Electronic Supplementary Material (ESI) for Biomaterials Science.

Antibiotic functionalised polymers reduce bacterial biofilm and bioburden in a simulated infection of the cornea.

N. Doroshenko, S. Rimmer, R. Hoskins, P.Garg, T. Swift, H. L.M. Spencer, R. M. Lord, D. Pownall, S. MacNeil, C.W.I. Douglas, J. Shepherd

Supplementary Material

Contents

Polymer Chemical Analysis

The chemical identity (chain end functionalisation) of PNIPAM polymers was analysed by FTIR (Table S1) and ^{1}H NMR (Table S2). These analyses demonstrated the reaction of the acid chain ends as the vancomycin ligands were covalently attached to the polymer chain ends.

Table S1: FTIR Spectra of PNIPAM Polymers

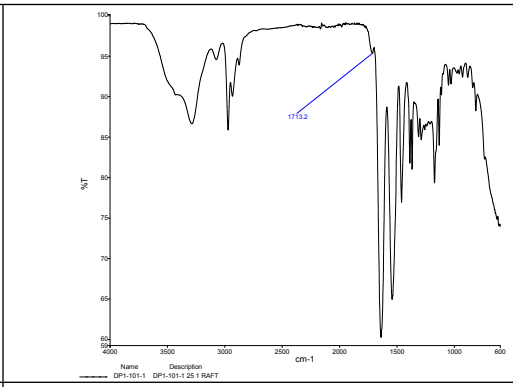
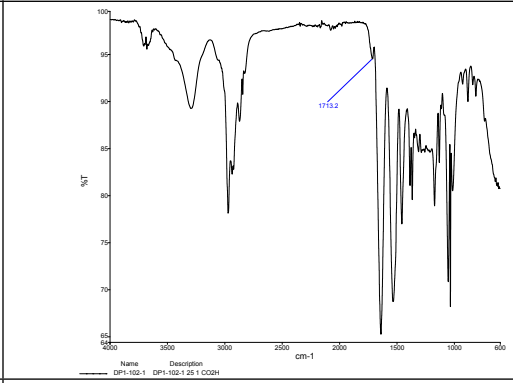
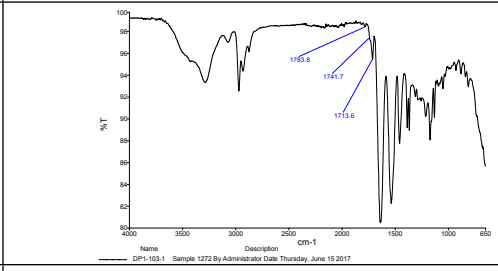
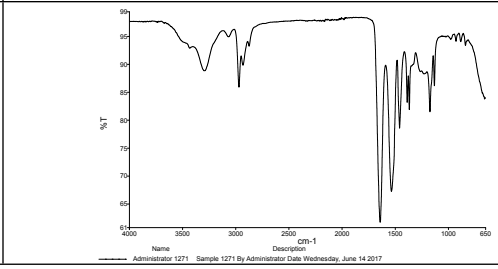
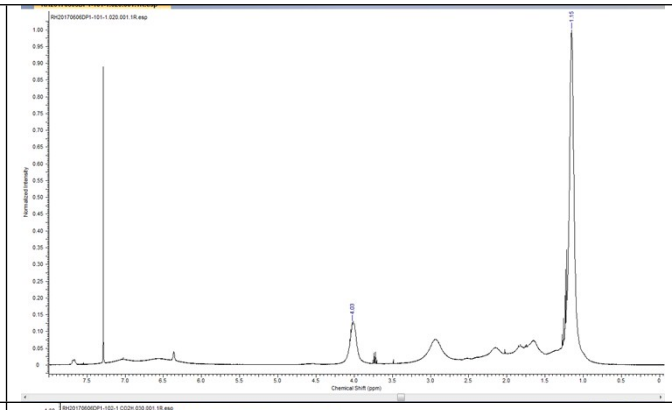
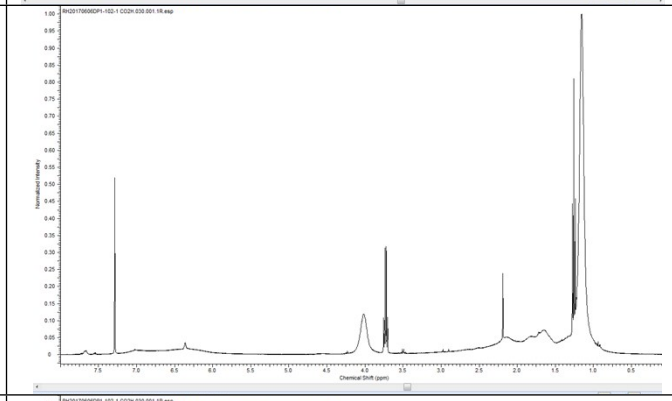
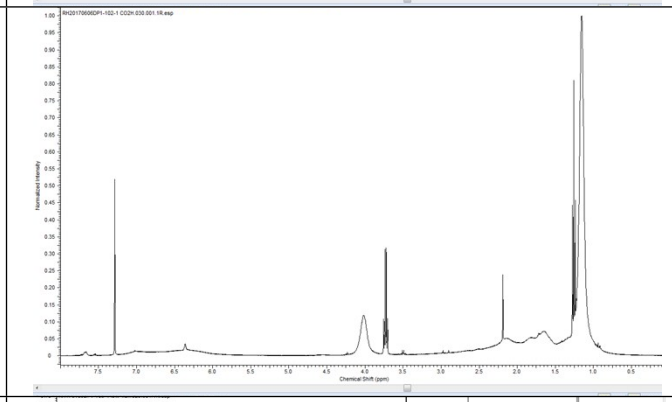
