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Waterborne physically crosslinked antimicrobial nanogels

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Supplementary Information

SI 1. Experimental section:

SI 1.1. Materials: Decylamine (99%, Aldrich), epichlorohydrin (99%, Merck), piperazine (99+%, Aldrich) and poly(ethylene imine) (99%, Sigma Aldrich) were used as received. Mili Q water was used as solvent for all the reactions. For determination of the antimicrobial activity, amphiphilic compounds and amphiphilic polymers were tested against the Gram negative bacteria *Escherichia coli* (ATCC 23716 and ATCC25922) and *Pseudomonas aeruginosa* (ATCC 27853) and the Gram positive bacteria *Staphylococcus aureus* (*ATCC 6538*) and *Staphylococcus epidermidis (ATCC 12228).*

SI 1.2. Measurements:

SI 1.2.a. NMR : ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 FT-NMR spectrometer at 400 and 100 MHz, respectively. Deuterium oxide (D_2O) and deuterated methanol (CD_3OD) were used as solvents.

SI 1.2.b. SEC: Size exclusion chromatography analyses (SEC) were carried out using water (containing 0.1 M NaCl, 0.1% TFA, 0.01% NaN_3) as eluting solvent at 30^{o}C with a flow rate of 1 mL/min using a high pressure liquid chromatography pump (Agilent 1100) and refractive index detector (Wyatt, Optilab DSP). Three columns with PSS Novema gel were applied. The length of each column was 300 mm, the diameter was 8 mm, the diameter of the gel particles was 10 µm and the nominal pore widths were 30, 3000 and 3000 Å. Calibration was achieved using Pullulan standards

SI 1.2.c. Dynamic light scattering (DLS) and Zeta Potential: The particle size was determined using both (i) commercial laser light scattering spectrometer (ALV/DLS/SLS-5000) equipped with an ALV-5000/EPP multiple digital time correlator and laser goniometry system ALV/CGS-8F S/N 025 with a helium-neon laser (Uniphase 1145P, an output power of 22 mW and a wavelength of 632.8 nm) as a light source and (ii) Malvern Zetasizer ZS. The same size was determined by ALV/DLS/SLS-5000, with a scattering angle of 90^o and Malvern Zetasizer Zs, which supports the accuracy of these measurements. Zeta potential (ζ) was measured using Malvern Zetasizer ZS. For reporting the final particle size, three batches were prepared and from each batch, samples were measured three times. Average size and error margin were determined. The error margins for the particle diameter were ± 3 nm, and for the zeta potential ± 5 mV, respectively.

SI 1.2.d. Static light scattering (SLS): SLS studies were performed using SLS instruments (Fica Goniometer) at a wavelength of 632.8 nm. Samples were measured at different scattering angles between 40^o -140^o, at an interval of 2^o. Each time three measurements were taken. Six different concentrations (between 3 mg/mL and 0.08 mg/mL) of each sample were measured to determine the radius of gyration (R_g) using the Berry plot (dn/dc values given in Table SI 7 were used).

SI 1.2.f. Cryo-TEM: Cryogenic TEM samples were prepared by plunge freezing of aqueous dispersion (1 mg/ mL) using a vitrobot system. Before that, solutions were filtered with a 200 nm mesh size PET membrane and dropped on a lacey TEM grid. The TEM grid was hydrophilized in a plasma oven for 45 seconds before use. Cryo-TEM micrographs were taken on a Carl Zeiss Libra 120 Microscope (Oberkochen, Germany) at -170 °C. The electron beam accelerating voltage was set at 120 kV.

SI 1.2.f. Cryo-SEM: Cryo-SEM images were taken using a Hitachi S-4800 field emission scanning electron microscope (FESEM) operating at 1-2 kV and 2-10 µA current. Samples for cryo-SEM were placed on a small tube and plunged in liquid nitrogen for freezing. The images were obtained after partly sublimation of the surface.

SI 1.2.g. AFM: The surface topology of polymer coated surface was studied using an atomic force microscopy (Nanoscope III). The tapping mode imaging was performed with standard silicon cantilevers (Nano world, NCH-W point probe).

SI 1.3. Synthesis:

SI 1.3.a. Bifunctional coupler and hydrophobic coupler: The bifunctional coupler and the hydrophobic coupler were synthesized as reported earlier and characterized similarly[1].

