Supplementary information

Poly(*N*-Vinylpyrrolidone) anti-malaria conjugates of membrane disruptive peptides

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I. RAFT mediated polymerization of NVP and deprotection of the heterotelechelic system.

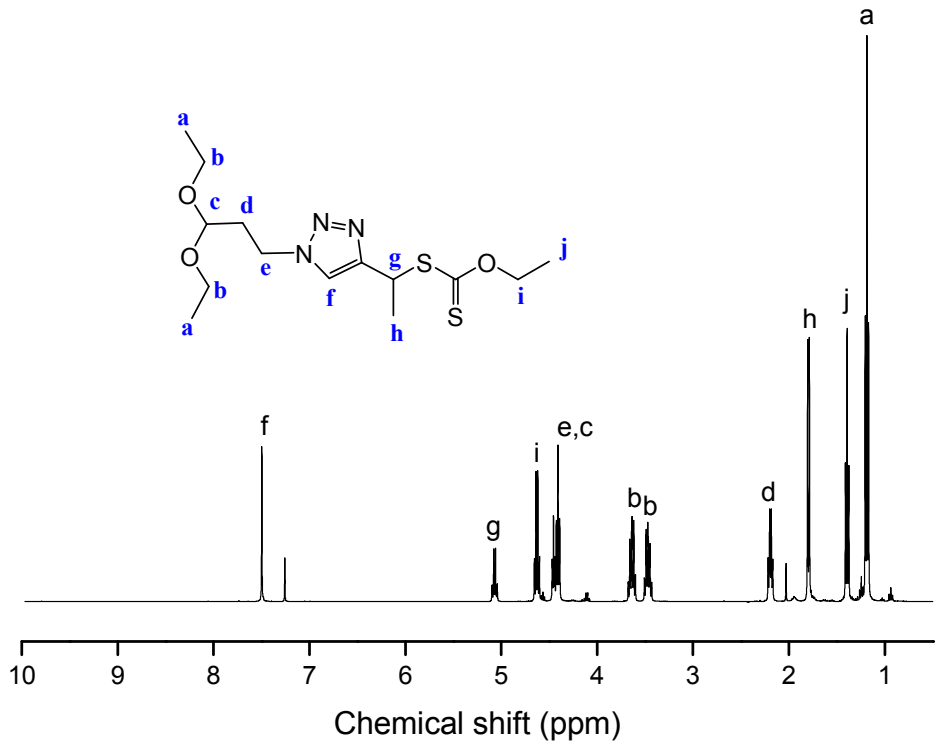


Figure S1: ^{1}H NMR spectrum of the acetal functional triazole based RAFT agent (XA1).

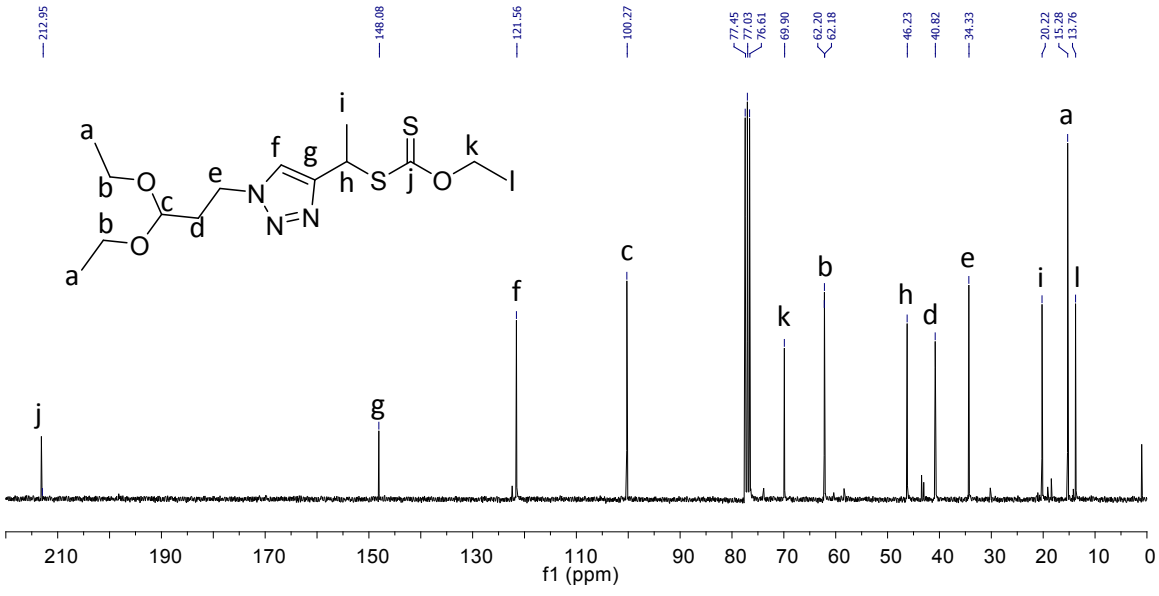


Figure S2: ^{13}C NMR spectrum of the acetal functional triazole based RAFT agent (XA1).

