. Previous work has shown that computational (logarithmic) partition coefficients (cLogP) could estimate hydrophilicity of synthetic polymers and correlate with their antimicrobial activity.[18, 28] The aim of the present study was to compare different charged groups in the same polymeric system. Because the cLogP does not account for different charges present in a molecule, we exploited the computational (logarithmic) distribution coefficient (cLogD), which allows an estimation of hydrophobicity of charged chemical species at a specific pH. Due to the conditions that would be used in subsequent experiments, we selected pH 4.0 and pH 7.4 as reference pH values for our cLogD calculations (**supplementary Table S2** (monomers) and **Table S3** (polymers)).

First, the cLogD of our previously reported polymer composition PA-50:25:25 was calculated at pH 4.0 (-29.7) and pH 7.4 (-18.7) as the targeted cLogD value for the polymers containing the other charged groups.

Based on these cLogD values of PA-50:25:25, the cLogD value of the other polymers not containing PA were adjusted by altering the monomer ratios (**supplementary Tables S1 and S3**, yellow shading for reference pH 4.0 and red shading for pH 7.4). Furthermore, a non-charged polymer composition only containing hydroxyethyl (75%) and heptyl (25%) was prepared as a control to check whether charge is required for antifungal activity (NC-00:75:25); alongside two variations of PA-50:25:25, which contained different ratios of PA (35% in PA-35:40:25 and 65% in PA-65:10:25) to investigate the optimal ratio of PA for antifungal activity and biocompatibility. For some polymers, the cLogD could not be adjusted to the value of the reference polymer PA-50:25:25. For those exceptions, compositions with a similar cLogP as PA-50:25:25 were designed (**supplementary Table S3**).

The characterisation of our polymers is summarised in **supplementary Table S3**. After PET-RAFT polymerisation for 16-20 h, the monomer conversions were determined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, confirming almost full conversion (α > 98%) for all prepared polymers, while number-average molecular weight (M_n) and dispersity ($Đ$) values of the polyacrylamides were measured by size exclusion chromatography (SEC) using poly(methyl methacrylate) (PMMA) standards and dimethyl acetamide (DMAc) as elution solvent (**supplementary Table S3**). After purification and, if necessary, post-modification, the success of post-modification and X_n were determined by ^{1}H NMR spectroscopy. The values obtained by ^{1}H NMR spectroscopy were generally in good agreement with the expected values (**supplementary Figures S9-S23**).

A discrepancy was observed between the experimental M_n (determined by SEC, ranging from 5,500 to 7,700 g/mol) and theoretical M values (before post-modification ranging from 2,800 to 5,400 g/mol, **supplementary Table S1**). This behaviour has been previously reported in our published ternary polymer libraries and was attributed to the differences in structure and hydrodynamic diameter between the PMMA standards and our amphiphilic polyacrylamides.[18] Apart from M_n, narrow and symmetrical molecular weight distributions were observed (**supplementary Figure S24**), also supported by a low $Đ$ (1.09-1.11, **supplementary Table S1**) for polymers belonging to the PA-, Gu-, and CA-families. Slightly higher $Đ$ values were observed for TA- and QA-containing polymers (1.23-1.37) which might result from additional electron

donation by the TA monomers and an increase in reaction rate during PET-RAFT polymerisation.[39] The Im-containing polyacrylamides were not analysed by SEC due to poor solubility in solvents such as DMAc and Milli-Q water.

The zeta potential (ζ) and hydrodynamic diameter (D_H) of the synthesised polymers dissolved in Milli-Q water was analysed after post-modification by dynamic light scattering (DLS) (**supplementary Table S3**). PA- and Gu-containing polymers showed high, positive ζ values (>+40 mV), resulting from their positively charged groups. TA-polymers showed neutral ζ values between -1 and +2 mV; after quaternisation with iodomethane the resulting QA-polymers showed higher ζ values between +27 and +44 mV, which supported successful quaternisation. Due to poor solubility, the Im-containing polymers were dissolved and measured in acidic phosphate buffer (pH 4.5) and consequently showed ζ values between +32 and +36 mV. CA-containing polymers showed more neutral ζ between -8 and +9 mV, whereas the non-charged control (NC-00:75:25) showed a negative ζ of -35 mV.

The PA-, TA-, QA-, Gu-, and Im-containing polymers displayed low D_H values of below 15 nm. An exception was Gu-30:40:30 for which potentially the higher content of HEA increased agglomeration resulting in a D_H of 49 nm. Additionally, a higher ratio of QA led to an increased D_H from 6.9 nm for QA-30:30:40 up to 41 nm for QA-50:25:25. The polymers containing CA and the non-charged control showed higher D_H values above 100 nm, indicating agglomeration potentially due to hydrogen-bonding.

Screening the initial set of ternary synthetic polymers for activity against *C. albicans* and biocompatibility

Activity against 'Candida albicans'

To investigate the antifungal activity of our library of ternary polymers, we performed a minimum inhibitory concentration (MIC) assay at pH 4.0 and pH 7.4 against the opportunistic fungal pathogen *C. albicans*. The MIC was determined photometrically after 24 h (pH 4.0) or 48 h (pH 7.4) (**Table 3**). Assays were carried out at pH 4.0 and pH 7.4 to investigate the influence of pH on the activity of our charged compounds. AmpB, caspofungin, and fluconazole, representatives of the three existing classes of antifungal drugs used in clinics

to treat systemic *Candida* infections (polyenes, echinocandins, and azoles, respectively), were included as quality controls and comparators for our polymers.

Table 3. Minimum concentrations of ternary polymers and antifungal drug controls required to inhibit *C. albicans* growth (MIC), induce haemolysis in red blood cells (HC), inhibit metabolic activity of murine fibroblasts (IC), and corresponding therapeutic index (taken as the ratio of IC and MIC (at pH 4.0)).

Compound	**$MIC_{90\%,24h}$ (pH 4.0, µg/mL)**	**$MIC_{90\%,48h}$ (pH 7.4, µg/mL)**	**$HC_{50\%,2h}$ (µg/mL)**	**$IC_{50\%,24h}$ (µg/mL)**	**Therapeutic index (IC_{MEF}/MIC_{pH4})**
PA-35:40:25	256-512	64-128	>2000	128	<1
PA-50:25:25	16-32	32	1000-2000	128	4-8
PA-65:10:25	16	16-32	500	64	2-4
TA-40:50:10	>512	>512	>2000	>1024	n.d.
TA-50:25:25	>512	>512	1000-2000	128-256	<1
TA-55:30:15	>512	>512	2000	256	<1
QA-30:30:40	>512	>512	1000-2000	64	<1
QA-40:25:35	>512	>512	1000-2000	64-128	<1
QA-50:25:25	>512	>512	1000-2000	128-256	<1
Gu-30:40:30[#]	512	>512	250	32-64	<1
Gu-35:35:30	256-512	512	250	32	<1
Gu-40:30:30[#]	64-128	64-128	250-500	32-64	<1
Gu-45:25:30[#]	32-64	32-64	250-500	32	<1
Gu-50:25:25	128-256	64	500-1000	32	<1
Gu-55:25:20	>512	>512	1000-2000	32	<1
Im-45:35:20	>512	>256	1000-2000	>1024	<1
Im-50:25:25	~512	>256	500	>1024	>2
Im-60:25:15[#]	256-512	>256	1000	>1024	>2