SI 1.3.b. Synthesis of multifunctional PEI (PEIHy$^{d}_{12.5}$C$^{az}_{12.5}$) nanogel:

Step 1: To a solution of PEI **1** (1 g, 23 mmol repeating unit) in distilled water (50 mL), the solution of the hydrophobic coupler (1.12 g, 2.87 mmol) in distilled water (10 mL) was added. The solution was stirred (stirring rate 1000 rpm) for 15 hours at 90^{o}C and then cooled down to room temperature. The solution contains the hydrophobically modified PEI **2**.

Step 2: To the solution containing hydrophobically modified PEI **2** (as prepared in step 1), a solution of the bifunctional coupler (0.78 g, 2.87 mmol) in distilled water (3 mL) was added. The solution was stirred for 3 hours at 90^{o}C (stirring rate 1000 rpm) and then cooled down to room temperature. After cooling, the solution was filtered using a normal filter paper to remove small amounts of large aggregates. The resultant solution contains multifunctional PEI **3 - PEI-H$^{d}_{12.5}$C$^{az}_{12.5}$.** All other multifunctional PEIs were prepared using the same procedure. The starting materials are given in table SI 1:

Table SI 1: Starting materials for preparing multifunctional PEI nanogels:

Multifunctional PEI	PEI (g) [mmol r.u.*]	Hydrophobic coupler (g) [mmol]	Bifunctional coupler (g) [mmol]
PEIHy$^{d}_{10}$C$^{az}_{15}$	1 [23.2]	0.9 [2.3]	0.95 [3.5]
PEIHy$^{d}_{12.5}$C$^{az}_{12.5}$	1 [23.2]	1.12 [2.87]	0.78 [2.87]
PEIHy$^{d}_{15}$C$^{az}_{10}$	1 [23.2]	1.37 [3.5]	0.62 [2.3]

***r.u. = repeating unit**

SI 1.4. Molecular coatings: Silicon cantilevers were coated via spincoating using an aqueous solution of **PEIHy$^{d}_{12.5}$C$^{az}_{12.5}$** (concentration = 0.05 mg/mL) at room temperature and dried thereafter. The coated surface was analyzed by AFM.

SI 1. 5. Biological studies:

SI 1. 5.a. Antimicrobial tests of polymer solutions:

The antibacterial activity of the amphiphilic polymers in solution was determined by measuring the minimum inhibitory concentration (MIC) using different test bacteria. Suspensions of strains with known colony forming units (CFU; $2x10^6$ CFU/mL) were incubated at 37°C in nutrient solutions (Mueller-Hinton Broth, MHB) with different concentrations of the polymer samples. The polymer samples were solubilized in bidistilled water and added to the nutrient solution at a constant ratio of 1:10. The growth of the bacteria was followed during the incubation over 20 h by measuring the optical density at 612 nm every 30 min and 1000 s shaking at 100 rpm per cycle of 30 min by using a microplate reader/incubator. The minimal inhibitory concentration (MIC) corresponds to the

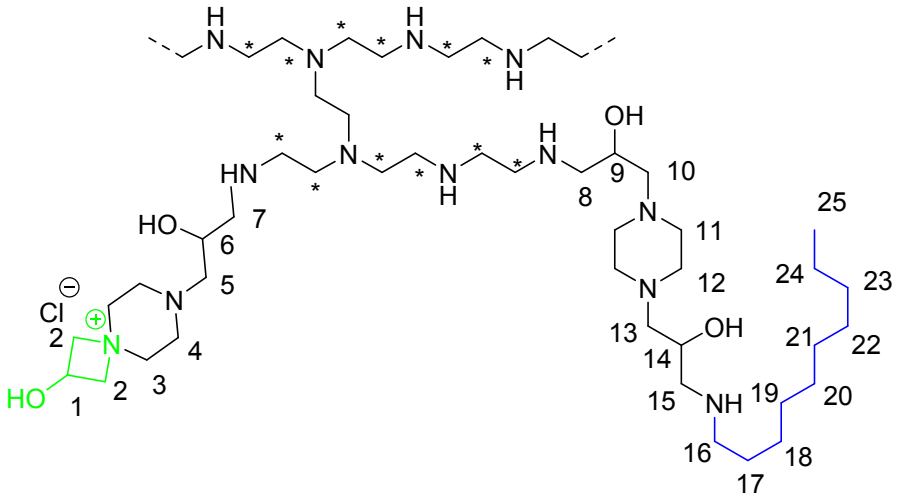
concentration of the test substance at which 100% reduction of the growth of the inoculated bacteria was observed by comparison with control samples without test substance.