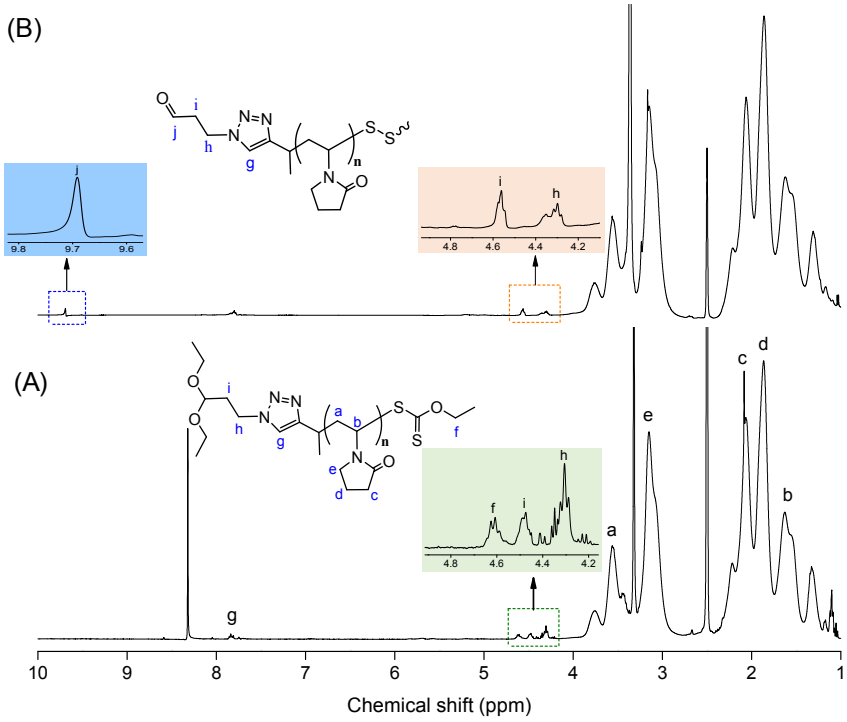


Figure S3: [1]H NMR spectra of (A) α-acetal, ω-xanthate heterotelechelic PVP and (B) α-aldehyde, ω-thiol heterotelechelic PVP.

Table S1: Relationship between molecular weight and dispersity before and after deprotection.

Polymer	M_n[a] (g·mol^{-1})	$Đ$[a]
PVP_{4K}	4000	1.29
PVP_{4K} deprotected	4900	1.44
PVP_{9K}	9300	1.24
PVP_{9K} deprotected	11400	1.33

[a]Molar mass and dispersity determined by SEC analysis in DMF + LiBr (PMMA as standards).

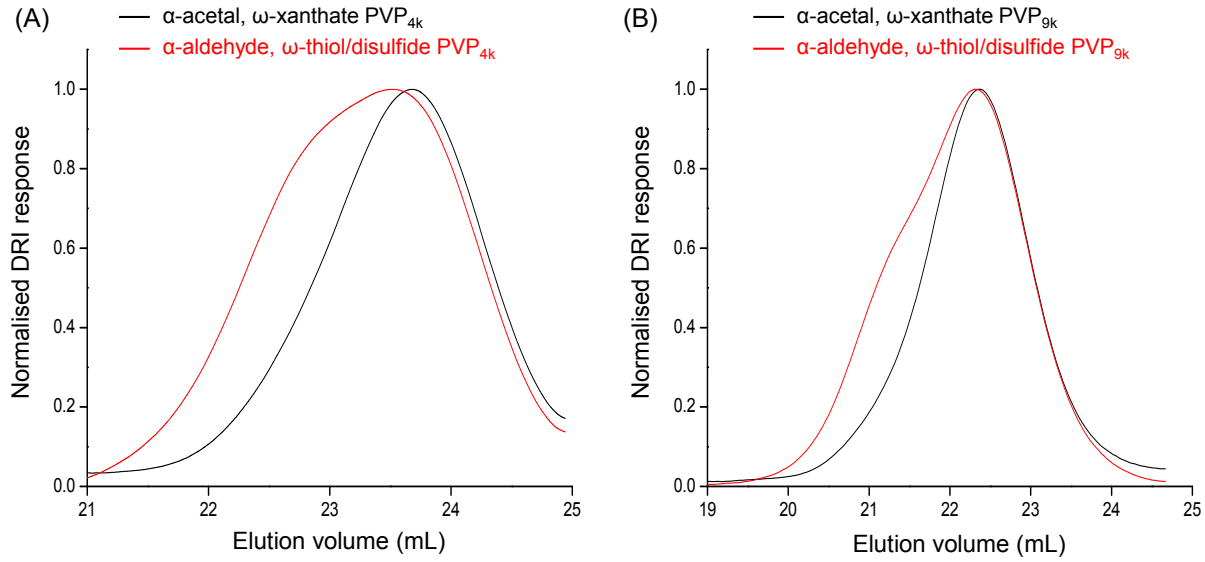


Figure S4: SEC elution profiles detailing the shifts in the PVP elugrams upon deprotection.

II. Conjugation of targeting ligand to PVP

Deprotected PVP_{4K} and PVP_{9K} polymers were functionalised with GSRSKGT (Figure S5) (1 mole equivalence) via reductive amination (Figure S6).