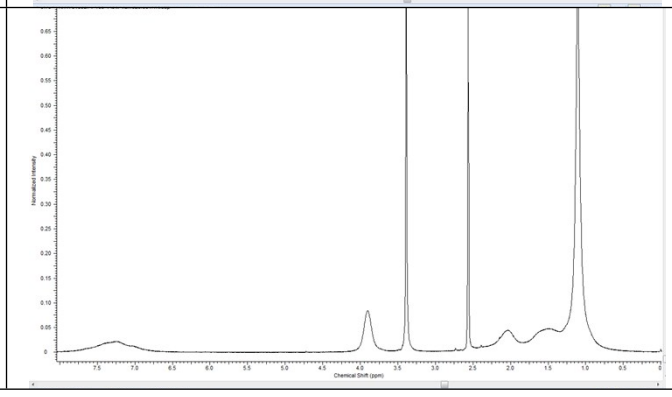
25:1 RAFT, CO2H from initiator peak at 1713 cm^{-1}	
DP1-102-1 25:1 CO2H functionalised, CO2H peak at 1713 cm^{-1}	
DP1-103-1 25:1 succinimide Peaks at 1741 cm^{-1} and 1783 cm^{-1} indicative of succinimide	
DP1-105-1 25:1 Vancomycin No carboxylic acid or succinimide peaks	

Table S2: ^{1}H NMR Spectra of PNIPAM Polymers

DP1-101-1 25:1 RAFT	
DP1-102-1 25:1 CO2H	
DP1-103-1 25:1 Succinimide	
DP1-105-1 25:1 Van	

Polymer size characterisation

The polymers used in this project were functionalised highly branched PNIPAM compounds that had additional pendent vancomycin ligands on the molecule chain ends. Their synthesis and characterisation has been previously described (Swift *et al.* 2017). The molar mass distribution (determined by Viscometric Methanol SEC) are shown in Figure S1, alongside the hydrodynamic radii distribution (Figure S3), determined by ^{1}H DOSY NMR (raw data Figure S2).