SI 1. 5.b. Hemolytic activity test:

The hemolytic activity was assessed as follows. Human erythrocytes (red blood cells (RBC), 0, Rh positive; citrate blood) were obtained by centrifugation (3000 rpm, 10 min) to remove plasma, washed 3 times in PBS and diluted in PBS to obtain a stock solution $2.6x10^8$/mL RBC. 250 µL of the stock solution was pipetted into solutions of defined polymer concentration in PBS up to 500 µL; the final amount of RBC being $1,3x10^8$ RBC/mL. The RBC were exposed for 60 min at 37 °C, thereafter centrifuged (4000 rpm, 10 min) and the absorption of the supernatant was determined at 414 nm in a 96 well plate. As reference solutions (i) PBS for determining spontaneous hemolysis and (ii) 0.5 % Triton X-100 for 100 % hemolysis (positive control) were used. Hemolysis was plotted as a function of polymer concentration and the hemolytic activity was defined as the polymer concentration that causes 50 % hemolysis of human RBC relative to the positive control (HC_{50}) and in comparison to that HC_{10} was given as the polymer concentration that causes 10% hemolysis of human RBC.

SI 2. Characterization of the modified PEIs:

SI 2.1. NMR of PEIHy$^{d}_{12.5}$C$^{az}_{12.5}$:



^{1}H NMR (D_2O + MeOD [1:2], 400MHz): δ = 4.80 (m, H^1), 4.60 & 4.13 (m, H^2), 3.9 – 3.3 (H^3, H^6, H^9, H^{14}), 3.2 - 2.1 (H^4, H^5, H^7, H^8, H^{10}, H^{11}, H^{12}, H^{13}, H^{15}, H^{16}, H^*), 1.8 – 1.1 (H^{17}, H^{18}, H^{19}, H^{20}, H^{21}, H^{22}, H^{23}, H^{24}), 0.85 (H^{25}) ppm.

Table SI 2: Expected and calculated molar ratio of hydrophobic (C-10 chain) to cationic (azetidinium group) groups within the multifunctional polymers:

Polymer	hydrophobic/cationic molar ratio (expected)	hydrophobic/cationic molar ratio (calculated)
PEIHy$^{d}_{10}$C$^{az}_{15}$	1: 1.5	1: 1.48
PEIHy$^{d}_{12.5}$C$^{az}_{12.5}$	1: 1	1: 0.98
PEIHy$^{d}_{15}$C$^{az}_{10}$	1.5: 1	1.5 : 1

The ratio of hydrophobic/cationic groups was calculated using the integration ratio of the protons at δ = 4.15 ppm (H^2) and δ = 0.8 ppm (H^{11}) (see Figure SI 1B).

SI 2.2. SEC: The molecular weight of the newly prepared polymers was determined by SEC using water as eluting solvent and compared with the starting non-functionalized PEI.

Table SI 3: M_w values for the starting material and multifunctional PEIs

Polymer	M_w
PEI	24600 (reported 25000 by Sigma Aldrich)
$PEIHy^{d}_{10}C^{az}_{15}$	27900
$PEIHy^{d}_{12.5}C^{az}_{12.5}$	26900
$PEIHy^{d}_{15}C^{az}_{10}$	24200

SI 3. Characterization of the nanogels:

SI 3.1. NMR studies:

For full conversion of hydrophobic and azetidinium coupler the expected integration ratio of the protons H^2 : H^{11} in PEI-$Hy^{d}_{12.5}C^{az}_{12.5}$ was 4: 3. In deuterated water the ratio H^2 : H^{11} was found to be 4: 0.9 (Figure 2A); in deuterated water and deuterated methanol (1:2) the ratio was found to be 4: 2.9 (Figure 2B). This indicates that when the multifunctional polymer was in pure water the alkyl chains aggregate and the collapsed core does not show signals in the ^{1}H NMR. However, when deuterated methanol is added the core shell structure opens and molecular solutions are observed.