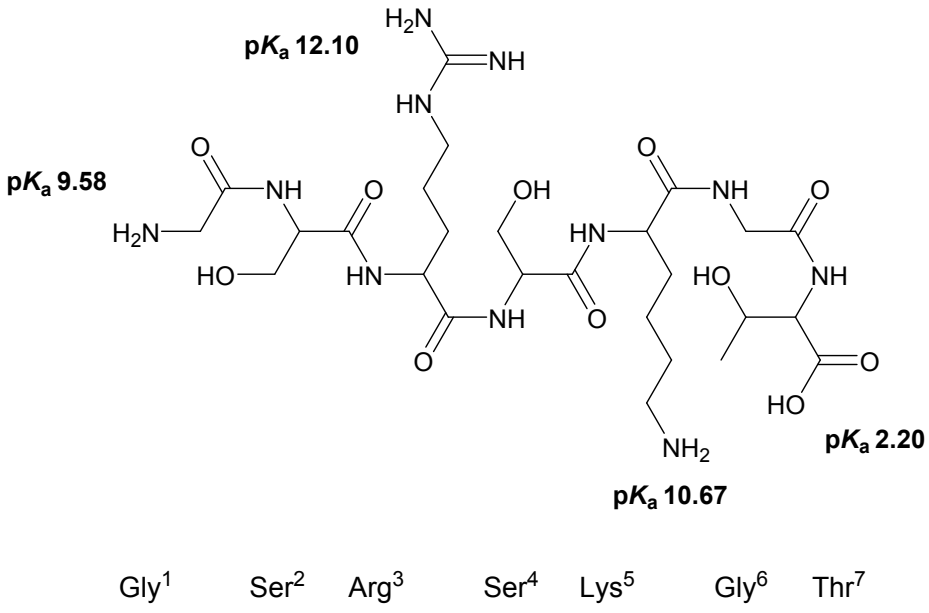
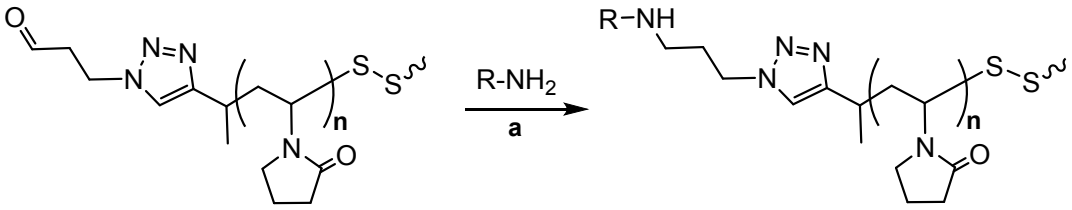


Figure S5: Malaria infected red blood cells targeting ligand (GSRSKGT) structure.



R = GSRSKGT (1%) and *n*-propylamine (99%)

Figure S6: Reaction scheme for the introduction of targeting ligands: (a) $NaBH_3CN$, sodium borate buffer (pH = 9.7).

To clarify the presence of the targeting ligand within the polymers, a qualitative Kaiser test was performed to determine the presence of primary amines.[1] Test tube 1 was made up of Kaiser test reagents with DMF, as a control. Test tubes 2 and 4 comprise of terminal aldehyde polymers PVP_{4K} and PVP_{9K} (no primary amine residues) with Kaiser reagents. Test tubes 3 and 5 contain Kaiser reagents together with PVP_{9K} and PVP_{4K} functionalised with the malaria targeting ligand and purified via dialysis. In all three control reactions, test tubes 1, 2 and 4, the colour remained lime, affirming that no primary amines were present. However, test tubes 3 and 5 gave a blue to green positive result for the presence of primary amines.

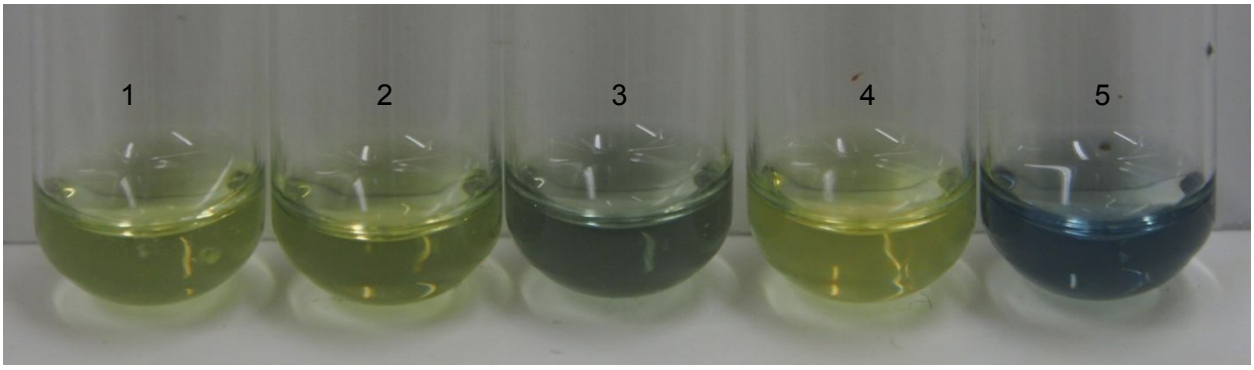


Figure S7: Results of the Kaiser test performed on the targeting ligand functionalised PVP_{4K} and PVP_{9K}. (1) Control – DMF and Kaiser reagents; (2 and 4) Control – terminal aldehyde functional PVP_{4K} and PVP_{9K}; (3 and 5) – targeting ligand functionalized polymers.

III. Tyrocidine production and purification

Table S2: Summary of tyrocidines and analogues determined by substituents on the variable positions.