Im-65:20:15	256	>256	1000-2000	>1024	>4
Im-75:10:15[#]	128-256	>256	2000	>1024	>4
CA-30:50:20	~512	>512	1000-2000	512-1024	1-2
CA-35:25:40	~512	>512	1000-2000	128-256	<1
CA-50:25:25	~512	>512	1000-2000	512-1024	1-2
NC-00:75:25	>512	>512	2000	256	<1
AmpB	1-2	1-2	16	4	2-4
Fluconazole	0.25-0.5	0.25-0.5	>128	>32	>125
Caspofungin	0.125-0.25	0.125-0.25	~128	>32	>250

Note: MICs are defined as the minimum concentration required to inhibit >90% *C. albicans* growth after incubation at 35 °C for 24 h (pH 4.0) and 48 h (pH 7.4). The HC is defined as the minimum concentration at which >50% of lysed sheep red blood cells were observed after 2 h incubation at 37 °C. IC is defined as the concentration inhibiting >50% of metabolic activity (IC) of murine fibroblasts after 24 h, as determined by an alamarBlue assay. The therapeutic index was calculated for each polymer as the ratio of IC (MEFs) and MIC (*C. albicans*, pH 4.0). Colour-coding of polymers (left-hand column) represents their partition coefficient adjustment at either pH 4.0 (yellow) or pH 7.4 (red), while [#] symbolises additionally synthesised compositions. MICs, HCs, ICs and therapeutic indexes are color-coded from green (good performance) to red (poor performance).

Briefly, TA- or QA-containing polymers showed no activity against *C. albicans* at either pH value (MIC >512 µg/mL at both pH values). Similarly, polymers containing CA (mimicking glutamic acid) showed no activity at pH 7.4, and very minor *C. albicans* growth inhibition at pH 4.0 (MIC ~512 µg/mL). In contrast, polymers composed of PA (mimicking lysine) showed a PA-ratio dependent activity against *C. albicans*, with PA-65:10:25 displaying the lowest MIC (16 µg/mL at pH 4.0, 16-32 µg/mL at pH 7.4) in the entire library, while PA-35:40:25 showed less favourable antifungal activity (256-512 µg/mL at pH 4.0, 64-128 µg/mL at pH 7.4). Since polymers with Gu as a charged group (mimicking arginine) showed antifungal activity at a Gu-

ratio from 35-50%, additional polymer compositions were added to the library with a fixed amount of heptyl (30%) and a varied ratio of Gu and hydroxyl (Gu-30:40:30, Gu-40:30:30, Gu-45:25:30). The resulting composition with the highest inhibitory effect against *C. albicans* was Gu-45:25:30 with an MIC of 32-64 μg/mL at both pH. Polymers containing Im groups (mimicking histidine) showed activity against *C. albicans* depending on the ratio of Im. Increasing Im from 45% to 65% led to an increase in antifungal activity at pH 4.0 (MIC >512 μg/mL vs. 256 μg/mL). However, these polymers were not active at pH 7.4, potentially due to a decrease in charged groups in the Im-containing polymers at that pH since the pKa of imidazole is in the acidic range.[40] Nonetheless, two additional compositions (Im-60:25:15 and Im-75:10:15) were prepared to further investigate the influence of changing the ratio of Im. Polymer Im-75:10:15 demonstrated the lowest MIC (128-256 μg/mL at pH 4.0) among the Im-containing polymers.

In addition to absorbance-based MIC determination which measures fungal growth inhibition, we plated an aliquot of samples which did not show growth after each MIC assay on YEPD plates to assess whether the activity of our polymers and the antifungal drug controls was fungicidal, *i. e.*, resulted in cell death (**supplementary Table S4**). In agreement with previous studies,[18] the polymers belonging to the PA family showed fungicidal activity at concentrations ≥ 4×MIC at pH 4.0 and 7.4. Similarly, the most antifungal Gu-compositions (Gu-40:30:30, Gu-45:25:30 and Gu-50:25:25) showed fungicidal activity at concentrations above the MIC, independent of pH. The other polymer compositions did not show fungicidal activity and therefore only inhibit growth at concentrations up to 2×MIC (512 μg/mL), possibly because of their low antifungal activity (MICs of 256 μg/mL or higher) which precluded testing of concentrations higher than 2×MIC. As expected, the antifungal drug fluconazole showed fungistatic behaviour at pH 4.0 and 7.4 even at concentrations up to 8 μg/mL (>16×MIC). AmpB was fungicidal at concentrations above the MIC (2-4×MIC) at pH 4 and 7.4. Caspofungin was fungistatic at pH 4.0, but showed fungicidal activity at pH 7.4 at concentrations above 2-8×MIC.

Haemolysis and cytotoxicity

In addition to antifungal activity, biocompatibility with host cells is a key parameter for the clinical application of therapeutics. To test the biocompatibility of our ternary polymers with different charged groups, we

evaluated their capability of lysing sheep red blood cells (RBCs) by measuring the haemolytic concentration (HC) of polymer required to lyse >50% RBCs after 2 h in PBS (**Table 3**). Additionally, we also investigated the polymers' biocompatibility with viable mammalian cells, namely murine embryonic fibroblasts (MEFs), by measuring the inhibitory concentration (IC) of polymer required to decrease metabolic activity of MEFs by >50% after 24 h using the alamarBlue® assay (**Table 3**).

Polymers with the worst biocompatibility with MEFs in our library, *i. e.*, displaying low HC and IC values, belonged the Gu-family (arginine-like) with IC values of 32-64 μg/mL and consequently a low therapeutic index below 1. Gu-45:25:30, the most antifungal composition within the Gu-polymer family (MIC 32-64 μg/mL), was also relatively haemolytic with a HC of 250-500 μg/mL. The only acceptable composition regarding haemolysis was Gu-55:25:20 with a HC of 1000-2000 μg/mL, however, this polymer also showed no antifungal activity, with both of these effects possibly due to the low ratio of hydrophobic heptyl groups. For PA-containing polymers, an increased ratio of PA led to both increased haemolysis and cytotoxicity to MEFs. TA-, QA-, and CA-containing polymers were biocompatible with RBCs as they displayed HCs of 1000-2000 μg/mL or higher. However, the increase in hydrophobic group ratio also increased their toxicity to MEFs (for example, the IC of CA-50:25:25 was 512-1024 μg/mL, while for CA-35:2540 the IC was 128-256 μg/mL), supporting previous observations in ternary polymers with different hydrophobic groups and degree of hydrophobicity.[18] Im-containing polymers were the most compatible polymer compositions with MEFs, with no detected cytotoxicity for any of the tested compositions up to a concentration of 1024 μg/mL. Im-containing polymers with a lower ratio of hydrophobic groups and a higher ratio of Im also displayed favourable haemocompatibility properties (for example, the HC of Im-50:25:25 was 500 μg/mL vs. Im-75:10:15 which was 2000 μg/mL). As expected, the antifungal drug controls caspofungin and fluconazole displayed high biocompatibility, while AmpB was relatively haemolytic (HC 16 μg/mL) and cytotoxic (IC 4 μg/mL), which is in agreement with previous reports.[18]