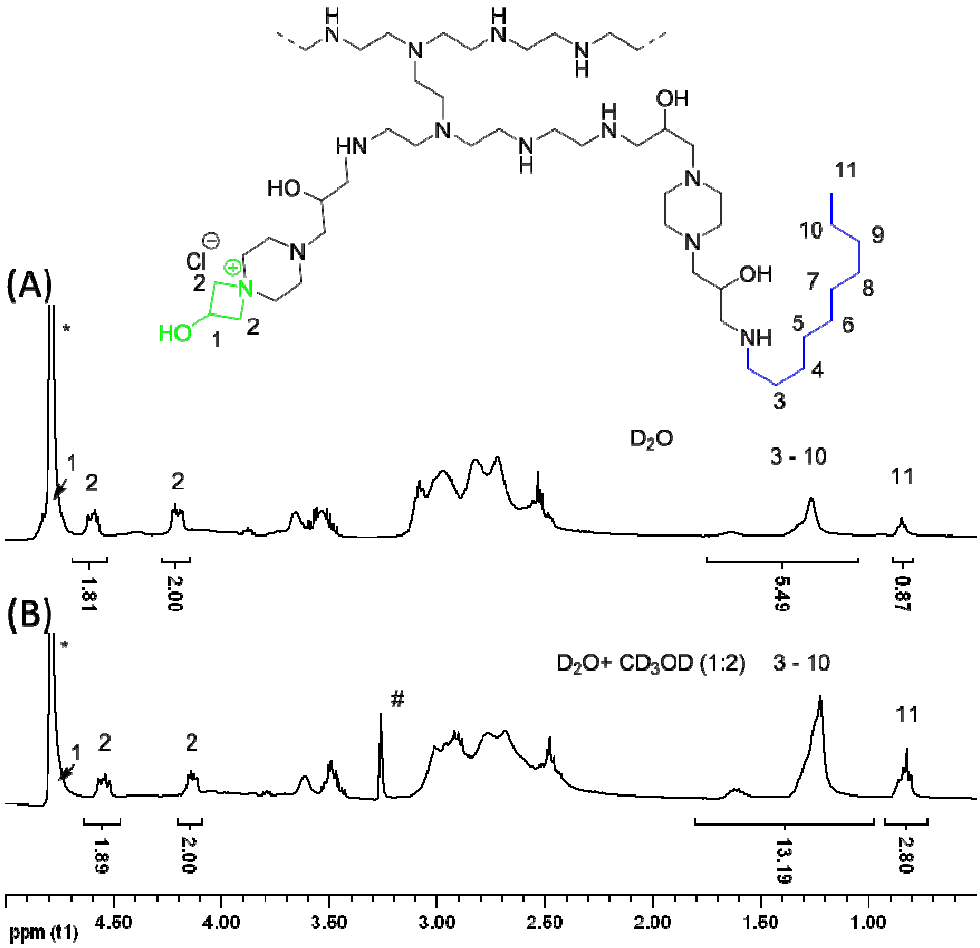


Figure SI 1: ^{1}H NMR analysis of the multifunctional PEI (for PEIHy$^{d}_{12.5}$C$^{az}_{12.5}$) –proving the core-shell structure (* and # indicates the solvent peaks [water and methanol respectively]).

SI 3.2. Cryo- SEM images of the PCNGs:

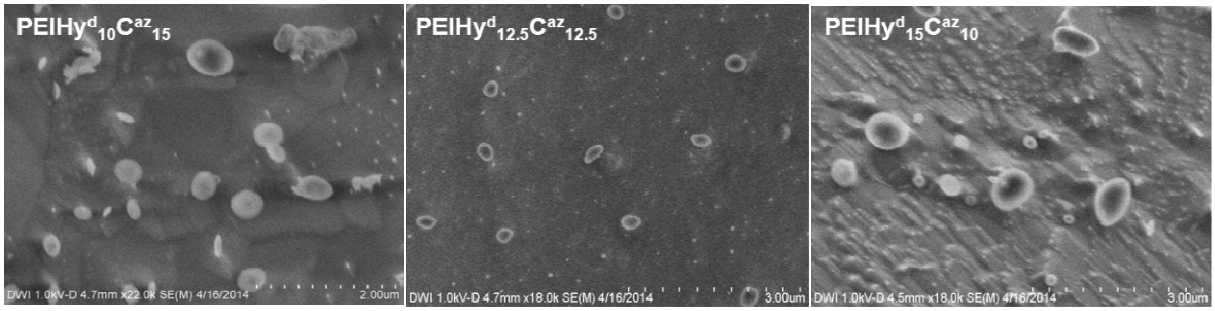


Figure SI 2: Cryo-SEM images of PEIHy$^{d}_{10}$C$^{Az}_{15}$, PEIHy$^{d}_{12.5}$C$^{Az}_{12.5}$ and PEIHy$^{d}_{15}$C$^{Az}_{10}$: showing the morphology of the soft nanoparticles:

SI 3.3. Dynamic light scattering (DLS) studies:

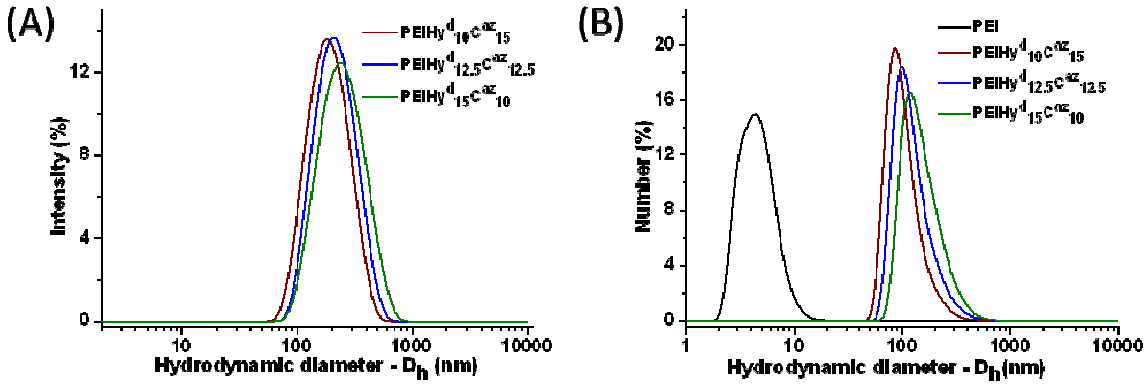


Figure SI 3: Results of DLS measurements in water at T = 25°C for the starting material (PEI) and multifunctional PEIs, showing the formation of particles, with low dispersity: (A) intensity average distribution, (B) number average distribution.

Table SI 4: DLS measurements: Determination of the particle diameter at different concentrations in water at T = 25°C. (error margin: diameter = ± 3 nm, PDI = ±0.02)

Polymer concentration (mg/ mL)	$PEIHy^{d}_{10}C^{az}_{15}$ diameter (nm) [PDI]	$PEIHy^{d}_{12.5}C^{az}_{12.5}$ diameter (nm) [PDI]	$PEIHy^{d}_{15}C^{az}_{10}$ diameter (nm) [PDI]
2	167 [0.17]	198 [0.20]	230 [0.19]
1.5	165 [0.17]	194 [0.20]	225 [0.17]
1	160 [0.16]	190 [0.21]	220 [0.18]
0.8	162 [0.17]	186 [0.22]	217 [0.16]
0.5	155 [0.18]	194 [0.19]	217 [0.18]
0.2	153 [0.22]	185 [0.18]	216 [0.18]
0.02	151 [0.19]	186 [0.21]	214 [0.18]

Table SI 5: DLS measurements: Determination of the particle diameter in water at T = 25°C after increasing storage time (polymer concentration = 1 mg/mL): stability of the PCNGs. (error margin: diameter = ± 3 nm, PDI = ±0.02)

Time (days)	Diameter (nm) [PDI] ($PEIHy^{d}_{12.5}C^{az}_{12.5}$)
1	192 [0.21]
15	190 [0.18]
60	190 [0.21]
120	192 [0.20]

Table SI 6: Determination of the particle diameter in water at T = 25°C and different salt (NaCl) concentrations (polymer concentration = 1 mg/mL): effect on the stability of the PCNGs at different salt concentration. (error margin: diameter = ± 3 nm, PDI = ±0.02)

NaCl Concentration (wt%)	Diameter (nm) [PDI] ($PEIHy^{d}_{12.5}C^{az}_{12.5}$)
0	182 [0.20]
3	182 [0.21]
5	186 [0.18]
10	190 [0.19]