Name	Abbreviation	Position 3	Position 4	Position 7	Position 9
Tyrocidine A	Trc A	L -Phe	D -Phe	L-Tyr	L-Orn
Tyrocidine B	Trc B	L -Trp	D -Phe	L-Tyr	L-Orn
Tyrocidine C	Trc C	L -Trp	D -Trp	L-Tyr	L-Orn
Tyrocidine A_1	Trc A_1	L -Phe	D -Phe	L-Tyr	L-Lys
Tyrocidine B_1	Trc B_1	L -Trp	D -Phe	L-Tyr	L-Lys
Tyrocidine C_1	Trc C_1	L -Trp	D -Trp	L-Tyr	L-Lys
Tryptocidine A	Trp A	L -Phe	D -Phe	L -Trp	L-Orn
Tryptocidine B	Trc B	L -Trp	D -Phe	L -Trp	L-Orn
Tryptocidine C	Trc C	L -Trp	D -Trp	L -Trp	L-Orn
Tryptocidine A_1	Trc A_1	L -Phe	D -Phe	L -Trp	L-Lys
Tryptocidine B_1	Trc B_1	L -Trp	D -Phe	L -Trp	L-Lys
Tryptocidine C_1	Trc C_1	L -Trp	D -Trp	L -Trp	L-Lys

The tyrothricin complex obtained from the bacterial cultures is known to contain tyrocidines and their analogues along with linear gramicidin (VGA), which constitutes between 10% - 20% of the extract.[2] Gramicidin has been reported to be a potent anti-malaria agent with known haemolytic properties.[3] However, the structure of VGA is not compatible with the intended chemistry for this study. For this reason, it was necessary to purify the extract further in an acetone/ether (1:1 v/v) solvent mixture. This mixture was selected to wash out the more hydrophobic VGA. Ultra-

performance liquid chromatography (UPLC) system coupled to an electron spray ionization detector was employed in the analysis of tyrothricin extract from the bacterial cultures along with the purified tyrocidine decapeptide preparation. The elution profiles obtained are represented in Figure S6.

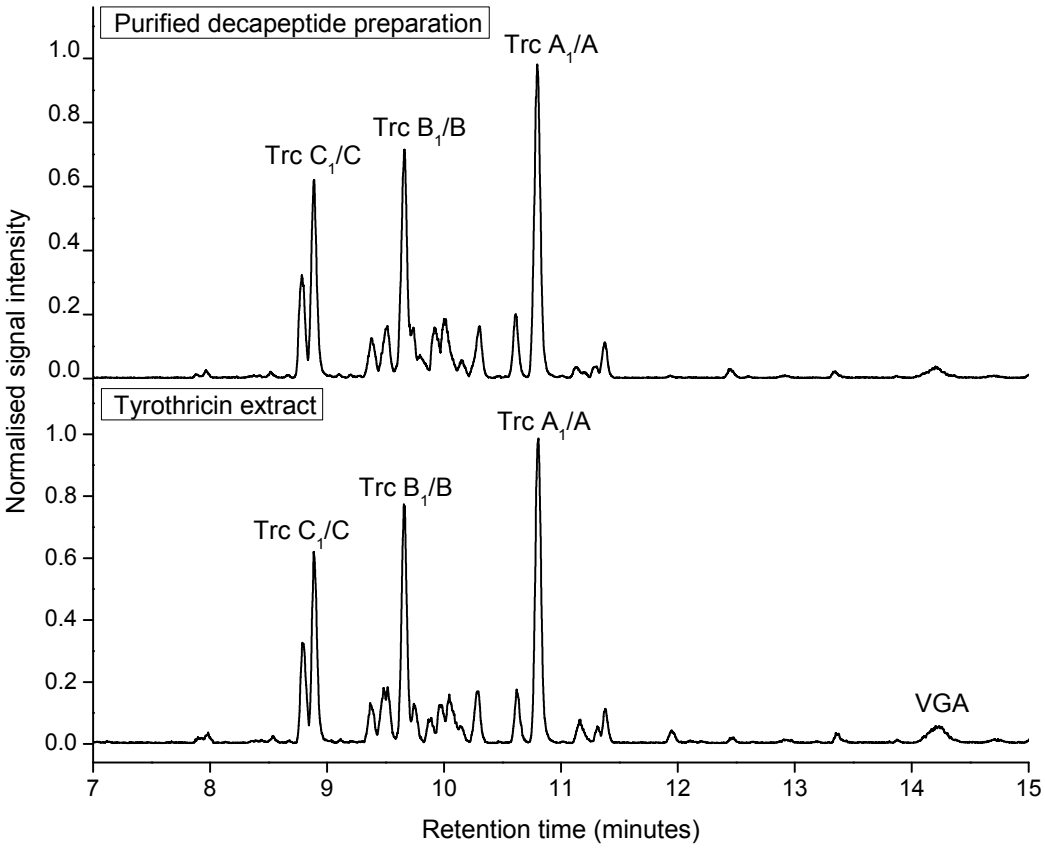


Figure S6: UPLC-MS elution profiles depicting tyrothricin extracted from *Br. parabrevis* (bottom) and gramicidin depleted decapeptide preparation**.**

The UPLC chromatograms represented a typical elution profile for tyrocidines and their respective analogues, in which the Trc C elutes first, followed by Trc B and lastly Trc A. The double peaks for C_1/C, B_1/B, and A_1/A were attributed to tyrocidine analogues bearing either L-Lys or L-Orn on position 9 of the decapeptide structure. Separation of the tyrothricin components was largely influenced by size as well as chemical composition (hydrophobicity depending on Trp:Phe). Trc