To estimate *in vitro* selectivity of the polymers and antifungal drugs, the therapeutic index was calculated by taking the ratio of IC (MEFs) and MIC (*C. albicans*, pH 4.0), as described previously.[18] The therapeutic index of the approved antifungal AmpB is 2-4. A therapeutic index below 2 indicates poor selectivity either due to

poor antifungal activity or relatively high cytotoxicity to MEFs. Polymers from our library composed of TA, QA, CA, or Gu had a therapeutic index below 2. However, the polymers PA-50:25:25, Im-65:20:15, and Im-75:10:15 have a therapeutic index of >4, indicating that the concentration of polymer required to inhibit *C. albicans* is more than 4 times higher than the concentration of the polymer that results in more than 50% inhibition of MEF cells' metabolic activity. Therefore, PA-50:25:25, Im-65:20:15, and Im-75:10:15 outperform AmpB. Indeed, the clinical use of AmpB is limited due to its known toxic side effects for patients, especially nephrotoxicity.[2]

Considering all results together, PA-containing polymers remained the most promising structures due to their antifungal activity at pH 4.0 and 7.4, and relatively low cytotoxicity and haemolytic activity. Nonetheless, Gu is an interesting functional group since Gu-containing polymers were antifungal at pH 4.0 and pH 7.4 with the disadvantage of being cytotoxic at the same time, a known issue of Gu-containing polymers.[20] In contrast to the pH-independent bioactivity of PA- and Gu-containing polymers, Im-containing polymers were slightly active against fungal cells at pH 4.0, but not at pH 7.4 where they were also non-toxic for MEFs at concentrations up to 1024 µg/mL.

Overall, it became obvious that the cLogP and cLogD calculations did not provide an estimate of antifungal activity or biocompatibility of our new polymeric compounds. This can be attributed to the random nature of our compounds after polymerisation which is not considered in Marvin (Chemaxon). Furthermore, it is likely that different charged groups trigger slightly different modes of action and target different cellular features, making a prediction of their activity more difficult. Nonetheless, the partition coefficient calculations helped to rationally create an initial library, from which additional compositions were prepared if antifungal activity was observed (Gu- and Im-families). Additionally, the ζ potential of the ternary polyacrylamides was not predictive for their biological activity in our library, although an overall positive ζ generally seemed to be necessary to obtain antifungal activity. This indicates that the biological activity is governed by the physicochemical properties of the overall polymer (such as charge density), which are largely dictated by the ratio of hydrophilic to hydrophobic length and polymer X_n. Furthermore, a small D_H below 20 nm was

favourable for antifungal activity. This was expected because low-X_n polymers with a higher D_H indicate the formation of aggregates, resulting in lower bioavailability.

Combining charged groups with antifungal activity within one polymer

In the previous sections, we described the design, synthesis and activity of ternary polymers composed of a single type of charged group (PA or TA or QA (lysine-like), or Gu (arginine-like), or Im (histidine-like), or CA (glutamic acid-like), combined with uncharged hydrophilic groups (hydroxyethyl) and hydrophobic groups (heptyl). In most naturally occurring antifungal peptides, various positively charged groups are combined with other hydrophilic, uncharged and hydrophobic amino acids.[23] Additionally, altering charged groups in the antifungal peptide histatin 5 has been shown to affect antifungal activity.[41] Hence, we thought to combine our most active positively charged moieties (primary amine, guanidium, imidazole) in our ternary polyacrylamides (**Figure 4** and **Table 4**), with the intention of improving the biocompatibility and antifungal activity of our ternary polyacrylamides. We also combined PA and CA as charged units of a polymer to check if the negative charge of CA could improve the biocompatibility of PA-50:25:25, as the incorporation of carboxylic acid residues into a positively charged homopolymer has demonstrated potential to decrease host cell toxicity.[20]

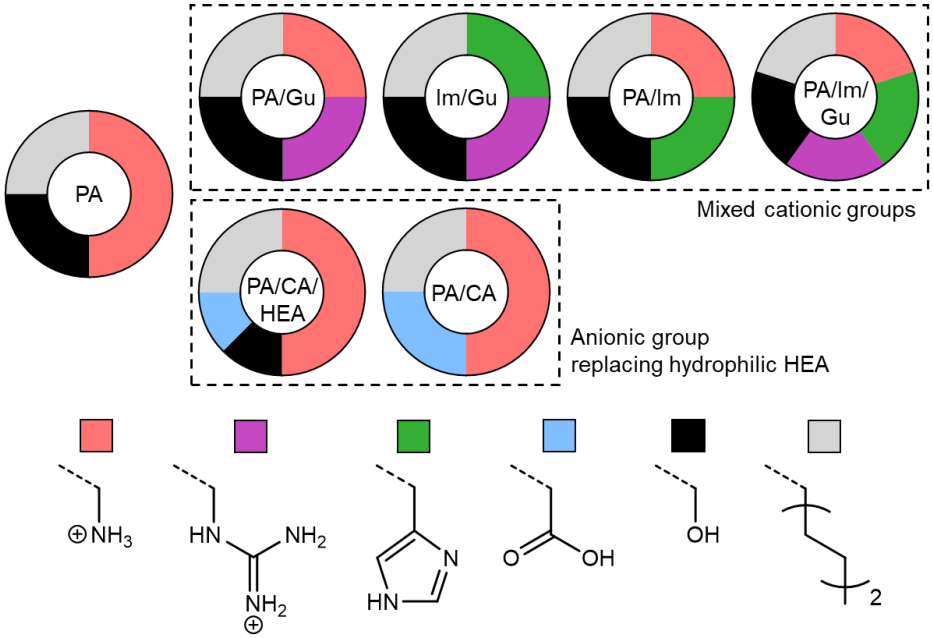

Figure 4. Composition of additional polyacrylamides with mixed charged functional groups in comparison to PA-50:25:25 (PA, left). Red – primary ammonium (PA, lysine-like); purple – guanidinium (Gu, arginine-

like); green – imidazole (Im, histidine-like); black – hydroxyl (HEA, hydrophilic unit); grey – heptyl (hydrophobic unit).