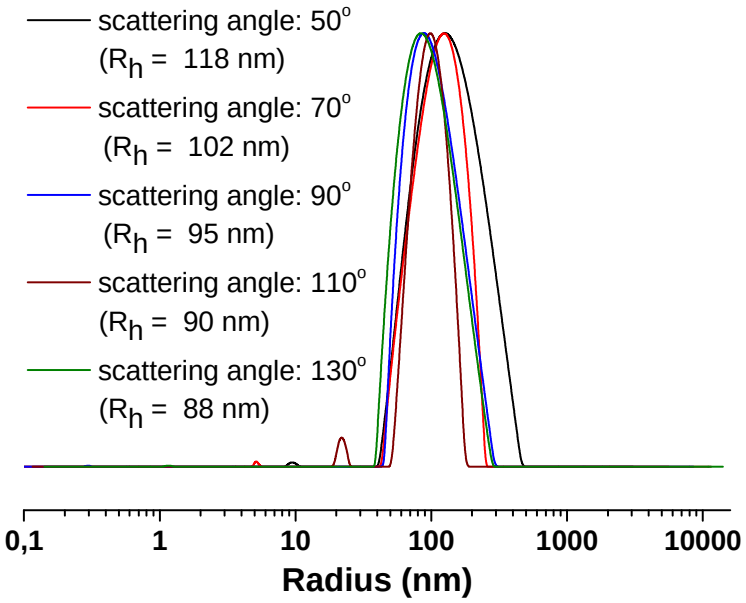


Figure SI 4: CONTIN plots (DLS) in water at T = 25°C for $PEIHy^{d}_{12.5}C^{az}_{12.5}$ at different scattering angle.