C, Trc B and Trc A separation was based on size. The larger decapeptides eluted first followed by the smaller ones. In the case of gramicidins, specifically VGA, the separation was largely influenced by hydrophobicity. The larger more hydrophobic VGA was retained longer on the C_{18} column. Purification with acetone/ether extraction was intended to remove the bulk of the hydrophobic gramicidin fraction and more than 95% of this pentadecapeptide fraction was removed. A small amount of VGA remained in the purified sample. A more detailed depiction of the peptides in the tyrothricin extract is given as the extracted ion chromatograms in Figure S7. A summary of the retention times and respective *m/z* values detected from ESI-MS is shown in Table S3.

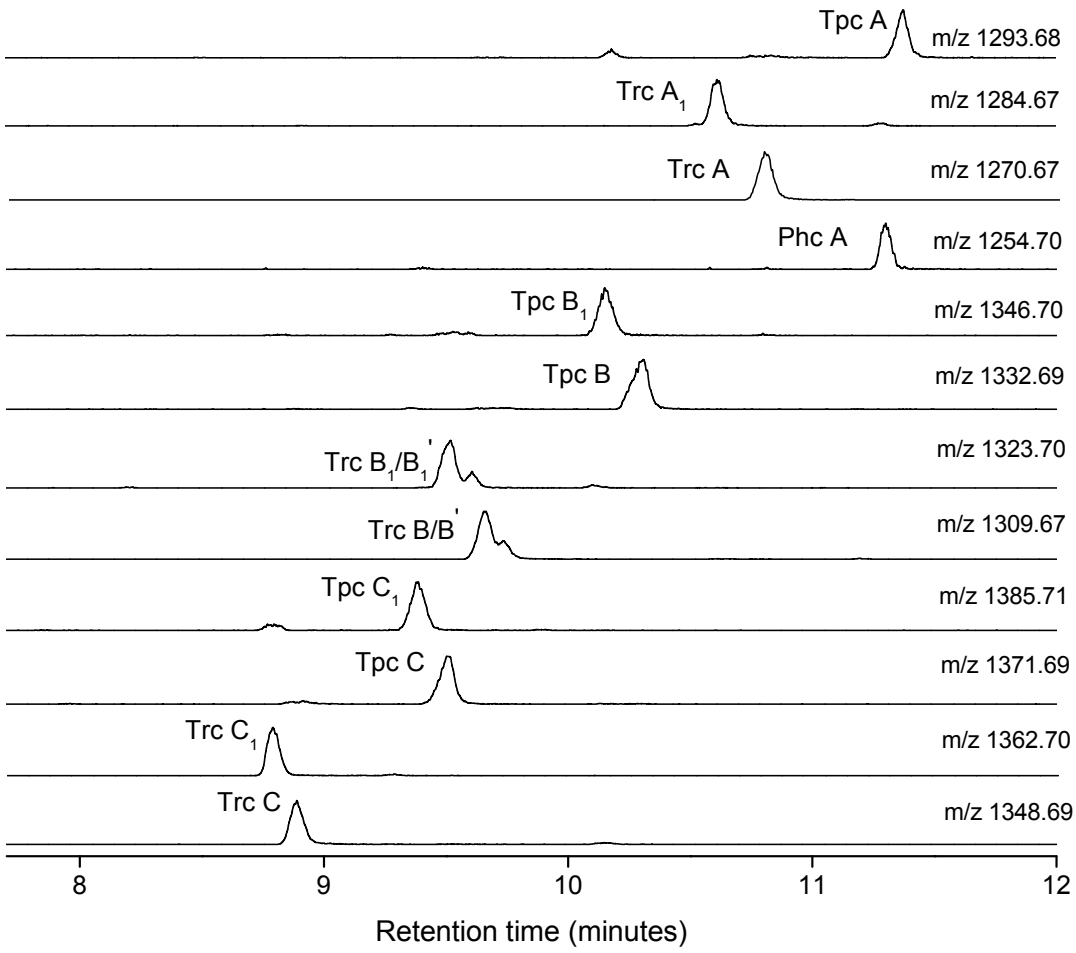


Figure S7: Extracted ion chromatograms (A-M) from the UPLC-MS analysis of tyrothricin extract. Corresponding *m/z* values of the extracted ion chromatograms are given on the right side of the chromatograms.

Table S3: Summary of the peptides and their UPLC retention times and confirmation of their identity via high resolution ESI-MS of purified tyrocidines and tryptocidines in the tyrothricin preparation.