Table 4. Ternary polyacrylamides with a combination of charged monomers and analysis of their compositions (^{1}H NMR), theoretical molecular weight values (M_{theo}); number-average, experimental molecular weight values ($M_{n,\,exp}$, SEC); dispersity values ($Đ$, SEC); hydrodynamic diameters (D_H, DLS) and zeta potentials (ζ, DLS).

Polymer	**Polymer composition[a]**					X_n [b]	M_{theo} (g/mol)	$M_{n,\,exp.}$ (g/mol)[c]	$Đ$[c]	D_H (nm)	ζ (mV)
	% PA	**% Im**	**% Gu**	**% HEA**	**% Heptyl**						
PA/Gu	24.2	-	25.0	24.8	26.0	21.6	4,500	7,700	1.09	20.5	+37.5
Im/Gu	-	26.5	24.2	25.6	23.7	22.0	4,300	5,700	1.14	1.3	+54.1
PA/Im	25.8	24.9	-	24.4	24.9	19.4	3,600	6,100	1.14	1.3	+53.2
PA/Im/Gu	20.3	20.0	19.4	19.9	20.4	18.6	2,900	7,800	1.25	0.7	+35.4
	% PA	**% CA**		**% HEA**	**% Heptyl**						
PA/CA/HEA	51.0	12.3		12.6	24.1	18.8	4,400	6,500	1.11	1.5	+37.0
PA/CA	50.1	24.7		-	25.2	18.6	4,000	6,400	1.12	1.1	+31.0

Note: [a] determined by ^{1}H NMR spectroscopy at t_0; [b] determined by ^{1}H NMR spectroscopy based on final purified sample; [c] determined by DMAc-SEC, based on poly(methyl methacrylate) standards; X_n – degree of polymerisation

The additional polymers were synthesised as previously described, and their composition and characterisation are displayed in **Figure 4**, **Table 4**, and **supplementary Figure S25** (SEC traces). The targeted ratios for combining two different positively charged groups were adjusted to 25% each to yield 50% cationic group, plus 25% HEA and 25% heptyl. These polymers were called PA/Gu, Im/Gu, and PA/Im, referring to the respective monomer combination. When combining all three positively charged monomers (PA/Im/Gu), we targeted a ratio of 20% for each monomer (including HEA and heptyl) and this was approximately achieved.

When combining PA and CA, HEA was replaced entirely by CA (PA/CA) or combined with HEA at equal ratios (12.5% and 12.5%) such that the ratio of PA was still 50% and 25% for heptyl. The targeted X_n for all polymers was again 20. All polymers showed a positive ζ in Milli-Q water (**Table 4**) with the minimum for PA/CA (+32 mV) as expected, whereas maximum ζ values were observed for Im/Gu (+54 mV) and PA/Im (+53 mV). The highest D_H was observed for PA/Gu in Milli-Q water (21 nm). The other polymers displayed D_H values below 2 nm, indicating well dispersed polymers.

The new polymers with a combination of charged groups were then tested for their antifungal activity against *C. albicans* with an MIC assay at pH 4.0 and pH 7.4, haemolytic activity, and cytotoxicity against MEFs (**Table 5**). As in **Table 3**, the therapeutic index was calculated by using IC (MEFs) and MIC (*C. albicans* at pH 4.0).

Table 5. Minimum concentrations of polymers with mixed charged groups required to inhibit *C. albicans* growth (MIC), induce haemolysis in red blood cells (HC), inhibit metabolic activity of murine fibroblasts (IC), and corresponding therapeutic index.

Polymer	**$MIC_{90\%,24h}$ (pH 4.0, µg/mL)**	**$MIC_{90\%,48h}$ (pH 7.4, µg/mL)**	**$HC_{50\%,2h}$ (µg/mL)**	**$IC_{50\%,24h}$ (µg/mL)**	**Therapeutic index (IC_{MEF}/MIC_{pH4})**
PA-50:25:25	16-32	32	1000-2000	128	4-8
PA/CA	128-256	>512	>2000	256-512	1-4
PA/CA/HEA	64-128	512	1000-2000	128-256	1-4
PA/Gu	16-32	128-256	1000	64	2-4
Im/Gu	128	256-512	500	128	1
PA/Im	64	128-256	1000	128-256	2-4
PA/Im/Gu	16-32	64	1000-2000	128	4-8

Note: MICs are defined as the minimum concentration required to inhibit >90% *C. albicans* growth after incubation at 35 °C for 24 h (pH 4.0) and 48 h (pH 7.4). The HC is defined as the minimum concentration at which >50% of lysed sheep red blood cells were observed after 2 h incubation at 37 °C. IC is defined as the concentration inhibiting >50% of metabolic activity (IC) of murine fibroblasts after 24 h, as determined by an alamarBlue assay. The therapeutic index was calculated for each polymer as the ratio of IC (MEFs) and MIC

(*C. albicans*, pH 4.0). MICs, HCs, ICs and therapeutic indexes are color-coded from green (good performance) to red (poor performance).

Combining the positively charged PA with a carboxylic acid (CA) as a second hydrophilic moiety instead of HEA (PA/CA) led to a decrease in activity against *C. albicans* at both pH 4.0 (MIC 128-256 µg/mL) and pH 7.4 (MIC >512 µg/mL), when compared to PA-50:25:25. Similarly, when testing a polymer with 50% PA, 12.5% CA, 12.5% HEA, and 25% heptyl (PA/CA/HEA), the MIC against *C. albicans* was lower than PA-50:25:25 at pH 4.0 (64-128 µg/mL) and pH 7.4 (512 µg/mL). Hence, the positive charge in the ternary polymer composition PA-50:25:25 plays a key role in the antifungal activity, and it seems to be negated by CA in PA/CA and PA/CA/HEA (as also observed by a decreasing ζ). PA/CA outperformed PA-50:25:25 in terms of biocompatibility with RBCs and MEFs, while PA/CA/HEA was in between, which agrees with the results of a previous study incorporating negatively charged groups in a positively charged peptide.[20] Correspondingly, the therapeutic index of polymers containing PA and CA was not improved due to the substantially lower antifungal activity of CA-containing polymers compared to PA-50:25:25.

When PA was combined with Gu (PA/Gu), the antifungal activity decreased at pH 7.4 (MIC 128-256 µg/mL) and remained the same at pH 4.0, as compared to PA-50:25:25. Furthermore, the haemolytic activity (HC of 1000 µg/mL) and cytotoxicity to MEFs (IC of 64 µg/mL) increased. Therefore, PA/Gu is not as promising as PA-50:25:25. Issues with increasing toxicity to RBCs and MEFs *in vitro* has similarly been reported for a synthetic peptide when replacing lysine (mimicked here by PA) with arginine (mimicked here by Gu).[38] A combination of Im and Gu (Im/Gu) or PA and Im (PA/Im) led to an increase in MICs against *C. albicans* (or decrease in antifungal activity) and decreasing biocompatibility, resulting in a lower therapeutic index. Interestingly, when PA, Im, and Gu (20% each) were all combined in one ternary polymer (20% hydroxyethyl, 20% heptyl; PA/Im/Gu), similar MICs against *C. albicans* (16-32 µg/mL at pH 4.0, and 64 µg/mL at pH 7.4) were obtained, and biocompatibility was the same as for PA-50:25:25, resulting in the same therapeutic index. Moreover, PA/Im/Gu showed a slightly increased minimum fungicidal concentration at both pH 4.0 and pH 7.4 (**supplementary Table S5**).