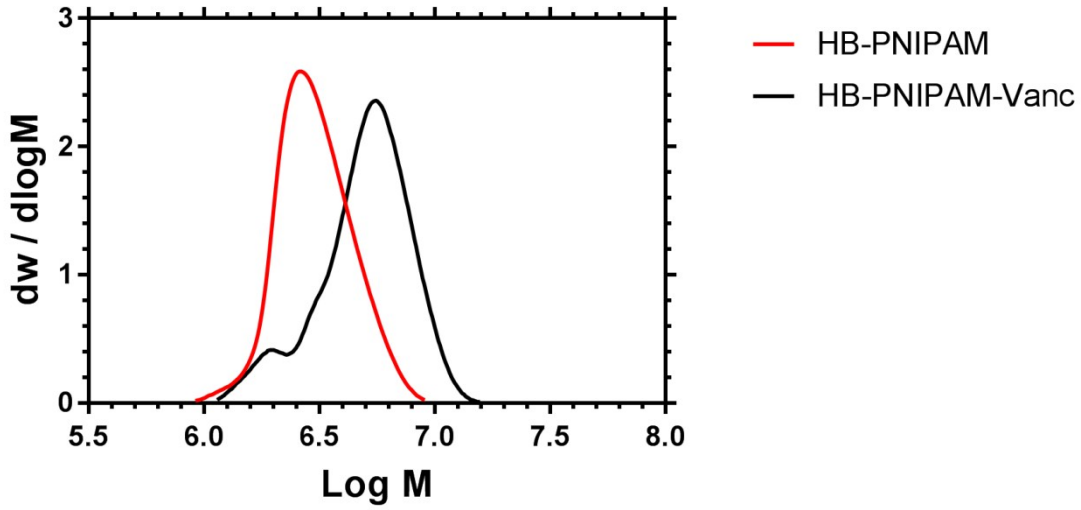


Figure S1: Molar mass distributions of HB-PNIPAM and vancomycin functionalised HB-PNIPAM-van