Peptide identity	Retention time (minutes)	Doubly charged *m/z* detected	*m/z* experimental (*m/z* theoretical)	Mr experimental (Mr theoretical)	Mass error (ppm)	Mole Contribution[a] %
Trc C_1	8.78	681.8550	1362.6953 (1362.6999)	1361.6943 (1361.6921)	1.61	6.6
Trc C	8.89	674.8493	1348.6917 (1348.6842)	1347.6830 (1347.6764)	4.89	13.7
Tpc C_1	9.38	693.3624	1385.7133 (1385.7159)	1384.7091 (1384.7080)	0.79	2.9
Tpc C	9.51	686.3549	1371.6885 (1371.7002)	1370.6941 (1370.6924)	1.24	5.0
Trc B_1	9.66	662.3505	1323.6958 (1323.6890)	1322.6853 (1322.6812)	3.09	6.1
TrcB_1’	9.73	662.3505	1323.6958 (1323.6890)	1322.6853 (1322.6812)	3.09	0.8
Trc B	9.51	655.3453	1309.6733 (1309.6733)	1308.6749 (1308.6655)	7.18	17.0
TrcB’	9.61	655.3453	1309.6733 (1309.6733)	1308.6749 (1308.6655)	7.18	2.8
Tpc B_1	10.16	673.8628	1346.6954 (1346.7050)	1345.7099 (1345.6971)	9.51	2.7
Tpc B	10.30	666.8533	1332.6871 (1332.6893)	1331.6909 (1331.6815)	7.05	5.2
Trc A_1	10.61	642.8410	1284.6835 (1284.6781)	1283.6663 (1283.6703)	3.11	4.2
Trc A	10.80	635.8410	1270.6672 (1270.6624)	1269.6563 (1269.6546)	1.33	20.9
Tpc A_1	11.17	654.3508	1307.6987 (1307.6941)	1306.6859 (1306.6862)	0.23	0.7
Phc A	11.28	627.8395	1254.6699 (1254.6675)	1253.6629 (1253.6597)	2.53	1.1
Tpc A	11.38	647.3438	1293.6829 (1293.6784)	1292.6719 (1292.6706)	1.01	3.0
VGA	14.19	941.5469	1883.0104 (1882.0862)	1881.0781 (1881.0784)	0.16	1.6
Other cyclic decapeptides	-	-	-		-	5.7

[a]The mole contributions were deduced from peak integrals and mass error of the respective peptides was calculated from the theoretical *m/z* and confirmation of their identity via high resolution ESI-MS, with the assumption that the ion response factors are similar for the peptides in the tyrothricin complex. The average M_r of the Trc preparation were calculated from the mole contribution as 1325.8.

IV. Tyrocidine modification

Table S4: Summary of the peptides and the UPLC retention times of modified tyrocidine and tryptocidines.

Peptide identity	Retention time (minutes)	Doubly charged *m/z* detected	*m/z* experimental (*m/z* Theoretical)	Mr experimental (Mr theoretical)	Mass error (ppm)	Mole Contribution %
Mod-Trc C_1	10.84	708.8555	1416.7037 (1416.7104)	1415.6953 (1415.7026)	5.15	2.0
Mod-Trc C	10.95	701.8508	1402.6948 (1402.7025)	1401.6859 (1401.6870)	0.78	18.5
Mod-Tpc C	11.64	713.3618	1425.7083 (1425.7108)	1424.7079 (1424.7030)	3.44	8.8
Mod-Trc B	11.92	682.3454	1363.6848 (1363.6839)	1362.6751 (1362.6761)	0.73	20.6
Mod Trc B’	12.19	682.3454	1363.6848 (1363.6839)	1362.6751 (1362.6761)	0.73	2.6
Mod-Tpc B	12.65	693.8569	1386.7050 (1386.6999)	1385.6981 (1385.6921)	4.33	7.4
Mod Tpc B’	12.81	693.8569	1386.7050 (1386.6999)	1385.6981 (1385.6921)	4.33	1.0
Mod-Trc A/A_1	13.55	662.8450	1324.6652 (1324.6730)	1323.6743 (1323.6652)	6.87	20.2
Mod-Tpc A	14.23	674.3501	1347.6975) (1347.6890)	1346.6845 (1346.6812)	2.45	4.5
VGA	14.12	941.5469	1882.0970 (1882.0862)	1881.0781 (1881.0784)	0.16	1.0
Unidentified and unreacted decapeptides	-	-	-		-	8.8
Contaminants	-	-	-		-	8.2

V. Aggregation/oligomerisation of tyrocidines and analogues

Post peptide modification, it was important that very little of the Trc character be altered. Their oligomerisation behaviour was needed to drive self-assembly of polymer-peptide conjugates into nano-particles. Furthermore, loss of oligomerisation could also result in loss of activity, as to formation of dimers are thought to be essential for membrane activity and oligomerisation in

membranes.[4] The oligomerisation properties were analysed by determination of the tendency of Trc A, B and C to form oligomers following acrylate modification. The deconvoluted mass spectra of Trc A, B and C acrylate derivatives are given in Figure S8. The deconvolution of the peptide ion mass spectra using the MaxEnt 3 algorithm (a maximum entropy algorithm to deconvolute ESI-MS spectra) from MassLynx V4.1 allows the detection of oligomeric species before and after acrylate modification of tyrocidine.