In sum, combining different charged groups into our ternary polymer system did not outperform PA-50:25:25, which was solely composed of PA as the charged moiety. Only a combination of PA, Im, and Gu in polymer PA/Im/Gu achieved comparable antifungal activity (at pH 4.0) and biocompatibility compared to PA-50:25:25.

We subsequently selected the most antifungal compositions in the three families of our initial polymers (**Table 3**; PA-50:25:25, Gu-45:25:30, and Im-75:10:15) as well as our derivative with three cationic groups (PA/Im/Gu) to further investigate their *C. albicans* killing kinetics, interactions with selected antifungal drugs (AmpB, fluconazole, caspofungin), and activity against other clinically relevant fungi and clinical fungal isolates.

Fungal killing kinetics of most promising antifungal polymers

One way to classify antifungal compounds is by their killing behaviour over time. If an antifungal drug kills the fungus, it is classified as a fungicidal drug and this will be reflected in a reduction of viable cell counts, such as colony forming units (CFUs). A compound which only inhibits growth without reducing CFUs is called fungistatic. Both types of drugs are applied in clinic, however, fungicidal drugs which kill the fungus quickly are associated with improved clinical success due to lower likelihood of the emergence of resistant strains and potentially decreased period of treatment.[2]

To test for potential differences in the ability of our most fungicidal polymers (PA-50:25:25, Gu-45:25:30, Im-75:10:15, and PA/Gu/Im) to kill *C. albicans* over time, a time-kill-assay was performed. A log-phase *C. albicans* culture was diluted to 2×10^5/mL and incubated at 2×MIC of the respective polymer or antifungal drug for 4 h at pH 4.0 or 7.4. Samples were plated after 0.25, 0.5, 1, 2, and 4 h, and CFUs were counted (**Figure 6**).

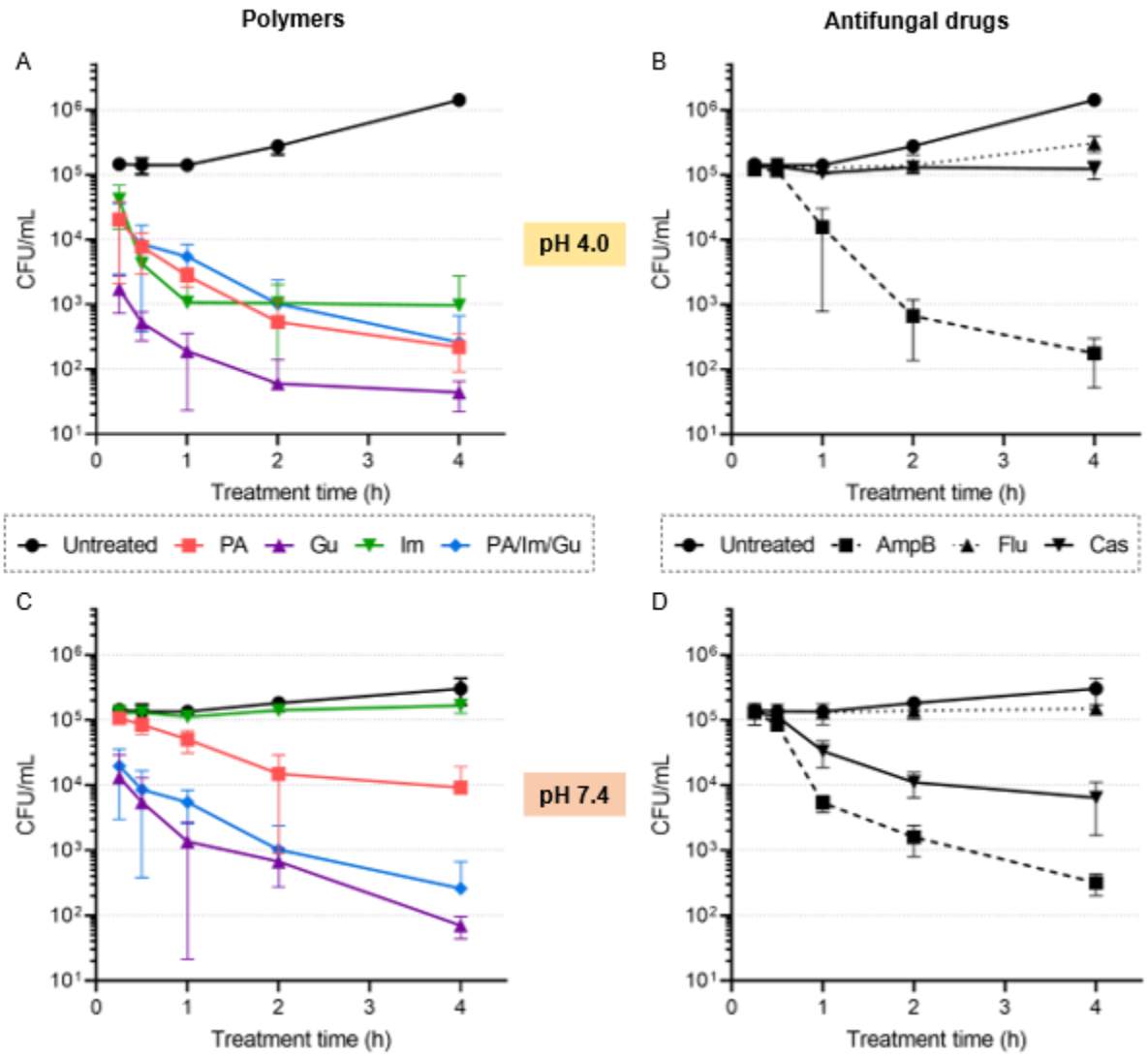

Figure 6. Time-kill behaviour of selected polymer compositions and antifungal drugs. Polymers PA-50:25:25 (PA, red), Gu-45:25:30 (Gu, purple), Im-75:10:15 (Im, green), and PA/Im/Gu (blue) were tested (A, C), alongside the antifungal drugs (B, D) amphotericin B (AmpB), fluconazole (Flu), and caspofungin (Cas) over 4 h at 35 °C at pH 4.0 (A, B) and pH 7.4 (C, D). Assays were carried out at 2×MIC of polymer or drug, except for Im which was assayed at 256 µg/mL at pH 7.4 (MIC>256 µg/mL) since it precipitated in the medium at higher concentrations. Graphs show the mean CFU/mL for n=3 replicates. Error bars represent the standard deviations.