SI 3.4. Static Light Scattering

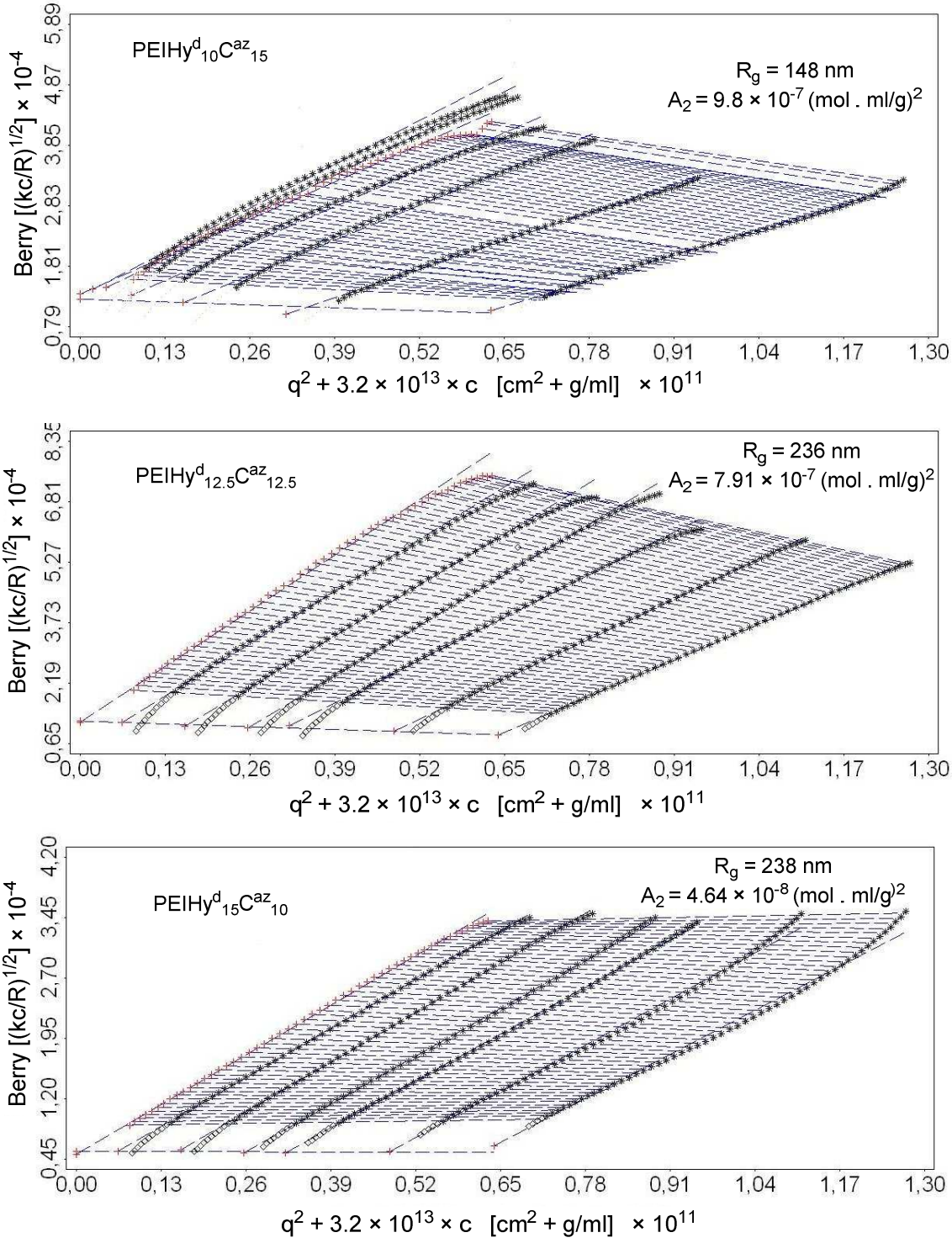


Figure SI 5: Berry plots for different polymer samples: (Solvent: Water, T = 25 ^{o}C).

Table SI 7: Values of dn/dc in water for different polymers.

Polymer	Temperature (^{o}C)	dn/dc (ml/ g)
$PEIHy^{d}_{10}C^{az}_{15}$	25	0.208
$PEIHy^{d}_{12.5}C^{az}_{12.5}$	25	0.211
$PEIHy^{d}_{15}C^{az}_{10}$	25	0.218
$PEIHy^{d}_{12.5}C^{az}_{12.5}$	50	0.204

SI 4. Temperature dependent studies: (error margin: diameter = ± 3 nm, PDI = ± 0.02)

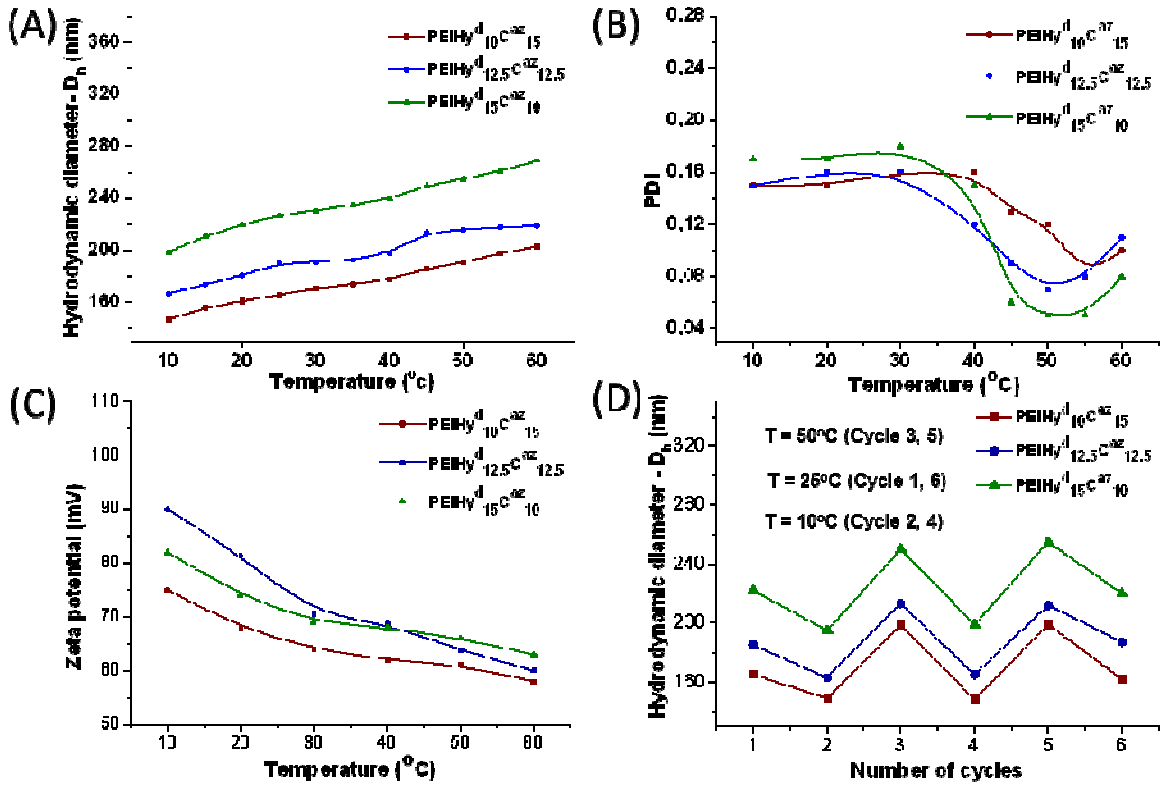


Figure SI 6: Effect of temperature on the size and zeta potential of PCNG in water: (A) size vs. temperature plot, (B) size distribution vs. temperature plot, (C) zeta potential vs. temperature plot, (D) reversible size change at different temperatures (heating/ cooling cycles).