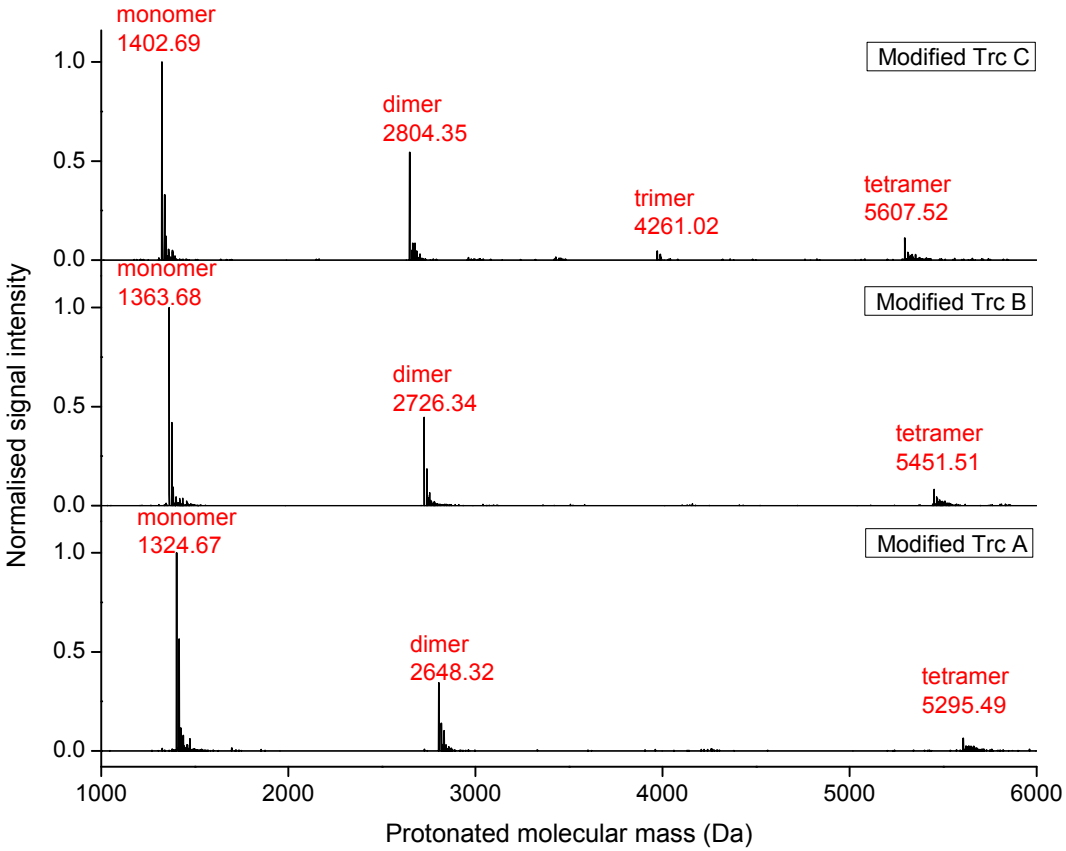


Figure S8: MaxEnt deconvoluted mass spectra of the Trc A, B and C and their acrylate modified analogues showing their oligomers that are stable in the mass spectrometer.