At slightly acidic conditions (pH 4.0, **Figure 6A and B**), all selected polymers showed fungicidal activity. Gu-45:25:30 was the most fungicidal, killing faster and quantitatively most *C. albicans* cells (>99.9% after 4 h). PA and PA/Im/Gu showed similar killing behaviour over time (>99% killing after 4 h). Im-75:10:15 was

the least fungicidal overall (>90% after 4 h), but the dynamics of killing were similar to PA-50:25:25 and PA/Im/Gu up to the 1 h point. The untreated control showed growth after 2 h incubation. Out of the antifungal drug controls, only AmpB showed fungicidal activity at pH 4.0, killing >99% of *C. albicans* after 2 h. Caspofungin and fluconazole showed fungistatic behaviour at acidic pH.

Performing the same assay at pH 7.4 revealed some differences in the antifungal activity of the polymers and antifungal drugs (**Figure 6C and D**). Overall, the polymers killed *C. albicans* cells more slowly at pH 7.4 compared to pH 4.0. Gu-45:25:30 remained the fastest killing compound with >99.9% *C. albicans* killing after 4 h. PA/Im/Gu showed a very similar behaviour, however, killed slightly fewer *C. albicans* cells after 4 h (>99%). PA-50:25:25 was less fungicidal at pH 7.4 compared to pH 4.0, killing >90% *C. albicans* cells after 4 h (compared to >99% at pH 4.0). Im-75:10:15 precipitated at concentrations above 256 µg/mL at pH 7.4 and an MIC could not be determined (**Table 3**). Therefore, Im-75:10:15 was used for killing kinetic experiments at pH 7.4 at a concentration of 256 µg/mL. No fungicidal activity was observed for Im-75:10:15 at pH 7.4, most likely due to the deprotonation of imidazole at pH 7.4; the pKa of structurally similar histamine is around 6.0.[40] This result demonstrates the necessity of a positive charge for antifungal activity in our ternary polymer system. Regarding antifungal drug controls, AmpB showed fungicidal activity, like its behaviour at pH 4.0, and killed >99% of *C. albicans* cells after 4 h. Fluconazole demonstrated fungistatic properties (as at pH 4.0), while caspofungin killed >90% of *C. albicans* cells after 4 h which makes it a fungistatic compound at slightly acidic pH and moderately fungicidal at physiological pH.

Comparing the performance of polymers to the antifungal drugs, polymer PA-50:25:25, PA/Im/Gu, and Gu-45:25:30 outperformed AmpB in terms of the speed of *C. albicans* killing at pH 4.0 since the first fungicidal activity was observed after 0.25-0.5 h for the mentioned polymers, while killing by AmpB was observed after 1 h. This observation is in agreement with the previously performed killing kinetic assay in slightly acidic, diluted YEPD broth for similar ternary, PA-containing polymers in comparison to AmpB.[18] At physiological pH 7.4, PA-50:25:25 did not perform as well as at pH 4.0 and did not outperform AmpB, but instead was comparable to the moderate fungicidal activity of caspofungin. Gu and PA/Gu/Im still outperformed AmpB at pH 7.4 in terms of the speed of killing and showed similar fungicidal activity after 4 h. Altogether, these

results demonstrate the potential of the ternary polymers PA-50:25:25 and PA/Im/Gu since they outperform the currently applied antifungal drug AmpB in terms of their fungicidal kinetics at certain pH and are also more biocompatible.

The pH-dependence of the antifungal activity further highlights its importance in biological assays with respect to the intended final application. *C. albicans* can colonise and survive environments in the human body with ambient pH values ranging from acidic (*e. g.*, vagina or skin, pH 4 to 5),[42] to more neutral (*e. g.*, oral mucosa or gut with pH values between 6 and 7,[43] and the bloodstream, pH of 7.4[5, 44, 45]), and also alkalinises its microenvironment during infection.[45] Therefore, assaying antifungal activity at a wide range of pH values is desirable. For our polymers, that means that PA-50:25:25, Gu-45:25:30, and PA/Im/Gu could be suitable for application at acidic and neutral conditions. Notably, Im-75:10:15 would only be effective in acidic environments but also showed the best biocompatibility with MEFs (and RBCs), which makes Im a particularly interesting functional group for topical applications.

Assessment of polymer-drug interactions

In addition to fungicidal activity, the application of drug combinations in a synergistic manner is another option to increase effectiveness of the respective compounds and prevent rapid emergence of drug-tolerant or -resistant fungal strains, because ideally, multiple fungal targets can be hit simulataneously.[2, 10, 46] Hence, we tested the interactions between the most promising ternary polymers (PA-50:25:25, Gu-45:25:30, Im-75:10:15, and PA/Im/Gu) with three established antifungal drugs (AmpB, fluconazole, and caspofungin) which belong to three distinct antifungal drug classes. This was achieved by carrying out an *in vitro* checkerboard assay at MIC assay conditions (pH 4.0) and calculating the fractional inhibitory concentration index (FICI) for the respective drug-polymer combinations (**Figure 7**). The FICI relates the MIC of the drug when applied in combination to the MIC of the drug when used alone. In this study, we used the commonly accepted definition of FICI.[47] An FICI value below 0.5 indicates synergism, an FICI value between 0.5 and 1.0 indicates additive drug-drug interactions, 1.0 to 4.0 indicates indifferent drug-drug interactions, and an FICI value above 4 indicates antagonism. The individually measured MICs for the compounds alone and in combination are shown in **supplementary Table S6**.

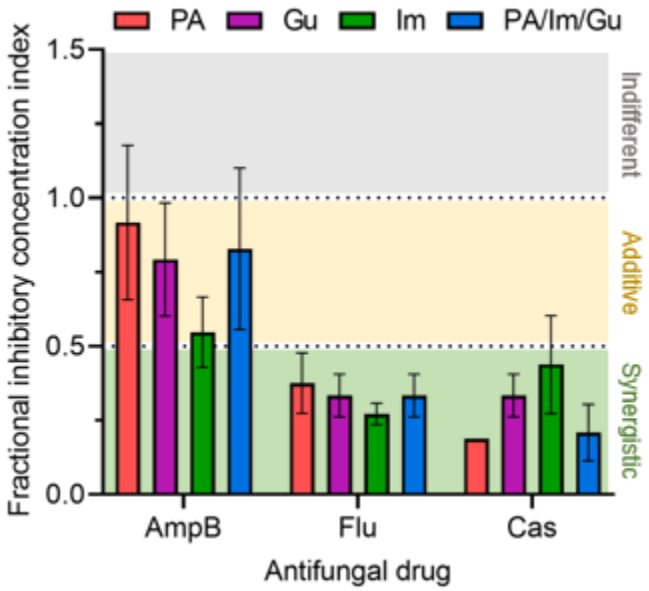

Figure 7. Fractional inhibitory concentration index (FICI) for drug-polymer combinations against *C. albicans* at MIC assay conditions (pH 4.0). Standard deviations are shown as error bars. Grey shading indicates indifferent drug interaction, yellow additive interaction, and green synergistic interaction. PA – PA-50:25:25 (red bars); Gu - Gu-45:25:30 (purple bars); Im – Im-75:10:15 (green bars); AmpB – amphotericin B; Flu – fluconazole; Cas – caspofungin.