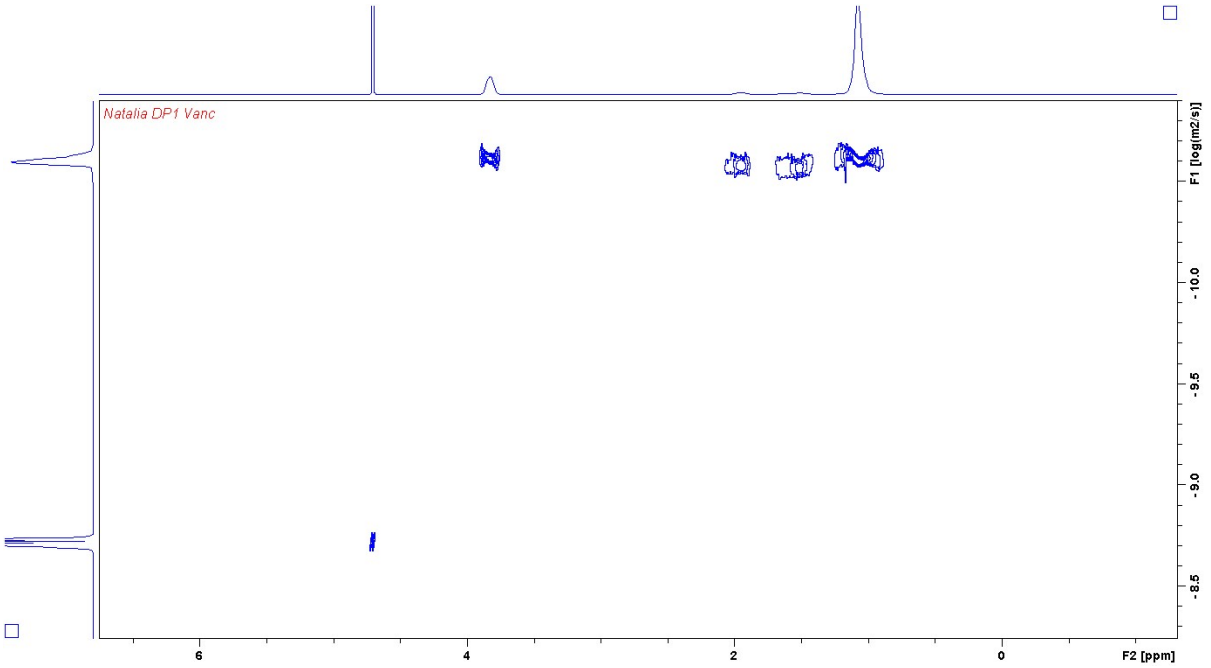


Figure S2: DOSY analysis of HB-PNIPAM-van in D_2O at 298 K.

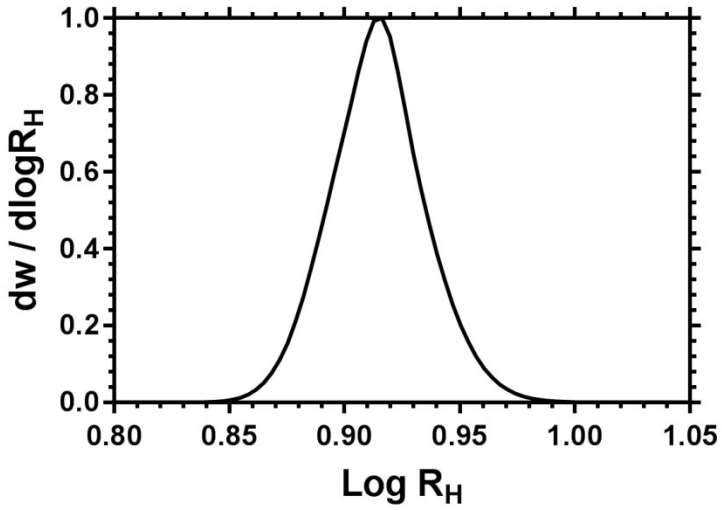


Figure S3: DOSY R_H distribution of HB-PNIPAM-van in D_2O at 298 K.