SI 5. Biological studies:

Table SI 8: Antimicrobial activity (MIC: lowest polymer concentration resulting in 100 % growth inhibition during 20 h) and hemolytic activity of the cationic nanogels:

Polymer	MIC_{100} (µg/mL)				HC_{50} (µg/mL)
	E.coli (ATCC 25922)	*P.aeruginosa (ATCC 27853)*	*S.aureus (ATCC 6538)*	*S.epidermidis (ATCC 12228)*	
$PEIHy^{d}_{10}C^{az}_{15}$	20	200	7	3	90
$PEIHy^{d}_{12.5}C^{az}_{12.5}$	20	200	7	2	100
$PEIHy^{d}_{15}C^{az}_{10}$	10	200	5	2	70
Unmodified PEI	400	70	20	3	1000

SI 6. Calculation of the hydrodynamic radius of single $PEIHy^{d}_{12.5}C^{az}_{12.5}$ molecule at 25°C in water:

The hydrodynamic radius of the PCNG (R_h): 95 nm.

No of molecules associates to form a PCNG (N_{agg}) :2271

Hydrodynamic radius of single $PEIHy^{d}_{12.5}C^{az}_{12.5}$ chain (R): $[(95)^3 /2271]^{1/3}$ = 7.2 nm

This value correlates to the hydrodynamic radius of the water soluble non modified PEI (starting material), R_g = 4 (± 1) nm. The small increase in the hydrodynamic radius is due to mainly the hydrophobic and cationic functionalization. This value is very close to the values reported for the hydrodynamic radius of modified single polymer PEI chains, with comparable hydrophobic and cationic building blocks. [2]

SI 7. Calculation of the density of the PCNG at 25°C ($PEIHy^{d}_{12.5}C^{az}_{12.5}$):

The hydrodynamic radius of the PCNG (R_h): 95 nm = 95×10^{-8} dm.

Molecular weight of the aggregates = 61,100,100 g. / mol.

Weight of a single PCNG (M) = [61,100,000 /6.023×10^{23}] g.

Volume of a single PCNG (V) = 4/3 π $(95 \times 10^{-8})^3$ dm^3

Density of the PCNG (D) = M/V = 0.354 g /dm^3 = 0.0354 g / L.

SI 8. Earlier reported modified PEI (from reference 41 and 42), used as reference for comparing antimicrobial properties of the current polymers:

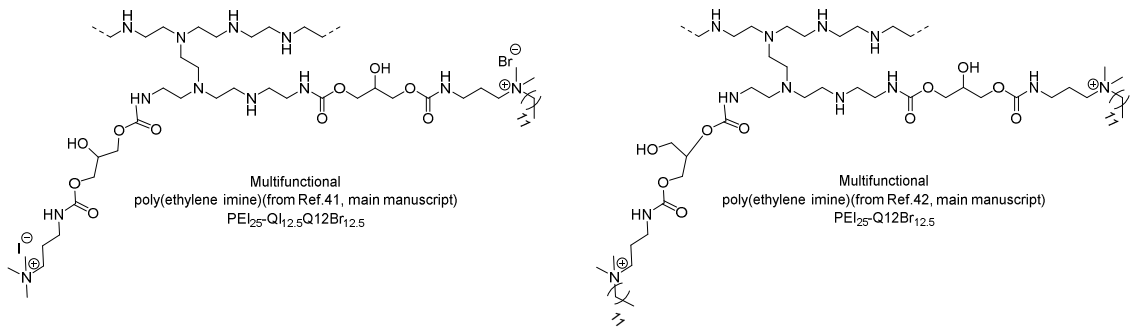


Figure SI 7: Chemical structure of earlier reported modified PEIs (reference 41 and 42 from the main manuscript).

References:

1. Chattopadhyay S., Keul H., Möller M., *Green Chem.* 2013, 15, 3135
2. Pasquier N., Keul H., Heine E., Moeller M., Angelov B., Linser S., Willumeit R., *Macromol. Biosci.* 2008, 8, 903.