VI. PVP-tyrocidine bioconjugation

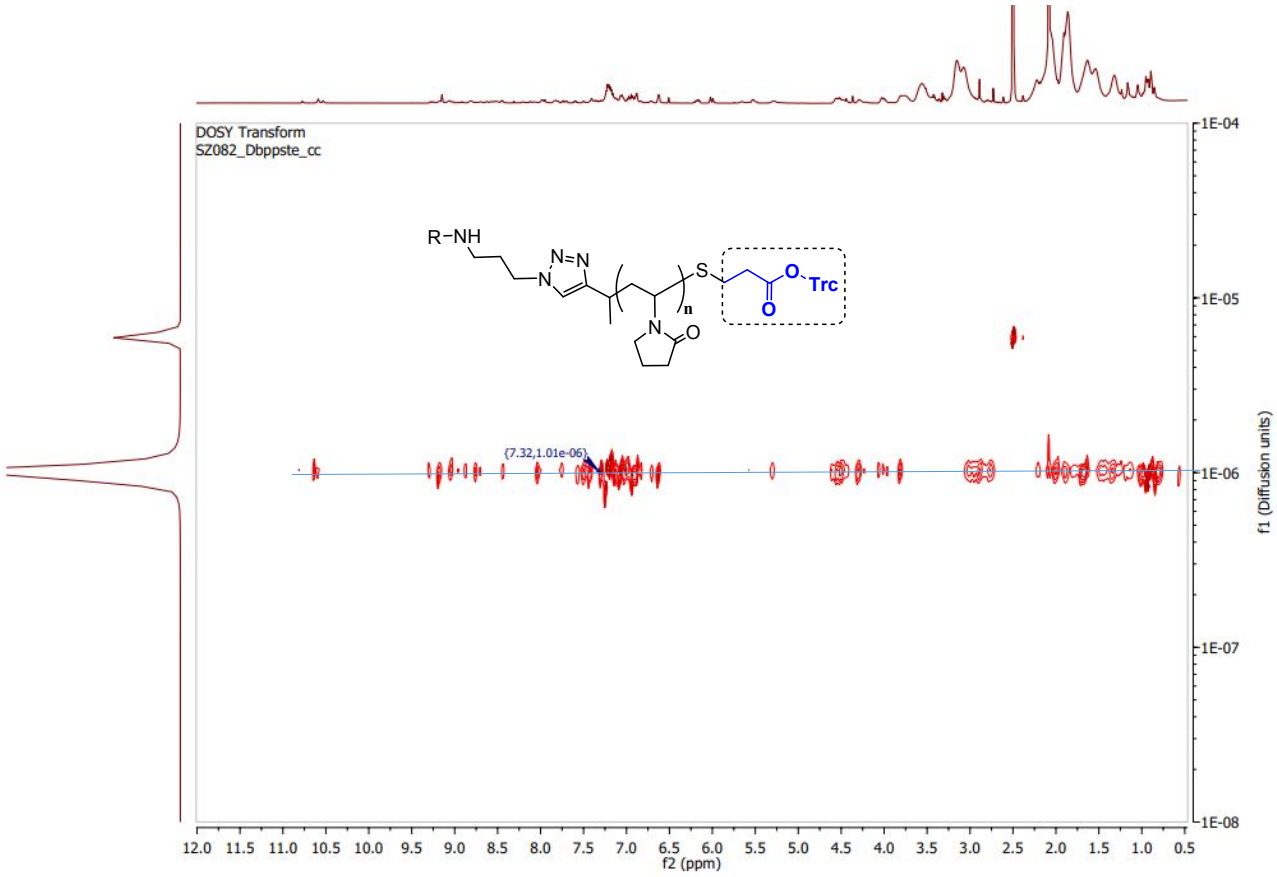


Figure S9: Diffusion ordered NMR (DOSY) spectrum of PVP_{4K}-trc conjugate.

VII. Cytotoxicity assays

Trc and PVP-Trc conjugates were screened for *in vitro* Cytotoxicity on HepG2 cells using the LDH assay. The results are represented as a measure of cell viability and cytotoxicity (Figure S10). Each polymer conjugate was tested at a concentration ≥10× the previously determined IC_{50}. The concentrations used are included in Table S5 and given as the concentration of the polymer conjugate as well as the drug equivalent, assuming all of the drug is released. All conjugates were determined to be non-toxic to HepG2 cells at 10 $\mu g \cdot mL^{-1}$.

Table S5: *In vitro* cytotoxicity of compounds on HepG2 cells, obtained at concentrations of 10 $\mu g \cdot mL^{-1}$. The data is represented as a measure of cell viability.

	Concentration ($\mu g \cdot mL^{-1}$)	Trc ($\mu g \cdot mL^{-1}$)	Viability ± SEM (%)
Trc	-	10.0	61.9 ± 7.6
Conjugate 1[a]	37.0	10.0	97.1 ± 3.0
Conjugate 3[b]	37.0	10.0	95.7 ± 3.0
Conjugate 2[c]	79.4	10.0	99.7 ± 0.3
Conjugate 4[d]	79.4	10.0	100 ± 0.0

*Data are means of three independent biological replicates ± SEM for the proliferative and haemolysis assays. [a]α-propyl-PVP_{4K}-Trc, [b] α-$GSRSKGT_{(2\%)}$, $propyl_{(98\%)}$-PVP_{4K}-Trc, [c]α-propyl-PVP_{4K}-Trc, [d]α-$GSRSKGT_{(2\%)}$, $propyl_{(98\%)}$-PVP_{9K}-Trc.

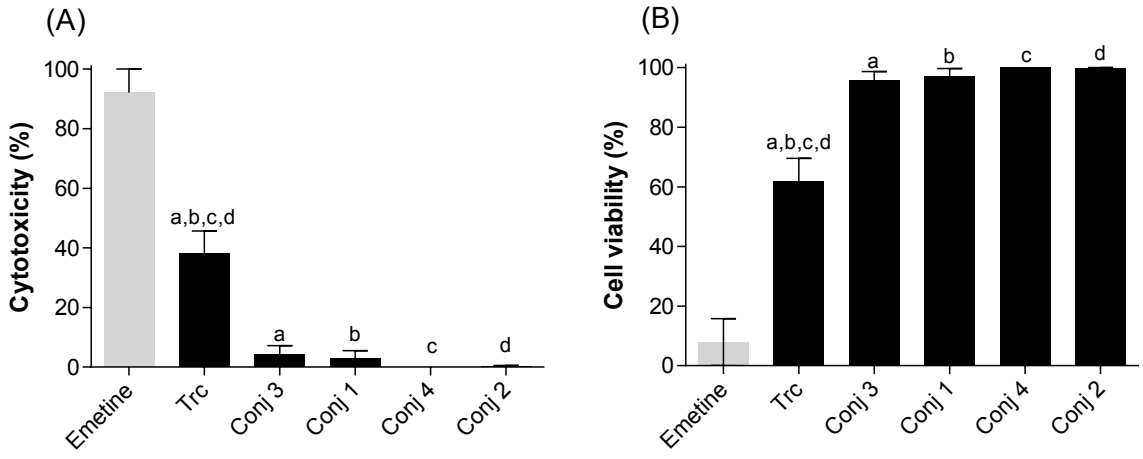


Figure S12: *In vitro* viability of HepG2 cells following treatment with polymer set (n=3, ± SEM). Data are represented as cytotoxicity (A) and viability (B) with emetine used as the positive control for cytotoxicity. Statistically significant differences were calculated using the paired Student's t-test where a, b, c, d- $P<0.05$.

VII. References

1. Jarre, G.; Heyer, S.; Memmel, E.; Meinhardt, T.; Krueger, A., Synthesis of nanodiamond derivatives carrying amino functions and quantification by a modified Kaiser test. *Beilstein J. Org. Chem.* **2014,** 10, (1), 2729-2737.

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3. Otten-Kuipers, M. A.; Franssen, F. F.; Nieuwenhuijs, H.; Overdulve, J. P.; Roelofsen, B.; den Kamp, J. A. O., Effect of tryptophan-N-formylated gramicidin on growth of Plasmodium berghei in mice. *Antimicrob. Agents Chemother.* **1997,** 41, (8), 1778.

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