Confocal microscopy analysis of infected corneas

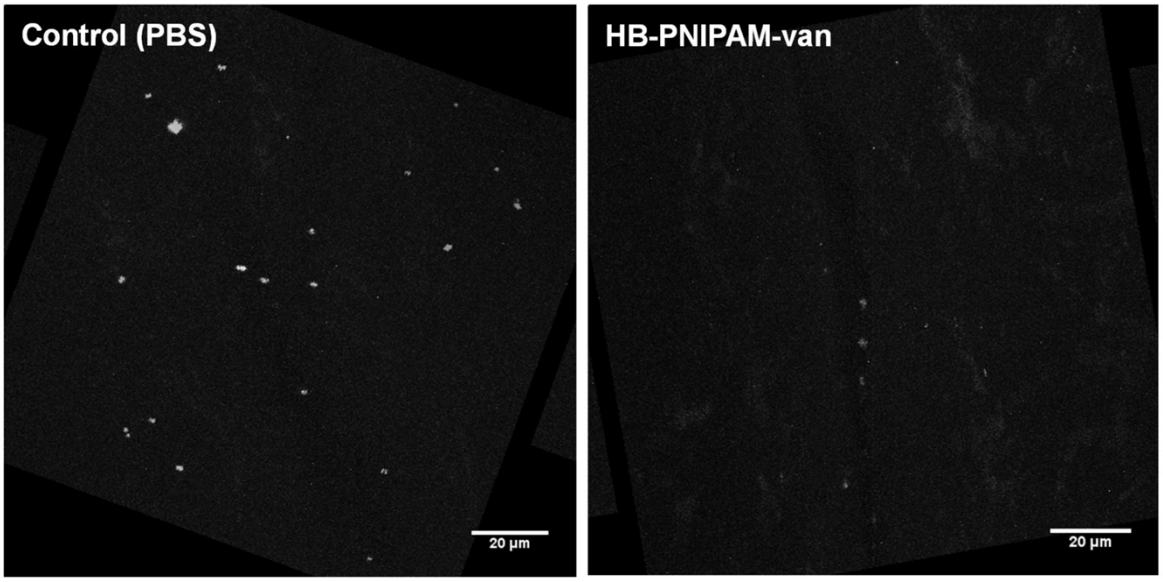


Figure S4: Confocal microscopy analysis of infected rabbit corneas. Raw confocal microscopy images of infected *ex vivo* rabbit corneas following a 4-h treatment with HB-PNIPAM-van or PBS (control). Background fluorescence is auto-fluorescence from the cornea. Bright circular aggregates are GFP-labelled *S. aureus*.

SEM Analysis of Polymer and Bacteria Samples

Preparation of the polymer solutions for SEM analysis involved cooling (to 4°C) and subsequent re-heating (to room temperature) of a solution of the dehydrated polymers in dH_2O at a concentration of 0.5 mg ml^{-1}. These control experiments show aggregated polymer particles that formed during the sample drying process. These aggregates are visually distinct from the bacteria-aggregates seen in the presence of *S. aureus* (Figure S5).

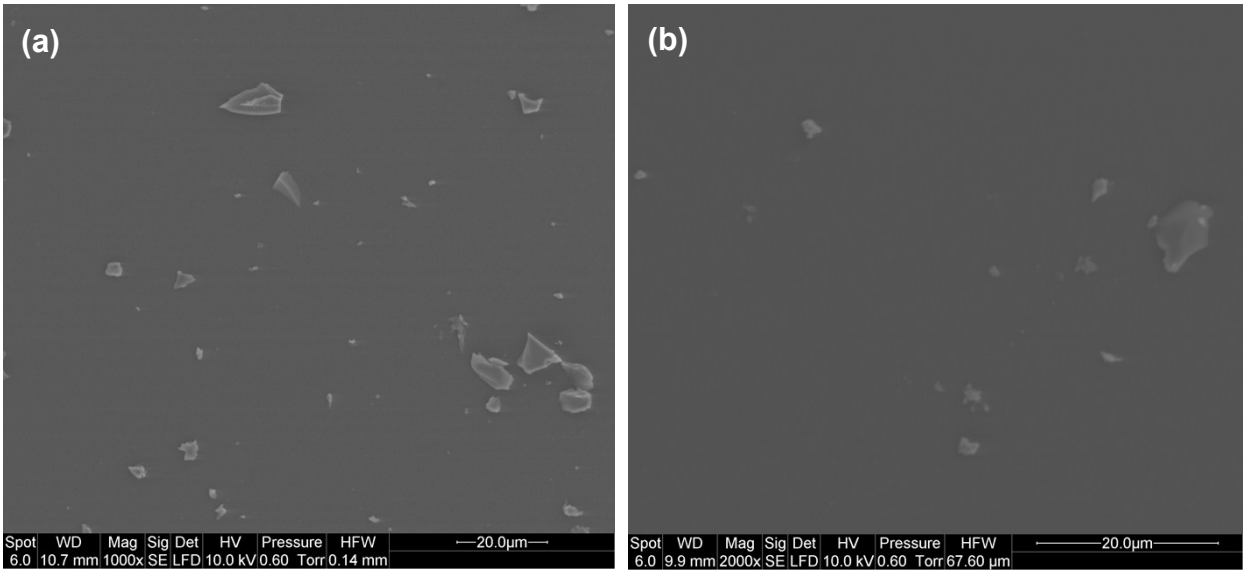


Figure S5: Scanning electron micrographs of HB-PNIPAM and HB-PNIPAM-van. HB-PNIPAM (a) formed larger desolvated aggregates that varied in size from approximately 1-100 µm. HB-PNIPAM-van (b) formed smaller aggregates measuring approximately 1-10 µm. Scale bar: 20 µm.