As indicated by the FICIs, all tested polymers were synergistic in combination with fluconazole or caspofungin, and additive in combination with AmpB. This behaviour has been previously reported for PA-50:25:25.[48] Gu-45:25:30 and PA/Im/Gu showed very similar FICIs to PA-50:25:25. Therefore, the drug-interactions of the selected positively charged polymers appear to be independent of the specific charged moiety. Im-75:10:15 displayed slightly different FICIs compared to the other three positively charged polymers. This, together with its promising biocompatibility is worthy of further investigation. Overall, our results demonstrate that the positively charged group in our ternary, amphiphilic polyacrylamide library influences drug interactions, activity against *C. albicans* and biocompatibility. However, our results also highlight the potential for their application as antifungal compounds in combination with other approved antifungal drugs to increase efficiency and decrease the emergence of drug resistance, as demonstrated for PA-50:25:25.[48]

Activity against clinical isolates of *Candida* species and *Cryptococcus neoformans*

In addition to *C. albicans*, other commonly detected strains in candidaemia patients include *Candida glabrata* (now: *Nakaseomyces glabrata*), *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* (now: *Pichia kudriavzevii*) and *Candida dubliniensis*.[5] For this reason, we wanted to test if our most antifungal polymers were active against clinical isolates of these *Candida* spp. obtained from bloodstream infections (grey in **Table 6**). Additionally, we also tested a lab strain of *C. glabrata* (ATCC® 2001), and *Cryptococcus neoformans* (ATCC® 208821) which is another member of WHO's critical group of fungal priority pathogens.[7] We performed an MIC assay as described previously at pH 4.0, over 24 h for *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. dubliniensis* (**Table 6**). The incubation time for *C. glabrata* and *C. parapsilosis* was increased to 48 h since after 24 h no visual growth was detected for the untreated control and absorption measurements indicated virtually no growth. *C. neoformans* was incubated for 72 h before MIC determination. The MICs obtained for AmpB, fluconazole and caspofungin against our fungal strains were interpreted based on clinical breakpoints suggested by CLSI for the respective *Candida* species, excluding *C. dubliniensis* and *C. neoformans* for which breakpoints are not available.[34]

Table 6. Minimum inhibitory concentration (MIC) of selected polymers and antifungal drugs against *Candida* and non-*Candida* fungi. Clinical isolates are highlighted in grey, while no shading indicates lab strains.

Fungal species	**Strain**	**$MIC_{90\%,pH\ 4.0}$ (µg/mL)**						
		Polymers				**Antifungal drugs**		
		PA	Gu	Im	PA/Im/Gu	AmpB	Flu	Cas
Candida albicans	SC5314*	16-32	32-64	128-256	16-32	1-2	0.25-0.5	0.125-0.25
	AM2003/0191 (CI 1)	16	32	64-128	32-64	1-2	0.25-0.5	0.5
	b30708/5 (CI 2)	128-256	128	256	256	0.5-1	0.5	0.5
	s20122.073 (CI 3)	4-8	4	8	8	0.5-1	0.25	0.25-0.5
Candida parapsilosis	SCSXM67507	256	64-128	>512	512	1-2	2-4	0.5
Candida tropicalis	SCS501148	16	8-16	64	32	1	8-16	0.125
Candida dubliniensis	SCS171549690	8	8	16	8-16	0.5-1	1-2	0.5-1
Candida glabrata (*Nakaseomyces glabrata*)	ATCC® 2001	8-16	8-16	64-128	16-32	1-2	128	0.25-0.5
	SCSBB415201	>512	128-256	512	>512	1-2	2-4	1
Candida krusei (*Pichia kudriavzevii*)	AMR29-01	16-32	32	64-128	16-32	1	8-16	0.125
Cryptococcus neoformans var. *grubii*	ATCC® 208821	2	2	4	2-4	1	4-8	ND

Note: The MIC was determined at pH 4.0 and 35 °C after 24 h (*C. albicans*, *C. tropicalis*, *C. krusei*, *C. dubliniensis*), 48 h (*C. parapsilosis*, *C. glabrata*), or 72 h (*C. neoformans*). The MIC was defined as the minimal observed concentration to inhibit >90% fungal growth; Yellow and red shading indicates dose-dependent susceptibility and resistance, respectively, according to clinical breakpoints for antifungal drugs as endorsed by CLSI [34] (not available for *C. neoformans* and *C. dubliniensis* for the tested antifungals). Orange shading indicates increased tolerance of the respective fungal strain to the respective polymer resulting in an

MIC >512 µg/mL; CI – clinical isolate; PA – PA-50:25:25; Gu – Gu-45:25:30; Im – Im-75:10:15; AmpB – amphotericin B; Flu – fluconazole; Cas – caspofungin; ND – not determined. *Data for *C. albicans* SC5314 copied from **Table 3 and 5**.

The clinical isolates of *C. albicans* and *C. krusei* were susceptible to all tested antifungal drugs. Although the MIC of the *C. krusei* isolate appeared elevated (8-16 µg/mL) compared to *C. albicans* (0.25-0.5 µg/mL), *C. krusei* is known to be intrinsically resistant to fluconazole and CLSI guidelines suggest to not interpret MICs of fluconazole against this fungal strain.[4, 34]

The *C. parapsilosis* bloodstream isolate was found to be more tolerant to fluconazole (MIC 2-4 µg/mL) compared to *C. albicans* (MIC 0.25-0.5 µg/mL), but this was still considered to be clinically susceptible (dose-dependent).[34] The *C. parapsilosis* isolate was susceptible to caspofungin and AmpB, as usually reported for *C. parapsilosis* strains.[4]

The *C. tropicalis* bloodstream isolate, was susceptible to caspofungin and AmpB, in line with reports that most clinical isolates of *C. tropicalis* are drug-susceptible.[4] However, results obtained for the *C. tropicalis* isolate (MIC of 8-16 µg/mL) indicates that the clinical isolate is resistant to fluconazole.[34]

C. glabrata is known for its intrinsic fluconazole tolerance,[4] and this was the case for the lab strain we tested (ATCC® 2001, MIC 128 µg/mL). While the *C. glabrata* bloodstream isolate was more susceptible to fluconazole (MIC 2-4 µg/mL, indicating dose-dependent susceptibility) than the lab strain, an increased MIC to caspofungin was observed (bloodstream isolate: 1 µg/mL; lab strain: 0.25-0.5 µg/mL). Based on the CLSI breakpoints,[34] this indicates that the *C. glabrata* clinical isolate is resistant to caspofungin. AmpB was effective against both *C. glabrata* strains.