The aggregates formed between *S. aureus* and HB-PNIPAM-van (Figure S6) are visually distinct from the aggregates formed by the polymer alone (Figure S5).

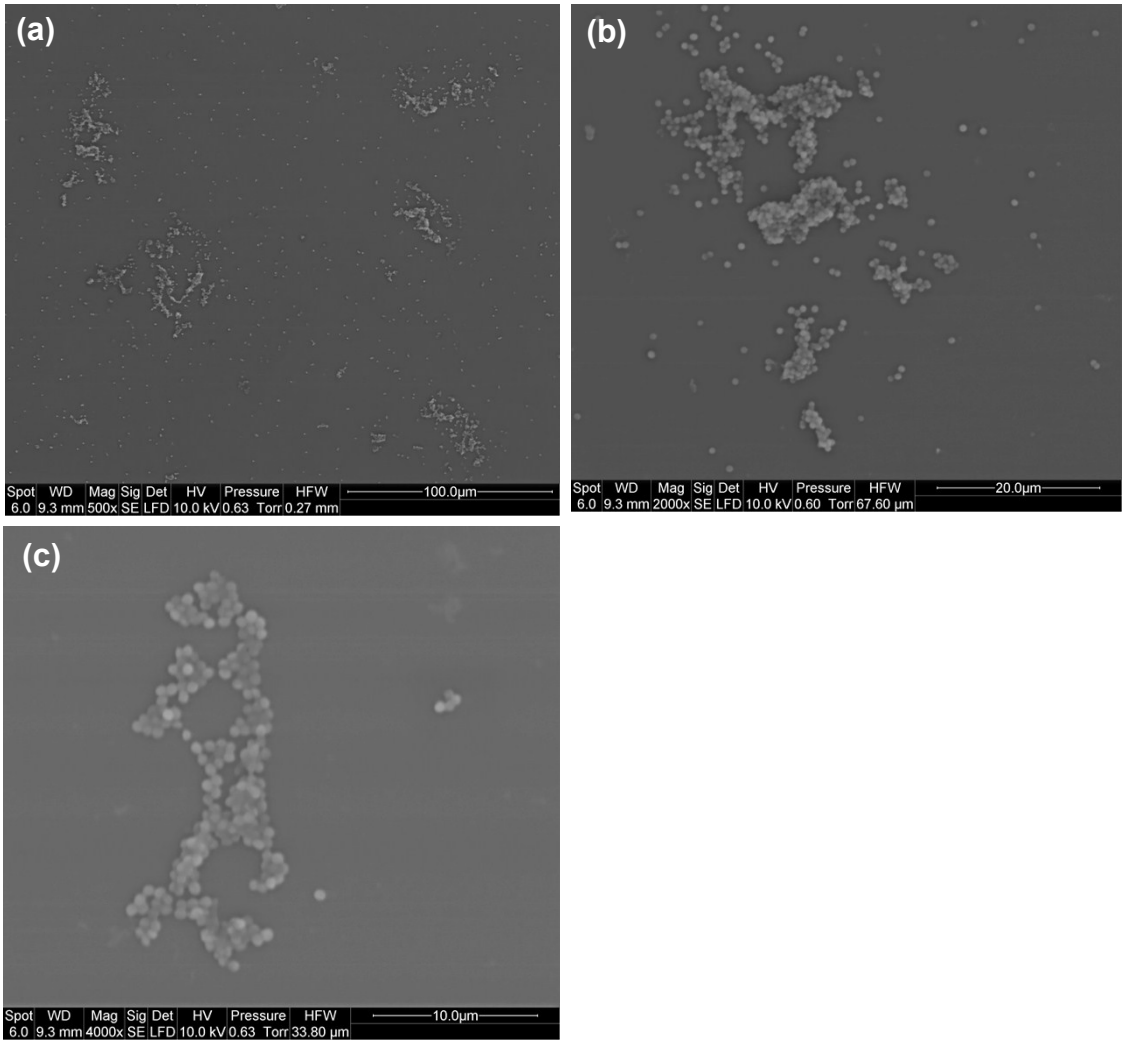


Figure S6: Scanning electron micrographs of HB-PNIPAM-Van and *S. aureus* aggregates; (a) 500X magnification, (b) 2000X magnification, and (c) 4000X magnification.

Bacterial concentration and fluorescence intensity

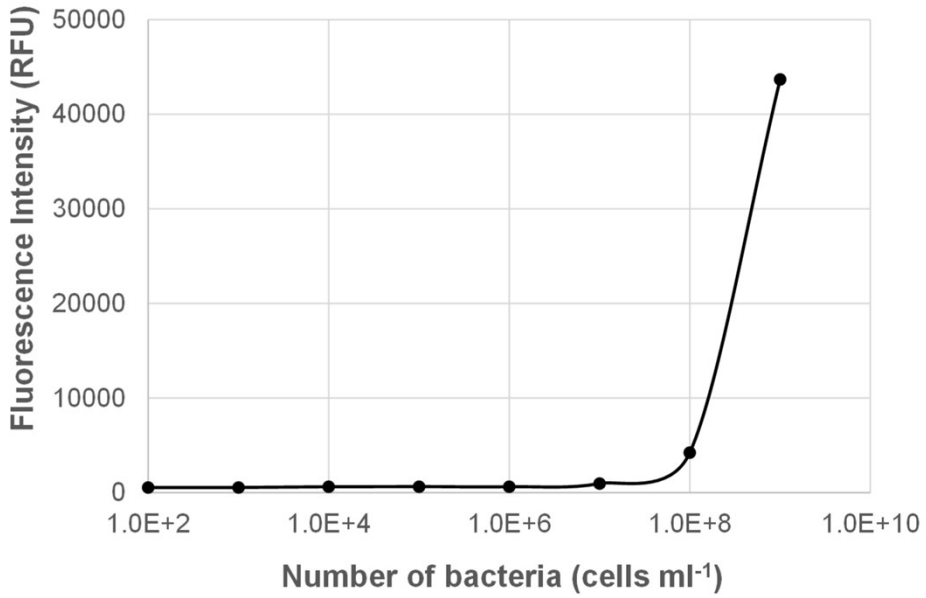


Figure S7: The effects of bacterial concentration on fluorescence intensity. Fluorescence Intensity of different concentrations of *S. aureus* in a 96 well microplate, following a 2 hour attachment incubation period. Bacteria were stained with SYTO® 9 as described in methods: attachment assays section of the paper.

Cell Viability Results

	IC_{50} values ± SD (mg/mL)			
	ARPE-19	**HCT116 +/+**	**HCT116 -/-**	**A549**
N-isopropylacrylamide	>0.1	>0.1	>0.1	>0.1
RAFT Agent	0.021 **±** 0.003	0.038 **±** 0.004	0.035 **±**- 0.003	0.03 **±**- 0.05
PNIPAM linear	>0.1	>0.1	>0.1	>0.1
PNIPAM branched	>0.1	>0.1	>0.1	>0.1
PNIPAM-Vanc	>0.1	0.092*	>0.1	>0.1

** from a single run*

Table S3 – Cytotoxicity values ± SD (mg/mL) for the monomers and polymers against ARPE-19 (human epithelial retinal cells), HCT116 +/+, p53-wildtype and HCT116 -/-, p53-null (human colorectal carcinomas) and A549 (human lung carcinoma)