The lab strain of *C. neoformans* showed an MIC of 1 µg/mL and 4-8 µg/mL against AmpB and fluconazole, respectively, while caspofungin was not tested because echinocandins are not effective against *Cryptococcus* spp. *in vivo*.[49]

Altogether, the examined fungal isolates were susceptible to AmpB, however, some isolates showed increased tolerance or resistance to fluconazole (*C. glabrata*, C. *tropicalis* and *C. parapsilosis*). The *C. glabrata* clinical isolate was the only strain found to be caspofungin-resistant.

Next, we determined the MICs of the selected polymers (PA-50:25:25, Gu-45:25:30, Im-75:10:15, and PA/Im/Gu) against our clinical isolates of *Candida* spp. and the lab strains of *C. glabrata* and *C. neoformans*.

Polymer PA-50:25:25 was active against the clinical isolates of *C. albicans* (16 and 4-8 µg/mL for clinical isolate (CI) 1 and CI 3, respectively), *C. tropicalis* (MIC 16 µg/mL), *C. dubliniensis* (8 µg/mL), *C. krusei* (16-32 µg/mL), and a lab strain of *C. neoformans* (2 µg/mL). The clinical isolate of *C. parapsilosis* and *C. albicans* CI 2 showed elevated MICs of 256 and 128-256 µg/mL, respectively. Although PA-50:25:25 did not inhibit more than 90% growth of the clinical *C. glabrata* isolate after 48 h (MIC >512 µg/mL), it still inhibited 50% growth of that isolate as shown in **supplementary table S7** ($MIC_{50\%}$ 128 µg/mL). These results are consistent with those from our previous study which showed that PA-50:25:25 was active against *C. glabrata*, *C. krusei*, and a *C. neoformans* clinical isolate.[18] We have also shown PA-50:25:25 to be active against drug-resistant or -tolerant clinical *C. albicans* isolates.[48]

Polymer Gu-45:25:30 showed comparable MICs to treatment with PA-50:25:25 for all tested fungal isolates except for the *C. glabrata* clinical isolate which was susceptible at lower concentrations considering both $MIC_{90\%}$ (64-128 vs. >512 µg/mL, **Table 6**) and $MIC_{50\%}$ (32-64 vs. 128 µg/mL, **supplementary table S7**). Similarly, the polymers Im-75:10:15 and PA/Im/Gu displayed activity against the clinical isolates of *C. albicans*, *C. tropicalis*, *C. dubliniensis*, and *C. krusei*, and also against the lab strains of *C. glabrata* and *C. neoformans*. Only one *C. albicans* clinical isolate (CI 2), and the clinical isolates of *C. glabrata* and *C. parapsilosis* showed increased tolerance to Im and PA/Im/Gu with MICs of 256 µg/mL or above.

Overall, the ternary polymers remained active against clinical isolates and lab strains of *Candida* spp. and *C. neoformans*, demonstrating their promise as broad-spectrum antifungal agents. Although tolerance to some of our polymers was increased in *C. albicans* CI 2, *C. parapsilosis* and the *C. glabrata* clinical isolate, compared to the *C. albicans* lab strain, the antifungal activity of the polymers against clinical isolates of *C. albicans* (CI 1) and *C. tropicalis* was approximately equal to the *C. albicans* lab strain. We also saw better performance of our polymers against the *C. albicans* clinical isolate CI 3, *C. dubliniensis*, and *C. neoformans* which were more susceptible to our antifungal polymers than the *C. albicans* lab strain. The differences in antifungal activity of our polymers against isolates of the same fungal species with comparable susceptibilities to known antifungal drugs (*e. g.*, the set of *C. albicans* strains that were tested) demonstrates the importance of testing multiple fungal isolates to estimate the potential of novel antifungal compounds.

Testing the polymers for their antifungal activity against different species that are tolerant or resistant to antifungal drugs with known modes of action can indicate whether a novel formulation has the same or a different mode of action. The *C. glabrata* lab strain, *C. tropicalis*, and *C. krusei* displayed decreased susceptibilities to fluconazole (as expressed by higher MICs compared to *C. albicans*). Resistance or increased tolerance to fluconazole has been attributed to upregulation of drug efflux pumps, but also mutation and upregulation of the target (14α-demethylase, an enzyme involved in ergosterol biosynthesis).[4, 50] The activity of our ternary polyacrylamides against those three fungal species was not hampered. This indicates a mode of action of the polymers which is different from fluconazole and is not affected by potential drug-tolerance mechanisms affecting the efficacy of that antifungal drug. This has also been demonstrated previously for PA-50:25:25.[48]

The increased tolerance of the caspofungin-resistant *C. glabrata* isolate to our polymers indicates a potentially overlapping antifungal mechanism. However, the synergy experiment (**Figure 7**) suggests different modes of action because synergy with caspofungin was observed in combination with the polymers. The mode of action studies and activity of PA-50:25:25 against caspofungin-resistant strains in a previous study further supports different targets of caspofungin and the synthetic polymer.[48] Altogether, considering the haemolytic activity, toxicity towards MEFs and activity against various fungal species, PA-50:25:25, Im-75:10:15 and PA/Im/Gu displayed the most promising potential as antifungal lead compounds.

Conclusions

Novel alternatives for treating fungal infections are urgently required. One such alternative could be to mimic the properties of antimicrobial peptides synthetically to efficiently produce antifungal compounds. In the present study, we synthesised 29 amphiphilic polyacrylamides with a focus on different charged groups mimicking naturally occurring amino acids (lysine, arginine, histidine, glutamic acid), and screened them for their activity against the opportunistic fungal pathogen *C. albicans* and biocompatibility with RBCs and MEFs.

Briefly, an overall positive charge promoted activity against *C. albicans*, and host cell toxicity could be balanced by adjusting the ratio between charged, hydroxyl, and heptyl groups. Polymers containing a primary ammonium (mimicking lysine), imidazole (mimicking histidine), or a combination of the three most active functional groups (primary ammonium, imidazole, and guanidinium – arginine mimic) displayed the most promising antifungal properties and outperformed amphotericin B in terms of therapeutic index. In addition, these promising polymer compositions demonstrated a fast and pH-dependent *C. albicans* killing behaviour. Synergistic action with the well-established antifungal drugs fluconazole and caspofungin and antifungal activity against other clinically relevant and drug-resistant *Candida* species and *Cryptococcus neoformans* underline the significant potential of our synthetic, amphiphilic polymers as novel antifungal treatment options. Prospectively, the most promising functional groups (primary ammonium, guanidinium, imidazole) which mimic natural amino acids could be employed in follow-up studies about the effect of sequence-specific incorporation of these subunits or blocks in a polymer.

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Supporting Information

^{1}H NMR spectra of monomers, RAFT agent, selected polymers, SEC chromatograms of polymers, minimum fungicidal activity of polymers, MIC values for drug-polymer combinations (**Figures S1-S25** and **Table S1-S7**) are available in supporting information.

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