The cytotoxicity of both monomers and polymers were determined using the MTT assay, which assesses that metabolic activity of cells, and gives the concentration required to inhibit 50% of the cells (IC_{50}). The results are displayed in Table S3, and shows the IC_{50} values obtained against ARPE-19 (human epithelial retinal cells), HCT116 +/+, p53-wildtype and HCT116 -/-, p53-null (human colorectal carcinomas) and A549 (human lung carcinoma). For the purpose of this study, 100 mg/mL stock solutions (in DMSO) were produced for all monomers and polymers, and they were further diluted with complete media to achieve final concentrations ranging from 0.1 mg/mL – 0.39 □g/mL. No dose-dependent cytotoxic effects were observed for any of the monomers or polymers the maximum tested concentration (0.1 mg/mL). However, the reversible addition-fragmentation chain transfer (RAFT) agent which accounts for approximately 0.4% w/w of HB-PNIPAM is cytotoxic to all 4 cell lines, however has no toxic effects when incorporated into the highly branched PNIPAM structure.

Cell Viability Experimental

The *in vitro* chemosensitivity tests were performed at the University of Bradford, in a sterile Class II Biological Safety Cabinet fitted with a HEPA filtered laminar airflow. Cellular assays were conducted against ARPE-19 (human epithelial retinal cells), HCT116 +/+, p53-wildtype and HCT116 -/-, p53-null (human colorectal carcinomas) and A549 (human lung carcinoma). ARPE-19 cells were obtained from the American Type Culture Collection, and provided by the University of Huddersfield (Professor Roger Philips and Dr Simon Allison). All cell lines were cultured from cryopreserved vials, removed from liquid N_2, thawed, centrifuged and re-suspended in complete media. Cancer cell lines were routinely maintained as monolayer cultures in appropriate complete medium, and maintained in either T-25 or T-75 flasks at 37 °C and 5% CO_2. ARPE-19 cells were culture in high glucose DMEM-F12 medium containing 10% foetal calf serum. HCT116 +/+ and HCT116 -/-, were cultured in RPMI 1640 supplemented with 10% foetal calf serum, sodium pyruvate (1 mM) and L-glutamine (2 mM). A549 were cultured in low glucose DMEM supplemented with 10% foetal calf serum and L-glutamine (2 mM). Prior to chemosensitivity studies, cell monolayers (once 50-60% confluent), were passaged using Trypsin-EDTA and diluted to a concentration of 1 x 10^4 cells/mL. All assays were conducted using 96-well plates, in which 100 □L of the 1 x 10^4 cells/mL cell suspension was added to each well and incubated for 24 hours at 37 °C and 5% CO_2 (column 1 contains just media to serve as a blank). After 24 hours, 100 □L of the monomer and polymer dilutions (0.2 mg/mL – 0.78 □g mL) were added to the plates in columns 3-12 (column 2 contains 100% cells to serve as a control), and then incubated for a further 24 hours at 37°C and 5% CO_2, giving final drug concentrations of 0.1 mg/mL – 0.39 □g/mL. After 24 hours the media was removed via pipette and fresh complete media added to each well, the plates were then incubated for 96 hours at 37 °C and 5% CO_2 to serve as a recovery period.

After the recovery period, 20 □L MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL) was added to each well and incubated for 3 hours at 37°C and 5% CO_2. Reduction of MTT, by NADH-dependent cellular oxidoreductases, to insoluble formazan provides an adequate representation of the number of viable cells contained within each well. All solutions were then removed from the 96-well plates via pipette and 150 µL of DMSO added to each well in order to dissolve the purple formazan crystals. After mixing well, the absorbance of each well was measured at 540 nm using a ThermoFisher Multiskan FC spectrophotometer microplate reader. Using the blank and 100% cell control, the % cell viability was determined ((abs of compound containing wells – abs media wells)/abs 100% cell control) and from this the % cell death (100%- % cell viability) can be calculated. On a logarithmic scale, the percentage cell death was plotted against the concentration of monomer, polymer or compound, and from this the half maximal inhibitory concentration (IC_{50}) value was determined. Each of the

experiments was performed in triplicate with mean values being stated as the IC_{50} ± Standard Deviation (SD).